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The Clinical and Pathological Spectrum of Idiopathic Inflammatory Myopathies

*Implications for pathogenesis, classification
and diagnosis*

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Cover: Drawing by Björn Danielsson of cross-sectioned muscle.

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In memory of my father, Gunnar Danielsson,

in gratitude to my mother, Britt,

with love to my family:

Ingela, Björn, Elsa

and Dag

“Let us not talk falsely now, the hour is getting late”

An elusive Nobel laureate

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ABSTRACT

Background: Idiopathic inflammatory myopathies (IIM) constitute a heterogeneous group of diseases with severe consequences for the life of affected patients. Dermatomyositis, polymyositis and inclusion body myositis (IBM) are the classical representatives of this group. The treatments given today often have limited effects, and are taken at the cost of side effects. Major obstacles in the search for more effective treatments are; (1) an incomplete understanding of the disease mechanisms, (2) difficulties to delineate homogeneous disease groups for clinical studies and (3) the sometimes challenging task to diagnose these diseases.

Aims: We addressed a number of “loose ends” in the areas of pathogenesis, classification and diagnosis; mechanisms of muscle fiber degeneration in IIM, with a focus of programmed cell death (apoptosis) and invasion of muscle fibers by inflammatory cells (partial invasion); protecting and mediating factors present in muscle; the association of other diseases with IIM, in particular celiac disease ; the evaluation of two classification systems and laboratory methods for increased diagnostic performance.

The studies: We included 106 patients, diagnosed at the Neuromuscular unit in Linköping, Sweden, with pathological muscle findings consistent with IIM. The incidence in the county of Östergötland (during 5 years) was 7.3 per million/year (3 patients each year). Of 88 patients with confirmed IIM 4 (4.5 %) had celiac disease, 33 (38%) had an associated systemic inflammatory disease and 5 (5.7 %) had a malignancy. Ninety-nine patients were included for a comparison of two classification systems using criteria of the European Neuromuscle Centre (Amato/ENMC), and the widely used Bohan and Peter classification, both with the addition of IBM according to *Griggs et al.* Using the Amato/ENMC criteria the most prevalent diagnostic group after IBM (30%) was non-specific myositis (23%), followed by polymyositis (20%) and dermatomyositis 17%). A substantial number of patients meeting Bohan and Peter (or Griggs) criteria were excluded by Amato/ENMC criteria, most (21/23) due to lack of detectable muscle weakness. Extended muscle sectioning increased the sensitivity of a muscle biopsy by 15 % and the specificity by 22%, and showed an overlap between disease groups. Muscle biopsies from patients with IIM and controls were used to investigate pathological findings considered specific for disease groups, and for the presence of programmed cell death (apoptosis) and disease protecting and mediating factors in muscle. The presence of apoptotic muscle fiber nuclei was detected in muscle with partial invasion (however not in the invaded fibers) in the presence of granzyme B and CD8⁺ cytotoxic T cells. The major apoptosis inhibiting protein Bcl-2 was shown to be constitutionally expressed in healthy muscle but weakened in IIM.

Conclusion: We present apoptosis as a possible disease mechanism in parallel with partial invasion of fibers. Furthermore, partial invasion may not be a suitable distinguishing feature in the pathogenesis, or for classification and diagnosis of IIM. We also introduce the anti-apoptotic Bcl-2 as a possible relevant muscle fiber protecting factor. A more extensive pathological work-up improves classification and diagnosis of IIM. The proposed Amato/ENMC creates a substantial portion of patients with non-specific or unclassified myositis. Associated diseases are common in IIM, and also include celiac disease.

SVENSK SAMMANFATTNING

Kroppens muskler (tvärstrimmig muskulatur) kan drabbas av en mängd olika, men ofta ovanliga, sjukdomar. En sjukdomsgrupp som kallas idiopatiska inflammatoriska muskelsjukdomar (IIM) orsakas av en kronisk inflammation i musklerna. De som drabbas lider ofta av en långvarig funktionsnedsättning i form ett rörelsehandikapp som orsakas av försvagade muskler. Dermatomyosit, polymyosit och inklusionskroppsmyosit (IBM) är de mest kända sjukdomarna inom gruppen. Det mesta talar för att dessa sjukdomar har en *autoimmun* orsak, vilket innebär att kroppens immunförsvar angriper den egna kropps vävnaden, i detta fall musklerna. Den behandling som finns tillgänglig idag har begränsad effekt och medför ofta biverkningar. En orsak till detta är att sjukdomsmekanismerna bara delvis är kända. Dessutom försvåras behandlingsforskning av osäkerhet om hur sjukdomarna skall klassificeras (det vill säga delas in i tillräckligt likartade sjukdomar) för att lämpliga behandlingsstudier skall kunna genomföras. Förutom att sjukdomarna är ovanliga så är också symtomen ibland svåra att känna igen. Detta tillsammans med att sjukdomarna ofta uppträder samtidigt med andra sjukdomar medför att det ofta är svårt att ställa diagnosen.

Vi tog fasta på att antal ”lösa trådar” inom tre forskningsområden; sjukdomsmekanismer (patogenes), klassifikation och diagnostik, som sedan blev föremål för våra studier. Ett område som studerades var mekanismer för olika typer av muskelfiberdöd (muskelfibrer är de ”jätteceller” som bygger upp muskler och ”skapar rörelse”). Vi studerade programmerad celldöd (apoptos) och en angreppsform av celldödande inflammatoriska celler som invaderar muskelfibrer som kallas partiell invasion. Samtidigt undersökte vi skyddande och sjukdomsorsakande ämnen (proteiner) i muskelvävnad. För att förbättra lämplig indelning i sjukdomsgrupper och diagnostik gjorde vi en utvärdering av ett nytt klassifikationssystem och undersökte lämpligheten av partiell invasion som ett klassifikationskriterium, samt utvärderade en laboratoriemetod för förbättrad diagnostik. Vi undersökte också hur många som varje år insjuknar med IIM och om glutenintolerans (celiaki) är vanligare i denna patientgrupp än hos folk i gemen.

Ett hundra sex patienter deltog i studierna. De hade alla konstaterats ha muskelinflammation i vävnadsprov (muskulbiopsi), som var analyserade vid Neuromuskulära enheten i Linköping. Under fem år (1997-2001) insjuknade varje år tre personer i Östergötland, vilket motsvarar 7,3 per 1 miljon invånare och år. Glutenintolerans undersöktes med blodprovsscreening, och av 88 patienter med fastställd IIM hade 4 (4,5 %) glutenintolerans. Detta visade sig vara klart vanligare (statistiskt signifikant skillnad) än i den övriga befolkningen (ca 1 %). Trettio tre patienter (38 %) hade samtidigt en inflammatorisk systemsjukdom (inom den reumatiska sjukdomsgruppen) och 5 (5,7 %) hade en samtidig cancersjukdom. Detta är

sjukdomar som man vet har en koppling till IIM, men uppgifterna om hur vanligt det är varierar.

Nittionio patienter blev efter ett antal fastlagda kriterier indelade i sjukdomsgrupper enligt en nyligen föreslagen klassifikation (Amato/ENMC) och enligt en mer beprövad (men delvis kritiserad) (Bohan och Peter). För sjukdomsgruppen IBM användes i bägge fallen en särskild klassifikation (Griggs). När kriterierna för Amato/ENMC användes var IBM (30 %) den vanligaste sjukdomsgruppen, följt av gruppen *ospecifik* myosit (muskelinflammation) (23 %), följt av polymyosit (20 %) och dermatomyosit (17 %). Anmärkningsvärt var att många patienter som kunde klassificeras enligt Bohan och Peter-klassifikationen inte ”fick plats” i Amato/ENMC klassifikationen (exkluderades). Den vanligaste orsaken (21 av 23) var att dessa patienter inte hade någon tydlig kraftnedsättning; också anmärkningsvärt var att partiell invasion, som används som särskiljande kriterium av Amato/ENMC, förekom i olika sjukdomsgrupper. En tillämpad metod på Neuromuskulära enheten för snittning och färgning av muskelvävnad, som inte tidigare utvärderats vetenskapligt, visade sig kunna öka antalet fall med diagnostiserad IIM med 15 % och antalet rätt klassificerade diagnoser med 22 %.

Vävnadsprov från muskel användes också för undersökning av mekanismer som skyddar mot eller leder till celldöd. Vi kunde påvisa att programmerad celldöd förekommer i muskler från patienter med IIM, även om detta tidigare har ifrågasatts. Denna typ av celldöd fann vi nästan uteslutande i muskel där det också påvisades partiell invasion. Partiell invasion orsakas av att immunceller ($CD8^+$ mördar T celler) invaderar muskelfibern. Dessa immunceller bildar ett enzym som kan utlösa programmerad celldöd (granzym B). I mikroskop kunde vi se dessa mördarceller, med granzym B i ”närkontakt” med muskelfibrer med apoptostecken. Vi kunde också påvisa att muskler hos friska uttrycker ett sjukdomsskyddande protein, Bcl-2, som kan förhindra att muskelceller dör, medan Bcl-2 var försvagat i muskler hos patienter med IIM.

Slutsatser: Vi har visat att programmerad celldöd (apoptos) förekommer i muskelfibrer vid IIM. Resultaten ger stöd för att immunceller ($CD8^+$ ”mördar” T celler) samtidigt ger upphov till både apoptos och partiell invasion i muskler vid dessa sjukdomar. Vi introducerar också i detta sammanhang det apoptosskyddande proteinet Bcl-2 som en möjlig skyddande faktor. Resultaten visar också att man kan ifrågasätta användandet av partiell invasion som ett särskiljande vävnadsfynd, både för beskrivning av sjukdomsprocessen och som grund för klassifikation och diagnos. Den nyligen introducerade klassifikationen enligt Amato/ENMC skapar en stor patientgrupp som inte kan klassificeras, vilket kan innebära ett problem för behandlingsstudier. Patienter med IIM löper en ökad risk för systeminflammatoriska sjukdomar och cancer, och vi kunde bekräfta att de även har en förhöjd risk för glutenintolerans (celiaki). Den undersökta snittnings- och färgningsmetoden leder till förbättrad diagnostik av IIM och bör användas i större utsträckning.

LIST OF PAPERS

The thesis is based on the following papers, which will be referred to by their roman numerals.

- I. Danielsson O, Lindvall B, Gati I, Ernerudh J. Classification and Diagnostic Investigation in Inflammatory Myopathies: a study of 99 patients. *The Journal of Rheumatology* 2013;40:1173-1182.
- II. Danielsson O, Nilsson C, Lindvall B, Ernerudh J. Expression of apoptosis related proteins in normal and diseased muscle: A possible role for Bcl-2 in protection of striated muscle. *Neuromuscular Disorders: NMD* 2009;19:412-7.
- III. Olof Danielsson, Bo Häggqvist, Liv Gröntoft, Karin Öllinger and Jan Ernerudh. Partial invasion and apoptosis in idiopathic inflammatory myopathies; parallel processes mediated by CD8⁺ cytotoxic T cells.
Manuscript
- IV. Olof Danielsson, Björn Lindvall, Claes Hallert, Magnus Vrethem and Charlotte Dahle. Increased prevalence of celiac disease in idiopathic inflammatory myopathies – a retrospective cohort study of incidence and of associated immune-mediated diseases and malignancy.
Manuscript

ABBREVIATIONS

ADCC	Antibody dependent cell-mediated cytotoxicity
AGA	Anti-gliadin antibodies
ANOVA	Analysis of variance
APAF-1	Apoptotic protease activating factor-1
ASS	Antisynthetase syndrome
ATP	Adenosine triphosphate
Bcl-2	B-cell lymphoma 2
CD	Cluster of differentiation
CK	Creatinine kinase
COX	Cytochrome oxidase
DAB	Diamino-benzidine
DD	Death domain
DM	Dermatomyositis
ELISA	Enzyme-linked immunosorbent assay
EMA	Endomysium antibodies
EMG	Electromyography
ENMC	European Neuromuscular Centre
ER	Endoplasmic reticulum
FAS-L	FAS-ligand
FADD	FAS-associated death domain
FLICE	FADD-like ICE
FLIP	FLICE inhibitory protein
FSHD	Fascio-scapulo-humeral dystrophy
HMGCR	Hydroxy-methylglutaryl-Coenzyme A reductase
HLA	Human leucocyte antigen
IAP	Inhibitor of apoptosis

IBM	Inclusion body myositis
ICE	Interleukin-1 β converting enzyme
ICOS-L	Inducible co-stimulator ligand
IIM	Idiopathic inflammatory myopathies
ILD	Interstitial lung disease
JDM	Juvenile DM
LGMD	Limb-girdle muscular dystrophy
MAA	Myositis associated autoantibodies
MAC	Membrane attack complex
MALT	Mucosa-associated lymphoid tissue
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MUAP	Muscle unit action potential
mRNA	Messenger ribonucleic acid
MSA	Myositis specific antibodies (see Table 1 for specific MSAs)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PM	Polymyositis
RT-PCR	Real-time (originally reverse transcriptase) PCR
SDS	Sodium dodecyl sulphate
SLE	Systemic lupus erythematosus
TdT	Terminal deoxynucleotidyl transferase
tTG	Tissue transglutaminase
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
TUNEL	TdT-mediated dUTP-biotin nick end labeling

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INTRODUCTION

Idiopathic inflammatory myopathies (IIM) constitute a heterogeneous group of rare diseases, which often have severe consequences for the life of affected patients. The treatments available today often have limited effect and given to the cost of adverse reactions. There are some major issues that need to be addressed to facilitate the conduction of treatment studies and improved care of patients.

There is an incomplete understanding of the disease mechanisms causing these diseases, and study of *pathogenesis* may identify new targets for treatment. It has further been difficult to delineate homogeneous disease groups, which has resulted in controversies concerning which *classification* is best suited as a basis for clinical studies. It is also worth emphasizing that *diagnosis* of these diseases sometimes is a challenging task, and to avoid delay of diagnosis is an important factor for improved treatment and care of patients.

The Neuromuscular unit in Linköping has had an interest in IIM that reaches four decades back in time, and it is my privilege to continue this tradition. The founder of the unit, Dr. KG Henriksson presented his thesis [1] in 1980, which included a large cohort of patients with IIM, showing important findings concerning classification, prevalence and associated diseases. Dr. Henriksson used the, at the time, well accepted Bohan and Peter classification [2], but noticed several shortcomings and proposed some changes [1]. Subsequently, inclusion body myositis emerged as the most common inflammatory myopathy in the elderly [3], and diagnostic criteria were presented by Griggs *et al.* [4]. In addition, the characteristic pathology of polymyositis and dermatomyositis [5], and of immune-mediated necrotizing myopathy [6], were described and included in a new classification presented by the *European Neuromuscular Centre* (ENMC) [7]. This classification had however not been applied in a clinical context, when it was introduced. There are also remaining controversies concerning classifications of IIM. Some

experts still prefer the Bohan and Peter classification [8, 9], with the addition of IBM.

Dr Henriksson also, for the first time, highlighted a possible association of IIM with celiac disease. The prevalence of celiac disease has, in recent general population screening studies, turned out to be considerably higher [10] than what was known at the time, and no study has definitely confirmed a higher prevalence of celiac disease in IIM patients. My predecessor in leading the unit, Dr. Björn Lindvall, presented his thesis [11] in 2002. His findings showed the co-occurrence of myositis in Sjögren syndrome and highlighted the expression of immunologically important proteins in healthy muscle and patients with inflammatory myopathies, especially major histocompatibility complex class I (MHC I), membrane attack complex (MAC) and adhesion molecules.

The intent of my thesis was to increase the knowledge of IIM, by studies addressing pathogenesis, classification and diagnosis of these diseases. It is my hope that the background chapters of the thesis may be of use for colleagues, seeing patients with IIM. Since selected parts may be of interest to different readers the chapters were written as rather independent sections. My desire to avoid cumbersome cross-references was therefore given priority to the cost of some repetition. The background chapter is meant to present an overview of the present state of knowledge in this field and to put the studies in perspective. I have however refrained from the daunting task to summarize the basic immunology discussed in the papers, and readers not so acquainted to this field are referred to standard textbooks [12, 13]. In the methods section, mainly the principles of the used methods are explained. The most important results are then presented and discussed regarding their “close range” implications, followed by a condensation to a number of conclusions. Finally, the results are put into a broader context with the ongoing research, in this truly exciting field.

BACKGROUND

Muscle

Striated muscle constitutes a vast end organ of the nervous system and makes up more than a third of the body weight in most humans. It allows locomotion, balance and indispensable movements for manipulation of objects, eating, and verbal as well as non-verbal communication. It has further to meet a vast spectrum of demands, with the capacity to generate great force quickly and less forceful movements with endurance, but also of fine-tuned small amplitude movements of the eyes, hands and vocal organs. This explains the rather extensive areas of the central nervous system dedicated to motor functions. The motor neurons for voluntary and involuntary motor activities converge on the soma of the lower motor neuron, in the motor nuclei of the brainstem and the ventral horn in the spinal cord. The axons of the lower motor neuron exit the central nervous system and forms peripheral nerves after redistribution of axons in nerve roots and fascicles.

The term *motor unit* was coined by Sherrington [14] to emphasize the close functional properties of the lower motor neuron and its adjoined muscle fibers, connected at the neuromuscular junctions. When an action potential reaches the axon ending of the motor neuron, the transmitter acetylcholine is released in the synaptic cleft. When binding to its receptor at the postsynaptic membrane, a postsynaptic membrane potential is generated, and if enough quanta are bound at the postsynaptic membrane, an action potential is released, traveling along the muscle fiber membrane and into tube-like invaginations, to the interior of the fiber. These processes are dependent on the functioning of several membrane ion channels.

The functional units of muscle tissue are longitudinally arranged bundles, called muscle fibers. Each fiber is surrounded by a scant connective tissue called the *endomysium* (Figure 1), which contains capillaries with tight junctions, nerve endings and scant numbers of adjacent macrophages [5, 15]. The muscle is separated from surrounding tissue by a fibrous sheath, called the *epimysium*. Within the muscle, the *perimysium* forms bundles of connective tissue, containing nerves, blood vessels and lymph, and encloses (often) several hundreds of fibers, in fascicles. The understanding of microcirculation of human muscle rests on inference from *in vivo* studies of small mammals, visualizing a network of arcade arterioles in the perimysium, fed by vessels in the epimysium [16]. The arcades give off transverse arterioles, which penetrate the endomysium and divide and yield terminal arterioles, which, in turn, give rise to capillaries, running parallel to the fiber. The transverse arterioles do not intercommunicate and an obstruction of blood flow at this level cannot be compensated for [17]. One microvascular unit irrigates and drains a cylinder of muscle tissue of 750 – 1000 μm length in the studied animals [18].

Figure 1. A schematic illustration of a neuromuscular unit is shown.

Figure 2. The left picture (a) shows an electromicrograph of longitudinally sectioned muscle where the striation is apparent. The left white bracket indicates the length of the actin filaments; the middle indicates the length of a sarcomere and the right, the length of the myosin filaments. A Z-disc is indicated with an asterisk. The white arrows indicate two of several mitochondria. The right picture (b) shows a micrograph of transversally sectioned muscle, stained with 9.6 ATPase (adenosine triphosphatase, preincubated at pH 9.6) the dark brown type 2-fibers and the lighter brown type-1 fibers can be distinguished. The black bracket shows the borders of a fascicle, surrounded by the perimysium (large black arrow). The small black arrow indicates the endomysium, surrounding a fiber.

The muscle fiber

Muscle fibers are giant cells with syncytial properties, reaching several centimeters in length, with a diameter normally ranging from 40 to 80 μm , and containing several hundreds of postmitotic nuclei. Each fiber is enclosed by a cell membrane, normally adherent to a basal membrane. As the two membranes cannot be differentiated from each other in light microscopy, they are collectively called the *sarcolemma*. The regenerative capacity of muscle tissue is mainly dependent on muscle resident stem cells, called satellite cells, which are located in niches between the cell membrane and the basal membrane, for review see [19]. The liquid compartment inside the fiber is called the *sarcoplasm*. Each fiber contains hundreds of nuclei, which are typically located directly beneath the cell membrane. The interior of the fiber mostly comprises longitudinally arranged myofibrils, containing serially arranged *sarcomeres*, which form the functional units of muscle contraction.

