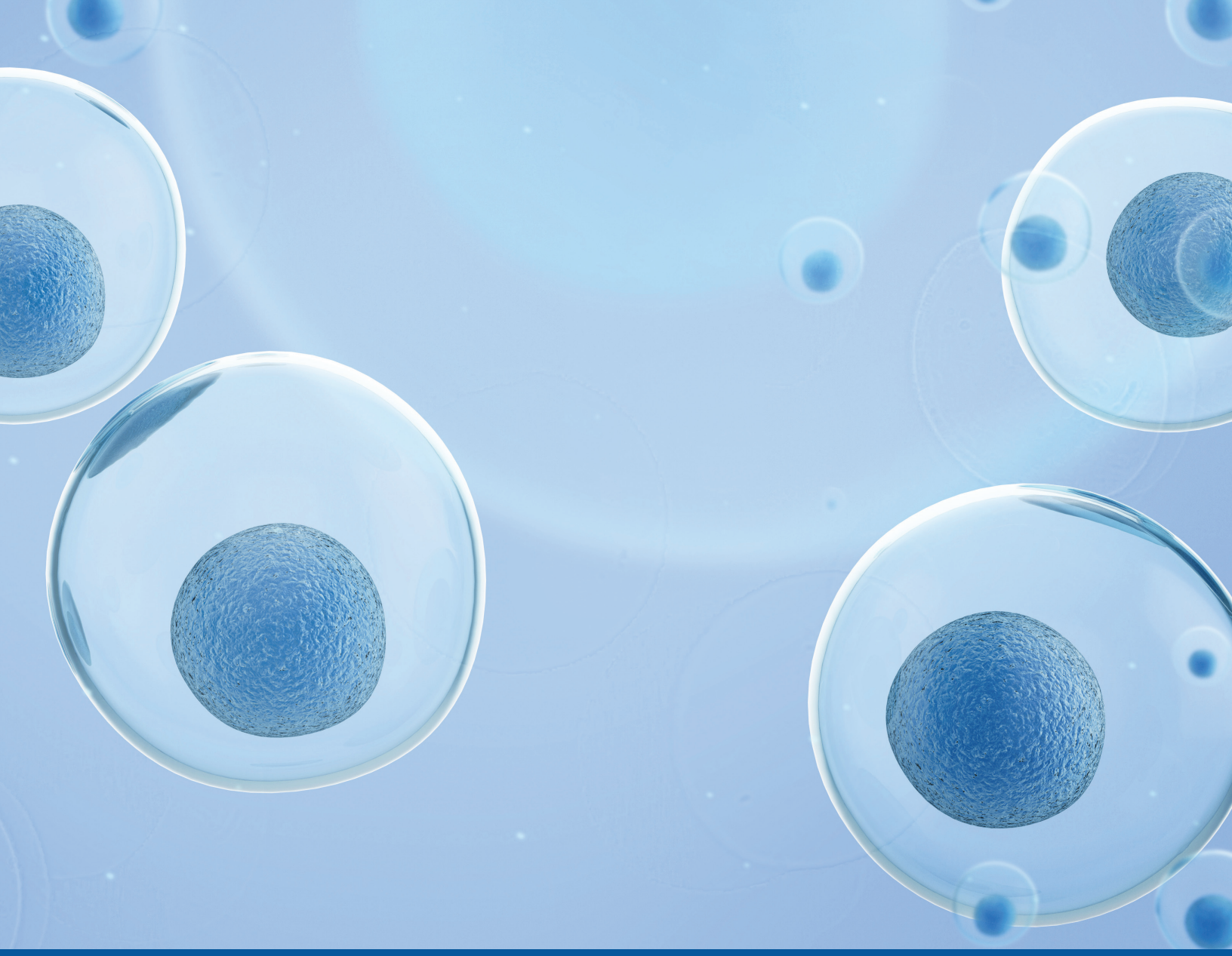

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Contents

Introduction

Cellular therapy involving the transplantation of stem cells collected from bone marrow (BM) or peripheral blood (PBSCs) are key to treating diseases of the hematopoietic system. To do so, there needs to be a careful and reliable measurement of the viability and quantity of stem cells available for transplantation and the outcomes post-transplantation. Measurement of the hematopoietic stem cells (HSC) via the marker CD34+ has become is a key indicator for their use, commonly done using the International Society of Hemotherapy and Graft Engineering (ISHAGE) protocol. However, methodology even when using the ISHAGE can varying, with single and dual platform approaches that can lead to reproducibility and standardization issue. Properly configured flow cytometry equipment combined with reliable labelling of CD34+ cells using reagents such as the BD™ Stem Cell Enumeration kit with the BD FACSLyric Flow Cytometer have greatly improved the evaluation of HSCs collected from bone marrow or peripheral blood collection.

The goal of this article collection is to educate readers as to the importance for clear and accurate measurements of CD34+ stem cells in the process of collecting and enumerating PBSCs for transplantation-based therapeutics. To open the collection, a review by Durán et al. (2019) serves to educate the reader on the importance of hematopoietic stem cells (HSCs) for clinical therapies to hematological diseases and the difficulties in maintaining and expanding HSCs *in vitro*. They discuss recent methods to reprogram somatic and pluripotent stem cells into clinically viable HSCs and provide insight into the current state of the field. In Rimac and Bojanić (2022), an overview on the role of flow cytometry in evaluating the purity and viability of HSC for cellular therapies is presented. A discussion of existing approaches and challenges is given, as well as good practices for validation and quality control of flow cytometry. In cellular therapies, the efficient collection of mononuclear cells after stem cell mobilization is crucial to the success of peripheral blood cell stem cell (PBSC) transplantation. Chung et al. (2022) compares the collection efficiency of two different cell separator systems by measuring the CD34+ using the BD Stem Cell Enumeration kit. They report that while the collection efficiency between the Spectra Optia and Amicus system was not significantly different, the Spectra Optia has increased platelet retention and therefore clinics should optimize apheresis device selection based on the patient profile. During collection of PBSCs, a high patient white blood cell count can negatively impact the ability to collect CD34+ cells. Boehlen et al. (2019) describes the development and optimization of a novel collection protocol for increasing the yield of mononuclear cells (MNCs) from peripheral blood, reducing the need for repeated apheresis sessions for patients. Lastly, we present a BD Bioscience white paper reporting the multi-site evaluation of the BD Stem Cell Enumeration kit combined with the BD FACSLyric and BD FACSCanto II flow cytometer. The assay kit was found to perform equally well based on CD34+ and CD45+ cell counts from each of the two flow cytometers.

The precise and reproducible enumeration of CD34+ stem cells is critical to the success of cellular therapies for the hematopoietic system. By introducing readers to the advantages of clinical flow cytometry combined with the BD Stem Cell Enumeration kit, we hope to empower users to investigate the use of this technology to address their specific diagnostic goals. For more information and resource, we encourage you to visit the BD Biosciences Stem Cell Enumeration Kit product page and explore the resources provided there.

By Jeremy Petravicz, PhD, Senior Editor,
Current Protocols

References

Barcia Durán, J.G., Lis, R. and Rafii, S. Haematopoietic stem cell reprogramming and the hope for a universal blood product. *FEBS Lett.* 2019; 593: 3253-3265. https://doi.org/10.1002/1873-3468.13681

Rimac, V, Bojanić, I. Role of flow cytometry in evaluation of the cellular therapy products used in haematopoietic stem cell transplantation. *Int J Lab Hematol.* 2022; 1- 8. https://doi.org/10.1111/ijlh.13849

Chung, Y, Kong, JH, Hu, Y, et al. Comparison of spectra optia and amicus cell separators for autologous peripheral blood stem cell collection. *J Clin Apher.* 2021; 36: 28– 33. https://doi.org/10.1002/jca.21835

Boehlen, R, Fernandez, P, Bargetzi, M, Sigle, J-P. A new protocol for improving efficiency of autologous peripheral blood stem cell collection in patients with high white blood cell counts. *J Clin Apher.* 2020; 35: 217– 223. https://doi.org/10.1002/jca.21782

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REVIEW ARTICLE

Haematopoietic stem cell reprogramming and the hope for a universal blood product

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Haematopoietic stem cells (HSCs) are the only adult stem cells with a demonstrated clinical use, even though a tractable method to maintain and expand human HSCs *in vitro* has not yet been found. Owing to the introduction of transplantation strategies for the treatment of haematological malignancies and, more recently, the promise of gene therapy, the need to improve the generation, manipulation and scalability of autologous or allogeneic HSCs has risen steeply over the past decade. In that context, reprogramming strategies based on the expression of exogenous transcription factors have emerged as a means to produce functional HSCs *in vitro*. These approaches largely stem from the assumption that key master transcription factors direct the expression of downstream target genes thereby triggering haematopoiesis. Both somatic and pluripotent cells have been used to this end, yielding variable results in terms of haematopoietic phenotype and functionality. Here, we present an overview of the haematopoietic reprogramming methods reported to date, provide the appropriate historical context and offer some critical insight about where the field stands at present.

Keywords: haematopoietic stem cells; pluripotent cell reprogramming; somatic cell reprogramming

The notion that exogenous gene expression could alter a cell's transcriptional programme to the extent of bestowing the phenotypic profile and even the functionality of an altogether different cellular identity was first shown in 1987 in *Drosophila melanogaster*. Conditional overexpression of *antennapedia* – a homoeotic gene that specifies the second thoracic segment of the fly – gave rise to legs in the place of antennae of treated specimens [1]. Two years later, Weintraub *et al.* added to this finding with the development of the first mammalian conversion experiments in the form of a myogenic model. Exogenous expression of a single gene, *MYOD1*, delivered by retrovirus or DNA transfection into primary or transformed chicken, rat or human fibroblasts resulted in myotube formation, cell fusion and expression of myosin heavy chain and desmin, namely a muscle phenotype [2]. This and further studies earned MYOD1, now known as a transcription factor (TF), the moniker of 'master regulator' of myogenic fate maintenance and acquisition.

Landmark work by the group of Thomas Graf brought the concept of TF reprogramming to the field

Abbreviations
AGM, aorta–gonad–mesonephros; CFU, colony-forming unit; EC, endothelial cell; EB, embryoid body; eHB, expandable haemangioblasts; EHT, endothelial-to-haematopoietic transition; EMP, erythro-myeloid progenitor; hESCs, human embryonic stem cells; HSC, haematopoietic stem cell; HSPC, haematopoietic stem and progenitor cell; HUVEC, human umbilical vein EC; iPSC, induced pluripotent stem cells; MAF, mouse adult fibroblast; MEF, mouse embryonic fibroblast; mESC, mouse embryonic stem cell; MOI, multiplicity of infection; NHP, nonhuman primate; NOD, nonobese diabetic; NSG, NOD-SCID;*Il2rg*$^{-/-}$; PCS, pluripotent cell sources; rtTA, reverse tetracycline trans-activator; SCNT, somatic cell nuclear transfer; TF, transcription factor.

of haematology. Their efforts to untangle the TF networks that determine haematopoietic lineage decisions often consisted of exogenous expression of a gene of interest as a means to drive a haematopoietic progenitor population towards a specific fate. Following this methodology, Kulessa *et al.* [3] studied the role of *Gata1* in megakaryocytic lineage determination and cemented the role of Pu.1 (henceforth referred to as Spi1) in the earliest stages of myelopoiesis [4]. In 2002, Xie *et al.* isolated $CD19^+$ B cells from the spleen and bone marrow of mice, respectively, and transduced them with a retrovirus encoding *Cebpα*. Exogenous *Cebpα* expression was found to inhibit *Pax5* and to synergize with *Spi1*, resulting in the upregulation of macrophage-specific genes, such as *CD11b*. Primary B cells were thus reprogrammed into phagocytotic macrophages, a transdifferentiation event from a lymphoid to a myeloid fate. Remarkably, the study proposed that the conversion had taken place in two discrete stages: $CD19^+CD11b^-$ cells became $CD19^+CD11b^+$ before losing B-cell identity in favour of a $CD19^-CD11b^+$ phenotype [5].

In the light of the multiple investigations showing the plasticity of haematopoietic cells [6], and the ever-increasing demand for a scalable blood product, the manipulation of TF networks became a potential source of HSCs *in vitro*. In 2014, Riddell *et al.* reported the reprogramming of mouse pre-/pro-B and common myeloid progenitor cells, respectively, using a cocktail of lentiviruses encoding eight TFs: *Runx1t1*, *Hlf*, *Lmo2*, *Prdm5*, *Pbx1*, *Zfp37*, *Mycn* and *Meis1* (Fig. 1, purple labels) [7]. Two days after viral transduction, the cells were transplanted into adult congenic mice, allowing reprogramming to take place *in vivo*. Serially transplanted recipient mice showed multilineage donor chimerism in the peripheral blood for up to 18 weeks. Notably, the authors also observed unique V(D)J junction sequences in reprogrammed B and T cells, granulocytes and macrophage/monocytes that were common to the four cohorts of pre-/pro-B cells subjected to reprogramming. Reasoning that Ig heavy-chain rearrangements from the original pre-/pro-B cells could be used as an internal barcode to track the clonal origin of reprogrammed HSCs, they showed that a single reprogrammed cell could gain multilineage differentiation potential. As such, using a 'backward' reprogramming strategy, Riddell *et al.* showed that transient expression of eight transcription factors in lineage-restricted B-cell progenitors conferred the functional hallmarks of HSCs, including clonal multilineage reconstituting potential, the capacity to reconstitute bone marrow stem and progenitor cell compartments, and long-term self-renewal. Further, they found that partially reprogrammed HSCs had been unable to properly upregulate *Mpl* or *cKit* receptors, suggesting that a failure to integrate signals in response to angiocrine factors produced by the perivascular and vascular niches may explain incomplete reprogramming events.

Other than the work by Riddell *et al.*, every major approach to HSC reprogramming has made use of nonhaematopoietic cells at the outset. The objective of these 'forward' strategies has been the same: to generate long-term engrafting HSCs that are able to home in and populate the bone marrow of serially transplanted recipients. Attempts that rely on either somatic or pluripotent cells have been put forward, each with compelling advantages and serious drawbacks. To date, cellular reprogramming has yet to yield a bona fide HSC in a remotely scalable manner, calling into question the feasibility of the task, the tests used for its validation or both. The following is a summary of the most prevalent approaches, accompanied by a discussion of their respective achievements.

Direct conversion of somatic cells to haematopoietic stem and progenitor cells

Most attempts to reprogramme somatic cells into HSCs capitalize on the ubiquity and ease of culture of fibroblasts, which constitute a loosely defined cell type under the umbrella of connective tissue. Fibroblasts from the embryonic mouse [8–10], human neonatal foreskin [11,12] and human adult dermis [11,13] are most commonly used in these conversion efforts, though two or more types of fibroblasts are rarely compared in the same haemogenic approach. The numerous sources whence fibroblasts can be obtained make them particularly sensitive to batch-to-batch effect, too. Still, some of the following efforts yield short-term engraftable, multipotent reprogrammed haematopoietic cells. Several even offer some mechanistic insight into the process of transdifferentiation that converts connective into haematopoietic tissue. In the end, fibroblasts would serve as very cost-effective, noninvasive cell sources for reprogramming-based haematopoietic cell therapies, even if functional HSCs have proven tough to produce.

The first attempt to generate HSCs using TF overexpression in fibroblasts was published in 2010 by Szabo *et al.* [11] (Fig. 1, grey labels). *OCT4* overexpression alone, aided by a combination of cytokines in the media that consisted of SCF, G-CSF, FLT3, IL3, IL6 and BMP4, was used to reprogramme human dermal fibroblasts into $CD45^+$ cells *in vitro*. Functional tests included colony-forming unit (CFU) assays that gave rise to granulocytic, erythrocytic, monocytic and megakaryocytic

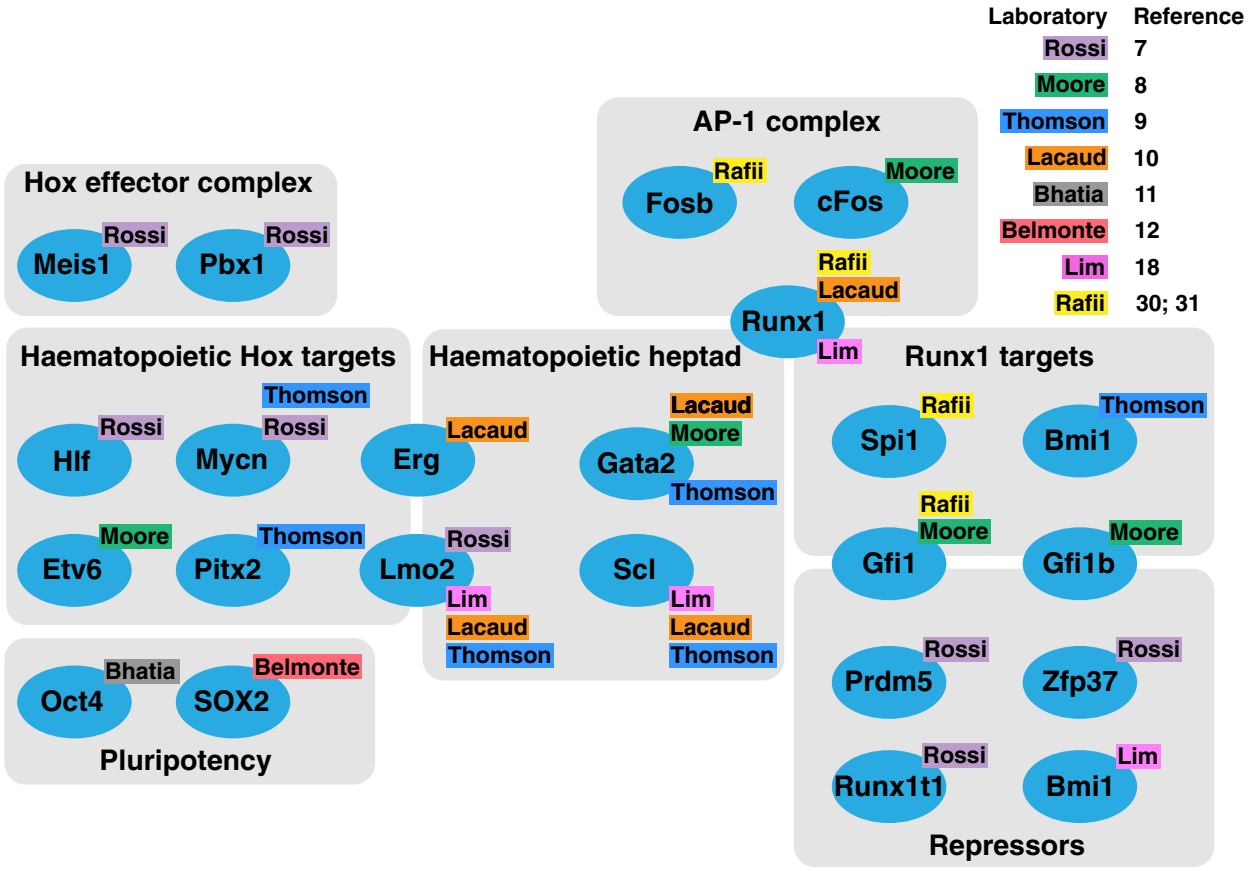

Fig. 1. Many somatic cell reprogramming approaches towards haematopoietic stem cell fates have drawn from transcription factor (TF, blue ovals) networks known to interact (adapted and updated) [115]. The TFs employed in eight reprogramming approaches have been classified into categories based on the literature as well as laboratory head. The haematopoietic heptad refers to the seven TFs described to physically interact in Wilson *et al.* [14].

colonies *in vitro*. The *in vivo* engraftment data, however, showed a $CD45^{+}$ shift two orders of magnitude larger for the cord blood-derived positive controls. In addition, *OCT4* did not grant fibroblasts any lymphopoietic ability *in vivo*. Despite a follow-up report by the same group suggesting that exogenous *OCT4* expression would be necessary for most, if not all, somatic cell reprogramming strategies [13], haemogenic studies going forward have largely focused on optimizing a cocktail of HSC-specific TFs to drive conversion.

Transcriptomics data obtained from bulk populations or single cells have greatly expanded our knowledge and provided important insights into the gene regulatory networks involved in HSC generation. These studies, for instance, have discerned a TF heptad – consisting of SCL, LYL1, LMO2, GATA2, RUNX1, ERG and FLI1 – that is essential for haematopoietic fate determination [14]. The more recent HSC reprogramming efforts have taken advantage of these findings to introduce the expression of pro-haematopoietic TFs and so endow somatic cells with haematopoietic transcriptional programmes. A notable exception is a 2016 study that used *SOX2* and *miR125b* overexpression in human foreskin fibroblasts [12] (Fig. 1, red labels). Following a similar strategy to that of Riddell *et al.* [7], the investigators placed the transduced cells within the bone marrow niche of NSG mice to undergo reprogramming *in vivo*; still, this effort yielded cells of the myeloid lineage only.

A key attempt at reprogramming fibroblasts into HSCs was reported in 2013 by Pereira *et al.* [8] Overexpression of *Gata2*, *Gfi1b*, *cFos* and *Etv6* led to induction of a haemogenic programme in mouse embryonic fibroblasts (MEFs; Fig. 1, green labels). Said programme was based on positive signal from cells isolated from a *CD34-rtTA*;*TRE-H2B-GFP* reporter mouse. Inducible *H2B-GFP* transgenic mice are useful in pulse-chase studies of long-term repopulating haematopoietic stem and progenitor cells (HSPCs), as the GFP signal becomes diluted with each cell division and only the most immature cells remain brightly

labelled. The investigators crossed this mouse strain with one carrying a reverse tetracycline trans-activator (*rtTA*) transgene under the control of the human *CD34* promoter. This second strain was created precisely to induce expression of heterologous genes, such as *H2B-GFP*, in HSPCs. While Gata2, Gfi1b and cFos were sufficient to activate the reporter, Etv6 markedly increased the efficiency of haematopoietic reprogramming. Transduced fibroblasts segued into circular, endothelial-like structures that produced phenotypically marked haematopoietic cells harbouring HSC gene expression. The resulting reprogrammed cells displayed colony-forming potential after placental reaggregation culture. However, the human *CD34* promoter also marks mouse endothelial cells (ECs) [15]. For this reason, the transcriptional programme that *Gata2*, *Gfi1b*, *cFos* and *Etv6* are said to induce in these fibroblasts is likely that of mixed haematopoietic and endothelial fates.

