INTRODUCTION

Microparticles (MP) are plasma membrane vesicles of 0.1–1 μm in diameter, mainly composed of lipids, proteins and nucleic acids, actively released from cells upon activation, apoptosis, malignant cell transformation and stress ***(Ahn, 2005).***

Elevations in the levels of these circulating microparticles in plasma of cancer patients are frequently reported, particularly MP of platelet origin. This phenomenon can be related to activation of the different vascular cells (i.e. platelet, endothelial cells and monocytes) frequently observed. ***(Falanga et al., 2009)***

In blood, platelet-derived MP (PMP) constitute the majority (>80%) of the pool of MP. There is considerable interest in evaluating the biological relevance of alterations in blood-borne MP populations, and several techniques have been developed with the aim to detect and quantify MP levels in circulating blood ***(Falanga et al., 2012).***

AIM OF THE STUDY

Our aim was to study the effect of childhood cancer on the level of platelet microparticles and its relation to remission (response) status and to study the effect of the level of plasma microparticles on the thrombotic complications of childhood cancer patients.

Chapter One
MICROPARTICLES

Introduction:

Many cells, including platelets, endothelial cells, leukocytes, and erythrocytes, shed small fragments of their plasma membranes into the circulation. There is increasing evidence that these submicron fragments, termed microparticles, have important physiological roles. Platelet microparticles are the most abundant microparticles in the bloodstream constituting approximately 70% to 90% of circulating microparticles ***(Italiano et al., 2010).***

Historical background:

In the 1940s it was known that platelets support clotting, because platelet-containing plasma clotted faster than platelet-poor plasma (PPP). High-speed centrifugation of PPP further prolonged the clotting time, suggesting that PPP contained another (subcellular) factor that facilitated clotting ***(Nieuwland et al., 2007).***

In 1967, Wolf reported that activation of platelets resulted in the generation of "platelet dust" and that these platelet-derived MPs supported thrombin generation in platelet poor plasma ***(Wolf, 1967).***

Platelet dust consists of small vesicles that are mainly platelet derived (platelet-derived microparticles, or PMPs) and explain the coagulant activity of PPP ***(Nieuwland et al., 2007).***

Crawford extended these observations and concluded that these microparticles were formed by in vivo fracture of membrane buds from extended platelet pseudopods ***(Crawford, 1971).***

Microparticle Structure:

A. Types:

In 1999 Heijnen et al. reported that two different types of microparticles are released after stimulation of platelets: microvesicles, and exosomes. (figure 1) ***(Heijnen et al., 1999).***

a) Microvesicles: They are 100 nm to 1 μm in diameter. Their upper size limit may therefore be almost that of a platelet. They are formed by surface shedding upon platelet activation. Formation of microvesicles has been demonstrated in vitro using flow cytometry. Analysis of the microparticles obtained from the releasate of activated platelets showed that the microvesicles contained predominantly plasma membrane glycoproteins. ***(Heijnen et al. 1999).***

b) **Exosomes:** Exosomes are derived from exocytosis of multivesicular bodies and α-granules. Exosomes are smaller in size than microvesicles measuring 40 to 100 nm in

diameter. Exosomes could be detected using Western blot but not flow cytometry probably because of their small size. Exosomes were shown to be selectively enriched in the endocytic marker CD63 ***(Heijnen et al. 1999).***

