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Reference

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Review

Unraveling modulators of platelet reactivity in cardiovascular patients using omics strategies: Towards a network biology paradigm

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Abstract
Platelets play an important role in the pathogenesis and the ischemic complications of atherosclerosis. Platelets may be activated by several different agonists, promoting the release of their granule contents and subsequent aggregation and thrombus formation; this leads to ischemic events such as myocardial infarction or stroke. Aspirin, the most popular antiplatelet agent, is a cornerstone in the treatment and prevention of ischemic events in cardiovascular 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 preserved platelet function. This phenotype is defined as “high on-treatment platelet reactivity”. It is a risk factor for the recurrence of ischemic events, particularly in acute vessel injury settings. The determinants of platelet reactivity in these patients remain unclear, but previous studies, including healthy subjects, suggested that it is genetically determined.

Abbreviations: ADP, adenosine diphosphate; ADRA2A, adrenoceptor alpha 2A; Bcl-3, B-cell lymphoma protein-3; CV, cardiovascular; Cox-1, cyclooxygenase-1; CYP450, cytochrome P-450; DMS, demarcation membrane system; FG, functional genomics; GWAS, genome-wide association study; GP, glycoprotein; GP6, glycoprotein VI; IP3, inositol triphosphate; mTOR, mammalian target of rapamycin; mRNA, messenger ribonucleic acid; miRNA, micro RNA; MAPK, mitogen-activated protein kinases; PAI-1, plasminogen activator inhibitor; PEAR1, platelet endothelial aggregation receptor-1; PFA-100, platelet function analyzer-100; RNA, ribonucleic acid; SNPs, single nucleotide polymorphisms; TGS, targeted-gene study; TxA2, thromboxane A2; TxB2, thromboxane B2; TP, TxA2 receptor; VASP, vasodilatator-stimulated phosphoprotein phosphorylation; vWF, von willebrand factor.

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Over the last decade, technological improvements have led to the development of highly efficient omics strategies. High-throughput genomics, transcriptomics and proteomics have the potential to dissect fine metabolic modulations. However, the bioinformatics management of these large data sets remains a challenging issue. Network biology approaches permit the integration of different omics data sets and the identification of mutual interactions between gene products and/or molecules. The inherent topology of the network can be then explored at a pathway level rather than at a gene level. Network biology constitutes an efficient tool to further explore platelet metabolism and defects, such as modulators of platelet reactivity in cardiovascular patients.

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1. Introduction

Platelets are small anucleated cell fragments also called thrombocytes [1]. With a diameter of 2–4 \( \mu \text{m} \), they are the smallest cellular components of the bloodstream. Although platelets do not contain a nucleus, they can alter their protein profile by splicing and translation of a limited batch of messenger ribonucleic acids (mRNA) inherited from their parent cell, the megakaryocyte [2–4]. Megakaryocytes are highly specialized hematopoietic cells [5], which derive from hematopoietic stem cells. They become polyploid (4–64N) through several cycles of endomitosis [6–8]. In addition, a cytoplasmic maturation occurs involving the formation of a demarcation membrane system (DMS) and the accumulation of cytoplasmic proteins and secretory granules [6,9]. The DMS is supposed to function as a membrane reserve for pro-platelet formation and constitutes the open canalicular system in mature platelets [5]. Granule number increases with megakaryocyte maturation. With an average lifespan of 10 days before being phagocytosed by the spleen or the liver [10], approximately \( 10^{11} \) platelets must be produced every day (around 10% renewal) [8] to ensure an average concentration of 150–400 \( \times 10^9 \) platelets/L [11].

Platelet production is mainly regulated by thrombopoietin. This humoral factor is produced in the liver and the kidneys, and stimulates the production of thrombocytes in bone marrow, from their hematopoietic cell of origin, the megakaryocyte [5,8]. Cell apoptosis is also involved in the regulation process since pro-platelets contain active caspase-3 and 9 and their production is decreased by anti-caspase agents [11,12].

Mature platelets have 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 under electronic microscopy, together with glycogen particles and a large cytoskeleton [5].

1.1. Platelets and atherothrombosis

Platelets are usually activated following vessel injury, but they are also involved in the progression of atherosclerotic plaques in high shear stress conditions (Fig. 1) [13]. Cardiovascular (CV) diseases, including arterial thrombosis, are the most common cause of mortality in developed countries [14] and platelets are key-targets for the treatment and the prevention of ischemic events. Upon inflammation [15], endothelial cells are activated and recruit platelets to the site of atherosclerotic plaque formation [10,13], together with adhesive molecules which stimulate migration of smooth muscle cells and monocytes [16]. In mice, deletion of the TxA2 receptor delays plaque development, illustrating the role of platelets in atherosclerotic plaque formation [13,17]. Moreover, activated platelets also release adhesive molecules in the nascent plaque, thus enhancing the effect of endothelium activation on plaque progression. For instance, \( \alpha \)-selectin and chemokines released from platelets activate monocytes that migrate into the plaque and increase the local inflammation process (Fig. 1).
Fig. 1 – Atherosclerotic plaque formation. Platelets are involved in the atherosclerotic process; they secrete factors, which induce monocyte recruitment, enhance inflammation and cell proliferation, and promote thrombus formation. All together, these processes favor plaque expansion.

