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ORIGINAL ARTICLE – OPEN ACCESS

Exosomes' Profile in Ankylosing Spondylitis: A Preliminary Study

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ABSTRACT

Objective: Ankylosing spondylitis (AS) is a chronic systemic inflammatory disease that leads to structural and functional impairments and reduced quality of life, with heterogeneous manifestations. The origin and possible role of extracellular vesicles represented by exosomes (EVexo) in the pathogenesis of AS were examined in this study.

Materials and Methods: Extracellular vesicles (EVs) were isolated from serum from ten AS patients and ten healthy controls through Izon qEV2/35 nm columns. After assessing the isolate purity by bicinchoninic acid assay (BCA) and Enzyme-Linked ImmunoSorbent Assay (ELISA), the relationship between EVexo concentration and AS was tested by the BCA method. The EVexo surface markers were analyzed by flow cytometry (FC) to verify EVexo presence and reveal its origin.

Results: In FC analysis, CD86+TSG101+ and CD3+TSG101+ exosome percentages of AS group were significantly higher than the control group ($p<0.05$). A significant difference was found between the AS and control groups in terms of CD3+IL17+ and CD3+IFNg+ and CD86+TNFα+ and CD86+IL12(p35)+ exosome percentages ($p<0.01$).

Conclusion: The exosomes whose ratio increased in the AS process were derived from T cells expressing increased levels of IL-17A and IFNg in their membranes, and macrophages expressing increased levels of TNFα and IL-12(p35) in their membranes. The EVexo profile did not change according to the AS course.

Keywords: Exosome, extracellular vesicle, ankylosing spondylitis, cell surface markers

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INTRODUCTION

Ankylosing spondylitis (AS), mainly affecting the sacroiliac, axial and peripheral joints, manifesting as arthritis, dactylitis, and enthesitis of varying severities but also leading to severe bone deformity, including bamboo spine deformity in a limited number of patients (1), is the prototype of seronegative spondyloarthropathies, and is a chronic, progressive, systemic and heterogeneous inflammatory disease leading to a decrease in the quality of life due to structural and functional impairment. Due to delay in diagnosis and the clinical heterogeneity of AS in terms of both axial and peripheral joints and extra-articular involvement, such as ocular, cardiac, intestinal, and skin involvement, there is a need for prognostic markers to differentiate patients according to the expected prognosis at the time of diagnosis.

Extracellular vesicles, which can be released nearly from any type of cell (2), are represented by exosomes (EVexo) are small vesicles that function as carriers for transcription factors, cell surface receptors, proteins, and several types of RNAs. Tumor susceptibility gene 101 protein (TSG101), Ras-related protein Rab-11B (RAB11B), charged multivesicular body protein 2a (CHMP2A), CD63 and CD81 antigens are examples of primary markers of these structures that help in understanding their functional properties. Many diseases, including rheumatoid arthritis, have been associated with EVexo (3). Studies show that exosome presence in synovial fluid exosomes can cause inflammation and joint damage (4). We hypothesized that there might be a relationship between AS disease process and exosomes. Therefore, differences in the ratio of peripheral blood mononuclear cell (PBMC)-derived exosomes, their origin and their cytokine expression profiles in AS patients were compared to healthy controls.

MATERIALS and METHODS

Study Design

This single-center, a two-arm study was performed between February 2019 and August 2020. The study was approved by the Marmara University School of Medicine Clinical Research Ethics Committee in February 2019.

Table 1. Clinical characteristics of patients and healthy volunteers

	AS (n=10)	Mild AS (n=4)	Severe AS (n=6)	HC (n=10)
Age (mean±SD)	41.3±9.51	35.5±7.68	45.17±9.09	40.08±7.19
Sex (men/women) (%)	90/10	75/25	100/0	90/10
BASDAI score (mean±SD)	4.16±1.71	2.3±0.37	5.40±0.68	–
Level of fatigue (mean±SD)	4.2±2.29	2.25±1.26	5.50±1.87	–
Level of neck, back, waist or hip pain (mean±SD)	6.1±3.14	2.75±0.96	8.33±1.50	–
Level of pain/swelling in other joints (mean±SD)	5.6±2.95	2.25±0.5	7.83±0.75	–
Level of discomfort in the touch-sensitive area (mean±SD)	4.80±2.44	2.25±1.25	6.50±1.05	–
Level of morning stiffness (mean±SD)	5.1±2.33	2.5±0.58	6.83±0.75	
Duration of morning stiffness (mean±SD)	2.9±1.52	1.5±0.57	3.83±1.17	

AS: Ankylosing spondylitis; HC: Healthy control; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; SD: Standard deviation

Study Groups

The study groups were composed of 10 AS patients all fulfilling both Assessment of SpondyloArthropathies Study Groups and New York Study Group criteria, 6 with severe disease (sAS) either newly diagnosed or unresponsive to non-steroidal anti-inflammatory drugs with >4 points in Bath AS Disease Activity Index (BASDAI) score and 4 with mild disease (mAS) with <2.8 BASDAI score, and ten healthy controls (HC) who had no diagnosis or symptoms of autoimmune/autoinflammatory diseases and were not taking any immunosuppressive drugs at the time of the study (Table 1). All patients and controls gave informed consent.

Sample Preparation and EVexo Isolation

Peripheral venous blood was collected via a 21G needle into 8.5 ml of serum separator gel in a tube and turned up and down six times gently to bring the blood into contact with silica particles in the tube's wall. After waiting for spontaneous clotting for 1 hour, the tubes were centrifuged at 2500 g for 15 minutes two times, and serum was transferred into tubes and frozen at -80°C until bicinchoninic acid assay (BCA), Enzyme-Linked ImmunoSorbent Assay (ELISA) and flow cytometry (FC) analysis.

