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TESI DI LAUREA

CHARACTERIZATION AND CLINICAL CORRELATES OF EXTRACELLULAR VESICLES IN IDIOPATHIC INFLAMMATORY MYOPATHIES: DATA FROM A MONOCENTRIC COHORT

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ABSTRACT

Background: Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of rare and chronic immune-mediated diseases for which few options of disease markers are available to date.

Extracellular vesicles (EV) are ubiquitously expressed subcellular nanoparticles that play a key role in cell-to-cell signaling and modulation of the immune response. Growing evidence suggests that circulating EV may be considerably altered across several autoimmune conditions, yet their role in IIM is still unclear.

Aim of the Study: This study aimed at characterizing the circulating EV pool and at exploring EV potential as a disease and treatment biomarker in the context of IIM. Specifically, we aimed at developing an innovative and reliable methodology for the isolation and characterization of plasma EV and at investigating correlates between features of circulating EV and clinical and laboratory parameters of IIM patients.

Patients and Methods: Adult (≥18 years old) IIM patients consecutively followed-up at the Rheumatology Unit of Padua University Hospital and age- and sex-matched healthy donors (HD) were included in this single center observational study. IIM diagnosis was performed by an experienced rheumatologist on clinical, laboratory and histology data. Upon obtainment of informed consent, a 5 ml whole blood sample was collected in sodium-citrate tubes from every participant.

EV were isolated through size exclusion chromatography (SEC) and subsequent ultra-filtration; particles' morphology was observed via transmission electron microscopy (TEM). Nanoparticle tracking analysis (NTA) was performed to evaluate the concentration and size of EV. The characterization of EV surface markers was conducted through imaging flow cytometry (IFC).

Clinical and laboratory data were retrieved from the intra-hospital network health records. Data were analyzed in a cross-sectional fashion; parametric Student-t test and one-way ANOVA with Bonferroni correction tests were used for comparisons.

Results: We included 45 IIM patients (female:male ratio 1.8:1; mean age±SD 60.0±12.7 years) and 45 age and sex-matched HD (female:male ratio 3:1; mean age±SD 56.6±14.2 years). Fourteen patients (31.1%) were affected with dermatomyositis; 8 with polymyositis (17.8%); 2 with inclusion body myositis

(4.4%); 8 with cancer-associated myositis (CAM,17.8%); 12 with anti-synthetase syndrome (22.2%); 3 with unspecified myositis (6.7%).

Isolated EV concentration values reached ~10^{10} EV/mL at NTA. TEM images confirmed the intact and roundish morphology of EV and IFC highlighted expression of EV surface markers CD63, CD81, and CD9.

IIM patients displayed significantly increased EV concentrations compared to healthy controls ($1.95 \times 10^{10} \pm 1.47 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 7.82 \times 10^{9}$; $p = 0.025$). Interstitial lung disease (ILD) or CAM was associated with significantly higher EV concentrations compared to HD only or to HD and non-CAM patients, respectively (ILD vs HD: $2.12\times10^{10}\pm1.61\times10^{10}$ vs $1.45\times 10^{10}\pm0.78\times10^{10}$, $p = 0.021$; CAM vs non-CAM: $3.28\times10^{10}\pm2.84\times10^{10}$ vs $1.67\times10^{10}\pm7.7\times10^{9}$, $p = 0.004$; CAM vs HD $3.28\times10^{10}\pm2.84\times10^{10}$ vs. $1.45\times10^{10}\pm0.78\times10^{10}$ $p<0.001$).

Moreover, EV concentrations were significantly higher in patients with a shorter (≤6 months) disease duration (short duration vs long duration: $3.20\times10^{10}\pm2.42\times10^{10}$ vs $1.80\times10^{10}\pm1.80\times10^{10}$; $p = 0.042$) and in treatment-naïve patients compared to HD ($2.83\times10^{10}\pm2.89\times10^{10}$ vs $1.45\times10^{10}\pm0.78\times10^{10}$; $p = 0.010$), which tended to be numerically decreased in IIM patients undergoing immunosuppressive treatment ($2.83\times10^{10}\pm2.89\times10^{10}$ vs $1.81\times10^{10}\pm1.12\times10^{10}$; p=ns). Patients bearing an antibody positivity showed numerically increased levels of circulating EV (seropositive vs seronegative: $2.09\times10^{10}\pm1.63\times10^{10}$ vs $1.49\times10^{10}\pm0.63\times10^{10}$; $p = 0.063$).

Conclusions: Our study supports the reliability of the protocol exploited for the isolation and characterization of plasma EV both in IIM patients and in HD.

We show that IIM patients display significantly increased concentrations of circulating EV compared to HD, especially in recent-onset, seropositive and treatment-naïve patients.

In summary, our findings provide evidence of remarkable abnormalities in the circulating EV pool in IIM patients which may associate with disease phenotypes and treatment response, thereby substantiating the role of plasma EV as a potential biomarker in the surveillance of IIM patients.

RIASSUNTO

Background: Le miopatie infiammatorie idiopatiche (IIM) sono un gruppo eterogeneo di rare malattie autoimmuni per cui ad oggi vi è scarsità di biomarker.
Le vescicole extracellulari (EV) sono nanoparticelle ubiquitariamente espresse implicate nel signaling intercellulare. Dati recenti suggeriscono che le EV circolanti siano alterate in diverse condizioni autoimmuni; tuttavia, il loro ruolo nelle IIM non è ancora stato chiarito.

Scopo dello studio: Questo studio mira a caratterizzare il pool di EV circolanti e ad esplorare il potenziale delle EV come biomarcatore di malattia e di trattamento nel contesto delle IIM. In particolare, intendiamo sviluppare una metodologia innovativa ed affidabile per l'isolamento e la caratterizzazione delle EV plasmatiche e indagare le correlazioni tra le caratteristiche delle EV circolanti e i parametri clinici e di laboratorio dei pazienti affetti da IIM.

Materiali e metodi: In questo studio osservazionale monocentrico sono stati inclusi pazienti adulti (≥18 anni) affetti da IIM in follow-up presso l'U.O.C. di Reumatologia dell'Azienda Ospedaliera di Padova e donatori sani (HD) appaiati per età e sesso. La diagnosi di IIM è stata effettuata da un reumatologo esperto sulla base di dati clinici, di laboratorio e istologici. Previo consenso informato, è stato prelevato un campione di 5ml di sangue intero da ogni soggetto.
Le EV sono state isolate mediante cromatografia ad esclusione dimensionale (SEC) e successiva ultrafiltrazione; la morfologia delle particelle è stata osservata mediante microscopia elettronica a trasmissione (TEM). L'analisi di tracciamento delle nanoparticelle (NTA) è stata eseguita per valutare la concentrazione e le dimensioni delle EV. La caratterizzazione dei marcatori di superficie delle EV è stata condotta mediante citofluorimetria (IFC).
I dati clinici e di laboratorio sono stati ottenuti dalle cartelle cliniche della rete intraospedaliera. I dati sono stati analizzati in modo trasversale; per i confronti sono stati utilizzati i test parametrici t di Student e one-way ANOVA con correzione di Bonferroni.

Risultati: Sono stati inclusi 45 pazienti con IIM (rapporto femmine/maschi 1,8:1; età media±SD 60,0±12,7 anni) e 45 HD appaiati per età e sesso (rapporto femmine/maschi 3:1; età media±SD 56,6±14,2 anni). Quattordici pazienti (31,1%)

erano affetti da dermatomiosite; 8 da polimiosite (17,8%); 2 da miosite a corpi inclusi (4,4%); 8 da miosite associata al cancro (CAM, 17,8%); 12 da sindrome anti-tRNAsintetasi (22,2%); 3 da miosite non specificata (6,7%).

I valori di concentrazione delle EV isolate hanno raggiunto ~10^{10} EV/mL al NTA. Le immagini TEM hanno confermato la morfologia intatta e tondeggiante delle EV e l'IFC ha evidenziato l'espressione dei marcatori di superficie delle EV, CD63, CD81 e CD9. I pazienti affetti da IIM hanno mostrato concentrazioni di EV significativamente maggiori rispetto ai controlli sani ($1.95 \times 10^{10} \pm 1.47 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 7.82 \times 10^{9}$; $p = 0.025$). La presenza di interstiziopatia polmonare (ILD) o di CAM è stata associata a concentrazioni di EV significativamente più elevate rispetto ai soli HD o rispetto a HD e pazienti non-CAM, rispettivamente (ILD vs HD: $2{,}12\times10^{10}\pm1{,}61\times10^{10}$ vs $1{,}45\times10^{10}\pm0{,}78\times10^{10}$, $p = 0{,}021$; CAM vs non-CAM: $3{,}28\times10^{10}\pm2{,}84\times10^{10}$ vs $1{,}67\times10^{10}\pm7{,}7\times10^{9}$, $p = 0{,}004$). Inoltre, le concentrazioni di EV erano significativamente più elevate nei pazienti con una durata della malattia più breve (≤6 mesi) (breve durata vs lunga durata: $3{,}20\times10^{10}\pm2{,}42\times10^{10}$ vs $1{,}80\times10^{10}\pm1{,}80\times10^{10}$; $p = 0{,}042$) e nei pazienti naïve al trattamento rispetto ai HD ($2{,}83\times10^{10}\pm2{,}89\times10^{10}$ vs $1{,}45\times10^{10}\pm0.\ 78\times10^{10}$; $p = 0{,}010$). Minori differenze sono state registrate tra pazienti trattati e HD ($1{,}81\times10^{10}\pm1{,}24\times10^{10}$ vs $1{,}45\times10^{10}\pm0{,}78\times10^{10}$; $p = 0{,}084$). I pazienti con positività anticorpale hanno mostrato livelli numericamente maggiori di EV circolanti (sieropositivi vs sieronegativi: $2{,}09\times10^{10}\pm1{,}63\times10^{10}$ vs $1{,}49\times10^{10}\pm0{,}63\times10^{10}$; $p = 0{,}063$).

Conclusioni: Il nostro studio supporta l'affidabilità e la validità del protocollo adottato per l'isolamento e la caratterizzazione delle EV plasmatiche sia nei pazienti affetti da IIM che nei controlli sani. Abbiamo dimostrato che i pazienti affetti da IIM presentano concentrazioni significativamente maggiori di EV circolanti rispetto ai controlli sani, soprattutto nei pazienti di recente insorgenza, sieropositivi e naïve al trattamento. In sintesi, i nostri risultati forniscono evidenza di notevoli anomalie nel pool di EV circolanti nei pazienti affetti da IIM che possono associarsi ai fenotipi di malattia diversi e alla risposta al trattamento, avvalorando così il ruolo delle EV plasmatiche come potenziali biomarcatori nel monitoraggio dei pazienti affetti da IIM.

1. INTRODUCTION

1.1 IDIOPATHIC INFLAMMATORY MYOPATHIES

1.1.1 DEFINITION

Idiopathic inflammatory myopathies (IIM) are a highly heterogenous group of rare and chronic conditions that share the common feature of immune-mediated injury primarily targeting the striated muscle.

Due to the differentiated connective nature of the muscular tissue, IIM fall under the broader category of autoimmune connective tissue diseases.

IIM generally present as a chronic and progressive weakness that predominantly affects the proximal muscles in a symmetrical fashion. It is variably accompanied by extramuscular manifestations typically involving the skin, joints and the pulmonary interstitium, potentially leading to severe disability and life-threatening complications. (1)

Although a classical pattern of presentation exists, IIM heterogeneity is rooted in the presence of several disease subtypes that display a vast array of diverse clinical manifestations, histopathologic findings as well as serologic profiles and treatment responses.

1.1.2 CLASSIFICATION

The elaboration of a widely accepted and reliable classification has proven to be an all-time challenging task.

As a result, the use of these criteria is limited to research purposes, with the sole intention of providing uniformity throughout clinical trials.

The current *EULAR/ACR classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups* were developed in 2017 and recognize the following subtypes: polymyositis (PM); inclusion-body myositis (IBM) dermatomyositis (DM); amyopathic dermatomyositis (ADM); juvenile dermatomyositis (JDM); non-JDM juvenile myositis.(2)

Due to the insufficient sample size, the following subgroups could not be acknowledged as independent entities: immune-mediated necrotizing myositis (IMNM) that falls under the spectrum of PM; hypomyopathic dermatomyositis; juvenile polymyositis (JPM). Neither cancer-associated myositis (CAM) nor anti-synthetase syndrome (ASyS) were included.

Although less performing in the context of rarer subsets, these criteria were shown to reach a maximum sensitivity of 93% and a specificity of up until 88% providing that the histology is available (otherwise restricted to 87% and 82%).

Indeed, clinical, serologic and histopathologic findings are accounted for as 16 variables are assessed through a web-based calculator which assigns a likelihood score allowing for the stratification of patients into “definite”, “probable” and "possible" IIM. *(See Table 1)*

A classification algorithm is also provided.

(See Figure 1)

Table 1 . EULAR/ACR classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups.

Table 2 The European League Against Rheumatism/American College of Rheumatology (EULAR/ACR) classification criteria for adult and juvenile idiopathic inflammatory myopathies (IIMs)

When no better explanation for the symptoms and signs exists, these classification criteria can be used

Variable	Score points		
	Without muscle biopsy	With muscle biopsy	Definition
Age of onset			
Age of onset of first symptom assumed to be related to the disease ≥18 years and <40 years	1.3	1.5	18≤Age (years) at onset of first symptom assumed to be related to the disease <40
Age of onset of first symptom assumed to be related to the disease ≥40 years	2.1	2.2	Age (years) at onset of first symptom assumed to be related to the disease ≥40
Muscle weakness			
Objective symmetric weakness, usually progressive, of the proximal upper extremities	0.7	0.7	Weakness of proximal upper extremities as defined by manual muscle testing or other objective strength testing, which is present on both sides and is usually progressive over time
Objective symmetric weakness, usually progressive, of the proximal lower extremities	0.8	0.5	Weakness of proximal lower extremities as defined by manual muscle testing or other objective strength testing, which is present on both sides and is usually progressive over time
Neck flexors are relatively weaker than neck extensors	1.9	1.6	Muscle grades for neck flexors are relatively lower than neck extensors as defined by manual muscle testing or other objective strength testing
In the legs, proximal muscles are relatively weaker than distal muscles	0.9	1.2	Muscle grades for proximal muscles in the legs are relatively lower than distal muscles in the legs as defined by manual muscle testing or other objective strength testing
Skin manifestations			
Heliotrope rash	3.1	3.2	Purple, lilac-coloured or erythematous patches over the eyelids or in a periorbital distribution, often associated with periorbital oedema
Gottron's papules	2.1	2.7	Erythematous to violaceous papules over the extensor surfaces of joints, which are sometimes scaly. May occur over the finger joints, elbows, knees, malleoli and toes
Gottron's sign	3.3	3.7	Erythematous to violaceous macules over the extensor surfaces of joints, which are not palpable
Other clinical manifestations			
Dysphagia or oesophageal dysmotility	0.7	0.6	Difficulty in swallowing or objective evidence of abnormal motility of the oesophagus
Laboratory measurements			
Anti-Jo-1 (anti-histidyl-tRNA synthetase) autoantibody present	3.9	3.8	Autoantibody testing in serum performed with standardised and validated test, showing positive result
Elevated serum levels of creatine kinase (CK)* *or* lactate dehydrogenase (LD)* *or* aspartate aminotransferase (ASAT/AST/SGOT)* *or* alanine aminotransferase (ALAT/ALT/SGPT)*	1.3	1.4	The most abnormal test values during the disease course (highest absolute level of enzyme) above the relevant upper limit of normal
Muscle biopsy features—presence of:			
Endomysial infiltration of mononuclear cells surrounding, but not invading, myofibres		1.7	Muscle biopsy reveals endomysial mononuclear cells abutting the sarcolemma of otherwise healthy, non-necrotic muscle fibres, but there is no clear invasion of the muscle fibres
Perimysial and/or perivascular infiltration of mononuclear cells		1.2	Mononuclear cells are located in the perimysium and/or located around blood vessels (in either perimysial or endomysial vessels)
Perifascicular atrophy		1.9	Muscle biopsy reveals several rows of muscle fibres, which are smaller in the perifascicular region than fibres more centrally located
Rimmed vacuoles		3.1	Rimmed vacuoles are bluish by H&E staining and reddish by modified Gomori trichrome stains

*Serum levels above the upper limit of normal.

Table 2 . EULAR/ACR classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups. Lundberg I et al., Arthritis & Rheumatology, 2017. (3)

Figure 1. EULAR/ACR classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups.

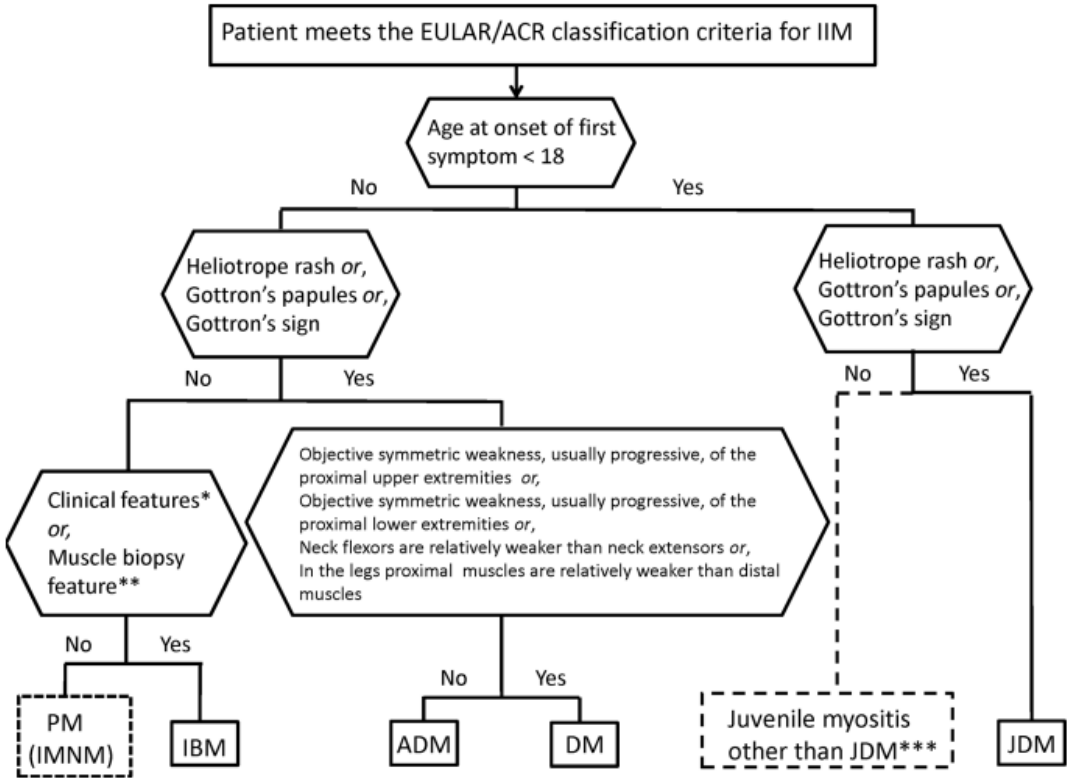

Figure 2 Classification tree for subgroups of IIM. A patient must first meet the EULAR/ACR classification criteria for IIM (probability of IIM ≥55%). The patient can then be subclassified using the classification tree. The subgroup of PM patients includes patients with IMNM. For IBM classification, one of the following, *finger flexor weakness and response to treatment: not improved, or **muscle biopsy: rimmed vacuoles, is required for classification. ***Juvenile myositis other than JDM was developed based on expert opinion. IMNM and hypomyopathic DM were too few to allow subclassification. ACR, American College of Rheumatology; ADM, amyopathic dermatomyositis; DM, dermatomyositis; EULAR, European League Against Rheumatism; IBM, inclusion body myositis; IIM, idiopathic inflammatory myopathies; IMNM, immune-mediated necrotising myopathy; JDM, juvenile dermatomyositis; PM, polymyositis.

