Cord Blood Haematopoietic Stem Cell Units for Transplantation

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ACADEMIC DISSERTATION

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Jos emme muuta suuntaa,
joudumme sinne,
minne olemme menossa.

To my family; Tita, Leevi and Auni

1 ABSTRACT

During the cord blood banking process vast amounts of data are gathered on obstetric and infant related factors, in addition to information from laboratory analyses and the blood processing itself. Current information technology offers the means to explore ways of transforming these data into valuable information for further development of cord blood transplantation. The aims of this study were to explore the association between cord blood nucleated cell and $CD34^+$ cell content through the standardised banking process and to analyse associations of infant and obstetric characteristics with human leukocyte antigens as well as with cord blood cell concentrations, in order to clarify the factors affecting the quality of cord blood transplants.

Cord blood collections from healthy term infants (N=1999) were processed, analysed and frozen for use as haematopoietic stem cell transplants in the Finnish Cord Blood Banking programme between January 1999 and September 2003. Data on obstetric and infant characteristics, as well as from cord blood bank laboratory analyses and processing were collected prospectively and entered in a spreadsheet application specifically developed for this purpose. Descriptive analysis, the Mann-Whitney U-test, binary decision tree, and simple and multivariate linear regression were the statistical methods used to compare groups and control possible confounding effects. A method of comparing extreme low and high centiles was additionally used to test the hypothesis of internal associations between infant characteristics and cord blood cellular contents.

The volume reduction process was shown to be predictable. Correlation between whole cord blood CFU concentration and cord blood unit $CD34^+$ cell concentration was excellent, suggesting that the methods as applied can be used for evaluating the haematopoietic potential of cord blood transplants. No associations between nucleated or $CD34^+$ cell concentrations and ABO, Rh or Kell blood groups were observed. HLA DRB1*13 was shown to be over-represented in infants with the highest birth weights, suggesting a possible role for HLA molecules or some unknown factor linked to the HLA DRB1 region of chromosome 6 in normal intrauterine growth and development. The positive association with HLA DRB1*13 remained when the birth weight was corrected for varying gestational age according to gender (relative birth weight). As DRB1*13 has been associated with protection from e.g. infectious diseases, the mechanism could be via molecular host responses. The possible association between tissue types and haematopoietic progenitor and stem cell concentration of normal infant may not be observed in future cord blood bank material, as cord blood collections are currently focused on to yield mainly transplants with high cell counts. Birth weight was shown to be the single most important factor for predicting higher nucleated and $CD34^+$ cell concentrations, as well as collected volumes. Thus cord blood from high birth weight infant predicts the highest total cell contents in collection. Female infants had higher nucleated cell concentrations, although this consisted mainly of higher neutrophil concentrations. Instead, male infants were shown to have higher $CD34^+$ cell and colony forming unit concentrations. Particularly, cord blood from male infants was shown to have, in caesarean section

deliveries, more abundant early series haematopoietic progenitors than cord blood from female infants.

In conclusion, obstetric and infant related factors may affect the quality of haematopoietic stem cell transplants. Progenitor and stem cells, measured as $CD34^+$ or colony-forming cells in our study, may have a more central role in intrauterine growth and development than has been reported earlier, which also suggests possible differences in the growth potential of other stem cell lineages. High birth weight, especially high relative birth weight of term infant, and male gender were shown to be associated with higher concentration of hematopoietic progenitor and stem cells.

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2 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV).

I Aroviita P, Teramo K, Westman P, Hiilesmaa V, Kekomaki R. Associations among nucleated cell, CD34+ cell and colony-forming cell contents in cord blood units obtained through a standardized banking process. Vox Sanguinis 2003;84(3):219-27.

II Aroviita P, Partanen J, Sistonen P, Teramo K, Kekomaki R. High birth weight is associated with human leukocyte antigen (HLA) DRB1*13 in full-term infants. Eur J Immunogenet 2004;31(1):21-6.

III Aroviita P, Teramo K, Hiilesmaa V, Westman P, Kekomaki R. Birthweight of full-term infants is associated with cord blood $CD34^+$ cell concentration. Acta Paediatrica 2004;93(10):1323-9.

IV Aroviita P, Teramo K, Hiilesmaa V, Kekomaki R. Cord blood haematopoietic progenitor cell concentration and infant gender. Transfusion 2005;45 (In Press).

In addition, some unpublished data on blood groups, HLA, and collection volumes are presented.

3 ABBREVIATIONS

ALL	acute lymphoblastic leukaemia
BFU-E	burst forming unit – erythroid
BMDW	Bone Marrow Donors Worldwide
CAFC	cobblestone area-forming cell
CFC	colony forming cell / colony, see CFU
CFU	colony forming unit, equivalent to CFC
CFU-	Bas, basophil; E, erythrocyte; Eos, eosinophil; G, granulocyte; GEMM, granulocyte-erythrocyte-macrophage-megakarycyte (equivalent to CFU-Mixed); GM, granulocyte-macrophage; M, macrophage; Meg, megakarycyte;
CFU-TOT	sum of CFU-GM, CFU-GEMM and BFU-E
CI	confidence interval
c-kit	c-kit ligand, mast/stem cell growth factor, steel factor
CRU	competitive repopulating unit
DFS	disease-free survival
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EFS	event-free survival
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macropahge colony-stimulating factor
GMP	good manufacturing practice
GVHD	graft-versus-host disease
HBs-Ag	hepatitis B surface antigen
HCV	hepatitis C virus; anti-HCV, antibodies against HCV
HIV1/2	human immunodeficiency virus 1/2; anti-HIV1/2, antibodies against HIV1/2
HLA	human leukocyte antigen
HPP-CFC	high proliferating potential CFC
HTLV-I/II	human T-cell lymphotrophic virus I/II
LTC-IC	long-term culture-initating cell
MHC	major histocompatibility complex
NMDP	National Marrow Donor Program
NOD/SCID	non-obese diabetic / severe combined immunodeficiency
n.s.	non-significant
OS	overall survival
PBSC	peripheral blood stem cell
SOP	standard operational procedure
SRC	NOD/SCID mouse repopulating cell
TRM	transplant related mortality
WMDA	World Marrow Donor Association

4 INTRODUCTION

The potential of bone marrow derived cells to prevent radiation induced death was recognised in the 1950s, first in animal experiments and later also in humans (Jacobson LO *et al.*, 1949, Lorenz E *et al.*, 1951, Thomas ED *et al.*, 1957, Thomas ED, 1999). The first bone marrow transplantations were performed in the late 1960s (Gatti RA *et al.*, 1968, Thomas ED, 1993), and by the following decade haematopoietic reconstitution using bone marrow derived cells was an established procedure (Thomas ED *et al.*, 1975a, Thomas ED *et al.*, 1975b). The 1990 Nobel Prize in Medicine was given to E. Donnall Thomas for developing methods to control graft-versus-host disease using the cytotoxic drug methotrexate, thus enabling allogeneic bone marrow transplantations (Thomas ED, 1993).

Bone marrow transplantations began in Finland in the early 1970s (Volin L *et al.*, 1984). The first related allogeneic paediatric transplantation was performed at the Children's Hospital 1974 (Makipernaa A *et al.*, 1995), and at Meilahti Hospital on an adult patient in 1981 (Ruutu T, 2004, personal communication). In the 1990s the use of unrelated bone marrow donors began, in parallel with the development of donor registries in several European countries (Cleaver SA, 1993, Oudshoorn M *et al.*, 1994).

Haematopoietic stem cells are also present in the blood of newborn children and can be safely collected from the umbilical cord and placenta after birth (Knudtzon S, 1974, Broxmeyer HE *et al.*, 1989, Gluckman E, 2000, Broxmeyer HE, 2004). As a third to a quarter of patients in need of haematopoietic stem cell transplantation lack a suitable donor, additional sources of haematopoietic stem cells have been sought (Gluckman E *et al.*, 1989).

The haematopoietic progenitor cell content of cord blood is considered different from adult haematopoietic progenitor cells. In the 1990s, cord blood became widely accepted as a source of stem cells for allogeneic haematopoietic reconstitution, and lately also for adult patients, although the available cord blood cell dose is smaller than that which can be collected from adult donors. Until a direct laboratory test for stem cells is developed, final validation of the suitability of a cord blood transplant as source of potent stem cells can only be obtained through clinical transplantation. For secure and prompt procurement of cord blood units for transplantation, cord blood banks with frozen repositories of well-characterised cord blood units have been established all over the world (Rubinstein P *et al.*, 1994, Armitage S *et al.*, 1999b, Mugishima H *et al.*, 2002, Rebulla P, 2002).

In this study the vast amount of data gathered in the cord blood banking process was utilised to analyse both the cord blood units as well as various relevant infant physiological phenomena in order to characterise and improve the quality of cord blood stem cell transplants.

5 REVIEW OF THE LITERATURE

5.1 Cord blood haematopoietic stem cells

Human and mice haematopoietic stem cells bear close resemblance, and understanding of the human haematopoietic system is fundamentally based on studies in mice (Manz MG *et al.*, 2004, Orkin SH and Zon LI, 2002).

5.1.1 Haematopoiesis

Blood formation begins in embryonal mesoderm, from where primitive extraembryonic haematopoiesis in yolk sac and definitive intraembryonic haematopoiesis in aorta-gonad-mesonephros (AGM) region are derived (Godin I *et al.*, 1995, Orkin SH and Zon LI, 2002). Primitive erythroblasts and $CD34^+$ haematopoietic cells are first detectable in the human yolk sac after 18.5 days of development, and cells with both lymphoid and myeloid potential have been detected in the AGM region between 24-34 days (Galloway JL and Zon LI, 2003). The haematogenic endothelium found in the dorsal aorta is thought to originate from the AGM region (Robin C *et al.*, 2003). Association of haematopoiesis and angiogenesis is close, suggesting a common precursor cell known as the haemangioblast (Shalaby F *et al.*, 1995, Choi K, 2002). Stem cells migrating via the blood stream are belived to be responsible for the transition of haematopoiesis from the human yolk sac or, more probably, from the AGM region, to liver (Migliaccio G *et al.*, 1986, Orkin SH and Zon LI, 2002). At the 7^{th} week of gestation, the liver is the main haematopoietic organ (Galloway JL and Zon LI, 2003). The long-term contribution of primitive haematopoiesis is controversial and may be species-dependent (Orkin SH and Zon LI, 2002). Definitive normoblastic erythropoiesis accounts for more than 90% of the circulating erythrocytic cells by 10^{th} week of gestation (Brugnara C and Platt OS, 2003). During the third month of gestation, haematopoiesis can also be detected in spleen, thymus and lymph nodes. Haematopoiesis begins in bone marrow during the fourth to fifth months of gestation. From the sixth month of gestation, bone marrow is the principal site of haematopoiesis, although blood cell formation can be detected in liver and spleen until the first postnatal week (Brugnara C and Platt OS, 2003).

Morphologically, haematopoietic stem cells are medium-sized mononuclear cells with a high nuclear-cytoplasmic ratio, basophilic cytoplasm with no granules, and prominent nucleoli; they cannot, however, be classified based on the appearance under a light microscope (Clark SC *et al.*, 2003).

Lineage plasticity of the haematopoietic stem cells. In classical models of haematopoiesis (Figure 1), pluripotent stem cells self-replicate while occasionally differentiating into a stage of more differentiated progeny and thus losing their multipotency (Till JE *et al.*, 1964, Clark SC *et al.*, 2003). Recently, non-classical models of haematopoiesis – e.g. unified stem model (Quesenberry PJ *et al.*, 2002) and phase space model (Kirkland MA, 2004) – have been introduced. These models propose, instead of the irreversible hierarchical differentiation of haematopoietic stem cells that these cells may, under appropriate circumstances,

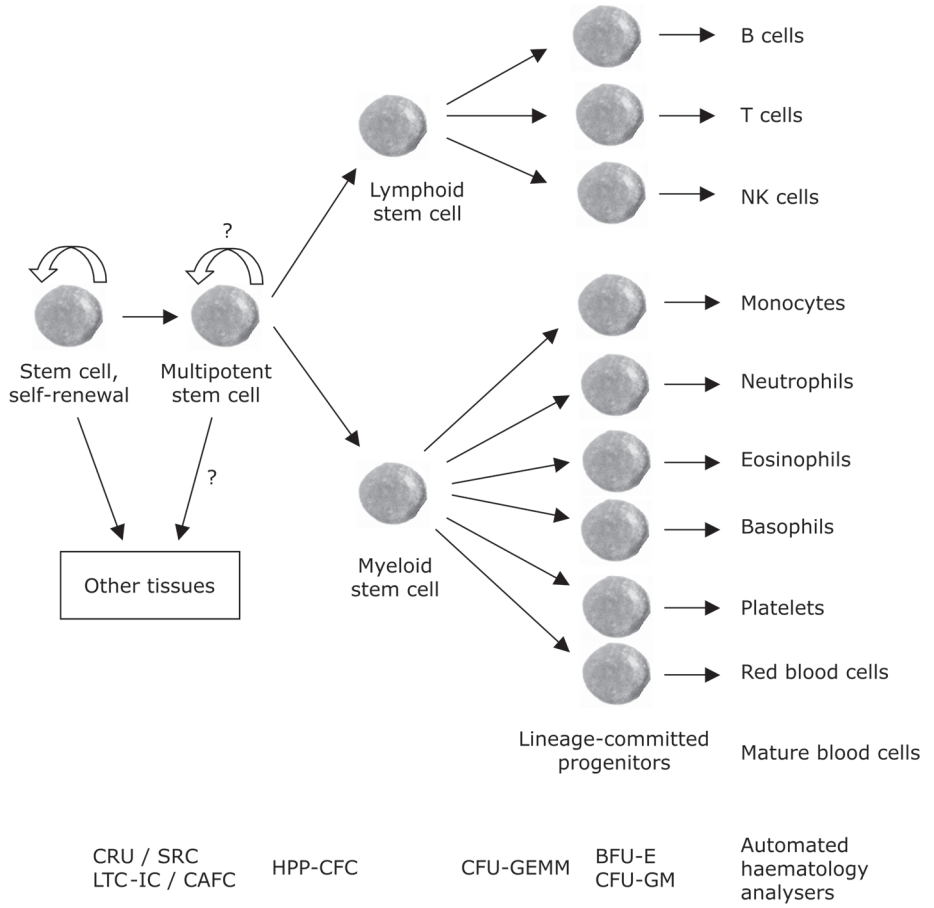

Figure 1 A simplified representation of the hierarchical differentiation of haematopoietic stem cells. Laboratory assays have been developed to identify cells at various levels of differentiation; for abbreviations, please refer to chapter 3.

re-differentiate exceeding even the embryonic-derived lineage specific boundaries (Martin-Rendon E and Watt SM, 2003). Mechanisms involved in proposed lineage plasticity of stem cells include multiple factors, e.g. cytokine and growth factor networks in the microenvironment, niches, of the stem cells (Spradling A *et al.*, 2001), as well as chromatin modulation (Quesenberry PJ *et al.*, 2002) and changes in gene expression (Cai J *et al.*, 2004) during the cell cycle. However, until now, several studies on stem cell plasticity and transdifferentiation have reported controversial findings (Wagers AJ and Weissman IL, 2004).

Multipotent mesenchymal stem cells capable of differentiating into cell types of all three germ layers have also been detected in cord blood (Lee OK *et al.*, 2004). Further, possibilities of differentiating cord blood mesenchymal cells into, e.g., neural or muscle cells are being studied (Bicknese AR *et al.*, 2002, Gang EJ *et al.*, 2004).

Foetal haematopoietic stem cells

Haematopoietic stem cells have been detected in foetal tissues in early gestation. Hann and collaborators studied haematopoietic progenitor cells (CFU-GEMM, CFU-GM and BFU-E) in clonogenic cultures from human foetal samples between 12 and 23 weeks of gestation. CFU-GEMM colonies were observed in liver in all fetuses, whereas they were first detected in bone marrow at 15 weeks and in spleen at 18 weeks of gestation, foetal thymus showing no haematopoietic activity. In blood samples of four foetuses between 13 and 21 weeks of gestation high levels of 9-50 CFU-GEMM, 80.5-400 CFU-GM and 119-364 BFU-E colonies per 10^5 plated cells were observed (Hann IM *et al.*, 1983). Linch and collaborators studied foetal blood samples between 12.5 and 19 weeks of gestation. The increase in foetal CFU-GM growth was greater when placental conditioned medium was added to provide exogenous colony-stimulating factors, compared to the growth of CFU-GM obtained from adult bone marrow; in addition, foetal BFU-E were more sensitive to erythropoietin than BFU-E obtained from adult peripheral blood (Linch DC *et al.*, 1982). In samples of foetal blood, Zauli and collaborators observed that the sensitivity of BFU-E at 18-22 weeks of gestation to suboptimal concentrations of erythropoietin was approximately 10-15-fold higher than the sensitivity of adult BFU-E (Zauli G *et al.*, 1994). Haematopoietic progenitor cell concentration, measured as CFC, is approximately 0.134/μl during weeks 10-11 of gestation reaching a peak value of 65/μl at 18 weeks and declining thereafter to 10/μl at the time of birth (Mayani H *et al.*, 2003). Thus, foetal haematopoietic stem cell concentration appears to be high in mid-gestation and to decline thereafter.

5.1.2 Methods to analyse haematopoietic stem cells

Full blood counts

Nucleated cell content has been used to predict haematopoietic stem cell potential, both in bone marrow and cord blood transplantations (Lim F *et al.*, 1999, Rocha V *et al.*, 2002). Nucleated cells of blood samples are routinely enumerated, using electrical impedance method, by automated haematology analysers (Dacie JV and Lewis SM, 1985), which provide the concentration of nucleated cells, red cells and platelets, as well as the differential relative count of nucleated cells from which the mononuclear cell fraction can be estimated. Depending on the equipment, an enumeration of nucleated red blood cells may also be available (Stevens CE *et al.*, 2002, Wang FS *et al.*, 2003). Using a combination of electric impedance and electric capacitance detection, modern haematology analysers can provide a more direct estimate of the immature cell content, representing possibly haematopoietic stem cells (Takekawa K *et al.*, 1998, Creer MH, 2003 [abstract], Wang FS *et al.*, 2004). As the measurement principles of haematology analysers vary greatly, nucleated cell counts from different laboratories may not always be comparable (Eichler H *et al.*, 2004).

Flow cytometric analyses

Numerous immunophenotypes to identify haematopoietic stem cells have been studied. Hector and Mayani in their review conclude from several studies that

immunophenotype $CD34^+$ $CD38^-$ $CD45RA^{low}$ $CD71^{low}$ Thy-1^+ c-kit^{low} Rh^{low} represents a primitive cord blood haematopoietic progenitor cell (Mayani H and Lansdorp PM, 1998). Detecting high alhehyde dehydrogenase activity as a sign of conserved stem cell function has also been used (Hess DA *et al.*, 2004). Despite the intensive research on other markers, however, CD34 remains the widest used surface marker of haematopoietic progenitor and stem cells in clinical practice.

CD34. CD34 is a highly O-glycosylated transmembrane protein with a molecular weight of 115 kDa (Satterthwaite AB *et al.*, 1992). The gene is located at band 1q32 in the long arm of chromosome 1. The CD34 gene extends 26kb and eight exons code the protein. The CD34 protein consists of five domains: an N-terminal part, a region rich in glycosylation sites, a membrane-proximal domain, a transmembrane region and a cytoplasmic tail (Simmons DL *et al.*, 1992). The cytoplasmic tail of human CD34 has the highest degree of homology, 92%, with that of the murine CD34, suggesting an important functional role (Sutherland DR and Keating A, 1992, Krause DS *et al.*, 1996). This region contains known or potential protein kinase target sites (Fackler MJ *et al.*, 1992).

CD34 was identified twenty years ago using antibodies against a human myeloblastic leukemic cell line (Civin CI *et al.*, 1984, Tindle RW *et al.*, 1985, Katz FE *et al.*, 1985). Different antibodies detected distinct, non-overlapping epitopes (Watt SM *et al.*, 1987, Lanza F *et al.*, 2001). CD34 is expressed on developmentally early lymphohaematopoietic progenitor and stem cells, small-vessel endothelial cells and embryonic fibroblasts (Krause DS *et al.*, 1996) and also in some leukaemic cell lines (Bahia Kerbauy DM *et al.*, 2003), but not generally in solid tumours (Krause DS *et al.*, 1996).

$CD34^+$ cells comprise approximately 1.5% of bone marrow mononuclear cells. The surface expression of CD34 decreases to undetectable levels by the stage that maturing haematopoietic cells lose their capacity to form colonies in cultures (Strauss LC *et al.*, 1986). The $CD34^+$ cell population includes virtually all haematopoietic progenitors analysed using colony-forming assays (Sutherland DR and Keating A, 1992). Purified bone marrow $CD34^+$ cells have also been demonstrated to be able to reconstitute all haematopoietic lineages after myeloablative therapy (Berenson RJ *et al.*, 1991).

Roles of CD34 in leukocyte adhesion on vascular endothelium (Fina L *et al.*, 1990, Baumheter S *et al.*, 1993, Baumhueter S *et al.*, 1994), in progenitor and stem cell localisation and adhesion in bone marrow (Healy L *et al.*, 1995), and in maintenance of the haematopoietic stem/progenitor phenotype (Fackler MJ *et al.*, 1995) have been suggested. However, despite vast amounts of scientific work, the biological function of CD34 has remained elusive (Lanza F *et al.*, 2001).

Cord blood CD34+ cells. Sensitive flow-cytometric methods to detect $CD34^+$ cells also from cord blood have been developed . In these methods, DNA dye positive cells are further classified to separate $CD45^{negative\text{-}week}$ $CD34^+$ cells from mature $CD45^+$ $CD34^{neg}$ leukocytes. $CD34^+$ cells can be detected either using a single or dual platform protocol. Dual platform refers to a protocol, where the $CD34^+$ cell concentration in the sample is counted using the flow-cytometrically obtained relative $CD34^+$ cell content within nucleated cell or white blood cell count, which in turn is obtained from a haematology cell analyser. In a single

platform protocol a known number of highly fluorescent beads are added to the known sample volume and the bead count is used to calculate the analysed sample volume and then its $CD34^+$ cell concentration (Gratama JW *et al.*, 1999). Mean relative contents of $CD34^+$ cells in cord blood of 0.25-0.42% have been reported (Surbek DV *et al.*, 2000c, Solves P *et al.*, 2001).

As the result of colony-forming assays is obtained only after approximately two weeks, and as the $CD34^+$ cell content of cord blood correlates well with the colony-forming cell content (Law P *et al.*, 1993, Encabo A *et al.*, 2003), $CD34^+$ cells are now routinely analysed in cord blood banks as a surrogate marker of the haematopoietic progenitor and stem cell content of cord blood transplants. Although haematopoietic progenitors have also been reported among CD34 negative cells (Nakamura Y *et al.*, 1999), the improtance of CD34 in current practice cannot be overemphasised.

A median number of transplanted $CD34^+$ progenitor cells of 1.2-1.5 $*10^5$ per kilogram of patient weight has been repored (Thomson BG *et al.*, 2000, Laughlin MJ *et al.*, 2001). Wagner and collaborators reported better myeloid engraftment, lower treatment-related mortality and higher survival in patients who had received more than 1.7 $*10^5$ cord blood $CD34^+$ cells per kilogram (Wagner JE *et al.*, 2002). However, as the interlaboratory replicability of $CD34^+$ cell analyses is still not standardised (Barnett D *et al.*, 1998), the $CD34^+$ cell content alone cannot be used to select a cord blood transplant for a patient.

Neonatal $CD34^+$ cells. Neonatal $CD34^+$ cell concentration has been shown to decline from 19.3 /µl to one third between two and 48 hours of life, the rate of decline being greatest during the first four hours (Li K *et al.*, 1999). Reason for this phenomenon is not known. Delivery stress mediated mechanisms may contribute to high cord blood $CD34^+$ cell counts at birth. However, as only minor differences between the cord blood $CD34^+$ cell content in vaginal and caesarean section deliveries have been reported (Sparrow RL *et al.*, 2002, Solves P *et al.*, 2003c), regulation during fetal maturation probably plays a more important role.

CD133. CD133, a glycoprotein with a molecular weight of 120kDa, is selectively expressed only on $CD34^+$ human haematopoietic progenitor cells and not on other blood cells, umbilical vein endothelial cells, fibroblast cells or the myeloid leukaemia cell line used to originally identify CD34 (Yin AH *et al.*, 1997). Recently, cord blood $CD133^+$ cells have been isolated on a clinical scale and shown to have repopulating activity in NOD/SCID mouse, and mesenchymal potential in vitro (Bonanno G *et al.*, 2004). Accumulating clinical transplantation data will hopefully clarify the relevance of CD133 as a useful potential transplantation antigen.

Cell cultures

Colony-forming cells (CFC; colony-forming unit, CFU). Haematopoietic progenitor cells have been analysed using colony-forming cell cultures (Ma DD *et al.*, 1987). In these cultures nucleated cells plated on a semi-solid medium in the presence of cytokines are incubated in a humidified environment at +37°C with 5% CO_2 for 14 days (Broxmeyer HE *et al.*, 1989), after which the BFU-E, CFU-GM and CFU-GEMM colonies are enumerated under a light microscope (Eaves C and Lambie K, 1995). Lowered O_2 tension has also been studied in order to boost the

incidence of detectable progenitor cells (Smith S and Broxmeyer HE, 1986, Broxmeyer HE *et al.*, 1989, Ivanovic Z *et al.*, 2000).

Difficulties in standardising colony forming culture techniques has lead to problems in interpreting uneven results between laboratories (Lumley MA *et al.*, 1999, Lamana M *et al.*, 1999). However, these methods may be used in internal comparisons until better standardisation of colony forming cell assays has been achieved.

Cord blood CFC. The existence of haematopoietic progenitor colonies in human cord blood was demonstrated in 1974 by Søren Knudtzon, who reported 122 granulocytic colonies per $2*10^5$ cord blood nucleated cells plated compared with 3 colonies per $2*10^5$ adult peripheral blood nucleated cells plated (Knudtzon S, 1974) (Figure 2).

Concentrations of haematopoietic progenitor cells in cord blood and bone marrow have been in the focus of several studies, and primitive CFU-GEMM and BFU-E colonies have been reported to be more abundant in cultures of cord blood (Hows JM *et al.*, 1992, Kasai M and Masauzi N, 1998, Mayani H *et al.*, 1998). Haneline and collaborators studied cord blood samples collected after the birth of normal term and preterm infants at 23-41 weeks of gestation. They reported concentrations of 3.9/μl of HPP-CFC (see Section Long-term cultures), 11/μl of CFU-GEMM/BFU-E and 10.6/μl of CFU-GM at 23-31 weeks of gestation, compared with 1.1/μl, 3.2/μl and 2.9/μl of respective CFC at 32-41 weeks (Haneline LS *et al.*, 1996). However, Migliaccio and collaborators reported a two-fold higher CFC concentration of 41/μl in term cord blood compared with 18/μl at 17-32 weeks of gestation. In their study, foetal blood samples were obtained via umbilical vein puncture, whereas cord blood samples were obtained after birth (Migliaccio G *et al.*, 1996). Sample techniques and culture conditions probably affect the observed CFC levels. Ogawa and collaborators showed that cord blood contains blast cell colonies with high replating frequency, thus indicating the presence in cord blood of primitive haematopoietic progenitor cells (Nakahata T and Ogawa M, 1982, Leary AG and Ogawa M, 1987). Broxmeyer and collaborators analysed the properties of cord blood in a banking setting and reported comparable frequencies of haematopoietic progenitor cells with those reported for successfull engraftment with bone marrow cells (Broxmeyer HE *et al.*, 1989). The concentration of colony-forming cells in cord blood samples obtained from full-term deliveries is approximately 7-8/μl for BFU-E, 13-24/μl for CFU-GM and 1-11/μl for CFU-GEMM (Abboud M *et al.*, 1992, Traycoff CM *et al.*, 1994).