In vivo functional tests were reported in the human system in 2018, when the same group used three of the original four TFs – *GATA2*, *GFI1B* and *FOS* – to attain an engraftable population of HSPCs [16]. This follow-up report also provided mechanistic insight into the reprogramming of fibroblasts into haematopoietic cells *in vitro*. GATA2 is shown to sit atop the hierarchy of TFs, especially during the early stages of reprogramming. Integrating RNA-seq and ChIP-seq analyses, the investigators observed that GATA2 and GFI1B cooperate at the chromatin level, where the AP-1 motif appeared as the most enriched target. Yet another improvement to this method is published in this issue of FEBS Letters [17]. This time, addition of GFI1 to the TF cocktail and coculture of the transduced fibroblasts with a stromal cell line of mouse origin increased the yield of reprogramming even further. This approach has now accomplished perhaps the most faithful conversion of fibroblasts into haematopoietic cells with multilineage engraftment potential to date.

Shortly after Pereira *et al.* [9], another group used *Gata2*, *Lmo2*, *Mycn*, *Pitx2*, *Sox17* and *Scl* to reprogramme MEFs into expandable haemangioblasts (eHBs), that is tripotent cells that give rise to ECs, haematopoietic cells with multilineage potential, and smooth muscle cells (Fig. 1, blue labels). In this study, each TF was cloned under a doxycycline-inducible promoter, which gave the investigators the ability to control their exogenous expression at will. Directed differentiation of eHBs under doxycycline-free conditions yielded tubulogenic ECs and smooth muscles cells. With stimulation by defined cytokines, such as Epo and Tpo, eHBs generated phenotypic haematopoietic cells, such as $CD71^{+}Ter119^{+}$ erythrocytes and $CD41^{+}CD42d^{+}$ megakaryocytes, respectively. Haematopoietic function was assayed by means of CFU assays, which showed, albeit quite inefficiently, burst-forming unit-erythrocytic (BFU-E), CFU-granulocytic/monocytic (CFU-GM) and CFU-granulocytic/erythrocytic/macrophagic/megakaryocytic (CFU-GEMM) ability among the reprogrammed cells.

Using a similar TF combination – *Erg*, *Gata2*, *Lmo2*, *Runx1c* and *Scl* – Batta *et al.* [10] reprogrammed MEFs and mouse adult fibroblasts (MAFs) into haematopoietic progenitor cells (Fig. 1, orange labels). This conversion strategy also entailed the acquisition of endothelial traits before haematopoietic commitment, at least as measured by gene expression profiling. MEFs and MAFs from $p53^{-/-}$ mice yielded more efficient reprogramming than the wild-type counterparts, but the engraftment potential of the resulting cells remained negligible. More recently, a group used *Scl*, *Lmo2*, *Runx1* and *Bmi1* overexpression in MEFs to generate multipotent haematopoietic cells with some B lymphoid potential (Fig. 1, pink labels) [18]. By means of small-molecule inhibition, this study also showed that signalling through Bmp4 and Mapk/Erk pathways is required for their reprogramming platform, shedding some light on the mechanism of fibroblast-to-haematopoietic conversion.

The various approaches to reprogramme fibroblasts into multilineage, long-term engraftable HSCs using exogenous TF expression have several commonalities that go beyond the prevalence of *Gata2* and *Lmo2* (Fig. 1). Some have overexpressed *OCT4* or *SOX2* to disrupt the entire transcriptional programme of human fibroblasts. Others have made use of $p53^{-/-}$ mice towards a similar end: genetic plasticity. These approaches, more akin to directed differentiation than reprogramming, are particularly susceptible to genetic instability and, potentially, tumour formation. Some fibroblast-based technologies involve a requisite endothelial intermediate state prior to haematopoietic commitment, to an extent that they seem to succeed at reprogramming fibroblasts into ECs [8,9]. Some even incorporate canonical endothelial TFs like Erg [19] and Sox17 [20]. But the haemogenic capacity of the resulting cells is still lacking, as long-term engraftment or the semblance of a functional lymphoid compartment has yet to be reported from the direct conversion of mouse or human fibroblasts into haematopoietic cells. In addition, the fibroblastic provenance of reprogrammed haematopoietic cells can be ascertained by genomic methylation patterns alone [21,22].

With all of these drawbacks in mind, and being aware that epigenetic memory is an issue, ECs themselves emerged as a potential starting point for HSC reprogramming. A separate review published in this issue of FEBS Letters is concerned with the provenance of

haematopoietic cells during development, especially regarding the spatio-temporal expression patterns of key transcription factors [23]. Here, it suffices to say that the earliest measurable HSCs emerge from a specialized vascular tissue, haemogenic endothelium, located in the dorsal aspect of the aorta–gonad–mesonephros (AGM) region of the fish [24], amphibian [25], avian [26] and mammalian [27,28] mesoderm by a process termed endothelial-to-haematopoietic transition (EHT) [29]. Efforts to generate HSCs have thus sought to capitalize on the endothelial ontogeny of haematopoietic cells. In Sandler *et al.* [30], human umbilical vein ECs (HUVECs) as well as adult dermal ECs were transduced with *FOSB*, *GFI1*, *RUNX1* and *SPI1* and placed in coculture with a model of the vascular niche. *FGRS*-transduced ECs were thus reprogrammed into multipotent haematopoietic progenitors (Fig. 1, yellow labels). These reprogrammed cells displayed a VE-Cadherin^{-}CD45^{+} immunophenotype and engrafted long-term in the bone marrow of adult, immunocompromised mice after primary and secondary transplantation. The age of recipient animals affected this functional readout, as transplantation into two-week-old NSG mice was shown to enhance B lymphoid differentiation. Still, while the transcriptome of the reprogrammed cells was comparable to that of CD34^{+} cord blood cells, the former were unable to differentiate into functional T cells *in vivo*.

Reasoning that SPI1 interfered with lymphopoiesis, Lis *et al.* [31] showed that transient *FGRS* overexpression in primary, adult murine lung, liver and brain ECs yielded haematopoietic cells that displayed long-term, multilineage repopulation potential, including T-cell subsets with adaptive immune function upon serial transplantation into congenic animals (Fig. 1, yellow labels). Further, inhibition of TGF-β and CXCR7 or activation of BMP and CXCR4 signalling was shown to favour HSC reprogramming. Although this updated strategy yielded cells that harbour all hallmarks of HSCs, the reprogramming efficiency was still very low (~ 0.6%). Recent work on the heterogeneous origin of the cells that comprise adult blood vessel endothelium may offer some clues to explain such a low reprogramming rate. In Plein *et al.*, the vasculature of the embryo is shown to develop from two distinct lineages: in addition to mesoderm-derived ECs, the authors observed that a complementary source of endothelium is recruited into the pre-existing vasculature through transdifferentiation of erythro-myeloid progenitors (EMPs) that emerge in the yolk sac [32]. This distinct lineage persists into adulthood, raising the possibility that vascular endothelium acts as a functional mosaic, where a distinct lineage may be more amenable to haematopoietic reprogramming than others.

Whether an elite group of ECs, fibroblasts or any somatic cell used in reprogramming is better suited for haematopoietic fate acquisition than its phenotypic equivalents remains to be shown. Either way, the goal and basis for direct conversion techniques will remain the development of personalized, nonimmunogenic haematopoietic cell transplantation therapies where somatic cells are isolated from patients themselves. Methods that employ fibroblasts and ECs offer contrasting advantages in this context. While connective tissue cells are easily derived and maintained *in vitro*, ECs need to be purified and may be outgrown by other cells in culture, fibroblasts among them. The procurement of human ECs alone, unlike that of fibroblasts, may pose an insurmountable challenge for technologies based on primary endothelium to reach the clinic. But haematopoietic and ECs have a shared ontogeny, and this vestigial identity is reflected in the faithful yet scarce reprogramming of ECs into HSCs, at least in the mouse. Reprogramming of fibroblasts, on the other hand, has yet to produce a serially transplantable haematopoietic cell. The clinical viability of personalized medicine approaches to HSC transplantation is also put into question by the sheer cost of carrying out cell isolation and reprogramming on a case-by-case basis. Pluripotent cell reprogramming has emerged almost in response to the drawbacks of more intuitive approaches like the ones discussed above. Genetic manipulation and clonal expansion of pluripotent cells is not only possible but almost routine practice. As a result, pluripotent stem cell technologies, in contrast to direct conversion methods that rely on somatic cells, are tied to the promise of a universal, off-the-shelf blood product that would significantly reduce the cost and maximize the chance of translation of haematopoietic reprogramming to a clinical setting.

Pluripotent cell reprogramming

Not unlike haematopoietic ontogeny, the process of maturation of any somatic cell is often depicted as a downward trajectory along one of many branches that stem from levels of more potency on top. The fertilized oocyte is said to be totipotent, as it has the potency to give rise to every other cell type in the body of the organism, including those of extra-embryonic tissues like the yolk sac and the placenta. Once the blastocyst is formed, the inner cell mass – from which human embryonic stem cell (hESC) lines are derived – is pluripotent; that is, it can no longer generate extra-embryonic tissues. Instead, these cells have the potency to differentiate into all three germ layers of the embryo proper. As cells continue to differentiate *in utero* and

through birth, they lose potency. Somatic cells such as hepatocytes or cytotoxic T cells are said to become terminally differentiated; adult stem cells, including haematopoietic stem cells (HSCs), are able to retain their multipotent quality. Somatic cell fate was considered irreversible until seminal work by Sir John Gurdon in *Xenopus leavis*. In 1962, he reported that the nucleus from a terminally differentiated intestinal cell of a tadpole, when introduced into an enucleated oocyte of the same species, would yield normal adult frogs [33]. Sir Gurdon's method and results would not be replicated in a mammalian species for another 35 years [34], but his insight had immediate effect: the process of differentiation was no longer assumed to involve irreversible nuclear changes.

As a result, the seeming plasticity of the nucleus allowed researchers to delve into more mechanistic questions long before the cloning of a Finn Dorset ewe [35]. As soon as 1969, cell fusion was proposed as an alternative method to nuclear transfer. The earliest studies in cell fusion suggested the existence of *trans*-acting repressors, or 'some special feature,' that possessed tumour-suppressing capacity [36]. Later, hybridization of two mammalian somatic cells showed that the nuclei of one could somehow activate genes not normally expressed in the nuclei of the other [37]. Still, it was through somatic cell nuclear transfer (SCNT) that Hochedlinger *et al*. [38] showed unequivocally that 'terminally differentiated' T and B cells could gain totipotency in 2002.

Transdifferentiation efforts between different somatic fates precede this accomplishment by a long margin: *MYOD1* was known to induce a myogenic programme since the 1980s [2,39]. Still, cell reprogramming based on transcription factor (TF) overexpression would only reach its apex when Shinya Yamanaka was able to approximate with four genes what was accomplished by SCNT four years prior in Hochedlinger *et al*. [40] *Oct4*, *Klf4*, *Sox2* and *cMyc* overexpression converted mouse fibroblasts into 'induced pluripotent stem cells' (iPSCs) that contributed to the germline and gave rise to embryos through tetraploid complementation. One year later, the same group generated human iPSCs [41]. Until then, pluripotent cell-derived reprogramming strategies had relied more ethically fraught approaches, using either primary or SCNT-derived hESCs [42]. An *in vitro* system such as Yamanaka's that would generate pluripotent cells from somatic ones likely propelled more investigators to pursue cellular reprogramming as a viable avenue of research. iPSCs could be generated in a patient-specific manner from any tissue of the body and then converted to the tissue of choice, with or without gene correction [43]. By 2008, several groups had adopted the technique, generating iPSCs that were considered epigenetically [44] and developmentally [45] indistinguishable from ESCs of murine [46], nonhuman primate (NHP) [47] and human origin [48,49]. Concern that *cMyc* would predispose iPSC-derived cells to malignant transformation was partially quashed when the TF was found to be dispensable to attain pluripotency [50]. To this day, pluripotent stem cell approaches to regenerative medicine have been proposed to address afflictions from cystic fibrosis [51–53] to diabetes [54–56] to third-degree burns [57–59].

Haematopoietic conversion strategies from pluripotent cell sources (PCS) making use of both iPSCs and ESCs have been reported. Generally, instead of TF overexpression, these approaches consist of directed differentiation by exposure to signalling molecules like BMP4 and Activin in order to guide pluripotent cells first towards a mesodermal, then a haemogenic endothelial and finally a haematopoietic fate [60,61]. The earliest attempts relied on mouse embryonic stem cells (mESCs) cultured as three-dimensional aggregates called embryoid bodies (EBs). In 1993, Keller *et al*. [62] reported that mouse EBs could be differentiated into primitive haematopoietic cells with some *in vitro* erythro-myeloid progenitor (EMP) potential after Epo and Scl treatment. Later, hybrid strategies that combined TF overexpression and directed differentiation of EBs from mESCs were employed to show that *Hoxb4* could be used to obtain haematopoietic cells with *in vivo* multilineage potential [63], though these results did not extend to the human system [64].

Since then, it was reported that hESCs undergo an endothelial intermediate state prior to haematopoietic commitment, that is *in vitro* reprogramming from PCS somewhat mirrors *in utero* EHT [65]. The first cells to acquire haematopoietic identity were predominantly erythro-megakaryocytic, while cells at a later time point displayed mostly myeloid potential. More recently, PCS studies have reported findings *via* TF overexpression in both iPSCs and ESCs (Fig. 2). One group employed different TF combinations to induce two distinct haematopoietic fates, respectively, parting from both human iPSCs and ESCs. *GATA2* and *SCL* overexpression generated EMPs (Fig. 2, blue labels), while *ETV2* and *GATA2* produced cells with myeloid potential (Fig. 2, orange labels) [66]. Similarly, work on both human iPSCs and ESCs revealed that inhibition of the WNT/β-catenin pathway was required for primitive haematopoiesis, while definitive HSC emergence occurred only upon WNT/β-catenin activation [67]. Manipulation of the WNT pathway was later used to produce phenotypic ECs with some *in vitro*

haemogenic ability from hESCs [68]. While none of these approaches generated haematopoietic cells with *in vivo* function, they contributed to our understanding of EHT as it takes place in the embryo.

A more successful attempt to obtain HSPCs with *in vivo* function from PCS used *HOXB9*, *ERG*, *RORA*, *SOX4* and *MYB* overexpression on EMPs that were dedifferentiated to a pluripotent state and then converted to haematopoietic cells (Fig. 2, pink labels). Still, the resulting reprogrammed cells only displayed short-term engraftment and under 1% chimerism [69]. Perhaps the most promising effort to generate HSCs from PCS was later reported by the same group in Sugimura *et al.* [70]. In that investigation, doxycycline-inducible overexpression of seven transcription factors – *ERG*, *HOXA5*, *HOXA9*, *HOXA10*, *LCOR*, *RUNX1* and *SPI1* – was reported to give rise to HSPCs parting from both human iPSCs and ESCs, respectively (Fig. 2, green labels). The cells underwent reprogramming *in vivo*: after lentiviral transduction, they were delivered intrafemorally into recipient NSG mice, which were treated with doxycycline in the diet. Human chimerism was assayed 12 weeks later, showing multilineage engraftment of the reprogrammed cells. In addition, the TCR repertoire of phenotypic T cells isolated from engrafted recipients showed high combinatorial diversity. This method thus yielded not only long-term chimerism, but also a T-cell pool capable of undergoing TCR recombination at rates that were comparable to those of native human HSPCs.

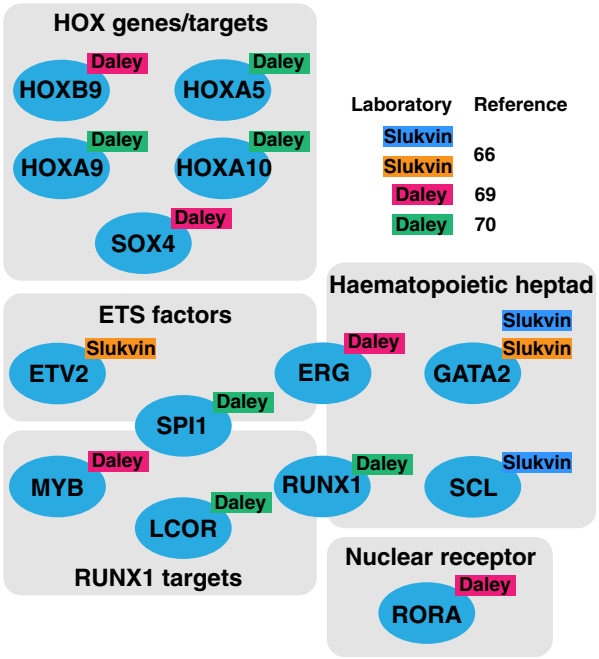

Fig. 2. Haematopoietic reprogramming using pluripotent cell sources has relied on transcription factor (TF, blue ovals) networks that are active in early development (adapted and updated) [115]. The TFs employed in three pluripotent reprogramming approaches have been classified into categories based on the literature as well as laboratory head. The haematopoietic heptad refers to the seven TFs described to physically interact in Wilson *et al.* [14].

Pluripotent cell sources alone may lack the appropriate environmental cues to support haematopoietic commitment, which would explain why Sugimura *et al.* rely on the bone marrow niche of the mouse to reprogramme human pluripotent cells. In Riddell *et al.* [7], reprogrammed somatic cells were also transplanted within days of viral transduction for *in vivo* reprogramming. Most of the haematopoietic conversion platforms reviewed here are designed to take place *in vitro* instead, which would offer an obvious advantage in the translation of any technology to the clinical setting. Some make use of coculture systems like a model of the vascular niche to provide support for the nascent haematopoietic cells; other direct reprogramming strategies involve passage through an endothelial intermediate state prior to haematopoietic commitment, which may serve the same purpose [8,68,71]. In Sugimura *et al.* [70], the investigators purposefully induce a haemogenic endothelial state, and a gene that is constitutively expressed by endothelium, *ERG*, is part of their TF cocktail. Given the endothelial ontogeny of haematopoietic cells, the ubiquity of fibroblasts and mounting evidence showing the genetic and epigenetic instability of iPSC technologies [21,72], it would seem that somatic, rather than pluripotent, cells are the ideal starting material for the generation of HSCs *in vitro*. After all, the translation of iPSCs to the clinic remains, at most, in the form of tractable *in vitro* models for drug testing [73] and modelling primitive haematopoiesis [65,67]. But iPSCs have ignited the promise of universal cell therapies at zero ethical cost and so, despite drawbacks, they remain the most likely cell source for an off-the-shelf blood product. All in all, it is only clear that no one approach to HSC reprogramming is more cost-effective, scientifically sound or prudent than the other, at least not at the moment.

Conclusions and perspectives

Developmental biologists have made great progress towards disentangling the intricacies of haematopoiesis, a process that is now understood less as a single phenomenon and more as a series of events. Three waves of haematopoiesis are thought give rise to blood cells with starkly different characteristics and

functions, from circulating nucleated erythrocytes to erythro-myeloid progenitors to HSCs [74,75], though a fourth, late wave of *de novo* fetal haematopoiesis has been reported in perinatal chicken and mouse models [76]. Haematopoietic cells can emerge from a common mesodermal progenitor or VE-Cadherin^{+} ECs. They appear in the yolk sac and the dorsal aorta, but with the onset of circulation, they are able to migrate to and interact with multiple niches [77,78].

Yet, it is uncontroversial that much more complexity is likely to belie developmental haematopoiesis, especially when it comes to the self-renewal capacity of HSCs. To date, the earliest HSCs that have been found to yield long-term, serial, multilineage engraftment in irradiated adult mice arise from the AGM on or around embryonic day 10.5 *via* EHT. Whether HSCs can be expanded *ex vivo* remains a question. A recent paper reported the 899-fold *in vitro* expansion of mouse HSCs over the course of one month using cytokine supplementation and polyvinyl alcohol in the medium [79]. Human HSCs, however, were only shown to expand reliably for 7 days following the same method.

The challenges to manipulate and reprogramme HSCs are many. Researchers have drawn from the field of developmental haematology to induce a haemogenic fate in multiple cell types. Our group and others have found optimal TF combinations for somatic and pluripotent cell reprogramming, devised systems that provide supportive signals, and measured the capacity of the converted cells to both engraft in the long term and give rise to myeloid and lymphoid progeny in mice. Most researchers have pruned a list of TFs after various iterations, arriving at multiple different combinations [8–10], including our laboratory [30]. Others have resorted to *in vivo* reprogramming as a means to provide a pro-haematopoietic microenvironment [7,69,70]. Some have drawn from an arsenal of pluripotency-based strategies [11,12,68,70]. But despite the approach, the challenge – to produce fully functional haematopoietic cells efficiently and reliably – remains unattainable in the human system.

Reasonable hypotheses that account for the failure to produce human HSCs *in vitro* abound. Perhaps another TF is necessary; the lentiviruses are integrating at unfavourable locations in the genome; the multiplicity of infection (MOI) is suboptimal; the stoichiometry of the factors is incorrect; the starting population is heterogeneous; a small molecule will resolve the issue; and so on. In the field of iPSCs, polycistronic delivery of the TFs [80], tuning the MOI [81], or using transgenic mice [82–84] still only converts < 1% of the starting population to pluripotency. In a study by Biddy *et al.* [80], the authors carefully tracked the reprogramming trajectory of single fibroblasts towards a multipotent endodermal fate. Predictably, they identified a gene, *Mettl7a1*, that was summarily overexpressed in the original population to double the output of conversion. These forms of experimental design continue to fail to improve reprogramming efficiency in any significant way.

Some corners of HSC biology remain unexplored. For instance, low oxygen tension has been reported to aid HSC maintenance through HIF1α-mediated reduction of ROS [85,86]. Expansion of primary HSPCs is also known to improve with the supplementation of small molecules like SR1 [87] and UM171 [88], both of which interact with the aryl hydrocarbon receptor (AhR) pathway. HIF1α and AhR compete for HIF1β [85,89,90], so inhibition of AhR and the consequent increase of HIF1α signalling would promote HSC maintenance, a mechanism that may extend to the generation of haematopoietic cells *in vitro*. Energy metabolism, too, may offer a way to improve the haemogenic capacity of current reprogramming methods. Oxidative phosphorylation is preferentially used by progenitors to produce ATP, while HSCs favour glycolysis [85,91,92]. Some have reported that ECs themselves are mostly glycolytic, too [93–95]. Whether differential energy metabolism is a hallmark of EHT remains to be shown, but recent advances on small-molecule regulation by HSCs certainly allow for this and other possibilities. Modulation of calcium [96] and retinoic acid [97] *in vitro*, for example, may bring about significant improvements to current reprogramming platforms. Researchers have also begun to untangle the regulation of histone methylation as it affects HSC metabolism [98] and even multipotency [99], so the opportunities for hypothesis building and experimentation in the field of reprogramming are far from scarce.

