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Microparticles and their Roles in Inflammation: A Review[§]

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Abstract: Microparticles (MPs) were long dismissed as "platelet-dust", cell debris, with no functional significance. Theyare anucleated vesicles (0.1μm to 1μm), enclosed in a membrane, secretedinto the circulation by cells during cell activation or apoptosis. They have now emerged as mediators and markers of inflammatory diseases and autoimmune disorders. They are distinctly different from exosomes and apoptotic bodiesand are released from nearly every cell type, the most abundant being platelets, leukocytes and endothelial cells. MPs can be detected using flow cytometry and more recently by nanotechnology, which is more accuratein detecting, quantifying and phenotypingMPs.

MPs are instrumental in the pathogenesis of various cardiovascular diseases (thrombotic and atherosclerotic) through their pro-inflammatory and pro-coagulant properties. Their levels are significantly elevated in chronic inflammatory disorders such as rheumatoid arthritis and multiple sclerosis. However, increasing evidence suggests they also possess anti-inflammatory and anti-coagulant roles which could confer immunoprotection. MPs transport various lipids, proteins, mRNA and microRNA (miRNA) which may influence activities of receiving cells. Particularly the miRNAspecies delivered by MPs have been shown to modulate inflammation.

In vitro and *in vivo* studies are being conducted to bioengineer MPs to facilitate delivery of therapeutic compounds to desired location safely, specifically and more effectively, with fewer side effects. More research is required to understand the composition, origin, mechanisms of formation and release as well as their clearance from the circulation to pave way for gaining greater pharmacological benefits by controlling MP-mediated cellular responses.

Keywords: Autoimmune disease, biomarkers, cardiovascular disease, disease activity, inflammation, microparticles, phosphatidylserine, thrombosis.

1. INTRODUCTION

This review aims to highlight current understanding ofmicroparticles(MPs) and the basis of their likely modulatory role in inflammation, drawing attention to the still incomplete picture of their involvement in the onset, development or resolution of inflammatory conditions. There is now a consensus on their size and origin and significant advances have been made in methods of their detection. This review will characterise the inflammatory response as a condition relevant to MPs, giving clinical examples of acute and chronic inflammation within cardiovascular disease as well as autoimmune disorders such as rheumatoid arthritis, multiple sclerosis and anti-phospholipid syndrome. An overview of evidences will be provided in support of the argument that MPs have dual functionality: they may exert pro- and anti-inflammatory/coagulant effects. Finally, novel areas of research relating to MPs (such as miRNA) and their importance into determining functional properties of MPs as well as the potential use of MPs as drug-delivery vehicles will be discussed.

1.1. Definition and Origin of MPs

Many cells shed small particles or vesicles in a regulated manner, which are involved in cell signalling and communication. Previously considered as cell debris, or "platelet dust" with no specific role [1], MPs have now emerged as anucleated mediators or markers of inflammatory diseases and have moved into the limelight in an attempt to understand better pathogenic processes in a variety of acute and chronic diseases. Their elevated levels and cell signalling roles have been linked to an increasing range of diseases.

First discovered in 1946 [2], MPs are small, intact, membranous vesicular structures, lacking nucleus and ranging in size from 0.1- 1μm [3]. While platelets are the principle source of MPs, multiple cell types can release MPs. They originate from plasma membranes of endothelial cells, vascular smooth muscle cells, leukocytes or white blood cells (neutrophils, lymphocytes, macrophages and monocytes), erythrocytes, epithelial cells and tumour cell lines.

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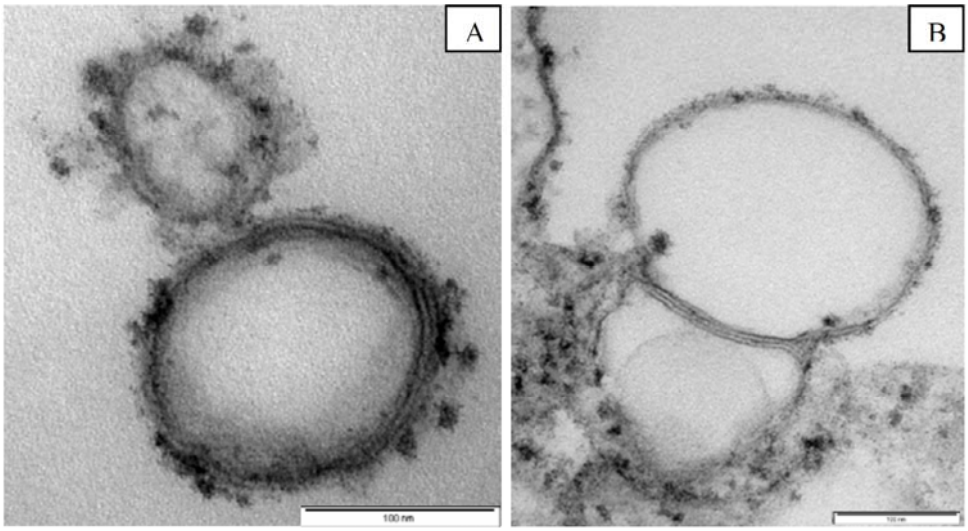

Fig. (1). Typical transmission electron micrographs of microparticles isolated from normal plasma in our lab. These sections were taken from prepared MP pellets after embedding in 3% agar and Spurr's resin. The two images (**A**, **B**) demonstrate distinct membrane bound vesicles of <1 μm with a typical lipid bilayer appearance and no intracellular structures. Bars indicate 100nm.

Platelet derived MPs are the most abundant sub-type measured in human plasma [4] (Fig. **1**: an example from our experimental data). The MPs released from RBCs are generally smaller compared to those released from other origins [5]. MPs carry various nuclear components such as RNA and DNA. They are released from cells during activation, apoptosis or necrosis, circulate in the blood and play an emerging role in inflammation, coagulation, thrombosis, atherosclerosis, malignancy and infection (Fig. **2A**). In other words, despite their presence in peripheral blood of healthy individuals, their levels rise significantly in disease states characterised by cell activation and death. This suggests a link between MPs and inflammation [4]. Their role in transferring bioactive molecules such as antigens and receptors away from their site of origin, to where they are needed, has been discovered [6]. Because of their role in various disease states they have become a popular therapeutic target for various drugs, which will be described later.

1.2. Composition of MPs

MPsare rich in phospholipid and protein and also contain cytoplasmic components. In intact cells, the lipid bilayer has an asymmetrical composition of phospholipids, where phosphatidylethanolamine and phosphatidylserine (PS) are present in the inner leaflet of the membrane and the outer layer consists of phosphatidylcholine, phosphatidylinositol, sphingomyelin and various glycolipids. This asymmetric distribution is disrupted upon MP release. They express phosphatidylserine on their outer membrane surface along with surface proteinsthat reflect their cell of origin e.g. CD42a for platelets, CD3 for T cells, or CD144 for endothelial cells (Fig. **2**). Such properties of antigen expression also make it easier for them to be detected and distinguished as, platelet, lymphocyte orendothelial-derived MPs, respectively [7]. Phosphatidylserine on the outer surface of the released MPs acts as an assembly point for prothrombinase complex thus activating a coagulation cascade [5] (Fig. **2D**).

The biological effectof MPs is dependent on various factors such as cell origin, stimulus and thecircumstances of release including pathophysiological conditions. Dependent upon these factors, variable amounts of lipids and proteins are present in the MP membrane [5]. Although the phospholipid composition of MPs may be different from their parental cell, the protein composition dependsentirely on the parental cell [5]. However, this protein composition may vary in response to different agonists and cell surface molecules expressed on MPs may differ from the parental cell. For instance, in contrast to resting cultivated endothelial cells, IL-1α activated-endothelial cell cultures release MPs that express increased levels of E-selectin and endothelial cell adhesion molecule 1 [8]. The evidence of transfer of genetic material between cells *via* MPs comes from the fact that they contain mRNA and miRNA. MiRNA are post-transcriptional regulators usually involved in suppression of target cells.

