Journal of Autoimmunity 139 (2023) 103073

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Extracellular vesicles opsonized by monomeric C-reactive protein (CRP) are accessible as autoantigens in patients with systemic lupus erythematosus and associate with autoantibodies against CRP

Jesper Karlsson [a,*], Jonas Wetterö [a], Lawrence A. Potempa [b], Rafael Fernandez-Botran [c], Yasmine O'Neill [d], Lina Wirestam [a], Fariborz Mobarrez [d], Christopher Sjöwall [a]

[a] Department of Biomedical and Clinical Sciences, Division of Inflammation & Infection, Linköping University, Linköping, Sweden
[b] Roosevelt University, College of Science, Health and Pharmacy, Schaumburg, IL, United States
[c] Department of Pathology & Laboratory Medicine, University of Louisville, Louisville, KY, United States
[d] Department of Medical Sciences, Division of Clinical Chemistry, Uppsala University, Uppsala, Sweden

ARTICLE INFO

Handling Editor: M.E. Gershwin

Keywords:
Systemic lupus erythematosus
C-reactive protein
Extracellular vesicles
Anti-CRP antibodies
Immune complexes

ABSTRACT

The pentraxin C-reactive protein (CRP) is a pentameric protein now known to be able to undergo dissociation into a monomeric, modified isoform, referred to as mCRP. In carefully assessing the bioactivities of each isoform, mCRP has strong pro-inflammatory activities while pCRP has mild anti-inflammatory activities. Systemic lupus erythematosus (SLE) is a disease characterized by a vast number of autoantibodies, including *anti*-CRP autoantibodies which have been associated with SLE disease activity and lupus nephritis. The origin of these autoantibodies is currently unknown. Extracellular vesicles (EVs) have been implicated in SLE pathogenesis as they can expose nuclear antigens on their outside surface, thereby being a potential adjuvant for the generation of autoantibodies. Herein, we studied exposure of both pCRP and mCRP on EVs in SLE plasma and the implications of each in disease activity, organ damage and clinical manifestations. We used flow cytometry to detect CRP isoforms on EV surfaces in 67 well-characterized SLE patients and 60 sex- and age-matched healthy controls. Autoantibodies against mCRP were measured using ELISA. We found an abundance of both pCRP and mCRP on SLE EVs compared to controls. Furthermore, $mCRP^+$ but not $pCRP^+$ EVs were elevated in patients with active disease and in *anti*-CRP positive patients. The proportions of $mCRP^+$ EVs were lower in patients with acquired organ damage, especially in patients with lupus nephritis (LN), and displayed an inverse relationship with disease duration in LN and patients with active disease. Speculatively, these data suggest EV-bound mCRP as a relevant factor in SLE pathogenesis, which could contribute to development of *anti*-CRP autoantibodies by stimulating an immune response.

1. Introduction

Quantifying C-reactive protein (CRP) in blood is a widely used test to assess the degree of inflammation associated with tissue damage. Studies have shown that CRP concentration can help distinguish bacterial from viral infections, and increased levels reflect risks of ischemic cerebro- and cardiovascular disease [1]. The complex role of CRP in systemic lupus erythematosus (SLE) has for long been a matter of debate [2]. Unlike most diseases, in SLE, CRP appears to be an unreliable marker of disease activity. Still, association between CRP levels and irreversible organ damage has been demonstrated [3].

That CRP found in blood is a pentameric, liver-derived protein of identical 23-kDa subunits arranged in cyclic symmetry around a central void (referred to as pCRP). Under certain conditions as may exist in damaged tissues associated with the activation of host defense responses, CRP is now known to dissociate into individual monomers which spontaneously undergo a conformational change, becoming a distinctive isoform described as a monomeric, modified isoform (i.e., mCRP) which has very distinctive antigenic, solubility and biologic properties compared to pCRP [4,5]. To date, the majority of studies

* Corresponding author. Department of Biomedical and Clinical Sciences, Division of Inflammation and Infection, Linköping University, Campus US, SE-581 85 Linköping, Sweden.
E-mail address: jesper.karlsson@liu.se (J. Karlsson).

https://doi.org/10.1016/j.jaut.2023.103073
Received 20 February 2023; Received in revised form 30 May 2023; Accepted 6 June 2023
Available online 23 June 2023
0896-8411/© 2023 The Author(s). Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

involving CRP do not distinguish the contributions of each distinctive isoform in the data presented. However, it is now shown that mCRP is a strongly pro-inflammatory isoform while pCRP is a weakly anti-inflammatory isoform [2,6,7]. Our group has previously shown that the relative relationship between pCRP and mCRP can discriminate between active and quiescent SLE [8]. In addition, immunoglobulin G (IgG) autoantibodies against mCRP have been reported in SLE, as well as in additional conditions, and appear to associate with renal involvement and increased disease activity and long-term prognosis [9–12]. Still, detailed knowledge of the source of autoantigens and mechanisms eliciting these autoantibodies in SLE is lacking.

Extracellular vesicles (EVs) are membrane-bound particles exerted into circulation from activated cells, apoptotic blebbing or other types of cell death, although their origin is not fully elucidated [13]. The process of blebbing include translocation of nuclear components into a protrusion of the plasma membrane, which is thereafter separated from the rest of the membrane [14,15]. EVs are secreted as a type of intercellular communication [16]. The nomenclature has changed over the years, and the term EVs now includes secreted vesicles of all sizes and origin [17]. EVs can be derived from a multitude of cell types and their diameter ranges from approximately 100–1000 nm (previously known as microparticles or microvesicles). They are distinguished from apoptotic bodies based on the size and contents, as apoptotic bodies are larger and contain remnants from apoptotic cells [18]. However, they can be difficult to separate since both can have common components and originate from apoptotic processes. EVs have been shown to be important in the pathogenesis of SLE, attributed to their ability to expose nuclear antigens such as DNA and histones on their surface [18,19]. As recently reviewed, EVs may expose mitochondrial damage-associated molecular patterns, such as mitochondrial DNA, on their surface, promoting inflammatory responses [20]. Due to this, EVs might suffice as autoantigens and adjuvants, and consequently contribute to the formation of immune complexes (ICs) and generation of autoantibodies.

Of relevance to this study, the primary ligand onto which pCRP binds is phosphocholine, which is prominently found on the surface on EVs which were formed with sloughed phosphatidyl choline and sphingomyelin lipids. pCRP can bind to the surface of EVs. When brought in a juxtaposed position with the apolar EV microenvironment, pCRP can be induced to dissociate and convert into mCRP. Hence, any EVs produced naturally, and which may contact acute phase serum, can associate with either or both pCRP and mCRP. Because of the distinctive bioactivities of each CRP isoform, a key question is how each CRP–EV isoform complex may contribute to disease [21–23].

In this study, we asked whether the CRP isoforms are accessible on EVs as autoantigens in SLE and whether they are associated with different disease manifestations, disease activity or organ damage. To pursue this, we investigated CRP isoforms on EVs by flow cytometry and *anti*-CRP antibodies by ELISA in appurtenant samples from well-characterized patients with SLE and matched controls.

