Circulating Microparticles in Systemic Lupus Erythematosus

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PUBLICATIONS

This Ph.D. thesis is based on the following papers:

I. Distinct features of circulating microparticles and their relationship to clinical manifestations in systemic lupus erythematosus, Nielsen CT, Østergaard O, Johnsen C, Jacobsen S, Heegaard NH. Arthritis & Rheumatism, Vol. 63, No. 10, October 2011, pp 3067–3077 (1).

II. Quantitative proteome profiling of normal human circulating microparticles, Østergaard O*, Nielsen CT*, Iversen LV, Jacobsen S, Tanassi JT, Heegaard NH. *) Have contributed equally to the study. Journal of Proteome Research, 2012 Apr 6;11(4):2154-63 (2).

III. Increased IgG on cell-derived plasma microparticles in systemic lupus erythematosus is associated with autoantibodies and complement activation, Nielsen CT*, Østergaard O*, Stener L, Iversen LV, Truedsson L, Gullstrand B, Jacobsen S, Heegaard NH. *) Have contributed equally to the study. Arthritis & Rheumatism, 2012 Apr;64(4):1227-36 (3).

AIMS OF THE STUDY

A hallmark of systemic lupus erythematosus (SLE) is the production of autoantibodies against nuclear components. These components become accessible as nuclear debris on the surface of dying (apoptotic) cells and are also contained in membranous vesicles termed apoptotic bodies as part of normal physiology. An increased risk of the development of SLE has been observed in a variety of innate immune deficiencies affecting the clearance of dead and dying cells and their debris. Thus it has been hypothesized that an improper disposal of the apoptotic cells and bodies may cause a persistent autoantigen overload triggering antinuclear autoimmunity. When tolerance first has been broken these extracellular autoantigens may also take part in immune complex formation contributing to inflammation and sustained autoimmunity. In the blood a heterogeneous pool of subcellular membranous vesicles collectively termed microparticles (MPs) is circulating. These MPs are released constitutively and may increase as a result of cellular activation and apoptosis, i.e. apoptotic bodies only constitute a subfraction of the circulating pool of MPs. As these MPs may reflect the state of the parental cells and tissues, MPs have been proposed as potential biomarkers of e.g. pathological processes that are difficult to access, likely to be reflected in changes in their concentrations, cellular origins and composition.

The overall aims of the studies included in this thesis were to develop and apply quantitative and qualitative methods to characterize MPs from SLE patients and control subjects and to correlate these findings with clinical and serological characteristics of SLE patients to explore the putative roles of MPs in inflammation and SLE disease pathogenesis. Accordingly, the specific aims of the studies in this thesis were to:

1. Characterize the size, morphology and ultrastructure of circulating MPs in SLE patients and healthy controls using electron microscopy.

2. Characterize the protein composition of circulating MPs using mass spectrometry in SLE and healthy controls.

3. Quantify and compare the cellular origins, concentrations and distributions of circulating MPs in SLE patients with healthy controls.

4. Quantify, characterize and compare the concentrations of annexin-binding and non-binding MPs in SLE patients with healthy controls.

5. Quantify and compare the cargo of surface-bound immune complex components on SLE-MPs with control MPs.

6. Explore associations between MP-findings and clinical manifestations and serological measures in the SLE patients.

1. INTRODUCTION

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease presenting with a wide array of clinical manifestations and incompletely understood pathogenesis. SLE is characterized

by alterations in both the innate and adaptive immune system ultimately leading to the loss of immunologic tolerance and occurrence of autoantibodies against nuclear material (4). In the blood a heterogenous pool of subcellular vesicles circulate, collectively termed microparticles. These circulating microparticles are potential biomarkers, carriers of nuclear autoantigens and triggers of autoimmunity in SLE.

The focus of this review is to elucidate the putative connections between SLE and SLE pathogenesis and circulating microparticles as a framework and point of reference for the experimental work carried out in the ph.d.-study.

1.1. SYSTEMIC LUPUS ERYTHEMATOSUS

1.1.1. Systemic lupus erythematosus – a heterogeneous clinical disease

SLE is a disease with a female to male ratio of 9 to 1 primarily affecting women in the fertile age and of non-Caucasian origin (5). The incidences and prevalences of SLE vary considerably depending on ethnicity. However, a recent Danish population-based study reported a prevalence and yearly incidence of SLE of 20-30 and 1 per 100,000, respectively. (6).

SLE is a clinically heterogenous disease that may affect most organ systems. Some of the common manifestations include non-erosive polyarthritis, malar rash, cutaneous hypersensitivity to sunlight, serositis and nephropathy in addition to haematological abnormalities (7). Patients may also experience symptoms from the central nervous system, lungs and the heart including vascular manifestations either representing as vasculitis, thromboses as part of having a secondary anti-phospholipid syndrome and Raynaud's phenomena. More than ninety-five percent exhibit the serological hallmark of SLE, antinuclear antibodies (ANA), and activation of the complement system is also found (7). Since no symptoms or laboratory measurements are specific for SLE a set of classification criteria with a specificity and sensitivity of 96 % has been devised (8;9). SLE is a serious chronic disease characterized by alternating periods of disease flares and remissions, and it is associated with a decreased quality of life, physical disability and, although life expectancy has improved, premature death. The mortality in SLE is primarily related to active disease, disease-associated organ damage, infections and cardiovascular events (10).

1.1.2. Genes, the environment and SLE

The etiology of lupus is still elusive. Triggering of lupus in some individuals e.g. by sun-exposure or drugs and the striking female predominance have suggested that environmental factors acting on a genetic susceptible individual contribute to the development of chronic autoimmunity.

Epidemiological studies have identified a number of environmental factors suspected of modulating susceptibility to SLE and some of these include viruses such as Epstein-Barr virus, hormones, smoking, alcohol intake and exposure to ultraviolet light, aromatic amines, pesticides, silica, heavy metals, organic solvents including various drugs like hydralazine and INF-α (11).

A genetic predisposition to SLE was initially recognized by observations of sibling risk ratios ranging from 8 to 29 and concordance rates of 25 % for monozygotic twins and 2 % for dizygotic twins (12;13). Unbiased large-scale genome-wide association studies have identified more than 20 risk loci associated with SLE, most of them with modest odds-ratios (1.15-2.4) (14). These risk genes are mainly involved in biological processes and pathways participating in antigen presentation, immune complex (IC)

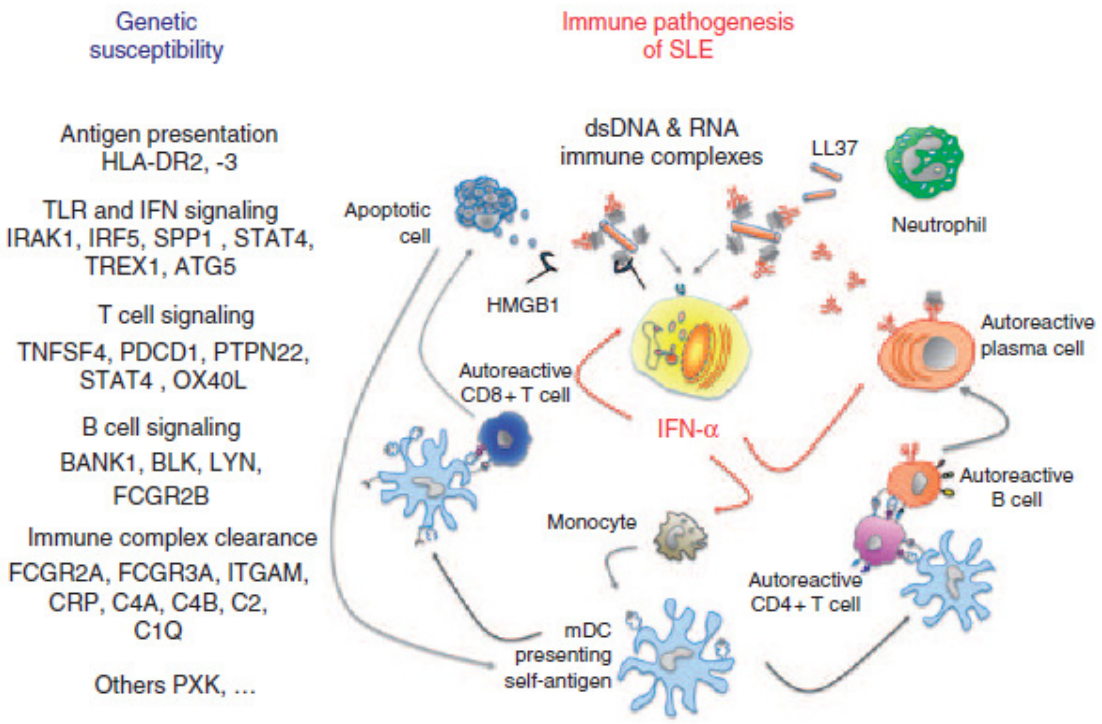

Figure 1
Schematic overview of SLE pathogenesis. (Obermoser G et al.. The interferon-α signature of SLE. Lupus (2010), 19, 1012-1019) (16).

and cell clearance, TLR-function, interferon-α production, B- and T-cell signalling as outlined in Figure 1 (15). These findings help understand the genetic contribution to SLE susceptibility, but more interestingly they highlight pathways of importance in SLE pathogenesis, many of which have already been found disturbed in SLE patients. Additionally, these unbiased approaches have also identified novel loci with no obvious connection to known pathways pointing in new directions for exploring unrecognized mechanisms involved in SLE pathogenesis (14).

1.1.3. Pathogenesis of systemic lupus erythematosus

As the immune system is constantly challenged, the ability to sense and handle self- and non-self-antigens properly is essential to avoid autoimmune disease. A characteristic feature of SLE is the loss of immunological tolerance against self and the occurrence of autoantibodies against nuclear components, antinuclear antibodies (ANA) (7). Initiation and propagation of autoimmunity is a result of intricate innate and adaptive immunological interactions. It is believed that an increased production of type I interferons prime and promote the triggering of autoreactive T- and B-cells by autoantigens from improperly cleared apoptotic cells and activated neutrophils and hence the formation of autoantibodies and immune complexes (16). The focus of the first section of this review is therefore on the role of improperly cleared dying cells as sources of autoantigen-containing particles and endogenous triggers of autoimmunity.

1.1.3.1. Dying cells as a source of autoantigens in SLE

The observations that nuclear autoantigens are generated and exposed on cell surface blebs in vitro during the processes of programmed cell death, apoptosis, have fostered the notion that the apoptotic cell could be a major endogenous source of autoantigens triggering antinuclear autoimmunity in SLE (17). As these autoantigen-containing blebs are released from the cells as vesicles termed apoptotic bodies, they may putatively circulate in a particulate form as part of the heterogeneous pool of microparticles in the blood. In the pre-autoimmune state apoptotic cells and bodies may “fuel” and direct the immune system towards antinuclear autoimmunity. In the setting with broken immunological tolerance MPs may serve as antigenic targets and participate in the formation of immune complexes, which contribute to the inflammatory and autoimmune reactions observed in the SLE patients.

1.1.3.2. Apoptosis

Apoptosis is an important part of maintaining homeostasis in multicellular organisms and involves as cascade of controlled events ultimately leading to the rapid non-inflammatory removal of unwanted cells (18). Early morphological features of apoptosis are nuclear changes such as chromatin condensation and fragmentation and clustering of heterogeneous ectopic RNP-derived structures in addition to vigorous cell membrane blebbing (19-21). Once the apoptotic programme is initiated, the apoptotic cells quickly release “find me” signals to attract professional phagocytes that recognize surface-exposed “eat me” signals such as phosphatidylserine (PS) and subsequently engulf and digest the apoptotic corpses in an immunologically silent manner (22). Apoptosis and the safe removal of dying cells are tightly regulated. If early apoptotic cells are not efficiently cleared they may progress into later stages of apoptosis and secondary necrosis resulting in a loss of membrane integrity, the release of damage-associated molecular patterns (DAMPs) and removal associated with inflammatory reactions (23).

1.1.3.3. SLE autoantigens are exposed on apoptotic cells and bodies

Casciola-Rosen and colleagues made the initial observations that during UV-induced apoptosis of keratinocytes nuclear and cytoplasmic antigens were generated and relocated into two sets of blebs or apoptotic bodies, i.e. smaller blebs (1.3 µm) containing fragmented endoplasmic reticulum, ribosomes and Ro and larger blebs (2.7 µm) containing nucleosomal DNA, Ro, La and small nuclear ribonucleoproteins (17). Subsequent studies using different cell-lines and inducers of apoptosis have confirmed the relocation of the endoplasmic reticulum, nucleosomes and ribonucleoproteins into blebs or apoptotic bodies during both early and later stages of apoptosis (19;24-28). Blebbing and autoantigen relocation is a dynamic and regulated process. It depends on effective caspase-mediated activation of DNA fragmentation and activation of ROCK-I, and DNA and RNA seem to be distributed into separate apoptotic bodies (26;29-32). Importantly, nucleosomal antigens are detectable on the surface of apoptotic cells and bodies and thus directly accessible to the immune system (24;25;27;28). Also PS, the major “eat me” signal for professional phagocytes and an established marker of onset of apoptosis, is exposed on the surface of apoptotic cells and bodies (17;24;33). However, PS-exposure appear not to be a universal feature of apoptotic cells and bodies formed in vitro, including apoptotic bodies containing nucleic acids (24;26).

Observations of SLE autoantibodies targeting protein antigens that undergo post-translational modifications or proteolytic cleavage during apoptotic cell death further substantiate the apoptotic cell as a source of autoantigens (34;35). Post-translational modifications, such as phosphorylations, acetylations, methylations and citrullinations, and proteolytic cleavage of proteins are essential parts of the normal regulation of apoptosis. Utz et al. observed that ANA positive SLE sera compared with control sera precipitated specifically phosphorylated proteins derived from apoptotic cells (36). Specific modifications of ribonucleoproteins as determinants of antigenicity have been described in SLE (34). One of the most studied modified autoantigen is the spliceosomal U1-70K snRNP, which is both phosphorylated and cleaved by caspases during apoptosis (37). It was shown that only the apoptotic-modified protein compared with the intact protein was recognized by autoantibodies from SLE mice and human subjects (38). Additionally the modified U1-70K snRNP have also been found capable of triggering autoreactive B-cell and T-cells in murine models (39;40).

Also, SLE neutrophils have recently been shown to be prone to NETosis, i.e. neutrophil cell death characterized by the release of neutrophil extracellular traps (NETs) containing chromatin strands, antimicrobial peptides and other neutrophil proteins and peptides upon immune complex stimulation. These structures are also antigenic targets of autoantibodies and potent stimulators of IFN-α release from plasmacytoid dendritic cells that are chronically activated in SLE patients (41;42)

To extrapolate these in vitro observations suggestive of accessible autoantigens on apoptotic bodies, Pisetsky et al. recently showed that IgG from SLE plasma and not from controls recognize plasma microparticles from healthy donors. However, the binding was not reduced by DNase treatment and no correlation with anti-dsDNA in the SLE plasma was found, suggesting that other antibody specificities than anti-dsDNA could account for the antibody binding (28). Additionally an increased number of IgG-carrying events with particle properties was detected in SLE pa-

tients compared to healthy controls and was associated with serum anti-dsDNA levels.

Altogether these findings demonstrate that the antigenic targets of SLE-specific autoantibodies may be expelled from neutrophils and apoptotic cells in an accessible particulate form.

1.1.3.4. Exposure to apoptotic cells may trigger autoimmunity

The notion that apoptotic cells could serve as substrates fuelling autoimmunity have been tested in murine models. Mevorach et al. injected syngeneic irradiated thymocytes into naïve healthy mice and observed that the majority of the mice developed transient ANA and antibodies against single-stranded DNA and cardiolipin. Upon repetition of the injections a second rise in the antibody levels was observed (43). The mice appeared healthy and showed no signs of kidney disease, but all had IgG deposits in the kidneys several months after immunization. Similarly, exposing normal mice to apoptotic cells combined with β2-glycoprotein I resulted in the generation of anti-phospholipid antibodies (aPL) and lupus anticoagulant activity while either apoptotic cells or β2-glycoprotein I alone did not have this effect (44). Bondanza et al. vaccinated normal and SLE prone mouse strains with dendritic cells that had phagocytosed apoptotic thymocytes and observed that all mice developed ANA and anti-dsDNA (45). The serological autoimmunity was only transient in the normal mice and did not lead to pathology. In contrast, autoimmunity was maintained and kidney disease accelerated and survival shortened in the susceptible mice. Lastly, in a mutant mouse model that lacked the ability to fragment DNA, relocation of nuclear components into apoptotic bodies was impaired, and when challenged with pristane, an inducer of SLE-like autoimmunity in mice, antibodies developed against cytoplasmic antigens and phospholipids but not against nuclear components as opposed to the non-mutant mouse (31). These data suggest that relocation of antigens to blebs may direct the immune response against the particular antigens in the blebs.

Taken together these mechanistic studies show that increased loads of apoptotic cells and their associated autoantigens are capable of triggering autoimmunity in both the normal and susceptible subject, but that persistent autoimmunity relies on a SLE prone genetic background.

1.1.3.5. Defective clearance of apoptotic cells and SLE

Since the healthy immune system efficiently recognizes, removes and tolerates the apoptotic cells billions of times a day, several fundamental mechanisms regulating the immune system and fate of the apoptotic cells must be altered to trigger autoimmunity. Efficient phagocytosis of apoptotic cells depends on surface-exposed markers on the dying cells, soluble adaptor proteins, receptors on the phagocytes and functional phagocytes (22). Several human and murine observations including genome wide association studies indicate that defective clearance of apoptotic cells and debris is an important mechanism in the breakdown of immunological tolerance in SLE. These observations include deficiencies of opsonising molecules, defects of extracellular DNA degradation and phagocyte deficiencies (23).

Apoptotic cell clearance relies on a redundant system of soluble opsonising molecules that facilitate recognition and rapid phagocytosis by professional phagocytes. The molecules that recognize surface-exposed molecules on apoptotic cells include members of the two protein families, the collectins (C1q, mannose-binding lectin (MBL), ficolins, surfactant A and D) and the pentraxins (serum amyloid P, C-reactive protein (CRP), pentraxin 3) (23). Accordingly, the finding of an increased prevalence of autoimmune responses in patients and mice lacking or having decreased functions of one or more of these proteins has added credibility to a potential link between defective clearance of apoptotic material and autoimmunity (46). The most prominent example is the increased risk of developing SLE, when lacking functional components of the early parts of the classical complement cascade particular C1q and C4 (47;48). Individuals lacking C1q are almost all going to develop SLE. The SLE-like systemic autoimmunity has been replicated in mice homozygotic deficient for C1q. These mice developed ANA, anti-histone antibodies and severe proliferative glomerulonephritis with electron dense deposits and multiple extracellular apoptotic bodies in the glomeruli (49). In humans also variant alleles of mannose-binding lectin have been associated with an increased risk of SLE, but although MBL deficient mice had compromised apoptotic cell clearance, spontaneous autoimmunity did not develop (50;51). It has also been shown that serum amyloid P binds chromatin, and mice deficient in serum amyloid P develop nuclear autoimmunity and glomerulonephritis. CRP may also have a protective role (52). Low concentrations of CRP in humans appear to predispose to SLE (53). Additionally, CRP injected into SLE mice appear to attenuate autoimmunity, and disease onset was delayed in mice expressing a human CRP transgene (54;55).

