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Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop

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ON BEHALF OF THE ISTH SSC WORKSHOP

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Microparticles (MPs) are sub-micrometer sized vesicles released from cell membranes in response to activation or apoptosis [1]. MPs originating from several cell sources have been described in human plasma. Among them, platelet-derived MPs (PMPs) are believed to account for the majority of circulating MPs in healthy subjects [2]. Their levels are increased in several prothrombotic and inflammatory disorders [1]. In these clinical settings, PMP counts may be useful for identifying patients at risk for vascular disorders and for monitoring response to treatment [3]. However, their clinical use is not fully established, because standardized methodologies for PMP counting are lacking.

A previous International Society on Thrombosis and Haemostasis (ISTH) Vascular Biology Subcommittee survey indicated that approximately 75% of laboratories use flow cytometry (FCM) to enumerate MPs in clinical samples. However, a wide variety of preanalytic variables and analytic variables have been reported in the literature, resulting in a wide range of PMP values in platelet-free plasma (PFP) of healthy subjects (100–4000 PMPs μL^{-1}). This lack of consensus stresses the need for standardization [4].

Three ISTH Scientific and Standardization Subcommittees (SSC Vascular Biology, DIC, and Haemostasis & Malignancy) have initiated a project aimed at standardizing the enumeration of cellular MPs by FCM. A first collaborative workshop was set up, to: first, establish the resolution and the level of background noise of the flow cytometers currently used in laboratories with respect to the strategy requirements; and second, define the interinstrument reproducibility of PMP enumeration in human plasma. This strategy was based on the use of fluorescent

calibrated sub-micrometer beads (Megamix beads; BioCytex, Marseille, France), which allow the window of MP analysis to be reproducibly set [5].

The study included 40 laboratories accounting for 59 flow cytometers, and was performed in two stages (Fig. S1): in stage A, participating laboratories received Megamix beads and were asked to set up the FCM protocol and to validate an instrument protocol adapted from a previously described method [5]. On the basis of forward scatter (FS)/FS channeling (FSC) resolution and background characteristics, stage A results led to acceptance or rejection of the tested instruments, with some time being allowed for technical intervention in order to improve any deficient performance. In stage B, selected laboratories received PFP samples prepared as frozen aliquots by the core laboratory, and were asked to analyze them with the previously validated instrument(s), common reagents, and the FCM protocol established in stage A. A detailed description of the methodology is available in the Supporting Information (Data S1).

The purpose of this initial phase was to check whether the instrument to be used to enumerate PMPs demonstrated the required performance with a blend of fluorescent beads with well-known sizes and relative amounts. The instruments were validated on the basis of their capacity to discriminate between 0.5- μm and 0.9- μm Megamix beads using the FS/FSC parameter, as well as their background noise. Instruments detecting < 0.1% of fluorescent bead events among total events were rejected, because such a level of background may impede the electronics functions and induce a major loss of events owing to coincidences and electronic aborts (Fig. S2A,B). Analysis of the results demonstrated that instruments were heterogeneous with respect to FS/FSC resolution and background noise. Furthermore, the level of performance could vary over time (Fig. S2C). Some of the parameters affecting FS/FSC resolution were identified with Megamix beads. Among them, FS/FSC gain, FS/FSC mode, neutral density (intensity scavenging)

