Original Article

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Quantitative Analysis of CD34+ Haematopoietic Stem Cells using Flow Cytometry: A Cross-sectional Study

Pathology Section

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ABSTRACT

Introduction: For a successful transplant, it is significant to precisely count haematopoietic stem and progenitor cells (CD34+) to make sure the graft is big enough. Standardised protocols are essential for consistent results across biological samples. Accurate enumeration of CD34+ Haematopoietic Stem Cells (HSC) is essential for successful transplantation, but variability in flow cytometric methods and lack of standardisation can affect reliability. Standardised protocols are therefore necessary to ensure reproducible and clinically meaningful results.

Aim: To standardise the method of enumeration of CD34 counting in flow cytometry and its correlation with quality controls.

Materials and Methods: The present cross-sectional study was done in the Pathology Department, Flow Cytometry Section, at BLDE (Deemed to be University), Shri BM Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, India, from March 2024 to October 2025. Total 30 samples (10 from each source i.e., bone marrow, cord blood as well as peripheral blood samples) were analysed using a bead-based, single-platform flow cytometric assay (BD Stem Cell Enumeration Kit) on a BD FACSCanto™ II. The primary outcome was the absolute count of viable CD34+ cells/µL measured by single-platform bead-based flow cytometry. Secondary parameters included CD34+ percentage, precision (CV), linearity, bead recovery and acquisition quality indices. Statistical analysis was accomplished by Statistical Package for Social Sciences (SPSS) version 20. Continuous variables were summarised as mean±SD or median (IQR) based on distribution assessed by the Shapiro-Wilk test. Group comparisons (PB, CB, BM) used ANOVA with appropriate post-hoc analysis.

Results: Cord blood showed the highest CD34+ viability (94.73%), followed by bone marrow (73.20%) and peripheral blood (57.28%). CD34+ counts were highest in bone marrow (509.05 cells/µL), followed by peripheral blood (231.5 cells/µL) and cord blood (46.84 cells/µL). CD34 count showed a negative correlation with viability (r=-0.351, p=0.047) and a positive correlation with CD45% (r=0.466, p=0.009).

Conclusion: Mono-platform flow cytometry is reliable in the enumeration of CD34+ in various biological specimens. The analysis of a counts, viability and CD45 simultaneously increases the accuracy of the test, as it aids in making correct clinical decisions in the transplant of stem cells.

Keywords: Cell viability, CD45 marker, Flow cytometric analysis, Haematopoietic progenitor cells, Stem cell transplantation

INTRODUCTION

The HSCs are a very rare population of multipotent cells possessing the ability for self-renewal as well as differentiation into every blood cell type in the body. Enumeration and viability analyses of HSCs are very essential in clinical haematology, especially in HSC transplantation, since they are used as guides in apheresis and assessment of transplant success [1]. CD34 is presently used to measure the number of transplantable progenitors quantitatively, although it does not specifically stain all stem cells [2]. Mobilisation strategies attempt to maximise the number of CD34+ cells in the peripheral blood so that they can be collected effectively, while haematological analysis after harvesting is performed to ensure a satisfactory number of live grafts [3].

Flow cytometry is the gold standard for CD34 cell enumeration, although technical difficulties such as low number of events, pre-analytical variables, gating, or variability among laboratories may influence the result [4]. Single-platform methods using fluorescence beads or viability dyes such as 7-Aminoactinomycin D (7-AAD) offer absolute quantification and have been demonstrated to be accurate, reliable and consistent for a range of specimens, including peripheral blood, bone marrow, or umbilical cord blood [5,6]. Commercial kits, such as the BD stem cell enumeration kit according to International Society of Haematotherapy and Graft Engineering (ISHAGE) recommendations, would make analysis even more reliable, although validation studies or proper quality control would be crucial in low- or tertiary-level laboratories [7,8].

