Common and distinct immunological aspects in acquired inflammatory myopathies and inherited muscular dystrophy

D I S S E R T A T I O N
zur Erlangung des akademischen Grades
D o c t o r r e r u m n a t u r a l i u m

(Dr. rer. nat.)

im Fach Biologie

eingereicht an der Lebenswissenschaftlichen Fakultät
der Humboldt-Universität zu Berlin

von
Preuße
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Tag der Disputation: 26. November 2014

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ABSTRACT

The heterogeneous group of myopathies can affect the function of the skeletal muscle, as well as other organ systems, like the connective tissue, the heart or the lung. Diseases of voluntary muscles comprise a huge number of different entities, which may be inherited or acquired. The correct diagnosis may be difficult to obtain and the patients' clinical history, laboratory results and muscle biopsies are helpful to distinguish between different entities. Therefore, the classification of muscle diseases is constantly changing and getting more detailed. Acquired myopathies are potentially treatable, but there are often only unspecific treatment options. In contrast to acquired myopathies, there is no causative cure for inherited forms of myopathies. Nevertheless, treatment is warranted to increase the quality of life and life expectancy in patients. Therefore, the knowledge of underlying pathological and immunological mechanisms in these diseases is highly important. Macrophages, due to their multifunctional capacities, are involved in a variety of reactions in the muscle, and it has been shown in many studies, that during muscle injury, macrophages are involved in clearing pathogens and perform a switch from pro-inflammatory to alternatively activated macrophage phenotypes. Moreover, macrophages are important players in autoimmune reactions in myopathies and might therefore be an interesting target for specific therapies.

In the current thesis, three different entities were analyzed. They all share common aspects of the immune response, but also feature distinct immunological aspects as well. All entities have an inflammatory part in common, which is mainly regulated by influx of immune cells into the muscle and adjacent connective tissue. However, the composition of these infiltrates was varying between the diseases and the involvement of e.g. lymphocytes or macrophages was variable. In addition, the respective cytokine milieu in the muscle tissue was also highly specific in the examined inflammatory myopathies and in muscular dystrophies. Thus, the aim of the study was to precisely examine interactions between immune cells, and also analyze characteristic pathological phenomena, such as hypoxia, inflammation and fibrosis.

Necrotizing myopathies have an immune-mediated background or showed a toxic aetiology. In this work I demonstrated that both sub-groups of necrotizing myopathy can clearly be distinguished by their morphological characteristics, as well as certain immune aspects, such as presence of autoantibodies, B cell invasion and also the underlying cytokine milieu. The immune reaction in patients with immune-mediated necrotizing myopathies was defined by a T_H1-like, pro-inflammatory milieu, with up-regulation of IFN-γ and TNFα. Interestingly, beside muscle fibre necrosis, both groups showed a clear inflammatory infiltrate with macrophages invading muscle fibres. Macrophages are the predominant cell population and are spread throughout the muscle. In addition, considerable amounts of $CD4^+$ and $CD8^+$ T cells are found in the infiltrates of patients with an immune-mediated disease, while only few patients showed presence of B cells and plasma cells and expression of the B cell chemoattractant *CXCL13* was low. Furthermore, a clear expression of MHC class I molecules and C5b9 deposition was detected in these cases, while none of these features were found in patients with a toxic aetiology, which clearly distinguished both subtypes of necrotizing myopathies.

Analyses of the clinical and morphological picture in patients suffering from dermatomyositis showed that juvenile patients have very characteristic symptoms, like dysphagia and calcifications of skeletal muscles that are not part of the disease in adults. On the other hand, both diseases are considered inflammatory vasculopathies, although clear vasculitis with destruction of vessel walls is not apparent. A typical perifascicular pattern of atrophy was identified, as well as effects of hypoxia, and both features are more pronounced in juvenile patients, as compared to adults. Since these hypoxic changes are most salient in the perimysium, as well as in adjacent atrophic muscle fibres, these areas were analyzed by qPCR after laser microdissection. Up-regulation of molecules involved in hypoxia was found in the perifascicular area, while the centro-fascicular tissue of the same patients showed no regulation of hypoxia-associated molecules. In line with these findings, the loss of capillaries in dermatomyositis patients was determined. The quantification of capillaries per fibre showed that capillary loss was also more prominent in juvenile patients. Taken together, these findings showed that hypoxia is involved in development of perifascicular atrophy and that the features described are in general more pronounced in juvenile dermatomyositis than in the adult form.

A number of inherited myopathies are known to also harbor significant inflammatory infiltrates. Among these Duchenne muscular dystrophy is the most prominent. Here the development of fibrosis was a major feature of skeletal muscle degeneration and directly correlates with early loss of ambulation. Patients develop fibrosis, as demonstrate by EvG and collagen VI staining, and expression levels of molecules involved in this process, such as *TGFβ* or the collagen synthetase (*P4HA1*) were significantly elevated in comparison to those of healthy controls. Macrophages are not only strong players in inflammatory processes but also tightly control fibrotic processes. In line with this, I showed that over time both, inflammation and fibrotic tissue was changing characteristically. Quantification of macrophages demonstrated that the highest number of invading cells was seen in patients with an age of about four years. However, after loss of ambulation the intensity of inflammatory processes decreased. This was monitored by reduced numbers of all inflammatory cells, as well as reduced expression of molecules driving inflammation. A computer-based algorithm was used to quantify fibrosis, and I demonstrated that the amount of connective tissue increased with the age of patients, while at late stage of disease fatty transformation was an additional important issue. Quantifications revealed that the ratio between fibres and connective tissue was reduced in Duchenne muscular dystrophy in comparison to Becker muscular dystrophy and female carriers of the *DMD* gene mutation.

ZUSAMMENFASSUNG

Die heterogene Gruppe der Myopathien kann sowohl die Funktion des Muskels beeinflussen, als auch verschiedene andere Organsysteme des Körpers, wie zum Beispiel das Bindegewebe, das Herz oder die Lunge. Erkrankungen der Skelettmuskulatur können entweder erworben oder vererbt sein und die korrekte Diagnose der Erkrankung ist aufgrund von Ähnlichkeiten der Symptome oft schwer zu stellen. Daher sind neben der Patientenanamnese, sowohl weitere laborchemische Tests, als auch eine Muskelbiopsie äußerst hilfreich. Durch neue Erkenntnisse und Einsatz neuer Analysemethoden ist die Klassifikation der Myopathien daher in einem dauerhaften Wandel.

Die erworbenen Muskelerkrankungen sind theoretisch behandelbar, jedoch stehen zumeist nur sehr unspezifische Behandlungsoptionen zur Verfügung. Im Gegensatz dazu, sind für vererbte Formen der Muskelerkrankungen bisher keine kausalen Therapiemöglichkeiten bekannt, jedoch ist auch hier die Entwicklung neuer Therapien von größter Bedeutung um sowohl die Lebensqualität, als auch die Lebenserwartung der Patienten zu verbessern. Um dies zu erreichen, ist das Wissen über die zugrundeliegenden pathologischen und immunologischen Mechanismen von großer Bedeutung. Durch ihre multifunktionalen Eigenschaften sind Makrophagen nicht nur an verschiedene Reaktionen im Muskel beteiligt, sondern es wurde auch gezeigt, dass sie eine wichtige Rolle bei Verletzungen der Skelettmuskulatur spielen. Neben ihrer Rolle bei der Entfernung von Pathogenen, wurde gezeigt, dass Makrophagen hier von einem pro-entzündlichen zu einem eher alternativ aktivierten Phänotyp wechseln. Des Weiteren sind sie wichtige Mediatoren von Autoimmunreaktionen im Muskel und könnten daher ein interessantes Ziel für zukünftige Therapien sein.

In der vorliegenden Arbeit wurden drei verschiedene Muskelerkrankungen untersucht. Alle Entitäten zeigen entzündliche Phänomene, zeichnen sich zusätzlich aber durch spezifische immunologische Phänomene aus. Gemeinsam ist ihnen ein jeweils charakteristischer Einstrom von Entzündungszellen in den Muskel und das angrenzende Bindegewebe. Die Zusammensetzung dieses Zellinfiltrates war jedoch bei den verschieden Erkrankungen unterschiedlich und die Beteiligung von z.B. Lymphozyten oder Makrophagen war unterschiedlich stark ausgeprägt. Weiterhin unterscheidet sich das zugrunde liegende Zytokinmilieu innerhalb des Muskelgewebes und dieses ist höchst spezifisch für die einzelnen untersuchten entzündlichen Myopathien und die Muskeldystrophie. Daher war es Ziel der Arbeit, die genauen Interaktionen zwischen den Immunzellen zu untersuchen, sowie die charakteristischen Phänomene der Erkrankungen (z.B. Hypoxie, Entzündung und Fibrose) zu untersuchen.

Nekrotisierende Myopathie können sowohl durch eine immun-vermittelte Genese, als auch durch Kontakt mit toxischen Substanzen ausgelöst werden. In der vorliegenden Arbeit konnte gezeigt werden, dass beide Subgruppen klar durch morphologische Kriterien, als auch durch spezielle Immunaspekte, wie beispielsweise das Vorkommen von Autoantikörpern, B Zell-Invasion, als auch das zugrunde liegende Zytokinmilieu, gekennzeichnet sein. Die Immunreaktion von Patienten mit immun-vermittelter Genese war weiterhin gekennzeichnet durch ein T_H1-basiertes, pro-entzündliches Milieu mit erhöhter Expression von IFN-γ und TNFα. Interessanterweise wiesen beide Gruppen, neben

Muskelfasernekrosen, klar definierte entzündliche Zellinfiltrate mit Makrophageninvasion in das Muskelgewebe auf. Makrophagen waren dabei die vorherrschende Zellpopulation und im gesamten Muskel verteilt. Des Weiteren fanden sich in den Infiltraten $CD4^+$ und $CD8^+$ T Zellen bei Patienten mit immun-vermittelter Genese. Bei diesen Patienten waren jedoch selten B Zellen oder Plasmazellen im Muskelbiopsat zu finden und die Expression des B Zell Chemokins *CXCL13* war gering. Auch konnte eine klare Expression von MHC Klasse I Molekülen und eine Ablagerung von Komplement gefunden werden, während Patienten mit einer toxischen Genese keine dieser Merkmale aufwiesen. Daher können die Subgruppen mit nekrotisierender Myopathie klar voneinander abgegrenzt werden.

Die Analyse des klinischen und morphologischen Bildes von Patienten mit Dermatomyositis zeigte, dass juvenile Patienten im Gegensatz zu adulten Patienten, sehr charakteristische Symptome aufwiesen, wie beispielsweise Schluckbeschwerden und Kalzifizierung der Skelettmuskulatur. Andererseits werden beide Erkrankungen als entzündliche Vaskulopathie eingestuft, wobei eine genuine Zerstörung der Gefäßwände nicht vorliegt. Ein typisches perifaszikuläres Atrophiemuster konnte ebenso wie hypoxische Effekte identifiziert werden, wobei beide Phänomene deutlich ausgeprägter bei juvenilen Patienten vorkamen. Da die hypoxischen Veränderungen meist im Perimysium auftraten, sowie in den angrenzenden Fasern, wurden diese Bereiche mittels Laserdissektion ausgeschnitten und im Anschluss per qPCR analysiert. In diesen Bereichen konnte eine Hochregulation an Hypoxie-assoziierten Molekülen gefunden werden, während die zentralen Bereiche des Gewebes derselben Patienten keine verstärkte Expression aufwiesen. Passend zu diesen Ergebnissen konnte ein Verlust von Kapillaren gemessen werden. Die Quantifizierung der Kapillaren pro Faser hat gezeigt, dass der Kapillarverlust bei juvenilen Pateinten verstärkt vorkam. Dies zeigt, dass Hypoxie bei der Entwicklung der perifaszikularen Atrophie beteiligt ist, und dass die beschriebenen Effekte besonders bei juvenilen Patienten vorkommen.

Einige erbliche Myopathien können ebenfalls signifikante entzündliche Infiltrate aufweisen, wie beispielsweise die Muskeldystrophie Duchenne. Die Entwicklung von Fibrose in der Skelettmuskulatur ist dabei ein Hauptkriterium der Muskelfaserdegeneration und korreliert direkt mit einem frühen Gehverlust. Die Färbung mit EvG und Kollagen VI zeigte diese Fibroseentwicklung und Expressionsmolecule wie beispielsweise *TGFβ* oder die Kollagensynthetase (*P4HA1*) waren signifikant erhöht im Vergleich zu gesunden Kontrollen. Makrophagen sind in Entzündungsprozessen involviert und kontrollieren außerdem fibrotische Prozesse. In der vorliegenden Arbeit konnte gezeigt werden, dass sich sowohl Entzündung, als auch fibrotische Prozesse charakteristisch verändern. Die Quantifizierung der Makrophagen zeigte, dass die stärkste Zellinvasion bei Patienten mit einem Alter von vier Jahren gesehen wurde. Im Gegensatz dazu sind die Entzündungsprozesse nach dem Verlust der Gehfähigkeit rückläufig. Diese Veränderungen sind durch verminderte Zahlen an Entzündungszellen, sowie durch reduzierte Expression von Molekülen die Entzündungsprozesse vorantreiben gekennzeichnet. Ein neu entwickelter computer-basierter Algorithmus wurde dabei genutzt, um die Entwicklung der Fibrose zu quantifizieren. Die Menge an Bindegewebe steigt mit dem Alter der Patienten, während bei älteren Patienten außerdem ein fettgewebiger Umbau ein wichtiger Aspekt der Pathologie war. Die Quantifizierung zeigte weiterhin, dass das Verhältnis zwischen Fasern und Bindegewebe bei Patienten mit Muskeldystrophie Duchenne im Vergleich zu Muskeldystrophie Becker und weiblichen Überträgerinnen des mutierten *DMD* Gens reduziert ist.

CONTENTS

ABBREVIATIONS

aDM	adult dermatomyositis
APC	antigen presenting cells
ATP	adenosine triphosphate
BCR	B cell receptors
BMD	Becker muscular dystrophy
C5b9 / MAC	membrane-attack-complex of the complement system
CCR	C-C chemokine receptor
CK	creatine kinase
CMD	congenital muscular dystrophy
COPD	chronic obstructive pulmonary disease
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DGC	dystrophin-glycoprotein complex
DM	dermatomyositis
DMD	Duchenne muscular dystrophy
fC	female carrier of *DMD* gene
HIF-1α	hypoxia-inducible factor 1 alpha
HLA	human leucocyte antigen
HMG-CoA	3-hydroxy-3-methylguanyl-coenzyme A reductase
HPF	high power field
HSC	hematopoietic stem cells
IL	interleukin
IMNM	immune-mediated necrotizing myopathy
IVIG	intravenous Immunglobulines
jDM	juvenile Dermatomyositis
LCM	laser capture microdissection
LGMD	limb girdle muscular dystrophy
LPS	lypopolysaccharide
M1 / CAM	classically activated macrophages
M2 / AAM	alternatively activated macrophages
MAA	myositis-associated autoantibodies
MHC	major histocompatibility complex
MHCn	myosin heavy chain neonatal
MIF	macrophage migration inhibitory factor
MMP	matrix metalloproteinases
MRI	magnet resonance imaging
MSA	myositis-specific autoantibodies
MTX	methotrexate
MD	muscular dystrophy
NAM	necrotizing autoimmune myopathy
NC	normal controls
NCAM	neural cell adhesion molecule
nIMNM	non-immune mediated necrotizing myopathies
NM	necrotizing myopathy

NOS	nitric oxide synthase
pDC	plasmacytoid dendritic cells
PM	polymyositis
PRR	pattern recognition receptors
qPCR	quantitative real-time polymerase chain reaction
ROI	region of interest
RT	room temperature
sIBM	sporadic inclusion body myositis
SCID	severe combined immunodeficiency
SRP	signal recognition particle
STAT	signal transducer and activator of transcription
TAM	tumor-associated macrophages
Tc cells	cytotoxic T cells
TCR	T-cell receptors
TGF	transforming growth factor
T_H cells	T helper cells
T_H1	T helper cell 1
T_H2	T helper cell 2
T_H17	T helper cell 17
TIMP	tissue inhibitor of metalloproteinase
TNF	tumor necrosis factor
T_{reg}	regulatory T cells
VEGF	vascular endothelial growth factor

1 INTRODUCTION

Entities of the heterogeneous group of myopathies not only affect the skeletal muscle, as well as the connective tissue, but also the cardiac muscle, the respiratory system, the skeleton, the skin and a multitude of other organ systems. This may give rise to rather mono-symptomatic diseases, but also to complex 'system diseases'. Myopathies can either be inherited (recessive, dominant, e.g. muscular dystrophies) or acquired (e.g. autoimmune diseases, exposure to toxins etc.). Even though myopathies are in general relatively rare, they comprise a huge variety of subgroups, adding up to more than 800 different forms of neuromuscular diseases (classification according to Walton). Exceptions to the exceedingly rare forms are inherited myopathies, like the Duchenne muscular dystrophy (DMD), with an incidence of about 1:5.000 male new-borns.

Theoretically, acquired myopathies are treatable, but there are often only non-specific therapeutic options, and some forms of myopathies are completely resistant to therapy (with e.g. corticosteroids). In contrast to acquired myopathies inherited forms of myopathies are not curable with current medication; nevertheless, treatment is warranted to increase the life expectancy and quality of life. To understand the natural course of these diseases, the knowledge of underlying pathological and immunological mechanisms is highly important.

1.1 Autoimmune processes and skeletal muscle diseases

A number of inflammatory diseases of the skeletal muscle were termed immune inflammatory myopathies (IIMs). These comprise the so called classical entities dermatomyositis (DM), polymyositis (PM), necrotizing myopathy (NM) and sporadic inclusion body myositis (sIBM), which are discussed in more detail below. However, the contribution of an autoimmune pathogenesis to these entities is much debated. Especially in sIBM, two pathomechanisms are concurring, inflammatory processes and degenerative processes, and it is to date not clear, which one is the first process and which one is a subsequent event. On the other hand, an autoimmune pathogenesis is generally accepted for the entities DM and autoimmune NM. To understand the underlying immunology of myopathies and the cells involved in these complex mechanisms is highly important, since it is the prerequisite for development of new and targeted therapies.

In general, autoimmunity is defined as an attack by cells of the immune system, which is directed against their own cells or organs instead of foreign cells [1]. The underlying pathomechanisms of autoimmunity are under intense investigation and are very complex, involving a multitude of different cells of the immune system. Examples for autoimmunity are rheumatoid arthritis, lupus erythematodes, Wegener´s granulomatosis or the Sjögren´s syndrome.

For understanding the specific diagnosis and also treatment options of these autoimmune muscle diseases, the detection of autoantibodies is crucial. A causative link has been established to the pathogenesis of certain myopathies [2]. It is possible to identify certain autoantibodies which are linked to specific diseases makes it very likely that autoimmunity plays a major role in their pathogenesis. Furthermore, recognition of autoantibodies serves as s diagnostic tools. To give an example, anti-

signal recognition particle (SRP) antibodies have been identified in a subtype of autoimmune NM. Although their role in myopathies is not clear, but it was recently shown that their levels directly correspond to disease activity and also paralleled the creatine kinase levels in affected patients, thus their monitoring serves as a very useful tool to monitor therapeutic efficiency [3]. Some of the most commonly found antibodies in the entities, which are also investigated in the current study, are summarized in Table 1. Beside this intrinsic involvement of autoimmunity, also e.g. immunosuppressive medication can lead to immunodeficiency and a subsequent shut down of the immune mechanisms [4,5], resulting in possibly considerable complications, such as opportunistic infections.

Table 1: Most common autoantibodies in necrotizing myopathy and dermatomyositis

Entity	Disease associated
Necrotizing myopathy	HMG-CoA, SRP, Jo1, PL-7, PL-12, Ro52
Dermatomyositis	Mi-2, Jo1, p155/140, MDA5, PM-Scl

To classify autoimmune diseases a general organization depending on the involved organs or organ systems is used. Hence, diseases are grouped into organ-specific ones, such as multiple sclerosis or myasthenia gravis, or into those comprising systemic involvement, e.g. rheumatic arthritis or Sjögren´s syndrome.

In autoimmunity, a correlation to genetic predisposition, as well as to environmental triggers is assumed [6,7]. Regarding the genetic predisposition, studies with twins revealed that there is a high variation of a genetic association in different autoimmune diseases and that further research is needed to pin down specific involved genetic molecules [8]. In addition, genome-wide search studies found correlations of gene variations which are related to diseases [9]. Many studies showed especially the importance of the human leucocyte antigen (HLA) gene complex, e.g. HLA- DQA1*0301 in DM [10,11] or HLA-DRB1*01 in macrophagic myofasciitis [12]. Nevertheless, genetic predisposition seems to be only a minor factor for development of autoimmune diseases and environmental triggers may be involved frequently [13]. Dysregulation of the immune system in reaction to toxins or pathogens can initiate an inflammatory response mainly leading to activation of T cells. The starting signaling cascade then leads to immune reactions with antibody production and tissue injury [14]. To date, numerous studies have conclusively demonstrated that autoimmune reactions/diseases are induced by various toxins [15].

Interestingly, autoimmune processes have also been discussed to be involved in the pathogenesis of genetic disorders of the skeletal muscle like Duchenne muscular dystrophy. Mendell *et. al.* have shown that autoreactive T cells directed against revertant fibres in this disease [16]. The authors argue that truncated or dysfunctional dystrophin protein is recognized as ‘non-self’ and therefore elicits an autoimmune response. However, it remains to be established if this happens as a regular phenomenon in all of the affected boys and if this autoimmune response is somehow silenced during the disease course. Although not part of the present thesis, it needs to be stressed that these questions will certainly be addressed and will be important in future therapeutic options.

1.1.1 Macrophages

The bone marrow gives rise to haematopoietic stem cells (HSC), differentiating into monocytes, which reside in various lymphoid and nonlymphoid tissues (e.g. liver: Kupffer cells, lung: alveolar macrophages), but can also circulate in the body's blood stream. Macrophages are distinguished by their morphology and specialized functions. Furthermore, some specialized monocytes, as microglia in the central nervous system (CNS) are not regenerated via bone marrow derived cells but probably renew themselves from local resident cells. [17,18] (Figure 1 A)

Macrophages are involved in steady-state homeostasis, where they phagocytose or engulf cellular debris and dying cells [19]. Via receptors like the scavenger receptor or CD14, pathogens are recognized and phagocytosis is initiated. The debris is actively encapsulated into phagosomes, which are acidophil. To further digest cellular debris/pathogens, the phagosome fuses with lysosomes containing enzymes and proteins for cell lyses. Lysozyme, toxic free radicals and the acid milieu subsequently digest the debris. In response to inflammation, macrophages stimulate lymphocytes via secretion of cytokines [19–21] and stimulate tissue fibrosis and wound healing [22]. Each of these functions is later discussed in more detail.

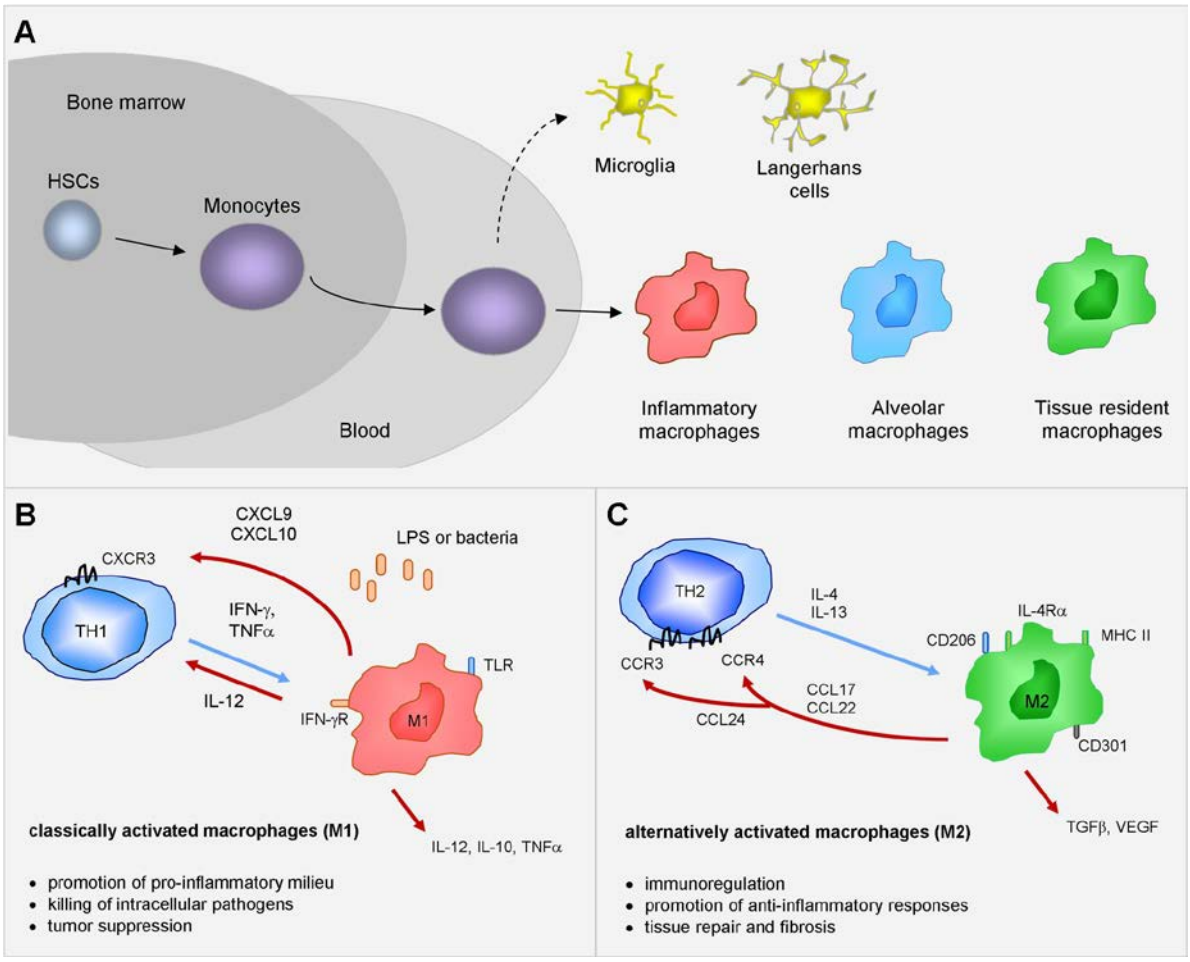

Figure 1: Macrophage development and function

A: Development of macrophages from hematopoietic stem cells and their differentiation into tissue-resident cells; B, C: Polarization of naive macrophages is dependent on external stimuli. B: Classical activation of macrophages (M1) after LPS or IFN-γ stimulation and C: alternative activation of macrophages (M2) through stimulation with IL-4 or IL-13. The figure is adapted for A: from Geissmann *et. al.* Science, 2010 [19] and for B, C: from Biswas & Mantovani, Nature Immunology, 2010 [23]

Depending on their function and expression profile, macrophages are divided into different subsets, but the exact definition of these phenotypes is controversially discussed. The polarization of macrophages is not a straight forward development; on the contrary, it has been reported that various intermediate macrophage phenotypes exist and changing stimuli might redirect macrophage polarization. Hence, macrophages are probably not terminally differentiated, instead they show a gene expression, which is linked to environmental triggers und stimuli. [18,24–31] The two main subclasses of macrophages are the classically activated macrophages (CAM, M1, Figure 1 B), which act pro-inflammatory, and the alternatively activated macrophages (AAM, M2, Figure 1 C) [32], which react more anti-inflammatory. In addition, certain 'disease-associated' macrophages and 'regulatory' macrophages exist [20]. Each subgroup plays an important and partly opposing role in immune responses and tissue homeostasis and might actively contribute to development or progression of disease, e.g. in promoting tumor angiogenesis [33–36] or in enhancing the process of liver fibrosis [22].

Classically activated macrophages are generated after IFN-γ– or LPS-induction by T helper cell 1 (T_H1) stimulation and STAT1 signalling to regulate the cellular immune reaction against pathogens and are involved in early resolution of inflammatory processes. They are able to produce high amounts of pro-inflammatory cytokines (e.g. IL-12, IL-1β or TNFα) and mediators such as reactive oxygen species. Molecules like IL-12, iNOS and COX are therefore used to classify macrophages as CAMs. Since these species are also be harmful for neighbouring healthy tissue, the reaction of these macrophages needs to be controlled to prevent extensive tissue damage by e.g. down-regulation of their pro-inflammatory activity through AAMs. [20]

In contrast to CAMs, AAMs are stimulated through a T_H2-induced signalling via IL-4 or IL-13. They up-regulate CD206, CD301 and MHC class II molecules, and secrete factors such as TGFβ and VEGF. AAMs have first been studied in worm and parasite infections, as well as in allergic reactions, but they also participate in humoral immune responses and tissue repair. The activation of genes in AAMs is mediated through the common IL-4Rα chain and STAT6 signalling. [30,37,38]

Both subgroups can further be regulated simultaneously or subsequently by the same factors, e.g. as it has been shown in mice, where adenosine is down-regulating CAMs, while at the same time AAMs are up-regulated [39]. Current transcriptome analyses add significantly to the knowledge about the expression of various factors in human macrophages and identified for example new gene clusters, as well as new CAM- or AAM-associated cell surface molecules [36].

To study macrophage activity and their function in human immune responses is challenging. It is not easy to obtain a macrophage cell line with remained macrophage profiles from tissue-resident macrophages, because isolating cells from the tissue might influence cell activity or activation. Therefore, studies are mostly performed on isolated monocytes primed with different stimuli. Mouse studies on the other hand are also intensely studied, but results are often hard to translate into the human system. [32]

For example, the role of arginine in mouse and human macrophages is highly discussed. The importance of the enzyme arginase and therefore the metabolisation of L-arginine to L-ornithine and urea in mice has been shown in various studies, e.g. the expression of inducible nitric oxide synthase

(iNOS) in mice [40,41]. On the other hand, its relevance in the human situation is questionable [41–43]. The same is true for the discussion of the role of expression of *arg1*, which is found in mouse macrophages and neutrophils and is an important mediator of T cell differentiation and macrophage priming into an alternative activation status [40]. In contrast, *arg1* is only expressed in human neutrophils and not in macrophages [42]. Nevertheless, it is supposed to be involved in the repair of the extracellular matrix [18,44], but seems to be no suitable molecule for macrophage classification in humans.
Various other processes are also associated with macrophage polarization, e.g. macrophages accumulate in obesity and secrete pro-inflammatory cytokines. In this disease the transition from anti- to pro-inflammatory macrophages promotes insulin resistance [45–48]. Beside their protective functions, macrophages are also involved in the pathogenesis of respiratory diseases such as asthma or in chronic obstructive pulmonary disease (COPD). The quantities between different macrophage subtypes is influencing the course of the disease, since CAMs in moderate asthma are less numerous than AAMs, but the number of CAMs in severe forms of asthma is strongly increased [49].

1.1.2 Macrophages in muscle diseases

In human skeletal muscle, macrophages are essential, since they contribute to the immune reactions and are potent regulators of distinct pathological reactions. For the investigated groups of muscle diseases, the processes of hypoxia and fibrosis are of special interest, as well as the regeneration of skeletal muscle.

Insufficient oxygen supply within the tissue is called hypoxia. It may be caused by pulmonary diseases, heart failure or different forms of vasculopathy and affects single or multiple tissues [50–54]. It has been shown in humans and mice that macrophages accumulate in areas of low oxygen and changes in the oxygen supply lead to distinct changes in macrophage gene expression. The up-regulation of genes involved in macrophage surviving/migration (e.g. *VEGF*, *MIF*) or in recruitment of other cells [55] affects the polarization of macrophages [56]. In addition, expression of various cytokines/chemokines is influencing the polarization by e.g. exertion of pro-inflammatory phenotypes through increased expression of the macrophage migration inhibitory factor (MIF) has been shown in acute respiratory distress syndrome [57]. Inflammation, healing of wounds and bacterial infections or tumors [58] can also cause hypoxia within the respective tissue. Many of the hypoxia-associated genes are regulated by the hypoxia-inducible factor 1 alpha (HIF-1α) and directly influence the efficiency of macrophages [56]. The vasculopathy in DM results in hypoxia, therefore this entity is a good example for macrophages reacting to hypoxic stress and changes in macrophage gene regulation is a crucial feature to investigate.

A further important aspect is development of fibrosis, which may be involved in very diverse cellular reactions as tissue remodeling, wound healing and replacement of specific mesenchymal tissue. It is mainly driven by AAMs and fibroblasts. The communication between macrophages and fibroblasts normally regulates wound healing and enhances fibrogenesis, but under varying conditions

macrophages may also cause reduced fibrosis [29]. In different fibrotic diseases, like idiopathic pulmonary fibrosis, systemic sclerosis or DMD, fibrosis is one of the predominant pathologies. The process of fibrosis can theoretically be targeted by specific therapy.

