Università di Pisa

Scuola di Specializzazione in Patologia Clinica

Tesi di Specializzazione:

Particulate matter induces the generation of procoagulant microparticles by human mononuclear and endothelial cells

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Index

ABSTRACT

Background: Particulate airborne pollution is associated with increased cardiopulmonary morbidity. Microparticles are extracellular vesicles shed by cells upon activation or apoptosis involved in physiological processes such as coagulation and inflammation. We investigated the hypothesis that particulate matter causes the shedding of microparticles by human mononuclear and endothelial cells.

Methods: Cells were isolated from the blood and the umbilical cords of normal donors with standard techniques and cultured in the presence of particulate from a standard reference. Microparticles were assessed in the conditioned medium as posphatidylserine concentration. Microparticle-associated tissue factor was assessed by a one-stage clotting assay. Nanosight technology was used to confirm the main results.

Results: Particulate matter induces a dose- and time- dependent, rapid (1 h) increase in microparticle generation in both cells. These microparticles express functional tissue factor. Particulate matter increases intracellular calcium concentration and phospholipase C inhibition reduces microparticle generation. Nanosight analysis confirmed that upon exposure to particulate matter both cells express particles with a size range consistent with the definition of microparticles (50-100 nm).

Conclusions: Exposure of mononuclear and endothelial cells to particulate matter upregulates the generation of microparticles at least partially mediated by calcium mobilization from intracellular storage pools. This observation might provide a further link between airborne pollution and cardiopulmonary morbidity.

CHAPTER 1: INTRODUCTION

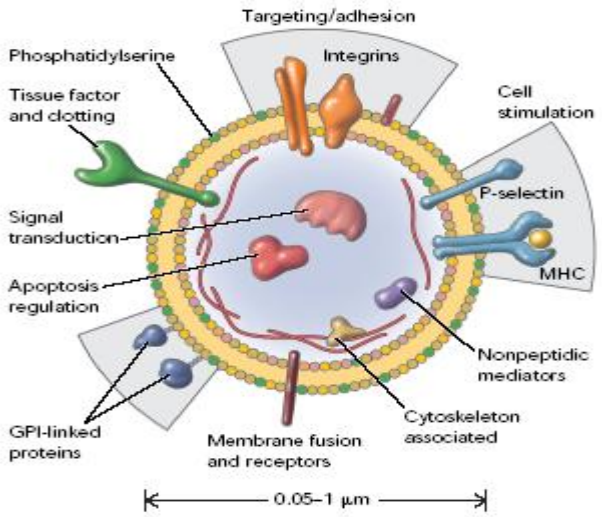
1.1 Microparticles

Microparticles (MP) are heterogeneous cell-derived microvesicles released through a process of exocytic budding of the plasma membrane by different vascular cell types such as platelets, endothelial cells, neutrophils, monocytes and lymphocytes.[1]

MP are heterogeneous in nature, varying in both size and content; they are typically defined as 0.05–1 μm in size and they are composed of a phospholipid bilayer that expose membrane proteins (trans-membrane proteins and receptors) and enclose cytosolic material (enzyme, transcription factors and nucleic acids) derived from their cell of origin.[2] (*Figure 1)*

Originally described as inert "cellular dust" and devoid of physiological significance, in the recent years several studies have shown that MP are important players in different biological processes, including blood coagulation and inflammation and are involved in the pathogenesis of several diseases.[3]

Figure 1: Cellular microparticle



1.2 Mechanisms of formation of microparticles

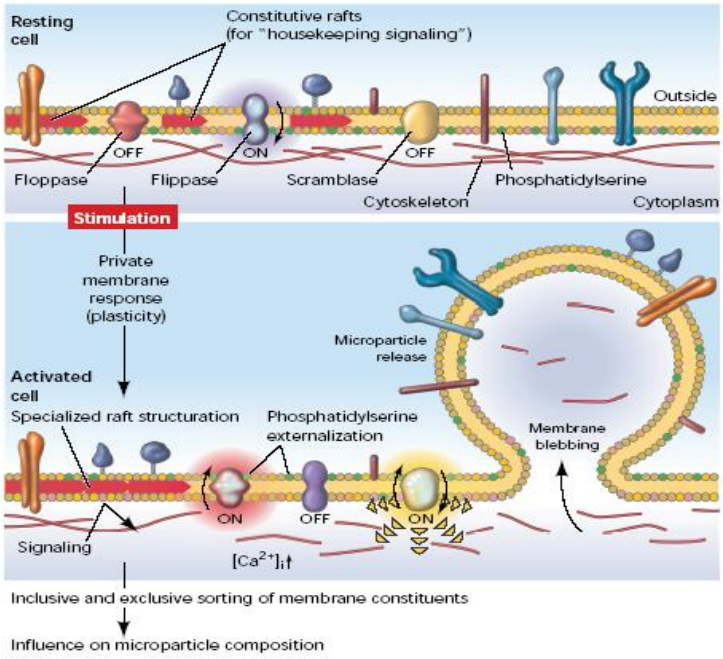
There are two well-known cellular processes that can lead to the formation of MPs: chemical and physical cell activation (by agonists or shear stress, respectively), and apoptosis (through the action of growth factor deprivation or apoptotic inducers)[3]. Although the precise molecular mechanisms of MP formation are not know, cytoskeletal reorganization and alterations in phospholipid asymmetry of the plasma membrane are essential to formation.[1]

In normal conditions, each of the two leaflets of the membrane bilayer has a specific lipid composition. Aminophospholipids (phosphatidylserine (PS) and phosphatidylethanolamine) are segregated in the inner leaflet, while phosphatidylcholine and sphingomyelin are exposed in the external one. There are three specific enzymes that control the lipid distribution in the plasma membrane: flippase, floppase and scramblase. Flippase promotes the traslocation of PS and phosphatidylethanolamine towards the inner membrane in an ATP-dependent manner; whereas floppase catalyses the transport of PS to the outer membrane. Finally scramblase is ATP-independent and promotes unspecific bidirectional redistribution across the bilayer.[2]

During MP formation this asymmetric distribution of phospholipids is lost; membrane remodeling is associated with a significant and sustained increase of cytosolic Ca^{2+} accompanying cell stimulation that may lead to the collapse of the membrane asymmetry by stimulating/inhibiting transporters. The most prominent change in lipid distribution is the surface exposure of PS, followed by MP release allowed by cytoskeleton degradation by Ca^{2+}-dependent proteolysis.[2] (*Figure 2*)

By contrast, several studies showed that not all MPs contain PS, suggesting that the mechanisms implicated in MP formation may be more complex than suspected and could require supplemental intracellular pathways.[4] In this regard, it has been shown that of several proteins linked to cytoskeleton reorganization, such as Rho kinase II, are involved in the MP formation by endothelial cells through a mechanism involving caspase-2 [5].Alternatively, other Authors have shown the involvement of caspase 3 and Rho kinase I in MP formation.[6]

Figure 2: mechanism of formation of microparticles



1.3 Membrane Microparticles in Blood Coagulation

MPs are elevated in hypercoagulative disorders and this relationship is probably a result of their active participation in the coagulation process. The pro-coagulative properties of MPs are largely linked to their physical characteristics with two specific surface features thought to be responsible for this pro-coagulant activity: the externalization of phosphatydilserine and the exposition of tissue factor (TF).

