14.

Potential Clinical Applications for Haematopoietic Progenitor Cell (HPC) Quantitation on the SYSMEX XE-2100 Automated Haematology Analyser

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Introduction

My primary objective is to present some of the data obtained at the Saint Louis Cord Blood Bank which was used in the submission to the Food and Drug Administration in the United States for approval to use the HPC (Haematopoietic Progenitor Cell) parameter clinically. Dr Thiede has already explored and discussed some of the potential clinical applications of HPC. This presentation will describe other applications and will particularly look at the information that HPC measurement may provide about cord blood stem cell content. Rather remarkable advances have occurred over the past few years in the therapeutic use of stem cell therapy with cells being collected from bone marrow, umbilical cord blood or peripheral blood. The products collected are minimally manipulated and then infused directly into patients for the treatment of a wide variety of malignant and non-malignant diseases and a great deal of success has been achieved with these approaches.

At the present time there is a serious need for a rapid, reliable method to measure HPC content in order to guide HPC product selection, to identify the optimal timing of apheresis HPC collection, to evaluate HPC recovery during stem cell processing and to monitor engraftment following HPC transplantation. This information is vital to determine if the products have sufficient concentration of therapeutically active cells for the patient, and to determine whether or not these cells have the characteristics necessary to achieve optimal therapeutic benefit. At the present time, the methods that we have for characterising progenitor cell content in these products are expensive, laborious and time consuming.

They are also limited because, for many years, the primary approach used to identify haematopoietic stem cells has been based on information obtained by immunophenotyping using flow cytometry to identify and count cells presumed to be stem cells. The evidence which suggests that this approach is useful is based on studies that actually measure the functional properties of these stem cells. At the present time, the information that tells us that CD34, the most widely used marker, actually measures stem cells, is based on the correlation of CD34 with functional assays. There are many functional assays (**figure 1**). The assay that we perform routinely in the umbilical cord blood bank is in the category of functional assays called “colony forming unit assays” and these predominantly measure lineage committed progenitor cells. These assays do not do a very good job in determining the actual content of the long-term haematopoietic reconstituting cells that have the predominant therapeutic benefit. So, at the present time, there really is no readily available gold standard to identify long-term engrafting haematopoietic stem cells. The best we can do with any new method

is simply to look at how it correlates with what we think we currently know. This was the overall goal we attempted to achieve in our evaluation of the HPC parameter.

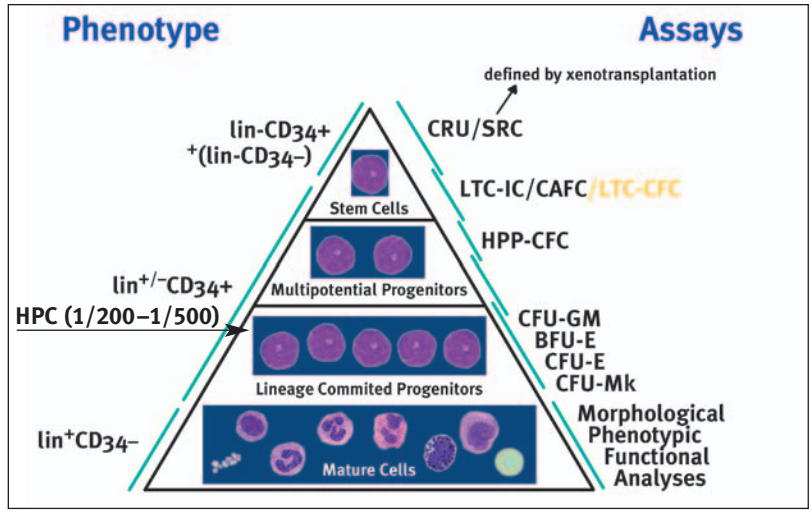

Figure 1
Characterisation of umbilical cord blood derived haemopoietic stem cells. Phenotypes and functional assay procedures.

The HPC Parameter on the SYSMEX XE-2001 – What is it?