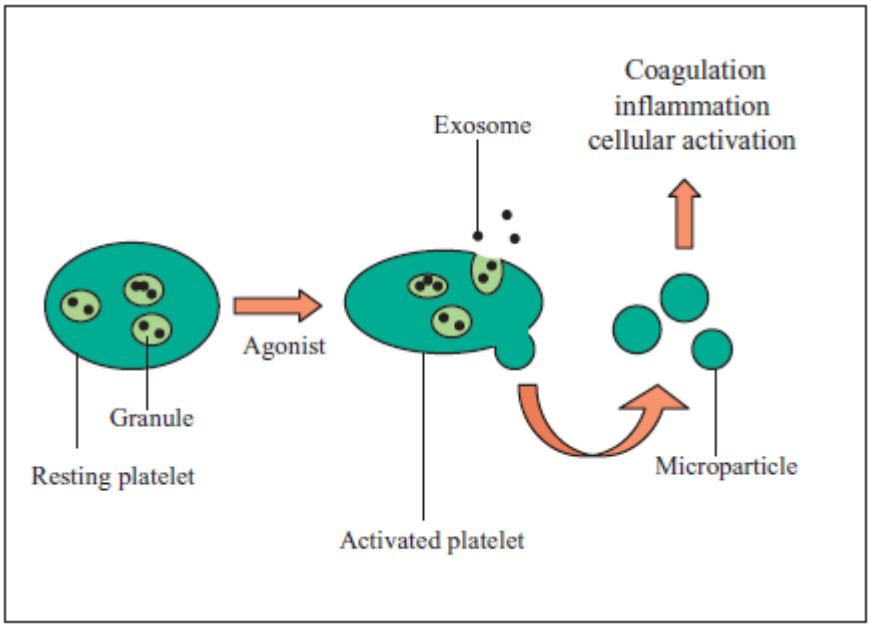

Figure (1): Platelet-derived microparticles (PMP) and exosomes. A *resting platelet* (left) contains α granules in which exosomes are stored. The activated platelet releases microparticles (right) which are 'blebbed' from the plasma membrane. When the activated platelet secretes its granule contents, granule membranes fuse with the plasma membrane and their contents, including the *exosomes*, are released ***(Nieuwland et al., 2007).***

B. Platelet microparticles glycoprotein:

Platelets and PMP share glycoprotein (GP) receptors, such as GPIb (CD42b), platelet–endothelial cell adhesion molecule-1 (CD31), and the integrin αIIbβ3 (GPIIb-IIIa,

CD41/CD61). In addition, subpopulations of PMPs can expose activation markers, including P-selectin (CD62P), and may bind fibrinogen. The antigenic composition of PMP and their functions are dependent on the mechanisms underlying their release. For example, PMP released from platelets activated by collagen and thrombin expose integrin αIIbβ3 that binds fibrinogen, whereas PMP from complement C5b-9- activated platelets expose αIIbβ3, which does not bind fibrinogen ***(Nieuwland et al., 2007).***

C. Platelet microparticles phospholipids:

MPs enriched in phosphatidylserine (PS) and phosphatidyl-ethanolamine (PE) exposed on their outer surface are released. MPs isolated from blood (mainly PMPs) comprise 60% phosphatidylcholine, 20% sphingomyelin, 9% PE, the remainder being minor quantities of other phospholipids ***(Burnier et al., 2009).***

D. Other contents of platelet microparticles:

Platelet microparticles contain molecules in addition to glycoproteins and phospholipids, such as platelet activating factor (PAF), β-amyloid precursor protein and Ca2+-dependent protease calpain ***(Nomura, 2001).***

Mechanism of platelet microparticles Production:

Microvesicles are shed from the cell surface of normal healthy or damaged cells and 'hijack' from these cells both membrane components and engulfed cytoplasmic contents. Shedding of membrane-derived microvesicles is a physiological phenomenon that accompanies cell activation and growth. Interestingly, rapidly growing cell lines tend to secrete more microvesicles than slowly growing ones. Generally, the number of microvesicles shed from cells increases upon (i) cell activation, (ii) hypoxia or irradiation, (iii) oxidative injury, (iv) exposure to proteins from an activated complement cascade and (v) exposure to shearing stress. Microvesicles' shedding depends on an increase in cytosolic Ca^2 and degradation of the membrane skeleton. Exosomes originate from the endosomal membrane compartment after fusion of secretory granules with the plasma membrane, where they exist as intraluminal membrane-bound vesicles ***(Ratajczak et al., 2006).***

In the steady state the cell membrane is asymmetric in its composition with phosphatidylcholine and sphingomyelin located in the outer layer whereas phophatidylserine (PS) and phosphatidylethanolamine (PE) present in the inner layer. This asymmetric distribution in the membrane is maintained by a group of two ATP dependent enzymes namely *flippase, floppase* as well as a bidirectional ATP-independent *scramblase*. Flippase

specially translocates PS and PE from the outside to the inside of the bilayer membrane. Floppase transports phospholipids and cholesterol from the inner to the outer leaflet. Scramblase whose role is thought to be the transportation of phospholipids between the two monolayers of the cell membrane, is inactive in steady state. Following stress or under physiological conditions, an increase in intracellular calcium, a subsequent loss of phospholipid asymmetry following the inactivation of flippase and activation of floppase and scramblase, and disruption of the cytoskeletal apparatus occurs leading to MP vesiculation (figure 2) ***(Jaiswal et al., 2012).***

Successive mechanisms initiate MP (*ectosome*) formation during cell activation or other cell processes including apoptosis and senescence:

1. Calcium is released by the endoplasmic reticulum.
2. Calcium inactivates flippase and activates floppase and scramblase, inducing the loss of phospholipids asymmetry between the inner and the outer leaflets. Contacts between aminophospholipids and cytoskeleton are then disrupted.