The necessity to control extracellular DNA to avoid autoimmunity was further supported by observations of a lower activity of DNase1 in SLE sera and by the observations that DNase1 deficient mice developed ANA positive SLE-like disease with glomerulonephritis (56).

Additionally, mice with defects in MER, a receptor for endocytosis, and defects in the intracellular pathways of apoptotic cell degradation exhibit signs of systemic autoimmunity (23).

In some SLE patients deficient macrophage clearance appears to be a prominent feature. It was demonstrated that cultured monocyte-derived macrophages isolated from SLE patients had an impaired uptake of apoptotic cells compared with healthy and disease controls (57). Accumulation of apoptotic cells have been detected both in the bone marrow and in skin upon UV-radiation in SLE patients (58;59). In a subset of SLE patients compared with disease controls more undigested apoptotic nuclei were detected in lymph node germinal centers (60). These apoptotic nuclei were not taken up by tingible body macrophages and instead they were found associated with the surface of follicular dendritic cells.

Thus, the proper control of the clearance of cellular debris seems highly important to avoid autoimmunity. As many of the mentioned defects are rarely found in patients, the extent of the contribution from clearance defects in the initiation and propagation of autoimmunity in vivo in humans is not known. It should be noted that the observed autoimmunity in the single component deficiencies may also reflect other immune regulatory roles of these molecules than apoptotic cell clearance. As an example C1q may influence the negative selection of B-cells in the bone marrow and has recently been shown to attenuate INF-α production from IC-stimulated pDCs (61;62). Moreover, in the setting of already broken peripheral tolerance, circulating autoantibodies influence handling and immunogenicity of apoptotic remnants. Autoantibodies may opsonise and facilitate the phagocytosis of apoptotic and secondary necrotic cells in a complement-dependent manner thereby inticing inflammation (63-65). The role of autoantibodies in this respect is not finally settled as Reefmann et al. found that autoantibodies inhibited phagocytosis as may also be the case regarding NETs (66;67).

1.1.3.6. Autoantibodies, autoantigens, clearance and triggering of INF-α production

INF-α seem to be a critical cytokine in SLE pathogenesis, and patients exhibit an ongoing production of INF-α (68). Autoantigen-containing ICs are potent inducers of INF-α from pDCs and several of the genes associated with SLE involve TLR-function and interferon pathways (16). Apoptotic bodies and NETs are major suspects as autoantigen sources of these immune complexes contributing to the sustained INF-α production (Figure 1).

Normally, type I interferons are produced by pDCs in response to viral infections (69). pDCs express constitutively high amounts of TLR-7 and TLR-9 and these cells are capable of producing vast amounts of INF-α upon stimulation (16). Characteristically, SLE patients display signs of sustained INF-α production with higher cytokine concentrations in the blood and increased expression of INF-α inducible genes in circulating leucocytes, the latter referred to as the INF-α signature (70-72). The pronounced production of INF-α observed in SLE is likely to be caused by stimulation by endogenous nucleic acids together with autoantibodies acting through both TLR and non-TLR pathways. Apoptotic bodies with bound autoantibodies engage FcγRIIa receptors (CD32) on pDCs and are internalized to the endosomal compartment where TLR-7 and TLR-9 are stimulated (73;74). Activated neutrophils release NETs that contain complexes of antimicrobial peptides and self-DNA and are also capable of stimulating INF-α production from pDCs through TLR-9 (41;42). During apoptosis molecules associated with the autoantigens may increase INF-α production, e.g. high mobility group box 1 (HMGB1) associated with nucleosomes and the antimicrobial peptide LL37 bound to self-DNA in NETs (42;75). INF-α have widespread immunomodulatory effects that may contribute to the breakdown of peripheral tolerance and perpetuation of autoimmunity. Briefly, INF-α promotes the maturation of monocyte dendritic cells affecting antigen presentation, upregulates T-cell co-stimulatory molecules (CD80 and CD86) influencing autoreactive CD4+ and CD8+ T-cells, promotes TLR-7 and –9 on pDCs, mDCs and macrophages increasing nucleic acid responsiveness and promotes the differentiation of B-cells into antibody producing plasmablasts (16;69).

Thus autoantigens from dying cells, putatively in the form of microparticles, may serve as precursors of circulating immune complexes capable of stimulating immune responses. In vitro studies suggest that the presentation of autoantigens in membranous vesicles may modulate and even increase the immunostimulatory properties of the formed MP-ICs. Recently, the immuno-stimulatory properties of ICs and autoantigen-associated proteins and peptides like HMBG1 and LL37 was shown (42;75). It is likely that other proteins and peptides in circulating MPs may have similar effects and additionally MPs may have adjuvant properties due to their liposomal structure, a principle used in vaccines (76). Furthermore, MPs carry adhesion molecules on the surface that may affect their adhesion to cells and sequestration of MPs and ICs in the tissues.

1.2. MICROPARTICLES IN THE BLOOD

1.2.1. Definition of microparticles in the blood

The presence of circulating membranous vesicles have been known for decades and they were originally thought to be inert debris, blood dust (77). These vesicles are heterogeneous in terms of size, origin, cargo, and stability and may be defined as all membrane-enclosed bodies smaller than 1 µm in diameter found in the blood (78). Subcellular vesicles are released from cells both constitutively and during the processes of cellular activation, apoptosis and necrosis (Fig. 2). As described, apoptotic bodies may constitute a source of circulating autoantigens involved in inflammation and autoimmunity in SLE. Apoptotic bodies are likely to constitute only a minor fraction of the pool of circulating subcellular membranous vesicles in the blood and may be larger particles overlapping in size with platelets (Fig. 3) (79). Definitions of the various subfractions of vesicles vary and no consensus exists (78). In this thesis we collectively use the word microparticles but this term is often used more narrowly to classify the subfraction of membrane vesicles that is derived from plasma membranes, of which MPs released from activated cells may also be called ectosomes (Fig. 2) (80;81). Another important type of membrane vesicles are exosomes, small vesicles that originate from intraluminal vesicles in intracellular multivesicular bodies and subsequently released by exocytosis after fusion of multivesicular bodies with the plasma membrane (80). Additionally, the circulating membrane vesicle fraction contains other membrane-enclosed species, e.g. organellar remnants of demised cells, nanoparticles, and other membranous particles (80;82;83).

1.2.2. Formation of plasma microparticles

In the resting cell the membrane bilayer maintains a specific asymmetric lipid composition with PS and phosphatidylethanolamine predominantly present on the inner leaflet and sphingomyelin and phosphatidylcholine enriched on the outer leaflet (87). This asymmetry is controlled by an inward-directed pump ("flippase"), aminophospholipid translocase, specific for PS and phosphatidylethanolamine, an out-directed pump ("floppase"), and scramblase, an enzyme that contributes to unspecific bidirectional movements of lipids between in the bilayer (88). Following e.g. cellular activation or apoptosis the cytosolic calcium concentration rises and triggers the collapse of the membrane asymmetry, relocation of antigens and proteolytic degradation of the cytoskeleton leading to dynamic blebbing, surface-exposure of PS and ultimately release of membrane-enclosed MPs (20;88). A wide spectrum of bioactive molecules such as lipids, proteins and nucleic acids are released with these MPs, but the mechanisms controlling the MP cargo are as yet elusive.

1.2.3. Cellular origin of plasma microparticles

The origin of cell-derived MPs can be determined by their expression of cell-specific surface markers (89). Several markers have been used to determine the origins of MPs and some are considered as constitutive cell markers while others are more sensitive to stimulation (90;91). Typically, a minor panel of antibodies against specific epitopes is used to identify MPs from erythrocytes (CD235a), platelets (CD42a/b, CD41a/b and CD61), leukocytes and subsets (CD45, CD14, CD3, CD4, CD8, CD20 and CD66b) and endothelial cells (CD146, CD144, CD51, CD105, CD31+/CD42a–, and CD62E) (89;90). As PS is exposed on the MP surface during formation, PS-binding probes - primarily annexin V - have been used as a marker of a definite cell-derived MP. To date no marker have been identified that can discriminate between apoptotic-derived MPs or MPs from activated cells.

While the absolute MP concentrations may differ between studies, solid evidence has accumulated to state that the majority of circulating membrane-shedded MPs derives from platelets and erythrocytes while the remainders originate from leukocytes, endothelial and even tumour cells (87;92).

1.2.4. Microparticles as mediators of intercellular information

Circulating MPs are not only garbage but may also serve as a large reservoir of bioactive molecules (lipids, proteins and nucleic

acids) that can be readily mobilized and act locally or over long distances. In vitro and ex vivo studies have provided insights into the potential biological significance of MPs. MPs seem to exert physiological functions in a variety of ways. For example, MPs may act through: 1) the release of molecules to their environment, 2) the activation of enzymatic cascades such as the coagulation system by surface-exposed molecules, 3) the direct interactions with target cells, and 4) the transfer of molecules like

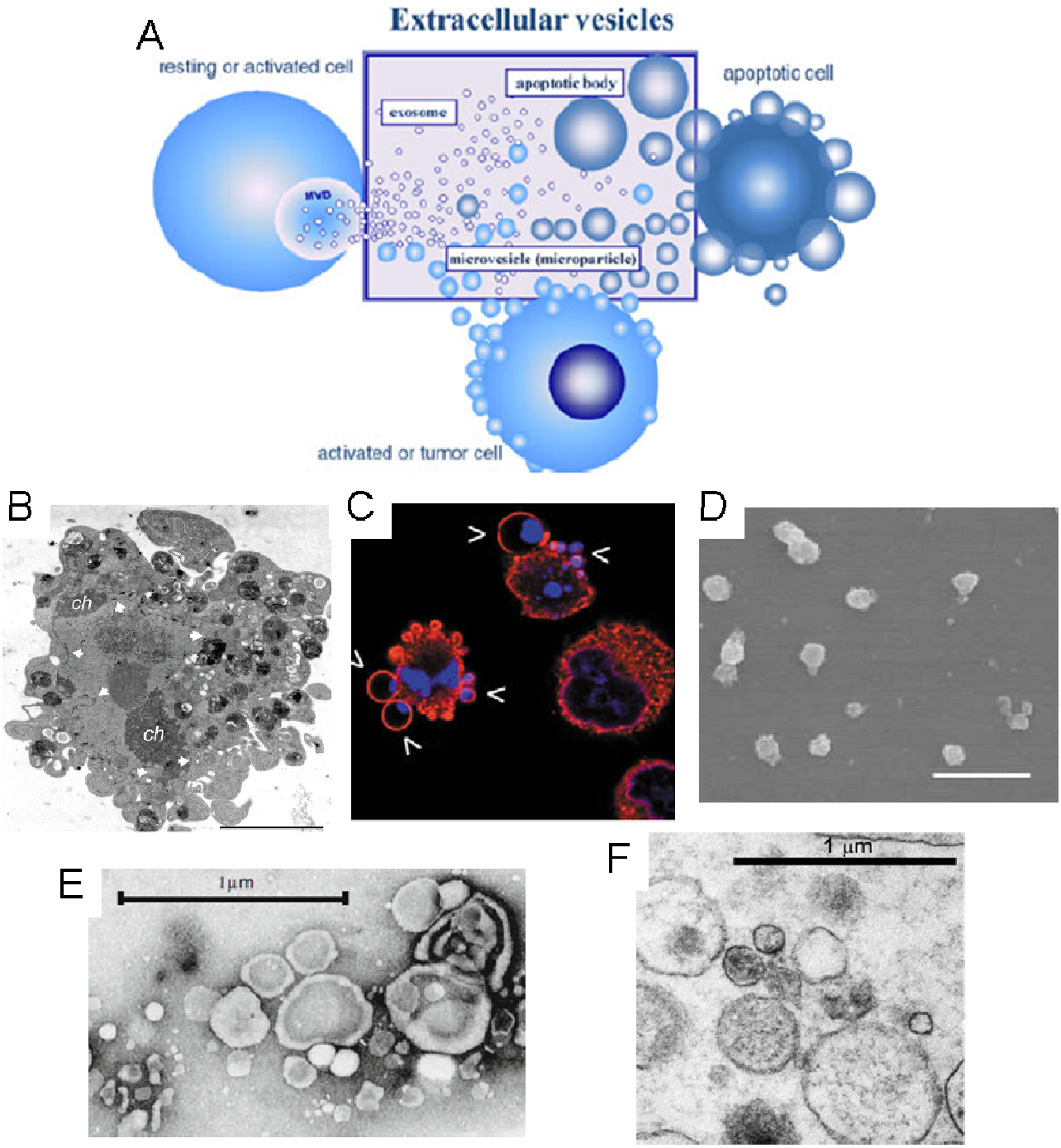

Figure 2
Illustration of cellular contributions to the pool of circulating MPs and examples of apoptotic cells, bodies and plasma microparticles. A, Illustrates the cellular contributions to the heterogenous pool of particles in the blood (79). B-C, Transmission electron microscopy and confocal microscopy of apoptotic cells with numerous surface blebs in vitro (white markers). Red stain = endoplasmic reticulum; blue stain = nucleic acids (26). D, Scanning electron microscopy of isolated plasma MPs (84). E, Transmission electron microscopy of plasma MPs in suspension using negative staining (85). F, Transmission electron microscopy of embedded plasma MPs (86).

arachidonic acid, microRNA, and surface receptors from MPs to acceptor cells (78;93). Plasma MPs as traffickers of bioactive molecules are thus emerging as important for intercellular communication with pleiotropic roles in the regulation of coagulation, angiogenesis and immune reactions (93). Below, some important findings are highlighted, while a complete review of the field is beyond the scope of this thesis (93-95).

The field of coagulation and haemostasis has attracted particular interest with respect to MPs. Circulating MPs are capable of triggering coagulation by exposing PS and tissue factor. The exposure of PS on MPs provides binding sites for the coagulation factors II, Va, and Xa, serving as a platform for the assembly of the prothrombinase complex on the surface. The prothrombinase complex then catalyzes the conversion of prothrombin to thrombin. This has led to the notion of MPs being highly procoagulant (95). Also, tissue factor (TF), the principal trigger of the extrinsic pathway coagulation on platelets has been detected on circulating MPs capable of initiating the coagulation processes and serving as a vehicle of TF (95). MPs also carry large von Willebrand multimers contributing to platelet aggregation, and P-selectin glycoprotein ligand 1 involved in intercellular adhesion (95). The overall importance of MP formation and PS exposure in coagulation have been corroborated by the rare clinical condition, Scott's syndrome, where a defective scramblase activity compromises PS-exposure and MP formation ultimately causing a severe bleeding disorder (87).

MPs display both pro- and anti-inflammatory functions in vitro. MPs from granulocytes (ectosomes) had no effect on the release of IL-8 and tumor necrosis factor (TNF)-α, but triggered the release of transforming growth factor-β from macrophages suggesting an anti-inflammatory role of shedded MPs in early inflammation (81). Another potential way of MPs to downregulate immunity is by inducing apoptosis as demonstrated in macrophages, circulating angiogenic cells, and in T- and B-cell lines, the latter by MPs displaying FasL (78;96;97). As an example of their pro-inflammatory properties, MPs may induce IL-1β release from monocytes and IL-6, CC-chemokine ligand-2 and TNF-α from endothelial cells (93).

In rheumatoid arthritis MPs appear to directly contribute to synovial inflammation. MPs in synovial fluid are more abundant than in plasma and are primarily of granulocytic and monocytic origin (98;99). Berckmans et al. found that isolated synovial fluid MPs stimulated the release of pro-inflammatory chemokines and cytokines, MCP-1, IL-8, RANTES, IL-6, ICAM-1 and VEGF from synovial fibroblasts (100). Distler et al. also observed that MPs from T-cells and monocytes were capable of triggering the release MMP-1, MMP-3, MMP-9 and MMP-13 from synovial fibroblasts (101). Recently, Boilard et al. showed that platelet-derived MPs in synovial fluid may amplify arthritis (102).

These observations clearly suggest regulatory roles of MPs involved in disease pathogenesis, however, we still have little understanding of the functions of MPs in health and disease and ultimately why cells shed MPs (103).

1.2.5. Plasma microparticles as potential biomarkers

As microparticles are formed constitutively during normal physiology and change numbers, phenotypes and composition in pathology, MPs have been proposed as future diagnostic, prognostic or predictive tools in a multitude of diseases (79;104-106). MPs have attracted particular interest since they may provide information on the state of not easily accessible tissues (105). In asymptomatic individuals leukocyte-derived MPs were found associated with inward carotid remodelling and subclinical atherosclerosis (107). In specific pathological states, plasma MPs have been found elevated in a coagulopathies, cancer, cardiovascular, metabolic, infectious and autoimmune diseases (79;87;93). Circulating EMPs may serve as predictors of cardiovascular mortality and adverse cardiovascular events in patients with coronary artery disease, pulmonary hypertension or end-stage renal disease (105). In some cross-sectional studies MP levels correlated with disease activity measures suggesting their potential as monitors of disease activity. Erdbruegger et al. found that the level of EMPs was associated with disease activity and that their numbers dropped significantly during remission in patients with anti-neutrophil cytoplasmic antibody associated small-vessel vasculitis (108). In contrast, van Eijk et al. observed elevated numbers of C1q-positive MPs in patients with active RA with no change despite clinical and paraclinical remission (109). No studies to date have investigated the diagnostic potential of MPs in any disease.

1.2.6. Plasma microparticles in SLE

Plasma MPs have been previously been quantified in both systemic connective diseases and vasculitides (reviewed in (93)). Hypothetically, improperly cleared apoptotic cells and increased inflammation with cellular activation would lead to increased concentrations of MPs, changes in subpopulation distributions and antigenic composition. The studies on MPs to date in patients with SLE and both primary and secondary anti-phospholipid syndrome (APS) have, however, been contradictory. In SLE patients increased levels of MPs from various origins, i.e. platelet-, leukocyte- and endothelial cell-derived, have previously been reported (110-115). However, others did not find any difference in total MP concentrations (AnxV-binding) or levels of platelet-derived MPs compared to healthy controls (28;116;117). Alterations in MP-phenotypes and antigenic composition of MPs in SLE patients have not been investigated. The only study to explore the potential association between MP concentrations, autoantibody profiles and disease activity measures found a negative correlation between PMPs and the anti-dsDNA levels in a limited number of samples (115). Also the cargo of C1q and IgG have been explored on SLE MPs. Nauta et al. showed that C1q was present in equal proportions in SLE and healthy controls, and Pisetsky et al. observed that SLE patients had increased concentrations of IgG carrying particles suggesting that MPs may be antigenic targets (28;117). However, these particles were not labelled with a cell-specific probe or antibody and may thus in part reflect soluble immune complexes (28). Thus the concentrations of MPs, their roles and MPs as antigenic targets in SLE and inflammation have only been studied previously to a very limited extent.