Activation of endothelial cells and the expression of tissue factor increase the thrombogenic potential of plaques [13]. In addition, platelets release TxA2, which increases inflammation by its vasoconstricting action [5] and promotes platelet aggregation and local platelet recruitment (Fig. 1) [15]. The lesion is then covered by a fibrous cap. Rupture of an atherosclerotic plaque occurs by ulceration or erosion, under the effect of inflammation and/or enzymes released by immune cells. Platelets are key components in the subsequent thrombus formation, which can occlude the artery and results in organ infarction [16].

1.2. Antiplatelet drugs

Since platelets play a major role in atherosclerosis and thrombus formation, antiplatelet agents belong to the first line of treatment in CV diseases [18]. The main oral antiplatelet drugs target two important amplification pathways of platelet activation: TxA2 production and the action of adenosine diphosphate (ADP, Fig. 2).

Aspirin (acetylsalicylic acid) is the oldest anti-platelet drug used in CV diseases [19]. It irreversibly acetylates platelet cyclooxygenase-1 (Cox-1) serine 529 and inhibits TxA2 production, thus impairing platelet activation [5] (Fig. 3). A low dose of aspirin (75–325 mg/day) is usually prescribed because it induces an almost complete inhibition of platelet Cox-1. However, in nucleated cells, such as endothelial cells, cyclooxygenase causes 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 with low doses of aspirin. Thus, endothelial cell-derived eicosanoid production is barely affected by low doses of aspirin. Moreover, using lower doses of aspirin minimizes the inhibition of prostaglandins and its related gastrointestinal tract side effects [18]. Several clinical studies showed that aspirin is associated with a 25% relative decrease in the risk of vascular death, stroke and myocardial infarction in high risk patients [20].

Clopidogrel belongs to the anti-P2Y12 thienopyridine family, which are pro-drugs metabolized into an active compound by several P-450 cytochromes (CYP450) in the liver. It acts on the ADP receptor P2Y12 (Fig. 2), by covalent modifications of two cysteine residues. The P2Y12 receptor is important for the amplification of the platelet activation process, not only when platelets are stimulated with ADP, but also with other agonists such as collagen [5]; it also plays a major role in thrombus formation in high shear stress conditions [21]. At the maintenance dose of 75 mg/day, maximal pharmacodynamic effect is reached between days 5 and 7 [22]. This delay between drug intake and antiplatelet effect can be partially overcome by the administration of an initial loading dose (600 mg).

Patients at high risk of ischemic event (for instance after an acute coronary syndrome and/or percutaneous coronary intervention) are usually treated by using a dual anti-platelet therapy with aspirin and an anti-P2Y12 drug for between 1 and 12 months. Although it combines the advantages of both drugs, the efficacy of this treatment may be limited by compensatory platelet activation pathways partially restoring platelet reactivity [18]. Contrary to acute settings, the dual
antiplatelet therapy is generally not recommended in stable cardiovascular patients [22]. The delay and the variability of the pharmacodynamic effect of clopidogrel promoted the development of more efficient anti-P2Y12 drugs, such as prasugrel, a third generation thienopyridine drug, and ticagrelor, a non-thienopyridine molecule. Other platelet receptors or pathways are targeted by antiplatelet drugs. Integrin αIIbβ3, for instance, is antagonized by several compounds (eptifibatide, abciximab or tirofiban), which are administered intravenously (Fig. 2). These treatments are often prescribed to patients in acute clinical situations [18,22]. Phosphodiesterase inhibitors, such as cilostazol and dipyridamole, increase levels of cyclic adenosine monophosphate, inhibiting platelet activation (Fig. 2) [22]. These latter drugs have specific side effects that limit their use in daily practice. Other antiplatelet drugs with new targets, such as the thrombin receptor PAR-1 or the collagen receptor GPVI, are in development [23,24].

