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TESI DI LAUREA

CHARACTERIZATION OF THE CARGO OF CIRCULATING EXTRACELLULAR VESICLES IN PATIENTS AFFECTED WITH IDIOPATHIC INFLAMMATORY MYOPATHIES AND EVALUATION OF CLINICAL CORRELATES IN A CROSS-SECTIONAL COMPARATIVE ANALYSIS FROM A MONOCENTRIC COHORT

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Sommario

ABSTRACT

Background idiopathic inflammatory myopathies (IIM) are a heterogenous group of systemic autoimmune disorders affecting primarily the striated muscle, yet encompassing different additional clinical manifestations. Distinct epigenetic features are likely to influence the disease course, partially accounting for differences across IIM subsets. The research field on extracellular vesicles (EVs) is growing, highlighting the crucial role of their biologically active cargo, including proteins and nucleic acids like micro-RNAs (miRNAs), in intercellular communication and antigen trafficking. miRNAs are post-transcriptional gene expression regulators capable of modulating the immune response and are likely involved in the development of several systemic autoimmune diseases. Unravelling of any epigenetic influence in IIM pathogenesis may shed light on their pathogenic mechanisms and provide translational implications.

Aim of the study This study aims to investigate the circulating EV pool across different IIM subsets and healthy donors (HD) through EV isolation and characterization of the miRNA content, ultimately assessing the potential role of circulating EVs as pathogenetic players and IIM biomarkers.

Materials and methods A monocentric study was conducted including consecutive adult (≥18 years old) IIM patients (classified according to the EULAR criteria) and followed up at the Rheumatology Unit of Padua University Hospital, and age- and sex- matched HD. Clinical and laboratory assessment of IIM patients was performed by an experienced rheumatologist. EVs from patients and HD were isolated from platelet-free plasma through size exclusion chromatography followed by ultrafiltration and quantified by nanoparticle tracking analysis (NTA). EV-miRNA cargo was investigated through Next-Generation Sequencing (NGS). Differences in EV distribution and miRNA expression between IIM and HD and across IIM subsets were assessed. Additionally, changes in the EV pool according to clinical parameters and response to treatment in IIM patients were investigated.

Results Sixty-four consecutive IIM patients and sixty-five HDs were included in the study. NTA measurements of EVs concentration showed a significantly higher mean concentration of circulating EVs in IIM patients than in HD ($p=0.0073$).

Across IIM subsets, patients affected with cancer associated myositis (CAM) displayed the highest levels of circulating EVs compared to no CAM patients (p=0.0060) and to HD (p=0.0004). Patients with circulating myositis-associated autoantibodies displayed significantly higher EV levels than HD (p=0.0363). Patients in clinical remission displayed higher levels of circulating EVs compared to those with active disease (p=0.0087). Moreover, the EVs levels were significantly reduced in IIM patients treated with rituximab (RTX) than in patients receiving other treatments (p<0.0001). NGS analysis detected EV-miRNAs with different expression profiles between IIM (n=47) and HDs (n=49): miR-223-3p (p=0.019), miR-15a-5p (p=0.0189), miR-451a (p=0.0074), miR-486-5p (p=0.0052), miR-32-5p (p=0.0146), and miR-222-3p (p=0.0282) were up-regulated in IIM, while miR-141-3p (p=0.0313), miR-142-3p (p=0.0244), and let-7a-5p (p=0.0003) were down-regulated in IIM patients vs. HDs. Other EV-miRNAs expression varied across IIM subsets: CAM patients displayed up-regulated expression of miR-143-3p compared to non-CAM patients (p=0.0085), while miR-148a-3p (p=0.0171) and miR-335-5p (p=0.0171) were up-regulated in dermatomyositis vs. polymyositis/ inclusion body myositis/anti-synthetase syndrome patients. Patients characterized by active disease displayed an up-regulated expression of miR-222-3p (p=0.002) and miR-151-3p (p=0.0233) and down-regulated expression of miR-363-3p (p=0.0001), miR-374a-5p (p=0.0258), miR-144-3p (p=0.0170), miR-181a-5p (p=0.0037) compared to those in clinical remission. Moreover, IIM patients receiving only glucocorticoids (GC) reported up-regulated expression of miR-4433b-5p (p=0.0439), miR-92a-3p (p=0.0111), let-7f-5p (p=0.0304), and down-regulated expression of miR-27a-3p (p=0.0486) compared to patients receiving GC in combination with immunosuppressants (IS).

Conclusions Our study showed significantly increased concentration of circulating EVs in IIM patients compared to HD, with differences within specific disease subsets. EV-miRNAs exhibited a differential expression profile between IIM and HD and significant differences were outlined across IIM subsets. These preliminary results suggest the involvement of EVs in IIM pathogenesis and we speculate a potential role of EVs and EV-miRNAs as novel biomarkers for diagnosis, disease subset characterization and treatment response evaluation.

RIASSUNTO

Introduzione Le miopatie infiammatorie idiopatiche (IIM) sono un gruppo eterogeneo di malattie sistemiche autoimmuni che coinvolgono principalmente il muscolo striato, oltre a presentare ulteriori e variabili manifestazioni cliniche. Elementi epigenetici specifici influenzano il decorso clinico e spiegano in parte le marcate differenze riscontrabili tra sotto-gruppi di pazienti. La ricerca sulle vescicole extra-cellulari (EVs) è in espansione, e sottolinea il ruolo del loro cargo biologicamente attivo, che include proteine e acidi nucleici come i micro-RNA (miRNA), nei processi di comunicazione intercellulare e presentazione antigenica. I miRNA sono importanti regolatori post-trascrizionali dell'espressione genetica e sono coinvolti nella patogenesi di numerose patologie autoimmuni, data la loro capacità di influenzare diversi aspetti della risposta immunitaria. Chiarire gli elementi epigenetici coinvolti nelle IIM potrebbe fornire informazioni utili riguardo la fisiopatogenesi di queste patologie suggerendo importanti implicazioni traslazionali.

Scopo dello studio Questo studio ha l'obiettivo di caratterizzare le EV e il loro contenuto di miRNA in pazienti con IIM, per individuare differenze tra pazienti e soggetti sani (HD) e tra pazienti con diverse caratteristiche, al fine di esplorarne il ruolo come fattori patogenetici e come biomarcatori.

Materiali e metodi E' stato condotto uno studio monocentrico che ha incluso pazienti consecutivi adulti (>18 anni) con diagnosi di IIM (classificati secondo i criteri EULAR) e seguiti presso il reparto di Reumatologia del Policlinico Universitario di Padova, comparandoli con soggetti sani appaiati per età e genere. La valutazione clinica e laboratoristica è stata condotta da un reumatologo esperto. Le EVs sono state isolate da plasma privo di piastrine di pazienti e HD tramite cromatografia ad esclusione dimensionale seguita da ultrafiltrazione e quantificate tramite Nanoparticle Tracking Analysis (NTA). Il cargo di miRNA delle EV è stato caratterizzato tramite Next-Generation Sequencing. Sono stati confrontati i livelli di EV e l'espressione dei miRNA tra pazienti e soggetti sani e tra diversi sottogruppi di pazienti.

Risultati 64 pazienti consecutivi con IIM e 65 HD sono stati inclusi nello studio. La valutazione tramite NTA ha dimostrato concentrazioni di EV circolanti

significativamente maggiori nei pazienti rispetto a HD (p=0.0073). Tra i sottotipi di pazienti, quelli affetti da IIM associata a neoplasie presentavano livelli più alti di EV rispetto agli altri pazienti (p=0.0060). La concentrazione di EV era significativamente superiore nei pazienti con autoanticorpi rispetto ai HD (p=0.0363) e nei pazienti in remissione clinica rispetto ai pazienti con malattia attiva (p=0.0087). Inoltre, la concentrazione di EV era significativamente inferiore nei pazienti trattati con Rituximab rispetto ai pazienti trattati con altri farmaci (p<0.0001). L'analisi NGS ha rilevato livelli di miRNA associati alle EV diversi tra pazienti (n=47) e HD (n=49): miR-223-3p (p=0.019), miR-15a-5p (p=0.0189), miR-451a (p=0.0074), miR-486-5p (p=0.0052), miR-32-5p (p=0.0146) e miR-222-3p (p=0.0282) erano incrementati nei pazienti, mentre miR-141-3p (p=0.0313), miR-142-3p (p=0.0244), e let-7a-5p (p=0.0003) erano diminuiti nei pazienti rispetto ai HD. Altre differenze sono state rilevate tra sottogruppi di pazienti. Pazienti con IIM associata a neoplasie presentavano maggiore espressione di miR-143-3p rispetto ai restanti pazienti (p=0.0085), mentre pazienti con dermatomiosite presentavano una maggiore espressione di miR-148a-3p (p=0.0171) e miR-335-5p (p=0.0171) rispetto ai pazienti con polimiosite + miosite a corpi inclusi + sindrome da anticorpi anti-sintetasi. Pazienti con malattia clinicamente attiva presentavano una maggiore espressione di miR-222-3p (p=0.002) e miR-151-3p (p=0.0233) e una minore espressione di miR-363-3p (p=0.0001), miR-374a-5p (p=0.0258), miR-144-3p (p=0.0170), miR-181a-5p (p=0.0037) rispetto a pazienti in remissione clinica. Inoltre, pazienti trattati esclusivamente con glucocorticoidi presentavano una maggiore espressione di miR-4433b-5p (p=0.0439), miR-92a-3p (p=0.0111), let-7f-5p (p=0.0304), e una minore espressione di miR-27a-3p (p=0.0486) rispetto ai pazienti trattati con un'associazione di glucocorticoidi e altri immunosoppressori.

Conclusioni Questo studio dimostra che la concentrazione di EV circolanti è significativamente maggiore nei pazienti con IIM rispetto a HD, con differenze anche tra gruppi di pazienti con diverse caratteristiche. Inoltre, i miRNA presentano profili di espressione diversi tra pazienti e HD e tra diversi sottogruppi di pazienti. Questi risultati preliminari suggeriscono il ruolo di EV e miRNA come nuovi biomarcatori utili nella diagnosi di IIM, nella distinzione di diversi sottotipi di malattia e nella valutazione della risposta al trattamento.

IDIOPATHIC INFLAMMATORY MYOPAHTIES

DEFINITION

Idiopathic inflammatory myopathies (IIM) are a heterogeneous class of rare systemic autoimmune disorders characterized by chronic muscle inflammation and weakness. Extra-muscular manifestations, such as skin rash, arthritis, interstitial lung disease, display variable frequency across different IIM subtypes, and in some patients these can dominate the clinical picture, with minimal or absent muscle weakness. [1]

Along with signs and symptoms, myositis-specific and associated autoantibodies are associated with distinct clinical patterns and disease phenotypes. Occurrence in overlap with other connective tissue diseases and the association with malignancies are possible in IIM.

CLASSIFICATION

Several classification systems for IIM have been developed through the decades, however frequently biased by being based on personal experience of small monocentric cohorts and often not including all significant IIM subtypes. [2]

The first system to provide a somewhat rigorous framework for IIM classification was provided by Bohan and Peter in 1975. Key aspects of their criteria that distinguished them from prior attempts were: the requirement first to exclude all other forms of myopathy; an approach to estimate the certainty of diagnosis of both PM and DM by defining possible, probable and definite disease criteria; the first inclusion of “characteristic rashes” to distinguish DM; detailed descriptions of the criteria; and definitions for five subgroups of IIM, including polymyositis (PM), dermatomyositis (DM), juvenile PM/DM, overlap myositis and cancer-associated myositis. [3]

In the following years, along with the discovery of other subsets of disease and antibodies specific to IIM, several modifications were proposed to the Bohan and

Peters criteria by different groups, shifting emphasis on varying features of clinical, serological, pathological nature. For instance, in 1991 Love et al. suggested a classification based on several specific autoantibodies that defined subgroups of patients with their clinical features, response to therapy and prognosis. In 1997 Targoff et al. proposed a system retaining the Bohan and Peters designation of definite, probable and possible disease with minimal change, except for the addition of one criterion based on the presence of myositis specific antibodies. [4]

This ongoing process led to EULAR-ACR (European Alliance of Associations for Rheumatology-American College of Rheumatology) publishing in 2017 a new classification system for adult and juvenile IIM aiming to serve as a practical tool to distinguish IIM from mimicking conditions with high sensitivity and specificity as well as characterizing major subgroups of IIM. This effort involved international experts in adult and pediatric rheumatology, neurology, dermatology, epidemiology and biostatistics. The following IIM subgroups were defined: polymyositis (PM), inclusion body myositis (IBM), dermatomyositis (DM), amyopathic dermatomyositis (ADM), juvenile myositis other than JDM. [5]

In the process of classification development, statistic models were tested on selected candidate criterion items from expert opinions and existing criteria for IIM. In this model, 16 variables were identified, and each variable was given a score on the basis of its predictive ability. The sum of weighted scores from each criterion item is converted to IIM probability using a mathematical formula, differing whether muscle biopsy information is present or not. A probability cut-off of ≥55% was defined as the minimum probability needed to classify a patient as having an IIM. These criteria were demonstrated to reach a maximum sensitivity of 93% and a specificity of up to 88% when providing biopsy data, in its absence restricted to 87% and 82% respectively. Patients with a score that reaches minimum threshold probability of having IIM can then be subclassified into major IIM subgroups in a stepwise manner using a classification tree. Through this tree, patients with juvenile or adult dermatomyositis, amyopathic dermatomyositis, IBM or polymyositis could be identified. [2,5]

Variable	Score points	
	No biopsy	Biopsy
Age of onset of first related symptoms		
18–40	1.3	1.5
≥40	2.1	2.2
Muscle weakness		
Objective symmetric weakness, usually progressive, of proximal upper extremities	0.7	0.7
Objective symmetric weakness, usually progressive, of proximal lower extremities	0.8	0.5
Neck flexors are relatively weaker than neck extensors	1.9	1.6
In the legs, proximal muscles are relatively weaker than distal muscles	0.9	1.2
Skin manifestations		
Heliotrope rash	3.1	3.2
Gottron's papules	2.1	2.7
Gottron's sign	3.3	3.7
Other clinical manifestations		
Dysphagia or esophageal dysmotility	0.7	0.6
Laboratory measurements		
Anti-Jo-1 (anti-histidyl-tRNA synthetase) autoantibody positivity	3.9	3.8
Elevated serum levels of creatine kinase (CK)* *or* lactate dehydrogenase (LDH)* *or* aspartate aminotransferase (ASAT/AST/SGOT)* *or* alanine aminotransferase (ALAT/ALT/SGPT)*	1.3	1.4
Muscle biopsy features		
Endomysial infiltration of mononuclear cells 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

Figure 1 Score points for the European League Against Rheumatism/American College of Rheumatology classification criteria for adult and juvenile idiopathic inflammatory myopathies to be used when no better explanation for the symptoms or signs exists

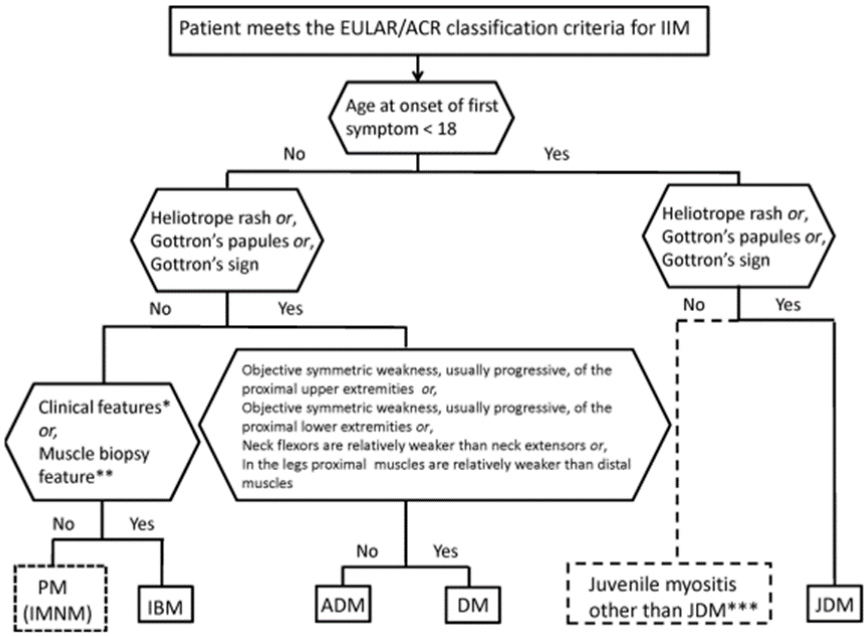

Figure 2 Classification tree for subgroups of idiopathic inflammatory myopathies (IIMs). A patient must first be classified as having IIM using the European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria. The patient can then be subclassified using the classification tree. The mixed (dotted outlined box) subgroup of patients with PM includes patients with IMNM. Juvenile myositis other than JDM was developed based on expert opinion and extrapolation from adults. IMNM and hypomyopathic DM were too few to allow subclassification. ADM, amyopathic dermatomyositis; DM, dermatomyositis; IBM, inclusion body myositis; IMNM, immunemediated necrotising myopathy; JDM, juvenile dermatomyositis; PM, polymyositis

This classification system, albeit constituting a rigorous and integrated approach in IIM diagnosis and classification, still presents several limitations. For instance, it lacks a clear frame of reference for recognized and frequent clinical-nosological entities such as anti-synthetase syndrome, cancer associated myositis, immune mediated necrotizing myopathy (IMNM) and overlap myositis.

Furthermore it doesn't include several autoantibodies that are widely regarded as confirmatory diagnostic tools, contributing to the definition of specific disease subsets, leading to unsatisfactory performance in some instances . This is the case, for example, in anti-MDA5 positive cases of dermatomyositis which are known to have distinct clinical and prognostic profiles and are not defined in the EULAR-ACR system.[6]

For these reasons, the definition of other recognized entities like the anti-synthetase syndrome (ASyS) must rely on other independent classifications like the one proposed by Connors in 2010. This system allows to diagnose cases of ASyS on the basis of the presence of serum positivity for an anti-aminoacyl-tRNA-synthetase antibody, plus one or more of several clinical features, including Raynaud's phenomenon, arthritis, interstitial lung disease, fever and mechanic's hands. [7] The process of disease classification is constantly undergoing rounds of update, in keeping up with novel phenotypes with prominent or exclusive extra-muscular manifestations being characterized.

EPIDEMIOLOGY

A wide range of estimates of incidence and prevalence of IIM have been published. Globally, albeit with a majority of studies from Asia, Europe and North America, the incidence estimated range is 11-660 new cases/1,000,000 person/year and prevalence 3-34 patients/100,000 people.[8]

The global incidence peaks around 50–60 years of age, though PM and DM may also present in juvenile forms (11-16 years). The overall female-to-male ratio is

about 2:1 with the remarkable exceptions of IBM and CAM, where the male-to-female ratios are 3:1 and 2:1, respectively.

The incidence and prevalence of DM are estimated at 1.4/100,000/year and 5.8/100,000 respectively.[9] A female preponderance is reported with an overall ratio of 2:1 [10] and average age of onset is between 45 and 64 years. [11]

JDM has an estimated prevalence of 3.2/1,000,000 children.[9] It appears to be more common among girls and age of onset peaks around 5-14 years.[11]

Age- and gender- adjusted incidence of PM is approximately 3.8/100,000/year and prevalence 9.7/100,000.[9]

IBM is considered to be the most frequently acquired myopathy after the 50th year of life, with a prevalence around 9.3/1,000,000 in the general population and 51.3/1,000,000 in people aged over 50 years[9]. It affects twice as many men as women.[12] The prevalence of IBM displays a degree of geographical variability consistent with the frequency of specific haplotypes in the populations studied, corroborating data regarding the role of specific genes in IBM.[13]

ASyS has an estimated global prevalence around 9 cases/100,000, with its defining autoantibodies being reported in around 11-40% of total IIM cases. It affects more frequently females than males with an estimated ratio of 7:3 and average age of disease onset is 48 ±15 years, similar to some estimates for DM and PM but younger than IBM and IMNM (immune mediated necrotizing myopathy). [14]

IMNM cases with anti-SRP or anti-HMGCR antibodies account for less than 10-15% of adult IIM cases, being more frequent in women over 40 years of age. These autoantibodies are also reported in juvenile patients, albeit less frequently. [15]

PATHOLOGY FINDINGS

With recent advances in serological testing and imaging modalities, diagnosis of IIM in clinical practice is becoming less reliant on muscle histology. Nevertheless, it is an invaluable tool for confirming IIM subtypes and is still considered the gold standard for IIM diagnosis.[16]

DM

In dermatomyositis, the inflammation is predominantly perivascular or in the interfascicular septae and around rather than within the fascicles. Early in the inflammatory process, there is activation of the complement leading to the formation and deposition of the C5b-C9 complement membrane attack complex (MAC) on or around the endomysial blood vessels. The inflammatory infiltrate consists primarily of B cells, macrophages and T CD4+ cells. The intramuscular blood vessels show endothelial hyperplasia with tubuloreticular profiles, fibrin thrombi, especially in children, and obliteration of capillaries resulting in reduction of capillary density. Muscle fibers undergo phagocytosis and necrosis, commonly in groups involving the periphery of the fascicle, resulting in perifascicular atrophy. This pattern of atrophy, characterized by two to ten layers of atrophic fibers at the periphery of the fascicles, is diagnostic of dermatomyositis, even in the absence of inflammation. Muscle fibers, especially in the perifascicular area, are reported to aberrantly express MHC class I complex, while MHC II is expressed on surrounding vascular cells. The skin lesions show perivascular inflammation with CD4-positive cells in the dermis; in chronic stages there is dilatation of superficial capillaries. Notably Skin histopathology in DM is not clearly discernible from cutaneous lesions in SLE.[10,17–19] Some features of DM pathology, like perifascicular atrophy, can be present in ASyS, especially the cases with anti-jo-1 antibodies. New markers have been proposed to aid pathology evaluation, like sarcoplasmic MxA expression, which has shown a 98-100% specificity and is proposed by some authors as diagnostic hallmark for seronegative DM. [5]

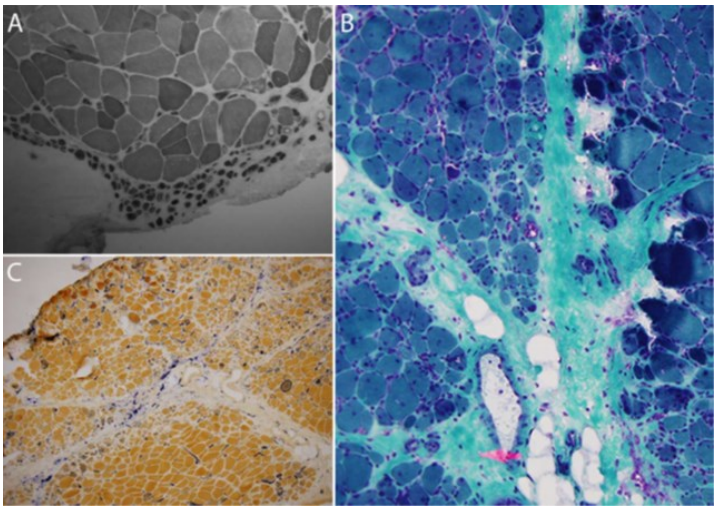

Figure 3 Key myopathologic features of DM. (A) ATPase stain and (B) Gomori trichrome stain show perifascicular atrophy. (C) Alkaline phosphatase staining (blue) shows primary involvement of perimysial connective tissue[54]

IBM

Muscle biopsies from patients with sporadic IBM are histologically unique in that they often include coexisting inflammation, mitochondrial dysfunction, and abnormal protein aggregation. The inflammatory infiltrate is comprised of CD8+ T cells that surround and invade non-necrotic fibers. An increased number of cytochrome oxidase-negative muscle fibers and the presence of so-called ragged-red fibers suggest that mitochondrial damage has an important role in sporadic inclusion-body myositis. Rimmed vacuoles, best visualized by Gomori-trichrome staining, are considered a histological hallmark. Nuclear membrane proteins are found within rimmed vacuoles, suggesting that these vacuoles could be the remnants of degenerated myonuclei. Another study showed that proteins accumulating in rimmed vacuoles are related to protein folding and autophagy, suggesting that impaired autophagic function could be implicated in their formation. Several other cytoplasmic inclusions, which are patho-physiologically relevant and important for diagnosis, are found in muscle biopsies of patients with inclusion-body myositis. The tubofilamentous inclusions seen by electron

microscopy gave rise to the name inclusion-body myositis. Cytoplasmic accumulations of amyloid can be visualized using Congo red and polarized light. These structures include β-amyloid-related and amyloidogenic-related molecules β-secretase and γ-secretase, which are increased in the plasma of patients with sporadic inclusion-body myositis. Cytoplasmic aggregations of other proteins, including phosphorylated neurofilaments, p62, and TAR DNA-binding protein can also be found in muscle biopsies of patients with sporadic IBM.[12,19]

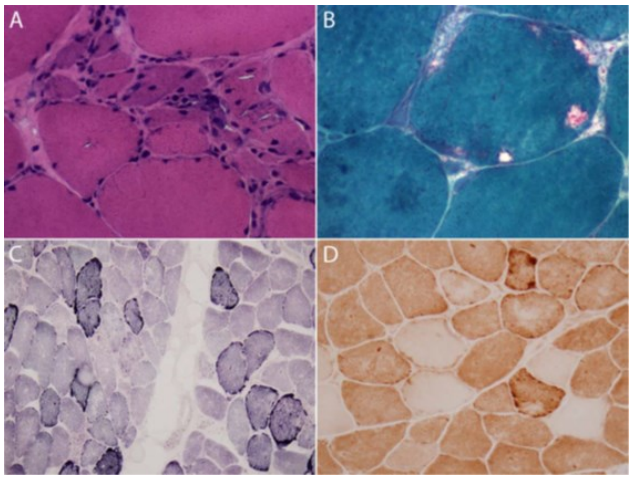

Figure 4 Histologic features differentiating IBM from PM. (A) H&E and (B) Gomori trichrome stains show rimmed vacuoles in sIBM. (C) Succinate dehydrogenase (SDH) stain demonstrating increased irregular uptake of blue dye in fibers that would be considered ragged red fibers on Gomori trichrome, and (D) cytochrome oxidase (COX) stain demonstrating several COX-negative fibers. These findings are predictive of steroid non-responsiveness and a clinical picture of sIBM. In addition, a number of COX-positive fibers can be seen, consistent with ragged red fibers[54]

ASYS

ASyS muscle biopsies can reveal perifascicular atrophy similar to dermatomyositis. However, compared with dermatomyositis, the muscle biopsies from patients with antisynthetase syndrome can show an increased number of perifascicular necrotic fibers. Furthermore, most of these biopsies show nuclear actin

aggregation, an electron microscopy feature that is not seen in other inflammatory myopathies.[12]

Macrophagocytosis is another characteristic finding in muscle biopsies. Perimysium, predominantly areas around the vessels, is infiltrated by macrophages and CD8 lymphocytes. Alkaline phosphatase activity is highly expressed in the perimysium. Contrary to polymyositis and inclusion body myositis, no infiltrates in the endomysium are observed. Increased expression of major histocompatibility complexes class I and II (MHC I and MHC II) can be observed in the cytoplasm and on the sarcolemma of myofibers, predominantly in the perifascicular region.[14] Other features, such as perimysial connective tissue fragmentation, are seen in anti-Jo-1, anti-OJ, and anti-PL-7 ASyS. Sarcolemmal MAC deposition in nonnecrotic fiber is observed, frequently with perifascicular pattern in anti-Jo-1 ASyS.[5]

IMNM

Histological features required for IMNM diagnosis include: Individualized necrotic myofibres scattered throughout the muscle biopsy presenting different stages of necrosis, myophagocytosis and regeneration; Paucilymphocytic infiltrates composed predominantly of macrophages. Other characteristic finding are MHC class I expression on the sarcolemma of non-necrotic muscle fibers with regional focal differences in intensity; Deposition of complement (C5b–9) on the sarcolemma; Atrophic myofibers with signs of fibrosis and fatty tissue replacement; Coarse staining of many fibers in the sarcolemma with NADH-tetrazolium reductase stains and enlarged vessel walls. An interesting diagnostic hallmark of IMNM is the constant presence of several myofibers with fine granular and homogeneous staining of the autophagy marker p62 in the sarcoplasm.[15]

Although paucilymphocytic infiltrate is specific, lymphocytic infiltration can be seen in approximately 20% of the antibody-positive patients. [5]

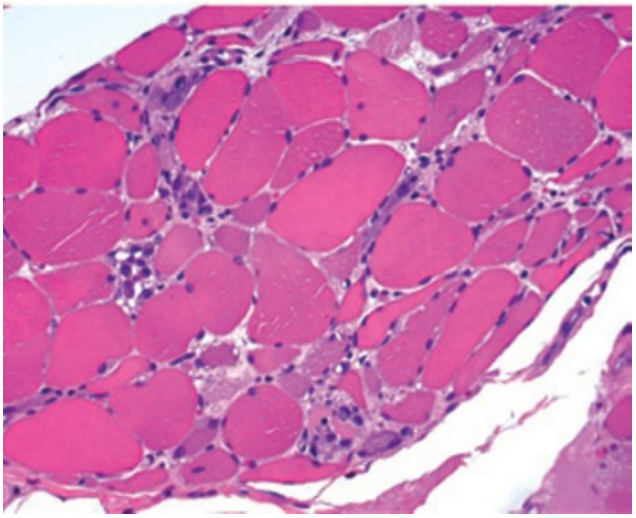

Figure 5 Degenerating, necrotic and regenerating muscle fibers are a characteristic feature of muscle biopsies from patients with immune-mediated necrotic myopathy[41]

PM

In polymyositis, multifocal lymphocytic infiltrates surround and invade healthy muscle fibers. The inflammation is primary, a term used to indicate that lymphocytes (CD8 positive cells) invade histologically healthy muscle fibres expressing MHC class I antigens. This lesion is also referred to as the CD8/MHC-I complex. In chronic stages, connective tissue is increased and may react with alkaline phosphatase. [17]

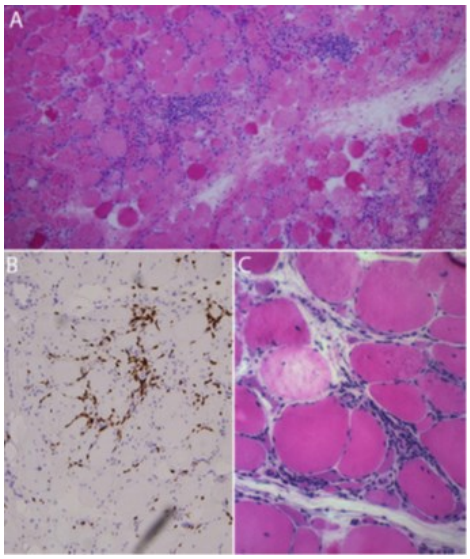

Figure 6 Key myopathologic features of PM. (A) Muscle from a patient with PM, demonstrating fiber size variation with rounded atrophic fibers, increased internal nuclei, widespread fiber necrosis with phagocytosis, basophilic fibers undergoing regeneration, and perivascular and endomysial inflammatory infiltrates with mononuclear cell invasion of non-necrotic myofibers (H&E). (B) Immunohistochemical stain of CD3 identifying T cells invading the endomysium from a patient with PM. (C) Myopathologic features common to both PM and sIBM, including fiber size variability, myofiber necrosis with phagocytosis, regenerating fibers, and perivascular and endomysial inflammatory cell infiltrates (H&E)[54]

SEROLOGY

Autoantibodies specific for IIM (myositis-specific antibodies or MSA) are clinically useful biomarkers to help the diagnosis of these conditions. Autoantibodies that are found in IIM are often classified into myositis-specific autoantibodies (MSA) and myositis-associated autoantibodies (MAA). MSAs are almost exclusively found in IIM, although some antibodies such as anti-aminoacyl transfer RNA (tRNA) synthetases (ARS) are also found in patients classified as idiopathic ILD. Classic MSAs include anti-ARS, anti-MI2, anti-SRP, anti-HMGCOAR, anti-TIF1γ, anti-MDA5, anti-SAE, anti-SUMO.[20]

The majority of IIM patients present with myositis specific antibodies (MSA) or myositis associated antibodies (MAA). Approximately 70% of patients with dermatomyositis have a dermatomyositis specific autoantibody, most of which are associated with a distinct clinical phenotype, making them useful to predict organ

involvement and prognosis. Around 20–30% of patients, classified as seronegative IIM, have no known autoantibodies.[8,12]

Patients with IIM may also have autoantibodies that are present in other autoimmune disorders such as systemic lupus erythematosus (SLE), systemic sclerosis or Sjögren syndrome. These autoantibodies are often named myositis-associated autoantibodies (MAAs) and the most frequent are anti-Ro52, anti-PM-Scl, anti-Ku and anti-U1RNP.[8]

Anti-synthetase antibodies (ARS) are directed against cytoplasmic aminoacyl-tRNA synthetases that catalyze the ATP-dependant reaction of single amino acid attachment to its specific tRNA and ensure proper protein synthesis. Although 20 aminoacyl-tRNA synthetases can be distinguished, antibodies have been detected against eight of them, including anti-Jo-1 (histidyl-tRNA synthetase), anti-PL-7 (threonyl), antiPL-12 (alanyl), anti-EJ (glycyl), anti-OJ (isoleucyl), anti-KS (asparaginyl), anti-Zo (phenylalanyl) and anti-Ha (tyrosyl). ARS can be found in approximately 30% of IIM patients with anti-Jo-1 being the most common type. Anti-Jo-1 specificity is detected in approximately 20-30% myositis patients, while each of the other ARS occurs in not more than 5% of patients. ARS are generally considered to be mutually exclusive, yet cases of ARS co-occurrence have been described. Antibodies against Ro (including Ro52) are considered as the most common type of associated antibodies in ARS-positive patients, occurring in 30-65% of cases. Presence of anti-Ro52 is prevalent in anti-Jo-1 patients and reported to be associated with earlier development of arthritis, mechanic's hands, and dermatomyositis-specific skin findings.[5]

Patients with anti-ARS have a similar clinical condition known as anti-synthetase syndrome characterized by a combination of symptoms such as myositis, arthritis, interstitial lung disease, Raynaud phenomenon, mechanic's hands and fever. Although some of the symptoms are more commonly seen in patients with defined ARS type, patients with ASS develop in general alike clinical presentation. The prevalence of each symptom varies depending on the study.[14]

Several recent studies suggest that antibodies to non-Jo-1 ARS are associated with earlier and more severe interstitial lung disease (ILD) and poor prognosis

compared with anti-Jo1 positive patients. Also, non-Jo1 anti-ARS patients are more likely to have ILD without typical myositis. One recent study reported that 10 % of patients with idiopathic ILD had anti-ARS, more commonly anti-PL12 and anti-KS.[20]

Based on a large meta-analysis that included 27 studies and 3,487 patients, the prevalence of antisynthetase antibodies (ARS-Abs) in patients with dermatomyositis or polymyositis is 20% and 29%, respectively, making them the most common autoantibodies in patients with IIM. [21]

Anti-SRP SRP antigen is a complex of 7SL RNA and several proteins, playing a role in regulating the translocation of proteins across the endoplasmic reticulum. The majority of literature supports that anti-SRP is specific for PM and associated with treatment-resistant severe myopathy, histologically characterized as necrotizing myopathy. [20]

Anti-HMGCR The antigen targeted by this class of autoantibodies is recognized as 3-hydroxy-methil-glutaril-CoA-reductase, a key enzyme in cholesterol biogenesis pathway and pharmacological target of statins. [22] Although anti-HMGCR antibodies were originally identified as statin-related, it is also present in statin-naıve patients especially if younger or from Asian countries. [5]

