Microparticle subpopulations are potential markers of disease progression and vascular dysfunction across a spectrum of connective tissue disease


Objective: Microparticles (MPs) are membrane-bound vesicles derived from vascular and intravascular cells such as endothelial cells (EMPs) and platelets (PMPs). We investigated EMP and PMP numbers across a spectrum of autoimmune rheumatic diseases (AIRDs) with the aim of comparing the levels of, and relationship between, EMPs and PMPs.

Methods: Patients with Systemic Lupus Erythematosus (SLE) (n = 24), Systemic Sclerosis (SSc) (n = 24), Primary Raynauds Phenomenon (RP) (n = 17) and “other CTD” (n = 15) were recruited. EMPs and PMPs were quantified using flow cytometry. Associations between MP levels and objective functional vascular assessments were evaluated.

Results: SLE patients had significantly higher EMPs and PMPs than healthy controls and SSc patients. Higher PMP levels were noted in SSc and primary RP when compared to healthy controls and ‘other CTD’ patients. A modest correlation was noted between EMP and PMP levels in healthy controls (Spearman r = 0.6, p = 0.017). This relationship appeared stronger in SLE (r = 0.72, p < 0.0001) and other CTD patients (r = 0.75, p < 0.0001). The association between EMPs and PMPs was notably less strong in SSc (r = 0.37, p = 0.15). A significantly lower EMP/PMP ratio was detected in SSc/RP patients in comparison to both healthy controls and SLE/other CTD patients. Higher EMP and PMP levels were associated with higher digital perfusion following cold challenge in SSc. In contrast, higher PMP (but not EMP) levels were associated with lower digital perfusion at both baseline and following cold challenge in primary RP. Higher PMP levels were associated with greater endothelial-independent dilation in patients with SLE.

Conclusion: MP populations differ across the spectrum of AIRDs, possibly reflecting differences in vascular cell injury and activation. MP levels are associated with functional assessments of vascular function and might have a role as novel vascular biomarkers in AIRDs.

Significance and innovations: Levels of circulating endothelial and platelet microparticles differ between SSc/primary RP compared with SLE and other CTDs (UCTD, MCTD and Primary Sjogrens). MP release may occur within different vascular sites across these disease groups (macrovacular and microvascular).

The association between circulating MP levels and objective assessment of macro- and microvascular dysfunction within these disease areas suggests that MPs might have a useful role as novel circulating biomarkers of vascular disease within the CTDs.

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1. Introduction

Microparticles (MPs) are membrane-bound vesicles that display biological activities which impact on normal physiology as well as the pathogenesis of immune-mediated diseases [1]. MPs arise from cells undergoing activation or apoptosis, detaching from the membrane as...
formed structures. MPs are considered important mediators of cellular cross-talk in the regulation of inflammation, vascular function, cell apoptosis and proliferation. Thus MPs might contribute to the immunopathogenesis of various diseases via their role in the regulation of inflammation, thrombosis and angiogenesis [2]. High MP levels have been reported in diseases associated with vascular damage, such as atherosclerosis and coronary artery disease, whilst low levels have been observed in sepsis. This suggests diverse effects of MPs across a spectrum of disease, which may be in part, dependent on the disease and/or stimulus they encounter [3,4]. MP phenotype has also been demonstrated to be transformed depending on the underlying disease and patient population. For example in chronic heart failure (CHF) body mass index has been demonstrated to be a significant predictor for impaired phenotype of circulating endothelial microparticles (EMPs) in patients [5,6]. Similarly development of CHF in metabolic syndrome patients may be closely related to altered balance between activated endothelial cell-derived MPs and apoptotic endothelial cell-derived MPs. This phenomenon of impaired phenotype of circulating MPs has been suggested as a precursor to both clinically significant endothelial dysfunction and resultant CHF [5,6]. Studies in patients with a wide variety of rheumatic diseases have generally reported elevation in MP numbers compared with healthy control populations. These elevations are most notable in conditions with a strong vascular component and primarily involve platelet MPs (PMPs), with elevations of EMPs having also been documented [7,9].

The pathological hallmark of Systemic Sclerosis (SSc) is microvascular injury, leading to ischaemia and tissue remodelling (fibrosis). Endothelial cell activation is one of the potential pathological mechanisms in SSc [10]. Platelets have long been considered a potentially important cell mediator in the pathogenesis of SSc and release a large number of vasoactive mediators (e.g. thromboxane, serotonin) and growth factors (TGF-β, PDGF) that might contribute to tissue ischaemia and remodelling fibrosis [11]. However, the precursor molecular mechanisms underlying disease remain ill-defined and the contribution of EMPs and PMPs to disease remains unclear. Previous studies describing MP levels in SSc have reported conflicting results [7,12].

