

# The Cord Blood Separation League Table: a Comparison of the Major Clinical Grade Harvesting Techniques for Cord Blood Stem Cells

Christina Basford<sup>1</sup>, Nicolas Forraz<sup>2</sup>, Saba Habibollah<sup>1</sup>, Kendal Hanger<sup>1</sup>, Colin McGuckin<sup>2</sup>

<sup>1</sup>Newcastle Centre for Cord Blood, Institute of Human Genetics, Newcastle University, Newcastle upon Tyne, NE1 3BZ, United Kingdom, <sup>2</sup>CTI-LYON, Cell Therapy Research Institute, Parc Technologique de Lyon-St Priest, Cèdre 1, 97 Allée Alexandre Borodine, 69800 SAINT PRIEST LYON, France

**Background and Objectives:** Well over 1 million Umbilical Cord Blood units (UCB) have been stored globally in the last 10 years. Already, over 20,000 transplants been performed using UCB for haematopoietic reconstitution alone, now this potential is joined by regenerative medicine. However, more needs to be known about processing of this stem cell source for it to reach full potential.

**Methods and Results:** In this study we evaluated five separation methods: plasma depletion, density gradient, Hetastarch, a novel method known as PrepaCyte-CB and an automated centrifugal machine. Sepax gives the highest recovery of nucleated cells, an average of 78.8% (SD±21.36). When looking at CD34+ haematopoietic stem cells PrepaCyte-CB provided the greatest recovery at 74.47% (SD±8.89). For volume reduction density gradient was the most effective leaving  $0.03 \times 10^6$  RBC/ml, 8 times more efficient than its nearest competitor PrepaCyte-CB ( $p < 0.05$ ). Finally PrepaCyte-CB processing left samples with the highest clonogenic potential after processing and more significantly after cryopreservation: 9.23 CFU/ $10^8$  cells (SD±2.33), 1.5 fold more effective than its nearest rival Sepax ( $p < 0.05$ ).

**Conclusions:** PrepaCyte-CB was the most flexible method; the only processing type unaffected by volume. Results indicate that processing choice is important depending on your final intended use.

**Keywords:** Stem cells, Bioprocessing, Umbilical cord blood, Transplantation

## Introduction

The global rise in Umbilical Cord Blood (UCB) as a transplant source, has been amazing, over 20,000 transplants already having taken place in haematopoietic reconstructions alone (1). It has become a real alternative to bone marrow (BM) and peripheral blood (PB) as a

source of adult stem cells to treat multiple diseases. The first properly validated transplant took place in Paris in 1989 and was performed by Gluckman et al. They were successful in reconstituting the haematopoietic system of a child with Fanconi's anaemia with UCB from an HLA-identical sibling rather than BM (2). However, we believe the success of UCB for transplantation can be traced back to 1970, when a young male with acute leukaemia, received a multiple (eight) cord blood unit transplant, remaining disease free for twelve months. After the triumph of these early infusions of UCB more than 85 conditions can currently be treated using this stem cell source, such as the previously mentioned Fanconi's anaemia, a BM failure disorder (2), metabolic disorders like Krabbe's disease (3) and immune defects like severe combined immune de-

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Correspondence to **Colin McGuckin**

CTI-LYON, Cell Therapy Research Institute, Parc Technologique de Lyon-St Priest, Cèdre 1, 97 Allée Alexandre Borodine, 69800 SAINT PRIEST LYON, France

Tel: +33-4-26 03 01 29, Fax: +33-4-26 03 01 29

E-mail: c.mcguckin@conoworld.com

ficiency (SCID) (4).

UCB has become such a popular adult stem cell source for many reasons, not least because over 130 million births worldwide per annum represents the largest, easily available stem cell source. It also allows for storage of units from ethnic minorities not easily possible within BM registries (5). This potentially allows for an increase in the rate of matched unrelated donor allogeneic transplants (6). It has also been found that there is a lower risk of graft versus host disease (GvHD) when transplanting UCB when compared to BM (7). This could be due to the fact that the cells transplanted from UCB are more naïve and have lower human leukocyte antigen (HLA) protein expression (6). UCB has been shown to contain a higher frequency of early progenitor cells than PB or BM (8). Further findings show that term and pre-term UCB contain significantly higher number of early and committed progenitor cells, and that they are better able to form colony-forming-unit granulocyte-macrophage (CFU-GM) when compared to adult PB (9). Other studies show that as well as being a source of haematopoietic progenitor cells, UCB also contains non-haematopoietic stem or progenitor cells including mesenchymal and endothelial precursors (10).

Even more recently UCB is becoming a real player in the regenerative medicine field. There are many groups looking to develop tissues for either transplantation or drug testing with many successes. For example, the McGuckin and Denner groups, working in Texas, have succeeded in being able to generate insulin producing islet cells from UCB with the hope to be able to treat diabetes in the future (11). Another group have managed to produce functioning neural cells (12) showing great potential in the treatment of neurological disorders.

So, although there are many benefits to using UCB for transplantation there is one major limitation: the total nucleated cell (TNC) count and cell number recoverable from a single unit. This is affected by unit size, maternal factors such as number of previous pregnancies and age of mother (13), limited volumes available from each sample, but not least the processing method used. Together, these factors highlight the need to make processing as efficient as possible (14) to make cord blood banking a real option. Many methods are currently available and the purpose of this study was to evaluate five of the main types. Techniques were varied; from density gradient separation, like Lymphoprep or Ficoll-Paque (15), rouleaux formation using Hetastarch (HES), a starch based method causing red cells to clump (16), plasma depletion; a simple volume reduction method which avoids the addition of any chemicals and simply removes the plasma (17), a novel closed

separation kit, closed separation kit, known as PrepaCyte-CB which offers rapid and specific cell separation (18). In this study we used a PrepaCyte-CB kit designed specifically for UCB units, to allow recovery of other non-erythroid subsets such as leukocytes, thrombocytes and stem cells, including both hematopoietic stem cells (HSC) and multi-lineage progenitor cells, without them needing to be bound to unwanted chemicals (19) or particles. The final separation system chosen was also the only fully automated system, provided by Biosafe, known as the 'Sepax' machine (20).

## Materials and Methods

### Inclusion criteria

Samples were collected from the Royal Victoria Infirmary Maternity Unit, Newcastle upon Tyne, UK, only after written and informed consent was gained from the parents. The protocol followed, was reviewed and approved by the National Health Service local ethics committee. A negative viral profile and infection status was required. UCB units were processed and cryopreserved within 24 hours of collection and only if the sample exceeded 40 ml.

### Collection

For logistical and reasons of homogeneity of treatment, samples were only collected from caesarean section births. Collection took place post partum, after the placenta was delivered. The cord was clamped in line with currently used protocols at the hospital, and was performed by the surgeon. Under no circumstance was the birthing procedure adjusted or changed due to our study. Two clamps were used: one placed close to the placenta and one close to the baby. The placenta was then hung in a cone shaped collection vessel with the cord hanging through the underside. Collection bags used contain citrate phosphate dextrose adenine (CPD-A) anticoagulant and had a needle attached (Baxter PL146-CPDA-1-35 ml Deerfield IL, USA). This was spiked into the bottom of the cord allowing the blood to drain into the collection bag. Only blood from the umbilical cord vein was collected, not from the placenta. Gravity and natural movement were used to drain the cord. Once sample collection was complete, the unit was transported back to the laboratory and stored at room temperature until processing was initiated.

### Processing

All umbilical cord blood samples used in this study were processed within 24 hours of collection. For each

unit, analysis samples were taken pre and post processing and post cryopreservation/thaw.