1.3. IN VITRO CHARACTERIZATION OF PLASMA MICROPARTICLES

In general, the characterization of MPs is technically challenging because of their small size, heterogeneous densities and sizes and overlap with other particulate structures and platelets (Fig. 3). The majority of studies on circulating MPs have aimed to determine their concentrations and cellular origins, in addition to testing their properties ex vivo (89). The methods of choice have been solid-phase assays and light scatter flow cytometry. Light microscopic techniques such as confocal microscopy have served as complementary techniques to flow cytometry and to determine localization of cellular structures and molecules. Due to the wave-length of light MPs with sizes below 200-300 nm are undetected in light microscopy and light scatter flow cytometry (118). Novel technologies such as atomic force microscopy and fluorescence nanoparticle tracking analysis capable of antibody-specific

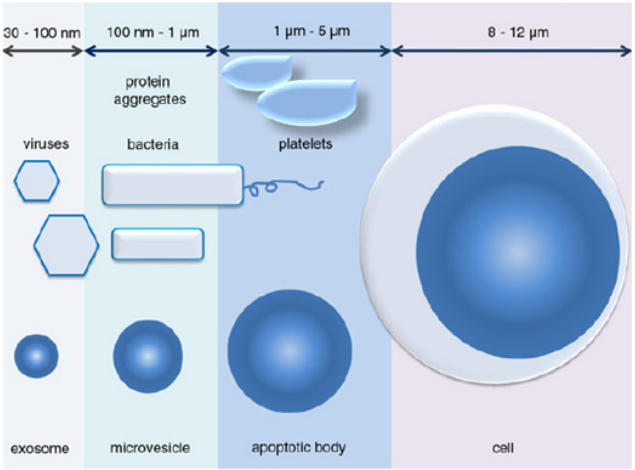

Figure 3
Illustration of the size overlaps of various particle fractions in the blood (79). Apoptotic cells may also release membrane-shedded particles smaller than 1 µm. Lipid vesicles are not shown. (Gyorgy G et al.. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell. Mol. Life Sci. (2011) 68:2667–2688) (79).

enumeration of the entire pool of MPs including obtaining size measures are promising future approaches (119;120). To characterize the size, morphology, ultrastructure and surface of MPs from below 50 nm and above both transmission and scanning electron microscopy have reached widespread use, but due to their labour-intensiveness only small sample sets have been analysed (118). Analyses of the MP composition have primarily focused on the proteome and not the nucleic acid or lipid composition (118). Mass spectrometry can provide an unbiased comprehensive coverage of the proteins in MPs and have been applied in a few studies of plasma MPs (104;121-124).

The main strategies and approaches to characterize MPs are briefly described in the following with emphasis on the technologies on which this thesis is based.

1.3.1 ENUMERATION AND CELLULAR ORIGIN OF MPS

Enumeration of MPs and characterization of their cellular origins have primarily been obtained using antibody-dependent solid-phase capture assays or light scatter flow cytometry. Both technologies can provide quantitative and qualitative MP measurements (89;118).

1.3.1.1. Solid-phase capture assays

Solid-phase capture assays are based on pre-coating of microtiter plate wells with a specific antibody or probe to capture the MPs of interest followed by the detection using either conjugated antibodies or by exploiting functional properties of the MPs primarily related to their exposure of PS or tissue-factor (118). The most widely used capture assay, the prothrombinase-assay, is flexible regarding capture antibodies or probes and can thus be used to quantitate MPs from different cellular origins. Here, captured MPs are quantified based on their ability to convert prothrombin to thrombin which in the assay depends on the amount of MP-expressed phosphatidylserine serving as a relative measure of the amount of captured MPs in the sample (89;125). PMP, LMP and total MP levels measured with the assay were recently reported to correlate with the corresponding MP concentrations quantified by flow cytometry (115).

Solid-phase assays are attractive since they allow high throughput analyses of untreated MPs directly in plasma. However, the assays are limited by being dependent on antibody specificity and affinity and by the possible interference from soluble antigens and influence from residual platelets in the starting material. Moreover, the prothrombinase-assay uses PS-exposure as a measure of MPs even though PS-exposure may be affected by pre-analytical handling of plasma samples and not all of the different subsets expose PS. As an example more than 90 % of PMPs expose PS in frozen samples as compared to less than 70 % for LMPs and EMPs (126;127).

1.3.1.2. Light scatter flow cytometry

Light scatter flow cytometry is a technology where fluorescently stained cells or particles in a fluid stream pass through a laser beam, and size measures, surface-marker expression, numbers and distributions of populations are obtained from the scattered and emitted light (128). Flow cytometry of submicron MPs compared with cells presents a number of challenges primarily related to the small size of MPs. Flow cytometry is the methodology of choice in our laboratory for MP-enumeration and antigen characterization as it provides more and specific information than the solid-phase assays.

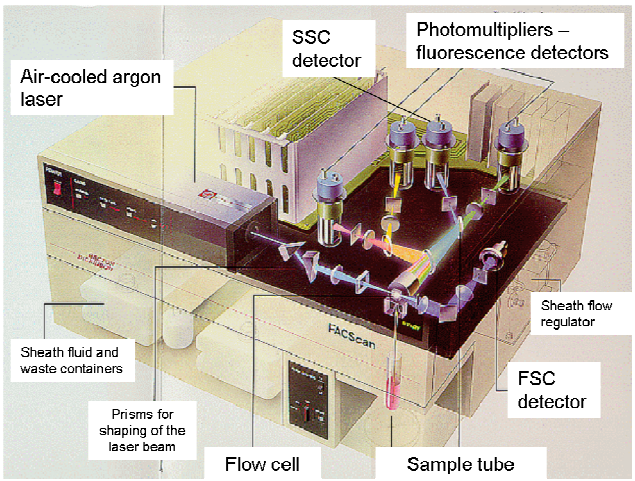

Figure 4
Illustration of a typical flow cytometer. Samples are placed on a tube holder and driven through the flow cell by the sheath fluid. In the flow cell the MPs/cells in suspension pass the laser beam and the forward and sideward scattered (FSC and SSC) and fluorescent emitted light are detected. (Flow Cytometry - A Basic Guide. Graham Leslie)

The general principles of flow cytometry of MPs are briefly described in the following (128). Samples are initially prepared by incubating either isolated MPs or PPP with one or more optimally titrated fluorescent antibodies or probes and then analysed immediately. The samples enter the flow cytometer driven by the sheath fluid allowing MPs to pass the laser light one at a time in the flow cell (Fig. 4). As MPs pass the laser beam the scattered and emitted light from each particle is recorded. Additionally the flow rate of the instrument is measured. Thus data is obtained on a large numbers of individual MPs enabling MPs and their cellular

subpopulations including those characterized by other markers to be identified and quantified using specialized software. Here, MPs are first identified by size using the forward and sideward scatter signals from standard polystyrene size beads and/or from MPs from in vitro activated cells. Then the identified MPs can be analysed by their fluorescent staining setting a detection threshold based on the signal from negative controls. In addition to identification and enumeration, relative measures of abundances of the stained markers, the fluorescence intensities, are also obtained.

Flow cytometry excels in providing specific MP measurements of several markers simultaneously on a large number of MPs based on both size and antibody-staining. However, MP flow cytometry is technically challenged by the small size of MPs. Improving the discrimination of MPs from other particulate structures in plasma and impurities in buffers and reagents is of paramount importance and may be achieved by washing of MPs, sterile filtration of buffers and sheath fluid and centrifugation of antibodies and probes (126). Weak fluorescent staining signal as a result of small sized MPs and/or few epitopes is often encountered rendering the use of strong fluorescent stains necessary and multi-marker staining challenging. Additionally since MPs are in the size range of and smaller than the wavelength of the laser light, small-sized MP populations are undetected by the flow cytometer and MP-sizing is restricted to the sideward scatter light signals as opposed to forward scatter used for cells (92;120).

Published flow cytometry MP protocols differ largely regarding platelet-poor plasma isolation, the choice of analyzing on washed MPs or MPs directly in plasma, cell-surface-markers used to identify the MP-populations, the used instruments, settings and data analyses. This renders comparisons between studies difficult and may to a large degree explain discrepant results between different studies (89;118). Analyses are either performed directly on PPP or on isolated and washed MPs using high-speed centrifugations (118). Washing MPs reduces abundant plasma proteins and background noise rendering analyses of MP-associated

plasma proteins such as immunoglobulins possible. However, washing of MPs may also result in MP-losses (99;126). Attempts to standardize MP measurements have recently been made. In the first large multicenter-study using the same protocols of sample preparation, instrument and gate setting in different laboratories using different instruments showed that comparable results across laboratories could be obtained, but some instruments failed to qualify and particular instruments from Becton Dickinson had a larger variation using the investigators strict gating protocol (129;130).

1.3.2. ELECTRON MICROSCOPY

Electron microscopy is a microscopy technique that provides high-resolution images with much higher magnifications than normal light microscopy and thus is suitable for the characterization of nano-sized structures like microparticles. Two supplementing variants, transmission electron microscopy (TEM) and scanning electron microscopy (SEM), excel in providing detailed characterization of the morphology and composition particular the ultrastructure (TEM) and surface topography (SEM) (131). Briefly, in TEM a beam of electrons is transmitted through an ultrathin specimen and the interactions between the electrons and the specimen form an image which is magnified, focused and transformed into a digital image (131). In SEM the electron beam scans the surface of the specimen pre-coated in an electro-conductive material, and the deflected and newly formed electrons are detected and assembled into a digital image (131). Electron microscopy can also be combined with gold-labelled antibodies or probes, termed immune electron microscopy.

EM techniques are highly informative and visually impressive, but are also limited due to the large amount of sample materiel required, and to the tedious and harsh preparation steps which may affect the morphology of the investigated material and complicate interpretation.

1.3.3. PRINCIPLES OF SHOTGUN/BOTTOM-UP PROTEOMICS OF MICROPARTICLES

MP proteomics is the study of the MP protein composition, the proteome, i.e. identification, characterization and quantification of the proteins present in MPs. The major analytical tool in this analytical field of science is mass spectrometry. Since the MP cellular origins and numbers change in pathology, and since the MP composition reflects different processes such as cellular growth, activation, apoptosis, and necrosis, it is likely that the characterization of the MP proteome could prove highly useful in both basic and clinical science. Individual proteins or global protein profiles may provide novel insight into MP-biology and lead to the discovery of new potential therapeutic targets along with diagnostic, prognostic, and predictive tools in a multitude of diseases. However, it is crucial for comparison of MP proteomes to develop standardized and reproducible proteomics approaches.

Mass spectrometry is an analytical technology that provide an unbiased comprehensive identification of the proteins and quantification of their relative abundances in complex biological samples (132). Different proteomic strategies can be applied depending on the type of study (133). When the purpose of the study is to obtain unbiased global protein profiles in complex samples such as the plasma MP-proteome, a so-called label-free shotgun or bottom-up proteomic approach using nano-liquid chromatography coupled to tandem mass spectrometry (nano-LC-MS/MS) of enzymatically digested samples can provide the identification and quantification of the whole set of proteins with high accuracy, sensitivity and resolution (Fig. 5) (132-135).

Shotgun proteomics typically involves a series of steps outlined in Figure 4. In brief, the complexity of the material is reduced by purification or gel-electrophoresis separation before the proteins are proteolytically digested, separated by nano-sized liquid chromatography, ionized and subjected to mass analyses and peptide fragmentation in the mass spectrometer. The proteins are identified using database searches where measures of relative protein abundances are also obtained.

Accordingly, in Paper II and III MPs are purified by repeated ultracentrifugation steps to reduce plasma proteins and also to increase the sensitivity and detection of low abundant species that may otherwise be overshadowed by more highly abundant signals (135). The proteins are then proteolytic digested using trypsin, a serine protease that efficiently cleaves peptide chains at the C-terminal side of arginine and lysine residues (134). To secure optimal digestion an additional protease may be added. As tryptic digestion is highly efficient and predictable, computer generated theoretical peptides fragment masses can be obtained and used to match the experimental spectra in the database searches in the protein identification process (136). Peptide digests are typically separated on a low flow miniaturized reverse phase liquid chromatography system (nano-LC) by hydrophobicity to fractionate the complex mixture of peptides before electrospray ionization (ESI) and mass analyses. In the ESI-source, the peptides in liquid solution are subjected to high voltage and sprayed forming small droplets that quickly vaporize before the

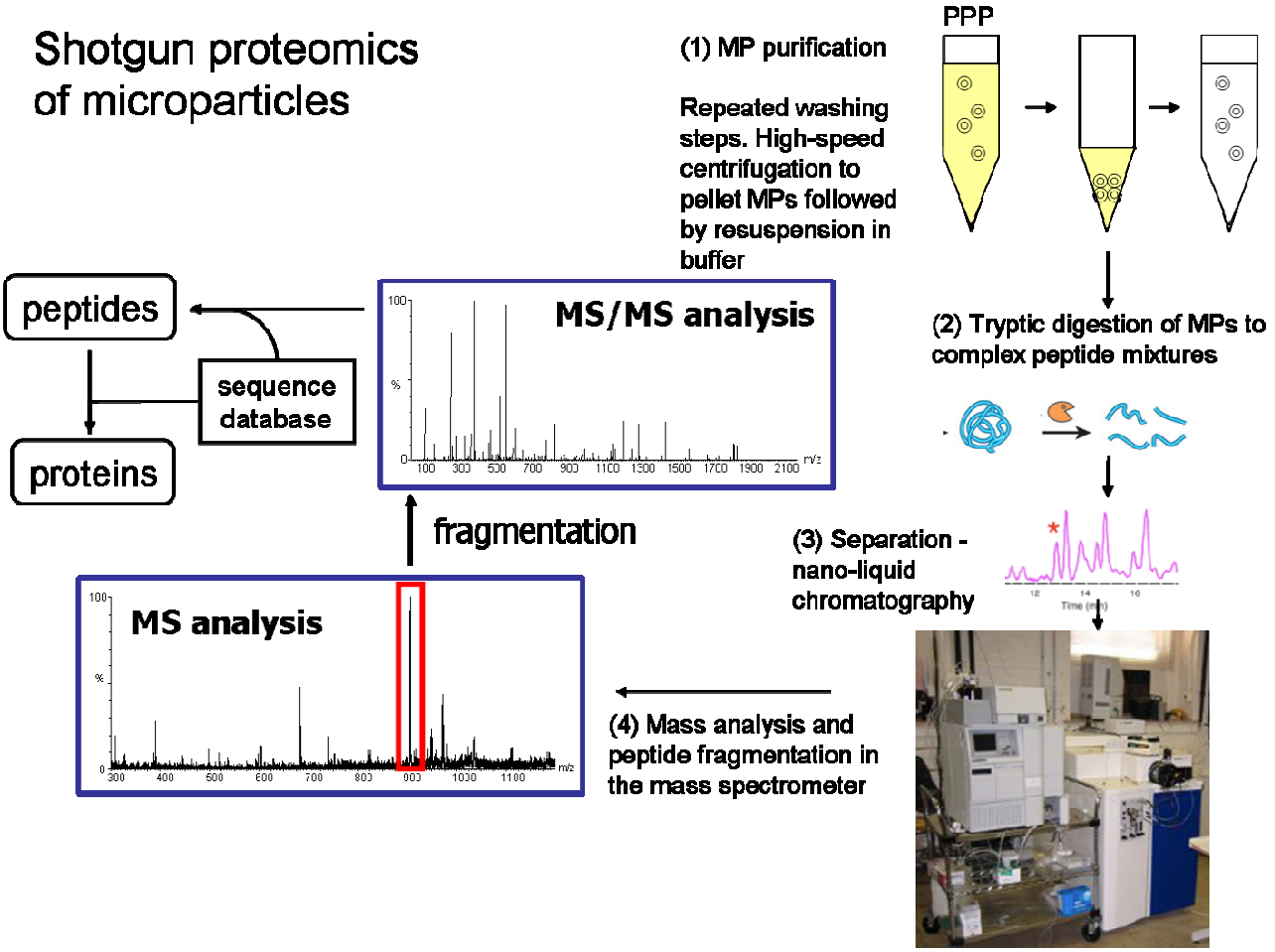

Figure 5
Protocol outline for a shotgun proteomic approach of microparticles. MPs = microparticles; PPP = platelet-poor plasma; MS = mass spectrometry (Adopted from and with courtesy of Dr. Juan Vizcaino).

ionized peptides in gas phase enter the mass analyzer (132). Mass spectrometers measure the mass to charge (m/z) ratio of a given analyte (here: peptides). Different types of mass spectrometers can be used for tandem mass spectrometry, i.e. mass spectrometry where both the parent ion and its fragmentation pattern are recorded (135). The LTQ Orbitrap XL mass spectrometer used in our experiments is a hybrid instrument composed of both an Orbitrap and linear ion trap. The instrument has a high resolution, mass accuracy, and sensitivity (135). MS/MS data are recorded in parallel in a data-dependent mode, i.e. initially peptide mass analysis generate a peptide mass spectrum, MS1, in the linear ion trap, and the most abundant ions (charge state +2 or higher) are then fragmented by collision-induced dissociation in the Orbitrap generating peptide fragment spectra, MS2. For protein identification these experimental peptide fragment spectra are matched with theoretical peptide fragments, peptide spectral matches (PSMs), based on database searches (136). Different criteria for the protein identification process can be set-up prior to the database search. Measures of relative protein abundances are obtained during the protein identification processes e.g. ion intensities and spectral counts (SCs) that are derived from the total numbers of PSMs for the protein and can thus be used for relative quantitation between samples (123;137;138).

2. METHODS AND MATERIALS

2.1. STUDY DESIGN AND APPROVALS

The 2 clinical studies (Paper I and III) were conducted using a cross-sectional design. Inclusion of out- and in-patients and controls was performed consecutively and simultaneously from May 2008 to March 2009 at the Departments of Rheumatology (SLE and RA), Rigshospitalet, and Dermatology (SSc), Bispebjerg, both Copenhagen University Hospital. These patient and control samples comprised the basis for the three papers included this thesis (Table 1). The studies were all approved by the local ethics committee (approval number H-B-2007-130) and carried out in accordance with the principles of the Declaration of Helsinki. All participants were included after giving written informed consent. Registration of personal data was approved by Datatilsynet, Copenhagen (approval number 2007-54-0373).

2.2. PATIENTS AND CONTROLS

2.2.1. Systemic lupus erythematosus

Seventy SLE-patients all fulfilling the 1997 revised American College of Rheumatology criteria for SLE were included and subjected to large-scale MP-analyses in Paper I (n = 70) and III (n =

Table 1. Overview of the patient and control samples and microparticle analyses in Papers I-III.

	Paper I				Paper II		Paper III		
	TEM	Flow cytometry		LC-MS/MS	Flow cytometry	LC-MS/MS	Flow cytometry		LC-MS/MS*
		AnxV+/- MPs, PMPs, LMPs, EMPs	Fresh vs. Frozen: AnxV+/- MPs		AnxV+ MPs		IgG+ MPs**	IgG+, IgM+ and C1q+ MPs**	
SLE	3	70	3	-	-	-	68	12***	12***
HC	3	29	3	-	12	12	38	12***	12***
SSc	-	-	-	-	-	-	-	6	6
RA	-	-	-	-	-	-	-	6	6

*12 pools of 3 samples were analysed.
**All samples were also labeled with AnxV.
***12 of the 68 SLE/38 HC samples were also analysed for their respective MP-cargo of IgG, IgM and C1q with flow cytometry and LC-MS/MS.
TEM = transmission electron microscopy; LC-MS/MS = liquid chromatography tandem mass spectrometry; AnxV = annexin V; PMPs = platelet-derived MPs; LMPs = leukocyte-derived MPs; EMPs = endothelial cell-derived MPs; SLE = systemic lupus erythematosus; HC = healthy controls; SSc = systemic sclerosis; RA = rheumatoid arthritis

68) (Table 1) (8). Additional 3 SLE patients were included to compare flow cytometry analysis of fresh and frozen plasma. Two additional individuals were primarily included but subsequently excluded since they only classified as having a primary anti-phospholipid syndrome. Patients taking medication including disease-modifying antirheumatic drugs were allowed in the study. Patients with a history of thrombosis and/or anti-phospholipid syndrome with the most recent thrombotic event occurring more than 6 months prior to study inclusion were allowed in the study. Patients with cancer or pregnancy were not included.