2. Patients and methods

2.1. Patients and healthy controls

During 2018, we included samples from patients classified with SLE ($n = 67$) based on the 1982 American College of Rheumatology (ACR82) and/or the 2012 Systemic Lupus International Collaboration Clinics (SLICC) criteria sets [24]. All subjects were part of a regional research quality register designated *Clinical Lupus Register in North-Eastern Gothia* (Swedish acronym 'KLURING') at the University Hospital in Linköping, Sweden [25].

Disease activity was assessed by the SLE disease activity index 2000 (SLEDAI-2K) and clinical SLE disease activity index 2000 (cSLEDAI-2K; exclusion of items for low complement and *anti*-dsDNA antibodies) [26,

Table 1
Characteristics of included patients, healthy controls, and the patients stratified in clinical phenotypes.

	SLE: All ($n = 67$)	HC ($n = 60$)	SLE: APS ($n = 20$)	SLE: LN ($n = 22$)	SLE: Skin & Joint ($n = 25$)
Background variables					
Age, mean (standard deviation [SD])	43 (12)	43 (11)	45 (12)	40 (11)	43 (12)
Female sex, *n* (%)	58 (87)	52 (87)	15 (75)	20 (91)	23 (92)
Current or previous tobacco smokers, *n* (%)	15 (22)	0 (0)	5 (25)	4 (18)	6 (24)
Body Mass Index, mean (SD)	25.8 (4.4)	23.9 (3.4)	25.6 (4.0)	25.8 (4.3)	25.9 (4.9)
Disease variables					
Disease duration in years, mean (SD)	12 (9)	N/A	16 (12)	10 (8)	10 (7)
SLEDAI-2K, median (interquartile range [IQR])	2 (0–4)	N/A	2 (0–2)	2 (0–4)	2 (0–5)
cSLEDAI-2K, median (IQR)	0 (0)	N/A	0 (0)	0 (0)	0 (0–2)
SDI, median (IQR)	1 (0–1)	N/A	1 (0–3)	0 (0–1)	0 (0–1)
Ongoing pharmacotherapy					
Azathioprine, *n* (%)	4 (6)	N/A	0	3 (14)	1 (4)
Belimumab, *n* (%)	2 (3)	N/A	0	1 (5)	1 (4)
Glucocorticoids, *n* (%)	38 (57)	N/A	11 (55)	14 (64)	13 (52)
Hydroxychloroquine, *n* (%)	60 (90)	N/A	17 (85)	21 (95)	22 (88)
Methotrexate, *n* (%)	6 (9)	N/A	1 (5)	1 (5)	4 (16)
Mycophenolate mofetil, *n* (%)	17 (25)	N/A	6 (30)	5 (23)	6 (24)
Rituximab, *n* (%)	5 (7)	N/A	1 (5)	2 (9)	2 (8)
Other conventional immunosuppressants, *n* (%)	4 (6)	N/A	2 (10)	1 (5)	1 (4)
Clinical features (ACR-82 definitions)					
Malar rash, *n* (%)	26 (39)	N/A	6 (30)	8 (36)	12 (48)
Discoid rash, *n* (%)	3 (5)	N/A	0 (0)	1 (5)	2 (8)
Photosensitivity, *n* (%)	31 (46)	N/A	10 (50)	10 (45)	11 (44)
Oral ulcers, *n* (%)	13 (19)	N/A	2 (10)	6 (27)	5 (20)
Arthritis, *n* (%)	51 (76)	N/A	13 (65)	17 (77)	21 (84)
Serositis, *n* (%)	26 (39)	N/A	8 (40)	14 (64)	4 (16)
Renal disorder, *n* (%)	25 (37)	N/A	2 (10)	22 (100)	1 (4)
Neurologic disorder, *n* (%)	6 (9)	N/A	4 (20)	1 (5)	1 (4)
Hematologic disorder, *n* (%)	45 (67)	N/A	14 (70)	14 (64)	17 (68)
Immunologic disorder, *n* (%)	42 (63)	N/A	13 (65)	20 (91)	9 (36)
IF-ANA, *n* (%)	67 (100)	N/A	20 (100)	22 (100)	25 (100)

APS – antiphospholipid syndrome; cSLEDAI-2K – clinical SLE Disease Activity Index 2000; HC – healthy controls; IF-ANA – antinuclear antibodies analyzed by immunofluorescence on HEp-2 cells; LN – lupus nephritis; N/A – not applicable; SDI – SLICC/ACR damage index; SLE – systemic lupus erythematosus; SLEDAI-2K – SLE Disease Activity Index 2000.

27]. We defined high disease activity (active disease) as SLEDAI-2K ≥ 5 and low disease activity as SLEDAI-2K ≤ 4. The SLICC/ACR damage index (SDI) was used to estimate irreversible organ damage in 12 separate domains [28]. The patients were selected based on their SLE phenotype; 20 with lupus nephritis (LN) without antiphospholipid syndrome (APS), 20 with skin and joint involvement only, and 20 with APS, meeting the Sydney criteria, without renal involvement [29]. As comparators, 60 non-smoking sex- and age-matched blood donors without ongoing medication were available as healthy controls (HC). In addition, 7 patients with SLE showing active disease (SLEDAI-2K ≥ 5) were included. Characteristics of the patients and HC are detailed in Table 1.

2.2. Routine laboratory assessments

Clinical routine analyses were performed at the Clinical Chemistry Laboratory, University Hospital in Linköping. Plasma CRP (detection limit 0.15 mg/L) and complement proteins C3 and C4 were measured using high-sensitive turbidimetry and nephelometry, respectively. Plasma interleukin 6 (IL-6) was assessed using a colorimetric immunoassay (detection limit 1.5 ng/L).

2.3. Measurement of plasma mCRP

Plasma levels of mCRP were measured using an in-house sandwich enzyme-linked immunosorbent assay (ELISA) previously described in detail [8]. In brief, 96-well plates were coated overnight with goat *anti*-human mCRP polyclonal antibody diluted in PBS. After blocking with PBS containing 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), recombinant mCRP of different concentrations and plasma samples were added to the plate and incubated for 2h at room temperature. Mouse *anti*-human mCRP monoclonal antibody (8C10) were then added and incubated for 90min in room temperature before incubation with a goat anti-mouse IgG antibody conjugated with horseradish peroxidase (Abcam, Cambridge, UK). After an hour incubation in room temperature, substrate solution (3,3′,5,5′ tetramethylbenzidine [TMB]; Sigma) was added. The reaction was stopped using 1 M H_2SO_4 and optical density measured at 450 nm. Plasma concentrations of mCRP were calculated based on the mCRP standard curves.

