Novel biomarkers of changes in muscle mass or muscle pathology

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NOVEL BIOMARKERS OF CHANGES IN MUSCLE MASS OR MUSCLE PATHOLOGY

PhD THESIS

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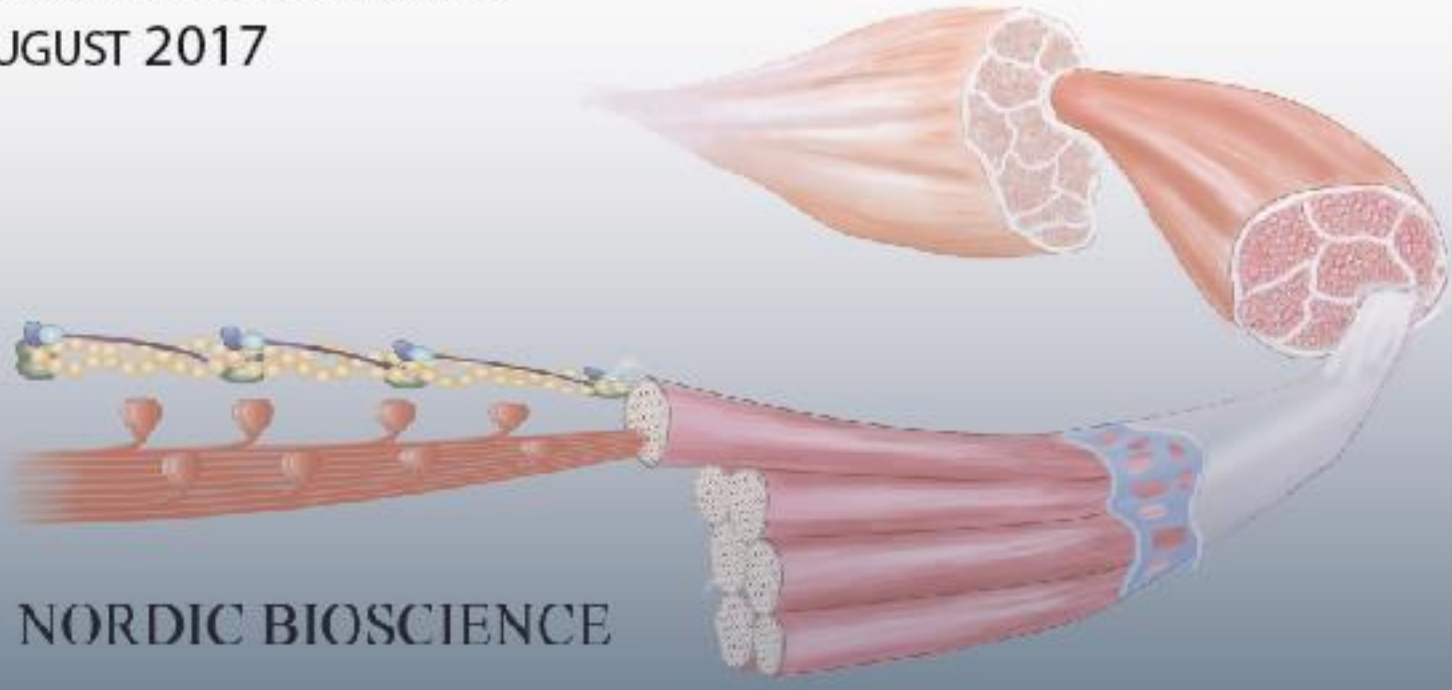

NORDIC BIOSCIENCE

PREFACE

This thesis is the result of a PhD project under the supervision of Professor Birte Svensson at DTU and Kim Henriksen Ph.D. at Nordic Bioscience. Furthermore, Professor Susanne Jacobsen, Associate Professor Per Hägglund at DTU and Anders Nedergaard, PhD at Nordic Bioscience acted formerly as supervisors in the project. All the experiments were conducted at Nordic Bioscience, Herlev, Denmark from January 2014 to August 2017.

This work includes an introduction to the field and current relevant topics, in addition to the laboratory work performed in three clinical studies, as described in the included manuscripts. In the discussion section the different aspects of the experimental work are presented and discussed in line with relevant literature, concluding with limitations and possible future steps.

PAPERS AND MANUSCRIPTS

I. Neo-epitope Peptides as Biomarkers of Disease Progression for Muscular Dystrophies and Other Myopathies

Arvanitidis A, Henriksen K, Karsdal MA, Nedergaard A.. J Neuromuscular Dis 2016; 3: 333–346.

II. Collagen turnover biomarkers assist in differential diagnosis of different forms of myositis

Athanasios Arvanitidis, Xiang Guo, Brandon W. Higgs, Christopher Morehouse, Philip Z. Brohawn, Morten A. Karsdal, Kim Henriksen, Wendy I White, *Submitted to Journal of biotechnology*

III. Serum C-terminal slow skeletal troponin T as a potential biomarker of muscle wasting and rehabilitation

Arvanitidis, A, Sun, S, Belavý, DL, Felsenberg, D, Rittweger, J, Armbrecht, G, Nedergaard, A, Lønbro S, Karsdal, MA, Henriksen, K, *Submitted to Journal of cachexia, sarcopenia and muscle*

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ABSTRACT

Muscle protein turnover is a dynamic equilibrium that regulates the body composition and homeostasis through various cytokines and proteases. When the balance between protein synthesis and protein degradation is altered, proper muscle function and regeneration is being hampered, affecting patient's quality of life. In these conditions, a constellation of symptoms as inflammation, fibrosis and muscle wasting has been widely observed despite different onsets. Many of these pathologies are incurable and can only be treated symptomatically.

A wide array of biomarkers has been used to monitor qualitative and quantitative changes in muscle. Unfortunately, there has not been an ideal panel of biomarkers that can be readily applied in studies and assist with prognosis of the disease or response to treatment. Protein biomarkers in serum are easily obtainable, not as invasive as other methods and can be used as targets for sensitive antibody-based assays.

The overall hypothesis is that both the ECM and myofibrillar biomarkers are released in circulation of people with muscle pathologies and can be used to develop bioassays. We wanted to test if those protein fingerprint biomarkers can characterize and distinguish between healthy individuals and patients with different myopathy diseases, describe the underlying mechanisms of muscle conditions and possibly putative response to an intervention. There were three different studies where biomarkers were applied in this thesis.

Study I involved 51 myositis patients (28 Dermatomyositis, DM and 23 Polymyositis, PM) compared to a control group. A range of biomarkers derived from cleavage of collagens I (C1M and PINP), III (C3M and PRO-C3), VI (C6M) and C-reactive protein (CRPM) was applied to distinguish between the diseases in this cross-sectional cohort. Both DM and PM significantly affect several of the biomarkers levels measured in this study, most prominently CRPM and PINP, indicative of significantly altered turnover of extracellular matrix components and CRPM. C3M correlated with Interferon gene score, in PM and DM, and CRPM with MMT8 score in DM.

We further developed an assay directed at the C-terminal of troponin T1 (TNNT1) that was measured in studies II and III.
In study II, a group of cancer patients after radiotherapy was admitted to a resistance-training program alongside to a control group that followed the same training regime. Serum samples were obtained right after radiotherapy, before and during the training period. TNNT1 levels were significantly elevated in the patient group compared to the control group, even before engaging in any form of physical activity. After engaging in physical training, the biomarker levels further increased through time, reaching a significant difference both compared to the patients baseline (T24vsT0, $p<0.05$) as well as to the control group (T1 and T24 vs control, $p<0.0001$).

In study III, healthy subjects were put in 56 days of bed rest, split in a group with resistance vibration exercise as a countermeasure and a group with no countermeasure at all. After the

bed rest period, both groups entered the same rehabilitation process for a period of 128 days. There was a significant difference between the two groups in the bed rest stage that demonstrates a distinct response to the RVE counter measure. The increased levels of circulating TNNT1 for the RVE group in this study could be explained by the unloading of troponin from the muscle. During the remobilization stage, the TNNT1 levels were increased significantly in both groups in a very similar manner, compared to the baseline as well as the levels during the bed rest period. In day 28 of recovery were the maximum levels of TNNT1 observed and by the time of training completion, the levels were almost returned back to baseline.

The results of this thesis point to the fact that that a panel of biomarkers could fill in the need to characterize complex processes in rare neuromuscular diseases. Addressing the main manifestation of the diseases in well-described clinical cohorts could expedite pharmaceutical trials and provide valuable information on the pathology of the disease.

DANSK RESUMÉ

Muskel protein turnover er en dynamisk ligevægt der regulerer kropssammensætningen og homeostase gennem cytokiner og proteaser. Når ligevægten mellem proteinopbygning og proteinnedbrydning bliver forskudt, medfører dette en hæmning af muskel funktion og regenerering, hvilket påvirker livskvaliteten for det enkelte individ. Uafhængigt af årssagen til denne lidelse, er der hos patienterne en kombination af symptomer såsom inflammation, fibrose og tab af muskelmasse. Flere af disse patologiske ændringer er uhelbredelige, og patienterne modtager primært symptombehandling.

Et bredt panel af biomarkører blev benyttet til at måle kvalitative og kvantitative ændringer i musklerne. Dog er der på nuværende tidspunkt ikke udviklet et ideelt panel af biomarkører som kan benyttes til udvikling af lægemidler, forudsige progression af sygdommen eller registrere respons på en given behandling. Protein biomarkører i serum er let tilgængelige, non-invasive og kan benyttes som mål for sensitive antistof-baserede assays.

Hypotesen i denne PhD afhandling er at både extracellulær matrix (ECM) og myofibrillære proteinfragmenter bliver udskilt i blodbanen hos individer med forskellige muskel sygdomme. Disse fragmenter kan benyttes til udvikling af bioassays. Hvorvidt disse forskellige fragmenter (protein fingeraftryk) kan karakterisere og skelne mellem raske individer og patienter med forskellige myopatier, beskrive den underliggende mekanisme af muskel sygdommen eller respons på en given intervention, vides endnu ikke.

Tre forskellige studier blev målt til evaluering af biomarkører i forbindelse med dette projekt.

Studie I bestod af 51 myositis patienter (28 dermatomyositis (DM) og 23 polymyositis (PM)) sammenlignet med en kontrol gruppe. Forskellige biomarkører af kollagen type I (C1M og PINP), III (C3M og Pro-C3), VI (C6M) og C-reaktivt protein (DRPM) var målt, for at undersøge hvorvidt disse kunne skelne mellem DM, PM og kontrol gruppen i et tværssnitsstudie. I både DM og PM blev ændrede biomarkør niveauer detekteret i sammenligning med kontrollerne. Hvoraf de bedste markører CRPM og PINP indikerede en signifikant ændring i proteinturnover af ECM komponenter. C3M korrelerede med Interferon gen-scoren, i PM og DM, og CRPM med MMT8 scoren i DM, hvilket indikerer en potential anvendelse af disse markører i fremtidige studier af interventioner i PM og DM.

Ydermere udviklede vi et assay rettet mod C-terminalen af troponin T1 (TNNT1), dette assay blev målt i studie II og III.

Studie II bestod af en gruppe cancer patienter, som modtog strålebehandling og herefter blev inkluderet i et træningsprogram sideløbende med en kontrolgruppe. Serum prøver blev opsamlet umiddelbart efter strålebehandlingen før og under træningsperioden. TNNT1 niveauet var signifikant forhøjede i patient gruppen i forhold til kontrol gruppen, før påbegyndelse af fysisk træning. Efter fysisk træning var niveauet af biomarkøren yderligere forhøjet, hvilket resulterede i en signifikant forskel sammenlignet med både patienter ved påbegyndelsen af studiet (T24vsT0, $p<0.05$) og kontrol gruppen (T1 og T24 vs kontrol gruppen, $p<0.0001$).

Studie III bestod af raske individer som var sengeliggende i 56 dage, og inddelt i to grupper hvoraf den ene udførte modstands vibrations træning (RVE) og den anden ikke udførte træning. Efter at være sengeliggende deltog begge grupper i den samme rehabiliterings proces i 128 dage. Der var en signifikant forskel mellem de to grupper som var sengeliggende, hvilket demonstrerer et forskelligt respons til RVE counter målingen. Det forhøjede niveau af TNNT1 i blodet for RVE gruppen i dette studie skyldes frigivelse af troponin fra musklen. I forbindelse med remobiliseringen af patienterne var TNNT1 niveauet forhøjet signifikant i begge grupper, sammenlignet med påbegyndelsen af studiet og gennem perioden hvor individerne var sengeliggende. Det højeste niveau af TNNT1 var observeret på dag 28 efter genoptræningen, hvorefter niveauet ved afslutningen næsten var normaliseret til det samme niveau som ved påbegyndelsen af studiet.

Resultatet af dette projekt viser at et panel af biomarkører kan benyttes til at imødekomme behovet for karakteriseringen af processerne i sjældne neuromuskulære sygdomme. Sammenhængen mellem disse biomarkører og primære manifestationer af sygdommene bør karakteriseres i velkarakteriserede kohorter. Dette ville kunne bidrage til at validere biomarkørernes egenskab til at reflektere sygdommens patologi, og derfor om de ville kunne bruges i farmaceutiske studier som forsøger at påvirke disse mekanismer.

ABBREVIATIONS

ADP	Air Displacement Plethysmography
BBR	Berlin Bed Rest study
BIA/BIS	Biological Impedance Analysis/Spectroscopy
BMD	Becker's muscular dystrophy
C1M	MMP degraded type I collagen
C3M	MMP degraded type III collagen
C6M	MMP degraded type VI collagen
CAPN	Calpains
CK	Creatine Kinase
CRP	C-reactive protein
CRPM	MMP degraded CRP
CT	Computed Tomography
CTX-1	Cross-linked biomarker of collagen type I
DAHANCA	Danish Head and Neck Cancer Group
DEXA	Dual-energy X-ray absorptiometry
DM	Dermatomyositis
DMD	Duchenne's muscular dystrophy
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
FSHD	Facioscapulohumeral Muscular Dystrophy
HNSCC	Head and Neck Squamous Cell Carcinoma
IBM	Inclusion body myositis
IGF-1	insulin growth factor-1
IL-	Interleukin-
IMCT	Intramuscular Connective Tissue
LBM	Lean Body Mass
LDL	Lower Detection Limit
LGMD	Limb-Girdle Muscular Dystrophy
MAFbx	Muscle Atrophy F-box
Mb	Myoglobin
MMP	Matrix Metalloproteinase
MMT8	Manual Muscle Testing 8
MRI	Magnetic resonance imaging
MuRF1	Muscle RING finger 1
NF-κB	Nuclear factor-kappa beta
OA	Osteoarthritis
PINP	Formation of type I collagen
PM	Polymyositis
PRO-C3	Formation of type III collagen
PRT	Progressive Resistance Treatment
QC	Quality Control
RA	Rheumatoid Arthritis
RVE	Resistance Vibration Exercise
STAT3	Signal transducer and activator of transcription 3
TGF	Transforming Growth Factor
TMB	Tetramethylbenzinidine
TNF-α	Tumour Necrosis Factor–α
TNNT1	Troponin T1 c-terminal biomarker

1. BACKGROUND

Skeletal muscle consists of aligned myofibers, which are multinucleated myocytes. Within each myocyte lies an organized bundle of contractile proteins that is collectively named as the sarcomere. The sarcomere comprises thin and thick filaments, which are responsible for muscle contraction. Main constituents of the thick filament are the light and heavy myosin chains. On the thin filament, actin monomers create a functional scaffold on which troponins and tropomyosin reside and take part of the regulation of muscle contraction [1–3]. Larger proteins as titin, nebulin and obscurin form a structure that aligns the sarcomere and provide tension and elasticity [4].

Myofibers can be further classified into two different types, depending on expression of different myosin heavy chain isoforms. The two groups are known as type I and type II myofibers or slow-twitch and fast-twitch fibers [5]. Type I muscle fibers express type I myosin and are producing energy in a slow rate, through oxidative phosphorylation. Due to a higher protein synthesis and a higher oxidative capacity [6], there is a distinctively higher protein turnover rate in slow fibers[7]. Conversely, type II fibers express type II myosin, produce energy in rapid bursts and express metabolic enzymes favoring glycolytic/anaerobic metabolism. Type II fibers generate more power while type I fibers are favored in long activities that require endurance.

Force is generated through muscle fiber contraction as regulated through the tropomyosin and troponin complex that resides on the thin filament. The troponin complex is composed by three different troponins: TnI, TnC and TnT, each serving different functionalities. TnC binds calcium ions and activates a conformational change that makes the inhibitory protein TnI to reveal the myosin binding sites on the actin filament, which enables muscle contraction. TnT serves as the anchor of the whole troponin complex by binding to tropomyosin and the other troponins through the C-terminus. The calcium-dependent muscle contraction is inhibited by TnT by suppressing myosin ATPase activity inactivating fiber contraction. Mutations in TnT can cause cardiomyopathies or nemaline myopathy which is a rare congenital muscle disease characterized by general weakness and hypotonia[8]. Complete absence of troponin T diminishes the Ca^{2+} dependent activation, while deletions in the last 14 or 28 amino acids in the C-terminus lead to ATPase activity [9]. Irrespectively to presence of Ca^{2}, truncation of the C-terminus prohibits full muscle relaxation[10]. Presence of slow skeletal troponin T in serum can reflect training-induced changes in muscle and more specifically the troponin complex or preferential modification caused in slow-twitch fibers. Exercise-induced damage has been shown to allow release of skeletal troponin I into the blood stream[11].

Muscle fibers are ensheathed in a cell membrane called the sarcolemma, which is the basement membrane and is mainly comprised of type VI collagen, dystroglycans, agrin and laminins. The basement membrane is attached to the fiber cytoskeleton via transmembrane proteins as dystrophin and integrins. It is further surrounded by the endomysium which is a layer of type III and IV collagen, further associating with collagen V[12]. The endomysium constitutes the inner part of the extracellular matrix (ECM) in the muscle, occupying the space between the sarcolemma of adjacent muscle fibers. Furthermore, the basement membrane

adjacent to the sarcolemma is mostly consisting of type IV and VI collagen[13,14]. Often times in literature, there is no distinction between the basement membrane and the sarcolemma. Fibers are further organized in bundles called fascicles and are further surrounded by an ECM layer called perimysium. Collagen type I dominates the perimysium associating with type V collagen cores. Fibril associated collagens type XII and XIV are also localized in the perimysium. The whole muscle is surrounded by the epimysium, which is a connective tissue layer in line with the tendons, indirectly attaching to the bones. Type III collagen is found in the epimysium. A schematic overview of the muscle structure in the different levels can be seen in *Figure 1.*

The intramuscular ECM is also referred to as intramuscular connective tissue (IMCT). The structure and organization of the IMCT can be visualized electron micrograph scans after digestion of the muscle fibers with NaOH. A schematic representation can be seen in figure 1. Collagen thickness and composition of the IMCT varies between different muscles, but generally type I collagen content is higher than type III. Collagens in the IMCT facilitate structural integrity and flexibility for mechanical force transduction, but are also implicated in cellular signaling.

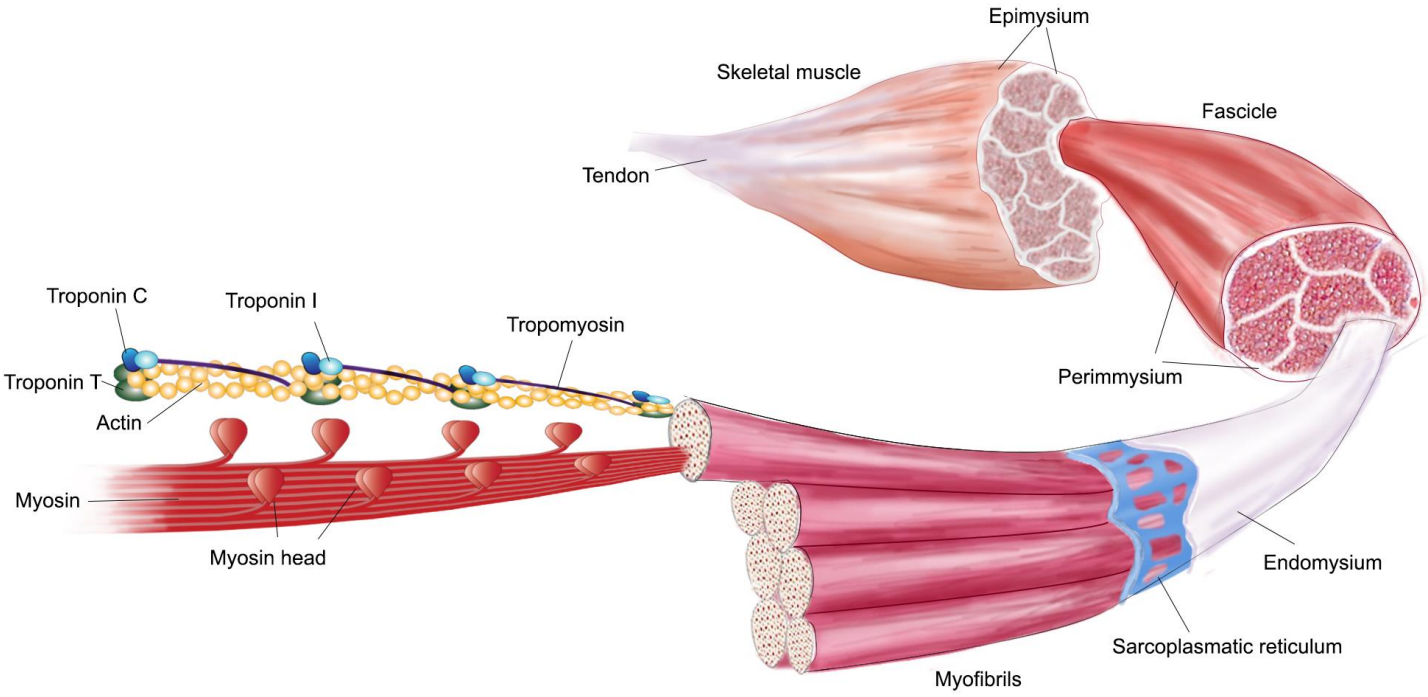

Figure 1: *The architecture of skeletal muscle. Myofibrils of many actin and myosin molecules constitute the muscle fibers that are ensheathed in the endomysial layer. These fibers bundle together to form fascicles that are in turn covered by the perimysium. Fascicles are further banded and held together by the epimysium, connecting to the tendon and forming the muscle.*

Muscle protein turnover is a dynamic equilibrium that regulates the body composition over time. The basic metabolic rate is a net outcome affected by insulin and glucagon secretion during food intake, exercise and many other hormones such as estrogens and androgens,

growth hormone etc. Corticosteroids and immune mediators, such as interleukin-1 (IL-1), tumor necrosis factor, and interleukin-2 (IL-2) and can also have an impact on body composition, through modulation of appetite, food intake and direct effects on skeletal muscle signaling [15].

1.1 Mechanisms of Muscle Loss

When the dynamic equilibrium between protein synthesis and protein degradation is negatively skewed, muscle wasting ensues. Muscle wasting can have different onsets that vary in pathology characteristics and molecular pathways. At any given point, it can manifest rapidly (as cachexia under certain conditions as cancer or radiotherapy), moderately (in atrophy caused by disuse and immobilization) or progressively (because of aging in sarcopenia) (See *Figure 2)*.

1.1.1 Cachexia, Disuse and Sarcopenia

Cachexia is characterized by a generalized inflammatory state leading to protein wasting and energy store depletion because of a disease[16]. Protein synthesis is defective but the major cause of muscle loss in cachexia is considered to be protein degradation. Many inflammatory mediators may play a role in muscle wasting during cachexia; IL-6 has emerged as a critical factor related to the maintenance of body mass during disease.