Anti-SRP and anti-HMGCR are widely reported in literature to be associated with necrotizing immune mediated myopathies (IMNM), with around two thirds of patients with IMNM having autoantibodies recognizing either SRP or HMGCR.[12] Among seropositive cases, Anti-SRP IMNM is associated with more severe muscle weakness, higher risk of cardiac involvement, and poorer treatment response compared to anti-HMGCR IMNM. Of note, seronegative IMNM shows frequent occurrence of associated connective tissue disorders and significantly higher rates of extra-muscular disease activity. Seronegative IMNM is also reported to be associated with increased risk of cancer, while the correlation is not univocal for anti-HMGCR.[5]

Anti-MI2 Mi2 antigen is identified as nucleosome remodeling deacetylase complex (NuRD). The NuRD complex regulates transcription via histone deacetylation and ATP-dependent nucleosome remodeling and the Mi-2 subunit

of the NuRD complex has been shown to act as a DNA-dependent, nucleosome-stimulated ATPase that acts primarily as a transcriptional repressor. Anti Mi-2 antibodies are found in 10-30% of IIM patients, especially in DM. Clinical studies are consistent in showing that anti-Mi-2 is associated with classic features of DM including Gottron's papules, heliotrope rash, shawl sign, and V-sign. The risk to develop clinically significant ILD is low and cancer is uncommon. Although these patients typically have more-severe skin rashes, they also have a good response to steroid therapy and a generally good prognosis.[20,22,23]

MDA5 MDA5 was initially identified in 2002, as a type I IFN inducible gene in human melanoma cells, and the first function described for this protein was to induce the death of cancer cells. Since this first description, MDA5 is now considered as a key sensor of viral infection, mediating the production by the infected cell of type I IFN and the induction of other genes that collectively establish an antiviral host response[24] through the recognition of viral ds-RNA. Most reports of anti-MDA5 positive patients describe a high prevalence of clinically amyopathic dermatomyositis (CADM) and rapidly progressing interstitial lung disease (RPILD) leading to a poor prognosis.[20] The prevalence of antiMDA-5 antibodies ranges from 6.9% to 40.6% in patients with dermatomyositis and is more common in both Asian cohorts and patients with clinically amyopathic dermatomyositis (CADM) compared with those with classic dermatomyositis.[21] The heterogeneity of clinical features and outcomes among patients with anti-MDA5 DM prompted to divide the clinical spectrum of anti-MDA5+ DM into three distinct clinical subgroups. Patients with a clinical picture dominated by RP-ILD, often in association with mechanic's hand, have the worst prognosis. Patients with predominant skin involvement are considered to have intermediate outcomes, while those with predominant arthralgia seem to have the best prognosis.[24]

Anti-TIF1 TIF1α, TIF1β, and TIF1γ belong to the TIF family of transcription cofactors and are part of a tripartite motif superfamily (TRIM24, TRIM28, and TRIM33, respectively), being involved in the regulation of the P53 oncosoppressor gene. Anti-TIF1 autoantibodies can be identified in 22–32% of patients with juvenile DM and in 7–31% of adult patients with DM. Initially anti- TIF1γ/α was reported in 75 % of patients with cancer-associated myositis. The association of anti-TIF1γ/α with

DM, in particular with cancer-associated DM has been confirmed in many reports from several countries. However, it should be noted that association of antiTIF1γ/α with cancer does not seem to apply to children or young adults affected by DM. Conversely Juvenile patients with anti-TIF1 serology appear to have more severe cutaneous involvement. [20,22,23,25]

Anti-NXP2 These antibodies recognize a ~140-kD nuclear protein called nuclear matrix protein 2 (NXP-2; also known as MORC3), which plays an important role in diverse nuclear functions such as RNA metabolism and maintenance of nuclear architecture. In the first study of a cohort of Argentine pediatric myositis patients, anti-NXP-2 antibodies were the most prevalent specificity (25 % of cases), associated with muscle contracture, atrophy, and significant compromise of the functional status.[20] Furthermore both adult and juvenile patients with anti-NXP2 specificity are reported to have higher risk of developing calcinosis [22] and young patients tend to present more severe disease than antiTIF1-g JDM and anti-MDA5 JDM.[5]

Anti-SAE The target antigens of this class of autoantibodies were identified as small ubiquitin-like modifier-activating enzyme A subunit (SAE1) and the SUMO-1 activating enzyme B subunit (SAE2), respectively. These are enzymes involved in the post-translational modification of specific proteins known as sumoylation. Anti-SAE are considered an uncommon MSA, being present only in DM patients, in less than 10% of cases. Among patients with anti-SAE, a high prevalence of cutaneous lesions including heliotrope and Gottron rash were identified. Most patients had systemic features (82 %), and dysphagia was noted in 78%. A majority of them presented with skin disease prior to onset of myositis.[20,26]

MAAs Anti-Pm/Scl and anti-Ku are two MAAs usually found in patients with overlap myositis and concurrent features of systemic sclerosis or systemic lupus erythematosus. Both are associated with characteristic clinical findings frequently seen in patients with ARS antibodies, including arthralgias, fevers, and Raynaud's phenomenon. The rate of ILD in patients with polymyositis-Scl antibodies is reported to range from 38% to 78%; the rate in patients with anti-Ku antibodies may be as high as 27% based on one large meta-analysis. [21] Rigolet et al. reported

steroid resistant ILD in IIM patients with anti-Ku serology, despite a favorable response of myositis to treatment.[26] The anti-SS-A 52-kD (anti-Ro52) IgG antibody is a MAA frequently found in patients with IIM, with an estimated prevalence exceeding 20%, and higher rates in patients demonstrating ILD. Co-occurrence of SS-A 52-kD IgG antibody with ARS and MDA5 is not uncommon and its presence has been linked to more aggressive pulmonary and extrapulmonary disease.[21] Autoantibodies recognising cytosolic 5'-nucleotidase 1A (NT5C1a) are present in 30–60% of patients with sporadic inclusion-body myositis but these types of autoantibodies can also be found in 5–10% of patients with polymyositis, 15–20% of patients with dermatomyositis, 10% of patients with systemic lupus erythematosus and 12% with Sjögren's syndrome. Unlike other types of inflammatory myopathies, sporadic inclusion-body myositis is not associated with any myositis-specific autoantibody. Although not specific to sporadic inclusion-body myositis, anti-NT5C1a autoantibodies have been associated with increased severity and mortality in these patients.[12]

Of the MSAs, anti-TIF1γ, anti-NXP2 and anti-HMGCR autoantibodies have been reported to be associated with an increased risk of cancer in patients with IIM. Anti-TIF1γ autoantibodies stand out, with up to 84% of anti-TIF1-c-positive adult patients having cancer-associated DM .[25]

AETIOLOGY

In comparison with healthy individuals, higher prevalence of autoimmune disease, such as systemic lupus erythematosus, autoimmune thyroid disease or T1DM, have been reported concurrently in patients with IIM, as well as in first-degree relatives of both adult and juvenile patients with IIM. Conversely, a nationwide study in Taiwan that investigated co-aggregation of autoimmune disease in the families of individuals with systemic lupus erythematosus and systemic sclerosis identified a higher relative risk of IIM in these families than in the general population and a national study in China suggested that relatives of patients with systemic sclerosis have an increased risk of IIM and certain other autoimmune

diseases. Such aggregation of autoimmune diseases within families of patients with IIM suggests that shared genetic and/or environmental factors might contribute to disease risk.[27]

Genetic Factors

The major histocompatibility complex (MHC), also known as the human leukocyte antigen (HLA) region, has been shown consistently to be the strongest genetic risk factor for autoimmune disease. In IIM, the strongest association is with the 8.1 ancestral haplotype (8.1 AH), a large common haplotype in Caucasian populations that confers susceptibility to many other autoimmune or immune-mediated diseases. [28]

In a combined IIM analysis including 2,566 patients from 14 countries, the most associated variants were classical HLA alleles, with HLA-DRB1*03:01, part of the 8.1 AH, being the most significant allele. After conditioning on the effects of HLA-DRB1*03:01, a strong association was found with HLA-B*08:01 suggesting that there is an independent effect within this locus.[29]

Associations with the 8.1 ancestral haplotype were found with anti-Jo-1 (HLA-B*08:01 and HLA-DRB1*03:01), anti-PM/Scl (HLA-DQB1*02:01) and anti-cN1A autoantibodies (HLA-DRB1*03:01). Associations independent of this haplotype were found with anti-Mi-2 (HLA-DRB1*07:01) and anti-HMGCR autoantibodies (HLA-DRB1*11). A strong HLA association was observed in patients with anti-TIF1 autoantibodies with the HLA-allele group DQB1*02. The association at the HLA-DQB1 locus differed between adult-onset and juvenile-onset patients. In adult-onset IIM, the strongest HLA association was with HLA-DQB1*02:02. In contrast, in juvenile-onset patients, a strong association with HLA-DQB1*02:01 was observed, but the strongest association was with HLA-DRB1*03:01, which is on the same haplotype as HLA-DQB1*02:01.[30]

Another instance of differing HLA associations between adult and juvenile phenotypes is the case with anti-3-hydroxy-3-methylglutaryl-coA reductase

(HMGCR) autoantibodies, which are associated with HLA-DRB1*11:01 in adults and HLA-DRB1*07:01 in juvenile patients with IIM.[27] Several case-control studies have supported that DRB1*11:01 is an immunogenetic risk factor for anti-HMGCR myopathy. This allele is present in about 70% of people with anti-HMGCR autoantibodies, but only in about 15% of the general population.[12]

Analyses of the relationship between type II HLA alleles and anti-MDA5 DM in Chinese cohorts demonstrated a higher frequency of HLA-DRB1*04:01, *12:02 and *12:01 alleles in Chinese patients with anti-MDA5 DM.[24]

It has been hypothesized that risk within MHC genes can be explained by differences in the structure of the peptide-binding pocket affecting the ability to bind antigenic peptides. Key amino acid positions within HLA genes may be responsible for the risk in these genes. For anti-Jo-1, anti-PM/Scl and anti-cN1A autoantibodies, an arginine at position 74 of HLA-DRB1 conferred the strongest risk. In patients with anti-PM/Scl and anti-cN1A autoantibodies, an arginine conferred all of the risk at this position, whereas in patients with anti-Jo-1 autoantibodies there was also evidence of risk attributable to other amino acids.[28,30]

Other specific amino acid associations in the HLA region, such as position 57 of HLA-DQB1, position 77 of HLA-DRB1*03:01, and positions 26 and 11 of HLA-DRB1*03:01, differentiate dermatomyositis, polymyositis and IBM, respectively, and suggest different predominating patho-physiologies in different clinical subgroups. [27]

Although many of the largest studies in IIM have been conducted in Caucasian populations, other populations can have unique risk HLA haplotypes in IIM. For example, HLA-DRB1*08:03 confers risk of IIM in the Japanese population, while HLA-DQA1*01:04 and HLADRB1*07 alleles are associated with an increased risk of dermatomyositis in the Chinese population. Although these haplotypes differ, they may share common features that confer risk.[28]

In IIM, substantial genetic risk hence resides within the MHC; however, large studies are beginning to reveal associations outside this region that overlap with other seropositive autoimmune diseases suggesting common etiologies and

pathways. In addition, there is evidence of non-HLA associations that differentiate between clinical subgroups of IIM.[28]

Among the several non-HLA loci associated with IIM is PTPN22; this locus was associated with polymyositis, but not with adult or juvenile dermatomyositis, suggesting that different clinical subgroups have different pathophysiology.[27]

Other genes, including STAT4, TRAF6 and UBE2L3 in various subtypes of IIM; PLCL1 in dermatomyositis; and CCR5 in IBM, have been associated with disease, reaching a suggestive level of significance. In IBM, sequencing of candidate genes involved in related neuromuscular or neurodegenerative diseases and whole-exome sequencing of genes encoding proteins overrepresented in the skeletal muscle rimmed vacuoles of patients with IBM identified rare variants in VCP, SQSTM1 and FYCO1, suggesting impaired autophagy as a mechanism of IBM pathogenesis.[27]

A variant in BLK was associated with dermatomyositis and is known to be involved in B cell activation. Other associated genes such as RGS1 and IL18R1 in polymyositis, and GSDMB in dermatomyositis suggest novel mechanisms that may differentiate between these diseases. [28]

Although the role of complement in IIM is not well defined, in dermatomyositis there is evidence of complement-induced vascular damage and muscle fiber ischemia. Many components of the complement system are encoded within the MHC, and notably the 8.1 AH is strongly associated with a genetic deficiency of complement C4 because of the presence of only a single copy of C4B and the absence of C4A. Researchers found that C4A deficiency and HLA-DRB1 03:01 were both risk factors for JDM; however, the strongest effect was noted with their concurrent presence.[28]

One non-HLA locus, an intronic variant of WDFY4, has been recently associated with the anti-MDA5 DM in Japanese patients[24]

Environmental Factors

Exposure to potential inhalatory environmental triggers such as dust, fumes or tobacco smoke has been statistically linked to ASyS, particularly in anti-JO1 positive patients.[14] In a population study of Canadian patients, the likelihood of having a systemic autoimmune disease increased with the amount of fine particulate air pollution. Moreover, an analysis of the geospatial distribution of DM patients in the Philadelphia metropolitan area found that point sources of airborne pollutant matter, such as emissions from industrial and commercial sources, were associated with the presence of clinically amyopathic DM, but not classic DM. Cigarette smoking is associated with varying phenotypic characteristics of DM. Smokers are more likely to have anti-ARS antibodies, and anti-Jo-1 antibody, while they have anti-TIF1γ antibodies less frequently. The association between smoking and anti-jo-1 antibodies appears to be even more significant in the presence of HLA-DRB1*03.[11]

Several reports suggest that herbal supplements and drugs may be linked with DM onset or flare in susceptible patients. Drugs more frequently reported include hydroxyurea, TNF-α inhibitors, interferons and penicillamine even though most evidence is based on case reports.[11]

Toxic myopathy in the setting of HMG-CoA (3- hydroxy-3-methylglutaryl–coenzyme A) reductase inhibitor (statin) use is well-described. [31] Musculoskeletal symptoms such as myalgia and cramps are quite common in patients taking statins, but they are usually mild. By contrast, rhabdomyolysis is a well-known severe adverse event associated with statin use. Fortunately, however, this adverse event occurs rarely, at a rate of around 0.4 per 10,000 patient years. In most cases, statin-associated muscle complaints improve when the treatment is discontinued, and complete recovery can be expected within a few weeks or months after discontinuation of the drug. Nevertheless, over the past decades, numerous case reports have indicated that statins might cause IIM in some patients. The identification of inflammatory cells in muscle biopsies from these patients, and the fact that they only improved on initiation of immunosuppressors supported this hypothesis. A specific antibody was found to be significantly more

represented in IIM patients exposed to statins than their statine-naïve age-matched IIM counterparts. The target antigen was later identified as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR)—the pharmacological target of statins. Statins are known to dramatically upregulate HMGCR protein levels; thus, in some patients, increased HMGCR expression could trigger anti-HMGCR autoimmunity. Moreover, regenerating human muscle fibers also express high levels of HMGCR. This finding suggests that after statin medications are discontinued, high levels of HMGCR expression in regenerating muscle tissue might continue to drive the autoimmune response.[22]

On the basis of case series and animal models, vaccines (especially those containing aluminum hydroxide) have been hypothesized to be triggers of polymyositis and dermatomyositis and focal forms of myositis.[27]

In genetically susceptible individuals, viral, bacterial, and parasitic infections have been suspected to induce autoimmunity or exacerbate existing autoimmune conditions. Epidemiological data shows that subsets of PM and DM patients defined by various myositis-specific antibodies tend to develop disease at specific times of the year. This seasonal clustering of disease onset could be indicative of a common environmental trigger such as infection.[11]

For instance, epidemiological studies on anti-MDA5+ DM highlighted a seasonal distribution of the disease. Classically, anti-MDA5 DM onset follows a seasonal repartition with a peak in late winter and spring and a dip in summer, following respiratory virus infections epidemiology. It is also interesting to note that a geographical distribution of the disease has also been reported in Japan, with an increased prevalence of anti-MDA5 DM in patients living in rural areas as opposed to urban areas.[24] Viral complement-fixation antibodies to Coxsackie B1, B2, and B4 were found significantly more frequently in the sera of JDM patients, who were tested within four months of JDM onset, than in the sera of their matched juvenile rheumatoid arthritis controls and from pediatric controls hospitalized for viral syndromes. A case-control study investigated the presence of EBV, which has been linked to several autoimmune disorders, in IIM patients. It showed higher frequencies of anti-Epstein-Barr nuclear antigen 1 (EBNA1) antibodies at the onset

of DM/PM and the EBV genome was detected at a higher frequency in DM/PM patients than in their matched healthy control counterparts.[11]

Dalakas et Al. reported four cases of IBM in HIV-positive patients and demonstrated that clonally expanded and viral-specific CD8 T cells are recruited within the muscle to surround or invade muscle fibers. The observations suggest that in IBM viruses (HIV may be the first identified example) can trigger a viral-specific inflammatory response that may lead to disease initiation.[32]

A number of specific infectious agents have been implicated in IIM pathogenesis on the basis of reported occurrences of suspected infection-induced disease or biological plausibility from animal models. Examples include hepatitis B virus in polymyositis and dermatomyositis; hepatitis C virus in IBM; retroviruses, particularly HIV and human T-lymphotropic virus-1, in polymyositis, dermatomyositis and IBM; Toxoplasma spp. and Borrelia spp. in polymyositis and dermatomyositis; and influenza, picornavirus and echovirus in polymyositis, dermatomyositis and juvenile dermatomyositis. [27]

Cutaneous features of DM including persistent erythema, increased prevalence in sun-exposed areas, and photoaggravation suggest that UV radiation plays a strong role in disease pathogenesis. The prevalence of DM increases significantly with decreasing geographical latitude from northern Europe to southern Europe, suggesting that UV exposure may be implicated in DM pathogenesis. Okada et al. demonstrated that among 13 geoclimatic variables, studied in a population of 919 consecutive IIM patients from 15 locations, surface ultraviolet radiation intensity was the strongest contributor to DM and was strongly related to the proportion of anti-Mi2 antibodies. Additionally, increases in the anti-TIF1-γ antibody have been reported with decreasing latitudes, further highlighting potential mechanisms explaining the increased prevalence of DM closer to the equator.[11,33]

PHYSIOPATHOGENESIS

DM

Atrophic, degenerating and/or regenerating myofibers identified in perifascicular regions are characteristic features of dermatomyositis. Selective depletion of capillaries in these perifascicular regions has been proposed to result in hypoxia and observed myofiber pathology. The possibility that vasculopathy could contribute to dermatomyositis is supported by research showing that tubulo-reticular inclusions are found within capillary endothelial cells in dermatomyositis. Further evidence indicating that vascular damage may contribute to dermatomyositis pathology comes from a study of dermatomyositis muscle biopsy specimens. This study showed that intermediate-sized blood vessels are unevenly distributed within muscles, and often have pathological features including perivascular inflammation.[22]

The vascular damage, hypnotized by some as the driving force in DM pathology, depends largely on complement. Complement C5b-9 membranolytic attack complex is activated early (before the destruction of muscle fibers is evident) and deposited on the endothelial cells, leading to necrosis, reduction of the density of endomysial capillaries, ischemia, and muscle fiber destruction resembling microinfarcts.[34]

DM etiopathogenesis has not been completely elucidated yet but recent in-vivo and in-vitro transcriptomic studies of blood and target tissues (like skin and muscle) have highlighted the key role of IFNs in inducing and maintaining disease manifestations, with different signatures associated with diverse clinical phenotypic subtypes. Despite the heterogeneity in clinical studies in terms of number of analyzed genes, tissue origin, and IIM subgroup, it has been demonstrated that the IFN-scores consistently discriminated IIM patients from healthy controls, exhibiting some degree of correlation with disease severity. Among all IIM subtypes, growing evidence has shown a role for IFN in sustaining the pathogenesis of several manifestations in DM.[35]

The marked overproduction of type 1 interferon–inducible transcripts and proteins in muscle is unique to dermatomyositis compared with all other muscle diseases studied. Microarray gene expression studies of muscle biopsy specimens measuring about 18,000 transcripts in muscle biopsy samples from patients with a wide range of myopathies showed that only dermatomyositis samples with perifascicular atrophy have marked elevation of type 1 interferon–inducible transcripts.[36]

Two type 1 interferon–inducible proteins are similarly highly specific biomarkers of dermatomyositis muscle. MxA is uniquely (compared with other muscle diseases) abundant in dermatomyositis myofibers with perifascicular atrophy and in dermatomyositis muscle capillaries. ISG15, a ubiquitin-like modifier, is furthermore attached to many other proteins in dermatomyositis muscle. Exposure of human skeletal muscle cell cultures to interferon-α or interferon-β produces a similar picture of ISG15 conjugation present in human dermatomyositis samples.[36] Studies of dermatomyositis skin have also shown overexpression of MxA protein in the epidermis and inflammatory cells. [37]

IFN is not only responsible for muscle damage but also plays a role in its maintenance by affecting myotube regenerative capacity. Accordingly, recently published data support the role of type I IFN overproduction in impairing proliferation of myoblasts in DM patients, thereby leading to defective muscle repair. Moreover, type I IFN was shown to cause mitochondria damage in myotubes by increasing reactive oxygen spices production which is among the non-immunologic mechanisms involved in muscle damage in IIM. [35]

Plasmacytoid dendritic cells (pDCs), infiltrate dermatomyositis muscle and skin. These cells can rapidly produce large amounts of type 1 interferons after stimulation by Toll-like receptor-7 or Toll-like receptor-9 agonists. Through an auto-stimulatory type 1 interferon receptor–mediated mechanism, the accumulation of these cells in tissues exponentially amplifies their efficiency at producing type 1 interferons. Although pDC infiltration in dermatomyositis skin and muscle suggests they are a source of type 1 interferons, this finding does not exclude other potential sources, such as fibroblasts.[36]

Although the effects of these cytokines on other immune cells, which then act as effectors of tissue injury, have been suggested, it is possible that sustained inappropriate intracellular production of type 1 interferon–inducible molecules could be the mechanism giving rise to endothelial injury, myofiber injury, and the vacuolar changes of basal epidermal keratinocytes seen in dermatomyositis.[36]

Some autoantibodies reported in IIM, including those against Mi-2 and Jo-1, have been emphasized as targeting proteins overproduced by regenerating myofibers. Because regeneration of myofibers implies previous injury, such findings suggest that production of these autoantibodies is a mechanistic event downstream of myofiber injury.[36]

The hypothesis of a viral infection as a possible trigger for autoimmunity appears particularly compelling in anti-MDA5 positive cases. The scenario could involve an activation of MDA5 in infected cells, leading to IFN-I production and increased levels of MDA5, followed by an excessive local apoptosis favored by a specific genetic background (intronic variant of WDFY4). The release of the MDA5 antigen into the microenvironment following cell lysis could be the cause of a loss of tolerance towards MDA5, resulting in the production of antiMDA5 antibodies.[24] Anti-MDA5 autoantibodies may participate to formation of immunocomplexes (ICs) and NET release therefore exacerbating the vicious cycle of IFN production via TLR7 sensing and neutrophil activation. Anti-MDA5 ICs could further contribute to a dysregulated IFN secretion being potent and direct IFN-α inducers. The results of recent studies on patients with anti-MDA5+ DM have demonstrated that the levels of IFN signature and of circulating anti-MDA5 autoantibodies correlate with disease severity, with special reference to ILD and cutaneous manifestations. The importance of type-I IFN in anti-MDA5+ DM is ascertained also in ILD pathogenesis, at the extent that some authors proposed the use of serum IFNα as disease biomarker. [35]

Serum IFN-γ appears to be deeply involved in the physiopathology of RP-ILD. A study by Ishikawa et al. reported high serum IFN-γ and the presence of IFN-γ-positive histiocytes in the lung in patients with DM RP-ILD. These results suggest that IFN-γ seems to have a pathological influence in DM RP-ILD by activating

macrophages and accelerating inflammation. Furthermore, serum IFN-γ levels correlated with ground glass opacities, as evaluated by CT-scan. Considered together, the results suggest the relevance of IFN-γ involvement in the pathophysiology of DM, specifically in the formation of pulmonary lesions seen in RP-ILD.[38]

IBM

Inclusion-body myositis is a complex disorder because, in addition to autoimmunity, it features an important degenerative component, highlighted by the presence of congophilic amyloid deposits within some fibers. Similarly to what is seen in Alzheimer's disease, these deposits comprise amyloid precursor protein, amyloid-β42, apolipoprotein E, α-synuclein, presenilin, ubiquitin, and phosphorylated tau, which indicates the presence of protein aggregation. [34]

Despite initial reports of relative absence of lymphocyte infiltration, subsequent studies have demonstrated the presence of B cells, plasma cells and immunoglobulin transcripts in the muscle of patients with IBM, indicating a humoral component in this disorder.[27]

In IBM, CD8+ cytotoxic T cells surround and invade nonnecrotic muscle fibers that aberrantly express MHC class I. MHC class I expression, which is absent from the sarcolemma of normal muscle fibers, is probably induced by cytokines secreted by activated T cells.[34]

Analysis of T-cell receptor molecules expressed by the infiltrating CD8+ T cells reveals clonal expansion of T-cell receptor chains and conserved sequences in the antigen-binding region, which suggests an antigen driven T-cell response. This is further supported by the expression of costimulatory molecules and up-regulation of adhesion molecules, chemokines, and cytokines. The up-regulation and overload of MHC class I may also cause glycoprotein misfolding, which stresses the endoplasmic reticulum of the myofibres.[34]

However, the largest body of evidence for non-immune-mediated mechanisms in IIM is available for IBM. The pathogenesis of IBM includes many pathways involved in protein homeostasis and cell stress mechanisms, and several of these pathways

seem to be linked directly to inflammation. In IBM, intracellular accumulation of abnormal proteins is hypothesized to cause or aggravate cell stress pathways, thus leading to structural damage and weakness. Several lines of evidence have demonstrated malfunction of the autophagic machinery in IBM. Proteomic analysis of vacuolated fibers identified the autophagic adaptor protein FYVE domains and coiled coil domain-containing protein 1 (FYCO1) as a novel component in the rimmed vacuoles. The accumulation of FYCO1 was associated with a missense variant of FYCO1 in 11% of patients with IBM, which supports the hypothesis of impaired autophagic activity in IBM. Collectively, these data indicate that autophagy is a relevant mechanism in IBM pathology. Apart from inflammation and protein accumulation, mitochondrial abnormalities, such as cytochrome c oxidase deficiency in muscle fibers, are hallmarks of IBM. Several mitochondrial defects have been demonstrated in the muscle tissue of patients with IBM. Such mitochondrial changes are associated with oxidative damage, production of inflammatory mediators, and impairment of muscle strength.[27]

In IBM patients, the lack of significant response to conventional immunosuppressant regimens seems to support the importance of the degenerative mechanisms in its physio-pathogenesis. [12]

Conversely, recent findings suggest that the infiltrating T-lymphocytes in IBM present a peculiar phenotype ($CD28^-$, $CD5^-$, $CD16^+$, $CD94^+$, $CD57^+$), similar to that observed in T-LGLL (large granular lymphocyte leukemia), characterized by a poor response to steroids as well. This suggests that immunological features specific to IBM could also explain the less than desirable response to immunosuppressive treatments in these patients. [39]

IMNM

The pathogenesis of IMNM has been progressively clarified over the past years, with convergent data demonstrating that anti-HMGCR and anti-SRP autoantibodies are pathogenic; however, the pathogenesis of seronegative IMNM still remains unclear. [15]

In seropositive patients CPK levels, a biomarker of muscle fiber necrosis, correlates with muscular strength, and the titers of anti-HMGCR and anti-SRP correlate with muscular strength and CPK level. [15,40]

The presence of sarcolemmal complement deposits, as well as signs of classical complement pathway activation, suggest that muscle fibre necrosis is antibody- and complement-dependent, whereas no or few cytotoxic T cells and/or natural killer cells are observed in muscle biopsies from patients with IMNM.[15]

HMGCR levels are up-regulated by statins and during muscle regeneration after injury. This observation suggests that the first instance of muscle damage and regeneration (for example, the myotoxicity of statins) may induce an immune response against muscle antigens in patients with genetic susceptibility. Accordingly, some MHC class II DRB1 alleles confer an immunogenetic predisposition to anti-HMGCR-positive IMNM.[15]

Even after discontinuing statins, the presence of high levels of HMGCR in regenerating muscle fibers may perpetuate the immune response. [41]

One in vitro study demonstrated that both anti-SRP and anti-HMGCR antibodies induce muscle fiber atrophy with an increase in the transcription of genes encoding atrophic factors such as MAFBX and the E3 ubiquitin-protein ligase TRIM63. In addition, co-culture of muscle fibers with anti-SRP or anti-HMGCR autoantibodies was associated with high levels of inflammatory cytokines (such as TNF and IL-6) and reactive oxygen species, showing that, even in the absence of complement, the binding of these autoantibodies to their targets induces cellular stress in myofibres.[15]

The role of IL-6 in inducing MAFbx and Trim63 seems to be determinant. TNF is also a well-known inflammatory cytokine that leads to atrophy via p38 mitogen-activated protein kinase to stimulate MAFbx expression in skeletal muscle. In vitro, muscle cells cultured in the presence of TNF increased cell levels of ceramide, which leads to muscle atrophy, suggesting synergistic effects of these proinflammatory molecules. TNF also induces oxidative stress via an increase of mitochondrial super oxide production, leading to atrophy.[42]

Immunization of mice with recombinant SRP54 or HMGCR also decreases muscle strength, confirming that breakage of tolerance to each of the two IMNM target antigens provokes acquired muscle disease. The observation of complement deposition at the surface of myofibers in biopsies from patients with IMNM, prompted an evaluation of the role of complement in mice. IgG from patients with seropositive IMNM was markedly less pathogenic in complement-deficient mice than in wild-type mice. In line with this observation, as mice have lower complement levels than humans, and human IgG activates human complement more efficiently than mouse complement, supplementing recipient mice with fresh human complement augmented the in vivo pathogenicity of IgG from patients with seropositive IMNM. Together, these data underscore the key role of the complement cascade in seropositive IMNM.[15]

ASyS

ASyS seems to be initiated in the respiratory system. As a consequence of tissue aggression by different environmental factors and infectious agents, cellular stress occurs. This leads to cell death with microparticle release and danger signal pathways activation. As a result, innate immune cells, like NK and neutrophils, are activated. Their activation could lead to further tissue damage, increasing release of particles (proteins, enzymes, cytokines and nucleic acids), which have the capacity of damaging the tissues, potentially causing the exponential release of antigens. ARSs themselves display chemo-attractant properties. Sera from anti-Jo-1 positive patients induce the expression of intracellular adhesion molecule 1 in human endothelial cells, which allows immune cells to reach target tissues. After cleavage by proteases, ARS fragments can act as interleukin-8 like cytokine, bind CXC chemokine receptor or mimic endothelial-monocyte activating polypeptide. In this scenario, inflammation-induced proteolytic enzymes, such as Granzyme-B, could split ARS into immunogenic peptides. Once the immunogenic peptide is produced, dendritic cells might be attracted within the lungs, where they further mature and activate the antigen presentation process. Successive events include

antigen presentation, efficiently favored by the HLA-B*08.01 genetic background, CD8 T-cell priming and CD4 helper T-cell–B-cell crosstalk. These phenomena finally lead to autoantibody production. The disease propagation is linked to the circulation of these specific cells, autoantibodies, and production of proinflammatory cytokines. Although not proven yet, it is speculated that disease propagation could follow a second independent hit to target tissues, such as mechanical injury affecting the muscles, vascular disorders (potentially mediated by ARS themselves) or infectious diseases, which could amplify ARS and MHC-I expression in muscle, thus allowing local access to immune effectors. [43]

ASyS inflammatory milieu in muscle biopsy is outlined by an upregulation of type II IFN inducible genes, responsible for the increased perifascicular expression of human leukocyte antigen (HLA)-DR molecules that represent a distinctive finding in ASyS biopsies. A differentiating element from DM is also the absence of sarcoplasmic MxA, that further confirms the marginal role of type I IFN in this subgroup. RNA-sequencing analysis in a cohort of anti-Jo1+ patients with ILD, compared to healthy controls and subjects with idiopathic pulmonary fibrosis (IPF), showed higher levels of IFNγ inducible chemokines (CXCL9 and CXCL10) responsible for the recruitment of activated T cells and attesting the relevance of IFNγ in ILD pathogenesis in this disease subset.[35]

CAM

It has been demonstrated that some tumors (breast, lung adenocarcinoma, and hepatocellular carcinoma), but not the corresponding normal tissues, express high levels of myositis autoantigens. Notably, the expression of these autoantigens by tumor cells as well as by regenerating myoblasts, indicate a possible antigenic similarity between the two cell populations. Therefore, it is possible that an immune response initially directed against cancer cells cross-reacts with regenerating muscle cells, subsequently triggering the development of the disease in genetically predisposed individuals. It has also been demonstrated that cells at early stages of neoplastic transformation and below the limits of clinical detection, can provide co-stimulatory danger signals that promote the activation of innate immune cells.[44]

A possible pathogenetic hypothesis may be that factors of tumor origin targeting the skeletal muscle induce muscle damage and regeneration, which results in an initial subclinical myopathy. This hypothesis is supported by a report providing histochemical evidence for subclinical myopathy in clinically healthy muscle tissue of patients with colorectal cancer. In the particular subset of individuals genetically predisposed to autoimmunity, regenerating myofibers overexpressing myositis-specific autoantigens, may induce an autoimmune response which is then perpetuated by the deposition of immune complexes within the affected skeletal muscle, further progressing towards a clinically relevant cancer associated autoimmune myositis.[44,45]

TIF1γ has been found to act as both a tumor suppressor and promoter in different cancers. Through its involvement in the TGF-b pathway, TIF1γ can hinder tumorigenesis mainly by inhibition of TGF-b-induced epithelial-to-mesenchymal transition via mono-ubiquitination of SMAD4.[25]

In anti- TIF1γ positive, cancer-associated DM, cancer is hypothesized to be the trigger for DM, with TIF1γ possibly functioning as a tumor autoantigen. Indeed, some studies have identified genetic mutations in TIF1γ genes in CAM patients, confirming this hypothesis. Alterations of TIF1γ may lead to neoantigen formation, activating the adaptive and innate anti-tumor immune response. This anti-tumor response could lead to eradication of the tumor or elimination of only the clones expressing this antigen. This could explain the occurrence of TIF1γ autoantibodies in adult patients with DM without cancer. In the latter case, tumor cells lacking the altered TIF1γ could be selected by a T cell-dependent immunoediting process. The immune response to altered TIF1γ is thought to cross-react with native TIF1γ. A large TIF1γ antigen supply in the muscle and skin could explain why the anti-tumor response leads to the clinical manifestation of DM.[25]

Non-immune mechanisms

In recent years, the hypothesis that non-immune mechanisms could participate in muscle damage and dysfunction in autoimmune myositis has been investigated. This hypothesis is supported by some disease features, above all the observation

that the extension of muscle inflammation widely varies from patient to patient and does not correlate with the severity of muscle damage and of clinical manifestations. [44]

In general, these mechanisms can fuel inflammation via a positive feedback loop, affect muscle contraction and cause muscle weakness, imbalance muscular protein homeostasis and lead to atrophy and irreversible structural damage of muscle fibers. [27]

The endoplasmic reticulum (ER) stress response pathway is one of the best known non-immune-mediated mechanisms contributing to muscle fiber damage and weakness in IIM.[46] When stressing stimuli alter the complex homeostasis of ER, causing the accumulation of unfolded/misfolded proteins in ER, an ER stress response is triggered. [44] ER stress mechanisms include the unfolded protein response (UPR) and the ER overload response (EOR). The unfolded protein response is characterized by upregulation of several factors, with the collective function of reducing the protein overload and subsequent accumulation of unfolded proteins in the ER. The ER overload response, modulates inflammation by upregulating NF-κB signalling. Both ER stress pathways are activated in the muscle in all forms of IIM, including IBM.[27] UPR and EOR are elicited by the MHC-I overexpression observed in many IIM patients. The NF-κB pathway activated in EOR leads to the transcription of numerous target genes, including proinflammatory cytokines, adhesion molecules, and also MHC class I antigens, which in turn feeds the ER stress response in a self-sustaining loop mechanism.[44] When ER stress is further prolonged, apoptotic pathways are triggered by CHOP, a proapoptotic transcription factor, and the activation of caspase 12, an ER-resident caspase.[46]

