Platelet flow cytometry in diabetes mellitus and in other metabolic-vascular pathologies

Citometría de flujo de plaquetas en la diabetes mellitus y otras patologías metabólico-vasculares

M. Labiós Gómez, M. Martínez Silvestre, F. Gabriel Botella
Internal Medicine Service, University Clinical Hospital of Valencia. Flow cytometry Unit. Clinical Analysis Service. University Hospital “La Fe”, Valencia

Abstract
In recent years flow cytometry has experienced a great development, which confirms it as an important tool to work with a great clinical and analytical potential. Its major advantages derive from the fact that we work directly with whole blood, without the risk of artefactual activation that often occurs by the manipulation of the sample when working with washed platelets. In addition, flow cytometry is able to detect simultaneously different antigens on well-defined cell subpopulations. This methodology permit the study of circulating spontaneously activated platelets and evaluate the effect of several drugs, in vitro and ex vivo, on platelet function in numerous pathologies. Despite its advantages, flow cytometry is currently not used usually in the study of diabetes and other metabolic-vascular pathologies.

Keywords: flow cytometry, platelet activation, clinical utility.

Resumen
En los últimos años, la citometría de flujo ha experimentado un gran desarrollo que la confirma como una importante herramienta de trabajo con gran potencial clínico y analítico. Sus principales ventajas derivan del hecho de utilizar directamente sangre entera –sin el riesgo de activación artifactual que frecuentemente se produce por la manipulación de la muestra cuando se trabaja con plaquetas lavadas– y de poder detectar simultáneamente diversos antígenos en distintas subpoblaciones celulares bien identificadas. Esta metodología permite estudiar plaquetas circulantes activadas espontáneamente y valorar el efecto in vitro y ex vivo de diversos fármacos sobre la función plaquetaria en numerosas patologías. A pesar de sus ventajas, actualmente la aplicación de la citometría de flujo en el estudio de la diabetes y otras patologías metabólico-vasculares está lejos de ser un hecho habitual.

Palabras clave: citometría de flujo, activación plaquetaria, utilidad clínica.

Flow cytometry and platelet activation markers

Flow cytometry
It is an analytical technique that allows assessing different parameters in individualized cells that flow through a sensor point. Thanks to the technology of the monoclonal antibodies and to the use of different fluorochromes, the density and the distribution of several cell antigens can be studied. The application of the flow cytometry has experienced during the last years a great development, allowing studying several cell subpopulations simultaneously.

At the beginning of the 70s the argon laser started to be used for the fluorescence measurements. Multiple commercial flow cytometers were developed during the following decade (Coulter Cytometry, Ortho Diagnostics, Becton Dickinson, Bio-rad Laboratories Inc, Cytomation Inc., Partec AG, Sysmex Corporation, etc.) which have been incorporating the technology developed dur-
ing the last years in several fields, therefore more versa-
tile, accurate and fast flow cytometers have been com-
mercialized.

At present, there are cytometers able to measure multiple
cell parameters of dispersed light and conjugated fluo-
rescence with specific antibodies as from a cell suspension
or particles (cores, organelles, chromosomes). These sys-
tems, unlike other analysis techniques that measure the
mean population of a determined property, are able to
measure, store, process and represent the parameters of
thousands of cells in a customized manner. Basically,
these systems collect the dispersed light and the one
emitted by such particles, as the radiation from the argon
laser with a wave length of 488 mm affects them.

The critical essential step in flow cytometry is to count
with a suspension of individual cells, representative of
the original population. Such suspension is marked with
conjugated antibodies with fluorescent colors and after
an optimal incubation period, it is injected into the cy-
tometer, in which the sample is focused hydro dynami-
cally in the flow chamber. In such chamber, the cells in-
teract with the monochromatic light shaft, causing
diffraction. Once the cell interacts with the radiation, the
system measures several parameters and relate them
with determined cells characteristics. Such cytometric
parameters are as follows:

- Frontal dispersion (forward scatter): related to the cell
  size.
- Lateral dispersion (side scatter): related to the density
  and cell rugosity.
- Fluorescence intensity: related to the amount of conju-
gated antibodies with a fluorochrome, bound to a spe-
cific antigen.

