

**Title: Endothelial microparticles: pathogenic or passive players in endothelial dysfunction in autoimmune rheumatic diseases?**

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**Running Title:** Endothelial Microparticles in Rheumatic Disease

## **Abstract**

Autoimmune rheumatic diseases are characterised by systemic inflammation and complex immunopathology, with an increased risk of cardiovascular disease, initiated by endothelial dysfunction in a chronic inflammatory environment. Endothelial microparticles (EMPs) are released into the circulation from activated endothelial cells and may therefore, reflect disease severity, vascular and endothelial dysfunction, that could influence disease pathogenesis via autocrine/paracrine signalling. The exact function of EMPs in rheumatic disease remains unknown, and this has initiated research to elucidate EMP composition and function, which may be determined by the mode of endothelial activation and the micro environment. To date, EMPs are thought to play a role in angiogenesis, thrombosis and inflammation by transferring specific proteins and microRNAs (miRs) to target cells. Here, we review the mechanisms underlying the generation and composition of EMPs and the clinical and experimental studies describing the involvement of EMPs in rheumatic diseases, since we have previously shown endothelial dysfunction and an elevated risk of cardiovascular disease are characteristics in systemic lupus erythematosus. We will also discuss the potential of EMPs as future biomarkers of cardiovascular risk in these diseases.

## Abbreviations

APC	Antigen Presenting Cells
ANCA	Anti-Neutrophil Cytoplasmic Antibody
AECA	Anti-Endothelial Cell Antibodies
APL	Anti-phospholipid
APS	Anti-phospholipid syndrome
BVAS	Birmingham Vasculitis Activity Score
CTD	Connective Tissue Disease
DC	Dendritic Cell
ECD	Endothelial Cell Dysfunction
EMP	Endothelial microparticle
eNOS	endothelial nitric oxide synthase
EPC	Endothelial Progenitor Cell
FMD	Flow Mediated Dilation
FOI	Fluorescent Optical Imaging
HC	Healthy Control
HSP	Henoch-Schonlein Purpura
ICAM	Intracellular Adhesion Molecule-1
KD	Kawasaki Disease

LAC	Lupus Anticoagulant
MV	MicroVesicle
NO	Nitric Oxide
PH	Pulmonary Hypertension
PMP	Platelet Microparticle
PMR	Polymyalgia Rheumatica
PS	Phosphatidylserine
RA	Rheumatoid Arthritis
SLE	Systemic Lupus Erythematosus
SSc	Systemic Sclerosis
TF	Tissue Factor
TNF- $\alpha$	Tumour Necrosis Factor alpha

## **Need for new biomarkers in rheumatic disease**

Large epidemiology studies have demonstrated that cardiovascular events are a leading cause of morbidity and mortality in rheumatic diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (1-3). This enhanced risk of premature atherosclerosis is independent of traditional risk factors and is partly attributable to persistent systemic inflammation, atherogenic therapies such as glucocorticoids, and a procoagulant environment (4). Autoimmune rheumatic diseases such as SLE, RA and systemic sclerosis (SSc) have presented challenges in the clinic because of their unclear disease aetiology and their unpredictable disease progression, demanding a need for improved and more accurate assessment of disease activity, prognosis and co-morbidity, with a view to ultimately allow a more personalised approach to therapy.

There are several reports linking elevated endothelial microparticles (EMPs) with the immunopathogenesis of rheumatic diseases, via their potential role in the regulation of inflammation, thrombosis and angiogenesis (5). EMPs are of particular interest in SLE and related connective tissue diseases (CTDs), given the importance of the endothelium in both inflammatory disease manifestations and the role of endothelial dysfunction in the early stages of atherosclerosis. Endothelial cell dysfunction (ECD), characterised by a shift of the actions of the endothelium toward reduced vasodilation, a proinflammatory state and enhanced prothrombotic properties, is emerging as the common denominator for diverse and highly prevalent cardiovascular diseases. Mechanisms that participate in the reduced vasodilatory responses in endothelial dysfunction include reduced nitric oxide generation and oxidative excess. Upregulation of adhesion molecules, generation of chemokines such as macrophage chemoattractant peptide-1, and production of plasminogen activator

inhibitor-1 participate in the inflammatory response and contribute to a prothrombic state.

