

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



E.M. McCarthy^{a,b,c}, D. Moreno-Martinez^c, F.L. Wilkinson^c, N.J. McHugh^{d,e}, I.N. Bruce^{a,b}, J.D. Pauling^{d,e}, M.Y. Alexander^c, B. Parker^{a,b,*}

^a Centre for Musculoskeletal Research, Institute of Inflammation and Repair, Manchester Academic Health Science Centre, The University of Manchester, United Kingdom

^b NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester University Hospital NHS Foundation Trust and Manchester Academic Health Science Centre, Manchester, United Kingdom

^c Healthcare Science Research Institute, Manchester Metropolitan University, Manchester, United Kingdom

^d Department of Rheumatology, Royal National Hospital for Rheumatic Diseases, Bath, United Kingdom

^e Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom

ARTICLE INFO

Article history:

Received 18 August 2016

Received in revised form 5 November 2016

Accepted 7 November 2016

Available online 10 November 2016

Keywords:

Endothelial microparticles

Platelets

Systemic lupus erythematosus

Systemic sclerosis

ABSTRACT

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” ($n = 15$) (Primary Sjogrens Syndrome, UCTD or MCTD) as well as 15 healthy controls 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 compared with 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 ($r = 0.45$, $p = 0.014$) and RP ($r = 0.37$, $p = 0.15$). 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 (macrovascular 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.

© 2016 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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

* Corresponding author at: Centre for Musculoskeletal Research, The University of Manchester, Stopford Building, Oxford Road, Manchester, United Kingdom.
E-mail address: Benjamin.Parker@manchester.ac.uk (B. Parker).

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 $\leq 1/160$ 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) a cold challenge (hands submerged in waterbath at 15 °C for 1 min) and at 5 min intervals during 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 μ l aliquots at -80 °C until analysis at a central site.

2.3.2. Flow cytometry

Sizing beads of 0.16 μ M, 0.2 μ M, 0.24 μ M and 0.5 μ M (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 μ M–1 μ M. Following appropriate control staining and compensation adjustment EMPs and PMPs were enumerated. We added 50 μ l of PPP and 50 μ l of 10 μ m diameter counting beads (Flow Count Fluorospheres; Beckman Coulter, UK), used to count a standard number of events, to 900 μ l 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 μ l of phycoerythrin (PE)-conjugated anti-human CD31 (BD Pharmingen 55546), 2.5 μ l of allophycocyanin (APC)-conjugated anti-human CD42b (BD Pharmingen 551061) and 5 μ l of fluorescein isothiocyanate (FITC)-conjugated anti-human annexin-V marker (BD Pharmingen 51-65874X). Flow cytometry was performed on prepared samples using a FACSVerser flow cytometer (BD Biosciences). Analysis was stopped once 1000 counting beads had been

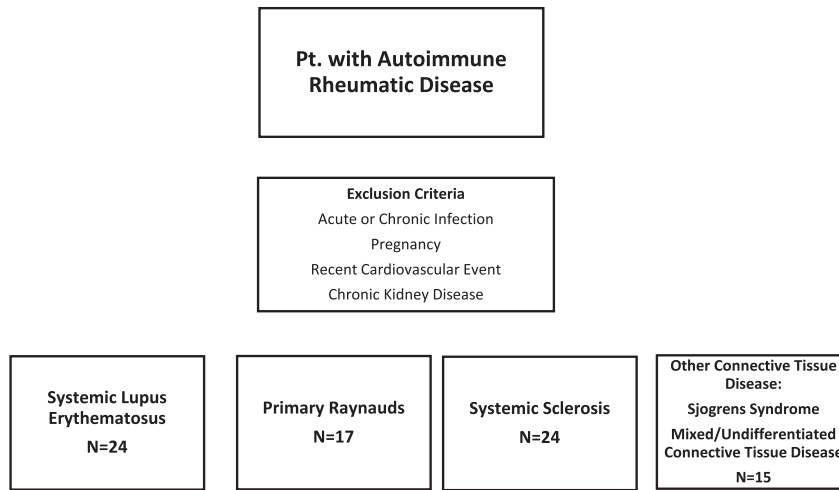


Fig. 1. Flow chart of patient recruitment and exclusion criteria.