What is HPC measuring and why should it correlate with CD34+ assays and with CFU assays? It is very complicated, as Dr Thiede has already pointed out. What I would like to demonstrate is that the ways in which the HPC signals are generated in the SYSMEX device actually correlate with the functional features of the cells. Now it is known that the very early stem cells and the very early haematopoietic progenitor cells are smaller than the lineage committed progenitors, and as they mature, they gradually first increase in size and then progressively decrease in size. Accompanying this are marked alterations in the cytoplasm, characterised predominantly by loss of the diffuse blue cytoplasmic staining, the latter representing the extensive protein synthesizing endoplasmic reticulum. The cells then focus their metabolic activities towards intracellular granule formation, and so the cytosol undergoes a number of characteristic changes predominantly due to the accumulation within these cells of large numbers of granules. By the end of the maturation sequence, the cytoplasm is extremely granular. In addition, the chromatin structure of the nucleus changes tremendously. In the early developmental stages, the chromatin network is extremely open and nucleoli are easily visible. With maturation the nuclear chromatin becomes more condensed and, in the case of the neutrophil, lobulated.

The surface membrane of the early progenitor cells in the marrow is specifically adapted to promote adherence to the supporting stromal cells which is necessary for maintenance of self-renewal properties and to guide differentiation. These adherent cells do not readily leave the marrow. In fact, they are so tightly adherent under most conditions that they have to alter their surface membrane

characteristics in order to become mobilised from the marrow and enter the circulation. These membrane alterations also cause the cells to become more deformable allowing them to move through the vascular endothelium and finally into tissue spaces. Membrane changes are accompanied by alterations in the content of surface membrane proteins and the manner in which these proteins interact with the cytoskeleton. These changes also dramatically alter the sensitivity of the surface membrane to lysis by non-ionic detergents.

What is most fascinating about the SYSMEX HPC approach to stem cell characterisation is that it uses a combination of direct current (DC) resistance and radio frequency (RF) impedance signals and selective detergent lysis. The DC signal identifies changes in cell size as these cells first get larger, and then get smaller again and the RF signal changes in response to alterations in chromatin structure and cytoplasmic granularity (**figure 2**). The trends in the location of cell clusters that are visible in the IMI channel (RF signal versus DC signal) scattergram identify the changes that occur during the cell maturation sequence. This provides a visual display of how the cells are changing during maturation. The feature, which is probably the least well understood, is the selective detergent lysis issue. The early progenitor cells have a more rigid membrane structure and these early progenitors are not susceptible to lysis by non-ionic detergents. As the cells mature, the membrane becomes more fluid and deformable and when maturation is complete, the detergent ruptures the cell membrane. HPC analysis on the SYSMEX, then, is based on the simultaneous detection of RF and DC impedance signals with the subsequent application of adaptive cluster analysis to define the specific region in the IMI channel scattergram that, based on the maturation sequence described, identifies haematopoietic progenitor cells.

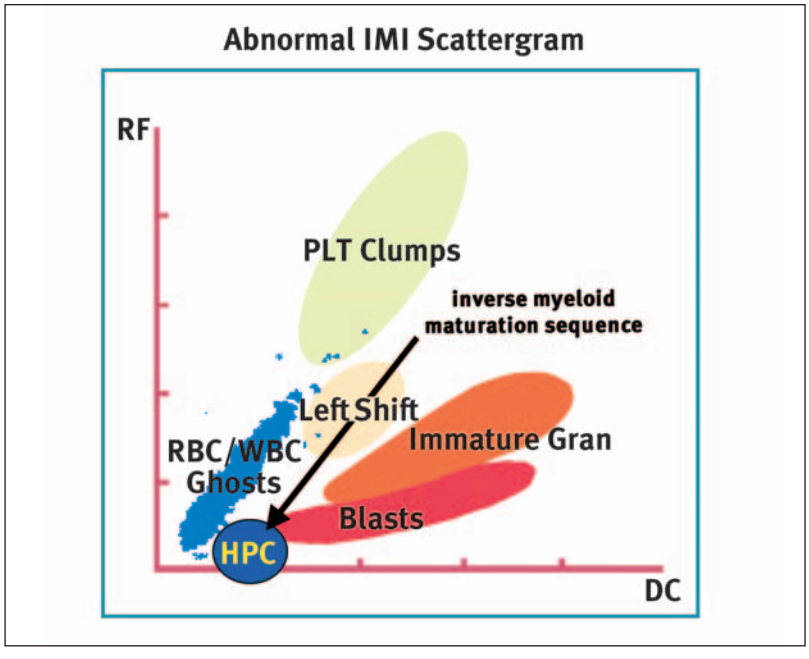

Figure 2
The IMI scattergram showing the location of the various cell types including HPC.