CD34 enumeration can also be used for other purposes, including engraftment assessment, assessment of the efficacy of mobilisation and analysis of the pool of progenitor cells for scientific research [8]. Unfortunately, the lack of local standardisation for CD34 enumeration in many Indian labs can limit its utility for clinical decision-making [9]. Therefore, establishing a validated, standardised single-platform CD34 counting protocol- including local quality control limits, operator consistency and specimen-specific normative data- is crucial for reliable clinical and research applications [10].

The above study aimed to implement and validate a unified CD34 enumeration method using the BD stem cell enumeration kit on a BD FACSCanto II system, assess analytical performance, define local QC limits and collect normative data across peripheral blood, bone marrow, as well as cord blood samples. The objectives of the study were to perform enumeration of CD34+ HSC and assess their correlations for quality control and clinical applicability.

The novelty of this study lies in the simultaneous evaluation of CD34+ cell count, viability and CD45 expression using a standardised single-platform method across multiple biological samples, along with establishment of local quality control parameters.

MATERIALS AND METHODS

The present cross-sectional study was conducted in the Pathology Department, Flow Cytometry Section, at BLDE. (Deemed to be University), Shri BM Patil Medical College, Hospital and Research Centre, Vijayapura, Karnataka, India, from March 2024 to October

2025. Approval from the Institutional Ethics Committee (IEC) was obtained earlier starting the research BLDE (DU)/IEC-SBM PMC/126/2023-24, dated-10/02/2024. Informed consent had been taken from the individuals.

Sample size selection: Thirty samples were selected on the basis of inclusion and exclusion criteria for the study. According to research conducted by Lemos NE et al., with "quantification of peripheral blood CD34+ cells prior to stem cell harvesting by leukapheresis: a single center experience" Sample size computation using G*Power ver. 3.1.9.4 software, the correlation between absolute CD34+ count/µL from peripheral blood (r=0.596, p-value <0.001), this study requires a sample size of 30 [11].

Inclusion criteria:

- Peripheral blood from healthy adult volunteers with normal haematological parameters {Complete Blood Count (CBC), serum creatinine, uric acid, no Hepatitis B surface Antigen (HBsAG), no Human Immunodeficiency Virus (HIV), normal random blood sugar} like healthy donors (normal CBC) with no history of haematological disorders, malignancy, or chronic systemic illness and not on any medications affecting bone marrow function.
- Umbilical cord blood from healthy term deliveries with obstetric clearance and informed consent from mother.
- Bone marrow aspirates from patients with non-malignant conditions and no clinical suspicion of leukaemia.
- Properly labelled specimens collected in appropriate anticoagulants and received within the defined pre-analytical time frame.

Exclusion criteria:

- Suspected or known haematologic malignancies.
- Cord blood from neonates with low birth weight or significant perinatal complications.
- Samples with haemolysis, clots, labelling errors, or improper transport conditions.
- Specimens exceeding the acceptable time to analysis or failing pre-analytical or run-time quality control checks.

Study Procedure

All samples were handled according to a locked Standard Operating Procedure (SOP) throughout the whole process. Upon receiving, the samples were checked regarding their identity, volume, type of anticoagulant used and transport conditions. Pre-analytical data was recorded. Staining was performed using the BD® Stem Cell Enumeration Kit or equivalent (anti-CD45, anti-CD34 and availability dye). The lyse-no-wash method was performed, however, in case of particular matrix-specific debris the lyse-wash step was applied; the erythrocyte lysis time and buffer volumes were kept constant. The bead tubes were vortexed as per the respective instructions to prevent clumping and to get uniform bead distribution.

The instrument set-up was confirmed by the use of daily cytometer Quality Control (QC) beads; detector voltages, area scaling and compensation matrices were taken from the locked template. Single-stained controls were used for compensation verification. Doublet discrimination was applied using Front Scatter-Height (FSC-H) versus Front Scatter Area (FSC-A) {and, if necessary, Side Scatter -Height (SSC-H) versus Side Scatter Area (SSC-A)}. For every tube, the laboratory's goal was to get at least 75,000 total and 1,000 bead events. If the bead events were below the minimums or the bead recovery ranged outside the acceptance (commonly±10% of the assigned bead count), the acquisition was repeated. Data stability was monitored using time-versus-fluorescence plots.