For EVexo isolation, first, centrifugation and size exclusion chromatography (SEC) methods make use of the physical properties of EVexo, and then the affinity-based isolation method, which makes use of their chemical properties, were used. In the SEC method with Izon Science's qEV2/35 nm columns (Christchurch, New Zealand), sodium azide was removed by washing with 60 ml 1X Dulbecco's phosphate-buffered saline (wisent, 311-425CL, St. Bruno, QC, Canada). Two milliliters of serum that was previously centrifuged at 10.000 g for 10 minutes was loaded into the reservoir, and the obtained EV-rich filtrate was aliquoted.

EVexo in SEC-derived preparations were captured with magnetic beads 3 µm in diameter coated with tetraspanin antibodies CD9, CD63, and CD81 found on the surface of EVexo derived from almost every cell type using the ExoCapTM Streptavidin CD9/CD63/CD81 kit (MBL Life Science, Woburn, MA, USA, MEXSA). After adding 660 µl bead mixture into three separate 2 ml tubes and holding on a magnetic stand for 1 minute, supernatants were removed, and 1 ml washing/Dilution buffer was added to each tube. Then, 6.6 µg (33 µl) CD9, CD63 and CD81 capture antibodies were added to tubes and incubated in the rotator for 60 minutes at room temperature. After holding on the magnetic stand for 1 minute, and after which a 1.98 ml bead cocktail was created by taking an equal volume (0.66 ml) from each bead population, and a 12.5 µl antibody-coated bead cocktail was added to a 2 ml tube with 50 µl treatment buffer to minimize nonspecific binding and mixed, the supernatants were removed, and the beads were washed three times. Finally, the beads were suspended in 660 µl washing/dilution buffer, and CD9-, CD63- and CD81-covered bead populations were obtained. After all the above, 50 µl filtrate was added and incubated for 24 hours at room temperature with gentle mixing. It was suspended in 250 µl staining buffer (BD Biosciences, 554657, Wokingham, UK).

BCA and ELISA Analyzes

BCA and ELISA evaluated the purity of filtrates obtained with SEC-based qEV2/35 columns. First, the total protein concentration of the unprocessed serum and filtrate were compared with a PierceTM BCA Protein Assay Kit (Thermo Scientific, 23227, Agawam, MA) according to the instructions and absorbance was detected at 562 nm. Two wells were used in separate plates for each sample and incubated for 30 and 90 minutes. While according to the instructions, the albumin concentration of unprocessed serum and filtrate were compared with the Human Albumin ELISA Kit (Abcam, ab227933, Cambridge, UK) by the sandwich ELISA method, in addition, radioimmunoprecipitation assay (RIPA) lysis buffer was not used in BCA analysis.

Change in EVexo concentration during AS process was assessed by the BCA method by measuring the total exosomal protein concentration with a PierceTM BCA Assay Kit (Thermo Scientific, 23227). Two hundred microliters of filtrate and lysis buffer (RIPA buffer containing protease inhibitor cocktail; sc24948, Santa Cruz, Dallas, USA) were incubated at room temperature for 5 minutes, then sonicated on ice 3x5 seconds each. Following a similar protocol used for the purity control, microplates were incubated for 30 and 90 minutes at 37°C, and absorbance was measured at 562 nm.

FC Analysis of Markers on the EVexo Surface

FC analysis of markers on the EVexo surface was performed to confirm the presence of EVexo. The change in EVexo concentration, the exosomes' origin, and cytokine profile have been analyzed.

Table 2. Purity analysis of filtrate and serum with the BCA (without lysis)

	Total protein concentration in filtrate and serum for each incubation time (μg/mL)			
	Filtrate Median (min–max)	**Serum Median (min–max)**	**p_1**	**Cohen's d**
AS				
30 min	115.8 (61.6–268.3)	4875 (4615–5398.3)	**0.005**	**26.092**
90 min	74.1 (6–341)	5222.3 (4869.75–5593.5)	**0.005**	**29.689**
p_2	**0.093 (Hedges' g: 0.313)**	**0.013 (Hedges' g: 1.485)**		
HC				
30 min	110.8 (25–535)	4848.33 (4570–9505)	**0.008**	**4.216**
90 min	44.75 (1–457.25)	5047.25 (4952.25–8522.25)	**0.008**	**5.461**
p_3	**0.017 (Hedges' g: 0.597)**	**0.173 (Hedges' g: 0.057)**		
Difference made by incubation time				
in AS	-38.2 (-117.3; 174.3)	331 (-209.8; 565.2)	**0.004**	**0.989**
in HC	-75.0 (-186.5; 65.6)	206.4 (-982.7; 456)	**0.034**	**0.538**
p_4	**0.096 (Cohen's d: 0.609)**	**0.288 (Cohen's d: 0.543)**		

BCA: Bicinchoninic acid assay; Min: Minutes; Min–max: Minimum–maximum; p_1: Filtrate and serum comparison; p_2: Incubation time comparison in AS group; p_3: Incubation time comparison in HC group; p_4: AS and HC group cpmparison; Cohen's d and Hedges' g: Effect size; 1,2,3: Wilcoxon Signed Ranks test; 4: Mann-Whitney U-test

Table 3. Exosomal total protein concentration (with lysis)

	Total protein concentration in AS and HC for each incubation time (μg/mL)			
	AS Median (min–max)	**HC Median (min–max)**	**p**	**Cohen's d**
30 min	66.84 (5.31–374.5)	26.46 (9.15–116.08)	**0.049**	**0.905**
90 min	70.67 (15.39–223.72)	44 (4.28–141.5)	**0.190**	**0.502**
diff	-0.08 (-0.42/0.21)	-0.07 (-0.18/0.08)	**0.406**	**0.370**