**Hetastarch (HES):** The UCB was transferred to 150 ml transfer bags (Baxter, R4R2001) then HES (Baxter B5084 6% in 0.9% Sodium Chloride) was added to the UCB at a concentration of 20% blood volume. The bag was centrifuged at  $125\times g$  for 10 min, with the brake off, (Jouan CR422. St-herblain, France), to stop disruption of the RBC pellet. Using a plasma expresser (Fenwal BM-1. Lake Zurich IL, USA), the supernatant containing the desired nucleated cells was expressed off into a second transfer bag. The second bag was then centrifuged at 400 to  $500\times g$  for 10 min (Jouan CR422). Again using a plasma expresser, the supernatant was removed into a third transfer bag and this time discarded, leaving the pelleted nucleated cells in the second bag, where they were then resuspended in Human Serum Albumin (HSA) (Bio Products Laboratory PL08801/006. Elstree, UK).

**PrepaCyte-CB:** Before adding the UCB unit to the PrepaCyte-CB device it was thoroughly mixed using a plate rocker (Genesis Blood Collection Mixer CM-735. Hackensack NJ, USA). The UCB unit was then spiked with the connecting tube from the PrepaCyte-CB system ([www.BioE.com](http://www.BioE.com)) allowing the blood to drain into it. For optimal recovery it was recommended to drain a portion of the reagent-cord blood mixture back into the collection bag, mix and transfer bag into the processing bag. The tubing between UCB collection bag and the bag set was heat sealed and the collection bag discarded. The bag set containing the reagent-blood mixture was rocked for 3~5 min, 15~20 rocks per min. After mixing, the bag set was hung on a plasma expresser (Fenwal BM-1) for 30 min to allow the unwanted cells to aggregate and sediment. After sedimentation, using the plasma expresser, the TNC-rich supernatant was pushed into the next bag for centrifugation at 400 to  $500\times g$  for 10 min, with a low break to avoid disruption of the pellet (Jouan CR422). After centrifugation the TNC and stem cell portion was pelleted allowing the unwanted second supernatant to drain back through the system into the first bag with the unwanted RBC portion of the sample. The stem cell fraction then continued into the cryopreservation bag or equally it could be used in the laboratory for tissue culture purposes.

**Plasma depletion:** The collection bag was connected to a 300 ml transfer bag (Baxter) and the product flowed through. It was then heat sealed and the tubing and collection bag were removed. The bag was spun for 10 min at 2,400 rpm (temp. 15~26°C). The bag was carefully removed from the centrifuge and hung on a plasma expresser, where the unwanted plasma drained into a 150

ml transfer bag (Baxter). After removal of plasma, the bags were heat sealed and removed. The UCB unit was thoroughly mixed to resuspend the cells in the residual plasma. The cryobag was then attached, using sterile technique and the UCB was allowed to flow into it. The unwanted bag and tubing was then heat sealed and removed.

**Biosafe's sepax:** This fully automated, functionally closed and sterile system, was controlled by computer software. It was possible to isolate the HSC rich buffy-coat of a UCB unit to a final volume of 10 to 90 ml. Each unit was separated as per the user guide with a single use kit, which was inserted into the machine. Briefly, the UCB was added to the machine where it filled the central chamber. Whilst filling, the system simultaneously sedimented the UCB unit. The sample was spun at speeds of up to  $1,900\times g$  and the blood components were separated concentrically. Then, using optical sensors and motorised stopcocks the blood components were directed to individual blood bags and extracted from the UCB unit (<http://www.biosafe.ch>). This method was recently validated by the Besançon Cord Blood Bank in France (20).

**Density gradient; ficoll-paque/lymphoprep:** Ficoll is a neutral, highly branched, high-mass, hydrophilic polysaccharide which can readily dissolve in aqueous solutions. The UCB was diluted 50 : 50 with a buffered saline solution (PBS) and was then layered over the Ficoll. The sample was centrifuged at  $800\times g$ , for 20 min, to accelerate the density gradient separation, as per manufacturers guide (<http://www.axis-shield-density-gradient-media.com>). The buffy layer of WBCs was at the interface between the sample and the medium, and was collected using a sterile Pasteur pipette. The buffy coat was then washed with PBS.

### Sample analysis

**Enumeration by differential cell counting:** The cell counts were performed using the CellDyn4000 Analyser, a mechanical method. A sample of 1 ml was applied to the machine at each of the three points during processing. The WBC count and differential determine the number of white blood cells and the percentage of each type of WBC in each unit of blood.

**Colony forming unit assays:** A cell suspension was prepared with the appropriate number of cells; at  $0.25\times 10^5$ /plate. Colony counts must then be multiplied by 4 to give the number of colonies per  $10^5$  cells. The relevant number of cells was added to make up a volume of IMDM (Gibco/Invitrogen 21980-032. Paisley, UK)+20% FCS+ supplements to 0.5 ml, then added to a 3.5 ml aliquot of methylcellulose culture medium, (Stem cell Technologies

04534, USA), making a final volume of 4 ml. Samples were plated in triplicate in small, gridded Petri dishes (Nunc brand, VWR 734-2114 Westchester PA, USA) 1ml per plate. The plates were incubated at 37°C with 5% CO<sub>2</sub> for 14 days. After incubation the number of granulocyte-macrophage colonies and other lineages present were assessed. An average colony number from the three test plates was then calculated to give colony numbers per 10<sup>5</sup> cells plated.

**Flow cytometric analysis:** Flow cytometric analysis was carried out using a Becton Dickinson FACS Caliber machine. The samples were prepared as follows; 100 µl of blood was added to each tube and 50 µl of antibody cocktail (For antibody, fluorochromes and supplier details see Table 1). The tubes were then incubated at room temperature, in the dark for 20 min. The cells were then lysed and washed on the BD FACS Lyse/Wash Assistant, after which the samples were run on the flow cytometer.

**Cryopreservation of umbilical cord blood:** When the UCB was ready for cryopreservation it was cooled to 4°C before being transferred to a cryostore bag (Quest Biome-

dical CS250n, Solihull, UK.), where dimethyl sulphoxide (DMSO) (Origen 210002, Austin TX, USA) mixed with Dextran 40 (Baxter Healthcare B5043), also cooled to 4°C, was added at a concentration of 10%. The sample was cryopreserved in a controlled rate freezer (Planar Kryo 560-16, Sunbury-on-Thames, UK) using the following protocol: Start temperature=4°C, step I=hold @ 4°C for 10 min, step II=-2°C/min to -5°C, step III=-1°C/min to -40°C, step IV=-5°C/min to -100°C. The sample was then transferred to the gaseous phase of a liquid nitrogen Dewar. Samples were frozen for a minimum 14 days before thawing.

**Thawing of umbilical cord blood:** This protocol was based on Rubinstein's method (14). After thawing, the sample was then resuspended in Dextran/Albumin to its original post processing volume.

