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(Citation)
Rheumatology,61(4):1669-1679

(Issue Date)
2021-07-23

(Resource Type)
journal article

(Version)
Accepted Manuscript

Title

Identification of Plexin D1 on circulating extracellular vesicles as a potential biomarker of polymyositis and dermatomyositis

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Abstract

Objectives. We aimed to identify disease-specific surface proteins on extracellular vesicles (EVs) as novel serum biomarkers of polymyositis and dermatomyositis (PM/DM).

Methods. We performed liquid chromatography-tandem mass spectrometry (LC/MS) on purified EVs from sera of 10 PM/DM, 23 patients with other autoimmune diseases and 10 healthy controls (HC). We identified membrane proteins preferentially present in EVs of PM/DM patients by bioinformatics and biostatistical analyses. We developed EV sandwich ELISA for directly detecting serum EVs expressing disease-specific membrane proteins and evaluated their clinical utility using sera of 54 PM/DM, 24 rheumatoid arthritis (RA), 20 systemic lupus erythematosus (SLE), 13 systemic sclerosis, 25 Duchenne and Becker muscular dystrophy (DMD/BMD) patients, and 36 HC.

Results. LC/MS analysis identified 1,220 proteins in serum EVs. Of these, Plexin D1 was enriched in those from PM/DM patients relative to HC or patients without PM/DM. Using a specific EV sandwich ELISA, we found that levels of Plexin D1-positive EVs (Plexin $D1^+$ EVs) in serum were significantly greater in PM/DM patients than in HC, RA or SLE, or DMD/BMD patients. Serum levels of Plexin $D1^+$ EVs were greater in those PM/DM patients with muscle pain or weakness. Serum levels of Plexin $D1^+$ EVs were significantly correlated with levels of aldolase (r_s=0.481), white blood cells (r_s=0.381), neutrophils (r_s=0.450), and platelets (r_s=0.408) in PM/DM patients. Finally, serum levels of Plexin $D1^+$ EVs decreased significantly in patients with PM/DM in clinical remission after treatment.

Conclusion. We have identified levels of circulating Plexin $D1^+$ EVs as a novel serum biomarker for PM/DM.

Key words

Polymyositis, dermatomyositis, biomarker, extracellular vesicles, Plexin D1

Key messages

Comprehensive proteomic analysis of serum EVs is useful for identifying biomarkers of autoimmune diseases.

LC/MS analysis and ELISA identify Plexin D1-positive EVs as a novel serum biomarker for PM/DM.

Plexin D1-positive EVs may play a role in the pathogenesis of muscle lesion of PM/DM.

Introduction

Polymyositis (PM) and dermatomyositis (DM) are systemic autoimmune diseases characterized by chronic inflammation of the muscles, lungs, skin, and several other organs [1, 2]. Recently, certain myositis-specific autoantibodies have been proposed as useful diagnostic markers for idiopathic inflammatory myopathies including PM/DM [3, 4], but there are few reliable specific markers for disease activity, prognosis or treatment responses of patients with refractory PM/DM [5, 6].

Extracellular vesicles (EVs), including exosomes and microvesicles, are small membrane vesicles released by almost all cell types and are found circulating in blood and other body fluids. Recent studies have demonstrated that EVs are involved in numerous physiological processes by mediating cell-to-cell communication through transfer of their cargoes (miRNAs, mRNAs, and proteins) [7]. In particular, EVs derived from both immune and non-immune cells have pivotal roles in regulating the immune system. Through their surface immune regulatory proteins, including MHC molecules, co-stimulatory molecules, signaling molecules and adhesion molecules, EVs activate the immune response but can also drive pathological inflammatory and autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [8-12]. Although studies on the roles of EVs in idiopathic inflammatory myopathies are still limited, there is some evidence supporting the involvement of EVs in promoting muscle inflammation, fibrosis, loss of function and weakness in PM/DM [13-16].

In addition to their biological effects, circulating EVs are also promising as a novel type of systemic biomarker for various different diseases because their cargoes reflect their cellular origin and function [17, 18]. While the detection of miRNAs and internal proteins in EVs requires time-consuming purification steps such as ultracentrifugation, surface proteins on EVs can be detected directly from blood and other body fluids without purification steps, allowing clinical application using existing immunocapture-based techniques [18]. Interestingly, increasing evidence suggests that surface proteins (e.g., membrane proteins) on EVs, especially exosomes, are altered in pathological conditions and useful as non-invasive biomarkers for cancer diagnosis and prognosis [19-22]. Previous studies have reported that microvesicles derived from immune cells, including T cells (CD3-positive and CD4- or CD8-positive), B cells (CD19-positive), or monocytes (CD14-positive), have been identified as potential biomarkers for a variety of autoimmune diseases such as RA [11], SLE [12], and PM/DM [13, 14]. However, such microvesicles are not disease-specific because their origin is from common pathogenic immune cells. On the other hand, disease-specific surface proteins on circulating exosomes derived from pathogenic cells may be useful as specific biomarkers for

diagnosis, assessing disease activity, prognosis, and treatment response in patients with autoimmune disease. Nonetheless, they have rarely been employed as novel biomarkers. In the present study, we identified Plexin D1 as a disease-specific surface protein on serum EVs from PM/DM patients using comprehensive proteomic analysis. We also developed a specific EV sandwich ELISA for directly detecting serum Plexin D1-positive EVs (Plexin $D1^+$ EVs). Furthermore, we evaluated the clinical utility of serum Plexin $D1^+$ EVs as a novel biomarker, focusing on their associations with clinical symptoms, disease activity, and their changing levels after treatment.