AS: Ankylosing spondylitis; HC: Healthy control; Min: Minutes; Min–max: Minimum–maximum; diff: Amount of difference made by incubation time; p: Significance value-Mann–Whitney U-test; Cohen's d: Effect size

Different antibody concentrations were tested before EVexo staining to obtain the strongest signal. Then, 12.5 µl Human TruStain FcX (Biolegend,422302) was added to 250 µl EVexo solution (captured EVexo) and incubated for 10 minutes, leading to Fc receptor blockage, and the samples were labeled with the four different antibody cocktails given below. Specific amounts of Ab cocktail (TSG101/FITC: sc7964 from Santa Cruz Biotechnology (Dallas, USA), CD3/PE-Cy7: 300420 and CD86/PE-Cy7: 374210 from Biolegend (San Diego, CA, USA), IL-17A/PE: IC3171P, IL-12/IL-35(p35)/PE: IC2191P and TNFα/PE membrane form: FAB210P-025 from R&D Systems (Minneapolis, MN, USA), Mouse IgG2a(k)/FITC: 11-4724-82, Mouse IgG1(k)/PE-Cy7: 25-4714-42 and Mouse IgG1(k)/PE: 12-4714-81 from Thermofisher (Agawam, MA), IFNγ/PE: 855.002.019 from Diaclone (Besancon, France)) were added to 25 µl EVexo-bead complex and brought to 100 µl with buffer. After incubation for one hour at room temperature, the cells were washed three times with staining buffer, and 250 µl staining buffer was added. The same procedure was also performed for the isotype control. Finally, FC analysis of EVexo labeled with four Ab cocktails was performed with the BD FACSCalibur platform and analyzed via unstained and isotype negative controls.

a) Antibody cocktail-1: TSG101/FITC, CD3/PE-Cy7, IL-17/PE

b) Antibody cocktail-2: TSG101/FITC, CD3/PE-Cy7, IFN-γ/PE

c) Antibody cocktail-3: TSG101/FITC, CD86/PE-Cy7, TNF-α/PE

d) Antibody cocktail-4: TSG101/FITC, CD86/PE-Cy7, IL-12/PE

e) Isotype control: (IgG2a(k)/FITC, IgG1(k)/PE and IgG1(k)/PE-Cy7

For accurate gating, Fluorescence Minus One controls were prepared for all fluorophores and antibodies. Unstained controls and isotype controls were used to determine autofluorescence and non-specific binding, respectively.

Statistical Analysis

Statistical analyzes were performed using SPSS Statistics for Windows, version 22.0 (SPSS Inc., Chicago, Ill., USA). The general and clinical features of the participants were defined using mean, standard deviation, and percentage (Table 1). The normal distribution of values was evaluated with the Kolmogorov-Smirnov test, and $p<0.05$ was considered statistically significant.

Table 4. Determination of albumin concentration

	Albumin concentration (10^8) (pg/mL)			
	AS Median (min–max)	**HC Median (min–max)**	**p_1**	**Cohen's d**
Filtrate	0.52 (0.00001–14.0)	0.13 (0.0001–2.6)	**0.089**	**0.579**
Serum	199.17 (174.7–236.3)	183.75 (161.1–234.9)	**0.280**	**0.373**
p_2	**0.005 (Cohen's d: 13.220)**	**0.005 (Cohen's d: 10.828)**		

AS: Ankylosing spondylitis; HC: Healthy control; Min–max: Minimum–maximum; p_1: AS and HC comparison-Mann–Whitney U-test; p_2: Filtrate and serum comparison-Wilcoxon Signed Ranks test; Cohen's d: Effect size

Table 5. Exosomal surface marker analysis according to study groups

	AS Median (%) (min–max)	**Mild AS Median (%) (min–max)**	**Severe AS Median (%) (min–max)**	**HC Median (%) (min–max)**	**p_1 (Cohen's d)**	**p_2 (n^2)**
TSG101	11.5 (0–24.5)	11.5 (0–14.4)	10.5 (4.3–24.5)	0.30 (0–34.1)	**[4]0.023 (d: 0.702)**	**[1]0.064 (n^2: 0.395)**
CD86	54.4 (37.6–57.2)	58.2 (54.2–58.8)	52.7 (37.6–57.2)	11.9 (4.2–23.4)	**[4]0.001 (d: 5.614)**	**[1]0.001 (n^2: 3.236)**
IL-17A	43.7 (0–49.2)	41.1 (0–48.2)	43.7 (40.2–49.2)	14.7 (4.1–29.2)	**[4]0.002 (d: 2.103)**	**[1]0.007 (n^2: 0.922)**
IL-12(p35)	40.39 (0–59.5)	27.4 (0–59.5)	40.4 (17.1–46.5)	0.92 (0–14.3)	**[4]0.002 (d: 2.160)**	**[1]0.009 (n^2: 0.843)**
IFN-γ	57.86 (39.6–68.8)	44.96 (39.6–57.4)	58.97 (57.6–68.8)	50.15 (39.52–60.56)	**[4]0.247 (d: 0.563)**	**[1]0.014 (n^2: 0.820)**
	Mean±SD	**Mean±SD**	**Mean±SD**	**Mean±SD**		
CD3	54.33±9.04	57.68±6.90	52.11±10.18	19.59±7.36	**[3]0.001 (d: 4.336)**	**[2]0.001 (n^2: 2.113)**
TNFα	37.92±12.46	31.59±18.76	42.13±3.90	14.88±9.53	**[3]0.001 (d: 2.077)**	**[2]0.001 (n^2: 0.978)**

AS: Ankylosing spondylitis; HC: Healthy control; SD: Standard deviation; Min–max: Minimum–maximum; p_1: AS group vs. HC group; p_2: mild AS, severe AS and HC group; 1: Kruskall Wallis test; 2: Oneway ANOVA test; 3: Student t test; 4: Mann-Whitney U-test; n^2: Eta Squared

BCA and ELISA results were not normally distributed for total protein and albumin concentration. Thus, median (minimum–maximum) values were given (Table 2–4). Wilcoxon signed-rank test was used to compare serum and filtrate measurements at 30th and 90th minutes (Table 2, 4). AS-HC group comparison was made with the Mann–Whitney U-test (Table 3, 4).