Disuse atrophy is a broad definition encompassing the mechanical unloading of the muscle, during immobilization, bed rest or even spaceflight. Compared to cachexia, disuse atrophy does not normally include recruitment of inflammatory cytokines but there is noted increase in the production of catabolic cytokines and cortisol, based on experiments in rodents [17]. During the initial stages of disuse related atrophy, type-I fibers are affected to a greater extend [18], conversely to most wasting diseases where type II fibers are generally more susceptible. Studies of cast-immobilization in humans have revealed that out of total decrease in leg volume, 46% was due to type I fiber size decrease and only 37% type II fiber decrease [19]. This mass loss and phenotype switch is also accountable for the increased fatigue observed based on the glycolytic nature of fast fibers compared to the oxidative capacity of slow fibers.

Sarcopenia is a syndrome that involves skeletal muscle mass and strength loss. Although mainly connected to aging, it can be also a result of poor nutrition, lack of exercise or poor lifestyle factors [20,21]. Decreased levels of protein synthesis and degradation of controlling motor neurons and muscle fibers in sarcopenia, are partly attributed to decreased levels of IGF-1 and induced cell apoptosis[22]. This is further augmented by increased levels of inflammatory cytokines TNF-α and TNF-β, IL-6 and IL-1 that can diminish the IGF-1 effects and accelerate muscle loss.
Interestingly, there is also a noted switch of fiber type profiling in ageing muscle, where loss of type II fibers seems to be more favorable. In part, this can be caused after denervation of type II fibers, followed by reinnervation by slow motor neurons, inducing the phenotype switch. Fiber type degradation preference also occurs in satellite cell impairment. It is found to be more pronounced in type II fibers relative to satellite cells in type-I fibers, diminishing the repair ability in type-II fibers.

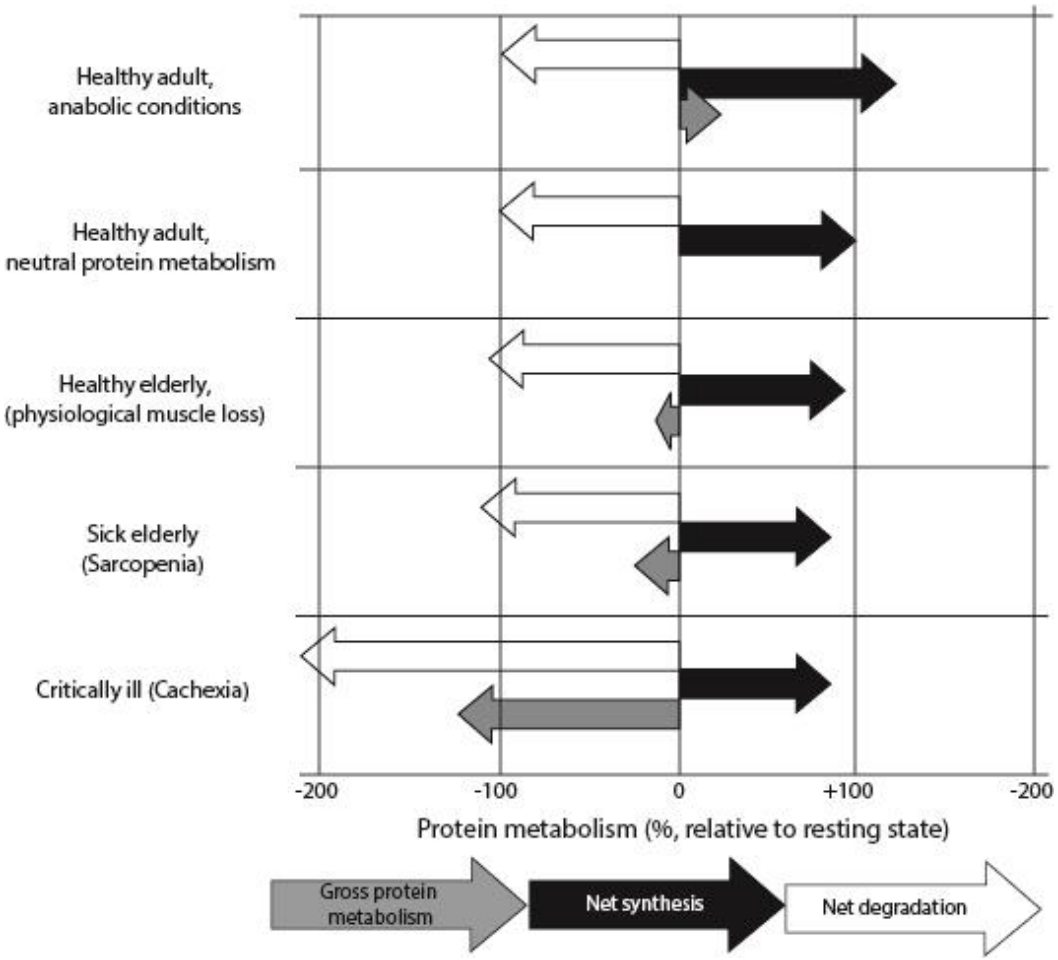

Figure 2 *Overview of net and gross protein synthesis and degradation rates in muscle during various conditions or states. The figure clearly shows that in all but the most critically ill, net protein metabolism (whether it is net synthesis or net degradation) is vastly smaller than gross degradation or synthesis. Hence, a good biomarker or panel of biomarkers should reflect net degradation or synthesis (Figure taken from: Nedergaard et al, 2013)* [23]

Furthermore, there is a range of neuromuscular pathologies or diseases that can cause muscle deterioration and compromise quality of life through impaired metabolic homeostasis and hindered freedom of motion. Muscular dystrophies, congenital and inflammatory myopathies and myositis are diverse groups of pathologies that affect more than 2 million people in Europe alone and are characterized by loss of muscle mass and function, frequently accompanied by fibrosis and/or inflammation possibly as a secondary phenomenon (further detail on epidemiology: http://neuromuscular.wustl.edu/).

These diseases are mostly chronic and manifest at varying ages, from infancy to adulthood. Most are progressive and the severe ones are associated with significantly shortened lifespan, while others cause lifelong disability [24].

1.1.2 Neuromuscular Disorders

One heterogeneous group of muscle disorders is the inflammatory myopathies also called myositises that can cause muscle atrophy, weakness and site-specific inflammation [25]. This group includes polymyositis (PM) and dermatomyositis (DM), two very similar manifestations

of that can be separated by the skin rash that patients with DM develop through calcium deposits in the muscle or skin. The disease can be fully or partially reversed if treated early, but in some cases can lead to irreversible calcification of the subcutaneous tissue and muscles, pulmonary fibrosis or respiratory failure and death [26].

While the various forms of myositis are idiopathic, mainly inflammatory conditions, muscular dystrophies and congenital myopathies are genetic disorders that affect the structural integrity or critical signalling cascades controlling the muscle fibres [27]. Dystrophies manifest a range of effects including, fatty tissue invasion and muscle wasting through an abnormal dynamic between necrosis and regeneration, thereby affecting the metabolic fitness, fibrosis and/or inflammation [23,28]. The most common dystrophies are Duchenne and Becker muscular dystrophies (DMD and BMD) Limb-Girdle Muscular Dystrophy (LGMD) and facioscapulohumeral muscular dystrophy (FSHD) are summarized in *table 1*. DMD and BMD are two similar pathologies that constitute the most common causes of pathological muscle loss, aside from sarcopenia and cachexia. Absence of a critical subsarcolemmal protein, dystrophin, initiates a pathophysiological cascade eventually leading to leakage, disruption and death of the muscle fibres, generating sustained regeneration cycles[29]. The exposure of intracellular proteins to the extracellular environment may elicit immunological reactions and thereby contribute to chronic inflammation. Over time, abnormal regeneration leads to progressive endomysial fibrosis [30–32] and displacement of muscle by fat or connective tissue [33], prompting fatal respiratory and cardiac implication by the third decade of the patients life.
Likewise, LGMD is another group of heterogeneous muscle dystrophies that are also characterized by degenerating myofibers, and later on infiltration of T-cells and macrophages, leading to a secondary inflammation [34,35].
Lastly, FSHD is an autosomal dominant inherited neuromuscular disorder[36–38] and predominantly affects face muscles, upper arms and the scapula. FSHD leads to premature disability and loss of independence despite not reducing life expectancy severely. Part of the disease consists of perivascular inflammation with observed fiber necrosis, phagocytosis and increased tissue regeneration [39].

1.2. Common Characteristic in Muscle Wasting Pathologies

Despite being conditions or diseases with different aetiologies, muscle-wasting mechanisms share several in their pathways and eventual degradation. Overlapping and non-overlapping traits among these conditions permit distinction between pathologies but also identification of mutual monitoring potential. Abnormal muscle turnover is the common denominator in all the pathologies, with inflammation or fibrosis playing a role in the production of potential peptide biomarkers [40–42] (*Table 1*).

1.2.1. Protein Synthesis/Degradation Imbalance

Playing a major role in hindered muscle synthesis is the increased production of myostatin as result of a disease or muscle disuse [43]. In normal protein turnover, dietary ingestion induces insulin response which in turn activates the the PI3K- Akt-mTOR pathway through the insulin growth factor-1. (IGF-1). IGF-1 induced response triggers an anabolic response that can

stimulate protein synthesis and proliferation of satellite cells, while suppressing protein degradation at the same time. During fasting or atrophy, suppression of the PI3K- Akt-mTOR pathway occurs possibly via autocrine signaling inhibitors as myostatin, leading to reduced protein synthesis. Myostatin (growth-differentiation factor 8, GDF8) belongs to transforming growth factor β (TGF-β) superfamily and can inhibit satellite cell differentiation and muscle protein synthesis [44,45]. Studies regarding 25 days of bed rest in humans resulted in 12% greater serum myostatin levels , which resulted in fiber atrophy and proteolysis[46].

Table 1: Prevalence of Myopathies and Dystrophies; histological and biochemical disease characteristics

Condition	Biochemical imbalance	Phenotype	Prevalence per 100.000	References
Muscular dystrophies				
Duchenne Muscular Dystrophy (DMD)	Increased CK, MMP1, MMP2, MMP7, MMP9, fibronectin Cathepsins H and L	Fibrosis, inflammation, muscle wasting, fat substitution	8 - 29 (males)	24,47–50
Becker Muscular Dystrophy (BMD)			7 - 29 (males)	
Limb-Girdle Muscular Dystrophy (LGMD)	**Heterogeneous** (absence of proteins, dysfunctional interactions etc.)	Atrophy, endomysial fibrosis, inflammation in some cases	0.8- 2.3	51–53
Facioscapulohumeral dystrophy	DUX4 expression in muscle	Fibrosis, perivascular infiltration, endomysial inflammation	5	36,39,54,55
Inflammatory Myopathies				
Polymyositis (PM)	Pro-/inflammatory markers increased	Acute inflammatory onset (responsive to immunomodulation), muscle fiber atrophy, lung fibrosis	6.3-7.1	56–58
Dermatomyositis (DM)			6.3	
Inclusion Body Myositis (IBM)		Chronic inflammation, muscle fiber atrophy, lung fibrosis (rare)	1.5 (general population) 5.1 (people >50 years old)	59
Congenital myopathies	**Heterogeneous**	Central nucleated fibers, Type I muscle fiber prevalence. rare inflammation	**6 (per live births)**	60,61

In pathophysiological muscle wasting, the calcium-activated system involving the calpains, the ubiquitin system, and the lysosomal system involving the cathepsins, consist the main processes for protein degradation. A collective illustration of the processes being described hereon can be seen in *Figure 2*.

Calpains (CAPN) are calcium-dependent proteases that have demonstrated a more complex role in muscle homeostasis. Calpains have been linked to muscle degradation through both apoptosis and necrosis [62] . One LGMD variation is caused due to mutations on the *CAPN3* gene which is exclusively expressed in skeletal muscle [53]. It is believed that it also shares a protective role in exercise induced stress and some calpains can inactivate upstream caspases [63]. Caspases are a family of proteases and their activity is related to muscle atrophy in various muscle wasting models involving inflammation and myonuclear apoptosis [64]

Muscle wasting can also occur through ubiquitination as it has been identified under atrophy conditions or disuse reducing muscle fibre size, increasing loss of myonuclei and proteolysis[65,66]. The muscle RING finger 1 (MuRF1), muscle atrophy F-box (MAFbx)/atrogin-1 nuclear and factor-kappa B (NF-κB), have been proposed to be muscle specific E3 ubiquitin ligases. Dysregulation of the normal conduct of intracellular signaling channels through the NF-κB mitogen- activated protein kinases (MAPK) pathways in skeletal muscle has been correlated to damage on the dystroglycan complex [67,68]. Furthermore, activation of NF-κB and AP- transcription factors [69]also upregulates MMP-9 expression in a positive feedback mechanism observed in dystrophic muscle.
In sarcopenia, MMP-2 and MMP-9 have a prominent role regard ECM degradation and muscle loss. They are very active in proteolysis of collagen IV that comprises the basement membrane in skeletal muscle, contribute to muscle degradation in immobilization [13,70]. MMP-9 can also degrade collagen I, III, and V present in skeletal muscle ECM [71,72]. Both MMP-2 and MMP-9 were upregulated in a rat hind limb immobilization studies [73]. MMP-2 has also shown intracellular activity and the ability cleave muscle specific proteins [74].
Likewise, in mouse and dog muscular dystrophy models the dystrophic phenotype was associated with increases of MMP expression and when MMP expression or activity were inhibited, amelioration of the dystrophic phenotype was noted [75,76]. Involvement of increased MMP levels in pathogenesis were supported by experiments held on *mdx* mice [77].
Moreover, various cathepsin mRNA isoforms were upregulated in atrophying muscle of rats [78,79]. Cathepsin and caspase groups are known to be proteolytically active alongside with MMPs partaking in dystrophies and myositis.

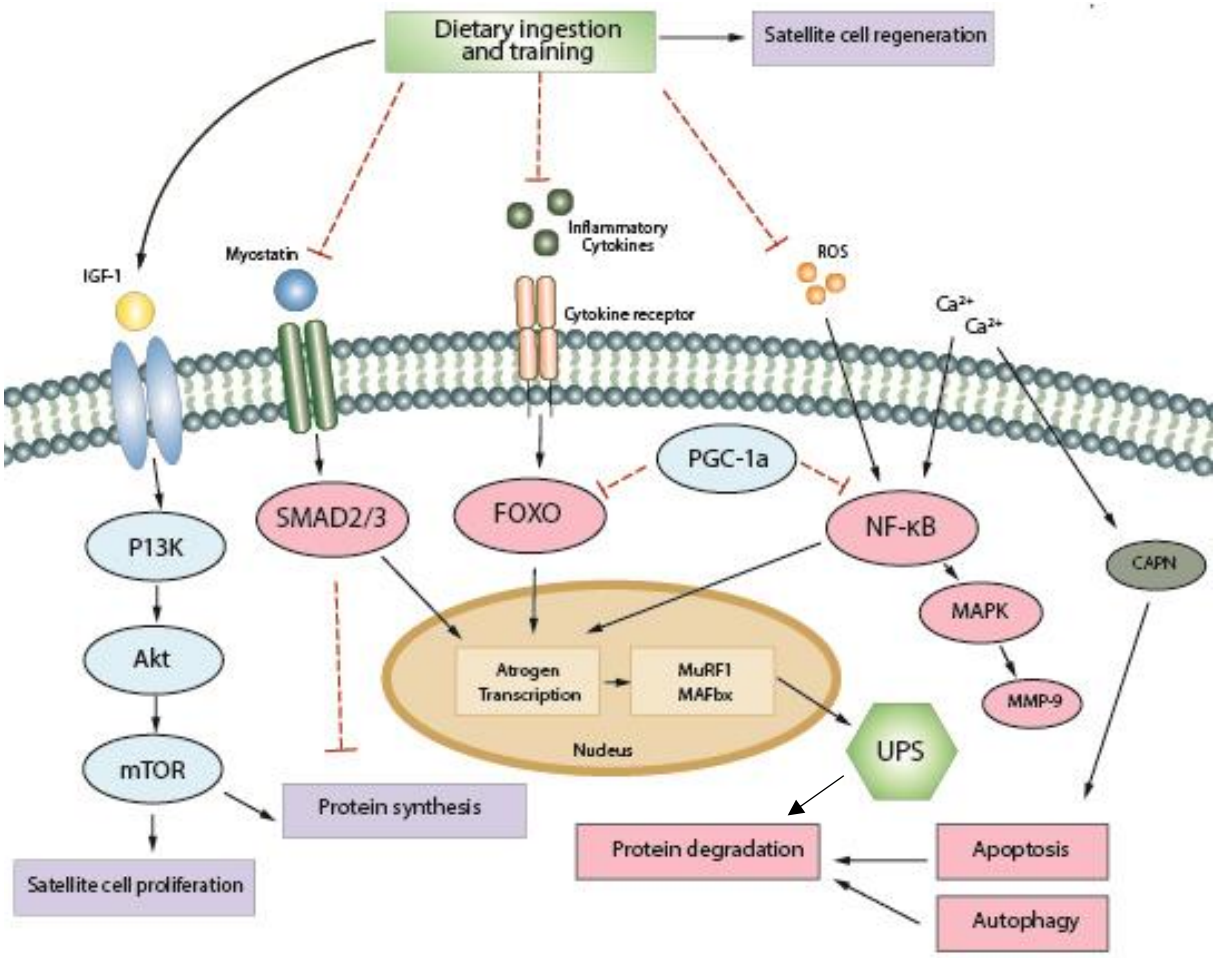

Figure 3 : *Schematic representation of the signaling pathways involved in a. protein synthesis and satellite cell proliferation after food intake and training, and b. muscle wasting through ubiquitination, apoptosis and autophagy. Anabolic pathways mediated through insulin-like growth factor 1 (IGF-1), can also be impaired in sarcopenia and cachexia caused by lower IGF-1 levels.*

1.2.2 Inflammation

In many dystrophies, compromised structural integrity is a prevalent characteristic leading to leakage of their contents. Increased expression of major histocompatibility complex type II is shown in both dystrophies and myositis and a consistent T lymphocyte invasion is seen in both human and animal models of muscular dystrophy. Among the main inflammatory cytokines that have been implicated in wasting diseases are interleukin-6 (IL-6), tumour necrosis factor–α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ)[80,81]. TNF-α has been shown to upregulate MMP-9 in muscle cells, again through the NF-κB pathway[69], augmenting ECM and muscle catabolism through the ubiquitin- proteasome system [101].

In studies with transgenic mice, IL-6 overexpression resulted to skeletal muscle atrophy that could be diminished by administration of IL-6 receptor inhibitors [99]. Aberrant expression of cytokines transpire the altered protein metabolism in sarcopenia. Increased levels of proinflammatory cytokines TNF-α and IL-6 can down-regulate GH/IGF-1 expression[65] and lead to targeted myofibril protein catabolism[69,82,83] . In cachectic cancer patients IL-6 mediates

skeletal muscle wasting signaling through its receptor (i.e. glycoprotein 130), which activates STAT3, p38, FoxO3, and atrogenes[100].

Collagen degradation mediated through MMP activation has been very well described in other inflammatory diseases. Inflammation and fibrosis are sometimes interconnected as the proliferation of inflammatory cells leads to overexpression of cytokines that stimulate collagen accretion, i.e. TGF-β, and push native cells towards fibrogenic phenotypes (TNF, TGF-β1), contributing to scar tissue formation[84–86].

1.2.3. Fibrosis

Fibrosis occurs after repeated degradation and synthesis cycles affecting the ECM. In dystrophies, development of significant intramuscular fibrosis and functional muscle tissue replacement has been seen consistently. Intramuscular collagen deposition leads to cardiac or respiratory dysfunction which are prominent contributors to mortality in dystrophy patients [87]. Conversion of satellite cells to myofibroblasts or fibroblasts promotes accumulation of interstitial collagens (types 1 through 6) and proteoglycans, which in turn results in changes in quality and quantity of ECM[88]. Continuous elevation of MMP-9 hinders myofibrilar regeneration, promotes inflammation and fiber necrosis leading to fibrosis. Dystrophic fibrosis has been also associated with higher expression of MMP-1, and -29 [47] that are responsible for modifying the ECM. Under normal circumstances the intramuscular connective tissue is well-ordered but in fibrosis it expands, taking the place of degraded muscle fibers with possible alterations in the organization of collagens (isoform ratios or crosslinking) [89]. Between the different cytokines, TGF-β1 stands out as the main promoter of fibrosis through augmentation of collagen synthesis [90]. TGF-β1 can boost growth and differentiation of fibroblasts, while also reducing the expression of matrix degrading proteases [91].
Collectively, the aberrant protein turnover, fibrosis and inflammation within the muscle, are symptoms that can be observed in many of the neuromuscular disorders. These symptoms can be potentially gauged through the use of different biomarkers.

1.3. Biomarkers

A biomarker is defined as a characteristic that can be objectively measured and evaluated as "an indicator of normal biological processes, pathogenic processes or pharmacological responses to therapeutic or other health care interventions" [92]. Biomarkers can be qualitative or quantitative (e.g. histological assessment vs. bone mineral density). They can be investigative, diagnostic, prognostic, describe disease burden or treatment efficacy. Biomarker classification facilitates the selection of surrogate endpoints in a clinical study setting. Proper selection of endpoints is vital in clinical cohorts, to expedite and enhance the process by deselecting non-responding individuals earlier in the study or promote better initial selection of candidates, e.g. fast progressors or otherwise at-risk populations. Avoiding inclusion or prolonged involvement of unsuitable patients in the trials can mitigate unnecessary time and fund consummation, accelerating the trial process [93].

1.3.1 - Existing biomarkers used in myopathies

Regarding diagnosis of many dystrophies, gene testing is available, but despite the identification of specific mutations verified by molecular methods [94], there is still a uncertainty in certain diagnoses [95,96]. Muscle biopsies are often mandatory, employing immunohistochemical assays to identify the specificity of the pathology, the extent of degeneration and fatty tissue replacement. While diagnosis is somewhat adequately covered, assessing burden of disease and making qualified prognoses for disease trajectory is hampered by the severe lack of specific, robust or practical biomarkers.

When characterizing dystrophic patients, both the current and projected quantity and quality of the muscle is of interest. A range of biophysical techniques as air displacement plethysmography (ADP), biological impedance analysis/spectroscopy (BIA/BIS), magnetic resonance imaging (MRI), computed tomography (CT), dual energy x-ray absorptiometry (DEXA) can all be used to assess muscle volume, while MRI and micro CT can even provide information about fibrosis [97,98]. However, there are problems with availability, cost, radiation exposure, accuracy, discrepancies between clinicians or machines that limit the use of all of these tools in routine clinical practice [99–103]. Imaging techniques (DXA, MRI and CT) are rarely used in monitoring of muscle diseases as the information they provide is considered of limited clinical utility, compared to the expense and possible radiation exposure associated with their use.

Stable isotope based techniques have been employed in an attempt to measure protein metabolism. Besides also requiring repeated muscle biopsies, the validity of measuring of 3-methylhistidine in serum or urine as a muscle protein degradation marker has been questioned. As 3-methylhistidine can originate from non-skeletal muscle tissue, total levels in serum and release kinetics can interfere with obtaining the true values [104].

Muscle function or quality is usually assessed through functional testing. Isometric grip strength, sitting and rising from a chair or six minutes walking tests are common examples of the functional tests used in clinical studies and settings [105]. However, strength and endurance tests (sitting/rising, walking test) suffers from high variability and risk of intra-rater differences. Most dystrophies and myopathies cause muscle weakness, increased incidence of muscle fiber degeneration and regeneration and increase levels of Creatine Kinase (CK) levels in serum. Although CK and myoglobin (Mb) in serum are used as indicators of general muscle injury or degradation, neither are capable of indicating the source or the extent.

