A network-based approach to elucidate determinants of platelet reactivity in aspirin-treated cardiovascular patients

ZUFFEREY BAKOS, Anne

Abstract

High residual platelet reactivity (PR) in aspirin-treated cardiovascular patients (CV) is at risk for ischemic events. The determinants of this phenotype are unknown but suggested to be genetically determined. PR was assessed in 110 CV patients treated with aspirin 100mg/day. CV patients with extreme high or low PR were selected for ‘omic analysis, characterizing about 1000 platelet proteins and 6000 transcripts differentially expressed. A genome-wide association study of single nucleotide polymorphisms allowed mapping genetic variants between extreme patients. These datasets were integrated using a network biology approach. The total network was 2077 nodes and 722380 edges. We filtered it by selecting correlations observed at least twice among the different datasets. This resulted in a network with 99 nodes and 309 edges. Preliminary analysis showed a significant enrichment of genes involved in platelet activation, cytoskeleton and glucose metabolism. This may delineate new targets for the prevention of ischemic events in CV patients.

Reference


URN : urn:nbn:ch:unige-290118
DOI : 10.13097/archive-ouverte/unige:29011

Available at: http://archive-ouverte.unige.ch/unige:29011

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A Network-Based Approach To Elucidate Determinants Of Platelet Reactivity In Aspirin-Treated Cardiovascular Patients

THÈSE

présentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention interdisciplinaire

par

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de

Anniviers (VS)

Thèse n°4509

GENÈVE

2012
Doctorat ès sciences
Mention interdisciplinaire

Thèse de Madame Anne ZUFFEREY BAKOS

intitulée :

"A Network-based Approach to Elucidate Determinants of Platelet Reactivity in Aspirin-treated Cardiovascular Patients"

La Faculté des sciences, sur le préavis de Messieurs J. C. SANCHEZ, professeur associé et directeur de thèse (Faculté de médecine, Département des sciences des protéines humaines), P. FONTANA, docteur et codirecteur de thèse (Hôpital Universitaire de Genève, Département des spécialités de médecine), D. HOCHSTRASSER, professeur ordinaire et codirecteur de thèse (Section des sciences pharmaceutiques), P.-A. CARRUPT, professeur ordinaire (Section des sciences pharmaceutiques) et Madame A. ANGELILLO-SCHERRER, professeure associée (Service et laboratoire central d'hématologie, Centre hospitalier universitaire vaudois, Lausanne, Suisse), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

Genève, le 20 décembre 2012

Thèse - 4509 -

Le Doyen, Jean-Marc TRISCONE

N.B. - La thèse doit porter la déclaration précédente et remplir les conditions énumérées dans les "Informations relatives aux thèses de doctorat à l'Université de Genève".
A Viktor.

A mes parents.
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REMERCIEMENTS

Tout d’abord, je souhaiterais remercier les Professeurs Anne Angelillo-Scherrer et Denis Hochstrasser pour leur soutien et pour avoir accepté d’évaluer mon travail de thèse.


Un très grand merci à Séverine Nolli. C’est un plaisir de travailler avec toi, tant par la qualité de ton travail que par ta constante bonne humeur et ta motivation à apprendre de nouvelles choses.

Je remercie aussi Jean-Luc Reny. La pertinence de tes réflexions lors de nos discussions ont été une grande source d’inspiration pour le projet, mais aussi dans ma compréhension des plaquettes.

Un très grand merci à Ioannis Xenarios et Mark Ibberson. Le projet n’aurait définitivement pas été le même sans votre contribution. Votre disponibilité (Mark, tu as dû répondre à des centaines de mails de questions) et votre compétence ont rendu cette collaboration particulièrement productive et instructive pour moi.

Je souhaite aussi remercier les Professeurs Henri Bounameaux et Philippe De Moerloose pour leur soutien tout au long du projet, y compris lors des moments de financement qui furent parfois compliqués.

Merci à tous les « patients cardiovasculaires stables » qui ont répondu à notre annonce et ont accepté de participer à cette étude.

Je souhaiterais particulièrement remercier Natacha et Virginie. Votre soutien et nos discussions m’ont été particulièrement précieux durant ces années. C’est un bonheur de travailler avec des amies.

Un grand merci aux locataires du 9028 et à tous les « doctorants & Co. ». Domitille, merci de m’avoir initiée à l’art subtil du gradient de sucre. Vanessa, Xavier, Hui Song, Didia, Francesco, et plus récemment à Cindy, Leire et Florian, merci pour l’atmosphère tellement agréable - et calorifique - du bureau. Merci aussi à Natalia, Florent et Alex pour toutes les bonnes soirées de rires et d’histoires... d’urgences ou de pharmacie. Le week-end à Londres restera un magnifique souvenir !

Merci à tous les autres membres du BPRG. C’est un plaisir de travailler dans une équipe aussi ouverte et compétente.

Merci aussi à mes collègues d’angiologie et particulièrement Sylvie, Karim, Céline, Anne-Sophie et Bert. Votre gentillesse et vos remarques constructives m’ont été d’une grande aide.
Un grand merci aux membres (à vie) du GPG, dont Josianne, Virginie et Youssef. Le dessin d’un logo, un soir de congrès en Italie, a mené à la formation d’un groupe très stimulant intellectuellement, mais aussi à de très agréables soirées, toujours intéressantes culinairement.

Merci aussi à Christian Staub et Olivier, sans qui, je n’aurais jamais tenté cette aventure.

Un grand merci aux plus merveilleux amis du monde. Merci à Nathalie, Béa, (re-)Virginie, Greg, Anais, Harris et Eli, pour nos repas du vendredi et nos soirées chez Loichot ! Et vive les protéines hydrophobes !! Merci aussi à Lisa pour les cafés et les séances de course !

Finalement, un merci particulier à ma famille.

Maman et Papa, merci pour votre soutien inconditionnel et pour m’avoir toujours poussée à rebondir, à ne jamais me reposer sur mes acquis.

Merci à ma très chère « GM ». Tu t’es toujours montrée enthousiaste quant à ma formation. Merci aussi pour les longues soirées passées à essayer de comprendre mon projet et, plus largement, mes études.


Un merci particulier à Aline, Quentin et tout récemment à Julien, ma famille de cœur. Votre soutien et votre gentillesse sont irremplaçables.

Un immense merci à Viktor. Merci d’avoir cru plus que moi, à certains moments, que j’arriverais au bout de ma thèse. Merci pour ton soutien inconditionnel, pour les petits plats après les longues journées de labo, pour être venu me chercher au retour de congrès (donc très souvent, ces derniers temps), merci de trouver « normal » que je travaille autant, bref merci pour tout ce que tu es.
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<td>Two-dimensional gel electrophoresis</td>
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<td>Diacylglycerol</td>
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<td>bp</td>
<td>DNA base pairs</td>
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<td>ESI</td>
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<td>Endoplasmic reticulum</td>
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<td>GPF</td>
<td>Gas-phase fractionation</td>
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<td>Higher energy collision dissociation</td>
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<td>LC</td>
<td>Liquid chromatography</td>
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<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein phosphorylation</td>
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<td>vWF</td>
<td>Von Willebrand factor</td>
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ABSTRACT

Platelets play an important role in the pathogenesis and the complication of atherosclerosis. They are involved not only in the thrombus formation but also in the pathogenesis of atherosclerotic plaques. Platelets may be activated by several agonists, promoting the release of their granule contents, platelet aggregation and also favoring atherosclerotic plaque rupture. This process favors the occurrence of ischemic events such as myocardial infarction or ischemic stroke. Aspirin, the most popular antiplatelet agent, is cornerstone in the treatment and prevention of ischemic event in cardiovascular (CV) patients. It inhibits a particular amplification pathway of platelet activation, based on thromboxane A2 (TxA2) generation. However, despite a consistent inhibition of TxA2 production, a substantial proportion of patients display a preserved platelet function. This phenotype is defined as “high on-aspirin treatment platelet reactivity (PR)”. High PR in aspirin-treated CV patients is a risk factor for recurrence of ischemic events, at least in acute settings such as acute coronary syndromes. The determinants of PR in these patients remain unclear but previous studies mostly including healthy subjects suggested that it is genetically determined.

To better understand this phenomenon, we designed a project aiming to characterize the molecular mechanisms of the modulation of PR in CV patients. PR was assessed twice in 110 stable CV patients treated with aspirin 100 mg/d by light transmission aggregometry using several agonists. A composite variable, called PR index was derived from this analysis, summarizing all the platelet function tests. We showed that PR in CV patients is stable over time and global, as opposed to agonist specific. The PR index was used to rank the subjects and patients located at the extreme end of this phenotype (high vs. low PR patients) were selected for ‘omic analysis. Three complementary strategies were applied. Transcriptomic study as well as quantitative proteomic analysis of platelets and platelet subfractions allowed the characterization of gene products differentially expressed in patients with extreme PR phenotypes. The proteomic analysis was performed on three sample preparations: the whole platelet,
the releasate and the secretory granules. During the development of the secretory granule enrichment method, we took advantage of gas-phase fractionation mass spectrometry method to increase the characterization of the platelet secretory granule proteome reaching more than 800 identified proteins. Finally, a genome-wide association study (GWAS) of single nucleotide polymorphisms (SNPs) allowed mapping genetic variants between extreme patients. These datasets were integrated using a network biology approach, aiming to explore the results at a pathway level rather than at a single gene level. We quantified more than 1000 platelet proteins and about 2000 transcripts. In addition, around 1.5 million of SNPs were characterized. The total network issued from the integration of all these datasets was of 2077 nodes and 722380 edges, representing genes most correlated with the difference of PR in both extreme groups of patients. We filtered it for more detailed analysis by selecting correlations observed at least twice among the different datasets. This resulted in a network with 99 nodes and 309 edges. Preliminary analysis showed a significant enrichment of genes involved in platelet aggregation, cytoskeleton dynamics and glucose metabolism. However, this interpretation must be further conducted in order to identify pathways of interest which will be verified and validated using several specific approaches. This identification of candidate pathways may finally delineate new targets for the prevention and the treatment of ischemic events in CV patients.
RÉSUMÉ

Les plaquettes jouent un rôle prépondérant dans la progression et les complications des lésions d’athérosclérose. Elles peuvent être activées par de nombreux agonistes et, après activation, libérer leur contenu granulaire pour finalement s’agréger, former un thrombus et également rendre les plaques d’athérosclérose plus instables, ce qui favorise la survenue d’événements ischémiques. L’aspirine est considérée comme une pierre angulaire dans la prévention et le traitement des événements ischémiques chez les patients cardiovasculaires (CV). Elle inhibe une boucle d’amplification particulière de l’activation des plaquettes, qui est basée sur la génération de thromboxane A2 (TxA2). Toutefois, malgré une inhibition avérée de la production de TxA2 par les plaquettes, une proportion substantielle de patients traités par l’aspirine présente une fonction plaquettaire préservée. On définit ce phénomène en tant que « réactivité plaquettaire (RP) élevée sous traitement à l’aspirine ». Les patients qui présentent ce type de RP élevée sont à risque en ce qui concerne une récidive d’événement ischémique, en particulier lorsque cette RP élevée est présente en phase aigüe. Les facteurs déterminant la RP chez ces patients demeurent peu connus, toutefois plusieurs études, incluant pour la plupart des sujets sains, suggèrent un rôle prépondérant de la génétique.

Afin de mieux comprendre ce phénomène, nous avons conçu un projet qui a pour but la caractérisation des mécanismes moléculaires responsables de la variation de RP parmi les patients traités par aspirine. La RP de 110 patients CV stables sous traitement d’aspirine (100 mg/jour) fut mesurée 2 fois à 2 semaines d’intervalle par agrégométrie à transmission lumineuse, en utilisant plusieurs agonistes différents. Cette analyse déboucha sur l’établissement d’une variable composite, nommée indice de RP, qui résume les différents tests fonctionnels effectués. Nous avons montré que la RP chez les patients CV est stable dans le temps et globale, c’est-à-dire qu’elle n’est pas spécifique à un agoniste particulier. L’indice de RP fut utilisé pour classer les patients et ceux qui présentaient une RP extrême, soit forte, soit faible, ont été sélectionnés pour de plus amples analyses à haut débit « ’omics ». Trois stratégies
complémentaires furent choisies. Une première analyse combinant les données de transcriptomique et une analyse protéomique quantitative sur les plaquettes entières, le produit de sécrétion des plaquettes et les granules de sécrétion a permis la caractérisation des produits de gènes différemment exprimés chez les 2 groupes de patients avec une RP extrême. Lors de l’établissement du protocole d’enrichissement des granules de sécrétion, nous avons tiré avantage de la méthode de spéctrométrie de masse par fractionnement en phase gazeuse pour améliorer la caractérisation du protéome des granules de sécrétion des plaquettes, l’amenant à plus de 800 protéines identifiées.

Finalement, une étude d’association sur l’ensemble du génome (GWAS) de polymorphismes génétiques (SNP) nous a permis de répertorier les variants génétiques entre les patients extrêmes. Ces données furent intégrées aux données de transcriptomique et protéomique sur la base d’une approche de biologie en réseau (network biology), qui a pour but d’explorer les résultats en tant que voies métaboliques, plutôt qu’en se focalisant sur chaque gène de manière individuelle. Nous avons quantifié plus de 1000 protéines plaquettaires et presque 2000 transcrits. En outre, environ 1.5 million de SNPs furent caractérisés. Au total, un réseau de 2077 gènes et 722380 liens représente les gènes les plus corrélés avec la différence de RP entre les 2 groupes de patients. Nous avons ensuite filtré le réseau en sélectionnant les corrélations observées au moins 2 fois parmi les différents jeux de données, afin d’aller plus loin dans l’analyse. Il en résulte un réseau de 99 gènes et 309 liens. Une interprétation préliminaire montre un enrichissement significatif de gènes impliqués dans l’agrégation plaquettaire, la dynamique du cytosquelette et le métabolisme du glucose. Cependant, cette interprétation doit être poursuivie afin d’identifier les voies d’intérêt qui seront vérifiées et validées par la suite, en utilisant des approches spécifiques. L’identification de voies candidates pourrait finalement aboutir à l’identification de nouvelles cibles pour la prévention et le traitement des événements ischémiques chez les patients CV.
INTRODUCTION
I. Platelets

A. Platelet structure and synthesis in megakaryocytes

Platelets are small cell fragments also called thrombocytes [1]. With a diameter of 2 to 4 µm, they are the smallest component of the bloodstream. They do not contain any nucleus, however they can make extranuclear splicing and translation from a limited batch of mRNA inherited from their cell of origin, the megakaryocyte [2-4]. Megakaryocytes are highly specialized hematopoietic cells [5], which derive from hematopoietic stem cells. They become polyploid (4 to 64N) through several cycles of endomitosis [6-8]. During this phase, the mitosis process is stopped at the late cytokynesis step [8]. In addition, a cytoplasmic maturation occurs involving the formation of demarcation membrane system (DMS) and the accumulation of cytoplasmic proteins and secretory granules [6, 9]. DMS is an extensive network of membrane channels, made of tubules and flattened cisternae (figure 1). It is derived from plasma membrane invagination, being in contact with external milieu. Moreover, it is supposed to function as a membrane reserve for pro-platelet formation and constitutes the open canalicular system (OCS) in mature platelets [5]. Granule number increases with megakaryocyte maturation, as described in more details in the paragraph I.B.

![Figure 1](image_url) "Electron micrograph of a thin section through a mature megakaryocyte having a well-defined demarcation membrane system (DMS). The DMS is a smooth membrane system organized into a network of narrow channels homogeneously distributed throughout the cytoplasm (≈5000). The DMS has been proposed to originate from the invagination of plasma membrane and to function as a membrane reservoir for proplatelet formation or as a mechanism to subdivide the megakaryocyte cytoplasm into "platelet fields." From Michelson AD. PLATELETS, Second Edition. Oxford, UK: ElsevierInc. 2002:1343."
relies on microtubules and actin cytoskeleton [5]. Platelet maturation occurs at proplatelet termini [11], where microtubules form a coiled ring which is then typical of resting discoid platelets [5]. With an average lifespan of 10 days before being phagocytosed by spleen or liver [12], approximately, $10^{11}$ platelets must be produced per day (around 10% of renewing) [8] to ensure an average count of 150 to $400 \times 10^{10}$ platelets [13].

![Figure 2 Megakaryocyte maturation and production of platelets. From Battinelli EM, Hartwig JH, Italiano JE, Jr. Delivering new insight into the biology of megakaryopoiesis and thrombopoiesis. Curr Opin Hematol. 2007;14:419-26](image)

Platelet formation takes place mainly in the bone marrow. Megakaryocytes are found in the extravascular space of sinus endothelial cells. However, megakaryocytes are also present in lungs where they release platelets in the pulmonary circulation [5]. In addition, 5 to 20% of circulating platelets are megakaryocyte fragments which can form platelet-like structures. Bloodstream is thus proposed to be the site of proplatelet maturation [5].

Platelet production is mainly regulated by trombopoietin, which is secreted in the bone marrow environment [5, 8]. However, cell apoptosis [14] and microRNA [15] play also an important role in this process. Indeed, several studies showed that pro-platelets contain active caspase-3 and 9 and their production is decreased upon anti-caspase agents [13]. MicroRNAs are small RNA molecules of around 22 nucleotides. They have both positive and negative effect on
thrompoiesis by acting on numerous different targets at each step of megakaryocyte differenciation (figure 3) [15].

![Figure 3 Role of microRNAs in regulation of megakaryocytopoiesis](image)

**Figure 3 Role of microRNAs in regulation of megakaryocytopoiesis** Red rectangles correspond to microRNAs, which increase during megakaryocytopoiesis, whereas green ones stand for decreased microRNAs. Adapted from Li H, Zhao H, Wang D, Yang R. microRNA regulation in megakaryocytopoiesis. Br J Haematol. 2011;155:298-307.

Mature platelets do not have any nucleus, but a large number of secretory granules. They also contain endoplasmic reticulum and Golgi, as well as translation machinery. In addition, a few mitochondria are also visible in electronic microscopy together with glycogen particles and an important cytoskeleton [5].

**B. Role in haemostasis: importance of secretion**
Platelets are involved in many physiological mechanisms, such as inflammation and wound healing, but also angiogenesis and tumor metastasis [16]. However, the main role of platelets is to stop hemorrhage due to vessel damage by forming a thrombus (primary haemostasis), and triggering the coagulation cascade (secondary haemostasis). When an injury occurs in a vessel, several components of the extracellular matrix are exposed to resting platelets. These components, such as von Willebrand factor (vWF), collagen, laminin, thrombospondin and fibronectin, trigger platelet activation by their interaction with receptors located in the plasma membrane [5]. The pathways triggered upon agonist interactions rely mainly on G-proteins and tyrosine kinase (figure 4). They lead to the cytoskeleton reorganization, cell shape changes and finally the release of secretory granules, to recruit a second wave of platelets [17].
Among these agonists, collagen is the most potent, due to its strong activating effect on platelets and its ability to link vWF [17]. It interacts with 2 receptors, GPVI and the integrin αIIβ1. GPVI triggers a strong tyrosine kinase-based activation pathway, whereas the mode of action of αIIβ1 is still not well understood.

vWF is a large multimeric protein complex synthesized by megakaryocytes and endothelial cells. A small proportion binds to exposed collagen, whereas the rest, released in plasma, interacts with the surface of activated platelets or endothelial cells, by self-association. It is recognized by the glycoprotein Ibα (GPIbα), which is a member of the glycoprotein Ib-V-IX complex and activates a tyrosine kinase cascade [17]. Several other receptors play a role in platelet adhesion. Alpha IIbβ3 (figure 4) is the most abundant integrin and recognizes vWF and fibrinogen. Receptors of vitronectin (αVβ3), laminin (α6β1) and fibronectin (α5β1) seem also involved in this process, but their exact role – probably in term of activation modulation, due to their low density – remains unknown [17].

Platelet activation is triggered mainly either via tyrosine kinase cascades or protein-G transduction (figure 4) [17, 18]. The tyrosin kinase pathway is triggered upon phosphorylation of the activated receptor by a Src family kinase (SFK) member (Fyn or Lyn) (figure 4, left panel). This triggers Syk activation, which phosphorylates several targets ending up to the phosphorylation of phospholypase C γ (PLCγ). Through the intermediary of mitogen-activated protein kinases (MAPK), thromboxane A2 (TxA2) synthesis is promoted and thus amplifies platelet activation.

Alpha IIbβ3 can link fibrinogen thanks to conformational changes and thus, makes bridges between platelets (figure 4) [17]. Four subfamilies of protein-G coupled receptors are expressed in platelets: Gq, G12/G13, Gi/Gz and Gs (figure 4, right panel). They are all coupled to platelet receptors of important agonists in the regulation of the activation process (TxA2 (TP receptor), serotonin (5HT), ADP (P2Y1 and P2Y12 receptors), thrombin (PAR-1 receptor), PGI2 (IP receptor) for example). Despite the large number of receptors and activation cascades, almost all pathways trigger activation of PLC (figure 4), which induces diacylglycerol (DAG) and inositol triphosphate (IP3) production [18]. IP3 then increases intracellular calcium release, which activates numerous targets in platelets, as nitric oxide (NO) synthase and TxA2 production [18]. These activation signaling cascades are associated with a drastic cytoskeleton rearrangement, which induces shape changes and a complete reorganization of plasma membrane receptors [19]. Secretory granules coalesce in the platelet center and are released in the OCS [18], which makes platelet activation irreversible. However, it is not clear yet whether cytoskeleton acts as a barrier for secretion in resting platelets or actively promotes it during its reorganization [19].
The activation process is thus enhanced by an auto- and paracrine secretion. Platelets contain 3 types of secretory granules that are released upon the complex action of the secretory machinery [20]. The most abundant and largest ones are α granules [17]. They are 50 to 80 per platelet and range a size of 200 to 500 nm, which corresponds to around 10% of the total platelet volume. These granules contain many important proteins for thrombosis, such as chemokines, pro- and antithrombotic proteins and activation receptors [21], making their membrane composition close to the plasma membrane one [22]. Depending on the proteins, their cargo is synthesized in megakaryocyte (vWF, platelet factor 4) or endocytosed in the platelet (finbrinogen, albumin, factor V). Moreover, many recent publications suggest that their heterogenous content undergoes differential release depending on the stimulus. For instance, vWF and fibrinogen do not seem to be present in the same granules [23] or in the same part of the granule [24] and are differentially released with anti- and proangiogenic factors, respectively [23, 25, 26]. This sorting seems to rely on different granule structure [17]. Packaging into different zone of the granules [24] and/or different composition in vesicles-associated proteins, notably VAMP-7 may be involved in this process [27]. The second class of secretory granules is the dense granules (also called δ granules). They are smaller and fewer than α granules, with only 3 to 8 granules per platelet [28]. Their content is very different from α granules, with only a few proteins, but a high level of small molecular weight molecules, such as ADP (a major platelet activator), calcium ions, ATP and polyphosphates [17, 29]. Finally, lysosomes (λ granules) are also secreted upon platelet activation. Platelets only have a very few lysosomes (only 1 is often
visible by electron microscopy) [5]. They contain several enzymes, such as cathepsin D and E, carboxypeptidase and galactosidase [28]. Their role in haemostasis is not clear, but they are suggested to play a role in thrombus stabilization, since a defect in lysosome secretion induces a less stable thrombus, as in Hermansky-Pudlack syndrome [30, 31]. Finally, platelet aggregation allows forming a fibrinogen-rich thrombus at the site of the damage. Three mechanisms are involved in this last step, the importance of which depends on the shear stress of the environment, i.e. the kind of vessel and the location where the injury occurs. At low shear stress (<1000/s, veins for example), fibrinogen-αIIbβ3 is the main motor of platelet aggregation, whereas at higher shear stress (between 1000/s and 10 000/s), vWF and fibrinogen are responsible of a reversible platelet aggregate, which is stabilized by agonist secretion. Above 10 000/s (arteries), platelet aggregation relies mainly on vWF-GPIbα [17].

This phenomenon is tightly controlled by the platelets themselves and vascular endothelial cells. NO is produced by endothelial cells from L-arginin and diffuses through the platelet plasma membrane. At low concentration, it is known to stimulate granule secretion (figure 4) [18]. However, at higher concentration, it binds to a soluble receptor called soluble guanylyl cyclase (sGC), increasing intracellular cGMP and stimulating protein kinase G (PKG). This has several effects, among which phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and IP3R-associated to cGMP kinase substrate. This results in the inhibition of intracellular calcium release, dense granule secretion, integrin activation and cytoskeleton re-arrangement [17]. Prostacyclin (PGI2) is a compound secreted by endothelial cells, in response to shear stress increase and TxA2 secretion by platelets. This paracrine secretion binds to a G-protein coupled receptor on the platelet plasma membrane, called prostacyclin receptor (IP), which stimulates adenyl cyclase (AC) and increases cAMP level in the platelet (figure 4). cAMP activates protein kinase A (PKA), which phosphorylates many regulatory proteins, among which VASP [17]. In this context, there is a very fine co-regulation between TxA2 and PGI2 secretion to control haemostasis. Finally, the thrombus is stabilized during wound healing [32]. Mechanisms behind this stabilization are not clearly understood, but they might involve secretion of several proteins in the clot, such as CD40 ligand, soluble CD40, Eph-kinase and growth arrest-specific gene 6 (gas-6). In addition, fibrin makes links between platelets and stabilizes aggregates. Clot retraction occurs upon actin-myosin interaction and move platelets closer, enhancing the stabilization process [33].
C. Platelets and atherosclerosis

Platelets are activated by vessel lesions but also by atherosclerotic plaques in high shear stress conditions and make them more instable. Arterial thrombosis is the most frequent cause of mortality in developed countries [34] and platelets are key-targets for the treatment and the prevention of ischemic events. Indeed, platelets are involved in the development of atherosclerosis (figure 5). Upon inflammation, endothelial cells secrete vWF, which recruits platelets to the site of the atherosclerotic plaque formation (figure 5A) [12, 35], together with adhesive molecules, which stimulate migration of smooth muscle cells and monocytes [36]. In mice, deletion of platelet TxA2 receptor delays plaque development, which seems to enforce that platelets increase their rate of formation [35]. Moreover, activated platelets also release adhesive molecules in the nascent plaque, called fatty-streak (figure 5B), enhancing the effect of endothelium activation. For instance, P-selectin and chemokines activate monocytes, moreover, tissue factors and CD40 increase inflammation and activation of endothelial cells [35]. In addition, platelets release TxA2, which increases inflammation, by its vasoconstricting action and by activating a second wave of platelets. The plaque derives in atheroma, formed from lipids, cells, necrotic debris and connective-tissue components, and becomes irreversible [37]. The artery wall becomes thicker and compensates this phenomenon by gradual dilatation [36]. The lesion is then covered by a fibrous cap (figure 5C). Ruptures of atherosclerotic plaques occur by ulceration or erosion, under the effect of inflammation and/or enzymes released by immune cells. It triggers the formation of a thrombus, which can occlude the artery (figure 5D) and results in infarction [36].
D. Platelets and antiplatelet drugs

Since platelets play a major role in atherosclerosis and thrombus formation, antiplatelet agents belong to the first line of treatment in cardiovascular diseases [38].

Aspirin (acetylsalicylic acid) is the oldest anti-platelet drug used in cardiovascular (CV) disease treatment [39]. It irreversibly acetylates platelet Cox-1 serine 529 and thus inhibits paracrine and autocrine TxA2 production and thus impairs platelet activation [5]. A low dose of aspirin (75-325mg/day) is usually prescribed because it induces a complete inhibition of platelet Cox-1, whereas cyclooxygenase in endothelial cells presents a minimal level of acetylation due to its higher turnover. In addition, Cox-2, which is predominantly expressed in endothelial cells, presents a limited level of acetylation at low dose of aspirin. Moreover, low doses of aspirin minimize side effects on the gastrointestinal tract [38]. Several clinical studies showed that aspirin is associated with a relative decrease of 25% in the risk of vascular death, stroke and myocardial infarction in high risk patients [40].

Clopidogrel belongs to the thienopyridines family, which are pro-drugs metabolized by several cytochromes P-450 (CYP450) in the liver into an active compound. It acts on the ADP receptor P2Y12 by covalent modifications of two cysteine residues. As previously mentioned, the P2Y12
receptor is important for the amplification of the platelet activation process [38]. However, 85% of the pro-drug is first metabolized by esterase before CYP450 in an inactive component [38]. The necessity of drug activation is associated with a delay between drug intake and antiplatelet effect, which is partially overcome by the administration of a loading dose (600 mg). At the maintenance dose of 75 mg/day and without an initial loading dose, the maximal pharmacodynamic effect is reached between day 5 and 7 [41].

Patients at high risk of ischemic event (for instance after an acute coronary syndrome and/or percutaneous coronary intervention) are usually treated by a dual anti-platelet therapy with aspirin and an anti-P2Y12 drug for a duration between 1 and 12 months. Even though it combines advantages of both drugs, the efficacy of this treatment may be limited by compensatory platelet activation pathways partially restoring platelet reactivity [38]. Contrary to acute settings, the dual antiplatelet therapy is generally not recommended in stable disease and most of stable cardiovascular patients are on aspirin as their sole antiplatelet drug [41]. The delay and the variability of the pharmacodynamic effect of clopidogrel promoted the development of more efficient drugs, particularly affecting the P2Y12 receptor, such as prasugrel, the third generation thienopyridine drug and ticagrelor, a non-thienopyridine anti-P2Y12 drug.

Other platelet function players are targeted by antiplatelet drugs. Integrin αIIbβ3, for instance, is antagonized by several compounds (eptifibatide, abciximab or tirofiban), which are administered intravenously. These treatments are often prescribed to patients in acute clinical situations [38, 41]. Phosphodiesterase inhibitors, as cilostazol and dipyridamole, raise levels of cyclic adenosine monophosphate, inhibiting platelet activation [41]. These latter drugs have specific side effects that limit their use in daily practice.

E. Evaluation of platelet reactivity: platelet function assays

Several ex vivo platelet function assays exist to assess platelet reactivity. Regarding the evaluation of platelet reactivity in patients with antiplatelet drugs (on-treatment platelet reactivity), we can divide platelet function assays into 2 categories: 1) assays that are specific for the antiplatelet drug target and 2) assays that evaluate platelet reactivity more globally, using platelet agonists not directly dependent of the drug target. The categorization of platelet function assays is described in Table 1. Pre-analytical parameters are of utmost importance when assessing platelet function [42]. For instance, blood container should be polypropylene to avoid platelet aggregation on the tube wall. Temperature of storage plays also an important
role, as well as the anticoagulant (often citrate), the needle size, the presence of a tourniquet and the necessity to discard the first ml of the collected blood [42, 43].

The most common method for assessing platelet function is light transmission aggregometry (LTA) [42]. Platelet-rich plasma (PRP) is obtained from whole blood by centrifugation (typically, 10 minutes at 150g). A small volume of PRP is poured into a cuvette containing a magnetic stir bar and then placed in the aggregometer, where PRP is stirred by the bar at 37°C. A ray of light goes through the PRP and a detector measures the decrease of turbidity after the addition of a platelet agonist. Several agonists can be tested simultaneously by using several cuvettes, one for each agonist. Epinephrine, ADP, collagen, thrombin receptor-activating peptide (TRAP), U46619 (mimetic of TxA2) and arachidonic acid (AA) are the most commonly used agonists [42]. The results of LTA can be influenced by plasma lipid levels and platelet count (below 100 x 10^9 platelets per ml the result is not reliable). ADP is considered as a weaker agonist when compared to other agonists such as collagen or TRAP. In a low calcium concentration medium such as in citrated PRP, ADP usually induces an irreversible aggregation profile due to the artefactual generation of TxA2 [44]. Thus, at least when using a relatively low concentration of ADP (10 µM or lower), aspirin intake impairs the second and irreversible wave of the aggregation profile. A reversible aggregation profile is also obtained when treated with an anti-P2Y12 drug such as clopidogrel. Epinephrine is also a weak agonist, which strongly requires TxA2 production to induce aggregation [42]. It is often considered as an activation “potentiator” more than an agonist [45]. TRAP is also called SFLLRN, corresponding to a peptide sequence which activates the PAR-1 thrombin receptor. Its effect poorly relies on TxA2 and is considered as almost not sensitive to the aspirin effect. Collagen induces an aggregation characterized by a prolonged lag time and it also necessitates TxA2. As previously detailed, AA is converted to PGH2 by Cox-1, which is the substrate of thromboxane synthase that produces TxA2 in platelets. AA-induced platelet aggregation is thus abolished by aspirin, whereas U46619, which mimics TxA2, bypass Cox-1 and induces platelet aggregation independently of aspirin treatment [42]. Platelet aggregation can also be measured in diluted whole blood by impedance aggregometry [42]. Two electrodes are placed into the sample, and activated platelets adhere to them upon the addition of an agonist. The system records the extent and rate of increase in impedance. This assay avoids preparing PRP, but is also sensitive to low platelet counts.

Platelet Function Analyzer-100 (PFA-100®, Siemens, Marburg, Germany) is a commercial automated device, which also allows using whole blood [42]. In a cartridge under vacuum, sample is forced to go from one compartment to another through a hole in a nitrocellulose membrane coated with a combination of agonists (collagen-epinephrine or collagen-ADP).
Agonists and high shear stress trigger platelet aggregation. PFA-100® then measures the time necessary for the aperture to be closed by the platelet plug (closure time). This test is rapid and requires a small volume of sample, but it is influenced by high concentration of anticoagulant (usually citrate), platelet count and hematocrit [42].

The test can also be performed by mixing whole blood with beads coated with agonists. The VerifyNow assay (Accumetrics, San Diego, USA) is based on light transmission variations due to platelet agglutination on fibrinogen-coated beads. It is a bedside test and several specific cartridges allow evaluating the biological response to aspirin, anti-P2Y12 and anti-αIIbβ3 [46].

Flow cytometry can also be used to assess platelet function, with the advantages of using very small amount of material, less sensitive to thrombocytopenia and using whole blood or PRP. Platelets are generally hybridized with 2 antibodies conjugated with different and compatible fluorochromes, against a platelet identifier and an epitope related to the pathway of activation of interest. Then the cytometer measures fluorescence with a laser beam and the system records the proportion of both events. Platelet activation can be monitored using antibodies detecting αIIbβ3 conformational changes, such as PAC1, or the secretion of P-selectin [42]. Despite a low volume of whole blood needed for this test, it requires complex sample preparation and experienced technicians [47]. There is still a commercial standardized flow cytometry kit (PLT VASP/P2Y12, Biocytex, Marseille, France) that is designed to specifically assess the inhibition of the P2Y12 receptor through evaluation of the phosphorylation status of the Vasodilatator-stimulated phosphoprotein (VASP) and is largely used to assess the potency of anti-P2Y12 drugs such as clopidogrel.

