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CHARACTERIZATION OF IMMUNE SPECIFICITIES IN IDIOPATHIC INFLAMMATORY MYOPATHIES

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“Are Medusa's eyes the ones that turn you into stone? What if she looked at her reflection? Weary and helpless, she would self-lash out, and then, would she break, or would she come back stronger?”

Maribel Galindo ‘🔔’

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Characterization of immune specificities in idiopathic inflammatory myopathies

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my mom, my family and all my friends,
To **the patients** suffering from autoimmune diseases,
You have inspired me and have kept me going,
This wouldn't have been possible without all of you.

"Once a disease has entered the body, all parts which are healthy must fight it: not one alone, but all. Because a disease might mean their common death. Nature knows this, and Nature attacks the disease with whatever help she can muster."

Theophrastus Bombastus von Hohenheim (Paracelsus)

POPULAR SCIENCE SUMMARY OF THE THESIS

“It's not about just learning what to attack, but learning what to tolerate.”
Sally Bloomfield

The human immune system has many faces, connections and roads that collectively work to respond to an infection, eliminate cancer and even maintain good metabolism. This inflammatory response evolved to protect our bodies from foreign substances, viruses, bacteria and even tumour cells. After the initial damage, the body resolves the inflammation in an active and choreographed manner to repair the tissue and remove foreign pathogens and cells. However, subtle changes disrupting its ability to distinguish a pathogen from the host or even an overzealous response can cause our immune system to instead target our own body.

Just the idea of an organism attacking itself with a system made for its own protection was too far-fetched in 1901 when Paul Ehrlich described the “horror autotoxicus”. Nowadays, there is no doubt about the existence of autoimmunity, with over 80 different autoimmune conditions affecting millions of people worldwide. As varied as the cell types in our body, so are the faces of autoimmunity. The cause of autoimmune diseases is still a mystery and due to their variability, they are a challenge to study.

In many autoimmune diseases, we can find autoantibodies, proteins from the immune system that act like misguided soldiers, marking a tissue from the host for destruction. Autoantibodies are produced by B cells and B cells are helped by T cells. Autoantibodies and the immune cells are tied together by their target and won’t stop before their target is eliminated. This can cause longstanding debilitating disease, for example when the target is associated with muscles.

This thesis aims to study a particular group of autoimmune diseases called myositis. This entity is characterized by the infiltration and attack of the muscle by immune cells and autoantibodies. But myositis is not limited to the muscle, often the lungs and skin are also involved. Sometimes the inflammation is mild and with a low pace, but in other cases, it can be devastating, causing damage and loss of organ function. Generally, patients experience weakness, rash, muscle and joint pain. But in other occasions, the only manifestation might be a lack of air or difficulty breathing.

The studies presented in **Paper I** and **IV** of this thesis focus on the helper T cells. The mechanism of why these T cells attack the lung has remained elusive. In **Paper I** we found that a protein (called HisRS) can activate T cells from the airways of myositis patients. These activated T cells can potentially activate B cells in the nearby lung tissue and thereby start the autoimmune disease. In **Paper IV,** we use modern molecular biology techniques to take T cells from human muscle tissue and measure their activity. This information can also help us understand why these T cells are attacking the host and possibly, in the future, could help us to develop specific therapies to control them.

T cells are activated in a special way. They require their activating protein to be presented to them by another cell on top of the, so-called, *HLA*. In **Paper III**, we studied the genetical variations in the *HLA* and we showed that specific autoantibodies are associated with specific variants of *HLA*. One of the muscle-specific autoantibodies is called anti-FHL1. In **Paper II** and **V**, we focused our studies on anti-FHL1 autoantibodies and showed anti-FHL1 association with disease manifestations in myositis patients in Sweden and Australia.

In summary, autoimmune diseases result from the immune system being misdirected. This thesis progresses the knowledge of myositis in terms of the pathophysiology of HisRS, epitope presentation and T cell activation; in terms of the clonality and function of muscle T cells; in terms of the variations in *HLA* and diversity of autoantibodies, and in terms of the applicability and clinical relevance of the anti-FHL1 muscle-associated autoantibody. This thesis unravels aspects of the disease mechanism in myositis that could be used for the development of new diagnostic tools and new treatments.

ABSTRACT

Idiopathic inflammatory myopathies (IIM or myositis) are a group of rheumatic autoimmune diseases characterised by skeletal muscle inflammation and weakness, and are associated with high morbidity and mortality. The diagnosis integrates several subgroups of muscular disorders with autoimmune backgrounds, where identifying myositis-specific and associated autoantibodies play an essential role. The spectrum of these autoantibodies may reflect different clinicopathological mechanisms involved in the disease for the various subgroups of IIM. The high heterogeneity and complexity in myositis represent a clinical challenge and due to the rarity of the disease, the pathophysiological mechanism behind IIM remains elusive. The aims of this thesis were to 1) study the mechanisms of disease associated with the group of IIM and anti-Jo-1 autoantibodies in terms of T cell functionality and local autoantibody production; 2) to gain a deeper understanding of T cell infiltrate in the muscle tissue at a single-cell level; 3) to characterise the autoantibody subgroups and identify new associations of risk *HLA* alleles, and 4) to study the prevalence of anti-FHL1 autoantibody and clinical manifestations in cross-sectional and longitudinal cohorts.

In **Paper I**, we identified histidyl–tRNA synthetase (HisRS) reactive $CD4^+$ T cells in the bronchoalveolar lavage fluid and germinal centre-like structures in the lungs of patients with IIM/anti-synthetase syndrome and the *HLA-DRB1*03* allele. Thereby, we identified a potential link between autoreactive T cells and the formation of autoantibodies originating from the lung. In **Paper II** and **V**, we detected anti-four-and-a-half-LIM domain 1 (FHL1) autoantibodies in patients with IIM from Australia and Sweden in a cross-sectional and longitudinal approach. In **Paper V,** we also evaluated the autoantibody levels against FHL1 over time and reported the correlation between FHL1 levels and disease severity. We found that anti-FHL1 autoantibodies were present in patients with IIM who were previously autoantibody-negative, thus adding a novel autoantibody that supports IIM diagnosis. In **Paper III**, we classified IIM autoantibody co-expression patterns and determined the underlying *HLA* allele variations. In **Paper IV,** we used single-cell mRNA sequencing to explore T cell infiltration in the muscle of patients with IIM. We identified a muscle T cell signature containing genes associated with tissue homing and tissue residency**.** Moreover, we identified immunosuppression-resistant T cell clones in blood and muscle.

In conclusion, this thesis contributes to the understanding of the pathophysiology of IIM from the perspective of the tissue-specific T cells, *HLA* variations, and autoantibodies diversity. Our findings also propose new avenues for further research, which could facilitate the development of a personalised treatment or new biomarkers.

LIST OF SCIENTIFIC PAPERS

I. **Pro-inflammatory histidyl-tRNA synthetase-specific CD4$^+$ T cells in the blood and lung of patients with idiopathic inflammatory myopathies**. **Galindo-Feria AS***, Albrecht I*, Fernandes-Cerqueira CS, Notarnicola A, James E, Herrath J,Dastmalchi M, Sandalova T, Ronnblom L, Jakobsson P, Fathi M, Achour A, Grunewald J, Malmstrom V, Lundberg IE.
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**Equal contribution*

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**Equal contribution, °Equal contribution*
Manuscript

V. **Anti-FHL1 autoantibodies in adult myositis: a longitudinal follow-up analysis.**
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**Equal contribution*
Manuscript

LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

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CONTENTS

LIST OF ABBREVIATIONS

aaRS/ARS	Aminoacyl-tRNA synthetase
Ab	Antibody
ABD	Anti-codon binding domain
AD	Autoimmune disease
ACR	American College of Rheumatology
AENEAS	American, European NEtwork of Anti-synthetase Syndrome
AGER	Advanced glycosylation end-product specific receptor
ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
ASAT	Aspartate aminotransferase
ANA	Antinuclear antibodies
Anti-Jo-1	Anti hystidyl-tRNA synthetase autoantibodies
Anti-RNP	Anti-ribonuclear protein antibodies
Anti-SSA	Anti-Sjögren's-syndrome-related antigen A autoantibodies
Anti-SSB	Anti-Sjögren's-syndrome-related antigen B autoantibodies
ASS/ASyS/ASSD	Anti-synthetase syndrome
APC	Antigen presenting cell / Allophycocyanin
Arg	Arginine
ATP	Adenosine triphosphate
ATS	American Thoracic Society
AU	Arbitrary units
AUC	Area under the curve
BAFF	B cell activating factor
BALF	Bronchoalveolar lavage fluid
BLK	B lymphocyte kinase
BSA	Bovine serum albumin
BV	Brilliant violet
C15ORF	CDAN1 interacting nuclease 1
C4A	Complement component 4A
CADM	Clinical amyopathic dermatomyositis
CALHN	Central Adelaide Local Health Network
CAM	Cancer-associated myositis
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDAN1	Codanin-1
CI	Confidence interval

CK	Creatine kinase
CLIP	Class II-associated invariant chain peptide
COL6A2	Collagen alpha-2 (VI) chain
COPD	Chronic obstructive pulmonary disease
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T lymphocyte associated protein 4
cN-1A	Cytosolic 5′-nucleotidase 1A
CXCL	C-X-C motif chemokine ligand
CXCR	C-X-C motif chemokine receptor
Cy	Cyanine
Da	Dalton
DC	Disease control
DCs	Dendritic cells
DM	Dermatomyositis
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
E. coli	Escherichia coli
EJ	Glycyl-tRNA synthetase
ER	Endoplasmic reticulum
ENMC	European Neuromuscular Centre
ELISA	Enzyme-linked immunosorbent assay
EMG	Electromyography
EPRS	Glutamyl-prolyl-tRNA synthetase
ESR	Erytrhocyte sedimentation rate
eQTL	Expression quantitative trait locus
EULAR	European Alliance of Associations for Rheumatology
Fab	Fragment antigen-binding
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallizable region
FcR	Fc receptor
FDA	Food and Drug Administration
FDR	False discovery rate
FITC	Fluorescein isothiocyanate
FHL1	Four-and-a-half-LIM domain 1
FL	Full length
GBM	Glomerular basement membrane
GC	Germinal center
GrB	Granzyme B

GRS	Glycyl-tRNA synthetase
GWAS	Genome-wide association study
HAQ	Health assessment questionnaire
HC	Healthy control
Hg38	Human genome build 38
H&E	Haematoxylin and eosin
His	Histidine
HisRS	Histidyl tRNA synthetase
HLA	Human leukocyte antigen
HMGB1	High mobility group box protein 1
HMGCR	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
HR	Hazard ratio
HRCT	High-resolution computer tomography
HSP	Heat shock protein
IBM	Inclusion body myositis
IC	Immune complex
ICAM	Intracellular adhesion molecule
IFI35	Interferon-induced protein 35
IFL	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IIM	Idiopathic inflammatory myopathies
IM	Institute of Myology
IL	Interleukin
ILD	Interstitial lung disease
IL-1R	Interleukin-1 receptor
IMAC	Immobilized metal affinity chromatography
IMACS	International Myositis Assessment & Clinical Studies group
IMCCP	International Myositis Classification Criteria Project
IMNM	Immune-mediated necrotizing myopathy
iNOS	Inducible nitric oxide synthetase
IP	Immunoprecipitation
IPF	Idiopathic pulmonary fibrosis
IQR	Interquartile range
IRFs	Interferon regulator factors
ISG	Interferon-stimulated gene
IVIg	Intravenous immunoglobulins
JAK	Janus kinase
JDM	Juvenile dermatomyositis

KLRG1	Killer cell lectin-like receptor G1
KS	Asparaginyl-tRNA synthetase
LDH	Lactate dehydrogenase
LIM	Lin-11, Isl-1, Mec-3
LR	Likelihood ratio
MAAs	Myositis-associated autoantibodies
MaBP	Maltose-binding protein
MAC	Membrane attack complex
MDAAT	Myositis disease activity assessment Tool
MDA5	Melanoma differentiation-associated protein 5
MDI	Myositis Damage Index
MRC	Medical Research Council
MRS	Methionyl-tRNA synthetase
MHC	Major histocompatibility complex
Mi-2	Nuclear DNA helicase
MMT-8	Manual muscle test-8
mRNA	Messenger RNA
MSAs	Myositis-specific autoantibodies
MyoNet	Global Myositis Network
MW	Molecular weight
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor-kappa B
NOD	Nucleotide-binding oligomerization domain
NLR	NOD-like receptor
NLRP3	NLR family pyrin domain containing 3
NOD	Nucleotide-binding oligomerization domain
NS	Not significant
NT5C1a	Cytosolic 5'nucleotidase 1A
NXP-2	Nuclear matrix protein-2
OD	Optical density
OJ	Isoleucyl-tRNA synthetase
OM	Overlap myositis
OR	Odds ratio
PAM	Partition around medoids
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween 20
pDCs	Plasmacytoid dendritic cells
PE	Phycoerythrin

PerCP	Peridinin-chlorophyll-protein complex
PL-7	Threonyl-tRNA synthetase
PL-12	Alanyl-tRNA synthetase
PM	Polymyositis
Pm-Scl	Nucleolar protein complex
PPV	Positive predictive value
PRDX3	Peroxiredoxin 3
ProDERM	Progress in DERMatomyostis
PRRs	Pathogen recognition receptors
PSMB8	Proteasome 20S subunit beta 8
PSMB9	Proteasome 20S subunit beta 9
PTGES3L	Prostaglandin E synthase 3 like
PTM	Post-translational modifications
PTPN6	Tyrosine-protein phosphatase non-receptor type 6
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis
RB	Reducing buffer
RCT	Randomized controlled trial
RefSeq	Reference sequence
RNA	Ribonucleic acid
RNP	Ribonucleoprotein
RPKM	Reads per kilobase million
SAE	Small ubiquitin-like modifier activating enzyme
SAMD	South Australian Myositis Database
scFv	Single chain variable fragment
SciLifeLab	Science for Life Laboratory
SCRAN	Single Cell Rna-seq ANalysis
SD	Standard deviation
SEB	Staphylococcal enterotoxin B
SEC	Size exclusion chromatography
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGC	Structural Genomics Consortium
SLAMF1	Signaling lymphocytic activation molecule family 1
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SNP2HLA	Imputation of Amino Acid Polymorphisms in Human Leukocyte Antigens
SPRTN	SprT-like N-terminal domain
SRP	Single recognition particle

SRQ	Swedish Rheumatology Quality Register
SS	Sjögren's syndrome
SSc	Systemic sclerosis
STAT	Signal transducer and activator of transcription
STAR	Spliced Transcripts Allignment to a Reference
SUMO-1	Small ubiquitin-like modifier-1
SweMyoNet	Swedish Myositis Network
T1DGC	Type 1 Diabetes Genetics Consortium
TBS	Tris-buffer saline
TCR	T cell receptor
TGF	Transforming growth factor
T_{FH} cells	T follicular helper cells
T_H cells	T helper cells
T_{RM}	Tissue-resident memory T cells
TIF1γ/α	Transcription intermediary factor 1γ/α
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
TRAF6	TNF receptor associated factor 6.
tRNA	Transfer RNA
Tregs	T regulatory cells
TRIM21	Tripartite motif-containing protein 21
t-SNE	t-Distributed stochastic neighbour embedding
TWEAK	TNF-related weak inducer of apoptosis
U1RNP	U1 small nuclear protein
UBE2L4	Ubiquitin conjugating enzyme E2 L4
UCSC	University of California Santa Cruz
UMAP	Uniform manifold approximation and projection
VAS	Visual analogue scale
WHEP	WRS HisRS EPRS domains
WB	Western blot
WRS	Tryptophanyl-tRNA synthetase
YPS/HA	Tyrosyl-tRNA synthetase
ZFAT	Zinc finger and AT-hook domain containing
ZO	Phenylalanyl-tRNA synthetase

1 INTRODUCTION

A critical role of the functioning immune system is the ability to discriminate self from non-self [1]. Essentially, the immune system has evolved to deal with the endogenous and exogenous challenges in our environment by successfully destroying cells infected by pathogens while protecting the integrity of the host [2, 3]. This protective system safeguards our biological identity and offers protection against a wide diversity of pathogens while maintaining tolerance to self [4]. The immune system relies on self-tolerance, which is the adequate homeostatic balance between the strong response needed when combating foreign pathogens while avoiding eliciting destructive anti-self-responses [5]. On the contrary, the breakdown of such physiological tolerance mechanisms, characterized by an inability to adapt to the constant presence of an antigen and further react by attacking autologous tissue, is what we call autoimmunity [3].

Several doctrines consider that “physiological autoimmunity” could play a possible regulatory role or a compensatory phenomenon, such as the “theory of immunological clearance”, “the theory of autoimmune regulation of cellular functions”, and the “doctrine of cytotoxins” [6-9]. Nevertheless, the transition from “physiological” autoimmune response to autoimmune disease (AD) is more nuanced. It can range from a “low level” of autoreactivity required for lymphocyte selection and immunological homeostasis; to an “intermediate level” expressed by the presence of autoantibodies and tissue infiltrates not associated with clinical manifestations, and finally to a “pathogenic autoimmunity” present for example, in autoimmune diseases [10]. In this last stage, there is a clear immune-mediated association with injury or dysfunction orchestrated by signals from the targeted host tissue [11].

The standard definition of autoimmune disease includes the presence of dysregulation of the immune system leading to loss of tolerance to self-antigens, immune cell stimulation with the appearance of autoantibodies and/or autoreactive T cells, inflammation, and tissue injury [12]. The transition from physiological to pathogenic autoimmunity is often multifactorial, suggesting that multiple pathways could be involved in breaking the tolerance and the development of AD [13]. Indeed, the spectrum, severity, and duration of ADs result from complex interactions between genetic, hormonal, environmental, and stochastic factors with the contribution of both humoral and cellular immune responses [14]. This combination of factors involved in the induction and/or exacerbation of autoimmune conditions was referred to as the “Mosaic of autoimmunity” in 1989, which may explain the high clinical heterogeneity of AD even within the same group of patients, and in 1991 the “kaleidoscope of autoimmunity” also denoted the possibility that more than one disease may coexist in the same individual or family [15, 16].

In the last three decades, more attention has been focused on the geoepidemiology of AD since they are associated with a significant cause of morbidity and mortality and high healthcare costs, comparable to those of cancer and heart disease [17-21]. So far, more than 80 autoimmune diseases have been identified, with an increasing incidence over the years in different countries ranging from 5-30%, a cumulative prevalence of 5%, and a higher frequency of autoimmunity in females (7.1% for females and 3% for males) [19, 22]. Depending on the extent of the clinical and pathological manifestations, AD can be classified as systemic or organ-specific. In systemic AD, the targets are self-antigens that are ubiquitously expressed. On the contrary, in organ-specific AD, the targets are commonly locally-expressed self-antigens. In both scenarios the injury can be mediated by autoantibodies and/or T cells [11].

In clinical practice, the detection of serum autoantibodies plays a central role in diagnosing and classifying AD. However, the pathogenicity of many of the autoantibodies has been a matter of debate and further research, since some autoantibodies can also be found in cancer, tissue damage, and healthy subjects, indicating that the presence of autoantibodies is not specific to AD [23]. The complexity of AD thus requires research from different areas such as immunology, allergy, and rheumatology, among others, to interpret the transition of the immune system from a physiological to a pathological state [6].

This Ph.D. thesis focuses on a group of systemic AD denominated “idiopathic inflammatory myopathies” (IIM) with emphasis on the identification of new T cell self-epitopes, characterization of the T cell repertoire at the site of the affected organ or tissue (muscle, lung, and blood) and the clinical relevance of a newly identified “muscle-specific” autoantibody.

2 LITERATURE REVIEW

2.1 IDIOPATHIC INFLAMMATORY MYOPATHIES

The idiopathic inflammatory myopathies (IIM, systemic autoimmune myopathies, or myositis) are rare chronic autoimmune disorders that possess a large spectrum of clinical phenotypes [24-26]. Despite the name, IIM are characterized by a systemic immune response that can affect not only the skeletal muscle but also a large variety of extra-muscular organs. The involvement of joints, skin, heart, gastrointestinal tract, and/or the lung denote the complex nature of this disease [27]. Still, the exact mechanism of the autoimmune response in this disease is not fully understood and diagnosing this group of patients can be particularly challenging.

For the accurate diagnosis of IIM, it is essential to combine the clinical symptoms and signs, histopathological findings upon muscle biopsy, MRI pattern, serum levels of muscle enzymes, and autoantibody profiles [28, 29]. Currently, IIM are classified into different subgroups: polymyositis (PM), dermatomyositis (DM, including amyopathic dermatomyositis), juvenile dermatomyositis (JDM), anti-synthetase syndrome (ASyS), immune-mediated necrotizing myopathy (IMNM), inclusion body myositis (IBM) and overlap myositis (OM) [29-38]. PM is one of the most controversial subgroups, which initially included a heterogeneous and large IIM subset. Still, the identification of new autoantibodies has narrowed down the spectrum of this phenotype [39].

Patients with IIM can have varying degrees of symmetric, proximal muscle weakness, myalgia, and low muscle endurance. These symptoms can gradually worsen over time [40]. In DM, typical skin rashes can help distinguish from other myositides [41]. Additionally, IIM may manifest with extra-muscular symptoms such as dysphonia, dysphagia, arthritis, cardiomyopathy, interstitial lung disease (ILD), Raynaud's phenomenon and constitutional symptoms such as fever, weight loss, and fatigue [42]. In some patients, the extra-muscular manifestations can dominate the symptomatology with low or even absent muscle weakness, which may represent a diagnostic challenge [29]. The differential diagnosis of muscle disease is extensive and should be considered throughout the evaluation, including muscular dystrophies, neuromuscular disorders, metabolic and endocrine myopathies, drug-induced myopathies, and infections [43].

2.2 EPIDEMIOLOGY

Although IIM have a low incidence (11/1 000 000 cases per person/year) and prevalence (14 cases per 100 000 habitants), a systematic review and a register-based study showed an increase in IIM cases between 1966 and 2013 [44, 45]. This increment might reflect a

more significant recognition of this entity and better management of the registers over time. The peak age range for IIM disease onset is 5-15 years in children and 45-60 years in adults, and it is more frequent in women (ratio 2:1), except for the subgroup IBM, which is more frequent in men (3:2) [46, 47].

The mortality rates may vary according to the population studied, with a reported 10-year survival rate between 20%-90% [48-52]. A subsequent study reported that the highest mortality is present in the adult form within the first year of diagnosis and is associated with pulmonary or circulatory diseases, and malignancies [46]. In particular, interstitial lung disease (ILD) represents major cause of mortality and disability in patients with IIM [53].

2.3 RISK FACTORS

The pathogenic mechanisms identified so far are related to specific interactions between environmental and genetic risk factors. However, despite the advances in immunological, genetic and clinical studies, the details of these associations and the drivers of subsequent immune activation remain poorly understood.

2.3.1 Environmental risk factors

Although the association of environmental factors is complex and entails a high variability between the subgroup of patients with IIM, some broad commonalities exist. This list includes a variety of factors such as seasonal and geographic clustering and a history of exposure to infectious and non-infectious agents (e.g., smoking, ultraviolet light exposure) [54-56]. In particular, the history of smoking was associated with PM (OR 2.24), ASyS (OR 1.93), and the development of autoantibodies such as anti-Jo-1 (OR 1.94) [55]. An increased risk was found with the presence of heavy smoking and the *HLA-DRB1*03:01* allele in Caucasian population [55]. However, recent *HLA* association studies have not been able to replicate this finding [57]. The lack of significant interaction between smoking and *HLA DRB1*03:01* when modelling the risk for anti-Jo-1 autoantibodies could be potentially to the lack of power [58], and requires further research.

In 2017, a nationwide population-based case-control study reported associations between IIM and respiratory and gastrointestinal tract infections, where individuals with a history of infection had an increased risk of developing IIM by 50% [54]. Several infectious agents, such as viruses, bacteria, and parasites, have been implicated in the pathogenesis of IIM based on clinical observation and animal models [59]. Some examples include the co-occurrence of cytomegalovirus in PM [60, 61], hepatitis B virus in PM and DM [62, 63], hepatitis C virus in IBM [64], Epstein-Barr virus in DM and JDM [65, 66], HIV and human T-lymphotropic virus-1 in PM, DM and IBM [67-70]; influenza, picornavirus and echovirus in PM, DM and JDM [71]. However, no evidence has been confirmed for a causal relationship between infections and IIM despite these indications [71].

2.3.2 Genetic risk factors

In the last decades, there have been considerable efforts between multiple centres to study the complexity of genetics in autoimmunity, including IIM. The strongest disease association with IIM, in terms of odds ratio, was identified within the human leukocyte antigen (*HLA*) region on chromosome 6, according to two genome-wide association studies in adult and juvenile DM and PM [72, 73]. The documented non-*HLA* loci associated with IIM reported have been *PTPN22*, *STAT4*, *TRAF6*, *BLK*, and *UBE2L4* [74-76]. Recently, a strong association was reported for a decrease in *C4A* copy number among patients with IIM [77]. Furthermore, a recent gene-based aggregate testing identified the *PRDX3*, *SLAMF1*, *ZFAT,* and *PTPN6* genes as potential risk factors involved in the development of muscle weakness and damage through different mechanisms such as autophagy, Toll-like receptor signalling and oxidative stress [78-81].

***HLA* loci associated with IIM.** The Myositis Genetics Consortium (MYOGEN) demonstrated an association with alleles from the *HLA* 8.1 ancestral haplotype *HLA-DRB1*03:01* and *HLA-B*08:01* for DM and PM in the Caucasian population [75]. Regarding the autoantibody subgroups, this ancestral haplotype association was identified for the anti-histidyl-tRNA synthetase (anti-Jo-1), anti-Pm-Scl and anti-cN1A in IIM [82].

Further conditional analysis within the study indicated that multiple variants of this haplotype could independently contribute to the risk of the IIM subgroups. Different haplotypes have been associated with IIM in other ethnic groups, such as *HLA-DRB1*08:03* in Japanese and *HLA-DQA1*01:04*, *HLA-DRB1*07*, *HLA-DRB1*09:01*, *HLA-DRB1*12:01* in Chinese populations [83-85].

Other two genetic studies identified a strong association between *HLA-DRB1*03:01*, and the presence of anti-Jo-1 autoantibody, suggesting a greater genetic involvement in a subset of IIM, which in turn is driven by adaptive immunity [86, 87]. An independent study of 2566 Caucasian IIM cases indicates that the association of IIM with *HLA-DRB1*03:01* can be explained by the presence of asparagine at position 77 and arginine (Arg) at position 74 [75]. Of note, Arg-74 of HLA-DRB1 lies within the peptide-binding groove, potentially influencing the antigen recognition and presentation of auto-antigenic peptides to the immune system.

Despite significant advances in the identifications of genetic risk variants for autoimmune diseases obtained from genome-wide association studies (GWAS) and genome sequencing studies [88], the challenge remains for translational genomics. For instance, to understand the mechanism of presentation of autoreactive self-epitopes and the production of specific autoantibodies in IIM. Variations across different ethnic groups have been reported in the

HLA alleles associated with autoantibody phenotype [82, 89-91] (**Table 1**). These differences could account for a smaller cohort size and a lack of autoantibody harmonization testing [92]. New insights into the relationship between *HLA* associations with autoantibody-defined subgroups will be discussed in detail in the results of **Paper III** in **Chapter 5** and in the discussion in **Chapter 6**.

TABLE 1. MSA Associations with *HLA* alleles [92, 93]

MSA	*HLA-DR*	*HLA-DQ*	Population
Anti-Jo-1	*DRB1*03:01*		Caucasian, African-American
Anti-Mi2	*DRB1*07:01*	*DQA1*02*	Caucasian, African-American
	*DRB1*03:02*	*DQB1*02*	
Anti-MDA5	*DRB1*01:01*		Japanese
	*DRB1*04:05*		
	*DRB1*12:02*		Korean
Anti-TIF1γ		*DQA1*03*	Caucasian
		DQB1	
Anti-SAE	*DRB1*04*	*DQA1*03*	Caucasian
		*DQB1*03*	
Anti-HMGCR	*DRB1*11:01*		European American and African-American
	*DRB1*07:01*		Juvenile myositis
Anti-SRP		*DQA1*01:04*	European American and African-American
		*DQA1*01:02*	

Legend: Mi2, nucleosome-remodelling deacetylase complex; MDA5, melanoma differentiation-associated gene 5; TIF1γ, transcription intermediary factor 1; SAE, small ubiquitin-like modifier activating enzyme; HMGCR, hydroxy-3-methylglutaryl-CoA reductase; SRP, signal recognition particle.

Heritability. Due to the rarity of IIM, there are few reports of heritability and familiar occurrence [94, 95]. The heritability estimates based on GWAS reported a phenotypic variance for PM of 8.3% and 5.5% for DM [76]. However, a recent nationwide family-based study in Sweden reported an overall heritability for IIM among any first-degree relative of 22% (CI 12%-31%) and an OR of 3.99 [96]. The discrepancies among the studies can be due to an increased number of patients with IIM in the latter, the heterogeneity of IIM and that an SNP-based method could underestimate heritability when the disease is rare, suggesting the involvement of rare genetic variants [97]. Still, this report shows that IIM has a lower risk of familial aggregation than other autoimmune diseases such as rheumatoid arthritis (RA) or coeliac disease, indicating a stronger role of the environmental risk factors [98, 99].

Rare genetic variation. The presence of rare genetic variants, defined as a minor allele frequency <1%, has recently been addressed in a large Scandinavian myositis cohort, using targeted DNA sequencing to analyse the contribution of rare and common genetic variation in 454 IIM cases [78]. Among the relevant findings was the identification of a significant novel genetic risk locus for DM, the interferon-induced protein 35 (*IFI35*), induced by type I IFN and associated with the regulation of the innate immune response [100]. A possible regulatory effect was also found with an expression quantitative trait locus (eQTL) of the skeletal muscle-specific gene prostaglandin E synthase 3 like (*PTGES3L*). Additionally, aggregate genetic associations to the proteasomal genes *PSMB8/9* and advanced glycosylation end-product specific receptor (*AGER*) were identified in ASyS patients. Together, these findings support the notion of a contribution of rare genetic variations to disease susceptibility in IIM.

2.4 PATHWAYS IMPLICATED IN IIM PATHOGENESIS

2.4.1 Immune-mediated and non-immune-mediated disease mechanisms

The precise mechanisms that initiate and maintain the chronic inflammation of IIM are still unknown. Several studies have highlighted the importance of the adaptive and innate immune system in the chronic response to self-antigens and inflammation. The autoimmune origin of IIM is supported by evidence of T cell-mediated myocytotoxicity, the presence of autoantibodies, overexpression of the human leukocyte antigen (*HLA*) type I (and occasionally type II) on muscle fibres, and the strong association with certain *HLA-DR* genotypes [56, 101, 102].

Although there is a general pattern of self-immunity, few specific antigens have been found, and the diversity of the clinical phenotypes suggests that distinctive molecular pathways may be involved in different subgroups of patients with IIM [103]. Non-immune mechanisms, such as 1) endoplasmic reticulum stress; 2) autophagy; 3) hypoxia; 4) metabolic and mitochondrial dysfunction; 5) upregulation of TRAIL; 6) expression of heat-shock proteins (HSP70 and HSP90), and 7) HMGB1 are also likely to be involved in the pathophysiology of IIM [102, 104-107]. Major details involving the aforementioned non-immune mechanisms are beyond the purpose of this summary and will not be discussed.

2.4.2 Innate immune cells and inflammatory mediators

Myeloid and plasmacytoid dendritic cells and M1 and M2 macrophages have been identified in the muscle inflammatory infiltrate [108]. These antigen-presenting cells and immature muscle cell precursors play a central role in the inflammatory process. This mechanism is suggested through the up-regulation of HLA class I and II, cytokine secretion by myoblasts, activation of the toll-like receptor (TLR) pathway and inhibition of myotube differentiation with

regeneration impairment [109, 110]. Activation of TLR can lead to NF-kB signalling and pro-inflammatory cytokine and chemokine secretion. Inflammatory cells and cytokine expression in the muscles are associated with histopathological changes in muscle fibre atrophy, necrosis and regeneration [102]. In recent years, the expression of NLRP3 inflammasome in muscles has been shown in patients with DM and PM, activation of caspase-1 and secretion of IL-1β and IL-18 [111]. The latter cytokines have also been associated with the initiation and progression of PM and DM [112-114].