Table 2: Summary of current biomarker classes regarding monitoring changes in skeletal muscle

Biomarker class	Examples	Advantages	Disadvantages	Refs
Biophysical	ADP, BIA/BIS, MRI, CT DXA	Generally good sensitivity and on par specificity with other methods	Problems with availability on site, cost, required expertise and exposure to radiation, rarely used for longitudinal studies	97–103
Functional test	Isometric grip strength 6 minute walking Sit and rise	Easy to implement in most studies, reflecting quality of life	Affected by many variables that cannot be controlled, intra- and inter- test variation	105
Biopsy based	Immunohistochemistry stable isotope 3-methylhistidine	Very good and robust methods, high sensitivity	Require muscle biopsy which is not sustainable method over time, biopsy site bias, possible specificity problems	104
Serum	CK, Mb, MMP-9 Collagen markers	Easily obtained through routine blood sampling, applicable in sensitive ELISA assays, low volume needed, less invasive, cost effective in large studies	Specificity problems with the biomarkers routinely applied (CK, Mb), other biomarkers although promising, have not been fully validated in large cohorts	106

ADP: Air Displacement Plethysmography, BIA/BIS Biological Impedance Analysis/Spectroscopy, MRI: Magnetic Resonance Imaging CT: Computed Tomography, DXA: Dual X-ray Absorptiometry, CK: Creatine Kinase, Mb: Myoglobin, PIIINP: Pro-Collagen III, MMP-9: Matrix metalloproteinase-9

Creatine Kinase is so far the main biomarker for assessing disease activity ("burden of disease") and is a marker of sarcolemma disruption and membrane leakage that is routinely used in the past decades. Despite being obligatory for diagnosis of some dystrophies as BMD [106] values of CK in serum strongly vary between and within patients. Generally there is a strong increase of CK up to 200-fold in DMD/BMD patients, but the levels lower with age, fibrosis progression and the extensive loss of muscle mass that takes place[107] [108]. Therefore, CK cannot provide viable information on the extent of the damage and biopsies cannot be repeated over time to facilitate sufficient monitoring over the course of a treatment.

More importantly, little or no robust protein based biomarkers are available for prognosis of the disease or early indicators of response to a treatment. Therefore, there is a lack of biochemical markers that can predict or monitor tissue turnover. It is of significant interest to find biomarkers reflecting protein metabolism, which will be more sensitive in monitoring tissue loss than current techniques.

In clinical trials, easily obtainable biomarkers that can detect treatment response at an early stage can assist in selecting better-suited candidates for cohorts or exclude patients that show no progression. Serum biomarkers have demonstrate such abilities in clinical studies as potential candidates for prognosis, stratification or response to treatment [109–113]. Considering the size of the muscle mass, the quantitative and qualitative changes in protein turnover associated with these pathologies and the degree of leakiness in healthy and diseased muscle

fibres, muscle pathologies can result in release of pathology-specific peptides that can be used as serological or urinary biomarkers [114,115].

Many bioinformatic approaches as protein sequencing, MS/MS and large-scale screening have been followed in order to identify possible protein candidates that can potentially serve as biomarkers for sarcopenia, cachexia or muscle dystrophy.

A comparatory MS analysis between 5 DMD patients and 5 healthy controls revealed that many N- or C- terminal sequences of titin could be observed and confirmed by western blot analysis [116]. However, the biomarker did not demonstrate strong specificity and was not validated in a larger cohort regarding the efficacy as a non-invasive monitoring method in dystrophies. However, a cohort of this sort would still be bound to the vast differences in an age-matched control group. As DMD is affecting children, the healthy subjects would be in a constant growing stage in different rates. Growth naturally affects the turnover of many of the proteins of interest in children of that age, inducing difficulties in a comparative study.

Proteases as MMP-9 have also been proposed as a specific biomarker of monitoring DMD progression as it has been found to be significantly upregulated compared to healthy subjects. In a study involving ambulant and non/ambulant males suffering from DMD differences in serum MMP-9 levels were observed compared to a control group of healthy boys[117]. It has to be noted that the natural MMP-9 inhibitors were also upregulated in the patient group, making the effect of MMP-9 values alone hard to interpret. Lastly, levels of fibronectin in serum have been studied as a method of determining fibrosis based on a polyclonal antibody competitive ELISA [50]. Although demonstrating mixed diagnostic potential it did not have success in the longitudinal part of the study.

1.3.2 Neoepitope biomarkers

One possible type of biomarkers showing promise regarding specificity are neoepitope peptide biomarkers. These are biomarkers in which pathology-specific post-translational modifications on distinct proteins, generate disease-specific epitopes. Any peptides whose production in vivo is pathology-related may therefore be used as biomarkers. For instance, in some dystroglycanopathies there is an aberrant glycosylation of α-dystroglycan that has served as an epitope for immunohistological examination of biopsies[118]. Unfortunately, this abnormality is observed in a small subset of dystrophic patients and although it is an interesting target, the neoepitope might not be available in blood circulation. A good neo-epitope biomarker needs to be applicable in as many patients in a specific disease as possible and readily available in bodily fluids. In this respect, neoepitope peptides produced through pathology-specific proteolytic cleavage are particularly interesting, because proteolytic activity is well known to be modulated in a large number of pathologies[119–121]. As the sarcolemma becomes permeable, smaller peptides produced upon proteolysis can leak into the circulation more easily than do intact proteins, making them measurable in serum and plasma or even urine [122].

In drug development, neo-epitope biomarkers can improve the clinical trial process by other generating early data in pre-clinical drug tests or facilitating completion of endpoints in terms of efficacy or response to treatment. Indeed, the most common reason for in clinical development is lack of proof of efficacy or unacceptable toxicity. Biomarkers can be used in preclinical studies to early address potential toxicity issues or help select the most promising

compounds[123]. Neoepitope biomarkers have been successful in advanced clinical trials, demonstrating a timely response to treatment [124,125].

1.3.3 Existing neo-epitope biomarkers

A stellar example of a highly specific neoepitope biomarker is the cathepsin K cleaved carboxyl-terminal collagen cross-linked biomarker of collagen type I (CTX-I) that has become the gold standard of bone turnover [126,127]. This is a result of the near exclusivity of osteoclasts expressing the protease in combination with the high abundance of type I collagen in bone. Thusly, any fragment detected in circulations is tightly associated with total osteoclast activity, hence related to the process of bone loss/turnover. Such a strong and specific correlation allows for early detection of bone loss, compared to conventional methods of quantification e.g. DEXA scanning. CTX-I has been quite successful at prognosing response to therapy interventions regarding the bone turnover within a quite short time frame [128].

Similarly, MMPs or ADAMTS (A-Disintegrin And Metalloproteinase) can function as disease specific proteases that can interact with relevant tissues as the already discussed collagen rich ECM to monitor aberrant turnover [129]. A range of proteolytic peptides derived from collagens 1 to 6 produced by MMPs, calpains or caspases has worked as biomarkers of many ECM-related diseases as ankylosing spondylitis [130] , OA [131] , RA [132], kidney fibrosis [133], lung fibrosis and liver fibrosis [134]. Also, some of these collagen peptide biomarkers as well as proteolytic fragments of C-reactive protein (CRP), namely MMP-cleaved CRP (CRPM), have shown to be biomarkers of the tissue inflammation[135]. Because of the shared molecular origins of these markers and the proteases involved in the previously described muscle pathologies there is a high likelihood that several of these biomarker can find use in neuromuscular disorders (Table 2).

In skeletal muscle, currently, the N-terminal pro-peptide of type III collagen (Pro-C3) is a promising biomarker since it has shown to correlate with the anabolic response to hormone treatment [136]. Understanding the muscle protein-turnover systems will help us identify potential biomarkers. Several proteolytic systems are involved in the muscle protein turnover, most notably the calcium dependent pathways (caspases and calpains), the lysosomal pathways and ubiquitin-proteasome system pathways [137–139]. The specific muscle turnover products generated by proteolytic systems would change along with the altered protein metabolism and possibly reflect the muscle loss process.

With reference to the muscle ECM in clinical immobilization studies, changes of MMP-generated collagen VI levels were found to be associated with muscle regrowth following immobilization [140]. In skeletal muscle, Pro-C3 has also been demonstrated to be a potential biomarker reflects the anabolic response to therapy in old people [142].

In the case of skeletal muscle extracellular matrix, there is a complex distribution of the different collagens in the various layers of the ECM and the connective tissue, while the effect depends on the nature of the specific disease [144]. As mentioned before, fibrosis is by definition the consequence of abnormal ECM turnover, resulting in excessive deposition of ECM proteins, especially collagens I, III and VI [143]. Fragments of these constituents or their corresponding propeptides detected in circulation have been shown to reflect the extent of fibrosis and ECM remodeling in rats [145] and even demonstrate response to antifibrotic treatment [146,147]. Indeed, Pro-C3 has been proposed in assessing collagen III formation in fibrosis models [141] (*Table 3*).

This has also been demonstrated as the case for myopathies and dystrophies, where endo- and perimysial fibrosis in the form of accumulated collagen I, III, IV and fibronectin predominates [148,149]. In DMD, endomysial fibrosis has been one of the strongest predictors of motor deterioration. Myostatin inhibitors and several putative antifibrotic treatment routes are being looked into. Previous preclinical and in vitro studies have documented that myostatin inhibitors downregulates markers of fibrosis, but this has not been shown conclusively in clinical models yet [150,151].

Table 3: *Neoepitope biomarkers with relevance to neuromuscular disorders covering a spectrum of processes connected to the disease*

Type of marker	Related to process	Application in muscle	Refs
C1M, C3M	Collagen 1,3 degradation	Inflammation in muscle tissue	[152]
PINP, C1M	Collagen I synthesis, ECM remodeling	Fibrosis	[145,153]
CAF	Agrin fragmentation	Functional disintegration in neuromuscular junction, sarcopenia	[154]
Titin	Muscle metabolism	Muscle turnover	[116,155]

An interesting neo-epitope describing fragmentation of the C-terminus of agrin by neurotrypsin in the neuromuscular junction has been recently presented [154]. The 22kD C-terminal agrin fragment correlates with neurogenic sarcopenia. This marker aims at assessing the underlying condition at the neuromuscular junction, which correlates with the decline of muscle mass in males.

Similarly, muscle atrophy is also associated with proteolytic cleavage of internal proteins and progressive tissue loss. An MMP-2 generated titin fragment biomarker has been previously developed and applied in disuse cohorts [155]. Even though titin is a very big protein, metabolic byproducts from intracellular protein degradation could enter the circulation, exhibiting a very early response of disuse atrophy. Compared to the terminal biomarkers discussed before, this neo-epitope biomarker holds the specificity advantage as it will only be available upon interaction of the substrate and the protease. Generated peptides can be possibly used as biomarkers of the myofibrillar proteins turnover in the muscle and thus provide information about ongoing muscle catabolism or anabolism. Indeed, serum Troponin I has been shown to be increased in dystrophic patients [156]. These intracellular proteins, particularly from the myofibrillar fraction as they display the greatest degree of tissue specificity, show promise as biomarkers of muscle turnover.

The focus of this thesis is to develop an assay based on a good muscle specific protein candidate and assess the assay validity in relevant muscle wasting models. Furthermore based on the information provided in the previous chapters, already existing collagen based biomarkers were of interest regarding their application in muscle pathologies.

2. RESEARCH PURPOSE, HYPOTHESES AND AIMS

As established in the background section, there is a need for well-characterized biomarkers that can serve to observe changes in muscle and reflect parts of the pathology of diseases. In *paper I,* the specific needs for monitoring muscle wasting, inflammation and fibrosis in drugs that are currently in advanced clinical trials are described. Existing biomarkers to describe progression or improvement in these pathologies at an early state have yet to be found. Research groups have taken different approaches addressing this need, varying from repeated anthropometric measurements to immunohistology in biopsies and recently micro RNA biomarkers.
Protein biomarkers in serum are easily obtainable, not as invasive as other methods and can be used as targets for antibody-based assays. In our disposal, we had a panel of already validated protein biomarkers, mainly targeting the collagens in the ECM, which have demonstrated potential in describing this processes in other diseases. We wanted to test if those protein fingerprint biomarkers could be released through inflammatory mechanisms of myopathy patients. If so, a second question would whether these biomarkers can characterize and distinguish between healthy individuals and patients with different myopathy diseases (*manuscript II*). In that study, biochemical tests describing the inflammatory component as well strength and functional tests were available. Using those tests, we could test the ability of biomarkers to correlate with the outcomes of those tests. Furthermore, we wanted to examine the possibility of developing novel biomarkers to address the degradation and release processes of muscle specific proteins in wasting models or therapeutic interventions (*manuscript III*).

The overall hypothesis is that both the ECM and myofibrillar biomarkers are released in circulation of people with muscle pathologies and can describe the underlying mechanisms of the muscle conditions, as well as the putative response to an intervention. The premise of the individual hypotheses is described below, separately for each study.

2.1 Idiopathic Inflammatory Myopathies (Study I)

In myositis, inflammation is part of the cause of the pathology, in contrast to congenital dystrophies, where it is a secondary phenomenon. Inflammation contributes to the symptoms and declined functionality, making it a relevant pathological mechanism to treat. Glucocorticoids have been shown to slow loss of locomotor function or even provide temporary improvements and are recommended as first-line treatment in both myositis [157–160] Biological anti-inflammatory drugs like inhibitors of TNF-alpha, IL-2, IL-6 or type I interferon have already shown promise in this regard in both inflammatory myopathies [161–164].
Activation of inflammatory pathways and increased proteolytic rates in myositis have been shown to be associated with overexpression of MMPs, localized in the muscle fibers [165]. Thus, proteolytic fragments generated by MMPs appear to be good biomarker candidates. Assays directed at monitoring changes in the aforementioned pathologic manifestations have been successful in providing information for developing therapies for similar diseases during clinical trials. In study I, there was a group of dermatomyositis patients, a group of polymyositis patients and a control group. In addition to serum samples obtained from all the participants, trained physicians performed muscle strength assessment.

Individual aims for this study were:
Aim 1a: Test if inflammatory biomarkers and ECM based neo-epitopes can be used to distinguish diseased populations from control group or populations with different diseases
Aim 1b: Determine whether ECM based biomarkers can reflect functionality or force generation capacity of affected muscle groups.

2.2 Cachexia, Disuse Atrophy and Training (Studies II and III)

As loss of muscle tissue and associated muscle function is a trait of most myopathies, therapeutic approaches aimed at restoring functional muscle mass are under examination.
There is a long record of cancer patients undergoing rapid muscle loss with or without any chemotherapy or radiation treatment. Patients are submitted to rehabilitation programs including resistance or other training sometimes together with dietary changes and pharmaceutical supplementation. Training has been also proposed as both a counter measure during long periods of bed rest or as a method of restoring loss of muscle back to normal levels.
As discussed previously, myofibrillar proteins and protein fragments, such as the previously described MMP-cleaved Titin fragment, can enter the circulation and may show promise as biomarkers of muscle protein turnover and by extension, catabolism or anabolism. However, these peptide biomarkers have yet to show promise in terms of being biomarkers of anabolism, or catabolism and by extension anabolic treatment response.
In study II, a group of cancer patients after radiotherapy was admitted to a resistance-training program alongside to a control group that followed the same training regime. Serum samples were obtained right after radiotherapy, before and during the training period.
In study III, healthy subjects were put in 56 days of bed rest, split in a group with resistance vibration exercise as a countermeasure and a group with no countermeasure at all. After the bed rest period, both groups entered the same rehabilitation process for a period of 128 days. Throughout the entire study, frequent blood samples were collected.
We developed an assay directed at the C-terminal of troponin T1 (TNNT1) that was measured in both of the mentioned studies.

The specific aims were:
Aim 2a: Demonstrate that the TNNT1 biomarker can be released and detected in blood circulation under muscle damage in a distinct manner compared to normal physiology.
Aim 2b: TNNT1 can follow the catabolic/anabolic effects induced by the disuse model and the training intervention in both studies.

3. BIOMARKER POTENTIAL IN DRUG DISCOVERY FOR NEUROMUSCULAR DISEASES

Manuscript I

Neo-epitope peptides as biomarkers of disease progression for muscular dystrophies and other myopathies

Aim

This review paper covers the specific needs for monitoring muscle wasting, inflammation and fibrosis in neuromuscular diseases. Possible methods of using biomarkers for monitoring those processes are discussed. Clinical trials in advanced stage at the time are mentioned and methods to benefit from serum biomarkers are proposed.

Conclusions

Neoepitope peptide biomarkers hold great potential in this respect, as they have been successful in characterizing localized pathological protein turnover, fibrosis and inflammation in a range of other diseases.

Current treatments are mainly targeted on dealing with the inflammation or fibrosis either directly (anti-inflammatory, anti-fibrotic) or indirectly (exon skipping). By using neoepitope biomarkers, improved characterization of these traits could strengthen or speed up ongoing clinical trial efforts as they can contribute to disease progression monitoring, treatment efficacy and stratification/selection for participating patients.

Note

Parts of this review paper were discussed in the introduction part of this thesis.

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Review

Neo-epitope Peptides as Biomarkers of Disease Progression for Muscular Dystrophies and Other Myopathies

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Abstract. For several decades, serological biomarkers of neuromuscular diseases as dystrophies, myopathies and myositis have been limited to routine clinical biochemistry panels. Gauging the pathological progression is a prerequisite for proper treatment and therefore identifying accessible, easy to monitor biomarkers that can predict the disease progression would be an important advancement. Most muscle diseases involve accelerated muscle fiber degradation, inflammation, fatty tissue substitution and/or fibrosis. All these pathological traits have been shown to give rise to serological peptide biomarkers in other tissues, underlining the potential application of existing biomarkers of such traits in muscle disorders. A significant quantity of tissue is involved in these pathological mechanisms alongside with qualitative changes in protein turnover in myofibrillar, extra-cellular matrix and immunological cell protein fractions accompanied by alterations in body fluids. We propose that protein and peptides can leak out of the afflicted muscles and can be of use in diagnosis, prediction of pathology trajectory and treatment efficacy. Proteolytic cleavage systems are especially modulated during a range of muscle pathologies, thereby giving rise to peptides that are differentially released during disease manifestation. Therefore, we believe that pathology-specific post-translational modifications like cleavages can give rise to neoepitope peptides that may represent a promising class of peptides for discovery of biomarkers pertaining to neuromuscular diseases.

Keywords: Muscular dystrophies, myopathies, biomarkers, prognosis

BACKGROUND

Loss of muscle mass and function compromises health through impaired metabolic homeostasis and quality of life via hindered freedom of motion. Besides being an issue in the form of cachexia and sarcopenia, it is additionally important in a range of neuromuscular diseases, namely muscular dystrophies, congenital myopathies and myositis. These are a diverse group of pathologies affecting more than 2 million people in Europe alone that are characterized by loss of muscle mass and function, frequently accompanied by fibrosis and/or inflammation possibly as a secondary phenomenon (further detail on epidemiology: http://neuromuscular.wustl.edu/).

These diseases are mostly chronic and manifest at varying ages, from infancy to adulthood. Most are progressive and the severe ones are associated with significantly shortened lifespan, while others cause lifelong disability [1].

While the various forms of myositis are idiopathic, mainly inflammatory conditions, muscular dystrophies and congenital myopathies are genetic disorders that affect the structural integrity or critical signalling cascades controlling the muscle fibres [2].

Duchenne's muscular dystrophy is the most common childhood dystrophy, where the absence of a

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critical subsarcolemmal protein, dystrophin, initiates a pathophysiological cascade eventually leading to leakage, disruption and death of the muscle fibres, generating sustained regeneration cycles [3]. The exposure of intracellular proteins to the extracellular environment, may elicit immunological reactions and thereby contribute to chronic inflammation which leads to abnormal regeneration and endomysial fibrosis [3, 4].

Considering the size of the muscle mass, the quantitative and qualitative changes in protein turnover associated with these pathologies and the degree of leakiness in healthy and diseased muscle fibres, muscle pathologies can result in release of pathology-specific peptides that can be used as serological or urinary biomarkers [5, 6].

DISEASE DESCRIPTION

The term muscular dystrophy covers more than 30 inherited diseases, associated with skeletal muscle degeneration and causing progressive weakness. Dystrophies manifest a range of effects including, fatty tissue invasion and muscle wasting through an abnormal dynamic between necrosis and regeneration, thereby affecting the metabolic fitness, fibrosis and/or inflammation [7, 8]. Since it is unfeasible to describe all of the diseases in a single review, the focus will be only on the most frequent ones and on their similar characteristics.

Duchenne and Becker muscular dystrophies (DMD and BMD) are two similar pathologies that constitute the most common causes of pathological muscle loss, aside from Sarcopenia and cachexia. Both are characterized by progressive fibrosis [4, 9, 10], inflammation, and displacement of muscle by fat or connective tissue [11]. This is more pronounced in DMD, usually leading to death in the third to fourth decade of life, while the milder and more variable BMD result in a life expectancy shortened by 0–20 years.

Limb-Girdle Muscular Dystrophy (LGMD) is a group of heterogeneous muscle dystrophies. Although they are clinically quite diverse, most manifestations are also characterized by degenerating myofibers, and later on infiltration of T-cells and macrophages, leading to a secondary inflammation [12, 13].

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant inherited neuromuscular disorder ranking third in muscular dystrophy frequency [2, 14–16]. This muscular dystrophy predominantly affects face muscles, upper arms and the scapula and leads to premature disability and loss of independence despite not reducing life expectancy severely. Part of the disease consists of perivascular inflammation with observed fiber necrosis, phagocytosis and increased tissue regeneration [17].

Inflammatory myopathies or myositis cause muscle atrophy and weakness through inflammation processes [18]. Furthermore, myositis can lead to pulmonary fibrosis, respiratory failure and death [19].

Shared pathological traits

Despite being different diseases with different etiologies, dystrophies and myositis share several traits that provide us with a common ground in which to search for biomarkers. Overlapping and non-overlapping traits between the individual myopathies are of great interest from a monitoring perspective, as they permit stratification. Shared traits for the myopathies are the presence of abnormal rates of turnover of muscle fiber components, inflammation or fibrosis, resulting in the production of peptide biomarkers [20–22] (Table 1). Although varying in manifestation and extent, monitoring these traits in each disease is useful, both for clinical assessment as well as prognosis of the disease course or treatment efficacy. Based upon experiences from inflammatory and degenerative pathologies in other tissues, we believe that detecting and assessing the presence or levels of these indicators in serum is a viable method to diagnose or determine the extent of the disease.