Detachment and apoptosis of endothelial cells are associated phenomena (6).

We and others, have associated elevated EMPs with cardiovascular diseases and a correlation with high atherosclerotic plaque grade (7-10). Furthermore, EMP composition may reflect as yet unidentified important biological functions in disease pathogenesis and vascular dysfunction, and contribute to the increased cardiovascular risk present in a number of inflammatory diseases. Our own studies have shown the number of circulating EMPs correlates with the inflammatory status of SLE, and inversely relates to endothelial function in this patient group, raising the interest in the potential use of EMPs as a biomarker for the increased cardiovascular risk in SLE (11).

### **Characteristics of endothelial microparticles**

MPs, also referred to as microvesicles (MVs), may originate from different vascular cell types including platelets, monocytes, endothelial cells, red blood cells, and granulocytes. In health, it has been reported that >80% of circulating MPs express membrane antigens that suggest a platelet origin(12). MPs form when the asymmetrical distribution of lipids between the inner and outer leaflets of a plasma membrane is lost (13). Under resting conditions, phosphatidylserine (PS) is located almost exclusively in the inner monolayer (14). When cells undergo activation or apoptosis, PS translocation to the outer leaflet is the initial event that will ultimately lead to the shedding of MPs that are therefore regarded as markers of cell stress (13). The dynamic balance of cell stimulation, cell proliferation and death within the vessels is reflected by the formation and release of MPs that may thus represent a vascular storage pool of bioeffectors.

With regard to EMP release disruption of the endothelium occurs following activation or apoptotic cell death. This may be initiated and propagated by a variety of triggers (such as classic cardiovascular risk factors, inflammatory cytokines and complement activation), leading to conformational changes of the plasma membrane and the release of phosphatidylserine(PS)-exposing microparticles (15, 16). EMPs are 100nm-1µm vesicular, anuclear structures comprising of proteins, micro-RNAs, mRNAs and enzymes specific to the cell from which they originate (17) , enabling paracrine and autocrine actions on cells of the vascular system (18).

EMPs are elevated in numerous cardiovascular-related diseases with an impaired endothelial component including coronary artery disease (7-9), carotid artery disease (10), stroke (19), familial hypercholesterolaemia (20), pulmonary hypertension (21), myocardial ischemia, preeclampsia, diabetes, metabolic syndrome as well as rheumatic diseases, suggesting that these microparticles represent a surrogate marker of ECD. To date it has been impossible to identify the specific vascular bed from which EMPs are derived due to a lack of specific surface markers; however, the total EMP population is thought to reflect the overall health of the endothelium. It should be noted that low levels of EMPs are also detected in healthy controls (10, 19, 21), suggesting that EMPs may also have a potential role in the homeostatic regulation of the healthy vascular endothelium.

## **Endothelial microparticle function**

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 (22). 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 (23). In order to interrogate the role played by EMPs in the context of inflammation, and endothelial dysfunction, EMP release and composition under inflammatory conditions and their subsequent effects have been studied using *in vitro* models. Much of this investigative work is performed on cells derived from large vascular beds such as Human Aortic Endothelial cells (HAoEC) and Human umbilical vein endothelial cells (HuVECs) (24, 25).

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 (26). Taken together, these findings suggest that there are distinct mechanisms for the formation of EMPs in apoptotic and activated cells (27), with several studies suggesting that these types of EMPs have different functions in vascular diseases (28, 29). Thus, we suggest EMPs may provide a measure of the health of the underlying endothelial. However, their utility as biomarkers of disease must also take into account potential differences between the healthy and disease states.



## **Pathogenic effects of endothelial microparticles on the endothelium**

A number of studies have shown that TNF- $\alpha$ , a key cytokine involved in the pathogenesis of a number of rheumatic diseases, activates endothelial cells and induces release of EMPs (24-26). EMPs produced under these conditions have been used in a number of functional studies. High levels of the surface antigens E-selectin, intracellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) have been detected on EMPs derived from activated endothelial cells and can mediate adhesion of monocytes to endothelial cells *in vitro* (30). These data suggest that EMPs may be both a consequence and a cause of the inflammatory response.