Proof of Concept

Having defined the HPC region, the next step is to prove that progenitor cells lie in this region. A number of different experimental approaches may be used, however the most direct is to pre-select stem cells and then send them through the IMI channel of the SYSMEX XE-2100, and ask the question, do they show up in the HPC region? The method used for CD34+ cell isolation [**1**] included positive selection with magnetic beads with surface-bound anti-human CD34 antibody and negative selection with magnetic beads coated with multiple antibodies to remove lineage positive cells. When the CD34+ cells were analyzed on the XE-2100, they were consistently found in the narrow area within the HPC region of the IMI channel scattergram. Virtually all these cells cluster in that region where haematopoietic progenitor cells had previously been identified. There may also be a small number of lineage-committed cells and some of those appear to be detergent sensitive and do not cluster in the HPC region. The reason for this is not known; it may be the result of in-vitro cell manipulation. What I think is most interesting is that the fraction of the CD34+ cells that are actually the true stem cells lack an antigen called CD38, and we capture a higher percentage of the CD34+ CD38– cells in the HPC region than are found in the total CD34+ cell population. However, clearly there is going to be a strong correlation between CD34 content and HPC following prior CD34+ selection and subsequent HPC analysis on the SYSMEX instrument.

With regard to assessing the functional activities of HPC identified by the SYSMEX, there is really no easy way to select the cells because the analysis of HPC is destructive; the cells are killed as they go through the analyser. The only method is to establish a correlation between functional activities and HPC content in various products. The correlation between HPC and Colony Forming Unit (CFU) assay ($r = 0.86$) in cord blood is shown in **figure 3**. This study involved approximately 80 different cord blood samples collected at various gestational ages and processed at various times after collection.

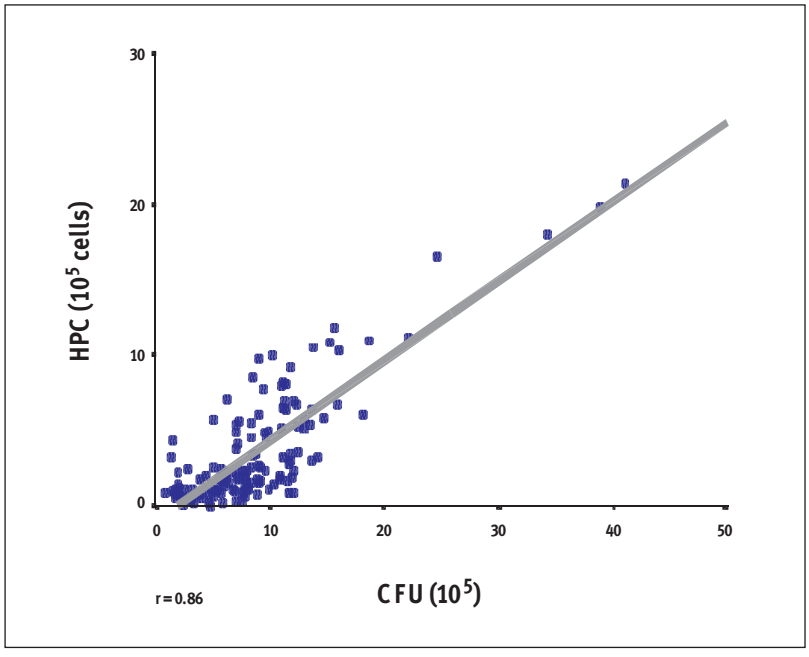

Figure 3
Characterisation of umbilical cord blood derived haemopoietic stem cells. Phenotypes and functional assay procedures.

It is known, however, that the correlation differs in different stem cell populations. Dr. Thiede has pointed out very clearly that the correlation differs in normal patients given growth factor compared with cancer patients given chemotherapy and then growth factor. There is a very different stem cell mobilization response when the bone marrow has already been modified by prior chemotherapy and this may affect the characteristics of the HPC. Cord blood differs yet again from peripheral blood and bone marrow. Accordingly, although HPC correlates well with CFU, the strength of the correlation and the linear regression slope will likely differ for cord blood, peripheral blood and bone marrow. What I find quite interesting is that CFU correlates better with HPC than does the CD34+ cell count or total nucleated cell count (TNC), suggesting that HPC may actually be providing a better marker of functional activity than TNC and CD34+ cell count which are essentially surrogate markers for stem cell function.

