UNIVERSITÀ di VERONA
Scuola di MEDICINA E CHIRURGIA

DEPARTMENT OF

MEDICINE

PHD SCHOOL

LIFE AND HEALTH SCIENCES

PHD IN

CLINICAL AND EXPERIMENTAL BIOMEDICAL SCIENCE

WITH THE FINANCIAL CONTRIBUTION OF

ATENEO - UNIVERSITY OF VERONA

Cycle XXXIII, year 2020-2021

PLATELETS BEYOND ATHEROTHROMBOSIS

S.S.D. (Disciplinary Sector): BIO/12

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PhD thesis
Verona, September 2021

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1. Summary (Italian)

Le piastrine sono oggigiorno sempre più studiate per meglio caratterizzare il loro ruolo in diversi processi fisiopatologici. La tesi comprende studi che hanno affrontato l'analisi della piastrina attraverso metodiche in grado di esplorare differenti aspetti funzionali. La NAFLD (Non-Alcoholic Fatty Liver Disease) è un'epatopatia cronica che degenera nella sindrome NASH (Non-Alcoholic Hepatitis) e, nel peggiore scenario, nel cancro al fegato. Usando la piattaforma Cellix di Microfluidica con microchip rivestiti con collagene e la Citofluorimetria a flusso (FACS) per identificare le microvescicole di origine piastrinica (PMVs), si evidenzia un maggiore rilascio di PMV nei pazienti in esame, verosimilmente dovuto ad uno stato di pre-attivazione piastrinica.

Un'attivazione piastrinica elevata, che porta ad aggregati piastrinici più ampi, si è riscontrata dopo incubazione con C6O4 (100-200 ng/mL), un nuovo composto appartenente alle sostanze Perfluoro-alchiliche (PFAS), usato in detersivi e rivestimento di pentole ecc. Acido acetilsalicilico 100µM (ASA) ha dimostrato un'attività neutralizzante le proprietà pro-trombotiche di C6O4. Risultati simili si osservano in analisi FACS su PMVs indipendentemente dalla co-stimolazione con ADP 7.5µM, TRAP 10µM o in condizione di riposo; mentre l'incubazione con C6O4 250ng/mL induce un più alto rilascio di PMVs, acido acetilsalicilico (ASA) controbilancia questo Per studiare la produzione piastrinica e la regolazione della funzionalità piastrinica è stato analizzato il canale ionico K_{Ca}1.1. L'analisi degli effetti sull'aggregazione piastrinica di BMS191011 (20µM), un apertore del canale K_{Ca}1.1 usando concentrazioni crescenti di diversi agonisti (ADP, U46619 e Collagene), ha dimostrato a uno spostamento verso destra della curva dose-risposta indicativo di un effetto antipiastrinico. Combinando ASA (o PGE1 2.5nM con U46619) e BMS191011, lo spostamento è più accentuato. Il recettore del Fibrinogeno (GPIIb/IIIa), è stato analizzato mediante FACS, usando l'immunoglobulina PAC-1 assieme al marker di secrezione dagli α-granuli di P-Selectina (CD62) in piastrine stimolate con agonisti solubili. Gli apertori del canale K_{Ca}1.1 (BMS191011 (5-20µM), NS11021 (5µM) and NS1619 (5µM)) hanno mostrato una selettività nell'attività inibitoria. Nessun effetto è stato riscontrato in piastrine stimolate con U46619 (0.5-2µM), mentre è stata osservata una riduzione

nell'espressione degli epitopi di attivazione in piastrine stimolate con ADP. Nell'analisi in Microfluidica su collagene immobilizzato, trattamenti con BMS191011 (5-20µM), NS11021 (5µM) e NS1619 (5µM)) hanno ridotto in modo significativo la formazione di trombi e area di adesione rispetto al controllo non trattato. Dal punto di vista quantitativo, sono stati osservati effetti inibitori simili con ASA (100µM) e 11,12-acido epossieicosatrienoico (EET) (1µM), un eicosanoide inibitore delle piastrine. In condizioni statiche BMS191011 non altera l'adesione e la diffusione piastrinica, indipendentemente se la matrice extracellulare usata fosse collagene o fibrinogeno, dato che indica che l'apertura del canale $K_{Ca}1.1$ inibisce l'adesione piastrinica in condizioni di flusso attraverso l'inibizione dei segnali intracellulari dell'ADP.

1. Abstract (English)

Platelets are nowadays more and more investigated to better characterise their role in many different pathophysiological processes. The present thesis summarises explore NAFLD (Non-Alcoholic Fatty Liver Disease) is a chronic epatopathy degenerating in NASH syndrome and in the worst scenario to liver cancer. Using a Cellix Microfluidic platform with collagen coated microchip and a Flow Cytometry (FACS) on Platelet derived Microvesicles (PMVs) essay, a stronger platelet activity and higher release of PMVs in such patients, is highlighted, probably due to a pre-activation platelet status.

A platelet activation, leading to wider platelets aggregates, is observed after incubation with C6O4 (100-200 ng/mL), a new compound belonging to Perfluoro-alkyl substances (PFAS), used in detergent, coatings for cookware etc. Aspirin 100µM (ASA) shows a neutralisation of C6O4 procoagulant properties. Similar results are observed in FACS analysis on PMVs, regardless the co-stimulation with ADP 7.5µM, TRAP 10µM or in resting condition; while C6O4 250 ng/mL incubation induces higher PMVs released, ASA counterbalances this PFAS compound.

To investigate the mechanisms of platelet production and function regulation, the ion channel $K_{Ca}1.1$ is analysed. Aggregation analysis on the channel opener BMS191011 (20µM), using increasing concentration of different vehicles (ADP, U46619 and Collagen) causes, generally, a rightward shift of the concentration-response curve. Combining ASA (or PGE1 2.5 nM with U46619) and BMS191011, the slide is more enhanced. No alteration is induced by BMS191011 when is used U46619 as agonist.

The Fibrinogen receptor (GPIIb/IIIa), was analysed by FACS, using the immunoglobulin PAC-1 and the α-granules secretion using as marker P-Selectin (CD62) in platelets stimulated with soluble agonists. $K_{Ca}1.1$ channel openers (BMS191011 (5-20µM), NS11021 (5µM) and NS1619 (5µM)) show selectivity in the inhibitory activity. No effects in platelets stimulated with U46619 (0.5-2µM). A reduction in expression is observed in ADP-stimulated platelets. Treatment with BMS191011 (5-20µM), NS11021 (5µM) and NS1619 (5µM), significantly decreases thrombus formation and area coverage with respect to untreated control

in Microfluidic analysis on immobilized collagen.

Quantitatively similar effects are observed with ASA (100μM) and 11,12-EETs (1μM). Under static condition BMS191011 does not alter platelet-adhesion and spreading, regardless if the extracellular matrix is collagen or fibrinogen indicating that opening of the $K_{Ca}1.1$ channel reduces platelet adhesion under flow by inhibiting ADP signalling system.

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2. Introduction

Blood is essential for life because it delivers fundamental substances like oxygen and nutrients to all the body's cells. Blood cells consist of three basic units: Red Blood Cells (RBCs) or Erythrocytes, White Blood Cells (WBCs) or Leukocytes and Platelets (Plts) or Thrombocytes (Alberts, et al., 2002), which are the main topic of the thesis.

2.1. General introduction

Platelets are the smallest blood cells, numbering 150 to 350 x 10^9 /L of blood in healthy individuals (Michelson, Platelet - 3rd Edition, 2013). Bizzozero in the 19th century was the first to describe the ability of platelets to adhere to an injured vessel wall and form aggregates (de Gaetan & Cerletti, 2002); (Brewer, 2006). Platelets play a crucial and long-established roles in thrombosis and haemostasis (Ruggeri, 2002); (Davì & Patrono, 2007). Overtime they were increasingly recognized as pivotal players in numerous other pathophysiological processes including inflammation and atherogenesis (Lindemann, Krämer, Seizer, & Gawaz, 2007), antimicrobial host defence (Engelmann & Massberg, 2013), and tumour growth and metastasis (Gay & Felding-Habermann, 2011). Consequently, profound knowledge of platelet structure and function is becoming more important in research and in many fields of modern medicine. For this and many other reasons, human platelets are amongst the most widely studied cells in the body.

Platelet structure have an average diameter of 2 to 5 µm, a thickness of 0.5 µm, and a mean cell volume of 6 to 10 fl. Platelets are anucleated cells. The structure of the platelet can be conceptually divided in four regions: a peripheral zone, a sol-gel zone, an organelle zone, and membrane systems (White, 2013).

2.1.1. Megakaryocytopoiesis – a short overview

In recent years the study of megakaryocyte (MK) differentiation has generated considerable interest. Due to the fact that it is now widely recognized that changes in platelet reactivity are determined in the MK during thrombopoiesis and that MKs change in pathological condition (Erusalimsky, 1993). For a long time, substantial progress in the study of MK differentiation has been hampered by both the unavailability of specific megakaryocyte colony-stimulating factors (MK-CSF) and

by difficulties in obtaining pure population of both mature MKs and their progenitors in ample supply. The later problem has been partially circumvented by the application of techniques which do not require a large number of cells, such as flow cytometry, dynamic video imaging, in situ hybridization, and the polymerase chain reaction. In addition, the use of megakaryoblastic cell lines, although not the real thing, provide enough homogeneous material for biochemical analysis.

MKs originate in the bone marrow from pluripotent stem cells through a complex differentiation process involving stem cell commitment, mitotic amplification of committed progenitors, nuclear polyploidization, and cytoplasmic maturation leading to the production of platelets. This process is physiologically controlled by a serum factor which stimulates the bone marrow to produce mature MKs in response to platelet consumption (Hoffman, 1989). The existence of a humoral regulator of megakaryocytopoiesis has been postulated for a long time, but its identity has been established only recently (de Sauvage, et al., 1994); (Lok, et al., 1994); (Kaushansky, et al., 1994); (Wedling, et al., 1994). This factor, known as c-Mpl ligand or thrombopoietin, stimulates haematopoietic progenitors to proliferate and differentiate into mature MKs and, when injected into animals, increases platelet production dramatically. In addition to c-Mpl ligand, a variety of pleiotropic haematopoietic growth factors, including interleukin (IL)-3, IL-6, IL-11, granulocyte/macrophage colony-stimulating factor (GM-CSF), leukaemia inhibitory factor, oncostatin M, stem cell factor, and erythropoietin (EPO), synergistically promote the growth and maturation of human MKs (reviewed in refs (Hoffman, 1989) and (Gordon, 1992)). *In vitro*, phorbol ester, in combination with cytokines which stimulated proliferation, also stimulate MK maturation (Long, Smolen, Szczepanski, & Boxer, 1984); (Long, Hutchinson, Gragowski, Heffner, & Emerson, 1988). Furthermore, phorbol esters cause terminal megakaryocytic differentiation of erythroid and mekaryoblastic leukaemic cell lines.

A distinctive feature of MK differentiation is the process of nuclear polyploidization. After cessation of proliferation and before cytoplasmic fragmentation takes place, MKs undergo a variable number of endomitotic cycles. As in normal mitosis, during MK endomitosis the nuclear membrane breaks down, mitotic spindles are formed, and condensed chromosomes can be identified in

metaphase. After this stage, however, there seems to be a division from the normal mitotic pathway which results in the restitution of the nuclear membrane and the inhibition of nuclear and cellular division. Thus, the cells become polyploidy by allowing many rounds of DNA replication without completion of the intervening mitosis.

The ploidy of bone marrow MKs usually ranges from 4n to 6n, with 16n being the modal ploidy under normal physiological conditions in all the mammals studied. In human this ploidy distribution may change under pathological conditions, as shown in patients with cancer, platelet disorders, and cardiovascular disease (reviewed in ref. (Erusalimsky, 1993)).

2.1.2. Functional responses: secretion

Platelets contain four distinct intracellular storage granule (Mc Nicol & Gerrard, 1995):

a. Alpha granules contain adhesive molecules (including fibrinogen, fibronectin, thrombospondin), growth factors (β-transforming growth factor, platelet-derived growth factor), coagulation factors (V, VII), and other proteins (platelet factor 4, platelet basic protein, β-thromboglobulin, connective tissue activating protein III, neutrophil activating peptide 2).
b. Dense granules contain 5-HT, ATP, ADP, calcium, and pyrophosphate.
c. Lysosomes contain acid proteases, acid glycosidases, acid phosphatases, and aryl sulfatases.
d. Peroxisomes contain catalase.

With the possible exception of peroxisomes, platelet activation by a number of agonists is associated with release of granule contents. Methods to stimulate the specific release from the individual granule species have been devised (Lages, 1986); (Kaplan, 1986); (Nishibori, et al., 1993), although overlap does occur. Release can be measured in many ways. It can be measured from platelets in PRP or washed preparations. Studies can be done in conjunction with aggregation or simply by incubation in a shaking water-bath at 37°C. The latter case is less time-consuming and gives similar results.

2.1.3. Platelets role in the haemostatic process and in the pathological thrombus formation.

Platelets plays a pivotal role not only in the physiological haemostatic clot formation, but also in the pathological thrombus formation, particularly along the arteries with high blood flow rate (Ross, 1993); (Fuster, Badimon, Badimon, & Chesebro, 1992). In physiological conditions, platelets do not interact with the vascular wall but, after an intimal injury, the antithrombotic features of endothelial cells are lost and the adhesive sub-endothelium molecules are exposed collagen and fibronectin, along with the von Willebrand factor (vWF), allowing their interaction with blood. The adhesion of platelets to the damaged vascular wall it is the first step in haemostasis and in thrombosis (Sixma & de Groot, 1994).

Platelets in the early stage, bind to the vWF through the adhesive receptor GPIb-IX-V in correspondence with the injury site. The vWF therefore mediates the interaction between platelets and sub-endothelium. Subsequently the collagen α2β1 and GPVI receptors modulate a stronger adhesion with a further platelet activation. Those starting interactions induce the release from dense granules (ADP) and from the α-granules (fibrinogen, factor V, P-Selectin) (Jackson, Nesbitt, & Kulkarni, 2003). Mediators released from platelet granules enforce platelets activation and are also important for the coagulative and inflammatory responses, by means of the released factor V and P-selectin exposure on the platelet membrane surface. After stimulation with an activating agonist, platelets hydrolase arachidonic acids from phospholipids – via phospholipase A_2, converting it in thromboxane A_2 (TXA_2) by means of cyclooxygenase and thromboxane A_2 synthase (Samuelsson, et al., 1978). $P2Y_1$ and $P2Y_{12}$ platelets receptors are activated by ADP released from dense granules (Kunapuli, et al., 2003), while thromboxane A_2 activates α thromboxane receptors on platelets membrane (Jackson, Nesbitt, & Kulkarni, 2003). Platelets interacting with these mediators have undergone a “shape change”, a process leading to the reorganisation of the cytoskeleton's actin, from a discoidal conformation in to a spherical shape with filopodia emission (Siess, 1989); (Fox, 1993). Meanwhile the Tissue Factor (TF) is available and coagulation factors are released, entailing thrombin formation and thus a coagulative response. Thrombin activates platelets through PAR-1 and PAR-4 receptors (proteases activated) and

converts fibrinogen in fibrin stabilizing the platelet clot where the vascular wall is damaged. The fibrinogen binds to platelets by means of its activated receptor and this "cross-linking" between platelets and fibrinogen leads to platelets aggregates stockpile and thus the arrest of the vascular injury site bleeding. Hence the platelets activation is the consequence of several signals originating from many receptors, each of them contributing to the platelet clot formation Figure 1).

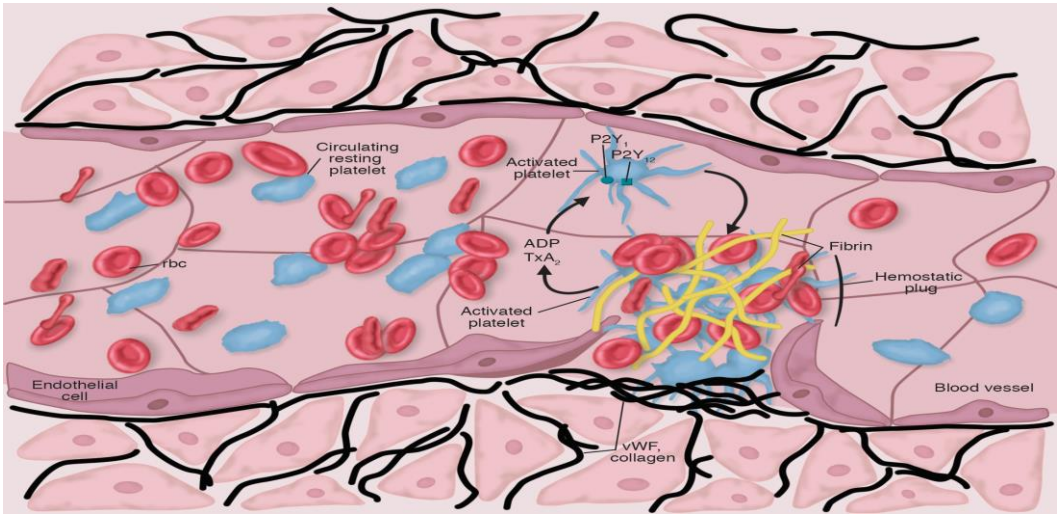


Figure 1: The haemostatic process. When a vessel injury occurs, platelets roll and bind to the vessel wall, interacting with vWF and collagen (black strands). The consequence is a platelet shape change, index of platelet activation. ADP release from dense granules and thromboxane A2 (TxA2) generation take ten place. The agonist ADP and TxA2 cause further platelet activation and accumulation of platelets at the site of injury. The tissue factor is exposed and it catalyses the coagulation response, resulting in the formation of thrombin. Platelets are further activated and thrombin cleaves fibrinogen to form fibrin. At the site of damage, the interaction among fibrin and activated platelets forms a stable haemostatic plug that arrests bleeding. (Dorsam & Kunapuli, 2004).

2.1.3.1. Haemostasis

The physiological process that stops bleeding at the site of vascular injury while maintaining the normal blood flow elsewhere in the circulation is known as haemostasis. The formation of a haemostatic plug blocks the leaking of blood. Under physiological condition, the endothelium coates the internal lumen of the blood vessels exhibiting an anticoagulant surface essential to maintain blood in its fluid state. When the blood vessel is damaged, components of the subendothelial matrix are then exposed to the bloodstream. Numerous of these components activate the two main processes of haemostasis leading to blood clot formation composed primarily of aggregated platelets and fibrin. Due to a tightly regulated mechanism, the process is activated within seconds after damage and remains localized to the site of injury (Gale, 2011).

2.1.3.1.1. Primary Haemostasis: platelet adhesion

It is well known in the primary haemostasis platelets adhere to the vessel wall at the injury site. This process takes place through signals depending from glycoproteins (GP) expressed on the surface of platelets.

The intact endothelium produces prostacyclin, nitric oxide and expresses ectonucleoside triphosphate diphosphohydrolase-1 (CD39), elements with inhibitory function towards haemostatic process: this role decrease following to a vessel injury, since extracellular matrix components, such as collagen, are in direct contact with blood.

Platelet adhesion diverges in according to the circulation shear stress level. When the shear stress is high, circulating von Willebrand factor (vWF) lays down at the level of the exposed subendothelial collagen; this determines the deployment of vWF which, normally inactive in the circulation, shows multiple binding sites for GP1b (CD42), a member of the vWF receptor complex GP1b-V-IX. This process drops down the platelets flow speed, triggering the rolling of platelets and the interaction between GPVI and collagen (Koupenova, Clancy, Corkrey, & Freedman, 2018).

Under low shear stress, platelets mainly adhere on collagen by means of the glycoprotein GP1a/2a (integrin α2β1) and, to a lesser extent, through GPVI,

components of the collagen receptor. Actually, α2β1 has a role in the tardive stabilization of the thrombus even in high shear stress situation.

GPVI, differently from α2β1 and vWF, has scarce adhesive activity, but it is important for the platelets activation and for the exposition of α2β1 and αIIbβ3.

An additional pro-adhesive factor is P-selectin, which is stored in the platelets α-granules and released when platelets become activated, and is expressed on the surface of activated endothelial as well. P-selectin binds to PSGL-1 (P-selectin glycoprotein ligand-1) sited on leukocytes and it mediates the rolling on the stimulated endothelial cells; additionally, P-selectin is responsible for the formation of aggregates composed of activated platelets and leukocytes, the aggregation of activated platelets on neoplastic cells and platelet adhesion to the endothelium. The cellular interactions mediated by P-selectin are thus fundamental in the inflammation processes, thrombosis, tumour growth and metastasis (Chen & Geng, 2006).

The adhesion phase leads to physiological and cytoskeletal changes in the platelet (platelet activation) allowing the development of the subsequent aggregation's phase; among these, one of the first changes taking place is the transition of the platelets from a discoidal shape to an activated status with several pseudopods.

2.1.3.1.2. Primary Haemostasis: platelet aggregation

The initial platelet adhesion and activation is followed by the recruitment of other platelets from the circulation and by the formation of three-dimensional aggregates, processes guided by important molecular interactions.

The recall of other platelets, their activation and aggregation is mediated by the generation and release of endogenous pro-aggregating substances, namely TXA_2 by the platelet thromboxane-synthase, ADP released from δ granules, the main platelet agonist, and thrombin produced via coagulation cascade. Endogenous aggregating substances determine the conformational changes of the platelet receptor for fibrinogen, αIIbβ3, already exposed allowing the aggregation process (Primary aggregation). The release of the α-granules and δ granules is essential to reinforce the aggregation and to make it irreversible (Secondary wave of aggregation).

The adhesion to the endothelial cells occurs through the interaction between αIIbβ3 and endothelial αVβ3 (receptors for vWF, fibrinogen, fibronectin) and ICAM-1 (via fibrinogen); alternatively, αIIbβ3 might interact with endothelial GPIb-IX-V (via vWF) (Nuyttens, Thijs, Deckmyn, & Broos, 2011) (Figure 2)

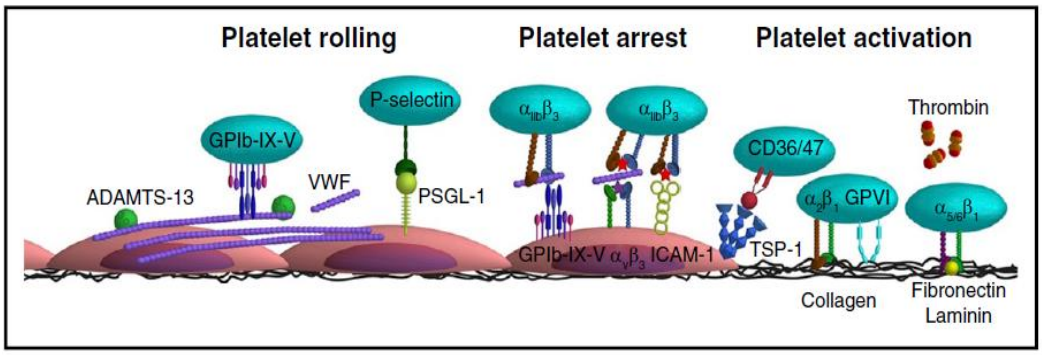


Figure 2: Schematic summary of different interaction among platelets' molecules and endothelium. Platelets interact with endothelium through the following mechanisms:

- *Platelet rolling via interaction between platelet GPIb-IX-V and endothelial vWF with the help of the axis P-selectin/PSGLI;*
- *Platelets adhesion onto the endothelium through the interaction between αIIbβ3 and endothelial αVβ3 (by means of vWF, fibrinogen, fibronectin) and ICAM-1 (via fibrinogen); alternatively, αIIbβ3 might interact with endothelial GPIb-IX-V (thanks by vWF);*
- *Platelet activation between the thrombin stimulus and Thrombospondin 1 axis (TSP-1)/CD36;*
- *Platelet adhesion blocks at the collagen thanks to α2β1* (Coenen, Mastenbroek , & Cosemans, 2017)

2.1.3.1.3. Main soluble platelet agonists

Thrombin modifies via hydrolysis PAR-1 and PAR-4 receptors and makes them able to induce a transmembrane signal. Both PAR-1 and PAR-4 bind to Gαq and to $G\alpha_{12/13}$ activating the fibrinogen receptor independently from Gαqi, stimulated by the secreted ADP (Kim, et al., 2002).

Epinephrine binds to adrenergic receptor α2A causing Gαz activation which leads the inhibition of adenylyl cyclase (Yang, et al., 2002). Stimulation of this receptor alone is not sufficient to induce neither dense granules secretion nor αIIbβ3 activation in washed platelets, but it is able to enhance both the secretion and the

aggregation induced by other agonists (Steen, Holmsen, & Aarbakke, 1993); (Lanza, et al., 1988).