2.4. Anti-CRP antibody assay

Plasma concentrations of *anti*-CRP antibodies were measured using a modified version of a previously described in-house enzyme-linked immunosorbent assay (ELISA) [10]. Costar® 96-well high binding polystyrene flat-bottomed half-area plates (Corning, Corning, NY, USA) were coated with 0.5 μg/mL human CRP from human fluids (Sigma, St. Louis, MO, USA) diluted in carbonate/bicarbonate-buffer (pH 9.6) over night in 4 °C and then blocked for 2h at room temperature (RT) with Pierce™ Protein-Free (PBS) blocking buffer (Thermo-Fisher Scientific, Waltham, MA, USA). Samples diluted 1:50 in PBS-Tween (0.05%) were added and incubated for 1h at RT, followed by 1h RT incubation of rabbit anti-human immunoglobulin IgG H&L (alkaline phosphatase) secondary antibody (Abcam, Cambridge, UK) diluted 1:2000 in PBS-Tween. SIGMAFAST™ *p*-Nitrophenyl Phosphate (PNPP; Sigma) was added and optical density (OD) was analyzed at 405 nm (630 nm reference wavelength). The plates were measured at several time points until an OD of 1.5 was obtained for the highest value of the standard curve. The plates were washed 4 times with PBS-Tween between every addition to the plate. One patient sample with previously known high levels of *anti*-CRP antibodies was used as standard and the samples were expressed as percentage of the standard. Cut-off limit for *anti*-CRP positivity was based on the 95th percentile of the 60 age- and sex-matched HC samples included. One plasma sample was continuously included on every plate to control for inter-assay variability. Intra-assay and inter-assay variabilities <20% were accepted.

2.5. EV analysis

An EV-enriched pellet was isolated from platelet poor plasma (anticoagulated by citrate) by centrifugation at 2000*g* for 20 min at room temperature. The upper part of the supernatant was then centrifuged at high-speed at 20 800*g* for 45 min [18]. Most of the exosome-rich supernatant was discarded before vortexing to obtain an EV-enriched pellet. 20 μL sample was added to a 96-well plate and incubated for 20 min in the dark with 5 μL monoclonal *anti*-mCRP (3H12) in-house conjugated with Dylight 488 (Thermo-Fisher Scientific, used at 1:100 dilution) or 5 μL monoclonal *anti*-pCRP (1D6) in-house conjugated with Dylight 633 (Thermo-Fisher Scientific, used at 1:100 dilution) antibody. The samples were then diluted with 100 μL CytoFLEX Sheath Fluid (Beckman Coulter, Brea, CA, USA) before flow cytometry analysis by CytoFLEX flow cytometer (Beckman Coulter). To determine the gating for EV analysis, 0.13 μm, 0.22 μm, 0.45 μm, 0.88 μm, and 1.35 μm Nano fluorescent Yellow Particles (Spherotech, Lake Forest, IL, USA) was used to detect a range in size between roughly 0.2 μm and 1.0 μm (Fig. 1A, B, D). The lower EV gate was set to 0.2 μm beads, as the background noise around 0.13 μm is quite prominent and may interfere with accurate measurements. In addition, the gating strategy was controlled using labeled and unlabeled EVs as beads and EVs have different refracting index. Unstained EVs, isotype controls, single fluorochrome stained EVs, and EVs stained as fluorescence-minus-one (FMO) controls were used to set-up the instrument (Fig. 1C). The threshold of the instrument was set to violet side scatter. Presented results represent the proportion of mCRP-positive or pCRP-positive EVs (events of antibody positive EVs divided by total amount of events in the gate).

An *in vitro* experiment was performed to explore the potential association between *anti*-CRP antibody positivity and CRP on EVs. EVs were isolated from one HC as described above. The EVs were incubated with 50 μl (in duplicates) of either *anti*-CRP antibody negative plasma from a HC, *anti*-CRP antibody negative SLE plasma, *anti*-CRP antibody positive SLE plasma, or Sheath Fluid (Beckman Coulter). The samples were incubated shaking for 3h in 37 °C before incubating them with *anti*-mCRP (FITC-conjugated) and *anti*-pCRP (APC-conjugated) and prepared for flow cytometry analysis as described above. Unstained EVs, FMO, and blanks were used as controls.

2.6. Statistical analyses

SPSS Statistics 28 (IBM Corp., Armonk, NY, USA) was applied for statistical analyses. Due to lack of data normality, non-parametric tests were carried out. Differences between groups were tested using either Mann Whitney *U* or Kruskal-Wallis 1-way ANOVA tests, depending on the number of groups. Spearman's Rho was used for correlation analysis. A *p*-value of <0.01 was considered statistically significant.

2.7. Ethics considerations

Oral and written informed consent was obtained from all patients and healthy controls. The study protocol was performed according to the Declaration of Helsinki and approved by the Regional Ethics Review Board in Linköping (ref. M75–08, and ref. 2017/572–32).

3. Results

3.1. Higher abundancy of mCRP$^+$ EVs in patients with active SLE

More pCRP$^+$ EVs were present in samples from patients with SLE compared to HC (Fig. 2A), but the proportions did not differ between the low and high disease activity groups. mCRP$^+$ EVs were more abundant in SLE patients with high disease activity compared to HC and low disease activity but did not differ significantly between HC and SLE patients without active disease (Fig. 2B). There was no significant difference in plasma mCRP levels between SLE phenotypes, HC or between