Systemic Lupus Erythematosus (SLE) is associated with features of macrovascular disease such as atherosclerosis, attributable in part, to chronic systemic inflammation. As with SSc, the function of MPs derived from specific tissues or cells in SLE remains unclear. Most studies have found elevated numbers of circulating MPs in SLE patients [13,14]. However, others have reported either unchanged or lower levels of total and PMPs [9]. Previously we have demonstrated that EMP levels are related to measures of vascular damage and endothelial dysfunction in patients with active SLE [15]. Few studies have evaluated EMP and PMP levels across a spectrum of connective tissue diseases. Such data may help delineate their contribution to disease pathogenesis in addition to providing an attractive circulating biomarker of vascular injury and disease activity. The principal objectives of this study were to evaluate EMP and PMP levels across a spectrum of connective tissue diseases, with known varying degrees of macrovascular and microvascular involvement. Where possible, we have explored and shall report associations between EMP and PMP levels with objective assessment of macro- and microvascular function.

2. Patients and methods

Patients with SLE were recruited from The Kellgren Centre for Rheumatology, Central Manchester NHS Foundation Trust (CMFT) and East Lancashire Hospitals NHS Trust. Although originally recruited for a complementary study, an additional cohort of patients, defined within the manuscript as “other CTD” were also recruited from CMFT. Other CTD is defined as: Primary Sjogrens Syndrome, Undifferentiated Connective Disease or Mixed Connective tissue disease (without features of SSC/SLE). Healthy controls were recruited from The University of Manchester and CMFT. Patients aged between 18 and 75 years with SSC or primary RP were approached for inclusion in the study from the Department of Rheumatology, Royal National Hospital for Rheumatic Diseases, Bath. Patients with SSc fulfilled either the American Rheumatology Association (ARA) and/or the LeRoy and Medsger classification criteria for early SSc. Primary RP was defined as at least 2 episodes of fingertip localized notable blue and/or sequential white/blue discoloration, in conjunction with pain upon cold exposure or emotional stress within one week of examination and anti-nuclear autoantibody titre ≥ 1/160 on HEp-2 cell immunofluorescence. We excluded subjects with a recent acute infection (<1 month), recent cardiovascular event (<3 months), any chronic infection, pregnancy and patients with chronic kidney disease (Fig. 1). In addition SSc/RP patients were excluded from the study if they were taking an endothelial receptor antagonist or anticoagulant.

All participants provided informed written consent and the study received prior approval from the relevant institutional ethics review boards.

2.1. Clinical assessment

Participants underwent history and exam at the time of study visit. Clinical features of disease and medication use, were recorded for all patients. All subjects were asked to abstain from smoking on the day of study.

2.2. Additional vascular measures

Flow mediated dilatation (FMD) of the brachial artery was performed using B mode ultrasound and automated edge-tracking software in SLE patients only [15]. Peripheral microvascular perfusion was evaluated only in SSc and primary RP by measuring digital perfusion at the volar aspect of the distal left middle finger using Laser Speckled Contrast Imaging (LSCI). Digital perfusion was assessed at baseline (B), immediately following (t0) and 15 min after reperfusion (t5, t10 and t15 respectively) as previously reported [16].

2.3. Assessment of endothelial and platelet microparticle levels

2.3.1. Sample collection

Peripheral venous blood was collected from the study participants in a 5 ml citrated vacutainer. Platelet-poor plasma (PPP) was generated using a two-step centrifugation process. The venous sample was initially centrifuged at 1700g for 10 min at 4 °C to generate plasma which was harvested and centrifuged at 20,000g for a further 10 min at 4 °C. The PPP was harvested and the final sample frozen in 100 μl aliquots at −80 °C until analysis at a central site.