Figure 2. EULAR/ACR classification criteria for adult and juvenile idiopathic inflammatory myopathies and their major subgroups. Lundberg I et al., Arthritis & Rheumatology, 2017.(3)

The EULAR/ACR criteria replaced the former Bohan and Peter classification criteria (dating back to 1975) that had increasingly fallen out of favor due to the extensive limitations in the pathological entities identified (only including PM, DM, CAM and overlap myositis) and the failure to encompass key extramuscular manifestations such as interstitial lung disease (ILD). Moreover, they heavily relied on experts' opinions and were derived exclusively from monocentric studies. (4)

The Bohan and Peter's classification focuses on the evaluation of five criteria: 1) symmetrical proximal muscle weakness upon physical examination; 2) cutaneous eruption consistent with DM; 3) elevation of serum muscle enzymes; 4) myopathic

changes on electromyography (EMG); 5) characteristic muscle biopsy abnormalities and the absence of histopathological signs of other myopathies. (5)(6) As a result, they can formulate the diagnosis of: 1) definite DM if at least four criteria were met including the dermatological one; 2) definite PM in the presence of all four criteria except for the dermatological one; 3) possible or probable DM or PM when no exclusion criteria were met yet not all necessary criteria were positive.

The sensitivity reaches 94% at the expense of a 29% specificity.

Table 3. Recent developments in classification criteria and diagnosis guidelines for idiopathic inflammatory myopathies.

Criteria	Description
A	Proximal and symmetrical muscle weakness of the pelvic and scapular girdle, anterior flexors of the neck, progressing for weeks to months, with or without dysphagia or involvement of respiratory muscles
B	Elevation of the serum levels of skeletal muscle enzymes: creatine kinase, aspartate aminotransferase, lactate dehydrogenase and aldolase
C	Electromyography characteristic of myopathy (short and small motor units, fibrillation, positive pointy waves, insertional irritability and repetitive high-frequency firing)
D	Muscle biopsy showing necrosis, phagocytosis, regeneration, perifascicular atrophy, perivascular inflammatory exudate
E	Typical cutaneous changes: (1) Heliotrope rash with periorbital oedema and violaceous erythema (2) Gottron's sign: vasculitis in the elbow, metacarpophalangeal and proximal interphalangeal joints
Polymyositis	(1) Definite – all of A–D (2) Probable – any three of A–D (3) Possible – any two of A–D
Dermatomyositis	(1) Definite – E plus and three of A–D (2) Probable – E plus and two of A–D (3) Possible – E plus and one of A–D

Table 4. Recent developments in classification criteria and diagnosis guidelines for idiopathic inflammatory myopathies. Oldroyd, A. et al, Current Opinion in Rheumatology, 2018. (7)

It was not until 2003 that revised diagnostic criteria were introduced by Dalakas and Holhfeld, which emphasize histology and immunologic pathology data to discriminate between IIM subgroups.(8)

A comparison between former IIM classification models is reported herein.

Table 5. The clinical features, diagnosis and classification of dermatomyositis.

Comparison among three classification criteria.

Authors [Ref]	Point of strength	Concerns	Sensitivity[a]	Specificity[a]
Bohan & Peter 1975 [5,6]	Useful as screening tool. Used until now for enrolling patients in clinical trial.	Unable to differentiate PM from IBM or dystrophies. Imaging and autoantibodies are not considered.	0.943	0.294
Dalakas & Hohfeld 2003 [3]	Very high specificity. Patients with ADM are considered. Definition of histological features is precise.	Low sensitivity of some items. Imaging and autoantibodies are not considered.	0.771	0.999
ENMC 2004 [30]	Imaging and autoantibodies are considered. Definition of histological features is precise.	Overlap and paraneoplastic DM are not considered. Complex to use in clinical practice.	0.714	0.824

DM: dermatomyositis; PM: polymiositis; IBM: inclusion body myopathy; ADM: amyopathic dermatomyositis; ENMC: Amato/European Neuromuscular Centre Workshop.
[a] Sensitivity and specificity are reported by Linklater Het al. [22].

Table 6. The clinical features, diagnosis and classification of dermatomyositis. Iaccarino L et al., Journal of Autoimmunity, 2014. (9)

None of the abovementioned criteria accounted for anti-synthetase syndrome (ASyS), a recently recognized IIM subtype characterized by the presence of autoantibodies (e.g., antiJo1, antiPL7, antiPL12, OJ, EJ, Ks, Ha, Zo) directed against one of many aminoacyl-tRNA synthetases configuring a peculiar clinical picture.

Indeed, ASyS stands out for the occurrence of the so-called mechanic's hand and Raynaud's phenomenon alongside the classical clinical triad that entails a high burden of ILD (often more severe and rapidly progressive), non-erosive arthritis and myositis. Skin rashes, sicca syndrome and constitutional symptoms may concur as well. (10) However, only 19% of cases exhibit the full-on clinical triad at the disease onset, the most common presentation being an isolated manifestation followed by a second or third triad component within one year, making it extremely challenging to achieve an early and correct diagnosis.

The precise serologic profile may aid the diagnosis and provide useful information about the overall disease expression and prognosis of these patients.

Considering all of the above, two main nosographic classifications have been proposed, namely the Connors' (2010) and the Solomon's (2011) criteria.(11) (12) The Connors' criteria for ASyS are centered around the necessary evidence for a tRNA synthetase autoantibody and at least another clinical symptom whilst

Solomon's criteria are renowned for being stricter, requiring at least two major or one major and two minor criteria on top of the positive serology.

Table 7. The Diagnosis and Treatment of Antisynthetase Syndrome.

Connors et al. (2010) [1]	Solomon et al (2011) [10]
Required: Presence of an anti-aminoacyl tRNA synthetase antibody	**Required:** Presence of anti-aminoacyl tRNA synthetase antibody
PLUS *one or more* of the following clinical features:	**PLUS** *two major* or *one major and two minor criteria*:
• Raynaud's phenomenon	Major:
• Arthritis	1. Interstitial Lung Disease (not attributable to another cause)
• Interstitial lung disease	2. Polymyositis or dermatomyositis by Bohan and Peter critieria
• Fever (not attributable to another cause)	
• Mechanic's hands (thickened and cracked skin on hands, particularly at fingertips)	Minor:
	1. Arthritis
	2. Raynaud's phenomenon
	3. Mechanic's hands

Table 8. The Diagnosis and Treatment of Antisynthetase Syndrome. Witt L et al., Clinical Pulmonary Medicine, 2016. (13)

Likewise, Overlap Myositis (OMs) had long been overlooked until in 2005 Troyanov et alii (14) identified this new clinical and serologic entity resulting from the simultaneous intersection of at least one overlap clinical manifestation and overlap antibody positivity.

In fact, IIM can be accompanied by or appear over the course of other related rheumatic diseases, above all systemic sclerosis (SSc) and systemic lupus erythematosus (SLE). Various degrees of overlap are observed in 11% of patients.

IMNM, instead, made its debut in 2003 (along with non-specific myositis) on the occasion of the ENMC international workshop where its peculiar histologic features

of necrotic myofibers in the relative absence of inflammatory infiltration were finally acknowledged with classification dignity. (15)

1.1.3 EPIDEMIOLOGY

IIM are considered rare diseases having a prevalence ranging from 3 to 34 cases per 100.000 inhabitants and an incidence between 11 and 660 patients per a million person-years.(16)

Despite the significant increase in the reported incidence of IIM, it remains unclear which proportion of the newly registered cases should be attributed to a real rise in the disease frequency rather than to the improved diagnostic capabilities over the years.

Akin to most other autoimmune diseases, IIM displays a preference for the female gender with an overall female to male ratio of about 2:1 with the remarkable exceptions of IBM and CAM where the male to female ratios are found to be 3:1 and 2:1 respectively. Interestingly, gender preferences appear to be strongly attenuated in the setting of juvenile forms. (9)

A North to South geographic gradient has been observed, probably suggesting a role of vitamin D deficiency and/or UV exposure in the development of the disease.

Unlike IBM that displays a mean age at disease onset of 67 years, IIM can be found among all age groups with adult DM and PM reporting a mean age at disease onset of 52 and 56 years, respectively.

1.1.4 ETIOLOGY

The etiology of IIM varies greatly across different subtypes but does always recognize a complex interplay of both genetic and environmental factors as the main cause of these disorders.

Studying the risk factors for IIM presents many obstacles due to the rarity of the diseases in addition to the inconsistencies in the classification criteria having resulted in major misclassifications and general incompatibility of data over time.

GENETIC FACTORS

Compelling evidence from the *Myositis Genetics Consortium*'s study (boasting an unprecedented sample size of 2.556 Caucasian patients) points to human leukocyte antigen (HLA) genes on chromosome 6 being the strongest genetic risk factors. (17)

Specific HLA-alleles of the 8.1 ancestral haplotype (8.1AH) are arguably associated with characteristic clinical phenotypes, e.g., HLA-DRB1*03:01 and HLA-B*08:01alleles to PM and DM respectively.

However, such links might be mediated by the even stronger association that exists between HLA alleles and the serologic profile exhibited by the patient which, in turn, tightly correlates with the clinical phenotype.

Moreover, a few other non-HLA loci, either through exonic or eQTL SNPs, may determine a higher risk of IIM: e.g., PM has been tied to PTPN22 (involved in TCR signal transduction), IL18R1 (belonging to the IL1R family) and RGS1 (a regulator of G-protein signaling); DM is associated with GSDMB (Gasdermin-B whose gene family is implicated in cancer and apoptosis regulation in epithelial cells) while other studies link it to PLCL1(phospholipase C-like 1) and BLK (nonreceptor tyrosine-kinase of the src family of proto-oncogenes also playing a role in B-cell receptor signaling and B-cell development); IBM is matched to CCR5 (also known as co-receptor for entry of macrophage-tropic viruses like HIV), VCP along with SQSTM1 and FYCO1 which suggest autophagy dysregulation. Other significantly mutated genes to be mentioned are STAT4 (for TCR signaling transduction) TRAF6 and UBE2L3 (both involved in NF-kB pathway in B cells).

Overlap forms are plausibly due to a shared immunopathologic background.

ENVIRONMENTAL FACTORS

Current opinion regarding the environmental factors of IIM views them as the trigger for an underlying genetic predisposition. (16)

Many triggers have been recognized over the years, the paramount ones being viral infections, statin use (along with other drugs), smoke, UV radiation and exposure to dust or organic solvents.

The pathogenetic role of viruses in IIM seems to be independent from a direct infection of the muscle tissue but rather appears to be mediated by other indirect mechanisms such as cross-reactivity. This hypothesis is corroborated by the absence of viral sequences in myositis muscles and by the documented co-occurrence of hepatitis B virus infection in PM patients. Additionally, HIV and HTLV1 retroviruses were associated to a consistent number of PM, DM, and IBM cases whereby virus-specific $CD8^+$ T lymphocyte clones were found to be responsible for muscle invasion.

As far as lipid-lowering agents are concerned, it is important not to confuse the temporary effects of the direct myotoxicity commonly encountered in the daily clinical practice with the long-standing and irreversible damages of IMNM. As a matter of fact, either a brief or a chronic use of lipid-lowering agents is thought to powerfully upregulate the ectopic expression of HMGCR on the cell membrane leading, in turn, to the loss of immune tolerance. In overt IMNM the immune response is no longer driven by the drug exposure and progresses despite statin discontinuation. Up to 65% of HMGCR-positive IMNM patients have a history of statin exposure.

Other medications such as TNF-inhibitors, IFN-α and IFN-β alongside checkpoint-inhibitors are suspected to be involved in the development of IIM although it is often difficult to distinguish the effect of the drug from the disease itself that required the drug in the first place.

Smoking is also a prominent trigger of ASyS possibly mediated by an increase in anti-Jo1 antibody positivity.

UV radiation may be to blame for an increased risk of DM on account of the photo-sensitive induction of Mi2 expression in keratinocytes, potentially at the base of the development of anti-Mi2 antibodies positivity.

Conversely, low levels of immune-modulating vitamin D are seen to be anticipating a diagnosis of DM, PM or IBM in the majority of such patients.

Interestingly, a significant rise in IIM was noticed amid World Trade Center survivors and rescuers arguably due to the extensive exposure to dust particles.

A higher risk of IIM is also found in patients with prior occupational exposure to organic solvents.

A considerable proportion of IIM is either closely preceded or followed by the diagnosis of a neoplasm (usually within two years) with a higher occurrence in DM (25%) especially among elderly males. These entail lung, breast, ovary and stomach cancer alongside non-Hodgkin lymphomas.

1.1.5 PATHOGENETIC MECHANISMS

The IIM share the feature of immune-mediated muscle injury although the precise mechanisms underpinning muscle injury are incompletely understood and vary widely across different subtypes.

DM PATHOGENESIS

An earlier pathogenic model has for years explained the dermatomyositis pathogenesis and it stemmed from the distinct histopathological features observed in these patients such as the perifascicular myofiber atrophy alongside the capillary and perimysial abnormalities. (9)

In this model an antibody and complement-mediated microangiopathy (with early deposition of membrane attack complexes -MACs- on endothelial cells) determines ischemia and microinfarctions consequently resulting into perifascicular myofiber atrophy.

The regenerating muscle fibers would then go on to express surface antigens associated with regeneration (i.e., MYH8, vimentin, N-CAM upon immunohistochemistry testing) including adhesion molecules that further trigger the infiltration of macrophages, lymphocytes and dendritic cells into the muscle tissue.
A major humoral component was supported by considerable B lymphocyte infiltrates yet myositis specific antibodies (MSAs) are not necessary to IIM genesis.

However, a growing body of evidence based on transcriptomic and proteomic data points to IFN-β being the keystone of DM pathogenesis. (18–20)

In this paradigm shift, fibroblasts, myofibers and keratinocytes are responsible for the massive and sustained production of type 1 IFN (IFN-β in particular) leading to intracellular accumulation of type 1 IFN-inducible transcripts and proteins. This causes injury to myofibers and keratinocytes through ill-defined mechanisms which may include the endoplasmic reticulum overload response (EOR) and the unfolded protein response (UPR). The ER stress may also lead to oxidative damage through the production of reactive oxygen species (ROS) by the dysregulated mitochondria in a calcium-rich intracellular environment. The immune response is further amplified by the release of pro-inflammatory cytokines.(21)

It is worth emphasizing that type1 IFN-inducible transcripts are the single most upregulated pathway in the muscle of DM patients. (8)

This could explain the efficacy of JAK-STAT inhibitors in the treatment of DM as they target this pathway.

PM PATHOGENESIS

The lack of uniform classification criteria for IIM has paved the way for the incompatibility of data amongst research groups, ultimately undermining our comprehension of the pathogenesis of polymyositis.

As a matter of fact, PM has historically been the only known alternative to DM and all the subsequent pathological entities within the IIM spectrum gradually branched off from it.

Nevertheless, the primary mechanism underpinning PM and IBM pathogenesis seems to be $CD8^+$ cytotoxic T cell invasion of myofibers.

$CD8^+$ T cells unexplainably migrate within the muscle to target MHC-I expressing myofibers. They then gain access to the myofiber through the release of metalloproteinases and perforin granules that injure the myofiber to necrosis.(16)

IBM PATHOGENESIS

Inclusion-body myositis' pathogenesis has not been fully elucidated yet, partly due to the lack of valid animal models for this disease.(22)

However, genetic studies highlight a significant association with HLA DRB1*03:01 and HLA-B*08:01.

Additionally, histology data show the presence of highly differentiated cytotoxic T cells, myeloid dendritic cells and macrophages surrounding and invading myofibers.(23) The clonal expansion of plasma cells (and that of cytotoxic T cells) within the muscle tissue, hints at the antigen stimulation being the primary cause of such proliferation leading most patients to have a positive serology for anti-CN1A antibodies (against the 5' cytosolic nucleotidase IA).

The abovementioned $CD8^+$ cytotoxic T cells display a particular asset of CD markers ($CD28^-$ and $CD5^-$; $CD16^+$, $CD94^+$ and $CD57^+$) consistent with a terminal differentiation in reaction to unidentified specific antigens which are often shared among various IBM patients.(24,25)

This exact population of T cells is also clonally expanded in the peripheral blood resulting in the frequent overlap with the diagnosis of T cell large granular lymphocytic leukemia (TLGLL). This seems coherent with their shared glucocorticoid resistance due to the apoptosis resistance granted by such differentiation profile.

Interestingly, rimmed vacuoles and P62 and TDP43 protein inclusions are a hallmark of this subtype, insinuating the existence of a degenerative component to

IBM, conceivably mediated by the ER stress response to these aggregates whose toxicity still needs to be confirmed by further investigations.(26)

IMNM PATHOGENESIS

There are three subtypes to the immune-mediated necrotizing myositis: seronegative, HMGCR-positive and SRP-positive IMNM, the first one having the most obscure pathogenesis. (27)

Indeed, the seropositive forms demonstrate an antibody- and complement-mediated myofiber injury with substantial MAC deposition and MHC-I exposure along the sarcolemma of non-necrotic myofibers.

Both anti-SRP and anti-HMGCR autoantibody titers are in close correlation with muscle weakness and elevated CPK. In addition, their passive transfer to immunodeficient mice is a sufficient condition to generate the disease despite a wild-type complement system.

The humoral response is mounted against SRP (signal recognition particle) and HMGCR (3-hydroxy-3-methyl-glutaryl-coenzyme A reductase) which are proteins ectopically localized on the cell membrane of these patients.

Anti-HMGCR positive IMNM potentially arises when a genetically susceptible individual (associated with MHCII allele DRB1*11:01) upregulates HMGCR especially after being exposed to statin drugs. The trigger for the other subtypes is less understood. (28)

CAM PATHOGENESIS

At least two main hypotheses may provide a viable explanation to the association between cancer and IIM and they are not mutually exclusive.

Indeed, the exposure on the cell membrane of neo-antigens either by the neoplastic cells or the regenerating myofibers may initiate the immune response by molecular mimicry.

Moreover, the loss of self-tolerance may be triggered by the prolonged antigen presentation or the post-translational modification of self-proteins in the context of an altered tumor microenvironment (TME).(29)

The myopathy could be then regarded on a par with a paraneoplastic manifestation as corroborated by the subsequent regression of the IIM after tumor remission. (30)

ASyS PATHOGENESIS

The mechanisms underlying anti-synthetase syndrome still need to be clarified.

Nonetheless, reactivity against one of many aminoacyl-tRNA-synthetases seems to be the cornerstone of ASyS pathogenesis, hence its name. (10)

Even though clonally expanded T $CD4^+$ lymphocytes in both blood and lungs were found to be reactive against histidyl-tRNA synthetase, the development of autoantibodies against the various isoforms of this enzyme remains the defining feature of ASyS.(31)

Indeed, said autoantibodies (whose primary example is anti-Jo1) target self-epitopes whose titers seem in tune with disease activity and may predate the diagnosis by months.

Interestingly, immunization with histidyl-tRNA synthetase was able to determine muscle and lung infiltration in mice following the development of autoantibodies but failed to reproduce the involvement of other usually affected tissues like the joints.

Antigen-driven class switching and affinity maturation were also noted.

Other hypotheses envision the histidyl-tRNA synthetase functioning as a chemokine or tend to underscore the role of innate immunity.(32,33)

PATHOGENESIS OF LESSER-KNOWN IIM

Eosinophilic myositis (occasionally considered as a form of PM) is associated to calpain-3 mutation (the mutated gene in the LGMD 2A). Chronic parasitic

infections play a pivotal role in the genesis of this IIM subtype as evidenced by the conspicuous eosinophilic infiltrate.

Granulomatous myositis: can be idiopathic or be related to either sarcoidosis (granulomas are detected on biopsy) or GvHD where microangiopathy could be the main culprit. Up to 50% of these patients eventually evolve into an IBM diagnosis.

1.1.6 CLINICAL MANIFESTATIONS

IIM do not manifest through a single presentation. Instead, every patient displays a variable degree of expression of the disease on the muscular and extramuscular level thus delineating three different clinical profiles: exclusively muscular, exclusively extramuscular and mixed involvement.

MUSCULAR MANIFESTATIONS

As previously stated, the acute (within days) or subacute (over several months) development of proximal limb and girdle weakness is the hallmark of most IIM.

The patients may be noticing a progressively increasing difficulty in performing trivial daily actions such as climbing a flight of stairs or carrying the groceries.

Interestingly, the muscular disease can be subclinical as well and only manifest upon muscle enzyme testing. (34)

Deltoids, hip flexors, abductors and extensors are mostly affected alongside neck flexors. On the other hand, neck extensors tend to be relatively spared. A distal pattern is frequently and early noticed in IBM. Asymmetry is an unusual finding. (35)

PM, IBM and IMNM classically share this presentation.

Oculomotor involvement is an uncommon finding in IIM and should prompt investigation of other diagnosis in the first place; face muscles are not impaired except for IBM. (36)

Muscle atrophy (40%) and contractures are rare and restricted to severe and longstanding disease. Muscle tenderness can sometimes be elicited upon palpation which together with myalgia affects over 50% of PM patients. (1)

Up to one third of patients (especially in IBM) can suffer from proximal dysphagia due to either oropharyngeal or superior esophageal sphincter weakness often putting the patient at risk of life-threatening aspiration pneumonias and critical malnourishment.(37,38) Therefore, severe or refractory dysphagia always demands full investigation and aggressive management. Positioning of a nasogastric tube may be required. Overlap with Sjögren syndrome may further aggravate the dysphagia.