At the inclusion the disease history was obtained and clinical assessment was made followed by blood collection. Patient characteristics and demographics are presented in Table 2. Disease history including clinical, serological and pharmacological data was recorded into an SLE cohort database (7). Disease activity was scored with the Safety of Estrogens in Lupus Erythematosus: National Assessment (SELENA) version of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (139). Cumulative organ damage was assessed using the Systemic Lupus International Collaborating Clinics (SLICC) Damage Index score (140). Baseline clinical manifestations were recorded according to the definitions of SLEDAI.

The large SLE cohort was used for flow cytometric studies in both Paper I and III. In Paper III, a subset of 12 SLE patients were selected and compared with 12 matched healthy controls, 6 patients with rheumatoid arthritis and 6 patients with systemic sclerosis for characterization of the MP-associated IgG, IgM and C1q with both flow cytometry and mass spectrometry (Table 1). For the LC-MS/MS experiments 4 homogenous pools of 3 SLE patients were made based on their history of nephritis and disease activity: SLE-01) previous and present biopsy-proven nephritis, high SLEDAI (> 12), SLE-02) previous biopsy-proven nephritis with present quiescent disease, low SLEDAI (= 0), SLE-03) no history of nephritis with present active disease other that nephritis, high SLEDAI (> 10) and SLE-04) no history of nephritis with quiescent disease, low SLEDAI (= 0).

2.2.2. Rheumatoid arthritis

In Paper III, 6 patients with rheumatoid arthritis full-filling the ACR classification criteria from 1987 were included to make two homogenous groups, RA in remission and with active disease, respectively (Table 1) (141). The first RA group of 3 patients (2 female, 1 male) had non-erosive disease and were in remission. Disease activity was assessed by the Disease Activity Score, using a 28-joint score (DAS28) and C-reactive protein (CRP) with a group mean of 1.4 (142). All patients were anti-CCP and IgM-rheumatoid factor negative. The second group of 3 patients (2 female, 1 male) had active disease (DAS28-CRP = 4.2 (mean), one had radiographic erosions, all were anti-CCP and IgM rheumatoid factor positive).

Table 2. Clinical characteristics of 70 patients with systemic lupus erythematosus.*

	SLE (n = 70)
Disease manifestations**	
Renal disease	12 (17)
Vasculitis	5 (7)
Arthritis	7 (10)
Rash	4 (6)
Alopecia	5 (7)
Mucosal ulcers	6 (9)
Serositis	2 (3)
Leukopenia	8 (11)
Thrombocytopenia	3 (4)
SLEDAI, mean ± SD (range)	5 ± 5 (0-21)
Autoantibodies and complement**	
Anti-dsDNA	31 (44)
Anti-histone***	48 (71)***
Anti-ENA***	14 (21)***
Anti-C1q***	17 (25)***
Low C3	33 (49)
Low C4	46 (68)
Low C1q***	17 (25)***
Medication**	
Prednisolone ≤ 7.5 mg daily	15 (21)
Prednisolone > 7.5 mg daily	15 (21)
Anti-malarials	13 (19)
Azathioprine, MTX or mycophenylate mofetil	30 (43)
SLICC/ACR Damage Index, mean ± SD (range)	1 ± 1 (0-8)
Anti-phospholipid syndrome	16 (23)

* Values depict number (percent) of subjects unless otherwise stated
** At time of inclusion.
***Only determined in the 68 SLE patients included in Manuscript III

2.2.3. Systemic sclerosis

In Paper III, also 6 female patients with systemic sclerosis were included, 3 with diffuse cutaneous systemic sclerosis and 3 with limited cutaneous systemic sclerosis, all fulfilling the ACR classification criteria (Table 1) (143). At inclusion the group with limited disease had anti-centromere antibodies and modified Rodnan skin scores of 4, 6, and 11 (144). None had X-ray verified lung fibrosis. The patients with diffuse systemic sclerosis all had anti-Scl-70 antibodies and digital ulcers with modified Rodnan skin scores of 26, 30 and 30. One patient had X-ray verified lung fibrosis.

2.2.4. Healthy controls

Twenty-four female and five male non-medicated healthy individuals with a median age of 42 years (range 22-71) were included as controls in Paper I (Table 1). In Paper III, 33 female and 5 male non-medicated healthy Caucasian individuals with a median age of 45 years (range 24-62 years) were included. In both Paper II and III, the same 12 HC samples were used and had been selected to age- and gender-match the SLE, SSc and RA patients analysed in Paper III. In Paper II these HC samples were subjected to an in-depth proteomic profiling, identification of MP-normalizers analyzing both single and pooled samples in addition to addressing methodological issues. In Paper III the MP-associated IgG, IgM and C1q were determined by flow cytometry on single samples and by LC-MS/MS on 4 sample pools.

2.3. ISOLATION OF PLATELET-POOR PLASMA AND SERUM

Venipuncture was performed with a 21-gauge needle. The first tube obtained after releasing the tourniquet was always used for serological analyses. Blood for MP preparation was collected into 9 mL citrate tubes (Vacuette sodium citrate 3.8%, Greiner Bio-One, Kremsmünster, Austria). Immediately after collection, blood cells were removed by centrifugation (1800 g, 10 min, 21 °C) followed by a second centrifugation step (3000 g, 10 min, 21 °C) to remove platelets. The resulting platelet-poor plasma (PPP) was divided into 250 µL aliquots, snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

In Paper I, platelet-poor plasma from 3 SLE patients and 3 healthy controls was also collected to compare AnxV-binding MPs in fresh and frozen samples. MPs from the fresh plasma were isolated, washed and labeled for immediate FACS-analysis, and the remaining plasma was snap-frozen in 250 µl aliquots for subsequent analysis later the same day.

2.4. SEROLOGY, ROUTINE BIOCHEMISTRY AND SOLUBLE CD62P

To characterize the patient baseline status at inclusion routine clinical biochemistry parameters were obtained for all samples at the Departments of Clinical Biochemistry, Rigshospitalet and Bispebjerg, Copenhagen University Hospital. Additionally, antinuclear antibodies (ANA) were detected by indirect immunofluorescence on HEp-2 cells in the SLE and SSc patients. In the SLE patients antibodies against double-stranded DNA, histones, ribonucleoproteins, C1q, CCP (also RA), Ro60, Ro52, La, cardiolipin IgG/IgM, and β-2 glycoprotein-I IgG/IgM were determined. Anti-Scl-70 antibodies were only assessed in the scleroderma patients. Complement components C3, C4 and C1q were also quantified in the SLE patients. Soluble CD62P (sCD62P/P-selectin) in plasma was measured in duplicate using a solid-phase capture ELISA according to the manufacturer's instructions (not included in the Papers) (R&D Systems, Inc., Minneapolis, MN, USA).

2.5. ELECTRON MICROSCOPY

Transmission electron microscopy of MPs both in suspension and sections of embedded MPs were performed (Paper I) (1). Briefly, TEM on MP sections were aquired after a week-long preparation, where MPs from 25 mL fresh plasma had been isolated, washed, fixated and embedded in resin (Epon) followed by negative staining with osmium tetroxide and uranyl acetate and subsequent ultrathin sectioning. TEM of MPs in suspension were prepared and aquired on the same day as the MPs were isolated and washed from 15 mL fresh plasma and negatively stained with sodium silicotungstic acid (1). Samples were examined mounted on formvar-carbon coated copper grids in a Morgagni 248D (Epon-sections) or a Philips CM100 BioTWIN (MP-suspensions) transmission electron microscope.

Supplementary to TEM, scanning electron microscopy (SEM) of MPs was also attempted in a few preliminary experiments (not included in the Papers). Here, fresh platelets and washed MPs from one healthy control in addition to washed MPs from frozen samples from one healthy control and one SLE patient were isolated and washed using the protocol for flow cytometry (presented in the next section) and immobilised on graphite filters under vacuum followed by coating of the surface with a 10 nm thick layer of gold. SEM was performed on a Zeiss Ultra55 instrument.

2.6. FLOW CYTOMETRY

In the Papers two different flow cytometric protocols were applied. Analyses were made either directly on MPs in crude heparinised PPP (Paper II) or on isolated and washed MPs in suspension (Papers I and III).

Flow cytometric analysis on washed MPs (Papers I and III) was adapted from Nieuwland et al. and reduces background noise including plasma proteins that may interfere with the measurements (89;145). Briefly, 250 µl plasma aliquots were thawed on ice for one 1 hr. While samples were thawed, buffers were produced and 0.1 µm filtered (Sartorius Stedim Biotech AS). A volume of 230 µl of the plasma was transferred to a new tube and adjusted to 250 µl in low phosphate buffered saline (PBS; 154 mmol/L NaCl, 1.4 mmol/L phosphate, pH 7.4) containing 10.5 mM trisodium citrate (PBS-citrate). The MPs were then pelleted by ultracentrifugation (18,890 g/30 min/22 °C). Then 225 µl of supernatant was removed and replaced by 225 µl PBS-citrate buffer before resuspension of the pellet. This was succeeded by a second centrifugation step (18,890 g/30 min/22 °C) and again 225 µl supernatant was removed and discarded. Finally 75 µl PBS-citrate buffer was added and the MPs resuspended. The volume of the final MP-suspension was measured and adjusted to 100 µl in citrate buffer. The plasma protein concentration is reduced 40 times by this approach.

After preparation of the MP suspension, MPs were labelled with fluorescent antibodies or AnxV as presented in Table 3. Generally monoclonal antibodies and fluorescent conjugates with a high staining index (e.g. R-Phycoerythrin) were preferred. The optimal concentrations for antibodies and probes were always tested. In Paper I, MPs were single-labeled and the concentrations of the cellular MP-subsets or AnxV-binding MPs were determined by adding 5 µl MP-suspension to 45 µl calcium-containing PBS (PBS-Ca, 2.5 mM CaCl2) and then 5 µl pre-diluted AnxV-FITC (Becton Dickinson), anti-CD42a-FITC (Becton Dickinson), anti-CD45-PE (Becton Dickinson) or anti-CD146-FITC (Abd Serotec). In Paper III, the MPs were double-labeled with 5 µl AnxV-allophycocyanin (APC) and 5 µl anti-human C1q-FITC (Ce-

Table 3. Overview of the microparticle antigens detected by flow cytometry in Papers I-III.

Antigen	CD-marker	Antibody/probe	Cellular origin of MPs	Paper
Platelet glycoprotein IX (GP9)	CD42a	Antibody	Platelets	I
Leukocyte common antigen (LCA); Protein tyrosine phosphatase, receptor type, C (PTPRC)	CD45	Antibody	Leukocytes	I
Melanoma cell adhesion molecule (Mel-Cam); S-endo 1 endothelial-associated antigen; Cell surface glycoprotein MUC18	CD146	Antibody	Endothelial cells*	I
Phosphatidylserine	-	Annexin V	-	I, II, III
IgG1	-	Antibody	-	III
IgM	-	Antibody	-	III
Complement component C1q	-	Antibody	-	III

*The antigen has also been detected on melanoma cells and a small subfraction of circulating T-cells

darlane Laboratories) or unconjugated anti-human IgG or IgM (Sanquin) that were detected with 10 µL of R-phycoerythrin anti-mouse IgG conjugate to the improve staining intensity. In all the tubes PBS-Ca was added to a final incubation volume of 55 µl. For control experiments the same concentrations of isotype-matched control antibodies or AnxV in PBS-citrate were used since AnxV binding to PS is calcium dependent (127). Samples were left to incubate in the dark for 30 minutes, adjusted to 955 µl in either PBS-Ca or PBS-citrate and analysed on the flow cytometer.

In the direct approach used in Paper II the PPP was thawed on ice for 1 hour. To prepare one sample tube 5 µl of PPP was heparinised by adding 5 µl of 10 % w/v sodium heparin (Sigma-Aldrich) and 5 µl pre-diluted AnxV-allophycocyanin (Becton Dickinson) to a final volume of 955 µl in PBS-Ca or PBS-citrate. Samples were left to incubate in the dark for 60 minutes.

Post-incubation the analytic steps were equal for the two approaches. The samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson) with both forward (FSC) and side scatter (SSC) recorded with logarithmic gain. Acquisition time was 60 seconds allowing absolute quantification of the MPs based on measurement of the instrument flow rate and net dilution of the samples. All samples were processed for analysis in a random fashion. The SSC was calibrated prior to each run using 1 µm beads (Flow Cytometry Size Calibration Kit, Molecular Probes). These daily bead measurements were also used for setting the upper limits of the MP-gate in both FSC and SSC signals. A lower limit was placed to exclude buffer noise. The MP gate was validated by showing that the majority of AnxV+ MPs induced from platelets using the calcium ionophore A23187 (Sigma-Aldrich) were detected in this gate (Fig. 7) (145;146). Size gated MPs were further analyzed in SSC/FL-1 and SSC/FL-2 (Paper I) or FL1/FL4 and FL2/FL4 (Paper III) plots to discriminate labeled particles from unlabelled particles using a fluorescence threshold determined by the fluorescence level of the MPs stained using the isotype-matched control antibodies and AnxV negative controls. The AnxV+ and AnxV– MPs were backgated to a FSC/SSC plots (Fig. 13). Data analysis was performed using FlowJo software (Treestar, Inc., Ashland, OR, USA).

2.7. SDS-PAGE AND IMMUNOBLOTTING

In Paper II the effect of using different numbers of MP washings on the removal of plasma proteins was evaluated using standard protocols. The pellets and wash supernatants were separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis, NuPAGE Bis-Tris 4-12%, Invitrogen) and separated proteins were subsequently evaluated both with silver-staining and immunoblotting with antibodies against actin, albumin (HSA), integrin alpha-2B (ITGA2b or ITGAIIb) and transthyretin (TTR) (2).

2.8. MP PROTEOMICS

A shotgun proteomic approach was developed to characterize the MP proteome in healthy controls (Paper II) as a platform for subsequent studies (Paper III). The general principle of this approach is outlined in the background section (Fig. 5).

In Paper II, firstly, the number of washing steps needed to reduce plasma protein was evaluated using SDS-PAGE, immunoblotting and LC-MS/MS. In all experiments MPs were isolated from PPP and washed by repeated ultracentrifugations at 18,990 g, 30 min, 22 °C. Briefly, five 1000 µl aliquots of PPP from a healthy control were washed 2, 4, 6, 8 and 10 times, respectively, and the final supernatants and MP pellets were evaluated by SDS-PAGE, silver staining and immunoblotting detecting the contents of human serum albumin (HSA), integrin alpha-2b (ITGA2b), actin and transthyretin (TTR). The MP-pellet proteome was also characterized using LC-MS/MS. Secondly, reproducibility experiments of the whole approach were performed by LC-MS/MS, and potential MP-load normalizers for data analyses were identified. Here, the MPs from two HCs were washed 5 times each day for 3 days, digested and frozen on the day. LC-MS/MS analyses to compare the six MP proteomes were then performed on the same day to minimize technical variation and assess procedural variation.

Thirdly, using the 5 washing step-protocol we characterized the proteome in 12 HCs and compared the individual proteomes with the proteomes from 4 pools of 3 HC samples each. Lastly, correlation analyses were made between flow cytometrically determined plasma concentrations of AnxV-binding MPs from crude plasma and the proteome abundances of a number of selected proteins reflecting plasma proteins (HSA and transthyretin) and cellular material (myosin-9 and integrin alpha-2b).

Recorded raw-files were analyzed using the MaxQuant software (Paper II) or MSQuant (Paper III) for peptide quantitation by intensity and for protein identification using the Andromeda (Paper II) or Mascot (Paper III) search engines.

In Paper III the optimized shotgun proteomics approach was applied analyzing 12 pools of 3 individuals, i.e. HCs and patients with rheumatoid arthritis, systemic sclerosis and systemic lupus erythematosus selected to make homogenous clinical pools. HCs were sex and age-matched. Here, the relative abundances of IgG, IgM and C1q to myosin-9 were evaluated using spectral counts.

2.9. STATISTICS

The flow cytometric plasma concentrations of microparticles were non-normally distributed. Comparisons between two sample groups were performed with the Mann-Whitney U-test. In Paper III, 4 groups were compared with one-way ANOVA (LC-MS/MS and flow cytometry), however, the intergroup differences and presented p-values regarding the flow cytometric data were calculated with the Mann-Whitney U-test. Univariate Spearman's rank correlation analyses were used to explore associations between flow cytometric MP-measurements and clinical and serological variables (Paper I and III). In Paper II comparisons of protein intensities and rank consensus in repeat analyses were performed using Pearson correlation in OriginPro v. 7.5. To assess whether the protein composition in pooled samples reflected that of the single samples, principal component analysis (PCA) was performed. PCA is a statistical tool that can estimate the contribution to the variance of all the variables, protein identifications and abundances in this case, in large datasets. The two components (termed F1 and F2) that contribute most to the variance are typically plotted. In Paper II, PCA was employed to explore if the single samples and pools were grouped by the two principal components that contribute most to the variance in the datasets in addition to assessing the variance from these two components. Prior to principal components analysis, the intensities calculated by MaxQuant for each identified protein in the samples were normalized by the intensity of myosin-9 and log2 transformed. The datamatrix was then subjected to principal components analysis using XLSTAT 2011. GraphPad Prism statistical software was used if nothing else is stated. p-values ≤ 0.05 were considered significant.

3. RESULTS AND DISCUSSION

As microparticles are formed constitutively during normal physiology and change in pathology, MPs could serve as future diagnostic, prognostic or predictive tools in a multitude of diseases. In SLE the prominent features of innate and adaptive immune activation in combination with defective clearance of apoptotic material may be reflected in changes of numbers, distributions and the antigenic composition of MPs. Thus the overall aims were to set-up and apply antibody and antibody-independent approaches to characterize the circulating MPs in SLE with regard to numbers, origins, AnxV-binding, composition and their cargo of immune complex components and with regard to associations with clinical and serological parameters.

3.1. CHARACTERIZATION OF MICROPARTICLES

3.1.1. Electron microscopy of microparticles

EM is a potentially highly informative technique providing information on the size, morphology and composition (ultrastructure) of microparticles isolated from plasma.

Using two different protocols for MP preparation of HC- and SLE-MPs, transmission electron microscopy of MPs in suspension and of sections of Epon-embedded MPs confirmed that plasma microparticles are very heterogeneous both in size and density, ranging from below 50 nm, i.e. below the detection limit of a flow cytometer, to above 1 µm in line with observations by other investigators (Fig. 6A-J) (85;86;147-149). In the preparations with MPs in suspension, free but also aggregated MPs were observed (Fig 5 G-J). Aggregation may reflect the presence of MP-MP complexes in plasma, however, it is more likely a result of poor resuspension of pelleted MPs, since MPs from 15 mL plasma were washed and finally resuspended in only 10 µl buffer to yield high enough concentrations (> 109 MPs/µL, final concentration). In the sectioned material residual small platelets and non-platelet-like vesicles larger than 1 µm were also observed. The majority of vesicles were in the submicron range. However, some of the vesicle-like structures may represent sectioned platelet protrusions. Comparison between SLE- and HC-MPs showed no apparent differences.