Evaluation of HPC

Stability: Stability may prove to be the most difficult issue of all in the application of the HPC measurement. The membrane lysis event induced by the reagent system is not fully understood as yet but may have an important effect on specimen stability over time. We have assessed alternative methods of anticoagulation of the blood specimen in order to determine conditions under which the HPC concentration is optimally maintained. Data derived from adult autologous peripheral blood stem cell (PBSC) collections into EDTA, heparin and citrate anticoagulants are illustrated in **figure 4** [**2**]. Immediately following autologous stem cell collection EDTA and heparin produced comparable numbers of HPC. Collection into citrate, however, resulted in a significantly lower HPC concentration. Over time,

the HPC lose the ability to cluster in the appropriate region and so the count falls. Fortunately, the HPC count remains stable in EDTA for up to 4 hours. Measurements are unreliable after this time. What is very interesting is that the CD34 and CFU content do not change significantly over the same time period. This indicates that there is not a loss of viable stem cells but that these stem cells are losing the ability to remain resistant to detergent lysis. HPC stability studies on umbilical cord blood collected into citrate (CPD) anticoagulant demonstrate less than 10% loss of cells on storage at room temperature for up to 4 hours (**figure 5**). Overall the main limitation of HPC may be this specimen-related issue of HPC stability.

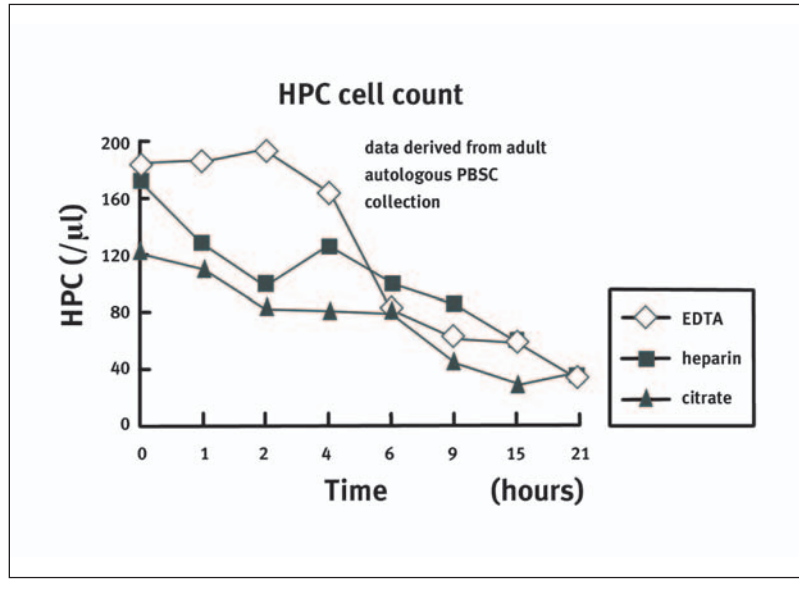

Figure 4
*Effect of anticoagulant for adult. Data derived from autologous PBSC collection [**2**].*

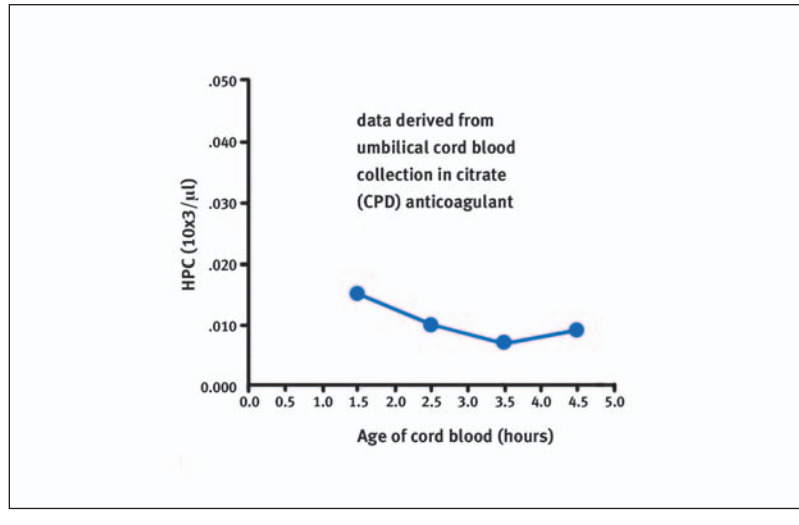

Figure 5
Effect of anticoagulant for cord blood

Linearity: Linearity studies were carried out both on undiluted peripheral blood and in specimens concentrated by removal of autologous plasma (**Table 1**).