Depending on their phenotype and external stimuli macrophages react pro- or anti-fibrotic [59–61]. Activated macrophages promote fibrosis by secretion of cytokines such as TGFβ or IL-1β and stimulate fibroblast proliferation and survival. As it was shown in liver fibrosis blocking of those macrophages slow down the disease process [59,62]. In contrast, stimulation of the immune response additionally enhances fibrosis via secretion of anti-inflammatory cytokines and polarization of macrophages into wound healing phenotypes.

Since activation of fibrosis is necessary for wound healing, but overactive processes also lead to negative effects in various diseases, anti-fibrotic therapy could contribute to slowing down the progress of disease. Thereby the challenge is to identify the point of time for administration, since activation of macrophages at varying stages can be either helpful or detrimental [22,59,62]. Therefore, the timeline of action in the specific entity and the involved mediators are of high importance. A good example is DMD, where macrophage activation and polarization is time-dependent.

1.2 Skeletal muscle

Muscles can be categorized into three major subtypes, namely cardiac muscle, smooth muscle and skeletal muscle. While the movement of cardiac and smooth muscle (e.g. intestinal organs, blood vessels) is involuntarily, function and activity of the skeletal muscles are controlled by the central and the peripheral nervous system and are necessary for active movement of the body.

1.2.1 Structure and function of the skeletal muscle

During development, the long, cylindrical, multinucleated myofibres (muscle cells/muscle fibres/myocytes) are formed from progenitor myoblast cells through the process of myogenesis. The muscles of the extremities are anchored to the bones by tendons on each end.

The functional basis for muscle contraction are repeated actin and myosin filaments, so called sarcomeres, which can contract to reduce the muscle length (Figure 2). Thousands of myofilaments form myofibrils, which in turn build up myofibres. They are located in the sarcoplasm (= cytoplasm) and are encased by collagenous connective tissue, the endomysium, when coating a single fibre, and perimysium, when the tissue is located around a whole muscle bundle. The myofibrils are laminated with mitochondria and myonuclei are located close to the sarcolemma (= plasma membrane). Furthermore the fibrils are surrounded by a sarcoplasmic reticulum, which serves as calcium reservoir for the muscle contraction. The contraction of actin and myosin is due to the regulatory proteins troponin and tropomyosin, which are associated with actin. After stimulation, initiated by the motor neurons and transmitted by peripheral nerves to the motor endplate, calcium ions (Ca^{2+}) are released from the sarcoplasmic reticulum and troponin/tropomyosin undergo conformational changes to expose actin and facilitate the interaction with myosin. The contraction of the muscle is ATP-dependent. The energy is produced through an aerobic process in the mitochondria from carbohydrates or fat, or

anaerobically via glycolysis. Furthermore, to some extent ATP is also stored in the muscle and can be quickly released for muscle contractions. A major factor of this reaction is creatine kinase (CK), which catalyzes the conversion of ADP and phosphocreatine to creatine and ATP, restoring the energy source in muscle cells.

The skeletal muscle fibres can be divided into so called slow twitch and fast twitch fibres. The type I, slow twitch, red fibres are packed with mitochondria and myoglobin. The contraction is slow, with little force and lasts for a long time. The type II, fast twitch, white muscle fibres, contract much faster and generate high power. But in contrast to the slow fibres they fatigue rapidly.
To distinguish between slow and fast muscle fibres the staining with ATPase is quite common. In addition the determination of the myosin heavy chain (MHC) type of the fibre (MHC fast, slow, neonatal or developmental, various subtypes) is helpful. Healthy muscles contain a mixture of fibre types, and their proportions between muscles and between species vary. A type I fibre is surrounded by type II fibres and *vice versa,* producing a checkered pattern in the healthy muscle.

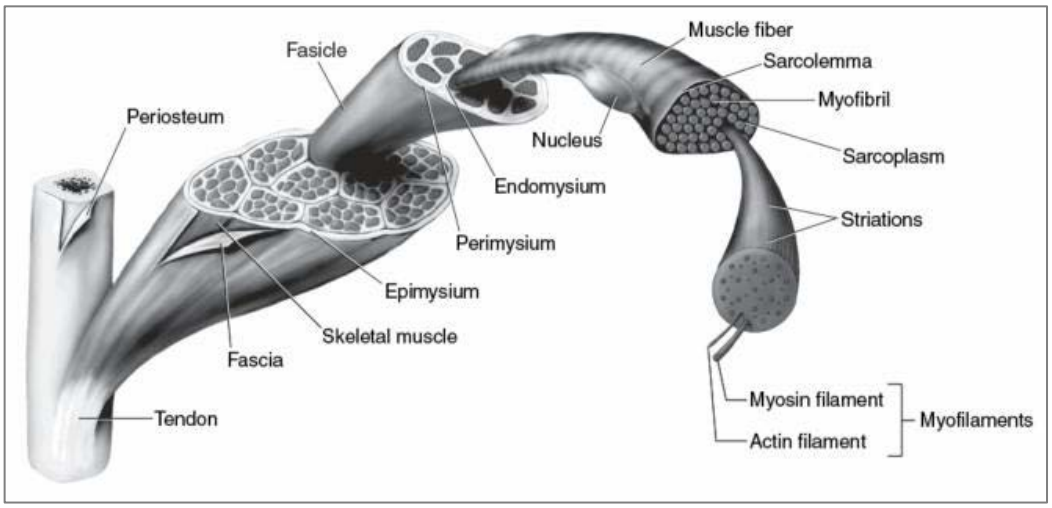

Figure 2: Organization of skeletal muscle, figure from W. Whiting and S. Rugg, 2005, Dynatomy

1.2.2 Interaction of skeletal muscle with other cells

Besides their function in movement and body stability, the cross-talk of muscle cells with other cells of the body is intense. Various immune cells reside within the muscle and can react to environmental changes. They are also active in repair mechanisms or secrete growth factors. Especially macrophages are important for the clearance of debris after muscle injury, but also the influx of other immune cells is necessary. For these processes a functioning cross-talk is vital and cells need to be in close contact to coordinate the reactions inside the muscle [63].
In case of injury or inflammation a well-orchestrated cascade is initiated. First, muscle cells secrete e.g. cytokines and chemokines (also termed myokines) which attract monocytes to the site of inflammation [64–66]. Accumulated monocytes, as well as resident cells are activated, resulting in the secretion of additional attracting factors. In addition to local accumulation, myokines can circulate in the body and reach other tissues. When the muscle is exercised, IL-6 is induced and influences glucose production [64]. After activation, the receptors for IL-1 or IFN-γ, as well as CCR2 and CCR4 are

also strongly up-regulated in muscle cells [67], hence leading to an expression of pro-inflammatory mediators such as TNFα or IL-1β. Following the activation of cells and a local pro-inflammatory phase a variety of myokines are released. After the 'resolution' of the inflammation, muscle regeneration is initiated, a crucial mechanism which involves differentiation of satellite cells and growth to myoblasts [29,68]. These myoblasts then fuse to myotubes and further differentiate to myofibres. For these processes of regeneration the switch from CAM to AAM is highly important, since CAMs are pro-inflammatory and stimulate the first resolution of inflammation (till around 2 days after injury), while AAMs support muscle regeneration and growth (from 2 days after injury on) [29,69,70]. In this context is has been reported that impaired generation of anti-inflammatory macrophage phenotypes lead to reduced regeneration rates [71]. In addition, endothelial growth factors such as IGF-1 and VEGF are essential for angiogenesis. Moreover, satellite cells and macrophages are also important producers of these factors, again demonstrating their involvement in muscle repair [72]. The continuously repetitive cycle of damage, repair and fibrosis in myopathies may lead to satellite cell exhaustion and fibre degeneration [68,73] as well as insufficient tissue remodeling. Therefore, targeting involved immune cells might be a strategy for modulating the course of disease.

1.3 Myopathy

According to the definition by the World Health Organization, myopathy is defined as a primary disease of the skeletal muscle that leads to a dysfunctional static. The different forms of myopathies are formally classified according to the 'International Classification of Diseases' and are divided into primary myopathies, other myopathies and myopathies with other underlying diseases (ICD: International Statistical Classification of Diseases and Related Health Problems; current version: ICD-10).

1.3.1 Diagnosis

To accurately diagnose a patient with myopathy various criteria are important. Acquired and inherited myopathies may display similar symptoms, but a correct diagnosis is of outmost importance for the decision on an adequate treatment [74].

Next to careful examination of the patient other points, like a complete clinical history, additional symptoms (e.g. cardiac involvement, involvement of other organs) and localisation of the affected muscle (via e.g. MRI - magnetic resonance imaging) should be considered.

Nowadays, muscle biopsies are less often performed, since an increased understanding of the underlying mechanisms and the development of new techniques (e.g. whole genome sequencing) make them less vital. Especially inherited muscle diseases are sometimes only identified via genetic techniques. Nevertheless, biopsies of 'skeletal muscle' are of outmost importance to get a detailed diagnosis. The same is true for conditions where the underlying gene is not identified yet. Furthermore, the expression pattern of certain relevant proteins involved in pathogenesis can be determined on muscle sections. With histology, enzyme and immune histochemistry and electron microscopy it is possible to identify structural abnormalities and a multitude of pathological processes.

1.3.2 Symptoms

A classical symptom of all myopathies is muscle weakness, but the distribution can strongly vary between different entities or single patients with the same entity. Further symptoms are stiffness, muscle atrophy or swelling, as well as cramps or myalgia. Problems with walking long distances, climbing stairs or difficulties with swallowing and speaking are described by the patients. Additionally, symptoms may be very complex, further affecting other organ systems as the heart, lungs or the skin. However, for each entity disease-characteristic symptoms have been defined and described.
The investigation of the serum may be helpful as well, because increased CK levels, lactate levels, liver enzymes etc. and/or elevated antibody titers are helpful. For some subgroups of myopathy autoantibodies are found and may be characteristic, although their precise pathogenetic relevance is still unclear in inflammatory myopathies. The muscle-related symptoms can arise through e.g. allergic reaction, side effects of drugs or exposure to toxic substances and previous autoimmune diseases trigger a secondary inflammatory reaction inside the muscle.

1.4 Idiopathic inflammatory myopathies (IIM)

Idiopathic inflammatory myopathies classically comprise three ‘related’ entities, which are Polymyositis (PM), Dermatomyositis (DM) and sporadic Inclusion body Myositis (sIBM). Recently IIMs have been extended by two further entities, which are the non-specific myositis [75] and the necrotizing myopathy (NM)/necrotizing autoimmune myopathy (NAM) [76]. Additionally, further reclassifications are constantly discussed [77,78] and the definition of specific entities, e.g. PM is not consistent [79–81]. With an incidence of 5-10 per 100,000 IIMs outnumber other rare inflammatory myopathies like eosinophilic myofasciitis, granulomatous myositis, focal myositis or macrophagic myofasciitis.
The five groups of IIMs share classical symptoms such as myalgia, muscle weakness and inflammatory infiltrates, however each group is also characterized by specific clinical and morphological features [77,80,82]. In some patients, myositis-associated (MAA) or myositis-specific autoantibodies (MSA) can be found.

The treatment of IIMs mostly includes medication with corticosteroids and physical therapy [76,82]. The efficacy varies between the groups. While DM and PM show relatively good responsiveness to immunosuppressive therapies, sIBM generally displays a slower development of symptoms and shows no substantial response to immunosuppressive therapy [74,83–88]. Most treatment regimens were not even tested in a controlled and randomized/standard fashion. They are often used in analogy to other treatment regimens for defined, presumably related inflammatory autoimmune diseases. This fact makes discussion and consensus description about effectiveness of ‘new’ options difficult and controversial.

1.4.1 Necrotizing myopathy (NM)

In recent years, patients with necrotizing myopathies [83,89] have been thoroughly described and discussed [90] and based on pathological criteria of immune and inflammatory myopathies [76] NM was

added to the conventional IIMs. In this classification, immune-mediated NM (IMNM) was described as an immune polymyopathy, whereas unique morphological features are mentioned. The most important criteria for diagnosis were myofibre necrosis, regeneration and a poor mononuclear cell infiltrate. Additionally, varying transsarcolemal major histocompatibility complex class I (MHC class I) positivity was described [91,92]. In contrast to the IMNM group a second subgroup of NM was postulated where a toxic agent causes the disease [93].

Patients with IMNM suffer from severe symmetrical proximal muscle weakness, myalgia, elevated serum CK levels and they show myofibre necrosis in their muscle biopsy [94]. Additional symptoms are fever, cardiac or pulmonary involvement and weight loss may occur in some IMNM patients but not in toxic-mediated NMs. The specific skin rash as seen in dermatomyositis is absent in IMNM. As for the entities DM and PM, the determination of MSAs and MAAs is an important part of the diagnostic work ups, and recently some NM-specific antibodies were found [95–98]. First, the detection of SRP-antibodies, a ribonucleoprotein, should be mentioned, because a correlation between the severity of clinical symptoms, serum CK levels and the level of SRP autoantibodies was seen [3]. Secondly, the 3-hydroxy-3-methylguanyl-coenzyme A reductase (HMGCoA) antibody is of interest. The latter was found in a subgroup of immune-mediated statin-induced NMs (also termed statin-induced immune NAM) [99]. Furthermore patients with anti-synthetase antibodies such as Jo1, PL7, PL12 or antibodies associated with paraneoplasia present a muscle pathology of NM [95,100–103]. These findings are particularly relevant since they indicate that the inflammation in the muscle is a secondary event, which follows a primary immune reaction against e.g. a tumour [104].

Under immuno-suppressive/-modulatory treatment, muscle symptoms decline, which further supports the significant involvement of immune reactions in development of disease [105,106]. Since repetitive disease flares are frequent in NM, patients may develop strong reactions to the treatment with corticosteroids and therefore additional therapeutic options are mandatory [105,107–109]. To date, methotrexate (MTX), intravenous immunoglobulins (IvIGs), plasma exchange and/or Rituximab are proposed, but are not evaluated in randomized controlled trials [108,110–112]. In contrast to IMNM, patients with a toxic aetiology do not display any additional clinical or morphological signs of an immune reaction, despite the presence of typical necrotic myofibres and myophagocytosis. Only the removal of the triggering agent contains a therapeutic character [99,113].

In a morphological context, various reports showed, that necrosis and myophagocytosis are predominant features in the muscle biopsies and that invading immune cells are found amongst the inflammatory infiltrate. These infiltrates mainly consist of macrophages and only few lymphocytes are found, whereas B cells are relatively sparse [75]. Furthermore an up-regulation of the membrane-attack-complex (C5b9) in muscle biopsies has been demonstrated, as was positivity for MHC class I on the sarcolemma. However, different authors reported other results for MHC class I and C5b9 expression [98,106,108,114].

1.4.2 Adult and juvenile Dermatomyositis

As part of the IIMs classical clinical symptoms of myopathies are found in dermatomyositis. These symptoms include weakness or pain, but in addition discriminating features, such as a very specific skin involvement is also common. The skin can show a characteristic rash on the décolleté, the eyelids and the cheeks, Gottron´s papules or calcifications on elbows and knees. [115]

The juvenile dermatomyositis (jDM) is the most common inflammatory myopathy in children with the incidence being 3-4/1,000,000 children. JDM is defined as DM with an onset of disease before the age of 16, with girls being more affected than boys [116–118]. Comparable to the classical symptoms of adult DM (aDM), juveniles show the following symptoms: Joint pain, fatigue and dysphagia. However, some symptoms and signs of jDM are unique and are not found in aDM such as calcification of the skeletal muscle. In addition, the children withdraw from their environment and have a slightly elevated body temperature. In acute cases complications like arthritis, intestinal perforation, pneumonia, myo- and/or pericarditis, cardiac arrhythmias, kidney failure or CNS involvement with central vasculitis have been described. In these cases the fast start of an adequate treatment is crucial for prognosis and progression of the disease [119–121]. Diagnostic evaluation comprises laboratory values, with the blood often showing increased CK values; even though the values can also be in the normal range. In about 40 % of DM cases myositis-specific or -associated antibodies are detected [10,122] such as anti-Mi-2 or anti-PL12. Some of these autoantibodies display a correlation to the clinical development, which hints at a connection between antibodies and immunogenesis [123], e.g. Mi-2 autoantibodies have not been associated with cancer and indicate a rather ‘benign’ course of disease.

For treatment, immune-modulating medication is used. Current steroid treatment, methotrexate (MTX) and intravenous gamma-globulins are combined. However, some patients need further therapy or treatment of drug-induced side effects. Anti-TNF antibodies or B cell blockers (e.g. Rituximab) have been used with varying success, but until today no double-blind studies have been performed to prove the efficacy of these treatments or concerning the molecular basis of the disease. [124–126]

Since the aetiology of DM is unclear, current studies address a broad variety of hypotheses concerning predisposition for and development of the disease. Amongst these, certain genetic predispositions [11], and a correlation with specific human HLA alleles were found (e.g. HLA-DRB1*03 or HLA-DQA1*0501) [11,127]. Furthermore, an association with polymorphisms of the *TNF* gene, encoding for the pro-inflammatory T_H1-related cytokine TNF-α, was suspected [128] and in jDM maternal microchimerism may be an influencing factor: During pregnancy, cells are exchanged between mother and child influencing biological processes of the foetus, and studies of Reed *et al.* show that children affected with jDM have higher amounts of maternal cells than their siblings. The cells were located within muscles and the peripheral blood [129] and are mostly either dendritic cells or B cells of the maternal peripheral blood [130]. Moreover, some studies show a link to infection of e.g. the respiratory tract or gastrointestinal symptoms [117,131]. Some studies focus on aspects of the immune response as the contribution of chemokines and cytokines, especially IFN-associated ones. It has been shown, that type I interferons e.g. IFN-α and IFN-β, are involved in the formation of tubuloreticular structures within endothelial cells and may be of diagnostic value [116,132]. Furthermore, they promote various cellular functions that are

involved in DM pathogenesis, e.g. MHC class I expression [133,134] or T cell activation [135]. Various genes regulated by type I interferons are up-regulated in jDM and aDM patients [136,137]. Additionally, type II interferons may contribute to DM and were also focus of different studies [138]. Other lines of research concentrate on the role of dendritic cells, especially plasmacytoid dendritic cells (pDC). These cells seem to be important for various reasons: first, they are a cellular source of huge amounts of type I interferons and second, mature cells are mostly located in the areas of lymphocytic infiltrates [139,140]. Additionally, T and B cells are found as well as significant amounts of macrophages [141,142]. However, the composition of various subtypes of inflammatory cells has not yet been precisely illuminated; neither have their specific contribution to certain pathomechanisms.

On muscle biopsy, typical perifascicular atrophy is seen, as well as infiltration by immune cells into the perimysium and the adjacent muscle tissue. Today it is commonly accepted that this perifascicular muscle fibre atrophy and ischemia as a result of the vasculopathy, are hallmarks of the disease [94,126,143,144]. However, the contribution of each of these features and their interrelationship are not well characterized and source of controversies [135]. By using 3D reconstructions Gitiaux and coworkers have elegantly shown that inflammation in larger arcade arteries may be more relevant to hypoxia-related pathology than capillary loss [145]. Various proteins were up-regulated on muscle fibres, which may have been a consequence to the hypoxic events.

1.4.3 Animal models in immune inflammatory myopathies

The use of animal models in the last years has increased our knowledge about various diseases and processes including myopathies [146–148]. Unfortunately most of the latter, such as DM or NM, can still not be accurately modeled in the mouse until today. Instead most current mouse models only mimic single aspects of IIMs and accompanying systemic features are often not displayed. For example a mouse model, where a viral infection is leading to development of muscle symptoms (e.g. macrophage accumulation, complement activation in muscle cells), but no skin involvement, only mimics parts of the pathogenesis of DM [146,149]. Another model to simulate PM is induced by immunization of mice with myosin, which led to infiltration of T cells and macrophages, but not of B cells [147].

To investigate the various aspects of myopathies an animal model would have the advantage to modify molecular pathways that were identified as relevant, but developing of models is still ongoing.

1.5 Muscular dystrophy (MD)

In contrast to inflammatory myopathies, muscular dystrophies are caused by mutated genes, which lead to defective muscle proteins and accompanied by a subsequent degeneration and/or necrosis and death of cells/tissue. The major forms of muscular dystrophy are classically grouped into X-linked dystrophies (e.g. Duchenne muscular dystrophy, Becker muscular dystrophy, Emery-Dreifuss muscular dystrophy), limb-girdle muscular dystrophies (various autosomal dominant and recessive forms) and others (e.g. congenital, distal, myofibrillar, fascioscapulohumeral and myotonic muscular dystrophies).

Symptoms frequently arise in early childhood and are getting more severe over time. The diagnosis of muscular dystrophies is based on evaluation of muscle biopsies, protein analyses, electrocardiography and genomic DNA analyses. [150–153] The diseases manifest as multi-system disorders involving different parts of the body such as the heart, gastrointerstinal system, the peripheral nervous system or brain. Next to symptoms of a myopathy, common signs of the disease are scoliosis, cardiomyopathy [154,155] and respiratory difficulties[156] .

1.5.1 Dystrophin

Muscular dystrophies can be caused by mutations of various proteins involved in the dystrophin-glycoprotein complex (DGC) that provides structural stability to the muscle cell by connecting the sarcomeres to the muscle membrane (Figure 3).

The gene *DMD*, which encodes for the dystrophin protein, is the largest known human gene and located on the short arm of the X-chromosome (Xp21.2). Mutations of dystrophin and subsequent disruption of the protein complex leads to progressive instability, fibre necrosis, inflammation, degradation and fibrosis. Some of these processes are linked to each other and may contribute to disease progression in DMD. Since the heart and respiratory muscles are also affected by muscle fibre loss, most of the patients die due to pneumonia or cardiomyopathy (approximately 10-40 % of all DMD patients [154,157]).

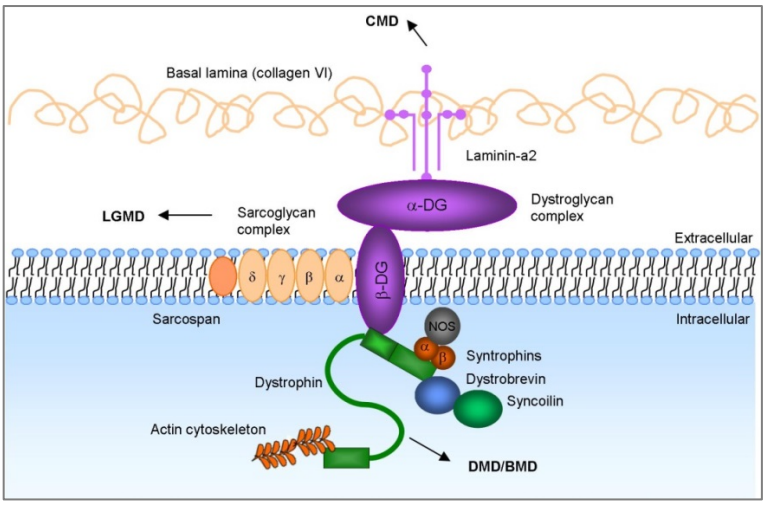

Figure 3: Organization of the dystrophin-glycoprotein complex
BMD: Becker muscular dystrophy; CMD: congenital muscular dystrophy; DG: dystroglycan; DMD: Duchenne muscular dystrophy; LGMD: Limb girdle muscular dystrophy; NOS: nitric oxide synthase; picture from 'Pharmacological strategies for muscular dystrophy' Khurana and Davies, Nature reviews 2008 [158]

1.5.2 Duchenne muscular dystrophy (DMD)

Duchenne muscular dystrophy is the most common form of the muscular dystrophies and worldwidely affects 1:5,000 male new-borns [94]. The recessive X-chromosomally inherited disease leads to a non-functional dystrophin protein followed by muscle necrosis, degeneration, fibrosis and a premature death. While both sexes can carry the gene mutation (60-70 % deletion, 15-30 % point mutations,

~10 % duplications) only males exhibit severe symptoms. In contrast, females rarely show any signs of the disease. Usually the symptoms appear before the age of three years and are often detectable during early infantile development. The first signs are proximal muscle weakness of legs and pelvis accompanied by a loss of muscle mass. The development of motor milestones is delayed and jumping or running is not possible at all. Eventually, weakness spreads and the symptoms grow stronger demonstrated by the classical Gowers´ sign [159]. At the age of 10-12 years most of the boys are wheelchair bound due to the developing fibrosis of the skeletal muscle and subsequent muscle weakness. Steroid treatment can only delay these changes. Symptoms are enhanced by use of the wheelchair and scoliosis and bone deformation develop. At night assisted ventilation is often used and boys may be later dependent on constant respirator supply. Additionally, mild cognitive impairment can be found frequently [160], since dystrophin also has a neuronal isoform. The average life expectancy of patients before modern respiratory assistance was around 20 years, but due to improved supportive therapy, it increased up to 40 years or even older in the last decade.

Treatment for DMD is mostly confined to supportive treatment of symptoms, which is of outmost importance and has been doubled the life expectancy. Since there is no direct cure for DMD until today, current therapies involve prednisolone or other corticosteroids, as well as the afore mentioned support of breathing, walking, orthopaedic- and physiotherapy. Consequently, new and more specific therapies are needed and various approaches are currently under investigation. One promising opportunity may be ‘exon skipping’, meaning skipping the mutated part of the gene, which results in a truncated but still functional dystrophin protein. However, clinical trials are currently beginning and the treatment may not be accessible worldwide and is probably only useful in a defined number of patients with specific gene mutations [161,162]. Additionally, other pharmacological approaches [163,164] are under investigation and new targets are tested in mouse models. As an example, studies using a double mutant mouse model showed that restoration of 15-20 % of dystrophin can enhance the muscle strength and protect the muscle fibre from exercise-induced damage [165]. Furthermore, increasing the muscle mass by insulin-like growth factors and improving calcium homeostasis [158,166] are goals of the research. Associated secondary processes, such as the role of mast cells [167] or the deregulation of the neuronal nitric oxide synthetase (nNOS) [168] are also of interest.

It has been shown many times that immune inflammatory processes are strongly involved in the pathogenesis of DMD and that these processes are disease-specific. They are due to a dysregulation of inflammatory pathways, since it is clear that the influx of mononuclear cells and granulocytes occurs early in dystrophic muscles of mice [156,169,170]. In addition to cellular influx, various cellular processes, e.g. endomysial fibrosis, are developing over time. Thereby, the amount of fibrosis within the muscle correlates with a bad outcome and an early loss of ambulation, which has been shown in a clinical study of DMD patients and a 10-year follow-up period. [171–174] Both processes, inflammation and fibrosis, and their interrelationship are not well understood and need to be investigated in more detail [169]. In some muscle biopsies so called ‘revertant’ fibres are found that by definition ‘re’-express the dystrophin protein. Alternative splicing leads to a shift inside the reading frame of the *DMD* gene and therefore a truncated but functional dystrophin protein can be detected [175,176]. These fibres might be

targeted by immune cells [16]. Up-regulation of utrophin in the sarcolemma, a homolog of dystrophin, is also characteristic in DMD patients. In this regard, mechanisms involved in up-regulation of utrophin and utrophin-associated genes is also currently under investigation [177] .

1.5.3 Animal models of Duchenne muscular dystrophy

In contrast to the other myopathies, where no appropriate animal models exist to date, in DMD, various animals are used to study the disease hallmarks. Although there are some distinctions between the mouse and human situation the *mdx* mouse is widely used as 'standard' model to analyse inflammation, fibrosis and new targets for therapy [178–181]. In the *mdx* mouse, which carries a spontaneous genetic deficiency and is not genetically engineered, fewer/less severe symptoms occur and with increasing age the phenotype overall decreases [178]. Since it was shown that utrophin diminishs the effects of dystrophin loss, the utrophin knockout mouse model is also used (*utrn*$^{-/-}$) [182], even though, it is not directly a model of DMD. Other mouse models are the *utrn*$^{-/-}$*mdx* double knockout or *utrn*$^{+/-}$*mdx* heterozygous knockout mouse, where the development of disease is more similar to the human situation than in the respective single knockout. In these two mouse lines increased fibrosis and stronger clinical symptoms such as cardiomyopathies develop, and additionally, a reduced life expectancy is observed. [183,184]

Various projects underline the importance of DMD mouse models for the testing of new therapies and for increasing the understanding of involved processes. As an example, the arginine metabolism is under investigation, because arginine is often used as dietary supplement in DMD patients without a clear understanding of the long term effects. In mice, short-term treatments seem to be beneficial [185], but after a long-term use of arginine supplements, an increase of fibrosis due to an enhanced alternative polarization of macrophages was shown in *mdx* mice [186]. In line with these findings the reduction of nitric oxide synthase (NOS) exacerbates muscle inflammation and fibre damage in mice [187]. Therefore, the competition and balance between both macrophage subtypes are of outmost importance in progression of disease in the mouse model [188,189]. Furthermore, associated symptoms of DMD have to be studied. Since cardiomyopathy is one of the main causes of death, various groups have performed experiments to improve cardiac health. In this field Schinkel *et. al.* generated an adeno-associated virus to increase the expression of microdystrophin inside the heart and to protect intact cardiac morphology [190].

Next to the mentioned mouse models, further model organisms are used to analyse the disease. There are various canine models, such as the golden retriever (GRMD), Rottweiler or German short-haired pointer. The development of disease is comparable to that in humans and many of the characteristic features of DMD are found (e.g. fibrosis, regeneration, cardiomyopathy, respiratory distress). Additionally, a feline model of DMD exists, but the development of disease and its severity are different from that in the human situation. [178,191,192]

1.5.4 Becker muscular dystrophy

Becker muscular dystrophy (BMD) is a milder variant of muscular dystrophies (genetically allelic to DMD) caused by a reduced expression of dystrophin leading to a shortened but functional isoform of dystrophin. This muscular dystrophy is occurring in approximately 3 - 6:100,000 male new-borns and is therefore much less common than DMD.

BMD patients mostly exhibit weak symptoms at an age of 10-25 years; however, there are highly variable courses of the disease. Some patients develop walking problems in their adolescence and others remain ambulant until their fifties or sixties [193]. In addition, the life span of these patients may be reduced or in the normal range. The risk of sudden cardiac death due to cardiac insufficiency (hypertrophic cardiomyopathy) is higher and patients are also prone to develop rhabdomyolysis. In the muscle biopsy a reduced expression of dystrophin is found, whereas often only the N-terminal fragment of the dystrophin protein is affected.

1.5.5 Female carrier of mutations of the *DMD* gene

Apart from spontaneous mutations (~$^1/_3$ of patients [94]) DMD and BMD are X-chromosomally inherited. In girls who carry the mutated gene, the normal, not-mutated gene can compensate for the affected one. Female carriers have a 50 % chance of passing the mutated gene on to their offspring [194] (Figure 4).

The affected women are mostly asymptomatic but few (between ~3-8 %) can show symptoms in varying degrees [194]. Those can be weak or range up to DMD-like symptoms including myalgia, elevated CK-levels, cramps and cardiomyopathies. [194] In the biopsy a mosaic pattern of dystrophin expression is found due to the scattered X-inactivation in the cells.

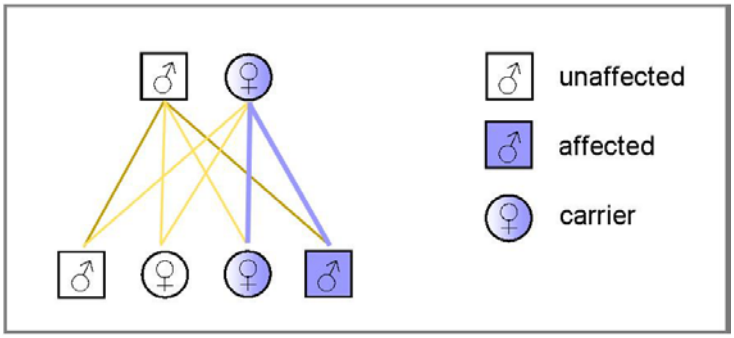

Figure 4: X-linked recessive inheritance of the defective *DMD* gene
Mother are carriers (fC) of the defective gene and statistically pass the disease to 50 % of her offspring.

1.6 Aim of the project

In the human body numerous and variable interactions occur between different key molecules of the immune system, such as lymphocytes or macrophages, and different tissues, including the connective tissue, blood vessels and the skeletal muscle.