Additional cytokines have been identified with a varying frequency in muscle biopsies from patients with IIM, such as tumour necrosis factor (TNF), interferon-gamma (IFNγ), IL-12 and IL-2 (T helper cell type 1 cytokines); IL-4 and IL-13 (T helper cell type 2 cytokines); IL-17, IL-22, IL-23, TNF-related weak inducer of apoptosis (TWEAK) and IL-6 (Th-17 cytokines); IL-10 and transforming growth factor-β (TGFβ) (Treg cytokines); IL-1α, IL-1β and type I interferons (IFNα and IFNβ) (innate immune cytokines) [59]. Likewise, CXCL9, CXCL10, CCL2, CCL3, CCL4, CCL9, and CCL21 have been identified in muscle tissue of patients with IIM, suggesting a local sustaining of the inflammatory response [115].

2.4.3 Adaptative immune response

T cells in IIM. T lymphocytes are likely to play a central role in idiopathic inflammatory myopathies (IIM) since they are found in the skin, muscle tissue and bronchoalveolar lavage fluid (BALF) of affected patients with PM, DM, IBM or ASyS [116-119]. $CD4^+$ and $CD8^+$ T cells may be present in the muscle of patients with DM, PM, and IBM, but the T cell subset and localization differ among the subgroups [120, 121]. In 1990 the gamma-delta (γδ) T cell population was found in muscles of patients with PM; subsequently, it was described that these cells could recognize aminoacyl-tRNA synthetases [122, 123].

The mechanism of how T cells infiltrate the muscle remains elusive, and their role in the pathogenesis of the disease remains to be clarified. This has led to the question of whether the presence of T cells in different tissues of patients with myositis results from a specific response or a bystander effect. Nevertheless, the presence of lymphocytes expressing restricted T cell receptor (TCR) families such as Vα2 and Vβ3 in muscle, together with the identification of selective TCR-V gene usage in muscle and lung tissue, suggest the presence of clones capable of recognizing autoantigens and possibly participating in the pathophysiology of the disease [119, 124].

So far, few studies have identified potential T cell epitopes and looked at antigen-specific responses [125, 126]. Still, it has been established that the presence of T cells and specific T cell subsets, such as $CD28^{null}$ T cells of $CD4^+$ and $CD8^+$ phenotype contribute to the

pathogenesis of the disease [116]. In particular, proinflammatory, highly cytotoxic and apoptosis-resistant T cells such as $CD28^{null}$ T cells ($CD4^+$ or $CD8^+$) are associated with poor outcomes [117]. New insights from microarray data showed that patients with IBM had a particular muscle signature of T cell cytotoxicity [127]. These cytotoxic and highly differentiated T cells were $CD8^+$ and killer cell lectin-like receptor G1 (KLRG1) positive.

Due to the myocytotoxic effect, granzymes and perforin are of particular interest in T cells and their role in IIM. In particular, granzyme B has been associated with a putative role in creating neo-epitopes upon specific cleavage of autoantigens [128]. Furthermore, it is documented that cytotoxic $CD8^+$ T cells expressing granzyme B and perforin are often present in the endomysial space surrounding the muscle fibres in patients with PM and IBM [120, 128, 129].

Despite decades of research in human and animal models, the antigen specificity of T cells in IIM is unknown. The development of MHC-II tetramer for the detection of antigen-specific $CD4^+$ T cells and T cell receptor (TCR) analysis will allow us to answer a current knowledge gap in myositis. These studies are currently under performance and will be described more in detail in the material and methods in **Chapter 4**.

B cells in IIM. B cells, plasma cells, and immunoglobulin transcripts in muscle biopsies of patients with DM, PM, and IBM have been described in the literature [130-132]. In particular, muscle-infiltrating B cells exhibited memory and plasma B cell phenotype, with evidence of selection, clonal diversification and somatic hypermutation of their B cell receptors [130, 133, 134]. Recently, a group analysed the presence and characteristics of anti-Jo-1 binding B cells, revealing an enrichment for the autoimmune-prone $CD21^{lo}$ B cell phenotype, which are reported to have different functional properties of anergic, recent activated or memory B cells [135].

Besides, the recent finding of germinal centre-like structures in the lung of patients with ILD/ASyS or in the muscles of patients with IIM might suggest a local production of autoantibodies in the muscles or the lungs of some patients [125]. The presence of autoantibodies can be identified in at least half of the IIM population [93]. Nonetheless, the pathogenic role of autoantibodies causing organ damage is still controversial since most of the targeted antigens are intracellular.

Although the relationship between cause and effect is still unclear, the clinical associations of the autoantibodies with specific disease phenotypes, disease severity, organ damage and response to treatment may indicate a role in the pathogenesis of subgroups with the

respective autoantibodies [136]. State of the art regarding the possible pathogenic role of anti-Jo-1 autoantibodies will be discussed in the specific background of the paper.

2.5 CLASSIFICATION CRITERIA

Many classification criteria have been proposed to define homogeneous disease groups, allowing better diagnosis and research possibilities [24]. In 1975, Bohan and Peter proposed a set of criteria for diagnosing and classifying PM and DM, but IBM was not included (**Table 2**) [137, 138]. These criteria include clinical (progressive and symmetrical proximal muscle weakness) and skin manifestations, laboratory, electrodiagnostic and histopathological (evidence of inflammation on muscle biopsy) features.

Table 2. Bohan and Peter's diagnostic criteria for PM and DM [137, 138]

First, consider other forms of myopathies as a differential diagnosis.
1. Symmetrical proximal muscle weakness.
2. Skeletal muscle enzyme elevation: CK, LDH, aldolase, transaminase
3. Abnormal electromyography:
a) Polyphasic, short, small motor-unit potentials.
b) Fibrillation, positive sharp waves, increased insertional irritability.
c) Bizarre, high-frequency, repetitive discharges.
4. Muscle biopsy abnormalities
5. Typical skin rash of DM: Gottron sign, heliotrope rash
Possible PM: two of the first four criteria.
Possible DM: criterion five (skin rash) plus any two criteria.
Probable PM: any three of the first four criteria.
Probable DM: criterion five (skin rash) plus any three of the first four criteria.
Definite PM: four of the first four criteria.
Definite DM: criterion five (skin rash) plus all four other criteria.

Legend: EMG: electromyography; CK, creatine kinase; LDH, lactate dehydrogenase.

IBM was incorporated decades later in the Dalakas diagnostic criteria of 1991 [139]. In 1995, a clinical-pathological expert-based criteria for IBM were postulated as the Griggs criteria [140], followed by the European Neuromuscular centre (ENMC) in 2000, the Medical Research Council (MRC) in 2010, the Institute of Myology (IM) in 2010 and the ENMC 2013. A machine learning approach was used to analyse IBM diagnosis categories and construct unbiased diagnostic criteria, identifying that from 24 IBM diagnostic categories proposed since 1987, only 12 had a sensitivity of >95% but with variable specificities between 11%-84% [141].

Among the low-performance items were 1) the use of pathologic features not performed frequently in clinical practice and 2) the comparative strength criteria concerning knee extension and hip flexion. The presence of antibodies targeting cytosolic 5'-nucleotidase 1A (cN1A) could help diagnose this subgroup since their frequency has been reported between 33%-72% of patients with IBM in different cohorts [142-144]. Nevertheless, the anti-cN1A autoantibody is not considered in the clinicopathological IBM ENMC 2013 since the autoantibody was characterized after the criteria were published.

The need for a better discrimination tool between IIM and mimicking conditions, together with a more sensitive identification of subgroups without manifest muscle symptoms, led to the development of the 2017 EULAR/ACR classification criteria for adult and juvenile IIM and their major subgroups [145, 146]. This classification uses two probability-score models (with and without muscle biopsy data) and determines the probability of IIM and four major subgroups: PM, (J)DM, IBM and amyopathic DM. These classification criteria contain 16 variables from six categories, including the presence of anti-Jo-1 autoantibody. The International Myositis Classification Criteria Project (IMCCP) proposes a cut-off of at least 55%, which had the best sensitivity (87-93%) and specificity (82-88%). According to the recommendations of the steering committee, a "probable IIM" corresponds to a probability $\geq$55% and <90% and "definite IIM" to a probability $\geq$90% [145]. (**Table 3**).

2.6 AUTOANTIBODIES IN IIM

The identification of the autoantibodies in the diagnostic approach to autoimmune diseases has been fundamental in the last decades [147]. Their relevance in autoimmunity can be broad, as seen in the case of antinuclear antibodies (ANAs) and systemic autoimmunity; or organ-specific, as in anti-glomerular basement membrane autoantibody in anti-GBM disease (also known as Goodpasture syndrome), anti-myelin basic protein in multiple sclerosis, etc. [148, 149]. It is important to mention that the association and clinical relevance with specific autoimmune diseases require confirmation of antigen specificity. Additionally, understanding the physiologic role of the autoantibodies and their mechanism of action could enlighten the scientific community in developing new therapeutic approaches.

The classification and subtyping of IIM have considerably evolved in the last decades due to myositis-specific autoantibodies (MSA) identification (**Figure 1**).

Table 3. EULAR/ACR classification criteria for IIM [145]

Variable	Score	
	Muscle biopsy	
	–	+
Age of onset of first symptoms assumed to be related to disease ≥18 and ≤40 years	1.3	1.5
Age of onset of first symptoms assumed to be related to disease ≥40 years	2.1	2.2
Muscle weakness:		
Objective symmetric weakness, usually progressive, proximal upper extremities	0.7	0.7
Objective symmetric weakness, usually progressive, proximal lower extremities	0.8	0.5
Neck flexors are relative weaker than neck extensors	1.9	1.6
In the legs, proximal muscles are relatively weaker than distal muscles	0.9	1.2
Skin manifestation:		
Heliotrope rash	3.1	3.2
Gatton's papules	2.1	2.7
Gorton's sign	3.3	3.7
Other clinical manifestation:		
Dysphagia or oesophageal dysmotility	0.7	0.6
Laboratory measurements:		
Presence of anti-Jo-1 autoantibody	3.9	3.8
Elevated serum levels of CK or LDH or ASAT/AST/SGOT or ALAT/ALT/SGPT	1.3	1.4
Muscle biopsy features - presence of:		
Endomysial infiltration of mononuclear cell surrounding but not invading myofibres		1.7
Perimysial and/or perivascular infiltration of mononuclear cells		1.2
Perifascicular atrophy		1.9
Rimmed vacuoles		3.1

Legend: CK, creatine kinase; LDH, lactate dehydrogenase; ASAT/AST/SGOT, aspartate aminotransferase; ALAT/ALT/SGPT, alanine aminotransferase. If biopsies are included, a cut-off of at least 55% corresponds to a sore of ≥5.5 or ≥6.7. A "definite IIM" corresponds to a total aggregate score of ≥7.5 without muscle biopsy and ≥8.7 with muscle biopsy.

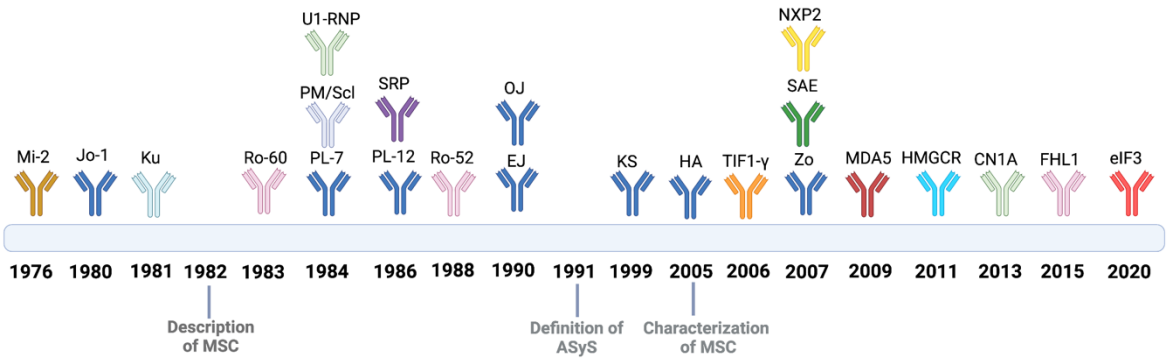


Figure 1. Timeline of the discovery of MSA and MAA. Multi-tRNA synthetase complex (MSC); ASyS: anti-synthetase syndrome. Created with Biorender.com

The MSAs have contributed not only to the diagnosis of IIM but also to giving information regarding their subtype and prognosis of the disease [150]. The presence of myositis-specific (MSA) or associated (MAA) autoantibodies has been reported in 50-70% of children and adults with IIM [93, 151]. Their discovery has improved the diagnosis and treatment, allowing correlations with distinct clinical phenotypes [152-155]. Until now, there are about 16 known MSA, and previous studies have reported that more than one MSA rarely coexist in the same patient [29, 156, 157]. The explanation for this monospecificity is not understood. The MSA include the following antigens: aminoacyl-tRNA synthetases (ARS), signal recognition particle (SRP), transcription intermediary factor 1γ/α (TIF1γ/α, p155/140, TRIM33), melanoma differentiation-associated protein 5 (MDA5), nucleosome remodelling deacetylase complex (Mi-2), nuclear matrix protein 2 (NXP2), Small ubiquitin-like modifier activating enzyme (SAE), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), and anti-eIF3 [93]. The frequency and IIM subgroups associated with the MSA autoantibodies are presented in **Table 4**.

On the contrary, the MAA are not specific since they can be present in other connective tissue diseases but may be found in IIM such as exosome complex 75kDA/100kDa (PM-Scl), Ku complex 70kDA/80kDa (Ku), small ribonucleoprotein (U1-RNP), Ro52 (TRIM21), and cytosolic 5´-nucleotidase 1A (cN1A). The frequency of the MAA autoantibodies and their association to IIM subgroups and other rheumatological diseases are presented in **Table 5.** Recent discoveries regarding the possible pathogenic role of a new muscle-specific autoantibody, four-and-a-half-LIM domain protein-1 (FHL1), will be discussed in 2.10.2 Specific background of Papers II and V and in **Chapter 3**.

Table 4. Myositis-specific autoantibodies (MSA)

Autoantibody	Autoantigen	Prevalence in IIM	IIM subgroup
Anti-Jo-1	Histidyl-tRNAs*	15-30%	ASyS
Anti-PL-7	Threonyl-tRNAs*	5-15%	ASyS
Anti-PL-12	Alanyl-tRNAs*	5-10%	ASyS
Anti-EJ	Glycyl-tRNAs*	<5%	ASyS
Anti-OJ	Isoleucyl-tRNAs*	<5%	ASyS
Anti-KS	Asparaginyl-tRNAs*	1-8%	ASyS
Anti-Zo	Phenylalanyl-tRNAs*	<1%	ASyS
Anti-YRS/HA	Tyrosyl-tRNAs*	<1%	ASyS
Anti-Mi2	Nucleosome remodelling deacetylase complex	4-20%	DM
Anti-SRP	Signal recognition particle	5-15%	IMNM
Anti-MDA5	Melanoma differentiation-associated protein 5	13-30%	DM, CADM
Anti-SAE	Small ubiquitin-like modifier activating enzyme	<10%	DM, CADM
Anti-NXP2	Nuclear matrix protein 2	3-24%	DM, CADM
Anti-TIF1γ	Transcriptional intermediary factor 1, TRIM33	10-20%	DM, JM
Anti-HMGCR	3-hydroxy-3-methylglutaryl CoA reductase	6-10%	IMNM
Anti-eIF3	Eukaryotic initiation factor 3	<1%	PM

Legend: ASyS: anti-synthetase syndrome; DM; dermatomyositis; CADM, clinically amyopathic dermatomyositis; IMNM, immune-mediated necrotizing myopathy; JM; juvenile myositis; PM, polymyositis. *tRNA synthetase.

Table 5. Myositis-associated autoantibodies (MAA)

Autoantibody	Autoantigen	Prevalence in IIM	IIM subgroup
Anti-Ro52	E3 ubiquitin-protein ligase TRIM21	10-40%	PM, ASyS, often second antibody present
Anti-Ro60	RNA-binding protein Ro60	6-10%	PM, ASyS
Anti-PM-Scl	Human exosome protein complex	8-10%	PM, DM, overlap with scleroderma
Anti-U1RNP	U1 small nuclear RNP	10%	MCTD
Anti-Ku	Regulatory subunit of DNA dependent protein kinase	<2%	ILD, overlap with scleroderma
Anti-CN1A	Cytosolic 5′-nucleotidase 1A	4-21%	IBM, pSS, SLE
Anti-FHL1	Four-and-a-half-LIM domain 1	14-27%	PM, DM, IBM, ASyS, IMNM

Legend: RNP: ribonucleoprotein, MCTD; mixed connective tissue disease; ILD: interstitial lung disease; IBM: inclusion body myositis, PM, polymyositis; DM; dermatomyositis; ASyS: anti-synthetase syndrome; IMNM; immune-mediated necrotizing myopathy; pSS; primary Sjögren's syndrome; SLE: systemic lupus erythematosus.

2.7 CLINICAL AND SEROLOGICAL PROFILE OF IIM SUBGROUPS

From a clinical perspective, the identification of autoantibodies has played a central role in diagnosing and classifying patients with IIM. A subgrouping based on this approach has led to a more homogeneous classification characterized by common clinical, serological and histopathological features. This approach allows a better stratification of patients and assists the physician in selecting treatment and monitoring the disease and prognosis. I will discuss the pathogenesis of the major subgroups of IIM: PM, DM, IBM, IMNM, OM and ASyS. (**Figure 2**).

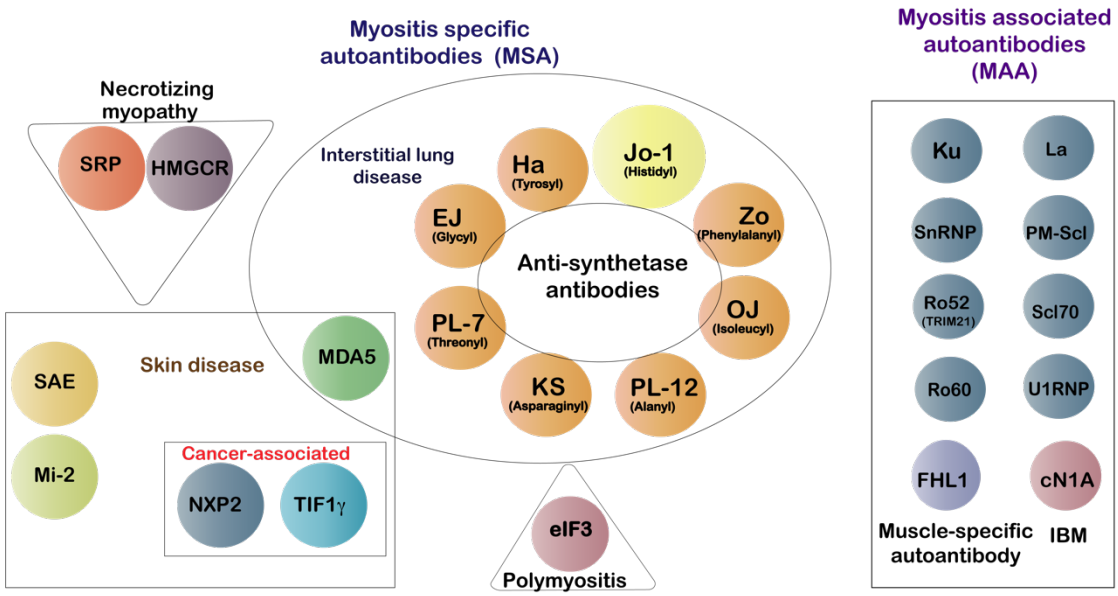


Figure 2. Clinical subgroups of IIM are associated with MSA and MAA. The identification of myositis-specific and myositis-associated autoantibodies has allowed the classification of the myositis subgroups into homogeneous clinical phenotypes. Jo-1, histidyl-tRNA synthetase; OJ, isoleucyl-tRNA synthetase; PL-12, alanyl-tRNA synthetase; PL-7, threonyl-tRNA synthetase; KS, asparaginyl-tRNA synthetase; Zo, phenylalanyl-tRNA synthetase, EJ, glycyl-tRNA synthetase; Ha/YRS, tyrosyl-tRNA synthetase; MDA5, melanoma differentiation-associated gene 5; Mi-2, nucleosome-remodeling deacetylase complex; NXP-2, nuclear matrix protein-2; HMGCR, hydroxy-3-methylglutaryl-CoA reductase; SRP, signal recognition particle; TIF1γ, transcription intermediary factor 1; Pm-Scl, polymyositis/systemic sclerosis overlap syndrome; SnRNP, small nuclear ribonucleic protein; U1RNP, U1 small nuclear RNP; cN1A, Cytosolic 5′-nucleotidase 1A; FHL1, Four-and-a-half-LIM domain 1.

2.7.1 Polymyositis

The frequency of PM has varied largely upon the discovery of new MSA and validations of classification criteria and is now considered a rare form of IIM [39]. Currently, PM is regarded as an inflammatory muscle disease, without skin association, that involves proximal muscles and the presence of endomysial infiltrates of $CD4^+$ and $CD8^+$ T cells in the muscle biopsy [29]. Most patients previously identified as PM have been re-classified as part of the ASyS, IBM, IMNM or OM. So far, one autoantibody has been recently described in Caucasian patients with PM, the anti-eukaryotic initiation factor 3 (eIF3) autoantibody. Anti-eIF3 was

reported as a good prognostic marker since it was associated with a favourable response to treatment [158]. Still, a high percentage of the MSA-negative group falls into the PM definition, indicating the need for further studies in this group [159].

2.7.2 Dermatomyositis

DM is the most common subtype of IIM, with a frequency of 30-40% [160]. DM can occur in children (JDM) with an average age of onset of 7 years old and adults, with the highest incidence between the 4^{th}-6^{th} decade [161]. The presence of skin disease characterizes this subtype. Several characteristic skin lesions have been documented, such as skin rashes on the eyelids (heliotrope sign), over the neck, shoulders (shawl sign), upper chest (V sign), hips (Holster), papules or erythema over the surface of the finger joints, elbows, and knees (Gottron's papules or sign) [41]. Furthermore, the nail folds, eyelids and gums can show evidence of small-vessel inflammation as telangiectasias of the capillary loops [162].

Besides the proximal muscle weakness and elevation of muscle enzymes, the clinical presentation can involve respiratory, digestive and circulatory system damage. The histopathological characteristics suggest the possibility of immune-mediated vascular damage and include the presence of perifascicular myofibre abnormalities, including atrophy and necrosis; reduced number and size of endomysial capillaries and positive staining for activated membrane attack complex (MAC) in endothelial cells [163]. The cellular infiltrate consists mainly of B cells, macrophages, dendritic cells and $CD4^+$ T cells [160].

Serologically, this subgroup is characterized by the presence of the following MSA: anti-Mi-2, anti-TIF1γ, anti-NXP2, anti-MDA5 and anti-SAE, which can be found in approximately 60% of the patients [29]. Patients with DM and anti-Mi-2 autoantibodies usually have a good prognosis with a favourable disease course characterized by classical DM skin manifestations and mild muscle inflammation. For some MSA such as anti-TIF1γ and anti-NXP2, the clinical manifestations differ depending if there is a juvenile or adult DM form. In JDM, anti-TIF1γ, anti-MDA5 and anti-NXP2 autoantibodies are the most common and strongly associated with cutaneous involvement and not with malignancy [164]. Calcinosis can be present in both groups of JDM and DM and is frequently associated with anti-NXP2 autoantibodies [165].

On the contrary, in the adult form of DM, one important feature found in several population-based studies is the association with cancer (cancer-associated DM) [166, 167]. Up to 50% of patients with anti-TIF1γ autoantibodies developed cancer within three years of myositis onset. A longitudinal study demonstrated that the autoantibody levels declined after a successful response to cancer therapy [168, 169].

A particular group corresponding to patients with characteristic cutaneous disease in the absence of muscular involvement is denominated clinically amyopathic DM (CADM), accounting for approximately 20% of all cases [160]. This group is generally associated with anti-MDA5 or anti-SAE autoantibodies [93]. Anti-MDA5 autoantibodies were identified in patients with CADM and associated with rapidly progressive ILD [170]. Patients with DM anti-MDA5 autoantibody can present three distinct phenotypes regarding the predominance of lung involvement, skin and/or articular manifestations or vascular symptoms [171]. The cumulative 100-month survival rate for patients with anti-MDA5+ DM is 66%, with the highest frequency of fatal outcomes within the first six months since diagnosis [172]. The change on anti-MDA5 autoantibody levels after treatment can help predict the course of the disease, where patients that responded to treatment had a significant decrease in the levels compared to those without response or fatal outcomes [173].

2.7.3 Inclusion body myositis

IBM has a disease onset usually after the age of 50 years. A gradual disease onset characterizes the clinical presentation with a slow progression over years. The muscle weakness is often asymmetric of proximal and distal muscles, with frequent involvement of quadriceps and long finger flexors [174]. CK serum levels can vary; usually, they are mildly elevated but can occasionally be high [175]. Other non-specific symptoms include myalgia, cramping and dysphagia [176]. This entity is particularly resistant to immunosuppressive treatment, with a progressive disability associated with patients who use wheelchairs between 37.5%-47% in 12-year follow-ups [177].

Clinically, it has been associated with Sjögren's syndrome (SS), sarcoid myopathy, systemic lupus erythematosus (SLE) and other connective tissue diseases [29]. Serologically, the cytosolic 5´-nucleotidase 1A autoantibody (anti-cN1A) was identified in 2013, and the target is highly expressed in skeletal muscle [178]. The prevalence of anti-cN1A ranges from 30-76% [179], and this autoantibody has also been reported in SLE and Sjögren's syndrome but rarely in other subgroups of IIM [144]. The clinical associations between IBM and anti-cN1A have been inconsistent; some of them have reported an association with a severe clinical picture such as bulbar involvement [142, 180], and high mortality associated with lung complications [179]; but there are also reports with no clinical association [181].

The histological muscle findings of IBM include endomysial T cell infiltrates invading non-necrotic fibres (predominantly $CD8^+$ T cells). Other histological findings that can help in the differentiation of IBM are the presence of rimmed vacuoles (Congo red staining), the mitochondrial changes including ragged red fibres (Gomori trichrome stain modified), ragged blue fibre (succinate dehydrogenase stain) and the presence of fibres negative for

cytochrome C oxidase [176]. Other features such as myonuclear degeneration, myofibrillar cytoplasmic aggregates and mitochondrial pathology can be seen in the biopsy, suggesting a degenerative component [182]. The presence of anti-cN1A has been reported in the perinuclear regions in muscle fibres and rims of the vacuoles, indicating that these autoantibodies might be associated with myonuclear degeneration [183]. Nevertheless, the presence of highly differentiated and apoptotic resistant cytotoxic T cells supports an autoimmune component of the pathophysiology of IBM [127]. Additionally, a recent study described the presence of a "neoplastic-like" T cell expansion characterized by aberrant populations of large granular lymphocytes and possibly contributing to the refractoriness of the disease [184].

2.7.4 Immune-mediated necrotizing myopathy

IMNM is a subgroup characterized by proximal and symmetric muscle weakness, high serum levels of muscle enzymes (up to 20-50 fold the normal upper limit), myopathic pattern in the electromyography, and muscle histopathology with myofibre necrosis or regeneration and minimal lymphocytic infiltrate (pauci-immune) [29]. The presence of extra-muscular manifestations is rare. This entity has been divided into three subgroups according to their MSA: anti-HMGCR IMNM, anti-SRP IMNM and autoantibody-negative IMNM [185].

Anti-HMGCR autoantibodies were initially associated with a group of adult patients with myositis, elevated CK levels and a history of statin exposure [186]. These autoantibodies have been reported in adult (6-12%) and juvenile myositis (1-17%), with a higher prevalence in Asian populations [187, 188]. The target of this autoantibody is HMGCR, a glycoprotein essential in the biosynthesis of cholesterol [189]. Although histopathological analysis of muscle of patients with anti-HMGCR autoantibody has revealed overexpression of HMGCR in regenerating myofibres [186], several studies have shown a high variability in the degree of statin exposure, suggesting the existence of other factors in the production of this autoantibody [187, 188]. The presence of scattered non-necrotic fibres of $CD68^{+}$ macrophage infiltrates in conjunction with membrane attack complex (MAC) expression in the muscle fibre membrane suggests the presence of an antibody-dependent cell-mediated toxicity pathway [136]. Additional clinical studies have correlated the anti-HMGCR levels with elevated CK at baseline [190, 191]. However, the relationship between anti-HMGCR autoantibodies and disease activity needs further investigation.

Anti-SRP autoantibodies are present in 4% of juvenile and 5-18% of adult patients with IIM and are associated with a more severe degree of muscle weakness than anti-HMGCR [185, 188]. These autoantibodies are associated with high CK levels, and some reports have documented QTc prolongation and a varying degree of ILD (6-22%) [192-195]. Similarly to

anti-HMGCR autoantibodies, a single-centre study identified a positive correlation between levels of anti-SRP autoantibodies and CK serum levels [196].

The autoantibody-negative IMNM group has been associated with cancer and a higher rate of cardiac abnormalities such as prolonged QTc and left ventricular diastolic dysfunction in more than 50% of the cases [195, 197]. A recent study found a high frequency of extra-muscular manifestations, female predominance and higher rates of associated connective tissue disease; however, the small sample size limits these conclusions and more extensive prospective studies are required to confirm these findings [198].

2.7.5 Overlap myositis

Myositis can occur with other autoimmune diseases such as systemic sclerosis (SSc) [199, 200], SLE [201], SS [202] or RA [203]. The overlap myositis group is heterogeneous and can present with different histological findings. This group can present several MAA, such as anti-Pm-Scl, anti-Ku, anti-U1-RNP, anti-Ro, and anti-La. One subgroup of overlap myositis with SSc is referred to as scleromyositis and is characterized by head drop, distal weakness and extra-muscular manifestations [29].

2.7.6 Anti-synthetase syndrome.

Aminoacyl-tRNA synthetases (aaRSs) are enzymes responsible for the first step of protein synthesis. These enzymes perform their function by attaching a sequence of amino acids to the corresponding cognate tRNA [204]. The end product, the tRNA amino-acylated, will continue to the ribosome and the consequent steps of protein synthesis [205]. In humans, there are twenty aaRS that include both cytosol and mitochondria aaRSs [206, 207]. In addition to this function, the individual aaRS have different cytoplasmic, nuclear and extracellular functions, which increases the complexity of understanding the mechanism of how and why aaRSs become autoantigens [208].

Diagnosis, definition and classification criteria. Autoantibodies targeting aaRS are the most frequent type of MSA in patients with IIM, which are found in 23-35% [209-213]. To date, recognition by autoantibodies of eight out of 20 ARSs has been described, and the association with a distinct clinical phenotype was reported for the first time in 1990 by Marguerie et al. [214], and later named “Anti-synthetase syndrome” (ASyS) by Targoff et al. in 1992 [215]. Two decades later several sets of criteria for the diagnosis of ASyS were proposed, such as Connors et al. in 2010, Salomon et al. in 2011 and Lega et al. in 2015 [38, 216, 217]. A summary of these criteria is presented in **Table 6**.

A recent systematic review of the literature analysed these criteria and found that Connor’s criteria was the most widely used [218]. However, the lack of consensus in this entity has

not allowed the development of a validated definition for ASyS, which highlights the need for standardised and validated classification criteria. Such project is ongoing as a collaboration between the American College of Rheumatology (ACR) and European Alliance of Associations for Rheumatology (EULAR)

According to the American European NEtwork of Anti-synthetase Syndrome (AENEAS) study group, the classic triad of ASyS corresponding to the presence of myositis, arthritis, and ILD, is present in up to 20% of the patients at disease onset [219, 220]. Similarly, other groups have reported that at disease onset, the common clinical manifestations were isolated lung disease, seronegative arthritis or myositis, suggesting that the diagnosis of this entity requires a high level of awareness of the disease, especially at early stages [212]. It is important to highlight the findings of several epidemiological studies where the results show that ILD is the most severe and often the sole manifestation of ASyS [221]. ILD can be present in up to 90% of patients and is associated with high morbidity and mortality [27, 222].