Cord blood haematopoietic progenitor cells also tend to grow in cultures with limited external cytokine addition when compared with adult cells (Valtieri M *et al.*, 1989). When colony forming cultures of samples from fetuses (17-32 weeks of gestation), term cord blood and adults were compared, the number of cytokines required to observe maximal colony formation increased along with the ontogenetic stage of the cells (Migliaccio G *et al.*, 1996). Carow and collaborators showed that replated cord blood CFU-GEMM colonies gave rise to CFU-GEMM, BFU-E and CFU-GM colonies in secondary cultures, whereas bone marrow CFU-GEMM produced mainly CFU-GM colonies (Carow CE *et al.*, 1991). In the study of Migliaccio and collaborators, even tertiary replatings were possible from foetal CFU-GM and CFU-Mixed, as well as from cord blood CFU-Mixed colonies, but

not from cultured adult samples (Migliaccio G *et al.*, 1996). These findings suggest that cord blood haematopoietic progenitor cells have an elevated proliferation/ expansion potential compared with cells from adult sources.

Published data concerning the assessment of cord blood colony forming units in the transplant setting are scarce. Migliaccio and collaborators reported stronger association of myeloid engraftment, platelet engraftment and post-transplantation events with a transplanted cord blood CFC dose than with a nucleated cell dose in a multivariate model (Migliaccio AR et al., 2000). The association between CFU-GM dose and time to engraftment has been demonstrated using other stem cell sources, e.g. autologous or allogeneic bone marrow, or peripheral stem cells (Spitzer G et al., 1980, Douay L et al., 1986, Ma DD et al., 1987, Schwartzberg L et al., 1993). Stronger correlation between cord blood $CD34^+$ cell and colony forming cell concentration than between cord blood nucleated cell and colony forming cell concentration has been reported (Lim F et al., 1999), supporting the hypothesis that colony forming cells are a true predictor of clinical success.

Neonatal blood CFC. Compared with cord blood, neonatal blood contains haematopoietic progenitor cells - measured as CAFC and LTC-IC (see Section Long-term cultures) - at equivalent levels shortly after birth (Zhang XB *et al.*, 2002). High levels of colony forming cells have been detected in cord (3050 CFC/ ml) and neonatal blood during the first month of life (330 CFC/ml), the levels declining from the second month onwards to the level detected in adults (second month 88 CFC/ml vs. adults 60 CFC/ml) (Gabutti V *et al.*, 1975). Also, Geissler and collaborators reported 26-fold higher levels for CFU-GM, seven-fold for BFU-E and five-fold for CFU-Mixed colonies in infants aged between one day and 10 weeks, compared with adult values (Geissler K *et al.*, 1986). Therefore, as neonatal blood contains haematopoietic progenitor cells measured both as colony forming and $CD34^+$ cells, it has been studied in cord blood transplantation (Li K *et al.*, 1998).

Long-term cultures. Colony-forming cells with high proliferative potential (HPP-CFC) have been described in human bone marrow samples after an extended culture of 28 days in 10% CO_2 / 7% O_2 conditions (McNiece IK *et al.*, 1989). Cells with high proliferative potential have also been found in cord blood after 21 days of culture in 5% CO_2 / 5% O_2 in approximately eight-fold higher frequency than in bone marrow, and are believed to represent more primitive progenitor cells than CFU-GEMM (Lu L *et al.*, 1993). A long-term culture-initiating cell (LTC-IC), an even more primitive cell type, has been characterised using long-term cultures of five weeks to prevent the growth of differentiated clonogenic progenitor cells (Sutherland HJ *et al.*, 1989, Sutherland HJ *et al.*, 1990). Pettengell and collaborators reported equivalent proportions of cord blood and bone marrow LTC-IC (1/35000 and 1/34000, respectively) after an eight-week culture of mononuclear cells, the proportion of leukapheresis LTC-IC being 1/13000 (Pettengell R *et al.*, 1994). Primitive haematopoietic clones can also be analysed in phase-contrast microscopy as they form phase-dark cobblestone-like areas (Breems DA *et al.*, 1994). Cord blood derived $CD34^+$ cells have been reported to contain six-fold higher numbers of these cobbelstone area forming cells (CAFC) than corresponding cells from bone marrow (Theilgaard-Monch K *et al.*, 1999). Thus, cord blood appears to contain more primitive haematopoietic progenitor and stem cells compared with adult sources.

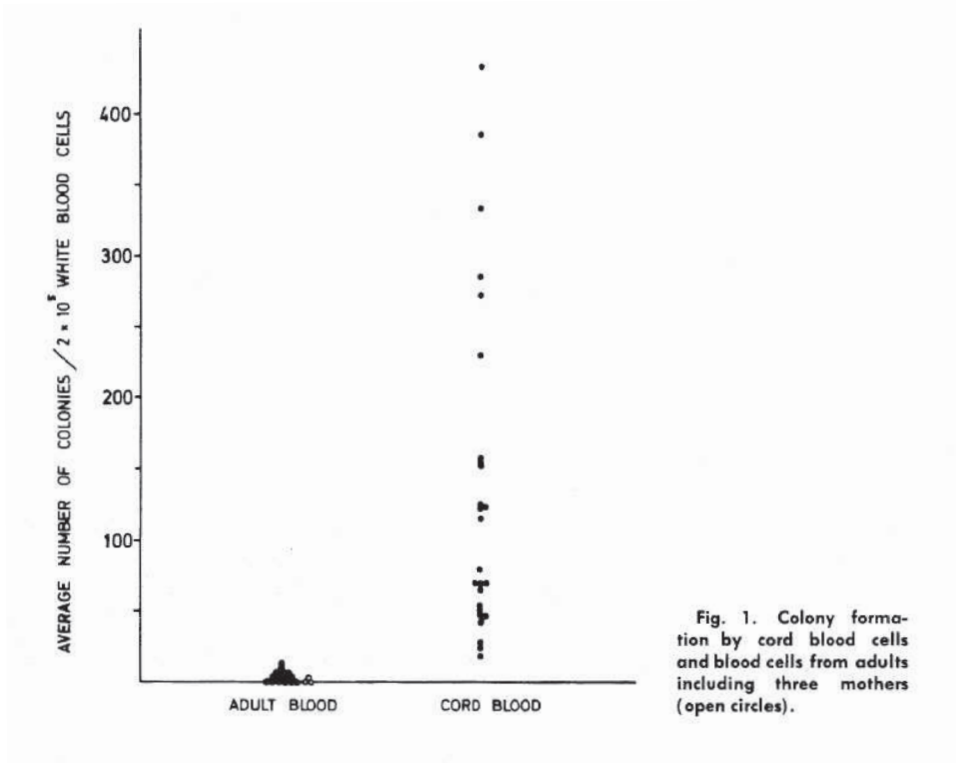

Figure 2 The first report on colony forming colonies in human cord blood. From: Knudtzon S. In vitro growth of granulocytic colonies from circulating cells in human cord blood. Blood 1974;43(3):357-61. Copyright of American Society of Hematology, used with permission.

Viability

The permeability of the cell membrane of dying cells increases. Non-viable cells can thus be estimated by using reagents staining the damaged cells (Kaltenbach JP *et al.*, 1958, Freshney RI, 2000); e.g. 7-amino-actinomycin (7-AAD) and Trypan-Blue have been used in assessment of cell viability of cord blood samples (Xiao M and Dooley DC, 2003). Measurements of 7-AAD negative $CD34^+$ cells by flow cytometry are expected to yield an accurate gauge of viable $CD34^+$ cells in cord blood.

In vivo assays

An estimation of even more primitive haematopoietic stem cell content has been developed experimentally using severe combined immunodeficiency (SCID) mice in an in vivo assay (McCune JM *et al.*, 1988, Vormoor J *et al.*, 1994). In SCID mice repopulating cell (SRC) or competitive repopulating unit (CRU) assays, the haematopoietic reconstituting capacity of stem cells is evaluated by transplanting the cells using limiting dilution in SCID mice strain (Conneally E *et al.*, 1997). The frequency of SRC in cord blood has been found to be $1/9.3*10^5$ cells compared with $1/3.0*10^6$ in bone marrow and $1/6.0*10^6$ in mobilised peripheral blood (Wang JC *et al.*, 1997).

5.2 Major histocompatibility complex (MHC)

The existence of a histocompatibility locus controlling the rejection of foreign tissues was first demonstrated in mice (Snell GD and Higgins GF, 1951, Amos DB *et al.*, 1955). Human major histocompatibility complex (MHC) was recognised a few years later in studies of leukocyte antibodies of multiple transfused patients (Dausset J, 1954). Leukocyte antibodies were also detected in sera of multiparous women (Van Rood JJ *et al.*, 1958). Epitopes of these antibodies, HLA antigens or HLA molecules, participate in the immune response and are encoded by genes in MHC area of the short arm of chromosome 6 (Mickelson E and Petersdorf EW, 2004).

HLA molecules are devided in two classes, I and II, which differ somewhat in their structure as well as function (Klein J and Sato A, 2000). HLA molecules of both classes feature in their structure a peptide-binding groove and a transmembrane region binding the molecule to cell membrane. Foreign antigens are prosessed intracellularly and bound to the peptide-binding groove of an HLA molecule. The HLA molecule-peptide complex is then transported to cell surface, where it is presented to T-cells for eliciting an immune response. Class I HLA molecules bind and subsequently present peptides originating from intracellularly produced foreign proteins after e.g. viral infection of a cell. Thus, the expression of class I molecules by virtually all somatic cells is understandable. By contrast, class II HLA molecules bind and present peptides derived from extracellular proteins after endocytosis and intracellular processing and are expressed predominantly in the cells of the immune system. T-cell recognition of the HLA molecule-peptide complexes is the basis of auto- and allorecognition.

There are several gene loci in the HLA system, of which class I A and B and class II DRB are considered the most important in tranplantation immunology (Klein J and Sato A, 2000). The HLA system is extensively polymorphic (Marsh SG *et al.*, 2002), the number of different alleles identified in loci A, B and DRB being more than 1300 (Turner D, 2004).

5.2.1 Disease associations

HLA alleles have been associated with protection from or susceptibility to numerous diseases, e.g. autoimmune diseases such as diabetes and coeliac disease, and also malignant diseases (Tiwari JL and Terasaki PI, 1985, Posthuma EF *et al.*, 1999, Lechler R and Warrens A, 2000).

HLA DRB1*13, in particular, has been associated with protection from infectious diseases, such as malaria (Hill AV *et al.*, 1991), human papillomavirus-associated cervical carcinoma (Apple RJ *et al.*, 1994), and chronic hepatitis B virus infection (Thursz MR *et al.*, 1995).

5.2.2 HLA match

A close match between donor and recipient HLA antigens has been considered of fundamental importance for the success of haematopoietic stem cell transplantation (Thomas ED, 1999, Morishima Y *et al.*, 2002). Based originally on HLA types obtained using serological methods, HLA matching is currently

perfomed from DNA samples on an appropriately defined level using molecular methods (Mickelson E and Petersdorf EW, 2004). The donor and recipient have been at least matched for the A and B antigens as well as for DRB1 allele groups (6/6 match). Matching for other alleles/antigens, such as HLA C and so-called minor histocompatibility antigens, probably also influences the transplantation results (Elia L *et al.*, 1999, Kogler G *et al.*, 2002, Flomenberg N *et al.*, 2004).

In cord blood transplantations, compared with bone marrow transplantations, less stringent HLA matching between patient and the donor has yielded favourable outcomes (Tables 1-3). Even 4/6 matches are accepted, provided the total transplanted cord blood cell dose is adequate (See Chapter 5.3.1 for Dose). For example, Rubinstein and collaborators reported only 7% of 6/6 matches in their study on 562 unrelated cord blood transplantations (Rubinstein P *et al.*, 1998). Recently, in adult double cord blood transplantation, favourable outcomes with relatively low graft-versus-host disease have been reported. In these transplantations, an HLA match of 4/6 between the patient and both grafts as well as between the two grafts has been accepted (Barker JN et al., 2005). However, although HLA match requirements in cord blood transplantation might be more permissive than in transplantation of haematopoietic stem cells from adult donors, better HLA matching has been associated with better results also in cord blood transplantation (Rubinstein P and Stevens CE, 2000).

Haematopoietic stem cell transplants are primarily selected also according to ABO blood groups. As cord blood does not contain isoagglutinins, minor blood group discrepancy is not an issue in cord blood transplantation if the donor mother is not ABO immunised. Associations between blood groups and haematopoietic stem cell content of the graft have not been reported.

5.3 Allogeneic unrelated haematopoietic stem cell transplantation

Although excellent results in the treatment of malignant haematopoietic disorders with intensive chemo-radiotherapy regimens are being achived (Saarinen-Pihkala UM *et al.*, 2004), allogeneic haematopoietic stem cell transplantation is integrated in the therapeutic plan of many malignancies as well as severe non-malignant haematopoietic disorders (Gratwohl A, 2004). In addition to the characteristics of the haematopoietic transplant itself - e.g. cell content, ABO- and HLA compatibility - the results of haematopoietic stem cell transplantation are affected by diagnosis, treatment, phase of the disease, timing of transplantation, and conditioning of the patient for the transplantation, as well as by the experience of the transplantation team.

Data on the results of haematopoietic stem cell transplantations have been collected and analysed in international efforts starting in the 1970s (Goldman JM and Horowitz MM, 2002, Gratwohl A, 2004). The yearly worldwide number of autologous haematopoietic stem cell transplantations has not increased since the 1990s, while the number of allogeneic haematopoietic stem cell tranplantations has increased steadily. In 2002, 20 207 haematopoietic stem cell transplantations were performed in Europe, of which 6 915 were allogeneic, i.e. transplanted stem cells were collected from another individual, and 13 292 autologous, i.e. transplanted stem cells were collected previously from the same individual (Gratwohl A, 2004).

In 2003, 1 011 bone marrow and 1 188 peripheral blood stem cell transplants, as well as 963 unrelated cord blood units from unrelated donors were provided for transplantation, indicating a high use of cord blood for transplantation (Annual report 2003 of the World Marrow Donor Association) (Marry E and Oudshoorn M, 2004, Oudshoorn M and Foeken-van Goozen L, 2004). About one-third of the almost 3000 patients receiving cord blood transplantation to date through Netcord network (see 5.4.9 Cord blood banks and networks) have been adults (Netcord inventory and use, www.office.de.netcord.org, accessed July 2004).

5.3.1 Haematopoietic stem cell transplantation - concepts

Haematopoietic stem cell collection

Bone marrow. Haematopoietic stem cells can be harvested by bone marrow aspirations under general or spinal anesthesia (Thomas ED and Storb R, 1970, Buckner CD *et al.*, 1984). An average of 9.5 ml of bone marrow per kilogram donor weight, containing 2.2 $*10^8$ nucleated cells, was harvested from an adult donor (Buckner CD *et al.*, 1984). Thus, a bone marrow transplant collected from a donor weighing 70 kg would contain 154 $*10^8$ nucleated cells. The risk of serious, life-threatening complications to bone marrow donors has been reported to be 0.3% (Bortin MM and Buckner CD, 1983, Stroncek DF *et al.*, 1993).

Peripheral blood stem cells. Peripheral blood contains increasing numbers of haematopoietic stem cells during the recovery phase of cytotoxic treatment or after growth factor stimulation (Goldman J, 1995). Collection after G-CSF or GM-CSF mobilisation of marrow haematopoietic stem cells using an apheresis device has been increasingly used in allogeneic transplant settings (Russell N *et al.*, 1996, Champlin RE *et al.*, 2000). Peripheral blood stem cells differ from those of bone marrow, e.g. the $CD34^+$ cell content of a peripheral blood stem cell graft may be five-fold (Russell N *et al.*, 1996). Two-to-3 $*10^6$ $CD34^+$ cells per kilogram of patient weight has been regarded as a minimum dose for allogeneic transplantation (Russell N *et al.*, 1996). The apheresis procedure is considered as safe as marrow harvesting (Anderlini P *et al.*, 2001, Favre G *et al.*, 2003). Use of peripheral blood from minor donors has also been studied (Lipton JM, 2003).

Conditioning

To diminish the leukaemic cell burden and to enable engraftment of transplanted haematopoietic stem cells by suppressing the immunological defense of the recipient, a conditioning treatment is given to patients prior to transplantation. The treatment consists of cytotoxic regimens and irradiation, either alone or in varying combinations depending on the diagnosis and the phase of the disease (Bensinger WI and Spielberger R, 2004). Recently, non-myeloablative conditioning regimens emphasising the immunologic effects of the transplant have also been studied in cord blood transplantations (Rizzieri DA *et al.*, 2001, Barker JN *et al.*, 2003, Alyea EP *et al.*, 2004, Barker JN *et al.*, 2004 [abstract]). The possibilities of modifying conditioning regimens are numerous but may have remained somewhat unexplored due to the many variables in clinical patient care.

Dose

As a quick and reliable characterisation of haematopoietic progenitor and stem cells is not yet possible, the haematopoietic cell content of a transplant is generally estimated according to its total nucleated cell count. A higher number of infused adult nucleated cells per kilogram of body weight has been associated with increased speed of engraftment as well as with improved survival of the patient due to reduction in the incidence of graft rejection in related (Niederwieser D *et al.*, 1988) as well as in unrelated (Sierra J *et al.*, 1997) bone marrow transplantations.

In cord blood transplantations, higher transplanted nucleated cell dose has been positively correlated with the speed and probability of myeloid and platelet engraftment (Rubinstein P *et al.*, 1998, Gluckman E *et al.*, 2004). The nucleated cell number is seldom a limiting factor in adult donor settings, as adequate cell numbers can usually be collected from the donor (Buckner CD *et al.*, 1984). Generally, 2-3*10^8 bone marrow nucleated cells have been given per kilogram of patient body weight (Davies SM *et al.*, 2000, Rocha V *et al.*, 2002). Notably, in cord blood transplantation, one log lower dose of 2 *10^7 cord blood nucleated cells per kilogram is used (Gluckman E, 2001).

Myeloid and platelet engraftment

A marker of initial success of haematopoietic stem cell transplantation is myeloid and platelet engraftment. Engraftment refers to the time when haematopoietic stem cells infused into the circulation during transplantation begin producing the blood cells. Myeloid engraftment is normally defined as the first of three consecutive days with an absolute neutrophil count $>0.5*10^9$/l and platelet engraftment as the first of seven consecutive days with a platelet count $>50*10^9$/l without platelet transfusions (Bryant E and Martin PJ, 2004). Myeloid engraftment is typically expected after two weeks and platelet engraftment 1-2 weeks later (Champlin RE *et al.*, 2000). Adult haematopoietic stem cell transplants, especially if collected from peripheral blood, engraft earlier than cord blood transplants (Rubinstein P *et al.*, 1998, Champlin RE *et al.*, 2000).

Chimerism

The level of engraftment of myeloid and lymphoid cells in a patient can be evaluated by studying chimerism, i.e. the proportion of donor derived cells in the blood versus cells of patient origin. In these analyses based on molecular technologies, several differences, e.g. tandem repeats, HLA, gender and blood groups, can be used (Bryant E and Martin PJ, 2004).

Acute and chronic graft-versus-host disease (GVHD)

Further success of a transplant is related to the occurence and degree of acute and chronic graft-versus-host disease (GVHD). GVHD is caused by immunologically reactive T cells in the donor graft, which recognise and attack host tissues (Korngold R and Sprent J, 1987). Principal target organs of GVHD are skin, gastrointestinal tract, liver and lymphoid tissue (Glucksberg H *et al.*, 1974).

Acute GVHD is divided in four grades depending on the extent and severity of skin rash, serum levels of bilirubin and volume of diarrhea (Thomas ED *et al.*, 1975b, Przepiorka D *et al.*, 1995).

Although chronic GVHD with associated immune-deficiency state typically develops after day 100 of post-tranplantation, the distinction between acute and chronic GVHD cannot be made solely according to the time from transplantation. Chronic GVHD commonly involves skin, mouth, liver and eye, and more seldom the gastrointestinal tract, lungs, oesophagus and joints (Socie G, 2004). The most commonly used clincal grading system of chronic GVHD is division between limited (localised skin involvement with or without hepatic dysfunction) and extensive (generalised skin involvement or limited chronic GVHD and liver, eye, salivary gland, oral mucosa or other organ involvment) (Shulman HM *et al.*, 1980).

The incidence of GVHD seems lower after cord blood transplantation than after transplantation with adult haematopoietic stem cells (Rocha V *et al.*, 2001).

Manipulation of the graft

Excess plasma or red cells of a fresh haematopoietic stem cell graft can be removed in case of blood group discrepancy between donor and the patient (Saarinen UM *et al.*, 1992, O'Donnell MR, 2004). Graft can also be immunologically manipulated; e.g. T-cell depletion of adult stem cell grafts is used to diminish the incidence and severity of GVHD (Ho VT and Soiffer RJ, 2001).

Cord blood which is stored frozen has been protected by DMSO. Practice varies as to the washing of the transplant immediately prior to transplantation. A small absolute quantity of DMSO in cord blood may be allowed to other than the smallest recipients to avoid loss of haematopoietic stem cells (Rubinstein P *et al.*, 1995, Rowley SD, 2004).

Graft failure

Primary graft failure refers to a situation where engraftment does not happen and the graft does not start producing new blood cells (Martin PJ, 2004). Secondary graft failure indicates a situation where the engraftment has taken place but the function of the graft fails later.

Graft-versus-leukaemia effect (GVL) and donor lymphocyte transfusion (DLT)

Studies on patients with or without GVHD after unrelated adult stem cell transplantation have suggested that the relapse rate of leukaemia may be higher in patients with no or milder GVHD. This suggests an antileukaemic effect of graft, called graft-versus-leukaemia (GVL) effect (Weiden PL *et al.*, 1979, Horowitz MM *et al.*, 1990). GVL effect, possibly a separate phenomenon from GVHD, has been elicited by using donor lymphocyte transfusions (DLT), i.e. infusing immunoreactive lymphocytes collected from the original donor after diagnosis of a post-transplant relapse (Mackinnon S *et al.*, 1995, Saarinen-Pihkala UM *et al.*, 2003). After cord blood transplantation this procedure is not applicable, if not expanding lymphocytes from the small compartment of the unit (Shpall E *et al.*, 2004 [abstract]).

Outcome research

In addition to using concepts of engraftment, GVHD, graft failure, relapse (recurrence of the original disease) and transplant related mortality (TRM), the results of haematopoietic stem cell transplantation studies are analysed using event-free, disease-free and overall survival (EFS, DFS and OS, respectively). These refer to the proportion of patients free of disease-related events at certain

time points after transplantation (Lee SJ, 2004). Haematopoietic stem cell transplantation, especially in children, also has long-term consequences on the quality of life, e.g. diminished fertility (Salooja N *et al.*, 2001) and poorer growth (Hovi L *et al.*, 1999, Sanders JE *et al.*, 2004).

5.3.2 Bone marrow transplantation

The outcome of bone marrow transplantation can be affected by numerous variables, e.g. diagnosis, age of the patient, phase of the disease at time of transplantation, and conditioning treatment.

Based on a large database of more than 16 600 unrelated haematopoietic transplants facilitated by the National Marrow Donor Program in the USA, 26%, 23% and 19% of transplants have been provided for patients suffering from chronic myelogenous leukaemia, acute myelogenous leukaemia and acute lymphoblastic leukaemia, respectively (www.marrow.org/MEDICAL/distribution.html, October 2004). The most common non-malignant disease has been severe aplastic anemia in 4% of all transplants. If the transplantation is perfomed in an early phase of the disease, the five-year survival of adult patients has been up to 32-43%, depending on the diagnosis (www.marrow.org/MEDICAL/disease_outcome_data.html, October 2004). Survival after unrelated bone marrow transplantation is reportedly better in younger patients (McGlave PB *et al.*, 2000), probably because they tolerate better the intensive treatment accompanying haematopoietic stem cell transplantation.

An overview of the results of unrelated bone marrow transplantations in children and adults is presented below (Table 1).

A comparable median time to myeloid engraftment of 18 days has been reported both in adults and children (Davies SM *et al.*, 2000, Bunin N *et al.*, 2002). The probability of initial myeloid engraftment – routinely between 90% and 100% - has not been a clinical problem. Compared with myeloid engraftment, a slower median time to platelet engraftment (23-32 days) has been reported (Balduzzi A *et al.*, 1995, Davies SM *et al.*, 2000).

In addition, the probability of platelet engraftment - evaluated from all of the patients participating in the study - has been only 47-50%, as many of the patients have succumbed from early transplant related events (Balduzzi A *et al.*, 1995, Davies SM *et al.*, 2000).

Severe acute grade III-IV GVHD has been reported in 47-49% of patients after strictly HLA-matched unrelated bone marrow transplantation (Kernan NA *et al.*, 1993, Balduzzi A *et al.*, 1995). Severe acute GVHD has been reported to be more frequent with increasing patient age (Kernan NA *et al.*, 1993) and in HLA-mismatched transplantations (Woolfrey AE *et al.*, 2002). Extensive chronic GVHD has been reported in 35-39% of patients (Kernan NA *et al.*, 1993, Balduzzi A *et al.*, 1995, Bunin N *et al.*, 2002).

Vettenranta and collaborators reported less GVHD and more relapses in bone marrow transplantation recipients who received a T-cell depleted graft compared with those who received an unmanipulated graft (Vettenranta K *et al.*, 2000). The authors stated that as the event free survival was similar in the two groups, the higher risk of transplant related toxic complications in paediatric recipients of

Table 1 Unrelated donor bone marrow transplantation results in children and adults.