Scanning electron microscopy of fresh platelets and MPs from a healthy control and MPs isolated from frozen plasma from 1 HC and 1 SLE-patient was performed in a few preliminary experiments not presented in any of the Papers (Fig. 6K-R). In the MP-preparations several submicron particles were observed. Two types of particles in the size range from 0.1-0.3 µm were observed. One type were small edged particles found both freely and associated with platelets in fresh platelet preparations (asterisk, Fig. 6K-L), whereas the other type of particle was more round detected in the MP SLE sample (Fig. 6O-Q). Additional observations were tree-like structures possibly representing precipitates (black arrows) and large flattened structures (white arrows) that could be platelets affected by the vacuum during sample preparation (Fig. 6M-N). While we observed submicron particles that resembled cell-derived MPs, particularly the round type (O-Q), additional experiments are needed to confirm this (84;111;150;151).

Altogether electron microscopy of MPs showed that circulating MPs isolated from plasma comprise a very heterogeneous pool of submicron vesicles. EM also showed that not all platelets are removed from the platelet-poor plasma preparations. Due the low sample number and the complexity of their morphological presentations no conclusions regarding differences between SLE- and HC-MPs could be made.

3.1.2. Flow cytometry of microparticles

As determined by electron microscopy the pool of circulating subcellular particles in the blood is a heterogenous population. No clear consensus on the definition and measurement of membranous MPs exists (78;118). Cell-derived microparticles are most often operationally defined as particles smaller than 1 µm that bind a cell-specific antibody or probes such as annexin V. In experimental set-ups MPs are often defined as the fraction of particles isolated from plasma using high-speed centrifugations. Due to the small size and overlaps of membranous MPs with other particulate fractions such as lipoproteins, protein complexes and

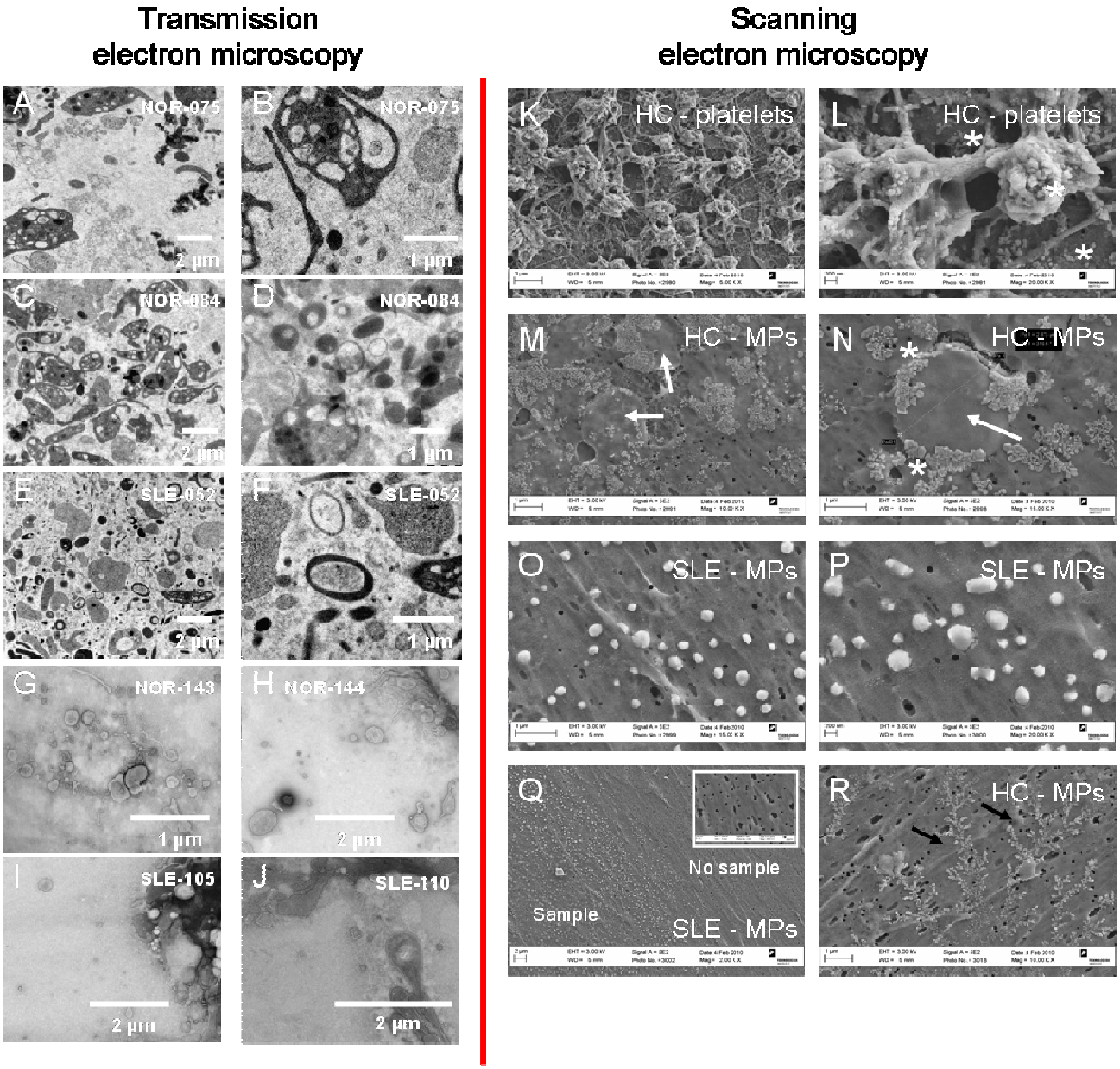

Figure 6
Electron microscopy of plasma microparticles from healthy controls and patients with systemic lupus erythematosus (data not published). A-F, Transmission electron microscopy of sections of Epon-embedded MPs and , G-J, of MPs in suspension showing submicron vesicles and small platelets. K-R, Scanning electron microscopy of fresh platelets and MPs from a healthy control and a SLE sample. O-Q, Submicron MPs were detected in a SLE sample in the size range 100-300 nm resembling MPs observed in other SEM studies. Q, Depict the border between sample and no sample (background). The asterisks highlight submicron particles found both free and associated with platelets that could resemble cell-derived MPs or precipitates. White arrows = large flattened structures most likely platelets. Black arrows = suspected precipitates. NOR/HC = healthy control; SLE = systemic lupus erythematosus.

also smaller platelets, no entirely satisfactory method for MP quantitation in the blood exists.

3.1.2.1. Identification of MPs by size in flow cytometry – principles and issues

We adopted and optimized a protocol for MP analysis, where MPs were isolated using a two-step ultracentrifugation step (18,890 g/30 min/22 °C) before flow cytometric analysis as described by Biro et al. (89). An MP-gate based on size in FSC/SSC plots that classify events smaller than 1 µm as microparticles and excluding larger particles and platelets and background noise from buffers and reagents was set-up (Fig. 7). This MP-gate was used for the characterization of the cellular origins, AnxV-binding and the surface cargo of IgG, IgM and C1q presented in this thesis (Table 3).

The upper MP-gate limit was set using polystyrene standard beads (Fig. 7A), which were also used for the instrument calibration prior to every flow cytometry experiment. To validate the

MP-gate (Fig. 7G-I), platelets in platelet-rich plasma were stimulated with the calcium ionophore A23187, and the majority of the induced events was found to be located in the MP-gate and stained positive for a platelet marker (data not shown) (145;146). The lower limits of the gate were set to exclude background noise from buffers and reagents. As observed by Ayers et al., 0.1 µm filtration of freshly produced buffers and centrifugation of fluorescent reagents reduced background noise (data not shown) (126).

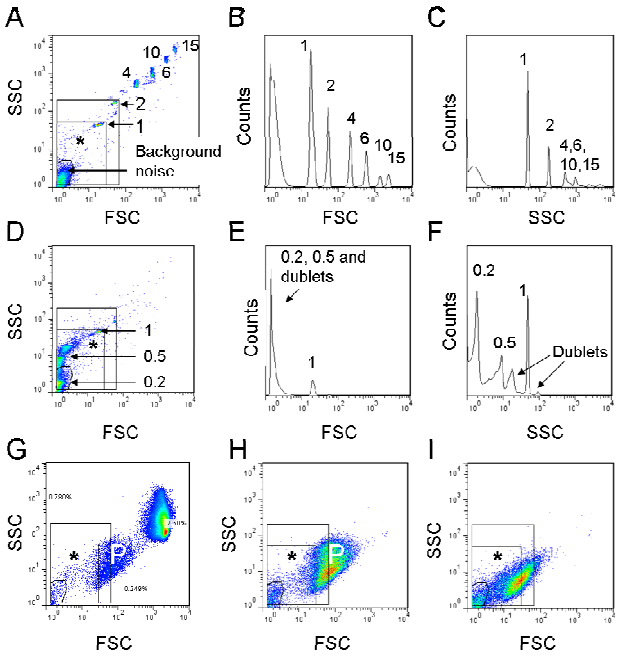

Figure 7
Flow cytometric set-up of MP-gates using polystyrene beads and in vitro induction of platelet-derived microparticles. A, D, G-I, Forward/sideward scatter plots with two MP gates set with upper limits of 1 (asterisk) and 2 µm polystyrene beads, respectively, and a lower limit to exclude background noise. All numbers in the plots refer to the size of the beads in µm. A-C, FSC and SSC depict polystyrene beads ranging from 1-15 µm. D-F, FSC/SSC density plots and their respective histograms showing standard beads of 0.2 µm, 0.5 µm and 1.0 µm. Comparing E and F shows clearly that submicron beads can only be distinguished in SSC reflecting that particles with the same size or smaller than the wavelength of light cannot be discriminated in FSC. G-I, FSC/SSC density plots of whole blood (G) and platelet-rich plasma (H, unstimulated and I, stimulated) show that platelets (P) in platelet-rich plasma form submicron platelet-derived microparticles when stimulated with the calcium ionophore A23187. FSC = forward scatter; SSC = sideward scatter.

Defining MPs as being smaller than 1 µm is an operational definition and does not respect the biology and heterogeneity of MPs (17). Setting the upper gate limit to 2 µm increased the numbers of MPs compared to using the MP-gate < 1 µm (percentage increase: median; 5-95th percentiles) estimated in 99 samples from both healthy controls and SLE patients: AnxV+ MPs (31%; 8-51%), PMPs (30%; 9-51%)s, LMPs (70%; 21-143%) and EMPs (56%; 17-307%, 6 samples were excluded due to negative results). Since the events in the larger gate are likely to comprise a mixture of single MPs (apoptotic bodies), platelets, platelet-MP or MP-MP complexes, the MP-gate < 1 µm was chosen for further MP analysis (116). Including the background noise events in the MP-gate increased the total numbers of events more than 100 % with a 18-43% (range of medians for all the MP subsets) increase regarding the specifically labelled populations while compromising the resolution of the weakly stained MP populations (19 samples had to be excluded for EMP analysis due to a higher background signal). Thus gating out the background noise leads to an underestimation of the number of MPs, but makes the MP analysis less sensitive to impurities in buffers, protein-complexes, lipoproteins, cellular debris and unspecific binding of the antibodies or probes.

Moreover, light scatter flow cytometers have an estimated lower detection limit of approximately 200-300 nm due to the wavelength of light leaving the smallest fraction of MPs undetected per se (92;120). Other technologies (besides electron microscopy) with lower detection limits have recently been tested for MP analysis (152). Impedance flow cytometry and the use of highly fluorescent and specific stains in light scatter flow cytometry increased the number of tected MPs (92;153). However, the smallest subfractions of MPs (< 100 nm) were still undetected. Atomic force microscopy and technologies based on the brownian motions and light scatter of particles in suspension (dynamic light scatter and fluorescence nanoparticle tracking analysis, NanoSight®) can determine the size and/or the size distributions and quantify the concentrations of the smallest MPs (119;120;154). Atomic force microscopy is highly labour intensive and is characterized by low throughput. Dynamic light scatter technologies provide only size distributions and do not resolve small and large particles well. The fluorescence nanoparticle tracking analysis is a much more promising tool for future MPs studies being applicable in larger sample sets and applicable for immunodetection potentially providing a comprehensive quantitative characterization of the cellular origins and concentrations of both small and large MPs in crude diluted plasma (119).

3.1.2.2. Detection of surface markers on microparticles

Although failing to detect all MPs, flow cytometry provides otherwise difficult accessible information on the phenotypes of thousands of MPs from the binding of antibodies and probes. Panels of antibodies against surface epitopes of MPs derived from erythrocytes (CD235a), platelets (CD42a and CD61), leukocytes (CD45, CD14, CD3, CD4, CD8 and CD66b) and endothelial cells (CD146, CD144 and CD62E) were tested and titrated on plasma MPs with high numbers of the relevant MP phenotypes. No specific marker for apoptotic bodies have been identified and AnxV was used as a generic marker of PS-exposure on MPs (17). Subphenotyping of leukocyte-derived MPs was compromised by low abundances and weak staining signals and ultimately abandoned (155). Setting the fluorescence thresholds were generally challenged by weak staining signals reflecting few surface epitopes and the small size of MPs. A reduced panel of MP-antigens was ultimately chosen for analysis in the studies presented in this thesis (Table 2). The presence of CD146 on melanoma cells and a small subset of circulating T-lymphocytes was not likely to affect our results (113).

3.1.2.3. Quantification of Annexin V-binding MPs - reproducibility

Enumeration of MPs using flow cytometry and setting an MP-gate of 1 µm underestimates the entire MP-pool measuring only the "tip of the iceberg". Flow cytometry, however, provides an

operational and standardized tool for MP identification, enumeration and characterization of MPs in larger sample sets. Recently a flow cytometric protocol using strict gate setting by beads have proved applicable for the standardization of MP-measurements across laboratories and instruments (129). In our laboratory using the protocol for washed MPs (Paper I and III) the intraday (8 tubes) and interday (12 days, single tubes) variations of the concentrations of AnxV-binding MPs were 9% and 35%, respectively. Using the protocol directly in heparinised crude plasma (Paper II) the intraday (8 tubes) and interday (10 days, 8 tubes) variations of AnxV-binding MPs concentrations were 10% and 20%, respectively. While the latter protocol is more reproducible, washing of MPs may be necessary to avoid interference from plasma proteins and lipid vesicles (Papers I and III).

3.1.3. Development of a standardized proteomics protocol for microparticle characterization

As the MP composition changes and may reflect the state of the parental cells, MPs possess the ability to provide novel insight into disease pathogenesis and serve as biomarkers of disease (91;156;157). To exploit this information in-depth characterization of the MP-proteome for an unbiased and comprehensive coverage of proteins and their relative abundances was applied. Previous studies using proteomic characterization of plasma MPs are few and comparisons across studies are difficult (104;121-124). To ensure comparable data, standardized protocols with known characteristics are needed. In Paper II such a protocol is presented and subsequently applied in clinical samples in Paper III (2;3;158).

ontology analysis showed a substantial overrepresentation of extracellular proteins, lipoprotein particles and protein-lipid complexes in addition to the expected overrepresentation of cytoskeletal, cytoplasmic, and organellar proteins (Paper II, Supplemental Fig. 2S). This underlines that MPs are extremely heterogenous and that the pelleted fraction of MPs from plasma may include lipo-vesicles (82).

3.1.3.3. Reproducibility and technical variation

Two healthy control samples were purified on 3 consecutive days, tryptic digested and frozen. All samples were then analysed in triplicate with nano-LC MS/MS on the same day. Little technical variation (<10%) between total protein intensities in the triplicate runs of each sample was detected (Fig. 9A). This would allow for a reduction in the number of repeated analyses of each sample and larger biological sample sets in the future.

In contrast the interday variation in total protein intensities were up to two times or more (Fig. 9A). The identification and ranking of proteins (Fig. 9C-E) was, however, preserved with more than 90% (300 out of 332) of the same proteins identified in samples processes on all 3 days (Fig. 9B). Individual protein intensities spanned 4-5 decades (Fig. 9C-E) and had a higher variation in the less abundant proteins as expected (data not shown).

Since the samples are loaded based on equal volumes and not by equal total protein concentration the variability must be due to different loss and/or dilution of MP material during the washing process. To account for this in the comparison between samples, we proposed that the data set may be normalized by cytoskeletal protein intensities, e.g. myosin-9, as cytoskeletal

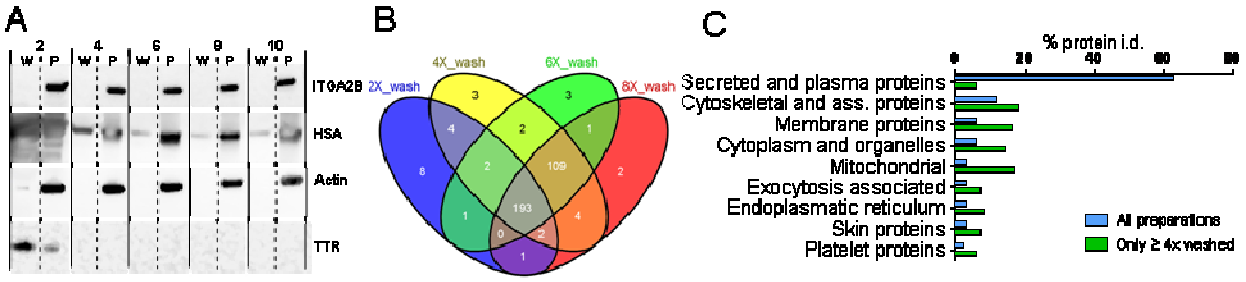

Figure 8
Impact of the number of washing steps on the, A, detection of specific proteins using immunoblotting, B, number of proteins identified using LC-MS/MS and , C, enrichment of non-secreted proteins. Adopted from Figure 1, Paper II.

3.1.3.1 Preparation and purification of plasma MPs

Purification of MPs is necessary to explore the MP-compartment in the blood. Purification of MPs by at least 4 repeated washes removed soluble plasma proteins (Fig. 8A), made it possible to reveal more than 100 new protein identities (Fig. 8B) and enriched the samples for non-secreted proteins (Fig. 8C). Albumin was not completely removed by washing compared to another soluble plasma protein transthyretin (Fig. 8A) indicating that albumin may be associated with MPs. Using more than 4 washing-steps did not provide additional information.

3.1.3.2. Proteomic profiling of 12 healthy controls

Next, proteomic profiling of MPs from 12 healthy controls (single samples and 4 pools of 3) identified a total of 536 individual proteins, and 334 (63%) of these proteins were detected in all samples, termed the core proteome (Suppl. Protein list). A gene

proteins are abundant MP constituents in most proteomic reports of MPs either from plasma or cultured cells (104;121-124;156;159). Normalization of the total intensity data by myosin-9 intensity (compare Figure 10A and B) may help identify outlier samples (95_d3) in repeatedly processed samples and assign proteins to the MP fraction because bona fide MP proteins (b-actin) correlates significantly with myosin-9 in linear plots while no such correlation is found with proteins not known to be associated with MPs such as transthyretin (Fig. 10C-D).