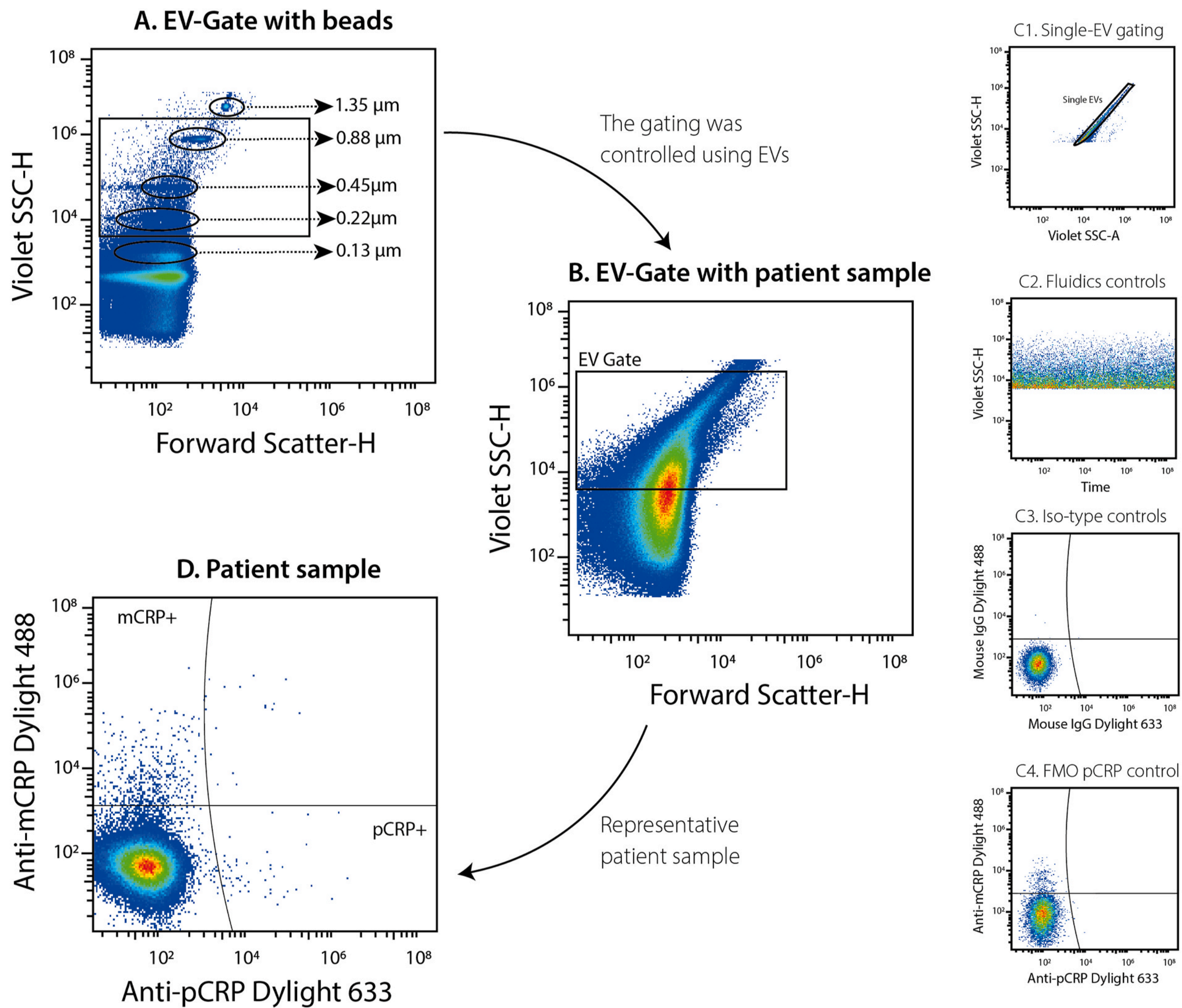

Fig. 1. Representative plots of flow cytometric analysis of extracellular vesicles (EVs). (A) Dot-plot of Nano fluorescent Yellow Particles used to determine the EV gate. (B) Gating strategy for all EVs based on size and complexity. The EV-protocol consisted in addition to EV-gates as seen in B, also gates for single-EVs (C1; Hight vs Area for Violet SSC) and also a fluidics control to ensure optimal sample analysis (C2; Violet SSC vs time). Unstained EVs, Iso-type controls, single fluorochrome stained EVs, and EVs stained as fluorescence-minus-one (FMO) controls were used to set-up the machine (C3-4) (D) Representative systemic lupus erythematosus patient sample demonstrating EVs labeled with *anti*-mCRP (3H12 clone) Dylight 488 and *anti*-pCRP (1D6 clone) Dylight 633.

SLE patients with or without active disease. The proportion of mCRP$^+$ EVs were higher in patients with APS and skin & joint involvement, but not in LN, when comparing to HC (Supplementary Fig. 1A). All three SLE phenotypes had a higher proportion of pCRP$^+$ EVs compared to the controls (Supplementary Fig. 1B). Regarding the ratios of mCRP$^+$/pCRP$^+$ EVs, a higher ratio was observed in patients with high disease activity compared to those with low disease activity, while no difference was found comparing SLE to HC (Fig. 2C), or HC to any of the SLE phenotypes (Supplementary Fig. 1C). In addition, the proportions of mCRP$^+$ EVs did not correlate with SLEDAI-2K scores (rho = 0.25, p = 0.04), but correlated significantly with cSLEDAI-2K (rho = 0.41, $p < 0.001$). Proportions of pCRP$^+$ EVs did not reach significance with SLEDAI-2K (rho = 0.11, p = 0.40) or with cSLEDAI-2K (rho = 0.26, p = 0.04). No significant differences in the abundancy of mCRP$^+$ and pCRP$^+$ EVs or their ratios were found between the different SLE phenotypes (i.e., LN, skin and joint involvement, or APS). Furthermore, subjects with a history of oral ulcers showed more mCRP$^+$ EVs than patients without ($p < 0.01$), but no significant difference in pCRP$^+$ EVs. No significant association of mCRP$^+$ EVs, pCRP$^+$ EVs, or their ratios, with plasma mCRP (Supplementary Fig. 2), plasma CRP, IL-6, C3, C4, age, sex, BMI, smoking habits, or any types of ongoing pharmacotherapy were observed.

3.2. Anti-CRP positive subjects display a higher abundancy of mCRP$^+$ EVs

Fourteen of the 67 (20.9%) patients with SLE were *anti*-CRP antibody positive in the circulation, whereof 7/14 (50%) patients showed high disease activity. *Anti*-CRP positivity was associated with higher cSLEDAI ($p < 0.01$) but not SLEDAI (p = 0.04) scores. Furthermore, *anti*-CRP antibody positive patients displayed a significantly higher amount of mCRP$^+$ EVs compared to *anti*-CRP negative individuals (Fig. 3B); *anti*-CRP positive patients did not have significantly higher proportions of pCRP$^+$ EVs, although the median value of pCRP$^+$ EVs were higher in *anti*-CRP positive compared to *anti*-CRP negative patients (0.8×10^{-3} vs. 0.52×10^{-3}, respectively) (Fig. 3A). When EVs were isolated from one healthy individual and exposed to plasma from *anti*-CRP positive and negative patients, respectively, the proportion of mCRP$^+$ EVs were more pronounced than for pCRP$^+$ EVs (Supplementary Fig. 3). There was no significant difference in plasma mCRP levels between *anti*-CRP positive and *anti*-CRP negative patients (not shown). The ratio of mCRP$^+$/pCRP$^+$ EVs was not significantly altered between *anti*-CRP positive and negative patients (Fig. 3C). No significant differences were found for the presence of *anti*-CRP antibodies between the LN, skin and joint involvement, and APS disease phenotypes.