Activation of ER stress pathways can alter cellular respiration and induce mitochondrial dysfunction leading to an overproduction of reactive oxygen species (ROS). In a model of mouse with chronic inflammation, muscular atrophy was shown to be related to production of ROS, mitochondrial damage, and caspase overexpression, which were downregulated following supplementation

with red grape polyphenols, thereby suggesting a role for oxidative stress in IIM progression.[46]

Denervation can occur as consequence of inflammation as TNF-a and IL-6 are negative regulators of neurotrophic factors. In addition, an important trigger for destabilization of the neuro-muscular junction is the constitutive expression of MHC-I, raising the possibility that the increased MHC-I expression drives aberrant synapse elimination in mature muscle fibers, thereby affecting muscle trophism and function.[46]

Muscle tissues of IIM patients also show fewer capillaries per area than healthy individuals, and it has been postulated that lower oxygen levels lead to muscle weakness. Gene profiling of muscle biopsies showed a simultaneous co-expression of promoters (HIF-1a, CD146, fibronectin) and inhibitors of angiogenesis (CXCL10, TGFß, angiopoietin 2), denoting an active capillary remodeling in patients with IIM. Transcription factor HIF-1a is a direct sensor of hypoxia, and its proteins have been shown to accumulate in IIM patients, being not detected in healthy controls. A recent study suggests that hypoxia triggers the production of interferon-I in vitro. RIG-I is an IFN-inducible gene overexpressed in perifascicular areas of muscle fibers in dermatomyositis. Hypoxia induces an increase in expression of RIG-I in myotubes through HIF-1a stabilization, and it has been seen that RIG-I overexpression triggers IRF3 phosphorylation leading to activation of IFN.[46]

CLINICAL MANIFESTATIONS

Skeletal muscle involvement

The most common clinical feature associated with IIM is symmetrical proximal muscle weakness that progresses over a period of weeks to months. Patients may complain difficulty rising from chairs, climbing stairs, or washing their hair. In severe cases of autoimmune myopathy, diaphragmatic weakness may also occur and require mechanical ventilation. Although autoimmune myopathies are most

frequently characterized as ‘painless weakness’, some patients do have considerable myalgia.[22]

Tasks requiring distal muscles, such as buttoning or holding objects, are affected early in inclusion-body myositis but only in advanced cases of polymyositis, dermatomyositis, and necrotizing autoimmune myositis. The ocular muscles are spared in all subtypes, but facial muscles are commonly affected in inclusion-body myositis. In all disease subtypes, neck-extensor and pharyngeal muscles can be involved, which results in difficulty holding up the head (head drop) or in dysphagia. Muscle atrophy is detected early in inclusion-body myositis, with selective atrophy of the quadriceps and forearm muscles, but it develops in all subtypes if the weakness is severe and chronic. [34]

Compared to other IIM subtypes, IBM features several specific traits. IBM disease is slowly progressive, with weakness occurring over the course of years. In other inflammatory myopathies, progressive weakness can occur over weeks or months. Many patients with IBM have an asymmetric pattern of muscle weakness while symmetric weakness is the rule in patients with other inflammatory myopathies. Patients with sporadic inclusion-body myositis usually have prominent knee extensor weakness along with distal weakness; arm abductors and hip flexors can also be affected but are frequently stronger than more distal muscles. [12]

Patients with IMNM have proximal muscle weakness that predominantly involves the lower limbs. Frequently, patients with anti-SRP-positive IMNM have concomitant dysphagia (30–70%) and dysphagia was also observed in up to a quarter of patients with anti-HMGCR-positive IMNM. Patients with anti-SRP-positive IMNM appeared to suffer from more severe muscle weakness than those with anti-HMGCR-positive IMNM and to more frequently experience muscle atrophy.[15]

Extra-muscular manifestations

Skin

A violaceous periorbital, often oedematous, rash known as a heliotrope rash and/or an erythematous rash over the extensor surfaces of the metacarpophalangeal, proximal interphalangeal and distal interphalangeal joints referred to as Gottron papules are both highly specific features of dermatomyositis. Indeed, patients with these features who lack muscle involvement are referred to as having 'amyopathic' dermatomyositis. Although less specific, patients with dermatomyositis might also present with skin atrophy, dyspigmentation, and telangiectasias on the upper back (the 'shawl sign') or the upper chest (the 'V-sign'). Changes in the nail beds, including periungual telangiectasias and cuticular hypertrophy, might be seen in patients with dermatomyositis. Of note, dermatomyositis rashes can be exacerbated by exposure to ultraviolet light. Finally, patients with dermatomyositis may develop painful subcutaneous calcifications, especially in juvenile cases.[12,22]

Anti-MDA5 DM is associated with a more specific cutaneous phenotype, that includes palmar papules and skin ulcerations with punched out borders. Unlike Gottron's papules, palmar papules are often located on the palmar surface or lateral sides of the fingers, especially over metacarpophalangeal and interphalangeal joints. Many of these lesions have a central ivory coloration, and they are frequently painful. Palmar papules can be associated with hyperkeratosis, and complicated by ulcerations. Other less specific mucocutaneous lesions have been described, including oral ulcers, panniculitis, alopecia, and flagellate erythema.[21,24]

Patients with dermatomyositis who have autoantibodies recognizing small ubiquitin-like modifier activating enzyme or melanoma differentiation-associated gene 5 tend to have more substantial skin than muscle involvement. In fact, most patients with anti-MDA5 autoantibodies are hypomyopathic or amyopathic.[12]

Mechanic's hands (MH), defined as fissured, scaly, non-itching hyperkeratotosis, located at the palmar and lateral sides of the hands and fingers, are one of the hallmarks of ASyS. However, distinguishing those lesions from other cutaneous

conditions may be challenging. Depending on the study, prevalence ranges from 19% to 56.5% of patients with anti-ARS. International literature reports more MH prevalence in patients with anti-JO1 and anti-PL12 antibodies compared to other anti-ARS specificities.[14]

Raynaud's Phenomenon (RP) was reported to be more frequent in patients with polyarthritis in the course of ASyS as compared to patients with myositis and/or ILD. The subgroup with anti-PL-12 antibodies appears to be most frequently affected by RP.[14]

Abnormalities in nailfold capillaroscopy in the form of giant capillaries, avascular areas, microhemorrhages or ramifications are reported, especially in ASyS patients. A scleroderma-resembling pattern has been described in over 35% of cases, predominantly those with longer disease duration or anti-Jo-1 antibodies, yet it did not correlate with the presence of RP.[14]

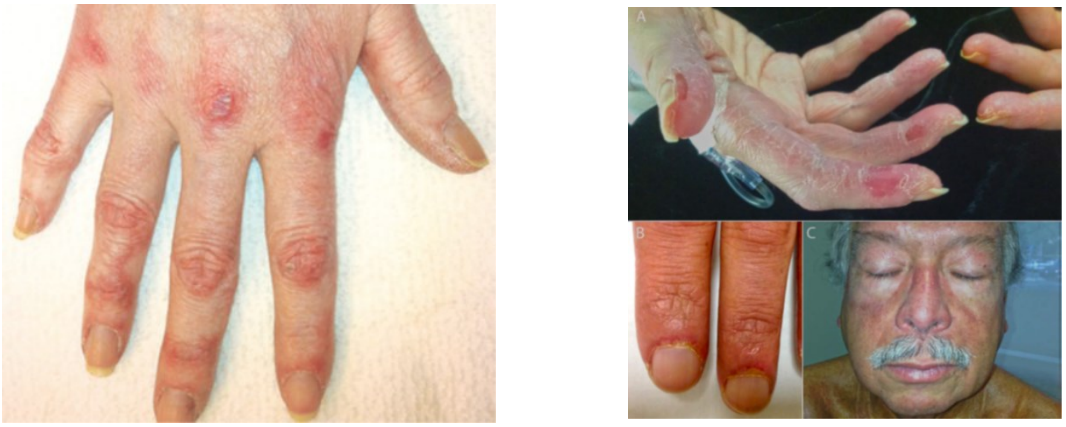

Figure 7 Gottron's papules; mechanic's hands, nailfold abnormalities, heliotrope rash are common cutaneous manifestations in IIM patients.

Respiratory system

Interstitial lung disease (ILD) is a common complication of IIM, with a reported prevalence reaching 42.6%, and even higher in the presence of certain antibodies. Although it can occur at any point in the course of disease, ILD frequently precedes manifestations of myositis, with a presentation that ranges from subclinical to fulminant. Although many patients respond favorably to immunosuppressive

therapy, the risk of relapse and long-term progression remains significant, occurring in up to one-third of all patients. Moreover, ILD is the primary cause of death and hospitalization in patients with inflammatory myositis.[21] The mortality rate of patients who develop RP-ILD is reported to be approximately 50%, with most deaths occurring during the very early stages of the illness.[24] ILD related to IIM is not easily distinguished from other forms of interstitial pneumonia based on imaging and histopathology.[26]

The presentation of ILD in patients with IIM can range from asymptomatic, incidentally discovered lung abnormalities to rapidly progressive respiratory failure mimicking ARDS. In cases of the latter, patients often demonstrate a progressive cough or dyspnea that is often initially misdiagnosed. By the time they seek treatment, they frequently show a significant oxygen requirement necessitating hospital admission. Dyspnea in patients with severe myositis occasionally may also be caused by respiratory muscle weakness, and interpretation of spirometric values must be taken in the context of the muscle disease of such patients. [26]

By far, the MSAs with the greatest pulmonary implications include the anti-synthetase antibodies and those directed against MDA-5. The prevalence of a parenchymal lung abnormality can range from 56% to 100%, depending on the specific autoantibody in question and the method for determining the presence of lung disease. Furthermore, evidence suggested that the frequency of each lung disease pattern may be influenced by the underlying serologic profile.[21]

In patients with anti-PL-7 and anti-PL-12, ILD is usually more severe with lower FVC and DLCO and more prominent fibrosis as compared to anti-Jo-1-positive patients. Black race was found to be an independent risk factor of severe ILD during the course of ASyS. Ventilatory impairment due to skeletal muscle weakness is more frequently observed in anti-Jo-1 positive patients. It is noteworthy that ILD can occur in patients with no signs of myopathy, which contributes to delayed diagnosis. Cases of acute respiratory distress syndrome are not infrequent, even as an initial presentation of ASyS. Acute onset of ILD was observed in as many as

74.1% of patients with anti-EJ. Rapid progression of ILD was statistically more frequent in the anti-PL-7-positive group.[14]

A radiographic pattern of UIP in ASyS is associated with increased risk of disease progression compared with other imaging patterns. UIP pattern on CT imaging appears to be more common with anti-PL-7, which may contribute to the increase in reported disease severity.[26]

ILD is also a distinct feature of antiMDA-5 related DM. The frequency of ILD with anti-MDA-5 antibodies varies but seems to be higher in Asian populations.[26] These patients frequently develop a rapidly progressive and sometimes lethal form of interstitial lung disease. [12]

Interstitial lung disease is frequently associated also with anti-SRP seropositivity, and is present in 23–38% of patients on systematic CT. However, its presence has fewer clinical implications, as most of these patients with ILD do not complain of dyspnea, and pulmonary functional tests remain normal.[15] In several cohorts NSIP appears to be the most reported radiographic pattern.[47]

Reports of ILD frequency with anti-SAE antibodies have varied largely in the literature; select reviews have suggested little or no association, whereas individual case series, largely in Asian populations, have reported ILD in up to two thirds of cases. When detected, the severity of ILD is reportedly minor, with patients often being asymptomatic. Organizing pneumonia seems to be a common radiographic pattern on CT imaging. [47]

Radiologic Findings of the parenchymal abnormalities in patients with IIM tend to have predominantly subpleural and basilar distribution, although patchy or diffuse disease can be seen.[21] Nonspecific interstitial pneumonia is the most common pathologic pattern in patients with myositis-associated ILD. Characteristic CT imaging features of NSIP include ground-glass opacity, reticulation, and traction bronchiectasis, most often involving the lower lung zones.[26]

While patients presenting with acute ILD tend to have ground glass opacities and consolidations, patients presenting with a more chronic course of ILD have more

evidence of reticulation and honeycombing. Interestingly, ground glass and reticular opacities can be associated with fatal cases of ILD.[48]

By contrast, consolidation was recognized as the principal pattern more frequently in non-fatal ILD. According to the results of a joining cluster analysis, DM-ILD HRCT pattern can be categorized into 3 groups. Of these, the group with dominant GGA and/or reticular opacity without honeycombing, subpleural bands, and traction bronchiectasis was closely related to fatal ILD. In this series, most consolidant-dominant patients presented an acutely progressive disease that however responded to immunosuppressive therapy. In this study, acute respiratory symptoms were more frequent in fatal ILD than in the other groups. Based this HRCT classification, patients with a consolidant-dominant pattern had the lowest CK level, suggesting that low CK level could be a hallmark of acutely progressive ILD responsive to treatment.[49]

Patients with ILD are at additional risk for developing pulmonary hypertension. This can in turn substantially increase morbidity and overall mortality.[50]

Spontaneous pneumomediastinum is a rare complication of IIM, associated with significant morbidity and mortality. One review reported that one-quarter of patients died in the 1st month, with a cumulative survival rate of only 64% at 1 year and 55% at 2 years. Although pneumomediastinum in patients with PM has been reported, the vast majority of cases have occurred in patients with DM, especially MDA5 related. The pathogenesis of pneumomediastinum in patients with myositis is poorly understood, though it is speculated to result from the rupture of subpleural blebs that occur with ILD, leading to dissection of air around perivascular sheaths and into the mediastinum.[26,48]

Hearth

Heart involvement comprises myocarditis, inflammatory infiltration in the cardiac conduction system and replacement fibrosis. Only ~10% of patients with IIM have clinically overt cardiac disease; subclinical involvement is more frequent (up to 75% of patients). The initial description that cardiac involvement was more common in patients positive for anti-SRP auto-antibodies is now controversial and

those with overlap myositis and systemic sclerosis are considered more likely to be affected.[8]

Other extra-muscular features

Arthritis and arthralgia are common signs in ASyS with the prevalence varying significantly from 20 to 88% in different cohorts of patients. Joint inflammation in the course of ASyS usually manifests as symmetrical polyarthritis affecting predominantly hand or wrist joints. Larger joints, such as the knees, elbows, shoulders, ankles or hips are less frequently involved. In a minority of patients, oligoarticular or asymmetrical arthritis is observed. Synovitis, periarticular calcifications, or isolated arthralgia are also reported. Arthritis appears to occur more frequently in anti-Jo-1 patients as compared to those with other ARS subtypes, often being the initial symptom. The course of arthritis may differ depending on the time of appearance. Researchers suggest that if arthritis is an initial manifestation, it is more likely to develop as rheumatoid arthritis-resembling type with symmetrical and polyarticular involvement. Prevalence of anti-CCP antibodies in ASyS patients with arthritis ranges from 4.96% to 13.5%, while RF might be found in up to 31.5% of patients. Patients with anti-ARS and anti-CCP antibodies are at risk of developing severe arthritis as compared to anti-CCP-negative group. [14]

During the last decade many studies reported an increased prevalence of arthritis (42-82%) and arthralgia also in patients with anti-MDA5+ DM. The arthritis described in the patients closely resembles that found in rheumatoid arthritis, potentially leading to misdiagnosis in the absence of other DM symptoms. Articular involvement is usually associated with morning stiffness and is typically symmetric, affecting small joints of the hands and wrists or the ankles.[24]

Fever is another typical feature of Asys, with a prevalence between 25.5% and 60.9%, with reports describing it more commonly in the anti-PL-12- positive subset.[14]

cancer

Cancer is a major cause of mortality in idiopathic inflammatory myopathies. Prevalence of cancer in patients with IIM varies between 6.7 and 32% and is mainly documented in patients with DM and to a lesser extent in immune-mediated necrotizing myopathy. This association is considered not to be fortuitous when it occurs within 3 years, before or after, the diagnosis of idiopathic inflammatory myopathies. [25,51]

Given the strong association between malignancy and some IIM subsets, especially in patients older than 50 years, a rigorous screening workup and surveillance in the first 3 years from disease onset are warranted. [25,34]

The MSA most specifically linked to cancer is anti-TIF1γ. As previously noted, anti-TIF1γ autoantibodies are rather frequent in juvenile DM, but the association with cancer is restricted to adults with DM, suggesting a different pathophysiology. Using anti-TIF1γ to predict cancer-associated DM is therefore only useful in adults, especially over 39 years of age. Of the other MSAs anti-NXP2 is clearly associated with malignancy as well. Clinical risk factors for cancer-associated DM in general include older age, male sex, dysphagia and cutaneous necrosis.[25]

An increased risk of malignancy is also described in IMNM, particularly in seronegative and anti-HMGCR positive patients. In these groups, malignancy occurred mainly after the age of 50, and mostly within 3 years of the diagnosis of IMNM. [51]

The most common cancers linked to IIM are ovarian cancer, breast cancer, colon cancer, melanoma, nasopharyngeal cancer (in Asians), and non-Hodgkin's lymphoma, while some authors have also reported associations with lung and bladder cancer.[34,45]

DIAGNOSIS

The diagnosis of IIM is based on clinical symptoms such as subacute development of symmetrical muscle weakness and muscle fatigue, most prominent in proximal muscles, supported by laboratory analysis of muscle damage biomarkers, serologic profile, imaging techniques, muscle biopsy and electromyography.[3]

Laboratory investigations

Elevated serum levels of muscle enzymes such as creatine phospho-kinase (CPK), aldolase, lactic acid dehydrogenase, aspartate transaminase and/or alanine transaminase are present in at least 90% of patients with autoimmune myopathy. Although elevated levels of creatine phospho-kinase are believed to be the most sensitive and specific marker of muscle damage, patients with autoimmune myopathies can present with elevated serum aldolase levels without an accompanying increase in serum creatine kinase levels.[22]

Autoantibody testing has become an important tool for diagnosis of IIM and also to identify subgroups of IIM with different clinical phenotypes and prognosis. Assessment should include myositis-specific autoantibodies (MSA) and myositis-associated antibodies (MAA) such as anti-Ro52, anti-Ro60, anti-La, anti-U1RNP, PM-Scl and anti-Ku. They can be present in other systemic autoimmune diseases, like systemic lupus erythematosus, Sjögren´s syndrome or systemic sclerosis; Although not specific for myositis, their presence may be helpful to distinguish an inflammatory myopathy from a non-auto-immune myopathy and provide prognostic information. Anti Ro52/SSA-52 abs are an example: when coexpressed with anti-MDA5 aAbs seem to imply more prevalent RP-ILD and less favourable outcomes. [3,24]

In some cases, monitoring serologic titer of MSAs could provide useful prognostic insights. For instance in anti-MDA5 DM, aAbs titers appear to be significantly lower in surviving patients compared to deceased ones. Anti-MDA5 titers also correlate with the severity of the disease, and more specifically with the severity of ILD and

cutaneous symptoms. Furthermore, the value of the anti-MDA5 Abs could also be useful for the evaluation of the response to treatment. In a Japanese cohort, patients with anti-MDA5 Abs levels greater than 500 units/mL (positivity threshold at 8 units/mL) were resistant to treatment by glucocorticoids/cyclophosphamide or intravenous immunoglobulins. Inversely, patients with anti-MDA5 Abs levels lower than 500 units/mL had less severe lung lesions and cutaneous symptoms improved after treatment. [24] Notably, in anti-MDA5 DM, serum ferritin and CPK levels have also been deemed useful biomarkers to predict outcomes, with patients having increased ferritin and lower CK levels often featuring a worse prognosis. [26]

Imaging

T2-weighted magnetic resonance imaging (MRI) with fat suppression or short tau inversion recovery sequences (STIR) are the most sensitive and specific routine methods in IIM imaging.[52] On short tau inversion recovery imaging, increased signal intensity within muscle tissue is consistent with the presence of muscle necrosis, degeneration, and/or inflammation. As a result, this finding has been incorporated into contemporary diagnostic criteria for autoimmune myopathies. MRI can also identify when chronic muscle damage has resulted in fatty replacement of skeletal muscle; this finding, if extensive, might predict poor immunosuppressive therapy response. Since autoimmune myopathies can result in 'patchy' muscle involvement, some authors have suggested that MRI-guided muscle biopsies might improve diagnostic accuracy of pathology evaluation.[22] STIR MRI can also be used to examine the cutaneous, subcutaneous and fascial manifestations of juvenile dermatomyositis. Up to 85% of juvenile dermatomyositis patients have subcutaneous edema. Furthermore, MRI changes often precede the development of clinical apparent calcinosis.[52]

Ultrasound may be a cost-effective alternative to MRI, with contrast-enhanced ultrasound also permitting the assessment of muscle vascularization. PET sensitively detects increased muscle metabolism and simultaneously screens for underlying malignancies.[52]

Pathology evaluation

Muscle biopsy with histopathological evaluation of frozen muscle tissue by an experienced muscle pathologist is the most definitive and most invasive step to make the correct diagnosis. It often is the only way to distinguish between different subtypes of IIM. It is also often crucial to rule out muscular dystrophy, other forms of hereditary disease or other myopathies. To achieve this, the selection of a representative muscle for the biopsy is crucial: usually, this muscle should demonstrate moderate paresis and MRI can help to locate sites of inflammation or end-stage changes of tissue alteration. However, since the distribution of inflammatory infiltrates is often patchy, a normal muscle biopsy does not necessarily exclude IIM.[3,9]

Electromyography

Electromyography (EMG) is another tool to detect myopathies and some changes may also distinguish between necrosis and denervation, such as the size, shape, and recruitment pattern of the motor units' potential, although there are no specific EMG findings for myositis. [3] Needle EMG of affected muscles usually displays a myopathic pattern, with features of 'irritable myopathy', such as spontaneous activity (fibrillation potentials and positive sharp waves) and/or complex repetitive discharges. A retrospective Dutch study with 98 patients with myositis revealed that none of the patients with the diagnoses DM, PM or IBM had a normal needle EMG. [9,22] Presence of spontaneous activity can also help to distinguish active disease from steroid-induced myopathy.[17]

Further investigations

Once a diagnosis of IIM has been made, further investigations are recommended to clarify extra-muscular organ involvement as it may deeply affect management and prognosis. Particularly the lungs and the gastro-intestinal tract are frequently involved. Cardiologic involvement may also occur, and ECG and ECHO-cardiogram are recommended to screen for subclinical heart muscle disorders [3]. Cardiac MRI is a sensitive technique that can detect inflammation in the myocardium or irreparable changes very early, even when no symptoms are present. Serum cardiac troponin I is a sensitive and specific measure of cardiac involvement,

whereas troponin T levels may be elevated also owing to inflammation in non-cardiac striated muscles.[8]

Adult patients with myositis, and especially those with dermatomyositis have an increased risk of having an associated malignant disease. Therefore, it is a generally recommended to screen for malignancies in patients with dermatomyositis, particularly if the patients have anti-TIF-1γ, anti-NXP2 or anti-HMGCR antibodies, are older and/or if they have a poor response to conventional immunosuppressive treatment.[3]

Given the high prevalence of ILD in patients with IIM, it is warranted conducting full pulmonary function tests (PFTs) when IIM is diagnosed and annually, even in the absence of overt respiratory symptoms. High-resolution CT may be performed, particularly in patients with anti-synthetase autoantibodies and anti-MDA5 CADM. PFTs are known to have a suboptimal sensitivity for detecting the presence of ILD in patients with an underlying connective tissue disease, and any significant change in respiratory symptoms or appreciable decline in FVC or diffusing capacity of the lungs for carbon monoxide (DLCO) should prompt the acquisition of chest CT scan imaging. [21]

Dyspnea in patients with severe myositis may occasionally be caused by respiratory muscle weakness, and interpretation of spirometric values must consider the context of the muscle involvement. On occasion, MIPs or MEPs may provide insight into underlying diaphragm weakness. Pulmonary hypertension is a recognized complication in ASyS patients and is associated with increased mortality. Echocardiography should be considered as a screening tool in the following situations: a change in respiratory symptoms not readily explained by progressive interstitial changes on CT scan imaging, dyspnea out of proportion to the degree of ILD, pulmonary artery enlargement on CT scan imaging, a decline in DLCO not proportional to the decline in FVC, an initial DLCO of > 40% predicted, or any physical examination findings suggestive of pulmonary hypertension (eg, peripheral edema, jugular venous distension, or loud second heart sound). [21]

While dysphagia is reported to be highly frequent in IBM, patients with other IIMs also often display signs of swallowing difficulties. A high incidence of self-reported

dysphagia in patients with myositis is found, suggesting that every examination should include questions regarding this topic. When dysphagia is reported, additional diagnostics may be conducted, including videofluoroscopy or flexible endoscopic evaluation of swallowing.[9]

CLINIMETRY

To adequately evaluate patients, assess their response to therapy and track long-term outcomes it is essential to assess disease activity and disease damage. These are two of the core set measures identified by the International Myositis Assessment and Clinical Studies Group (IMACS) and the Paediatric Rheumatology International Trials Organisation (PRINTO) in the effort of standardizing well validated measures for the evaluation of IIM patients. Disease activity refers the type, extent and severity of reversible pathological manifestations while disease damage is related to persistent changes from previously active disease, like scarring, atrophy and fibrosis.

In clinical practice, one the most fundamental tools for disease activity assessment is the manual muscle test (MMT-8), validated for dermatomyositis, polymyositis and juvenile dermatomyositis. It manually quantifies, on a scale of 1 to 10, isometric contraction of 8 standardized muscle groups, including neck flexors, deltoids, biceps, wrist extensors, glutes (medius and maximus separately), quadriceps, dorsal ankle flexors. The score of each district is then compounded to a total, with a maximum of 150/150, allowing to gauge overall improvement of progression. This technique is practical and time efficient, and it demonstrated adequate sensitivity and reliability if performed by a trained practitioner. [53]

The skin is a prominent and responsive feature of dermatomyositis, and thus, the ability to measure disease activity and damage in the skin is important for clinical use and investigative studies. The Cutaneous Dermatomyositis Disease Area and Severity Index (CDASI), a one-page instrument that measures key features of disease activity (erythema, scale, erosion and/or ulcerations and nailfold telangiectasias) and damage (poikiloderma, calcinosis and scarring), has excellent

reliability, as well as responsiveness. A second modified version of CDASI, has also been validated, showing similar reliability to the initial version.[2]

Magnetic Resonance imaging (MRI), even though a standardized evaluation protocol hasn't yet been validated, is a useful modality to assess disease damage. It's ability to differentiate between acute and chronic muscle damage pathology makes it particularly useful in clinical practice, allowing to clarify whether a patient is flaring, requiring therapeutic adjustment, or the inflammation has resolved and the weakness is a result of purely atrophy and scarring. [53]

PROGNOSIS

Early case series on IIM patients' survival, before the adoption of glucocorticoids, showed poor prognosis with a rate of 5 years survival < 50%.[54] In more recent years, although survival has drastically improved, it is reported that only 20% to 40% of treated patients will achieve remission, whereas 60% to 80% will experience a polycyclic or chronic, continuous course of the disease. IIMs further continue to have a great impact on life quality in medium- and long-term follow-up, as up to 80% of treated patients develop disability (using Health Assessment Questionnaire scores).[55] IBM patients, due to the specific features of the disease, appear to be particularly prone to developing significant disability with a time from disease onset until the first use of a wheelchair ranging from 14 to 16 years. After a median time of 24 years, all IBM patients are completely wheelchair dependent.[9]

More recent cumulative mortality estimates in IIM patients show large variations; 10-year survival rates vary between 20% and 90%, depending on study design and patient selection. It has been suggested that the overall mortality in IIM is highest during the first year after diagnosis. The main causes of death are lung disease, malignancies and cardiovascular disease.[8]

Most frequently reported causes of death are known to differ among disease subsets. For instance in IMNM, cancer is more common in seronegative and anti-HMGCR+ patients, while cardiopathy is more frequent in anti-SRP+ patients; [15]

cancer is more common in DM than PM, while in ASyS a fatal outcome, mostly related to ILD, is more frequent in anti-pl-7 and anti-pl-12 than in anti-jo-1 positive patients, even though the latter experience complete remission less often. [14]

Recognized predictive factors for poor prognosis in IIM include older age; subset of IIM and specific MSA type; male sex; non-Caucasian ethnicity, long duration of symptoms; skin ulcers; respiratory involvement; cardiac involvement; cancer; dysphagia.[55]

TREATMENT

Treatment of IIM patients is largely empiric [56] and often based on personal experience [57], with evidence for specific approaches being mostly relatively weak [8]. Particular subtypes of IIM vary in their responsiveness to treatment [58] and a major challenge in the clinical management of these conditions is predicting which medications are going to be effective for a specific patient. [8]

In establishing a treatment plan, several factors should be considered. Immunosuppressive treatment should be initiated as soon as possible as any delay can contribute to worse outcomes. Early detection and management of any extra-muscular involvement is crucial, as conditions like malignancy, ILD or myocarditis can significantly affect survival. Long-term side effects of medication should also be considered, especially in relation to the attributes, comorbidities and needs of specific patients. [57]

The current standard of care is initiating with high-dose **corticosteroids**, starting with prednisone at 1 mg/ kg/day with eventual taper to a minimal dose anywhere from 4 weeks to several months after initiation. Patients with severe disease, such as ILD, dysphagia, or profound weakness, are typically started on 1 g/day intravenous methylprednisolone for 3-5 days before switching to 1 mg/kg/day of oral prednisone for several months. Many patients feel immediate improvements after starting corticosteroids, but strength increases over 2-3 months. In the case

of “steroid-responsive” patients, the goal is to reduce the dose to the smallest, most effective amount. [57]

After initial stabilization, which may take 4–12 weeks, the steroid dose can be tapered every 1 or 2 weeks by 10 mg until 20 mg/day is reached. Subsequently, the taper is slowed to a reduction by 5 mg until 10 mg daily and to 2.5 mg thereafter. The rate of dosage reduction is dependent upon the patient’s response. If the condition worsens in the process, the taper should be stopped, or the dosage increased. [59]

Therapeutic response evaluation should be guided by improvements in strength, which are generally matched by reduction of CPK levels. It’s important to note that reported subjective improvement or a drop in CPK alone, without increased strength should not be regarded as objective proxies of adequate response to the treatment regimen. [60]

If the patient relapses while tapering steroid treatment or doesn’t improve in 3-6 months, a second line immunosuppressive agent should be added. These agents may also be started immediately in patients with rapidly progressive disease, respiratory muscle failure or dysphagia, and in patients with extra-muscular involvement such as ILD [57], who require intensive combination immunosuppressive treatment as early as possible. [61] They should also be considered as steroid sparing agents if, upon steroid tapering, a patient shows dependance on daily doses of steroids >15mg. [57]

The choice of second line immunosuppressant medication depends widely on comorbidity and experience of the physician. [62]

Methotrexate (MTX) inhibits folic acid and purine metabolism and adenosine signaling. Several retrospective series have found response rates ranging from 71% to 82% in PM and DM. [63] It is generally well tolerated, and its beneficial effects can be seen in 2–3 months. [57] MTX **i**s administered in a weekly single dose of 5–20 mg, followed by a high dose of folic acid on the subsequent day, to reduce toxicity and side effects. Common side effects include increase of the liver enzymes. [59]

Azathioprine is a purine analogue that can block T-cell and B-cell proliferation. It's demonstrated to allow reduction in glucocorticoid dose over a several years period with better functional outcomes and has also shown efficacy for the treatment of myositis-associated ILD [63] as an adjunctive therapy, particularly in patients with corticosteroid-resistant disease. [50] Furthermore it's also considered relatively safe during pregnancy. [1] Treatment with azathioprine is started with a daily dosage of 50 mg over 1 week. The dosage is increased weekly and monitored by the number of lymphocytes, which should be 600–1000/µl. A major potential side effect is bone marrow suppression, especially in patients with thiopurine methyltransferase deficiency. Thus, the activity of this enzyme should be measured before initiation of the treatment. A further frequent side effect is an increase of liver enzymes. Thus, frequent controls of blood count and liver enzymes are essential during treatment. [59]

Mycophenolate mofetil is a prodrug that inhibits T- and B-cell proliferation by inactivation of inosine monophosphate dehydrogenase, an important enzyme in purine synthesis. [63] The use of mycophenolate in IIM has been increasing over the years with more retrospective and prospective studies supporting its efficacy in this disease as well as in associated conditions such as ILD and refractory DM rashes. It is typically used as a second-line agent except in patients with moderate to severe myositis associated with ILD, in whom it can be the first-line agent. [1] The therapy is started with a dosage of 500 mg twice daily and can be increased to 2 g or even 3 g per day. Side effects are less frequent compared to MTX or azathioprine and include kidney and liver toxicity, nausea, diarrhea/colitis, hypertension, hyperglycemia and cytopenias. [59]

Other substances, such as cyclosporin or cyclophosphamide, are used less often, due partly to a higher risk of side effects. However, these drugs can be useful for escalation therapy in individual patients, particularly when all other treatments have failed.[59]

The **calcineurin inhibitors** tacrolimus and cyclosporine exert their effects via inhibition of T cells. Tacrolimus has both greater potency and an improved safety profile when compared to cyclosporine, so may be the preferred calcineurin

inhibitor in anti-synthetase syndrome-ILD. [50] Both are used as second-line agents and their toxic effects require an aggressive monitoring plan, including monitoring blood levels periodically. [1] Tacrolimus is initiated at 1mg twice daily and increased by 1–2 mg weekly until blood levels reach 5–8 ng/mL. Final doses are typically 1 mg to 5 mg, twice daily. Providers should closely follow blood pressure, renal function, electrolytes, lipids, and complete blood count. Adverse events in patients treated with tacrolimus include renal dysfunction, hyperkalemia, hyperglycemia, hypertension, tremor, and headache. If used as add-on therapy in addition to another immunosuppressive agent, it is advocated that tacrolimus should be the first agent to stop after prednisone once a patient has entered a clinical remission, given the risk of renal dysfunction with the long-term use.[50]

The use of **cyclophosphamide** is limited to severe refractory muscle weakness, rapidly progressive ILD or systemic vasculitis associated with PM or DM. [1] Concerns over the cytotoxic effects of cyclophosphamide often limit its administration to severe and refractory anti-synthetase syndrome-ILD. The use of cyclophosphamide has been linked to malignancies, sterility, hemorrhagic cystitis, congestive heart failure, cytopenias, and opportunistic infections. Cyclophosphamide should be prescribed only by providers familiar with its adverse effects and need for frequent monitoring. Patients should have complete blood counts, renal function, and urinalysis prior to every administration of cyclophosphamide (usually every 1– 2 weeks).[50]

Intra-venous Immunoglobulins (IVIG) can be used as an alternative when the side effects from immunosuppressants outweigh their clinical benefit. They can also be used as an add-on treatment during a relapse or when immunosuppressants are not sufficiently effective. In addition, IVIG is a treatment option when immunosuppression is not wanted, e.g. in child-bearing women or adolescent patients.[59] IVIG may also be used as a short-term measure to rapidly decrease the dose of steroids, if excessive steroid-related side effects are occurring. [57] Their efficacy has been proven in a randomized, double-blind study. [62] Several mechanisms have been proposed to explain the effects of IVIG in immune mediated disease, through their interaction with innate and adaptive immune system.[64] The exact mechanism of action of IVIG is unknown and they are

considered immunomodulatory rather than immunosuppressive. [63] Interruption of complement activation that contributes to muscle inflammation and damage has been specifically postulated as one possible mode of action.[65] Additionally, they're hypothesized to also increase the suppressive function of Treg cells on self-reactive T cells.[64]