The sarcomeres are scaffolds of structural proteins, which allow controlled movements of filaments relative to each other. The normally strict anatomic parallel alignment of myofibrils, containing the approximately 2 μm long sarcomeres, gives muscle its striated appearance, most evidently seen in electron microscopy (Figure 2). The contraction comes about when actin filaments, anchored in parallel to protein complexes (Z-discs) on both sides of the sarcomere, slide along the larger myosin filament. This is coupled with an ATP-driven process, triggered by calcium release in the sarcoplasm, and results in a shortening of the sarcomere. The sarcomeres are in turn anchored to each other, by intermediary filaments that are in continuity with the basal membrane by mechanically resistant structural protein complexes. The combined shortening of the myofilaments are thus laterally conveyed to neighboring fibers, connective tissue and, finally, muscle tendons [20, 21].

Another important functional unit in the muscle fiber is known as the *sarcotubular* system. The above mentioned invaginations of the cell membrane are called T-tubules, which penetrate the muscle fiber and become closely opposed to the *sarcoplasmic reticulum*, which is the muscle variant of the endoplasmic reticulum, specialized in calcium release. These areas of contact, called triads, couple a cell membrane depolarization with a calcium release to the sarcoplasm, causing a contraction. This process is called the *excitation-contraction coupling*. For a summary of the assembly and ultrastructural anatomy of muscle fiber see [22].

Considering the properties and major tasks assigned to muscle tissue, it is not surprising that it is the most energy demanding tissue of the body. In the intermyofibrillar network of the fiber, there is an abundance of mitochondria and large stores of glycogen and lipid droplets. To meet the needs of quick force generation as well as endurance work, the human adult muscles are further equipped with different fiber types in varying proportions. The vast majority are either slow-twitch oxidative, fast twitch oxidative or fast twitch glycolytic, usually corresponding to Type 1, Type 2a and Type 2b fibers, respectively, as visualized by routine ATPase (adenosine triphosphatase, pre-incubated at different pH) stains.

Development of muscle

During embryonal development, the tissue layer formed between the dorsal *exoderm* and the ventral *entoderm*, the *mesoderm*, increases in relative size, bilaterally along the neural tube and forms the paraxial mesoderm. Parallel to the overall segmentation of the embryonal body axis of vertebrates, a cyclic expression of specific mRNAs (messenger ribonucleic acids), define the timed formation of epithelial cell clusters, called somites [23, 24]. The cells of the somite form clusters of the progenitor cells for the skin and the supporting tissue of the body, the *dermatome* and *sclerotome*, respectively, and cells committed to a myoblast lineage, constituting the *myotome*, are in an intermediate position (Figure 3). Already

destined to specific anatomical sites, these cells egress from their respective sites of origin, above (epaxial) or below (hypaxial) the neuroaxis, and migrate as *myoblasts*. The myoblasts fuse in different stages, at their target sites, to homogeneous appearing myotubules, where a cross striation later evolves [25].

Figure 3. A schematic drawing of a transversally sectioned mammalian embryo, at an approximate age of 12 days, is shown. The myogenic precursor cells migrate from the hypaxial myotome to form muscles of the trunk muscles (left arrow) or the extremities (right arrows). The cells of epaxial origin contribute to axial skeleton muscles (not shown). Modified after Pownall *et al.* [26]

Muscle diseases

Disturbances of all the described processes, as well as other conditions, can be the cause of muscle disease. The number of described diseases is immense, but most fall into a limited number of groups, the most important are summarized below:

- Muscular dystrophies
- Congenital myopathies
- Metabolic diseases
- Neuromuscular junction diseases
- Ion channel diseases
- Inflammatory muscle diseases

Muscular dystrophies are caused by genetic mutations that lead to a progressive destruction of muscle fibers. The debut age ranges between before birth and late adulthood. The dominating symptom is a progressive, usually proximal, muscle weakness. Many of the affected genes code for structural proteins, needed for mechanic purposes. The *congenital myopathies* are inherited diseases present at birth, which do not primarily cause destruction of fibers, but rather an impaired development and function. Most affected genes code for proteins involved in the functional units of force generation. The diseases often manifest as a generalized weakness that may vary from slight disability to severe symptoms, present at birth. Because of the energy demands and the storage functions of muscle, it is often affected by *metabolic diseases* (mitochondrial disorders, glycogen storage and lipid oxidation diseases). The signal transmission is hampered in the *neuromuscular junction diseases*, either by acquired autoimmune processes or genetic mutations of synaptic proteins. Symptoms from these diseases often vary in time, and may have a muscle fatiguing quality, *myasthenia*. The proper function of a

vast array of ion channels is essential, both for the neuromuscular signal transmission, the muscle action potential and excitation-contraction coupling. *Diseases of ion channels* often have an episodic character and may result in spontaneous muscle contractions. *Inflammatory muscle diseases* can be caused by secondary inflammatory reactions provoked by a number of infecting organisms or toxic agents. A group of diseases with a presumed autoimmune pathogenesis can be labelled primary inflammatory myopathies, although they are more commonly called *idiopathic inflammatory myopathies (IIM)* and they constitute the major topic of this thesis.

Muscle immunology

Organs and tissues in the body show differences in their immunologic activity, but also in their accessibility to the immune system, for overview see [27]. To avoid potential harmful antigen exposure, the organs constantly exposed to environmental antigens, are equipped with protecting factors, such as epithelial barriers and commensal flora of bacteria [28]. The gastrointestinal and respiratory systems have developed their own secondary lymphatic organs, called the mucosa-associated lymphoid tissue (MALT), and the skin has special local immunological properties [29, 30], in order to deal with the large amounts of antigens it is exposed to.

In contrast, some organs have a high vulnerability to the potential collateral damage, caused by inflammatory responses, and have developed ways to avoid inflammatory intrusion. The brain is protected by a blood-brain barrier with tight endothelial junctions, preventing the passive transfer of large molecules, and neurons of the central nervous system normally lack MHC I expression [31], which is a prerequisite for many immune reactions. The testes [32], the anterior chamber of the eye [33], nucleus pulposus of the intervertebral disc [34] and the

trophoblast of the placenta [35] express FAS-Ligand (FAS-L) to fend off activated lymphocytes, by binding to the FAS-receptor (FAS), inducing apoptosis of these cells. These organs show limited rejection of transplant grafts, and have been designated *immune privileged sites* [32-35]. The trophoblast of the placenta also express HLA-G [36], which counteracts an immune response to the fetus.

Like neurons in the brain, muscle fibers normally do not express MHC I [37], and their feeding capillaries also have tight junctions [15]. Muscle fibers have further been shown to, similar to trophoblast cells, express HLA-G [38], and thus share several features with immune privileged sites. Although muscle tissue normally shows signs of immune protection, it evidently becomes activated in inflammatory disease [39]. In this scenario muscle fibers not only up-regulate MHC I, but have also been shown to take active part in immunoreactions, expressing several immune stimulatory as well as protective molecules [40]. The muscle endothelial cells express adhesion molecules allowing the entry of inflammatory cells [41], which are attracted by chemokines and cytokines, secreted by the muscle fibers [42]. Fibers can produce a large amount of cytokines [43] and molecules necessary to present cytosolic antigens and form an immunologic synapse with CD8⁺ T lymphocytes [40].

Earlier investigations have shown contradictory results concerning the expression of MHC II in muscle fibers in IIM, for compilation see [44]. It has however recently been repeatedly reported, and its detection is now recommended for IIM diagnostics [44], and has further been suggested as a distinctive marker for a specific IIM subgroup [45]. The classical co-stimulators (B7-1, B7-2) have not been detected in muscle fibers, although fibers have in IBM been reported to express the inducible co-stimulator ligand (ICOS-L), which may serve a similar purpose [46]. CD68⁺ macrophages are normally present in the endomysium and increase in numbers in IIM [5]. In summary, muscle tissue in the normal state, is relatively inaccessible to the immunologic system, but can up-regulate its own immunologic repertoire and, with its own particular profile, muster and interact with re-

sponses of the immunologic system, and there seems to be a delicate balance between mediating and protecting factors.

The emerging concept of idiopathic inflammatory myopathies (IIM)

The first scientific attention of IIM dates back to the eighteenth century (1863), when Wagner [47] described the first case of dermatomyositis (DM) and Unverricht [48] some decades later summarized the characteristic features of patients with muscle weakness and pain in combination with erythema of the skin. Although Hepp [49], at the time, had already described a case of polymyositis, it took until 1954, until Eaton clearly distinguished this disease from dermatomyositis [50], and nearly another two decades, until the first support for an autoimmune basis was presented [51]. The special characteristics of childhood dermatomyositis and an already noted DM/PM-association with other systemic inflammatory diseases and malignancy formed the diagnostic groups defined by the classification of Bohan and Peter in 1975.

Even though several criteria were left for interpretation [52], this classification was generally well accepted, and implemented by clinicians and scientists. In the following decade it became clear that a therapy resistant group of patients was linked to a specific muscle pathology: the presence of rimmed vacuoles and inclusion bodies, which had earlier been described by Yunis and Samaha [53], hence the name inclusion body myositis (IBM). A typical clinical syndrome became evident in these patients, with particular affection of the quadriceps and distal arm flexors. It was also noted that the characteristic pathology and clinical syndrome were not always found in the first investigations, as concluded in the review by Griggs *et al.* [4].

In a series of studies by Arahata and Engel, the different types of muscle pathology in DM, PM and IBM were described [5, 54-57]. Their findings also suggested that PM and IBM are CD8⁺ cytotoxic T cell mediated diseases, where the primary target is a muscle fiber antigen, and that DM is a humorally mediated disease, primarily involving vessel endothelia. These concepts have found further support in later studies [58-60]. Concomitantly, another type of immune pathology was more frequently reported, characterized by less inflammatory cells but more necrotic fibers [61, 62]. This type of pathology, later named *immune-mediated necrotizing myopathy*, was observed to correspond to a more sudden disease onset with severe symptoms.

The association of IIM to systemic inflammatory disorders had been established earlier, but a new development was due to an increasing number of myositis specific autoantibodies (MSA) and myositis associated autoantibodies (MAA), detected in blood of IIM patients. The autoantibodies appear to be closely linked to the HLA-haplotype of the patients, but also correspond to different clinical and pathological syndromes, and have added valuable information for diagnosis and comorbidity [63, 64]. The antisynthetase syndrome [65] and mixed connective tissue disease (MCTD) [66] are early examples of diseases, where autoantibodies have helped to define clinical syndromes, including myositis. Subsequently others have followed [67], and will be discussed in the next section.

The inflammatory myopathies

Epidemiology and clinical presentation

The reported annual incidence of IIM have shown great variations, ranging from 1.16 to 19 per million/ year and the prevalence from 2.4 to 33.8 per 100 000 [68]. Females are more commonly affected than males, but the ratio varies in different studies from 1.5:1 to 8:1 [68]. In the county of Gävleborg, Sweden, an annual incidence of 7.6 per million was found [69], and a prevalence rate of 5.9 cases per 100 000 (1: 17 000) was reported in an area in southeast Sweden [1]. The variations of the reported incidence and prevalence of the subgroups are even greater [68], and it would be misleading to present an estimate.

Of note is, however that the proportion of diagnosed IBM patients within the IIM group has increased the last decades, and in a recent Dutch national registry study, it was the most prevalent type of IIM in the older age group. Earlier, the male to female ratio was estimated to 3:1 [3], but in 2 recent population-based studies it was found to be 3:2 [70, 71]. A higher prevalence of IBM was further found in Olmstead county, USA, than in Western Australia, the Netherlands and Turkey, which was consistent with the frequency of the HLA-DR3 allele in the respective populations [68]. The relative incidence of DM seems to show a latitudinal gradient in the northern hemisphere, being more common closer to the equator [68]. In juvenile DM (JDM), two studies have reported a clustering of cases in spring, and two other studies showed that there were a history of infection in more than a half of the affected patients 3 months prior to disease onset, for references see Meyer *et al.* [68].

Most patients with IIM present with a mainly symmetrical proximal muscle weakness, evolving over weeks to months, sometimes accompanied by pain [72]. This makes getting up from low chairs, climbing of stairs and lifting objects over the shoulders, a challenge for patients. In most cases, blood enzymes of muscle origin, in particular creatine kinase (CK), are elevated and electromyography

(EMG) shows signs of myopathology and spontaneous muscle activity in affected muscles [1, 73]. A muscle biopsy usually confirms the diagnosis, showing inflammatory infiltrates and muscle fiber necrosis [74]. There are however several differences between the disease groups in terms of response to therapy [75], association with MSA and MAA (Table 1), comorbidity [67], and the muscle pathology shows signs of diverging pathogenic processes [5].

Dermatomyositis

Dermatomyositis (DM) has, since its first descriptions [47, 48], commonly been understood as a disease, characterized by a skin rash and proximal weakness, due to inflammation in muscle. Presently DM is considered as a microangiopathy affecting both muscle and skin [76]. The presence of a similar skin rash, without signs of muscle disease, is known under the name *dermatomyositis sine myositis*. Experts in the field have also created a diagnostic group, designated *possible dermatomyositis sine dermatitis* [7], for a type of IIM with pathological muscle findings typical of DM, but without a skin rash.

It is more common that the skin rash occurs simultaneously or precedes the muscle weakness, than the opposite [77]. The rash often affects sun exposed (heliotrope) parts of the body and there may be a general photosensitivity of the skin [78]. The rash is often red-purplish in the face, with an edema involving the eyelids as well as the loose tissue surrounding the eyes [79]. When it affects the anterior side of the neck, shoulders and upper part of thorax, it is called the “V-sign”, while it is called the “shawl-sign”, when it occurs on the posterior side. A red macular rash often affects the extensor surfaces of the knees and elbow joints, and the malleoli (Gottron’s sign) and a scaly eruption may affect the knuckles (Gottron’s papules) [79]. The heliotrope rash and Gottron’s papules are held to be pathognomonic for DM [9], and the appearance of both indicate a DM without overlap features [79]. Dilated capillary loops and overgrowth of the proximal nail bed (the cuticle) are often seen under close inspections of fingernails, as in other systemic inflammatory diseases [77]. In addition to the proximal muscle weak-

ness, the swallowing function may also be affected, but not as often as in IBM patients [80]. Inflammatory involvement of the heart has been documented [81], likewise have cardiac diastolic dysfunction, but it has not been clearly established as a cause of heart failure [82]. Arrhythmias and different types of cardiac conduction abnormalities have been repeatedly reported, but the clinical significance of this is presently unknown, for review, see [82]. Respiratory muscles are seldom involved to a degree that it causes symptoms [83], whereas an interstitial lung disease (ILD) is a common feature of an antisynthetase syndrome [83, 84].

DM occurring in childhood (JDM) has some important differences compared to the adult form. The disease is more commonly accompanied by a vasculitis [85], which may cause spontaneous bleeds from the gastrointestinal mucosa, and the children often suffer from generalized symptoms. Calcinosis is more common than in the adult form and may cause eruptions of the skin and joint contractures, sometimes resulting in major disability [86].

There is an association with malignancy among adults with DM [87]. The malignancy most commonly affects the abdominal or pelvic organs, the lungs, the breast and lymphatic cells [88]. The neoplastic disease may be present before the DM diagnosis, or may become apparent several years later [87]. An active search for a malignancy is recommended when DM is diagnosed, and repeated yearly during 3 years [77]. There are only rare reports of malignancy in childhood DM, and an association of malignancy with childhood IIM has not been confirmed [89]. A number of MSA are found in serum of DM-patients [84]. Some MSA correlate with a good prognosis and responsiveness to therapy (MI-2) [90], other with dysphagia, severe skin affection (SAE) [91] or interstitial lung disease (MDA-5) [92]. Calcinosis and contractures in children, or malignancy in adults, are associated with antibodies against NXP-2 [93, 94], and a strongly enhanced risk for a malignancy is seen in adults with Tif- γ -antibodies [93]. See Table 1 for nomenclature, target antigens and clinical association of MSA.

Table 1. Myositis specific antibodies (MSA)

MSA	Immune target	Function	Clinical association
Anti- *	aminoacyl tRNA synthetases	Aminoacetylation	Antisynthetase syndrome**
Jo-1	Histidyl tRNA synth.	Protein synthesis	Predominant myositis
PL-7	Threonyl tRNA synth.	Same as above	Mild myositis
PL-12	Alanyl tRNA synth.	Same as above	ILD predominant
EJ	Glycyl tRNA synth.	Same as above	Predominant myositis
OJ	Isoleucyl tRNA synth.	Same as above	ILD predominant
KS	Asparaginyl tRNA synth.	Same as above	ILD predominant
Zo	Phenylalanyl tRNA synth.	Same as above	ILD predominant
Ha	Tyrosinyl tRNA synth.	Same as above	ILD predominant
Anti-Mi-2	NuRD subunit	Gene transcription	"Classic DM" Good therapeutic response
SRP	Signal recognition particle	Protein transport across endoplasmic reticulum	Necrotizing myopathy
TIF-γ	Transcriptional intermediary factor	Ubiquitination, Gene transcription	DM, photo sensibility Cancer association
NX-2	Nuclear matrix 2 protein	Gene transcription	Severe DM, Cancer association
MDA5	Melanoma differentiation associated protein	Innate antiviral response	Amyopathic DM, ILD, poor prognosis
SAE	SUMO-1 activating enzyme	Protein sumoylation	DM, initially amyopathic, dysphagia
HMGR	3-Hydroxy-4-methylglutaryl-Co-enzyme A reductase	Cholesterol biosynthesis	Necrotizing myopathy, Previous statin exposure
FHL-1	Four-and-a-half LIM domain 1	Multiple roles in muscle	Severe myopathy
cNIA	cytosolic 5' nucleosidase	Degradation of DNA	Inclusion body myositis

* The nomenclature of anti-aminoacyl tRNA synthetases, like many other antibodies, is primarily based on the initials or the name of the index patient [95].

** Antisynthetase syndrome: The clinical constellation of myositis, ILD, inflammatory arthritis, fever, mechanic's hands and Raynaud phenomenon expressed to variable degrees, in combination with autoantibodies to RNA synthetases.

Abbreviations: MSA; myositis specific antibodies. ILD: Interstitial lung disease, DM; dermatomyositis.

Inclusion body myositis (IBM)

IBM patients have a more slowly evolution of symptoms than other IIM patients. They have often had problems with stumbling or difficulties to get up from chairs and climb stairs for several months, or even years, when they seek medical attention [96, 97]. IBM is one of a handful of known muscle diseases, where the weakness may be asymmetric, and it affects both proximal and distal muscles [98]. Two other uncommon features are that muscle atrophy (usually the

medial vastus) may be seen earlier than the weakness becomes prominent, and that a slight weakness may be seen in the upper face muscles [98]. Swallowing difficulties are more common than in other types of IIM [99]. The knee extensors are almost always weaker than the hip flexors, and the finger flexors weaker than the arm abductors [100].

However, not uncommonly, patients present without the classic IBM distribution of weakness [101], and some patients seek medical attention because of swallowing difficulties [102, 103]. Biopsy and EMG findings reminiscent of neurogenic disease and case reports of concomitant axonal neuropathies [104, 105] earlier led to a suspicion that the disease affected both nerve and muscle, but this has not found support in later studies [97]. An association with systemic inflammatory diseases has been reported [71, 106], but not with malignancy [107]. An autoantibody has recently been detected in patients with IBM (cNIA), see Table 1. [108-110]. Its clinical use will probably become a valuable adjunct for diagnosing IBM.