1.3.1 MP and haemostasis: role of phosphatydilserine

The externalization of phosphatidylserine results in a negatively charged surface; this negatively charged surface allows for interaction with cationic domains in clotting proteins, the subsequent assembly of coagulation factors and ultimately thrombin formation.[7] In particular, PS is an

essential component of the multi-molecular complexes tenase and prothrombinase, which activate factor X and factor II (prothrombin) into factor Xa and factor IIa (thrombin), respectively, as well as of the tissue factor (TF)/factor VII(a) complex.[8]

While it is still generally assumed that activated platelets represent the main source of PS in the coagulation process, the possible contribution of MP as sources of PS has long been recognized[9] and is now becoming more appreciated. [10]

MPs shed from activated platelets constitute the main circulating population. They harbor major membrane glycoproteins, including functional adhesive receptors, and consequently disseminate a pro-coagulant potential that can be targeted according to the nature of counterligands[11] . They can bind to soluble or immobilized fibrinogen and aggregate with platelets [12]

The pro-coagulant potential of MPs is not, however, restricted to platelet MPs. MPs from monocytes, lymphocytes, or endothelial cells also present PS at their surface.

Scott syndrome is the direct evidence of the key role of MPs and PS in hemostasis. This inherited disorder, characterized by hemorrhagic complications, is linked to the lack of surface exposure of plasma membrane PS and to the inability of platelets and other blood cells to generate MPs. [13]

1.3.2 Tissue factor and MP

The second surface feature that promotes the pro-coagulant activity of MPs is the exposition of tissue factor. TF is a critical component of the early stages of coagulation where it forms a complex with Factor VII/VIIa, ultimately leading to the initiation of coagulation. *(7)*

TF expression is not restricted to the subendothelium but can also originate from stimulated monocytes or endothelial cells. [14]

At the stimulated monocyte cell surface only 10–20% of the total extractable TF activity is expressed. Thus most of its activity is latent or encrypted. [15]

TF activity is highly dependent on its lipid environment.[16]The phenomenon whereby TF activity is increased in the presence of negatively charged phospholipids, known as decryption, could be mediated through the secretion of TF-rich MP by monocytes. Such MPs can bind activated platelets or platelet-derived MPs and facilitate fusion events between monocytes (or monocyte MPs) and platelets (or platelet MPs) leading to MP rich in decrypted, active TF .[17]Moreover, in experimental thrombi, TF was predominantly co-localized at the surface of membrane vesicles, frequently clustered adjacent to platelet surfaces.[15]

An alternative pathway of blood coagulation in which circulating MP bearing both TF and the adeshion receptor P-Selectin glycoprotein ligand (PSGL-1) are recruited to the site of vascular damage by PSGL-1 ligand, P-Selectin, has been described.[18] The importance of the interaction between P-selectin and PSGL-1 was confirmed in vivo in a mouse model of hemophilia A in which soluble P-selectin was able to induce the generation of TF positive MPs and correct hemostasis .[19] Functional competence of blood-borne TF could be expressed when MPs and platelets adhere to neutrophils.[20] Finally, in vitro interaction of endothelial MPs with monocytic cells was also shown to induce TF-dependent pro-coagulant activity.[21]

1.3.3 Cellular sources of procoagulant MPs in blood

Monocytes

Many evidences indicate that monocytes are likely to be the major source of TF+MPs in health and disease. Several studies showed that lipopolysaccaride (LPS) stimulation of monocytes increases the TF expression and the release of TF+ MPs. [22-25]. Another study reported that THP-1 cell derived MPs were enriched in TF and PSGL-1.[26] Importantly, plasma from patients with meningococcal sepsis contained MPs that expressed TF and the monocyte marker CD14 [27], and more recently Aras and colleagues reported a transient increase of MPs that express both TF+ and CD14 in a human endotoxemia model. [28]

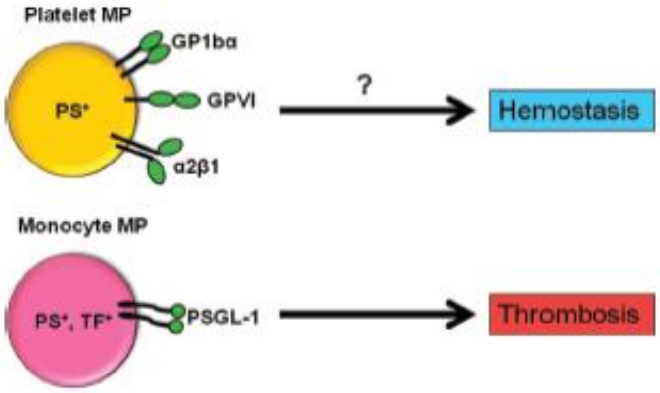
Endothelial Cells

Cultured endothelial cells express TF in response to a variety of agonists, including cytokines and LPS.[29] Moreover endothelial cell-derived MP (EMP) were observed in sickle cell patients in crisis and expressed both TF and endothelial marker CD144.[30] The pro-coagulant activity of EMP was confirmed by the demonstration that EMP from activated cells triggered TF-dependent thrombin formation *in vitro* and thrombus formation *in vivo*.[31]

Platelets

Several studies indicate that platelets are the major source of PS+ MPs in blood and represent 70%-90% of all circulating MPs .[32,33] In contrast, they may express very low levels of TF, but it seems unlikely that they provide a major contribution to the pool of TF+ MPs present in healthy individuals and patients. Therefore, it has been proposed that platelets derived MPs (PMPs) may play a role in hemostasis. Conversely, PS+,TF+ MP derived from monocytes may contributes to thrombosis and have a minor role in hemostasis. (*Figure 3*)

Figure 3: Proposed role of PMPs and MMPs in hemostasis and thrombosis



1.3.4 Microparticles, thrombosis and inflammation

Pathological dysregulations lead to variations in the nature or proportion of circulating MPs (qualitative and quantitative aspects), and more frequently, an increase of the number of circulating pro-coagulant MPs, result in an increased thrombotic propensity.