More urgently, the conventional methods to assess HSC function are also far from ideal. Increasingly immunocompromised mouse strains on the nonobese diabetic (NOD) genetic background have been developed as a means to improve long-term engraftment of both normal and malignant haematopoietic cells [100–103]. To gauge the long-term, multilineage repopulation potential of human blood cells, haematologists have long relied on such models. This review, too, has regarded successful xenotransplantation as a decisive criterion to evaluate the myriad haematopoietic reprogramming methods reported to date. Today, many of these methods call for experiments using substrains of NOD-SCID;*Il2rg*$^{-/-}$ (NSG) mice, the hallmark of which is a lack of mature T, B or NK cells. How can

the lymphopoietic capacity of a potential HSC, let alone its own immunogenicity, be assessed in an immunodeficient system? Further, how does long-term, serial engraftment constitute evidence of self-renewal? The initial experiments showing multilineage human haematopoietic engraftment of immunocompromised mice were thought of as research and development of *in vivo* tools to study blood malignancies, not HSCs [102,104,105]. SCID mice, derived from a spontaneous mutation on the *Pkrdc* locus that resulted in a marked decrease in T and B cells [106], were in fact instrumental for the discovery of initiating cells in acute myeloid leukaemia [107]. More recently, NSG mice have been used to identify the clonal heterogeneity of the same cells [108]. Overall, the field of normal haematopoiesis is likely to have grown because of, and not despite, the development of immunocompromised mouse models, but demanding that they be used to test human HSC reprogramming is ultimately promoting an unsustainable standard when the recipient animal is itself incapable of adaptive immunity.

The concept of an HSC was born with the idea of its self-renewal ability, something that was hypothesized by way of CFU–spleen assays [109]. Serial transplantations, especially in a limiting-dilution manner, are the superior assay. The logic behind them is sound in the murine system, as transplantations of homozygous mutants for the pan-haematopoietic marker CD45 into congenic recipients allow reliable tracking of donor cells without risk of developing GVHD. Similarly, autologous transplantation of nonhuman primates (NHPs) offers ideal experimental set-ups [110–112]. In both cases, the innate and adaptive immune cells from the donor mouse or NHP would not interfere adversely with those of the host and vice versa. The expense and ethical implications of work on NHPs are as glaring as are the limitations of xenotransplantations. Other researchers have already brought up similar points [113,114]. Whether TF reprogramming of somatic or pluripotent cells into bona fide HSCs is possible and scalable, and how, is a moot question without better tools or different standards to assay human tissue function. This is a first-degree barrier to translational research that has yet to be reconciled; to do so will require a restructuring from the bottom up, starting with experimental design.

References

1 Schneuwly S, Klemenz R and Gehring WJ (1987) Redesigning the body plan of Drosophila by ectopic expression of the homoeotic gene Antennapedia. *Nature* **325**, 816–818.

2 Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB and Miller AD (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD (muscle regulatory gene/MyoD retrovirus). *Dev Biol* **86**, 5434–5438.

3 Kulessa H, Frampton J and Graf T (1995) GATA-1 reprograms avian myelomonocytic cell lines into eosinophils, thromboblasts, and erythroblasts. *Genes Dev* **9**, 1250–1262.

4 Nerlov C and Graf T (1998) PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes Dev* **12**, 2403–2412.

5 Xie H, Ye M, Feng R, Graf T and York N (2004) Stepwise reprogramming of B cells into macrophages. *Cell* **117**, 663–676.

6 Graf T (2013) Differentiation plasticity of hematopoietic cells. *Blood* **99**, 3089–3101.

7 Riddell J, Gazit R, Garrison BS, Guo G, Saadatpour A, Mandal PK, Ebina W, Volchkov P, Yuan GC, Orkin SH *et al.* (2014) Reprogramming committed murine blood cells to induced hematopoietic stem cells with defined factors. *Cell* **157**, 549–564.

8 Pereira CF, Chang B, Qiu J, Niu X, Papatsenko D, Hendry CE, Clark NR, Nomura-Kitabayashi A, Kovacic JC, Ma'ayan A *et al.* (2013) Induction of a hemogenic program in mouse fibroblasts. *Cell Stem Cell* **13**, 205–218.

9 Vereide DT, Vickerman V, Swanson SA, Chu LF, McIntosh BE and Thomson JA (2014) An expandable, inducible hemangioblast state regulated by fibroblast growth factor. *Stem Cell Rep* **3**, 1043–1057.

10 Batta K, Florkowska M, Kouskoff V and Lacaud G (2014) Direct reprogramming of murine fibroblasts to hematopoietic progenitor cells. *Cell Rep* **9**, 1871–1885.

11 Szabo E, Rampalli S, Risueño RM, Schnerch A, Mitchell R, Fiebig-Comyn A, Levadoux-Martin M and Bhatia M (2010) Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature* **468**, 521–526.

12 Pulecio J, Nivet E, Sancho-Martinez I, Vitaloni M, Guenechea G, Xia Y, Kurian L, Dubova I, Bueren J, Laricchia-Robbio L *et al.* (2014) Conversion of human fibroblasts into monocyte-like progenitor cells. *Stem Cells* **32**, 2923–2938.

13 Mitchell R, Szabo E, Shapovalova Z, Aslostovar L, Makondo K and Bhatia M (2014) Molecular evidence for OCT4-induced plasticity in adult human fibroblasts required for direct cell fate conversion to lineage specific progenitors. *Stem Cells* **32**, 2178–2187.

14 Wilson NK, Foster SD, Wang X, Knezevic K, Schütte J, Kaimakis P, Chilarska PM, Kinston S, Ouwehand WH, Dzierzak E *et al.* (2010) Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell* **7**, 532–544.

15 Radomska HS, Gonzalez DA, Okuno Y, Iwasaki H, Nagy A, Akashi K, Tenen DG and Huettner CS (2002) Transgenic targeting with regulatory elements of the human CD34 gene. *Blood* **100**, 4410–4419.
16 Gomes AM, Kurochkin I, Chang B, Daniel M, Law K, Satija N, Lachmann A, Wang Z, Ferreira L, Ma'ayan A *et al*. (2018) Cooperative transcription factor induction mediates hemogenic reprogramming. *Cell Rep* **25**, 2821–2835.e7.
17 Daniel MG, Sachs D, Bernitz JM, Fstkchyan Y, Rapp K, Satija N, Law K, Patel F, Gomes AM, Kim H-S *et al*. (2019) Induction of human hemogenesis in adult fibroblasts by defined factors and hematopoietic co-culture. *FEBS Lett* **593**, 3266–3287.
18 Cheng H, Ang HY-K, A El Farran C, Li P, Fang HT, Liu TM, Kong SL, Chin ML, Ling WY, Lim EKH *et al*. (2016) Reprogramming mouse fibroblasts into engraftable myeloerythroid and lymphoid progenitors. *Nat Commun* **7**, 13396.
19 Birdsey GM, Shah AV, Dufton N, Reynolds LE, Osuna Almagro L, Yang Y, Aspalter IM, Khan ST, Mason JC, Dejana E *et al*. (2015) The endothelial transcription factor erg promotes vascular stability and growth through Wnt/β-catenin signaling. *Dev Cell* **32**, 82–96.
20 Schachterle W, Badwe CR, Palikuqi B, Kunar B, Ginsberg M, Lis R, Yokoyama M, Elemento O, Scandura JM and Rafii S (2017) Sox17 drives functional engraftment of endothelium converted from non-vascular cells. *Nat Commun* **8**, 1–12.
21 Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, Apostolou E, Stadtfeld M, Li Y, Shioda T *et al*. (2010) Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. *Nat Biotechnol* **28**, 848–855.
22 Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LIR *et al*. (2010) Epigenetic memory in induced pluripotent stem cells. *Nature* **467**, 285–290.
23 Menegatti S, Kruijf M, Garcia-Alegria E, Lacaud G and Kouskoff V (2019) Transcriptional control of blood cell emergence. *FEBS Lett* **593**, 3304–3315.
24 Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY and Traver D (2010) Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**, 108–111.
25 Ciau-Uitz A, Walmsley M and Patient R (2000) Distinct origins of adult and embryonic blood in Xenopus. *Cell* **102**, 787–796.
26 Jaffredo T, Gautier R, Eichmann A and Dieterlen-Lièvre F (1998) Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development* **22**, 4575–4583.
27 de Bruijn MFTR, Speck NA, Peeters MCE and Dzierzak E (2000) Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J* **19**, 2465–2474.
28 Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E and Robin C (2010) In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**, 116–120.
29 Kissa K and Herbomel P (2010) Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**, 112–115.
30 Sandler VM, Lis R, Liu Y, Kedem A, James D, Elemento O, Butler JM, Scandura JM and Rafii S (2014) Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. *Nature* **511**, 312–318.
31 Lis R, Karrasch CC, Poulos MG, Kunar B, Redmond D, Duran JGB, Badwe CR, Schachterle W, Ginsberg M, Xiang J *et al*. (2017) Conversion of adult endothelium to immunocompetent haematopoietic stem cells. *Nature* **545**, 439–445.
32 Plein A, Fantin A, Denti L, Pollard JW and Ruhrberg C (2018) Erythro-myeloid progenitors contribute endothelial cells to blood vessels. *Nature* **562**, 223–228.
33 Gurdon JB (1962) Adult frogs derived from the nuclei of single somatic cells. *Dev Biol* **4**, 256–273.
34 Wilmut I, Schnieke AE, McWhir J, Kind AJ and Campbell KHS (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature* **385**, 810–813.
35 DiBerardino MA (1988) Genomic multipotentiality of differentiated somatic cells. *Cell Differ Dev* **25**, 129–136.
36 John HA, Birnstiel ML and Jones KW (1969) Suppression of malignancy by cell fusion. *Nature* **223**, 582–587.
37 Blau HM, Chiu CP and Webster C (1983) Cytoplasmic activation of human nuclear genes in stable heterocaryons. *Cell* **32**, 1171–1180.
38 Hochedlinger K and Jaenisch R (2002) Monoclonal mice generated by nuclear transfer from mature B and T donor cells. *Nature* **415**, 1035–1038.
39 Davis RL, Weintraub H and Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987–1000.
40 Takahashi K and Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663–676.
41 Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K and Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861–872.
42 Müller LUW, Daley GQ and Williams DA (2009) Upping the ante: recent advances in direct reprogramming. *Mol Ther* **17**, 947–953.
43 Inoue H, Nagata N, Kurokawa H and Yamanaka S (2014) IPS cells: a game changer for future medicine. *EMBO J* **33**, 409–417.

44 Maherali N, Sridharan R, Xie W, Utikal J, Eminli S, Arnold K, Stadtfeld M, Yachechko R, Tchieu J, Jaenisch R *et al.* (2007) Directly reprogrammed fibroblasts show global epigenetic remodeling and widespread tissue contribution. *Cell Stem Cell* **1**, 55–70.
45 Okita K, Ichisaka T and Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. *Nature* **448**, 313–317.
46 Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE and Jaenisch R (2007) In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* **448**, 318–324.
47 Liu H, Zhu F, Yong J, Zhang P, Hou P, Li H, Jiang W, Cai J, Liu M, Cui K *et al.* (2008) Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. *Cell Stem Cell* **3**, 587–590.
48 Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R *et al.* (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917–1920.
49 Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW and Daley GQ (2008) Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* **451**, 141–146.
50 Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N and Yamanaka S (2008) Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol* **26**, 101–106.
51 Hohwieler M, Perkhofer L, Liebau S, Seufferlein T, Müller M, Illing A and Kleger A (2017) Stem cell-derived organoids to model gastrointestinal facets of cystic fibrosis. *United European Gastroenterol J* **5**, 609–624.
52 Conese M, Beccia E, Castellani S, Di Gioia S, Colombo C, Angiolillo A and Carbone A (2018) The long and winding road: stem cells for cystic fibrosis. *Expert Opin Biol Ther* **18**, 281–292.
53 Pollard BS and Pollard HB (2018) Induced pluripotent stem cells for treating cystic fibrosis: state of the science. *Pediatr Pulmonol* **53**, S12–S29.
54 Maehr R, Chen S, Snitow M, Ludwig T, Yagasaki L, Goland R, Leibel RL and Melton DA (2009) Generation of pluripotent stem cells from patients with type 1 diabetes. *Proc Natl Acad Sci USA* **106**, 15768–15773.
55 Dadheech N and James Shapiro AM (2018) Human induced pluripotent stem cells in the curative treatment of diabetes and potential impediments ahead. *Adv Exp Med Biol* **1144**, 25–35.
56 Yabe SG, Fukuda S, Nishida J, Takeda F, Nashiro K and Okochi H (2019) Induction of functional islet-like cells from human iPS cells by suspension culture. *Regen Ther* **10**, 69–76.
57 Fu S, Ding J, Liu D, Huang H, Li M, Liu Y, Tu L and Liu D (2018) Generation of human-induced pluripotent stem cells from burn patient-derived skin fibroblasts using a non-integrative method. *Int J Mol Med* **41**, 87–94.
58 Kim Y, Park N, Rim YA, Nam Y, Jung H, Lee K and Ju JH (2018) Establishment of a complex skin structure via layered co-culture of keratinocytes and fibroblasts derived from induced pluripotent stem cells. *Stem Cell Res Ther* **9**, 1–10.
59 Cossu G, Birchall M, Brown T, De Coppi P, Culme-Seymour E, Gibbon S, Hitchcock J, Mason C, Montgomery J, Morris S *et al.* (2018) Lancet commission: stem cells and regenerative medicine. *Lancet* **391**, 883–910.
60 Wahlster L and Daley GQ (2016) Progress towards generation of human haematopoietic stem cells. *Nat Cell Biol* **18**, 1111–1117.
61 Blaser BW and Zon LI (2018) Making HSCs in vitro: don't forget the hemogenic endothelium. *Blood* **132**, 1372–1378.
62 Keller G, Kennedy M, Papayannopoulou T and Wiles MV (1993) Hematopoietic commitment during embryonic stem cell differentiation in culture. *Mol Cell Biol* **13**, 473–486.
63 Kyba M, Perlingeiro RCR and Daley GQ (2002) HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. *Cell* **109**, 29–37.
64 Wang L, Menendez P, Shojaei F, Li L, Mazurier F, Dick JE, Cerdan C, Levac K and Bhatia M (2005) Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic *HOXB4* expression. *J Exp Med* **201**, 1603–1614.
65 Rafii S, Kloss CC, Butler JM, Ginsberg M, Gars E, Lis R, Zhan Q, Josipovic P, Ding BS, Xiang J *et al.* (2013) Human ESC-derived hemogenic endothelial cells undergo distinct waves of endothelial to hematopoietic transition. *Blood* **121**, 770–780.
66 Elcheva I, Brok-Volchanskaya V, Kumar A, Liu P, Lee JH, Tong L, Vodyanik M, Swanson S, Stewart R, Kyba M *et al.* (2014) Direct induction of haematoendothelial programs in human pluripotent stem cells by transcriptional regulators. *Nat Commun* **5**, 1–11.
67 Sturgeon CM, Ditadi A, Awong G, Kennedy M and Keller G (2014) Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nat Biotechnol* **32**, 554–561.
68 Ng ES, Azzola L, Bruveris FF, Calvanese V, Phipson B, Vlahos K, Hirst C, Jokubaitis VJ, Yu QC, Maksimovic J *et al.* (2016) Differentiation of human embryonic stem cells to HOXA + hemogenic

vasculature that resembles the aorta-gonad-mesonephros. *Nat Biotechnol* **34**, 1168–1179.
69 Doulatov S, Vo LT, Chou SS, Kim PG, Arora N, Li H, Hadland BK, Bernstein ID, Collins JJ, Zon LI *et al.* (2013) Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. *Cell Stem Cell* **13**, 459–470.
70 Sugimura R, Jha DK, Han A, Soria-Valles C, da Rocha EL, Lu YF, Goettel JA, Serrao E, Rowe RG, Malleshaiah M *et al.* (2017) Haematopoietic stem and progenitor cells from human pluripotent stem cells. *Nature* **545**, 432–438.
71 Navarro-Montero O, Ayllon V, Lamolda M, López-Onieva L, Montes R, Bueno C, Ng E, Guerrero-Carreno X, Romero T, Romero-Moya D *et al.* (2017) RUNX1c regulates hematopoietic differentiation of human pluripotent stem cells possibly in cooperation with proinflammatory signaling. *Stem Cells* **35**, 2253–2266.
72 Ma H, Morey R, O'Neil RC, He Y, Daughtry B, Schultz MD, Hariharan M, Nery JR, Castanon R, Sabatini K *et al.* (2014) Abnormalities in human pluripotent cells due to reprogramming mechanisms. *Nature* **511**, 177–183.
73 Morris SA and Daley GQ (2013) A blueprint for engineering cell fate: current technologies to reprogram cell identity. *Cell Res* **23**, 33–48.
74 Ciau-Uitz A, Patient R and Medvinsky A (2016) Ontogeny of the hematopoietic system. *Encycl Immunobiol* **1**, 1–14.
75 Dzierzak E and Bigas A (2018) Blood Development: Hematopoietic Stem Cell Dependence and Independence. *Cell Stem Cell* **22**, 639–651.
76 Yvernogeau L, Gautier R, Petit L, Khoury H, Relaix F, Ribes V, Sang H, Charbord P, Souyri M, Robin C *et al.* (2019) In vivo generation of haematopoietic stem/progenitor cells from bone marrow-derived haemogenic endothelium. *Nat Cell Biol* **21**, 1334–1345.
77 Wei Q and Frenette PS (2018) Niches for hematopoietic stem cells and their progeny. *Immunity* **48**, 632–648.
78 Daniel MG, Rapp K, Schaniel C and Moore KA (2019) Induction of developmental hematopoiesis mediated by transcription factors and the hematopoietic microenvironment. *Ann N Y Acad Sci*, https://doi.org/10.1111/nyas.14246.
79 Wilkinson AC, Ishida R, Kikuchi M, Sudo K, Morita M, Crisostomo RV, Yamamoto R, Loh KM, Nakamura Y, Watanabe M *et al.* (2019) Long-term ex vivo haematopoietic-stem-cell expansion allows nonconditioned transplantation. *Nature* **571**, 117–121.
80 Biddy BA, Kong W, Kamimoto K, Guo C, Waye SE, Sun T and Morris SA (2018) Simultaneous single-cell profiling of lineage and identity in direct reprogramming. *Nature* **564**, 219–224.
81 Trokovic R, Weltner J, Nishimura K, Ohtaka M, Nakanishi M, Salomaa V, Jalanko A, Otonkoski T and Kyttälä A (2014) Advanced feeder-free generation of induced pluripotent stem cells directly from blood cells. *Stem Cells Transl Med* **3**, 1402–1409.
82 Carey BW, Markoulaki S, Beard C, Hanna J and Jaenisch R (2010) Single-gene transgenic mouse strains for reprogramming adult somatic cells. *Nat Methods* **7**, 56–59.
83 Stadtfeld M, Maherali N, Borkent M and Hochedlinger K (2010) A reprogrammable mouse strain from gene-targeted embryonic stem cells. *Nat Methods* **7**, 53–55.
84 Abad M, Mosteiro L, Pantoja C, Cañamero M, Rayon T, Ors I, Graña O, Megías D, Domínguez O, Martínez D *et al.* (2013) Reprogramming in vivo produces teratomas and iPS cells with totipotency features. *Nature* **502**, 340–345.
85 Suda T, Takubo K and Semenza GL (2011) Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell Stem Cell* **9**, 298–310.
86 Semenza GL (2012) Hypoxia-inducible factors in physiology and medicine. *Cell* **148**, 399–408.
87 Boitano AE, Wang J, Romeo R, Bouchez LC, Parker AE, Sutton SE, Walker JR, Flaveny CA, Perdew GH, Denison MS *et al.* (2011) Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science* **329**, 1345–1348.
88 Fares I, Chagraoui J, Gareau Y, Gingras S, Ruel R, Mayotte N, Csaszar E, Knapp DJ, Miller P, Ngom M *et al.* (2014) Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science* **345**, 1509–1512.
89 Chan WK, Yao G, Gu Y and Bradfield CA (1999) Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways. *J Biol Chem* **274**, 12115–12123.
90 Lindsey S and Papoutsakis ET (2012) The evolving role of the aryl hydrocarbon receptor (AHR) in the normophysiology of hematopoiesis. *Stem Cell Rev Rep* **8**, 1223–1235.
91 Simsek T, Kocabas F, Zheng J, Deberardinis RJ, Mahmoud AI, Olson EN, Schneider JW, Zhang CC and Sadek HA (2010) The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell Stem Cell* **7**, 380–390.
92 Takubo K, Nagamatsu G, Kobayashi CI, Nakamura-Ishizu A, Kobayashi H, Ikeda E, Goda N, Rahimi Y, Johnson RS, Soga T *et al.* (2013) Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell Stem Cell* **12**, 49–61.