1.3. MPs, Exosomes and Apoptotic Bodies

Importantly, MPs differ in size, subcellular origin, composition and content from structures such as exosomes and apoptotic bodies [4]. Exosomes are generally smaller than MPs (<100nm), and unlike MPs, they do not externalise phosphatidylserine (PS). This is because they arise from invaginations of the endosomal membrane, leading to encapsulation of cytoplasmic material. When this structure fuses with the cell membrane, exosomes are released from the cell. This has been described for epithelial, B and T, dendritic cells and platelets. Exosomes are found in all biological fluids such as saliva, breast milk, urine and plasma [9]. On the other hand, apoptotic bodies are relatively larger than MPs(500 nm–3 μm), externalise PS and unlike MPs, their membrane is permeable, making it easier for their nuclear content to be stained for visualising [10]. PS present

in the outer leaflet of apoptotic bodies directs activity of phagocytic cells [5].

1.4. Formation and Release of MPs

Various stimuli lead to MP release: upon activation by thrombin (serine protease of the clotting cascade), calcium ionophore A23187, high shear stress and ADP (adenosine diphosphate) plus collagen, platelets release MPs [5]. These platelet-derived MPs express the plasma membrane glycoproteins GPIb, IIb, and IIIa as well as membrane receptors that target coagulation factor Va indicating a procoagulant potential of these MPs [11]. Platelets are fragments of megakaryocytes, so platelet-derived MPs can also be derived from megakaryocytes during megakaryopoiesis [12]. Both types are found in circulation and can be distinguished on the basis of their surface markers. MPs derived from platelets express markers such as CD62P and lysosome-associated membrane protein-1, whereas megakaryocyte-derived MPs lack these markers and contain full length filamin A (a marker that is cleaved during platelet formation) [12].

TNF-α was the first stimulus that was shown to result in the release of endothelial-derived MPs fromhuman umbilical vein endothelial cells [13]. Activation by bacterial lipopolysaccharide, inflammatory cytokines, formation of the terminal complex of complement activation (C5b-9) or reactive oxygen species leads to MP release by endothelial, vascular smooth muscle cells and monocytes [5] (Fig. **2A**, **B**). MP release upon agonist stimulation is a calcium-dependent process, which can be inhibited by calcium chelators.

Recent *in vitro* studies have given some insight into the mechanisms of MP formation, whereas, the *in vivo* mechanisms largely remain unknown. A few key steps involved in this process that have been recently suggested are: plasma membrane budding; loss of membrane phospholipid asymmetry; and activation of three enzymes, flippase, floppase and scramblase. Flippase maintains the normal asymmetric distribution of phospholipids between the membrane leaflets which has been described earlier, whereas floppase is involved in the ATP-dependent transient translocation of phosphatidylserine to the outer leaflet upon

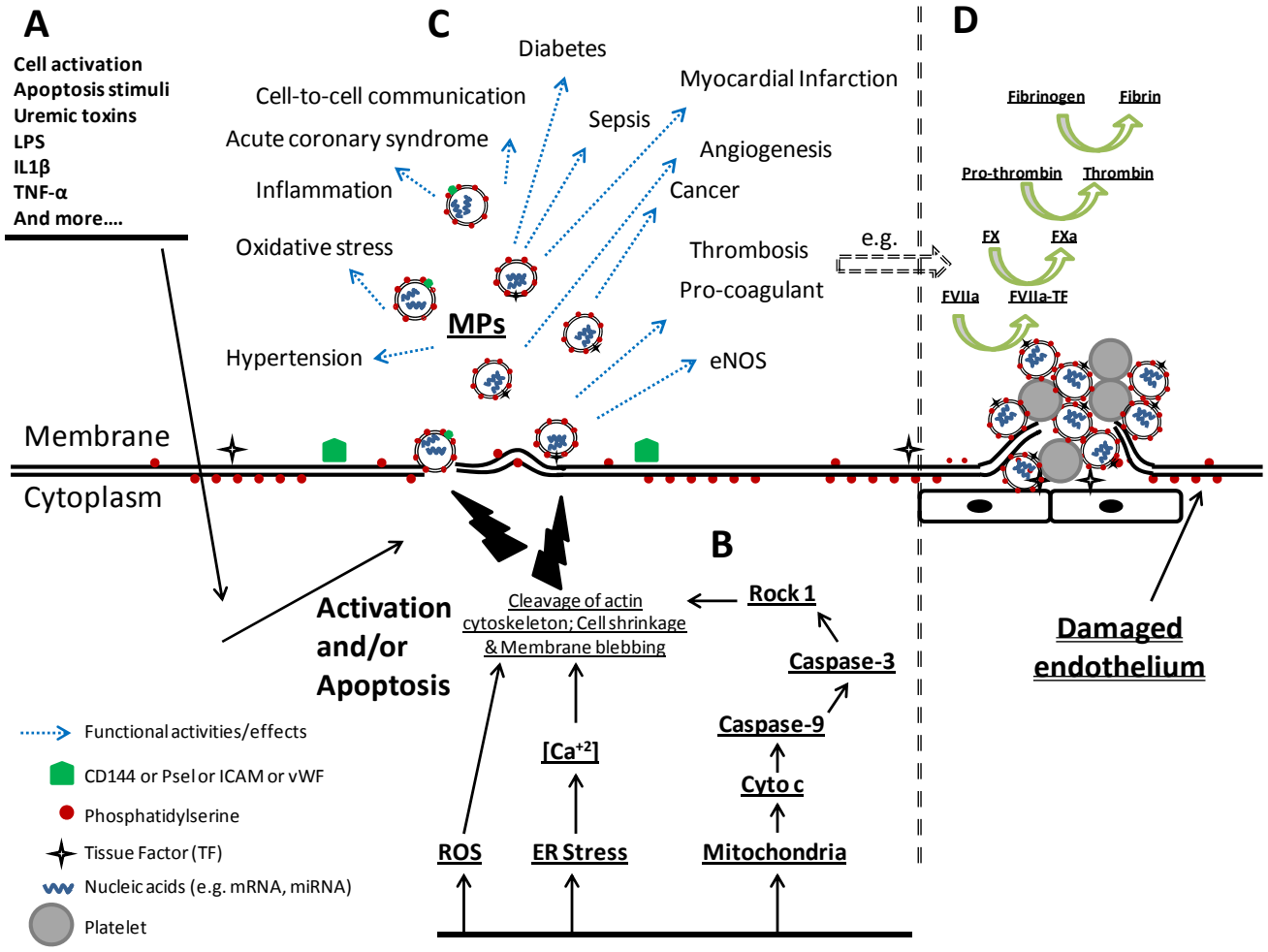

Fig. (2). Microparticle formation and a possible mechanism of action. (**A**) Stimulation of either a receptor dependent or an independent intracellular pathway results in (**B**) cell activation and/or apoptosis through a number of mechanisms. (**C**) Released MPs express surface markers from their parent cells and contain cytoplasm, nucleic acids, mRNA and miRNA, which all play a role in cell-to cell communication, angiogenesis, thrombosis, inflammation and more. (**D**) As an example, TF/PS positive MPs implicate in an accelerated progression of coagulation activation and thrombin generation.

cell activation [14]. Scramblase, as the name suggests, promotes membrane randomisation by enabling all phospholipids to flow down their concentration gradients (scrambling). Patients with Scott syndrome have defective scramblase, and as a result PS is not transported to the outer leaflet of plasma membrane and MP shedding is inhibited leading to abnormal haemostatic response [15].

Upon activation, calcium influx inhibits flippase activity and activates calcium-sensitive enzymes, such as calpains (protease which cleaves talin and α-actin of the cytoskeleton) and gelsolin (actin-binding protein). Membrane budding leads to shedding of MPs in a process called vesiculation [14] (Fig. **2B**). Recent studies show that PS translocation to the outer leaflet involves mitochondrial membrane depolarisation [14]. The relationship between calpain activation and MP shedding was first demonstrated by Fox *et al.* when inhibiting calpain resulted in inhibition of MP shedding [16]. However, calcium-dependent mechanisms for MP release other than calpain-dependent exist as well. Moreover, the phenotype and quantity of MPs formed by activation may be different to those formed by apoptosis. They may differ in terms of their size, phospholipid composition and the surface proteins, as well as their physiological function [17]. This would have important implications for their functionality.