Assessment of serum thromboxane B2 (TxB2) level, the stable metabolite of TxA2, is a specific way to evaluate platelet ability to produce TxA2. It is particularly appropriate to test aspirin potency [42, 46]. This can be done by ELISA in serum samples, in which platelets release TxA2 under endogenous thrombin.

This list is not exhaustive but highlights the variety of platelet function tests and their limitations. This explains also the large variations regarding the incidence of antiplatelet drug poor responsiveness (see next paragraph).

F. Biological variability in antiplatelet drug response

Using platelet function tests described above, several studies showed that the biological response of antiplatelet drugs can vary between patients and a significant proportion of treated patients are deemed “non responders”, “poor responders” or “resistant” since their platelet reactivity is similar to the one of patients without antiplatelet drug treatment [48, 49]. Even
though the lack of compliance is the most common cause of non response, the variability of on-treatment platelet reactivity still exists in documented compliant patients [48]. The term “resistance” to a drug should be used when a drug is unable to hit its pharmacological target [49] i.e. when aspirin is unable to inhibit platelet-derived COX-1-dependent TxA2 production, or when clopidogrel is unable to inhibit the platelet P2Y12 receptor. This definition refers to specific assays (Table 1). Since there is no validated universal cut-off value for the definition of “resistance”, the term “poor response” is more in line with the continuous biological response. [50]. The term “high on-treatment platelet reactivity” relates more to platelet function assessed with non-specific assays that provide a more global evaluation of platelet reactivity (Table 1).

The prevalence of high on-treatment platelet reactivity in aspirin-treated patients has been described as very heterogeneous and largely dependent on the biological tool used to assess aspirin potency. Indeed, the heterogeneity of this prevalence relates at least in part to the specificity of the method in assessing aspirin inhibition of TxA2 synthesis by platelets. When using specific assays, the prevalence of poor aspirin response is very low demonstrating that Cox-1 is largely inhibited in the vast majority of the patients [49]. When using non specific assays, the prevalence of high on-treatment platelet reactivity is much higher averaging 30% in aspirin-treated patients [49].

Little is known about the determinants of high on-treatment platelet reactivity in CV patients treated with aspirin. A study in healthy subjects showed that platelet hyperreactivity is found in 14% of this population of more than 300 volunteers [51]. Moreover, it has been shown that this phenomenon in hereditary, global (not agonist-specific), independent of aspirin intake, stable over time and barely affected by cardiovascular risk factors [51-54]. The strong hereditary feature of platelet reactivity in subjects treated with aspirin points to a genetic control modulating platelet reactivity.

The biological variability of clopidogrel response was mostly investigated with relatively specific assays and around 30-40% of clopidogrel-treated patients are deemed poor responders [55]. The causes of clopidogrel non response relate to the variability of the activation of the pro-drug through liver cytochromes. Although this phenotype was shown to be highly hereditary [56], clinical determinants also play a minor role [57, 58].
Platelet function assays | Assay specificity for the drug target
---|---
| Aspirin | Anti-P2Y12 drugs |
| LTA or whole blood aggregometry (agonist): | |
| AA ± specific | Non specific |
| ADP Non specific | ± specific |
| Col Non specific | Non specific |
| U46619 Non specific | Non specific |
| TRAP Non specific | Non specific |
| Epi Non specific | Non specific |
| Serum TxB2 Specific | Non specific |
| Flow cytometry (VASP assay) Non specific | Specific |
| VerifyNow® ASA cartridge ± specific | Non specific |
| VerifyNow® P2Y12 cartridge Non specific | ± specific |
| PFA-100® Col/Epi cartridge Non specific | Non specific |
| PFA-100® Col/ADP cartridge Non specific | Non specific |

Table 1 Categorization of platelet function assays commonly used for the evaluation of antiplatelet drug potency.
The terms “resistance” or “poor response” refer to specific assays (that evaluate the target of aspirin or anti-P2Y12 drugs) and the term “high on-treatment platelet reactivity” or “high platelet reactivity” refers to non specific assays (assay that is barely or not affected by the antiplatelet drug).

G. Platelet reactivity as a determinant of recurrence of ischemic events in cardiovascular events

Increased platelet reactivity in aspirin-treated patients was repeatedly associated with recurrence of ischemic events, at least in acute event settings such as acute coronary syndromes (ACS) or percutaneous coronary intervention [59-61] while it does not seem to affect cardiovascular outcome in stable patients [62]. Interestingly, it has been suggested that, in order to identify cardiovascular patients at risk of ischemic events, platelet reactivity should be evaluated with a panel of methods exploring different aggregation pathways such as AA, ADP and collagen-induced platelet aggregation [63]. Altogether, these data suggest that platelet reactivity modulates the risk of recurrence of ischemic events in cardiovascular patients, independently of the method of platelet function evaluation.

The identification of the modulators of platelet reactivity is of utmost importance since it may delineate new targets for the prevention of recurrence of ischemic events, and help to tailor the therapy according to the characteristics of each patient.

In conclusion, an evaluation of the platelet reactivity of patients under aspirin prescription could help to optimize their treatment. Moreover, a better knowledge of the molecular mechanisms responsible of high on-treatment platelet reactivity could allow finding new therapeutic targets for anti-platelet medication.
II. ‘omics strategies in the frame of platelet reactivity

Recent improvements in bioinformatics, high-throughput genetic, transcriptomic and proteomic techniques have advanced our knowledge in platelet physiology. These techniques allow testing several samples at the same time for hundreds of gene products, considering complex processes that underlie platelet function [64-66].

A. Genetics of single nucleotide polymorphism

Single nucleotide polymorphisms (SNPs) are single DNA base pairs (bp) where several alleles exist in normal subjects of a given population [67]. Moreover, they correspond to around 90% of human genetic polymorphism [68]. In humans, SNPs are in a large majority bi-allelic, meaning that nucleotide X is exchanged by Y (X \ \rightarrow \ Y). The most abundant SNP is the C \ \rightarrow \ T variety, probably partially due to the frequent reaction of 5-methylcytosine deamination [67]. The typical frequency of SNP is 1 for 1000 bp and at least 1% of the studied population. However, the frequency of SNPs can drastically vary in given region of the genome [68], such as some HLA non-coding regions, which are found as “hotspots” [67]. The reasons for this non-random SNPs distribution are not fully understood, but several factors are known to be associated with SNPs rate, such as the recombination frequency and microsatellite occurrence [68].

Platelet reactivity has been shown to vary between individuals, but is strongly inherited, implying a genetic contribution to platelet function [53, 69, 70]. Several genetic studies were made focusing on rationally chosen genes (targeted approach), which are known to be involved in platelet function (see table 2) [71-73]. In this context, the choice of SNP is of high importance to ensure reproducibility and should be done according to possible linkage and haplotype definition [72]. In addition to the biological test used to assess platelet reactivity and other parameters are crucial in such studies. The size of the cohort included in the project must be large enough to guarantee its statistical power. The ethnicity of the cohort is also highly important, because of “ethnic outliers” that may bias the results. Finally, SNPs are often linked into haplotypes and thus, a single SNP does not systematically represent the causative genetic variation [72]. Functional genomics is based on technical improvement in DNA sequencing and allowed to test many SNPs from panels of targeted genes on larger cohorts (see table 3) [71].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein identification</th>
<th>Chromosomal location</th>
<th>dbSNP or Chr. location</th>
<th>MAFa</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADRA2A</td>
<td>α2A-adrenergic receptor</td>
<td>10q24-q26</td>
<td>chr10: 112839380b</td>
<td>0.41</td>
</tr>
<tr>
<td>F2R</td>
<td>Coagulation factor II (thrombin) receptor; protease-activated receptor 1 (PAR-1)</td>
<td>5q13</td>
<td>rs168753</td>
<td>0.14</td>
</tr>
<tr>
<td>FCGR2A</td>
<td>IgG Fc receptor type IIa</td>
<td>1q23</td>
<td>chr1: 161479745c</td>
<td>0.44</td>
</tr>
<tr>
<td>GNB3</td>
<td>Guanine nucleotide-binding protein β-3 subunit variant</td>
<td>12p13</td>
<td>rs5443</td>
<td>0.45</td>
</tr>
<tr>
<td>GP1BA</td>
<td>Platelet glycoprotein Ibα</td>
<td>17pter-p12</td>
<td>rs2243093</td>
<td>0.15</td>
</tr>
<tr>
<td>GP6</td>
<td>Platelet glycoprotein VI</td>
<td>19q13.4</td>
<td>rs1613662</td>
<td>0.16</td>
</tr>
<tr>
<td>ITGA2</td>
<td>Integrin subunit α2</td>
<td>5q23-q31</td>
<td>rs1126643</td>
<td>0.38</td>
</tr>
<tr>
<td>ITGA2B</td>
<td>Integrin subunit αIIb</td>
<td>17q21.32</td>
<td>rs5911</td>
<td>0.41</td>
</tr>
<tr>
<td>ITGB3</td>
<td>Integrin subunit β3</td>
<td>17q21.32</td>
<td>rs5918</td>
<td>0.17</td>
</tr>
<tr>
<td>P2RY1</td>
<td>Purinergic receptor P2Y1</td>
<td>3q25.2</td>
<td>rs1065776</td>
<td>0.05</td>
</tr>
<tr>
<td>P2RY12</td>
<td>Purinergic receptor P2Y12</td>
<td>3q24-q25</td>
<td>rs809699</td>
<td>0.14</td>
</tr>
<tr>
<td>PTGS1</td>
<td>Prostaglandin-endoperoxide synthase 1; cyclooxygenase-1 (COX-1)</td>
<td>9q32-q33.3</td>
<td>rs842787</td>
<td>0.06</td>
</tr>
<tr>
<td>TXA2R</td>
<td>Thromboxane A2 receptor</td>
<td>19p13.3</td>
<td>rs1131882</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Genome-wide association study (GWAS) is a non-targeted method allowing automated screening of the whole genome for SNPs, using commercial chips. This approach has been applied to platelet characteristics, such as mean platelet volume or platelet count [72, 73]. The first GWAS meta-analysis of platelet function was published in 2010 [74]. Two European ancestry cohorts of 4000 subjects in total were tested for aggregation to epinephrine, ADP and collagen. Seven loci were found to be associated to platelet aggregation results in both cohorts with variable effect depending on agonists (see table 4). These loci were also tested in an independent cohort of African ancestry and all but one could be replicated in this latter cohort.
Several genes are found in common using these approaches, despite SNPs are different (even affecting the same locus): PEAR1, GP6 and ADRA2A. Platelet endothelial aggregation receptor-1 (PEAR1) is phosphorylated upon platelet activation and takes place in amplification of αIIbβ3 activation [75]. It has been shown to be related to epinephrine response, but also to ADP and collagen responses [74]. Glycoprotein VI (GP6) is a collagen receptor and, as expected, is associated with collagen activation. The reported SNP (producing a H322N) may decrease the interaction of GPVI with its downstream effectors, Fyn and Lyn pathways and thus collagen response [73]. Adrenoceptor α 2A (ADRA2A) is the major epinephrine receptor in platelet [73]. It is thus biologically coherent to find it related to epinephrine-induced aggregation [74]. These examples corroborate each other and enforce the plausibility of an influence of these SNPs on
platelet reactivity. SNPs allow having a different point of view on platelet reactivity, revealing new key regulators or new functions of known regulators. In this context, GWAS represents a tool of choice to increase cohort size for large-scale screening.

B. Transcriptomics

Platelets are anucleated cell fragments, but they contain rough endoplasmic reticulum (ER) and ribosomes. Several studies showed that protein synthesis occurs in platelets [3, 76]. Moreover, platelets contain a stable pool of mRNAs, which is involved in platelet function and life-span, hemostasis and inflammation [77]. In addition, this pool decreases with the platelet age and is thus an indicator of platelets turnover [77]. mRNA is estimated of around 5000 different transcripts in platelets [77] (in line with the present work [78]) and covers approximately half of the megakaryocyte transcriptome. The content of mRNA also varies with platelet activation or certain diseases, such as systematic lupus erythematosus [77, 79]. Platelet mRNAs are translated in different manner depending on the final protein and its role (figure 6). A small number of mRNAs are highly abundant and constitutively translated into proteins. These include actin, αIIbβ3 and vWF (figure 6.1). On the other hand, B-cell lymphoma protein-3 (Bcl-3), which is involved in clot retraction, is translated upon thrombin activation and under mammalian target of rapamycin (mTOR) regulation, as shown in figure 6.2. Thrombin activation also increases synthesis of continuously translated proteins, such as plasminogen activator inhibitor (PAI-1). Finally, protein synthesis can also occur by a functional spliceosome, which has been found in platelet [4]. Indeed, pre-mRNAs exists in platelets and are spliced upon platelet activation (figure 6.3). Tissue factors and interleukin 1β are examples of such regulation.

These different mechanisms of regulation are made possible by a strong interaction of mRNAs and protein synthesis machinery with cytoskeleton and the presence of translation factors, such as protein eukaryotic initiation factor, which is constitutively expressed. Platelet activation triggers a drastic cytoskeleton remodeling, which changes the localization of the different partners of protein synthesis.

An important regulation way of mRNA in platelet is microRNA (miRNA). These small nucleotides (around 22 bp) can induce mRNA degradation, delay or promote translation [80]. Several mRNAs and their modulating microRNAs were recently associated with PR in healthy subjects [81]. Among the 284 miRNAs expressed by platelets, 74 were differentially expressed in association with different PR to epinephrine. These data were combined to quantitative transcriptomic results on the same cohort, to obtain a list of couple micorRNA-mRNA with binding site at the
3’ untranslated region (UTR) of mRNA. Among them, 3 pairs were interesting in term of role and could be validated at the level of protein expression.

The transcriptome was tested in the context of platelet reactivity. Two-hundred-and-eighty-eight healthy individuals were assessed for RNA expression using microarray [82]. VAMP8/endobrevin was found as associated with high platelet reactivity in LTA, being higher in high platelet reactivity subjects. In addition, a SNP, rs1010, and a miRNA, miRNA-96, were shown as key player of VAMP8 modulation. Since VAMP8 is a v-SNARE involved in the targeting and fusion of secretory granules to plasma membrane, this study links platelet reactivity variations with granule release.

![Figure 6 The different mRNA regulation pathways in platelets.](image)

C. Proteomics

The proteome is defined by a cellular or tissue protein content at a particular moment. It covers a dynamic range of $10^6$ to $10^{11}$ in human cells and biofluids, respectively [83]. Proteomics is the domain of molecular biology, which studies and characterizes proteomes. A typical proteomic workflow implies 4 phases (figure 7): sample preparation, sample separation, liquid chromatography (LC) mass spectrometry (MS) or tandem-MS analysis and protein identification and/or quantification by bioinformatics. Finally, a last step consists in results validation using an orthogonal method. In most cases, “bottom-up” shotgun proteomics is used: trypsin digestion prior to LC-MS/MS sequencing and identification. The workflow can be designed for
identification of a protein dataset or to quantify differences among 2 conditions [78, 84]. In every case, reproducible sample preparation, in term of cell lysis and proteins solubilization, is of high importance and will influence accuracy and precision of the results.

Samples can be fractionated to decrease dynamic range and thus increase the sensitivity of the MS/MS analysis. This can be done by focusing on a given organelle (subcellular fractionation [85-87]). It is also possible to concentrate on a subset of proteins presenting the same physico-chemical property (LC, multidimensional protein identification technology (MudPIT), which combines several chromatographic separations [88] or combined fractional diagonal chromatography (COFRADIC) [89]) or the same affinity for a given component (an example is nucleotide affinity chromatography [90]). There are also in-gel separations, such as sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) or 2-dimensional gel electrophoresis (2D-gel).

Finally, off-gel electrophoresis (OGE) allows an isoelectric separation and proteins are recovered in solution for further analysis [91].

In addition, platelets have also the ability to aggregate, which allows separating platelet releasates (the supernatant which include the content of secretory granules) from the platelet plug [92] and constitutes a subcellular fractionation.

Trypsin is used in the vast majority to perform protein digestion. Indeed, trypsin cuts proteins after lysine or arginine residues (excepted when preceded by a proline) with high site specificity. It produces basic peptides, which favor an efficient ionization process. However, other enzymes can be used in particular cases, such as endoproteinase Glu-C for glycated proteome studies [93]. Moreover, depending on the physico-chemical properties of the sample, digestion protocols can be optimized by adding organic solvents, chaotropes, surfactants or using physical methods, such as sonication [84, 87]. After digestion, peptides can be fractionated using the same methods than proteins to decrease sample complexity and thus increase proteome coverage [83, 89].
Mass spectrometers are composed of 3 components [94]. The source is the part where peptide ionization occurs, either in solid phase (matrix-assisted laser induced desorption ionization (MALDI)) or in solution (electrospray ionization (ESI)). ESI is used most of the time due to its easiness to couple with LC and its production of multi-charged ions. Then the mass analyzer separates ions, which are finally reported by the detector [94]. MS/MS (also called tandem MS) is based on 2 sequential MS analysis, either in time (in the same analyzer) or in space (in 2 analyzers). The first analysis allows selecting precursor ions, which are then fragmented during the second analysis. Among the different tandem mass spectrometers on the market, the most sensitive and accurate one is the LTQ Orbitrap [95]. This hybrid instrument is composed of a linear trap quadrupole (LTQ), where precursors are separated, and of a C-trap, which injects ions.
into the orbitrap. This last allows peptide analysis based on Fourier transform with very high resolving power and sensitivity [95]. In addition, it permits several types of peptide fragmentation, among which collision induced dissociation (CID) and higher energy collision dissociation (HCD). They may be used for peptide identification and isobaric quantification, respectively, as explained below [96]. Fractionation of ions is also possible in the mass spectrometer itself using an approach called gas-phase fractionation (GPF) [97, 98]. During each injection of a given sample, only a window of mass-to-charge ratios (m/z) is considered for precursor selection. This allows considering only a subset of the total ions and thus increases the number of fragmented precursors. However, it necessitates several injections of the same sample to cover the whole m/z spectrum.

Finally, MS spectra are analyzed by bioinformatics tools. The most largely used method is peptide fragment fingerprint (PFF), which consists in a direct comparison of the experimental spectrum with theoretical sequences in a database. Statistical models allow evaluating the level of confidence of the retrieved match [94]. Several available tools exist, such as Sequest®, Mascot® or EasyProt [94, 99].

Numerous quantitative approaches suitable for cell lines (metabolic labeling as stable isotope labeling by amino acids in cell culture (SILAC) [86, 100]), biofluids or tissue (chemical labeling [83]) were developed the last decade. Among the chemical labeling, isobaric relative quantification is a highly versatile method [101, 102]. It relies on the peptide derivatization by tags. It labels free amino-terminus groups and lysine. Each peptide sample to be compared is labeled with a different tag (figure 8). All tags produce isobaric derivatization, which allows mixing different samples together for optional peptide separation [103] and LC-MS/MS analysis. This decreases the technical variation of the analytical workflow. During MS analysis, tags are fragmented (often by HCD) and fragments appear as different peaks in MS spectra, whom intensity permits relative quantification. On the other hand, peptide CID fragments are used to identify the corresponding peptide [104]. Isobaric tag for relative and absolute quantification (iTRAQ) allows the simultaneous quantification of 8 different samples, whereas tandem-mass-tag (TMT) enables 6 quantifications per experiment (figure 8).

Platelets were extensively analyzed by proteomics, as explained in details in chapter 3 ([66, 105]). Indeed, since platelets are enucleated and contain a limited amount of mRNA, their proteome is a target of interest to study their physiology. Moreover, there is a low recovery between platelet proteome and transcriptome [2, 64], which highlights the benefit of combining several strategies to learn more about platelets.
Figure 8 TMT labeling workflow. Six samples are labeled with 6 different isobaric tags (1), which allows their pooling (2) and common MS analysis (3). Spectra HCD and CID are merged by bioinformatics to enable protein identification and quantification (4). Adapted from Dayon L, Hainard A, Licker V, Turck N, Kuhn K, Hochstrasser DF, et al. Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. Anal Chem. 2008;80:2921-31

D. Integrative approach: network biology

The molecular biology paradigm assuming an unidirectional and direct relationship between proteins has been recently challenged by the emergence of network biology paradigm, which takes into account the contextual links between gene products, but also other molecules (figure 9) [106]. Indeed, a linear pathway implies that downstream function unilaterally affected by upstream modulation, but not the opposite. Network biology goes beyond this linear pathway representation with a view allowing the representation of mutual influences between interactors. It is depicted as nodes representing molecules of interest (gene products, but also co-factors, small molecules or drugs), which are linked by edges standing for any kind of direct and indirect relationship (physical interaction, functional connection or expression level correlation). These networks are thus at the interface between genotype and phenotype [106]. Therefore, they necessitate a more global point of view on biological processes (achieved by large scale quantitative ‘omics methods) and the development of new approaches and new tools to integrate datasets of different origins.
Considering pathways instead of single gene products as being affected between 2 phenotypes is particularly adapted to dissect fine metabolic modulations particularly in experimental settings associated with high biological variation [107], as with human samples. Moreover, network biology better reflects the physiological situation, where the modulation of a given molecule of interest affects many different factors, topologically visible as clusters (figure 9). This integration allows exploiting the complementary aspects of different datasets, going one step further than considering common gene product regulation among mRNA and proteins. Known protein-protein interactions and pathway databases information can also be used to weight experimental edges and complement the network. Then, interpretation of the network can be done using gene set or gene ontology (GO) enrichment analysis [108] or bioinformatics tool [109]. Finally, validation of such results can be done in vivo or using biological models, reproducing the same phenotype by modulating the pathway of interest [106]. This approach has never been applied to platelet reactivity characterization.

Figure 9 Pathway visualization. Molecular biology (left scheme) stands as linear downstream relationships among proteins, whereas network representation (right scheme) includes different molecules of interest and types of bidirectional links. Adapted from Bensimon A, Heck AJ, Aebersold R. Mass spectrometry-based proteomics and network biology. Annual review of biochemistry. 2012;81:379-405.
III. References

Chapter 1


IV. **Aim of the project**

Platelets are key-players in haemostasis, and are at the forefront of the pathogenesis of atherothrombosis as well as the occurrence of arterial ischemic events. In this context, aspirin is the most prescribed anti-platelet drug. However, despite a pharmacological effectiveness of the drug (inhibition of TxA2 production), a substantial proportion of patients have a preserved platelet function, defining high on-treatment platelet reactivity. The precise cause of this phenomenon in cardiovascular patients is largely unknown, but high on-treatment platelet reactivity has been shown to be heritable, stable over time and global (as opposed to agonist-specific) in healthy individuals.

**Working hypothesis:** Aspirin-treated cardiovascular patients with extreme on-treatment platelet reactivity phenotype are characterized by differences in the expression of gene products involved in relevant platelet activation pathways.

**Aim:** The aim of this project is to integrate the phenotype – platelet reactivity – with genetics, transcriptomics and proteomics characterization of cardiovascular patients with extreme platelet reactivity using a bioinformatic network biology approach, in order to identify candidate pathways modulating this phenotype.

The chapter 2 is a research article published in *Thrombosis and Haemostasis*. It describes the characterization of platelet reactivity (PR) in a population of 110 consecutive cardiovascular patients treated with aspirin 100 mg/day and attending two visits, 2 weeks apart for the evaluation of PR. This clinical work allowed selection of patients with extreme high or low platelet reactivity. These latter patients attended a third visit for the collection of samples dedicated to proteomics analysis. The chapter 3 is a review published in *Mass Spectrometry Reviews*, which describes state-of-the-art and recent advances in the field of platelet proteomics. It also mentions several pitfalls and quality controls for such type of experiment.

An important step of this project was to set up the subcellular fractionation to recover secretory granule-enriched fraction for proteomic analysis. This procedure is presented in the chapter 4, with the different quality controls applied in this experiment and the advantages of proteomic analysis of this subcellular fractionation compared to analysis of the whole platelet proteome.

The chapter 5 describes a preliminary interpretation of a first series of patients’ proteomic results. Secretory granule-enriched fractions from 6 patients (3 low vs. 3 high platelet reactivity patients) show possible involvement of cytoskeleton dynamics in the PR phenotype.
The final aim of this work is the identification of platelet activation pathways that modulate PR in aspirin-treated cardiovascular patients using a network biology approach. The chapter 6 describes the integrative approach of data issued from transcriptomics, genetics and proteomics together with the PR phenotype that is used to build the network. Chapter 6 also shows the candidate pathways derived from the network analysis that are most likely implicated in PR modulation. Finally, the chapter 7 is a discussion of the results and describes examples of future experiments that will be developed to validate the pathways of interest.
PLATELET REACTIVITY IS A STABLE AND GLOBAL PHENOMENON IN ASPIRIN-TREATED CARDIOVASCULAR PATIENTS
PLATELET REACTIVITY IS A STABLE AND GLOBAL PHENOMENON IN ASPIRIN-TREATED CARDIOVASCULAR PATIENTS

This chapter is a research article published in “Thrombosis and Haemostasis” (IF = 5.044) in 2011.

The first step of the project was to characterize platelet reactivity (PR) in cardiovascular patients. We derived a PR index, which integrates the evaluation of different platelet function pathways through different platelet function assays. We showed that PR is stable over time and is a global phenotype, as previously shown in healthy subjects. This index was used to select the most extreme PR patients (high vs. low), to further study them in the following steps of the project.

I contributed to the phenotypization of the patients and in the interpretation of the statistical results. In addition, I participated to the writing of the manuscript.
Platelet reactivity is a stable and global phenomenon in aspirin-treated cardiovascular patients

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Summary

In healthy subjects, platelet hyperreactivity is a global phenomenon – as opposed to agonist-specific – and epinephrine-induced platelet aggregation (EPA) is a reliable marker of this phenotype. Few data are available on platelet reactivity and the relationship between EPA and aggregation induced by other agonists in cardiovascular patients. It was the objective of this study to characterise platelet reactivity in stable cardiovascular patients treated with aspirin and to derive a composite index integrating several aggregation pathways, suitable for selecting patients with extreme phenotypes for further proteomics analysis. Platelet reactivity to agonists was assessed in 110 patients twice, two weeks apart. Factorial analysis was used to determine whether the results obtained with the different agonists could be summarised in a single composite index. EPA correlated with the aggregation values obtained with each of the other agonists, with correlation coefficients of 0.44 to 0.55 (p<0.001). We constructed a composite “platelet reactivity” index that included 60% of the information provided by each agonist. The results obtained at the first patient visit were consistent with those obtained at the second visit (r=0.78, p<0.01). No clinical or biological parameters correlated with the composite index. The extreme phenotypes of six selected subjects were confirmed 12 months after the second visit. In conclusion, platelet reactivity in aspirin-treated cardiovascular patients is a global phenomenon that can be summarised by a composite index based on the aggregation responses to various agonists and integrating several activation pathways. This index is not dependent on clinical or biological variables, suggesting that genetic factors regulate platelet reactivity in these patients.

Keywords
Platelet, aspirin, aggregation, atherothrombosis

Introduction

Platelet activation plays a key role in acute ischaemic events associated with atherothrombotic disease, and aspirin inhibition of platelet activation has an antithrombotic effect in cardiovascular patients (1). Increased platelet reactivity as assessed with the aggregation response to agonists such as arachidonic acid (AA) (2, 3), adenosine diphosphate (ADP) (3–5) and collagen (6), is associated with recurrent ischaemic events (7). Interestingly, it has been suggested that, in order to identify cardiovascular patients at risk of such events, platelet reactivity should be evaluated with a panel of methods exploring different aggregation pathways such as AA, ADP and collagen-induced platelet aggregation (8).

Epinephrine-induced platelet aggregation (EPA) in platelet-rich plasma (PRP) is particularly useful for characterising platelet reactivity, notably identifying a subset of healthy subjects (around 14%) with platelet “hyperreactivity” (9, 10). EPA was especially stable over several years, and epinephrine-hyperreactive healthy subjects are more likely to exhibit hyper-function with other agonists. This suggests that platelet reactivity is a global or intrinsic phenomenon, rather than agonist-specific, and that a common mechanism might be at work (9–11). Studies of healthy subjects have shown moderate to strong heritability of platelet function phenotypes, tested in the presence (12) or absence of aspirin (13), further pointing to genetic modulation of platelet reactivity (14–16).

Whether platelet reactivity in cardiovascular patients treated with antiplatelet agents is also a global phenomenon is an important issue. Indeed, identification of common factors contributing to platelet reactivity to various platelet agonists in this population might reveal new targets for the prevention of recurrent ischaemic
events. Peace et al. reported the first attempt to assess platelet reactivity in this setting, in patients treated with both aspirin and clopidogrel (17), and found that platelet reactivity differed from that in untreated healthy subjects. Interestingly, they found no relationship between the aggregation induced by epinephrine and that induced by other agonists such as AA and collagen in PRP. However, we suspected that clopidogrel co-administration might have masked such associations, and therefore suggested that this issue might best be addressed by studying cardiovascular patients treated with aspirin alone (18).

The aim of the Platelet Hyperreactivity Project (NCT00976196) is to identify gene products influencing platelet reactivity in aspirin-treated cardiovascular patients by using a proteomic approach. The first part of this study that is presented here is the characterisation of platelet reactivity in this population and the identification of patients with extreme platelet phenotypes. We demonstrate for the first time that, similarly to what has been found in healthy subjects, EPA is associated with platelet aggregation responses to other agonists in cardiovascular patients treated with aspirin, suggesting that platelet reactivity is a global phenomenon in this population.

Methods

Patients

Between January 2009 and January 2010, patients with documented symptomatic atherothrombotic disease (coronary artery disease [CAD], ischaemic cerebrovascular disease and/or peripheral artery disease) treated with 100 mg/day non enteric-coated aspirin were recruited through advertisements in the local press. The remaining 113 patients were invited to attend two outpatient visits (V1 and V2), for a physical examination and blood sampling, two weeks apart, at least one month after the last acute ischaemic event and the beginning of aspirin treatment. All the subjects denied taking non-steroidal anti-inflammatory drugs and had noted no symptoms of infection or inflammation in the previous 10 days. Smokers were instructed not to smoke for 24 hours (h) before blood sampling. The patients were specifically questioned on their adherence to aspirin treatment during face-to-face interviews at both visits. If they had omitted at least one dose of aspirin during the previous 10 days, they were qualified as non-adherent and excluded from the study. One hundred ten of the 113 patients attended both visits and were included in the final analysis. All patients gave their written informed consent, and the study protocol was approved by the Central Ethics Committee of University Hospitals of Geneva.

Blood collection

Venous blood was collected in resting (>15 min) patients with a 19-gauge needle and no tourniquet, after an overnight fast, into tubes containing either EDTA, or lithium heparin, or 0.105 M sodium citrate (1 vol/9 vol) or no anticoagulant (BD Vacutainer, Becton Dickinson, Meylan, France).

Six selected patients attended a third visit where venous blood was collected using an 18-gauge needle into citrated tubes and in tubes containing ACD solution (25 g of trisodium citrate dehydrate, 14 g of citric acid monohydrate, and 20 g of anhydrous D(+)-glucose in 1 L of H2O; 1 volume of anticoagulant/6 volumes blood). PRP was obtained by centrifugation at 150 g for 15 min. Washed human platelets were prepared as described elsewhere (19) and resuspended at a density of 250 G/l in Tyrode’s buffer (pH 7.3) containing albumin (0.35%), apyrase (0.02 U/ml), CaCl2 (2 mM) and MgCl2 (1 mM). The choice to select six patients was imposed by the use of tandem mass labelling for subsequent proteomic analysis, that allows a maximum of 2 x 3 samples (20).

Platelet function evaluation

Platelet aggregation

Platelet aggregation was evaluated in the entire population on both occasion (V1 and V2) and repeated at V3 for the six selected patients. Platelet aggregation was performed in stirring conditions (1,000 rpm) in unadjusted PRP, as recommended by the International Society on Thrombosis and Haemostasis (ISTH) sub-committee on Platelet Physiology (Cairo, Egypt, May 2010, document available at: http://www.isth.org/default/assets/File/SSCMminutes/2010_MINUTES.pdf), within 3 h after blood collection, or in Tyrode’s buffer adjusted to 250 G/l for washed platelets in the six selected patients (V3), on an eight-channel aggregometer (TA-8V, SD Medical, Hillecourt, France). For PRP, we used 0.4 µM and 10 µM epinephrine (Sigma-Aldrich, Buchs, Switzerland, intra-assay coefficient of variation [CV]: 32.2% and 10.2%, respectively) and other agonists, reactivity to which has been shown to be predictive of ischaemic events in cardiovascular patients, namely AA 1 mM (Bio/Data Corporation, Horsham, PA, USA, CV: 8.4%), Horm collagen 0.5 and 1 µg/ml (Nycomed, Linz, Austria, CV: 7.6% and 10.8%, respectively) and ADP 2 µM (Sigma-Aldrich, CV: 11.1%) (2, 4, 6, 8). For washed platelets, we used epinephrine 10 µM, AA 0.1 mM (CV: 8.0%), ADP 2 and 5 µM (CV: 11.8% and 12.8%, respectively) and collagen 1, 2 and 5 µg/ml (CV: 6.2%, 8.9% and 9.1%, respectively). We recorded the maximal aggregation response to all the agonists, and the collagen lag time, i.e. the interval between collagen addition and the onset of detectable aggregation.
Serum TxB2 assay

TxA2 production in response to endogenous thrombin was evaluated by allowing a 6-ml tube of whole blood to clot at 37°C for 1 h, as previously described (21). Serum was stored at −80°C and TxB2, the stable breakdown product of TxA2, was assayed within three months with an ELISA kit (GE Healthcare, Glattbrugg, Switzerland), blinded to all other test results. TxB2 assay was performed at two dilutions, each in duplicate, and samples with a CV of >8% were re-tested.

Statistical analysis

Data are reported as medians and interquartile range (IQR) or as frequencies and percentages. The individual subjects’ V1 and V2 values were compared with Spearman’s test. Correlations between aggregation in response to epinephrine and to the other agonists was assessed with Spearman’s test and represented on scatter plots. Trends across quartiles of EPA were tested with Cuzick’s non-parametric trend test (22).