Table 6: Summary of Anti-synthetase criteria

Connors et al. [38]	**Solomon et al. [216]**	**Lega et al. [217]**
Presence of **one anti-aaRSs antibody** plus one or more of the following: - Myositis [a] - ILD [b] - Arthritis - Raynaud's phenomenon - Fever - Mechanic's hands	Presence of **one anti-aaRSs antibody** plus <u>Two major criteria</u> or <u>One major plus two minor criteria</u> **Major criteria** - Myositis [a] - ILD [b] **Minor criteria** - Arthritis - Raynaud's phenomenon - Mechanic's hands	Presence of **one anti-aaRSs antibody** plus one or more of the following: - Myositis - ILD [b] - Arthritis or arthralgia <u>Or two among:</u> - Raynaud's phenomenon - Fever - Mechanic's hands

Legend: ILD: interstitial lung disease. [a] According to Bohan and Peter's criteria. [b] by American Thoracic Society (ATS) criteria.

2.7.7 Anti-synthetase autoantibodies

Anti-aaRSs autoantibodies are directed towards histidyl- (also known as Jo-1), threonyl- (PL-7), alanyl- (PL12), glycyl- (EJ), isoleucyl- (OJ), tyrosyl- (Ha), asparagyl- (KS), and phenylalanyl- (ZO) tRNA synthetases. The clinical spectrum of ASyS associated with the different anti-aaRSs autoantibodies is heterogeneous. The specific clinical characteristics of the anti-synthetase autoantibodies are presented in **Table 7**.

Table 7 Anti-synthetase autoantibodies in ASyS [223]

Auto-antibody	tRNA synthetase	Protein name	Prevalence in IIM	ILD	Myositis	Clinical manifestation
Jo-1	Histidyl	HisRS	15-30%	++	++	ILD (50-90%), fever (27-70%) Arthritis (58-75%), myositis (57%), muscle weakness (59-78%), mechanic hands (20-56%), Gottron's sign (44%), RP (19-60%). [224-226]
PL-7	Threonyl	ThrRS	5-15%	++	+	ILD (55-76%), fever (34%), myositis (48%), muscle weakness (40-52%), arthritis (31%), Gottron's sign (41%), RP (38%). [224, 225, 227]
PL-12	Alanyl	AlaRS	5-10%	++	+	ILD (69-89%), fever (36-44%), pulmonary hypertension, oesophageal involvement (20%), myositis (36%), muscle weakness (17%), arthritis (22-35%), Gottron's sign (33%), RP (44%) [224-227]
EJ	Glycyl	GlyRS	<5%	++	+	ILD (73-84%) NSIP, fever (39-60%), arthritis (24%), myositis (40%), muscle weakness (39-55%), Gottron's sign (45%), RP (13%) [224, 225, 228]
OJ	Isoleucyl	IleRS	<5%	++	+	ILD (44->90%), fever (13%), myositis (40-80%), muscle weakness (25%), arthritis (13-60%), mechanic hands (40%), Gottron's sign (13-30%), RP (13%).[224, 225]
KS	Asparaginyl	AsnRS	1-8%	++	+	ILD (>90%), fever (5-8%), arthritis (26-31%), mechanic hands (30%), muscle weakness (7%), Gottron's sign (8%), RP (31%).[224, 229]
Zo	Phenylalanyl	PheRS	<1%	+	+	Arthralgia, ILD (14-57%) NSIP, RP. [230]
YRS/HA	Tyrosyl	TyrRS	<1%	+	+	Rash, arthritis, ILD

Legend: ASyS: Anti-synthetase syndrome, ILD: Interstitial lung disease; RP: Raynaud phenomena; NSIP: Nonspecific interstitial pneumonia, UIP: usual interstitial pneumonia. *Reprinted after permission from Elsevier. Galindo-Feria AS et al. Autoantibodies: pathogenic or epiphenomenon. Best Pract Res Clin Rheumatol 2022.*

Multiple research groups have questioned whether the aaRSs autoantibodies have a role in the pathogenesis of IIM or are merely an epiphenomenon. In this thesis I will only focus on the role of anti-Jo-1 and the potential pathogenesis of IIM/ASyS.

2.7.8 Anti-HisRS (anti-Jo-1) autoantibodies in IIM

Autoantibodies targeting HisRS (also known as anti-Jo-1 antibodies) are the most common among the MSA, with frequencies between 20-30% in patients with IIM [209, 211, 231]. HisRS consists of three main domains: the WHEP domain located in the N-terminal region, the catalytic domain (CD) in the internal part of the protein and the anti-codon binding domain (ABC) in the C-terminal region [232]. Previous studies have shown that anti-Jo-1 autoantibodies are directed towards several epitopes located particularly within the WHEP domain [233-235]. A more detailed characterization of the relationship between anti-Jo-1 autoantibodies and autoimmunity will be presented in 2.10.1 Specific background of the paper.

Worldwide epidemiological studies have shown that patients with anti-Jo-1 autoantibodies can develop ILD in up to 90% of the cases [27, 212, 236, 237] and these autoantibodies

have been associated with a variable degree of myositis, arthritis, mechanic's hands, Raynaud's phenomenon and fever [223]. A report from the AENEAS cohort of anti-Jo-1 positive patients identified that at disease onset, 50% of the patients had ILD, and 84% developed lung disease after an 80-month follow-up [219]. Additional associations with disease activity in patients with anti-Jo-1 autoantibodies and incomplete ASyS during follow-up were the presentation of novel clinical manifestations such as Raynaud's phenomenon, mechanic's hands and fever. [238].

Anti-Jo-1 autoantibodies, clinical manifestations and disease activity. To understand the role of anti-Jo-1 autoantibody in the pathogenesis of IIM, several studies have been performed to analyze the relationship of this autoantibody with the presence of different clinical associations, biomarkers and disease activity tools.

Clinical observations since 1984 have reported that the production of anti-Jo-1 autoantibodies can occur before disease onset [214, 239]. The current identification of high affinity and high reactivity autoantibodies against all HisRS domains at diagnosis suggest that the affinity maturation and epitope spreading could have happened before the onset of symptoms [240]. Still, this hypothesis needs to be confirmed.

A retrospective study analysed the presence of clinical manifestations and anti-Jo-1 autoantibody levels in patients with IIM. Patients with high anti-Jo-1 levels presented with either complete or incomplete ASyS, being the most common involvement of muscle (92%), lung (62%), or joints (69%) [241]. In this study, the intermediate or low levels of anti-Jo-1 autoantibodies were associated with other autoimmune connective tissue diseases, infection or cancer, indicating that the levels of anti-Jo-1 autoantibody at diagnosis could be a helpful indicator of ASyS when presenting with myositis, ILD or arthritis [241]. A recent study showed that patients who developed ILD and arthritis presented the highest levels of anti-Jo-1 IgG antibodies at diagnosis. Additionally, poor pulmonary function was associated with the reactivity of anti-WHEP IgG in BALF [240].

In a cross-sectional study in 2007, Stone K. et al. reported a correlation between anti-Jo-1 autoantibody levels and disease activity measured by the MDAAT tool; and a moderate correlation in longitudinal serum samples between the anti-Jo-1 autoantibody levels and CK, lung and global disease activity, arthritis and myositis [242]. The association between anti-Jo-1 levels and progression of ILD was also reported in a longitudinal cohort and was associated with high reactivity and affinity of the full-length HisRS autoantibodies from an early disease course up to 3 years during follow-up, despite treatment in some individuals [240].

However, there are still questions regarding these results since the clinical associations related to disease activity in longitudinal serum samples have not been replicated in other cohorts, probably due to the variability and lack of standardization of the autoantibody testing methods [241, 243]. Therefore, the question regarding the pathogenicity of the anti-Jo-1 autoantibody remains. Further studies will be necessary for studying sera from patients before the onset of disease (pre-clinical) as well as longitudinal clinical studies in larger cohorts and *in vitro* experiments to characterize this autoantibody as pathogenic or mere epiphenomenon [223, 243].

Another biomarker reported to play a role in the prognosis of the disease in association with anti-Jo-1 autoantibodies is the B cell activating factor (BAFF). A study showed that increased BAFF levels in serum correlated with CK levels ($r=0.365$, $p<0.001$) [244] and was reported as a good predictor marker in patients with ILD and anti-Jo-1 autoantibodies compared to other patients with IIM without ILD ($p<0.05$) [245]. An analysis of serial serum samples from cohorts in the Czech Republic and Sweden revealed that anti-Jo-1 levels became negative in association with a significant decrease in BAFF levels in 17% (n=40) of the patients within 0.5-4.3 years of follow-up [245]. Additionally, the results from the rituximab in myositis trial (RIM) demonstrated that anti-Jo-1 serum levels decreased after treatment with rituximab [246].

Pathogenesis of anti-Jo-1 autoantibodies: experimental results. Anti-Jo-1 autoantibodies bind to sites spread across the entire HisRS protein and include linear and conformational epitopes [240, 247]. Among the various epitopes analysed, the N-terminal portion containing amino acids 1-60 of HisRS was identified as the immunodominant epitope [234]. In addition, the presence of class switching, epitope spreading and affinity maturation of the anti-Jo-1 autoantibodies point to a contribution of antigen-specific $CD4^+$ T cells in developing these autoantibodies [248-251]. Detailed information regarding current *in vitro* studies will be presented in 2.10.1 Specific background of the paper.

Animal models: Several mice experiments have been performed to study the role of HisRS in the pathogenesis of IIM. The most relevant results can be summarized as follow:

- The production of anti-HisRS autoantibodies requires a species-specific immunisation to elicit an antigenic immune response [233].
- Inoculation with cDNA of the N-terminal domain from human HisRS can induce inflammatory infiltrates in the muscle of mice along with detectable anti-HisRS autoantibodies, indicating that different immune responses occur upon antigen presentation [252].

- Mice immunized with murine N-terminal HisRS elicited an early T cell infiltration response in the muscle. Furthermore, the mice developed IgG class-switched autoantibody responses that persisted for at least seven weeks [253].
- The immunisation with murine N-terminal HisRS was capable of breaking tolerance in an adjuvant-based model, with the presence of anti-HisRS autoantibodies causing muscle and lung inflammation [254].
- The immunological response was significantly higher in mice after immunization with murine-WHEP-domain instead of the murine-full-length HisRS protein, with high levels of anti-HisRS autoantibodies detected in BALF and serum [255]. In this mice model, a challenge with cardiotoxin or bleomycin resulted in immune cell invasion in muscle and lung, respectively [255].
- HisRS plays a role in the innate immune response through MyD88 signalling pathways and contributions of TLR2 and TLR4. This could alter NF-κB-mediated gene expression profiles leading to muscle dysfunction [256].
- The immunisation of IFNα/βR-null mice with murine N-terminal HisRS and a TLR-7/8 agonist promoted upregulation of MHC I at the injection site and in uninjected muscles. This experimental autoimmune myositis developed anti-HisRS autoantibodies, endomysial and perimysial leukocyte infiltration, and myofibre regeneration, indicating that TLR7/8 is important for the induction and maintaining of the systemic autoimmune response [257].

Collectively, these studies indicate that HisRS, in certain contexts, is capable of inducing myositis. Though these models have attempted to clarify the link between innate and adaptive immune responses in myositis, until now, they have only provided indirect evidence that anti-Jo-1 autoantibodies contribute to the disease pathogenesis in IIM.

2.8 ASSESSMENT OF DISEASE ACTIVITY AND DAMAGE

For an adequate evaluation of the patient with IIM, it is necessary to consider the disease activity, response to treatment and long-term outcomes. It is essential to reach a consensus on activity and damage definitions. Disease activity can be defined as the type, extent and severity of pathological manifestations of IIM and can be reversible. Disease damage includes persistent changes from an active disease such as scarring, atrophy or fibrosis. Other items to be considered are the complications of long-term therapy, comorbidity, patient-reported outcomes (PROM) and health-related quality of life (HRQoL). Since 2001, several standardised and well-validated assessment measures have been developed through international collaborative groups, such as the International Myositis Assessment and Clinical Studies Group (IMACS) and the Paediatric Rheumatology International Trials Organization (PRINTO) [258, 259].

To assess the disease activity in adult patients with DM or PM, a group of validated core set measures (CSM) are used for the assessment of the disease and include the following [260]:

1. Physician's global disease activity measured by a 10 cm visual analogue scale (VAS) (0-10, with 10 being the maximum disease activity).
2. Patient’s global disease activity measured by a 10 cm VAS.
3. Muscle strength evaluated with the Manual muscle test 8 (MMT-8), with a score of 80 being the maximum on one side and score of 150 with bilateral scoring.
4. Health assessment questionnaire (HAQ) of activities of daily living, where 3.0 is the maximum score indicating severe disability.
5. Serum levels of muscle enzymes: CK, LDH, ASAT, ALAT.
6. Extra muscular activity score evaluated by the Myositis Disease Activity Assessment Tool (MDAAT): constitutional, cutaneous, pulmonary, gastrointestinal, cardiac and skeletal activity.

The assessment of disease damage is based on the Myositis Damage Index score (MDI) of the IMACS [261], which evaluates the presence and severity of changes for at least six months in eleven organ systems: cutaneous, cardiovascular, vascular, endocrine, muscle, infection, skeletal, gastrointestinal, pulmonary, ocular and malignancy. In addition, this index includes the evaluation of global damage that can be scored by physicians and patients using VAS and other damage (e.g. death). The MDI index is validated for IIM and is sensitive to change [260].

While an indication of high disease activity on the MDAAT may encourage escalation of therapy, an indication of disease damage on MDI allows a comprehensive longitudinal evaluation of the effects of both disease and therapy in skeletal and other organ systems (**Figure 3**).

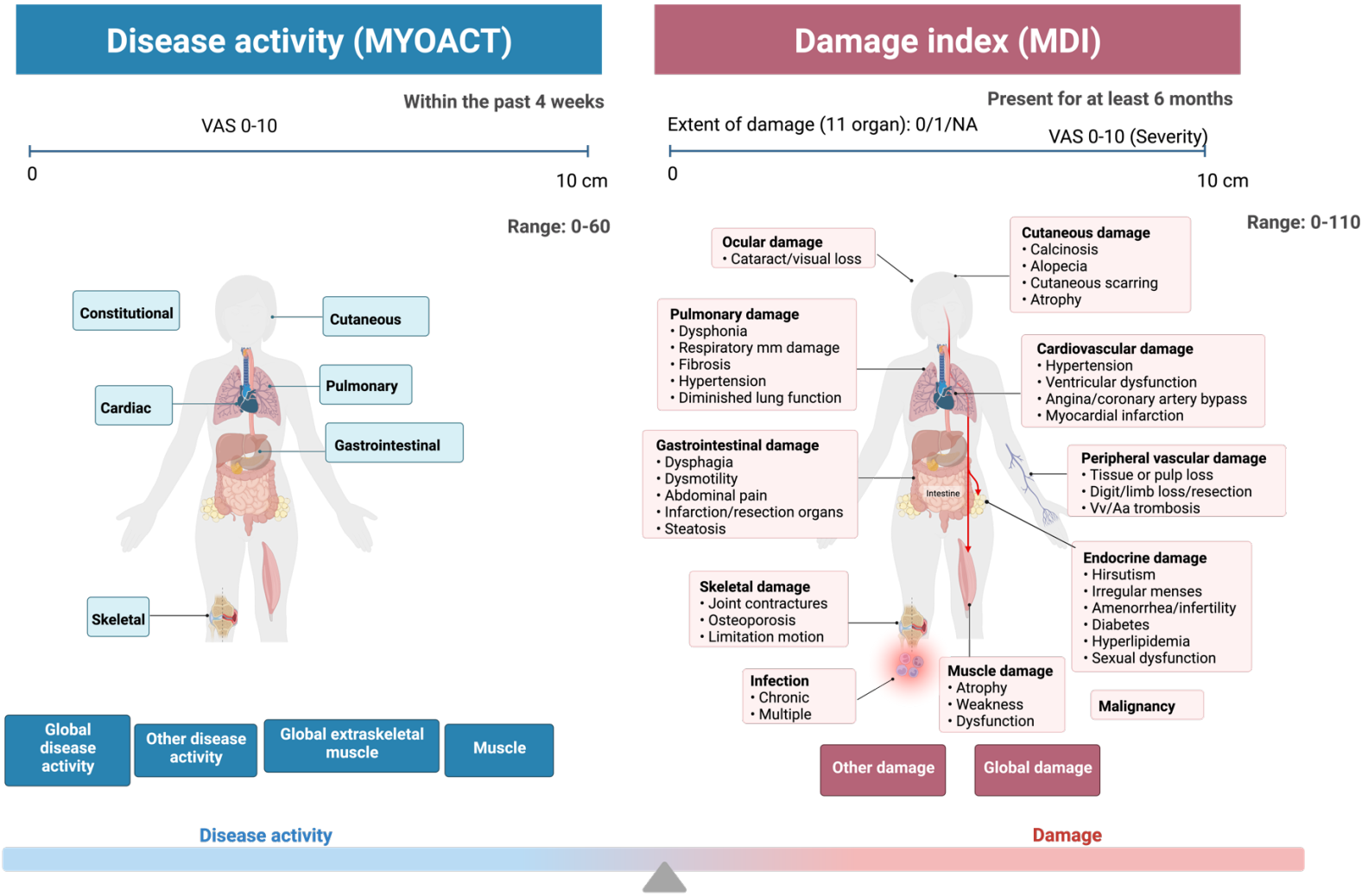


Figure 3. Myositis disease activity and myositis damage index. MYOACT, Myositis Disease Activity Assessment Tool; MDI, Myositis Damage Index; VAS, visual analogue scale; NA, non-available. Created with Biorender.com

2.9 TREATMENT

Current treatment for IIM remains challenging due to the few published randomized controlled trials, the rarity of these diseases, the presence of distinct clinical phenotypes and possible systemic complications such as severe muscle weakness, skin rash or life-threatening organ involvement [262]. There has been particular interest in leveraging specific pathophysiological mechanisms and molecular targets as potential therapeutic targets in IIM [263].

Still, few randomized, double-blind clinical trials have been published [264-267]. A recent study highlighted the importance of identifying the presence of MSA early in the disease. The results showed that patients who were positive for autoantibodies associated with DM (anti-MDA5, anti-TIF1γ, anti-Mi2, anti-SAE) had a higher frequency of achieving a moderate response compared to ASyS (anti-Jo-1, anti-PL7, anti-PL12, anti-EJ), IMNM (anti-SRP and anti-HMGCR), MAA without any MSA (anti-Pm-Scl, anti-U1 anti-RNP, anti-Ro52, anti-Ku), and seronegative group (negative to the previous autoantibodies) [268].

The traditional pharmacological approach is based on high doses of glucocorticoids in combination with immunosuppressive agents such as methotrexate or azathioprine as first-line therapy. Second-line therapy includes mycophenolate mofetil, cyclosporine or tacrolimus; other drugs such as cyclophosphamide and biological agents are considered a third-line therapy [262]. Biologic agents such as rituximab (anti-CD20) constitute an alternative for specific subgroups of patients with IIM, such as ASyS or refractory disease [267, 269]. Intravenous immunoglobulins (IVIg) were approved for the treatment in patients with adult DM by the U.S Food and Drug Administration (FDA) in 2021 based on the randomized control trial “ProDERM”, that evaluated its safety and long-term efficacy [270]. The IVIg has also been increasingly used as first-line treatment in IMNM, and currently, they are used as second- or third-line therapy in combination with glucocorticoids or other immunosuppressants in various subgroups of patients refractory to the conventional immunosuppressive treatment [29, 270].

Additional immunomodulatory agents have been included, such as abatacept (fusion protein containing the extracellular domain of CTLA4) [271]; anakinra (recombinant IL-1 receptor antagonist) [272]; tocilizumab (IL-6 receptor antagonist) [273, 274]; JAK inhibitors such as tofacitinib [275] and baricitinib [276]; or belimumab [277].

Other drugs have also found a niche in this group of diseases related to specific organ involvement. Some examples include basiliximab (anti-CD25) for interstitial pneumonia of CADM [278] and pirfenidone as an antifibrotic agent, though its role in IIM remains to be elucidated [279]. For refractory skin disease, a placebo-controlled phase II study for lenabasum (cannabinoid receptor type 2 agonist) and a clinical trial for apremilast (phosphodiesterase-4 inhibitor) for cutaneous disease in patients with recalcitrant DM is being conducted [280].

An alternative approach based on the pathophysiology of the disease has been undertaken by targeting the type 1 IFNα signature in PM and DM. Sifalumab (anti-IFNα) has been reported to inhibit IFN-protein and transcripts; however, the clinical relevance of this response still has to be evaluated [281]. Other novel treatments that are under study for use in PM and DM are KZR-616 (a selective inhibitor of the immunoproteasome), low-dose IL-2 in combination with standard therapy [282], and human umbilical cord mesenchymal stem cell-derived allogenic mitochondria (PN-101) [283].

Unfortunately, some therapeutic approaches have not been successful, such as the use of sodium thiosulfate for calcifications in DM, which was investigated in an RCT with negative results [284]. Other treatments tested without positive results in myositis were the toll-like receptor 7/8/9 antagonist (IMO-8400) for treating skin in DM [285], zilucoplan (which inhibits the cleavage of C5 and the subsequent formation of the membrane attack complex) [286] and siponimod in DM (BAF 312, oral sphingosine-1-phosphate 1/5 modulator) [287].

Within the non-pharmacological intervention, exercise has been introduced as an adjuvant therapy with minor adverse effects [288]. The important role of exercise as part of the treatment is strengthened by the expression of muscle-derived cytokines (also known as myokines) with an anti-inflammatory effect in the skeletal muscle in response to exercise [289, 290].

2.10 SPECIFIC BACKGROUND OF PAPERS

2.10.1 Paper I and IV: Histidyl-tRNA synthetase (HisRS) in IIM

HisRS is a homodimeric enzyme that is ubiquitously expressed and mainly located intracellularly [291]. It is responsible for synthesizing histidyl-tRNA in a two-step process, which interacts with the ribosome (HisRS: $tRNA^{His}$ complex) to incorporate histidine into a growing peptide [291]. Histidine is an essential amino acid that is important in many catalytic functions of enzymes with antioxidant, antisecretory, and anti-inflammatory properties with a role in suppressing proinflammatory cytokine expression through the NF-kB pathway [292].

HisRS has an essential role in several regulatory mechanisms of cell metabolism and immune responses [293]. It is present as a homodimeric protein and consists of three main domains: the WHEP domain located in the N-terminal region, the catalytic domain in the internal part of the protein and the anti-codon binding domain (ABD) at the C-terminal region [232]. The N-terminal or catalytic domain includes the residues 1-320 and it is responsible for the aminoacylation of tRNA, while the C-terminal domain is essential for the dimeric structure of the protein. The WHEP domain has been associated with noncanonical activities of the proteins and is present in five aaRSs. It is named after **W**RS (tryptophanyl-tRNA synthetase), **H**isRS (histidyl-tRNA synthetase), and **EP**RS (glutamyl-prolyl-tRNA synthetase). GRS (glycyl-tRNA synthetase) and MRS (methionyl-tRNA synthetase) also contain a WHEP domain [294]. Until now, two splice variants (SV) have been identified, a monomeric splice variant comprising the WHEP and ABD domain [295], and a WHEP domain composed of the first 60 amino acids [296] (**Figure 4**).

HisRS and its N-terminal domain may play an important role in initiating and maintaining the inflammatory response, specifically in muscle and lung tissue. This is suggested upon the identification of highly expressed HisRS on bronchial epithelial cells and in regenerating muscle fibres, with an increased expression during inflammation [297]. Additional *in vitro* studies documented the secretion from the cytosol into the extracellular environment from diverse cell lines, including lung and muscle, of the full-length HisRS and WHEP domain [296]. The presence of extracellular HisRS in patients with anti-Jo-1 autoantibodies could potentially lead to tissue-damaging neutrophil extracellular traps (NETs) formation, associated with a lower DNase activity and impaired clearance, especially in patients with ILD [298]. However, another study could not replicate the formation of NETs, possibly due to the low number of patients with ILD [299]. An interesting result showed that in normal subjects, the role of extracellular HisRS could be homeostatic [255]. Still, the finding of reduced levels of extracellular HisRS in circulation in anti-Jo-1 patients prompts several questions regarding its role in the disease mechanism.

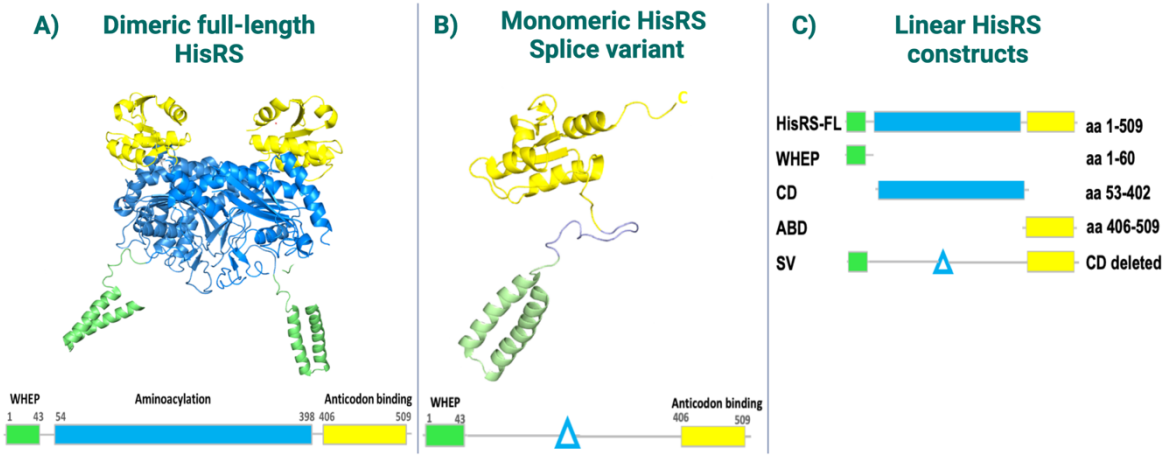


Figure 4. Structure of Histidyl-tRNA synthetase. (A) Dimeric structure of the full-length Histidyl-tRNA synthetase (HisRS). **(B)** Monomeric HisRS structure of the splice variant. **(C)** Graphical linear representation of HisRS antigens. For **A-B**, visualization of the structure of HisRS was performed using PDB ID:4G84 and 2LW7 [296]. For **A-C**, the following colour code was used: WHEP-domain, green; internal catalytic domain (CD), blue; anticodon binding-domain (ABD), yellow. aa; amino acids, FL; full length, SV; splice variant. *Adapted and reprinted after permission from John Wiley and Sons, Galindo-Feria AS et al. Anti-Jo-1 autoantibodies, from clinic to the bench. Rheumatology & Autoimmunity. 2022; 1-12 [243].*

Several *in vitro* studies have shown that anti-Jo-1 autoantibodies act as competitive inhibitors for the aminoacylation reaction between the catalytic enzyme ATP/histidine, binding close to the ATP-histidine binding sites of the HisRS [300, 301]. Anti-Jo-1 autoantibodies can bind to free and $tRNA^{His}$-bound HisRS [302], with high affinity to the HisRS: $tRNA^{His}$ complex [252].

Further *in vitro* studies performed in immune cells and sera from myositis patients suggest that anti-Jo-1 autoantibodies might be involved in the disease pathogenesis of ASyS, particularly in the clinical subset with ILD. [233, 242].

Among the relevant human *in vivo* findings that support this hypothesis are the following:

1. HisRS is highly expressed in the bronchial epithelial cells of the lung and in regenerating muscle fibres. This could explain the organ selection for the immune reaction in patients with IIM and anti-Jo-1 autoantibodies [297].
2. HisRS is a substrate of the protease granzyme B, which is linked to the formation of neo-epitopes [128]. HisRS possesses a granzyme B cleavage site (LGPD) in its N-terminal domain [303]. These N-terminal fragments ($HisRS_{1-48}$) possess chemotactic properties by interacting with CCR5, which can induce the migration of inflammatory cells [129].
4. Anti-Jo-1 autoantibodies can recognize a conformation of HisRS that is “granzyme B-sensitive” in lung tissue [303].

5. Anti-Jo-1 autoantibodies present a high avidity and affinity of towards the WHEP domain, which contains the granzyme B cleavage site. This reactivity was overrepresented among anti-Jo-1 autoantibodies in circulation and BALF [240].
6. The presence of $CD4^+$ T cells reactive against HisRS full-length and/or HisRS fragments of approximately 150 amino acids was reported in peripheral blood of patients with IIM [304], suggesting that HisRS protein or its fragments could be released from damaged muscle or lung and may be involved in the recruitment of activated T cells, including HisRS-activated T cells, to inflammatory sites such as the lungs or muscles.
7. An epidemiological study reported an association between *HLA DRB1*03:01* genotype, smoking and anti-Jo-1 autoantibodies [56].

Epitope prediction in HisRS. Currently, new prediction models are being used to identify HisRS-specific $CD4^+$ T cell epitopes restricted to HLA-DRB1*03:01. One type of prediction of a $CD4^+$ T cell epitope was based on the crystal structure of the HLA-DRB1*03:01 molecule in a complex with Class II-associated invariant chain peptide (CLIP) [305]. HLA-DRB1*03:01 T cell epitopes are described to commonly consist of an aliphatic residue in position 1, an acidic or polar residue in position 4, and either a basic residue at position 6 paired with a small residue at position 9 (submotif 1) or a non-basic residue at position 6 paired with a large residue at position 9 (submotif 2) [306].

Upon identifying the relevant pockets in the peptide-binding groove, it is possible to predict the fitting of amino acids from the selected peptide(s) sequence. (**Figure 5**). This approach was used in **Paper I** for prediction of a HisRS epitope, which contained 2 such motifs and had a high probability of containing a DRB1*03:01 epitope

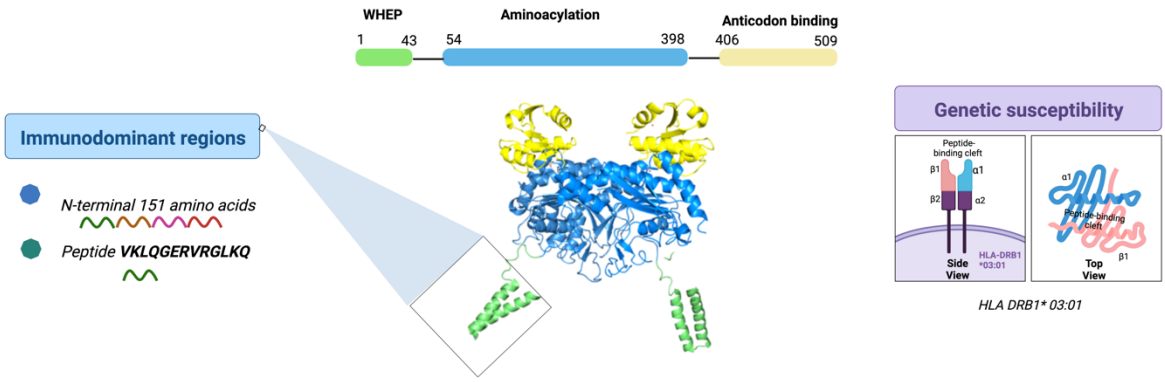


Figure 5. Epitope prediction of HisRS. Visualization of the structure of HisRS was performed using PDB ID:4G84. An immunodominant region was determined within the WHEP domain corresponding to the N-terminal 151 amino acids. A 13-mer peptide was located within the immunodominant region. The predicted epitope contained two motifs that can bind the HLA-DRB1*03 molecule. Created with Biorender.com

Hypothesis of the pathophysiology of anti-Jo-1 in IIM. The role of anti-Jo-1 autoantibodies and their relationship between the immune system and the pathogenesis of IIM has been studied by several groups. Additional *in vitro* studies have shown that sera from anti-Jo-1 positive patients can induce interferon production from plasmacytoid DCs [307]. Moreover, N-terminal fragments of HisRS have chemotactic properties and induce migration of CCR5-expressing T cells, IL-2, activated monocytes, and immature DCs [129]. Experiments from human sera and mice showed that anti-Jo-1 autoantibodies presented over time rising affinity maturation, broadening spectrotyping, persisting high-affinity and inhibition of HisRS aminoacylation activity [233].