Gating followed ISHAGE guidelines: Initial FSC/SSC gating to exclude debris, CD45 versus SSC gating to identify leukocytes, CD34 versus SSC gating within singlet leukocytes and viability gating to enumerate viable (7-AAD$^-$) CD34$^+$ cells. The Fluorescence Minus One (FMO) controls were intermittently used for verification of the thresholds set in gating, especially during validation and reagent lot changes. The precision and linearity experiments were carried out using duplicate acquisition and dilutions of samples [6].

All the data was collected and descriptive statistics were given for product-oriented observations regarding CD34$^+$ cells/kg [8].

STATISTICAL ANALYSIS

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) (version 20). Continuous variables were expressed as mean±SD or median (IQR), while categorical data were presented as frequencies and percentages. Normality was assessed using the Shapiro-Wilk test. Comparison between sample types was done using One-way Analysis of Variance (ANOVA) with Tukey's post-hoc test. Correlation between variables was evaluated using Pearson's correlation coefficient and a p-value <0.05 was considered statistically significant.

RESULTS

Out of total 30 cases, majority of patients were new born (10 cases) and males were predominant (17 cases) in the study [Table/Fig-1,2]. Cord blood showed the highest and most consistent viability (94.73±4.68%; IQR 6.05; range 85.6-99.38). Bone marrow had the moderate viability (73.20±18.13%; IQR 28.98; range 37.8-90.9) followed by Peripheral blood (57.28±6.17%; IQR 8.67; range 48.3-72.67) [Table/Fig-3].

Age group	Frequency
20-40	7
41-50	7
51-60	6
New born	10
Grand total	30

[Table/Fig-1]: Age group distribution of study participants (N=30).

Gender	Frequency
Male	17
Female	13
Grand total	30

[Table/Fig-2]: Gender distribution of study participants (N=30).

Sample type	Mean±SD	IQR	Min-Max
Bone marrow	73.20±18.13%	28.98	37.8-90.9
Cord blood	94.73±4.68%	6.05	85.6-99.38
Peripheral blood	57.28±6.17%	8.67	48.3-72.67

[Table/Fig-3]: CD34 cell viability (%) across different sample types.

Bone marrow showed the highest mean CD45 count (0.51±0.22%; median 0.49; range 0.07-0.86). Cord blood had a lower mean (0.46±0.19%; median 0.44; range 0.08-0.82), while peripheral blood showed a similar mean (0.48±0.21%; median 0.47; range 0.06-0.84) [Table/Fig-4].

Sample type	Mean±SD	Median	IQR	Min-Max
Bone marrow	0.51±0.22%	0.49	0.28	0.07-0.86
Cord blood	0.46±0.19%	0.44	0.25	0.08-0.82
Peripheral blood	0.48±0.21%	0.47	0.27	0.06-0.84

[Table/Fig-4]: CD45 count (%) across different sample types.

Bone marrow showed the highest mean CD34 count (509.05±395.5 cells/µL; IQR 584.9; range 26-1360), followed by peripheral blood (231.5±103.25 cells/µL; IQR 206.8; range 25-438). Cord blood had the lowest counts (46.84±39.19 cells/µL; IQR 25.5; range 20.4-148) [Table/Fig-5].

Sample type	Mean (cells/µL)	Standard Deviation (SD)	IQR	Min-Max
Bone marrow	509.05	395.5	584.9	26-1360
Peripheral blood	231.5	103.25	206.8	25-438
Cord blood	46.84	39.19	25.5	20.4-148

[Table/Fig-5]: Absolute CD34 cell count (cells/µL) across different sample types.