3.1.3.4. Quantitative validation

If sample preparations do not lead to large variations in the degree of sample loss, the procedure for data analysis and normalization by myosin-9 may be validated by correlating the quantity of MP-associated proteins with the number of input MPs determined by independent flow cytometric analysis of the crude PPP samples (Fig. 10E-F). Thus, protein intensities of myosin-9 and

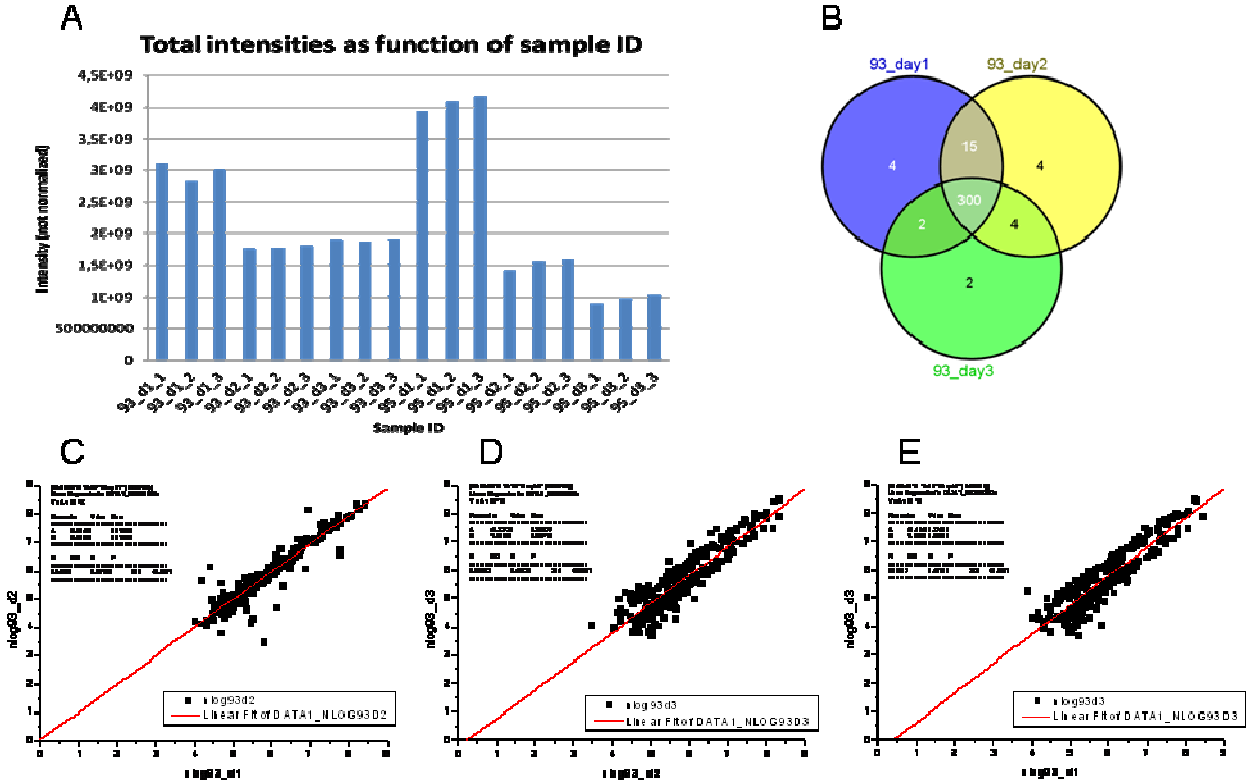

Figure 9
Reproducibility and technical variation. A, Total protein intensities for two samples processed on 3 different days. B, For one of the samples the overlap of proteins identifications and, C-E, the association between individual protein intensities (ranking) on the 3 days were shown. Plots are adapted from Figure 3 in Paper II.

integrin alpha-2B (not shown) correlated statistically significantly (r2 ≥ 0.85) with the concentrations of input MP while the plasma proteins transthyretin and serum albumin (not shown) showed no such correlation. This supported myosin-9 (and integrin alpha-2B) as a quantitative measure of the analysed amount of MPs in a sample and as a normalizer for valid comparisons of the MP protein composition when comparing samples from different groups, e.g. of patients and controls.

3.1.3.5. Pooled samples versus single samples

Single samples were compared with pooled samples and showed a large degree of overlap in the number of identified proteins. Since the capacity to analyse large number of samples usually is a bottle-neck in proteomics studies pooling of homogenous samples may be a feasible approach to obtain data on a larger number of individuals.

3.1.3.6. Proteomics of circulating microparticles

Although highly informative, studies of both in vitro generated MP and plasma MP proteomes have been limited. The notion that MP protein composition may reflect the state of the parental cell was supported by changes in the proteome of platelet-, mononuclear- and endothelial cell-derived MPs depending on the triggering stimuli (156;157;159-161).To date the plasma PMP or MP proteome has only been characterized in a few studies in healthy controls, and recently also in a biomarker discovery study in patients with deep venous thrombosis (104;121-124). Jin et al. was the first to explore the plasma platelet-derived MP proteome identifying more proteins in MPs than plasma and discovering proteins that have not been described before in plasma in addition to enrichment of certain proteins like IgM and IgA J-chain in MPs (122). Subsequently Smalley et al. described a selective enrichment of proteins, particular complement and immunoglobulins proteins in plasma MPs compared studies of isolated platelet-derived MPs (124). To extend this study the plasma proteome of forty-two individuals were characterized identifying a core proteome of 130 proteins (defined here as proteins found in 50% of the samples) and higher total numbers of identified proteins (458) than previously (123). The only clinical MP proteomics study to date identified 151 proteins (104). Galectin-3-binding protein and alpha-2-macroglobulin were found enriched in patients with deep venous thrombosis compared with disease and healthy controls illustrating the potential of proteomics in biomarker discovery (104). Recently, we have also observed that particular galectin-3-binding protein was enriched in SLE MP-samples using the protocol presented here (158). However, the protocols, instruments and data analysis software used in these studies differed considerably making comparisons between protein identifications and abundances difficult.

Using high-resolution tandem mass spectrometry in Paper II we identified more than 500 proteins in total with 334 being present in all 12 healthy control samples serving as a reference dataset for subsequent clinical studies (158). Normal plasma MPs were enriched in cytoplasmic, cytoskeletal and organellar proteins as shown in other reports but also immune complex components and lipoproteins were found (104;121-124). A recent

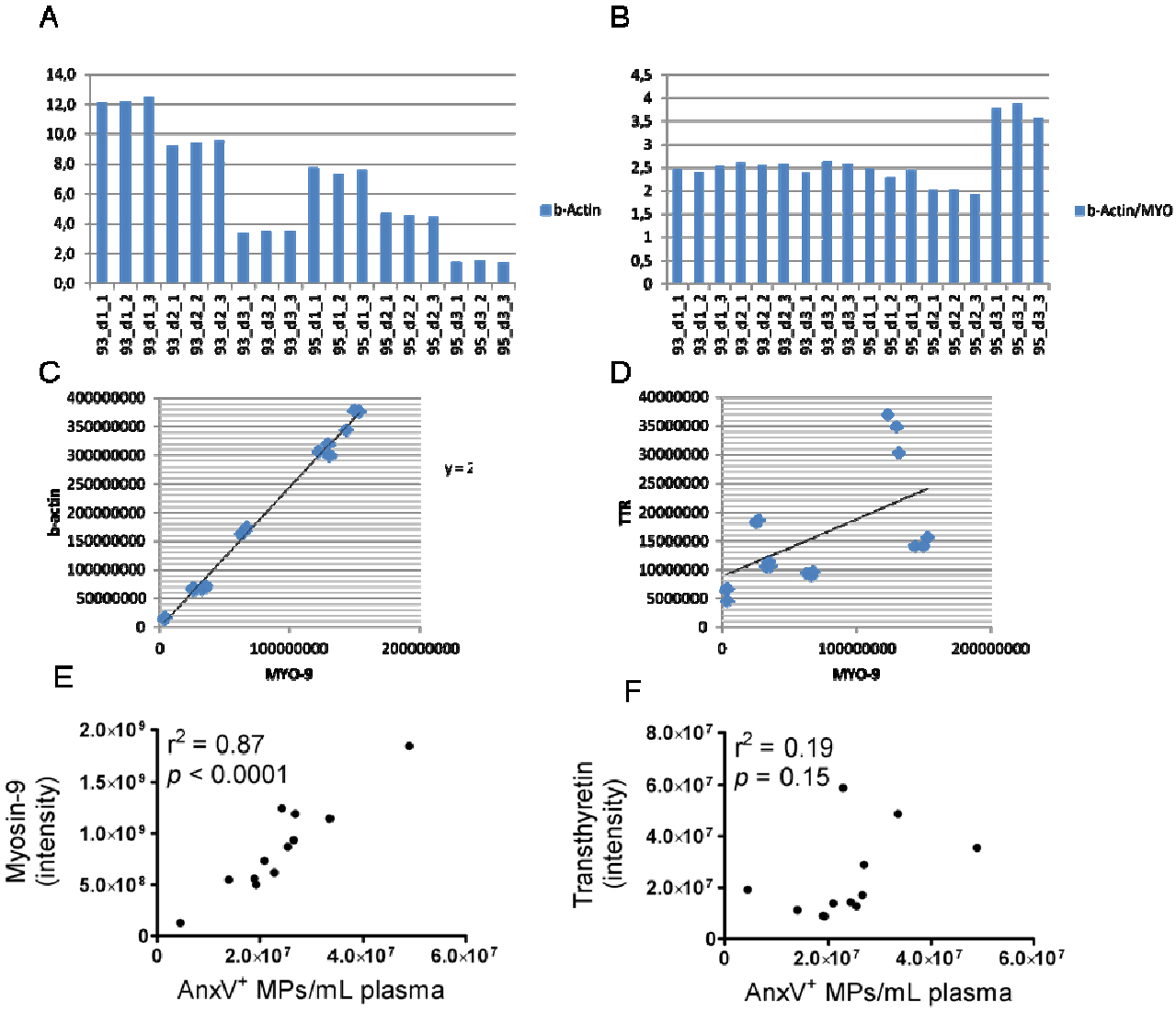

Figure 10
Impact of myosin-9 normalization on β-actin protein intensities (A-B), associating proteins to MPs by significant correlations with myosin-9 (C-D) and association of myosin-9 and transthyretin with the input plasma concentrations of AnxV+ MPs determined with flow cytometry (E-F).

interesting study of atherosclerotic plaque MPs revealed great immunoglobulin abundances inside MPs (162). We demonstrated that MP protein identification is compromised and overshadowed by abundant plasma proteins if the MPs are not washed well, and that MP purification above a certain dilution of plasma proteins did not reveal additional proteins. A major drawback of the protocol was the high degree of procedural variation as may be suspected in the other MP proteomics protocols where ultracentrifugation also have been used to isolate MPs. Reproducibility is, however, not stated in any previous studies (104;121-124). Cytoskeletal proteins appear to constitute some of the most abundant proteins in MPs (20;156). We observed that the relative abundance of myosin-9 reflected the amount of analysed MPs in our samples and this protein thus was proposed as a normalizer of MP proteomic datasets to accommodate procedural variation and enable valid comparisons across samples and studies.

Using this protocol in Paper III we were able to show that SLE patients had more MP-associated IgG, IgM and C1q relative to myosin-9 analyzing sample pools of 3 individuals from 12 SLE, 6 SSc, 6 RA and 12 HCs. These are results are described more in detail in Section 3.2.6.

3.2. QUANTITATIVE AND QUALITATIVE CHARACTERISTICS OF PLASMA MICROPARTICLES IN SLE

3.2.1. Total numbers and cellular origins of microparticles

Two of the primary objectives of these studies were to enumerate the total numbers of MPs and specific MPs derived from platelets, leukocytes and endothelial cells. No perfect marker capable of detecting all MPs have been found and since the most frequently used marker, annexin V, misses some MPs, the concentration of identified MPs in the MP-gate may the best measure of the whole population (90). Several previous studies in

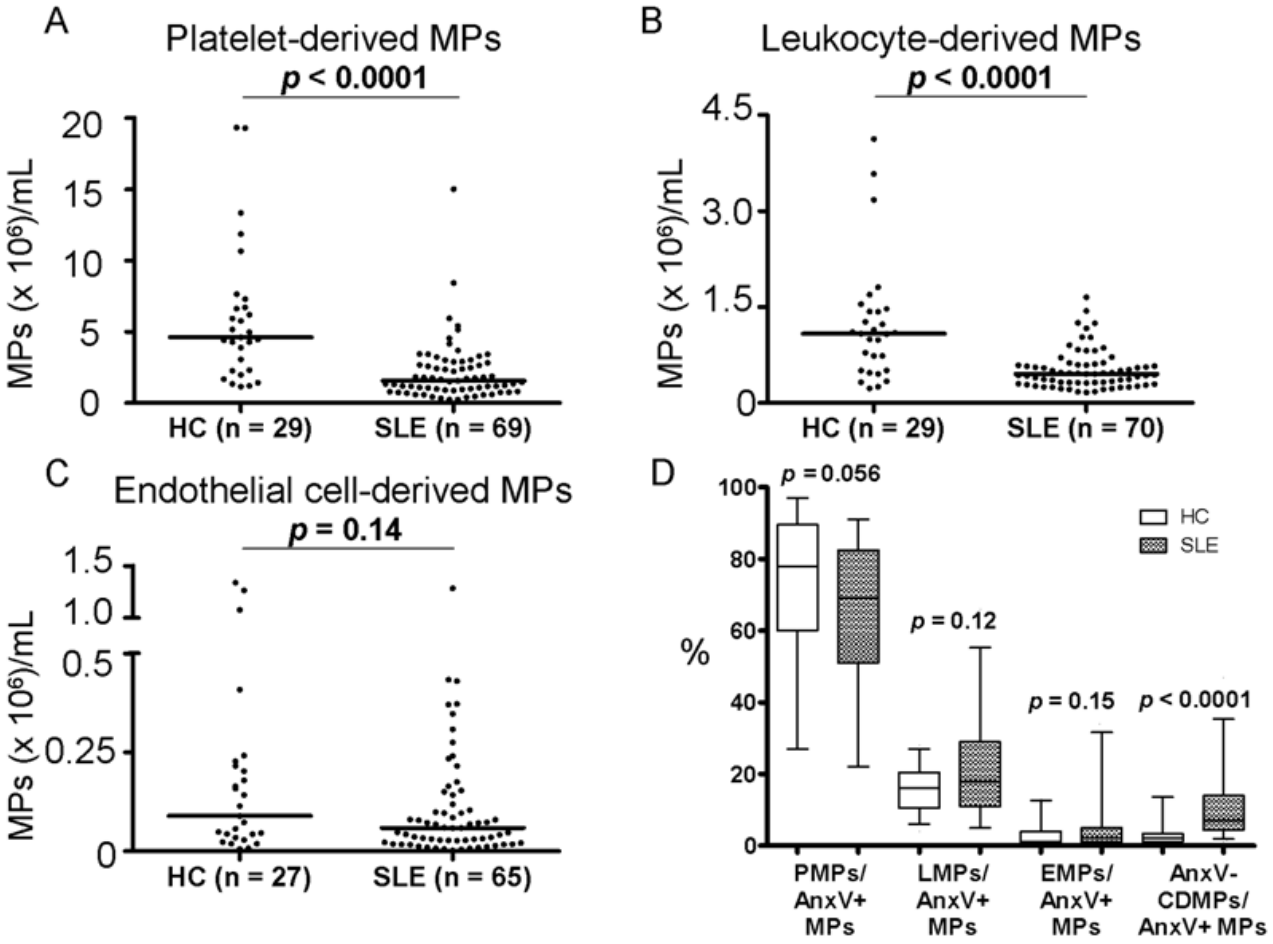

Figure 11
Plasma levels of cell-derived MPs. A-C, platelet-, leukocyte- and endothelial cell-derived MPs and their relative distribution in SLE patients and healthy controls. Horizontal lines depict the medians. D, relative distribution of PMPs, LMPs, EMPs and AnxV– CDMPs as a fraction of the total AnxV+ MP numbers. Boxes depict the median (central line) and 25-75th percentile (border of the box) and the whiskers the 5-95th percentile. Comparisons between the groups were performed with the Mann-Whitney U-test. Sample numbers lower than 29 (HC) and 70 (SLE) reflect analysis failures (A and C). HC = healthy controls; SLE = systemic lupus erythematosus. Adopted from Figure 4, Paper I.

systemic autoimmune diseases and vasculitides have found elevated levels of plasma MPs (93). Contrary to expectation, we observed highly significantly reduced total numbers of all MPs, platelet- and leukocyte-derived MPs in the SLE patients compared with healthy controls as shown in Figure 11A-B and Figure 13A. The levels of MPs positive for endothelial cell markers showed a lower trend in the SLE samples but for these MPs the difference did not reach statistical significance (Figure 11C).

The decreased concentrations in the investigated MP populations in our SLE patients were a surprising finding. As mentioned above studies in both acute and chronic inflammatory conditions have reported elevated concentrations of circulating cell-derived MPs. The combination of observations of increased M

P concentrations, increased cellular activation and apoptosis during inflammation and a reduced clearance capacity has led to the notion of there being generally high levels of microparticles in SLE and in other inflammatory diseases (28;108;111;112;114;115;155;163-166). However, our results do not support this, and a recent small study in SLE patients by Pisetsky et al. found no difference in MP concentrations between SLE patients and controls (28). An additional example are the studies of samples from RA patients published by the Nieuwland-group using the same protocols as in our work where both equal, elevated and decreased MP-levels, respectively, have been reported (99;163;167). Besides the RA-studies decreased MP levels were also observed in multiple sclerosis, during infections and sepsis and in the elderly (164;168;169). Thus the link between plasma MPs and inflammation is complicated. These apparent discrepancies may partly reflect technical aspects and influences from variation in pre-analytical and analytical protocols but also an insufficient understanding of the complexity of MP biology including their formation, clearance, tissue deposition and role in homeostasis.

Increased cellular activation may be linked to increased formation of MP concentrations and be reflected in the levels of activation markers in plasma. We therefore quantified the platelet activa ion marker, soluble CD62P (sCD62P, P-Selectin) to elucidate a relationship with the most abun- dant MP population

(106). No difference in the plasma concentrations of sCD62P was detected between the SLE patients and healthy controls (Fig. 12, not included in any of the thesis papers). These results oppose an increased platelet activation state in SLE as previously observed or more hypothetically, an exhausted state of platelets as a cause for compromised PMP production (115).

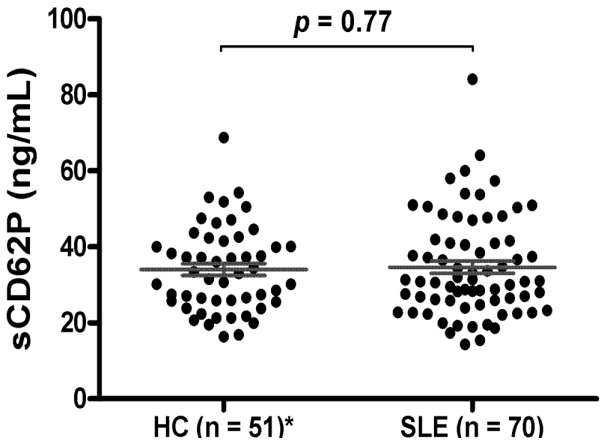

Figure 12
Plasma levels of soluble CD62P (P-selectin) in healthy controls and SLE patients measured with ELISA. Central line = mean, error bars = standard error of the mean. Unpaired t-test was used for comparisons. *Additional healthy controls were tested than included in the Paper I. HC = healthy controls; SLE = systemic lupus erythematosus.

Inflammation and tissue damage may increase inflammatory removal, destruction or deposition and ultimately lower levels of MPs. Decreased levels were found during infection, and an inverse relationship between inflammatory markers or more extensive disease and the levels of AnxV+ MPs and/or PMPs in SLE, systemic sclerosis and sepsis was observed (115;155;168;169). While no such inverse relationships between disease activity measures and the MP-levels were detected in our cohort, increased quantities of IgG on SLE-MPs were associated with complement activation in serum and overall lower AnxV+ MP. This is discussed more in detail Section 3.3. Also enzymatic degradation triggered by inflammation, e.g. by secretory phospholipase A2, have been proposed as a potential significant contributor to plasma MP concentrations (115).