ADP is a pivotal component of platelets dense granules and an important agonist that stimulate platelets binding to three receptors: $P2Y_1$ coupled to Gαq, $P2Y_{12}$ coupled to Gαi and $P2X_1$ calcium channel which potentiates "shape change" induced by agonists (Kunapuli, Dorsam, Kim, & Quinton, 2003). Currently it is well established that the concomitant signal - via $P2Y_1$ and $P2Y_{12}$ - is necessary and sufficient to activate the integrin GPIIb/IIIa (Jin & Kunapuli, 1998); (Daniel, et al., 1998); (Hollopeter, et al., 2001); (Foster, et al., 2001). Furthermore, also the selective activation of Gαi coupled to the selective stimulation of $G\alpha_{12/13}$ thanks to other agonists gives rise to platelets aggregation even in Gαq absence (Nieswandt, Schulte, Zywietz, Gratacap, & Offermanns, 2002); (Dorsam, Kim, Jin, & Kunapuli, 2002). The $P2Y_{12}$- Gαi signal is crucial for the dense granules secretion and TXA_2 generation; it is also required for platelets aggregation induced by ADP and the TXA_2 analogue U46619 (Dangelmaier, Jin, Smith, & Kunapuli, 2001); (Jin, Quinton, Zhang, Rittenhouse, & Kunapuli, 2002); (Paul & Kunapuli, 1999). Moreover, $P2Y_{12}$ receptor stimulation induces through the α subunit the adenylyl cyclase inhibition (Yang, et al., 2002), while through the βγ subunit the activation the phosphatidylinositol (PI) 3-kinases γ (Hirsch, et al., 2001); (Jackson, Yap, & Anderson, 2004); (Lian, et al., 2005) and AKT (Kim, Jin, & Kunapuli, 2004); (Woulfe, et al., 2004), inducing the enhancement of platelet secretion leading to an irreversible aggregation (Li, et al., 2003), and of RapIb, small GTP-asi which increase the bond agonist-induced of ligands to αIIbβ3 having an effect on the integrin affinity (Woulfe, Jiang, Mortensen, Yang, & Brass, 2002); (Bertoni, et al., 2002). The stimulation of $P2Y_1$ induces PLCβ activation with subsequent generation of inositol 3,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 mobilizes calcium from the intracellular stores, inducing "shape-change", while DAG activates the protein kinase C (Brass, Woolkalis, & Manning, 1988) (**Errore. L'origine riferimento non è stata trovata.**).

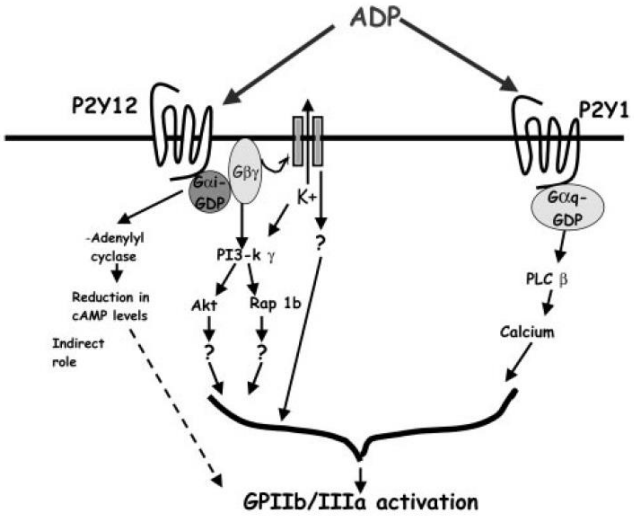


Figure 3: Schematic of possible effectors downstream of the P2Y12 receptor. The figure depicts the signalling events that occur on ADP-induced P2Y12 receptor stimulation. PI3-kinase γ, Akt, and Rap 1b are known effectors, which contribute to P2Y12 receptor-mediated GPIIb/IIIa activation. The βγ subunit of the Gi released on receptor stimulation binds to and activates GIRK channels. GIRK channel activation results in the efflux of K^+ and in the generation of an intracellular signalling cascade leading to integrin activation. Functional transducers mediating this signal are yet to be identified. (Shankar, et al., 2004)

TXA2 is a potent platelet agonist with an important role *in vivo* in the haemostasis and thrombosis processes. It binds to TPα and TPβ receptors subtypes and its action is performed via heterotrimeric G-proteins of two types, Gαq and $G\alpha_{12/13}$ (Raychowdhury, et al., 1994); (Offermanns, Laugwitz, Spicher, & Schulz, 1994); (Djellas, Manganello, Antonakis, & Le Breton, 1999). Doses able to stimulate a fully platelets activation, TXA_2 triggers responses Gαq mediated, activating PLCβ, which has an essential role in the platelets activation (Lian, et al., 2005); (Offermanns, Toombs, Hu, & Simon, 1997). There are more and more evidences that this signal pathway is implicated, beyond the activation of the αIIbβ3, in the secretion of ADP which amplifies the platelet response through $P2Y_{12}$ Gαi-coupled receptor (Dorsam & Kunapuli, 2004); (Paul & Kunapuli, 1999). A pivotal component of this signal pathway Gαi-dependent is undoubtedly PI3-kinaseγ (see ADP). Furthermore, it has been found a PI3-kinaseγ role downstream of Gαq either

in platelets than in other cell types (Woulfe, Jiang, Mortensen, Yang, & Brass, 2002); (Goel, Phillips-Mason, Gardner, Raben, & Baldassare, 2004).

The signal transduction through $G\alpha_{12/13}$ activation is important in the "shape change" phenomenon. Some studies demonstrated that Gαq deficient platelets did not aggregate in response to TXA_2 and Thrombin, while "shape change" occurred normally (Klages, Brandt, Simon, Schultz, & Offermanns, 1999). The $G\alpha_{12/13}$ signal pathway, acting together with Gαi signal mediated, by ADP or epinephrine, leads to platelet aggregation and degranulation (Nieswandt, Schulte, Zywietz, Gratacap, & Offermanns, 2002); (Dorsam, Kim, Jin, & Kunapuli, 2002). $G\alpha_{13}$ plays an essential role mediating platelet responses, as proved by the strong reduction in the haemostatic responses and in the protection against *in vivo* arterial thrombosis in $G\alpha_{13}$ deficient mice (Moers, et al., 2003).

2.1.3.1.4. "Inside-out" and "Outside-in" signals

Interaction among platelets and agonist such as collagen, ADP, epinephrine, TXA_2, thrombin, vWF and other arachidonic acid metabolites, induce an intracellular platelet signal that leads to αIIbβ3 activation, which is the heterodimeric integrin as acting as fibrinogen receptor (Brass, Manning, Cichowski, & Abrams, 1997). Following stimulation with an agonist, αIIbβ3 modifies its conformational status from low affinity to high affinity to fibrinogen (Shattil, Kashiwagi, & Pampori, 1998). The reaction between fibrinogen and αIIbβ3 is strictly controlled. Agonists transduce the signals inside the cell leading to the interaction between Talin, important cytoskeletal protein with regulatory ligand function, and the cytoplasmic β subunit of αIIbβ3 (Tadokoro, et al., 2003). This interaction induces conformational changes in the αIIbβ3 extracellular domain, resulting in increased affinity to adhesive ligands ("inside-out signalling") (**Errore. L'origine riferimento non è stata trovata.**- Left side).

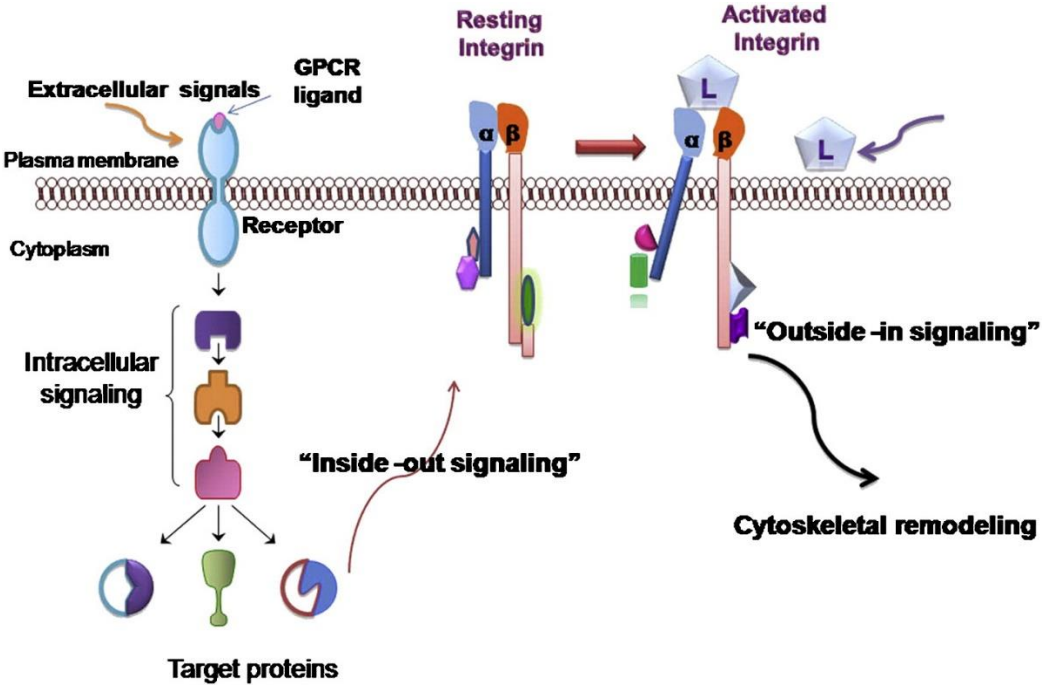


Figure 4: Bidirectional signalling across integrins. Signalling pathway: Agonist stimulation, signalling via G protein coupled receptors (GPCRs) and/or growth factor receptors can lead to "inside-out" signalling of integrins. Extracellular domains of resting integrins open up to bind extracellular matrix ligands. This leads to "outside-in" signalling of integrins resulting in cytoskeletal remodelling and downstream signalling cascades. (Das, Ithychanda, Qin, & Plow, 2014)

Furthermore, the initially reversible extracellular ligand interaction, becomes progressively irreversible and promotes the integrin "clustering" and further conformational changes transduced via intracellular signal pathways. All these events lead to the recruitment and/or activation of enzymes (i.e. tyrosine kinase), adapters (i.e. Grb2) and effectors (i.e. Rac1, RhoA, cdc42) to assemble the signals complex which determines integrin activation ("outside-in signalling") (**Errore. L'origine riferimento non è stata trovata.**- Right side) (Shattil & Newman, 2004).

2.1.3.1.5. Tyrosine Kinases in platelet activation

Tyrosine kinases (TK) are enzymes playing essential role in many cell functions, such as cell signalling, growth, and division (Hubbard & Till, 2000). These enzymes are classified in two categories on the basis of their cellular location: trans-membranous receptor (RTK) or intracellular (NRTK) (Robinson, Wu, & Lin,

2000). When TKs are active, they catalyse ATP and adding a phosphate residue to other amino acids or peptides, induce a conformational changes that can affect the function of their target; by means of an auto-phosphorylation, TK can self-induce a modification of its structure and function (Lemmon & Schlessinger, 2010).

Few examples of RTKs are EGFR, epidermal growth factor receptor, PDGFR, platelet-derived growth factor receptor and FGFR, fibroblast growth factor receptor.

While RTKs are essential in the "Outside-in" signal, there are studies showing the activation of NRTKs which turned out to be involved in platelet and integrin activation if stimulated with soluble agonists (Minuz, et al., 2018). In 2006 it was described how stimulating washed platelets with TXA_2 analogue, in presence of the ADP scavenger apyrase under non-aggregating conditions, either Rho kinase-induced phosphorylation of myosin light chain (MLC) and tyrosine phosphorylation signal were induced. Both pathways are implicated in triggering platelet shape change, without affecting neither secretion nor aggregation (Minuz, et al., 2006).

Platelets are the keystone of primary haemostasis as described in the paragraph 2.1.3.1. Haemostasis. They can carry out this function thanks to highly regulated mechanisms mediated by surface receptors and downstream messengers, and one of them is indeed the Tyrosine Kinase. Inducing platelet conformational changes, TKs contribute in the formation of a solid fibrin rich plug or thrombus (Sater, Gandhi, Dainer, & Pantin, 2017).

2.1.3.1.6. Secondary Haemostasis

Clot stabilization, described as secondary haemostasis, is characterized by the consolidation of the platelet mass through the formation of fibrin's fibres structure, thanks to the activation of the thrombin complex, and by means of the actin/myosin mediated platelet retraction.

There are two activation pathways of the pro-thrombin complex: one is defined extrinsic, due to the release of the Tissue Factor (TF), and the other one is called intrinsic, given by the factor XII activation that occurs when the blood gets in touch with the extracellular matrix. The two pathways are in communication, since both

pathways lead to the activation of factor X and the cofactor V activation, that bind to the negative charged platelet surfaces, where they generate the prothrombin complex. This activates prothrombin to thrombin which is responsible for the proteolytic activation of fibrinogen assembling polymeric fibrin (a calcium dependent mechanism) which, stabilized by the factor XIII, determines the clot development.

2.1.3.1.7. Haemostatic plug

In the haemostatic plug formation, it has been proposed that there are regional differences in platelet activation and these reflect the regional dissimilarities in the distribution of platelets agonists. Plug can be differentiated indeed into a central region (core) composed mostly of activated platelets which are well compact and a peripheral area (shell) characterized by platelets only partially activated and more loosely adherent, followed by a tail, which made of platelets, oriented according to the blood flow (Stalker, et al., 2013).

Platelets sitting in the core are P-Selectin positive: they thus underwent exocytosis of α granules and are strongly activated; the outer layers are instead P-Selectin negative and platelets are less adhered to each other. The extent of platelet activation in the outer layers has not yet been defined, although it is quite dubious that those platelets might be completely quiescent. The discovery that only P-Selectin negative platelets embolize suggests that a more stable adhesion depends on platelet activation. Further studies showed an accumulation of discoidal platelets in the vascular injury *in vivo*, supporting the hypothesis that the shell region is composed by less adherent platelets.

While collagen is limited at the vascular wall, thrombin, ADP and TXA_2 are not. Studying fibrin disposition, it was observed that its formation takes place mainly in the core region near to the site of damage. When thrombin diffuses outward, it meets important inhibitors limiting its function. Thrombin inhibition inhibits core formation: hirudin blocks the central region formation, leaving intact some parts of the shell (Stalker, et al., 2013).

TXA_2 and ADP are instead small molecular weight compounds, able to diffuse outwards compared to the region where platelets are enough activated to produce

them: produced in the core, act on the inactivated shell platelets too, but their effect is limited overtime (thromboxane) by the hydrolysis (ADP) and dilution. ADP concentration decrease below a critical threshold might be one of the factors limiting the shell size and it wards off from a post injury vascular occlusion. This explain as well the P2Y12's antagonists' capacity to reduce the platelets accumulation, in blood leaking-free. Aspirin produces the same effect, inhibiting the generation of TXA_2 (Figure 5).

Beyond α-granules degranulation, the central region is characterized by reduced porosity and increment of packing density of platelets.

This increase of density reduces the room among adjacent platelets, due to platelets shape change, cohesion and eventual clot retraction.

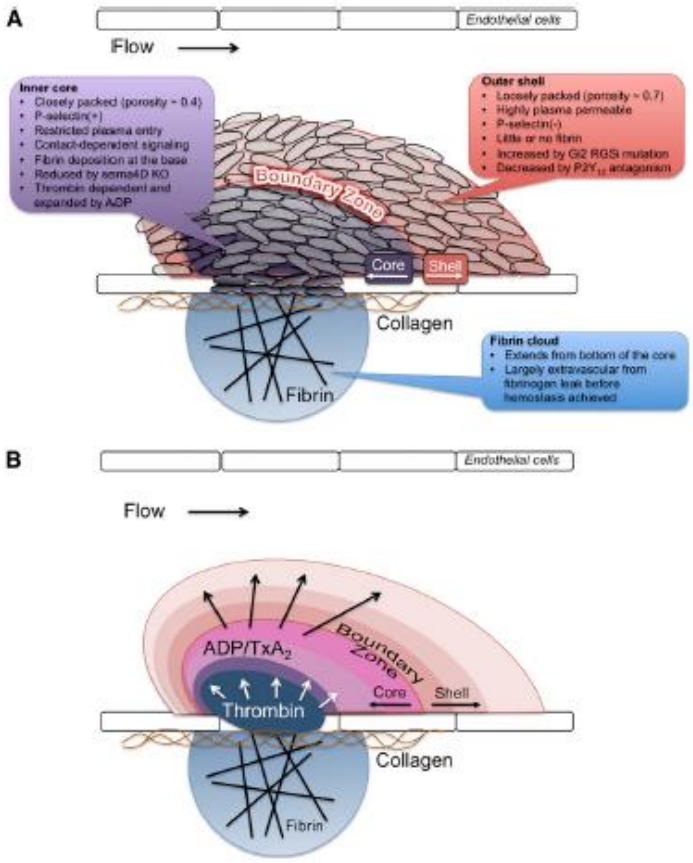


Figure 5: Haemostatic plug formation model, instructed by the interaction of local conditions with the platelet signalling network. The presented data suggest that during the initial formation and up to 1-hour post-injury, a haemostatic plug is composed of distinct regions defined by the platelet activation degree and by the packing density (Panel A). This proposed architecture is a result of a soluble agonists' gradient produced at the injury's site (Panel B). Thrombin generation at the injury site drives the activation of the platelets in the core region (blue to purple/pink), but its propagation away from the vessel wall is limited. ADP gradient and potentially TxA2 springing from activated platelets (purple to red) farther extends and leads to the recruitment of additional platelets developing the shell region. Additional platelet signalling, such as contact-dependent signalling pathways, emphasizes the architecture through local positive feedback. (Stalker, et al., 2013).

2.1.3.2. Thrombosis

Thrombus formation on arteriosclerotic plaque diverges, partially, from the simple haemostatic plug. It is thought platelets have a central role in the arterial thrombus development due to the fast flow conditions; actually, the arterial thrombus originating above an ulcerated plaque, show a large fibrin amount associated to platelet aggregates.

Platelet activation is the first step in thrombus generation and it is linked to adhesion events and platelets aggregation akin to those originated in the haemostatic plug.

The coagulative activation pathway plays, on other hand, an important role in thrombus formation. Adventitial fibroblasts, macrophages and smooth muscle cells display large TF amount. TF expression in the arteriosclerotic plaque is induced by several factors, such as pro-inflammatory cytokines, bacterial endotoxins and modified LDL. This proves the TF expression carries out an important pro-coagulant task in the thrombus formation (Asada, Yamashita, Sato, & Hatakeyama, 2018).

It was also demonstrated that in patients with cardiovascular risk factors a higher amount of circulating TF is present in the circulation bound to microvesicles; these are recalled inside the thrombus by platelet P-Selectin ligand PSGL-1 binding to microvesicles (Falati , et al., 2003).

Thrombus formation requires the important contribution of fibrin, which is associated to the platelet constituent developing a structure able to grow faster and more firmly compared to the haemostatic plaque (Figure 6).

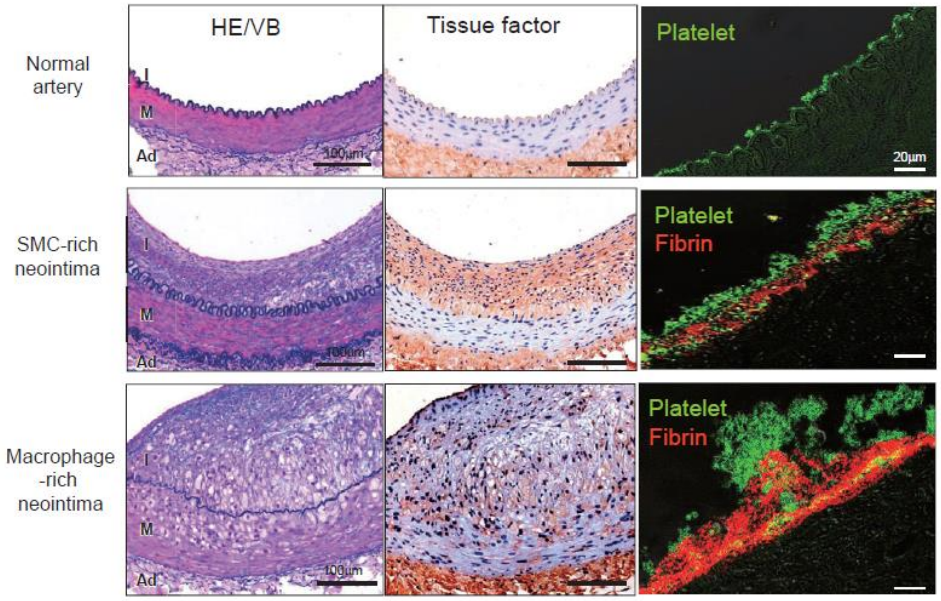


Figure 6: Immunohistochemistry analysis of tissue factor (TF) and thrombus formation in femoral arteries in normal and atherosclerotic rabbit. (Left column) representative tissue section of normal femoral artery, smooth muscle cell rich neointima artery (SMC-rich neointima) and macrophage-rich neointima artery (Macrophage-rich neointima); (Central column) Tissue Factor expression in SMC- and macrophage-rich neointima and in adventitia; (Right column) Thrombus composition 15 minutes post balloon injury. In normal artery there are only small aggregated platelets, while on SMC-neointima thrombus are shown a mixture of platelets and fibrin. Thrombus on macrophage-rich neointima is way larger (Asada, Yamashita, Sato, & Hatakeyama, 2018).

2.1.4. Platelet-derived microvesicles

Platelets that are activated *in vitro* by thrombin, collagen, thrombin plus collagen, or C5b-9 (the membrane attack complex of complement) undergo a vesiculation reaction that correlates temporally with the development of a procoagulant platelet surface. The latter is defined operationally by:

a. An increased transbilayer movement of negatively charged, anionic phospholipids from the inner leaflet to the outer leaflet of the plasma membrane.
b. Vesiculation from the platelet surface membrane of "microvesicles" with an average diameter of 0.1 μm.

c. An increase in the number of membrane binding sites for coagulation factor VIIIa and Va on platelet microvesicles.

The binding of factors VIIIa and Va is required for optimal assembly of the intrinsic "tenase" and "prothrombinase" enzyme complexes, respectively. Thus, development of a procoagulant surface on activated platelets and microvesicles enables the efficient catalysis of factor X to factor Xa and prothrombin to thrombin (Sims, Faioni, Wiedmer, & Shattil, 1988); (Bevers, Comfurious, & Zwaal, 1991); (Schroit & Zwaal, 1991) and (Gilbert, et al., 1991).

The molecular basis for the agonist-induced exposure of anionic phospholipids and platelet vesiculation is unclear, although calcium influx is required (Wiedmer, Shattil, Cunningham, & Sims, 1990). Platelet aggregation is not essential, but it can potentiate the process in a manner that appears dependent on activation of platelet calpain (Fox, Austin, Reynolds, & Steffen, 1991). Although granule secretion supplies factor Va for the prothrombinase reaction, platelet secretion is not required for reorientation of phospholipids or microvesicles formation (Wiedmer, Shattil, Cunningham, & Sims, 1990). Under certain experimental conditions, platelet procoagulant activity can be induced while microparticle formation is inhibited, indicating that, while the two processes are usually linked, vesiculation is not required for the initial development of the procoagulant surface (Dachary-Prigent, Freyssinet, Pasquet, Carron, & Nurden, 1993).

Platelets microvesicles are extracellular vesicles (EV) that can represent useful biomarkers in pathophysiologic processes mediated by platelets and suitable mediators of intracellular communication, but above all they are the vehicle of the communication between platelets and a variety of target cells in several diseases contests (Burger, et al., 2013).

The International Society for Extracellular Vesicles (ISEV) proposed few guidelines, The MISEV2018 guidelines (Théry, et al., 2018), which include suggestions about the nomenclature, collection, separation, characterization and EV concentration.

ISEV defines as extracellular vesicle a particle released by a cell that is delimited by a double lipid layer without a functional nucleus. The EV can be classified in according to their physical characteristics like dimension (< 100 nm small EV, 100

– 200 nm medium/large EV, >200 nm large EV), density (low, medium, high), biochemistry composition (i.e. EV with CD63+/CD81+, EV with Annexin A5), cellular origin (podocytes EV, hypoxic EV, wide oncosomes, apoptotic bodies).

Due to the isolation difficulty, the contexts' multidisciplinarity where EV have been studied and the different classifications, does not exist nowadays a nomenclature agreement. Currently EV can be classified in three typologies: exosomes, microvesicles (microparticles) and apoptotic vesicles (DeLeo & Ikezu, 2018). In the lack of classification, the term extracellular vesicle can be considered appropriate.

While exosomes are the smallest vesicles (30-150nm), the microvesicles (MVs), also called microvesicles, are extravascular vesicles (EV) with a diameter of 0.05-1.00 µm representing fragments released by plasmatic membranes of different cells (erythrocyte, leukocytes, enterochromaffins cells, smooth muscular cells), with a 70-90% of plasmatic deviation (Wang, Wang, & Hu, 2016); (Diamant, Tushuizen, Sturk, & Nieuwland, 2004). It does not still exist anyway a unique definition of microvesicles.

Data collected from experimental and clinical studies suggest that MVs detach from the cellular membrane immediately after the activation or the apoptosis. Phosphatidylserine (PS) flopping on the plasmatic membrane is the key event that leads to the MVs formation: following the cellular activation intracellular Ca^{2+} increases, flippase is inactivated, while the floppase become activated. These events determine the collapse of the mechanism that maintains the lipid asymmetry of the membrane allowing the budding of the membrane and the release of annexin V positive MVs. The process by which this all happens is complex and it has not yet been entirely explained, although there might be evidences on the crucial role of free intracellular calcium, mitochondria and caspases.