Rho-associated kinase (ROCK I) plays a crucial role in apoptopic MP formation. This kinase is activated by a caspase-mediated mechanism and facilitates myosin light-chain phosphorylation (Fig. **2B**). It also promotes actin-myosin filaments coupling to the plasma membrane causing membrane contraction and thus initiates cytoskeletal rearrangement and apoptopic MP formation, characteristic of the executive phase of apoptosis [15].

1.5. Detection Techniques

The origin of MPs can be traced using flow cytometry with the help of antigenic markers of their parental cell that they present on their surface.

Standardised and optimised methods are important to detect MPs so that no false estimates are made, MPs can be distinguished from cell debris, and the data from different independent studies can be compared [7]. This has proven difficult. Various techniques such as Dynamic Light Scattering (DLS), enzyme linked immune-sorbent assays (ELISA), ultracentrifugation, adsorption to latex beads followed by flow cytometry, and electron microscopy (EM), are used to quantify and characterise the properties of these MPs [18]. Most of these techniques require extensive sample preparation and are not all necessarily quantitative [19]. Not all particles present can be captured by ELISA, a technique which cannot distinguish with accuracy between MPs, exosomesand soluble antigens [19]. Dynamic light scattering (DLS), can measure particles in suspension but mixtures of MPs and exosomes cannot be resolved using this technique.

1.5.1. Flow Cytometry

The technique used most commonly is flow cytometry or fluorescence-activated cell sorting (FACS)[6]. However, a universal protocol for the processing of the samples has not been agreed upon by all despite a number of consensus meetings. PS on the outer leaflet of the membrane of MPs is detected by Annexin V which in turn acts as a probe to detect MPs and quantify them [4]. Some MPs, however, are smaller than the threshold of detection for FACS, which is currently set at0.3 μm [19]. The various sub-populations of MPs can be detected and categorised through fluorescently labelled antibodies that are generated specific to the cell surface antigens [4]. Cells are aligned in laminar flow and a laser is shone across a particle one at a time. Some of the light scatters on the side (a measure of granularity) and somescatters forward (a measure of particle size). Hence, MPs show typical forward (FSC) and side (SSC) scatter patterns (Fig. **3**: an example from our experimental data). Using size-calibrated fluorescent beads (Megamix, BioCytex of three diameters; 0.5, 0.9, and 3μm) provides the advantage of having MPs counted and analysed with both intra- and interinstrument reproducibility.

Alternate detection methods have been proposed, such as binding assays in a solid-phase or microtiter plate format [4]. These techniques exploit secondary antibodies and give the amount of MP-related material in the specimen, but no size or number of MPs. Moreover, analysis of MP can be affected by the type of technique used, as well as other factors such as centrifugation, diameter of the needle used to collect blood, filtration of buffer and freezing methods [4].

1.5.2. Nanoparticle Tracking Analysis

Research for better equipment and methodologies to detect and assay MPs, has led to the development of newer technologiesfor sizing and phenotyping MPsknown as nanoparticle tracking analysis (NTA). It overcomes the limitations of the existing fluorescence-based technologies for MP detection. Using NTA, MPs andexosomes in the range of 30—1000nm can be visualised in liquid, directly and individually, as well as counted in real-time, providing highly resolved particle size distribution profiles as well as concentration measurements [20]. NTA is not only easier to use than the existing methods, but is also faster, more sensitive and precise as well ascost effective. Using various excitation wavelengths, fluorescently labelled particles can be characterised more efficiently and accurately. Specificity of analysis of particular types of MPs can be achieved when combined with fluorescent labelling.

NTA measures the Brownian motion of the particles and relates it to particle size. The smaller the particles, the faster their locomotion. The sample is sufficiently diluted and these particles in solution exhibiting Brownian motion can be observed using a light microscope as they have different light-scattering characteristics of laser light, exploited by NTA. A video (usually 60 seconds long) is takenof these particles, with 30 frames per second, and is analysed using the NTAsoftware [19]. It tracks the motion of these particles on a frame-by-frame basis and calculates the mean squared displacements for each particle displaying a particle size distribution (Fig. **4**: an example from our experimental data).

2. FUNCTION OF MPs

Depending on their origin, MPs may have various physiological and pathophysiological functions (Fig. **2C**), the main and most studied roles being in coagulation, haemostasis, and thrombosis (Fig. **2D**). As mentioned

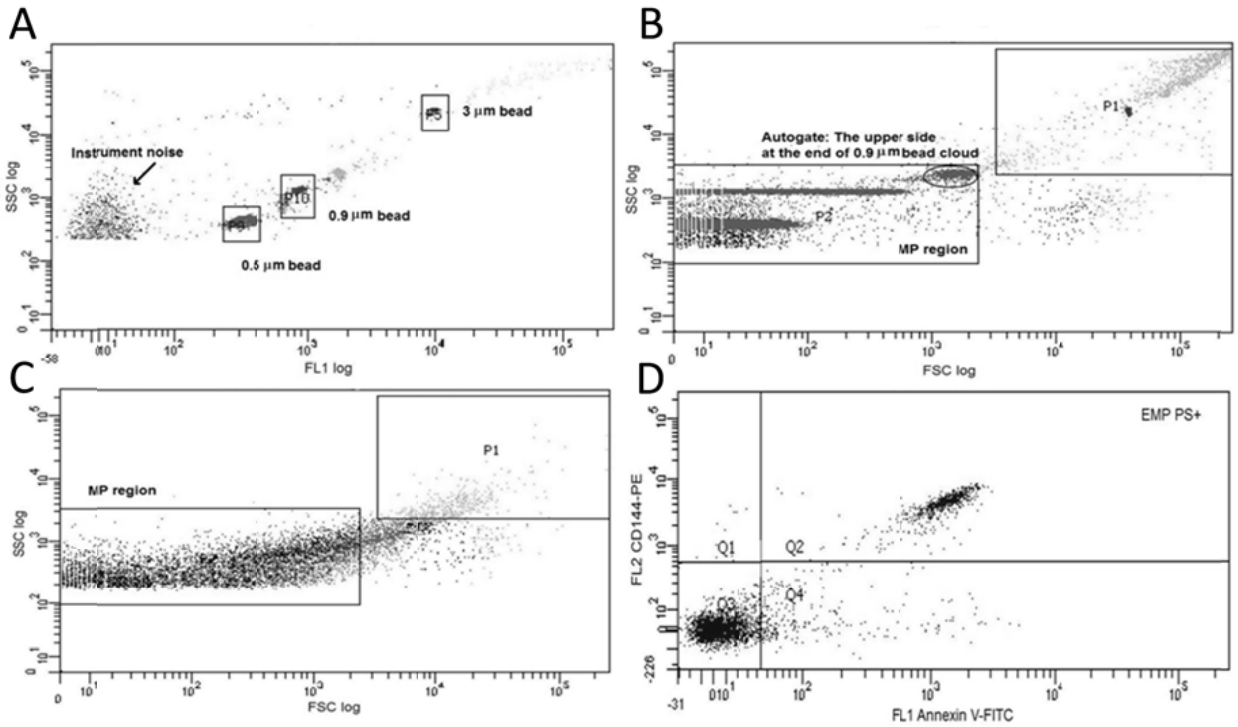

Fig. (3). Gating and detection of MPs using flow cytometry (BD FACSAria™ II; Becton Dickinson, BD Bioscience, San Joes, USA)**.** (**A**) Side scatter log (SSC log) versus FL1 fluorescence properties of beads of 0.5, 0.9, and 3μm diameter (Megamix) to adjust forward scatter (FSC) parameter. (**B**) Determination of MP analysis region by setting up an autogate around events between 0.5μm and 0.9μm fluorescent beads. (**C**) An example of endothelial microparticle (EMP) size distribution (**D**) dual labelling of MPs using CD144-phycoerytherin (PE) (indicative of MPs' endothelial originality) and Annexin V-FITC (indicative of PS positivity).

before, MPs play an emerging role in inflammation, angiogenesis and vascular reactivity as well [4].