In order to obtain this information, the optics of the cy-
tometer is placed in such a way to allow collecting the
dispersed light in the same direction than the incident
light (forward), the dispersed light with an 90° angle as
regards to the other one (side) and the light emitted by
fluorescence with this same angle. From the light col-
clected from the side, through dichroic mirrors, the differ-
ent wave lengths, which reach the corresponding detec-
tors with a determined intensity. In these detectors, the
light signal turns into an electric signal; it is amplified
conveniently and processed in a computer with software
able to transform the electric signals in graphic and nu-
merical results.

Especially in the platelets, the flow cytometry is able to
assess the in vivo activation condition of the circulating
platelets. Besides being able to determine the natural
condition of the platelet function, the inclusion of an ex-
ogenous activator allows assessing the platelet reactivity
in vitro or the ex vivo action of a drug. The main advan-
tages of the flow cytometry of total blood for the analy-
sis of the platelet activation are depicted in table 1.

The principal inconveniences of this technique are the
high cost of the equipment and the antibodies, and the
need of counting with well-qualified staff, making its use
difficult in a standard clinical laboratory.

**Activation markers**

It is well known that the platelets are essential cells in
the normal hemostatic process. The lesion of the vascu-
lar wall originates the exposure and release of several
agonists that activate platelets. Such activation consists
mainly of the exposure of binding sites for adhesion
molecules – as the fibrinogen and the Von Willebrand
factor – and of the secretion of the intracellular granules
content, that ensure the growth of platelet aggregates,
with which the formation of the hemostatic clot starts.

It is known that the glycoprotein complex IIb/IIIa (GP-
IIb/IIIa) of the platelet membrane plays an essential role
in the process of platelet aggregation, interacting with
the plasmatic fibrinogen when the conditions of the
blood flow are of low shearing speed, and with the Von
Willebrand factor when the shearing speed is high. The
platelet at rest does not adhere itself neither to the vascu-
lar endothelium nor to the leucocytes, but when it is activated, the adhesion to both increases due to the morphologic and functional change, in a first step, which the GPIIb/IIIa experiences, that will be represented as GPIIb/IIIa in the activation process. Another glycoprotein that is only expressed in the surface of the activated platelet is the P-selectin (CD62) that regulates the platelet adhesion to the neutrophils and monocytes and to the endothelium, stabilizes the initial interaction GPIIb/IIa-fibrinogen and allows the formation of big platelet aggregates and the possible development of a thrombo. At the same time, the platelet-monocyte complex and platelet-endothelium, measured by the CD62 induces the activation of the tissue factor and stimulates the deposition of fibrin at the vascular lesion site. On the other hand, the expression of CD62 is correlated positively with the thickness and rigidity of the arterial wall, and is associated to the pathological changes that can be observed. These findings suggest that the CD62 expressed in the surface of the activated platelets, is involved in the initial process of the in vivo atherosclerotic lesions. Another indicator of degranulation reaction is the exposure of the liposome CD63 antigen on the platelet surface, which constitutes also a sensitive marker of platelet activation.

It is known that the circulating platelets might get stimulated spontaneously inside the vessels, forming platelet micro aggregates (PMA) with potential to form major thrombos, able to occlude vessels. Therefore, the detection of the PMA turns out to be relevant as first step indicator of the platelet aggregation, which is a phenomenon that constitutes a thrombosis risk factor.

Besides the indicated processes, another response of the platelet in the activation process is the exposure in the membrane of the phospholipid phosphatidylserine (PS), which provides an adequate catalytic surface for the formation of the calcium-binding depending prothrombinase complex, which transforms the prothrombin in thrombin. This exposure of PS in the external hemilayer of the platelet membrane is associated, when caspase family is activated, to the formation of micro particles or micro vesicles from the platelets, that are released to the environment and that present also a pro-coagulant activity due to the formation of prothrombinasa complex on the surface, thanks to the exposure of PS on it.