Inflammation

One of the traits of dystrophies is impaired structural integrity of muscle fibres leading to leakage of their contents, provoking an immune response. Increased expression of major histocompatibility complex type II is shown in both dystrophies and myositis and a consistent T lymphocyte invasion is seen in both human and animal models of muscular dystrophy. Likewise, it has been shown that genes coding for cytokines which induce apoptosis and inflammation (TNF-α, TGF-β, NF-κB and IL-6) are over-expressed in DMD patients. [3, 23, 24]. Fragments of MMP-degraded collagen I and III have in these pathologies been shown to characterize the inflammatory phenotype and in the case of osteoarthritis even separate patient with predominant inflammatory (synovitis) and mechanical osteochondral defects [25, 26]. Correspondingly, it has been

Table 1
Prevalence of Myopathies and Dystrophies; histological and biochemical disease characteristics

Condition	Biochemical imbalance	Phenotype	Prevalence per 100.000	References
Muscular dystrophies				
Duchenne Muscular Dystrophy (DMD)	Increased CK, MMP1, MMP2, MMP7, MMP9, fibronectin Cathepsins H and L	Fibrosis, inflammation, muscle wasting, fat substitution	8–29 (males)	[1, 34, 36, 37, 112]
Becker Muscular Dystrophy (BMD)			7–29 (males)	
Limb-Girdle Muscular Dystrophy (LGMD)	**Heterogenous** (absence of proteins, dysfunctional interactions etc.)	Atrophy, endomysial fibrosis, inflammation in some cases	0.8–2.3	[43, 113, 114]
Facioscapulohumeral dystrophy	DUX4 expression in muscle	Fibrosis, perivascular infiltration, endomysial inflammation	5	[14, 17, 115, 116]
Inflammatory Myopathies				
Polymyositis (PM)	Pro-/inflammatory markers increased	Acute inflammatory onset (responsive to immunomodulation), muscle fiber atrophy, lung fibrosis	6.3-7.1	[117–119]
Dermatomyositis (DM)			6.3	
Inclusion Body Myositis (IBM)		Chronic inflammation, muscle fiber atrophy, lung fibrosis (rare)	1.5 (general population) 5.1 (people >50 years ol d)	[120]
Congenital myopathies	**Heterogeneous**	Central nucleated fibers, Type I muscle fiber prevalence. rare inflammation	**6 (per live births)**	[121, 122]

shown in mouse and dog muscular dystrophy models that the dystrophic phenotype is associated with increases of MMP expression and that inhibition of MMP expression or activity actively ameliorates the dystrophic phenotype [27, 28].

Inflammation and fibrosis are sometimes interconnected as the proliferation of inflammatory cells leads to overexpression of cytokines that stimulate collagen accretion, i.e. TGF-β, and push native cells towards fibrogenic phenotypes (TNF, TFG-β1), contributing to scar tissue formation [29]. In the myocardium, this fibrosis leads to cardiac dysfunction which a prominent contributor to mortality in DMD patients [30].

Fibrosis

Fibrosis has been consistently reported to be present in various dystrophies after repeated regeneration cycles. Particularly with dystrophies, development of significant intramuscular fibrosis and replacement of functional muscle tissue with collagenous tissue is a problem. The satellite cells contributing to the formation of new muscle fibers, gradually lose their myogenic lineage and switch towards a fibrogenic phenotype, becoming myofibroblasts or fibroblasts. This leads to accumulation of interstitial collagens (types 1 through 6) and proteoglycans, which in turn results in changes in quality and quantity of extra-cellular matrix tissue (ECM)[4, 31]. The arrangement and distribution of the extracellular matrix around the contractile fibers is complex and differentially affected by muscle diseases. Collagen I dominates the perimysium while type III collagen is found both in endomysium and epimysium, further associating with collagen V [32]. Thus, the normally well-ordered intramuscular connective tissue expands by taking the place of degraded muscle fibers with possible alterations in the organization of collagens (isoform ratios or crosslinking) [33]. Although describing the most aggressive development seen in DMD, the initial events in this process manifest in most dystrophies. Furthermore, dystrophic fibrosis has been shown to be associated with increased expression of gelatinases and collagenases MMP-1, -2, and -9 [34].

Muscle protein turnover

It is obvious from a macroscopic perspective of the muscle that the loss of functional muscle cells is associated with a change in muscle protein turnover. Stable isotope studies have shown that baseline

protein synthesis does not appear to be impaired in dystrophic patients [35]. However, considering that 2–400 grams of muscle is turned over a day in healthy adults, a small percentile deficit in the synthesis and degradation ratio can easily amount to several kilos of muscle per year. Such a difference is adequate to explain the observed muscle loss with even the most aggressive dystrophies, but it would be almost impossible to detect using stable isotope methodology.

In dystrophies and myositis, proteases from the MMP, calpain, caspase and cathepsin groups are known to contribute to muscle catabolism. Upregulation of MMP-7 and cathepsins H and L were demonstrated in DMD patients [36] along with a significant upregulation of MMP-2 expression [37] while progressive increase of MMP-9 levels was more recently noted in serum from DMD patients [38]. Findings were supported by experiments held on mdx mice, demonstrating increased MMP activity and involvement in pathogenesis [39]. Moreover, various cathepsin mRNA isoforms were upregulated in atrophying muscle of rats [40, 41]. Calpains (CAPN) are calcium-dependent proteases that have been linked to muscle degradation and necrosis [42] but mutations on the *CAPN3* gene which is exclusively expressed in skeletal muscle leads to LGMD [43], possibly because of a protective role of the CAPN3 protease activity in exercise induced stress [44]. Lastly, caspase activity is related to muscle atrophy in various muscle wasting models involving myonuclear apoptosis [45].

BIOMARKERS

BIPED (Burden of disease, Investigative, prognostic, efficacy and diagnostic) Classification

A biomarker is defined as a characteristic that can be objectively measured and evaluated as "an indicator of normal biological processes, pathogenic processes or pharmacological responses to therapeutic or other health care interventions" [46]. Biomarkers can be qualitative or quantitative (e.g. histological assessment vs. Bone mineral density) and are commonly classified as "dry" or "wet". The latter is everything biochemical and the former is everything that is not, including imaging techniques (such as MR or PET), questionnaires, clinical descriptions, etc.

Biomarkers can serve different purposes and thus have different inherent uses and restrictions. Diagnosis, description of the disease severity/progression or the impact of a potential treatment are some of the areas where biomarkers can be utilized. A suggested classification of different markers, the BIPED criteria, was given by the Osteoarthritis Biomarkers Network, providing practical requirements and recommendations for each category [47]. This nomenclature can be applied to muscle pathologies. In the BIPED criteria, there are five categories of markers: Burden of disease, Investigative, prognostic, efficacy and diagnostic which constitute the acronym of the proposed nomenclature (BIPED). For each of these classifications, requirements with respect to sensitivity, specificity and robustness are provided (Table 2). Different categories are not mutually exclusive and candidate biomarkers can belong to more than one category. Biomarker classification facilitates the selection of surrogate endpoints in a clinical study setting. Proper selection of endpoints is vital in clinical cohorts, to expedite and enhance the process by either deselecting non-responding individuals earlier in the study or promote better initial selection of candidates, e.g. fast progressors or otherwise at-risk populations. Avoiding inclusion or prolonged involvement of unsuitable patients in the trials can mitigate unnecessary time and fund consummation, accelerating the trial process [48].

Existing wet and dry biomarkers used in myopathies

Gene testing is available for a number of dystrophies, but despite the marked progression in the identification of specific mutations that can be verified by molecular methods [49], not all genes implicated have yet been discovered, limiting the sensitivity of genetic diagnostics [50, 51]. Muscle biopsies are often employed to identify the specificity of the pathology, the extent of degeneration, fatty tissue replacement or to be used in immunohistochemical assays. While diagnosis is thus adequately covered, assessing burden of disease and making qualified prognoses for disease trajectory is hampered by the severe lack of specific, robust or practical biomarkers.

When characterizing dystrophic patients, both the current and projected quantity and quality of the muscle is of interest. A range of biophysical techniques as air displacement plethysmography (ADP), biological impedance analysis/spectroscopy (BIA/BIS), Magnetic resonance imaging (MRI),

Table 2
BIPED criteria for muscular dystrophy/myopathy. Adapted description of the BIPED criteria from the initially proposed by the osteoarthritis Network [47]

	Burden of disease	Investigative	Prognostic	Efficacy	Diagnostic
Definition	Biomarker associated with the extent or severity of muscle loss	Biomarker not meeting criteria for another category	Predicts onset or progression	Indicative of treatment efficacy	Differentiates diseased groups from non-diseased
Subjects	Must manifest muscular dystrophy/myopathy	NA	With and/or without diagnosed muscular dystrophy/myopathy[1]	With muscular dystrophy/myopathy	With and or without muscular dystrophy/myopathy
Design	Cross-sectional, case control	NA	Longitudinal	Controlled trial	Cross-sectional or case-control
Outcomes	Extent of severity of muscular dystrophy/myopathy	NA	New or worsening muscular dystrophy/myopathy	New or ameliorated muscular dystrophy/myopathy	Muscolar dystrophy vs no muscular dystrophy/myopathy
Criteria	Significant association between marker and extent or severity of muscular dystrophy/myopathy	NA	Significant association between marker and onset or progression of muscular dystrophy/myopathy	Significant association between marker and treatment effect	Significant association between marker and muscular dystrophy/myopathy diagnosis
Examples	Creatine Kinase, strength tests, biopsy		None or very limited selection	Muscle mass, strength, endurance	DNA test, biopsy histochemistry, imaging

Computed Tomopgraphy (CT), Dual X-ray Absorptiometry DXA) can all be used to assess muscle volume, while MRI and microCT can even provide information about fibrosis [52, 53]. However, availability, cost, radiation exposure, accuracy, validation and to some extent culture amongst clinicians have limited the use of all of these tools in routine clinical practice [7, 54–58]. Practically, muscle function or quality is assessed through functional testing Isometric grip strength, sitting and rising from a chair or six minutes walking tests are common examples of the functional tests used in clinical studies and settings [59].

Most dystrophies and myopathies cause muscle weakness, increased incidence of muscle fiber degeneration and regeneration and increase levels of Creatine Kinase (CK) levels in serum. Presence of CK and myoglobin (Mb) in serum are indicators of general muscle injury or degradation, but neither are capable of indicating the source or the extent.

Creatine Kinase is so far the main biomarker for assessing disease activity ("burden of disease") and has been extensively used as a marker of sarcolemma disruption and membrane leakage during the past 50 years. Aberrant CK levels are obligatory for diagnosis of some dystrophies as in BMD [60]. As a biomarker though, CK is far from ideal, as it varies strongly between and within patients [11]. In DMD/BMD, CK is increased up to 200-fold but the levels lower with age, fibrosis progression and loss of muscle mass [11, 61]. Asymptomatic individuals with abnormal dystrophin genes but normal CK levels are also known to exist [62]. In Limb-girdle muscular dystrophy (LGMD), elevation of CK is not observed in general but can be very high in the autosomal recessive forms that tend to have an earlier onset as well. Levels of CK can also be increased in Facioscapulohumeral dystrophy (FSHD) albeit rarely, as with congenital myopathies and myositis [61].

Therefore, CK cannot provide viable information on the extent of the damage and biopsies cannot be repeated over time to facilitate sufficient monitoring over the course of a treatment. Imaging techniques (DXA, MRI and CT) are rarely used in monitoring of muscle diseases as the information they provide is considered of limited clinical utility, compared to the expense and possible radiation exposure associated with their use, while strength and endurance tests (sitting/rising, walking test) suffers from high variability and risk of intra-rater differences [6].

More importantly, little or no robust protein based biomarkers are available for prognosis of the disease or early indicators of response to a treatment. In clinical trials, it is of paramount importance to

Table 3
Neoepitope biomarkers with relevance to neuromuscular disorders covering a spectrum of processes connected to the disease

Type of marker	Related to process	Application in muscle	Refs
C1M, C3M	Collagen 1,3 degradation	Inflammation in muscle tissue	[123]
PINP, C1M	Collagen I synthesis, ECM remodeling	Fibrosis	[84, 103]
CAF	Agrin fragmentation	Functional disintegration in neuromuscular junction, sarcopenia	[82]
Titin	Muscle metabolism	Muscle turnover	[87, 124]

have biomarkers that can detect treatment response at an early stage. The ability to exclude patients that show no progression or better yet select better-suited candidates for cohorts is a key factor for successful clinical studies. It has to be noted that there are studies that have demonstrated the utility of some serum biomarkers as potential candidates for prognosis, stratification or response to treatment [5, 63–67].

Neoepitope biomarkers

One possible type of biomarkers showing promise in this regard is neoepitope peptide biomarkers. These are biomarkers in which pathology-specific post-translational modifications to distinct proteins generate disease-specific epitopes. Peptides whose production *in vivo* is pathology-related may therefore be used as biomarkers. In this respect, neoepitope peptides produced through pathology-specific proteolytic cleavage are particularly interesting, because proteolytic activity is well known to be modulated in a large number of pathologies [3, 68–70]. As the sarcolemma becomes permeable, smaller peptides produced upon proteolysis can leak into the circulation more easily than do intact proteins, making them measurable in serum and plasma or even urine [71].

Existing neo-epitope biomarkers

An example of this is the carboxy-terminal collagen crosslinked biomarker of collagen type I (CTX-I) cleaved by Cathepsin K, that has become one of the primary biomarkers of bone turnover [72, 73]. As collagen type I is the predominant matrix protein in bone and Cathepsin K is almost exclusively expressed by osteoclasts, the abundance of the fragment is tightly associated with total osteoclast activity, thus related to the process of bone loss/turnover. This feature enables the CTX-I assay to detect changes in bone loss much earlier than e.g. DXA. CTX-I has been successful at prognosing changes in the bone turnover [74].

On a similar note, recent research has shown that fragments of collagen produced through cleavage of MMP (Matrix metalloproteinase) or ADAMTS (A Disintegrin And Metalloproteinase) can function as biomarkers in conditions of aberrant ECM turnover [75]. The rationale is that altered abundance or activity of several MMP, ADAMTS, calpain and caspase proteases have been shown to be involved in protein turnover changes in many connective tissue pathologies. A range of proteolytic peptides derived from collagens 1 to 6 has shown to work as biomarkers of ankylosing spondylitis [76], OA [77], RA [78], kidney fibrosis [79], lung fibrosis [75] and liver fibrosis [80]. Also, some of these collagen peptide biomarkers as well as proteolytic fragments of C-reactive protein (CRP), namely MMP-cleaved CRP (CRPM), have shown to be biomarkers of the tissue inflammation associated with arthritic conditions and the response to anti-inflammatory treatment in these conditions [25, 81]. Because of the shared molecular origins of these markers and the proteases involved in the previously described muscle pathologies there is a high likelihood that several of these biomarker can find use in neuromuscular disorders (Table 3).

Clinical experience and potential in muscle pathologies

An interesting neo-epitope describing fragmentation of the C-terminus of agrin by neurotrypsin in the neuromuscular junction has been recently presented [82]. The agrin fragment correlates with neurogenic sarcopenia. This marker aims at assessing the underlying condition at the neuromuscular junction, which correlates with the decline of muscle mass in males.

In clinical immobilization studies, changes of MMP-generated collagen VI product levels were found to be associated with muscle regrowth following immobilization [83]. Other MMPs and collagen combinations studied for liver or kidney fibrosis, including biomarkers as collagen I fragment generated by MMP -2, -9 and -13 (C1M) [84] or the MMP-9 proteolytically revealed neo-epitope of type III collagen, (C3M) [85] can be used as a biomarker relating to muscle protein turnover. Also, an immunoassay using antibodies against the collagen type III propeptide before the interaction with N-terminal proteases

has been also proposed in assessing collagen III formation in liver fibrosis models [86].

Based on the progressive nature of fibrosis as the disease escalates (e.g. DMD, BMD), markers predicting fibrosis propensity by measuring the continuous synthesis/degradation processes of collagen in other tissues can be evaluated on their potential against muscle diseases.

As it has been shown that intracellular proteins such as creatine kinase and myoglobin can leak into the extracellular compartment and thus work as biomarkers of muscle disruption, it seems likely that other proteins or protein fragments from the muscle can enter the circulation. An MMP-degraded fragment of titin has been shown to be detectable in serum from healthy young men [87]. This indicates that metabolic byproducts from intracellular protein degradation can enter the circulation. Generated peptides can be possibly used as biomarkers of the myofibrillar proteins turnover in the muscle and thus provide information about ongoing muscle catabolism or anabolism. Indeed, serum Troponin I has been shown to be increased in dystrophic patients [88]. These intracellular proteins, particularly from the myofibrlliar fraction as they display the greatest degree of tissue specificity, show promise as biomarkers of muscle turnover.

POTENTIAL FOR NEOEPITOPE BIOMARKERS IN EXISTING AND EMERGING THERAPIES

As previously explained, myopathologies are either congenital or occult/idiopathic and in general there are currently no FDA-approved disease-modifying therapies. In most cases, treatment is symptomatic and aims at maintaining the self ambulatory ability for patients [60, 62]. Current scientific efforts are being directed towards several different treatment modalities, with the dominant approaches being based on anabolic/anticatabolic, anti-fibrotics or anti-inflammatory therapies, underlining the need for biomarkers monitoring these individual aspects of the diseases. The current pipeline for drugs targeting few of the most common causes of muscle wasting can be seen in Fig. 1.

While routine clinical biochemistry biomarkers provide measures of inflammation, e.g. IL-6, TNF-α or C-reactive protein, they are less well-suited for characterization of muscle fibrosis and catabolism. Since existing biomarkers have proven inadequate to detect subtle changes in the pathology, assessing treatment effects becomes challenging. A panel of varying peptide biomarkers of both formation and degradation could be of value in characterizing the fibrotic aspects of inflammatory myopathies and dystrophies and thereby contribute to the clinical characterization of subjects. Neoepitope biomarkers have demonstrated potential in describing these processes.

Antiinflammatory

In myositis, inflammation is part of the cause of the pathology, whereas in congenital dystrophies, it is a consequence of the chronic muscle disruption. In both cases, this contributes to symptoms and declined functionality, making it a relevant pathological mechanism to treat. Glucocorticoids have been shown to slow loss of locomotor function in DMD or even provide temporary improvements and is recommended as first-line treatment in both myositis and dystrophies [89–92] Biological anti-inflammatory drugs like inhibitors of TNF-alpha, IL-2, IL-6 or type I interferon have already shown promise in this regard in both inflammatory myopathies and dystrophies [93–97].

Likewise, in inflammatory myopathies, disproportionate activation of inflammatory pathways and increased proteolytic rates have been shown to be associated with overexpression of MMPs, localized in the muscle fibers [68, 98]. Muscle and tendon are weightbearing connective tissues with considerable expression and turnover of collagens, and inflammation in muscle pathologies is associated with increases in MMP activity. Thus, proteolytic fragments generated by proteases induced by inflammation-driven proteases, such as MMPs, appear to be good biomarker candidates. It has to be noted that some common medical interventions for those diseases (e.g. corticosteroids) could create a masking effect when trying to use collagen biomarkers some compounds induce cartilage resorption, tendon degradation or modify protein expression [99, 100]. Although it would create an extra burden in detecting significant differences in patient's biomarker levels it could still be viable as an indicator within a larger biomarker panel.

Antifibrotic

Fibrosis is by definition the consequence of abnormal ECM turnover, resulting in excessive deposition

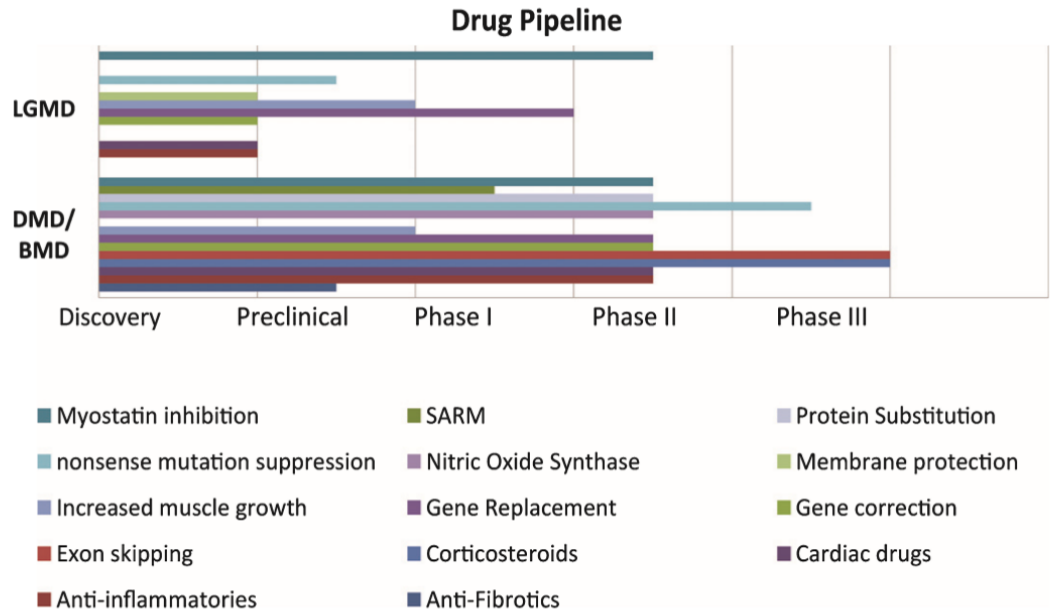

Fig. 1. Ongoing clinical trials for DMD/BMD and LGMD diseases. The current stage at the time of writing for the most advanced in each category is illustrated. Indicatively "Exon skipping" drugs is a promising class, in which Drisapersen/PRO051 (Prosensa), Eteplirsen/AVI-4658 (Sarepta) are currently in phase III [65] Ataluren/PTC-124 has received marketing approval in Europe (but not in US) under the name Translarna mda.gov.

of ECM proteins, especially collagens I, III and VI [101]. It has to be noted that the distribution of collagen isoforms in the various layers of the extracellular matrix of skeletal muscle tissues and the arrangement of the connective tissue associated with contractile fibers is extremely complex and differentially affected in muscle diseases [102]. Nevertheless, fragments of these constituents or their corresponding propeptides detected in circulation have been shown to reflect the extent of liver fibrosis and ECM remodeling in rats [84, 86, 103] and even demonstrate response to antifibrotic treatment [104, 105].

This has also been demonstrated as the case for myopathies and dystrophies, where endo- and perimysial fibrosis in the form of accumulated coallagen I, III, IV and fibronectin predominates [106, 107]. In DMD, endomysial fibrosis has been one of the strongest predictors of motor deterioration [31]. Therefore, early identification of loss in muscle strength or mass can be achieved by following closely the extent of fibrosis in the muscle tissue. Inhibiting the fibrotic processes may contribute to improved muscular function and this is the purpose of several current and previous pharmacological trials.

Myostatin inhibitors and several putative antifibrotic treatment routes are being looked into. Previous preclinical and *in vitro* studies have documented that myostatin inhibitors downregulates markers of fibrosis, but this has not been shown conclusively in clinical models yet [108, 109].

The most advanced in terms of clinical trials is HT-100, a delayed-release Halofuginone preparation. It exerts its effect through inhibition of fibrosis and inflammation, in part through blocking of TGF-beta signaling [110], while it also directly inhibits collagen type I synthesis. At the time of writing it has initiated Phase II trials (by Akashi, NCT02525302) for DMD treatment.

Assays directed at monitoring changes in the aforementioned pathologic manifestations have been successful in providing information for developing therapies during antifibrotic clinical trials. Biomarker characterization in a Halo/Akashi trial of Halofuginone trial helped reveal fibrosis collagen turnover, incidentally through use of neoepitope collagen peptide fibrosis biomarkers, i.e. PINP and C3M [110] and can be applied in future clinical cohorts.

Anabolic

As loss of muscle tissue and associated muscle function is a trait of most myopathies, therapeutic approaches aimed at restoring functional muscle mass are under examination, with particular focus on pharmacological interventions. While the efficacy of

exercise is questionable, anabolic drugs have shown some promise, although none of these have reached FDA approval yet.

Essentially, there are only two classes of drugs that display consistent myoanabolic properties and those are anabolic androgenic s and inhibitors of the endogenous cytokine myostatin. Inhibition of myostatin (also known as GDF-8) has been shown to result in muscle hypertrophy and myostatin inhibitor drugs are at the time of writing in clinical trials for myositis (Novartis' bimagrumab in phase III, NCT01423110) and Duchenne (PF-06252616 for Pfizer, phase II, NCT02310763).