EMPs have also been shown to play a role in thrombosis (31). Phosphatidylserine (PS) is expressed on released EMPs, which confers a procoagulant property to EMPs, due to the ability of PS to bind and activate coagulation factors (32). It has been reported that EMPs also contain tissue factor (TF), which can initiate the extrinsic coagulation pathway (24, 33). This thrombogenic activity of EMPs has been confirmed by the demonstration that EMP-triggered TF-dependent release, promotes thrombin formation *in vitro* and thrombus formation *in vivo* (34).

EMPs impair vasorelaxation and bioavailability of endothelial-derived nitric oxide (NO) production by aortic cells in healthy animal models in a concentration-dependent manner, suggesting that circulating EMPs may directly affect endothelial-dependent vasodilatation and thus, not only act as a marker for ECD, but also potentially aggravate pre-existing ECD (35).

EMPs isolated from end-stage renal disease patients with cardiovascular disease have been shown to impair the release of nitric oxide from vascular cells and platelet-derived MPs can

act as a source for thromboxane A<sub>2</sub>, which increases vascular contraction (36). EMPs may also be involved in cross-talk with the smooth muscle layer. Recent work has shown that EMPs produced under inflammatory conditions were able to induce vascular calcification in smooth muscle cells, thus contributing to vessel stiffness (37).

### **Restorative effects of endothelial microparticles**

In contrast to their proposed role in ECD, we and others have detected a functional endothelial nitric oxide synthase (eNOS) in EMPs (38). In our *in vitro* study, EMPs were able to prevent lipid-induced endothelial dysfunction via Akt/eNOS signalling and attenuation of oxidative stress in ECs *in vitro* (Mahmoud et al 2015 under review) suggesting a protective role of EMPs in endothelial function. Indeed, Hussein *et al* demonstrated that EMP release could prevent apoptosis by diminishing levels of caspase-3 in cultured endothelial cells, by trapping caspase-3 within the released MPs (39). Thus, endothelial-derived MPs play a role in homeostasis and contribute to the sorting of several pro-apoptotic factors, preventing cell detachment and apoptosis.

Furthermore, induction of endothelial repair mechanisms have been demonstrated using EMPs produced under different conditions. Tissue factor-containing EMPs produced under physical stress conditions, were able to promote neovascularisation by stimulating CCL20 via  $\beta$ 1-integrin signalling, while high glucose-induced EMPs were able to transfer miR-126, promoting vascular repair via SPRED1 (40). The plasmin generation capacity of EMPs may be pivotal in maintaining vascular patency, since the proteolytic capacity of the plasminogen activation system has been shown to affect the angiogenic potential of endothelial progenitor cells *in vitro*, via extracellular matrix degradation and the release of growth factors (41). However, in contrast, cell culture-derived EMPs have also been shown to inhibit

angiogenesis in mouse models of atherosclerosis (42). In clinical studies, EMPs act in a vasculoprotective manner in conditions associated with acute vascular stresses, including septic shock (43). The various functions exhibited by EMPs are likely to reflect particular membrane and surface proteins specific to their parental cells and underpinned by a particular stimuli or environment thus, contributing to downstream regulatory mechanisms on target cells in a controlled and defined manner. Therefore, rather than being inert markers of injury, EMPs may act as downstream delivery systems for pro-inflammatory products that are vasculoprotective in acute inflammatory conditions, whilst also having the capacity to, not only perpetuate further vascular dysfunction in chronic disease, but also act as surrogates of vascular dysfunction (11, 44-46) . How this translates to the effects observed in patients remains to be determined.

## **The role of endothelial microparticles in rheumatic diseases**

### Rheumatoid arthritis

It has been reported in a range of studies in RA patients that EMP (and total MP) numbers are increased in comparison to healthy controls (HC), as well as being associated with disease specific features such as disease activity (47). MPs isolated from RA patients demonstrate potent ability to activate the endothelium *in vitro*, whilst having a deleterious effect on endothelial cell function, thus supporting their role as a marker of vascular damage in disease (47). Similarly, our own group has previously reported that at a cellular level, a clinically available TNF- $\alpha$  inhibitor (Certolizumab) prevents TNF- $\alpha$  induced activation of the NF- $\kappa$ B pathway and prevents MP production by activated endothelial cells, suggesting a potential novel mechanism by which anti-TNF therapy may improve cardiovascular outcomes in inflammatory arthritis patients (25).