It is well determined that if the platelet is at rest, membrane turns out to be impermeable to the Ca\(^{2+}\) ion, which can be found in a higher concentration in the external environment than in the intraplatelet environment. During the activation process, the concentration of cytosolic free calcium increases due to the diffusion of such ion from the external milieu, through specific calcium channels, and the mobilization of the internal intraplatelet stores, mainly of the dense tubular system. The mobilization of the Ca\(^{2+}\) is fundamental regarding to the platelet response to the activation process and proceeds to other changes experimented by platelets: morphology, aggregation, secretion and expression of the pro-coagulating activity.

As we can observe in this revision, the platelet parameters indicated up here (GPIIb/IIIa*, CD62, CD63, PS, Ca\(^{2+}\) mobilization, micro particles and micro aggregates) are useful to assess the condition of platelet activation and function, and all of them can be determined by means of flow cytometry.

**Application of flow cytometry to the record of antiplatelet therapeutics**

The platelets play a critical role in patients with cardiovascular disease, as they increase the risk of thrombosis events. It is known that the antiplatelet drugs might reduce the incidence of ischemic cardiovascular events, therefore it is interesting to register with the aim of stating the most adequate customized doses in each case, and reduce the risk of hemorrhage and thrombosis recurrence.

Since the acetylsalicylic acid (ASA) has an inhibitory effect on the platelet only through the cyclooxigenase route 1 (COX-1), whose inhibition impedes the generation of thromboxane A\(_2\), it has been observed that a great number of patients, in spite of being treated with this drug, show recurrence of thrombosis events attributable to the platelet activation through different routes than the one inhibited by the ASA, or because of patients who respond badly or have developed a resistance to such drug. In these cases, the thrombosis events might be due to the in vivo exposure of the platelets to strong concentrations of agonists, as collagen or thrombin or adenosine diphosphate (ADP) released by the platelets themselves or by the thromboxane A\(_2\) of non platelet origin, that might cause its activation. It has been communicated that the expression of GPIIb/IIIa, CD62 and
CD63 in circulating platelets, or in platelets activated with ADP or thrombin, is not modified with the ASA, therefore several working groups have studied the efficiency of the combined therapy of ASA with other drugs, as ticlopidine and clopidogrel, that exert their action through a different mechanism, consisting of the blockade of the P2Y12 receptor of the ADP. In this type of analysis, the flow cytometry allows assessing, as platelet activation markers, the expression and induction through ADP of CD62, CD63 and GPIIb/IIIa*, it can be proved that in patients who respond adequately, clopidogrel prevents the expression of such markers of activation after the stimulation with ADP or thrombin, and that the addition of ASA improves its inhibitory effects ex vivo. With the use of the flow cytometry it can also be determined the minimum doses of ASA that is necessary to potentiate the effect of clopidogrel in each patient, as well as the detection of patients who do not respond adequately to the treatment with this drug.

The post-surgery thromboembolic ictus is frequently associated to a hyperactivity of the platelets to the ADP, and clopidogrel prevents the expression of such markers of activation after the stimulation with ADP or thrombin, and that the addition of ASA improves its inhibitory effects ex vivo. With the use of the flow cytometry it can also be determined the minimum doses of ASA that is necessary to potentiate the effect of clopidogrel in each patient, as well as the detection of patients who do not respond adequately to the treatment with this drug.

The new antiplatelet drugs (abciximab, tirofiban, eptifibatide, SR121566A) block the GPIIb/IIIa receptors, limiting the binding of the fibrinogene and the Von Willebrand factor to them; therefore, they inhibit the aggregation of the platelets, with independence of which one was the route that started the activation process. As group, the anti-GPIIb/IIa drugs show a wide range of applications, included the primary prevention of the cardiovascular disease and the treatment of patients with acute coronary syndrome, stents implantation and angioplasty and ictus, providing, in general, an immediate antiplatelet action, higher than the combination of ASA and clopidogrel.