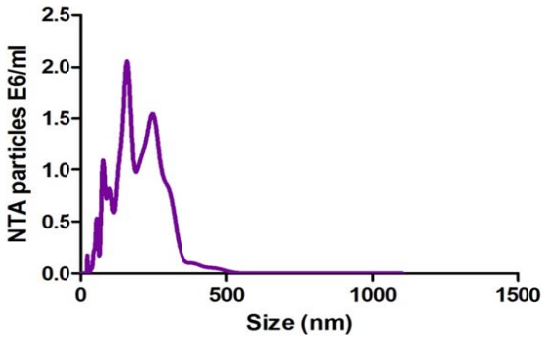

Fig. (4). NTA measurement of particle size (x-axis) and concentration (y-axis). Endothelial-derived microparticles measurement by nanoparticle tracking analysis from our experimental data.

2.1. MPs and Inflammation

By releasing cytokines as well as being involved in cell-cell interactions, MPs play an emerging role in inflammation. The pro-inflammatory potential of MPs is well established in both *in vitro* and *in vivo* studies.

During inflammation, the anticoagulant pathways are down regulated and inflammatory response is promoted. One of the stimuli for release of endothelial MPs is oxidative stress [21]. The endothelial MPs have oxidised membrane phospholipidson their surface due to this oxidative stress which cause adhesion of monocytes to endothelial cells as well as activation of neutrophils *in vitro* [22]. Leukocytes are attracted to the inflammatory site by adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin, which are expressed by stimulated endothelial cells. Leukocyte-derived MPs also express ligands for these adhesion molecules and thus these MPs are also attracted to the adhesion molecules. This leuko-endothelial adhesion leads to the release of cytokines and growth factors into the innermost layer of endothelial cells [17]. This is an important early event in inflammation.

Sepsis is characterised by the body's overshooting systemic inflammatory response to infection. Activated protein C (APC) used in treatment of sepsis inhibits coagulation factors Va and VIIIa by degrading them. This degradation is further enhanced by cofactor of APC called protein S and thus helps limit thrombin generation [23]. Monocytes and endothelial cells express endothelial protein C receptor (EPCR). Upon activation by APC and in the presence of protease activated receptor 1 (PAR1), endothelial cells as well as monocytes release EPCR-containing MPs. These EPCRs are not truncated like the soluble EPCR and thus are full length. In patients suffering from sepsis, these endothelial cell derived MPs may confer benefits [23]. In septic patients, TNF-α release EMPs in a

greater number which could explain why the benefit of anti-TNF-α treatment was limited in these patients [23].

2.2. MPs and Coagulation

The pool of bioactive effectors that MPs store account for their proinflammatory (secretion of IL-β) as well as prothrombotic properties around their site of formation [24]. There is a strong molecular link between inflammation and coagulation as the latter is stimulated by the former. As mentioned before, the PS exposed on the outer membrane leaflet has a high procoagulant activity. Inflammation leads to the expression of intravascular tissue factor (TF). MPs express TF on their outer membranes just as they express PS. PS along with TF acts as a site for assembly of tenase complexes of the coagulation cascade which is then activated forming thrombin. This further affirms the role of MPs in procoagulation [17] (Fig. **2D**). Large von Willebrand factor multimers has been detected on endothelial MPs which play a role in aggregation of platelets [4].

2.3. MPs and Cell Signalling

Cellular communication both locally and distant from the point of MP releaseis mediated by two mechanisms. Firstly, the bioactive molecules (ligands) that MPs carry activate receptors on the target cell. Secondly, they are involved in a host of cellular functions by transferring their content e.g. receptors, active lipids or RNA (mRNA) to recipient cells [15]. These membrane-associated receptors can be transferred where needed, proteins and active lipids released, and genetic information exchanged by transfer of RNA [25]. The MP surface molecules enhance the efficiency and specificity of this transfer system. They act as adhesion molecules making the interaction with the target cells easier and more specific. These surface molecules also regulate the release of MP contentfacilitating cell signalling [15].

2.4. MPs and Vascular Function

Endothelium-derived MPs have been shown to stimulate vascular repair *in vitro*. Platelet-derived MPs can transfer arachidonic acid to endothelial cells. This has several effects such as increased adhesion of monocytes to endothelial cells as well as vasodilation [26]. The former is achieved by inducing expression of intercellular adhesion molecule 1 (ICAM-1) in endothelial cells and stimulating lymphocyte function–associated antigen 1 and macrophage antigen 1 in monocytes. This involves protein kinase C-mediated pathways [26]. The latter is achieved by stimulating expression of cyclooxygenase 2 (COX-2) and thus increased production of prostaglandins which in turn induces vasodilation of arteries [21].

However, increasing amount of evidence suggests possible deleterious effects of MPs in certain pathological conditions. This is due to amplification of inflammation and vascular injury through increased production of inflammatory cytokines and chemokines, as well as increased endothelial adhesion molecule expression [24]. In cardiovascular diseases, endothelial dysfunction leads to vascular function being diminished: Endothelium-derived NO mediates relaxation of heart in healthy conditions. In acute myocardial infarction, MPs that are shed in peripheral blood impair the endothelial nitric oxide (NO) transduction pathway specifically [27].

2.5. Anti-Coagulant, Anti-Inflammatory Activity of MPs and Immunoprotection

As early as 1991, Tans and colleagues suggested an anticoagulant activity of MPs. Upon platelet activation, the released MPs accounted for 25% of the anticoagulant activity possessed by platelets [28]. Because they demonstrated similar levels of procoagulant activity by platelets and platelet derived MPs, they inferred that the same platelet-activating stimuli can give rise to procoagulant or anticoagulant activities *in vivo* [28].

MPs can induce apoptosis in immunocompetent cells by expression of Fas ligand (FasL) on their surface [29]. Although this kind of response could lead to control of overwhelming inflammation, it could also lead to devastating effects; e.g., MP-associated FasL derived from tumour cells in malignancy could induce apoptosis in T-cells thus providing an escape route for tumour from the immune system [30]. They block the entrance of immunocompetent cells (lymphocytes etc.) in cancerous lesions and prevent them from exerting their antitumor effects [30].

Recent studies show the protective effects of MPs in health and disease. Downregulation of proinflammatory mechanisms in the earlier stages of severe inflammation could be attributed to leukocyte-derived MPs [24]. This in turn confers protection, such as immunosuppression induced by erythrocyte-derived MPs, which may arise on prolonged storage of blood products [31]. These leukocyte-derived MPs harbour an endogenous anti-inflammatory protein called Annexin 1 (AnxA1) and release anti-inflammatory cytokine transforming growth factor β1 (TGF-β1) at early stages of inflammation itself. They also inhibit proinflammatory cytokines such as IL-8 and tumour necrosis factor α (TNF-α) in a dose-dependent manner in turn inhibiting macrophage activation and thus inflammation [32]. However, later in inflammation, there may againbe MP-induced release of proinflammatory cytokines such as IL-1β and IL-6.

However, certain methodologic differences to previous studies may have resulted in this piece of information; one of them being the time period of stimulation of cells. As opposed to the conventional several hours-long stimulation, Gasser and Schifferli stimulated cells for only 20 minutes. Thus due to this shorter stimulation period, the composition of MPs collected for this study may have been different to those produced at later time points [26].

After release from donor cells, MPs are transferred to acceptor cells. While studying the mechanisms of MP transfer to monocytes and B cells, Koppler *et al*. found that this transfer renders B cells less active and induces anti-inflammatory cytokine profile in monocytes [33]. There is a downregulation of LPS-mediated pro-inflammatory cytokine (e.g. TNF-α) release from these monocytes and IL-10 (anti-inflammatory cytokine) release is substantially enhanced. MP-induced inhibition on monocytes was found to be dose-dependent which suggested that *in vivo* inhibitory strength and efficacy of MPs is dependent on their local production and concentration [33].