Range tested	Regression equation	r^2	Product source
0–106/µL	y = 1.000x–4.20	.99	Peripheral blood
0–609/µL*	y = 1.002x–11.92	.99	Cord blood

Table 1
*Linearity studies on HPC parameter. * Indicates prepared concentrated sample.*

Reproducibility: Precision studies on the HPC parameter were performed on peripheral blood collected in EDTA. The results are shown in **table 2**. HPC analysis precision studies in the range 4–99 cells/µL demonstrated an average coefficient of variation of 15%.

HPC	Mean	SD	CV %	Median	Min.	Max.
10^3/µL	.004	.001	25	.0045	.003	.008
	.011	.002	18	.0105	.008	.014
	.042	.005	11	.042	.035	.053
	.084	.008	9	.084	.071	.098
	.099	.012	12	.102	.076	.114

Table 2
Reproducibility studies on HPC parameter.

Comparability: In previous collaborative studies, we have found that CD34+ cells isolated from peripheral blood are predominantly recovered in the HPC region of the IMI scattergram and that there is a strong correlation between HPC and positively selected CD34+ content (1). In umbilical cord blood, we have characterized the relationship between HPC, total nucleated cell count (TNC), CD34+ cell content and limiting-dilution clonal assays of haematopoietic cell function (i.e., CFU activity). There is a significant correlation between HPC and TNC (r = 0.72, n = 396), CD34+ cell count (r = 0.74, n = 396) and CFU activity (r = 0.79, n = 95) in processed cord blood. There is also a significant correlation between HPC and TNC (r = 0.60), CD34+ (r = 0.62) and CFU activity (r = 0.86) in autologous peripheral blood stem cells collected by apheresis following growth factor stimulation. The stronger correlation between HPC and stem cell functional (CFU) activity in cord blood and apheresis products suggests that HPC may detect both CD34+ and CD34– haematopoietic progenitors with colony forming cell activity.

Reference ranges in health: Under normal conditions paediatric and adult subjects have virtually no haematopoietic progenitor cells detected in the peripheral circulation.

Practical Clinical Points

Is it possible to use HPC to determine CD34+ cell content in autologous or allogeneic peripheral blood samples? We have addressed this issue in paediatric patients undergoing autologous bone marrow transplantation after peripheral blood stem cell collection. Following growth factor mobilisation, we found a very significant correlation between peripheral blood HPC and peripheral blood CD34+ but with a slightly larger number of HPC than CD34+ (**figure 6**). This is very similar to Dr. Thiede's data. Patients from whom we could collect an adequate CD34+ cell dose within a single collection day are shown in red; green are those patients requiring two days for adequate collection; and those requiring three or more days for adequate collection are shown in blue.

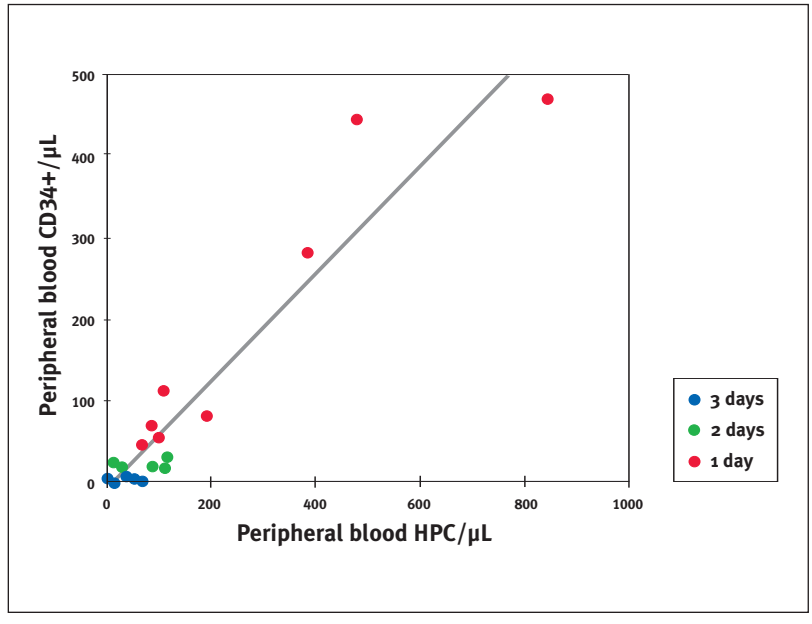

Figure 6
Correlation between mobilised peripheral blood HPC and CD34+ count (paediatric patients)

