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Circulating microparticles remain associated with complement activation despite intensive anti-inflammatory therapy in early rheumatoid arthritis

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ABSTRACT

Objectives Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterised by synovitis and joint destruction. The pathogenesis of RA is not clear, but is considered to be an immune-mediated inflammatory disorder, in which the complement system plays an important role. Although cell-derived microparticles (MPs) have been associated with inflammation and complement activation, it is unknown whether MPs are either cause or consequence. Therefore, we investigated whether circulating MPs differ between patients with very early as yet untreated arthritis and healthy controls, and whether intensive anti-inflammatory treatment of such patients affects circulating MPs.

Methods Patients with RA (n=24) and controls (n=15) were included. Nine patients with RA were re-evaluated after 8 weeks of intensive treatment with a combination of drugs ('COmBination therapy in Rheumatoid Arthritis' (COBRA) scheme). Disease activity was measured by erythrocyte sedimentation rate (ESR), C reactive protein (CRP) and Disease Activity Score for 28 joints (DAS28). Flow cytometry was used to study MPs and exposure of complement activator molecules and complement components.

Results At baseline, concentrations of MPs exposing C1q, CRP or serum amyloid-P (SAP) were all significantly elevated in patients with early RA compared to controls (p=0.003, p=0.002 and p=0.003, respectively). Upon treatment, DAS28 score, ESR and CRP levels significantly decreased (p=0.008, p=0.008 and p=0.012), but the concentrations of circulating MPs and MPs exposing complement components or activator molecules were unaffected.

Conclusion Circulating MPs exposing complement components or activator molecules are elevated in early RA. Since a strong anti-inflammatory therapy suppressed inflammation in patients with early RA but not levels of circulating MPs, it is unlikely that inflammation is the main underlying cause of MP release in these patients.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease with a complex pathogenesis, characterised by synovitis leading to cartilage, tendon and joint destruction.^{1,2} Although the pathogenesis of RA is not clear, it is considered to be an immune-mediated inflammatory disorder, in which the complement system plays an important role.

Recently, cell-derived microparticles (MPs), which are small membrane vesicles released from blood cells or endothelial cells upon activation or during apoptosis, were shown to be associated with

complement activation, inflammation and coagulation in various diseases, including inflammatory diseases.³⁻⁷ Although inflammation causes release of MPs and in turn MPs may induce or enhance inflammation, it remains unknown whether circulating MPs merely reflect ongoing inflammation or whether MPs actually contribute to the disease development.

In vitro studies and animal models indicate that inflammatory mediators such as tumour necrosis factor α (TNF α) and interleukin (IL)1 trigger MP release.⁸⁻¹⁰ Several other observations, however, suggest a more active role for MPs in development of rheumatoid inflammatory activity. Leucocyte-derived MPs, present in synovial fluid of patients with RA, expose tissue factor and trigger coagulation,¹¹ and induce the production and release of chemokines and cytokines by fibroblast-like synoviocytes, which in turn may further contribute to synovial inflammation and angiogenesis.¹² Circulating platelet-derived MPs (PMPs) were reported to be elevated in patients with RA, compared to controls, and these PMPs were associated with disease activity as measured by the Disease Activity Score in 28 joints (DAS28).³ Finally, synovial MPs may also be involved in complement activation in patients with RA, since we recently demonstrated the presence of bound complement components C1q, C3 and C4 as well as complement activator molecules on circulating MPs from patients with RA, further supporting their role in complement activation.⁴

To determine whether circulating MP numbers are associated with inflammatory activity in patients with RA, we compared MPs in patients with very early as yet untreated arthritis and healthy controls. Additionally, we determined the effects of changes in disease activity upon intense anti-inflammatory therapy with the 'COmBination therapy in Rheumatoid Arthritis' (COBRA) strategy¹³ on MP numbers and composition.