Factorial analysis was used to determine whether combinations of the platelet function tests (yielding four different models) performed at each visit could be summarised on a single axis representing a composite index. Factorial analysis – a method that was already used to combined platelet function test results (23) – is a statistical tool for investigating whether a number of variables of interest are linearly related to a smaller number of unobservable factors. In other words, factorial analysis tests the hypothesis that, for example, variations in five observed variables (e.g. five different platelet function test results) mainly reflect the variations in one unobserved variable that can be summarised as one axis. Thus, the five platelet function tests would contribute to the variation of an unobserved variable in a shared fashion and each test would add individual information from a given platelet activation pathway. The alternative is that there is no or only partial relationship between these platelet function test results, thus yielding at least two and up to five separate axis thus arguing for the absence of a global reactivity phenomenon. Axes are constructed by linear combination of the five standardised platelet function test results (mean of 0 and variance of 1) as recommended for factorial analyses (24). The retained axis is that which summarises a relevant amount of the information (the criterion used here was an eigenvalue > 1 [24]). On this basis only one axis per model was retained, indicating that the five platelet aggregation test results can be summarised in a single variable (referred to below as the “platelet reactivity” composite index, PR).

The correlation between the PRs of each model was assessed with Spearman’s test. The capacity of PR to summarise the aggregation results observed with the different agonists is expressed as the percentage of variance. To evaluate the robustness of this approach, we separately constructed PR with platelet function test results obtained at V1 and at V2, with one concentration of each agonist (collagen and epinephrine) in each model to avoid overestimating the goodness-of-fit of the summarised axis due to the obvious strong correlation of the same parameter (maximal aggregation to a given agonist) at two different concentrations. The PR values obtained at the two visits were averaged for further analysis.

Associations of clinical and biological parameters with the PRs specific to each model were tested with the Mann-Whitney or Kruskall-Wallis test for categorical variables and Spearman’s test for continuous variables.

As PR is an index derived from a combination of standardised platelet function test results, it has no units. It nonetheless allows patients to be ranked and selected. After averaging PR values obtained at V1 and V2, six patients were randomly selected among the 12 patients with the most extreme values and were asked to attend a third visit (V3). The stability of their respective extreme phenotypes was estimated graphically by calculating their PR values according to the coefficients obtained at each visit (V1 and V2).

Results

Demographic data are shown in Table 1. Most of the patients were men, and CAD was the most frequent qualifying disease. The patients had been taking aspirin 100 mg/day for a median of 105 months (IQR: 37 – 176), and the 71 patients who had had at least one acute ischaemic event were evaluated a median of 120 months (IQR: 38 – 168) after the last event. Face-to-face interviews identified no “non-adherent” patients. Good adherence was further supported by low TxB2 levels (3.9 ng/ml [IQR: 2.4 – 7.2] and 3.7 ng/ml [IQR 2.2 – 6.5] at V1 and V2, respectively) and by low AA-

Table 1: Demographic data.

<table>
<thead>
<tr>
<th>n</th>
<th>110</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>69 (63–75)</td>
</tr>
<tr>
<td>Sex (% male)</td>
<td>82.7</td>
</tr>
<tr>
<td>Qualifying disease:</td>
<td></td>
</tr>
<tr>
<td>CAD (%)</td>
<td>77.3</td>
</tr>
<tr>
<td>PAD (%)</td>
<td>11.8</td>
</tr>
<tr>
<td>ICD (%)</td>
<td>10.9</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>10.0</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>11.8</td>
</tr>
<tr>
<td>Previous smokers (%)</td>
<td>69.0</td>
</tr>
<tr>
<td>Hypercholesterolaemia (%)</td>
<td>55.4</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>44.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.1 (23.6 – 27.3)</td>
</tr>
<tr>
<td>β-blockers (%)</td>
<td>39.1</td>
</tr>
<tr>
<td>Statins (%)</td>
<td>81.8</td>
</tr>
<tr>
<td>ACEI (%)</td>
<td>17.3</td>
</tr>
<tr>
<td>ARB (%)</td>
<td>20.0</td>
</tr>
</tbody>
</table>

ACEI: angiotensin-converting enzyme inhibitors; ARB: angiotensin receptor blockers; BMI: body mass index; CAD: coronary artery disease; ICD: ischaemic cerebrovascular disease; PAD: peripheral artery disease.
Figure 1: Correlation between platelet aggregation induced by 10 µM epinephrine (EPA) and other agonists. AA: arachidonic acid; ADP: adenosine diphosphate; EPA: epinephrine-induced platelet aggregation.

<table>
<thead>
<tr>
<th></th>
<th>1st quartile</th>
<th>2nd quartile</th>
<th>3rd quartile</th>
<th>4th quartile</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>28</td>
<td>27</td>
<td>28</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Epinephrine 10 µM (%)</td>
<td>19.6 (17.6 – 21.6)</td>
<td>24.4 (23.9 – 26.0)</td>
<td>30.0 (28.6 – 31.7)</td>
<td>39.6 (36.1 – 44.4)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AA 1 mM (%)</td>
<td>4.4 (3.0 – 6.8)</td>
<td>5.0 (3.6 – 7.6)</td>
<td>8.6 (4.7 – 10.0)</td>
<td>8.8 (6.9 – 11.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ADP 2 µM (%)</td>
<td>53.8 (44.7 – 59.3)</td>
<td>56.4 (51.6 – 59.5)</td>
<td>56.5 (52.1 – 60.1)</td>
<td>60.4 (56.4 – 64.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Collagen 0.5 µg/ml (%)</td>
<td>14.2 (11.2 – 20.7)</td>
<td>21.0 (13.7 – 30.6)</td>
<td>21.7 (15.8 – 31.1)</td>
<td>30.2 (24.4 – 33.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Collagen 0.5 µg/ml lag time (s)</td>
<td>50.5 (43.5 – 57.4)</td>
<td>45.8 (36.3 – 54.6)</td>
<td>39.7 (32.0 – 52.7)</td>
<td>32.3 (30.7 – 36.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Collagen 1 µg/ml (%)</td>
<td>38.1 (35.3 – 48.6)</td>
<td>51.7 (40.8 – 59.6)</td>
<td>48.4 (37.0 – 57.3)</td>
<td>56.4 (51.9 – 61.9)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Collagen 1 µg/ml lag time (s)</td>
<td>38.7 (33.3 – 43.6)</td>
<td>32.5 (25.3 – 39.7)</td>
<td>29.7 (22.9 – 41.3)</td>
<td>24.4 (19.7 – 27.7)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

AA: arachidonic acid; ADP: adenosine diphosphate.
induced aggregation (6.7% [IQR: 4.1 – 9.2] and 6.5% [IQR: 4.5 – 8.8] at V1 and V2, respectively). None of the patients had AA-induced aggregation ≥20% at either visit (2). Correlation coefficients for the aggregation responses at V1 and V2 ranged from 0.42 (ADP 2 µM, p<0.001) to 0.74 (epinephrine 10 µM, p<0.001). As expected in patients taking aspirin, aggregation profiles were often reversible and displayed a single-wave. Graphical inspection of the distribution of aggregation response showed no bimodal distribution as the one observed in healthy volunteers with no aspirin treatment (9).

EPA correlates with the aggregation response to other agonists in aspirin-treated patients

The associations between 10 µM EPA and aggregation observed with the other agonists at V1 are shown in Figure 1, and the aggregation results are described in Table 2 according to 10 µM EPA quartiles. EPA 10 µM correlated with the aggregation responses to all the other agonists, with correlation coefficients ranging from 0.37 to 0.53 (p<0.001 for all agonists). Similar results were obtained with 0.4 µM EPA, with correlation coefficients ranging from 0.43 to 0.75 (p<0.001 for all agonists). Similar correlations were obtained at V2 (data not shown). Of note, the biological effect of aspirin, as assessed with serum TxB2 assay, did not correlate with EPA at either concentration (correlation coefficient = 0.03, p = 0.75 and correlation coefficient = –0.08, p = 0.42 with 10 and 0.4 µM, respectively).

A common underlying factor influences platelet reactivity to different agonists

Four different models, all including the EPA, AA, ADP and collagen responses at different concentrations, were studied independently at V1 and V2. Only one axis per model (the “platelet reactivity” composite index, PR) was retained with factorial analysis, suggesting a single underlying component summarising the platelet function test results. The capacity of PR to summarise the platelet function test results observed with the different agonists in each model is shown in Table 3. At V1, PR contained between 58.0% and 63.8% of the information provided by the platelet function test results observed with the different agonists at V2.

Table 3: Factorial analysis of platelet aggregation responses in aspirin-treated patients at V1. The coefficients represent the contribution of each parameter to the construction of the “platelet reactivity” composite index (PR). PR is the linear combination of the aggregation results, weighted by the coefficients indicated in this table. Similar results were obtained with platelet aggregation observed at V2.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model 1</td>
</tr>
<tr>
<td>Epinephrine 10 µM</td>
<td>0.25</td>
</tr>
<tr>
<td>Epinephrine 0.4 µM</td>
<td>-</td>
</tr>
<tr>
<td>AA 1 mM</td>
<td>0.27</td>
</tr>
<tr>
<td>ADP 2 µM</td>
<td>0.22</td>
</tr>
<tr>
<td>Collagen 0.5 µg/ml</td>
<td>0.28</td>
</tr>
<tr>
<td>Collagen 0.5 µg/ml (lag time)</td>
<td>-0.26</td>
</tr>
<tr>
<td>Collagen 1 µg/ml</td>
<td>-</td>
</tr>
<tr>
<td>Collagen 1 µg/ml (lag time)</td>
<td>-</td>
</tr>
<tr>
<td>% of variance explained</td>
<td>60.5</td>
</tr>
</tbody>
</table>

Figure 2: Correlation between the composite “platelet reactivity” index (PR) obtained with model 1 at V1 and V2. PR was calculated for each patient based on their individual aggregation results adjusted with the coefficients described in Table 4. Patients identified with a star were selected for further analysis. PR: platelet reactivity index. V1 and V2: first and second visits.
results, depending on the model. Similar results were obtained at V2, with PR containing 56.1% to 63.2% of the information. In all the models the platelet function test results made roughly equal contributions to PR, based on the beta coefficients. The correlation coefficients between the different PRs derived from each of the four models ranged from 0.95 to 0.99 (p<0.001) at each visit, and the correlation coefficients between V1 and V2 ranged from 0.74 to 0.78 in the different models. Figure 2 depicts the correlation of the model 1 PR between V1 and V2.

Determinants of the "platelet reactivity" index (PR)

The association between selected parameters and PR, as assessed with model 1, is shown in Table 4. No relevant association was found. Similar results were obtained with the PRs derived from the other three models. Model 1 was arbitrarily used for further analysis.

Platelet function at the extreme ends of the "platelet reactivity" index (PR)

PR results at V1 and V2 were averaged, and six patients with values located at the extreme ends of this averaged variable (depicted with a star in Figure 2) were asked to attend a third visit 12 ± 2 months after V2. As shown in Figure 3, their respective extreme phenotypes were maintained at V3, as estimated graphically by calculating their PR values according to the beta coefficients obtained at the two previous visits. Comparison of the percentage of the mean difference between these extreme patients in terms of the aggregation results obtained with PRP and washed platelets showed a consistent difference between the two groups, although it was slightly less marked with washed platelets. Relative difference between mean aggregation results obtained at V3 in the two groups of three patients ranged from 50% (ADP 2 µM) to 300% (collagen 0.5 µg/ml) in PRP and from 42% (ADP 5 µM) to 120% (collagen 5 µg/ml) in washed-platelet conditions. Of note, there was no difference in AA-induced aggregation with washed platelets (1.6 ± 0.6% in both groups) and, as expected (25), epinephrine 10 µM did not induce platelet aggregation in washed platelets condition (2.3 ± 1.1% in our six subjects).

Table 4: Association between co-variables measured at V1 and the platelet reactivity (PR) index.

<table>
<thead>
<tr>
<th>Covariables (categorical)</th>
<th>P</th>
<th>Covariables (continuous)</th>
<th>Rho</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>0.97</td>
<td>Age</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.62</td>
<td>BMI</td>
<td>0.03</td>
<td>0.79</td>
</tr>
<tr>
<td>Current smokers</td>
<td>0.89</td>
<td>WBC count</td>
<td>0.10</td>
<td>0.29</td>
</tr>
<tr>
<td>Previous smokers</td>
<td>0.06</td>
<td>Platelet count</td>
<td>−0.06</td>
<td>0.53</td>
</tr>
<tr>
<td>Hypercholesterolaemia</td>
<td>0.11</td>
<td>MPV</td>
<td>0.17</td>
<td>0.08</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.27</td>
<td>Haemoglobin</td>
<td>−0.11</td>
<td>0.27</td>
</tr>
<tr>
<td>β-blockers</td>
<td>0.56</td>
<td>CRP</td>
<td>0.00</td>
<td>0.99</td>
</tr>
<tr>
<td>Statins</td>
<td>0.12</td>
<td>Fibrinogen</td>
<td>−0.02</td>
<td>0.85</td>
</tr>
<tr>
<td>ACEI</td>
<td>0.62</td>
<td>vWF</td>
<td>0.11</td>
<td>0.27</td>
</tr>
<tr>
<td>ARB</td>
<td>0.97</td>
<td>TxB2</td>
<td>0.07</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Figure 3: Location of the selected patients (closed dots) in the distribution of the PR phenotype at V1 and V2, and according to the aggregation results obtained at V3 when the PR value was calculated according to the beta coefficients obtained at V1 and V2. PR: platelet reactivity index, V1, V2 and V3: first, second and third visits.
Discussion

Platelet function is crucial for normal haemostasis, while enhanced platelet responsiveness (i.e. platelet hyperreactivity) is linked to an increased risk of thrombotic events (6, 26–29). Platelet responsiveness is a continuous trait that appears to be both constant in a given individual (30–35) and inherited (13, 36, 37). Studies of healthy subjects suggest that platelet hyperreactivity is quite frequent (around 14% of the healthy population) and that it is a global phenomenon (9, 10). Although the need for studies of cardiovascular patients was underlined several years ago (29), data are still scarce.

Here, we show for the first time that, in stable cardiovascular patients treated with aspirin 100 mg/day, maximal platelet aggregation induced by epinephrine as well as with other agonists correlate with each other. These results differ from those reported in patients treated with both aspirin and clopidogrel (17), probably biased by the variability of clopidogrel potency (18). Aggregation responses can thus be summarised in a single index (PR), in line with the concept of a global phenomenon described in healthy subjects (9, 10).

Adherence to aspirin treatment was excellent in our population, based on face-to-face interviews and TxB2 levels far below those measured in 96 subjects without aspirin exposure (38). This excellent adherence may have been due to heightened awareness of the importance of adherence to antiplatelet drug treatment, given the stated focus of the study. Similar excellent adherence was noted in two studies focusing on the biological response to antiplatelet drugs (39, 40). The low TxB2 levels in our study are consistent with the concept of a low prevalence of aspirin “resistance” as measured with specific tests (41) and are in line with the concept of residual platelet reactivity – as assessed with non-specific tests – being a risk factor for recurrence of ischaemic events (42–45).

We further analysed platelet reactivity by using factorial analysis of platelet function test results obtained with various agonists. Among the possible aggregation inducers, we arbitrarily chose agonists that have been predictive of worse clinical outcome in cardiovascular patients. Indeed, we postulated that the selection of patients based on the combination of aggregation results with these agonists would increase the confidence of identifying clinically relevant mechanisms with the upcoming proteomics analysis compared to the selection of patients based on a combination of aggregation responses that included agonists without evidence of an association with clinical events or based on EPA alone. Factorial analysis led to the construction of a composite index, designated PR, which summarises a large proportion of the information provided by each agonist and therefore integrates several activation pathways. This significantly strengthens the concept that a common mechanism may account for global platelet reactivity, as suggested in healthy subjects (10), and further favours the selection of patients based on a composite index that integrates aggregation results of several agonists. PR was reproducible at two visits two weeks apart. Interestingly, TxB2 values did not correlate with PR, confirming that residual TxA2 generation has no major impact on PR, as suggested by an independent study that included 50 cardiovascular patients (23).

We found no association between PR and clinical or biological co-variables, probably due to the fact that our population was stable and that we addressed the issue of the association of clinical or biological co-variables with a composite variable – PR – rather than with a single aggregation response. This latter finding is in keeping with the modest total variance of the post-aspirin platelet phenotype attributable to measured co-variables in healthy subjects (12). However, Peace et al. did show an association between EPA and cardiovascular risk factors such as diabetes, smoking history and hypertension (17). This discrepancy with our results may be explained by the definition of platelet reactivity (PR index vs. EPA alone) and the antiplatelet drug treatment (aspirin alone vs. dual therapy). Our findings point to a role of genetic variations in inter-individual differences in platelet reactivity.

The inclusion of EPA in this platelet reactivity index is questionable, as it has been reported that other “true” platelet agonists (such as thrombin) are required to initiate aggregation, and that epinephrine merely potentiates their effects (25). Indeed, the mean aggregation response to epinephrine 10 μM was only 2.3 ± 1.1% with washed platelets in our six subjects. However, the results obtained in our selected patients at the extreme ends of the platelet reactivity phenotype in washed-platelet conditions support the existence of a true difference in platelet function between the two extremes. Of note, AA-induced aggregation did not differ between the two extremes, probably owing to the very low aggregation responses in washed-platelet conditions with this agonist in patients with aspirin treatment.

This is the first study to demonstrate that platelet reactivity is a global phenomenon in stable cardiovascular patients treated with aspirin. Our data show that the platelet response to various agonists is consistent and is not affected by common clinical and biological variables. Together, these findings support the genetic regu-

What is known about this topic?

- High platelet reactivity is associated with recurrence of clinical events in cardiovascular patients treated with antiplatelet drugs.
- In healthy subjects, platelet hyperreactivity (around 14% of the population) is a global phenomenon – as opposed as agonist-specific – and epinephrine-induced platelet aggregation is a reliable marker of this phenotype.
- A previous study suggested that characteristics of platelet reactivity in cardiovascular patients do not follow the same pattern as in healthy subjects.

What does this paper add?

- Platelet reactivity in cardiovascular patients treated with aspirin is a global and stable phenomenon and epinephrine-induced platelet aggregation does actually correlate with other aggregation responses.
- The correlation between all the aggregation responses allows the construction of a composite index that integrates several activation pathways, being thus an interesting candidate for selection of patients for further proteomic analysis.
lation of platelet reactivity in aspirin-treated cardiovascular patients. Identification of gene products modulating platelet reactivity may have major clinical implications in identifying new target for the treatment of cardiovascular and/or haemorrhagic diseases. Pioneering studies with high-throughput technologies such as proteomic, genome-wide association and transcriptomic approaches have highlighted proteins associated with cytoskeleton and energetic metabolism (46) and genes coding for PEAR1, GP6, VAV3, ITPR1 (35, 47) and VAMP8 (48) in healthy subjects. Whether these latter genes modulate PR in cardiovascular patients is unknown.

Acknowledgments
The authors thank C. Gachet for helpful discussions, S. Nolli for excellent technical assistance and S. Gueddi, P. Urban, J. Mascarini and S. Zaza for patient recruitment.

Conflict of interest
P. Fontana has received grant support from Evolva and honoraria from CSL Behring. None of the other authors declares any conflict of interest.

References


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Thrombosis and Haemostasis 106.3/2011
PLATELET PROTEOMICS
Platelets were extensively studied by proteomics, using numerous analytical approaches, as referred in this review published in “Mass Spectrometry Reviews” (IF = 10.461) in 2011. Indeed, platelets play a key role in haemostasis, but also in different pathogenic processes, such as atherosclerosis and bleeding disorders. The characterization of their proteome, i.e. their content of proteins at a given moment and in a given situation, allows a better understanding of platelet physiology including activation and aggregation processes and role in diseases.

I entirely wrote this review.
Platelets are small cell fragments, produced by megakaryocytes, in the bone marrow. They play an important role in hemostasis and diverse thrombotic disorders. They are therefore primary targets of antithrombotic therapies. They are implicated in several pathophysiological pathways, such as inflammation or wound repair. In blood circulation, platelets are activated by several pathways including subendothelial matrix and thrombin, triggering the formation of the platelet plug. Studying their proteome is a powerful approach to understand their biology and function. However, particular attention must be paid to different experimental parameters, such as platelet quality and purity. Several technologies are involved during the platelet proteome processing, yielding information on protein identification, characterization, localization, and quantification. Recent technical improvements in proteomics combined with inter-disciplinary strategies, such as metabolomics, transcriptomics, and bioinformatics, will help to understand platelets biological mechanisms. Therefore, a comprehensive analysis of the platelet proteome under different environmental conditions may contribute to elucidate complex processes relevant to platelet function regarding bleeding disorders or platelet hyperreactivity and identify new targets for antiplatelet therapy. © 2011 Wiley Periodicals, Inc., Mass Spec Rev 31:331–351, 2012

Keywords: platelet; proteomics; sample preparation

I. INTRODUCTION

One of the smallest cellular compounds of blood is cell fragments, called platelets. They play an important role in hemostasis, wound repair and thrombotic disorders such as atherothrombosis (George, 2000; Davi & Patrono, 2007). Indeed, antiplatelet drugs are a cornerstone in the treatment of atherothrombosis by decreasing recurrence of ischemic events by one fifth (Baigent et al., 2009).

Platelets are produced from giant, polyploid cells called megakaryocytes, which are rare myeloid cells found in the bone marrow (Fig. 1; Italiano et al., 1999; Patel, Hartwig, & Italiano, 2005). After reaching around 100 μm by several endomitosis (Battinelli, Hartwig, & Italiano, 2007), these cells tailor their cytoplasm and membranes, forming a demarcation membrane system (DMS). DMS is interconnected with the plasma membrane. It works as a membrane reservoir for the platelet biogenesis and is based on a dense tubular network. During this step, several platelet-specific proteins, such as von Willebrand factor or fibrinogen receptor, are produced. They are then packed into secretory granules or integrated into the plasma membrane. Other proteins, such as fibrinogen, albumin or IgG, are endocytosed or pinocytosed from plasma. These proteins are included into secretory granules derived form the Golgi. Lysosomes, mitochondria, spliceosomes, mRNA, secretory granules and the protein translation machinery are finally distributed within DMS.

Under cytokine regulation, for instance thrombopoietin (Broudy et al., 1996), megakaryocytes form pseudopodia-like structures, called pro-platelets, which correspond to DMS evagination. Pro-platelets detach from the megakaryocyte and are released in blood, where, after fragmentation, they become functional platelets: small (2–4 μm), round cell fragments. Each megakaryocyte is estimated to produce 1,000 platelets (Patel, Hartwig, & Italiano, 2005).

When a vessel damage occurs, the extracellular matrix is exposed. G-protein-coupled membrane receptors of platelets, together with integrin and immunoreceptor tyrosine-based activation motif (ITAM) receptors can specifically recognize agonists present in the extracellular matrix, such as collagen, laminin, von Willebrand factor or ecto-ADP (adenosine diphosphate) (Smyth et al., 2009; Li et al., 2010). This recognition triggers platelet adhesion followed by platelet shape change, forming long dendritic extensions (Ruggeri & Mendolicchio, 2007). These cytoplasmic extensions are facilitated by the open canalicular system (OCS) that represents a reservoir of membrane that can be evaginated following activation and shape change to increase surface area. This new shape facilitates platelet recruitment and aggregation, until they constitute a platelet plug (George, 2000; Ruggeri & Mendolicchio, 2007; Fig. 2). The adhesion between platelets...
FIGURE 1. Video-enhanced light microscopy of a mouse megakaryocyte forming proplatelets in vitro. During the initial stages of proplatelet formation, the megakaryocyte spreads and its cortical cytoplasm begins to unravel at one pole. (This zone of erosion is labeled with a white asterisk in the first panel.) As the cell spreads, the cytoplasm at the erosion site is remodeled into large pseudopodia (white arrow at 2 hr) that lengthen and thin over time, forming narrow tubes of 2–4 μm in diameter. Proplatelet extensions frequently bend (white arrow at 2 hr), and bending sites subsequently bifurcate to generate new proplatelet processes. In this manner, the entire cytoplasmic volume of the megakaryocyte is converted into branched proplatelet extensions, and proplatelet ends are dramatically increased. Proplatelets also develop segmented constrictions along their length that impart a beaded appearance. The process of proplatelet elaboration ends in a rapid retraction that separates strands of proplatelets from the residual naked nucleus (asterisk in lower right at 10 hr). The scale bar is 20 μm (from Italiano et al., 1999). ©1999 Rockefeller University Press. Originally published in J. Cell Biol. 147(6):1299–312.

and sub-endothelium structures is mediated via glycoprotein (GP) receptors that bind to specific agonists, such as von Willebrand factor (VWF; GPIb–V-IX), fibrinogen (GPIb–IIIa) or collagen (GPIa–IIa and GPVI) (Angiolillo, Ueno, & Goto, 2010). They can be constitutively active (GPIb–V-IX) or activated by conformational modifications upon platelet activation (GPIIb–IIIa; George, 2000). Although these receptors are of crucial importance for platelet function, they are present at relatively low abundance. For instance, there is an average of only 5000 GPIb-V-IX and GPIIb–IIa receptors per platelet, whereas less than 5000 GPIaIIa and PAR-1 receptors per platelet are found (Michelson, 2002).

The activation and aggregation processes are amplified by the release of platelet secretory granules. This wave of secretion induces a second generation of platelet activation, adhesion and aggregation in an autocrine and paracrine manner.

There are three types of secretory organelles in platelets, which are morphologically distinguishable (Fig. 3). First, α-granules (200–500 nm) are the most abundant (80 per platelet (King & Reed, 2002)) and secrete a large variety of proteins, mainly involved in other platelet recruitment and activation, coagulation, wound repair, and cell adhesion. As mentioned previously, these activators can be either synthesized by the megakaryocytes or recovered from the plasma. Interestingly, it has been shown that α-granule content is not homogenous and that it can be selectively and specifically released according to particular G-protein-mediated stimulations (Italiano et al., 2008). By studying platelet-induced angiogenesis, Italiano and coworkers showed that pro- and anti-angiogenic factors are found in different α-granules. This observation has also been made for von Willebrand factor and fibrinogen (Italiano et al., 2008). The second type of granules is called dense granules. They are fewer (3–9 per platelet (King & Reed, 2002)) and smaller in size. They contain low molecular weight molecules, such as ADP, ATP or serotonin, and ions, as calcium or magnesium. This content enhances and stabilizes platelet aggregation and is involved in vasoconstriction. Finally, activated platelets also trigger a lysosomal secretion. Only a few lysosomes are found in platelets and they are typically 180–250 nm diameter. Corresponding to the post-multivesicular body step in the endocytic pathway, they are more heterogeneous than other granules in term of nature and composition. They release lysosomal enzymes such as cathepsins and hexosaminidases, which may be involved in clot remodeling processes (King & Reed, 2002; Ren, Ye, & Whiteheart, 2008). Depending on the agonist, the amount and the type of released granules vary. For instance, ADP which contributes to a major amplification pathway is only a weak agonist and triggers only α- and dense granule secretion, whereas thrombin induces the release of all vesicle content, even at low doses (Coppinger & Maguire, 2007). Activated platelets also induce an inflammatory response, via inflammatory and mitogenic substances released from α-granules (e.g., P-selectin or Platelet factor 4) or directly synthesized (tissue factor or interleukin-1β (IL-1β)) (Denis et al., 2005; Davi & Patrono, 2007; Weyrich et al., 2009). Platelet activation and secretion induce the formation of a stable platelet plug, and further recruit and activate other platelets. This plug allows the injury to be covered, thus preventing bleeding until complete scarring (Fig. 2).

Although platelets are anucleated cell fragments, they still contain 3,000–6,000 mRNAs. Among these transcripts, some of them are constitutively expressed and very abundant, such as those coding for membrane GPs and von Willebrand factor. Others are translated during platelet activation, involving an alternative splicing. This last phenomenon concerns inflammatory proteins such as IL-8 or Bcl-3 (Denis et al., 2005; Dittrich et al., 2000).

If they are not activated, platelets circulate in blood 10 days on average, with a proportional decrease in functional ability, before being degraded by the spleen (George, 2000).

Since these cell fragments are enucleated and contain a limited amount of mRNA, a key approach to study their biochemistry is to focus on their protein content. Indeed,
proteomic experiments provide information about biological processes by studying protein structure, localization, interaction, modification, and quantification. Proteomics helps to consider hundreds to thousands of proteins at a time. A comprehensive analysis of platelet proteome may contribute to elucidate complex processes relevant to platelet function regarding bleeding disorders or platelet hyperreactivity and may identify new targets for antiplatelet therapy (Senzel, Gnatenko, & Bahou, 2009).

The aim of this review is to describe recent improvements in platelet proteome studies in normal and pathological conditions, but also to depict the main pitfalls associated to platelet proteomics.

II. PLATELET PROTEOMICS: SAMPLE PREPARATION

Sample preparation is the key step of any proteomic analysis (Fig. 4). Analytical methods based on mass spectrometry (MS), electrophoresis and liquid chromatography (LC) are well established and versatile, but they are sensitive to contaminations. In case of platelets isolation, other blood cells are the major contaminants.

A. Platelet Isolation

Platelet isolation is a challenging step performed by apheresis (buffy-coat) or centrifugation of the whole blood. Indeed, several pre-analytical factors may modify platelet structure and function and consequently the final proteomic profile. These factors include the recent administration of drugs interfering with platelet function such as aspirin or non-steroid anti-inflammatory drugs (Patrono & Rocca, 2009), quick isolation of platelets after blood collection (Thon, Schubert, & Devine, 2008a) or, in case of platelet concentrate bags, their storage (Snyder et al., 1987) and age (Egidi et al., 2010) and temperature of the platelet suspension (ideally at 37°C). These isolation features may induce protein modifications, such as glycosylations (Wandall et al., 2008), and thus bias the results. This is of high importance when platelets are isolated from medical blood transfusion products. Indeed, apheresis or whole blood units used in research settings are usually out-of-date. In these conditions, storage duration or temperature are not driven by the experimental workflow, but by transfusion guidelines and preliminary tests should be done to ensure the quality of the platelet suspension (Snyder et al., 1987; Thon, Schubert, & Devine, 2008a; Thon et al., 2008b). For instance, it has been shown by Hoffmeister et al. (2003a,b) that cooling platelets induces a rapid clearance of the platelets, which can be of high importance in case of transfusion, but also for platelet functional studies from medical blood product. Actually, platelets upon cold cluster von Willebrand factors and are thus recognized by complement type 3 receptors of the hepatic macrophages, which phagocytose them. The same authors showed that this phenomenon is blocked by enzymatic galactosylation of the chilled platelets.
If fresh blood is taken from a donor, the type of anticoagulant used to prevent clot formation in the collection tube is also important. Citrate-containing anticoagulants (acid–citrate–dextrose (ACD), acid–citrate–phosphate) are usually used to preserve platelet function. Heparin stabilizes efficiently the plasma by interaction with antithrombin. However, it also interacts with other proteins, such as platelet factor 4 or IL-8, and may induce unexpected side effects, such as platelet activation (Capila & Linhardt, 2002). EDTA acts by chelating calcium ions necessary for many enzymatic actions. Due to its general effect on enzymes, it influences plasma protein MS profile, probably by triggering protein modifications (Luque-Garcia & Neubert, 2007).

Platelet separation is usually performed by centrifugation of the whole anticoagulated blood, allowing the recovery of the platelet-rich-plasma (PRP). The aim of this sample preparation is to recover a platelet suspension as pure as possible, avoiding contaminations from other blood cells and preventing platelet activation. Only the upper third of the PRP after centrifugation should be taken. However, PRP can still contain blood cells and an additional centrifugation is needed to get a satisfactory platelet pellet that will be further washed and resuspended in an appropriated buffer, such as Tyrode’s buffer (Raghavachari et al., 2007). Some authors use antibody coated magnetic beads to remove residual white blood cells as well as a red cell lysis buffer or discontinuous gradients (Dittrich et al., 2008) to further increase the purity of the platelet suspension. In order to avoid platelet activation during the centrifugation processes, inhibitors of platelet function may be used (Prostacyclin PGI2 for example), with the potential drawback that these inhibitors may induce protein changes that will interfere with proteomics analysis. However, PGI2 has a short half-life and the changes in platelet physiology induced by PGI2 are usually reversible (Garcia et al., 2005a; Higuez et al., 2008). The quality and purity of the final platelet suspension should be evaluated. Several tools such as automatic counter, microscopy (light or electronic (White, 2004); Fig. 5) and platelet aggregation tests should be used.

Another key-step in proteomic sample preparation is the cell disruption. In this context, platelets are particular due to their small size and their absence of nucleus. Several methods can be used depending on the purpose of the experiment (Canas et al., 2007). In the early seventies, Barber and coworkers triggered platelet lysis by hypotonic shock on a glycerol gradient to isolate plasma membrane on continuous or discontinuous sucrose gradients and characterized it (Barber & Jamieson, 1970). One year later, they compared several methods for platelet lysis before isolating platelet membranes (Barber, Pepper, & Jamieson, 1971). They performed a mechanical homogenization with a motorized Teflon pestle, a sonication, an osmotic lysis, a nitrogen decompression (applying a high pressure on the cells; the pressure release induces cavitations in the platelets) and a glycerol lysis of platelet suspension with and without plasma membrane stabilizing agents (fluorescein mercuric acetate or zinc ion method).

Authors then compared the methods in term of effectiveness considering the sucrose gradient bands enzymatic activity, by hemagglutination inhibition and electronic microscopy. They concluded from this work that none of the methods was fully satisfying in this context, although glycerol lysis appeared to be the easiest, the most reproducible and the most efficient (85% of the platelets). Moreover, the use of stabilizing agent results in a strong decrease of lysis and is incompatible with certain biochemical assays. These lysis methods and others were applied to platelets in many different contexts, as nitrogen decompression (Broekman, Westmoreland, & Cohen, 1974, Gogstad, 1980), pressure homogenization in a French pressure cell (French & Holme, 1974), potter homogenizer (Marcus et al., 2000), sonication (Ruiz et al., 2004; Maynard et al., 2007, 2010) and freeze/thaw cycles (sometimes in combination with sonication (Kaiser et al., 2009)). Noteworthy, sonication induces local heat that may be responsible of the loss of given biological properties. It is also possible to disrupt platelets with other strategies, such as enzymes (van der Meulen, Bhullar, & Chancellor-Maddison, 1991) or detergents (Pieroni et al., 2010), but they can impact a proteomic analysis, respectively by adding exogenous proteins to the sample or being incompatible with the following analytical step.

Although these verifications are time-consuming, they have to be done to ensure reliable set of results. It is indeed
necessary to compare different platelet isolation protocols or lysis methods according to the context of the experiment and to define which one is the most appropriate.