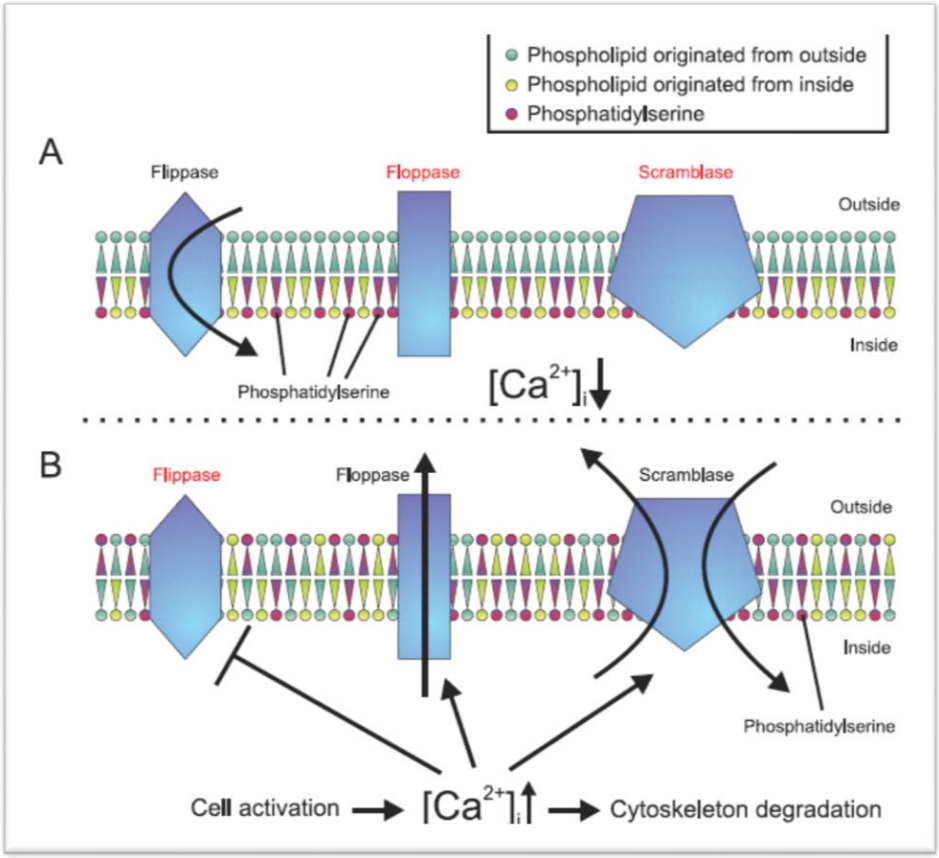

Figure (2): Maintenance of the asymmetric distribution of phospholipids in the membrane by a three piece enzyme system: flippase, floppase and scramblase. A) Resting cell, characterized by a low cytoplasmic calcium concentration and active flippase. B) The formation of microparticles (MPs) is associated with the loss of the plasma membrane asymmetry. This is due to the exposure at the outside surface of phosphatidylserine (in red), as a consequence of the activation of floppase and scramblase and inhibition of flippase by an increase of intracellular calcium concentration ***(Burnier et al., 2009).***

3. In addition, calcium release leads to activation of two enzymes: *calpain* and *gelsolin*. Calpain hydrolyzes actin binding proteins that decreases association of actin with membranes glycoproteins, while gelsolin (only in platelets) is involved in the cleavage of the actin capping proteins.