### Systemic Lupus Erythematosus

EMPs have also been shown to be elevated in SLE, in both active and low disease activity states (11, 48). Anti-endothelial cell antibodies (AECAs) do not seem to be the main cause of endothelial dysfunction in this population, with EMPs instead being released from the endothelium of activated and apoptotic cells (48). In active SLE, EMP numbers are significantly related to objective measures of endothelial dysfunction, as assessed by flow mediated dilatation (FMD) (11). Similar to inflammatory arthritis, Parker et al demonstrated that improvement in disease control and suppression of inflammation was associated with improvement in both absolute EMP numbers and endothelial dysfunction in patients, suggesting that better control of active inflammatory disease may contribute to improved

cardiovascular risk in patients with SLE, further supporting the proposal that an EMP measure may be a useful surrogate marker (11).

In addition to being associated with endothelial dysfunction, EMPs may contribute directly to the pathogenesis of disease, linking the endothelium to both disease aetiology and development of premature atherosclerosis. SLE patients exhibit a population of annexin V<sup>+</sup>/CD31<sup>+</sup> /CD45<sup>-</sup> endothelial microparticles, not present in healthy subjects, RA or SSc patients, which contain apoptosis-modified chromatin. This microparticle subpopulation, when isolated from the plasma of SLE patients, increases the expression of co-stimulatory surface molecules and production of pro-inflammatory cytokines IL-6, TNF- $\alpha$  and IFN- $\alpha$  by blood-derived plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs), as well as priming blood-derived neutrophils for NETosis. These results underline the important role of apoptotic endothelial microparticles in driving the autoimmune response in SLE patients (49). This novel activation pathway may be implicated in various additional inflammatory disorders suggesting endothelial microparticles could be an important immunomodulatory therapeutic target (50).

### Sjogren's Syndrome

Similar to SLE, EMP numbers are elevated in Sjogren's Syndrome and have been shown to be directly correlated with disease duration. Interestingly, early EPCs (CD34<sup>+</sup>CD309<sup>+</sup>CD133<sup>+</sup>) demonstrate an inverse correlation with disease duration, suggesting that the reparative potential of the endothelial layer is preserved in the earliest stages of disease, with progressive exhaustion of the precursor endothelial cell pool occurring as disease progresses. In turn, this likely leads to a defective vascular layer restoration, manifesting as endothelial dysfunction (51). A similar mismatch in damage and repair mechanisms has

been observed in patients with Polymyalgia Rheumatica (PMR), where an increased EMP/EPC ratio has been noted, compared with controls, because of both increased EMP (CD31<sup>+</sup>/CD42<sup>-</sup>) numbers and reduced EPC (CD34<sup>+</sup>/KDR<sup>+</sup>) levels. Notably improvement in disease activity following corticosteroid therapy leads to a parallel decline in the EMP/EPC ratio, adding strength to the proposal that an EMP/EPC ratio could act as a biomarker for response to treatment (52).

### Systemic Sclerosis

Systemic Sclerosis (SSc) is characterised by microvascular involvement early in the disease course, potentially contributing, via tissue ischaemia, to the widespread fibrosis characteristic of this condition. Platelet-derived microparticles also make an important contribution to this disease upon activation, as their secretome contains a range of vasoactive mediators favouring vasoconstriction (e.g. thromboxane, serotonin) and growth factors (TGF- $\beta$ , PDGF) that may contribute to fibrosis (53). The elevated levels of EMPs in SSc patients appear to correlate with organ involvement; eg. Annexin V non-binding EMP concentrations correlate negatively with lung function parameters (DLco and FVC) in limited and diffuse cutaneous subsets of SSc (54). Similarly, the elevated EMP levels detected in pulmonary hypertension (PH), the leading cause of mortality in SSc patients, could reflect an increased vascular pro-coagulant and inflammatory state, which might be related to thrombo-embolic complications as well as PH progression (55). Finally, EMPs also correlate with objective measures of vascular function/damage in SSc, as Jung et al have identified a strong association between EMPs and perivascular inflammation, quantified by Fluorescence Optical Imaging (FOI) in SSc and Raynaud's patients and have suggested that

EMP measurement may be a potential biomarker of both diagnosis and response to therapy (56).