Normally distributed FC values are presented as mean±standard deviation and those not normally distributed as median (minimum–maximum) (Table 5, 6). The values of AS and HC groups were compared with student's t test. For multiple group analysis, one-way ANOVA test was used, where Levene's test was used to determine the homogeneity of variances. The post-hoc tests were either Tukey HSD or Tamhane's T2 according to the homogeneity of variance.

With the effect size being calculated by Cohen's d in two-group, Hedges' g for the within-group dependent sample, and n2 (Eta squared) values in three-group comparison, Mann–Whitney U-test was used for comparing AS and HC groups and the Kruskal-Wallis test compared mild and severe AS and the control groups.

RESULTS

BCA and Exosomal Total Protein Concentrations

Serum protein concentration in AS and HC groups, after both 30 and 90 minutes of incubation, was found significantly higher with a strong effect size than the filtrate obtained after isolation ($p<0.01$ and Cohen's-d >0.8 for all, Table 2). In the AS group, the incubation time affected the serum protein concentration but not in the filtrate ($p_2<0.05$ and Hedges' g>0.8). While the difference between the AS and HC groups was not found to be significant ($p_4>0.05$), the difference created by the incubation period in serum and filtrate within AS and HC groups was significant (p=0,004 and Cohen's d=0.989 for AS; p=0.034 and Cohen's d=0.538 for HC, Table 2).

The exosomal total protein concentration of the AS group was higher than that of the HC group after 30 minutes of incubation with slight significance (p=0.049 and Cohen's d=0.905, Table 3). The protein concentrations of AS and HC groups after long incubation period in the presence of lysis were not significantly different (p>0.05). Protein concentration was significantly higher in the absence of lysis after both 30 and 90 minutes of incubation in the HC group ($p<0.05$, Cohen's d>0.8), while lysis showed no significant difference at 30 or 90 minutes in the AS group.

ELISA and Albumin Concentrations

The serum albumin concentration in both the AS and HC groups was significantly higher compared to the filtrate group ($p<0,01$ and Cohen's d>0.8 for all, Table 4). While there was no significant difference between serum albumin levels of AS and HC groups, a moderate effect size was found between filtrate albumin levels.

Table 6. Double positivity analysis of exosomal surface markers according to study groups

	AS Median (%) (min–max)	Mild AS Median (%) (min–max)	Severe AS Median (%) (min–max)	HC Median (%) (min–max)	p_1 (Cohen's d)	p_2 (n^2)
IL-17A+ TSG101+	23.7 (1.5–44.9)	21.1 (1.5–35.1)	28.5 (17.3–44.9)	2.6 (0–30.7)	[4]**0.002 (d:1.739)**	[1]**0.009 (n^2: 0.890)**
IFNγ+TSG101+	10.3 (0–31.8)	9.8 (0–25.1)	10.3 (0.8–31.8)	4.1 (0–33.8)	[4]**0.165 (d:0.574)**	[1]**0.316 (n^2: 0.301)**
	Mean±SD	**Mean±SD**	**Mean±SD**	**Mean±SD**		
CD3+TSG101+	20.99±14.18	17.47±10.82	23.35±16.59	7.91±13.98	[3]**0.048 (d:0.929)**	[2]**0.132 (n^2: 0.490)**
CD86+TSG101+	22.90±13.08	16.33±10.76	27.28±13.46	6.07±13.87	[3]**0.012 (d:1.248)**	[2]**0.022 (n^2: 0.723)**
TNFα+TSG101+	19.36±10.74	15.04±13.06	22.25±8.97	5.01±8.26	[3]**0.004 (d:1.498)**	[2]**0.008 (n^2: 0.737)**
IL-12(p35)+ TSG101+	18.68±10.33	12.22±13.36	22.99±5.42	3.51±6.04	[3]**0.001 (d:1.793)**	[2]**0.001 (n^2: 0.937)**
CD3+IL-17A+	43.13±4.06	40.49±2.97	44.88±3.89	26.47±5.07	[3]**0.001 (d:3.627)**	[2]**0.001 (n^2: 2.081)**
CD3+IFNγ+	44.67±2.27	43.19±1.58	45.67±2.19	32.88±6.47	[3]**0.001 (d:2.432)**	[2]**0.001 (n^2: 1.473)**
CD86+TNFα+	41.31±4.96	39.6±5.72	42.44±4.56	22.86±6.42	[3]**0.001 (d:3.216)**	[2]**0.001 (n^2: 1.650)**
CD86+IL12(p35)+	29.94±9.34	28.92±13.88	30.62±6.33	11.69±8.78	[3]**0.001 (d:2.013)**	[2]**0.002 (n^2: 0.899)**

AS: Ankylosing spondylitis; HC: Healthy control; SD: Standard deviation; Min–max: Minimum–maximum; p_1: AS group vs. HC group; p_2: mild AS, severe AS and HC group; 1: Kruskall Wallis test; 2: Oneway ANOVA test; 3: Student t test; 4: Mann-Whitney U-test; n^2: Eta Squared