The aim of the study is the examination and morphological description of those interactions but also the analyses of specific and characteristic pathological phenomena, e.g. hypoxia or fibrosis. Since the latter features are hallmarks of distinct human muscle diseases, the human skeletal muscle is a

perfectly suitable system to study these various aspects and their interplay. In this work I have focused on specific disease entities that have an 'inflammatory part' in common. However they all exhibit different, but very specific features that can be described as pathological patterns (e.g. fibrosis, atrophy, necrosis, hypoxia …). These phenomena are suggested to be directly or indirectly linked to specific inflammatory processes.
For the analyses of the entities different hypotheses were leading the investigation. In general, I assumed that macrophages hold an important role in the development and progression of all investigated diseases and therefore their role is addressed in detail.

Hypothesis I:
In the necrotizing myopathies acute immune processes lead to the development of disease in autoimmune NM in contrast to toxic NM. I postulate that it is possible to differentiate the immune response in the immune-mediated cases, compared to the toxic aetiology and that there are distinct subtypes. Lymphocytes and macrophages should be important in IMNM, while I expect only low numbers in nIMNM. I further assume that the different subtypes are distinguishable through their cytokine profile and that these can putatively serve as diagnostic tools to distinguish the different aetiologic backgrounds.

Hypothesis II:
Based on the well described morphological criteria for dermatomyositis and recent work about vessel organization, I investigated the severity of the hypoxia-related pathology. My particular interest is the effect of hypoxia on perimysial and centrally located muscle fibres in jDM in comparison to aDM. Since children are known to display a stronger phenotype of DM in comparison to adults, I expect to find differences with respect to inflammatory infiltrates and mechanisms of hypoxia. These results would also support the assumption, that inflammation and hypoxia are linked and that macrophages play a central part in both processes.

Hypothesis III:
The development of fibrosis describes a major feature of skeletal muscle degeneration in Duchenne muscular dystrophy. Since alternatively activated macrophages have been linked to fibrosis in several diseases, I assume that in DMD the influx and the amount of anti-inflammatory and wound-healing macrophages is stage-dependent.

Little is known about the exact contribution of macrophages to these various pathomechanisms in human skeletal muscle diseases. However, the knowledge about molecular differences, especially concerning events in the progression and severity of the disease triggered by macrophages are of outmost importance. New insights on pathogenesis of the entities could create a way to find novel therapeutic approaches to treat inflammatory myopathies or muscular dystrophies.

2 MATERIAL

2.1 Laboratory equipment, commercial kits and buffer

Table 2: Laboratory Equipment

Product	Purchased from
Agilent 2100 Bioanalyzer	Agilent Technologies, USA
PCR Cycler	VWR, Germany
PCR Cycler	Eppendorf, Germany
Kryostat HM560	Microm Laborgeräte, Germany
Microplate Reader TECAN Infinite 200	Tecan, Germany
Olympus BX50 Microscope & DP25 Camera	Olympus, Japan
Zeiss Observer.Z1 Microscope	Zeiss, Germany
Multifuge SR3+	Thermo Scientific, Germany
Real-Time PCR System 7900 HT	Applied Biosystems, USA
Pannoramic 250	3DHistech, Hungary

Table 3: Commercial kits

Product	Purchased from
AEC Staining Kit (AEC101)	Sigma-Aldrich, Germany
BCA Protein Assay Kit	Pierce, USA
DNAfree Kit Ambion	Ambion, Germany
High-Capacity cDNA Archive Kit	Applied Biosystems, USA
TaqMan Fast Universal PCR Master Mix (2x)	Applied Biosystems, USA
Liquid DAB+ Substrate Chromogen System	DAKO, Germany
PEN Membrane Glass Slides	AlphaMetrix, Germany
CapSure® LCM Cap	AlphaMetrix, Germany
HistoGene® LCM Frozen Section Staining Kit	AlphaMetrix, Germany

Table 4: Chemicals and buffer

Product	Purchased from
Aceton	Merck, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany
Trizol	Peqlab, Germany
Glycogen	Invitrogen, Germany
Chloroform	Merck, Germany
Isopropanol	Roth, Germany
EtOH abs.	Roth, Germany
Citric acid	Merck, Germany
Elastica Solution (resorcin fuchsin)	Th. Geyer GmbH & Co. KG, Germany
Eosin-Y	Brunschwig Chemie, Netherlands
Xylol	Baker, Germany
FCS (Fetal Calf Serum)	PAA, Austria
Nuclease-Free Water	Quiagen, USA
Kernecht-Aluminium sulfate solution	Merck, Germany
Lugol's Solution	Merck, Germany
Mayers Hematoxylin Solution	Merck, Germany
Methyl violet	Merck, Germany
Normal serum goat	Vektor Laboratories, USA
Normal serum mouse	Jackson Immunoresearch, USA
Normal serum rabbit	Vektor Laboratories, USA
ROTI-HISTOKITT II	Roth, Germany
2. AK Kit	Ventana/Roche, Switzerland
Tissue Tek	Thermo Sientific, Germany
Bovine serum albumin (BSA)	Sigma-Aldrich, Germany
Dulbecco's Phosphate buffered saline (PBS) w/o Ca^{2+}/Mg^{2+}	Biochrom, Germany
RPMI	PAA, Austria

2.2 Antibodies

Table 5: Antibodies used for immunohistochemistry

Specificity	Host	Clone	Dilution	Purchased from
αB-Crystallin	mouse	G2JF	1:10	Novocastra, Great Britain
C5b9	mouse	aE11	1:200	DAKO, Germany
CD4	rabbit	SP35	undiluted	ZYTOMED Systems GmbH, Germany
CD8	mouse	144B	1:100	DAKO, Germany
CD11c	rabbit	EP1347Y	1:50	Abcam, Great Britain
CD20	mouse	L26	1:400	DAKO, Germany
CD31	mouse	7C70A	1:25	DAKO, Germany
CD45	mouse	2B11	1:400	DAKO, Germany
CD56	mouse	monoclonal	1:200	GeneTex Inc, USA
CD68	mouse	KP1	1:10,000	DAKO, Germany
CD79a	mouse	7CB117	1:100	DAKO, Germany
CD138	mouse	MI15	1:30	DAKO, Germany
CD206 / MRC1	mouse	7-450	1:500	Acris, Germany
CD301	mouse	125A10.03	1:200	Dendritics, USA
Collagen VI	mouse	VI-26	1:200	Merck, Germany
Dystrophin	ascites fluid	MANDYS8	1:10	Sigma-Aldrich, Germany
Factor VIII	rabbit	Polyclonal	1:1000	DAKO, Germany
HIF-1α	mouse	H1alpha67	1:40	Abnova, Taiwan
iNOS	rabbit	polyclonal	ready to use	GeneTex Inc, USA
Laminin-α5	mouse	4C7	1:30,000	Chemicon, Germany
MHCneonatal	mouse	NB-MHCn	1:20	Novocastra, Great Britain
MHC class I	mouse	W6/32	1:1000	DAKO, Germany
MHC class II (anti-HLA-DM)	mouse	CR3/43	1:100	DAKO, Germany
Utrophin	rabbit	polyclonal	1:10	Sigma-Aldrich, Germany
VEGF	rabbit	15D2	1:50	CellSignaling, USA
Secondary antibodies	**Host**	**Clone**	**Dilution**	**Purchased from**
AF 633	Goat anti rabbit	A-21070	1:100	Invitrogen, Germany
Cy3	Goat anti rabbit	111-166-003	1:100	Dianova, Germany
Cy3	Goat anti mouse	115-166-003	1:100	Dianova, Germany
AF 488	Goat anti rabbit	A-11008	1:100	Invitrogen, Germany
AF 488	Goat anti mouse	A-11001	1:100	Invitrogen, Germany
AF 647	Donkey anti mouse	A-31571	1:100	Invitrogen, Germany
FITC	Goat anti mouse	115-096-003	1:100	Dianova, Germany
FITC	Goat anti rabbit	111-096-003	1:100	Dianova, Germany

2.3 Quantitative Real-Time PCR Assays

Table 6: TaqMan Assays from Applied Biosystems, USA

TaqMan® Gene Exp Assays	Assays number
TaqMan® Gene Exp Assay *ARG1*	Hs00968979_m1
TaqMan® Gene Exp Assay *CCL17*	Hs00171074_m1
TaqMan® Gene Exp Assay *CCL18*	HS00268113_m1
TaqMan® Gene Exp Assay CD206 (*MRC1*)	Hs00267207_m1
TaqMan® Gene Exp Assay CD301 (*CLEC10a*)	HS00197107_m1
TaqMan® Gene Exp Assay cox2 (*PTGS2*)	Hs00153133_m1
TaqMan® Gene Exp Assay *CXCL13*	Hs00757930_m1
TaqMan® Gene Exp Assay *HIF1A*	Hs00153153_m1
TaqMan® Gene Exp Assay iNOS (*NOS2*)	Hs01075529_m1

TaqMan® Gene Exp Assay *IFNA*	Hs00265051_s1
TaqMan® Gene Exp Assay *IFNG*	Hs00989291_m1
TaqMan® Gene Exp Assay *IL1B*	Hs01555410_m1
TaqMan® Gene Exp Assay *IL4*	Hs00929862_m1
TaqMan® Gene Exp Assay *IL4R*	Hs00166237_m1
TaqMan® Gene Exp Assay *IL5*	Hs00174200_m1
TaqMan® Gene Exp Assay *IL6*	Hs00985639_m1
TaqMan® Gene Exp Assay *IL10*	Hs00961622_m1
TaqMan® Gene Exp Assay *IL12p40*	Hs01011518_m1
TaqMan® Gene Exp Assay *IL13*	Hs99999038_m1
TaqMan® Gene Exp Assay *IL17a*	Hs00174383_m1
TaqMan® Gene Exp Assay *IL21*	Hs00222327_m1
TaqMan® Gene Exp Assay *IL27*	Hs00377366_m1
TaqMan® Gene Exp Assay *IL27R*	Hs00175472_m1
TaqMan® Gene Exp Assay *MIF*	Hs00236988_g1
TaqMan® Gene Exp Assay *PGK1*	Hs99999906_m1
TaqMan® Gene Exp Assay *P4HA1*	Hs00990001_m1
TaqMan® Gene Exp Assay *RORG*	Hs01076122_m1
TaqMan® Gene Exp Assay *SOCS1*	HS00705164_s1
TaqMan® Gene Exp Assay *STAT1*	Hs01013989_m1
TaqMan® Gene Exp Assay *STAT2*	Hs01013123_m1
TaqMan® Gene Exp Assay *STAT3*	Hs00374280_m1
TaqMan® Gene Exp Assay *STAT6*	Hs00598625_m1
TaqMan® Gene Exp Assay *TGFB1*	Hs00998133_m1
TaqMan® Gene Exp Assay *TNFA*	Hs00174128_m1
TaqMan® Gene Exp Assay *VEGFA*	Hs00900055_m1

3 METHODS

3.1 Patients and biopsy specimens

Biopsied muscle tissue of all patients and patients' muscle specimens that served as normal controls were analyzed retrospectively. Biopsies were taken for diagnostic reasons; no post-mortem samples were used. Based on specific clinical and laboratory parameters patients were assigned to certain entities (IIM) or diseases (DMD, BMD, fC).

Patients with necrotizing myopathies were further divided into two subgroups (16 patients with IMNM, 8 with nIMNM). Assignment to the immune-mediated aetiology was based on at least one of the following parameters: myositis-specific (MSA) or myositis-associated (MAA) autoantibodies or autoantibodies associated with paraneoplastic disease, and/or defined autoimmune disease. In contrast, the non-immune-mediated subgroup did not display any autoantibodies nor autoimmune disease or neoplasia. By definition, a causative toxic agent was identified in each patient suffering from nIMNM, whereas none of the patients with IMNM received statins prior to the muscle biopsy. Other conditions such as muscular dystrophy, especially dystrophinopathy or dysferlinopathy, have been carefully excluded in the study about NM.

For the dermatomyositis study all patients showed clinical signs of dermatomyositis diagnosed according to international clinicopathological consensus criteria [115,195], e.g. skin involvement, dysphagia and muscle pain or weakness. Furthermore, electron microscopy showed tubular reticular formations. Groups were further defined depending on the age of patients: age <16 years: juvenile (15 patients), >16 years: adult (11 patients).

Patients with Duchenne muscular dystrophy were clearly diagnosed on the typical clinical picture and a muscle biopsy. For most of the patients' gene analyses were available to determine the distinct *DMD* gene mutations (25 patients). Control groups with Becker muscular dystrophy (6 patients) and female carriers of the mutated *DMD* gene (5 patients) were analyzed and genetically proven.

Normal controls constituted skeletal muscle biopsies derived from neurologically healthy patients of different age and sex, as well as without any histological or ultrastructural abnormalities, including absence of inflammatory infiltrates or MHC class I up-regulation, and absence of all relevant parameters associated with myositis (e.g. autoantibodies, signs of systemic inflammation). Normal control individuals were biopsied due to diffuse and rather nonspecific complaints, such as myalgia or fibromyalgia.

All biopsies were single biopsies and the area analyzed did not significantly differ between the groups (measured with an Olympus BX50 microscope and the cell^D software). The entire section of each biopsy was analyzed, and available clinical information of patients is given in Supplemental Table 1 - Supplemental Table 4. Informed consent was obtained from all patients, and the Charité ethics committee has approved the studies (inflammatory myopathies (necrotizing myopathy and dermatomyositis): No. EA1/204/11, Duchenne muscular dystrophy: No. EA1/170/11). All muscle biopsy specimens had been cryopreserved immediately after removal at -80 °C prior to diagnostic work-up.

3.2 Histology, enzyme histochemistry, and immunohistochemistry

All stainings were performed on 7 µm thick cryosections, produced with the 'Microm Cryo-Star HM 560' (Microm Laborgeräte, Germany). The sections were stored unfixed at -20 °C or fixed at -80 °C until staining. Fixation was performed by incubation in acetone for 10 min.

The 7 µm thick cryostat sections were stained with various antibodies (detailed list see below and for information about antibodies see section 2.2) using the iview-Ventana DAB Detection Kit with appropriate biotinylated secondary antibodies and diaminobenzidine (DAB)-visualization of the peroxidase reaction product on a Benchmark XT immunostainer in a standardized manner (both Ventana, Arizona, USA). For double staining of two different cellular fractions the protocol was repeated for the second primary and secondary antibody. Visualization with AEC (3-amino-9-ethylcarbazole, red) was used for the second reaction. Omission of primary antibodies in control sections resulted in absence of any cellular labelling.

Additional stains and staining for immunofluorescence were performed in staining chambers with or without fixation in acetone. The sections were then blocked with the appropriate serum (1:10 in PBS) dependent upon the source of the secondary antibody and incubated with the afore-mentioned primary antibodies over-night at 4 °C or for 1h at RT. After a washing step, the secondary antibody was added for 1h. For double immune staining, with the purpose to show co-localisation of two cellular or subcellular structures, the above-mentioned protocol was performed for the first antibody and afterwards the same protocol was repeated with the second primary antibody and appropriate secondary antibody. After a final washing step, the sections were mounted and stored at 4 °C.

Photomicrographs were taken with the Zeiss Observer.Z1 Microscope (immunofluorescence) with the Axiovision 4 Software (Zeiss, Germany) or with the Olympus BX50 microscope, digital camera DP25 and the Cell^D software (Olympus, Germany).

Histochemistry:

Hematoxylin and eosin (H&E): Cell nuclei are stained in blue, while cytoplasm, connective tissue and other extracellular substances are colored in pink by eosin.

Gömöri trichrome: cell nuclei are stained in red-purple, normal muscle myofibrils in green-blue, intermyofibrillar muscle membranes are red and interstitial collagen is stained in green.

Elastica Verhoeff's van Gieson (EvG): Collagen is stained in pink or deep red and other forms of connective tissue are colored yellow and elastic fibrils in vessels are black.

Immunohistochemical demonstration of cells expressing:

CD4, CD8, CD11b, CD20, CD31, CD45, CD68, CD79a, CD138, CD206, CD301, deposition of complement C5b9 (MAC), endothelial staining by Factor VIII (Von-Willebrand-Factor), expression of major histocompatibility complex (MHC) class I / II or myosin heavy chain neonatal on the sarcolemma, HIF-1α, Laminin-α5, vascular endothelial growth factor (VEGF-A) and presentation of CD56 (NCAM) and αB Crystallin.

Antibodies, secondary antibodies, as well as other reagents used for histochemistry were stored according to the manufacturer at 4 °C or -20 °C.

3.3 Electron microscopy

Ultrastructural analyses of all muscle specimens was performed for diagnostic after fixation in 2.5 % glutaraldehyde for 48 hours at 4 °C, post fixation in 1 % osmium tetroxide and embedding in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, after 3-5 blocks had been generated from each sample. Electron microscopy was performed with a Zeiss P902 electron microscope (Zeiss, Germany).

3.4 Laser microdissection

Laser microdissection was performed in cooperation with the 'Neuromuskuläres Zentrum Ruhrgebiet' in Bochum, Germany. The technique was applied as a method to specifically cut out regions of interest and perform comparative cytokine/chemokine expression level analysis via quantitative real-time polymerase chain reaction (qPCR) after laser dissection. Cryostate slices (15 µm thick) were prepared on nuclease and human nucleic acid free PEN-membrane Slides from MicroDissect GmbH (Leica, Germany). After H&E staining with diethylpyrocarbonate (DEPC) treated, autoclaved H_2O the slides were air-dried.

Areas of interest within the muscle, as well as adjacent 'normal appearing' tissue from patients with dermatomyositis and healthy tissue from control patients was isolated using a laser microdissection system (Leica LMD6500). From each biopsy 500,000 μm^2 were collected into a tube cap by focal melting of the membrane through the laser. Immediately after the dissection the tissue was resolved in 500 µl trizol and stored at -80 °C until RNA isolation.

3.5 Quantifications

3.5.1 Semi-quantitative score

We defined a semi-quantitative scale to compare patients with IMNM and nIMNM. All cases were graded from 0 (none) to 4 (maximum) based on different histological stainings, and characterized two times in a blinded manner by three independent myopathologists. Mean values from both scaling rounds are displayed.

MHC class I expression (see Figure 5)

0	no sarcolemmal staining
1	> 10 % of fibres with sarcolemmal staining
2	> 30 % of fibres with sarcolemmal staining
3	> 60 % of fibres with sarcolemmal staining and scant sarcoplasmic staining
4	> 90 % of fibres with sarcolemmal and strong sarcoplasmic staining

C5b9 complement (MAC) deposition	
0	no capillaries stained
1	> 10 % capillaries (some/few)
2	> 30 % of capillaries (focal)
3	> 60 % of capillaries (large areas)
4	> 90 % of capillaries show MAC deposition

$CD56^+$ and MHC $neonatal^+$ fibres	
0	no fibres stained
1	> 10 % of fibres stained
2	> 30 % of fibres stained
3	> 60 % of fibres stained
4	> 90 % of fibres stained

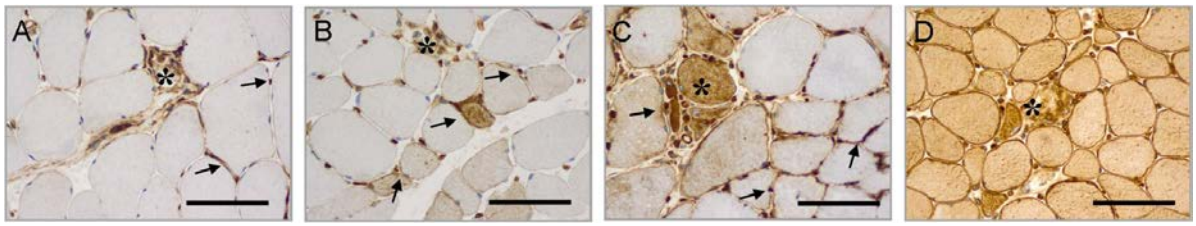

Figure 5: Semi-quantitative score of MHC class I immune reactivity in muscle biopsies from NM patients
The photomicrographs illustrate the classification of cases with different degrees of MHC class I expression. For details on the score see above; A = > 10 %; B = > 30 %; C = > 60 %; D = > 90 %; scale bar = 100 µm

3.5.2 Cell counts

For quantification of $CD4^+$ or $CD8^+$ T cells, $CD20^+$ or $CD79a^+$ B cells, $CD138^+$ plasma cells, as well as $CD68^+$ macrophages cell counts of 10 high power fields (HPF; one high power field is defined as 0.16 mm^2) in whole biopsy sections of each patient were performed. The average number of cells/HPF per case is displayed.

3.5.3 Quantification of collagen VI

For quantification of fibrosis an automatic analysis of entire whole slide images (WSI) of collagen VI stained muscle biopsies was performed with the Pannoramic 250 (3DHistech, Hungary). A frameless tessellation of WSIs at 10x magnification was applied to quantify regions of interest (ROI) piecewise with the 'DMD Quantifier' Plugin for the Cognition Master Software [196]. ROIs were defined using manual annotation. Each slide must contain at least one positive region (pROI) to be included. Each pROI may contain multiple negative ROIs, which are excluded (nROI).
For each image tile of 2,000 x 2,000 pixels a color deconvolution using one color vector triplet over the entire collective is applied to separate muscle fibres and connective tissue as an initial step. Subsequently minimum threshold is applied to the muscle layer and Otsu threshold is used for the

connective tissue layer for segmentation. Subsequently muscle segments are created using subtraction of initial connective tissue and muscle layer. Resulting segments are refined using morphological operations and filtered by area, form factor of contour (e.g. round, angular or elongated) and standard deviation of pixel intensities to remove artifacts. Finally areas of objects for both classes are assessed, summarized and their ratio was calculated. During processing of entire WSIs, all analyzed tiles together with a classified false-color image (Figure 6) and calculated results for each tile are saved for review and further usage.

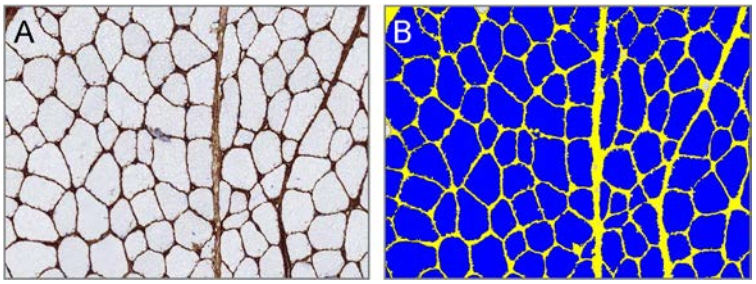

Figure 6: Quantification of fibrosis by Collagen VI staining of muscle biopsies from DMD patients
A: Original ROI; B: processed picture of ROI; blue is defined as are of muscle cell, yellow was assessed as connective tissue

3.5.4 Capillary density

To determine the capillary density and number of muscle fibres in patients with DM, staining with laminin-α5 was manually counted with the ImageJ software on 10 HPF in whole biopsy sections of each patient. The average ratio of capillaries/fibres per case is displayed.

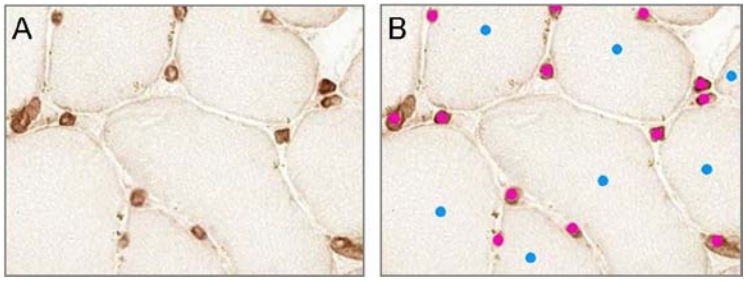

Figure 7: Analysis of capillary density in DM patients
A muscle biopsy specimen with DM was stained with an antibody directed against laminin-α5; determination of capillaries (red) and fibre number (blue) and both structures were counted manually.

3.6 Molecular biological methods

3.6.1 RNA isolation and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from the muscle tissue samples using the trizol/chloroform method according to the manufacturer's instruction (Invitrogen, USA). Thereafter, RNA was resuspended in DEPC-treated water and the concentration of RNA was photometrically determined with a TECAN® fluorescence plate reader 'infinite M200' (Tecan, Switzerland). Due to limited amounts of tissue after

isolation with laser microdissection the amount of all chemicals were reduced to ¼ of the normal amount.

To eliminate contaminating DNA a DNase free Kit (Applied Biosystems, USA) was used. The RNA was then reversely transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) according to the manufacturer's protocol by usage of 1 µg or 2 µg total RNA per sample [93]. In a next step, the expression levels of gene-transcripts and of the endogenous control gene, phosphoglycerate kinase 1 (*PGK1*), were analyzed by qPCR using the 5'-nuclease technology on an ABI PRISM® 7900HT Sequence Detection System and the human TaqMan® pre-developed assay reagents (both: Applied Biosystems, USA). The expression level of the housekeeping gene *PGK1* was comparable in all analyzed samples and unaffected by duration of the various diseases. The assay identification numbers are as listed in Table 6.

The qPCR reactions were prepared in a final volume of 20 µl, with the TaqMan® Fast Universal PCR Master Mix (Applied Biosystems, USA), containing 20 ng cDNA. All analyzes were performed in triplicate, the baseline was set automatically and the threshold cycle (Ct) was determined. The figures display either the reciprocal ΔCt (= Ct_{target} - $Ct_{endogenous\ control}$) to show gene expression of the respective molecules compared to normal controls or the log10 of RQ-values (fold-change = $2^{-\Delta\Delta Ct}$) demonstrating the regulation of patient expression levels as fold-change compared to the normal controls ($\Delta\Delta$Ct = ΔCt_{sample} - $\Delta Ct_{normal\ control}$). In these cases 0 demonstrates an expression on level of healthy controls, 1 ≙ 10-fold increase, 2 ≙ 100-fold increase, etc. Evaluation of results was performed with the Applied Biosystems SDS 2.3 & 2.4 software, as well as with the RQ Manager 1.2.1 (both Applied Biosystems, USA).

3.7 Statistics

For analyses of the semi-quantitative score of histological stains, cell counts and capillary density the Mann-Whitney *U*-test was used. Furthermore, the Kruskal-Wallis one-way analysis of variance with Dunns correction was applied for analyses of quantitative differences of mRNA transcripts. Data are presented as means with standard deviation. The level of significance was set at $p < 0.05$. Statistics were calculated with the software GraphPad Prism 5.02 (GraphPad Software, USA).

4 RESULTS

4.1 Summary of patients enrolled in the project

All groups of patients were characterized and grouped according to the underlying disease. For the project, different numbers of patients with necrotizing myopathy (NM), dermatomyositis (DM) or Duchenne muscular dystrophy (DMD) were analyzed (see below). Control groups comprised healthy controls (NC), patients with Becker muscular dystrophy (BMD) and female carriers of the mutated *DMD* gene (fC).

Beside the classical symptoms of muscle disease each entity displayed characteristic features, which are detailed in Supplemental Table 1 - Supplemental Table 4 and discussed in the respective sections of each entity. Before starting analyses, all patients were evaluated for reviewing the clinical data, diagnostic laboratory findings and morphological characteristics. Patient biopsies not fitting the criteria were excluded from the study and afterwards remaining patient biopsies were histologically characterized and the local cytokine milieu in the muscle tissue was examined.

Entity	**Subgroups**	**n =**
Necrotizing myopathy (NM)	immune-mediated NM (IMNM)	16
	toxic-mediated NM (nIMNM)	8
Dermatomyositis (DM)	juvenile DM (jDM)	15
	adult DM (aDM)	11
Duchenne muscular dystrophy (DMD)	early phase (0-3 years)	10
	middle phase (4-8 years)	10
	late phase (> 8 years)	5
Healthy controls (NC)		9
Becker muscular dystrophy (BMD)		6
Female Carrier (fC)		5

4.2 Histology of healthy control patients

As control for all entities biopsy specimens of neurologically healthy patients of different age and sex were used. Muscle biopsies were performed due to nonspecific complaints of the patients to exclude the possibility of an inflammatory disease. The muscle biopsies were thoroughly checked for morphological changes and ultrastructural abnormalities. Patients with inflammatory infiltrates or MHC class I up-regulation, as well as other parameters associated with myositis (e.g. autoantibodies, signs of systemic inflammation) were excluded from the study. For future comparisons with the investigated diseases, the healthy, normal picture for various stainings of cellular features and resident cells is shown in Figure 8.

The biopsy from healthy muscle showed multinucleated, polygonal muscle cells (Figure 8 A). Staining with MHC class I (Figure 8 B) or MHC class II of capillaries (Figure 8 C) demonstrated a regular distribution of capillaries. Lymphocytes (CD45, Figure 8 D) and macrophages (CD68, Figure 8 E) were not seen in healthy patients. Regeneration as shown with MHCn staining (Figure 8 F) was not detected in healthy patients. Expression of dystrophin (Figure 8 G) was strong on all muscle fibres, while expression of utrophin was confined to capillaries and other vessels (Figure 8 H). In addition the sarcolemma of muscle cells were positive stained with collagen VI (Figure 8 I).

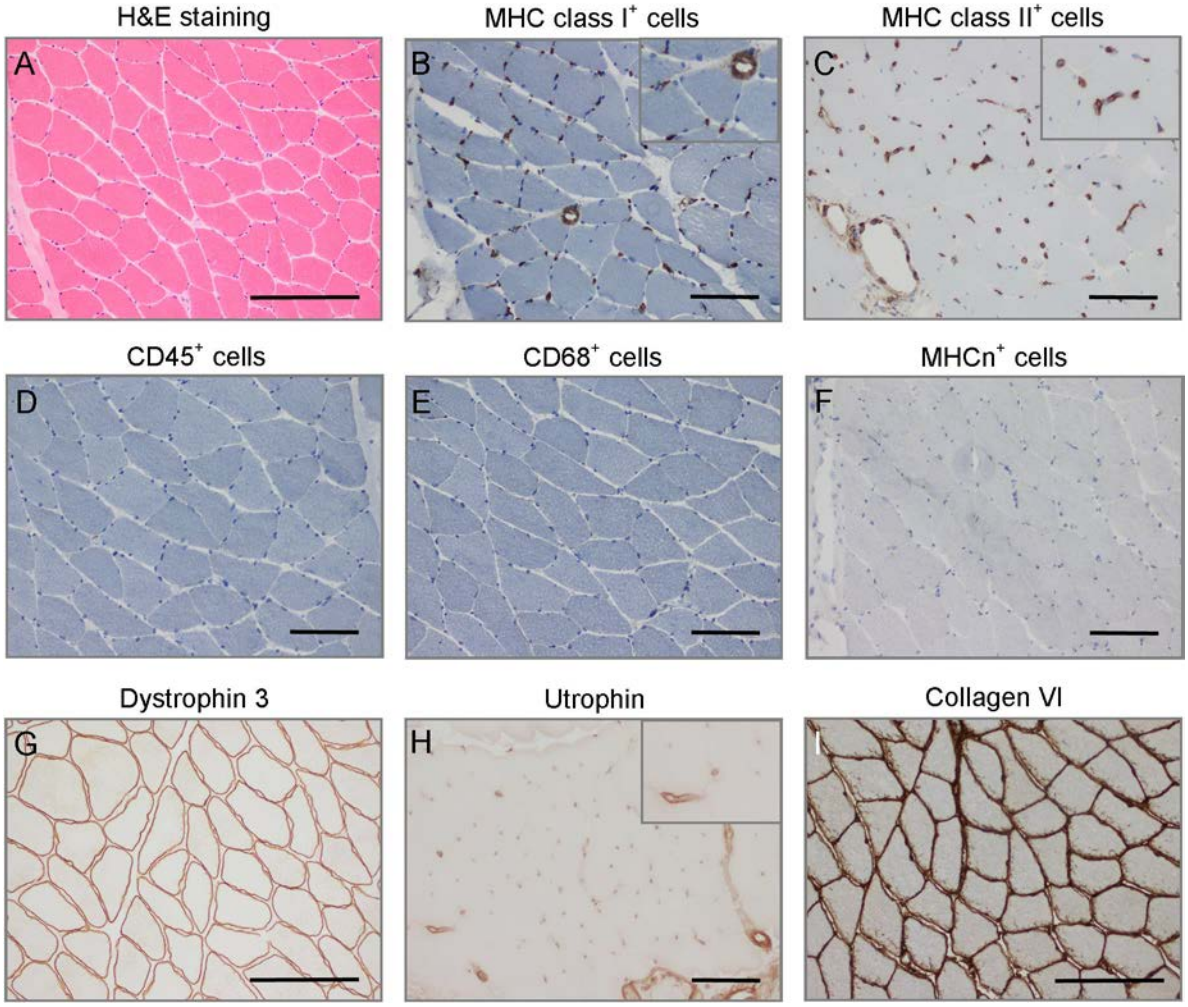

Figure 8: Representative staining in muscle biopsies from healthy control patients revealed homogenous expression pattern and lack of immune cells

A: H&E staining show multinucleated, polygonal muscle cells and additional staining with B: MHC class I on capillaries and C: MHC class II on capillaries. D: $CD45^+$ lymphocytes and E: $CD68^+$ macrophages are not seen in healthy patients. F: regeneration in healthy adult patients is not detectable. G: Staining with dystrophin shows a uniform staining pattern, while H: staining with Utr is confined to capillaries and other vessels. I: Collagen VI staining on the sarcolemma also shows a physiological distribution and staining intensity. Scale bar in A, G, I = 200 µm and in B-F, H = 100 µm

4.3 Necrotizing myopathy (NM)

4.3.1 Clinical information and general morphology of NM patients

Patients with necrotizing myopathies can be divided into two main subgroups depending on the disease triggering event/agent. Patients were classified as either immune-mediated NM (IMNM, 16 patients) or as non-immune-mediated NM (nIMNM, 8 patients) based on clinical and laboratory data. IMNM patients had MSA or MAA autoantibodies and/or an autoimmune disease, while the nIMNM patients did not have any autoantibodies or any autoimmune disease. Instead, a toxic agent was identified in patients suffering from nIMNM. The precise parameters are also listed in the methods section on page - 21 -.