Platelets, like all cells, release different types of extracellular vesicles in response to cellular activation and apoptosis, including microvesicles (PMVs) and exosomes. Generally, the platelets microvesicles are vesicles of 30-800 nm and, for their size and for their low volume, are often difficult to isolate and to properly characterize. The first evidence of the platelets microvesicles dates back to 1967 thanks to Wolf (Wolf & Ziyadeh, 1997)who called them “platelet dusts”, even if they have been

quickly highlighted the properties of the factors promoting the clot formation (van der Pol, Böing, Harrison, Sturk, & Nieuwland, 2012).

Microvesicles deriving platelets and from other cell types circulating physiologically in the blood and detectable in the biologic fluids, carry out several functions, some of which not yet identified. Moreover, have been observed relationships between the circulating microvesicles number and the clinical expression of a few pathologies such as mellitus diabetes, chronic renal disease, preeclampsia and severe hypertension (Burger, et al., 2013).

Microvesicles mediate the communication cell-cell delivering information through surface receptors: mRNA, microRNA and perhaps DNA too from the originating cell to the target cell (Burger, et al., 2013). Microvesicles carry out than important roles in thrombosis, inflammation and angiogenesis and, for this reason, they have been studied with increasing interest in the last years ((Morel, Morel, Freyssinet, & Toti, 2008); (Vajen, Mause, & Koenen, 2015); (Todorova, Simoncini, Lacroix, Sabatier, & Dignat-George, 2017)).

2.1.4.1. PMVs and inflammation

Two types of observations have so far been conducted concerning the link between PMVs and inflammation: the PMVs laden can interact with inflammatory cells and in inflammation-associated disorders the levels of PMVs are increased. This entails that PMVs determine the activation of immune and endothelial cells, intensification of the trans endothelial migration of leukocytes and the amplification of the cell-cell communication, increasing as well the pro-inflammatory cytokines (Słomka, Urban, Lukacs-Kornek, Żekanowska, & Kornek, 2018).

Specifically, it has been demonstrated that PMVs regulate:

- Expression of cyclooxygenase-2 (COX-2) and prostacyclin (PGI_2) in endothelial cells (Barry, Pratico, Lawson, & FitzGerald, 1997);
- Monocytes and endothelial cells interaction by means of the expression of ICAM-1 (Smith, Marlin, Rothlein, Toman, & Anderson, 1989);
- Aggregation and accumulation of neutrophils through the expression of P-Selectin and IL-1 (Forlow, McEver, & Nollert, 2000);

- Production of pro-inflammatory molecules: CD40L, IL-1, IL-6 and TNF-α (Cloutier, et al., 2013);
- C reactive protein (CRP) production, enhancing the local inflammatory response via activation of the classic complement pathway (Braig, et al., 2017).

The inflammatory role of PMVs has been observed in several pathologies, particularly in chronic inflammation, typically connected to tissue damage, such as in cardiovascular diseases, rheumatoid arthritis, anti-phospholipid antibody syndrome, mellitus diabetes (Beyer & Pisetsky, 2010).

PMVs role in the inflammation is not so easy as appeared at the beginning; indeed, further studies showed PMV anti-inflammatory skills as well. Actually these characteristics have been investigated just in few experimental researches (Sadallah, Eken, Martin, & Schifferli, 2011); (Tang, et al., 2010).

There is, moreover, a strong molecular bond between inflammation and coagulation since the latter one is stimulated by the first one: the inflammation leads to a higher TF's exposure on the outside membrane of the PMVs.

2.1.4.2. PMV and thrombosis

An increase of microvesicles deriving platelets concentration is associated to a higher thrombotic events incidence; for instance, this increment can be observed in patients with Paroxysmal Nocturnal Haemoglobinuria whose cells are particularly sensible to complement mediated lysis (Wiedmer, et al., 1993); (Hugel, et al., 1999) and in patients with heparin induced thrombocytopenia (Hughes, et al., 200).

These microvesicles display on their surface the majority of the proteins and receptors that, before PMV detachment, were organized on the platelet plasmatic membrane. Especially, PMVs present on their surface the receptors like GPIb, GPIIb/IIIa and P-Selectin, Phosphatidylserine (PS), Tissue Factor (TF), negative charged phospholipids and also they show a catalytic surface for the complex of the prothrombinase (**Errore. L'origine riferimento non è stata trovata.**).

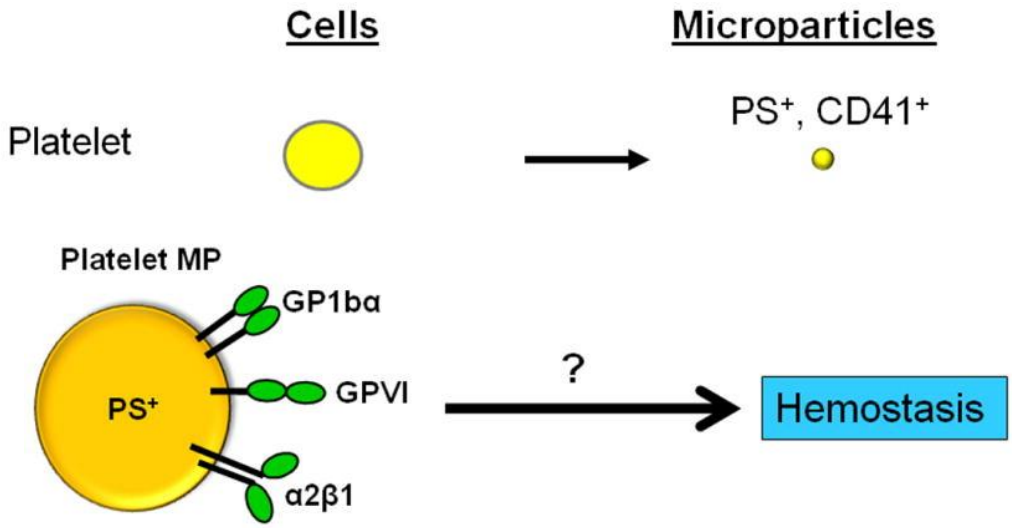


Figure 7: Platelets deriving Microvesicles (PMVs) markers and surface receptors (Owens 3rd & Mackman, 2011).

PMVs expressing both PS and TF have the higher procoagulant activity. Vesicles, particularly, expressing the Tissue Factor, activate the thrombus formation (Biró, et al., 2003)and they have been shown in human atherosclerotic plaques (Suades, Padró, Vilahur, & Badimon, 2012). It is highlighted however that TF, differently to PS, is not only expressed by PMV, but also from microvesicles deriving from endothelium, neutrophils and monocytes (Owens 3rd & Mackman, 2011).

2.1.4.3. Platelets microvesicles analysis

In vivo and *in vitro* studies on platelets derived microvesicles (PMVs) are challenging and not unvarying since there are different methods for isolation, characterization and PMVs quantification. Several techniques offer different parameters analysis; for this reason, a multi-parameter analysis use might be a solution but, in order to be used, it requires an accurate selection and the use of diverse methodologies in combination (Kailashiya, 2018).

Microvesicles study occurs in three steps: harvesting of the specimen and processing, isolation/separation and concentration and last but not least, detection.

To minimize the platelet activation meanwhile the blood is withdrawn it is recommended to use large needles (19-22 Gauge) and to use theophylline-

adenosine-dipyridamole, sodium citrate or acid-citrate-dextrose as anticoagulants instead of heparin and EDTA (Kailashiya, 2018).

The MISEV2018 guidelines (Théry, et al., 2018) defined that EV can be isolated based on their biophysics and biochemical properties. The protocol choice depends on the successive analysis and each isolation or separation technique conditions the EC concentration, presenting advantages and limitations. Techniques used are the differential centrifugation, the chromatography, the multidimensional exclusion (Xu, Greening, Zhu, Takahashi, & Simpson, 2016), the ultracentrifugation and the immuno-captured (Obeid, et al., 2017).

The survey and classification of microvesicles aim to reach significant results and, in order to do that, they use Flow Cytometry techniques. With this method indeed is possible:

- Discriminate individual platelets and PMVs by means of light scattering;
- Measure the binding of factors Va and VIIIa bond to platelets and to microvesicles through the use of monoclonal antibodies marked with fluorochromes (Sims, Faioni, Wiedmer, & Shattil, 1988);
- Measurement of anionic phospholipids amount outwardly orientated using annexin V marked with a fluorophore (Thiagarajan & Tait, 1990);
- Monitoring of the phospholipids movements, like the phosphatidylserine, through the lipid double layer.

There are, nevertheless, other techniques among which the more important are the electronic microscopy, ELISA, Biosensor, Resistive pulse sensing (RPS) and further functional techniques (Kailashiya, 2018).

2.1.5. Platelets' role in the genesis of organ damage

Platelets carry out an important role in the damage tissue restoration: this function is partly conducted via haemostasis mechanism, whose platelets are those cells mainly involved, although, platelets take part to this process also through regeneration and remodelling tissue (Eisinger, Patzelt, & Langer, 2018). When platelets are active exhibit on their surface several membrane receptors in active state and they release a great variety if mediators: these mechanisms allow platelets to interact with several different cellular types and consequentially platelets supply these cells the capacity to take part to functions, such as

inflammation, vascular homeostasis and cellular proliferation. Platelets role has to be evaluated in a wider context esteem to the only haemostatic function.

In response to the damage, platelets accumulation determines the immunity cells recall, either innate or adaptive (Koupenova, Clancy, Corkrey, & Freedman, 2018). An important mediator in this role seems to be P-Selectin expressed on the platelets surface which through the bound with the glycoprotein PSGL-1, expressed in leukocytes, induces a pro-inflammatory signal. This role is necessary and essential in the tissue damage subsequently to infective processes, since it determines the immunity response activation and for the elimination of the causal agent (Figure 8).

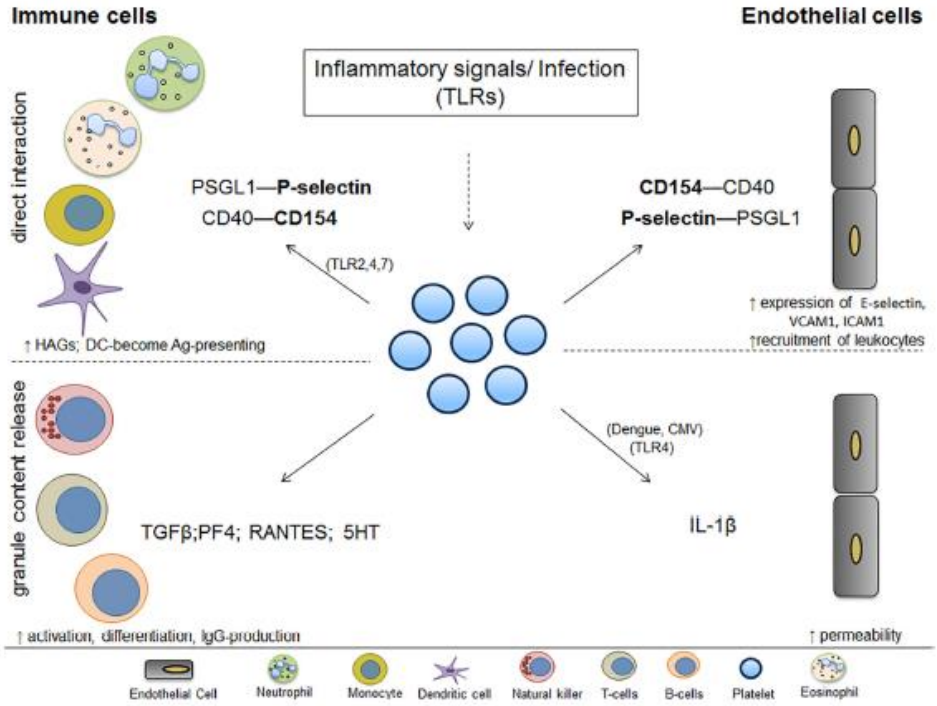


Figure 8: Platelet-mediated interactions with vascular or circulating cells. Platelets interact with endothelial cells and circulating immune cells, establishing the response to microbes, inflammatory stimuli and vascular damage. By means of the TLR, platelets react to inflammatory signals, changing he receptors' surface expression and releasing of substances which allow the interaction with different immune cells. Platelets can activate the innate or adaptive immune response, activate the endothelium making it more permeable and mediating the leukocytes trafficking. (Koupenova, Clancy, Corkrey, & Freedman, 2018).

Different studies demonstrated the role of platelets in the angiogenesis control as result to damage tissue: platelets support angiogenesis at the early stages, encouraging endothelial cells in the development of new vessels, while they are involved in the stabilization and maturation of the vessels themselves at more advanced stages. A central role in this scenario has been addressed to VEGF and bFGF, but the hypothesis concerning which are the real mediators of this process are several. Nevertheless, the angiogenesis control by the platelets appears to perform a central role in the tissue damage reparation.

Less clear is, instead, which might be the relationship between platelets and the apoptosis control; whereas in some instance apoptosis is stimulated, in others it has been clearly seen an anti-apoptotic effect. Are then necessary further studies to define if in this situation the platelets activity promotes the reparation or worsen the injury. However, platelets are responsible of the tissue regeneration, stimulating of cell proliferation as it happens in hepatic or pulmonary damage models.

In the contest of tissue damage reaction, it has been proved the serotonin release from platelets stimulates the production and the deposition of the extracellular matrix and the restoration of connective tissue thanks to fibroblasts. Moreover, the fibroblasts activity could be fostered by anti-apoptotic signals of platelets origin. All these processes synergic act to allow the tissue reshaping. While in acute circumstances these processes are indispensable in the wound healing and in the tissue homeostasis maintaining, at long term, instead, they can be responsible for a tissue damage aggravation. As a matter of fact, an excessive recall and activation of inflammatory cells by platelets, it may contribute to the damage; besides, there are evidences to support that the platelets activation are responsible for the fibrosis insurgence and hypertrophy: this was demonstrated in some studies regarding hepatic damage and myocardial infarction.

Beyond the haemostatic and inflammatory response, the platelets activation induces the platelets microvesicles (PMVs) formation which have a role in the haemostasis and immunity.

2.1.6. Blood Collection

Many drugs are known to interfere with platelet function and therefore it is crucial to ensure that potential blood donors have not ingested such drugs during the previous 14 days (Weiss, 1972). Several antiplatelet drugs such as aspirin (Leist, 1974), other non-steroidal anti-inflammatory drugs, and anti-histamines are available in over the counter preparations and donors often do not volunteer this information unless specifically asked.

The blood should be drawn in a relaxed atmosphere. A busy laboratory is often not the ideal environment, especially for a first time, tense donor. Several investigators advocate that blood be drawn from fasting donors. Severe lipaemia may interfere with the apparent aggregatory response of platelets (Zucker, 1989). However, if plasma-free platelet suspensions are to be used fasting is not necessary.

During the preparation procedure, the platelets should only be manipulated with, and stored in, polyethylene, polycarbonate, or siliconized glass. In the case, the silicone should be applied to ensure complete and irreversible coverage of the glassware, which should then be thoroughly washed before use (Mustard, 1989).

It is preferable to maintain platelets either at room temperature or at 37°C during all manipulations.

In common with all bodily fluids, blood products should be regarded as potentially hazardous. Gloves should be worn when drawing blood and during all manipulative procedures. Blood products should be regarded as biohazardous and handled according to individual institute's regulations.

Blood may be drawn into syringes. The appropriate amount of anticoagulant should be added to the syringe before attachment of the Butterfly-type needle. Butterfly-type, Vacutainer brand blood collection sets (Becton Dickinson) with 21-gauge needle are suitable for this method. Contamination of the sample with thrombin generated during venipuncture may be reduced by discarding the first 2 ml of blood drawn (Mustard, 1989). The blood should be drawn into the syringes in an even manner to avoid vortexing, and thoroughly and continuously mixed with the anticoagulant. To facilitate mixing 20 ml, or larger, syringes should be used. If larger quantities of blood are required, the syringes must be changed and during this

procedure it is important to compress the tubing to prevent loss. Available anticoagulants include liquid EDTA, powdered EDTA, heparin, and sodium citrate. To avoid any form of turbidity, platelets may be obtained by simply allowing the blood to flow freely into an anticoagulant.

2.1.6.1 Anticoagulants

A number of anticoagulants are available. The appropriate choice depends largely on what aspect of platelet function is to be ultimately examined.

Heparin, for example, has been widely used as an anticoagulant. However some heparin preparations cause platelet aggregation (Eika, 1972); (Eika, 1972).

Inhibition of coagulation by chelating divalent cations is the method of choice for most investigators. EDTA has been used but is not ideal as it both affects cell surface adhesive receptors and may deplete intracellular calcium stores. Any one of three citric acid based anticoagulants may be used:

- sodium citrate
- citrate-citric acid-dextrose (CCD)
- acid-citric acid-dextrose (ACD)

  All three are made in distilled water and may be stored at 4°C for several weeks. Suitable ratios of blood to anticoagulant are:

- blood: sodium citrate (9:1, v/v)
- blood: CCD (9:1, v/v)
- blood: ACD (8.1:1.9, v/v)

Platelet-rich plasma (PRP) prepared from blood using either sodium citrate or CCD as the anticoagulant may be used directly in functional studies. In contrast, pH of ACD (approximately PH 4.5) lowers that of the PRP to level which is incompatible with aggregation, and in this case the platelets must be isolated into a plasma-free, washed suspension.

2.1.7. Important notion on platelet isolation and activation

The best way to measure the platelet procoagulant response is to work with washed platelets in order to exclude any contribution of plasma factors other than the prothrombinase components or platelet activators required for the assay. In is of

note, that the isolation procedure may be accompanied by minor platelet activation, affecting the procoagulant activity of the "non-activated" platelets. The best results are obtained when isolation is performed using acidified washing buffers. Each washing step requires careful and gentle resuspension of the platelet pellet and it is recommendable to work quickly, in order to avoid prolonged presence of the platelets in acidified medium. After completion of the isolation procedure, the platelet suspension is allowed to stand 10-15 minutes before starting experiments. One may judge the quality of the platelet preparation by the visual appearance of the Schlieren affect when the suspension is swirled. The presence of fatty acid-free (human) serum albumin in the washing buffers and in the final platelet suspension is essential to maintain a low "basal" prothrombinase activity of platelets. Storage of washed platelets in buffers without albumin will give rise to gradual increase of procoagulant activity in time.

A variety of physiological agonists are known to cause platelets to change shape, express fibrinogen binding sites with subsequent aggregation, and to cause secretion of granule contents. Only a few of these agonists can evoke a significant procoagulant response. Non-physiological compounds such as calcium ionophore and sulfhydryl reagents can also cause a procoagulant response. In all cases, the presence of extracellular Ca^{2+} during activation is essential for expression of procoagulant activity; some agonists also require stirring for maximal stimulation of the procoagulant response. For agonists that cause platelets to aggregate, it is recommended to perform platelet activation and measurement of the prothrombinase activity in the same incubation tube, i.e. to avoid subsampling procedures. For agonists which do not cause platelet aggregation, activation may be carried out at higher platelet concentrations, followed by subsampling to the prothrombinase system.

2.1.8. Use of fluorescent indicators to measure intracellular Ca^{2+} and other ions.

Changes in cytosolic calcium ion concentration ($[Ca^{2+}]i$) play a major role in platelet activation. Elevation of $[Ca^{2+}]i$ alone con stimulate shape change, aggregation, and secretion, although physiologically other second messengers such as diacylglycerol may contribute to these events. As with other non-excitable cells,

platelet agonists stimulate a rinse in $[Ca^{2+}]i$ by promoting the entry of Ca^{2+} into the cytosol from two sources. Ca^{2+} is released from intracellular stores in the dense tubular system, a structure analogous to the endoplasmic reticulum of other cells, and Ca^{2+} enters the platelet across the plasma membrane.

Agonists release Ca^{2+} from internal stores by binding to surface receptors and activating phospholipase C via a GTP binding protein. Phospholipase C cleaves the membrane phospholipid, phosphatidylinositol 4,5-triphosphate. The diffusible messenger, inositol 1,4,5-triphosphate, then binds to receptors on the intracellular Ca^{2+} store, opening a Ca^{2+}-release channel.

The mechanisms by which agonists stimulate Ca^{2+} entry across the plasma membrane are poorly understood and this is an intensive area of investigation in platelets and other non-excitable cells, which are generally agreed to lack voltage-operated Ca^{2+} channels. Three types of so-called receptor-mediated Ca^{2+} entry (RMCE) are envisaged:

a. Receptor-operated channels (ROCs), where agonist-receptor binding is closely coupled to channel opening, either through subunits of the receptor protein, or via a GTP binding protein.
b. Second messenger-operated channels (SMOCs), where agonist-receptor binding results in the formation of a diffusible second messenger, which in turn binds to a receptor on the cytosolic face of the plasma membrane to open channel.
c. Store-regulated Ca^{2+} entry, where depletion of the intracellular Ca^{2+} store is somehow signalled to the plasma membrane to activate Ca^{2+} entry.

In platelets, ADP has been shown to activate a receptor-operated, non-selective cation channel which can conduct a Ca^{2+} into the cells. It is still uncertain whether other agonists can generate Ca^{2+} entry directly via ROCs or SMOCs. Depletion of the platelet intracellular Ca^{2+}store, for example by using inhibitors of the store Ca^{2+}ATPase such as thapsigargin, stimulates Ca^{2+} entry in the absence of changes in other second messengers. Hence store-regulated Ca^{2+} entry appears to occur in platelets and any agonist which discharges stored Ca^{2+} is likely to promote Ca^{2+} influx by this route.

2.1.9. Monitoring platelet aggregation by light transmission

Aggregation can be monitored by measuring light transmission through a stirred platelet suspension (Born, 1968). Following the addition of an agonist, platelet activation proceeds in a multi-step process. Several agonists initially cause the platelets to change shape which decreases light transmission (Figure 9). As aggregation occurs, the platelets form small clumps leading to an increase in light transmission. The aggregation profile can sometimes be divided into two phases:

- Primary aggregation, which is a reversible process.
- Secondary aggregation, which is irreversible and for weak agonists (e.g. ADP, 5-HT) is dependent on granule release and thromboxane production.

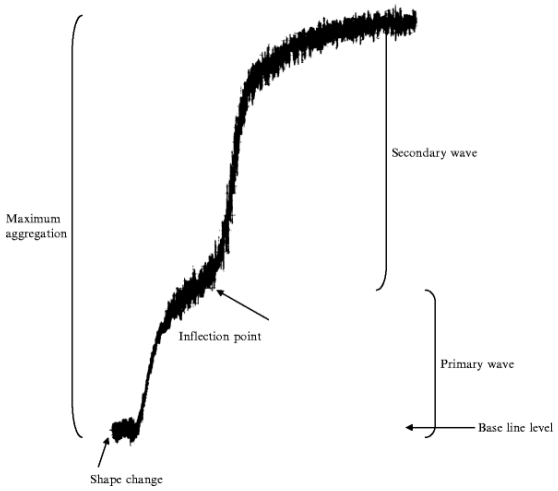


Figure 9: Components of platelet aggregation as monitored by light transmission. A platelet sample is stirred in an aggregometer and light transmission is measured. Following the addition of an agonist the light transmission transiently decreases, consistent with platelet shape change. The subsequent increase in light transmission reflects platelet aggregation (1° aggregation) is due to the effects of the initiating agonist. A small plateau may be observed before 2° aggregation occurs. The 2 aggregation is associated with granule release and thromboxane production. (Frontroth, 2013).*

Conventionally, aggregometers are calibrated so that there is about 10% transmission though the unactivated platelet suspension and about 90% transmission through the blank.

2.1.10. Platelet-leukocyte interactions

Activated platelets are known to interact with leukocytes via a number of glycoprotein receptors (e.g. P-Selectin, CD63, αvβ3). Such an interaction can be characterized by observing the numbers of activated platelets resetting around leucocytes. In rats, platelets and PMNs are obtained by drawing blood via a heart puncture. Platelet-leukocyte interactions can then be tested in the presence or absence of peptides or monoclonal antibodies directed against glycoproteins that are thought to play a role in such cell-cell interactions.

2.1.11. The use of flow cytometry to study platelet activation

Many common clinical conditions have been reported to be associated with platelet hyperreactivity and/or circulating activated platelets, such as coronary artery disease, diabetes mellitus, stroke, deep vein thrombosis, hyperlipoproteinaemia, etc. However, the role of platelet activation in clinical setting is controversial, in part because the methods used to detect platelet activation (e.g. platelet aggregation and radioimmunoassays of plasma β-thromboglobulin (βTG) and/or platelet factor 4 (PF4)) have major methodologic problems (Kinlough-Rathbone, Packham, & Mustard, 1983); (Kaplan & Owen, 1983); (Levine, 1986); (George & Shattil, 1991); (Shattil, Cunningham, & Hoxie, 1987) and (Abrams & Shattil, 1991). Platelet aggregometry may show whether a particular clinical condition results in changes in platelet reactivity, but cannot determine whether the condition directly activates platelets. In contrast, radioimmunoassays of plasma βTG and PF4 concentrations may indirectly determine that a clinical condition activates platelets, but cannot measure changes in platelet reactivity associated with the condition. None of these assays can measure the extent of activation of individual platelets nor can they detect distinct subpopulations of platelets. Platelet aggregation studies are semi-quantitative and subject to standardization problems (Kinlough-Rathbone, Packham, & Mustard, 1983) and (George & Shattil, 1991). As a result of the plasma

separation procedures required, radioimmunoassays of plasma βTG and PF4 concentrations are particularly vulnerable to artefactual *in vitro* platelet activation (Kaplan & Owen, 1983) and (Levine, 1986). Clinical studies that utilize flow cytometric assays of washed platelets (Ejim, Powling, Dandona, Kernoff, & Goodall, 1990) and (Wehmeir, et al., 1991) are also susceptible to artefactual *in vitro* platelet activation as a result of the washing procedures.