Several soluble biomarkers have already been shown to follow the degradation and formation processes in muscle remodeling [86]. Loss of muscle strength and reconstitution of muscle mass was described during an immobilization/remobilization study by measuring collagen VI turnover [83]. It has also been shown that circulating collagen III fragments measured in human plasma can define formation of muscle mass [86]. However, there is a scarcity of biomarkers of change in muscle mass, with stable isotope-based measurement of protein synthesis (and/or degradation) being the currently only viable method.

As discussed previously, myofibrillar proteins and protein fragments, such as the previously described MMP-cleaved Titin fragment, can enter the circulation and may show promise as biomarkers of muscle protein turnover and by extension, catabolism or anabolism. However, these peptide biomarkers have yet to show promise in terms of being biomarkers of anabolism, or catabolism and by extension anabolic treatment response.

FUTURE BIOMARKER DEVELOPMENT

While serological biomarkers of muscle mass would be of utility in characterization of myopathic or dystrophic patients in clinical care, biomarkers indicative of myoanablism or catabolism would be of great benefit in identifying at-risk populations in many different clinical scenarios and treatment efficacy in anabolic drug trials. Discovering peptide biomarkers of myoanabolism should be a priority to the biomarker research niche.

Suggested parent proteins for peptide fragment biomarkers could be proteins from the Dystrophin-associated protein complex (DAPC), endo- or perimysial ECM, the sarcolemma or the muscle contractile apparatus itself, i.e. sarcomeric proteins (Fig. 2).

If peptides exist that have multiple pathology-specific PTMs, this allows for higher specificity of the individual biomarker and possibly for further stratification capabilities. Therefore, identification of peptides from relevant parent proteins with secondary pathology-specific PTMs is important as this can give rise to more specific biomarkers. Relevant PTMs in this context could be increased oxidative nitrosylation or carbonylation, citrullination, crosslinking or cleavage mediated by proteases upregulated as part of a disease's pathology, e.g. over expression of proteases. As these PTMs are related to the pathological *process*, (e.g. defective ECM remodeling), peptides containing them may translate into biomarkers that respond earlier to pathology changes in contrast to biomarkers that are related to the outcome of the process (e.g. imaging techniques or histopathology in the case of fibrosis). Indeed, this has been shown to work for other connective tissue pathologies like osteoporosis, rheumatoid arthritis, osteoarthritis and several types of organ fibrosis [111]. Hence, due to the inherent qualities of neoepitopes, there is potential in using them as prognostic biomarkers as well as in monitoring disease progression.

CONCLUSION

Dystrophies and inflammatory myopathies are serious diseases with limited treatment options. Despite the differences in the pathologies of the diseases, a common thread can be identified through muscle fiber degeneration, inflammation and/or fibrosis. Several routes for developing pharmacological interventions against one or all of these traits are being explored.

Neoepitope peptide biomarkers hold great potential in this respect as, they have been successful in characterizing localized pathological protein turnover, fibrosis and inflammation in a range of other diseases. With both dystrophies and myositis, more muscle fibres are being degraded and built up at any one time point, than in the healthy condition. This situation is dissimilar to the normal steady state turnover of muscle proteins and we hypothesize that this difference produces characteristic peptides derived from the muscle proteins that are measures of diseases activity.

Treatments are mainly targeted on dealing with the inflammation or fibrosis either directly

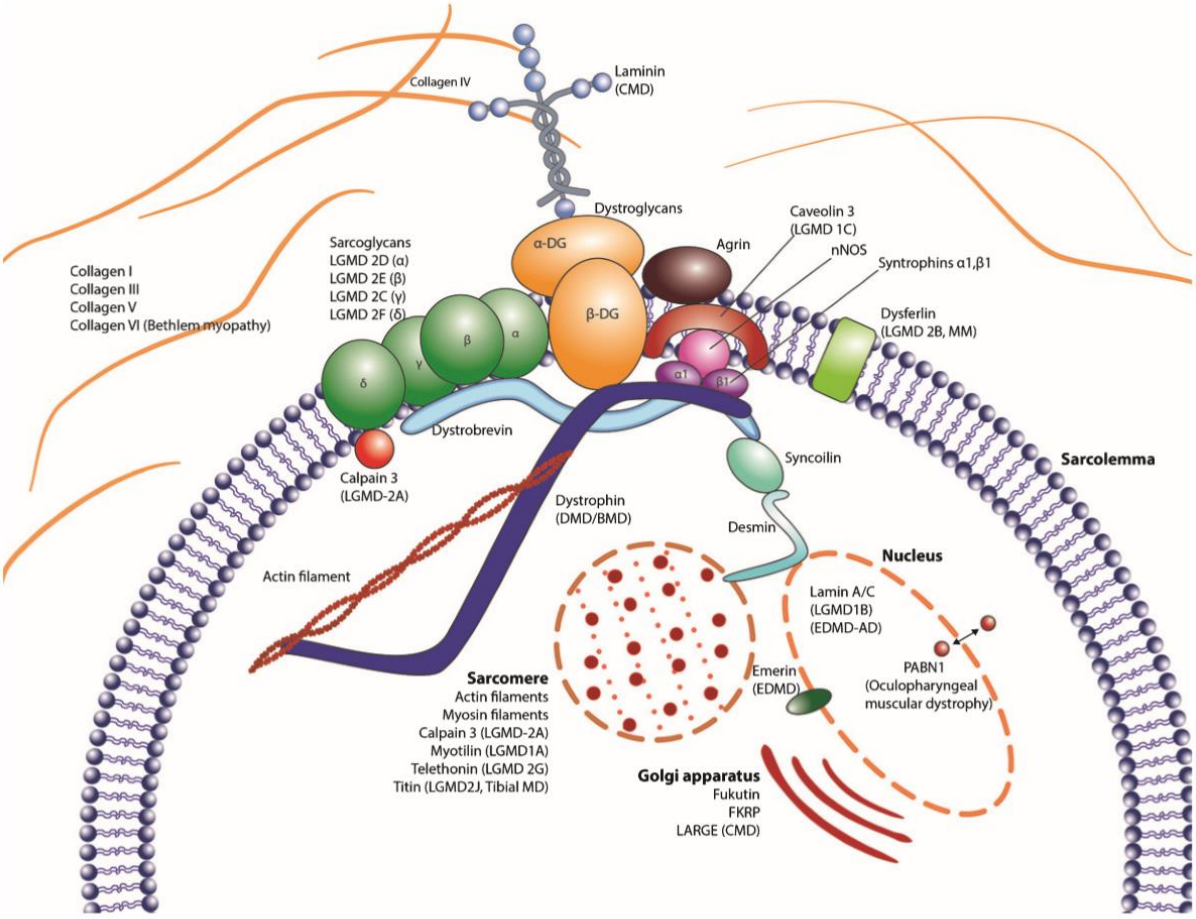

Fig. 2. Anatomy of the muscle structure: The extracellular matrix, the sarcolemma and the intracellular domains are illustrated, depicting the relations between the different constituents. Proteins that are affected by mutations or deletions have the respective disease indicated in parentheses.

(anti-inflammatory, anti-fibrotic) or indirectly (exon skipping). Suggested biomarkers (C1M, PINP etc.) have shown capability in describing and monitoring the extent of these clinical features. We believe that by using neoepitope biomarkers, improved characterization of these traits could strengthen or speed up ongoing clinical trial efforts as they can contribute to disease progression monitoring, treatment efficacy and stratification/selection for participating patients. Assessment of a pharmaceutical entity's potential will be much easier for both the industry as well as the medical personnel, leading to higher quality submissions to the regulatory agencies.

CONFLICT OF INTERESTS

The authors are involved in protein based biomarker research but are otherwise impartial to the technologies and approaches discussed.

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4. PROTEIN FINGERPRINT BIOMARKERS OF THE EXTRACELLULAR MATRIX

Manuscript II

Collagen turnover biomarkers assist in differential diagnosis of different forms of myositis

Aim

Inflammatory myopathies, or myositis, cause pain and loss of musculoskeletal mass and function. Inflammation is associated with dysregulation of extracellular matrix (ECM) giving rise to neo-epitope peptide biomarkers that have shown promise as diagnostic or prognostic tools in several other inflammatory and degenerative conditions. The purpose of the current study was in a prospective manner to elucidate to what extent ECM turnover markers correlated with diagnosis and physical function in patients with dermatomyositis (DM) and polymyositis

Introduction to the cohort

In a cohort of 51 myositis patients (28 DM, 23 PM), we measured a range of neo-epitope biomarkers derived from cleavage of collagens I (C1M and PINP), III (C3M and PRO-C3), VI (C6M) and C-reactive protein (CRPM). We then related these to PM and DM diagnosis. Secondly, we performed Spearman's correlation analyses of the Interferon gene score in both muscle and blood, as well as Manual muscle testing (MMT8) score to the assessed biomarkers.

Main findings

The levels of the biomarkers CRPM and PINP were downregulated in DM and PM patients relative to healthy controls, whereas PRO-C3 was found to be increased in DM. In DM only, we found that C1M and C3M were upregulated relative to controls.
Secondly, biomarker panels correlated with Interferon gene scores in blood for PM and DM; however, the correlations were seen for different markers in the two diagnostic groups. Thirdly, we found that CRPM correlated well with the MMT8 score for DM.

Conclusions

Both DM and PM significantly affect several of the biomarkers measured in this study, most prominently CRPM and PINP, indicative of significantly altered turnover of extracellular matrix components and CRPM. C3M correlated with Interferon gene score, in PM and DM, and CRPM with MMT8 score in DM. The biomarker panels could be of use in myositis diagnosis, functional patient characterization and monitoring of treatment efficacy.

Collagen turnover biomarkers assist in differential diagnosis of different forms of myositis

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Abstract

Background
Inflammatory myopathies, or myositises, are serious inflammatory conditions affecting muscle, causing pain and loss of musculoskeletal mass and function. Inflammation is associated with dysregulation of extracellular matrix (ECM) turnover giving rise to neo-epitope peptide biomarkers that have shown promise as diagnostic or prognostic tools in several other inflammatory and degenerative conditions. Thus, the purpose of the current study was to elucidate to what extent ECM turnover markers correlated with diagnosis and physical function in patients with dermatomyositis (DM) and polymyositis (PM).
Materials and Methods
In a cohort of 51 myositis patients (28 DM, 23 PM), we measured a range of neo-epitope biomarkers derived from cleavage of collagens I (C1M and PINP) and III (C3M and PRO-C3), C-reactive protein-M (CRPM) and C6M. We then related these to PM and DM diagnosis, as well as levels in matched healthy controls. Secondly, we performed Spearman's correlation analyses of the Interferon gene score in both muscle and blood, as well as Manual muscle testing (MMT8) score to the assessed biomarkers.
Results
We found that the levels of the biomarkers CRPM and PINP were reduced in DM and PM patients relative to healthy controls, whereas PRO-C3 was found to be increased in DM. In DM only, we found that C1M and C3M were increased relative to controls.
Secondly, we found that C3M levels correlated with Interferon gene scores in blood for PM and DM; however, the correlations were seen for different markers in the two diagnostic groups. Thirdly, we found that CRPM correlated well with the MMT8 score for DM.
Discussion/Conclusion
We have found that both DM and PM significantly affects several of the biomarkers measured in this study, most prominently CRPM and PINP, indicative of significantly altered turnover of extracellular matrix components and CRPM. Furthermore, we found that C3M correlated with Interferon gene score, in PM and DM, and CRPM with MMT8 score in DM. Thus, a combination of neo-epitope biomarkers could be of use in myositis diagnosis, functional patient characterization and monitoring of treatment efficacy.

Keywords
Myositis, biomarker, Collagen, Dermatomyositis, Polymyositis, Function

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Background

Myositises are for the major part comprised by the idiopathic inflammatory myopathies: polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM). The prevalence of inflammatory myopathies have been reported ranging from 10 to 100 per million, making them a fairly rare group of diseases. Their prevalence increases with age and is higher in women than in men as is the case with most inflammatory conditions[1,2].

Diagnosis is normally made through the presence of muscle weakness, elevated Creatine Kinase (CK), abnormal EMG finding and abnormal histological findings in tissue samples. If there are dermatological symptoms, then the diagnosis is usually DM. From a clinical perspective, PM and DM are often grouped together as they share several pathological traits and both respond to anti-inflammatory treatment. While IBM may appear similar to PM, it is unresponsive to normal anti-inflammatory treatment, making the unresponsiveness to steroid treatment one of the main factors separating it from PM. For all the myositis manifestations, autoimmunity plays an important part in the pathology [1,2].

Treatments of myositises are symptomatic and when considering anti-inflammatory biologicals, treatment choices of myositisis are limited and new drug development is needed. However, biomarkers of disease activity are not well developed and easily assayed biomarkers monitoring disease aspects, such as inflammation, muscle integrity and response to treatment, would benefit both patients and clinical development.

Chronic inflammation in all tissues is associated with dysregulation of turnover of extracellular matrix components, most prominently in the form of upregulation of expression and activity of proteases and sometimes parallel increases in expression of extracellular matrix constituents. The increase in proteolytic activity gives rise to peptide fragments that can be measured in serum. This has been shown particularly with MMP-cleaved fragments of collagens I, II, III, IV and VI in a range of pathologies, where local upregulation of MMPs is playing a part in the pathology [3–9].

In a similar way, during synthesis of the fibrillar type I and III collagens, propeptides are secreted as part of the maturation of the protein and can thus be used as a surrogate measure of synthesis of these proteins, thereby providing us with surrogate measures of both synthesis and degradation of extracellular matrix constituents [9–12].

In both PM and DM, increased expression and activity of Matrix Metallo-Proteinase (MMP) enzymes from the gelatinase (MMP-2 and MMP-9) and collagenase (MMP-1) families have been reported in muscle [13].

As 30-40 % of the body mass consists of muscle and collagens I, III, IV and VI are highly abundant in the epi,- peri- and endomysial fascia layers surrounding muscle fibres [14] [15]. The combined abundance of these collagens and increased activity of especially MMP-enzymes in muscle makes turnover markers of these proteins possible biomarkers of myositis conditions.

Lastly we decided to include a metabolite of C-reactive protein (CRP), in the screening panel. CRP has been shown to be upregulated in some myositis cases, particularly DM [16]. The CRPM peptide fragment produced from MMP-cleaved CRP has previously been shown to be associated with localized inflammation in both osteo- and rheumatoid arthritis, indicating that it is a locally produce metabolite of CRP[5,17,18]. Given the inflammatory nature of myositis, and the previously discussed involvement of MMP proteases, we decided to examine whether also this biomarker could be indicative of pathology in DM or PM myositis.

Thus, the purpose of the current study was, to elucidate if the biomarkers C1M, C3M, C6M, PINP, PRO-C3, and CRPM either by themselves or in combination would be related to myositis diagnosis, or functional status in the form of IFN gene score and MMT8 score in a cohort of PM and DM subjects.

Materials and Methods

Subjects

51 myositis patients (23 with PM, and 28 with DM, diagnosed according to the Bohan and Peter criteria) and 30 healthy age- and gender matched controls were included in a clinical trial described previously (NCT00533091) [19,20]. Subjects had blood drawn, clinical characterization including manual muscle testing (MMT8) and muscle biopsy specimens taken. The results and data points used in this paper are derived exclusively from the baseline measurements, and the demographics have previously be published [19,20].

Interferon gene signature

The IFNGS score was calculated using 13 type I IFN-inducible genes (IFI27, RSAD2, IFI44L, IFI44, OAS1, IFIT1, ISG15, OAS3, HERC5, MX1, ESPTI1, IFIT3, and IFI6) and reported as a median fold change relative to a pool of normal control samples, as described previously [21]. The effects on IFN score in this trial have been reported before [19–21].

Muscle testing

Muscle testing was done by manual assessment of strength in a subset of 8 muscles, i.e. neck flexors, deltoids, biceps, wrist extensors, gluteus maximus and medius, quadriceps and ankle dorsiflexors, as previously specified in the MMT8 muscle inventory [22].

Neoepitope biomarkers

Biomarker measurements of serum peptide biomarkers C1M, C3M, C6M, P1NP, PRO-C3, and CRPM were performed in serum samples as described previously[3,8,23–26]. All biomarkers were measured in duplicates and when the CV in a duplicate measurement was above 15%, the sample was re-measured.

Statistics

When we tested for differences in biomarker levels between healthy subjects, DM and PM patients, we did a one-way ANOVA across groups with post hoc testing using Tukey's correction for multiple testing (done in Prism 6 for Mac OS X).

To assess correlations between IFN scores, MMT8 and the biomarkers, Spearman's ranked correlation analysis was performed with no adjustment for multiple comparisons.

Results

Biomarkers in relation to diagnosis

The baseline demography has been published previously, and showed that the cohort was balanced from and age and gender point-of-view [21].

For the type I collagen synthesis biomarker PINP, a statistically significant lowering was observed in dermatomyositis and polymyositis, and there appeared to be no difference in the levels between the two groups (Figure 1A). For the biomarker of MMP-mediated degradation of type I collagen, we observed a different pattern, with the DM group being significantly higher than the healthy group as well as the PM group, which was not different from the controls.

For synthesis of type 3 collagen, we measured Pro-C3, and as seen in figure 1C, Pro-C3 levels were elevated in the DM group, when compared to the healthy controls. In the PM group the Pro-C3 levels appeared to be somewhat elevated compared to the controls, but this did not reach statistical significance. For the type 3 collagen degradation marker C3M, the pattern was very similar, with significantly high levels in the DM group compared to healthy controls, and a trend towards an increase in the PM group compared to controls.

For CRPM (CRP degraded by MMPs), we found that the levels were significantly lower in the two myositis groups than in the healthy controls. Finally, for type 6 collagen degradation (C6M), we observed no differences between the groups.

Biomarkers in relation to IFN signatures and MMT8 score.

To assess the relationship between the collagen turnover biomarkers and the IFN signature in the blood and muscle, we assessed Spearman's ranked correlations between the biomarkers and the two IFN signatures as well as MMT8 scores at baseline (Table 1).

For DM we found a significant positive correlation between the IFN gene signature assessed in blood and C3M, as well as a significant positive correlation between MMT8 and CRPM levels. In DM, no other significant correlations were observed.

In PM, significant positive correlations between CRPM levels and IFN blood, as well as C6M levels and IFN in blood were noted, while no other significant correlations were observed.

No correlations between biomarkers and IFN scores in muscle were observed, despite a significant correlation between the two IFN scores (Spearman's r of 0.7, $p<0.001$).

Table 1: Spearman's r correlation coefficients for the biomarkers, the IFN scores and the muscle output.

		C1M	PINP	C3M	Pro-C3	CRPM	C6M
DM	IFN Blood	0,002	0,33	0,40*	-0,02	0,32	-0,18
	IFN Muscle	-0,18	0,10	0,14	0,15	-0,03	-0,27
	MMT8	-0,01	-0,17	0,38	-0,03	0,46*	0,21
PM	IFN Blood	0,24	0,11	0,40	0,25	0,45*	0,42*
	IFN Muscle	0,05	0,04	0,16	0,22	0,12	0,13
	MMT8	-0,20	0,01	-0,36	-0,34	-0,37	-0,38

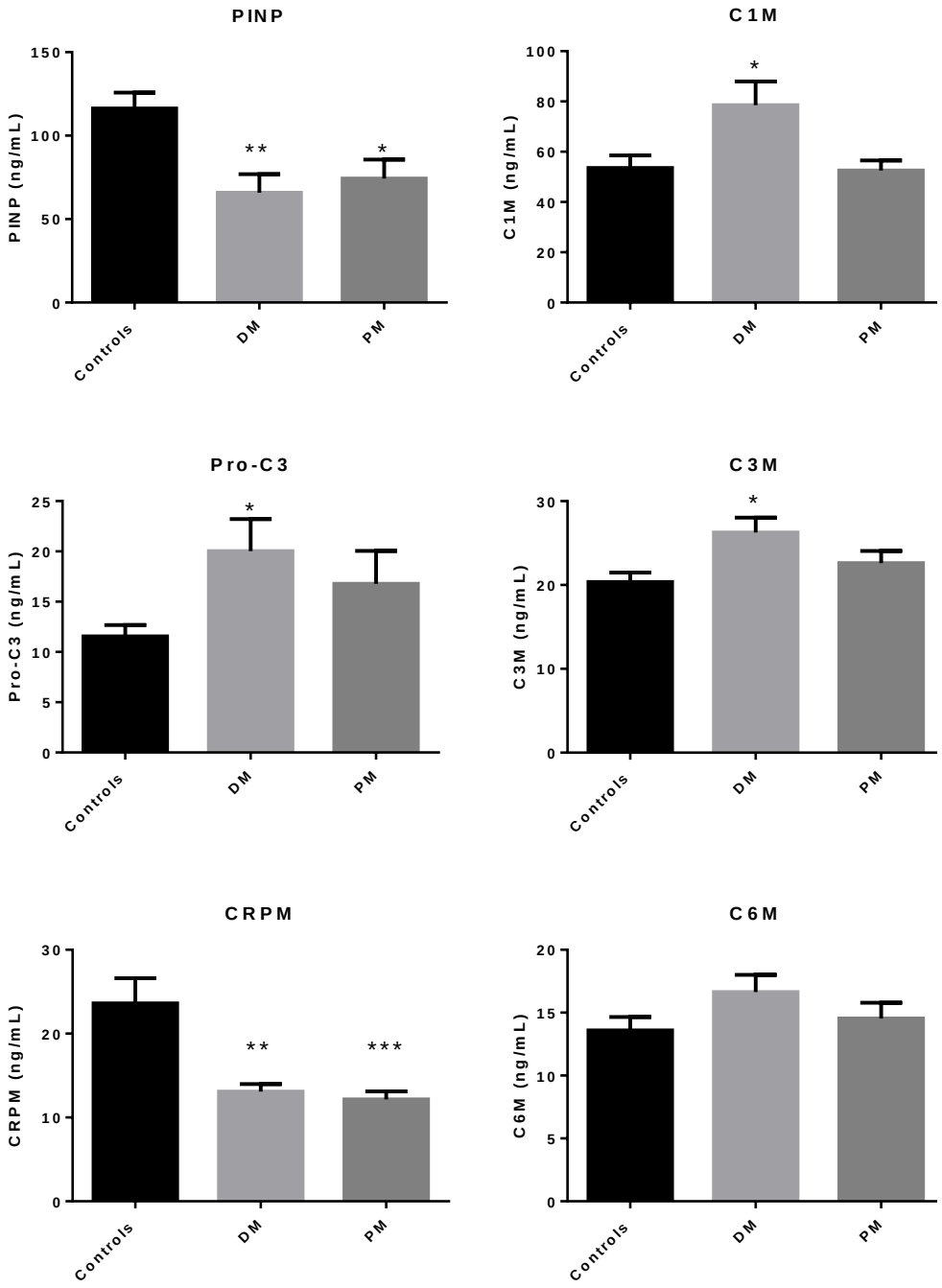

Figure 1: Biomarker levels in the three diagnostic groups.

Discussion

Biomarkers across PM and DM.

We here present data indicating that differential diagnosis of PM and DM can be possibly facilitated using collagen turnover biomarkers. Furthermore, in this small study we were able to identify relationships between the biomarkers and both inflammatory indicators, such as IFN score in blood, as well and the functional score MMT8. Data which clearly indicate that the collagen turnover biomarkers are of relevance when developing therapies for the different forms of myositis.

We found that either type of myositis was associated with substantial decreases in CRPM and PINP biomarkers relative to controls, whereas DM alone was associated with increases in PRO-C3, C1M and C3M.

