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Letter to the Editor

Problems with single platforms for CD34+ quantification: How aware are Brazilian hematologists and transplant specialists about them?

Dear Editor,

I will start with a short anecdote. At the last XXVIII Congress of the Brazilian Society of Bone Marrow Transplantation, held in August 2024, I met an old friend, an experienced hematologist with decades of experience in the study of CD34 cells with their many facets (quantification, collection, cryopreservation, etc.). During our conversations, I mentioned that, on reviewing the entire scientific program of the event, I did not find a single roundtable, symposium, or lecture devoted to the discussion of CD34 cells. In effect, during the entire Congress, there was only one presentation of a single study that addressed the quantification of $CD34^+$ cells and its relationship with the success of leukapheresis. Considering the significance and centrality of CD34 cells in the execution of both autologous and allogeneic stem cell transplants, this observation was not just a curiosity – it was worrying, I told my friend. He agreed with me and said that, in fact, it would be advisable that at least a single symposium or roundtable should be devoted to the discussions on CD34 cells. "Yes," I replied, "especially because, although very precise for $CD34^+$ quantification, the modern single-platform templates that use microbeads for the enumeration of $CD34^+$ cells are not free from problems." Suddenly, he turned to me with a face that betrayed a certain surprise at my information and asked: "What problems?".[1] His response immediately made me think about how much Brazilian hematologists and transplant specialists are aware of the problems involving the quantification $CD34^+$ cells.

Flow cytometry single-platform assays to enumerate $CD34^+$ hematopoietic stem cells ($CD34^+$ HSC) are the best methodology we currently have for the accurate and reliable determination of how many $CD34^+$ HSC there are in each leukapheresis product intended for transplantation. In effect, over the past two decades, the single-platform technique became the 'gold standard' strategy for the quantification of $CD34^+$ HSC for autologous and allogeneic hematopoietic stem cell transplantations (HSTC), surpassing the traditional International Society of Hematotherapy and Graft Engineering (ISHAGE)-based dual platform. As widely recognized, the principal advantage of the single-platform technique is its reduced variability as it excludes the need of white blood cell counts on automated hematology analysers [1,2].

Notwithstanding, single-platform assays are not without problems. In 2001, Bruno Brando et al. [3] described for the first time an uncanny phenomenon occurring with the single-platform method. The authors perceived that some of the microbeads present in the flow cytometry tube just vanished when phosphate-buffered saline (PBS)-diluted leukapheresis samples were vortexed before acquisition in the flow cytometer. As a result, the phenomenon generated artifactually high $CD34^+$ HSC counts. They concluded that, when microbeads were resuspended in saline media, the vortex agitation almost invariably induced what they called the 'vanishing counting beads' (VCBs) phenomenon. Nevertheless, although worrying, the problem of VCBs is easy to solve: the addition of small amounts of protein (1% bovine serum albumin or 10% human pooled plasma) completely prevents the phenomenon. In order to avoid the VCBs, the authors then advised that sample suspensions containing microbeads for single-platform analysis be resuspended in media containing protein supplements [3]. This guarantees precise $CD34^+$ counting, preventing the realization of HSTC with a dose of $CD34^+$ HSC that is below ideal.

After briefly explaining these points to my friend, I started to wonder whether Brazilian laboratories involved in the quantification of $CD34^+$ HSC cells routinely supplement their samples with proteins and, furthermore, whether transplant physicians are aware of the possibility that the $CD34^+$ HSC report they receive from general laboratories may contain inaccuracies due to the occurrence of VCBs. So, preliminarily, my first intention with this letter is to share these concerns with other hematologists and transplant colleagues. But the

[1] If the readers are at this moment asking themselves the same question, then a careful reading of this "letter to the editor" is of utmost importance.