Regarding the antibody characteristics of anti-Jo-1 autoantibodies, a study reported that anti-Jo-1 autoantibodies displayed IgG Fc-glycans, which are associated with inflammation [308]. Still, further studies using glycan manipulation are required to comprehend the functional role of the Fc-glycans in disease pathogenesis.

Taken together, these studies suggest that increased amounts of granzyme B triggered by pro-inflammatory environmental stimuli such as infections or smoke exposure [309, 310] may contribute to the release of N-terminal HisRS fragments and the formation of neo-epitopes that can be recognized by anti-HisRS autoantibodies (anti-Jo-1) in patients with IIM/ASyS [311, 312]. HisRS protein or fragments released from damaged muscle or lung may be involved in the recruitment of T and B cells to inflammatory sites in the lungs or muscles [313]. Additionally, the finding of high affinity and avidity autoantibodies at the time of diagnosis indicates that the process of affinity maturation, epitope spreading and broadening spectrotyping happened before the onset of symptoms [240]. This first step of breaking the tolerance emphasizes the role of the lung in the specific immune response and the need to elucidate further HisRS-specific T cell responses in patients with lung involvement in IIM/ASyS**.** (**Figure 6**).

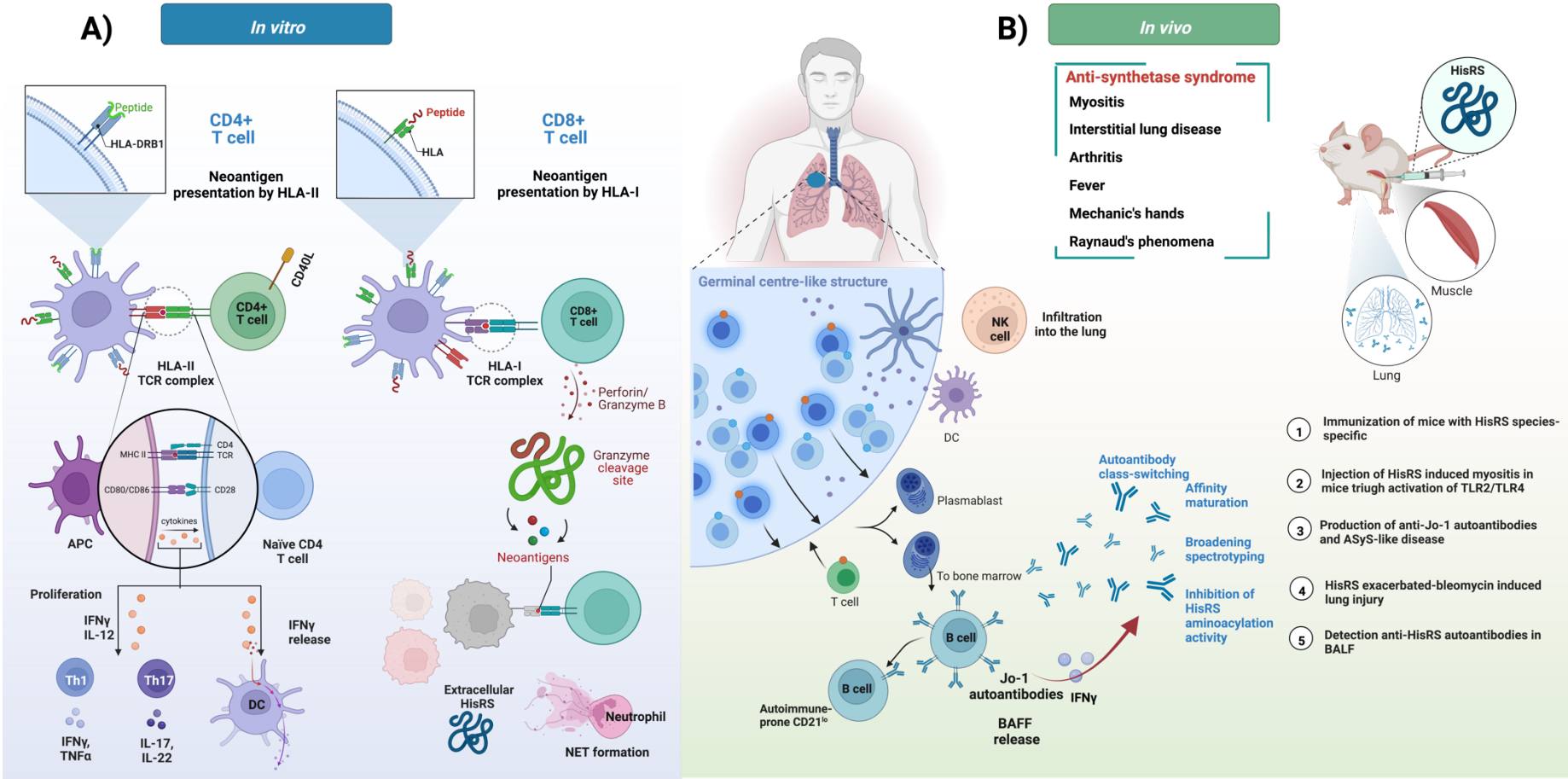


Figure 6. Development of anti-Jo-1 autoantibodies and the effects of HisRS on immune cells. **(A) Effects of HisRS on T cell activation *in vitro***. Upon uptake of HisRS by DCs, peptides are presented to CD4+ and CD8+ T cells on MHC Class I and II. Activation of $CD4^+$ T cells through interaction with MHC Class II leads to proliferation of T cells and secretion of pro-inflammatory cytokines. Activation of CD8+ T cells by MHC Class I interaction leads to the secretion of perforin and granzyme B, resulting in cleavage of HisRS and production of neoantigens. Extracellular HisRS induces the formation of NETs. **(B) Effects of HisRS *in vivo* and clinical features of ASyS**. Activated DCs and primed T cells infiltrate into germinal-centre-like structures in the lungs, where they activate B cells leading to the production of anti-Jo-1 antibodies and BAFF release. Activated NK cells are also found in lung infiltrates. *In vivo* mice models have confirmed the pathogenic effects of anti-Jo-1 autoantibodies and HisRS, where immunisation of HisRS led to ASyS-like disease with production of anti-Jo-1 autoantibodies in sera and BALF. ASyS, anti-synthetase syndrome; BAFF, the TNF family member B cell activation factor; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; GZMB, granzyme B; HisRS, histidyl-tRNA synthetase; MHC, major histocompatibility complex; NET, neutrophil extracellular trap; PRF, perforin; TCR, T cell receptor. *Adapted and reprinted after permission from John Wiley and Sons, Galindo-Feria AS et al. Anti-Jo-1 autoantibodies, from clinic to the bench. Rheumatology & Autoimmunity. 2022; 1-12 [243]*

2.10.2 Paper II and V: Anti-FHL1 autoantibodies

The second most prevalent group of autoantibody types in patients with IIM are the anti-FHL1 autoantibodies, discovered in 2015 in patients with IIM from a Swedish cohort [314]. Anti-FHL1 autoantibodies are directed toward the Four-and-a-half LIM domain 1 (FHL1) protein, which is a muscle-specific antigen found predominantly in skeletal and heart muscle [315], consisting of four and a half highly conserved LIM domains [314, 316]. In the cell, FHL1 is involved in protein-protein interactions, mainly with cytoskeletal proteins, and functions as a regulator of transcriptional factors (e.g. NFATc1) [315]. Several mutations in the *FHL1* gene (position Xq26.3) have been described, being the most relevant those associated with X-linked myopathies, such as reducing body myopathy, X-linked myopathy with postural muscle atrophy, scapuloperoneal myopathy, Emery–Dreifuss muscular dystrophy, among others [314, 316] **(Figure 7).**

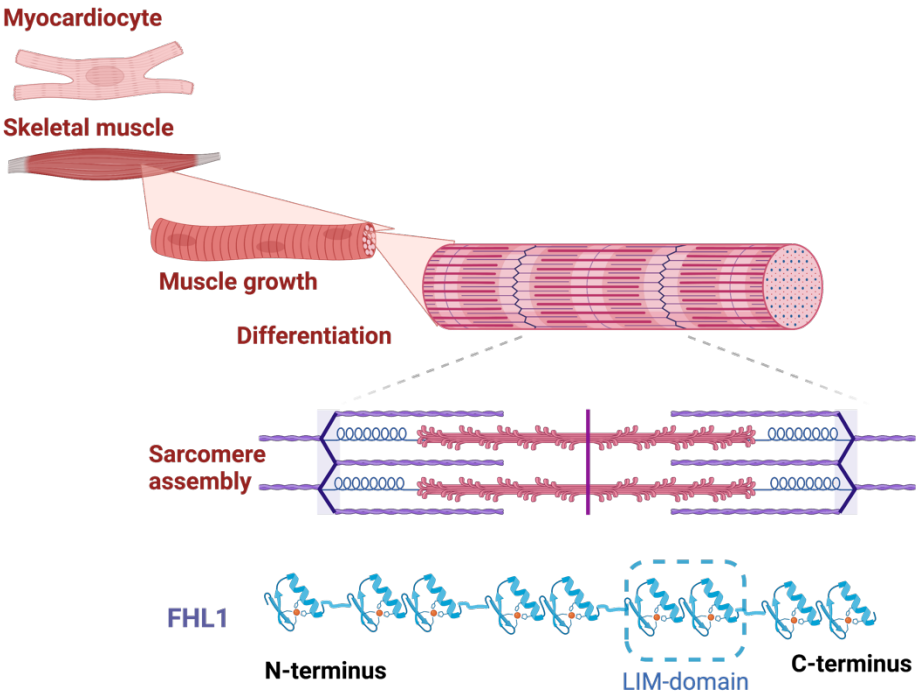


Figure 7. **FHL1 protein structure and location in the muscle**. FHL1, Four-and-a-half-LIM domain 1. Created with Biorender.com.

Mouse experiments. To analyse the role of anti-FHL1 autoantibodies in IIM, an MHC class I transgenic mice immunised with the FHL1 protein (FHL1-MaBP) or a control (MaBP) was used. In this model, immunisation with FHL1-MaBP in myositis susceptible double-transgenic (HT) mice, but not in the single-transgenic control mice (H), was associated with muscle inflammation, weakness of the forelimbs and hind limbs, and weight loss nine weeks after the immunisation. The HT-mice immunised with FHL1-MaBP had an average reduction, of their forelimb grip strength of 29% and hind limb strength of 13%, compared

with the H-control group. The presence of anti-FHL1 autoantibodies was detected in both HT- and H-immunised mice groups after the immunisation with FHL1-MaBP but not in the MaBP immunised H-control group. The FHL1-MaBP immunised HT mice group had a significantly lower survival of 12.5% compared to HT-MaBP of 50%, with a hazard ratio of 2.2. The histopathology showed significant muscle damage with IgM depositions. Additionally, there was increased mRNA expression of *CD4, CD8a, Gzmb,* and *Prf1* in the hind-limb tissue of FHL1-MaBP immunised HT mice, indicating the presence of T cell infiltrates and suggesting a link between anti-FHL1 responses and muscle damage [314] **(Figure 8).**

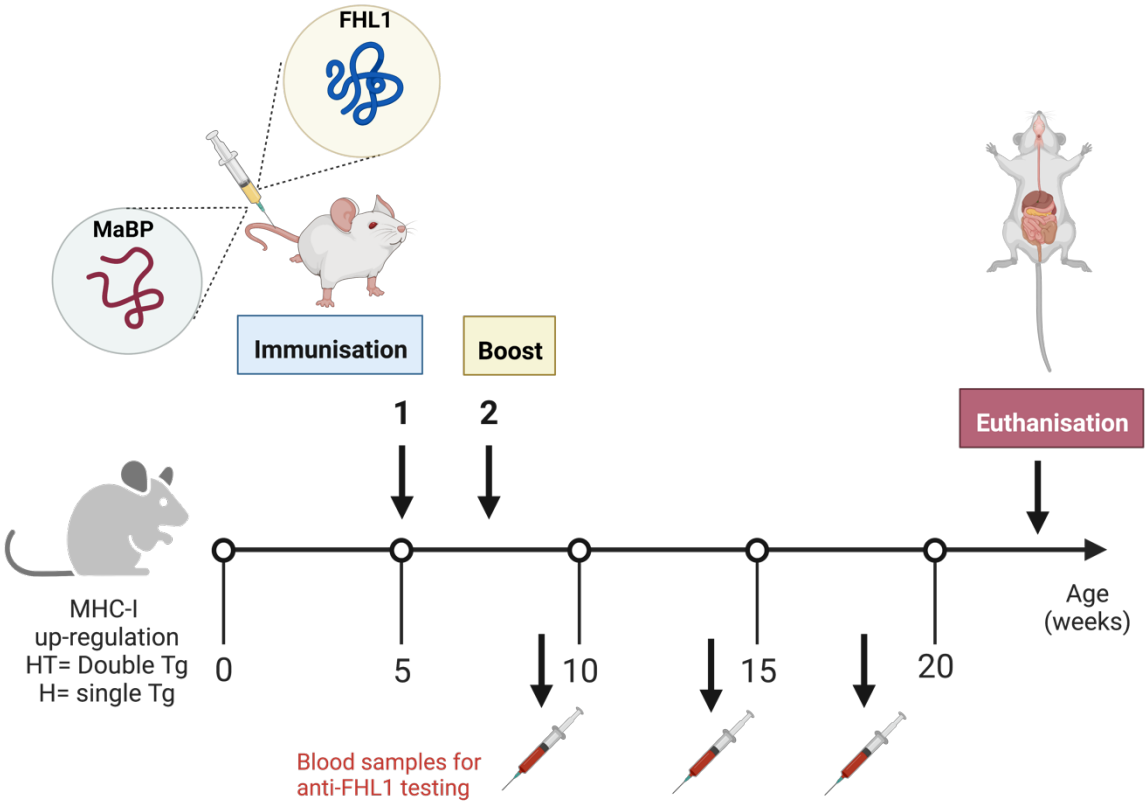


Figure 8. Mouse model for FHL1 experiments. FHL1, Four-and-a-half-LIM domain 1; MaBP, Maltose binding protein; Tg, transgenic**.** Created with Biorender.com.

The frequency of this antibody in other cohorts is unknown and will be the subject of my studies. One of the main focus will be to elucidate the functional role of anti-FHL1 antibodies in IIM and other autoimmune diseases. It will be necessary to correlate the presence and levels of the antibody with disease activity, organ damage, outcome measures and response to treatment. A longitudinal study evaluating autoantibody levels in relation to disease activity and damage will also be included in my thesis

3 RESEARCH AIMS

The overall aim of this thesis was to study T cells and autoantibodies potentially involved in the pathogenesis of IIM and ASyS. There is still a gap of knowledge concerning autoreactivity of T cells and their epitopes in IIM. A better understanding and characterization of T cells could shed light on the pathogenesis and potential new therapeutic options. In addition, we wanted to explore the mechanisms involved in T cell migration and persistence in the skeletal muscle of patients with IIM.

Since this autoimmune disease is multifactorial and different genetic and environmental mechanisms are likely to play a role in the disease, we also wanted to include the relationship between the *HLA* and myositis-specific autoantibodies in the study. Our research group has previously investigated the presence of a muscle-specific autoantibody in IIM, the anti-FHL1 autoantibody. Therefore, we aimed to validate its presence and determine the clinical relevance of anti-FHL1 in an independent cohort. Furthermore, we investigated the relationship between the anti-FHL1 autoantibody levels and disease activity in a longitudinal cohort of patients with IIM in Sweden.

Specific aims of the studies included in the thesis:

In **Paper I**, we aimed 1) to study the presence, reactivity and phenotype of HisRS $CD4^+$ T cells in PBMCs; 2) to evaluate the presence of antigen-specific T cells in the BALF, and 3) to assess the specific presence of anti-Jo-1 autoantibodies in the lungs of patients with IIM/ASyS.

In **Paper II**, we aimed to determine the prevalence and associations of autoantibodies targeting anti-FHL1, a muscle-specific autoantigen, in South Australian patients with histologically-confirmed IIM.

In **Paper III**, we aimed to investigate the co-occurrence of autoantibodies in patients with IIM and to estimate the relationship between autoantibody-defined subgroups, clinical manifestations and *HLA* alleles.

In **Paper IV,** we aimed to investigate the presence and phenotype of local infiltrating T cells in patients with IIM using single cell mRNA sequencing.

In **Paper V**, we aimed to determine the prevalence and clinical associations of anti-FHL1 autoantibodies in a Swedish cohort of patients with IIM. Additionally, we evaluated the presence of anti-FHL1 autoantibodies levels across the course of the disease

4 MATERIALS AND METHODS

4.1 PATIENT COHORTS, DATA SOURCES AND CLINICAL INFORMATION

In **Paper I** we included peripheral mononuclear cells (PBMC), sera and BALF from patients with IIM, sarcoidosis and healthy controls (HC). Additionally, lung biopsies from patients with IIM and chronic obstructive pulmonary disease (COPD) were analysed. The samples were obtained from patients diagnosed in Sweden and attending Karolinska University Hospital. The lung biopsies were retrieved from regional biobanks.

Paper II consists of samples from patients with IIM from the South Australian Myositis Database (SAMD) managed at the Royal Adelaide Hospital Rheumatology Unit in South Australia. This cohort consists of patients with a histologically confirmed diagnosis of IIM. It also includes a group of systemic sclerosis (SSc) as comparators, obtained from the Australian Scleroderma Cohort Study. Additionally, HC sera was provided by the Department of Human Immunology, South Australia Pathology.

Paper III includes patients from the MyoNet registry (formerly called Euromyositis) from recruiting centres from 5 European countries: Salford Royal NHS Foundation Trust (Manchester, United Kingdom), Institute of Rheumatology (Prague, Czech Republic), Karolinska University Hospital (Stockholm, Sweden), Copenhagen University Hospital (Copenhagen, Denmark) and Oslo University Hospital (Oslo, Norway).

Paper IV includes PBMC and muscle biopsy from patients diagnosed with IIM attending Karolinska University Hospital.

For **Paper V**, samples from consecutive patients diagnosed with IIM in Sweden and attending Karolinska University Hospital or belonging to the Swedish Myositis Network (SweMyoNet).

For **Papers I, III, IV** and **V**, clinical information was collected by extracting data from the Swedish Rheumatology Quality Register (SRQ) [317], and the MyoNet registry, and by reviewing patient medical charts [318].

4.1.1 Ethical approvals and considerations

For **Papers I, III, IV and V**, the studies were approved by the Regional Ethical Committee of Stockholm and performed according to the Declaration of Helsinki guidelines on studies with human subjects. All subjects provided written informed consent. For **Paper II**, the study was approved by the Human Research Ethics Committee and the Central Adelaide Local Health

Network (CALHN) Governance. Patients and controls included in the study provided written informed consent for the use of their serum for research purposes. For **Paper III**, each centre received ethical approval according to the Helsinki declaration, and patients gave informed consent to participate in this study.

4.1.2 Classification

In **Paper I,** the classification of IIM was according to the Bohan and Peter criteria [137, 138]. **Paper II,** the diagnosis of IIM and its subgroups was based on the histopathological findings in muscle biopsies. The criteria used by the Anatomical Pathology laboratory at South Australia Pathology were applied according to ENMC and validated criteria [36, 319-322]. The SAMD also includes patients with myositis not-otherwise-specified (MNOS) who have skeletal muscle inflammation though insufficient features to satisfy a diagnosis of PM, DM, IBM, or IMNM. **Paper III, IV and V,** the classification of IIM was done according to the 2017 EULAR/ ACR classification criteria [146]. Griggs criteria were applied for IBM [140].

4.1.3 Definition of clinical data

Myopathic muscle weakness was defined as objective subacute symmetric muscle weakness, proximal more than distal, sparing the eye and facial muscles. Muscle involvement was considered if at least one of the following were present: muscle weakness (MMT-8 <80 or impaired muscle endurance by functional index-2), increased muscle enzymes (CK, LDH, ASAT, ALAT), pathological muscle biopsy or myopathic electromyography (EMG). DM rashes included the presence of heliotrope rash or Gottron's papules or signs. The treating physician also assessed the presence of ulceration, calcinosis, Raynaud's phenomenon, and arthritis. Dysphagia was defined as difficulty in swallowing or objective evidence of abnormal motility of the oesophagus. Diagnosis of interstitial lung disease (ILD) was based on the American Thoracic Society criteria (ATS) [323], and was considered present if described on a chest radiograph or computed tomography with abnormal pulmonary function testing.

Anti-synthetase syndrome (ASyS) was defined as anti-tRNA synthetase positivity and $\geq$1 of the following: myositis, Raynaud's phenomenon, arthritis, ILD, fever or mechanic's hands [38]. Immune-mediated necrotizing myopathy (IMNM) was defined according to the 2017 ENMC criteria with the presence of anti-HMGCR or anti-SRP autoantibodies, elevated CK levels and proximal muscle weakness [324]. For the seronegative IMNM, a muscle biopsy with dominant myofibre necrosis and a paucity of muscle inflammation was required [324].

Overlap myositis was defined as DM or PM diagnosed in the presence of another connective tissue disease. Myositis not-otherwise specified (MNOS) includes patients with skeletal muscle inflammation but who do not satisfy the diagnosis of PM, DM, IBM, ASyS or IMNM.

The diagnosis of mixed connective tissue disease (MCTD) was defined according to the Alarcón-Segovia diagnostic criteria consisting of the presence of anti-U1RNP autoantibodies and ≥3 of the following: hand oedema, synovitis, myositis, Raynaud's phenomenon or acrosclerosis [325].

For patients followed at the Rheumatology clinic, Karolinska University Hospital from 2003 onwards, information on disease activity and damage was evaluated prospectively at regular clinical visits following diagnosis of IIM and recorded in the SweMyoNet register. Disease activity was assessed as proposed by the International Myositis Assessment Collaborative Studies Group (IMACS), including patient global disease activity (PGA), physician global disease activity (PhyGA), serum levels of creatine kinase, manual muscle test-8 (MMT-8), health assessment questionnaire (HAQ) and extra-muscular disease activity using the Myositis Disease Activity Assessment Tool (MYOACT). A visual analogue scale from 0-10 cm (where 10 was the worst), was used for measuring PGA and PhyGA. For assessment of damage the Myositis Damage Score (MYODAM) was used [260, 261].

Myositis-specific autoantibodies (MSAs) (anti-Jo-1, anti-PL7, anti-PL12, anti-OJ, anti-EJ, anti-Mi2, anti-MDA5, anti-NXP2, anti-TIF1γ, anti-SAE1, anti-SRP) and myositis-associated autoantibodies (MAAs) (anti-Pm-Scl, anti-Ro52, anti-Ro60, anti-Ku, anti-cN1A and anti-U1RNP) were analysed using one or more of 1) immunoprecipitation, 2) Euroline myositis panel 4 by Euroimmun, Lübeck, Germany or 3) enzyme-linked immunosorbent assays. Smoking status was defined as ever or never smoker. Treatment information was recorded at the time of sampling.

4.1.4 Cohorts included in each study

Papers I, II, III and **V** are retrospective studies and **Paper IV** is a prospective study. For **Papers III** and **V**, clinical data were prospectively collected in registries and then retrieved for this study.

Samples for **Paper I** and **V** included serum samples collected in our biobank. For **Paper I**, BALF was retrospectively identified from individuals with IIM, sarcoidosis and HC from the Division of Respiratory Medicine, Karolinska Institutet according to a previously published protocol [326]. **Paper II** included serum samples collected from the South Australian Myositis Database. For **Paper IV**, fresh samples from blood and muscle were collected, processed, sorted and stored within 6 hrs in recently diagnosed patients or at follow-up after treatment. All samples were stored at -80°C.

Paper I Cohort: PBMCs were obtained from IIM/ASyS anti-Jo-1$^+$ (n=15; 6 male, 9 female) and anti-Jo-1$^-$ (3 female) patients. From six of these patients, bronchoscopy with paired BALF and BALF cell pellets were obtained (3 anti-Jo-1$^+$ and 3 and anti-Jo-1$^-$). Additional cell depleted BALF samples were obtained from four anti-Jo-1$^+$ (1 male, 3 female) and four anti-Jo-1$^-$ (1 male, 3 female) IIM/ASyS patients. Paired PBMCs and BALF cell pellets samples were retrieved from sarcoidosis patients (n=7; 6 male, 1 female).

Sera from IIM/ASS patients with anti-Jo-1$^+$ (n=17; 6 male, 11 female) and anti-Jo-1$^-$ (n=7; 1 male, 6 female) were obtained. Additional sarcoidosis patients with paired samples of sera and cell depleted BALF were collected (n=9; 4 male, 5 female). Fresh PBMCs were obtained from buffy coat from healthy donors that expressed at least one copy of the *HLA-DRB1*03* allele (n=12; 3 male, 9 female). Lung tissue specimens were retrieved from regional biobanks in Sweden from patients with IIM/ASS (n=14, 4 anti-Jo-1$^+$ and 10 anti-Jo-1$^-$) and comparator cases (n=10; chronic obstructive lung disease, COPD). A graphical representation of the cohort from **Paper I** is presented in **Figure 9.**

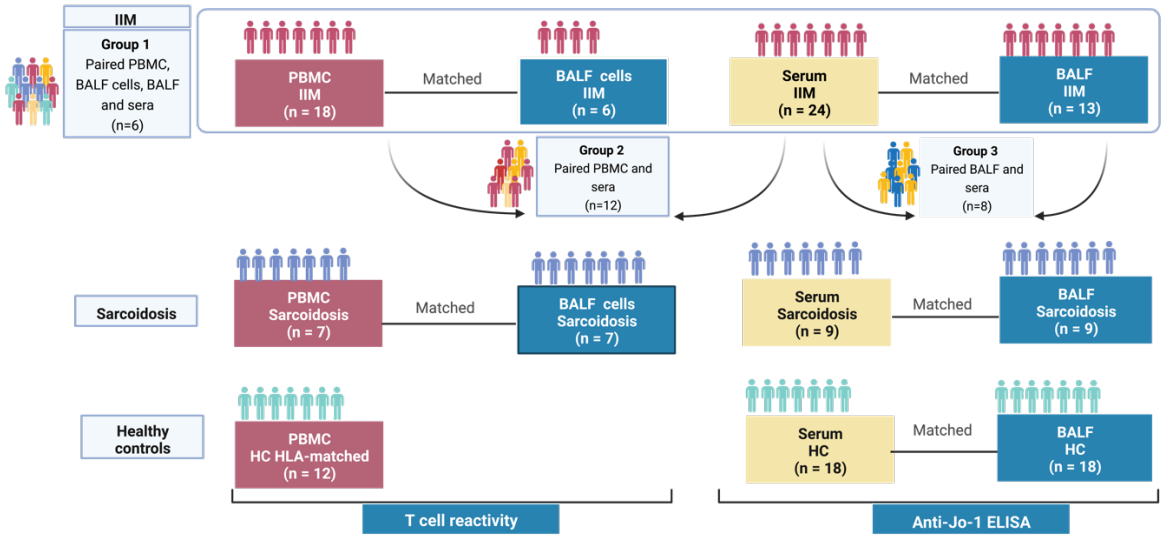


Figure 9. Cohort description of Paper I. PBMC, Peripheral blood mononuclear cell; BALF, bronchoalveolar lavage fluid; IIM, idiopathic inflammatory myositis; HC, healthy controls. Created with Biorender.com

Paper II Cohort: The study comprised sera and clinical data retrospectively collected from 267 adult patients diagnosed with IIM. As a comparator group sera and clinical data from 174 patients with SSc who fulfilled the 2013 ACR/EULAR classification criteria [327]. Additionally, 100 HC provided by the Department of Human Immunology, SA Pathology were included.

Paper III Cohort: We analysed sera, *HLA* alleles and clinical data from 1348 patients withIIM patients that presented complete autoantibody profiles (14 measured autoantibodies) from 5 European countries: United Kingdom, Czech Republic, Sweden, Denmark and Norway.