Elevated levels of circulating MPs have indeed been reported in various disorders characterized by thrombotic complications and vascular injury, particularly in cardiovascular diseases.[3,34]

Plasma levels of MPs are increased in diseases that involve a degree of vascular injury. In this regard several groups have reported elevations in plasma levels of EMPs, PMPs and MMPs in diabetes. [35,36]

Additionaly, elevations in plasma levels of MPs predict cardiovascular morbidity and mortality in atherosclerosis, pulmonary hypertension and heart failure. [37-39]

The implication of MPs in inflammation is also well documented. For instance, the presence of interleukin-1β (IL-1 β) was observed in shed MPs[40]and MPs are a source of aminophospholipid substrates of secretory phospholipase A2 for the generation of lysophosphatidic acid, a potent proinflammatory mediator and platelet agonist.[41]

Mesri and Altieri reported that MPs derived from polymorphonuclear leucocytes promote the release of inflammatory cytokines IL-6 and MCP (monocyte chemotactic protein) in cultured endothelial cells.[42]

Additionally, T-cell-derived MPs have been reported to promote production of TNF-α, and IL-1β by monocytes [43]

Monocyte-derived MPs have been shown to stimulate production of IL-8 and MCP in human airway epithelial cells [44,45] and both monocyte and endothelial MPs stimulate IL-6 and MCP production in podocytes.[46]

Furthermore, MPs were shown to favor endothelial activation and monocyte-endothelium interactions, the two initial steps of the atherosclerotic plaque formation.[47]

Finally, atherosclerotic lesions accumulate large amounts of MPs that may support thrombus propagation upon plaque rupture .[48]

1.4 Particulate matter

Particulate matter (PM) is a complex mixture of small particles and liquid droplets, which vary in size, composition, and origin. PM exposure has wide effects on health [49] and a large body of evidence has consistently shown that both short and long term exposure to PM are associated with increased cardiovascular and pulmonary related morbidity and mortality [50,51]

However, the mechanisms behind this association are not fully understood.

1.4.1 PM: physical-chemical characteristics

PM is an air-suspended mixture of solid and liquid particles that vary in number, size, shape, surface area, chemical composition, solubility, and origin.[52]

1.4.1.1. Size

Among all these characteristics the size of particles is the most important and is the one used for the classification of PM, because it is directly linked to their potential to cause health problems, as it is in turn directly linked to the ability of particles to penetrate more or less deeply into the respiratory system. The atmospheric particles are not spherical and their size is described by the aerodynamic equivalent diameter (AED) : the diameter of a sphere of density of 1 g/cm^3 that would have the same physical behavior when suspended in the air.

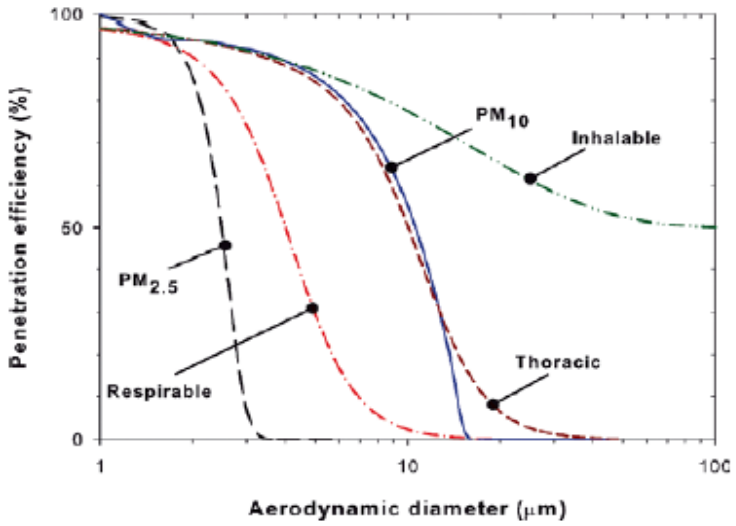
The Total Suspended Particulate (TSP) consists of all particles that remain suspended in the atmosphere and have a diameter ranging from less than 10 nm up to 100 μm. In the years, different methods or conventions have been developed to classify particles by size: 1) modes, based on the observed size distributions and formation mechanisms; 2) occupational health sizes, based on the

entrance into various compartments of the respiratory system; 3) cut point, based on the 50% cut point of the specific sampling device .[53]

The size-selective sampling method is the one used for definition of air quality standards and legislature and it is the most widely used in literature, especially in toxicological studies that sample PM in the atmosphere. This method is based on PM samplers efficiency curves and particle size is usually defined by the 50% cut point. Thus PM10 refers to all the particles collected by a sampling device that collects the 50% of 10 μm particles.

The concentration of particles with a diameter of less than 10 μm (PM10) and of particles with a diameter of less than 2.5 μm (PM2.5) have been selected as standard sizes to a regulatory aim, on the basis of health considerations. PM10 was chosen in order to consider particles small enough to enter the thoracic region of the human respiratory tract and PM2.5, or fine PM, comprehends a part of the respirable PM, capable of deeper penetration and higher deposition in airways and lungs. (Figure 4)

Figure 4: PM2.5, PM10, inhalable, thoracic, and respirable particulate matter sampling conventions promulgated by US-EPA [54]



PM2.5 also comprises ultrafine particles having a diameter of less than 0.1 μm. In most locations in Europe, PM2.5 constitutes 50–70% of PM10.

1.4.1.2 Chemical composition

PM is a mixture with physical and chemical characteristics varying by location. Common chemical constituents of PM include sulfates, nitrates, ammonium, other inorganic ions such as ions of sodium, potassium, calcium, magnesium and chloride, organic and elemental carbon, crustal material, particle-bound water, metals (including cadmium, copper, nickel, vanadium and zinc) and polycyclic aromatic hydrocarbons (PAH). In addition, biological components such as allergens and microbial compounds are found in PM.

PM components can derive from anthropic and natural sources including combustion processes (traffic, warming, waste and industrial incineration, fires), traffic-related abrasion processes (road and car brake debris), dusts resuspension from soil or soil erosion and biological material.[55]

Ultrafine and fine PMs are mainly generated by combustion sources and observed under an electron microscopy appear to be composed by aggregates of small round-shaped particles of 20-40 nm. Coarse PM is mainly generated by mechanical degradation of materials and contains particles of different shapes, regular if derived from crystalline structure or irregular.[52]

However, the physico-chemical properties of particles not only vary with size but also with time, season and location; in fact differences in meteorology, emission sources and topography contribute to the dispersion, transformation and removal of PM from the atmosphere, resulting in variations of particles concentrations, composition and properties [55,56, 57].

1.4.2 PM exposure and health effects

The adverse effects of PM on human health is by now well established [55,58,59] and can be divided into local effects, that rise in the lung, and systemic effects, that impact on the cardiovascular system.[60]

Numerous epidemiological studies have reported consistent associations between PM atmospheric concentrations and onset/exacerbation of respiratory and cardiovascular diseases as well as mortality in the population.