1.3. Determinants of platelet reactivity and risk of recurrence of ischemic events

Biological evaluation of platelet reactivity in CV patients treated with antiplatelet drugs shows that the efficacy of the drugs can vary between patients and that a significant proportion of treated patients are deemed “non-responders”, “poor responders” or “resistant”. This is because their platelet reactivity is higher and can even reach a level similar to that of patients without antiplatelet drug treatment [25]. Although lack of compliance is the most common cause of non response, variability of on-treatment platelet reactivity still exists in documented compliant patients. The term “resistance” to a drug should be used when a drug is unable to hit its pharmacological target [25] i.e. when aspirin is unable to inhibit platelet-derived Cox-1-dependent TxA2 production, or when clopidogrel is unable to inhibit the P2Y12 platelet receptor. As a consequence, with regard to aspirin response, resistance refers to assays evaluating TxA2’s stable breakdown product (serum TxB2). With regard to clopidogrel response, resistance refers to the specific evaluation of P2Y12 receptor inhibition (using quantification of the phosphorylation status of the vasodilator phosphoprotein [VASP assay]) [25]. The term “high on-treatment platelet reactivity” relates more to platelet function assessed with non-specific assays (aggregation-based assays) that provide a more global evaluation of platelet reactivity.

Several genetic and non-genetic factors have been associated with the variability of antiplatelet drug response [26], but these factors explain only a small proportion of the observed variability. There is however a major difference between the causes of the variability of aspirin response in comparison to clopidogrel response. The biological response to the latter antiplatelet drug is mainly mediated by the efficiency of the metabolization of the pro-drug and thus by the concentration of the active metabolite that is driven by esterases and liver CYP [27]. Clopidogrel response is thus mostly determined by liver-related factors. Conversely, specific assays revealed that aspirin has a much more homogeneous effect, with more than 95% of TxA2 production being inhibited in the vast majority of patients [25]. However, when using aggregation-based assays, a significant proportion of CV patients (around 30%) displayed preserved platelet function despite adequate inhibition of platelet-derived TxA2 production [28]. This finding points to platelet-related factors that may overcome aspirin’s inhibition of the TxA2 pathway. Aspirin may thus reveal compensatory mechanisms that allow platelet aggregation to occur despite TxA2 inhibition, and cardiovascular patients treated with aspirin as their sole antiplatelet drug are of particular interest for the identification of these compensatory pathways [29].

The platelet activation pathways that might modulate platelet reactivity in aspirin-treated CV patients are not known. Pioneering studies addressed the issue of the heterogeneity of platelet reactivity in healthy subjects. They showed that a phenotype of “platelet hyperreactivity” is found in around 14% of this population [30]. Moreover, it has been

**Fig. 3 – TxA2 production pathway.** Phospholipids in the plasma membrane and arachidonic acid are substrates of the TxA2 synthesis pathway. Cox-1 metabolizes 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 autocrin and paracrin interaction of TxA2 with its receptor (TP receptor) triggers a G-protein activation cascade. Aspirin inhibits this pathway by acetylation of Cox-1.
shown that this phenotype is strongly heritable, global (not agonist-specific), stable over time and barely affected by CV risk factors [30–33]. Moreover, platelet hyperreactivity was shown to be independent of aspirin intake [34], i.e. subjects with platelet hyperreactivity without aspirin treatment still displayed platelet hyperreactivity on treatment. The strong hereditary feature of platelet reactivity [32] points to a genetic control.

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, stroke or percutaneous coronary intervention [35–37]. However, it does not seem to affect cardiovascular outcome in stable patients [38]. Overall, there is a 2- to 4-fold increased risk of a recurrent ischemic event in patients with a high on-aspirin or on-clopidogrel platelet reactivity. 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 [39]. Altogether, these data suggest that platelet reactivity modulates the risk of recurrence of ischemic events in cardiovascular patients in acute vessel injury settings, independently of the method of platelet function evaluation. This strengthens the hypothesis that a common factor modulates platelet reactivity.

2. Omics strategies relevant to platelet reactivity

Recent improvements in high-throughput genetic, transcriptomic and proteomic techniques, as well as in bioinformatics methods, have advanced our knowledge of platelet reactivity physiology. These tools have allowed the analysis of hundreds of gene characteristics and products at the same time and can give a picture of all the actors of a given functional pathway [40–42]. Automated lab-on-a-chip methods in transcriptomics and genetics make possible large scale studies of human samples [43]. Moreover, highly sensitive mass spectrometers, such as Orbitrap [44], coupled with an efficient separation method such as off-gel electrophoresis [45], can detect small amounts of proteins in complex samples. These strategies are complementary and versatile. Moreover, there is a small overlap between platelet proteome and transcriptome information [2,40], which highlights the benefit of combining several strategies to learn more about platelet physiology.

2.1. Genetics of single nucleotide polymorphism

Platelet reactivity has been shown to vary between individuals, but is strongly inherited, implying a genetic contribution to platelet function [32,46,47]. Several genetic studies were made based on a candidate gene approach, targeting genes known to be involved in platelet function (Table 1) [48,49].

The association studies regarding putative genetic variants and platelet reactivity face several challenges. These include the number of subjects, the ethnic homogeneity of the population and the biological assay to assess platelet reactivity. The first attempts to identify the genetic causes of the modulators of platelet reactivity used a candidate gene approach–targeting genes known to be involved in platelet activation processes (Table 1). Over the last decades, to better discriminate the DNA loci implicated in phenotypic variability, genome-wide association studies (GWAS) were performed. These studies aimed to identify associations between millions of genetic variants and platelet reactivity. Their major drawback was the management of false positive results due to the large number of associations tested.