CRPM has previously been described as a biomarker of local inflammation, which was elevated in conditions, such as rheumatoid arthritis and in subgroups of osteoarthritis patients [17,18]. We were intrigued to find that CRPM was strongly reduced in both DM and PM, relative to healthy subjects. This contrasts with the previous findings, as myositis is characterized by increased inflammatory status as well as increased MMP activity. One possible explanation could be a difference in MMPs involved in myositis and arthritis, and hence a different processing of CRP locally in the tissues. Alternatively, it could relate to the progression status of the disease; however, this remains to be elucidated.

Our reported reduction of PINP matches with a previous myositis study where PINP was found to be reduced, while PICP, the carboxyterminal fragment of collagen type I was upregulated [27].

In the literature, both of these biomarkers are associated with synthesis and maturation of collagen I, so these findings might be reflecting more on the disease activity which leads to the abnormal turnover of type I collagen. In fact, immunohistochemical studies in polymyositis patients have shown increases in the amount of collagenous tissue, e.g. fibrosis [28]. This could possibly be a basis for our findings, as fibrosis is associated with not just increased amounts of collagenous tissue, but also qualitative changes in turnover [3] [8].

For PINP, the observed downregulation in both types of myositis was similarly hard to understand. Our reported downregulation of PINP matches with a previous myositis study where PINP was found to be downregulated, while PICP, the carboxyterminal fragment of collagen type I was upregulated[27]. In the literature, both of these biomarkers are associated with synthesis and maturation of collagen I, so these incongruent findings must reflect specific turnover anomalies in myositis that have not yet been characterized. Thus, normally we would interpret a decrease in PINP as a marker of reduction in collagen I synthesis, but given the findings from the Kubo study, this warrants more consideration. Immunohistochemical studies in polymyositis patients have shown increases in the amount of collagenous tissue, e.g. fibrosis[28]. This does not necessarily contradict our findings, as fibrosis is associated with not just increased amounts of collagenous tissue, but also qualitative changes in turnover [3,8].

For the collagen type 3 turnover biomarkers (C3M, ProC3), we found increased levels in the DM group, indicating that type 3 collagen turnover is upregulated in DM. Furthermore, a trend towards increased levels was observed in the PM group. Interestingly, a case-study conducted using another assay measuring the total pro-peptide of collagen type 3 (not exclusively the matured collagen III as Pro-C3) also found elevated levels in myositis compared to control values, supporting the findings presented here [29].

Neither C1M nor C6M has been measured previously in myositis. As for C1M we think that the increase of C1M and C3M in DM, but not in PM, could possibly be explained by the involvement of skin in DM. This would imply that the C1M and C3M peptides found in DM but not in PM are actually produced in the skin; however, this will obviously need to be confirmed in other studies. As for C6M, no significant changes manifested between groups and none were expected.

While these data are obviously generated in a cohort of somewhat limited size, it is very encouraging that the collagen turnover markers, especially the collagen type I and collagen type 3 markers, are able to aid the separation of DM from PM, something that has not been before. This indicates that implementation of these biomarkers in which could potentially help in development optimized treatments for the various forms of myositis in the future.

Neoepitope biomarkers vs. IFN gene signature

A range of biomarkers, wet and dry, have been proposed in relation to myositis. As the IIMs are inflammatory by definition and PM and DM are responsive to anti-inflammatory treatments, one of the wet biomarkers that have been used extensively in recent years is the interferon gene signature. This has been shown to correlate (R^2 of 0.3-0.4) with global and muscle-specific soreness as well as several other myositis biomarkers[30,31].

This formed the basis for our desire to compare the neoepitope biomarker panels with Interferon gene signatures in both blood and muscle.

Interestingly, the biomarker C3M showed a significant correlation (Spearman's $r=0.4$, $p<0.05$) with IFN score in blood in DM, and a borderline significant correlation in PM (Spearman's $r=0.4$, $p=0.056$), indicating that C3M which is considered to be a marker of systemic inflammation is related to the IFN score in blood. Interestingly, in the PM group CRPM and C6M were also correlated to the IFN score in blood, while this was not observed for DM. In fact, if anything the C6M marker, albeit not reaching statistical significance, was inversely correlated to IFN score in blood in DM, again supporting that the neo-epitope biomarkers provide differential output in the two myositis groups.

For the IFN score in muscle, we observed no correlations to any of the serum biomarkers.

Neoepitope biomarkers correlate with MMT8 score

Next, we wanted to find out if the biomarkers we measured had any relationship with the functional status of the subjects in the form of the MMT8 measurement obtained in this study.

For DM, the biomarker CRPM was positively correlated to MMT8, which agrees well with the fact that myositis appears to be associated with decreases in CRPM. Interestingly, and as seen for the correlations to IFN blood scores, the trends towards correlations observed in the PM group were inverse of those observed in the DM group.

These data indicate that, if validated in larger cohorts, the neoepitope biomarkers could provide important information in relation to clinical trials.

Limitations

There are several limitations to our findings. Ideally, our biomarker panels should have been discovered in one cohort and validated in another, but the size of this trial was inadequate for that. We are intent on validating both the full and reduced biomarker panels in other myositis trials.

Furthermore, we did not have MMT8 scores in the age- and gender matched controls. This would have been immensely useful to have to demonstrate that the biomarker relationships observed in myositis patients are related specifically to their pathologies and not a generic trait of the biomarkers. Additionally, no reliability measurement of the MMT8 raters was done in this study, and test-retest reference data for the MMT8 inventory are scarce in the literature, but a previous study with MMT hints at a test-retest reliability of 67-100 % in the hands of experienced raters across the various items, but an integrated reliability is not provided in this study [32]. However, these data indicate that an overall reliability of 80-90% should be expected, which agrees well with the reported reliabilities of similar manual measures [33].

Regarding the MMT8 score, many of the other papers in the literature about myositis biomarkers have correlated various wet biomarkers with pain scores in the form of muscle VAS [31,34]. In this study, we did not have access to muscle VAS scores. However, the muscle VAS score is also a subjective score with reliability in the same range as the MMT8 score. It must be considered that MMT8 integrates both a degree of pain measure, as pain compromises movement, and structural damage to or loss of muscle tissue.

Finally, we did not take into account the potential effect of the glucocorticoid and/or baseline methotrexate therapy, and although a previous study showed that the IFN score was unaffected by this [20], it is of interest for future assessments of inflammatory biomarkers in myositis.

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Finally, we did not take into account the potential effect of the glucocorticoid and/or baseline methotrexate therapy, and although a previous study showed that the IFN score was unaffected by this [20], it is of interest for future assessments of inflammatory biomarkers in myositis.

Conclusion

Thus, we can confirm that our biomarkers can help differentiate between DM and PM, an important feature, as it is attractive to treat the two forms of myositis with different regimens.

We also found that neo-epitope biomarkers correlated with IFN gene score in blood for both PM and DM, and we found an indication of differential correlations to muscle function measured using the MMT8 inventory.

Together, these findings suggest that biomarker panels consisting of ELISAs against Collagen type I and III fragments and MMP-degraded CRP are promising biomarkers in myositis in terms of diagnosis, functional characterization and likely monitoring of treatment efficacy.

Author contributions:

Xiang Guo2, Athanasios Arvanitidis1 and Brandon W. Higgs (developed plan and key in write up), Christopher Morehouse (analyzed data and initial interpretation), Philip Z. Brohawn (generated data in lab)
Morten A. Karsdal1, Kim Henriksen1, Wendy White2 (Participated in discussions and aided in the writing)

All authors have read and approve this manuscript

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5. BIOMARKER DEVELOPMENT, TARGETING INTRACELLULAR CONTRACTILE PROTEINS

Title:

Serum C-terminal slow skeletal troponin T as a possible biomarker of muscle wasting and rehabilitation

Introduction to the cohorts:

DAHANCA: 41 HNSCC patients that have been through radiotherapy with or without chemotherapy as well as a weight-, gender- and age-matched control group of 21 healthy individuals. The patients were subjected to a training regime of their choice for 12 weeks and then to progressive resistance training for 12 weeks more. Measurements of lean body mass (LBM) and blood collection took place at baseline right after progressive resistance treatment (PRT) (T0) right after the first part of training (T12) and after completion of the training period (T24). Serum samples were collected before, during and after the training period.

BBR: 20 healthy young men underwent 8 weeks of strict bed rest. They were randomized into two groups, i.e. resistive vibration exercise group (RVE) and control (CTRL) groups. The RVE group was assigned to resistive vibration exercise 11 times per week. No exercise was performed in CTRL group. After 8 weeks of bed rest, subjects were re-ambulated and observed in the hospital for another 5 days. Serum samples were collected 2 days before bed rest (BDC-2), in the bed rest procedure (BR+) and in recovery period (R+).

Main findings

DAHANCA: TNNT1 levels were significantly elevated in the patient group compared to the control group, even before engaging in any form of physical activity. After engaging in physical training, the biomarker levels further increased through time, reaching a significant difference both compared to the patients baseline (T24vsT0, $p<0.05$) as well as to the control group (T1 and T24 vs control, $p<0.0001$).

BBR: There was a significant difference between the two groups in the bed rest stage that demonstrates a distinct response to the RVE counter measure. The increased levels of circulating TNNT1 for the RVE group in this study could be explained by the unloading of troponin from the muscle. During the remobilization stage, the TNNT1 levels were increased significantly in both groups in a very similar manner, compared to the baseline as well as the levels during the bed rest period. In day 28 of recovery were the maximum levels of TNNT1 observed and by the time of training completion, the levels were almost returned back to baseline.

Conclusions

We have developed a robust and specific biomarker targeted at the C-terminus of Troponin T1. The biomarker demonstrated differences across the two studies. Part of the biomarker response could reflect a process in training. In the immobilization study, findings were in accordance with previous research

Title

Serum C-terminal slow skeletal troponin T as a potential biomarker of muscle wasting and rehabilitation

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Abbreviations

ATP	adenosine triphosphate
BBR	Berlin bed rest study
BSA	bovine serum albumin
CK	creatine kinase
DAHANCA	Danish Head and Neck Cancer Group
DEXA	Dual-energy X-ray absorptiometry
ECM	extracellular matrix
EDL	extensor digitorum longus muscle
ELISA	enzyme-linked immunosorbent assay
HNSCC	head and neck squamous cell carcinoma
HRP	Horse radish peroxidase
LBM	lean body mass
MMP	Matrix metalloproteinases
MRI	magnetic resonance imaging
PBS	Phosphate-buffered saline
RVE	resistance vibration exercise
SEM	standard error of the mean
SOL	soleus
TMB	tetramethylbenzinidine
TNNT1	troponin T1 c-terminal biomarker
TnT	Troponin T

Abstract

A competitive enzyme-linked immunosorbent assay (ELISA) directed at the C-terminus of fast skeletal troponin T was developed (TNNT1). The biomarker was technically and biologically validated and further evaluated in two muscle wasting and rehabilitation models. Extraction of tissues confirmed expression solely in skeletal muscle. The clinical cohorts comprised cancer patients exposed to resistance training after radiotherapy and as well as subjects from a longitudinal bed rest study, with and without countermeasure followed by rehabilitation. In the cancer study, the TNNT1 was found to be significantly elevated compared to both the control group and the patient's baseline levels. Additionally, there was a distinct pattern of serum levels of TNNT1 between patients with and without countermeasure under bed rest, followed by a distinct increase in serum during the remobilization period. This biomarker could hold the potential to demonstrate changes in the skeletal muscle under wasting conditions or training interventions.

Keywords: biomarker, ELISA, troponin T, skeletal muscle, bed rest, cachexia, muscle wasting, TNNT1

Background

Skeletal muscle loss happens naturally during aging but under circumstances as cancer and radiotherapy it can be significantly accelerated [1,2]. Muscle loss can also occur in the younger population as a primary component of a musculoskeletal disorder[3] or as a secondary outcome of another disease, inactivity, injury or medication[4]. The changes in skeletal muscle caused by these pathologies or the counter effects from therapeutic interventions against them have been challenging to monitor. The methods regularly used to assess or diagnose changes after muscle damage include biopsies or isotope labeling[5], Magnetic Resonance Imaging (MRI) or Dual-energy X-ray absorptiometry (DEXA) scans, all of which have significant limitations [6–9].

Serum biomarkers are widely applied as they are an easier and much cheaper approach in monitoring tissue-specific alterations. Serum markers of skeletal muscle changes, creatine kinase (CK), creatinine myoglobin and lactate dehydrogenase have been broadly used, but they lack in specificity regarding the tissue of origin and there are doubts about their diagnostic significance[10,11].

No matter the trigger, muscle loss is a consequence of an imbalance between protein synthesis and degradation, accompanied by loss of structural integrity in the sarcolemma and changes in the extracellular matrix (ECM) [12,13]. Quantitative changes in muscle protein metabolism, in combination with increased permeability of the sarcolemma can result in release of protein fragments into the circulation. The protein fragments generated due to the underlying pathology form a unique proteomic profile. This new distinct proteomic profile in serum is of interest, with respect to identifying biomarkers that can be used to monitor changes in muscle mass and integrity with better precision**.** Moreover, muscle pathologies can demonstrate preference in fast or slow twitch fiber types as It has been shown that certain diseases demonstrate a favoritism for specific fiber types [14,15]**.** Such information is lost when using markers that lack any fiber type-specificity. Additionally, besides muscle wasting, different patterns in either fiber type have been observed during muscle regeneration. Matrix metalloproteinases that facilitate muscle reparation by resolving the collagenous fibrotic tissue (mainly MMP-9 and MMP-2) demonstrate differential expression and activity in fast- and slow-twitch rich muscles [16].

In skeletal muscle, the troponin complex resides on the thin filament of the sarcomere and is composed by fast and slow fiber specific isoforms of TnI, TnC and TnT proteins, each serving different functionalities.

Overall the troponin complex together with tropomyosin bind directly to actin and regulate muscle contraction. Troponin as a biomarker, is mainly known in the form of the cardiac TnI isoform biomarker that has been in use for decades as the gold standard in monitoring myocardiac infarctions[17] Specifically, TnT serves as the anchor of the whole troponin complex by binding to tropomyosin and the other troponins through the C-terminus. Calcium-dependent muscle contraction is inhibited by TnT by suppressing myosin ATPase activity inactivating fiber contraction. Mutations in TnT can cause cardiomyopathies or nemaline myopathy which is a rare congenital muscle disease characterized by general weakness and hypotonia[18]. Complete absence of troponin T diminishes the Ca2+ dependent activation, while deletions in the last 14 or 28 aminoacids in the C-terminus lead to ATPase activity [19]. Irrespectively to presence of Ca^2 truncation of the C-terminus prohibits full muscle relaxation[20]. Exercise-induced damage has been shown to allow release of skeletal troponin I into the blood stream[21]. Presence of slow skeletal troponin T in serum can reflect training-induced changes in muscle and more specifically the troponin complex or preferential modification caused in slow-twitch fibers.

We have raised a monoclonal antibody using a decapeptide, which is unique to the sequence for the C-terminus of the slow isoform of skeletal troponin T (hereon referred to as TNNT1), that was used to develop an ELISA assay. The biomarker was technically validated and applied in two different cohorts. In the first study, a group of head and neck squamous cell carcinoma (HNSCC) patients after radiotherapy was admitted to a resistance training program alongside to a control group that followed the same training regime. In the second study healthy young men were exposed to 56 days of bed rest, split in a group with resistance vibration exercise as a countermeasure and a group with no countermeasure at all. After the bed rest period both groups entered the same rehabilitation process for a period of 128 days.
Overall, the aim was to develop a robust assay following the changes of the TNNT1 biomarker in serum samples. Furthermore, we wanted to determine if the biomarker could distinguish changes in the skeletal muscle brought on by injury, inactivity or intervention

Materials and Methods

Reagents

All compounds and reagents mentioned are high purity chemicals, provided by widely recognized manufacturers. Most chemicals are from Merck (Whitehouse Station, NJ) and Sigma Aldrich (St. Louis, MO). The synthesized peptides used for monoclonal antibody production were provided from the Chinese Peptide Company, Beijing, China.

Immunization procedure

Six 4–6 week-old Balb/C mice were immunized subcutaneously in the abdomen with 200 µL emulsified antigen (KLH-CGG-GKGRVGGRWK, 50 µg per immunization), using Freund's incomplete adjuvant. Immunizations were performed at two-week intervals until stable titer levels were obtained. At each bleeding, the serum titer was investigated and the mouse with the highest titer was selected for fusion. The selected mice were boosted intravenously with 50 µg immunogen in 100 µL 0.9% sodium chloride solution three days before isolation of the spleen for cell fusion.

Fusion and antibody screening

The fusion procedure has been described elsewhere[22]. Briefly, mouse spleen cells were fused with SP2/0 myeloma fusion partner cells. The hybridoma cells were raised in 96 well plates and incubated in 37C

incubator. After 7-10 days, supernatants were screened using an indirect ELISA, while the biotinylated peptide Biotin-GKGRVGGRWK was used as a catcher peptide on streptavidin-coated microtitre plates

Competitive ELISA description

When not stated otherwise, all the ELISA assays used are based on competitive binding inhibition of the antibody to the coater. In brief, the coater is a biotinylated form of the standard peptide that interacts with the streptavidin-coated plate wells. On the incubation step the antibody is inserted in solution with the standard peptide or the serum sample. The standard peptide or the analyte existing in the serum sample is competing with the coater against binding to the antibody. In the consequent washing step only the excess of antibody that managed to bind to the coater will remain while the antibodies bound to the free peptide will be washed away. Inhibition of the signal translates to the existence of higher concentration of the analyte in the original serum samples.

Characterization of clones

Clones were tested against the selection peptide (GKGRVGGRWK), a non-sense peptide, and an elongated peptide (GKGRVGGRWKA). The clones with the best selection peptide inhibition were chosen for subcloning. Clone selection was done at least 3 times by limited dilution, then the supernatant of monoclones were purified using Protein G columns according to manufacturer's instructions (GE Healthcare Life Science, Little Chalfont, and Buckinghamshire, UK). Isotyping of the monoclonal antibodies was performed using the Clonotyping System HRP kit, cat. no. 5300-05 (Southern Biotech, Birmingham, AL). Native reactivity and peptide binding of the monoclonal antibodies was evaluated by displacement of human serum, plasma and urine; rat serum and urine; and mouse serum, plasma and urine, in a preliminary competitive ELISA using 10 ng/mL biotinylated peptide coater on a streptavidin coated microtitre plate and the supernatant from the growing monoclonal hybridoma.

Assay protocol

A 96-well streptavidin plate was coated with biotinylated synthetic peptide Biotin-GKGRVGGRWK dissolved in assay buffer (50 mM PBS, 1% BSA, 0.1% Tween-20, 4g/L NaCl, pH 7.4 adjusted at 20°C) and incubated 30 min at 20°C. 20 µL of standard peptide or sample were added to appropriate wells, followed by 100 µL of monoclonal antibody and incubated overnight at 4°C. Then, 100 µL of Horse radish peroxidase (HRP) conjugated rabbit anti-mouse polyclonal antibody solution (Jackson immunoresearch, Baltimore USA, product code: 315-035-045) were added, and the plate was left to incubate at 20°C for 1h. Finally, 100 µL tetramethylbenzinidine (TMB) (Kem-En-Tec cat. no. 438OH) was added and the plate was incubated 15 min at 20°C in the dark. All the above incubation steps included shaking at 300 rpm. After each incubation step the plate was washed five times in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). The TMB reaction was stopped by adding 100 µL of stopping solution (1% HCl) and measured at 450 nm with 650 nm as the reference. A standard curve was produced through a serial 1:2 dilution of the calibrator decapeptide and plotted using a 4-parameter fit mathematical fit (y =(A-D)/(1 + (x/C^B) + D)) model in Softmax Pro version 6.3 (Molecular Devices, Sunnyvale, CA, USA). The fit was used to calculate the analyte level in all samples and controls through curve regression.

Technical evaluation

Human and rat serum quality control (QC) samples were used to determine dilution recovery from 2-fold dilutions. Linearity was calculated as a percentage of the serum recovery values from the undiluted sample. The lower limit of detection (LDL) was estimated by running 21 blank samples (assay buffer) and determined as the mean+3xSD. The inter and intra assay variations were determined by running 10 independent assay runs with 5 QC samples, in three different days and in different time points. All sample assessed in every assay run were in double determination.

Tissue lysate preparation

Rat tissues from heart, brain, soleus and extensor digitorum longus were snap-frozen in liquid nitrogen and pulverized using mortar and pestle. The residue was transferred to a vial and weighted. Extraction buffer was added (250mg tissue/mL buffer). For clearing the lysate, 5-6 shots of 5 sec sonication each every 10 min were applied until the lysate was homogenous. In between the lysate was kept on ice. After sonication the debris was span down at 4°C /15 min / 13000rpm – supernatant was collected and stored at -80°C.

Extraction buffer: 50mM Tris-HCl (Sigma Aldrich cat T3253) 50mM HEPES (Sigma Aldrich cat H3375) 15% glycerol (Merck cat 1.04094) 1mM EDTA (Merck cat 1.08418) 0.5% sodium deoxycholate (Sigma Aldrich cat D6750) final pH 8.3 -plus the protease inhibitor cocktail (from Roche, cat no 05 056 489001), 1 tablet to 50 ml buffer (Stored at 2-8°C). For protein determination the DC Protein Assay from BioRad was used and protein concentration was adjusted before tested in the ELISA assay.

Clinical samples

The Danish Head and Neck Cancer Group (DAHANCA) 25B trial

Patient plasma samples and data obtained from the DAHANCA 25B randomized controlled trial (RCT) (registered clinicaltrials.gov identifier: NCT01509430) were used for this analysis. The DAHANCA 25B is described in full detail elsewhere[23]. Briefly, the trial involved 41 HNSCC patients that have been through radiotherapy with or without chemotherapy as well as a weight-, gender- and age-matched control group of 21 healthy individuals which were included and described in a previous study (Lønbro et al., https://www.ncbi.nlm.nih.gov/pubmed/23964657) . The patients were randomly assigned to 12 weeks of progressive resistance exercise initiated either immediately after the two-month post-treatment follow-up (EE group) or initiated with a 12-week delay (DE group). Radiotherapy and chemotherapy was conducted according to the DAHANCA guidelines (www.dahanca.dk) at the oncological departments of Aarhus University Hospital and Odense University Hospital. The exercise protocol comprised 2-3 weekly session with seven exercises addressing the large muscle groups of the body. . Assessments of lean body mass (LBM) and blood collection took place at baseline before the initiation of progressive resistance training (PRT) (T0), just after the first 12 weeks of PRT completion of the EE group with the DE group acting as controls (T12) and again after the last 12 weeks of PRT completion of the DE group with the EE group acting as controls (T24). All blood sampling, testing and training took place and repeated at the same local facilities for each patient. Lean body mass was found to be increased equally by approximately 4.3% in both the EE and DE groups after PRT. Collective data from the patient and control groups at baseline can be seen in Table 1.

Table 4: Anthropometric measurements of the subjects involved in the DAHANCA study.