Effects have been related both to short-term and long-term exposures to PM; short-term exposures have been reported to induce exacerbation of airways disease in patients with asthma and Chronic Obstructive Pulmonary Disease (COPD) [61,62], onset of cardiovascular diseases [63] and increased mortality for heart attacks and respiratory causes.[60]Several studies reported an increase of mortality of a value ranging from 0.2 to 0.6% for 10 μg/m^3 of PM10.[64,65]

Long-term exposure epidemiological studies showed associations with PM2.5 but not with larger size fractions; fine PM has been linked with increases of risk of cardiovascular mortality [52,66,67] and of lung cancer [67,68].

In particular, long-term exposure to PM2.5 is associated with an increase in the long-term risk of cardiopulmonary mortality by 6–13% for 10 μg/m^3 of PM2.5.[69]

Accordingly to this observation, both daily and annual average concentration threshold for PM2.5 and PM10 have been indicated by the World Health Organization [70] to protect the health of the population. In particular, guideline values have been chosen on the basis of evidences reported in literature:

• for PM2.5: 10 μg/m3 for the annual average and 25 μg/m3 for the 24-hour mean (not to be exceeded for more than 3 days/year);

• for PM10: 20 μg/m3 for the annual average and 50 μg/m3 for the 24-hour mean.

1.4.3 PM toxicity: molecular mechanisms

The molecular mechanisms on the basis of the toxicity of PM are not fully understood. In this regard*in vitro* models are of fundamental importance in understanding the molecular pathways activated by PM exposure at cellular level, which in turn might explain the biological outcomes observed in humans.

The models used for PM toxicity analysis include various alveolar and bronchial epithelial cell lines and monocyte/macrophage cells; endothelial cell lines and co-coltured systems of these models have been used to investigate possible cardiovascular effects.[71,72]

Several studies indicated an association between the PM exposure and the release of several mediators of inflammations (cytokines) in different cell cultures.

It has been demonstrated that alveolar macrophage-derived IL-6 mediated pro-coagulation effects in mice exposed to PM10,[73] providing a link between lung cytokines and systemic responses. Moreover, many in vitro and in vivo studies have reported the release of IL-6 and IL-8in cell lines exposed to various PMs.[73-75]

Brown et al showed that the exposure to PM induces oxidative stress in lung cells and promotes a release of TNF-a from pheripheral blood mononuclear cells.[76,77]

Other studies have also shown that PM10 stimulate an increase of intracellular Ca2+ .[78]

Another important effects of PM exposure on the cells is the genotoxicity. In particular, fine PMs usually have a higher genotoxic potential compared to coarse fractions, in in vitro and in vivo models.[79,80] The high genotoxicity of these PMs is due to the particles composition and source, since particles derived from combustion processes have been found to be rich in metals and organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), which are notorious genotoxic elements.[81] Generation of ROS and consequent oxidative stress is widely recognized as an important toxicological mechanism in PM induced DNA double strand breaks/oxidation and possibly lung cancer.[82] Moreover, polycyclic aromatic hydrocarbons and volatile compounds

(benzene) contained in fine PM can form bulky DNA adducts, leading to double strands breaks of DNA. [82]

Finally, in cells exposed to PM both necrotic and apoptotic processes have been widely reported. [55,58]

Necrosis is often related to a severe inflammatory status or oxidative stress characterized by high levels of released cytokines and ROS formation.

Intrinsic and extrinsic apoptotic pathways have been described especially for PM2.5; this fraction has been found to induce apoptosis through the TNF-α-dependent pathway and by the mitochondrial pathway. Both pathways have been related to severe DNA damage and ROS formation. [83,84]

CHAPTER 2: AIM

Elucidating the cellular pathways activated in response to PM exposure is a key step for to the understanding of the clinical outcomes in the PM exposure-related diseases.

We investigated the hypothesis that particulate matter causes the shedding of TF-bearing, pro-coagulant microparticles by human mononuclear and endothelial cells, thus providing a novel link between airborne pollutants and cardiopulmonary risk.

CHAPTER 3: MATERIALS AND METHODS

3.1 Materials

3.1.1 Reagents and kits

RPMI 1640 medium, penicillin, streptomycin, L-glutamine, fetal bovine serum (FBS), trypan blue, phosphate buffered saline (PBS), Ficoll-Hystopaque, dextran T500 and U73122 were obtained from Sigma (Milano, Italy). Thromboplastin standard was obtained from Beckman Coulter (Milano, Italy). Human anti-TF antibody was obtained from America Diagnostica (Instrumentation Laboratory, Milano, Italy). The Zymuphen MP-Activity kit was obtained from Hyphen BioMed (Neuville-sur-Oise, France). The Fluo-4 NW Calcium Assay kit was obtained from Molecular Probes (Invitrogen, Milano, Italy). Standard reference material 1648a Urban Particulate Matter (SRM1648a) was obtained from National Institute of Standard Technology (Gaithersburg, MD, USA).

3.1.2 Cell isolation and culture

3.1.2.1 HUVEC

HUVEC were isolated from humbilical vein cords as described [85] by digestion with 0.1% collagenase (specific activity: 316 U/mL, Gibco, Invitrogen) and grown to confluence at 37°C in 5% CO_2 humidified incubator, on 25-cm^2 tissue culture flasks previously coated with 1% gelatine in supplemented culture medium (M199 with 10% heat inactivated foetal calf serum, 100 U/mL penicillin, 100μg/mL streptomycin, 2mM l-glutamine, 10mM HEPES pH 7.4, heparin 12 IU/mL,

1% retinal derived growth factor, all from Sigma). Following trypsin treatment, the cells were detached from the flasks and final mono-layers were prepared by seeding HUVEC on gelatine-precoated culture plates and then incubated for 24–48 h to ensure confluence. HUVEC were identified by their typical cobblestone morphology and immunofluorescence staining by monoclonal antibodies against von Willebrand Factor (Immunotech, Milano, Italy). Cells up to the fourth passage were used for all experiments.

The investigation conformed with the principles outlined in the declaration of Helsinki for use of human tissue.

3.1.2.2 PBMC

PBMC were isolated either from fresh buffy coats obtained from the local blood bank or from the peripheral blood of normal volunteers as described.[45] Briefly, a fresh buffy coat was mixed gently with an equal volume of 2,5% Dextran T500, and left for 40 minutes for erythrocyte sedimentation. Ten mL of leukocyte-rich supernatant was recovered and layered over 5 mL of Ficoll-Hystopaque and centrifuged for 30 minutes at 350 x g at 4°C. The PBMC-rich ring was recovered and washed twice in PBS. PBMC were then resuspended in RPMI supplemented with 100 U/mL penicillin, 100µg/mL streptomycin, 2mM l-glutamine and allowed to adhere for 30 minutes at 37°C on 24-well plates (2x106 cells/well). Then the cells were washed two times with pre-warmed PBS and resuspended in RPMI, 100 U/mL penicillin, 100µg/mL streptomycin, 2mM l-glutamine, 5%FBS and incubated overnight at 37°C.