Polymyositis (PM)

PM, as a *stand-alone entity*, has been reported as a rare disease, and that follow-up often reveals another diagnosis (commonly IBM or an overlap syndrome) [62, 77]. Careful clinical-pathological differentiation has however showed that PM, although not as common as previously thought, certainly is the most appropriate diagnosis for a substantial group of IIM patients [111]. PM can be considered an inflammatory myopathy with a predominant CD8⁺ cytotoxic T cell mediated muscle pathology, occurring in patients without a skin rash and no clinical or pathological signs of IBM [89]. The pattern of weakness in PM is similar to DM, in line with the historic concept of PM/DM [1]. The muscle enzymes were in the retrospective study by Chahin and Engel [111] more elevated than in IBM, and the treatment response to immunotherapy usually good. Compared to DM, there is a lower risk of malignancy in PM, although an association has been reported for non-Hodgkin lymphoma, lung and bladder cancers [88].

In contrast to DM, PM in childhood is rare [112]. The autoantibodies signal recognition particle (SRP) and hydroxy-methylglutaryl Coenzyme A reductase (HMGCR) have in particular been associated with an aggressive IIM, with pathology consistent with an immune-mediated necrotizing myopathy [113-115], although, at least in the case of (SRP), there is a pathological heterogeneity, where several patients meet even strict criteria of PM [116, 117]. Immune-mediated necrotizing myopathy, also sometimes referred to as *necrotizing autoimmune myopathy* should probably be considered a (clinico) morphological syndrome rather than a circumscribed disease [118]. The clinical phenotype is characterized by a more sudden disease onset with severe muscle weakness, myalgia and high CK-levels, associated with several underlying etiologies [119].

Overlap syndromes and associated diseases

It is difficult to draw a sharp line between syndromes overlapping with IIM, *i.e.* two diseases sharing certain symptoms and signs [76], and an increased association of diseases to IIM. In a publication by Troyanov *et al.* [120], the association of IIM to a connective tissue disease was reported to be 24%, when using strict (Bohan and Peter) criteria, and 60%, when “overlap features” were considered. An overlap feature was defined as one or more of the following items: a clinical sign of an associated systemic inflammatory disease, a malignancy or the presence of MSA or MAA. The difficulty of using only a MAA for separating an overlap syndrome from an increased association of diseases, is illustrated by a study of 159 patients with systemic lupus erythematosus (SLE), scleroderma, myositis or Sjögren’s syndrome [121]. Thirty-six patients had Ku-antibodies (ranging between 9 and 20% in the disease groups), but only one of these patients had a clinical myositis overlap (SLE). This indicates that clinical or pathological signs need to support the presence of an overlap syndrome. Regardless the chosen distinction, both groups have certainly grown [67], since Bohan and Peter stated diseases, with a known potential to overlap [2, 74]. One reason for the increase is the discovery of several MSA and MAA (Table 1), although there is a

call for caution in interpretation, given the importance of a valid cut-off, defining a pathological level of antibodies.

Mixed connective tissue disease (MCTD) has no widely accepted diagnostic criteria, but myositis has been considered to belong to the core manifestations of the disease [66], and presence of anti-ribonucleoprotein antibodies (U1-RNP) is considered mandatory for diagnosis. The proportion of MCTD patients who have myositis has been variously reported, ranging from 25 to 75% [122]. The myopathies associated with scleroderma constitute a heterogeneous group, where the full spectrum of muscle involvement needs further study [123]. Most patients with autoantibodies against the nucleolar antigen Pm-Scl have features of both myositis and scleroderma, including myositis, sclerodactyly, proximal scleroderma, Raynaud phenomenon and pulmonary involvement [124].

In a recent study of 500 patients from an SLE registry, 44 (8.8%) had been diagnosed with myositis, but only 15 were subjected to biopsy. Of these, 7 patients had findings consistent with IIM, without signs of vasculitis. These patients did not differ in other clinical or serological features from the other SLE patients. The antisynthetase syndrome (ASS) is now accepted as an overlap syndrome [65], with well described clinical features, including interstitial lung disease (ILD), myositis, non-erosive arthritis, Raynaud phenomenon, “mechanic hands”, skin rashes, sicca syndrome, constitutional symptoms and presence of autoantibodies against aminoacyl-tRNA synthetases [65].

Some patients with Sjögren syndrome suffer from muscle weakness and muscle pain, and many of them have been shown to have a subclinical myositis [125]. Several patients with Sjögren syndrome have also been diagnosed with IBM [126], and in some of these cases, the muscle weakness was reported to respond to therapy [127, 128]. It has further been suggested that IBM patients with Sjögren syndrome may represent a subset of IBM [11, 128], that may improve when treated early [127]. In a study of patients with rheumatoid arthritis, a sub-

group of patients with myositis, either had a raise of erythrocyte sedimentation rate, not accounted for by synovitis, or of creatinine phosphate (CK), where muscle tissue of these patients was reported to exhibit *de novo* synthesis of rheumatoid factor and IgM [129], which was interpreted as evidence of a pathogenic overlap in these diseases.

DM by itself, considered a microangiopathy affecting both muscle and skin [76], could be looked upon as an overlap syndrome. The occurrence of dermatomyositis *sine* myositis and possible dermatomyositis *sine* dermatitis (based on pathological features) is in line with the view of DM as an overlap syndrome, where each subcomponent can occur separately. The question arises whether the extent of the disease is due to protecting or mediating factors in patients, or if patients have different disease entities, as indicated by the description of several new syndromes associated with autoantibodies [84]. Thyroid disease and diabetes mellitus are diseases that may have an autoimmune pathogenesis, and both have been reported to be associated with IIM [130, 131]. These diseases, however, need to be further subdivided with respect to autoimmune pathogenesis or not, before conclusions can be made. Celiac disease, with its immune-mediated mechanisms, is a disease that deserves a closer investigation.

Celiac disease

Celiac disease is an immune-mediated enteropathy induced by ingestion of gluten, derived from wheat, barley and rye, in genetically susceptible individuals. This genetic susceptibility is mainly conferred by the HLA-DQ locus [132], carrying the alleles HLA-DQ2 or HLA-DQ8 [133]. Patients usually experience gastrointestinal symptoms or suffer from malabsorption, but celiac disease may also be clinically silent and associated with a large number of inflammatory disorders, including diseases of the skin, liver, endocrine organs, thyroid gland, heart and connective tissue [134]. The reported prevalence has varied in different populations, but is estimated to 1% in most western countries [135].

The standard investigation for confirming the diagnosis is small bowel biopsy [135]. The histology of the intestinal mucosa typically shows a loss of villi and crypt hyperplasia, together with intra-epithelial inflammatory infiltrates [136]. In celiac disease, antibodies against different epitopes of the gliadin molecule (the alcohol soluble fraction of gluten) are detected in patient serum. The detection of serum antibodies has increasingly been used for screening investigations. IgA-antibodies against gliadin (IgA-AGA) were early recommended for screening, but have recently been shown to have low sensitivity and specificity for celiac disease, and are no longer recommended as a screening test. In contrast, both IgA-antibodies against endomysium (anti-EMA) as well as IgA antibodies against tissue transglutaminase (anti-tTG) are regarded as both sensitive and specific markers for celiac disease [137, 138]. With increasing reliability of antibody screening and genetic testing, intestinal biopsy is presently suggested not to be necessary in all patient groups [139].

It is important to diagnose celiac disease because most patients will show clinical improvement, usually within weeks [140], and several diseases associated with celiac disease have been reported to improve, after introduction of gluten free diet [134]. In 1976, Henriksson *et al.* [141] reported a case with concomitant celiac disease and polymyositis, where the patient recovered from the muscle disease when gluten-free diet was introduced, and the same group later described 5 patients with celiac disease in a cohort of 119 IIM patients [142]. Similar case descriptions have followed [143]. An increased prevalence of celiac disease in IIM has however only found support in one study, where screening for autoantibodies was followed by intestinal biopsy [144]. Three patients were diagnosed with celiac disease from a population of 51 IIM patients, and the authors concluded that celiac disease probably is more prevalent in IIM patients than in the general population [144].

Pathogenesis

Genetics

The etiology of IIM is unknown. A viral origin has been suspected, but repeated PCR investigation have failed to detect viral RNA in muscle of affected patients [145, 146]. The etiopathogenesis is likely a result from an interaction of genetic and environmental factors working together [147, 148]. Familial cases are scarce, but have been reported for the major subgroups [149-151]. Most evidence of immunogenetic associations is found for the MHC-region [152]. The rarity and heterogeneity of IIM have made the interpretation of genetic association studies difficult, and stratification with respect to ethnicity, disease group and the presence of MSA/MAA are important to allow conclusions to be made [153].

However, shared HLA-susceptibility between ethnic groups have also been found, which indicates that there may be an affinity to common antigenic peptides [153]. Studies done on polymorphisms in the HLA-region in Caucasians, have shown an overrepresentation of *HLA-DR B1*0301* [154]. The MHC common ancestral haplotype 8.1 (*HLA-A1, B8*0301-DQA1*0501-C4A*Q0*) (AH.8.1) was reported to confer an increased risk for IIM [155]. This was confirmed in a recent genome-wide association study, which also indicated that that gene variants within AH8.1, carried nearly all of the genetic risk in the major myositis phenotypes in Caucasians [152], with the strongest individual allelic association for *HLA-DRB1*03:01* for DM and JDM and *HLA-B*08:01* for PM and anti-Jo-1 autoantibody-positive myositis. For a summary of the profound impact of AH8.1 on the immune response and association with autoimmune diseases see [156]. When comparing a large cohort of Australian IBM patients with controls, carriers with the *HLA-DRB1*03/*01* were shown to have an increased risk for the development of IBM [157]. These alleles also correlated with a more severe phenotype [157]. An important result of many genetic association studies

is that HLA alleles are more strongly associated with developing MSA/MAA in IIM than showing an association to traditional clinical subgroups [153].

Immunopathogenesis

An autoimmune pathogenesis is implicated in most diseases within the IIM group, but an additional degenerative component is also present in IBM. The pivotal immune histochemical and ultrastructural investigations by Arahata and Engel [5, 54-56] demonstrated that DM, on one hand, and PM and IBM on the other, had different immune pathological profiles, strongly supporting different immune effector mechanisms in DM compared to PM and IBM.

In DM the primary target of the immune attack is considered to be the endomysial capillary endothelium. The formation of the membrane attack complex (MAC), comprising complement C5b-C9, is detected in capillaries before inflammatory and structural changes are seen [59]. This is followed by a swelling and vacuolization of the capillaries, resulting in necrosis and perivascular inflammation [158]. The main hypothesis is that the ensuing fiber damage is caused by ischemia, due to the loss of capillaries, and that the characteristic perifascicular atrophy is caused by hypoperfusion of the perifascicular zones [159].

In PM and IBM, evidence support an antigen driven process, mediated by cytotoxic CD8⁺ T cells, towards muscle fibers, presenting an, yet unknown, antigen on MHC I molecules [5, 40, 54-56]. Endomysial macrophages have been reported to express markers of myeloid dendritic cells, capable of presenting antigens to naïve T cells [160]. Muscle from patients with polymyositis (PM) and inclusion body myositis (IBM) characteristically shows invasions of MHC I expressing muscle fibers by an inflammatory infiltrate, dominated by CD8⁺ cytotoxic T cells [5]. This scenario is commonly referred to as a *partial invasion* [7]. In IBM there is a parallel degenerative process, characterized by the presence of fibers with rimmed vacuoles and fibers with amyloid deposits. In addition to amyloid, several aggregated proteins have been detected in these fibers, similar to what is

found in neurons in Alzheimer disease [161]. For decades, there has been an on-going debate, whether the immunologic or the degenerative process is the primary event in IBM [161, 162]. In the absence of representative animal models, the cause, as well as the order of events, remain unknown [163]. Thus, the answer to the question “*if the hen or the egg came first*” is still: “*the mouse*”.

Many questions still remain about the pathological process of partial invasion. The invaded fiber does not show classical signs of apoptosis or necrosis, rather a gradual disintegration and displacement by inflammatory cells [54]. This type of cell degeneration is not found in other tissues, and is rarely seen in other muscle diseases [5]. The general effector mechanism mediated by cytotoxic CD8⁺ T-cells is to induce apoptosis, either by means of granzyme/perforin secretion, or by binding of FAS-ligand (FAS-L) to FAS receptors (FAS) of target cells [164-167]. Expression of granzyme B and perforin in inflammatory cells [168, 169], as well as the expression of FAS in muscle fibers [170, 171], have been reported in inflammatory myopathies, but signs of apoptotic fiber nuclei have not been observed in FAS-expressing fibers [172].

Furthermore, in spite of reports of detected apoptotic myonuclei in IIM [173, 174], signs of apoptosis in muscle fibers has been considered rare, and unlikely of importance for the pathogenesis in IIM [173, 175, 176]. A lack of apoptosis has also been noted for the inflammatory cells in IIM, which has been interpreted as a lack of efficiency to terminate the inflammatory reaction, possibly contributing to the chronicity of these diseases [175, 177]. A summary of the phenomenon and importance of apoptosis is relevant for this thesis, and given under a separate subheading below.

Necrosis is seen in all types of IIM [89], although necrosis of single fibers is not regarded to be explained, neither by CD8-mediated pathology in PM and IBM, nor by the microcirculation theory in DM. As to the hypothesis in DM, of a compromised microcirculation, several necessary steps remain unsubstantiated [178],

and an alternative hypothesis, involving up-regulation of Type 1 interferon-inducible proteins, has been presented [179]. The recently added group to the IIM family, immune-mediated necrotizing myopathy, is characterized by necrotic muscle fibers and absent or sparse inflammatory infiltrates [7]. The pathogenesis of this disease is largely unknown, but an antibody dependent cell-mediated cytotoxic (ADCC) process has been hypothesized [77].

In a different approach to the study of pathogenesis of IIM, it was reported that myositis can be induced by experimental up-regulation of MHC I in muscle fibers of mice [180]. MHC I up-regulation of muscle fibers is a common and early event in IIM [181], with the possible exception of immune-mediated necrotizing myopathy [114]. It was also reported that the mice with transgenically up-regulated MHC I in muscle showed similar signs of endoplasmic reticulum (ER) stress response, as observed in biopsies from patients with IIM [182]. The ER-stress response is a cell protecting mechanism, which can be activated in several ways, including ischemia or accumulation of misfolded proteins [183, 184]. The response includes diminished production of proteins, up-regulation of the NF- κ B-pathway and ER chaperone proteins, but may also induce programmed cell death (apoptosis), when the functions of the cell are impaired [185]. This indicates that there is a parallel processes in IIM-muscle, with the potential to cause fiber death [182], which may also be involved in the formation of vacuoles in IBM [186]. Involvement of the ER-stress response with its dual potential to protect as well as damage muscle fibers, directs the attention to the vulnerability of muscle, and presence of protecting factors in general and in IIM in particular.

Apoptosis

Considering its fundamental importance in biology, the phenomenon of programmed cell death was surprisingly recently defined by Kerr *et al.* in 1972 [187]. Apoptosis is morphologically characterized by cell shrinking, nuclear condensation and cell membrane convolution, also referred to as “blebbing” [187].

Its relative late discovery may be due to the short time frame of its appearance, estimated to 1-3 hours [188], and its lack of perturbation of surrounding tissue [189]. Macrophages are seen to dispose of apoptotic cells, without triggering an inflammatory reaction [190], which is commonly the case during necrotic cell deaths. If the macrophage clearance is delayed, the immune system may be exposed to cellular, normally hidden, antigens, which has recently been implicated as a triggering factor for autoimmune diseases [191].

Apoptosis, as a form of cell demise, unraveled the earlier unexplained mechanism behind the molding of organs, seen in the embryo, and the involution of tissue in adult animals [189]. Apoptosis is an evolutionally conserved process, observed in all multicellular animals, where the basic pathways are similar in nematodes, insects as well as in mammals [189]. Apoptosis was early reported to have a crucial importance in the primary lymph organs in the positive and negative selection of maturing effector cells [192] and for the abrogation of immune responses [193], where dysfunction soon was connected to immune diseases or malignancies [194, 195]. Apoptosis has also been shown to have a role in degenerative diseases of the nervous system [196-198] and in diseases in other organs such as liver, heart and kidneys [199-201].

Two major apoptotic pathways have been defined, the *extrinsic* and the *intrinsic pathways* [202]. The extrinsic pathway is induced by ligand-receptor-binding at the plasma membrane. The tumor necrosis factor (TNF) receptor family consists of several members, including the death receptors: FAS-, TNF- and TRAIL (tumor necrosis factor related apoptosis inducing ligand) -receptors, all with their specific ligand [203, 204]. FAS-L and FAS are well studied prototypes, where ligand-binding causes a trimerization of the receptor, which brings the cytoplasmic tails, containing a *death domain*, in close proximity with an adapter protein called FAS-associated protein with death domain (FADD) [32, 205, 206]. This protein acts as a scaffold for the main effector proteins of apoptosis, the *caspases* [207]. The caspases are proenzymes (zymogens), and binding to the receptor-

protein-complex mediates dimerization and activation of caspase 8. Caspase 8 in turn, activates downstream executional caspases (3, 6 and 7), by cleavage of their prodomain [208].

The mitochondria constitute the major cellular compartment involved in the induction of apoptosis by the intrinsic pathway [209]. The Bcl-2 family proteins are critical for regulating cell death, mediated along this pathway [210, 211]. This protein family, with homologues in the well-studied nematode *Caenorhabditis elegans*, was early described also in mammals and humans, and is considered to have a central importance in several apoptotic pathways [212]. The proteins of this family share a sequence homology in four prodomains. The anti-apoptotic prototype Bcl-2 possesses all domains, while the proapoptotic members (*e. g.* Bax) lack the apoptotic-repressing fourth domain [213]. Bcl-2 is a main inhibitor of apoptosis by its ability to prevent protein release from the mitochondrion. However, it has also been shown to inhibit other apoptotic pathways, protect against necrosis and other, more recently described forms of cell death [214-221].

Proapoptotic members of the Bcl-2 family including Bax and Bak, bind to the outer mitochondrial membrane, which promote a release of cytochrome c and other pro-apoptotic proteins from the inner membrane space [210]. Cytochrome c together with apoptotic protease activating factor 1 (APAF-1) then forms a scaffold called the *apoptosome* [222] for the activation of a homodimer of caspase 9, executing the activation of caspase 3, 6 and 7, which in turn execute the downstream cell death program, including the activation of endonucleases that degrade DNA through internucleosomal cleavage [223]. Beside Bax, several proapoptotic members of the Bcl-2 family, containing only the third homology domain, play a crucial role by promoting mitochondrial membrane permeabilization through interaction with Bcl-2 or Bax. Bid belongs to this group, and is activated by caspase 8-mediated proteolytic truncation. In its active form Bid is able to transduce the apoptotic signal from the extrinsic pathway to engage also the in-

trinsic pathway [224, 225], and thus allows “cross-talk” between the two pathways. There are several other pro- and anti-apoptotic proteins, which all are part of a delicate “life-death-balance”, where cells and tissues differ in their vulnerability to inducing mechanisms [226].

In addition to Bcl-2 and other proteins of the same family, two other anti-apoptotic proteins, with relevance to the studies, will be briefly mentioned. The proteins of the IAP (inhibitor of apoptosis) protein family are able to inhibit most caspases, using different mechanisms, and are also involved in several other cellular pathways [227]. FLIPs (FLICE [Fas-associated death-domain-like IL-1 β -converting enzyme]-inhibitory proteins) have structural similarity to caspases and inhibits caspase 8, and thus apoptosis by the extrinsic pathway [228]. Similar to IAPs, FLIPs are also affecting cellular pathways, not inducing apoptosis [228].