The Pearson's correlation coefficient was found to be r=-0.351. The association was statistically significant (p=0.047) when 30 samples were analysed, thus signifying a negative relationship between the CD34 cell count as well as the corresponding percentages of viability measured throughout the dataset [Table/Fig-6].

Variables	Pearson's correlation (r)	p-value	n
CD34 count (absolute) vs. CD34 viability (%)	-0.351	0.047	30

[Table/Fig-6]: Internal consistency (standardisation check) between CD34 absolute count and CD34 viability (N=30).

A moderate, statistically significant positive association was observed between absolute CD34 count and CD45 percentage (r=0.466, p=0.009; n=30) [Table/Fig-7].

Variables compared	Pearson's correlation (r)	p-value	n
CD34 count (absolute) vs. CD45 (%)	0.466	0.009	30

[Table/Fig-7]: Linearity/biological validity (standardisation) between CD34 absolute count and CD45 percentage (N=30).

One-way ANOVA showed significant differences in absolute CD34 counts (F=7.845, p=0.001) and CD34 viability (F=6.856, p=0.01) among bone marrow, cord blood, as well as peripheral blood samples, whereas CD45% did not differ significantly (F=0.986, p=0.386) [Table/Fig-8].

Parameters	Bone marrow (mean±SD)	Cord blood (mean±SD)	Peripheral blood (mean±SD)	F-value	p-value
CD34 count (absolute)	509.05±395.50	46.84±39.19	231.5±103.25	7.845	0.001
CD34 viability (%)	73.20±18.13%	94.73±4.68%	57.28±6.17%	6.856	0.01
CD45 (%)	0.51±0.22%	0.46±0.19	0.48±0.21	0.986	0.386

[Table/Fig-8]: Consistency across sample types (standardisation across matrices) using One-way ANOVA.

Tukey HSD showed significant differences in absolute CD34 counts between bone marrow and cord blood (mean difference 462.21 cells/µL, p=0.001) and between cord blood and peripheral blood (-184.66 cells/µL, p=0.015), while between bone marrow and peripheral blood (mean difference 277.55 cells/µL, p=0.072). [Table/Fig-9]. Tukey post-hoc analysis showed significant differences in CD34 viability among bone marrow as well as cord blood (-21.53%, p=0.004) and between cord blood and peripheral blood (37.45%, p=0.026), while bone marrow and peripheral blood shows (15.92%, p=0.031) [Table/Fig-10].

Comparison	Mean difference	p-value
Bone marrow vs cord blood	462.21	0.001
Bone marrow vs peripheral blood	277.55	0.072
Cord blood vs peripheral blood	-184.66	0.015

[Table/Fig-9]: Tukey HSD post-HOC comparison between sample types for absolute CD34 count.

The average difference between the samples of bone marrow and cord blood was found to be 0.05% (p=0.624), while the average difference between bone marrow and peripheral blood was 0.03% (p=0.781). A mean difference of -0.02% (p=0.845) was found between cord blood and peripheral blood which suggests that CD45 expression remains relatively consistent across different sources [Table/Fig-11]. ANOVA presented that was a significant difference in the number of absolute CD34 cells (F=7.845, p=0.001). Viability of CD34 cells was also significantly different (F=6.856, p=0.01) [Table/Fig-8].

Comparison	Mean difference	p-value
Bone marrow vs cord blood	-21.53%	0.004
Bone marrow vs peripheral blood	15.92%	0.031
Cord blood vs peripheral blood	37.45%	0.026

[Table/Fig-10]: Tukey HSD post-HOC comparison between sample types for CD34 cell viability (%).