A whole blood flow cytometry assay that circumvents many of the problems associated with assay of βTG, PF4, and platelet aggregation, has been described by Shattil, Cunningham and Hoxie in 1987. In this assay (Shattil, Cunningham, & Hoxie, 1987), platelets are directly analysed in their physiological milieu of whole blood (including red cells and white cells, both of which affect platelet activation) (Santos, et al., 1991) and (LaRosa, et al., 1994). The minimal manipulation of the samples limits artefactual *in vitro* activation and potential loss of platelet subpopulations (Shattil, Cunningham, & Hoxie, 1987); (Abrams & Shattil, 1991) and (Michelson, et al., 1991). Both the activation state of circulating platelets and the reactivity of circulating platelets can be determined. The flow cytometric method permits the detection of a spectrum of specific activation-dependent modifications in the platelet membrane surface. A subpopulation of as few as 1% of partially activated platelets can be detected in whole blood by this method (Shattil, Cunningham, & Hoxie, 1987) and (Kestin, et al., 1993). Only minuscule volumes (~2 µl) of whole blood are required (Shattil, Cunningham, & Hoxie, 1987) and (Michelson, et al., 1991), making whole blood flow cytometry particularly advantageous for neonatal studies (Rajasekhar, et al., 1994). The platelets of patients with profound thrombocytopenia can also be accurately analysed.

Addition to whole blood of thrombin, one of the most physiologically important platelet activators (Hansen & Harker, 1988); (Eidt, et al., 1989) and (Kelly, et al., 1991), results in a fibrin clot, thereby precluding the use of thrombin as an agonist in the whole blood assay. Furthermore, thrombin is a potent inducer of platelet to platelet aggregation, which precludes analysis by flow cytometry of activation-dependent changes in individual platelets. Alan D. Michelson has recently described a flow cytometric assay that enables platelet activation by thrombin to be directly measured in whole blood (Michelson, et al., 1991); (Kestin, et al., 1993)

and (Michelson, 1994). In this method, the synthetic tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline (GPRP) is used to inhibit both fibrin polymerization and platelet to platelet aggregation (Michelson, et al., 1991); (Kestin, et al., 1993) and (Michelson, 1994). GPRP corresponds in part to the amino terminal sequence of the α chain of fibrin and is an analogue of the polymerization sites involved in fibrin polymer formation (Laudano & Doolittle, 1978). GPRP therefore competitively inhibits fibrin polymerization (Laudano & Doolittle, 1978). In addition, GPRP inhibits fibrinogen binding to its platelet receptor, thereby partially inhibiting platelet aggregation (Plow & Marguerie, 1982) and (Adelman, Gennings, Strony, & Hanners, 1990). However, GPRP does not block platelet activation (Michelson, et al., 1991). GPRP is stable, resistant to proteolytic agents (including thrombin), and does not suppress thrombin activity (Laudano & Doolittle, 1978).

An alternative to the use of thrombin and GPRP in the whole blood flow cytometric assay is the use of a thrombin receptor agonist peptide (TRAP), a peptide fragment of the "tethered ligand" receptor for thrombin (Vu, Hung, Wheaton, & Coughlin, 1991). TRAP directly activates platelets, without resulting in a fibrin clot. The advantage of TRAP is that it can be used in the absence of GPRP. The disadvantage of TRAP is that it may not reflect all aspects of thrombin-induced platelet activation, because the "tethered ligand" receptor may not be the only platelet receptor for thrombin (Yamamoto, et al., 1991).

The advantages of whole blood cytometry are:

a. Platelet activation can be studied in the more physiological milieu of whole blood.
b. Activation-dependent changes in multiple surface receptors can be detected.
c. High degree of sensitivity for the detection of platelet subpopulations.
d. Both the activation state of circulating platelets and the reactivity of circulating platelets can be determined.
e. Minimal manipulation of samples prevents artefactual *in vitro* platelet activation and potential loss of platelet subpopulations.
f. Only~2 µl of blood is required for the assay.
g. The platelets of patients with profound thrombocytopenia can be accurately analysed.

2.1.12. Monoclonal antibodies

Monoclonal antibodies can be used to measure the expression of any platelet surface antigen. However, there has been particular interest in the use of monoclonal antibodies that are "activation-dependent", i.e. antibodies that bind only not activated platelets not to resting platelets.

The two most widely studied activation-dependent antigens are P-selectin and the GPIIb-IIIa complex. P-selectin, also referred to as GMP-140, PADGEM protein, and CD62P, is a component of α granule membrane of resting platelets that is only expressed on the platelet surface membrane after α granule secretion (Mc Ever, 1990). Therefore a P-selectin-specific IgG monoclonal antibody such as S12 (developed by Dr Rodger P. McEver, University of Oklahoma, Oklahoma City, OK) only binds to degranulated platelets, not to resting platelets (Figure 10 and Figure 11B).

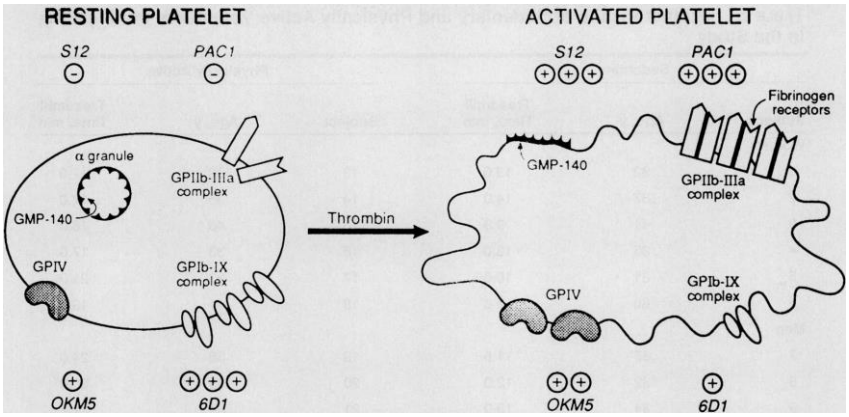


Figure 10: Effect of platelet activation on monoclonal antibody binding. The cartoon depicts the binding of monoclonal antibodies (in italics) to resting platelets and the relative change in the binding of these antibodies after thrombin activation. S12 is directed at the α granule membrane protein P-selectin. P-selectin is not detectable on the surface of resting platelets. After thrombin activation, P-selectin is translocated to the platelet plasma membrane. Thus, S12 only binds to the surface of activated platelets. PAC1 is directed at the fibrinogen binding site on the glycoprotein (GP) IIb-IIIa complex. The fibrinogen binding site is not exposed on resting platelets. Thrombin stimulation results in a conformational change in the GPIIb-IIIa complex that exposes the fibrinogen binding site. Thus, PAC1 only binds to the surface of activated platelets. OKM5 is directed at an epitope on GPIV which may be a thrombospondin binding site. OKM5 binds to resting platelets but binding is increased following thrombin stimulation. 6D1 is directed at the von Willebrand factor binding site on GPIb. In contrast to the other monoclonal antibodies, the binding of 6D1 is markedly reduced following thrombin stimulation. Reproduced from Kestin et.al. (Kestin, et al., 1993).

P-selectin mediates adhesion of activated platelets to neutrophils and monocytes. The GPIIb-IIIa complex is a receptor for fibrinogen, von Willebrand factor, fibronectin, and vitronectin that is critical for platelet aggregation (Phillips, Charo, Parise, & Fitzgerald, 1988). Whereas most monoclonal antibodies directed against the GPIIb-IIIa complex bind to resting platelets, PAC1 is directed against the fibrinogen binding site exposed by a conformational change in the GPIIb-IIIa complex of activated platelets (Shattil, Hoxie, Cunningham, & Brass, 1985). Thus, PAC1 only binds to activated platelets, not to resting platelets (Figure 10 and Figure 11B). While native PAC1 is an IgM, a recombinant Fab fragment of PAC1 produced in a baculovirus expression system also binds to platelets in an activation-

dependent manner (Abrams, Deng, Steiner, O'Toole, & Shattil, 1994).

In addition to monoclonal antibodies that bind only to activated, not resting, platelets, some investigators have used monoclonal antibodies that bind to resting platelets but have increased binding to activated platelets, e.g. GPIV-specific monoclonal antibodies (Figure 10) (Kestin, et al., 1993).

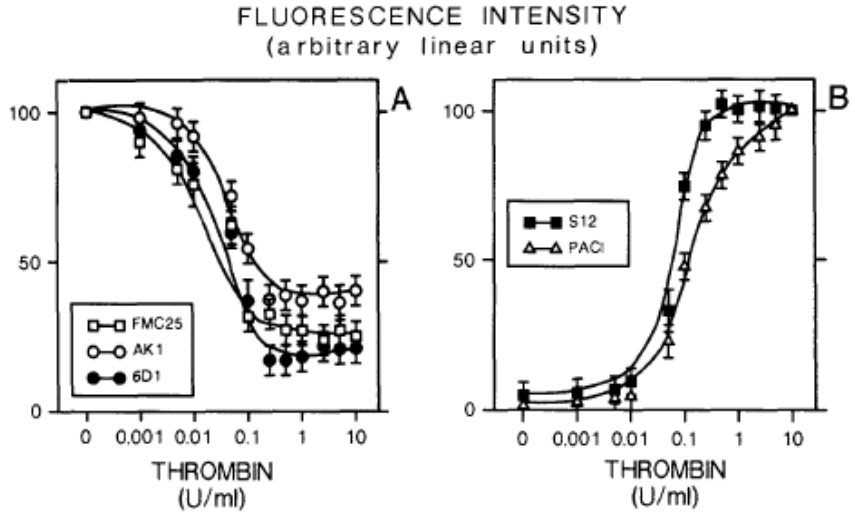


Figure 11: Effect of thrombin on the binding of monoclonal antibodies to the platelet surface, as determined by whole blood flow cytometry. (A) Monoclonal antibodies 6D1, FMC25, and AK1 are directed agonist GPIb, GPIX, and the GPIb-IX complex, respectively. For each antibody in panel A, the fluorescent intensity of resting platelets was assigned 100 units. (B) Monoclonal antibodies S12 and PAC1 are directed agonist P-selectin and the GPIIb-IIIa complex, respectively. For each antibody in panel B, the fluorescence intensity of maximally activated platelets was assigned 100 units. Data from panel A and B were obtained from the same three experiments (mean ± S.E.M.). Reproduced from Michelson et al. (Michelson, et al., 1991).

In contrast to the "activation-dependent" monoclonal antibodies and the monoclonal antibodies that bind resting platelets but have increased binding to activated platelets, the binding of GPIb-IX-specific monoclonal antibodies to activated platelets is markedly decreased compared to resting platelets (Figure 10 and Figure 11A) (Michelson, et al., 1991); (Michelson & Barnard, 1987) and (Michelson, 1992). The activation-induced decrease in the platelet surface expression of the GPIb-IX complex appears to be the result of a translocation of GPIb-IX complexes to the membranes of the open surface canalicular system

(Hourdille, et al., 1990). The GPIb-IX complex is a receptor for von Willebrand factor that is critical for platelet adhesion to damaged blood vessel walls (Ruggeri, 1991). The activation-dependent decrease in the binding of monoclonal antibodies to the platelet GPIb-IX complex may be a very sensitive marker of platelet activation *in vivo*. For example, using a whole blood flow cytometric assay, we recently demonstrated that strenuous exercise in sedentary subjects, but not physically active subjects, resulted in both platelet activation and platelet hyperactivity (Kestin, et al., 1993). However, these changes were more readily detected with monoclonal antibodies directed against GPIb (CD61, developed by Dr Barry S. Coller, Mount Sinai Medical Center, New York, NY) and, to a lesser extent, GPIV (OKM5, Ortho Diagnostic Systems) rather than with monoclonal antibodies directed against the GPIIb-IIIa complex and P-selectin (Kestin, et al., 1993). This study (Kestin, et al., 1993) also illustrates the importance of analysing activation-dependent alterations by use of a panel of monoclonal antibodies directed against different platelet surface antigens.

Platelet-specific monoclonal antibodies can often be purchased conjugated to either biotin or fluorescent isothiocyanate (FITC). Alternatively, antibodies can be FITC-conjugated by the method of Rinderknecht (Rinderknecht, 1962) or by a kit method (e.g. QuickTag FITC conjugation kit, Boehringer Mannheim). Antibodies can be biotinylated as described by Shattil et al. (Shattil, Cunningham, & Hoxie, 1987) or by following the biotin manufacturer's directions.

The saturating concentration of each antibody for platelet binding is typically between 0.5 and 40 µg/ml. In addition, when two monoclonal antibodies are used in the same assay, it is necessary to determine that they do not interfere with each other for platelet binding.

2.1.13. Flow cytometry analysis

The fluorescence of FITC and phycoerythrin are detected using band pass filter (e.g. 525 nm for FITC and 575 nm for phycoerythrin in a Coulter EPICS Profile Cytometry).

Platelets are identified in the diluted whole blood samples by:

- Setting the discriminator (Coulter) or threshold (Becton Dickinson) on the identifying FITC-conjugated monoclonal antibody
- Setting a gate on the platelet light scatter region

The binding of the biotinylated activation-sensitive antibody is discriminated by analysing 5000-10 000 individual platelets for phycoerythrin fluorescence.

Platelets can be identified in whole blood by light scatter only. However, under certain experiment conditions, some of the particles falling within the light scatter gate for platelets may not bind any platelet-specific monoclonal antibody.

2.1.14. Methodological issues – Expression of antibody binding

Antibody binding should not be reported as mean channel number, because this is a log scale. Antibody binding can be expressed as mean particle fluorescence intensity (in linear units) or as the per cent of particles staining positive for a particular antibody.

It is very important to realize that “antibody positive” platelets may have very little antigen expressed on their surface.

Mean fluorescence intensity is therefore the preferred method of data presentation if the goal is to determine the total amount of platelet surface antigen. For activation-dependent antibodies, inclusion of a control of platelets maximally activated by thrombin, TRAP, or phorbol myristate acetate, assists in the quantification of the amount of surface antigen per platelet.

2.1.15. Number of antibody binding sites

Although flow cytometry does not result in a measure of the absolute number of binding site, Shattil et al. (Shattil, Cunningham, & Hoxie, 1987) and Johnston et al. (Johnston, Pickett, McEver, & George, 1987) used monoclonal antibodies double labelled with 125I and biotin to demonstrate a direct linear relationship between the number of antibody binding sites per platelet as determined by 125I-labelled and

(after incubation with phycoerythrin-streptavidin) fluorescent labelled antibody. Once this relationship is known for a given monoclonal antibody, it is possible to use subsequent batches of the biotinylated or FITC-conjugated antibody for binding site quantification, provided that the molar ratio of fluorescein to antibody is known. The lower limit of detection of antibody binding by flow cytometry is approximately 500 antibody molecules per platelet.

2.1.16. Flow cytometry of washed platelets

The ability of a reagent to directly affect platelets in the absence of plasma, white cells, and red cells can be evaluated by flow cytometric analysis of washed platelets. Washed or gel filtered platelets are processed and analysed in a similar way to whole blood samples. The platelets can be identified solely on the basis of their characteristic forward and orthogonal light scattering profile. However, as for whole blood assays, depending on the experimental conditions, a proportion of the particles falling within the light scatter gate for platelets may not bind a platelet-specific monoclonal antibody. Each investigator should therefore confirm that a fluorescent labelled monoclonal antibody as a "platelet identifier" is necessary for the specific experimental conditions.

Methods of sample preparation for the minimization of platelet aggregate formation, should use a platelets dilution to < 50 000/µl prior to activation.

2.1.17. mAb which recognize platelet activation antigens

Platelet activation by physiological agonists gives rise to degranulation and the incorporation of both granule and lysosomal membranes into the cell surface membrane. Integral proteins of granule and lysosomal membranes thus become exposed at the cell surface and may be used as markers of activation. mAb to these proteins may be used to monitor the activation process.

An example of mAb which recognize platelet activation antigens is CD62 antigen, otherwise known as P-selectin, GMP 140, or PADGEM. The CD62 antigen is an integral membrane protein of 140 kDa found in the α granules of resting platelets and in the Weibel-Palade bodies of endothelial cell (Mc Ever, 1991). It is a member of the selectin family of adhesion molecules and recognizes carbohydrate ligands

related to sialyl Lex. Activation of platelets with either strong agonists, such as thrombin, or week ones, such as ADP, causes a rapid translocation of CD62 to the cell surface where it mediates adhesion, principally to neutrophils. Anti-CD62 mAb have been used to monitor platelet activation and some, but not all, are able to inhibit platelet adhesion to neutrophils.

2.1.18. New prospective: Microfluidic

In vitro platelet activation study results to be wanting since some processes befalling *in vivo* during haemostasis and thrombosis, are not reproducible. The statistic of the generally used models, as matter of fact, does not allow to evaluate the blood flow effect and consequently the events like adhesion, aggregation and von Willebrand Factor activity depending on the shear stress and on elements like coagulation, fibrin formation and the fibrinolysis subjected to shear stress cannot be properly studied. Furthermore, the endothelium absence does not allow to evaluate the important role of promotion and inhibition of the clot formation that the endothelium itself has in the haemostatic process.

The introduction of tests based on models reproducing flow conditions has tries to solve these physiological flow condition in order to evaluate the interaction between blood cells and vessels. More than forty years ago already have been introduced methodologies characterized by big flow chamber such as cone-and-plate viscometer and parallel-plate flow chamber assays (Zhang & Neelamegham, 2017). Microfluidic techniques take place within the context of these methodises aiming to recreate controlled blood flows through polydimethysiloxane chip built in lithography on which are recreated blood vessel models. These systems consist of reactive surfaces (collagen, collagen / kaolin or collagen / tissue factor (TF)), flow transport regimes and optical imaging systems (Zhu, et al., 2015) (Figure 12).

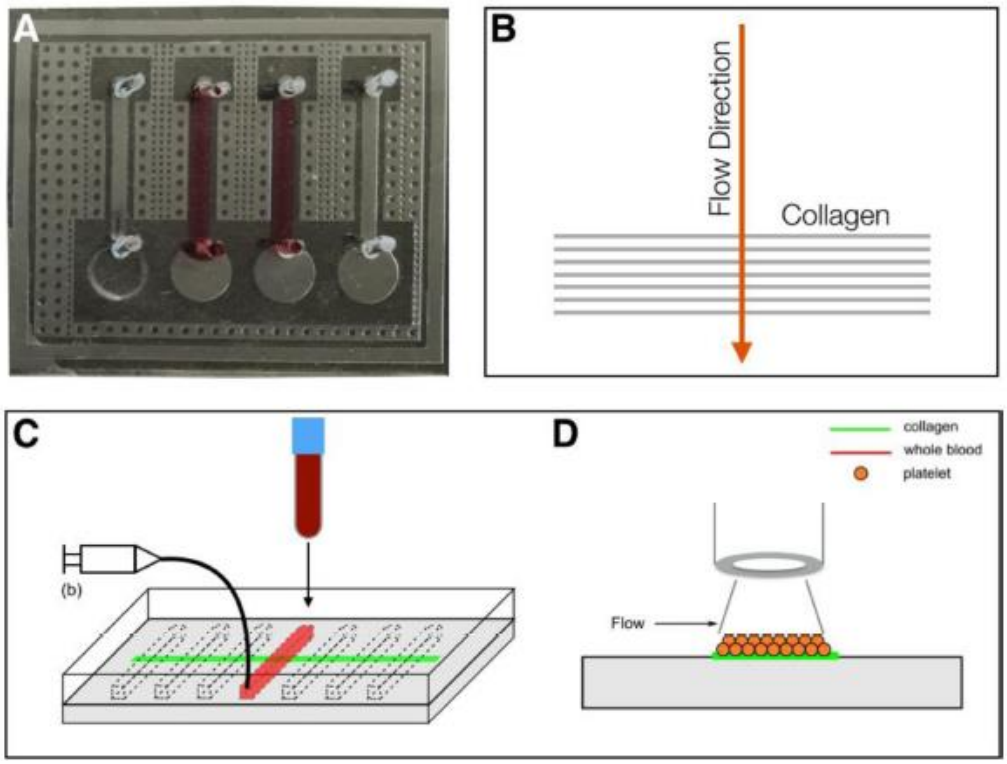


Figure 12: Representative images of a microfluidic device.

a. *Representative image of a PDMS device in Microfluidic with four channels, two of them filled with a red dye;*
b. *Schematic example explaining the disposition of the collagen with perpendicular flow;*
c. *The sample is perfused in the channels through a syringe allowing to create a desired shear stress value;*

Transversal section of a channel where it can be evaluated the platelets deposition by means of microscopy (Branchford, Ng, Neeves, & Di Paola, 2015).

Microfluidics techniques solve some of the limitations of high flow chambers methodises, using channels reproducing *in vitro* the vessel structure, using small volumes of blood (less than 50µL) and shear stress wider variations.

Microfluidic is a highly dynamic technique that allows to recreate different situation occurring *in vivo*; for instance, it can be simulated pattern of vascular injury or it can be studied flow variations by pro or anti-haemostatics factors.

Microfluidic studies can generate information concerning the platelet activation modulation and the subsequent coagulation cascade that leads to the fibrin deposition and to the clot formation; furthermore, they can be used to identify

characteristics of haemostatic and thrombotic pathologies and they can help to determine the effects on patient of specific therapies amending the coagulative processes (Branchford, Ng, Neeves, & Di Paola, 2015).

The limits of this method are due to materials costs to create Microfluidic structures, mechanisms of image capture and analysis apparatus. Moreover, are problematic the systems complexity and the currently lack of methods standardization.

Developments in Microfluidic studies concern the employment of endothelial vasculature and models of vascular stenosis. The aim would be to use this kind of technique in clinical and therapeutic field.

2.2. Topics of the research

2.2.1. Introduction on PFAS: PFOA and C6O4

Cheryl A. Moody and Jennifer A. Field published in August 2000 and Erik Kissa described in his book "Fluorinated Surfactants and Repellents" what Perfluoro-alkyl substances (PFAS) are, which are and their chemical-physical stability and surfactant properties as well as their environmental implication. PFAS are synthetic compounds that have been widely used in products such as fire-extinguisher foams, soil-extraction additives, house-hold detergents, films, waterproof clothing, and coatings for cookware (Kissa, 2001); (Moody & Field, 2000). Due to their properties, PFAS overtime acquired a high environmental persistence and during the last twenty years a several studies showed a consistent accumulation of these compounds either in the environment and in the biota (Giesy & Kannan, 2001); (Lanza, et al., 2017); (Andrady, 2017); (Navarro, et al., 2017). Perfluoro-octanesulfonic acid (PFOS) and perfluoro-octanoic acid (PFOA), two long-chain legacy PFAS, have been associated with potential health issues following an environmental exposure leading to a bioaccumulation in humans has. Epidemiological studies showed an increased incidence of reproductive disorders, metabolic derangements and cardiovascular mortality in those populations living where plants of PFOS and PFOA are produced (Geiger, et al., 2014); (Liu, Wen, Chu, & Lin, 2018); (Shankar, Xiao, & Ducatman, 2012); (Huang, et al., 2018); (Di Nisio, et al., 2019); (Di Nisio, et al., 2020); (Pitter, et al., 2020). Lin and his research group described in two publications, in 2013 and in 2016, the possible pathogenic link between exposure to legacy PFAS and cardiovascular risk and its plausible involvement in the atherosclerosis process (Lin, et al., 2013); (Lin, et al., 2016). Recently, in this current year, it was shown that PFOA accumulates quite easily on preferential site on the platelets membrane, inducing an defaced downstream signalling of platelet's activation and subsequent aggregation (De Toni, et al., 2020).

Due to the potential effects of legacy PFAS on human health, the production of PFOS and PFOA has been ceased by primary manufacturers. To bypass this issue a new compound, the fluorinated compound acetic acid, 2,2-difluoro-2-((2,2,4,5-tetrafluoro-5(trifluoromethoxy)-1,3-dioxolan-4-yl)oxy)-, ammonium salt (1:1),

known as C6O4, has been proposed as a suitable substitute of legacy PFAS and subsequently in 2012,approved by the European Chemical Agency (Lohmann, et al., 2020), since its lower environmental and tissue persistence due to the novel cycle di-ether structure was highlighted. Therefore, the possible effects of C6O4 on platelet's activation profile investigation is a relevant topic to examine. With this purpose, human platelets from healthy donors were incubated with C6O4 at different concentrations and the consequences on the activation, production and phenotype of platelets micro-particles and aggregation under-flow were evaluated. It has been also explored the possible preventive use of acetyl-salicylic acid (ASA) as plausible therapy against the platelet pro-aggregation profile associated with C6O4.

2.2.2. Introduction to platelet K channel

Along the cell plasmatic membrane there are several channel types. Among them, a specific group of transmembrane proteins playing important roles in the regulation of cell functions and amending the ionic cell permeability, is known as Ion channel. Generally speaking, endogenous ion flows control proliferation, differentiation and migration of numerous cell types and ionic currents and channels play essential roles during myoblast, cardiomyocyte and neural stem cell differentiation (Biagiotti, et al., 2006); (Konig, et al., 2004); (van Kempen, et al., 2003). Focusing the attention on both megakaryocytes and platelets, the ions flow regulation was shown to influence platelet production and function. Nevertheless, a whole characterization of ion channel expression and function is still not available in the human megakaryocyte-platelet lineage. Therefore, there is the need to clarify how these electrical signals act as functional biophysical control mechanisms in stem cell biology (Ge, et al., 2014).