**B. Subcellular Fractionation**

The platelets proteome is a highly complex protein mixture, generating large experimental data difficult to interpret. Several approaches have been developed to alleviate this complexity. They constitute an important part of proteomics, due to their high influence on the sensitivity, the selectivity and the reproducibility of the experiments. Sometimes, fractionation is necessary to get rid of the most abundant proteins, which increase de background noise and hide the low abundant proteins. Most abundant platelet proteins are involved in cytoskeleton (mainly actin), cell signaling and protein processing (Garcia et al., 2004a).

Prior to a proteomics analysis, a prefractionation step allows isolating an organelle of interest or a given chromatographic or electrophoretic fraction (Boschetti & Righetti, 2009). The oldest organelle prefractionation method is based on density gradients centrifugation. The cell lysate is resuspended in a sugar or a polyol (sugar–alcohol) solution, such as sucrose, metrizamide or sorbitol, or in a colloidal silica solution, like percoll. The separation is performed according to organelle density, shape and size. Classically, organelles are collected in a enriched fraction and their content checked by methods focusing on a particular organelle-specific marker, such as calreticuline for endoplasmic reticulum or cathepsin D for lysosomes (Righetti et al., 2005; Brunner et al., 2009). Several platelet organelles have been prefractionated using this density gradient strategy. For instance, platelet α-granules have been isolated by sucrose (Marcus et al., 1966; Broekman, Westmoreland, & Cohen, 1974; van der Meulen, Bhullar, & Chancellor-Maddison, 1991; Maynard et al., 2007), ficoll (Siegel et al., 1971) or metrizamide gradients (Gogstad, 1980; Berman et al., 1986) and by a metrizamide-percoll double gradient (Seiffert & Schleef, 1996). However, it is important to consider the level of contamination by adjacent fractions in gradients. Moreover, each of these substances can, in particular conditions, react with organelles. Sucrose induces an osmotic stress on organelles (Siegel et al., 1971) and can induce their aggregation. Adding EDTA can reduce this last drawback (Broekman, Westmoreland, & Cohen, 1974). Ficoll, despite it is osmotically inert, tends to form complexes with cellular organelles or components, such as fibrinogen (Siegel et al., 1971).

Organelles can also be isolated by immunopurification; however, it is sometimes difficult to obtain a specific antibody against certain organelles. This method can be used with a preliminary centrifugation to eliminate non-desirable organelles (Brunner et al., 2009). This strategy has been chosen by Niessen and coworkers in combination with antibody coated magnetic beads. They could isolate very pure fractions of α-granules, dense granules and membranes respectively (Niessen et al., 2007).
Organelles can also be separated according to the difference of their global electric charge in an electric field. This approach constitutes the basis of free-flow electrophoresis method and has been applied to platelet plasma membrane enrichment by Crawford et al. (Crawford, Authi, & Hack, 1992) and, more recently, by Senis et al. (2007).

Whatever the sub-fractionation chosen, it is crucial to evaluate potential contaminations by other organelles, as well as the functional and morphological integrity of the isolated organelle, to ensure the specificity and the validity of the approach. This purpose is traditionally achieved by respectively Western blot, enzymatic assay and electron microscopy, but often necessitates the difficult selection of organelle-specific markers (Andreyev et al., 2010).

**C. Platelet Protein Separation, Detection, and Identification**

A key step in a classical platelet proteomics workflow is the separation of the prepared protein suspension in order to further decrease its complexity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) relies on a separation according to polypeptide size in a polyacrylamide gel. This method enables a low to medium separation, since every band corresponds to tens of proteins. Protein separation is more accurate with a two-dimensional gel electrophoresis (2-DE), which takes advantage of 2 protein physiochemical properties: protein isoelectric point and size. Among the first investigators who studied platelet proteome by 2-DE gel were Clemetson, Capitanio, and Luscher (1979). These authors used whole platelets and platelet membrane to performed 2-DE gels in reducing and non-reducing conditions. They could identify proteins and glycoproteins by comparison with purified samples or plasma proteins. The same method was used by Snyder to define the protein alterations due to platelets concentrate storage (Snyder et al., 1987). Researchers analyzed samples from platelet concentrates on days 1, 7, and 21 by 2-DE gels, using computer-assisted comparison of a silver-staining coloration, a fluorescent and radioactive labeling. They detected reproducible changes in 30 different spots of the 789 matched spots between the 14 2-DE gels that they performed to build a composite map at days 1 and 7. This study clearly shows how storage conditions may bias proteomics results. These differences tend to increase with the storage duration, since at day 21 the patterns were more different from days 1 and 7. Authors suggest that this is mainly due to proteolysis, but also to changes in membrane proteins and possible partial activation. In 1995, Gravel and coworkers have published a reference 2-DE gel map of human platelets in their basal state (Gravel et al., 1995; Fig. 6). The authors identified more than 25 spots, with the help of different methods (matching with other 2-DE gel reference map, N-terminal sequencing and immunoblotting with chemoluminescence detection), corresponding to cytoskeletal proteins, G-protein subunits and proteins common to the human liver, erythrocytes and plasma.

Since 1990s, technical improvements in mass spectrometry instruments changed dramatically the analysis of proteins (Table 1). Starting by a protein separation step, the analysis of the sample by MS allows the identification, the characterization and the quantification of proteins with a higher sensitivity and specificity. The first step of this process is the digestion of proteins into peptides, often using trypsin as protease. The peptides are then analyzed by mass spectrometry (Domon & Aebersold, 2006; Ahrens et al., 2010). This consists first in the generation of ions from the peptides either by electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI). They are called “soft” ionization methods, because they do not induce any severe fragmentation of the analyte.
TABLE 1. Summary of the proteomic analysis performed on human platelets

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<th>Authors</th>
<th>Analyzed samples</th>
<th>Protein separation</th>
<th>Mass spectrometry analysis</th>
<th>Number of identified proteins</th>
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Ions go through the mass analyzer, which sorts and determines their mass-to-charge ratio \( (m/z) \). It is tuned to acquire proteomic data with the highest sensitivity, mass accuracy and resolution to generate mass spectra. Four types of mass analyzers are used in proteomics: the time-of-flight (TOF), the ion trap (IT), the quadrupole (Q) and the Fourier transformation cyclotron (FT-ICR). The TOF measures the velocity of the ions to reach the detector. The IT traps and selects ions in a three dimensions electric field, before ejecting them in their \( m/z \) order. The Q performs separation by applying a quadrupole electric field to ions. The FT-ICR applies a magnetic field, which induces ions cyclotronic movements. Ions mass is then calculated based on a Fourier transform by detecting the image current of the ions. These analyzers can be combined in “hybrid” instruments. For instance, TOF and Q analyzers can be used in tandem (Q-TOF) achieving the ion selection in the quadrupole and its fragmentation in a collision cell with an inert gas. Ion fragments go through the TOF to be recorded by the detector, producing a MS/MS spectrum. More recently, another analyzer was developed and used in proteomics. The Orbitrap is a high performance tandem MS type in which ions are trapped, orbiting around a spindle-like electrode (Makarov, 2000). Their oscillation is characteristic of their \( m/z \) and produces a mass spectrum based on a Fourier transform (Han, Aslanian, & Yates, 2008). The Orbitrap provides MS and MS/MS data with a high resolving power, dynamic range, mass accuracy, and sensitivity. These features make it an instrument of choice for proteomic applications (Garcia et al., 2005a; Perry, Cooks, & Noll, 2008) and contributed to very large scale studies of platelet proteome (Piersma et al., 2009; Qureshi et al., 2009; Yu et al., 2010; Table 1).

Identification of proteins from MS spectra is then performed by several bioinformatic methods relying on database search. MS/MS spectra are applicable to complex protein mixtures, because each spectrum corresponds to independent information. A molecular weight fingerprint measured from a digested protein or most often tandem mass spectra of peptide can be used for the database identification. Experimental spectrum is compared with theoretical ones defined from protein database sequences. A score is assigned depending on the algorithm used for the database search to define the quality of the retrieved “match”. This method is called peptide fragment fingerprint. Several softwares achieve this task, such as SEQUEST®, PeptideSearch®, Mascot®, or Phenylx® (Sadygov, Cociorva, & Yates, 2004). The second main approach to identify MS/MS spectra is the de novo sequencing and compares the complete or partial sequence extracted from the spectrum with theoretical sequences by sequence similarity. This method is used in identification algorithms such as SeqMS® or PEAKS® (Hernandez, Muller, & Appel, 2006).

Marcus et al. made the first platelet proteome analysis by MS, using a MALDI-TOF machine after a 2-DE gel separation. They took advantage of the MALDI post-source decay for the cytosolic protein identification and tyrosine phosphorylation characterization (Marcus et al., 2000). They could identify almost 190 proteins.

The first proteomic analysis of platelet microparticles (MP) was performed by Garcia et al. (2005b) using IT. MPs are small fragments of cell membrane present in plasma (Burnier et al., 2009). They are released by different cells, including platelets, and are associated with inflammation and thrombosis. Authors used SDS–PAGE and LC-MS/MS strategies to identify 578 proteins, out of which 380 not found in previous platelet studies (Garcia et al., 2005b).

O’Neil and coworkers increased platelet protein identification to reach 284 proteins, from 123 genes, by 2-DE gel and Q-TOF MS/MS analysis, focusing on the more acidic fraction of the sample (isoelectric point \( (pI) \) 4–5). Identified proteins were mainly cytoskeletal or involved in signal transduction (O’Neill et al., 2002). This study has been completed by Garcia et al. (2004a). They used the same strategy looking at the \( pI 5–11 \) region of the platelet proteome, and identified 760 protein features, corresponding to 311 open reading frames (ORFs), whom major group was signaling proteins. One year later, Claeyts, Geering, and Meyer (2005) focused their work on multimeric proteins of platelets cytosolic and micosomal fraction by 2-DE Blue Native/SDS gels electrophoresis. This method consists in a first dimension separating the monomeric and the multimeric proteins in their native state, followed by a second denaturing dimension. Spots were then cut and proteins were identified by Q-TOF MS/MS. They identified 63 proteins from 58 spots, among them 9 were shown as platelet proteins for the first time by proteomics and they could identify several known cytosolic and membrane complexes. However, despite of its sensitivity and its high resolution, 2-DE gel experimental procedure presents some limitations. A major problem, whatever studied sample is that big, very basic and/or hydrophobic proteins tend to be lost during a 2-DE gel separation. This drawback has been partially overcome by gel-free techniques of peptides mixture.

The combined fractional diagonal chromatography, or COFRADIC™, is an example of gel-free methods presenting a high separation power (Gevaert et al., 2002). A complex peptide mixture is run on a reverse-phase liquid chromatography (RP-LC) and collected as fractions. Each of them is then modified by oxidation of the methionine (Met) and run again on RF-LC with the same method. Modified peptides contain thus a Met and present an altered retention time, due to the increase hydrophobicity of the newly formed sulfoxide group. This alteration is used to select these peptides and perform MS and MS/MS analysis using a Q-TOF. This approach was again used in 2003 by the same group, but based on a different chemistry to sort platelet N-terminal peptides (Gevaert et al., 2003). Authors derivatized N-terminus parts of the proteins before the trypsin digestion. Then they separated the peptide mixture by reverse phase LC and blocked internal N-terminus parts with a strong hydrophobic group. This induces a shift of hydrophobicity, which allows to separate the N-terminal peptides from the rest of the peptide. Every protein is thus represented by one peptide only, which strongly decreases the sample complexity. They could identify 264 proteins of cytosolic and membrane skeleton fractions of human platelets. Three years later, Martens et al. used three different COFRA-DIC™ techniques to isolate amino-, cysteinyl, and methionyl terminal peptides. They identify 641 proteins, among them, 404 novel proteins in COFRADIC™ studies. Many of them were hydrophobic proteins, such as CD36 antigen or sarcoplasmic/endo-plasmic reticulum calcium ATPase 2 and 3 (Martens et al., 2005). Nevertheless, COFRADIC™ does not give any information concerning peptide \( pI \) and thus is complementary to 2-DE gel, without replacing it.
Multidimensional protein identification technology (MudPIT) is also a gel-free approach coupling one or more LC steps with MS analysis. It provides multidimensional separation according to the kind of chromatographic methods used and can be directly interfaced with ion sources (Kislinger et al., 2005). Coppinger et al. (2004) have applied MudPIT to platelet releasate characterization in 2004 providing an alternative to 2-DE gel-based analysis.

Finamore et al. (2010) recently compared a platelet proteomic analysis using either a chromatographic (nano ultra performance liquid chromatography, nUPLC) or 2-DE gel protein separation. They identified more than 500 spot with the in-gel strategy and 114 proteins by the UPLC shotgun approach. They found a higher representation of cytoskeletal and receptor proteins from the gel-free results, whereas proteins involved in cell metabolism were more predominant in the 2-DE gel list. This study highlights the differences resulting from the choice of the analytical workflow.

Great advantage can be taken from combining several proteomic approaches. Qureshi et al. (2009) combined three strategies to identify a total of 1,507 platelet proteins, which constitutes the most complete proteomic studies of resting platelets. They apply a SDS–PAGE LC-MS/MS workflow to platelets from 10 independent samples to identify 956 proteins. Then, focusing on membrane proteins, they found 182 additional proteins (see below). Finally, using an immobilized metal affinity column (IMAC), they identified 262 phosphorylated proteins and 104 acidic co-eluting non-phosphorylated proteins. The same year, Lewandrowski combined SDS–PAGE, MudPIT and COFRADICTM after a two phase partitioning to study platelet membrane global proteome (Lewandrowski et al., 2009). The two-phase partitioning consists in a polyethylene glycol/dextran system, which separates the plasma membrane thanks to its hydrophobicity (higher than the other vesicles membranes). Authors performed an SDS–PAGE and MudPIT together allowing the identification of 1,202 proteins whereas they identified 498 proteins with the COFRADICTM approach. This last is based on a methionine, cysteine, and N-terminal peptide isolation contributing to 247, 219, and 160 proteins, respectively. Among them, 16 were identified by the three COFRADICTM approaches. Considering the Gene Ontology (GO) terms, the identified proteins were mainly involved in signal transduction and in cellular adhesion. However, authors also found other groups of membrane proteins, such as several tetraspanins or ion transporters. They also performed a relative quantification of the proteins by exponentially modified Protein Abundance Index (emPAI), a method relying on the spectral count, that is, assuming that the abundant peptide are more often detected, producing a higher number of spectra. The highest emPAI indices were obtained for known abundant proteins of platelets, such as integrin IIbIIIa or GPIb-IX-V complex. Authors also observed a general accordance between thin relative quantification and the number of copies of the proteins found in literature. They also developed an elegant approach to enable a quality control of their data, by combining the emPAI index with the GO term. Actually, considering the 100 most abundant proteins, 76 were characterized as membrane proteins and 52 as plasma membrane located. Moreover, they also evaluated the level of contamination by other blood constituents, by focusing on the emPAI index of given markers, such as CD45 for the leukocytes. Finally, authors made an evaluation of the known interactions between the 1,282 proteins using STRING (http://string.embl.de). A cluster of kinase and adhesion-mediating receptors was clearly observable, which confirmed the enrichment of plasma membrane proteins. Yu et al. (2010) combined several protein extractions with multiple gel-free (isoelectric focusing (IEF) on peptides and 2D-LC) and in-gel (SDS–PAGE) separations, different MS approaches (Q-TOF and Orbitrap) and two identification bioinformatics algorithms (Mascot® and SEQUEST®) in order to perform a global analysis of the platelet proteome from human and rat. They identified 1,053 human platelet proteins, including 114 novel proteins, and 837 rat proteins. Among them, 386 pairs of gene sequences were found as orthologous in human and rat.

Platelet protein identification can be further completed by a quantitative step. Quantitative proteomics is a very efficient approach to compare two or more conditions with different phenotypes or environmental stimuli. Recently, Mateos-Caceres et al. (2010) compared two populations of cardiovascular patients, presenting a different platelet response to aspirin. They selected 51 patients treated with aspirin and performed a quantitative 2-DE gels analysis followed by the identification of spots by MALDI-TOF/TOF. They identified 11 proteins differentially expressed involved in energetic metabolism, oxidative stress, cytoskeleton or cell survival, such as, 1,6-bisphosphate aldolase, glutathione-S transferase or chloride intracellular channel isotype 1.

Gel-free quantification methods have been also developed. The quantitative measurement is thus achieved in the mass spectrometer. Isotope-based quantifications can be done with platelet samples, for instance, by isotope-labeled internal standards added to the sample, enzymatic labeling with 18O or, in case of cell culture experiments (based on platelets production by megakaryocytes cell culture (Battinelli et al., 2001)), by stable isotope labeling by amino acids (SILAC). Platelet proteomes from SILAC mice (wild-type and mutants) were analyzed by Krüger et al. (2008). After 4 weeks of SILAC feeding wild type mice, 86% of the 241 quantified proteins were labeled. Human platelet samples were used by Staes and coworkers to optimize a new O18 labeling method, which ensures a more stable labeling (Staes et al., 2004). To decrease the sample complexity, they performed a COFRADIC peptides separation in order to consider only N-terminal peptides shared among 12 fractions. They identified 31 peptides corresponding to 26 proteins within one fraction and extrapolated the identification of around 260 proteins considering the entire set of fractions.

Derivatisation of digested peptides with isobaric tags can also be performed (isobaric tags for relative and absolute quantification, iTRAQ, or tandem mass tag, TMT) (DeSouza et al., 2005; Dayon et al., 2008; Wilm, 2009). These strategies aim to distinguish each sample after their mixing to analyze them within the same process and thus with the same technical variation. iTRAQ and iCAT (isotope-coded affinity tag) methods have been used to study platelet function (Thon et al., 2008). Recently, iTRAQ derivatisation has been used on blood MP proteins. Ramacciotti et al. used iTRAQ labeling to compare a population of patients with a deep venous thrombosis (DVT) with healthy controls. They found by MALDI-TOF-TOF 2 highly expressed proteins (Galectin-3 binding protein and α-2 macroglobulin) in DVT patients. Nine proteins were...
less expressed in patients, among which fibrinogen β and γ chains. These proteins are involved in inflammation, inhibition of fibrinolysis, plug formation, and cell shedding (Ramacciotti et al., 2010).

Wong, McRedmond, and Cagney (2009) used a nucleotide affinity chromatography to isolate nucleotide-binding proteins from resting and TRAP-activated platelets (Wong, McRedmond, & Cagney, 2009). The quantification between both platelet preparations was achieved by a MS label-free method called spectral count. The rational behind this quantification approach is that the more abundant are given peptides, the higher would be the number of their MS/MS spectra (Neilson et al., 2011). They identified 175 proteins as ATP-binders, 17 as cAMP-binders and 24 as cGMP-binders. Moreover, 10 of the ATP-binding proteins showed a significant different expression level upon TRAP-activation. Almost all of them were associated with the cytoskeleton.

These examples show the versatility of the different proteomic sample preparations and the technical improvements in MS instruments (Table 1). They also highlight the benefit of combining several approaches to study a biological process. Indeed, a multiple proteomic strategy is the best way to enlarge our point of view on a topic of interest, by combining different sources of results. This becomes of higher importance concerning cellular compartments, due to the lower amount of material.

III. STUDYING PLATELET COMPARTMENTS

A. Platelet Releasate and Activation/Signaling Cascade

As mentioned previously, one of the major events in the platelet aggregation process is the release of the granules content. Among agonists, thrombin is particularly efficient to trigger platelet secretion of all types of granules (Smyth et al., 2009). This activation relies on the cleavage of thrombin receptors, called protease-activated receptors 1 and 4 (PAR-1 and PAR-4), by the thrombin itself and on its interaction with another receptor GPIbα (Sadler, 2003). PAR cleavage yields a new amino terminus, initiated by the sequence SFLLRN and called thrombin receptor-activating peptide (TRAP). Agonists can also be used to study activation cascade proteomes, such as the phosphoproteome.

Maguire et al. (2002) studied the platelet phosphoproteome upon thrombin stimulation, based on phosphoprotein enrichment by immunoprecipitation followed by 2-DE gel (Maguire et al., 2002). They could detect 67 phosphorylated proteins spots after thrombin activation. They identify 10 of them by MALDI-TOF MS and Western blot, of which the signaling protein MAPKKK or the ATP receptor P2X1. Two years later, Coppinger et al. (2004) used the same agonist to activate platelets and study their releasate in atherosclerosis context. They separated proteins by 2-DE gel and analyzed the spots by MALDI-TOF. They completed this experiment by a MudPIT characterization. They could identify more than 300 proteins by MudPIT; with 81 present in 2 or 3 replicated experiments. Among them, 63% (51 proteins out of the 81) were not known to be secreted by platelets. Interestingly, the 2-DE gel approach allowed them to identify only 9 proteins. It illustrates the importance of combining several proteomic strategies and the improvements provided by MudPIT.

TRAP alone is sufficient to cause PAR-1 activation and to produce platelet secretion, even if the induced morphological changes are less extensive than upon thrombin exposure (Fuste et al., 2002). Several characterizations of the released proteome from TRAP-activated platelets have been performed. In 2009, Piersma performed a proteomic analysis of the TRAP-activated platelet releasate of three healthy volunteers. They identified 716 proteins with 225 present in the three samples (Piersma et al., 2009). This study was based on a GeLC-MS/MS experiment, including an SDS–PAGE separation, followed by a nano-liquid chromatography separation of the digested peptides and a LTQ-FT hybrid mass spectrometer analysis. This approach allowed the authors to dramatically increase the list of identified proteins and the confidence of their results, since they come from a triplicate. These results were compared with previous publications concerning the proteome of the platelet microparticles and α-granules (Coppinger et al., 2004; Garcia et al., 2005b; Maynard et al., 2007). They found a large overlap (60% and 73% respectively), using a data mining approach for gene ontology and testing the presence of secretion signal peptide (Signal IP) in the 716 proteins.

Moreover, platelet agonists can also be used to study their effect on the platelet signaling pathway. Garcia et al. (2004b) took advantage of TRAP receptor-specificity and used a 2-DE gel followed by a LC-MS/MS strategy (Q-Tof) to compare the PAR-1 signaling proteome of resting and activated platelets. They focused on twofold changes in protein expression intensity on 2-DE gels and phosphorylation modifications. Sixty-two proteins were detected as having at least a twofold change in expression level upon TRAP stimulation. Forty-one of the 62 were identified by MS, corresponding to 31 ORFs. They belong to cytoskeletal, signaling, or protein processing group of protein. Eight of them, such as transgelin 2, endoplasmic reticulum protein 29 or D-prohibitin, were described in the platelet for the first time. Garcia and coworkers could also identify signaling proteins, which were not previously shown to be regulated by phosphorylation, Dok-2, RGS10, and RGS18. In this context, phosphorylation corresponds to the main post-transcriptional modification triggered by activation. The RGS proteins are known to transmit signal by increasing GTPase activity of the G-protein coupled receptors. Dok-2 was shown in platelet for the first time in platelet in this work. Authors speculate that Dok-2 phosphorylation may highly contribute in thrombus formation. Moreover, they found only 10 common proteins with the study of Maguire et al. (2002). This difference may be due to differences of strategy, such as immunoprecipitation of phosphoproteins in the first study. Two years later, Garcia studied tyrosine-phosphorylated proteins upon GPVI activation by collagen-related peptide (CRP) (Garcia et al., 2006). They performed two analytical approaches, a 2-DE gel and a phosphotyrosine immunoprecipitation followed by a SDS–PAGE. They identified 96 proteins phosphorylated by this activation. Eleven of which were novel platelet proteins, such as SPIN90 or the transmembrane protein G6f. This last presented a specific phosphorylation to CRP, constituting a further step in the understanding of GPVI platelet activation pathway. More recently, Schulz et al. made a complementary work on this pathway using an monoclonal antibody to activate platelets and a quantitative 2-DE gel to find protein abundance differences induced GPIV activation. They identified by MS 8 functionally relevant proteins related
to cell signaling, metabolism, cytoskeleton, and membrane trafficking, such as aldose reductase of pleckstrin.

In 2008, Zahedi et al. combined 2 LC methods to enriched proteomic samples in phosphorylated proteins (Zahedi et al., 2008). They isolated platelets from blood and, after lysis and trypsin digestion, they used an IMAC and a SCX chromatography to isolate phosphopeptides. By MS/MS or precursor ion scanning, they could identify 564 phosphorylation sites on 278 proteins, among them several kinases and kinase substrates, as Rap1-GAP2 and PDE5, together with known platelet proteins, including GP1bapla. The both LC methods presented an overlapping of the phosphopeptides of only 22%, highlighting the interest of combining them. This work constituted the first “liquid” (as opposed to in-gel) approach to study phospho-subproteome.

Senis et al. (2009) aimed to further explore the alphaIIb-beta3 signaling pathway (Senis et al., 2009). As in their GPVI study, they immunoprecipitated proteins presenting phosphotyrosine after activating platelet with fibrinogen before. A SDS–PAGE-MS analysis allowed them to identify 27 proteins, among them 17 were never shown as involved in this signaling cascade, such as Dok-1, Dok-3, or G6f.

Releasates and platelet activation are specific characteristics reflecting platelet physiology, but these highly depend on the platelet preparation and quality. Agonist utilization is also a crucial parameter when releasates are considered. It specifically links to a given receptor, triggering a particular activation pathway. This will most likely influence the released proteome.

B. Platelet Membrane Proteome

Membrane proteins are often associated to the first step of many activation cascades important for cell metabolism. They are the targets of many drugs. However, they are understudied by traditional proteomics methods, because they are difficult to solubilize, to separate and to identify (Tan, Tan, & Chung, 2008). Indeed, these proteins are low abundant, structurally heterogeneous and very hydrophobic. These features make them underrepresented in many platelet proteomic profiles, because solutions, which allow their proper resuspension are usually incompatible with their subsequent separation by electrophoresis methods, digestion, and MS analysis. For instance, in the case of 2-DE gels, membrane proteins have difficulties to enter in the immobilized pH gradient (IPG) gel and tend to precipitate during the IEF process. Moreover, trypsin cleavage sites are fewer and less accessible in the transmembrane domains. This decreases their presence in downstream experiments. As a consequence, specific strategies have been designed to study membrane proteins of platelets.

Kinoshita, Nachman, and Minick (1979) published a platelet adaptation of a method enriching the plasma membrane of erythrocytes and HeLa cells with polylysine beads (Kinoshita, Nachman, & Minick, 1979). Platelets were attached to the beads by their surface membrane and were then lysed by sonication. They tested the efficiency of this strategy first by observing the beads before and after sonication by microscopy, to assess that the beads were covered by platelets and that sonication broke the large majority of the cells. Electronic microscopy showed that membrane fragments were still attached to the beads. They then evaluated the purity of the recovered fraction by iodination labeling of the plasma membrane and radioactive measurement the fractions and by enzymatic studies. They also performed a analysis by SDS–PAGE to further study the enriched fraction and could isolate a large number of surface glycoproteins.

Moebius et al. (2005) published the first MS analysis focused on platelet membranes (Moebius et al., 2005). They prepared a membrane fraction by pre-cleaning the platelet lysis solution on a sorbitol gradient, to remove other organelle contaminations. They extracted hydrophobic proteins from the lipid bilayer with Triton X-114 partition. The authors observed an increase of membrane protein amounts, whereas the content of cytoskeletal proteins was decreased. Proteins were then separated on a 16-BAC/SDS–PAGE. In this procedure, proteins are separated according to their electrophoretic mobility using two detergents, the cationic 16-BAC in the first dimension, and the anionic SDS in the second. It allows recovering proteins focused into spots, increasing the separation resolution compared to traditional SDS–PAGE. Finally, authors analyzed digested proteins by LC-MS/MS (ESI-Q-TOF) and identified 297 different species, among which plasma membrane proteins was the major subpopulation. In this study, Moebius and coworkers could identify 6 times more membrane proteins than in previous 2-DE gel-based studies. They also combined two database search algorithms, SEQUEST and Mascot, and only proteins identified by both programs were selected, increasing the confidence in these results.

Since a large proportion of surface and secreted proteins is glycosylated, Lewandrowski et al. (2006) established an approach focusing on the analysis of N-glycosylation sites on human platelet proteins. They combined a lectin affinity chromatography with a chemical trapping glycoproteins. Concanavalin A was used to trap N-glycans, before trypsin digestion and removal of the non-glycosylated peptides by a second lectin affinity chromatography. Lectin binds carbohydrates and represents thus a suitable method for glycoproteins or glycopeptides enrichment. Peptide are then derivatized with the englycosidase, PNGaseF, to remove the glycosyl groups.

The second method consists in the derivatization of the glycan residues, creating a reactive aldehyde, before trapping the proteins on a hydrazide-functionalized agarose beads. The elution is finally performed using PNGaseF. The samples were then analyzed by LC-MS/MS. Authors identified 70 different glycosylation sites present on 41 proteins.

In their proteomics and transcriptomics study of platelet membranes, Senis et al. (2007) used several membrane protein enrichment techniques. They performed a lectin affinity chromatography and eluted proteins with N-acetylgalcosaminose. This method substantially increased the level of protein purification. They also tested an alternative chromatography method based on the derivatization of exposed lysine by biotin, before purification with avidin beads. This approach was able to detect a higher number of membrane proteins than the first one, according to the ratios GPIβ/actin and aIIbβ3. Finally, in order to distinguish between surface proteins and proteins located in internal membranes, the authors treated platelets with neuraminidase, an enzyme which removes sugars from the outer plasma membrane, and separate them by Free Flow electrophoresis. This method generated a charge difference between both pools of proteins and allowed a separation with less than 5% of contamination. In total, Senis and coworkers
identified 136 membrane proteins after LC-MS/MS analysis (46 plasma membrane proteins, 68 internal membrane proteins and 22 transmembrane proteins of unknown localization), based on FT spectra analysis by SEQUEST and Mascot.

The same year, Lewandrowski et al. (2007) went deeper in the platelet membrane proteome analysis focusing on glycosylated platelet membrane proteins. Their approach was based on a gel-free strategy called N-glycosylation site analysis using strong cation exchange enrichment (ENSAS). Platelet plasma membrane proteins were enriched by aqueous two-phase partitioning, after lysis, in a polyethylene glycol/dextran polymer system. Proteins were recovered from the polyethylene phase and trypsin digested. The resulting glycosylated peptides are then purified by strong cationic exchange chromatography. It retains non-glycosylated peptides, relying on the assumption that most of external N-glycans contain sialic acid residues, which decrease the global positive net charge of the peptides. These peptides can be collected in the first fraction. An enzymatic deglycosylation and a simultaneous deamidation are performed to change the glycosylated asparagine to aspartic acid. This step permits the recognition of glycosylation sites with a NX(S/T) consensus sequence by mass spectrometry. The authors identified 148 glycosylation sites on 79 different proteins. This approach presents several advantages. First, being gel-free, it suits to the analysis of hydrophobic proteins and is compatible with many proteomics techniques. Second, the level of purification of chromatographic collected peptides is high, reducing at the same time the complexity and the range of the peptide mixture.

More recently, Tucker et al. (2009) used a sulfo-NHS-biotin affinity purification, which combines cell surface biotinylation and affinity purification, to isolate cell surface proteins before and after thrombin stimulation. This process does not affect the phosphorylation state of each condition. The authors reduced the sample complexity by a 2-D analysis (liquid IEF and SDS–PAGE) and identified proteins by FT-ICR MS. They found 88 proteins important for thrombocyte function using SEQUEST. Among them, they described many new platelet proteins, such as Lck or KSR1, or novel proteins in the thrombocyte and affinity purification, to isolate cell surface proteins before and after thrombin stimulation. This process does not affect the phosphorylation state of each condition. The authors reduced the sample complexity by a 2-D analysis (liquid IEF and SDS–PAGE) and identified proteins by FT-ICR MS. They found 88 proteins important for thrombocyte function using SEQUEST. Among them, they described many new platelet proteins, such as Lck or KSR1, or novel proteins in the thrombin stimulation pathway, such as HIP-55.

The same year, Qureshi et al. (2009) identified 182 membrane-associated proteins. They enriched the fraction of interest by centrifugation to precipitate membranes and protein resuspension in a glucopyranoside/guanidium buffer. They performed then a SDS–PAGE fractionation and a LC-MS/MS analysis. As mentioned previously, this analysis was part of a comprehensive platelet proteomic study. Lewandrowski published one of the largest platelet membrane studies in 2009 (Lewandrowski et al., 2009). They combined three analytical approaches with the two-phase partitioning previously mentioned: a SDS–PAGE, a MudPIT and a COFRADIC step (see above for a detailed description of these methods). A total of 1,282 membrane proteins were identified with 418 proteins as common between the shotgun (SDS–PAGE and MudPIT) and the COFRADIC methods.

Plasma membrane proteome constitutes a particular cellular proteomic compartment, due to its hydrophobicity. In most of the cases, they have to be studied using dedicated protocols, which are complementary to traditional proteomic workflows. However, plasma membrane receptors and related proteins are key-components of the platelet biology, making this compartment of high importance in the platelet proteomic field.

C. Platelet Granules

As explained before, platelets contain three types of secretory vesicles: α-granules, dense granules, and lysosomes (Fig. 3). These vesicles are released in the extracellular medium, when platelets are activated. They are also involved in several platelet-related diseases, such as gray platelet syndrome (decreased number of α-granules) and Hermansky–Pudlack syndrome (dense granule deficiency, albinism and defect in lysosome-derived organelles). However, genes causing these syndromes are poorly defined, pointing out granule characterization as an important step in the understanding of these diseases (Salles et al., 2008).

There are different ways to isolate platelet granules, but a particular attention must be paid to the assessment of fraction purity. Co-isolation of contaminating organelles, such as mitochondria, dramatically increases aspecific results.

Berger, Masse, and Cramer (1996) showed, by electron microscopy, that several plasma membrane proteins of the platelets are also found in α-granules (Berger, Masse, & Cramer, 1996). They focused their study on glycoproteins GPIb, GPIX, and GPV in resting platelets. They immunolabeled platelets from patients with gray platelet syndrome with the same markers and found that this syndrome is characterized by a severe decrease of α-granules without any defect in α-granule membrane composition. Indeed, the few present α-granules were correctly labeled with antibodies directed against specific membrane GPs.