This approach has been applied to investigate the genetic basis determining platelet morphology, such as mean platelet volume or platelet count [48,49]. The first GWAS meta-analysis of platelet function was published in 2010 [50]. 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 with platelet aggregation results in both cohorts, with variable effect depending on the agonists (Table 1). These loci were also tested in an independent cohort of African ancestry and all but one of the seven loci was replicated in it.

Several common genes were found (PEAR1, GP6 and ADRA2A for example) using GWAS and a candidate gene approach, although the SNPs may be different within the same locus. Platelet endothelial aggregation receptor-1 (PEAR1) is phosphorylated upon platelet activation and plays a role in the amplification process of αIIbβ3 activation [51]. It has been shown to be related to epinephrine response, but also to ADP and collagen responses [50]. Glycoprotein VI (GP6) is a collagen receptor and, as expected, is associated with collagen-induced platelet activation. The reported SNP (producing a H322N, rs1671152) may decrease the interaction of GPVI with its downstream effectors, Fyn and Lyn pathways, and thus the subsequent collagen response [49]. The adrenoceptor α2A (ADRA2A) is the major epinephrine receptor in platelets [49]. This latter gene is of particular importance since epinephrine-induced platelet aggregation is considered the most reliable marker of platelet reactivity [33]. Despite some plausibility related to the function of this gene, genetics alone can only explain a minority of the variance of parameters in cardiovascular diseases, such as mean platelet volume [52] or platelet reactivity [53].