2.3.2. Flow cytometry

Sizing beads of 0.16 μM, 0.2 μM, 0.24 μM and 0.5 μM (Megamix-Plus SSC, Stago, Marseille) were used to prepare the assay, with the threshold being set on SSC as recommended by the manufacturer, for maximum accuracy to detect MPs in the size range of 0.1 μM–1 μM. Following appropriate control staining and compensation adjustment EMPs and PMPs were enumerated. We added 50 μl of PPP and 50 μl of 10 μM diameter counting beads (Flow Count Fluospheres; Beckman Coulter, UK), used to count a standard number of events, to 900 μl of calcium-rich buffer (Annexin V Apoptosis Detection Kit, BD Pharmingen, UK 556547). Simultaneous incubation for 15 min with fluorescent antibodies was performed using 2.5 μl of phycoerythrin (PE)-conjugated antihuman CD31 (BD Pharmingen 55546), 2.5 μl of allophycocyanin (APC)-conjugated anti-human CD42b (BD Pharmingen 551061) and 5 μl of fluorescein isothiocyanate (FITC)-conjugated anti-human annexin-V marker (BD Pharmingen 51-65874X). Flow cytometry was performed on prepared samples using a FACsVerse flow cytometer (BD Biosciences). Analysis was stopped once 1000 counting beads had been
counted and gates were set to exclude artefact and beads. Absolute EMP and PMP counts per milliliter of plasma were then calculated. The formula for calculation of the MP levels is as follows:

\[ V = \frac{z}{x/y} \]

where \( z \) = total volume of sample (1000 μl); \( x \) = total number beads added (50 × number of beads per μl); \( y \) = number of beads counted (1000); and 20 is the dilution factor. Once the volume of plasma analysed is known, the total number of dual positive events (Annexin V+/CD31+/CD42b−) is multiplied by (1000/v) to generate a number of EMPs (or PMPs) per 1000 μl of plasma. Events positive for Annexin V (microparticle marker) and CD31 (endothelial marker) and negative for CD42b (platelet-marker) (Annexin V+/CD31+/CD42b−) were defined as EMPs. PMPs were defined as the sum of Annexin V+/CD31+ or Annexin V+/CD31−/CD42b+ events.

### 2.4. Statistical analysis

Significance of between group differences was assessed using the Mann-Whitney U test for continuous variables and chi-squared test for categorical variables. Non-parametric data is presented as median (Interquartile Range (IQR)). Correlation between measures was

| Table 1 Baseline demographics, disease characteristics and medication use in each group. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Control | SLE | Systemic sclerosis | Primary Raynauds | Other CTD |
| No. of participants | 15 | 24 | 24 | 17 | 15 |
| Gender | | | | | |
| Female | 14 (93.3) | 23 (95.8) | 19 (79.1) | 13 (76.5) | 15 (100) |
| Male | 1 (6.7) | 1 (4.2) | 5 (20.8) | 4 (23.5) | 0 |
| Age (years), mean (SD) | 40.7 (10.7) | 43.8 (13.1) | 60.5 (8.9) | 50.4 (14.1) | 45.1 (12.5) |
| Disease duration | 8.68 (7.3) | 6.86 (6.6) | 26.4 (16.36) | | |
| Smoker | 14 (93.3) | 21 (87.5) | 21 (87.5) | 16 (94.1) | 14 (93.3) |
| No | 1 (6.7) | 3 (12.5) | 3 (12.5) | 1 (5.9) | 1 (6.7) |
| Medication use | | | | | |
| Oral corticosteroid | 23 (95.8) | 5 (20.8) | | | 8 (52.3) |
| Anti-malarial | 17 (70.8) | 0 (0) | | | 3 (20) |
| Additional imm. | 11 (45.8) | 6 (25) | | | |
| Anti-platelet | 7 (29.1) | 5 (20.8) | | | |
| Disease features | | | | | |
| ACR criteria | Malar 16 (66.7) | DD or DP 12 (50) | | | |
| Sonography Ulcer 16 (66.7) | Ulcer 5 (20.8) | PAH 2 (8.3) | | | |
| Arthritis 19 (79.2) | Telangiectasia 18 (75) | | | | |
| Photosensitivity 18 (75) | Calciosis 9 (37.5) | | | | |
| Raynauds 14 (58.3) | Raynauds 24 (100) | Raynauds 17 (100) | Raynauds 5 (33.3) | |

Unless otherwise stated, values are n (%). UCTD = Undifferentiated Connective Tissue Disease. MCTD = Mixed Connective Tissue Disease. ACR = American College of Rheumatology. CNS = Central Nervous System. Additional Immun. = Additional Immunosuppressive therapy. DU = Digital Ulcer, DP = Digital Pitting, ILD = Interstitial Lung Disease. PAH = Pulmonary Arterial Hypertension.
assessed using Spearman’s correlation coefficient. Data analyses were performed using the Prism and SPSS 21.0 software packages.