N	Age	Sex M/F	Diagnosis	HLA Match	Engraftment		GVHD		TRM	Relapse	Survival	**Authors**
					Myeloid	Platelet	Acute	Chronic				
	median (y) [range]				median[a] (d) [range] incidence [95%CI]	median[b] (d) [range] incidence [95%CI]	median (d) [range] incidence [95%CI]	median (d) [range] incidence [95%CI]	median (d) [range] incidence [95%CI]	median (d) [range] incidence [95%CI]	median (d) [range] incidence [95%CI]	Data, y
363	9 [0-19]	N/A	ALL CR2	6/6 76% 5/6 24%	18 [9-52] 98% [97-99] for patients surviving >d21	N/A [N/A]	Gr II-IV 47% [42-52] Gr III-IV 29% [N/A]	at 2-year 39% [33-45]	at d100 27% [22-32] at 5-year 42% [36-48]	at 5-year 22% [18-26]	5-year LFS 36% [30-42] 5-year OS 38% [32-44]	**Bunin N *et al.*, 2002** 1988 - 2000
88	<18	44/44	ALL CR1 10 CR2 34 CR3 10 Rel 34	6/6 56 <6/6 32	N/A [N/A]	N/A [N/A]	Gr III-IV HLA match 43% Mismatch 59%	Extensive HLA match 32% Mismatch 38%	N/A [N/A]	3-year CR1 0.10 CR2 0.33 CR3 0.20 Rel 0.50	3-year LFS CR1 0.70 CR2 0.46 CR3 0.20 Rel 0.09	**Woolfrey AE *et al.*, 2002** 1987 - 1999
28 URD	N/A [0.2-20]	16/12	ALL CR2	6/6	Graft failure 4%	N/A [N/A]	Unrelated vs. Sibling Gr II-IV 64% 38% Gr III-IV 32% 14%	Unrelated vs. Sibling Limited or extensive 57% 26% Extensive 22% 13%	N/A [N/A] 11% [N/A]	N/A [N/A] 36% [N/A]	5-year EFS N/A [N/A] 54% [N/A]	**Saarinen-Pihkala UM *et al.*, 2001** 1990 - 1997
37 Sibl		26/11	ALL CR2	6/6	5% N/A [N/A] 100% [N/A]				N/A [N/A] 19% [N/A]	N/A [N/A] 40% [N/A]	N/A [N/A] 39% [N/A]	

5246	29 [0-66]	3075/ 2171	Malignant 4712 Non- malignant 534	6/6 3928 5/6 1267 4/6 51	18 [N/A] 84% [N/A] by d28	32 [N/A] at d100 47% of all patients 70% of those surviving at d100 at 2 years 55% of all patients 95% of those surviving at 2 years	N/A	N/A	N/A	N/A	N/A	**Davies SM *et al.*, 2000** 8/1991-6/1999
30 T-r[c]	7.3 [0.4-17]	15/15	ALL 24 AML 6	6/6	20 (mean) [11-41]	N/A [N/A]	Gr III-IV 27% [N/A]	Extensive 8% [N/A]	13% [N/A]	15% [N/A]	OS 67%	**Vettenranta K *et al.*, 2000** 1993 - 1998
30 T-d	7.7 [0.2-16]	15/15	ALL 24 AML 6	6/6	17 [11-78]	N/A [N/A]	0% [N/A]	0% [N/A]	0% [N/A]	37% [N/A]	63%	
88	9 [0.5-17]	43/45	ALL 43 AML 13 CML 18 SAA 4 Other 10 of which 4 non- malignant	6/6 46 5/6 42	21 [11-37] 93% [N/A]	23 [20-79] 50% [N/A]	Gr II-IV 13 [5-49] 90% [N/A] Gr III-IV N/A [N/A] 49% [N/A]	Extensive 170 [100-256] 37% [N/A]	84 [14-777] 28% [N/A]	83 [13-407] 36% [N/A]	DFS 844 [276-2948] 38% [N/A] 3-year DFS CML 75% ALL $\leq$2R 47% ALL adv 10% AML 46% Other 29%	**Balduzzi A *et al.*, 1995** 1985 - 1993
62	9.3 [0.6-17]	39/23	ALL 32 AML 13 SAA 11 SCID 2 Other 4 of which 17 non- malignant	Sibling 6/6 50 3/6 1 Parent 6/6 8 3/6 1 Registry 6/6 2	21 [8-33] 79% [N/A] G-CSF post- transplant N=5 Graft failure 6.5%	N/A	Gr III-IV N/A [N/A] 21% [N/A]	Extensive N/A [N/A] 10% [N/A]	N/A	29%	OS 47%	**Makipernaa A *et al.*, 1995** 1974 - 1992

462	26 [0.3-54.5]	290/ 172	Malignant 390 Non- malignant 72	6/6 5/6 >5/6	306 153 3	22 [6-84] by d100 0.94 [0.91-0.97]	N/A	Gr II-IV 0.64 [0.59-0.69] Gr III-IV 0.47 [0.41-0.53]	Limited or extensive 0.55 [0.48-0.62] Extensive 0.35 [0.28-0.42]	N/A	N/A	2-year DFS in patients with leukaemia prognosis good 0.40 [0.32-0.48] poor 0.19 [0.13-0.25]	**Kernan NA *et al.*, 1993** 1987 - 1990

N/A, not available; CR1/2/3, first/second/third complete remission; AL, acute leukaemia; ALL, acute lymphatic leukaemia; AML, acute myeloid leukaemia; CLL, chronic lymphatic leukaemia; CML, chronic myeloid leukaemia; d, day; DFS, disease-free survival; EFS, event-free survival; G-CSF, granulocyte-colony stimulating factor; Gr, gradus; GVHD, graft-versus-host disease; HLA, human leukocyte antigen; LFS, leukaemia-free survival; OS, overall survival; Rel, relapse; SAA, severe aplastic anaemia; SCID, severe combined immunodeficiency; Sibl, sibling donor; TRM, transplant-related mortality; URD, unrelated donor; y, year

[a] Absolute neutrophil count (ANC) $>0.5*10^9$/l, first of 3 consecutive days.
[b] Platelet count $>50*10^9$/l, first of 7 consecutive days without transfusion support.
[c] T-r, T-replete, Helsinki, Finland; T-d, T-deplete, Bristol, UK.

unmanipulated grafts appears to be balanced by an increased risk of relapse among recipients of T-depleted grafts.

Three-year disease-free survival of 40-47% in low risk leukaemia and of 10-19% in advanced leukaemia has been reported (Kernan NA *et al.*, 1993, Balduzzi A *et al.*, 1995). Unrelated donors offered at least equal five-year event-free survival compared with sibling donors (54% vs. 39%, respectively) in children with ALL in second remission (Saarinen-Pihkala UM *et al.*, 2001). In a recent report on Nordic high risk ALL patients a nine-year event free survival of 61% and overall survival of 74% were reported (Saarinen-Pihkala UM *et al.*, 2004).

Comparable results have been reported from 62 Finnish children who received allogeneic bone marrow transplantation between 1974 and 1992 (Makipernaa A *et al.*, 1995).

5.3.3 Peripheral blood stem cell transplantation

The first succesful peripheral blood stem cell (PBCS) transplantations from HLA identical sibling donors were reported in 1995 (Bensinger WI *et al.*, 1995, Korbling M *et al.*, 1995, Schmitz N *et al.*, 1995). Studies comparing allogeneic PBSC transplantations with bone marrow transplantations are, however, scarce (Schmitz N, 2004). Ringdén and collaborators compared peripheral blood transplants (N=45) with bone marrow transplants (N=45) (Ringden O *et al.*, 1999). They reported faster median myeloid (16 days vs. 20 days) and platelet engraftment (23 days vs. 29 days) in the PBSC transplant group compared with the bone marrow transplant group, whereas acute grade II-IV GVHD (30% vs. 20%), one-year TRM (27% vs. 21%) or overall survival (54% vs. 53%) respectively, did not differ statistically significantly between the groups. Fast haematopoietic recovery and relative ease to the donor of stem cell collection have boosted the shift from bone marrow to PBSC collection, and in 2002 already 54% of allogeneic unrelated transplantations were performed using PBSCs (Gratwohl A, 2004).

5.3.4 Cord blood transplantation

Transfusion of multiple cord blood samples for the treatment of lymphangiosarcoma was tried already in the beginning of 1960s with no apparent effect on the course of the disease (Ende M, 1966). The same authors reported a transient haematopoietic engraftment in a patient with acute lymphoblastic leukaemia after a series of eight transfusions of cord blood performed in 1970 (Ende M and Ende N, 1972, Bandini G *et al.*, 2003). In 1974, Knudtzon reported in vitro growth of granulocytic colonies from human cord blood and suggested the use of cord blood for restoration of bone marrow function in humans (Knudtzon S, 1974). After that began the scientific development of the clinical use of cord blood for haematopoietic reconstitution (Broxmeyer HE *et al.*, 1989, Gluckman E *et al.*, 1989, Rubinstein P and Stevens CE, 2000).

The first human allogeneic cord blood transplantation was performed in 1988 from a sibling donor to a patient suffering from Fanconi's anemia (Gluckman E *et al.*, 1989). The use of related cord blood for transplantation then expanded (Wagner JE *et al.*, 1995, Rocha V *et al.*, 1998). Successful results were followed by foundation of the first cord blood banks for storage of unrelated cord blood units

Table 2a Unrelated donor cord blood transplantation results (pre-transplant).

Number of patients	Age median (y) [range]	Weight median (kg) [range]	Sex Male/ Female	Diagnosis Malignant/ Non-malignant	HLA match	Conditioning	GVHD prophylaxis	**Authors** Data, y
23	24 [13-53]	73 [48-120]	13/10	ALL 8 AML 13 CML 2	double cord blood transplantation both cord blood units at least 4/6 match to the patient and to each other	myeloablative conditioning using CY (120mg/kg) and total body irradiation (1320 cGy), immunsuppression with CsA	ATG + MP N=2 fludarabine + mycophenolate mofetil N=21	**Barker JN *et al.*, 2005** 2000 - 2003
57	31 [18-58]	70 [46-110]	33/24	malignant 55 genetic disorder 2	6/6 2 5/6 8 4/6 44 3/6 3	varied; TBI, busulfan, ATG	Cy + MP	**Long GD *et al.*, 2003** 1996 - 2002
95	6y [4mo-16y]	21 [4.4-78]	N/A	AML CR1 20 AML CR2 47 AML >CR2 5 relapse 28	6/6 9% 5/6 47% 4/6 33% <4/6 11%	varied; TBI, busulfan, ATG	varied; Cy, P, MTX, tacrolimus	**Michel G *et al.*, 2003** 1994 - 2002
102	7.4 [0.2-56.9]	25.9 [5.0-107.5]	60/42	65/37	6/6 14 5/6 44 4/6 42 3/6 2	varied; CY (120mg/kg) + TBI (1320-1375 cGy) for malignant diseases; all patients received anti-thymocyte globulin	CsA+MTX N=2, CsA+MP N=100	**Wagner JE *et al.*, 2002** 1994 - 2001
22	29 [18-46]	69.5 [41-85]	12/10	all malignant	6/6 1 5/6 13 4/6 8	thiotepa, busulfan, CY, ATG N=21 thiotepa, fludarabine, ATG N=1	Cy + P	**Sanz GF *et al.*, 2001** 1997 - 2000

68	31.4 [17.6-58.1]	69.2 [40.9-115.5]	N/A	54/14	6/6 2 5/6 18 4/6 37 3/6 11	total-body irradiation based regimen N=51, busulfan based regime N=14	CsA or CsA+MP	**Laughlin MJ *et al.*, 2001** 1995 - 1999
27 Number of transplants N=30	4.85 [0.4-17.1]	18.4 [5.65-71.4]	14/13	28/2	6/6 3 5/6 17 4/6 10	TBI + CY + ATG N=23 busulfan + melphalan + ATG N=7 (N=3 second TX with TBI+CY+ATG in first TX, N=2 non-malignant disease, N=2 TBI in a prior autologous TX)	CsA + MTX N=23 CsA + MP N=7	**Thomson BG *et al.*, 2000** 1994 - 1999
102			58 / 44	102 / 0 ALL N=70 AML N=32		varied; TBI-containing regimens / Chemo-therapy based regimens	CsA N=20/5 (related / unrelated), CsA+steroid N=3/41, CsA+MTX N=13/5, CsA+MTX+steroid N=2/3, CsA±steroid±ATG/ALG or Mab N=4/4, FK506+MTX N=-/2	**Locatelli F *et al.*, 1999** 1990 - 1997
						22 / 20		
42 related	5.5 [1.7-14]	19.5 [10-46]	25/17	good poor ALL 15 15 AML 9 3	6/6 30 5/6 1 4/6 3 3/6 7 2/6 1			
						34 / 26		
60 unrelated	5.5 [0.2-15]	20.0 [4.4-83]	33/27	good poor ALL 28 12 AML 14 6	6/6 6 5/6 27 4/6 22 3/6 4 2/6 1	ALG/ATG or monoclonal antibody: related N=10, unrelated N=50		
66 good risk	5.0 [0.5-15]	19 [6.5-67]	35/31	ALL/AML 43/23				Good risk: TX during 1st or 2nd CR, Poor risk: others
36 poor risk	6.3 [0.3-14]	21 [4.4-83]	23/13	27/9				
562	<2 114 2-5 127 6-11 137 12-17 82 ≥18 102	<10 77 10-19 148 20-39 152 40-59 91 ≥60 94	324 / 238	404 / 158	6/6 40 5/6 218 4/6 261 3/6 37 2/6 3	transplant centres used their own protocols.	transplant centres used their own protocols.	**Rubinstein P *et al.*, 1998** 1992 - 1998

143								**Gluckman E *et al.*, 1997**
78 related	5 [0.2-20]	19 [5-50]	N/A	46 / 32	6/6 60 5/6 3 4/6 5 3/6 9 2/6 1	varied; CY (or other chemotherapy) + TBI, CY + busulfan; antithymocyte globulin or monoclonal anti-T-cell antibody was given to patients receiving an HLA mismatched transplant	CsA, CsA+P, CsA+MTX	1988 - 1996
65 unrelated	9 [0.3-45]	30 [4-90]		49 / 16	6/6 9 5/6 43 4/6 11 3/6 2			
18	2.7 [0.1-21.2]	15.4 [3.3-78.8]	10 / 8	13 / 5	6/6 7 5/6 7 4/6 3 3/6 1 2/6 0	varied in the two transplant centre participating in the study.	CsA N=1, CsA+MP N=14, CsA+MTX N=3	**Wagner JE *et al.*, 1996** 1994 - 1995
25	7.0 [0.8-23.5]	18.4 [7.5-79.0]	18 / 7	19 / 4	6/6 1 5/6 20 4/6 3 3/6 1	varied	Cs+MP+MTX N=11, Cs+high-dose MP N=8, Cs+low-dose MP N=2, Cy N=4	**Kurtzberg J *et al.*, 1996** 1993 - 1995

Table 2b Unrelated donor cord blood transplantation results (post-transplant).

Number of patients	Dose[a]	Engraftment		GVHD		Relapse	TRE	TRM	Survival	**Authors**
		Myeloid	Platelet	Acute	Chronic					
	median [range]	median[b] (d) [range] incidence [95%CI]	median[c] (d) [range] incidence [95%CI]	median (d) [range] incidence [95%CI]	median (mo) [range] incidence [95%CI]	median (d) [range] incidence [95%CI]	incidence [95%CI]	incidence [95%CI]	incidence [95%CI]	Data, y
23	3.5 [1.1-6.3] larger unit 1.9 [0.6-3.6] smaller unit 1.4 [0.5-4.7]	23 [15-41] - [-] 21 patients engrafted: d21 single unit N=16 d60 double unit N=2 d100 17 evaluable patients: double unit N=0	- [-] by d180 71% [47-95]	Gr II-IV - [-] 65% [42-88] Gr III-IV - [-] 13% [0-26]	extensive - [-] 23% [6-40]	N/A	N/A	6 month 22% [5-39]	1y DFS 57% [35-79] follow up median 10 months [3.5mo-2.5y]	**Barker JN et al., 2005** 2000 - 2003
57	1.5 [0.54-2.78]	26 [12-55]	84[d] [35-167]	32 [13-86] N=17 (30%) Gr II 14% Gr III-IV 15% N=24 no acute GVHD	8/25	N/A	N/A	100d non-relapse 50%	median (d) [range] EFS/OS 87/91 [10-2251] alive N=11 after follow-up (d) of 1670 [67-2251] 3-year OS 19%	**Long GD et al., 2003** 1996 - 2002
95	4.4 [0.4-36]	26 [12-57] 78%±4%	52 [17-171] 58%±5% CR1/2 66%±6% advanced 39%±10%	- [-] 35%±5%	- [-] 15%±5%	2y - [-] 29%±5%		100d 20%±4%	2y OS 49%±5% 2y LFS 42%±5% CR1 59%±11% CR2 50%±8% advanced 21%±9%	**Michel G et al., 2003** 1994 - 2002

102	3.1 [0.7-57.9]	23 [9-54] 0.88 [0.81-0.95]	86 [29-276] 0.65 [0.53-0.77]	35 [8-86] N/A [N/A] Gr II-IV 0.39 [0.29-0.49] Gr III-IV 0.11 [0.05-0.17] None N=39 Gr I N=24 Gr II N=28 Gr III N=8 Gr IV N=3	5 [2-7] Extensive 0.09 [0.04-0.14]	at 2 years 196 [21-672] 0.37 [0.24-0.50] ALL: standard-risk 0.10 [0.00-0.29] high-risk 0.43 [0.17-0.61] high-risk AML: 0.47 [0.25-0.69]	N/A [N/A]	0.30 [0.21-0.39]	0.58 [0.49-0.70] at 2 years 0.47 [0.36-0.57]	**Wagner *et al.*, 2002** 1994 - 2001
22	1.71 [1.01-4.96]	22 [13-52]	69[d] [49-153]	9 [4-14] Gr II-IV 32%	121 [100-325] 9/10 extensive in 4	N/A	N/A	d100 43% [21-65]	1-year DFS 53% [31-75] patients 30-years old or younger, 1-year DFS 73% [45-100]	**Sanz GF *et al.*, 2001** 1997 - 2000
68	1.6 [0.6-4.0]	27 [13-59] 0.90 [0.85-1.0]	99 [42-228] N/A [N/A]	N/A [N/A] N/A [N/A] Gr II-IV 0.60 [0.49-0.71] Gr III-IV 0.20 [0.11-0.29] Gr 0-I N=22 Gr II N=22 Gr III N=7 Gr IV N=4	N/A [N/A] from d100 until the last follow-up 0.38 [0.23-0.52] N=12 of 33 of which 1 extensive	N/A [N/A] N/A [N/A]	N/A [N/A]	N/A [N/A]	19 of 68 as of Aug 2000 follow-up 22mo [11-51]	**Laughlin MJ *et al.*, 2001** 1995 - 1999

27 Number of transplants N=30	3.63 [0.95-13]	27 [12-60] 89.3% [76.5-100] by d60 100%	75 [33-158] by d60 28.6 [9.1-48.1]	any GVHD 57.1% [37.7-76.6] Gr II-IV 37.2% [16.7-57.7] Gr III-IV 8.8% [0-20.6] Gr III N=1 (liver) Gr IV N=0	Not detected	Relapse as cause of death in N=10	N/A	20% [4-36]	52.3% [34.1-70.5] DFS 54.7% [34.5-74.9] EFS 49.6 [29.9-69.4]	**Thomson BG *et al.*, 2000** 1994 - 1999
102 42 related 60 unrelated	 3.2 [0.7-10] 4.4 [0.9-36]	 27 [14-49] by d60 84% 33 [12-56] 79%	 56[d] [17-180] 85% 85 [16-159] 78%	 Gr II-IV Gr III-IV 41% 16% 37% 23%	 at 2 years 13% (2/25) 28% (3/25)	 42% 40% good risk 31%±9% poor risk 77%±14%	N/A 	44% 35% 52%	42% 2-year EFS 34% 39% 30% good risk 49% poor risk 8%	**Locatelli F *et al.*, 1999** 1990 - 1997
562 65 121 198 162	 ≥10[e] 5.0-9.9 2.5-4.9 0.7-2.4	28 [10-120] 0.81 [0.77-0.85] Per cell dose 0.91 [0.84-0.98] 0.86 [0.79-0.93] 0.79 [0.72-0.86] 0.74 [0.66-0.82]	90 [16-250] 0.85 [0.79-0.91]	Gr 0-I 54% Gr II 24% Gr III-IV 23%	25% of the patients surviving ≥6mo	14% Effect of the stage of disease (ALL, AML, CLL) on the relapse incidence at 1 year: early 19%, interm. 24% advanced 35%	46%	N/A	N/A	**Rubinstein P *et al.*, 1998** 1992 - 1998

143 78 related 65 unrelated	3.7 [0.7-30]	30 [8-56] by d60 82% 79% 87%	56[d] [9-180] 52% 62% 39%	Gr≥II 27% 18% 40%	 for patients surviving >d100 14.3% (8/56) 0% (0/23)	23% (22/95) 26%[f] (10/38) 14% (7/49)	N/A	N/A	 63% 29%	**Gluckman E *et al.*, 1997** 1988 - 1996
18	4.1[g] [1.4-40.0]	24 [16-53] by d60 100% for 13 patients who survived >30d	67 [55-120]	Gr II-IV 0.50±0.13 Gr III-IV 0.11±0.08	N/A	N/A	N/A	N/A	at 6 months 0.65±0.12	**Wagner JE *et al.*, 1996** 1994 - 1995
25	3.0 [0.7-11.0]	22 [14-37]	82 [N/A]	Gr II-IV 43% (9/21) None N=4 Gr I N=8 Gr II N=7 Gr III N=2 Gr IV N=0	9.5% (2/21) (liver or skin)	13% (1/8)	N/A	N/A	48%[h] (12/25)	**Kurtzberg J *et al.*, 1996** 1993 - 1995

N/A, data not available; ALG/ATG, anti-lymphocyte-globulin / anti-thymocyte-globulin; ALL, acute lymphatic leukaemia; AML, acute myeloid leukaemia; cGy, centigray; CI, confidence interval; CLL, chronic lymphatic leukaemia; CML, chronic myeloid leukaemia; CR, complete remission; CsA/Cs, cyclosporine (A); CY, cyclophosphamide; d, day; DFS, disease-free survival; EFS, event-free survival; Gr, gradus; Mab, monoclonal antibody; mo, month; MP, methylprednisolone; MTX, methotrexate; P, prednisolone; TBI, total body irradiation; TRE, transplant-related events; TRM, transplant-related mortality; TX, transplantation; y, year

[a] Total nucleated cells $*10^7$/kg patient weight infused.
[b] Absolute neutrophil count (ANC) $\geq 0.5*10^9$/l, first of 3 consecutive days.
[c] Platelet count $\geq 50*10^9$/l, first of 7 consecutive days without transfusion support.
[d] Platelet count $\geq 20*10^9$/l, first of 7 consecutive days without transfusion support.
[e] Number of leukocytes before cryopreservation.
[f] Of the 46 patients who received a related graft to treat cancer, 10 of 38 with leukemia relapsed. Of the 49 patients who received an unrelated graft to treat cancer, 7 relapsed.
[g] Not specified whether the given number refers to the cell content of the cord blood unit before cryopreservation or to the dose infused to the patient.
[h] Event free survival at 6/1996.

Table 3 Comparison of overall results of unrelated cord blood and bone marrow transplantation.

Number of patients	Age	Dose[a]	Diagnosis	HLA match	Engraftment		GVHD		TRM	Relapse	Survival	**Authors**
					Myeloid	Platelet	Acute	Chronic				
CBT BMT	median (y) [range]	median [range]	Malignant/ Non-malignant		median[b] (d) [range] incidence[d] [95%CI]	median[c] (d) [range] incidence [95%CI]	incidence [95%CI]	incidence [95%CI]	incidence [95%CI]	incidence [95%CI]	incidence [95%CI]	Data, y
68	36 [16-53]	2.47 [1.1-5.29]	all malignant	6/6 0% 5/6 21% 4/6 54% 3/6 22% 2/6 3%	22 [16-41] 92 [85-99]	48 [30-263] 89% [82-88]	N=60 Gr II-IV 50% Gr III-IV 7%	N=54 limited + extensive 78% extensive 24%	1-year 9% [2-16]	2-year 16% [7-25]	2-year DFS 74% [63-85]	**Takahashi S *et al.*, 2004** 1996 - 2003
45	26 [16-50]	33.0 [6.6-50]		6/6 87% 5/6 13%	18 [12-33] 100% [N/A]	28 [16-113] 89% [80-100]	N=45 Gr II-IV 67% Gr III-IV 27%	N=35 limited + extensive 74% extensive 40%	29% [15-42]	25% [12-37]	44% [30-59]	
							Gr III-IV, hazard ratio 0.09 [0.01-0.58] P=0.01	no difference P>0.18	hazard ratio 0.32 [0.12-0.86] P=0.02	no difference P=0.73	hazard ratio 0.27 [0.14-0.51] P<0.01	

150 CBT mismatched	- [16-60] CBT patients were younger than BMT patients	2.2 [1.0-6.5]	CBT patients were more likely to have advanced leukaemia at trans-	4-5/6	27 95CI [25-29] - [-]	60 95CI [54-71] - [-]	Gr II-IV 61/150 (41%)	35/69 (51%) extensive 33%	95/150 (63%)	26/150 (17%)	3-year LFS 23% [17-30]	**Laughlin MJ *et al.*, 2004** 1996 - 2001
83 BMT-mm mismatched		22 [0.1-58]	plantation than BMT patients	5/6	20 95CI [18-22] - [-]	29 95CI [27-34] - [-]	43/83 (52%)	17/43 (40%) extensive 71%	54/83 (65%)	12/83 (14%)	19% [12-27]	
367 BMT-m matched		24 [0.2-170]		6/6	18 95CI [18-19] - [-]	29 95CI [27-30] - [-]	176/367 (48%)	86/243 (35%) extensive 52%	169/367 (46%)	83/367 (23%)	33% [28-37]	
					P<0.001 cumulative incidence at 1 year, difference between CBT and BMT-mm P=0.29 BMT-m P<0.01	P<0.001 cumulative incidence at 1 year, difference between CBT and BMT-mm P=0.16 BMT-m P<0.01	no difference in CBT vs. BMT-m, hazard ratio 0.81 [0.59-1.10] less likely after CBT vs. BMT-mm, hazard ratio 0.66 [0.44-0.99]	rate higher after CBT than BMT- m, hazard ratio 1.62 [1.08-2.42] rate similar after CBT and BMT- mm, hazard ratio 1.12 [0.63-2.02] proportion of extensive lower after CBT P=0.03	rate lower after BMT-m P<0.001	no differences in rates P>0.16	higher after BMT-m P=0.001	
98	24.5 [15-55]	2.3 [0.9-6]	AML 46% ALL 54%	6/6 6% 5/6 51% 4/6 39% 3/6 4%	26 [14-80] d60 75% [66-84]	N/A	Gr II-IV 26% [14-38] Gr III-IV 13%	2-year 30% [20-40]	2-year 44%	23%	LFS 33% OS 36%	**Rocha V *et al.*, 2004** 1998 - 2002
584	32 [15-59]	29 [<10-90]	AML 54% ALL 46% disease status at transplant more advanced: CBT 52% BMT 34% P<0.002	6/6 100%	19 [5-72] 89% [87-91]		Gr II-IV 39% [31-47] Gr III-IV 19% Gr II-IV relative risk 0.57 [0.37-0.87] P=0.01	46% [44-48] relative risk no difference P=0.11	38% relative risk no difference P=0.50	23% relative risk no difference P=0.93	LFS 38% OS 42% LFS relative risk no difference P=0.70	