2.2. Transcriptomics

Platelets are anucleated cell fragments, but they do contain rough endoplasmic reticulum and ribosomes. Several studies showed that protein synthesis occurs in platelets [3,54]. Moreover, platelets contain a stable pool of mRNAs, which is involved in platelet function and life-span, hemostasis and inflammation [55]. In addition, this pool decreases with platelet age and is thus an indicator of platelet turnover [55]. Platelets are estimated to contain around 5000 different mRNA transcripts [55] covering approximately half of the megakaryocyte transcriptome. The content of mRNA also varies with platelet activation or certain diseases, such as systemic lupus erythematosus [55,56]. Platelet mRNAs are translated in different modes depending on the final protein and its role (Fig. 4). A small number of mRNAs are highly abundant and constitutively translated into proteins. These include actin, αIIbβ3 and von Willebrand factor (vWF) (Fig. 4(1)). On the other hand, B-cell lymphoma protein-3 (Bcl-3), which is involved
<table>
<thead>
<tr>
<th>Entry name</th>
<th>Protein names</th>
<th>Gene names</th>
<th>Method of identification</th>
</tr>
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<tbody>
<tr>
<td>ADA2A_HUMAN</td>
<td>Alpha-2A adrenergic receptor (Alpha-2 adrenergic receptor subtype C10)</td>
<td>ADRA2A ADRA2R</td>
<td>GWAS, TGS</td>
</tr>
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<td>(Alpha-2A adrenoreceptor) (Alpha-2A adrenoceptor) (Alpha-2AAR)</td>
<td>ADRAR</td>
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<td>AKT2_HUMAN</td>
<td>RAC-beta serine/threonine-protein kinase (EC 2.7.11.1) (Protein kinase Akt-2)</td>
<td>AKT2</td>
<td>FG</td>
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<td>CD36_HUMAN</td>
<td>Platelet glycoprotein 4 (Fatty acid translocase) (FAT) (Glycoprotein IIb)</td>
<td>CD36 GP3B GP4</td>
<td>FG</td>
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<td></td>
<td>(GPIIb) (Leukocyte differentiation antigen CD36) (PAS IV) (PAS-4) (Platelet collagen receptor) (Platelet glycoprotein IV) (GPIV) (Thrombospondin receptor) (CD antigen CD36)</td>
<td>CD36 GP3B GP4</td>
<td>FG</td>
</tr>
<tr>
<td>FCG2A_HUMAN</td>
<td>High affinity immunoglobulin epsilon receptor subunit gamma (Fc receptor gamma-chain) (FcRgamma) (Fc-epsilon RI-gamma)</td>
<td>FCER1G</td>
<td>FG</td>
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<td>GNB3_HUMAN</td>
<td>Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-3 (Transducin beta chain 3)</td>
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<td>TGS</td>
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<td>Guanine nucleotide-binding protein G(a) subunit alpha (G(a) alpha chain) (G2-alpha)</td>
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<td>FG</td>
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<td>GP1B_HUMAN</td>
<td>Platelet glycoprotein Ib alpha chain (GP-Ib alpha) (GPib-alpha) (GPibA) (Glycoprotein Ibalpha) (Antigen CD42b-alpha) (CD antigen CD42b)</td>
<td>GP1B</td>
<td>TGS</td>
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<td>GPV1_HUMAN</td>
<td>Platelet glycoprotein V1 (GPV1) (Glycoprotein 6) (Integrin alpha-2 (CD49 antigen-like family member B) (Collagen receptor) (Platelet membrane glycoprotein 1a) (GpIa) (VLA-2 subunit alpha) (CD antigen CD49b)</td>
<td>GP6</td>
<td>GWAS, FG, TGS</td>
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<td>JAK2_HUMAN</td>
<td>Tyrosine-protein kinase JAK2 (EC 2.7.10.2) (Janus kinase 2) (JAK-2) (JAK-2)</td>
<td>JAK2</td>
<td>FG</td>
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<tr>
<td>JMD2C_HUMAN</td>
<td>Probable JmC domain-containing histone demethylation protein 2 C (EC 1.14.11.1-) (Jumonji domain-containing protein 1 C) (Thyroid receptor-interacting protein 8) (TR-interacting protein 8) (TRIP-8)</td>
<td>JMD2C</td>
<td>GWAS</td>
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<td>MK14_HUMAN</td>
<td>Mitogen-activated protein kinase 14 (MAP kinase 14) (MAPK 14) (EC 2.7.11.24) (Cytokine suppressive anti-inflammatory drug-binding protein) (CSAID-binding protein) (CSBP) (MAP kinase Mxi2) (MAX-interacting protein 2) (Mitogen-activated protein kinase p38 alpha) (MAP kinase p38 alpha) (Stress-activated protein kinase 2a)</td>
<td>MAPK14 CSBP CSBP1 CSBP2 CSBP1 Mxi2 SAPK2A</td>
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<td>MP2K_HUMAN</td>
<td>Dual specificity mitogen-activated protein kinase 2 (MAP kinase 2) (MAPK 2)</td>
<td>MAP2K2 MEK2 MKK2 PRKMK2</td>
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<td>MP2K4_HUMAN</td>
<td>Dual specificity mitogen-activated protein kinase 4 (MAP kinase 4) (MAPK 4)</td>
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<td>FG</td>
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<tr>
<td>MRV1_HUMAN</td>
<td>Protein MRV1 (inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate) (JAK1-related protein MRV1)</td>
<td>MRV1 IRAG JAW1L</td>
<td>GWAS</td>
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<td>P2RY1_HUMAN</td>
<td>P2Y purinoreceptor 1 (P2Y1) (ATP receptor) (Purinergic receptor)</td>
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<td>TGS</td>
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<td>P2Y12_HUMAN</td>
<td>P2Y purinoreceptor 12 (P2Y12) (ADP-glucose receptor) (ADP-G-R) (P2T(AC)) (P2Y(AC)) (P2Y(cyc)) (P2Y12 platelet ADP receptor) (P2Y(ADP)) (SP1999)</td>
<td>P2RY12 HORK3</td>
<td>FG, TGS</td>
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**Table 1 (Continued)**

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<td>Proteinase-activated receptor 1 (PAR-1) (Coagulation factor II receptor) (Thrombin receptor)</td>
<td>F2R Cy2R PAR1 TR</td>
<td>TGS</td>
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<td>PEAR1_HUMAN</td>
<td>Platelet endothelial aggregation receptor 1 (hPEAR1) (Multiple epidermal growth factor-like domains protein 12) (Multiple EGF-like domains protein 12)</td>
<td>PEAR1 MEGF12</td>
<td>GWAS, FG</td>
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<td>PGH1_HUMAN</td>
<td>Prostaglandin G/H synthase 1 (EC 1.14.99.1) (Cyclooxygenase-1) (COX-1) (Prostaglandin H2 synthase 1) (PGH synthase 1) (PGHS-1) (PHS 1) (Prostaglandin-endoperoxide synthase 1)</td>
<td>PTGS1 COX1</td>
<td>TGS</td>
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<td>PK3CG_HUMAN</td>
<td>Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit gamma isoform (PI3-kinase subunit gamma) (PI3K-gamma) (PtdIns-3-kinase subunit gamma) (EC 2.7.1.153) (Phosphatidylinositol 4,5-bisphosphate 3-kinase 110kDa catalytic subunit gamma) (PtdIns-3-kinase subunit p110-gamma) (p110gamma) (Phosphoinositol 3-kinase catalytic gamma polypeptide) (Serine/threonine protein kinase PIK3CG) (EC 2.7.11.1) (p120-PI3 K)</td>
<td>PIK3CG</td>
<td>GWAS</td>
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<td>RAF1_HUMAN</td>
<td>RAF proto-oncogene serine/threonine-protein kinase [Cleaved into: Sonic hedgehog receptor N-product; Sonic hedgehog protein C-product] (Proto-oncogene c-Raf) (cRaf) (Raf-1)</td>
<td>RAF1 RAF</td>
<td>FG</td>
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<tr>
<td>SHH_HUMAN</td>
<td>Sonic hedgehog protein (SHH) (HHG-1) (Prostaglandin-endoperoxide synthase 1) (Hedgehog)</td>
<td>SHH</td>
<td>GWAS</td>
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<td>TA2R_HUMAN</td>
<td>Thromboxane A2 receptor (TXA2-R) (Prostaglandin TP receptor) (Platelet activator)</td>
<td>TBXA2R</td>
<td>TGS</td>
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<tr>
<td>VAV3_HUMAN</td>
<td>Guanine nucleotide exchange factor VAV3 (VAV-3)</td>
<td>VAV3</td>
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in clot retraction, is translated upon thrombin activation and under mammalian target of rapamycin (mTOR) regulation, as shown in Fig. 4(2). Thrombin activation also increases synthesis of continuously translated proteins, such as plasminogen activator inhibitor (PAI-1). Finally, protein synthesis can also occur via a functional spliceosome, which has been found in platelets [4]. Indeed, pre-mRNAs exist in platelets and are spliced upon platelet activation (Fig. 4(3)). Tissue factors and interleukin 1β are examples of such regulation.