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:

$$V = z/(x/y)/20$$

where z = total volume of sample (1000 μ l); x = total number beads added ($50 \times$ number of beads per μ l); 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 μ l 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 +/CD42b + 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 lcSSc = 2 dcSSc = 2	17	15 Primary Sjogrens = 6 (40) UCTD = 5 (33.3) MCTD = 4 (26.7)
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)
Yes					
Medication use					
Oral corticosteroid		23 (95.8)	5 (20.8)		
Anti-malarial		17 (70.8)	0 (0)		8 (52.3)
Additional immun.		11 (45.8)	6 (25)		3 (20)
Anti-platelet		7 (29.1)	5 (20.8)		
Disease features		ACR criteria	Organ involvement		Organ involvement
		Malar 16 (66.7)	DU or DP 12 (50)		Arthritis 9 (60)
		Discoid 12 (50)	ILD 5 (20.8)		Skin 6 (40)
		Ulcer 16 (66.7)	PAH 2 (8.3)		Sicca 5 (33.3)
		Serositis 7 (29.2)	Telangiectasia 18 (75)		
		Arthritis 19 (79.2)	Calcinosis 9 (37.5)		
		Photosensitivity 18 (75)			
		Renal 10 (41.7)			
		CNS 6 (25)			
		Immunological 11 (45.8)			
		Raynauds 14 (58.3)	Raynaud's 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 EMPs compared with healthy controls [median (IQR) 293,889/ml (118,024, 797,734) vs 132,171/ml (55,426, 195,349), $p = 0.03$] and patients with SSc [median (IQR) 293,889/ml (118,024, 797,734) vs 161,271/ml (111,732, 199,346), $p = 0.04$]. 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

Significantly higher PMP levels were noted in SSc (median (IQR) 186,348/ml (103,025, 486,144)) compared with healthy controls (median (IQR) 41,085/ml (25,969, 74,806), $p < 0.001$) and patients with "Other CTD" (median (IQR) 58,178/ml (16,528, 142,296), $p < 0.001$).

Similarly, higher PMP levels were noted in the primary RP compared to healthy controls [median (IQR) 246,758/ml (161,822, 339,580) vs 41,085/ml (25,969, 74,806), $p < 0.001$] and patients with "other CTD"

[median (IQR) 246,758/ml (161,822, 339,580) vs 58,178/ml (16,528, 142,296), $p < 0.001$].

No significant differences were observed in PMP levels between SLE patients and those with either SSc or primary RP; nor was any difference detected between the SSc and primary RP groups (Fig. 2B). SLE patient PMP levels were not higher than healthy control PMP levels [median (IQR) 78,410 (23,246, 395,859) vs 41,085/ml (25,969, 74,806), $p = 0.12$].

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-

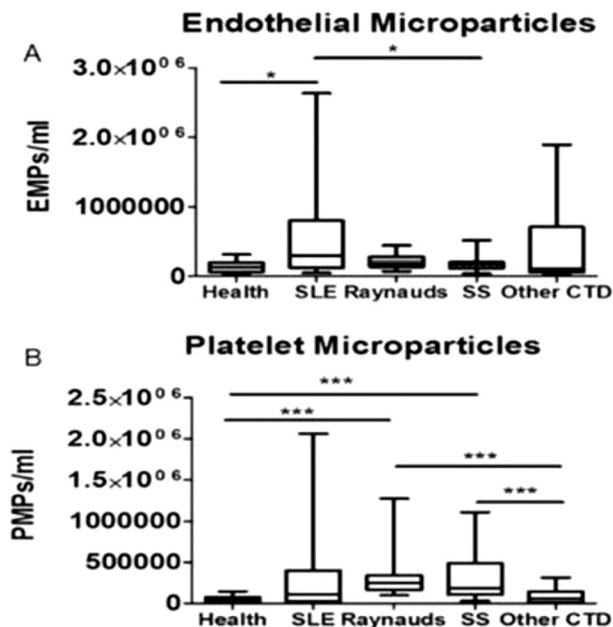


Fig. 2. The levels of EMPs and PMPs in SLE, Primary Raynauds, Systemic Sclerosis and other CTD patients. (A) The number of EMPs/ml (Annexin V +/CD31 +/CD42b- events) and (B) PMPs/ml (Annexin V +/CD31 +/CD42b+ or Annexin V +/CD31 -/CD42b+ events) were assessed by flow cytometry in SLE ($n = 24$), SSc ($n = 24$), Primary Raynauds ($n = 17$), other CTD patients ($n = 15$) as well as healthy controls ($n = 15$). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