The evaluation of clinical data from all patients with NM showed no significant difference in the following basic parameters: the average age in both groups was similar (56.9 $^{+}$/$_{-}$ 16.9 years in IMNM and 59.5 $^{+}$/$_{-}$ 13.4 years in nIMNM), as was the female/male ratio (both ~60:40) (both features Figure 9 A). However, nIMNM patients may develop higher CK levels as compared to those of IMNM patients (Figure 9 B and Supplemental Table 1). Patients with immune-mediated NM were mostly treated with steroids (12/16) and most of them described improvement of symptoms (e.g. reduction of muscle pain or weakness) during the following period of observation. Only in two cases this therapy had no beneficial effect. In contrast, in nIMNM the triggering agent (e.g. medication) was removed and mostly no further data about development of the disease were available. (Supplemental Table 1)

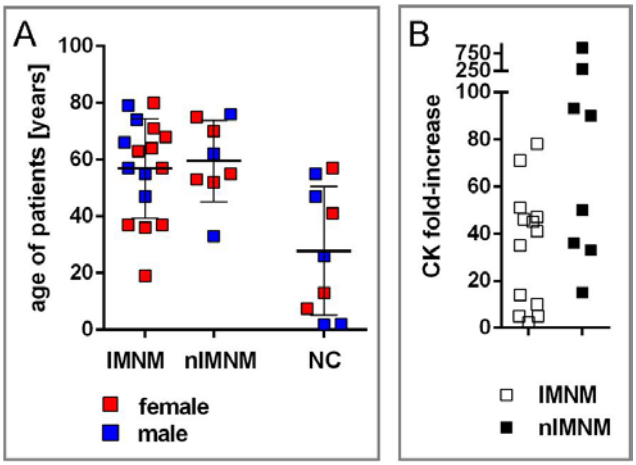

Figure 9: Age, sex and CK level are comparable in both subgroups of patients with NM
A: Age of patients with IMNM and nIMNM in comparison to NC is shown; furthermore, the number of female and male patients is displayed by colored squares. B: Fold-increase of CK values in both subgroups of NM. The nIMNM patients develop higher CK levels than IMNM patients. Statistical analysis in A and B were performed with Mann-Whitney *U*-test, $p < 0.05$ was considered significant.

The general picture of the disease shown by routine histochemical staining with H&E staining (Figure 10 A, B) and Gömöri trichrome (Figure 10 C, D) was basically comparable in both subgroups, with regard to myophagocytosis and myofibre necrosis. Both elements were detectable, independently from disease aetiology.

To exclude features of other diseases the biopsies were analyzed for the presence of perifascicular pattern of atrophic fibres and for a gradient of increasing capillary density towards the center of the

fascicles, as it is found in dermatomyositis patients, but neither one was a feature in the IMNM muscle specimens. Electron microscopic investigation further ruled out the presence of undulating tubules in endothelial cells, which is another ultrastructural feature of DM (Figure 10 E, F). [93]

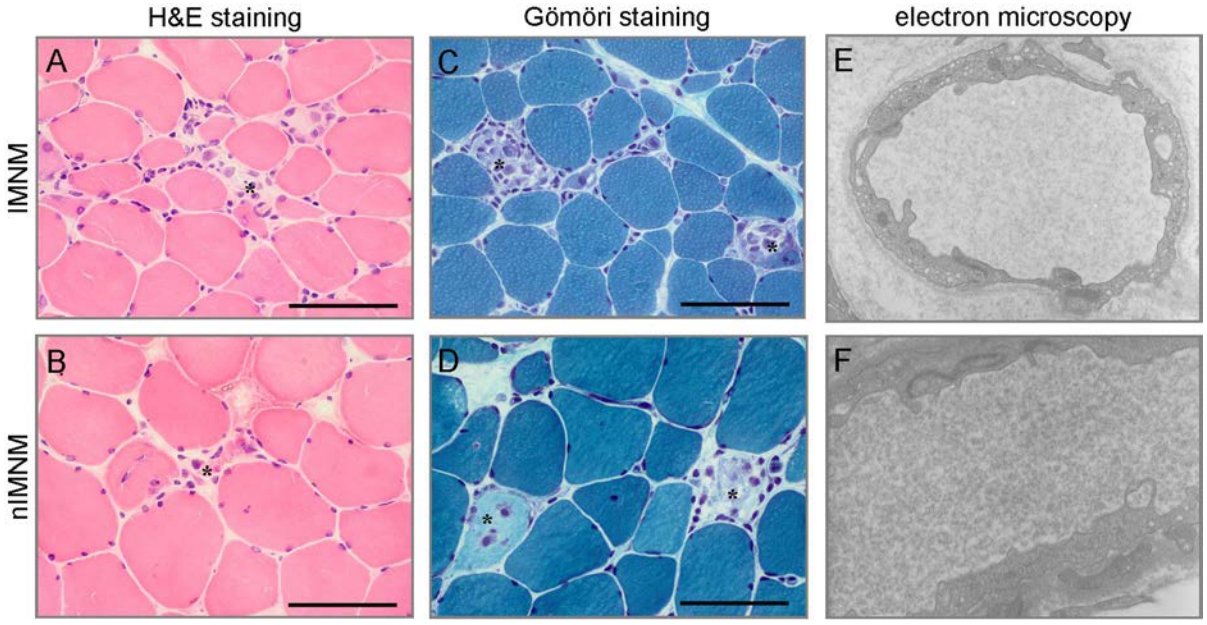

Figure 10: Representative illustration of morphological characteristics in both subgroups of NM patients
A, B: H&E staining and C, D: Gömöri trichrome stainings illustrate the general picture of disease and show myophagocytosis and necrosis of muscle fibres; E, F: representative pictures of electron microscopy show no undulating tubules in endothelial cells of capillaries in either IMNM nor nIMNM patients. * = necrotic muscle fibre; scale bar = 100 µm, EM magnification x30,000

4.3.2 Number and distribution of immunological cells in inflammatory infiltrates differs between IMNM and nIMNM patients

To differentiate the subtypes of NM and to characterize the inflammatory infiltrate in more detail, additional immunohistochemistry and ultrastructural analyses were performed.

It has been stated by many researchers that IMNM, especially anti-SRP-associated NM, is characterized by a 'poor cellular infiltrate' [91,92]. Therefore, not only the presence but also the specific distribution of interstitial immune cells were investigated. Using immunohistochemistry it was shown that the inflammatory infiltrate in IMNM muscle tissue (Figure 11 A), but not in nIMNM muscle tissue (Figure 11 B), consists of a substantial amount of macrophages (Figure 11 A, B). Histologically these macrophages were either iNOS$^+$ (Figure 11 C, D) or CD206$^+$ (Figure 11 E, F). The analysis of mRNA expression levels showed expression of *NOS2* (iNOS) in both, IMNM and nIMNM, of the same level as healthy controls (Figure 11 H), while CD206 (*MRC1*) expression was significantly elevated as compared to NC (Figure 11 H). In addition, the quantification of immune cells using cell counts on serial sections in the biopsy samples showed that the CD68$^+$ macrophages quantitatively constituted the dominant mononuclear cellular infiltrate in IMNM (Figure 11 G), independent of myophagocytosis. IMNM muscle tissue had huge numbers of CD68$^+$ macrophages in the endomysium and perimysium, while these cells were significantly fewer and exclusively confined to necrotic fibres in patients with nIMNM (Figure 11 G).

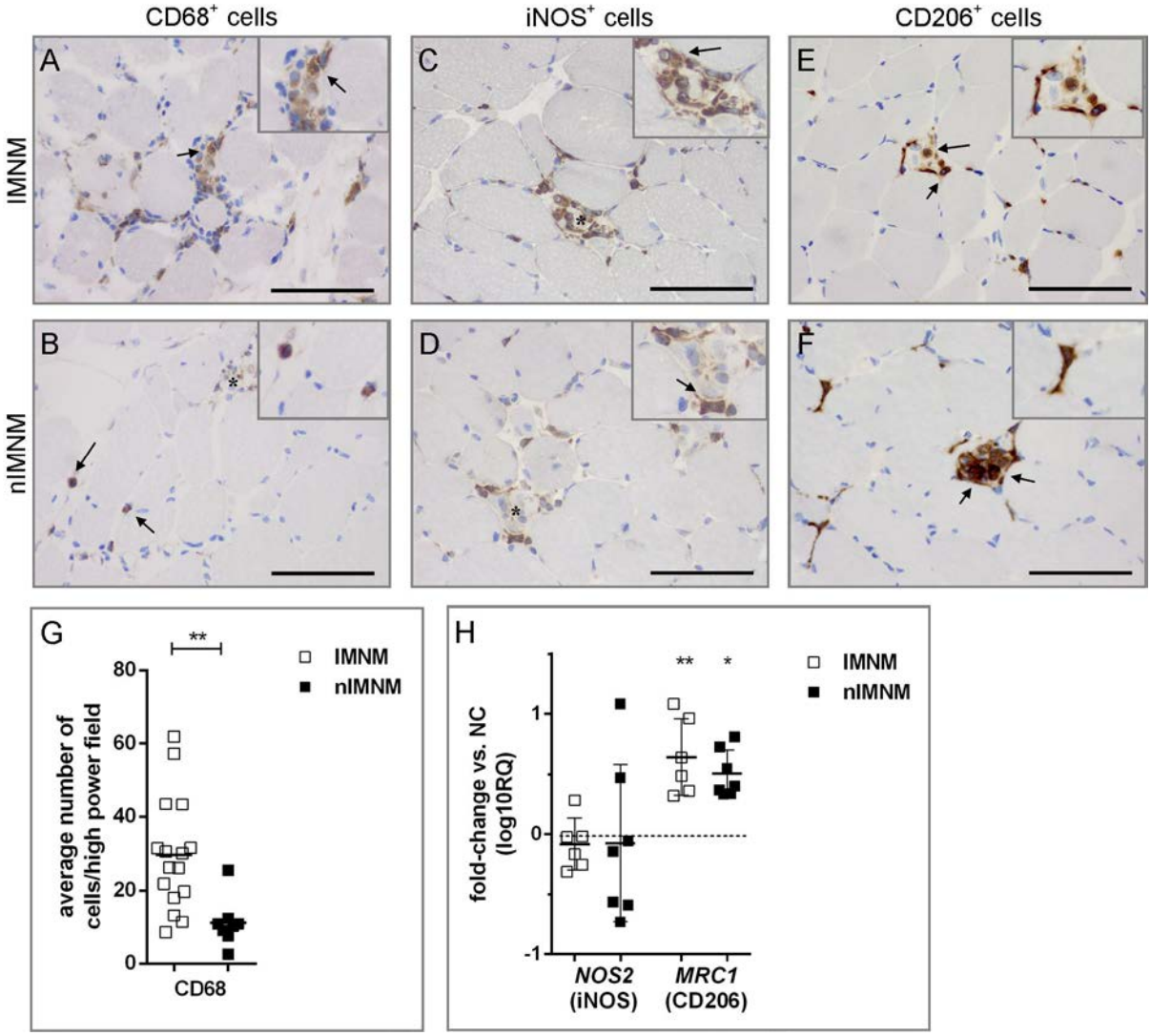

Figure 11: Numbers of macrophages in cellular infiltrates of patients with IMNM are significantly higher when compared to nIMNM

A, B: Histologically stained CD68$^+$ cells in NM and C, D: staining of biopsy specimens for iNOS. E, F: as well as with CD206. G: Cell count of CD68$^+$ cells in both subgroups of NM. IMNM patients have higher numbers of macrophages, while the number of cells in nIMNM is low. H: qPCR analysis showed expression of the pro-inflammatory cytokine iNOS (*NOS2*) and the AAM molecule CD206 (*MRC1*) when compared to that of NC (1 ≙ 10-fold increase). Statistical analysis in G was performed with Mann-Whitney *U*-test and in H with Kruskal-Wallis one-way ANOVA with Dunn´s multiple comparison test, $p < 0.05$ was considered significant (* ≙ $p < 0.05$, ** ≙ $p < 0.01$). Arrows indicate immunoreactive cells of the respective subtype; A-D: * = necrotic muscle fibre; scale bar = 100 µm

Using staining for CD4 and CD8, the T cells within the muscle biopsies were analyzed. As shown in Figure 12 the inflammatory infiltrate in IMNM muscle tissue comprised CD4$^+$ (Figure 12 A) and CD8$^+$ T cells (Figure 12 C) and in some cases, the T cells were also identified in a perivascular location. In contrast, nIMNM biopsies displayed only scant T cells in the endomysium and in proximity to fibre necrosis and myophagocytosis (Figure 12 B, D). As already seen in histological sections, the quantification of T cells showed a considerable amount of T cells in IMNM, while numbers of T cells in nIMNM were low (Figure 12 E). In comparison to CD68$^+$ macrophages, the numbers of T cells in IMNM is lower (Ø 30 macrophages vs. Ø 18 T cells, Figure 11 E, Figure 12 E).

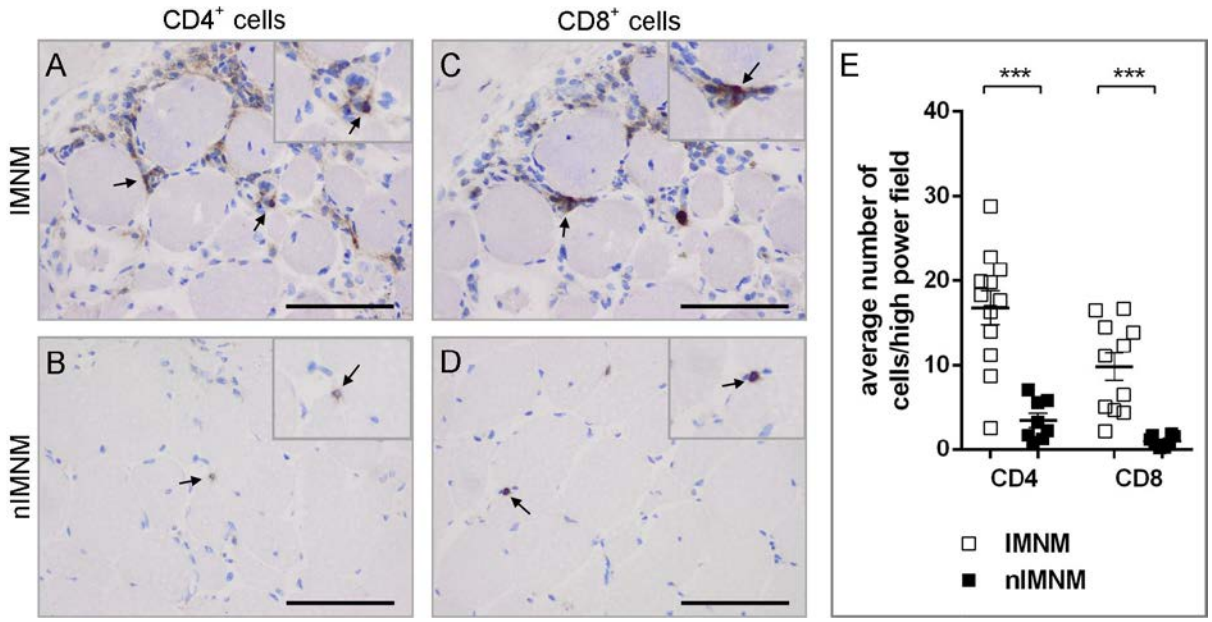

Figure 12: Numbers and distribution of T cells in cellular infiltrates differ between IMNM and nIMNM patients

A, B: $CD4^+$ cells stained in immunohistochemistry in NM patients and C, D: staining with an antibody against CD8. E: Cell count of $CD4^+$ and $CD8^+$ cells in both subgroups of NM. IMNM patients have higher numbers of T cells, while the number of cells in nIMNM is low. Statistical analysis in E was performed with Mann-Whitney *U*-test, $p < 0.05$ was considered significant (*** ≙ $p < 0.001$), Arrows indicate immunoreactive cells of the respective subtype; * = necrotic muscle fibre; scale bar = 100 µm

In addition to macrophages and T cells, the role of B cells in inflammatory myopathies was of interest, since their contribution in IIMs is still unclear. Nevertheless, in a few severely ill patients with anti-SRP-autoantibodies, B cell depletion therapy with Rituximab has been used with only little effect [89,197]. Therefore, the participation of B cells was assessed in more depth and molecular molecules for B cell recruitment into the skeletal muscle were analyzed.

Three out of the 16 muscle biopsy specimens showed few $CD79a^+$ B cells and in some cases $CD138^+$ plasma cells were detected in the endomysium (Figure 13 A, C), while the nIMNM biopsies did not display any substantial numbers of B cells or plasma cells (Figure 13 B, C). The molecular analysis of the B cell chemoattractant *CXCL13* [198,199] in muscle specimens further revealed significantly higher expression of this chemokine in IMNM as compared to nIMNM, as well as in comparison to NCs (Figure 13 D). Accordingly, patients with increased expression of *CXCL13* were also those with histologically proven presence of B cells. Since *CXCL13* expression is also seen in muscle tissue of IMNM biopsies without histologically proven B cells, the recruitment in these patients might just have started. It was further shown, that beside follicular dendritic cells, also macrophages are able to produce CXCL13 [200], again demonstrating their involvement in immune cell recruitment. In addition, some of the patients with B cells or high expression of *CXCL13* were positively tested for myositis-antibodies (color coded in Figure 13 C, D, anti-Jo1- or anti-SRP-autoantibodies). This might hint at an association between antibodies and e.g. prognosis of disease or responsiveness to treatment, since correlations between antibodies and clinical findings have been reported in various studies [3,110,201]. However, the number of B cells in IMNM muscle tissue overall was very low rendering the importance of these cells for therapeutic approaches questionable.

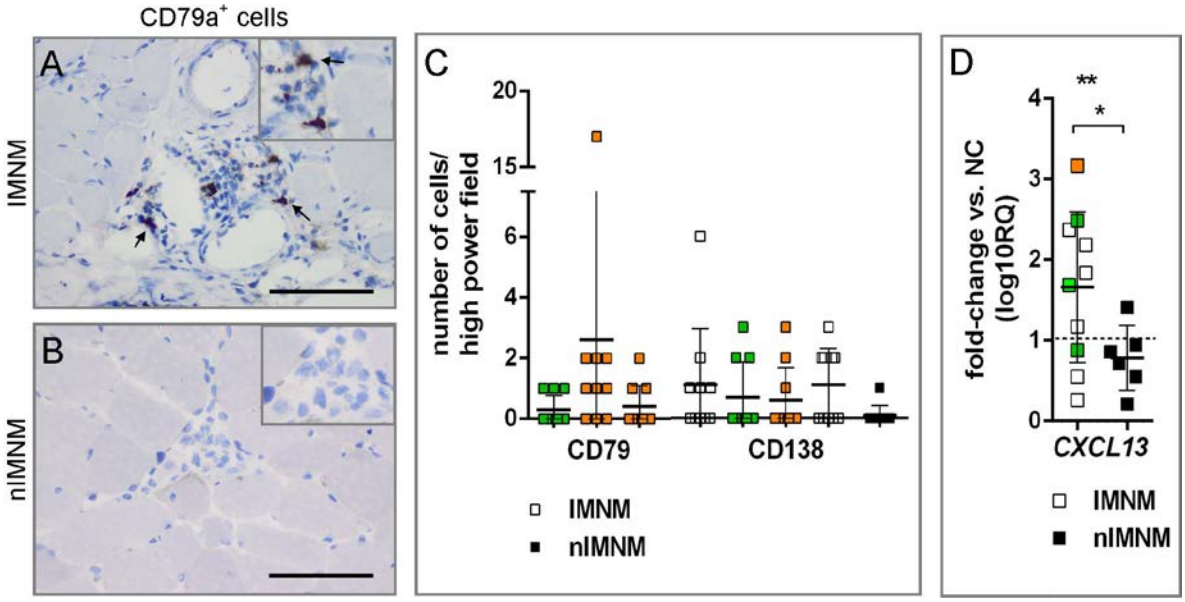

Figure 13: Histological and molecular analyses of B cells in both subgroups of NM
A, B: Immunohistochemical staining with CD79a in NM muscle tissue. C: Cell counts revealed single cases with B cells or plasma cells in IMNM patients. D: The expression of the B cell chemoattractant *CXCL13* is illustrated in NM as compared to NC (1 ≙ 10-fold increase, 2 ≙ 100-fold increase). Some patients with B cell infiltration also showed increased expression levels of *CXCL13* (marked by colored squares: orange ≙ anti-Jo1 Ab; green ≙ anti-SRP Ab). Statistical analysis in D with Kruskal-Wallis one-way ANOVA and Dunn´s multiple comparison test, $p < 0.05$ was considered significant (** ≙ $p < 0.01$). Arrows indicate immunoreactivety of the $CD79a^+$ cells; scale bar = 100 µm

4.3.3 Additional histological features allow differentiation of IMNM and nIMNM patients

Next I investigated the distribution of MHC class I molecule expression by immunohistochemistry, because conflicting results were published concerning 'the intensity of its expression and the expression pattern' [97,98,111,202]. Hengstman *et. al.* described a case series, where MHC staining in muscle biopsies was completely absent [98], while Christopher-Stine *et. al.* showed sarcolemmal MHC class I staining on 50 % of the evaluated patients with HMGCoA antibodies [97]. In the present study, IMNM patients in general exhibited a prominent expression of MHC molecules on a large number of non-necrotic muscle fibres (Figure 14 A). The expression was thereby diffusely distributed and particularly strong in biopsies derived from patients with anti-SRP or anti-synthetase antibodies and in paraneoplastic NMs. The investigations of muscle biopsies from nIMNM patients revealed MHC class I expression confined only to the sarcoplasm of necrotic fibres, as well as a scant sarcolemmal staining on few adjacent fibres (Figure 14 B). As expected no significant or diffuse up-regulation of MHC molecules on non-necrotic fibres was found in nIMNM biopsies (Figure 14 B) and these myofibres are therefore not targeted by a potential T cell response.
Since necrotic fibres are a hallmark in patients with NM, and the membrane attack complex is involved in cell decay, the complement deposition of the membrane attack complex (MAC; C5b9) was studied. As demonstrated by histology complement deposition was particularly prominent especially on capillaries in IMNM biopsies (Figure 14 B), again especially in patients where anti-SRP or anti-synthetase autoantibodies were found. The same was true in biopsies derived from patients with paraneoplastic aetiology. Semi-quantitative scores of the stainings further showed a significantly stronger expression of MHC class I and C5b9 in IMNM as compared to nIMNM (Figure 14 E). The complement deposition on capillaries in the nIMNM group was insignificant or completely absent (Figure 14 D, E).

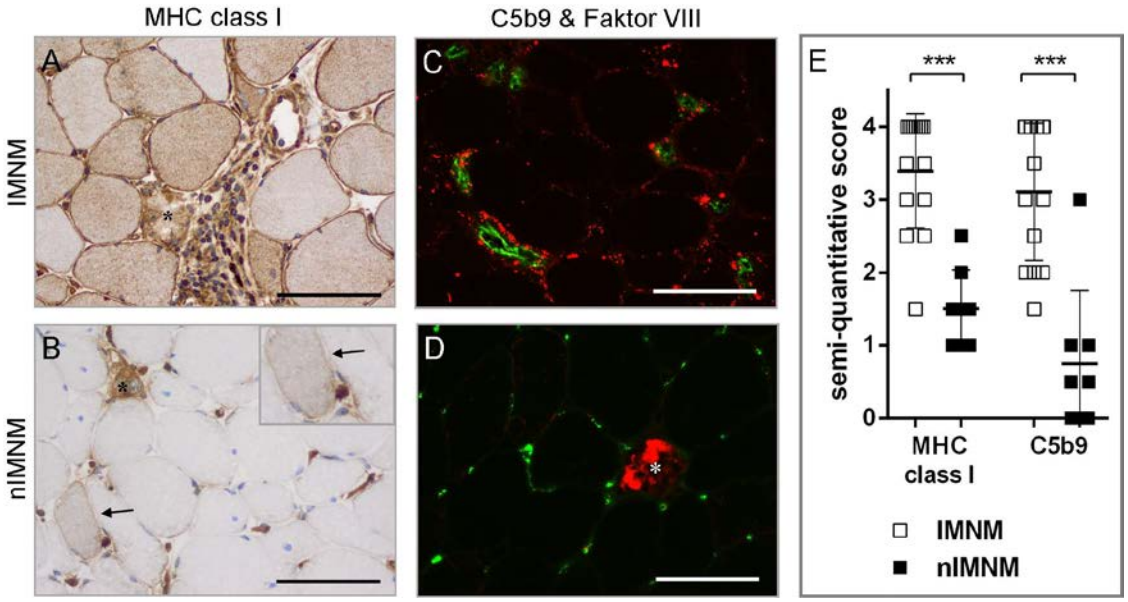

Figure 14: Analyses of sarcolemmal MHC class I expression and complement deposition showed differences between the NM subgroups

A, B: Histological staining of MHC class I and C, D double immunofluorescence of complement C5b9 (red) and factor VIII (green) in NM patients. E: Quantification of histology showed the amount of MHC class I expression, as well as of C5b9 deposition. For definition of scale see methods: 0 = min; 4 = max. Statistical analysis in E was performed with Mann-Whitney-*U*-test, *p* value < 0.05 was considered significant (*** ≙ *p* < 0.001); ***** = necrotic muscle fibres, scale bar = 100 µm

In addition to the mentioned complement deposition on capillaries, which was also previously described [1], a further interesting and discriminating feature of severe IMNM cases was the decoration of the sarcolemma of non-necrotic fibres by MAC (Figure 15 A). This feature was consistently absent in biopsies from patients with nIMNM (Figure 15 B). With the characteristic sarcoplasmic labelling with αB-crystallin, some of these fibres were identified as so called X fibres [203] (Figure 15 C), which appeared otherwise normal by trichrome or H&E stain (Figure 10 A, C). These findings may indicate that the X fibres are committed to be attacked by the immune system. X fibres were consistently absent in nIMNM specimens (Figure 15 D).

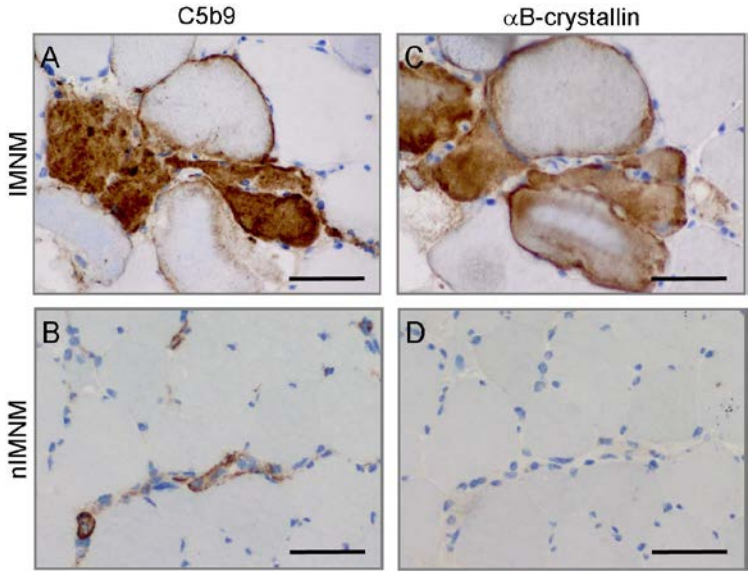

Figure 15: Deposition of complement and labelling of X fibres is prominent in IMNM patients

A: Sarcolemmal decoration of muscle fibres with complement in IMNM and B: staining of nIMNM patients. C: In IMNM αB-crystallin staining was seen, D: while no staining was seen in nIMNM. scale bar = 50 µm

Considering the different aetiologic backgrounds I hypothesized that regenerating processes would possibly differ between muscle biopsy specimens from patients with an immune background, compared to patients with toxic aetiology. To identify regenerating fibres I used antibodies against neural cell adhesion molecules (NCAM; CD56), which are involved in cell-cell adhesion, and myosin heavy chain neonatal (MHCn), a molecule expressed by newly formed myotubes. In IMNM the number of regenerating fibres was significantly more pronounced as indicated by the presence of numerous $NCAM^+$ (CD56) or $MHCn^+$ muscle fibres (Figure 16 A, C), whereas these molecules were less frequently detected in biopsies from patients with nIMNM (Figure 16 B, D). Semi-quantitative score of the histology revealed that regeneration in IMNM is enhanced as compared to nIMNM, but not on a significant level (Figure 16 E). The increased regeneration rates in IMNM argue for a high turnover of necrotic and inflamed muscle fibres and potential restoration of healthy tissue. This meets the observation that patients might be cured after therapy wherby no muscle symptoms remain.

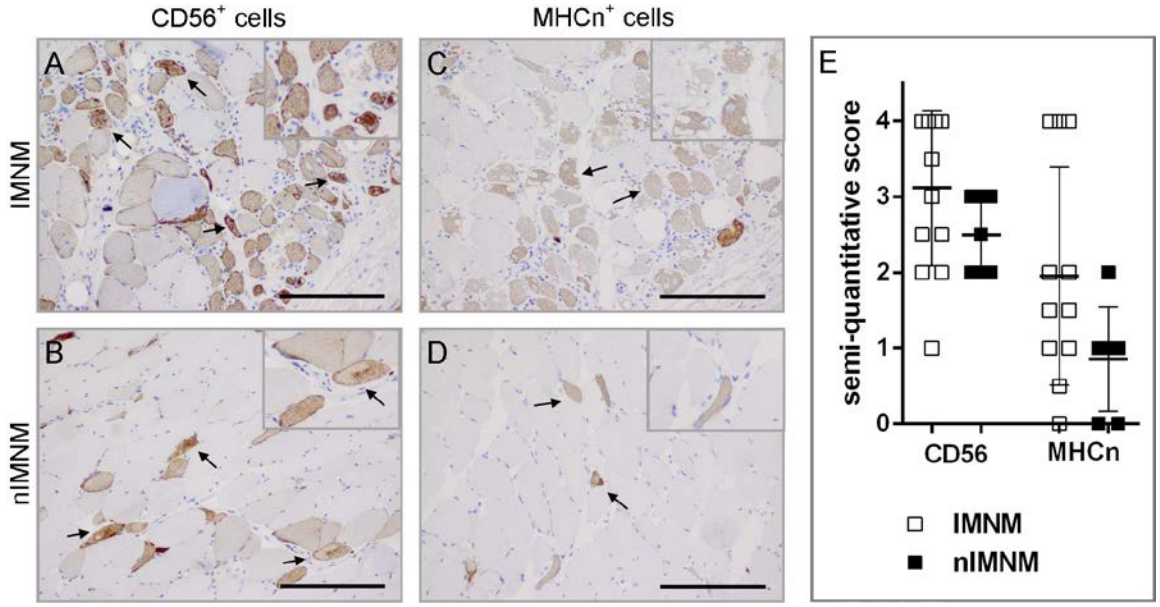

Figure 16: Regeneration processes are elevated in IMNM patients when compared to nIMNM
A, B: Illustration of regeneration in NM patients by staining with CD56 and C, D: MHCneonatal. E: Semi-quantitative score of immunohistochmical stainings showed increased regeneration in IMNM as compared to nIMNM, however without statistical significance. Statistical analysis in E were performed with Mann-Whitney *U*-test, $p < 0.05$ was considered significant; arrows indicate immunoreactive cells of the respective subtype; scale bar = 100 µm

4.3.4 Differential expression of cytokines/chemokines revealed differences between IMNM and nIMNM patients

Based on the above mentioned quantitative studies concerning the inflammatory mononuclear infiltrates in NM biopsy specimens, I hypothesized that the immune pathogenesis of the disease might be essentially regulated by macrophages and T cells. Therefore, I analyzed key molecules of the pro- and anti-inflammatory immune milieu by qPCR, as well as mediators for classical and alternative activated phenotypes of macrophage polarization [18,26,28,32].