Additionally pre-analytical and analytical issues in MP analysis have also attracted increasing interest (118). Our pre-analytical protocol may affect the absolute MP numbers rendering direct comparisons with other studies difficult. However, we have followed the same strict protocol for all samples, patient as well as healthy control, and analyzed samples in a random fashion and batch-wise making pre-analytical issues less likely to contribute to the observed significant intergroup differences.

3.2.4. Annexin V binding and non-binding microparticles in SLE

During MP formation phosphatidylserine flip-flops across the cell membrane and becomes exposed on the surface. The exposure of PS on the surface of MPs has several physiological implications including triggering of the coagulation cascade and potentially "flagging" MPs for macrophage clearance (22;95). However, PS-exposure is not a universal physiological feature of cell-derived MPs including apoptotic bodies (24;91;126;127;170). The degree of PS-exposure may depend on the cellular origin and state of the parental cell and appears susceptible to changes during sample processing (91;118). In the analytical setting PS-exposure has been extensively used as a marker to identify cell-derived MPs and as a tool to quantify the total MP-population e.g. by using AnxV-binding to MPs in flow cytometry.

In Paper I we also wanted to characterize the entire population of submicron particles by their AnxV-binding capabilities. Previous studies on MPs have focused on MPs that bind cell-specific antibodies and/or AnxV (111;114;115;170). Characterization of MPs by their degree of PS-exposure may provide novel insight into PS-exposing and non-exposing MP phenotypes which potentially reflect pathology and altered MP functionalities. The results in Paper I have been the first published attempt, to our knowledge, to explore the entire MP-population by their AnxV-binding capabilities comparing a large disease group with healthy controls.

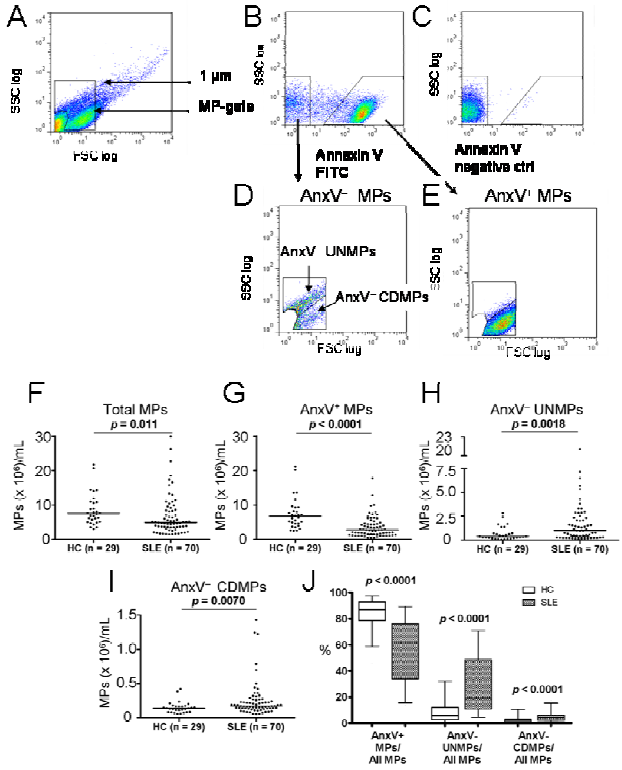

Figure 13
Representative flow cytometry density plots from a SLE patient and plasma concentrations of annexin V-binding and non-binding MPs in SLE patients and healthy controls. A-B, MPs stained with AnxV FITC were identified (B). D-E, Backgating of the AnxV-binding and non-binding MPs showed non-binding MPs located in two separate populations. One population is localized in the same region as the AnxV+ MPs and likely cell-derived, termed the AnxV– cell-derived MPs, AnxV– CDMPs. The other population is termed AnxV– UNMPs, AnxV– MPs on unknown nature, most likely composed of a mixture of cell-derived MPs, chylomicrons and protein complexes. A-E, Depict the plasma concentrations and distributions of the identified MP-populations. The figures are adapted from Paper I.

We observed that SLE patients had highly significantly decreased concentrations of AnxV-binding MPs and increased concentrations of AnxV non-binding MPs compared with healthy controls (Fig. 13B-E). The majority of all MPs in both groups were AnxV-binding MPs. The distributions, however, were skewed towards a larger proportion of AnxV non-binding MPs in SLE patients (Fig. 13J). Interestingly, backgating the AnxV– MPs to a FSC/SSC plot revealed that the AnxV non-binding MPs represented at least two different populations based on their biophysical properties, i.e. morphology and/or composition (Fig. 13D). The SSC-low population was located in the same region as AnxV+ MPs (Fig. 13E) as well as in the same region as PMPs formed in the induction experiments (Fig. 7I). Based on these observations we defined two separate populations (gates) in our MP-plots for quantification, i.e., AnxV– cell-derived MPs (AnxV– CDMPs) and AnxV– MPs of unknown nature (AnxV– UNMPs). Both AnxV– CDMPs and AnxV– UNMPs were significantly increased in the SLE patients. Since the AnxV– CDMPs may be a better estimate for AnxV– non-binding MPs of cellular origin, the significant 3-fold higher ratio of AnxV– CDMP:AnxV+ MP in the SLE patients indicated an increase in the fraction of circulating cell-derived MPs in SLE, that are not exposing PS or are having PS-blocked for binding (Figure 10D).

Recently Gyorgy et al. showed that circulating immune complexes may be classified as MPs in flow cytometry due to shared biophysical properties (82). It is likely that the AnxV– MP populations included a mixture of different types of particles including true cell-derived MPs, protein complexes and lipid vesicles. Since MPs were not labelled with a cell-specific antibody and Annexin V the cellular origin of the AnxV-binding and non-binding MPs could not be determined with certainty. The association profiles, however, provided hints to the nature and origin of these particles (Table 4).

The finding of highly significant associations between AnxV+ MPs concentrations PMP and LMP levels (Table 4) suggested that the majority of AnxV+ MPs originated from platelets and leukocytes. Previous double-labelling experiments with cell-specific antibodies and AnxV in frozen plasma samples have showed that the majority of PMPs and LMPs bound AnxV, but differences in the AnxV-binding between healthy and disease groups have not been explored (126;170).

The AnxV– CDMPs were associated with EMP levels, while the AnxV– UNMPs were associated with cholesterol and triglyceride levels in the blood. The fact that AnxV– CDMPs were associated with the levels of EMPs suggested an endothelial origin of the majority of these MPs (90;91). Recently EMPs have been shown to bind AnxV to a lesser degree than PMPs and LMPs in plasma (126). Additionally, in vitro culture studies have suggested that EMPs may be less prone to PS flip-flop, and that the degree of flip-flop depended on the inducing stimuli (91). Interestingly the SLE patients in our study had increased levels of the AnxV– CDMPs and besides being associated with EMPs the AnxV– CDMPs were also associated with glomerulonephritis, disease activity measures and vascular dysfunction, discussed more in detail in section 3.3 (Table 5). Thus phenotyping of EMPs by their AnxV-binding may be a potential biomarker of vascular pathology.

The AnxV– UNMPs were highly significantly associated with cholesterol and triglyceride levels suggesting that that some MPs may circulate bound to lipid vesicles, and/or that lipid vesicles, including chylomicrons, may be co-isolated or insufficiently reduced during the preparatory washing steps. Chylomicrons have previously been known to interfere with platelet analysis in flow cytometry (171). Additionally, the lipid metabolism is disturbed in SLE, and patients express dyslipoproteinemia with altered chylomicron metabolism, higher fasting levels of VLDL and triglycerides and lower levels of HDL (172-175). Thus finding increased AnxV– UNMPs could be a reflection or a direct measure of these phenomena.

Altogether characterization of whole pool of submicron MPs by their AnxV-binding indicated that the pool of MPs is comprised

Table 4. Correlation analysis. Lipid profile, MP cellular origins and AnxV-binding and AnxV non-binding MPs with Spearman's rank correlation coefficients (R) and *p*-values. Spearman's rho P-values ≤ 0.05 are highlighted.*

		$AnxV^+$ MPs		$AnxV^-$ CDMPs		$AnxV^-$ UNMPs	
Clinical variables	N	R	*P*	R	*P*	R	*P*
Cholesterols, total	68	-0.03	0.82	0.21	0.09	**0.27**	**0.03**
HDL	68	0.05	0.70	0.10	0.40	0.08	0.51
LDL	68	-0.11	0.38	0.07	0.56	0.04	0.72
Triglycerides, total	67	-0.04	0.75	**0.29**	**0.02**	**0.38**	**0.002**
PMPs	70	**0.80**	**< 0.0001**	0.16	0.17	0.18	0.13
LMPs	70	**0.55**	**< 0.0001**	0.02	0.84	0.23	0.06
EMPs	70	0.17	0.15	**0.43**	**0.0002**	0.14	0.24

*Extract of Table 2 from Manuscript I.

of different types of particles with altered concentrations and distributions in SLE compared with healthy controls. From a general perspective finding elevated numbers of AnxV non-binding in SLE using flow cytometry is not surprising and could be a measure reflecting the increased circulating immune complexes, the disturbed lipid metabolism, and the altered cellular clearance mechanisms frequently observed in SLE. The causes for the increased numbers of AnxV non-binding MPs may be lack a surface exposure of PS per se, however, other factors affecting the PS-AnxV interaction may also include calcium concentrations in the analytical preparations, enzymatic degradation of PS e.g. by higher activity of plasma secretory phospholipase A2, blockage of PS by endogenous ligands or immunoglobulins or neutralisation of the added AnxV by soluble ligands (24;91;115;127;169;176). PS-exposure is important in hemostasis and cell clearance, whereas the physiological implications for the altered distributions of AnxV non-binding cell-derived MPs in SLE are not clear.

3.2.6. AnxV-binding and non-binding MPs in fresh and frozen plasma

An unresolved issue in MP analysis is the impact from pre-analytical handling. PS-exposure is particularly influenced by sample processing. As platelets are activated by lowering temperature and since short-term freezing may induce microparticles, it is possible that MP concentrations, PS-exposure and expression of different surface markers may be affected by sample processing (118;177;178). In order to clarify if findings in frozen plasma reflect fresh plasma, we compared AnxV MP measurements in fresh and frozen plasma (Fig. 14, from the supporting material for reviewers of Paper I). Here, samples from 3 SLE patients and 3 healthy controls were all collected within 30 minutes and prepared and analysed on the same day. In both groups freezing of the samples resulted in lower levels of total MPs, AnxV– UNMPs and AnxV– CDMPs, whereas the concentrations of AnxV+ MPs did not change (Figure 13). In the SLE patients we found higher levels of AnxV– UNMPs and AnxV– CDMPs in both fresh and frozen plasma. Thus freezing of samples lowered the absolute numbers of AnxV non-binding MPs and tended to diminish intergroup differences, but the observed differences found in frozen plasma still reflected those found in fresh plasma in this small study. It cannot be excluded, however, that finding almost unchanged levels of AnxV+ MPs upon freezing may conceal that some AnxV-binding MPs are lost and that AnxV-non-binding MPs, in particular AnxV– CDMPs, expose PS and change their phenotype to AnxV-binding MPs (126;127). Using frozen material is suboptimal, but it also has the great advantage of providing information on a large number of uniformly treated patient and control samples analysed batch-wise with minimal technical variation.

Few methodological studies have addressed the influence of pre-analytical and analytical steps on MP measurements (118). The focus of those studies has been the impact on MP the detected concentrations, particularly PMPs and EMPs, but not on the expression of different surface-markers or changes in AnxV-binding. Hypothetically, formation of MPs as a result of the freezing process would relate to activation of insufficiently removed "contaminating" platelets in platelet-poor plasma. Nevertheless data do not consistently confirm this notion, which again may reflect differences in experimental set-ups, most importantly in the centrifugation protocols used for preparation of PPP. It is also important whether flow cytometric analysis was performed directly or on washed MPs. Additionally, other factors such as thawing conditions and sample storage time may influence results

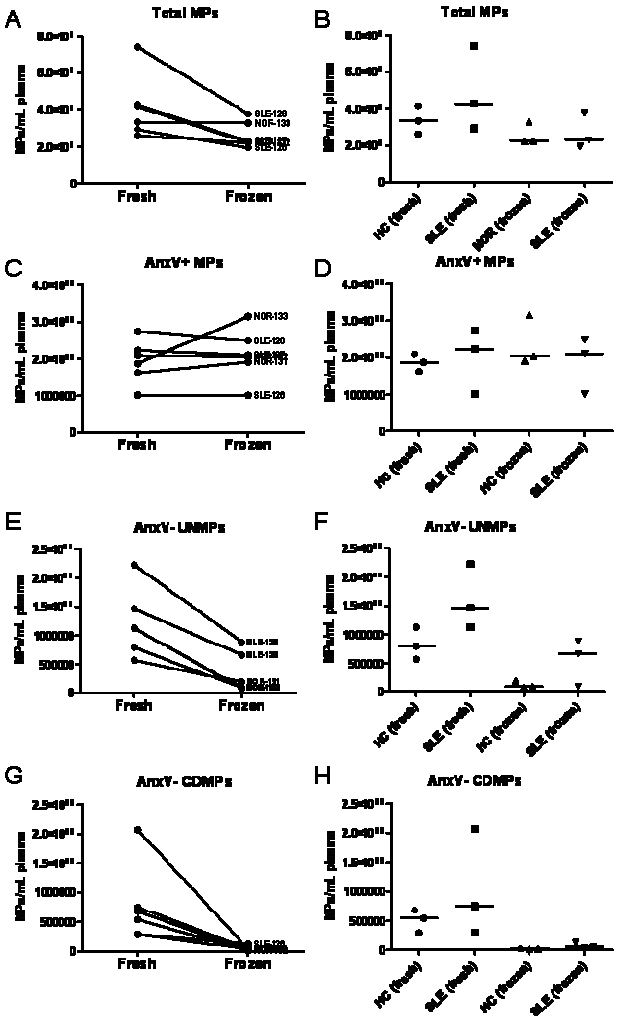

Figure 14
AnxV-binding and non-binding MPs in fresh and frozen plasma from 3 SLE patients and 3 healthy controls. Plots depict plasma concentrations of Total MPs, AnxV-binding and non-binding visualizing changes for each sample (A, C, E and G) and absolute concentrations of MPs in each groups (B, D, F and H).Horizontal bars depict the median. AnxV+ MPs = Annexin V binding MPs; AnxV– UNMPs = Annexin V non-binding MPs of unknown nature; AnxV– CDMPs = Annexin V non-binding cell-derived MPs.

(126;179-183). Particular relevant to the studies in thesis, Ramaciotti et al. reported no differences in their MP-results in both MS and FACS of fresh and frozen samples, but the data were not shown (104). To minimize the pre-analytical impact on control and patient samples a strict sampling protocol was followed in our studies. Additionally, control and patient samples were collected consecutively during the same period of time (equal storage time), prepared and analysed randomly and batch-wise within a short range of time. Thus pre-analytical steps are not likely to explain the 2-3 fold reduced concentrations of the MP-populations detected in our SLE samples.

3.2.6. Increased MP-associated immune complex components in SLE

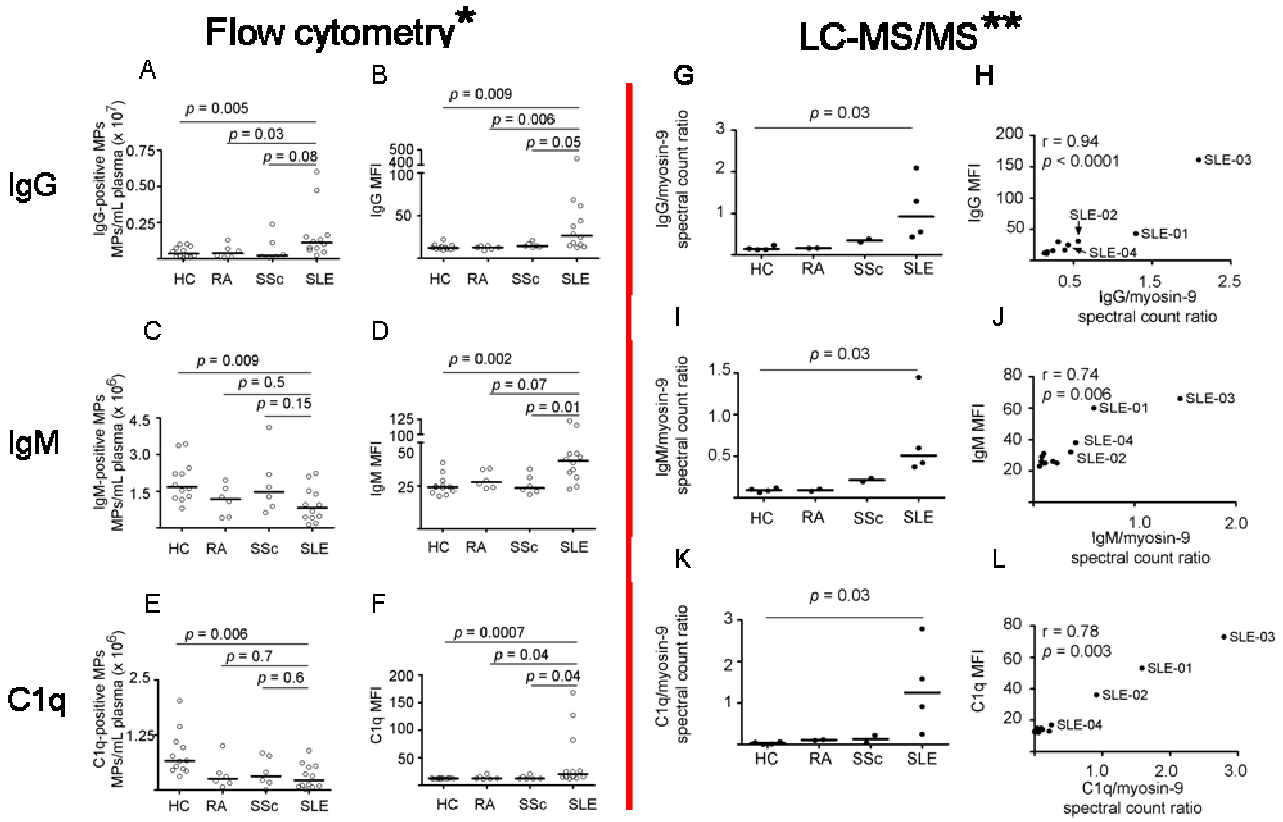

Figure 15
MP concentrations and loads of MP-associated IgG, IgM and C1q determined with flow cytometry and LC-MS/MS in patients with SLE, SSc and RA and healthy controls. A-F, Flow cytometric concentrations of IgG/IgM/C1q and AnxV double positive MPs and their average loads per MP, MFI. H, J and L, Depict the univariate Spearman's rank correlation analyses between the MFI values and relative protein abundances. Bars = median. Mann-Whitney U-test (A-F) and one-way ANOVA (G, I, K) were used for statistical comparisons. All plots are adapted from Paper III. *The plots of IgG-, IgM- and C1q-negative AnxV-positive MPs have been removed. **The plots depicting the correlation between spectral counts of myosin-9 and integrin-α IIb and the concentrations of AnxV-binding MPs have been removed.