A further study was performed to assess whether or not the HPC and CD34+ cell content varied during the first and subsequent blood volumes processed during the apheresis stem cell collection procedure. The actual content of HPC per µL of blood remains relatively stable over the entire collection time period so that the percentage actually recovered with each volume of blood processed showed an even stair-case pattern (**figure 7**), indicating that if you use HPC measurement to monitor patients throughout collection, you could use the HPC count to determine the need for processing successive volumes of peripheral blood.

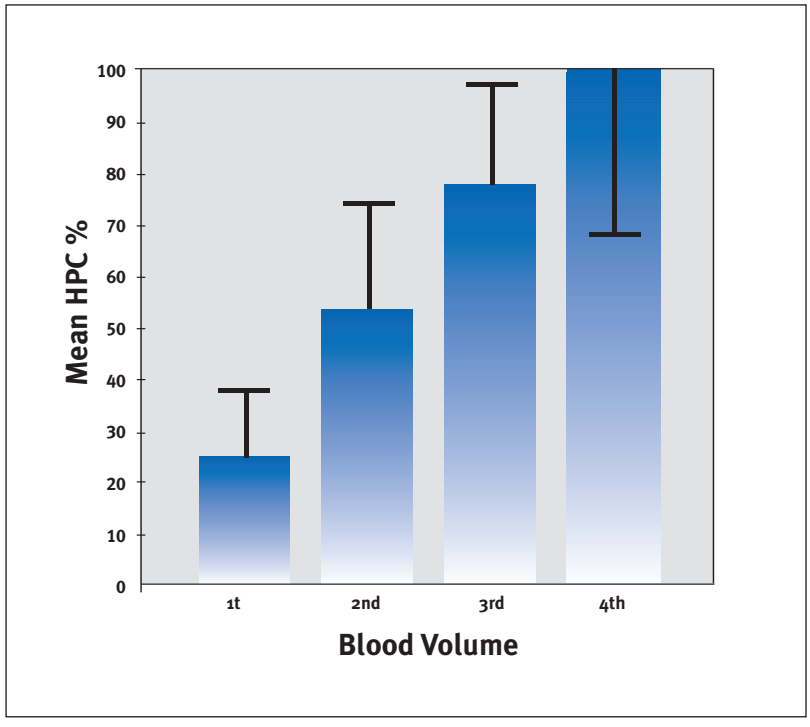

Figure 7
Stem/progenitor cell collection efficiency during consecutively processed blood volumes.

To determine the optimum cut-off for HPC to predict whether to proceed with apheresis stem cell collection we have used the correlation between HPC and CD34, and our previous observation that adequate stem cell collections could be obtained when patients had at least 10 CD34+ cells/µL. We did not perform a formal receiver operator characteristic (ROC) curve analysis, but picked just this one operating point (**figure 8**). The resulting square regions on the plot were defined as: A = discordant negative (CD34+/HPC negative [false negative]); B = concordant positive (CD34+/HPC [true positive]); C = concordant negative (CD34–/HPC negative [true negative]; and D = discordant positive (CD34–/HPC positive [false positive]). For this analysis, the CD34+ cell count was selected as the reference method (i.e., the CD34+ cell count was accepted as defining the "true" stem cell content). At a cut-off of 20/µL for HPC, we did not have a single instance of high CD34+ where the HPC was not also elevated. The data is presented in **table 3**.

Table 3
Utility of the HPC parameter to identify optimal timing of apheresis.
TP = true positive;
FP = false positive;
FN = false negative;
TN = true negative.