As illustrated in Figure 17 the cytokine expression level of muscle tissue derived from IMNM patients was characterized by a significant up-regulation of the pro-inflammatory master cytokine *IFNG*, as well as the cytokines *TNFA*, *IL12p40* and the transcription factor *STAT1* [28,204] as compared to NCs (Figure 17 A, B). In

nIMNM samples, the respective cytokines were expressed at lower levels when compared to those in IMNM (Figure 17 A, B), indicating a lower pro-inflammatory response in patients with toxic-mediated aetiologies. Furthermore, the effector molecule cox2 (*PTGS*) was not differentially regulated in either group, which may mirror comparable up-regulation in the muscle tissue (Figure 17 A) and in addition they were only insignificantly elevated when compared to NCs. Expression of the T cell stimulating cytokine *IL12p40* was increased in IMNM as compared to nIMNM and NCs (Figure 17 B).

Gene expression measurements of the master cytokines of T_H2 immunity, *IL4* and *IL13*, revealed no significant expression (Figure 17 D). In line with this finding, the gene expression of the common IL-4 receptor alpha (*IL4RA*; CD124), which mediates IL-4 and IL-13 signaling, as well as expression of the macrophage mannose receptor (*MRC1*; CD206) were expressed at similar levels in both groups (Figure 17 C). Both were significantly elevated when compared to NCs in IMNM and in case of CD206 also in nIMNM. In addition, *TGFB* and *CCL18* were elevated in IMNM patients, in case of *CCL18* on a significant level (Figure 17 C). The expression of these molecules demonstrates an involvement of anti-inflammatory reactions in NM patients in addition to the up-regulation of pro-inflammatory cytokines.

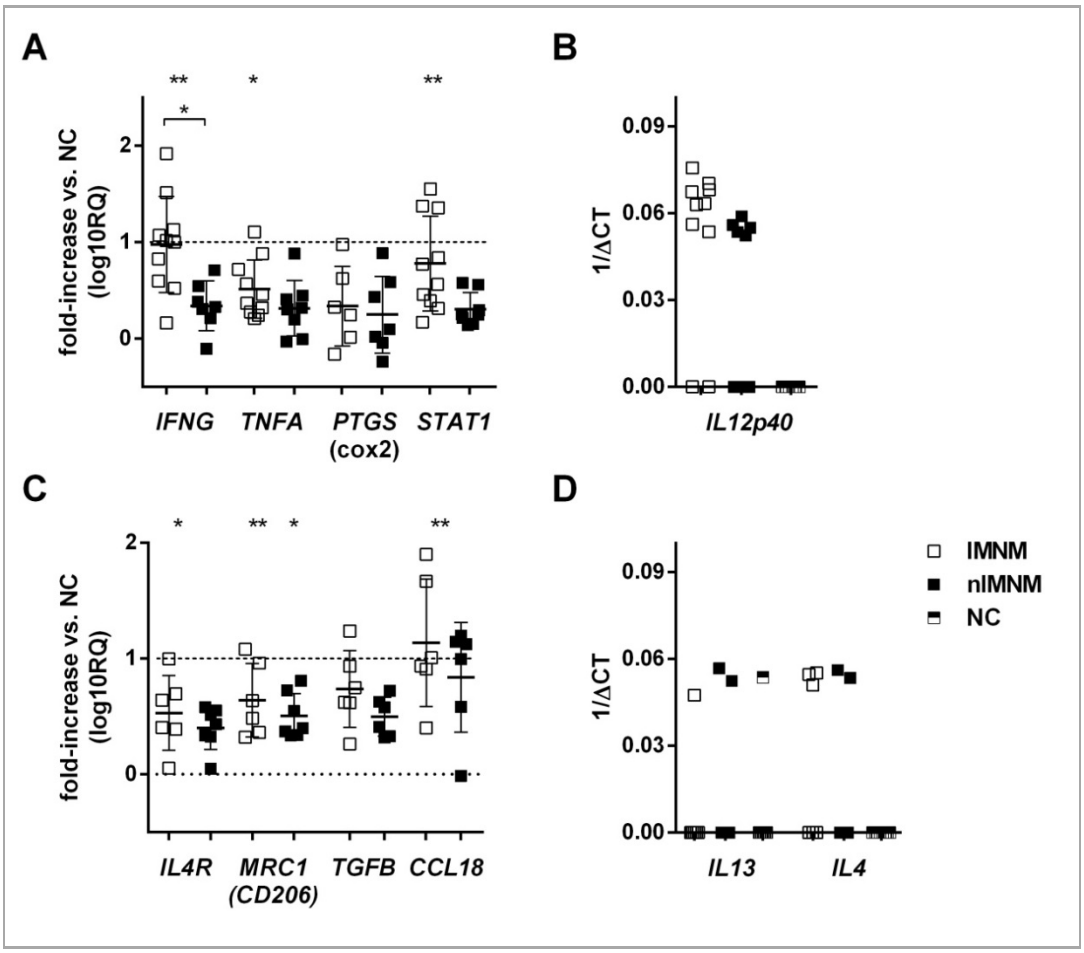

Figure 17: Expression of pro-inflammatory cytokines NM patients

A: Expression of pro-inflammatory mediators in IMNM and nIMNM as compared to NC (1 ≙ 10-fold increase, 2 ≙ 100-fold increase). B: The pro-inflammatory molecule *IL12* is not expressed in NM; nor in NC. C: Expression of anti-inflammatory molecules is not differentially regulated in both groups and D: anti-inflammatory cytokines are not expressed. Statistical analysis was performed with Kruskal-Wallis one-way ANOVA with Dunn´s multiple comparison test**.** It was not possible to perform statistics for the expression of *IL12p40*, *IL4* and *IL13*, because the respective cytokines were not expressed in NC; *p* value < 0.05 was considered significant (* ≙ $p < 0.05$, ** ≙ $p < 0.01$).

4.3.5 Recapitulation

Patients suffering from necrotizing myopathy can be clearly divided into two distinct subgroups depending on laboratory and histological parameters. The clinical background and medical history gave already hints for a correct classification into immune- or non-immune NM, which illustrates the importance of a comprehensive clinical evaluation of the patient history.

Moreover, histological analyses were further identified as tools to clarify the nature of the respective disease subtype. The immune reaction in patients with IMNM is defined by a T_H1-like, pro-inflammatory milieu, with up-regulation of *IFNG* and *TNFA*. Using quantification it was clearly demonstrated that macrophages were the predominant cell population and spread throughout the muscle. In addition, considerable amounts of T cells were found in the inflammatory infiltrates of IMNM patients. Furthermore, a clear expression of MHC class I molecules and C5b9 deposition were detected in IMNM, while none of these features were detected in NMs with an toxic aetiology adding additional features to distinguish the different subtypes of NM.

4.4 Dermatomyositis (DM)

4.4.1 Clinical information and general morphology of DM patients

Patients with dermatomyositis were analyzed with respect to various clinical data, summarized in Supplemental Table 2. One of the most important diagnostic characteristics was the presence of undulating tubules in endothelial cells of capillaries of skeletal muscle, which were found by electron microscopy (Figure 19 E, F). For evaluation the group of DM patients was split into juvenile patients (jDM, 15 patients, age of onset < 16 years) and adult patients (aDM, 11 patients).

In the jDM patients the gender distribution between girls and boys was 50:50, whereas in the aDM more women were affected than men (70:30) (Figure 18 A). The mean age in juvenile patients was 7.0 $^{+}/_{-}$ 4.3 years, whereas the adult patients were 61.0 $^{+}/_{-}$ 14.7 years old (Figure 18 A). These age-frames fit perfectly with previously published data [116,117,131].

Therapeutic strategies were different from patient to patient, but glucocorticoid treatment was the most common therapy, with methotrexate or azathioprine as a long-term immune modulator. The success of therapy also varied strongly between patients. Overall, jDM patients showed a better response to steroid treatment. Most of the patients experienced partial or complete remission of symptoms (11/15 remission and 4/15 remitting-relapsing). In aDM patients, fewer individuals showed improvement/partial improvement (5/11) and in two patients no effect of the therapy was seen. For the remaining patient's no further information was available about long-term development of the disease.

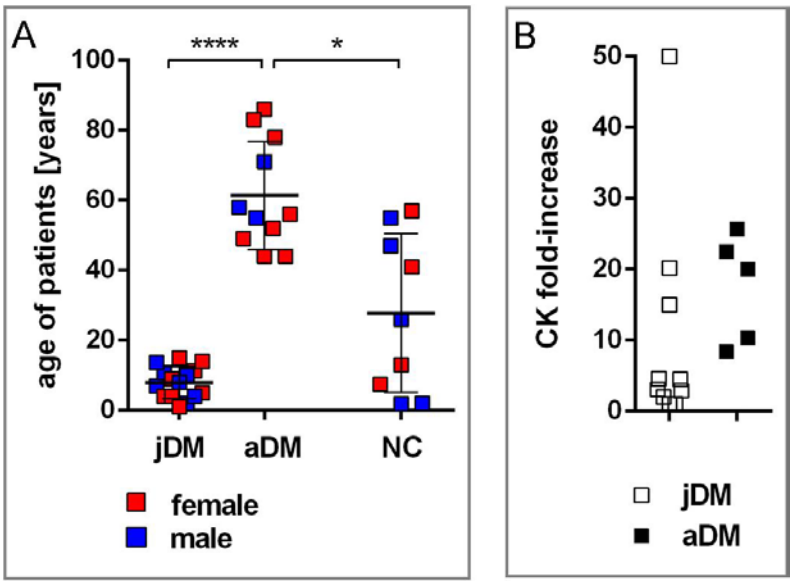

Figure 18: Age, sex and CK levels in jDM and aDM patients
A: Age of patients with jDM and aDM in comparison to that of NC is shown; furthermore, the number of female and male patients is displayed by colored squares. B: Fold-increase of CK values in both groups of DM. Statistical analysis in A, B were performed with Mann-Whitney *U*-test, $p < 0.05$ was considered significant (*≙ $p < 0.005$, **** ≙ $p < 0.001$).

Histological stainings with H&E or Gömöri trichrome revealed the general picture of the disease with clear inflammatory infiltrates, especially in perimysial areas. Biopsies from jDM and aDM patients displayed the same morphological picture of DM with widened perifascicular areas, inflammatory infiltrates and muscle fibre atrophy predominantly in the perimysial area (Figure 19 A-D). These effects were also described before [205] confirming the correct classification of this patient cohort.

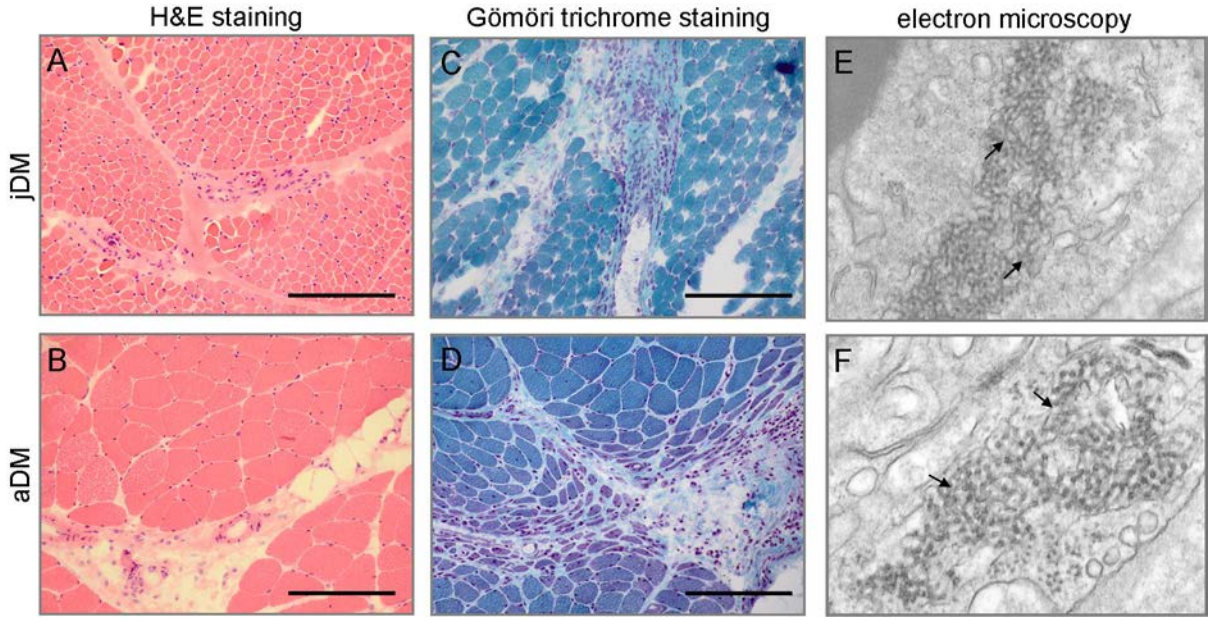

Figure 19: Representative staining of morphological characteristics in jDM and aDM patients
A, B: The general overview for aDM and jDM patients is displayed with H&E staining and C, D: Gömöri trichrome. A typical perifascicular atrophy pattern is seen. E, F: Another hallmark of DM is the presence of undulating tubuloreticular structures in endothelial cells (indicated by arrows), which are shown by electron microscopy. Scale bar = 200 µm, EM magnification x30,000

4.4.2 Composition of infiltrates is similar in both subgroups of DM patients

In the biopsy specimens of patients with DM $CD4^+$ and $CD8^+$ T cells were detected in the endomysium, as well as in the perimysium of all patients (Figure 20 A-D). In contrast to T cells, only few $CD79a^+$ B cells were observed in both groups (Figure 20 E, F). Quantification of lymphocytes revealed that there was no significant difference in the number of $CD4^+$ / $CD8^+$ T cells (Figure 20 G) or B cells (Figure 20 H) in both subgroups. Cell counts of $CD138^+$ plasma cells showed that there were only low numbers in both groups; however some aDM patients exhibited high numbers of plasma cells increasing the mean value for the whole group to a significant level between aDM and jDM (Figure 20 H).

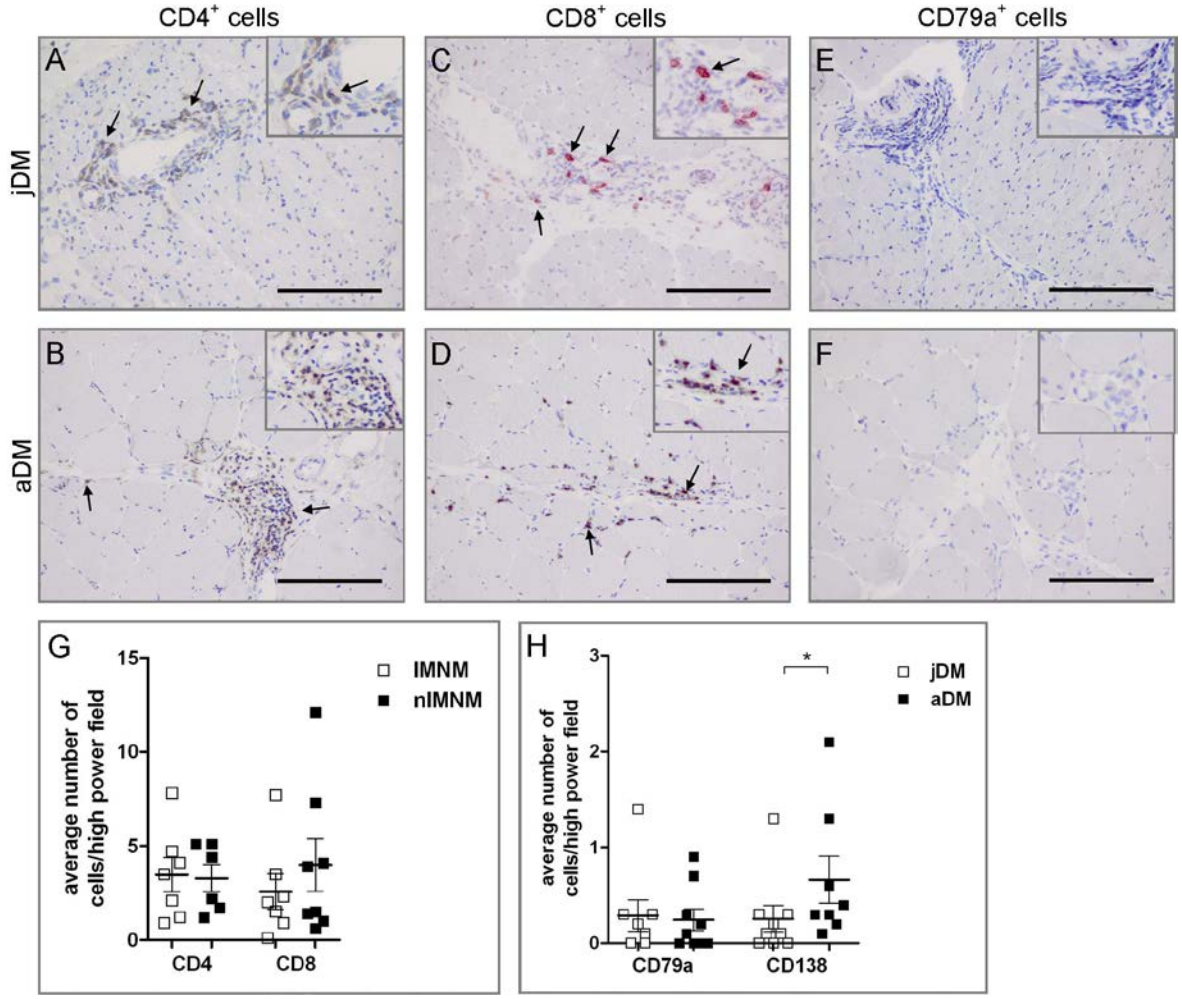

Figure 20: Composition of the inflammatory infiltrate in DM patients is similar
A, B: $CD4^+$ T cells are found perifascicular, as well as C, D: $CD8^+$ T cells. E, F: $CD79a^+$ B cells are scant and mostly not detected in jDM; nor aDM patients. G: Quantification of T cells in DM patients and H: quantification of B cells in jDM and aDM. Statistial analysis was performed with Mann-Whitney *U*-test, $p < 0.05$ was considered significant. Arrows indicate representative immunoreactive cells of the respective subtype; scale bar = 200 µm

The most prevalent population of inflammatory cells throughout the biopsies of DM patients were $CD68^+$ macrophages with a large proportion of CD206 positivity and with a clear accumulation in the perifascicular region (Figure 21 A, B). In addition, macrophages were identified in or around necrotic fibres (Figure 21 A, B). Macrophages were either $iNOS^+$ (Figure 21 C, D) or $CD206^+$, while the histological staining was more intense for CD206 staining (Figure 21 E, F). Overall, there was no significant difference in the number of immune cells of jDM as compared to aDM patients, as shown by counting of $CD68^+$ cells (Figure 21 G). On the molecular level, jDM patients showed a trend for up-regulation of the cytokine cox2 (*PTGS*), while expression of these molecules in aDM was on the level of NCs (Figure 21 H). In contrast to that, the expression of CD206 was significantly elevated in aDM patients when compared to jDM, as well as to that of NC (Figure 21 H). This speaks for different prevalent milieus and therefore activation pathways in jDM in contrast to that of aDM.

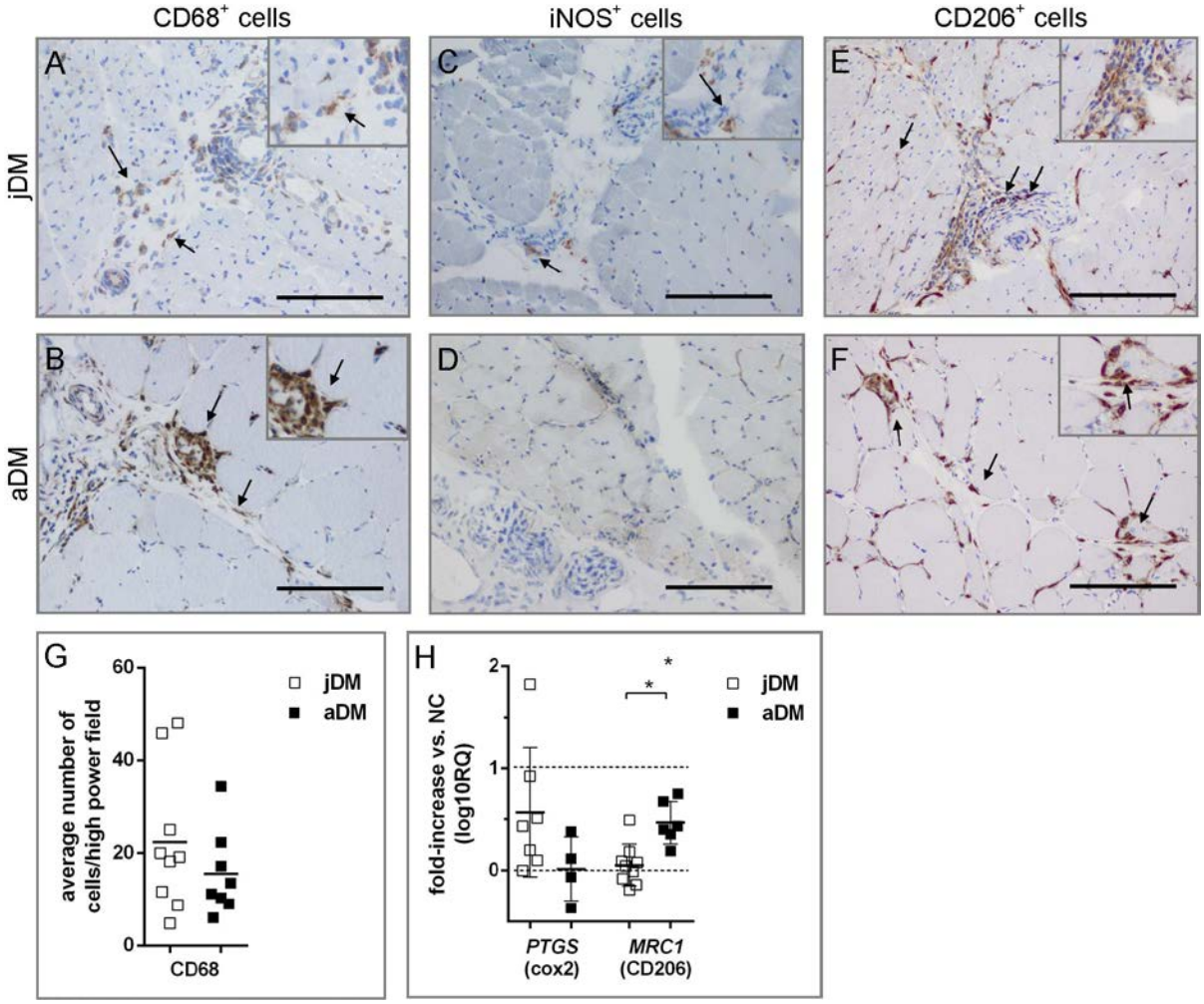

Figure 21: Involvement of CD68$^+$ macrophages in cellular infiltrates of patients with DM
A, B: CD68$^+$ cells in DM patients and C, D: staining of biopsy specimens with iNOS and E, F: CD206. G: Quantification of macrophages in patients with DM showed no difference between the groups. H: qPCR analysis of DM patients showed expression of the pro-inflammatory cytokine cox2 (*PTGS*) and the molecule for AAMs CD206 (*MRC1*) when compared to NC (1 ≙ 10-fold increase). Statistical analysis in G were performed with Mann-Whitney *U*-test and in H with Kruskal-Wallis one-way ANOVA with Dunn´s multiple comparison test, $p < 0.05$ was considered significant (* ≙ $p < 0.05$, ** ≙ $p < 0.01$). Arrows indicate immunoreactive cells of the respective subtype; scale bar = 200 µm

4.4.3 Perifascicular atrophy and regenerative processes are prominent in jDM patients

In addition to the inflammatory infiltrates, a difference in the histological staining pattern of various proteins in jDM as compared to aDM was seen. A typical perifascicular pattern was identified by various stainings in DM patients, and exemplarily shown here by MHC class I and MHC class II expression (Figure 22 C, D). The up-regulation of MHC class I and MHC class II might not only be a reaction to the inflammatory infiltrates, but rather a separate pathological event in DM [206]. In terms of expression intensity of both MHCs no differences were observed between jDM and aDM patients.

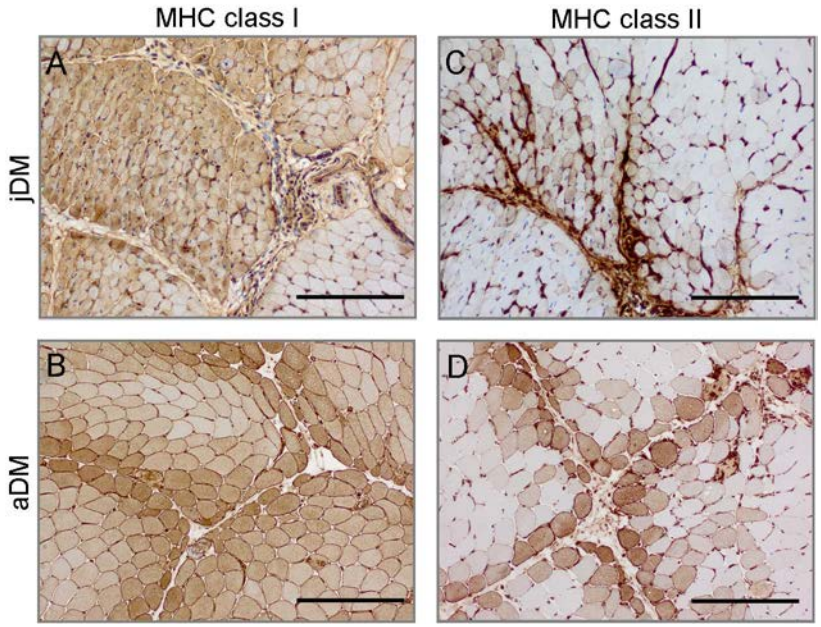

Figure 22: Expression of MHC class I and MHC class II is strong in patients with DM
A: Expression of MHC class I was strong on muscle fibres in jDM, as well as B: in aDM. The same picture is seen in C, D: expression of MHC class II. Scale bar = 200 µm

Furthermore, the perifascicular pattern demonstrates atrophy and regeneration, which was more prominent in jDM patients (Figure 23). Expression of CD56 (Figure 23 A) was high in jDM and also expression of MHCn (Figure 23 C) was frequently detected. In contrast to the pronounced regeneration in jDM, the up-regulation of proteins in aDM patients was less strong (Figure 23 B, D). Regenerative processes seemed therefore less involved in the pathogenesis of the disease in adults when compared to those in juvenile patients.

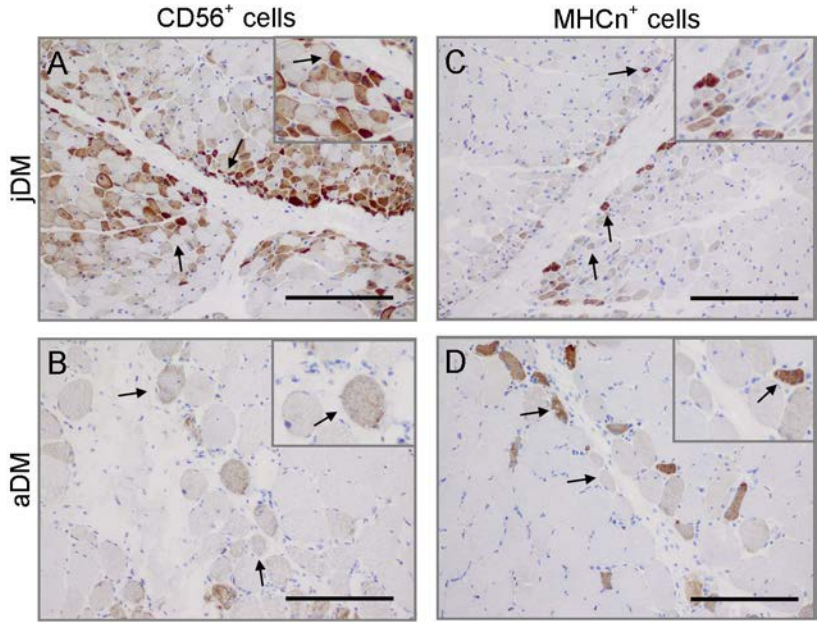

Figure 23: Regenerative processes are increased in patients with jDM
A: Regeneration is detected in jDM patients by staining of $CD56^+$ cells and C: $MHCn^+$ fibres. B, D: Expression of the molecules CD56 and MHCn is weaker in aDM. Arrows indicate immunoreactive cells of the respective subtype; scale bar = 200 µm

4.4.4 Hypoxia and prominent capillary loss is more pronounced in jDM as compared to aDM patients

It is known that hypoxic events occur in DM patients, but its impact on pathogenesis is unclear, as well as its different contribution in the juvenile or adult form of the disease. Therefore hypoxic key molecules were analyzed by protein expression and on molecular level in both sub-entities. Hypoxic reactions demonstrated by expression levels of the hypoxia-inducible factor 1α (HIF1α, Figure 24 A, B) or induction of the vascular endothelial growth factor (VEGF, Figure 24 C, D) was observed in DM patients. Regulations of hypoxia might be due to up-regulated expression of HIF-1α and its target genes. Hypoxia was also mostly found in the perifascicular, atrophic areas of the muscle in jDM (Figure 24 A). In contrast to these results, none of the aDM patients showed extensive positive staining for HIF-1α (Figure 24 B). In addition, VEGFA expression was strong in jDM patients (Figure 24 C) and less intense in aDM (Figure 24 D).

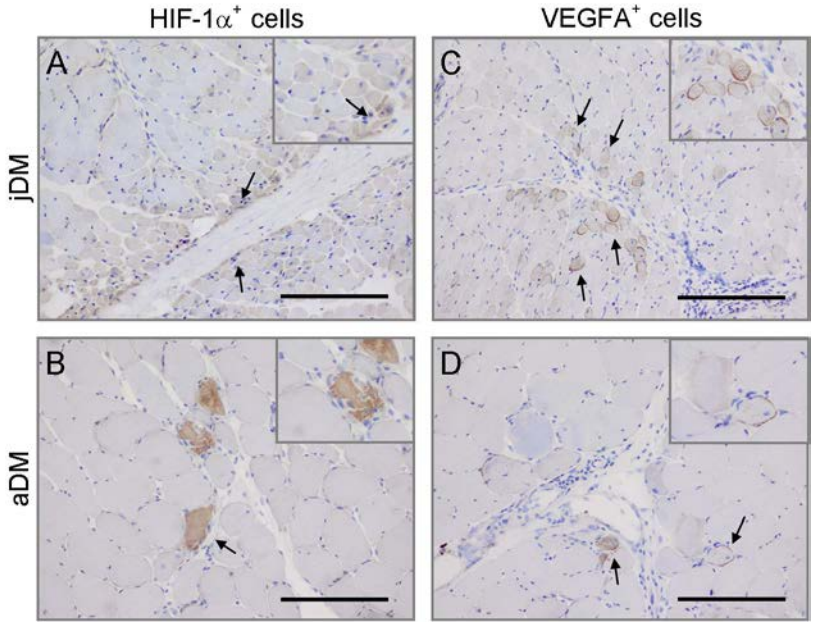

Figure 24: Signs of hypoxia are prominent in jDM patients, but not in aDM patients
A, B: Staining with HIF-1α is found with a perifascicular distribution in jDM, while aDM patients show only low immunoreactivity. C, D: The same picture is found for the vascular endothelial growth factor (VEGFA). Arrows indicate representative immunoreactive fibres of the respective subtype. Scale bar = 200 µm

Another distinct feature of patients with DM is a loss of capillaries, mostly in the centre of the fascicle, which leads to decreased oxygen supply and enhanced effects of hypoxia [145]. However, until now no comparative studies has been performed between jDM and aDM, to determine differences or similarities in the amount of capillary loss. Therefore, density and distribution of capillaries in biopsies with DM were determined. As shown in Figure 25 most patients with DM were affected by capillary loss (Figure 25 A, B, E). Nevertheless counting of single capillaries and muscle fibres demonstrated that jDM patients are more strongly affected by capillary loss than aDM patients. As visualised, the ratio between capillaries and fibres was significantly decreased in jDM in comparison to aDM and NC (Figure 25 E), while this reduction was only seen in some aDM patients (Figure 24 E). Another striking phenomenon was the spatial proximity of macrophages to the capillaries and bigger vessels. This close association of macrophages and vessels was seen in both, jDM and aDM (Figure 25 C, D), indicating a close interaction between activated macrophages and endothelial cells.