METHODS

Patients

Consecutive untreated patients with RA (n=24) were included and venous blood was collected at baseline in the fasting state. Of these patients, nine were enrolled in a trial addressing the effects of tight control and intensified COBRA combination treatment in early RA and were treated with COBRA treatment comprising sulfasalazine, methotrexate and high-dose step-down prednisolone at 60 mg/day (week 1), 40 mg/day (week 2),

30 mg/day (week 3), 20 mg/day (week 4), 15 mg/day (week 5), 10 mg/day (week 6) and 7.5 mg/day thereafter.¹⁴ Criteria for inclusion in that study were active disease defined by a DAS28 score >3.2. Of these nine patients, an additional fasting blood sample was collected after 8 weeks of treatment. Furthermore, fasting blood was collected from healthy age-matched controls (n=15). All patients fulfilled the criteria of the American College of Rheumatology (ACR) for RA.¹⁵ All participants gave written informed consent and the study protocol was approved by the Institutional Ethics Committee of the Slotervaart Hospital, Jan van Breemen Institute and BovenIJ Hospital.

Collection of blood samples

Participants were asked to refrain from beverages other than water (particularly no caffeine-containing beverages or alcohol), smoking, medication and meals from midnight prior to the testing day. Blood was collected from the antecubital vein in tubes containing 0.5 ml of 3.2% sodium citrate (BD, San Jose, California, USA). Cells were removed by centrifugation (20 min at 1550 g and 20°C) within 10 min after collection. Aliquots of cell-free plasma (250 µl) were snap frozen in liquid nitrogen for at least 15 min and stored at -80°C.¹⁶

Isolation of MPs

MPs were isolated from plasma aliquots (250 µl) after thawing on melting ice by centrifugation (30 min at 18 890 g and 20°C). After centrifugation, MP-free supernatant (225 µl) was removed. The remaining MP pellet was washed with 225 µl phosphate-buffered saline (PBS) containing (0.32% w/v) trisodium citrate (pH 7.4). After centrifugation, the supernatant was removed and the MP pellet was resuspended in PBS-citrate (75 µl).

Labelling of MPs

Aliquots of MPs (5 µl) were diluted in 35 µl of PBS containing 2.5 mmol/litre CaCl₂ (PBS/Ca, pH 7.4). Subsequently, 5 µl allophycocyanin (APC)-labelled annexin V (Caltag Laboratories, Carlsbad, California, USA) was added and combined with either fluorescein isothiocyanate (FITC)-labelled CD61 (DakoCytomation, Glostrup, Denmark) plus phycoerythrin (PE)-labelled CD62p (P-selectin) or CD63 (glycoprotein 55; both antibodies from Immunotech, Fullerton, California, USA), or FITC-labelled CD144 (Alexis, San Diego, California, USA) plus E-selectin (CD62e-PE; Ancell, Bayport, Minnesota, USA). For appropriate settings of fluorescence thresholds, MPs were incubated with isotype-matched control antibodies, that is, PE-labelled IgG₁ and/or FITC-labelled IgG₁ (BD), or FITC-labelled Ig (IQP, Groningen, The Netherlands). MPs were labelled for 15 min at room temperature, and labelling was stopped by addition of PBS/calcium (900 µl) to each tube. Samples were analysed for 1 min by fluorescence-activated cell sorting (FACS) on a FACS Calibur device (BD) and data were analysed using Cellquest Pro (V.4.0.2; BD).¹⁷

Alternatively, MPs (5 µl aliquots) were incubated for 30 min at room temperature with anti-C1q, anti-C3-15, anti-C reactive protein (CRP) 5G4, anti-serum amyloid-P (SAP)-14, anti-IgM, anti-IgG (gift from Sanquin, Amsterdam, The Netherlands) or isotype-matched control antibodies IgG₁ and IgG_{2a} (Pharmacia, Montlingen, Switzerland) in a final volume of 50 µl of PBS containing 2.5 mmol/litre CaCl₂ (PBS/Ca, pH 7.4). After labelling, MPs were washed with PBS/calcium (200 µl). Subsequently, PE-labelled F(ab')₂ and APC-labelled annexin V were added and the mixtures were incubated for 30 min at room temperature. For setting of fluorescence thresholds, MPs were incubated

with isotype-matched control antibodies, that is, PE-labelled IgG₁ and/or FITC-labelled IgG₁ (BD), or FITC-labelled Ig (IQP). Finally, PBS/calcium (400 µl) was added to each tube and samples were analysed for 1 min on a FACS Calibur device. Data were analysed using Cellquest Pro.⁶ All antibodies and control antibodies used were tested and titrated using purified cells and MPs before use.