The diaphragm and the thoracic muscles may also be affected in IIM thus contributing to the occurrence of restrictive pulmonary disease in these patients. The extent of the muscular weakness in pulmonary function tests (PFTs) can be gauged through MIF (mean inspiratory flow) and MEF (mean expiratory flow) gradual deterioration. Mechanic ventilation may be necessary. (1,16)

EXTRAMUSCULAR MANIFESTATIONS

Clinically amyopathic dermatomyositis (CADM), post-myositis DM, hypomyopathic DM and occasionally ASyS can exhibit an exclusively extramuscular clinical phenotype.

The extramuscular disease burden comprises a broad range of manifestations that spans from inflammatory arthritis, interstitial lung disease (ILD) and vasculitic signs to distinctive cutaneous features.

Systemic, nonspecific features such as fever, arthralgia and weight loss may also be present.

ARTICULAR MANIFESTATIONS

True arthritis is relatively uncommon although it may accompany disease flares or overlap forms; in this regard, ASyS and a subset of anti-MDA5+ DM exhibit the most considerable rate of arthritis, which primarily affects the small joints of the hands in a symmetrical yet non-erosive fashion, thereby only partially mimicking rheumatoid arthritis. (9,13) Overall, a variable articular involvement can be documented in up to 20% of patients.

PULMONARY MANIFESTATIONS

Myositis patients frequently develop a pulmonary involvement which is the single most common extramuscular manifestation encountered in IIM hence representing a significant cause of morbidity and mortality among these patients. This justifies the extensive pulmonary testing (i.e., chest X-ray, HRCT scan, pulmonary function tests with DLCO) they undergo upon diagnosis. (12,39)

The pulmonary involvement may be the consequence of several mechanisms: 1) the above-mentioned respiratory failure determined by the diaphragm's and thoracic muscles' impairment; 2) the recurring infections these patients contrive as a result of either the immunosuppressive medication or the dysphagia; 3) the direct inflammatory damage to the pulmonary interstitium in the setting of interstitial lung disease (ILD). Pneumomediastinum might also occur in these patients.

ILD affects as many as 80% of DM and PM patients with a strong association to the serologic profile. (40)

It outlines a variable clinical picture which encompasses both asymptomatic and symptomatic patients ranging from nonproductive cough and dyspnea to rapidly progressive and fatal ILD. Cough, clubbing of the nails and coarse crackles on auscultation are a classical triad of the disease.(11)

An abnormal restrictive pattern on PFTs and reduced gas diffusing capacity upon DLCO testing are considered quite typical.

The temporal relationship between the IIM diagnosis and the onset of ILD varies widely across different patients occasionally anticipating the official diagnosis by years.

Of note, ILD can be classified into various subcategories based upon histopathologic and HRCT radiologic features though combined entities also exist: 1) nonspecific interstitial pneumonia (NSIP); 2) organizing pneumonia (OP); 3) usual interstitial pneumonia (UIP).

NSIP is usually characterized by bilateral and grossly symmetrical ground-glass opacities with a basal predominance. Fine reticulations can be observed alongside pulmonary volume loss and subsequent traction bronchiectasis.

An apicobasal gradient is detected and the involvement tends to be mainly subpleural. However, immediate subpleural sparing is highly specific for NSIP.

NSIP has a relatively favorable prognosis compared to UIP displaying a 90% 5YOS for the cellular subtype and over 60% 5YOS for the fibrotic subtype due to the former's better response to corticosteroids. Mycophenolate mofetil (MMF) may provide benefit in this circumstance.(41)

OP radiologically appears with multifocal ground glass opacities (GGOs) and/or patchy consolidations with a predominant subpleural or peribronchial distribution. Heterogeneously sized nodules can also be seen. Bronchial wall thickening is an inconsistent finding. It has mostly a benign prognosis due to its satisfactory response to corticosteroids. (42)

Rapidly-progressive ILD (RP-ILD) is a distinctive feature of the most severe anti-MDA5+ DM subset manifesting radiologically as a combination of NSIP and OP patterns with a rapidly progressive expansion of alveolitis and septal consolidations starting with a subpleural, basal distribution with a quick invasion of the lung parenchyma.(43)

UIP's most distinctive HRCT feature is honeycombing, particularly if the parenchyma involved is greater than 5%.

An apicobasal gradient of honeycombing and immediate subpleural reticular opacities is a useful feature in the differential diagnosis from NSIP and concurrent emphysema. Traction bronchiectasis are plausible elements. UIP is burdened with an ominous prognosis and is fortunately a rare condition. (44)

CUTANEOUS MANIFESTATIONS

Skin lesions are the cardinal sign of DM (hence its name) but can also feature in ASyS. Cutaneous manifestations may concur but more often predate the muscular disease by months or even years. They tend to be pruritic.(9)

DM cutaneous manifestations can be subdivided into pathognomonic, highly characteristic and compatible skin lesions according to Euwer and Sontheimer's classification.(45)

PATHOGNOMONIC SKIN LESIONS OF DM

Gottron's papules: visible in up to 30% of DM patients, they are erythematous to violaceous papules covering bony prominences particularly the dorsal-lateral aspect of MCP and IP joints in a symmetric fashion.

Gottron's sign: symmetrical macular erythema with a violaceous hue overlying the extensor surfaces of MCP, IP, elbows, knees or ankles. Either edema or ulceration can occasionally be evident. Instead, when scaling is present, it can mimic psoriasis and lichen planus.

HIGHLY CHARACTERISTIC SKIN LESIONS OF DM

Heliotrope rash: it is a violaceous erythema (with or without swelling) located in the periorbital area. The upper eyelid tends to be more involved than the lower one. It is recognizable in about 25% of DM patients.

Periungual erythema with or without dystrophic cuticles: interestingly, the extent of cuticular involvement is seen to be matching the level of cutaneous disease

activity as if it were a direct representation of active vasculopathy. Capillaroscopy reveals anomalous capillary bed loops with alternating zones of dilation and dropout. In addition, hypertrophic cuticles may give rise to hemorrhagic infarcts therein.

Photosensitive and symmetrical macular erythema: it has a violaceous shade and overlies the face (midfacial erythema with no nasolabial fold sparing), the trunk (V-neck sign on the upper chest) and the extensor surfaces of the limbs (shawl signs over the posterior neck and shoulders).
When scaling occurs, it can resemble cutaneous lupus erythematosus or seborrheic dermatitis.

COMPATIBLE SKIN LESIONS OF DM

Poikilodermatomyositis: it is a cutaneous manifestation consisting of alternating areas of hypopigmentation and hyperpigmentation with associated telangiectasia. The V-area of the anterior neck and chest, the posterior shoulders and lower back are preferential sites of involvement. Poikiloderma in the lateral aspects of the thighs is referred to as the holster's sign.

Calcinosis cutis: it is a sign straddling the border between cutaneous and vasculitic signs which are more frequent across juvenile groups (e.g., subcutaneous calcinosis, periungual infarctions and digital ulcerations). Insoluble calcium salt deposition occurs at the muscle fasciae's level giving rise to palpable subcutaneous nodules that occasionally erupt into ulcers to release their calcareous content. Overlap myositis together with anti-PM-Scl, anti MJ anti antiNXP2 positive patients are more prone to developing this severe and challenging manifestation. Colchicine, diltiazem and bisphosphonates are being employed for this purpose with varying outcomes.

OTHER COMPATIBLE YET ASPECIFIC MANIFESTATIONS

Mechanic's hand: it is not considered a cutaneous manifestation in the strict sense. Mechanic's hand is a term referring to a hyperkeratotic and fissured skin on the palmar and lateral aspects of the fingers which is a characteristic sign of ASyS.

Raynaud's phenomenon is a vasculitic sign which is displayed in 35% of cases. It is common amongst idiopathic DM and overlap conditions.

Figure 3. Cutaneous involvement in dermatomyositis.

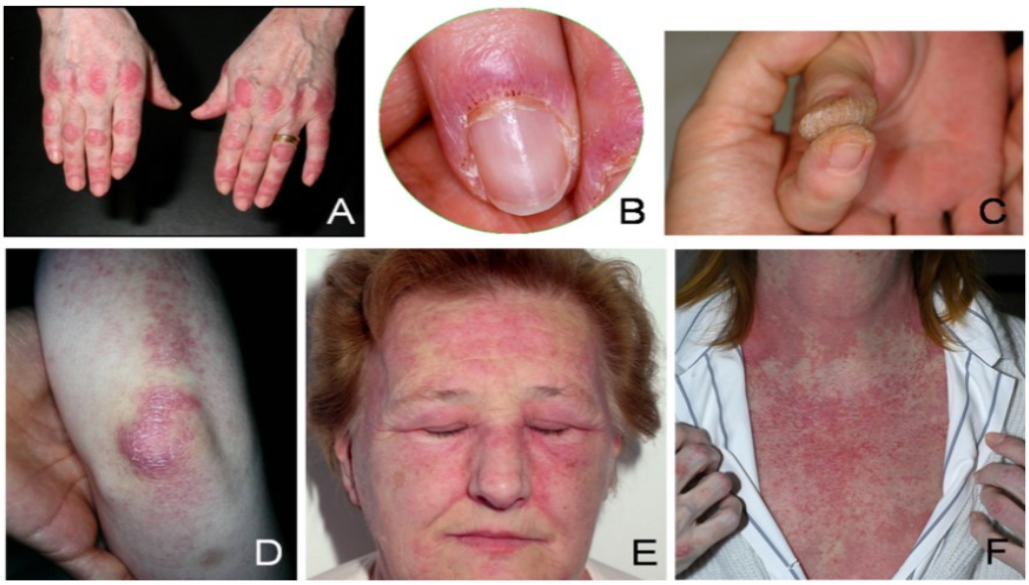

Figure 4. Cutaneous involvement in DM. A. Gottron's papules; B. Periungueal teleangiectasia with cuticular hemorrhage and dystrophy; C. Mechanics' hand; D. Gottron's sign over the elbow; E. Heliotrope rash; F. Poikilodermatomyositis. Adapted from (9)*, with kind permission of Iaccarino et alii.*

CARDIAC INVOLVEMENT

Cardiac abnormalities are commonly identified (in 13-72% of patients) yet usually as asymptomatic findings. (46)

Inflammatory infiltration in the heart may lead to subclinical myocarditis. In addition, replacement fibrosis within the conduction system could take place, leading to asymptomatic arrhythmias in 30-80% of patients with a preference for the ventricular conduction system. (47)

The odds ratio for acute myocardial infarction in this population is comprised between 3 and 4. (48)

Cardiac involvement may also include congestive heart failure (CHF, in 10-15% of cases), left ventricular diastolic dysfunction, and hyperkinetic left ventricular contraction. (9)

1.1.7 DIAGNOSIS

The first step in an IIM diagnosis is a high clinical suspicion based on the typical muscular and extramuscular manifestations of the disease. The clinical suspicion must, however, be confirmed by a wide array of further testing on the grounds of laboratory, imaging and histopathologic findings. Lastly, other similar medical conditions should be ruled out through a comprehensive differential diagnosis. (7,9,13,36,49,50)

LABORATORY FINDINGS

IIM patients show several biochemical abnormalities, some of which are aspecific in nature (e.g., CRP, ESR and alpha2globulins) while others are rather specific (muscle enzymes and serologic markers).

MUSCLE ENZYMES

Most IIM patients will demonstrate elevation of at least one muscle-derived enzyme, classically CPK (i.e., creatine phosphokinase), which stands out as the primary enzyme in routine monitoring for muscular activity. (16) Indeed, it has proven to be highly sensitive in this context even though it does not necessarily correlate with disease severity. In 30% of patients, though, it may fall within normal range particularly at disease onset or in the case of advanced muscle atrophy. IMNM and IBM yield the highest and the lowest CPK levels among IIM subtypes, respectively.

Myoglobin (MB) is found to be elevated in over 70% of patients with active myositis and hardly ever leads to acute kidney injury in these disorders due to the higher threshold for tubular toxicity. Its sensitivity for muscle flares is on par with CPK's but with a closer temporal relationship. (51)

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are also employed in muscular activity monitoring. However, γ-glutamyltransferase (GGT) might also be needed in case a liver dysfunction had to be ruled out.

Lactate dehydrogenase (LAD) and aldolase are occasionally on the panel too, the latter having a low sensitivity counterbalanced by a great specificity for perimysial involvement.(52)

SEROLOGIC MARKERS

Ascertaining the serologic profile of IIM has a high prognostic value and contributes to the identification of the clinical phenotype that better suits the patient but has limited diagnostic significance.

The antibodies found in IIM can be subdivided into myositis specific antibodies (MSAs) and myositis associated antibodies (MAAs) depending on whether they appear almost exclusively in IIM patients (i.e., MSAs) or are shared with other rheumatic diseases (i.e., MAAs), typically demarcating overlap myositis.

MSAs and MAAs are normally measured via ELISA, but unfortunately are not widely available. Additionally, many commercially available myositis panels have inconsistent levels of accuracy.

Nevertheless, over 70% of IIM patients test positive for some autoantibody and around 45% of them are positive for an MSA.

Said antibodies target ubiquitous self-antigens that carry out various functions within the intracellular space, notably protein transcription and gene regulation.(53)

MSA

AntiMi2 is an autoantibody directed against a helicase with a role in transcriptional activation. It is the standard antibody for DM appearing in almost 30% of patients and implies a lower risk of malignancy. It is linked to a relative acute onset of DM. Shawl or V-neck sign can be noticed in these patients. It is renowned for having a good response to treatment.(54)

Anti-tRNA synthase antibodies whose major representative is anti-Jo1 (anti histidyl t-RNA synthetase) are the cornerstone of ASyS. Anti-PL12, anti-OJ, anti-PL7, anti-EJ, anti-KS, anti-Zo and anti-Ha belong to this category. While anti-Jo1 is present in over 20% of patients, the other ones are far more infrequent. Anti-Jo1 and anti-PL12 are associated to an ILD form who lack proper evidence of myositis.

Anti-MDA5 antibodies target the RNA helicase encoded by the melanoma differentiation-associated gene 5. It usually determines a peculiar phenotype consisting of rapidly progressive ILD, arthritis, CADM, and occasionally ulcerations over Gottron's sign and papules plus non-scarring alopecia. The prognosis is ominous. (55)

AntiTIF1-γ antibodies strike transcriptional intermediary factor 1 gamma and bear a heightened risk of malignancy alongside characteristic skin findings that encompass hyperkeratotic papules on the palms and soles, psoriasis-like lesions and "red-on-white" patches. (56)

Anti-SRP-positive patients frequently but not exclusively develop IMNM. The involved antigen is the signal recognition particle which engages in the translocation of newly synthesized proteins into the ER. Cardiac involvement is not surprising in this population with a subsequently severe prognosis also owing to its scarce response to treatment. (57)

An anti-HMGCR-positive serology suggests an IMNM associated to exposure to statins which also recognize the 3-hydroxy-3-methylglutaryl coenzyme A reductase. The presentation can be similar to that of anti-SRP^{+} cases. (28)

Anti-NT5c1A antibodies are present in over 50% of IBM cases where they do have some significance as a diagnostic biomarker. They target the 5'cytosolic nucleotidase IA. (58)

AntiSAE antibodies are directed against the small ubiquitin-like modifier activating enzyme which mediates the regulation of gene transcription. Neoplasms are more frequent among these patients than in the general population. Dysphagia and cutaneous manifestations are common and anticipate by far the development of myopathy. (59)

Anti-NXP2 antibodies recognize nuclear matrix protein 2 which is a transcriptional regulator. It is commonly found among JDM patients with severe disease, edema and calcinosis. It is associated with malignancy in males and adults. (56)

MAAs

AntiRo/SSA+ (often accompanied by anti-La/SSB antibodies) patients mostly overlap with ASyS and have a higher risk of ILD. The immune response is mounted against protein antigens associated with poorly understood small RNA molecules known as hY-RNAs.

An antiSm positivity may imply an overlap with SLE; they target the common core of small nuclear ribonucleoprotein (snRNP) particles.

Positivity to anti-U1RNP (anti-U1-small nuclear RNP) is encountered in MCTD.

Anti-PM/Scl antibodies (against the human exosome protein complex) and anti-Ku (against the regulatory subunit of DNA-dependent protein kinase) hint at the coexistence of SSc. (60)

IMAGING

IN MUSCLE ASSESSMENT

The MRI is the gold standard for muscle imaging in the framework of IIM as it allows for a detailed and synoptic view of the extent of muscular involvement. Indeed, T2-weighted and short tau inversion-recovery sequences (STIR) reveal muscle edema during myositis active flares. On the other hand, T1-weighted sequences are best suited for the visualization of fibrous-adipose replacement areas and muscular atrophy in severe and advanced disease. Occasionally, calcifications might be visible. Its sensitivity outweighs the sensitivity of the biopsy in that it avoids sampling errors that may come with the latter. Unfortunately, it is not much specific as several conditions may present with an undistinguishable radiologic appearance (e.g., rhabdomyolysis, muscular dystrophy and metabolic myopathy). The MRI can be used to identify candidate regions apt to undergo biopsy.

The musculoskeletal ultrasound with power Doppler is an affordable and rapid technique for IIM assessment. It is non-invasive but is remarkably operator-dependent. On ultrasound, active myositis imbibition of the inflamed tissue results in a hypoechoic and enlarged muscle tissue. Hyperemia on power Doppler is evident in active early disease. Muscle volume shrinkage and hyperechogenicity are indicative signs of later stages. Despite its low specificity, the ultrasound may be a valid alternative to the MRI in the assessment of muscle activity and area selection for biopsy.(16,49)

PULMONARY ASSESSMENT

Chest X-rays can be performed in every IIM patient as a baseline screening tool. HRCT is a non-invasive and sensitive imaging method suitable for detection and monitoring of ILD, which should be promptly performed in case of clinical suspicion of ILD or just in presence of ILD-associated serological abnormalities, e.g. anti-t-RNA synthetase or anti-MDA5 antibodies, even in absence of overt clinical symptoms. (11,12,39)

To minimize the exposure to radiation, lung ultrasound can be performed as a first-level screening examination, yielding a higher sensitivity than chest X-ray for interstitial abnormalities, yet being still burdened by a high inter-operator variability.

ELECTROMYOGRAPHY AND NERVE CONDUCTION STUDIES (EMG AND NCS)

EMG proves to be extremely useful in the evaluation of the functional aspects of muscle involvement. In fact, it can be used to identify the muscle group best suited for biopsy, yet muscle biopsy should not be performed on the same muscle that has recently undergone EMG testing.

Although nonspecific, abnormalities may be observed in around 80% of patients. The literature reports the existence of an electromyographic triad consisting of: 1) short, small, polyphasic motor unit potentials; 2) fibrillation potentials, positive sharp waves, and insertional activity; 3) complex repetitive discharges.

EMG along with NCS is useful for the differential diagnosis between myopathic and neuropathic causes of motor weakness. Nonetheless, IBM may display a denervation pattern upon testing. (61)

MUSCLE AND SKIN BIOPSY

A muscle and skin biopsy should be performed whenever clinical and laboratory data fail to provide a clear diagnosis, possibly before a therapy is undertaken.

Despite an unparalleled specificity, the sensitivity remains disappointing (between 80-90%) for CAM in particular.

Therefore, to avoid sampling errors the area should be carefully chosen on the grounds of a reduced MMT-8, an EMG and/or MRI appearance indicating inflammatory activity. Quadriceps, deltoids and biceps are preferably selected.

On this occasion, biochemical assays to rule out metabolic myopathies are also performed and immunohistologic tests are executed to evaluate mutant proteins responsible for muscle dystrophies.

Shared histologic features amongst IIM include necrosis, degeneration and regeneration of the myofiber in addition to inflammatory infiltration.

However, characteristic findings allow to distinguish the disorders from one another. A comparison table ensues. (16)

Table 9. Overview of the histopathological findings across different IIM subtypes.