Paper IV Cohort: Muscle biopsies and peripheral blood from 15 patients with clinical signs of muscle weakness and suspicion of IIM were collected at time of the diagnostic work-up at the Karolinska Hospital Rheumatology Clinic. Out of the fifteen patients, one was later diagnosed with *COL6A2* myopathy, and one patient was diagnosed with Becker muscular dystrophy based on whole exome sequencing. In six patients with IIM and one patient with *COL6A2* myopathy, the presence of immune cell infiltrates in muscle tissue was confirmed by flow cytometry and histology and included for further analyses. (**Figure 10**)

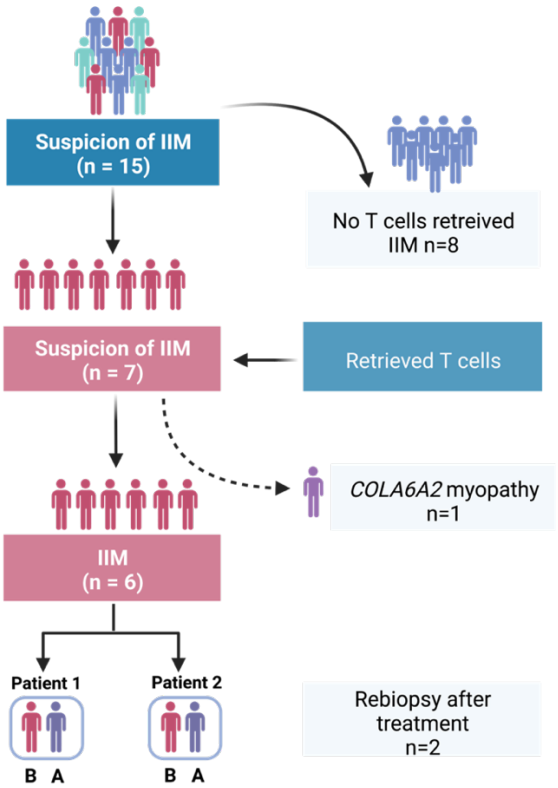


Figure 10. Cohort description of Paper IV. Isolation of T cells from PBMCs and muscle tissue. From 15 patients, available T cells were obtained from seven patients: patients with suspicion of IIM n=7, and 1 patient with *COL6A2* myopathy. Samples were retrieved after treatment in n=2 IIM patients. IIM, idiopathic inflammatory myositis. PBMC, Peripheral blood mononuclear cell. B, before; A, after. Created with Biorender.com

Paper V Cohort: The study included 449 adult patients with IIM from the Swedish myositis registry (SweMyoNet). For the disease comparator groups, we included serum samples from patients with Sjögren's syndrome (SS) n=100 according to the 2002 American-European consensus Group (AECG) and 2016 ACR/EULAR classification criteria for primary Sjögren's syndrome [328, 329]; systemic sclerosis, (SSc) n=10 who fulfilled the 2013 ACR/EULAR classification criteria [327]; rheumatoid arthritis (RA) n=10 according to the ACR/EULAR 1987 or 2010 criteria [330, 331]; and systemic lupus erythematosus (SLE) n=10, according to the 1997 ACR criteria [332]. In addition, we included patients with neuromuscular

disorders (NMD) n=16, with the following diagnoses: spinal muscle atrophy type 2 (n=1), limb girdle muscle atrophy (n=3), mitochondrial disease (n=1), myotonic dystrophy type 1 (n=3), congenital myasthenia (n=1), and motor neuron disease (n=1) and non-specified (n=6) from the Division of Neurology, Department of Biomedical and Clinical Sciences, Linköping University. Furthermore, we included population-based controls without a rheumatic disorder (HC, n=100). Longitudinal samples were obtained from patients with IIM from anti-FHL1+ (n=57) and anti-FHL1- (n=30) autoantibodies. In a subgroup of 42/57 anti-FHL1+ and 25/30 FHL- IIM patients, longitudinal clinical outcome measurements and histopathological features in muscle biopsies at baseline were analysed (**Figure 11**)

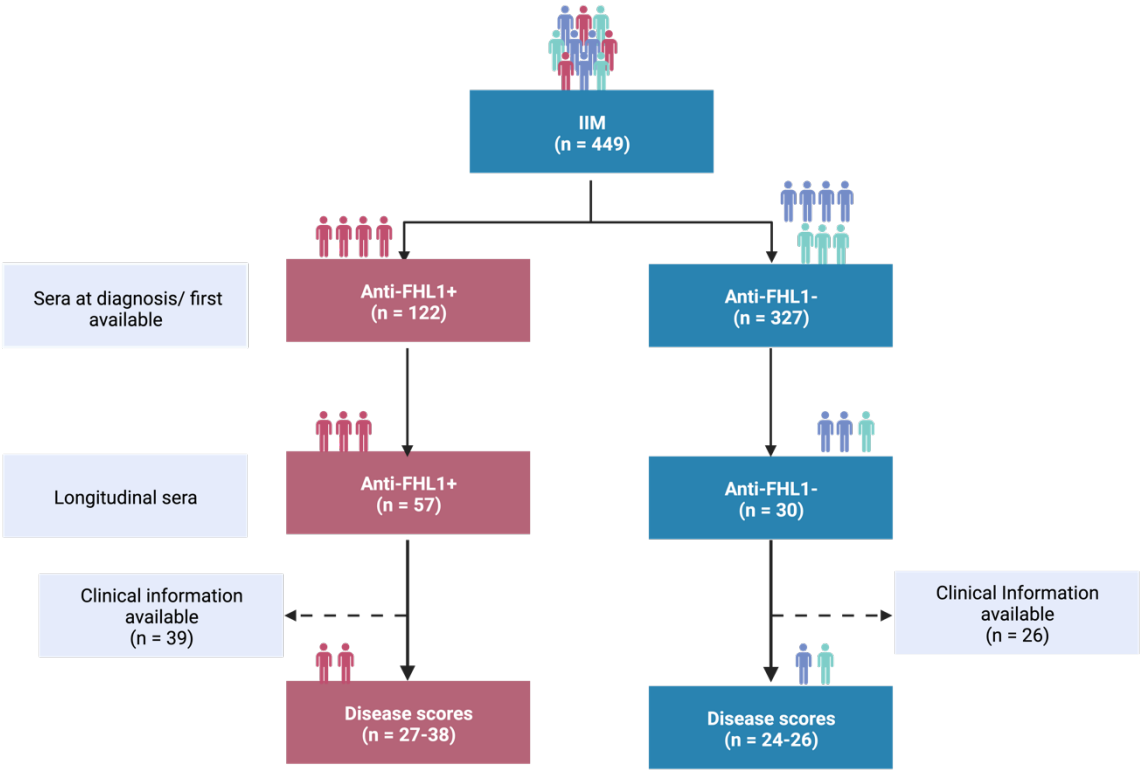


Figure 11. Cohort description of Paper V. Baseline and longitudinal analysis of IIM patients for the presence of anti-FHL1 autoantibodies. IIM, idiopathic inflammatory myositis. Created with Biorender.com

4.2 METHODOLOGY OF *IN VITRO* EXPERIMENTS

Functional T-cell Assay (Paper I). Fresh samples were used for all the analyses. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood of healthy donors, IIM and sarcoidosis patients. The samples were prepared by centrifugation over Ficoll-Hypaque gradients. PBMCs were re-suspended in 96 well plates in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum (1×10^6 cells/well) according to an adapted protocol [333]. Cells were incubated for five days with recombinant human full-length HisRS (20 µg/ml, Bio Supply), with the candidate peptide $HisRS_{11-23}$ (20 µg/ml, sequence: VKLQGERVRGLKQ, GenScript) or with medium as a negative control. Cells were re-stimulated on day 5 for 8 hours, and Brefeldin-A (10 µg/ml, Sigma-Aldrich) was added for the last 6 hours. Stimulation with Staphylococcal enterotoxin B (SEB, 10 µg/ml, Sigma-Aldrich) was used as a positive control.

Following stimulation, cells were stained with LIVE/DEAD Fixable Green Dead Cell Kit, antibodies towards CD14, CD3, CD4, CD40L, IFNγ, IL-2, and IL-17A. In some experiments, CXCR3, CCR5 and CCR6 antibodies or blocking antibodies for HLA-DR or HLA-DQ were used. Details on antibodies used are specified in **Table 8**. Cells were run on a Gallios Analyser (Beckman Coulter), and data were analysed using FlowJo software, version 7.5.1 (Tree Star, Inc). The gating strategy is presented in **Figure 12**.

Table 8. Antibodies used for flow cytometry analysis in functional assays

Product	Clone/Catalog No.	Source
BD cytofix/cytoperm		BD Biosciences
Brefeldin A	B6542	Sigma-Aldrich
CCR5 PE	45523	R&D Systems
CCR6 APC	53103	R&D Systems
CXCR3 BV421	49801	R&D Systems
CD14 FITC	345784	BD Biosciences
CD3 Pacific blue	300431	BD Biolegend
CD4 APC-H7	RPA/T4	BD Pharmigen
CD40L PE	555700	BD Pharmigen
IFNγ PE-Cy7	4S.B3	Life Technologies
IL-2 Alexa Fluor 647	MQI-17H12	Biolegend
IL-17A PerCP-Cy5.5	BL168	Biolegend
$HisRS_{11-23}$ peptide		Genscript
LIVE/DEAD Fixable Green Dead Cell Stain Kit	L23101	Life Technologies
Recombinant human full-length HisRS	ENZ-268	Bio Supply UK Limited
Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus*	S4881	Sigma-Aldrich

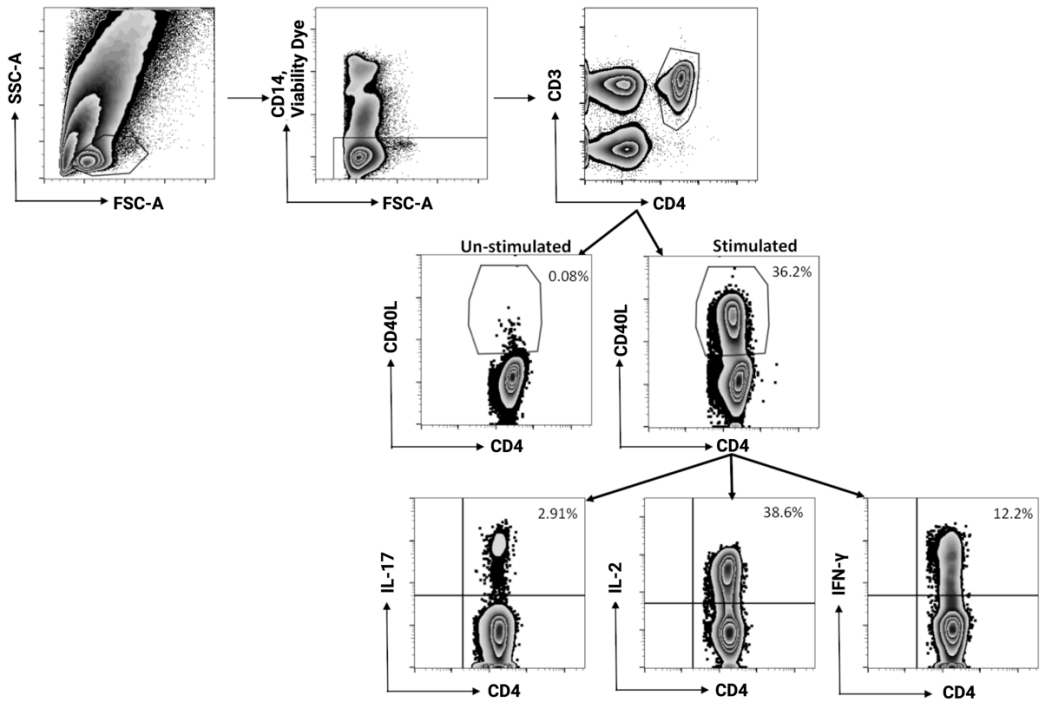


Figure 12. Gating strategy. Different leukocyte populations were assessed using a forward/sideward scatterplot (FSC/SSC). A gate was set around the leukocytes, followed by the removal of monocytes ($CD14^+$) and dead cells. The T cell gate was obtained by gating $CD4^+$ versus $CD3^+$ cells; activated cells ($CD40L^+$) were obtained in a third gate within the $CD3^+$ $CD4^+$ population. HisRS protein and $peptide_{11-23}$ specific T cell cytokine responses were determined as signal above the signal background as determined on the un-stimulated cells of the same individual. *Supplementary Figure 1 from* ***Paper 1***. *Reprinted after permission from John Wiley and Sons.*

4.3 IMMUNOHISTOCHEMISTRY AND HISTOPATHOLOGIC EXAMINATION (PAPER I)

Paraffin-embedded lung tissue retrieved from Swedish regional bio-banks of anti-Jo-1^+(n=4), anti-Jo-1^-(n=10) IIM/ASS patients and comparative tissues of COPD patients (n=10; chronic obstructive lung disease) were analysed by immunohistochemistry according to a previously published protocol [334]. Deparaffinized slides from lung tissue biopsies were treated with Tris-EGTA buffer for 15 min in the microwave for antigen retrieval, followed by endogenous peroxidase blocking with 3% H_2O_2 in Tris-buffer saline (TBS) for 20 min. After that, slides were treated with Avidin-Biotin blocking solutions (Dako) and blocked with 3% skim milk/poly-L-lysin/TBS for 1hr at room temperature. Primary antibodies against CD3 (Thermo Fisher), CD138 (Dako), HisRS-C-terminal (Abcam), CXCR3 (R&D Systems), CCR5 (R&D Systems) or isotype controls rabbit IgG (Cell Signaling), IgG2b (Dako), IgG1(R&D Systems) were diluted in 0.5% skim milk/TBS and incubated overnight at 4ºC followed by adding biotinylated secondary antibodies for 1hr at room temperature. Vectastain ABComplex (Vector Laboratories) in TNB buffer (TBS containing Du Pont Blocking Reagent, PerkinElmer) was added for 30min followed by the addition of tyramide signal amplification reagent (TSA Perkin-Elmer) for 6 min and finally Vectastain ABComplex for 30min (all steps at room temperature). Development was done with 3,3′-Diaminobenzidine (Sigma), and nuclei were visualized by hematoxylin staining.

4.4 HLA TESTING (PAPERS I AND III)

HLA alleles and amino acid determination: HLA-DRB1, HLA-DQA1, HLA-DQB1 alleles were directly genotyped by sequence-specific primer PCR assay (DR low-resolution kit; Olerup SSP)[335] or genotyping by Illumina's protocols in the UK (Centre for Genetics and Genomics Versus Arthritis, University of Manchester, UK) [336]. Additionally, amino acid positions for the former molecules and *HLA-B*, *HLA-C* and *HLA-A* were imputed with the SNP2HLA software using single nucleotide variations extracted from target sequencing by The Dissect Consortium and The Immunoarray Development Consortium [78, 337, 338] using the T1DGC study as reference panel [339]. The amino acid imputation was available only for the UK and Scandinavia (i.e., Sweden, Denmark, and Norway).

4.5 RECOMBINANT PROTEIN PRODUCTION (PAPERS I, II AND V)

The study of proteins and their biotechnological application frequently involves their isolation from other cellular components. Recombinant proteins can be produced in different expression systems, including bacteria, yeast or mammal cells, by the insertion of the coding sequence for the protein of interest. Currently, the production of proteins into a prokaryotic host, such as *E. coli*, is one of the preferred methods due to its low cost and high yield of production [340]. However, it is important to consider some post-translational modifications will not be present, since these are generally not performed by bacteria [341].

From gene cloning to protein purification, numerous alternatives are available that, in the end, might affect the functionality of the recombinant protein. Many factors can influence this outcome, such as protein toxicity to the expression host, aggregation in inclusion bodies or misfolded protein [340]. In this thesis, FHL1 has been recombinantly produced in *E. coli* and mammalian cells by collaboration with Structural Genomics consortium (SGC) and Euroimmune. To further extend our analysis, we performed some experiments with FHL1 produced in yeast. Also, biotinylated recombinant HisRS protein was produced using a bacterial expression system. Additional cloning and protein purification were produced during this PhD project, such as HLA-DRB1*03 tetramers in collaboration with SciLifeLab and Monash University.

Recombinant full-length human HisRS protein (Paper I): The recombinant HisRS full-length (amino acids 1-509) was expressed in *E.coli* (expression strain BL21(DE3)R3 pRARE2). The construct was subcloned by ligation-independent cloning into expression vector pNIC-Bio3 (Genebank no. JN792439) and contained an Avi-tag enabling site-specific biotinylation using co-expression of BirA and hexahistidine (His_6) tag to enable immobilized metal affinity chromatography (IMAC) purification [342]. The protein was purified using a two-step method including IMAC followed by size exclusion chromatography. Mass

spectrometry and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) confirmed a purity >95% and biotinylation levels [342]. The production of recombinant HisRS full-length is described in a previous published protocol [343].

Recombinant full-length human FHL1-protein (Paper II and V): The recombinant, full-length human His-tagged, MaBP-tagged and biotinylated FHL1-protein (amino acids 1-280, isoform 1) were produced in *E. coli* (expression strain BL21(DE3)Ripl). For His-tagged FHL1 and MaBP-tagged FHL1, the construct was cloned with restriction enzymes into expression vector pET28a (Novogen) or pMAL-cRI (New England Biolabs). Biotinylated FHL1 was produced following the same protocol as HisRS. The proteins were purified using a two-step method including IMAC followed by size exclusion chromatography. Mass spectrometry and SDS-PAGE confirmed a purity >80-95% [342]. Recombinant full-length human His-tagged FHL1 protein expressed in *yeast* was acquired from Mybiosource, Inc (San Diego, CA, USA) and Recombinant HEK293 His-tagged FHL1 protein was provided by Euroimmune (Luebeck, Germany).

Internal control proteins: We evaluated the reactivity against irrelevant proteins with a molecular weight similar to FHL1 and underwent a similar purification process. The controls proteins used were: a) Sacsin (SACS, amino acids 1-339 in vector pNIC-Bio3), b) SprT-like N-terminal domain (SPRTN, amino acids 1-216 in vector pNIC-Bsa4, including a His-tag), and c) CDAN1 interacting nuclease 1 (C15ORF41A, amino acids 1-281 in vector pNIC-Bsa4, including His-tag).

HLA-DRB1*03 tetramer production: Another approach to detect the presence of antigen-specific $CD4^+$ T cells has been through the use of peptide-HLA monomers or peptide-HLA tetramers, which can bind to T cell receptors. This methodology increases the sensitivity in the detection of autoreactive T cells, where some studies have reported a low frequency as 0.02% [344]. Since peptide-HLA monomers can be more unstable and may exhibit a higher degree of dissociation, we decided to produce peptide-HLA-class II (HLA-DRB1*03) tetramers. Moreover, peptide-HLA tetramers have a superior avidity compared with peptide-monomers [344]. For the production of HLA-DRB1*03, several experiments were undertaken to achieve the highest yield and stability. Different cell lines were tested, including the expression in S2 insect cells [345], *E. coli* [346] and HEK293 cells [347]. Various peptides from HisRS were examined, and the construct with the highest binding affinity to the HLA-DRB1*03 was selected. As an internal control, the $tetanus_{506-525}$ peptide was used (NYSLDKIIVDYNLQSKITLP). Peptides were synthesized by GenScript, reconstituted in ultra-pure H_2O at a concentration of 10 mg/ml, and stored at -20 C in aliquots until used.

The study of low-affinity peptide and HLA-peptide-TCR interactions in this autoimmune disease has been challenging and required standardization of several protocols [348]. For the evaluation of the presence of *in vivo* and *ex vivo* antigen specific-T cells, isolated recombinant α and β chains were expressed with the antigenic peptides (HisRS peptide and tetanus$_{506\text{-}525}$) covalently-bound to increase the stability of the HLA-peptide complex. HLA molecules were associated with different fluorochromes and the T cell samples were analysed by flow cytometry according to a previously published protocol [349]. This approach will allow us to characterize the phenotype of the antigen-specific T cells and, at a single level, allow the characterization of the TCR sequence.

4.6 ELISA (PAPER I, II AND V)

From a clinical perspective, identifying autoantibodies has a central role in diagnosing and classifying autoimmune diseases [350]. The recognition of an antigen/autoantibody depends on the interaction between the antibody and its antigen. Several methodologies have been used for this purpose such as enzyme-linked immunosorbent assay (ELISA). One of the common types of ELISA is the so-called “Sandwich ELISA”, which initially involves the immobilization of an antigen to a solid surface and the addition of a specific antibody that can bind to the target antigen. The specific antibody can be linked to an enzyme or can be detected by a secondary antibody conjugated with an enzyme. In the final step, an enzymatic substrate is added so the enzyme can convert to a colorimetric signal that can be measured with a spectrophotometer. Between these steps, several washing procedures are performed to remove the excess in either antigen and/or antibodies, non-specifically bound proteins and other molecules that could give a false signal.

Both native and recombinant autoantigens can be used for ELISA. They can be immobilized on a capture plate via surface adsorption, capturing antibodies or other molecules (e.g. streptavidine), and then incubated with the samples of interest. This technology offers several advantages: a small amount of antigen is needed, multiple sera in the same plate can be analysed, and the antigen-antibody reaction is detected by spectrometric reading [350]. The different types and characteristics of the proteins used during this PhD are described in **Table 9**.

Table 9. Protein information regarding expression, amino acid coverage and molecular weight of proteins used in ELISA

Protein ID	Expression	UniProt ID	Amino acid coverage	Molecular weight (Da)
HisRS full-length	*E.coli*	P12081	M1-C509	60 019
His-tagged FHL1	*E.coli*	Q53FI7	M1-L280	35 714
MaBP-tagged FHL1	*E.coli*	Q53FI7	M1-L280	62 000
biotinylated FHL1	*E.coli*	Q53FI7	M1-L280	36 838
His-tagged FHL1	Yeast	Q13642	M1-P323	40 300
SPRTN	*E.coli*	Q9H040	M1-K225	28 925
Sacsin	*E.coli*	Q9NZJ4	M1-K339	43 348
C15ORF41A	*E.coli*	Q9Y2V0	M1-A281	34 802
biotinylated C15ORF41A	*E.coli*	Q9Y2V0	M1-E324	37 193

4.6.1 Detection of anti-HisRS full-length IgG autoantibodies by ELISA (Paper I)

HisRS ELISA was used to detect anti-HisRS full-length IgG autoantibodies in serum and BALF by adding recombinant full-length HisRS protein at 0.5 µg to 96 well plates. Bound antibodies were detected by alkaline phosphatase–conjugated anti-human IgG (Anti-human IgG-AP Dako). Phosphatase substrate tablets (Sigma) dissolved in diethanolamine buffer, pH 9.6, were used as a substrate and incubated for up to 60 minutes to detect IgG. Absorbance was measured at 405 nm (A_{405}, SoftMax Pro v4.8, Molecular Devices). All steps were performed at room temperature except for coating. To quantify the anti-Jo-1 autoantibody levels, an anti-Jo-1 IgG purified standard curve from a pool of sera from 38 patients with IIM and anti-Jo-1 autoantibodies was employed.

4.6.2 Detection and validation of anti-FHL1 IgG autoantibodies by ELISA (Paper II and V)

FHL1 ELISA was used to detect anti-FHL1 autoantibodies in serum. Recombinant, full-length human His-tagged FHL1-protein was coated at 0.25µg on a 384-well high-binding plate (Corning). Plates were thereafter incubated overnight at 4°C with reducing buffer, pH 6.8 (100 mM Tris-HCl, 50 mM NaCl, 10 mM dithiothreitol); washed with phosphate-buffered saline (PBS) 0.05% Tween (PBST) and blocked with PBST 0.1% bovine serum albumin (BSA, lyophilized powder, Sigma-Aldrich). Patient sera (diluted 1:500 in PBST 0.1%BSA) was added after washing the plates and incubated overnight at 4°C. Polyclonal rabbit anti-human IgG-alkaline phosphatase was added as the secondary antibody and incubated for 2 hrs (1:2000 in PBST, Dako). Phosphatase substrate tablets (Sigma) dissolved in diethanolamine buffer, pH 9.6, were used as substrate, and the optical density (OD) was measured every hour until five hours at an absorbance of 405nm using SpectraMax Plus

384 microplate reader (SoftMax Pro v4.8, Molecular Devices). The schematic presentation for the ELISA protocols is presented in **Figure 13**.

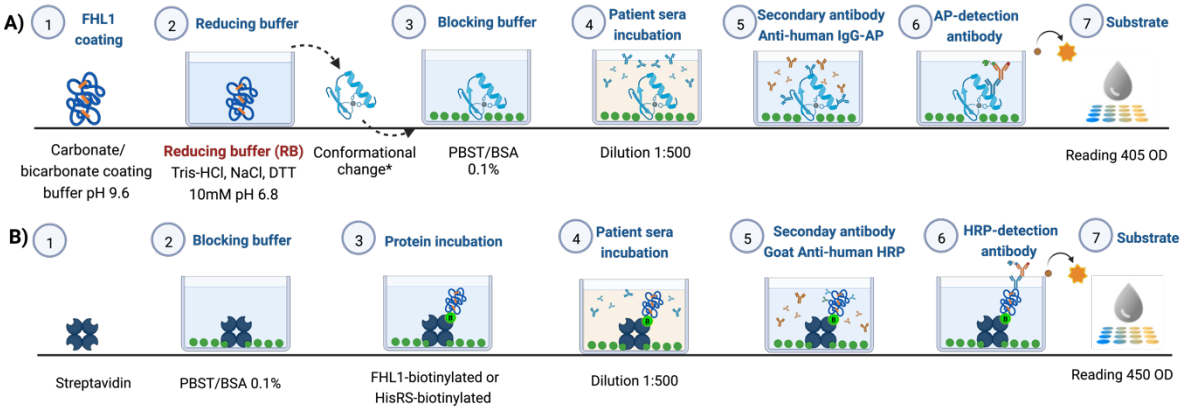
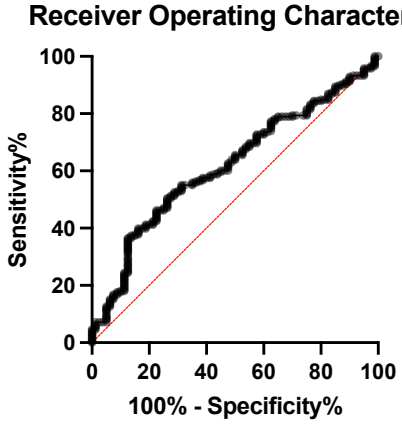


Figure 13. ELISA protocols used. **(A)** Protocol used for analysing His-tagged and MaBP-tagged proteins including reducing buffer **(B)** Protocol for analysing biotinylated proteins. PBS, phosphate-buffered saline; BSA, bovine serum albumin; DTT, dithiothreitol; AP, alkaline phosphatase, RB, reducing buffer. Created with Biorender.com

The OD values were transformed to Arbitrary Units (AU) by interpolating a sigmoidal 4 parameter $\log_{10}(x)$ standard curve consisting of 0.05 to 10 AU, where 1 AU=1:500 dilution of a standard serum sample that was used in all ELISA plates. We used a receiver operating characteristic (ROC) curve, including the HC, to specify a cut-off value for anti-FHL1 autoantibodies (**Figure 14**). The cut-off was set at 1.069 AU according to the Youden index [351]. The performance of the test shows a high specificity, positive predictive value (PPV) and likelihood ratio (LR) when performing the test on a patient with high suspicion of IIM.



Sensitivity: 15.52%
Specificity: 98%
PPV: 95.5%
NPV: 30.3%
LR+: 7.52
LR-: 0.86
AUC: 0.61
95% CI: 0.55-0.68
P value: 0.001

Figure 14. FHL1 ELISA ROC curve. Legends: IIM, idiopathic inflammatory myopathy; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio; AUC, area under the curve; CI, confidence interval.

Optimization and validation of FHL1 ELISA: To evaluate the specificity of the FHL1 ELISA, we compared the performance of the FHL1 protein produced under different conditions: a) recombinant full-length human His-tagged, MaBP-tagged, and biotinylated-FHL1-protein expressed in *E.coli*, b) recombinant full-length human His-tagged FHL1-protein expressed in HEK-293 cells (provided by Euroimmune, Lübeck, Germany), c) recombinant full-length human His-tagged FHL1-protein expressed in yeast (Mybiosource,

Inc, San Diego, CA, USA). Additionally, we tested the ELISA performance in different buffers and purification protocols to control for the presence of protein aggregates and misfolding after freezing/thawing events. Several internal control proteins with similar molecular weight and purification procedures were included. All the results were validated by western blot (WB).

Different anti-FHL1 reactivity according to the protein purification procedure: When comparing the ELISA reactivity between different purification procedures for the His-tagged FHL1, we found no reactivity in the high purity fraction (>90%). Nevertheless, when we tested the "standard" fractions with a purity of 80-85%, the correlation was high between His-tagged and MaBP-tagged FHL1 ($r=0.95$, $p<0.0001$). The WB demonstrated the presence of a band for the His-tagged FHL1 and MaBP-tagged FHL1 upon incubation with sera from IIM FHL1+ patients, suggesting that the loss of reactivity could be associated with a process of aggregation or changes in the conformational structure of the protein during protein purification.

Anti-FHL1 autoantibodies are directed to a conformational epitope: We aimed to evaluate the possibility of restoring the original conformation by using reducing buffers that could allow the protein to regain the original conformation. For this, we tested two different buffers that have been reported to be helpful in the use of proteins with rich cysteine-cysteine interactions and finger domains [352, 353]: Reducing buffer (RB) 1 contained 100 mM Tris–HCl, pH 6.8, 50 mM NaCl, 10 mM dithiothreitol and RB 2: 20mM HEPES, 300 mM NaCl, 5% Glycerol, 0.5mM TCEP. The results showed increased reactivity in the ELISA when the protein was incubated with either one of these two buffers at 4°C overnight. We then compared an FHL1-biotinylated batch with the His-tagged FHL1 to analyse the difference in reactivities in IIM FHL1+ patients and included a recombinant anti-FHL1 single-chain variable fragment (scFv) generated in-house according to a previously published protocol [343]. As expected, the highest reactivity was present in the His-tagged FHL1 incubated with RB and with the anti-FHL1 scFv that was used as an internal control, indicating that the RB could restore the conformation and will be helpful in detection of a signal in ELISA.

Validation of anti-FHL1 ELISA in different systems: We then tested the reactivity of IIM and control samples using the FHL1 produced from *E.coli* and HEK 293 cells, and evaluated their correlation. HEK 293-FHL1 was tested with and without RB. We included IIM FHL1+ ($n=12$), FHL1- ($n=8$), NMD ($n=10$) and HC ($n=15$) that were paired and tested simultaneously for all the conditions. The correlation between *E.coli* and HEK-FHL1 without RB was good ($r=0.62$, $p<0.01$), and it increased after incubation with RB ($r=0.79$, $p<0.001$). When we analysed the performance of the ELISA test between the two conditions in the HEK-FHL1,

we found a lower performance in the non-RB HEK-FHL1 ELISA (Sensitivity 47% vs 58%, specificity 90% vs 72%, LR 2.17 vs 5.17). Interestingly, the comparison between *E.coli* and HEK-FHL1 proteins demonstrated a similar specificity between these two proteins but higher sensitivity and LR in the *E.coli* FHL1-RB (Sensitivity 67%, specificity 90%, LR 7.3).

Additionally, we tested the reactivity of IIM and control samples using the FHL1 produced from yeast and our control FHL1-R from *E.coli*. We included IIM FHL1+ (n=6), FHL1- (n=6), NMD (n=7) and HC (n=9) that paired and tested simultaneously for all the conditions. The correlation between *E.coli* and yeast-FHL1 with and without RB was excellent in both conditions (r=0.96, p<0.0001 and r=0.94, p<0.0001).

4.7 ISOLATION OF TOTAL IgG USING DEVELOPED IN-HOUSE AND PREPARED AFFINITY COLUMNS

Serum samples from three patients with anti-FHL1+ autoantibodies were centrifugated and diluted 1:5 (volume/volume) in PBS. Total IgGs from serum were purified on HiTrap Protein G HP columns (GE Healthcare, Stockholm, Sweden). IgG was eluted with 0.1M glycine-HCl buffer (pH 2.7) and the pH was immediately neutralized with 1M Tris (pH 9). The buffer of eluted IgGs was exchanged to PBS using Dialysis Slide-A-Lyzer (10 000 MWCO, 2hrs), and the concentrations were measured by nanodrop (ThemoFisher Scientific). Isolation protocol and SDS-PAGE image of protein fractions collected are visualized in **Figure 15**.

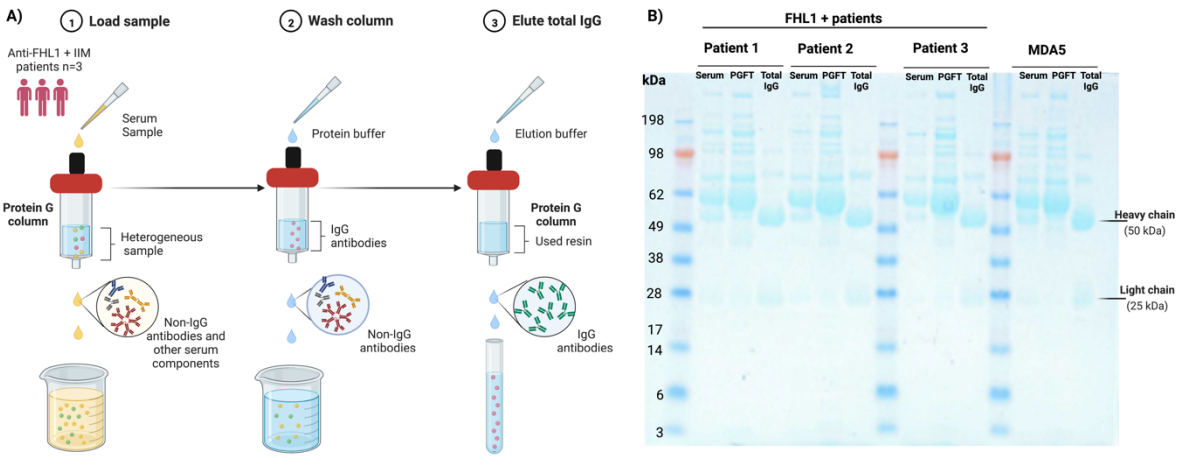


Figure 15. Diagram of the total IgG purified from anti-FHL1+ patients. (A) Purified total IgG from patients with IIM and anti-FHL1+ autoantibodies. 10-12,5 mL of sera was obtained from n=3 patients with anti-FHL1+ autoantibodies. Total IgG was eluted from the protein G column. **(B)** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) image of sera, protein G flow-through (PGFT) and total IgG fraction purification of n=3 anti-FHL1+ and n=1 anti-MDA5 + patient as a control. Bands are visualized by Coomassie blue staining. Created with Biorender.com

4.8 WESTERN BLOT (PAPER V)

Western blot (WB) is a widely used analytical technique for detecting protein expression, analysis of protein-protein interaction and can also be used for searching new biomarkers [354]. This technique uses monoclonal or polyclonal antibodies to identify target proteins through specific antigen-antibody reactions. For this technique, the protein of interest should be run on SDS-PAGE. The negatively charged and denatured proteins by SDS are separated into a gel matrix according to their molecular weight before being transferred into a nitrocellulose or PVDF membrane sheet. After blocking the membrane, a primary antibody is added for specific detection of the target. Then, a secondary antibody conjugated with horseradish peroxidase or alkaline phosphatase and the substrate is added for the detection. Washing steps are needed between the incubation processes to remove the excess antibodies and limit a high background signal. WB was used to test the reactivity of the anti-FHL1 autoantibodies from sera and total IgG from patients with IIM to the different recombinant FHL1 proteins.