To corroborate the ion channel relevant roles and further understand the electrical signal regulation in biophysical mechanisms, some specific analysis was carried out in platelets to better describe and characterize these transmembrane proteins, valuating the platelets response to the ion channel's openers or to the blockers. The ion channel that was better studied is a large-conductance calcium and voltage-activated potassium channel, called in according to the nomenclature $K_{Ca}1.1$ (also known as Maxi-K, BK, slo1).

The $K_{Ca}1.1$ channel is a calcium (Ca^{2+}) activated, voltage-operated potassium (K^{+}) channel; although it is widespread distributed in human cells its function in megakaryocytes and platelets is still unknown.

In 2012 Singh, Stefani and Toro showed that, in other tissues, $K_{Ca}1.1$ channels is involved in cell excitability, smooth muscle contractility, Ca^{2+} homeostasis, and cancer cell proliferation. Moreover, blocking $K_{Ca}1.1$ channel function or expression, a large Ca^{2+} transient flux is induced and it bears to an increase of the β1 integrin expression and function, causing the modulation of the cell-matrix interaction (Tanner, Pennington, Laragione, Gulko, & Beeton, 2017); (Hu, et al., 2012). Whenever the combination of intracellular Ca^{2+} concentration changes and the alteration of the membrane potential occurs, the $K_{Ca}1.1$ channel may activate an important negative feedback system, generating a liaison between increases in

intracellular Ca^{2+} concentration to outward hyperpolarizing K^+ current. A plausible consequence of this hyperpolarisation of cell membranes is the cell excitability decrease, occurring for instance during the smooth muscle relaxation. (Bentzen, Olesen, Rønn, & Grunnet, 2014). Thanks of their structures composition, the activity of K^+ channels can be modulated by means of selective channel blockers and openers, making them potential therapeutic targets. $K_{Ca}1.1$ channels are both Ca^{2+} and voltage sensitive, and several studies showed their propensity to interact with seven transmembrane G protein-coupled receptors, such as β-adrenergic receptors and thromboxane (TX) A2 receptors, two major players in platelet activity (Ge, et al., 2014); (Singh, Stefani, & Toro, 2012); (Toro, et al., 2014). In agonist-stimulated platelets, K^+ transmembrane flux increases, whereas it is petty under resting conditions (Mahaut-Smith, Rink, Collins, & Sage, 1990); (Mahaut-Smith, 1995). Channels and intermediate/small conductance K^+ channels and intermediate/small conductance Ca^{2+}-activated K^+ channels turned up to be opened in platelets stimulated with agonists, leading to a platelet response strengthening (Shankar, et al., 2004); (Shankar, et al., 2006); (Wolfs, et al., 2006). However, up to now, there are no information on $K_{Ca}1.1$.

In this work, was investigated the $K_{Ca}1.1$ channels' contribution to functional responses post modulation with soluble agonists and the behaviour underflow of adhesion-induced platelet aggregation and examined the role of $K_{Ca}1.1$ in platelet formation. The outcomes displayed consistent evidence of $K_{Ca}1.1$ openers' modulatory role, in platelet formation and function.

2.2.3. NAFLD Introduction

NAFLD (Non-Alcoholic Fatty Liver Disease) is the chronic hepatopathy most common in adults. Commonly associated to insulin resistance and metabolic syndrome.

It is defined as the liver fat accumulation (usually higher than 5% of the organ weight) in secondary causes absence like for example the increase of the fat intake, chronic hepatic pathology and alcohol abuse.

Even though the majority of patients aren't in risk of death due to this disease, they display higher morbidity and mortality risk.

NAFLD spectrum generally goes from steatosis to Non-Alcoholic Steato-Hepatitis (NASH), to develop into fibrosis and finally to cirrhosis with its complications, such as the ascitic decompensation and the Hepatocellular Carcinoma (HCC).

A Dutch study developed between 2006 and 2013, focused its attention on 50.704 occidental Europe patients' cohort, with an age between 18 and 91 years old, with the goal to evaluate in an exhaustive manner the NFALD prevalence. The suspicion of NAFLD was observed in the 22% (8.259 participants) of the studied cohort. Previous European studies investigating the NAFLD prevalence in the general population reported results between 17.9% and 29.9% (van den Berg, et al., 2017). Among them, 15-20% of the patients are NASH and generally speaking they represent the 3-4 % of the population.

2.2.3.1. NAFLD pathophysiology

The liver central role in the carbohydrate and lipids homeostasis control together with other tissues, makes the organ vulnerable to body's metabolic alterations.

The Non-Alcoholic Hepatic Steatosis is the hepatic expression of the metabolic syndrome.

Several studies linked a higher risk of chronic hepatic disease with obesity, particularly visceral adiposity. Visceral fat excess is associated to lipid metabolism alterations, chronic sub clinic phlogosis and oxidative stress which are commonly recognized as the main aspects of the physiopathology of the metabolic syndrome.

Main changes in the lipid and carbohydrate metabolism at the hepatic level in the metabolic syndrome are predominantly related to insulin pathway alterations (Tilg

& Moschen, 2008): the metabolic tissues inability to react to insulin stimulus induces an increase in circulating lipids levels resulting in liver triglycerides retention producing steatosis. If this mechanism is maintained might develop towards to an inflammatory condition (NASH), necroinflammation, loss of function scarring (cirrhosis) and potential hepatocellular carcinoma.

The pathophysiological theory mostly accepted is the "Two Hints" theory: the insulin-resistance and the lipid accumulation in the liver are taken as the first "hint" and they are the pivotal pathogenetic factor in the hepatic steatosis development, while the second "hint" is given by the hepatocytes damage, phlogosis and fibrosis. Other factors implicating the second "hint" activation, are the oxidative stress and the subsequent lipids peroxidation, pro-inflammatory cytokines, adipokines and mitochondrial dysfunction (Day & James, 1998). The outcomes are the cellular degeneration, necrosis, apoptosis, stellate cells activation and fibrogenesis (Figure 13: Working hypothesis on the possible mechanisms of liver damage in HCV or metabolic syndrome patients).

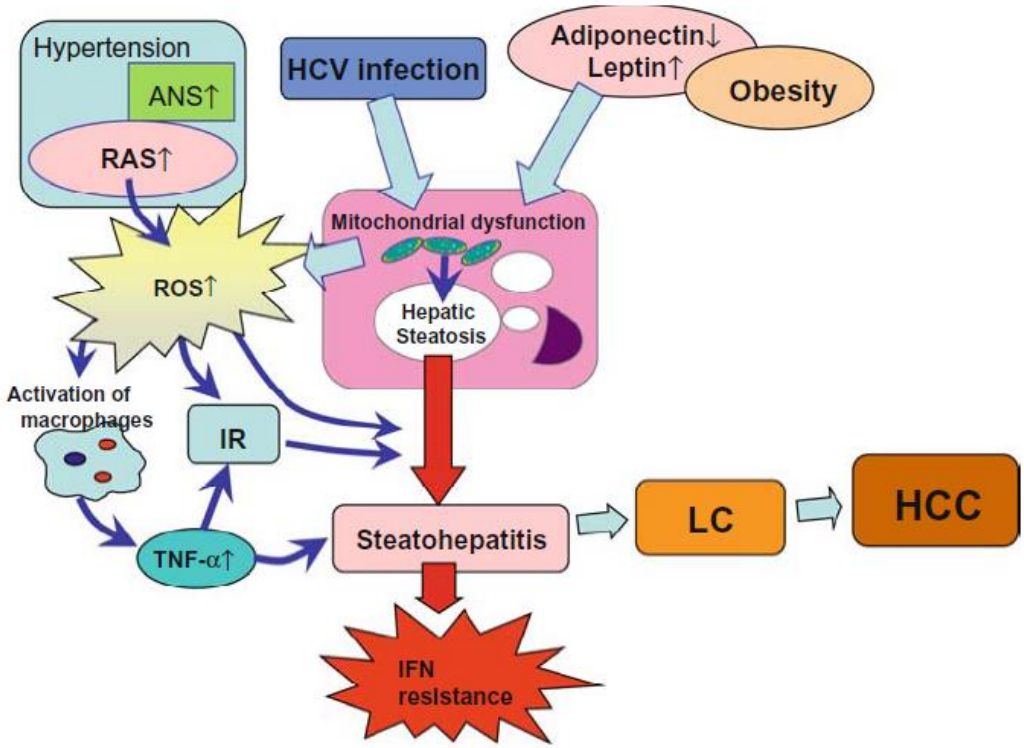


Figure 13: Working hypothesis on the possible mechanisms of liver damage in HCV or metabolic syndrome patients (Watanabe, Yaginuma, Ikejima, & Miyazaki, 2008)

The association of inflammation markers and the NAFLD occurrence has been reported in several studies (Foroughi, et al., 2016). This relationship might be described by means of the visceral adipose tissue growth which induces a pro-inflammatory status. Furthermore, the hepatic fatty acids oxidation gives rise to oxygen radicals with consequently lipid peroxidation, cytokine induction and mitochondrial dysfunction which promotes inflammation and it causes the hepatocyte apoptosis and cellular damage (van den Berg, et al., 2017).

In the pathogenesis of hepatic damage, the hypertension role is more marginal: NAFLD has a 50% prevalence in hypertensive individuals; the relationship between the two factors is, though, complex, since NAFLD is an important risk factor for hypertension, while hypertension itself is an independent predictive factor of NAFLD. The pathophysiologic mechanisms responsible for this correlation seems to be the induction of insulin-resistance through the renal sodium retention, the activation of the sympathetic nervous system, the RAA system activation and the hepatic fibroblasts stimulation from the angiotensin II (Oikonomou , et al., 2018).

2.2.3.2. Platelets in NAFLD

Increasing evidences shown platelets play an active role in hepatic diseases; it has been also demonstrated that activated platelets contribute in the hepatic damage mediated by cytotoxic T lymphocytes in a viral hepatitis model (Iannacone, et al., 2005).

The platelets role in NAFLD's parenchymal damage has not yet clearly understood, but the relationship between platelets and NAFLD is quite undeniable: NAFLD patients have a significant increase of PCT, PDW and MPV (Alempijevic, et al., 2017), whereas plasmatic values of platelets are normal (Malehmir, et al., 2019) o reduced (Alempijevic, et al., 2017). It has to be taken into consideration a high value of MPV suggests a greater enzymatic and metabolic activity, while an increase PDW value shows a higher reticulate platelets production and a platelets activity gain. In addition, NAFLD patients have a significant platelet increment in the liver compared to healthy controls (Malehmir, et al., 2019).

Malehmir et al. demonstrated Kupffer cells have a pivotal role in the platelets recruitment in the NAFLD's premature and advanced stages, in the borderline and advanced NASH. In this process has not been found any evidence of the GPIIb/IIIa role, while they seem to be important in the onset NASH and CD44 borderline that binds hyaluronic acid and GPIbα in tardive NASH. GPIbα represents the first step in the platelets adhesion and platelets activation processes, encouraging the binding of the von Willebrand Factor (vWF) at the damaged endothelium level even under high shear stress conditions. This suggests there are alteration of platelets adhesion and activation, while the aggregation does not play any main role in this process.

It has been reached thus to the conclusion that GPIbα is required for the NASH induction. Platelets instead are essential for NASH development. Sure enough, in the response to the adhesion and to the cellular activation, platelets release bioactive factors from the intracellular α-granules that are essential for the recall of inflammatory cells and for the tissue damage progress.

Normally the platelets activation and degranulation are the important components of the physiological response to the tissue damage which determines the closure of the wound and reparation but, during a chronic inflammation, a fast platelets activation might lead to an irregular fibrotic response.

In this perspective, platelets activity could be considered a therapeutic target to prevent the progression in NAFLD parenchymal damage. Together with the aspirin's pleiotropic effects, its antiplatelet activity has been recognised useful in this pathology and it has been suggested a regular assumption of aspirin is associated to a reduction in NAFLD prevalence in male patients and older (Shen, Shahzad, Jawairia, Bostick, & Mustacchia, 2014).

Later on, Z. Gordon Jiang et al. demonstrated the daily consumption of aspirin at low dosage for a month lead to a reduction of the hepatic fibrosis progression in patient with probable viral hepatitis, NASH and alcoholic hepatopathy (Jiang, et al., 2016).

Recently Malehmir et al. verified the use of aspirin reduces the cytokines' hepatic expression and the interactions platelets-hepatic endothelium and those between platelets and immune cells, represent a possible therapeutic approach for patients with NASH and also to prevent or to revert the transaction from NASH to HCC.

The using of others drugs instead of aspirin led to different results: while the Ibuprofen (Shen, Shahzad, Jawairia, Bostick, & Mustacchia, 2014) or Sulidinac (Malehmir, et al., 2019) use appear to be not only non-therapeutic, but actually they seem to be related to a higher NAFLD and fibrosis incidence, Ticagrelor (Malehmir, et al., 2019) showed results comparable with those obtained with aspirin. Furthermore, antibodies specific against GPIbα represent a potential new treatment.

2.2.3.3. Platelets in renal damage

As it was shown in the hepatic damage, platelets appear to play a role in the renal damage. Particularly, platelets were found in the glomerular structures in cases of glomerulonephritis (Cameron, 1983). In these circumstances, platelets are pro-inflammatory and pro-thrombotic status mediators, which emphasise the renal damage; in diabetes mellitus patients it looks like these mechanisms are involved in the progression and acceleration of the nephropathy. Studies on this topic are still few.

In the diabetes nephropathy platelets appear to be linked to the amplification of microvascular damage already present for other causes. In this scenario, platelets are activated and their secretor products are released in the glomerular structure; moreover, platelets release cytokines and growth factors that can straight influence the proliferation of glomerular mesangial cells, cell migration and extracellular matrix's proteins synthesis.

Proof of platelets' central role in the renal damage, was demonstrated the anti-platelets drugs' therapy leads to a reduction of renal proteins' excretion and a mild deterioration of the glomerular filtration (Donadio Jr., Ilstrup, Holley, & Romero, 1988). The use of anti-platelets drugs has not yet been codified.

Different researches demonstrated also the positive correlation between high PMV and PDW values and diabetic nephropathy compared to diabetic patients without nephropathy, showing a platelet hyperactivity scenario (Liu, et al., 2018). The right association between MPV and PDW and diabetic nephropathy has to be further investigated.

3. Material and Methods

3.1. Blood withdraw

The blood withdraw from the patient's arm is performed paying attention to specific expedients in order to limit as much as possible platelet activation due to swirling motions and to shear stress. In this respect, it is used a Safety-Multifly®-Needle SARSTEDT 0.9x19mm (

Figure 14) which is connectable with a bayonet system to two syringe Vacutainers SARSTEDT Monovette® in succession.

Figure 14: Safety-Multifly®-Needle SARSTEDT 0.9x19mm. (Own photo).

The first syringe Vacutainer has a final volume of 4.5mL which will be considered as waste, since following the trauma due to the needle insertion, it will collect the first blood mL carrying those platelets more suitable to activation. Subsequently, the second syringe Vacutainer, this time with a final volume of 9mL, is connected to the needle and it will gather the blood that will be used for the experiments.
A further expedient is to take out the blood pulling the syringe Vacutainer's piston as slow as possible, always to limit every kind of stress to platelets.
Both the syringes Vacutainer SARSTEDT Monovette® upon the withdraw, must contain an anticoagulant solution of Sodium Citrate 3.2% equal to 10% of the total capacity of the syringe Vacutainer. Furthermore, it is important to remove the haemostatic tourniquet once the needle has been inserted in the patient's arm and before to take out the blood.
After the withdraw, the Vacutainers must be inverted 3-4 times to let the anticoagulant interact with the withdrawn blood. Finally, the collected blood must rest for 30 minutes at Room Temperature (RT), which is an additional precaution to minimize the platelets' unwanted activation.

3.1.1. Preparation of platelet suspension. Platelet-rich-plasma (PRP)

In many experiments, citrated plasma is not an ideal medium in which to study platelet function due to factors such as plasma protein content, calcium chelation, and platelet agglutination by the action of Thrombin. Under such circumstances plasma-free platelet preparation are preferred.
The preparation of PRP from whole blood is achieved by centrifugation. Reported centrifugation conditions vary widely, although 800g for 5 min and 200g for 20 min at room temperature give reproducibly good separation (Giacomazzi, Degan, Calabria, Meneguzzi, & Minuz, 2016). The PRP should be aspirated using a plastic pipette and transferred to a fresh plastic tube. Care should be taken not to disturb either the buffy coat or red cells to prevent contamination of the platelet preparation. Platelet counts in PRP are normally 200'000 to 500'000 platelets/µl.
Human whole blood was donated by healthy volunteers in accordance with the Ethics Committee for clinical research of the Verona and Rovigo provinces, and the principles of the Declaration of Helsinki.

In the experiments conducted for this work, the procedure proposed was as describe below.

After the resting time 500µL of blood are used to perform the haemachrome, in order to have a general healthy condition of the patient and in particular on the platelets state.

The remaining blood (~ 8,5 mL) is centrifuged at 180g for 15 minutes at RT, setting the centrifuge at the maximum speed (level 5 out of 5) and with minimum deceleration (level 1 out of 5), to have two phases in the 9mL syringe Vacutainer SARSTEDT Monovette®.

The yellowish supernatant corresponds to the Platelets Rich Plasma (PRP) of our interest; the reddish bottom layer contains instead Red Blood Cells (RBCs) and White Blood Cells (WBCs), both of them physiologically bigger and heavier than platelets, that are discarded.

The obtained PRP is transferred to a 4 mL tube, paying attention in the pipetting step to proceed as careful as possible and leaving about half centimetre of PRP from the interphase inside the Vacutainer to avoid to pick up RBCs and WBCs residues. From the obtained PRP, further 500 µL are used to perform another haemachrome study to verify the isolated PRP pureness and to know the platelet count.

3.1.2. Platelets Poor Plasma (PPP) production

Half of the isolated PRP is additionally centrifuged at 1600g for 20 minutes at RT with maximum acceleration (level 5 out of 5) and with minimum deceleration (level 1 out of 5), to pelletize the platelets in suspension and to obtain a supernatant made of Platelets Poor Plasma (PPP). This latter is used to dilute PRP to obtain a final platelet concentration of about $100x10^3$ platelets per mL, which is the platelet concentration required to perform Microfluidic experiments and for the isolation of the Platelets derived Microvesicles. Adjusting platelet concentration to $100x10^3$ platelets per mL allows to standardize the experimental conditions and make and reduce inter-assay variability reproducible. PPP was used to regulate the final platelet concentration avoiding changes in the viscosity of the samples, according to the Newtonian fluids formula (Equation 1):

$$\tau = \eta \frac{\partial v}{\partial z}$$

Equation 1

where τ is the fluidic shear stress, η is the viscosity (g/cm-s = Poise) and $\frac{\partial v}{\partial z}$ is the velocity gradient or shear rate (s-1),

Platelet poor plasma (PPP), was also used in the aggregation test as blank.

3.1.3. Washed Platelet production

As already given advanced notice in paragraph "3.1.1. Preparation of platelet suspension. Platelet-rich-plasma (PRP)", sodium citrate might not be the right anticoagulant to choose for this purpose. In the preparation of washed platelet *in vitro*, acid/citrate/dextrose mixture (ACD anticoagulant, sodium citrate 14mM/citric acid 11.8mM/dextrose 18mM) is preferred since it better preserves the structural and physiological properties of platelets also after two or more hours post blood collection (Macey, et al., 2002). Depending on the experiment requirement and condition, aspirin (ASA) 100µM (to inhibit platelet COX-1 activity) and apyrase 0.4 U/mL (to avoid platelet ADP receptor desensitization) should be added to the anticoagulant before get the blood drawn. Washed platelet were obtained by centrifugation at room temperature (RT) of blood sample for 10 minutes at 200g without breaks to obtain PRP (see paragraph "3.1.1. Preparation of platelet suspension. Platelet-rich-plasma (PRP)"). This was followed by another centrifugation at 700g for 15 minutes at RT without breaks. The pellet on the Eppendorf tube bottom was then resuspended in pH 7.4 Hepes buffer (NaCl 145 mM, KCl 5 mM, NaH_2PO_4 0.5 mM, HEPES 10mM and dextrose 6mM). After that, the platelet suspension must rest for at least 10 minutes before to be used (Minuz, et al., 2018).

3.2. Flow Cytometry (FACS - Fluorescence-activated cell sorting)

Once obtained the PPP (see paragraph "3.1.2. Platelets Poor Plasma (PPP) production"), pipette 1 mL of it in a 1,5 mL Eppendorf and perform a centrifuge at 15.000g for 5 minutes at RT with the deceleration system set on "soft" in order to obtain the so called Platelets Free Plasma (PFP).

Using a new 1,5 mL Eppendorf, dispense 140 µL Binding Buffer, 5 µL of Annexin V with annexed FITC fluorophore, 5 µL of CD41 (or CD61) with annexed PE fluorophore and 40 µL of the sample PFP under investigation picked up from the supernatant post centrifugation. Carry out a 30 minutes' incubation at RT in the dark.

After the staining time, further 500 µL of Binding Buffer must be added to stop the reaction and transfer the whole volume (~690 µL) into a Flow Cytometry TruCount tube (carrying counting beads equal to a specific amount like for instance 48150 beads per tube declared by the production company) and read the sample at the BD FACS Canto machine (Figure 15).

Figure 15: BD FACS Canto machine, instrument used to perform Flow Cytometry analysis. (Own photo).

The value of interest will be the one concerning the Microvesicles (MVs) double positive for Annexin V and CD41 (or CD61).

Set the sample reading at "low" speed and arrest the detection once 1000 TruCount (TC) beads are recorded which will be used as normalizer. Through the following equation (Equation 2) it is calculated the MVs number per µL.

$$\frac{MVs\ number\ read\ at\ the\ machine}{1000\ TC} \times \frac{48150\ TC}{40\mu l\ specimen} = MVs/\mu l$$

Equation 2

3.2.1. Flow-cytometry analysis of fibrinogen receptor activation and alpha granule secretion

Flow-cytometry was assayed on PRP diluted to 50,000 platelets/µL in PBS. It was incubated at 37°C for 15 min with or without K_{Ca}1.1 channel openers BMS-191011 (5 and 20 µM), NS1619 (5 µM) and NS11021 (5 µM). After incubation, PE-labelled anti-CD62P and the FITC-labelled monoclonal antibody PAC-1, a ligand mimetic monoclonal antibody that specifically binds to the active form of integrin αIIbβ3 complex, were added to platelet suspension with ADP (5 µM) or scalar dose of U46619 (from 0.1 to 2 µM), respectively, for 15 minutes at room temperature. Samples were analysed by flow cytometry (FACSCanto, BD), using dual-color fluorescence. Platelets were identified on the basis of their Forward Scatter and Side Scatter properties. In all the experiments, two isotype-matched irrelevant mouse FITC- IgG1 and PE-IgG1 were included as the negative control. Cells exhibiting positive FITC or PE signals were considered activated platelets.

3.3. Platelet adhesion and aggregation under flow

Microfluidics experiments were performed on a Microfluidic Cellix platform using 8-canal biochips (Vena8 Fluoro+) coated with 200 µg/ml equine tendon collagen (Horm collagen, Takeda), as described in paragraph "3.3.1. Microfluidic". Experiments were performed in parallel channels maintaining the temperature stable at 37°C and a constant shear stress of 10 dynes. Different K_{Ca}1.1 channels openers were investigated: NS1619 (5 µM), NS11021 (5 µM), BMS-191011 (5 and 20 µM), epoxyeicosatrienoic acid (EET)-11,12 (1 µM). Some experiments were also performed in the presence of acetylsalicylic acid (ASA) (100 µM) to block the cyclooxygenase-1 activity or Ticagrelor (1 µM) to block the P2Y12 receptor or ADP (0.5 µM) as a co-agonist to collagen. Analysis was performed using DucoCell software (Cellix Ltd) as described in "3.3.1. Microfluidic" paragraph. Data were expressed as platelet count-normalized area (2 µm). Cinematic recording of platelet adhesion and aggregation (20 s) was performed with the camera centred in the middle of each channel.

3.3.1. Microfluidics

To evaluate the platelets adhesion, the assay is conducted using the "Microfluidic Cellix platform" (Cellix Ltd., Ireland), composed of an optic microscope (Leica DM IRB, objective magnification 20X, numerical aperture 0.30), a Mirus Evo Nanopump, a Q Imaging Exi Blue video camera (1392x1040pixels, 800Mb/s bandwidth capacity, 15 frames per seconds full resolution at 14 bits, 30 MHz, EXi Blue Q IMAGING), a Cellix manifold and a Biochip multichannel Vena8Fluoro+ Cellix (Cellix Ltd) (Figure 16).

Figure 16: Biochip multichannel Vena8Fluoro+ Cellix. (Own photo).