Myositis subtype	Muscle fibres and tissue	Inflammatory cell infiltrates	MHC I expression	MAC depositions	Other specific findings
Dermatomyositis	Perifascicular atrophy, reduced number of capillaries	Perivascular, perimysial, T cells, B cells, macrophages, plasmacytoid dendritic cells	Perifascicular fibres	Small blood vessels	Sarcoplasmic MxA expression
Polymyositis	Degeneration, necrosis, regeneration	Endomysial inflammatory infiltrate with T cells often surrounding and/or invading non-necrotic muscle fibres	Diffuse distribution	No specific findings	Absence of rimmed vacuoles
Immune-mediated necrotizing myopathy	Necrotic fibres with scattered distribution, different stages of necrosis and myophagocytosis and regeneration, endomysial fibrosis and proliferation	Macrophage predominant, paucilymphocytic infiltrates	Diffuse distribution, sometimes only faint	Sarcolemmal and/or on small blood vessels	No specific findings
Antisynthetase syndrome	Oedematous and/or fragmented perimysium that stains with alkaline phosphatase, sometimes perifascicular myofibre necrosis	Scattered perimysial $CD68^+$, $CD4^+$, $CD8^+$ cells	Perifascicular predominance	Fibres adjacent to the perimysium, sarcolemmal on non-necrotic fibres	Myonuclear actin filament inclusions in electron microscopy, absence of MxA expression
Inclusion body myositis	Rimmed vacuoles, ragged red fibres, cytochrome oxidase-negative fibres, groups of atrophic fibres	Endomysial inflammatory infiltrate with mainly $CD8^+$ cells surrounding and/or invading non-necrotic muscle fibres	Diffuse distribution	No specific findings	TDP43, p62 aggregates, 15–18 nm filaments in electron microscopy

Table 10. Overview of the histopathological findings across different IIM subtypes. Abbreviations: MAC, Membrane Attack Complex; MxA, Myxovirus resistance protein 1. Adapted from Lundberg I et alii, Nature Reviews Disease Primers, 2021. (16)

Upon DM muscle biopsy perifascicular atrophy and fibrosis are characteristic findings. Perimysial abnormalities represent the signature of DM, displaying a conspicuous perivascular inflammatory infiltrate, MAC deposition on endothelial cell wall of endomysial capillaries leading to vessel destruction and ischemia, resulting in histologically visible vasculitis.

Unlike PM and IBM, invasion of non-necrotic fibers is a minor component.

Immunohistochemistry reveals expression of regeneration associated proteins (MYH8, vimentin, NCAM) along with myxovirus resistance protein A (MxA), a type 1 interferon-inducible protein, with plausible pathogenetic significance. MACs are detectable on vessel walls before an inflammatory infiltrate is noticeable. (62,63)

PM specimens usually display an endomysial involvement whereby inflammatory cells (mainly $CD8^+$ cytotoxic T cells) infiltrate the single myofibers with a predilection for the central ones. No immune-complex deposition is reported. Widespread myofiber necrosis and regeneration is visible. (63)

IMNM's signature finding is a considerable amount of necrosis despite a relative scarcity of inflammatory infiltrations. Necrotic and regenerating fibers are scattered throughout the tissue. (62)

IBM's pathognomonic features consist of rimmed vacuoles, Congo-red^{+} amyloid material or p62^{+} inclusions. Ragged red fibers (RRF) are absent and cytochrome-C oxidase fibers (COX) are negative. Invasion of myofibers by infiltrating inflammatory cells (mainly CD8^{+} cytotoxic T cells, myeloid dendritic cells and macrophages) is pronounced, moreover they are seen to lump in clusters in the perimysial space. (63,64)

ASYS's biopsy samples show perifascicular necrosis with a higher frequency than DM's (79% and 35% respectively). A population of clonally expanded CD4 T cells against histidyl-tRNA synthetase is seen to infiltrate the endomysium. Additionally, it can be found in the lungs and blood of ASyS patients.(63,64)

DIFFERENTIAL DIAGNOSIS OF IIM

Many conditions may mimic IIM, most notably: muscular dystrophies; metabolic myopathies; mitochondrial myopathies; endocrine myopathies; drug-induced myopathies; infectious myopathies; motor neuron disease; myasthenia gravis. (36)

None of these conditions is associated to the cutaneous manifestations of DM, but the differential diagnosis of IIM subtypes with an exclusively muscular presentation is far more challenging.

Certainly, a detailed medical history and physical examination, followed by EMG and laboratory testing in addition to muscle biopsy (and occasionally genetic testing) are needed to formulate a correct diagnosis of IIM.

Table 11. Differential diagnosis of muscle disease.

Inflammatory myopathies	Noninflammatory myopathies
Infectious myopathies Bacterial (egs. Staph, Strep, M. tuberculosis) Viral (egs. Influenza, EBV, HBV, HCV, HIV, HTLV-1) Fungal (egs. Candida, Coccidiomycosis) Protozoal (egs. Toxoplasma, malaria) Cestode (egs. Cysticercosis) Nematode (egs. Trichinosis) **Toxic myopathies** (egs. Cocaine/heroin, cimetidine, D-penicillamine, adulterated rapeseed oil, amiodarone, L-tryptophan, colchicine) **Lipid-lowering agents** **Myositis associated with graft versus host disease** **Myositis ossificans** **Myositis associated with the vasculitides** **Macrophagic myofasciitis** **Inflammatory dystrophies** (fascioscapulohumeral dystrophy (FSHD), dysferlin deficiencies) **Idiopathic inflammatory myopathies** (egs. PM, DM) **Malignant hyperthermia** **Myotonia** **Neuromuscular junction disorders** (egs. Eaton-Lambert Syndrome, myasthenia gravis) **Periodic paralyses** **Rhabdomyolysis** **Tendonitis-fasciitis syndromes**	**Congenital** (nemaline rod, central core) **Mitochondrial** (acid maltase, McArdle's, phosphofructokinase (PFK), carnitine and carnitine palmityltransferase deficiency) **Endocrine** (hypo- & hyperthyroidism, acromegaly, diabetes, hypo- & hyperparathyroidism) **Toxic** (drugs – egs. Ethanol, AZT, corticosteroids) **Nutritional** (vitamin E deficiency, malabsorption syndromes) **Muscular dystrophies** (egs. Duchenne's, Becker's, FSHD, LGMD (limb-girdle muscular dystrophy), Distal, Oculopharyngeal) **Neuropathies** Denervating conditions (spinal muscular atrophy, amyotrophic lateral sclerosis) Proximal neuropathies (Guillan-Barre syndrome, autoimmune polyneuropathy, diabetic plexopathy, acute intermittent porphyria) **Overuse syndromes** **Paraneoplastic syndromes** (egs. carcinomatous neuropathy, cachexia, myonecrosis) **Rheumatic syndromes** (egs. Giant cell arteritis/ polymyalgia rheumatica (GCA/PMR), granulomatosus with polyangiitis, polyarteritis nodosa (PAN), fibromyalgia syndromes) **Trauma**

Table 12. Differential diagnosis of muscle disease. Castro C. et al., Diagnosis and treatment of inflammatory myopathy: issues and management, Therapeutic Advances in Musculoskeletal Disease, 2012(49)

1.1.8 POST DIAGNOSTIC EVALUATION

It is important to perform a multidisciplinary assessment of newly diagnosed IIM patients. Chest X-rays are requested in all cases and if any abnormalities are found or based on a high clinical/serologic suspicion, a HRCT is also solicited.

Dysphagic patients need to undergo a thorough evaluation.

Age-appropriate cancer screening tests are also performed according to the clinical and serologic phenotype of the patient.

1.1.9 CLINIMETRICS

A correct quantification of disease activity (evaluating the extent and severity of reversible manifestations) and damage (organic and functional irreversible modifications) is paramount to IIM management.(9)

Although many scores have been proposed over the years, Manual Muscle Strength Testing (MMT) is the most widespread one. It gauges the strength of 15 different muscle groups on a scale of one to ten and their sum represents the final score. It is highly operator-dependent.

As far as the quality of life is concerned, the Medical Outcomes Study Short Form (MOS SF-36) is commonly used.

To this end, the International Myositis Assessment and Clinical Studies Group (IMACS) and the Pediatric Rheumatology International Trials Organization (PRINTO) have developed their own standardized measures.

1.1.10 PROGNOSIS

Early diagnosis and treatment are essential to avoid or delay muscle atrophy and functional loss.
The major prognostic indicators in IIM include the disease subtype, the serologic markers, the diagnostic delay, the age at disease onset, the disease severity at onset, the extent of the extramuscular disease burden and underlying malignancy.

Almost 40% of patients achieve clinical remission thanks to immunosuppressive therapy while the rest of patients are bound to a remitting-relapsing disease course.

Mortality estimates show large variations across different populations with 10YOS rates ranging from 20 to 90%, with the highest mortality peak within the first year after diagnosis. (16)

Malignancy, cardiovascular disease and ILD account for the majority of death causes in IIM.

1.1.11 THERAPY

NON-PHARMACOLOGICAL TREATMENT

Physical therapy is an important complement to the pharmacological treatment and it should be undertaken as soon as the clinical status of the patient allows it.

No underload exercises should be made during the active phases of the disease in which passive mobilizations are preferred in order to avoid contractures. Active workouts, possibly under the supervision of a physical therapist, are encouraged during the phase of remission. (65)

Intriguingly, physical exercise seems to activate a vast array of molecular pathways that ultimately promote capillary growth and muscle remodeling while dampening the immune response.

A strong association between physical exercise and the self-reported quality of life, is observed in these patients.

Moreover, patients should be educated about the benefits of photoprotection from the beginning as it may influence the course of the disease at any level.

Aspiration precautions and speech therapy evaluations play a pivotal role in the management of patients with severe dysphagia thus lessening the impending risk of aspiration pneumonias.(49)

PHARMACOLOGICAL TREATMENT

Figure 5. Guidelines for the treatment of idiopathic inflammatory myopathies.

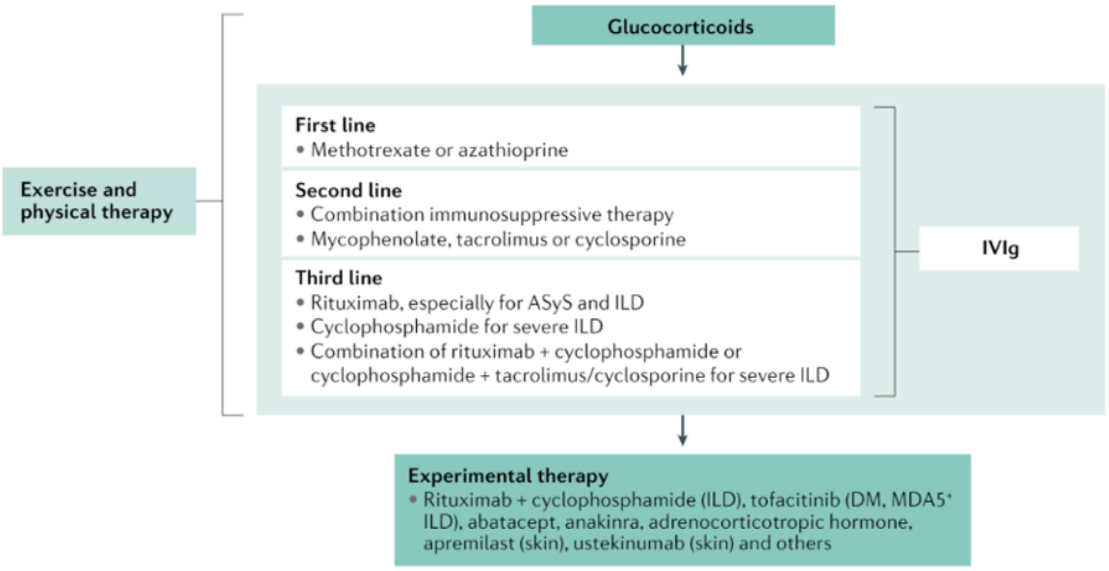

Figure 6. Guidelines for the treatment of idiopathic inflammatory myopathies. Abbreviations: ASyS, antisynthetase syndrome; DM, dermatomyositis; IBM, inclusion body myositis; IIM, idiopathic inflammatory myopathies; ILD, interstitial lung disease; IVIg, intravenous immunoglobulin; MDA5, melanoma differentiation-associated gene 5. Lundberg I et alii, NATURE REVIEWS | DISEASE PRIMERS (2021) (16)

GLUCOCORTICOIDS

The rare and diverse nature of IIM makes them unsuitable for large clinical trials resulting in the general lack of consensus regarding the treatment of myositis.

However, glucocorticoids are unanimously acknowledged as the first line drug especially in the setting of severe ILD or muscle weakness.
A high loading dose of 1 mg/kg/day should be administered for a period of 4-6 weeks with subsequent tapering in tune with disease monitoring. An oral dose of 80 mg per day should not be exceeded.

Providing that the clinical picture is permissive, the prednisone should be gradually tapered by 20% each month until reaching the lowest effective dose (around 5-10 mg/day) over a total of 9-12 months.

A significant improvement in muscle strength is most expected over the course of the second semester after treatment initiation.

If disease control is unsatisfactory, immunosuppressants should be added.

An intravenous methylprednisolone pulse may be required in severe cases of myositis or ILD at a dose of 500-1000 mg/day for three consecutive days. (66)

Even though glucocorticoids may achieve biochemical and clinical improvement of IIM manifestations, they are tied to numerous long-term side effects alongside higher flare rates and partial response.

Therefore, these patients may benefit from the association with an immunosuppressant. (65)

IMMUNOSUPPRESSANTS

Immunosuppressive drugs target different disease pathways and have a glucocorticoid-sparing action. Indeed, they enable glucocorticoid dose lowering and are efficient at reducing disease recurrences.

However, they are quite slow-acting, requiring around three months to achieve the desired effect.

They can be initiated either at the time of diagnosis or when corticosteroids alone are unable to yield the expected result. (67)

METHOTREXATE

Methotrexate (MTX) is the first immunosuppressant of choice in the initial treatment of myositis with a recommended dose of 10-20 mg/week. MTX is particularly useful in the management of juvenile forms. Unfortunately, it might by burdened with hepatotoxicity and myelodepletion. (68) MTX should also be discarded if the patient has a known TPMT (thiopurine methyltransferase) deficiency. Moreover, it is a known teratogen.

AZATHIOPRINE

Azathioprine (AZA) is relatively safe for use in pregnancy and is the immunosuppressant of choice in patients with an underlying liver or lung condition

but also in alcohol addiction. (66) The oral dose is 2-3 mg/kg/day and it allows for better functional outcomes in the long term than glucocorticoids alone. (69,70) Due to its risk of bone-marrow suppression, it requires a CBC monitoring within the first two weeks of administration and is otherwise well tolerated except for mild gastrointestinal disturbances.

MYCOPHENOLATE MOFETIL

Mycophenolate mofetil (MMF) is a second line agent except in cases of moderate to severe myositis associated with ILD where it can serve as the first line drug. MMF dosage should be progressively raised until a target dose of 500-1000 mg *bid* is reached. Its adverse effects bear a strong resemblance to AZA's thus requiring a similar monitoring. (67)

CYCLOSPORINE AND TACROLIMUS

Cyclosporin A (CsA) and Tacrolimus are second-line agents belonging to calcineurin inhibitors that suppress T cell activation. The recommended dose for oral CsA is 3-5 mg/kg/day, while tacrolimus should aim for plasma levels of 8-10 ng/ml. Their use is gaining room in refractory or severe myositis with or without an associated ILD, requiring a tight monitoring. (67)

CYCLOPHOSPHAMIDE

Cyclophosphamide (CYC) is a powerful immunosuppressive agent useful in the treatment of refractory or severe disease such as in the event of rapidly progressive ILD, severe myositis or systemic vasculitis.

It is available both in intravenous (500 mg pulses every 15 days for 6 months or monthly 0.75g/m2 until target dose) and oral formulation (1,5-2 mg/kg/day).

Regrettably, its use is limited by a considerable multisystem toxicity that restricts it to dire clinical situations. (66)

INRAVENOUS IMMUNOGLOBULINS

Intravenous immunoglobulins (IVIg) are second or third-line agents which display anti-inflammatory and immunomodulating properties. They are used either concomitantly with other therapies or subsequently to their failure.

They are known to accelerate the response to treatment and are safe in pregnant, oncologic or infected patients.

In case of resistance to treatment, IVIg can be administered at the high dose of 2g/kg for 2-5 days per month according to the patient's response, usually not exceeding 3-4 courses.

Their pharmacodynamics encompasses several immunomodulatory mechanisms, their use is well tolerated and results in an improvement of the self-reported quality of life. (71)

BIOLOGICS

Rituximab (RTX) is a biologic medication that acts via a depletion of CD20+ B cells which are likely involved in the pathogenesis of several IIM subgroups. It is relatively safe and well tolerated allowing for a significant steroid reduction as well as musculocutaneous improvement in patients with otherwise refractory IIM. The most dramatic clinical improvements are observed among Jo1+ or Mi2+ patients and JDM. RTX should be administered on two separate occasions, two weeks apart, at the dose of 1000 mg. (72,73)

Anti-TNF therapies (i.e., Etanercept and Infliximab) have brought mixed results, possibly due to the ambiguous function TNF-alpha exercises in the muscular *milieu* impairing muscle contraction and myogenesis on one hand while promoting myotube development on the other. (74,75)

Tocilizumab (anti-IL6 biologic medication) is still a long way from featuring in the standard regimen but has so far shown promising results according to a few RCTs. (76)

Abatacept is a fusion protein deriving from the combination of CTLA4 and IgG1's Fc portion which leads to an inhibition of T-cell co-stimulation. Interestingly, it is associated to strong signs of improvement both from the clinical and histology standpoint. It is administered intravenously for 30 minutes at a dose between 500 and 1000 mg depending on the patient's weight. (77)

PLASMA EXCHANGE (PEX)

In outstandingly refractory disease, PEX can be performed in association with glucocorticoids, immunosuppressants or biologics. It might be exploited as a rescue therapy in life-threatening RP-ILD, usually associated with anti-MDA5+ DM. (78)

DISCONTINUATION OF IMMUNOSUPPRESSIVE THERAPY

After the first round of treatment, a gradual and careful tapering of glucocorticoids (followed by the tapering of the glucocorticoid-sparing agents) can be attempted.

The abovementioned discontinuation of immunosuppressive therapy can be undertaken only provided that the clinical scenario is permissive and no disease recurrence is detected under close follow-up.

1.1.12 GENERAL RECOMMENDATIONS

In the context of an unusually stubborn resistance to treatment, an alternative explanation should be thoughtfully sought. Other plausible scenarios are an incorrect original diagnosis, a glucocorticoid-induced myopathy or a misrecognized malignancy.

Glucocorticoid-induced osteoporosis is a relevant cause of long-term treatment morbidity and mortality therefore demands accurate prevention measures. Vitamin D monitoring along with prescription of anti-resorptive medications is indicated.

Prophylaxis against opportunistic infections is also one of the strongholds of IIM disease management.

In case of desire of pregnancy, it is important to stress that being diagnosed with an auto-immune disease intrinsically entails a higher risk of delivery complications along with birth defects, IUGR, miscarriage and stillbirth.
An additional risk of congenital heart block (CHB) in the offspring of Ro52+ mothers is described in the literature. A complication-free pregnancy is still achievable provided that a few conditions are met: the conception is planned far enough in advance; the conception is postponed until a satisfactory degree of disease control is achieved (disease activity negatively impacts the outcome of pregnancy); all teratogenic medications (folate antagonists like MTX e.g.) are discontinued in good time and pregnancy-safe medications (steroids and AZA) have proven reliable in preventing disease recurrence in that patient; a close follow-up for high-risk pregnancies is soon established; stress-dose glucocorticoids are administered during childbirth if needed, especially in chronically treated patients with axis suppression.

1.2 EXTRACELLULAR VESICLES

1.2.1 DEFINITION AND CLASSIFICATION

"Extracellular vesicles" (EV) represents a generic term for a vast array of lipid-bilayer nanoparticles which enclose cytoplasmic contents except for a functional nucleus and are naturally released into the extracellular space by virtually all cells under specific circumstances (for instance, apoptosis or cell activation). (79)

Although soluble factors have dominated the landscape for decades, recent years have seen an emerging interest in the investigation of EV role as key actors in intercellular communication owing to their ability to effectively convey active biomolecules such as proteins, lipids and nucleic acids while also protecting them from the otherwise inevitable degradation in the extracellular *milieu*.

An expanding body of research is shedding light on their relevant contribution to both physiologic and pathologic processes (notably coagulation, tumorigenesis and immune-modulation) as they also appear to be highly conserved across all domains of life, including prokaryotes.

EV can be found in nearly every biological matrix therefore are promising candidates as disease biomarkers despite also having a potential for extensive therapeutic use in light of their function as high-precision intercellular vehicles, able to cross biological barriers.(80)

Interestingly, EV display a significant degree of heterogeneity in terms of biogenesis, size, density and biochemical composition in addition to cellular origin and release conditions.