Another pathway of relevance for this thesis is the main effector mechanism of cytotoxic CD8⁺ T cells (and NK-cells). These cells induce apoptosis by secreting perforin and granzymes, where perforin forms a pore in the membrane of the target cell, which enables the entry of granzymes with the capacity to cleave caspases [229-231]. Interestingly this pathway can be inhibited by Bcl-2 [232]. The other described effector mechanism of CD8⁺ cytotoxic T cells is the membrane expression of FAS-L, inducing apoptosis by binding to its receptor FAS [166].

Apoptosis has repeatedly been detected in muscle from patients with spinal muscle atrophy [233, 234], although, interpreted to be a secondary event, due to the death and loss of stimulation of its connected motor neuron [234]. Other than that, reports of apoptosis in most other muscle diseases, including IIM, has been rare or conflicting [175]. Several anti-apoptotic proteins have been detected in muscle disease, while the pro-apoptotic APAF-1 was reported to be absent [235-238]. The authors of these studies interpreted the results to indicate that muscle is resistant to both the extrinsic, intrinsic, as well as the CD8⁺ T cell induced apoptotic pathways. In contrast to this, other studies have documented apoptosis in

IIM [173, 174]. Contributing factors for the conflicting reports, could be that there is a lack of an accepted golden standard for detecting apoptosis in muscle, or alternatively, that experiences from apoptosis in other cells are not easily conferred to the study of muscle [175]. A schematic illustration of the mentioned apoptosis pathways is shown in Figure 4.

Apoptotic pathways simplified

Figure 4. The granzyme/perforin-mediated, the extrinsic and intrinsic pathways are schematically shown, with the major participating proteins. To the upper left, a CD8⁺ cytotoxic T cell is shown to secrete a granule, containing granzyme/perforin for initiation of thus named pathway. Above, in the middle, another CD8⁺ cytotoxic T cell docks with its FAS ligand components to the FAS receptor, effectuating a trimerization, with the potential to execute the extrinsic pathway. The major components of the intrinsic pathways (cytochrome c, APAF 1 and caspase 9) are shown below. The full arrow signifies the nucleus as one destination of the cleaved caspase 3, and the dashed arrow the uncleaved caspase 3, as a potential target of the not yet activated caspase 8 and 9. To the right, the names of the figures, representing proteins, are given.

Abbreviations: CD; cluster of differentiation, FADD; FAS-associated death domain, APAF-1; apoptotic protease activating factor-1.

Classification

An early classification of IIM was presented by Walton and Adams 1958, after detailed analysis of 40 cases and review of literature [72]. They distinguished between PM, DM, and PM associated with *collagen disease*, and PM/DM with malignancy. They also discriminated acute and chronic forms, and by severity. Interestingly, they also distinguished between cases where the muscle weakness dominated the clinical picture, from cases where an associated collagen disease affected the health of the patient more. They however considered PM and DM to be essentially the same disease process. In 1975 Bohan and Peter published their classification [2, 74], which has been the framework for clinicians and researchers up to this day [239].

The authors motivated their classification with the lack of general accepted criteria, and stated what they considered were “*facts, fancies and fiction*” in the views of IIM, but admitted that their criteria were “empirically derived”. They favored a “splitting”, and separated 5 diagnostic groups: I; primary, idiopathic PM, II; primary, idiopathic DM, III; DM or PM associated by neoplasia, IV; childhood DM (or PM) associated with vasculitis, V; DM or PM associated with collagen-vascular disease. They also presented five major diagnostic criteria: I; progressive, proximal, symmetrical weakness, II: Muscle biopsy findings of necrosis and inflammatory infiltrates, III; Elevated muscle enzymes in serum; IV; Electrophysiological triad of small polyphasic units, signs of denervation and complex repetitive discharges, V; Typical skin rash involving eyelids and periorbital area as well as a scaly erythematous rash (Gottron’s sign). Exclusion criteria were also given for findings indicating neurogenic diseases, hereditary and toxic muscle diseases, and secondary inflammatory myopathies. A more thorough description is given in the appendix 1 (Paper I).

In a study from 1980 of 107 patients in southeast Sweden, using the Bohan and Peter classification, the authors noted problems with both the sensitivity and specificity of the stated criteria [1, 52]. The adult DM and PM patients did not differ in terms of weakness pattern, associated diseases or response to therapy; and in their presented classification, PM and DM could preferably be lumped together as PM/DM [1].

The classification of IBM presented by Griggs *et al.* [4] states that for a *definitive* diagnosis all characteristic pathological features need to be detected. These are: endomysial inflammatory infiltrates that invade non-necrotic muscle fibers, vacuolated fibers and either intracellular amyloid deposits, detected by light microscopy, or 15-18 nm tubulofilaments detected by electron microscopy. If endomysial infiltrates invading non-necrotic fibers are found in the biopsy, but not the other stated pathologic features, then a diagnosis of *possible* IBM is allowed when the weakness pattern is “classic”, that is finger flexors are weak, hand flexors are weaker than extensors and quadriceps muscles are at least moderately weak, and muscle enzymes, electromyography findings and the age of the patient (> 30 yrs.) are consistent with the diagnosis (in detail described in appendix 1, Paper I).

With the increasing awareness that IBM constituted a major part of the cases that were earlier diagnosed as PM, and that immune-mediated necrotizing myopathy seemed to be the cause of many other cases, several leading scientists felt that the Bohan and Peter classification was outdated, and should be replaced by a classification based on more recent knowledge. Dr. Amato presented a new classification that was modified and adopted by the European Neuromuscular Centre (ENMC) in 2003 [7]. The Amato/ENMC classification defined 5 major groups, affecting muscle: 1; Inclusion body myositis, 2; PM, 3; DM, 4; non-specific myositis and 5; immune-mediated necrotizing myopathy. A progressive symmetric and proximal weakness was required, and, except for DM, an elevated creatine kinase (CK) level in blood. In addition, there were specific pathological criteria

for a definite diagnosis of PM and DM. In the case of PM, endomysial inflammatory infiltrates were required, with inflammatory cells surrounding and invading non-necrotic muscle fibers, whereas in DM, a perifascicular atrophy was required for diagnosis. Immune-mediated necrotizing myopathy was diagnosed, based on a predominance of necrotic fibers, compared to inflammatory infiltrates. In addition, a new diagnostic group, possible dermatomyositis *sine* dermatitis was introduced, for patients without a rash, where the biopsy revealed a perifascicular atrophy. The Griggs classification was referred to for the diagnosis of IBM. For a more complete description of the Amato/ENMC classification see appendix 1 (Paper I).

A major progress in the field was the discovery of an increasing number of MSA and MAA in serum of many IIM patients. Several of these antibodies have helped to delineate syndromes with clinically useful implications, for prognosis and association with systemic inflammatory diseases, and malignancy. In 1997 Targoff *et al.* [63] presented a classification, based on Bohan and Peter criteria, but with the addition or possible substitution of these criteria by MSA or MAA. This approach was taken one step further when Troyanov *et al.* [120] in 2005 [62] presented and compared the outcome of using three classifications of 100 adult patients, fulfilling Bohan and Peter criteria, although excluding IBM patients. In addition to the Bohan and Peter classification, they introduced a modification where they required one *clinical overlap feature* for the diagnosis of overlap myositis. They also introduced a *clincoserologic* classification, where the clinical overlap feature could be substituted for an MSA or an MAA. An interesting finding was that the number of overlap myositis exceeded 60%, when the clinicoserological classification was applied, and that the number of PM patients decreased markedly, as follow-up was extended, and additional patients were diagnosed with an overlap syndrome or IBM.

The discussion of PM as a “pure” disease entity had been highlighted 2 years earlier in a study of van der Meulen *et al.* [62]. This retrospective follow-up

study of 165 patients with earlier diagnosed IIM found only a handful of PM patients, often with atypical features, when a strict pathological definition (mononuclear cells surrounding and, preferably, invading non-necrotic muscle fibers) was applied, and patients with connective tissue diseases were excluded. Following this report, the extinction of PM, as a pure form was proclaimed in an editorial [240]. However, another retrospective follow-up study of 107 patients with biopsy features of PM or IBM, revealed 27 patients with PM, where only 3 had an associated systemic inflammatory disease, at a mean follow-up at 6 years [111]. In a study of DM, addressing both clinical and pathological features, 44 DM patients were identified in a cohort of 100 IIM patients. Of these 44 DM, 24 were classified as “pure” DM and 20 as overlap DM [79]. In addition, clinical, serological and pathological findings, useful for differential diagnosis, were identified.

There was early awareness that there is a delay in diagnosis of IBM, when the pathology based Griggs criteria were applied [7]. More recently, it became evident that there are several patients with clinical, but not pathological, features of IBM, and these patients also lack a response to immunotherapy similar to IBM patients, meeting strict Griggs criteria. [111]. Therefore, a new set of criteria was presented, that, besides the classical pathological criteria for definite diagnosis, also presented a group of *clinically defined* and another group labelled *probable* IBM, where the latter 2 groups mainly relied on the typical clinical picture, especially the weakness pattern [100].

Diagnosing IIM

Diagnosing IIM may be equally challenging as classifying these diseases. The prime purpose of classification criteria is to allow comparison between different studies of the same disease, and is not meant for clinical diagnosis [241]. Despite the inherent risks, they have, however, often served as guides for clinicians.

Differential diagnosis

Muscle weakness is the dominating symptom of most patients with suspected IIM, and, like in other myopathies, a weakness affecting the limb-girdle muscles is the most common presentation. Therefore, all patients with a *limb-girdle syndrome* should be included in the differential diagnosis. Most of these diseases are muscular dystrophies, like Duchenne muscular dystrophy in childhood, Becker or limb-girdle muscular dystrophies of adults. A clue to diagnosing dystrophies may be a family history of a similar disease. However, most dystrophies are recessive diseases, or caused by X-chromosome mutations, hence the parents are seldom affected, and, statistically, only every fourth sibling. The muscular dystrophies usually have a slower progress, although limb-girdle muscular dystrophy (LGMD) type 2b (dysferlinopathy), is known to sometimes present subacutely. On the other hand, patients with a dysferlinopathy usually also have a distal weakness, with difficulties standing on tiptoe [242]. Commonly there is an evident wasting of muscles in dystrophic patients, and many patients show scapular winging or calf hypertrophy [243].

A progressive proximal muscle weakness is also the most common muscle finding in mitochondrial diseases. Even though it may be the only symptom, it is usually overshadowed by other neurological signs as part of a “mitochondrial syndrome” [244]. Other metabolic myopathies may also present with this type of weakness, especially one group of the glycogen storage diseases, including *Pom-*

pe disease [245]. Neuromuscular junction diseases should also be considered. *Lambert-Eaton myasthenic syndrome*, which often is a paraneoplastic disease, commonly causes a proximal leg weakness accompanied by autonomic disturbances. An uncommon feature of this disease is that strength may increase immediately after exercise (facilitation). Rarely, patients with myasthenia gravis or congenital myasthenia may present with a limb-girdle weakness. A muscle fatiguing component is however often found in *myasthenic disorders*. A neuromuscular transmission failure can usually be detected in this group of diseases by *repetitive nerve stimulation studies* (see below) [246]. Toxic myopathies are often painful with an acute onset and may lead to a prominent elevation of CK (necrotizing myopathy) [247], whereas endocrine myopathies, depending on the cause, may have a quite variable clinical picture [248]. However, both disease groups often present with a proximal muscle weakness [247, 248].

Because of the predominant muscle involvement and rather slow progress of IBM, its differential diagnosis has to consider other diseases. The combination of prominent weakness of knee extensors and finger flexors seldom occurs in LGMD or in the more uncommon *distal dystrophies*. *Myotonic dystrophy* is an autosomal dominant disease, which also affects proximal and distal muscle. A distinguishing feature is that the weakness of face muscles in these patients often affects masticatory muscles, commonly accompanied by a temporal atrophy [249, 250]. The weakness commonly also affects the neck flexors, proximal arm and distal leg muscles more than in IBM [251]. Adult patients with myotonic dystrophy typically exhibit a disturbance of muscle relaxation, a *myotonia*.

Fascio-scapulo-humeral dystrophy (FSHD) is an autosomal dominant disease, which shares the unusual traits of IBM that the muscle weakness can be asymmetric, and affect face muscles. The weakness of the face and shoulder girdle muscles is however usually more prominent in patients with FSHD, who in most cases also exhibit a scapular winging [252]. *Motor neuron disease* is an important differential diagnosis to IBM. It may present with asymmetric weakness

of proximal and distal muscles, or affect bulbar muscles, causing dysphagia [253]. It is however uncommon that the knee extensors and the long finger flexors are involved early. Wasting of muscles is usually more prominent, and *fasciculations* are often seen when inspecting the tongue, extremities or the trunk. Electromyography (EMG) is useful to detect signs of denervation and impaired recruitment in motor neuron disease [254].

There are also patients with IIM, who show only slight or even absent weakness [125]. How frequent this is, is presently unknown. Elevated muscle enzymes or typical EMG-findings (see below) may also be reasons to suspect an IIM. For patients with a DM-like dermatitis or patients with a suspected overlap syndrome, a subclinical muscle involvement may be important to detect. The diseases that have to be considered in the differential diagnosis motivate that a detailed disease and family history is taken. A general medical examination is important to find signs of concomitant diseases. The exposure to myotoxic drugs (especially statins), the earlier level of physical activity and the presence of swallowing problems should be noted, and a close inspection of the skin, nails and muscles may reveal important signs.

A complete neuromuscular examination is advised on the first visit of the patient, where disturbances of sensibility or reflex abnormalities point to a neurogenic disorder. The most important part of the examination is that of muscle strength. This testing preferably includes a grading, for example the scale presented by Kendall [255], and including the 10 muscles/muscle groups recommended by ENMC [7]. It is also advisable, at least on the first visit, to examine both sides of the body and also include eye and face muscles, as well as the finger flexors. For follow-up it is useful to select at least two muscles (or muscle groups) for a more objective method of force measurement. Using a scheme for the examination as shown in the Figure 5, may be helpful for future comparisons.

Figure 5. In the top left column some specific symptoms and signs can be checked or negated. Below, results of objective measurements of grip strength and selected muscle groups are documented. The middle figure serves as a “blueprint” for the muscle groups indicated by circles. The right figure shows the results of strength testing of the 10 muscle groups (with relevant additions), as recommended by the European Neuromuscular Centre (ENMC) [7], using the Kendall scale [255], with grading from no muscle activity (0) to normal strength (10).

A broad laboratory testing of blood may detect abnormalities causing a secondary myopathy, *e.g.* a disorder of thyroid function, or of elevated muscle enzymes. Testing for MSA and MAA in blood has become increasingly important [84], and should be considered in most cases. *Magnetic resonance imaging* can be helpful in selected cases, to detect muscle edema or selectively involved muscle in IBM, or when myofasciitis is suspected [77]. It may also guide the choice of biopsy muscle, but the sensitivity was reported to a modest 80% [256].

Electromyography and nerve conduction studies

Electromyography (EMG) is the registration of the electrical activity of muscle. The registered signal represents a sum of the muscle action potentials within the “pick-up range” of the electrode. The activity is studied at the insertion, with slight activation, at rest and during maximal contraction. Following insertion there is in viable muscle a short burst of electrical activity due to injury and me-

chanical stimulation. This initial burst may be absent, extended or distorted in diseases that inhibit or disturb the muscle action potential. During slight muscle activity the compound action potentials of fibers belonging to one muscular unit, a muscle unit action potential (MUAP), can be singled out on the screen, and the waveform, amplitude and duration be judged. Denervation/reinnervation produces polyphasic, and in chronic denervation, high amplitude MUAPs with long duration. In muscle disease one observes polyphasic, low amplitude MUAPs with short duration. Changes in muscle are sometimes patchy; therefore it is necessary to investigate a representative numbers of muscle units in the chosen muscle. For review and references see [257].

At rest the EMG pattern is normally silent. Spontaneous activity such as *fibrillations* and *positive sharp waves* are emanating from single muscle fiber action potentials and typically seen in neurogenic disorders. It is however also often seen in myopathies with ongoing degeneration/regeneration of muscle fibers [73]. *Complex repetitive discharges* are trains of high-frequency action potentials and are observed in patients with chronic partial denervation, muscular dystrophies and is also commonly found in IIM [258]. *Myotonic discharges* often occur together with visible spontaneous muscle contractions (myotonia), provoked by mechanical provocation, cold or contraction, and are reduced by repeated contractions. They may on the screen resemble other forms of spontaneous activity, but typically “wax and wane”, and the sound they produce on the loudspeaker is similar to that of a (*sic*) dive-bomber. During maximal muscle activation the recruitment pattern of motor units can be studied. In neurogenic diseases, due to denervation/reinnervation (sprouting of nerves), larger motor units and decreased recruitment pattern are seen, whereas in myopathies, there is, until late stages of disease, an increased recruitment pattern of MUAPs [73].

Nerve conduction studies register the time and amplitude of evoked responses of supramaximal electrical nerve stimulation. Peripheral nerve disorders, affecting fast conduction sensory and motor nerves can usually be reliably detected. In

muscle diseases, upon stimulation of a peripheral motor nerve, the MUAPs may be of low amplitude because of muscle atrophy, but nerve conduction velocity remains within normal ranges. *Repetitive nerve stimulation*, using different frequencies, allows electrical assessment of the neuromuscular transmission and detection of diseases where this is disturbed. By using small needle-electrodes and restricted filter settings, the action potentials of single fibers can be recorded (single-fiber EMG), which increases the sensitivity of the investigation for nerve transmission diseases [259].

An electrophysiological examination will detect most neurogenic disorders, causing muscle weakness [73]. EMG has a high sensitivity to detect a myopathy in IIM patients, and the typical combination with spontaneous activity and myopathic pattern of the MUAPs is seen in the majority of patients with active disease [1, 73]. EMG is useful to distinguish a relapse of IIM from a steroid induced myopathy, where no spontaneous activity is recorded [73], and for selecting a biopsy muscle. It further allows comparison with later follow-up investigations [1, 260], and may thus influence therapeutic decisions.

Muscle biopsy

A muscle biopsy is the single most important investigation, for diagnosing IIM or detect alternative causes of a muscle weakness [77]. There are different methods to obtain a biopsy. An open biopsy has some advantages, where the muscle can be inspected *in situ* and enough tissue can easily be obtained. However, compared to other procedures it is more time consuming, and not well suited for repeated biopsies. Needle biopsies cause less discomfort and are easily repeated, although the tissue is often too small or traumatized for morphological studies. Another safe and easily performed method that gives less traumatized material, is the “semi-open” biopsy, using an *alligator forceps*, which is the routine method at our unit [261]. As indicated above, the selection of biopsy muscle is important and can be guided by EMG or MRI-imaging; other commonly used methods are ultrasound or the selection of a moderately weak muscle [262]. In the experience

of our unit, the anterior tibial muscle is usually well suited, when a suspected IIM affects the lower extremities and the deltoid muscle when the upper extremities are more affected.

Biopsy findings in IIM

The classical biopsy findings in IIM are inflammatory infiltrates and necrosis of muscle fibers (Figure 6a and 6b, respectively). Some exceptions are allowed and will be discussed further below. An inflammatory infiltrate, is characterized by more than just a handful of aggregated inflammatory cells. In IIM the inflammatory infiltrate comprise almost exclusively mononuclear cells. More than occasional eosinophilic granulocytes may indicate another type of disease, such as fasciitis or a granulomatous disease. In the latter type of diseases the infiltrate shows a higher grade of organization and the presence of epithelioid and (sometimes) giant cells. In IIM there are usually several infiltrates and dispersed inflammatory cells seen in the biopsy.