Protein anchorage to the cytoskeleton is therefore disrupted, resulting in membrane budding and microparticles shedding ***(Burnier et al., 2009).***

Activated platelets release PMP *in vitro* when agonists (first messengers), such as collagen and thrombin, bind to their surface receptors. These receptors then transduce signals across the cell membrane and generate changes in levels of intracellular second messengers such as Ca^{2+}, leading to the release of PMP. Alternatively, platelets release PMP when compounds are added that directly affect second messenger levels (e.g. calcium ionophores and phorbol esters). One of the essential steps in platelet activation is the elevation of cytosolic calcium levels. This elevation is a prerequisite for PMP release during activation, and results in the activation of several enzymes that are calcium-dependent for their activity, such as calpains and protein kinase C (PKC). Calpains are calcium-dependent proteases that facilitate PMP formation by degrading structural proteins including actin-binding protein, talin, and the heavy chain of myosin. Increased levels of Ca^{2+} is also a cofactor of various isoforms of PKC — serine–threonine kinases that phosphorylate a wide array of intracellular proteins, including signal transduction elements and structural proteins. Platelets release PMP upon direct activation of PKC by phorbol esters. Also, platelet agonists, such as thrombin and adenosine diphosphate, induce the release of PMP at least partially via activation of PKC ***(Nieuwland et al., 2007).***

In vivo, some PMP may originate directly from megakaryocytes rather than from platelets. In vitro, cultured human bone marrow cells that matured to megakaryocyte progenitor cells not only produced platelets but also numerous PMP. The contribution of megakaryocytes to the pool of PMP in the circulation is currently unknown ***(Nieuwland et al., 2007).***

Microparticle Function:

A. Coagulation

Coagulation factors bind to exposed aminophospholipids via Ca2+ ions. This binding facilitates the formation of tenase and prothrombinase complexes. PMP are enriched in binding sites for activated factor V (factor Va), factor VIIIa, and factor IXa, and provide the membrane surface for thrombin formation. MP in the plasma of healthy humans are mainly (>80– 90%) of platelet origin. When PMP are removed from the PPP by high-speed centrifugation, the (MP-depleted) plasma fails to clot after recalcification. This experiment illustrates that the presence of PMP is essential for clotting in vitro ***(Nieuwland et al., 2007).***

MP-depleted plasma fails to clot even in the presence of artificially prepared phosphatidylserine -containing phospholipid vesicles. Thus, PMP are likely not only to support, but also to initiate, coagulation. PMP isolated from human plasma samples may utilize both the extrinsic (tissue factor- and factor VIIa-

dependent) and contact activation (intrinsic, factor XIIa-dependent) pathways ***(Nieuwland et al., 2007).***

B. Inhibition of Coagulation

Because PMPs are subject to the same platelet stimulation reactions, PMPs possess both pro and anticoagulant properties. The relative distributions of pro- and anticoagulant activities in platelets and PMPs are similar. Furthermore, a study reported that protein C inhibitor, a member of the serpin family selected from activated platelets, binds preferentially to the phosphatidylethanolamine in platelet membranes and PMPs and efficiently inhibits phospholipid-bound activated protein C. PMPs do not always display procoagulant phospholipids, possibly due to an incomplete flip-flop of phospholipids between the membrane leaflets or to scramblase activity. These observations underscore the heterogeneity of PMPs ***(Nomura, 2001).***

Activated protein C (APC) inhibits both factor Va and factor VIIIa, and (activated) platelets and PMP facilitate this inactivation. APC resistance is associated with PMP, and inverse correlations between levels of circulating PMP and prothrombin fragment F1+2 or thrombin–antithrombin complexes have been reported ***(Nieuwland et al., 2007).***

C. Adhesion

In vitro, PMP bind via integrin αIIbβ3 to the subendothelial matrix. Once bound, PMP promote platelet and leukocyte adhesion under flow conditions. In a rabbit model of arterial injury, PMP adhered mainly at the site of injury. Evidently, PMP facilitate binding of blood cells to the (damaged) vessel wall ***(Nieuwland et al., 2007).***

D. Cell-to-cell communication

Microparticles may affect target cells either by stimulating them directly via surface-expressed ligands or by transferring surface receptors from one cell to the other. Since microparticles engulf cytoplasm during their formation, they acquire proteins and RNA that originate from the cytosol of the parent cell. An increasing body of evidence suggests that after attachment or fusion with target cells, microparticles deliver cytoplasmic proteins and RNA to recipient cells. This process can be mediated either through receptor-ligand interactions or through internalization by recipient cells via endocytosis. This phenomenon may contribute to the reprogramming of target cells ***(Italiano et al., 2010)*** (figure 3)**.**