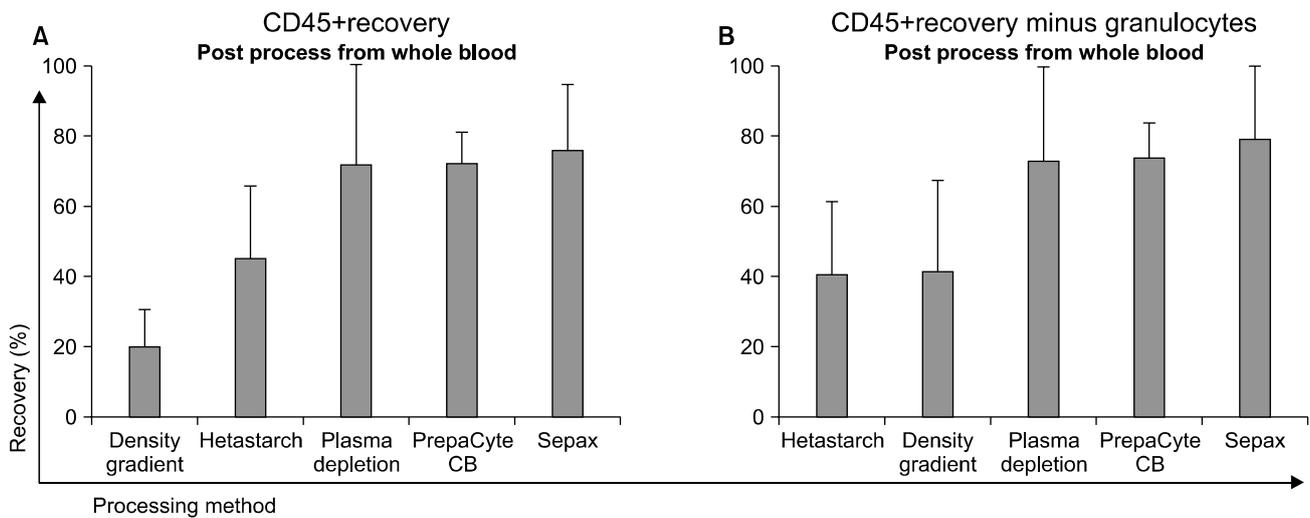
### Statistical analysis

Statistical analysis was carried out using the statistical software programme, Minitab (Version 15, 2006, University of Pennsylvania, USA). The data was analysed using

**Table 1.** Antibody cocktails used for cell separation assessment: flow cytometry

Antibody	Type	Volume (µl)	Cat. no.	Distributor	Tube no.
CD45 (APC Cy7)	IgG <sub>1</sub>	5	557833	BD Biosciences	1, 2, 3, 4
CD235a (PE)	IgG <sub>2b</sub>	5	555570	BD Pharmingen	1, 2, 3, 4
7AAD	IgG <sub>1</sub>	5	559925	BD Pharmingen	1, 2, 3, 4
CD14 (Pacific Blue)	IgG <sub>1a</sub>	5	558121	BD Biosciences	1, 2
CD33 (PerCP-Cy5.5)	IgG <sub>1</sub>	20	333146	BD Biosciences	1, 2
CD7 (FITC)	IgG <sub>1</sub>	20	555360	BD Pharmingen	1, 2
CD4 (Alexa 700)	IgG <sub>1</sub>	5	557922	BD Pharmingen	1, 2
CD25 (PE-Cy 7)	IgG <sub>1</sub>	5	557741	BD Pharmingen	1, 2
CD3 (Biotin) <sup>c</sup>	IgG <sub>2a</sub>	20	555338	BD Pharmingen	1
CD8 (Alexa 647)	IgG <sub>1</sub>	5	557708	BD Pharmingen	1
CD19 (Biotin) <sup>c</sup>	IgG <sub>1</sub>	20	555411	BD Pharmingen	2
CD56 (APC)	IgG <sub>1</sub>	20	555518	BD Pharmingen	2
CD34 (PE Cy7)	IgG <sub>1</sub>	5	348811	BD Sciences	3
CD133 (APC)	IgG <sub>2b</sub>	5	130-090-854	Miltenyi Biotec	3
CD38 (Biotin) <sup>c</sup>	IgG <sub>1</sub>	5	ab30418	AbCam	3
CD90 (FITC)	IgG <sub>1</sub>	5	555595	BD Biosciences	3
CD117 (unconjugated) <sup>b</sup>	IgG <sub>1</sub>	5	313202	Bio legend	3
CD41 (unconjugated) <sup>a</sup>	IgG <sub>1</sub>	5	ab15021	AbCam	3
Lineage 1 (FITC)	IgG <sub>1/2b</sub>	20	340546	BD Biosciences	4
CD123 (PerCP-Cy5)	IgG <sub>2a</sub>	20	558714	BD Pharmingen	4
CD11c (PE)	IgG <sub>1</sub>	5	555392	BD Pharmingen	4
HLA-DR (Alexa 700)	IgG <sub>2a</sub>	5	307626	Bio legend	4
HLA-DQ (Biotin) <sup>c</sup>	IgG <sub>2a</sub>	5	ab24265	AbCam	4
HLA-ABC (Pacific Blue)	IgG <sub>2a</sub>	5	311418	Bio legend	4
HLA-G (unconjugated) <sup>a</sup>	IgG <sub>1</sub>	5	ab7758	AbCam	4
CD16 (PE-Cy7)	IgG <sub>1</sub>	5	335823	BD Biosciences	4

Unconjugated antibodies were labelled using a Zenon labelling complex from Invitrogen; Alexa405 z25313<sup>b</sup> or Alexa430 z25301<sup>a</sup>. The biotinylated antibodies were labelled using Quantum dots 605, also from Invitrogen, conjugated to streptavidin<sup>c</sup>.



**Fig. 1.** Sepax gives an increased recovery of nucleated cells than all other methods. (A) Sepax offers the highest recovery of CD45+ cells at 75.79% (SD±18.58) compared to PrepaCyte CB at 72.03% (SD±8.48), Plasma depletion at 71.38% (SD±29.35), Hetastarch at 44.94% (SD±20.06), and Density Gradient at 19.13% (SD±10.88). Sepax was only significantly more efficient than Hetastarch and density gradient methods ( $p < 0.005$ ). (B) After exclusion of granulocytes recovery with Sepax is still the greatest at 78.8% (SD±21.36) compared to other methods. These results were determined using differential cell counts coupled with flow cytometric analysis of CD45+ cells, viability of CD45+ cells. Exclusion of granulocytes was also determined in this way to give a more accurate picture of cell recovery. Fluorochromes used were PE Cy5 for CD45+ cells, PE for CD14 +/- cells and 7AAD for viability.

non parametric, two-tailed Mann-Whitney U-testing to determine significance. A  $p < 0.05$  was considered to be statistically significant.

## Results

For this study, 80 UCB units were processed. Of those samples, 46% were from female infants ( $n=37$ ) and 54% were from male infants ( $n=43$ ). The birth weights of the infants involved ranged from 2.41 kg to 4.42 kg with an average of 3.52 kg (SD±0.38). The volume of blood collected ranged from 46.6 ml to 194.3 ml with an average volume of 98.72 ml (SD±31.56).