During the preparation step, the Biochip multichannel Vena8Fluoro+ Cellix must be coated with 200 µg/mL equine tendon collagen (Horm collagen, Takeda) at 4°C in a humid chamber overnight. The day after, each channel is incubated at RT for 30 minutes with Bovine Serum Albumin (BSA) 10µg/mL (BSA, Sigma Aldrich) in Hepes buffer (saline solution NaCl 9g/L), to block eventually unspecific binding and successively each channel has to be washed with Hepes buffer. This washing step has to be done using a constant shear stress dictated by the Mirus Evo Nanopump of 5 Dyne for 2 minutes to get rid of the excessing BSA and to comb the collagen filaments constituent of the channel coating of the Biochip multichannel Vena8Fluoro+ Cellix.

Thanks to the Q Imaging Exi Blue video camera, we could record short videos that allow, using PRP but not whole blood, to study the kinetics and modality of platelet adhesion and thrombus formation under shear stress. This offers additional information to that obtained through the classical analysis of the area covered by platelets adherent to the collagen surface that is necessarily performed under static condition right after perfusion is stopped and the microchannel washed with Hepes

Buffer to get rid of non-adherent cells. Nevertheless, since the main advantage of the use of the microfluidic system is the possibility to manipulate the sample as less as possible and to mimic more closely the physiological conditions of platelet adhesion whole blood microfluidic analysis using fluorescent dyes could be used to get information on the biological events that characterize the process of thrombus formation over the time.

Subsequently, ~ 90µl of PRP sample are perfused with 10 Dyne shear stress for 150 seconds, keeping the Biochip multichannel Vena8Fluoro+ Cellix temperature at 37°C. The choice of 10 Dyne shear stress perfusion was dictated by the interest in the analysis of platelet adhesion under arterial shear stress condition (that typically ranges between 10 and 70 Dyne), rather than the effect on venous shear stress condition of platelet adhesion (between 1 and 6 Dyne) or pathological arterial shear stress conditions (above 100 Dyne) (Paszkowiak & Dardik, 2003). At the end of sample perfusion, a washing step with Hepes buffer is performed with 5 Dyne shear stress for 2 minutes to get rid of the eventually unattached platelets. During the washing step, 20 photos along the whole channel length are taken by means of the Q Imaging Exi Blue video camera (Figure 17).

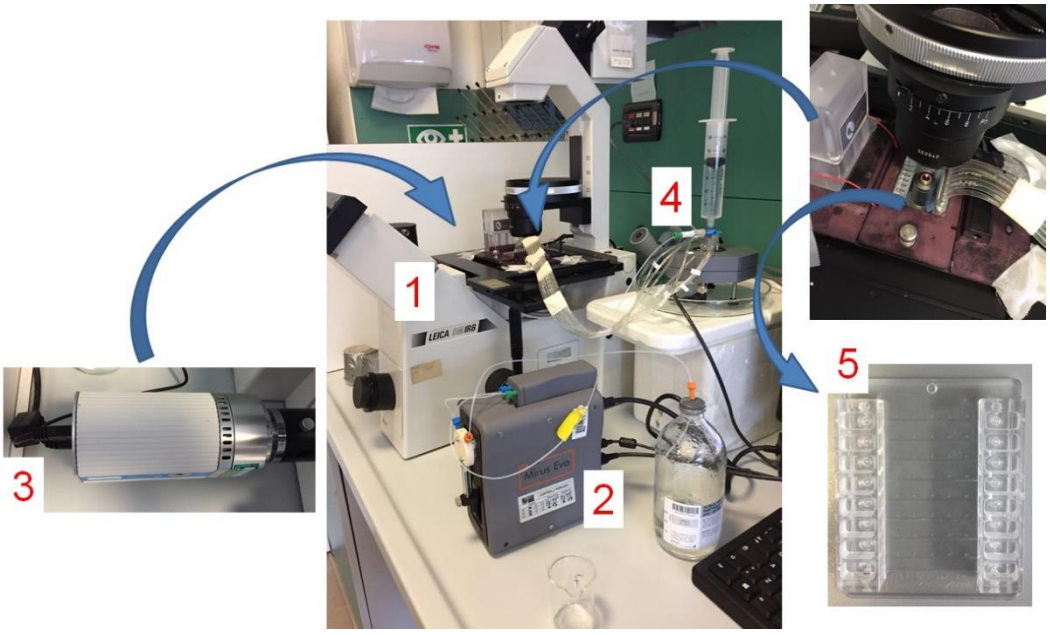


Figure 17: Microfluidic Cellix Platform composed of an optic microscope Leica IRB (1), a Mirus Evo Nanopump (2), a Q Imaging Exi Blue video camera (3), a Cellix manifold (4) and a Biochip multichannel Vena8Fluoro+ Cellix (5). (Own photo).

These photos will be later on analysed with DucoCell software (Cellix Ltd, Dublin, Ireland), able to provides cell counting and analysis of morphological parameters of cells including area, diameter, perimeter, ellipticity, form-factor. Platelets adhesion on collagen surface was calculated and expressed as areas occupied by cells aggregates (Arbitrary Units, AU).

3.4. Platelet aggregation

To carry out platelet aggregation analysis a four-channel aggregometer (APACT 4004 LABiTec, Germany) (Stratmann, Karmal, Zwinge, & Miesbach, Jan-Dec 2019), according to the turbidimetric method of Born was used. Briefly, the rate of platelet aggregation was calculated as the change in percentage of transmitted light (%T), monitored for 5 minutes after the addition of the agonist to PRP at 37 C° under stirring (1000 rpm). The 0% of aggregation was determined by transmitted light of PRP before the addition of the agonist, while 100% aggregation was determined by transmitted light of PPP. Were tested scalar doses of the following agonists to generate dose-response curves: collagen (0.5 to 10 µg/mL), ADP (0.3 to 10 µM) and the TXA2 analogue U46619 (0.05 to 20 µM). All the experiments were performed both in the presence and the absence of the $K_{Ca}1.1$ channel opener BMS-191011 (20 µM). To evaluate the effects of ADP and collagen in absence of endogenous TXA2, aggregation tests were performed either in the presence or absence of ASA (100 µM), added to PRP. Platelet aggregation induced by U46619 was tested also in the in presence or absence of the platelet inhibitor PGE1 (2.5 nM), to modulate platelet response. In each experiment, a single PRP sample was used with one of the agonists to generate dose-response curves in all the different experimental conditions. Cumulated data were fitted in sigmoidal dose-response curves and EC50s were calculated.

4. Statistical analysis

Collected data were analysed with GraphPad Prism software v.5.03 (GraphPad Software, San Diego, CA, USA). Kolmogorof and Smirnov test was preliminary applied to evaluate normality of data distribution. Adhesion-induced platelet aggregation under flow was analysed using one-way ANOVA followed by Tukey's test. Repeated measures one-way ANOVA and Tukey's test for post hoc analysis were applied to compare aggregation curves. Friedman's test and Dunn's test for post hoc analysis were used in the comparison of PMVs release in each experimental condition. Data are expressed as individual data, or mean and standard error (Mean±SEM) as indicated in figure legends or mean and standard deviation (Mean±SD). Where indicated, it was calculated the percent coefficient of variation (CV) as the ration between the standard deviation and the mean of the analysed values. The inferential analysis was performed using the Student t test, analysing pair data and Wilcoxon test. Concerning the evaluation of the effects of agonists and antagonists on platelet aggregation, it was performed a non-linear regression analysis to generate dose-response curves and to calculate EC50. In case of dose response curve's shift, it was calculated the EC50 ratio. Student's t-test was applied when two groups were compared. ANOVA, followed by the post hoc Bonferroni t-test, was applied in multiple comparisons. Data are expressed in the text as mean of differences with 95% confidence intervals or median and interquartile range. A statistical significance (P) value <0.05 was set.

5. Results

5.1. PFAS substances: PFOA and C6O4

All data presented here below are unpublished data and currently under revision. The embargo (academic publishing) ensures their secrecy.

5.1.1. Peripheral blood platelet sample preparation

PRP was obtained as described in the paragraph "3.1.1. Preparation of platelet suspension. Platelet-rich-plasma (PRP)" and then incubated with or without increasing concentrations of C6O4 (1, 10, 100, 200 and 500 ng/mL) for one hour at 37°C and then tested in microfluidic analysis of adhesion-induced platelet aggregation.

A wide range of C6O4 concentrations, comparable with those observed in human blood with other PFAS, were tested. Our attention was focused only on a concentrations subgroup that could be representative of the bioactivity of C6O4. Being the first study on the new PFAS short chain compounds, we therefore evaluated the biological effect on platelets of high relatively concentrations C6O4, even though in the same order of magnitude detected in biological materials.

5.1.2. Platelet adhesion and aggregation underflow

PRP treated with increasing doses of C6O4 was diluted with 9 g/L NaCl saline solution to obtain a final concentration of $1 \cdot 10^8$/ml platelets. Each sample was then fluxed through the channels with a shear stress of 10 Dyne for 3 mins.

Some experiments were also performed using PRP preincubated with ASA (100 µmol/L) to block cyclooxygenase-1 activity and prevent Thromboxane A_2 (TXA_2) generation.

During the experiment the temperature of the multichannel Biochip Vena8 Fluoro + was kept at 37°C. After the perfusion 20 images for each experiment were taken along the whole channels and analysed with DucoCell software (Cellix Ltd, Dublin, Ireland) which provides cell counting and analysis of morphological parameters of cells including area, diameter, perimeter, ellipticity, form-factor. Platelets adhesion on collagen surface was calculated and expressed as areas occupied by cells aggregates (Arbitrary Units, AU).

5.1.3. Release of platelets-derived microvesicles.

The PMVs release was determined in PRP as described in 2016 by Giacomazzi et al. (Giacomazzi, Degan, Calabria, Meneguzzi, & Minuz, 2016). To maintain the physiological calcium concentration in plasma, PRP was obtained from 100 µmol/L PPACK anticoagulated blood. Treatment with C6O4 250 ng/mL as described above was carried out. A stimulation for 30 min at room temperature under low shear stress conditions using GyroMiniTM Nutating Mixer (Labnet Int. Edison, NJ, USA) was performed, incubating 300µl aliquots of treated PRP with various agonists. As control, 9 g/L NaCl saline solution was used instead of agonists for the resting conditions. To block platelet cyclooxygenase type 1 activity, some experiment (in PRP treated with C6O4 250 ng/ml) were conducted also in presence of ASA 100 µmol/L. The agonists used were the following: 7.5 µmol/L ADP, 10 µmol/L TRAP, 5 µg/mL collagen and 1.5 mmol/L AA. After the agonists incubation, PRP samples were centrifuged at 13.000 x g for 5 min at room temperature without break to obtain platelet-free plasma (PFP).

For PMVs labelling, 40 µL of PFP was incubated in annexin V binding buffer in presence of annexin V-FITC and anti-human CD41-PE. A PE-Mouse IgG1 antibody was used as isotype control. After 30 min of incubation in the dark at room temperature, the reaction was stopped by dilution in annexin V-binding buffer and transferred in BD Trucount™ tubes (Becton Dickinson, Italy) which contain a predefined number of beads. PMVs were finally analysed by flow cytometry as CD41 and CD41-PE/annexin V-FITC-positive events in the PMVs region which was determined by fluorescent beads of three diameters (0.5, 0.9 and 3 µm, Megamix, Stago, Biocytex, Marseille, France) (Robert, et al., 2009).

5.1.4. Effects of C6O4 exposure on platelet adhesion and aggregation under flow.

To evaluate possible effects of C6O4 on platelet adhesion and aggregation under flow, PRP was incubated for 30 minutes at 37°C with C6O4 at the concentrations 0, 100 or 200 ng/mL, in the presence or absence of ASA 100 µmol/L. Platelets were seeded on a microchip pre-coated with collagen and then subjected to microfluidics analysis under predefined shear rate at 37°C. Under these experimental conditions, we preliminarily evaluated the inter-assay and intra-assay variability (17.7% and

7.3%, respectively). All the experiments were performed testing, in parallel channels and in a single chip, two different conditions: PRP pre-incubated without C6O4 or ASA (control sample) and PRP pre-incubated with C6O4 and/or ASA. Data are expressed as the ratio between collagen-adhering platelet aggregates generated from treated PRP, and the untreated PRP.

As shown in Figure 18, platelets firmly adhering generate small aggregates, which increase in size upon the interaction and adherence to collagen fibres through the recruitment of flushed platelets. This process leads to aggregates, covering large areas in the micro-vessels, that can be quantified after flushing the system with saline solution.

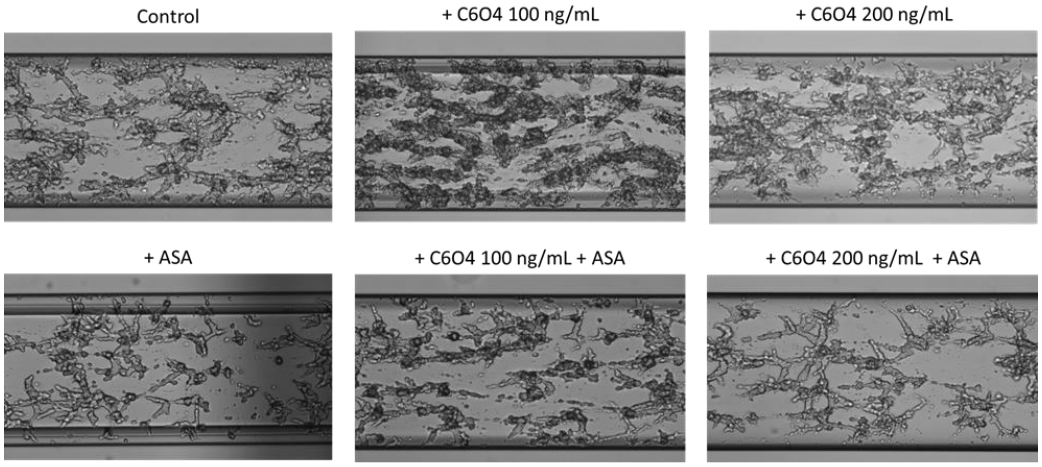
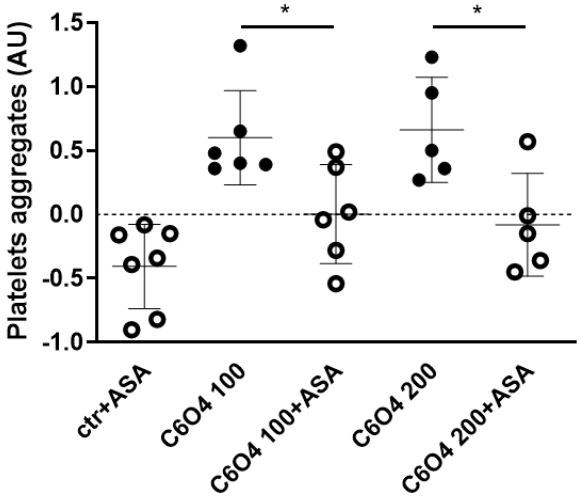


Figure 18: Microfludics analysis of platelet adhesion and aggregation: Representative images showing platelet adhesion to immobilized collagen under different experimental conditions and platelet thrombus formation. Flowing in collagen-coated microchannels adhering platelets recruit circulating platelets to generate large stable aggregates. Prior to the analysis PRP was incubated for 30 minutes at 37°C without or with 100 or 200 ng/mL C6O4, in the presence or absence of ASA 100 µmol/L.

Using this methodological approach, we observed that the platelet aggregates were significantly increased by the pre-incubation with C6O4 100 ng/mL (+ 0.60 AU, 95% CI: +0.21 to +0.99 AU; $P<0.05$ vs control) and 200 ng/mL (+ 0.66 AU, 95% CI: +0.15 to +1.17 AU; $P<0.05$ vs control). Incubation of PRP with ASA 100 µmol/L significantly reduced platelets adhesion and aggregation under flow to immobilized collagen, both when PRP was not pre-treated with C6O4 (-0.40 AU,

95% CI: -0.11 to -0.71 AU; P<0.05) and when was treated with 100 ng/mL C6O4 (-0.60 AU, 95% CI: - 0.11 to -1.08 AU; P<0.05 or 200 ng/mL C6O4 (-0.74 AU, 95% CI: -0.06 to -1.48 AU; P<0.05) (Graph 1).



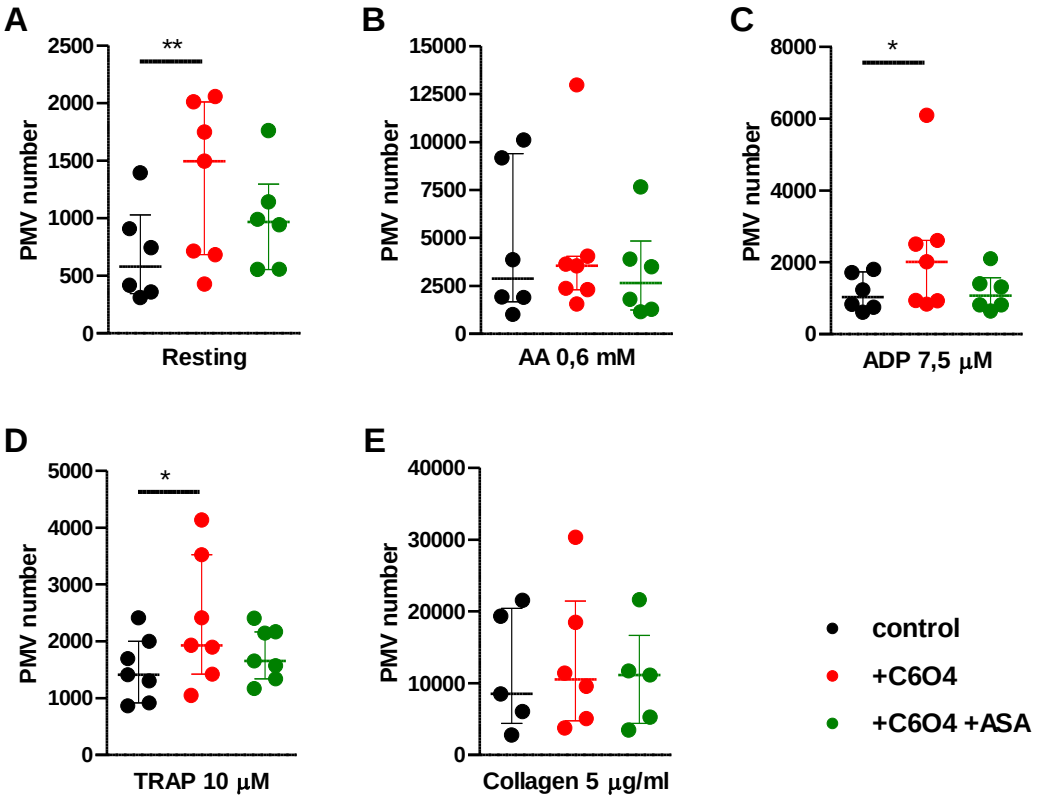
*Graph 1: Quantitative analysis of the area covered by adhering platelets under control conditions and in the presence of C6O4 with and without acetylsalicylic acid (ASA). The ratio of collagen-adhering platelet aggregates generated from treated PRP to aggregates from untreated PRP flowing at the same time in two parallel microchannels are calculated. Data are presented as individual values normalized versus control conditions representing platelet-covered area. Mean and standard deviation (Mean ±SD) are shown. One-way ANOVA followed by Tukey's test as post hoc analysis were applied. * indicates P<0.05.*

This study therefore demonstrated for the first time the effect of ASA modulation either on the already known platelet aggregation and on platelet adhesion, which represents the first initial step of the process leading to the platelet thrombus formation. Thus ASA incubation operates on the premature platelet activation phase.

5.1.5. Effects of C6O4 exposure on platelets microvesicles release

The *in vitro* generation of pro-coagulant PMV was assessed measuring particles in the range of 0.1- 1 µm expressing CD41 and binding annexin V (double positive PMV). The assay was performed applying a low intensity shear stress to PRP, stimulated or not with a platelet agonist (Graph 2). All the tested agonists increased

PMV generation, particularly AA 1.5 mmol/L and collagen 5 μg/mL represented the strongest stimulus for *in vitro* PMV generation.



*Graph 2: In vitro generation of procoagulant platelet-derived microvesicles: In vitro generation of procoagulant platelet-derived microvesicles (PMV) in unstimulated (A) platelet rich plasma (PRP) and in PRP stimulated with arachidonic acid 1.5 mmol/L (B), ADP 7.5 µmol/L (C), TRAP 10 µmol/L (D) and collagen 5 µg/mL (E). A single relatively high concentration (250 ng/mL) of C6O4 was tested as preliminary investigation. (A) The number of PMV generated in PRP pre-treated with C6O4 was 250 ng/ml (n=7) is compared with untreated PRP (n=6) and ASA-treated PRP (n=6). Increased PMV generation is observed in the presence of C6O4. (B, E) No significant difference in PMV release after stimulation respectively with arachidonic acid 1.5 mmol/L (n=6) and collagen 5 µg/ml (n=5) is observed between PRP treated with C6O4 and untreated PRP. (C, D) The number of procoagulant PMV generated in PRP pre-treated with C6O4 250 ng/ml compared with untreated PRP are higher in platelets stimulated respectively with ADP (n=6) and TRAP (n=7). The presence of ASA 100 µmol/L, tends to reduce MV generation but is not statistically significative. Friedman's test followed by Dunn's test as post hoc analysis were applied. Individual data are shown, * indicates P<0.05, ** indicates P<0.01.*

As shown in Graph 2A, pre-treatment of PRP with C6O4 250 ng/mL was associated with increase in pro-coagulant PMV from resting PRP in the absence of any platelet agonists. (median: 581 events, interquartile: 347 - 1030 events *vs* 1622 events, interquartile: 707 – 2022 events; P<0.05). No significant difference in PMV release after stimulation with AA 1.5 mmol/L was observed between platelets exposed to C6O4 compared to naïve sample (n=6) (median 2898 events, interquartile 1682-9403 events *vs* 3601 events, interquartile 2179-6281 events, P=n.s. Graph 2B or 5 μg/mL collagen (n=5) (median 11414 events; interquartile 6668-24393 events vs median 8514 events; interquartile 4406-20440 events; P= n.s. Graph 2E). Statistically significant differences were also observed testing platelets stimulated with either ADP (n=6) (control: median 1032 events, interquartile 717-1732 events vs 250 ng/mL C6O4: median 2259 events, interquartile 912-3476 events; P<0.05, Graph 2C) and TRAP (n=7) (control: median 1552 events, interquartile 1205-2103 events *vs* C6O4: median 2171 events, interquartile 1778-3672 events; P<0.05,Graph 2D). In the presence of ASA 100 μmol/L, a trend towards reduced MV generation was observed in all the tested conditions, including PRP not pre-treated with C6O4.

5.2. Platelet $K_{Ca}1.1$ channel

On February 16th 2021 the article "Expression and functional characterization of the large-conductance calcium and voltage-activated potassium channel K ca 1.1 in megakaryocytes and platelets" was published. Here below I present my contribution to such publication. Further information can be visualized in the above mentioned article (Balduini, et al., 2021).

5.2.1. Effects on the pharmacological modulation of the $K_{Ca}1.1$ channel on platelet function *in vitro*

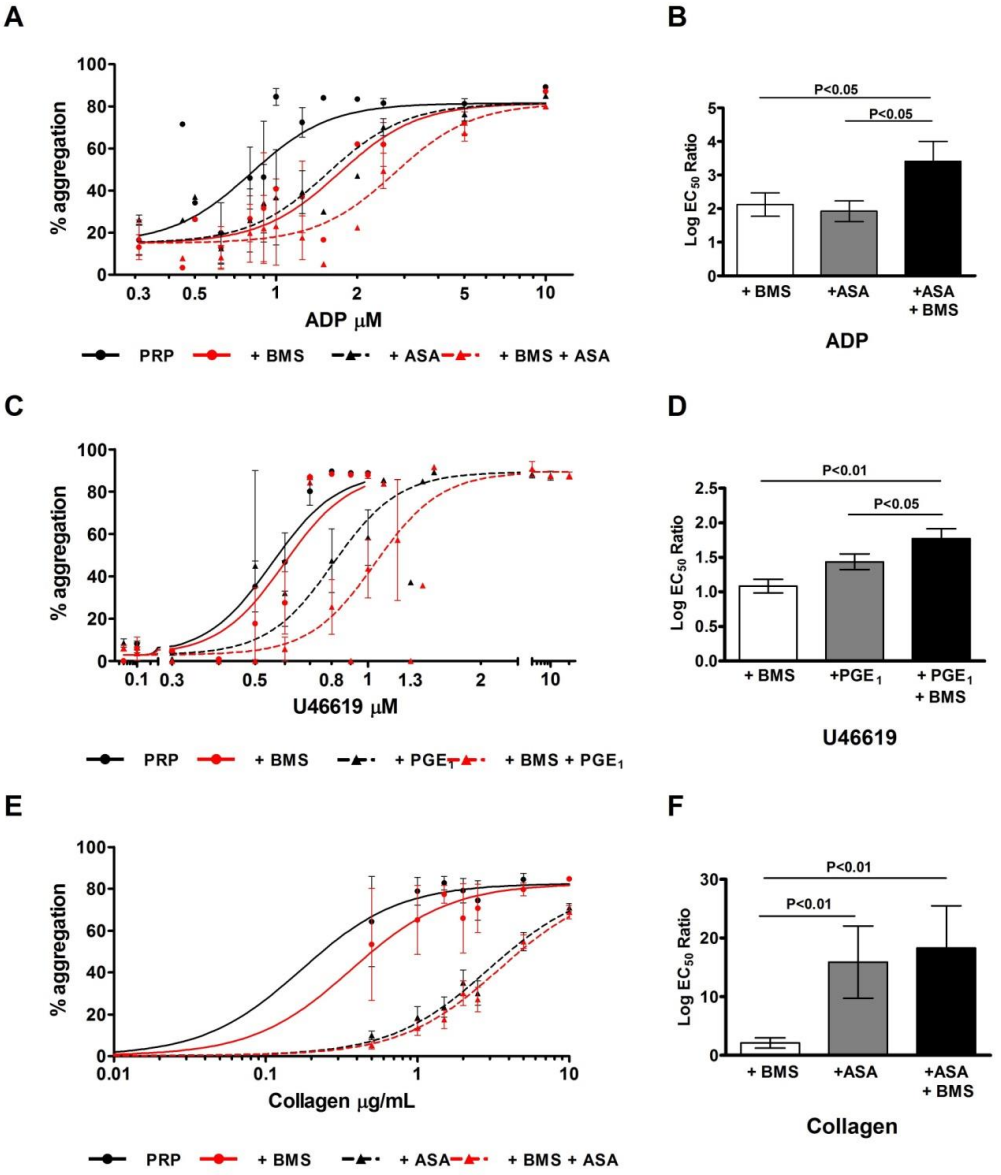
Platelets sensitivity to agonists was tested in a wide range of concentrations of platelet agonists in the presence or absence of the $K_{Ca}1.1$ channel opener BMS-191011. In the Graph 3(panels A-F) are reported the concentration-response curves and the ratio of the Log10 EC50 values (concentration of the agonist giving the half-maximal platelet aggregation) which were recorded in the presence of the tested compounds versus vehicle (control conditions).