### Anti-Phospholipid Syndrome

The antiphospholipid syndrome (APS) refers to persistent anti-phospholipid antibodies (aPL) associated with thrombotic and/or obstetrical complications. Because of their procoagulant and proinflammatory properties, EMPs have been implicated in the pathogenesis of antiphospholipid syndrome (APS). A number of studies have compared the levels of MPs in the blood of patients with primary APS, SLE patients with secondary APS, SLE patients with or without antiphospholipid antibodies (aPL) in the absence of secondary APS, and healthy individuals with or without aPL in the absence of thrombotic events. In general, these studies have reported elevated numbers of EMPs in patients with APS, lending credence to links of EMPs with thrombotic events and pregnancy complications (57-59). However, the precise contribution to thrombosis remains unclear. Interestingly, only APS plasma induced the release of EMP with procoagulant activity suggesting that generation of EMP in APS and SLE patients results from an autoimmune process involving aPL (60).

In support of this, EMP levels were also associated with Lupus Anticoagulant (LA). In contrast however, Jy et al described elevated EMP counts in individuals with aPL, although this increase was independent of a history of thrombosis; PMP counts in contrast were increased only in patients with aPL and a thrombotic event (61). These findings suggest that aPL might cause chronic endothelial cell activation or injury, leading to the enhanced shedding of EMPs. Interestingly, aPL-positivity in SLE has also been associated with the presence of carotid plaque, supporting their role in chronic endothelial injury (62). In contrast, only certain aPL specificities might cause the activation of platelets and the release

of PMPs, which increase the risk for thrombosis. Therefore, the EMP/PMP ratio could be key to successful stratification within this patient group.

### Vasculitides

Increased numbers of EMPs during the acute phase of vasculitides have also been reported, with patients in remission displaying normal levels (63). The numbers of EMPs correlate with the Birmingham Vasculitis Activity Score (BVAS) in children with different types of vasculitides and in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides in adults (64). Comparison of blood EMP levels with those of circulating endothelial cells, another potential biomarker for vasculitides, indicates that whilst both markers correlate with disease activity in ANCA-associated vasculitides, EMP levels decline faster after induction of remission (65). Similar findings have been demonstrated in paediatric vasculitis. In Kawasaki Disease (KD), when compared with healthy controls, there were significantly greater numbers of CD144<sup>+</sup>/CD42b<sup>-</sup>, CD62E<sup>+</sup>, and CD105<sup>+</sup> EMPs in patients with negative correlations observed between the values of FMD and EMPs in the three phases of KD (66). Similar observations were made in Henoch-Schonlein Purpura (HSP) with EMP levels falling following treatment. Despite this reduction, patients in remission still exhibited higher EMP levels in comparison to healthy controls again suggesting their potential as a marker of subclinical inflammation in HSP (67).



## **Barriers to the use of endothelial microparticles as biomarkers.**

EMPs are identified and enumerated via flow cytometry using a panel of markers; most commonly Annexin V<sup>+</sup> to indicate PS-positivity. Endothelial cell markers used vary between groups and the combination of CD31<sup>+</sup>/CD42b<sup>-</sup> is widely used (9, 68) but remains an imperfect choice (44, 64). Platelet cell adhesion molecule (PECAM-1 or CD31) is present on both endothelial and platelet-derived MPs (PMPs), therefore, CD42-negativity is used to exclude PMPs. Other combinations used to detect EMPs, include CD144 (VE-cadherin), CD54 (intracellular adhesion molecule-1 (ICAM)), and CD62e (E-selectin) (44, 64). Again, these markers remain problematic as they only detect a sub-population of EMPs (for example, from activated endothelial cells only) and therefore may only be present at lower levels (23, 69). The process of identifying and quantifying EMPs involves several distinct stages - blood collection, centrifugation, antibody detection of cell surface antigens, and flow cytometry – and there is significant variation at all stages, and there is a growing consensus that for accurate assessment of microparticles, a firm set of guidelines is needed (70, 71). Previous reviews have extensively highlighted the difficulties, methods and potential characteristic markers that may be used in this process (72). It may be that additional methods should be employed to enable accurate phenotyping, sizing, and enumeration of the wide range of MPs generated from different vascular beds and different cell types, in order to fully understand the biology of these microparticles. This may also remove variation between sample handling between different laboratories. These additional methods may include electron and atomic force microscopy, together with a full RNA, lipid, and protein profiling exercise.