Methods

Experimental design and patients

The experimental design for novel biomarker discovery in PM/DM patients is shown in Figure 1. A total of 64 patients with PM/DM, 42 with RA, 25 with SLE, 13 with systemic sclerosis (SSc), 25 with Duchenne and Becker muscular dystrophy (DMD/BMD) and 46 healthy controls (HC) was enrolled at Kobe University Hospital. Patients with PM/DM were diagnosed according to the definite or probable criteria of Bohan and Peter, and patients with other autoimmune dieseases met the established criteria for each disease [2, 23-27]. The clinical diagnosis of DMD/BMD patients was confirmed by identification of mutations in the *DMD* gene and/or immunohistological examination using muscle biopsy samples. Blood samples were obtained from the inpatient and outpatient Departments of Kobe University Hospital and from hospital staff between 2012 and 2021. Paired samples from 24 pre- and post-treatment PM/DM patients were collected during the follow-up period, during which 21 patients had achieved clinical remission and three had not. Blood samples without anti-coagulant were allowed to clot at room temperature for 10 minutes. Sera were then separated by centrifugation at 3,000 rpm for 7 min at room temperature and stored frozen at -80°C until use. Clinical symptoms (rash typical of DM, interstitial lung disease (ILD), muscle pain, muscle weakness, dysphagia and malignancy) and laboratory data were collected from the medical records. The rash typical of DM was defined as heliotrope rash, Gottron sign, and Gottron papules. Diagnosis of ILD was based on high-resolution computed tomography of the chest, pulmonary function tests, or blood tests. Muscle pain was defined as spontaneous or experienced when gripping something. Muscle weakness was defined as manual muscle testing score <4 in proximal limb muscles and neck flexors. Diagnosis of dysphagia was accomplished by otolaryngologists using videoendoscopic evaluation of swallowing. Patients who were diagnosed with at least one cancer were defined as PM/DM patients with malignancy. Characteristics of PM/DM patients and control groups are summarized in Table 1. This

study was approved by the Ethics Committee of Kobe University Hospital and complied with the principles of the Declaration of Helsinki (approvals number B200020 and B200021). All participants provided written informed consent or were provided information disclosure and opportunities to opt out.

EV sandwich ELISA

Nunc MaxiSorp flat-bottom 96 well plates (Thermo Fisher Scientific, Waltham, MA, USA) were coated with 125 ng/well of anti-CD9 antibody (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) in carbonate buffer (pH 9.6), overnight at 4°C. After thrice washing with PBS/0.05% Tween 20 (PBS-T), the plate was blocked with PBS/5% BSA for 2 hours at 37°C. After washing x3 again with PBS-T, 10 µl serum or standard control sample in PBS/1% BSA containing 10 µg/ml TRU Block (Meridian Life Science, Inc., Memphis, TN, USA) was added and plates were incubated overnight at 4°C. After washing x5 with PBS-T, the plate was incubated with biotinylated anti-Plexin D1 antibody (125 ng/ml, R&D Systems) or biotinylated anti-CD63 antibody (125 ng/ml, BioLegend, San Diego, CA, USA) in PBS/1% BSA for 60 minutes at room temperature. After again washing x5 with PBS-T, the plate was incubated with polyHRP-conjugated streptavidin (Thermo Fisher Scientific) diluted 1:50,000 in PBS/1% BSA for 60 minutes at room temperature. After the final x5 wash with PBS-T, TMB substrate was added (also Thermo Fisher Scientific). The reaction was stopped with 2N HCl and optical density (OD) at 450 nm was immediately measured. The standard control sample was prepared from pooled serum from healthy controls. The ratio of the OD of the serum sample to a standard control sample was calculated, yielding the "ELISA index" to quantify serum Plexin $D1^+$ EVs.

Statistical analysis

Results were expressed as median values. Differences were analyzed using Wilcoxon rank-sum testing for comparison of two groups, Wilcoxon signed-rank testing for comparison of paired data and Kruskal-Wallis testing with Dunn's post hoc testing for comparison among multiple groups. Spearman's rank correlation coefficient was used for correlation analysis. All analyses were performed with GraphPad Prism version 7.0 software (GraphPad Software, San Diego, CA, USA). A P value of <0.05 was considered statistically significant.

For details, please see online supplementary methods (Supplementary Data S1, available at *Rheumatology* online).

Results

Plexin D1 in serum EVs is a novel candidate biomarker for PM/DM patients

The experimental design for identification of novel biomarkers is shown in Figure 1. To identify all proteins in serum EVs from a screening set (Table 1), we used size exclusion chromatography (SEC) to isolate the EVs from 10 PM/DM patients, 23 patients without PM/DM (5 SLE and 18 RA) and 10 HC. Purified EVs were then individually analyzed on an LC/MS system able to distinguish a total 1,220 proteins most commonly detected in PM/DM patients, HC and the other patients (Supplementary Figure S1A, available at *Rheumatology* online). We performed DAVID gene ontology analysis on these 1,220 proteins and found that extracellular exosome proteins (37.1%), plasma membrane proteins (29.2%) and cytosolic proteins (21.1%) were enriched in serum EVs (Supplementary Figure S1B, available at *Rheumatology* online). Of these 1,220 proteins, 599 (49.1%) had been previously reported in the online Exocarta database (Supplementary Figure S1C, available at *Rheumatology* online). These results indicated that purification by SEC can separate serum EVs from abundant serum-proteins such as albumin. Next, we visualized the membrane proteins up- or down-regulated on serum EVs from PM/DM patients using volcano plots combining DAVID gene ontology and UniProtKB/Swiss-prot analyses (Figure 2A). This revealed that the following 5 membrane proteins were significantly up-regulated in the serum EVs from PM/DM patients relative to HC or other patients: CYBB (cytochrome b-245 heavy chain), ICAM5 (intercellular adhesion molecule 5), NEO1 (neogenin), PLXD1 (Plexin D1), and SLC1A2 (Solute Carrier Family 1 Member 2). Of these, we selected Plexin D1 for further testing as a candidate biomarker in PM/DM patients because it is not only expressed on different immune cells [28-31] but also muscle tissues from patients with juvenile DM [32]. Indeed, LC/MS analysis confirmed that Plexin D1 levels were significantly enriched in serum EVs from PM/DM patients compared to HC ($p=0.0237$) and tendentially from the other patients ($p=0.0593$) (Figure 2B). Furthermore, we verified the expression of Plexin D1 in serum EV-containing eluates (fractions 7 to 9) from PM/DM patients using SEC and Western blotting (Figure 2C). We next developed a specific EV sandwich ELISA for directly detecting serum Plexin $D1^+$ EVs. To directly detect serum CD9- and Plexin D1-double-positive EVs ($CD9^+$ Plexin $D1^+$ EVs), we designed EV sandwich ELISA using anti-CD9 antibody as the capture antibody for serum EVs and biotinylated anti-Plexin D1 antibody as the detection antibody (Figure 1). High purity $CD9^+$ Plexin $D1^+$ EVs were confirmed in serum EV-containing eluates from PM/DM patients (Figure 2D).