The $K_{Ca}1.1$ channel opener BMS-191011 (20 µM), in the presence of increasing concentrations of ADP, caused a rightward shift of the concentration-response curve of platelet aggregation (EC50 0.91 µM, CI 95% 0.43-1.92, for the control conditions, and EC50 2.67 µM, CI 95% 0.97-7.29, BMS-191011, respectively) (Graph 3A). In the presence of ASA (100 µM), the concentration-response curve was shifted to the right, drawing a trend close to the one described above (Graph 3A). A further shift to the right of the concentration-response curve of ADP was induced by the co-incubation of ASA and BMS-191011 (Graph 3A-B). In the presence of ASA and BMS-191011 the EC50 value (6.18 µM, CI 95% 2.11-18.09) was approximately 6-fold higher than that found in control conditions (PRP only). A statistically significant increase in the EC50 ratio between BMS-191011 or ASA versus BMS-191011 plus ASA was observed (Graph 3B).

Increasing the concentrations of U46619, an analogue of TXA2, a step response of platelet aggregation was observed. As shown in Graph 3C, 20 µM BMS-191011 does not alter the slope of the concentration-response curve, which replicates that of the control conditions. In the presence of the stable prostacyclin analogue 2.5 nM PGE1 (which acts by inducing cAMP generation), a rightward shift of the

concentration-response curve of U46619 was observed (Graph 3C). When a co-incubation of PGE1 with BMS-191011 was performed, the shift of the curve was significantly enhanced (Graph 3C and D), leading to a further reduction of the platelet sensitivity to U46619 (Graph 3C). Moreover, the co-incubation of PGE1 with BMS-191011, significantly increased the EC50 ratio when compared with BMS-191011 or PGE1 alone (Graph 3D). This suggests that cAMP-dependent signalling increases platelet response to openers of the $K_{Ca}1.1$ channel.

Platelet aggregation in response to collagen showed a shift to the right of the concentration-response curve although it was not significantly altered by 20 μM BMS-191011 (Graph 3E). As expected, the concentration-response curve shifted to the right when incubated with ASA (100 μM) (Graph 3E, F) and the maximal platelet aggregation was reduced (Graph 3E). Nevertheless, no significant changes of these effects were observed co-incubating ASA with BMS-191011 (Graph 3E and F).



Graph 3: Effects of the $K_{Ca}1.1$ channel opener BMS-191011 on platelet aggregation. Effects of BMS-191011 (20 µM) on the response curves of platelet aggregation (monitored for 5 minutes) induced by increasing concentrations of ADP (A) (n=36 in each curve), the TXA2 analogue U46619 (C) (n= 39-75 in each curve), or collagen (E) (n= 33-53 in each curve). Aggregation induced by ADP (A and B) or collagen (E and F) was tested also in the presence of ASA (100 µM) and ASA plus BMS-191011. Platelet aggregation induced by U46619 was tested also in the presence of the stable prostacyclin analogue PGE1 (2.5 nM) and PGE1 plus BMS-191011 (C and D). The ratio of Log10 EC50 was calculated comparing values obtained with the platelet agonist alone with those obtained in the presence of the tested compounds (B, D and F). Data are expressed as mean ± SEM.

5.2.2. Fibrinogen receptor activation and secretion of alpha granule

As Shattil reported in 1985, the activation of the fibrinogen receptor can be assessed using flow cytometry. Particularly, it was used a pentameric IgM, known as PAC-1, able to recognize an epitope on the GPIIb-IIIA complex, which is located near the fibrinogen-binding domain (Shattil, Hoxie, Cunningham, & Brass, 1985). The analysis was performed in platelets stimulated with soluble agonists and showed a selectivity in the inhibitory activity of different $K_{Ca}1.1$ channel openers BMS-191011 (5-20 μM), NS1021 (5 μM), NS1619 (5 μM). In fact, a tendency towards reduction in the surface expression of active fibrinogen receptor and P-Selectin (CD62), a maker of alpha-granule secretion, was observed in ADP-stimulated platelets (

Table 1), although a statistically significance was not reached.

Treatment	PAC1 +	P-SELECTIN +
U46619 (2μM)	69.9 ± 11.7 (n=4)	63.3 ± 5.1 (n=4)
+ BMS-191011 (20μM)	48.1 ± 15.0 (n=4)	41.1 ± 12.0 (n=4)
U46619 (1μM)	61.5 ± 7.7 (n=9)	58.7 ± 5.5 (n=8)
+ BMS-191011 (20μM)	50.0 ± 8.9 (n=9)	47.1 ± 7.8 (n=8)
U46619 (0.5μM)	42.5 ± 11.8 (n=6)	47.0 ± 9.5 (n=5)
+ BMS-191011 (20μM)	38.7 ± 11.5 (n=6)	44.3 ± 10.1 (n=5)
U46619 (0.2μM)	25.6 ± 10.6 (n=6)	33.8 ± 12.0 (n=5)
+ BMS-191011 (20μM)	26.3 ± 11.8 (n=6)	28.0 ± 11.1 (n=5)
U46619 (0.1μM)	4.8 ± 2.2 (n=9)	9.9 ± 6.5 (n=8)
+ BMS-191011 (20μM)	7.2 ± 3.3 (n=9)	12.5 ± 6.5 (n=8)
ADP (5μM)	19.8 ± 6.0 (n=7)	46.0 ± 7.0 (n=7)
+ BMS-191011 (20μM)	13.3 ± 4.8* (n=7)	30.6 ± 6.8** (n=7)
+ NS1619 (5μM)	11.8 ± 3.8** (n=7)	30.7 ± 6.7** (n=7)
+ NS11021 (5μM)	9.9 ± 3.4** (n=7)	28.7 ± 6.2** (n=7)

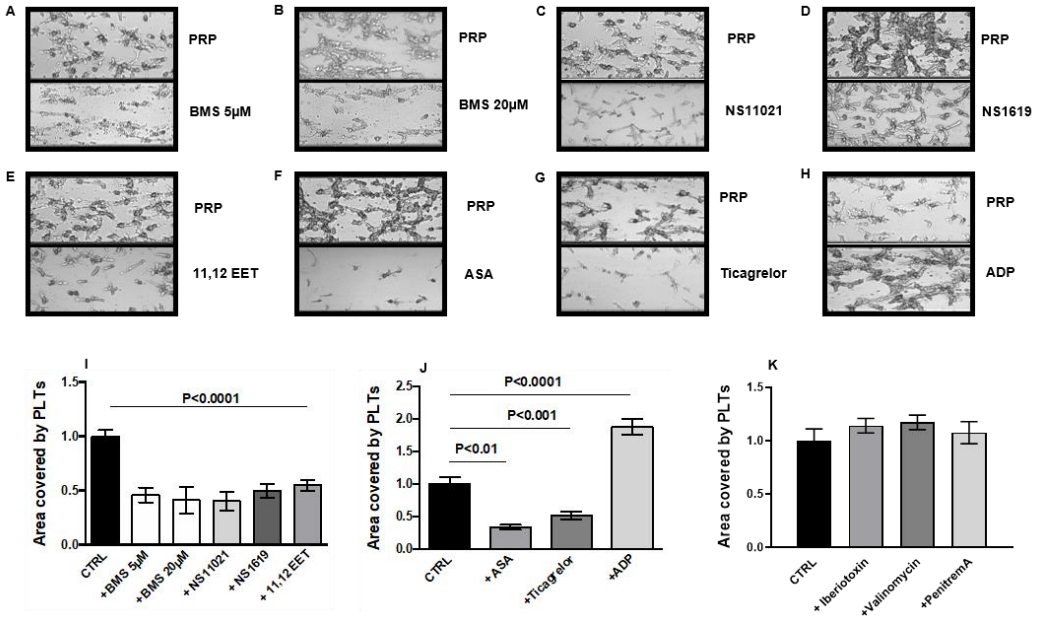
Table 1: Expression of active GPIIb-IIIa complex and P-selectin on activated platelets in presence/absence of $K_{Ca}1.1$ channels openers. U46619 or ADP-stimulated platelets positive for the active fibrinogen receptor (as detected using PAC1 mAb) and P-selectin were expressed as percentage of the total number of platelets. Data are

*expressed as mean ± SEM. Statistically significant differences are indicated by *P<0.01 and **P<0.001 respect to PRP + ADP alone.*

5.2.3. Platelet interaction with the extracellular matrix are altered when $K_{Ca}1.1$ channel is treated with its own openers

Platelet adhesion and spreading, are induced by extracellular matrix components. Using a microfluidics apparatus, platelet adhesion and thrombus formation on immobilized collagen under flow were analysed. Treatment with BMS-191011 (5 and 20 µM), NS11021 (5 µM), and NS1619 (5 µM) significantly blunted ($P<0.0001$) thrombus formation (Graph 4A-D), and area coverage with respect to untreated controls (Graph 4I). Similar effects were observed with ASA (100 µM), and 11,12-EETs (1 µM), a major product of CYP-epoxygenase-dependent arachidonic acid metabolism (Graph 4E, F, I and J), which was shown to act through the activation of $K_{Ca}1.1$ channels (Krötz, et al., 2004). As reported in Graph 4G, H and J, ADP (0.5 µM) enhanced thrombus formation while the P2Y12 antagonist Ticagrelor (1 µM) inhibited it.

Under static conditions, regardless the adopted extracellular component, BMS-191011 did not alter platelet adhesion and spreading, neither with collagen (control 34.51 ± 2.6%, n=7; BMS-191011 33.5 ± 2.8%, n=7) nor with fibrinogen (control 14.02 ± 4.4%, n=5; BMS-191011 13.2 ± 2.9%, n=5).



Graph 4: Platelet adhesion and thrombus formation in microfluidic experiments. (A-H) Representative images showing platelet adhesion and thrombus formation under flow to immobilized collagen (200 µg/mL). Images were collected using Leica DM IRB optical microscope (magnification 20X, numerical aperture 0.30). Five images (1 out of 4 fields) from the whole length of each channel were taken 3 minutes after the beginning of the perfusion assay conducted at 37°C. Analysis of the area of surface coverage in each of the tested conditions was performed using DucoCell software (Cellix Ltd). Data were expressed as platelet count-normalized area (2 µm). Control conditions (CTRL) and the effects of K_{Ca}1.1 openers BMS-191011 (5 µM n=6, 20 µM n=3), NS1619 (n=9), NS11021 (n=6) and 11,12 EET (n=5) are shown (A-E) along with the effects of ASA (n=4), the P2Y12 inhibitor Ticagrelor (n=3) and ADP 0.5 µM (n= 3) (F, G and H). (I and J) Quantitative analysis of the area covered by thrombi under control conditions and in the presence of different functional agonists of the K_{Ca}1.1 channel, EETs, ASA, Ticagrelor and ADP 0.5 µM. Data are expressed as mean ± SEM.

5.3. Non Alcoholic Fatty Liver Disease (NAFLD)

All data presented here below are unpublished data and the embargo (academic publishing) ensures their secrecy.

In this pilot study, to better understand NAFLD pathophysiology, two main approaches were performed: Microfluidic and Flow Cytometry analysis (3.3.1. Microfluidic and 3.2. Flow Cytometry (FACS - Fluorescence-activated cell sorting).

The blood obtained by patients selectively chosen as previously described in the paragraph 3.1. Blood withdraw. The blood was centrifuged and PRP was than isolated (see paragraph "3.1.1. Preparation of platelet suspension. Platelet-rich-plasma (PRP)").

The first step was to verify if the methods and technique used were reproducible, repeatable and adjustable.

5.3.1. Microfluidic analysis and NAFLD patients

5.3.1.1. Intra-essay variability

To study intra-essay variability, the PRP specimen belonging to the same subject was used to recreate four independent experiments on four different channels of the Biochip multichannel Vena8 Fluoro+ Cellix. The results were compared one to another.

In the following columns graph (Graph 5: Area of platelet adhesion of the single experiments using one single subject to estimate the Intra-essay variability.) is shown the average area of platelet adhesion of the single experiments with the related standard deviation, comparing them to the mean value of the four channels (dotted red line).

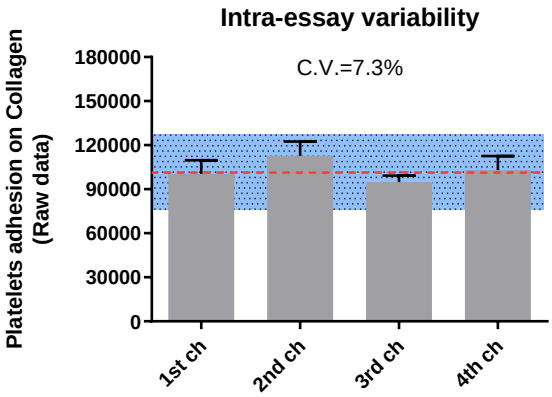


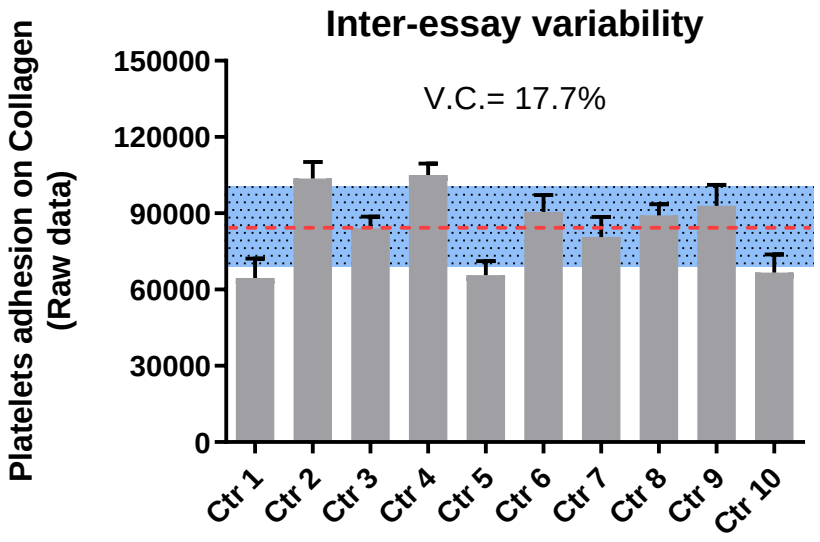
Image N°	1st ch	2nd ch	3rd ch	4th ch
1	149565	144376	94108	142821
2	146446	146590	67390	122068
3	94294	148345	110353	126877
4	91074	128660	109012	130455
5	89807	98272	101427	65664
6	73236	86196	85814	99301
7	89953	85954	90109	92506
8	93481	102340	97237	72171
9	75741	73866	98356	75383

Graph 5: Area of platelet adhesion of the single experiments using one single subject to estimate the Intra-essay variability.

Graph 5: Area of platelet adhesion of the single experiments using one single subject to estimate the Intra-essay variability. shows that the values obtained are similar one to another and the percent coefficient of variation (CV) is 7,3%. This essay is then suitable to be reproducible.

5.3.1.2. Inter-essay variability

To study inter-essay variability, the PRP specimens belonging to the ten different healthy subjects were used to recreate ten independent experiments on ten different channels of the Biochip multichannel Vena8 Fluoro+ Cellix.
In the following columns graph (Graph 6: Area of platelet adhesion of the single experiments using ten different healthy subjects to estimate the Inter-essay variability.) is shown the mean area of platelet adhesion of the single experiments with the related standard deviation, of the ten experiments (dotted red line).



Graph 6: Area of platelet adhesion of the single experiments using ten different healthy subjects to estimate the Inter-essay variability.

Graph 6: Area of platelet adhesion of the single experiments using ten different healthy subjects to estimate the Inter-essay variability. shows that the values of the percent coefficient of variation (CV) is 17.7%. The obtained values are required to define which are the normality values and the controls variability to define the significance cut-off when healthy controls are compared with patients.

5.3.1.3. Modulation of platelet response

To study the modulation by agonists and antagonists, the PRP sample obtained from the same subject was incubated with ASA (100 µM), ADP (0,5 µM) and Ticagrelor (1 µM), prior to perfusion in the channel.

The obtained values were normalized, displayed in a column graph and compared with the mean value recorded with PRP only (Graph 7).

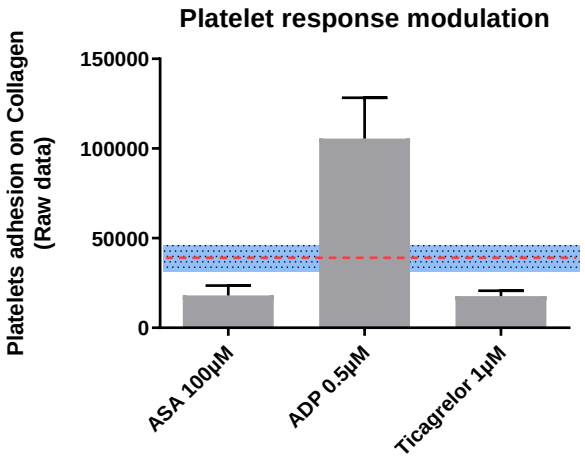


Image N°	ASA 100µM	ADP 0.5µM	Ticagrelor 1µM
1	12108,47	40605,95	16095,94
2	38179,72	151114,6	28966,24
3	9662,442	113683,4	14385
4	8268,968	155560,2	10883,25

Graph 7: Area of platelet adhesion obtained post incubation with ASA, ADP and Ticagrelor.

The collected results with the three compounds modulating the platelet response added to the PRP, show clear differences compared to the sample with PRP only: in the Graph 7 can be noticed the significant reduction of the area of platelet adhesion following the incubation with ASA and Ticagrelor and a significant increment of the area of platelet adhesion post incubation with ADP.

The system therefore, seems to be enough sensible to pharmacological modulation and suitable for testing agonists, recreating *in vitro* conditions similar to an *in vivo* scenario.

5.3.1.4. Case-control study: hypertensive patients

A priori assumption that the microfluidic techniques are efficient for the *in vivo* representation of the patient's conditions, hypertensive subjects' and healthy controls' values are reported in a scatter graph; on these data the mean value and the respective standard deviation were calculated (Graph 8).

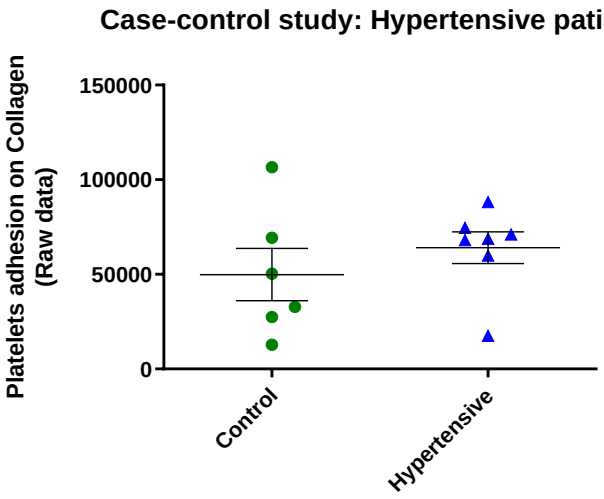


Image N°	Control	Hypertensive
1	12765	59916
2	27428	17599
3	50302	68846
4	32816	88139
5	69301	68002
6	106622	74594
7		71039

Graph 8: Comparison between platelets adhesion's area of healthy controls and hypertensive patients.

In the Graph 8 is shown a higher propensity of platelets adhesion on Collagen in a group of hypertensive patients compared to platelets from healthy controls. This investigation suggests a higher platelet adhesiveness in hypertensive subjects compared to healthy individuals.

5.3.1.5. Case-control study: NAFLD patients

The same study was performed with NAFLD subjects and healthy controls. Data are reported in a scatter graph; on these data was calculated the mean value and the respective standard deviation (Graph 9).

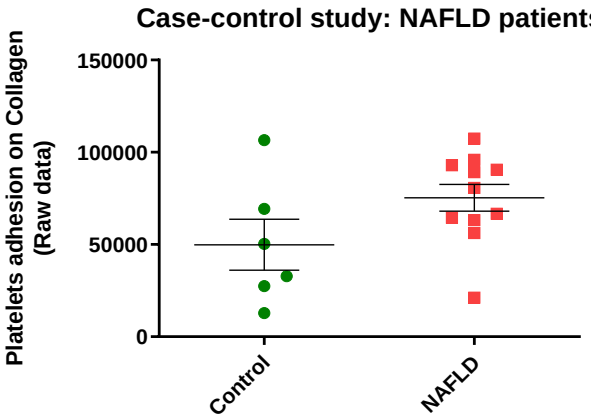


Image N°	Control	NAFLD
1	12765	21164
2	27428	56126
3	50302	64579
4	32816	107433
5	69301	95936
6	106622	63179
7		90596
8		80687
9		89151
10		92963
11		66741

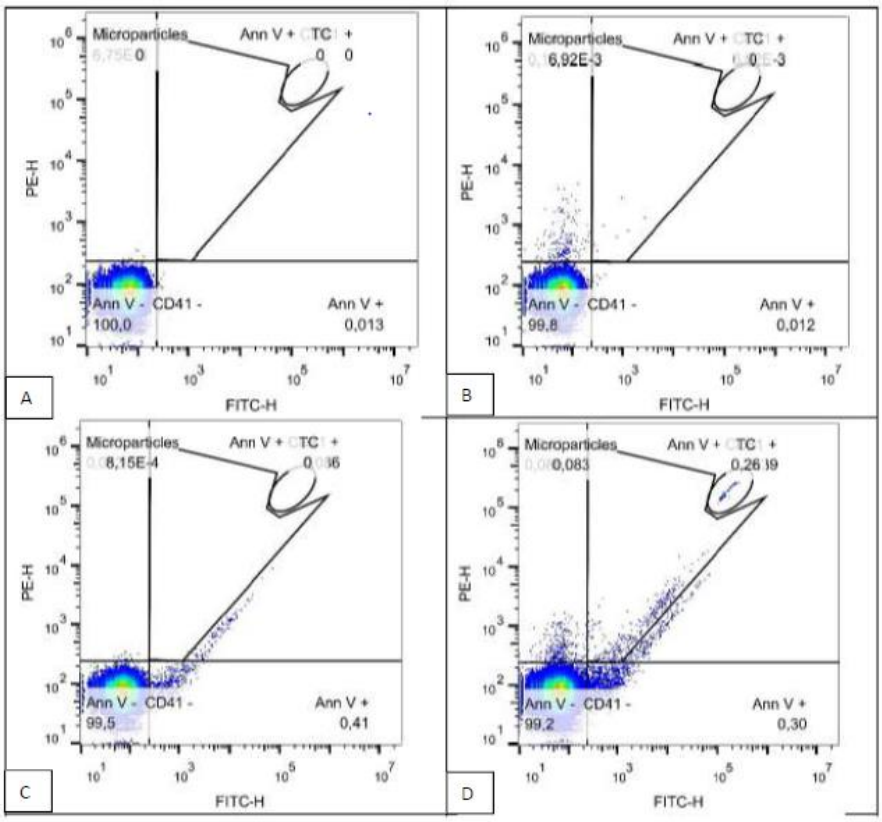
Graph 9: Comparison between platelets adhesion's area of healthy controls and NAFLD patients.

Graph 9 shows a higher propensity to platelets adhesion on collagen NAFLD patients compared to platelets from to healthy controls. This investigation suggests a higher platelet adhesiveness in NAFLD subjects compared to healthy individuals similar to what observed in hypertensive patients.

5.3.2. Flow Cytometry (FACS - Fluorescence-activated cell sorting) and NAFLD patients

5.3.2.1. Platelets derived microvesicles (PMVs) study on healthy control

Graph 10 are reported data obtained through a flow cytometry analysis of blood sample from a single healthy subject. The values of Annexin V are shown on the abscissa and those of CD41 on the ordinate.



