In recent years, proteomics has contributed to the development of platelet research both at basic and translational level. This led to the identification of novel receptors, signaling proteins, and anti-thrombotic drug targets\(^1\). In the early days, researchers tried to map the platelet proteome, convinced that this should provide relevant information on platelet biology, especially given the fact platelets do not have a nucleus and so genomic-based approaches are hampered. Initial studies were based on two-dimensional gel electrophoresis (2-DE) for protein separation and mass spectrometry (MS) for protein identification\(^2\). In that way, hundreds of proteins were identified, many of which were not previously known to be present in platelets, including a high proportion of signaling proteins. This was very encouraging because platelet signaling is a very relevant event in platelet biology. Indeed, platelet receptor stimulation triggers the activation of signaling pathways that eventually lead to integrin signaling and platelet aggregation. Moreover, unwanted platelet activation and aggregation is responsible for many diseases where platelets play a role, such as thrombosis and acute coronary syndromes. In this context, platelets constitute an ideal anti-thrombotic drug target.

Our group had always a great interest on dissecting the main platelet signaling cascades by proteomics\(^3\). To do so, the proteome of basal and activated platelets is compared. Most of our studies were based on 2-DE for protein separation. However, we recently combined immunoprecipitations with SDS-PAGE to improve the coverage of selective groups of low abundant proteins and those more difficult to analyze by 2-DE (e.g. very hydrophobic proteins, such as membrane proteins). Our first study was in 2004, when we analyzed the PAR-1 signalling cascade by comparing the proteome of basal platelets to that of platelets stimulated with thrombin-receptor activating peptide (TRAP)\(^4\). This initial study was based on narrow range pI 4-7 and 6-11 high resolution 2D gels (18 × 18 cm) for protein separation, and fluorescent staining, and led to the identification of 62 differentially regulated protein features. From these features, 41 were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and were found to derive from 31 different genes, most of which corresponded to signalling proteins. Several of the proteins identified had not previously been reported in platelets, including the adapter downstream of tyrosine kinase 2 (Dok-2). Further studies revealed that the change in mobility of Dok-2 was due to tyrosine phosphorylation. This report set the stage for future studies and illustrated the potential of 2-DE-based proteomics to study platelet signalling.

Following the success of the above study, we carried out a similar platelet signalling proteomics study, this time by analyzing the proteome of collagen-related-peptide (CRP)-activated platelets compared to the proteome of un-stimulated platelets\(^5\). Glycoprotein VI (GPVI) is the major activation inducing receptor for collagen in platelets and CRP is a specific agonist. Since tyrosine kinases and phosphatases play a fundamental role in the GPVI signalling cascade, the study of the tyrosine phosphoproteome was one of the main objectives of the study. We used two main approaches for protein separation in order to address the question above: 2-DE (pI 4-7 and 6-11) of whole cell lysates; and phosphotyrosine immunoprecipitations (with agarose-conjugated 4G10 monoclonal antibody) followed by 1D-PAGE. In both cases, proteins were identified by LC-MS/MS. By using this global approach, 96 proteins were found to vary in response to CRP in human platelets. Interestingly, the phosphotyrosine immunoprecipitation-based approach led to the identification of most of the GPVI signalling proteins known so far, and 3 additional novel proteins, one of which was the type I transmembrane protein G6f. Interestingly, G6f was found to be specifically phosphorylated on Tyr-281 in response to platelet activation with CRP, providing a docking site for the adapter Grb2. All of the above data were validated by traditional biochemical approaches (e.g. immunoprecipitations and western blotting), which provided valuable information on the novel proteins identified and led to more detailed mechanistic studies.