93 Mertens S, Noll T, Spahr R, Krutzfeldt A and Piper HM (1990) Energetic response of coronary endothelial cells to hypoxia. *Am J Physiol Circ Physiol* **258**, H689–H694.
94 Dagher Z, Ruderman N, Tornheim K and Ido Y (2001) Acute regulation of fatty acid oxidation and AMP-activated protein kinase in human umbilical vein endothelial cells. *Circ Res* **88**, 1276–1282.
95 De Bock K, Georgiadou M, Schoors S, Kuchnio A, Wong BW, Cantelmo AR, Quaegebeur A, Ghesquière B, Cauwenberghs S, Eelen G *et al.* (2013) Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* **154**, 651–663.
96 Luchsinger LL, Strikoudis A, Danzl NM, Bush EC, Finlayson MO, Satwani P, Sykes M, Yazawa M and Snoeck HW (2019) Harnessing hematopoietic stem cell low intracellular calcium improves their maintenance in vitro. *Cell Stem Cell* **25**, 225–240.e7.
97 Cabezas-Wallscheid N, Buettner F, Sommerkamp P, Klimmeck D, Ladel L, Thalheimer FB, Pastor-Flores D, Roma LP, Renders S, Zeisberger P *et al.* (2017) Vitamin A-retinoic acid signaling regulates hematopoietic stem cell dormancy. *Cell* **169**, 807–823.e19.
98 Yang Z, Shah K, Khodadadi-Jamayran A and Jiang H (2019) Control of hematopoietic stem and progenitor cell function through epigenetic regulation of energy metabolism and genome integrity. *Stem Cell Rep* **13**, 61–75.
99 Vo LT, Kinney MA, Liu X, Zhang Y, Barragan J, Sousa PM, Jha DK, Han A, Cesana M, Shao Z *et al.* (2018) Regulation of embryonic haematopoietic multipotency by EZH1. *Nature* **553**, 506–510.
100 Goyama S, Wunderlich M and Mulloy JC (2015) Xenograft models for normal and malignant stem cells. *Blood* **125**, 2630–2640.
101 Beer PA and Eaves CJ (2015) Modeling normal and disordered human hematopoiesis. *Trends Cancer* **1**, 199–210.
102 Abarrategi A, Mian SA, Passaro D, Rouault-Pierre K, Grey W and Bonnet D (2018) Modeling the human bone marrow niche in mice: from host bone marrow engraftment to bioengineering approaches. *J Exp Med* **215**, 729–743.
103 Skelton JK, Ortega-Prieto AM and Dorner M (2018) A Hitchhiker's guide to humanized mice: new pathways to studying viral infections. *Immunology* **154**, 50–61.
104 Lai Y, Wei X, Lin S, Qin L, Cheng L and Li P (2017) Current status and perspectives of patient-derived xenograft models in cancer research. *J Hematol Oncol* **10**, 1–14.
105 Almosailleakh M and Schwaller J (2019) Murine models of acute myeloid leukaemia. *Int J Mol Sci* **20**, 453.
106 Bosma MJ and Carroll AM (1988) The SCID mouse mutant: definition, characterization, and potential uses. *Curr Top Microbiol Immunol* **137**, 197–202.
107 Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA and Dick JE (1994) A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645–648.
108 Clappier E, Gerby B, Sigaux F, Delord M, Touzri F, Hernandez L, Ballerini P, Baruchel A, Pflumio F and Soulier J (2011) Clonal selection in xenografted human T cell acute lymphoblastic leukemia recapitulates gain of malignancy at relapse. *J Exp Med* **208**, 653–661.
109 Till JE and Mcculloh EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**, 213–222.
110 Watts KL, Adair J and Kiem HP (2011) Hematopoietic stem cell expansion and gene therapy. *Cytotherapy* **13**, 1164–1171.
111 Radtke S, Chan YY, Giese MA, Rongvaux A, Adair V and Kiem HP (2017) A novel highly conserved hematopoietic stem/progenitor cell population for stem cell transplantation and gene therapy. *Blood* **130**, 3339.
112 Humbert O, Peterson CW, Norgaard ZK, Radtke S and Kiem HP (2018) A nonhuman primate transplantation model to evaluate hematopoietic stem cell gene editing strategies for β-hemoglobinopathies. *Mol Ther Methods Clin Dev* **8**, 75–86.
113 Eaves CJ (2015) Hematopoietic stem cells: concepts, definitions, and the new reality. *Blood* **125**, 2605–2614.
114 McRae HM, Voss AK and Thomas T (2019) Are transplantable stem cells required for adult hematopoiesis? *Exp Hematol* **75**, 1–10.
115 Ebina W and Rossi DJ (2015) Transcription factor-mediated reprogramming toward hematopoietic stem cells. *EMBO J* **34**, 694–709.

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REVIEW

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Role of flow cytometry in evaluation of the cellular therapy products used in haematopoietic stem cell transplantation

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Abstract

Cellular therapy nowadays includes various products from haematopoietic stem cells (HSC) collected from bone marrow, peripheral blood, and umbilical cord blood to more complex adoptive immune therapy for the treatment of malignant diseases, and gene therapy for inherited immune deficiencies. Broader utilization of cellular therapy requires extensive quality testing of these products that should fulfil the same requirements regarding composition, purity, and potency nevertheless they are manufactured in various centres. Technical improvements of the flow cytometers accompanied by the increased number of available reagents and fluorochromes used to conjugate monoclonal antibodies, enable detailed and precise insight into the function of the immune system and other areas of cell biology, and allows cell evaluation based on size, shape, and morphology or assessment of cell surface markers, as well as cell purity and viability, which greatly contributes to the development and progress of the cell therapy. The aim of this paper is to give an overview of the current use and challenges of flow cytometry analysis in quality assessment of cellular therapy products, with regard to basic principles of determining HSC and leukocyte subpopulation, assessment of cells viability and quality of thawed cryopreserved HSC as well as the importance of validation and quality control of flow cytometry methods according to good laboratory practice.

KEYWORDS
cellular therapy, flow cytometry, haematopoietic stem cell transplantation

1 | INTRODUCTION

The field of cellular therapy has been constantly evolving since the first bone marrow (BM) transplantation in the 1950s, and currently has been successfully implemented as a treatment for patients with malignant, congenital, or acquired diseases of the haematopoietic system.[1] Cellular therapies that initiated with haematopoietic stem cell (HSC) transplantation, now are becoming more complex including adoptive immune therapy for malignant diseases and gene therapy for the treatment of inherited immune deficiencies.[2] The novel promising cellular therapies are immune effector cells (IECs), such as gene-modified T cells and natural killer (NK) cells.[3] Broader utilization of cellular therapy requires extensive quality testing of these products that should fulfil the same requirements regarding composition, purity, and potency nevertheless they are manufactured in various centres.[4] In order for cellular therapy products to be exported from one centre to another for further clinical use, it is critical to have mechanisms in place to ensure that the cell collection and processing procedures yield safe, effective, and comparable products at all centres.

Initially, the evaluation of the quality of BM graft was limited to enumeration of total nucleated cells and colony forming unit (CFU) testing, while nowadays various assays have been used for assessing the quality of cellular therapy products. The assessment of the cell type may involve evaluations based on cell size, shape, and morphology, or the evaluation of cell surface markers by flow cytometry. The purity of the cells is also often evaluated by flow cytometry, as well as

cell viability measured by dye exclusion assays. Flow cytometry has applications in various fields such as immunology, cellular biology, bacteriology, virology, cancer biology and infectious disease monitoring. It has seen dramatic advances over the last 30 years, allowing detailed and precise insight into the function of the immune system and other areas of cell biology.[5] This paper aims to give an overview of the current use and challenges of flow cytometry analysis in the quality assessment of cellular therapy products used in HSC transplantation setting.

2 | FLOW CYTOMETRY: PRINCIPLES, INSTRUMENTS, REAGENTS

Flow cytometry is a technique utilized in many different settings, both in the routine laboratory and in research facilities. With the advancement of technology, the field of flow cytometry is also evolving, so today special types of instruments have been designed for specific purposes, for example, system that combines microscopy and flow cytometry or flow cytometry with mass spectrometry. This technique allows simultaneous analysis of cell characteristics of mixed cell population from peripheral blood (PB) and BM as well as solid tissues that can be dissociated into single cells, with cell sorting for further analysis, which is one of the main application of flow cytometry.[5]

Technical improvements of the flow cytometer are accompanied by an increase in the number of available reagents and fluorochromes used to conjugate monoclonal antibodies which results in the complexity of the analysis and requires the use of newer cluster data analysis algorithms. All that improves methods of data mining allow useful information to be extracted from the high-dimensional data now available from flow cytometry.[5]

In addition to immunophenotyping, which is the most used application in flow cytometry, apoptosis analysis, cell cycle analysis and cell sorting are also used in the quality assessment of cellular therapy products.[5–8]

3 | ENUMERATION OF HAEMATOPOIETIC STEM CELL

HSC sources currently used for transplantation are BM, mobilized PB and umbilical cord blood (UCB).[9] Each type of HSC graft requires a different method of collection and processing, and has its advantages and disadvantages. BM has been almost completely replaced as a source of HSC with peripheral blood stem cells (PBSC), due to easier collection with leukapheresis procedure without the need for general anaesthesia and more rapid haematopoietic reconstitution after transplantation.[10,11] HSCs from UCB have a high clonogenic potential and because they are immunologically naive and immature, can be transplanted with only partial histocompatibility. But due to their limited volume, UCB is mainly applicable in transplantation of paediatric patients.[9]

Regardless of the source of HSCs, haematopoietic stem and progenitor cells appear morphologically as either small lymphocytes in the case of the earliest stem cells or in the case of maturing progenitors, as blast forms. Therefore, they can be identified only by functional assays or by immunophenotypic surface marker analysis. Phenotype of HSC is $CD34^{+}/CD45^{dim}/SSC^{low}/FSC^{low\ and\ intermediate}$.[12]

Later progenitors may be functionally assayed in soft agar culture systems. When supported by the proper growth factors, they form colonies of their progeny that can be enumerated and expressed as a particular number of CFUs per total number of cells plated. Functional assays for enumerating different species of HSCs require several weeks of sterile culture incubation. Therefore, these assays have some disadvantages, and the most significant being poor intra- and inter-laboratory reproducibility, non-standardization, and long turnaround time.[13,14] Hence, functional haematopoietic cell assays in transplantation clinical practice are generally limited to quality-control procedures of cryopreserved cells and are rarely used in routine laboratory testing.

The measurement of CD34+ cells by flow cytometry has, however, become the universal assay for measuring the potency of HSC products. The evaluation of absolute CD34+ cell count in patient PB is also important for the decision of optimal timing to start the leukapheresis procedure. According to the Joint Accreditation Committee of International Society of Cellular Therapy (JACIE) standards, enumeration of viable CD34+ cells must be performed in fresh PBSC products to access the graft quality.[15] Minimal CD34+ cell count for one transplantation is $\geq 2 \times 10^6$ CD34+ cells/kg of recipient's body weight, while the optimal is 5×10^6 CD34+ cells/kg of body weight, which is associated with faster recovery of neutrophil and platelet count after transplantation.[16,17]

Although laboratories for quality control in transplant centres use different protocols for determining CD34+ cells, the protocol of International Society of Hemotherapy and Graft Engineering (ISHAGE) is the most commonly used (Figure 1).[18] This protocol has been continuously updated, by introducing counting beads and including viability dyes.[12] However, there are still differences between laboratories using the ISHAGE protocol: some use single platform method, while others dual platform.[19] The benefit of the single platform is the simultaneous determination of the percentage and absolute count of CD34+ cells using fluorescent microspheres (beads), which can be in liquid phase or in lyophilized form. The main advantage of lyophilized beads is that the tube contains an exact number of beads which allows the calculation of the absolute cell count. Furthermore, there is a difference in the cell labelling protocol which depends on whether the single or dual platform method is used. Some laboratories use lyse-wash method (dual platform), while others use lyse-no wash method (single platform) which lasts shorter and potential cell loss is prevented. Although the ISHAGE protocol for CD34+ cell determination is used in many laboratories, the mentioned differences indicate that standardization is necessary.

Although according to EBMT guidelines, CD34+ evaluation is not mandatory for the assessment of BM many centres include it in quality testing.[20] If the laboratory performs determination of viable

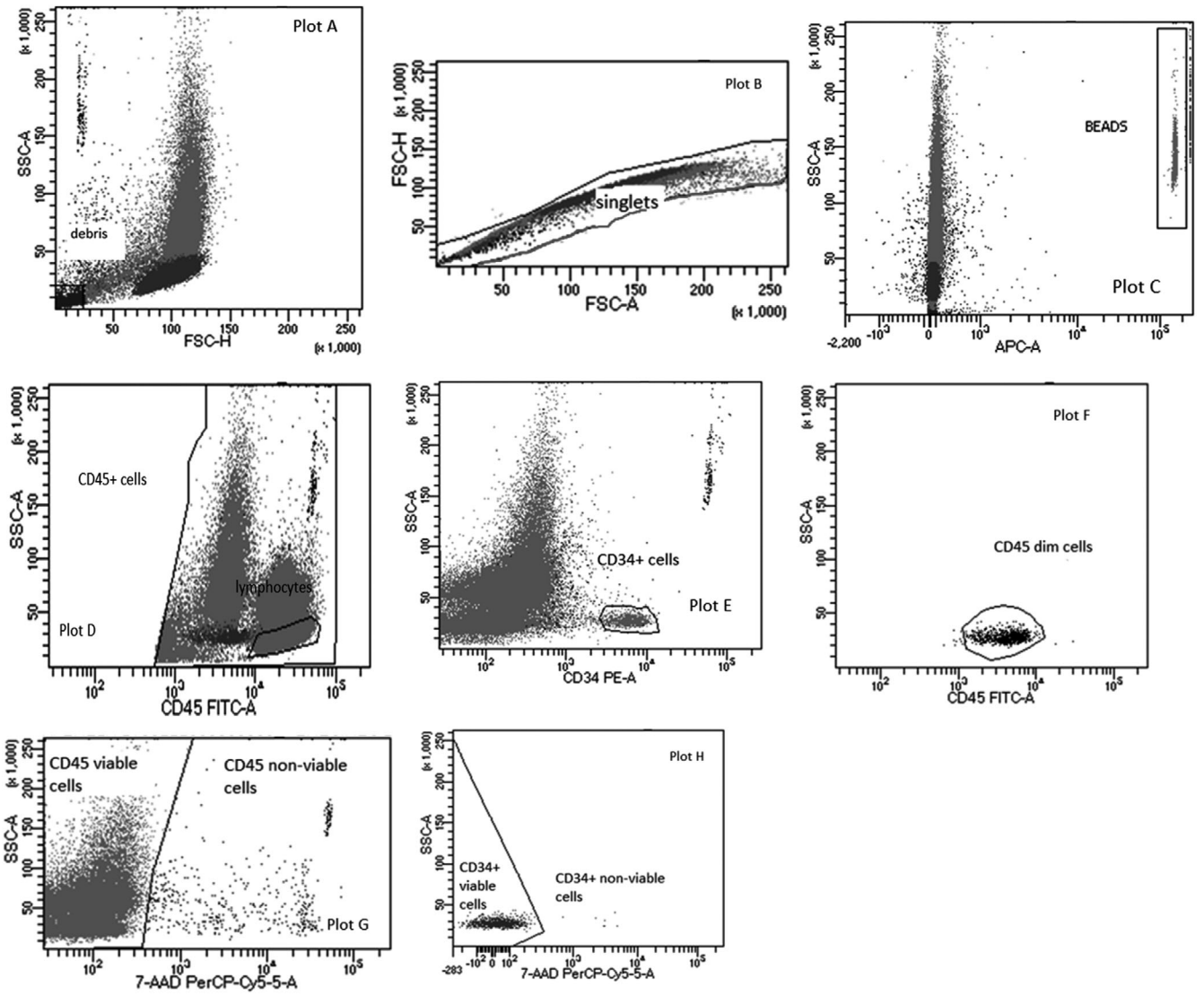

FIGURE 1 Gating strategy for analysis of CD34+ cells haematopoietic stem cells for single platform using modified ISHAGE protocol. ISHAGE, International Society of Hemotherapy and Graft Engineering

CD34+ cells in BM samples, it is necessary to carefully set up protocols and templates for cells analysis on flow cytometer. Compared to CD34+ cells enumeration in PB or in PBSC graft, the analysis of BM samples is challenging because they contain high red blood cell count and may contain fat, cell clumps which all complicate the analysis. Therefore, the settings on the flow cytometer must be adjusted to reduce debris and allow a more accurate determination of the population of CD34+ cells.

4 | ASSESSMENT OF CELLS VIABILITY

The viability of cells was firstly evaluated using the dye exclusion tests, based on the principle that live cells possess intact cell membranes that exclude certain dyes, whereas dead cells do not. The cell suspension is mixed with dye and then visually examined to determine whether cells take up or exclude dye. The most common dyes used for performing dye exclusion tests are trypan blue, eosin Y, acridine orange, or propidium iodide. Dye exclusion is a simple and rapid technique, but the limitation of the method is that viability is determined indirectly from the assessment of cell membrane integrity. It is important that the test be performed accurately because a small amount of dye uptake indicative of cell injury may go unnoticed.[21,22]

As already mentioned, in addition to the number of CD34+ cells, according to the JACIE standards it is necessary to determine the viability of collected and processed/cryopreserved cells. In the assessment of CD34+ cells viability, the most commonly used dye is 7-aminoactinomicin D (7-AAD), which binds to GC region of the cell DNA, and allows the determination of necrotic cells and those in late apoptosis (7-AAD positive) using flow cytometer.[22] Besides 7-AAD, some laboratories still used dye excluding tests in routine work, but because 7-AAD has become a standard part of the ISHAGE protocol,

most laboratories use it to determine the viability of CD34+ cells.[21] The main disadvantage of the method with 7-AAD, as well as the previously mentioned dye exclusion tests, is a failure to detect cells in early apoptosis, which may lead to overestimation of graft cell viability.[23] Several studies have shown that the method using Annexin V (Ann V) is suitable for detection of cells in early apoptosis.[14,24] Annexin V is a protein that has a high affinity for negatively charged phospholipids, such as phosphatidylserine, which are characteristic of the cytoplasmic side of the viable cell membrane. But in early apoptosis phosphatidylserine becomes exposed on the cell surface due to cell membrane asymmetry and can be detected using a reagents containing Ann V protein and Ann V binding buffer with calcium, which allows binding Ann V to negatively charged phospholipids. It may be useful to evaluate the apoptotic status of progenitor cells before beginning ex vivo manipulation procedures such as stem cell expansion or gene therapy.[25] Other assays for the determination of apoptotic cells that are used in research settings are method with DNA binding dye Syto 16, method for detecting activation of caspases, TUNEL (TdT dUTP Nick End Labeling) assay for detection of endonuclease digestion of DNA, and method for detection of mitochondrial apoptosis using dyes that determine mitochondrial membrane potential and chromation condensation in the nucleus using method with specific dye.[5,26]

5 | ASSESSMENT OF QUALITY OF THAWED CRYOPRESERVED HSC

Fresh HSCs, once harvested, are only viable for several hours to a few days, limiting their use and geographical reach. Currently, HSCs and other cell therapies are cryopreserved using the same techniques with cryoprotectant dimethyl sulfoxide (DMSO) at a slow cooling rate.[27] Cryopreservation of HSCs allows their transportation from the site of processing to the site of clinical use, creates a larger window of time in which cells can be administered to patients, and enables sufficient time for quality control and regulatory testing. Despite these benefits, during processing, cells are exposed to some factors (e.g., centrifugation, condition of storage, and thawing process) that can lead to the reduction of the cell count in graft, but also to a decline in cell viability after thawing.[27]

The gold standard for cryopreservation for HSCs is still DMSO. The timing of cell exposure to DMSO is very important because DMSO can directly impact cellular function by affecting metabolism, enzyme activity, apoptosis, and cell cycle. In addition, the effect of DMSO depends on the type of cells, the stage of cell differentiation, the duration of exposure, and DMSO concentration.[28] Since DMSO has the toxic effect on the cells in thawed graft and could also cause adverse reactions during the infusion, in some transplant centres it is removed from thawed HSCs before transplantation.[27] However, the question is how many cells are lost and damaged by this process, because it is known that the number of viable CD34+ cells in a thawed product is actually a real number transplanted to the patient.[11,29] In addition to the DMSO, some other factors affect the recovery of the cells after cryopreservation, such as cell concentration, pre-freeze storage conditions, freezing rate, and storage temperature, which also affect cell viability.[27]

It is well known that 'single platform' method in combination with 7-AAD represents the reference method for determining the count of viable CD34+ cells in fresh samples. However, for analysing thawed cryopreserved samples modification of the method is necessary. It is recommended to adjust the gates Side scatter versus Forward scatter and CD45 versus SSC in order to allow the acquisition of a higher number of dead and live cells. Therefore, analysis of thawed samples requires the adaption of gatting strategy and acquisition settings for the purpose of more precise and accurate analysis of HSC.[11,30]

According to NetCord – Foundation for the Accreditation of Cellular therapy (FACT) standards for Cord Blood Banks the post-thaw CD34+ cell viability should be ≥70%.[31] There are no such recommendations for minimal CD34+ cell post-thaw viability of PBSCs, and it is only required that the viability of nucleated cells should be >50% after freezing and thawing of apheresis product.[32] It is therefore questionable how to perform the quality control of the PBSC graft after thawing and which assays and methods should be used. Although some studies evaluated the methods for post-thaw viability of PBSC and CB, as well as attempted to standardize process from sample preparation to acquisition on flow cytometer, still there are no guidelines defining each step in the process after thawing of cryopreserved samples, for example, exact conditions of sample thawing, is it necessary to wash cells, dilute the samples and remove cryoprotectant before labelling, and acquisition on flow cytometer.[33,34]

In quality specifications for UCB, post-thaw CFU is still one of the requirements, but due to the before mentioned disadvantages of CFU assay, in routine work it would be desirable to use assay with higher reproducibility and a shorter turnaround time for faster assessment of graft quality.[35,36] Although 7-AAD is the most commonly used dye for the determination of cell viability on flow cytometer in HSC transplants, the lack of a test is that it cannot determine cells in early apoptosis. Since it is known that in early apoptosis the functioning of the cell is impaired, the question is whether such damaged cells have the possibility of proliferation. Thus, it would be useful to determine the viability of cells, in addition to the method with 7-AAD use the method for determining cells in early apoptosis, such as the method with annexin V. Few studies showed that for UCB samples, assay using 7-AAD and Ann V was a feasible method for prediction of CFU results.[14,24] Duggleby and coworkers[24] showed that significant numbers of CD34+ AnnV+ events were found within the 7AAD-gated population on their custom protocol for determining viable CD34+ cells. In their study, the measured results indicated a good correlation between nonapoptotic cells (CD34+ AnnV−) and CFU results, so they confirmed the fact that the current standard enumeration of CD34+ viable cells does not fully reflect potency after -thawing because standard enumeration with 7-AAD does not measure the early apoptotic cell. Radke and coworkers[14] in their study presented that in comparison to the standard ISHAGE protocol, the method with AnnV resulted in similar good correlations between CFU and CD34+ cells seeded, which leads to an improved conformability

with the theoretically expected colony formation. The results of these studies were especially important because the Ann V method can be used in the case the number of viable cells needs to be determined as soon as possible before transplantation.