Amounts of Plexin D1$^+$ EVs in the serum are increased in PM/DM patients in association with muscle pain or weakness

We next evaluated the clinical utility of serum Plexin D1$^+$ EVs using 172 sera from a validation set (Table 1). Serum levels of CD9$^+$ Plexin D1$^+$ EVs were significantly higher in PM/DM patients than in HC ($p=0.0016$), RA ($p<0.0001$) and SLE ($p<0.0001$), suggesting that they could be detected by EV sandwich ELISA as well as by LC/MS (Figure 3A). To confirm whether the elevation of CD9$^+$ Plexin D1$^+$ EVs in sera is due to muscle destruction, we also assessed serum levels of CD9$^+$ Plexin D1$^+$ EVs in DMD/BMD patients. Muscular dystrophy patients have more massive muscular destruction than PD/DM patients, leading to higher muscle enzyme levels in sera than those in patients with PM/DM. We found that amounts of CD9$^+$ Plexin D1$^+$ EVs in DMD/BMD patients were similar to those in HC and significantly lower than those in PM/DM (Figure 3B). These results indicate that elevation of CD9$^+$ Plexin D1$^+$ EVs in sera may not simply be the result of muscle destruction. In the receiver operating characteristic (ROC) analyses, serum levels of CD9$^+$ Plexin D1$^+$ EVs were able to distinguish PM/DM from HC (AUC=0.756; $p<0.0001$), other autoimmune diseases (AUC=0.784; $p<0.0001$), or DMD/BMD (AUC=0.748; $p=0.0004$) with good diagnostic accuracy (Supplementary Figure S2, available at *Rheumatology* online). On the other hand, there were no significant differences in serum levels of CD9$^+$ Plexin D1$^+$ EVs between clinical subtypes or the myositis-specific antibody (MSA)-associated subtype of PM/DM patients (Figure 3C, 3D). We then assessed associations with clinical symptoms and found that serum levels of CD9$^+$ Plexin D1$^+$ EVs were more highly elevated in PM/DM patients suffering muscle pain or weakness ($p=0.0428$), while there was no significant difference between patients with or without ILD, dysphagia, malignancy, and fasciitis (Figure 3E-H, Supplementary Figure S3 available at *Rheumatology* online).

Serum levels of Plexin D1$^+$ EVs are correlated with increased aldolase, white blood cells, neutrophils, and platelets in PM/DM patients

We next analyzed correlations between serum levels of CD9$^+$ Plexin D1$^+$ EVs and disease activity markers or peripheral blood cells in pre-treatment PM/DM patients, in order to avoid confounding effects of drugs such as prednisolone [33]. Serum levels of CD9$^+$ Plexin D1$^+$ EVs were significantly correlated with the muscle enzyme aldolase ($r_s=0.481$, $p=0.027$) as a marker of disease activity, as well as with white blood cell counts (WBC) ($r_s=0.381$, $p=0.046$), neutrophils ($r_s=0.450$, $p=0.019$), and platelets (PLT) ($r_s=0.408$, $p=0.031$) as markers of systemic inflammation, but not with creatine kinase (CK) or other disease activity markers (Figure 4A-J). Intriguingly, serum levels of CD9$^+$ Plexin D1$^+$

EVs had no correlation with the muscle enzymes in DMD/BMD patients (Supplementary Figure S4, available at *Rheumatology* online), and with other inflammatory markers (WBC, neutrophils, and PLT) in patients with other autoimmune diseases (Supplementary Figure S5, available at *Rheumatology* online). These results suggested that only in PM/DM patients serum $CD9^+$ Plexin $D1^+$ EVs was specifically increased and has specifically correlation with disease activity.

Serum levels of Plexin $D1^+$ EVs are decreased in PM/DM patients after treatment

Finally, we evaluated changes of serum levels of $CD9^+$ Plexin $D1^+$ EVs after treatment in 24 PM/DM patients. These were found to be significantly decreased in 21 PM/DM (9 PM, 7 DM and 5 clinically amyopathic DM (CADM)) patients who achieved clinical remission after treatment ($p<0.0001$) (Figure 5A). In contrast, levels of $CD9^+$ Plexin $D1^+$ EVs tended to increase in 2 of the remaining 3 PM/DM (3 DM) patients who did not respond (Figure 5B).

Discussion

In this study, we identified levels of serum Plexin $D1^+$ EVs as a novel biomarker of PM/DM patients. They were significantly more elevated in PM/DM patients with muscle pain or weakness and were positively correlated with several markers of disease activity or systemic inflammation. Reciprocally, we demonstrated that serum levels of Plexin $D1^+$ EVs were significantly decreased in PM/DM patients who achieved clinical remission after treatment.

To the best of our knowledge, this is the first report presenting a comprehensive proteomic analysis of serum EVs from PM/DM patients. Here, we aimed to identify disease-specific surface proteins (e.g., membrane proteins) on serum EVs for further clinical application. From the volcano plots combining different bioinformatics analyses (DAVID gene ontology and UniProtKB/Swiss-prot), we identified the membrane proteins CYBB, ICAM5, NEO1, PLXD1, and SLC1A2 as significantly up-regulated in serum EVs from PM/DM patients relative to those from HC or RA and SLE patients. Of these 5 up-regulated membrane proteins, we focused on Plexin D1, which is not only expressed on various immune cells [28-31] but also muscle tissues from patients with juvenile DM [32]. In addition, we found that the peptide fragment detected by LC/MS was located in the cytoplasmic domain of Plexin D1 (data not shown), suggesting that this molecule in serum may be derived from EVs rather than soluble proteins.

Plexin D1 is a single-pass transmembrane receptor for several semaphorin ligands. It has been reported to play important roles in the development of the vasculature and central

nervous system [34] and the immune system [28-31]. It has also been implicated in cancer metastasis [35]. Notably, recent studies have demonstrated that Plexin D1 regulates various immune responses such as Th2/Th17 differentiation of T cells [28], germinal center formation and humoral immune responses of B cells [29], IL-12/IL-23p40 production by dendritic cells [30], and the migration of neutrophils [31]. In the context of autoimmune disease, Plexin D1 has been reported to be involved in dysregulation of angiogenesis in systemic sclerosis [36] and chronic inflammation of muscle in juvenile DM [32]. However, there appear to be no reports on the involvement of Plexin $D1^+$ EVs in the pathogenesis of autoimmune diseases including PM/DM.