When MPs released by Annexin A1-positive human leukocytes were delivered intravenously, they inhibited the recruitment of neutrophils to an IL-1β-inflamed air pouch. This, alongwith other experiments done by Dalli *et al*.,

showed that the anti-inflammatory properties exhibited by leukocyte-derived MPs bearing active Annexin A1, could be helpful in dampening down the inflammatory response in microcirculation [34].

Monocyte-derived MPs have been shown to have anti-inflammatory properties by enhancing the expression of proteins effective in anti-inflammation, such as peroxisome proliferator-activated receptor γ (PPAR-γ) protein [35]. The anti-coagulant role of activated protein C (APC) has already been discussed before (in sepsis). It binds to EPCR on MP surface and degrades coagulation factors V and VIII. Recombinant human APC (rhAPC) is therefore not only used in treatment of severe sepsis (as mentioned before), but also provides neuroprotection in ischemia [25]. Rise in the levels of APC expressing MPs in septic patients after treatment with rhAPC proves that the APC-mediated cytoprotective effects such as cytokine modulation and antiapoptotic activity are partly due to these MPs [36]. Such MPs can play a role in balancing haemostasis by opposing the thrombotic effects of procoagulant MPs [37]. Thus APC infusion could be useful to release anticoagulant MPs that inhibit coagulation induced by MPs released upon cell apoptosis, in conditions such as sepsis or heatstroke (characterised by leukocyte-derived MP release and their procoagulatory effects in vessels) [25]. Thus MPs can play opposing roles, based upon the proteins they carry. They can be mediators of cell death as well as cell survival.

The anticoagulant activity of the MPs which is opposite to the normal coagulant potential discussed before could be due to several factors. One of them is an approximate 10-fold higher PS concentration exposed on these MPs [25]. Additionally, based on their parental cell, various proteins present on the MP surface such as anticoagulant thrombomodulin, EPCR, also indicate the anticoagulant nature of these MPs [25].

3. MPs AND INFLAMMATORY DISEASES

Acute inflammation has a rapid onset but is not very long lasting (several days) and is caused by release of vasoactive amines such as histamine, serotonin and bradykinin, and complement components -all classical mediators of acute inflammation -, causing the signs and symptoms associated to it (in vascularised tissue: warmth, redness, swelling, pain, reduced function). If the inflammation ends, the tissue is healed; otherwise it may develop into chronic inflammation. Clinical examples of acute inflammation are asthma, acute respiratory distress syndrome, septic shock and vasculitis. The role of MPs in determining the outcome of sepsis is beginning to be dissected [24].

Chronic inflammation has a delayed onset and is longer lasting (months to years). It is caused by continued or prolonged presence of antigens. Cytokines, reactive oxygen species, growth factors and hydrolytic enzymes are the mediators that cause the symptoms linked to this type of inflammation: Chronic inflammation usually causes irreparable damage in that it leads to the tissue destruction. As MPs carry various immunologically active molecules, they are considered inflammatory mediators in diseases like multiple sclerosis and various cardiovascular diseases. Of note, there may be acute exacerbations in chronic inflammatory disease, and the role of MPs is beginning to be investigated in these [38].

3.1. Cardiovascular Diseases (CVD)

Cardiovascular disease is one of the leading causes of mortality in developing as well as developed countries. Inflammation along with coagulation and vascular dysfunction, underlines the mechanisms involved in pathogenesis of various cardiovascular diseases (ischemic heart disease, cerebrovascular and peripheral vascular disease). The role of MP in coagulation, vascular dysfunction as well as inflammation has been established in the previous paragraphs. The link between clinical CVD and increased MP levels, along with their proinflammatory and procoagulant potential renders them harmful. It is also hypothesised that endothelial cell-derived MP levels are higher during active plaque formation rather than in mature plaques [39]. Thus there is plenty of evidence that links MP levels to CVD, however, there is not enough evidence of their direct pathophysiological link to CVD [39].

MPs can be strongly linked to endothelial damage, coagulation, activation of platelet, all of which relate to the risk factors of cardiovascular disease, such as thrombosis [39]. Platelet MPs have been extensively studied for their role in clotting.

Cardiac events such as acute myocardial infarction and sudden cardiac death are a principle cause of mortality in patients with end-stage renal disease (ESRD). Thrombotic events are commonly seen in ESRD patients, in whom endothelial cells are dysfunctional. Patients undergoing long-term dialysis are also chronically inflamed with endothelial dysfunction, both known to be significant cardiovascular risk factors [40]. In maintenance dialysis patients, increased TNF-α and IL-6 levels - both of which are inflammatory mediators- and increased levels of C-reactive protein (CRP) suggest inflammation as a risk factor. CRP is a risk factor for CVD in healthy populations as well as ESRD patients [40]. Data published by this group has shown elevated levels of pro-coagulant endothelial and platelet derived MPs in patients with ESRD on dialysis compared to controls [41]. Oxidative stress which is one of the stimuli for the release of MPs is increased in ESRD patients and even more so in patients with CVD [40].

Atherosclerosis occurs when fatty acids build up in the intima making the arteries harder and narrower. The endothelial vasodilatory mechanism is disrupted by decreasing prostacyclin and NO production [42]. Proinflammatory cytokines and cell adhesion molecules are upregulated leading to adherence of monocytes, neutrophils and platelets to the inflamed endothelium.

Patients with subclinical atherosclerosis are shown to have increased levels of leukocyte-derived MPs, identified by their affinity for CD11a [43]. The abundance of macrophage and leukocyte MPs within plaque samples also accounts for significant TF activity [4].

3.2. Coronary Artery Disease (CAD)

A progressive increase in endothelial cell-derived MP levels was seen from patients with unstable angina to acute myocardial infarction as well as acute coronary syndrome

(ACS) compared to healthy subjects: While investigating the correlation between endothelial cell-MPs and coronary artery disease (CAD), a group found that levels of two different populations of endothelial cell-MPs ($CD31^+$ and $CD51^+$) were elevated in acute and chronic stable phases of ischemia [44]. CD31 and CD51 are platelet-endothelial cell adhesion molecules. Interestingly, this study showed that there was a difference between endothelial cell-MPs released during acute and chronic phases of ischemia: There was a pronounced increase in $CD31^+$ EMP levels in ACS patients but not patients with stable angina pectoris when compared with the control group. On the other hand both ACS and patients with stable angina had elevated $CD51^+$endothelial cell-MP levels which meant that while increased $CD31^+$ endothelial cell-MP levels correspond to the acute phase (ACS), $CD51^+$ endothelial cell-MP corresponded to all CAD [44].

ACS and stable angina differ from each other in terms of the composition of plaque, and extent of inflammation, hypoxia and thrombosis. Hypoxia results in inflammation and this was suggested as the reason for the difference between $CD31^+$ endothelial cell-MP levels in ACS and SA relative to healthy subjects [44]. Although platelet MPs levels are also elevated in CAD, endothelial cell-MPs are considered better markers than platelet MPs because these did not discriminate stable angina patients relative to control as $CD31^+$endothelial cell-MP did [44].

High levels of endothelial cell-derived MPs are found in coronary artery events [45]. In parallel, apoptotic bodies are generated from atherosclerotic endothelium [42].

3.3. Autoimmune Diseases

In autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and antiphospholipid syndrome, the body's immune regulation is compromised leading to destruction of self by T-cell and/ or antibody mediated processes.

3.3.1. Multiple Sclerosis (MS)

Multiple sclerosis, a demyelinating disease affecting the central nervous system, shows elevated levels of endothelial cell-derived MPs, which may act as molecular biomarkers of endothelial dysfunction even before seeing lesions [38]. *In vitro*, these endothelial cell-derived MPs ($CD54^+$EMP and $CD62E^+$EMP)promote monocyte adhesion to and their migration through human brain microvascular endothelial cells and its monolayers (particularly monolayer of cerebral ECs) [46], thus providing a mechanism by which the blood-brain barrier is disrupted in disease [23] and contributing to the pathogenesis of MS by facilitating the formation of demyelinated lesions. $CD31^+$endothelial cell-derived MPs in circulation are associated with contrast-enhancing lesions on brain MRI and can be assayed using flow cytometry, in turn giving an estimate for their elevation in MS patients compared to healthy control subjects [46].