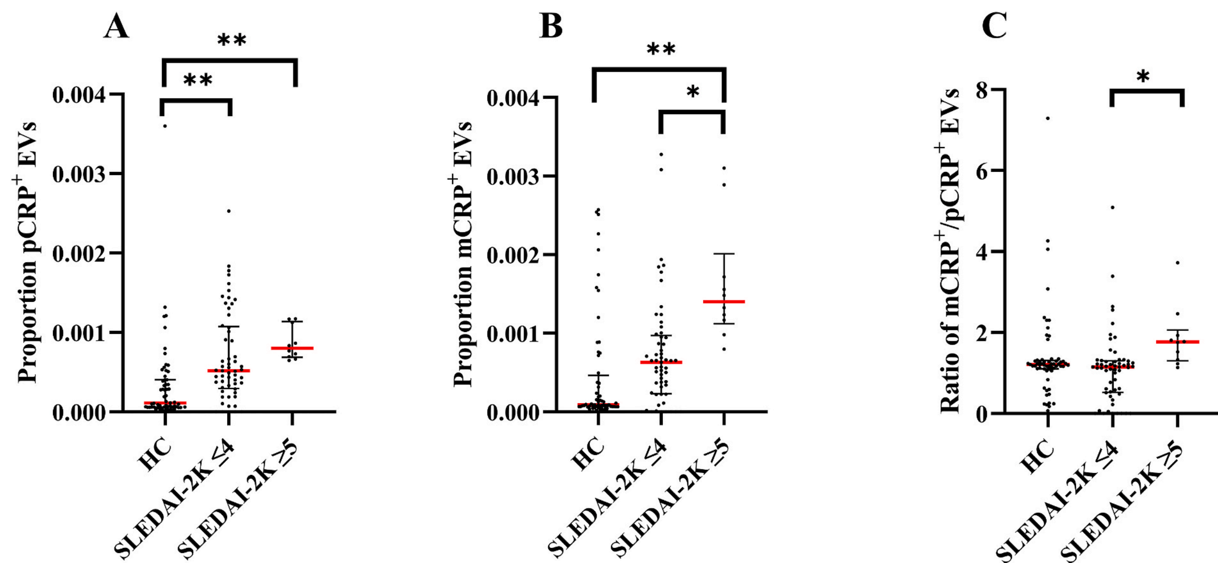

Fig. 2. Graph A and B show proportions of pentameric C-reactive protein (pCRP; A) and monomeric C-reactive protein (mCRP; B) positive extracellular vesicles (EVs) from patients with systemic lupus erythematosus *versus* sex- and age-matched blood donors (HC; $n = 60$). The subjects are divided into low (SLEDAI-2K ≤ 4; $n = 57$) and high (SLEDAI-2K ≥ 5; $n = 10$) disease activity. Graph C displays the ratios of $mCRP^+$ and $pCRP^+$ EVs between the same groups as indicated in A and B. The Y-axis represents the proportion of $mCRP^+$ or $pCRP^+$ EVs (antibody positive events divided by total number of events of the selected gate). ns, not significant; * = $p < 0.01$; ** = $p < 0.001$.

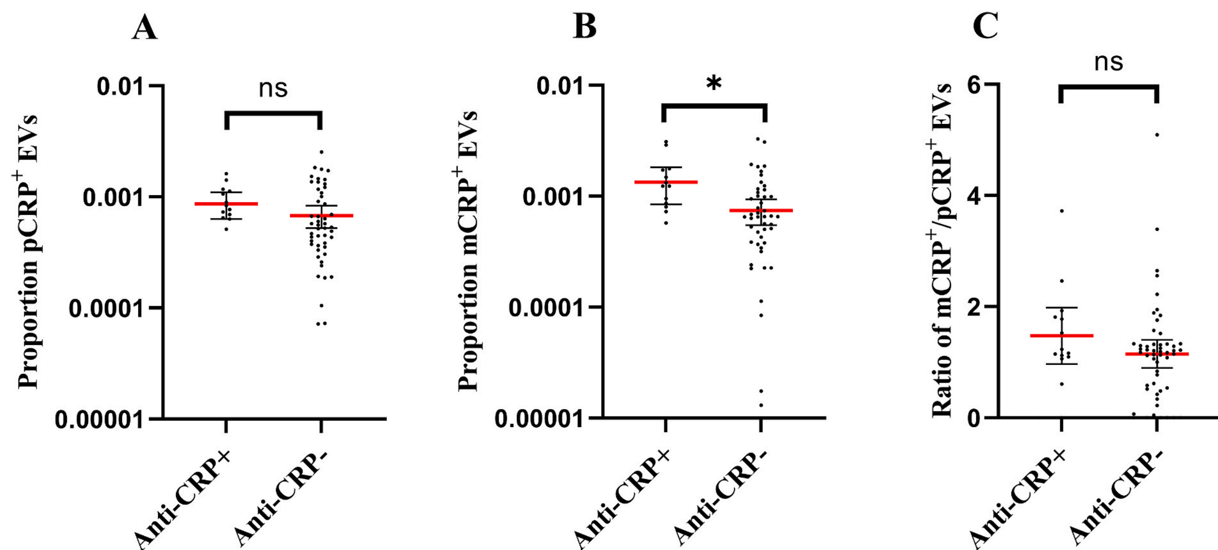

Fig. 3. Graph A and B illustrate proportions of pentameric C-reactive protein (pCRP; A) and monomeric C-reactive protein (mCRP; B) positive extracellular vesicles (EVs) from subjects with systemic lupus erythematosus. The patients are divided into *anti*-CRP antibody positive ($n = 15$) and negative ($n = 52$). Graph C shows the ratios of $mCRP^+$ and $pCRP^+$ EVs between the same groups as in A and B. The Y-axis represents the proportion of $mCRP^+$ or $pCRP^+$ EVs (antibody positive events divided by total number of events of the selected gate). ns, not significant; * = $p < 0.01$.

3.3. Less $mCRP^+$ EVs in plasma of patients with aquired organ damage

When CRP on EVs were compared between subjects with existing organ damage (SDI ≥ 1) and those without damage (SDI $= 0$), significant differences were observed for mCRP but not pCRP. $mCRP^+$ EVs were relatively fewer in samples from individuals with acquired damage assessed by SDI than those without (Fig. 4B). This observation was related to global damage only and not to damage in any specific domain. No significant difference was found for $pCRP^+$ EVs regarding organ damage (Fig. 4A). Plasma levels of mCRP did not differ significantly between the groups (not shown). Furthermore, the ratio of $mCRP^+$/$pCRP^+$ EVs achieved a higher value in patients without any damage (Fig. 4C). No significant correlations were observed between the proportion of neither $pCRP^+$ nor $mCRP^+$ EVs and SDI or SLE duration. Moreover, the proportion of $mCRP^+$ EVs correlated with the proportions of $pCRP^+$ EVs for both SLE and HC (rho = 0.78, $p < 0.001$ and rho = 0.82, $p < 0.001$, respectively).

The difference between the proportion of $mCRP^+$ EVs in patients with and without accrued damage remained only for patients with LN (without damage $n = 12$, with damage $n = 8$, $p < 0.01$), and not for the patients with skin and joint involvement, APS, or active disease when dividing them into phenotypes. Moreover, in patients with LN, $mCRP^+$ EVs were inversely correlated with global SDI (rho = −0.61, $p < 0.01$). In addition, an inverse correlation between $mCRP^+$ EVs and disease duration was achieved among patients with LN (rho = −0.58, $p < 0.01$) but no such significant correlation was observed in subjects with APS