We established a specific EV sandwich ELISA for directly detecting serum Plexin $D1^+$ EVs, and confirmed that Plexin D1 levels on serum EVs were significantly increased in PM/DM patients compared to HC, RA and SLE, and DMD/BMD patients. Although lower levels of serum Plexin $D1^+$ EVs from CADM or anti-MDA5 antibody-positive patients were observed, there was no significant difference between clinical subtypes or myositis-specific antibody (MSA)-associated subtypes in PM/DM patients. Interestingly, we found that serum Plexin $D1^+$ EVs in PM/DM patients were associated with muscle pain or weakness, the most common myositis symptoms, but not with any other clinical symptoms. Furthermore, we also found that serum levels of Plexin $D1^+$ EVs in PM/DM patients were correlated with the muscle enzyme aldolase as a marker of disease activity, as well as with white blood cell counts, neutrophils, and platelets as markers of systemic inflammation. Finally, we demonstrated that serum levels of Plexin $D1^+$ EVs were significantly decreased in patients with PM/DM, including PM, DM, and CADM, who achieved clinical remission after treatment. In contrast, they tended to be increased in patients who did not enter remission. These results indicate that serum levels of Plexin $D1^+$ EVs may reflect common pathological changes in PM/DM patients rather than any specific for any particular heterogeneous PM/DM subtype.

It is not clear why there is no significant correlation between serum levels of Plexin $D1^+$ EVs and the muscle enzyme CK. Although CK and aldolase are both enzymes that are derived from damaged skeletal muscle [37], some patients with myopathy or eosinophilic fasciitis have normal CK but high aldolase levels [38, 39]. Our results showed that serum levels of Plexin $D1^+$ EVs were not associated with fasciitis in PM/DM patients who underwent magnetic resonance imaging (MRI) examinations, suggesting a different mechanism for altered levels in the blood. Importantly, the association between serum Plexin $D1^+$ EVs and aldolase was specific to PM/DM patients and not to DMD/BMD patients. A previous report demonstrated that serum aldolase but not CK is a potential biomarker of damaged early regenerating muscle cells in myositis patients [40]. That

report also indicated that the aldolase protein is expressed prior to CK during in vitro muscle regeneration. Moreover, aldolase is highly expressed in early regenerating muscle cells not only in an *in vivo* mouse model of muscle damage and repair but also in muscle biopsies from myositis patients. Furthermore, other studies revealed that regenerating muscle cells in biopsies from these patients expressed high levels of several myositis autoantigens, suggesting that immune-mediated mechanisms mediated by pathogenic immune cells or autoantibodies may contribute to the pathogenesis of inflammatory myopathy [41, 42]. Together with our results and those of previous studies, we suggest that serum Plexin $D1^+$ EVs may be a potential biomarker for immune-mediated inflammatory myopathies including PM/DM.

The limitation of this study was that we did not evaluate the origin and pathological function of Plexin $D1^+$ EVs due to research design or methodology. As serum EVs contain whole body-derived EVs, it is difficult to identify EVs derived from specific cells or tissues. Although there are no results from histopathological or cellular experiments, our finding showed that serum levels of Plexin $D1^+$ EVs in DMD/BMD patients were lower than those in PM/DM patients, suggesting that serum Plexin $D1^+$ EVs may not simply reflect muscle destruction and not be derived from muscle tissues. On the other hand, our findings also showed that the association between serum Plexin $D1^+$ EVs and inflammatory markers such as WBC, neutrophils or PLT was specific to PM/DM patients and not to patients with other autoimmune diseases. Seto *et al.* recently reported that circulating pathogenic neutrophils may play an important role in idiopathic inflammatory myopathies through their ability to injure muscle tissues [43]. Movassagh *et al.* reported that the migration of peripheral blood human neutrophils is negatively regulated via Semaphorin 3E/Plexin D1 axis [31]. The association between pathogenic neutrophils and Plexin D1 have not been explained and need to be investigated in the future.

Considering the function of Plexin $D1^+$ EVs, it remains unknown whether they are involved in pathogenesis of PM/DM by acting on neighboring cells or distant organs. It is well known that ligands on EVs activate intracellular signaling by ligand-receptor interaction [8, 9, 18], but the role of receptors on EVs is not well understood. However, recent studies have demonstrated that several types of receptors on EVs can activate ligand-mediated reverse signaling [44-46]. Although reverse signaling has been reported to play an important role in cell-to-cell communication by Semaphorin and Plexin family [47, 48], there are currently no reports of reverse signaling in Plexin D1 and its ligands, Semaphorin 3E and Semaphorin 4A. Further research is needed to elucidate the involvement of Plexin $D1^+$ EVs in the pathogenesis of PM/DM.

In summary, we have identified Plexin $D1^+$ EVs as a novel type of biomarker and

documented that higher serum levels of Plexin $D1^+$ EVs are associated with muscle pain or weakness in PM/DM patients. We also demonstrated that serum levels of Plexin $D1^+$ EVs change in response to pathological conditions. Although further studies are needed to elucidate the origin and pathological function of Plexin $D1^+$ EVs, our study suggests that serum Plexin $D1^+$ EVs are potential biomarkers of PM/DM.

Acknowledgements

K.U. designed the study, performed the experiments, analysed the data, and drafted the manuscript. K.U. performed LC/MS experiment and data analysis. K.A., S.T., T.I., H.A. and A.M. provided patient samples and clinical data. T.O., Y.N., S.K. and J.S. made substantial contributions to the study concept and design, the analysis and interpretation of data, drafting the article and revising the manuscript. All authors read and approved the final manuscript. We thank Shino Tanaka-Natsui, Department of Rheumatology and Clinical Immunology, Kobe University Graduate School of Medicine for providing technical assistance.

Funding: This work was supported by the JSPS KAKENHI Grant Numbers JP18H00468, JP20H01087.

Disclosure statement: The authors have declared no conflict of interest.

Data availability statement

Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information. Additional data are available upon reasonable request.

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Figure legends

Figure 1. Overview of experimental design for biomarker discovery
Serum EVs were purified by size exclusion chromatography using sera from 10 patients with PM/DM, 18 with RA, 5 SLE patients, and 10 HCs as the screening set (n=43). LC/MS was applied to identify all proteins contained in the serum EVs. Several biomarker candidates were selected by bioinformatics and statistical analyses. Plexin D1 was identified as a novel biomarker candidate by verification using Western blotting. Further, we established a specific EV sandwich ELISA for detecting Plexin D1-positive EVs and evaluated the clinical utility of such EVs using sera from 54 PM/DM, 24 RA, 20 SLE, 13 SSc, 25 DMD/BMD patients, and 36 HCs as the validation set (n=172).
DMD/BMD: Duchenne and Becker muscular dystrophy; EV: extracellular vesicle; HC: healthy control; LC/MS: liquid chromatography/mass spectrometry; PM/DM: polymyositis and dermatomyositis