6 | ASSESSMENT OF LEUKOCYTE SUBPOPULATION

In addition to CD34+ cell determination, the quality assessment of allogenic HSC graf must include enumeration of other leukocyte subpopulation, especially CD3+ T cells. Besides T cells, for different types of cell therapy it is necessary to determine leukocytes subpopulation in the graft. Nowadays, flow cytometers used in laboratory work enable the detection of several cell markers at once, depending on the laser and detector configuration of flow cytometer (e.g., 10-colour flow cytometer with three lasers [violet 405 nm, blue 488 nm, and red 638 nm]). Therefore, it is possible to determine leukocytes subpopulations using monoclonal antibodies (mAb) in one test tube, reducing the price and time to test results. Leukocyte subpopulations are usually detected using anti-CD 19 mAb for B-cells, anti-CD 16 and anti-CD 56 for NK cells, anti-CD3, anti-CD 4 and anti-CD8 mAb for subpopulation of T-cells and anti-CD 14 and anti-CD16 for monocytes (Table 1).

When the laboratory defines the protocol for detecting cells of interest, it is necessary to choose the antibodies (Abs) and fluorochromes which will allow accurate cell identification. This is especially important when performing multiple analysis in one step (e.g., enumeration of lymphocytes subpopulation). In addition to individual Ab, there are commercial kits that contain predefined Abs labelled with fluorochromes and reagents for determining cells of interest, for example, for determining HSC or lymphocyte subpopulations.[16,37,38] The advantage of these kits is that they contain pre-assembled Ab panels, which facilitates the creation and implementation of protocols in laboratories that have no experience with setting up in-house methods. Furthermore, some manufacturers offer software for acquisition and analysis which have an auto-gating algorithm for isolation of the cells of interest, which also contributes to more user-friendly cell analysis, for example, BD FACSCanto Clinical software (BD Bioscience, San Jose, CA).[39]

TABLE 1 The antibodies most commonly used in the evaluation of the cellular therapy products intended for haematopoietic stem cell transplantation

Antibody	Cell line	References
Anti-CD34	Haematopoietic stem cells	[14,17,18,23,24,33,34,37,39, 43,45,47,48,54–56]
Anti-CD3	T cells	[38,40,45,47,48,50,52]
Anti-CD19 Anti-CD20	B cells	[38,40,43,45,47,48,50,52]
Anti-CD14	Monocytes	[50]
Anti-CD56/CD16	NK cells	[38,40,48,50]

Transplantation of allogeneic HSC is sometimes followed with additional administration of donor lymphocytes, for example, donor lymphocyte infusion (DLI). Lymphocytes for DLI could be collected during allo-HSC collection procedure or from unstimulated PB, and cryopreserved as a simple and effective therapeutic option for patients in case of disease relapse.[40,41] The purpose of DLI is to enhance donor T-cells potency against leukaemic cells (graft-vs.-tumour effect), that is, to treat disease relapse and improve immune recovery.[42]

In the fresh apheresis products which will be cryopreserved for DLI, CD3+ cells are determined on flow cytometer. Since there are only a few reports on how this cell population tolerates the cryopreservation and thaw process, and because of difference among laboratories in cryopreservation protocols, each centre should determine the viability of CD3+ cells in the thawed sample before infusion.[38]

In the case of partial HLA matching, the HSC graft could be manipulated before administration in order to remove unwanted cells. The transplantation of HSC graft obtained from haploidentical donors carries an increased risk of developing graft-versus host disease (GvHD). In order to avoid that potential complication after transplantation, immunomagnetic techniques can be used prior to the infusion of the graft: indirect T cell depletion by the enrichment of CD34+ cells or the depletion of unwanted CD3+ T cells and CD19+ B cells.[43–45] The method of enrichment of target CD34+ cells is based on magnetic isolation technique using anti-CD 34 mAb conjugated to superparamagnetic iron dextran particles and magnetic cell separator.[45] After selection, the number of viable CD34+ and CD3+ cells is determined using flow cytometer. The challenge in the analysis is to determine the count of CD3+ cells in the positive fraction after selection. The residual number of CD3+ cells is usually less than 1% and for analysis is needed special protocol for the rare number of T cells. In addition, multigating strategy protocol should be implemented.[46] Nowadays, two different T-cell depletions are used in haploidentical HSC transplantation: in vitro T-cell depletion of PBSC and post-transplantation cyclophosphamide for in vivo T-cell depletion.[47] Immunological techniques for depletion of T-cell receptor alpha/beta and CD19+ cells is even more demanding than enrichment techniques. After the depletion process, very few residual TCR$\alpha\beta^{+}$ and CD19+ cells must be determined using flow cytometry.[48] For the analysis of the products obtained using immunological techniques, it is very important to devise gating strategies, set up a protocol on flow cytometer and then accurately analyse the cells of interest, because regardless of their very small number in the product, the effect of the procedure is assessed according to result from flow cytometer.

7 | IMMUNE EFFECTOR CELLS

Adoptive cellular therapy with IECs is an exciting and rapidly developing field that is evolving from a clinical manufacturing model, generally occurring in academic institutions, to an industry model with centralized manufacturing. Immune effector cells currently comprise cells that express broad cytotoxicity against tumours or targeted

cytotoxicity against tumour-associated antigens, as well as the cells that induce tolerance by suppressing inflammatory responses or enhancing immune recognition. Their identity, enumeration, and viability are critical information for initiating manipulation and releasing the products for infusion.[49,50]

Despite rapid development, IECs therapy still faces several challenges such as manufacturing processes, logistic and coordination aspects and toxicity profiles. Therefore, the Immune Effector Cell Task Force was created the standards and accreditation program for IEC.[51] The standards clearly state that relevant and validated assay should be employed to evaluate cellular therapy products undergoing manipulation that alters the function of the target cell population, where multi-colour flow cytometry is the technology of choice for cell surface marker detection, viability and enumeration. Accurate quantification of viable absolute numbers of cells is a prerequisite for several activities: standardization of input cellular material for CAR-transduced T or NK cell manufacturing, cell selection, in-process quality controls and dosing of IEC.[50,51]

CAR-T cell therapy is increasingly applied in clinical practice, in which genetic modification optimizes the T cells to actively proliferate and recognize cancer cells.[49] As with CD34+ cells collection for HSC transplantation, obtaining a sufficient concentration of T-cells is a critical part of the collection process. It is therefore important to perform quality control of leukapheresis products using anti-CD3 mAb, because quality of the final product depends on the quality of the starting material used in CAR-T cells manufacturing.[42,52] After the manufacturing process, a sample of the CAR-T cell product is taken for quality control, which includes, among other things, phenotyping, viability and purity of effector cell population.[42]

In addition to the determination of the quality of the leukapheresis product, flow cytometry is used in the monitoring of expansion and persistence of the therapeutic cells after the infusion and in the evaluation of treatment response in patients who received graft with CAR-T cells.[53]

8 | QUALITY CONTROL AND VALIDATION OF FLOW CYTOMETRY METHODS

Before the introduction of new methods in routine work, it is necessary to perform validation that usually includes assessment of precision and accuracy, linearity, limit of quantification, method comparison between two or more flow cytometers and carryover, and sometimes a sample stability study.[37,39,50]

8.1 | Stability of the cellular therapy product samples

Sample stability study is an important part of validation of the protocol used for quality assessment, especially when implementing a new type of cell therapy product. Several studies have examined the stability of fresh leukapheresis samples and thawed cryopreserved UCB samples.[37,39,54,55] The results showed that fresh leukapheresis samples were stable up to 24 h stored at 4°C.[37,39] The results of stability studies of thawed UCB samples were conflicting. Lee et al.[54] in their study showed that thawed UCB samples were stable up to 6 h after thawing, regardless of whether samples were stored at room temperature or at 4°C. On the other hand, Huang and colleagues reported that CD34+ cell viability decreased significantly after only 20 min when thawed UCB samples were stored at RT.[55] Krasna et al.[56] evaluated the stability of the HSC products after immunoselection and reported that CD34+ cells after selection were less stable than CD34+ cells in leukapheresis product during refrigerated storage up to 6 days. Since the results of the studies conducted so far have varied depending on the type of cellular therapy products and storage conditions, each laboratory should perform a sample stability study as part of the validation protocol.

Before performing validation, it is necessary to optimize the settings of clones combined with the best possible fluorochromes and also antibody concentration to minimize nonspecific background fluorescence.

In routine work, after initialization and start up procedure, lasers on flow cytometer need to be adjusted and commercial reagents (fluorescent beads) are most commonly used for this purpose. If necessary, spectar overlap compensation can be performed.[57]

8.2 | Internal and external quality control

According to good laboratory practice, it is necessary to provide quality control for the methods used in routine laboratory work. Internal quality control should be performed every day before routine work, for which commercial controls are used, usually from the same manufacturer as the reagents. Beside internal control, it is also important to participate in external quality assessment (EQA). Samples for EQA are usually analysed several times per year, and laboratories decide in which scheme will participate (e.g., for CD34 count, or for lymphocyte immunofentoyping). Laboratories that are part of HSC transplantation program usually participate in the UK NEQAS quality scheme and/or in national schemes.[58,59] It is very important that samples from EQA are processed in the same way as routine samples, because the external control allows periodic verification of the method, and at the same time checks the technical performance of the test. Validation and verification of the methods, internal and EQA are part of the accreditation procedure.[60] If the laboratory wants to meet the requirements for accreditation, it is necessary to implement the mentioned procedures.

9 | CONCLUSION

Flow cytometry methods nowadays open new views and insights into the quality of various cellular products used in the field of HSC transplantation which greatly contributes to the development and progress of cell therapy. They are now in routine use in clinical as well as in research laboratory work, and application of good laboratory practice,

constant education and training are needed for the implementation and maintenance of the methods in this fast-growing field of laboratory testing.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

All data from this study are available from the corresponding author upon reasonable request.

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REFERENCES

1. Chabannon C, Kuball J, Bondanza A, et al. Hematopoietic stem cell transplantation in its 60s: a platform for cellular therapies. *Sci Transl Med*. 2018;10:1-10.
2. Kohn DB. Historical perspective on the current renaissance for hematopoietic stem cell gene therapy. *Hematol Oncol Clin North Am*. 2017;31:721-735.
3. Maus MV, Nikiforow S. The why, what, and how of the new FACT standards for immune effector cells. *J Immunother Cancer*. 2017;5:36.
4. Stroncek DF, Jin P, Ren J, et al. Quality assessment of cellular therapies: the emerging role of molecular assays. *Korean J Hematol*. 2010;45:14-22.
5. McKinnon KM. Flow cytometry: an overview. *Curr Protoc Immunol*. 2018;120:5.1.1-5.1.11.
6. Murray C, Pao E, Jann A, Park DE, Di Carlo D. Continuous and quantitative purification of T-cell subsets for cell therapy manufacturing using magnetic ratcheting cytometry. *SLAS Technol*. 2018;23:326-337.
7. Desoutter J, Ossart C, Lacassagne MN, Regnier A, Marolleau JP, Harrivel V. Cryopreservation and thawing of hematopoietic stem cell CD34-induced apoptosis through caspase pathway activation: key role of granulocytes. *Cytotherapy*. 2019;21:612-618.
8. Passegué E, Wagers AJ, Giuriato S, Anderson WC, Weissman IL. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J Exp Med*. 2005;202:1599-1611.
9. Panch SR, Szymanski J, Savani BN, Stroncek DF. Sources of hematopoietic stem and progenitor cells and methods to optimize yields for clinical cell therapy. *Biol Blood Marrow Transplant*. 2017;23:1241-1249.
10. Kong JH, Hu Y, Kong SY, et al. Analysis of laboratory parameters for optimal autologous peripheral blood stem cell collection from lymphoma and myeloma patients. *J Clin Apher*. 2021;36:135-142.
11. Lanza F, Saccardi R, Seghatchian J. New horizins on stem cell cryopreservation through the artificial eyes of CD34+, using modern flow cytometry tools. *Transfus Apher Sci*. 2020;59:102785.
12. Whitby A, Whitby L, Fletcher M, et al. ISHAGE protocol: are we doing it correctly? *Cytometry B Clin Cytom*. 2012;82:9-17.
13. European Committe (Partial Agreement) on Organ Transplantation. *Haematopoietic Progenitor Cells from Bone Marrow and Peripheral Blood. Guide to the Quality and Safery of Rissues and Cells from Human Application*. European Directorate for the Quality of Medicine and Health Care (EDQM), Councile of Europa; 2019:267-288.
14. Radke TF, Barbosa D, Duggleby RC, Saccardi R, Querol S, Kögler G. The assessment of parameters affecting the quality of cord blood by the appliance of the Annexin V staining method and correlation with CFU assays. *Stem Cells Int*. 2013;2013:823912.
15. FACT-JACIE. *International Standards for Hematopoietic Cellular Therapy. Product Collection, Processing, and Administration*. 7th ed. FACT-JACIE; 2021. Accessed April 20, 2021. www.ebmt.org/jacie-standards
16. Mohammed RN, Ahmad D, Khoshnaw N, et al. Leukapheresis cell concentration adjustment required for a successful recovery of HSC after cryopreservation. *Cryobiology*. 2020;92:21-25.
17. Jansen EM, Hanks SG, Terry C, et al. Prediction of engraftment after autologous peripheral blood progenitor cell transplantation: CD34, colony-forming unit-granulocyte-macrophage, or both? *Transfusion*. 2007;47:817-823.
18. Gratama JW, Kraan J, Keeney M, Sutherland DR, Granger V, Barnett D. Validation of the single-platform ISHAGE method for CD34(+) hematopoietic stem and progenitor cell enumeration in an international multicenter study. *Cytotherapy*. 2003;5:55-65.
19. Murugesan M, Nair CK, Nayanar SK, Pentapati KC. Flow cytometric enumeration of CD34+ hematopoietic stem cells: a comparison between single- versus dual-platform methodology using the International Society of Hematotherapy and Graft Engineering protocol. *Asian J Transfus Sci*. 2019;13:43-46.
20. Gorin NC. Bone marrow harvesting for HSCT. In: Carreras E, Dufour C, Mohty M, Krögel N, eds. *The EBMT Handbook Hematopoietic Stem Cell Transplantation and Cellular Therapies*. European society for blood and marrow transplantation; 2019:109-115.
21. Takanashi M, Selogie E, Reems JA, et al. Current practices for viability testing of cryopreserved cord blood products: an international survey by the cellular therapy team of the biomedical excellence for safer transfusion (BEST). *Collaborative Transfusion*. 2018;58:2184-2191.
22. Varan HD, Bay M, Ozturk A, Dalva K, İlhan O. Comparison of the methods evaluating post thawing viability of peripheral blood stem cell graft. *Transfus Apher Sci*. 2019;58:192-195.
23. Watz E, Remberger M, Ringden O, et al. Quality of the hematopoietic stem cell graft affects the clinical outcome of allogeneic stem cell transplantation. *Transfusion*. 2015;55:2339-2350.
24. Duggleby RC, Querol S, Davy RC, et al. Flow cytometry assessment of apoptotic CD34+ cells by annexin V labeling may improve prediction of cord blood potency for engraftment. *Transfusion*. 2012;52:549-559.
25. Anthony RS, McKelvie ND, Cunningham AJ, Craig JI, Rogers SY, Parker AC. Flow cytometry using annexin V can detect early apoptosis in peripheral blood stem cell harvests from patients with leukaemia and lymphoma. *Bone Marrow Transplant*. 1998;21:441-446.
26. Sparrow RL, Komodromou H, Tippett E, Georgakopoulos T, Xu W. Apoptotic lymphocytes and CD34+ cells in cryopreserved cord blood detected by the fluorescent vital dye SYTO 16 and correlation with loss of L-selectin (CD62L) expression. *Bone Marrow Transplant*. 2006;38:61-67.
27. Hornberger K, Yu G, McKenna D, Hubel A. Cryopreservation of hematopoietic stem cells: emerging assays, Cryoprotectant agents, and technology to improve outcomes. *Transfus Med Hemother*. 2019;46:188-196.
28. Shu Z, Heimfeld S, Gao D. Hematopoietic SCT with cryopreserved grafts: adverse reactions after transplantation and cryoprotectant removal before infusion. *Bone Marrow Transplant*. 2014;49:469-476.
29. Lee S, Kim S, Kim H, Beak EJ, Jin H, Kim HS. Post-thaw viable CD34 (+) cell count is a valuable predictor of haematopoietic stem cell engraftment in autologous peripheral blood stem cell transplantation. *Vox Sang*. 2008;94:146-152.
30. Lanza F, Mangianti S, Accorsi P, et al. Manipulation, and cryopreservation of autologous peripheral blood stem cell products in Italy: a survey by GITMO, SIDEm and GIIMA societies. *Transfus Apher Sci*. 2020;59:102753.
31. Foundation for the Accreditation of Cellular Therapy, ed. *NerCord-FACT International Standards for Cord Blood Collection, Banking, and*

Release for Administration. 7th ed. Foundation for the Accreditation of Cellular Therapy; 2019.
32. Wuchte P. Processing, cryopreserving and controlling the quality of HSCs. In: Carreras E, Dufour C, Mohty M, Kröger N, eds. *The EBMT Handbook Hematopoietic Stem Cell Transplantation and Cellular Therapies*. European society for blood and marrow transplantation; 2019: 127-130.
33. Fournier D, Lewin A, Simard C, et al. Multi-laboratory assay for harmonization of enumeration of viable CD34+ and CD45+ cells in frozen cord blood units. *Cytotherapy*. 2020;22:44-51.
34. Castelhano MV, Reis-Alves SC, Vigorito AC, et al. Quantifying loss of CD34+ cells collected by apheresis after processing for freezing and post-thaw. *Transfus Apher Sci*. 2013;48:241-246.
35. European Committe (Partial Agreement) on Organ Transplantation. *Umbilical Cord Blood Progenitors. Guide to the Quality and Safery of Rissues and Cells from Human Application*. European Directorate for the Quality of Medicine and Health Care (EDQM), Councile of Europa; 2019:289-298.
36. Quarol S, Rocha V. Procurement and management of cord blood. In: Carreras E, Dufour C, Mohty M, Krögel N, eds. *The EBMT Handbook Hematopoietic Stem Cell Transplantation and Cellular Therapies*. European society for blood and marrow transplantation; 2019:131-136.
37. Omana-Zapata I, Oreizy F, Mosqueda F, et al. Performance of a novel BD stem cell enumeration kit on two flow cytometry systems. *Int J Lab Hematol*. 2013;35:393-399.
38. Berens C, Heine A, Müller J, et al. Variable resistance to freezing and thawing of CD34-positive stem cells and lymphocyte subpopulations in leukapheresis products. *Cytotherapy*. 2016;18:1325-1331.
39. Rimac V, Bojanić I, Gojčeta K, Golubić ĆB. Evaluation of the BD stem cell enumeration kit on the BD FACSCanto II flow cytometer using bd facscanto clinical and bd facsdiva software. *Int J Lab Hematol*. 2021; 43:61-67.
40. Worsham DN, Reems JA, Szczepiorkowski ZM, et al. Clinical methods of cryopreservation for donor lymphocyte infusions vary in their ability to preserve functional T-cell subpopulations. *Transfusion*. 2017;57: 1555-1565.
41. Castagna L, Sarina B, Bramanti S, Perseghin P, Mariotti J, Morabito L. Donor lymphocyte infusion after allogeneic stem cell transplantation. *Transfus Apher Sci*. 2016;54:345-355.
42. Mukherjee S, Reddy O, Panch S, Stroncek D. Establishment of a cell processing laboratory to support hematopoietic stem cell transplantation and chimeric antigen receptor (CAR)-T cell therapy. *Transfus Apher Sci*. 2021;60:103066.
43. Haastrup E, Ifversen MRS, Heilmann C, Fischer-Nielsen A. Depletion of αβ+ T and B cells using the CliniMACS prodigy: results of 10 graft-processing procedures from haploidentical donors. *Transfus Med Hemother*. 2019;46:446-449.
44. Li Pira G, Biagini S, Cicchetti E, et al. Immunoselection techniques in hematopoietic stem cell transplantation. *Transfus Apher Sci*. 2016;54: 356-363.
45. Stroncek DF, Tran M, Frodigh SE, et al. Preliminary evaluation of a highly automated instrument for the selection of CD34+ cells from mobilized peripheral blood stem cell concentrates. *Transfusion*. 2016; 56:511-517.
46. Schuum M, Lang P, Handgretinger R. Graft manipulation. In: Carreras E, Dufour C, Mohty M, Krögel N, eds. *The EBMT Handbook Hematopoietic Stem Cell Transplantation and Cellular Therapies*. European society for blood and marrow transplantation; 2019:137-142.
47. Foell J, Kleinschmidt K, Jakob M, Troeger A, Corbacioglu S. Alternative donor: αß/CD19 T-cell-depleted haploidentical hematopoietic stem cell transplantation for sickle cell disease. *Hematol Oncol Stem Cell Ther*. 2020;13:98-105.
48. Schumm M, Lang P, Bethge W, et al. Depletion of T-cell receptor alpha/beta and CD19 positive cells from apheresis products with the CliniMACS device. *Cytotherapy*. 2013;15:1253-1258.
49. Karanu F, Ott L, Webster DA, Stehno-Bittel L. Improved harmonization of critical characterization assays across cell therapies. *Regen Med*. 2020;15:1661-1678.
50. Mfarrej B, Gaude J, Couquiaud J, Calmels B, Chabannon C, Lemarie C. Validation of a flow cytometry-based method to quantify viable lymphocyte subtypes in fresh and cryopreserved hematopoietic cellular products. *Cytotherapy*. 2021;23:77-87.
51. Foundation for the Accreditation of Cellular Therapy (FACT). Standards for Immune Effector Cells, First Edition, Version 1.1, March 2018.
52. Demaret J, Varlet P, Trauet J, et al. Monitoring CAR T-cells using flow cytometry. *Cytometry B Clin Cytom*. 2021;100:218-224.
53. Maryamchik E, Gallagher KME, Preffer FI, Kadauke S, Maus MV. New directions in chimeric antigen receptor T cell [CAR-T] therapy and related flow cytometry. *Cytometry B Clin Cytom*. 2020;98:299-327.
54. Lee YH, Koh H, Nam E, Kim YJ. Cryopreserved cord blood mononuclear cells in DMSO are healthy for at least 6 hours after thawing. *Transfus Apher Sci*. 2020;59:102603.
55. Huang L, Song GQ, Wu Y, Wang J, Sun ZM. Optimal length of time of cryopreserved umbilical cord blood infusion after thawing. *Hematology*. 2014;19:73-79.
56. Krasna M, Malicev E, Rozman JZ, Vrtovec B. Assessment of stability of CD34+ cell products enriched by immunoselection from peripheral blood mononuclear cells during refrigerated storage. *Transfus Apher Sci*. 2017;56:566-570.
57. Matos DM. Standardization of a compensation matrix for the use in an ISHAGE-based single-platform for CD34+ stem cell quantitation on the FACSVia™ flow cytometer. *Int J Lab Hematol*. 2020;42: 198-205.
58. Bainbridge J, Rountree W, Louzao R, et al. Laboratory accuracy improvement in the UKNEQAS leucocyte Immunophenotyping immune monitoring program: an eleven-year review via longitudinal mixed effects modeling. *Cytometry B Clin Cytom*. 2018;94:250-256.
59. Levering WH, Preijers FW, van Wieringen WN, et al. Flow cytometric CD34+ stem cell enumeration: lessons from nine years' external quality assessment within the Benelux countries. *Cytometry B Clin Cytom*. 2007;72:178-188.
60. International Organization for Standardization (ISO). *EN ISO 15189-Medical Laboratories_Requirements for Quality and Competence*. 3rd ed. ISO; 2012.