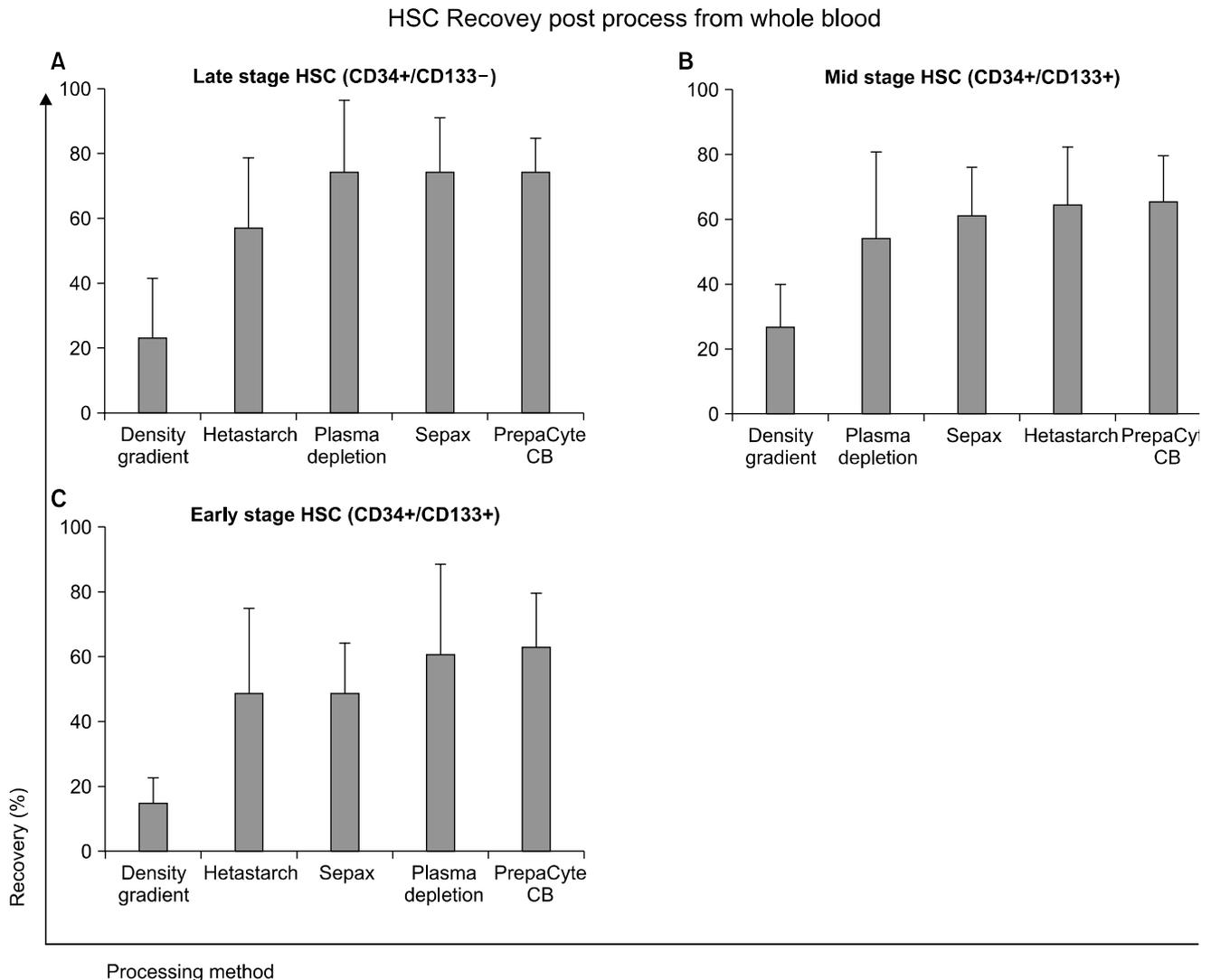
### Recovery of nucleated cell fraction

When looking at TNC recovery, Sepax gives the highest recovery at 75.79% (SD±18.58). This is significantly greater than all other methods tested ( $p < 0.05$ ) apart from PrepaCyte-CB ( $p=0.90$ ) (Fig. 1A). After exclusion of granulocytes recovery with Sepax is still the greatest at 78.8% (SD±21.36) compared to other methods, however data is only significant when compared to density gradient separation where recovery is 40.28% (SD±20.97) ( $p < 0.01$ ). These results were determined using differential cell counts coupled with flow cytometric analysis of CD45+ cells, viability of CD45+ cells. Exclusion of granulocytes was also determined in this way to give a more accurate picture

of cell recovery. Fluorochromes used were PE Cy5 for CD45+ cells, PE for CD14 +/- cells and 7AAD for viability. We also examined the effect initial cord blood volume had on recovery of nucleated cells. Hetastarch, Sepax (not significant) and plasma depletion processing exhibited a negative correlation; as the volume of the cord blood increased, the recovery of nucleated cells decreased ( $p < 0.05$ ) (Fig. 6A, D, E). However when processed with PrepaCyte-CB there was no significant correlation and recovery remained constant regardless of volume (Fig. 6B). Density gradient processing became more efficient as UCB volume increased ( $p < 0.01$ ) (Fig. 6C).

### Recovery of haematopoietic stem cells

We looked at three different populations of HSC as previously described; early stage (CD34-/CD133+), mid stage (CD34+/CD133+) and late stage (CD34+/CD133-) (13). When looking at recovery of late stage haematopoietic stem cells results show that PrepaCyte-CB gives the greatest recovery at 74.47% (SD±8.89). Although this result is higher than all other methods it is only significantly more effective than Hetastarch, whose recovery was low at 56.48% (SD±21.99), providing an average increase of 17.99% ( $p=0.024$ ) (Fig. 2A). Again we looked at the effect initial cord blood volume had on recovery of CD34+ cells. As was shown with nucleated cell recovery, Hetastarch, Sepax and plasma depletion processing ex-

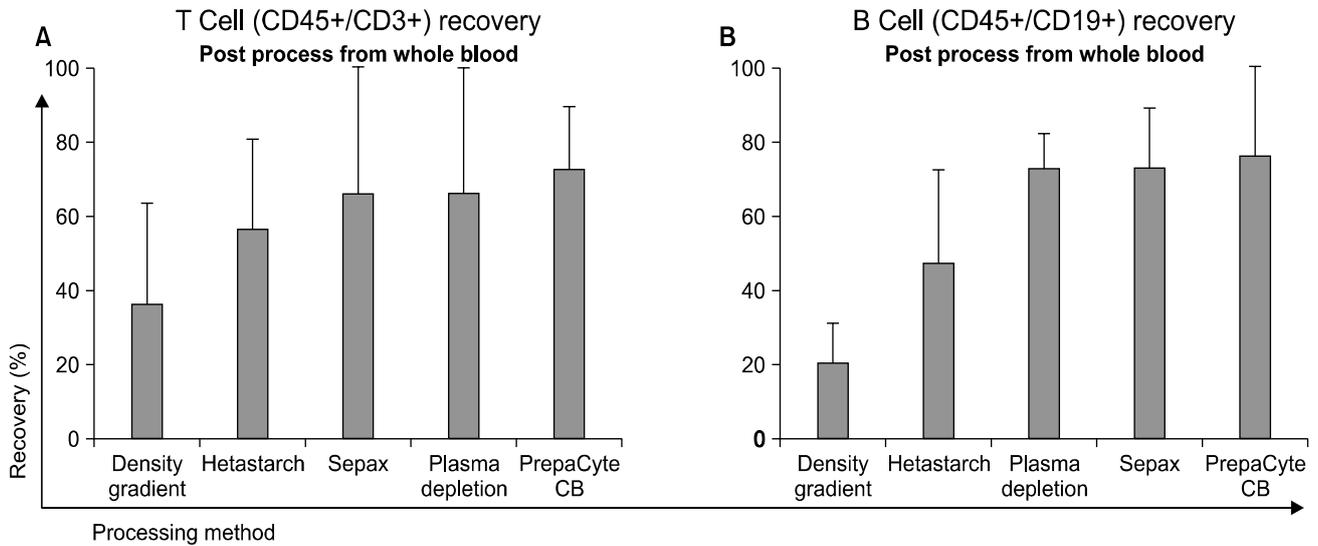


**Fig. 2.** PrepaCyte-CB provides the most efficient recovery of haematopoietic stem cells than other methods tested. (A) Recovery of late stage haematopoietic (CD34+/CD133-) stem cells was greater with PrepaCyte-CB at 74.41% (SD±8.89) than Sepax; 73.91% (SD±16.86), plasma depletion; 73.88% (SD±22.26) Hetastarch; 56.48% (SD±21.99) and density gradient separation methods 22.83% (SD±18.56). However, results are only significant when compared to HES ( $p=0.05$ ). (B) Recovery of mid stage haematopoietic (CD34+/CD133+) stem cells stem cells was greater with PrepaCyte-CB at 65.83% (SD±14.22) than Hetastarch; 64.21% (SD±17.89), Sepax; 60.98% (SD±14.90), plasma depletion; 54.30% (SD±26.79) and density gradient separation methods 27.43% (SD±13.37). However, results are only significant when compared to density gradient ( $p<0.01$ ). (C) Recovery of early stage haematopoietic (CD34-/CD133+) stem cells was greater with PrepaCyte-CB at 62.59% (SD±16.23) than plasma depletion; 60.10% (SD±28.5), Sepax; 48.50% (SD±15.06) Hetastarch; 48.40% (SD±26.2) and density gradient separation methods 13.06% (SD±9.36). However, results are only significant when compared to density gradient ( $p<0.01$ ). CD34+ cell numbers and viability were calculated using flow cytometric analysis of CD34 antibody and 7AAD uptake. Fluorochromes used were FITC for CD45+ cells, PE for CD34+ cells and 7AAD for viability.