	› 10 CD34/µL	‹ 10 CD34/µL	Total
› 20 HPC/µL	16 (57.1%) [TP]	8 (28.6%) [FP]	24 *(85.7%)*
‹ 20 HPC/µL	0 [FN]	4 (14.3%) [TN]	4 *(14.3)*
Total	16 *(57.1)*	12 *(42.9%)*	

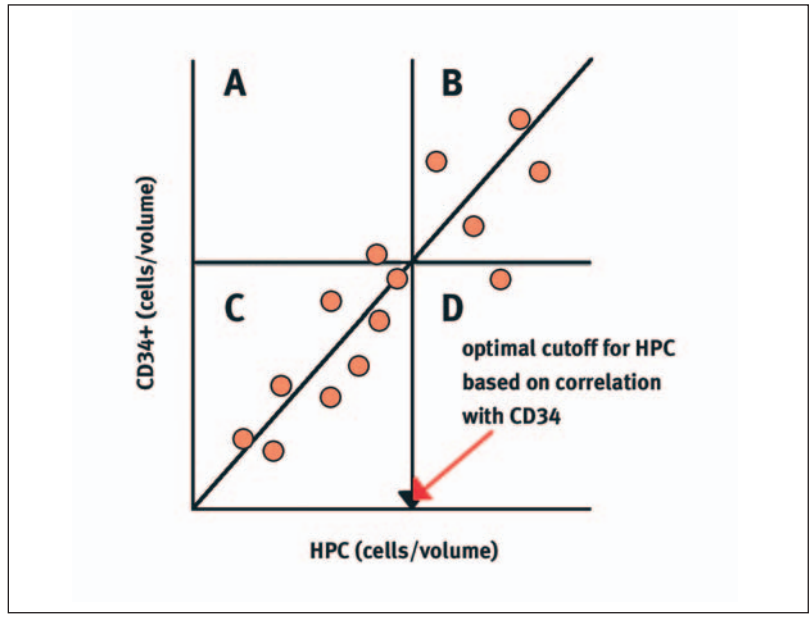

Figure 8
Defining the optimal cut-off for HPC to predict apheresis collection based on correlation with CD34+ cell count.

The experimental series consists of 28 autologous peripheral blood collections from paediatric patients following G-CSF mobilization. Overall agreement is 71%, however, sensitivity is 100% with a negative predictive value of 100%. Thus, whenever the HPC count is low, the CD34+ cell count is also low indicating that collection should be delayed. The positive predictive value is 67%. Thus, when the HPC count is high, 67% of the time the CD34+ cell count will also be high and apheresis collection should proceed. About 1/3 (33%) of the time that the HPC count is high, the apheresis collection may require processing additional volumes of blood to ensure collection of an adequate CD34+ cell dose. HPC quantitation is therefore a useful screening test to exclude potential candidates for apheresis peripheral blood progenitor cell collection when the stem cell content is low. When the HPC count is high, we recommend that the HPC and CD34+ cell count be repeated after collecting 4 blood volumes to ensure that an adequate collection has been achieved.

Future Studies

The ultimate determination of what HPC means should be based on a clinical endpoint and not on a surrogate marker of what a stem cell is thought to be. In the author's opinion, what needs to be established is the correlation of HPC with survival after transplant. At the present time, the most characteristic feature of umbilical cord blood transplants is the fact that therapeutic benefit is very dose dependent and dose limited. For cord blood stem cell transplants, CD34+ cell count and TNC show strong correlation with survival probability, even though they are not ideal ways of determining stem cell content. If transplant outcomes are strongly correlated with the HPC content of the cord blood product determined by analysis on the SYSMEX device at the time of collection, we would have a better

way of defining and identifying the best cord blood products for processing. So our hope is to use the HPC measurement prospectively and at some point in time to actually have cord blood transplant outcomes data that we can correlate with HPC.

Conclusion

A laboratory method that could rapidly estimate circulating haematopoietic progenitor cells (HPC) in peripheral blood on the day of planned apheresis would be invaluable in deciding whether to begin the procedure and for allocation of resources in both the apheresis unit and the progenitor cell processing laboratory. Our experience at St Louis University in peripheral blood haemopoietic stem cell collections following growth factor mobilization demonstrates that the HPC parameter can identify products with high CD34+ progenitor cell content. Another potential utility of the HPC parameter is the estimation of stem cell content in umbilical cord blood products. Analysis of HPC in a specimen of freshly collected EDTA-anticoagulated cord blood allows identification of products with high CD34+ cell count and colony-forming (CFU) activity. The ability to identify units with high content of viable haematopoietic progenitor cells that would otherwise not meet the minimum criteria for processing would be a tremendous advantage to an umbilical cord blood bank.

References

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[2] **Saigo K., Sugimoto T., Narita H.,** *et al (1999)*
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