These different regulation mechanisms are facilitated by a strong interaction of mRNAs and protein synthesis machinery with the 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.

Platelet transcriptome was investigated in the context of the variability of platelet reactivity. RNA expression was assessed in 288 healthy individuals using microarray [57]. The expression level of VAMP8/endobrevin was positively associated with high platelet reactivity, as assessed with light transmission aggregometry. In addition, a SNP (rs1010) and a microRNA (miRNA-96) were shown to be key players in VAMP8 modulation. Since VAMP8 is a v-SNARE involved in the targeting and fusion of secretory granules to the plasma membrane, this study linked platelet reactivity variability to granule release.

Recent data suggest that microRNA (miRNA) play an important role in mRNA regulation in platelets. These small nucleotides (around 22 base pairs) can induce mRNA degradation or delay or promote translation [58]. Several miRNAs and their modulating miRNAs were recently associated with platelet reactivity in healthy subjects [59]. Among the 284 miRNAs expressed by platelets, 74 were differentially expressed in different platelet reactivity categories. These data were combined with quantitative transcriptomic results on the same cohort, to obtain a list of paired miRNAs-miRNAs with a binding site at the 3’ untranslated region (UTR) of mRNA. Among them, 3 pairs were of particular interest and could be validated at the level of protein expression.

Although mRNAs and miRNAs play a role in the modulation of platelet function by transcriptomics, their exact role at the proteomic level, as well as their functional impact, remain unclear.

**2.3. Proteomics**

Platelets have been extensively analyzed using proteomics [42,60]. Indeed, since platelets are anucleated and contain a
limited amount of mRNA, their proteome is interesting for the study of their physiology. Recently, the platelet proteome was dramatically extended to reach almost 4000 proteins and 2500 phosphorylation sites [40]. In order to cover as much of the platelet proteome as possible, the authors combined several strategies: this involved 2 complementary phosphopeptide enrichments and the inclusion of a data set of membrane proteins from a previous study [61]. They also estimated the copy number of 3718 proteins in their sample, using a normalized spectral abundance factor; this reflects the spectral count of a protein versus its length as a measure of its abundance. This estimation ranged from 2.2 × 10^6 to less than 500 proteins. In addition, they also assessed the proteome variation by relative quantitative mass spectrometry in platelets isolated from 4 different donors. They concluded that 85% of the 1900 proteins quantified showed almost no biological variation. This type of work represents a baseline for any project dedicated to the study of platelet function. Of note, data mining is an essential step after proteomic analysis and the integration of the protein–protein interactions to construct the identified pathways is called systems strategy and allows identifying clusters, i.e. groups of proteins, for further functional validation [62]. Proteomics has been used to study several diseases triggered by genetic variants and affecting platelet reactivity, such as gray platelet syndrome [63] or cystic fibrosis [64]. Other pathologies associated with platelet function modulation were also explored, such as arterial thrombosis [65] or acute coronary syndrome [66]. Proteomics was also used to investigate the impact of aspirin or clopidogrel on platelet function [67] [68]. However, there is limited proteomics data regarding the investigation of platelet reactivity variability.

The proteins involved in the cytoskeleton (gelsolin precursor isotype 2 and 3, and F-actin capping protein isotype 1) were found by 2-dimensional gel electrophoresis down-regulated in stable cardiovascular patients under aspirin treatment and presenting a high platelet reactivity. This had been assessed using a Platelet Function Analyzer 100 (PFA-100™, Siemens, Marburg, Germany) [69]. These patients also showed a modulation of proteins involved in glycolysis (GAPDH and 1,6-bisphosphate aldolase) and in oxidative stress (heat shock protein 71 and 60, and glutathione S-transferase), which could lead to an increased turnover of platelets and might explain a poor response to aspirin treatment.