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RESEARCH ARTICLE

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Comparison of spectra optia and amicus cell separators for autologous peripheral blood stem cell collection

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Abstract

Introduction: Autologous peripheral blood stem cell (PBSC) transplantation has become a standard treatment option for many oncology patients. The aim of this study was to evaluate the performance of two cell separators, Spectra Optia (Terumo BCT, Japan) and Amicus (Fresenius-Kabi) for autologous PBSC collection.

Methods: We retrospectively evaluated 56 apheresis by Spectra Optia with Continuous Mononuclear Cell Collection (cMNC) from 20 patients, and 50 apheresis by Amicus from 27 patients between December 2018 and December 2019. CD34+ collection efficiency (CE2) and platelet (PLT) loss were evaluated.

Results: There was no significant difference in CD34+ CE2 between Spectra Optia with cMNC (median, 28.8%) and Amicus (median, 33.1%; $P = 0.537$). PLT loss was significantly lower in Amicus (median, 28.6%) than in Spectra Optia with cMNC (median, 37.8%; $P = 0.009$).

Conclusion: CD34+ CE2 was comparable between Spectra Optia and Amicus, and PLT loss was significantly lower in Amicus. To the best of our knowledge, this is the first report comparing autologous PBSC collection of the Spectra Optia and Amicus. These results may provide general guidance with regard to device selection to apheresis clinics that use both separators for optimal outcomes depending on each patient's characteristics.

KEYWORDS

apheresis, cell separator, collection, peripheral blood stem cell

1 | INTRODUCTION

Autologous peripheral blood stem cell (PBSC) transplantation has become a standard treatment option for many oncology patients.[1,2] Stem cell-rich blood cells are generated through enforced mobilization with granulocyte-colony-stimulating factor (G-CSF) with or without chemotherapy. After stem cell mobilization, PBSCs are collected by leukapheresis using blood cell separators. The leukapheresis procedure is one of the critical factors affecting the outcome of PBSC transplantation. Optimal outcomes require efficient stem cell collection and minimal platelet (PLT) attrition.

There are several blood cell separators used for PBSC collection. And the currently available separators worldwide are the Spectra Optia (Terumo BCT, Tokyo, Japan) and Amicus (Fresenius-Kabi, Lake Zurich, IL). The Spectra Optia system is a recently introduced apheresis

system, replacing the COBE Spectra (Terumo BCT). Unlike its predecessor, the Spectra Optia automatically detects and maintain buffy coat interfaces.[3] The Continuous Mononuclear Cell Collection (cMNC) protocol in Spectra Optia is a single-stage procedure in which target cells are continuously flushed into a collection container.[4,5] The Amicus which has been used for PLT apheresis, with the additional development of software for cell separation, replaced the CS-3000+ which has been used for years for leukapheresis.[6] The Amicus is a continuous-flow system with cyclic cell harvests, which is also equipped with automated collection protocols.[7] The aim of this study was to evaluate the performance of these two cell separators for autologous PBSC collection.

2 | MATERIALS AND METHODS

2.1 | Patients and ethics statement

We retrospectively evaluated a total of 106 apheresis data from 42 adult patients whose PBSC apheresis procedures were performed at the National Cancer Center between December 2018 and December 2019. Specifically, 56 apheresis from 20 patients using the Spectra Optia with cMNC, and 50 apheresis from 27 patients using the Amicus. Five patients underwent procedures using both the Spectra Optia and Amicus. The study was approved by the Institutional Review Board at the National Cancer Center, Korea (No. NCCNCS13745).

2.2 | Apheresis procedure

PBSCs were collected according to the institution's mobilization protocol using chemotherapy plus mobilization agents (G-CSF with or without plerixafor) or mobilization agents only. It was recommended that PBSCs be collected on the day that the CD34+ cell count was ≥5/μL or the white blood cell (WBC) count was >3.0 x10^9/L,[8-10] however, it was ultimately determined through a clinical decision. Patients undergoing PBSC collection were assigned to a Spectra Optia or Amicus in a random manner. Settings as per the manufacturer's recommendations were used. For apheresis by Spectra Optia, collect rate was set at 1.0 mL/min. For apheresis by Amicus, the MNC and RBC offset settings were 1.5 and 6.8. For all PBSC collections (Spectra Optia and Amicus), a blood flow rate was 60-70 mL/min. The whole blood to anticoagulant (3000 IU heparin with 500 mL of acid-citrate-dextrose solution) ratio was routinely 24:1. The end point was a total processed volume (TPV) of 3-5 times the patient's estimated blood volume. The CD34+ cell dose per kg body weight was determined. The apheresis target dose for one autologous PBSC transplantation was ≥3 × 10^6 CD34+ cells/kg body weight before freezing.

WBC, PLT, mononuclear cell (MNC), and hematopoietic progenitor cell (HPC) counts in the peripheral blood and PBSC products were determined using an XE-2100 hematology analyzer (Sysmex Corporation, Kobe, Japan) with differential counts determined by manual counting. CD34+ cells were also counted in the peripheral blood pre-apheresis only and PBSC products by flow cytometry (FACS Canto II, Becton, Dickinson and Company, San Jose, CA) using a single-platform assay (BD stem cell enumeration kit, Becton Dickinson and Company, Franklin Lakes, NJ).[11,12]

2.3 | Collection efficiency and platelet loss

The collection efficiency (%) was calculated using a well-known "CE2" model as follows: (total CD34+ cells obtained from apheresis ×100)/[peripheral CD34 + cells/μL × apheresis blood volume processed (μL)]. PLT loss during apheresis was calculated using the formula: PLT loss (%) = [(pre-apheresis peripheral PLT/μL-post-apheresis peripheral PLT/μL)/pre-apheresis PLT/μL] × 100.[13,14] Patients who received PLT transfusion prior to PBSC collection were excluded from the PLT loss (%) calculation.

2.4 | Statistical analysis

Descriptive data are presented as a median with range. The significance of the observed differences between Spectra Optia and Amicus was assessed with the Chi-squared test or Mann-Whitney *U* test, as appropriate. A Pearson correlation was applied to determine the relation between pre-apheresis peripheral markers by an automatic hematologic analyzer and the CD34+ cell count by flow cytometry. SPSS Statistics 24 (IBM SPSS Inc., Chicago, IL) was used for statistical analyses and a 2-tailed *P* value of 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Patient characteristics

Of the 42 patients, 26 (61.9%) were male and 16 (38.1%) were female with a median age of 58 years (range, 22-64). Diagnosis of patients were as follows: multiple myeloma, 22 (52.4%); diffuse large B-cell lymphoma, 10 (23.8%);

TABLE 1 Demographic and clinical characteristics of patients who underwent peripheral blood stem cell (PBSC) collection by Spectra Optia and Amicus

	Spectra Optia with cMNC	Amicus	*P* value
No. of patients	20	27	
Sex			.889
Male	13 (65.0%)	17 (63.0%)	
Female	7 (35.0%)	10 (37.0%)	
Age (years, median)	58 (40-64)	58 (22–64)	.325
Diagnosis			.154
Multiple myeloma	14 (70.0%)	11 (40.8%)	
Diffuse large B-cell lymphoma	4 (20.0%)	7 (25.9%)	
Non-Hodgkin lymphoma	2 (10.0%)	6 (22.2%)	
Others	0 (0.0%)	3 (11.1%)	
Mobilization regimens			.084
Chemotherapy plus mobilization agents	6 (30.0%)	15 (55.6%)	
Mobilization agents only	14 (70.0%)	12 (44.4%)	

Abbreviation: cMNC, continuous mononuclear cell collection.

TABLE 2 Laboratory parameters (medians) of pre-apheresis peripheral blood and collected products

	Spectra Optia with cMNC	Amicus	*P* value
No. of apheresis	56	50	.132
On the first or second day[a]	30 (53.6%)	34 (68.0%)	
On or after the third day[a]	26 (46.4%)	16 (32.0%)	
Pre-apheresis peripheral blood parameters			
WBC ($x10^9$/L)	34.0 (2.0-94.5)	32.7 (1.5-97.5)	.790
PLT ($x10^9$/L)	82 (28-279)	90 (35-269)	.597
MNC ($x10^9$/L)	4.0 (0.9-25.5)	4.2 (0.5-32.7)	.924
HPC ($x10^9$/L)	13 (0-183)	16 (0-233)	.657
CD34+ cells (/uL)	18 (1-144)	17 (1–191)	.798
Total processed blood volume (mL)	15 926 (12233-19 845)	14 411 (10959-19 090)	**< .001**
Collected products			
Total volume (mL)	270 (204-322)	234 (157-310)	**<.001**
WBC ($x10^9$)	37 (13-84)	35 (7-59)	.161
PLT ($x10^9$)	70 (34-335)	62 (22-173)	.173
MNC ($x10^9$)	25 (4-68)	21 (2-46)	**.031**
Hematocrit (%)	2.0 (0.6-4.0)	4.6 (1.1-10.5)	**<.001**
CD34+ cells ($x10^6$)	78 (5-1654)	77 (2-691)	.695
Performance			
Collection efficiency, CE2 (%)	28.8 (4.0-79.7)	33.1 (5.7-132.4)	.537
Platelet loss (%)[†]	37.8 (7.1-53.9)	28.6 (3.6-54.2)	**.009**

Abbreviations: cMNC, continuous mononuclear cell collection; WBC, white blood cell; PLT, platelet; MNC, mononuclear cell; HPC, hematopoietic progenitor cell; PBSC, peripheral blood stem cell.
Bold shows the statistical significance with *P* value less than .05.
[a]The day of collection as autologous PBSC collection is performed as consecutive daily procedures. [†]PLT loss (%) were analyzed for 50 cases of Spectra Optia with cMNC and 45 cases of Amicus. Patients who received PLT transfusions prior to PBSC collection were excluded from the PLT loss (%) calculations.

non-Hodgkin lymphoma, 7 (16.7%); and others, 3 (7.1%). Nineteen patients (45.2%) were mobilized with chemotherapy plus mobilization agents and 23 patients (54.8%) with mobilization agents only. There were no significant differences in sex, age, diagnosis, or mobilization regimen between the Spectra Optia and Amicus groups (Table 1).

3.2 | Characterization of apheresis

For the 106 apheresis conducted, the median pre-apheresis peripheral blood parameter values were as follows: WBC, 33.8×10^9/L (range, 1.5-97.5); PLT, 84×10^9/L (range, 28-279); MNC, 4.1×10^9/L (0.5-32.7); HPC by hematology analyzer, 15×10^9 L (range, 0-233); CD34+ cells by flow cytometry, 18/μL (range, 1-191). The median TPV was 15 510 mL (range, 11 416-19 885) (Table 2). The number of apheresis underwent with pre-apheresis peripheral CD34+ cells <5/μL was 11 for Spectra Optia, and 10 for Amicus. And the number of apheresis mobilized with plerixafor was 12 for Spectra Optia, and 4 for Amicus.

There were no significant differences in pre-apheresis values for the peripheral WBC count, PLT count, MNC count, HPC count by the hematology analyzer, and CD34 + cell count by flow cytometry between the Spectra Optia and Amicus groups. The TPV (mL) was significantly larger in the Spectra Optia group (median, 15 926) than in the Amicus group (median 14 411, $P < .001$). There was no significant difference in the frequency of pre-apheresis peripheral CD34+ cells <5/μL between the two groups ($P = .963$). The frequency of mobilization with plerixafor was higher in the Spectra Optia group (21.4%) than in the Amicus group (8.0%) with marginal significance ($P = .055$).

3.3 | Collection efficiency and platelet loss

There was no statistically significant difference in CD34+ CE2 (%) between the Spectra Optia group (median, 28.8) and the Amicus group (median, 33.1, $P = .537$). There also were no significant differences in CD34+ CE2 (%) between the two systems when further analysis was performed excluding apheresis with pre-apheresis peripheral CD34+ cells <5/μL ($P = .107$), or excluding apheresis mobilized with plerixafor ($P = .878$).

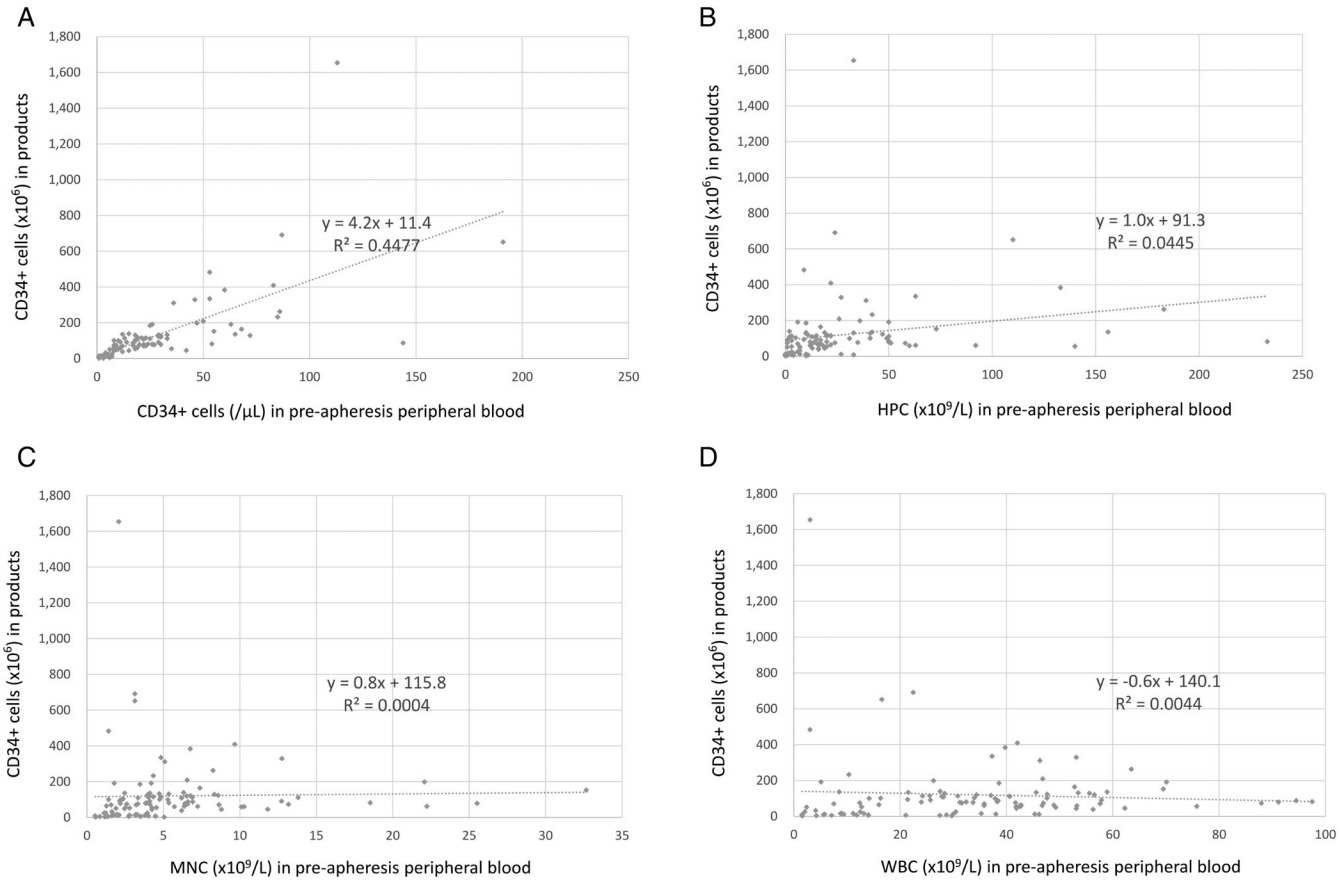

FIGURE 1 Scatter plots are presented between CD34+ cells ($x10^6$) in products and A, CD34+ cells (/μL), B, HPC ($x10^9$/L), (C) MNC ($x10^9$/L), and (D) WBC ($x10^9$/L) in pre-apheresis peripheral blood. HPC, hematopoietic progenitor cell; MNC, mononuclear cell, WBC, white blood cell

PLT loss (%) were analyzed for 50 cases of Spectra Optia with cMNC and 45 of Amicus excluding cases with PLT transfusions prior to PBSC collection. PLT loss (%) was significantly higher in the Spectra Optia group (median, 37.8) than in the Amicus group (median, 28.6, $P = .009$) (Table 2).

3.4 | Correlation between parameters of peripheral blood and CD34+ cell count

When the correlation between each parameter (WBC count, MNC count, HPC count by the hematology analyzer, and CD34+ cell count by flow cytometry) in the pre-apheresis peripheral blood and final CD34+ count in the products was analyzed, the CD34+ cell count exhibited the best correlation ($r = .669$, $P < .001$), followed by HPC ($r = .211$, $P = .030$). MNC ($r = .020$, $P = .835$), and WBC ($r = -.066$, $P = .499$) showed no significant correlation (Figure 1). When the correlation between each parameter by an automated hematology analyzer and CD34+ cell count in the pre-apheresis peripheral blood was analyzed, the best surrogate marker for CD34+ cell count was HPC ($r = .497$, $P < .001$), followed by WBC ($r = .306$, $P = .001$), and MNC ($r = .232$, $P = .017$).

4 | DISCUSSION

In this study, we compared the autologous PBSC collection performance between two apheresis devices which are currently in use, Spectra Optia and Amicus. With regard to the Spectra Optia, we evaluated the cMNC protocol as it was developed later than Mononuclear Cell Collection (MNC) protocol and is currently in use worldwide.

Collection efficiency was comparable between the Spectra Optia with cMNC and the Amicus groups. Our results were different from that of a recent report which compared the Spectra Optia with MNC protocol and Amicus in leukapheresis for adoptive immunotherapies; it was concluded that collection efficiency was higher for the Spectra Optia with MNC than the Amicus in non-cytokine stimulated donors.[15] In terms of PLT loss, Amicus yielded lower attrition. This result was in line with prior studies which reported the Amicus generated products with a lower PLT content than other cell separators, the Spectra Optia with MNC[15] or the previous CS-3000 + .[6,16]

It is well known that the circulating CD34+ cell/μL can be correlated with the product CD34+ cell.[17,18] This study also showed a high correlation of CD34+ cell counts in pre-apheresis peripheral blood with those in the products. However, the CD34+ cell count using flow cytometry was impractical for many reasons. It has not yet been automated and therefore took considerable time and labor, including experienced technicians and laboratory personnel. Moreover, CD34+ cells in peripheral blood are sparse relative to other nucleated cells, making it difficult to correctly count such a small cell population. Among the parameters that could be easily obtained by an automatic hematology analyzer, the HPC count showed a better correlation with the CD34+ cell count and was a better predictor than WBC or MNC counts. In clinical laboratories where CD34+ count by flow cytometry is not available, the HPC count by a hematology analyzer can be monitored as a surrogate marker for CD34+ cell counts.

The limitation of this study is that we were not able to calculate the CE1 which is a more accurate parameter than CE2, as post-apheresis peripheral CD34+ cell counts are not routinely performed at our center. In addition, the nonmatched retrospective character of the present analysis imposed inborn limitations.

In conclusion, our results demonstrated that collection efficiencies were comparable between the two systems and PLT loss was significantly lower in the Amicus. It would be considerate to choose a cell separator based on the patient's characteristics in apheresis clinics that operate both systems. From these results, our apheresis clinic favorably applied the Amicus in patients with low PLT counts. To the best of our knowledge, this was the first report regarding autologous PBSC collection that compared the Spectra Optia and Amicus. This result may be of particular interest for apheresis clinics using both separators and may give general advice with regard to device selection for an optimal outcome depending on each patient's characteristics. Further reports with prospective, randomized controlled clinical trials could provide additional insight for choices of cell separators for PBSC collection.

CONFLICT OF INTEREST

All authors declare no conflict of interest.

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REFERENCES

1. Barlogie B, Shaughnessy J, Tricot G, et al. Treatment of multiple myeloma. *Blood*. 2004;103(1):20-32.
2. Li DQ, Hou YF, Wu J, et al. Gene expression profile analysis of an isogenic tumour metastasis model reveals a functional role for oncogene AF1Q in breast cancer metastasis. *European Journal of Cancer*. 2006;42(18):3274-3286.
3. Brauninger S, Bialleck H, Thorausch K, Seifried E, Bonig H. Mobilized allogeneic peripheral stem/progenitor cell apheresis

with spectra Optia v.5.0, a novel, automatic interface-controlled apheresis system: results from the first feasibility trial. *Vox Sang*. 2011;101(3):237-246.