3.2 Methods

3.2.1 MP generation and purification

PBMC and HUVEC were washed twice with pre-warmed PBS. For MP release analysis, SRM1648a was added; after the time of incubation at 37°C the supernatants were recovered, cleared by centrifugation at 14,000 x g for 5 min at room temperature to remove dead cells and big cell fragments that might have detached during the stimulation and immediately used for further experiments. In selected experiments, MP were further purified by ultracentrifugation (100,000 x g for 2hours, 4°C); the pellet was resuspended in 250 μL of normal saline and used in a one-stage clotting assay to measure TF-dependent coagulation. For Nanosight analysis, an aliquot of cell medium was centrifuged at 4°C at 1000g for 15 minutes, at 2000g for 15 minutes and then at 3000g for 15 minutes. Supernatants were then submitted to ultracentrifugation at 110,000 x g for 2 hours at 4°C.

3.2.2 Measurement of MP-associated PS

PS-positive MP in each sample were detected using the Zymuphen MP activity kit (Hyphen BioMed, Neuville-sur-Oise, France). Briefly, an aliquot of cell medium containing MP, supplemented with calcium, Factor Xa and thrombin inhibitors, is introduced into one of the microplate wells coated with streptavidine and biotinylated annexin V, then incubated. In particular, following a washing step, the Factor Xa-Va mixture containing calcium, then the purified prothrombin, are introduced. The microparticles present in the tested sample bind to annexin V and expose their phospholipids surface, thus allowing to FXa-FVa, in presence of calcium, to activate prothrombin in thrombin. The phospholipids concentration is the limiting factor. There is a direct relationship between the phospholipids concentration and the amount of thrombin generation, which is measured via a colorimetric reaction. Results are expressed according to the values of absorbance

at 405 nm and microparticles concentrations are calculated using a calibration curve and expressed in nanomoles of phosphatydilserine.

3.2.3 Nanosight detection of MP

The number and dimension of MP were assessed by nanoparticle tracking analysis (NTA). Using a Nanosight LM10-HS system (NanoSight Ltd., Amesbury, UK), MP were visualized by laser light scattering. Briefly, MP-enriched pellets were resuspended in 300 µL of 0.1 µm filtered sterile PBS and five recordings of 30 seconds were performed for each sample. Collected data were analyzed with NTA software, which provided high-resolution particle size distribution profiles and concentration measurements of the vesicles in solution.

3.2.4 Measurement of intracellular calcium concentration

Molecular Probes Fluo-4 NW Calcium Assay kit was used to measure the changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) of PBMC and HUVEC. Prewashed PBMC (0.33×10^6 cells/well) or HUVEC (15×10^3 cells/well) were loaded with 100 µL of the dye loading solution containing Fluo-4 NW dye and probenecid, according to the manufacturer's instructions.

The 96-well plate was incubated at 37 °C for 45-60 min in the dark and SRM1648a (500µg/mL) was added to the cells. The changes in Fluo-4 NW fluorescence were measured by the Wallac 1420 Victor 2 (PerkinElmer, Milan, Italy) at λex 494 nm and λem 516 nm. Calcium mobilization was observed over time (up to 150 sec) and analyzed by the Wallac 1420 Software version 3 (PerkinElmer Life and Analytical Sciences, Wallac, Milan, Italy). The increase in $[Ca^{2+}]_i$ fluorescence was expressed as relative fluorescence units (RFU).

3.2.5 Assessment of MP-bound TF activity

TF activity was measured in MP generated in vitro from PBMC and HUVEC by a one-stage clotting time assay as described, except that the normal human plasma was made MP-poor by ultracentrifugation (100,000xg for 2 h, 4°C). Briefly, disrupted MP (100 μL) were mixed with 100 μL of MP-poor normal human plasma at 37°C; 100 μI of 25 mM $CaCl_2$ at 37°C was added to the mixture and the time to clot formation was recorded. The results were expressed in arbitrary milliunits of pro-coagulant activity by comparison with a standard curve obtained using a human brain thromboplastin standard. This preparation was assigned a value of 1,000 arbitrary mU for a clotting time of 30 s. An anti-human TF antibody (American Diagnostica, Instrumentation Laboratory, Milano, Italy; 30 μg/mL) largely inhibited the pro-coagulant activity, thus confirming its identity with TF. [86] (data not shown).

3.2.6 Data presentation and statistical analysis

Unless otherwise indicated, data are shown as mean±SEM from n independent, consecutive experiments; comparisons among groups were made by either ANOVA for repeated measures followed by Tukey's analysis or Student's paired t-test, as appropriate, using Prism Software (GraphPad, San Diego, CA, USA). Values of $p<0.05$ were considered statistically significant.

Chapter 4: RESULTS

4.1 PM induces MP generation by PBMC and HUVEC

To investigate whether PM induces the release of MP, PBMC and HUVEC were stimulated with increasing concentrations of SRM1648a for 1 hour. Both PBMC and HUVEC generated MP upon stimulation with SRM1648a. The effect was concentration dependent and reached a plateau at approximately 250 and 125 µg/mL for PBMC and HUVEC, respectively (Figure 5).

Due to the relatively high dispersion of data, we used 500 µg/mL SRM1648a, a concentration that caused a consistent increase in MP generation, for all subsequent experiments with both cell types. As shown in Figure 6, the increase in MP generation was statistically significant with both PBMC and HUVEC.