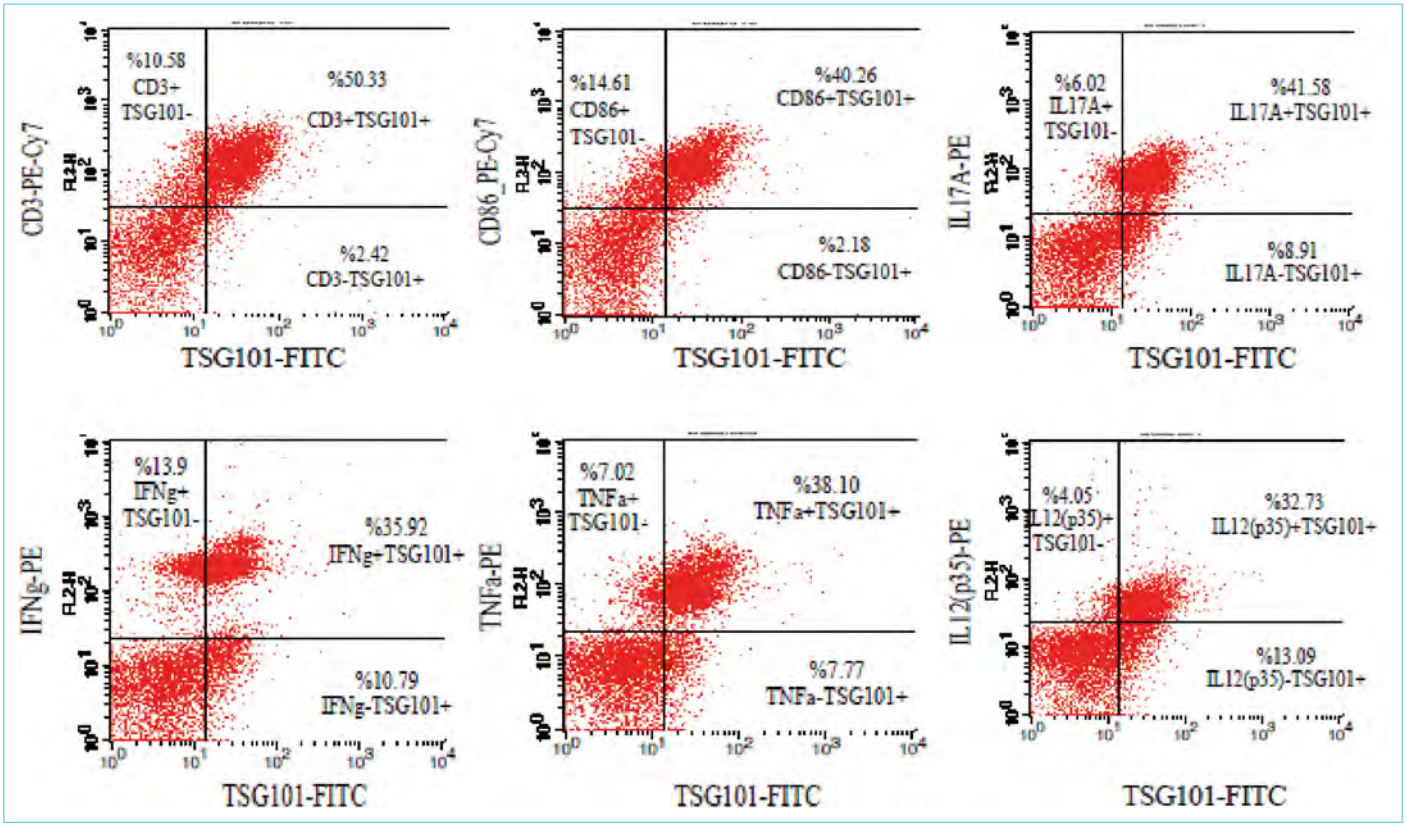

Figure 1. Double positivity analysis confirming that the structures active in the AS process are EVexo

FC Analysis of Markers on The EVexo Surface

The isolated EVexo were stained with four antibody cocktails, and FL1, FL2 and FL3 histograms and FL1-FL2, FL1-FL3 and FL2-FL3 dot plots were obtained. To confirm that the structures active in the AS process are exosomes and to show these exosomes' origin and cytokine profile, the double positivity of surface markers was evaluated (Table 6), where further, a detailed quantitative analysis of surface markers according to the severity of AS and study groups is shown in Table 5.

TSG101 and CD3 positivity were significantly different between AS and HC groups (p=0.023 and 0.001, respectively,