After moving to the University of Santiago de Compostela in 2006, we collaborated with former colleagues at Birmingham (UK) and Oxford (UK) to carry out a detailed proteomic analysis of integrin αIIbβ3 outside-in signalling in human platelets\(^6\). Since the integrin αIIbβ3 signalling pathway is based on tyrosine phosphorylation events —as it happens with the GPVI cascade—the chosen experimental approach was also based on phosphotyrosine immunoprecipitations. Washed platelets were plated on either a BSA- or fibrinogen-coated surface for 45 min at 37 °C. Proteins were immunoprecipitated with an antiphosphotyrosine antibody (4G10, agarose-conjugated) and resolved on 4%-12% NuPAGE Bis-Tris gradient gels (Invitrogen). Following gel staining with a fluorescent dye equivalent to SYPRO Ruby, bands of interest were excised, proteins trypsin digested, and analyzed by MS. The approach led to the identification of 27 proteins, 17 of which were not previously known to be part of a tyrosine phosphorylation-based signalling complex downstream of αIIbβ3. The group of proteins identified included the novel immunoreceptors G6f,
and G6bb-B, and two members of the Dok family of adapters, Dok-1 and Dok-3, which underwent increased tyrosine phosphorylation following platelet spreading on fibrinogen. We focused on Dok proteins for further mechanistic and functional studies, showing that tyrosine phosphorylation of Dok-1 and Dok-3 was primarily Src kinase-independent downstream of the integrin. Moreover, both proteins inducibly interacted with Grb2 and SHIP-1 in fibrinogen-spread platelets(9). Based on previous reports from B and T cells, we hypothesized that Dok-1 and Dok-3 participate in a multi-molecular signalling complex, together with SHIP-1 and Grb2, which may negatively regulate αIIbβ3 outside-in signalling.

In 2006 we participated in the discovery of the novel platelet receptor CLEC-2(1). CLEC-2 is a C-type lectin-like type II transmembrane receptor expressed in platelets with an important activator potential. Although its physiological function has yet to be defined it has been speculated as a plausible target for antithrombotic drugs. CLEC-2 mediates platelet activation by the snake venom rhodocytin. We are currently carrying out a global proteomics approach to study the proteome of rhodocytin-activated platelets with the aim of discovering new key players in the CLEC-2 signalling cascade (Pariguína et al., unpublished). We collected platelet samples from healthy donors and performed two complementary proteomic techniques for protein separation: phosphotyrosine immunoprecipitations followed by 1-D-PAGE, and two-dimensional Differential In-gel electrophoresis (2D-DIGE). After protein visualization, a detailed image analysis for further mechanistic and functional studies, showing that tyrosine phosphorylation of Dok-1 and Dok-3 was primarily Src kinase-independent downstream of the integrin. Moreover, both proteins inducibly interacted with Grb2 and SHIP-1 in fibrinogen-spread platelets(9). Based on previous reports from B and T cells, we hypothesized that Dok-1 and Dok-3 participate in a multi-molecular signalling complex, together with SHIP-1 and Grb2, which may negatively regulate αIIbβ3 outside-in signalling.

In conclusion, during the last decade proteomics has contributed to a better understanding of platelet biology. Novel platelet receptors and signalling proteins have been identified, some of which might play relevant roles in platelet activation and aggregation. In addition, clinical proteomics has been recently applied to the study of platelet-related diseases, helping to identify novel platelet biomarkers and drug targets that might eventually improve the diagnosis and treatment of those pathologies where platelets play a relevant role(13). The initial expectations of platelet proteomics researchers have been fulfilled and, although there will always be challenges ahead, the future looks promising.

Sources of funding
The authors would like to acknowledge the support given by the Spanish Ministry of Economy and Competitiveness (MINECO) [grant No. SAF2010-22151, co-funded by the European regional development fund (ERDF)], the Galician Government (ConSELLeria de Educación, Xunta de Galicia, Spain) [grant No. INCITE09PXIB203145PR], and the Fundación Mutua Madrileña (Spain). Dr. García is a Ramón y Cajal Research fellow (MINECO), and Mr. Pariguína is a FPI pre-doctoral fellow (MINECO).

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