26	6.0 [0.6-14.9]	5.8[e] [12-160]	ALL CR1 21 CR2 28	6/6 15.4% 5/6 38.5% 4/6 42.3% 3/6 3.8%	29 [21-35] 88% [80-98] at d60	51 [32.5-59] 77% [56-91] at d180	Gr II-IV 19% [7-39]	33% [15-57]	d100 19% [7-39]	15% [4-42]	3-year OS 65% [46-83]	**Jacobsohn DA *et al.*, 2004** 1992 - 2003
23 Related[f]	4.4 [0.5-15.7]	25 [60-450]		6/6 87% 5/6 13%	15.5 [13-21] 96% [78-100]	24 [17-35] 96% [78-100]	22% [7-44]	40% [19-64]	13% [3-34]	25% [8.7-49]	64% [45-84]	
8	38.5 [21-51]	2.43 [2.02-3.00]	AML, ALL	5/6 1 4/6 5 3/6 2	20 [18-25]	48 [31-56]	Gr0 0 GrI 2 GrII 6 GrIII 0 GrIV 0	limited 3/8 extensive 2/8	N/A	0	2 year DFS 85.7%	**Ooi J *et al.*, 2002** 1995 -
8	23 [17-36]	29.4 [22.0-39.0]		6/6 8	15 [14-21] P=0.0028	25.5 [21-138] P=0.0005	Gr0 2 GrI 1 GrII 4 GrIII 0 GrIV 1	limited 2/7 extensive 0/7		1	75.0% P=0.51	2001
296	<16	N/A	AL, CL, JCML, MDS	6/6 62% 5/6 32% 4/6 6%	hematologic recovery slower in CBT group	N/A	less	similar	similar higher if >1 mismatch	similar	DFS and OS similar	**Rubinstein P *et al.*, 2001** 1995
210	<16			6/6 6% 5/6 41% 4/6 49% 3/6 4%							lower if >1 mismatch	- 1998
26 CBT	4.5 [0.2-17.9]	3 [1-28]	17/9	6/6 5 5/6 12 4/6 8 3/6 1	88% [75-100]	72% [50-94]	42% [23-61]	5% [0-13]			53% [31-75]	**Barker JN *et al.*, 2001** 1991
26 BMT	4.7 [0.6-17.7]	20 [19-40]	17/9	6/6 26	96% [89-100]	76% [54-98]	35% [17-53]	20% [5-35]			41% [22-60]	- 1999
31 CBT	5.8 [0.2-17.9]	3 [1-28]	12/19	6/6 4 5/6 22 4/6 5	85% [72-98]	84% [64-100]	36% [19-53]	7% [0-16]			52% [30-73]	
31 T-BMT	6.8 [0.5-1.7]	5 [2-20]	12/19	6/6 31	90% [80-100]	84% [64-100]	35% [18-52]	13% [1-25]			56% [38-79]	

99	6	3.8	Acute	6/6 8	(at60d)	(at180d)	at100d	at 2 years	at100d	at2y	2 years OS/EFS	**Rocha V *et al.*, 2001**
CBT	[2.5-10]	[2.4-36]	leukaemia	5/6 43	[95CI]		Gr III-IV				35%	
				4/6 40	32	81					[25-45]	
				3/6 6	[11-56]	[16-159]					31%	1994
				2/6 1	80%	90%	22%	25%	39%	38%	[21-41]	-
					[70-90]	[80-100]	[14-30]	[1-17]	[29-48]	[25-53]		1998
											49%	
262	8	42		6/6 211	18	29					[43-55]	
BMT	[5-12]	[30-60]		5/6 46	[10-40]	[8-141]					43%	
				4/6 1	96%	85%	30%	46%	19%	39%	[37-49]	
					[95-97]	[79-91]	[24-36]	[37-53]	[14-24]	[32-46]		
											41%	
180	8	38		6/6 97	16	29					[33-49]	
T-BMT	[6-12]	[14-56]		5/6 61	[9-40]	[8-165]					37%	
				4/6 10	90%	85%	8%	12%	14%	47%	[30-44]	
				3/6 2	[84-96]	[77-93]	[0-16]	[6-17]	[9-20]	[39-55]		

N/A, not available; AL, acute leukaemia; ALL, acute lymphatic leukaemia; AML, acute myeloid leukaemia; BMT, bone marrow transplantation; CBT, cord blood transplantation; CI, confidence interval; CL, chronic leukaemia; CR1/2, first/second complete remission; DFS, disease-free survival; EFS, event-free survival; GVHD, graft-versus-host disease; JCML, juvenile chronic myeloid leukaemia; LFS, leukaemia-free survival; m, matched; MDS, myelodysplastic syndrome; mm, mismatched; OS, overall survival; T-BMT, T-cell depleted BMT; TRM, transplant-related mortality; y, year

[a] Total nucleated cells $*10^7$/kg patient weight infused.
[b] Absolute neutrophil count (ANC) $\geq 0.5*10^9$/l, first of 3 consecutive days.
[c] Platelet count $\geq 50*10^9$/l, first of 7 consecutive days without transfusion support.
[d] Incidence denotes incidence or probability.
[e] Total nucleated cells $*10^7$ infused.
[f] Matched sibling donor N=19, syngeneic donor N=1, parental 5/6 HLA match graft with a class I mismatch N=3; in two of these patients the graft was T-cell depleted. Bone marrow N=20, peripheral blood stem cells N=3.

(Rubinstein P *et al.*, 1994), allowing for the first unrelated cord blood transplantations to be reported a few years later (Kurtzberg J *et al.*, 1996, Wagner JE *et al.*, 1996). Recently, favourable results on cord blood transplantation also to adult patients have been reported (Laughlin MJ *et al.*, 2001, Sanz GF *et al.*, 2001, Laughlin MjM, 2004, Rocha V *et al.*, 2004).

An overview of the results of allogeneic unrelated cord blood transplantations is presented in Table 2. No prospective studies comparing cord blood and bone marrow transplantation have been published until now. Published retrospective studies have reported parallel results overall for cord blood and bone marrow transplantation (Grewal SS *et al.*, 2003, Benito AI *et al.*, 2004) (Table 3).

Median times to myeloid engraftment of 22-23 days after cord blood transplantation have been reported (Kurtzberg J *et al.*, 1996, Wagner JE *et al.*, 2002), although longer times of up to 32-33 days have also been observed (Locatelli F *et al.*, 1999, Rocha V *et al.*, 2001). The probability of myeloid engraftment by day 42 after cord blood transplantation has been 79-89% (Locatelli F *et al.*, 1999, Thomson BG *et al.*, 2000). Notably, Rubinstein and collaborators reported a clear positive association between rising dose of infused nucleated cells and incidence of myeloid engraftment (Rubinstein P *et al.*, 1998).

The median time to platelet engraftment in paediatric patients has been 75-85 days (Locatelli F *et al.*, 1999, Thomson BG *et al.*, 2000). In their early study of 18 patients Wagner and collaborators reported a shorter median platelet engraftment time of 67 days (Wagner JE *et al.*, 1996), which, however, is still definitively longer than after stem cell transplants from adult sources. The probability of platelet engraftment by day 180 after cord blood transplantation has been 65-85% (Rubinstein P *et al.*, 1998, Wagner JE *et al.*, 2002).

Thus, both the initial incidence and speed of engraftment of myeloid cells and platelets have been inferior to those of transplants from adult donors if the matched adult transplantations are directly compared with mismatched unrelated cord blood transplantations (Laughlin MJ *et al.*, 2004, Rocha V *et al.*, 2004).

Severe acute GVHD has been reported in 9-11% of patients (Thomson BG *et al.*, 2000, Wagner JE *et al.*, 2002), although a higher incidence (23%) has been reported by some authors (Locatelli F *et al.*, 1999, Rubinstein P *et al.*, 1998).

Data on the incidence of chronic GVHD vary; the condition having been reported in 9.5-28% of patients (Kurtzberg J *et al.*, 1996, Rubinstein P *et al.*, 1998, Locatelli F *et al.*, 1999). A 9% incidence of extensive chronic GVHD was observed by Wagner and collaborators (Wagner JE *et al.*, 2002), while some authors have not detected chronic GVHD at all (Gluckman E *et al.*, 1997, Thomson BG *et al.*, 2000).

The probability of severe acute GVHD after cord blood transplantation has remained lower than after stem cell transplantion from adult sources, despite HLA incompatibilities of 1-3 mismatches out of six in a substantial proportion of cord blood transplantations (Rocha V *et al.*, 2001).

In their large study of 562 unrelated cord blood transplantation recipients, Rubinstein and collaborators reported a 46% incidence of transplantation-related events by day 100 (Rubinstein P *et al.*, 1998). Treatment-related mortality at one year after transplantation has been 20-30% (Thomson BG *et al.*, 2000, Wagner

JE *et al.*, 2002), although an incidence of 52% has also been reported (Locatelli F *et al.*, 1999). In recent studies, overall survival at one year has been 52-58% (Thomson BG *et al.*, 2000, Wagner JE *et al.*, 2002).

Seven related and seven unrelated cord blood transplantations have been reported in the Children's Hospital in Helsinki, with an overall survival of 78.5% (Vettenranta K, 2004, personal communication). Six of these transplants have been reported earlier (Vettenranta K *et al.*, 1997a, Vettenranta K, 2000). Two of the five patients received three unrelated cord blood transplantations, in which the time to myeloid engraftment was 23-35 days, and the time to independency of platelet transfusions 16-55 days.

In cord blood transplantation studies patients without a compatible sibling or unrelated adult donor have received transplanted cord blood with the best available HLA match often as a last resort. A higher degree of HLA disparity between patient and cord blood transplant has been associated with lower incidence of myeloid and platelet engraftment and slower recovery (Gluckman E *et al.*, 2004), as well as with elevated transplant-related events (Rubinstein P *et al.*, 1998). HLA disparity in cord blood transplantations has been more a rule than an exception, as the total number of cord blood transplants available in banks is still less than 200 000 compared with more than 9 000 000 unrelated donors in registries (http://www.bmdw.org/Database/Donors.htm, September 2004). Patients with varying malignant and non-malignant diseases have also been analysed together, and as cord blood transplantation has been used as a second alternative for bone marrow transplantation, patients in studies have represented more advanced phases of disease (Rubinstein P *et al.*, 1998). Conditioning regimens have varied between transplant centres as well (Rocha V *et al.*, 2001). The main problem in cord blood transplantation has been slower engraftment of the platelet compartment than in bone marrow transplantation; however, in favour of cord blood is the reported lower incidence of acute and chronic GVHD (Madrigal JA *et al.*, 1997, Rocha V *et al.*, 2000) and better tolerance of HLA mismatch between patient and cord blood unit (Rubinstein P *et al.*, 2001 [abstract]).

Haematological recovery after cord blood transplantation

The delayed haematopoietic recovery observed with cord blood compared to bone marrow transplantation may be due to the intrinsic difficulty of cord blood cells to undergo differentiation. Frassoni and collaborators reported higher frequency of CFU and LTC-IC colonies in the bone marrow of children who had received cord blood transplantation a median time of one year earlier compared with those who had received bone marrow tranplantation, although the transplanted bone marrow cellular dose was 1-log higher (Frassoni F *et al.*, 2003). Cord blood haematopoietic stem cells may thus provide for a better haematopoietic reconstitution.

Immunological recovery after cord blood transplantation

Although higher early mortality (<100 days) after cord blood transplantation from bacterial and viral infections compared with bone marrow transplantation has been reported (Rocha V *et al.*, 2001), immunological recovery after cord blood

transplantation may exceed that after bone marrow transplantation. Talvensaari and collaborators reported higher T-cell receptor diversity and T-cell receptor rearrangement excision circle (TREC) values, an ex vivo measure of thymic function, two years after cord blood transplantation compared with bone marrow transplantation (Talvensaari K *et al.*, 2002). Human foetal and cord blood lymphocyte progenitors also have similar potential to those of adult blood for generating B cells with a diverse immunoglobulin repertoire (Kolar GR *et al.*, 2004). These studies suggest that, although immunologically naive, cord blood cells are likely to have a great potential to fully reconstitute the immune system.

GVL effect after cord blood transplantation

Cord blood transplantation probably elicits an adequate graft-versus-leukaemia (GVL) effect, as relapse rates in clinical studies have not been unusually high as was earlier feared (Rubinstein P *et al.*, 1998, Gluckman E and Rocha V, 2004, Broxmeyer HE, 2004).

Experimental cord blood transplantation

Multiple cord blood transplantation. To overcome the low cell dose in an average cord blood unit, infusion of multiple cord blood units has been studied. However, early and more recent studies on multiple cord blood unit transfusion have yielded only transient improvements (Ende M, 1966, Ende M and Ende N, 1972, Shen BJ *et al.*, 1994, Weinreb S *et al.*, 1998). Infusion of leukocytes to treat life-threatening infections after profound post-transplant neutropenia has been reported to yield prompt and sustained myeloid recovery (Saarinen UM *et al.*, 1995). Co-transplantation of third-party haploidentical mobilised peripheral blood $CD34^+$ cells with an unrelated cord blood unit has also been reported to enhance engraftment, thereby shortening post-transplant neutropenia (Fernandez MN *et al.*, 2004 [abstract]). These results suggest a boosting mechanism, possibly cytokine mediated, in the haematopoietic stem cell milieu.

More recently, double cord blood transplants have been shown to yield inital double chimerism of the recipient (Barker JN *et al.*, 2001b, De Lima M *et al.*, 2002). Barker and collaborators reported a low incidence of severe GVHD (15%) and TRM (23%) in high-risk adults receiving 11 single and 29 double cord blood unit transplantations, with no significant differences in transplant outcomes between single and double unit recipients (Barker JN *et al.*, 2004 [abstract]). Although both transplanted units initially engrafted, it was not possible to predict by the amount of transplanted nucleated or $CD34^+$ cells, or by the HLA match, which of the units was responsible for long-term haematopoietic reconstitution. Interestingly, a higher $CD3^+$ cell dose in the predominating cord blood unit has been reported recently (Barker JN *et al.*, 2005). These promising results suggest that combining two units may render cord blood satisfactory for larger proportion of patients requiring stem cell transplantations, especially for adult patients.

Ex-vivo expansion. $CD34^+$ selection and expansion of cord blood haematopoietic progenitor and stem cells in the laboratory has been extensively studied for use in gene therapies as well as to facilitate provision of adequate cell doses for transplantation for adult patients (Traycoff CM *et al.*, 1995, Dorrell C *et al.*, 2000, Shpall EE *et al.*, 2004). Expanded cells are now infused together with the non-expanded part of the graft, thus proving the safety of the procedure.

Other stem cells. As cord blood has become accepted as a source of haematopoietic stem cells, also presence of stem cells of other cell lineages has been explored. Recently, multipotent mesenchymal stem cells capable of differentiating into cell types of all three germ layers have been detected in cord blood (Lee OK *et al.*, 2004). Also, studies on differentiating cord blood mesenchymal cells into e.g. neural or muscle cells are under way (Bicknese AR *et al.*, 2002, Gang EJ *et al.*, 2004).

Potential problems with cord blood

Small cell content. Although cord blood has been used with success in allogeneic haematopoietic reconstitution even in adult patients (Laughlin MJ *et al.*, 2001), the majority of currently available cord blood units contain relatively low total nucleated cell numbers (Oudshoorn M and Foeken-van Goozen L, 2004), possibly inadequate for transplantations as single units. Recently, promising results using two cord blood units from different allogeneic unrelated donors have been reported (Barker JN *et al.*, 2005).

Single dose. Compared with the possibility of a donor lymphocyte transfusion in case of relapse after adult donor haematopoietic stem cell transplantation (Dazzi F *et al.*, 2000), after cord blood transplantation haematopoietic or other cells from the same donor are not available. However, cord blood banks may have frozen the unit in more than one compartment, enabling separate future use. Possibilities to expand specific cell populations from cord blood, e.g. NK cells or cytotoxic T-lymphocytes, are being explored (Ayello J *et al.*, 2004 [abstract], Satwani P *et al.*, 2004 [abstract]).

Maternal cell contamination. Maternal cells may contaminate infant blood due to transplacental cell transfer (Schroder J and de la Chapelle A, 1972). Scaradavou and collaborators reported 38% of cord blood collections positive for maternal DNA (Scaradavou A *et al.*, 1996). Recently, minor H antigen HA-1 specific T-cells, potentially supporting the GVL effect, have been detected in cord blood (Mommaas B *et al.*, 2004). Cord blood standards do not require testing of units for maternal cell contamination (NETCORD and FAHCT, 2001), and neither has maternal cell engraftment been a problem in cord blood transplantation.

Origin of leukaemia. The aetiology of leukaemia remains largely unknown. Identification of chromosome translocations in neonatal blood spots of later identified leukaemia patients has led to the suggestion that childhood ALL is frequently initiated *in utero* (Wiemels JL *et al.*, 1999). Infant leukaemia is, however, a rare disease (Biondi A *et al.*, 2000). In addition, a cord blood unit is re-evaluated if information concerning the usability of the unit for tranplantation purposes is obtained.

5.4 Cord blood banking and networks

The probability of finding a related or unrelated haematopoietic stem cell donor, despite the huge number of nine million or more stem cell donors in international registries, is only approximately 50-75% (Confer DL, 1997). Thus, additional sources for haematopoietic stem cells are needed. Since the first successful human transplantation of cord blood (Gluckman E *et al.*, 1989), cord blood banks have been established world wide during the 1990s (Rebulla P, 2002).

In principle, cord blood is abundantly available, being a byproduct of delivery and considered as a waste material. The genetic background of any population may be well represented in a cord blood bank. Once identified and subjected to additional testing in a bank, a cord blood transplant can be shipped frozen to the transplant centre with minimal delay. Most banks collect cord blood from healthy volunteer donor mothers with no remuneration for use in unrelated allogeneic tranplantation.

Cord blood may be collected as a directed cord blood unit after the birth of a healthy sibling. The unit may then be stored in a bank if there is already an affected sibling in need of possible cord blood transplantation in the family (Reed W *et al.*, 2003). Hospitals may also take care of banking of the directed cord blood unit.

In a commercial setting cord blood can be cryopreserved, even without a family disease history, in case of a possible future autologous or allogeneic need – albeit extremely improbable – for haematopoietic stem cell reconstitution (American Academy of Pediatrics. Work Group on Cord Blood Banking, 1999). Autologous and directed, as well as commercial cord blood banking, are not further discussed in this review.

5.4.1 Quality management systems

The importance of implementing good manufacturing practice (GMP) and quality systems in blood banking was emphasized in mid 1990s. The cord blood community has developed standard policies and procedures to enable and improve both evaluation of the quality of cord blood transplants and of clinical cord blood transplantation results (Fraser JK *et al.*, 1998, American Association of Blood Banks, 2001). Other quality systems have also been adapted in cord blood banking, as reported by Sirchia and collaborators (ISO) (Sirchia G *et al.*, 1998b, Sirchia G *et al.*, 1998a) and Armitage and collaborators (GMP/GLP) (Armitage S *et al.*, 1999b). Netcord/FAHCT standards (www.unmc.edu/Community/fahct), adopted by the European Group for Blood and Marrow Transplantation (EBMT) and the World Marrow Donor Association (WMDA), among many organisations, specify detailed requirements for the whole cord blood banking process from donor recruitment through collection, processing, banking and testing to selection and release of a cord blood unit for clinical use (NETCORD and FAHCT, 2001). These standards require all cord blood bank policies and processes be defined in detail as written standard operational procedures (SOP).

5.4.2 Safety aspects of the cord blood transplant

Recruiting and selecting the donor mother

In cord blood banking programmes, mothers are routinely informed about the possibility of donating cord blood during the antenatal period (Navarrete C *et al.*, 1998). The aim of this phase of recruiting is to ensure safe transplants by preselecting healthy mothers for donation.

Medical history and maternal blood sample

The mother is interviewed using a standard questionnaire covering the personal and family history of genetic diseases, malignancy, transmittable infectious diseases and autoimmune diseases, as well as disorders of unknown aetiology (Armitage S *et al.*, 1999b). Netcord/FAHCT standards require each cord blood donor mother to be tested for anti-HIV-1/2, HIV-1-Ag (or HIV-NAT), anti-HTLV-I/II, HBsAg, anti-HBc, anti-HCV, and a serological test for syphilis (NETCORD and FAHCT, 2001). A cord blood unit sample also has to be tested for any additional required future infectious disease markers prior to release for transplantation. The maternal blood sample is drawn as part of the informed consent process (Armitage S *et al.*, 1999b).

It is obvious that the infectious tests required will increase in number as new diseases appear. Archived samples are stored to allow for future tests, e.g. genomic testing for inherited diseases, during long-term storage of the cord blood unit. Once stored, the evidence of safety of cord blood units can be accumulated, contrasted to living stem cell donors.

5.4.3 Cord blood collection

Informed consent

Informed consent may be obtained orally before cord blood collection and complemented in written after successful collection (Armitage S *et al.*, 1999b). The collections can also be directed only to mothers who have given full consent beforehand and who fullfill the predefined collection criteria (Elias M *et al.*, 2003). However, as a marked proportion of cord blood collections is known not to yield adequate volume for further use, in this model a substantial effort is wasted overall in the labour intensive consent process.

Collection principles of cord blood

In contrast to the risks associated with bone marrow and blood stem cell collections, cord blood *ex utero* collection methods pose no risk to the donor mother or infant (Fasouliotis SJ and Schenker JG, 2000, Fasouliotis SJ and Schenker JG, 2000). Cord blood banks perform routinely a high number of collections, a substantial portion of which are not acceptable for further storage due to e.g. low collection volume, low cell content or adverse medical history of the mother (Armitage S *et al.*, 1999b).

Several methods of collecting cord blood were analysed during the development of cord blood banking programmes in the early 1990s (Wagner JE *et al.*, 1992, Hirsch I *et al.*, 1993, Harris DT *et al.*, 1994, Bertolini F *et al.*, 1995). Open collections where cord blood was drained from the dissected cord directly into a container or aspirated by syringes were associated in elevated rate (12.5%) of bacterial contamination compared with closed collections (3.5%) using standard sterile closed blood collection bag sets (Bertolini F *et al.*, 1995). An aseptic technique is important in cord blood collection: rates of bacterial contamination have diminished from 28% to 4% with accumulating experience (Armitage S *et al.*, 1999b), and even lower contamination rates are possible.

Cord blood collections are performed either by trained cord blood bank personnel or delivery unit midwives or obstetricians (Wall DA *et al.*, 1997). Blood

can be collected while the placenta is still *in utero*, or *ex utero* after the birth of the placenta in a separate collection area (Lasky LC *et al.*, 2002). After the infant is born the umbilical cord is double clamped and dissected. The umbilical vein is then cannulated and, to avoid haemolysis, cord blood is drawn by gravity into the collection bag, where it is continuously mixed with the anticoagulant (Donaldson C *et al.*, 2000).

Although several obstetric practices, e.g. positioning the infant before clamping of the umbilical cord, and timing of clamping, affect the obtained cord blood volume and cell contents, attempts to modify the course of delivery in order to increase the cellular yield in cord blood collections are currently not accepted. Also, especially in unrelated setting, *in utero* collections may be experienced as more disturbing than collections after the placenta is born.

5.4.4 Selection of cord blood collections for banking

Cord blood volume obtained from the umbilical vein during collection may vary between 0 and 255ml depending on the collection technique and mode of delivery (Armitage S *et al.*, 1999b, Donaldson C *et al.*, 2000, Elchalal U *et al.*, 2000). Banks routinely use a cut-off limit of collected cord blood, e.g. 40ml, to increase the cell yield available for banking (Armitage S *et al.*, 1999b). Depending on the selected cut-off limit, an average collected cord blood volume reported has been 68-107ml (Wall DA *et al.*, 1997, Armitage S *et al.*, 1999b, Donaldson C *et al.*, 2000, Lasky LC *et al.*, 2002).

Collected total cell amount naturally also varies according to the collected volume. Total nucleated cell counts, such as 102-169 $*10^7$/unit, have been reported (Armitage S *et al.*, 1999b, Lasky LC *et al.*, 2002, Wall DA *et al.*, 1997, Elchalal U *et al.*, 2000), which may reflect the actual cut-off limits set for collected volume and nucleated cell concentrations analysed by the individual banks (Eichler H *et al.*, 2004).

5.4.5 Short-term liquid storage of cord blood before processing

Maintaining the quality of cord blood during transport from collection site to the laboratory and at the laboratory before processing is of utmost importance. In particular, fluctuations in temperature before long-term storage have to be minimised (NETCORD and FAHCT, 2001).

When cord blood storage in +4°C or +22°C was compared, 88-93% of $CD34^+$ cells, 85-95% of CFC and 89-91% of CAFC were viable at 48 hours in either temperature (Bertolini F et al., 1998). Storing of cord blood at +4°C or +22°C resulted in comparable recoveries of $CD34^+$ cells and nucleated cells at 72 hours . Neither did storing cord blood for 24 hours before cryopreservation adversely influence the post-thaw cell recovery of nucleated cells, $CD34^+$ cells or CFC (Hubel A *et al.*, 2003).

Most collection facilities are located near the processing laboratory, but transporting the cord blood from remote locations has also produced adequate results (Wada RK *et al.*, 2004). Cord blood can be placed between thermoregulatory elements (e.g. pre-chilled whole blood bags) and the

temperature of the shipment may be electronically monitored throughout transport (Hubel A *et al.*, 2004). Thus, as long as the ambient conditions are carefully monitored, cord blood can be stored cooled or at room temperature (+17°C - +20°C) before processing, although the risk of bacterial growth may increase during storage at higher temperatures.

Liquid storage of cord blood for other usages

Blood bank stored cord blood was used in allogeneic blood transfusions as far back as the 1930s (Halbrecht J, 1939). More recently, cord blood has also been studied for autologous transfusion in preterm infants to correct anaemia (Surbek DV *et al.*, 2000a, Eichler H *et al.*, 2000). As cord blood contains haematopoietic progenitor and stem cells, the feasibility of blood bank stored liquid cord blood for haematopoietic reconstitution in undeveloped countries has been studied (Ende N *et al.*, 1999). However, the established practice for long-term maintenance of cord blood haematopoietic stem cells is storage in the frozen state (see Section 5.4.7).

5.4.6 Processing: whole blood and volume reduction of cord blood

As storage of thousands of cord blood collections as whole blood would require substantial additional economic resources for greatly increased freezer capacity, methods to reduce the volume of the collection still maintaining the haematopoietic stem cell content have been studied (Harris DT *et al.*, 1994, Denning-Kendall P *et al.*, 1996, Bertolini F *et al.*, 1996). In 1995, Rubinstein and collaborators published their widely adapted volume reduction protocol involving two sentrifugation phases (Rubinstein P *et al.*, 1995). In the first phase, the red cell sedimentation rate is enhanced by adding hydroxyethyl starch to the cord blood prior to centrifugation. The separated leukocyte and platelet rich supernatant with the haematopoietic stem cells is further centrifuged and excess plasma is removed to obtain a cord blood unit of exactly desired volume (Rubinstein P *et al.*, 1995).

Another volume reduction protocol adapted by cord blood banks uses the triple bag system, a so called top-and-bottom bag in which, after centrifuging, red cells and excess plasma are extracted in one step leaving the buffy coat layer containing the haematopoietic stem cells in the original bag (Armitage S *et al.*, 1999a). In this system, adjusting the end volume has been problematic.

Using these methods, recoveries of up to 91-92% of nucleated cells have been reported on experimental scale (Rubinstein P *et al.*, 1995, Armitage S *et al.*, 1999a). Several other technical solutions to separate the cord blood leukocyte fraction are also being developed.

5.4.7 Cryopreservation

Since cryopreservation is the only feasible method for long-term storage of cord blood haematopoietic stem cells, and as the number of cells in a given collection is limited, it is of the greatest importance that the cryopreservation protocol optimises cell recorery, viability and function (Armitage S, 2000). Cord blood megakaryocytic progenitor cells have been reported to be more sensitive to the

stresses of cryopreservation than the respective myeloid progenitor cells (Xu Y *et al.*, 2004). Cord blood cryopreservation techniques have been adapted from those developed for bone marrow and peripheral blood stem cells (Rowley SD, 1992, Donaldson C *et al.*, 1996). Recently, theoretically optimised methods to improve cryopreservation protocols have been developed (Woods EJ *et al.*, 2003). A crucial issue in tissue cryopreservation is controlling the formation of ice crystals during freezing. At rapid rates of cooling intracellular ice crystals may form, resulting in mechanical cell injury. At slow cooling rates ice crystal formation tends to occur in extracellullar space, resulting in increased osmolality as free water is incorporated in growing ice crystals. Extreme extracellular hyperosmolality may then lead to dehydration injury (Rowley SD, 2004). Controlled-rate freezing profiles aim to minimise the formation of ice crystals.