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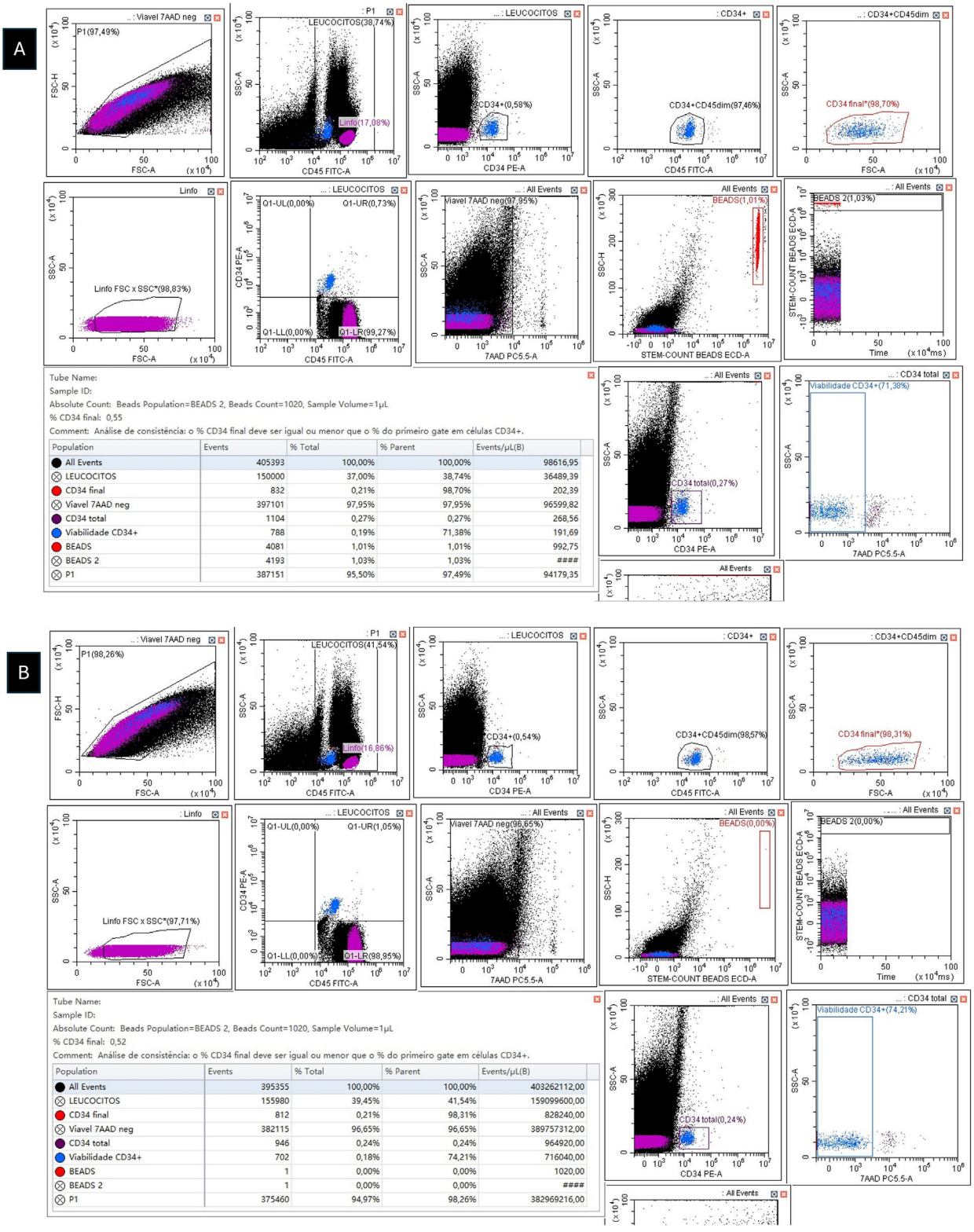

Figure 1 – A) Enumeration of viable CD34⁺ cells with the single-platform ISHAGE protocol (Stem-Kit) on a DxFLEX flow cytometer (3-laser and 13-color detection) (Beckman Coulter). Leukapheresis sample incubated with CD34 PE, CD45 FITC, and 7-AAD plus 2% human albumin (dilution factor = 7). viable CD34⁺ = 1.416 cells/mm³. B) Enumeration of viable CD34⁺ cells with the dual-platform ISHAGE protocol (Stem-Kit) on a DxFLEX flow cytometer (3-laser and 13-color detection) (Beckman Coulter). Leukapheresis sample incubated with CD34 PE, CD45 FITC, and 7-AAD plus 2% human albumin (white blood cell count = 143.700/mm³). CD34⁺ viable = 748 cells/mm³. The dual-platform assay showed that the single platform overestimated the viable CD34⁺ count, confirming the protein-resistant VCB phenomenon. Notice that a simple way to suspect the protein-resistant VCB phenomenon is to use what we call 'internal dual-platform' derived from the single-platform template. In practice, what we do is

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issue is not so simple because, even if Brazilian laboratories are already adding proteins to avoid this problem, VCBs are like the Hydra from Greek mythology: they have many heads... or at least two heads.

In fact, we recently described a new problem with single-platform assays, a phenomenon we called 'protein-resistant VCBs'. In this case, VCBs occur even in protein-supplemented samples [4,5]. Although still awaiting the exclusion of local confounding variables that could be impacting this phenomenon, it appears that protein-resistant VCBs are a very real, albeit rare, phenomenon, whose presence greatly increases the complexity of single-platform analyses. Therefore, it is important that Brazilian flow cytometry laboratories and transplant centers check whether they have encountered cases of classic (non-protein resistant) and of protein-resistant VCBs and share their experience with the scientific community. Until the phenomenon is better defined and, more importantly, until we figure out how to eliminate it, we recommend that, in the presence of protein-resistant VCBs, the dual-platform assay should be used for determining $CD34^+$ HSC counts (Figure 1) [4,5].

Back to my friend. When I explained to him about the existence of VCBs, he commented that he did not know how many physicians in transplant centers and flow cytometry laboratories involved in $CD34^+$ cell quantification in Brazil were aware of this problem concerning single-platform assays. I told him that I had no idea either. I hope, with this letter, that Brazilian hematologists and transplant specialists become aware about the need to substantially increase their attention when dealing with $CD34^+$ HSC quantification platforms that use bead-based methods.

Conflicts of interest

The author declares no conflicts of interest.

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to compare the value of $CD34^+$ cells/mm^3 of the single-platform method with the calculated value of $CD34^+$ cells/mm^3 of the 'dual platform' that is intrinsically present in single-platform studies. The formula is: Internal **dual-platform** = ($CD34^+$ events ÷ $CD45^+$ events) x WBC (mm^3)**. In this case, the internal dual-platform viable $CD34^+$ cell count was (832 ÷ 150.000) x 143.700 = 797 cells/mm^3. This result is quite consistent with that obtained in the dual-platform assay (for more details, see Matos DM.[5]).**

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