Several open-label studies have reported safety and efficacy of **Rituximab** in patients with severe and refractory IIM. A large, randomized, double-blind, placebo-controlled clinical trial (Rituximab in Myositis (RIM) Trial) in DM, PM and juvenile DM failed to meet its primary or secondary endpoints. However, 83% of patients who were refractory to multiple immunosuppressive agents showed clinical improvement in muscle disease as well as glucocorticoid reduction within 1 year. Furthermore, rituximab was considered relatively safe and well tolerated in this patient population. The presence of anti-ARS antibodies, anti-Mi2 auto-antibodies, juvenile DM and low disease damage at trial entry were strong predictors of a beneficial response to rituximab. [1] Post hoc analyses of the RIM trial demonstrated that the presence of a myositis autoantibody was most strongly associated with improvement. Interestingly, when skin manifestations of DM were specifically analyzed post-hoc in the RIM trial, rituximab also significantly reduced the frequency of skin rashes. [63] Rituximab is also increasingly being used in myositis-associated ILD, especially in ASyS, with positive outcomes in retrospective and prospective studies [1] even though most of the limited literature on the use of rituximab for myositis-associated ILD is in combination with other immunosuppressant agents for refractory cases. [21] Rituximab targets CD20 positive cells, effectively depleting B lymphocyte population over the course of several weeks. [63] However the exact mechanism of action of Rituximab in IIM patients remains elusive, as the sustained production of MSA and MAA by long lived plasma cells (CD20 negative) is unaffected by treatment, despite evident clinical benefits. It is likely that other B cell-related mechanims, like disturbance in antigen presentation and B-T cell costimulatory interaction, and non-B cell-related mechanisms might be involved. Indeed, CD20 expression has also been described in T and NK cells, which can then be affected by Rituximab, as demonstrated in

rheumatoid arthritis patients. [66] Common side effects of rituximab include headache, nausea and vomiting, diarrhea and infection. Other serious adverse events include cytopenias and serious infusion reactions. Providers should ensure that patients do not have viral hepatitis before choosing rituximab and should monitor complete blood counts before every rituximab infusion. [50]

Considering the importance of IFN signaling pathways in DM, **inhibitors of Janus Kinases** (JAK) appear to be a promising treatment. [24] Janus kinases are cytoplasmic protein tyrosine kinases, critical for type I interferon signal transduction. [63] JAK Inhibitors, differing in their selectivity for specific JAK isoforms, are a class of small, orally administered molecules capable of interfering with this pathway. Several studies reported the efficacy of tofacitinib, an oral JAK 1/3 inhibitor, in relapsing or refractory patients, [24] with efficacy in the management of DM skin and muscle involvement. [63] Moreover, numerous studies have reported on the beneficial effects of Tofacitinib in the management of anti-MDA5+ DM related ILD. Particularly, anti-MDA5+ patients with early stage ILD were treated with combined steroids and Tofacitinib in an open label trial, showing higher 6-month survival rates compared to historical controls treated conventionally. [67] Tofacitinib seems well tolerated in most patients, but one study reported high rates of cytomegalovirus reactivation, varicella-zoster virus reactivation and bacterial respiratory infections.[24] The principal role in ILD patients however seems to be played by the JAK2/STAT3 axis. STAT 3 is overexpressed in lung macrophages, endothelial cells, myofibroblasts and neutrophils in idiopathic pulmonary fibrosis (IPF) and systemic sclerosis patients, allowing the deposition of extracellular matrix leading to fibrosis. Conversely, Transforming growth factor β (TGFβ) is recognized by JAK2-coupled receptors that are highly expressed in fibroblasts, hyperplastic alveolar epithelial type II cells, and in the small pulmonary arteries of IPF patients. For these reasons Baricitinib, selectively acting on JAK2, appears particularly promising in the treatment of severe forms of IIM RP-ILD. This is further supported by the positive experience gathered treating SARS-CoV-2 related severe interstitial pneumonia, which seems to share strikingly similar molecular features with IIM related RP-ILD. [67] Tofacitinib and Baricitinib also seem

to be effective in calcinosis, as described in numerous real-life experiences.[67] Based on this evidence, several trials are assessing the role of JAKi in IIM and numerous case reports/series have already attested their successful use in clinical practice. [35]

Calcinosis is one of the most severe and challenging presentation of DM. Control of active disease with traditional immunosuppressive agents is necessary but is often not sufficient for the treatment of calcinosis. Bisphosphonates, diltiazem, rituximab, IVIG and sodium thiosulfate have been tried with some success in patients with calcinosis.[1]

Skin symptoms in DM sometimes do not respond adequately to systemic immunotherapy, even once myositis symptoms subside. Due to increased sensitivity to UV rays, adequate sun protection is essential. Topical glucocorticoids or calcineurin inhibitors are effective. If topical therapy and immunosuppressive therapy for myositis prove inadequate in treating skin lesions, oral treatment with antimalarial drugs is indicated (e.g. hydroxychloroquine < 6.5 mg/kg BW/d). Its efficacy can be increased (if necessary) by combining hydroxychloroquine with mepacrine.[62]

Unlike other forms of IIM, IBM is typically refractory to immunotherapy. [1] Prednisone, the standard therapy of the other subtypes of myositis, is usually not effective in IBM; however, individual patients may experience at least a temporary improvement.[59] IVIG might slow disease progression, but its long-term effectiveness remains unclear. Exercise is currently the only treatment which has consistently shown some degree of benefit in IBM, although the optimal type of exercise program is yet to be determined.[1]

Pilot studies of arimoclomol, which co-induces the heat shock response by prolonging the activation of heat shock factor 1 and may promote normalization of protein handling within muscle, and rapamycin (sirolimus), which inhibits protein kinase that regulates several intracellular processes, including survival, protein synthesis and autophagy, have shown encouraging results but these have not been confirmed. Unfortunately, a phase II/III clinical trial of arimoclomol in

IBM failed to meet its primary endpoint; however, a phase III clinical trial of rapamycin is ongoing.[8]

Emerging data support the safety and effectiveness of exercise in adults with IIM.[1] Thus, regular physiotherapy is regarded as an essential part of the treatment of these conditions.[9] Exercise has been shown to be safe and to improve aerobic capacity and muscle strength in IIM patients. In a randomized, controlled trial of aerobic exercise in DM and PM, patients' isometric peak force, exercise tolerance, and anaerobic threshold intensity improved, whereas muscle enzymes remained unchanged. In severe cases of IIM, passive range-of-motion exercises may be prescribed until strength and CK start to improve, at which point a strengthening program can be started.[57] The efficacy of physical exercise is also supported by investigations on gene expression and proteomics of muscle samples of IIM patients, reporting beneficial effects on protein synthesis, mitochondrial activity, and anti-inflammatory response. [68]

EXTRACELLULAR VESCICLES

DEFINITION

Extracellular vesicles (EVs) is the generic term used for particles naturally released from the cells that are limited by a lipid bilayer and cannot replicate, i.e. do not contain a functional nucleus. [69] EVs are released by almost all cell types, are present in virtually every body fluid[70] and their secretion mechanisms appear to be highly conserved throughout evolution.[71] The content, or cargo, of EVs consists of lipids, nucleic acids, and proteins.[72]

EVs secretion was initially proposed as a mechanism through which cells eliminate unnecessary proteins.[73] However, it has since been understood that EVs are involved in a wide range of intercellular communication processes and the ability of these EVs to influence the recipient cell has been well demonstrated.[72]

According to the biogenesis and dimensions, EVs are traditionally classified into:

- exosomes (30–150 nm)
- microparticles (MPs; 100–1000 nm)
- apoptotic bodies (1000–5000 nm)[70]

MPs, exosomes, and apoptotic bodies are characterized by peculiar protein profiles due to their different biogenesis. Exosomes tend to be enriched in glycoproteins compared to the secreting cells, however, MPs are thought to contain proteins with higher levels of post-translational modifications, such as glycosylation and phosphorylation, compared to exosomes, which is potentially useful to distinguish the nanoparticles based on their content rather than size. However, substantial overlap of protein profiles is often observed.[72]

Exosomes form by inward budding of the limiting membrane of early endosomes, which mature into multivesicular bodies (MVBs). Endosomes are are involved in protein sorting, recycling, storage, transport, and release. Exosomal formation and MVB transportation are regulated by endosomal sorting complex required for

transport (ESCRT) complexes and their accessory proteins (Alix, TSG101, HSC70, and HSP90β). However exosome biogenesis can also occur throught ESCRT-independent mechanism. In such cases, exosome release is thought to depend on sphingomyelinase enzyme instead of ESCRT. The transmembrane proteins belonging to tetraspanins family (CD63, CD9, CD81) are constitutive proteins on the surface of exosomes and are often enriched in EVs compared to the lysate cell. However, these proteins have since been identified in MPs and apoptotic bodies. [72]

Microparticles, also called microvescicles (MVs), are EVs that form by direct outward budding of the cell's plasma membrane. The route of MVs formation is not yet well understood, however, it is thought to require cytoskeleton components, such as actin and microtubules, along with molecular motors (kinesins and myosins), and fusion machinery (SNAREs and tethering factors). MVs contain mainly cytosolic and plasma membrane associated proteins, especially proteins known to cluster at the plasma membrane surface, including tetraspanins. It has been reported that such proteins can have 100-fold higher concentration in MVs compared to the cell lysate. Other proteins commonly identified in MVs include cytoskeletal proteins, heat shock proteins, integrins, and proteins containing post translational modifications, such as glycosylation and phosphorylation. Interestingly, the glycan binding proteins on the surface of MVs are considered a potential key factor in understanding how MVs are targeted to recipient cell and interact with other cells.[72]

Apoptotic bodies are released by dying cells into the extracellular space. Unlike exosomes and MVs, apoptotic bodies contain intact organelles, chromatin, and small amounts of glycosylated proteins. Indeed, the proteomic profiles of apoptotic bodies and cell lysate are quite similar, whereas there are stark differences in the proteomic profiles between exosomes and cell lysate.[72]

The Minimal Information for Studies of Extracellular Vesicles (MISEV) 2018 guidelines encourages the use of the generic term "extracellular vesicles" in the place of the terms exosomes and microvesicles, which are historically burdened

by both contradictory definitions and inaccurate expectations of unique biogenesis. The characterization of EVs should refer to:

- physical characteristics, such as size ("small EVs" (sEVs) and "medium/large EVs" (m/lEVs) or density (low, middle, high,) with each range defined;
- biochemical composition (CD63+/CD81+- EVs, Annexin A5-stained EVs, etc.);
- descriptions of conditions or cell of origin (podocyte EVs, hypoxic EVs, large oncosomes, apoptotic bodies).[69,74]

BIOGENESIS

Exosomes Endocytic vesicles arise in lipid raft domains of the plasma membrane through endocytosis, leading to the intracellular formation of early endosomes. The intraluminal vesicles (ILVs) formed by inward budding of the early endosomal membrane sequester proteins, lipids, and cytosol that are specifically sorted. [71] The early endosomes become late endosomes and ILV accumulate in their lumen during this process, transforming late endosomes in multivesicular bodies (MVBs). [75] Subsequently, MVBs fuse with either lysosomes in which the ILVs are degraded, or with the plasma membrane, which results in the release of their internal vesicles, i.e., exosomes, into the extracellular space and the incorporation of the peripheral MVB membrane into the plasma membrane.[75]

The exact mechanisms of sorting ILVs to secretion or degradation are still unclear. However, it has been demonstrated that the fate of a particular MVB might also depend on the level of cholesterol in its membrane. Specifically, a cholesterol rich vesicle was secreted while a morphologically identical vesicle that lacked cholesterol was sent to the lysosome for degradation.[72]

The best-described mechanism of exosome biogenesis is the ESCRT-dependent manner. ESCRT complex is composed of approximately thirty proteins that assemble into four complexes (ESCRT-0, -I, -II and -III) with associated proteins (VPS4, VTA1, ALIX). The ESCRT-0 complex recognizes and sequesters ubiquitinated transmembrane proteins in the endosomal membrane, whereas the ESCRT-I and -II complexes appear to be responsible for membrane deformation into buds with sorted cargo, and ESCRT-III components subsequently drive vesicles scission. Finally, the dissociation and recycling of the ESCRT machinery require interaction with the AAA-ATPase VPS4. The mechanisms of inclusion of soluble cytosolic proteins into ILVs are still not very well understood, but a role for HSC70 has been proposed. The chaperone binds to soluble cytosolic proteins containing a KFERQ sequence and to PS on the MVB outer membrane and thus enters ILVs in a TSG101- and VPS4- dependent manner. However, some evidence demonstrates that MVBs and ILVs can form in absence of ESCRT function.[71]

This suggests that there might be different machineries for exosomes biogenesis that lead to different types of exosomes. At the current state of the art, it still cannot be excluded that depending on the cell type and/or the conditions (mature/immature, stimulated/ unstimulated, etc.) different machineries may be required. [76]

Other mechanisms have been proposed to contribute to exosome biogenesis; for instance, membrane macrodomains rich in ceramide can induce a curvature of the endosomal membrane and facilitate its budding. Another contributor in exosome biogenesis are tetraspanins enriched plasmatic membrane domains, specialized in compartmentalization of receptors and protein signals.[77]

Once formed, MVBs must fuse with the plasma membrane to release their content and secrete exosomes. [76] Members of the Rab family play well-established roles in transferring vesicles between intracellular compartments and in MVB trafficking to the plasma membrane for exosome release through interactions with the cytoskeleton.[73] A recent study showed that the actin cytoskeletal regulatory protein cortactin also plays an important role in regulating exosome secretion. It was found that cortactin, Rab27a, and coronin 1b coordinate to control the

stability of cortical actin docking sites in MVBs, thus contributing to exosome secretion.[75] Similarly, the microtubule network is likely required for the transport of MVBs to the plasma membrane.[73]

The final step for exosome secretion to the extracellular compartment requires the fusion of the MVB to the PM. This process is mediated by the complexing of SNARE proteins to SNAPs between the two membranes allowing them to merge, releasing the exosomal content of the MVB.[71,76]

Microvescicles The exact mechanisms of MV biogenesis are still unclear. The specific steps and components involved might be cell-type or function specific and hence various distinct mechanisms have been reported in the literature. [78]

MVs are formed through outward budding of the PM. The formation of ectosomes at the PM primarily involves membrane constituents and their rearrangement, the cytoskeleton, and recruited proteins involved in membrane abscission. [78]

A rise in intracellular calcium induces the activation of the protease calpain, which cleaves cytoskeletal proteins and thus remodels the cytoskeleton. [71] The interaction between cytoskeletal proteins and the PM is then gradually lost. In this way, an initial delamination of the PM from the cortical cytoskeleton occurs. [78] Concomitantly, the lipid translocase floppases, enzymes that are involved in the exchange of lipids between the inner and outer leaflet of the membrane bilayer, are activated to induce changes within the bilayer favoring budding and membrane abscission. In particular, externalization of the phospholipid phosphatidylserine (PS) occurs. The extrafacial enrichment of PS drives PM curvature induction and thus vesicle formation. [78]

Proteins that promote cytoskeleton contraction have been implicated to aid in vesicle budding and abscission. In particular, the GTP-binding protein ADP-ribosylation factor 6 (ARF6) initiates a signaling cascade by activating phospholipase D (PLD). Hydrolysis of phosphatidylcholine (PC) by ARF-activated PLD produces membrane-bound phosphatidic acid (PA), which in turn recruits extracellular-signal-regulated kinase (ERK) and molecules that affect vesicle curvature. ERK then phosphorylates myosin light-chain kinase (MLCK), which in turn phosphorylates the myosin light chain and leads to actomyosin contraction

and subsequent pinching off of the ectosome in a process orchestrated by the small GTPase RhoA.[73,78]

Of interest, the ESCRT machinery have important functions in PM-associated processes. In fact, recent research has shown that ESCRT components may play a key role in the biogenesis of MVs. Studies have demonstrated that budding at the PM is driven by the interaction of the TSG101 subunit of ESCRT-I with the tetrapeptide PSAP motif of arrestin domain-containing protein 1 (ARRDC1). Finally, ESCRT-III and Vps4 ATPase are recruited and assembled to allow pinching-off of the ectosome and recycling of the vesicle-forming machinery. [78]

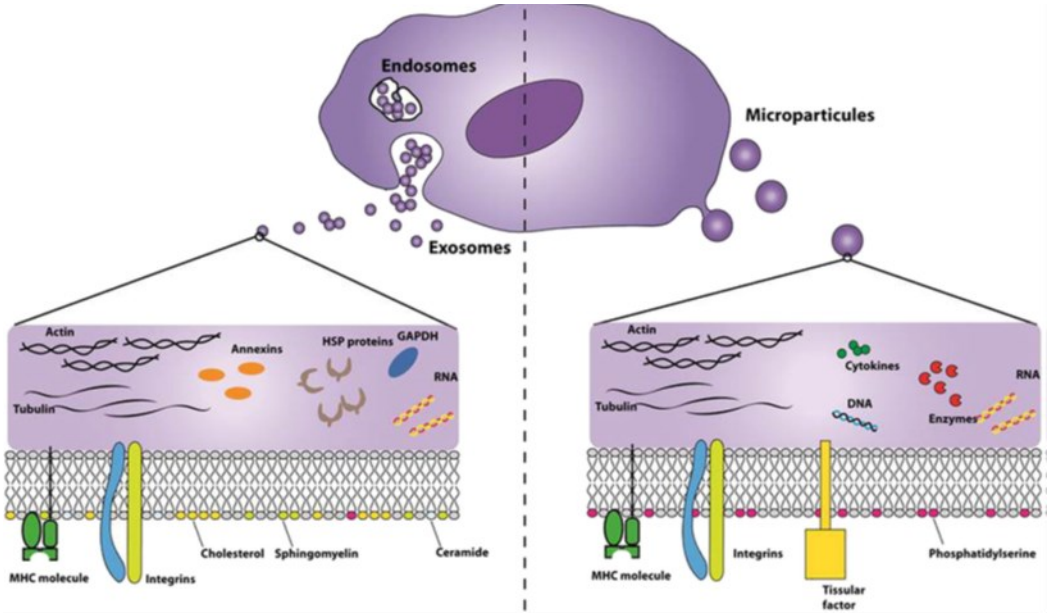

Figure 8 Mechanism of biogenesis of extracellular vesicles (exosomes and microvesicles or microparticles) from donor cell.[142]

EVs UP-TAKE AND BIOLOGICAL FUNCTIONS

Once secreted, EVs can bind to neighbouring cells or the extracellular matrix, or passively traffic through body fluids[79] to reach recipient cells. Delivering their content, EVs can elicit functional responses and promote phenotypic changes affecting the physiological status of cells in different ways. [80]

EVs released into the circulation appear to have a half-life of a couple of minutes to a few hours, depending at least partially to their cellular origin. [81]

Signals are transferred from EVs to recipient cells by mainly three methods: receptor-ligand interactions, direct membrane fusion, and endocytosis/ phagocytosis.[75]

Specific EV proteins such as MHC I and II, transferrin receptors and tetraspanins are active in the downstream signaling pathways of target cells by triggering, for example, integrins and calcium signaling, mitogen-activated protein kinase (MAPK) activation or natural killer group 2D (NKG2D) signalling.[82]

Target cell specificity is likely to be determined by specific interactions between proteins enriched at the surface of extracellular vesicles and receptors at the plasma membrane of the recipient cell.[80]

The presence of specific adhesion molecules involved in exosome binding to recipient cells, such as integrins and intercellular adhesion molecule 1 (ICAM1), depends on the lineage and the activation stage of parent cells.[79]

Many proteins that facilitate EVs uptake have been found, such as the tetraspanin membrane proteins CD9 and CD81 and heparan sulfate proteoglycans (HSPGs).[75]

Proteomic profiling of EVs identified different types of glycan-binding proteins or glycosylation patterns, which appear to determine the target cells or influence interactions with them. For example, B-cell-derived EVs contain α-2,3-linked sialic acid, which can be captured by macrophages through sialoadhesin (CD169). [83] Similarly, CD62 on the surface of activated platelets derived EVs allows them to bind to target cells via its classical P-selectin glycoprotein ligand-1 (PSGL-1) ligand. [82]

The specific composition of EVs will also influence their fate. The presence of amyloid precursor protein on one exosome subtype from neuroblastoma cells specifically targets them to neurons, in contrast to a CD63-enriched exosome subtype that binds both neurons and glial cells.[80]

Once they have bound to recipient cells, EVs may remain at the plasma membrane or may be internalized by clathrin-mediated or clathrin-independent endocytosis, such as macropinocytosis and phagocytosis, as well as through endocytosis via caveolae and lipid rafts.[80]

Cargo delivered by EVS can also activate various responses and processes in the recipient cell after internalization. For example, in dendritic cells, protein cargoes of exosomes can be processed in the endocytic compartment similarly to antigens and then used in antigen presentation. EVs could also fuse directly with the plasma membrane or endocytic membrane of recipient cells. Such processes are required to release intraluminal content in the cytoplasm of recipient cells, a key step to support the release of micro-RNA and messenger-RNA from EVs into recipient cells to regulate gene expression. Direct fusion of extracellular vesicles with the membrane of recipient cells also enables the exchange of transmembrane proteins and lipids.[80]

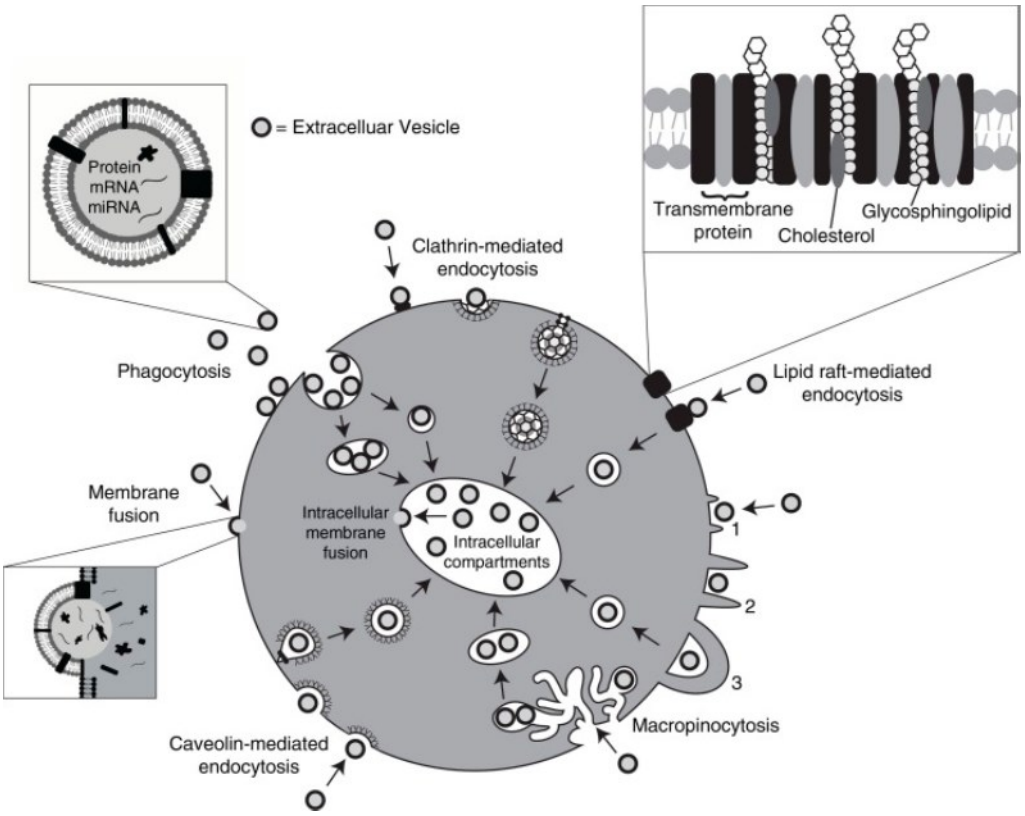

Figure 9. Mechanism of EVs-uptake into receptor cell.[84]

EVs CARGO

Proteins

Initial proteomic studies showed that EVs contain a specific subset of cellular proteins, some of which depend on the cell type that secretes them, whereas others are found in most EVs regardless of cell type. These observations highlight the biogenesis of nanoparticles and show that they represent a specific subcellular compartment and not a random array of cell fragments.[71]

Extracellular vesicles can transfer functional receptors to target cells, for example CCR5, through which the target-cell phenotype can be modified or intracellular signaling pathways can be influenced. [81,83]

Moreover, EVs can transport cytokines, chemokines, and growth factors to neighboring or distant cells, resulting in modulation of the target cell, as well as functional channels, such as aquaporin 2. [81]

EVs can also act through their enzymatic content. For example, in RA, cartilage erosion is related in part to the secretion inside EVs by synovial fibroblasts, of metalloproteinases and glycosidase enzymes degrading the cartilage matrix. [85]

Upon uptake, EVs are frequently targeted to lysosomal degradation and this pathway would provide a relevant source of metabolites to the recipient cells.[80]

EVs antigens (Ag) have also been proved to activate the complement cascade. For instance, in synovial fluid of RA patients, significantly increased levels of leukocyte-derived MVs were detected with complement components bound on their surface, potentially contributing to inflammation and fibrin deposition. However, exosomes derived from APCs also appear capable of escaping from complement-mediated lysis, through the expression of the GPI-anchored complement regulators CD55 and CD59.[83]

EVs represent a source of Ag for antigen presenting cells (APCs) and they participate in Ag presentation to T lymphocytes. The presence of biologically active molecules, such as TNF, within the EVs might also affect the immune response, and recently, an increased number of TNF family proteins has been identified on EVs derived from dendritic cells.[86]

As an example, tumor-derived EVs containing native tumor antigens can be efficiently taken up by DCs for antigen processing and cross-presentation to tumor-specific CTL.[79]

Furthermore, EVs also carry on their surface MHC I and II molecules, depending on their parental cell, together with co-stimulatory and adhesion molecules indicating that they can participate in direct antigen presentation. Peptide–MHC complexes of extracellular vesicles that are attached to APC surfaces can also be directly presented to T cells without the need for peptide–MHC reprocessing — through a mechanism known as cross-dressing.[79]

Mature DC-derived EVs appear to have increased stimulatory function, at least partly due to their higher ICAM1 content, which could increase binding of the EVs to APCs or could enhance T cell binding and/or activation during APC–T cell interactions. Indeed, DC-derived mature EVs transfer the ability to activate naive T cells to non-professional APCs.[79]

Increasing evidence suggests that extracellular vesicles also transport signals that may promote the activation of the acceptor cells into immunogenic APCs. This adjuvant-intrinsic effect may explain why EVs are more efficient than soluble peptides at transferring antigens between APCs.[79]

In other cases, EVs can participate in immune tolerance mechanisms.

For instance, Treg derived-EVs have a suppressive nature. This function has been attributed to the presence of the ectoenzyme CD73. Indeed, the expression of both CD39 and CD73 on Tregs contributes to immune suppression through the production of the anti-inflammatory mediator adenosine. Binding of this molecule to adenosine receptors A2aR on activated T effector cells (Teffs), triggers intracellular cAMP leading to the inhibition of cytokine production, thereby limiting T cell responses.[87]

In addition, in pregnancy the immune-tolerant microenvironment required to protect the fetus appears dependent on the increased secretion of immunosuppressant placental EVs. Several proteins enriched in placental EVs, such as human ligands of the activating natural killer (NK) cell receptor NKG2D,

FAS ligand, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) appear crucial in this regard.[88]

Lipids

Several specific lipids have been suggested to play a role in the formation of EVs and their lipidic composition might in fact condition their fate and functions in the organism.[82,86,89]

Although differences in the lipid composition of EVs derived from different sources have already been described, EVs are generally enriched in sphingomyelin, cholesterol, PS, ganglioside GM3, and ceramide or its derivatives, compared to their parent cells. Similarly to other biomolecules, lipids are specifically sorted into EVs. For instance, their high content in sphingolipids and cholesterol provides structural rigidity to EVs and an elevated resistance to physico-chemical changes.[82]

Besides the essential structural role of lipids in formation of EV membranes, bioactive lipids, such as sphingosine 1-phosphate, arachidonic acid, eicosanoids, fatty acids and cholesterol, can be transferred between cells by EVs. Vesicle-bound lysophosphatidylcholine has been proposed to play a role in the maturation of DCs and triggers lymphocyte chemotaxis via the G protein-coupled receptor. In addition, vesicle-bound prostaglandins trigger prostaglandin-dependent intracellular signaling pathways within target cells and EV lipids impact Notch signaling and induced cell death in pancreatic tumoral cells.[79,81,82]

Remarkably, even non-antigen harboring EVs are described to activate the complement cascade, where C1q plays an important role by simply binding to lipid membranes through electrostatic interactions rather than in an antibody-dependent manner.[83]

Nucleic acids

Extracellular vesicles are enriched in nucleic acids, which can be transferred to recipient cells, thereby changing their phenotype. Most studies on the EVs cargo of nucleic acids describe the major presence of mRNA and small RNA, including miRNA with low or undetectable levels of DNA and ribosomal RNA.[71]

The RNAs transported by extracellular vesicles are protected from degradation by RNase, allowing them to reach distant sites. [79]

Interestingly, it has been demonstrated that miRNAs administered to pDCs in the absence of liposomal vehicles cannot induce IFN-α secretion. This is in line with the prevailing view that EVs-associated microRNAs are responsible for intercellular communication, while free microRNAs may represent cell byproducts devoid of biological function. [90]

Some mRNAs and miRNAs are detected in both extracellular vesicles and parent cells, whereas others are identified in either extracellular vesicles or in parent cells, which suggests a preferential sorting of certain RNA sequences into extracellular vesicles.[79] An enrichment of RNA containing specific nucleotide motifs has been documented in EVs. The loading of miRNAs into EVs has been shown to be controlled by heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1. hnRNPs are a family of ubiquitous protein with roles in RNA trafficking and function. hnRNP-A2B1 recognizes the EXOmotif (GGAG tetranucleotide) in miRNAs and controls the loading of these miRNAs into EVs. hnRNP-A2B1 inside the EVs is sumoylated; this post-translational modification is necessary for the loading of miRNAs into EVs. Similarly, mRNA species also show a selective enrichment into EVs. Evidence suggests that a consensus sequence within the 3'-UTR of a number of mRNAs enriched in EVs may act as a zipcode sequence that targets mRNAs into EVs, similar to the EXOmotif of miRNAs. This zipcode consists of a 25 nt sequence which contains a short CTGCC core domain on a stem-loop structure and carries a miR-1289 binding site.[82]

Some selected mRNAs present in EVs could be translated into proteins in target cells. For example, EVS shed by endothelial progenitor cells induce activation of quiescent endothelial cells and stimulate angiogenesis by transfer of mRNA. In the kidney, mesenchymal stem cell derived EVs transfer mRNAs, inducing transcription and proliferation of tubular epithelial cells after injury. [76,81]

The miRNA content of EVs also plays a crucial role in modulating recipient cell functions. For instance, tumor-derived EVs containing specific miRNAs have been

demonstrated to elicit profound impact in tumor progression, by supporting immunosuppression or transferring oncogenic capacity. [83]

EVs cargo of miRNAs also appears to have a relevant role in the immune response modulating capabilities of EVs. For instance, in the presence of bystander T-cells, DC secrete small EVs enriched in miR-30b, miR-146a, and miR-155 which functionally promote further CD8+ T cell activation.[91]

Conversely, Treg-derived EVs containing several miRNAs such as miR-142–3p and miR-150–5p suppress the activity of other immune cells. Their uptake by DCs induces a decrease in proinflammatory cytokine IL-6 expression and an increase in immunosuppressive cytokine IL-10 expression, thus inhibiting immune activation.[91]

RELEVANCE OF EVs IN CANCER

EVs have emerged as prominent regulators of several key processes in cancer biology, as well as crucial factors in immune response during tumor progression.[91] Tumor products can be delivered via EVs to neighboring cells and distant organs with a wide array of effects. [92] Tumor derived-EVs can transfer oncogenic molecules between cells in the primary tumor leading to cancer development in originally non-cancerous cells. Examples exist in literature regarding transfer of epidermal growth factor receptors variants as well as miRNAs able to inhibit mRNAs of key tumor-suppressors like PTEN. [88]

Tumor-derived EVs have been reported to both stimulate and suppress immune responses. These EVs can mediate immunosuppression through several mechanisms. Containing tumor specific antigens, of which they are often enriched, they can participate in anti-tumor response stimulation. However, data suggest they have mostly immunosuppressive effects that support tumor progression and dissemination. For example, tumor-derived EVs are enriched for CD95L, TRAIL or galectin 9 which can promote T cell apoptosis. Similarly, they suppress the

immunological activity of natural killer cells by inhibiting their proliferation and compromising their cytolytic activity.[88]

Tumor-derived EVs are also considered crucial in metastatic disease spread. At a pre-metastatic stage EVs from the primary tumor can affect distant sites creating niches with conditions that favor dissemination as reported for the most common sites of metastases like lung, liver and bone marrow.[92] This process involves several EV-dependent mechanisms like endothelial permeabilization (through endothelial tight junctions downregulation via miR-105 [93]), extracellular matrix modification (through EV metalloproteinases [71] and TGF-β dependent fibroblast activation[92]), as well as recruitment of myeloid derived suppressor cells in target organs creating an immune-inhibitory niche. [92]

RELEVANCE OF EVs IN AUTOIMMUNE DISEASES

RHEUMATOID ARTHRITIS

The involvement of EVs in many physio-pathological processes, including rheumatological autoimmune diseases has been recognized. Several studies have investigated their role in RA.