However, the antagonists of GPIIb/IIIa are expensive and they also require the administration of an intravenous bolus followed by the continuous infusion of the drug during certain period of time, therefore, before implanting them, it would be convenient to study their efficiency compared to ASA, clopidogrel or ticlopidine and their possible combinations. The efficiency of the anti-IIb/IIIa drugs depends to a great extent to their dosage, which might be based in measuring the inhibition of the platelet aggregation. Though it is not well determined, it is accepted that an optimal level of platelet inhibition has been achieved for an efficacious prevention of thrombosis events after coronary surgery when the inhibition of 80% of the aggregation induced by the ADP is achieved. However; this inhibition depends on certain factors, as the concentration of used agonist, the number of platelets, the intake of food, the concomitant medication and the inter-individual variation. Thus, the aggregometry is not recommended in order to undertake a follow-up of this group of drugs as it turns insensitive to the extreme values of GPIIb/IIIa receptor activity by the drug and to the small inter-individual variations. On the contrary, the flow cytometry allows registering the treatment with these measurements, without the inconveniences of the aggregometry, when quantifying directly, through the antibodies. LYP18 and 4F8, the total and free number of sites of the, GPIIb/IIa receptors, in the steady phase of the treatment. Knowing these data, the number of sites occupied by the drug can be estimated. An optimal therapeutic dose is considered the one that produces an 80% of activity by the drug of the 50.000-80.000 GPIIb/IIa sites that are on the platelet surface. With this level of activity, the aggregation of the platelets is inhibited, as it remains only 20% of sites free, suitable to bind to the fibrinogens. With this dose, the intention is to obtain the maximum inhibition without inducing an excessive risk of bleeding in the patient. This therapeutic range, relatively narrow, together with the great variability of inter-individual response, makes the follow-up of these drugs advisable in order to avoid the hyper/hypo treatment risk as possible.

It has been described that the patients with an acute myocardial infarction, who underwent thrombosis with reteplase, show an increase in the expression of GPIIb/IIa* 24 hours after the thrombolysis. Said phenomenon
is not observed in patients treated with alteplase. These data that have been obtained by means of cytometry, indicate objectively the convenience of administering an anti-GPIIb/IIIa to the patients treated with reteplase in order to avoid the reocclusion of the rechanneled arteries. This clinical usefulness of the flow cytometry is not offered by any of the other preconized techniques at present in order to evaluate the platelet function (optical aggregometry, PFA-100, Verify Now, etc.).

**Circulating activated platelets in pathologies with a high atherothrombotic risk**

It is known that the platelet activation plays an important role in the mechanisms of the arterial disease which includes the myocardial infarction, the ictus and the peripheral artery disease. In the acute coronary syndrome and in the myocardial infarction, the presence of circulating activated platelets and platelet hyperactivity have been described, as well as the increase of monocytes-platelet circulating aggregates and circulating micro-particles. The presence of such circulating aggregates has been reported in patients with stable and unstable angina, and in those who underwent percutaneous coronary interventions. Other authors have communicated that the expression of glycoproteins of platelet membrane in circulating blood is associated to the increase of risk of suffering an ischemic event after the angioplasty and the stent implantation.

A relevant increase is observed in the ictus of the circulating activated platelets, as well as changes in the morphology and increase of circulating GPIIb/IIIa platelets. Other work groups describe a higher number of circulating micro-particles in the patients than in the control group. However, it is interesting to point out the great variation of platelet activation response in patients with ictus, that paradoxically might show even a lower activation than the control group.

In the peripheral vascular disease, an increase has been described in the percentage of positive circulating CD62 and in the positive GPIIb/IIIa platelets, as well as in the number of micro-aggregates and micro-particles of platelet origin, confirming the platelet hyper-activity in these patients by means of flow cytometry.

**Diabetes, hyperlipemia and blood pressure**

Several authors have suggested that diabetes, hyperlipemia and the blood pressure have a relevant influence on the platelet function, increasing the reactivity of such cells.