Such diversity has resulted in major nomenclature issues until the 2018 MISEV guidelines were published in an endeavor to provide an organized and standardized classification of EV as well as the unification of minimal laboratory protocols for EV study.

On the basis of their biogenesis, EV can be subdivided into two main subtypes, exosomes and microvesicles (i.e., MV, ectosomes or microparticles). The formers

are generated via inward budding of the endosomal membrane to form intraluminal vesicles (ILVs) contained inside multivesicular bodies (MVBs) which then fuse with the plasma membrane to release the ILVs as exosomes. On the other hand, the latters directly shed from the plasma membrane outwards into the extracellular space.

EV collectively range from 40 nm to 1 μm in size: specifically, exosomes are comprised between 40 and 150 nm whereas MVs have a diameter between 100 nm and 1 μm. As there is significant overlap between the two subtypes (especially in the 100-150 nm range) and the exact biogenesis cannot usually be pinpointed, EV is the generic term endorsed by the ISEV. They are conventionally subdivided into small EV (sEV) or medium/large EV (m/l EV) using a 200 nm cut-off for diameter.

Additionally, they can be subdivided into low, medium or high density.

Their biochemical composition can be explored through the analysis of surface markers (such as tetraspanins CD63 and CD81) via flow cytometry or through lysis and subsequent cargo analysis (e.g., via Western blot to reveal the protein content thereof like TSG101, Alix).

However, operational denominations referring to cell of origin or releasing conditions are also accepted such as apoptotic bodies (up to 5 μm), oncosomes (up to 10 μm), hypoxic EV, migrasomes and others.(81,82)

1.2.2 BIOGENESIS AND RELEASE

EV biogenesis is a highly regulated and complex process whose mechanisms vary based on the cargo, the cell type or stage of differentiation. It is also influenced by the physiological or pathological state of the donor cell alongside external cues conveyed through molecular pathways. (83)

Despite such variety, the cornerstones of EV biogenesis remain approximately identical among EV subtypes: 1) the future cargo (which acts as a primary regulator in this context) is targeted towards discrete microdomains along the limiting endosomal membrane for exosomes or the plasma membrane for MVs; 2) said

microdomains which are enriched in particular sphingolipids and transmembrane proteins, participate in the clustering of additional cargo components with the aid of sorting machineries in a step-wise fashion; 3) the sorting process induces a curvature of the endosomal or plasma membrane causing it to bud inwards or outwards respectively; 4) the exosomes undergo fission inside the lumen of the now called MVBs (which encircle many ILVs) while MVs undergo direct release from the plasma membrane after losing leaflet asymmetry; 5) both release mechanisms are calcium-dependent and imply varying degrees of cytoskeletal involvement. (84)

EXOSOME BIOGENESIS

The membranous portion of the exosome cargo may originally derive from the trans Golgi network (TGN) or from early endosomes formed by invagination of the plasmalemma.

The early endosomes are then routed to the degradative, recycling or maturation pathway whereby they undergo fusion with lysosomes, recycling of their components towards the plasma membrane or mature into late endosomes, correspondingly.

In exosome biogenesis the late endosome membrane is reorganized to form Tetraspanin-Enriched Microdomains (TEMs) which function as the landmark of exosome production: the main tetraspanins involved herein are CD9 and CD63.(85)

Furthermore, endosomal sorting complexes are recruited to the production site such as the Endosomal Sorting Complexes Required for Transport (ESCRT) or alternate machineries like the syndecan-syntenin-ALIX pathway for cargo clustering and membrane budding. The fission, on the other hand, is going to be uniquely performed by elements of the ESCRT. It is vital to emphasize the crucial role these machineries play in determining the composition hence the functional properties and the ultimate fate of these vesicles.

The lipids scheduled for packaging inside exosomes can become embedded in the membrane at the production site owing to their affinity for the discrete lipid microdomains therein present.

Nucleic acids are another relevant component to the exosome cargo and even though some molecules appear to be passively encapsulated in the vesicle as is the case for numerous fragments of DNA or RNA material, there is unmistakable evidence that functional RNA molecules are selectively loaded into exosomes and EV in general. Short sequence motifs in the 3'-UTR of mRNAs were found to act as a zipcode for association with miR1289, leading to enrichment of said mRNAs in the EV. Similarly, other short nucleotide sequences were found to drive loading of miRNAs into exosomes which also rely on AGO2 (belonging to the RNA-induced silencing complex, RISC) for importation into EV. (86)

Soluble cytoplasmic proteins can be escorted into the exosomes by chaperones HSP70 and HSC70. Moreover, anchored proteins (for instance GPI-anchored proteins) display remarkable affinity for lipid rafts and sphingolipid-enriched domains on endosomes. Some post-translational modifications like farnesylation or ubiquitination are associated with a higher enrichment of such proteins in exosomes. Indeed, the ESCRT machinery is composed of four complexes the first of which, ESCRT-0, clusters ubiquitinated proteins. This allows ESCRT-I to bind the ubiquitinated cargo to the exosome and activate ESCRT-II, thus initiating vesicle budding. ESCRT-II in turn recruits the last complex, ESCRT-III, which completes the budding inducing fission. The interaction between ESCRT-I and ESCRT-III is mediated by ALIX which serves as an intermediary between the TSG101 and CHMP4A components of ESCRT-I and III, respectively. (86)

As previously mentioned, the syndecan-syntenin-ALIX pathway is an alternative sorting machinery. This pathway depends on the presence of syndecan, a single transmembrane domain proteoglycan with a heparan sulfate chain that can bind specific cargo (i.e., FGFR1) consequently leading to syndecan clustering. These assemblies recruit syntenin1 which promotes syndecan internalization in the exosome production site whereby the endosomal heparinase digest the heparan sulfate chain. The heparan sulfate chain processing facilitates further clustering and ALIX binding (an accessory component of the ESCRT machinery) subsequently supporting membrane budding and later fission via ESCRT-III. (87)(88)

It is worth noting that GTPase ARF6 and phospholipase PLD2 are likewise responsible for MVBs formation.

It should not be overlooked either that the mechanical deformation of the endosomal membrane renders an important contribution to the inward budding required for exosome biogenesis as in the case of ceramide produced by neutral sphingomyelinase or cone-shaped tetraspanins which also play a role in selective cargo loading (i.e., integrins).

It should be stressed that vesicle fission leading to ILVs and MVB formation is always an ESCRT-dependent process since it requires ESCRT-III complex which is later going to be disassembled by AAA-ATPase suppressor-of-potassium-transport-growth-defect-1 protein (SKD1) to be recycled.

EXOSOME RELEASE

MVBs can follow two alternative paths: they can either take the degradative route that entails fusion with the lysosomes or they can take the secretory pathway whereby the MVB limiting membrane fuses with the plasma membrane.

The balance between the two ultimately determines the fate of the MVB, which is more frequently lysosome degradation. Destination to autophagosomes for degradation is also a viable option.

This balance can be altered in many circumstances for instance in lysosomal degradation defects or tetraspanin 6 expression triggering the syndecan-syntenin-ALIX pathway which seem to favor exosome secretion over degradation; conversely, expression of ubiquitinated MHC II molecules or ISGylated TSG101 proteins label the MVBs scheduled for lysosome degradation. Both options require MVBs to undergo transport along the cytoskeleton and later fusion with either the plasma membrane or the lysosomes.

The degradative pathway involves retrograde transport on microtubules through dynein motor proteins with the aid of RAB GTPase molecular switches while

anterograde transport with conventional motor proteins is needed for secretion, at least in the context of the immunological synapse. (86)

Docking and fusion are the last steps of exosome release which require the joint action of RAB GTPases, actin and calcium-sensitive SNARE protein complexes.

MICROVESICLE BIOGENESIS

The investigation of the exact mechanisms underlying MV biogenesis is still underway.

Although some machineries and hallmarks may be shared with exosomes, the rearrangement in the asymmetry of membrane phospholipids is an undoubtedly unique process of MV biogenesis.

Indeed, intracellular calcium elevations trigger calcium-dependent translocases such as flippases and floppases along with scramblases and calpain which are responsible for the flipping of lipid molecules across the plasma membrane leading to the exposure of phosphatidylserine on the outer leaflet which mechanically promotes outward budding. (84)

MICROVESICLE RELEASE

The outpouching of the plasma membrane implies a profound remodeling of the underlying acto-myosin cytoskeleton whose ring-shaped contraction is a key step to MV release and is tightly regulated by the RHO GTPase signalling pathway and RHO-associated protein kinase (ROCK).

Accumulating evidence indicates that ARF6 is the initiator of the ARF6-PLD-ERK cascade that culminates in the phosphorylation of the myosin light chain kinase (MLCK) with activating effects.

Metabolic changes induced by hypoxia or Warburg effect promote microvesicle budding and release by alternative mechanisms.

Figure 7. Overview of extracellular vesicles' biogenesis.

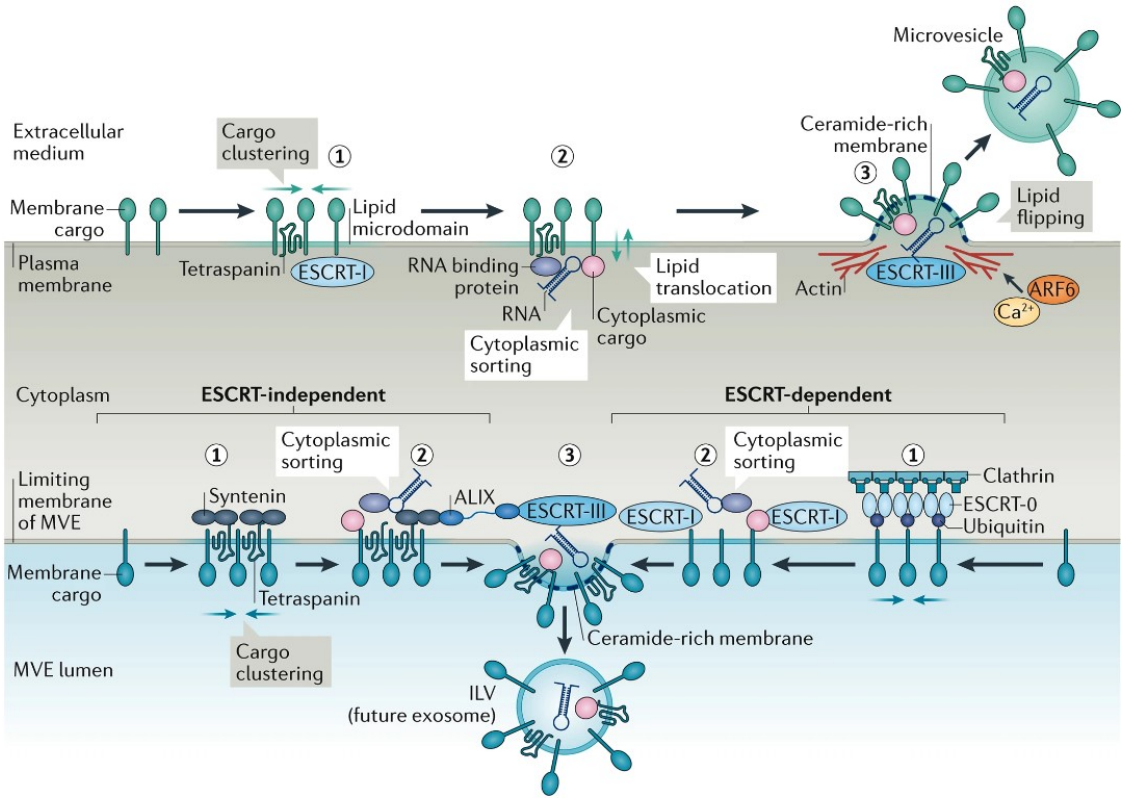

Figure 8. Overview of extracellular vesicles' biogenesis. Abbreviations: ESCRT, Endosomal sorting complexes required for transport; ARF6, ADP-ribosylation factor 6; MVE, multivesicular endosome; ALIX, ALG-2 interacting protein; ILV, intraluminal vesicle. Adapted from Van Niel G et alii, Nature Reviews Molecular Cell Biology (2018) (86)

1.2.3 TARGETING TO RECIPIENT CELLS AND UPTAKE

Interestingly, the target of EV release (recipient cell) may be the donor cell itself as EV can even perform autocrine functions.

EV targeting is a highly specific process that hinges on several variables such as the recipient cell type and physiologic state, pH conditions, surface markers exposed and ultimately the EV's function.

Specific interactions between ligands on the EV surface and corresponding receptors at the recipient plasma membrane are responsible for this tropism resulting in selective docking and potential uptake of EV. Tetraspanins, integrins, ICAM, lectins, heparan sulfate proteoglycans and ECM components are just some of the numerous mediators of such interactions. (80,86)

Once the EV is docked onto the recipient cell membrane, different scenarios open up: 1) the EV could remain bound to the surface and this contact may initiate a signal transduction cascade (e.g., antigen presentation and EGFR signalling); 2) the EV cargo might be released directly into the cytoplasm following fusion with the plasma membrane (e.g., miRNA delivery); 3) the EV may be engulfed through various mechanisms such as macropinocytosis, phagocytosis, clathrin-mediated endocytosis, caveolin or lipid raft-mediated endocytosis.

As soon as the EV is internalized, it can merge with endogenous MVBs and therefore undergo lysosome digestion to deliver valuable nutrients to the recipient cell or escape via back fusion with the limiting MVB membrane or recycling to the plasma membrane.

Figure 9. Overview of extracellular vesicles' fate in the recipient cell.

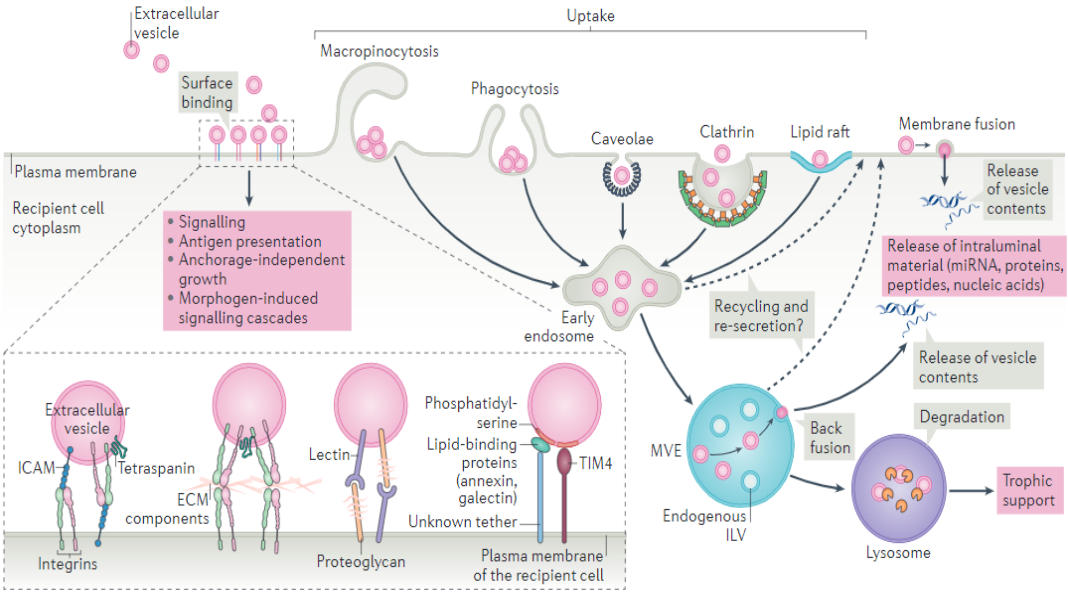

Figure 10. Overview of extracellular vesicles' fate in the recipient cell. Abbreviations: ICAM, Intercellular Adhesion Molecule; ECM, Extracellular matrix MVE; TIM4, T-cell membrane protein 4; Multivesicular endosome; ILV, Intraluminal vesicle. Adapted from Van Niel G et alii, Nature Reviews Molecular Cell Biology (2018) (86)

1.2.4 CARGO

Due to the lack of standardization in the isolation techniques and the inconsistency in the cell types and culture conditions used, inferring the actual biochemical composition of EV is an extremely arduous task.

PROTEIN CARGO

The protein cargo enclosed in EV is extremely diverse since it includes both cytosolic and plasma membrane proteins in the relative absence of the ones commonly expressed in intracellular organelles or in the nucleus.

Proteins involved in EV biogenesis (e.g., ALIX, TSG101 and other ESCRT components) and release (e.g., RABs and ARF6), response to thermic shock (i.e., HSP70 and HSC70) and vesicular trafficking are frequently co-isolated in these vesicles. Signal transduction proteins like EGFR and other transmembrane proteins such as LAMP1 or TfR are also commonly enriched in EV compared to the donor cell. Transcription factors, enzymes and extracellular matrix proteins contribute to the cargo composition as well.

Notably, some four transmembrane α-helices proteins belonging to the tetraspanin superfamily serve as high specificity markers for EV, the main ones being CD9, CD63, CD81 and CD82. Initially deemed exclusive to exosomes, tetraspanins have been found in MVs and apoptotic bodies as well. Other less specific markers are 14-3-3 regulatory proteins, MHC molecules and HSPs. (84,85)

Interestingly, the protein composition varies across EV subtypes too: on one hand, exosomes tend to have a significantly higher glycoprotein content compared to the donor cell whereas, on the other hand, MV tend to be more enriched with post-translationally modified protein cargos with a predilection for glycosylations and phosphorylations.

LIPID CARGO

As previously mentioned, lipids are fundamental actors in EV biogenesis endowing the membranous microdomains with greater rigidity compared to the donor cell, conferring increased resistance to the extracellular environment.

This denotes a significant discrepancy between the lipid composition of the donor cell and the EV's: phosphatidylcholine and diacylglycerol are, indeed, decreased in

EV in comparison to the cell of origin's plasma membrane; moreover, sphingomyelin, cholesterol and GM3 ganglioside alongside phosphatidylserine and ceramide are signature lipid molecules whose increased concentration is associated with the EV cargo.

As a matter of fact, saturated fatty molecules such as cholesterol or sphingolipids allow for lipids' tighter packing onto the endosomal or plasma membrane resulting in greater vesicle stability.

However, far from being mere structural components, lipids play a fundamental role in the actual fulfillment of EV biological functions. The presence of phosphatidylserine on the outer leaflet of MV may, indeed, facilitate their internalization by recipient cells. Moreover, exosomes with surface-bound lysophosphatidylcholine (i.e., LPC or lysolecithin) acquire the ability to successfully interact with DC causing them to undergo maturation and to perform antigen presentation more efficiently.(84)

NUCLEIC ACID CARGO

Although some authors report finding both genomic and mitochondrial DNA within the EV cargo, it is universally acknowledged that the most prominent nucleic acid molecules enriched in EV are small RNAs, rarely exceeding 200 bp in length.

Of note, there is a clear and strong relationship tying the RNA cargo to the physiologic or pathologic condition of the cell of origin. However, this does not imply that the EV cargo should mirror the RNA composition of the donor cell as to the RNA types and their relative concentration. Rather, they display a substantial discrepancy.

Said RNA molecules may most probably appear in the form of non-functional fragments though functional RNA molecules with clear or putative functions are certainly present in EV. They may also be stably bound to ribonucleoproteins

(RNPs), for instance Argonaute 2 (AGO2) or high- and low-density lipoproteins (HDLs and LDLs).

Many of the RNAs found therein engage in protein synthesis like tRNA fragments, 18S or 28S rRNA subunits and mRNAs. Nonetheless, a considerable portion of the RNA cargo consists of regulatory RNAs such as miRNAs, sncRNAs, lncRNAs, and piRNAs. SnoRNA and Y RNA which participate in RNA post-translational processing and DNA replication, have also been detected in EV. CircRNAs and vaultRNAs whose functions have not been fully elucidated yet, are likewise located inside EV. (86,89)

1.2.5 PHYSIOLOGIC FUNCTIONS

Growing evidence supports EV as major players in cell-cell communication on a par with mediators of the caliber of cytokines, hormones or neurotransmitters.

EV ability to shield their cargo from the degradative action of the enzymes in the extracellular *milieu* renders them appropriate vehicles for the transport of fragile molecules such as RNAs, specific lipids and proteins while also acting as a valid platform for ligand (or antigen) presentation. (90)

Thus, EV are able to convey meaningful external cues by triggering signal transduction pathways otherwise directly through the transfer of donor receptors to the recipient cell membrane.

EV may influence the recipient cell's behavior by additional mechanisms like the transfer of regulatory or messenger RNAs or already functional proteins.