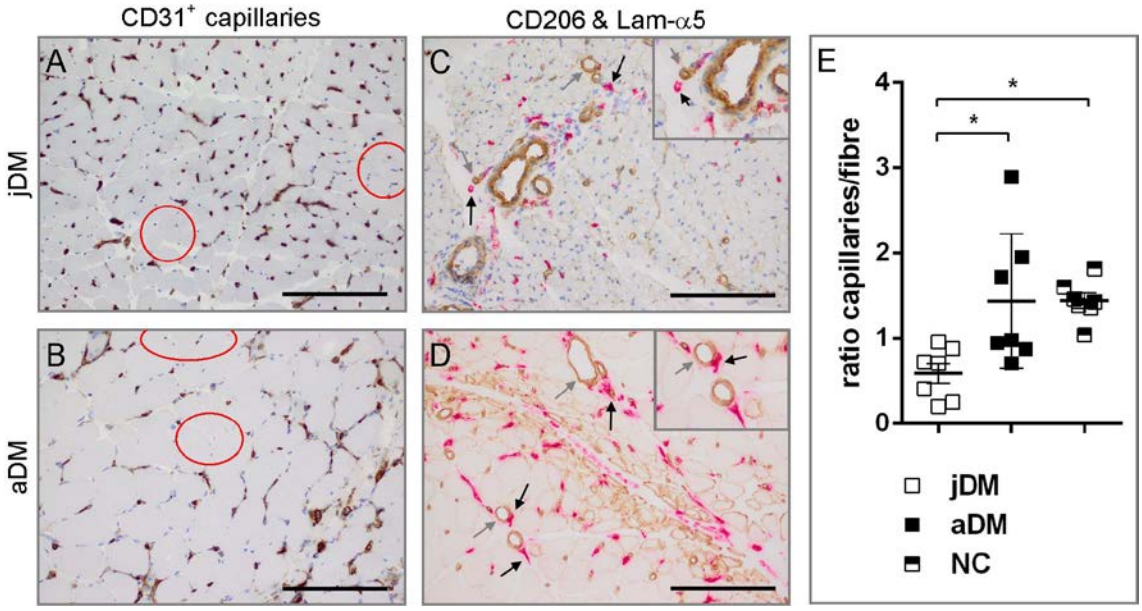

Figure 25: Loss of capillaries is significant in jDM, but not in aDM patients
A, B: Staining of capillaries in DM patients; C, D: Double staining of CD206$^+$ macrophages (red, black arrows) with Lam-α5 for capillaries (brown, grey arrows). E: Counting of muscle fibres and capillaries demonstrated a significantly reduced capillary/fibre ratio in jDM patients compared to aDM and NC; statistical analysis in E with nonparametric t-test and Mann-Whitney post-test, *p* value < 0.05 was considered significant; scale bar = 200 µm

4.4.5 Pro- and anti-inflammatory cytokine expression is found in subgroups of DM

For investigation of the underlying cytokine milieu, RNA from whole tissue was used for analysis by qPCR. Pro- and anti-inflammatory cytokines were not expressed at different levels between the groups and were not significantly regulated when compared to NC (Figure 26 A-D). However, some pro-inflammatory molecules, such as *IL6, IL1B* and *TNFA*, seemed to be more highly expressed in jDM than in aDM, but without statistical significance (Figure 26 A). In contrast, IL-17-asscociated molecules were not expressed in either group of DM, indicating that IL-17-associated immune responses were not prevalent in DM patients (Figure 26 B). The same was true for anti-inflammatory molecules, such as *IL4*, *IL13* and *IL5* (Figure 26 B).

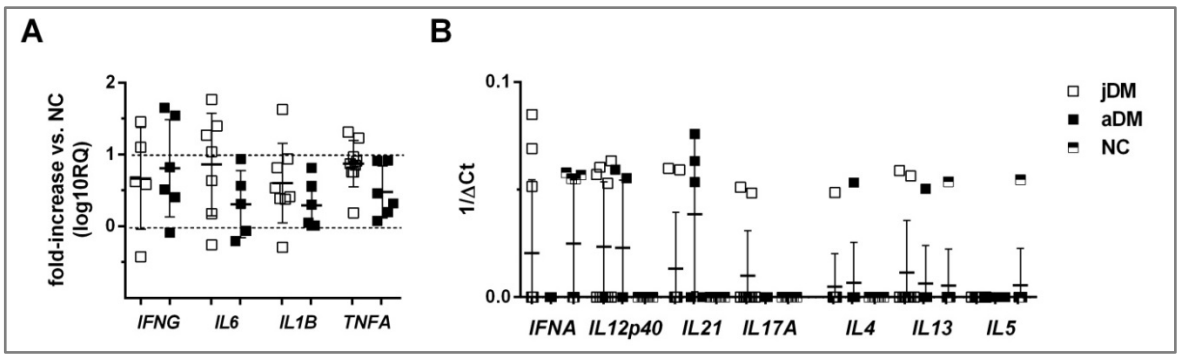

Figure 26: Cytokine profile in jDM and aDM patients is similar to those of NCs
A: Pro-inflammatory cytokines and molecules of classically activated macrophages in DM patients. Regulation is not significantly changed when compared to NC (1 ≙ 10-fold increase, 2 ≙ 100-fold increase). B: Pro- and anti-inflammatory molecules are not differentially expressed in jDM or aDM patients. Statistical analysis was performed with Kruskal-Wallis one-way ANOVA with Dunn´s multiple comparison test. It was not possible to perform statistics for the expression of *IFNA*, *IL12p40, IL21, IL17A*, *IL4, IL13* and *IL5*, because the respective cytokines were not expressed in NC, $p < 0.05$ was considered significant.

4.4.6 Enhanced hypoxia in DM patients is predominantly located in perifascicular areas

In addition to the pro- and anti-inflammatory pathways, molecules known to be involved in hypoxic reactions were investigated. Factors, activated under hypoxic conditions like *VEGFA*, *HIF1A* and *MIF*, were increased when compared to NC (Figure 27 A). However, only gene expression of *TGFB* and *MIF* showed a significant elevation. It has been shown, that TGFβ activity up-regulates HIF-1α, which in turn up-regulates various proteins as well as transcription factors [207]. Therefore the up-regulation of *TGFβ* fits with these data.

To further dissect the reactions within the muscle fibres hypoxic perimysial area as well as 'normal appearing' adjacent muscle fibres of the central region of fascicles were isolated via laser microdissection. With this technique the investigation of hypoxic reaction without a potential masking effect of the surrounding tissue was possible. Perimysial regions are important in DM, since inflammatory infiltrates are located in these areas and atrophy is found at the rim of the fascicles. The center of the fascicles appeared normal in histological stainings and differences in gene expression levels could give insights into regions of pathological reactions in DM. The latter is interesting, since widened perimysium is a hallmark in DM, while it is not seen in other myopathies. As expected, molecular expression of hypoxic molecules was elevated in perimysial areas, whereas expression was not detectable in non-atrophic, centro-fascicular tissue of the same patient (Figure 27 B). Especially *HIF1A*, *MIF* and *VEGFA* were increased, while expression of *TGFB1* was not detected. In adjacent 'normal appearing tissue' the DM patients did not show any expression and neither was the case in NC (Figure 27 B).

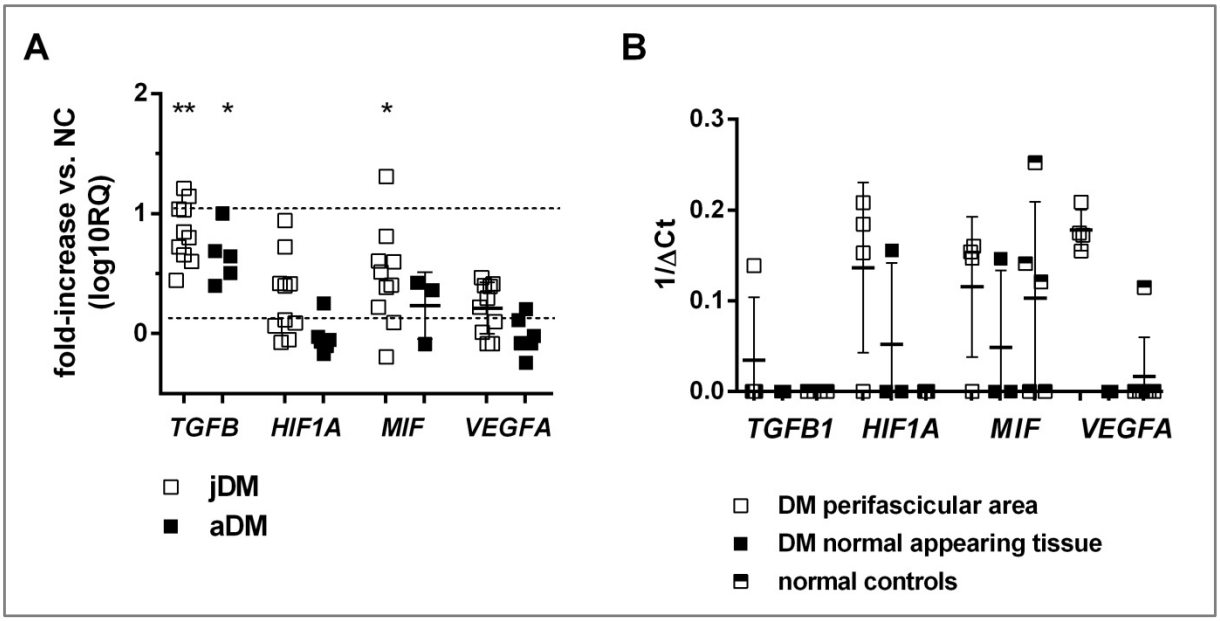

Figure 27: Gene expression levels are elevated in whole tissue and LCM-isolated tissue from DM patients
A: Gene expression levels for molecules associated with hypoxia from whole muscle tissue were analyzed. B: Normal appearing tissue and the perifasicular area of patients was LCM isolated and compared to LCM isolated tissue from NC. Hypoxia-associated molecules were investigated. Statistical analysis in A were performed with Kruskal-Wallis one-way ANOVA with Dunn´s multiple comparison test, $p < 0.05$ was considered significant (* ≙ $p < 0.05$, ** ≙ $p < 0.01$), while due to the low number of samples after LCM isolation it was not possible to perform statistical analysis.

4.4.7 Recapitulation

Combining analyses of the clinical and morphological picture I demonstrated that the patient cohort showed previously published features of DM. Besides infiltrates, containing T cells, B cells and macrophages an up-regulation of MHC class I and MHC class II was seen in all patients independent of their age. However, comparative analyses between jDM and aDM had not been performed before, and these analyses demonstrated that the composition of inflammatory infiltrates in both sub-entities was comparable. In addition, the typical atrophic, perifascicular pattern was identified, as well as effects of hypoxia. Both features were more pronounced in jDM as compared to aDM patients. In addition, these effects were confirmed by qPCR analyses, where factors associated with hypoxia were found up-regulated in jDM and aDM as compared to NC. Through laser microdissection I further demonstrated, that the effects of hypoxia were based on reactions located in the perifascicular area, while the adjacent tissue of the same patients showed no hypoxia-associated regulation. This may be the key for understanding of pathological mechanisms in DM. In line with these findings, the loss of capillaries in DM muscle tissue was determined. The quantification of capillaries per fibre showed, that capillary loss was also more prominent in jDM patients and co-staining of macrophages with capillaries further demonstrated a close spatial proximity between these cells.

4.5 Duchenne muscular dystrophy (DMD)

4.5.1 Clinical information in patients with DMD and basic histology of the control groups (BMD, fC)

For analysis of patients with Duchenne muscular dystrophy (25 patients) biopsy samples were analyzed depending on the age of patients at time of biopsy and other available information, such as mutation in the *DMD* gene, development of symptoms and therapy. All information is given in Supplemental Table 3.

Affected boys were mostly asymptomatic at the age of 0-3 years (early phase), but proximal muscle weakness and inflammation were developing in the following years (4-8 years, middle phase). The late phase of the disease (older than 8 years) was marked by increasing weakness, fibrosis and loss of ambulation. Further problems such as heart failure, breathing problems and scoliosis were emerging. Beside these signs the CK value was often extremely elevated and can be increased up to 200-fold [208], as also shown in Figure 28 A for available information. At time of biopsy all of these typical developments were found in the examined patients (Supplemental Table 3). Gene mutations comprised mostly gene deletions, but in addition duplications and point mutations were detected (Figure 28 C).

As control groups, besides NC, patients with Becker muscular dystrophy (BMD, 6 patients) were analyzed additionally. The male patients suffering from BMD were overall less strongly affected than DMD patients, but walking problems or cardiomyopathy may be found. Female carriers of the mutated *DMD* gene (fC, 5 patients) were also examined, where carriers are often asymptomatic. Information for these groups is given in Supplemental Table 3.

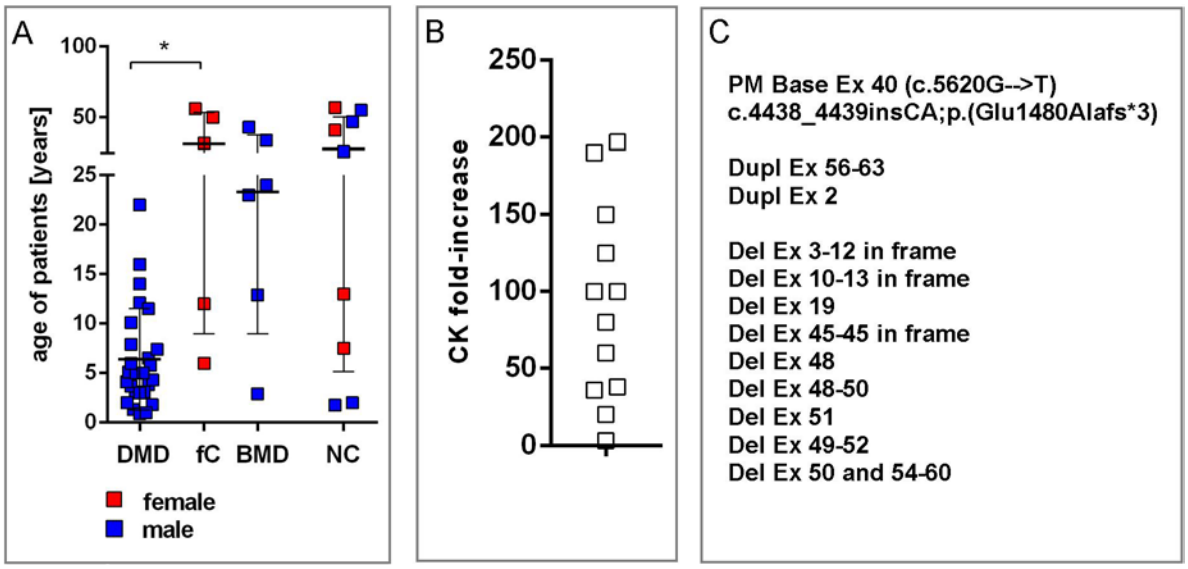

Figure 28: Clinical information of patients with DMD, BMD and fC

A: Age of patients at time of biopsy with DMD in comparison to control groups (BMD, fC, NC) is shown; furthermore, the number of female and male patients is displayed by colored squares. B: Fold-increase of available CK values in patients with DMD. C: Summary of known gene mutations in patients with DMD. Statistical analysis was performed with Mann-Whitney *U*-test, $p < 0.05$ was considered significant (* ≙ $p < 0.05$).

Expression of dystrophin was absent in patients with DMD (Figure 29 A), while in fC the expression of dystrophin was only diminished in a mosaic pattern, as seen in Figure 29 B. For patients with BMD a similar picture was seen, expression of dystrophin was reduced, but not completely absent (Figure 29 C). In contrast, expression of utrophin was up-regulated in DMD and BMD (Figure 29 D, F), while fC had lower sarcolemmal expression of utrophin in general (Figure 29 E).

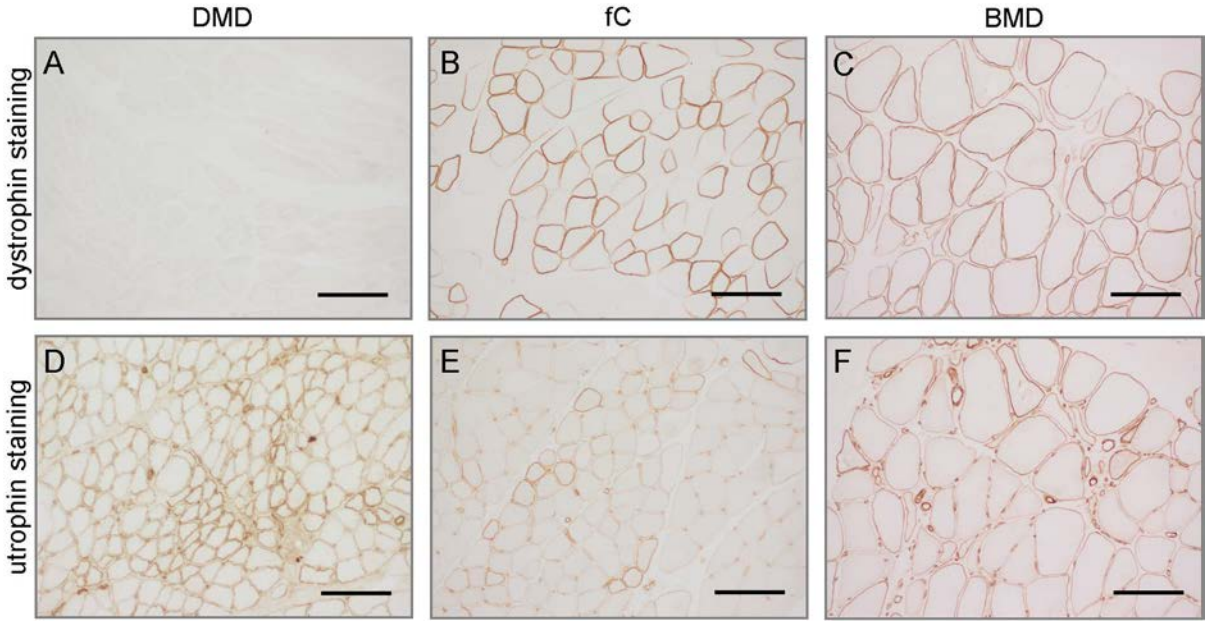

Figure 29: Expression pattern of dystrophin and utrophin differs between DMD patients and control groups
A: Expression of dystophin in DMD patients is completely absent, while expression in B: fC and C: BMD is still detectable. D: Expression of utrophin is strong in DMD, while E: fC show lower expression and F: BMD patients harbor some increased expression. Scale bar = 100 µm

4.5.2 Morphology is characterized by cellular infiltrates, inflammation and fibrosis

The morphological picture in DMD patients is quite variable, depending on the age of the patient and therefore the stage of disease. Variation of the mutation leads to different levels of dystrophin expression and influences not only the morphological picture but also progression of the disease. The starting point of treatment with corticosteroids also differs between patients, additionally influencing the development of disease. Nevertheless, to get an overview about the morphology patients were grouped according to current stage of disease to display development of the disease over time (Figure 30 A-C). The morphological picture was also depicted for BMD and fC (Figure 30 D, E). As seen in the H&E stainings (Figure 30 A-E) and Gömöri trichrome (Figure 30 F-J) increasing muscle pathology was observed with increasing age of patients and especially the first 8 years were minted by inflammation and cellular cell infiltration (Figure 30 A-B & F, G). Starting around the time when DMD patients have to rely on wheelchairs (> 10 years), the amount of inflammation was reduced, while development of fibrosis and replacement of fibres by fatty tissue dominated the morphological picture (Figure 30 C). Development of fibrosis was nicely visualized by EvG staining, where collagen is stained in pink and skeletal muscle is stained in dark yellow. In the control groups, the same features were found, however with a different degree of severity and Figure 30 demonstrated inflammatory infiltrates in BMD and fC, while fibrosis was weaker in BMD patients as compared to DMD (Figure 30 O), but may be substantial in fC (Figure 30 N).

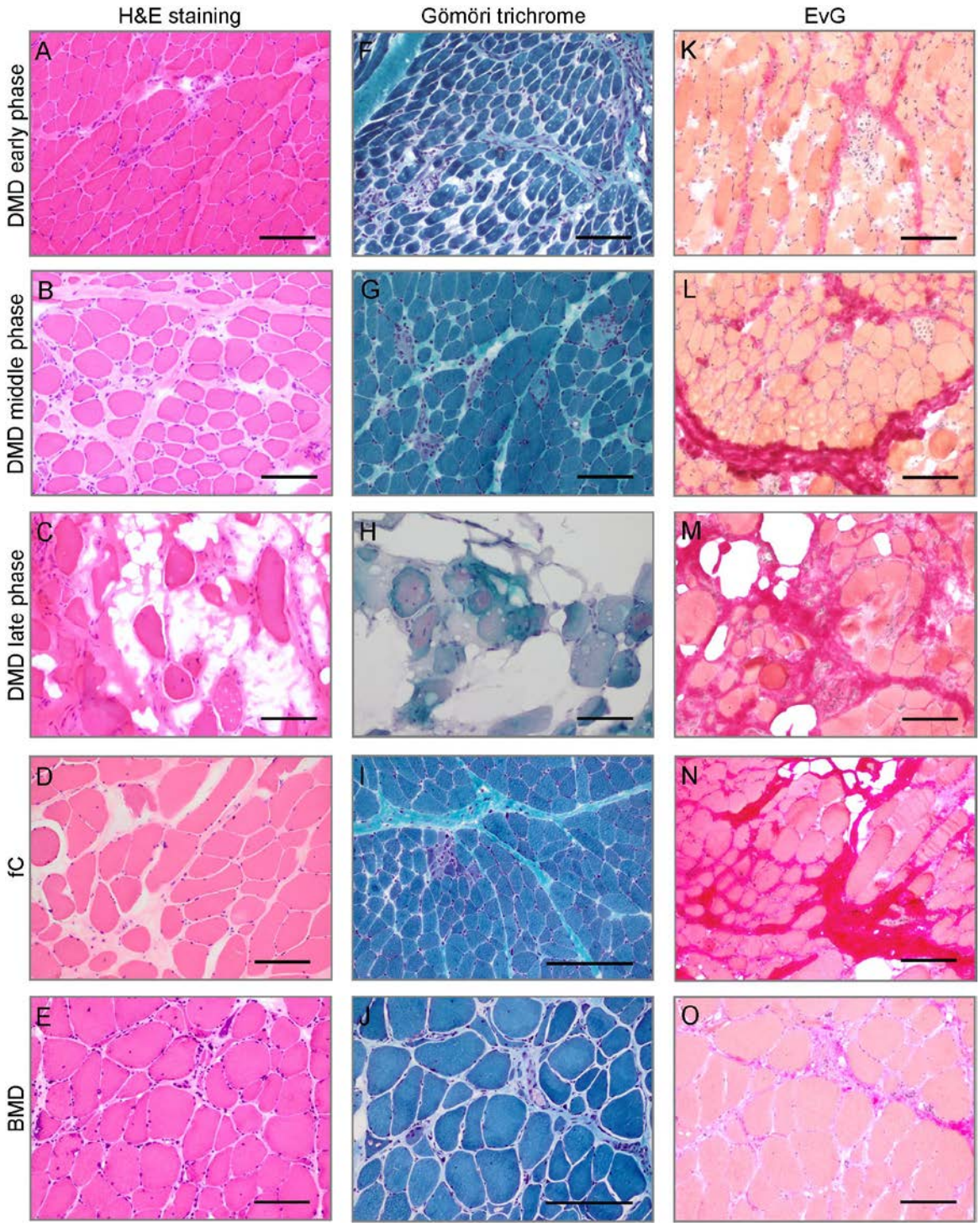

Figure 30: Pathological changes dominate the morphological picture in patients with *DMD* gene mutations

A-E: Representative illustration of the disease at different stages, inflammation was resolving until an age of 10 years and in older patients´ fibrotic tissue and fatty degeneration was dominant. Control groups showed less affection. F-J: Gömöri trichrome stain showed the same features and K-O: EvG staining illustrated fibrotic tissue. Scale bar = 100 µm

4.5.3 The number of macrophages decreases with progression of the disease in DMD

Cellular infiltration, especially by macrophages, was seen in the first decade of life in DMD patients (Figure 31 A, B, F, G), while these cells were only scant in the fibrotic, fatty muscle tissue of older patients (Figure 31 C, H). In BMD patients and in fC, infiltrates can also occur and if infiltrates were present, macrophages

were the prevalent cell population (Figure 31 D, E, I, J). However, except from macrophages in infiltrates they were absent in the muscle tissue from BMD and fC. Expression levels of molecules associated with macrophage polarisation revealed that expression levels in BMD and fC were similar to those of NC (Figure 31 K). Nevertheless, in DMD patients the expression level of CD301 (*CLEC10A*) was significantly elevated when compared to NC and in cox2 (*PTGS*) a trend for up-regulation was identified (Figure 31 K). Dissected into the different sub-groups, DMD patients showed a stage-dependent expression of the molecules CD301, CD206 (*MRC1*) and cox2. While the latter was especially expressed in older patients, molecules of AAMs were more strongly expressed in younger patients (Figure 31 K).

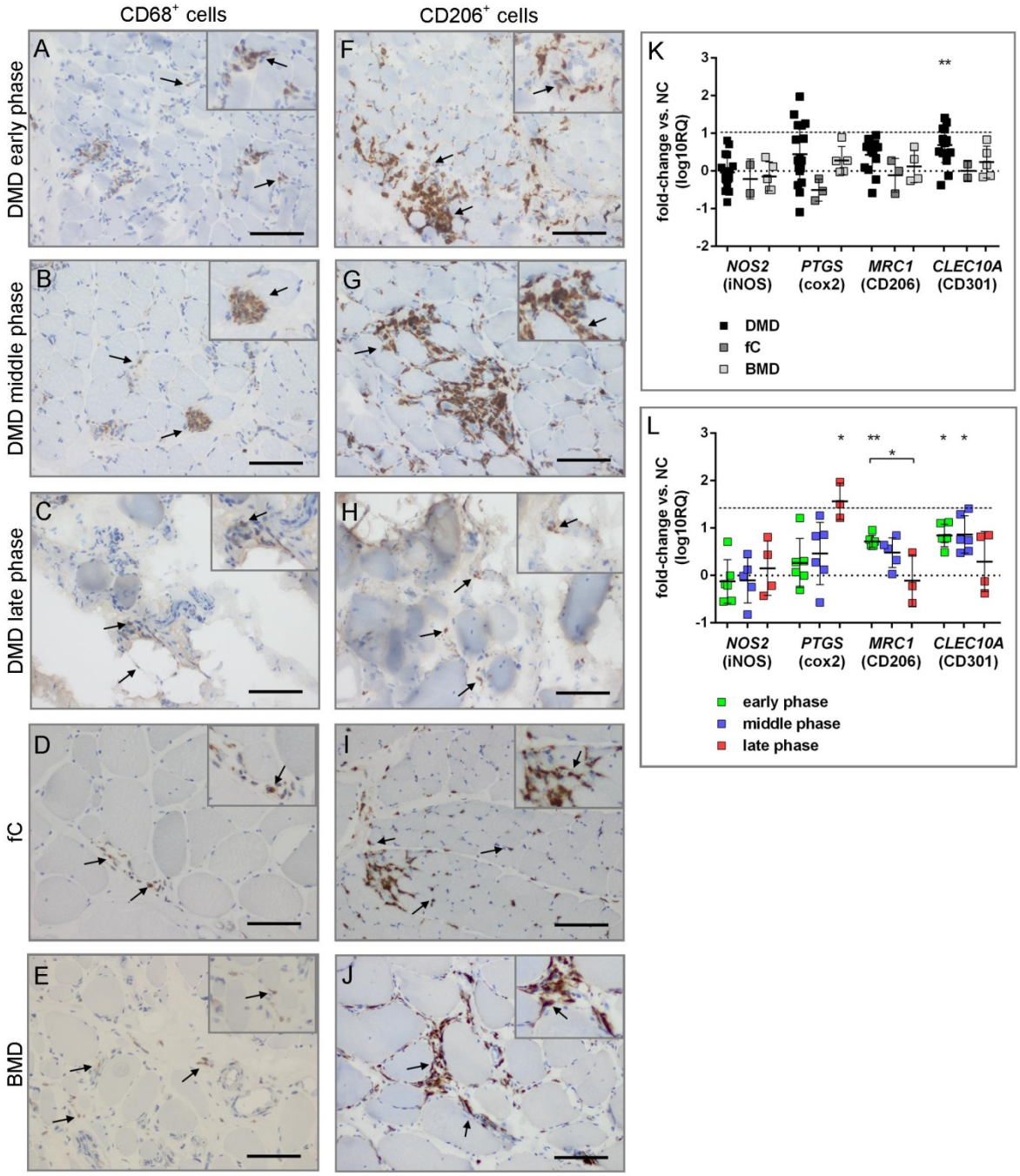

Figure 31: Macrophages are the predominant cell population in cellular infiltrates of DMD patients
A-E: CD68$^+$ cells in patients with DMD, fC and BMD. F-J: Staining with CD206. K: Expression levels of molecules associated with macrophages in comparison to NC (1 ≙ 10-fold increase). L: Molecules are further assessed in sub-groups of DMD patients. Statistical analysis in K, L were performed with Kruskal-Wallis one-way ANOVA with

Dunn´s multiple comparison test, $p < 0.05$ was considered significant (* ≙ $p < 0.05$, ** ≙ $p < 0.01$). Arrows indicate representative immunoreactive cells of the respective subtype. Scale bar = 100 µm

To further analyze the involvement of macrophages in progression of the disease, the number of macrophages was counted in the whole biopsy and their distribution was analyzed. Between the patients high variations in the number of macrophage were seen, as shown by numbers of macrophages in single patients with DMD (Figure 32 C). In addition, the distribution of macrophages, e.g. confined to inflammatory infiltrates (high numbers/high power filed) or scattered within the muscle tissue (low numbers/high power field), varied strongly between patients, as well as inside a single patient (Figure 32 C). Nevertheless number of macrophages peaked in patients around the age of 4 years, as seen in mean number of macrophages per high power field (Figure 32 A). Due to the high variation of macrophage distribution within the muscle biopsy of patients, as well as between the patients the correlation coefficient (R^2) was only 0.1948. In Figure 32 B, the quantifications for patients with BMD, as well as fC are displayed together with DMD patients (fC R^2: 0.7886; BMD: R^2: 0.3573). In control groups macrophages were only scant and numbers were more or less unchanged at different ages. However, younger patients showed slightly higher number of macrophages than older patients (Figure 32 B).

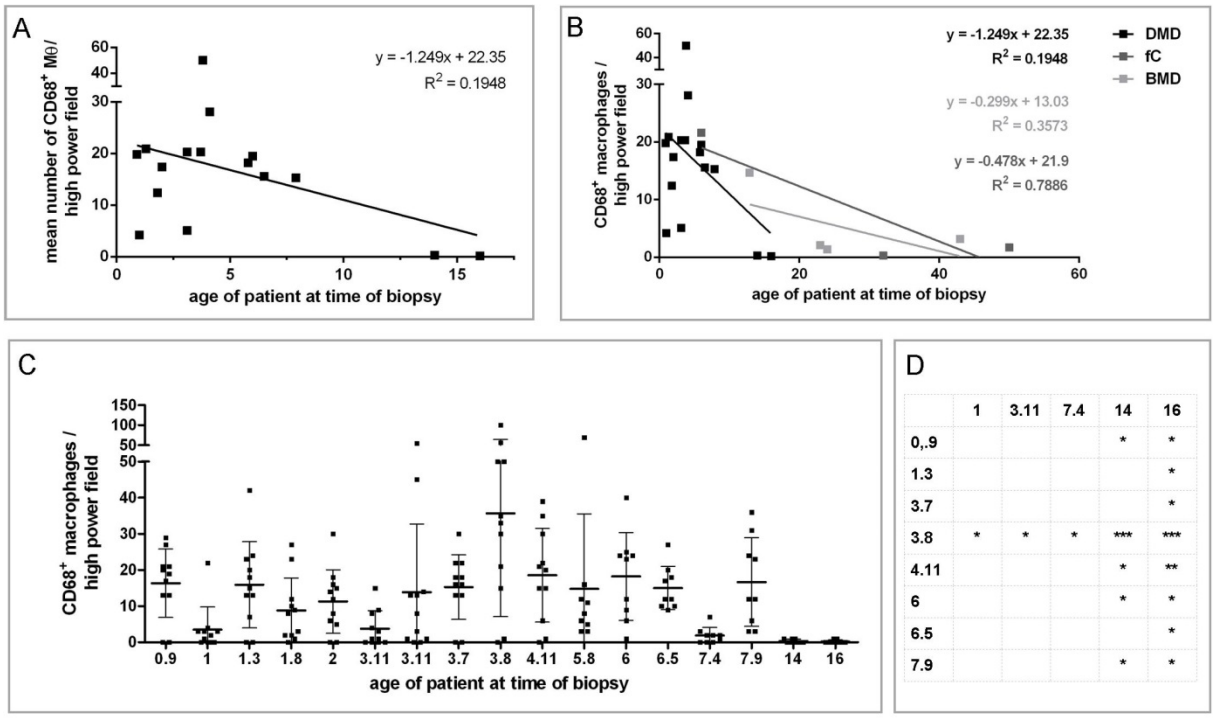

Figure 32: Numbers of macrophages changes with age of DMD patient, but not in BMD and fC
A: The cell count in patients with DMD at various ages shows that amount of macrophages peaks at an age of around four years and numbers decrease over time. Macrophages are completely absent in older patients (~ 15 years); B: $CD68^+$ cells were additionally counted in BMD and fC, where the number of cells is low; C: The distribution of cells in individual DMD patients is displayed, the distribution varies strongly between patients, as well as within the biopsy of a single patient; D: Statistical evaluation of $CD68^+$ macrophage distribution in DMD patients was done with Dunn´s multiple comparison test, p value < 0.05 was considered significant (* ≙ $p < 0.05$, ** ≙ $p < 0.01$, *** ≙ $p < 0.001$). For cell count the average number of cells per 10 high power fields is shown.