In PM and IBM the inflammatory cells have a predominant endomysial location, and typically surround and invade non-necrotic muscle fibers (Figure 7a) [56]. When applying immunohistochemistry, the majority of cells are stained with CD8 or CD 68 [5], identifying them as cytotoxic CD8⁺ T cells and macrophages, respectively. The muscle fibers stain for MHC I in the sarcolemma and often also in the sarcoplasm [37, 263]. In DM, the infiltrates are predominantly perivascular and perifascicular, but some endomysial infiltration is not uncommon. Inflammatory cells are sometimes scant in a DM biopsy, and are presently not required for diagnosis [7]. When immunohistochemistry is applied, a great share of the inflammatory cells comprises macrophages; but among the lymphocytes, CD4⁺ cells and B-lymphocytes dominate [5]. The MHC I expression is on the average not so strongly up-regulated as in PM or IBM [11], but often has a perifascicular predominance. The most typical finding (considered pathognomonic) in DM is a perifascicular atrophy (Figure 7b) [7], where several layers of fibers are small and show pathological changes [1]. These fibers also stain for regenerative mark-

ers [264], but the implication of this is unknown. Sometimes there are several vacuoles (without a rim) in these fibers [262]. MAC (membrane attack complex) often stains enlarged capillaries, particularly in the region of perifascicular atrophy [59]. Importantly, it was reported that in a substantial portion of IIM cases, the pathological findings did not fit into these disease specified descriptions [62].

Necrosis is a feature of all types of IIM [2, 4, 7, 265]. The most common finding is necrotic fibers, which are invaded by macrophages (Figure 6b), but earlier stages are also seen, where the striation of the fiber is lost and it takes on a pale appearance in most routine stains [262, 266]. In contrast, MAC is reported to be invariably expressed in necrotic fibers [267]. In rare DM cases (particularly the childhood form) necrosis of a multitude of connected fibers (infarction) can be seen [159]. In immune-mediated necrotizing myopathy, necrosis is the predominant finding, and inflammatory cells are scarce or even absent [7]. Since also MHC I up-regulation of muscle fibers have been reported to be absent [114], it may be difficult to differentiate this disease from myotoxic fiber necrosis, without the detection of MSA [119]. To this date, only a few muscle labs have reported a larger number of cases with elaborate pathological descriptions of immune-mediated necrotizing myopathy, and recent publications have also in this subgroup noted a pathological overlap [117, 268]. Our own experience is limited to a dozen of cases, where MHC I up-regulation and small infiltrates were found (when multi-level sectioning were applied, see methods section).

In IBM, there is, in addition to the predominant endomysial infiltrate and partial invasion, also fibers with rimmed (autophagic) vacuoles, with granular material and inclusions of protein aggregates, forming amyloid substance that can be visualized with Congo-red stain, which produces “apple-green” birefringence, when exposed to polarized light (Figure 8 a, b) [4]. These inclusions can also be seen in electron microscope as 15-18 nm long tubular filaments present in the nuclei or the sarcoplasm [53, 269]. These inclusions are often sparse and not always detected [100, 111]. This was the main reason why the most recent classification

presented a *clinically defined* diagnostic group for IBM [100, 270]. It is also common to find mitochondrial changes in IBM [271]. These manifest as an increased number of fibers that do not stain for cytochrome oxidase (COX-negative fibers), or as presence of aggregated mitochondria in myofibers, seen in the Gomori trichrome staining as *ragged-red fibers*.

Figure 6. The left picture (a) shows paraffin-embedded longitudinally sectioned muscle (all other micrographs are cross sectioned, frozen). It is stained with elastin-van Gieson (VG), which visualizes connective tissue (red) and elastic fibers (brown), while muscle fibers are yellow. An inflammatory infiltrate is seen between fibers (arrow), but the infiltrate is bordering to the perifascicular area, where normal blood vessels are seen. The right picture (b) shows a necrotic muscle fiber with invading mononuclear cells, stained with hematoxylin-eosin (HE).

Figure 7. The left picture (a) shows an inflammatory infiltrate, which invades two adjacent muscle fibers (partial invasion). The right picture shows a perifascicular atrophy (arrows), where small fibers also show pathological signs. Both sections are stained with hematoxylin-eosin (HE).

Figure 8. The pictures (a, b) show a vacuolated fiber stained with Congo red (CR), seen through a microscope with a polarized lens. When the lens is turned (90° between the pictures) amyloid substance becomes apparent by shifting color from red to “apple green”.

There are some other pathological conditions that may mimic IIM [272]. Muscular dystrophies are usually characterized by major unspecific changes in the pathology, including variation of fiber size and increased number of internal myonuclei, but there are commonly more characteristic findings, such as an increased amount of endomysial connective tissue, splitting of fibers, interspersed fat cells, necrosis and regenerating fibers [262].

In some dystrophies, it is not uncommon to see inflammatory infiltrates and an up-regulation of MHC I, which is particularly the case in Duchenne muscular dystrophy [5]. In this disease the dystrophic features are so prominent that a suspicion of IIM seldom arises. In FSHD and Dysferlinopathy, inflammatory infiltrates are not uncommon [272], and they have also been noted in LGMD type 2i [273]. Dysferlinopathy can be diagnosed with immunohistochemistry, and the clinical picture of FSHD is usually distinct from IIM [243, 252]. The progressive symptoms and signs in LGMD 2i will in most cases be typical for a muscle dystrophy, but interestingly, rare patients with LGMD 2i have been reported with a good clinical response to corticosteroids [273].

There are many specific and unspecific pathological findings that should be judged in unison with the clinical picture. Sometimes pathological findings are unevenly distributed in muscle [274], and an extensive sectioning of tissue can reveal important rare findings. If the clinical picture does not conform to the pathological findings, a new biopsy is often indicated [275]. Access to relevant clinical information is therefore essential, when reading a biopsy [276].

Therapy

There is at this point no evidence to support the use of immunosuppressive treatment in IBM [77, 277]. The exception may be as a diagnostic test for patients where the diagnosis is still uncertain, or when a temporary effect on a severe dysphagia is considered desirable [77]. Although a placebo controlled study has never been made, glucocorticoid therapy is generally considered the mainstay of therapy for the other types of IIM [84]. The recommended starting dose is 0.75-1.00 mg/kg body weight, which is then tapered over several months [277].

Methotrexate, azathioprine and cyclosporine are considered as first line adjuncts or as steroid-sparing agents [84]. Intravenous immunoglobulins have also been shown to be efficient in refractory DM, in a randomized study [278]. Mycophenolate and Rituximab have also shown efficacy [279, 280], but are considered third-line treatments [84, 281]. For patients with a coexistent interstitial lung disease cyclophosphamide or tacrolimus are recommended [282, 283]. Structured exercise programs have shown positive short term effects on muscle strength, and also to have favorable effects on gene expression and capillary density in muscles, of IIM patients [284, 285].

AIMS OF THE STUDY

A general aim was to increase the knowledge of IIM by addressing several, in the opinion of the author, “loose ends” regarding pathogenesis, classification and diagnosis of these diseases. Classification of IIM has been an area of long ongoing debate. Because of the rarity and heterogeneity of the diseases within the group, there has been a quest to construct homogeneous subgroups for randomized treatment studies. A better understanding of the pathogenesis of these diseases would help to identify targets for more specific and efficient treatments. Presently used treatments have limited efficacy and are often given at the cost of adverse effects. Diagnosing IIM is afflicted with many pitfalls that may delay treatment or result in unnecessary therapies with potential adverse effects. Some key issues for improving diagnosis were therefore identified and studied.

The specific aims of this thesis were to:

evaluate the consequences of applying the diagnostic groups formed by the Amato/ENMC classification compared to those of the previously established classification according to Bohan & Peter (Paper I),

identify and characterize key clinical and pathological criteria stated in the classification of IIM according to Amato/ENMC (Paper I, III),

find out, by using strict criteria, the relative number of patients with “pure polymyositis” (Paper I),

use the serial sectioning procedure to identify and characterize key pathological findings, in order to improve classification and diagnosis, and also for the study of pathogenesis of IIM (Paper I, III),

investigate the presence of programmed cell death, apoptosis, in IIM, and possible protective or mediating factors (Paper II, III),

estimate the incidence of IIM and the prevalence of associated diseases, with a focus on celiac disease. (Paper IV).

MATERIAL AND METHODS

The studies are in this section, for brevity, referred to as the studies of classification (Paper I), Bcl-2 (Paper II), partial invasion (Paper III) and celiac disease (Paper IV), respectively.

Patients and cases

There were altogether 132 patients and 34 controls included as 166 cases in the studies (Table 2).

Table 2. The number of cases included in the studies (Paper I-IV)

Not shown are 2 patients, included only for the investigation of incidence.

Patients	Classification	Bcl-2 study	Partial invasion	Celiac disease
	Paper I	Paper II	Paper III	Paper IV
82	X			X
6	X	X		X
10	X		X	X
1	X			
1		X		X
7				X
37		X		
20			X	
Σ 164	99	44	30	106

Paper I and IV

The same procedure was used to recruit patients with IIM to the studies investigating classification (Paper I) and prevalence of celiac disease (Paper IV). Eligi-

ble for both studies, were patients registered at the Neuromuscular unit in Linköping, whose muscle biopsy had shown findings consistent with an inflammatory myopathy during the years 1997-2002, and included were those who met the classification criteria of either Bohan and Peter or Griggs, or Amato/ENMC. Exempted were cases with the required findings, but where another muscle disease had been judged as the obvious cause (*e.g.* Duchenne muscular dystrophy).

The biopsies of the cases were re-reviewed for confirmation, and the address of the patients was retrieved from the Swedish national register. We sent a letter to the patients, asking them to have a blood sample drawn, with the purpose to screen for celiac disease (Paper IV), and also for permission to review their medical records (Paper I, IV). Ninety-nine patients gave their written consent and were classified according to the Bohan and Peter or Griggs (IBM), and Amato/ENMC (Figure 9).

Inclusion of patients

Figure 9. The inclusion process is shown.

The classification was based on review of medical records (median follow-up 7 yrs.), including laboratory data, results of neurophysiological studies, queries to patients and treating physicians, and personal examination of 44 patients. The participating patients were from different parts of the country, but the majority from southeast Sweden. There was a particular high coverage of participating patients living in the county of Östergötland, during the years 1997 to 2001, which allowed a calculation of incidence of IIM (Paper IV). Of eligible patients for this investigation, all except one could be judged with respect to inclusion criteria. The criteria were: at least probable IIM according to Bohan and Peter or possible IBM according to Griggs.

First identifying 99 cases for Paper I and IV, we subsequently decided to extend the inclusion for the study of celiac disease (Paper IV), to patients biopsied during 1995 and 1996. This allowed the inclusion of an additional 8 patients. One patient, who had consented to participation and was included in the classification study, never had a blood sample drawn for the detection of antibodies, and was thus excluded from the celiac disease study, hence a total of 106 patients were included in this study.

Paper II

For the Bcl-2 study (Paper II), stored muscle biopsies were used. Patients were included, based on biopsy coding in the register of the unit, followed by review of biopsies. Ten cases with a biopsy diagnosis of polymyositis, 20 healthy volunteer biopsies, 10 cases with Duchenne muscular dystrophy, and 4 control biopsies from children (boys) were selected.

Paper III

The results of serial sectioning in the classification study (Paper I), identified cases for the partial invasion study (Paper III). There were 36 identified cases, meeting Amato/ENMC criteria of partial invasion (Paper I). In 10 of these cases, the partial invasion could be detected in at least 3 HE stained biopsy sections,

with 50 µm gap between. This allowed further investigation of stored unstained sections, sectioned between the HE sections, containing this type of pathology (Paper III). See Figure 10 for inclusion of patients and Figure 12 for sectioning procedure.

Figure 10. The distinguishing pathological findings in the classification study (Paper I) was the basis for selecting the Pi (partial invasion) cases for the partial invasion study (Paper III). The sum of the different findings exceeds the number of cases due to “pathological overlap”.

* Of 99 eligible patients, 83 met classification criteria of Bohan and Peter or Griggs.

** The dermatomyositis (DM) and control cases were selected by searching the register of the Neuromuscular unit, identifying cases with the morphological code indicating DM or normal biopsies. The 10 most recent cases of each group were selected, where available clinical and laboratory data were sufficient, to confirm the diagnosis of DM or rule out a neuromuscular disease in the controls.

Classification

The diagnostic groups of the classifications according to Bohan and Peter, Griggs, and Amato/ENMC were briefly described in the classification section, for the explicit criteria see appendix 1 (Paper I).

Biopsies

There were in total 233 muscle biopsies analyzed from the 132 patients (1-4 biopsies per patient) and the 34 controls. The tissue had been stored at -70°C in a biobank, managed by the unit. For muscle biopsy diagnostics, it is essential to obtain unfixed frozen muscle tissue. The main reason for this is that the enzyme activity needs to be preserved for histochemical stains. Some antigenic epitopes are also better preserved for immunohistochemistry when unfixed. When extensive sectioning is needed, formalin-fixed paraffin-embedded tissue is however a valuable complementary method. It allows the more convenient sectioning with a microtome, and the morphology of the fibers is better preserved for some purposes. Ultrastructural investigations of muscle were not used in these studies. Preparation for electron microscopy requires special handling and fixation technique and will not be described or discussed.

For most muscle biopsies in these studies, the “semi-open” technique with an alligator forceps in local anesthesia was used [261]. Commonly 4-6 muscle pieces, taken in different directions, were removed. At least one piece was oriented under a microscope and snap frozen in isopentane, cooled in liquid nitrogen, and later cryosectioned for histology, histochemistry and immunochemistry [286]. Two pieces were formalin-fixed and paraffin embedded for multi-level serial sectioning. One to three pieces were frozen and saved for the option of repeated stains, enzyme investigation or western blot.

We also evaluated an added value for diagnosis and classification of a sectioning technique (multi-level sectioning) (Paper I) that is routinely practiced at the unit, to detect sparse pathological findings. In the planning stages of the study, we developed a second sectioning procedure (single level sectioning), to detect and further characterize diagnostically important pathological findings (mentioned in the patients and cases section). These techniques are therefore described in some detail below.

Sectioning techniques in the studies

Multi-level serial sectioning (Figure 11) was performed on formaldehyde-fixed and paraffin-embedded muscle tissue with the following procedure: four sections were collected, 50 µm of muscle tissue was then discarded and another 4 sections were collected. This procedure was repeated at least 16 times, where two sections stained with hematoxylin-eosin (HE) were repeatedly followed by two stained with elastin-van Gieson (VG). These sections were then screened for inflammatory infiltrates, necrosis, vascular changes or other types of pathology.

Figure 11. The sections (shown as numbers) were placed on glass slides in consecutive order and stained with hematoxylin-eosin (HE) alternated with elastin-van Gieson (VG). The arrows indicate the continuation of the process until at least 84 sections were prepared.

In single-level sectioning (Figure 12), 35 sequential sections were made from unfixed frozen tissue. Every 7th section was stained with modified (King-Engel) Gomori trichrome (GT) and viewed under a microscope. The staining of adjacent sections was guided by findings in the GT stained section; when vacuoles were detected the adjacent 6 sections were stained with Congo red (CR), under the assumption that vacuolated fibers more commonly contain inclusion bodies than other fibers do. A similar procedure was performed with HE stained sections (also every 7th section). When a partial invasion was found, judged by morphology, the flanking sections were stained with MHC I, CD8 and MAC to verify the required criteria for a partial invasion.

Single level sectioning –
frozen sections

Figure 12. The sections were placed on glass slides in consecutive order. The denotation consists of a digit to the left, followed by a letter, and on the slides with immunostains (Immunostain) or Congo red (Congo) stain, another digit followed. Each *unit-of-seven* sections was ordered numerically with the left digit. The letter in the middle denotes the slide order, and the right digit the sequential number of the sections on the slide. The “b-slide sections” were stained with hematoxylin-eosin (HE), followed by immunostainings of the “a- and c-slide sections”, of the same *unit-of-seven*, where the sought pathology (partial invasion) was detected (in microscope). The same procedure was followed using Gomori trichrome staining of “b-slide sections”, followed by Congo red staining of the “a- and c-slide sections”, when the sought pathology (vacuolated fibers) was detected.

Reading the biopsy

There is a large histologic variation in biopsies from healthy muscle and muscle affected by disease. Rare findings in muscle are specific for certain diseases, but most findings have to be viewed in the overall pathologic context, and together with the clinical picture [275]. When reading a biopsy all findings are therefore put in relation to what is judged as the variation of normal muscle. The routinely performed stains were: hematoxylin-eosin (HE), van-Gieson (VG), modified (King-Engel) Gomori trichrome (GT), nicotinamide adenine dinucleotide tetrazolium reductase (NADH), myosin adenosine trinucleotide phosphatase, pre-incubated at pH 9.4 and 4.6 (ATPase 9.6 and ATPase 4.6, respectively), non-specific esterase (NSE), acid phosphatase (AP), periodic acid-Schiff (PAS) myophosphorylase (MP), Oil-Red O (OR), myoadenylate deaminase (MA), major histocompatibility class 1 (MHC I) and membrane attack complex (C5-C9) (MAC). The sections were sequentially stained in the mentioned order, with each stain applied for at least 2 sections. A *strictly* normal biopsy in 9 of these stains is shown and commented in Figure 13.

Figure 13. The normal muscle histology in the HE (a) stain shows polygonal cross-sectioned fibers of almost equal size and a peripheral location of one, or several, myonuclei. The fibers are only separated by a barely detectable endomysial tissue. Mitochondria give the GT stain (b) an appearance of minute red dots, with some smaller aggregates under the sarcolemma. The NADH-stain (c) shows an evenly distributed oxidative activity in the interfibrillar network. The ATP stains (d, e) show a *checkerboard* appearance of the fiber types (type 1 light and type 2 dark brown in ATPase 9.4 (d), which is reversed in ATPase 4.6, where type 2a fibers are white and type 2b beige), and the NSE stain (f), shows no signs of sarcoplasmic esterase. A normal MP stain (g) excludes deficiency of this enzyme (McArdle disease), but is also sensitive sign of the post vital quality of the tissue. The stain for neutral fat (OR) (h) shows evenly distributed red lipid droplets. There is no MHC I- expression in the muscle fibers, but clearly in the endothelial cells of the surrounding capillaries (i). See text above for abbreviations.

Immunohistochemistry

Immunohistochemistry relies on the principle that enzyme labelled antibodies bind specific antigens in the tissue, where the enzyme catalyzes a reaction from an uncolored substrate (chromogen) to a visually detectable product, which can be observed in a microscope. In the direct method the primary antibodies are la-

belled with the enzyme. This method is easily performed and has rarely non-specific binding, but is nowadays seldom used, because of limited sensitivity.

Instead, techniques including an enhancement step, using soluble *enzyme-anti-enzyme immune complexes* are now more commonly applied. The most common method used in our studies, take advantage of the high-affinity bindings between streptavidin and biotin, where the application of the primary (antigen-specific) antibodies is followed by the application of secondary, biotinylated antibodies, and a solution of biotin-avidin-enzyme (horse radish peroxidase) complex. This is concluded with the substrate solution containing diamino-benzidine (DAB), which in the presence of the enzyme, turns into a yellow-brown end product.

This method gives rise to a multitude of enzymatic binding sites and results in a high sensitivity. Care needs to be taken, to avoid unspecific binding or cross-reactivity, which includes blocking of unspecific tissue reactions. The protocol needs to be adapted for the particular tissue; and the incubation time, temperature and grade of dilution have to be tested for each antibody clone, before use. Negative controls without application of primary antibodies are stained in parallel and positive controls are also needed when the presence of the antigen in the tissue is uncertain. The methods are illustrated in Figure 14.

Immunohistochemistry – direct and indirect method

Figure 14. Two primary antibodies, attached to their respective antigens, are schematically shown. The upper primary antibody is labelled with an enzyme that turns a colorless substance (chromogen) into a colored product (direct method). Bound to the lower primary antibody (also acting as an antigen) is a secondary antibody labelled with biotin. Bound to biotin is a complex of biotin-avidin with a multitude of attached enzymes, catalyzing the same reaction as above; several fold amplified (indirect method).