Cryoprotection

The increase of the osmolality and the effect of that increase on cells are controlled by adding cryoprotectant solutions to the cord blood unit before cryopreservation. The cryoprotectant properties of dimethylsulfoxide (DMSO) were described already in 1959 (Lovelock JE and Bishop MW, 1959). DMSO is the favored agent for cryopreservation of haematopoietic stem cells because of its rapid diffusion through the cell membrane and relative non-toxicity during infusion to patients (Takahashi T *et al.*, 1985, Rowley SD, 2004). The optimal concentration of DMSO for cryoprotection of haematopoietic stem cells is approximately 10%, concentrations of 5%-15% having been studied. Macromolecular non-penetrating cryoprotectants, such as hydroxyethyl starch (HES) and dextran, as well as protein solutions, have been studied in haematopoietic stem cell cryopreservation, especially in combination with penetrating cryoprotectants. However, studies comparing DMSO alone or in combination with e.g. HES in cryopreservation of haematopoietic stem cells are scarce (Rowley SD, 2004).

Characterisation of a cord blood transplant prior to cryopreservation

Several laboratory samples have to be taken from the cord blood unit for to analyse its cellular composition and haematopoietic potential and for HLA typing for matching purposes (NETCORD and FAHCT, 2001). As the volume of a cord blood collection is restricted, the sample volume is kept at a minimum and sampling of the side products of the process (plasma, residual nucleated cells in red cell fraction) are used when applicable. A haematology analyser is used to analyse samples for full blood count and differential (Section 5.1.2.1). A smear can be prepared and stained for later analysis, e.g. of nucleated red cells. $CD34^+$ cells (5.1.2.3) and haematopoietic progenitor cells are also enumerated (5.1.2.2), and aerobic and anaerobic microbiological cultures taken (Armitage S *et al.*, 1999a).

Controlled-rate freezing

Freezing the cord blood unit using a rate-controlled programmed freezer has been widely adopted by cord blood banks to provide maximum protection for haematopoietic stem cells (Armitage S, 2000). To protect the primary storage bag from breakage, it is enclosed in a metal canister for freezing. The optimal cooling rate depends on the cell type and on the concentration of cryoprotectant.

For human haematopoietic stem cells suspended in 10% DMSO, an optimal freezing rate of 1-3°C/minute has been reported (Ma DD *et al.*, 1982). After initial freezing of water, higher cooling rates may be used (Rowley SD, 2004). Although controlled-rate freezing is preferred by the majority of cord blood banks, parallel results using insulating material to protect the cord blood unit have been reported when the unit has been damped to -80°C freezer and after passive freezing transferred to -196°C for further storage (Itoh T et al., 2003). After controlled-rate freezing in a separate device, the canister is transferred to another container for long-term storage. To avoid transient warming events (Dobrila L *et al.*, 2001 [abstract]) during the transfer of frozen cells between two tanks, automated robotic devices where both controlled-rate freezing and long-term storage take place in the same device can be used (e.g. BioArchive, Thermogenesis, Rancho Cordova, CA).

Long-term storage

Cord blood haematopoietic stem cells are stored either in mechanical electric freezers or in the liquid or vapour phase of nitrogen below –135°C to prevent possible progressive growth of ice crystals, as water migrates form smaller to larger crystals at warmer freezing temperatures (Rowley SD, 2004).

Liquid phase of nitrogen (-196°C) provides the most stable environment for long-term storage and is regarded safest, as temperature changes in the vapour phase of up to 100°C have been reported (Rowley SD and Byrne DV, 1992). Contamination with hepatitis B virus of the liquid phase of nitrogen in a cryopreservation tank has been reported (Tedder RS *et al.*, 1995). Microbial contamination is also possible in the vapour phase of nitrogen (Fountain D *et al.*, 1997). Thus, cord blood haematopoietic stem cell units should be either quarantine stored before transfer to the main tank or double bags should be used to protect the unit and reduce the risk of contamination of the nitrogen tank (Khuu HM *et al.*, 2002).

Cryopreservation of cord blood in liquid nitrogen of at least six months as whole blood or separated mononuclear cells did not impair the clonogenic capacity of haematopoietic stem cells (Almici C *et al.*, 1997). Harris and collaborators reported only minimal effects of cryopreservation of seven years on the ability of cord blood cells to become cytotoxic effector cells when stimulated with interleukin-2 (Harris DT *et al.*, 1994). Broxmeyer and collaborators studied the quality of cord blood samples after extended storage. They reported immature cord blood cells stored frozen for 15 years with high proliferative, replating, ex vivo expansion and NOD/SCID mouse engrafting ability (Broxmeyer HE *et al.*, 2003). Also, cord blood CFU-GM, CFU-GEMM and BFU-E recovery rates of more than 97% have been reported after storage for up to 12 years (Mugishima H *et al.*, 1999). Thus, cord blood units probably remain effective for clinical transplantation after long-term frozen storage.

After review of clinical, processing and laboratory data against predefined acceptance criteria, cord blood units are entered in international search registries (Section 5.4.9).

Transportation in frozen state

The temperature should be maintained at -135°C or below during the

transportation of the cord blood unit from bank to transplant centre (NETCORD and FAHCT, 2001). For this purpose "dry shippers" can be used. In these devices liquid nitrogen is absorbed into the wall of the container, enabling a stable environment below –135°C in the container for 7-10 days (Rowley SD, 2004).

5.4.8 Thawing

Only minor to moderate infusion-associated adverse reactions have been described during infusion of haematopoietic stem cell grafts (Davis JM et al., 1990). Thus, haematopoietic stem cells are often infused without removing DMSO, as the removal procedure has been feared to hamper the haematopoietic quality of the product. As the storage procedures and conditions have been optimised for mononuclear cells, thawing of the unit yields lysis of the neutrophils and red cells (Rubinstein P *et al.*, 1995).

Reducing the DMSO content of a cord blood unit after thawing by diluting and washing the cell suspension one or more times with human albumin and dextran solution has been suggested to improve the cord blood cell viability (Rubinstein P *et al.*, 1995). Although post-thaw total leukocyte yields of non-washed and washed cord blood units did not differ (94% and 92%, respectively), the post-thaw yields of viable leukocytes in the study of Rubinstein and collaborators were 35% and 61%, respectively. However, in one study, wash-out of the DMSO did not improve the speed of engraftment after cord blood transplantation (Nagamura-Inoue T *et al.*, 2003).

Even when the cell yields are optimised, washing may cause a certain loss of cells and might also hamper their viability, especially if validated devices and procedures are not in use. $CD34^+$ cell and CFU yields exceeding 100% are seen (Armitage S *et al.*, 1999a), possibly because of cell activation. Correct identification of the cord blood unit throughout the thawing procedure using unique numbers is also important (such as e.g. ISBT 128, ICCBBA Inc) (Ashford P, 2002).

5.4.9 Cord blood banks and networks

The first cord blood bank was established in New York Blood Center by Dr Pablo Rubinstein in February 1993 (Rubinstein P *et al.*, 1994) (National Cord Blood Program). Cord blood banking programmes have since been initiated at several places in United States (Alonso JM, III *et al.*, 2001, Lasky LC *et al.*, 2002), Europe (Lazzari L *et al.*, 1996, Querol S *et al.*, 1998, Navarrete C *et al.*, 1998, Dal Cortivo L *et al.*, 1998, Jacobs HCJM and Falkenburg JHF, 1998, Pojda Z *et al.*, 1998, Armitage S *et al.*, 1999b, Kogler G *et al.*, 1999, Ordemann R *et al.*, 1999, M-Reboredo N *et al.*, 2000, Donaldson C *et al.*, 2000, Rendine S *et al.*, 2000, Proctor SJ *et al.*, 2001), Australia (Vowels MR and Lam-Po-Tang PR, 1997) and Asia (Mugishima H *et al.*, 2002, Lee TD, 2002, Elias M *et al.*, 2003, Wacharaprechanont T *et al.*, 2003). Forty cord blood banks or registries in 24 countries participate in WMDA (Oudshoorn M, Foeken-van Goozen L, 2004).

To enable an HLA type-based search of donors from bone marrow and peripheral blood stem cell donor registries across the world, Bone Marrow Donors Worldwide (BMDW) was established by EBMT (Oudshoorn M *et al.*, 1994). BMDW

relased its first listing of 156 000 donors in February 1989. Currently, more than 9 million potential donors are listed. Large national registries in the USA, e.g. the National Marrow Donor Program (www.nmdp.org) and the Caitlin Raymond International Registry (www.crir.org), also participate in BMDW. Although BMDW was first established to allow for international searches of tissue typed adult donors, cord blood units are also currently listed. At present, 37 cord blood registries in 21 countries (with a total of 172 550 cord blood units) participate in BMDW (www.bmdw.org/Database/Donors.htm, as of July 2004).

Netcord, founded in May 1998, is an international non-profit joint effort of leading cord blood banks which has issued statutes and guide-lines with the primary aim of improving the quality of cord blood transplants for clinical cell therapy on the international level (Wernet P *et al.*, 2001 [abstract], NETCORD and FAHCT, 2001) (www.unmc.edu/Community/fahct). To enable efficient cord blood unit data exchange and allocation, the Netcord inventory is available for searches in a virtual office via the internet (office.de.netcord.org). In addition, cord blood banks in Asia are now associated under the name of Asiacord (Mugishima H *et al.*, 2002).

To enable coordinated management of complicated international haematopoietic stem cell donor search processes, search activities in each country have been centralised to national hubs.

5.4.10 Ethics

Ethical issues surrounding the developing cord blood banking technology and practices have been addressed (Lind SE, 1994). Among other matters, the obligation to notify parents and children of positive results of testing for infectious diseases has raised some discussion (Sugarman J *et al.*, 1995). The need to maintain linkage between the donor and the banked cord blood has also been debated (Sugarman J *et al.*, 1997). In the US, a policy of not maintaining the linkage between cord blood donor mother identity and donated cord blood information has been applied earlier (Rubinstein P *et al.*, 1994). According to the Netcord/FAHCT standards the linkage has to be maintained (NETCORD and FAHCT, 2001). Another issue is whether unrelated *in utero* cord blood collections interfere with the labour process to such a degree that it should be considered unethical. Delivery practices are not routinely modified by any means in order to increase the collected cord blood volume. Moreover, the newborn is not touched at all and no blood sample is taken from him/her. All blood samples needed for infectious disease testing are drawn from the volunteer mother.

In the ideal informed consent process, persons who have adequate decision-making capacity are given accurate and relevant information about the risks, benefits and alternatives of a proposed intervention. They then use this information to make a voluntary decision about the intervention that is consistent with their own values (Sugarman J *et al.*, 1997). The quality of the informed consent procedure has been discussed as well; even if it fullfills the current ethical requirements, the procedure needs continuous optimisation regarding the donor mother's need for information (Sugarman J *et al.*, 2002).

5.5 Cord blood transplant

Several gestational as well as mother- and infant-related factors have been reported to affect both the cord blood quality and cell contents, although total cell counts have mainly been used in analyses (Shlebak AA *et al.*, 1998, Donaldson C *et al.*, 1999, Ballen KK *et al.*, 2001, Jones J *et al.*, 2003).

5.5.1 Birth weight of donor infant

Foetal growth and birth weight standards

Human growth is a complicated process characterised by numerous unique features, including extremely rapid foetal growth which slows down immediately after birth (Rosenfeld RG, 2003). Specific growth standards are needed to evaluate birth weight as an indicator of intrauterine growth. As male fetuses weigh more than female, at least from the 35th week of gestation on, growth standards have to be applied to gender (Tanner JM and Thomson AM, 1970). Also, as the varying genetic background of populations and multiple geographical and social factors affect birth weight (Gruenwald P, 1966), the standards need to be population-specific (Pihkala J *et al.*, 1989, Arbuckle TE *et al.*, 1993). Birth weight data sets are, however, cross-sectional by nature and, at a given gestational age, births are not a random sample of all intaruterine fetuses. Classifications such as "small for gestational age" (SGA) and "large for gestational age" (LGA) have been traditionally defined as infants below the 10th or above the 90th percentile, respectively, at each gestational age, and the term intrauterine growth retardation (IUGR) has been used almost as a synonyme for SGA (Wilcox AJ, 2001). There has also been considerable variance as to how macrosomia (too high a birth weight for the gestational age and gender) has been defined.

Birth weight, which depends both on gender and gestational age, can be analysed using a standardised deviate (z score) (Oken E *et al.*, 2003). The standard normal distribution (z distribution) is a normal distribution with a mean of 0 and a standard deviation of 1. Any point (x) from a normal distribution can be converted to the standard normal distribution (z) with the formula z = (x-mean) / standard deviation. Z for any particular x value shows how many standard deviations x is away from the mean for all x values (Armitage P *et al.*, 2002). Thus, a birth weight z score (relative birth weight) of 0 for a child indicates that the birth weight of that child is exactly as expected regarding the gestational age and gender of that child. Correspondingly, a relative birth weight of 1 indicates that the birth weight of that child is one standard deviation higher than its gestational age and gender-specific expected weight.

Although the actual level of accuracy in predicting birth weight is far from that described theoretically above, higher birth weight has been reported to yield higher total nucleated and CD34^{+} cell amounts in cord blood collections (Ballen KK *et al.*, 2001), whereas the relationship between cord blood CD34^{+} cell concentration and birth weight has not been described.

Placental weight

The relationships between placental weight, birth weight and obstetric factors have been studied for decades (Sedlis A *et al.*, 1967, Friedman EA and Sachtleben

MR, 1969). Placental weight and birth weight correlate positively (Courcy-Wheeler R and Wolfe C, 1992, Altman DG *et al.*, 1992). Higher placental weight has also been reported to correlate positively with collected cord blood volume (Jones J *et al.*, 2003).

Maternal diabetes

Studies on possible associations between maternal diabetes and cord blood quality are scarce. As the ideal intrauterine growth potential of an individual is not known, a registered 'normal' birth weight may even reflect abnormal (pathological) growth, especially in diabetic pregnancies (Schwartz R and Teramo KA, 1999).

Gestational diabetes predisposes the newborn infant to macrosomia (Schwartz R *et al.*, 1994). Two-thirds of women diagnosed with gestational diabetes using oral glucose tolerance test are treated only by diet (Suhonen L and Teramo K, 1993) and could thus participate in a cord blood banking programme. As birth weight correlates with the collected cord blood volume (Jones J *et al.*, 2003), cord blood collections from gestational diabetic pregnancies would be expected to yield higher total cell counts.

Gender

Differences between female and male fetuses are traditionally thought to originate under estrogen and androgen hormonal influence during pregnancy (Dennis C, 2004). Recently, however, differences in gene expression of early female and male mice fetuses have been reported before gonadal influence (Dewing P *et al.*, 2003).

Studies on the effect of gender on haematopoietic stem cells are scarce. Female infants have been reported to have higher cord blood nucleated cell total content (Ballen KK *et al.*, 2001) and concentration (Nakagawa R *et al.*, 2004).

5.5.2 Obstetric factors

Gestational age

The haematopoietic progenitor cell content of cord blood reportedly diminishes during pregnancy. Cord blood CFU-GM concentration in infants born after 25-31 weeks of gestation was 10/µl compared with 3.8/µl and 3.0/µl in infants born after 32-36 and 38-41 weeks of gestation, respectively (Clapp DW *et al.*, 1989). BFU-E colonies have been found three-fold ($160/10^5$ cells vs. $48/10^5$ cells) (Forestier F *et al.*, 1991) and seven-fold ($484/10^5$ vs. $69/10^5$ cells) (Jones HM *et al.*, 1994) higher in second trimester foetal blood compared to term cord blood.

$CD34^+$ cell content as percentage of lymphocytes (and as concentration) has been reported to diminish from a mean of 11.1% ($9.2*10^7$/l) at 13 weeks of gestation to 1.0% ($3.0*10^7$/l) at 38 weeks (Thilaganathan B *et al.*, 1994b). Surbek and collaborators reported a significantly higher relative $CD34^+$ cell content in second trimester compared to term cord blood (2.57% vs. 0.7%, respectively) (Surbek DV *et al.*, 1998). Parallel results were obtained by Shields and Andrews, who reported a 4.9-fold higher frequency of $CD34^+$ cells in early foetal blood at 17-24 weeks of gestation compared with that at term (6.4% vs 1.3%, respectively).

The frequency of $CD34^+$ cells in their study also declined linearly with gestational age (Shields LE and Andrews RG, 1998).

In contrast, leukocyte concentration has been reported to increase from 18^{th} week of gestation to term (Clapp DW *et al.*, 1989, Forestier F *et al.*, 1991).

Factors related to delivery

Stress during delivery has been associated with elevated cell numbers in cord blood. In a univariate model, Lim and collaborators observed that a prolonged first stage of labour resulted in increased numbers of nucleated cells, granulocytes, $CD34^+$ cells and haematopoietic progenitor cells in cord blood (Lim FT *et al.*, 2000). In their multivariate model where the explanatory variables were the Apgar score, umbilical venous pH, durations of first and second stages of labour and whether the labour was assisted or not, the explained variance (R^2) for cord blood nucleated cell, $CD34^+$ cell an CFC content was 30%, 15% and 7%, respectively. Aufderhaar and collaborators have reported a positive association between lower umbilical arterial pH, indicating a prolonged labour, and higher cord blood nucleated, $CD34^+$ cell and colony forming colony contents (Aufderhaar U *et al.*, 2003).

Mode of delivery. Cord blood nucleated cell concentration has been reported to be higher in collections from vaginal deliveries compared with that in collections from caesarean sections (mean 15.0 vs. 10.3 $*10^9$/l, respectively) (Vettenranta K *et al.*, 1997b). This is possibly due to cytokine interactions or oxidative sterss elicited by various stress factors during vaginal delivery (Lim FT *et al.*, 2000, Aufderhaar U *et al.*, 2003, Raijmakers MT *et al.*, 2003). However, as the collected volume is higher in collections from caesarean section deliveries than from vaginal deliveries (76 vs. 63 ml, respectively) (Sparrow RL *et al.*, 2002), the total nucleated cell content obtained does not markedly depend on the mode of delivery.

In utero versus ex utero cord blood collection. Cord blood collection *in utero* before the birth of the placenta has been reported to yield higher volumes than collection *ex utero* after the birth of the placenta (mean 93 vs. 66 ml, respectively) (Surbek DV *et al.*, 2000c), and thus larger total nucleated cell, $CD34^+$ cell and colony forming cell numbers (Solves P *et al.*, 2003b, Solves P *et al.*, 2003a, Solves P *et al.*, 2003c). Wong and collaborators studied paired cord blood samples and reported 9.5% higher concentration of nucleated cells and 11.6% higher concentration of CFU-TOT colonies in samples collected *in utero* compared with paired samples collected *ex utero* (Wong A *et al.*, 2001). Discrepant data on the microbial contamination rate in *in utero* and *ex utero* cord blood collections have been reported (Lasky LC *et al.*, 2002, Solves P *et al.*, 2003a), probably due to differences in obstetric practice or collection techniques. The procedures in cord blood banking for unrelated transplantation are designed not to disturb the delivery practices when the collections are performed *ex utero*.

6 AIMS OF THE STUDY

The general aim of this study was to evaluate and develop methods for using the vast amounts of data gathered in the cord blood banking process to analyse various infant physiological phenomena and to improve the quality of the cord blood haematopoietic stem cell transplant.

The specific aims were:

- to study the association between cord blood nucleated cell and $CD34^+$ cell content through the standardised banking process (I),

- to analyse the association between infant characteristics and human leukocyte antigens in order to clarify the multiple factors affecting intrauterine growth (II),

- to study the association between infant characteristics and cord blood cell concentrations in order to elucidate the effect of cord blood haematopoietic progenitor and stem cell content to intrauterine growth (III), and

- to study the association between infant gender and cord blood cell concentrations in order to reveal the independent effect of gender on cord blood haematopoietic progenitor and stem cell content (IV).

7 MATERIALS AND METHODS

The methods developed for the Finnish Cord Blood Bank programme were reported in study I. The same methods were used to obtain the material for the other studies (II-IV). The later studies included the material of the former. Although the study materials thus overlapped, the accumulating number of infants included in the studies during the developement of the banking programme improved the reliability of the analyses. The Netcord/FAHCT standards (NETCORD and FAHCT, 2001) were applied wherever applicable in the cord blood banking programme.

7.1 Study subjects

7.1.1 Ethics

The procedures for the cord blood banking programme were accepted by the Ethical Committees of the City of Helsinki and Vantaa (recruiting) and of the Helsinki University Central Hospital (collections), and were carried out according to the provisions of the Declaration of Helsinki. The mothers of cord blood donor infants gave written informed consent for their participation in the cord blood banking programme.

7.1.2 Material of the original studies

Cord blood collections for clinical use were began at the Department of Obstetrics and Gynaecology, Helsinki University Central Hospital, in January 1999. From January 1999 to October 2000 cord blood collections were performed at a single collection hospital. After October 2000 cord blood was collected using identical standards in two hospitals. One cord blood collection site was closed in November 2003. Altogether, 1999 cord blood collections were processed, frozen and transferred to international search registries for clinical use as haematopoietic stem cell transplants by September 2003 (Table 4).

Between January 1999 and October 2000, 3176 healthy pregnant women registered voluntarily as prospective cord blood donors (I). Of these, 1067 (34%) delivered in circumstances where the cord blood could be collected. The exclusion of 313 (29%) of the 1067 collections from processing (Figure 3) was mainly due to a low collected volume (N=284). Other reasons for discarding the collections from processing were adverse maternal or infant health information (N=10) as well as process development and validation and technical problems (N=19). Of the 754 (71%) units accepted for processing, 93 were not processed further because of a too low total nucleated cell number. Technical problems precluded the further processing of 29 units. Of the 632 (59%) frozen units 33 (3%) were thawed for process development and validation or because of technical problems during freezing. Thus, 599 (56%) cord blood units were further evaluated. $CD34^+$ cell results were not available from seven units and for four additional units some other data were missing. A uniform set of data was available for 588 (55%) cord blood units. CFU results were available for 88 (15%) cord blood units. 526

Table 4 Material of original publications.

Study	N	Study period	Cord blood bank material
I	588	January 1999 – October 2000	Cord blood bank process from collection to cryopreservation and long-term storage
II	1381[a]	January 1999 – December 2001	Collected cord blood units with HLA DRB1 (N=1263), $CD34^+$ and nucleated cell data
III	1368[a,b]	January 1999 – December 2001	Collected cord blood units at term with $CD34^+$ and nucleated cell data
IV	1999[a]	January 1999 – September 2003	Cryopreserved cord blood units accepted for transfer to international search registries

[a] The study materials partially overlapped with the previous studies.
[b] N=13 pre-term (gestational age <37 weeks, range [34 weeks 4 days – 36 weeks 5 days]) cord blood collections were excluded from the material of N=1381.

cord blood units were included in international search registries by the end of May 2002. As typical in cord blood banking, only approximately half of the 1067 collections performed could be succesfully stored as clinical cord blood transplants.

A complete set of HLA, $CD34^+$ and nucleated cell data was available for 1381 cord blood collections performed between January 1999 and December 2001. Of these, HLA DRB1 was investigated from 1263 samples (II) at a later phase of the cord blood banking process. Apgar scores were seven or more in 98.4% of the infants at one minute of age, and in all of them at 10 minutes of age. All infants received normal care in the maternity unit. The birth weight of seven (0.5%) infants was less than 2500 g. Forty-six infants (3.3%) were classified as macrosomic (birth weight z-score >2SD).

In study III, 1368 term deliveries with gestational age 37 weeks or more were analysed out of the total of 1381 cord blood collections. Of these collections, 1342 (98.1%) were from pregnancies between 37 and 42 weeks, and 26 (1.9%) from pregnancies longer than 42 weeks. Fifty-four infants (3.9%) were classified as macrosomic.

In study IV, 1999 cord blood collections further processed, frozen and transferred to international search registries for clinical use as haematopoietic stem cell transplants between January 1999 and September 2003 were analysed. CFU results were available from 321 (16.1%) cord blood collections. The median birth weight of the male infants (N=1059, 3715g, range [2425-5310]) was, as expected, higher than that of female infants (N=940, 3570g, [2315-5410], P<0.0001). There was no difference between male and female median relative

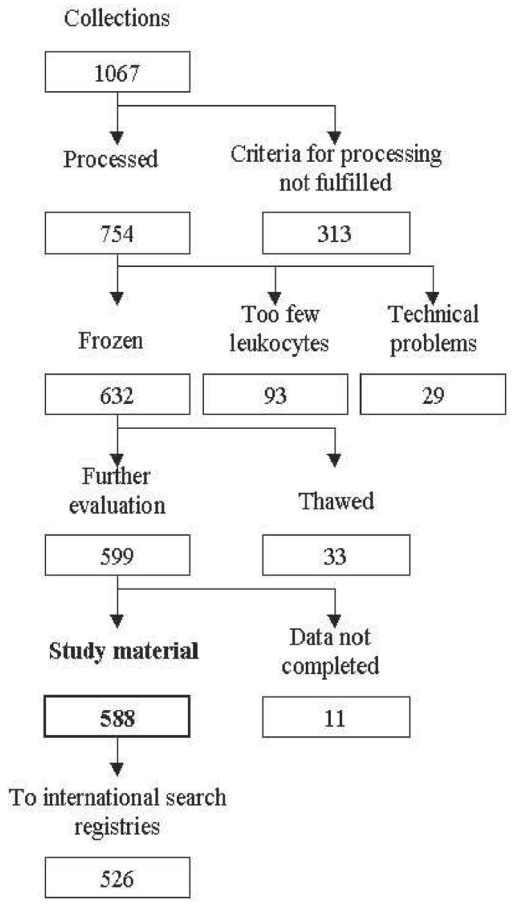

Figure 3 Study material: from collections to cord blood units accepted into the international search registries (I). Reasons for non-acceptance of cord blood units for transfer to search registries varied from medical reasons to technical errors. Aroviita et al. Vox Sanguinis 2003;84(3):219-27. Copyright of Blackwell Publishing, used with permission.

Table 5 Descriptive statistics of cord blood donors.

	N	Median	Range	Mean	SD
Birth weight, g	588	3695	2350-5100	3694	466
	1381[a]	3660	2300-5160	3687	473
	1999[a]	3650	2315- 5410	3678	460
Relative birth weight	588	0.135	-2.59-4.83	0.190	0.97
	1381	0.086	-2.59-4.83	0.157	0.97
	1999	0.054	-2.59-4.83	0.136	0.95
Gestational age, d	588	278	242-298	279	8.1
	1381	278	242-298	279	8.0
	1999	278	242-298	279	7.7

[a] The material N=1999 includes partly the material of N=1381, which includes partly the material N=588

birth weights (0.05, [-2.33–4.83] vs. 0.06, [-2.59–4.08], respectively, P=0.43). The median gestational age of male infants (278d, [242-298]) did not differ from that of female infants (277d, [255-298]), P=0.35).