The diabetes mellitus is one of the most frequent chronic diseases in the developed countries. It is known that the diabetes increases the risk of coronary heart disease, ictus and peripheral arterial disease, as consequence of the greater incidence of blood pressure, obesity, dyslipidemia compared to the non diabetic patients. These risk factors associated to the diabetes are closely related to the atherosclerosis and thrombosis processes, which are so frequent in these patients, so the basic objectives in the treatment of diabetes should be the control of the blood pressure and plasmatic lipids besides the normalization of the glycemia level.

There is a tendency to associate the microvascular risk to the T1D and the macrovascular risk to the T2D, but both types of complications are frequent in both types of diabetes; thus, the cardiac disease appears also frequently in the T1D.

It is well determined that in the angiopathic complications associated to diabetes, the platelets exert a key role and that platelet function might be altered through several mechanisms, among which the hemorrhagic disturbances that these patients show frequently should be pointed out. Such rheological disturbances depend to a great extent on the changes in the lipid situation of the red blood cells membrane, reducing the deformability and increasing the shearing tension of the circulating blood in certain areas of the circulatory system, causing a greater platelet activation. Thus, diabetes is associated to multiple metabolic, cellular disturbances and to the blood flow, which might lead to vascular complications. The activated platelets form circulating micro-aggregates that might contribute to the development of angiopathy by micro-embolization of the capillaries of the in vivo micro-circulation and to the development of great sized thrombosis.

Notwithstanding that several studies suggest a direct association between platelet aggregation and vascular and atherosclerotic complications in diabetes, the role of platelets in such complications are not clearly deter-
The presence of platelet-leukocyte circulating aggregates has been reported in diabetes, use, in general, unable methods to detect the presence of subpopulations of platelet aggregates formed spontaneously in the circulating blood. Most of the used methods require the addition of high concentration of stimulating agents, they use plasma rich in platelets as sample and measure the changes produced in the light transmission during the aggregation process. With the addition of agonists, the capability of platelets of being stimulated by an external agent is assessed, but the spontaneous activation that the platelets experience in the circulation cannot be assessed. On the other hand, the platelet response is more physiological when it is assessed in whole blood, in the presence of the rest of blood cells, than when it is isolated, as in the cases in which it is used for the analysis of the plasma rich in platelets. Finally, the changes in the transmitted light are only noticeable when the platelet aggregates are bigger, but they do not allow detecting the presence of small platelet aggregates or micro-aggregates. All these reasons, among others, support the performance of studies that use the flow cytometry of whole blood as technique.

The presence of platelet-leukocyte circulating aggregates has been reported in diabetes, but the presence of platelet-platelet aggregates is not described, either its formation is related to the spontaneous platelet activation or to the level of glycemia control. It has either been described if the changes in the distribution of phospholipids of the platelet membrane—with exposure to the phosphatidylserine and formation of the consequent prothrombinsa complex that transforms the prothrombin in thrombin—are involved in the formation of PMA in diabetic patients.

Diabetic patients are characterized for showing a higher number of GPIIb/IIIa receptors, a higher percentage of positive platelets CD62, CD63 and GPIIb/IIIa and more circulating micro-particles than the control group, constituting a risk situation of suffering acute vascular events. It has also been communicated that diabetic patients show an postprandial increase of the platelet reactivity. Since platelets have insulin receptors in the membrane and the hormone increases platelet activation, it might be speculated that the beneficial effects that the insulin exerts on platelet function are more related to the improvement of the metabolic control than with the direct normalizer effect on the platelets. In this sense, it has been reported that an adequate metabolic control of the disease reduces the activation of platelet markers.

With the aim of reducing the risk of suffering thrombosis events, it would be convenient to determine adequate anti-platelet strategies in these patients. Even after the dual treatment with ASA/clopidogrel, the diabetic patients show a greater residual activity in some markers of platelet activation than the normo-glycemic patients, and are benefited to a great extent of the anti-thrombosis prevention with ASA. The greater platelet activation and the greater response to the agonist action in diabetic patients have been attributed to the high glucose levels that cause a greater osmolarity leading platelets to be more reactive through different mechanisms.