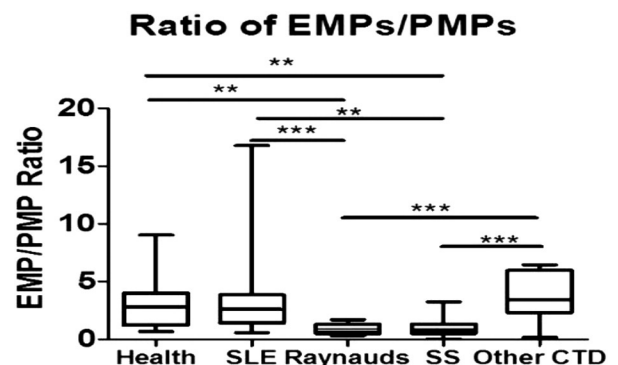


Fig. 3. The EMP/PMP ratio distinguishes SLE from healthy controls, Primary Raynauds, and Systemic Sclerosis. The ratio of EMPs/ml (Annexin V +/CD31 +/CD42b- events) to PMPs/ml (Annexin V +/CD31 +/CD42b+ or Annexin V +/CD31 -/CD42b+ events) was calculated following flow cytometry in SLE ($n = 24$), SSc ($n = 24$), Primary Raynauds ($n = 17$), other CTD patients ($n = 15$) as well as healthy controls ($n = 15$). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

independent vasodilation (Spearman $r = 0.56$, $p = 0.06$), which reflects vascular smooth muscle function. No association was observed between PMP levels and FMD.

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.

Microparticles are increasingly recognised as contributing to the pathogenesis of a wide spectrum of disease, although their precise function remains to be elucidated. Here we report that higher EMP/PMP levels appear to differentiate SLE/other CTD from SSc/primary RP, suggesting that this ratio is a potential novel biomarker to help inform clinicians as to the potential evolution of undifferentiated connective tissue disease. Whilst this may reflect the higher inflammatory burden observed in SLE, the inclusion of an additional other CTD cohort, on minimal immunosuppression, suggests an effect beyond disease activity. Work is currently ongoing within our group aimed at following a cohort of uCTD patients longitudinally to further investigate this observation.

The relationship between EMPs and PMPs appears dysregulated across the diseases assessed. Both SLE and SSc/primary RP are characterised by differing degrees of macrovascular and microvascular complications respectively. Thus differences in the observed numbers of MPs between groups, in particular SLE patients having higher EMP numbers with SSc patients having high PMPs, may reflect the involvement of different vascular beds and/or differential activation of platelets. In addition, factors which affect the health of the vascular bed may contribute to the observed differences. Chronic exposure to oxidative stress in microvascular endothelial beds may result in the gradual exhaustion of antioxidant protective mechanisms and destruction of the tissue bed, thus preventing the release of potentially protective EMPs. In support of this, Bartoloni et al. have recently demonstrated a

reduction in the number of potentially reparative circulating endothelial progenitor cells as disease progresses in primary Sjogrens Syndrome [8]. Similarly EMP numbers were not elevated in our SSc cohort thus leaving potentially harmful PMPs to exert their function unopposed. In addition, high shear stress like that observed in the microvasculature, has been demonstrated to increase PMP release, an effect that is further enhanced with prolonged duration of stress [17]. It is increasingly recognised that EMP function is more nuanced than previously considered, and the micro-environment stimulating EMP release appears to be a key contributor to the functional properties exhibited by EMPs [18]. In particular, the insult or injury that results in EMP release, namely cell apoptosis or activation, results in EMPs having distinctive and differing roles in disease, as highlighted by Jimenez et al. [19]. Proteomic analysis of cell-culture derived EMPs has shown that one third of the proteins found on EMPs are specific to the stimulus initiating their release, not only demonstrating the plasticity of these vesicles but also revealing the complexity of the mechanisms governing their formation [20]. Taken together, these findings suggest that there are distinct mechanisms for the formation of EMPs in apoptotic and activated cells [21], with several studies suggesting that these types of EMPs have different functions in vascular diseases potentially explaining the different EMP/PMP ratios seen across the diseases in our study [22,23]. Thus, differences in vascular territories, conditions that stimulate EMP and PMP release and varying EMP/PMP phenotype may account for some of the differences observed in the EMP/PMP relationship between SLE/other CTD and SSc/RP patients, suggesting that this ratio is a marker of disease evolution and the health of the vasculature bed territories involved in disease.