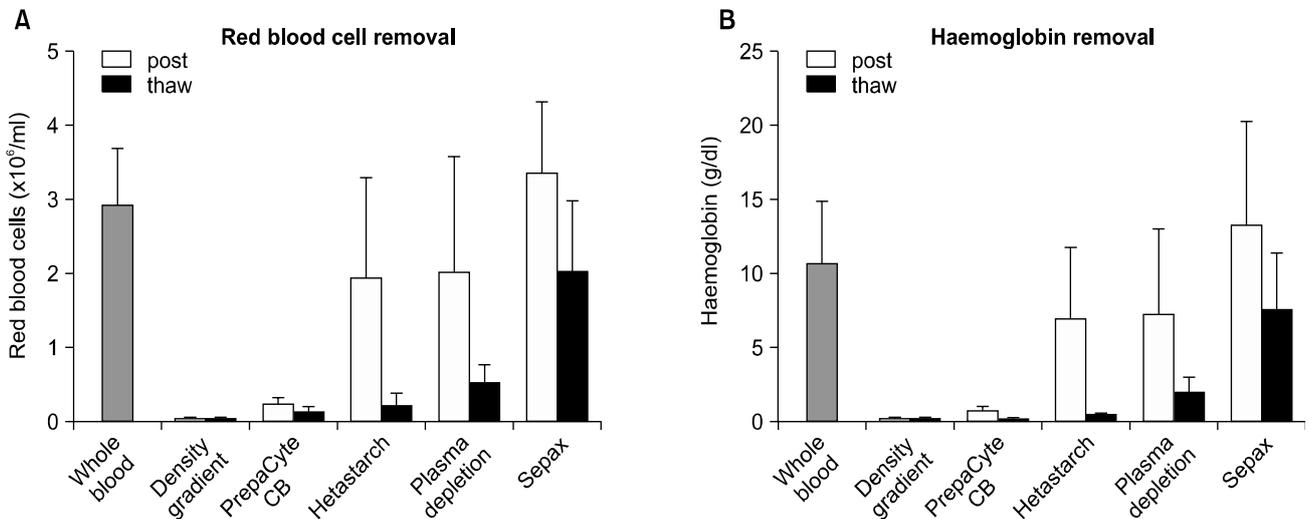
hibited a negative correlation; as the volume of the cord blood increased, the recovery of CD34+ cells decreased ( $p<0.05$ ) (Fig. 6F, I, J). However when processing with PrepaCyte-CB there was no significant correlation and recovery remained constant regardless of volume (Fig. 6G). Density gradient processing became more efficient as UCB volume increased ( $p<0.01$ ) (Fig. 6H).

Next we looked at recovery of mid stage HSC. PrepaCyte-CB gave the highest recovery at 65.83% (SD±14.22); this was greater than all the others methods evaluated but only significantly higher than density gradient processing which gave a recovery of 27.43% (SD±13.37) (Fig. 2B).

Finally when looking at recovery of early stage, it was



**Fig. 3.** PrepaCyte-CB gives the greatest recovery of T Cells (CD45+/CD3+) and B Cells (CD45+/CD19+). (A) Recovery of T Cells (CD45+/CD3+) was greater with PrepaCyte-CB at 71.84% (SD±18.06) than plasma depletion; 65.54% (SD±35.0), Sepax; 65.26% (SD±35.19) Hetastarch; 56.85% (SD±23.79) and density gradient separation methods 36.62% (SD±26.32). (B) Recovery of B Cells (CD45+/CD19+) was greater with PrepaCyte-CB at 75.68% (SD±24.0) than Sepax; 71.75% (SD±16.84), plasma depletion; 71.74% (SD±8.66) Hetastarch; 46.47% (SD±25.69) and density gradient separation methods 19.13% (SD±10.88). However, results are only significant when compared to HES (p= <0.05).



**Fig. 4.** Density gradient separation is the most effective method for volume reduction by removal of either; (A) Red blood cells and (B) Haemoglobin. (A) Compared to whole blood, which has an average of  $2.9 \times 10^6$  cell/ml (SD±0.75), post processing, density gradient is the most effective method for the removal of red blood cells, it leaves  $0.03 \times 10^6$  cell/ml (SD±0.02), PrepaCyte-CB leaves  $0.24 \times 10^6$  cell/ml (SD±0.08), Hetastarch leaves  $1.93 \times 10^6$  cell/ml (SD±0.22), plasma depletion leaves  $2.01 \times 10^6$  cell/ml (SD±1.53) and Sepax leaves  $3.33 \times 10^6$  cell/ml (SD±0.95), all results are significant (p= <0.05). Post thaw density gradient is again the most effective method for the removal of red blood cells, it leaves  $0.02 \times 10^6$  cell/ml (SD±0.01), PrepaCyte-CB leaves  $0.11 \times 10^6$  cell/ml (SD±0.10), Hetastarch leaves  $0.22 \times 10^6$  cell/ml (SD±0.16), plasma depletion leaves  $0.54 \times 10^6$  cell/ml (SD±0.22) and Sepax leaves  $2.01 \times 10^6$  cell/ml (SD±0.94), all results are significant (p= <0.05). (B) Compared to whole blood, which has an average of 10.58 g/dl (SD±4.11), post processing, density gradient is the most effective method for the removal of haemoglobin, it leaves 0.17 g/dl (SD±0.12), PrepaCyte-CB leaves 0.68 g/dl (SD±0.31), Hetastarch leaves 6.94 g/dl (SD±4.74), plasma depletion leaves 7.23 g/dl (SD±5.56) and Sepax leaves 13.07 g/dl (SD±7.03), all results are significant (p= <0.05). Post thaw PrepaCyte-CB is the most effective method for the removal of haemoglobin, it leaves 0.10 g/dl (SD±0.04), density gradient leaves 0.13 g/dl (SD±0.05), Hetastarch leaves 0.46 g/dl (SD±0.29), plasma depletion leaves 1.94 g/dl (SD±1.05) and Sepax leaves 7.57 g/dl (SD±3.70), all results are significant (p= <0.05).

again PrepaCyte-CB which gave the greatest recovery 62.59% (SD±16.23). This is significantly higher than separation using Sepax and density gradient ( $p < 0.05$ ) (Fig. 2C).

### Recovery of other cells types within UCB

When looking at T cells (CD45+/CD3+) recovery, PrepaCyte-CB gives the highest recovery at 71.84% (SD±18.06). This is significantly higher than Hetastarch ( $p < 0.05$ ) (Fig. 3A). For B cells (CD45+/CD19+) recovery, plasma depletion gives the highest recovery at 75.68% (SD±24.0). This is significantly higher than PrepaCyte-CB and Hetastarch ( $p < 0.05$ ) (Fig. 3B).