On the other hand, they may take the lysosomal route and deliver important cargo to the recipient cells in the form of metabolites, if necessary.

They are also instrumental in aiding cells to dispose of unwanted molecules such as beta-amyloid and alpha-synuclein aggregates or in case of impaired lysosome activity. (86)

Furthermore, EV shedding may serve as an alternative mechanism to eliminate dangerous portions of the plasma membrane like the cytolytic MAC components.

Platelet-derived EV, originally termed “platelet dust”, display potent pro-coagulant activity necessary for efficient hemostasis. (91)

Secretory EV importance in reticulocyte maturation via recycling of transferrin and its receptor, has historically been known for decades. (92,93)

The long-distance transfer of morphogens in the developing organism is another enticing function of EV in embryonic development; intriguingly, Drosophila studies suggest EV may facilitate the establishment of Wnt and Hedgehog gradients thus supporting proper development of limbs and anatomical axes. (94)

Similarly, microvesicles derived from embryonic stem cells (also owing to the extracellular matrix metalloproteinase inducer -EMMPRIN- released simultaneously) promote maternal tissue invasion necessary for trophoblast implantation while regulating angiogenesis and tissue remodeling in the context of placental growth. (95)

Moreover, tolerogenic EV appear in higher concentrations among pregnant women compared to non-pregnant ones as EV clearly contribute to the establishment of an immune-privileged microenvironment required for gestation.(96)

1.2.6 EV IN CARCINOGENESIS

It is beyond doubt that EV-mediated tumorigenic mechanisms are among the most florid fields of investigation in all EV biology.

Indeed, they represent key mediators in the complex interplay between tumor cells, stromal cells and the tumor microenvironment thus influencing primary tumor growth and paving the way for metastatic progression.

Part of such complexity resides in the multidirectional crosstalk these nanoparticles participate in, as stromal and tumor cells engage in a two-way communication while different tumor subpopulations also exchange meaningful cues within the primary tumor's niche. Additionally, many of such interactions rely on profound remodeling of the ECM to support local and distant invasion.

For instance, EGFRvIII constitutes one of the oncogenic molecules that are being shared amongst primary tumor subpopulations via EV exchange resulting in MAPK and subsequent AKT triggering.(97)

As previously mentioned, tumor-secreted EV interact with stromal fibroblasts and endothelial cells consequently inducing angiogenesis and cell-reprogramming to myofibroblasts correspondingly. In addition, non-transformed fibroblasts exposed to tumour-derived microvesicles which bind to surface integrins by means of vesicular fibronectin, subsequently undergo anchorage-independent growth.

Conversely, stromal cells have been demonstrated to have a dual role in the EV-mediated interaction with tumor cells: on one hand they confer apoptosis- and chemo-resistance alongside invasive properties to cancer-cells through different pathways (e.g. Wnt upregulation and PTEN downregulation due to specific miRNAs) as shown in numerous studies; on the other hand, they display anti-tumor activities for instance by casting metastatic cells into a state of dormancy. (98)

Likewise, tumor-secreted EV perform counterintuitive and opposite functions when interacting with the immune system resulting in both increased immune-tolerance (e.g., via Fas ligand and TRAIL induction of cytotoxic T cell apoptosis) and immune-stimulation (e.g., via exposure of MHC I and II molecules alongside T cell costimulatory molecules) against the tumor. (99)

Evasion of the immune system and consequent facilitation of metastatic dissemination may additionally be achieved by establishing a hypercoagulable state also via TF- and PSGL1-loaded EV. Platelet-secreted microvesicles' membranes additionally expose negatively charged lipids displaying an overall 50- to 100-fold higher procoagulant activity compared to activated platelets. (100)

An immune-suppressive microenvironment is established in melanoma and glioblastoma also by means of tumor-derived PD-L1^{+} EV which activate the PD-PD-L1 immune checkpoint on CD8+ T cells. (101)

Epithelial mesenchymal transition (EMT) is another key step in tumorigenesis that is being partly regulated by EV. In particular, vimentin- and MMP- loaded vesicles were demonstrated to promote acquisition of the mesenchymal phenotype with migratory and invasive properties. (102)

However, to successfully metastasize circulating tumor cells (CTCs) need to adhere to a favorable pre-metastatic niche (PMN) whose formation is also supported by EV through a variety of mechanisms. (103)

Endothelium-targeted EV first promote loss of endothelial barrier's integrity causing vascular leakiness (due to EV-mediated destruction of tight-junction proteins via specific miRNAs), effectively opening the door to other populations of EV who target the resident parenchymal cells in an effort to prepare the PMN.(104,105)

MMP proteolytic action initiates active remodeling of the ECM leading for instance to α3-integrin, fibronectin and ADAM-10 enrichment at the expense of cadherin-17 depletion; such alteration in the composition of the ECM together with VEGF-induced angiogenesis and metabolic changes like inhibition of glucose usage by neighboring stromal cells are only a few of the EV-mediated mechanisms known to be implicated in the preparation of the PMN.

1.2.7 EV IN AUTOIMMUNE RHEUMATIC DISEASES

EV have been discovered to underpin the pathogenesis of a wide variety of autoimmune conditions such as Systemic Lupus Erythematosus (SLE), Sjögren's Syndrome (SS), Systemic Sclerosis (SSc) and Rheumatoid Arthritis (RA) to cite a few.

EV IN SLE

Notoriously, SLE is characterized by the formation of Immune-Complexes (ICs) consisting of antibodies bound to the targeted self-antigens.

To this regard, EV have been found to be a crucial source of self-antigens for instance exposing dsDNA on their surface potentially activating DNA-specific B cells. (106)

A reduced clearance of apoptotic bodies is a hallmark of numerous autoimmune conditions, notably SLE.

Indeed, $CD46^+$, $CD55^+$ and $CD59^+$ EV are shielded from MAC assembly hence causing impaired clearance of apoptotic bodies and their subsequent accumulation. (107)

Some apoptotic-derived EV subpopulations were shown to trigger NET-osis in dendritic cells (DCs) of SLE patients further encouraging anti-dsDNA antibodies formation. (108)

Additionally apoptotic bodies from the serum of SLE patients were demonstrated to trigger cGMP-AMP Synthase (cGAS) which in turn activates Stimulator of Interferon genes (STING) thus increasing IFN I secretion. Similarly, exosomal miR-574 is responsible for the upregulation in the expression of IFN I among the pDCs of these patients. (109)

Extravesicular miR-21 activates STAT-1 and TLR-8 with immune-stimulatory effects. (110)

Interestingly, a significantly higher concentration of EV was isolated from the plasma of SLE patients compared to healthy controls (HCs) in several studies. (111)

More specifically, CD14+ monocyte-derived EV appear to be significantly more concentrated among SLE patients with active disease compared to HCs.(112)

The concentration of endothelium-derived MVs appear to be in tight correlation with the degree of vascular involvement in SLE patients. (113)

Podocyte-derived EV (especially $HMGB1^+$ EV) collected from urine samples of Lupus Nephritis (LN) patients, exhibit a strong association with glomerular damage and degree of LN activity with correlation with clinical parameters such as SLEDAI (SLE Disease Activity Index), ESR and proteinuria. (113)

Likewise, exosomal miR-146 is a promising urinary marker for kidney sclerosis. (113)

The EV isolated from SLE patients have a peculiar protein composition: they appear to be enriched in immunoglobulins and complement compared to HCs' and are relatively poor in microtubular and cytoskeletal proteins. (114)

EV IN RA

In alignment with the findings from the SLE field, some unconfirmed studies report that the overall EV concentration in peripheral blood is considerably increased in RA patients compared to HCs and the levels seem to correlate with disease activity and progression.(115)

Accordingly, specific EV subpopulations are elevated in this context such as PDMP (i.e., Platelet-derived microparticles) within the synovial fluid which lead to angiogenesis to sustain the chronic inflammation by facilitating further recruitment of inflammatory cells to the affected joint.(115) (84)

Likewise, FLS-MP (i.e., Fibroblast-like synoviocyte microparticles) appear to be raised: they participate in B cell stimulation within the intra-articular *milieu* and contain hexosaminidase D, a glycosidase with joint-damaging effects (especially elevated in the AR destructive subtype).(116)

EV originating from $CD8^+$ T cells are selectively concentrated in these patients.(117)

Citrullinated peptide-containing EV secreted by FLS are probably implicated in the development of anti-citrullinated protein antibodies (ACPA) with a primary role in the pathogenesis of RA.(118)

FLS-released EV carrying TNF-α with stimulating effects on the AKT and NFκB pathways (leading to apoptosis resistance and activation of T cells) were isolated in these patients as well. (119)

FLS-EV also contain miR-155 that inhibits MMP-1 and 3 expression.(120)

PLT-derived $CD41^{+}$ mpIC (i.e., microparticles containing IC) are particularly enriched in the synovial fluid of these patients where they prompt neutrophils to release leukotrienes.(121)

Macrophage-derived microvesicles which enclose DAMP (e.g., S100A9, HMGB1 and oxidized membrane phospholipids) directly bind and activate TLR2/4 and may similarly carry cytokines such as RANK-L, IL-15 IL-1β and TNF-α eliciting potent pro-inflammatory effects. (84)

It is worth noting that the cytokines RANK-L and IL-15 reach their peak efficiency in the membrane-bound form rather than in their soluble form. (84)

EV SSc

Enticingly, a dysregulated concentration of serum exosomes in scleroderma patients was noted to be detrimental: a rise in their concentration is associated with disease flares but when it plummets, the lack of collagen results in poor wound healing and skin ulcers. (122)

Pathogenetic dermal fibroblasts likely demonstrate traces of EV uptake as they express prominent levels of CD9, CD63 and CD81, namely specific vesicular markers. (123)

Cultured scleroderma fibroblasts release exosomes capable of inducing type 1 collagen expression in normal recipient fibroblasts.

Besides, collagen miRNAs are dysregulated in concentration within said exosomes.(122)

EV IN SS

Conversely, Sjogren patients have an MP concentration that has a counterintuitive relationship with disease activity: on average, MP are more increased among SS patients than amongst HCs, however, the most severe patients experience a drop in the MP concentration, probably due to consumption or sequestration in target tissues.

Interestingly, exosomes released by the epithelial cells located in the salivary glands of Sjögren patients have been found to deliver cargo proteins such as Ro/SSA and La/SSB which are key antigens in the pathogenesis of this syndrome. (124)

Consistently, the concentration of endothelial microvesicles correlates with disease activity in these patients.

An altered protein and miRNA composition has been detected in the cargo of EV isolated from the tears and saliva of SS patients.(124)

EV POTENTIAL APPLICATIONS IN RHEUMATIC DISEASES

EV are almost ubiquitous: their presence has been detected in countless biological fluids, most notably plasma, urine, saliva and synovial fluid. (79)

They represent promising disease biomarkers in the context of SLE, RA, SSc and SS for all the reasons listed above.

Owing to their precision as nano-scaled vehicles and to their unparalleled stability, they are also candidate for the replacement of “whole” MSCs in MSC therapy (mesenchymal stem cells).

Indeed, MSC therapy has garnered attention over the past few years for its anti-inflammatory and regenerative properties in advanced and seemingly irreversible joint destruction mainly in the setting of RA and OA (osteoarthritis).

Conventional MSC therapy, however, is burdened with serious limitations: 1) MSCs might undergo malignant transformation in the recipient patient; 2) they are unable to cross anatomical barriers such as the blood-brain barrier; 3) large scale production would be extremely costly and technically challenging.

MSC-derived EV, conversely, do not possess any of the above limitations. Rather, they entail a lower risk of graft rejection while also allowing for higher success rates, enhanced profitability and easier production and preservation. (125)

1.2.8 EV IN IIM

A fairly complex and bidirectional relationship exists between EV and IIM: on one hand, the formers are pathologically released in the context of several myopathies that share intracellular calcium dysregulations, on the other, IIM' pathogenesis is profoundly influenced by EV multifaceted contribution.

As mentioned earlier, EV are significant sources of autoantigens and ICs but they also deliver myokines, a combination of miRNA sequences and peptides. (21)

In vitro studies reveal that inflamed myotubes release myokines that produce inhibition of myogenic signals. (126)

Consistently, immune cell-secreted EV were found in greater concentrations among IIM patients than HCs. (21)

MSAs (e.g., anti-JO1 and antiMi2 antibodies) and MAAs (e.g., antiPM/Scl antibodies etc.) are usually included in PM EV. (111)

Additionally, platelet-derived EV showed concentrations that correlated with the degree of systemic inflammation in IIM and responded to glucocorticoid treatment. (127)

The EV function heavily relies on the RNA cargo itself: different RNA cargoes have been obtained, indeed, from untreated JDM patients compared to HCs and

uptake of such vesicles from aortic endothelial cells led to significant transcriptional abnormalities *in vitro* culminating in endothelial dysfunction in the form of impaired intercellular junction assembly and cell– cell adhesion formation. (128)

These mechanisms and the ones mentioned earlier in the context of other autoimmune conditions, hint at EV being main players in the development of IIM in particular.

EV AS BIOMARKERS IN IIM

The assessment of EV' potential as biomarkers in the setting of IIM represents an uncharted territory of investigation to this day.

A study conducted on PM and DM patients that aimed at characterizing plasma PDMP through ELISA and inferring clinical correlates, yielded encouraging results: PDMP concentration appeared to be raised in patients with active disease or who were undergoing treatment compared to HCs whereas said concentration turned out to be lower after glucocorticoid treatment; an increased PDMP/platelets ratio was associated with active disease (and CRP trend) compared to the treatment group but showed no relationship with CK levels; there were no significant differences in the levels of PDMP across the DM and PM subtypes; no significant correlation was detected with the degree of disease severity or histopathologic findings. (129)

Another study on PM and DM patients by means of IFC revealed that $CD3^+$, $CD14^+$ and $CD19^+$ EV i.e., T cell-, monocyte- and B cell derived EV, were significantly elevated amidst PM/DM patients as opposed to HCs. (130)

The raised overall concentration of EV alongside a specific increase in monocyte- and B cell-derived EV was observed to correlate with muscle weakness as gauged by MMT. No other clinical or laboratory correlations were identified but a positive anti-Jo1 serology and pulmonary involvement which were associated to significantly higher levels of monocyte-, T cell- and B cell-derived EV.

Morphological analysis through TEM illustrated how EV drawn from HCs' samples have the tendency to display a roundish shape and to be more heterogeneous in size and density whereas PM/DM patients exhibit EV with an amorphous structure and greater homogeneity in terms of size and density. (130)

2. AIM OF THE STUDY

This study aimed at characterizing the circulating EV pool and at exploring EV potential as a disease and treatment biomarker in the context of IIM. Specifically, we aimed at: 1) developing an innovative methodological approach for the isolation of EV from venous blood samples and to confirm its validity through Transmission Electron Microscopy (TEM) analysis of EV' integrity; 2) providing characterization of the isolated EV' features i.e. size, concentration through Nanoparticle Tracking Analysis (NTA) and surface markers through Imaging Flowcytometry (IFC); 3) analyzing EV-related features in IIM patients and matched healthy controls and assess their possible association with clinical and laboratory parameters obtained in an observational cross-sectional study.

3. PATIENTS AND METHODS:

3.1 PATIENTS

Adult IIM patients (≥18 years old) and age- and sex-matched healthy controls were consecutively enrolled from the inpatient or the outpatient clinic of Rheumatology Unit of Padua University Hospital between March 2020 and December 2021.

The eligibility criteria for patients entailed an age over 18 years and a documented diagnosis of any IIM subtype performed by an experienced rheumatologist on the grounds of clinical, laboratory and histology data, if available.

Patients were excluded if they were affected with additional disabling diseases which required immunosuppression and/or rehabilitation physical treatment.

The study received the approval of the Hospital Bioethics Committee (protocol number 0042610) and was conducted in compliance with Helsinki regulations.

Written informed consent was obtained for both patients and healthy controls group allowing for collection of a whole blood sample and full access to clinical and laboratory data from the intra-hospital network health records.

The retrieved data were exported to Microsoft Excel V.2019 (Microsoft, Redmond, Washington, USA) for storage and analysis.

Based on the literature, 55 key clinical variables were chosen and reviewed for each patient in a cross-sectional fashion, turning to parallel retrospective data only for referencing purposes.

Clinical (*Table n.7*), laboratory (*Table n.8*) and serologic (*Table n.9*) variables analyzed at the time of sampling are reported herein. The variables are expressed dichotomically if not specified otherwise.

Table 13. Clinical variables assessed.

	CLINICAL VARIABLES
1.	IIM subgroup, (n.)
2.	Disease duration, (yy)
3.	Raynaud's Phenomenon
4.	Gottron Sign Over Elbows/Knees
5.	Gottron 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.	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.	Neoplastic Diagnosis
24.	Acute Myositis
25.	Cutaneous Activity
26.	Articular Activity
27.	Muscular Activity
28.	Pulmonary Activity
29.	MMT-8, (/150)
30.	Type of Immunosuppressor, (n.)
31.	Prednisone Equivalent Dose, (mg/d)

Table 14. Clinical variables assessed.

Table 8. Laboratory values assessed.

	LABORATORY VALUES (U/L)
1.	CPK
2.	Aldolase
3.	Myoglobin
4.	LAD
5.	AST
6.	ALT

Table 15. Laboratory values assessed.

Table 9. Serologic markers assessed.

	SEROLOGIC MARKERS
MSAs:	
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
MAAs:	
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

Table 16. Serologic markers assessed.

3.2 METHODS

3.2.1 DISCLAIMER

The anonymity of the samples was ensured every step of the way by using a randomly generated personal identification code.

3.2.2 SAMPLE COLLECTION

A 6 mL venous blood sample was collected in sodium-citrate tubes from every participant and was stored at +4°C before being processed within 1 hour.

3.2.3 PLATELET-FREE PLASMA (PFP) PROCESSING

The blood samples were then centrifuged at 1500g for 20 minutes to separate the plasma from the cells. Plasma supernatants were collected in clean 15 mL tubes to undergo centrifugation twice at 3000g for 15 minutes at room temperature.

The supernatant platelet-free plasma (PFP) obtained leaving a plasma residue above the pellet area was finally collected in 1,5 mL microtubes and stored at -80°C until the next step.

3.2.4 SIZE EXCLUSION CHROMATOGRAPHY (SEC)

The PFP samples were thawed at room temperature to perform the size exclusion chromatography (SEC) step.

The SEC procedure was performed using qEV Original®/70 nm columns (Izon Science) endowed with a sepharose molecular sieve range which allows recovery particles of 70 to 1000 nm range in size following the manufacturer's instructions.

Briefly, the Izon column was removed from +4 °C and the storage solution was allowed to run. The column was equilibrated with Phosphate Buffered Saline (PBS)

(pH 7.4; ThermoFisher Scientific) filtered through 0.22 µm filters unit (Millex – GP; Merck Millipore) (fPBS), and 0,5 mL of PFP sample was added on the top.

The eluate was subsequently subdivided into 25 fractions (Fr) of 0,5 mL volume each. Fr. 1-6 were the void volume, which was disposed of, Fr. 7-10 containing the vesicular fraction, were collected in a clean 5 mL tube for further processing, and Fr. 11-25 containing the protein fraction, were eliminated.

Every column could be used up to five times according to the manufacturer, but each of them was utilized only up to three times as a precautionary measure after thorough cleaning steps with PBS and bacteriostatic solutions and preservation at +4 °C.

3.2.5 ULTRAFILTRATION (UF)

The EV fraction collected from the SEC step was enriched through ultrafiltration (UF) using the Amicon® Ultra-4 mL 100 kDa centrifugal filter unit (Merck Millipore). Each filter was sterilized with 1 mL of 70% ethanol by centrifugation at 2800g for 1 minute. The ethanol residues were removed with 2mL of fPBS by centrifugation at 2800 g for 2 minutes.

EV fractions (2 mL + 1 mL of fPBS) were added above the filter and centrifuged at 4000g for 10 minutes according to the manufacturer's instructions to collect the samples held on the filter. The collected samples were adjusted to 0,5 mL volume, aliquoted in 1,5 mL microtube, and frozen at -80°C.

3.2.6 TRANSMISSION ELECTRON MICROSCOPY (TEM)

A 25 microliters EV sample was placed onto a 400-mesh holey film grid and incubated with uranyl acetate 2% stain for 2 minutes in order to reveal the EV membrane.

The sample was observed with a Tecnai G2 (FEI) transmission electron microscope operating at 100 kV.

Images were captured with a Velata® (Olympus soft imaging system) digital camera to confirm the presence of EV in the sample and their structural integrity.