In support of this, we have also demonstrated that measurement of EMPs and PMPs may reflect the health of a patient's vasculature. PMP levels correlate moderately with endothelium independent vasodilation in SLE, reflecting vascular smooth muscle tone, adding to previous work by our group, which highlighted a relationship between EMPs and endothelial vascular function [15]. Similarly, we have identified that EMPs and PMPs reflect digital perfusion following localized cold challenge in SSc. Of note whilst PMPs were elevated in both SSc and RP patients they were observed to have contrasting relationships with small vessel digital perfusion. Further work is necessary to elucidate the exact function of PMPs in these diseases. Such work would benefit from simultaneous investigation of EMPs given that they were observed to relate directly with perfusion in SSc but not RP suggesting the diverse effects of EMPs between these two conditions. It is possible that the balance between EMPs and PMPs, rather than either MP subpopulation alone, dictates digital perfusion with a rise in EMP numbers post cold challenge potentially leading to improvements in digital perfusion. Thus these results suggest that, not only may EMP and PMP measurement act as a novel biomarker allowing prediction of disease progression, but also that MPs may serve as potential circulating biomarkers of patients' underlying vascular health. Whilst further validation studies are required, the observed relationship between MPs and vascular function suggests that MPs may be a simple non-invasive biomarker of

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

			Baseline	t 0	t 5	t 10	t 15
SSc	EMPs	Spearman r	0.05	0.42	0.42	0.46	0.46
		p value	0.82	0.04	0.04	0.03	0.02
	PMPs	Spearman r	0.19	0.42	0.52	0.54	0.64
		p value	0.38	0.05	0.01	0.01	0.00
Primary RP	EMPs	Spearman r	-0.34	-0.33	-0.13	-0.39	0.09
		p value	0.15	0.2	0.65	0.13	0.75
	PMPs	Spearman r	-0.52	-0.33	-0.69	-0.65	-0.49
		p value	0.04	0.23	0.00	0.00	0.06

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, vasodilation 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 large 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/primary RP, in comparison to SLE in particular, suggests differential release/production 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