Comparison	Mean difference	p-value
Bone marrow vs cord blood	0.05%	0.624
Bone marrow vs peripheral blood	0.03%	0.781
Cord blood vs peripheral blood	-0.02%	0.845

[Table/Fig-11]: Tukey HSD post-HOC comparison between sample types for CD45 (%).
Negative values in the mean difference column indicate that the first group in the comparison had a lower mean value than the second group

DISCUSSION

The above study shows how standardised analysis of CD34 cells by flow cytometry stays a valid and consistent technique of stem cell analysis in bone marrow, cord blood and peripheral blood samples. The results of significant source-specific differences in both CD34 count and viability emphasise the need for a standardised approach to avoid any misunderstanding of stem cell concentration and quality when compared directly in the absence of a standardised process. The examination of relationships between CD34 count and viability and CD45 values in relation to ANOVA and post-test results help validate biological differences obtained in stem cell sources.

The cohort ranged from neonates to adults, allowing comparison across biologically distinct age groups. Newborns were 33.3% of the participants, while adults were evenly distributed across age ranges, with none being predominant. This distribution was appropriate, as neonatal sampling predominantly yielded cord blood, whereas adult sampling provided bone marrow and peripheral blood. Yu H et al., demonstrated high reliability of viable CD34 enumeration in peripheral blood and leukapheresis samples (R^2=0.99, CV 3.49-9.51) [12] and similar reproducibility has been reported for cord blood by Trépanier P et al., [13]. These findings indicate that heterogeneous cohorts, whether neonatal- or adult-focused, can yield consistent CD34 counts using standardised methods. In this study, the larger adult proportion alongside a neonatal subgroup enabled matrix-level assessment while maintaining clinical representativeness, reflecting real-world sampling variability.

The gender ratio showed weak male domination in the study sample, 17 males (56.7%) and 13 females (43.3%) of 30 participants. This trend represents the normal conditions of clinical recruitment and not the extreme sex imbalance and justifies the interpretability of the enumeration products in both sexes.

CD34 viability showed a clear matrix dependence, highest and most stable in cord blood (94.73±4.68%, IQR 6.05), intermediate in bone marrow (73.20±18.13%, IQR 28.98), followed by peripheral blood (57.28±6.17%, IQR 8.67). Yu H et al., reported high reproducibility of CD34 viability with standardised workflows (R^2=0.99, CV 3.49-9.51) [12], Trépanier P et al., showed strong correlation between AI-based and manual gating [13] and Sawant RB emphasised the importance of harmonised protocols to limit interlaboratory variability [14]. The observed hierarchy-cord blood > bone marrow > peripheral blood-corresponds with existing literature and emphasises the necessity for uniform, viability-inclusive enumeration.

CD45% demonstrated low variability among bone marrow (0.51±0.22%, median value=0.49, range=0.07-0.86) cord blood (0.46±0.19%) and peripheral blood (0.48±0.21%) and were

relatively stable with low values. This is in agreement with Rimac V et al., who proved CD45 to be a consistent gating marker and Elsayed AH et al., who observed that bone marrow possesses immunphenotypical variability [15,16].

The overall median absolute counts of CD34+ cells were highest in bone marrow aspirate (509.05±395.50 cells/µL, IQR 584.9), intermediate in peripheral blood collection (231.5±103.25, IQR 206.8) and the lowest in cord blood though with the greatest consistency of all tissues analysed here, at (46.84±39.19, IQR 25.5) respectively. Murugesan M et al., reported great repeatable demonstrated outstanding linearity across enumeration platforms (r=0.85-0.92) [17], Yu H et al., demonstrated excellent linearity (R^2=0.99) and low CV (3.49-9.51%) [12], while Grommé M et al., noted that flow cytometry remains the reference at low counts [18].

The internal consistency analysis revealed a moderate negative correlation between absolute CD34 counts and viability (r=-0.351; p=0.047), however, the underlying cause of this association cannot be determined from the present data. This supports findings by Yu H et al., and Rimac V et al., that reproducible and accurate CD34 viability assessment is feasible by standardised protocols [12,15]. The results confirm the relevance of expressing results separately for absolute counts as well as viability values that provide vital information for the assessment of stem cells through combined multi-parametric analyses.