3.2.7 IMAGING FLOW-CYTOMETRY (IFC)

EV samples were diluted 1:100 in filtered PBS (0,1 micrometers filters; Merck Millipore) to avoid coincidence risk and then incubated for 30 minutes in the dark at room temperature with the following fluorophore-conjugated antibodies previously centrifuged at 20000 g for 10 minutes at 10 °C to prevent the formation of antibody-antibody aggregates: anti-human CD63 (Brilliant Violet 421; Biolegend clone H5C6,); anti-human CD81 (FITC; Biolegend, clone 5A6); anti-human CD9 (Alexa Fluor 647; Exbio, clone MEM-61); anti-human CD11c (PE ; Biolegend, clone 3.9).Technical controls included: labelled EV with Triton X-100 0,1% (Merck), unlabelled EV, single antibody-labelled EV, and mixed samples of single antibody-labelled EV, fPBS, fPBS with the addition of fluorophore-conjugated antibodies.

IFC acquisitions of EV samples were performed using Amnis ImageStreamX MkII (ISx; EMD Millipore, Seattle, WA, USA) instrument for a fixed time of 2 minutes setting low-speed fluidics, magnification at 60X, maximum laser power, core size 7 μm, the numerical aperture of 0.9, and the “Remove Beads” option unchecked. EV gating strategy was limited to the area under the Speed Beads. Upon each startup, the instrument calibration tool ASSIST® was performed to optimize performance and consistency. Data analyses and spectral compensation matrices were performed using IDEAS® software (version 6.3). The advanced ISx fluidic control coupled with the continuously running Speed Beads enable particles enumeration using the “objects per mL” feature within the IDEAS® software.

3.2.8 NANOPARTICLE TRACKING ANALYSIS (NTA)

EV quantification and size were measured in samples diluted in fPBS to the concentration range of 10^6 - 10^8 particles/mL as specified by the manufacturer

using the NanoSight® NS300 instrument (Malvern Panalytical) for optimal assessment of particle concentration and size distribution. The ideal detection threshold was determined to include particles with the restriction concentrations of 20 - 120 particles per frame while indistinct particles were limited to 5 per frame. According to the manufacturer's manual, camera level was increased until all particles were distinctly visible not exceeding a particle signal saturation over 20% (level 11-12). Autofocus was adjusted so that indistinct particles were avoided.

For each sample, particles moving under Brownian motion were recorded on 3 videos of 60 seconds each with a 20X magnification captured at 25°C, with syringe speed 40 μL/s; laser 45 mW at 488 nm.

EV concentration and size were calculated by NTA software (version 3.4) based on the dilution factor and hydrodynamic diameter using Einstein-Smoluchowski equation. To minimize data skewing based on single large particles, the ratio between total valid tracks and total complete tracks was always ≤ 1:5.

3.2.9 STATISTICAL ANALYSIS

After checking for normality of the distribution of EV concentration values, the statistical analysis was performed applying Student's t test and one-way ANOVA with Bonferroni correction to compare the quantitative data regarding EV among patients' subgroups and healthy controls. Continuous variables are expressed as mean±SD; median (interquartile value, IQR) is used for non normal continuous distributions. Data were analyzed in a cross-sectional fashion. GraphPad Prism® version 9 software and SPSS software 23 (Chicago, IL) were used to perform the analysis. A p value ≤0.05 was considered for significance.

4. RESULTS

BASIC PATIENT CHARACTERISTICS

During the study period, a total of 66 adult IIM patients was reviewed. Among these, 21 did not fulfil the inclusion criteria and were not included in the final analysis.

We included 45 IIM patients and 45 age- and sex-matched healthy donors (HD) in the present study. IIM patients were distributed across different disease subsets and their main clinical and demographic features are reported in *Table 10* and *Table 11*.

Table 10. Composition of IIM cohort according to IIM subtypes.

Diagnosis (see key)		Frequency, n	Percentage, %	Valid Percentage, %	Cumulative Percentage, %
Valid	DM	14	15.6	16.1	16.1
	PM	8	8.9	9.2	25.3
	IBM	2	2.2	2.3	27.6
	CAM	8	8.9	9.2	36.8
	ASyS	10	11.1	11.5	48.3
	HD	45	50.0	51.7	100
	Total	87	96.7	100	
Remaining Unspecified*		3	3.3		
Total		90	100		

*Table 17. Basic cohort composition. The distribution across various disease subtypes is herein. * 3 subjects were diagnosed with an idiopathic inflammatory myopathy whose exact subtype could not be ascertained due to the lack of histologic data at the time of sampling. Abbreviations: DM, dermatomyositis; PM, polymyositis; IBM, inclusion body myositis; CAM, cancer-associated myositis; ASyS, antisynthetase syndrome; HD, healthy donors.*

Figure 11. Ring diagram representing cohort composition.

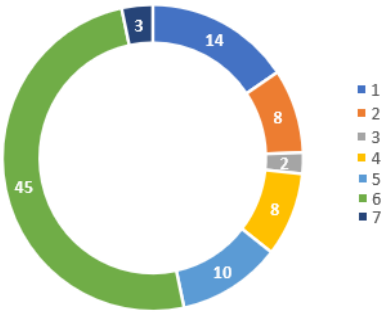

Figure 12. Ring diagram representing cohort composition. Key: (1) DM, Dermatomyositis; (2) PM, Polymyositis; (3) IBM, inclusion body myositis; (4) CAM, cancer-associated myositis; (5) ASyS, antisynthetase syndrome; (6) HD, healthy donors; (7) Unspecified subtype.

Table 18. Demographic and clinical features of IIM patients

Total number of patients	45 (100 %)
Females	29 (64.4 %)
Males	16 (35.6 %)
Age at diagnosis, mean ± SD, years	55.5 ± 13.6
Disease duration at the time of sampling, mean ± SD, years	4.9 ± 5.9
MMT-8 score, median (IQR)	145.5 (135.8-150)
IIM diagnosis	
DM	14 (31.1 %)
PM	8 (17.8 %)
IBM	2 (4.4 %)
CAM*	8 (17.8 %)
ASyS	10 (22.2 %)
Other**	3 (6.7 %)
Serology (ever)	
Myositis-specific autoantibodies	26 (57.8 %)
Anti-Mi2	7 (15.6 %)
Anti-t-RNA synthetase***	12 (26.7 %)
Anti-SRP	2 (4.4 %)
Anti-MDA5	1 (2.2 %)
Anti-Tif1gamma	5 (11.1%)
Myositis-associated antibodies	16 (35.6%)
Anti-SSA	11 (24.4 %)
Anti-SSB	3 (6.7%)
Anti-Ku	3 (6.7%)
Anti-PM/Scl-100	2 (4.4%)
Other †	5 (11.1%)
Clinical manifestations (ever)	
Cutaneous	28 (62.2 %)
Gottron's sign and papules	15 (33.3 %)
Heliotropic rash	14 (31.1 %)
Mechanic's hand	10 (22.2 %)
Raynaud's phenomenon	11 (24.4 %)
Other ‡	22 (48.9 %)

Arthritis	12 (26.7 %)
Myositis	37 (82.2 %)
ILD	24 (53.3 %)
Dyspnea	15 (33.3 %)
Cough	9 (20.0%)
Dysphagia	13 (28.9 %)
Clinical manifestations (upon sampling)	19 (42.2%)
Cutaneous activity	15 (33.3%)
Gottron's sign and papules	7 (15.6%)
Heliotropic rash	4 (8.9%)
Mechanic's hand	5 (11.1%)
Other §	12 (26.7%)
Articular Activity	1 (2.2%)
Muscular Activity	14 (31.1%)
Pulmonary Activity	1 (2.2%)
No Activity	26 (57.8%)
Ongoing treatment	
Oral corticosteroids	28 (62.2%)
Daily prednisone dose, mean ± SD, mg	13.2 ± 18.7
Immunosuppressant drugs	28 (62.2 %)
Mycophenolate mofetil	11 (24.4 %)
Methotrexate	13 (28.9 %)
Azathioprine	1 (2.2 %)
Cyclosporine A	1 (2.2 %)
Rituximab	3 (6.7 %)
Abatacept	1 (2.2 %)
No current treatment	6 (13.3 %)

Table 19. Demographic and clinical features of IIM patients. 1 case of high-grade serous ovarian carcinoma (2.2%), 1 case of cholangiocarcinoma (2.2%), 1 case of mucinous colorectal adenocarcinoma (2.2%), 2 cases of IPMN (4.4%), 1 case of low-grade colon adenoma (2.2%), 1 case of SCLC (2.2%) and 1 case of squamous-cell skin cancer (2.2%); ** 3 cases had an unspecified diagnosis of IIM in the temporary lack of histologic data; *** 8 cases of antiJO-1 positivity (17.8%), 3 cases of antiPL-12 positivity (6.7%), 1 case of antiPL-7 positivity (2.2%), 0 cases of anti-EJ (0%) or anti-OJ positivity (0%); † 1 case of antiPM1 positivity (2.2%); 1 case of antiU1RNP positivity (2.2%), 1 case of anti PM/Scl75 positivity (2.2%), 1 case of antiScl70 positivity (2.2%) and 1 case of antiSMA positivity (2.2%); ‡ 16 cases of shawl sign (35.6%), 7 cases of nailfold changes (15.6%), 2 cases of holster's sign 4.4%), 1 case of onychopathy (2.2%), 1 case of poikilodermatomyositis (2.2%), 1 case of wide-spread rash (2.2%); § 8 cases of shawl sign (17.8%), 2 cases of nailfold changes (4.4%), 2 cases of holster's sign 4.4%), 0 cases of onychopathy (0%), 0 cases of poikilodermatomyositis (0%), 1 case of wide-spread rash (2.2%). Abbreviations: DM, dermatomyositis; PM, polymyositis; IBM, inclusion-body myositis; CAM, cancer-associated myositis; ASyS, anti-synthetase syndrome; ILD, interstitial lung disease.*

4.1 EV SEPARATION

4.1.1 MORPHOLOGICAL ANALYSIS VIA TEM

TEM-acquired images in *Figure 7* confirm the presence in the analyzed samples of intact EV which retain their characteristic cup-shaped morphology. The prevalence of small EV (sEV) over medium/large sized EV (mEV) is also visible in these pictures.

Figure 13. Representative transmission electron microscopic images of extracellular vesicles derived from myositis patients versus healthy donors.

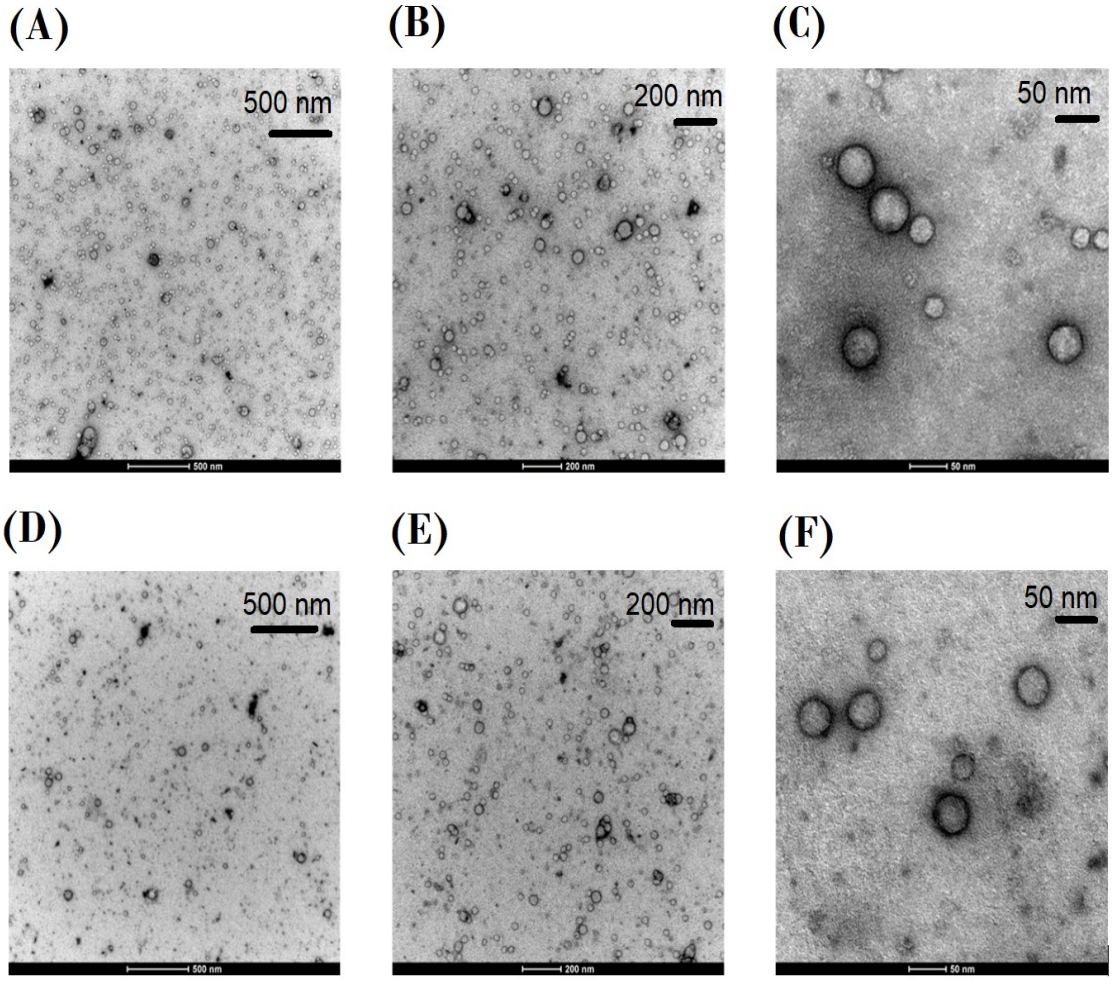

Figure 14. Representative transmission electron microscopic images of extracellular vesicles derived from myositis patients versus healthy donors. (A-C) myositis sample; (D-F) healthy donor's sample. Scale bars are also shown. Dilution ratio in PBS is 1:10 for every sample.

4.1.2 EV CHARACTERIZATION VIA IFC

Imaging flow cytometry (IFC) analysis demonstrates our samples contain nanoparticles that exhibit an expression profile of surface markers consistent with EV, especially those belonging to the small size range.

Indeed, the instrument shows the acquisition of representative particles emitting a positive fluorescent signal in the presence of a selection of tetraspanins (i.e., CD9, CD63 and CD81) and integrin CD11c *(see Figure 8).* While such tetraspanins have been selected by virtue of their relatively high specificity as surface markers for small EV of endosomal origin, integrin CD11c is employed as a generic EV surface marker, associated to the plasma membrane and/or endosomes in a non-tissue specific manner.

IFC characterization performed on several samples illustrates that our EV are mostly small-sized as evidenced by the predominant exposure of tetraspanins in the relative scarcity of integrin CD11c. Moreover, the absence of visible particles in the Bright Field column further corroborates that our EV belong to the lower end of the size spectrum as they fall under the resolution power of the instrument. Additionally, the lack of signal in the Bright Field column indicates that no clustering of multiple EV is likely to be occurring, as is desirable.

Figure 15. Characterization of EV by imaging flow cytometry.

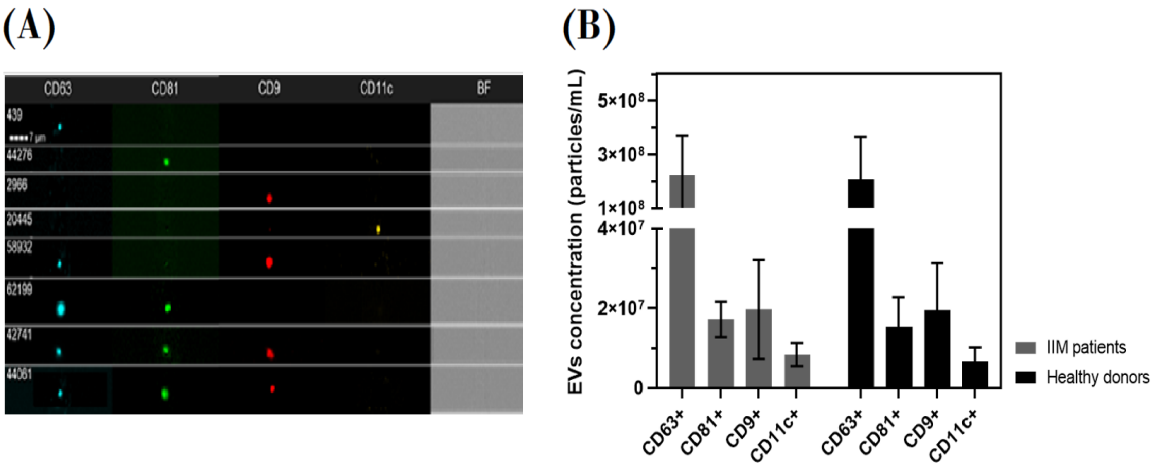

Figure 16. Characterization of EV by imaging flow cytometry. (A) Representative images of EV subpopulations as acquired by the instrument ImageStream Mark II™. The picture shows a clear positivity for the vesicular surface markers of interest and no signal in the bright field column. (B) Fluorescence profiling of tetraspanin and integrin expression. Different EV concentrations are reported for each EV subpopulation showing our EV lean toward a small EV profile. Abbreviations: BF, Bright field; IIM, idiopathic inflammatory myopathies.

Both patients (n=30) and healthy donors (n=30) characterized in our study were found to display a prevalence of CD63+ EV (EV/mL; values expressed as mean ± SD): ($2.91 \times 10^8 \pm 2.07 \times 10^8$ versus $2.40 \times 10^8 \pm 1.85 \times 10^8$; $p = 0.3272$) over CD81+ EV ($1.62 \times 10^7 \pm 2.06 \times 10^7$ versus $1.44 \times 10^7 \pm 1.73 \times 10^7$; $p = 0.7093$), CD9+ EV ($5.89 \times 10^7 \pm 4.78 \times 10^7$ versus $6.46 \times 10^7 \pm 4.83 \times 10^7$; $p = 0.6472$) and CD11c+ EV ($7.00 \times 10^6 \pm 7.78 \times 10^6$ versus $5.9 \times 10^6 \pm 6.68 \times 10^6$; $p = 0.5413$).

4.1.3 NTA ASSESSMENT OF EV CONCENTRATION AND SIZE

Nanoparticle tracking analysis (NTA) allowed us to gather precise information on the concentration and size of the EV contained in our samples, thus providing additional validation regarding the quantitative aspects of the isolated vesicles.

The isolated EV concentrations reached ~10^{10} EV/mL therefore are consistent with the standard values described in the literature.

NTA exact results will be reviewed in the following section.

4.2 FINDINGS OF EV CONCENTRATION AND SIZE IN IIM PATIENTS COMPARED WITH HD

NTA analysis revealed that IIM patients displayed significantly increased EV concentrations compared to HD ($1.95 \times 10^{10} \pm 1.47 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 7.82 \times 10^9$; $p = 0.025$), as shown in *Figure 9.*

Moreover, circulating EV were found to be smaller in size among IIM compared to healthy controls (median, IQR: 197.3 (183.5-211.9) vs 207.6 (195.8-216.6); $p = 0.029$.

Remarkably, upon patient stratification according to disease activity, the patients without any evidence of clinical activity in either domain (i.e., musculoskeletal, articular, cutaneous) had higher levels of circulating EV in comparison with healthy donors ($2.17 \times 10^{10} \pm 1.72 \times 10^{10}$ vs $1.75 \times 10^{10} \pm 0.78 \times 10^{10}$; $p = 0.022$).

On the other hand, clinically active patients in at least one domain had values that fell in between the two (clinically active patients compared to quiescent ones: 2.17 × 10^{10} ± 1.72 × 10^{10} versus 1.75 ×10^{10} ± 1.18 × 10^{10}; p = 0.034). *(See Figure 9)*

Figure 17. Comparison of EV concentrations between IIM patients and HD as measured by NTA.