Maynard et al. (2007) isolated α-granules to perform a proteomics analysis of their content using an LC-MS/MS following an in-gel digestion of sections of SDS–PAGE (Maynard et al., 2007). The organelle separation was based on a linear sucrose gradient, which allowed authors to recover a fraction enriched in P-selectin and von Willebrand factor α-granule markers. From this fractionated sample, they could identify 219 proteins. Thirty-nine were known to be from α-granules, 50 as part of the releasate and 44 potential new proteins. These proteins are involved in Alzheimer’s disease (aldehyde α-1 or clusterin), in vesicle transport (Rab11B or Rab27B), cell shape and motility (F-actin capping protein) and in atherosclerosis (endothelin-converting enzyme 1). In this context, electron microscopy and Western blot were performed to ensure the identification and integrity of the granules after the sucrose gradient separation, as well as protein location in the cell fractions. These validation methods showed that mitochondria and endoplasmic reticulum (ER) markers were present in multiple fractions. Actually, by MS, the authors found 65 proteins from mitochondria and 12 from ER in addition to the 219 proteins belonging to α-granules. Although these proteins could be a contamination from other organelles, they also could be present in granules. A functional study could answer this question, with the aim to identify interacting partners of these proteins or their precise localization, for instance by colocalization with a α-granule marker (such as von Willebrand factor or fibrinogen) by immunofluorescence microscopy, as done by Italiano et al. (2008). Maynard et al. used the same approach in 2010 to compare α-granules from healthy individuals and gray platelet
syndrome patients. They could identify a list of 586 proteins and validated some differentially expressed by electron microscopy. They also found that gray platelet syndrome patients present a lower amount of α-granule soluble proteins than healthy individuals, whereas endocytosed proteins were moderately affected by the pathology and membrane-bound proteins were similar between the two groups. They identified a series of unknown proteins in this context, including emillin-1, complement C3 and apolipoprotein E. Due to the rarity of gray platelet syndrome patients, they could study only one patient, which highlights the difficulties to obtain biological replicates in given cases (Maynard et al., 2010).

Dense granules are difficult to study, because they content a high level of non-protein compounds and their number is low (3–9 per platelet). However, Hernandez-Ruiz et al. (2007) could identify 40 dense granule proteins. Among them they focused on 14-3-3ζ, in atherosclerotic conditions. They used expired platelet units to isolate dense granules by Histodenz density gradient. Purity of the fraction of interest was validated by electron microscopy and Western blot analysis. They identified 18 proteins by 2-DE gel separation and MALDI-TOF MS analysis. In order to further characterize soluble proteins, they performed an LC-ESI MS/MS analysis. This second strategy identified 35 proteins. Both approaches lead to the identification of 40 different proteins, with 11 common to both methods. Only one of these 40 proteins, GPlIb, was known to be located in dense granules. Proteins were involved in cytoskeleton, platelet metabolism, glycolytic pathway, cell signaling, and protein folding.

Lysosomes are well known to play a key role in the endocytic pathway. However, their function in the secretory pathway has also been demonstrated, particularly during membrane reparation of fibroblasts. Lysosome defect is responsible of several pathologies, such as albinism or immunodeficiency (Stinchcombe, Bossi, & Griffiths, 2004). Platelet lysosome defect is one of the symptoms of the Hermansky–Pudlak syndrome. This syndrome is genetically heterogeneous but is characterized by an ocularcutaneous albinism and a platelet storage defect causing a bleeding disorder. Depending of the subtype, it can also induce pulmonary fibrosis, granulomatous colitis, and congenital neutropenia (Wei, 2006). Genes involved in this disease play a role in protein and membrane trafficking and are ubiquitously expressed, affecting a large variety of specialized cells, including pigment cells (melanocytes or pigment epithelial cells), T cells or platelets. Moreover, several proteomics studies have been performed on lysosomes from other cell types, based on centrifugal subcellular fractionation or affinity purification (Lubke, Lobel, & Sleat, 2009). To our knowledge, there is no large-scale proteomic analysis of platelet lysosomes published so far. These approaches could be applied to lysosomes to learn more about their specificity and their role in platelet function.

In summary, despite several points of concern, proteomics thus is particularly appropriated to study functional aspects of platelets. These examples show how a proteomic workflow can be dedicated to learn more about platelet secretion or specific activation pathways. By combining these approaches, proteomics highly contributed to increase our knowledge not only concerning the proteins present in platelets, but also on their function in given contexts.

### IV. ROLE OF PROTEOMICS IN UNDERSTANDING PLATELET-MEDIATED DISEASES

Since small genotypic variations may induce dramatic phenotypic effects (Macaulay et al., 2005) and knowing the pivotal role of platelets in blood hemostasis, proteomics is a tool of choice to investigate inherited or acquired dysfunction and may identify new potential drug targets. As previously mentioned, Maynard et al. (2010) compared platelet secretory granule proteomes from gray platelet syndrome patients and healthy subjects. They identified proteins previously unknown in this context, which not only constitute new insights in this pathology understanding, but also could be focused in innovative therapeutic strategies.

The only DNA contained by platelets is the mitochondrial DNA, which is actively transcribed. However, several diseases involve genetic variants of megakaryocyte nucleus DNA affecting platelet function. In most of the cases, they are rare and imply a single gene. These inherited disorders are of varying severity, depending on the affected function. For instance, Bernard–Soulier syndrome is an autosomal recessive disorder characterized by thrombocytopenia, giant platelets and a prolonged bleeding time. It presents a prevalence of 1 case in 1 million people. This disease is caused by qualitative or quantitative abnormalities in the GPIb-IX-V complex. Patients affected by a Bernard–Soulier syndrome present gingival and mucocutaneous bleeding, hemorrhage after trauma and epistaxis (Salles et al., 2008). Maurer-Spurej et al. (2008) have published the first report of the identification of a familial platelet defect by proteomics, in 2008. They studied the platelet proteome of 4 family members presenting severe bleeding problems. Their platelets revealed variable reduced response to ADP, collagen, and epinephrine in aggregation tests. The disorder autosomal dominant inheritance and associated symptoms could not be correlated with a known genetic disease. Using a SDS–PAGE LC-MS/MS strategy, they showed that patients’ platelets had a reduced amount of specific α-granule proteins (multimerin, fibrinogen, and trombospondin-1). They found that urokinase was responsible of the degradation of alpha-granules, leading to the discovery of the Quebec Platelet Disorder.

Acquired platelet dysfunction is another group of pathologies affecting platelet function. Their clinical presentation, as in many genetic platelet disorders, usually implies mucosal bleeding, bruises, or epistaxis. They are often resulting from a medication (antiplatelet drugs, but also non-steroidal anti-inflammatory drugs or serotonine recapture inhibitors) or a systemic disorder, such as uremia or liver disease (Shen & Frenkel, 2007).

Platelet function is also linked to other diseases (Davi & Patrono, 2007), notably, which combine modulation of thrombus formation and complex interactions between genes and environment. Targeted modulations in platelet physiology induced by antiplatelet drugs, such as aspirin or clopidogrel, have been shown to substantially influence the risk of cardiovascular diseases (Fontana & Reny, 2007; Angiolillo, Ueno, & Goto, 2010). The biological impact of drugs can be evaluated by proteomics. Coppinger et al. used SDS–PAGE LC-MS/MS (confirmed by antibody array results) to study the release of over 100 proteins upon ADP, collagen, or TRAP activation in presence or absence of aspirin (Coppinger et al., 2007). They
showed that the strength of the agonist influences the amount of secreted proteins and the secretion profile, and that aspirin decreases the protein expression of platelet releasates. Their data supports a two-wave model of secretion. According to this model, an initial wave is triggered by agonist stimulation. Thromboxane A2 generation enhances the activation process in an autocrine manner, by inducing platelet secretion and the second wave of aggregation. This second wave is inhibited by aspirin, causing a decrease of secreted proteins. The biological effect of aspirin has been reported to vary between patients treated with this drug that may induce different outcomes (Bonvini et al., 2009; Kasotakis, Pipinos, & Lynch, 2009).

This issue was investigated by quantitative proteomics (2-DE gel LC-MS/MS) by Mateos-Caceres et al. (2010). They found a series of differentially expressed proteins involved in mechanisms related to aspirin response, such as oxidative stress, cytoskeleton, enzymatic metabolism, and cell survival.

Arias-Salgado et al. (2008) used the same type of workflow to compare protein expression in two populations, 29 patients presenting an arterial thrombosis and 24 control subjects. They found seven proteins differentially expressed (three decreased and four increased in patients). These proteins are all associated with the cytoskeleton, possibly resulting in its disruption in these patients (Arias-Salgado et al., 2008). This kind of studies helps to better understand molecular and cellular consequences of a given pathology, to determine new drug targets and test their efficacy. Last year, Fernández Parguña et al. (2010) performed a 2-DE gel proteomic analysis of platelet from patients presenting a non-ST segment elevation acute coronary syndrome. They compared the proteome of this population at different time points after the acute event with stable coronary artery disease patients. They identified 22 different proteins, mainly members of signaling or cytoskeleton proteins. They also showed that this number decreases with the time after the initiation of the symptoms.

Pieroni published in 2010 a proteomic comparison of platelets from 9 healthy volunteers and 6 cystic fibrosis (CF) patients (Pieroni et al., 2010). Platelets are suggested to play a role in CF inflammation. Their analytical approach combined nUPLC and 2-DE gel separation, followed by MS. The in-gel strategy detected 1900 spots, among which 24 differentially expressed with a ratio >1.5, whereas the shotgun proteomics part of the workflow identified 65 differentially expressed proteins. Bioinformatics analysis showed a preponderant part of proteins implied in cellular movement, proliferation, and signaling.

Platelet proteomics focused on sub-cellular compartments thus emerges as a method of choice to study clinical symptoms and understand their biological mechanisms (Garcia, 2010).

V. PLATELET INTERACTOME

By combining proteomics, molecular biology methods, such as immunoprecipitation, and bioinformatics resources, it is possible to identify interacting platelet proteins and their associated metabolic pathways. Qureshi and coworkers determined the known interactome of their set of platelet proteins from the available Human Protein Reference Database (HPRD). They expanded it using information from their phosphoproteome study with a focus on integrin pathway (Qureshi et al., 2009). They lead to a protein–protein interaction network consisting of 1,034 proteins and 2,993 interactions.

Taking advantage of platelet specific proteins and mRNA, Dittrich et al. (2008) established a comprehensive proteome and transcriptome database of human platelets. They collected data from large-scale proteome and transcriptome studies and annotated databases and inferred interaction data from various human cell lines. They found a higher connectivity for platelet proteins involved in the reorganization of the actin cytoskeleton and in the signal transduction. This highlighted the pivotal role of these physiologic pathways in platelets.

In their publication comparing platelet proteomes from healthy donors and CF patients, Pieroni used the Ingenuity Pathway Analysis software (Ingenuity® System) in order to provide a functional network reflecting the metabolic relationships between the differentially expressed proteins and their statistical significance (Pieroni et al., 2010). Technical improvements could be applied to platelet interactome study (Blanco-Colio et al., 2009), such as surface plasmon resonance coupled with MS detection (Buijs & Franklin, 2005). This allows to directly fish and identify interacting proteins with a candidate of interest from a complex mixture of proteins, as shown by Ravanat et al. (2009). The authors used this strategy to find the prey of an antibody called ALMA.7 from platelet membrane lysate. They identified a 67-kDa protein, CD226, and verified their result by immunossay. The automation of such methods will dramatically scale the discovery of interacting proteins up. Moreover, a combination of different experimental approaches and data mining from literature allows thus having complementary results describing the phenomenon of interest. It guarantees a more complete comprehension of the process in a cellular context and decreases the risk of mis-interpretation of the results.

VI. PLATELET PROTEOMICS PITFALLS

As every experimental step can induce a bias, it is crucial to establish efficient quality control and validation methods during the design of the workflow.

The first problem to consider is the biological variation between samples of different subjects. This necessitates a precise and rigorous selection of blood donors. In human beings, these differences are due to various factors, such as sex, lifestyle, age, genetic background, or health status of the tested subject. Winkler et al. (2008) tested the extent of biological variation in a group of 20 healthy volunteers by 2-DE gel using an internal standard. Each platelet sample was separated on an acidic (pH 4–7) and a basic gel (pH 6–9). Each gel showed nearly 500 spots present in at least 90% of the gels. The total variation of these spots between patients was lower than expected (18%). The use of internal standard most likely increased the result confidence and reproducibility. From this study, two other conclusions were highlighted. First, it is important to adapt the sample size with the number of proteins that are investigated. This means that scientists must have an estimation of the number of proteins that they want to consider. Second, the technical variation induces also a bias in the results, which can influence the study conclusion. The importance of the bias varies depending on the experimental method and must be evaluated. In this study, the authors performed 3 acidic and 3 basic electrophoresis from the same
sample to evaluate the electrophoretic technical variation. They found a very low variation (below 10%). Eidelman et al. (2010) published a study of the platelet proteome considering the gender of the donor (Eidelman et al., 2010). A 2-DE gel MS (MALDI-TOF) workflow was combined with an antibody microarray and validated by Western blots on 16 platelet samples. They identified at least 2800 proteins on 2-DE gels, based on discovery studies with 2-DE gels and MS (but without reporting the proportion of unique proteins), and quantified 573 proteins with the microarray. The authors found that only 8 of the high abundant proteins (detected by 2-DE gel) and 161 of the low abundant proteins (detectable by microarray) were identified as gender-dependant, such as synapsin II or RAB5A. These differences could constitute a biological basis to explain the gender discrimination in cardiovascular diseases.

Blood storage is another step to consider in the case of platelets. Thon and coworkers showed that the concentration of 503 proteins was modified over 7-day storage. They combined several proteomic approaches (2-DE gel, DIGE, ICAT and iTRAQ) (Thon et al., 2008b). They identified mainly storage-associated protein changes, but only 5 proteins out of the 503 were common to every proteomic approach. This phenomenon is supported by the observation that, although platelets are still viable, they present morphological (decrease of α-granule content, shape change or decrease of platelet volume), biochemical (altered glycoprotein composition) and functional modifications (increase procoagulant activity). It is thus of interest to adapt blood collection and, if needed, storage to the aim of the study and proteomic workflow.

Platelet storage can also influence protein identification. Qureshi et al. (2009) showed that talin, a signaling protein, is degraded upon platelet pellet storage in SDS-containing lysis buffer. To limit this protein degradation, they stored isolated platelets at −80°C as a suspension, rather than as pellets. As explained above, the level of contamination in the platelet suspension has to be evaluated, as this step is the first of any proteomic workflow. This can be done using several methods, such as flux cytometry (identification of the blood cells thanks to their size), Western blot (specific marker of blood cells) or microscopy.

When enrichment and/or separation methods are used on protein samples, attention must be paid to the reproducibility of the approach. A technical coefficient of variation should be calculated on the entire workflow, to ensure a reliable result. Moreover, the quality of the preparation should be addressed, not only in term of contamination, but also in term of quantitative and qualitative loss. Actually, any sample manipulation can induce a technical bias influencing the peptides and thus the results. In the case of enrichment, the level of contamination by other cell component will determine the ability to detect low abundant proteins specific to the studied compartment. However, in this context, it can be difficult to distinguish between some organelles of platelets: many proteins of the plasma membrane are also found in secretory granules, it is thus hard to evaluate the level contamination of such organelles in a plasma membrane preparation.

It is often necessary to concentrate proteins, in order to perform a proteomic analysis. An efficient concentration method should discard the non-protein content (nucleic acid, lipids or salts) and avoid protein degradation. Protein precipitation is a method of choice for this purpose. The protein solubility depends on the solution pH, its ionic strength, its temperature and the protein concentration. Several methods have been established to precipitate proteins, but their efficiency varies with the type of sample. Zellner et al. (2005) investigated the quantitative and qualitative recovery of two platelet sample precipitation methods: ethanol and tri-chloroacetic acid (TCA) precipitation. Both methods precipitated almost the same amount of proteins, but the precipitation induces changes in protein composition, such as the lost of actin-associated proteins.

The list and number of identifications varies largely between proteomic studies even on the same sample preparation (see Table 1). This can be explained by the differences in technical resources among the different labs, for instance the choice of the spectrometer will dramatically influence the sensitivity of the detection and thus the results. However, several parameters have to be taken into account to ensure reliable identifications. Only proteins identified with at least 2 unique peptides should be considered, to avoid false positive identifications. Several identification algorithms use a false discovery rate (FDR) calculation based on the number of identifications in a reverse database as confidence evaluation of the data. The FDR should be typically of 1%. Peptides should be injected in at least three times in the mass spectrometer to increase the sample coverage and thus the confidence in the results and authors should mention the distribution of the identification per injection. Among these analyses, several features should be assessed such as the level of material per analysis and the reproducibility of the LC profile, in LC-MS analysis. The choice of the identification algorithm is also an important point. Authors should be aware of the differences between them, in term of isoforms management (isoform detection versus most probable hit) and protein database, for instance. This last parameter is of high importance, even if the most commonly used is UniProt/SwissProt. Researchers should take care of using a database manually curated, to ensure a high level of data quality. It is often useful to compare the results from different algorithms.

In a quantitative proteomic approach, differentially expressed candidates are identified by MS analysis and their relative abundance should be verified by an orthogonal method. In the case of the quantitative 2-DE gels, several proteins can be identified per spots. It thus highly important to specifically determine which one has to be considered as responsible of the difference. A method of choice for the verification is immuno-based strategies of the different samples. It is thus of high importance to normalize the different conditions in order to compare each other, which might be difficult. Zellner et al. (2008) recently showed that the use of fluorescent labeled secondary antibodies combined with a non-specific staining of all transferred proteins in Western Blot allows a simultaneous visualization of the total protein content on the membrane and a specific quantification of given markers. It is thus possible to normalize the targeted signal by the whole protein content, improving the quantification accuracy.

As in transcriptomics, the use of repositories to share proteomic raw data, such as PRIDE (http://www.ebi.ac.uk/pride/) is actually the only really efficient method to guarantee a complete and efficient comparison between published results and ongoing studies. This allows to trait the data with the
absolutely identical bioinformatic process and parameters. Otherwise, it remains very difficult to compare proteomics data.

In immuno-based methods (for instance to assess the level of enrichment of a subcellular fractionation), the selection of the marker is a crucial step, because it has to be specific of the organelle of interest, and distinguishable of potential isoforms. As Andreyev et al. (2010) recently showed, this specificity must be tested in preliminary experiments and a combination of several markers can increase the quality of the verification. Moreover, there are several other limitations of the immuno-based strategies. The choice of positive and negative controls is crucial for the results interpretation. It is also important to consider the amount of sample which is tested, together with the concentration of the marker in the sample. Loading controls can be set up to bypass this problem, such as the quantification of protein not affected by the studied phenotype. These controls can further be used to normalized the data. In this context, it is also crucial to quantify all the samples in the same run, to avoid any technical bias, such as the development times, which may influence the results.

Statistical power is another point to address. Indeed, proteomics studies often identify and, sometimes, quantify hundreds proteins in 1–10 samples. In this king of context, statistics are often limited, because of the low degree of freedom allowed by such experiment. Moreover, statistical tests are difficult to implement on data presenting large variable features. For example, every protein is measured by a different number of peptides, depending on its size and its physico-chemical properties. We can thus assume a potential significant level of false positive and false negative in the results. Biological evidence (expected pathways or several proteins part of the same biological process) can help to be confident with results, but it is always mandatory to verify those candidates.

In the case of PTMs, researchers should provide information detailed enough to permit a unambiguous assignation of each site. Spectra are thus necessary to be provided, together with a precise description of the methods used to study modifications.

As previously mentioned, platelets contain mRNA, whom part are translated during activation. An alternative to verify proteomic results is thus to analyze the transcriptome. However, mRNA translation is not only related to its transcription level, but also to its half-life and its translation rates. That is why level of protein expression and level of mRNA transcription do not always correlate. Another platelet feature to consider is that those anucleate fragment cells have a limited translation pool and endocytose certain proteins, without expressing them, such as fibrinogen. McRedmond et al. (2004) compared the transcriptomic and proteomic analysis of platelet secretory material. They found that 69% of the platelet secreted proteome correlates the transcriptome. The 30% difference corresponds to the temporal displacement between the platelet formation from megakaryocyte and mature platelet circulation. Platelet maturation and senescence induce protein expression or endocytosis from the plasma and mRNA degradation. Furthermore, translated mRNAs are only a part of the platelet transcriptome and the function of the untranslated transcripts is still not known. Moreover, transcriptomic information is limited, without giving any clues about post-translation modifications, localization, activation state or interactions of the gene products.

VII. CONCLUDING REMARKS

Platelets are particular blood components in being anucleated cell fragments. Many specific techniques have been developed to study the platelet proteome (Table 1).

In the future, improvements in proteomic workflows together with a network biology approach that integrate data from transcriptomic, metabolomic, proteomic, and bioinformatic experiments combined to clinical information will allow to delineate mechanisms of platelet function implicated in diseases and thus identify new targets for pharmacotherapy or biomarkers. Overall, the integration of high throughput “omics methods” on a large panel of individuals with clear clinical phenotypes using standardized protocols opens a new area in the understanding of platelet-driven diseases and their treatments. These results should be combined with previously published data. In such context, it will be more and more necessary to develop resources allowing pooling the data, such as transcriptomics and proteomics repository, in order to be able to have a global view of functional processes. Pitfalls of this approach include mRNA stability, technical limitation in the detection of low level of transcripts/proteins and correlation between the magnitude of the mRNA or protein level and its importance in cellular physiology (Senzel, Gnatenko, & Bahou, 2008).

VIII. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2-DE gel</td>
<td>two-dimensional gel electrophoresis</td>
</tr>
<tr>
<td>ACD</td>
<td>acid–citrate–dextrose</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>CF</td>
<td>cistic fibrosis</td>
</tr>
<tr>
<td>COFRADIC™</td>
<td>combined fractional diagonal chromatography™</td>
</tr>
<tr>
<td>CRP</td>
<td>collagen-related peptide</td>
</tr>
<tr>
<td>DMS</td>
<td>demarcation membrane system</td>
</tr>
<tr>
<td>DVT</td>
<td>deep venous thrombosis</td>
</tr>
<tr>
<td>EDTA</td>
<td>2,2’,2”-(ethane-1,2-diyldinitrilo)tetraacetic acid</td>
</tr>
<tr>
<td>ENSAS</td>
<td>enhanced N-glycosylation site analysis using strong cation exchange enrichment</td>
</tr>
<tr>
<td>emPAI</td>
<td>exponentially modified Protein Abundance Index</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
</tr>
<tr>
<td>FT-ICR</td>
<td>Fourier transform-ion cyclotron</td>
</tr>
<tr>
<td>GeLC-MS/MS</td>
<td>SDS–PAGE LC-MS/MS analysis</td>
</tr>
<tr>
<td>GO</td>
<td>gene ontology</td>
</tr>
<tr>
<td>GP</td>
<td>glycoprotein</td>
</tr>
<tr>
<td>HPRD</td>
<td>human protein reference database</td>
</tr>
<tr>
<td>iCAT</td>
<td>isotope-coded affinity tag</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focusing</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity column</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
</tr>
</tbody>
</table>
PLATELET PROTEOMICS

ion trap
immunoreceptor tyrosine-based activation motif
isobaric tag for relative and absolute quantitation
liquid chromatography
mass-to-charge ratio
matrix-assisted laser desorption ionization
methionine
microparticle
myeloid-related protein 14
mass spectrometry
multidimensional protein identification technology
nano ultra performance liquid chromatography
open canalicular system
off-gel electrophoresis
open reading frame
protease-activated receptors
prostacyclin
isoelectric point
platelet-rich-plasma
post-translational modification
quadrupole
sodium dodecyl sulfate–polyacrylamide gel electrophoresis
tri-chloroacetic acid
tandem mass tag
time-of-flight
thrombin receptor-activating peptide

The authors thank Dr. Michelangello Foti, from the University of Geneva, for electron microscopy pictures of the platelet preparations. All authors belong to the Geneva Platelet Group.

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Mass Spectrometry Reviews DOI 10.1002/mas


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Pierre Fontana obtained his M.D. in 1994 (University of Lausanne, Switzerland), and his Ph.D. in 2003 (University Paris V, France) in the field of platelet function. He is now head of the laboratory of Special Hemostasis at the University Hospitals of Geneva. Research activities cover platelet physiology and antiplatelet drugs.

Jean-Luc Reny had an initial biochemistry training and then pursued a medical and scientific curriculum with an M.D. degree (1993, University of Grenoble, France), a certification in internal medicine and vascular medicine (1998 and 2000, University of Paris VI, France) and a Ph.D. degree (2002, University of Paris V, France). His research interest is currently focused on the mechanisms and clinical consequences of the biological response to antiplatelet drugs.

Severine Noll is laboratory technician and works on platelet function since 2003. She is actively involved in platelet preparation and techniques development regarding proteomics.

Jean-Charles Sanchez has been working since 1989 in the field of proteomics. He obtained his Ph.D. in biochemistry at the Buckingham University (UK) in the field of proteomics and diabetes. Since 1995, he has been the head of the Biomedical Proteomics Group at the Faculty of
Medicine, Geneva University. He is a founder of the Swiss Proteomics Society (SPS) and the European Proteomics Association (EuPA). The activities in his group cover the discovery of biomarkers associated to (1) brain damage disorders and (2) impaired insulin secretion. Jean-Charles Sanchez is the author or co-author of more than 130 papers in refereed journals as well as 30 book chapters and 30 patents.
TOWARD A BETTER UNDERSTANDING OF THE PLATELET SECRETORY GRANULES PROTEOME
TOWARD A BETTER UNDERSTANDING OF THE PLATELET SECRETORY GRANULES PROTEOME

This project implies several sample preparation for the proteomic analysis of patient’s platelets by isobaric labeling. A particular attention was paid to secretory granules, because platelet secretion is shared by numerous agonists and constitutes thus a potential candidate for the modulation of platelet reactivity.

Secretory granules were enriched by sucrose gradient and analyzed by mass spectrometry in gas phase fractionation mode. This dramatically increased the number of identifications compared with previous reports, including many proteins shown for the first time in this compartment. The presence of these new proteins was verified in the sucrose fraction, but still needs to be validated by immunofluorescence microscopy co-localisation with granule markers as von Willebrand factor.

The establishment of the secretory granule enrichment constituted a large part of my work on this project. I performed the whole analysis development and wrote the chapter.
Toward a better understanding of the platelet secretory granule proteome

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\textit{Keywords: platelet, secretory granules, sucrose gradient, proteomics, gas-phase fractionation}
Abstract

Platelets are anucleated cell fragments involved in haemostasis. They contain 3 types of secretory granules, which are different in terms of morphology, number and content: α granules, lysosomes and dense granules. They are all secreted upon platelet activation. The present study aims to further characterize the protein composition of these granules. We used a strategy relying on subcellular fractionation by sucrose gradient prior to mass spectrometry analysis in gas-phase fractionation (GPF) mode. We identified more than 800 proteins, the vast majority being involved in pathways ranging from platelet granule biogenesis to secretion. A pathway analysis comparing granule-enriched fraction with a reference data set from whole platelet showed a strong enrichment in ERK1/2 and MHC I related pathways in the granules. Western blot confirmed the presence of new proteins in the platelet granules-enriched fraction, such as Lyn and Syk. To our knowledge, this work constitutes the largest characterization of the platelet secretory granule proteome.
**Introduction**

Platelets are anucleated cell fragments derived from megakaryocytes in bone marrow [1, 2]. They play an important role in haemostasis, being activated by agonists released when vessel damage occurs. Agonists, such as ADP or collagen, activate platelets through their interaction with specific receptors and trigger platelet aggregation on the damage area, forming a plug and favoring wound repair [3, 4].

Platelets contain 3 types of secretory organelles, which are released during aggregation. Granules are formed in the megakaryocytes, deriving from multivesicular bodies (MVB) during the megakaryocyte maturation. Typical proteins from MVB, such as von Willebrand factor (vWF), are specifically sorted into α-granules, which derive from the trans-Golgi network. On the other hand, CD63 is stored in separate MVB and directed to dense granules and lysosomes [5]. Trafficking between different granules also occurs, with the presence of common markers in α and dense granules, such as P-selectin or integrin αIIbβ3. Together with other organelles, such as mitochondria, endoplasmic reticulum and Golgi apparatus, granules are transported into cytoplasmic pseudopode-like structures, the proplatelets. These are then released in the blood circulation and become mature platelets [5, 6].

Granules differ in terms of molecular composition, size, ultrastructural morphology, kinetics and stimulus of release [5]. Alpha-granules are the main type of granules, being around 80 per platelet. They are 200-500 nm in size and contain chemokines, proteoglycans and proteins involved in platelet recruitment and activation, such as fibrinogen, P-selectin, αIIbβ3 or vWF [7]. However, their cargo appears to be heterogenous; for instance fibrinogen and vWF are differentially released and seem to be localized in different granule subpopulations in immunofluorescent studies [8, 9]. Moreover, these granule subpopulations have not the same vesicle-associated membrane protein composition and present a different localization during spreading [10]. Alpha granules contain molecules, which are produced by the megakaryocyte, but also others, such as albumin or fibrinogen, which are produced by other cells and
endocytosed by megakaryocytes and platelets [5]. The second class of secretory granules is dense granules. They contain small molecules and ions, such as serotonin, ADP or calcium, which induce vasoconstriction and stabilize platelet aggregation. Several membrane proteins, such as vacuolar proton pump, lysosome-associated membrane protein 1 and 2 or granulophysin are also characteristic from dense granules [11]. They are smaller in size and fewer, only 3 to 9 per platelet [5]. Finally, a few lysosomes are also released upon platelet aggregation. Typically of 180 to 250 nm of diameter, their content is more heterogeneous, due to their endocytosis origin. Their enzymatic cargo potentially plays a role in clot remodeling [5, 12].

Platelet granules contain inflammatory molecules, but also growth factors, which make platelet important in atherosclerosis progression [13]. Indeed, platelets adhere to atherosclerotic plaques, through collagen activation. This triggers their secretory granule release in the plaque, contributing to monocyte migration, smooth muscle cell proliferation, vasoconstriction, platelet activation and inflammation. This cascade of events finally favors the plaque instability [13], reason why antiplatelet drugs, such as aspirin or clopidogrel, may have a beneficial impact not only on the thrombus formation during the acute ischemic event but also on the stabilization of the atherosclerotic plaque [14].

Proteomics constitutes an efficient method to characterize platelet physiology [15, 16] and many publications showed the large variety of pre-analytical strategies in this field [17-19]. Among them, subcellular fractionation techniques allow enriching organelles of interest [20-22]. Here, we used a subcellular fractionation based on sucrose gradient, to obtain enriched secretory granules samples. We analyzed them by gas phase fractionation (GPF) [23] tandem mass spectrometry (MS/MS). We thus identified granule proteins that were previously not described in other proteomic studies [24], but also new proteins that may play a role in the secretory organelle physiology and possibly in diseases where platelets play a major role, such as atherosclerosis.
Material and Methods

Blood collection and platelet preparation

Venous blood was collected from a pool of normal, healthy subjects in anticoagulant (acid/citrate/dextrose, ACD) containing tubes under an institutional board-approved protocol. Platelet-rich plasma (PRP) was recovered by centrifugation at 150g for 10 min. An additional centrifugation procedure allowed to get rid of the remaining red (RBC) and white blood cells (WBC), as described elsewhere [25]. Platelets were then washed [26] and resuspended in a Tyrode-Hepes buffer [27] (NaCl 134 mM, KCl 2.9 mM, NaHCO₃ 12 mM, Na₂HPO₄ 0.34 mM, MgCl₂ 6H₂O 1 mM, Glucose 5mM, Heps 20mM pH 7.3) containing EGTA 1 mM. Morphological quality and contamination with other cells of the platelet suspension was evaluated by microscopic and electronic microscopy (EM) inspections. Western blot using CD11a as a marker of the white blood cells was also used with a rabbit monoclonal antibody provided by Epitomics at a dilution of 1:20000.

Subcellular fractionation

Platelets were suspended in Tyrode’s buffer containing 1:7 (vol/vol) of a protease inhibitor cocktail (Roche) and sonicated (Branson Sinifier Cell disrupterb-30/W350) to lysate the cells. Non-lyzed platelets were removed by centrifugation at 2000g, 15 minutes at 4°C. Sucrose gradients were prepared 24h before the fractionation and stored at 4°C to ensure their stabilization. Two sucrose solutions were prepared with 20 and 60% of sucrose and EDTA 5mM. Platelet suspension was carefully poured on the gradient and centrifuged at 96800g for 135 minutes (Beckman L60, rotor SW41). Eleven fractions were collected and stored at -80°C.

Western blot of the sucrose fractions

Ten percent of each fraction was aliquoted and precipitated with methanol-chloroform 4:1 (vol/vol). Laemmli’s buffer was used to solubilise the pellet and proteins were separated on a
12.5% T, 2.6% C polyacrylamide gel, prior blotting on a nitrocellulose membrane (Whatman). A rabbit polyclonal antibody directed to cathepsin D was provided by Millipore. Rabbit polyclonal antibody directed to calreticulin was a gift from Prof. K. H. Krause (University of Geneva, Geneva, Switzerland). Polyclonal rabbit antibodies anti-fibrinogen and anti-von Willebrand Factor conjugated with horseradish peroxidase were provided by DakoCytomation. Rabbit polyclonal antibodies against 14-3-3zeta and ERK2 and goat polyclonal antibody against CD42c were provided by Santa Cruz. Rabbit polyclonal antibody against glutamate dehydrogenase (GDH) was provided by Rockland. Markers were detected by immunoreactions enhanced by chemoluminescence using horseradish peroxidase-coupled secondary antibodies (Dako) and by ECL Western blotting system (GE Healthcare).

Transmission electron microscopy

Whole platelets were fixed in phosphate buffer (100 mM NaPO4, pH 7.4) containing paraformaldehyde 4% and glutaraldehyde 0.1% for 1 hour. The fixative was then removed by several washes with phosphate buffer. Fixed platelet pellet was infiltrated with sucrose and frozen in liquid nitrogen. Frozen sections of 45 nm were cut (FCS crytome, Leica) and transferred on grids for examination with a Technai™ 20 electron microscope (FEI Company) [28].