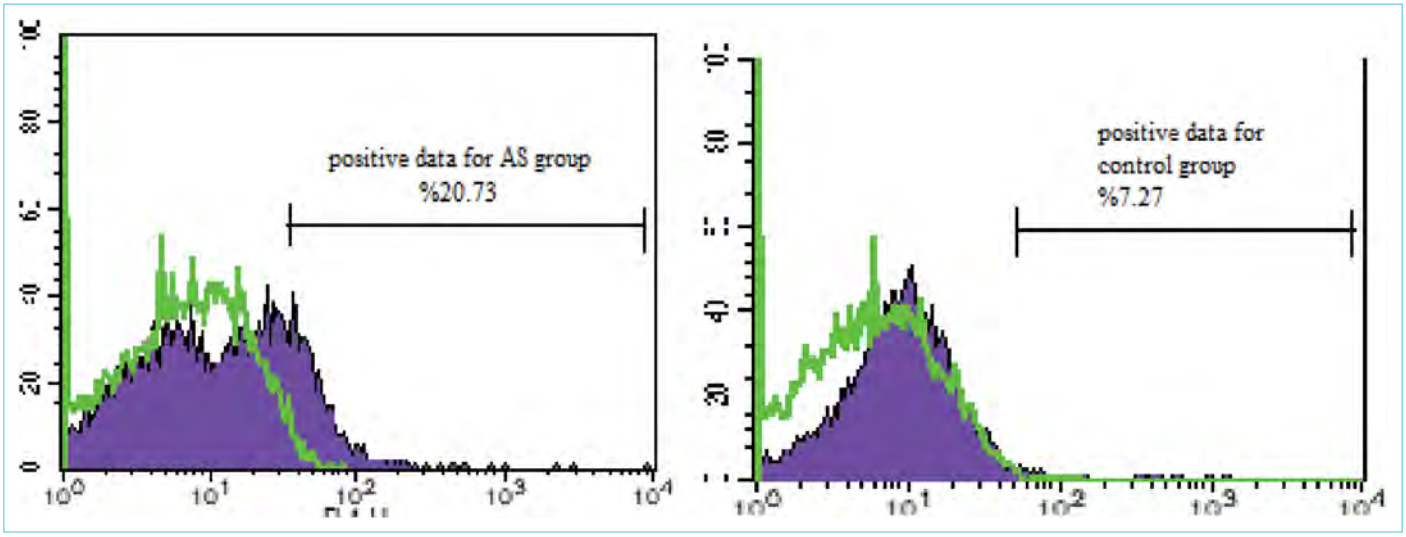

Figure 2. **The presence of TSG101+ EVexo was more common in AS patients than in controls**

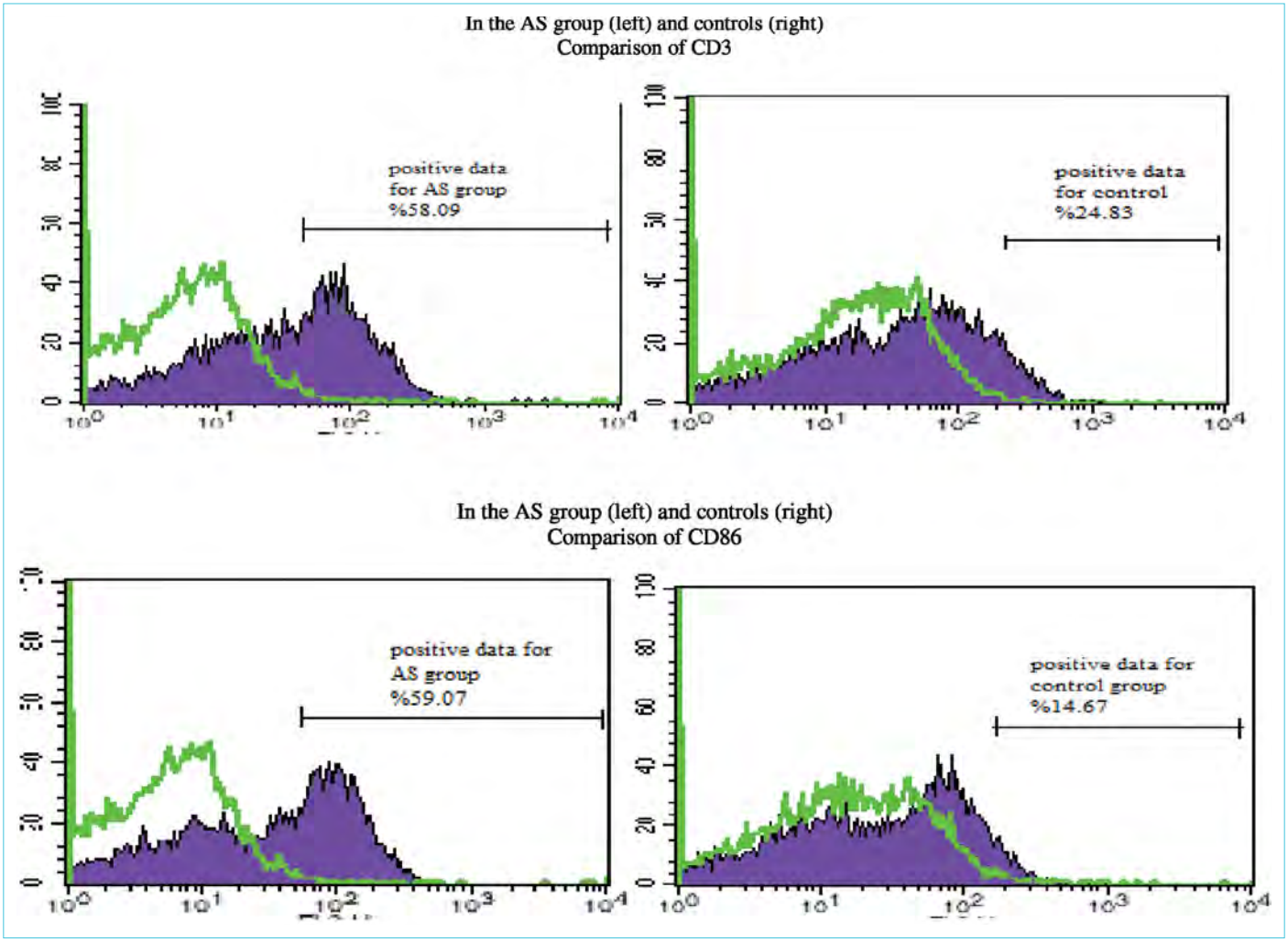

Figure 3. **Flow cytometry graphics of the origin of EVexo active in the AS process**

Table 5) which confirmed the increased EVexo presence in AS compared to HC. CD86 and TNFα levels were found significantly higher in the AS group (p=0.001 fpr both and Cohen's d>4.0, Table 5).

IL-17A/TSG101,CD86/TSG101,IL17A+TSG101+,TNFα/TSG101 and IL12(p35)/TSG101 double positivity levels were higher in the AS group (Table 6) and a strong effect size was obtained (p=0.002, 0.012, 0.004 and 0.001, respectively with Cohen's d>1.0 for all).