4.9 HUMAN MUSCLE DIGESTION AND SORTING (PAPER IV)

Muscle biopsies were collected in RPMI from vastus lateralis or tibialis anterior muscles using a semi-open approach previously described [355]. Fresh muscle biopsies were digested with liberase (Roche, 0.25 mg/ml) or collagenase A (4 mg/ml) and DNAse I (0.5 mg/ml Sigma Aldrich) for 60min at 37°C and stained with infra-red viability dye (Invitrogen) and fluorescently coupled antibodies. T cells isolated from the muscle ($CD45^+CD3^+$) and $CD3^+$ memory T cells from the blood (excluding naive $CCR7^+CD45RA^+$ T cells) were single-cell sorted using an influx sorter (BD) in the same plate to minimize batch effects.

4.10 SMART-SEQ2 SINGLE-CELL mRNA LIBRARY PREPARATION AND SEQUENCING

RNA-sequencing libraries were generated with the Smart-seq2 protocol as previously described [356] at the Eukaryotic Single-cell Genomics facility at Science for Life Laboratory in Stockholm, Sweden. Libraries were sequenced on an Illumina NovaSeq 6000 (2*100bp) at NGI Stockholm sequencing core facilities, Science for Life Laboratory in Stockholm, Sweden.

Sequence demultiplexing and quality controls were performed in the sequencing facility. The indexes of the reads were trimmed using TrimGalore v0.4.4. Reads were mapped using STAR v2.5.4b to the human genome assembly hg38 plus exon-exon junctions from gencode v21 (--sjdbGTFfile). We calculated reads per kilobase of the transcript, per million mapped reads (RPKM) expression values using RefSeq gene annotation (from the UCSC genome browser 10 Sep 2017), and the rpkmforgenes.py software (13 Mar 2015 version) with settings -minqual 255 -rmnameoverlap -fulltranscript. An additional layer of normalization

was applied on top of RPKM values using the R module scran v1.8.2, with a clustering size of 30. Cells with a negative size factor from scran, typically due to expressing very few genes, were filtered out. T cell receptor sequences were assembled using TraCeR v0.5.1[357]. A clone was defined by at least two cells sharing the same productive CDR3 amino acid sequence from both TCRα and TCRβ chains.

The pipeline of the experimental steps and bioinformatic analysis are represented in **Figure 16**.

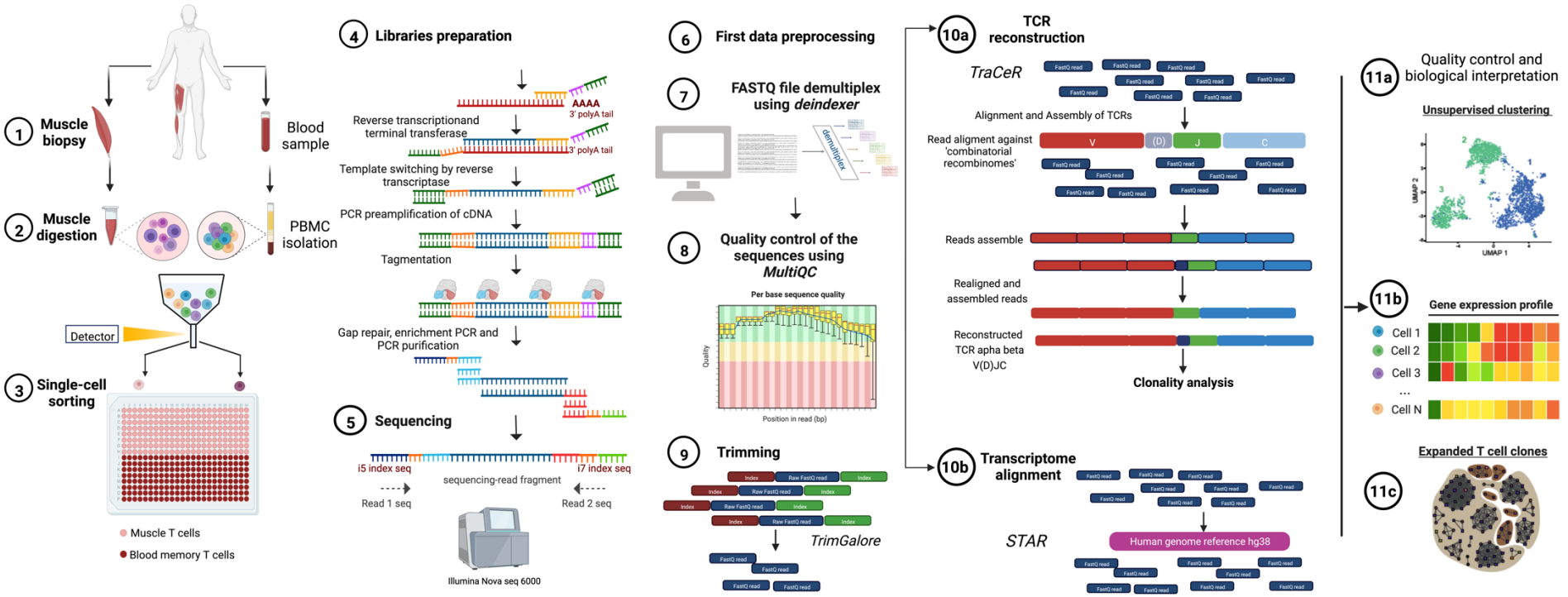


Figure 16. Single cell mRNA sequencing in blood and muscle T cell in patients with IIM. Steps (1-3): Technical workflow including isolation of memory T cells from PBMC (red) and muscle T cells (pink) from muscle biopsies, followed by cell sorting and plate acquisition. **Steps (4-5)**, Smart-seq2 single cell mRNA sequencing. **Steps (6-10):** Pipeline for analysis of data**.** The script for de-multiplexing of Fastq files was provided by Daniel Ramsköld. The pipelines were developed using MixCR [358] and TraCer [357]. (**10a)** Courtesy of Lina-Marcella Díaz-Gallo. **Step (11)**, analysis presented using **(11a)** unsupervised clustering and visualized using the uniform manifold (UMAP) method. **(11b)** Gene expression profile, and **(11c)** expanded cell clones. IIM, idiopathic inflammatory myopathies; PBMC, peripheral blood mononuclear cell; STAR, Spliced Transcripts Alignment to a Reference; TCR, T cell receptor.

4.11 STATISTICAL ANALYSIS

For **Papers I-V**, Descriptions of continuous variables are expressed as mean or median and standard deviation (SD) or interquartile range (IQR). Categorical variables are presented as frequencies and percentages. Differences between groups were analysed using Student's t-test or Mann-Whitney U test, depending on the normality of data for continuous variables, and Chi-squared or Fisher's-exact test for categorical variables. Wilcoxon's signed-rank test was used to compare different longitudinal samples and the baseline AU value. Kruskal-Wallis test with Dunn's correction was used for comparing the AU values of the different groups. Spearman correlation was used to correlate different FHL1 protein batches in ELISA reactivity and to analyse the longitudinal associations between FHL1 levels, MMT-8, CK ratio, disease activity and damage at baseline, second- and third-follow-up, follow-up at 3-5 years and last recorded visit. Pearson's correlation was performed to determine the association between anti-FHL1 autoantibody levels and laboratory parameters. The CK ratio was calculated using the CK value divided by the upper limit of the normal value. Two-tailed p-values <0.05 were considered statistically significant. The statistics were calculated with GraphPad Prism 9.0 (GraphPad Software), IBM SPSS Statistic v28.

In **Paper III**, the cluster analysis was performed with patients having at least one positive autoantibody. Patients without positivity to the included autoantibodies were considered a distinct subgroup. Autoantibody-based subgroups were identified using the Gower distance matrix and partition around medoids cluster calculation [359, 360]. The number of subgroups was chosen using the Silhouette metric and visualized with t-Distributed stochastic neighbour embedding (t-SNE). Logistic regressions adjusted for age, sex and centre were used to estimate the p-value, odds ratios (OR) and 95% confidence intervals (CI) of having specific clinical manifestations, *HLA-DRB1, HLA-DQA1, HLA-DQB1* alleles and imputed amino acid frequencies depending on the subgroup assigned.

The models for clinical associations were further adjusted for recruiting centres, while the models for genetic associations were applied separately by regions and subsequently meta-analysed. The Cochran-Mantel-Haenszel test was used to calculate the OR (95% CI) for the meta-analyses and heterogeneity calculated utilizing Cochran's Q test and Higgins' test (I^2). The genetic association p-values were adjusted by the Benjamini & Yekutieli (2001) step-up method for false discovery rate (FDR) [361]. We considered significant results at a 5% FDR. The regions were divided into the UK, Scandinavia (Sweden, Denmark, and Norway) and the Czech Republic. The genetic association analyses were performed using PLINK (version 1.9) [362]. All other statistical analyses were performed using R versions 3.6.1 and 4.1.1[363], notably with the cluster [364], survival [365] and survminer [366] packages.

In **Paper IV**, the bioinformatic analyses were performed in R (v4.1.1) with the Seurat package v4.1.0. Ribosomal reads were excluded from the data since they are often technical artifacts or housekeeping transcription activity. We filtered out cells expressing <1 000 or >8 000 genes, and <600 000 or >1 500 000 molecules. Cells with mitochondrial DNA representing >35% of the whole cell genome were also filtered out. Cells expressing *CD3E*, *CD3D* or *CD3G* less than 5 RPKM and *CD14*, *CD19*, *CD22* or *CD300E* more than 10 RPKM were considered non-T cell contaminants and were excluded. We also removed cells that expressed two TCRα or two TCRβ chains. Alpha, beta, gamma or delta chain of the T cell receptor genes were also filtered out to not introduce a bias during clustering. TCR V(D)J sequences were added into the single cell Seurat object as metadata. The expression values were normalized using "NormalizeData" function from Seurat.

Principal component analysis was performed for blood and muscle, and elbow plots were used to select the number of principal components included in the subsequent cluster analyses (n=20). Using these principal components, we corrected for patient specific batch effects with the harmony package (version 0.1.0). The data was divided based on the treatment status and cells from the second biopsy filtered for the first analysis. Clusters were identified with "FindClusters" function from Seurat Package using the Louvain algorithm. Clustering was visualized using the uniform manifold (UMAP) method. The differential gene expression was calculated between a given cluster and the rest of the cells to identify the genes that significantly contribute to cluster formation. Clusters were annotated manually based on the top 5 differentially expressed genes.

In **Paper V**, the overall longitudinal associations between anti-FHL1 autoantibody levels and clinical core set measures, disease activity and damage were tested by mixed-effect regression models using anti-FHL1 autoantibody levels as the dependent variable. Each core set measure, disease activity item, disease activity composite measure or damage composite measure respectively was considered as independent variable. The patient identity was considered as random effect in anti-FHL1+ patients. All models were adjusted for sex. Furthermore, we investigated if treatment moderated the association between anti-FHL1 levels and clinical outcome measures by including an interaction term between treatment and anti-FHL1 level in the mixed effect regression model.

Due to the non-normal properties of the included variables, the p-values in all mixed effect regression models were estimated by bootstrapping with 2000 repetitions. Two-tailed p-values <0.05 were considered statistically significant. The statistics were calculated with STATA 16 (STATACorp, LP, TX, USA) for data management and statistical analyses.

5 RESULTS

5.1 PAPER I: PRO-INFLAMMATORY HISTIDYL-tRNA SYNTHETASE-SPECIFIC CD4$^+$ T CELLS ARE PRESENT IN THE BLOOD AND LUNG OF PATIENTS WITH IDIOPATHIC INFLAMMATORY MYOPATHIES

Autoantibodies targeting histidyl-tRNA synthetase (HisRS, also known as anti-Jo-1) are one of the most common autoantibodies in IIM. Anti-Jo-1 autoantibodies are clinically associated with anti-synthetase syndrome (ASyS), which is characterized by the presence of interstitial lung disease (ILD) often at early stages of the disease. Multiple epidemiological studies have reported ILD as a leading cause of short-term mortality [52, 53, 367]. Insights from several studies, including rheumatoid arthritis [368], sarcoidosis [369] and multiple sclerosis [370], have suggested the lung as a place of antigen presentation and initiation of the disease. In **Paper I**, we wanted to address this question by studying the immune response in the lungs in genetically susceptible myositis patients with anti-Jo-1 autoantibodies.

HisRS T cell reactivity is present in peripheral blood of IIM/ASyS patients.
We demonstrated the presence of HisRS-specific CD4$^+$ T cells in the blood of patients with anti-Jo-1 autoantibodies. For these experiments, we used a predicted peptide (HisRS$_{11-23}$) that can bind to HLA-DRB1*03:01 according to previous analysis [305, 306]. The T cell reactivity was evaluated by the upregulation of CD40L in CD4$^+$ T cells from PBMCs following stimulation with HisRS full-length protein or HisRS$_{11-23}$ in patients from Group 1 and Group 2 (**Figure 17**).

Our results showed an upregulation of CD40L on CD4$^+$ T cells in 14/18 IIM/ASyS patients stimulated with HisRS full-length protein. A high CD40L upregulation (>10-fold) was identified in 6/14 reactive IIM/ASyS patients, with the highest responses in patients with anti-Jo-1 autoantibodies and at least one copy of the HLA-DRB1*03 allele compared to non-HLA-DRB1*03/anti-Jo-1 negative (**Figure 18**).

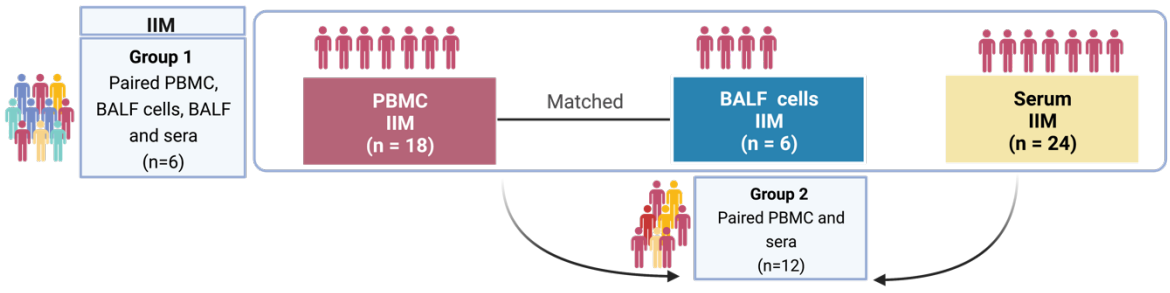


Figure 17. Graphical description of patients included in group 1 and group 2 for the T cell reactivity analysis. PBMC, Peripheral blood mononuclear cell; BALF, bronchoalveolar lavage fluid; IIM, idiopathic inflammatory myositis. Created with Biorender.com

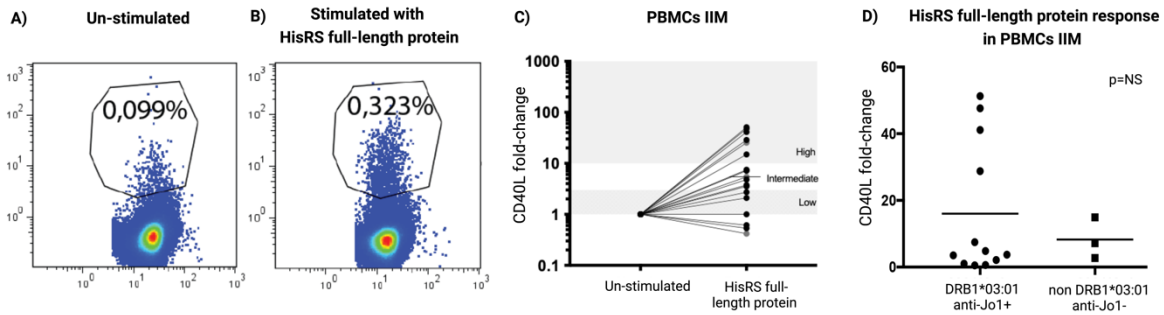


Figure 18. HisRS specific T cells in patients with IIM. Representative plot from flow cytometric analysis showing the CD40L upregulation in **(A)** un-stimulated or **(B)** stimulated with HisRS full-length protein. **(C)** CD40L fold-change on $CD4^+$ T cells from IIM/ASyS patients (n=18). **(D)** $CD4^+$CD40L fold-change upon stimulation with HisRS full-length protein according to *HLA* and autoantibody status. PBMC, peripheral blood mononuclear cell; HisRS, histidyl-tRNA synthetase; IIM, idiopathic inflammatory myositis; NS, non-significant. *Modified and adapted from Figure 1 and Supplementary Figure S4 in* ***Paper I****. Reprinted after permission from John Wiley and Sons.*

The stimulation with $HisRS_{11-23}$ on $CD4^+$ T cells from PBMCs demonstrated a CD40L upregulation in 11/14 IIM/ASyS patients. A $HisRS_{11-23}$ response >10-fold was observed in 3/11 IIM/ASyS. As a disease control group, we included patients with sarcoidosis, where the stimulation with HisRS full-length and $HisRS_{11-23}$ caused a CD40L upregulation in 2/7 patients. In the HLA-DRB1*03 HC group, the upregulation of the CD40L was detected upon stimulation with HisRS full-length in 1/12 HC and 5/12 HCs with $HisRS_{11-23}$. To evaluate the HLA-DR dependency on T cell responses, we demonstrated that anti-HLA-DR blocking abrogated up-regulation of CD40L upon stimulation with $HisRS_{11-23}$ (**Figure 19**).

HisRS-specific T cells are present in the lung of IIM/ASS patients and display a pronounced Th1 phenotype compared to PBMCs

To investigate the immune response in the lungs of antigen-specific $CD4^+$ T cells, we stimulated BALF T cells and corresponding PBMCs from anti-Jo-1^+ (n=2), anti-Jo-1^- (n=2) and sarcoidosis (n=7) patients with HisRS full-length or $HisRS_{11-23}$. For $CD4^+$ T cells from BALF and PBMC stimulated with HisRS full-length, there was a similar CD40L upregulation with a median fold-change of 3.6 and 4.58, respectively. Nevertheless, the $CD4^+$ T cell stimulation in BALF with $HisRS_{11-23}$ revealed the highest CD40L upregulation compared to the PBMC $CD4^+$ T cells, with a median fold-change of 88 vs 12.7, respectively. Additionally, we found an increased frequency of $CD4^+$CD40L+ T cells in BALF (median=7.83%) compared to PBMCs (median= 0.38%) after $HisRS_{11-23}$ stimulation, suggesting an enrichment of antigen-specific T cells in the lung compartment.

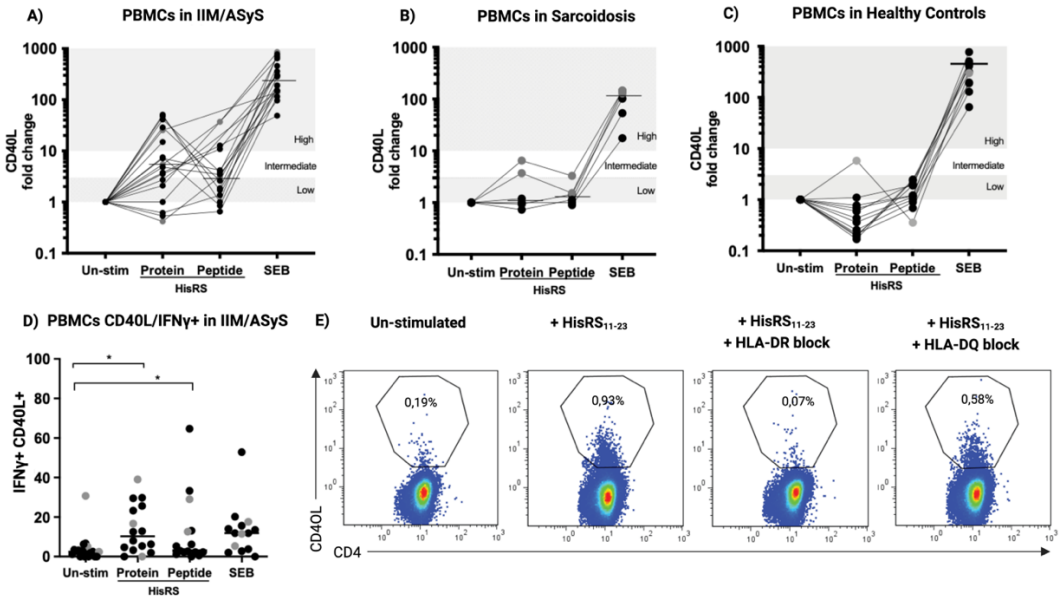


Figure 19. HisRS full-length and His$_{11-23}$ reactivity in CD4$^+$ T cells. CD40L fold-change on CD4$^+$ T cells from patients with **(A)** IIM/ASyS (n=18), **(B)** sarcoidosis (n=6), and **(C)** healthy controls (n=12). **(D)** IFNγ production assessed on CD4$^+$ CD40L+ T cells in PBMCs from patients with IIM/ASyS. **(E)** Representative plot from flow cytometry analysis showing the CD40L upregulation on CD4$^+$ T cells from IIM/ASyS patients after stimulation with HisRS$_{11-23}$. The CD40L upregulation was abolished by the presence of anti-HLA-DR but not with anti-HLA-DQ blocking antibodies. PBMC, peripheral blood mononuclear cell; HisRS, histidyl-tRNA synthetase; IIM, idiopathic inflammatory myositis; ASyS, anti-synthetase syndrome; SEB, Staphylococcal enterotoxin B. *,p-value <0.05. *Modified from Figures 1 and 2 in* ***Paper I****. Reprinted after permission from John Wiley and Sons.*

In un-stimulated cells from patients with IIM/ASyS, the presence of the Th1-associated chemokine receptors CXCR3 and CCR5 were highly increased in BALF CD4$^+$ T cells compared to their paired PBMCs (97% and 93% vs 46% and 50%, p<0.01 and p<0.05 respectively). Moreover, there was an enrichment of the Th17-associated chemokine receptor CCR6 in BALF T cells compared to the blood (60% vs 20%, p<0.01). Over 90% of CCR6$^+$ CD4$^+$ T cells in the lung compartment were positive for CXCR3, indicating that CD4$^+$T cells in BALF of patients with IIM/ASyS had a Th1/Th17 phenotype. The enriched expression of these chemokine receptors in BALF T cells could be associated to the regulation of antigen-induced T cell homing in lung diseases. These results add to the understanding of T cells homing, taken that CXCR3 upregulation has also been reported in patients with asthma [371], COPD [372], sarcoidosis [373] and healthy smokers [374].

Furthermore, BALF CD4$^+$CD40L+ T cells presented an increased production of IFNγ in response to stimulation with either HisRS full-length protein or HisRS$_{11-23}$ when compared to corresponding PBMCs (60% vs 10%, p<0.001). In the disease-control group, BALF cells from 2/7 sarcoidosis stimulated with HisRS full-length protein and HisRS$_{11-23}$ displayed high CD40L response with a median fold-change of 20 and 59, respectively. The CD40L upregulation in BALF CD4$^+$ T cells was four times higher than the corresponding PBMCs,

indicating that the location of the antigen-presentation might be or relevance for the immune response.

Anti-Jo-1 autoantibodies in the BALF

BALF samples from IIM/ASyS patients corresponding to Group 1 and Group 3, sarcoidosis patients (n=9) and HCs (n=18) were analysed for the presence of anti-Jo-1 autoantibodies. We detected anti-Jo-1 IgG autoantibodies in BALF from 6/7 anti-Jo-1$^+$ patients and 2/9 sarcoidosis patients, but not in HCs or anti-Jo-1$^-$ individuals. All anti-Jo-1$^+$ patients with reactivity in the BALF were also reactive in serum (**Figure 20**).

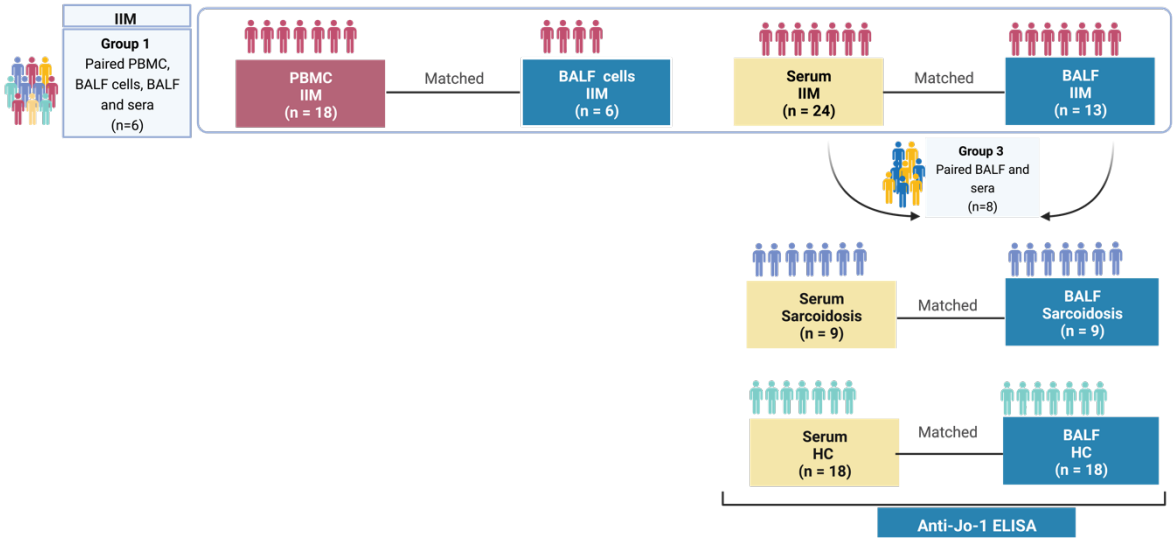


Figure 20. Graphical description of patients included in group 1 and group 3, sarcoidosis and HC for anti-Jo-1 autoantibody testing. PBMC, Peripheral blood mononuclear cell; BALF, bronchoalveolar lavage fluid; IIM, idiopathic inflammatory myositis; HC, healthy controls. Created with Biorender.com

Lung tissue of IIM/ASS patients is infiltrated by T cells and plasma cells

The analysis of lung tissue revealed the presence of germinal centre (GC)-like structures characterized by T cells and surrounding plasma cells in 2/4 anti-Jo-1$^+$ IIM/ASyS patients but in none of the tissues from anti-Jo-1$^-$ nor COPD patients. All patient groups presented infiltrating CD3$^+$ T cells, with a higher number in patients with COPD (p=0.009). Transbronchial lung biopsies on sarcoidosis patients did not show the presence of germinal centre formation.

In summary, in **Paper I** we demonstrated the presence of antigen-reactive CD4$^+$ T cells in PBMCs and BALF cells of patients with IIM/ASyS towards HisRS and a candidate peptide HisRS$_{11-23}$. We observed a high T cell reactivity against HisRS$_{11-23}$, enriched in the CD4$^+$ T cells from BALF. Our results demonstrated that the T cell activation with HisRS$_{11-23}$ was biased in HLA-DRB1*03:01. Furthermore, we confirmed the presence of anti-Jo-1 autoantibodies in BALF and serum. We identified the presence of GC-like structures in lung

tissue of IIM/ASyS patients. Nevertheless, $CD4^+$ T cell activation with HisRS and $HisRS_{11-23}$ was present in some patients without anti-Jo-1 autoantibodies and in a small number of sarcoidosis and HC, though to a substantially lower degree. The wide variety of T cell responses in these patients with different alleles indicates that other factors could also be implicated in the presentation of HisRS peptides and deserve further scrutiny.

The observations drawn from this study support the contribution of the lung as a potential site of primary T cell activation towards HisRS. We hypothesize that these proinflammatory T cells may promote B cell maturation, activation, GC-like structure formation and anti-Jo-1 autoantibody production. A continuous challenge persists regarding the study of other relevant peptides with broader coverage of HisRS antigenic sites and a deeper understanding of the phenotyping of local infiltrating autoreactive T cells. After the publication of **Paper I**, several different HisRS peptides were tested *in vitro* to continue the study possible T cells epitopes with HLA-II tetramers. Additionally, we have developed a methodology for studying infiltrating T cells in IIM patients, which will be presented in **Paper IV**.

5.2 PAPER II: AUTOANTIBODIES AGAINST FOUR-AND-A-HALF-LIM DOMAIN 1 (FHL1) IN INFLAMMATORY MYOPATHIES: RESULTS FROM AN AUSTRALIAN SINGLE-CENTRE COHORT

Four-and-a-half-LIM-domain 1 (FHL1) is a muscle-specific antigen present in skeletal and heart muscle. It is involved in the differentiation, intracellular protein-protein interactions and maintenance of structural elements. Anti-FHL1 autoantibodies were previously described to be muscle-specific. Our group proposed that they could be considered a biomarker of poor prognosis since they were associated with pronounced muscle fibre damage, muscle atrophy, dysphagia and vasculitis [314]. The presence of anti-FHL1 antibodies was documented in a cross-sectional study with a frequency of 25% of IIM patients in Sweden and the Czech Republic [314]. In **Paper II**, we wanted to determine the prevalence and clinical associations of anti-FHL1 autoantibodies in a South Australian cohort of patients.

This cross-sectional study included serum samples and clinical data from patients with IIM (n=267), systemic sclerosis (SSc, n=174) and HC (n=100) as comparators. Anti-FHL1 autoantibodies were detected by ELISA, and the results were transformed into arbitrary units (AU). The presence of anti-FHL1^{+} autoantibodies was detected in 37/267 (14%) patients with IIM, 12/174 (7%) patients with SSc, and 2/100 (2%) of HC. Patients with IIM and SSc had significantly higher levels of anti-FHL1 autoantibodies compared with HC. In the IIM group, the anti-FHL1 autoantibodies were more frequent within the subgroups of PM (15/37, 41%) and IBM (11/37, 29%). A higher frequency of anti-FHL1 autoantibodies was observed in patients with limited versus diffuse SSc.

In the anti-FHL1^{+} group, 78% of patients did not have any MSA, 77% were negative for MAA, and 65% were negative for both MSA and MAA. Anti-FHL1^{+} patients had a trend towards a higher frequency in *HLA* alleles *DRB1*07* and *DRB1*15*. Nevertheless, there was no significant difference in muscular or extra-muscular manifestations of IIM in patients with or without anti-FHL1 antibodies. In anti-FHL1^{+} IBM patients, there was a higher prevalence of atrophy in the muscle biopsy, lower MMT-8 scores and higher CK levels at baseline though these findings did not reach statistical significance. We identified seven anti-FHL1^{+} patients, which presented a higher degree of histological muscle atrophy, less CD68+ and CD45+ infiltration, and less myalgia, although this was not significantly different. SSc patients did not have a more frequent elevation of CK levels or more severe weakness and did not have a more frequent clinical diagnosis of myositis or atrophy in the muscle biopsy compared to anti-FHL1^{-} patients (**Figure 21**).