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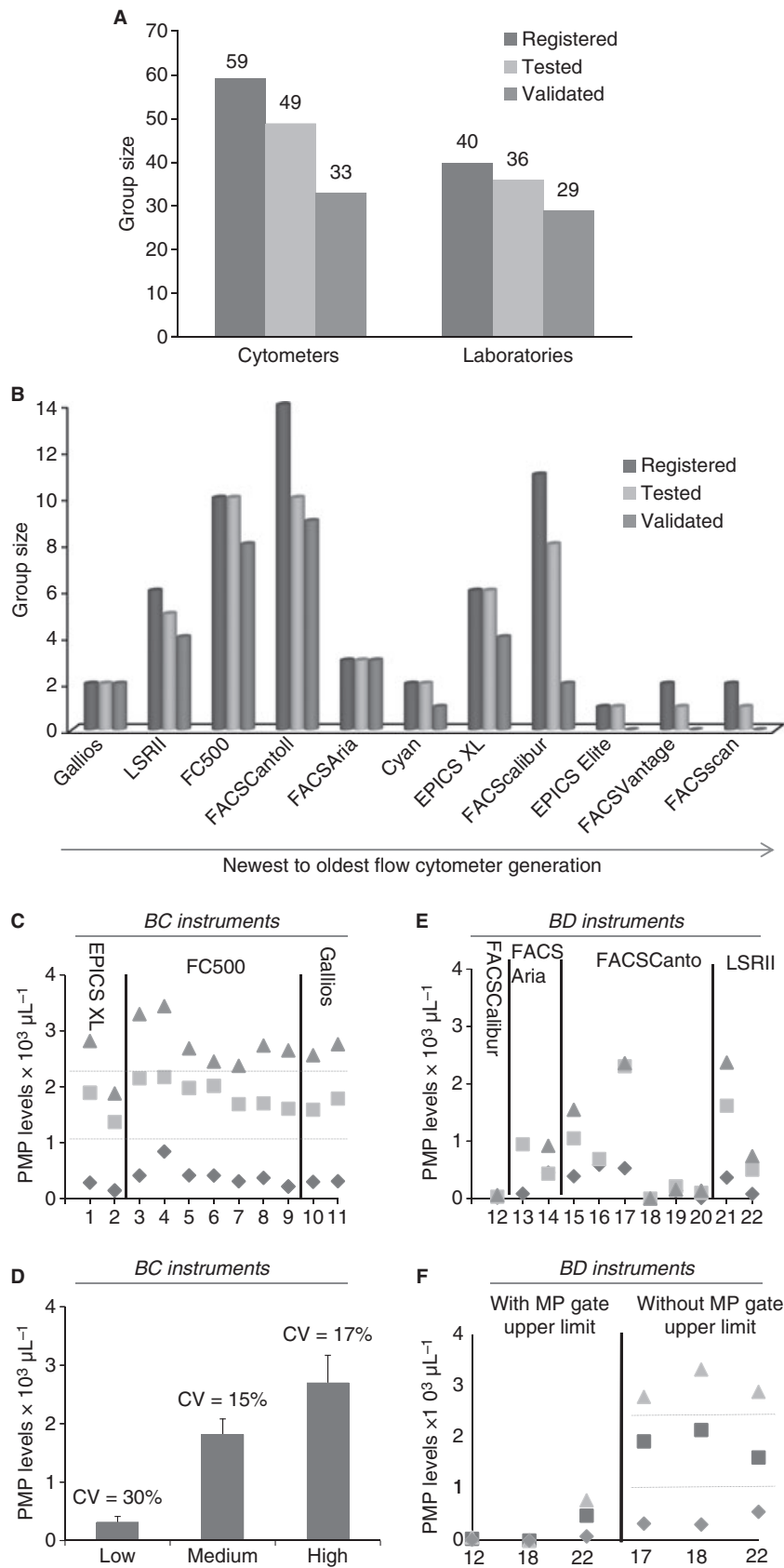


Fig. 1. Instrument validation (stage A) (A) Histogram showing the number of flow cytometers and laboratories that were registered in the workshop (black bars), were tested (light gray bars), and were validated (dark gray bars). (B) Histogram showing the number of flow cytometers that were registered in the workshop (black bars), were tested (light gray bars), and were validated (dark gray bars), according to their type, classified from newest to oldest generation. (C–F) Interlaboratory reproducibility (stage B). (C) Platelet-derived microparticle (PMP) levels obtained for the three tested platelet-free plasmas (PFP) (diamonds, low; squares, medium; triangles, high) for each validated Beckman Coulter (BC) instrument. (D) Histogram showing the interlaboratory reproducibility obtained for the three PFPs with BC instruments. (E) PMP levels obtained for the three PFPs (diamonds, low; squares, medium; triangles, high) for each validated Becton Dickinson (BD) instrument. (F) PMP levels for the three tested PFPs (diamonds, low; squares, medium; triangles, high) before and after removing the upper limit on the microparticle (MP) gate on three selected BD instruments. CV, coefficient of variation.

filters, flow rate and osmolarity of the Megamix suspension proved to be important (Fig. S3). Megamix bead analysis allowed easy optimization of these parameters.

Finally, among 59 registered instruments from 40 candidate laboratories, 49 were tested, and 33 (67%) from 29 laboratories were ultimately validated (Fig. 1A). As expected, a higher validation rate was obtained with instruments of later generations [Gallios from Beckman Coulter (BC), Miami, FL, USA; LSR II from Becton Dickinson (BD), Franklin Lake, NJ, USA; FC500 from BC; FACSCanto II from BD] than with instruments of older generations (FACSCalibur from BD; EPICS XL from BC): 84% vs. 35%, respectively. However, as shown in Fig. 1B, some of the most sophisticated instruments failed to qualify [FACSCanto II (1/10), LSR II (1/5), and FC500 (2/10)]. Conversely, some instruments of older generations reached the required criteria, as seen with EPICS XL (4/6) and FACSCalibur (2/8). This outcome shows that instrument response related to the FS/FSC parameter is highly variable, and depends on the status of an individual instrument, which needs to be recalibrated on a regular basis.