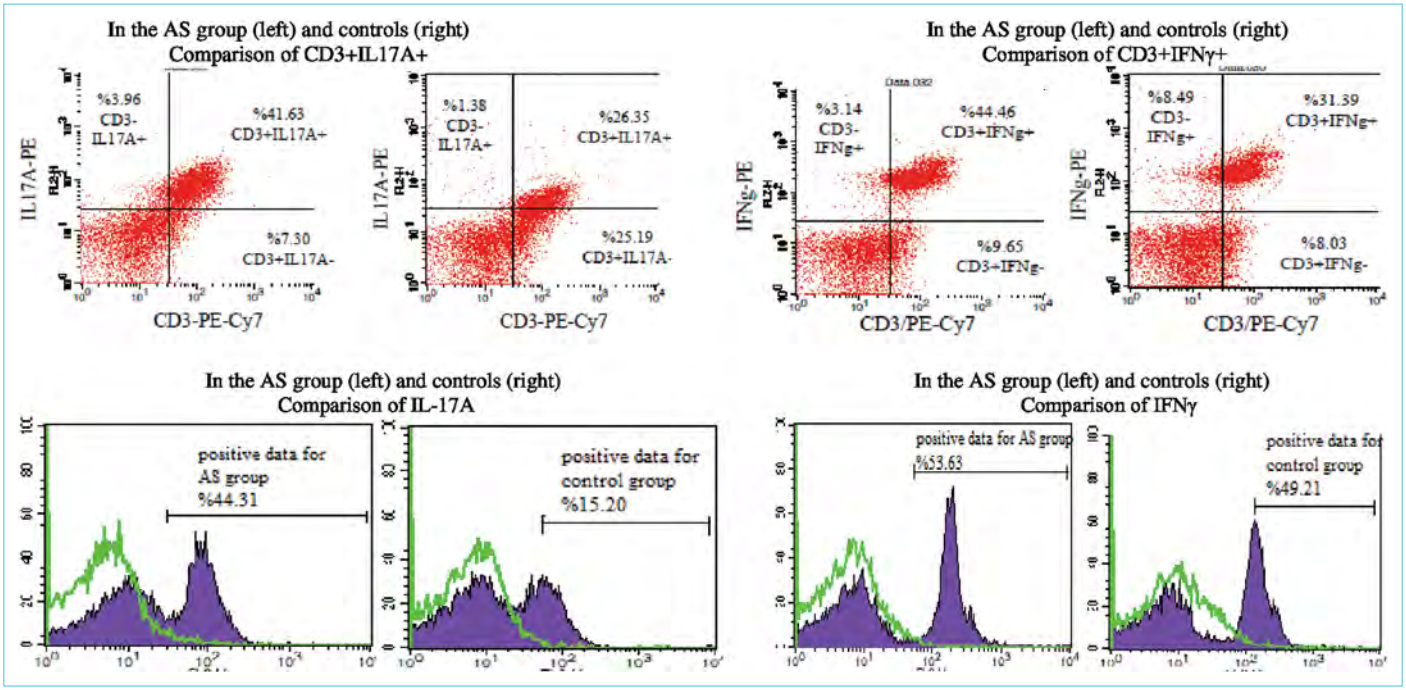

Figure 4. **Flow cytometry graphs of the role of EVexo in the AS process**

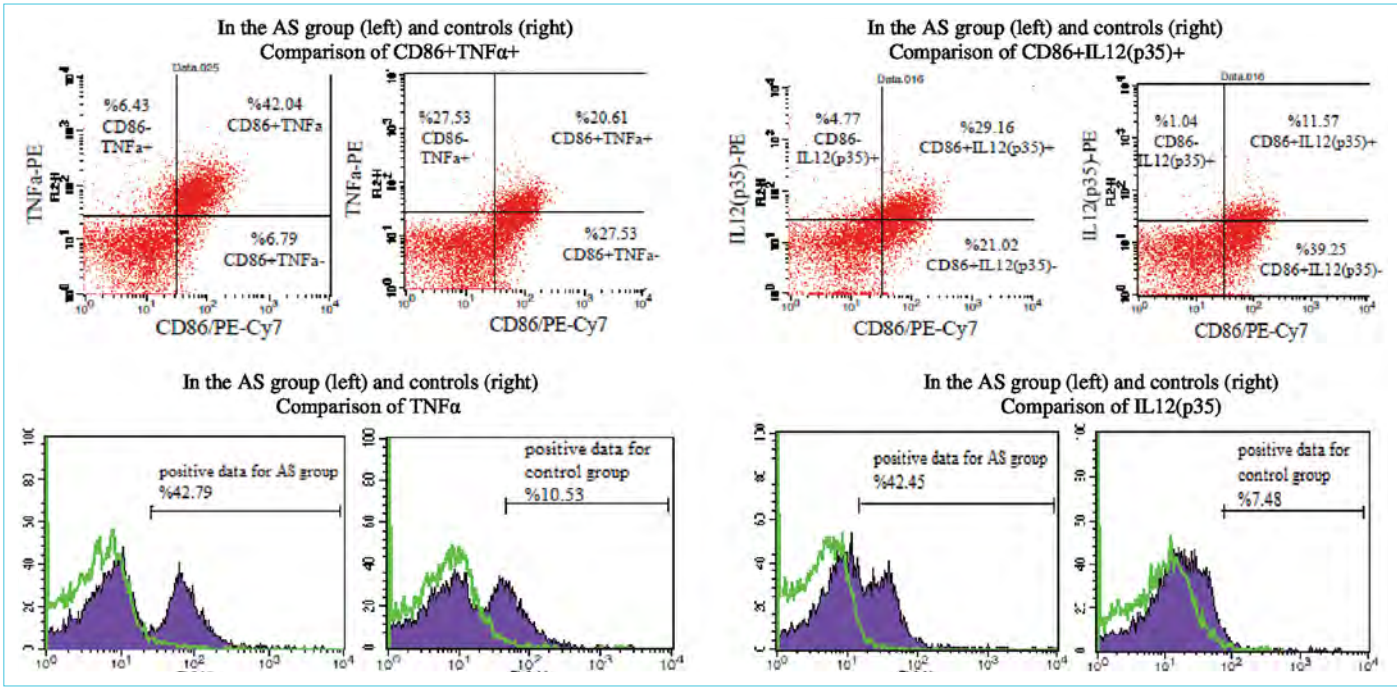

Figure 5. **Flow cytometry graphs of the role of EVexo in the AS process**

CD3/IL-17A, CD3/IFNγ, CD86/TNFα, CD86/IL-12(p35) double positivity levels were again higher in the patients (Table 6) and a strong effect size was obtained (p=0.001 for all and Cohen's d>2.0 for all).