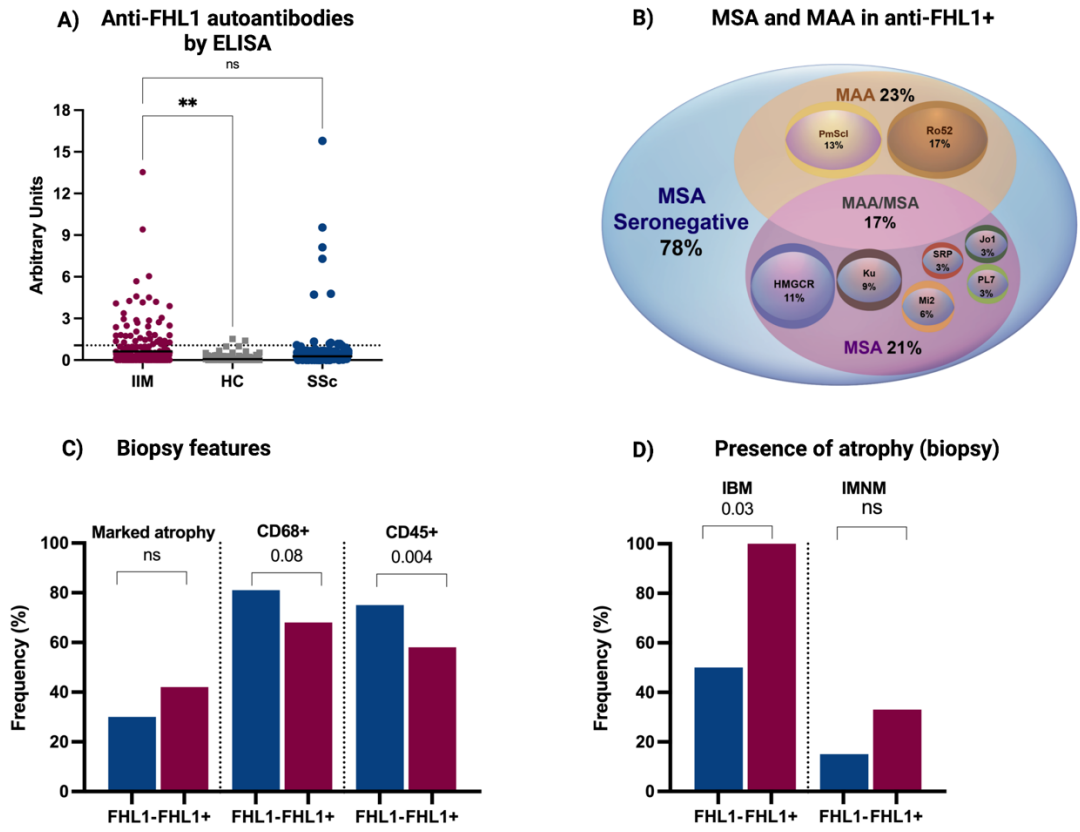


Figure 21. Serum and clinical characteristics of patients tested for anti-FHL1 autoantibodies. **(A)** Sera from patients with IIM, SSc and HC were analysed by ELISA using recombinant His-tagged FHL1. A cut-off value of 1.06 AU was calculated using a receiver operating characteristic (ROC) curve based on the HC. **(B)** Frequency of IIM patients presenting MSA and MAA in the anti-FHL1+ group. **(C)** Representative histological findings in the muscle biopsy regarding the frequency of marked atrophy and infiltration by CD68+ and CD45+ cells. **(D)** Comparison of the frequency of atrophy in the muscle biopsy by IIM subgroup in IBM and IMNM by anti-FHL1 status. HC: healthy controls; IIM: idiopathic inflammatory myopathy; IBM, inclusion body myositis; IMNM: immune-mediated necrotizing myopathy; MAA: myositis-associated autoantibodies; MNOS: myositis not-otherwise-specified; MSA: myositis-specific autoantibodies; ns, non-significant; SSc, systemic sclerosis. *Figure 1 in **Paper II**. Reprinted with permission from Oxford University Press.*

In conclusion, our study confirmed the presence of anti-FHL1 autoantibodies in patients with IIM but can also be present in other autoimmune diseases such as SSc.

The lack of a particular clinical phenotype and the co-existence with other MSA suggests this autoantibody, though muscle-specific, could be defined as MAA. Elucidating the functional role of anti-FHL1 antibodies in IIM and other autoimmune diseases will be needed. It will be necessary to correlate the presence and levels of the antibody with disease activity, organ damage, outcome measures and response to treatment. These questions will be addressed in **Paper V**.

5.3 PAPER III: *HLA* ASSOCIATIONS WITH AUTOANTIBODY-DEFINED SUBGROUPS IN IDIOPATHIC INFLAMMATORY MYOPATHIES

The discovery of myositis-specific and myositis-associated autoantibodies (MSA/MAA) has improved the diagnosis, clinical phenotyping and management of IIM. Furthermore, the identification of the *HLA* as the strongest genetic risk factor for autoimmunity has provided insights into the relationship between the innate and adaptive immune response [344, 375, 376]. Nevertheless, previous analyses regarding the association with the *HLA* and MSA have focused on uniform clinical manifestations, not fully capturing the heterogeneity of IIM [57, 76, 82]. Therefore, in **Paper III**, we explored the relationship between autoantibody-defined subgroups, including MSA/MAA and *HLA* genetic variants.

In this study, we included patients recruited from 5 European centres: Salford Royal NHS Foundation Trust (United Kingdom (UK), Institute of Rheumatology (Prague, Czech Republic), Karolinska University Hospital (Stockholm, Sweden), Copenhagen University Hospital (Copenhagen, Denmark) and Oslo University Hospital (Oslo, Norway). We analysed 1348 IIM patients with complete autoantibody profiles (14 autoantibodies), but only 829 (61%) patients presented positivity to at least one autoantibody. All centres had similar subgroups of IIM, except the Czech Republic, which had an increased prevalence of DM and Denmark of PM. The UK had the highest number of patients negative for all autoantibodies tested (58%).

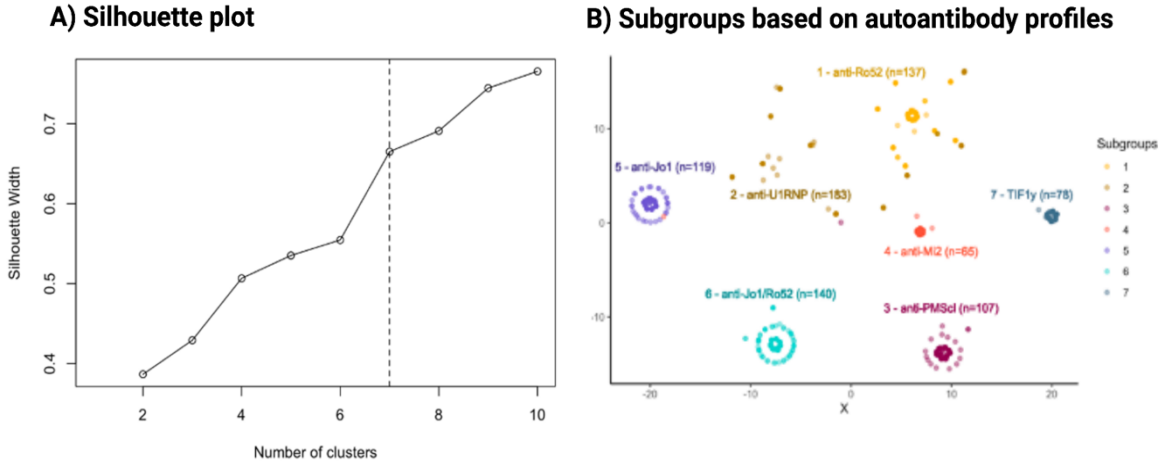


Figure 22. Autoantibody-based groups visualization. (A) Silhouette plot, **(B)** Subgroups based on autoantibody profiles. Each patient is represented by a dot. Individuals with no positivity for the autoantibodies included in this study (seronegative group) are not represented and correspond to the subgroup 8.

Seven autoantibody-defined subgroups were identified in addition to the subgroup of patients negative for all autoantibodies tested (**Figure 22**). The subgroups dominated by anti-Ro52 (subgroup 1) and anti-U1RNP (subgroup 2) were the most heterogeneous

according to their serological profiles. The other subgroups were relatively monospecific, except for subgroup 6, where all patients were positive for anti-Jo-1 and anti-Ro52. The subgroups dominated by anti-Pm-Scl (subgroup 3), anti-Mi2 (subgroup 4) and anti-TIF1γ (subgroup 7) were associated with DM rashes. Subgroup 6 was associated with Raynaud´s phenomenon. The subgroup negative for autoantibodies screened (subgroup 8) had lower odds of developing DM rashes and Raynaud´s phenomenon (**Table 10**).

Table 10. Association between clinical manifestations and subgroups

	Subgroups							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Medoids	**anti-Ro52**	**anti-U1RNP**	**anti-PM-Scl**	**anti-Mi2**	**anti-Jo-1**	**anti-Jo-1 /Ro52**	**anti-TIF1γ**	**none†**
ILD	1.0 [0.4-3.5]	1.3 [0.4-5.4]	1.4 [0.4-9.0]	0.6 [0.2-2.0]	1.6 [0.5-10]	3.8 [0.8-69]	**0.2 [0.1-0.4]***	1.4 [0.7-2.9]
Muscle weakness	0.7 [0.2-2.4]	0.6 [0.2-2.0]	0.6 [0.2-2.6]	0.5 [0.2-2.2]	0.7 [0.2-3.2]	3.6 [0.7-65]	0.7 [0.2-4.4]	2.4 [0.9-6.5]
Mechanic's hand	0.6 [0.3-1.3]	1.1 [0.5-2.5]	1.3 [0.5-4.0]	0.9 [0.3-3.2]	0.7 [0.3-1.5]	2.6 [1.0-8.1]	1.1 [0.4-4.9]	1.0 [0.6-1.6]
Heliotrope	1.3 [0.7-2.3]	0.9 [0.5-1.5]	**2.2 [1.1-4.8]***	**12 [3.6-75]****	1.1 [0.6-2.1]	**0.4 [0.2-0.7]***	**22 [4.5-385]***	**0.5 [0.4-0.8]***
Gottron's	1.3 [0.7-2.3]	0.8 [0.5-1.4]	**2.6 [1.3-5.9]***	**7.8 [2.8-33]****	1.3 [0.7-2.4]	**0.4 [0.2-0.7]***	**10 [3.1-65]****	**0.5 [0.4-0.8]***
Calcinosis	1.3 [0.6-2.5]	0.7 [0.3-1.3]	1.0 [0.4-2.1]	1.8 [0.8-4.2]	1.0 [0.4-1.9]	1.0 [0.5-2.2]	0.6 [0.2-1.7]	1.1 [0.7-1.6]
Raynaud	0.8 [0.4-1.7]	1.5 [0.7-3.8]	1.7 [0.6 -6.0]	0.9 [0.3-3.1]	1.4 [0.6-3.8]	**3.3 [1.2-11]***	0.9 [0.3-4.0]	**0.6 [0.4-1.0]***
Arthritis	0.7 [0.3-1.7]	0.8 [0.4-2.1]	0.7 [0.3-2.2]	0.6 [0.2-2.1]	2.7 [0.8-17]	2.1 [0.7-9.3]	0.5 [0.2-1.9]	1.1 [0.7-2.0]
Dysphagia	0.9 [0.4-2.2]	0.7 [0.3-1.5]	0.7 [0.3-2.1]	0.9 [0.3-3.9]	1.3 [0.5-4.0]	0.7 [0.3-1.6]	1.3 [0.4-8.1]	1.4 [0.9-2.5]

Legend: Represented are odd ratios [95% confidence interval]. ILD, interstitial lung disease. All models are adjusted for sex, age at diagnosis and recruiting centre. ***,** p-value <0.05**;** **, p-value <0.01; †Negative for all autoantibodies tested in this study. In bold are presented the significant associations.

Genetic associations

We observed non-significant associations in the subgroup dominated by anti-Ro52 (subgroup 1). The *HLA-DRB1*04, *11, *15, HLA-DQA1*03* and *HLA-DQB1*03* alleles were overrepresented in the subgroup dominated by anti-U1RNP (subgroup 2). The subgroup dominated by anti-Pm-Scl (subgroup 3) had significant associations with *HLA-DRB1*03, HLA-DQA1*05* and *HLA-DQB1*02*, pointing towards the ancestral haplotype 8.1 [377]. In contrast, the ancestral haplotype 57.1 [377] was more common in the subgroup dominated by anti-Mi2 (subgroup 4), with the strongest associations with *HLA-DRB1*07, HLA-DQA1*02* and valine at position 97 in HLA-B. Only *HLA-DRB1*03* was significantly associated with the subgroup dominated by anti-Jo-1 (subgroup 5). In contrast, the *HLA-DQA1*05, -DQB1*02* alleles and aspartic acid at position 9 of HLA-B were positively associated with the subgroup dominated by anti-Jo-1/Ro52 (subgroup 6) in addition to the *HLA-DRB1*03* association.

We detected positive associations between *HLA* class II and I with the subgroup dominated by anti-TIF1γ (subgroup 7), including the *HLA-DRB1*07*, **01*, *HLA-DQA1*02*, *HLA-DQB1*05* alleles and amino acids aspartic acid or glutamine at position 156 of HLA-C, and leucine at

position 62 of HLA-A. In the subgroup negative for the autoantibodies tested (subgroup 8), the *HLA-DRB1*13*, *HLA-DQA1*01, HLA-DQB1*06* alleles, tryptophan or arginine at position 152 of HLA-A were significantly more frequent. The results per centre of significant *HLA*-class II associations are presented in **Figure 23**.

In conclusion, our results showed that after considering the presence of multiple autoantibodies specificities, we could identify differential and novel *HLA* class II and class I associations. In particular, the subgroup dominated by anti-Ro52 was not associated with *HLA*; the subgroup dominated by anti-Jo-1 was associated with *HLA-DRB1*03*; and lastly, the subgroup by anti-Jo-1/Ro52 was associated with *HLA* class II and I variants. We could also identify novel associations with the subgroups dominated by anti-Mi2, anti-TIF1γ and the subgroup negative for the tested autoantibodies.

HLA alleles	Group Medoid	OR [95% CI]	P-value	FDR	Q	I^2
*DQA1*01*	8 none	1.6 [1.3-1.9]	0.001	0.006	0.86	0
*DQA1*02*	4 anti-Mi2	7.0 [6.3-7.7]	$2.8X10^{-8}$	$2.3X10^{-7}$	0.51	0
*DQA1*02*	7 anti-TIFy	3.6 [3.0-4.3]	0.0001	0.0004	0.63	0
*DQA1*03*	2 anti-U1RNP	1.7 [1.3-2.1]	0.01	0.04	0.39	0
*DQA1*05*	3 anti-PMScl	2.4 [1.9-2.9]	0.0006	0.003	0.88	0
*DQA1*05*	4 anti-Mi2	0.2 [0.0-0.9]	$4.8X10^{-6}$	$1.9X10^{-5}$	0.65	0
*DQA1*05*	6 anti-Jo1/Ro52	2.0 [1.6-2.4]	0.0007	0.005	0.41	0
*DQA1*05*	7 anti-TIFy	0.4 [0.0-1.0]	0.003	0.007	0.68	0
*DQB1*02*	2 anti-U1RNP	0.5 [0.1-1.0]	0.003	0.02	0.28	21.8
*DQB1*02*	3 anti-PMScl	5.2 [4.6-5.8]	$3.0X10^{-8}$	$3.0X10^{-7}$	0.64	0
*DQB1*02*	6 anti-Jo1/Ro52	2.1 [1.6-2.5]	0.001	0.005	0.20	37.3
*DQB1*02*	8 none	0.5 [0.2-0.9]	0.0001	0.001	0.77	0
*DQB1*03*	2 anti-U1RNP	1.8 [1.4-2.1]	0.003	0.02	0.33	8.9
*DQB1*05*	7 anti-TIFy	3.1 [2.5-3.6]	0.0001	0.0004	0.37	0
*DQB1*06*	7 anti-TIFy	0.2 [0.0-1.2]	0.0006	0.002	0.79	0
*DQB1*06*	8 none	1.5 [1.2-1.9]	0.009	0.03	0.37	0
*DRB1*01*	7 anti-TIFy	2.5 [2.1-2.9]	$3.7X10^{-5}$	0.0002	0.98	0
*DRB1*03*	2 anti-U1RNP	0.5 [0.1-0.9]	0.0003	0.002	0.1	56.4
*DRB1*03*	3 anti-PMScl	3.4 [3.0-3.8]	$2.4X10^{-8}$	$1.7X10^{-7}$	0.57	0
*DRB1*03*	4 anti-Mi2	0.1 [0.1-0.9]	$9.1X10^{-7}$	$4.5X10^{-6}$	0.66	0
*DRB1*03*	5 anti-Jo1	2.1 [1.7-2.5]	0.0001	0.001		
*DRB1*03*	6 anti-Jo1/Ro52	2.4 [1.7-3.1]	0.01	0.0001	0.04	69.3
*DRB1*03*	7 anti-TIFy	0.4 [0.2-0.9]	0.00019	$7.5X10^{-4}$	0.79	0
*DRB1*04*	2 anti-U1RNP	1.5 [1.2-1.9]	0.02	0.05	0.3	17.6
*DRB1*07*	4 anti-Mi2	8.8 [8.2-9.4]	$1.6X10^{-13}$	$1.6X10^{-12}$	0.58	0
*DRB1*07*	7 anti-TIFy	3.4 [2.9-3.9]	$4.0X10^{-6}$	$4.4X10^{-5}$	0.51	0
*DRB1*11*	2 anti-U1RNP	1.8 [1.4-2.3]	0.01	0.04	0.23	32.4
*DRB1*13*	8 None	1.7 [1.4-1.9]	0.0008	0.01	0.79	0
*DRB1*15*	2 anti-U1RNP	2.1 [1.7-2.5]	$2.5X10^{-4}$	0.002	0.24	29.0
*DRB1*16*	4 anti-Mi2	3.4 [2.4-4.4]	0.015	0.049	0.76	0

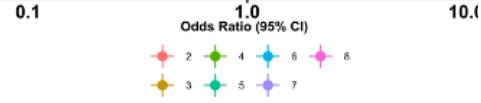


Figure 23. Significant associations of *HLA-DQA1, HLA-DQB1* and *HLA-DRB1* alleles with autoantibody-defined subgroups. Significant results from meta-analysing United Kingdom, Czech Republic and Scandinavia are shown. OR: odds ratio, 95% CI: confidence intervals, FDR: false discovery rate, Q: Cochran's Q test to measure heterogeneity across cohorts, I^2: Higgins' test to measure the degree of inconsistency and variation across the data.

5.4 PAPER IV: SINGLE CELL SEQUENCING UNCOVERS MUSCLE T CELL SIGNATURES AND CLONALLY EXPANDED TISSUE RESIDENT MEMORY T CELLS IN MYOSITIS

Although the causes of myositis are still unknown, genome-wide association studies (GWAS) and meta-analysis, as presented in **Paper III**, have identified *HLA* [72, 75, 82] as the strongest locus associated with the disease. This association is related to the crucial role of HLA class I and II molecules upon presentation of antigenic peptides to T cells, leading to the development of an antigen-specific immune response.

The presence of T cell infiltrates in muscle biopsies of patients with IIM has been described for decades [378-380], and together with the overexpression of HLA class I in myofibres, it has been associated with an inflammatory response leading to T cell-mediated cytotoxicity [381]. It has been suggested that specific T cell subsets, such as the of $CD4^+$ or $CD8^+$ $CD28^{null}$ T cells may contribute to the pathogenesis of the disease and can be associated with poor outcomes [116, 117]. Indeed, the identification of restricted TCR Vβ usage of $CD28^{null}$ T cells in the muscle of patients with IBM suggests that the presence of these T cells might provoke muscle damage.

Therefore, the identification of local inflammatory infiltrates in the muscle is important not only for the diagnosis and classification of IIM but also for understanding the pathogenesis of the disease [355]. However, the mechanism involved in T cell migration and persistence in tissues, as well as the understanding of the function and regulation of the tissue-resident memory T cells (T_{RM}) and circulating memory T cells, has been limited in humans [382] and in particular in the muscle tissue of patients with IIM.

In **Paper IV**, we aimed to explore these questions by performing an unbiased single cell mRNA profiling of paired muscle-infiltrating and blood-memory T cells in patients that underwent a study protocol for diagnosis of IIM at the Division of Rheumatology, Karolinska University Hospital. This study included 15 patients who underwent paired muscle and blood sampling. After enzymatic digestion of fresh muscle tissue, we could retrieve T cells from the seven patients which also presented muscle infiltrate by histopathological analysis: six patients with a confirmed diagnosis of IIM (IMNM n=2, IBM n=2, DM n=1 and ASyS n=1) and one patient with suspicion of IBM who was identified with a non-inflammatory myopathy during the follow-up. In this patient, a mutation in *COL6A2* was found. Muscle T cells were sorted based on the co-expression of $CD45^+$ and $CD3^+$ markers. Blood memory T cells were sorted based on the exclusion of naïve T cells ($CD45RA^+CCR7^+CD3^+$).

Then, we performed Smartseq2 single cell mRNA sequencing of the sorted T cells. After quality control filtering and normalization of the gene expression, we recovered 2805 T cells (muscle n=1,405 cells and blood n=1,400 cells) for the analysis. The unsupervised analysis identified three different clusters containing muscle T cells (cluster 1), blood $CD4^+$ T cells (cluster 2) and blood $CD8^+$ T cells (cluster 3) (**Figure 24 A and 24 B**). The transcriptomic analysis revealed a distinct signature between muscle T cells and blood memory T cells, with differentially expressed genes involved in transcription (ex: *FOS, CREM, JUNB*), migration (*CXCR4*), dephosphorylation *DUSP1, DUSP2, DUSP4* and *DUSP5*), cell cycle (ex: *RGCC, TNFAIP3, CDKN1A*), metabolism (ex: *TXNIP, SLC2A3, PER1*), cytoskeleton (*LMNA, SRGN, SLC38A2*) and signalling (*RGS1, RASGEF1B*).

After performing UMAP clustering, we identified the presence of 14 distinct of T cell clusters (**Figure 24 C)**. We found enrichment in the muscle compartment compared to blood for clusters 0, 1, 5, 7, 10 and 12 corresponding respectively to $HOBIT^+$ T cells, central memory $CD4^+$ T cells, cytotoxic T cells-2, $GZMK^+$ T cells, Tregs and proliferating T cells. All clusters were present in all patients except for cluster 10 (Tregs), which was not detected in the patient with ASyS. The characteristics of each cluster are presented in **Table 11.**

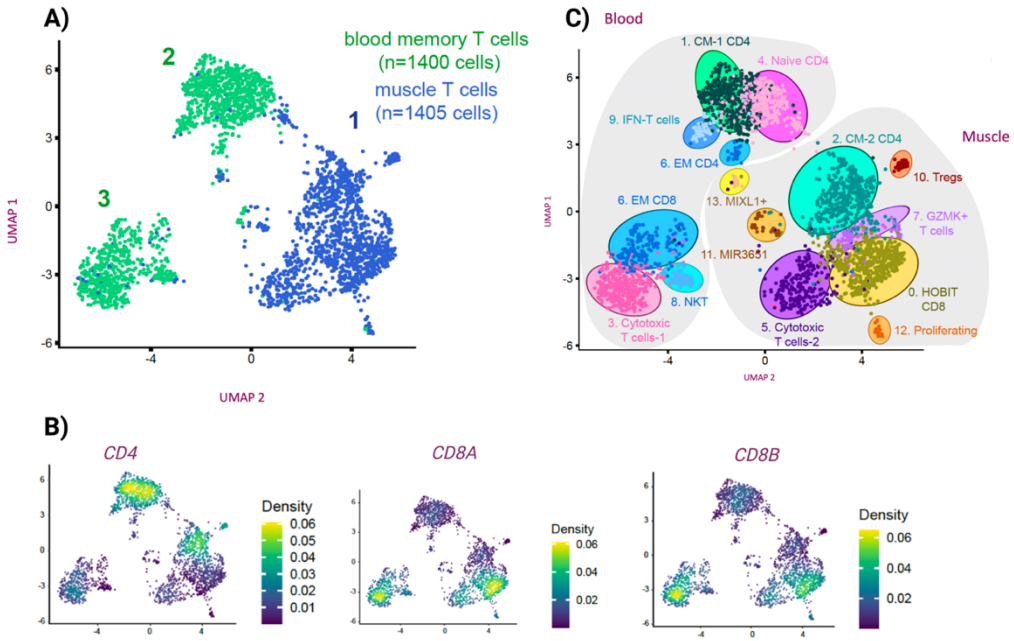


Figure 24. Cell clusters in myositis patients. **(A)** UMAP displaying three T cell clusters from n=7 patients in PBMCs (green, 1400 cells) and muscle (blue, n=1405 cells). **(B)** UMAP nebulosa plots displaying *CD4*, *CD8A* and *CD8B* gene density. **(C)** UMAP displaying 14 T cell clusters in the muscle and blood (n=2805 cells). UMAP, uniform manifold approximation and projection; CM, central memory T cells; EM, effector memory T cells.

Table 11. General characteristics of T cell clustering

Cluster	Tissue	T cell subtype	Gene expression
0	Muscle	$CD8^+$ T cells *	*ZNF683* (HOBIT), *CXCR6, XCL1*
1	Blood	$CD4^+$ Central memory (1) *	*MAL, CCR7, LEF1*
2	Muscle	$CD4^+$ Central memory (2)	*MAL, CCR7, LEF1*
3	Blood	Cytotoxic T cells (1)	*CX3CR1*, *ADGRG1* (GPR56), *FGFBP2*
4	Blood	Naïve $CD4^+$ T cells	*LTB, MYC*
5	Muscle	Cytotoxic T cells (2) *	*CX3CR1*, *ADGRG1* (GPR56), *FGFBP2*
6	Blood	Effector memory T cells	*KLRD1, IFIT3*
7	Muscle	$GZMK^+$ T cells *	*GZMK*
8	Blood	NKT-like cells	*FCGR3A, KLRF1, KLRD1*
9	Blood	IFN-T cells	*IFIT3*, *IFI44L*, *IFI6*
10	Muscle	Tregs *	*FOXP3*, *TNFRSF9, TIGIT*
11	Muscle	$MIR365^+$ T cells	*MIR365*
12	Muscle	Proliferating T cells *	*MKI67*
13	Muscle	$MIXL1^+$ T cells	*MIXL1*

Legend: Asterisk (*) indicates the enrichment in the muscle compartment compared to blood for clusters.

The evaluation of key T cell receptors and transcription factors identified that cluster 0 presented distinctive markers of genes associated with the phenotype of tissue-resident memory T cells: upregulation of *HOBIT*, *ITGA4, CXCR6*, *CD69*, *XCL1* and *XCL2;* and downregulation of *S1PR1*. Additionally, clusters 3, 5 and 8 expressed genes associated with cytotoxic T cell functions. In summary, our results show that in IIM, muscle infiltrating T cells are composed of cytotoxic, effector memory, tissue-resident, proliferating and regulatory T cells.

Clonally expanded T cells

As a clone, we defined two or more cells that share the same CDR3 amino acid sequence of the T cell receptor alpha (TCRα) and beta (TCRβ) chains. Expanded T cell clones were observed in the muscle of all patients. In the two patients with IMNM, most expanded clones were identified amongst cytotoxic $CD8^+$ T cells and were shared between blood (cluster 3) and muscle (cluster 5). In the patient with ASyS, we identified expanded cytotoxic $CD8^+$ T cell, and cytotoxic $CD4^+$ T cell clones shared between blood and muscle. Most expanded clones from the patients with IBM (patients 5 and 6) were amongst the $HOBIT^+$ $CD8^+$ T cell cluster and were present only in the muscle. The patient with *COL6A2* associated myopathy presented clones within cytotoxic $CD8^+$ T cells, which were shared between blood and muscle.

We found shared T cell clones between cytotoxic T cells-1 (cluster 3) and cytotoxic T cells-2 (cluster 5) in patients with IMNM, ASyS, IBM and *COL6A2* associated myopathy. T cell sequences sharing between proliferating (cluster 12) and $HOBIT^+$ T cells (cluster 0) were present in all six patients with IIM. The ASyS patient also had TCR sharing between effector

and cytotoxic CD4$^+$ T cells. Cytotoxic T cells sharing the same sequences were identified in muscle and blood. We could not detect blood T cells sharing TCR sequences with the HOBIT$^+$ muscle T cells.

Expanded T cell clones over time

Patient 2, with the diagnosis of IMNM and patient 4, with the diagnosis of ASyS, underwent a second muscle biopsy after nine months of treatment. TCR analysis revealed that the most expanded clones in muscle or blood were still expanded after treatment. In patient 2, the transcriptomic profile of the CD8$^+$ expanded clones showed downregulation of *CXCR3* and *HLA-DRB1* in the muscle after treatment. On the other hand, patient 4 presented more profound changes in the transcriptomic profile of expanded T cell clones, with downregulation of *CXCR4* in muscle and downregulation of genes related to the IFN pathway in muscle and blood after treatment.

In this paper we could show that infiltrating T cells in muscle of patients with myositis of different clinical and serological subsets present a specific muscle cell signature, including *CXCR4* expression. Additionally, we could differentiate the muscle T cell infiltrate into cytotoxic, effector, tissue-resident, Tregs and proliferating T cells, which provides a better characterization of T cells in this group of patients. This study revealed a distinct T cell cluster characterized by the expression of the transcription factor HOBIT and features associated with tissue resident memory T cells. Moreover, we could show that most clonally expanded clones were found amongst cytotoxic and/or HOBIT$^+$ T cells in the muscle of patients with IIM. Finally, we could identify the presence of expanded T cell clones in the muscle and blood after 9 months of extensive immunosuppressive treatment.

5.5 PAPER V: ANTI-FHL1 AUTOANTIBODIES IN ADULT MYOSITIS: BASELINE AND LONGITUDINAL FOLLOW-UP ANALYSIS

After validating the presence of anti-FHL1 autoantibody in other cohorts presented in **Paper II**, several questions remain unanswered. We aimed to increase our understanding regarding a possible association with a distinct clinical phenotype, the change of autoantibody levels through time and its clinical associations in a longitudinal analysis of IIM patients in Sweden **i**n **Paper V**.

This study included serum samples from 449 adult patients with IIM from the Swedish myositis registry (SweMyoNet) obtained close to diagnosis (baseline). Clinical data and serum samples were collected between 1981-2019. For the disease comparator groups, we included serum samples from patients with other disease controls (DC) such as Sjögren's syndrome (SS, n=100), systemic sclerosis (SSc, n=10), rheumatoid arthritis (RA, n=10) and systemic lupus erythematosus (SLE, n=10). Additionally, we analysed samples from patients with neuromuscular disorders (NMD, n=16) and population-based controls without a rheumatic disorder (HC, n=100). Samples for the longitudinal evaluation were selected from the anti-FHL1+ patients and the anti-FHL1- patients according to the availability of consecutive sera in the biobank. Anti-FHL1 autoantibodies were analysed by ELISA according to the protocol presented in **Paper II**.

Our results showed that anti-FHL1 autoantibodies were more frequent in patients with IIM (122/449, 27%) compared to DC (13/146, 9%, $p<0.001$) and HC (3/100,3%, $p<0.001$) (**Figure 25 A** and **25 B**). Moreover, the anti-FHL1 levels were higher in the IIM group than in the comparator groups. As reported previously, patients with SSc presented a higher frequency and autoantibody level of anti-FHL1 (5/10, 50%) within the DC group.

When comparing patients with or without anti-FHL1 autoantibodies in the IIM group at baseline, we did not find statistically significant differences in sex, ethnicity, IIM subgroup, or myositis-specific (MSA) autoantibodies. In 25% of patients with anti-FHL1+ autoantibody, there were no detectable MSA or MAA. HLA-DRB1*15 was more frequent in the anti-FHL1+ group (27% vs 14%, $p=0.02$). Erythrocyte sedimentation rate (ESR) was significantly higher at baseline in the anti FHL1+ group (median 40mm vs 18mm, $p=0.03$) and we found significant correlations between anti-FHL1 autoantibody levels and serum levels of LD ($r=0.58$, $p<0.001$), ASAT ($r=0.47$, $p=0.006$) and ALAT ($r=0.56$, $p=<0.001$).

We included longitudinal samples from patients with IIM that were anti-FHL1+ (n=57, median follow-up samples: 5) and anti-FHL1- (n= 30, median follow-up samples 4). The anti-FHL1+ patients were either positive in the baseline sample (F1, n=33, **Figure 25 C**) or became anti-

FHL1+ during follow-up (≥F2, n=24, **Figure 25 D**). In the 33 patients that were positive in the baseline sample, 20 patients (60%) became anti-FHL1 negative at the first follow-up sample after 36 months, and the anti-FHL1 autoantibody levels at first follow-up were significantly lower compared to the baseline (median AU 2.48 vs 0.76, p<0.0001, **Figure 25 E**). The 30 cases in the seronegative group remained seronegative through the observation period

Longitudinal clinical outcome measurements and histopathological features in muscle biopsies at baseline were available in a subgroup of 42/57 anti-FHL1+ and 25/30 FHL- IIM patients. The anti-FHL1+ group had a higher frequency of widespread MHC-I upregulation on the sarcolemma (91% vs 57%, p=0.02). There was no difference in the distribution of IIM subgroups or other clinical or laboratory features at baseline between anti-FHL1 positive or negative patients.