3.3.2. Rheumatoid Arthritis (RA)

Levels of platelet MPs have been shown to be elevated in plasma of patients with RA, whereas granulocyte and monocyte-derived MPs are more prominent in the synovial fluid. Other MPs present in the synovial fluid are T cell, B cell, platelet and erythrocyte-derived MPs [4]. These MPs induce TF/ factor VII-dependent thrombin production resulting in proatherogenicinflammation of arteries and formation of "rice bodies" (fibrin clots) in joints, despite lower levels of MPs in periphery [47]. Platelet-derived MPs are considered as directors of inflammation leading to RA [48]. After finding a mean of2×10^5 $CD41^+$MPs per microliter of synovial fluid from 20 RA patients, detectable levels in only 1/20 synovial fluid from patients with osteoarthritis, their pathophysiological significance in inflammatory arthritis was studied*in vivo* in mouse [48]. Collagen-activated platelets can form MPs (distinct from intact platelets)[49]. These platelets predominantly express glycoprotein VI (GPVI) (collagen receptor)[48] which, when activated generates these MPs [50]. These MPs stimulated RA fibroblast-like synoviocytes (the most abundantcell in the pathologic rheumatoid pannus tissue) to secrete IL-1β inducible cytokines Il-6 and 8 [48]. This can also be achieved by T-cell and monocyte-derived MPs, which, in addition, stimulate synthesis of matrix metalloproteinases in synovial fibroblasts [51]. Complement is instrumental in RA pathogenesis, and can be activated by MPs *in vitro via* the classical pathway [52]. All in all, MPs are a separate vehicle, apart from antibody / complement mediated effects, to amplify inflammation and tissue damage [26, 53].

3.3.3. Antiphospholipid Syndrome (APS)

APS (also called Hughes' syndrome) is an autoimmune disorder characterised by thrombosis, activation of apoptosis and the presence of high levels of pathogenic antiphospholipid antibodies (aPL). This is associated with several pregnancy-related disorders and complications including stillbirths, miscarriages and preeclampsia [54]. Platelet-derived MPs associate with one of these aPLs, β2-glycoprotein-1 (apolipoprotein H) antibodies, implying a direct pathogenic role of platelet-derived MPs in APS [4]. Additionally, levels of TF-exposing endothelial cell-derived MPs are elevated in APS patients compared to healthy control group [55]. TF initiates the coagulation cascade *via* the extrinsic pathway and its upregulation mediated by aPLs has been established in APS [56]. Thus a role of MPs in APS is evident. Inflammation not only serves as a link between the characteristic procoagulant phenotype and therefore thrombus generation observed in APS patients (Fig. **5**), but is also a vital mediator of placental injury [54]. Studies show that uncontrolled and amplified complement activation as well as inhibition of complement regulatory proteins upon binding of aPLs to trophoblasts, leads to inflammation in these patients. The recruited inflammatory cells (monocytes and neutrophils)and release of inflammatory mediators thus cause placental injury [57].

4. CLEARANCE OF MPs

Contrasting with the 10 days life span of platelets, platelet-derived MPs have a varying life-span. In mice, it can be up to 30 minutes whereas, in rabbits, as short as 10 minutes or less [23].

4.1. *In Vitro* Experiments

Rand and colleagues studied the clearance of platelet-derived MPs from circulation of rabbits. Platelet suspensions were labelled for detection by NHS-biotin and were

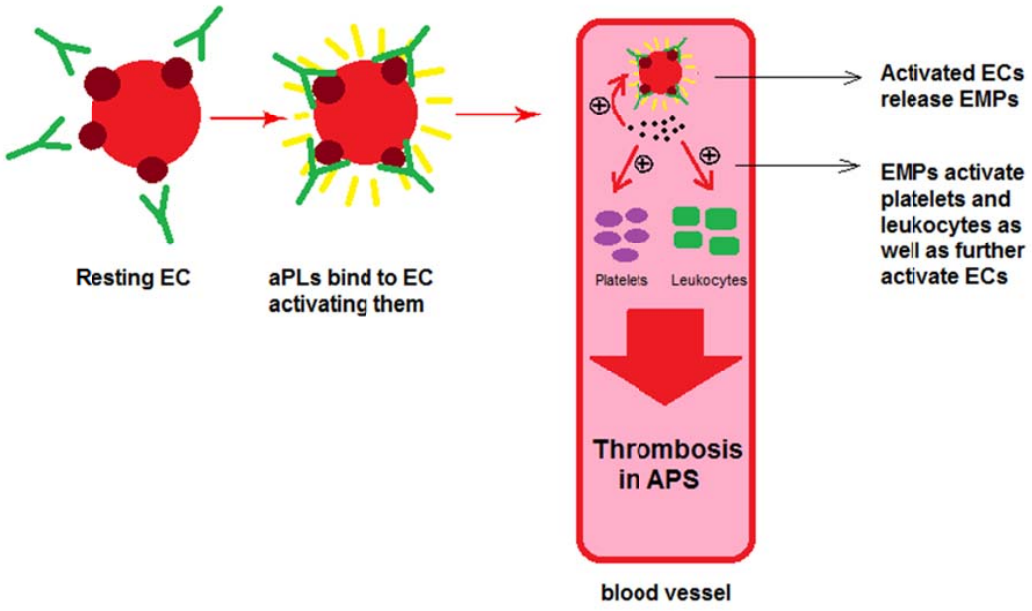

Fig. (5). Proposedmechanism of action of endothelial microparticles (EMPs) in thrombosis in Antiphospholipid syndrome (APS): aPLs (green) bind to resting endothelial cells (EC) (red) activating them. Activated ECs then release EMPs which stimulate platelets (purple), leukocytes (green) as well as further activate more ECs. Together these events lead to the development of thrombosis.

stimulated using calcium ionophore A23187for 1 hr to release MPs. Recipient rabbits were either injected with untreated biotinylated platelets (i.e. platelets that were not stimulated), or with platelet-derived MPs derived from the treated platelet suspension. Blood samples were taken, MPs isolated and analysed using flow cytometry. The percentage of MPs after 10, 30 and 60 minutes of injection was calculated. MPs were cleared within 10 minutes of injection and no MPs were detected within the next 50 minutes in the circulation [58]. It was also observed that in the absence of a clearance mechanism, platelet-derived MP levels can increase significantly in circulation. Both these observations imply that MPs are continuously produced in conditions where increased MP levels are detected [58].

4.2. Proposed Mechanisms of Clearance

MPs are ingested by the so-called macrophage-phagocyte system (formerly reticulo-endothelial system) macrophages in liver and spleen, as well as other tissues [59] by recognition of PS exposed on MPs. The direct mechanism involves the binding of PS with the receptors that recognise it (e.g. CD36, SR-A) and subsequent phagocytosis [60]. Alternatively, proteins such as growth arrest-specific gene 6 product (GAS6), protein S, lactadherin or complement are needed to bridge PS to the ingesting cell [59]. For instance, the most abundant complementopsonin on activation is C3b which opsonises platelet derived MPs. The C3b expressed on these PMPs results in their binding to complement receptors on RBCs (CR1), delivery to phagocytes in liver and spleen and subsequent clearance [23]. These mechanisms involve only clearance of cell-fragments that contain PS.

4.3. When it Goes Wrong

When this clearance procedure is compromised, various adverse effects, such as immunosuppression and increased thrombotic tendency occur [59], as explained hereafter.

4.3.1. Suppression of Immunity

Immune suppression seen after a blood transfusion could be attributed to elevated MPs. Erythrocyte-derived MPs were shown to suppress some of the properties of the immune system *in vitro*, for example by inhibiting activation of macrophages by zymosan A and lipopolysaccharide (LPS) indicated by a reduction in TNF-α and IL-8secretion [61].