Granules were fixed in Glutaraldehyde EM-grade 2%, formaldehyde 4% and CaCl2 2mM in sodium cacodylate 0.05M over night. Washes were performed with sodium-cacodylate 0.1M, before incubation in 0.8% K3Fe(CN)6 in sodium-cacodylate (0.1M). A second incubation in 0.8% K3Fe(CN)6 + 1% OsO4 in sodium-cacodylate (0.1M) was performed on ice. Washes were performed in sodium-cacodylate (0.1M) and in distilled water. Blocking and staining were done in uranylacetate 1% in H2O, 90 min at RT in the dark. Then, samples were dehydrated (15, 30, 50, 70, 90, 3x100% EtOH 7min each) and incubated in 100% propylenoxid (3x10 min). Embedding was propylenoxid-epon (1:1) overnight and in 100% Epon (3x 2h). Sections of 70 nm were cut and transferred on grids for examination with Tecnai™ G2 Sphera (FEI Company).
Proteomic sample preparation

Sucrose fraction samples were precipitated with methanol-chloroform 4:1 (vol/vol). All samples were suspended in a surfactant containing ammonium bicarbonate buffer (Rapigest, Waters) (pH 8.2) and homogenized by sonication in a tube sonicator (Hielscher) and by heating in accordance with the manufacturer instructions. In addition to a Bradford quantification (BioRad), 5% of the sample were analyzed by silver staining to relatively quantify the amount of material for further proteomic sample preparation. Briefly, samples were loaded on a SDS-PAGE, together with a range of platelet protein extract dilutions of known concentration. Gel was fixed in ethanol, acetic acid solution (4:1:5 vol/vol). Gel was incubated in 1% glutaraldehyde (vol/vol) 0.5M sodium acetate, followed by 2 washes in 2,7-naphtalene disulfonic acid. Then, it was incubated in the staining solution, composed of 0.8 g/l silver nitrate, 0.2% NaOH and 0.3% ammoniac. The gel was developed in 100 mg/l of citric acid 0.05% of 37% (vol/vol) formaldehyde. The reaction was blocked using a stop solution (0.05M Tris, 10% acetic acid). Relative quantification was done using TotalLab Quant by comparing entire lane intensities of evaluated samples to the reference platelet dilutions. In parallel, protein concentrations were determined by Bradford Assay (Bio-Rad), according to manufacturer guidelines. Fifteen µg of each sample was reduced with dichlorodiphenyltrichloroethane 5 mM and alkylated by adding iodoacetamide 15mM. Trypsin (1:20 weight/weight) was added overnight. Rapigest was then removed according to the Rapigest manufacturer protocol. Finally, samples were desalted using C18 microspin column (Harward) according to manufacturer instructions.

LC-MS/MS analysis

ESI LTQ-OT MS was performed on a LTQ Orbitrap velos from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters. Peptides were trapped on a home-made 5 µm 200 Å Magic C18 AQ (Michrom) 0.1 x 20 mm pre-column and separated on a home-made 5 µm 100 Å Magic C18 AQ (Michrom) 0.75 x 150 mm column with a gravity-pulled emitter.
The analytical separation was run for 65 min using a gradient of H2O/FA 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nL/min. For MS survey scans, the OT resolution was set to 60000 and the ion population was set to $5 \times 10^5$ with an m/z window from 400 to 2000. Five precursor ions were selected for collision-induced dissociation (CID) in the LTQ. For this, the ion population was set to $1 \times 10^4$ (isolation width of 2 m/z). The normalized collision energies were set to 35% for CID. Gas-phase fractionation (GPF) for data-dependant analysis was performed as published elsewhere [29]. Briefly, the m/z ranges for precursor ions selection were 400-521, 516-690, 685-986 and 963-2000 Thomsons.

**Protein identification**

Peak lists were generated from raw data using (ReadW). The monoisotopic masses of the selected precursor ions were corrected using an in-house script modified from a previously published report [30]. Briefly, the precursor ion m/z ratio was determined using Superhirn [31]. Then, miss-assigned precursor ions from the instrument control software were corrected using this value. In cases of ambiguity, both precursor m/z values were used for database searching. The corrected peaklist files were searched against the UniProt SwissProt database (2011_02 of 08-Feb-2011) using EasyProt [32]. Homo sapiens taxonomy was specified for database searching. The parent ion tolerance was set to 10 ppm. Variable amino acid modifications were oxidized methionine. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. Only one search round was used with selection of “turbo” scoring. The peptide p value was 1 E-2 for LTQ-OT data. False-positive ratios were estimated using a reverse decoy database [33]. All data sets where searched once in the forward and once in the reverse database. Separate searches were used to keep the database size constant. Protein and peptide scores were then set up to maintain the false positive peptide ratio below
1%. This resulted in a slight overestimation of the false-positive ratio [33]. For all analyses, only proteins matching two different peptide sequences were kept.

**Bioinformatic analysis and data mining**

Protein localization was retrieved from UniProt (http://www.uniprot.org/), by selecting “subcellular localization” field in the “customize” options. Human Protein Atlas (http://www.proteinatlas.org/) and PlateletWeb Systems Biology Workbench (http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de/plateletweb.php) were manually interrogated for each selected protein. The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/tools.jsp) was used to compare the 2 data sets in terms of BIOCARTA pathway enrichment. Default parameters were used for this analysis.

**Immunofluorescence confocal microscopy**

Immunofluorescence labeling was performed as published elsewhere [9]. Briefly, platelets were isolated from PRP and fixed in 4% paraformaldehyde. Fixed platelets were attached on polylysine-coated coverslips by centrifugation and permeabilized with 0.5% Triton X-100. The blocking procedure was performed using 1% bovin serum albumin (BSA, Sigma) and incubated with the primary antibody for 3 hours. Antibody directed to β-actin was provided by Santa Cruz. After several washing procedures, platelets were incubated with the appropriate secondary antibody for 1 hour. Finally, coverslips were mounted using Fluorocare antifade mountant (Biocare Medica) and stored in the dark at 4°C until analysis on a confocal microscope LSM700 (Carl Zeiss) (objective 63x). Images were acquired under the control of the Zen 2011 blue edition software (Carl Zeiss). Controls consisted in platelet incubation with the secondary antibodies in absence of primary antibodies.
Results

Samples were analyzed following the proteomic workflow depicted in figure 1. A sucrose gradient-based subcellular fractionation allowed getting a secretory granule-enriched fraction. This sample and whole platelets were trypsin-digested and analyzed by mass spectrometry. Protein identification was made using EasyProt [32] and protein lists were mined with several bioinformatic approaches.

Isolation of platelets, separation of platelet organelles and secretory granules enrichment

Platelet suspension was recovered from a pool of healthy subject. Its level of contamination by white blood cells and erythrocytes was assessed by several methods. A microscopic inspection yielded an average of 1 WBC for 1.5 million of platelets and 1 RBC for 540000 platelets (n=6), in line with previous reports [27, 34]. A western blot using CD11, a marker of the white blood cells showed no detectable band (Figure 2A and 2B). Finally, EM inspection of the morphology of platelets in the final platelet suspension showed non-activated, round-shaped morphology (Figure 2C).

Enriched fractions with platelet granules were obtained after sonication of the platelet suspension and organelle fractionation based on sucrose gradient (figure 1). Eleven fractions were collected and analyzed in term of purity and quality. Two bands were macroscopically visible. Figure 3A shows a typical western blot of each sucrose gradient fraction, using several organelle markers. Von Willebrand factor (vWF) and fibrinogen were used as specific α-granule markers and are present in the first, 8th and 9th fractions, together with 14-3-3 zeta and cathepsin D, which confirm the presence of dense granules and lysosomes in these fractions, respectively. The α-granule marker CD42c was found in the same fractions. Of note and as expected, several markers of other cellular compartments (from mitochondria (Glutamate dehydrogenase (GDH)) and to a lower extent from reticulum endoplasmic (ER) (calreticuline)) were also detectable. We concluded that fractions 8 and 9 were enriched in α granules, dense
granules and lysosomes. EM of these fractions showed granule-like structures (figure 3B). These fractions were pooled together for subsequent experiments.

Identification of granules proteins by LC-MS/MS

The granules-enriched fraction was precipitated to remove the sucrose and re-solubilized in surfactant containing buffer. Five percent of granules were analyzed by silver staining to relatively quantify the amount of material, in complement to the Bradford quantification. Fifteen micrograms of sample were then trypsin-digested. The sample was injected twice into an Orbitrap mass spectrometer for identification. Gas-phase fractionation (GPF) was used to acquire the data [23, 29]. This method allows increasing the coverage of the sample by selecting only ions from a window of given mass-to-charge (m/z) for tandem MS. In the window, the number of MS/MS spectra will thus increase compared with a non-GPF injection. However, this process necessitates injecting the same sample several times, in order to cover the entire m/z range, by sequential sliding of the window along the range. Here, 4 windows were used (GPF4) and the process was repeated twice.

Two samples of granules from a pool of donors were analyzed. The first GPF4 granule analysis allowed identifying 693 proteins, whereas the second GPF4 identified 678 proteins. Together, 803 potential granule proteins with a minimum of two peptides were found in our granule-enriched fraction (supplemental table 1). Compared to the data previously published [24], we identified 688 additional proteins (figure 4).

Bioinformatic interpretation of the granule proteome

Protein localization was retrieved from UniProt (“subcellular location” field) (figure 5). The missing or unclear information was manually completed using Gene Ontology (GO in supplemental table 2), PlateletWeb (PW in supplemental table 2) or Human Protein Atlas (HPA in supplemental table 2). Moreover, the location of proteins documented by GO being involved in
platelet activation, were systematically confirmed in PlateletWeb. Indeed, these proteins have a higher probability to be secreted during the platelet activation process and thus to be experimentally found in publications addressing secretion issues and listed in the PlateletWeb site. Finally, a location could be attributed to 789 out of 803 proteins of the secretory fraction. Figure 5 shows the distribution of the different locations among the data set. The main potential contamination, in accordance with the western blot results (figure 3A), came from mitochondria with 11% of proteins. Thirty-nine percent of the proteins were located in the cytoplasm. Many of them are found to interact with cytoskeleton, such as utrophin (P46939) or several tubulin isoforms (P68363, Q9BQE3, Q9H853, Q9NY65, Q9H4B7, Q13509, P68371, P07437 or Q3ZCM7). These proteins are not in granules but known to strongly interact with them and thus to co-enrich with the granules. The same is also possible for plasma membrane proteins (44 proteins). Indeed, many platelet receptors present at the cell surface are also found in α granules [35]. Granules are formed in megakaryocytes, but also in mature platelets, from ER, Golgi and clathrin-mediated endocytosis, depending on the cargo [7]. This particular feature explains the presence of 60 proteins located in ER and Golgi. Finally, around one third of the proteins are directly related to granules and grouped in the “vesicular proteins” class. This comprised several subcategories of locations which are related to secretion. The presence of lysosomes in granule-enriched fraction is in line with the western blot results (figure 3A) and constitutes an important platelet secretion component [36]. Peroxisome and phagosome proteins are related to endocytosis in general, reflecting the importance of endocytosis in platelet secretion, for instance in term of albumin or factor V secretion [36]. Melanosomes are a particular class of secretory granules, which are largely used to study secretory granule dynamics [37]. Many inheritated platelet secretion defects also affect melanosome secretion, due to numerous common physiological mechanisms [38]. “Granule-associated proteins” is a composite class grouping proteins directly interacting with granules, often at different places of the cell. For instance, ADAM10 is an endopeptidase involved in the cleavage of TNF-α at plasma membrane
and Golgi. It has also been found in secretory granules [39]. Finally, the large majority of the granule-associated proteins are secreted. Among them, as previously mentioned, some are well known such as Platelet factor 4 (P02776), and others were never shown before in platelet secretory granules, such as MHC-1 (P04439), Tyrosine-protein kinase SYK (P43405) or Tyrosine-protein kinase Lyn (P07948).

The vast majority of the proteins identified from the granule-enriched fraction are involved in platelet secretion, either at the level of granule biogenesis (ER, Golgi or endocytosis) or cargo (lysosomal or secreted proteins).

To assess the level of data quality improvement thanks to enrichment, we compared the number of unique peptides and of spectra of the granule-enriched fraction with a reference data set of 610 proteins from whole platelets prepared in the same conditions (same donor and same analytical workflow). Almost each category contains more proteins and average number of spectra and unique peptides per subcellular localization category in the granule-enriched fraction than in the whole platelet (Fig. 6).

An efficient enrichment strategy implies an over-representation of given information or pathways. In the context of secretory granules, secretion, granules and cytoskeleton would represent expected enrichments. The Database for Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/tools.jsp) was used as a platform for mining of protein results [40]. This resource allows mapping a large list of genes of interest to different information from databases, pathways and cellular functions, highlighting the enriched ones compared to the whole genome.

The 2 lists of identified proteins (secretory granules and reference whole platelet data sets) were submitted to DAVID and a pathway enrichment analysis was performed (Table 1). Among the 33 pathways in total, 17 were enriched only in the granule data set, whereas 3 were found specifically in the whole platelet (figure 6).
Three pathways were present only in the whole platelet data: “cell to cell adhesion signaling”, “endocytotic role of NDK, Phosphins and Dynamin” and “Free radical induced apoptosis”. They are enriched thanks to specific proteins identified in the whole platelet proteins list, which are discriminative for these pathways.

The granule data set shows 17 specific enriched pathways. Among them 12 pathways contained ERK (extracellular signal-regulated kinase)-signaling (figure 8). They all contain ERK1/2, which belong to the mitogen-activated protein kinases (MAPK) activation pathway [41]. As expected, many of the ERK-related pathways are integrin-related (figure 8). Two pathways are related to β-arrestin (“role of β-arrestins in the activation and targeting of MAP kinases” and “role of β-arrestin-dependant recruitment of Src kinases in GPCR signaling”), which triggers the internalization by endocytosis of G-protein coupled receptor (GPCR) upon their activation. “Fc epsilon receptor I signaling in mast cells” is also present only in the granule data set, thanks to the presence of MAPK activation pathway proteins such as LYN or SYK. In addition, “thrombin signaling and protease-activated receptors” and “PKC-catalyzed phosphorylation of inhibitory phosphoprotein of myosin phosphatase” contain common proteins in the granule data and refer to a platelet activation pathway and platelet formation, respectively. Finally, “Antigen processing and presentation” reflects the identification of HLA class I histocompatibility antigen (MHC1) and Antigen peptide transporter 1 and 2 in the platelet secretory granules, as known for other cell types.

Verification of granule localization of novel proteins

Many pathways related to ERK1/2 were found as enriched in the granule data set. This metabolic cascade constitutes a target of choice for verification, being identified in several organelles [42] but not formally in platelet secretory granules. ERK1/2 distribution among the different sucrose fractions was assessed during the quality control steps of the sucrose gradient. As shown in figure 3, ERK1/2 are present in fractions 8 and 9, the same fractions than granule
markers. This reinforces the possible presence of ERK1/2 in the platelet secretory granules. The antibody used recognizes both ERK1 and 2 in the fractions 8 and 9, but ERK2 (42 kDa) seems to be more specific to these fractions. Moreover, tyrosine-protein kinase SYK (P43405) and Tyrosine-protein kinase Lyn (P07948) were chosen to be confirmed in the granule. The first step was to confirm their presence in the granule-enriched fraction and to define their distribution along the sucrose gradient. A western blot of each sucrose fraction was performed with the fractions of interest (figure 8). Von Willebrand factor was used as a reference for granule presence. Lyn showed a similar distribution than vWF, i.e. in the upper sucrose fraction and in the fraction of interest (figure 8), whereas SYK was only detected in the granule-enriched fraction. This confirms the presence of these proteins in the granule-enriched fraction. To ensure their localization in the whole platelet, immunofluorescence co-localization experiments in confocal microscopy are ongoing, using granule markers, such as vWF or actin (figure 9).

**Discussion**

This study aimed to further characterize the platelet secretory granule proteome. High level of purity of the platelet suspension was demonstrated by several quality controls (QC), including western blot analysis with a white blood cell marker, CD11, and electron microscopy to ensure the resting state of the platelets (Figure 2). In addition, microscopic inspection yielded an average of 1 white blood cell for 1.5 million of platelet and 1 red blood cell for 540000 platelets (n=6). Then, secretory granules were enriched by sucrose gradient fractionation. Of note, EDTA was necessary in sucrose solution to avoid organelle aggregation and guarantee a reproducible separation. Here again, several QCs were used to identify the enriched fractions of interest (western blot using 8 organelle markers) and to evaluate their quality (electron microscopy to assess the granule-like structure presence) (Figure 3). Finally, samples were analyzed by mass spectrometry in GPF mode. This sub-cellular fractionation step aimed to increase the dynamic range covering the sample identification and detect less abundance granule proteins. We could
identify more than 800 proteins in the granule-enriched fraction. The presence of vWF, P-selectin or integrin αIIb among the most abundant proteins corroborates the presence of secretory granules. Maynard and coworkers published in 2007 the first proteomic study of platelet secretory granule [24]. They used a similar subcellular fractionation strategy than in this study. However, they separated proteins by SDS-PAGE, prior an in-gel digestion and an MS analysis using an ion trap. They identified 219 proteins (figure 4), among which one half is also identified here. In the present work, we identified around 690 additional proteins that were not previously described. Our approach dramatically increases the characterization of the platelet secretory granule proteome thanks to the enrichment procedure that we developed. This can be explained by the differences in analytical workflow and by the technical improvements. Indeed, the use of an Orbitrap, which presents a very high sensitivity compared to ion trap, and a GFP4 mode of data acquisition, which is known to strongly increase peptide detection and thus protein identification [29], are clear technical advantages.

The evaluation of granule data in terms of subcellular localization showed a possible slight contamination of mitochondria, also visible by western blot (figure 3). Manual exclusion of the known mitochondrial proteins has been performed in the Maynard’s study [24]. However, this option has not been chosen here, because even if the proteins could be mitochondrial, they also could be part of the secretory machinery, such as clusterin (P10909, see supplemental table) that is known to be in mitochondria and also belongs to the secretory machinery [43]. The other localizations represented in the granule data set were all related to platelet secretory machinery, including biogenesis and cargo secretion.

The benefit of subcellular fractionation is appreciable considering the higher number of spectra per category and of detected peptides to identify them in granule-enriched fraction compared with the whole platelet. In the particular case of platelets, which are anucleated small cell fragments, this approach increases both the number of identified proteins and the quality of
identification, by increasing the number of detected peptides and measurements, which reflects enrichment in low abundance proteins and a more reliable identification of each protein.

Pathway enrichment analysis was performed to compare the whole platelet and granule-enriched proteomes. Common pathways presenting similar enrichment in both data sets were related to integrin (“integrin signaling pathway”) or general platelet features (“Eph kinases and ephrins support platelet aggregation”). The most enriched pathway is “integrin signaling pathway” (figure 7). Integrins play a key role in platelet activation, being receptors for collagen (α2β1), von Willebrand factor and fibrinogen (αIIbβ3) or extracellular matrix component (αVβ3, α5β2, α6β1) [44]. They are known to be present in plasma membrane and α-granules [12], which are both analyzed here.

“Cell to cell adhesion signaling” and “endocytotic role of NDK, Phosphins and Dynamin” were found specifically enriched in the whole platelet list. They contain plasma membrane or membrane-associated proteins related to cell junction (PTK2 protein tyrosine kinase 2 (Q8IYN9) which is not present into granule-enriched fraction for “cell to cell adhesion signaling”) and to induced vesicle endocytosis (epidermal growth factor receptor substrate 15 (P42566) also present in whole platelet data set only for “endocytotic role of NDK, Phosphins and Dynamin”). The third whole platelet-enriched pathway is “free radicals induced apoptosis” and is located in the cytoplasm and in the mitochondria. These cellular compartments are decreased by the fractionation procedure. Indeed, superoxide dismutase 1 (P00441), glutathione synthetase (P48637) and glutathione reductase (P00390) are absent of the granule-enriched sample due to the fractionation. Finally, the granule proteins list is specifically enriched in 17 pathways, among which, a large majority is related to ERK signaling. It has been shown that ERK1/2 is involved in platelet activation by thrombin, vWF, ADP and collagen via internal calcium mobilization among other mechanisms [45, 46]. ERK phosphorylates many important proteins to induce gene expression modulations. It is known that ERK can be activated in the cytoplasm and induce signaling, however ERK presents different effects related to different localizations. This is made
possible by anchor and scaffold proteins, which interact with ERK, depending on its phosphorylation state. They target ERK towards various cell compartments in addition to nucleus, such as plasma membrane (integrins), Golgi (Sef) or late endosome (MAP kinase organizer 1) [42]. Two enriched pathways involve such anchor protein, β-arrestin: “role of β-arrestins in the activation and targeting of MAP kinases” and “role of β-arrestin-dependant recruitment of Src kinases in GPCR signaling”. β-arrestin plays a role in internalization of GPCR by endocytosis. Platelets express 7 different GPCRs, activation of which has an activating (fibrinogen, ADP) or resting effect (prostacyclin). It has been shown that β-arrestin takes part in the αIIbβ3 regulation in mouse [47], serves as plateform for MAPK activation [48] and triggers granule secretion in granulocytes [49]. Moreover, β-arrestin targets ERK to plasma membrane and endocytosis granules, which can be either recycled to the plasma membrane or fused with lysosomes [42]. “Fc epsilon receptor I signaling in mast cells” gives evidence of the presence of other MAPK activation pathway members, such as SYK and LYN (confirmed by western blot), which are known to interact together and with the collagen receptor glycoprotein VI at the plasma membrane [41]. This pathway describes the mast cell degranulation induced by Fc epsilon binding, phenomenon which is also triggered by platelet activation. Other pathways such as “antigen processing and presentation” are also related to secretory granules in other cells than platelets.

In conclusion, this study constitutes a step ahead in the understanding of the platelet secretion machinery, which is of high importance not only in the platelet physiology, but also in many pathologies involving platelets, among them atherosclerosis. Indeed, platelets are activated by atherosclerotic plaques and make them more instable, by secreting cytokines. Anti-platelet drugs such as aspirin and clopidrogel are often prescribed to decrease this phenomenon, but they are of variable efficiency. A better insight into the platelet secretory system could potentially help to define new and more efficient drug targets, mainly in the case of treatment failure.
Abbreviations

Bovin serum albumin (BSA)

Electronic microscopy (EM)

Extracellular signal-regulated kinase (ERK)

Gas phase fractionation (GPF)

Gene Ontology (GO)

Glutamate dehydrogenase (GDH)

HLA class I histocompatibility antigen (MHC1)

Human Protein Atlas (HPA)

Mass spectrometry (MS)

Mass-to-charge (m/z)

Multi vesicular body (MVB)

Platelet Web (PW)

Platelet-rich plasma (PRP)

Quality control (QC)

Volume (Vol)

Von Willebrand factor (vWF)
**Figure legends**

**Figure 1** Workflow of the proteomic analysis of whole platelet and granule-enriched fraction

Platelets were isolated form whole blood by centrifugation. To isolate secretory granules, a platelet lysate was loaded on a sucrose gradient. Samples were then trypsin-digested and analyzed by mass spectrometry in a GFP-4 mode. Finally, protein identification was performed by bioinformatics.

**Figure 2** Evaluation of the purity and the quality of the platelet suspension.

Ponceau-colored nitrocellulose membrane (A) and corresponding western blot of CD11 (180 kDa) (B) were performed on Molecular Weight Standards (1), 15 µg of white blood cells (2) and platelet suspension (3). No CD11 band could be detected in the platelet suspension. (C) Electronic microscopy picture allowed evaluating the resting state of the platelet after their isolation.

**Figure 3** Quality controls of the subcellular fractionation

(A) Western blot of the sucrose gradient fractions. Proteins were precipitated from the 11 sucrose fractions collected after the ultracentrifugation and loaded on western blots. Different markers were tested to assess the enrichment of the fraction 8 and 9 in granules. (von Willebrand factors (vWf), glutamate dehydrogenase (GDH)). (B) Electronic microscopy picture of the fraction of sucrose gradient, which is enriched in intact storage granules.

**Figure 4** Comparison between granule-enriched fraction and published data set

One-hundred and fifteen proteins were identified in common by comparing the granule proteome data of the study of Maynard and co-authors with granule-enriched data obtained here. Six hundred and eighty eight were not found in the previous work.

**Figure 5** Distribution of the granule-enriched protein subcellular localization

Subcellular localizations were retrieved from UniProt, Human Protein Atlas, Gene Ontology and PlateletWeb (see supplemental table 1) and represented in the right pie chart. “Vesicular proteins” corresponds to a groups of several subcategories depicted in the left pie chart. The number of proteins present in each category is written in brackets following the category name.

**Figure 6** Number of peptides and unique peptides detected in each subcellular localization category
The average number of peptides (A) and of unique peptides (B) was calculated for each category of subcellular localization depicted in the figure 5 and compared with the same results from a reference whole platelet protein list.

**Figure 7** BIOCARTA pathways enriched in the proteins identified from the whole platelet and granule analysis.

The 33 BIOCARTA pathways are represented on x-axis, whereas the percentage of the protein list present in the pathway on y-axis. Whole platelet pathways are in beige and granule pathways in pink. Among the 17 pathways enriched in granule data set, 12 rely on ERK1/2 activation cascades. Of these 12, 4 directly involve integrin signaling. The most enriched pathways is “Integrin signaling pathways” and present a similar representation between the 2 protein lists.

**Figure 8** Verification of SYK and Lyn presence in granule-enriched fractions

Western blot analysis of the sucrose gradient fractions shows the presence of SYK and Lyn in fraction 9 which are enriched in secretory granules (see figure 3).

**Figure 9** Immunofluorescence of vWf and actin

vWf (A) and actin (B) are examples of markers (α granule and cytoskeleton, respectively) in confocal immunofluorescence microscopy to establish the granular localization of newly identified proteins, such as SYK or Lyn. This work is still on progress.

**Table 1** BIOCARTA pathways enriched in whole platelet and granule-enriched data sets

Pathways are listed with the corresponding information for each data set, i.e. the number of proteins present in the pathway (count), the corresponding percentage (%) and the p-value associated calculated by DAVID.
Figures and tables

Figure 1

Whole blood → PRP → Platelets → Sucrose gradient → Secretory granules enrichment → In-solution digestion → MS/MS analysis: GPF-4 → Protein identification

Figure 2

A

250 kDa
150 kDa
100 kDa

B

250 kDa
150 kDa
100 kDa

C

5 μM
**Figure 3**

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**Figure 4**

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Granules

104 115 688
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Aknowledgements

We thank the Bioimaging Platform of NCCR Frontiers in Genetics and Prof. M. Foti (University of Geneva) for technical help in electronic microscopy of the secretory granules and whole platelet, respectively.
References


PLATELET SECRETORY GRANULES: A FIRST STEP TOWARD UNRAVELING PLATELET REACTIVITY IN ASPIRIN-TREATED CARDIOVASCULAR PATIENTS

Chapter 5
PLATELET SECRETORY GRANULES: A FIRST STEP TOWARD UNRAVELING PLATELET REACTIVITY IN ASPIRIN-TREATED CARDIOVASCULAR PATIENTS

The sample preparation described in the previous chapter was applied to 6 cardiovascular patients presenting a high vs. a low PR (3 vs. 3). Here, peptide fractionation was performed by off-gel electrophoresis. Around 190 proteins were identified and quantified among which 14 were differentially expressed. They were involved in cytoskeleton stability, exocytosis, glycosylation and platelet activation. This chapter constitutes a first step in the characterization of the molecular mechanisms modulating PR.

I was in charge of the whole analysis and data management and I wrote this chapter.
Platelet secretory granules: a first step toward unraveling platelet reactivity mechanisms in aspirin-treated cardiovascular patients

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Keywords: Platelet, platelet reactivity, aspirin, cardiovascular proteomics, tandem-mass-tag, cytoskeleton
Abstract

Platelets play a central role in haemostasis and are also involved in the pathophysiology of atherosclerosis process. They constitute thus a therapeutic target in cardiovascular patients and antiplatelet drugs are highly effective in this setting. Among these drugs, aspirin is the most prescribed antiplatelet agent. It decreases platelet reactivity by inhibiting cyclooxygenase-1. However, a significant proportion of patients displays a preserved on-treatment platelet reactivity despite inhibition of the cyclooxygenase-1. Little is known about the molecular mechanism responsible of this compensation. Here, a proteomic workflow was applied to platelets from 6 cardiovascular patients with high or low platelet reactivity under aspirin treatment. Fourteen proteins were found differentially expressed among the two groups. Among them, the majority are involved in cytoskeleton stability. The others take place in exocytosis, glycosylation and platelet aggregation. These results could point out a possible role of cytoskeleton reorganization in platelet reactivity, although functional validations are mandatory to confirm their implication. However, this work belongs to a larger project including additional proteomic analysis, transcriptomic quantification, and single nucleotide polymorphism characterization on a larger cohort of cardiovascular patients as well as a network biology approach in order to identify biological processes responsible of platelet reactivity.
Platelets, also called thrombocytes, are small discoid anucleated cell fragments released in blood circulation from megakaryocytes [1, 2]. When they are in contact with extracellular matrices due to vessel damage, particular receptors at their surface recognize specific circulating components, such as extra-cellular adenosine diphosphate (ADP) or collagen. Agonists trigger activation cascades ending up to platelet aggregation and formation of a plug on the damaged vessel wall [3, 4]. During this process, drastic changes occur in platelets. They become round and secretory granules, which are highly abundant in these cell fragments, are squeezed in the center. Then platelets form long filipods and granules are released in blood circulation to mobilize a second wave of platelets, increase inflammation and favor wound healing [5, 6]. There are three types of secretory granules: $\alpha$ granules, dense granules and lysosomes [7-9]. Alpha granules are the most abundant and the largest. They contain activating proteins, such as von Willebrand factor or fibrinogen, whereas dense granules contain mainly small molecules (ADP) and ions. Finally, a few lysosomes are also secreted during the platelet activation process. Their enzymatic content is supposed to play a role in plug remodeling. Platelets react to a variety of agonists, such as collagen, thrombin, ADP or thromboxane A2. Each of them triggers a particular activation pathway. However, several reports showed a variation of release threshold, depending on blood donors. This phenomenon is poorly understood and its evaluation is not obvious. The concept of platelet reactivity (PR) refers to the ability of platelets to aggregate upon activation of a given agonist at a given concentration [6, 10].

Platelets are of utmost importance not only in haemostasis after a vessel injury but also in atherothrombosis process [11, 12]. Platelets stick to atherosclerotic plaques and release inflammatory and mitogenic factors, such as chemokines. They increase inflammation, plaque instability and, as a consequence, favor plaque rupture and thrombus formation leading to acute ischemic events [11, 13]. Antiplatelet drugs are thus often prescribed to reduce the recurrence of ischemic events in cardiovascular (CV) patients, by decreasing PR [14]. Among them, aspirin is
the most common one [15]. It acetylates serine 529 of platelet cyclooxygenase-1 (Cox-1) [5]. This enzyme is implicated in the production of the prostanoid thromboxane A2 (TxA2) that interacts with a dedicated G-protein coupled receptor named TxA2 receptor (TP) (figure 1). The irreversible inhibition of Cox-1 by aspirin abolishes the production of TxA2 for the life span of the platelets [15, 16] (figure 1). Although the inhibition of the platelet-derived production of TxA2 reaches at least 95% in the vast majority of patients [17], there is a marked variability when PR is evaluated with more global platelet function assays. Yee and colleagues showed that a significant proportion (14%) of healthy subjects keep a high PR after aspirin intake and that this phenomenon is stable over time and global (not agonist-specific) [18, 19]. We have shown that PR is also stable and global in cardiovascular patients [20], but little is known about the molecular mechanisms at work to explain the variation of PR in these patients.

The aim of the present work is to identify gene products differentially expressed in 2 sets of extreme aspirin-treated patients (n=6) in term of platelet reactivity using a platelet proteomic workflow and to generate hypothesis for further functional validations. Platelet secretion is shared by numerous agonists and thus is an essential step of platelet aggregation. In addition, it is directly modulated by aspirin [21] and it is therefore a potential candidate in the explanation of the variability of PR. We thus decided to focus this study on secretory granule proteome. We used a proteomic workflow relying on subcellular fractionation and isobaric mass spectrometry (MS) quantification by tandem-mass-tag (TMT) to identify and quantify proteins from platelet secretory granules-enriched samples.
Material and methods

Granule-enriched sample preparation

Six cardiovascular patients presenting extreme high or low PR were selected as described elsewhere [20]. Platelets were isolated from whole blood and a subcellular fractionation based on sucrose gradient allowed to obtain fractions enriched in secretory granules [22]. Then fractions of interest were precipitated in methanol/chlorophorm and resuspended in a surfactant containing buffer (Rapigest, Waters) [22]. Quantification and quality control were performed as explained in [22]. When possible, two gradients were made per patient.

Sample digestion, TMT labeling and off-gel electrophoresis

Samples (10 µg per patient) were reduced by adding tris(2-carboxyethyl)phosphine (TCEP, Sigma-Aldrich) 2.5 mM and alkylated by iodoacetamide (Sigma-Aldrich) 40 mM. Trypsin (Promega) was used to digest proteins (1:20) overnight. Tandem-Mass-Tag (TMT) labeling was performed as previously described [23]. The 3 samples from patients with a high PR were labeled with the even tags (TMT-126, TMT-128 and TMT-130) and the 3 samples with from the low PR patients with the odd tags (TMT-127, TMT-129 and TMT-131) and pooled. Rapigest was then removed according to manufacturer information and sample was desalted using a C18 microspin column (Harvard). Finally, an off-gel electrophoresis (Agilent 31000 OFFGEL Fractionator Kit) was performed according to manufacturer guidelines.

Mass spectrometry analysis

Tandem mass spectrometry (ESI LTQ-OT MS) was performed on a LTQ Orbitrap velos from Thermo Electron (San Jose, CA, USA) equipped with a NanoAcquity system from Waters. Peptides were trapped on a home-made 5 µm 200 Å Magic C18 AQ (Michrom) 0.1 × 20 mm pre-column and separated on a home-made 5 µm 100 Å Magic C18 AQ (Michrom) 0.75 × 150 mm column with a gravity-pulled emitter. The analytical separation was run for 65 min using a
gradient of H2O/FA 99.9%/0.1% (solvent A) and CH3CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B at 55 min, and 20% A and 80% B at 65 min at a flow rate of 220 nL/min. For MS survey scans, the OT resolution was set to 60000 and the ion population was set to 5 × 10^5 with an m/z window from 400 to 2000. Maximum of 3 precursors were selected for both collision-induced dissociation (CID) in the LTQ and high-energy C-trap dissociation (HCD) with analysis in the OT. For MS/MS in the LTQ, the ion population was set to 7E3 (isolation width of 2 m/z) while for MS/MS detection in the OT, it was set to 2 × 10E5 (isolation width of 2.5), with resolution of 7500, first mass at m/z = 100, and maximum injection time of 750 ms. The normalized collision energies were set to 35% for CID and 60% for HCD. Each off-gel fraction was injected twice.