Identification and characterisation of MPs

MPs were defined according to size (forward scatter), side scatter and binding of annexin V, a protein that binds with high affinity and specificity to phosphatidylserine, as described previously.¹⁸ It should be mentioned, that the percentage of MPs binding annexin V increases by centrifugation and freeze-thawing. Under these conditions binding of annexin should be considered as a marker to identify MPs rather than reflecting the exposure of negatively charged phospholipids such as phosphatidylserine. The within-run coefficient of variation (CV) is 8% and the day-to-day CV is 13%. The presence of bound complement components (C1q, C3 and C4) as well as bound adapter molecules (CRP, SAP, IgM and IgG) was studied using flow cytometry as described previously.⁴

Statistical analysis

Data were analysed with SPSS for Windows V.16.0 (SPSS, Chicago, Illinois, USA). According to their distribution, the various parameters are expressed as mean (±SD) or median (interquartile range). Data with a non-Gaussian distribution was log transformed for analysis if possible. To compare the groups, Student t tests or Mann-Whitney U tests were used when appropriate. Furthermore, correlations between variables were analysed by using Pearson correlation or Spearman rho tests. Univariate linear regression analyses were performed on log-transformed data to investigate the influence of possible confounders (ie, sex, smoking status, systolic blood pressure and body mass index (BMI) on the results). The Wilcoxon signed-rank test was used to investigate the differences in values at baseline and at 8 weeks in the prospectively followed subgroup of patients (n=9). p Values less than 0.05 were considered statistically significant.

RESULTS

Characteristics and inflammatory measures

Baseline demographic and clinical characteristics of the patients with RA are summarised in table 1. The majority of patients with RA were IgM-rheumatoid factor and/or anti-citrullinated protein antibody (ACPA) positive. Their DAS28 scores (mean 5.2) reflect patients with a high disease activity. Erythrocyte sedimentation rate (ESR) and CRP levels were significantly elevated in patients compared to controls. The patient group comprised fewer women and had higher systolic blood pressure than the controls. The nine patients prospectively followed had similar ages, BMI levels, systolic blood pressure results and DAS28 scores, but higher CRP (p=0.04) and ESR (p=0.06) levels compared to the other patients with RA (n=15; see tables 1 and 3).

Elevated concentrations of MPs exposing complement components or activator molecules in early RA

The total number of MPs did not differ between patients and controls (table 2). In patients, the number of MPs exposing C1q, CRP and SAP were significantly elevated compared to controls (table 2 and figure 1). These results remained unchanged after adjusting for possible confounders (data not shown).

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Table 1 Baseline characteristics of the patients with rheumatoid arthritis (RA) (n=24) and healthy controls (n=15)

	Controls (n=15)	Patients (n=24)	p Value
Age, years	49±11	51±11	0.91
Female, n (%)	13 (87)	14 (58)	0.02
RF positive, n (%)	NA	16 (67)	NA
Anti-CCP, n (%)	NA	17 (71)	NA
DAS28	NA	5.2±1.3	NA
ESR	5 (3–8)	33 (15–48)	<0.001
CRP	1 (1–2)	13 (3–37)	<0.001
NSAID use, n (%)	NA	17 (77%)	NA
Smoking, %	7	30	0.13
Systolic blood pressure, mm Hg	119±7.8	131±22.8	0.03
Diastolic blood pressure, mm Hg	79±5.9	79±10.8	0.93
BMI	23.4±2.0	25.1±4.3	0.15

Values are presented as mean±SD or median (interquartile range), as applicable. Anti-CCP, anti-cyclic citrullinated peptide; BMI, body mass index (weight in kg/length in m²); CRP, C reactive protein; DAS28, Disease Activity Score in 28 joints; ESR, erythrocyte sedimentation rate; NA, not applicable; NSAID, non-steroidal anti-inflammatory drugs; RF, rheumatoid factor.