Of the whole material of 1999 infants, 1948 (97.4%) were delivered at term (37-41 weeks) and 940 (47%) were female. Forty-six percent of the deliveries were caesarean sections. Indications for elective caesarean sections included previous caesarean section, breech presentation and contractive pelvis. For logistic reasons (timing of the birth, immediate delivery of the placenta, low microbiological burden) caesarean section deliveries were preferred. The distribution of birth weights was normal. Logically, the median relative birth weight approached zero as the study material grew (Table 5). The median and distribution of gestational ages were identical in all studies.

7.2 Cord blood banking (I)

7.2.1 Recruiting donor mothers

Pregnant women attending routine maternity health care visits were informed of the possibility of donating cord blood and of the health requirements (preselection). Mothers were also recruited during outpatient clinic visits. Healthy volunteer mothers registered as prospective nonremunerated donors.

Baby health questionnaires, which were sent to well baby clinics, were used to study the success of the preselection process performed by maternity health care system and cord blood bank midwives in selecting cord blood units from healthy mother baby pairs.

7.2.2 Collection

Mother's written informed consent, health questionnaire and blood sample

Mother's consent was obtained prior to the delivery either using a registration form or in person by specially trained cord blood bank midwives. After delivery, the mother confirmed her informed consent in written within seven days (usually the day after delivery). The mother was also interviewed by cord blood bank midwives regarding her and her family's health history using standard questionnaires. The cord blood bank midwives drew the maternal blood samples and obtained the obstetric and perinatal data from the hospital records.

Cord blood collection

Cord blood collections were performed *ex utero* at the Department of Obstetrics and Gynaecology of the Helsinki University Central Hospital. The umbilical cord was clamped and dissected according to the normal procedure of the hospital delivery unit. After the placenta was delivered, it was wrapped in a sterile cloth and transferred by a cord blood bank midwife to a specially equipped collection room adjacent to the delivery rooms. The room was only used for cord blood collections, which were performed by specially trained cord blood bank midwives.

Equipment for collection
Placenta was placed in an autoclavable collection stand, which gives good support to the placenta. After careful disinfection of the umbilical cord, umbilical vein was cannulated and cord blood was collected into a cord blood collection bag (791-01U, Pall Medsep Corporation, Covina, CA; 25 ml CPD anticoagulant) in a closed system. The nominal volume of the collection bag was 150 ml, but in practice a maximum of approximately 110 ml of cord blood could be collected (see processing below). The collection time and the collected blood volume were registered with weighing blood mixer (Optimix, Baxter, Kista, Sweden). After collection, the tubing was stripped to mix all the blood with anticoagulant and sealed (Hematron 3, Baxter Fenwall, Kista, Sweden).

Transportation of cord blood between collection hospital and processing laboratory
Each collected cord blood was packed individually for transportation to the laboratory; it was placed between thermoregulatory elements (butanediol bags) in order to reach room temperature (17-26°C). Room temperature was chosen for transportation instead of refrigeration to avoid unnecessary temperature changes in the cord blood. The blood bag was then packed in an insulating container together with a continuously registering electronic thermometer (TinyTalk, Gemini Data Loggers, Dundee, Scotland; measurement every three minutes) and a standard thermometer and transported to the laboratory by car. On arrival at the laboratory the cord blood bag was placed on a platelet mixer (Helmer, Baxter, Kista, Sweden) to await processing at room temperature.

7.2.3 Processing

Volume collected
Cord blood volume collected was defined as the total volume received in the laboratory, excluding the volumes of the anticoagulant (25 ml) and the initial sample (5 ml) (I). In studies II-IV the volume collected was defined as the total volume received in the laboratory excluding only the volume of the anticoagulant (25 ml). The total volume received in the laboratory was measured by weighing the collection bag (AND EK-1200G, A&D Instruments, Oxford, UK) and reducing the weight of the empty bag (18 g). One gram was taken as one millilitre.

Processing premises
Processing was performed in the Finnish Red Cross Blood Service (FRC BS) Cord Blood Bank laboratory in special laminar air hoods. The positively pressurised premises were used only for cord blood processing. Microbial cultures from surfaces and air, as well as air particle measurements, were performed regularly by the FRC BS microbiology laboratory and clean room contamination control unit.

Acceptance criteria for processing
Cord blood collections with a collected volume of at least 50 ml with no adverse maternal or infant health history or technical problems were accepted for

processing. Units with volumes between 40 and 50 ml were processed if the total collected nucleated cell count exceeded 800 x10^6 / unit. The cord blood units were processed in 48 hours after collection.

Volume reduction and cryopreservation

The method described by Rubinstein and collaborators (Rubinstein P et al., 1995) was applied for volume reduction. At each transfer step of cord blood, exact calculations were performed to ensure a final volume of 20 ml. Briefly, hydroxyethyl starch (HESPAN®, 6% colloidal solution of hetastarch in 0.9% sodium chloride in water for injection, Fresenius Pharma, Graz, Austria) was added to the collection bag in 1:5 ratio relative to the volume of cord blood volume collected + anticoagulant. After the first centrifugation (46 g (400rpm), 18 min, 10°C, Sorvall RC3C, Kendro, Asheville, NC) the collection bag was attached to the cord blood processing bag set (791-02U, Pall Medsep Corporation, Covina, CA) using a sterile connecting device (SCD, Terumo, Leuven, Belgium), and leukocyte rich plasma was extracted to the processing bag. After the second centrifugation (400 g (1500 rpm), 13 min, 10°C, Sorvall RC3C) excess plasma was removed using a manual extractor (Fenwall 4R4414, Fenwall Laboratories, Deerfield, IL), and 5 ml mixture of cryoprotectant (55% w/v DMSO and 5% w/v Dextran 40, Pall Medsep Corporation) was added on cooling plates in a standardised way using an infusion pump (Ivac P600, Alaris Medical Systems, Hampshire, UK). The final product was transferred to the freezing bag, which is an integral part of the processing bag set. Segments were sealed to the freezing bag tubing and 20 ml and 5 ml chambers were also sealed (Sebra 1105, Sebra, Tuscon, AZ) to enable possible future separate use. The freezing bag was sealed (Fuji FS-315, Fuji Impulse, Osaka, Japan) into a quarantine pouch (Overrap Bags P/N 7-01-150, Thermogenesis, Rancho Cordova, CA) and placed in a metal canister (P/N 6-16-146, Thermogenesis).

Controlled rate freezing and storage of cord blood units

The canister was then inserted in a Controlled Rate Freezer (P/N 7-65-009, Thermogenesis) and the cord blood unit frozen using a specified freezing profile (pre-freeze 10°C/min; start-freeze –1°C; fan power 100%; end-freeze –11°C; post-freeze 2°C/min; end temperature –50°C) in a BioArchive system (Thermogenesis). After freezing, the robotic arm automatically stored the unit in the liquid phase of liquid nitrogen, where long-term storage takes place in a highly stable environment. The median viability of the cell contents after thawing (Rubinstein P *et al.*, 1995) was 87% (N=8/33, range 72-98, Trypan-Blue method).

7.2.4 Laboratory analyses

The cord blood bank laboratory performed the $CD34^+$ cell enumeration and haematopoietic progenitor cell assays. All other laboratory tests were performed in other laboratories of the FRC BS. The initial sample volume of 5 ml taken from whole cord blood at the beginning of processing was used for nucleated cell enumeration (0.5 ml), $CD34^+$ cell enumeration (0.5 ml, from which also blood smears for further morphology were archived), colony forming cell cultures (0.5 ml, from 15% of randomly selected cord blood units), HLA serological typing (3

ml, from which the plasma fraction was archived for further tests) and microbiological cultures (aerobic 0.5 ml, anaerobic 0.1 ml). Samples for nucleated cell and $CD34^+$ cell enumeration after volume reduction were drawn before the addition of the cryoprotectant.

Standardised concentration
The ratio of standard anticoagulant volume used to actual cord blood volume obtained in collection (without anticoagulant) varies. The result is a greater dilution of smaller collection volumes leading to inappropriately lower measured concentrations. This variable ratio was not corrected for in the cell concentration data in study I. To study the associations between infant characteristics and cord blood cell concentrations, the measured concentration was standardised by dividing the total cell number collected by the cord blood volume collected (excluding the anticoagulant). These actual values for $CD34^+$ and nucleated cell as well as for CFU concentrations were used in the analyses in studies III-IV as well as in this thesis.

Archived samples
Samples of cord blood native plasma, HES plasma, excess red cells, umbilical cord, DNA and blood smears, as well as maternal plasma and buffy coat were stored for future studies (data not analysed in the present study).

Nucleated cell, erythrocyte and platelet enumeration
Cells counts were obtained using an automated haematology analyser (Sysmex K1000, Sysmex Corporation, Kobe, Japan; method based on electric resistance detection) at the FRC BS component production quality control department. The nucleated red cell number was included in the total nucleated cell count. Lymphocyte, monocyte and neutrophil concentrations were calculated by multiplying the relative content of the respective cell type and the corrected nucleated cell concentration (IV).

$CD34^+$ cell enumeration
$CD34^+$ cell analyses were performed using a flow cytometer (FacsCalibur; Becton-Dickinson, San Jose, CA) and either a commercial single platform (ProCOUNT, Becton-Dickinson, 1/1999-3/2001) or dual platform (ISHAGE, 3/2001-9/2003) (Sutherland DR *et al.*, 1996) protocol. The performance of the $CD34^+$ cell analyses was evaluated six times a year in an external quality control scheme with successful results (UK NEQAS, National External Quality Assessment Service, Sheffield, UK; adult $CD34^+$ cells). Notably, the quality control samples were adult mobilised peripheral blood.

Haematopoietic progenitor cell enumeration
Colony forming cells (colony forming units, CFU) were cultured as statistical process control from 15% of randomly selected samples. $1x10^4$ and $2x10^4$ nucleated cells were plated in duplicate and cultured for 14 days in +37°C, 5% CO_2 without antibiotics using commercial reagents (Methocult GF H4434, StemCell Technologies, Vancouver, Canada). Total CFU content (CFU-TOT) was defined as the sum of granulocyte-macrophage (CFU-GM), burst-forming-unit-

eryhrocyte (BFU-E) and multipotential granulocyte-erythrocyte-macrophage-megakaryocyte (CFU-GEMM) colonies. Hemoglobinised colonies were analysed as the sum of CFU-GEMM and BFU-E colonies. The performance of the analyses was evaluated in an external quality control scheme biannually (Global Proficiency Testing Program for Hematopoietic Clonogenic Assays (cell thawing, inoculation and colony enumeration), StemCell Technologies, Meylan, France). The quality control samples were adult mobilised peripheral blood.

Microbial cultures
Aerobic and anaerobic microbial cultures (BacTAlert™, Organon Teknika Corporation, Durham, NC) were performed.

Infectious diseases testing
Blood donor infectious diseases screening tests (HIV1/2-antibodies, HCV-antibodies, HBs-antigen, HTLV1/2-antibodies, cardiolipin-antibodies) were performed on each maternal sample and cord blood collected as part of current infectious diseases screening system at the FRC BS.

HLA typing (II)
HLA A and B antigens were typed serologically using commercial typing trays (HLA ABC 144, Biotest AG, Dreieich, Germany). Low resolution DNA typing was used to define DRB1 allele groups (Innolipa, Innogenetics, Murex Biotech Limited, Kent, UK). DRB1*13 alleles were further typed using the DRB1*13 SSP high resolution typing kit (Olerup SSP AB, Saltsjöbaden, Sweden).

7.2.5 Acceptance of cord blood units to search registries

After thorough evaluation of the obstetric and infant-related data, as well as the data derived from the cord blood volume reduction process and laboratory analyses, against standards (NETCORD and FAHCT, 2001) and predefined internal criteria, acceptable cord blood units were included in international search registries.

7.3 Statistics (I-IV)

Descriptive analysis was used in all studies. In study I, simple linear regression (least squares equations) was used to analyse association between variables, all of which followed either normal or truncated normal distribution.

A method of comparing extreme low and high centiles was used to test the hypothesis of internal associations between infant characteristics and cord blood cellular contents (II-III). Cord blood donor infants were sorted according to $CD34^+$ cell concentration, nucleated cell concentration, relative birth weight and birth weight, and opposite groups were formed from infants belonging to respective opposite centiles (2.5^{th} vs. 97.5^{th} and 5.0^{th} vs. 95.0^{th}) (Figure 4). Non-parametric Mann-Whitney U-testing was used for opposite group comparisons (for HLA data analysis, see below).

Binary decision tree was used to control possible confounding effects such as delivery hospital, mode of delivery and $CD34^+$ cell enumeration protocol (III, IV). A multivariate linear regression model (best subset selection by examination of all possible regressions) was built to explain the independent effect of studied variables on cord blood $CD34^+$ cell (III, IV) and CFU-TOT concentrations (IV).

Data were handled using Microsoft Excel® and Access® software. To ensure coherent and reproducible data handling, Visual Basic for Applications programming was used to create large data tables (II). Statistical anaylses were performed using StatsDirect (StatsDirect Ltd, Sale, Cheshire, UK) software. Two-sided P values <0.05 were considered to be statistically significant.

7.3.1 HLA data analysis (II)

The data were analysed in phases. First, a screening was performed where the frequency of infants possessing an allele group in each of the opposite groups (see above) was compared with the frequency in the Finnish adult population (Siren MK *et al.*, 1996). As the frequency of many HLA alleles in the general population is low, only allele groups with frequencies >3% were considered for screening. If the frequency of infants possessing the allele group in either of the opposite groups exceeded, or, in order not to exclude possibly differing opposite groups due to rounding errors, coincided with the 95% confidence limits of the frequency in the Finnish adult population, the allele group was chosen for second phase, where the allele group frequencies in opposite groups were compared with each other using Fisher's exact test. If differences were observed, a further comparison against the expected frequency in an opposite group based on the frequencies in all cord blood donor infants was performed.

To test whether the Hardy–Weinberg equilibrium applies to the distribution of HLA phenotypes, an exact test using the Markov chain implemented in the Arlequin program package (Schneider et al., 2000) was employed. The frequencies of HLA A and B antigens were not compared between opposite groups, as the absence of deviation from the expected Hardy-Weinberg distribution of the phenotypes could not be shown ($P<0.05$). Instead, no deviation from the expected Hardy-Weinberg distribution of the DR phenotypes could be shown ($P=0.73$).

HLA DRB1 allele group frequencies were defined by the counting method as the percentage of cord blood donor infants carrying the allele group (Svejgaard A and Ryder LP, 1994). No attempt was made to evaluate the possible dose–response effect of HLA homotsygosity.

Eleven HLA DRB1 allele groups (Table 6) were initially screened for differences in allele group frequencies in opposite groups. Seven DRB1 allele groups (DRB1*13 found in comparisons of both relative birth weight and birth weight groups) whose frequency in opposite groups deviated from the 95% confidence interval of population frequency were selected for further comparisons using Fisher's exact test.

An improved Bonferroni procedure (Simes RJ, 1986, Armitage P *et al.*, 2002) was used to calculate the corrected error alfa levels for multiple HLA allele group comparisons. P values were ordered so that $P(1) < P(2) < \ldots < P(n)$, and a corrected error alfa level for each comparison was defined as $alfa_{corr}(x) = x * alfa / n$, where x is the ordinal number of the P-value for the corresponding allele

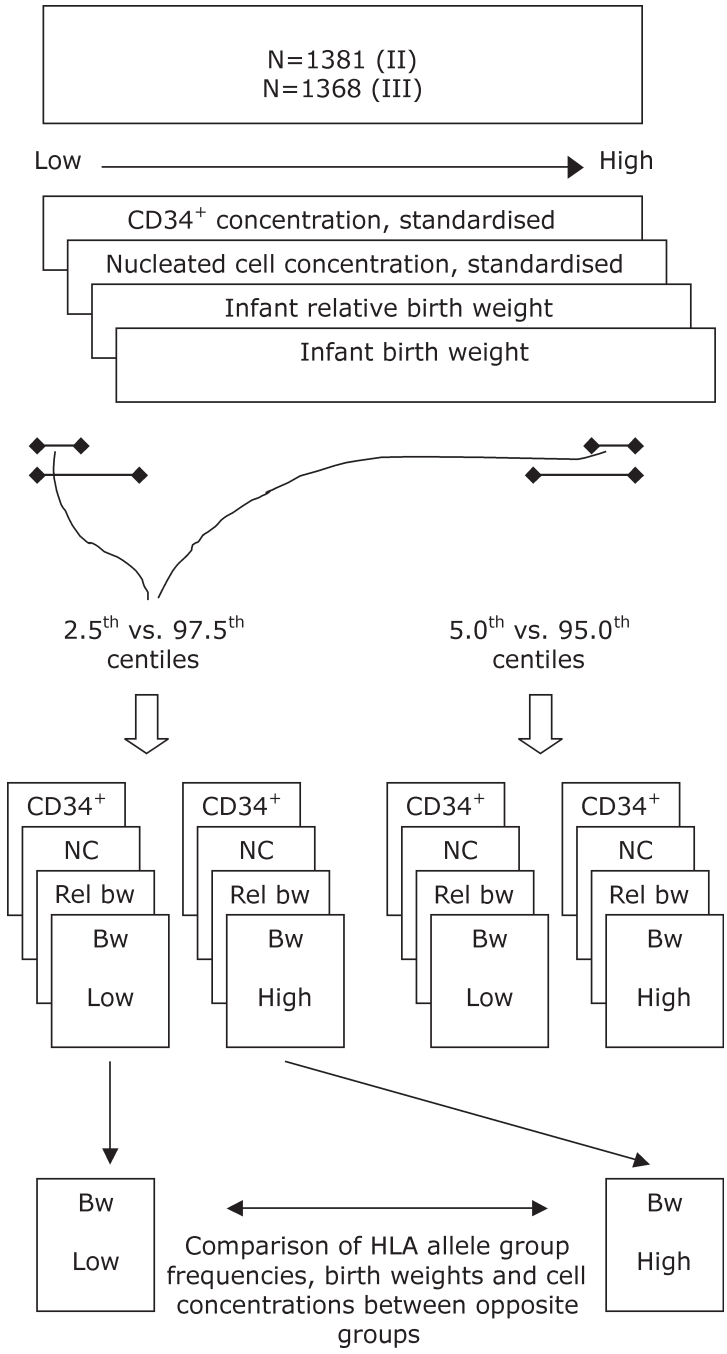

Figure 4 Opposite groups. Cord blood donor infants were ordered according to selected parameters ($CD34^+$ cell concentration, nucleated cell concentration (NC), relative birth weight (Rel bw) and birth weight (Bw)); respective opposite groups based on 2.5^{th} vs. 97.5^{th} as well as 5.0^{th} vs. 95.0^{th} centiles of each parameter were formed for comparisons.

Table 6a HLA A frequencies in cord blood donor infants (N=1381).

HLA antigen	Number of HLA antigens in infants			Population frequency, adjusted [a]	Infants possessing the HLA antigen			Population frequency [c]
	Frequency		95% conf. int.		Frequency (<3% shaded) [b]		95% conf. int.	
	N	%	%	%	N	%	%	%
A01	268	9.7	8.6 - 10.8	9.9	248	18.0	15.9 - 19.9	16.2
A02	923	33.4	31.7 - 35.2	30.0	770	55.8*	53.1 - 58.3	44.7*
A03	726	26.3	24.6 - 27.9	24.2	625	45.3*	42.6 - 47.8	37.1*
A09	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0.0
A23	15	0.5	0.3 - 0.8	0.4	15	1.1	0.5 - 1.6	0.7
A24	227	8.2	7.2 - 9.2	10.1	217	15.7	13.8 - 17.6	16.5
A10	5	0.2	0.0 - 0.3	0.0	5	0.4	0.0 - 0.7	0.1
A25	34	1.2	0.8 - 1.6	1.5	33	2.4	1.6 - 3.2	2.5
A26	47	1.7	1.2 - 2.2	1.9	47	3.4	2.4 - 4.3	3.3
A34	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0.0
A66	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0.0
A11	133	4.8	4.0 - 5.6	4.5	132	9.6	8.0 - 11.1	7.5
A28	125	4.5	3.4 - 5.6	5.2	119	8.6†	7.1 - 10.1	11.3†
A19	11	0.4	0.2 - 0.6	0.1	11	0.8	0.3 - 1.3	0.1
A29	13	0.5	0.2 - 0.7	0.7	13	0.9	0.4 - 1.4	1.2
A30	21	0.8	0.4 - 1.1	0.5	21	1.5	0.9 - 2.1	0.9
A31	99	3.6	2.9 - 4.3	4.7	96	7.0	5.6 - 8.3	7.9
A32	102	3.7	3.0 - 4.4	4.1	99	7.2	5.8 - 8.5	7.0
A33	9	0.3	0.1 - 0.5	0.3	9	0.7	0.2 - 1.1	0.4
A80	1	0.0	0.0 - 0.1		1	0.1	-0.1 - 0.2	
Total	2762	100			2464 [d]			

[a] Adjusted population frequencies counted from the population frequencies reported by Sirén *et al*. (1996).
[b] Only antigens with a frequency >3% were considered for testing.
[c] Population frequencies reported by Sirén *et al*. (1996). The total numbers are a sum of the subtypes and the main type.
* $p < 0.0001$
† $p = 0.0032$
[d] The number of homotsygous infants was 298.

Table 6b HLA B frequencies in cord blood donor infants (N=1381).

HLA antigen	Number of HLA antigens in infants			Population frequency, adjusted [a]	Infants possessing the HLA antigen			Population frequency [c]
	Frequency				Frequency (<3% shaded) [b]			
			95% conf. int.				95% conf. int.	
	N	%	%	%	N	%	%	%
B05	6	0.2	0.0 - 0.4	0.3	6	0.4	0.1 - 0.8	0.5
B51	158	5.7	4.9 - 6.6	4.7	156	11.3	9.6 - 13.0	8.7
B52	5	0.2	0.0 - 0.3	0.1	5	0.4	0.0 - 0.7	0.2
B07	400	14.5	13.2 - 15.8	13.7	375	27.2*	24.8 - 29.5	24.3*
B08	243	8.8	7.7 - 9.9	9.1	232	16.8	14.8 - 18.8	16.5
B12	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0.1
B44	227	8.2	7.2 - 9.2	7.7	217	15.7	13.8 - 17.6	14.1
B45	5	0.2	0.0 - 0.3	0.1	5	0.4	0.0 - 0.7	0.2
B13	84	3.0	2.4 - 3.7	3.3	82	5.9	4.7 - 7.2	6.2
B14	1	0.0	0.0 - 0.1	0.3	1	0.1	-0.1 - 0.2	0.6
B15	30	1.1	0.7 - 1.5	0.1	30	2.2	1.4 - 2.9	0.2
B62	327	11.8	10.6 - 13.0	12.4	302	21.9	19.7 - 24.0	22.1
B63	2	0.1	0.0 - 0.2	0.1	2	0.1	-0.1 - 0.3	0.1
B16	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0.0
B38	21	0.8	0.4 - 1.1	0.9	21	1.5	0.9 - 2.2	1.7
B39	81	2.9	2.3 - 3.6	4.1	80	5.8	4.6 - 7.0	7.6
B17	5	0.2	0.0 - 0.3	0.2	5	0.4	0.0 - 0.7	0.3
B57	29	1.0	0.7 - 1.4	1.3	28	2.0	1.3 - 2.8	2.4
B58	15	0.5	0.3 - 0.8	0.2	15	1.1	0.5 - 1.6	0.3
B18	138	5.0	4.2 - 5.8	5.1	136	9.8	8.3 - 11.4	9.5
B21	0	0.0	0.0 - 0.0	0.0	0	0.0	0.0 - 0.0	0.0
B49	5	0.2	0.0 - 0.3	0.1	5	0.4	0.0 - 0.7	0.3
B50	8	0.3	0.1 - 0.5	0.1	8	0.6	0.2 - 1.0	0.2
B22	4	0.1	0.0 - 0.3	0.4	4	0.3	0.0 - 0.6	0.8
B55	13	0.5	0.2 - 0.7	0.6	13	0.9	0.4 - 1.5	1.1
B56	46	1.7	1.2 - 2.1	1.3	44	3.2	2.3 - 4.1	2.4
B27	206	7.5	6.5 - 8.4	7.9	193	14.0	12.1 - 15.8	14.4
B35	398	14.4	13.1 - 15.7	13.8	362	26.2	23.9 - 28.5	24.4
B37	17	0.6	0.3 - 0.9	0.8	17	1.2	0.6 - 1.8	1.6
B40	16	0.6	0.3 - 0.9	0.8	16	1.2	0.6 - 1.7	1.5
B60	145	5.2	4.4 - 6.1	6.1	138	10.0	8.4 - 11.6	11.3
B61	72	2.6	2.0 - 3.2	1.8	72	5.2	4.0 - 6.4	3.5
B41	21	0.8	0.4 - 1.1	0.5	21	1.5	0.9 - 2.2	0.9
B42	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0
B46	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0
B47	15	0.5	0.3 - 0.8	0.7	15	1.1	0.5 - 1.6	1.4
B48	2	0.1	0.0 - 0.2	0.1	2	0.1	-0.1 - 0.3	0.2
B53	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0
B64	1	0.0	0.0 - 0.1	–	1	0.1	-0.1 - 0.2	–
B65	7	0.3	0.1 - 0.4	–	7	0.5	0.1 - 0.9	–
B67	2	0.1	0.0 - 0.2	–	2	0.1	-0.1 - 0.3	–
B70	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0
B71	1	0.0	0.0 - 0.1	0.0	1	0.1	-0.1 - 0.2	0
Total	2762	100			2625 [d]			

[a] Adjusted population frequencies counted from the population frequencies reported by Sirén *et al*. (1996.
[b] Only antigens with a frequency >3% were considered for testing.
[c] Population frequencies reported by Sirén *et al*. (1996). The total numbers are a sum of the subtypes and the main type.
[*] p=0.0215
[d] The number of homotsygous infants was n=137.

Table 6c HLA DRB1 frequencies in cord blood donor infants (N=1263). HLA DRB1 result was not available from all infants, as the typing was performed in a later phase of the cord blood banking process.

HLA allele group	Number of HLA allele groups in infants			Population frequency, adjusted [a]	Infants possessing the HLA allele group			Population frequency [c]
	Frequency				Frequency (<3% shaded) [b]			
			95% conf. int.				95% conf. int.	
	N	%	%	%	N	%	%	%
DRB1*01	486	19.2	17.7 - 20.8	17.5	436	34.5	31.9 - 37.1	29.7
DRB1*03	248	9.8	8.7 - 11.0	9.4	234	18.5	16.4 - 20.7	17.1
DRB1*04	373	14.8	13.4 - 16.1	14.2	344	27.2	24.8 - 29.7	22.8
DRB1*07	153	6.1	5.1 - 7.0	5.9	146	11.6	9.8 - 13.3	10.7
DRB1*08	217	8.6	7.5 - 9.7	9.6	206	16.3	14.3 - 18.3	17.7
DRB1*09	87	3.4	2.7 - 4.2	3.1	86	6.8	5.4 - 8.2	5.6
DRB1*10	25	1.0	0.6 - 1.4	0.9	25	2.0	1.2 - 2.7	1.7
DRB1*11	103	4.1	3.3 - 4.8	3.9	98	7.8	6.3 - 9.2	7.1
DRB1*12	68	2.7	2.1 - 3.3	2.1	68	5.4	4.1 - 6.6	3.8
DRB1*13	342	13.5	12.2 - 14.9	10.9	319	25.3	22.9 - 27.7	19.2
DRB1*14	49	1.9	1.4 - 2.5	1.2	49	3.9	2.8 - 4.9	2.3
DRB1*15	351	13.9	12.5 - 15.2	14.2	320	25.3	22.9 - 27.7	24.5
DRB1*16	24	1.0	0.6 - 1.3	0.2	24	1.9	1.1 - 2.7	0.3
Total	2526	100			2355 [d]			

[a] Adjusted population frequencies counted from the population frequencies reported by Sirén *et al*. (1996).
[b] Only allele groups with a frequency >3% were considered for testing.
[c] Population frequencies reported by Sirén *et al*. (1996). Total numbers are sums of the subtypes and the main type. As DRB1 results in our study were obtained using molecular methods instead of serological typing used by Sirén, DRB1 frequencies in infants were not compared with population frequencies.
[d] The number of homotsygous infants was n=171.

group in the opposite group pair tested, alfa the original level of type alfa error (0.05), and n the number of comparisons made. The number of HLA allele group frequency pairs selected for further testing in screening was used to correct the alfa error level for multiple comparisons in Fisher's exact test.