The discovery that nuclear autoantigens are exposed on apoptotic-derived MPs in vitro suggested that a subfraction of plasma MPs may carry circulating autoantigens serving as antigenic targets of antinuclear autoantibodies and capable of triggering MP-associated inflammation in SLE patients (17). Studies on the cargo of surface molecules on plasma MPs have been limited. Previously no difference in the concentrations of C1q-positive MPs was found in two studies comparing HCs with patients with RA and SLE, respectively (99;117). Recently ICs with particle properties were found increased in SLE-plasma using flow cytometry, however, without discriminating between soluble ICs and IgG on cell-derived MPs (28).

In the present work the IgG-, IgM- and C1q-positive MP concentrations were quantified using flow cytometry, and the loads of MP-associated IgG, IgM and C1q were determined using both flow cytometry and LC-MS/MS. To assess the disease specificity of the measurements 12 SLE patients with active or inactive disease were initially compared with patients with systemic sclerosis (n = 6) and rheumatoid arthritis (n = 6) and 12 healthy controls.

Using flow cytometry increased concentrations of IgG-positive MPs and increased MP-loads of IgG, IgM and C1q were detected suggesting increased loads of immune complexes on IgG-positive MPs in SLE (Fig. 15A-F). The MP-IgG and -C1q MFI values correlated significantly supporting that MPs carried ICs, however, triple-labelling with AnxV (or a cell-specific antibody), a-IgG and a-C1q would be preferable in future work. Only AnxV-binding MPs were accepted as cell-derived MPs and analysed to avoid the interference of soluble ICs in the analysis (82).

To verify these results 12 homogenous pools of 3 patient and control samples were analysed using the LC-MS/MS proteomics protocol presented in Sections 3.1.3. The input concentrations of AnxV+ MPs correlated with myosin-9 abundance not only in healthy controls (Paper II) but also in patient samples lending additional credibility to myosin-9 as a normalizer of MP-abundance (data not shown). The myosin-9 normalized IgG, IgM and C1q relative abundances were increased in all the SLE-pools and most pronounced in the 2 pools with patients with active disease (Fig. 15G, I and K). Thus patients with SLE carried more IgG, but SSc samples also showed more IgG than HCs and RA (possibly due to binding to anti-centromere or Scl-70 antigens). The few existing proteomics studies have also observed overrepresentation of immunoglobulins and complement proteins in/on plasma MPs and MPs from atherosclerotic plaques (104;124;162). Future studies in other diseases may shed light on the association between MP-associated immunoglobulins and inflammation.

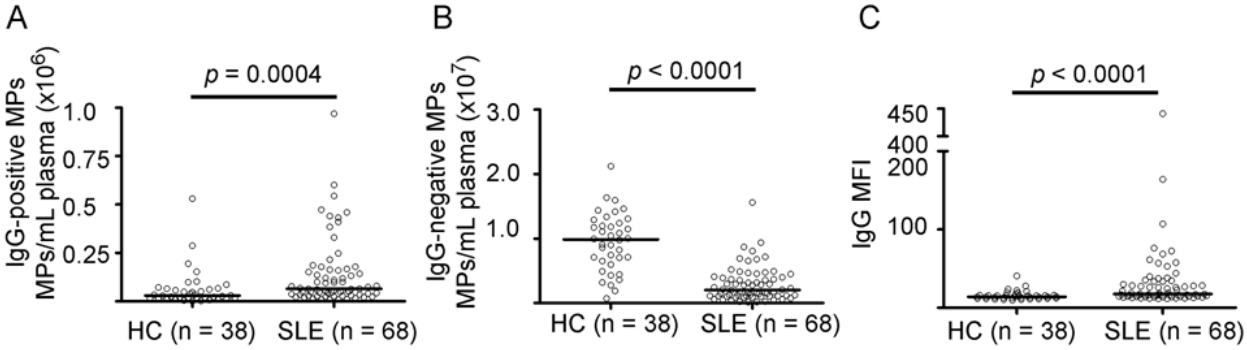

Figure 16
Flow cytometric determination of AnxV+ and IgG+/- MPs and the average load of IgG per MP comparing SLE patients with healthy controls. Bars = median. Mann-Whitney U-test was used for statistical comparisons.

Since MPs were pelleted by high-speed centrifugation a contribution to the results from soluble ICs cannot be excluded (82). However, the LC-MS/MS quantities correlated with the definite MP-associated IC quantities, i.e. the flow cytometrically derived MFI values (Fig. 15H, J and L).

As MP-IgG appeared to represent the load of MP-associated immune complex components, the study was expanded to a large number of SLE patients and healthy controls and these data verified the increased concentrations of IgG-positive MPs and increased MP-IgG loads (Fig. 16A and C). Altogether these results have provided the first specific evidence that SLE-MPs carry more IgG than controls – most likely as part of ICs.

3.3. CLINICAL AND SEROLOGICAL CORRELATES OF MICROPARTICLE CHARACTERISTICS IN SLE

In both Paper I and III MPs concentrations deviated significantly from controls. To explore possible disease-specific implications for the MP-measurements univariate Spearman's rank

Table 5. Correlation analysis. Clinical and AnxV-binding and non-binding MPs with Spearman's rank correlation coefficients (R) and *p*-values. Spearman's rho P-values ≤ 0.05 are highlighted.*

		AnxV⁺ MPs		AnxV⁻ CDMPs		AnxV⁻ UNMPs	
Clinical variables	n	R	*P*	R	*P*	R	*P*
Sex	70	-0.07	0.57	0.14	0.23	0.18	0.14
Age	70	**-0.23**	**0.05**	-0.17	0.16	-0.10	0.40
Disease duration	70	-0.01	0.91	-0.09	0.46	**-0.27**	**0.03**
APS	66	0.20	0.12	0.00	0.99	0.00	0.97
History of thrombosis, arterial	70	0.18	0.14	**0.30**	**0.01**	0.13	0.28
History of thrombosis, venous	70	0.08	0.49	-0.10	0.40	0.07	0.59
Nephritis*	70	-0.09	0.45	**0.33**	**0.01**	0.10	0.39
SLEDAI	70	0.06	0.62	**0.27**	**0.02**	0.14	0.25
SLICC/ACR DI	70	0.16	0.18	0.13	0.27	0.05	0.70
C-reactive protein	70	0.20	0.10	0.10	0.40	0.12	0.32
ESR	66	**0.34**	**0.005**	0.22	0.07	0.09	0.46
Medication	70	0.08	0.51	**0.25**	**0.04**	0.00	0.99
Hypertension	70	-0.08	0.48	**0.38**	**0.001**	0.19	0.11

*Extract of Table 2 from Manuscript I.

Table 6. Correlation analysis. Clinical and MP parameters with Spearman's rank correlation coefficients (r) and *p*-values. Spearman's rho *p*-values ≤ 0.05 are highlighted.

		IgG-positive MPs		IgG MFI	
Parameters*	n	r	*p*-value	r	*p*-value
AnxV$^+$ MPs total	68	0.32	**0.009**	-0.26	**0.03**
Active nephritis	68	-0.12	0.3	-0.0059	0.96
SLEDAI	68	0.11	0.4	0.11	0.4
Platelets	68	0.05	0.73	-0.11	0.4
Leukocytes	68	-0.25	**0.04**	-0.11	0.4
Plasma IgG	68	0.30	**0.01**	0.16	0.2
Anti-dsDNA	67	0.31	**0.01**	0.23	0.06
Anti-ENA	68	0.38	**0.001**	0.042	0.74
Anti-histone	68	0.29	**0.02**	0.08	0.50
Anti-C1q	68	0.19	0.1	0.39	**0.001**
C4	68	-0.17	0.2	-0.50	**< 0.0001**
C3	68	-0.21	0.09	-0.40	**0.0007**
C1q	67	-0.032	0.8	-0.25	**0.04**

correlation analyses were performed between the MP-concentrations and clinical and serological manifestations.

The main findings in Paper I were the identification and quantification of the two distinct AnxV– MPs populations. Generally, the levels of AnxV– CDMPs were very low compared to the AnxV– UNMPs and the AnxV+ MPs. While AnxV+ MPs were associated with decreasing age and increasing erythrocyte sedimentation rate and AnxV– UNMPs with disease duration, cholesterol and triglyceride levels, the AnxV– CDMPs had more disease specific associations (Table 4 and 5). As mentioned in Section 3.2.4 (Table 4) the concentrations of AnxV– CDMPs were associated with EMP levels, but also with disease activity, a history of arterial thrombosis, hypertension, high triglyceride levels, active nephritis, and the use of immunomodulatory drugs, but not a specific autoantibody profile (Table 5). The majority of the patients with active disease in the cohort had nephritis and likely to have pronounced endothelial and systemic inflammation which could explain these associations. EMPs have previously been found elevated in a number of inflammatory diseases including SLE and suspected of having a role in vascular dysfunction, angiogenesis and endothelial inflammation, while their biomarker potential have yet to explored (94;110;113;114). Although the observed associations in the present study may not be disease-specific to SLE, it could still be interesting to test the biomarker potential of AnxV– CDMPs and/or EMPs in lupus nephritis in future studies. Additionally, the biomarker potential of EMPs may reflect a more direct role of EMPs in disease pathogenesis as suspected in the anti-phospholipid syndrome (APS). Here, EMPs were found elevated in patients with anti-phospholipid antibodies (aPL) compared to relevant controls and most pronounced in patients with previous thrombotic events suggesting a link between the presence of aPL, triggering of EMPs and thrombosis (111;112;184). However, the notion of unique EMP triggering properties of aPL could not be confirmed in vitro and appeared to relate to the presence of autoantibodies in general. Plasma from patients with primary APS and SLE patients both with and without aPL had equal and higher triggering capabilities on EMP formation compared with plasma from healthy and non-autoimmune thrombotic controls (112). In the present study no correlations between AnxV– CDMPs and a history of venous thrombotic events or having a secondary anti-phospholipid syndrome were observed in our SLE cohort.

The major findings in Paper III were increased concentrations of IgG-positive MPs and MP-load of IgG. The binding of autoantibodies to MPs may have several physiological implications such as triggering immune complex formation and complement activation, altering MP turnover and providing co-stimulation and/or adhesive properties to ICs ultimately contributing to the inflammatory and immunological aberrations observed in SLE.

As presented in Table 6 (Table 2 from Paper III) the concentrations of IgG-positive MPs correlated with plasma IgG and autoantibodies against nuclear antigens (ENA, histones and dsDNA) indicating specific binding of immunoglobulins to accessible autoantigens on circulating MPs. However, SLE autoantigens in plasma MPs have yet to be detected. As an attempt to indirectly measure autoantigens on MPs, Pisetsky et al. incubated plasma MPs with SLE IgG (plasma) and observed a binding to the

MPs ex vivo, but the IgG-binding could not be removed by DNase treatment (28). In line with our findings the concentrations of IgG-positive particles were associated with anti-DNA levels in serum (28). The mode of antibody binding to plasma MPs is thus unclear and need to be established, i.e. whether the antibody is specifically bound to an antigen, to Fc- receptors or as part of ICs to complement-receptors (185;186).

The association profiles suggested that the concentrations of IgG-positive MPs and IgG MFI represented different measures. IgG MFI, as a measure of the average load of IgG per MP, may reflect the presence of MP-bound ICs. The IgG MFI was not associated with nuclear antibodies, but with lower levels of complement components in serum, i.e. activation of the complement system. In vitro studies have showed that binding of C1q to apop totic bodies and to ectosomes (MPs from granulocytes) were capable of triggering the classical complement cascade on the MP-surface (117;186). To extrapolate from this, our observations suggested that IgG binding to MPs triggered IC formation on the MP-surface that directly contributed to the complement activation measured in serum. Accordingly, determination of the proportion of circulating ICs that are in fact MP-associated and their contribution to serum complement activation in SLE would be highly interesting. The association of autoantigens and autoantibodies in MPs may also have other implications. As MPs are liposomal structures, MPs may have adjuvant properties enhancing the immuno-stimulatory properties of autoantigens and ICs on immune cells, e.g. the INF-α production from plasmacytoid dendritic cells (76). Measurement of plasma INF-α was attempted but below the detection limit in the SLE and control samples. Besides being associated with complement activation no significant associations between MP-IgG results and other disease activity measures or clinical manifestations were found.

Studies on the turnover of plasma MPs have been few, but the half-life of plasma MPs appears to be less than 6 hours (187-189). The binding of IgG to MPs may affect their turnover. Autoantibodies have in vitro been shown to opsonize and enhance phagocytosis of apoptotic and necrotic cells in a complement-dependent manner (63-65). However, contradictory to this Reefman et al. found an inhibitory effect of autoantibody binding to dying cells (67). In the SLE patients the IgG MFI was correlated negatively with the concentrations of AnxV+ MPs indicating that the presence of ICs on MPs may enhance MP clearance, degradation or deposition. Of note, we also observed that the concentrations of AnxV + MPs were associated with decreasing age suggesting a general age-associated decline in line with the observations by Forest et al. (168).

4. CONCLUSIONS AND PERSPECTIVES

In this thesis, plasma MPs from a large cohort of well-characterized SLE patients and controls were extensively characterized. Novel MP-populations were identified, significant quantitative and qualitative differences between the groups were demonstrated. Furthermore, both clinical and serological correlates to certain MP-subpopulations were in agreement with these MPs being directly involved in the pathogenesis of SLE.

1) Electron microscopy of plasma MPs showed a pool of submicron-size vesicles that were heterogeneous in terms of size, morphology and density. While presenting the first published EM photographs of SLE-MPs, the methodology proved unfit for comparing SLE and control samples. The heterogeneity of the MP-pool clearly stresses the need in future studies to isolate the relevant MP subpopulations prior to EM and/or use immune-gold labelling e.g. to demonstrate co-localization of MP-antigens and autoantibodies.

2) We presented the characteristization of the plasma MP proteome using label-free nano-LC-MS/MS in a shotgun proteomic protocol. Four washing steps enabled us to deplete the MP fraction for plasma proteins. Reproducibility experiments showed an excellent intra-assay variation but poor inter-day variation reflecting procedural variation. The cytoskeletal protein, myosin-9, was identified as a suitable MP-normalizer of MS-datasets to accommodate these procedural variations and enable comparisons across samples and studies. Serving as a standardized analytical platform, we were able to verify the increased quantities of MP-associated immune complex components observed on SLE-MPs with flow cytometry and to characterize the normal plasma MP proteome to serve as a reference dataset in future clinical studies (158).

3) Using flow cytometry optimized for detection of MPs (< 1 µm) we found decreased concentrations of MPs in general as well as in the fractions of MPs from platelets, leukocytes and endothelial cells in SLE patients. These data suggest that compromised formation of MPs, aggregation or increased clearance from the blood stream may be more predominant in SLE than healthy controls. Since other studies have reported increased MP concentrations in SLE, these findings need to be validated in an independent SLE cohort.

4) In the large SLE we were able to identify two AnxV non-binding MP-subpopulations which we termed AnxV– CDMPs and AnxV– UNMPs. The AnxV non-binding MPs were increased in frozen as well as fresh samples from SLE patients compared to HCs. The AnxV– CDMPs were most likely of cellular origin and correlated with EMP levels, disease activity measures and nephritis suggesting a role in vascular inflammation and lupus nephritis. Thus testing the biomarker potential of the AnxV– CDMPs in lupus nephritis seems warranted. As the causes and the implications of MP PS non-exposure are elusive, further characterization of the origin and composition and testing the functional effects of PS-exposure/non-exposure on MP clearance and immune cell activation is necessary to elucidate the role of MPs in the pathogenesis of SLE.

5) Using both flow cytometry and LC-MS/MS we were able to demonstrate increased quantities of immune complex components on MPs as well as increased concentrations of IgG-positive MPs in SLE patients compared to controls. These data demonstrate for the first time increased quantities of immune complex components associated with definite cell-derived MPs in SLE patients.

6) We also observed that the concentrations of IgG-positive MPs were associated with the levels of specific antinuclear antibodies in serum corroborating the notion that SLE-MPs may be antigenic targets of autoantibodies, i.e. carriers of autoantigens. However, SLE associated autoantigens in plasma MPs have yet to be detected, and isolation and further in-depth characterization of the IgG-positive MPs in SLE are needed to confirm the presence of autoantigens. We also observed that the increased loads of IgG on MPs were highly correlated with the consumption of serum complement components suggesting a direct contribution of IgG-positive MPs to the complement activation frequently observed

in SLE. The increased MP-IgG loads were also associated with lower concentrations of AnxV+ MPs suggesting that the presence of ICs on MPs may favour their clearance, degradation or deposition.

Overall, this study underlines the importance of MPs in SLE pathogenesis. MPs may harbour autoantigens, form ICs and trigger the classical complement cascade. Whether MP-ICs also act on immune cells and e.g. contribute to INF-α production or influence the turnover of MPs is unknown but highly relevant to SLE pathogenesis. Thus the observations in our SLE patients warrant experimental testing of the functional capabilities of MP-IgG and MP-ICs to understand and characterize the specific roles of MPs in SLE pathogenesis.

SUMMARY

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease presenting with a wide array of clinical manifestations and an elusive pathogenesis. A characteristic feature in SLE is the occurrence of autoantibodies against chromatin, double-stranded DNA, and RNA-binding ribonucleoproteins. Observations of defective clearance of dying cells in SLE combined with the generation and exposure of nuclear autoantigens during apoptosis have led to the hypothesis that improperly cleared apoptotic debris constitutes a source of autoantigens capable of triggering autoimmune disease. In blood, circulating, heterogeneous subcellular microparticles (MPs) are released from cells and platelets constitutively and upon cellular activation or apoptosis. Such MPs may reflect the state of their parental cells and tissues, and could serve as markers of pathology. Particular in SLE MPs may serve as carriers of autoantigens and constituents of immune complexes (ICs).

The purposes of this Ph.D. thesis were to develop and apply qualitative and quantitative methods to characterize circulating MPs with respect to numbers, cellular origins and composition in a large cohort of well-characterized SLE patients compared to healthy and disease controls and to explore associations with clinical, biochemical and serological parameters. The Ph.D. thesis consists of a review and three papers.

In the first paper we show that SLE patients have significantly decreased numbers of annexin V binding MPs and MPs from platelets, leukocytes and endothelial cells using flow cytometry. Two morphologically distinguishable populations of annexin V non-binding MPs were increased in the SLE patients. The annexin V non-binding MPs of most likely cellular origin were associated with the presence of lupus nephritis, markers of increased disease activity and levels of endothelial cell-derived MPs.

In the second paper we present the development of a proteomic method to characterize the protein composition of purified MPs using high-resolution mass spectrometry and establish a set of proteins which may serve as normalizers for MP protein quantitation enabling comparison across samples and studies. We identify a core proteome of more than 330 proteins in MPs from healthy individuals. The method enables an unbiased, comprehensive coverage of all proteins present in MPs irrespectively of the availability and utility of immunological reagents.

In the third paper we use the established flow cytometry and mass spectrometry platforms to show that SLE-MPs carry more surface-bound IgG, IgM and C1q indicating that SLE-MPs could be antigenic targets and constituents of ICs. Additionally, the numbers of MPs carrying IgG are also increased in SLE. The load of IgG on SLE-MPs was associated with markers of complement activation, indicators of disease activity in SLE.

In conclusion, using both antibody-dependent and –independent methods we demonstrate that SLE-MPs deviate distinctly from controls and may serve as precursors of ICs associated with complement activation and disease activity. This supports the hypothesis of MPs being directly involved in or reflecting tissue-specific or systemic inflammation in addition to carrying accessible antigens. Accordingly, further characterization of the proteome and functional properties of SLE-MPs seem highly warranted in future studies.

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