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease characterized by joint destruction and systemic manifestations. In recent years, a variety of evidence has pointed towards a potential pathogenetic role of EVs in RA. [85,94]

Several researchers have shown an increase in circulating levels of EVs in RA patients, with a significant correlation to disease activity. In RA patients with a high disease activity, the plasma concentration of monocyte, platelet, endothelial cell, and B lymphocyte-derived EVs has been shown to be significantly higher than in healthy controls. Patients with moderate disease activity showed enhanced production of plasma monocyte and endothelium-derived EVs, whereas there was

no difference versus healthy controls when activity indexes fell in the range of low disease activity.[94]

Furthermore, analysis of synovial fluid of RA patients revealed significantly higher concentrations of platelet and leukocyte derived EVs compared to their circulating levels. In the joint these EVs can act by promoting several processes strongly associated with the disease physio-pathogenesis. [85]

Of primary relevance, the synovial EVs have been demonstrated to include significant amounts of citrullinated peptides, established key antigens in the RA humoral immune response. [95]

The most abundant EVs in RA synovial fluid are those released by platelets. They have pro-inflammatory effects as well as promote migration, invasion and adhesion of fibroblast-like synoviocytes. [94] For example, platelet-derived EVs contain large amounts of IL-1β, involved in proinflammatory responses of synovial fibroblasts, thus enhancing joint inflammation by the release of IL-6 and IL-8. Particularly, synovial fibroblasts secrete EVs that upregulate matrix metallo-proteinase activity and, expressing TNFα at their surface, bind to autoreactive T lymphocytes and make them resistant to activation induced cell death (AICD) through the activation of akt and NFkB pathways. This evidence sustain the potential role of EVs in promoting the survival of autoreactive T lymphocytes.[85,86]

EVs associated miRNAs is also suspected to play a central role in RA pathogenesis. For instance, TNF DC-stimulation of RASF elicit a significant higher release of EVs harboring different miRNAs, including miR-155. This particular miRNA is known to facilitate inflammatory activation upon uptake in immune cells. [94]

A further pathogenetic element in RA is neoangiogenesis; the development of new blood vessels contributes to pannus development and represents a typical feature of inflamed synovial tissue. Of interest, it has been demonstrated that EVs from RA synovial fluid can stimulate angiogenesis.[94]

A potentially useful application of EVs in RA is the prediction of patient response to a specific treatment regimen. Evidence suggests that EV-derived miRNAs could be used as potential predictors of response to therapy in RA patients. Interestingly,

a specific plasma miRNA signature (miR-23 and miR223) has been identified as biomarker of response to anti-TNFα/DMARDs combination therapy. Specific miRNAs have also been identified in RA patients as predictors of response to adalimumab or etanercept, which are both monoclonal antibodies that inhibit TNF-α. [94]

SYSTEMIC LUPUS ERYTHEMATOSUS

Evidence strongly suggests a role of EVs in systemic lupus erythematosus (SLE) pathophysiology, based significant qualitative and quantitative differences in circulating EVs from SLE patients compared to controls. In particular, plasma from SLE patients appears to possess a population of nanoparticles with EVs of different size and granularity positive for annexin V and apoptosis-modified chromatin.[96]

Based on their autoantigen content, EVs are likely to participate in the formation of immune complexes. Indeed, it has been demonstrated that antibodies in plasma from SLE patients can bind to in vitro generated EVs from apoptotic cells. Furthermore, circulating EVs in SLE patients have been shown to bind to IgG, C1q and IgM to a lesser extent. The amount of EVs associated with IgG also correlates to plasmatic levels of anti-DNA antibody and to complement activation markers. [85]

It has been speculated that the EVs miRNAs content, acting as a damage associated molecular pattern (DAMP) molecule, activates toll-like receptor 7 (TLR7) signaling in pDCs, promoting their maturation and consequent further activation of lymphocytes through cytokines production. Thus, through TLR7 signaling, EVs could directly induce significant production of IFNα, considered a hallmark in SLE pathogenesis. [85,90] Moreover, Dieker et al. demonstrated that EVs isolated from the plasma of SLE patients can activate blood-derived pDCs and mDCs, while EVs from the plasma of healthy controls, RA, and SSc patients did not exhibit such an effect.[96]

Similar results emerged from a study by Lee et al., who demonstrated that the amount of IFN-α and TNF-α produced by a fixed number of EVs was higher in SLE than healthy controls, further highlighting the relevance of this mechanism in SLE.[97]

Endothelial activation and damage are commonly observed in SLE patients and are related to the development of nephropathies and vascular diseases. It has been observed that the circulating levels of endothelial-derived EVs (CD144+, VCAM+) and tissue leukocyte-EVs (TF+, CD45+) is higher in SLE compared to healthy controls, and these markers directly correlate with the degree of inflammation, glomerulonephritis, and vascular dysfunction. [94]

The protein signature of endothelial-derived EVs could then be used as biomarker of the activity and progression of SLE and of the presence of disease complications, especially in the kidney. The possibility of non-invasive detection of glomerular damage, for instance through urinary biomarkers, could offer significant advantages in clinical practice. It has been reported that urine miR-146a is markedly increased in SLE patients, and could be used to discriminate patients with active lupus nephritis from those without. Solè et al. investigated the expression level of miR-29c and found a negative correlation between urinary EVs miR-29c level and histological chronicity index and glomerular sclerosis. [94]

Several authors have investigated other possible correlations between EVs and clinical features of SLE patients. López et al. proved that total EVs, CD25+ EVs and platelet-derived EVs in the plasma of SLE patients associated with the increased disease duration and higher risk of cardiovascular disease. Fortin and colleagues revealed a positive correlation between CD41+ EVs harboring IgG and the SLE Disease Activity Index 2000, as well as a positive association between the concentrations of CD41– EVs harboring IgG and Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index. [70]

IDIOPATHIC INFLAMMATORY MYOPATHIES

Analysis of EVs expression in IIM patients has so far highlighted several interesting findings, contributing to furthering our understanding of this group of diseases.

Significantly elevated concentrations of monocyte (CD14+), T-lymphocyte (CD3 +) and B-lymphocyte (CD19+) -derived EVs have been described in the plasma samples of IIM patients compared to healthy controls. In particular, patients with anti-jo-1 antibodies and lung involvement appear to have significantly higher concentrations of lymphocyte and monocyte derived EVs compared to both controls and anti-jo-1 negative IIM patients. Furthermore, it has intriguingly been noted that levels of these EVs positively correlated with muscle strength, as evaluated by MMT-8. This somewhat counterintuitive data was speculated by the researchers to be associated with the activity of these EVs on metalloproteinases, which could counteract muscle fibrosis thus delaying muscle weakness. [98]

Increasing evidence has shown that platelets play an active role in a variety of immune and inflammatory disease. Their importance appears at least partly related to the secretion of platelet-derived EVs). Several observations suggest that interactions between endothelial cells, leukocytes, and platelets may be important in the pathogenesis of IIM. A study conducted by Shirafuji et al. demonstrated a significantly increased level of platelet-derived EVs in IIM patients without differences between IIM subtypes compared to controls. Furthermore, their levels were significantly higher in patients with higher levels of systemic inflammation (evaluated via CRP) and decreased after treatment with glucocorticoids. Several studies have reported platelet-derived EVs to play an active role in immune and inflammatory responses by modulating various aspects of vascular, leukocyte, and platelet functions. They can, for example, increase phagocytic activity in leukocytes, reduce endothelium-dependent arteriolar relaxation as well as deliver arachidonic acid to endothelial cells thereby increasing CD54 expression and leukocyte adhesion. [99]

More recent proteomic analysis on plasmatic EVs proteins in IIM patients revealed several interesting findings. Among the differentially expressed EVs proteins

shared by IIM patients, a significant enrichment was noted for proteins involved in the complement and coagulation cascade pathways, known to be involved in the pathogenesis of several autoimmune diseases like SLE. In DM, type I IFN (IFN-I) signaling could upregulate expression of EVs enriched in complement components like C1QB and C1QC. These could then promote the membrane attack complex (MAC) activation and damage in muscle capillaries endothelium, triggering the production of proinflammatory cytokines and chemokines, further increasing IFN production, thus creating a positive feedback loop perpetuating IFN release, complement activation and vascular damage. [100]

The role of EVs in vascular damage and disfunction in IIM was investigated in another study on JDM patients. Jiang et al. demonstrated that EV-associated miRNAs from JDM patients could influence aortic endothelial cells affecting their transcriptional activity, impacting permeability and cellular adhesion.[101]

EVs appear to also have an important role in muscle tissue homeostasis, vehiculating myokines secreted by myoblasts and myotubes to act in autocrine and paracrine fashion to influence several aspects of muscle cell function. In the past decade, several lines of evidence have elucidated the secretory capabilities of skeletal muscle. Myoblasts and myotubes secrete EVs containing more than 400 different signal molecules, including microRNAs and proteins, that are critical in regulating skeletal muscle myogenesis, metabolism, and development. For instance, after muscle injury, myoblasts secrete EVs rich in myogenic factors like Hepatocyte growth factor (HGF). HGF is a potent chemoattractant for muscle stem cell activation, acting in synergy with other myogenic factors, like IGF1 and TGFβ, to increase muscle repair. [102]

However, in the context of inflammation muscle EVs signaling could promote muscle atrophy through suppression of myogenic factors. A study conducted by Kim et al. demonstrated that myotubes treated with a mixture of cytokines, secreted EVs-associated myokines that could increase muscle atrophy, via upregulation of myostatin and ampk signaling and downregulation of the counteracting, myogenic, decorin. [103]

POTENTIAL CLINICAL APPLICATIONS OF EVs

A potential clinical application of EVs is their use as diagnostic and prognostic biomarkers. As mentioned above, EVs exist in body fluids and likely reflect the status of their parental cells. Therefore, EVs are ideal non-invasive biomarkers for disease diagnosis. For example, EVs that express CD63 and caveolin-1 in plasma samples can be considered biomarkers of melanoma and a novel tool for the clinical management of cancer patients[75]

EVs are currently receiving attention also as a promising therapeutic tool. As natural carriers of signal molecules, EVs possess many favorable properties including high stability, excellent biocompatibility, biological barrier penetration, and low toxicity, which make them an attractive vehicle for therapeutic delivery. Moreover, EVs may be less immunogenic, less cytotoxic, and non-mutagenic compared to other existing viral-based or liposome-based gene delivery vehicles. They use native mechanisms for cellular entry, internalization and trafficking, and therapeutics in the form of small RNA could benefit from EV delivery, including miRNAs, anti-inflammatory agents, and anti-cancer drugs.[83] These characteristics suggest that EVs may be developed as an ideal vehicle for therapeutic delivery.

Micro-RNAs

MicroRNAs (miRNAs) are single-stranded small non-coding RNAs ranging in length from 18 to 25 nucleotides (nt) that act as guide molecules in mRNA silencing mechanisms. [104] MicroRNAs are conventionally named using the "miR" prefix and a unique identifying number (e.g., miR-1, miR-2). The identifying numbers are assigned sequentially, with identical miRNAs having the same number, regardless of organism. Similar miRNA sequences within a species can also be given the same number, with their genes distinguished by letter and/or numeral suffixes, according to the convention of the organism.[105]

Each miRNA is predicted to regulate the expression of hundreds of target genes, thus acting as critical gene expression modulators,[106] involved in nearly every cellular development and differentiation process as well as in pathological contexts.

Many of the bilaterian animal miRNAs are phylogenetically conserved: around 55% of C. elegans miRNAs have homologues in humans, which indicates that miRNAs have had important roles throughout evolution.[107] It is estimated that humans express at least 2,300 mature miRNAs, whose genes constitute about 1-3% of the human genome. It is predicted that expression of more than 30% of human genes is directly regulated by miRNAs. [108] The role of miRNA has been elucidated in a variety of processes in human biology, including embryo development, cell differentiation, proliferation, apoptosis, signal transduction, and metabolism. [108]

Furthermore, miRNAs are considered crucial in the development and function of innate and adaptive immune cells. Several immunoregulatory genes, including transcription factors, cofactors and chromatin modifiers are miRNA targets and some even harbor binding sites for eight or more different miRNAs. Therefore, dysfunction of miRNAs or dysregulation of their expression is considered central in the pathophysiology of autoimmunity.[109]

miRNA BIOGENESIS

In humans, the majority of canonical miRNAs are encoded by introns of noncoding or coding transcripts, and only occasionally miRNAs can be encoded by exonic regions. Often, several miRNA loci are in proximity to each other, constituting a polycistronic transcription unit. The miRNAs in the same cluster are generally co-transcribed, but the individual miRNAs can be additionally regulated at the post-transcriptional level. While most miRNA promoters have not been characterized in detail, the few examples studied are similar in structure to protein coding gene promoters, including control elements and histone marks.[104,106]

The defining feature of all miRNA genes is the stem-loop precursor RNA structure, with one (or sometimes both) strands of the stem being the source of the mature miRNA. [106]

Canonical biogenesis pathway

Transcription of the miRNA host gene by RNA polymerase II leads to the primary miRNA (pri-miRNA) transcript formation. The pri-miRNA requires two endonuclease processing steps before it becomes a mature, active miRNA. [106]

The primary transcripts are usually several kilobases long and contain local stem-loop structures. The first step of miRNA maturation is cleavage at the stem of the hairpin structure, which releases a small hairpin that is termed a pre-miRNA. This reaction takes place in the nucleus by the nuclear RNase III-type protein Drosha. Drosha requires a cofactor, the DiGeorge syndrome critical region gene 8 (DGCR8) protein in humans. Together with DGCR8, Drosha forms a large complex known as the Microprocessor complex.[107] Dgcr8 is a double-stranded-RNA binding protein that stabilizes the association of Drosha with pri-miRNA and determines the precise location of the processing. Dgcr8 and Drosha cross-regulate expression of each other to maintain homeostatic control of miRNA biogenesis. Although Drosha and Dgcr8 comprise the minimum components of the microprocessor complex, many more proteins that interact with this structure have been identified, including the DEAD-box helicase proteins p68 and p72. In fact, it has been demonstrated that the deletion of either p68 or p72 proteins leads to

embryonic lethality highlighting their relevance in miRNA biogenesis mechanisms.[110]

The precursor is then exported out of the nucleus through a Ran-GTPase dependent manner by Exportin5. [106]

In the cytoplasm the second processing step occurs. The endonuclease Dicer cleaves the loop region of the precursor releasing the mature miRNA. Like Drosha, Dicer is also associated with an RNA binding protein, TRBP. The product of the Dicer reaction is a duplex RNA of approximately 21 nucleotides length. [106]

The specific nucleotide locations of Drosha and Dicer cleavage are usually narrowly defined, leading to mature miRNAs with clearly defined terminal ends. However, some miRNAs have heterogenous cleavage sites leading to multiple “isomiRs” of the mature miRNA that can vary by cell type and switch within diseases. This heterogeneity could lead to differential target gene repression and distinct biological functions. [106]

Alternative biogenesis pathways

While the majority of identified miRNAs are produced by the canonical pathway there are several examples of alternative biogenesis. The most common variation in biogenesis is the intronic miRNAs (mirtron) class of miRNAs. [106] Around 500 human mirtrons bypass DROSHA-dependent microprocessing. Intron splicing results in the formation of a circular molecule of RNA with a short tail, known as an intron lariat. Mirtron pre-miRNAs are derived directly from intron lariat through the enzyme Debranching RNA Lariats 1 (DBR1). Debranched mirtrons are analogous to canonical pre-miRNAs and join the canonical pathway at the cytoplasmic export stage by Exportin-5. Mirtrons are subsequently processed by DICER in the same manner as canonical miRNAs. Additionally, mirtron pre-miRNAs are not strictly bound by their intron boundaries. Mirtrons can be “tailed” by extra nucleotides at their 5p - or 3p -ends (or both), which are subsequently cleaved by nuclear exonucleases prior to pre-miRNA export. 5p-tailed mirtrons are the most abundant type of mirtrons (with 420 annotated sequences), followed by canonical mirtrons (33 annotated) and 3p -tailed mirtrons (18 annotated).[111]

Even though many mirtron can be transcribed independently of their host genes, their expression is most frequently coupled. Thus, they can exert synergistic or antagonistic functions to the gene they are spliced from. In fact, most of the mirtron whose function has been elucidated, are shown to act on biological pathways related to those of their host gene. Intronic miRNA can reduce the expression noise of their target host gene, by quenching expression when there is a low level of host gene promoter activity.[111]

Mirtrons evolve more quickly than canonical miRNAs. This aspect may in part be attributed to the fact that their expression only requires one cleavage event (DICER) instead of two as in the canonical mechanism. Thus, mirtrons require shorter hairpins and consequently a shorter stretch of self-complementary sequences than conventional miRNAs. From an evolutionary perspective, mirtrons are younger and less conserved across species. Thus, most mirtron haven't been functionally characterized. However, a small number of mirtrons are known to play important roles in multiple cancers' oncogenesis.[111]

Dicer-independent miRNA biogenesis has also been described. The biogenesis of miR-451 has been extensively studied. After transcription and Drosha cleavage the precursor directly binds Ago2, instead of Dicer, and Ago2 cleaves the star strand. The remaining loop sequence is trimmed back to yield the preloaded, mature miR-451. Several other miRNAs may follow the Dicer-independent pathway for their biogenesis.[106]

The existence of alternative pathways reflects the evolutionary flexibility of miRNA biogenesis. However, it is notable that the vast majority of functional miRNAs follow the canonical pathway for their biogenesis, and that only about 1% of conserved miRNAs are produced independently of Dicer or Drosha in vertebrates. Most other non-canonical miRNAs are low in abundance and poorly conserved. So, the functional relevance of non-canonical miRNAs should be interpreted with caution.[104]

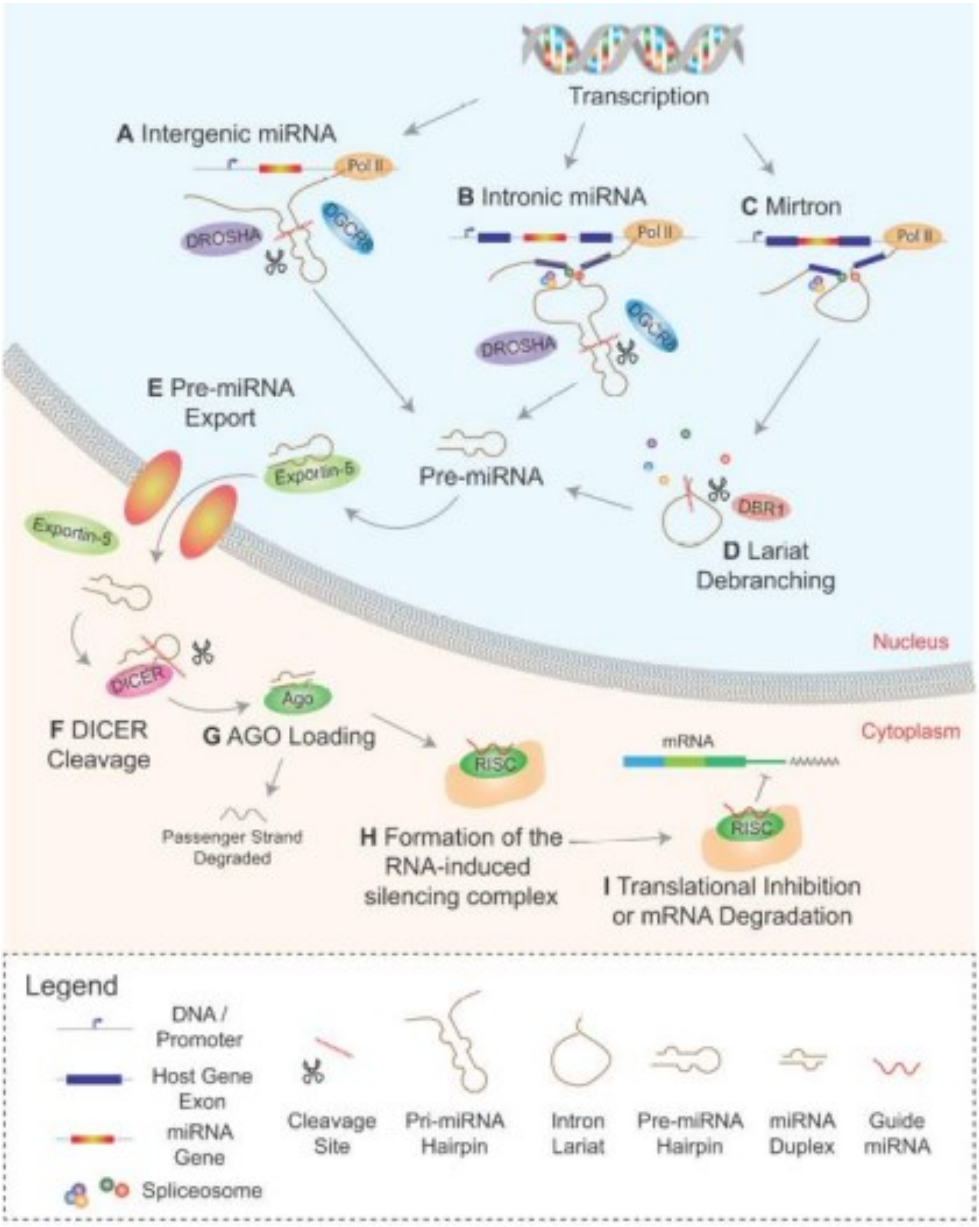

Figure 10 Overview of miRNA Biogenesis. (A) Intergenic primary miRNAs (pri-miRNAs) undergo co-transcriptional microprocessing via DGCR8/DROSHA to produce precursor miRNA (pre-miRNA). (B) In intronic miRNAs, microprocessing occurs during intron commitment for splicing and generally completes before the intron is spliced. (C) Mirtrons are a special class of intronic miRNAs whereby pre-miRNAs are derived directly from intron splicing and (D) lariat debranching by DBR1. (E) Canonical and mirtron pre-miRNAs join a common pathway of Exportin-5 mediated cytoplasmic export, after which (F) DICER cleavage produces a miRNA duplex. (G) miRNA duplexes are loaded onto Argonaute (Ago) proteins whereby strand selection occurs. (H) Retention of the miRNA guide strand by Ago is critical to the formation of the RNA induced silencing complex (RISC) which (I) targets protein-coding mRNAs promoting either transcript degradation or inhibiting translation. Pol II = RNA Polymerase II. [111]

miRNAs BIOLOGICAL FUNCTIONS

After Dicer cleavage, miRNA is incorporated into the RNA induced silencing complex (RISC complex). The RISC complex contains the essential protein

Argonaute (Ago), of which four family members have been identified in humans (Ago1–4). One strand of the duplex miRNA is loaded into RISC as a mature miRNA, while the other strand (or star strand) is typically degraded.

Argonaute directly binds the mature miRNA and seeks target mRNAs that have complementarity to the miRNA. In particular, nucleotides 2–7 of the miRNA, termed the "seed" region, are important for target association. The 3′ end of the miRNA can also contribute to target recognition, and centrally matched targets have been identified. [106]

If the miRNA duplex has complementarity in the central region, the star strand can be cut by Ago2 and further degraded by the nuclease complex C3PO. Most miRNA duplexes, however, lack central complementarity and therefore cannot participate in star strand cleavage. These miRNA duplexes rely on strand unwinding, and several helicases have been described to possess this activity.

However, for some miRNAs both strands may be loaded into RISC at similar frequencies. In this case, the strand from the 5′ end of the stem–loop is termed "5p" and the 3′ strand the "3p". [106]

The regulation of target gene by miRNAs involves several mechanisms, including RNA degradation, induced decapping, induced deadenylation, altered cap protein binding, reduced ribosome occupancy, and sequestration of the mRNA from translational machinery. These mechanisms are not mutually exclusive and some result in decreased mRNA levels, whereas others act only to decrease protein expression.[112]

The three major processes of mRNA repression appear to be endonucleolytic cleavage, mRNA degradation by deadenylation, and inhibition of translation initiation.[113]

The endonucleolytic cleavage of mRNA by miRNA requires complementarity between miRNA and the target mRNA. In that case, proteins within the RISC complex cleave the mRNA, leading to its degradation and silencing. Of note however, this mechanism of gene regulation rarely occurs in mammalian cells

because nearly all miRNA-mRNA interactions often have significant mismatches. [113]

Inhibition of translation initiation is another widely studied mechanism of miRNA-induced gene silencing. [113] The central domain of Argonaute proteins in fact exhibits sequence similarities to the cytoplasmic cap-binding protein eIF4E (eukaryotic translation initiation factor 4E), which is essential for cap-dependent translation initiation. AGO2 is capable of binding to the 5' cap structure of mRNA, competing with eIF4E and thus inhibiting translation initiation. [114]

Cellular localization is another mechanism by which miRNA might mediate repression of mRNA translation. Evidence suggests that ribonucleoprotein complex-bound mRNAs localize to p-bodies within the cytoplasm.[113] P bodies are aggregates of translationally repressed mRNPs with a conserved core that consists of the mRNA decapping machinery, including the decapping enzyme Dcp1p/Dcp2p, the activators of decapping Dhh1p/RCK/p54, Pat1p, Scd6p/RAP55, Edc3p, the Lsm1p-7p complex, and the 5p to 3p exonuclease, Xrn1p. For this reason, this proteins complex is referred to as the general repression/decay machinery.[115]

Even though most miRNAs are described to act to negatively regulate gene expression, some miRNA exhibit positive regulatory functions. For example, miR-373 has sequence complementarity to the promoter of E-cadherin and it can increase promoter occupancy by RNA Pol II, thereby increasing gene transduction. Single microRNAs can have opposing functions in different systems, illustrating the fact that microRNA communication is context dependent. One example is miR-125b in cancer, downregulated in multiple cancers such as hepatocellular, breast, and lung while overexpressed in colorectal, pancreatic, gastric, and some leukemias. These results indicate that miR-125b has both oncogenic and tumor suppressive ability subject to the tissue/environment.[112]

REGULATORY MECHANISMS of miRNA EXPRESSION

The control of miRNAs expression is crucial to maintain cellular functions. miRNAs expression dysregulation is often associated with human diseases, including cancer. miRNA biogenesis is controlled by multiple layers of feedback loops that involve the biogenesis factors, the miRNAs themselves and their targets.[107]

The transcription process is a major point of regulation in miRNA biogenesis through numerous Pol II-associated transcription factors . For instance, myogenic transcription factors, such as myogenin and myoblast determination 1 (MyoD1), bind upstream of miR-1 and miR-133 loci and induce the transcription of these miRNAs during myogenesis. The tumor suppressor p53 activates the miR-34 family of miRNAs, whereas the oncogenic protein MyC trans-activates or represses several miRNAs that are involved in the cell cycle and apoptosis. Epigenetic control also contributes to miRNA gene regulation; the miR-203 locus frequently undergoes DNA methylation in T-cell lymphoma but not in normal T lymphocytes.[107]

RNA decay enzymes might also target the precursors pri-miRNAs and pre-miRNAs. Once bound to Ago proteins, mature miRNAs seem to be more stable than average mRNAs, with the half-life of most miRNAs being greater than 14 hours. Moreover, certain miRNAs might be degraded more rapidly than others, suggesting a specific recognition of miRNA sequences by nucleases.[107]

RNA editing is another possible way of regulating miRNA biogenesis. The alteration of adenines to inosines, a process that is mediated by adenine deaminases (ADARs), has been observed in miR-142 and miR-151.[107]

Drosha constitutes a regulatory circuit together with DGCR8; Drosha downregulates DGCR8 by cleaving DGCR8 mRNA, whereas DGCR8 upregulates Drosha through protein stabilization.[107]

Post-translational modification can regulate the protein stability, nuclear localization and processing activity of Microprocessor. Phosphorylation of Drosha by glycogen synthase kinase 3β (GSK3β) is required for the nuclear localization of

Drosha, while acetylation of Drosha inhibits its degradation and stabilizes it. DGCR8 can be deacetylated by histone deacetylase 1 (HDAC1), which increases its affinity for pri-miRNAs and consequent expression regulation.[104]

Receptor-activated SMAD proteins (R-SMADs) SMAD1–3 and SMAD5 and p53 promote Microprocessor activity through their interaction with p68. [104]

EXP5 is ubiquitously expressed, but it has been shown that EXP5 is post-transcriptionally induced during cell cycle entry in a PI3K-mediated mechanism. In some tumours, XPO5 is mutated and the resulting C-terminal-truncated EXP5 cannot transport its pre-miRNA cargo, which globally reduces the level of mature miRNAs.[104]

Under hypoxia, epidermal growth factor receptor (EGFR) phosphorylates human AGO2, which results in the dissociation of human AGO2 from Dicer and the reduction of pre-miRNA processing for some miRNAs. Upon stress or viral infection, human AGO proteins are subject to poly-ADP-ribosylation, which inhibits their activity to repress targets.[104]

BIOLOGICAL RELEVANCE

miRNA molecules are important effectors of epigenetic regulation.[116] The biological importance of miRNAs in mammalian development was first demonstrated with the generation of mouse models deficient for key enzymes in miRNA biogenesis, Dicer and DGCR8. Loss of either step in miRNA biogenesis results in embryonic lethality. Similarly, tissue-specific knockout of either gene leads to developmental defects in that tissue, underscoring the requirement for miRNA function in most tissue types.[106]

In general, individual miRNAs are not required for specification of individual tissues, but they are often required for maintaining tissue homeostasis and differentiation state. Functional studies have demonstrated miRNA dysregulated expression as a causal factor in the progression of many diseases , including cancer and autoimmunity.[106]

miRNAs have been proven to play an important role in different aspects of cardiovascular disease progression, including acute myocardial infarction (AMI), hypertrophy, heart failure, arrhythmias, and atherosclerosis. [117] For example, during fibrosis of myocytes, miR-21 is significantly increased, resulting in cardiac hypertrophy. MiR-143/145 target several mRNAs that encode proteins involved in the proliferation and differentiation of vascular smooth muscle cells. Downregulation of the miR-143/145 family in mouse models results in hypertension and cardiac failure.[116] Extra collagen expression related to chronic cardiac stress is prevented by miR-29 family, mainly through its function in fibroblasts. In vitro studies showed that increasing activity of miR-29 induces hypertrophy of primary cardiac myocytes. Additionally, in a mouse model of cardiac pressure overload, miR-29 knockouts or anti-miR-29 infusion prevent cardiac hypertrophy and fibrosis and improve cardiac disease.[112]

miRNA profiling suggests that miRNAs are globally downregulated in tumor samples as compared to normal tissues. [110] To date only a few miRNA-mRNA interactions with importance for cancer pathogenesis have been proven, but the role of miRNA dysregulation in oncogenesis is rapidly gaining relevance. For example, the let-7 miRNA family is known to regulate RAS oncogenes and it has been demonstrated to decrease in lung cancer with an inverse relation to RAS. [118]

TGFβ signaling is considered an important contributor to cancer progression, promoting epithelial to mesenchymal transition. In fact, TGFβ, through SMAD proteins and their interaction with p68 (a component of the microprocessor complex), can increase miRNA production. For instance, its ability to upregulate expression of miRNAs like miR- 21 and 199a is demonstrated. MiR-21 is the most commonly over-expressed miRNA in cancers. Over-expression of miR-21 has been reported in more than 15 different malignancies. The oncogenic potential of miR-21 is largely attributed to its involvement in several intracellular signaling pathways, including the activation of AKT and the downregulation of the pro-apoptotic protein PDCD4. Expression of miR-199a has also been associated with cancers and one study suggests that leukemias with higher expression of miR-199a exhibit worse prognosis.[113]

One of the most studied proteins in cancer biology is the tumor suppressor p53. Mutations in p53 have been linked to several cancers and are often considered causal in the aberrant phenotype acquisition of malignant cells. It has been proven that its tumor suppressor functions are at least partially related to its ability to regulate miRNA expression via direct interaction with both p68 and p72. Several miRNAs upregulated by p53 can suppress cancer growth, like miR-15 and miR-16 targeting the antiapoptotic gene BCL2. In fact, these miRNAs are documented to decrease in several cancer types. [113] Furthermore, Bcl2 overexpression by miR-15a and miR-16-1 down-regulation seems to be the main regulatory mechanism involved in the pathogenesis of the majority of human B cell chronic lymphatic leukemias. [118]

It has also been suggested that secreted miRNAs could contribute to intercellular communication, both to neighboring and distant locations. For instance, mature miRNA duplex can be directly transferred through gap-junctions channels and target the mRNA in the neighbor cells. Conversely, non-small cell lung cancer cell lines secrete EVs containing miR-21/29a. These EVs can bind to human TLR8 in tumor-associated macrophages, leading NF-κB activation and secretion of the prometastatic inflammatory cytokines tumor necrosis factor α and interleukin 6.[116]

As master regulators of gene expression, miRNAs are involved in immune system development. Expression profiles of hematopoietic cells have identified several miRNAs differentially expressed in subsets of hematopoietic cells, with tight regulation during lineage differentiation steps.[119]

Indeed, miRNAs are regulators of B cell differentiation from naïve B cells into effector B cells.[119] and they fluctuate in expression throughout the different stages of B cell development in bone marrow, supporting their role in this process.[120]

The immune response in autoimmunity is characterized by increased immune cell activation and failed or inefficient immune regulation, with a functional imbalance between Treg and other lymphocyte subpopulations like type 17 T helper cells, as demonstrated in IIM patients.[19] The fundamental role of miRNAs in regulating this equilibrium has been shown in several studies. For example, miR-326 was

identified in lymphocytes as a factor enticing TH17 cell lineage polarization, and its expression correlates with disease activity and severity in multiple sclerosis and experimental autoimmune encephalomyelitis. [119]

Interestingly, it has been demonstrated that miRNAs involvement is indispensable for the function and homeostasis of mature T lymphocytes, particularly in the regulatory T (Treg) cell compartment. Indeed, mouse models conditionally deleted for Drosha and Dicer spontaneously develop inflammatory diseases, suggesting that miRNAs are essential for self-tolerance maintenance. [109] MiR-155 has been shown to be important for maintenance of suppressor cell activity. Interestingly miR-155 also downregulates lipopolysaccharide-induced inflammatory pathways in human monocyte-derived dendritic cells.[119] However, miR-155 has also been studied as one of the main regulators implied in the abnormal inflammation in rheumatoid arthritis joints, highlighting the complex and multifaceted role of miRNAs in autoimmune disease patho-physiology. [121]

Dysregulated miRNA biogenesis may also be the consequence of autoimmune conditions, partially explaining disease phenotypes. Intensified oxidative stress, elevated levels of proinflammatory cytokines and autoantibodies could affect the expression of miRNAs by interfering key components of the miRNA biogenesis machinery. For instance, treatment with type I interferon (IFN) and H2O2 post-transcriptionally represses protein level of Dicer and leads to miRNAs differential expression in different human cell lines. In addition, the autoreactive anti-Su antibodies in sera of patients with systemic rheumatic diseases has been found to cross-react with Ago2, potentially affecting the miRNA synthesis in autoimmune conditions.[109]

miRNAs IN AUTOIMMUNE DISEASE

RHEUMATOID ARTHRITIS

Evidence indicates important and complex roles for genetic and environmental factors in the etiology of RA. Joint destruction in RA is associated with progressive

proliferation of synovial macrophages and fibroblasts, followed by infiltration of lymphocytes through the vessels and proliferation of endothelial cells in the neighboring regions. Ultimately, inflamed synovial growth and cartilage and bone destructions occur. Overproduction of inflammatory cytokines, proteases, and growth factors leads to chronic inflammation, progressive joint destruction, immunity dysregulation, and systemic complications. In RA physiopathology, T cell, more particularly Th1, Th17, and regulatory T cells are important and coexist with stromal cells in the milieu of the inflamed joint. This microenvironment is rich in metabolic intermediates that are released into the extracellular space to shape cell–cell communication and the functional activity of tissue-resident cells. IL-17 plays a key role in this intercellular network. This cytokine stimulates the synthesis of tissue-degrading enzymes such as and matrix metalloproteinase and pro-inflammatory cytokines (i.e., TNF-a, IL-1) by fibroblast cells, chondrocytes, and macrophages. Regulatory T cells control immune responses and research has shown reduced Treg cell function and number in the peripheral blood of patients with RA. These cells, secreting IL-10 and TGFβ, can control the immune response, and inhibit inflammation.[121]

It has been reported that genetic variance of some miRNA genes such as miR-499, might predispose an individual to RA development. Additionally, altered expression of many miRNAs has been discovered in several cells, tissues and body fluids in patients with RA. MiRNAs expression also differs depending on disease's stage and activity.[122]

Most studies investigating the role of miRNAs in RA pathogenesis confirmed the increased expression of miR-155 in synovial tissue, rheumatoid arthritis synovial fibroblasts (RASFs), and synovial fluid (SF) of RA patients. The expression of miR-146 and miR-155 are also elevated in whole blood, PBMCs, and the population of SF CD14+ and synovium macrophages.[121] Mir-155 is one of the most important microRNAs playing a role in autoimmune diseases and can it target different mRNAs, including SHIP1 (Src homology-2 domain-containing inositol 5-phosphatase-1) in CD68+ macrophages and lymphocytes in RA patients and enhances production of pro-inflammatory cytokines such as TNF and IL-6, cytokines required for Th17 cell differentiation.[121]

Furthermore, higher intracellular levels of miR-155 appear to be crucial in the differentiation process of TH17 cells by targeting Suppressor of cytokine signaling 1 (SOCS1), thus allowing the upregulation of the IL-6/STAT3 pathway. [121]

miR-17-92 cluster regulates maturation of the immune system and acute phase response. Increased expression of this cluster leads to production of autoantibodies and differentiation of Th1 cells. miR-17 has been found increased in serum EVs of RA patients, associating with a decline in peripheral Tregs. Within the cluster, miR-18a exacerbates inflammation and joint damage by targeting TNFAIP-3, an established NF-kB target gene and negative regulator of NF-kB signaling. Additionally, miR-18a increases the expression level of important mediators such as IL-6, IL8, MCP, MMP1, and RANTES.[121,122]

In RA patients several epigenetic mechanisms transform synovial fibroblasts into RASFs, characterized by reduced apoptosis, increased proliferation, migration and invasion, enhanced proinflammatory cytokine production and production of bone matrix eroding enzymes, like matrix metalloproteinases. They also orchestrate the local invasion of several cells that result in tissue damage. Various intracellular pathways in RASFs, including Wnt, NF-κB, JAK/ STAT and TLRs appear to be affected by changes in miRNA expression. Most of the disturbed miRNA levels lead to secretion of pro-inflammatory cytokines or MMPs, increased proliferation and survival of RASFs, while few alterations oppose to the inflammatory milieu and the subsequent tissue damage.[122]

Low miR-27a levels in inflamed synovium lead to increased production of MMPs, which damage the cartilage matrix and allow RASFs to migrate and invade the cartilage. In addition, decreased levels of miR-30a in inflamed synovial tissues are correlated with reduced apoptosis and enhanced autophagy of RASFs and synovial macrophages. Low expression of miR-708 in synovial tissue of RA patients might amplify Wnt/β-catenin signaling and increase RASFs survival and migration. Finally, high levels of resistin in synovial fluid and synovial tissues of RA patients provoke a drop in miR-206 expression in endothelial progenitor cells. This drop leads to production of vascular endothelial growth factors that contributes to neovascularization.[122]

Maeda et al. discovered 12 dysregulated miRNAs in inflamed synovium that participate in bone metabolism, especially osteoblast and chondrocyte differentiation, such as Wnt and bone morphogenetic protein (BMP) pathways.[122]

Plethora of data indicates that the levels of circulating miRNAs differ among RA patients and healthy subjects. [122], suggesting that miRNAs could offer significant benefits in clinical practice such as diagnostics.