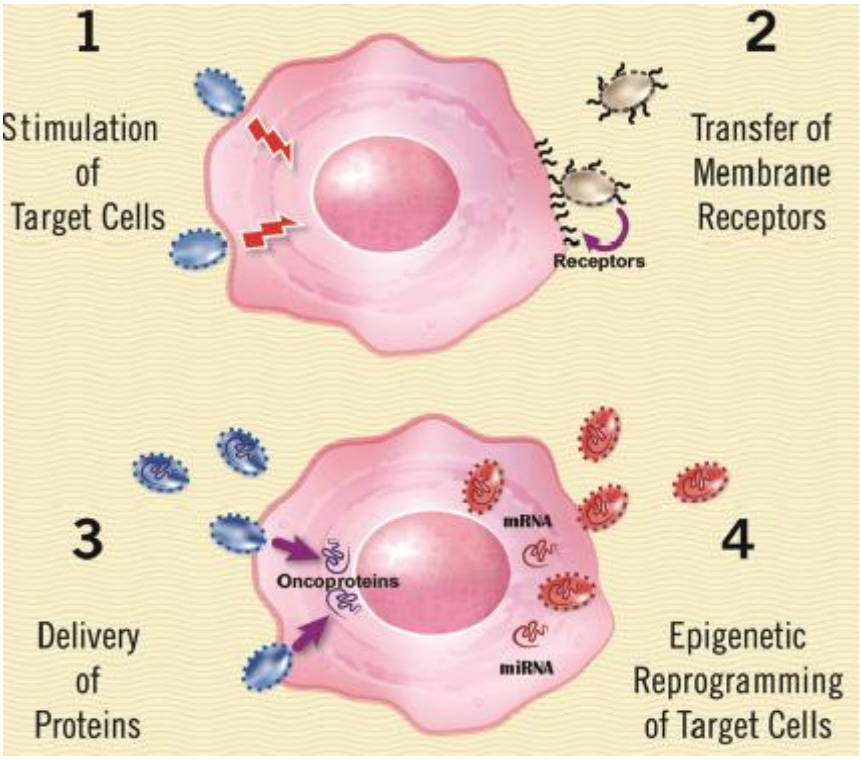

Figure (3): Schematic representation of MV-mediated cell-to-cell interaction. (1) MVs may signal through surface expressed receptors leading stimulation of target cells. (2) MVs may transfer receptors from the cell of origin to the target cell. (3) MVs may transfer oncogene products, transcription factors or infectious particles to target cells. (4) MVs may mediate a horizontal transfer of mRNA and microRNA (miRNA) inducing epigenetic changes in the target cell ***(Camussi et al., 2011).***

After activation, PMPs coated with tissue factor (TF) are able to interact with molecules, such as P-selectin, expressed on the surface of macrophages, polymorphonuclear neutrophils and platelets. PMPs remain on the surface of these cells and their phosphatydilserine enriched membranes provide a surface for the assembly of clotting factors. Moreover, PMPs may directly activate endothelial cells, polymorphonuclear

neutrophils and monocytes and influence the functions of normal and malignant human hemopoietic cells ***(Camussi et al., 2011).***

E. Carrier Function and Cell Activation

Secretory phospholipase A2-treated PMP contain the "bioactive lipid" arachidonic acid (AA). This AA can be transferred to platelets and endothelial cells, which become activated. PMP also contain other bioactive lipids, including platelet activating factor. Thus, PMP can modulate the activation status of "target" cells. Interestingly, MP from preeclamptic patients or from myocardial infarction patients impaired nitric oxide-mediated vasodilatation, suggesting that PMP may directly affect vascular tone in vivo. Whether this effect can be attributed solely to the delivery of AA, however, is unclear. In a rabbit artery model, PMP were also shown to contain cyclooxygenase that converted AA, produced by endothelial cells themselves, into the vasoconstrictor thromboxane (TX) A2 and its stable metabolite TXB2. Thus, PMP may modulate the vascular tone via various pathways, including the delivery of AA and the production of TXA2. PMP expose cell specific adhesion receptors, such as P-selectin, and therefore may act as "long-range carriers" (third messengers) that deliver bioactive molecules to specific target cells. For example, P-selectin binds to PSGL-1 on monocytes, and this interaction initiates expression of tissue factor and cytokines. Thus, PMP may not only support processes like coagulation directly, but also indirectly ***(Nieuwland et al., 2007).***