2.4. Omics text mining

As described above, several studies tried to identify genes potentially responsible for the variability of platelet reactivity in CV patients or in healthy subjects. They used several methods to select patients and several analytical approaches based on SNPs [32,48,49,70,71], proteins [69], or a combination of the two [57]. However, they all focused on gene products taken separately. In addition, apart from a few exceptions such as PEAR1 or GP6, patient samples from these different studies may show inconsistency at the gene product level, but more homogeneity at the level of the pathways they belong to. We mapped these published results onto a metabolic diagram in order to assess whether modulated pathways were similar (Fig. 5).

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 αIIβ3 (ITG2B and ITGB3) and 2 genes which may play a role in its activation (PEAR-1 [51] and PDIA3 [72]). Moreover, genes involved in the signaling pathways downstream of these receptors were also found to be affected, such as G proteins (GNAZ and GNB3) and mitogen-activated protein kinase (MAPK) related genes (AKT2, RAF1, MAPK14, MAP2K2, MAP2K4, VAV3, PIK3GC and JAK2). On the other hand, 2 genes responsible for intracellular calcium release were also found to be associated with platelet reactivity (IFPR1 and MRV1). In addition, a chloride channel (CLIC1) may also be involved in calcium homeostasis [69]. Going downstream in the process, platelet reactivity may also depend on cytoskeleton and cytoskeleton-related genes (CAPZ, GSN, IPCEF1 and GDR1), as well as glycolysis enzymes (ALDOA, GAPDH and LDHAL6A). It is of note that some of these glycolytic enzymes are known to physically interact with actin for modulation, such as GAPDH and ALDOA [73]. VAMP8, which is involved in secretory granule release, as well as MME, a secreted metalloprotease, were also identified as associated with platelet reactivity [57]. Protein synthesis is also an important phase of platelet activation and some genes, which may be involved at different levels of regulation were published (HP2C, ANKS1B, GLIS3, HSPA8, JMD1C AND SHH). Finally, 2 genes related to oxidative stress were associated with platelet reactivity variability (GSTP1 and HSPD1) (Fig. 5 and Table 2).

In summary, literature mining showed candidates of interest along several crucial pathways for platelet activation and aggregation, i.e. platelet activation, integrin αIIβ3 aggregation, signal transduction, calcium metabolism, glycolysis, cytoskeleton dynamics, oxidative stress, protein synthesis and secretory granule release. These pathways constitute possible modulators of platelet reactivity, however the exact role of each pathway and their effects on each other remain unclear and require further exploration.

3. Network biology: a new paradigm to dissect platelet reactivity

The molecular biology paradigm assuming a direct, one-way relationship between proteins has recently been challenged by the emergence of the network biology paradigm, which takes into account the contextual links between gene products, but also other molecules (Fig. 6) [74]. Indeed, a linear pathway implies that downstream function is unilaterally affected by upstream modulation, but not the opposite. Network biology goes beyond this linear pathway representation; it allows the representation of mutual influences between interactions. This can be depicted as nodes representing molecules of interest (gene products, but also co-factors, small molecules or drugs), which are linked by lines representing 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 [74]; they therefore require a more global view of biological processes (achieved by large scale, quantitative omics methods) and the development of new approaches.
Fig. 5 – Diagram of the metabolic pathways associated with platelet reactivity including the 2 selected clusters. The genes involved in the generic pathways (G protein, MAP kinase signaling pathway, oxidative stress, actin binding proteins and cytoskeleton dynamics, protein synthesis and glycolysis) are listed in Table 2 ([Ibfla: αIbβ3, composed of ITGA2B and ITGB3]).

and new tools to integrate data sets of different origins. In the platelet field, a web-based tool, called PlateletWeb (http://plateletweb.bioapps.biozentrum.uni-wuerzburg.de/plateletweb.php), has been developed as a database workbench centered on literature reviewing to study platelet signaling [75].