Antibodies

Polyclonal antibodies are obtained from immunized animals (most commonly rabbit), injected with immunogens together with adjuvants. Large amounts of sera can relatively easy be obtained. The sera need however be purified from cross-reacting antibodies. The obtained antibodies are directed to different epitopes of the immunogen, which may be an advantage for the sensitivity, but confers a greater risk of cross-reactions. A further disadvantage is posed by the differing antigen affinity of present antibodies, as the optimal dilution for each antibody may be different. There is also a risk of batch-to-batch variation.

Monoclonal antibodies are produced by an individual clone of plasma cells and are all directed against a specific epitope. Monoclonal antibodies are now availa-

ble for an increasing number of antigens. One potential disadvantage is that they only target a specific molecule, thus although highly specific, it is necessary to find a monoclonal antibody directed against a molecule that is clearly expressed in the tissue examined. However monoclonal antibodies over all have important advantages compared to polyclonal antibodies in terms of specificity and reproducibility, and should preferably be used when available. The antibodies used in the studies are shown in Table 3.

Table 3. Antibodies (abs.) used in the studies

Target	Clone / name	Primary ab.*	Source**	Paper
Bcl-2	100	IgG1	Santa Cruz	II, III
Bcl-2	124	IgG1	DAKO	II
CD8	DK25	IgG1	DAKO	I, IV
CD68	KP1	IgG1	Santa Cruz	III
CD163	GHI/61	IgG1	Santa Cruz	III
FAS	B-10	IgG1	Santa Cruz	II, III
Fas-L no1	c-178	polyclonal	Santa Cruz	II
Fas-L no2	NOK-1	IgG1	Santa Cruz	II
Fas-L no3	Q-20	polyclonal	Santa Cruz	II
Granzym B	GB11	IgG1	LS Bio	III
HSP70	C92F3A-5	IgG1	Santa Cruz	III
MAC (C5b-9)	aE11	IgG2a	DAKO	I, IV
Merosine 80	5H2	IgG1	Millipore	III
MHC I	W6/32(1)	IgG2a	DAKO	I, II, IV
MyHC-fast	WB-MHCf	IgG1	Leica	III
MyHC-slow	WB-MHCs	IgG1	Leica	III
Spectrin	RBC2/3D5	IgG2b	Leica	III
TRAIL	K-18	polyclonal	Santa Cruz	II

*FAS-L no 1 and 3 were rabbit and TRAIL goat polyclonal, all other were monoclonal abs.

**Santa Cruz (Biotech) Biotechnology Inc. Dallas TX; DAKO, Denmark A/S; LS Bio (LifeSpan Biosciences) inc. Seattle WA; Millipore Corp, Temecula CA; Leica Biosystems Nussloch GmbH, Germany.

TUNEL-method to detect apoptotic nuclei (Paper III)

TdT-mediated dUTP-biotin nick end labeling (TUNEL) is a method for *in situ* detection of DNA breaks, characteristic for apoptosis. The method was described by Gavrieli *et al.* [287]. Apoptotic cell nuclei are not easily distinguishable by morphological characteristics in light microscopy [188], and may only appear during a few minutes [288]. Therefore this method is needed to visualize apoptotic nuclei, which is done by detecting chemical binding to fragmented DNA. The method is based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3'-OH ends of DNA. TdT incorporates biotinylated deoxyuridin at sites of DNA breaks and the signal is amplified by avidin-peroxidase, which enables histochemical identification by light microscopy. The ApopTag® system, used in Paper III, differs from the standard method, in that it uses digoxigenin/anti-digoxigenin-binding (the antibody obtained from purified sheep polyclonal antibodies), instead of the described avidin/biotin system. This method has been shown to have an equal sensitivity compared to the conventional method [289, 290], and has been used for the study of apoptosis in cultured myocytes [291] and in the human heart [292].

Counting of Fibers and cells (Paper II, III)

In the studies of Bcl-2 (Paper II) and partial invasion (Paper III) the percentage of stained muscle fibers were calculated on digital images of stained tissue sections. In Paper II, the camera software was used to draw circles of equal size, usually containing more than 200 fibers. In the study of partial invasion (Paper III) the corresponding center point in all sequential sections was identified. This was needed to enable the investigation of the same muscle fibers in the different stains, in an area where at least one partial invasion was found. The number of inflammatory cells was related to 100 muscle fibers. Two photographed sections are shown in Figure 15.

Figure 15. The circled areas of two stained sections (from different biopsies) are shown, where each red mark denotes a fiber expressing fast myosin heavy chain (fMyHC) (which is the myosin heavy chain protein variant commonly expressed in type 2 fibers) (a), or Bcl-2 (b). The section to the left (a) is double stained with spectrin, which stains the fiber membrane and facilitates counting and localization of specific fibers. In the right section (b) the Bcl-2 expressing fibers are easily distinguished from non-expressing fibers. In several cases the difference was less distinct. A clearly detectable staining of the whole circumference of the sarcolemma, or a clearly detectable sarcoplasmic staining, distributed in more than a half fiber area, was chosen as a cut-off (similar to our previous study (Paper II)).

Western blot (Paper II)

Western blot is a technique for separation and identification of proteins. It also gives quantitative and some qualitative information about the protein. The proteins are extracted from muscle by homogenization and centrifugation and dissolved in the detergent sodium dodecyl sulphate (SDS) and separated by weight, using polyacrylamide gel electrophoresis (PAGE). The proteins are then transferred (blotted) to a nitrocellulose membrane, where the targeted proteins are detected by specific antibodies. Secondary antibodies, labelled with an enzyme, allow visualization. The appearance and localization (distance travelled in the electric field) are compared to controls, and semi quantitative information is inferred about the amount of the protein, relative to controls run simultaneously, as well as signs indicating if the protein is truncated.

RT-PCR (Paper II)

Reverse transcriptase (real time quantitative) polymerase chain reaction (PCR) was used to detect the mRNA expression in muscle of Bcl-2. Muscle is first homogenized and suspended in a RNA lysate buffer. The extracted RNA is then reversely transcribed to cDNA, using the enzyme *reverse transcriptase*. The method uses the PCR-reaction, where a heat-resistant DNA polymerase builds a complementary DNA template to a specific DNA sequence, flanked by added primers (short complementary strands). The DNA-strands are separated by heating and the procedure is repeated many times. The number of DNA-copies is doubled for each cycle, resulting in an exponential increase of the target sequence, until it is leveled off due to inhibiting factors. The large number of generated copies facilitates the detection of the probes, which is labelled with a reporter dye. An estimate of quantity of RNA in the sample and a comparison between different samples is possible by a measurement of generated products during the exponential face of the reaction, and comparison with a control sample (house-keeping protein) with a known amount of RNA.

Antibody screening for celiac disease (Paper IV)

Anti-gliadin antibodies ((IgA-AGA) and *anti-tissue transglutaminase antibodies* (IgA-tTG) were detected by enzyme-linked immunosorbent assay (ELISA). ELISA was originally described by Engvall and Perlman [293]. In this method the target antigen is attached to a solid support with the ability to absorb a certain amount of antigen. Blocking solution is added to prevent unspecific binding of antibodies and other proteins in serum. Specific antibodies present in patients' sera bind to the antigen, whereas unbound antibodies are washed away. A secondary antibody, labelled with an enzyme, is then applied, which binds already bound antibodies. In a final step a substrate solution containing a chromogen-substrate is added. The absorption of monochromatic light can then be detected as optical density and compared to a standard curve. The method allows separate detection of Ig-isotypes depending on the specificity of the secondary antibody.

Endomysium Antibodies (IgA-EMA) was assessed by indirect immunofluorescence microscopy. Fixed sections of monkey oesophagus were used as antigen source. The method is similar to immunohistochemistry, described above, with the difference that fluorescein-isothiocyanate-conjugated rabbit anti-human IgA antibodies are applied as secondary antibodies, and bound antibodies are visualized in a fluorescence microscope. As celiac disease in particular is associated with Ig-A antibodies, these were screened for in patient serum in Paper IV. It was therefore important to also screen for Ig-A deficiency in the patients. IgA in serum was detected by *turbidimetry*, a method measuring the transparency of a solution, which is lower when antigen-antibody-complexes form precipitates.

Statistics

The classification study (Paper I) was a descriptive study where raw data without statistics were presented. In the Bcl-2 study (Paper II), comparisons were made of the expression of Bcl-2 (percentage of positive fibers), between three groups, where a normal distribution of the data in the groups was not apparent. Therefore the non-parametric test of Kruskal-Wallis was used, followed by Dunn's test for multiple comparisons. Mann-Whitney U-test was used to compare the mRNA expression in two groups, again assuming non-normal data distribution. Further, the non-parametric Spearman rank test was used to investigate the correlation between the graded expression of MHC I to the percentage of fibers expressing Bcl-2.

In Paper III, three groups were compared regarding the percentage of fibers expressing the investigated proteins and the number of inflammatory cells (as a ratio to 100 muscle fibers) of particular subsets. Most group data were not normally distributed, why the Kruskal-Wallis' test was used, followed by Dunn's multiple comparison test. The Pearson normality test, however, confirmed normal dis-

tribution of the CD163/ CD68 ratio, hence the parametric ANOVA was used for this analysis, followed by Tukey's multiple comparisons test. The correlation between age and Bcl-2 expression was calculated in the combined control groups from this study and from the study in Paper II, using the Pearson test. This was done after the residuals from the regression curve were found to be normally distributed.

In Paper IV, the prevalence of celiac disease in a cohort of IIM patients was compared to the general population prevalence of celiac disease, found in another Swedish study, using a similar screening procedure. For calculating the confidence interval, the modified Wald method was used, as suggested by Agresti and Coull [294]. After the planned inclusion period was ended, a power calculation based on one sample proportion Wald z test indicated the need to include more cases, and the inclusion period was therefore extended.

Ethical considerations

The following ethical issues were considered before and during the studies:

A potential harm to the participating patients in the studies was the intrusion of their privacy and the risks and discomfort and trouble to have a blood sample drawn (Paper IV). Blood sampling is considered a safe procedure with little discomfort, and the right of the patients to deny participation, without any consequences in the further contact with health care, was explicitly stated in the invitation letter. Another potential harm to participating patients was that further work-up of their stored muscle tissue, could make later, clinically indicated, biopsy investigations impossible. We did however make sure that we did not use all the saved tissue. The handling of personal medical data contains the risk of breaching privacy. The unit has however long experience with coded patient data, and the risk of this type of harm to patients was considered minimal.

A potential ethical dilemma was that the investigation of patients' medical records could reveal health problems that needed professional attention. As the primary investigator is a physician used to this situation, this was not considered a risk, and several results could be used for the benefit of patients. We considered that there was an obvious scientific gain of the studies, which addressed methods of increasing the diagnostic accuracy and the relevancy of recommended classifications of IIM. A confirmed increased prevalence of celiac disease in IIM patients would result in an increased professional attention and possible lead to earlier diagnosis of this disease. In conclusion, we considered that the scientific gain outweighed the potential harm to patients, and the studies were approved by the regional ethical committee.

RESULTS AND DISCUSSION

Classification and diagnostic evaluation (Paper I)

We classified a consecutive cohort of patients ($n = 99$), with biopsy findings consistent with IIM, both according to the Amato/ENMC and the Bohan and the Peter or Griggs (IBM) classifications, based on medical record data, after a median follow-up time of 7 years. The numbers of patients meeting inclusion criteria of the three classifications are illustrated in Figure 16.

Number of patients meeting criteria of the three classifications

Figure 16. Ninety-nine patients gave their written consent to have their medical files reviewed. Sixteen patients did not have an inflammatory myopathy, as defined by the Bohan and Peter, Griggs or Amato/ENMC criteria. Eighteen patients had inclusion body myositis (IBM), 42 met the criteria of the defined inflammatory myopathies according to Amato/ENMC. The remaining 23 patients could be classified only according to Bohan and Peter.

Main differences of the classifications

One major difference between the classifications is that the Bohan and Peter criteria specifically creates groups for children and patients having another systemic inflammatory disease or a malignancy, disorders that may be pathogenically linked to an IIM, while the Amato/ENMC classification, acknowledges designation of associated disorders within the more pathologically specified groups. A brief description of the two classifications is given in the classification section. The explicit criteria are listed in appendix 1 and data of individual patients in Table 1 (both in Paper I).

When comparing the diagnostic groups formed by the two classifications (illustrated in Figure 17), the most important differences were: (1) Eighty three of the 99 patients could be included in the Peter and Bohan or Griggs classification, whereas only 60 fulfilled criteria of Amato/ENMC (also including Griggs). The main reason for this difference was that 21 of 23 excluded patients lacked a detectable muscle weakness, which is required for inclusion according to the Amato/ENMC. (2) The largest diagnostic group formed by the Amato/ENMC classification was *non-specific myositis*, while the largest group formed by Bohan and Peter or Griggs was *overlap syndrome*. In fact, one half of the patients with overlap syndrome lacked muscle weakness, and were thus among the excluded patients from the Amato/ENMC classification. PM, suggested to be an extremely rare disease [62, 240], was in fact the second largest specific diagnostic group after IBM.

Amato/ ENMC - Classification

Figure 17. The front side of the bars shows the numbers of patients diagnosed according to the Amato/ENMC- classification, and the lateral side shows the corresponding numbers of patients in these groups, classified according to Bohan and Peter. The insert explains the principle, that patients classified as polymyositis and dermatomyositis according to Amato/ENMC (front side) are divided into definite (D) and probable (P), and that the color coding on the lateral side shows the classification groups (given by numbers), according to Bohan and Peter. The Bohan and Peter groups 3, 4 and 5 (patients with associated cancer, children with IIM or overlap syndromes) could be viewed as a subgrouping within the Amato/ENMC classification, by adding these designations.

Abbreviations: PM: polymyositis, DM: dermatomyositis, DM sine D.: possible dermatomyositis *sine* dermatitis, Necr. M.: immune-mediated necrotizing myopathy, Non s. M: non-specific myositis, Not IIM: not idiopathic inflammatory myopathy, according to Amato/ENMC, however classified as probable or possible IIM, according to Bohan and Peter.

Serial sectioning

By using serial sectioning from single and multiple levels (described in the methods section), we could classify another 9 patients (15%) according to Amato/ENMC, and assign 13 patients (22%) a more specific diagnosis. When applying the Bohan and Peter classification (now without IBM), this method detected findings in 7 patients (11%) essential for diagnosing IIM, and allowed a more specific diagnosis in 15 (23%). Single level sectioning proved to be a valuable

tool to detect amyloid in IBM, and allowed a definite diagnosis in another 9 patients (50% of the group), compared to the conventional method.

The added value of serial sectioning

Figure 18. The height of the bars represents the number of patients in the diagnostic groups, according to the Amato/ENMC or Griggs classifications. The light gray fractions represent 7 patients, where multiple level serial sectioning was *needed for inclusion/classification*, and the black fractions 11 patients, where serial sectioning identified pathological findings that allowed a more *specific diagnosis*. The dark gray fraction represents 2 patients, where serial sectioning was needed for diagnosis, but also identified findings allowing a more specific diagnosis (that is, belonging to *both* groups). In total, 9 patients (15%) needed serial sectioning for diagnosis and 13 (22%) could be given a more specific diagnosis, owing to serial sectioning. Abbreviations: PM: polymyositis, DM: dermatomyositis, DM sine D: possible dermatomyositis *sine* dermatitis, Necr. M: immune-mediated necrotizing myopathy, Non s. M: non-specific myositis, IBM: inclusion body myositis.

Specific pathological findings

Concerning the specific pathological findings stated in the Amato/ENMC criteria, 36 patients had a partial invasion. These were not just IBM or polymyositis

patients, but also 1 patient with a definite dermatomyositis, 2 patients with possible dermatomyositis *sine* dermatitis and 6 patients who did not meet the inclusion criteria (no weakness). The partial invasions, identified in morphological stains, could all be confirmed as meeting stated criteria by immunohistological stains, except in one case (due to damaged tissue). Thus immunohistochemistry may not be necessary (as stated by criteria), if the morphology is straightforward. This observation may simplify the diagnostic procedure. Biopsies from 15 patients showed a perifascicular atrophy. Six were classified as dermatomyositis, 5 as possible dermatomyositis *sine* dermatitis and 4 patients did not meet the Amato/ENMC criteria. Notable, 4 patients with a perifascicular atrophy also had a partial invasion. One was diagnosed with definite dermatomyositis, 2 with possible dermatomyositis *sine* dermatitis and 1 did not meet criteria for specific diagnosis. A limitation of the study was that inclusion criteria, requiring a detected inflammatory infiltrate in the biopsies, may have excluded patients with immune-mediated necrotizing myopathy, which has recently drawn increased attention. Inflammatory infiltrates in this disease are sparse and claimed to be found in less than a half of the cases [6]. We diagnosed only one case, despite extensive sectioning.

Implications of the study

The Bohan and Peter classification has shaped the conception of IIM for a generation of researchers and clinicians, and, as concluded by D. Hilton-Jones in a review 2011 : “...acted as a framework for diagnosis and epidemiological studies ever since” [239]. The apparent differences compared to the Amato/ENMC classification may therefore have several implications that may affect the diagnostic process as well as the design of future research studies of IIM. As shown in our study, the sampling problem, that was already highlighted in the Bohan and Peter publication [74], is even more important when applying the Amato/ENMC classification, because of the weight given to specific pathology. Further, the extent of pathological work up is of great importance for correct diagnosis, and needs to be standardized for research studies. The largest groups created by Ama-

to/ENMC, nonspecific myositis and the group of excluded cases, fulfilling biopsy and laboratory criteria but lacking weakness, are important patient groups that also should be addressed in studies. The diagnostic group polymyositis, as defined by the Amato/ENMC classification (“pure polymyositis”), is not as common as IBM, but, as pointed out by Chahin and Engel [111], cannot be considered a rare IIM group.

Bcl-2 is constitutively expressed in healthy muscle (Paper II)

Muscle fibers do not normally express MHC class I, and they do express HLA-G [38], similar to the trophoblast of the placenta. Further, the endothelial cells of the lining capillaries are sealed with tight junctions [15]. These are features shared by immune privileged sites of the body [31, 36], hence developed to protect muscle fibers from injury and perhaps contributing to the rarity of inflammatory diseases in muscle. Polymyositis and IBM are considered to be autoimmune diseases mediated by CD8⁺ cytotoxic T cells, where evidence support that the muscle fiber is the primary target of the immune attack [295]. The main effector pathway of CD8⁺ cytotoxic cells is to induce apoptosis of the target cell. However, apoptosis has been a rare finding in IIM, and a pathogenic role has been considered unlikely [175]. Several studies have therefore explored the presence of apoptosis mediating and protecting factors in muscle, including the proteins Bcl-2, FAS-ligand (FAS-L) and FAS [38, 40, 235]. The earlier reports of the expression of Bcl-2 and FAS- L have been conflicting [170, 171, 296] and the detected presence of FAS, in the absence of apoptosis, has been given different interpretations [172, 174]. Using immunohistochemistry, we investigated the expression of Bcl-2, FAS and FAS-L in muscle biopsies from healthy controls, polymyositis, Duchenne muscle dystrophy and control children. We also investigated the expression of tumor necrosis factor related apoptosis inducing ligand (TRAIL), which is another protein that, similar to FAS-L, has a proclaimed role

in immune privileged sites by inducing apoptosis of infiltrating cells [297, 298].

The expression of Bcl-2

We found a constitutive expression in healthy muscle of Bcl-2, while the expression was much lower in muscle from patients with polymyositis and Duchenne muscular dystrophy (Figure 19). As the finding was in conflict with several studies, we performed western blot in 4 healthy controls and 3 cases of polymyositis, which confirmed the presence of Bcl-2 in muscle, and the bands were weaker in the polymyositis patients. Using RT-PCR, we also detected a higher mRNA expression of Bcl-2 in 3 healthy control cases compared to 3 polymyositis cases. When using immunohistochemical results, a reverse correlation was seen between the number of Bcl-2-expressing fibers and the grade of MHC I expression (see Figure 5 in Paper II). Interestingly, the expression of Bcl-2 in type 2 fibers even better distinguished between healthy and diseased muscle. The Bcl-2 expression in healthy muscle was particularly linked to type 2 fibers (Figure 19), suggesting a higher relative protection of type 2 fibers.