To avoid any preanalytic-linked variability, the core laboratory prepared and validated PFP samples (Fig. S4). All participants received six PFP aliquots from three different samples accounting for three different PMP levels (low, medium and high). PMPs were enumerated on validated instruments, with common reagents and the same FCM protocol that was previously calibrated with Megamix beads.

The analyses obtained from 22 instruments (11 BC and 11 BD) are shown in Fig. 1C,E. As illustrated in Fig. 1D, interlaboratory reproducibilities of 30% (low), 15% (medium) and 17% (high) were found among BC instruments with Megamix beads, whereas highly dispersed values were reported with BD instruments (Fig. 1E): – 24%, coefficient of variation (CV) = 78% (low); – 60%, CV = 87% (medium); and – 66%, CV = 91% (high).

A potential explanation for such a discrepancy between the two types of instrument was suggested by the observation of the location of the PMP population on the standardized size-related window of analysis. As shown in representative examples (Fig. S5A), the PMP population was located inside the MP gate defined by the Megamix beads for BC instruments, whereas it varied from being partly inside to entirely outside the MP gate on all BD instruments (Fig. S5B,C). Interestingly, as illustrated in Fig. 1F, when listmode files were reanalyzed without setting an upper limit on the MP gate with three BD instruments (no. 12, FACSCalibur; no. 18, FACSCanto II; and no. 22, LSR II), a similar range of PMP values

as determined by BC instruments was apparent. Taken together, these findings showed that PMP concentrations appeared to be consistent among instruments that measure the FS parameter with a relatively wide solid angle (1–19°, Beckman-Coulter; hereafter referred to as ‘wide FS platforms’). However, at present, this strategy could not be applied without substantial modifications for instruments with FSC signals collected with a lower solid angle (1–8°, Becton-Dickinson; so-called ‘low FS platforms’). These discrepancies between BD and BC instruments are also consistent with the notion that size-related information derived from plastic beads and that derived from biological particles are not comparable.

To conclude, these data indicate that standardization of PMP enumeration by FCM is feasible but is dependent on intrinsic characteristics of both the flow cytometer and the calibration strategy. Calibrated beads such as Megamix are useful standards that allow instrument qualification and follow-up. However, they do not constitute a universal biological standard for PMP enumeration. At present, this strategy has proved to be applicable for instruments that measure the FS parameter with ‘wide FS platforms’.

Moreover, additional questions are raised by this study. Is it adequate to focus only on MPs larger than about half a micrometer in size? If so, how representative is this ‘visible part of the MP iceberg’ for the clinically relevant biomarkers that we are seeking? Can we look forward to a newer generation or types of flow cytometer (or alternative technologies with similar immunologic capabilities) that would allow enumeration and characterization of particles of smaller sizes? Finally, a critical, but so far unaddressed, area is the impact of preanalytic conditions on MP enumeration. These questions constitute the basis for future SSC workshops.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Supplemental materials and methods.

Fig. S1. Design of the study.

Fig. S2. Resolution and background noise heterogeneity.

Fig. S3. Parameters affecting forward scatter (FS)/FSC resolution.

Fig. S4. Interquartile variability.

Fig. S5. Platelet-derived microparticle (PMP) location on the standardized size-related window of analysis between Becton Dickinson (BD) and Beckman Coulter (BC) instruments.

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Appendix

ISTH SSC Workshop: A. Leyte, M. van Schilfhaarde, A. Falanga, A. Vignoli, A. Enjeti, L. Lincz, A. Maraveyas, L. Madden, A. Bonnefoy, J. Rivard, K. Ben-Zion, J. Lopez, P. Davizon-Castillo, E. Maurer-Spurej, F. Mullier, J. Marvin, H. Kwaan, I. Weiss, I. Bosch, M. Woda, J. Pereira, P. Hidalgo, J. Antovic, F. Mobarrez, K. Ghosh, L. Macchi, G. Franck, M. Perez-Casal, M. G. Huisse, M. Macey, M. Harrison, A. Amirkhosravi, M. Davila, N. Li, N. Matijevic, W. Wang, P. Harrison, A. N. Boing, H. Büller, R. Nieuwland, W. Chandler,

W. Yeung, D. Stirling, S. Baird, S. Zahra, R. Krueger, R. Ross, J. Thom, S. Osanto, Y. Yuana, V. Martinez-Sales, V. Virtudes, V. Proulle, V. Latger-Cannard, M. De Carvalho, S. Susen, M. S. Carraway, and A. Charpentier.

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