At the heart of network biology is the concept that a particular clinical phenotype or disease trait is rarely the consequence of a single gene, but rather reflects the altered interactions of many interconnected genes [76]. The observation of such interactions and their representation in the form of graphs or networks, can allow scientists to gain a more systems-level view of an experiment or series of experiments. Many different types of molecular networks exist in biology. For example, protein interaction networks represent physical interactions between proteins [77,78]; metabolite networks link metabolites participating in the same biochemical reactions [79,80]; regulatory networks represent transcription

<table>
<thead>
<tr>
<th>Pathways</th>
<th>Genes</th>
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<tbody>
<tr>
<td>Signal transduction</td>
<td>G protein, MAPKinase</td>
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<td></td>
<td>GNAZ, GNB3</td>
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<tr>
<td>Oxidative stress</td>
<td>AKT2, RAF1, MAPK14, JAK2, MAP2K2, MAP2K4, VAV3, PIK3GC</td>
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<td></td>
<td>GSTP1, HSPD1</td>
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<tr>
<td>Protein synthesis</td>
<td>JHP2C, ANKSI1B, GLIS3, HSPA8, JMJD1C, SHH</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>CAP2, GSN, IPCEF1, GDIR1</td>
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<tr>
<td>Glycolysis</td>
<td>ALDOA, GAPDH, LDHAL6A</td>
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factors or miRNAs and their targets \[81,82\]; genetic networks connect genes together if there is evidence for gene–gene interaction or epistasis \[83\]; and phenotype networks, where genes with similar gene- or protein-expression profiles can be linked together and the resulting co-expression clusters, or modules, can be correlated with a phenotype \[84,85\]. The goal of many studies using networks is to discover modules of closely inter-connected genes that function together as a unit. Some functional gene modules are conserved across large evolutionary distances and are thought to represent the fundamental building blocks of molecular processes \[86\]. Discovery of such modules in human disease will therefore provide the building blocks for understanding disease progression and potential therapeutic intervention points. Cross-species conservation of gene modules can also identify relevant model organisms and assays for drug screening.

Networks have been successfully used to identify key genes involved in the pathogenesis of many diseases. A recent study on autism focused on trying to understand major pathways and molecular functions affected by the disease, by looking at rare variants in a network-based approach. By looking at the rare variants in the context of a network of known interactions, the authors were able to show that the functional clusters most often hit were related to a number of molecular processes, including actin network dynamics and reorganization, synaptogenesis and axonogenesis \[87\]. Networks have also been used for the study of somatic mutations occurring in metastatic melanoma. In a recent study, a large protein interaction network was used to find sub-clusters or modules of interacting proteins that were affected in tumors. Whilst the genes affected by somatic copy number variants were different in different tumors, they often occurred in the same modules of proteins, which were in turn associated with cell cycle and apoptotic functions \[88\].

These two examples used biological networks composed of known protein interaction and pathway data, and mapped genetic observations to these networks. An alternative approach is to generate a network from the data itself, rather than from additional functional information. The advantage of this approach is that the network reflects the data of a specific controlled experiment rather than data from many different experiments, often from many different cell types. Because the network does not rely on known relationships, observations made in such networks can lead to truly novel discoveries. A recent example of such a study used global gene expression profiles from human pancreatic islets and identified a network module containing Sfrp4, which was strongly over-expressed in non-insulin-dependent diabetes mellitus patients and affected insulin secretion \[85,89,90\]. Network theory has shown that the most connected genes within biological networks (the hub genes) are often the most essential \[76\]. In the abovementioned study, Sfrp4 was identified as a hub gene in the module, and was as such identified as an important putative target affecting insulin secretion. The identification of this gene would not have been possible without looking at the interconnectedness of the genes in the context of all the experimental data.

Considering networks of pathways (instead of single gene products) as being affected comparing 2 phenotypes is particularly adapted to the dissection of fine metabolic modulations, particularly in experimental settings associated with high biological variation \[91\], 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 (Fig. 6). This integration allows the exploitation of the complementary aspects of different data sets, going one step further than simply considering common gene product regulation among mRNA and proteins. Known protein–protein interactions and pathway database information can also be used to weight experimental relationships and complement the network. Then, interpretation of the network can be performed using gene-set or gene-ontology enrichment analysis \[92\], or other bioinformatics tools \[93\]. Finally, validation of such results can be performed in vivo or using biological models, reproducing the same phenotype by modulating the pathway of interest \[74\]. To the best of our knowledge, this type of approach has never been applied to identify the modulators of platelet reactivity. Of note, since current antiplatelet drugs mainly target the TxA2 and ADP pathways, the identification of other pathways modulating on-treatment platelet reactivity in cardiovascular patients could have a major impact on both our understanding of platelet physiology and on the management of platelet hyperreactivity in these high-risk patients.

4. Conclusion

The identification of the modulators of platelet reactivity is of utmost importance since it may define new targets for the prevention of recurrence of ischemic events, and help to tailor antithrombotic therapy according to the characteristics of each patient. Moreover, the identification of modulators of platelet reactivity may also be of importance in the investigation of patients with mild bleeding disorders \[94\]. The combination of several omic data sets is a promising approach to having a more global view of the candidate pathways modulating platelet reactivity. Network biology offers the powerful
tool necessary for the integration of those data sets of different origins. This is of particular interest when considering phenotypes relying on the study of very fine metabolic modulations in samples presenting biological variability, as human samples do. Furthermore, it allows us to work out the interactions between different pathways and is thus more representative of the physiological situation.

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