Recently, miR-146a-5p, miR-24-3p, and miR125a-5p were reported as potential biomarkers for RA diagnosis.[123]miR-146a-5p acts as an essential regulator in the differentiation and function of innate and adaptive immune cells. The expression levels of miR-146a have been studied in different samples of rheumatoid arthritis patients, including synovial fluid, articular tissue, and fibroblasts. Increased levels of miR146a have been reported in all these studies, suggesting the miR-146a as a suitable biomarker for RA diagnosis.[123]

miRNA expression also differs depending on the stage of the disease. Lower miR-16 and miR-223 levels have been found in the peripheral blood of patients with early RA compared to healthy individuals and patients with longstanding disease.

Murata et al. showed that higher levels of circulating miR-24 associates with more active disease.[122,124]

Increased levels of miR-16 and miR-146a in PBMCs of RA patients are associated with active disease, whereas low levels of these miRNAs correlate with remission of RA. [122]

Patients with RA associated with interstitial lung disease (ILD) demonstrate increased expression of serum miR-7 and miR-214 compared to RA patients without lung involvement. [122]

The expression levels of miRNAs were also found to be potential predictors of treatment response. For instance, it has been reported that high pre-therapy serum levels of miR-16, miR-125b and miR-223 in patients with RA were associated with better response to therapy with non-biologic DMARDs. Conversely pre-

therapy increased serum levels of miR-886 combined with low levels of miR-22 were highly predictive of response to treatment with adalimumab after twelve months. Likewise, RA patients with elevated serum miR-27a before initiation of ADA plus methotrexate combination were more likely to respond to this therapeutic regime, especially if miR-27a levels were diminished during the first 3 months of the treatment. In contrary, high baseline serum levels of miR-23 and miR-223 were inversely associated with response to anti-TNF agents.[122]

SYSTEMIC LUPUS ERYTHEMATOSUS

Systemic lupus erythematosus (SLE) is a severe autoimmune inflammatory disease with a broad range of clinical manifestations characterized by loss of tolerance to self-antigens, activation of dysregulated autoreactive T cells and B cells, production of autoantibodies and perturbed activity of several cytokines. Approximately 50% of SLE patients develop life-threatening complications, such as nephritis, vasculitis, pulmonary hypertension, interstitial lung disease, and cerebral stroke. [108]

Its pathogenesis is characterized by the induction of abnormal cell death pathways and clearance mechanisms, excessive externalization of modified cellular and nuclear debris, loss of tolerance to various self-antigens, and innate and adaptive immune disorders. The innate immune system might initiate and promote SLE autoimmunity and organ damage.[125]

Current studies suggest that the dysregulation of the innate and adaptive immune responses is likely rooted in the intricate interactions among environmental stimulants, sex hormone imbalance, genetic predisposition, epigenetic regulation, immunological factors, and other undefined factors. These complex interactions result in breach of self-tolerance characterized by uncontrolled activation and expansion of dendritic cells (DCs) and lymphocytes, coupled with the production of copious amounts of auto-antibodies.[108]In 2007, Dai and colleagues found differences in the miRNA expression profiles of SLE patients and healthy controls, as seven miRNAs were down-regulated (miR-196a, miR-L7-5p, miR-409- 3p, miR-

141, miR-383, miR-112, and miR-184) and nine were up-regulated (miR-189, miR-61, miR-78, miR-21, miR-142-3p, miR-342, miR-299-3p, miR-198, and miR-298), suggesting that miRNAs are potential diagnostic markers of SLE and may be important factors related to the pathogenesis of the disease.[108]

Collectively, there is a lack of distinct pattern in specific dysregulated miRNA expression in SLE in literature due to the variations in the ethnicity and stage of disease or treatment of recruited subjects, the sizes of screened populations and the types of biological sample tested as well as the detection methods in different studies. [109]

Overall, although it is difficult to identify a stable, specific and sensitive "miRNA signature" for SLE, it may be possible to pinpoint distinct groups of dysregulated miRNAs according to their shared functional consequences in SLE.[109]

One of the main pathophysiological mechanisms of SLE involves the production of antibodies against self-antigens by autoreactive B cells, such as anti-dsDNA antibodies. Studies of the aberrant expression of several miRNAs in the B cells of SLE patients have reported that miR7, miR-155, miR-30a, and miR-15a are up-regulated, while miR-1246 is down-regulated, compared to healthy donors. Although the mechanism of their induction still remains largely unknown, miR-155 is upregulated in peripheral blood mononuclear cells (PBMCs) and positively correlates with serological levels of anti-dsDNA. In fact, it appears to be involved in the production of anti-dsDNA autoantibodies. miR-155 has emerged as arguably the most important and well studied miRNA during immune response, acting as a master regulator, with both B and T cell intrinsic functions demonstrated to date. It participates in increasing autoantibody production by downregulating the transcription factor Ets-1, essential in the inhibition of plasma cell differentiation and antibody production. Overexpression of miR-155 can repress SOCS1 which increases STA phosphorylation and thus results in increased in IL-21 release with a consequent increase in antibody production. [108,120,126]

Long-lived and hyperactive B cells are the major sources for autoantibodies. Lyn is a key negative regulator of B cell activation, and is significantly downregulated

by mir-30a in SLE patients, leading to spontaneous proliferation and anti-dsDNA autoantibody production.[109]

B cell depletion therapy can have beneficial effects on patients with SLE, further highlighting the importance of B cells in the pathogenesis of autoimmune diseases.[108]

An imbalance between effector T cells (Teffs) such as Tfh and Th17 cells and regulatory T cells (Tregs) is also central to the pathogenesis of SLE. MiR-125a suppresses several factors of Teffs, including STAT3, IFN-g, and IL-13, and it has been reported to be commonly downregulated in the peripheral CD4+ T cells of patients with various autoimmune diseases, including SLE.[108]

Global DNA hypomethylation has been shown to be a consistent finding in CD4+ T cells of SLE patients and certain epigenetic biomarkers like the methylation levels of IFI44L have been proposed as a novel biomarker of SLE.[127]

The abnormal DNA hypomethylation observed in SLE T-cells is at least partially explained by the dysregulation of several miRNAs. For instance, miR-126 and miR-148a directly target DNA methyltransferase 1 (DNMT1), the key methyltransferase for maintaining DNA methylation during replication. [109] miR-21 is upregulated in SLE patients, and it indirectly inhibits DNMT1 expression by targeting the autoimmune gene RAS guanyl nucleotide-releasing protein 1. These data clearly show that abnormally expressed miRNAs in SLE patients have a critical functional link with the aberrant DNA hypomethylation in CD4+ T cells, resulting in the overexpression of autoimmune-associated methylation sensitive genes, such as those that encode CD70 (tumor necrosis factor ligand) superfamily.[108]

Among the up-regulated miRNAs associated with SLE, miR-21 has been identified in T cells where it regulates multiple pathways that lead to overall T and B cell hyperactivity, and it has been reported to have a strong correlation with SLEDAI score. miR-21 also directly suppresses the selective protein translation inhibitor PDCD4 expression, leading to an enhanced proliferation, increased IL-10 and CD40L expression in CD4＋ T cells, and in turn promoting the differentiation of plasma cells and IgG production. A similar regulatory pathway has been observed

in macrophages where miR-21-mediated suppression of PDCD4 favors the production of IL-10 upon TLR-4 stimulation via NF-κB pathway, supporting that miR-21 may promote inflammation through suppression of negative regulatory mediators. [109]

Abnormal chemokine expression appears to be a major component of SLE patho-physiology, as it is closely related to neutrophil extracellular traps (NETs) formation, and are closely related to the levels of inflammation and tissue damage in SLE. It has been demonstrated that miR-4512 is a critical factor in the abnormal transcriptome of PBMCs in SLE patients, and its downregulation promotes up-regulation of CXCL2 and TLR4, which further activates NF-kB-mediated chemokine and inflammatory cytokine expression, which in turn promotes neutrophils recruitment to inflammatory sites and formation of NETs, accompanied by the exposure of a large number of autoantigens able to induce or aggravate autoimmune response. [125]

Recent studies have described the involvement of let-7c, miR-155, miR-150 in regulating the functions of pDC in response to TLRs stimulation. B lymphocyte–induced maturation protein-1 (Blimp1) is identified as an important transcriptional repressor of let-7c miRNA, and its polymorphism has been described as a potential contributor for SLE development risk. In DCs, a loss of function of Blimp1 results in upregulation of let-7 miRNA, that results in a broad spectrum of proinflammatory modifications in DC phenotype, mediated in part through suppression of suppressor of cytokine signaling 1 (SOCS1) expression. [108]

Type I IFN is recognized as a central player in SLE pathogenesis and miRNAs with dysregulated expression in PBMCs appear to target key components of type I IFN signaling cascade.

The under-expression of miR-146a also appears correlated with SLE disease activity and IFN scores. This miRNA has been shown to be upregulated in normal PBMCs after TLR7/9 stimulation, and to function as a negative regulator of type I IFN production, acting in a negative feedback loop to maintain a normal IFN response. Recent genetic investigations identified a variant in miR-146a promoter region that can reduce its expression levels in PBMCs and increase SLE risk.[109]

The serum levels of miR-371b-5p and miR-5100 were significantly increased in SLE patients compared to the healthy population and RA patients. These miRNAs could also distinguish active SLE from inactive SLE disease, suggesting a potential role as biomarkers for SLE.[127]

IDIOPATHIC INFLAMMATORY MYPATHIES

In the past ten years, identification of several differentially expressed miRNAs in muscle biopsy samples from patients with inflammatory myopathies suggested miRNAs as potential molecular pathogenesis actors or prognostic biomarkers for disease development and progression.[128]

miRNAs are critical regulators of both inflammatory cytokines signaling and adult skeletal muscle differentiation and maintenance. For instance, miR-1, miR-133a, miR-133b, and miR-206 are critical regulators of myoblast-to-myocyte differentiation through regulation of multiple genes that influence myogenesis, including myogenic differentiation antigen 1, myogenin, serum response factor, and others. Recently, additional miRNAs have been shown to play a role in muscle differentiation, for example, miR-221/222 inhibit myoblast differentiation, while miR-26a, 378, and 762 drive myoblast differentiation in vitro. [129]

Myoblasts exposed to elevated levels of inflammatory cytokines like TNF and IL-1 downregulate the expression of myogenic miRNAs, like miR-1, miR-133 and miR-206, necessary to inhibit factors like histone deacetylase 4, serum response factor, neural polypyrimidine tract binding protein, which inhibit myoblast differentiation and muscle regeneration. In fact, comparison of muscle samples from IIM patients and healthy donors revealed an increased mRNA expression of inflammatory cytokines, like TNF, IFN and IL-1, in different subsets of IIM, accompanied by a decreased expression of the myogenic miR-1, miR-133s/b and miR-206.[129]

Specifically relevant to myoblast differentiation, miR-146a, miR-221, and miR-222 which inhibit myocyte differentiation were found to be overexpressed in IBM and PM samples compared to healthy donors. Furthermore, miR-378, a driver of

myoblast differentiation in growth medium, was found to be down-regulated in IIM muscle samples.[129]

The role of inflammatory signaling in IIMs has been studied extensively. Several inflammatory cytokines are significantly overexpressed in muscle biopsy samples from inflammatory myopathy patients, including IL-1 in IBM and IL-1 and IFN in PM. Like TNF, IL-1 activates NFkB and may suppress myogenic miRNAs following a similar mechanism to TNF. Conversely, IFNs signal through JAK/STAT, MAPK, p38 and other key pathways regulating myoblast-to-myocyte differentiation. Thus, targeting TNF alone, or even NFkB, may not provide clinical benefit for all IIM patients. These clinical observations suggest that only a subset of patients are characterized by a form of inflammatory myopathy driven by TNF alone and that a balance between TNF, IFNs, and other cytokines may contribute to overall disease pathophysiology and partially explain subtype-specific responses to various treatments. [129]

The down-regulated expression of miR-206 in IIM patients has been confirmed in both PBMCs and blood samples. It targets KLF4, a key positive regulator of Th17 cells differentiation. Indeed, Th17 cells were augmented in the peripheral blood of patients with DM compared to healthy controls, and a positive correlation between the percentages of Th17 cells and serum level of CPK has been reported supporting a role of Th17 cells in the pathogenesis of DM.[130]

Macrophage infiltration is a key feature in IIMs pathology and appears to be closely linked to TH17 signaling. In the context of IIM unresolved inflammatory infiltration facilitated by macrophages may lead to persistent muscle tissue destruction by immune cells or collagen deposition.[131]

Evidence suggests that miR-146a-5p plays an important role in regulating inflammatory response and macrophage migration in IIMs, and its expression has been shown to be significantly reduced in macrophages from IIM patients compared to healthy population. miR-146a-5p is induced by inflammatory responses and leads to the downregulation of its target genes regenerating islet-derived protein 3-alpha (REG3A), IL-1 receptor-associated kinase (IRAK) 1, and tumor necrosis factor receptor-associated factor 6 (TRAF6), thus serving as

negative feedback for immune activation. A study by Jiang et al. showed that IL-17A and IFN-γ are highly expressed in PBMCs from PM/DM patients with higher mRNA expression of REG3A and lower miR-146a-5p expression. This evidence supports the role of miR-146a-5p downregulation in the pathogenesis of IIMs, through macrophage migration and facilitation of NFkB signaling with consequent increase in inflammatory cytokines production. [128]

Other miRNAs have been investigated in their role of macrophage migration regulators. miR-409-3p has been widely reported in several diseases with a function in regulating cell migration and its expression is significantly reduced in the sera of PM patients. It acts through targeting and downregulating the chemokine receptor CXCR4. CXCR4 is a 7-transmembrane G-protein coupled receptor highly expressed in various cells, including lymphocytes, monocytes, macrophages, and neutrophils, and it plays a crucial role in biological processes through binding to its ligand CXCL12. Studies have shown that CXCR4 is upregulated in the muscles of DM and IIMs, and the CXCL12/CXCR4 signaling pathway acts in macrophage migration during PM/DM progression. Thus miR-409-3p downregulation appears of central relevance in IIM pathogenesis. [132]

Yutao et al. reported a downregulated expression of miR-381 in PM patients compared to healthy controls. Furthermore, it appeared to increase after treatment with glucocorticoid and traditional immunosuppressants, exhibiting an inverse trend with IL-17 and High-mobility group box 1 (HMGB1), which is considered to be one of its targets. HMGB1 controls the stability of nucleosomes, DNA recombination, replication, repair, and transcription in nucleus, it induces cytokine production in the extracellular environment, and is involved in the pathological processes of sepsis, cancer and arthritis. HMGB1 act critically in natural and acquired immunity, and it has been reported to be closely associated with the development of PM where it is significantly higher in patients compared with healthy controls. Furthermore, a survival analysis revealed that high HMGB1 levels in PM patients were inversely correlated to survival time. These data clearly indicate that miR-381 downregulation and its link with HMGB1 expression plays a critical role in the inflammatory response of PM. [133]

miRNA dysregulation has also revealed to be crucial in non-immune related mechanisms of IIM pathogenesis. For instance, Parkes et al. demonstrated that miR-96-5p is significantly upregulated in DM patients. miR-96-5p has mainly been studied as an oncogenic miRNA dysregulated in a variety of cancer types including gastric, breast, ovarian and colorectal cancer. However, it is able to bind and inhibit an enzyme that converts adenosine to adenosine monophosphate/diphosphate, the Adenosine Kinase (ADK) mRNA in skeletal muscle, thus contributing to mitochondrial dysfunction, an established non-immune related mechanism in IIM progression that results in modified reactive oxygen species production which can contribute to weakness in IIM. [134]

Other miRNAs, whose functions have yet to be clearly established in IIM pathogenesis, were found to be dysregulated in IIM. For instance, serum miR-23b-3p levels inversely correlate with CPK, but its role in the skeletal muscle damage in DM is still unclear. It has been reported that miR-23b-3p prevents multiple autoimmune diseases through the regulation of inflammatory cytokine pathways, such as NF-κB, TNF-α, IL-1β, and IL-17. This data suggests that the downregulation of miR-23b-3p may participate in muscle damage through targeting immune cells and inflammatory mediators.[128]

In addition to their role in relevant pathogenetic mechanisms, miRNAs expression profiles have also been studied as potential biomarkers associated with different subsets of IIM, supporting differential diagnosis and clinical course prediction. Investigating the relations between immune-related miRNAs and different phenotypes in DM patients, a decreased expression of serum miR-150-5p was found to specifically correlate with anti-MDA5 and anti-NXP2 autoantibodies in DM patients, while an up-regulated expression level of serum miR-146b-5p was reported in cancer associated DM (CADM) patients than non-CADM patients. Previous studies reported that expression of miR-146b-5p significantly increased in solid tumors. miR-146b-5p could downregulate the expression of its target genes to mediate the proliferation, invasion, and migration of cancer cells. This evidence indicates that miR-146b-5p may be to some extent a useful biomarker for cancer associated with DM. Moreover, serum levels of miR-146b-5p have also been found to inversely correlate with lung involvement in DM. In fact, miR-146b-

5p in DM patients with ILD were lower than those in patients without ILD.[128] Moreover, miR-7 is down-regulated in serum from IIM patients compared to healthy controls and appear to be even further reduced if ILD is present. [134]

Investigations on plasma and serum miRNA expression, conducted by Hirai et al., identified several miRNAs differentially expressed in PM compared to DM, as well as miRNAs whose levels were affected by treatment. Among these, miR-4442 was significantly higher in IIMs compared to RA or SLE and displayed a remarkable decrease after treatment with glucocorticoids in association with calcineurin inhibitors or cyclophosphamide pulse therapy. In addition, miR-3676, miR-3907, and miR-877* also showed significant association with disease activity. Serum miR-223 expression was reported to be decreased in PM/DM patients, particularly in CADM patients, compared with healthy controls, and it's expression also seems to increase after treatment.[135]

Zhong et al. conducted a study aimed at identifying differential expression profiles in circulating EV-miRNAs in specific subsets of DM patients. They revealed that circulating EV-miRNA profiles had a distinct pattern between serologically and clinically defined DM subsets and HCs. For instance, miR-4488 and miR-1228-5p were significantly upregulated in MDA5+ DM associated with ILD compared to other subtypes of DM and HC. Further analysis revealed that miR-4488 and miR-1228-5p may have crucial roles in the pathogenesis of ILD in MDA5+ patients through 112 and 60 candidate target genes, respectively. DDX39B was identified as one possible target genes of has-miR-4488, interacting with the pattern recognition receptor pathway to inhibit NF-κB signaling. This suggests that miR-4488 may contribute to systemic inflammation in DM-associated ILD by upregulating NF-κB signaling through repression of DDX39B. [136]

miRNAs AS A DIAGNOSTIC TOOL

An ideal biomarker should be measurable in a reproducible way, have high sensitivity and specificity for the clinical outcome of interest, and should reflect an important pathogenetic process. MiRNAs are exciting as potential biomarkers because they fulfill many of these criteria. [119]The extensive alterations in miRNA

expression in disease provide great potential for clinical diagnostics based on miRNA signatures. [106]

Indeed, recent studies have suggested that circulating miRNAs can be suitable for clinical use as diagnostic markers as they are stable in extracellular conditions in body fluids such as plasma and serum and protected from endogenous RNase activity. The circulating miRNAs are stable at room temperature for up to 24 hours and resistant to freezing and thawing from -80°C to room standard temperature.[123] Furthermore, miRNAs can be recovered from formalin fixed paraffin sections and other sources with low overall RNA quality. Current focus is on developing miRNA signatures for disease diagnosis, identifying cancers of unknown primary, and predicting response to therapy and drug resistance.[106]

Since many biological fluids can be easily obtained without invasive procedures, they offer tremendous potential as a source material for clinical diagnostics. However, while numerous studies have reported extracellular miRNA signatures for diseases it remains to be determined whether these signatures have acceptable accuracy and sensitivity for the clinic.[106]

For example, urinary miRNAs have been widely exploited for the diagnosis of urological cancer. Bryant et al. showed that miR-107 and 574-3p were significantly higher in the urine of prostatic cancer patients compared to healthy groups, and would perform even better than PCA3 normalized to PSA in identifying the presence of cancer. Other recent studies reported that urine miR-205 and miR-214 are significantly down-regulated in prostatic cancer patients with 80% specificity and 89% sensitivity.[117]

miRNAs are emerging as a potentially useful and accurate tool for molecular classification of breast cancer, a highly heterogenous and frequent disease. The original molecular classification of breast cancer subtypes is based on investigations on frozen tissue and is not applicable in conventional formalin fixed paraffin sections, limiting its degree of practicality in clinical practice. Recently several studies reported that dysregulated miRNAs expression was strongly associated to molecular breast cancer subtype, potentially aiding pathological evaluation. Furthermore, a combination of differentially expressed miRNAs in

plasma was reported to significantly and reliably identify some subtypes of breast cancer. [117]

An accurate determination of the tumor type significantly influences treatment decisions in patients with primary lung cancer. However, techniques and methods for lung cancer typing lack standardization. In particular, owing to limited tumor sample amounts and the poor quality of some samples, the classification of primary lung cancers using small preoperative biopsy specimens presents a diagnostic challenge using current tools. Gilad et al. were able to develop a miRNA-based assay to accurately differentiate between the four main lung cancer types from cytologic preoperative samples with performance comparable to histologic evaluation.[137]

Even though miRNAs appear particularly promising in oncology applications, their potential utility is becoming more clear in other relevant domains. For instance, Takotsubo cardiomyopathy is a type of non-ischemic cardiomyopathy and a major life-threatening condition. To date, there are no early biomarker for its diagnosis and its distinction from acute myocardial infarction (AMI). Jaguszewski et al. reported that a unique signature comprising miR-1, miR-16, miR-26a, and miR-133a is capable of distinguishing Takotsubo cardiomyopathy from healthy individuals (sensitivity 74.19%, specificity78.57%) and from AMI patients (sensitivity 96%, specificity70.37%). [117]

Several lines of evidence have highlighted a role for miRNAs in infectious disease with a high epidemiological relevance, such as HIV. It has been demonstrated that different miRNA profiles could correlate with parameters of HIV infection and progression, like viremia, levels of integration of HIV genetic material into host DNA, probability of rapid decrease of CD4 counts and response to therapy, among the others. Thus, researchers speculate that miRNAs could potentially serve as more sensitive, accurate and cost-efficient biomarkers for HIV-1 diagnosis and disease progression than those detected by currently available standard clinical assays, providing relevant information for optimizing treatment regimens. [138]

As mentioned, several lines of evidence suggest that specific miRNA footprints could could be implemented as useful novel biomarkers aiding the clinic in

diagnosis and management of autoimmune disease and a great effort is placed on the development of this research field, with numerous studies currently investigating this possibility.

AIM OF THE STUDY

This study aims to quantify and characterize the circulating extracellular vesicles (EVs) in IIM and investigate their cargo of miRNAs (EV-miRNA) to evaluate their potential role as biomarkers of IIM. More specifically,

- EVs were quantified via nanoparticle tracking analysis (NTA) to determine differences between IIM patients and healthy donors (HD) and evaluate correlations with clinical features or laboratory data among subsets of disease.
- the EV-miRNA was characterized via Next Generation Sequencing (NGS) to evaluate differential expression of miRNAs between patients and HD and among subsets of disease.

PATIENTS

Adult patients (age ≥18 years) with a diagnosis of IIM and age- and sex-matched healthy donors were enrolled at the Rheumatology Unit of Padua University Hospital between January 2020 and June 2023. Patients' eligibility criteria included age over 18 years and a documented diagnosis of any type of IIM, established by an expert Rheumatologist on the basis of clinical, laboratory and, if available, histological data. Patients with other serious health conditions requiring immunosuppressive therapy or physical rehabilitation treatment were excluded. The study obtained approval by the Hospital's Bioethics Committee (protocol number 0042610) and was conducted in conformity with the Helsinki standard guidelines. Patients and healthy donors signed informed consent. Based on scientific literature, 55 key clinical and laboratory variables (Tables Ia, Ib and Ic) were reviewed and selected for each patient cross-sectionally and parallel retrospective data was used for reference only.

Table Ia Clinical variables studied in IIM patients

CLINICAL FEAUTURES OF IIM PATIENTS

1	IIM subgroup (n.)
2	Disease duration (years) (n.)
3	Raynaud's Phenomenon
4	Gottron’s Sign Over Elbows/Knees
5	Gottron’s Sign Over Hands
6	Heliotrope Rash
7	Shawl Rash
8	Holster sign
9	Poikilodermatomyositis
10	Nailfold changes
11	Gottron's Papules
12	Midfacial Erythema 13
13	V-Neck Sign
14	Dysphagia
15	Dyspnea
16	Cough
17	Calcinosis
18	Mechanic's Hands
19	Myositis
20	ILD
21	Arthritis
22	HRCT pattern

23	Malignancy diagnosis
24	Acute myositis
25	Cutaneous activity
26	Articular activity
27	Muscular activity
28	Pulmonary activity
29	MMT-8 (/150)
30	Type of immunosuppressor agent (n.)
31	Prednisone equivalent dose (mg/d)

Table Ib Laboratory data studied in IIM patients

	LABORATORY VALUES ASSESSED
1	CPK
2	Aldolase
3	Myoglobin
4	LDH
5	AST
6	ALT

Table Ic, Serologic markers studied IIM patients.

SEROLOGIC MARKERS

	MYOSITIS SPECIFIC ANTIBODIES
1	Anti-Mi2 antibodies
2	Anti-SRP antibodies
3	Anti-HMGCR antibodies
4	Anti-MDA5 antibodies
5	Anti-Tif1gamma antibodies
	Anti-tRNA synthetase Ab
6	Anti-JO1 antibodies
7	Anti-PL12 antibodies
8	Anti-PL7 antibodies
9	Anti-EJ antibodies
10	Anti-OJ antibodies
	MYOSITIS-ASSOCIATED ANTIBODIES
11	Anti-SSA antibodies
12	Anti-SSB antibodies
13	Anti-Ku antibodies
14	Anti-PM/Scl-100 antibodies
15	Anti-PM antibodies
16	Anti-U1RNP antibodies
17	Anti-PM/Scl75 antibodies
18	Anti-Scl70 antibodies

MATERIALS AND METHODS

BLOOD SAMPLING AND PREPARATION

Sampling A venous blood sample of 6 ml was obtained from each recruited subject in a sodium-citrate tube and stored at +4˚C to be processed within 1 hour.

Processing Whole blood sample tubes were centrifuged at 1500g for 20 minutes to separate plasma and corpuscular matter. The supernatant was then collected into 15ml tubes to be centrifuged twice, at 3000g for 15 minutes, to yield platelet-free plasma (PFP). The PFP was then transferred into 1,5ml tubes and stored at -80˚C.

EVs ISOLATION AND ENRICHMENT

Size exclusion chromatography PFP samples were thawed and subjected to Size Exclusion Chromatography (SEC) to isolate EVs. SEC was performed using qEV original®/70 nm smart columns (Izon Science), which employ an internal sepharose matrix to separate particles with size between 70 nm and 1000nm. Breifly, SEC columns were initially washed with Phosphate Buffered Saline (PBS) (pH 7.4; ThermoFisher Scientific) filtered through 0.22 µm filters unit (Millex – GP; Merck Millipore) (fPBS). Then, 500 µL of PFP were added to run through the column and the eluate was collected in 25 fractions of 0.5 mL each. Fractions 1-6 were the void volume, which was disposed of, Fr. 7-10 containing the vesicular fraction were collected for further processing, and Fr. 11-25 containing the protein fraction were eliminated.

Ultrafiltration The vesicular fractions (2 mL) collected by SEC were enriched through ultrafiltration (UF) employing the Amicon® Ultra-4 mL 100 kDa centrifugal filter unit (Merck Millipore). Each filter was sterilized before use through centrifugation at 2800g for 1 minute with 1ml of 70% ethanol, and ethanol residue was later removed by centrifugation at 2800g for 2 minutes with 2ml of fPBS. The EVs fraction (2 mL + 1 mL of fPBS) was transferred on the filter unit and centrifuged

at 4000g for 10 minutes according to the manifacturer's instructions. The purified EVs retained on the filter were collected and adjusted to a total volume of 0.5 mL by adding fPBS, aliquoted in microtubes and stored at -80˚C.

EVs QUANTIFICATION AND CHARACTERIZATION

Nanoparticle tracking analysis EVs concentration and size were measured through nanoparticles tracking analysis (NTA) using the NanoSight® NS300 instrument (Malvern Panalytical). EVs samples were diluted in fPBS to the concentration range of 10^6 – 10^8 particles/mL for optimal measurement as specified by the manufacturer. The detection threshold was set to include particles with a restricted concentration of 20 - 120 particles per frame, while indistinct particles were limited to 5 per frame. Camera level was increased until every particle was distinctly visible, without exceeding a saturation of particle signal over 20% (level 11-12). Autofocus was adjusted to avoid most indistinct particles. For each sample, particles in Brownian motion were recorded in 3 separate 60 seconds videos with 20x magnification. Images were captured at a temperature of 25˚C, with a syringe speed of 40 µL/s, while the 45mW laser was set at 488 nm. EVs concentration and size were calculated by NTA software (3.4 version) through the dilution factor and hydrodynamic diameter using the Einstein-Smoluchowski equation. To reduce the distortion from single larger size particles, total complete tracks/total valid tracks ratio was always ≤ 1:5.

RNA EXTRACTION AND MICRO-RNA ANALYISIS

Total RNA extraction and miRNAs quantification Total RNA was extracted from the isolated EVs samples (350 µL) using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Germany), following manufacturer's instructions. The eluate collected from the RNeasy® UCP MinElute spin columns containing the total RNA was stored at -80 °C. The concentration of extracted miRNAs (ng/µL) was quantified by fluorometric spectroscopy through the Qubit® 3.0 Fluorimeter (Thermo Fisher Scientific, Massachusetts, USA) instrument using Qubit microRNA assay kit (Thermo Fisher Scientific) according to the manufacturer's manual.

SMALL NON-CODING RNA NGS LIBRARIES PREPARATION AND QUANTIFICATION

Libraries Preparation Sequencing libraries of small non-coding (snc)RNAs were generated using QIAseq miRNA Library kit (Qiagen, Germany) according to the manufacturer's manual. The adapters ligation step provides that a pre-adenylated DNA adapter is ligated to the 3' end of sncRNAs by incubating for 1 hour at 28°C, 20 min at 65°C, and finally at 4 °C the sample and the reaction mix containing the Ligation Activator buffer with enzymes.

The resulting product then proceeds for 5' adapter ligation in a similar manner. The 5' adapter is added with specific reagents to the product of the first reaction and the solution incubated for 30 minutes at 28˚C, 20 minutes at 65˚C, and 5 minutes at 4˚C.

Libraries reverse transcription and cDNA clean-up RNA molecules conjugated to the adapters undergo reverse transcription (RT) to obtain complementary DNA (cDNA). The primer for reverse transcription binds at the 3' end of the adaptor and it is combined with the short sequence UMI(Unique Molecular Index). During this step, each UMI is assigned to every sncRNA molecule to ensure accurate quantification of sncRNAs through NGS adding molecular tags to each molecule to eliminate the bias due to the number of reads that could result in an overestimation of expression. Moreover, a universal sequence is added to be recognized by the sample indexing primers during library amplification. During the bioinformatic analysis, the reads will be filtered to include only the sequences with the UMI. In the first RT reaction, the mix containing RT Initiator was added to the sample and incubated for 2 min at 75 °C, 2 min at 70 °C, 2 min at 65 °C, 2 min at 60 °C, 2 min at 55 °C, 5 min at 37 °C, 5 min at 25 °C, and then at 4 °C. The second step provides a reaction by incubating the sample with RT primer and enzyme for 1 hour at 50 °C, 15 min at 70 °C, and 5 min at 4 °C.

After the RT step, a cDNA clean-up was performed using a magnetic bead-based method, in order to enrich the samples in cDNA fragments with the adapters and remove the exceeded reagents.

Libraries amplification and clean-up The library amplification occurs through the use of a universal reverse primer and a specific forward primer containing a barcode index dried into a microplate to assign each sample a unique custom index. During the sequencing step, the barcode is recognized to collect the sequences derived from the same sample. The library amplification needs the primers and HotStartTaq DNA polymerase which was activated by incubation for 15 min at 95 °C. Consequently, 22 cycles of denaturation (at 95˚C for 15 seconds), annealing (at 60˚C for 30 seconds) and extension (72˚C for 15 seconds) were performed, followed by incubation for 2 min at 72 °C and 5 min at 4 °C.

After the amplification step, a sncRNA library magnetic bead-based method clean-up was performed to enrich the library with specific size RNA and to remove contaminants, adapter dimers, and inhibitors. Libraries were stored at -20°C.

Library DNA concentration assessment The sncRNAs libraries concentration(ng/µL) was quantified by fluorometric spectroscopy through Qubit® 3.0 Fluorimeter (Thermo Fisher Scientific) instrument using the Qubit 1X dsDNA HS kit (ThermoFisher Scientific) according to the manufacturer's manual.

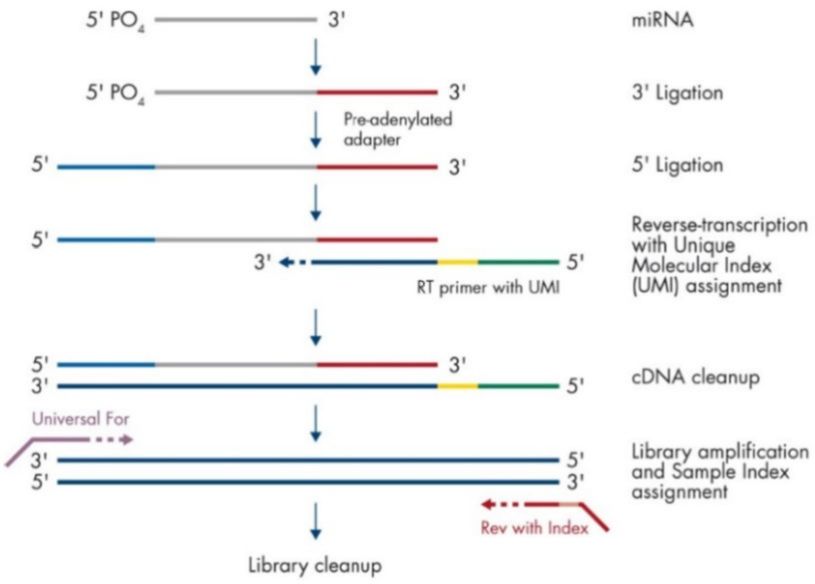

Figure 11 Libraries preparation, UMI assignment, cDNA clean-up, library amplification.