4. Spectra Optia Apheresis System n.d. https://www.terumobct.com/Spectra-Optia [Accessed May 25, 2017].
5. Punzel M, Kozlova A, Quade A, Schmidt AH, Smith R. Evolution of MNC and lymphocyte collection settings employing different spectra Optia([R]) Leukapheresis systems. *Vox Sang*. 2017;112(6):586-594.
6. Snyder EL, O'Donnell L, Dengler TJ, et al. Ex vivo evaluation of PBMNCs collected with a new cell separator. *Transfusion*. 2001;41(7):940-949.
7. Moog R. Comparison of two continuous-flow systems for the collection of peripheral progenitor cells. *Stem Cells Dev*. 2004;13(4):357-361.
8. Suh C, Kim S, Kim SH, et al. Initiation of peripheral blood progenitor cell harvest based on peripheral blood hematopoietic progenitor cell counts enumerated by the Sysmex SE9000. *Transfusion*. 2004;44(12):1762-1768.
9. Kim MK, Kim S, Jang G, et al. A randomized comparison of peripheral blood hematopoietic progenitor cell level of 5/mm3 versus 50/mm3 as a surrogate marker to initiate efficient autologous blood stem cell collection. *J Clin Apher*. 2007;22(5):277-282.
10. Lefrere F, Zohar S, Beaudier S, et al. Evaluation of an algorithm based on peripheral blood hematopoietic progenitor cell and CD34+ cell concentrations to optimize peripheral blood progenitor cell collection by apheresis. *Transfusion*. 2007;47(10):1851-1857.
11. Keeney M, Chin-Yee I, Weir K, Popma J, Nayar R, Sutherland DR. Single platform flow cytometric absolute CD34 + cell counts based on the ISHAGE guidelines. International Society of Hematotherapy and Graft Engineering. *Cytometry*. 1998;34(2):61-70.
12. Sutherland DR, Nayyar R, Acton E, Giftakis A, Dean S, Mosiman VL. Comparison of two single-platform ISHAGE-based CD34 enumeration protocols on BD FACSCalibur and FACSCanto flow cytometers. *Cytotherapy*. 2009;11(5):595-605.
13. Ford CD, Lehman C, Strupp A, Kelley L. Comparison of CD34 + cell collection efficiency on the COBE spectra and Fenwal CS-3000 plus. *J Clin Apher*. 2002;17(1):17-20.
14. Reinhardt P, Brauninger S, Bialleck H, et al. Automatic interface-controlled apheresis collection of stem/progenitor cells: results from an autologous donor validation trial of a novel stem cell apheresis device. *Transfusion*. 2011;51(6):1321-1330.
15. Steininger PA, Strasser EF, Weiss D, Achenbach S, Zimmermann R, Eckstein R. First comparative evaluation of a new leukapheresis technology in non-cytokine-stimulated donors. *Vox Sang*. 2014;106(3):248-255.
16. Jeanne M, Bouzgarrou R, Lafarge X, et al. Comparison of CD34 + cell collection on the CS-3000+ and amicus blood cell separators. *Transfusion*. 2003;43(10):1423-1427.
17. Wu FY, Heng KK, Salleh RB, et al. Comparing peripheral blood stem cell collection using the COBE spectra, Haemonetics MCS+, and Baxter amicus. *Transfusion Aphere Sci*. 2012;47(3):345-350.
18. Basquiera AL, Abichain P, Damonte JC, et al. The number of CD34(+) cells in peripheral blood as a predictor of the CD34 (+) yield in patients going to autologous stem cell transplantation. *J Clin Apher*. 2006;21(2):92-95.

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RESEARCH ARTICLE

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A new protocol for improving efficiency of autologous peripheral blood stem cell collection in patients with high white blood cell counts

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Abstract

Background: Collection efficiency (CE) of peripheral blood stem cell (PBSC) collections is negatively affected by increasing white blood cell (WBC) counts of the patient. This study compared a new optimized mononuclear cell (MNC) collection protocol (OPP) to the standard MNC collection protocol recommended by the manufacturer (STP) for PBSC collection in patients with WBC counts >35 000/μL.

Study design and methods: Single-center, retrospective, and observational study of 81 autologous PBSC collections on Fenwal Amicus cell separators in 70 adult patients.

Results: Median peripheral WBC count ($\times 10^3$/μL; 44.2 in OPP group vs 46.5 in STP group) and median $CD34^+$ count (105/μL in OPP group vs 40/μL in STP group) at the beginning of PBSC collection did not differ significantly. Median CE2 (45% vs 31%; $P < .001$) as well as $CD34^+$ yield of the apheresis product both with regards to median absolute $CD34^+$ content ($\times 10^6$; 793 vs 188; $P = .001$) as well as median $CD34^+$ content ($\times 10^6$)/kg body weight (8.93 vs 2.51; $P = .002$) were significantly higher for the OPP. Overall, 18/21 (86%) patients with the OPP obtained their target $CD34^+$ amount with a single apheresis session, compared to 25/50 (50%) with the STP ($P = .005$). PBSC collections using OPP lasted significantly longer (median 377 minutes vs 260 minutes; $P < .001$) than with the STP.

Conclusions: The OPP significantly improves CE2 for PBSC collections on Fenwal Amicus cell separators in patients with pre-apheresis WBC counts >35 000/μL and significantly reduces the necessity for multiple apheresis sessions. The OPP is therefore suited to reduce both patient burden and cost in autologous PBSC collection.

KEYWORDS

high WBC counts, improved collection efficiency, PBSC collection

1 | INTRODUCTION

Autologous hematopoietic stem cell transplantation (HSCT) has become standard of care for various hematological disorders in adult patients.[1] It involves the collection of peripheral blood stem cells (PBSC) by apheresis through automated cell separators after mobilization with granulocyte-colony stimulating factor (GCSF),

usually in combination with chemotherapy.[2] PBSC collection has to be both safe for the patient and efficient, that is, yielding an adequate amount of viable $CD34^+$ cells to ensure post-transplant engraftment.[3] Collection efficiency (CE) is a measure for the proportion of $CD34^+$ cells harvested per volume processed at a given $CD34^+$ cell count in the peripheral blood.[4]

Studies have demonstrated that CE is influenced by various factors such as the type of automated cell separator or the total processed blood volume (TPV) during apheresis.[5-8] A relevant patient-related factor with regard to CE is the patient's pre-apheresis white blood cell (WBC) count. High pre-apheresis WBC counts have a negative impact on CE, regardless of the type of apheresis device.[8-10]

With regard to the Fenwal Amicus cell separator, CE can be improved by adjusting various machine settings. According to the manufacturer, apheresis cycle volume can be adjusted from 1400 to 1000 mL/cycle, if the pre-apheresis WBC count exceeds 35 000/μL. The positive effect of a reduced cycle volume on CE has also been demonstrated for pre-apheresis WBC counts below 35 000/μL.[11] Poor CE can also be related to high flow rates, especially if WBC counts are elevated; therefore, limiting flow rates is another option to improve CE.[9,12] The offsets which control the beginning and end of mononuclear cell (MNC) transfer into the collection bag are also adjustable. Monitoring MNC transfer and adjusting offsets can lead to higher $CD34^+$ collection results.[13]

At our institution, a relevant number of patients with WBC counts >35 000/μL did not reach the target $CD34^+$ cell amount with a single apheresis procedure on the Fenwal Amicus cell separator, despite adjusting the cycle volume to 1000 mL/cycle. In order to improve CE and therefore reduce the necessity of repetitive PBSC collections and costs, we devised an optimized MNC collection protocol (OPP) with lower cycle volume and limited flow rate for the Fenwal Amicus cell separator. This study compares this new OPP to the standard MNC collection protocol recommended by the manufacturer (STP) for PBSC collection in patients with WBC counts >35 000/μL.

2 | MATERIALS AND METHODS

2.1 | Patient selection and stem cell mobilization

This is a single-center, retrospective, and observational study of autologous PBSC collection in adult patients with WBC counts >35 000/μL from 2010 to 2019. The study was approved by the local ethics committee. Patient eligibility and mobilization regime for autologous PBSC collection were at the discretion of the treating physician. All patients undergoing PBSC collection gave written informed consent. The $CD34^+$ cell dose per HSCT was 2.5×10^6 $CD34^+$ cells/kg body weight. The number of autologous HSCT defining the target amount of $CD34^+$ cells to be collected per individual patient was based on diagnosis and treatment protocol. Therefore, the target for PBSC collection was 2.5×10^6 (one autologous HSCT), 5.0×10^6 (two autologous HSCT), or 7.5×10^6 (three autologous HSCT) $CD34^+$ cells/kg body weight. PBSC mobilization was performed with GCSF (10 μg/kg/day) after administration of vinorelbine or following conventional chemotherapy (non-vinorelbine chemotherapy).[14] PBSC collection was initiated when peripheral $CD34^+$ cell count reached 20/μL. Plerixafor was used if patients failed to mobilize sufficient $CD34^+$ cells.

If the target amount was not obtained with a single apheresis procedure, GCSF mobilization was continued and PBSC collection repeated on consecutive days.

2.2 | Apheresis

All PBSC collections were performed on Fenwal Amicus apheresis devices (Fresenius-Kabi AG, Oberdorf, Switzerland; Version 2.5.15 until January 2011; Version 3.11 from February 2011 until April 2016; Version 4.4 since May 2016). Mode of vascular access (peripheral vs central venous) was at the discretion of the apheresis technician. Anticoagulant Citrate Dextrose Solution (ACD-A, Fresenius-Kabi) was used at a 1:12 ratio with an infusion rate of 1.5 to 2.0 mg/kg/min. To prevent citrate-related side effects, calcium gluconate 10% (B. Braun Melsungen AG, Melsungen, Germany), diluted in normal saline 0.9% (Sintetica SA, Mendrisio, Italy), was infused. The STP comprised a cycle volume of 1000 mL for 14 cycles, resulting in a TPV of 14 L for all patients, irrespective of total blood volume. The maximum whole blood flow rate for the STP was 80 mL/min.

For our OPP, we lowered cycle volume to 600 mL and increased cycle numbers to 24 to maintain TPV. The maximum whole blood flow rate was limited to 60 mL/min. All adjustments were in line with the manufacturer's specifications of the cell separator.[15] All other parameters were retained for both protocols (including MNC collection setpoint at 0.6). MNC offset volume was 2.3 mL, red blood cell (RBC) offset volume 6.5 to 6.8 mL. Exceptional adjustments of offsets for individual procedures according to Burgstaler et al. were at the discretion of the apheresis technician.[13]

2.3 | Blood counts and CD34+ measurement

Peripheral CD34+ cell counts prior to PBSC collection were measured by flow cytometry using the BD Stem cell enumeration Kit on BD FACSCalibur until 2013, thereafter on BD FACSCanto II instruments (BD Biosciences, San Jose, California) according to the manufacturer's instructions. The same protocol was used to quantitate viable CD34+ cells in the apheresis product within 24 hours after PBSC (storage temperature 4°C +/−2°C). Patients' blood counts before and after PBSC collection were determined on ADVIA 2120i (Siemens Healthcare, Zuerich, Switzerland), Sysmex KX-21 N (Sysmex Suisse, Horgen, Switzerland) or Sysmex XN-1000 (Sysmex Suisse). The apheresis product's cellular counts were obtained on Sysmex XN-5000 (Sysmex Suisse) or Sysmex XN-20 (Sysmex Suisse).

2.4 | Data collection and statistical analysis

Data acquisition was performed as part of our institution's routine quality control program for PBSC collections. Data recorded included: age, sex, total blood volume, body weight, diagnosis, mobilization regime, CD34+ target amount, vascular access, pre-apheresis complete blood and CD34+ cell count, CE, product cellular and CD34+ content, duration of apheresis, TPV (without correction for ACD), and product volume. CE was calculated as follows:

$$\text{CE2} = \frac{\text{absolute CD34}^+ \text{ content product} \times 10^6}{\text{whole blood volume processed [mL]} \times \text{pre-apheresis CD34}^+ \text{ count [/µL]} \times 10^3}$$

In addition, the rate of adverse events during PBSC infusion graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) was recorded. Data are reported as median with range. Statistical analysis was performed using Chi-squared test for categorical variables and Mann-Whitney-*U*-test for continuous variables. A *P*-value <.05 was considered statistically significant. Data were analyzed with MedCalc Statistical Software version 19.0.7 (MedCalc Software bvba, Ostend, Belgium; https://www.medcalc.org; 2019).

3 | RESULTS

We analyzed a total of 81 autologous PBSC collections in 70 adult patients with WBC counts >35 000/µL between May 2010 and April 2019. Between May 2010 and April 2016, we performed 59 collections according to STP in 49 patients. The OPP was used in 22 collections in 21 patients since May 2016.

Patient characteristics are given in Table 1. The distributions of sex, age, total blood volume, body weight, and diagnoses were similar between both groups. Eight patients had two PBSC collections according to STP on consecutive days; one patient had three procedures according to STP, the first PBSC collection after mobilization following non-vinorelbine chemotherapy/GCSF, the second and third collection on consecutive days after mobilization with vinorelbine/GCSF. One patient had two PBSC collections according to OPP on consecutive days. PBSC mobilization did not differ between groups with regard to GCSF administration following vinorelbine vs non-vinorelbine chemotherapy ($P = .46$). However, ten patients (20%) in the STP group received plerixafor as part of their mobilization regime, none in the OPP group. There was a significant difference with regard to central venous vascular access between both groups (70% STP group vs 33% OPP group; $P = .005$). For patients with multiple PBSC collections, the mode of vascular access (central venous vs peripheral) did not change between procedures. The target CD34+ amount of the individual patient (for one, two or three autologous HSCT) did not differ significantly between both groups ($P = .35$).

Table 2 displays the peripheral blood counts at the beginning of PBSC collection. WBC counts ($\times 10^3$/µL) did not differ significantly between study groups (46.5 in STP group vs 44.2 in OPP group; $P = .37$); CD34+ counts did also not significantly differ (105/µL in OPP group; 40/µL in STP group; $P = .11$). Median hematocrit was significantly higher in the OPP group (38% vs 32%; $P = .002$). The comparisons of the apheresis protocols are given in Table 2. CE2 (45% vs 31%; $P < .001$) and CD34+ yield of the apheresis products both with regards to absolute CD34+ content ($\times 10^6$; 793 vs 188; $P = .001$) and CD34+

TABLE 1 Patient characteristics

	STP	OPP	*P*-value
Number of patients (n)	49	21	
Sex male/female (n)	30 (61%)/ 19 (39%)	14 (67%)/ 7 (33%)	n.s.
Age[a] (years)	57 [20-70]	56 [30-72]	n.s.
Total blood volume[a] (L)	5.04 [3.08-7.84]	5.39 [3.29-9.80]	n.s.
Body weight[a] (kg)	72 [44-112]	77 [47-140]	n.s.
Diagnosis (n)			n.s.
Plasmacell Myeloma	21 (43%)	14 (67%)	
Hodgkin disease	2 (4%)	0	
Non-Hodgkin lymphoma	17 (35%)	3 (14%)	
Acute leukemia	6 (12%)	3 (14%)	
Solid tumor	3 (6%)	1 (5%)	
PBSC mobilization (n)[b]			n.s.
vinorelbine/GCSF	39 (78%)	18 (86%)	
Non-vinorelbine[c]/GCSF	11 (22%)	3 (14%)	
Vascular access for apheresis (n)[b]			.005
Peripheral access	15 (30%)	14 (67%)	
Central venous catheter	35 (70%)	7 (33%)	
$CD34^+$ target amount (n)[b]			n.s.
1 HSCT (2.5×10^6/kg bw)	28 (56%)	9 (43%)	
2 HSCT (5.0×10^6/kg bw)	18 (36%)	8 (38%)	
3 HSCT (7.5×10^6/kg bw)	4 (8%)	4 (19%)	

Abbreviations: bw, body weight; GCSF, granulocyte-colony stimulating factor; HSCT, hematopoietic stem cell transplantation; n.s., not significant; OPP, optimized protocol; PBSC, peripheral blood stem cells; STP, standard protocol.
[a]Reported as median [range].
[b]One patient in the STP group had two separate PBSC mobilizations.
[c]Includes R-CHOP, R-DHAP, paclitaxel, ifosfamide, high-dose chemotherapy according to HOVON protocols (Ara-C, daunorubicin).

TABLE 2 Comparison of apheresis protocols

	STP (n = 59)	OPP (n = 22)	*P*-value
WBC count pre-apheresis ($\times 10^3/\mu L$)	46.5 [35.1;123.1]	44.2 [35.4;62.3]	n.s.
Hematocrit pre-apheresis (%)	32 [19;43]	38 [26;47]	.002
Platelet count pre-apheresis ($\times 10^3/\mu L$)	172 [14;428]	214 [70;385]	n.s.
$CD34^+$ count pre-apheresis (/μL)	40 [3;851]	105 [17;360]	n.s
CE2	31% [3;74]	45% [21;77]	< .001
$CD34^+$ content product ($\times 10^6$)	188 [24;1689]	793 [104;2091]	.001
$CD34^+$ content product/kg bw ($\times 10^6$)	2.51 [0.38;28.62]	8.93 [1.21;41.00]	.002
WBC content product ($\times 10^9$)	52.3 [22.7;80.4]	97.7 [50.6;333.6]	< .001
Apheresis duration (min)	260 [213;402]	377 [231;419]	< .001
TPV (L)	16.05 [11.41;19.32]	17.39 [10.87;18.18]	.02
Average whole blood flow rate (mL/min)	62 [40;67]	46 [41;57]	< .001
Apheresis product volume (mL)	171 [140;225]	292 [159;312]	< .001

Note: Data are reported as median [range].
Abbreviations: bw, body weight; CE2, collection efficiency 2; n.s., not significant; OPP, optimized protocol; STP, standard protocol; TPV, total processed blood volume; WBC, white blood cell.

content ($\times 10^6$)/kg body weight (8.93 vs 2.51; $P = .002$) were significantly higher for the OPP. The significant effect of the OPP compared to the STP on CE2 and $CD34^+$ yield was maintained when patient subgroups according to peripheral WBC counts below or above $50 \times 10^3/\mu L$ were analyzed (Table 3). CE2 remained unchanged for the OPP across both subgroups (45% for subgroup WBC $<50 \times 10^3/\mu L$ vs 44% for subgroup WBC $\geq 50 \times 10^3/\mu L$; $P = .56$), whereas CE2 showed a significant decline for the STP (38% vs 24%, respectively; $P = .001$). Overall, 18/21 (86%) patients with the OPP obtained their target $CD34^+$ amount with a single apheresis session, compared to 25/50 (50%) with the STP ($P = .005$). This effect was significant in the subgroup of patients with a 2.5×10^6/kg body weight target (8/9 [89%] with OPP vs 11/28 [39%] with STP; $P = .01$); the effect did not reach significance in the subgroups with a target of 5.0×10^6/kg (7/8 [88%] OPP vs 12/18 [67%] STP; $P = .28$) and 7.5×10^6/kg (3/4 [75%] OPP vs 2/4 [50%] STP; $P = .49$).

PBSC collections using OPP lasted significantly longer (377 minutes vs 260 minutes; $P < .001$) and had a significantly higher TPV (17.39 L vs 16.05 L; $P = .02$) than with

TABLE 3 Comparison of subgroups according to peripheral WBC counts

	WBC count < 50 ×10³/μL			WBC count ≥ 50 ×10³/μL		
	STP (n = 38)	OPP (n = 14)	*P*-value	STP (n = 21)	OPP (n = 8)	*P*-value
CE2	38% [5;74]	45% [26;77]	.02	24% [3;36]	44% [21;56]	.003
$CD34^+$ content product ($\times 10^6$)	256 [26;1534]	623 [104;1657]	.02	136 [24;1689]	1025 [122;2091]	.03
$CD34^+$ content product/kg bw ($\times 10^6$)	3.30 [0.41;28.41]	8.16 [1.21;20.21]	.03	1.59 [0.38;28.62]	10.39 [1.49;41.00]	.02

Note: Data are reported as median [range]. *P*-values relate to comparisons within each WBC subgroup.
Abbreviations: bw, body weight; CE2, collection efficiency 2; OPP, optimized protocol; STP, standard protocol; WBC, white blood cell.

the STP (Table 2). Average whole blood flow rates (mL/min) were significantly lower with the OPP (46 vs 62; $P < .001$). Product volume (292 mL vs 171 mL; $P < .001$) and WBC content ($\times 10^9$) (97.7 vs 52.3; $P < .001$) were also significantly higher in PBSC collection products obtained with the OPP compared to products collected with the STP.

Median platelet content of OPP products was 1.95 ($\times 10^{11}$; range 0.20-5.20). WBC subgroup contents were available for 19 OPP products: Median granulocyte content 40.9 ($\times 10^9$; 9.1-181.4), median MNC content 59.6 ($\times 10^9$; 33.2-195.5). Median RBC contamination (21 OPP products) was 23 mL (5-55 mL) and 8% (2-18%) with respect to hematocrit. As the majority of patients' target $CD34^+$ amount was for more than one autologous HSCT in the OPP group, cross-cellular contamination per transplant dose was assessed for 18 patients of the OPP group who obtained their target $CD34^+$ amount with a single apheresis session: Median platelet content 1.08 ($\times 10^{11}$; range 0.07-2.90), median granulocyte content 20.1 ($\times 10^9$; 9.1-181.4; data of 16 products), median MNC content 35.9 ($\times 10^9$; 13.6-195.5; 16 products). Median RBC contamination per transplant was 11 mL (2-55 mL) and 4% (1-18%) with respect to hematocrit. At the time of data analysis, 57 and 33 autologous HSCT had been performed in patients of the STP and OPP group, respectively. Grade 1 to 2 adverse events during PBSC infusion occurred in 2/57 (4%) infusions of STP products and 4/33 (12%) infusions for OPP products ($P = .11$). No grade 3 or higher adverse events were observed.

4 | DISCUSSION

The collection of peripheral $CD34^+$ cells by automated cell separators is an integral part of the concept of autologous HSCT. To minimize patient burden and cost, PBSC collection has to be safe and yield sufficient numbers of viable $CD34^+$ cells with a minimum of apheresis sessions. CE is defined as the proportion of $CD34^+$ cells collected in relation to the TPV.[4] In our current study, we demonstrate that our OPP significantly improves CE2 for PBSC collections in patients with pre-apheresis WBC counts >35 000/μL.

According to the manufacturer, cycle volume for the Fenwal Amicus cell separator should be adjusted from 1400 to 1000 mL/cycle in case the patient's WBC count exceeds 35 000/μL. Nevertheless, a relevant number of our patients with WBC counts >35 000/μL did not reach the target $CD34^+$ amount with a single PBSC collection. We therefore devised a new OPP, which still complies with the manufacturer's specifications.[15] The rationale of reducing cycle volume to 600 mL was based on data by Burgstaler et al. who demonstrated a non-significant trend toward improved CE with a cycle volume of 750 mL.[16] A maximum flow rate of 60 mL/min was chosen according to Hartwig et al.[9] These modifications resulted in a significant improvement of CE2. We also observed a significantly higher $CD34^+$ yield of the apheresis product both with regards to absolute $CD34^+$ content ($\times 10^6$) as well as $CD34^+$ ($\times 10^6$)/ kg body weight. Significantly more patients obtained the target amount of $CD34^+$ cells with a single apheresis session. Thus, the necessity for repetitive PBSC collections was markedly reduced with our OPP.