	Cancer patients	Controls	Cancer stage	
n	41	21	N/A	7 (17%)
Age (years)	56+7	59+6	1	4 (10%)
Gender (M/F)	36M/5F	14M/7F	2	4 (10%)
Weight (kg)	72.7+13.7	76.3+13.2	3	4 (10%)
Height (m)	1.76+0.08	1.77+0.09	4	22 (54%)
BMI (kg/m2)	23.4+3.6	24.4+3.1		
LBM (kg)	53.2+8.7	54.9+12.5		

Berlin Bed Rest study

The study has been described elsewhere[24]. Briefly, 20 healthy young men underwent 8 weeks of strict bed rest. They were randomized into two groups, i.e. resistive vibration exercise group (RVE) and control (CTRL) groups. The RVE group was assigned to resistive vibration exercise 11 times per week. Vibration resistance exercises were performed by installing a vibration exercise apparatus at the end of the beds and pulling the subject towards the vibration plate with waist and shoulder straps and handles for the subjects to pull themselves towards the plate. No exercise was performed in CTRL group. After 8 weeks of bed rest, subjects were re-ambulated and observed in the hospital for another 5 days. The serum samples were collected 2 days before bed rest (BDC-2), in the bed rest procedure (BR+) and in recovery period (R+). Serum was thawn from −80 °C and TNNT1 was measured. The muscle mass of both groups were assessed by MRI and DEXA during the three periods. The study was approved by the ethics committee of the Charité Universitätsmedizin Berlin. Subjects gave their written informed consent

Statistical analyses

The biomarker data measured in the two clinical studies were initially subjected to a distribution analysis in order to determine whether any transformation was necessary. Where significant group effects were seen, manual testing, subjected to Bonferroni correction was performed. The analysis of variance was performed in Prism (v6.00 for Windows). Mean values and standard error of the mean (SEM) were calculated using GraphPad Prism, version 6 (GraphPad Software, San Diego, CA, USA). Subject values were analyzed using ordinary one- or two- way analysis of variance test appropriately, with Bonferroni correction for multiple comparisons.

Biomarker data from the control group and the HNSCC subjects were compared at baseline in a mixed models analysis of variance. In this analysis, all HNSCC subjects were pooled, as they were not separated by different intervention courses yet and thus biologically comparable.

In the BBR study Data were organized and transformed accordingly in Microsoft Excel for Windows (Microsoft Corporation, Redmond, WA, USA). Then the data were subjected to mixed models repeated measures ANOVA analysis, testing for Time and Treatment (CTRL vs. RVE) effects as well as Time*Treatment

interactions. If main effects were present, appropriate post hoc tests were performed, subjected to Bonferroni correction for multiple testing. Significance threshold is determined to be $p<0.05$ and data are presented as means ± SEMs where relevant.

Results

Technical performance

The lower limit of detection (LDL) for the assay was 0.63 ng/mL. Dilution recovery was within the acceptable range 100 ± 15% for human serum (overall mean 99.3%, Table 2) and the linearity followed closely the standard curve (Fig 1). The inter- and intra-assay variation was around or below 10% for assessments in both assays.

Table 5: Dilution recovery calculation in 5 serum samples

	HS-41	HS-60	HS-53	HS-71	HS- 89s
Undiluted	100%	100%	100%	100%	100%
1:2 Dilution	103	100	105	99	98
1:4 Dilution	96	89	104	99	99
1:8 Dilution	90	89	117	97	104
Mean	**96**	**93**	**108**	**98**	**100**

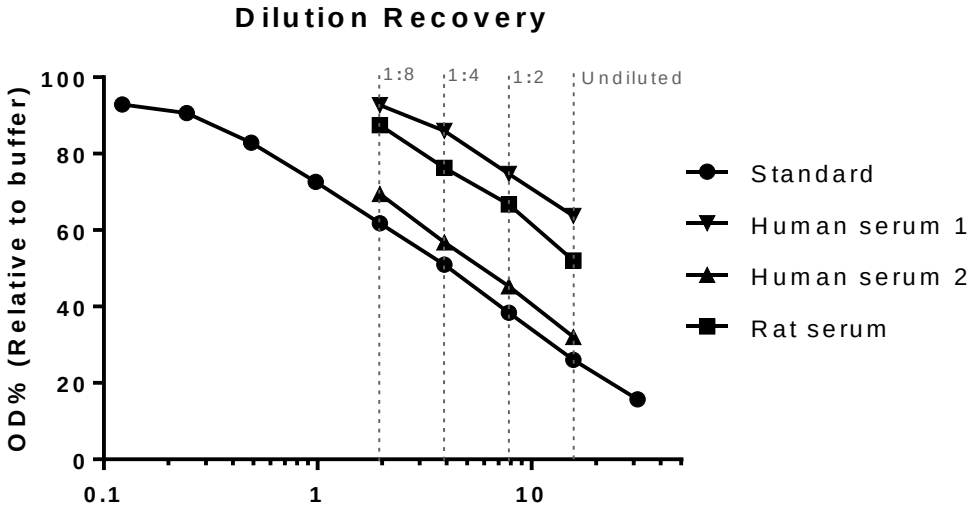

Figure 4: Linearity test between the standard curve and the serial dilutions of human and rat serum.

Rat muscle extracts

Extractions of rat tissues from three different animals were used to gauge skeletal muscle and isoform specificity of TNNT1. Type I fibers constitute about 96% of the soleus muscle (SOL), while extensor digitorum longus muscle (EDL) only contains 5.5% Type I fibers[25]. Heart was also tested for cross-reaction, while recombinant troponin T1 protein and rat brain extractions were used as a positive and negative control respectively. In figure 2 we observed as expected that the TNNT1 levels measured in SOL were much higher than EDL, while no signal was obtained in heart and brain tissue extracts.

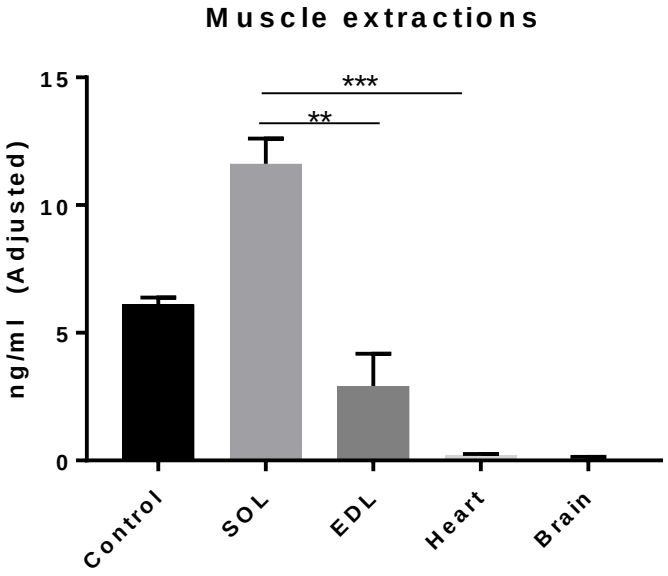

Figure 5: TNNT1 values as measured in tissue extractions from rats. Extraction buffer reported mean value was subtracted by all values. Soleus consists mainly of type I fibers while there is much less expression of type I fibers in the EDL The epitope was not detected in heart and brain lysates that were used as negative controls.

Clinical studies evaluation

DAHANCA

HNSCC patients are known to lose lean body mass consequently to both their disease and therapeutic intervention, forming a representative group where muscle turnover has been altered[26]. In the DAHANCA 25B trial, HNSCC patients that have underwent radiation with or without chemotherapy were assigned to progressive resistance training in order to recover from the muscle loss that they experienced as a secondary effect to the pathology or treatment[23]. During rehabilitation an average of 4.3 % regain of LBM was observed between the different timepoints, following progressive resistance training. By measuring the TNNT1 biomarker in this study, we aimed at monitoring the effect both the impact of the pathology and treatment of the patients at baseline compared to the control group, and of training regime through the different time points. Since no differences in TNNT1 serum levels were observed between the EE and DE groups following PRT, all patients were pooled together in one group for time points T0, T12 and T24.

As demonstrated in fig. 3 TNNT1 was found elevated in serum samples obtained right after treatment, compared to the control group (T0, $p=0.0124$). Furthermore, TNNT1 was significantly increased at both time points under training compared to the control group (T12 and T24, $p<0.0001$). Within the training period,

TNNT1 levels in serum were found progressively increased compared to the patients baseline with a significant difference between T24 and values at baseline (T24 $p<0.05$ compared to T0, marked with #).

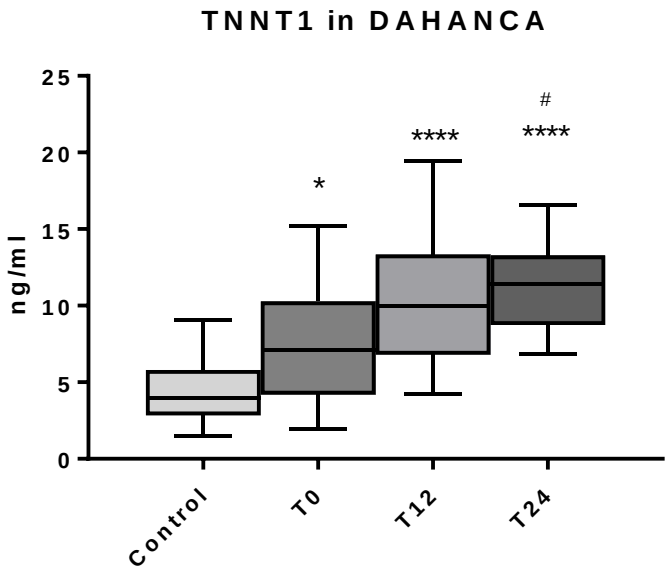

Figure 6: Serum sample measurement results from the DAHANCA study. Patient samples were retrieved at baseline (T0) before any training and at 12 weeks (T12) and 24 weeks (T24) later in the training regime. Significance to the control group is denoted by "" while significance to the patient baseline is denoted by "#"*

Berlin Bed Rest (BBR)

Prolonged periods of inactivity of the lower limbs during space flight missions or bed-rest have shown a negative impact to muscle mass as determined by DEXA and MRI imaging[24]. Significant muscle loss has occurred during bed rest studies but many different countermeasures have been tested for their potency as protective remedies [27,28]. Muscles that have a predominance of slow twitch fibers have demonstrated higher wasting rates during disuse in both rat human studies[13,29]. Resistance vibration exercise has been proposed as a good method of countering the muscle loss in extensive bed rest periods. The hypothesis of testing the levels of TNNT1 in a longitudinal bed rest study with and without counter-treatment is that there will be a difference, both between the two groups and between the different time points as changes in muscle occur[24].

Serum concentration of TNNT1 increased gradually initially up to 13.7% from baseline (± 5.53, BR26) and remained elevated throughout the bed rest period for the RVE group (fig 4). In contrast, the CTRL group had a marked drop in the first two weeks of immobilization, down to -7.12% (±2.11, BR5) before returning to normal levels by the end of the bed rest period. In the initial stages of bed rest, the difference between the CTRL and RVE groups was significant, reaching the highest separation at BR5 (CTRL vs RVE, $p=0.0005$). During the recovery stage, a significant increase of TNNT1in serum was observed in a very similar pattern for both the groups, which then returned to almost normal levels for both groups by the last day of recovery (Peak points: +32.97 ±4.98% for CTRL R28, $p=0.0037$ and 30.53 ±4.18% for RVE, $p=0.0048$).

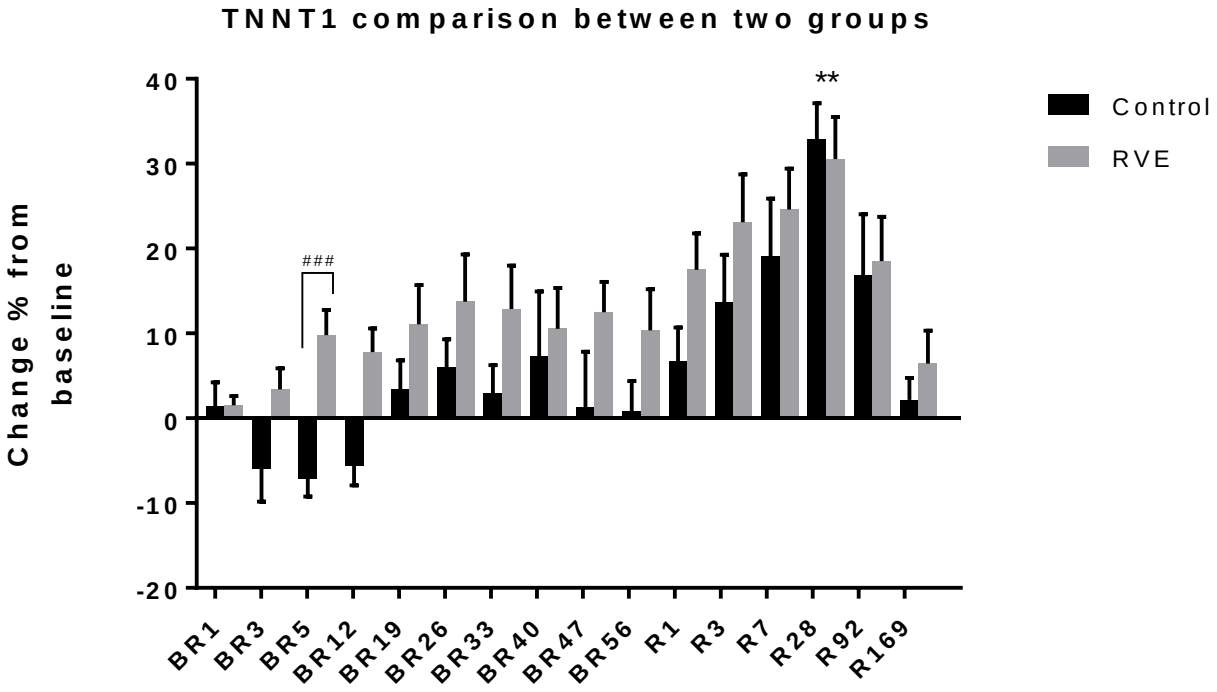

Figure 7 Comparison of the two different groups participating in BBR throughout the different time points. BR indicates the timepoints in the bed rest period of lower limb immobilization while R indicates the remobilization period timepoints measured in days. A distinctive pattern is observed between the control and RVE groups in the bed rest period indicating changes caused by the RVE countermeasure. This difference reached the highest point in BR5 where the TNNT1 serum levels were most significantly different (p=0.0005, noted with ###) In the recovery stage the response to remobilization is almost identical throughout the whole period. The levels of TNNT1 were significantly different for both groups compared to the baseline.

Discussion

Biomarkers reflecting muscle turnover, gain or loss are of big interest regarding diseases that affect muscle synthesis/degradation equilibrium as well as therapeutic interventions. Very few serum protein biomarkers have been strongly connected to those processes in human studies [30,31]. Notably, it has been shown that troponins can be released in both muscle specific pathologies and interventions[32–34]. We have developed an assay specifically directed at the C-terminus of the slow isoform of troponin T, TNNT1 to investigate its validity as a serum biomarker for changes in skeletal muscle.

Tissue specificity

Our analysis of the expression of TNNT1 in various tissues indicated that it is specifically expressed in skeletal muscles, whereas heart and other tissues appear not to express TNNT1 at detectable levels. This is consistent with previous findings on TNNT1 [14,15], and indicates that serum levels of TNNT1 will reflect changes in skeletal muscle.

DAHANCA Head and neck squamous cell carcinoma (HNSCC)

In order to assess the TNNT1 biomarker for a possible response to changes in catabolism/anabolism, we measured samples from HNSCC patients that after radiation treatment participated in muscle training rehabilitation. HNSCC patients are known to lose lean body mass as a consequence of both their disease and therapeutic intervention, forming a representative group where muscle turnover has been altered[26]. In the DAHANCA 25-b study, HNSCC patients that have underwent radiation with or without chemotherapy were assigned to progressive resistance training in order to recover from the muscle loss that they experienced as a secondary effect to the pathology or treatment. By applying the TNNT1 biomarker in this study, we aim at monitoring the effect of both the impact of the pathology and treatment of the patients at baseline compared to the control group, and of the training regime through the different time points

In this study we assessed the levels of TNNT1 in serum samples obtained by HNSCC patients at baseline right after primary treatment and at two follow-up time points when the patients underwent resistance training. Additionally, biomarker levels were measured in an age- and weight-matched healthy control group that did not participate in any training or physical activity. The purpose of this was to evaluate TNNT1 as a biomarker of response to changes in skeletal muscle.

We found that TNNT1 levels were significantly elevated in the patient group compared to the control group, even before engaging in any form of physical activity. It is known that cancer patients can sustain increased muscle degradation even after therapy. However, in this study it cannot be determined whether the amplified release of TNNT1 in the blood stream is a result of the catabolic state of the patients or of an increased protein turnover due to the disease or the treatment.

After engaging in resistance training, the biomarker levels further increased, reaching a significant difference both compared to the patients baseline (T24vsT0, $p<0.05$) as well as to the control group (T12 and T24 vs control, $p<0.0001$). This steady increase reflects a response to the resistance training independent of the disease and/or the treatment. During the training period of 24 weeks, patients gained an overall average of 4% in LBM as it has been described in previous papers (refs). This increase in LBM was accompanied by changes in biomarkers of MMP-based collagen turnover in the ECM[35]. The combination of the anabolic effect and increased turnover due to the training alongside to the ECM remodeling set the basis for the changes of TNNT1 as well. At this point unfortunately it is not feasible to isolate a precise origin for the biomarker changes.

Berlin Bed rest study (BBR)

Prolonged periods of inactivity of the lower limbs during space flight missions or bed-rest have shown to have a negative impact on muscle mass as determined by DEXA and MRI imaging[24]. Significant muscle loss has occurred during bed rest studies. Different methods have been tested for their potency as protective remedies was assessed[27,28]. Muscles that have a predominance of slow twitch fibers have demonstrated higher wasting rates during disuse in both rat human studies[13,29]. Resistance vibration exercise has been proposed as a good method of countering the muscle loss in extensive bed rest periods. The hypothesis of testing the levels of TNNT1 in a longitudinal bed rest study with and without counter-treatment is that there will be a difference, both between the two groups and between the different time points as changes in muscle occur. [24]

We observed that in the initial stages, the RVE and CTRL groups followed a different trend. The serum levels of TNNT1 in the RVE group were elevated from the baseline, while the levels in the CTRL group initially

decreased before reaching back to the baseline. The significant difference of the two groups in the bed rest stage (BR5 CTRL vs RVE, p=0.0002) demonstrates a distinct response to the RVE counter measure, regarding release kinetics of TNNT1 to the blood stream. It is documented that eccentric training is related to muscle injury and fiber type preferential damage, leading to release of protein fragments, including troponins, in blood circulation [33,36,37]. Repeated bouts of eccentric contractions under RVE training may be a confounding parameter for the elevated increase of TNNT1. In the CTRL group the initial drop could possibly be explained by the inactivity of the lower extremities and the lack of any countermeasure. It is not clear what the reason is for the later recovery and fluctuation in the TNNT1 levels during the bed rest process. There is the possibility that the TNNT1 releases into the bloodstream due to the followed atrophy and total muscle degradation induced by the complete inactivity. Unfortunately no specific dietary program was followed and food intake was not monitored to exclude the possibility of under-nutrition as a probable cause of atrophy in either BBR studies. In BBR-2 within the type I-rich SOL muscle the levels of TNNT1 were increased but were decreased in VL. This differential expression between the miscellaneous leg muscles in the CTRL group could be the reason for the variable pattern before the recovery stage.

In addition to the BBR study we used [24], in a very similar study named BBR2 the group of Salanova *et al* have demonstrated the changes in the muscle fiber composition and protein contents [38]. They divided the participants into three groups that had RVE training, resistance training or no counter measures and obtained biopsies from soleus and VL muscles at baseline and after 60 days of bed rest**.** According to their findings, there was a decrease in the size of type I fibers in the CTRL group for both SOL and VL, while the RVE group maintained fiber size. Additionally, there was a clear drop in the total number of type I fibers between the two biopsies for the CTRL group while in the RVE the negative change was not as pronounced. However, based on 2D DIGE quantification, TNNT1 was found in lower levels in the end biopsies of both muscles for the RVE group, while for the CTRL group it was increased in SOL but decreased in VL. In combination with their results, the increased levels of circulating TNNT1 for the RVE group in this study could be consequentially explained by the unloading of troponin from the muscle

During the remobilization stage, the TNNT1 levels were increased significantly in both groups in a very similar manner, compared to the baseline as well as the levels during the bed rest period. In day 28 of recovery were the maximum levels of TNNT1 observed and by the time of training completion, the levels were almost returned back to baseline. This common peak depicts that the biomarker reflects a response triggered by training. Various publications support the skeletal troponin release during training[34,39]. In previous publications collagen and titin biomarkers were applied in the same BBR study. Collagen III formation biomarker (Pro-C3) has demonstrated direct correlation with changes in LBM. The release pattern or Pro-C3 during the bed rest was very similar to TNNT1 in the control group, possibly indicating a close relation to the release of the TNNT1 and changes to the LBM [40]. The MMP-2 cleaved titin biomarker also followed the same initial decrease, again depicting the disuse-induced initial atrophy [41].

The pattern of the TNNT1 release kinetics does not seem to follow a clear anabolic or catabolic stimulus and in some cases the possibility for this could be masked by changes due to damage from eccentric exercise. In catabolic conditions, caspase-3 has been found to be activated in skeletal muscle, assisting to the degradation through the ubiquitin-proteasome system[42]. Caspase-3 activation produces a characteristic fragmentation of actin, while it has also been found to have a cleavage point in TnT that is conserved in the slow skeletal isoform[43]. This mechanism could at least partially explain the release of TNNT1 with no involvement of muscle injury in the case of cachexia in the cancer patients and the control group in the resting period in the BBR. As pointed out in the introduction, truncation of the C-terminus of TnT leads to inability of complete muscle

relaxation which can be associated to poor grip strength. It would be very interesting to test if progressive accumulation of TNNT1 in patients of muscle wasting can also correlate to changes in grip strength.

Limitations

Although the differences between the levels of TNNT1 are interesting, the study design does not allow for a pinpoint explanation regarding the kinetics or release mechanism into the blood stream. Both studies are also very small in number of participants which imposes the need of validation of the biomarker in a more extensive cohort. Furthermore, in the BBR study even though the patients were not using their lower limbs during the immobilization, they were compensating with their upper body for all the daily movements. Since no data were available for the changes in muscle of their upper torso and extremities, the total levels of the biomarker measured in serum could be a consequence of a mixed phenomenon rather than originating purely from the lower limbs. Moreover, when comparing with the results obtained from 2D gels from other studies, it needs to be kept in mind that those assays do not at all measure the same thing. In the gel system only the full protein can be identified while our assay targets the C-terminal part of TNNT1 which would indiscreetly measure the whole protein and/or any fragments that would include the epitope.

Conclusion and future perspectives

We have developed a robust and specific biomarker targeted at the C-terminus of Troponin T1. The assay was tested in two different cohorts, a study of HNSCC patients with resistance training in rehabilitation after treatment and a second immobilization study with and without training countermeasure. In both studies, we have observed significant changes in the biomarker levels in serum compared to baseline and the respective control groups. In both studies during the recovery stage after muscle loss or muscle atrophy, elevation of the biomarker was observed in serum. This increase could be attributed to an increased turnover during the recovery or to a response to the exercise. Furthermore, during the immobilization stage of the BBR study, a different pattern was observed between patients with training and the control group. The differences could be explained based on previous findings from other groups regarding the TNNT1 content in biopsies obtained during a similar immobilization study. Based on the collective findings we could not relate the biomarker to a specific anabolic or catabolic process as we have no clear indication for the mechanisms that it reflects regarding the protein turnover during training. As mentioned, one of the very interesting aspects of the fragmentation in the c-terminus of TnT is the impact on the proper muscle contraction and force generation. A very relevant disease for TNNT1 to be tested for its clinical significance, could by myotonic dystrophy (DM) which has a unique pathology associated with general stiffness and difficulties in relaxation of grip[44]. In addition to signs of possible dysregulation of the troponin system, myotonic dystrophy has also demonstrate profound selective atrophy of type I muscle fibers and isotype shifting.