4.3.2. Elevated thrombotic potential

Splenectomy is a common therapeutic practice when trauma occurs or in haematological disorders. In splenectomised immune thrombocytopenic purpura (ITP)patients, erythrocyte and leukocyte-derived MPs were shown to be elevated compared to the control (non-splenectomised ITP) patients [62]. This could also contribute to the increased thrombotic tendency in the splenectomised patients [59].

5. NOVEL AREAS OF RESEARCH

During the last two decades, an increasing interest has developed in MPs as potential biomarkers of several diseases and many studies have focused on their physiology and pathophysiology. They deliver various biological compounds to their target cells, thus bringing about a functional response. This is why they may have a potential as a novel class of drug delivery systems. The recent discovery of miRNA in MPs provides a means to dissect the effect of MPs on target cells.

5.1. MicroRNA

MicroRNAs (miRNA) are small, conserved, non-coding RNAs involved in regulation of translation of mRNAs and proteins thus regulating gene expression [63]. Their importance in health and disease may be under appreciated; their ability to regulate the transcription of not only their target genes but also indirectly of other genes in cell cycle

regulation and metabolism renders them of high significance [42]. Platelet-derived MPs carry miRNA that can act as a communication source between platelets and other vascular cells which can be crucial to inflammatory processes as well as vascular homeostasis [64]. For instance, miRNA-223; the most abundant miRNA in platelets is predicted to play a role in osteoclast, myeloid and granulocyte differentiation as well as hematopoietic stem cell proliferation [65]. MiRNA-223along with other miRNA (miR-96, miR-200b, miR-495and miR-107) is also instrumental in aggregation, secretion and adhesion of platelets [64].

The significance of MPs as biological vectors derives from the fact that a vast majority of plasma miRNA exists as packaged in MP. They are an efficient way of randomly packaging and transferring miRNA within the human body and acting as a source of communication between the cells transferring genetic content between them [63].

Involvement of miRNA-133 from platelet derived MPs has been indicated particularly in myocardial infarction [66]. Its levels were found to be elevated in plasma of patients with myocardial infarction corresponding to the increased MP levels [67]. Study on MP derived from monocytic leukaemia cell line THP-1 showed that by transferring miRNAs (e.g. miRNA-320) to cardiac cells, they can exert their proinflammatory effects by altering gene expression directly [68]. Compared to non-stimulated THP-derived MP, miRNA-21 in stimulated THP-derived MP is one of the highest to be up-regulated among other miRNAs. miRNA-21 upregulation is also observed in hypoxic vascular smooth muscle cells effecting endothelial inflammation and facilitating the endothelial to fibroblast transition during inflammation [69].

Examples of miRNAwhich exert anti-inflammatory effects are; miRNA-126, which inhibits VCAM-1 [70] and is found decreased in CVD patients [41] and miRNA-431, an inhibitor of macrophage migration inhibitory factor (MIF), which has a cardioprotective effect in ischemia reperfusion injury [68].

Over the years, miRNAshave been found not only intracellularly but also extracellularly in body fluids such as plasma, serum, urine and milk [71]. As well as acting as signalling molecules they can act as predictive diagnostic markers of disease. They can also be delivered therapeutically for disease treatment as pointed by several studies. MPs can be used for this purpose, and their potential in delivering compounds for therapy is discussed below.

5.2. MPs as Drug Delivery Systems

Conventional drugs are usually of small molecular weight and their delivery up to the target area is sufficient for them to carry out their functions. Biological drugs on the other hand are large and charged molecules. This makes them incapable of crossing plasma membrane, and therefore they require the complete delivery up to the target point. Therefore such carriers are required which ensure this delivery to the final destination [72]. Moreover, use of high drug doses to target inflammatory cells when treating inflammatory disorders could have undesired effects on other tissues. Although molecularly–selective drugs have been manufactured, but due to their lack of cell-type specificity they are of less therapeutic benefit *in vivo* [73]. The role of MPs as biological vehicles for transport of proteins, mRNA, miRNA and various other biologicals between different cells has been established by various studies as described above. The biologicals that MPs deliver play an important role in many disease states, most notably inflammation and cancer. Thus they have been proposed to be of therapeutic benefit by transferring exogenous drugs to target cells *in vivo*. With potentially less side effects, they can perhaps deliver anti-inflammatory drugs to activated inflammatory cells, by ensuring specificity for target cells [73]. Other advantages of using MPs as vehicles for drug delivery as opposed to the conventional systems might include [74]:

I). They can deliver functional RNA in cells.

II). As they are biological vehicles, they can be more stable in blood.

III). If MPs derived from the patient himself is used for drug-delivery, they would be more tolerant to the body's immune response thus ensuring quicker and safer delivery where required.

Activated myeloid cells derived from monocytes are key players in various chronic inflammatory diseases such as CVD or autoimmune disorders and cancers [73]. Acting as scavengers due to their phagocytic properties, they take up vesicles in peripheral blood including exosomes [73]. Thus these exosomes were used to deliver an anti-inflammatory polyphenol curcumin as a complex to activated myeloid cells which induced apoptosis in them. Due to its poor solubility and poor bioavailability, curcumin has not been of much clinical efficacy in humans. However, the study by Sun *et al.* on mice showed its increased stability *in vitro* and bioavailability *in vivo* as asignificant reduction was seen in the lipopolysaccharide (LPS)-induced inflammatory response [73]. Sun *et al.* were the first to report the successful loading of curcumin into exosomes for delivery *in vivo*.

This and more studies by the same group support a new method of drug delivery, and set stage for more experiments to be done using other therapeutic compounds that can be packaged in CMVs. Similar approaches can be used for MPs instead of nanoparticles (exosomes) to target inflammatory drugs to the desired location, the key factor being the size. The difference between their sizes leads to the various differences at multiple levels ranging from formulation to their use *in vivo* [75]. Two novel approaches that could exploit MPs for drug delivery are discussed below:

5.2.1. Engineering of Natural Cell Membrane-Derived Vesicles (CMV)

The cell derived microvesicles can be engineered to express the therapeutic compounds (drugs, miRNA, siRNA) and deliver them to a local cellular environment [71]. In such an approach, RNAi (interference) or antisense-approaches can be used to reduce expression, while miRNA or protein encoding plasmids can be used to transfect vesicles in order to increase expression levels [72].

Recently, by experimenting on mice Alvarez *et al.* proved that cell membrane derived vesicles can be used biotechnologically to our benefit [76]. The difficulty of targeting macromolecular drugs to brain cells due to the blood brain barrier is a well-established issue in pharmacology. Therefore, they used immature dendritic cell-derived exosomes from the bone marrow of mice and used them to transfer exogenous siRNA both *in vitro* as well as *in*

vivo. To ensure specific delivery of siRNA to the CNS and to avoid drug clearance mechanisms of the body, a protein expressed on the surface of exosome(lamp2b) was exploited to display a specific peptidewhich only binds nicotinic acetylcholine receptors (nAChR) in neuronal cells and blood brain barrier vascular endothelial cells. This was successfully demonstrated without any side-effects such as toxicity or unwanted immune reaction [76].

5.2.2. Artificial CMVs

Natural CMVs may contain components that may not all be necessary for them to act as drug-delivery vehicles. However, there are also certain specific lipids and proteins essential for cell targeting and completion of their designated functions. Utilising these pieces of information, artificial CMVs that mimic the actions of endogenous CMVs can be generated synthetically by using those specific components that are essential in MP in targeting and delivery.

To mimic endogenous MPs, it is necessary to know about their *in vivo* kinetics, tissue distribution and targeting behaviour that would make them suitable drug carriers [72]. Artificial liposome based systems have been in the limelight as a starting point [74]. This system uses two main components present in CMVs, PS and lactadherin. Lactadherin-opsonised microvesicles exposing PS on their surface were uptaken to a much greater extent by human umbilical vein endothelial cells [77]. Egg phosphatidyl glycerol (EPG) bearing vesicles were not taken up by endothelial cells as EPG is a phospholipid that lacks the lactadherin recognition signal [74]. Therefore this model supports the cellular interactions as in endogenous microvesicles.