In order to maintain target TPV at 14 L for all patients in analogy to the STP, the number of cycles for the OPP was set at 24 (24 cycles of 600 mL compared to 14 cycles of 1000 mL with the STP). After each MNC transfer, the collection chamber of the cell separator is prefilled to the MNC collection setpoint; this blood volume used for prefilling will be added to the cycle volume, thus the actual TPV increases, if cycle numbers increase. As a result, TPV was significantly higher with the OPP compared to the STP. It is therefore important to note that the formula for CE2 accounts for variations in TPV. The number of cycles affects the duration of PBSC collection, which was significantly higher with the OPP compared to the STP. Apheresis duration is a relevant factor with regards to patient comfort, especially when peripheral vascular access is used for PBSC collection. Predictive formulas for TPV incorporating target $CD34^+$

content of the apheresis product, peripheral $CD34^+$ count, and CE have been established.[17,18] They can be used to estimate the TPV of individual PBSC collections to obtain the target $CD34^+$ amount. From these formulas, it can be predicted that the observed 50% relative increase of CE2 with the OPP would allow for an approximately 30% reduction in TPV in order to obtain the same target amount of $CD34^+$ cells. The resulting reduction in TPV, and thus cycle numbers, may offset any prolongation of apheresis duration caused by the lower cycle volume of the OPP. Besides, longer duration of PBSC collections with the OPP must also be interpreted in the context that the OPP significantly reduces the necessity for repetitive PBSC collections.

Besides apheresis duration, product volume also significantly increased with the OPP. In general, the Fenwal Amicus cell separator generates lower product volumes compared to COBE Spectra.[19,20] The product volumes obtained with the OPP are in the range of product volumes reported by centers using the COBE Spectra and Spectra Optia.[20,21] They remained markedly below the product volumes that have been associated with increased risk of cardiac side effects during PBSC infusion.[22] In addition, the above-mentioned option to adjust TPV due to improved CE and the reduction of repetitive PBSC collections will result in a decrease of the final product volume for PBSC transfusion. With respect to cross-cellular contamination, platelet and RBC content of products obtained with the OPP were in the range that has been previously reported for PBSC collections with the Fenwal Amicus.[20,23] However, WBC content in the apheresis products was almost twice in the OPP group, despite comparable peripheral WBC counts at the beginning of PBSC collection. Therefore, compared to the literature, granulocyte content of OPP products tended to be higher even after adjustment for transplant dose.[23] Incidence and severity of adverse events during PBSC infusion have been related to granulocyte content.[10,24] However, we did not observe a significant increase in the rate of adverse events during PBSC infusion of OPP products. The lack of grade 3 or higher events in our study is in line with Camels et al. who reported a median granulocyte content of 28×10^9 for grade 1 and 2 adverse events and 58×10^9 for grade 3 and 4 adverse events during autologous PBSC infusion.[24]

Patient characteristics were not significantly different between study groups except for type of vascular access and pre-apheresis hematocrit. The decreased rate of central venous access in the OPP group can be attributed to institutional efforts during recent years to minimize the use of central venous access for PBSC collection. The type of access does not affect CE; therefore, any impact of this observation on our study results can be excluded.[25] With respect to hematocrit, previous studies have either demonstrated a lack of correlation or an inverse correlation between hematocrit and CE.[4,25,26] Therefore, the observed improvement in CE2 in the OPP group is unrelated to differences in hematocrit between study groups.

A major limitation of our study is that the upper range of pre-apheresis WBC counts observed in the OPP group is $62 \times 10^3/\mu L$. Therefore, we cannot draw any conclusions on CE2 for the OPP in patients with higher WBC counts. For the STP group, our data demonstrate that with increasing WBC counts CE2 decreases even further. In addition, our study only included patients for adult autologous PBSC collection. As TPV was not corrected for ACD used, CE2 is slightly underestimated in both study groups. We also cannot exclude an effect of the various software versions of the Fenwall Amicus apheresis devices on CE2, as all collections of the OPP were performed with the most recent version. Furthermore, the calculation of CE2 assumes stable $CD34^+$ concentrations in the patient's blood and does not account for changes in the peripheral $CD34^+$ count during apheresis.

5 | CONCLUSIONS

Limiting cycle volume to 600 mL and maximum flow rate to 60 mL/min during autologous PBSC collection significantly increases CE2 and $CD34^+$ yield in adult patients with WBC counts >35 000/μL. Our new OPP results in a significantly higher rate of PBSC collections, which obtain the target $CD34^+$ count/kg body weight with a single apheresis session. The resulting prolongation of apheresis duration and increased product volume can be alleviated by using validated formulas to adjust TPV of individual PBSC collections based on target $CD34^+$ amount and the improved CE. The new OPP is therefore suited to reduce both patient burden and cost in autologous PBSC collection.

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REFERENCES

1. Sureda A, Bader P, Cesaro S, et al. Indications for Allo- and auto-SCT for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2015. *Bone Marrow Transplant*. 2015;50:1037-1056.

2. Mohty M, Huebel K, Kroeger N, et al. Autologous haematopoietic stem cell mobilisation in multiple myeloma and lymphoma patients: a position statement from the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant*. 2014;49:865-872.
3. Faucher C, Le Corroller AG, Chabannon C, et al. Autologous transplantation of blood stem cells mobilized with filgrastim alone in 93 patients with malignancies: the number of CD34+ cells reinfused is the only factor predicting both granulocyte and platelet recovery. *J Haematother*. 1996;5:663-670.
4. Ford CD, Pace N, Lehman C. Factors affecting the efficiency of collection of CD34-positive peripheral blood cells by a blood cell separator. *Transfusion*. 1998;38:1046-1050.
5. Mehta J, Singhal S, Gordon L, et al. Cobe spectra is superior to Fenwal CS 3000 plus for collection of hematopoietic stem cells. *Bone Marrow Transplant*. 2002;29:563-567.
6. Ikeda K, Minakawa K, Muroi K, et al. Prospective randomized and crossover comparison of two apheresis machines for peripheral blood stem cell collection: a multicenter study. *Transfusion*. 2016;56:2839-2847.
7. Verlinden A, van der Velde A, Verpooten GA, et al. Determining factors predictive of CD34+ cell collection efficiency in an effort to avoid extended and repeated apheresis sessions. *J Clin Apher*. 2013;28:404-410.
8. Gidron A, Verma A, Doyle M, et al. Can the stem cell mobilization technique influence CD34+ cell collection efficiency of leukapheresis procedures in patients with hematologic malignancies? *Bone Marrow Transplant*. 2005;35:243-246.
9. Hartwig D, Dorn I, Kirchner H, Schlenke P. Recommendations for optimized settings of the amicus crescendo cell separator for the collection of CD34+ progenitor cells. *Transfusion*. 2004;44:758-763.
10. Sakashita AM, Kondo AT, Yokoyama APH, et al. The impact of preapheresis white blood cell count on autologous peripheral blood stem cell collection efficiency and HSC infusion side effect rate. *J Clin Apher*. 2018;33:331-341.
11. Osselaer JC, Vandenbossche S, van Hooydonk M, et al. Optimization of MNC collection efficiency depending on patient WBC precount with amicus. *Transfus Apher Sci*. 2013;48:175-176.
12. Burgstaler EA, Pineda AA, Winters JL. Effects of high whole blood flow rates and high peripheral white blood cell counts on CD34+ yield and cross-cellular contamination. *Cytotherapy*. 2003;5:445-446.
13. Burgstaler EA, Winters JL. Manual color monitoring to optimize hematopoietic progenitor cell collection on the Fenwal amicus. *J Clin Apher*. 2011;26:123-130.
14. Bargetzi MJ, Passweg J, Baertschi E, et al. Mobilization of peripheral blood progenitor cells with vinorelbine and granulocyte colony-stimulating factor in multiple myeloma patients is reliable and cost effective. *Bone Marrow Transplant*. 2003;31:99-103.
15. Fenwal Europe Amicus Zellseparator Band 3—Mononuclear Cell (MNC) Collection Software-Version 4.3 and Software-Version 4.4 REF 4R4580 und 4R4580R. Fenwal Europe 2012;3:1–234
16. Burgstaler EA, Winters JL. The effects of shorter cycles to improve CD34+ cell yields in patients with elevated circulating CD34+ cell levels using the Fenwal amicus. *Transfusion*. 2011;51(suppl):226A.
17. Rosenbaum ER, O'Connell B, Cottler-Fox M. Validation of a formula for predicting daily CD34(+) cell collection by leukapheresis. *Cytotherapy*. 2012;14:461-466.
18. Cousins AF, Sinclair JE, Alcorn MJ, H.A. Green R, Douglas KW. HPC-A dose prediction on the OptiaVR cell separator based on a benchmark CE2 collection efficiency: promoting clinical efficiency, minimizing toxicity, and allowing quality control. *J Clin Apher*. 2015;30:321-328.
19. Deneys V, Fabry A, Van Hooydonk M, et al. Efficiency of autologous stem cell collection: comparison of three different cell separators. *Transfus Apher Sci*. 2017;56:35-38.
20. Setia RD, Arora S, Handoo A, et al. Comparison of amicus and COBE spectra for allogenic peripheral blood stem cell harvest: study from tertiary care centre in India. *Transfus Apher Sci*. 2017;56:439-444.
21. Leberfinger DL, Badman KL, Roig JM, Loos T. Improved planning of leukapheresis endpoint with customized prediction algorithm: minimizing collection days, volume of blood processed, procedure time, and citrate toxicity. *Transfusion*. 2017;57:685-693.
22. Donmez A, Tombuloglu M, Gungor A, Soyer N, Saydam G, Cagirgan S. Clinical side effects during peripheral blood progenitor cell infusion. *Transfus Apher Sci*. 2007;36:95-101.
23. Burgstaler EA, Porrata LF, Markovic SN, Winters JL. Use of various offset settings in the Fenwal amicus during hematopoietic progenitor cell collection to increase lymphocyte yield and reduce cross-cellular contamination. *J Clin Apher*. 2010;25:301-309.
24. Calmels B, Lemarié C, Esterni B, et al. Occurrence and severity of adverse events after autologous hematopoietic progenitor cell infusion are related to the amount of granulocytes in the apheresis product. *Transfusion*. 2007;47:1268-1275.
25. Sarkodee-Adoo C, Taran I, Guo C, et al. Influence of preapheresis clinical factors on the efficiency of CD34+ cell collection by large-volume apheresis. *Bone Marrow Transplant*. 2003;31:851-855.
26. Mehta J, Oyama Y, Winter J, et al. $CD34^{+}$ cell collection efficiency does not correlate with the preleukapheresis hematocrit. *Bone Marrow Transplant*. 2001;28:597-601.

Multi-site evaluation of the BD® Stem Cell Enumeration Kit for CD34 cell enumeration on BD FACSLyric™ and BD FACSCanto™ II Flow Cytometers

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Background:[1-4]

Stem cell transplantation therapy is commonly used to treat hematopoietic disease, but the treatment relies on accurate enumeration of stem cells. What is the best way to approach this measurement?

Transplantation of hematopoietic stem cells (HSC):

Hematopoietic stem cells (HSCs) are CD34+ cells that can differentiate into mature blood cells:

HSC transplantation is an established therapy for malignant and non-malignant diseases that works by replacing or rebuilding a patient's hematopoietic system. Flow cytometric enumeration of CD34+ HSCs is used to assess HSC transplantation products, as CD34+ cell viability correlates with HSC engraftment.

Erythrocytes **Platelets** **Leukocytes**

Measuring hematopoietic stem cells:

The marker CD34 is used as a key indicator for measuring HSCs taken either from:

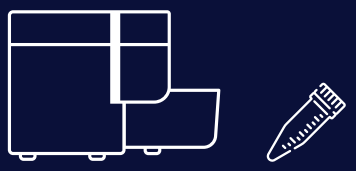
Bone marrow (BM)

Peripheral blood (PB)

Measurement helps to indicate viability and quantity:

Available for transplantation

Post-transplantation outcomes

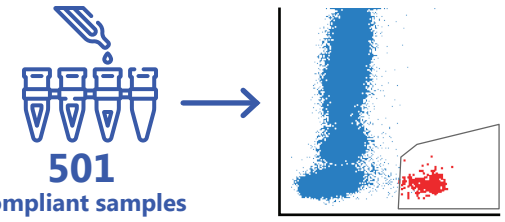


The International Society of Hematotherapy and Graft Engineering (ISHAGE) protocol is commonly used, but:

- Methodology using ISHAGE can vary
- There are single and dual platform approaches
- Reproducibility and standardization are issues
- Evaluation of HSCs is improved through standardization:
  - Correct configuration of cytometry equipment
  - Reliable labelling of CD34+ cells using reagents such as the BD® Stem Cell Enumeration kit

The study:

The BD® Stem Cell Enumeration Kit was evaluated through a study of 501 compliant samples across four sites. CD34+ HSCs were quantified as absolute counts and as percentage of leukocytes in various fresh and thawed specimens that were collected in different types of anticoagulants.



Method:

Specimens were tested using BD® Stem Cell Enumeration (SCE) Kit on the BD FACSLyric™ Flow Cytometer (FC) with BD FACSuite™ Clinical Application.

Instrument
BD FACSLyric™ Flow Cytometer

Assay
BD® Stem Cell Enumeration Kit with BD Trucount™ Tubes

Table 1. Viable CD34+ Bin Target and Current Enrollment

Viable CD34+ Absolute Count (cells/µL)	Target	Current
0 - 10	60	100
>10 and ≤100	130	242
>100 and ≤500	85	134
>500 and ≤1000	25	25
Total	**300**	**501**

Table 2. Enrolled Specimens

Specimen Type	Target	Current
Normal Peripheral Blood (NPB)	40	48
Mobilized Peripheral Blood (MPB)	60	63
Fresh Cord Blood (FCB)	56	61
Thawed Cord Blood (TCB)	56	71
Fresh Bone Marrow (FBM)	52	60
Thawed Bone Marrow (TBM)	52	61
Fresh Leukapheresis Product (FLP)	60	68
Thawed Leukapheresis Product (TLP)	60	69
Total	**436**	**501**

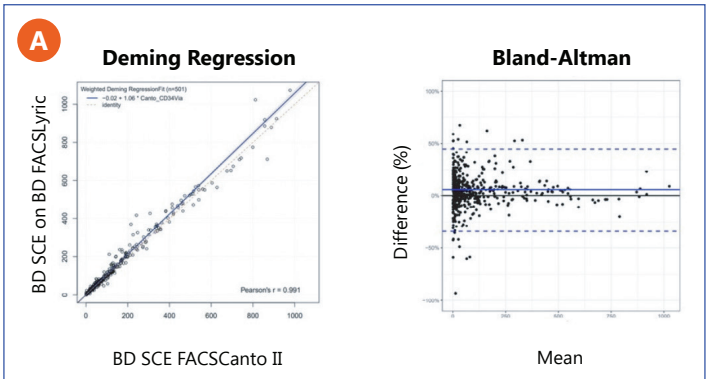
Table 3. Anticoagulant Type

Anticoagulant Type	Total
EDTA	202
Heparin	21
ACD-A	87
CP2D	60
CPD	49
CPDA	5
Mix (Heparin + ACD-A)	77
Total	**501**

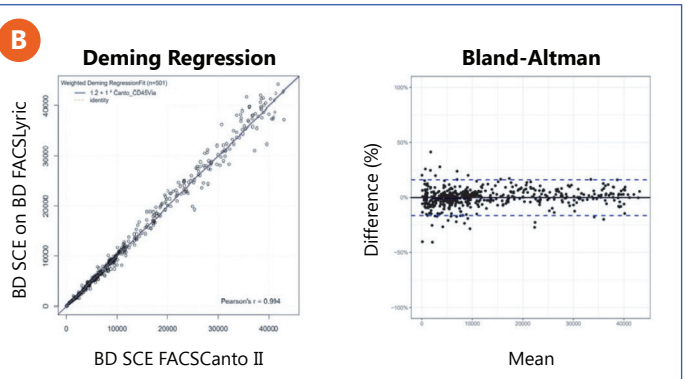
Tables 2 and 3: Fresh and thawed specimen in anticoagulant or anticoagulant combination were tested across four laboratories.

Results:

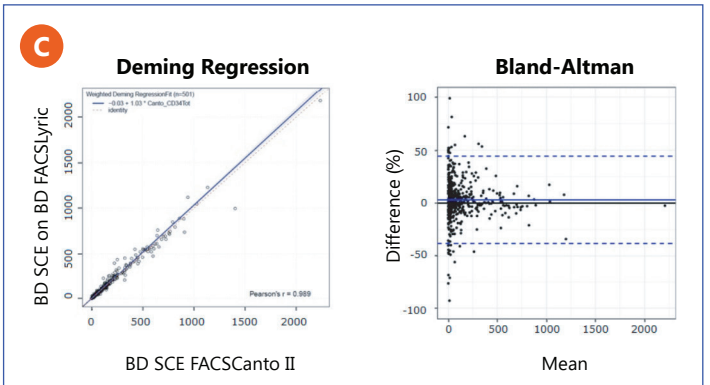
Viable CD34+ and CD45+ Absolute Counts



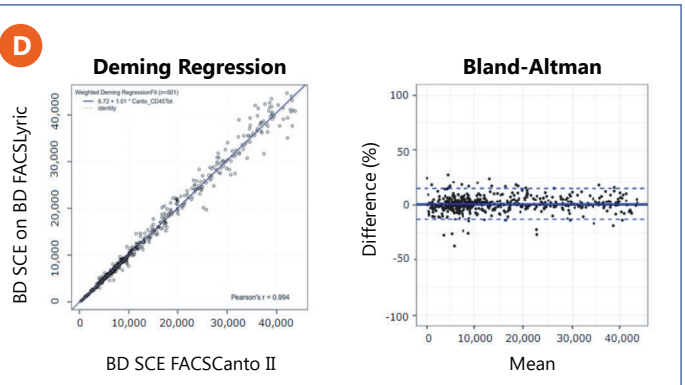
Illustrates the Viable CD34+ Absolute Counts Deming regression (Table 4) and bias results in Bland-Altman plots with 5.57% percent mean bias and 95% LOA between -33.78% and 44.92%.



Presents the Viable CD45+ Absolute Counts Deming regression (Table 4) and bias results in Bland-Altman plots with 0.16% percent mean bias and 95% LOA between -16.41% and 16.09%.



Shows the Total CD34+ Absolute Counts Deming regression (Table 4) and bias results in Bland-Altman plots with percent mean bias of 3.03% and 95% limits of agreement (LOA) between -38.21% to 44.28%.



Demonstrates the Total CD45+ Absolute Counts Deming regression (Table 4) and bias results in Bland-Altman plots with 1.19% percent mean bias and 95% LOA between -12.97% and 15.35%.

Table 4. Deming Regression Summary

Parameter	R2	Intercept (95% CI)	Slope (95% CI)
Viable CD34+ Absolute Count	0.983	-0.02 (-0.14, 0.10)	1.06 (1.04, 1.08)
Viable CD45+ Absolute Count	0.989	1.2 (-27.43, 29.84)	1.00 (0.99, 1.01)
Total CD34+ Absolute Count	0.977	-0.03 (-0.23, 0.16)	1.03 (1.01, 1.05)
Total CD45+ Absolute Count	0.987	6.72 (-30.34, 43.78)	1.01 (1.00, 1.02)

The SCE Deming regression results for viable CD34+ and viable CD45+ cells provided values for R2 > 0.983, intercept -0.02 and 1.2 and slope ≈1.0 (0.99, 1.08) when pooling all specimen types.

Table 5. Predicted Bias

Viable CD34+ Absolute Count at 10 Cells/µL Threshold		
Bias Type	CD34 Threshold	Bias (95% CI)
Proportional %Bias	10 cells/µL	5.67 (3.83, 7.52)
Absolute Bias	10 cells/µL	0.57 (0.38, 0.75)

Predicted bias was calculated for viable CD34 counts at 10 cells/µL threshold showing a proportional percent bias of 5.67 (3.83, 7.52) and absolute bias of 0.57 (0.38, 075).

Viable CD34+ cell counts predicted bias at 10 cells/µL showed values of 5.67% and 0.57 for proportional percentage bias and absolute counts.

Table 6. Agreement Analysis

Method		SCE Kit on BD FACSCanto™ II FC		
		Positive (≤10 cells/µL)	Negative (>10 cells/µL)	Total
SCE Kit on BD FACSLyric™	Positive (≤10 cells/µL)	93	6	99
	Negative (>10 cells/µL)	7	395	402
	Total	100	401	501
Agreement 95% CL (LCL, UCL)		**PPA[$]**	**NPA[&]**	**Overall**
		93% (86.3, 96.6)	98.5% (96.8, 99.3)	97.4% (95.6, 98.5)

$ = positive percent agreement (PPA). & = negative percent agreement (NPA)

Agreement analysis exhibited overall percent agreement of 97.4%, 93% PPA and 98.5% NPA. Thirteen samples showed discrepant results, eight samples had one or two cell differences; two showed three cell differences and the last three sample showed between 9 and 19 cell differences.

Conclusions

Different types of fresh and thawed specimens were collected in a variety of anticoagulants and successfully tested using the BD® SCE Kit by collecting and analyzing stained samples on both BD flow cytometry systems, the BD FACSCanto™ II with BD FACSCanto™ Clinical Software and test BD FACSLyric™ Flow Cytometer with BD FACSuite™ Clinical Application.

Equivalent performance of the BD® SCE Kit between the BD FACSLyric™ and BD FACSCanto™ II Flow Cytometers was demonstrated by the Deming regression results and predicted bias of viable CD34+ absolute counts at 10 cells/µL, by enumeration of viable CD45+ as well as total CD34+ and CD45+ absolute counts.

1. Rimac, V, Bojanić, I. Role of flow cytometry in evaluation of the cellular therapy products used in haematopoietic stem cell transplantation. Int J Lab Hematol. 2022; 1- 8. doi:10.1111/ijlh.13849
2. Chung, Y, Kong, JH, Hu, Y, et al. Comparison of spectra optia and amicus cell separators for autologous peripheral blood stem cell collection. J Clin Apher. 2021; 36: 28-33. https://doi.org/10.1002/jca.21835
3. Boehlen, R, Fernandez, P, Bargetzi, M, Sigle, J-P. A new protocol for improving efficiency of autologous peripheral blood stem cell collection in patients with high white blood cell counts. J Clin Apher. 2020; 35: 217-223. https://doi.org/10.1002/jca.21782
4. Rimac, V, Ines, B, Koraljka, G, Ćepulić Branka, G. Evaluation of the BD Stem Cell Enumeration Kit on the BD FACSCanto II flow cytometer using bd facscanto clinical and bd facsdiva software. Int J Lab Hematol. 2021; 43: 61-67. https://doi.org/10.1111/ijlh.13315