Figure 5:PM-SRM1648a induces the generation of MP in a concentration-dependent manner in HUVEC and PBMC

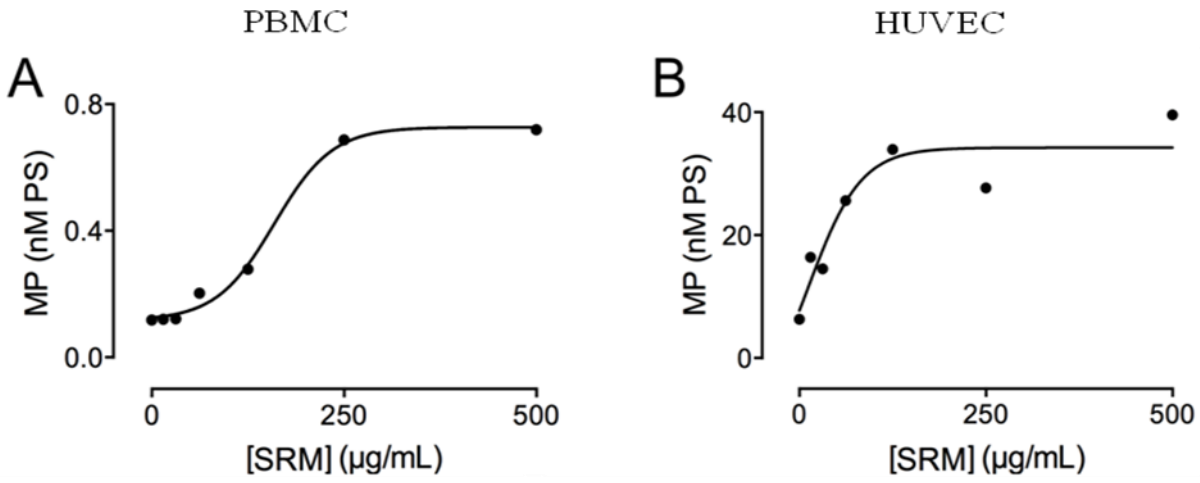
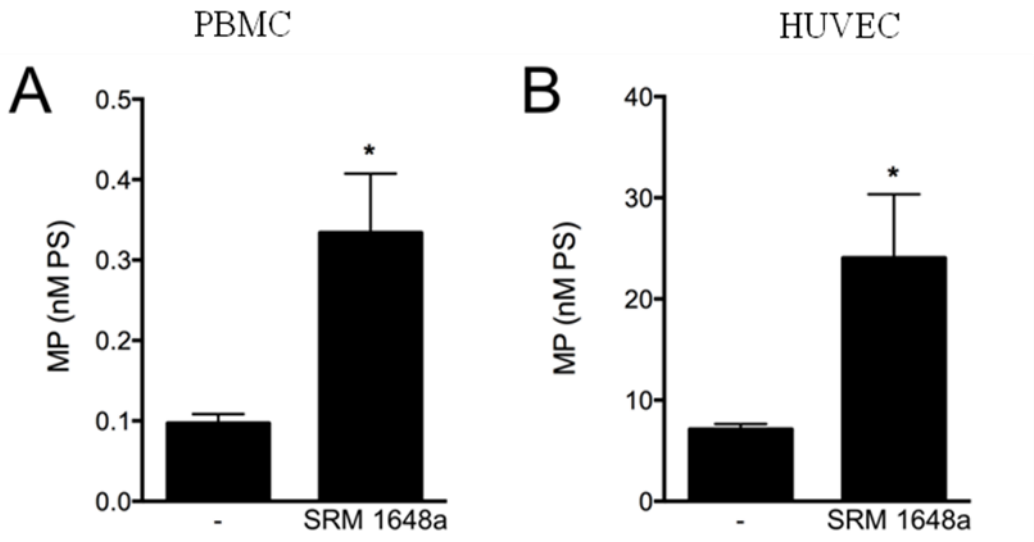


Figure 6: Increase of MP induced by SRM1648a

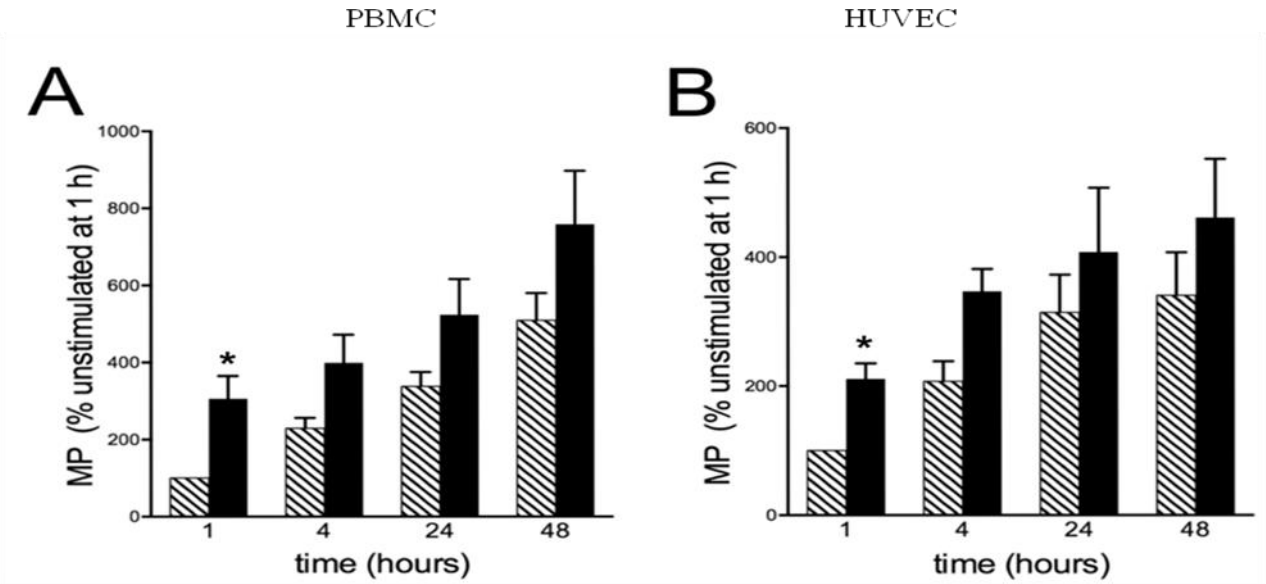


4.2 Kinetics of MP generation upon exposure to PM

MP generation can be caused by different mechanisms; accordingly, the kinetics vary depending, for example, on whether calcium mobilization, cell activation or apoptosis is involved.[3]

We treated cells with SRM1648a and assessed MP generation at different time points. As shown in figure 7, MP generation increased steadily with time both in the absence and in the presence of the agonist; however, the difference between SRM1648a treated and untreated cells tended to level off and was maximal at early time points (1 h) for both PBMC and HUVEC.