NEXT GENERATION SEQUENCING ON sncRNA LIBRARIES

Next Generation Sequencing (NGS) of the sncRNA libraries employed the NextSeq550 (Illumina) instrument and platform. Libraries to sequence in multiplexing were generated in equimolar amounts diluting each sample in RNAase-free water to reach the final pool concentration of 1.2 pM as recommended. The correct libraries concentration optimizes cluster density to avoid the phenomenon of "over-clustering" or "under-clustering" which would not discriminate the brightness of the reads. Then, libraries pool concentration was quantified through Qubit® 3.0 Fluorimeter (Thermo Fisher Scientific) instrument as previously described. After adequate washing and calibration of the system to set the number of cycles required for reading a sequence of 75 bp to make use of the UMIs, the identification indexes of every library were input, and the pool was loaded.

Briefly, a pool of NGS library fragments was denatured following the Illumina manufacturers' protocol and diluted as previously described. Fragments were flowed across a flow cell (NextSeqTM High Output Flow Cell v2.5, Illumina) and hybridized on complementary adapter oligos. Fragments were amplified via bridge amplification PCR, denaturated, and linearized by cleavage within an adaptor sequence, resulting in clusters of single-stranded templates for sequencing. During each sequencing cycle, a labelled deoxynucleoside triphosphate (dNTP) is added to the nucleic acid chain and it acts as a terminator for polymerization. The fluorescent dye is useful to identify the base and then enzymatically cleaved to allow incorporation of the following nucleotide. Base pairs are identified after laser excitation and fluorescence detection. Finally, the bioinformatic analysis was performed after the upload of the data of fragments sequences from each sample as FastQ files using the tool CLC Genomics Workbench software from NGS platforms.

ACLC Genomics Workbench normalizes and filters data, provides quality control, alignment, quantification, statistics to assess the Fold Change and determine miRNAs differential expression, and visualization tools.

STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism® software (version 9), applying Student's T test to compare the differences between parametric variables and one-way ANOVA with Bonferroni's correction when more than two groups were considered. Pearson's correlation was used to evaluate strength and direction of the correlation. Data are expressed as mean ± standard deviation (SD) for continuous variables and median with interquartile ranges were used for continuous non-normal distributions; categorical data are expressed as numbers and percentages. A two-tailed p-value (p) ≤ 0,05 was considered statistically significant.

RESULTS

Patients and HDs cohort

Sixty-four adult patients with a documented diagnosis of IIM (female:male ratio 2:1, mean age 60.81 ± 12.74 SD) and 65 healthy donors (HD; female:male ratio 2:1, mean age 49.94 ± 18.56) with muscle enzymes values within normality range were included in this study to undergo EVs isolation, quantification, and characterization. The IIM subtype distribution of patients and their clinical and demographic data are reported in table II. The EVs samples of 47 patients (female:male ratio 2:1, mean age 59.62 ± 13.55 SD) and 49 HD (female:male ratio 2:1, mean age 54.65 ± 16.86) following EV isolation and characterization, underwent total RNA extraction and micro-RNA analysis. The clinical and demographic data of IIM patients are shown in table III.

Table II ***Clinical and demographic features of IIM patients undergoing EVs isolation and quantification.***

Clinical and demographic features	**Values**
Patients (n)	64
Females (n, %)	42 (65.62)
Disease duration at sampling time (years, mean ± SD)	3.01±2.39
MMT-8 score median (IQR)	144 (134-150)
Serology (n, %)	
Myositis-specific autoantibodies (MSAs)	
Anti-Mi2	7 (10.94)
Anti-t-RNA synthetase *	17 (26.55)
Anti-SRP	3 (4.69)
Anti-MDA-5	5 (7.80)
Anti-TIF1-γ	5 (7.80)
Anti-HMGCoAR	1 (1.55)

Myositis-associated autoantibodies (MAAs)	
Anti-SSA	14 (21.87)
Anti-SSB	3 (4.69)
Anti-Ku	3 (4.69)
Anti-PM/Scl-100	5 (7.80)
Other **	8 (12.5)
Unknown	1 (1.55)
IIM subsets (n, %)	
Dermatomyositis	19 (29.69)
Polymyositis	10 (15.62)
Inclusion body myositis	2 (3.13)
Anti-synthetase syndrome	17 (26.55)
Cancer-associated myositis***	13 (20.30)
Unspecified****	3 (4.69)
Active clinical manifestations upon sampling (n, %)	
Cutaneous	28 (43.75)
Gottron's sign and papules	11 (17.19)
Heliotropic rash	6 (9.35)
Other *****	34 (53.12)
Arthritis	6 (9.35)
Myositis	15 (23.44)
ILD	7 (10.94)
Clinical remission	30 (46.87)
Myositis-related laboratory (U/L, mean ± SD)	
CPK	780.70 ± 1891.40
Aldolase	12.91 ± 22.70
GOT	54.55 ± 79.13
LDH	263.90 ± 92.95

Ongoing treatment	
Oral glucocorticoids (n, %)	44 (68.75)
Dose of prednisone (mg/day, mean ± SD)	13.57 ± 16.33
Pulse steroid	4 (6.25)
Immunosuppressant drugs (n, %)	42 (65.62)
Mycophenolate mofetil	15 (23.44)
Methotrexate	19 (29.69)
Azathioprine	1 (1.55)
Cyclosporine A/ Tacrolimus	2 (3.12)
Rituximab	4 (6.25)
Abatacept	1 (1.55)
Untreated	5 (7.80)

*Table II MMT, manual muscle test; IQR, interquartile range ILD, interstitial lung disease; *Anti-t-RNA synthetase: 11 cases of antiJO-1 positivity, 4 cases of antiPL-12 positivity, 1 case of antiPL-7 positivity, 1 case antiEJ-1 positivity. **Other : 1 case of antiPM1 positivity; 2 cases of antiU1RNP positivity, 3 case of anti PM/Scl75 positivity, 1 case of antiScl70 positivity and 1 case of anti-SMA positivity. ***CAM: 1 case of high-grade serous ovarian carcinoma, 1 case of cholangiocarcinoma, 3 cases of colorectal adenocarcinoma, 2 cases of IPMN, 1 case of low-grade colon adenoma, 1 case of small cell lung cancer, 1 case of lung adenocarcinoma, 1 case of squamous-cell skin cancer, 1 case of intestinal type adenocarcinoma of the nasal fossa, 1 case of extra-nodal non Hodgkin lymphoma, 1 case of urothelial carcinoma. ****Other: 3 patients with a diagnosis of IIM but without a specific subtype classification due to lack of histologic data at sampling time. *****Other : 11 cases with nailfold changes, 4 cases with holster sign, 1 case with poikilodermatomyositis, 2 cases with mid-facial erythema, 2 cases with V-neck sign , 14 cases with shawl sign, 1 case with livedo reticularis, 1 case with diffuse erythema, 1 case with purpura lower limbs.*

Table III ***Clinical and demographic features of IIM patients undergoing EVs isolation, quantification, and evaluation of miRNAs cargo by NGS analysis.***

Clinical and demographic features	**Values**
Patients (n)	47
Females (n, %)	30 (63.83)
Caucasians (n, %)	47 (100)
Disease duration at sampling time (years, mean ± SD)	4.70 ± 4.98
MMT-8 score median (IQR)	144 (100 – 150)
Serology (n, %) Myositis-specific autoantibodies (MSAs)	

Anti-Mi2	6 (12.76)
Anti-t-RNA synthetase *	15 (31.91)
Anti-SRP	3 (6.37)
Anti-MDA-5	4 (8.51)
Anti-TIF1-γ	4 (8.51)
Anti-HMGCoAR	1 (2.13)
Myositis-associated autoantibodies (MAAs)	
Anti-SSA	10 (21.28)
Anti-SSB	2 (4.25)
Anti-Ku	1 (2.13)
Anti-PM/Scl-100	3 (6.37)
Other **	4 (8.51)
Unknown	1 (2.13)
IIM subsets (n, %)	
Dermatomyositis	14 (29.79)
Polymyositis	6 (12.76)
Inclusion body myositis	1 (2.13)
Anti-synthetase syndrome	15 (31.91)
Cancer-associated myositis***	11 (23.39)
Unspecified	/
Active clinical manifestations upon sampling (n, %)	
Cutaneous	22 (46.81)
Gottron's sign and papules	10 (21.28)
Heliotropic rash	6 (12.76)
Other ****	23 (48.94)
Arthritis	5 (10.64)
Myositis	15 (31.90)
ILD	7 (14.88)
Clinical remission	40 (85.11)
Myositis-related laboratory (U/L, mean ± SD)	
CPK	869.52 ± 2186.46

Aldolase	12.93 ± 25.38
GOT	59.26 ± 90.36
LDH	267.13 ± 98.66
Ongoing treatment	
Oral glucocorticoids (n, %)	33 (70.20)
Dose of prednisone (mg/day, mean ± SD)	14.94 ± 17.28
Immunosuppressant drugs (n, %)	34 (72.34)
Mycophenolate mofetil	12 (25.52)
Methotrexate	12 (25.52)
Azathioprine	1 (2.13)
Cyclosporine A/ Tacrolimus	2 (4.25)
Rituximab	3 (6.37)
Abatacept	1 (2.13)
Untreated	3 (6.37)

*Table III MMT, manual muscle test; IQR, interquartile range ILD, interstitial lung disease. *Anti-t-RNA synthetase: 9 cases of antiJO-1 positivity, 3 cases of antiPL-12 positivity, 2 case of antiPL-7 positivity, 2 case antiEJ-1 positivity.**Other : 1 cases of antiU1RNP positivity, 3 case of anti PM/Scl75 positivity.***CAM: 1 case of high-grade serous ovarian carcinoma, 1 case of cholangiocarcinoma, 3 cases of colorectal adenocarcinoma, 1 cases of IPMN, 1 case of small cell lung cancer, 1 case of squamous-cell skin cancer, 1 case of intestinal type adenocarcinoma of the nasal fossa, 1 case of extra-nodal non Hodgkin lymphoma, 1 case of multiple myeloma. ****Other : 8 cases with nailfold changes, 3 cases with holster sign, 1 case with poikilodermatomyositis, 2 cases with mid-facial erythema, 3 cases with V-neck sign , 1 case with livedo reticularis, 13 cases with shawl sign*

Circulating EVs quantification

Circulating EVs in IIM patients and HD

NTA measurements of EVs concentration and size showed a significantly higher mean concentration of circulating EVs in IIM patients (n=64) than in HD (n=65) (mean ± SD: $1.73x10^{10}$ ± $1.30x10^{10}$ [EVs/mL] vs. $1.31x10^{10}$ ± $7.17x10^{9}$, p=0.0073) (figure 12) with EVs mean size of 198.7 ± 20.05 (nm ± SD) vs. 204.1 ± 17.78 and mode size of 151.8 ± 19.92 vs. 154.8 ± 16.89, respectively.

Among IIM, patients seropositive to any antibodies (n=55) (MSA, MAA or other) had significantly higher EVs concentration compared to HD ($1.80x10^{10}$ ± $1.38x10^{10}$ vs. $1.31x10^{10}$ ± $7.17x10^{9}$, p=0.0363) (figure12). Presence of myositis-specific antibodies (MSAs) ($1.78x10^{10}$ ± $1.51x10^{10}$) was associated with numerically higher

EV concentrations vs. HD (p=0.0639). However, significant associations between specific antibody types and EVs concentration were not found.

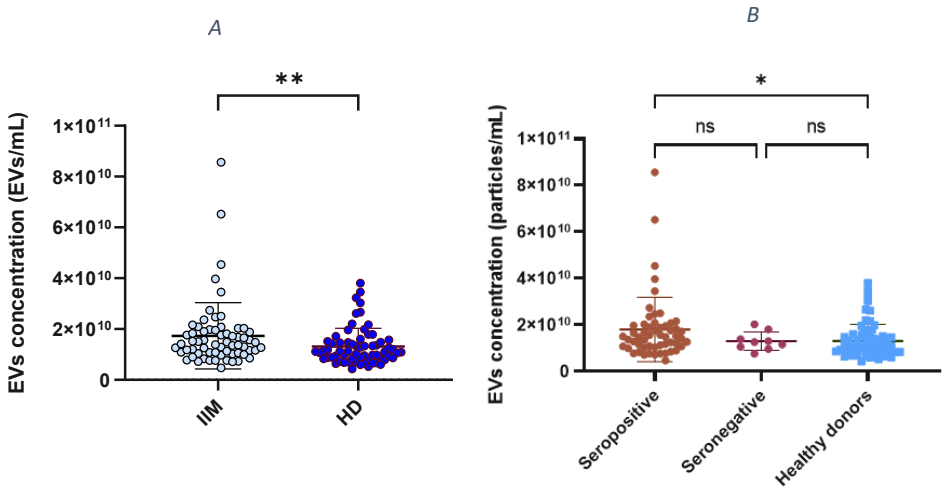

Figure 12 Graphs representing the EVs mean concentration [EVs/mL] ± SD in A) IIM patients (n=64) vs. HD (n=65). B) seropositive patients for autoantibodies (n=55) vs. seronegative (n=9) vs. HD

Circulating EVs in IIM subtypes

Patients affected with cancer-associated myositis (CAM; n=13) displayed highest levels of circulating EVs ($2.52x10^{10}$±$2.4x10^{10}$) compared to DM (n=19) and ASyS+PM+IBM (n=29) (DM, $1.62x10^{10}$±$9.87x10^{9}$; ASyS+PM+IBM, $1.44x10^{10}$ ± $5.53x10^{9}$) with a significant difference compared to ASyS+PM+IBM (p=0.0432)(figure 13A). CAM patients reported significantly higher levels of circulating EVs compared to non-CAM patients (n=51) ($2.52x10^{10}$ ± $2.40x10^{10}$ vs. $1.53x10^{10}$ ± $7.35x10^{9}$, p=0.0060) (figure 13B). We also found that CAM patients showed increased EVs concentration than HD (p=0.0004). Patients in clinical remission (n=30) displayed higher levels of circulating EVs compared to those with active disease (n=34) ($2.05x10^{10}$ ± $1.51x10^{10}$ vs. $1.44x10^{10}$ ± $1.02x10^{10}$, p=0.0087) (figure 13C). In patients receiving pharmacological therapy (n=57), those treated with glucocorticoids associated with immunosuppressants (n=36) appeared to have EVs concentrations lower than those of patients receiving glucocorticoids alone, but this difference did not reach statistical significance ($1.49x10^{10}$ ±

$7.25x10^9$ vs. $2.23x10^{10} \pm 1.99x10^{10}$, p=0.2245). Conversely, significantly reduced levels of circulating EVS were found in patients treated with rituximab (RTX) (n=12) compared to patients receiving any other immunosuppressant (n=45)($9.63x10^9 \pm 3.26x10^9$ vs. $1.96x10^{10} \pm 1.46x10^{10}$, p<0.0001)(figure 13D).

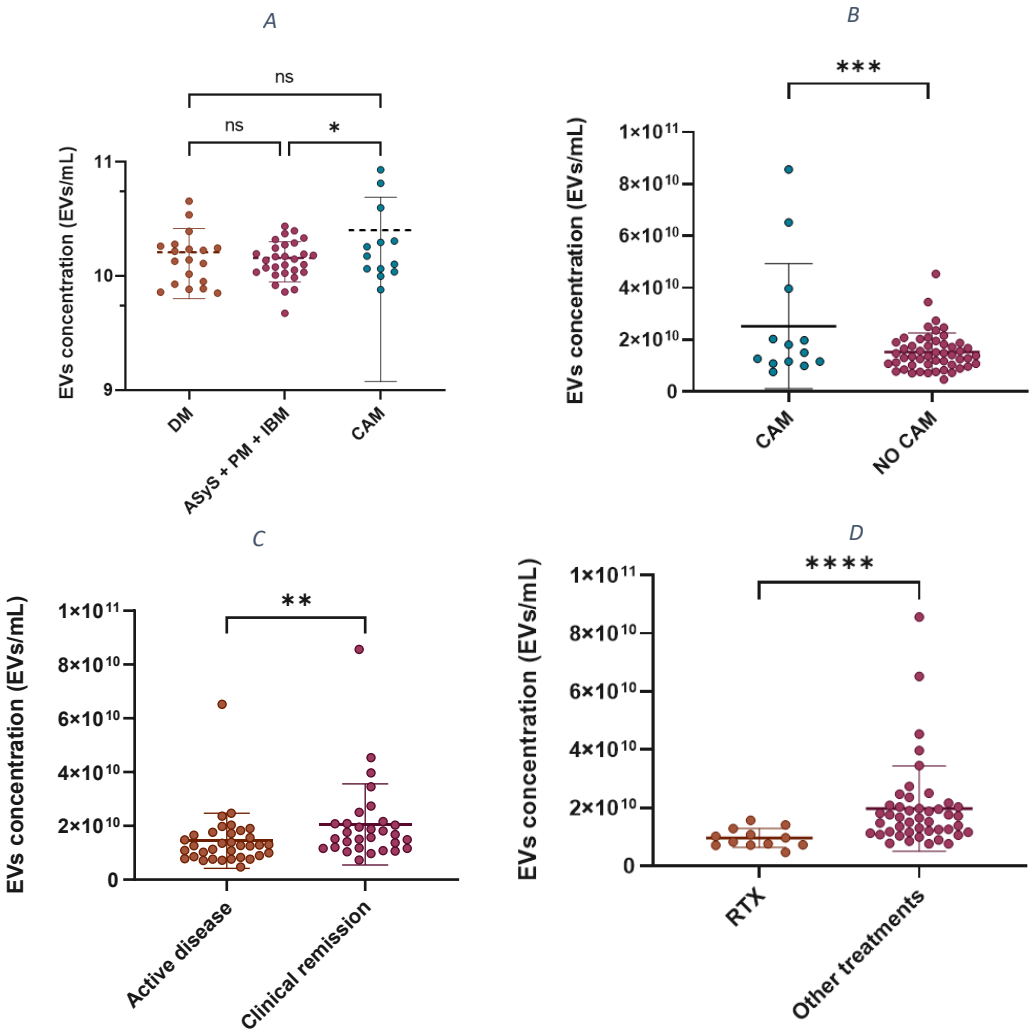

Figure 13 Graphs reporting the EVs concentration ([EVs/mL] ± SD) referred to A) DM (n=19) vs. ASyS + PM + IBM (n=29) vs. CAM (n=13); B) CAM (n=13) vs. no CAM (n=51); C), patients characterized by active disease (n=34) vs. clinical remission (n=30), and D) patients treated with RTX (n=12) vs. other treatments (n=45). DM: dermatomyositis; ASyS; anti-synthetase syndrome; IBM: inclusion body myositis; CAM: cancer-associated myositis; GC: glucocorticoids; IS: immunosuppressants; RTX: rituximab.

Micro-RNA expression analysis

EV-miRNAs expression in IIM patients and HD

NGS analysis of small non-coding RNAs (15-55 nucleotides in length) extracted from EVs samples of 47 IIM patients and 45 HD was performed, and sequences of 18-24 nucleotides in length corresponding to miRNAs were analysed. Among the 2632 miRNA aligned, the bioinformatics analysis showed that 2362 were expressed in both IIM and HD groups in at least 1 sample. After filtered correction, analysis covered 122 miRNAs to assess and compare their expression levels in EVs from patients and HD. Of these, differential expression between patients and HD was reported for 9 miRNAs with statistical significance. Six miRNAs (miR-223-3p, miR-15a-5p, miR-451a-5p, miR-486-5p, miR-32-5p, and miR-222-3p) were up-regulated, while three (miR-141-3p, miR-142-3p, and let-7a-5p) were down-regulated in IIM compared to HD (figure 14). Data are reported in table III.

EV-miRNAs dysregulated in IIM vs. healthy donors

	IIM patients (CPM, mean±SD)	HD (CPM, mean±SD)	P value
miR-223-3p	32678 ± 15122	26100 ±10849	0.0190
miR-15a-5p	2136 ± 1236	1554 ± 1093	0.0189
miR-451a-5p	28989 ± 24761	17948 ± 11054	0.0074
miR-486-5p	26253 ± 9538	21039 ± 7788	0.0052
miR-32-5p	1084 ± 559.9	804.9 ± 416.1	0.0146
miR-222-3p	738.1 ± 750.3	424.3 ± 264	0.0282
miR-141-3p	529.8 ± 808.9	1276 ± 1863	0.0313
miR-142-3p	58431 ± 37341	104668 ± 131644	0.0244
let-7a-5p	45411 ± 12869	58577 ± 19354	0.0003

Table III EV-miRNAs with significantly different expression in IIM patients and healthy donors; CPM: counts per million; SD: standard deviation.

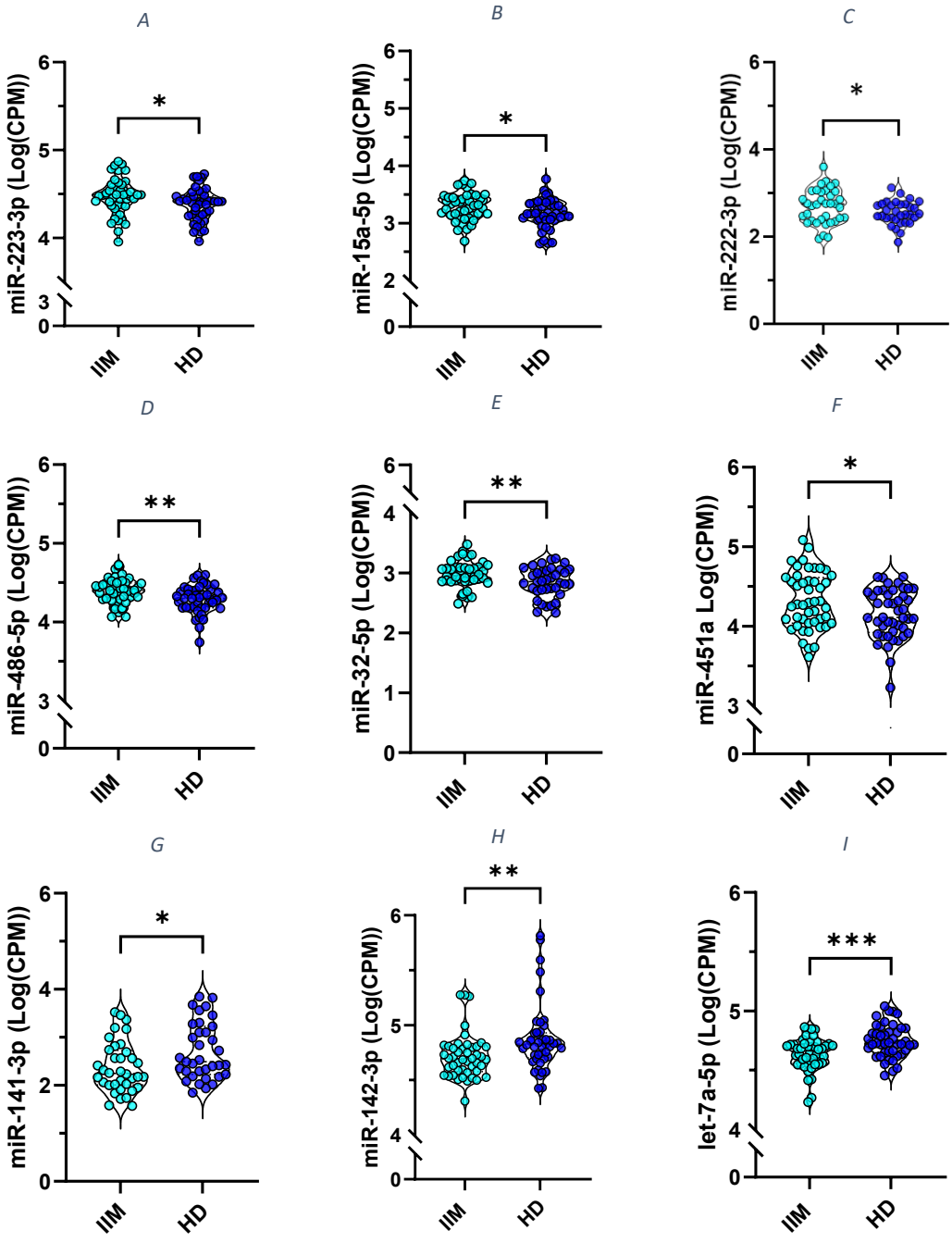

Figure 14 Expression levels of EV-miRNAs up-regulated miR-223-3p (A), miR-15a-5p (B), miR-222-3p (C), miR-486-5p (D),miR-32-5p (E), miR-451a (F), and down-regulated miR-141-3p (G), miR-142-3p (H) and let-7a-5p (I) in IIM patients (n=47) compared to HD (n=49).

miRNA expression in IIM subsets

Subsequently, miRNAs expression was evaluated within IIM subsets to detect any differential expression patterns between subgroups of patients. By comparing CAM (n=11) patients and noCAM (n=34) patients the expression levels of miR-143-3p were significantly down-regulated in CAM (CAM 1688 ± 503.3 CPM ± SD vs. NoCAM 2391 ± 1208; p= 0.0085) (figure 15).

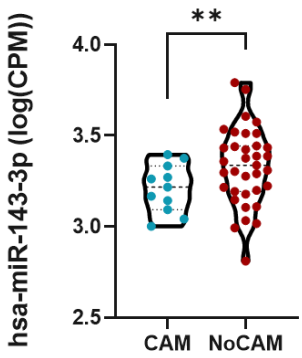

Figure 15 Graph representing the down-regulated expression of EV-miR-143-3p in CAM patients (n=11) vs. NoCAM patients (n=34). CAM, cancer associated myositis.

The differential EV-miRNA expression in IIM subset groups revealed that miR-148a-3p and miR-335-5p were significantly increased in DM group (miR-148a-3p n=14 and miR-335-5p n=13) compared to ASyS+PM+IBM group (miR-148a-3p n=21 and miR-335-5p n=17) (miR-148a-3p: 3699 ± 2513 vs 602.2 ± 303.8; p= 0.0171; miR-335-5p: 967.1 ± 348,4 vs ASyS+PM+IBM: 602.2 ± 303.8; p= 0.0171) (figure 16).

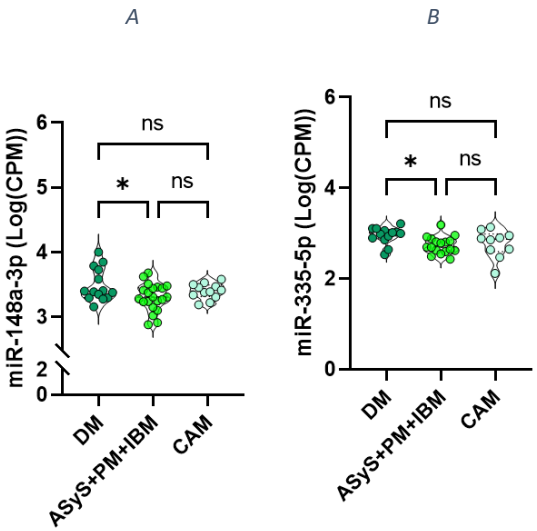

Figure 16 Graph reporting upregulated expression levels of miR-148a-3p (A) and miR-335-5p (B) in DM patients (A n=14 ;B n=13), compared to ASyS+PM+IBM patients (A n=21; B n= 17) and CAM patients (A n=12; B n= 10). DM, dermatomyositis; ASyS, anti-synthetase syndrome; PM, polymyositis; IBM, inclusion body myositis; CAM, cancer associated myositis.

Patients characterized by active disease displayed decreased expression of miR-363-3p, miR-374a-5p, miR-144-3p and miR-181a-5p compared to patients in clinical remission (miR-363-3p: 802.9 ± 416.2 vs 1674 ± 796.5, n= 23 vs 17; p= 0.0001; miR-374a-5p: 1265 ± 749 vs 1875 ± 978.4; n= 24 vs 19; p= 0.0258; miR-144-3p: 6794 ± 4129 vs 9867 ± 5023, n=26 vs 21; p= 0.0170; miR-181a-5p: 1518 ± 1078 vs 2359 ± 1223; n=25 vs 19; p= 0.0037) (figure 17 A-D). Conversely patients with active disease had up-regulated expression of miR-222-3p and miR-151a-3p (miR-222-3p, 984.9 ± 829 vs 555.3 ± 383.8, n=21 vs 12; p= 0.0020; miR-151a-3p: 2723 ± 999.1 vs 2081 ± 752.2, n=24 vs 20; p= 0.0233) (figure 17 E,F)

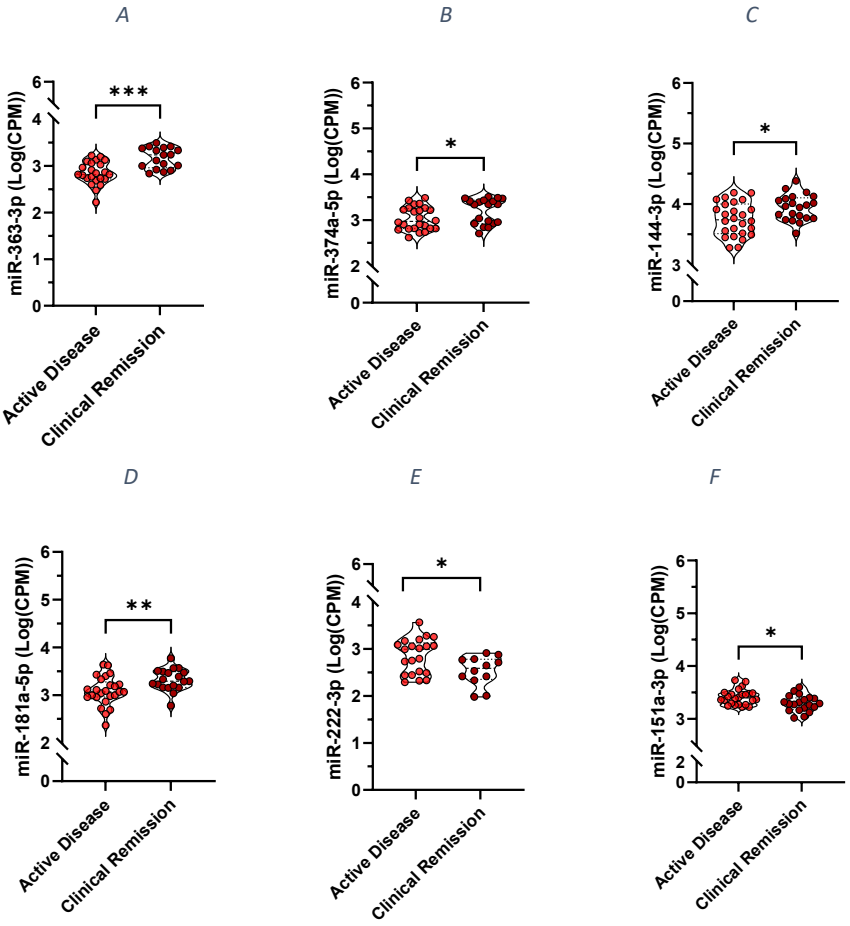

Figure 17 Expression levels of EV-miRNAs miR-363-3p (A), miR-374a-5p (B) and miR-144-3p (C) miR-181a-5p (D), miR-222-3p (E) and miR-151a-3p (F) in patients with active disease (A n=23; B n=24 ; C n=26 ; D n=25 ; E n=21 ; F n=24) compared to patients in clinical remission (A n=17 ;B n=19 ;C n=21 ;D n=19 ;E n=12 ;F n=20).

Other differences of EV-miRNAs expression were highlighted comparing patients treated with different medication regimens. Patients treated with glucocorticoids (GC) alone displayed a significantly increased expression of miR-92a-3p, let-7f-5p and miR-4433b-5p compared to patients receiving both glucocorticoids and other immunosuppressants(GC+IS) (GC vs GC+IS; miR-92a-3p: 16658 ± 4865 vs 12808 ± 3416, n= 15 vs 19 p= 0.0111; let-7f-5p: 43164 ± 14633 vs 33692 ± 12681, n=16 vs 20 p= 0.0304; miR-4433b-5p: 421.3 ± 313.3 vs 196.0 ± 95.36, n=11 vs 12 p= 0.0439) while miR-27a-3p was significantly down-regulated in patients treated only with GC than in patients receiving GC+IS (GC vs GC+IS; miR-27a-3p: 2055 ± 1029 vs 3090 ± 1492; n=14 vs 19; p= 0,0486) (figure 18).

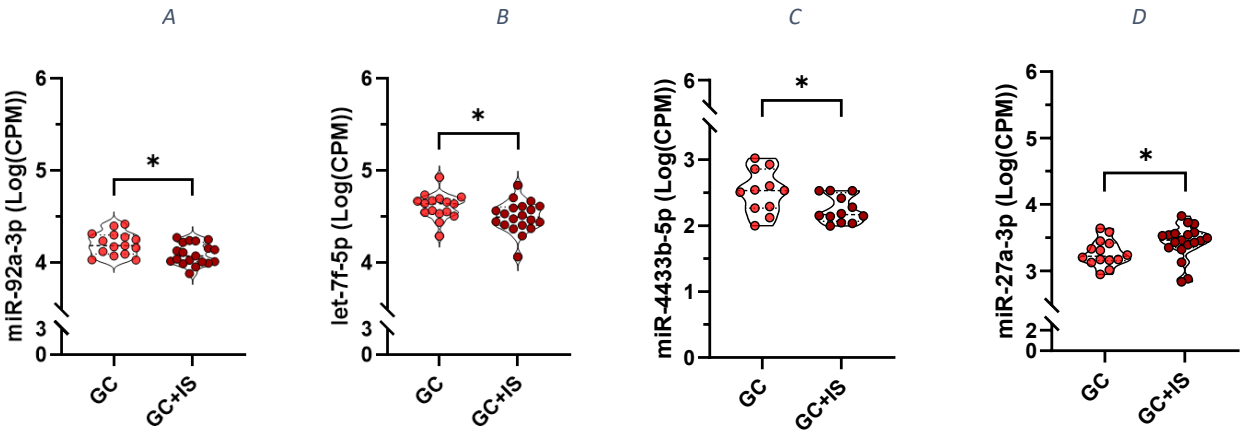

Figure 18 Expression levels of EV-miRNAs miR-92a-3p (A), let-7f-5p (B), miR-4433b-5p (C) and miR-27a-3p (D) in patients receiving only glucocorticoids (GC) (A n=15 ; B n=16 ; C n= 11 ; D n=14) compared to patients receiving glucocorticoids and immunosuppressants (GC+IS)(A n=19 ; B n=20; C n=12 ; D n=19).

DISCUSSION

In this cross-sectional study, we demonstrated that IIM patients exhibit abnormalities in the circulating EVs pool, which may be functionally linked to altered processes in IIM pathogenesis. Additionally, we report significant differences in the epigenetic footprint of patients and HD and also within IIM subsets, which may result in the extremely heterogenous clinical phenotype of these patients.

Idiopathic inflammatory myopathies (IIM) comprise a rare heterogeneous array of distinct clinical syndromes often featuring a multisystemic pathological involvement leading to severe detriments on quality of life and in some cases to a poor prognosis, often linked with extra-muscular involvement.

Although the understanding of the mechanisms underlying IIMs pathogenesis has significantly increased over the decades since Bohan and Peter outlined the diagnostic criteria for polymyositis and dermatomyositis almost 50 years ago, [22] the pathogenesis of these systemic autoimmune diseases remains largely enigmatic. [139]

Several clues have emerged portraying a multifaceted picture in IIM pathogenesis. Features of genetic predisposition, environmental factors, innate and adaptive immune response actors as well as non-immune mediated mechanisms appear to all be involved in a complex network, outlining a multifactorial etiopathogenesis process.

Although the landscape of IIM treatment is rapidly evolving with the advent of biological targeted drugs, in clinical practice these conditions are often treated with relatively nonspecific immunosuppressive therapies, frequently encumbered by significant tolerability issues, and often encountering difficulties in predicting adequate response to specific agents. This fact strongly warrants further investigations on the exact molecular mechanisms underlying these conditions, as the development of accurate explanatory models might provide novel and specific therapeutic targets, enabling significant improvements in the management of these patients. [22]

The field of extracellular vesicles (EVs) biology appears particularly promising in this context, as EVs are now recognized as central players in intercellular communication. Their ability to influence innate[83] and adaptive immune responses[79], to convey active mediators influencing several relevant biological processes and to directly elicit phenotypical changes in recipient cells have been extensively elucidated in a range of human diseases, with particular emphasis in cancer and autoimmunity. The relevance of EVs in SLE and RA, as discussed earlier, is particularly compelling and increasing evidence implies their crucial role in IIMs as well. Their ability to mediate interactions between neighboring as well as distant cells of diverse lineage could easily fit in the complex multifactorial etiopathogenesis of IIMs, in which several independent but interacting factors appear to act synergistically. Given their accessibility in biological fluids, EVs could also prove to be useful biomarkers in clinical practice.