Table 2 Complement component and activator molecule exposing MPs in patients and controls

MPs and complement components	Controls (n=15)	Patients (n=24)	p Value
Total MPs	103.6 (64.5–129.9)	108.9 (70.4–185.0)	0.27
C1q	4.7 (1.7–8.8)	9.4 (5.5–14.2)	0.003
C4	11.6 (7.0–15.8)	17.8 (7.6–25.0)	0.35
C3	11.3 (7.7–15.0)	9.1 (2.5–19.5)	0.34
CRP	3.0 (1.3–4.6)	7.3 (2.5–19.5)	0.002
SAP	47.9 (22.9–59.5)	95.3 (62.0–155.6)	0.003
IgM	18.4 (11.5–34.2)	24.4 (14.4–42.3)	0.43
IgG	3.6 (1.0–6.6)	3.3 (2.0–6.5)	0.64

Values are presented as median (IQR). p Values were calculated using the Mann-Whitney U test.

CRP, C reactive protein; MPs, microparticles (numbers×10⁴/ml); SAP, serum amyloid-P.

At baseline, ESR and CRP significantly correlated with MPs exposing C1q, CRP and SAP (for ESR: $r=0.37$, $p=0.02$; $r=0.54$, $p<0.001$ and $r=0.46$, $p=0.003$, respectively and for CRP: $r=0.39$, $p=0.02$; $r=0.52$, $p=0.001$ and $r=0.36$, $p=0.02$, respectively), confirming the association between ongoing inflammation and circulating MPs.

Intense inflammatory suppression does not alter MP composition

Upon treatment with intense anti-inflammatory therapy, DAS28, ESR and CRP values decreased significantly (table 3), but the concentrations of total circulating MPs and MPs exposing complement components or activator molecules were unaffected (table 3, figure 2). Numbers of MPs exposing C1q or CRP were still significantly elevated in the patients with RA after treatment compared to controls (data not shown). At 8 weeks we did not find correlations between DAS28 and CRP with total MP numbers or MPs exposing complement components or activator molecules.

DISCUSSION

This study demonstrates that MPs exposing complement components (C1q) or activator molecules (CRP or SAP) are elevated in early active RA. Although a strong anti-inflammatory therapy using a combination of disease-modifying anti-rheumatic drugs