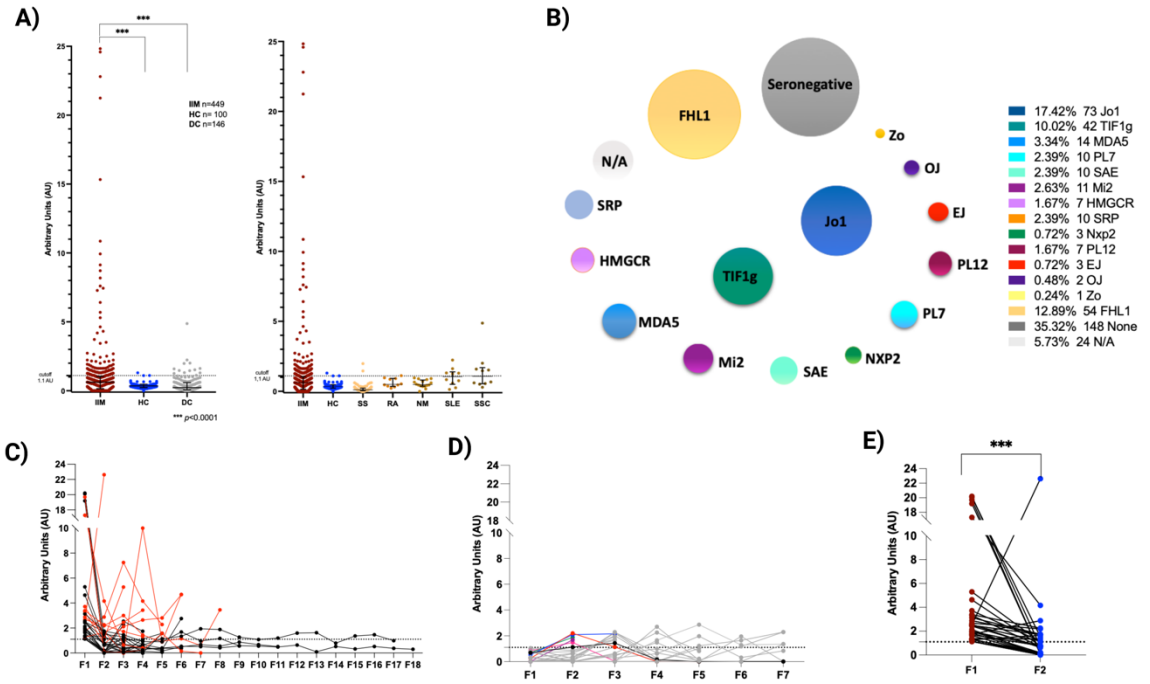


Figure 25. Anti-FHL1 autoantibodies in IIM patients and disease controls at baseline and longitudinal analysis. (A) Sera from patients with IIM (*n*=449), HC (*n*=100) and DC including SS (*n*=10), RA (*n*=10), NMD(*n*=16), SLE (*n*=10), and SSc (*n*=10) were analysed by ELISA using recombinant His-tagged FHL1. A cut-off value of 1.1 AU was calculated using a receiver operating characteristic (ROC) curve based on the HC. **(B)** Frequency of MSA in patients with IIM, including FHL1 autoantibody. **(C)** Group anti-FHL1+ at baseline (n=33), where the red line and symbols indicate the sera samples that had high anti-FHL1 autoantibody levels during follow-up. **F1** represents the sample closest to diagnosis (baseline) and **F2-F*n*** the longitudinal samples. **(D)** Group anti-FHL1- at baseline (n=24), where the coloured lines indicate individual samples that became positive in F2-F3. **(E)** Comparison of anti-FHL1 autoantibody levels at baseline (F1) and first follow-up (F2) in sera from patients belonging to the group C). IIM, idiopathic inflammatory myopathy; HC, healthy control; DC, disease controls; SS, Sjögren syndrome; RA, rheumatoid arthritis; NMD, neuromuscular diseases; SLE, systemic lupus erythematosus; SSc, systemic sclerosis.

Repeated measurements of the six-item core set measures for evaluation of myositis disease activity were available at baseline for twelve anti-FHL1+ and six FHL1-; and after 3-5 years for fifteen anti-FHL1+ and nine FHL1- patients with IIM. The patients in the anti-FHL1+ group had significantly higher PhyGA (median 33 vs 10, p=0.04) and a trend towards worse muscle strength at baseline as measured by MMT-8 compared with the anti-FHL1- group (median 73 vs 79, p=0.08). They also tended to have higher scores of the HAQ at baseline and more elevated CK (4.22 vs 0.59, p=0.07).

We found significant correlations between anti-FHL1 autoantibody levels at baseline and higher serum levels of CK (n=15, r=0.62, p=0.01), higher score of the disease activity composite measure MYOACT (n=14, r=0.71, p=0.004), and lower MMT-8 score (n=16, r=-0.59, p=0.01). There was an inverse correlation between anti-FHL1 autoantibody levels and global damage at the first follow-up (n=19 r=-0.44, p=0.05). At the last recorded visit there was a low correlation between higher anti-FHL1 autoantibody levels and cardiovascular damage (n=42, r=0.33, p=0.03) and an inverse correlation with CK-levels (n=42, r=-0.32, p=0.04).

Additionally, in the mixed effect regression models, we found a significant association in 39 anti-FHL1+ patients with IIM (observations during follow-up n=210) between higher levels of anti-FHL1 autoantibodies and younger age at onset of symptoms (b:-0.04, 95% CI -0.08;0.00, p=0.05) and younger age at diagnosis (b:-0.04, 95% CI -0.08;0.00, p=0.05).

In conclusion, in this study we report a higher frequency and autoantibody levels of anti-FHL1 in patients with IIM at the time of diagnosis or first available serum. Among the key findings of the study were: 1) anti-FHL1 autoantibody levels correlate with muscle weakness presented as lower MMT-8 score, higher CK index and higher activity index (MYOACT), 2) patients with anti-FHL1 autoantibodies presented earlier symptoms of the disease compared to patients that were anti-FHL1 negative and 3) anti-FHL1 autoantibodies are present in 25% of the patients that are negative for MSA and MAA autoantibodies. In the longitudinal analysis, we report a fluctuation in the anti-FHL1 levels over time. Still, the conclusions that can be drawn from these results are limited due to the low number of cases. Thus, whether these variations in the autoantibody levels are associated with the disease progression or the response to treatment remains to be determined.

6 DISCUSSION

This thesis aims to provide mechanistic insight into the role of T cells, *HLA* and autoantibodies in the pathogenesis of idiopathic inflammatory myopathies. Due to the heterogeneity of this autoimmune disease, we selected one of the most frequent groups of patients positive for anti-Jo-1 autoantibodies that carried a specific risk allele associated with the disease, the *HLA-DRB1*03*, for the study of antigen-specific T cells. We then broadened our scope by considering multiple specificities of autoantibodies and their associations with other *HLA* alleles that could be relevant to the disease. We also explored the presence and clinical associations of a muscle-specific autoantibody, the anti-FHL1, in cross-sectional and longitudinal cohorts. And finally, we developed a pipeline for isolating, analysing and characterizing single T cells from the muscle tissue of patients with IIM.

In **Paper I,** we provided mechanistic insights into three main findings in the pathophysiology of patients with IIM/ASyS: 1) the activation of antigen-reactive $CD4^+$ T cells in the blood and BALF cells by a HisRS peptide specific for the HLA-DRB1*03 molecule; 2) the presence of anti-Jo-1 autoantibodies in BALF, and 3) the presence of germinal centre-like structures in lung tissue. The high T cell reactivity observed in patients with IIM/ASyS indicates that the $HisRS_{11-23}$ epitope is a target of autoreactive T cells and that the antigen recognition site of relevance for the degree of T cell activation. In our opinion, the finding of an increased reactivity in BALF T cells was striking since this could support the contribution of the lung as a potential site of primary T cell activation towards HisRS. This finding is in line with previous reports describing a higher expression of HisRS in the lungs compared to other tissues [383] with higher sensitivity for granzyme B [312].

Our data showed that the study of HLA proteins encoded by risk alleles, such as *HLA-DRB1*03* for patients with anti-Jo-1 autoantibody, is essential for understanding the initiation of the antigen-specific immune response. Although different mechanisms could be involved in the generation and activation of autoreactive T cells (e.g., molecular mimicry, generation of neoepitopes, post-translational modifications, etc.), our finding on the identification of a potential epitope according to the binding predictions of HLA-DRB1*03:01 molecule has contributed to deepening our understanding of this association. Furthermore, the identification of T cell activation in sarcoidosis and healthy controls, although to a substantially lower degree, indicates that other *HLA* alleles could be implicated in the presentation of HisRS-derived peptides and represent potential avenues for future research.

A continuous challenge persists regarding studying of other relevant peptides with broader coverage of HisRS antigenic sites that could allow a better understanding and phenotyping of local infiltrating autoreactive T cells. As presented in material and methods, after the publication of **Paper I,** several HisRS peptides that were predicted as potential epitopes for HLA-DRB1*03:01 *in silico* were also tested *in vitro*. Additionally, we started developing HLA-II tetramers with selected peptides to continue the study of antigen-specific $CD4^+$ T cells. The use of deep sequencing technology, especially at the single level of these antigen-specific T cells, would enable us to characterize their TCR sequences. To uncover the process that shape TCR recognition can potentially offer targets for intervention, therapy or biomarkers for IIM.

The observations from **Paper I** allow us to hypothesize that after environmental stimuli associated with an inflammatory process (e.g., infection diseases, muscle damage or smoke exposure), there would be a release of granzyme B, which in the presence of HisRS, would lead to HisRS-cleavage and release of N-terminal HisRS fragments. This process would facilitate the formation and processing of neoepitopes, which may promote B cell activation and maturation, formation of GC-like structure and anti-Jo-1 autoantibody production. A schematic hypothesis of the proposed disease mechanism in anti-Jo-1 patients is presented in **Figure 26.**

The study of affected tissue such as lungs and muscle rather than only blood is highly relevant but challenging. In this thesis, we had the unique opportunity to access fresh blood, BALF and muscle samples from patients with IIM that allowed the study and characterization of lymphocytes at a tissue level. As presented in **Paper I**, the access to paraffin-embedded lung biopsies was limited, and the study in fresh or cryopreserved lung tissue would have been desirable. However, in **Paper IV,** we had the opportunity to develop a methodology for studying infiltrating single T cells in muscle and to study these cells using deep sequencing technology. This approach allowed us to analyse the TCR and clonality of the cells within blood and muscle. Furthermore, it allowed us to identify a distinct transcriptional subset enriched for features of tissue-residency, unravelling information of specific pathways that have not yet been reported. Moreover, the protocol used in this study is novel, and the feasibility of analysing sequential muscle biopsies of patients could be of clinical benefit in the future.

The discoveries of T cell infiltrates in the digested muscle, that sometimes could not be identified in the histopathology analysis suggests that this technique is more sensitive. In particular, the finding of T cells in patients with IMNM, that have low or no infiltrates (pauci-immune), has also raised some questions within the myositis community. Still, the number

of patients included in **Paper IV** was too few to draw definite conclusions for specific subgroups of patients with IIM. Nevertheless, our findings provide mechanistic insights into the tissue-resident T cells and potentially identify new biomarkers in patients with IIM.

In **Paper II** and **V**, we developed and validated an in-house ELISA protocol for the identification of anti-FHL1 autoantibodies. The use of different assays for detecting myositis autoantibodies in clinical practice is common; however, several studies have reported a high variability and difference in performance of these autoantibody assays [384, 385]. This high variability and lack of standardization have been of great controversy and a topic of further research for its harmonization and correct interpretation [150, 386]. In this thesis, the experiments performed on FHL1 proteins produced on different systems allowed us to gain more profound knowledge of the assay and to increase its performance. Additionally, the access to population-control samples that were age and sex-matched, without autoimmune diseases in Sweden or healthy controls in Australia, allowed us to compare, analyse and standardize cut-off points. Still, the lack of a commercially available kit limits access to clinical testing and further efforts are being made to have this ELISA test commercially available in the future.

As mentioned in the results part of **Paper II** and **V**, one of the key findings is the presence of anti-FHL1 autoantibodies in the group of patients corresponding to de “seronegative” for known MSA and MAA. The clinical manifestations of this group can be heterogeneous and require further characterization. Moreover, the frequency of patients without detected autoantibodies was significantly high in both cohorts tested (South Australia 47% and Sweden 35%), suggesting the need for identification of new autoantibodies or mechanism of disease in this subgroup of patients. Our results also showed the co-existence of anti-FHL1 with other MSA and MAA autoantibodies. The relevance of this latter finding and its effect on the immune response remains to be elucidated.

Likewise, the pathogenic role of this autoantibody is another pending question. One hypothetic mechanism is that the FHL1 protein, highly expressed in the muscle, upon cytotoxic CD8+ T cell infiltration and release of granzymes, could be cleaved by granzyme B (predicted site IGAD, D at amino acid position 50 [314]). In this scenario, FHL1 neo-epitopes could be released with further production of anti-FHL1 autoantibodies, contributing to the initiation and propagation of inflammation. The longitudinal results presented in **Paper V** have given us a better understanding of the behaviour of the anti-FHL1 autoantibody over time. The finding of the fluctuation of the anti-FHL1 autoantibody levels within the first three years from diagnosis or symptom onset indicates that this autoantibody could be relevant in initiating the disease. Another potential avenue of research will be to study if this

autoantibody is present before clinical manifestations, and to evaluate if the changes in the autoantibody level could be a useful prognostic marker for treatment response or damage. Still, future studies i*n vitro* and *in vivo* are required to understand the role of this autoantibody in autoimmune myopathies.

The results on **Paper III** allowed us to better understand the genetic predisposition, particularly within the *HLA* loci, of multiple specificities of autoantibodies in the subgroups of patients with IIM. This approach is of clinical relevance since, in common practice, it is not unusual to have patients with multiple autoantibody specificities. The original approach of narrowing the research questions to a specific group of patients with monospecific autoantibodies is necessary for untangling specific clinical associations with the disease and provides a basis for the tools needed to study each subset, for instance, in tetramer or peptide binding studies. However, the relevance of multiple autoantibody specificities in this heterogeneous group of patients remains unexplored.

Several epidemiological studies have identified differences in morbidity and mortality between patients positive for either anti-Jo-1 (subgroup 5 in **Paper III**) or the co-expression of anti-Jo-1 and anti-Ro52 (subgroup 6 in **Paper III**) [236, 387]. Clinically, the co-expression of anti-Jo-1/Ro52 autoantibodies has been associated with worse clinical outcomes associated with a more aggressive ILD [236], a predictor for relapse in PM and DM [388] or even a sub-phenotype characterized by female patients with arthritis, ILD and low prevalence of myositis [389]. A recent association study with *HLA* variants, clinical features and autoantibody profile in patients with primary Sjögren's syndrome reported a strong association signal with SNPs in the *HLA-DQA1* locus, only for the SSA/SSB autoantibody-positive subgroup [390], similarly to our results in subgroup 6 (co-expression of anti-Jo-1/Ro52). In the same line, a study in SLE revealed that the association with the *HLA-DRB1*03* (in high linkage disequilibrium with *HLA-DQA1* variants) in this disease could be attributed to the patients positive for anti-Ro/La [391].

In **Paper III**, we confirm associations that have been previously reported for anti-Mi2 (subgroup 4) in Caucasian and European American patients [392, 393]. Nonetheless, we identified new *HLA* class I and II risk alleles and amino acids for anti-TIF1γ (subgroup 7) that can potentially indicate a new research area regarding the association with cancer. Additionally, we report an overview of the risk alleles of [394] the group negative for other autoantibodies tested (subgroup 8), which can provide new avenues for future research. This last group could be partially composed by patients belonging to new autoantibodies, such as anti-FHL1.

The results of **Paper III** increase the evidence presented in other studies in autoimmune diseases, such as RA [395], SSc [396], SLE [391] and pSS [390], that there is an intrinsic relationship between given HLA alleles and the presence of specific autoantibodies. The mechanism behind *HLA* genetic variants and the production of MSA/MAA in IIM and other autoimmune diseases remains to be understood. Potential explanations include the "peripheral hypothesis" [397], where *HLA* risk alleles may increase the presentation of autoantigens to the immune system, as has been reported in RA [375, 395], type 1 diabetes [398] and celiac disease [399]. Another suggested mechanism, not mutually exclusive, is the "central hypothesis" [344, 376], where specific *HLA* alleles may influence thymic T cell selection, leading to an escape of negative selection and increased frequency in high-affinity autoreactive T cells. Some genetic polymorphisms also play a significant role in the transcriptional response and effect on immune cells possibly contributing to the risk of developing autoimmune diseases [400]. Uncovering such pathways would provide a better insight into the pathogenesis of the autoimmune disease and possibly indicate the use of potential biomarkers and therapeutic targets. A proposed mechanism for autoantibody production in IIM and its association with *HLA* is presented in **Figure 27.**

One of the main limitations of **Paper I** and **Paper IV** was the low number of patients, which restricted some conclusions. Still, some of the main results allowed us to highlight key findings, such as the presence of autoreactive T cells in BALF, clonal expansion and tissue-resident T cells in muscle. On the other hand, **Paper II, III** and **IV** consisted of a large number of patients, which is required when looking for the detection of new autoantibodies or possible new genetic associations. Nevertheless, even with the larger number of samples from the Australian cohort, the SweMyonet and the MyoNet registry, IIM is a rare and heterogeneous disease, limiting the statistical power of the analysis by autoantibody profile. These studies underpin the importance of research collaboration with multiple centres to keep studying the pathogenic mechanism of this rare disease.

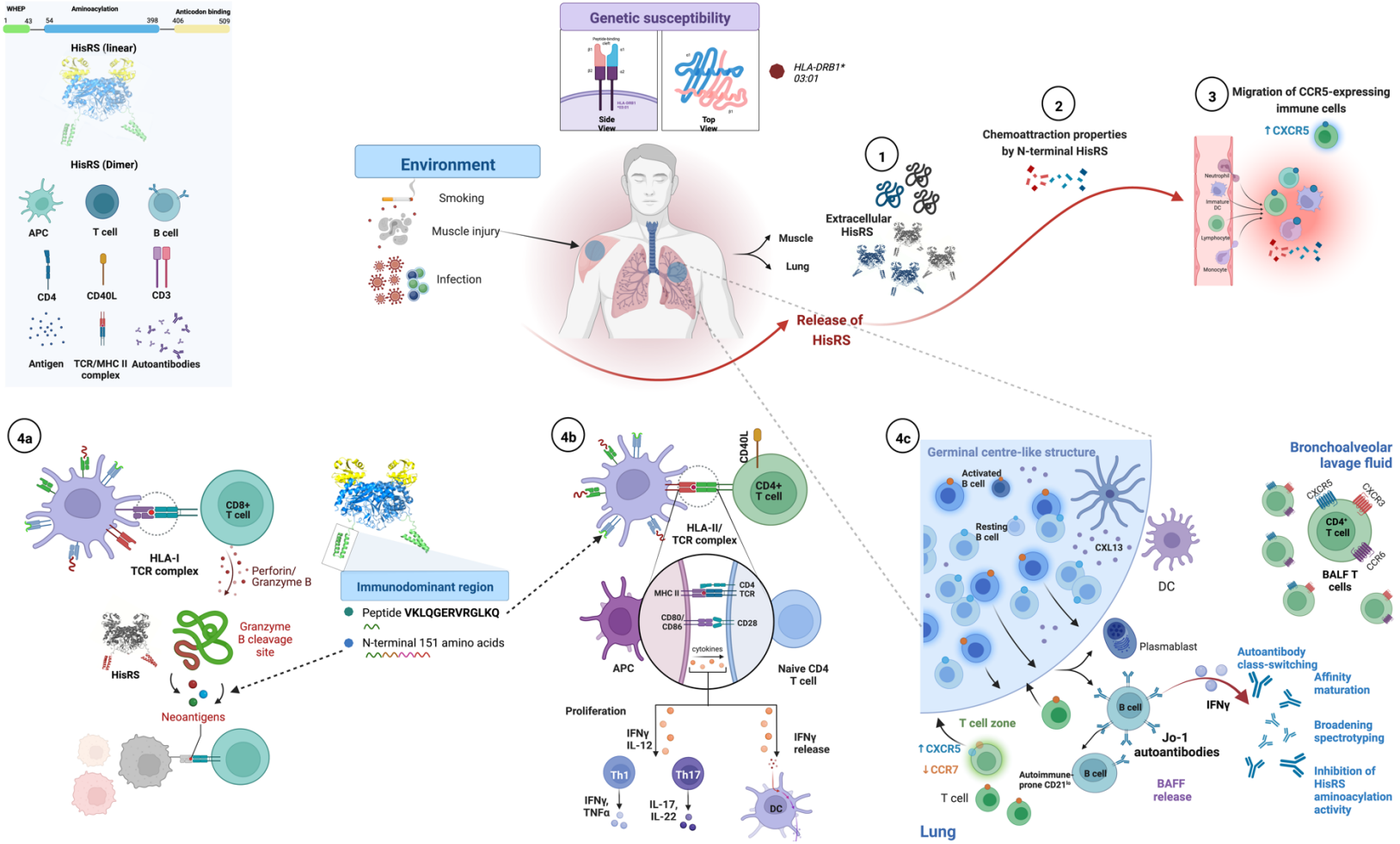


Figure 26. Hypothetical model of pathogenesis of patients with anti-Jo-1+ autoantibodies and ASyS syndrome. (1) The presence of an inflammatory environmental factor (e.g. smoking, muscle injury, infection, etc) would promote the release of HisRS, which is highly expressed in lung and muscles. In a susceptible host with *HLA*-gene risk allele (*HLA-DRB1*03:01)* this could lead to the activation of proinflammatory T cells. **(2)** The N-terminal HisRS fragment can induce the migration of CCR5-expressing immune cells and **(3)** would recruit T cells to the inflammatory site such as the muscle or lung. **(4a)** Proposed model of the antigen presentation from HLA-I $CD8^+$ T cell, granzyme B release and expressions of autoantigens. **(4b)** Proposed mechanism of antigen presentation from HLA-II $CD4^+$T cell according to our epitope prediction; activation and proliferation of antigen-specific T cells with further cytokine secretion. **(4c)** Hypothesis of the immune cell interactions in germinal centre-like structures in the lung, activation of B and T cells, local production of autoantibodies and autoimmune prone B cells. Created with Biorender.com

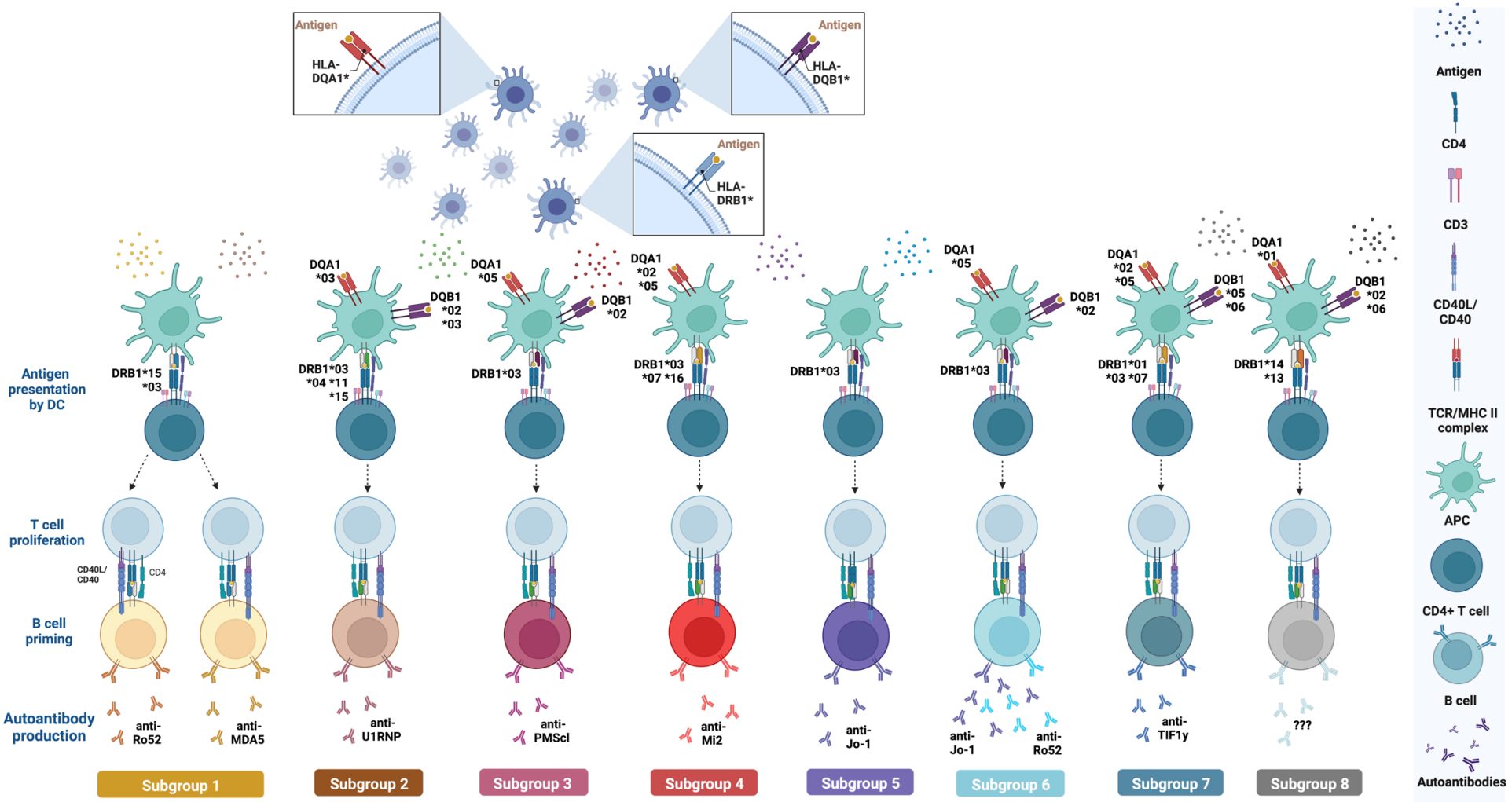


Figure 27. Suggested mechanisms for autoantibody production in IIM. Different HLA molecules, determined by their genetic variability, influence antigen presentation, T cell differentiation and proliferation, and priming of B cells, leading to specific autoantibody production in IIM. Based on the expression of particular *HLA* alleles, distinct MSA and/ or MSA are produced. In some cases, this differential expression of *HLA* class II would lead to B-cell priming and production of both MSA (for example: anti-Jo-1 or anti-MDA5) and MAA (for example: anti-Ro52) in the same individual (subgroup 1 or 6). Created with Biorender.com

7 CONCLUSIONS AND POINTS OF PERSPECTIVE

IIM is an active area of clinical and translational research. Through international collaborations and continuous research in the fundamental and clinical applied fields, we have contributed to the understanding of the pathophysiology of autoimmune myositis. The role of innate and acquired immunity (T cells, B cells, dendritic cells) with environmental factors and genetic predisposition has allowed a deeper study on specific epitopes which may be targets of new therapies and potential biomarkers for individuals at high risk.

In the last decades, the role of autoantibodies has been better elucidated, with the recognition that autoantibodies play an essential role not only in the establishment of the diagnosis but also in the prognosis and current response to treatment. The importance of this thesis is focused on two main autoantibodies: anti-Jo-1 and anti-FHL1. Given the characteristics of the humoral immune response against anti-Jo-1 (with spectratype broadening, epitope spreading and affinity maturation) as well as the clinical and epidemiological associations, it is necessary to identify the specific *HLA-DRB1*03:01*-restricted T cell epitopes and to define the relationship between anti-Jo-1 positivity and presumed T cell-driven, adaptive immune responses in IIM.

In **Paper I,** we report the identification of a specific HisRS-derived peptide in *HLA–DRB1*03*–positive individuals. Our approach to studying the response of a predicted epitope *in silico* and the identification of a potential epitope adds to the cumulative evidence supporting the presence of *HLA* risk alleles and their affinity for pathogenic antigens. This finding allows us to contribute to the hypothesis of the pathogenesis of anti-Jo-1 autoantibodies in IIM and suggest that the local activation in the lung may play a critical role in this disease, including the presence of germinal centre-like structures and local production of autoantibodies. A continuous challenge persists regarding studying other relevant peptides with broader coverage of HisRS antigenic sites and a deeper understanding of the phenotyping of local infiltrating autoreactive T cells. After the publication of **Paper I**, several HisRS peptides were tested *in vitro* to continue the study of antigen-specific $CD4^+$ T cells with HLA-II tetramers. Still, the question remains regarding the antigen-specificity of these $CD4^+$ reactive T cells and their phenotype, which suggest potential avenues as future research priorities. A major future goal is to determine the molecular and cellular basis of the antigen-specific T cells and TCRs involved in the disease, including circulating and tissue-resident $CD4^+$ and $CD8^+$ T cells.

In **Paper II** and **V**, we present a clinical characterization of the muscle-specific autoantibody, anti-FHL1, therefore improving our understanding of the clinical characteristics of this

autoantibody. **Paper II** showed that the presence of anti-FHL1 autoantibodies is more prevalent in IIM but can also be present in other autoimmune diseases such as SSc. However, there is a lack of a particular clinical phenotype, and although this autoantibody is muscle-specific, it could be defined as MAA. Elucidating the functional role of anti-FHL1 antibodies in IIM and other autoimmune diseases will be needed. In **Paper V**, we correlated the presence and levels of the antibody with disease activity, organ damage, outcome measures and response to treatment. Still a larger cohort analysis will be required due to a lack of power in the study. Additionally, future studies will be needed to confirm the risk *HLA* associations found in the study. We plan to develop an available assay to detect anti-FHL1-positive patients with standardised diagnostic tests, allowing a more accessible identification of this subset of IIM patients.

In **Paper III**, we presented differential and novel *HLA* class II and class I associations in autoantibody-defined IIM subgroups considering multiple specificities. We found relevant associations among the *HLA* and subgroups of patients defined by autoantibodies profile, particularly those dominated by anti-Jo-1/Ro52, anti-Mi2, TIF1γ and the seronegative group for the autoantibodies tested in the study. This approach sheds light on the presence of distinct pathogenic mechanisms, which will contribute to further understanding of autoantibody production in patients at risk. Longitudinal studies are needed to evaluate if these autoantibody-defined subgroups are associated with specific clinical manifestations, response to treatment or relapse.

The results depicted in **Paper IV** demonstrate the feasibility of using single-cell sequencing to explore T cell infiltrate in the local tissue, even when there is a low presence of immune cells detected by other standard methods. With this approach, we could identify a muscle T cell signature and receptors associated with homing and tissue residency. Moreover, the clonality analysis revealed that the T cell clones were mainly found within cytotoxic and tissue-resident T cell clusters and can resist therapy since the results showed their persistence after conventional immunosuppressive treatment. This finding could facilitate the development of personalized therapeutic targets and biomarkers in myositis. Still, it is necessary to include a larger sample of patients and study other affected tissues such as skin or lungs and preferably at different time points, such before and after treatment or disease flare. Increased knowledge of molecular pathways that may lead to chronic inflammation in the lungs and the muscle will be essential to exposing susceptibility patterns, identifying risk groups and developing new targets for therapy. Though the approach is highly desirable, at this moment, the cost would limit the inclusion of patients and the number of samples. Possibly this technique will be more accessible and automatized in the future,

including the pipeline to perform high-throughput analysis of integrated datasets that can combine genetic, epigenetic, transcriptomic, proteomics and clinical data.

In **Paper V**, the results provide a better understanding of anti-FHL1 autoantibody levels in adult patients with IIM over time, with the highest prevalence close to diagnosis and a high frequency of seroconversion to anti-FHL1 negative sera during follow-up. Additionally, we show that LD, ALAT, ASAT, CK ratio, MMT-8 and MYOACT correlate with the levels of anti-FHL1 autoantibodies, indicating a possible mechanism associated with disease activity and muscle involvement. Future studies *in vitro* and *in vivo* are required to understand the potential role of this autoantibody in the pathophysiology of autoimmune myopathies.

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