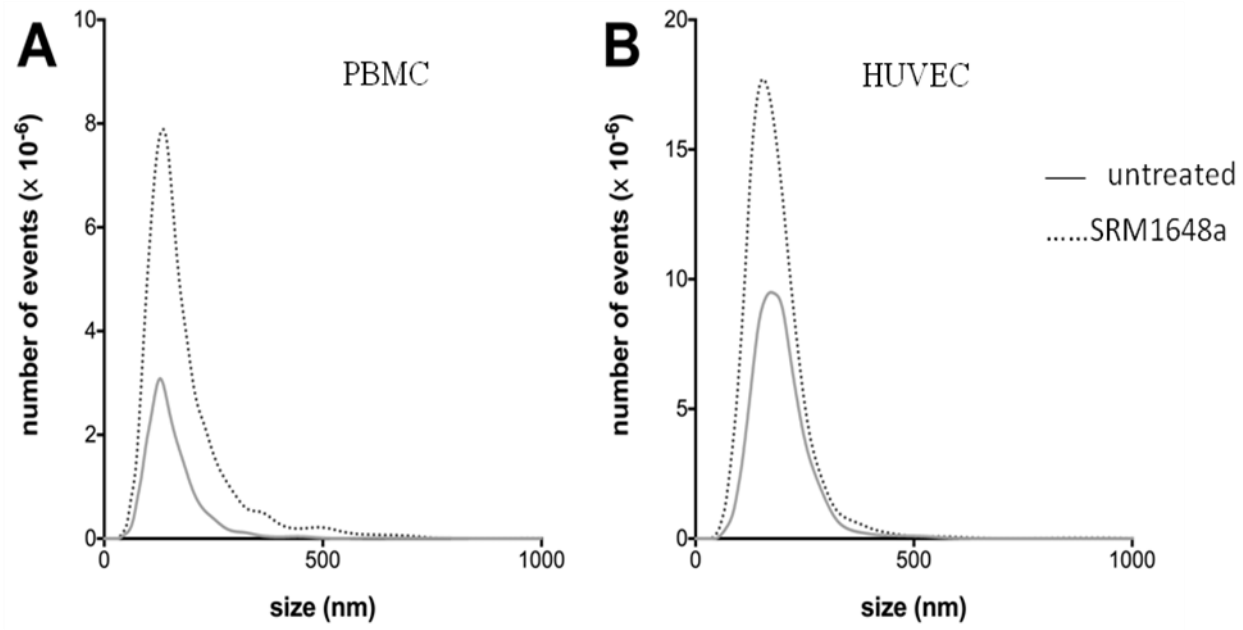
Figure 7: Kinetics of stimulation with SRM1648a



4.3 MP analysis by Nanosight

MP released in medium after SRM1648a treatment and in untreated control were isolated and their number and size distribution analyzed by Nanosight analysis. As shown in figure 8, both PBMC (4A) and HUVEC (4B) stimulated with SRM1648a showed an increased production of MP. In particular, for PBMC, the mean number of particles (mean of ten replicates) was $2.56 \cdot 10^8$/mL in untreated controls and $8.54 \cdot 10^8$/mL in SRM1648a stimulated cells while, for HUVEC cells, the mean number of particles was $11.44 \cdot 10^8$/ml and $22.25 \cdot 10^8$/ml for control and SRM1548a stimulated cells, respectively. MP size peaked at 120.6 nm and at 132.3 nm for control and SRM1648a-treated PBMC, and at 160.3 and at 156.1 nm for control and SRM1648a-treated HUVEC.

Figure 8: Distribution of MP analyzed by Nanosight analysis

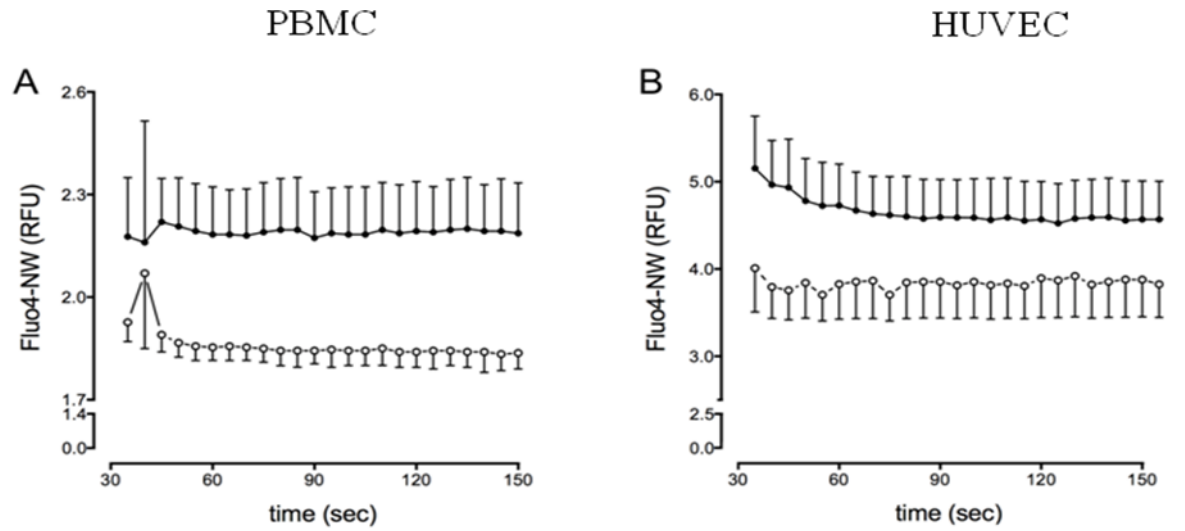


4.4 PM induces the mobilization of intracellular calcium in PBMC and HUVEC

PM has been shown to induce an increase in intracellular calcium concentration.[78]

Because calcium mobilization is involved in rapid MP generation[86,87,88] we investigated whether SRM1648a increases $[Ca^{2+}]_i$ in our experimental conditions. Figure 9 confirms that SRM1648a (500 µg/mL) induces a rapid and significant increase in $[Ca^{2+}]_i$ in PBMC (4A) and HUVEC (4B).

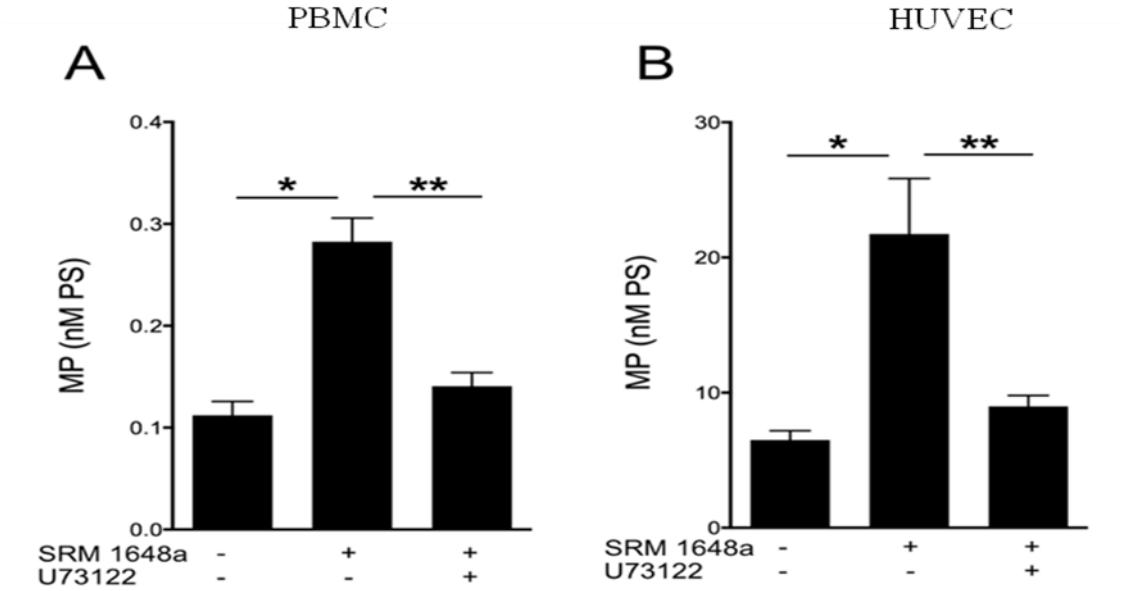
Figure 9:SRM1648a induces the mobilization of intracellular calcium in PBMC and HUVEC