In a study by Martinez-Lostao *et al*., a marked reduction was seen inexosomes carrying soluble, unconjugated death ligand APO2L/TRAIL in RA patients. This showed that rheumatoid synovial fluid T cells were sensitive to APO2L/TRAIL [78]. In a recent study by the same group, artificial lipid vesicles resembling exosomes were prepared and used to study the effectiveness of APO2L/TRAIL when conjugated to these liposomes in a rabbit model of antigen-induced arthritis (AIA). This conjugation increased the bioactivity of APO2L/TRAIL and was thus a more effective treatment when compared to the previous study by the same group, as shown by reduced inflammation in the joints [79].

Thus the advantage of using artificially prepared microvesicles is that the removal of those components which are not needed make the system less complex and makes it easier to replace them by other biological such as therapeutic cargo.

6. FUTURE PERSPECTIVES

With all the research being done come various challenging questions for future studies. The enumeration of MPs could be of vital significance as their increased number is an indicative of various disease states as discussed. This raises a question that whether or not does their absolute number relate to disease severity? Due to their increased number particularly in inflammation, can they be used as marker of inflammation and response to therapy? They express specific antigens on their surface and hence can they be targeted now that we know their surface expression? The microRNA that they carry can act as signalling molecules resulting in cell regulatory and metabolic functions, based on their type. How can we use this knowledge of the type of miRNA that MPs carry to gain therapeutic benefits? Which miRNA types are involved in regulation of critical genes responsible for inflammation? Are MPs filtered through a dialysis membrane in chronic renal patients? What is their relative importance in relation to cytokines- an interesting epiphenomenon or more? Are there other areas where MPs might be explored, e.g. transplantation? Do MPs modulate antigen presentation? These and many other questions are raised which require further experimental work.

Further refinement and validation of MP detection methods will contribute to more accurate quantification and characterisation of MPs. Use of specialised flow cytometers that can analyse particles as small as 0.1μm should be more commonplace for analysing human CMVs [38]. An extended knowledge of MP release, target-binding and pharmacological manipulation of MP generation would open insight into their paradoxical roles [10] (Fig. **6**).

Ultimately, questions about whether MPs are the cause or consequence of inflammation and whether they exacerbate or abrogate disease states, cannot be answered properly until there is a consensus on experimental methodologies so that results can be compared over different studies. Moreover, to assess the complete potential of MPs in drug delivery, knowledge of MP components essential for their durability in circulation, target specificity, and immunogenicity is required. Such biotechnological and artificial approaches have to be devised that can refine the MP production, targeting and loading methods, thus making MPs more suitable and reliable as drug delivery vehicles [74].

7. DISCUSSION/SUMMARY

This review has analysed the current knowledge on the composition, origin, formation, release and functions of MPs and in particular their link to inflammation and the related disorders. The understanding of the complexity of MPs remains incomplete and requires further in depth research both *in vitro* as well as *in vivo*. Various technologies have been used for detection, measurement and characterisation of MPs. Flow cytometry and nanotechnology have been discussed. NTA has proved to be more accurate compared to flow cytometry in sizing the MPs from plasma samples. Flow cytometry appears to be more appropriate for analysis of cells, not sub-cellular particles. Due to their small size, MPs fall at the edge of the reliable discrimination using conventional flow cytometers [39].

It has been established that MP are neither inert nor ‘dust’. They are not by-products of other cellular processes; instead, they are key players in various immune related disorders through their pro-inflammatory or anti-inflammatory properties. Their increasing significance in both physiological as well as pathological processes has been discussed in the scientific literature. They are novel signalling particles that can be generated during the two events that are elementary to inflammation; cell activation and cell apoptosis. They may be instrumental in coupling thrombosis and inflammation. Apart from initiating and amplifying inflammation and mediating cell death, they may also potentiate cell survival, confer immunoprotection and

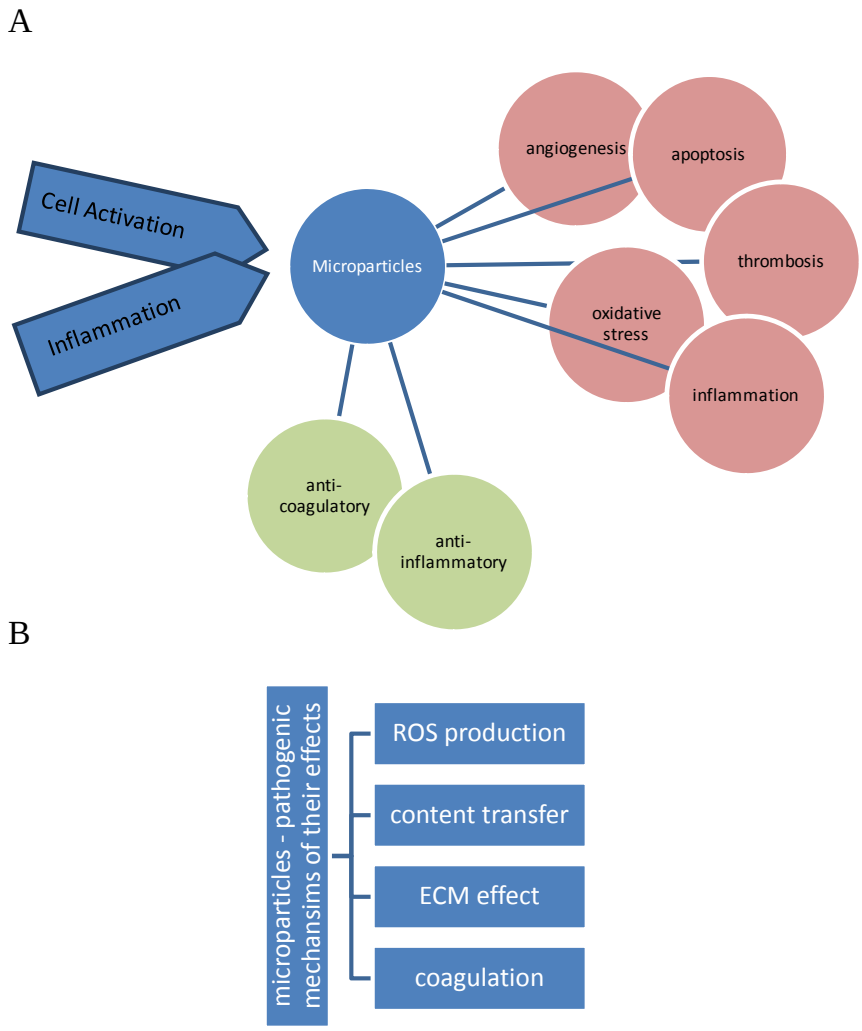

Fig. (6). Microparticles are significantly involved in pathophysiological mechanisms of disease. (**A**) Their roles are likely to qualitatitvely differ depending on the acuteness or duration of disease (arrow indicates arbitrary timeline). (**B**) Inflammation, coagulation, cell damage and transfer are generic features of proinflammatory MPs [3].

maintain endothelial integrity in early regulation of inflammation-induced coagulopathy. Their elevated levels in various inflammatory diseases such as cardiovascular disease, and autoimmune disorders such as rheumatoid arthritis, confirm their value as functional biomarkers of these and various other disorders. However, their composition and origin, mechanisms of stimuli leading to their formation and sites of formation determine their biological functions as friends or foes e.g. maintainers of vascular homeostasis or cause of vascular dysfunction [80].

Their role as conveyors of biological information is of particular importance as it can modulate different pathways at the same time in target cells. The proof of concept for this has come through recent studies that have exploited MPs biotechnologically for drug delivery. This approach can alter the pharmacokinetics as well the pharmacodynamics of different types of drugs by increasing their bioavailability, target specificity and providing protection from degradation in the circulation, thus ensuring their safe, controlled and sustained delivery to the target site. This reduces their undesired effects at sites other than their target site, and increases the therapeutic benefits that they have to offer.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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