### Volume reduction

For red blood cell removal, density gradient separation was the most effective method. The average number of red blood cells per ml of whole blood was  $2.92 \times 10^6$  cells (SD±0.75). After processing with density gradient methods the number of red blood cells was reduced to  $0.03 \times 10^6$  cells/ml of blood (SD±0.02). This reduction was significantly greater than with PrepaCyte-CB where RBC numbers were on average  $0.24 \times 10^6$  cells/ml (SD±0.08), a 12 fold decrease ( $p = 0.00005$ ), with plasma depletion where RBC numbers were on average  $2.01 \times 10^6$  cells/ml (SD±1.53), a 0.6 fold decrease ( $p = 0.00003$ ), with HES where RBC numbers were  $1.93 \times 10^6$ /ml (SD±1.32) a 1.5 fold decrease ( $p = 0.005$ ) and with Sepax where RBC numbers were  $3.33 \times 10^6$  cells/ml (SD±1.53), a 0.9 fold decrease ( $p = 0.000005$ ) (Fig. 4A). We also examined whether initial collected cord blood volume had an effect on RBC depletion but our data revealed no correlation. We also examined whether initial collected volume of UCB had an impact on RBC depletion but no correlation was observed.

For removal of haemoglobin density gradient was the most efficient. Whole blood had an average of 10.58 g/dl of haemoglobin (SD±4.11), after density gradient processing this was reduced to 0.17 g/dl (SD±0.12). Density gradient was 76 fold more efficient at removing haemoglobin than Sepax ( $p = 0.000009$ ) where post processing levels were 13.07 g/dl (SD±5.56), it was 43 fold more efficient at Haemoglobin removal than plasma depletion ( $p = 0.0008$ ) where post processing levels were 7.23 g/dl (SD±7.03), it was 41 fold more efficient than HES ( $p = 0.006$ ) where post processing levels were 6.94 g/dl (SD±4.74), and it was 4 fold more efficient than PrepaCyte-CB ( $p = 0.002$ ) where post processing levels were 0.68 g/dl (SD±0.31) (Fig. 4B).

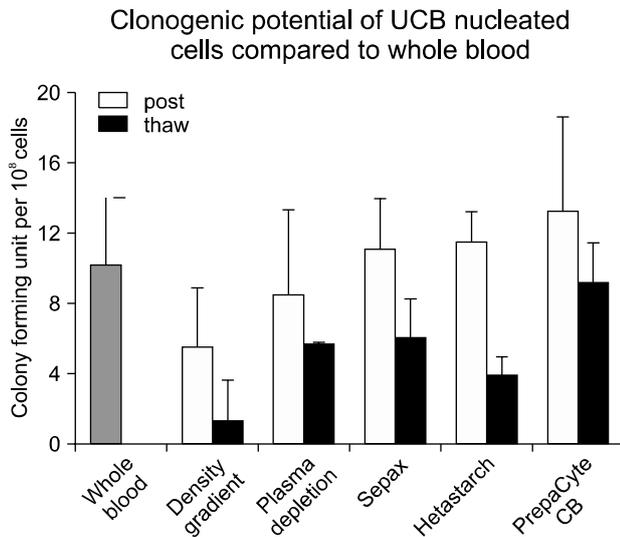
### Clonogenic potential

When processing with PrepaCyte-CB, UCB units are

left with the highest clonogenic potential compared to the other methods; 13.27 CFU/ $10^8$  cells (SD±3.33). This is significantly higher than with density gradient separation; where clonogenic potential was 5.54 CFU/ $10^8$  cells (SD±5.36), 2.3 fold greater ( $p = 0.0002$ ), plasma depletion; where clonogenic potential was 8.59 CFU/ $10^8$  cells (SD±1.65), 1.6 fold greater ( $p = 0.00003$ ), and Sepax; where clonogenic potential was 11.10 CFU/ $10^8$  cells (SD±2.89), 1.2 fold greater ( $p = 0.05$ ). Although PrepaCyte-CB also provided a higher clonogenic potential than Hetastarch these data was not significant ( $p = 0.17$ ). After thaw results are as follows: PrepaCyte-CB processing left UCB units 9.23 CFU/ $10^8$  cells (SD±2.31), the highest clonogenic potential compared to the other methods. This is significantly higher than with Sepax separation; where clonogenic potential was 6.08 CFU/ $10^8$  cells (SD±2.15), 1.5 fold greater ( $p = 0.0003$ ), plasma depletion; where clonogenic potential was 5.70 CFU/ $10^8$  cells (SD±1.06), 1.6 fold greater ( $p = 0.000003$ ), Hetastarch; where clonogenic potential was 3.94 CFU/ $10^8$  cells (SD±1.44), 6.4 fold greater ( $p = 0.000009$ ), and density gradient separation: where clonogenic potential was 1.33 CFU/ $10^8$  cells (SD±2.31), 6.9 fold greater ( $p = 0.000009$ ) (Fig. 5).

### Discussion

Recently there has been an explosion of clinical applications for UCB. This escalation leads to an increased need for optimising separation, whether to reduce volume to make storage more efficient or to increase stem cell yield for transplant. UCB potential is well documented, with enough clinical value to warrant further investigation. Since the advent of regular UCB transplantation in the 1990s, and the potential of the units increased, it was necessary to increase banking to meet the demand. It may also be linked to limitations of BM registries. The process of finding a donor can be long; potential donors may change address without notifying their registry; ethnicity can also be an issue with ethnic minorities often struggling to find a suitable donor. UCB can help towards this problem as it is easier to store samples from donors of many ethnicities (21). UCB is an accessible option, with samples already stored. However this increase is not in line with sample demand, and the contrast between these two resources is significant. There are currently 11 million patrons registered worldwide for BM donation, only an estimated 300,000 UCB units are publically banked. Some of these banks are struggling financially so it is of critical importance to find the most effective and economical processing, and storage methods (22). Many factors affect



**Fig. 5.** PrepaCyte-CB processing leaves samples with the highest clonogenic potential both post processing and post thaw. Whole blood has on average 10.22 CFU per  $10^8$  cells ( $SD \pm 3.80$ ). Post processing PrepaCyte-CB leaves the greatest number of CFU at 13.28 per  $10^8$  cells ( $SD \pm 5.36$ ) compared to Hetastarch which leaves 11.53 per  $10^8$  cells ( $SD \pm 1.65$ ), Sepax which leaves 11.10 per  $10^8$  cells ( $SD \pm 2.89$ ), plasma depletion which leaves 8.59 per  $10^8$  cells ( $SD \pm 4.74$ ), and density gradient which leaves 5.55 per  $10^8$  cells ( $SD \pm 3.36$ ). Samples processed with PrepaCyte-CB have a significantly greater clonogenic potential than all other methods except Hetastarch ( $p < 0.01$ ). Post thaw PrepaCyte-CB again leaves the greatest number of CFU at 9.23 per  $10^8$  cells ( $SD \pm 2.31$ ) compared to Hetastarch which leaves 3.94 per  $10^8$  cells ( $SD \pm 1.06$ ), Sepax which leaves 6.08 per  $10^8$  cells ( $SD \pm 2.15$ ), plasma depletion which leaves 5.70 per  $10^8$  cells ( $SD \pm 1.44$ ), and density gradient which leaves 1.33 per  $10^8$  cells ( $SD \pm 2.33$ ). Samples processed with PrepaCyte-CB have a significantly greater clonogenic potential than all other methods ( $p < 0.01$ ).