Analysis of linearity revealed a moderate positive correlation among CD34 cell counts and CD45% (r=0.466; p=0.009). This confirms the biological validity of CD34+ cell enumeration. This correlation can be attributed to the known presence of HSC expressing surface CD34+ antigens, Trépanier P et al., showed high concordance between automated and manual ISHAGE gating [13], Murugesan M et al., reported strong agreement across enumeration platforms [17]. Overall, the observed correlation reinforces the validity of standardised CD34/CD45-based enumeration.

One-way ANOVA showed significant differences between matrices for absolute CD34 counts (F=7.845; p=0.001) and CD34 viability (F=6.856; p=0.01), but not for CD45% (F=0.986; p=0.386). Bone marrow had the highest CD34 counts, followed by peripheral blood and cord blood, while CD34 viability was highest in cord blood, intermediate in bone marrow and lowest in peripheral blood. Previous studies showed by Trépanier P et al., and Rimac V et al., confirmed that, with standardised enumeration methods, observed differences reflect biological variability rather than analytical artifacts [13,15]. Thus, the ANOVA results support matrix-dependent differences in CD34 yield and viability, while stable CD45% validates the analytical standardisation across matrices.

Tukey HSD post-hoc analysis showed significant variances in CD34 counts among bone marrow and cord blood (mean difference 462.21 cells/µL; p=0.001) and between cord blood and peripheral blood (-184.66 cells/µL; p=0.015), while bone marrow and peripheral blood were not significant (277.55 cells/µL; p=0.072). This indicates that cord blood differs significantly from adult matrices in CD34 yield, consistent with biological expectations. Gromme M et al., reported that automated analysers align closely with flow cytometry at higher CD34 counts but rely on flow cytometry for accurate low-count measurements [18]; Murugesan M et al., demonstrated high consistency across platforms using standardised ISHAGE protocols [17].

Post-hoc analysis showed significantly higher CD34 viability in cord blood compared to bone marrow (mean difference -21.53%; p=0.004) and peripheral blood (37.45%; p=0.026), while bone marrow and peripheral blood all shows significance. This harmonises with the work of other research teams: Trépanier P et al., showed that AI-assisted gating allowed near-expert reproducibility for cord blood CD34 viability estimation, r≈0.9981, while Sawant RB highlighted the importance of the assessment of viability within the flow cytometry analysis [14]. These findings confirm the high and stable viability of cord blood and underscore the necessity of standardised, viability-inclusive stem cell enumeration.

The HSD analysis revealed no significant changes in CD45% among matrices (bone marrow vs. cord blood, p=0.624; bone marrow vs. peripheral blood, p=0.781; cord blood vs. peripheral blood, p=0.845), indicating stable CD45 expression despite variability in CD34 counts and viability. Rimac V et al., and Murugesan M et al., found CD34/CD45 quantitation highly reproducible between instruments [15,17], while Elsayed AH et al., indicated that gating variation is minimal within standardised gates defined on CD45 [16]. The CD45% is therefore a marker for analytical standardisation, while the CD34 values represent true biological variability. CD34+/CD45 gates calculations can provide a more accurate estimation.

The research has several strengths: balanced sampling regarding matrices and age distribution, standardised flow cytometry with multiparametric analysis and sophisticated statistical analysis techniques.

Limitation(s)

The limitations are small population size and cross-sectional research approach, uncontrolled pre-analytical variables, the lack of functional tests and insufficient subpopulation phenotyping which require careful interpretation and further research.

CONCLUSION(S)

The findings of the present study suggest that flow-cytometric stem cell enumeration could not only reliably detect the differences of CD34 yield and viability due to matrix but also maintain the CD45-related analytical stability, which calls for the matrix-aware interpretation of enumeration outputs and underlines that quantitative yield and cell integrity should be taken into consideration simultaneously for the complete assessment of the stem cell profiles across biological sources.

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