Figure 19. The fraction of all muscle fibers expressing Bcl-2 in healthy volunteers is higher than in patients with polymyositis and Duchenne muscular dystrophy. When investigating type-2 fibers separately, the differences between controls and patients became even more prominent. In controls almost all fibers expressed Bcl-2.

Modified after Danielsson *et al.*
Neuromusc Disord 2009;19:412-17

TRAIL, FAS and FAS-L

TRAIL was only detected in capillaries and invading inflammatory cells, not in any muscle fiber. FAS-L was in a pilot study investigated with a polyclonal antibody, indicating an expression in several muscle fibers, but when we used antibodies from another batch, no expression was found. Another FAS-L antibody, from the same manufacturer, was later shown to cross-react with tissues known not to express FAS-L [299], which underline to avoid polyclonal antibodies. When we repeated the staining using 2 different antibodies (polyclonal and monoclonal), no FAS-L staining in muscle fibers was found. FAS was not expressed in any fiber of healthy muscle, while in most cases with polymyositis and in all

with Duchenne at least some FAS expressing fibers were found. This is in line with earlier reports [170].

Implications of the findings

The results give strong support for the findings reported by Ibi *et al.* [296], indicating a constitutive expression of Bcl-2 in healthy muscle, although it was not clear in the Ibi study [296], if the controls were completely healthy (only abstract in English). Bcl-2 is a major inhibitor of apoptosis [215, 217, 221], but has been shown also to protect against several other types of cell death [216, 219, 220]. Of particular relevance to polymyositis and IBM is its potential to protect against granzyme B mediated apoptosis. Its inverse expression compared to MHC I in muscle is also of interest, as MHC I expression of muscle fibers is an early event in IIM, and a conditional up-regulation has by itself, been shown to induce a myositis in mice [180]. Further support for a protective role Bcl-2 in normal muscle has also been found in interventional animal studies [214], and a pathogenic role and possible target of therapeutic intervention may be addressed by further studies.

Although muscle tissue is normally relatively inaccessible to the immune system, the muscle tissue is activated in inflammatory disease, not only by up-regulating MHC I, but muscle fibers have also been shown to be an active part in immunoreactions, expressing several immune stimulatory as well as protective molecules [40]. However, FAS-L or TRAIL, both with potent immune protective capabilities, are not expressed in muscle.

Apoptosis in IIM with partial invasion (Paper III)

Muscle from patients with polymyositis (PM) and inclusion body myositis (IBM) characteristically shows invasions of major histocompatibility complex class I- (MHC I) expressing muscle fibers by an inflammatory infiltrate dominated by CD8⁺ cytotoxic T cells [5]. This scenario is commonly referred to as a partial invasion, and is included as a classification criterion for PM for research studies [7]. The invaded fiber does not show classical signs of necrosis, but rather a gradual disintegration and displacement by inflammatory cells [54]. This type of cell degeneration is not found in other tissues, and is rarely seen in other muscle diseases [5]. In dermatomyositis (DM), a disease which is considered as a humorally mediated microangiopathy affecting muscle and skin, the most characteristic pathologic muscle findings are perivascular inflammatory infiltrates and perifascicular atrophy [77].

We investigated sequential sections of muscle tissue containing a partial invasion, from 10 patients (pi-patients). In addition to histological staining (HE), we used a panel of immunohistological stains (MHC I, MAC, CD8, CD68, CD163, granzyme B, spectrin, Bcl-2, FAS, HSP70, spectrin, merosin 80, fast and slow myosin heavy chain). We also performed the TUNEL- assay to detect apoptotic nuclei. An area of equal size and with corresponding center point, containing at least one partial invasion, was photographed in all sections. The findings were compared to areas with typical pathology in 10 DM-patients and 10 controls. In 3 of the 10 pi-cases, 4 necrotic fibers were seen in the investigated area, whereas in 4 of the 10 DM-cases, 6 necrotic fibers were found. No necrotic fibers were seen in the 10 controls. All stained fibers and inflammatory cells within these areas were counted and related the number of present muscle fibers. The major pathological findings within these areas are summarized in Table 4.

Table 4 Data and results of patients with partial invasion

Case	Age (yrs)	Sex	Bohan and Peter *	Amato/ ENMC*	TUNEL+	Partial inv.	Necrotic fibers
1	66	m	IBM def	IBM def	1	1	0
2	62	f	Overlap IIM pr	non-IIM	0	3	1
3	60	f	Overlap IIM def	DM s. D	2	4	0
4	32	f	PM def	PM def	0	1	1
5	66	f	Overlap IIM pr	PM def	2	3	0
6	54	f	PM poss	non-IIM	0	1	0
7	60	m	IBM def	IBM def	0	1	2
8	73	m	IBM poss	IBM poss	5	3	0
9	70	m	IBM def	IBM def	3	1	0
10	75	f	PM pr	PM def	3	2	0

*The diagnoses were according to the classifications of Bohan & Peter [2] and Amato/ European Neuromuscular Centre (ENMC) [7]. In both, IBM diagnoses were according to Griggs [4]. Abbreviations: m; male, f; female, def; definitive, pr; probable, poss; possible, IBM; inclusion body myositis IIM; idiopathic inflammatory myopathy, non-IIM; patients without weakness and not IIM according to Amato/ENMC, PM; polymyositis, DM s. D; possible dermatomyositis *sine* dermatitis.

Number of stained fibers and cells in the circled areas

There was a lower fraction of fibers expressing Bcl-2 in the two groups with inflammatory myopathies compared to controls (Figure 20a), corroborating our previous findings in PM (Paper II), and extending them to include DM. The fraction of fibers expressing FAS as well as fibers expressing HSP70 was higher in the pi-patients than in the controls, but did not differ between DM-patients and the controls (Figure 20b and 20c). Both the numbers of CD68⁺ and CD163⁺ cells and the ratio between CD163⁺ and CD68⁺ cells were higher in the groups with inflammatory myopathies compared to controls (the ratios are shown in Figure 20d).

Figure 20 a-d. The graphs show the proportion of Bcl-2 (a), FAS (b) and HSP70 (c) stained fibers, as well as the CD163/ CD68 ratio (d) of the individual cases in controls (n = 10), dermatomyositis (DM, n = 10) and partial invasion (pi, n = 10) patients.

Because of different expression in the control groups in this and the previous study (Paper II), we plotted the Bcl-2-expression of the controls of both studies against age, and a significant correlation ($p < 0.05$, $r -0.66$) was found (Figure 21).

Figure 21. The age of the controls in the two studies are plotted against the Bcl-2 expression in muscle fibers. An inverse correlation is evident. The solid dots represent the healthy controls of the Bcl-2 study (Paper II) and the open rings the control patients in this study (Paper III).

Bcl-2 expression has been shown to protect against different kinds of cell death [219, 300-302], including pathways mediated by granzymes [232]. The present findings of a constitutive expression of Bcl-2 in healthy muscle, and a lower expression of Bcl-2 in IIM, confirm results from our previous study (Paper II) and the study by Ibi et al. [296]. Age could account for the difference in Bcl-2 expression in the controls, since there was a negative correlation between proportion of Bcl-2 expressing fibers and age in the controls, clearly evident when plotting data from both Bcl-2 studies (Paper II and III).

Another possible contributing factor could be the general health status, as the controls (Paper II) were all healthy volunteers, while the controls in Paper III were patients with an initial suspicion of muscle disease that was later excluded. Still, also in the control group of Paper III, the Bcl-2 expression declined with age, and it is likely that both factors (age and status of health) contribute. Another contributing factor could be methodological, since stainings were performed several years apart. However, the same method was used and most of all, the results in PM, being included in both studies, were similar. Considering the docu-

mented cell-protective effects of Bcl-2 [216, 219, 220], the differently expressed levels in patients and among controls might reflect a varying vulnerability to muscle disease, including IIM. The found correlation between Bcl-2 and the level of MHC I expression (Paper II), gives this hypothesis further support.

A few studies have reported a correlation between CD163⁺ macrophages (M2-type) in muscle to disease activity in IIM [303, 304]. We showed that CD163⁺ macrophages are sparse in normal muscle, but are increased in absolute and relative numbers in IIM. Further studies may clarify if CD163⁺ macrophages are recruited to counteract the inflammatory reaction or if they contribute to a parallel regenerative muscle reaction, or if present M1-macrophages possibly make a type switch in muscle during the disease process.

Partial invasion of muscle fibers

The inflammatory cells, surrounding and invading a fiber, in most cases (18 of 20), also invaded a neighboring fiber. Most inflammatory cells had the morphological appearance of lymphocytes or macrophages, but some cells had particularly large nuclei and a few cells a pale cytoplasm. The inflammatory cells were usually surrounded by a matrix, in some cases shared by a group of cells, in other cases by the whole infiltrate. In most of the cases (13/20) there was a single broad-based invasion of the fiber, but several other types of infiltrate/fiber engagements were seen. Four partial invasions are shown in Figure 22.

All the inflammatory infiltrates contained, in addition to CD8⁺ and CD68⁺ cells, also granzyme B⁺ and CD163⁺ cells, the latter mainly located in the surrounding infiltrate. However, no TUNEL⁺ myonucleus was found in the partially invaded fibers. Of the 20 partially invaded fibers, only one was Bcl-2⁺, while 8 were FAS⁺ and 9 HSP70⁺. For staining characteristics of all partial invasions see Paper III (Table 3). Sections of one case of partial invasion with 8 different stains are shown in the Figure 23.

Figure 22. Two cases with “broad-based” invasions of the fiber are shown (a, b), where the infiltrates are surrounded by a matrix. In one case (c), the infiltrate was (in several consecutive sections) encapsulated within the fiber. Another, uncommon, finding was a case with multiple sites of fiber invasion (d), flanked by a group of inflammatory cells, some with giant nuclei (arrows). All sections are stained with hematoxylin-eosin (HE).

Figure 23 a-h. One case of partial invasion is shown in 8 different stains (hematoxylin-eosin (HE), MHC I, CD8, granzyme B, spectrin, merosin 80, CD68, CD163). The invading infiltrate is broad-based in the HE-stained section (a), but splits the fiber which expresses MHC I in the sarcoplasm, in another (b). There are inflammatory cells stained with CD8 (c) and granzyme B (d), and the fiber cell membrane is pushed forward into the fiber by the invading cells (e), and the basal membrane is split (f, arrow) as, visualized by the spectrin and merosin stains, respectively. CD68⁺ (M1 and M2) macrophages are plentiful on both sides of the basal membrane (g), while CD163⁺ (M2) macrophages (h) are less numerous in the infiltrate.

Earlier investigations of partial invasion have, in agreement with our study, showed that the majority of the invading cells are CD8⁺ cytotoxic T cells and CD68⁺ macrophages [305]. Interestingly, it was reported that many of the macrophages were shown capable of producing inflammatory molecules, with the potential to contribute to the pathology [305]. Ultrastructural studies of partial invasion have shown that infiltrating cells push the morphologically intact cell membrane into the fiber [54]. The immunohistological findings in our study generally agree with this description, but also show that damage or abnormalities of the cellular and basal membranes can be visualized as discontinuities (Figure 23) in the spectrin and merosin stains.

TUNEL expressing muscle fibers

In 6 of the 10 partial invasion cases, there were, in the imaged areas surrounding a partial invasion, 16 fibers with a TUNEL⁺ myonuclei, but they were not present in the partially invaded fibers. In contrast to this, no TUNEL⁺ myonucleus was found in the imaged areas of the dermatomyositis cases or the controls. TUNEL⁺ inflammatory cells were seen in both partial invasion- and DM-patients, with a median of 0.36 and 0.35 per 100 fibers, respectively, but not in the controls. Ten of the 16 TUNEL⁺ fibers could be evaluated in all other stains. Examples of TUNEL⁺ fibers and inflammatory cells are shown in Figure 24.

Figure 24. The sections are stained with the TUNEL method [292], which stains the apoptotic nuclei brown. The normal nuclei were counterstained with methyl green to facilitate visualization. A fiber with one TUNEL⁺ myonucleus (a) is shown. Two TUNEL⁺ inflammatory cells (b) are surrounded by other inflammatory cells. A nucleus of one inflammatory cell is only partly stained (small arrow) and another is located close to the fiber membrane (large arrow) (c), where an adjacent section visualized the sarcolemma, which separated the TUNEL stained nucleus from the fiber (not shown). A fiber with 2 TUNEL⁺ myonuclei is shown (d).

All 10 fibers had up-regulated MHC I of the sarcolemma and 9 also in the sarco-
plasm. None of these fibers stained for Bcl-2 or FAS, but 5 for HSP70. All 10
fibers had CD8⁺ and CD68⁺ cells, 9 had CD163⁺, and 8 had granzyme B⁺ cells,
adhering to the sarcolemma (Figure 6, Paper III). No signs of partial invasion or
degeneration were found in these fibers.

Table 5. Immunohistochemistry of fibers with apoptotic nuclei (TUNEL⁺)

Stains are shown as positive (+) or negative (-).

Case	MHC 1	Bcl-2	HSP70	FAS	Fast	Slow	CD8**	Grz B**	CD68**	CD163**
1	+	-	+	-	+	+	+	+	+	+
2	+	-	+	-	-	+	+	+	+	+
2	+	-	+	-	+	+	+	+	+	+
4	+	-	-	-	-	+	+	-	+	+
4	+	-	-	-	-	+	+	+	+	+
5	+	-	+	-	+	+	+	+	+	+
5	+	-	+	-	+	+	+	+	+	+
8	+	-	-	-	-	+	+	-	+	+
8	+	-	-	-	+	-	+	+	+	+
9	+	-	-	-	+	-	+	+	+	-

* staining also of sarcoplasm

** adhering to sarcolemma, not invading the fiber.

Abbreviations: MHC I; major histocompatibility complex class I, HSP; heat shock protein; Fast; fast myosin heavy chain, Slow; slow myosin heavy chain, Grz; granzyme.

In order to provide a larger basis for quantification of TUNEL⁺ fiber nuclei, the entire area of the two TUNEL-stained sections of all patients were studied in light microscope. The fiber numbers in the sections ranged from 954-3669 in the pi-patients, 1019-3910 in the DM-patients and 864-2968 in the controls. When counting only TUNEL⁺ nuclei judged as definitive, 45 were unevenly distributed among all 10 pi-patients (range 1-13), compared to 4 in 3 of the DM-patients and none in the controls. It was noted that 2 of the 4 TUNEL⁺ fibers in the DM-cases were found in a section which also contained a (*sic*) partial invasion. Interestingly, in 3 pi-cases a fiber with two apoptotic nuclei, were found.

Implications of the findings

The presence of apoptosis in IIM has long been debated and its pathogenic role in these diseases considered unlikely [175]. Using the TUNEL-assay we detected apoptotic muscle nuclei in IIM, which is in line with some earlier studies [173, 174], but not all [235, 306]. We also showed that apoptotic nuclei were present

almost exclusively in biopsies with a partial invasion and that the presence of apoptosis was strongly associated with MHC I expressing muscle fibers that were not invaded, but had adherent CD8⁺ T cells, granzyme B⁺ cells and macrophages. Taken together, the findings lend support to an immune mediated mechanism leading to apoptosis, a process that seems to occur in areas affected by partial invasion, but constituting a parallel process, since apoptotic nuclei were not found in the pi-fibers. The absence of FAS in fibers with apoptotic nuclei, together with the presence of adherent granzyme B⁺ cells, favors a cytotoxic (CD8⁺ T cell) mode of apoptosis-induction rather than a FAS-mediated mechanism.

The prevalence of celiac disease is increased in IIM-patients (Paper IV)

Patients whose muscle biopsy, during 1995 and 2002, met the pathological criteria of inflammatory myopathy at the Neuromuscular unit, Linköping, were asked to have blood samples drawn, for analysis of the presence of IgA-antibodies against endomysium and gliadin, and for permission to review of their medical records. Of 127 eligible patients, 106 (83%) agreed to participate. Antibody positive patients were offered further investigation with small-bowel biopsy or investigation for the presence of antibodies against anti-tissue transglutaminase (t-TG). The patients were classified according to Bohan and Peter or Griggs [2], and the presence of celiac disease, systemic inflammatory and malignant diseases was documented. Consecutive patients diagnosed for the first time during 1997-2001, living in the county of Östergötland, were separately documented for calculation of incidence, as a comparison these patients were also classified according to Amato/ENMC.

Celiac disease

Review of medical records and biopsies, complemented with pathological and new clinical investigations in uncertain cases, allowed classification of 88 patients, of whom 3 patients already were diagnosed with a biopsy proven (definite) celiac disease. The screening procedure resulted in 3 patients positive for IgA-EMA and seven for IgA-AGA. One of the patients, who were positive for IgA-EMA, was diagnosed with definite (biopsy proven) celiac disease and another was diagnosed with probable celiac disease (refused biopsy). Both patients experienced relief of gastrointestinal symptoms after introducing gluten free diet. The third IgA-EMA-positive patient had no gastrointestinal symptoms, a normal jejunal biopsy and was negative for IgA-tTG antibodies, and thus considered to have false positive IgA-EMA. Celiac disease was excluded in all 7 patients with positive IgA-AGA. We compared the prevalence in classified IIM patients with the prevalence found in a general population study, reported 1999 by Ivarsson *et al.* [10], using a similar screening procedure. In agreement to this study, we only considered definite cases. Patient data are shown in Table 6.

Table 6. Data of patients with elevated antibody titers against endomysium (EMA-Abs) or gliadin (AGA-Abs) and of patients with a diagnosed celiac disease

Case	IgA-EMA	IgA-AGA	IgA-tTG	Jejunal biopsy	Gastrointest. symptoms	SID	Bohan and Peter or Griggs
1	1:160	Neg	—*	Positive**	Yes	ASS	IIM overlap def
2	Neg	>200	—	Normal	No	ASS	IIM overlap pr
3	Neg	42	—	Normal	No	No	Not IIM
4	Neg	43	Neg	—	No	Sjögren	IBM def
5	Neg	Neg	—	Positive	Yes	No	IBM def
6	1:40	Neg	Neg	Normal	No	RA	IIM overlap pr
7	1:80	Neg	—	—	Yes	No	Polymyositis poss
8	Neg	45	Neg	—	No	No	Polymyositis poss
9	Neg	Neg	—	Positive	Yes	Sjögren	IBM def
10	Neg	42	—	Normal	No	Sjögren	IIM overlap pr
11	Neg	>200	Neg	—	No	No	Polymyositis poss
12	Neg	>200	Neg	—	No	No	Not IIM
13	Neg	Neg	—	Positive	Yes	No	IBM def

* —: not performed

** : positive: pathology characteristic of celiac disease.

Abbreviations: IgA-tTG; anti-tissue transglutaminase antibodies, SID; systemic inflammatory disease, Neg; not elevated antibodies, ASS; antisynthetase syndrome, RA; rheumatoid arthritis, Sjögren; Sjögren syndrome, Poss; possible, Pr; probable, Def; definite.

In summary, 4 of 88 IIM patients (4.5%) had definite celiac disease, which is higher than the 0.53% prevalence found in the general population screening (CI 1.4% – 11%), and also higher than the presently *estimated* population prevalence approaching 1% [10, 135]. This confirmed the findings from the study by Henriksson *et al.* [142], indicating a prevalence exceeding 4% in IIM. Serology

screening in our study only identified one new case with definite (biopsy confirmed) and one with probable celiac disease. With the increased awareness of the prevalence in the general population, the presence of gastrointestinal symptoms should raise a suspicion of celiac disease, especially in the IIM population, with a now confirmed higher prevalence than in the general population. Of note is that 3 of 18 (17%) patients with IBM in our cohort had celiac disease. Recent studies have also drawn attention to the occurrence of celiac disease in IBM patients [144, 307], and that both diseases share an association with HLA B8-DR3 [307, 308]. A systematic screening for celiac disease may not be warranted for the whole IIM group, but screening in IBM seems clinically motivated, although it would be desirable to specifically address this topic in a new and preferably prospective study. It is important to be aware of possible false positive and negative antibody screening results.