7.3.2 Cord blood donor infants (II-IV)

Newborn infant data were obtained from hospital records. In addition to birth weight, which was measured immediately after birth as part of normal neonatal care, gestational age, mode of delivery and durations of the different stages of vaginal delivery were recorded.

Relative birth weight
Relative birth weight was expressed as birth weight z-score using the z distribution (Oken E *et al.*, 2003) and a Finnish reference population (Pihkala J *et al.*, 1989). Macrosomia was defined as birth weight z-score >2 SD. No attempt was made to exclude macrosomic infants from the study.

7.4 Contribution of the researcher

Pekka Aroviita has participated in applying all cord blood banking processes including recruiting, collection, processing, testing, storing and release of unrelated cord blood units. He has planned together with other collaborators the design of all studies. Pekka Aroviita has analysed the data and was the first author of all studies.

8 RESULTS

8.1 Cord blood banking process (I)

8.1.1 Recruiting the donor mothers

Methods used by the cord blood banking program for selecting units from healthy mother baby pairs

The success of the preselection process performed by cord blood bank midwives in selecting cord blood units from healthy mother baby pairs was tested by sending a health questionnaire to well baby clinics six months after the birth between January 1999 and September 1999. Ninety-five % (172/181) of the baby health questionnaires were returned in two months, and 166 infants were reported healthy (Aroviita P *et al.*, 2000 [abstract]). In three cases the reported diagnoses were coarctation of the aorta, infantile spasm and facial paresis. In the three other cases only symptoms and signs (vomiting, hearing defect, non-specific symptoms) but no definite diagnoses were available from the well baby clinic. The non-laboratory selection process was based on self-exclusion of the mother prior to registration, on obstetric records before and after collection, and on health questionnaires and interview of the mother. Only 3% of the babies were not reported healthy six months from birth, which may be used as a quality indicator of the selection process.

8.1.2 Collection of cord blood

Collection time

Cord blood bank midwives received the placenta immediately after its delivery. The median collection time of 2min 17s (range 1min 10s – 6min 2s) of the cord blood units frozen for further evaluation was uniformly short, as compared with conventional blood banking.

Collected cord blood volume

The median cord blood volume collected for processing was 69 ml (range 28-116, mean 72, SD 20). Five cord blood units were processed despite the blood volume collected being less than 40 ml (28-37); these units were not accepted for clinical use. The practice of measuring the nucleated cell content of the low volume (40-50 ml) units before further processing proved that only 10% (10/103) of these units contained a minimum target total nucleated cell number of 800 $x10^6$. The process was revised so that the nucleated cell content of all units was measured before the decison for further processing.

Maintaining a uniform temperature profile of cord blood during transportation between collection hospital and processing laboratory

To validate the cord blood transport procedure a sample of 107 shipments

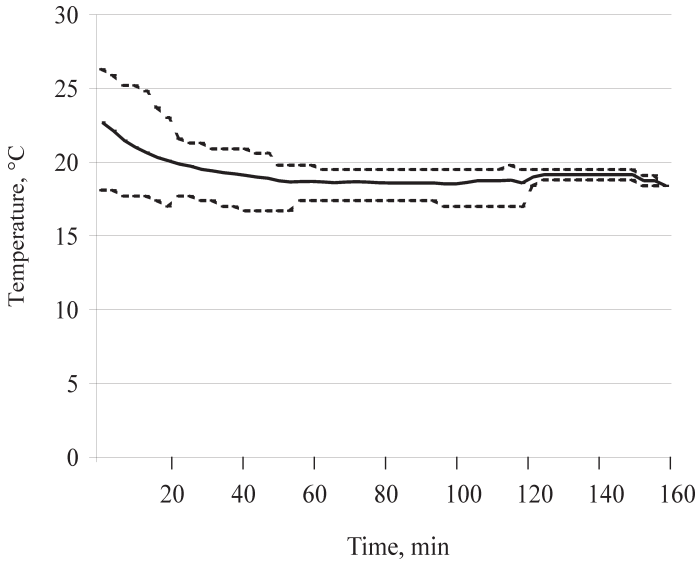

Figure 5 Temperature of cord blood during transportation between collection hospital and processing laboratory (N=107, continuous temperature monitoring every 3 minutes mean solid line, min/max broken lines). The median temperature at the collection site was +24°C (range 23–26). The temperature after 3 min of transportation was +23°C (range 18-26) and the profile reached the level of +18°C - +19°C after one hour of transportation. Due to variation in the actual transport times the number of observations is smaller in longer transportation time categories (median transportation time 36 min (range 9-156)). Aroviita et al. Vox Sanguinis 2003;84(3):219-27. Copyright of Blackwell Publishing, used with permission.

transported between November and August (median monthly temperatures in Helsinki, Finland +1.4°C — -5.7°C — +16°C) was analysed in detail (Figure 5).

The median temperature of the shipments on arrival at the laboratory was +19°C (range 17-22, mean 19, SD 0.9) (standard thermometer) and +19°C (range 17-23, mean 19, SD 1.1) (continuous monitoring). The minimum temperature during transportation was never less than +17°C.

Effect of storage time on nucleated cell and haematopoietic progenitor cell content of cord blood

The total nucleated cell number of collected cord blood or of cord blood units before cryopreservation did not differ between the storage time groups of <8h and 8-25h, and neither were the nucleated cell yields different (Aroviita P *et al.*, 2002 [abstract]). The median CFU-GM concentration of collected cord blood was lower in the group stored between eight and 25 hours (N=68, 7/µl, range [0.6-31]) than in the group stored less than eight hours (N=20, 11/µl [3-40], P<0.04). There were no differences in median CFU-GEMM (<8h: 10/µl [0-24] vs. 8-25h: 12/µl [0.6-47], P=n.s.) or CFU-TOT (<8h: 19/µl [3-56] vs. 8-25h: 19/µl [2-73], P=n.s.) concentrations between the storage time groups.

8.1.3 Cell yield and pre- versus post-processing correlations

Nucleated cells in cord blood units

The nucleated cell (NC) yield of the volume reduction process was 65% (range 34-83%, mean 64%, SD 7%). The two units (0.2%) in which the nucleated cell yield was less than 40% were not accepted for search registries. The correlation of the total nucleated cell numbers before and after volume reduction was strong (equation: $NC_{total\_after} = 0.61 \times NC_{total\_before} + 2 \times 10^7$, $r=0.96$, $R^2=0.92$, $P<0.0001$), suggesting a reliable and reproducible process.

$CD34^+$ cells in cord blood units

The $CD34^+$ cell yield of the volume reduction process was 74% (range 6-166%, mean 75, SD 15). The correlation of the total $CD34^+$ cell numbers before and after volume reduction was clear (equation: $CD34_{total\_after} = 0.70 \times CD34_{total\_before} + 9 \times 10^4$, $r=0.96$, $R^2=0.92$, $P<0.0001$). There was also a correlation between the $CD34^+$ cell and nucleated cell concentration in cord blood collected (equation: $CD34_{conc\_coll} = 3.2 \times NC_{conc\_coll} - 4.5$, $r=0.54$, $R^2=0.29$, $P<0.0001$) and between the total $CD34^+$ cell and nucleated cell numbers in cord blood units before cryopreservation (equation: $CD34_{total\_unit} = 0.0038 \times NC_{total\_unit} - 360000$, $r=0.64$, $R^2=0.41$, $P<0.0001$).

Haematopoietic progenitor cells in cord blood units

The CFU results were available from 88 cord blood collections as a result of random sampling (15% of cryopreserved units) for statistical process control. Of these, 68 (15%) were from 447 units whose processing was started the next day after collection (stored between eight and 25 hours before processing) and 20 (15%) from 130 units stored less than eight hours before processing. No CFU results were available from any of the units stored between 25 and 48 hours before processing (N=11). There were no differences regarding the volume collected or the concentrations of nucleated cells, $CD34^+$ cells or platelets in this group (N=88) when compared with the whole material (N=588) (data not shown).

The correlations between the total nucleated cell and CFU-TOT numbers and between the total $CD34^+$ cell and CFU-TOT numbers in collected cord blood were good (equation: $CFU\text{-}TOT_{total\_coll} = 0.0018 \times NC_{total\_coll} - 210000$, $r=0.69$, $R^2=0.47$, $P<0.0001$ and equation: $CFU\text{-}TOT_{total\_coll} = 0.57 \times CD34_{total\_coll} + 220000$, $r=0.87$, $R^2=0.75$, $P<0.0001$, respectively). Also, correlation between the total $CD34^+$ cell number in cord blood before cryopreservation and the total CFU-TOT number in collected cord blood was strong (equation: $CD34_{total\_unit} = 0{,}99 \times CFU\text{-}TOT_{total\_coll} + 220000$, $r=0.89$, $R^2=0.79$, $P<0.0001$).

Erythrocytes in cord blood units

The removal of erythrocytes in the volume reduction process was 73% (range 34-89, mean 70, SD 11).

Platelets in cord blood units

The removal of platelets in the volume reduction process was 68% (range 34-80, mean 68, SD 6).

Table 7 Comparison of the frequency of selected HLA allele groups between opposite groups formed by infant as well as cord blood characteristics.

Opposite groups formed by	HLA	Inf [a]	Exp[b]	Centiles			Fisher	
				5.0th		95.0th	P	*alfa* corr.[c]
Relative birth weight	DRB1*13	119	15	7[d]	vs.	19	0.015	*0.007* (1st of 7)
of which[e]	*1301			6		11	0.362	
	*1302			0		6	0.137	
	*1303			1		1	0.474	
Birth weight	DRB1*07	121	7	7	vs.	12	0.221	*0.036* (5th of 7)
	DRB1*08	121	10	13	vs.	4	0.034	*0.014* (2nd of 7)
	DRB1*13	121	15	11	vs.	20	0.063	*0.029* (4th of 7)
Nucleated cells[f]	DRB1*14	120	2	7	vs.	3	0.325	*0.05* (7th of 7)
$CD34^+$ cells[f]	DRB1*03	113	10	15	vs.	10	0.258	*0.043* (6th of 7)
	DRB1*12	113	3	0	vs.	5	0.057	*0.021* (3rd of 7)

[a] Inf, the total number of infants with HLA DRB1 result in the opposite groups; DRB1 result was available from 1263 of 1381 infants, as the typing was performed in a later phase of the cord blood banking process.
[b] Exp, the expected number of infants positive for the HLA allele in each opposite group based on the frequency in study material and on the size of the opposite group
[c] Corrected error *alfa* level for 7 multiple comparisons. In study II, the correction was performed using the total number of allele groups screened (N=11).
[d] The observed number of infants positive for the HLA allele in the respective opposite group (5.0^{th} or 95^{th} centile).
[e] High resolution HLA DRB1*13 typing was performed from 7 (5.0^{th} centile) and 18 (95.0^{th} centile) infants.
[f] Standardised concentration, where the concentration was corrected for dilution effect.

8.2 HLA and blood groups

8.2.1 Birth weight and HLA DRB1 (II)

HLA A and B antigen frequencies were not compared with published frequencies in Finnish population (Siren MK *et al.*, 1996), as the frequencies in the present study deviated from the expected Hardy-Weinberg distribution.

The frequency of HLA DRB1*13 positive infants in the study material was 25.3% (95%CI 22.9-27.7) (Table 6). HLA DRB1*13 was found more frequently among infants with the highest birth weights compared to those with the lowest birth weights. The association was statistically significant when relative birth weights were analysed (P=0.015) (Table 7), although it remained non-significant after correction for multiple comparisons. The same trend was also observed without correcting the birth weight for varying gestational ages (P=0.063). Over-expression of DRB1*13 in infants of higher relative birth weight was evident also in the 90.0^{th} vs. 10.0^{th} and 97.5^{th} vs. 2.5^{th} centile opposite groups (data not shown). The HLA DRB1*13 allele (*1301-03) frequencies in the opposite groups formed by relative birth weight did not differ.

Table 8 Association between blood groups and cord blood cell concentration (N=1999).

Blood group	N	%	Nucleated cells (*10^9/l) median	range	$CD34^+$ cells (/µl) median	range
O	641	32	13.5	6.7 - 38.1	32.1	1.9 - 237
A	864	43	13.7	6.0 - 32.7	30.9	5.4 - 174
B	342	17	13.5	6.0 - 32.7	30.2	4.1 - 208
AB	152	8	13.7	6.6 - 26.8	29.8	5.7 - 168
			0.99*		0.68*	
Rh(D) pos	1755	88	13.5	6.0 - 38.1	31.1	1.9 - 237
Rh(D) neg	244	12	13.8	6.8 - 30.3	31.7	6.6 - 208
			0.16*		0.31*	
Kell pos	95	5	14.3	7.4 - 23.3	29.3	5.7 - 187
Kell neg	1904	95	13.6	6.0 - 38.1	31.2	1.9 - 237
			0.68*		0.98*	

* Kruskal-Wallis test, P value; a non-parametric method for comparing several independent random samples

When the birth weights (or relative birth weights) of all infants positive (N=319) or negative (N=944) for the DRB1*13 were compared, the association was evident: the median birth weight (or relative birth weight) of HLA DRB1*13 positive infants was higher than that of HLA DRB1*13 negative infants (3690g vs 3650g, P=0.044; 0.186 vs 0.083, P=0.025; respectively).

The frequency of HLA DRB1*08 positive infants in the study material was 16.3% (95%CI 14.3-18.3). HLA DRB1*08 was found more frequently than expected among infants with the lowest birth weights (P=0.034, non-significant after correction for multiple comparisons). However, the difference between the median birth weights of HLA DRB1*08 positive (N=206) and negative (N=1057) infants was not statistically significant (median 3630 g vs 3670 g, P=0.566).

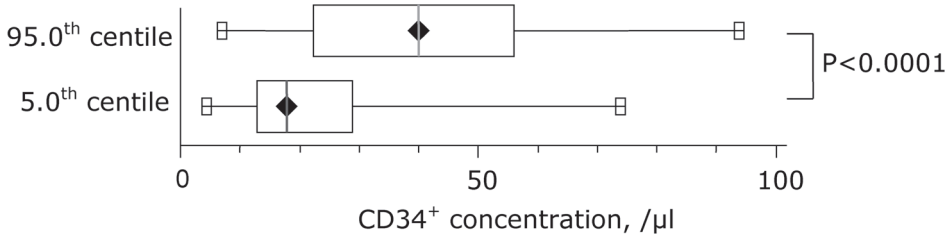

Figure 6 Infants in the highest 5.0% centile of relative birth weight had statistically significantly higher $CD34^+$ cell concentrations than infants in the lowest 5% centile (N=66 in both). Extreme values were removed (N=2 in both). Aroviita et al. Acta paediatrica 2004;93(10):1323-9. Copyright of Taylor & Francis, used with permission.

8.2.2 Blood groups (unpublished results)

The frequencies of the ABO and Rh blood groups were similar in 2490 cord blood collections (data not shown), in 1999 cord blood units accepted for clinical use (Table 8) and in Blood Service's entire collection of blood donors (Nevanlinna HR, 1972). In the 1999 cord blood units accepted for clinical use, no differences in nucleated cell or $CD34^+$ cell concentrations between A, B, AB and O, Rh(D) positive versus negative or Kell positive versus negative blood groups were observed (Table 8).

8.3 Cord blood transplant (I, III, IV)

8.3.1 Associations between infant and cord blood characteristics

Birth weight (III)

Nucleated cells. The correlation between birth weight and cord blood nucleated cell concentration was statistically clearly significant, although the R squared was low. This indicates that other factors also influence the cell concentration. Cord blood nucleated cell concentration did not correlate with relative birth weight or with placental weight.

$CD34^+$ cells. The correlation between birth weight and cord blood $CD34^+$ cell concentration was statistically clearly significant. Again, low R squared indicates that other factors also influence the concentration. The correlations between relative birth weight and cord blood $CD34^+$ cell concentration and between placental weight and $CD34^+$ cell concentration were similarly statistically significant with low R^2 values.

Infants with the highest birth weight or with highest relative birth weight (95.0^{th} centile) had roughly double the concentration of cord blood $CD34^+$ cells as compared to those of infants in the lowest birth weight groups (5.0^{th} centile) (median 38 vs 22/μl, and 45 vs 20/μl, respectively, $P<0.0001$ in both). Correcting the opposite groups for extreme $CD34^+$ cell concentrations by removing the lowest and highest values (N=68 —> N=66) did not diminish the level of statistical significance (Figure 6).

In multivariate linear regression analysis using a sample from one collection hospital where all $CD34^+$ cell analyses were performed using the ProCOUNT protocol (N=694), the correlation between birth weight and $CD34^+$ cell concentration was statistically highly significant after adjusting for the number of deliveries, length of gestation, mode of delivery, sex, and nucleated cell concentration (III) (Table 9).

Gender (IV)

Nucleated cell concentrations. The median cord blood nucleated cell concentration of female infants was higher than that of male infants (13.9 vs. 13.3 $*10^9$/l, respectively, P=0.0001). This difference was mainly due to the higher neutrophil concentration of female infants compared to that of male infants, as illustrated in the cumulative frequency graphs (Figure 7). The nucleated cell concentration in infants delivered vaginally was higher than that in infants

Table 9 Multivariate linear regression analyses (III, IV).

Predictor variables	Partial regression coefficients				P value
Analysis on the factors possibly associated with infant birth weight (III)[a]					
	Intercept	b0	-3572	grams	P<0.0001
Number of births	Continuous variable	b1	+85.6		P<0.0001
Length of gestation (days)	Continuous variable	b2	+25.4		P<0.0001
Mode of delivery	Caesarean section (1)[b] vs. Vaginal (0)	b3	+28.8		P=0.40
Gender	Male (1) vs. Female (0)	b4	+139		P<0.0001
Nucleated cell concentration (*10^9/litre)	Continuous variable	b5	-3.2		P=0.51
$CD34^+$ cell concentration (/µl)	Continuous variable	b6	+3.0		P<0.0001
Analysis on the factors possibly associated with cord blood $CD34^+$ cell concentration (IV)[a]					
	Intercept	b0	41.6	/µl	<0.0001
Mode of delivery	Caesarean section (1) vs. Vaginal (0)	b1	-0.26		0.89
Relative birth weight	Continuous variable	b2	4.08		<0.0001
Gender	Male (1) vs. Female (0)	b3	3.84		0.049
In the respective model for CFU-TOT concentration, there was a trend of higher CFU-TOT concentrations in male infants (N=321, partial regression coefficient "Gender Male" = 3.466, P=0.07)					

[a] Collections from one collection hospital and $CD34^+$ cell analyses performed using the ProCOUNT protocol (study III, N=694; study IV, N=637).
[b] "1" and "0", e.g., the expected effect of the predictor variable "Caesarean section (1)" on the analysed variable "Birth weight" is calculated: 1 * +28.8 = +28.8 (grams)

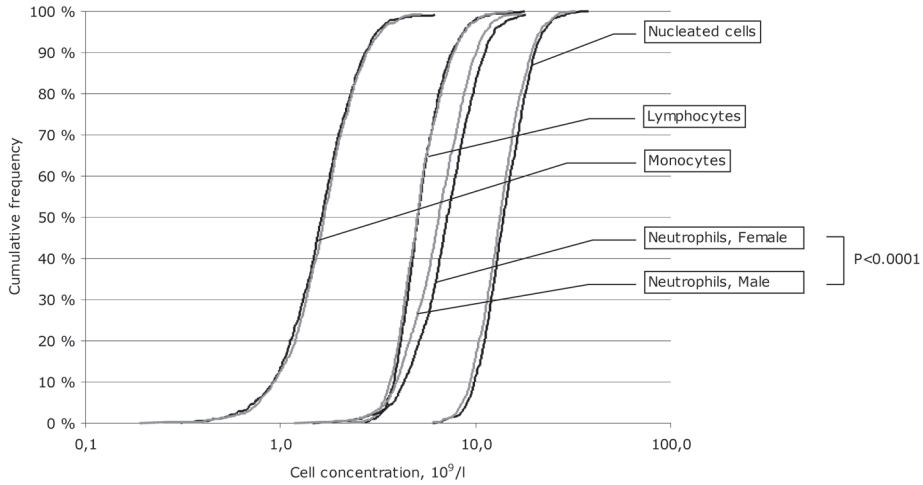

Figure 7 Cumulative frequency of cord blood nucleated cell concentrations of male (N=1059) and female (N=940) infants. Any given point in these graphs represents the percentage of infants (y axis) that have at least the respective cell concentration (x axis).

delivered by caesarean section. This difference consisted equally of all nucleated cell subgroup concentrations. Female infants from both caesarean section and vaginal deliveries had higher nucleated cell concentrations than the respective male infants, the difference in both modes of delivery consisting of the higher neutrophil concentration of female infants.

CD34⁺ cell concentrations. The median cord blood CD34⁺ cell concentration of all male infants (N=1059, 31.8/μl, range [1.9-208]) was higher than that of female infants (N=940, 30.2/μl [5.4-237], P=0.03). Although the difference in absolute numbers appears small, it was 5.3%. The difference was statistically significant also in a sample from one collection hospital with all CD34⁺ cell analyses performed with the ProCOUNT protocol (Figure 8).

Haematopoietic progenitor cell concentrations. The cord blood CFU results were available from N=321 of N=1999 infants (16%). The median CFU-TOT concentration of male infants (N=154, 21.9/μl, range [3.4-136]) tended to be higher than that of the female infants (N=167, 19.8/μl [0.5-94], P=0.10). Interestingly, the male infants had higher median CFU-GEMM concentration than female infants (11.1/μl [0.0-47] vs. 9.9/μl [0.0-53], respectively, P=0.03), while there was no difference in median CFU-GM concentrations (9.1/μl [1.4-56] vs. 8.0/μl [0.6-53], respectively, P=0.23).

*Multivariate linear regression analysis.*The difference in cord blood haematopoietic progenitor cell concentrations between male and female infants was also analysed using multivariate linear regression in a sample from one collection hospital where all CD34⁺ cell analyses were performed using the ProCOUNT protocol (N=637). In this model the male gender was associated statistically

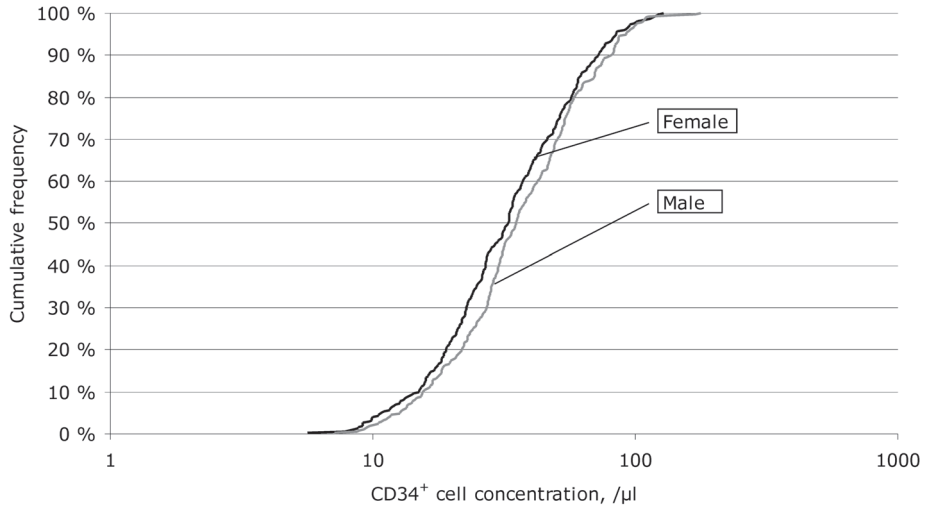

Figure 8 Cumulative frequency of CD34⁺ cell concentrations of male (N=336) and female (N=301) infants. All collections were from one collection hospital and the CD34⁺ cell analyses were performed using the ProCOUNT protocol. Male: median 35.1 /µl, range [7.2-177], mean 42.3, SD 26; Female: 32.9 /µl, [5.6-128], 38.5, 24, Mann-Whitney U-test P=0.04.

significantly with higher CD34⁺ cell concentration after adjusting for the mode of delivery and the relative birth weight (IV) (Table 9).

Gestational age (III)

Gestational age of term infants did not correlate with cord blood CD34⁺ cell concentration (Figure 9), in contrast to the positive correlation with the nucleated cell concentration. This explains the previously reported negative correlation between the percentage of CD34⁺ cells of nucleated cells and gestational age which was also apparent in this study.

Mode of delivery (I, III-IV)

Volume and nucleated cell content of cord blood (I). The median cord blood volumes collected from vaginal deliveries (N=326) and caesarean sections (N=262) were 61 ml (range 28-116, mean 66, SD 18) and 80 ml (range 42-114, mean 80, SD 19), respectively (P<0.0001). There were no differences in the total collected nucleated cell numbers in vaginal and caesarean section delivery groups (96.3 $x10^7$ (range 34.7-250, mean 104, SD 40) and 92.8 $x10^7$ (range 35.4-320, mean 102, SD 42), respectively, P=n.s.), as the nucleated cell concentrations of cord blood collected from vaginal and caesarean section deliveries differed in the opposite direction compared with the collection volumes (11.1 $x10^9$/l (range 5.1-22, mean 11.5, SD 3.2) and 9.1 $x10^9$/l (range 4.4-29, mean 9.7, SD 3.0), respectively (P<0.0001)).