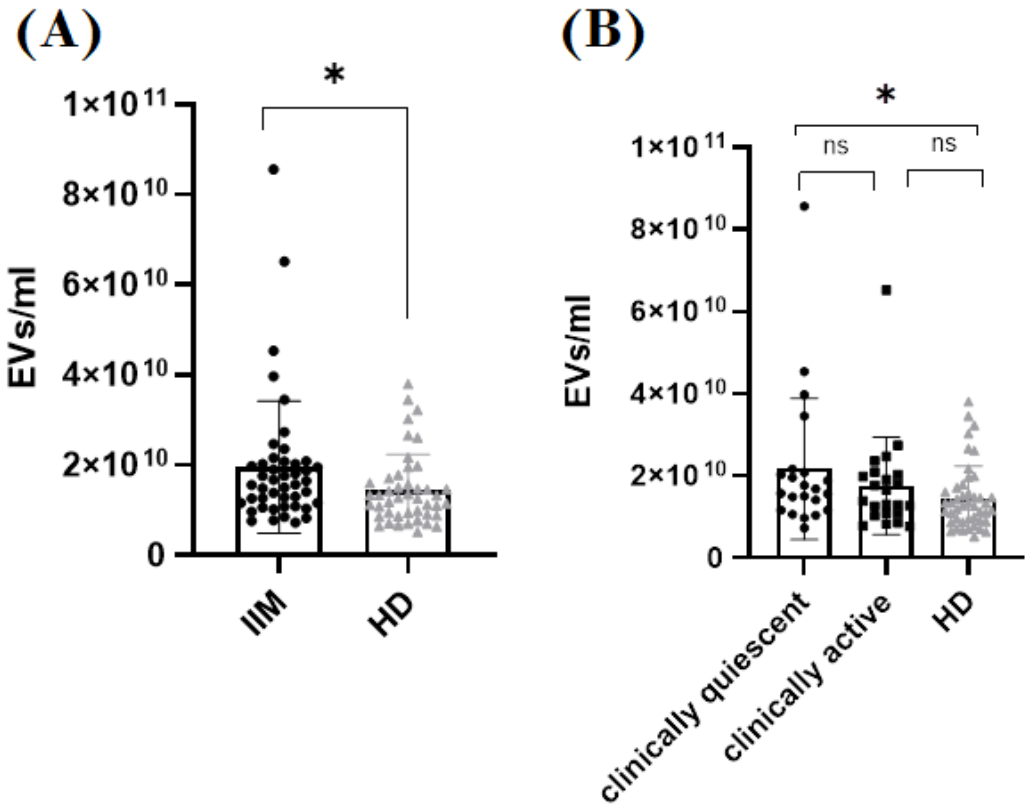

*Figure 18. Comparison of EV concentrations between IIM patients and healthy donors as measured by NTA. (A) Plasma EV concentrations among patients and healthy controls, (Student' s t test) *<0.05; (B) Circulating EV levels according to clinical activity, (one way ANOVA) *<0.05. Abbreviations: IIM, idiopathic inflammatory myopathies; HD, healthy donors.*

4.3 EV FINDINGS ACROSS IIM SUBSETS AND ACCORDING TO CLINICAL AND THERAPEUTIC FEATURES

After splitting IIM into individual subsets, CAM clearly stands out for the highest levels of circulating EV which are not matched by either HD or the other IIM subtypes even if taken separately or combined.

Indeed, EV levels associated to CAM ($3.28 \times 10^{10} \pm 2.84 \times 10^{10}$) were significantly higher than those detected across the no-CAM subgroups ($1.67 \times 10^{10} \pm 7.7 \times 10^{9}$; $p = 0.004$), as shown in *Figure 10.*

Besides, a concurrent ILD diagnosis was associated with a significantly greater concentrations of circulating EV compared to healthy controls ($2.12 \times 10^{10} \pm 1.61 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 0.78 \times 10^{10}$; $p = 0.021$).

No statistically significant differences were observed between ILD and non-ILD patients in this study; however, a numerically significant difference exists between the two subsets ($2.12 \times 10^{10} \pm 1.61 \times 10^{10}$ versus $1.72 \times 10^{10} \pm 1.26 \times 10^{10}$; $p = 0.37$) with non-ILD patients having intermediate concentrations interspersed between HD' and ILD patients' values. *(See Figure 10)*

Figure 19. Circulating EV plasma concentrations according to patient clinical manifestations and diagnoses.

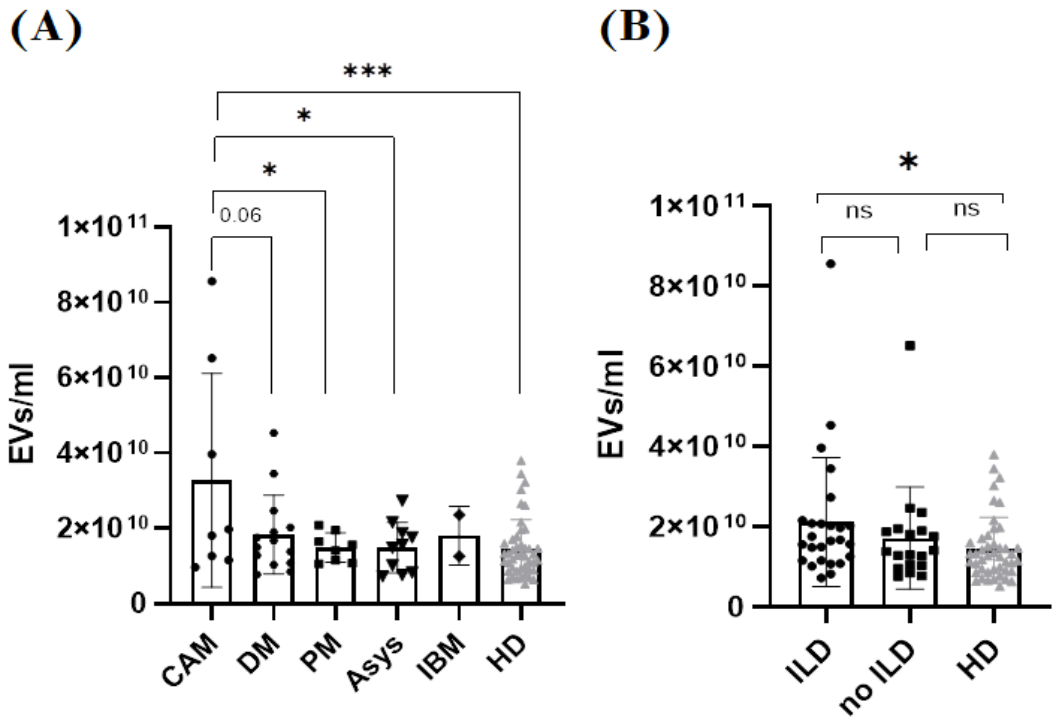

Figure 20. Circulating EV plasma concentrations according to patient clinical manifestations and diagnoses. (A) *Comparison of EV concentrations across different EV subsets and healthy controls. The chart shows a substantial increase in EV levels in the CAM subgroup compared to the others. CAM-IBM is not-assessable due to insufficient subgroup size; *<0.05, ***<0.001 (one way ANOVA); (B) Circulating EV levels stratified according to ILD presence. *<0.05, (one way ANOVA). Abbreviations: CAM, cancer-associated myositis; DM, dermatomyositis; PM, polymyositis; ASyS, anti-synthetase syndrome; IBM, inclusion-body myositis; HD, healthy donors; ILD, interstitial lung disease.*

ILD patients (n = 24) were found to display a relatively higher seropositivity rate than non-ILD patients (n = 21) (n = 16; 66.7% versus n = 11; 52.4%; $p = 0.33$). These estimates even gain slightly more strength if seropositivity towards ANA and ENA in ILD- as opposed to non-ILD patients is also taken into account (n=22; 91.7% versus n=17; 81.0%; $p = 0.29$).

Intriguingly, a positive serology was found to be associated with more elevated EV levels in comparison with seronegative patients ($2.09 \times 10^{10} \pm 1.63 \times 10^{10}$ versus $1.49 \times 10^{10} \pm 0.63 \times 10^{10}$; $p = 0.063$).

Nevertheless, no association to any antibody class nor any antibody specificity was identified within the broad panel analyzed.

It is worth emphasizing that patients with recent onset IIM (≤ 6 months, n=7) displayed higher circulating levels of EV compared to patients with a longer disease duration ($3.20 \times 10^{10} \pm 2.42 \times 10^{10}$ vs. $1.80 \times 10^{10} \pm 1.80 \times 10^{10}$; $p = 0.042$).

Regarding the stratification operated on the basis of treatment regimen at the time of sampling, it emerged that treatment-naïve patients (i.e., who had not undergone pharmacologic treatment either with glucocorticoids or immunosuppressants) exhibited greater EV concentration values ($2.83 \times 10^{10} \pm 2.89 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 0.78 \times 10^{10}$; $p = 0.010$) compared to HD. (*See Figure 11)*

Figure 21. Circulating EV levels according to treatment regimen at the time of sampling.

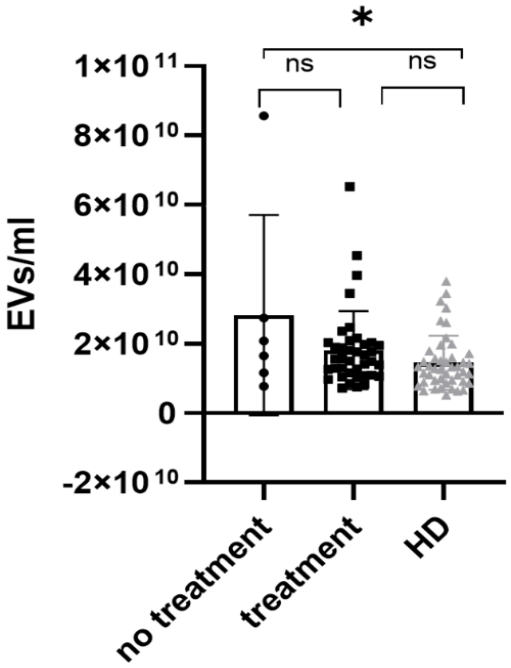

*Figure 22. Circulating EV levels according to treatment regimen at the time of sampling. As the SD is greater than the mean, negative Y axis values are also reported. *<0.05, (one way ANOVA). Abbreviation: HD, healthy donors.*

Despite the borderline statistical significance, a numerically relevant difference in circulating EV levels exists between treated IIM patients and healthy controls ($1.81 \times 10^{10} \pm 1.24 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 0.78 \times 10^{10}$; $p = 0.084$).

Even when glucocorticoid and immunosuppressant treatments are considered separately, the difference between treatment-naïve patients and healthy donors is maintained.

Indeed, both glucocorticoid-naïve (n = 17; $2.11 \times 10^{10} \pm 1.83 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 0.78 \times 10^{10}$; $p = 0.052$) and immunosuppressant-naïve patients (n=17; $2.43 \times 10^{10} \pm 2.14 \times 10^{10}$ versus $1.45 \times 10^{10} \pm 0.78 \times 10^{10}$; $p = 0.01$) appear to have higher EV levels than healthy controls.

Within the patient group, no statistically significant differences were highlighted between treated and untreated IIM patients with regards to circulating EV concentrations ($1.82 \times 10^{10} \pm 1.12 \times 10^{10}$ versus $2.83 \times 10^{10} \pm 2.89 \times 10^{10}$; $p = 0.12$).

Table 20. EV concentration and size comparison among HD and IIM patients stratified according to treatment regimen.

GLUCOCORTICOIDS					
	IIM GC-naïve	***p***	**HD**	***p***	**IIM non GC-naïve**
EV/ml (× 10^{10})	2.11 ± 1.83	0.052	1.45 ± 0.78	0.08	1.86 ± 1.22
Size (nm)	193.8 ± 14.4	0.017	205.9 ± 18.11	0.26	200.2 ± 21.89
IMMUNOSUPPRESSORS					
	IIM GC-naïve	***p***	**HD**	***p***	**IIM non GC-naïve**
EV/ml (× 10^{10})	2.11 ± 1.83	0.052	1.45 ± 0.78	0.08	1.86 ± 1.22
Size (nm)	193.8 ± 14.4	0.017	205.9 ± 18.11	0.26	200.2 ± 21.89

Table 21. EV concentration and size comparison among HD and IIM patients stratified according to treatment regimen. values are reported as mean ± SD for each category. (Student's t test). Abbreviations: IIM, idiopathic inflammatory myopathy; GC, glucocorticoid; p, p-value; HD, healthy donors; IS, immunosuppressant.

However, some numerically relevant differences can be noted, especially among the patients taking immunosuppressants compared to those who do not (IS naïve IIM patients versus non IS-naïve IIM patients: $2.43 \times 10^{10} \pm 2.14 \times 10^{10}$ versus $1.67 \times 10^{10} \pm 0.79 \times 10^{10}$, $p = 0.09$; glucocorticoid naïve IIM patients versus non GC-naïve IIM patients: $2.11 \times 10^{10} \pm 1.83 \times 10^{10}$ versus $1.86 \times 10^{10} \pm 1.22 \times 10^{10}$, $p = 0.58$). (*See Table 12).*

We found no significant difference in EV concentrations across the distinct types of immunosuppressants used. On the other hand, a medical history positive for treatment with the anti-CD20 monoclonal antibody Rituximab (ever, n = 8) was associated with significantly lower levels of EV compared to RTX-naïve patients ($1.11 \times 10^{10} \pm 0.29 \times 10^{10}$ versus $2.14 \times 10^{10} \pm 1.56 \times 10^{10}$; $p = 0.001$).

Interestingly, if a stratification is performed according to the received therapy, there are no relevant differences among the quiescent patients' group: in other words, a patient on clinical remission without treatment exhibits comparable levels of circulating EV to another patient who is maintained in clinical remission by means of a pharmacological treatment.

5. DISCUSSION

In recent years, EV have attracted increasing interest owing to their pivotal role as subcellular mediators of intercellular communication in a broad range of physiological and pathological contexts.

It is now being widely recognized that EV are promising candidates for use as biomarkers of diagnostic and prognostic value in several conditions, mainly in the oncological and rheumatological field. (124,131–133)

Additionally, an expanding body of research is aspiring to harness their enormous potential as therapeutic drug carriers and in the setting of vaccine development thereby leading some experts to coin the appropriate term of “exosome theranostics”. (134)

However, it is universally acknowledged that despite global efforts heading in this direction (79), a serious limitation to said applications is imposed by the lack of consensus within the scientific community around the preanalytical techniques of EV samples’ processing.

This is the context for the present study, whereby we propose a methodological approach not defined “gold standard” which proves to be reproducible and highly reliable for the isolation of circulating EV from the plasma of both IIM patients and healthy donors.

Unlike most wide-spread isolation protocols, we recommend performing SEC followed by ultrafiltration as opposed to ultracentrifugation to avoid damaging the delicate vesicular content of the sample.

Indeed, TEM-acquired images of our samples confirmed the presence and structural integrity of the EV thus isolated.

The vesicular nature of the observed nanoparticles has been further substantiated by characterization via IFC of specific surface markers.

NTA corroborated the validity of our approach, identifying a pool of small- to medium-sized EV whose measured concentrations are aligned with the order of magnitude commonly reported in the current literature (i.e., ~10^{10} EV/mL).

Hence, the collected data allowed to compare findings between healthy donors and IIM patients while also enabling to infer relevant clinical correlations among the latters.

In accordance with previous studies (129,130,135), we found that IIM patients display abnormally high plasma concentrations of EV compared to healthy donors, alongside a slightly decreased vesicular size. The functional implications of such observations still require further investigation; however, it may be speculated that the exosome subset is more represented across IIM patients, which could couple with different functional properties of the EV pool.

We also report lower EV levels among patients showing signs of clinical activity compared to quiescent IIM patients. In this regard, lower EV levels coinciding with peaks of disease activity are consistent with previous findings (136), thereby opening new avenues for speculation around the likely migration of EV within sites of inflammation during disease flares.

Nevertheless, further investigation is needed to validate such enticing hypotheses.

Within the patient group, our data point at CAM being the IIM subset associated with the highest EV concentrations which are unparalleled in comparison with any other IIM subtype.

We postulate such remarkable increase might be related to dysregulated antigen trafficking in paraneoplastic forms.

Indeed, EV are likely to play a pathogenetic role in this subtype, facilitating the spreading of (possibly post-translationally modified) self-antigens from the neoplastic *milieu* virtually by means of a prolonged and extensive antigen presentation which in turn triggers potent immune responses resulting in the development of the paraneoplastic myopathic manifestations.(29)

On the same note, a remarkable yet weak association between the serological status of the patient and EV levels has been detected in the present study: in fact, seropositivity regardless of antibody specificity appears to be linked to abnormally elevated plasma EV concentrations.

Although the resulting *p*-value is deemed borderline ($p = 0.06$), it provides us with a clinically significant indication; besides, a faint statistical significance may partly be attributable to an insufficient sample size, as is the case with such a rare disease.

Taken together, these findings hint at a notable pathogenetic relevance of EV across the various IIM subtypes (especially in CAM) in light of their function in antigen presentation and more broadly in the setting of immune regulation whereby they lead to immune activation determining a clear impact on the serologic profile of the patient.

To this date, it is not possible to exclude whether such relationship is bidirectional: actually, a pathological pool of active B cells may be at the root of both an increase in EV secretion and parallel abnormal antibody production.

Interestingly, we identified a statistically significant correlation between the presence of ILD and higher levels of circulating EV compared to healthy controls and of numerical significance compared to non-ILD patients.

It is worth noting that said results might be the consequence of a bias, due to ILD patients having higher seropositivity rates compared to non-ILD patients. Regrettably, our current sample size is not permissive for a multivariable analysis, leaving us unable to weigh the magnitude of such bias.

No other significant associations have emerged upon patient stratification according to the other clinical and laboratory data assessed, including levels of muscle enzymes as expression of inflammatory insult. Again, this may partly be explained by a still limited sample size as well as by the normalization of clinical and laboratory parameters induced by the pharmacological therapy undertaken by the vast majority of our patients (86.7%).

Noteworthy, no significant correlation between EV and creatin-kinase levels could be established by Shirafuji et al. either (129), despite an appropriate number of clinically active patients was included in that study.

We measured higher circulating levels of EV among patients with recent onset IIM compared with those with longer disease duration.

On the other hand, a previous work has highlighted that a longer disease duration positively correlated with EV concentration (135). However, it should be noted that this study solely assessed endothelial-derived microparticles (EMP), thus it focused on a different EV subtype given that our EV seem to fall mainly within the exosome category and may have a different cellular origin. Additionally, these analyses were conducted in a different pathological context i.e., chronic endothelial damage in primary Sjögren syndrome, therefore it is likely that a longer disease duration can be associated with cumulative endothelial injury which is reflected in the sustained increase of endothelial microparticle production over time.

Instead, our findings could be interpreted through the lens of an unsatisfactory disease management in newly diagnosed IIM patients compared with those further along in the disease course.

Indeed, stratifications based on treatment regimen yielded encouraging results indicating that untreated patients tend to score higher in terms of circulating EV than their treated or healthy counterparts.

Therefore, pharmacological therapy seems to bring considerable benefit to IIM patients effectively lessening their EV values to resemble those of healthy donors.

Of all the drugs considered, we show that the anti-CD20 monoclonal antibody Rituximab confers the most striking advantages which hints at the likely role of B cell-mediated release of our isolated EV.

Accordingly, extensive research (130,137,138) illustrates that B cells considerably engage in EV secretion but further investigations are underway to precisely identify the predominant cellular origin of EV in the setting of IIM.

Additional longitudinal studies could help to unravel whether treatment response is indeed supported by a decrease in B cell-derived EV. In this regard, a comparison between absolute B cell counts and B cell-derived EV in relation to IIM disease activity could yield essential insight as well.

On the same note, a longitudinal study may be an appealing future perspective as it could partly compensate for a limitation of the present study's design: in fact, a cross-sectional approach may not prove to be the most appropriate one in the setting of treatment response assessment over time.

Moreover, the observational design restricts our ability to infer causation in terms of the effect of treatment onto EV concentrations.

It is beyond doubt that extending our study to other institutions would endow it with much greater statistical power thus allowing us to perform multivariable analyses to ultimately reduce the burden of confounding factors on our results and improving its generalizability.

Lastly, cost/benefit analyses may be required to ascertain the feasibility of the implementation of EV measurement in the day-to-day clinical practice.

However, the strength of our study lies in the validity of the experimental method described as well as in the representative cohort included and, most importantly, in the originality of our findings.

Taken together, our data show that circulating EV are significantly increased in IIM patients, especially in recent-onset, seropositive and treatment-naïve disease making them a suitable candidate to aid in the diagnostic process and disease monitoring on a par with other routinely evaluated laboratory markers.

6. CONCLUSIONS

In conclusion, our study supports the reliability and validity of the protocol presented herein for the isolation and characterization of plasma EV both in IIM patients and in healthy controls.

Our findings provide evidence of significant abnormalities in the circulating EV levels in IIM which may associate with disease phenotypes and treatment response, thereby substantiating the role of plasma EV as potential biomarkers for early diagnosis and surveillance of IIM patients.

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