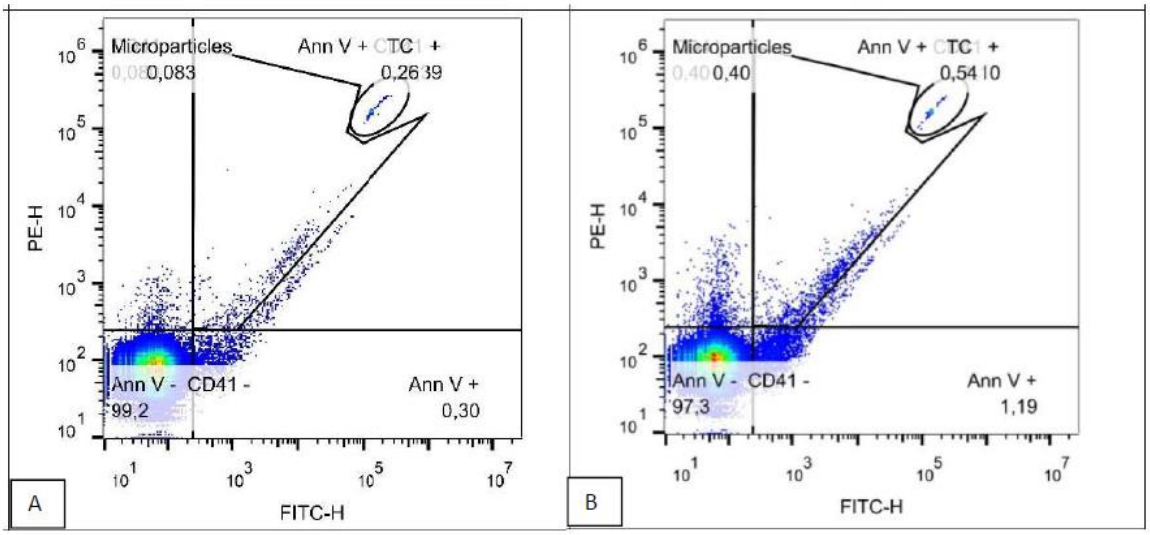
Graph 10: Flow Cytometry images: A. Control FITC-AnnV - _ PE-CD41 -; B. Control FITC-AnnV - _ PE-CD41 +; C. Control FITC-AnnV + _ PE-CD41 -; D. Control FITC-AnnV + _ PE-CD41 +.

Flow cytometry analysis was adjusted to read only those values that are within the pre-set limits referring to the size and granularity specific for platelets derived microvesicles. In the Graph 10, panels A, B and C are represented the PPP samples stained respectively for no antigen, CD41 and Annexin V.

The values of this study are reported in the panel D of the Graph 10, where are highlighted those particles presenting both positive signals for Annexin V and CD41. Among all positive signals in the Q2 region, a specific gate was defined to identify the microvesicles that are truly platelet-derived microvesicles (PMVs), since it was demonstrated that samples stained for Annexin V only presented positivity also in the region Q2. The obtained value was normalized using 1000 TruCount beads (TC).

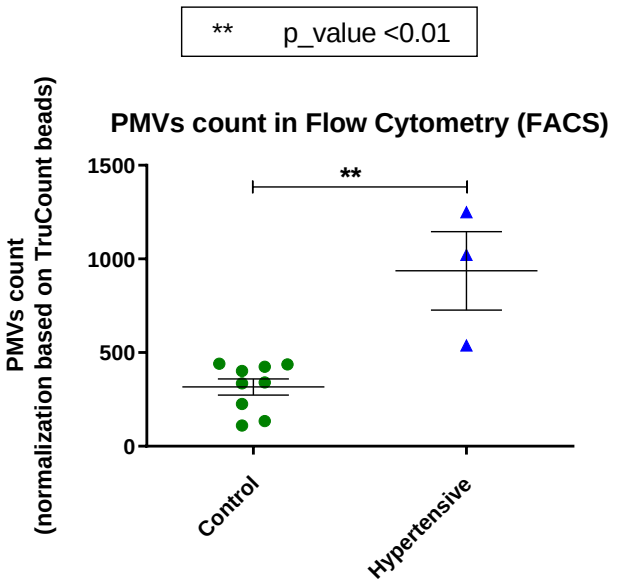
5.3.2.2. Case-control study: hypertensive patients

In the Graph 11 are compared the results of the PMVs count in Flow Cytometry of a healthy control and a hypertensive patient; both the values were normalized using 1000 TruCount beads (TC).



Graph 11: Comparison control vs hypertensive patient: A. Control FITC-AnnV + _ PE-CD41 +; B. hypertensive patient FITC-AnnV + _ PE-CD41 +.

A higher number of double positive signals (Annexin V+_CD41+) was observed in the hypertensive patient. This tendency is clearly shown in the following scatter graph (Graph 12) where the values obtained in the controls are compared with those of hypertensive patients.



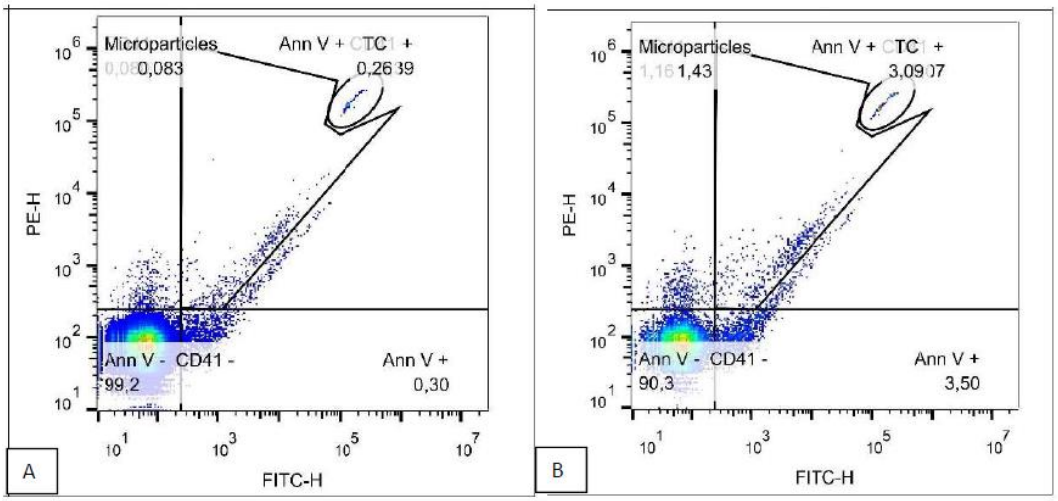
Speciment N°	Control	Hypertensive
1	340	1249
2	225	538
3	437	1022
4	335	
5	111	
6	134	
7	441	
8	402	
9	424	

Graph 12: Comparison of the PMVs number's distribution in healthy controls and hypertensive patients.

Although the study is preliminary, there is a statistically significant difference (p_value < 0,01) between the two groups. In particular, hypertensive patients present a higher number of circulating platelet-derived microvesicles.

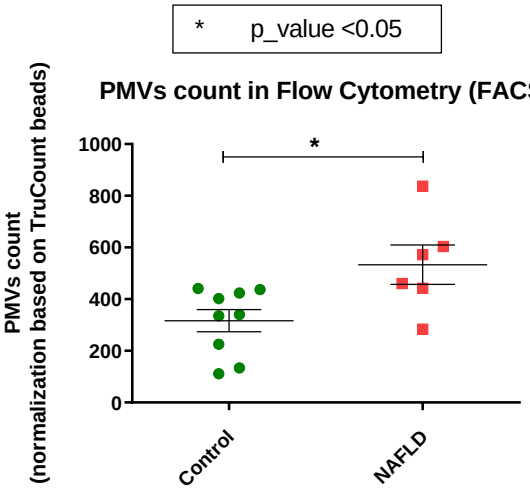
5.3.2.3. Case-control study: NAFLD patients

In the Graph 12 the results of the PMVs count in Flow Cytometry of a healthy control and a NAFLD patient are compared; both the values were normalized using 1000 TruCount beads (TC).



Graph 13: Comparison control vs NAFLD patient: A. Control FITC-AnnV + _ PE-CD41 +; B. NAFLD patient FITC-AnnV + _ PE-CD41 +.

A higher number of double positive signals (Annexin V+_CD41+) was observed in NAFLD patients. This tendency is clearly shown in the following scatter Graph *14* where the values obtained in the controls are compared with those from NAFLD patients.



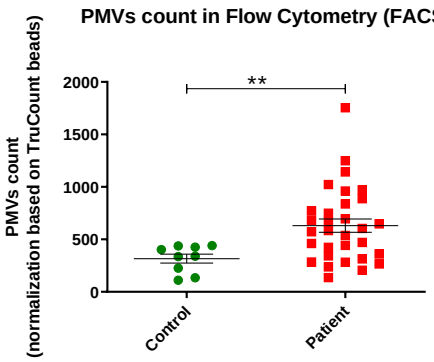
Speciment N°	Control	NAFLD
1	340	442
2	225	604
3	437	572
4	335	837
5	111	460
6	134	284
7	441	
8	402	
9	424	

Graph 14: Comparison of the PMVs number's distribution in healthy controls and NAFLD patients.

A statistically significant difference (p_value < 0,05) between the two groups was observed. In particular, NAFLD patients present a higher number of circulating platelet-derived microvesicles.

5.3.2.4. Case-control study: NAFLD and hypertensive patients

In the Graph 15 are compared the results of the PMVs count in Flow Cytometry of a healthy controls, NAFLD patients and hypertensive subjects; all the values were normalized using 1000 TruCount beads (TC).



Control	Patient (1/4)	Patient (2/4)	Patient (3/4)	Patient (4/4)
339	749	237	442	695
225	471	584	604	360
437	1754	682	770	205
335	1143	667	1249	284
111	888	648	538	
134	314	135	1022	
441	266	342	572	
402	960	971	837	
425	424	282	460	

** p_value ⩽ 0.005

Graph 15: Comparison of the PMVs number's distribution in healthy controls and NAFLD and Hypertensive patients.

The patients present a mean value which is statistically higher than the mean value of the healthy controls (Mann Whitney test: p_value ≤ 0,005).

6. Discussion and Conclusion

6.1.1. PFAS substances research study's aim

The study is focused on the evaluation of consequences of the interaction of the novel substitute compound of PFAS, named C6O4, with the platelets-plasma membrane; to reach the goal of this study, analysis on the platelet aggregation profile, either in static or dynamic conditions, and the extent of platelet microvesicles released, was performed.

6.1.2. PFAS substances research study's discussion and conclusion

C6O4 preferentially accumulates in plasma membranes of human platelet, leading to an alteration of the main biophysical properties of this cell, changing the fluidity and the electrostatic charge distribution. Looking at the platelet aggregation profile, either in static or dynamic conditions, and the extent of platelets microvesicles release, the detrimental consequences of C6O4 can be noticed. Notably, the use of acetyl-salicylic acid as antiplatelet drug showed a significant counteracting activity on this altered platelet function pattern. The present study represents the first one analysing the potential health consequences of the environmental exposure to this novel compound.

By means of a combined computational-experimental approach, the consequences of the interaction of C6O4 with platelets-plasma membrane, widely overlaps with what was observed with the legacy PFAS, taking into consideration the structural characteristics that differentiate the two classes of compounds as well, which are respectively cyclic and linear (De Toni, et al., 2020).

To investigate the possible effects of C6O4 on platelets, three different sets of experiments were performed; adhesion, aggregation and the release of microvesicles, the three main functional activities of platelet in haemostasis and thrombosis were explored. Fascinating, all these platelet activities were enhanced by C6O4. Particularly, I used a microfluidics apparatus to mimic the interaction between circulating platelets and vessels wall lacking of the endothelial layer; thus, PRP was perfused on collagen applying a constant laminar flow rate, to better reproduce what physiologically speaking occurs in human arteries.

Li, Grosser and Diamond in 2017, using the same experimental conditions used in this project, showed that platelet adhere to collagen through the engagement of

integrins (Li, Grosser, & Diamond, 2017), but further platelet adhesion and platelet recruitment, that will give rise to a platelet clot that will covers the collagen bed, depends on the release of TXA2 and ADP from activated platelets that will amplify the signals deriving from integrin's ligation ((Li, Grosser, & Diamond, 2017); (Ting, et al., 2019)). I observed that the whole process is significantly amplified by C6O4. To test the hypothesis that C4O6 could have some biological effects on platelets, relatively high concentrations of the chemical were tested, taking into consideration the range of concentrations detected in humans who were exposed to PFAS. In order to perform studies on the effects of C6O4 on platelets, resting platelets or agonists-stimulated platelets were used. Interesting and confirming the hypothesis, an increase in platelet aggregation was observed when platelets were stimulated with intermediate doses of AA or ADP or a low concentration of collagen. Taking into account all these events and the effects of ASA on platelets, an aggregation TXA2-dependent is induced, as also shown by Gremmel et al. in 2014 (Gremmel, Koppensteiner, Ay, & Panzer, 2014). Regarding PMVs, no effect C6O4 was appreciated when collagen and AA representing the strongest stimuli, were used. The experimental generation of platelet microvesicles can occurs thanks to the cooperative signals deriving from shear stress, a soluble agonist and the activation of an integrin (either aIIb/IIIa or the α2β1-GPVI complex) (Giacomazzi, Degan, Calabria, Meneguzzi, & Minuz, 2016); (Burger, et al., 2013). When platelets undergo a predefined shear stress alone and are stimulated with ADP and TRAP, a significant amplification of PMVs release is observed. To better understand the specific signalling pathways that are affected by this novel chemical, further studies must be carry out. It is important to highlight that in all the experimental condition we tested, effects of C6O4 were blunted by ASA The generation of TXA2, a crucial step in platelet adhesion, degranulation and partially in platelet aggregation and PMV release, is inhibited by ASA; this can explain what it was observed in the present experiments and is coherent with the ASA inhibitory effects obtained regardless the C6O4 exposition (Giacomazzi, Degan, Calabria, Meneguzzi, & Minuz, 2016); (Taus, Meneguzzi, Castelli, & Minuz, 2019). From a clinical point of view, a higher risk associated to platelet hyperactivity is probably attributable to C6O4 exposed patients. Due to the absence of scientific literature on

the C6O4 toxicology and based on the only available information that were provided by the European Chemical Agency producer, this new compound should have a way shorter half-lives and a reduced bioaccumulation compared to the legacy PFAS; this may exclude the major health worries due to the long serum half-life observed in human for PFOA and PFOS. (Olsen, et al., 2007). If this toxicological profile under analysis were to be confirmed by independent reports on the scientific literature, it might be sustained that, in order to get its biological effect on platelets, the interaction between C6O4 and platelet's membrane is not related to the bioaccumulation in the organism, but rather a short-term interaction might be enough to affect the platelet function.

In summary, the reported results provide the evidence of C6O4 biological effect, leading to a stronger platelet response to agonists. To address if this compound may exert its activity through a specific signalling pathway or primarily by skewing the membrane fluidity whenever a threshold concentration is reached, further investigation need to be carry out. Nevertheless, data suggesting an evident dose-response relationship in C6O4 activity on platelets were not observed. A possible *in vivo* consequence of C6O4 exposure is an increased risk of platelet-dependent thrombosis and potentially a higher risk of cardiovascular events.

6.2.1. K_{Ca}1.1 platelet channel research aim of the study

The study is focused on the expression and function analysis of the large-conductance calcium and voltage-activated potassium channel KCa1.1 in human platelets. Particularly, to investigate the KCa1.1 channel functionality, different channel agonists (BMS191011, NS1619, NS11021) and the epoxyeicosatrienoic acid isoforms 11,12-EET were investigated.

6.2.2. K_{Ca}1.1 platelet channel discussion and conclusion

Performing studies on platelet aggregation using the K_{Ca}1.1 channel activator BMS-191011, a selectivity of action was found, due to its ability to reduce the responses to ADP, but not to the TXA2 analogue U46619, or collagen. Noteworthy, in the presence of PGE1, BMS-191011 enhanced the inhibition of the aggregation response to U46619, implying the interference of the K_{Ca}1.1 channel activation with the cAMP signalling system.

Furthermore, BMS-191011 if co-incubated with ASA, induces a stronger inhibition of ADP-induced platelet aggregation, through an effect that was independent of the inhibition of TXA_2 production caused by ASA, since BMS-191011 did not affect the activity of platelet COX-1. Platelet aggregation induced by ADP is modulated by ASA and BMS191011 with different signalling pathways. It was then necessary to examine if Ca^{2+} signal was implicated in these mechanisms and hence it was measured, not by myself, the free intracellular Ca^{2+} in platelets and in megakaryocytes in relation to stimuli and membrane polarization. The rise in Ca^{2+} induced by ADP is actually reduced regardless of the tested K_{Ca}1.1 openers.

Using the microfluidic apparatus, platelet adhesion and aggregation to immobilized collagen is blunted by all the examined activators of the K_{Ca}1.1 channel and by 11,12 EET. When platelet adhesion under flow was investigated using ASA and the P2Y12 inhibitor Ticagrelor, similar inhibitory effects were observed indicating that platelet adhesion in this experimental condition is intensified by the local release of TXA2 and ADP from platelets. No changes in platelet adhesion and spreading onto immobilized collagen under static conditions in the presence of BMS-191011 were observed, though. Thus, activated K_{Ca}1.1 most probably inhibits platelet adhesion-aggregation induced by the binding to the immobilized collagen under the

physiological shear rate by hampering the ADP or TXA2-dependent amplificatory response. Altogether the results of the aggregation tests allow to conclude that antagonism towards the activity of released ADP accounts for most of the effects observed with $K_{Ca}1.1$ channel openers. These observations describe a specific modulatory role for $K_{Ca}1.1$ in platelets. Previous statement on G-protein-coupled internal corrective K^+ channels and intermediate/small conductance Ca^{2+}-activated K^+ channels in platelets denoted a role for these channels in strengthening of platelet aggregation, secretion, and procoagulant activity, and TXA2 biosynthesis (Shankar, et al., 2006); (Wolfs, et al., 2006).

To conclude, there are now evidence of the $K_{Ca}1.1$ expression and functionality in platelets. Additional investigation is necessary to better define the role of $K_{Ca}1.1$ *in vivo* and the potential of agonists and blockers of the channel in haemostasis and thrombosis. A plausible speculation on the pharmacological activation of $K_{Ca}1.1$ is that it may synergize with antiplatelet agents *in vivo*.

6.3.1. Non-Alcoholic Fatty Liver Disease (NAFLD) research study's aim

The study is focused on the identification of alteration at platelet level in patients Non-Alcoholic Fatty Liver Disease (NAFLD) affected, to corroborate preliminary studies highlighting the presence of a platelets hyperactivity responsible of organ damage platelet-mediated.

6.3.2. Non-Alcoholic Fatty Liver Disease (NAFLD) research study's discussion and conclusion

On this purpose, the application of two different methodologies, Microfluidic and Flow Cytometry, allowed to verify the presence of a higher platelet activity in such subjects, valuating respectively the presence of a pre-activation state and of a condition of activation *in vivo*. Data obtained indicate the presence of higher platelets adhesiveness and aggregability, suggesting the presence of a pre-activation showing an increase in the number platelet deriving microvesicles (PMVs). This was observed both in patients with NAFLD and arterial hypertension, posing for a stronger activation in both groups under investigation compared to healthy controls. The first hypothesis formulated suggests a pre-activation status of platelets of NAFLD and hypertensive patients, leading to a higher platelets adhesiveness and aggregability. To verify such hypothesis, methods able to accurately represent *in vitro* what occur *in vivo* were used. The choice fell on the Microfluidic since in literature there are different publications showing the advantages of this methodology, as a model for *in vivo* thrombus formation. The experiments performed proved the Microfluidic capability on this topic. Particularly, it was demonstrated that this technique shows reproducibility characteristics, since the intra-essay variability is roughly 24% (Graph 5), the inter-essay variability is less than 40% (Graph 6) and it is sensitive seen the ability to modulate with several stimuli, like ADP, ASA and Ticagrelor, the platelets adhesiveness and aggregability (Graph 7). Microfluidic can then be defined as a reliable and sensitive technique to identify changes at platelet activity level.

Based on its properties, Microfluidic is then a technique able to identify *in vitro* a state of increased platelet adhesiveness and aggregability. The results obtained from the performed experiments confirmed what just described and explained in the first

hypothesis, showing an increased platelet adhesiveness in hypertensive subjects (Graph 8) and NAFLD patients (Graph 9) compared to healthy control.

The Microfluidic experiments' demonstrated the presence of a platelet pre-activated state *in vivo*. Even though, some limitations can interfere with the outcome. For instance, the collagen on which platelets adhere, can produce a too strong signal that could partially mask the activation process: the plausible presence of circulating pre-activated platelets can just be a transient event followed by a completed activation, making therefore difficult the detection of pre-activated cells. Consequently, the obtained data, although consistent with the hypothesis, showed some limitations of the technique in the identification of a platelet pre-activated state. Further studies will be necessary to find out new markers able to provide for the lacks of the current methods.

The second hypothesis of a stronger platelet activation *in vivo* in the studied subjects. To examine platelet activation methodologies suitable for our purpose were developed. In the past, the measure of the urine metabolites of Thromboxane A2 (2,3-dinor-TXB2, 11-deidro-TXB2) as a biomarker of *in vivo* platelet activation was often used, although it has been demonstrated that this metabolite presents large inter-individual variability that force to enrol a high number of subjects in the study to have more significant and suitable results. Thus, such method was not applied in this preliminary study, carried out in a small cohort of subjects.

The methods then used is the platelets derived microvesicles count in Flow Cytometry. Several studies demonstrated the platelets derived microvesicles are released from the cells following activation processes; even if this process is still not completely understood and further studies are required, PMVs count seems to be a good marker of *in vivo* platelet activation.

Previous studies demonstrated that Flow Cytometry is a technique suitable for the microvesicles analysis. Experiments performed in this study confirmed the sensitiveness of the technique and more specifically its ability to detect platelet derived microvesicles, distinguishing them from other different cells derived microvesicles, thanks to the employment of specific markers for PMVs such as Annexin V and CD41/CD61 (Graph 10). The investigation of an ex vivo specimen

for the PMVs count, turned out to be reliable to evaluate the platelet activation state in the subjects.

The results obtained demonstrated the presence of a higher number of platelet derived microvesicles in hypertensive patients (Graph 12) and those NAFLD affected (

Graph *14*) if compared with healthy individuals.

Experiments in Flow Cytometry seems to demonstrate the activation of a higher platelets number *in vivo* in the studied patients. Such observation entails the studied subjects present a situation where platelets are more active and are more subjected to activation in the circulation. Thus, those patients are more predisposed to the develop thrombotic events.

The obtained results are in line with data reported in others studies which confirmed, through the measurement of Thromboxane A2 metabolites, the presence of a higher platelet activity in patients with hypertension and diabetes. The present investigation is the first concerning the patients with NAFLD Thromboxane A2 metabolites studies are able to define a positive correlation between the metabolites' values and the seriousness of the pathology taken into consideration (Minuz, et al., 2004). This raises the premise for the development of future studies valuating if it is possible to identify a similar relationship also in platelet derived microvesicles contest; in order to do so, it will be necessary to design studies characterized by a higher number of subjects, stratified according to the severity of the disease. Such studies will help to define the precise mechanisms by which microvesicles are released.

In summary, the present pilot study demonstrates platelet activation in subjects with hypertension and NAFLD compared to healthy controls. These results are, however, represent only preliminary data that need further confirmations. The obtained results, together with the evidences of the platelets role in inflammation and tissue repair, can be the prerequisites to hypothesize that the platelets activation in these subjects can be involved in the physiopathology of the organ damage in NAFLD patients and in hypertensive and diabetic nephropathy. The study was too limited to produce strong evidence of platelet activation in

NAFLD, but represent a model for clinical studies aiming to define the role of platelets in organ damage.

7. Bibliography

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8. Acknowledgment

First of all, I thank my Professor Pietro Minuz, without his supervision, his help but above all his support and his indiscriminate trust, I would not have been able to get to the end of this interesting and complicated journey into the world of PhD and research. I hope that all of his kindness and teaching from him have not been a waste of time and that his efforts have been rewarded. Thank you Prof.

I must thank Doc. Alessandra Meneguzzi as well that has always been ready to help me in calculation, stoichiometry and all those nasty part in the research field. Thanks Ale!

Of course I have to thank Doc. Andrea Dalbeni, Doc. Michele Bevilacqua and Doc. Cristiano Fava which were essential for this climbing toward the title of Doctor of Philosophy, you were amazing!

During my stay in London I had the chance to learn and perform studies I would never thought to do. It was amazing. Such experience was allowed only thanks to Prof. Krista Rombouts that always supervised me with kindness and patience and thanks to Prof. Massimo Pinzani, his generosity and willingness are rare to find elsewhere.

I met a myriad of amazing guys during my trip to London (Alex, Walid, Luca, Domenico, Eli, Marti, Yutaka and Paolo) and to my Winter Schools (Rox, Sion twins, and Gloria).

I cannot mention all LURM staff that willing or not, made my stay nicer and colourful. Just few names: GDC, Sissi, Marilì, Alby senior, Alby junior, Simo, Domenica, Lore, Denise, Fra and Ali. Cheers.

Those that I list here following did not actually contribute with my PhD, but all the coffee breaks were a panacea for my mood and mental health: Maria, Lucas, Angela, Lauretta and “nenna” Ila! Thank you!

Finally, last but not the least, I need to thank my family, a milestone of my life and my girlfriend Sara, the only real one that really had to stand all my bad moments, changing with her love, my scowl into happiness tears. I’m very grateful to you all! Thank you