Our study reports significantly increased circulating EVs in plasma from IIM patients compared to healthy donors, confirming previous observations[140] and suggesting their potential supporting role as a diagnostic biomarker.

The role of EVs has been elucidated in several domains of human biology and appears to be particularly relevant in specific processes of abnormal immune activation which are known to be crucial in IIM pathogenesis.[19] For instance, EVs have been shown to participate in complement activation[83], an established factor of Dermatomyositis pathology[10], arising before muscle infiltration and leading to vascular damage and contributing to the propagation of local inflammation in affected tissues. This appears particularly true in the context of increased interferon (IFN) signaling as observed in DM, where EVs are enriched in complement activating components, potentially triggering a self-sustaining loop of complement activation, tissue damage and interferon release.[141] Furthermore, as pertains to increased type I IFN signaling, RNAs specifically conveyed by EVs have been demonstrated to act as a TLR agonist in dendritic cells, which upon activation can release vast amounts of IFN, a known key player of DM pathogenesis.[142] EVs are also known to have a prominent role in antigen presentation mechanisms, acting independently or on the surface of cross-dressed APCs, and can also transport as part of their cargo several costimulatory molecules

which can increase efficacy of adaptive immune response activation[79]. Furthermore, EVs can contribute to macrophage activation.[116] Macrophages, in turn, are known to play an important role in IIM as they are necessary for clearing cellular debris in inflamed or necrotic tissues, but can also favor fibrosis and orchestrate myofiber regeneration, which can by itself increase expression of IIM target antigens, generating a feed-forward mechanism of antibodies production and immune-mediated damage.[44]

One of the most relevant factors determining IIM patients' prognosis is the frequent association with malignancy. [1] Since the first description of cancer associated myositis (CAM), numerous epidemiological studies have highlighted significant association between cancer and IIMs, particularly dermatomyositis. [143] Beside this association, there is an increasing recognition that neoplasia is associated not only with muscular-skeletal disorders but also with a wide range of other rheumatic symptoms, most of them paraneoplastic in nature.[44]

Although the link between cancer and autoimmune disease has been extensively explored, many uncertainties persist. It is estimated that among IIM patients, less than 20% have or will develop an associated malignancy. [144] Even if CAM patients usually display a good response to glucocorticoid therapy, retrospective studies evidence that they are burdened by a much worse prognosis than other IIM patients, with a 5-year survival rate <60% compared to >90%. [143]

The discovery that different types of cancerous cell lines express increased amounts of myositis antigens, not usually expressed in normal corresponding tissues, and that these same antigens are expressed at high levels only in regenerating muscle has led researchers to believe that an anti-tumour immune response might cross-react to damage other tissues. In fact, a study conducted by Zampieri et al. highlighted the presence of subclinical myopathy in patients with early colorectal cancer; the increased expression of target antigens in regenerating muscle fibres could then induce a self-sustaining mechanism of inflammation and muscle damage. This could then explain why, in some patients, autoimmune myopathy can persist even after successful eradication of the malignancy. Some researchers even speculated that the occurrence of immune-mediated myopathy

without cancer could represent instances of complete eradication of the primary cancer by the immune system, with bystander damage to regenerating muscle. [143–145]

These considerations place great importance on recognizing an underlying malignancy triggering autoimmune disease to refer patients for effective and timely treatment. [143] This can, however, prove to be very challenging, as malignancies might not be immediately evident and could require extensive diagnostic procedures.

Our results indicate higher levels of circulating EVs in CAM in comparison to both HD and other IIM subtypes. This observation fits in with current evidence in which EVs are purported to play a crucial role in several processes of cancer biology, by acting locally to influence the primary tumor micro-environment, but also systemically to regulate immune response and metastatic spread, affecting distant organs.EVs have been shown to possess complex functions in immune response against cancer. As a source of antigens and costimulatory molecules they can facilitate antigen presentation thus increasing adaptive immune response [79] against cancerous cells and potentially facilitating autoimmunity. Conversely, they have also been demonstrated to exhibit tolerogenic functions by inhibiting immune cells activation and response, highlighting their complex biological role.[88] Our results further confirm that EVs might have great relevance in regulating the complex mechanisms and interactions underlying CAM.

This evidence allows us to propose EVs as potential biomarkers of IIM and specifically of the CAM subset, thus aiding clinics in formulating earlier diagnosis of disease to guarantee more decisive treatment, potentially improving considerably these patients' outcomes.

By comparing different subsets of IIM patients, interesting differences in EVs levels emerged between patients with active disease and patients in clinical remission, with the latter having significantly higher concentrations of circulating EVs. This result is somewhat counterintuitive, but previous studies have reported similar findings of inverse relation between circulating EVs levels and disease severity and activity in other conditions, such as SLE, RA and Crohn's disease. Researchers

hypothesize that several mechanisms could underly this phenomenon, such as confinement or consumption of EVs in target tissues, sequestration on the surface of leukocytes or peripheral degradation by phospholipases like the sPLA2 in inflammatory conditions.[146]

Our results show that circulating EVs in IIM patients appear to decrease significantly after treatment with Rituximab. Previous investigations demonstrated that significantly elevated concentrations of monocyte (CD14 positive), T-lymphocyte (CD3 positive) and B-lymphocyte (CD19 positive) derived EVs are present in the plasma samples of IIM patients compared to healthy controls.[140] Rituximab targets CD20 positive cell lines effectively depleting B lymphocytes population. Since production of antibodies by plasmacells and long-lived B cells, not expressing CD20, is not directly affected by Rituximab, it's likely that other mechanisms are involved to determine its evident clinical benefits. Other B cell dependent mechanisms like disturbance in antigen presentation and B cell - T cell costimulatory interaction, and non-B cell-related mechanisms might be involved, as CD20 expression has also been described in T and NK cells. [66] Thus, even though further research is warranted, the effect of Rituximab on levels of IIM patients' circulating EVs could provide significant insights, testifying both to the relevance of EVs in IIM pathogenesis as well as helping to explain Rituximab response mechanisms and efficacy in autoimmune disease.

The ability of EVs to exert such complex biological functions resides largely on the biological activity of their cargo. Among its cargo, miRNAs appear to have significant relevance as prominent actors in epigenetic regulation, a field receiving growing attention by researchers in the post-genomic era. [139]

miRNAs conveyed by EVs have been shown to effectively modulate protein synthesis in recipient cells[80], eliciting a wide array of biological effects relevant in human biology.

The analysis of miRNAs EVs cargo reported 9 miRNAs exhibiting significantly different expression in IIM patients compared to healthy donors (HD), suggesting that a specific EV-miRNA footprint could be developed as a novel diagnostic biomarker for IIMs. Among the dysregulated miRNAs, 6 miRNAs (**miR-223-3p, miR-**

15a-5p, miR-451a-5p, miR-486-5p, miR-32-5p, and miR-222-3p) were up-regulated, while 3 (**miR-141-3p, miR-142-3p, and let-7a-5p)** were down-regulated in IIM compared to HD.

miR-223 has been studied extensively in the field of cancer biology, appearing to possess mainly tumor-suppressive functions, as its expression is down-regulated in a variety of cancer cell lines, such as acute myeloid leukemia, chronic lymphoid leukemia, and colon and breast cancer, even though upregulation of miR-223 has also been linked to metastatic gastric cancer cells, complicating our understanding of its role. [147]

More relevantly, miR-223-3p appears to have critical functions in inflammatory response regulation. This miRNA is crucial in TH1 and TH17 lymphocyte differentiation [148] at least in part by activating DCs to promote pathological TH17 lymphocyte differentiation. Furthermore, it has been demonstrated to be relevant in eliciting the effects of platelet derived EVs in inflammatory settings. [149]

Supporting its role in autoimmunity, it has been demonstrated that miR-223 is significantly upregulated in the serum of RA [149] and Crohn's disease patients, and in the latter to also correlate with disease activity indexes. [148]

Interestingly, in animal models of muscle injury miR-223 expression appears to be significantly upregulated. This has been demonstrated in muscle tissues after ischemic injury. [150] In a mouse model of inflammatory myopathy, NFKB was found to upregulate miR-223 which could target dystrophin expression, thus potentially contributing to muscle weakness. [151]

Conversely, miR-223 expression in macrophages appears to downregulate inflammation. Indeed, its expression has been demonstrated to negatively regulate NFKB activation and TNF, IL1, IL6 expression in macrophages by transcriptional inhibition of STAT3, thus probably acting in a negative-feedback loop to facilitate tissue inflammation resolution and damage restoration. [152]

Previous studies have investigated the expression levels of miR-223 in IIM patients, reporting significantly downregulated levels in cutaneous lesions of DM patients, thus suggesting its involvement in the keratinocyte hyperproliferation of

Gottron's papules. [147] Another study reported the down-regulated expression of miR-223 in the serum of IIM patients. [153] These evidence appear to conflict with our results, thus further research is warranted to understand the specific role of this miRNA in the patho-physiology of IIM.

miR-15/16 cluster are known to act as tumor suppressors. Expression of these miRNAs inhibits cell proliferation, promotes apoptosis of cancer cells, and suppresses tumorigenicity both in vitro and in vivo. miR-15a and miR-16-1 act by targeting multiple oncogenes, including BCL2, MCL1, CCND1, and WNT3A. The down-regulated expression of these miRNAs has been reported in chronic lymphocytic lymphoma (CLL), pituitary adenomas, and prostate carcinoma.[154]

However, in recent years, studies have found that miR-15a/16 also play a strong regulatory role in fibroblast differentiation, extracellular matrix synthesis and degradation, and the release of fibrotic mediators and that they are involved in various fibrotic diseases.[155] A large number of studies have confirmed the eminent role of TGF- β1 in promoting tissue fibrosis. SMAD7 is conversely the key negative regulator of TGF signaling and its downregulation has been linked to organ fibrosis in both human and animal models. miR-15a has been demonstrated to directly inhibit SMAD7 expression, thus indirectly facilitating tissue fibrosis, as demonstrated in myocardial models of fibrotic disease, where miR-15a levels were inversely correlated to SMAD7 expression and directly correlated with tissue fibrosis. [156] Our results indicate that miR-15a overexpression in IIM patients could have an important role, considering its mechanistic potential in contributing to muscle fibrosis and to ILD as well.

miR-451a is among the miRNAs we found to be upregulated in circulating EVs of IIM patients. This miRNA is highly conserved in vertebrates and has been demonstrated to regulate many biological processes like cell proliferation, invasion, and apoptosis in tumors. [157] It has been demonstrated to play a role in endothelial cell pathology, as its upregulation through the ampk/mTOR axis could promote endothelial to mesenchymal transition, for instance contributing to cardiac tissue fibrosis and hypertrophy in a diabetic cardiomyopathy model. [158] Its role has also been investigated in autoimmune arthritis were researchers

demonstrated it to be an essential regulator of neutrophil migration. Through its targets CPNE3 and Rab5a it could interfere with MAPK/p38 signaling thus inhibiting neutrophils migration to their end-target chemoattractants. In fact, in neutrophils isolated from RA patients miR-451 expression was found to be significantly downregulated compared to healthy donors. Furthermore, in an animal model of autoimmune arthritis inducing its upregulation could counteract tissue inflammation.[157] These data provide interesting information on the role of miR-451 in human pathology, but its interpretation in the context of autoimmune myopathy requires further investigations.

It has been reported that **miR-486-5p** is differentially expressed in human plasma or serum of patients affected by several pathological conditions including, solid tumor malignancies, sepsis, primary muscle diseases (e.g., Duchenne muscular dystrophy), cardiorespiratory diseases (e.g., chronic heart failure), diabetic kidney disease, osteoarthritis, neurological conditions (e.g., vascular dementia), and various endocrine disorders (e.g., metabolic syndrome, type 2 diabetes mellitus, polycystic ovary syndrome) and recurrent miscarriage. More than 300 predicted miR-486-5p targets have been identified, explaining the complexity of its role in human diseases.[159] In cancer biology, miR-486-5p was found to have a significantly aberrant expression in several solid malignancies such as hepatocellular cancer, non-small cell lung cancer, breast cancer, esophageal squamous cell carcinoma and pancreatic cancer. Its role appears to be highly pleiotropic, acting as both a tumor suppressor and oncogene in different contexts. [160]

Interestingly, through its ability to interact with PTEN and FOXO and inhibit their expression, miR-486-5p was found to be implicated in the skeletal muscle regeneration process, indirectly facilitating myoblast proliferation and myotube formation. This miRNA was found to be significantly down-regulated in muscle samples of muscular dystrophy patients and it is thus speculated to be essential in myocellular biology. However, its expression levels in response to muscle injury appear to exhibit complex dynamics, and abnormally elevated levels of miR-486-5p could also impair satellite cell functions in muscle regeneration, further highlighting the complexity of this regulatory network. [159] Nevertheless, the role of miR-486-5p in IIM is still unclear, and further research might unveil useful

implications that could further our understanding of immune-mediated muscle disease.

miR-32 is a highly conserved micro-RNA, expressed in a variety of tissues, including serum, liver, kidney, breast and brain. Its role has been studied mainly in oncologic disease where it was demonstrated to be differentially expressed in several cancer types. MiR-32-5p has been identified as an onco-miR in the majority of research, but it has also been described as a tumor-suppressor miRNA in other findings. Interestingly, it has been shown that this miRNA also plays a crucial role in many other non-neoplastic diseases, like vascular disfunction, atherosclerosis and calcification and in other biological processes like inflammation. [161] Intriguingly, it was found to significantly correlate with severity of COVID-19 infection, highlighting potential implications relevant to systemic inflammation and potentially to lung disease. [162]

miR-222-3p is a highly conserved miRNA, whose expression alteration have been illustrated in several biological processes and disease. Through its interaction with estrogen receptors, GLUT4 glucose transporters and Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), among the others, it is deemed to be a relevant regulator of energy metabolism in physiological as well as pathological conditions, for example in diabetes, polycystic ovary syndrome and cardiovascular disease. [163,164]

miR-222-3p also plays a relevant role in inflammatory conditions as it is an important negative regulator of suppressor of cytokine signaling 1 (SOCS1). One of SOCS1 main functions is to suppress the JAK/STAT signaling pathway, inhibiting systemic autoimmunity mediated by dendritic cells. Thus, miR-222-3p up-regulation can limit SOCS1 activity and indirectly amplify immune activation. [165] miR-222-3p is also considered to play a central role in skeletal muscle differentiation and regeneration. In fact, by inhibiting Insulin receptor substrate-1 (IRS-1) and consequently the PI3K/akt pathway, miR-222-3p can directly hinder myogenesis and it has been linked to skeletal muscle disease. [166]

Among the differentially expressed EV-miRNAs of IIM patients compared to HDs, those that were significantly downregulated included miR-141-3p, miR-142-3p and let-7a-5p.

miR-141-3p exhibits interesting functions in relation to its ability to interact with several key mediators of inflammation. In bone metastases of prostatic cancer miR-141-3p has been detected to be significantly reduced. Its levels appeared to be inversely correlated to NFKB signaling and TNF receptors TRAF5 and TRAF6, essential to promote epithelial to mesenchymal transition and cancerous spread, also linking its functions to inflammatory molecular cascades. [167] Other studies reported that decreased expression of miR-141-3p in inflamed intestinal tissues of patients with Crohn's disease correlated with increased recruitment of immune cells. Notably, miR-141-3p can modulate the expression of C-X-C motif chemokine (CXCL)12β, which is involved in promoting immune cell infiltrations, and is induced by TNFα. Furthermore, miR-141-3p has also been linked to TGFβ signaling, and its levels were inversely correlated to tissue fibrosis in inflammatory bowel disease mucosa. [148]

Conversely, miR-141-3p has also been shown to be specifically linked to TH17 cell differentiation, with levels significantly increasing in T cells during this process. Its effect on promoting TH17 differentiation appears to be explained by the inhibition of its target retinoic acid receptor beta (RARB) gene, whose product has been reported as a negative regulator of Th17 cell generation through inhibition of the JAK/STAT pathway genes.[168] Overall these data strongly imply that this miRNA could have relevant functions in the complex regulatory mechanisms of inflammatory response in IIM patients.

miR-142-3p appears to have a relevant effect on immune response in various physiological and pathological contexts. This is attributed to the fact that miR-142 can target nodal genes belonging to the immune response pathways, thereby having broad effects on multiple downstream signaling pathways. In particular, miR-142-3p expression levels appear to be altered in many diseases characterized by extensive inflammatory responses.

miR-142-3p is considered crucial in lymphocyte differentiation and functions, exhibiting central roles in regulatory T cell populations, effector T cell populations as well as B cell populations. Thus, miR-142-3p plays a pivotal role in maintaining immune homeostasis. Interestingly, in macrophages a functional target of miR-142-3p is IL-6. Studies have demonstrated that a decline in miR-142-3p levels is correlated with aging and that it could promote increased expression of this inflammatory cytokine, potentially linking this miRNA's under-expression with the pathogenesis of IIM. [169]

However, the specific functions of miR-142-3p are likely complex, as increased levels of this miRNA have been reported in autoimmune conditions like RA, whereas inhibition of miR-142-3p reduced the immune cell viability and increased apoptosis rate through regulating NF-κB signaling pathway.[170]

microRNAs belonging to **let7** family were originally discovered in the nematode Caenorhabditis elegans to control the timing of stem-cell division and differentiation. let-7 was subsequently the first described miRNA in human. let-7 and its family members are highly conserved across species in sequence and function, and dysregulated expression of let-7 leads to a less differentiated cellular state and the development of diseases such as cancer. [171] Its role has been investigated in several diseases, for instance in Glioma where it has been demonstrated that let-7 targets STAT3. The down-regulated -expression of let-7 would then up-regulate STAT3 which in turn increases cellular proliferation and decreases apoptosis and autophagy. [172] This interaction with STAT genes and inflammatory pathways is coherent with other observations in autoimmune conditions, such as multiple sclerosis. In this context, miRNAs of the let-7 family are able to regulate a variety of other miRNAs linked with the IL17 pathway, and, interestingly, levels of let-7 in the cerebro-spinal-fluid are inversely proportional to both central and peripheral inflammation and to disease severity as well. [173]

Of interest, let-7 has been found to be differentially expressed in other autoimmune conditions; for instance, it is upregulated in skin samples from systemic sclerosis patients. Other researchers have focused on this miRNA in the context of IIM with findings exhibiting similar trends to ours. In Gottron's papules

skin samples it was found to be undetectable in comparison to healthy skin. Moreover, its serum levels were also previously found to be lower in dermatomyositis, polymyositis and clinically amyopathic dermatomyositis (CADM) patients compared with controls. Although no specific mechanism of action has been tested as to how miR-7 might be involved in dermatomyositis pathogenesis, predicted targets of miR-7 include inflammatory mediators such as fibroblast growth factor 11 and CC chemokine ligand; therefore, reduction of miR-7 could elicit an inflammatory response.[147,174]

Our results obtained by the comparison of differentially expressed EV-miRNAs within IIM subtypes revealed several interesting differences. For instance, CAM patients exhibited a down-regulated expression of miR-143-3p than non-CAM patients . The future confirmation of these findings could provide interesting elements for developing clinically useful tools for CAM diagnosis, aiding the clinician in more appropriately directing efforts for cancer screening.

miR-143-3p has been shown to be involved in regulating different aspects of skeletal muscle cell biology; through its interaction with several factors like MyoD and Igfbp5 it has been connected with satellite cells profileration in muscle repair and regeneration processes and has been shown to be dysregulated in aging individuals. Furthermore, miR-143 also appears to have a role in vascular remodeling following injury [175] and has been directly connected with cardiovascular disease in human and animal models. [176]

The role of miR-143-3p has also been explored in the context of immune system homeostasis and inflammatory response modulation[177] as a negative regulator of NFkB signaling. [178] Interestingly, in intensive care unit patients, low circulating levels of miR-143 were demonstrated to be correlated to poor short- and long-term prognosis, irrespective of specific etiology, highlighting its role as a systemic inflammation biomarker. [179] It was also shown to be a prominent actor in determining T CD8+ memory cells differentiation. Indeed, an increase in miR-143 levels, via inhibition of GLUT-1 expression, could promote the metabolic changes necessary to elicit memory cells differentiation, thus decreasing T cell apoptosis and increasing inflammatory cytokines secretion. This aspect of its function was

investigated in cancer, where researchers could demonstrate an increased miR-143-3p expression to enhance anti-tumour activity of T cells against esophageal squamous cancer. [180]

In fact, the relevance of this particular miRNA has been mainly attributed to its role in cancer biology. The downregulation of miR-143 has been reported in malignancies like ovarian cancer, [181] colon cancer, prostate cancer, breast cancer, lung cancer, and gastric cancer, [180] in the latter having enough specificity and sensitivity to be proposed as a novel diagnostic biomarker. [182]

Its mechanisms as a tumor suppressor were investigated in non-small cell lung cancer, where researchers could determine Fibronectin Type III Domain Containing 1 (FNDC1) to be one of its relevant targets. Increasing evidence connects the upregulation of this protein as an important factor in progression and metastasis in several types of cancer and its inverse correlation with miR-143-3p has been demonstrated. [183] However, in other cancers, like colorectal cancer, miR-148-3p expression was found to be increased and to facilitate progression. Interestingly, colorectal cancer cells were demonstrated to elicit M2 macrophage phenotype differentiation, and that M2 machrophage cells release EVs enriched in miR-143-3p which could in turn influence malignant cells by promoting proliferation and migration. [184]

By comparing miRNAs expression among different subsets of IIM, we found significant differences in DM patients compared to PM+ASyS+IBM patients, with the expression levels of miR-148-3p and miR-335-5p being increased in DM patients. Numerous studies have shown that **miR-335-5p** is dysregulated in several types of cancer, such as breast cancer, lung cancer, colorectal cancer, and ovarian cancer. In different contexts it can act as both an oncogene or tumor suppressor, interacting with a variety of molecular pathways involved in proliferation, metabolism and apoptosis, such as those of BCL2 and PI3K/AKT/mTOR, eliciting opposing effects in different systems. [185] Through its interactions with regulators of cellular proliferation and protein synthesis this miRNA is also thought to be involved in myogenic differentiation and its modulation has also

been shown to be implicated in the skeletal muscle regenerative process after injury. [150]

miR-148a-3p has emerged as a crucial actor in immune response regulation. It is significantly upregulated in memory Th lymphocytes isolated from RA and SLE patients, where it is thought to target the pro-apoptotic BCL2, thus increasing survival of repeatedly stimulated T cells and contributing to the maintenance of autoimmune response. In addition to its role in T lymphocytes, miR-148a-3p is also considered relevant in B cell lineage differentiation and especially during plasma-cell phenotype acquisition, during which it is significantly upregulated. Research experiments conducted in animal models suggested that ectopic expression of this miRNA could alone break B cell tolerance thus eliciting an autoimmune reaction. [186] Furthermore, miR-148-3p has also been identified as a key downstream mediator of Notch signaling, necessary to promote the differentiation of circulating monocytes into macrophages and their polarization towards the M1 phenotype through PTEN/AKT mediated upregulation of NFKB. Thus, miR-148-3p through its effect on macrophages, could contribute to different elements involved in the pathogenesis of DM, like inflammatory cytokines secretion, increased antigen presentation and release of ROS. [187]

The comparison of EV-miRNAs between patients with active disease and patients in clinical remission also yielded some significant differences. For instance, **miR-363-3p** was over-expressed in patients in clinical remission. miR-363-3p has been described as a crucial negative regulator of TH17 differentiation, as it can downregulate several transcription factors, such as Rorct, Rora and Nfat5, necessary for TH17 phenotype acquisition. In fact, its expression levels have been observed to decrease upon the differentiation of TH17 cells, further confirming its role in immune system homeostasis. [188]

miR-374a-5p was also detected significantly higher in clinical remission patients than those characterized by active disease. In recent years, numerous studies have uncovered the role of miR-374 family members as essential regulators in cell growth and differentiation, as they have been demonstrated to participate in a variety of physiological and pathological processes. The targets of miR-374 family

members chiefly include: AKT, VEGF, PTEN, Wnt and Fas signalling pathways. Their relevance has been widely recognized in reproductive disorders and cancer, especially in the digestive system. [189] miR-374a-5p has also been demonstrated to indirectly regulate several transcripts implicated in inflammatory response, like in the IL17 induced, NFkB mediated, expression of CCL2, an important monocyte chemoattractant. [190] Interestingly, miRNAs of this family have been demonstrated to be differentially expressed in differentiating myotubes and to negatively regulate MRF4, an essential myogenic transcription factor. [189] Furthermore, these miRNAs have been shown to participate in TNFα induced dystrophin downregulation[191] potentially contributing to skeletal muscle impairment and, in animal models, to be upregulated by dexamethasone administration to facilitate adipocyte differentiation. [189]

Other miRNAs significantly upregulated in patients in clinical remission compared to patients with active disease included miR-144-3p and miR-181a-5p.

miR-144-3p has been shown to act as a tumor suppressor in lung adenocarcinoma, through its role in downregulating the oncogene WT1D, and in ovarian cancer, through Pre-B cell leukemia homeobox 3 (PBX3) suppression. [192] In osteoarthritis, miR-144-3p was demonstrated to reduce IL1β expression in synovial fibroblasts, thus negatively modulating the pathways of PI3K/AKT, NFkB and MAPK and participating in inflammatory response control. [192] Additionally, in a study conducted to investigate the circulating miRNAs footprint in anchylosing spondylitis, miR-144-3p was found to be among the significantly downregulated miRNAs in comparison to healthy controls. [193]

However, in other contexts miR-144-3p was shown to exhibit opposing functions, facilitating inflammation. For instance, in mycobacterial lung infections, miR-144-3p levels are directly correlated to inflammatory cytokines levels in macrophages [194], while serum EV-miR-144-3p was proposed as a reliable biomarker for Crohn's Disease activity, correlating to mucosal inflammation better than CRP. [195]

In addition, miR-144-3p is also considered an important factor in local response after myocardial infarction, with several studies demonstrating its role especially in muscle tissue remodeling following ischemic injury. In particular, this miRNA's

expression levels are upregulated in infarcted areas and upregulate mRNA and protein levels related to extra-cellular-matrix formation, including α-SMA, Col1A1, and Col3A1, thus contributing to scar formation and tissue fibrosis. [196]

miR-181a-5p appears to be relevant in several pathways related to immune response and inflammation. Through interaction with various target mRNAs its properties appear to be mainly anti-inflammatory in nature. For instance, it has been demonstrated to negatively regulate TH17 cell differentiation and functions, via targeting AKT3 and indirectly stimulating FOXO3, thus opposing the action of essential players in autoimmune disease. [197] Furthermore, bioinformatics analysis revealed that miR-181a-5p sequence has complementarity to JAK2 miRNA, making it potentially relevant in downregulating crucial inflammatory pathways in IIM pathogenesis. [198] In a model of lipopolysaccharide induced inflammation, miR-181a-5p was demonstrated to be able to downregulate expression of HMGB1, which can act as a damage associated molecular pattern (DAMP) molecule and further increase inflammatory mediators' production [199] and has also been documented to be relevant in IIM pathogenesis.[133]

Interestingly, miR-181a-5p expression in skeletal muscle has also been documented to increase following acute exercise and to decrease with ageing. [200] This might be linked to its role in the skeletal muscle regeneration process as this miRNA was documented to markedly increase during myoblast differentiation, downregulating targets like Hox-A11, a repressor of myoblast differentiation, and in turn upregulating the expression of Myf6, MyoD, and MyoG, necessary to induce myoblast differentiation. [201]

Conversely, miR-222-3p and miR-151-3p were significantly over-expressed in patients with active disease compared to patients in clinical remission.

The role of **miR-151-3p** has been mainly established in cancer biology, with several studies demonstrating its relevance in the progression of colon, breast and nasopharyngeal cancer as well as glioblastoma and osteosarcoma. In addition, augmented secretion of EVs enriched in this miRNA was also documented in gastric cancer patients, promoting metastatic niche formation in the liver and correlating to a poor prognosis. [202]

However, some data also link miR-151-3p to regulation processes relevant in inflammation and immune response as well as muscle biology. For instance, this miRNA was shown to target IL17a mRNA in endothelial cells, downregulating apoptosis induced by oxidized LDL, [203] and to target STAT3 in macrophage cells, reducing LPS induced secretion of IL6. [204]

Furthermore, miR-151-3p was also described among a small number of miRNAs that were robustly downregulated in peripheral circulation immediately after acute exercise, [205] suggesting potential selective clearance mechanisms from the bloodstream. Interestingly, this miRNA also appears to be relevant in the context of myoblast proliferation and differentiation, where an increased expression of miR-151-3p induces myoblast proliferation and regulates factors controlling muscle differentiation, like MHC-b/slow and slow muscle troponin I (TnI-S), thus enticing a fast twitch muscle fiber phenotype acquisition. [206]

These results, besides suggesting molecular mechanisms potentially relevant in IIM pathogenesis and disease resolution, could also be very promising in the clinical context. Currently available laboratory biomarkers of muscle damage in fact display limited utility in assessing disease activity and the practitioner must rely on approximate tools like the MMT-8. Thus, developing a specific measurable mean of determining disease activity could prove extremely useful.

The comparison of EV-miRNAs expression in patients treated with different pharmacological regimens also yielded significant results. For instance, **miR-27a-3p** was significantly higher in patients treated with glucocorticoids in combination with other immunosuppressants compared to patients treated with glucocorticoids alone. miR-27a has been shown to play a relevant role in regulating T lymphocyte homeostasis. In the Teff population it is considered a negative regulator of activation and cytokine production. Conversely, in Tregs many of the essential genes necessary for differentiation and suppressor function are directly targeted by miR-27a. In fact, overexpression of this miRNA in Tregs has been proven to impair their suppressive functions in animal models of autoimmune disease. [207] Other studies have investigated its role in the context of lung fibrosis, where miR-27a emerged as a key negative regulator at different levels of the TGFβ

pathway, directly targeting SMAD2, SMAD4, PTEN and PPAR-γ, thus contributing to the process of abnormal fibrosis, as suggested by its downregulation in systemic sclerosis patients compared to healthy controls. [208]

Conversely, other miRNAs, like miR-92a-3p and let-7f-5p where significantly overexpressed in patients treated only with glucocorticoids. **miR-92a-3p** has been mainly studied in the context of its cluster if miRNAs, the miR-17-92 cluster, comprising six different miRNAs. [209] Individual miR-17-92 miRNAs, albeit initially transcribed as one transcript, can have cooperative or opposing effects on different biological processes.[210]

The miR-17-92 cluster is extensively involved in the early development of B cells, but it is also crucial for the differentiation of germinal center B cells and the production of antibodies in antigen activation and the secondary antibody response. In another study, deletion of the miR-17-92 cluster results in a decrease of anti-DNA antibodies (both anti-double-stranded and anti-single-stranded DNA) in tyrosine phosphatase-deficient mice.[209] miR-17-92 is also reported to participate in the activation of T cells and it is considered a main factor for differentiation of Tfh, essential for antibodies production. [210] MiR-17-92 cluster has been found to be remarkably upregulated in the peripheral blood CD4+ T cells of patients with systemic lupus erythematosus, multiple sclerosis, or bronchial asthma. [209] Even though this co-transcribed cluster of miRNAs appears to act mainly facilitating inflammation and immune response activation, conversely miR-92 has been reported to suppress inflammatory responses by targeting the MKK4 kinase, subsequently reducing TNF-a and IL-6 production. [209] Interestingly, miR-92-3p has also been described as one of the main differentially expressed miRNAs in idiopathic pulmonary fibrosis and is considered a potential therapeutic target for the treatment of this disease.[211]

CONCLUSIONS

This study provides significant evidence to speculate the involvement of EVs in the patho-physiology of IIMs. We report significant differences in circulating EVs levels between patients and healthy donors and among specific disease subsets. Peripheral EVs concentrations varied significantly in relation to the presence of autoantibodies, association with cancer, disease activity and treatment regimens adopted. Considering the heterogeneous nature of IIMs and the numerous areas of uncertainty in IIM patho-physiology, our results provide interesting cues that might contribute to further the understanding of this complex group of diseases. Furthermore, the differences in EV levels are promising to propose novel potential biomarkers for IIM diagnosis and screening for associated malignancies. The investigation of EV-miRNAs cargo highlighted several specific miRNAs with altered expression patterns in IIM patients compared to healthy donors that significantly differentiate different IIM subsets as well. Several of these specific miRNAs are recognized as relevant actors in the regulation of complex biological processes like inflammation, innate and adaptive immunity, abnormal tissue fibrosis and myocellular regeneration and might be proven to be crucially involved in different aspects of IIM pathogenesis. Even though functional assays are required to further characterize the specific role of these EV-miRNAs in IIM, our results provide important preliminary data that could support the developement of novel biomarkers for an earlier diagnosis, more precise disease subtype assessment, and accurate management of IIM patients.

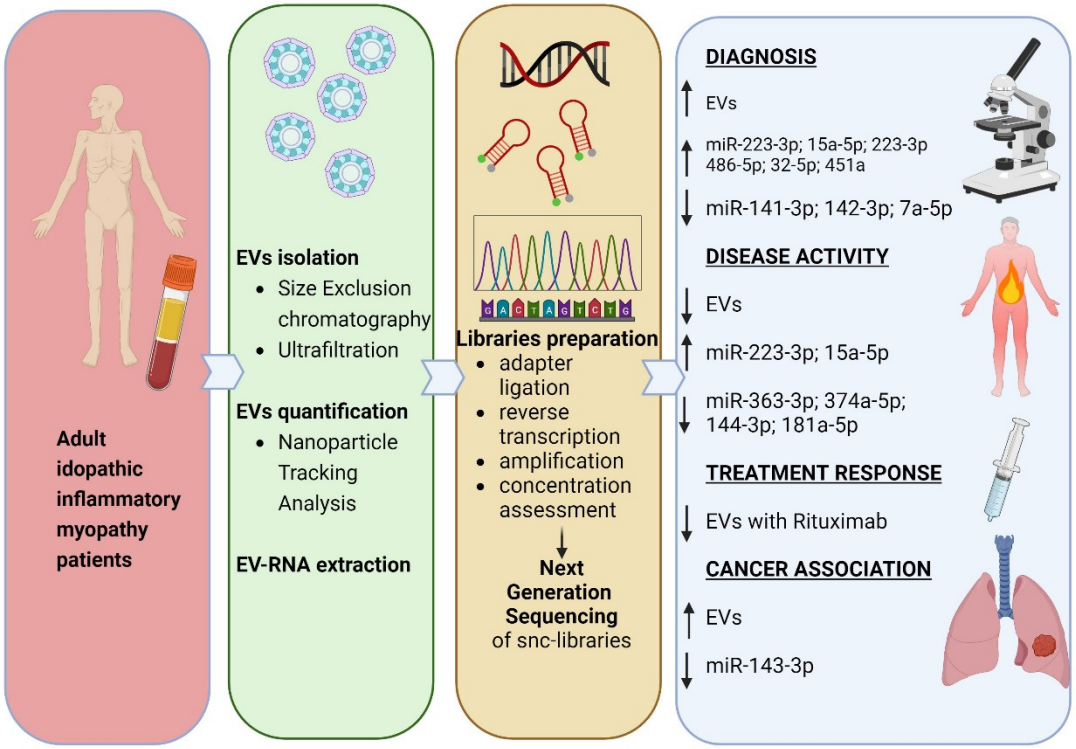

Figure 19 Graphical abstract, summarizing the methods and results of the present study.

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