**Protein identification**

Peak lists were generated from raw data using the embedded software from the instrument vendor (extract_MSN.exe). After peak list generation, the CID and HCD spectra were merged for simultaneous identification and quantification ([24] and http://www.expasy.org/tools/HCD_CID merger.html). The monoisotopic masses of the selected precursor ions were corrected using an in-house written Perl script [25]. The corrected mgf files, combined from the 24 analyzed off-gel fractions, were searched against UniProt database (2011_02 of 08-Feb-2011) using EasyProt [26]. Homo Sapiens taxonomy was specified for database searching. The parent ion tolerance was set to 20 ppm. Variable amino acid modifications were oxidized methionine. Trypsin was selected as the enzyme, with two potential missed cleavages, and the normal cleavage mode was used. Only one search round was used with selection of “turbo” scoring. The peptide p value was 1 E-2 for LTQ-OT data. False-positive ratios were estimated using a reverse decoy database [27]. All datasets where searched once in the forward and once in the reverse database. Separate searches were used to keep the database size constant. Protein and peptide score were then set up to maintain the false positive
peptide ratio below 1%. This resulted in a slight overestimation of the false-positive ratio [27]. For all analyses, only proteins matching two different peptide sequences were kept.

Relative protein quantification based on TMT labeling
The correction and normalization of the data were applied as described elsewhere [26]. Briefly, intensities of reporter tags of the peptides were corrected in term of isotopic distribution of each tag and normalized according to the total signal of each tag to correct any difference in the amount of each tagged sample. Finally, relative intensity of each reporter was calculated by dividing it by each peptide tags sum. Indeed, the different peptides of a single protein show variable intensities depending on the physico-chemical properties. This normalization allows thus making comparable all the peptides of a given protein each other. A libra ratio was then calculated from the mean intensity of all peptides of a given protein belonging to the same group. The computed ratio was high PR patients vs. low PR patients (ratio H/L). A p-value was calculated with a Student t-test to assess whether the 2 means are significantly different.

Results
A quantitative proteomic study was performed on secretory granules-enriched samples from platelet of CV patients presenting an extreme high or a low PR under aspirin treatment. Granule-enriched samples were prepared following the procedure described elsewhere [22]. The same quality controls were performed. Figure 2 shows a typical western blot of the 6 last sucrose fractions against von Willebrand factor and glutamate dehydrogenase (GDH) as marker of the α granules and mitochondria, respectively. As shown previously [22], subcellular fractionation relying on sucrose gradient allowed an enrichment in secretory granules.

TMT quantification results
One-hundred-and-eighty-eight proteins were identified and quantified from the MS analysis (supplemental table 1), which is in line with previous reports [28]. As expected, proteins known to be present in secretory granules were identified, such as von Willebrand factor (vWF), p-selectin or several integrins, together with proteins which play a role in vesicle trafficking (vesicle-associated membrane protein-associated protein A, Q9P0L0). Numerous cytoskeletal proteins were also found, as they are intimately linked to vesicles for secretion. Finally, a few mitochondrial proteins were also identified, suggesting a slight contamination. However other localizations could not be excluded.

We selected proteins presenting a ratio between the 2 groups of patients (high vs. low PR, H/L) equal or higher than 1.5 or equal or lower than 0.7. These thresholds were selected in an independent quantitative experiment assessing the technical variation of the workflow with 6 samples coming from the same donor (data not shown). The peptide mean coefficient of variation was 8% (± 6.5%). A threshold of 50% (3 times the highest technical variation) was picked to increase significance of the quantitative results. Fourteen proteins satisfied this criterion (table 1). A preliminary gene ontology (GO) analysis was performed using DAVID, to assess the cellular components present into this dataset (figure 3, supplementary table 2). The largest GO categories are “cytoskeleton” and “organelle envelope/membrane”, containing 5 proteins each. This is in line with the process of platelet secretory granule enrichment [22]. Indeed, cytoskeletal proteins are tightly linked to granules and platelet granule membranes contain many proteins [29]. The presence of “internal side of plasma membrane” reflects also the similarity between the platelet plasma membrane and secretory granule membrane.

To go one step further, each protein was independently investigated in terms of publications. Six of them are cytoskeleton proteins (figure 3). Tropomyosin-2 (Tpm2, ratio H/L = 1.8), inverted formin-2 (Inf2, ratio H/L = 0.5) and profilin-1 (Prof1, ratio H/L = 1.7) play a role in de-stabilization of actin filaments [30-32]. Moreover, TPM2 is the non-muscle isoform and may have a role in agonist-mediated receptor internalization, such as vitamin D receptor [33]. Heat
shock protein β 1 (HspB1, ratio H/L = 0.6) is involved in stress resistance [34], but also in actin organization [35, 36]. Kindlin-3, also called fermitin family homolog 3 (ratio H/L = 0.6), takes place in integrin-nediated platelet adhesion cascade [37]. Finally, tubulin β chain (Tbb, ratio H/L = 0.6, fig. 3) was also identified as differentially expressed. NAD(P) transhydrogenase (Nntm, ratio H/L = 0.6), ATP synthase (Atpg, ratio H/L = 0.5) and Coiled-coil domain-containing protein 109A (C109A, ratio H/L = 0.4) are mitochondrial proteins taking an active part in NADH production, ATP synthesis and intra-mitochondrial calcium uptake, respectively [38-40]. NADH-cytochrome b5 reductase 3 soluble form (B5R, ratio H/L = 0.7) is found in erythrocyte but it is identified by 2 unique sequences which are common to NADH-cytochrome b5 reductase 3 membrane-bound form, present in platelets. These isoforms result from alternative splicing [41] and vary in terms of localization, being found in mitochondria, but also in cytoplasm, Golgi and endoplasmic reticulum (ER) [42, 43]. In addition, neutrophil-activating peptide 2 (CXCL7, ratio H/L = 2.3) is known to be secreted and one of its isoforms is packaged into platelet α granules [44]. CXCL7 plays several roles in haemostasis, favoring thrombus formation, inflammation and coagulation [45]. Prostaglandin G/H synthase 1 is also known as cyclooxygenase-1 (Cox-1, ratio H/L = 0.5) and is the target of aspirin [14]. In platelets, Cox-1 produces TxA2 from arachidonic acid (figure 1). TxA2 has several effects, among them it induces platelet aggregation and vasoconstriction [46]. Dolichyl-diphosphooligosaccharide-protein glycosyltransferase 48 kDa subunit (DDOST, ratio H/L = 0.7) is a membrane protein, which is a member of the ligosaccharyltransferase complexe, in the lumen of ER [47, 48]. Finally, Ras-related protein Ral-B (Ral-B, ratio H/L = 0.7) acts as GTP sensor in GTP-dependant exocytosis of dense core vesicle (through its interaction with exocyst complex) and is crucial for cell survival pathways [49, 50].

**Discussion**

Aspirin-treated cardiovascular patients were selected depending on their PR [20]. Patients presenting a high PR are expected to have a compensatory mechanism allowing platelets to
aggregate upon small concentration of agonist, despite COX-1 inhibition. The major events occurring during platelet aggregation are the release of secretory granules to form a stable platelet plug [4, 51]. A particular focus was thus made on secretion-related pathways, by enriching secretory granules.

Among the 188 identified proteins, 14 proteins were found differentially expressed between the high and low PR patients. They correspond to as many possible hypotheses to validate. A preliminary GO analysis based on cellular components showed a large representation of cytoskeleton and organelle envelope proteins (figure 3). However, a deeper individual text mining strategy was performed to elucidate possible implications of these candidates in the phenotype of PR.

**Cytoskeleton proteins**

The largest category of proteins is related to cytoskeleton and contains 6 proteins (HSBP1, TBB, INF2, TPM2, FERMT3 and PROF1). As previously shown [22], cytoskeleton is tightly linked to secretory granules. It is then not surprising that these latter proteins are co-enriched together with platelet granule proteins. Cytoskeleton plays an important role in platelet activation. In resting state, actin filaments are distributed in the whole cytoplasm and connected with spectrin beneath plasma membrane. Microtubules (mt) constitute a ring maintaining the discoid shape of platelets. Agonist stimulation triggers a discoid-to-sphere shape change, followed by centralization of secretory granules in the center of the cells, formation of filipodia and exocytosis of secretory granules at the open canalicular system. All these steps rely on a complete reorganization of cytoskeleton [5]. This makes cytoskeleton-associated proteins of particular interest in the context of PR. Several candidates of interest play a role in actin filament dynamics. Inf2, on the cytosolic face the ER, favors the formation of highly transient actin filaments and could be involved in secretory pathways as Arp2/3 another actin-binding protein. It contains 2 domains, FH1 binding actin monomer and and FH2 interacting with barbed end and
it can thus induce both polymerization and depolymerization of actin filaments depending on the concentration of adenosine tri-phosphate (ATP) or phosphate, respectively [31, 52]. Its higher expression in low PR patients could trigger a less stable cytoskeleton reassembly upon agonist activation compared with high PR patients. HspB1 is well known to take place in oxidative stress response, but it also strongly influences actin cytoskeleton upon oxidative stress or cytokine stimulation and it stabilizes cytoskeleton in non-redox conditions [34-36]. A higher stabilization due to a higher concentration of HspB1 in low PR patients’ platelets could be partially responsible of a decrease of PR upon stimulation. Kindlin-3 is expressed in hematopoietic cells. Depending on the cell, they have different role, such as the maintenance of plasma membrane and cytoskeleton structure in erythrocytes or activation of leukocytes [37]. In platelets, kindlin-3 is involved in integrin-based activation, together with talin, even if the mechanism is not fully understood yet, but relying on their interaction with β integrin. Kindlin-3-/- knockout mice present cutaneous, cerebral, gastrointestinal and bladder hemorrhages and a significant bleeding time increase. Moreover, they express less integrin β 1, integrin β 3, CD9 and glycoprotein VI and their platelets do not aggregate upon ADP, TxA2 analog, thrombin and collagen. Fibrinogen induces also a reduced aggregation in these mice [53]. However, it has been shown that overexpression of kindlin-3 isoforms (kindlin-1 and 2) produces the same integrin inhibition in chinese ovary cells [54]. This leads to the possibility that kindilins act as scaffold for integrin activation [55]. Here, kindlin-3 is found more abundant in low PR patients. Assuming the scaffold hypothesis, an explanation could be that, as in the knockout mice, the larger amount of kindlin-3 decreases platelet aggregation rate to several agonists, including the ones used for PR index calculation [20]. Prof1 is involved in formin-dependant actin filament growth in several processes, such as cell motility, membrane trafficking or cytokinesis. However, it has been shown that, depending on its abundance and the level of actin monomer linked to ADP, Prof1 promotes polymerization or, on the contrary, depolymerization of actin filaments [32]. The difference of Prof1 expression among the 2 groups of patients could thus lead to a difference of
actin cytoskeleton stability and thus activation trigger. Tpm2 is involved in organelle transport, with myosin, and influences actin filaments making vary their flexibility depending on its phosphorylation state and the presence of severing proteins [30]. In addition, Tpm2 was found necessary to vitamin-D receptor internalization [33]. Interestingly, the vitamin D binding protein has been shown to be increased in plasma from aspirin-resistant coronary ischemic patients and incubation of healthy platelets with this protein reduced aspirin effect on TxA2 production in vitro [56]. Here also, Tpm2 is higher in high PR patients, who are considered as less sensitive to aspirin. This result reinforces a possible role of vitamin D metabolism in aspirin effect on platelets. Finally, Tbb is increased in low PR patients. This cytoskeletal protein is involved in intracellular transport, motility, cell shape and organelle transport [57]. A difference in Tbb surrounding secretory granules could reflect differences in their ability to be released upon stimulation. Taken together, these results indicate a multifactorial difference in platelet cytoskeleton stability, which could be part of the PR variation cause (figure 4).

**Mitochondria proteins**

Three mitochondrial proteins were also identified as differentially expressed. NNTM, ATPG and MCU are all increased in low PR patients. They could result of a random contamination of mitochondria during the subcellular fractionation of one of the samples. However, mitochondrial marker GDH presents a similar weak band in all the western blot of the sucrose fraction (data not shown).

**Glycosylation protein**

NB5R3 is a membrane protein with a multi-compartment distribution: ER, Golgi and mitochondria [42]. This protein is post-translationally inserted into the cytoplasmic face of membranes thanks to its N-myristoylation [43]. It plays a role in lipid metabolism in association with cytochrome b5 [43]. Lipidemia is known to influence PR. Indeed, hyperglycemic mice or
subjects having a low high-density lipoprotein level present an increased platelet activation and thrombosis [58].

**Secretory granule-associated proteins**

Four other proteins were found differentially expressed. RalB belongs to the RAS family of small GTPases [59]. It is ubiquitously expressed but highly abundant in testis, brain and platelet. Depending on the Ral effector it interacts with, RalB can have several functions. However, it is known to play an important role in exocytosis through its binding with the exocyst complex, phospholipase D and C and calmodulin [49]. Moreover, it is known to be located on secretory granules in many cell types and can have a positive or negative effect on secretion depending on the tissues and the bounded nucleotide [4]. The difference of expression among both groups of patients could thus favor secretion in patients with a high PR.

OST48 takes place in the rough endoplasmic reticulum (RER), being a sub-unit of the oligosaccharytranferase complex (OST). OST is responsible of the asparagines-linked glycosylation on secreted proteins [48], such as vWF [47]. The presence of OST48 in secretory granules could reflect either ER contamination or the presence of immature secretory granules containing ER or Golgi proteins. Moreover, it has been shown that glycosylation is crucial for secreted protein function [60] and for their secretion [61].

CXCL7 (also called Platelet-Factor 4, PF4) is a very abundant protein of α granules. Synthesized in megakaryocytes, it constitutes around 2% of the total platelet proteins [45]. It is known to have several actions, despite the involved pathways are not fully known. It has an important affinity for negatively charged molecules and, thus, interacts with heparin and negative charges at the surface of platelets, monocytes and endothelial cells. It favors platelet rich clot formation and stabilization, by neutralizing negative charges of platelet surface, which hamper interactions due to charge repulsion and by decreasing heparin-dependant thrombin inhibition and factor V consumption [44]. Moreover, it increases inflammation by activating neutrophils and endothelial
cells. It also plays a role in early stage of atherosclerosis, by producing low density lipoprotein (LDL) retention at endothelium surface [45]. CXCL7 was found increased in high PR patients compared to low PR patients. A possible effect of this over-expression could be to increased aggregation once the activation is triggered, favoring the formation of plug and increasing PR. Finally, Cox-1 is two time more abundant in low PR patients (figure 4). This observation is of particular interest, knowing that Cox-1 is targeted by aspirin for PR decrease [62]. A possible explanation could be that low PR patients express a large amount of Cox-1. As a consequence, their platelet activation relies in majority on TxA2 production. When they are treated with aspirin, this production dramatically decreased (as assessed by ELISA [20]) and platelets become significantly less reactive. On the other hand, patients with a high PR synthesize less Cox-1 in their platelets, leading to a smaller TxA2 dependency for platelet activation and thus a less important decrease of PR upon aspirin intake.

*Per se,* each difference in protein expression may not be sufficient to disturb cytoskeleton, metabolism and, as a consequence, PR. However, in addition to other cellular functions, they could constitute clues to unravel complex mechanisms responsible of PR variability in aspirin-treated cardiovascular patients. These data are preliminary results of a larger project, involving another series of 6 patients and integrating other ‘omics datasets such as quantitative proteomics of platelet releasates, proteomics of whole platelets, transcriptomics quantitation and genetics SNP characterization. Using a network biology approach, pathways of interest, more than single gene products only, will be considered for functional validation. *In vitro* experiments have to be performed, relying, for instance, on faint modulation of cytoskeleton and/or oxidative stress, to confirm their influence on aggregation tests. This step is crucial to confirm the implication of these candidates in PR modulations and thus identify potential new target to modulate PR in aspirin-treated cardiovascular patients.
### Abbreviations

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<tr>
<td>Accession number (AC)</td>
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<tr>
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Figure legends

Figure 1 TxA2 production pathway.
Phospholipids in the plasma membrane and arachidonic acid are substrates of the TxA2 synthesis pathway. Cox-1 metabolises arachidonic acid into prostaglandin G2, which is the substrate of hydroxyperoxidase. The product is prostaglandin H2, which is transformed into TxA2 by TxA2 synthase. Finally, an autocrine and paracrine interaction of TxA2 with its receptor (TP receptor) triggers a G-protein activation cascade. Aspirin inhibits this pathway by acetylation of Cox-1.

Figure 2 Western blot of a typical fractionation by sucrose gradient.
The last 5 fractions were precipitated and loaded on western blot as previously explained (see chapter 4). Antibodies against von Willebrand factor (vWF) and glutamate dehydrogenase (GDH) to verify the major content of these fractions. (Tot = Whole platelets, 5 µg)

Figure 3 Gene Ontology of the cellular compartments represented among the differentially expressed proteins
Almost 28% (5 proteins) of the proteins are present in the cytoskeleton or in organelle membranes. Three proteins (16.7%) were in mitochondria or in the internal side of the plasma membrane. Eleven percent of them, which corresponds to 2 proteins, are depicted as sarcomeric.

Figure 4 Role of Cox-1 and cytoskeleton-associated differentially expressed proteins between the 2 groups of patients.
Profilin-1, inverted formin-2 and HspB1 are involved in actin filament dynamics. Tpm2 plays a role in organelles movement and formation of actin lamellipodia. Moreover, it has been related to vitamin-D receptor, which is known to influence aspirin effect. Fermt3 is a scaffold protein which interacts with integrin β 1 and β 3 and modulates their activation. Finally, Cox-1 plays a crucial role in platelet activation and is targeted by aspirin, to inhibits TxA2 production.

Table 1 Proteins differentially expressed in secretory granules of patients presenting a high or a low PR.
Mean ratios are calculated from TMT reporters intensities of all the unique sequences belonging to each proteins. Student t-Test is calculated based on the hypothesis that means of both groups are identical and the associated p-value is reported. Swiss-Prot accession number (AC) and identifiers (ID) are also listed, as the number of measured peptides for quantification in each group (#Quantified peptides) and for identification (#Identified peptides) and, among them, the number of unique sequences (#Unique sequences in protein) in each protein.
Figures and tables

Figure 1

Figure 2
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<th>Description</th>
<th>#Identified peptides</th>
<th>#Unique sequences in protein</th>
<th>#Quantified peptides</th>
<th>Mean ratios</th>
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References


A NETWORK BIOLOGY STRATEGY TO BETTER UNDERSTAND PLATELET REACTIVITY IN CARDIOVASCULAR PATIENTS UNDER ASPIRIN TREATMENT
This chapter describes the final integration of the different ‘omics datasets. SNPs analysis, transcriptomic quantification as well as proteomic quantification of whole platelets, releasates and secretory granules led to a network of 99 nodes and 309 edges summarizing the most associated genes according to PR phenotype. We performed a preliminary interpretation of this network based on the mapping of databases-confirmed correlations. Two clusters presented a higher density of experimentally confirmed correlations. They contained genes involved in cytoskeleton dynamics, integrin αIIbβ3 aggregation and glucose metabolism. This mining will be further conducted to identify pathways of interest modulating PR.

I performed the proteomics analysis, whereas transcriptomic and genetic samples were prepared by S. Nolli and analyzed by M. Docquier. Data integration and network generation was made by M. Ibberson. Finally, I made the preliminary network interpretation, and I wrote the chapter.
A network biology strategy to better understand platelet reactivity in cardiovascular patients under aspirin treatment

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\textit{Keywords: Platelet, aspirin, platelet reactivity, atherothrombosis, SNP, proteomics, gene expression network biology}
Abstract

High platelet reactivity (PR) in aspirin-treated cardiovascular patients (CV) is a risk factor for recurrence of ischemic events. The determinants of PR in these patients are unknown but previous studies suggested that it is genetically determined. PR was assessed in 110 CV patients treated with aspirin using several agonists. Cardiovascular patients with extreme high or low PR were selected for further ‘omic analysis. Quantitative proteomic of platelets as well as transcriptomic analysis allowed the detection of gene products differentially expressed in these groups of patients. Moreover, a genome-wide association study (GWAS) of single nucleotide polymorphisms (SNPs) allowed mapping genetic variants between extreme patients. These datasets were integrated using a network biology strategy, which resulted in a core network of 99 nodes and 309 edges. A significant enrichment of genes involved in platelet activation, cytoskeleton and glucose metabolism was found. These candidate pathways may delineate new targets for the prevention of ischemic events in CV patients.
Introduction

Platelet function inhibitors such as aspirin are key medications in the prevention and the treatment of ischemic events in cardiovascular patients. Aspirin inhibits a particular enzyme in platelets, called cyclooxygenase-1 (Cox-1), which is crucial for the generation of thromboxane A2 (TxA2) that promotes platelet activation [1]. By inhibiting TxA2, aspirin decreases the platelet reactivity (PR), i.e. the ability of platelets to aggregate in response to a given agonist, and thus their tendency to make atherosclerotic plaques more instable [2, 3]. However, despite inhibition of TxA2 production, approximately 30% of cardiovascular patients present a high on-treatment PR [4], which was associated with the recurrence of ischemic events [5], at least in acute settings [6]. Moreover, this phenomenon was shown to be heritable [7], stable over time [8], global (as opposed to agonist specific) [9, 10], barely affected by cardiovascular risk factors [10] and independent of aspirin intake [8, 9]. Several attempts to delineate the mechanisms of the modulation of PR using a single ‘omic approach have yielded some results, but the findings of these studies are often not concordant [11-13].

A study was designed here to combine genetics, transcriptomics and proteomics using a network biology approach to evidence the pathways associated with PR variability in a cohort of stable cardiovascular patients treated with aspirin [10]. Indeed, each ‘omic approach gives complementary information regarding platelets, which are anucleated cell fragments containing mRNA and part of their proteins from their hematopoietic mother cell, the megakaryocyte [14]. The integration of these data sets was performed using a network biology approach. Network biology is an emerging field that represents biological processes as networks composed of nodes, which here stand for genes, and of edges, which correspond to relationships between nodes [15, 16]. The advantage of network-based over more traditional approaches (e.g. considering each data set in isolation) is that a gene can be represented in the context of its relationships with other genes. Often the context of a gene is important for identification of key pathways, thus a network-based approach can start to teach us something about the complete
system, rather than focusing on individual genes. The preliminary results of our network-based analysis showed a potential role of genes involved in cytoskeleton dynamics, glycolysis and integrin αIIbβ3 activation in the modulation of platelet function in cardiovascular patients.

**Material and methods**

*Patients’ population*

Cardiovascular patients were recruited as explained elsewhere [10]. Briefly, stable cardiovascular patients were assessed for PR using light transmission aggregometry with several agonists. A platelet reactivity index was derived from these analyses and used to classify patients. The 26 most extreme patients presenting an extreme high (13 patients) vs. extreme low (13 patients) were selected for ‘omics studies.

*‘omics step :*

**Genome-wide association study (GWAS) of single nucleotide polymorphism (SNP)**

DNA was extracted from citrate anticoagulated whole blood of the most extreme PR patients. Twenty-six samples were genotyped using the Illumina Omni 2.5 BeadChips. Genotype calls were generated using Illumina GenomeStudio software. SNPs with a minor allele frequency (MAF) <=0.01 and that were called in less than 90% of individuals were removed (1514827 SNPs remained after filtering). Samples with less than 90% of genotyped SNPs were also removed (25 samples remained after filtering).

Associations between SNPs and platelet reactivity (PR) were tested by assessing the correlation of each SNP genotype with the PR index as a continuous trait using PLINK software [17]. After correction for multiple comparisons, no single SNP was significantly associated with PR index. An alternative, more powerful approach is to correlate SNP genotypes with gene expression to identify expression quantitative trait loci (eQTLs). These eQTLs, are therefore loci where there is evidence that the observed gene expression is influenced by genetic variation. There are two
Chapter 6

types of eQTL, cis-eQTLs where the eQTL locus is located close to (in cis) with the gene for which the expression is evaluated, and trans-eQTLs, where the eQTL can be located anywhere in the genome, and may therefore be located within, for example, a distal regulatory element for the gene evaluated. In this study we focused only on cis-eQTLs. To calculate eQTLs we first identified SNPs located within a gene (-10Kbp upstream of the transcriptional start site to +10Kbp downstream from the end of the gene). Start and stop positions of the gene were from Ensembl 64 [18]. Following this step, 983724 SNPs remained. eQTLs were then calculated by correlating the genotypes of each SNP within a gene to the normalized expression of that gene as a continuous variable using PLINK software [17]

RNA profiling

EDTA-anticoagulated venous blood was collected and platelet-rich plasma (PRP) was recovered by centrifugation at 150g for 15 minutes. PRP was then centrifuge at at 750g for 8 minutes to pellet platelets. They were washed in Red Blood cell Lysis buffer (Roche) for 10 minutes to remove red blood cells contamination. Platelets were finally resuspended in PBS buffer (Gibco) and 5x10^8 platelets were stored in RNAlater® (Ambion) at -80°C, until RNA isolation with the mirVana™ miRNA isolation kit and conversion to cDNA by rt-PCR. Platelet cDNA was tested for quality by capillary electrophoresis (2100 Bioanalyzer, Agilent). Fifty ng of starting RNA were used for target preparation. Targets were amplified and labeled according to the 2 steps amplification protocol recommended by Affymetrix (Inc, Santa Clara, California) and hybridized on the Affymetrix GeneChip® Human Genome U133 Plus arrays. White blood cell contamination was estimated by comparing platelet-specific transcripts with white blood cell-specific transcripts after normalization in our sample and in published results from platelets, whole blood and white blood cells. Overall, this sample preparation showed a greater level of purity and enrichment compared to previous reports (supplemental figure 1). Data were RMA normalized and log2 transformed. Fold change differences between high PR and low PR patient

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groups was calculated using limma in R [19]. In order to filter out genes that showed very little difference in expression between high PR and low PR patients, only genes with mean log2 fold change (high PR vs. low PR) ≥ 0.3 or ≤ -0.3 were selected. There were 1949 genes after this filtering that were used to calculate gene-gene correlations.

Subcellular fractionations and proteomics isobaric quantification

Proteomics isobaric quantification was performed on the 12 most extreme high and low PR patients (6 vs. 6) using 3 proteomic preparations: whole platelet, secretory granule-enriched fractions and platelet releasate. Whole platelet suspension and secretory granule enrichment were prepared as previously described [20]. Purity of the platelet suspension was evaluated by microscopic inspection and confirmed by western blot as previously described [20]. Releasate samples were prepared based on a previous report [21] and using thrombin as the platelet agonist. Briefly, washed platelets were resuspended in tyrode buffer [10] at a concentration of \(7.6 \times 10^8\) plt/ml and incubated 1 hour at 37°C. Three-hundred-and-ninety-three µl were dropped in a cuvette of light transmission aggregometer (TA-8V, SD Medical, Heillecourt, France). A concentration of 1.8 mM CaCl2 was added. After 1 minute, 1 unit/ml of thrombin was added and aggregation was recorded for 6 minutes. Platelet aggregates were removed by round of centrifugation at 1000g for 10 minutes. Samples were ultracentrifuged (50’000g) for 1 hour at 4°C in order to discard microparticles. Finally, samples were stored at -80°C until mass spectrometry analysis, which was performed as previously mentioned [22]. Quality controls were performed by silver staining of 1D electrophoresis as previously described [20]. Ratios were calculated from the normalized isotope corrected values for each peptide quantified by mass spectrometry, following a well established procedure [22]. Peptides matching more than one protein were removed prior to the analysis. To generate high PR vs. low PR ratios per gene, NCBI annotations were used to identify the genes corresponding to each SwissProt accession code, and the ratios were averaged over all the peptides for a given gene.
proteins with high PR: low PR ratios \( \log_2(\text{ratio}) \geq 0.15 \) or \( \leq -0.15 \) were included in the subsequent network analysis.

**Network biology step**

**Gene set enrichment analysis (GSEA)**

GSEA was performed comparing high vs. low PR samples to rank genes from transcriptomic and proteomic data using the MSigDB V3 gene sets. In GSEA, a running score is calculated starting from the top of the list and going downwards by increasing it if the gene is found in a given gene set or decreasing it by a smaller amount if the gene is not present in the gene set. The enrichment score (ES) is calculated from this running score as the highest absolute score. A positive ES score indicates that the gene set is enriched for genes at the top of the ranked list (in our case those that are more highly expressed in high PR compared to low PR); a negative ES score indicates enrichment for genes at the bottom of the list (in our case those that are lower expressed in high PR compared to low PR patients) [23]. Raw p-values were calculated by permuting the gene labels in the ranked list 10,000x and recalculating the enrichment scores (ES score). Finally, the false discovery rate (FDR) was calculated from the raw p-values using the Benjamini Hochberg procedure [24]. For all of the significant gene sets, permutated ES scores greater than or equal to the actual ES score were never observed: the FDR are therefore marked as <0.001.

**Significance test for GSEA pathways**

In order to test the significance of results for individual enriched platelet pathways, a bootstrap test was performed by selecting 12 samples at random 1000X and repeating the GSEA against the 3 significant platelet-related pathways. An empirical p value (p) was then calculated and statistical significance was evaluated as p<0.05.

**Network construction**
Network biology is a method, which allows representing biological entities as nodes and relationships between them as edges in a graph. This approach was used to integrate different data sets in a network made of nodes, which stand for genes, connected by edges, which correspond to correlations between genes in a given quantitative data set.

Pearson correlations (pairwise complete observation) using data from all samples were calculated between the PR phenotype and pairwise between all peptides. In order to determine whether the correlations are biologically meaningful, log likelihood ratios (LLR) were calculated for each quantitative data set as described in [25]. The positive control gene pairs (N = 3206268) were defined as those genes either participating to the same pathway (PathwayCommons; http://www.pathwaycommons.org/) or whether there was evidence of either direct or indirect interaction in protein interaction databases (iRefIndex; [26]). Ten million negative control gene pairs were chosen by randomly sampling pairs of genes from the pathway and protein interaction databases. LLRs were calculated for a range of correlation values as the ratio between the fractions of pairs observed with this correlation value or higher in the positive control set compared to the fraction observed in the negative control set. Only positive correlations were tested. For each or the data sets the LLR was seen to increase with increasing correlation values but not over the whole range of correlations. This indicated that only correlations above a certain threshold are likely to be biologically relevant. The correlations above which the LLR was found to increase were: 0.7 (Gene), 0.6 (Protein from whole platelet, releasate and granules). These results indicate that gene-gene correlations above these values are potentially functionally relevant.

The significant correlations based on their LLR were then represented as a network. The two types of genetics data calculated for each gene (the SNP association with the PR index, and the eQTLs; see section above Genome-wide association study (GWAS) of single nucleotide polymorphism (SNP)) were mapped to the nodes of the network. Since each gene contains many SNPs, the SNPs showing the strongest association to the PR index or the expression level of the
gene (as assessed by the pvalue of association) were mapped to each gene. The genetic data are represented as node attributes in the network, allowing the degree of association to the PR index and the gene expression to be visualized and assessed together with all the other correlation data within the network. The network generated represents all the integrated data and could now be interrogated to explore the biology of the system. Since the network representing all the data is quite large, we decided to focus first on parts of the network where there was evidence from several experiments. To do this, we created a subnetwork containing only correlations present in at least 2 out of the 4 quantitative data sets (i.e. 1 transcriptomic and 3 proteomic analyses). This resulted in a sub-network, which was used as an initial starting point for further investigation.

In order to annotate the nodes of the network, 2 approaches were used: the functional annotation clustering tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID; http://david.abcc.ncifcrf.gov/tools.jsp [27]) and GOlayout (Cytoscape 2.8.2 [28]) for visualization of subnetworks partitioned by gene ontology (GO) category.

In addition, interactions from three databases, iRefIndex [26], Pathway Commons [29] and Platelet Web [30] were mapped on to the network to help highlighting clusters of interactions for which there is information that these proteins interact from the literature.

**Results**

Cardiovascular patients were recruited and classified according to their PR index as explained elsewhere [10]. The 26 patients, presenting the most extreme high and low PR index (13 vs. 13, figure 1), were selected for transcriptomics and genetics analysis allowing gene products characterization and/or quantification. Transcriptomics and proteomics data were then integrated during the network biology step.

`omics data step`
Genetics

Genome-wide association study (GWAS) of single nucleotide polymorphism (SNP) characterization was performed on the 26 most extreme patients (13 vs. 13, figure 1) and 1.5 million of SNPs were identified. Two analyses were made to assess the correlation with PR: first, individual SNPs were assessed for correlation with the PR phenotype; and second, putative expression quantitative traits loci (cis-eQTLs) were identified to assess the link between SNPs and gene expression.

Transcriptomics

A quantitative transcriptomic analysis was also performed on the same patients (13 vs. 13, figure 1). Around 5000 mRNAs were quantified, which is in line with previous reports of transcriptomic analysis in platelets [31]. Since platelets contain only a smaller amount of mRNA than nucleated cells, particular attention was paid to possible contamination from white blood cells. Our sample preparation showed a low level of contamination compared with published transcriptomic data from platelet [32], white blood cells [33] and whole blood [34, 35]. After filtering (see material and methods), 1949 genes were kept for the next step.

Proteomics

A quantitative proteomic analysis was performed on 2 series of 6 most extreme patients (6 vs. 6) depicted in figure 1 (supplementary file “proteomics”). Three types of samples were analyzed: “whole platelets”, “releasate” and “secretory granules”. In terms of purity, platelet suspension yielded an average of 1 white blood cell for 1.5 million of platelets and 1 red blood cells for 540000 platelets [20]. Nine-hundred-and-eighty-nine proteins were identified and quantified in the “whole platelet” sample. Releasates were prepared according to published method [21, 36], as shown in figure 2. A clear different pattern was obtained upon thrombin aggregation compared with control (without agonist) and with platelet-poor plasma, as described elsewhere [21]. We quantified 480 releasate proteins. Finally, secretory granules were analyzed [20] in 5
out of the 6 patients from each group (5 vs. 5). This analysis leads to the quantification of 201 proteins among the 10 selected extreme PR patients.

*Network biology step*

The large amount of comparisons between low and high PR of several ‘omic data sets prompts statistical correction for multiple testing. When applied to the different data sets taken individually (comparison of transcriptomics data only between low and high PR patients for example), no candidate of interest appeared as significantly different between the two extreme groups of patients. Indeed, the comparison of patients presenting the same pathology and the same medication, together with the large biological variability associated to the evaluation of human samples induces small ratios and low signal / noise ratios.

Network biology offers a new approach to study differences in populations with heterogeneous genetic background. This emerging field aims to graphically represent the molecules of interest as nodes connected by edges corresponding to any type of interactions [37]. Indeed, these networks constitute an intuitive way to represent whole systems of interacting gene products and allow to explore analytical results in terms of pathways instead of single protein or gene [38].