Figure 2. Plexin D1 in serum extracellular vesicles is a novel candidate biomarker of polymyositis and dermatomyositis
(A) Volcano plot showing up- or down-regulated proteins in serum EVs of PM/DM patients relative to HCs (left) or other patients (right). The x-axis indicates the log2 (fold-change) and the y-axis indicates the -log10 (P-value). Dashed lines indicate the thresholds, $p<0.05$ (unpaired Student's t-test) and fold change ≥ 2.0 or ≤ 0.5. The up- or down-regulated proteins were assessed for their membrane or non-membrane nature using DAVID gene ontology and UniProtKB/Swiss-plot. Black or white circles represent the up-regulated membrane or non-membrane proteins in serum EVs of PM/DM patients, respectively. White squares represent the down-regulated non-membrane proteins in serum EVs of PM/DM patients. CYBB, ICAM5, NEO1, PLXD1, and SLC1A2 are common up-regulated membrane proteins in serum EVs of PM/DM patients compared to HC and RA and SLE patients. **(B)** Plexin D1 levels in serum EVs of 10 HC, 10 PM/DM and 23 other patients were quantified by LC/MS analysis. The horizontal line represents the median value. The P-value was calculated using the Kruskal-Wallis test with Dunn's post hoc testing (multiple comparisons versus PM/DM patients). **(C, D)** Verification of Plexin D1 in serum EVs. The pooled serum of PM/DM patients was fractionated by size exclusion chromatography. The expression of Plexin D1 in serum EVs-containing eluates was confirmed by Western blotting (C) and EV sandwich ELISA for detecting CD9- and Plexin D1-double-positive EVs ($CD9^+$ Plexin $D1^+$ EVs) (D). Serum EVs-containing fractions were determined by EV sandwich ELISA detecting for CD9- and CD63-double-

positive EVs ($CD9^+$ $CD63^+$ EVs) and protein concentration by micro-BCA protein assay.
EV: extracellular vesicle; HC: healthy control; LC/MS: liquid chromatography/mass spectrometry; PM/DM: polymyositis and dermatomyositis

Figure 3. Serum levels of Plexin D1-positive extracellular vesicles are higher in PM/DM patients with associated muscle pain or weakness

Using 172 serum samples, serum levels of Plexin D1-positive EVs were measured by a specific EV sandwich ELISA for directly detecting serum CD9- and Plexin D1-double-positive EVs ($CD9^+$ Plexin $D1^+$ EVs). The index was calculated by optical density from serum samples and standard control sample. **(A)** 54 PM/DM, 24 RA, 20 SLE, 13 SSc and 36 HCs. **(B)** 54 PM/DM, 25 DMD/BMD, and 36 HC. **(C)** Clinical subtype of PM/DM patients (14 PM, 20 classic DM, 20 clinically amyopathic dermatomyositis (CADM)). **(D)** myositis-specific autoantibody (MSA)-associated subtype of PM/DM patients with anti-aminoacyl-tRNA synthetase (ARS) antibody (n=17), anti-melanoma differentiation-associated gene 5 (MDA5) antibody (n=16) and without either antibody (Others, n=22). Associations between serum Plexin D1-positive EVs and **(E)** ILD, **(F)** muscle pain or weakness, **(G)** dysphagia, and **(H)** malignancy in PM/DM patients. The horizontal line represents the median value. The P-value was calculated using the Kruskal-Wallis test with Dunn's post hoc testing (multiple comparisons versus PM/DM patients (A, B) or HC (C, D)) and Wilcoxon rank-sum testing (E-H).
DMD/BMD: Duchenne and Becker muscular dystrophy; EV: extracellular vesicle; HC: healthy control; ILD: interstitial lung disease; LC/MS: liquid chromatography/mass spectrometry; PM/DM: polymyositis and dermatomyositis

Figure 4. Serum levels of Plexin D1-positive extracellular vesicles correlate with amounts of aldolase, white blood cells, neutrophils, and platelets in PM/DM patients

Correlations between serum levels of $CD9^+$ Plexin $D1^+$ EVs and **(A)** C-reactive protein (CRP), **(B)** creatine kinase (CK), **(C)** aldolase, **(D)** Krebs von den Lungen-6 (KL-6), **(E)** ferritin, **(F)** white blood cells (WBC), **(G)** neutrophils, **(H)** lymphocytes, **(I)** monocytes, **(J)** platelets (PLT) in pre-treatment PM/DM patients. The black line shows the regression line. The Spearman rank correlation coefficients (r_s) and corresponding P-values are indicated on each scatter plot.
EV: extracellular vesicle; PM/DM: polymyositis and dermatomyositis

Figure 5. Serum levels of Plexin D1-positive extracellular vesicles are decreased in PM/DM patients after treatment.

Change of serum levels of $CD9^+$ Plexin $D1^+$ EVs in PM/DM patients after treatment in 21 PM/DM (9 PM, 7 DM and 5 clinically amyopathic dermatomyositis (CADM)) patients who achieved clinical remission **(A)** and 3 PM/DM (3 DM) patients who did not **(B)**. Black circles connected by a solid line represent data from one individual patient. The P-value was calculated using the Wilcoxon signed-rank test.
EV: extracellular vesicle; PM/DM: polymyositis and dermatomyositis

Screening set
PM/DM (n=10), RA (n=18), SLE (n=5), HC (n=10)

↓

Purification of serum EVs by size exclusion chromatography

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LC/MS analysis and protein identification

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Bioinformatics and statistical analyses