In addition to high numbers of macrophages, considerable amounts of lymphocytes were found in muscle tissue of young DMD patients (Figure 33 A, F, K), while there were only low numbers in tissue of older patients (Figure 33 C, F, M). B lymphocytes in general were not seen (Figure 33 K - M). In

control groups T lymphocytes were only scant (Figure 33 D, E and I, J) and B cells were also not detected (Figure 33 N, O). These pictures demonstrate that T cells seem to play only a minor role in progression of DMD pathology.

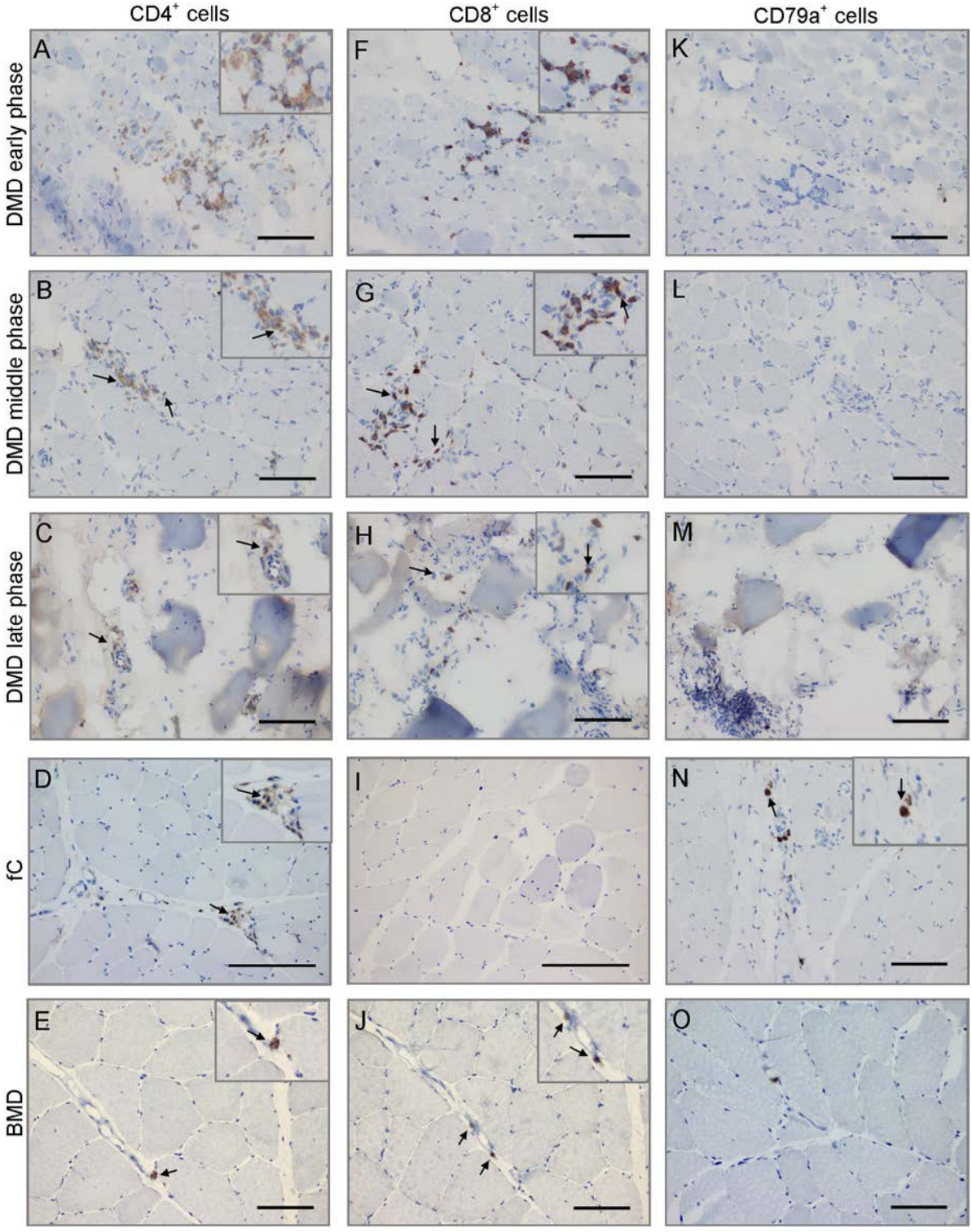

Figure 33: Absolute numbers of lymphocytes are low in patients with *DMD* gene mutations
A-E: Staining of $CD4^+$ T cells, as well as F-J: $CD8^+$ T cells and K-O: $CD79a^+$ B lymphocytes in patients with *DMD* gene mutations. Arrows indicate representative immunoreactive cells of the respective subtype. Scale bar = 100 µm

4.5.4 Development of fibrosis and muscle regeneration is strong in DMD, while control groups display only minor changes

Development of fibrosis is considered one of the hallmarks in DMD and it has been shown that fibrosis directly correlates with early loss of ambulation [171]. However, molecular analysis of expression levels of factors involved in the process has not been assessed in a systematic way yet. Automated whole slide image based quantification was used to analyse the development of fibrosis in patients over time (see methods p. - 24 -). Beside patients with DMD, BMD patients and fC were analysed and staining with collagen VI was used as basis for evaluation. Increasing amounts of fibrotic and fatty tissue were seen, as visualized by brown collagen staining and light, unstained fatty tissue (Figure 34 A-C). As especially observed in older patients the amount of fibrotic tissue was only one major part of muscle wasting, but the amount of fatty replacement was also considerably high (Figure 34 C). From the images, the ratio between muscle fibres and connective tissue was calculated. In DMD patients the ratio was changing over time, while in BMD and fC the ratio in all patients was similar (Figure 34 E, F, G). In DMD fibrotic tissue was increasing (Figure 34 A-C) and the variations in development of fibrosis within the group of DMD patients was high, depending on disease severity and age of patients. Additionally, the amount of fat was strongly increased in older patients, underlining the relevance of this process in muscle rebuilding (Figure 34 C).

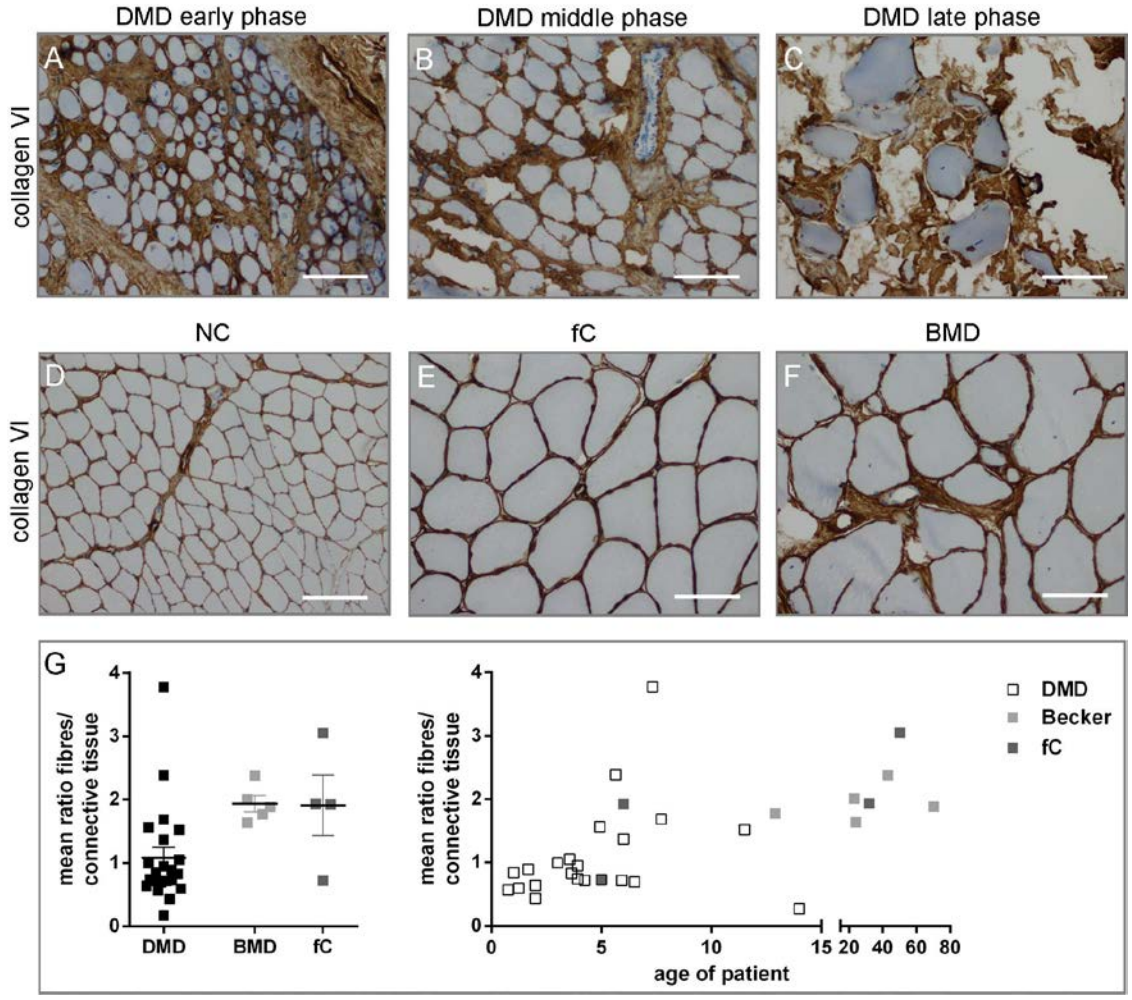

Figure 34: Increased fibrosis is seen in patients with DMD, but not in fC and BMD
A-C: The amount of fibrosis and fatty tissue increased with age of DMD patients. D: Muscle tissue derived from NC was regularly stained with collagen VI, as well as E: fC. F: The same is true for BMD patients. G: Quantification of fibrosis on whole slide images is shown as ratio between muscle fibres and connective tissue. Scale bar = 100 µm

In addition to analyses of fibrosis via immunohistochemistry, molecules involved in fibrosis, like *TGFB1*, Prolyl 4-hydroxlase 1 (*P4HA1*), which is involved in collagen synthesis, as well as the chemokine *CCL18*, were evaluated. Most analyzed molecules were strongly and significantly increased when compared to NC (Figure 35 A), while in BMD patients and in fC displayed expression levels were comparable to those of NC (Figure 35 A). When DMD patients are dissected into subgroups depending on the phase of the disease, a stage-dependent expression pattern of some molecules was seen (Figure 35 B). The molecule *CCL18*, which is involved in early development of fibrosis, was significantly elevated in patients between 4-8 years (Figure 35 B, blue squares), while expression was only at the level of NC in older patients (Figure 35 B, red squares). Reduced expression levels fit to the observation, that fibrotic tissue was already developed in older patients. Interestingly *SOCS1*, a suppressor of fibrosis, *P4HA1* and *TGFB1* were not differentially expressed in the different stages of the disease (Figure 35 B).

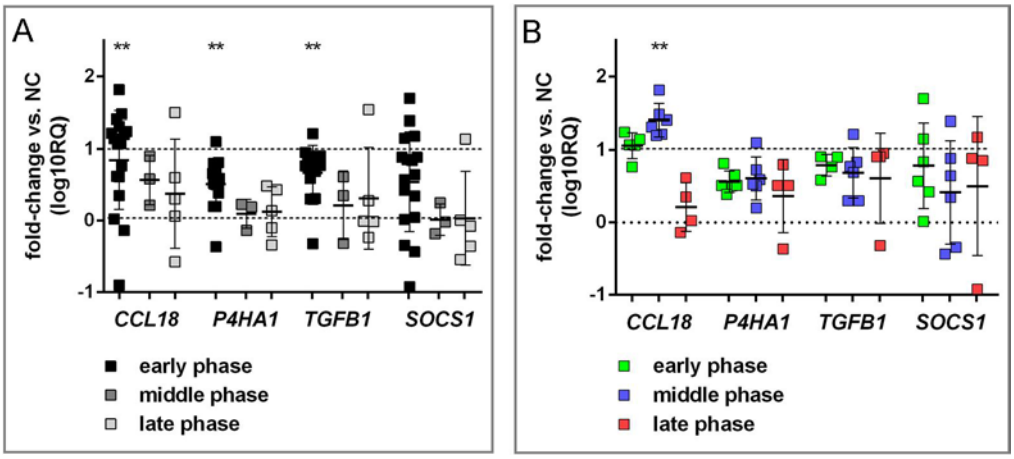

Figure 35: Molecules associated with fibrosis are elevated in patients with DMD, but not in control groups
A: Molecules involved in fibrosis as compared to NC are displayed. B: When DMD patients are grouped according to phase of disease, a phase-dependent expression is seen. Statistical analysis in A, B were performed with Kruskal-Wallis one-way ANOVA with Dunn´s multiple comparison test was performed; p value < 0.05 was considered significant (** ≙ $p < 0.01$)

4.5.5 Expression of cytokines/chemokines in DMD is dependent on the stage of disease

In addition to the molecules involved in fibrosis, cytokine and chemokine profiles of pro- and anti-inflammatory pathways were analyzed by qPCR to investigate potential activation in DMD.

High variation within the expression levels of DMD patients was seen, while the control groups with BMD and fC again displayed very similar expression levels and they were not different from expression levels in NCs (Figure 36 A). These results fit the mostly weak symptoms found in BMD and fC. In DMD patients various molecules were strongly regulated, as *IFNG*. Additionally, *IL6* and *IL1B* were significantly elevated in comparison to those in NC (Figure 36 A).

As for molecules of fibrosis, also key factors of pro- and anti-inflammatory pathways were evaluated in a stage-dependent manner; however the molecules were not differently expressed in the different sub-groups of DMD. Only *IL6* and *IL1B* showed a trend towards higher expression in older patients (Figure 36 B). In addition, interleukins *IL12p40*, *IL17* and *IL4* were exclusively expressed in DMD patients,

while no expression was detected in control groups (Figure 36 C), demonstrating an activation of both, pro- and anti-inflammatory pathways in DMD at a defined time frame during the disease.

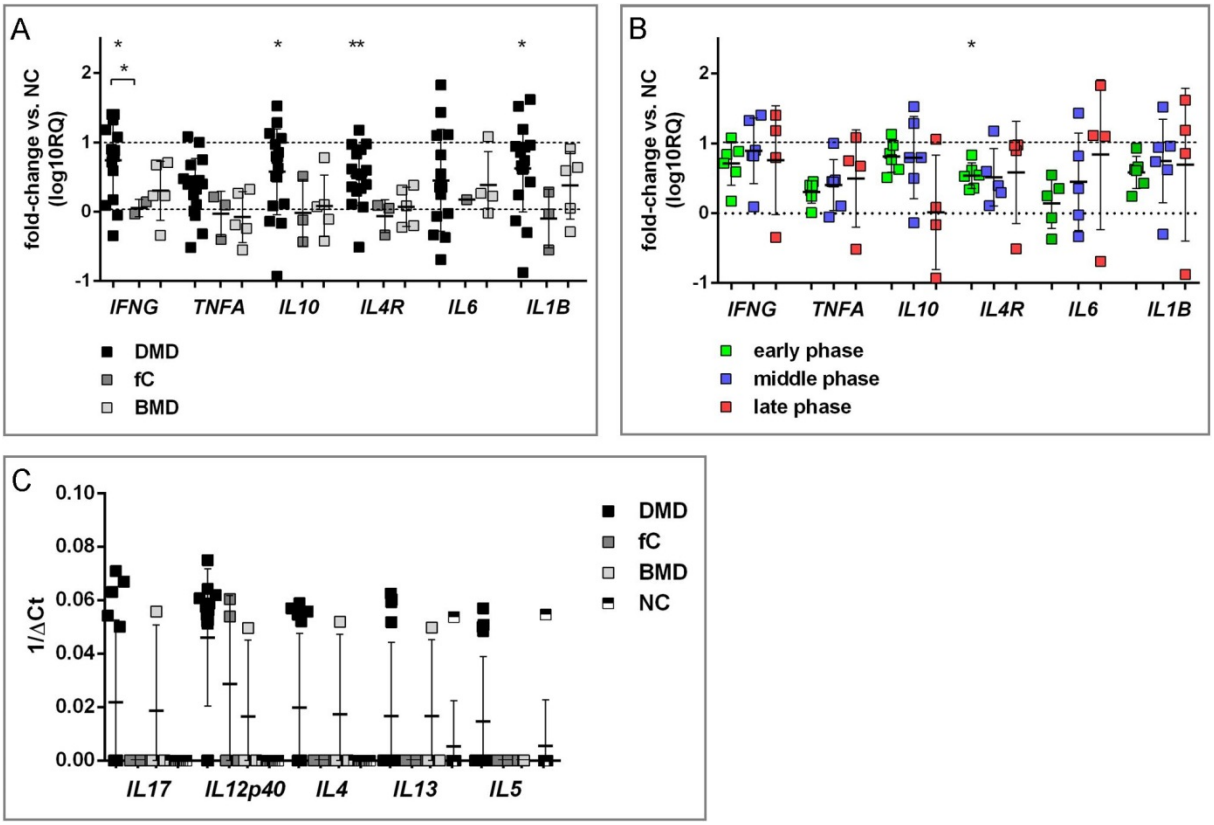

Figure 36: Cytokine and chemokine expression is stage-dependent in patients with DMD
A: Cytokines in DMD patients were elevated when compared to those in NC. B: Expression profiles of molecules split into subgroups of DMD patients. C: Expression levels of interferons in all groups of patients. Statistical analysis was performed with Kruskal-Wallis one-way ANOVA with Dunn´s multiple comparison test. It was not possible to perform statistics for the expression of *IL17*, *IL12*, *IL4, IL13* and *IL5*, because the respective cytokines were not expressed in normal controls; *p* value < 0.05 was considered significant (* ≙ $p < 0.05$, ** ≙ $p < 0.01$).

4.5.6 Recapitulation

Duchenne muscular dystrophy was characterized by increasing inflammation in young patients and by development of fibrosis in older patients. Patients with BMD or fC of the *DMD* gene mutation displayed less morphological alterations in general and can thus be used as control groups. Both groups harbored still some dystrophin expression, while inflammation, as well as development of fibrosis was weak. In contrast, DMD patients developed strong evidence of fibrosis, as it could be demonstrated by EvG and collagen VI staining. The ratio between muscle fibres and connective tissue/fat was decreasing in DMD patients, and on the molecular level, molecules involved in fibrosis, such as *TGFB1* or the collagene synthetase (*P4HA1*) were significantly elevated in comparison to those in NCs. In addition, the amount of macrophages was quantified and the strongest activity and highest number of invading cells were seen in patients at an age of four years. After loss of ambulation (around the age of 10 years) the number of macrophages (as well as lymphocytes) decreased again.

4.6 Inflammatory infiltrates in IIM and MD

The thorough evaluation of the inflammatory infiltrates in patients with NM, DM and DMD in the preceding chapters revealed overt differences in the composition of the inflammatory infiltrates in the entities IIM and MD. To give an overview about the composition, a semi-quantitative score of the involved cells is shown in Table 7.

Although there was heterogeneity in the different entities, the overall composition of the cellular infiltrate was very characteristic. The data clearly illustrated that macrophages were the prevalent cell population in all examined diseases; however, the mere number of macrophages was varying between the entities. IMNM patients had the highest number of macrophages in biopsied skeletal muscles, demonstrating a strong activation of these multifunctional cells. As shown in chapter 4.3.2 the macrophages were distributed across the whole muscle biopsy. In DMD the amount of macrophages can also vary strongly between individual patients. Here single macrophages were found scattered throughout the whole muscle biopsy, while a clear accumulation of cells in infiltrates was also seen. In addition to macrophages, T cells were also part of the infiltrate in all diseases, but their overall number is significantly lower in comparison to the number of macrophages. For example, IMNM patients had significant amounts of T cells, while they were less prevalent in nIMNM or DMD. Evaluation of B cells and plasma cells further revealed that these cells are only scant in all investigated entities, demonstrating a prevalence of T cell-mediated innate immunity. Involvement of the humoral immunity, in terms of pure numbers of B cells and plasma cells in the muscle tissue, is probably less relevant.

Table 7: Composition of inflammatory infiltrates in IIMs and MD

		$CD68^+$ macrophages	$CD4^+$ T cells	$CD8^+$ T cells	$CD79a^+$ B cells	$CD138^+$ plasma cells
NM	IMNM	+++	++	++	- / +	-
	nIMNM	+	+	- / +	-	-
DM	jDM	++	+	+	- / +	- / +
	aDM	++	+	+	- / +	- / +
DMD		+ / ++ / +++	+	+	- / +	-

- = no cells detected; + = only single cells detected; ++ = clear accumulation of cells (~20 cells); +++ = significant numbers of cells (>30 cells)

5 DISCUSSION

Human muscle diseases comprise a huge number of different entities, which may be inherited or acquired. Even if muscle diseases in general are relatively rare, all entities taken together affect thousands of people worldwide every year. The correct diagnosis is often difficult to obtain and in addition to the patients' clinical history, laboratory results and results of electromyography, muscle biopsy and histological analysis are helpful to distinguish between the different diseases that may harbor considerable overlapping features. In the last years, the classification of muscle diseases is constantly growing larger and more complicated. As far as the immune inflammatory myopathies are concerned, recent work has clarified that the classical groups of IIMs, meaning dermatomyositis and polymyositis, are now much more precisely defined. On the one hand new groups and subgroups of certain entities are emerged and on the other hand the entity 'PM', which has been studied for decades and defined on certain criteria, has seriously been questioned as being a separate entity [75–81,92,98]. Therefore, I believe that at present very interesting new data on pathogenesis and aetiology of the IIMs will probably lead to a more exact classification of these diseases. Hence, before starting an investigation as done in the present thesis, the clinical and morphological diagnosis of each individual patient and assignment to a specific group and subgroup based on the present knowledge are of high importance. The first goal of this work was therefore, to identify characteristics in each entity and to group patients accordingly. The present data will contribute to the discussion about classification and pathomechanisms of the myopathies that were studied here.

Muscle cells has been reported to react to injury or inflammation by secreting cytokines or chemokines (e.g. IL-6, IL-1, IFN-γ, CCL2) and attract immune cells to the site of injury [64,65]. Furthermore, they initiate local differentiation of satellite cells or myoblasts and therefore actively support muscle regeneration [68]. Macrophages, due to their multifunctional capacities, are also involved in a variety of reactions in the muscle. It has been shown, that during muscle injury macrophages are involved in clearing pathogens and perform a switch from pro-inflammatory to alternatively activated, wound healing-stimulating macrophage phenotypes [69,70]. Although, the concept of classically and alternatively activation of macrophages is important, it has become increasingly clear that macrophages can adopt a multitude of intermediate phenotypes and probably polyfunctional ones as well. Additionally, fibrosis is initiated by T_H17-like immune responses in interaction with regulatory macrophages [29]. Disturbance of these interactions is also influencing regeneration [71], as well as repair mechanisms [68,73], and a multitude of other functions. Hence, macrophages are important players in autoimmune reactions in myopathies and might therefore be an essential target for specific therapies.

Most of the examined inflammatory muscle diseases cannot be modeled in animal systems and even if there are existing animal models, they often only mimic single aspects of the disease. However, in 1974 the first model of 'allergic myositis' (EAM) [209,210] was established in the rat by immunization with myosin, which resembles the concept of other autoimmune models, such as the experimental autoimmune encephalomyelitis (EAE). EAE has been adopted by other researchers and transferred to the mouse, mimicking aspects of 'PM' [147]. One of the major drawbacks of this model is the fact that the

disease is not linked to a specific autoimmune antibody, which is known to be myositis-specific in humans [211]. Another very interesting approach has also been published by the group of Ascherman, who investigated the innate immune response in a mouse model for anti-synthetase myositis by injecting Jo1 antibodies into mice [212]. Even if this is a useful starting point to mimic a specific subgroup of myositis, to date, no convincing model is available. Therefore, using human skeletal muscle specimens has important advantages. The analyses open the possibility to directly correlate findings in the tissue to the current knowledge about the diseases themselves. In addition, as a future perspective, this approach might give reliable prognostic expectations with respect to a positive therapeutic response of patients and may help to develop personalized therapies. On the other hand, it is not possible to modulate molecular mechanisms only by analyzing human muscle samples; therefore the development and amelioration of murine models for myositis research are also of high importance. Although it is well known that certain pathomechanisms (especially the function of certain cytokines) in rats or mice may not have the same relevance as they do in humans.

In the current thesis three different entities were analyzed, which all share common aspects of their immune response, but feature distinct immunological aspects as well. All entities have an inflammatory part in common, which is mainly regulated by influx of immune cells into the muscle tissue and manifestation of inflammatory cell infiltrates. However, the composition of these infiltrates is varying among the diseases and the involvement of e.g. T cells or macrophages is variable. In addition, the respective cytokine milieu in the muscle tissue is also different in the IIMs and in muscular dystrophies. Therefore the complex interaction of immunity and the skeletal muscle was investigated in the entities, as well as their distinct secondary effects, such as hypoxia, necrosis and fibrosis.

5.1 Subgroups in NM can be distinguished by morphological features and differences in cytokine expression profiles

Necrotizing myopathy is since recently considered a sub-entity of the group of IIMs and hence shares the ‘inflammatory part’ with the other groups like ‘PM’, DM and sIBM [76,77,82]. Clear distinction of morphological alterations present in biopsies of these entities might sometimes be difficult and precise evaluation of characteristic features of each group is very important. Furthermore, in NM the general picture of the disease in patients with an immune-mediated background is basically similar to that in patients with a toxic-mediated aetiology, in terms of mere presence of necrotic muscle fibres. Nevertheless, reactions to therapy are quite different between these groups. Only patients with an immune-mediated background are treatable with immune-modulatory therapy, while patients with toxic aetiology would not benefit from these treatments in general [105,109]. Therefore, I addressed the crucial question, if it is possible to distinguish the two groups (IMNM and nIMNM) by molecular and histological analysis.

Interestingly, beside muscle fibre necrosis, both groups of NM showed a clear inflammatory infiltrate with macrophages invading muscle fibres. Macrophages were accompanied by $CD4^+$ and $CD8^+$ T cells in the infiltrate of IMNM patients, arguing for an interaction of macrophages and a T cell-mediated aetiology in most of the patients. However, some patients showed presence of B cells and plasma

cells, which in addition suggests a humoral immune component. This seemed not surprising, since many patients with autoimmune NM showed a strong positivity for myositis-specific autoantibodies (e.g. anti-SRP-autoantibodies). Current studies aim to clarify the humoral and also the T cellular part of the immune pathogenesis in *in vivo* and *in vitro* models (Benveniste and Boyer, personal communication). The presence of considerable amounts of immune cells found in this study are not in line with the results of many other researchers, who describe that NM is characterized by no or only a scant inflammatory infiltrate [91,92]. Nevertheless, quantification of cells in the present study revealed not only considerable numbers of macrophages, but also of T cells in the biopsies of most IMNM patients. On the other hand, paucity of immune cells in the nIMNM biopsies was a regular finding in all specimens. Interestingly, the macrophages were only confined to necrotic muscle fibres and T cells were found in the proximity of necrotic fibres and fibres with myophagocytosis. Since quantification of these cells revealed significant differences between the groups, I believe that the mere presence of lymphocytes in the context of NM can be considered an element in favor of an immune-mediated aetiology.

Since the common diagnostic strategy in myopathology involves the examination of presence or absence of certain patterns (such as inflammatory cells, expression of MHC molecules, complement deposition, fibrosis, vessel-associated pathology, mitochondrial function etc.), I included a thorough analysis of MHC class I molecules and complement deposition in NM patients. Under physiological conditions MHC class I molecules in the skeletal muscle are expressed on capillaries only; however, in IIMs up-regulation of MHC class I molecules on the sarcolemma proved involvement of the muscle cells in reactions of the immune system. In conjunction with several other molecules, e.g. ICOS, the so called immunological synapse between a $CD8^+$ T cell and a MHC class I molecule can be established. The results of recent studies differ between amount and distribution of MHC class I expression [97,99,114], and for MAC deposition even the absence of expression is described as a typical feature of NM [74,106,114]. MHC class I expression in IIMs has already been studied decades ago by Emslie-Smith and colleagues [213], and interestingly also by McDouall [214] in DMD. Since then MHC up-regulation in IIMs can be considered a paradigm of an ongoing immune response. Therefore, it seemed surprising that in an autoimmune disease, as NM, MHC class I expression was reported to be absent [98]. These authors also describe absence of inflammatory cells in their muscle specimens. In contrast to this, the data of the present study showed, that MHC class I expression in IMNM biopsies was robust and also found on non-necrotic muscle fibres. Although expression was heterogeneous in certain cases, it was regularly observed in distance of focal monocytic infiltrates. On the contrary, the expression in nIMNM patients was only low and confined to regions of myofibre necrosis or single necrotic and their adjacent fibres. The same picture was found for complement deposition. In IMNM patients the expression was widely distributed on the sarcolemma of muscle fibres and also detected on capillaries, while both features were completely absent in nIMNM cases. Remarkably, in IMNM especially patients with a paraneoplastic background and anti-SRP- autoantibodies or anti-synthetase-syndrome showed strong expression of MHC class I and MAC deposition. These results underline the importance of studying certain immune aspects in a standardized manner to be able to assign patients to different subgroups. The clear documentation of the antibodies and methodological procedures used for histology in these cases are essential, since different antibodies might vary in their binding

strength leading to different results. For example in nearly all studies detection of complement deposition has been done with an antibody against C5b9 [78,90,143]. This is of cause reasonable, but since the C5b9 complex is the endpoint of the complement cascade, the different activation pathways are not covered. The investigation of factors involved in different pathways (e.g. C1q, C3 or C4 antibodies) might therefore be also an interesting approach.

In addition to the above mentioned features, presence of B cells has not been described in autoimmune NMs. However, some studies with a small number of patients or single patients used B cell depletion therapies with e.g. Rituximab [89,197]. This approach was based on the concept that antibodies are involved in this disease entity and therefore B cells or B cell activation should theoretically play a role in the immune pathogenesis. However, a conclusive proof of this hypothesis has not been shown yet and the therapeutic success in these preliminary trials was heterogeneous. Therefore, I was interested in detection of B cells and plasma cells in the muscle biopsy specimens and also in the presence of associated B cell chemoattractants. In the present cohort of patients the analysis of B cells with immunohistology and molecular levels of the B cell chemoattractant *CXCL13* showed that B cells were only found in few patients with IMNM. Hence, the use of B cell blockers such as Rituximab or others [84,107,199] seems to be only reasonable in some patients, that can be identified by the features described here, but is probably of less importance in the majority of NM patients. Nevertheless, in refractory myopathies and in some NM patients with anti-synthetase syndrome or anti-SRP autoantibodies first encouraging results with Rituximab therapy were reported [201,215–217]. Therefore, a careful analysis of B cell immunology before treating with further medications is essential to avoid unnecessary use of medications and reduce possible side-effects or interactions with other therapies. Interestingly, patients with certain autoantibodies showed stronger expression of the B cell chemoattractant *CXCL13*, as well as strongly up-regulated expression of MHC class I or MAC deposition. Therefore, the evaluation of autoantibodies in the patients´ serum is certainly also very relevant [95,100,101,218]. However, it remains to be established, if patients endowed with specific autoantibodies relate to specific sub-groups defined by ENMC criteria and if they really have distinct immune pathomechanisms. Based on the findings of my work, ongoing investigations of IMNM patients are performed, with respect to presence of specific autoantibodies and special morphological or immunological features.