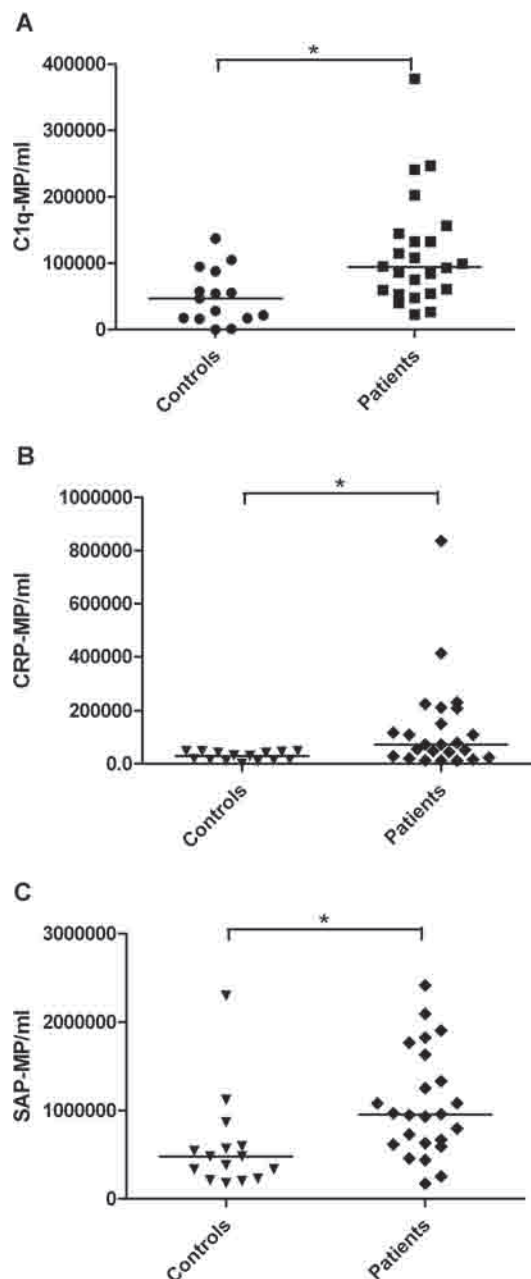


Figure 1 Concentration of circulating microparticles (MPs) exposing (A) C1q, (B) C reactive protein (CRP) or (C) serum amyloid-P (SAP) in plasma of patients with rheumatoid arthritis (RA) and healthy individuals. Individual values are shown, with the horizontal lines representing the median. Differences were analysed with the Mann-Whitney U test. * $p<0.001$.

(DMARDs) combined with high-dose prednisolone (under the COBRA scheme¹⁴) strongly suppressed inflammatory activity, circulating MPs were unaffected. Our present data may suggest that inflammation is not the underlying cause of MP generation in these patients.

Imaging studies have shown that synovitis is still apparent in the majority of patients with RA that are clinically in remission, indicating subclinical ongoing inflammation.¹⁹ The present findings suggest that MPs may be one of the factors that are actively involved in this sustained inflammation in RA. We cannot exclude, however, that a delay exists between normalisation of systemic inflammation and circulating MPs or their

Table 3 Inflammatory markers, complement component and activator molecule exposing MPs in a subgroup of patients treated with anti-inflammatory drugs at baseline and after 8 weeks

Disease scores and MPs	Baseline (n=9)	Week 8	p Value
DAS28	5.2 ± 0.7	2.2 ± 1.2	0.008
ESR	45 (17–62)	12 (6–27)	0.008
CRP	19 (9–69)	4 (1–8)	0.008
Total MPs	167 (73–239)	106.7 (36.9–266)	0.401
MPs+C1q	14.4 (8.4–24.4)	13.4 (4.2–39.8)	0.889
MPs+C4	22.2 (5.9–27.9)	19.1 (7.8–58.3)	1.0
MPs+C3	10.3 (4.1–21.4)	12.3 (1.9–37.8)	1.0
MPs+CRP	7.9 (3.4–31.2)	7.1 (2.9–28.1)	0.575
MPs+SAP	108.2 (53.4–172.8)	109.3 (29.7–214)	0.779
MPs+IgM	31.6 (13.7–41.4)	34.1 (15.1–63.1)	0.401
MPs+IgG	2.3 (0.3–6.0)	1.2 (0.0–3.2)	0.674

Values are median (IQR). p Values were calculated using the Wilcoxon signed-rank test. After total MPs, the other categories refer to the number of MPs exposing a certain complement component or activator molecules. CRP, C reactive protein; DAS28, disease activity score of 28 joints; ESR, erythrocyte sedimentation rate; MPs, microparticles (numbers × 10⁴/ml); SAP, serum amyloid-P.

composition. Alternatively, subpopulations of circulating MPs may be affected by normalisation of inflammation rather than the total population of MPs, as assessed in the present study. The biological relevance of MPs or subpopulations thereof to the pathology of early active RA may be questioned given the fact that inflammation and disease activity were both efficiently suppressed.

Recently, MPs have emerged as a new proinflammatory mediator. In fact, they are thought to amplify or disseminate inflammation. MPs are thought to trigger inflammation by several processes such as activation of endothelial cells and leucocytes, triggering production and release of chemokines and cytokines and by activating the complement cascade, which is thought to play a key role in the pathogenesis of RA.^{4 12 20–24}

By contrast, inflammation may trigger MP formation. For instance, in vitro studies showed that MPs are released from cells incubated with TNF α or IL1 and a study in mice showed that the number of PMPs in plasma markedly increased upon injection with TNF α .^{9–10} Data from the present study, however, implicate that MPs remain associated with complement activation in early RA despite aggressive anti-inflammatory therapy.