↓

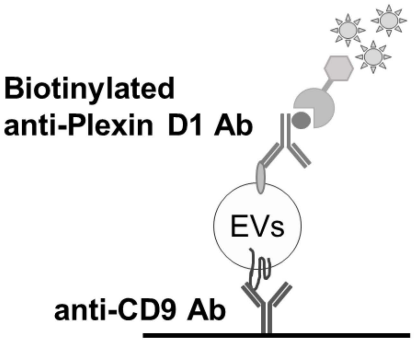
Plexin D1

↓

Western blot analysis

↓

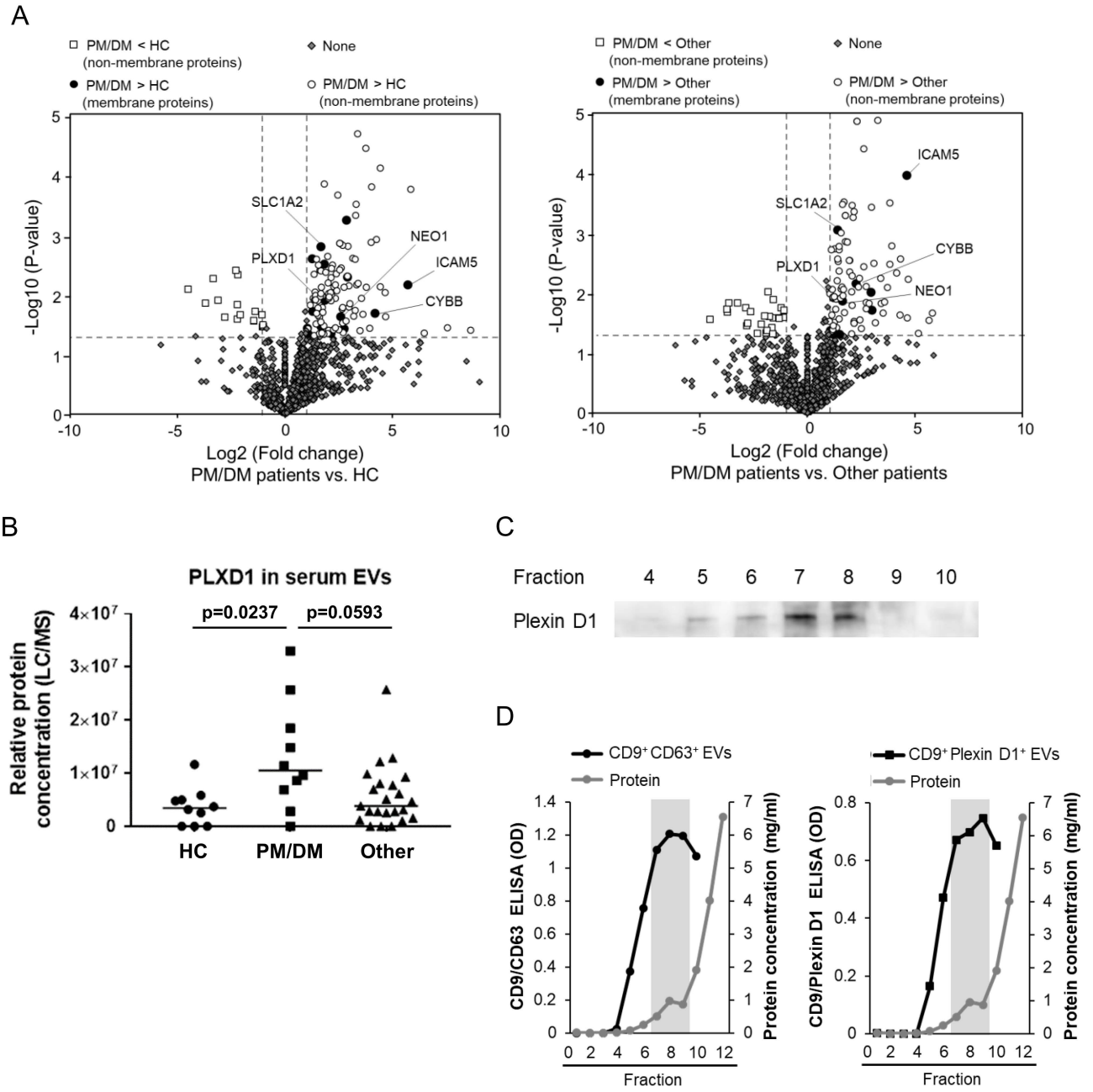
Establishment of EV sandwich ELISA for detecting Plexin D1-positive EVs