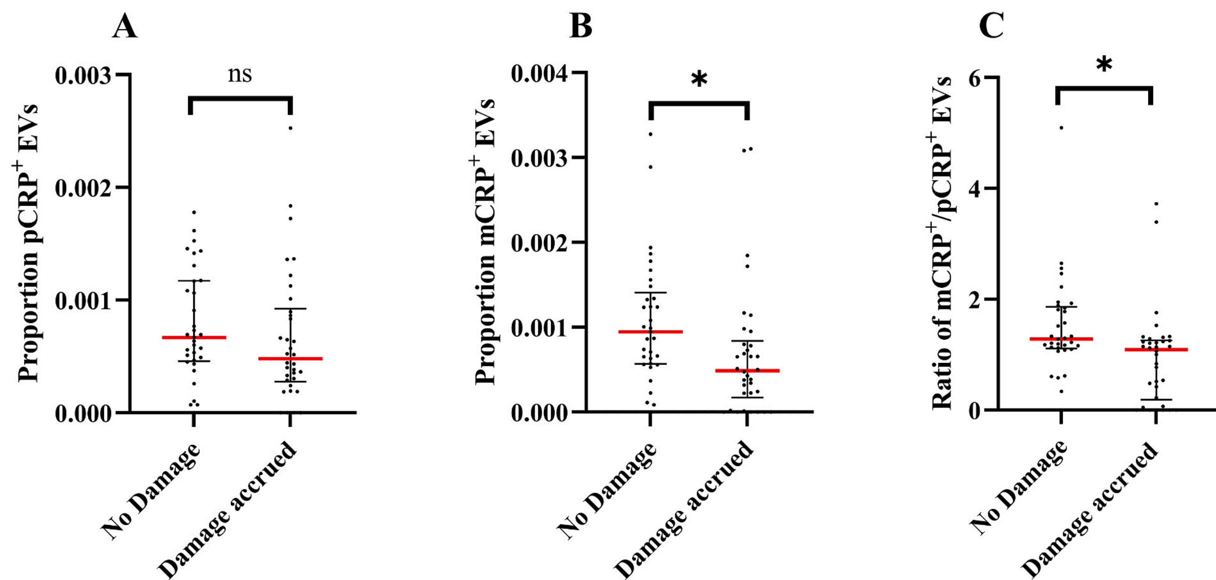

Fig. 4. Graph A and B demonstrate proportions of pentameric C-reactive protein (pCRP; A) and monomeric C-reactive protein (mCRP; B) positive extracellular vesicles (EVs) from patients with systemic lupus erythematosus. The subjects were divided according to damage accrual assessed by SLICC/ACR damage index; 34 patients with and 33 without any damage. Graph C displays the ratios of mCRP$^+$ and pCRP$^+$ EVs between the same groups as in A and B. The Y-axis represents the proportion of mCRP$^+$ or pCRP$^+$ EVs (antibody positive events divided by total number of events of the selected gate). ns, not significant; * = $p < 0.01$.

and skin and joint involvement. There was also a strong inverse correlation between mCRP on EVs and disease duration among subjects with SLEDAI-2K ≥ 5 (rho $= -0.88$, $p < 0.001$).

4. Discussion

This study is the first to investigate EV-bound CRP isoforms and *anti*-CRP autoantibodies in SLE. Our research group have previously evaluated the relationship of fluid-phase serum pCRP and mCRP in lupus [8]. Herein, we show that increased presence of mCRP$^+$ EVs is associated with high SLE disease activity. Based on the results of the current study, and our previously presented data on the relationship of fluid-phase pCRP and mCRP in lupus [8], it is tempting to speculate that mCRP on EVs may contribute to inflammation in patients with SLE and subsequently act as a complex of antigen and adjuvant leading to generation of mCRP autoantibodies, augmenting SLE progression [2,8,12,30].

Circulating pCRP remains an unreliable biomarker of inflammation in active SLE, although involvement of different organ domains may impact the relation. Herein, we found that pCRP on EVs failed to discriminate between active and non-active SLE. However, mCRP$^+$ EVs were found in higher abundancy in SLE with active, compared to those with quiescent, disease. Given the pro-inflammatory profile of mCRP and the increased abundancy of mCRP$^+$ EVs in patients with active disease, it is possible that mCRP together with the EVs may elevate the risk of developing *anti*-CRP autoantibodies and subsequent formation of ICs.

Zeller et al. recently showed that blocking the conversion of pCRP to mCRP can inhibit pro-inflammatory CRP-mediated effects [31], implicating the importance of CRP conversion in the inflammatory process. Furthermore, CRP$^+$ EVs have previously been reported to be elevated in sepsis and peripheral artery disease [32,33]. It has also been demonstrated that patients with SLE have increased numbers of IgG positive EVs and EVs with elevated amounts of C1q [34–36], which unveil that antibody responses towards EV surface antigens and/or opsonizing molecules transpires in lupus. Fortin et al. reported a positive correlation between IgG exposed CD41$^+$ EVs and SLEDAI-2K [37], further implicating the association of EVs, autoantibodies, and disease activity. Inflammatory exacerbation by EVs have also been suggested in other autoimmune disease such as rheumatoid arthritis, multiple sclerosis, primary Sjögren's syndrome, and type 1 diabetes [38]. Thereby, we augment the evidence of mCRP being a pro-inflammatory player with an important role in inflammation.

We further observed that patients with acquired organ damage (in any domain) did not display any difference in the proportion of pCRP$^+$ EVs when compared to those without damage, and that mCRP$^+$ EVs were seen in higher abundancy in patients without damage. Speculatively, this could reflect the importance of disease duration in damage accrual. As high disease activity is generally more frequently found in the early disease, and organ damage is irreversibly and incrementally accrued over time with increasing SLE duration, it is reasonable to assume that the decreased proportion of mCRP$^+$ EVs in patients with acquired damage is an indirect observation influenced by disease activity, as we observed more mCRP$^+$ EVs in active SLE. However, multiple factors could probably affect the decreased proportion of mCRP$^+$ EVs. As cardiovascular disease increases with age, another possible mechanism could be that CRP is located elsewhere, for instance on the endothelium [2]. Elevated CRP is associated with increased risk of cardiovascular disease, and CRP has been detected in endothelial atherosclerotic lesions [39–41]; and the mCRP isoform has been shown to mediate pro-inflammatory effects on vascular endothelium [41–43].

Furthermore, we observed an inverse relationship between the proportion of mCRP$^+$ EVs and the duration of the disease in patients with active disease and LN patients. Previously, we observed a negative correlation between serum mCRP and disease duration in active SLE [8]. Why this correlation only reside in LN and active disease requires further investigation, however, mCRP is of certain interest in lupus nephritis as *anti*-CRP antibodies mainly target mCRP and has been previously found in high prevalence in lupus nephritis flares [10,44]. Interestingly, there was no significant association with age, ruling out an age-related confounding effect. A constraint of this study is that the sampling was performed prior to the MISEV guidelines for reporting results on EVs [45,46]. While strengths of the study include the well-characterized SLE population with matched HC, the size of the study population and the lack of controls with other autoimmune diseases constitute limitations of the study. Nevertheless, despite a small study population, we show elevated mCRP$^+$ EV-proportions in active disease and in patients positive for *anti*-CRP antibodies, implying a possible role in autoantibody generation and SLE pathogenesis.

5. Conclusions

We demonstrate that EVs opsonized by mCRP, but not pCRP, are significantly increased in patients with active SLE as well as in *anti*-CRP antibody positive patients. Together with previously published data, our results suggest that mCRP on EVs could be implicated in SLE disease progression. Hypothetically, mCRP-opsonized EVs may constitute a platform for *anti*-CRP autoantibody generation by serving as adjuvant and autoantigen.