3. Results

3.1. Patient Populations

Twenty-four patients with SLE and SSc, seventeen with primary RP, fifteen with ‘other CTD’ and fifteen healthy controls were enrolled. Baseline characteristics of cases and controls are described in Table 1. Patients with SSc were significantly older than patients with SLE, other CTD and healthy controls ($p < 0.001$). No differences in terms of age were observed between healthy controls, SLE, RP or other CTD patients.

Corticosteroids, antimalarials and additional immunosuppressive therapies were used more frequently in the SLE group when compared to the other groups. The frequency of specific disease features in each of the cohorts is recorded in Table 1.

3.2. Relationship between microparticle levels and disease

3.2.1. Elevated levels of EMPs in SLE

Patients with SLE had significantly higher EMP levels compared with healthy controls [median (IQR) 293,889/ml (118,024, 797,734) vs 246,758/ml (161,822, 339,580), $p = 0.04$]. No other differences were observed in EMP levels across the patient groups investigated (Fig. 2A).

3.2.2. PMPs are elevated in SSc and primary RP

Similarly, higher PMP levels were noted in the primary RP compared to healthy controls [median (IQR) 58,178/ml (16,528, 142,296), $p < 0.001$]. No additional disease associations between MP levels and disease characteristics were identified in SSc patients.

3.2.3. The association of microparticles with disease-related features

SLE patients with active serositis had higher EMP levels than those without serositis [median (IQR) 808,839/ml (310,933, 1,210,000) vs 225,194/ml (100,000, 416,816), $p = 0.04$]. No difference in EMP or PMP numbers were observed for disease manifestations explored within the “other CTD” group. SSc patients with a history of digital ulceration/pitting (n = 12) had higher EMP levels [198,643/ml vs 144,840/ml, $p = 0.03$]. No additional disease associations between MP levels and disease characteristics were identified in SSc patients.

3.2.4. The EMP/PMP ratio distinguishes Primary Raynauds phenomenon and Systemic Sclerosis from healthy controls, SLE and other CTD patients

Next, we sought to investigate the relationship between EMPs and PMPs. A modest correlation was noted between EMP and PMP levels in health (Spearman $r = 0.6$, $p = 0.017$). The relationship between EMPs and PMPs was stronger in SLE ($r = 0.72$, $p < 0.0001$) and other CTD patients ($r = 0.75$, $p < 0.0001$). In contrast, the association between EMPs and PMPs was less strong in SSc ($r = 0.45$, $p = 0.014$) and primary RP ($r = 0.37$, $p = 0.15$).

When the EMP/PMP ratio was assessed across all groups, we noted that the ratio of EMPs:PMPs was 2.83 in healthy controls, which was comparable to the SLE and other CTD groups (2.83 vs 2.24 vs 3.4, $p = 0.55$). A significantly lower EMP/PMP ratio was seen in both primary RP and SSc patients, in comparison to both healthy controls and SLE/other CTD patients (RP = 0.64, SSc = 0.88, $p < 0.01$ vs healthy controls) (Fig. 3).

3.3. Association between MP levels and objective assessments of vascular function

3.3.1. Relationship between MP levels and measures of large vessel vascular function in SLE

We have previously reported on the relationship between endothelial function as assessed by FMD and EMPs in SLE patients [15]. Therefore, we sought to extend these studies and investigate the relationship between PMP levels and endothelial function in the same cohort of SLE patients. Baseline PMP levels showed a moderate correlation with endothelium-
3.3.2. Relationship between MP levels and digital microvascular response to cold challenge assessed using LSCI in patients with primary RP and SSc

Next, we sought to investigate the relationship of EMPs and PMPs with measures of digital perfusion in both SSc and primary RP. Consistent positive correlations between both EMP and PMP levels and digital perfusion following cold challenge (but not at baseline) were identified in patients with SSc (Table 2). These findings suggest that higher MP levels are associated with better digital perfusion. In contrast, there were consistent negative correlations between PMP (but not EMP) levels and digital perfusion at both baseline and following cold challenge in primary RP suggesting that the production of PMPs was associated with lower digital perfusion.

4. Discussion

In this study, we report different levels of EMPs and PMPs across a spectrum of AIRDs: SLE patients have higher levels of EMPs than healthy controls and SSc patients. In contrast, both SSc and primary RP are associated with elevated PMP levels. The EMP/PMP ratio appears to distinguish patients with SSC/primary RP from those with SLE, other CTD and healthy controls. In addition, we have identified that measurement of EMPs and PMPs reflects vascular function/perfusion across disease, suggesting that MPs may have a role as circulating markers of vascular health.