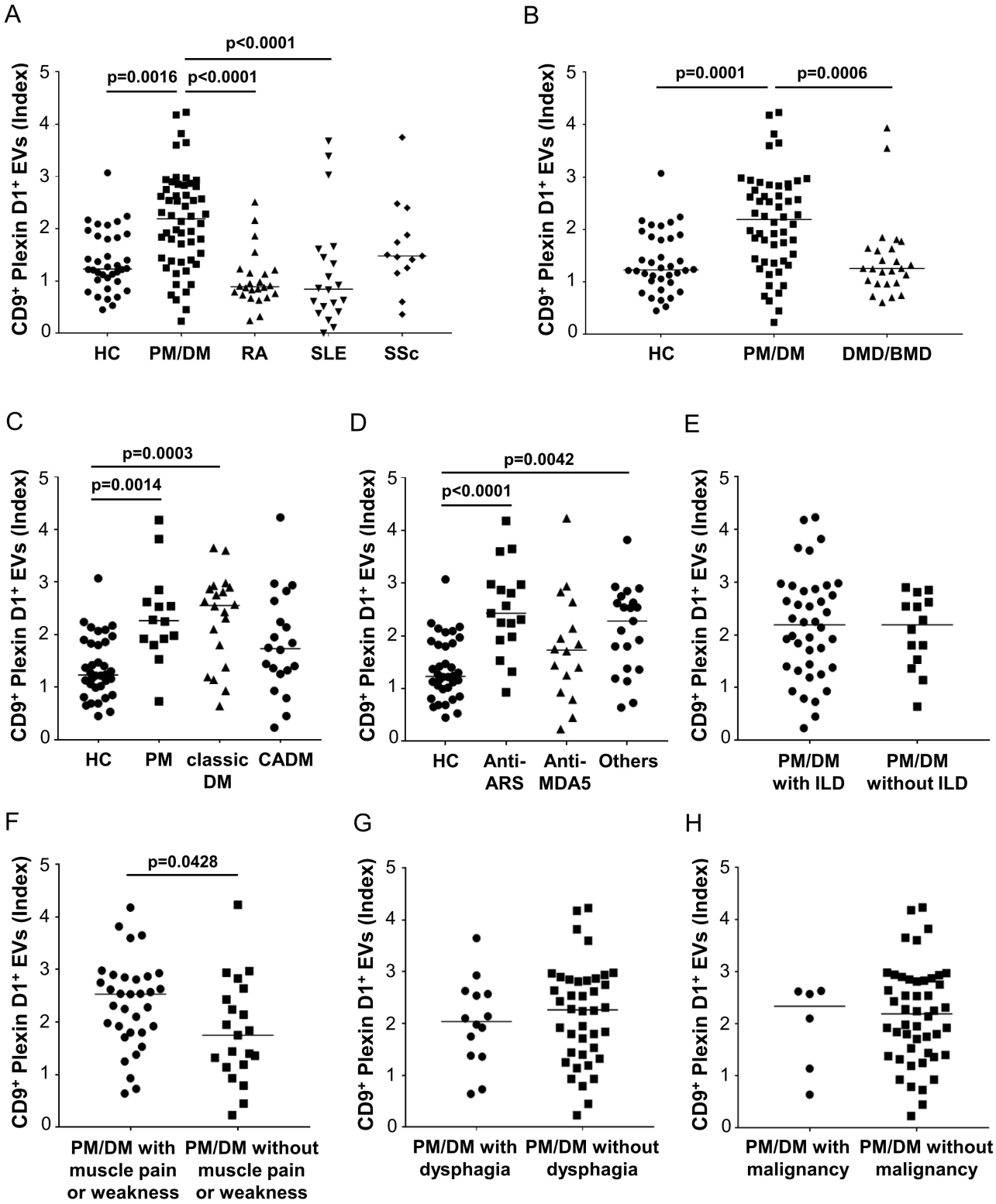


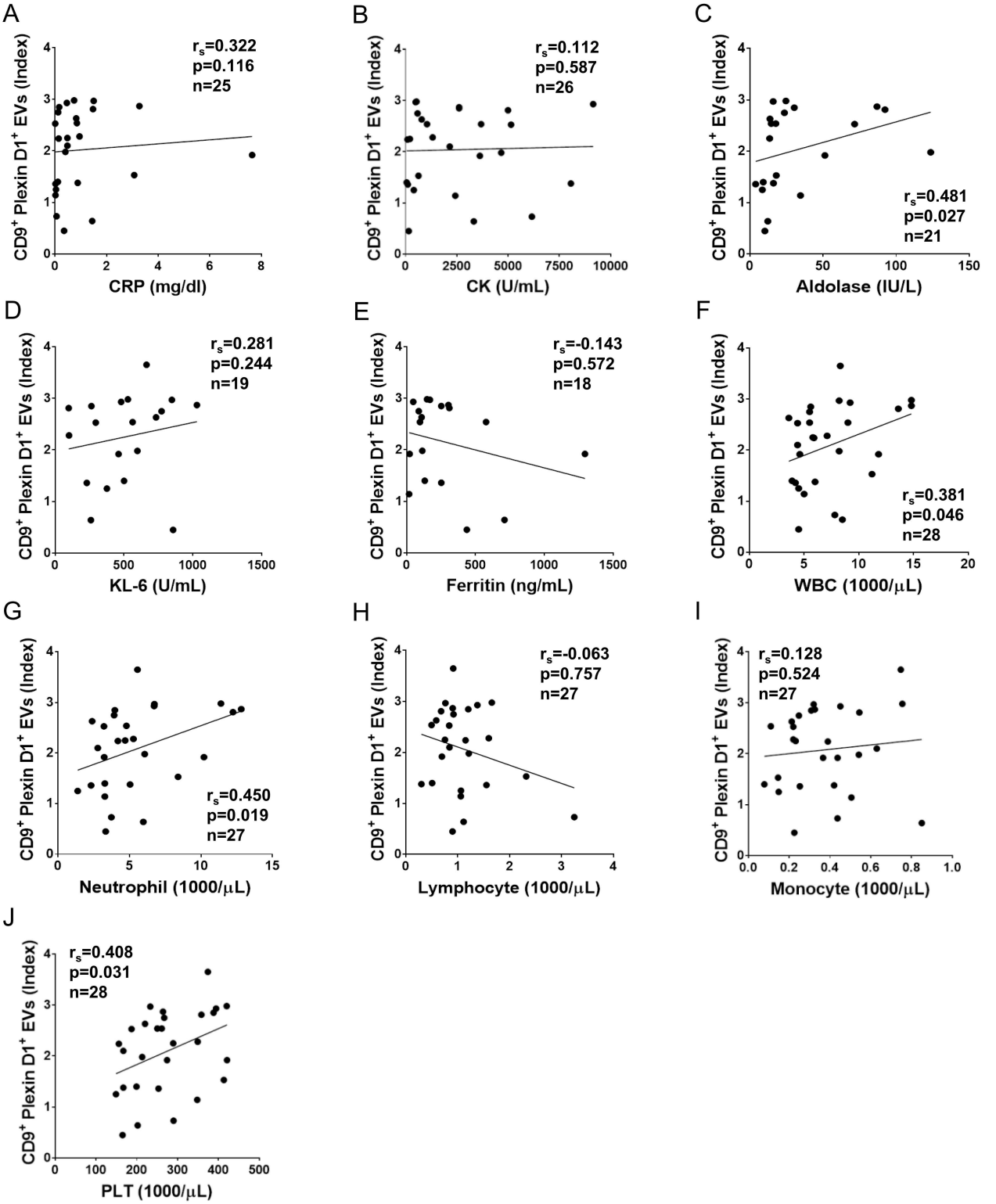
↓

Evaluation of clinical utility of Plexin D1-positive EVs

Validation set
PM/DM (n=54), RA (n=24), SLE (n= 20), SSc (n=13), DMD/BMD (n= 25), HC (n=36)


A
PM/DM < HC (non-membrane proteins)
None
PM/DM > HC (membrane proteins)
PM/DM > HC (non-membrane proteins)
-Log10 (P-value)
SLC1A2
NEO1
PLXD1
ICAM5
CYBB
Log2 (Fold change) PM/DM patients vs. HC
PM/DM < Other (non-membrane proteins)
None
PM/DM > Other (membrane proteins)
PM/DM > Other (non-membrane proteins)
-Log10 (P-value)
ICAM5
SLC1A2
CYBB
PLXD1
NEO1
Log2 (Fold change) PM/DM patients vs. Other patients
B
PLXD1 in serum EVs
p=0.0237
p=0.0593
Relative protein concentration (LC/MS)
HC
PM/DM
Other
C
Fraction 4 5 6 7 8 9 10
Plexin D1
D
CD9+CD63+ EVs
Protein
CD9/CD63 ELISA (OD)
Protein concentration (mg/ml)
Fraction
CD9+Plexin D1+ EVs
Protein
CD9/Plexin D1 ELISA (OD)
Protein concentration (mg/ml)
Fraction


A
p<0.0001
p=0.0016
p<0.0001
CD9+ Plexin D1+ EVs (Index)
HC
PM/DM
RA
SLE
SSc
B
p=0.0001
p=0.0006
HC
PM/DM
DMD/BMD
C
p=0.0003
p=0.0014
HC
PM
classic DM
CADM
D
p=0.0042
p<0.0001
HC
Anti-ARS
Anti-MDA5
Others
E
PM/DM with ILD
PM/DM without ILD
F
p=0.0428
PM/DM with muscle pain or weakness
PM/DM without muscle pain or weakness
G
PM/DM with dysphagia
PM/DM without dysphagia
H
PM/DM with malignancy
PM/DM without malignancy