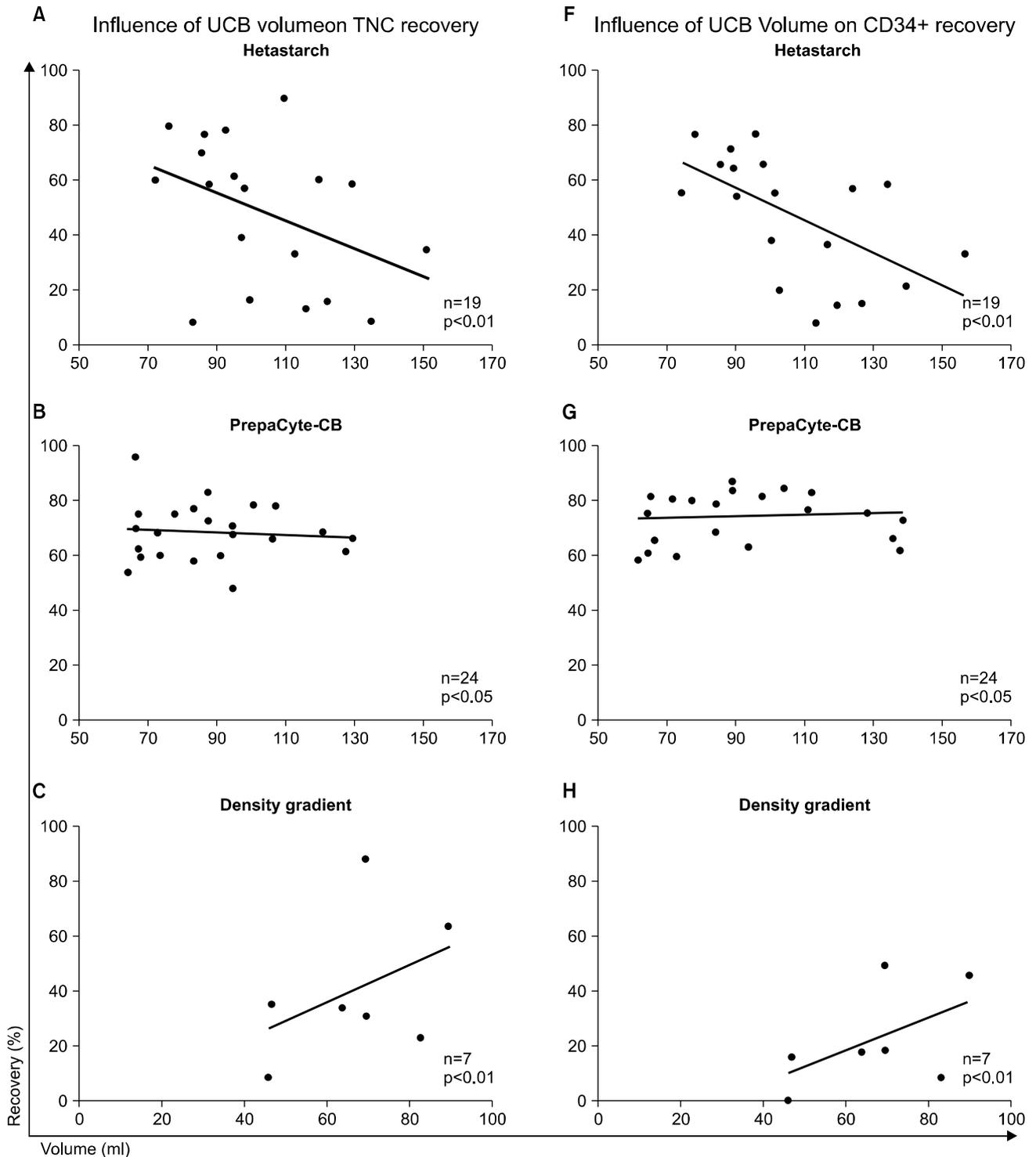
the success of processing, not least the physicality of normal birthing (13), so it remains an important priority to find the best possible methods of separation, so that even smaller units may prove clinically viable.

Our results show that Sepax depletion gives a higher recovery of nucleated cells, crucial for successful engraftment. However, recovery using Sepax is reduced as the size of unit processed increases. Hetastarch, density gradient and plasma depletion separation were also affected in this way, but PrepaCyte-CB processing was not affected by the initial volume of the collected unit, and recovery of both TNC and CD34<sup>+</sup> progenitor cells was as efficient with smaller volumes as it was with larger units. Density gradient separation shows a reverse correlation: as UCB volume increase, so does recovery. Although interesting, it does not fairly compare to the other methods as the maximum volume processed with density gradient was 90

ml and all other methods were routinely tested with units of over 100 ml. The National Cord Blood Program at the New York Blood Center suggests an average TNC count for a UCB transplant should be a minimum of  $2 \times 10^7$  cells per kg of body weight (23). Recent findings show that it is now as successful to transplant an adult patient with multiple UCB units as it is to transplant a child with a high dose single unit (24). HLA is also important when considering units for transplantation, ideally it would be a 6/6 match but, for 5/6 or less an increase TNC is essential to promote engraftment (25). From the results of this study we believe that Sepax, offers the best recovery of TNC, with PrepaCyte-CB and plasma depletion close behind. However, it should also be noted that the content of plasma depleted product is a concern for transplant in conditions other than haematopoietic reconstitution. Further, in our experiments it was not possible to differentiate plasma depleted product, even after further processing into hepatic lineages, despite this being possible for other processing systems such as PrepaCyte-CB, Sepax and density gradient (Hetastarch was not tested).

We next focused on recovery of haematopoietic stem cells of three different developmental stages; our study shows that PrepaCyte-CB offers the best methodology for optimum HSC numbers from all three stages, although again it is worth mentioning that Sepax recovery for both CD34<sup>+</sup> and TNC is diminished as the volume of the UCB increases. When the number of HLA mismatches is greater, then CD34<sup>+</sup> count is more critical (26). Suggested minimum numbers are  $1.7 \times 10^5$  per kg (27). The benefits of Sepax are that it is a fully automated system which allows for the mass processing of samples, which is suitable for larger cord blood banks.

Additionally we examined recovery of some immune cells: T and B cells. T cells which are involved in cell mediated immunity. They can be stimulated by antigens and have dual function, for example; T helper cells and cytotoxic T cells, or can act as regulatory T cells, which are involved in immunological tolerance by suppressing T cell mediated immunity at the end of an immune response and by halting auto-immune reactions (28). Also B cells, which are involved in the humoral immune response. These cells are activated in the presence of foreign antigen to make antibodies. However, this is a complex process and assistance is required from T helper cells (29). During this study we found superior recovery of CD45<sup>+</sup>/CD3<sup>+</sup> T lymphocytes with PrepaCyte CB which also gave the best results for CD10<sup>+</sup> B cells. Not much is known about whether these cells play a role in engraftment in humans but some mice models have shown that increased numbers



**Fig. 6.** Initial cord blood volume negatively correlates with recovery of CD45+ and CD34+ haematopoietic progenitor cells when processing with Hetastarch, Plasma Depletion and Sepax. For CD45+ cell recovery: (A) Hetastarch processing negatively correlates with initial volume ( $p < 0.01$ ). (B) Initial volume has no significant impact on recovery of TNC when processing with PrepaCyte-CB ( $p > 0.05$ ). (C) Density gradient processing has a positive correlation with an increase in collected volume ( $p < 0.01$ ). (D) Plasma Depletion separation negatively correlates with initial UCBV volume ( $p < 0.01$ ). (E) Sepax processing also negatively correlates with initial UCB volume but not significantly ( $p > 0.05$ ). For CD34+ recovery results are the same. (F) Hetastarch ( $p < 0.01$ ). (G) PrepaCyte-CB ( $p > 0.05$ ). (H) Density gradient ( $p < 0.05$ ). (I) Plasma Depletion ( $p < 0.01$ ). (J) Sepax processing negatively correlates with initial UCB volume ( $p < 0.05$ ).