The TSG101 and CD3/TSG101 double positivity level (%) of the AS group was higher than of the control ($p<0.05$). The effect size value was $0.5<d<0.8$ for TSG101, and $d>0.8$ for CD3+TSG101+. There was no significant difference between the mAS and sAS groups.

While the IFN-γ levels of the AS and control groups were not significantly different, a significant difference was found between the IFN-γ level of the sAS group and that of the mAS and control groups with a large effect value (eta squared=0.820) ($p<0.05$).

DISCUSSION

EVexo is associated with different inflammatory conditions, such as obesity, type-2 diabetes mellitus, kidney diseases, inflammatory bowel disease, several malignancies, and autoimmune

diseases, including rheumatoid arthritis (RA), systemic lupus erythematosus and Sjögren's syndrome. Considering their potential to carry different mediators to control inflammatory events (3, 5, 6). Studies have shown increased EVexo activity in RA, and the origin of EVexo is synovial fibroblasts, synovial fluid, and mesenchymal stem cells (7–10). Considering these results and the similarities and differences between RA and AS, we aimed to investigate the change in EVexo concentration, origin and cytokine profile of these EVexo in AS, which is assumed to have several pathogenic processes.

There are different methods for the isolation and subsequent biochemical and physical analysis of EVexo in the current literature. Immunoblotting of marker proteins, transmission electron microscopy (TEM) to analyze the structure, and nanoparticle tracking analysis to determine the number and size distribution of EVexo in a sample volume are the popular approaches in the current literature (11). However, none of these methods alone is sufficient for isolation and downstream analysis, as EVexo has a similar size, molecular content, and origin to many non-EV structures (11, 12). Therefore, in the literature, it is recommended to combine more than one method to isolate and characterize EVexo (11–18). We also used different methods (SEC, FC, BCA, and ELISA) that support and complement each other, based on the research question and downstream applications, to validate the results and design a validated algorithm for downstream studies. We preferred the SEC method, which is supported by the latest findings reported at the International Society for Extracellular Vesicles Virtual Conference 2020 (19–21) that it isolates both morphologically and functionally intact EVexo with lower contaminants from human body fluids, and, which detects body fluids and whether different proteins coexist on the same EVexo, FC as well.

Consistent with our expectation, according to BCA and ELISA analyzes, the filtrates obtained via SEC-based qEV2/35 nm columns were highly purer than serum for contamination of serum proteins that were not expected to be present in serum EVs but could be co-isolated with EVs. Although a longer incubation duration of 90 minutes did not significantly affect this concentration, probably due to buffering the pH or enaturation of proteins during more prolonged incubation, the AS group had a higher total protein concentration in BCA analysis after 30 minutes of incubation. We performed analysis both with and without lysis of the EV membrane, as the importance of lysis to define EV protein concentration was another methodological issue. In findings that are consistent with the results of Franquesa et al., 2014. (22), while some studies recommended lysis to obtain more accurate results, we observed no differences in protein concentration between lysed and not-lysed samples as in the AS group, and even observed that the lysis process denatured the proteins as in the HC group. This result might be related to the adverse effect of the sonication and RIPA buffer used for lysis.

By showing the presence of CD3, CD86, IL17, IFNg, TNFα, IL12 (p35) antigens together with transmembrane proteins such as CD9, CD63 and CD81, which are expected to be found in EVexo, and TSG101, which is involved in EVexo synthesis, we confirmed that the structures we examined in this study are EVexo (Fig. 1).

Consistent with BCA analysis, FC analysis showed increased TSG101+ EVexo in the AS groups than the controls, without a significant difference according to disease severity (Fig. 2). Double positivity analysis to identify cellular origins revealed that EVexo originated both from T lymphocytes with increased IFN-γ and IL-17A levels and macrophages with increased TNF-α and IL-12 (p35) levels, which is consistent with the immunologic nature of AS, including the effects of both adaptive and innate immunity (Fig. 3–5). However, individual differences caused by genetic diversity and environmental variables such as insomnia, diet/content, heavy metals intake, air pollution, which cause changes in cytokine production of PBMC cells (23–25), led to a significant standard deviation of the measurements.

The major limitation of this study was the relatively small sample size and methodologic uncertainty for EVexo isolation. Therefore, to obtain more accurate results, we performed both BCA and FC analysis of exosomal presence and obtained consistent results. Additionally, concerning the cross-sectional analysis of EVexo in both groups, possible dynamic variations in EVexo quantitation could not be revealed.

CONCLUSION

This study obtained the first preliminary results showing increased exosomal contribution in AS. Further studies with analyses of consecutive individuals, larger sample sizes, and different ethnic groups are necessary, where studies targeting this pathway might potentially elucidate the pathogenesis of AS and develop new treatment options. Additionally, while in addition, TEM images should be considered as the final proof of the existence of exosomes in further studies, performing this aforementioned method with synovial fluid might be another option to obtain additional clues about the local inflammatory response.

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