Acknowledgements

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6. General Discussion

Skeletal muscle is a very important tissue and constitutes 40% of the total bodyweight of an average adult human. It is important for structure, support, motion functionality and proper metabolism within a healthy organism. When affected by pathologies or diseases there are three main manifestations: continuous inflammation, progressive fibrosis and gradual muscle wasting. Since some of the muscle diseases have no cure available, a lot of effort has been put towards therapeutic interventions that can prevent or reverse these symptoms.
In inflammatory myopathies, excessive activation of inflammatory pathways and accelerated muscle degradation have been connected to overexpression of MMPs [165]. Targeted inhibition of the cytokines involved in the abnormal protease activation, e.g. TNF-α, IL-2, IL-6, has shown potential as treatment in inflammatory myopathies and dystrophies [161–164]. Administration of glucocorticoids is recommended as first-line treatment as it can slow down loss of locomotor function or even provide temporary improvements [157–160].
Fibrosis in the endo- and per-mysium is caused by accumulation of collagen I, III, IV and fibronectin [148,149]. In DMD, endomysial fibrosis has been one of the strongest predictors of motor function deterioration. Inhibiting fibrosis may improve muscular function, which is the purpose of several current and preceding pharmacological trials. Previous preclinical and in vitro studies have documented that myostatin inhibitors downregulate markers of fibrosis, but this has not been shown conclusively in clinical models yet [150,151]. The most advanced in terms of clinical trials is HT-100, a delayed-release Halofuginone preparation. It exerts its effect through inhibition of fibrosis and inflammation, in part through blocking of TGF-beta signalling [197], while it also directly inhibits collagen type I synthesis. At the time of writing, the drug has entered Phase II trials for DMD (by Akashi, NCT02525302), overseeing improvements in muscle function and gain. Other pharmaceutical approaches aimed at restoring functional muscle mass are under examination. Anabolic drugs have shown some promise, although none of these have reached FDA approval yet. Inhibition of myostatin has been shown to result in muscle hypertrophy and myostatin inhibitor drugs are at the time of writing in clinical trials for myositis (Novartis' bimagrumab in phase II completed in Feb 2017, NCT01423110) and Duchenne (PF-06252616 for Pfizer, phase II, NCT02310763).

Unfortunately, there is still an unmet need of monitoring tools directed at the progression of these manifestations. Progression rates regarding the disease itself or the response to the treatment could possibly be monitored with imaging or anthropometric methods but biomarkers that are more sensitive could provide faster assessment, biomarkers that have been widely used have not demonstrated clinically the required specificity in following the trajectory of these processes. No single biomarker has been effective and it is possible that a panel of biomarkers would be more capable of describing these complex symptoms.

Qualitative or changes in in muscle strength or function can be identified early, by monitoring fibrosis and inflammation within the muscle tissue through an array of serum biomarkers. Biomarkers have been successful in providing information for developing therapies during antifibrotic clinical trials. Biomarker characterization in a Halo/Akashi trial of Halofuginone

helped reveal fibrosis-collagen turnover, incidentally through use of fibrotic neoepitope collagen biomarkers, i.e. PINP and C3M.

The main objective of the thesis was to develop, characterize and test protein fingerprint biomarkers reflecting changes in muscle, resulting from different pathologies or conditions. One part of the objective was to test the potential of already existing biomarkers aiming at specific mechanisms involved in muscle pathologies. The other part was to develop a new biomarker based on a muscle protein with a structural as well as functional role in human skeletal muscle.
More specifically, we aimed at (1) assessing biomarkers aiming at the ECM for their ability to describe the common processes of the most muscle pathologies: inflammation and fibrosis. Then we selected troponin T1 as a candidate protein and aimed at (2) developing an ELISA assay targeting the C-terminus of troponin T1 (TNNT1). After validating the assay, we applied the TNNT1 biomarker in on one study involving patients under severe muscle loss and a second study where they were forced to inactivity that led to progressive atrophy. Both studies involved a rehabilitation period with exercise. The aim in those two studies was (3) to determine if TNNT1 is released from within the muscle fibers into the blood circulation and able to be measured, and (4) to test if the release kinetics can be identified and associated with a physiological process of the disease or the training intervention.

6.1. Assessing biomarkers aiming at the ECM for their ability to describe the common processes of the most muscle pathologies: inflammation and fibrosis

Idiopathic inflammatory myopathies are a group of, acquired systemic diseases that are characterized by elevated serum levels of muscle enzymes, inflammatory infiltrates and lead to progressive muscle weakness. Dermatomyositis (DM) and polymyositis (PM) are two very similar inflammatory myopathies, distinguished only by the involvement of the skin in DM pathology.

Here we present data indicating that differential diagnosis of PM and DM can be obtained using collagen turnover biomarkers. Furthermore, in this small study we were able to identify relationships between the biomarkers and both inflammatory indicators, such as IFN score in blood, as well and the functional score MMT8. Data, which clearly indicate that the collagen turnover biomarkers are of relevance when developing therapies for the different forms of myositis.

6.1.1: ECM fingerprint in inflammation

As expected, ECM biomarkers of formation and degradation were detected in serum samples of IIM. The biomarker levels were found to be significantly different both within the two myositis as well as compared to the control group. In detail, both types of myositis were associated with substantial decreases in PINP biomarkers compared to controls, while increased levels of PRO-C3, C1M and C3M were associated only with DM.

Collagen I formation marker PINP in both myositis matched the findings of a previous study in which the C-terminal fragment of collagen type I was on the contrary upregulated [189]. Both of

the markers are associated with synthesis and maturation of collagen I, which complicates a straightforward conclusion regarding the turnover process that they reflect. Although a trivial explanation would be that the biomarker decrease reflects a decrease in collagen I synthesis, in the Kubo study, polymyositis patients have shown increases in the amount of collagenous tissue, e.g. fibrosis [190]. This does not necessarily contradict our findings, as fibrosis is associated with not just increased amounts of collagenous tissue, but also qualitative changes in turnover [168,172]. Moreover, the disease can consist of different stages where fibrosis is not always actively ongoing, where collagen biomarker levels can depend on the sole sampling time point in a cross sectional study.

Increased degradation of collagen I as determined by C1M levels was only observed in DM. Similarly, C3M was found to be elevated in DM but not in PM. As described before, the major distinction between DM and PM is the involvement of skin in the pathology of DM. Collagens type I and III, are major constituents of the skin, which could explain the upregulation of those specific collagen types turnover in DM, but this needs to be verified experimentally. Our findings could be verified in a case study conducted using an old assay measuring the pro-peptide of collagen type III. Myositis patients compared to control values showed increased levels of the biomarker here[191].

In other inflammatory conditions such as rheumatoid arthritis and in subgroups of osteoarthritis patients, increased CRPM has been described as a biomarker of local inflammation [85,181]. Despite myositis being characterized by inflammation and heightened MMP activity, in this study CRPM was surprisingly decreased for both DM and PM, relative to healthy subjects. This could be in agreement with the assumption that parts of the disease were not active at the time of sampling as with the case of collagen I markers. It is also possible that the MMPs that are responsible for the fragment generation of CRP[198] are different than the ones accountable for the inflammatory status in myositis. If not so, further elucidation of the mechanisms involved in the progression of the disease would be required.

No matter what, the ECM fingerprint clearly demonstrated the potential to demonstrate differences in the collagen turnover between DM and PM, more so beyond the obvious distinction from the control group. Implementation of these biomarkers to a more extensive cohort would be very interesting, especially one that can provide info regarding anti-inflammatory treatment response.

6.1.2. Neoepitope biomarkers vs. IFN gene signature

As the IIMs are inflammatory by definition and PM and DM are responsive to anti-inflammatory treatments. One of the extensively used biomarkers in recent years is the interferon gene signature since it has been shown to correlate (R^2 of 0.3-0.4) with global and muscle-specific soreness as well as several other myositis biomarkers [193]. Comparison between the trends in ECM biomarkers and IFN gene signature allowed the assessment of the neoepitope biomarkers as markers of disease activity.

Interestingly, in the PM group C3M which is considered to be a marker of systemic inflammation, showed a borderline significant correlation in PM (Spearman's r=0.4, p=0.056) with the IFN score in blood, as did CRPM and C6M. In DM, C3M demonstrated a significant

correlation (Spearman's r=0.4, p<0.05) with IFN score in blood, but neither CRPM nor C6M did. In fact, in DM, C6M marker was inversely correlated to IFN score in blood in DM, albeit not reaching statistical significance.

These data support the claim of neo-epitope biomarkers providing differential output in the two myositis groups, and potentially indicating that, if validated carefully, markers such as C3M could replace the need for IFN gene scores.

6.1 3. Neoepitope biomarkers correlate with MMT8 score

In this study, we correlated the biomarker levels to MMT8 (Manual muscle testing) measurements obtained, in order to find out if there was any relationship with the functional status of the subjects in the form of the MMT8 measurement obtained in this study. In DM, there was a positive correlation observed between CRPM levels and MMT8, which is in good accordance with the fact that myositis appears to be associated with decreases in CRPM. It is Interesting, that PM follows opposite trends from DM in the case most biomarkers to MMT8. This pattern was also observed in correlations to IFN blood scores.

Table 6: (Taken from manuscript II) Spearman's r correlation coefficients for the biomarkers, the IFN scores and the muscle output.

		C1M	PINP	C3M	Pro-C3	CRPM	C6M
DM	IFN Blood	0.002	0.33	0.40*	-0.02	0.32	-0.18
	IFN Muscle	-0.18	0.10	0.14	0.15	-0.03	-0.27
	MMT8	-0.01	-0.17	0.38	-0.03	0.46*	0.21
PM	IFN Blood	0.24	0.11	0.40	0.25	0.45*	0.42*
	IFN Muscle	0.05	0.04	0.16	0.22	0.12	0.13
	MMT8	-0.20	0.01	-0.36	-0.34	-0.37	-0.38

These data indicate that, if validated in larger cohorts, the neoepitope biomarkers could provide important information in relation to muscle strength and possibly functionality. The clearly different levels and correlation patterns regarding the ECM biomarkers, among two very similar diseases is encouraging in the pursuit of defining minute changes in such pathologies.

6.2. Developing an ELISA assay targeting the C-terminus of troponin T1 (TNNT1)

Contractile protein based biomarkers reflecting muscle turnover, gain or loss are of big interest regarding diseases that affect muscle synthesis/degradation equilibrium as well as therapeutic interventions. There is a limited amount of such protein biomarkers strongly connected to those processes in human studies, but still none of them has been established clinically [136,142]. Troponins are known to be able to release in both muscle specific pathologies and interventions[199–201]. We have developed an assay specifically directed at the C-terminus of the slow isoform of troponin T, TNNT1 to investigate its validity as a serum biomarker for changes in skeletal muscle. The assay was validated technically for its specificity and robustness before being applied to two clinical cohorts. Using muscle lysates and control tissue, we demonstrated that the biomarker was muscle specific. We found that our biomarker could indeed be detected

in blood circulation in relation to muscle damage. The results presented in *manuscript III* will be discussed separately for the two studies.

6.2.1 TNNT1 serum levels in Head and neck squamous cell carcinoma (HNSCC) intervention study (DAHANCA)

HNSCC patients provided a good study system for afflicted protein turnover, as a consequence of both their disease and irradiation treatment, possibly under a state of cachexia [202]. Patients that have undergone radiation with or without chemotherapy were assigned to progressive resistance training in order to recover from the muscle loss that they experienced[203,204]. Amidst muscle regain, the anabolic stimulus following the catabolic state was the basis of our interest in applying the TNNT1 biomarker in this study.

Indeed, TNNT1 levels were significantly elevated in the patient group compared to the control group, at baseline. However, within the study design it is impossible to claim if amplified release of TNNT1 in the blood stream is a result of the catabolic state of the patients or of an increased protein turnover due to the disease or radiation.

After engaging in physical training, the biomarker levels further increased through time, reaching a significant difference both compared to the patients baseline (T24vsT0, $p<0.05$) as well as to the control group (T1 and T24 vs control, $p<0.0001$). The elevation of TNNT1 in response to resistance training was independent of the disease and/or the treatment. Although patients gained an overall average of 4% in LBM, we could not find a correlation between that gain and TNNT1 levels. It is more probable that TNNT1 follows an anabolic stimulus under training or an increased turnover because of the nature of the exercise. A higher turnover concurrently with the increase in lean mass is in accordance with the findings regarding MMP-based collagen turnover in the ECM[205]. The combination of the anabolic effect and increased turnover due to the training alongside to the ECM remodeling set the basis of justifying TNNT1 levels as well. At this point unfortunately, it is not feasible to define a precise origin for the biomarker changes.

6.2. 2. TNNT1 serum levels in disuse induced muscle loss (Berlin Bed rest study, BBR)

Measurements of muscle mass determined by DEXA and MRI have demonstrated the extent of muscle loss caused by disuse in prolonged periods of bed-rest [206]. In prolonged inactivity there is also a higher degradation rate of slow-twitch muscle fibers as verified in animal and human studies [17,207]. It is important to find possible counter measures and many regimes are have been studied, regarding their ability to prevent or reverse it muscle [208,209]. Resistance vibration exercise (RVE) is one of the methods proposed for countering the muscle loss. RVE is an eccentric form of exercise, meaning that at the time of force generation the muscle is in lengthening state. A common example of eccentric contraction occurs when running downhill. As the leg stretches to meet the ground, the muscle goes under load and generates force,

performing an eccentric contraction. It is documented that eccentric training is related to muscle injury and fiber type preferential damage, leading to release of protein fragments, including troponins, in blood circulation [200,210,211]. Studies have shown that although eccentric exercise leads to pronounced damage of muscle fibers, it also end in a greater hypertrophy, showing the complexity of muscle anabolism. In our hypothesis of testing the levels of TNNT1 in a longitudinal bed rest study with and without counter-treatment, we expected to find changes both between the two groups and between the different time points.

Interestingly, we have observed that at the initial stage of bed rest there was a different unloading process in the control (CTRL) group compared to the RVE. The difference was most pronounced in the fifth day of bed rest (BR5 CTRL vs RVE, p=0.0002). The initial drop could be possibly explained by the absence of any activity of the lower extremities, affecting normal degradation of protein as measured at the baseline. During the following days, it could be that atrophy ensues due to disuse and the turnover balance leans to muscle degradation. Similarly to the BBR study we used[206] Salanova *et al* have demonstrated the changes in the muscle fiber composition and protein contents in a very similar study named BBR2[212]. Participants were divided into three groups that had RVE training, resistance training or no counter measures and biopsies from soleus and VL muscles were obtained at baseline and after 60 days of bed rest. According to their findings, there was a decrease in the size of type I fibers in the CTRL group for both SOL and VL, while the RVE group maintained fiber size. Additionally, there was a clear drop in the total number of type I fibers between the two biopsies for the CTRL group while in the RVE the negative change was not as pronounced. These findings are in agreement of previous studies showing a favoritism of type II fiber wasting in eccentric training[201]. However, based on 2D DIGE quantification, TNNT1 specifically was found in lower levels in the end biopsies of both muscles for the RVE group, while for the CTRL group it was increased in SOL but decreased in VL. These data could explain the release of TNNT1 in circulation for the RVE group even when type I fiber wasting was not as not as marked as the CTRL group. Repeated bouts of eccentric contractions under RVE training may be a confounding parameter for the elevated increase of TNNT1. In the CTRL group, within the type I-rich SOL muscle the levels of TNNT1 were increased but were decreased in VL. This differential expression between the miscellaneous leg muscles in the CTRL group could be the reason for the variable pattern before the recovery stage.

During the remobilization stage, very similar release kinetics were observed in the two groups for the TNNT1 levels. As participants were regaining muscle, a steady elevation of TNNT1 was observed and peaked on day 28 of the recovery as it can be seen in fig 4 in *manuscript III*. Upon completion of the study period, TNNT1 levels were almost back to normal. This could reflect a systemic response triggered by training. Various publications support the skeletal troponin release during training[201,213]. In previous publications regarding the BBR study, collagen and titin biomarkers were also investigated[155,188]. Collagen III formation biomarker (Pro-C3) has demonstrated direct correlation with changes in LBM. The release pattern during the bed rest was very similar to TNNT1 in the control group, possibly indicating a close relation to the release of the TNNT1 and changes to the LBM. The MMP-2 cleaved titin biomarker also followed the same initial decrease as TNNT1, depicting the disuse-induced initial atrophy.

Within the confines of the study setting, it is difficult to be absolute on what is the cause for the release of TNNT1 in both the immobilization and reloading period. There is no clear anabolic or catabolic stimulus and in some cases, the possibility for this could be masked by changes due to damage from eccentric exercise. In catabolic conditions, caspase-3 has been found to be activated in skeletal muscle, assisting to the degradation through the ubiquitin-proteasome system[214]. TnT is found to be a substrate of caspase-3 with a distinct fragmentation site observed in cardiomyopathy studies [215]. The cleavage site is conserved in the slow skeletal isoform, which could be one of the possible ways that TNNT1 is detached from the protein complex. When conformational changes during muscle contraction and relaxation occur in the troponin system, lower association to the TNNT1 could set it free. We have established that truncation of the C-terminus of TnT leads to inability of complete muscle relaxation which can be associated to poor grip strength. It would be very interesting to test if progressive accumulation of TNNT1 in patients of muscle wasting can also correlate to changes in grip strength.

7. LIMITATIONS

There are several limitations to our findings that need to be taken into account when interpreting the results. All of the studies are relatively small and are more likely to serve as proof of concept rather that undisputed evidence. Furthermore, no information was obtained regarding collagen deposition and fibrosis within the muscle tissue. Fibrosis could directly affect the ECM fingerprint measurements in the myositis study, but also affect muscle regeneration capacity during training.

In the myositis study, it would be of relevance to have MMT8 scores in the age- and gender matched controls. This would allow verifying that differences between the patient groups and controls are related specifically to their pathologies and not a random intrinsic trait of the biomarkers. Furthermore, although the MMT8 has previously demonstrated good test-retest reliability in the hands of experienced raters across the various items, no reliability measurement of the MMT8 raters was performed in this study. It must be considered that MMT8 is affected by structural damage, loss of muscle tissue but also pain when it compromises movement. In many of the other papers in the literature about myositis biomarkers besides MMT8, correlations between biomarkers with pain scores in the form of muscle VAS were included [193,196]. Unfortunately, in this study, we did not have access to muscle VAS scores. However, the muscle VAS score is also a subjective score with reliability in the same range as the MMT8 score. Finally, we did not take into account the potential effect of the glucocorticoid and/or baseline methotrexate therapy, although a previous study showed that the IFN score was unaffected by this[183]. Future assessments of the biomarker panel in myositis studies required.

Regarding the BBR study, despite the similarities with the results obtained from 2D gels from other studies, it needs to be kept in mind that those assays do not measure exactly the same thing. In the gel system, only the full protein can be identified while our assay targets the C-terminal part of TNNT1, which would indiscreetly measure the whole protein and/or any fragments that would include the epitope.

8. CONCLUSION

In this thesis, we have demonstrated the needs for novel biomarkers in the field of skeletal muscle pathologies. Protein biomarkers in serum are easily obtainable, not as invasive as other methods and can be used as targets for antibody-based assays. We have tested biomarkers targeting both intracellular and extracellular proteins in muscle. We have found differential regulation of those markers between pathological and healthy groups as well as correlations to strength and function outputs.
To further specify, we found that:

(1) It is possible to use ECM based neo-epitopes and inflammatory biomarkers to distinguish between (a) populations suffering from different inflammatory myopathies and (b) a control group of healthy individuals. *(manuscript II)*
(2) ECM based and inflammatory biomarkers are able to mirror changes in functionality, force generation and disease activity in inflammatory myopathy patients. *(manuscript II)*
(3) TNNT1 is a good biomarker candidate, able to be released and detected in blood circulation under muscle damage in a distinct manner directed by the pathology. *(manuscript III)*
(4) It is unclear whether TNNT1 can follow the catabolic/anabolic effects induced by the disuse model and the training intervention in either study. However, there is evidence that TNNT1 follows fiber-type preferential wasting trends as it should and a possible mechanism for the release was discussed. *(manuscript III)*

The results of this thesis verify that a panel of biomarkers could fill in the need to characterize complex processes in rare neuromuscular diseases. Addressing the main manifestation of the diseases in well-described clinical cohorts could expedite pharmaceutical trials and provide valuable information on the pathology of the disease. Indeed, based on these findings, biomarker panels consisting of ELISAs against Collagen type I and III fragments and MMP-degraded CRP are promising biomarkers in myositis in terms of diagnosis, functional characterization and likely monitoring of treatment efficacy. Being able to recognize subtle differences between DM and PM is a valuable asset, as it is attractive to treat the two forms of myositis with different regimens. Since there is a lot of variation in the different forms of myopathies and dystrophies, the use of non-invasive serum biomarkers possibly assist in personalized care in the future.

Having biomarkers that have proven to describe the individual symptoms of inflammation, fibrosis and muscle wasting could also assist in developing other biomarkers. Since dystrophies are very rare diseases, it is hard to get access to large, well-planned cohorts to test potential biomarkers. Cell and animal-based models are usually employed but do not behave the same as the *in vivo* processes in human. Using pathological models in human studies that are at the same time clinically described through such biomarkers can generate data that will appealing enough to argue for the use of the biomarkers in the big cohorts.

Besides underlining the importance of the ECM in muscle pathologies, we also addressed the potential of intramuscular proteins to be used as biomarkers. Targeting the C-terminus of Troponin T1, we have developed a robust and specific biomarker assay. Although we could not verify the mechanisms that TNNT1 reflects regarding the protein turnover, it has been shown promising results. In order to improve the ability of the biomarker to demonstrate specific response in molecular pathways of wasting, it could be implemented in a neo-epitope assay. We already know that there is a conserved cleavage point for caspase-3 in skeletal TnT and that caspase-3 activation has biological significance in muscle pathologies. A sandwich ELISA could be developed targeting the caspase-3 cleavage site on the one end and the skeletal specific TNNT1 site on the other end. This approach would ensure that only fragments from skeletal muscle that have been affected by a very specific pathway are detected. It would be very interesting to see if such a setup would elucidate the involvement of TNNT1 in muscle pathologies.

As fragmentation in the c-terminus of TnT has an impact on the proper muscle contraction and force generation it would be very interesting to test TNNT1 in study that is directed at changes in force generation and proper relaxation. A very relevant disease for TNNT1 to be tested for its clinical significance, could by myotonic dystrophy which has a unique pathology associated with general stiffness and difficulties in relaxation of grip [216]. In addition to signs of possible dysregulation of the troponin system, myotonic dystrophy has also demonstrate profound selective atrophy of type I muscle fibers and isotype shifting.

Future perspectives

Despite these encouraging preliminary data, the cohorts that were used, were multivariate in regarding the changes in muscle. The biomarkers seem promising but will be much better characterized, if applied to simpler cohorts. For example a study with young men with and without anabolic stimulus (drug and/or training induced) would be a way to determine if the biomarkers respond to the anabolic conditions. It is also of our great interest to measure all the relevant protein fingerprint biomarkers in a big cohort of the most common dystrophies. Despite cross sectional studies being already interesting, a prospective study with different time points under a possible treatment including relevant primary and secondary outcomes (function tests, strength tests, LBM assessment etc.) would be the ideal cohort. Furthermore, a key area that we did not manage to test our biomarkers is regarding intramuscular fibrosis. The ECM has a paramount role in many of the dystrophies and a biomarker panel as the collagen fingerprint could give early insight on changes after treatment or other therapeutic intervention. This would give a well-rounded picture of the actual potential of protein fingerprint panels in neuromuscular disorders and help propel drug discovery.

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Thanos Arvanitidis

This thesis is dedicated to my grandmother

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