In the hypercholesterolemia, platelet activation increases in a parallel way to the high levels of the low density lipoprotein-cholesterol (LDL-C). In spite of the interest of this study regarding to platelet activation in hyperlipemia, the available information about these aspects is scarce and frequently contradictory. For example, it has been reported that the patients with high levels of LDL-C show a higher percentage of circulating platelets with the GPIIb/IIIa in its active form. Moreover, the platelets of such patients are more sensitive to the ADP action. It seems that there exists a correlation between the reduction of the plasmatic cholesterol concentration and the expression of CD62. In general, the observed impairments in platelet activation of hyperlipemic patients are normalized by means of the treatment with certain drugs, as the atorvastatin.

The lypemia induced by the diet makes the percentage of platelets expressing CD62 in the membrane to increase significantly during the postprandial period, both in vivo and after the stimulation with ADP. It is known that the hypertriglyceridemia is a cardiovascular risk factor associated to a hypercoagulability condition; although, the relation with platelet activation has not been determined. The available information in this sense is very scarce; thus, it has been described that the patients with hypertriglyceridemia show a higher percentage of positive CD63 platelets. However, such patients do not express CD62 more than the control group, which becomes difficult to understand, since CD63 is a platelet degranulation marker and the CD62 should also be expressed in this process.
The presence of a higher number of circulating microparticles\textsuperscript{43-50,83,84} has been described in the hypertension, whose membranes have a pro-coagulating nature due to PS exposure. The results of several studies indicate that the hypertensive patients constitute, at baseline, a population under atherothrombosis risk, characterized by the presence of circulating platelets activated spontaneously \textit{in vivo},\textsuperscript{47,48,50,51} and by relevant changes in the kinetics of the intra-platelet free calcium.\textsuperscript{48} Both parameters constitute important thrombosis risk factors and are assessable by means of flow cytometry. Many of the impairments described about platelet function, in hypertension, are normalized through an adequate antihypertensive treatment.\textsuperscript{47-51}

**Conclusion**

The platelet function is not yet assessed systematically in the clinical practice due to the work load that its performance suggests, because it is expensive and requires adequate instrumentation and space and the training of qualified staff. Besides these difficulties, the application of specific techniques, as the platelet aggregation in patients with cardiovascular disease is very arguable.\textsuperscript{85} However; the results of the consulted literature allow confirming that the flow cytometry is a well-determined powerful analysis technique, whose clinical application is increasing progressively. The cytometric parameter that have proved to be useful, both in the diagnosis and in the follow-up of antiplatelet treatments are depicted in table 2.

In conclusion, at present flow cytometry offers an important range of possibilities for the study of a great number of platelet activation markers in several pathologies, therefore it is expected to constitute not only an investigation tool but also of application in the usual clinical practice in the future.

**Table 2. Cytometric parameters with demonstrated clinical usefulness**

- Expression of GPIIb/IIla complex in its active form (GPIIb/IIla*)
- Exposure of P-selectin (CD62) in the platelet surface
- Exposure of phosphatidilserine (PS) in the platelet surface
- Exposure of CD63 lisosomal in the platelet surface
- Changes in the kinetics of the cytoplasmatic free calcium
- Formation of micro-particles and platelet micro-aggregates
- Formation of mixed aggregates platelet-leukocytes
- Quantification of the activity for anti-GPIIb/IIla drugs

**Practical considerations**

- The flow cytometry uses whole blood directly and in this way it allows to detect several antigens simultaneously in different well identified cellular sub-populations.
- This methodology allows studying the \textit{in vitro} and \textit{ex vivo} effect of several drugs on platelet function in several pathologies as diabetes, hyperlipemia and hypertension.
- The principal inconveniences of this technique are the high cost of the equipment and of the antibodies, as well as the need of well-qualified staff, therefore its use is quite difficult in a routine clinical laboratory.

**Declaration of potential conflict of interests**

M. Labiós Gómez, M. Martínez Silvestre and F. Gabriel Botella state that there are no conflicts of interest as regards to the content of this article.

**References**