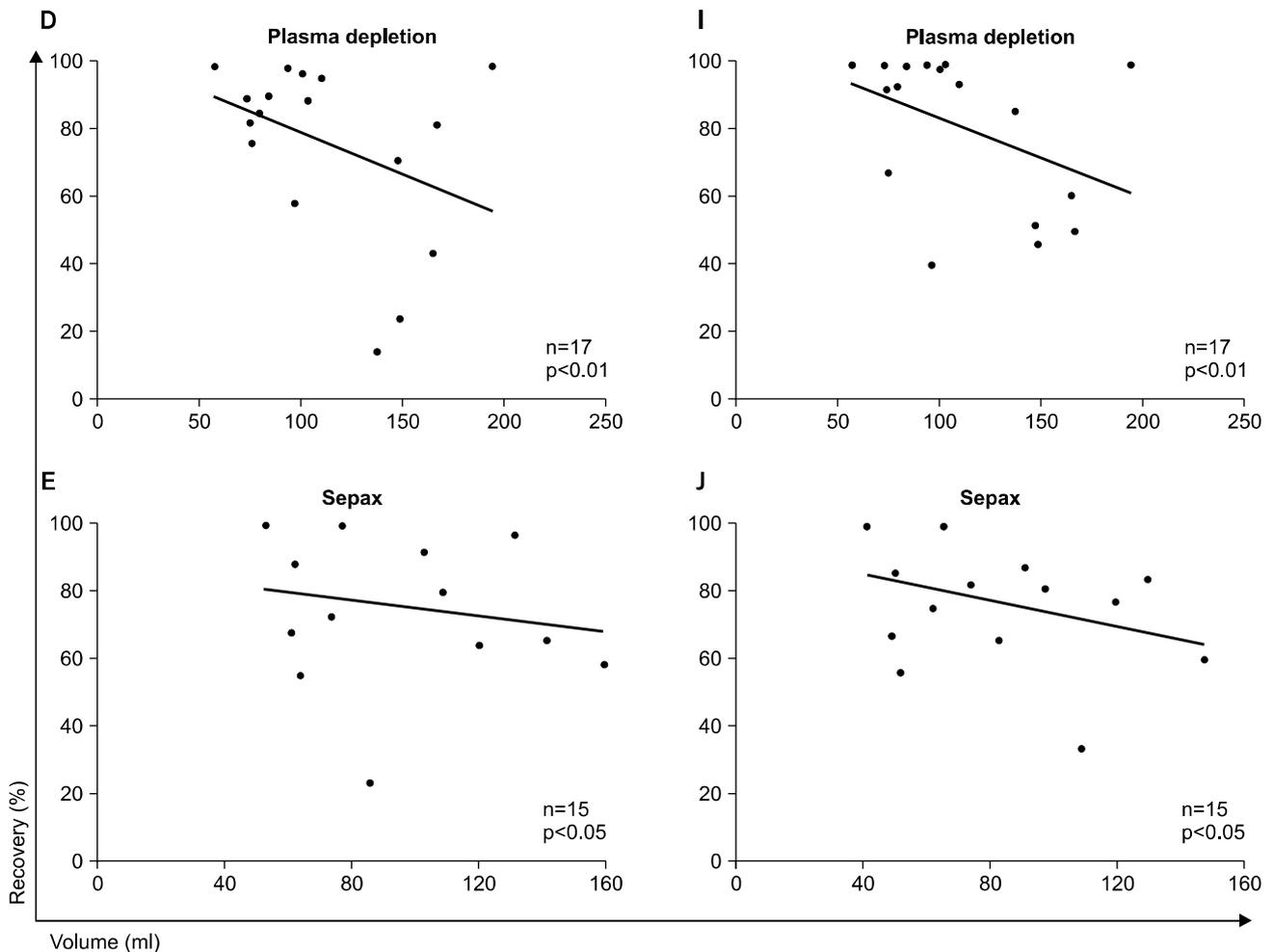


Fig. 6. Continued.

of T cells transplanted can increase bone marrow reconstitution and therefore haematopoiesis and also eliminate residual leukemic disease in the transplanted mice. However, it is important to get the fullest picture of the unit quality that we can.

When looking at the volume reduction of the physical size of the unit, it would seem that plasma depletion would be of particular benefit, as a smaller volume reduces the space needed for storage and also means less DMSO is added to the sample in preparation for cryopreservation (20). This means that it could even save the need to wash samples before infusion (for haematopoietic transplant only) as it has been previously shown that TNC recovery after cryopreservation is greater without a wash step (30). However, if you measure volume reduction as the ability to remove RBC and Haemoglobin; then it is actually a simple and economic density gradient separation which is the most efficient. Removal of RBC is also

a well documented way to reduce the bulk of UCB units (14). Ficoll-paque was the most successful at removing both RBC and haemoglobin. Banking of UCB units is a fiercely discussed topic at the moment with much focus on the debate of private versus public banks (31) so it is important to get the methods right, whichever style of banking the cord blood unit is destined for.

Finally we looked at clonogenic potential of the UCB units which was measured by the CFU assay. Traditionally, this is the most important test, since it gives the best possible readout for potential of the cord blood to be useful if used therapeutically. PrepaCyte-CB performed best in this test, not only post processing but also after cryopreservation and the subsequent thawing. The significance of post thaw CFU is critical for future therapeutic uses of UCB units as it is necessary to know that they will still be able to engraft after storage (32). This could be because PrepaCyte-CB is the second most efficient method for re-

**Table 2.** Processing league table

Method	Performance position & frequency			Points
	1st <sup>a</sup>	2nd <sup>b</sup>	3rd <sup>c</sup>	
1 PrepaCyte-CB	4	4	1	21
2 Sepax	2	3	2	14
3 Plasma Depletion	2	1	4	12
4 Density gradient	2	1	0	8
5 Hetastarch	0	1	3	5

<sup>a</sup>1st place is worth 3 points, <sup>b</sup>2nd place is worth 2 points, <sup>c</sup>3rd place is worth 1 point (9 methods of analysis).

moving RBC. A reduction in RBC numbers has previously been shown to have a advantageous effect on CFU (16, 33). The fact that recovery of CD34+ cells did not fit well with the clonogenic potential also fits with our previously reported theories that many CD34+ populations are relatively late for haematopoietic engraftment (such as CD34 +/45+) and that cells earlier than CD34 exist in cord blood units. This is an important point to consider when carrying out cord blood banking because it means that those banks, who only discuss CD34+ cells and/or TNC, are not representing the whole picture of cord blood.

We also analysed the cost of processing a single unit using each of the five methods. Costs were worked out by pricing the general plastic ware, the separation reagents and any special, non-usual laboratory equipment required. For some of methods where special kits were used both the list price option and the best available price (for example using a large number of kits of a yearly period) were taken into consideration. Plasma depletion followed by density gradient was the cheapest methods analysed. Sepax was the most expensive.

During the analysis no one method consistently came out best; so a league table was devised. Each method was rated for all nine analysis methods (as discussed above) and was awarded 3 points for 1<sup>st</sup> place, 2 points for 2<sup>nd</sup> place and finally 1 point for 3<sup>rd</sup> place (Table 2). Plasma Depletion has proved to be a cost effective, rapid and efficient method for volume reduction of UCB. Sepax was proven to be the most efficient method for TNC recovery. Only Hetastarch didn't perform best in any of the tests. This system showed that PrepaCyte-CB is consistently the best performer over the whole range of analysis. PrepaCyte-CB has other benefits as it is a closed system, reducing the risk of contamination. Not only is this factor useful to the USA and the rest of the developed world but may serve to help UCB storage a more viable option in less economically developed parts of the world where ac-

cess to a clean room may not be available. The PrepaCyte-CB system does not require expensive laboratory equipment and this is a significant issue in the developing countries where transplants are rarely carried out due to lack of necessary equipment. It is worth noting that UCB transplants are currently unavailable to large sections of the second and third worlds.

In conclusion we can say that, depending on your primary use for UCB, different processing methods may be more applicable than others, but that while 'futureproofing' is not easy in medical sciences, the choice of processing method must be carefully addressed so that patients have cord blood units available in a suitable form to treat their disease.

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### Potential Conflict of Interest

The authors have no conflicting financial interest.

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