Within this work I demonstrated that both sub-groups of NM can be clearly distinguished by their morphological characteristics, as well as by certain immune aspects, such as presence of autoantibodies, B cell invasion and also the underlying cytokine milieu. With knowledge of all of these factors, an adequate therapy for individual patients might be possible according to a defined immune profile, by e.g. TNF blocker, B cell blockers or other biologicals. [93]

5.2 Intrinsic hypoxia-associated pathways are more pronounced in jDM than in aDM patients

The characteristic fact in patients with dermatomyositis is an appearance of the disease either in early childhood (Ø 7 years of age) or in adults at an average age of 60 years, while one does not meet this

feature in NM, sIBM or 'PM'. It is well known that jDM and aDM share morphological features, as perifascicular atrophy of muscle fibres, complement deposition on capillaries and a characteristic inflammatory infiltrate, which have been reviewed and redefined in an international consensus conference recently [115]. On the other hand, jDM patients show a very characteristic disease picture with some features that are not part of the disease in adults, including frequent dysphagia and calcifications of skeletal muscles. In addition there is a well-studied link of adult DM patients to cancer, which is not the case in jDM [95].

Both diseases are considered inflammatory vasculopathies, although clear vasculitis with destruction of vessel walls (necrotizing vasculitis) is not apparent. Also it seems well established, that this vasculopathy leads to rarefication of capillaries, especially in perifascicular regions. One of the theories is that this is due to an endothelial damage and subsequent loss of capillaries of unknown cause. Ultrastructural analysis reveals tubuloreticular formations in these endothelia, which have been attributed to early stages of capillary loss. In a very recent work by Gitiaux and colleagues, it has elegantly been shown that vascular supply is profoundly impaired in DM patients [145]. They could demonstrate that DM patients lose whole microvascular units instead of single capillaries, resulting in reduced blood supply. I hypothesized, that hypoxia-related phenomena may be involved in this vasculopathy, which may ultimately lead to perifascicular atrophy. Also I speculated that molecular pathways involved in inflammation may be linked to hypoxia. A candidate linking the above mentioned processes are macrophages [22,58]. It was previously shown that macrophages in conjunction with T cells (mainly $CD4^+$ T cells) are clustering around small arterioles in the perifascicular region and extending into the perimysium. Thus, I further speculated that these macrophages might interact with the 'vessel compartment' in a specific, yet unknown way. Therefore, pathways involved in inflammatory vasculopathy and hypoxia and their specific contribution in jDM and aDM were a central focus of this work.

In this study I demonstrated that jDM patients are overall stronger affected and showed more symptoms than aDM patients. Therapeutic response to standard therapy (corticoid treatment and/or MTX treatment) was more successful in jDM than in aDM patients. In the studied cohort of aDM patients the therapeutic regime had often only little effect. Most patients described amelioration of symptoms, but complete and sustained remission was often not achieved.

Classical morphological hallmarks of DM are perifascicular atrophy and inflammatory cells, which are mostly located perimysially, but may be also scattered throughout the muscle. [143,144] In this study jDM patients were more strongly affected by perifascicular atrophy than aDM patients, which was visualized by various histological stains. This phenomenon has not been pointed out before and although I found it quite striking, it has to be verified in a larger cohort of patients in the future. Therefore this observation was taken as a starting point to link atrophy to hypoxia. Indeed I found prominent staining of HIF-1α and VEGF in skeletal muscle fibres within the perifascicular region in jDM, which was not the case in aDM.

Under normal conditions the whole body is continuously supplied with oxygen, but insufficient oxygen supply in single organs or tissues can be caused by e.g. pulmonary diseases, heart failure or different forms of vasculopathy [50–54]. In aDM patients it was shown by Pestronk *et .al.* that capillary

damage/loss are concentrated in regions distant from the perimysium [143]. However, by counting of single capillaries and muscle fibres I demonstrated that capillary loss is occurring in jDM patients in contrast to aDM where capillary loss was only seen in some patients. Correlation to the density of inflammatory infiltrates and degrees of perifascicular atrophy revealed that capillary loss is directly linked to these phenomena (Allenbach, Preuße, Stenzel, ongoing work). Inversely, muscle specimens displaying mild or no significant perifascicular atrophy and only scant inflammation showed no significant capillary reduction. As suggested by Probst-Cousin *et. al.* up-regulation of factors involved in hypoxia might be an adaption to the deprivation of the blood supply [144]. Various factors are involved in development of hypoxia or in regulating gene expression under hypoxic conditions (e.g. growth factors), one of the most important factors being HIF-1α. Up-regulation of this factor also seems to be important to mediate the deprivation of blood supply via angiogenesis [144]. In addition *VEGF*, which is involved in angiogenesis and deposition of extracellular matrix [52,219,220], or the growth factor TGFβ, which is involved in tissue remodelling and fibrogenesis [221] are elevated in hypoxic tissue as seen in the current study. Inverse regulatory mechanism between these molecules are also suggested [222]. In keeping with this, I showed an important involvement of hypoxia. Molecular analyses further confirmed these data, since molecules associated with hypoxia were strongly elevated on the mRNA level, especially in jDM patients. Here an elevated expression of *HIF-1α*, *VEGFA* and *MIF* was seen. Since these hypoxic changes are probably due to the reactions in the perimysium, as well as in adjacent atrophic muscle fibres, the isolation of these areas with laser microdissection was an elegant way to analyze the reactions without masking effects of neighboring 'unaffected' tissue. Although isolation of tissue with LCM is a useful technique to analyze specific regions/cells, it should be mentioned, that the laser isolation might affect the molecular profile of the isolated cells or tissue. Therefore, results from qPCR are quite interesting, but are not easy to compare to results from e.g. qPCR from whole-tissue isolates. For example, the amount of isolated RNA is lower and minor changes in cytokine profiles may not be correctly displayed. However, drastic changes in cytokine/chemokine expression were seen in LCM isolated tissue and especially prominent regulatory mechanisms can be revealed. By isolating the perifascicular area it is clear that effects of hypoxia and their regulation were confined to these atrophic areas in DM and non-atrophic, centro-fascicular tissue regions of the muscle fascicle are significantly less affected. Since morphological changes in DM were mostly confined to the perimysium, these results support the hypothesis that molecules involved in hypoxia indeed contribute to pathogenesis of jDM and in lesser extent to aDM.

Beside perifascicular atrophy, a characteristic and well described inflammatory infiltrate was found in DM. However, in contrast to NM, where inflammatory infiltrates are scattered throughout the biopsy, in DM the inflammation was mostly confined to the perimysial area. In this region, inflammatory cells accumulated, with a clear predominance of macrophages, but also lymphocytes were seen. However, their number was significantly lower than the number of macrophages. Linking alternative activation of macrophages to vessel pathology, I was able to show a prominent presence of $CD206^+$ macrophages in all of the examined specimens. This was mostly seen in the perifascicular region, while in the centro-fasciular region only scant $CD206^+$ macrophages were identified. Although this was not studied

in further detail in the present work, it is planned to study a putative interaction of macrophages and endothelia in e.g. an *in vitro* setting.

Comprising the findings, it became clear, that perifascicular atrophy and effects of the vasculopathy seemed to be the most important factors in progression of the disease. Since jDM patients are more strongly affected by vasculopathy and therefore by hypoxia and perifascicular atrophy, but react better to therapy, there might be a correlation between these phenomena. Is the higher regenerative capacity following atrophy the reason for a better responsiveness to therapy? Or do the immune cells react more strongly to therapy due to the young age of patients? These elements need to be further analyzed to ultimately find answers to these questions.

5.3 Influx of macrophages and development of fibrosis are stage-dependent processes in DMD

A number of inherited myopathies are known to also harbor significant inflammatory infiltrates, including the Duchenne muscular dystrophy, dysferlinopathies, and several others. The role of inflammation in these diseases is less well understood and also less studied than in IIMs, since it is generally believed that the inflammation is a secondary process. However, there are several lines of evidence, that this is not the whole truth, because already more than 20 years ago it was shown by McDouall and colleagues that MHC expression is strongly up-regulated, demonstrating an important influence of immune reactions in DMD patients [214]. In addition, corticosteroids are considered the therapeutic gold standard in DMD, indicating that immunosuppression has a beneficial effect in this disease [223], while the exact mode of action is not clarified in all details. Conversely, the same therapeutic approach was inefficient in dysferlinopathies, where also considerable immune reactions take place [224]. This indicates that the inflammatory reaction seems to be very specific in one entity and the putative therapeutic mechanism cannot be extrapolated to other diseases.

Development of fibrosis is a hallmark of DMD and since fibrosis is directly correlated with an early loss of ambulation, as it has been shown in a study with a 10-year follow-up [171], understanding this process can represent a good starting point for specific therapeutic intervention. Current studies mostly focus on restoration of dystrophin expression by e.g. exon-skipping approaches. Pharmaceutical companies as e.g. GSK or Prosensa are developing components to skip various exons. As an example, Prosensa announced that agents for skipping of exon 51 are already in a clinical phase III trial (Prosensa webpage, 2014). These approaches seem very reasonable, since it has been shown in the *mdx* mouse model, that a restoration of about 20 % of dystrophin expression already improves pathology and performance in motor tests and the muscle is protected from exercise-induced damage [165]. These findings are further supported by the morphological picture seen in patients with Becker muscular dystrophy or female carriers of the mutated *DMD* gene where a partial expression of dystrophin is found, which mainly prevents the DMD phenotype in these patients.

However, also reduction of fibrosis or slowdown of its development may be equally important to increase the quality of life of the patients. Therefore, in this project I focused on the process of fibrosis and tissue remodeling, including fatty transformation, and the involvement of macrophages in progression of disease. As mentioned in the introduction, macrophages are strong players in

inflammatory processes and control various reactions in early inflammation and in fibrotic processes. In line with this, in the present work, I showed that over time both, inflammation and fibrotic tissue/fatty tissue was developing in DMD. However, after loss of ambulation the intensity of inflammatory processes decreased again. This was monitored by reduced numbers of inflammatory cells, as well as reduced expression of molecules driving inflammation. This led to the hypothesis that there must be a point in time (or a period) with high macrophage activity and additionally a phase of acute and active fibrogenesis. These phases might be simultaneous or subsequent events. Although, individually both phenomena have been studied before, a chronological link between them has not been formally established.

Creating a new computer-based and specifically developed algorithm for tissue analysis I was able to quantify fibrotic tissue. In the analyzed cohort of patients at different ages, the amount of connective tissue increased with the age of the patients, while at late stage of disease (> 8 years) fatty transformation was an additional important issue. However, quantification of this fatty degeneration process proved to be complicated, since it is not easy to differentiate between fatty tissue (which is optically empty = white) and the translucent area beyond the tissue. Unfortunately, we did not find a valuable solution yet and therefore the precise quantification of fatty degeneration is part of ongoing investigations. Nevertheless, quantifications revealed that the ratio between muscle fibres and connective tissue was reduced in DMD in comparison to BMD and fC, demonstrating increased fibrosis in DMD patients. Older patients had a high ratio, since fibrotic and fatty tissue was the main components of the muscle, while muscle cells are only scant.

Since fibrosis and loss of ambulation are correlated, the time-point for loss of ambulation might be delayed if the development of fibrosis is delayed [225]. The process of fibrogenesis is probably tightly linked to the function and activation status of macrophages, since Desguerre *et. al.* link fibrosis to AAMs [171]. In keeping with this statement, I showed that the highest numbers of AAMs are seen in patients around the age of four years and. Expression levels of e.g. CD206 (*MRC1*) and CD301 were also highest in the early and middle phase, supporting the findings by histology. In patients around four years, the fibrogenesis was starting, while the process was already advanced in patients older than eight years, which was paralleled by increased expression levels of fibrosis-associated molecules, such as the Prolyl hydroxylase or *CCL18*. In addition, at late time points only scant lymphocytes or macrophages were observed. These findings are supported by results from studies with the *mdx* mouse, where the switch of pro- to anti-inflammatory macrophages is associated with increased regeneration and tissue repair [188]. Therefore, the balance between macrophage populations might influence the course of the disease. Despite the promising results in the *mdx* mouse model the correlation to the human situation is challenging, since the development of disease clearly differs between mice and humans. Not only is the progress of disease milder in *mdx* mice, the symptoms grow stable in older mice and neither the ‘clinical picture’ nor the morphological picture deteriorates further (reaching a plateau) [178]. Therefore, I think that the role of macrophages and their impact on fibrogenesis in mice differs from that in the human situation and both conditions have to be studied comparatively.

It is well known, that progression of the disease in individual patients is happening at different speed, which is probably dependent on the genetic background and consecutive production of residual amounts of dystrophin protein. Therefore, not only the age of patients, but also the information that can be gained from muscle biopsy, e.g. active mediators, which mirror the stage of disease, is essential to understand heterogeneity of the disease. With this knowledge, a therapy targeting specific mechanisms at certain time points may be developed. Although corticosteroids are well established and the present use of corticosteroids is clearly beneficial, in daily practice it is often unclear when to start therapy and when to stop it, causing troublesome discussions in individual families. Based on the results of the present work, it seems appropriate to start therapeutic intervention at an early time point with anti-fibrotic therapy, since up-regulation of pro-fibrotic molecules was already a very early process. These data also point to the fact that fibrosis is not continuously developing during the disease; moreover it seems to be limited to the ambulatory phase. I clearly show that quantitative amounts of fibrotic tissue was not further increasing after the age of ten years, but nevertheless there is significant tissue remodeling after this time point with remarkable fatty tissue degeneration. Interaction of adipocytes with inflammation is a further challenge in DMD and has not been addressed at all.

6 CONCLUSION

Comprehensive analyses of three human muscle diseases revealed that macrophages are underestimated cells contributing in different aspects of the immune reaction in myopathies. In all three entities macrophages are the predominant immune cell population and are involved in a variety of cellular processes, such as inflammation, hypoxia and fibrosis.

Due to the fact, that all diseases have an inflammatory part in common, reduction of the inflammatory processes might be beneficial in all entities. In IMNM increased expression of pro- and anti-inflammatory mediators was detected and infiltrates consist not only of macrophages but also of T cells, while toxic NM displayed a different morphology. Based on these results, the most interesting fact is that classification of patients into IMNM or nIMNM is possible by thorough morphological analysis, as well as by assessment of molecular mediators. In addition, IMNM patients often showed special features when specific autoantibodies were present. Future investigations should therefore focus on subgroups in NM with distinct autoantibodies, like anti-Jo1- or anti-SRP-autoantibodies. Here a correlation of antibody appearance and special clinical phenomena is an important research objective. Ongoing work in our group therefore focuses on analysis of $Jo1^+$ patients and it was shown that important morphological changes occurred inside the nucleus. We were able to illustrate that intracellular trafficking of actin is impaired. Additional investigations are needed to explain this specific pathophysiology.

To clarify the pathogenesis in patients with DM, beside evaluation of cellular infiltrates, the extent of hypoxia was an additional important feature. I demonstrated that jDM patients were more strongly affected by the vasculopathy. In addition, capillary loss was especially occurring in jDM or more strongly affected aDM patients. Localization of hypoxic molecules in the perimysium was demonstrated. Since AAMs were predominant in these areas, future research of our group will focus on the interactions between macrophages and vessels in an *in vitro* model.

The present data reveal that in DMD the infiltration of immune cells was a time-dependent process and was closely associated with fibrogenesis. In this case AAMs were the predominant cell population in the middle phase of the disease, and their activity was a crucial factor in disease development. Therefore the slow-down of fibrogenesis, leading to prolongation of the mobile phase, may be the key for increasing the quality of life of the patients. Continuing studies should therefore transfer these findings to a mouse model and correlate the disease stages of humans to the corresponding stages in mice. Here the *mdx* model or other mouse models, e.g. *mdx*/utrn$^{-/+}$, which more closely reflect the human situation [183], are notably. The use of mouse models might be helpful, since e.g. the process of fibrogenesis can be influenced through application of therapeutics, such as anti-fibrotic medication or by immune-modulation. Also crossing these mice with mice harboring a genetic modification of relevant molecules can be helpful to further dissect mechanisms that are important during development of the disease.

SUPPLEMENTAL DATA

Supplemental Table 1: Clinical findings of patients with necrotizing myopathy
Patients grouped as immune-mediated (16 patients), non-immune-mediated (8 patients)

	patient number	age	gender	CK (x-fold)	auto-Ab (MAA/MSA)	systemic disease (autoimmune or paraneoplastic)	treatment/ clinical follow-up
immune-mediated NM	1	57	f	14x	ANA 1:1280, PL-7, PL-12, Ku, Ro52	Sjögren's syndrome, antisynthetase syndrome	multiple cycles of treatment with steroids, 2 cycles of Rituximab, multiple cycles of IVIG; MTX still considerable dyspnoea, improved muscle strength
	2	74	m	5x	-	colon-cancer	treatment of colon carcinoma with resection and adjuvant chemotherapy
	3	80	f	10x	n.d.	primary biliary cirrhosis (PBC)	treatment with steroids
	4	36	f	46x	Ro-52	Raynaud's syndrome	treatment with multiple regimens of steroids multiple cycles of IVIG subjective improvement of Raynaud's syndrome and muscle weakness
	5	66	m	41x	SRP	renal cell carcinoma	treatment with steroids, tumor resection (nephrectomy) subjective improvement of muscle weakness
	6	57	m	n.d.	ASMA	squamous cell carcinoma	treatment of carcinoma with chemotherapy treatment with steroids without improvement
	7	19	f	78x	SRP	infection with Chlamydia, 6 months prior to muscle symptoms	Chlamydia treatment with Doxycyclin 1st line steroids: partial recovery; 2nd line IVIG & steroids: recovery; maintenance treatment: Azathioprine & low dose steroids complete cessation of any treatment due to pregnancy
	8	55	m	47x	n.d.	Non-Hodgkin lymphoma	lymphoma treatment with chemo- and radiotherapy
	9	63	f	n.d.	SRP	symptoms started after viral infection a few months earlier	treatment with steroids, MTX
	10	37	f	n.d.	ANA 1:2560, Ro-52		treatment with steroids mild improvement
	11	64	f	35x	Jo-1	rheumatoid arthritis, interstitial lung disease antisynthetase syndrome	1st line steroids: recovery; 2nd line IVIG & steroids: recovery maintenance treatment: IVIG & low dose steroids

	12	79	m	2,4x	AChR	myasthenia gravis, obstructive airway disease	treatment with steroids and Kalymin: without improvement cessation of Kalymin and high dose steroids followed by reduction of steroids and maintenance therapy with AZT
	13	68	f	71x	Jo1	Anti-synthetase syndrome	treatment with steroids
	14	37	f	51x	Jo1	Anti-synthetase syndrome	no information about treatment and follow-up
	15	71	f	45x		adenocarcinoma of the lung	tumor resection, treatment with steroids: mild improvement maintenance therapy of 30mg prednisolone required
	16	47	m	5x		chronic lymphatic leukemia stage A(I); polyneuropathy	CLL therapy: steroids, Rituximab, fludarabin, cyclophosphamide 3 cycles followed by high dose IVIGs and cyclophosphamide
	patient number	**age**	**gender**	**CK**	**auto-Ab (MAA/MSA)**	**toxic agent / clinical aspect**	**treatment / clinical follow-up**
non-immune-mediated NM	17	52	f	33x	n.d.	malarone	
	18	62	m	90x	-	simvastatin, Voriconazole	stop of medication with voriconazole and statins
	19	55	f	15x	-	simvastatin	stop of statins, with subjective partial improvement; addition of steroids
	20	76	m	900x	n.d.	acute renal failure, dialysis, diabetes mellitus	dialysis, no further information available
	21	53	f	50x	-	Quetiapine*	methylprednisolone, no further information about course of disease available
	22	75	f	300x	-	simvastatin	stop of statins, forced diuresis, CK drops to normal no further information about clinical long term course available
	23	33	m	36x	n.d.	alcohol excess	discharge from hospital with benzodiazepine and Paracetamol
	24	70	f	93x	n.d.	simvastatin	no further information about clinical long term course available

nd.: no data; mo: months; f: female; m: male; Ab: antibody; AChR: acetylcholine receptor; ASMA: alpha smooth muscle actin; IVIG: Immunglobulins; MTX: methotrexat

Supplemental Table 2: Clinical findings of patients with dermatomyositis
Patients grouped for juvenile DM (15 patients) and adult DM (11 patients)

	patient number	age	gender	tubuli	CK (x-fold)	symptoms	therapy	success of therapy
juvenile dermatomyositis	25	15	f	+	50x	mw, s, d, si	MTX, Cyclosporin A, high dose corticoisteroids (1mg/kg) later additionally Hydroxychloroquin	complete remission, no sign of calcification
	26	7	m	+		mp, si, d, mw	Prednisolon therapy (40mg/d 8we) after relapse MTX Methylprednisolon (3d/every 2we), IVIG 30g, 3/d Methylprednisolon	remission, relapse after dose reduction,
	27	2	m	+	3,1x	si, s	Prednisolon, MTX, folic acid	remission, relapse after dose reduction
	28	5	f	n.m.	4,5x	si, mw, mp	6-12 we Corticoids: Decortin and Prednisolon 20mg/d due to side effects, change in therapy: Prednisolon, Azathioprin	remission, side effects, change of therapy, clear remission
	29	4	f	n.m.	2x	mw, mp	Methylprednisolon-pulse, MTX	remission, slight relapse, then complete remission
	30	8J 10M	m	+	normal	mw, si, s	high dose Methylprednisolon IV, Cyclosporin A, Prednisolon due to side effects, change of therapy: Urbason, MTX	remission, side effects, change of therapy, complete remission
	31	13J 8M	m	+			Urbason, MTX	clear improvement
	32	11J 3M	f	n.m.	20,2x	mw, mp	Urbason, MTX	clear improvement
	33	3J 11M	f	+	4,6x	mw, mp, si, d	Methylprednisolon therapy	slight improvement
	34	9J 1M	f	+	2,9x	si, mw, mp	Urbason pulse 650mg over 3d	complete remission
	35	4	m	n.m.		mw, mp, fever, s, si	MTX, Prednison 4we cortison therapy, Methotrexat, Decortin	improvement relapse and change in therapy
	36	ca. 1J	f	n.m.			Urbason, decortin therapy 1mg/d	complette remission Urbason stopped, without problems

	37	10	m	+	normal	si, mw, mp	Prednisolon 20mg - 0 - 10mg, MTX 15mg/1/we; Urbason therapy 3x500mg on 3 consecutive days and 30mg IgG IV change in therapy due to muscle pain: MTX 15mg time of discharge from hospital Prednisolon 12,5mg/d, MTX 15mg 1x/we	clear remission slight relapse with pain change in therapy: remission
	38	8	m	n.m.		mw, si, a	Urbason pulse therapy 20mg/kg 3d, MTX 15mg/we	Remission Relapse after infection Remission
	39	14	m	n.m.	15x	mw, mp, si	Methylprednisolon therapy 1g/d on 3 consecutive days, MTX 25mg 1x/we, Vigantolette 500 I.E/d	First no effect, than remission, CK normal
	patient number	**age**	**gender**	**tubuli**	**CK (x-fold)**	**symptoms**	**therapy**	**success of therapy**
adulte dermatomyositis	40	44	f	+		mw, s, a	Azathioprin 125mg/d, Prednisolon 12,5g/d, MTX 1/we Quensyl/Urbasol Prednisolone reduced to 7,5mg/d	weakness decreased, skin unchanged change of therapy no remission, strong side effects Prednisolon reduced
	41	71	m	+	22,5x	si, mp, d	Methylprednisolon 500mg IV dosis reducsd	remission, CK normal relapse
	42	56	f	+	20x	mw	Prednisolon	CK decreased
	43	49	f	+		mw	Corticosteroids 20mg	
	44	58	m		8,4x	s	Prednisolon iV (250mg, 100mg)	remission
	45	86	f	+	10,3x	mw	Corticoid therapy	no effect
	46	78	f			t	Prednisolon 20mg	
	47	55	m		25,7x	mw, mp		
	48	52	f		normal	mp	MTX Prednisolon IV (250mg, 100mg)	no effect
	49	83	f	+	2.5x	mw, t, d, si	Prednisolon 2mg/kg reduced to 1mg/kg, Azathioprin	remission, CK normal
	50	44	f	+	normal	mw, mp, mya, a	MTX 7.5mg/we	

Symptoms: muscle weakness = mw, muscle pain = mp, arthralgia = a, skin involvement = si, dysphagia = d, swelling = s, tetraparesis = t; mya = myalgia; n.m. = no material for EM studies; nd.: no data; mo: months; f: female; m: male; IVIG: Immunglobulins; MTX: methotrexat

Supplemental Table 3: Clinical findings of patients with Duchenne muscular dystrophy and control groups
Displayed are all available information about symptoms, further diseases and treatment of the DMD patients (25 patients) and control groups (female carrier of the *DMD* gene: 5 patients, and Becker muscular dystrophy: 6 patients)

	patient number	age	gene mutation	symptoms	medication/therapy
Duchenne muscular dystrophy	51	3,11	Dup Ex 56-63	cardiomyopathy, respiratory insufficiency, mild mental retardation, wheelchair dependent	Enalapril 2x/d 3mg; Carvedilol 2x/d 6,25mg physiotherapy, swimming each 1x/we
	52	12,1	no information available	cardiomyopathy, wheelchair dependent, respiratory support at night, nutrition via PEG-tube	Metoprolol, Hydrochlorothiazid
	53	10,1	no information available		
	54	14,00	no information available	Scoliosis, cardiomyopathy, respiratory support, wheelchair dependent	Propranolol (Obsidian) 1x6,25mg, Enalapril 2x5mg, Hydrochlorotiazid 2x12,5mg, Movicol 1TL/d
	55	7,90	no information available		
	56	1,30	PM Base Exon 40 (c.5620G-->T)	migraine, decreasing muscle strength, initial wheelchair dependency, positive Gowers'-sign	Calcort 30mg/d, physiotherapy, logopaedics, psychotherapy, swimming each 1x/we
	57	3,70	Point mutation Ex 17	biopsy at ~4 years of age: CK 190-fold at age 11: loss of ambulation, multiple contractures	At age 11: Prednisolon, PTC study
	58	4,11	no information available	to small, to light, problems climbing stairs, positiv Gowers'-sign, muscle weakness proximal, gnome calves, mental retardation	
	59	3,80	no Del/Dup found	CK 197-fold, proximal weakness, walking only short distances, learning disability, aggression	no medication
	60	1,80	Del Ex 50 and 54-60	motor retardation, mother genetic proven carrier of *DMD* gene, mental retardation, speech development only rudimentary, proximal weakness, hypertrophic calves	Deflazacort
	61	11,50	no del/dup found	proximal weakness, wheelchair dependent since age 9, CK up to 100-fold	
	62	0,90	Del Ex 48-50	hepatomegaly, CK 60-fold, proximal weakness	
	63	3,11	Del 3-12 in frame	proximal weakness, CK 125-fold, positiv Gowers'-sign	L-Carnitin, Vit B Complex, Coenzym Q10
	64	6,50	Del Ex 48	biopsy at six years of age: muscle weakness, no cramps, no pain, no familial genetic background, hypertrophic calves, CK > 3,5-fold currently 14 years: adipose, loss of ambulation	Prednisolon
	65	5,80	Dupl Ex 2	positive Gowers'-sign, limited strength/motor capacities since age 3, CK > 100-fold	
	66	4,30	Del Ex 10-13 in frame	uncle died at 25 years of age, also genetically proven DMD, loss of ambulation at age 8, early development of scoliosis,	

				prominent contractures	
	67	7,40	Del Ex 45-45 in frame	CK > 20-fold	
	68	5,11	no information available		
	69	5	Del Ex 51	combined developmental disorder, starting heart insufficiency, wheelchair dependent, learning disorder, CK 36-fold, loss of ambulation at age 7	beta-blocking agents 2x0,5/d, Movicol 1 Btl/d cortison no effect
	70	3	no information available		
	71	1	Del Ex 49-52	CK 80-fold	
	72	2	Del Ex 19	CK 150-fold, mental retardation, proximal weakness, hypertrophic calves, positive Gowers'-sign	
	73	5	c.4438_4439insCA;p.(Glu1480Alafs*3)	proximal weakness, speech development delayed	Deflazacort
	74	22	no information available		
	75	6	no mutation found	proximal weakness, multiple contractures, hypertrophic calves, positive Gowers' sign	
	patient number	**age**	**gene mutation**	**symptoms**	**medication/therapy**
female *DMD* carrier	76	6	no information available		
	77	12	no information available		
	78	32	c.4438_4439insCA;p.(Glu1480Alafs*3)	cardiomyopathy	ACE inhibitors, beta-blocking agents
	79	50	no analyzes		
	80	56	no information available		
	patient number	**age**	**gene mutation**	**symptoms**	**medication/therapy**
Becker muscular dystrophy	81	12,90	Del Ex 45-47 in frame	proximal weakness, CK 30-fold, foot drop since age 6, reduced muscle strength, hypertrophic calves, positive Gowers' sign	
	82	23,00	no information available	increasing walking problems, reduction of breathing capacity, hip problems	
	83	2,90	no information available	cardiomyopathy, initial wheelchair dependency, weak scoliosis, positive Gowers'-sign, weakness of pelvic girdle	Calcort 6, 5/d
	84	24	no information available		
	85	43	c.650-3C>A hemizygous	no information available	
	86	34	no information available	no information available	

Del = deletion, Dup = duplication

Supplemental Table 4: Clinical information for healthy normal controls

	patient number	age	gender	time since onset of symptoms (mo)	CK	auto-Ab (MAA/MSA)	Symptoms/ treatment
normal controls	88	47	m	--	normal	-	diffuse myalgia & arthralgia, no treatment
	89	41	f	--	normal	-	myalgia, no treatment
	90	2	m	--	normal	-	*, no treatment
	91	57	f	--	normal	-	myalgia, no treatment
	92	55	m	--	normal	-	myalgia, no treatment
	93	13	f	--	normal	-	Epilepsy, medication Felbamat, Primidon
	94	1,75	m	--	normal	-	dystonic movement disorder
	95	7,5	f	--	normal	-	diffuse myalgia, no treatment
	96	26	m	--	normal	-	Recurring subjective weakness, no treatment

* Mother with a history of malignant hyperthermia; therefore, due to an upcoming operation of the heart in her child, the risk for malignant hyperthermia in the child was excluded, among other means by doing a muscle biopsy which was entirely normal

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DANKSAGUNG

An dieser Stelle möchte ich all denen danken, die mich in den Jahren meiner Doktorarbeit begleitet und unterstütz haben.

Mein besonderer Dank gilt:

… *Prof. Dr. Frank L. Heppner*, der es mir ermöglicht hat meine Arbeit in seinem Institut anzufertigen und für seine kritische Hinterfragung meiner Daten und Thesen.

… *Prof. Dr. Werner Stenzel* dafür, dass er mir gezeigt hat, was man alles unter einem Mikroskop sehen kann, wenn man weiß, wie es geht. Außerdem waren der Austausch und die Kooperation mit anderen Wissenschaftlern und die vielen, tollen Kongressreisen nur durch seine fortwährende Unterstützung möglich.

… *Prof. Hans-Hilmar Goebel* für seine andauernde Unterstützung und dafür, dass er seine langjährige Erfahrung auf dem Gebiet der Muskelerkrankungen mit mir geteilt hat.

… den *Mitgliedern meiner Prüfungskommission* dafür, die sich die Zeit genommen haben meine Arbeit zu lesen und zu begutachten.

… all denen, die unsere Projekte und meine Stelle finanziell unterstützt haben. Im Einzelnen: der *FAZIT-Stiftung*, der *Sonnenfeld-Stiftung*, der *action Benni & Co.* und der *DGM*.

… *meinen Kollegen*, für die wunderbare Zusammenarbeit und die vielen Anregungen für meine wissenschaftliche Arbeit. Nicht nur den Arbeitsalltag, sondern auch unsere vielen privaten Unternehmungen werden mir in guter Erinnerung bleiben!

EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, Corinna Preuße, dass die vorliegende Dissertation zum Thema: '*Common and distinct immunological aspects in acquired inflammatory myopathies and inherited muscular dystrophy*' selbstständig verfasst wurde und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet wurden.

Berlin, den 16. Juni 2014
Corinna Preuße