- [1] M. Diamant, M.E. Tushuizen, A. Sturk, R. Nieuwland, Cellular microparticles: new players in the field of vascular disease? *Eur. J. Clin. Invest.* 34 (6) (2004) 392–401.
- [2] J.H. Distler, D.S. Pisetsky, L.C. Huber, J.R. Kalden, S. Gay, O. Distler, Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases, *Arthritis Rheum.* 52 (11) (2005) 3337–3348.
- [3] L. Bernal-Mizrachi, W. Jy, J.J. Jimenez, J. Pastor, L.M. Mauro, L.L. Horstman, et al., High levels of circulating endothelial microparticles in patients with acute coronary syndromes, *Am. Heart J.* 145 (6) (2003) 962–970.
- [4] H.A. Mostefai, F. Mezziani, M.L. Mastronardi, A. Agouni, C. Heymes, C. Sargentini, et al., Circulating microparticles from patients with septic shock exert protective role in vascular function, *Am. J. Respir. Crit. Care Med.* 178 (11) (2008) 1148–1155.
- [5] A.E. Berezina, A.A. Kremzer, Impaired phenotype of circulating endothelial microparticles in chronic heart failure patients: relevance to body mass index, *Diabetol. Metab. Syndr.* 9 (4) (2015) 230–236.
- [6] A.E. Berezina, A.A. Kremzer, T.A. Samura, T.A. Berezina, P. Krzuziak, Impaired immune phenotype of circulating endothelial-derived microparticles in patients with metabolic syndrome and diabetes mellitus, *J. Endocrinol. Investig.* 38 (8) (2015) 865–874.
- [7] S. Guiducci, J.H. Distler, A. Jungel, D. Huscher, L.C. Huber, B.A. Michel, et al., The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis, *Arthritis Rheum.* 58 (9) (2008) 2845–2853.
- [8] E. Bartoloni, A. Alunno, O. Bistoni, S. Caterbi, F. Luccioli, G. Santoboni, et al., Characterization of circulating endothelial microparticles and endothelial progenitor cells in primary Sjogren's syndrome: new markers of chronic endothelial damage? *Rheumatology (Oxford)* 54 (3) (2015) 536–544.
- [9] C.T. Nielsen, O. Ostergaard, C. Johnsen, S. Jacobsen, N.H. Heegaard, Distinct features of circulating microparticles and their relationship to clinical manifestations in systemic lupus erythematosus, *Arthritis Rheum.* 63 (10) (2011) 3067–3077.
- [10] R. Sgonc, M.S. Gruschwitz, H. Dietrich, H. Recheis, M.E. Gershwin, G. Wick, Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma, *J. Clin. Invest.* 98 (3) (1996) 785–792.
- [11] J.D. Pauling, V.B. O'Donnell, N.J. McHugh, The contribution of platelets to the pathogenesis of Raynaud's phenomenon and systemic sclerosis, *Platelets* 24 (7) (2013) 503–515.
- [12] L.V. Iversen, O. Ostergaard, S. Ullman, C.T. Nielsen, P. Halberg, T. Karlsmark, et al., Circulating microparticles and plasma levels of soluble E- and P-selectins in patients with systemic sclerosis, *Scand. J. Rheumatol.* 42 (6) (2013) 473–482.
- [13] A.H. Lazarus, J. Ellis, J.W. Semple, M. Mody, A.R. Crow, J. Freedman, Comparison of platelet immunity in patients with SLE and with ITP, *Transfus. Sci.* 22 (1–2) (2000) 19–27.
- [14] J. Pereira, G. Alfaro, M. Goycoolea, T. Quiroga, M. Ocqueteau, L. Massardo, et al., Circulating platelet-derived microparticles in systemic lupus erythematosus. Association with increased thrombin generation and procoagulant state, *Thromb. Haemost.* 95 (1) (2006) 94–99.
- [15] B. Parker, A. Al-Husain, P. Pemberton, A.P. Yates, P. Ho, R. Gorodkin, et al., Suppression of inflammation reduces endothelial microparticles in active systemic lupus erythematosus, *Ann. Rheum. Dis.* 73 (6) (2014) 1144–1150.
- [16] J.D. Pauling, J.A. Shipley, D.J. Hart, A. McGrogan, N.J. McHugh, Use of laser speckle contrast imaging to assess digital microvascular function in Primary Raynaud phenomenon and systemic sclerosis: a comparison using the Raynaud condition score diary, *J. Rheumatol.* 42 (7) (2015) 1163–1168.
- [17] Y. Miyazaki, S. Nomura, T. Miyake, H. Kagawa, C. Kitada, H. Taniguchi, et al., High shear stress can initiate both platelet aggregation and shedding of procoagulant containing microparticles, *Blood* 88 (9) (1996) 3456–3464.
- [18] L.L. Horstman, W. Jy, J.J. Jimenez, Y.S. Ahn, Endothelial microparticles as markers of endothelial dysfunction, *Front. Biosci.* 9 (2004) 1118–1135.
- [19] J.J. Jimenez, W. Jy, L.M. Mauro, C. Soderland, L.L. Horstman, Y.S. Ahn, Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis, *Thromb. Res.* 109 (4) (2003) 175–180.
- [20] D.B. Peterson, T. Sander, S. Kaul, B.T. Wakim, B. Halligan, S. Twigger, et al., Comparative proteomic analysis of PAI-1 and TNF-alpha-derived endothelial microparticles, *Proteomics* 8 (12) (2008) 2430–2446.
- [21] N. Mackman, G.E. Davis, Blood coagulation and blood vessel development: is tissue factor the missing link? *Arterioscler. Thromb. Vasc. Biol.* 31 (11) (2011) 2364–2366.
- [22] P.J. Yong, C.H. Koh, W.S. Shim, Endothelial microparticles: missing link in endothelial dysfunction? *Eur. J. Prev. Cardiol.* 20 (3) (2013) 496–512.
- [23] P.E. Rautou, A.S. Leroyer, B. Ramkhalawon, C. Devue, D. Duflaut, A.C. Vion, et al., Microparticles from human atherosclerotic plaques promote endothelial ICAM-1-

- dependent monocyte adhesion and transendothelial migration, *Circ. Res.* 108 (3) (2011) 335–343.
- [24] V. Proulle, B. Hugel, B. Guillet, L. Grunebaum, T. Lambert, J.M. Freyssinet, et al., Circulating microparticles are elevated in haemophiliacs and non-haemophilic individuals aged <18 years, *Br. J. Haematol.* 131 (4) (2005) 487–489.
- [25] J. Sellam, V. Proulle, A. Jungel, M. Ittah, C. Miceli Richard, J.E. Gottenberg, et al., Increased levels of circulating microparticles in primary Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis and relation with disease activity, *Arthritis Res. Ther.* 11 (5) (2009) R156.