Since the start of this study IgA-AGA has been shown to have a low sensitivity and specificity for celiac disease, which is also evident from our results, and is no longer recommended as a screening test. In contrast, both IgA-EMA as well as IgA-tTG are regarded as both sensitive and more specific markers for celiac disease [137, 138], and are presently used as standard screening tests. In the study of Selva O'Callahan *et al.* [144], however, none of their IIM patients with confirmed celiac disease had elevated titers of IgA-EMA or IgA-tTG. Their diagnosed cases were 3 of 17 with elevated IgA-AGA. Different possible explanations for a lesser sensitivity of IgA EMA and tTG in this patient group were discussed in their report, and include suppressed titers of IgA-class antibodies in blood due to immunosuppressive treatment, or to the presence of an autoimmune disease, as has been reported to occur in patients with SLE and Sjögren syndrome [309, 310]. The results of their and our screening, and the diagnosed cases by Henriksson *et al.* [142], suggest that for IIM patients with gastrointestinal symptoms or signs of malabsorption, screening with a single autoantibody is not enough to exclude celiac disease, but if negative, should prompt a further investigation, including other antibodies, HLA-typing or a jejunal biopsy.

Classification

Eighty eight of the 106 included patients (83%) fulfilled the Bohan and Peter or Griggs criteria of at least possible IIM or possible IBM. Of these, 28 patients were diagnosed with “isolated” polymyositis, 4 with “isolated” dermatomyositis, 5 had an associated malignancy, 3 were diagnosed with IIM in childhood and 30 had an associated systemic inflammatory disease, and another 18 had IBM (Table 7).

Table 7. Characteristics of the study population – demography, follow-up time, number of biopsies and grades of diagnostic certainty

	PM n = 28	DM n = 4	Malignancy n = 5	Childhood n = 4	SID & IIM n = 30	IBM n = 18	Not IIM n = 18	Total n = 106
Female/male	19/9	2/2	5/0	3/0	24/6	6/12	8/10	67/39
Age median, yrs (range)	60 (18-84)	57 (42-60)	60 (47-72)	8 (6.5-16)	48 (24-80)	66 (48-76)	51 (26-84)	58 (6.5-84)
Follow-up, yrs (range)	7.5 (0.5-18)	9.2 (4.5-18)	5 (0.5-8.0)	13 (12-16)	8.2 (3.0-18)	7.5 (2.5-14)	2 (0.0-20)	7.4 (0.0-20)
Biopsies*	49	6	8	4	46	29	26	168
Definite#	14	1	4	3	12	16	-	50
Probable#	7	2	1	0	14	**	-	24
Possible#	7	1	0	0	4	2	-	14

* 1-4 muscle biopsies were analyzed from each patient.

** The Griggs classification does not have a category for probable.

The Bohan and Peter classification requires 2 criteria for possible, 3 for probable and 4 for definite diagnosis of IIM. The criteria are: biopsy findings, proximal muscle weakness, EMG-findings, raised muscle enzymes and, in the case of dermatomyositis, a typical skin rash.

Associated systemic inflammatory diseases

In the group of 30 patients having an associated inflammatory systemic disease, 15 had an antisynthetase syndrome (Jo-1 antibodies were detected in 13 of 15), 7 Sjögren syndrome, 3 SLE, 3 MCTD, 1 RA, and 1 systemic sclerosis. In the group diagnosed with IBM, 3 of 18 patients had concomitant Sjögren syndrome, resulting in a total of 33 IIM patients (38%) with an associated systemic inflammatory disease (Figure 25). Eleven of 22 patients (50%) lacking muscle weakness, also had a systemic inflammatory disease. In the 5 patients with an associated malignancy (3 with DM and 2 with PM), the primary tumor affected the lung, bladder,

ovary, breast and pancreas, respectively. A causal connection was obvious in the 3 DM-cases. In earlier cohorts, disregarding IBM, the reported frequencies of an associated malignancy were similar, as were frequencies of associated systemic inflammatory disease, ranging between 15 and 31%, when using similar criteria, but as high as 60%, when widening the definition, considering *overlap features*. [52, 62, 120, 311]. Taken together, active search for associated immune mediated disorders is important in IBM-patients and IIM patients lacking muscle weakness, and active search for malignancy is important, especially in DM-patients (3 of 7 in our cohort had a malignancy).

Idiopathic inflammatory myopathies associated with systemic inflammatory diseases

n = 33 patients*

Figure 25. The distribution of IIM patients with associated systemic inflammatory diseases is shown by the number of cases with the respective diagnosis. The patients were classified according to Bohan and Peter or Griggs (IBM).

*Three of 18 patients with IBM and concomitant Sjögren syndrome are also included.

Incidence of IIM

During the years 1997-2001 (5 years), 15 new patients (3 per year) living in the county of Östergötland were diagnosed with IIM. Available data were considered sufficient for classification of 28 of 29 patients, whose biopsy fulfilled biopsy criteria. Based on these numbers, the incidence was calculated to 7.3 per million/year. The population average in the county during these years was 412 592 (range 411 333 - 415 010), source Statistiska Centralbyrån: <http://www.scb.se>. Fourteen patients fulfilled the criteria according to Amato/ ENMC, resulting in the same crude incidence. The incidence of 7.3 per million/year is in agreement with studies using similar assessments and criteria [69, 312], and similar to the estimated incidence (7.98 per million/year) in a recent systematic review [68].

MAIN CONCLUSIONS

Pathogenesis

- Apoptosis of fiber nuclei is present in inflammatory myopathies, and occur chiefly in myositis associated with partial invasion. The affected fibers express MHC I, and are surrounded, but not invaded, by CD8⁺ T cells, macrophages and granzyme B⁺ cells. These processes occur in the same area, albeit not in the same fiber (Paper III).
- The findings collectively support that apoptosis, induced by cytotoxic CD8⁺ T-cells, may be a pathologic mechanism in IIM, in parallel with the fiber disintegration seen in partial invasion (Paper III).
- Bcl-2 is constitutively expressed in healthy muscle, but is lower in IIM. Its expression decreases with age and possibly with a compromised general health status, which may confer a vulnerability to muscle (Paper II, III).

Classification

- The Amato/ENMC classification excludes patients without muscle weakness and forms, despite an extensive diagnostic work up, a relatively large group with *unspecific* myositis. Polymyositis, as strictly defined, was the second largest specific diagnostic group, after IBM (Paper I).
- The frequency of detected partial invasions in muscle of IIM patients is to a great part a function of how extensive the search is. Partial invasion is not uncommonly found in other disease groups than IBM and polymyositis (Paper I).

Diagnosis

- Multilevel and single level serial sectioning increases the sensitivity and specificity of a muscle biopsy, when classifying and diagnosing IIM (Paper I).
- Associated systemic inflammatory diseases and malignancy are common in IIM, and have important implications for diagnosis and follow up. The detected incidence of IIM in Östergötland was 7.3 per million/ year. (Paper IV).
- The prevalence of celiac disease in patients with IIM is increased compared to the general population (Paper IV).

INTERPRETATION OF FINDINGS IN A BROADER CONTEXT AND FUTURE PERSPECTIVES

Pathogenesis

IIM are usually presented as a heterogeneous group of diseases with a presumed autoimmune pathogenesis [77]. Autoimmunity is defined as a breach of tolerance of self-antigens, causing a disease. What is the evidence supporting an autoimmune pathogenesis in IIM? It is well to remember the accompanying descriptive term, *heterogeneous*. What is true for one disease may not be true for another. Least is known about the most recently introduced group, immune-mediated necrotizing myopathy. The earlier claimed absence of inflammatory cells and MHC class 1 expression in muscle fibers [158, 313], has been challenged by a report that identified both inflammatory cells and MHC I up-regulation [268], which were distinguishing features compared to non-immune-mediated necrotizing myopathy (usually provoked by myotoxic agents). In the same study cell quantification and cytokine expression supported a Th1-M1 polarized immune profile. The initial report, describing this type of pathology with support for an immune-mediated mechanism, also noted MAC deposition in capillaries, which had a “pipestem” appearance [61]. One of the described patients had a carcinoma; and later reports support that strong complement deposition in capillaries may be a typical feature of a paraneoplastic subtype of immune-mediated necrotizing myopathy [268, 314].

Taken together, the immune pathogenesis of this disease is not yet settled, but several reports indicate that the associated pathology have diverse etiologies, for review see [119].

Factors in favor of an autoimmune pathogenesis in IIM include the common genetic background involving MHC class II, the association with other autoimmune diseases, the self-perpetual chronic nature, as well as response to immunomodulatory therapy [315]. Other supporting findings include myofiber up-regulation of MHC I in PM and IBM and the expression of co-stimulatory molecules with the potential to deliver a second signal, hence a scenario that could elicit an immune response by cytotoxic CD8⁺ T cells [46, 316]. Further, CD8⁺ T cells are in close proximity to the fiber membrane when invading muscle fibers; and they are oligoclonally expanded and secrete perforin and granzyme B, seemingly directed against antigens of the muscle fiber. However, findings of an anticipated apoptosis or classic necrosis have not been documented in these particular fibers, and the invading cells have not been observed to cross the cell membrane [54] (Paper III). On the other hand, we found that CD8⁺ T cells were visualized adjacent to apoptotic fibers, as were CD68⁺ macrophages that may be the source of, for example, cytotoxic oxygen radicals with the potential to damage the fiber [305].

Even though the process of partial invasion thus shows several signs of an antigen driven process and signs of a damaging effect of the fiber, this may not be the *major* fiber damaging pathway in cytotoxic CD8⁺ T cell mediated IIM. Partial invasion seems to be an integrated part of the pathology in IBM, but is a less frequent finding in PM, where it rather forms part of a continuum of endomysial inflammatory reactions, which also are observed in IIM with overlap and DM (Paper I).

Granzyme B may have a pathogenic role since it can cleave other enzymes than effector caspases (inducing apoptosis), and turning cryptic antigens into major immunogens, which can activate self-reacting T cells [317]. This has been a pro-

posed mechanism underlying the development of autoimmunity [318]. Indeed, many of the described autoantigens in IIM have been shown to be potential substrates for granzyme B [319], which is of particular relevance for the estimated 20-25% of all IIM patients, who have Jo-1 antibodies and an antisynthetase syndrome [277]. It was shown that mice immunized with Jo-1 replicated the human antisynthetase syndrome [320]. Using the same model, the authors also showed that a cleaved part of Jo-1, without adjuvant, could trigger an innate immune response [321]. Even though the cleavage site of mouse Jo-1 is not identical to its human counterpart, it gives support for Jo-1 as a potential autoantigen in IIM. The authors of this report interpreted the findings as strengthening of the concept of a coordinated pathogenic pathway: MHC class I expression, ER stress, release of HSP to generate lymphocytic invasion of muscle, as suggested by earlier reports [182]. With Paper III, we introduced apoptosis of myonuclei, as a possible parallel process in IIM, which was found to be particularly apparent in CD8⁺ cytotoxic T cell-mediated muscle pathology, which also can be induced by ER stress [185].

The detection of clonally expanded plasma cells in IIM muscle, followed by evidence for a local B cell maturation, also indicate a local antigen driven response in DM, IBM and PM [322, 323]. Interesting is also the reported formation in juvenile DM patients of extra-nodal lymphoid microstructures [324], which have also been detected in salivary glands and the synovium in Sjögren syndrome and rheumatoid arthritis, respectively [325, 326]. In IBM, an autoantibody was recently detected, which is directed against an enzyme, involved in hydrolysis and repair of DNA (cN1A) [108-110]. The same antigen was also detected in rimmed vacuoles [108]. This favors, but does not prove, an autoimmune process in IBM. Our observation of fragmented myonuclei (as seen in apoptosis) in CD8⁺ T cell-mediated IIM (Paper III), opens for a possible exposure of cryptic nuclear antigens in these diseases. The reports of a seasonal clustering of juvenile DM and its frequent disease onset after infections may indicate antigenic mimicry as a potential autoimmune inducing mechanism in this subgroup [68]. Taken together, alt-

though not proven, several lines of evidence indicate an autoimmune pathogenesis of most IIM groups.

A central question is if inflammatory autoimmune-like diseases can affect muscle tissue as the sole target organ. Several of the myositis conditions are part of a systemic inflammatory process, as seen in DM and the antisynthetase syndrome [327]. Therefore, the discussion of the existence of a “pure” polymyositis is of relevance, and based on the following findings: documented occurrence without association with other immune-mediated diseases [111] (Paper I), the time course and response to therapy [111] (Paper I), the shared haplotypes with other autoimmune diseases [155], presence of autoantigens [328] and oligoclonality of present CD8⁺ cytotoxic T cells [58], PM does seem to appear as a tissue specific autoimmune disease.

In addition, the detection of a disease specific autoantibody in IBM, where the antigen was shown to be localized in the most disease characteristic component, the rimmed vacuoles, and all of the above stated supporting factors concerning PM, except response to therapy, also support autoimmune mechanisms in IBM, hence, as a muscle-specific immune-mediated disease. It should also be noted that in several cases, although being a systemic disease, the muscle involvement is the major target. Nevertheless, the distinction between association and overlap syndrome is often difficult, but relevant, and it was therefore discussed/ problematized earlier in this thesis, under the subheading *overlap syndrome*. Further studies, both epidemiologic and pathogenic, are important to delineate the nature of IIM as muscle specific or part of general systemic inflammatory diseases, and whether this condition affects clinical management including treatment.

Although muscle has been regarded as an immunologically “inert” organ being inaccessible for the immune system, it is clear that it can also become a very active part in immune processes. Therefore, there are several protective mechanisms involved to promote muscle integrity, of which the expression of Bcl-2 is

an important factor identified by us. Bcl-2 expression was shown to be inversely correlated with the MHC class I expression (Paper I), indicating that Bcl-2 has a protective effect versus immune-mediated attack. Further, Bcl-2 expression was inversely correlated with age, in line with the fact that the frequency of several IIM increase with age. However, our finding could in part be explained by the source of muscle biopsies, including both healthy individuals (at a younger age) and patients deemed free of muscle disease (at a higher age). The role of age versus source of biopsy should be sorted out. The potential of protecting effects by Bcl-2 is interesting and should be the object for further mechanistic studies, and Bcl-2-associated pathways may constitute a possible target for therapeutic intervention.

The heterogeneity of patients also within disease groups, as illustrated by a case of PM where most T lymphocytes expressed $\gamma\delta$ -chains [329], further opens for specific etiologies in selected cases. The association of myositis with dysfunction of organs exposed to the environmental antigens is of interest for further studies about pathogenesis. The confirmed association with celiac disease (Paper IV) is therefore not only clinically relevant, but also of interest for studies concerning pathogenesis.

Classification

The evolution of classifications of IIM are built upon an increasing knowledge of these diseases, but it has also been an ongoing discussion about the usefulness of “lumping” and “splitting”. Different classifications have put different emphases on clinical features, association with other diseases, autoantibodies or muscle biopsy findings. The continued splitting has led to much useful scientific and clinical information, although some problems have become evident. The criteria for clinical, serological and pathological distinctions between the disease groups

do not form the same diagnostic groups [153] (Paper I). This may be accounted for: (1) by overreliance of pathological findings resulting from different immunologic effector mechanisms or sampling problems, (2) that autoantibodies may be an immunologic finger print of the individual patient, rather than disease specific, and (3) that many patients do not develop the whole spectrum of a syndrome.

A “purist” approach to disease entities may leave us with many small, defined, disease groups and many patients with an unspecific diagnosis. It may also bring the risk, as voiced by a senior scientist, that we “assume we know more than we do”, as echoed in the review in 2011, by Dr. David Hilton-Jones [239]. This could be an obstacle in the further search of the causes of IIM. An alternative approach, avoiding this, could be to untie the pathological diagnosis from the clinical syndromes, and delineate a limited set of pathological syndromes, as suggested by Pestronk [313].

From the perspective of the most recently presented classification [7], the results of our studies indicate two relevant conclusions: Partial invasion seems to be an integrated part of the pathology in IBM, but is a less frequent finding in PM, where it rather forms part of a continuum of endomysial inflammatory reactions. The frequency of detected partial invasions in IIM is to a great part a function of how extensive the search is (Paper I). In spite of the fact that we used the stricter Amato/ENMC criteria (Paper I), our findings were similar to those reported by Chahin and Engel [111]. The requirement of partial invasion for diagnosing the PM group may therefore be questioned. The group of patients with non-specific pathology, although consistent with myositis, and patients lacking muscle weakness together constitute a substantial part of patients with inflammatory muscle disease (Paper I). It is important that these patients also are considered in future studies.

New classification criteria by the *international myositis assessment and study group (IMACS)* are currently under review and will be published soon [241].

The criteria will be based on statistical analysis of more than 1500 patients, from 47 centers worldwide, investigating 93 variables. Sixteen variables have been identified, to best discriminate between IIM and non-IIM, and further 16 variables, with a “different importance”, which will be assigned another weight score [241]. This classification will certainly lay the ground for further research and understanding in the field.

Diagnosis

When diagnosing IIM, the criteria by Bohan and Peter [2] are still useful for the clinician, which may contribute to the tenacious popularity of this classification. The lack of an absolute requirement of a *muscle biopsy*, has however been considered a weak point of their classification [7], and was a main reason why we chose a muscle biopsy finding consistent with IIM as a requirement of our classification study (Paper I). Despite some held concerns of subjecting children with a seemingly obvious DM to an invasive muscle biopsy investigation, preliminary results indicate its clinical usefulness even in this subgroup [85]. In addition to confirm an IIM, a muscle biopsy will in the majority of cases detect pathological findings, consistent with a subgroup [62, 111] (Paper I). Due to sampling problems and pathological overlap, it is however important to interpret the pathology in conjunction with other laboratory data and clinical findings (Paper I). We further showed that an extended pathological work-up increases the diagnostic yield of a muscle biopsy.

The detection of elevated blood level of *muscle enzymes* (in particular CK) is a valuable supporting finding that was almost invariably present (80 of 83) in our cohort (Paper I). Except for detecting neurogenic diseases, EMG together with nerve conduction studies will in most cases detect findings suggesting an inflammatory myopathy (Paper I), and direct the choice of muscle for biopsy [1,

73]. The investigation of muscle strength is important to detect a *weakness pattern* common to IIM, with special attention to muscle groups typical for IBM or other muscle diseases [7, 100]. It is also important to be aware of the fact that some patients with an inflammatory muscle disease may lack detectable muscle weakness. In our cohort, a half of these patients had an associated systemic inflammatory disease (Paper IV). The common co-occurrence of other inflammatory diseases and malignancy is important to acknowledge, both at the time of diagnosis and during follow-up (Paper IV).

More recent added tools for diagnosing IIM are MRI of muscle and the detection of an increasing number of MSA and MAA in blood. So far there are relatively few publications of the utility of MRI to diagnose IIM. Characteristic muscle involvement of IBM can usually be detected, and it was shown to increase the yield of an MRT directed biopsy [256]. Another study reported that almost all investigated IIM patients showed abnormal findings in muscle, but no correlation was found between edema-like or fat abnormalities compared to muscle histology (inflammatory infiltrates) of the corresponding muscle [256]. More studies will certainly be needed to evaluate MRI as a diagnostic tool for IIM. Although auto-antibody screening was not addressed in our studies, its increasing importance for diagnosis, follow-up and choice of therapy has been confirmed by many reports, which have been highlighted and cited throughout the thesis. The future work in this field has the potential of elucidating pathogenic mechanisms and aid in the diagnosis and treatment of IIM.

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