We cannot answer the question yet as to whether these MPs really have proinflammatory properties and thus actively contribute to complement activation, or whether they merely reflect ongoing complement activation. In the 1980s, Sims and coworkers demonstrated that cells were protected from complement-induced lysis by the release of complement complex-enriched MPs.²⁵ Thus, the presence of elevated concentrations of complement-enriched MPs in early RA may also be a reflection of ongoing and uncontrolled activation of the complement system.

Our main finding that powerful inhibition of inflammation did reduce disease activity but did not disturb the association between circulating MPs and complement activation, suggests that MPs and complement contribute to the development of and/or the chronic character of inflammatory diseases such as RA.

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Competing interests None.

Ethics approval This study was conducted with the approval of the Institutional Ethics Committee of the Slotervaart Hospital, Jan van Breemen Institute and BovenIJ Hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

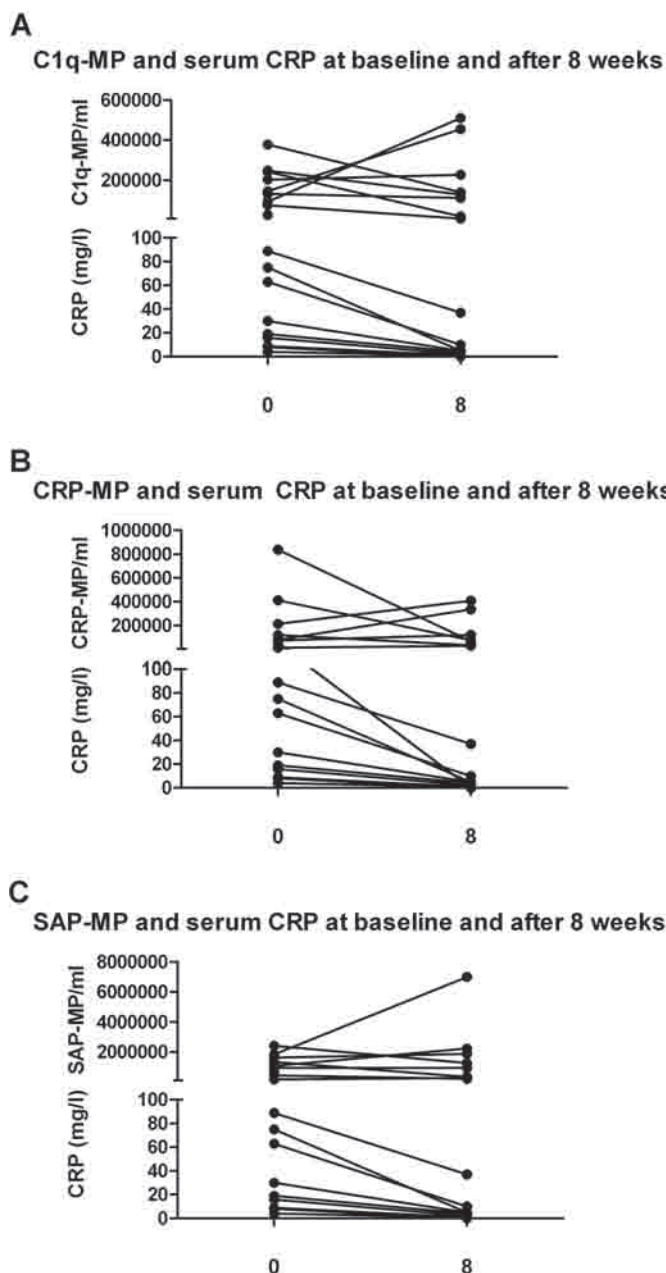


Figure 2 Depicted are the values for serum C reactive protein (CRP) and concentrations of microparticles exposing (A) C1q, (B) CRP or (C) serum amyloid-P (SAP) in plasma of patients with rheumatoid arthritis (RA) (n=9) at baseline and after 8 weeks of 'COmBination therapy in Rheumatoid Arthritis' (COBRA) treatment. Lines connect individual values at both time points.

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