A
r_s=0.322
p=0.116
n=25
CD9+ Plexin D1+ EVs (Index)
CRP (mg/dl)
B
r_s=0.112
p=0.587
n=26
CD9+ Plexin D1+ EVs (Index)
CK (U/mL)
C
r_s=0.481
p=0.027
n=21
CD9+ Plexin D1+ EVs (Index)
Aldolase (IU/L)
D
r_s=0.281
p=0.244
n=19
CD9+ Plexin D1+ EVs (Index)
KL-6 (U/mL)
E
r_s=-0.143
p=0.572
n=18
CD9+ Plexin D1+ EVs (Index)
Ferritin (ng/mL)
F
r_s=0.381
p=0.046
n=28
CD9+ Plexin D1+ EVs (Index)
WBC (1000/μL)
G
r_s=0.450
p=0.019
n=27
CD9+ Plexin D1+ EVs (Index)
Neutrophil (1000/μL)
H
r_s=-0.063
p=0.757
n=27
CD9+ Plexin D1+ EVs (Index)
Lymphocyte (1000/μL)
I
r_s=0.128
p=0.524
n=27
CD9+ Plexin D1+ EVs (Index)
Monocyte (1000/μL)
J
r_s=0.408
p=0.031
n=28
CD9+ Plexin D1+ EVs (Index)
PLT (1000/μL)

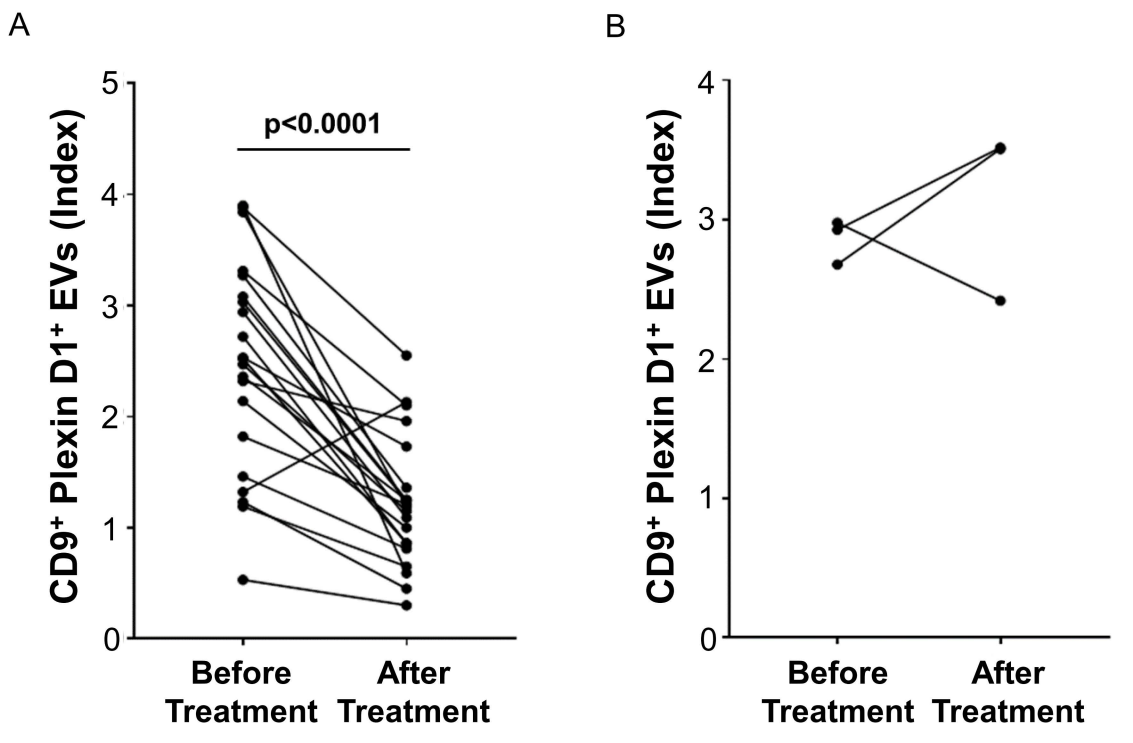

A
B
p<0.0001
CD9+ Plexin D1+ EVs (Index)
CD9+ Plexin D1+ EVs (Index)
0
1
2
3
4
5
Before Treatment
After Treatment
Before Treatment
After Treatment

Table 1 Characteristics of PM/DM patients and control group

	Screening set (n=43)				Validation set (n=172)					
		Control group				Control group				
	PM/DM	RA	SLE	HC	PM/DM	RA	SLE	SSc	DMD/BMD	HC
Number of samples	10	18	5	10	54	24	20	13	25	36
Age (years), mean (SD)	44.5 (18.6)	62.9 (12.6)	31.2 (10.8)	50.8 (6.0)	54.1 (15.7)	57.3 (11.3)	38.1 (16.9)	60.1 (9.2)	14.4 (6.4)	48.9 (7.2)
Female, n (%)	7 (70.0)	15 (83.3)	4 (80.0)	7 (70.0)	38 (70.4)	16 (66.7)	16 (80.0)	8 (61.5)	1 (4.0)	24 (66.7)
Subtype of PM/DM										
PM, n (%)	2 (20.0)				14 (26.0)					
Classic DM, n (%)	3 (30.0)				20 (37.0)					
CADM, n (%)	5 (50.0)				20 (37.0)					
MSA										
Anti-ARS, n (%)	2 (20.0)				17 (31.5)					
Anti-MDA5, n (%)	5 (50.0)				16 (29.6)					
Clinical symptoms										
Rash typical of DM, n (%)	8 (80.0)				40 (74.1)					
ILD, n (%)	8 (80.0)				40 (74.1)					
Muscle pain or weakness, n (%)	6 (60.0)				33 (61.1)					
Dysphagia, n (%)	2 (20.0)				14 (25.9)					
Malignancy, n (%)	0 (0)				6 (11.1)					

PM/DM: polymyositis/dermatomyositis; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SSc: systemic sclerosis; DMD/BMD: Duchenne and Becker muscular dystrophy; HC: healthy controls; CADM: clinically amyopathic dermatomyositis; MSA: myositis-specific autoantibody; ARS: aminoacyl-tRNA synthetase; MDA5: melanoma differentiation-associated gene 5; ILD: interstitial lung disease.