Author statement

Jonas Wetterö, Fariborz Mobarrez and Christopher Sjöwall designed the study. Jesper Karlsson, Yasmine O'Neill, Fariborz Mobarrez and Rafael Fernandez-Botran conducted the experiments. Christopher Sjöwall managed patient enrollment. Lawrence A. Potempa purified antibodies and provided reagents for the experiments. Jesper Karlsson, Jonas Wetterö, Lina Wirestam and Christopher Sjöwall analyzed the data. Jesper Karlsson wrote the initial draft. Jonas Wetterö, Lina Wirestam, Fariborz Mobarrez and Christopher Sjöwall supervised the study. All authors read and approved the final manuscript.

Funding

This work has been supported by grants through the Swedish Rheumatism Association (Grant nr R-939149), the Region Östergötland (ALF Grants), the Gustafsson Foundation, the King Gustaf V's 80-year Anniversary foundation (Grant nr FAI-2020-0663) and the King Gustaf V and Queen Victoria's Freemasons foundation (2017-21). The funders had no role in the design of the study or collection, analysis, or interpretation of data or in writing the manuscript.

Declaration of competing interest

None to declare.

Data availability

Data will be made available on request.

Acknowledgements

We are sincerely grateful to all the participating patients. Study nurse Marianne Petersson is acknowledged for biobank administration, and the clinicians at the Rheumatology Unit in Linköping for their efforts.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2023.103073.

References

[1] P. Povoa, L. Coelho, F. Dal-Pizzol, R. Ferrer, A. Huttner, A. Conway Morris, et al., How to use biomarkers of infection or sepsis at the bedside: guide to clinicians, Intensive Care Med. (2023) 1–12.

[2] H. Enocsson, J. Karlsson, H.Y. Li, Y. Wu, I. Kushner, J. Wetterö, et al., The complex role of C-reactive protein in systemic lupus erythematosus, J. Clin. Med. 10 (2021).

[3] S.S. Lee, S. Singh, K. Link, M. Petri, High-sensitivity C-reactive protein as an associate of clinical subsets and organ damage in systemic lupus erythematosus, Semin. Arthritis Rheum. 38 (2008) 41–54.

[4] S.R. Ji, Y. Wu, L. Zhu, L.A. Potempa, F.L. Sheng, W. Lu, et al., Cell membranes and liposomes dissociate C-reactive protein (CRP) to form a new, biologically active structural intermediate: mCRP(m), Faseb. J. 21 (2007) 284–294.

[5] L.A. Potempa, B.A. Maldonado, P. Laurent, E.S. Zemel, H. Gewurz, Antigenic, electrophoretic and binding alterations of human C-reactive protein modified selectively in the absence of calcium, Mol. Immunol. 20 (1983) 1165–1175.

[6] B. Molins, M. Figueras-Roca, O. Valero, V. Llorenc, S. Romero-Vázquez, O. Sibila, et al., C-reactive protein isoforms as prognostic markers of COVID-19 severity, Front. Immunol. (2023) 13.

[7] C. Svanberg, H. Enocsson, M. Govender, K. Martinsson, L.A. Potempa, I.M. Rajab, et al., Conformational state of C-reactive protein is critical for reducing immune complex-triggered type I interferon response: implications for pathogenic mechanisms in autoimmune diseases imprinted by type I interferon gene dysregulation, J. Autoimmun. 135 (2023), 102998.

[8] J. Karlsson, J. Wetterö, M. Weiner, J. Rönnelid, R. Fernandez-Botran, C. Sjöwall, Associations of C-reactive protein isoforms with systemic lupus erythematosus phenotypes and disease activity, Arthritis Res. Ther. 24 (2022) 139.

[9] S.A. Bell, T.W. Du Clos, G. Khursigara, J.J. Picazo, R.L. Rubin, Autoantibodies to cryptic epitopes of C-reactive protein and other acute phase proteins in the toxic oil syndrome, J. Autoimmun. 8 (1995) 293–303.

[10] C. Sjöwall, P. Eriksson, S. Almer, T. Skogh, Autoantibodies to C-reactive protein is a common finding in SLE, but not in primary Sjogren's syndrome, rheumatoid arthritis or inflammatory bowel disease, J. Autoimmun. 19 (2002) 155–160.

[11] Y. Shoenfeld, M. Szyper-Kravitz, T. Witte, A. Doria, A. Tsutsumi, A. Tatsuya, et al., Autoantibodies against protective molecules–C1q, C-reactive protein, serum amyloid P, mannose-binding lectin, and apolipoprotein A1: prevalence in systemic lupus erythematosus, Ann. N. Y. Acad. Sci. 1108 (2007) 227–239.

[12] S.S. Pesickova, R. Rysava, M. Lenicek, L. Vitek, E. Potlukova, Z. Hruskova, et al., Prognostic value of anti-CRP antibodies in lupus nephritis in long-term follow-up, Arthritis Res. Ther. 17 (2015) 371.

[13] R.E. Veerman, G. Gucluler Akpinar, M. Eldh, S. Gabrielsson, Immune cell-derived extracellular vesicles - functions and therapeutic applications, Trends Mol. Med. 25 (2019) 382–394.

[14] J.D. Lane, V.J. Allan, P.G. Woodman, Active relocation of chromatin and endoplasmic reticulum into blebs in late apoptotic cells, J. Cell Sci. 118 (2005) 4059–4071.

[15] M. Zirngibl, B.G. Furnrohr, C. Janko, L.E. Munoz, R.E. Voll, C.D. Gregory, et al., Loading of nuclear autoantigens prototypically recognized by systemic lupus erythematosus sera into late apoptotic vesicles requires intact microtubules and myosin light chain kinase activity, Clin. Exp. Immunol. 179 (2015) 39–49.

[16] N. Polyakova, M. Kalashnikova, A. Belyavsky, Non-classical intercellular communications: basic mechanisms and roles in biology and medicine, Int. J. Mol. Sci. 24 (2023).

[17] Y. Couch, E.I. Buzas, D. Di Vizio, Y.S. Gho, P. Harrison, A.F. Hill, et al., A brief history of nearly EV-erything - the rise and rise of extracellular vesicles, J. Extracell. Vesicles 10 (2021), e12144.

[18] F. Mobarrez, E. Fuzzi, I. Gunnarsson, A. Larsson, S. Eketjall, D.S. Pisetsky, et al., Microparticles in the blood of patients with SLE: size, content of mitochondria and role in circulating immune complexes, J. Autoimmun. 102 (2019) 142–149.

[19] E.I. Buzas, The roles of extracellular vesicles in the immune system, Nat. Rev. Immunol. 23 (2023) 236–250.

[20] T. Di Mambro, G. Pellielo, E.D. Agyapong, M. Carinci, D. Chianese, C. Giorgi, et al., The tricky connection between extracellular vesicles and mitochondria in inflammatory-related diseases, Int. J. Mol. Sci. (2023) 24.