### **Conclusion: Are endothelial microparticles friends or foes?**

In conclusion, EMPs can be considered as complex structures displaying a large repertoire of endothelial-derived molecules and biological functions, depending on their composition.

When the data are taken together, the involvement of EMP in vascular homeostasis appears to be more complex than initially thought. EMPs can play a major role in inflammation, thrombosis, angiogenesis and repair as summarised in Figure 1. However, depending on the physiological or pathological context, the mechanisms and sites of formation, EMPs are emerging as having favourable effects to maintain vascular homeostasis. Further studies are warranted to establish whether these different EMP “phenotypes” and paradoxical effects are also found *in vivo*.

The initial vision was that EMPs were noxious, supporting proinflammatory, procoagulant potential and inhibiting vascular repair. Increased levels of MPs of endothelial origin in various pathologies, such as atherothrombosis, vasculitis, and sepsis, also support this harmful potential. However, recent data have brought to light the beneficial effect of EMPs on endothelial integrity, such as stimulation of vascular repair, control of cell death mechanisms or cytoprotective activities supported by antigen presenting cells (APC), or induction of adaptive immunity. Therefore, we would do an injustice to the humble MP if we were to label them as either pathogenic or simple passive players in disease. It is clear that MPs may play a role in homeostasis and could be protective, and because of their modulation in disease conditions, remain open to the possibility of acting as novel biomarkers of disease.

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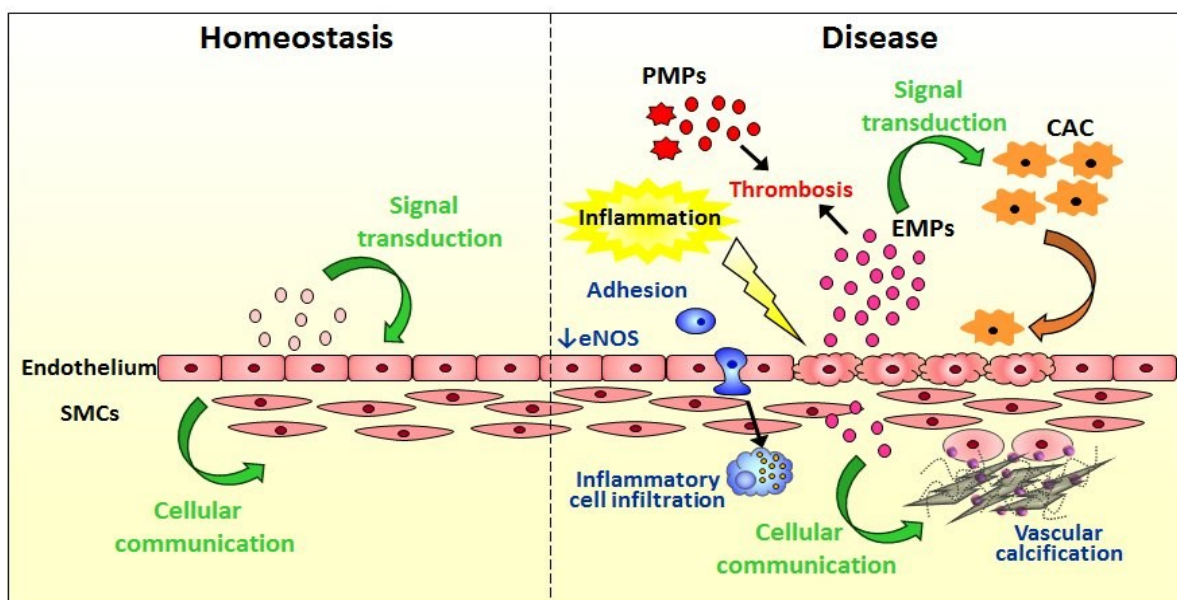
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**Figure 1.** An illustration to highlight the multiple roles that EMPs may play in the pathogenesis of autoimmune rheumatic diseases and the potential signalling pathways involved in repair of the vessel wall. PMPs; platelet microparticles, EMPs; endothelial microparticles, CAC; circulating angiogenic cells, eNOS; endothelial nitric oxide synthase, SMCs; smooth muscle cells.