The concordance of the modulated pathways among transcriptomics and proteomics data sets was assessed. This was made by gene set enrichment analysis (GSEA) [23]. Table 1 shows an example of the GSEA results for the transcriptomic and proteomic “releasate” data. The presence of common pathways, such as “platelet degranulation” or “platelet activation”, and of related pathways (“integrin cell surface interactions” and “integrin pathways”, for instance) is consistent with a putative common pathway modulating PR. More patients were available for the transcriptomic part of the project. These data were used to test the sample size on the GSEA results. A GSEA analysis was performed with 3 vs. 3, 6 vs. 6, 9 vs. 9 and 12 vs. 12 extreme patients. The highest enrichment for each of the 3 tested pathways was obtained with 6 samples.
from each group of patients (supplemental figure 2). This enrichment could be an artifact of selecting 12 samples rather than 6, 18 or 24. The small number of samples means that the 12 samples chosen based on high and low PR might be showing enrichment for platelet pathways by chance. To test this possibility a significance test was performed (see *Significance test for GSEA pathways*). The result of this test showed that the enrichment obtained for these pathways was statistically significant (\text{REACTOME\_PLATELET\_ACTIVATION}: p=0.036; \text{REACTOME\_FORMATION\_OF\_PLATELET\_PLUG}: p=0.025; \text{REACTOME\_PLATELET\_DEGRANULATION}: p=0.045)

A network of 2077 nodes and 722980 edges was generated from the different quantitative ‘omics results (1 transcriptomic dataset and the 3 proteomic datasets), using only the correlations predicted as biologically relevant according to the LLR. It was then filtered to a manageable size for more detailed interpretation. Edges were selected when correlations were observed in at least 2 out of the 4 analyses. A network of 99 nodes connected by 309 edges resulted from this filtering (figure 3 and supplemental figures 3A, 3B and 3C and high resolution pictures in supplementary file 1). A functional annotation was performed using functional annotation clustering from DAVID and subnetworks were furthermore partitioned by gene ontology (GO) category. Several GO categories were found enriched: “actin binding” and “cytoskeleton”, “platelet α granule”, “negative regulation of apoptosis”, “extracellular matrix binding”, “glycolysis”, “calcium binding proteins”, “cell motion and focal adhesion”. In addition, several subnetworks were visualized: “blood coagulation”, “platelet activation”, “platelet degranulation”, “signal transduction”, “carbohydrate metabolism” and “small molecule metabolic process” (a representative example is depicted in figure 3, the others are in the supplementary file 2). These annotations give insights regarding possible modulated pathways and constitute the basis for the interpretation of the network.

Correlations were also tested for literature-based relevance, by assessing their presence in databases (Pathways Commons, iRefIndex and Platelet Web). Two highly connected clusters
appeared to contain a higher density of literature confirmed correlations (Figure 4 and supplemental file “network”). The mining of the network started with those 2 clusters (Figure 5). The first cluster is composed of 8 genes among which 6 are related to actin binding and cytoskeleton (FLAN, ACTB, TLN1, DBN1, MYH9 and TPM3). The 2 remaining genes play a role in platelet activation (ITGA2B and THBS1). On the other hand, the second cluster is composed of a large majority of genes involved in glycolysis (ENO1, GAPDH, TPI1 and ALDOA), of 2 genes, which modulate actin cytoskeleton dynamics (HSBP1 and TWF2) and of SH3BGRL2.

Discussion
In this translational study and for the first time, an integrative approach based on network biology was used to identify pathways modulating PR in cardiovascular patients treated with aspirin [10]. Gene products from these patients were quantified using transcriptomics and proteomics and represented as a network, on which SNP results were mapped. The integration of the data was performed without setting a specific statistical threshold for each independent data set. Indeed, the statistical analysis was made on the calculated correlations and clusters of interest were finally re-enforced by mapping literature-confirmed interactions (figure 4). In addition, this approach overcomed the observed lack of concordance of the gene expression and protein results, the small number of patients selected for further analysis and the biological variability related to any human sample. Indeed, platelets presented an extremely low overlap between mRNAs and proteins [39]. Platelet mRNAs and many proteins originate from megakaryocytes and, since platelets are anuclear, they do not generate new transcripts (except in mitochondria) [31, 40]. Thus, a large part of the proteins do not come from the mRNAs present in platelets. For these reasons, transcriptomics and proteomics data may not correlate at a gene product level, but they may be more concordant at a pathway level. Our network biology approach provides a powerful tool to represent complex processes that underly platelet function and increase the power of detecting genes of a same pathway, by integrating several
‘omics datasets. In addition, even though transcriptomic and proteomic studies present similar affected pathways, they may generate in some instances opposite ratios among the extreme high and extreme low PR patients. A possible explanation for this observation is the regulation of protein expression by microRNAs (miRNA), which is of high importance in platelets [41].

A pathway analysis by GSEA was used to identify up- and down-regulated genes belonging to the same cellular pathway with respect to PR index, in order to test for enrichment of specific pathways in each individual dataset. A comparison of the GSEA results was made between the transcriptomic and “releasate” proteomic datasets. The pathways “platelet activation”, “platelet degranulation”, “hemostasis” and “formation of platelet plug” were identified in both datasets. Moreover, several pathways related to platelet activation and signaling were found (table 1). This result enforces the presence of several modulated pathways common between these data and thus the benefit of integrating these data sets.

A network was built from the correlations calculated between gene products in each data set. Then, the network was filtered for correlations present in at least 2 out of the 4 data sets (mRNAs, whole platelet, releasate and secretory granule proteins). This ended up with a network of 99 nodes and 309 edges. An interpretation was made with DAVID, by functional annotation clustering, showing an enrichment of the network in “actin binding and cytoskeleton”, “glycolysis”, “calcium binding proteins”, “cell motion”, “platelet α granule”, “negative regulation of apoptosis” and “focal adhesion”. This analysis was complemented by partitioning the network according to GO annotation. Six subnetworks were identified: “blood coagulation”, “platelet degranulation”, “signal transduction”, “carbohydrate metabolism”, “platelet activation” and “small molecule metabolic process”. These subnetworks reflect the possible implication of their related pathways in PR phenotype.

Furthermore, we mapped correlations that are indexed in literature-based interaction databases (IREFIndex, Pathways Commons and Platelet Web) (figure 4). Two clusters in the network (figure
4) were of particular interest because they presented a higher density of correlation and of literature-confirmed correlations (figure 5).

The first cluster contains ITGA2B, THSB1, FLNA, ACTB, TLN1, DBN1, MYHP and TPM3 (figure 5). ITGA2B codes for a protein called integrin α-IIb and belongs to a heterodimer receptor on the platelet surface, αIIbβ3 (also called integrin IIb-IIIa) [14]. This integrin plays a central role in platelet aggregation, being receptor of fibrinogen and von Willebrand factor (vWF). These molecules act as bridges between platelets for aggregates formation. Other molecules are also recognized by αIIbβ3, such as fibronectin, prothrombin and vitronectin (also present in the network, but not in this cluster), which contribute to platelet regulation or adhesion to extracellular matrix. Platelet activation by agonists, such as ADP, thrombin or collagen, trigger a structure change of this receptor, which increases its affinity for its ligand. This constitutes the “inside-out signaling”. Once the ligand interacts with αIIbβ3, the “outside-in signaling” is initiated, involving kinase signaling pathways and cytoskeleton remodeling [14]. Another gene of this cluster, THBS1 is expressed in CD47 protein (also called integrin-associated protein), which is the receptor of thrombospondin [14]. This protein modulates αIIbβ3 [42] and α2β1, a receptor to collagen [14]. ITGA2B interacts with many cytoskeletal proteins, including talin (TLN1), which is also present in the cluster [43], and vinculin. ITGA2B may interact with myosin (MYH9, in the cluster) depending on its phosphorylation level [14]. Indeed, activated integrin αIIbβ3 triggers drastic actin cytoskeleton reorganization through talin, promoting their clustering and the formation of focal contacts, regrouping additional cytosolic proteins, as actin (ACTB) and vinculin. The focal contact may mature into focal adhesions, which become signaling hubs, in contact with surrounding matrix [43]. DBN1 gene codes for a protein called drebrin, which is known to interact with actin cytoskeleton and to play a role in the stabilization of gap junction in neurones [44] and may thus be involved in this process. Finally, the cluster contains also 2 other genes involved in actin cytoskeleton dynamics: tropomyosin-3 (TPM3) and filamin-A (FLNA). This
cluster reflects the modulations in αIIbβ3 activation, clustering and its related cytoskeleton rearrangements between the low and the high PR patients.

The second cluster is composed of HSPB1, GAPDH, TPI1, TWF2, ALDOA, ENO1 and SH3BGRL2 (figure 5). Two genes of this cluster are also related to actin cytoskeleton. Hsp27 (HSPB1 gene) is known to be distributed to cytoskeleton in response to its phosphorylation by MAP kinase [45] and may be sufficient to trigger platelet secretion upon ADP activation [46]. In addition, twinfilin-2 (TWF2), is related to cytoskeleton and inhibits actin filament assembly in vitro [47], disfavoring actin cytoskeleton reorganization. On the other hand, several genes of glucose metabolism are also present in this cluster. Aldolase (ALDOA) catalyses the formation of glyceraldehyde-3-phosphate and dihydroxiacetone phosphate from fructose 1,6-biphosphate. Dihydroxiacetone phosphate is converted into glyceraldehyde-3-phosphate by the triosephosphate isomerase (TPI1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is responsible of its conversion to 1,3-biphosphoglycerate, with the production of one NADH molecule. Later in the process, enolase (ENO1) is responsible of the transformation of 2-phosphoglycerate into phospho-enol-pyruvate by the loss of 2 molecules of water [48]. This is of particular interest, because glycolysis is the main system of energy production in platelets [48]. Indeed, most of the glucose (99%) is anaerobically degraded into lactate in resting platelets. When platelets are activated and aggregate, glycolytic flux drastically increases. An up-regulation of lactate production is observed at low dose of thrombin, whereas at higher doses, mitochondrial respiration is increased [49]. Here, patients were recruited based on low doses of agonist [10], i.e. in conditions where glycolysis (as assessed by lactate release) is dominant.

In addition, GAPDH, TPI1 and ALDOA physically interact with actin cytoskeleton for glycolysis modulation [50], which makes a functional link with HSBP1 and TWF2. Finally, SH3 domain-binding glutamic acid-rich-like protein 2 (SH3BGRL2) is a homologue of the SH3 binding glutamic acid-rich protein, which may be involved in redox processes [51].
The modulation of these 2 clusters in patients presenting a high PR under aspirin reflects a modified inactivation process of αIIbβ3, actin cytoskeleton dynamics and glycolysis in such patients. Mateos-Caceres and co-workers also assessed proteome differences between 2 groups of stable cardiovascular patients treated with aspirin, presenting high or low PR as defined with the PFA-100 [11]. The authors identified differentially expressed proteins from cytoskeleton, glycolysis, oxidative stress and intracellular calcium regulation. They postulated that patients with a high PR, who present an altered cytoskeletal remodeling upon activation and energetic metabolism, together with a higher oxidative stress and a possible increase of intracellular calcium, may have a high platelet turnover and generate platelets more prone to aggregate (figure 6). However, this last conclusion is debatable in our model, since a higher turnover implies a more intense production of not inhibited platelets and thus an increased production of TxA2. However patients’ platelets produced a decreased level of TxA2, as assessed during the phase of patients characterization [10]. Even though our results are in line with this publication, they constitute only an encouraging preliminary interpretation of the network, and thus represent only a partial view of the causes of the variations of PR in this cohort of patients. Indeed, other genes of the network belong to these pathways (figure 3) and other pathways are represented, as suggested by the GO analysis. In addition, these pathways of interest may be slightly modulated together to end up with variation of PR under aspirin treatment. Their individual and collective implication in this phenomenon will have to be verified by in vitro test and validated on an independent cohort. This work constitutes thus the beginning of the mining of this network, which constitutes a large amount of information to further unravel the molecular mechanisms modulating PR.

In conclusion, we performed a genetic, transcriptomic and proteomic analyses of platelet samples from cardiovascular patients selected for their extreme high or low PR under aspirin-treatment. These results were integrated into a network, which allows exploring the data at pathway level instead of considering gene products separately. This network showed a possible
implication of glycolysis, integrin αIIbβ3 and cytoskeleton dynamics. However, this may not constitute an exhaustive list of the candidates of interest. Others studies will be also integrated in the mining of the network, as well as additional GO annotation. Moreover, further in vitro verification and validation are mandatory to assess their role in platelet reactivity variations.
Abbreviation

Cyclooxygenase-1 (Cox-1)

Expression quantitative traits loci (eQTL)

Gene ontology (GO)

Gene set enrichment analysis (GSEA)

Genome-wide association study (GWAS)

Kilo base pair (kbp)

Log likelihood ratio (LLR)

MicroRNA (miRNA)

Platelet reactivity (PR)

Platelet-rich plasma (PRP)

Single nucleotide polymorphism (SNP)

Thromboxane A2 (TxA2)

Von Willebrand factor (vWF)
Figures and tables legends

Figure 1 Distribution of the PR index at the first (V1) and second (V2) visit for the PHP population.

The 26 extreme PR patients who were selected for the ‘omics analysis are depicted by a red cross, whereas the rest of the population is identified with a blue diamond.

Figure 2 Releasate sample preparation

(A) Workflow of the samples preparation. Platelets aggregate upon thrombin (1 unit/ml) addition and supernatant is recovered by centrifugation. An additional ultracentrifugation step allows removal of microvesicles. (B) Quality control of the releasate preparation. Ten percent of the supernatant of platelet suspension with (+T) and without (-T) thrombin were loaded on a gel and stained by silver staining and compared with a sample of platelet-poor plasma (PPP) to assess the plasma contamination.

Figure 3 Carbohydrate metabolism subnetwork

Subnetwork related to glycolysis isolated using GOlayout in the network and represents thus a possible pathway of interest in PR variation.

Figure 4 Network of the differentially expressed gene products between extreme PR patients

The network of 99 nodes and 309 edges results from the integration of the transcriptomic and proteomics data sets. SNP association score is mapped on the nodes and their color reflects the log2 ratio observed in the releasates data. The blue correlations are observed in this study, whereas the red ones are confirmed in literature-based databases. The 2 clusters of interest used as basis for pathways interpretation are surrounded in grey.

Figure 5 Clusters of interest

The 2 clusters of interest selected from the whole network (figure 4), according to the literature-confirmed correlation. They constitute the starting point for the network interpretation. They contains genes involved in actin cytoskeleton (yellow), integrin αIIbβ3 aggregation (green) and glycolysis (blue) pathways.

Figure 6 Model of the possible role of pathways of interest in PR variations

Mateos-Caceres and co-workers postulated that modulation in glycolysis, platelet activation, oxidative stress and cytoskeleton remodeling could lead to platelet apoptosis in high PR patients and thus to a higher platelet turnover which would explain a lower effect of aspirin. Such model could represent a basis for drawing a working hypothesis from our result, but it will be enriched with the additional information provided by our integrative approach.
Table 1 Table of the selected pathway enrichment result

These pathways were found enriched by GSEA in transcriptomic and/or proteomic releasate data. The presence of common or related pathways suggests the modulation of common metabolic process regulation.

Supplemental figure 1 Estimation of the white blood cell contamination in the platelet sample for the transcriptomics analysis

Specific markers for platelet (A) and white blood cells (B) were selected. Colors are representative of the relative level of detection of each probe set. 1. Platelet data from this project 2. Published platelet data [32] 3. Whole blood analysis [34, 35] 4. Leukocyte analysis [33]. ITAG2B: αIIb, PF4: platelet-factor 4, ZAP70: tyrosin-protein kinase ZAP-70, ITGAL: CD11a, LILRB3: leukocyte immunoglobulin-like receptor subfamily B member 3.

Supplemental figure 2 Pathway enrichment score for 3 platelet function-related pathways

ES score were calculated for each pathway against the number of samples in high and low PR patients groups transcriptomic data. The maximum pathways enrichment is found when comparing 6 vs. 6 most extreme patients.

Supplemental figure 3 Network of the differentially expressed gene products between extreme PR patients

Colors of the nodes are defined according to the log2 ratios in the whole platelet (A), secretory granules (B) proteomic or transcriptomic (C) data.
Figures and tables

Figure 1

Figure 2

A

Platelet suspensions

Agonist

Centrifugation

Ultra centrifugation

Releasate

B

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Figure 3

extracellular region

plasma membrane

cytoplasm

Nucleus
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Supplemental figures

Supplemental figure 1

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Supplemental figure 2
References

17. Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, Maller J, Sklar P, de Bakker PI, Daly MJ, Sham PC: PLINK: a tool set for whole-genome association and population-based linkage analyses. *American journal of human genetics* 2007, 81:559-575.


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DISCUSSION, PERSPECTIVES AND CONCLUSION
The Platelet Hyperreactivity Project described in this thesis manuscript aims characterizing the molecular mechanisms modulating platelet reactivity (PR) in a cohort of cardiovascular (CV) patients. To achieve this goal, 110 stable CV patients treated with aspirin were tested for platelet function using several agonists. This led to the construction of a composite variable, the PR index, which combines the different platelet function test responses [1]. Patients were classified according to their PR index. Then, an integrative approach based on network biology was applied to genetic, transcriptomic and proteomic profiling of patients presenting an extreme high or low PR. In this chapter, the importance and the limitations of this project in the context of cardiovascular diseases, but also the following steps for pathways verification and validation will be discussed.

I. Platelet role in hemostasis and importance in CV diseases

Platelets are key player in hemostasis, but also in the progression and complication of atherosclerosis [2]. They can be activated by several agonists [3], among which components of blood vessel lesions [4] and atherosclerotic plaques [5]. Platelet activation triggers amplification pathways based on thromboxane A2 (TxA2) and adenosine diphosphate (ADP) release. Once activated, platelets adhere to the lesion, recruit other platelets through a paracrine process and promote thrombus formation and instability of the atherosclerotic plaque [5]. Platelet function inhibitors target platelet activation and are prescribed to CV patients. They mainly target the most important amplification pathways, TxA2 and ADP. Aspirin inhibits the cyclooxygenase-1 (Cox-1), which is crucial for TxA2 generation, and decreases the overall PR, i.e. the ability of platelets to aggregate upon a given agonist activation.

Platelet reactivity is expected to decrease in CV patients treated with aspirin. However, several studies showed that part of patients had a preserved platelet function, defining high residual on-treatment PR [6-9]. Indeed, these patients present a suppression of TxA2 production, meaning that aspirin reached its pharmaceutical target, but the PR remains preserved as assessed with
different non-specific platelet function assays. There is no validated cut-off value for the
definition of high on-treatment PR, which is rather a continuous biological response. However, a
higher PR has been associated with the recurrence of ischemic events, as characterized with
aggregation-based assays relying on arachidonic acid (AA) [10], ADP [11], collagen [12] or the
Platelet Function Analyzer 100 (PFA-100, Siemens, Marburg, Germany) [13], at least in acute
vessel injury [14]. Of note, the combination of several methods assessing different pathways of
platelet function is suggested to identify CV patients at risk of recurrence of ischemic events
[15].
The characteristics and determinants of high on-aspirin treatment PR were mainly studied in
healthy subjects, where it affected around 14% of the population. This phenotype was shown to
be global (not agonist-specific), heritable, independent of aspirin intake and stable over time [6,
16, 17]. In aspirin-treated CV patients, the prevalence of a high PR phenotype is around 30%
[18]. The characteristics of PR and the mechanisms modulating this phenotype are poorly
understood.
To study PR in aspirin-treated CV patients, we recruited a cohort of 110 CV patients under
aspirin treatment (100 mg/day). Their PR was assessed twice (two weeks apart) by light
transmission aggregometry (LTA) using several agonists at several concentrations. All agonists
were correlated with epinephrine, which was shown particularly stable and efficient for high PR
in healthy subjects [6, 16]. Then, the different platelet function tests were combined into a PR
index by factorial analysis. All agonists presented a homogeneous contribution of the different
assays. We concluded that, similar to healthy subjects, PR is a global phenomenon in aspirin-
treated CV patients. In addition, we observed a significant correlation between the PR index
assessed at both visits ($R^2 = 0.78$), suggesting that PR is stable over time. This was further
confirmed by a third test of 12 extreme PR patients (6 vs. 6) about 1 year later. We used the PR
index to select the most extreme PR patients for further ‘omics analysis.
This first part of the project allowed the characterization of PR in aspirin-treated stable CV patients and we showed that their PR presented similar features than healthy subjects, i.e. global, stable over time and not affected by biological variables or cardiovascular risk factors. Although recent data suggest that the variability of platelet reactivity influences clinical outcome in acute settings only, investigation of stable cardiovascular patients was preferred as compared to patients with acute coronary syndrome since we aim to identify the modulators of platelet reactivity at the platelet level, without the background noise that would have been associated with an acute setting. Indeed, platelet activation is modulated by inflammatory processes associated with acute thrombotic events, such as increased expression or secretion of prothrombotic and proinflammatory mediators, including CD40L, P-selectin and nuclear factor-κB. In addition, patients with acute coronary syndrome or recent stent placement have a P2Y12 inhibitors associated with aspirin that causes a major bias in platelet reactivity evaluation [19]. Moreover, although this constructed index (PR index) was never associated with recurrence of clinical events per se, each of the agonists used for the factorial analysis was individually shown to predict cardiovascular events [1]. In addition, the PR index reflects more the global PR than considering one agonist only, as epinephrine.

II. ‘Omnics analysis of the most extreme PR CV patients treated with aspirin

Extreme PR patients were selected for ‘omics analyses. Thirteen patients on each end of the PR distribution were tested in genome-wide association studies (GWAS) based on single nucleotide polymorphisms (SNPs). The same patients were also tested for gene expression and half of them for protein expression. Taking advantage of the proteomic versatility [20], 3 samples were prepared for isobaric tagging quantification [21] of platelet from the 12 most extreme PR patients (6 vs. 6): whole platelets, releasates and secretory granules enriched fraction (this last,
Granules-related fractions are of particular interest since platelet degranulation is a key step in the platelet aggregation process, independently of the activating agent. It is therefore a potential candidate in the explanation of the modulation of PR. The releasates are the content of the secretory granules which is released in response to platelet aggregation [22-24]. We applied a published method of granule-enriched sample preparation [25]. In this part of the project, we identified a large proportion of differentially expressed proteins involved in cytoskeleton dynamics, either promoting or decreasing actin polymerization. In addition, 2 proteins related to secretory granules were also identified. This suggests variations of secretion and autocrine and paracrine activation of platelets. The fact that several proteins belonging to these pathways were found differential suggests a modulation of these processes between both groups of patients, possibly in a coordinated manner. However, this work constitutes only a partial view of the molecular mechanisms at work in PR. Since the large majority of platelet organelles are secretory granules [4], the benefit of subcellular fractionation to enrich platelet secretory granules is evidenced in the enrichment of lower abundant proteins. Indeed, the relative number of pathways related to granules is much higher in the granule-enriched fractions than in the whole platelets dataset (among the 33 pathways identified in both data sets, 17 pathways were not found in whole platelets, whereas only 3 were specific to whole platelets) [26]. However the GO terms represented in this data set are similar to the expected ones from whole platelet. In addition, this sample preparation necessitates a relative large volume of blood sample (100 ml of whole blood in average) and enriched fraction may present contaminations from other organelles. Other fractionation methods, such as off-gel electrophoresis with a higher number of fractions and/or gas-phase fractionation, could be more efficient in quantifying low abundant proteins using the same amount of blood.

The proteomic and transcriptomic analysis of these datasets yielded an important number of gene products. Taken independently, each of them contained gene products biologically interesting in the context of PR. However, there was a lack of correlation between the results of
transcriptomics and proteomics data. The low overlap observed in platelets between mRNAs and proteins [27] may be due to the megakaryocytic origin of mRNAs and part of the proteins [4]. In addition, taken individually (i.e. comparison of proteomic data of high vs. low patients or transcriptomic data of high vs. low patients) none of the gene products were significantly different after correction for multiple testing. The relatively small number of patients studied at this step and the large biological variability observed with human samples could explain part of this poor correlation. In addition, very tiny differences in the quantitative results were found, which could be explainable by high patients similarity: they have the same pathology and the same treatment; the differences considered in this project are thus very fine metabolic changes. Actually, we cannot conclude from the ratios themselves that genes are up or down-regulated, because ratios are below the limit of significance. This is the accumulation of genes belonging to the same pathway which highlights the role of their related-pathway in PR modulation. However, defining that a pathway is up- or down-regulated necessitates further confirmation and no conclusion can be drawn from these results. Furthermore, the presence of common modulated pathways between the transcriptomic and proteomic data, as assessed by the Gene Set Enrichment Analysis (GSEA), was encouraging. It enforced the integrative approach that we eluted and the exploration of the results in terms of pathways instead of single gene products. The study of very fine physiological variations implies the development of tools allowing overcoming problems of significance related to the small number of patients, which are after present in ‘omics approaches. Here, bioinformatic integration of the ‘omics data was performed by network biology [28, 29]. This strategy allows considering the different data sets as a whole, which increases the power of detecting gene, and focusing on patterns or clusters of connected nodes affecting together PR [30]. This process ended up with a network of 99 nodes and 309 edges, which summarizes the most connected genes according to PR index. By mapping literature-confirmed information on the network, 2 clusters presented a higher density of experimentally confirmed edges. They constituted a basis for the mining of the network. These
two clusters contained genes involved in glycolysis, actin cytoskeleton and integrin αIIbβ3 aggregation. However, they did not correspond to an exhaustive list of the genes related to these pathways, nor all the pathways playing a role in this phenotype. Actually, they were only a preliminary interpretation.

III. Perspectives

The next steps of the project will be to select pathways of interest that will be further verified and validated. This selection is a critical step and necessitates a combination of literature mining, results verification and integration of new data. In addition, since several pathways are at work, the relative role of each one will be an issue.

First, in order to enrich the list of the clusters of interest, subnetworks drawn with GOlayout (Cytoscape) could be a tool of interest, because it allows partitioning the network according to gene ontology (GO) categories and thus identify other genes of the network of the same GO categories, but not belonging to the clusters.

Several studies tried to identify genes potentially responsible of PR variability in CV patients or in healthy subjects. They used several methods to select patients and several analytical approaches based on SNPs [17, 31-34], mRNA, proteins [35] or a combination of them [36]. However, they all focused on gene products taken separately. In addition, except few exceptions such as PEAR1 or GP6, patient samples from these different studies present heterogeneity at gene product level, due to biological and technical variability. However, they may show more homogeneity at the level of pathways. We mapped these results together with our 2 clusters on a metabolic schema, in order to assess whether modulated pathways were similar (figure 1).
Figure 1 Schema of the metabolic pathways associated with PR including the 2 selected clusters. The genes in purple are the genes that were identified both in the PHP study and in other independent works as associated with PR [31, 32, 34-38]. Genes in green are related to other pathways (platelet activation, signaling, calcium metabolism, protein synthesis and secretory granule release) that were identified in the PHP study only (in green). The genes involved in the generic pathways (G protein, MAP kinase signaling pathway, oxidative stress, actin binding proteins & cytoskeleton dynamics, protein synthesis and glycolysis) are listed in table 1 (ⅠibⅠa: αIIbβ3, composed of ITGA2B and ITGB3).

Many genes coding for platelet agonist receptors were found: TxA2 receptor (TP), epinephrine receptor (ADRA2A), ADP receptors (P2Y1, P2Y12), thrombin receptors (PAR-1), collagen receptors (GP6 and its co-factor FCER1G, ITA2), vWF receptor complex (GPIb-IX-V and FCG2A), heparin receptor (HSBP1), HSBP1 receptor (CD36), integrin αIIbβ3 (ITGA2B and ITGB3) and 2 genes, which may play a role in its activation (PEAR-1 [39] and PDIA3 [40]). In addition, genes
involved in the signaling pathways downstream to these receptors were also found affected, such as G proteins (GNAZ and GNB3) and MAPkinase related genes (AKT2, RAF1, MAPK14, MAP2K2, MAP2K4, VAV3, PIK3GC and JAK2). On the other hand, 2 genes responsible of intracellular calcium release were also found associated with PR (ITPR3 and MRVI1). In addition, a chloride channel (CLIC1) may also be involved in calcium homeostasis [35]. Going downstream in the process, PR may also depend on cytoskeleton and cytoskeleton-related genes (TLN, ACTB, FLNA, CAPZ, GSN, IPCEF1, TPM3, MYH9, DBN1, TWF2 and HSBP1), as well as glycolysis enzymes (ALDOA, GAPDH, LDHAL6A, ENO1 and TPI1). It is of note that some of these glycolytic enzymes are known to physically interact with actin for modulation, such as GAPDH, TPI1 and ALDOA. VAMP8, which is involved in secretory granule release, as well as MME, a secreted metalloprotease, were also identified as associated with PR [36]. Protein synthesis is also an important phase of platelet activation and some genes, which may be involved at different level of regulation were published (JHP2C, ANKS1B, GLIS3, HSPA8, JMJD1C AND SHH). Finally, 3 genes related to oxidative stress were associated with PR variability (GSTP1, HSPD1 and SH3BGRL2) (Figure 1 and table 1).

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Table 1 List of the genes associated with PR belonging to signal transduction, oxidative stress, proteins synthesis, cytoskeleton and glycolysis. The genes written in green were found in literature, the dark ones were identified in this project, whereas the purple ones were found in literature and in this project. *HSBP1 is a heat shock protein, but affects cytoskeleton dynamics and it is thus present in 2 categories.

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In summary, literature mining showed candidates of interest along several crucial pathways for platelet activation and aggregation, *i.e.* platelet activation, integrin αIIbβ3 aggregation, signal transduction, calcium metabolism, glycolysis, cytoskeleton dynamics, oxidative stress, protein synthesis and secretory granule release. This is in line with several pathways coming from our project; indeed, the preliminary interpretation of our network showed a possible implication of glycolysis, integrin αIIbβ3 and cytoskeleton. However, the exact role of each pathway and their relative effect on each other remains unclear. For instance, a decrease of glycolysis would influence the overall energetic production and thus the ATP-related processes, including signal transduction and cytoskeleton remodeling. Moreover, these patients show a normal global platelet physiology, implying very slight modulations of these pathways, which may not be sufficient *per se* to affect overall platelet function. Actually, the differences of phenotype may be due to a combination of pathway alterations, which, all together, change platelet response to low doses of agonist, as in physiological conditions.

The selection of candidate pathways for functional verification and validation is the next challenging issue of this project. Three complementary approaches will help us to identify the most pertinent pathways. First, microRNA (miRNA) analysis will be performed. MicroRNAs play a major role in platelet physiology [41, 42]. Recently, 284 miRNAs were identified in platelets and some of them were associated with PR in healthy individuals [43]. To further explore this possibility, we assessed whether the network was enriched in sequence targets for some miRNAs and whether they belong to this published list. Five miRNAs were predicted by TargetScan to have enriched targets in the network: miR-1, miR-133, miR182, miR-320 and miR-744 (figure 2). In addition, the 2 clusters of interest were found enriched in targets for miR-18a/b, miR-1827 and miR-145. All of them were also identified by Nagalla and co-workers [43]. Among these candidates, miR-320 is of particular interest, since it was found at higher levels in high PR samples [43]. These 8 miRNAs are interesting candidates to explain the difference of mRNA and protein expression. They should further be verified in our cohort of patients in order
to confirm a different level of their expression between both groups of patients and to assess their effective targets from the network.

Second, the genetic data were under-used so far. We will test SNPs within the genes of candidate pathways and to enrich this list with SNPs coming from the literature [17, 31, 32, 34, 37, 44]. This might identify specific SNPs (or loci) associated with expression variations and PR phenotype. Finally, we will functionally annotate groups of genes based on literature mining.

Then, the verification strategy will depend on the selected pathways. However, the first envisaged strategy relies on pathway mRNA quantification using the nanostring technology.
(nCoulter). This method allows an ultra-sensitive, highly multiplexed and reproducible detection of mRNAs. To do so, we will select 100 genes and miRNA sequences belonging to the pathways of interest. This will confirm the candidates, but also enrich the pathways with other candidate genes, increasing the confidence in the pathway selection. Moreover, we can perform this analysis simultaneously in 48 patients. The second approach is based on the quantification of metabolites resulting from selected pathways. MS-based metabolomics aims to characterize several pathway products in a functional manner. This strategy will be used focusing on a given set of metabolites, in a targeted approach. Plasma is the most common sample matrix, but in this project, other samples, such as releasates, could be also of interest. For instance, glycolysis pathway could be verified by targeting succinic acid, citric acid, fumaric acid and malic acid in plasma, but also in platelet suspension. Here, again, additional patients will be included in order to characterize less extreme phenotypes.

Validation of the selected pathways will imply a functional in vitro step, followed with an in vivo phase. First, pathways of interest will be modulated by drugs or antibodies directed to particular proteins in peripheral blood platelets, to assess their effect on PR. In addition, platelets derived from CD34+ cells [45] and Meg-01 [46] may complement these experiments, by the use of transfection [47].

Finally, we plan to validate the selected pathways using metabolomics or SNPs characterization on an independent cohort of CV patients treated with aspirin [48], who can be classified according to their PR index.
IV. Conclusion

PR variations were shown to determine the recurrence of ischemic events (at least in acute situations) and occurrence of bleeding events. We showed that PR is a global and stable phenotype in stable CV patients treated with aspirin, as it is in healthy subjects. This enforces a genetic contribution to this variability. However, studies aiming to identify genes of interest in this field are not concordant and they present a limited contribution to the phenotypic variations. We here combined several high-throughput approaches to identify pathways of interest involved in PR variability, instead of considering single gene products. To our knowledge, this project is the first integrative approach of platelet function in stable cardiovascular patients treated with aspirin, gathering genetics, transcriptomics and proteomics data according to PR index. This was made possible using a bioinformatic workflow based on network biology.

Preliminary data interpretation showed a potential role of cytoskeleton dynamics, glycolysis and integrin αIIbβ3 aggregation. These results are in line with previous reports, if pathways instead of genes are considered. However, the network interpretation will continue and be enriched by complementary data, such as targeted miRNAs quantifications and pathway verification by nanostring gene expression measurements. Finally, an important step of validation will be carried out, to functionally understand the role of the candidate pathways in PR and confirm their implication on an independent cohort of patients.

This project showed that a network biology approach could lead to identification of pathways, which may modulate PR in a population of CV patients treated with aspirin. This strategy increases the power of the analytical method aiming to characterize very fine differences in a highly variable population, such as humans. Furthermore, the results of this project may provide a better understanding of the molecular modulators of PR in cardiovascular patients. They may constitute new medication targets for the treatment and the prevention of ischemic events or for the treatment of platelet defects.
V. References