[21] J.D. McFadyen, J. Kiefer, D. Braig, J. Loseff-Silver, L.A. Potempa, S.U. Eisenhardt, et al., Dissociation of C-reactive protein localizes and amplifies inflammation: evidence for a direct biological role of C-reactive protein and its conformational changes, Front. Immunol. 9 (2018) 1351.

[22] I.M. Rajab, M.E. Olson, J.M.B. Addams, M.L. Choe, M.S. Nelson, L.A. Potempa, C-reactive protein in gallbladder diseases – diagnostic and therapeutic insights, Biophysics Report 6 (2020) 49–67.

[23] L.A. Potempa, W.Q. Qiu, A. Stefanski, I.M. Rajab, Relevance of lipoproteins, membranes, and extracellular vesicles in understanding C-reactive protein biochemical structure and biological activities, Front Cardiovasc Med 9 (2022), 979461.

[24] A. Ighe, O. Dahlström, T. Skogh, C. Sjöwall, Application of the 2012 Systemic Lupus International Collaborating Clinics Classification Criteria to Patients in a Regional Swedish Systemic Lupus Erythematosus Register, Arthritis Research & Therapy, 2015, p. 17.

[25] M. Frodlund, O. Dahlström, A. Kastbom, T. Skogh, C. Sjöwall, Associations between antinuclear antibody staining patterns and clinical features of systemic lupus erythematosus: analysis of a regional Swedish register, BMJ Open 3 (2013), e003608.

[26] D.D. Gladman, D. Ibanez, M.B. Urowitz, Systemic lupus erythematosus disease activity index 2000, J. Rheumatol. 29 (2002) 288–291.

[27] A.G. Uribe, L.M. Vila, G. McGwin Jr., M.L. Sanchez, J.D. Reveille, G.S. Alarcon, The Systemic Lupus Activity Measure-revised, the Mexican Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), and a modified SLEDAI-2K are adequate instruments to measure disease activity in systemic lupus erythematosus, J. Rheumatol. 31 (2004) 1934–1940.

[28] D. Gladman, E. Ginzler, C. Goldsmith, P. Fortin, M. Liang, M. Urowitz, et al., The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus, Arthritis Rheum. 39 (1996) 363–369.

[29] S. Miyakis, M.D. Lockshin, T. Atsumi, D.W. Branch, R.L. Brey, R. Cervera, et al., International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS), J. Thromb. Haemostasis 4 (2006) 295–306.

[30] Q.Y. Li, H.Y. Li, G. Fu, F. Yu, Y. Wu, M.H. Zhao, Autoantibodies against C-reactive protein influence complement activation and clinical course in lupus nephritis, J. Am. Soc. Nephrol. 28 (2017) 3044–3054.

[31] J. Zeller, K.S. Cheung Tung Shing, T.L. Nero, J.D. McFadyen, G. Krippner, B. Bogner, et al., A novel phosphocholine-mimetic inhibits a pro-inflammatory conformational change in C-reactive protein, EMBO Mol. Med. (2022), e16236.

[32] B. Fendl, R. Weiss, T. Eichhorn, I. Linsberger, T. Afonyushkin, F. Puhm, et al., Extracellular vesicles are associated with C-reactive protein in sepsis, Sci. Rep. 11 (2021) 6996.
[33] J.R. Crawford, J. Trial, V. Nambi, R.C. Hoogeveen, G.E. Taffet, M.L. Entman, Plasma levels of endothelial microparticles bearing monomeric C-reactive protein are increased in peripheral artery disease, J Cardiovasc Transl Res 9 (2016) 184–193.
[34] C.T. Nielsen, O. Ostergaard, O.P. Rekvig, G. Sturfelt, S. Jacobsen, N.H. Heegaard, Galectin-3 binding protein links circulating microparticles with electron dense glomerular deposits in lupus nephritis, Lupus 24 (2015) 1150–1160.
[35] C.T. Nielsen, O. Ostergaard, L. Stener, L.V. Iversen, L. Truedsson, B. Gullstrand, et al., Increased IgG on cell-derived plasma microparticles in systemic lupus erythematosus is associated with autoantibodies and complement activation, Arthritis Rheum. 64 (2012) 1227–1236.
[36] F. Mobarrez, I. Gunnarsson, E. Svenungsson, Altered beta(2) -glycoprotein I expression on microparticles in the presence of antiphospholipid antibodies, J. Thromb. Haemostasis 15 (2017) 1799–1806.
[37] P.R. Fortin, N. Cloutier, V. Bissonnette, E. Aghdassi, L. Eder, D. Simonyan, et al., Distinct subtypes of microparticle-containing immune complexes are associated with disease activity, damage, and carotid intima-media thickness in systemic lupus erythematosus, J. Rheumatol. 43 (2016) 2019–2025.
[38] W.C. Wu, S.J. Song, Y. Zhang, X. Li, Role of extracellular vesicles in autoimmune pathogenesis, Front. Immunol. 11 (2020), 579043.
[39] S.D. de Ferranti, N. Rifai, C-reactive protein: a nontraditional serum marker of cardiovascular risk, Cardiovasc. Pathol. 16 (2007) 14–21.
[40] B. Lindahl, H. Toss, A. Siegbahn, P. Venge, L. Wallentin, Markers of myocardial damage and inflammation in relation to long-term mortality in unstable coronary artery disease. FRISC Study Group. Fragmin during Instability in Coronary Artery Disease, N. Engl. J. Med. 343 (2000) 1139–1147.
[41] J.R. Thiele, J. Habersberger, D. Braig, Y. Schmidt, K. Goerendt, V. Maurer, et al., Dissociation of pentameric to monomeric C-reactive protein localizes and aggravates inflammation: in vivo proof of a powerful proinflammatory mechanism and a new anti-inflammatory strategy, Circulation 130 (2014) 35–50.
[42] S.R. Ji, L. Ma, C.J. Bai, J.M. Shi, H.Y. Li, L.A. Potempa, et al., Monomeric C-reactive protein activates endothelial cells via interaction with lipid raft microdomains, Faseb. J. 23 (2009) 1806–1816.
[43] T. Khreiss, L. Jozsef, L.A. Potempa, J.G. Filep, Conformational rearrangement in C-reactive protein is required for proinflammatory actions on human endothelial cells, Circulation 109 (2004) 2016–2022.
[44] C. Sjöwall, A. Zickert, T. Skogh, J. Wetterö, I. Gunnarsson, Serum levels of autoantibodies against C-reactive protein correlate with renal disease activity and response to therapy in lupus nephritis, Arthritis Res. Ther. 11 (2009) R188.
[45] J.A. Welsh, E. Van Der Pol, G.J.A. Arkesteijn, M. Bremer, A. Brisson, F. Coumans, et al., MIFlowCyt-EV: a framework for standardized reporting of extracellular vesicle flow cytometry experiments, J. Extracell. Vesicles 9 (2020), 1713526.
[46] K.W. Witwer, D.C. Goberdhan, L. O'Driscoll, C. Thery, J.A. Welsh, C. Blenkiron, et al., Updating MISEV: evolving the minimal requirements for studies of extracellular vesicles, J. Extracell. Vesicles 10 (2021), e12182.