4.5 PM-induced MP generation is mediated through phospholipase C (PLC)

To further characterize the role of calcium in the induction of MP by SRM1648a-stimulated cells, we used the PLC inhibitor, U73122, to investigate the role of calcium ions stored in the endoplasmic reticulum. Cells were pre-treated with U73122 (1μM) for 30 minutes prior to stimulation. Figure 10 shows that U73122 inhibits SRM1648a-induced MP generation in PBMC (A) and HUVEC (B).

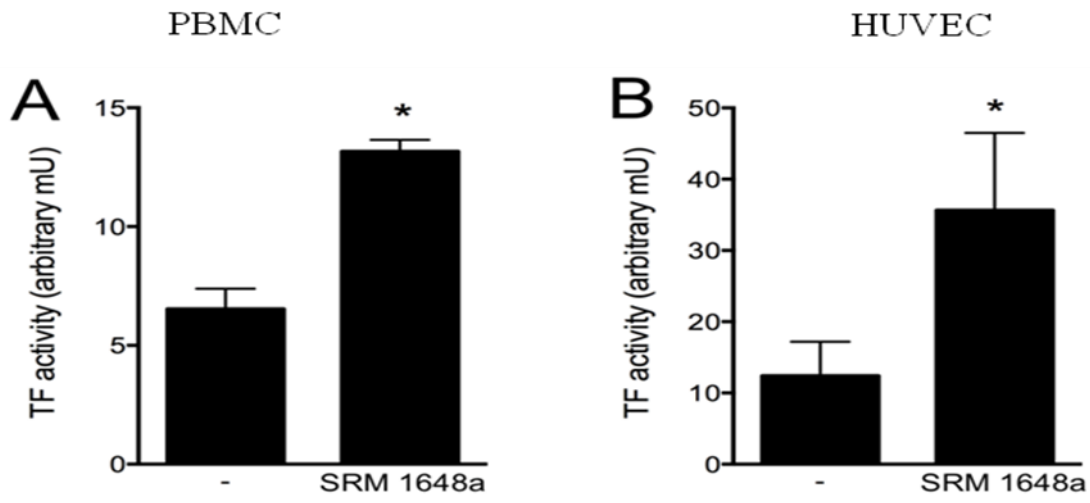
F*igure 10: PM-induced MP generation is mediated through phospholipase C (PLC)*



4.6 PM induces the expression of MP-bound TF

The pro-coagulant activity of MP is due to the exposure of PS on their surface and is also enhanced by the presence of functional TF (reference). To evaluate whether SRM1648a induces the generation of TF-bearing MP by PBMC and HUVEC, we analyzed the pro-coagulant activity of purified MP released by treated and untreated cells through a one-stage clotting test. As shown in Figure 11A and B respectively, SRM1648a induces a 2- to 2.5-fold increase in pro-coagulant activity of MP by PBMC and 3- to 3.5-fold increase in pro-coagulant activity of MP by HUVEC. A monoclonal antibody to TF (epitope specific for amino acids 1–25; American Diagnostica, Stamford, CT, USA; 30 μg/mL) inhibited most of the pro-coagulant activity (not shown), confirming its identity with TF.

Figure 12: SRM 1648a induces the expression of MP-bound TF



CHAPTER 5: DISCUSSION

The present study aimed at investigating the possible role of PM on the generation of MP by PBMC and HUVEC. Our data demonstrate that SRM1648a, a standard reference source of PM, is able to induce the generation of MP in both PBMC and HUVEC.

The observation that SRM1648a causes an increase in $[Ca^{2+}]_i$ in both cell lines is consistent with the hypothesis that PM stimulates MP shedding through calcium mobilization. The demonstration that the PLC inhibitor, U73122, largely inhibits the effect suggests a scenario whereby MP shedding requires calcium mobilization from intracellular storage pools that takes place via phosphatidylinositol (3,4,5) trisphosphate.[89]

The relatively rapid kinetics of MP generation is also consistent with a cell activation- rather than apoptosis-mediated mechanism.[3]

MP were detected and enumerated through the ability of the negatively charged phosopholipids expressed on the outer leaflet of their membrane to activate the prothrombinase complex. Therefore, these MP are by definition pro-coagulant. However, the pro-coagulant potential of MP of different origin may be increased by the expression of TF on their membrane.[7]

Our data demonstrate that MP generated upon exposure of PBMC and HUVEC to SRM1648a carry functionally active TF and can therefore trigger the activation of the extrinsic pathway of blood coagulation.

Flow cytometry is a well established method of MP analysis and has been used successfully by many Authors. [87,90,91] The approach, however, carries significant theoretical problems due to the small size of the vesicles that are of the same order of magnitude as the incident wavelength,[92] and it is likely that only bigger MP are accurately recognized. Furthermore, in the experiments reported

in this work large PM could not be consistently removed from the samples and clogging of the flow cytometer was observed. For these reasons, we used nanosight technology as an alternative approach to confirm the main data. The results indicate that both PBMC and HUVEC shed vesicles with a diameter that ranges form 50 to 500 nm, therefore consistent with MP, and that exposure to PM increase the number of events.

Experimental evidence indicates that peaks of particulate air pollution correlate with increased morbidity and mortality from respiratory and cardiovascular causes[50,51].

The molecular mechanisms on the basis of the toxicity of PM are not fully understood. Several studies showed that PM induces a pro-inflammatory response with an increase in the production of pro-inflammatory cytokines and an induction of oxidative stress.[73-77]

In this study we showed a role of the pro-coagulant and pro-inflammatory MP after exposure to PM and this observation might provide a further link between airborne pollution exposure and cardiopulmonary morbidity.

Furthermore, we indicated a specific molecular mechanism in the formation of MP. In this regard, a better understanding of the mechanisms of MP generation might prove useful for a pharmacological modulation of their detrimental effect.

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