Table 2
Correlation of EMPs and PMPs with cold challenge and reperfusion in Systemic Sclerosis and Primary Raynaud's Phenomenon patients.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>t 0</th>
<th>t 5</th>
<th>t 10</th>
<th>t 15</th>
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<td>SSc EMPs</td>
<td>Spearman r</td>
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<td></td>
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<tr>
<td>PMPs EMPs</td>
<td>Spearman r</td>
<td>0.19</td>
<td>0.42</td>
<td>0.52</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
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<td>0.05</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Primary RP EMPs</td>
<td>Spearman r</td>
<td>−0.34</td>
<td>−0.33</td>
<td>−0.13</td>
<td>−0.39</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.15</td>
<td>0.2</td>
<td>0.65</td>
<td>0.13</td>
</tr>
<tr>
<td>PMPs EMPs</td>
<td>Spearman r</td>
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<td>−0.33</td>
<td>−0.69</td>
<td>−0.65</td>
</tr>
<tr>
<td></td>
<td>p value</td>
<td>0.04</td>
<td>0.23</td>
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</table>

Small vessel vascular perfusion in SSc and primary RP patients was assessed by digital perfusion at the volar aspect of the distal left middle finger using Laser Speckle Contrast Imaging (LSCI) during a standardised local cold challenge (15 °C for 60 s). Digital perfusion was assessed at baseline, immediately following cold challenge (t0) and at 5 min intervals during reperfusion (t5, t10 and t15 respectively).
vascular involvement across CTD. Future studies should evaluate the sensitivity to change of MPs as biomarkers following therapeutic intervention (e.g. immunosuppression, vaso-dilation and anti-platelet therapies) across these disease groups.

4.1. Study limitations

Our study has a number of limitations. Whilst we have used a standard combination of markers to identify EMPs and PMPs (CD31 and CD42b), others have used different combinations. Replication of our results using additional markers would add strength to our findings as would elucidation and inclusion of Annexin V non-binding MPs. Similarly the use of markers that identify both apoptotic and activated MPs would allow for more detailed analysis of the phenotype of MP involved across the AIRDs assessed in this study. In addition, we acknowledge that the patients with SSC recruited, were older than those with SLE, although there was no difference in terms of disease duration. This is in part due to the different natural history of the diseases, with SLE commonly presenting earlier. There appears to be very few, if any studies carried out on the impact of age on MP levels except in subjects younger than 18 years old [24,25]. Finally due to a lack of availability of all assessment instruments across sites SLE patients were the only cohort in the study that had FMD measured whilst microvascular perfusion following cold challenge was measured in SSC and RP patients only.

4.2. Future directions for study

Future work would benefit from including both measures of vascular function in all those recruited to allow more precise interpretation and analysis of the relative contribution of the tissue beds to microparticle function. In addition larger replication studies are required, following patients over time, which also allow for additional regression analysis of the effects of environmental factors such as smoking etc. as well as disease activity and patient characteristics across the diseases. Such a study will also allow for the assessment of alteration in MP numbers and phenotype with change in therapy and improved disease control. Finally, additional functional experiments aimed at addressing the exact role of EMPs and PMPs derived from SLE and SSC patients, as well as the contribution of EMPs and PMPs derived from different vascular bed territories, are clearly required to determine the significance of the clinical observations we have identified.

Our study has found that the levels and relationship between EMPs and PMPs differ across the spectrum of AIRDs, with high PMPs seen in SSC/RP and high EMPs observed in SLE. The low EMP/PMP ratio in SSC/RP, in comparison to SLE in particular, suggests differential regulation of MPs according to the background vascular pathology and vascular bed involved. The relationship between MPs and vascular function also suggests that MPs may be a simple non-invasive biomarker of vascular involvement across CTD and may in the future assist in establishing the degree of vascular disease modulation produced by various therapeutic strategies.

Funding statement

This study was funded by Arthritis Research UK (18845) and the Raynaud’s and Scleroderma Association. This report includes independent research supported by the National Institute for Health Research Manchester Musculoskeletal Biomedical Research Unit. The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health.

Conflict of interest

None of the authors has received any financial support or other benefits from commercial sources for the work reported in this manuscript, nor do any of the authors have any other financial interests which could create a potential conflict of interest, or the appearance thereof.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

References