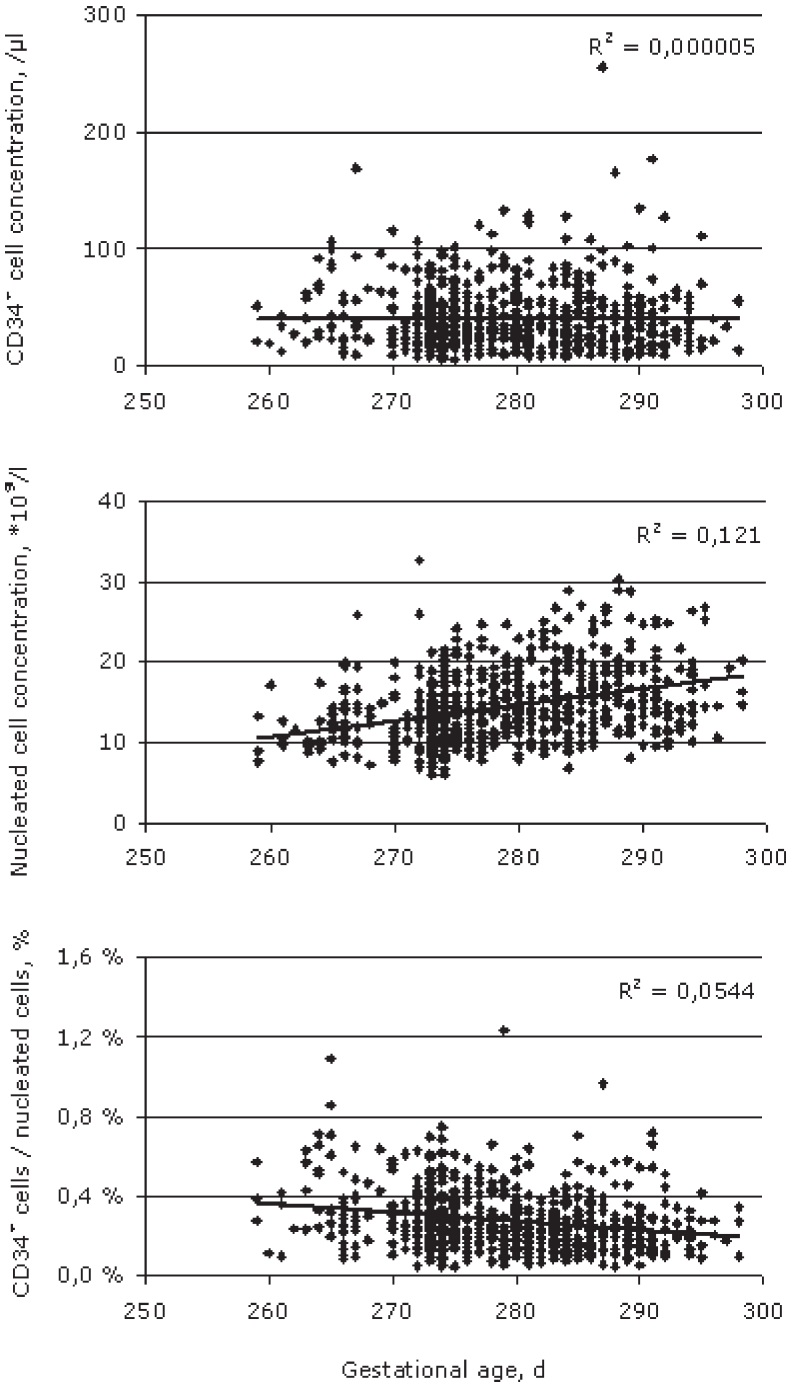

Figure 9 Correlations between gestational age and cord blood cell concentrations. CD34⁺ cell concentration: r=-0.002, P=0.95; nucleated cell concentration: r=0.35, P<0.0001; CD34⁺ cells / nucleated cells: r=-0.23, P<0.0001. Cord blood collections were from one collection hospital and CD34⁺ cell analyses were performed with ProCOUNT protocol (N=694). Aroviita et al. Acta paediatrica 2004;93(10):1323-9. Copyright of Taylor & Francis, used with permission.

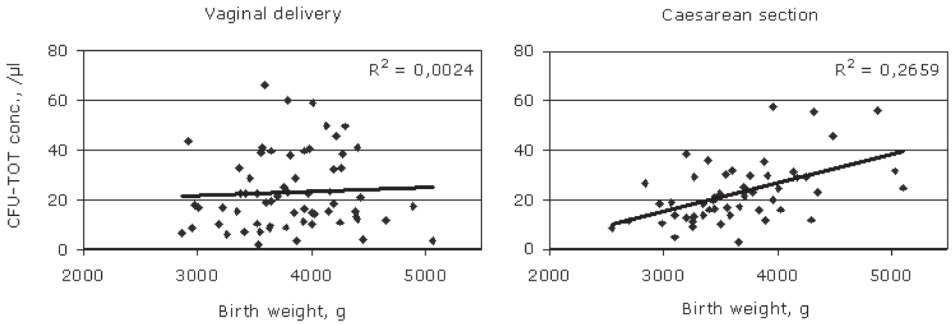

Figure 10 Correlations between birth weight and CFU-TOT concentration in vaginal (N=63) and caesarean section deliveries (N=51). Vaginal delivery: r=0.05, P=0.70; Caesarean section: r=0.52, P=0.0001. Modified from Aroviita et al. Acta paediatrica 2004;93(10):1323-9.

CD34⁺ cell concentration (IV). In the whole material of 1999 infants, there was no difference between the median CD34⁺ cell concentrations from caesarean section (N=916, median 30.8 /µl, range [1.9-181], mean 36.7, SD 24) and vaginal deliveries (N=1083, median 31.8 /µl, [4.1-237], 38.5, 27, P=0.24).
Birth weight and CFU-TOT concentration (III). Vaginal and caesarean section deliveries were analysed separately to discover whether possible associations

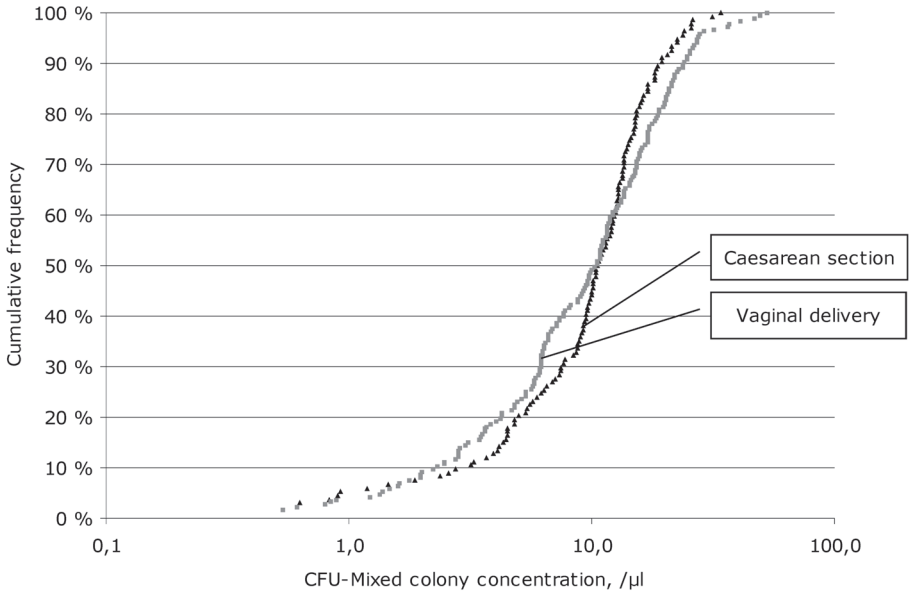

Figure 11 Cumulative frequency of cord blood CFU-Mixed colony concentrations in caesarean section (N=134) and vaginal (N=187) deliveries.

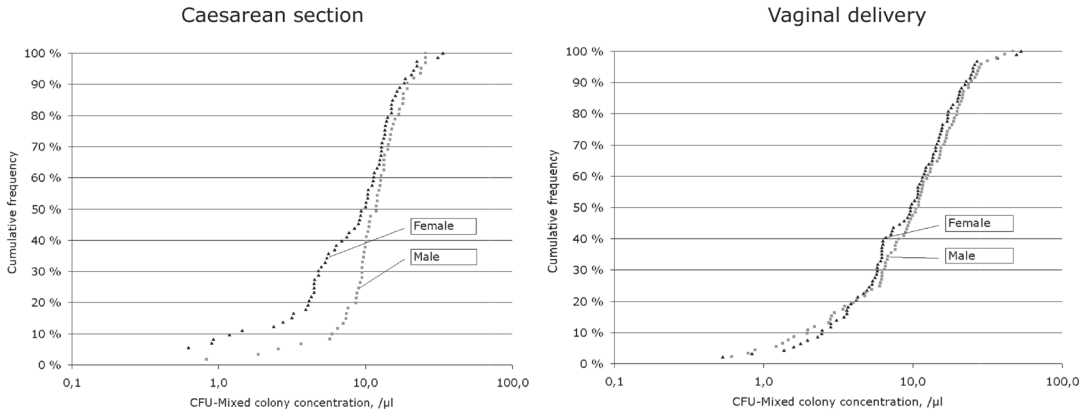

Figure 12 Cumulative frequency of cord blood CFU-Mixed colony concentrations of male (N=61) and female (N=73) infants in caesarean section deliveries and of male (N=93) and female (N=94) infants in vaginal deliveries. Modified from Aroviita et al. Transfusion 2005;45 (In Press).

between birth weight and cord blood CFU-TOT concentrations might vary according to mode of delivery. An association between birth weight and CFU-TOT concentration of cord blood was clear in infants delivered by caesarean section, while in infants delivered vaginally a statistically significant association was not observed (Figure 10).

Differences in the cumulative distributions of the CFU concentration according to mode of delivery and gender (IV). The cumulative frequency distributions of the CFU concentrations of caesarean section deliveries were clearly different from those of vaginal deliveries, the difference being most apparent in CFU-Mixed (equivalent to CFU-GEMM) concentrations (Figure 11), although no differences in CFU-TOT and CFU subgroup concentrations according to the delivery mode were detected in Mann-Whitney U-testing (P=0.55-0.98). The distribution of the CFU-Mixed concentrations of cord blood collected from caesarean section deliveries was more uniform than the distribution of the CFU-Mixed concentrations of cord blood collected from vaginal deliveries (25th-75th percentiles in caesarean section and vaginal deliveries: 6.4-14.4 vs. 5.4-17.0 /µl, respectively).

When caesarean section and vaginal deliveries were analysed separately, the difference between CFU concentrations of male and female infants was again most apparent in CFU-Mixed concentrations. The CFU-Mixed concentrations of male infants were statistically significantly higher than those of female infants in caesarean section deliveries (median 12.0/µl, range [0.8-26] vs. 10.0/µl [0.0-34], respectively, P=0.01) compared with CFU-Mixed concentrations in vaginal deliveries (11.0/µl [0.0-47] vs. 9.8/µl [0.0-53], respectively, P=0.45) (Figure 12).

Collected cord blood volume (unpublished results)

Collected volume and birth weight. Collected cord blood volume (N=1999) had

a statistically significant correlation with birth weight (r=0.27, 95%CI for r = 0.232 - 0.313, R^2=0.07, P<0.0001). The correlation was also clear between collected volume and relative birth weight (r=0.32, 95%CI for r = 0.277 - 0.356, R^2=0.10, P<0.0001). Correlation between collected cord blood volume and placental weight (r=0.39, 95%CI for r = 0.353 - 0.427, R^2=0.15, P<0.0001) was statistically significantly higher than the correlation between collected volume and birth weight based on comparison of the 95% confidence intervals for r. Placental weight had the strongest correlation (P<0.0001) with the collected cord blood volume also in a multivariate linear regression analysis after adjusting for the birth weight (P=0.23), relative birth weight (P=0.98), number of births (P=0.51) and gestational age (P<0.0001).

Collected volume and cell concentration. Correlations of collected cord blood volume (N=1999) with nucleated cell (r=-0.14, 95%CI for r = -0.18 - -0.10, R^2=0.02, P<0.0001) as well as with $CD34^+$ cell concentration (r=0.10, 95%CI for r = 0.06 – 0.15, R^2= 0.01, P<0.0001) were weak. No correlation of collected cord blood volume (N=321) with CFU concentration was observed (r=0.07, 95%CI for r = -0.04 – 0.17, R^2=0.00, P=0.24).

8.3.2 Haematopoietic cell content (I, III, IV)

Collected cord blood volumes as well as cord blood $CD34^+$ and nucleated cell concentrations of study III (N=1368) were comparable to those reported in studies I (N=588) and IV (N=1999). Cell contents in collected cord blood and in cord blood units after volume reduction (i.e. before cryopreservation) are presented in Table 10, Figure 13 and Figure 14.

Table 10 Cell content of cord blood collections and units (partly unpublished results).

Cell concentrations in collected cord blood and in cord blood units after volume reduction

	N	Median	Range	Mean	SD	
Collected cord blood[a]						
Nucleated cells	1999	13.6	6.0-38.1	14.3	4	$*10^9$/l
Lymphocytes	1998	38.4	13.7-75.5	38.7	8	%
Monocytes	1985	12.0	1.9-26.6	12.2	3	%
MNC	1998	50.6	23.2-82.3	50.8	8	%
Neutrophils	1985	49.4	17.7-76.8	49.1	8	%
Lymphocytes	1998	5.0	2.3-17.8	5.4	2	$*10^9$/l
Monocytes	1985	1.7	0.2-6.2	1.8	1	$*10^9$/l
MNC	1998	6.7	3.4-24.0	7.2	2	$*10^9$/l
Neutrophils	1985	6.8	1.2-18.0	7.1	3	$*10^9$/l
Erythrocytes	1999	4.4	3.2-8.8	4.5	0.5	$*10^9$/l
Platelets	1999	302	120-552	303	57	$*10^9$/l
After volume reduction						
Nucleated cells	1998	32.8	10.7-110	35.6	14	$*10^9$/l
Lymphocytes	1996	33.9	13.1-80.0	34.6	8	%
Monocytes	1917	11.9	2.6-26.9	12.0	4	%
MNC	1996	45.7	17.3-80.0	46.1	9	%
Neutrophils	1917	54.0	13.3-82.7	53.5	9	%
Lymphocytes	1996	11.2	3.6-37.7	11.9	4	$*10^9$/l
Monocytes	1917	3.9	0.5-16.4	4.2	2	$*10^9$/l
MNC	1996	15.0	4.8-47.3	15.9	6	$*10^9$/l
Neutrophils	1917	17.3	2.6-67.1	19.3	9	$*10^9$/l
Erythrocytes	1996	3.2	1.2-5.5	3.2	0.8	$*10^9$/l
Platelets	1998	345	75-750	352	85	$*10^9$/l

[a] Standardised concentration, see Methods.

Table 10 (cont.)

Total cell counts in collected cord blood and in cord blood units after volume reduction[a]

	N	Median	Range	Mean	SD	
Collected cord blood						
Nucleated cells	1999	101.4	32.7-358	109.9	42	$*10^7$
Lymphocytes	1998	38.8	11.2-356	42.1	19	$*10^7$
Monocytes	1984	12.3	1.9-51.5	13.4	6	$*10^7$
MNC[b]	1997	51.3	15.4-372	55.5	23	$*10^7$
Neutrophils	1985	49.6	2.3-339	54.6	25	$*10^7$
Erythrocytes	1997	0.3	0.2-0.8	0.3	0.1	$*10^9$
Platelets	1999	22.4	4.8-50.3	23.4	7.2	$*10^9$
After volume reduction						
Nucleated cells	1998	64.4	21.2-213	69.7	26	$*10^7$
Lymphocytes	1996	21.9	7.0-73.2	23.3	8	$*10^7$
Monocytes	1917	7.6	1.0-33.0	8.2	4	$*10^7$
MNC	1996	29.6	9.4-97.0	31.2	11	$*10^7$
Neutrophils	1917	34.1	5.5-133	37.7	17	$*10^7$
Erythrocytes	1996	0.06	0.02-0.1	0.06	0.02	$*10^9$
Platelets	1998	6.8	0.08-14.8	6.9	2	$*10^9$

[a] Total number of respective cells in a single cord blood collection or in a cord blood unit after volume reduction.
[b] Mononuclear cells were counted as sum of lymphocytes and monocytes.

$CD34^+$ cell content in collected cord blood and in cord blood units after volume reduction

	N	Median	Range	Mean	SD	
$CD34^+$ cell concentration						
Collected cord blood[a]	1999	31.2	1.9-237	37.7	26	/µl
After volume reduction	1996	86.8	9.1-942	110	87	/µl
$CD34^+$ cell number[b]						
Collected cord blood	1999	2.4	0.1-24.4	3.0	2	$*10^6$
After volume reduction	1996	1.7	0.2-18.3	2.1	2	$*10^6$

[a] Standardised concentration, see Methods.
[b] Total number of $CD34^+$ cells in a single cord blood collection or in a cord blood unit after volume reduction.

Table 10 (cont.)

Colony forming unit (CFU) content in collected cord blood

	N	Median	Range	Mean	SD	
CFU colony concentration[a]						
CFU-TOT	321	22.4	4.3-136	27.0	17	/µl
CFU-GM	321	9.0	1.6-56.4	11.1	8.2	/µl
BFU-E	164[b]	3.6	0.2-65.4	6.7	9.1	/µl
CFU-GEMM	316	11.0	0.6-53.1	12.6	8.2	/µl
CFU-Hemoglobinised[c]	321	13.5	0.9-82.1	15.8	10	/µl
CFU colony number[d]						
CFU-TOT	321	1.7	0.2-13.6	2.1	1.6	$*10^6$
CFU-GM	321	0.7	0.1-6.6	0.9	0.7	$*10^6$
BFU-E	164	0.3	0.01-6.9	0.5	0.8	$*10^6$
CFU-GEMM	316	0.8	0.03-5.4	1.0	0.7	$*10^6$
CFU-Hemoglobinised	321	1.0	0.07-7.0	1.2	0.9	$*10^6$

[a] Standardised concentration, see Methods.
[b] During the early study period (before 2001) the BFU-E colonies were not counted separately from the CFU-GEMM colonies. Thus, CFU-GEMM equals to CFU-Hemoglobinised during that period.
[c] CFU-Hemoglobinised were counted as sum of CFU-GEMM and BFU-E.
[d] Total number of CFU colonies in a single cord blood collection.

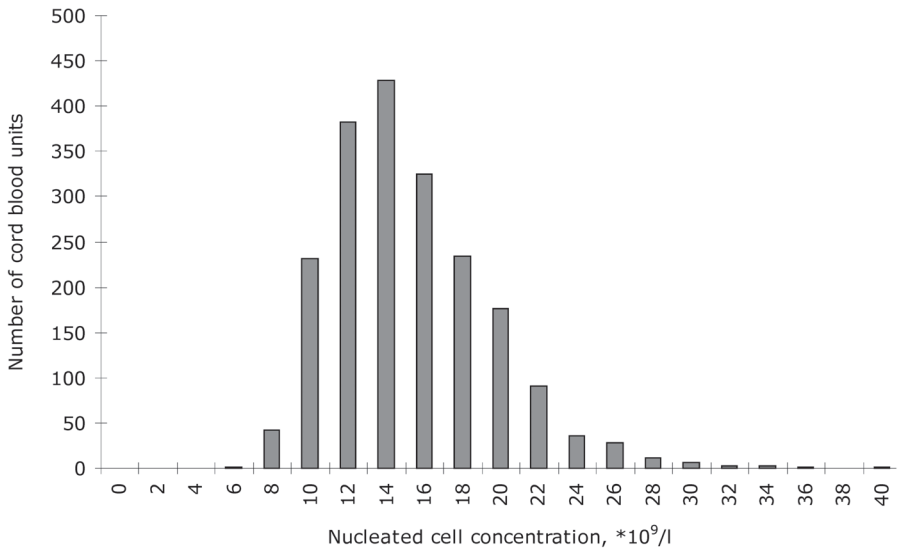

Figure 13 Histogram of the nucleated cell concentrations of collected cord blood (N=1999, median 13.6 $*10^9$/l, range 6.0-38.1).

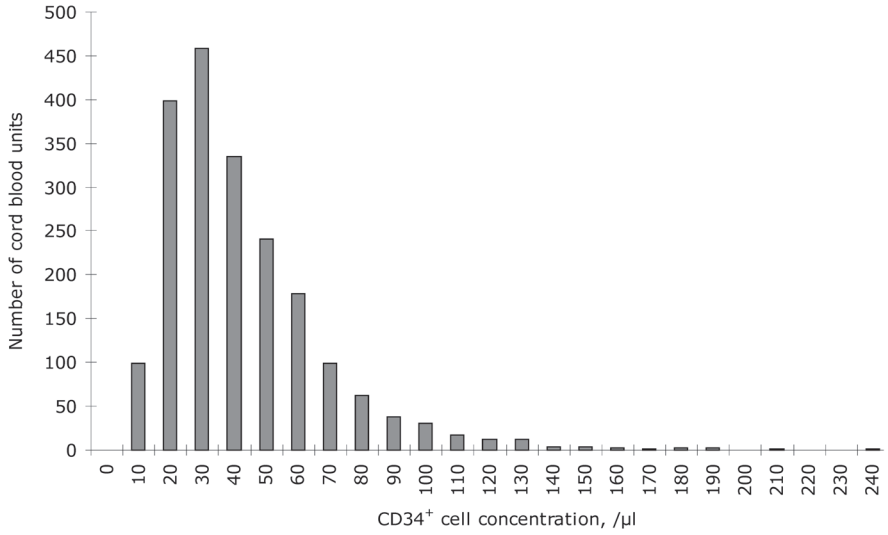

Figure 14 Histogram of the CD34⁺ cell concentrations of collected cord blood (N=1999, median 31.2 /µl, range 1.9-237).

9 DISCUSSION

Accepted methods of blood transfusion service practice, in addition to specific international cord blood bank standards, were adopted in the Finnish Cord Blood Bank programme, the methodology and results of which are described and discussed in studies I-IV. The Finnish Cord Blood Bank with all its activities (collection, processing, testing, banking, selection and release) was awarded accreditation by the FACT (Foundation for the Accreditation of Cellular Therapy) in September 2004. In the present study, infant and obstetric characteristics were collected prospectively in a uniform computerised system to allow analysis of selected internal relationships. Cord blood cell contents from a large uniform material of 1999 cord blood donors and cord blood units for transplantation were analysed.

A standard amount of anticoagulant is routinely used in cord blood collection, whereas the collected cord blood volume varies. This causes the blood/anticoagulant ratio in collection bags to vary, smaller blood volumes being diluted more than larger ones. Cord blood banks naturally calculate the total number of cells of a collection using the measured concentration data. However, the measured values in the cord blood banking process cannot be used as such to estimate the actual cord blood cell concentration. If the anticoagulant is deducted from the total volume and the measured concentration is corrected for this native blood volume, the corrected, or standardised, concentrations may be used to estimate the effects of gestation, labour and selected infant characteristics on the cell counts in cord blood. These standardised concentrations were used in studies III-IV as well as in this thesis.

In this study series, haemoglobin (Hb), haematocrit (Hkr), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean platelet volume (MPV) were not analysed. Erythrocyte and platelet yields were analysed in the volume reduction process (I). Thawing and potential washing of the cryopreserved cord blood unit before transplantation is normally the responsibility of the transplant centre. However, thawing validation data are available from the cord blood bank.

9.1 Nucleated cell content of cord blood transplants

Nucleated cell content has been used in graft selection to predict the haematopoietic potential of a cord blood transplant (Lim F *et al.*, 1999). Rubinstein and collaborators have reported faster myeloid engraftment in cord blood transplant recipients who were infused the highest nucleated cell numbers per kilogram of body weight (Rubinstein P *et al.*, 1998). Estimations of nucleated cell dose needed for successful cord blood transplantation vary (Gluckman E *et al.*, 1997, Gluckman E, 2000). Gluckman has suggested a dose limit of $>2*10^7$ cord blood nucleated cells per kilogram of patient body weight at the time of collection (Gluckman E, 2001). On the basis of this suggestion, 18% and 47% of the single units stored in the Finnish Cord Blood Bank by September 2003 would have been adequate for patients weighing 70kg and 50kg, respectively. A consistent cell dose threshold has not been set, although the significance of higher cell

dose for successful cord blood transplantation has been well documented (Gluckman E *et al.*, 2004). According to recent reports, cord blood units actually used for transplantation have contained high cell numbers, e.g. a median nucleated cell number of 142*10^7 (Oudshoorn M, Foeken-van Goozen L, 2004). Even if neutrophil engraftment has been rapid with a large total nucleated cell dose, this dose has had less influence on the speed of platelet engraftment.

White blood cell concentration of healthy newborn infants has a wide normal variation (mean$\pm$SD 7.71$\pm$4.99 *10^9/l) (Forestier F *et al.*, 1991). In our studies, too, a wide variation in nucleated cell concentrations in cord blood samples of term infants was observed (median 10.2 *10^9/l, range [4.4-26.6], Table 8), which is also in accord with normal reference values in haematological textbooks (Nathan DG *et al.*, 2003).

Cord blood nucleated red blood cell concentrations between 0.3 and 1.1 *10^9/l have been reported (Thilaganathan B *et al.*, 1994a, Stevens CE *et al.*, 2002). The nucleated cell concentration obtained from fresh cord blood samples with haematology analyser used in this study (Sysmex K1000) includes nucleated red cells. However, including nucleated red blood cells in the total nucleated cell count has been shown not to hamper the reliability of using the total nucleated cell content as an estimate of the haematopoietic potential of a cord blood transplant (Stevens CE *et al.*, 2002).

A proportion of newborn infants may have very high white blood cell concentrations (>30-40 *10^9/l). Supposing a maximum cord blood nucleated cell concentration of 40 *10^9/l and a collected volume of 250 ml, 1000 *10^7 nucleated cells could theoretically be collected. As the factors related to delivery and infant health underlying these high cell concentrations cannot be fully explored, it is not necessarily wise to aim for high cell concentration alone in the quest for high haematopoietic progenitor and stem cell contents. The extent to which high cell concentrations should be pursued remains to be determined on the basis of future cord blood transplantation results.

Good collaboration between the delivery room and cord blood collection staff is invaluable. In order to avoid clots in the cord blood unit, the collection should start as soon as possible after clamping of the cord and should last only two to three minutes, at most five minutes. Factors inherent in the cord blood banking process may affect the obtained cord blood volume; e.g the size of the collection bag may limit the maximum collected cord blood volume. To obtain cord blood collections with high cell content, several cord blood banks have adopted a selection process that captures only the highest collected cord blood volumes for banking. As the collected volume does not predict the nucleated cell concentration, the cell concentration of each collection is currently analysed before the decision to process further or not. However, this implies collecting a number of small cord blood units which will not end up in long-term storage.

Collected cord blood volume can also be increased by modifying the collection techniques, e.g. using flushing of the umbilical vein (Elchalal U *et al.*, 2000). These methods are more labour intensive and prone to microbial contamination and activation of coagulation, and may not be suitable for clinical cord blood banking. Collecting cord blood in utero before the birth of the placenta has been reported to yield higher volumes (Solves P *et al.*, 2003b). This, however, may

imply interfering with the delivery process.

Several obstetric factors possibly affecting the cord blood cell content, e.g. gestational age, birth order, mode of delivery and length of the stages of vaginal labor have been studied (Donaldson C *et al.*, 1999, Ballen KK *et al.*, 2001, Jones J *et al.*, 2003), among many others. In the present study, nucleated cell concentration of term infants was shown to correlate positively with gestational age of term infants (III). As birth weight, relative birth weight and placental weight correlated positively with the collected cord blood volume, higher expected birth weight, together with higher gestational age, could be used for predicting high volume cord blood collections, and cord blood collections could consequently be directed to deliveries of largest infants. Otherwise, the studied obstetric factors, although they can be assessed pre-delivery, have only marginal predictive value for the cord blood cell yield, and thus selecting infants for cord blood donation based on these factors is not generally feasible.

Adequate results in clinical transplantation have been obtained using the nucleated cell content to evaluate the haematopoietic potential of cord blood. Although the correlation between nucleated cell and colony forming cell concentration in cord blood is clear (r=0.58) (I), the nucleated cell content may not reflect optimally the full haematopoietic progenitor and stem cell content of a given unit. Thus, improved markers for predicting the haematopoietic potential of a cord blood transplant would be needed.

9.2 $CD34^+$ cells

The single platform ProCOUNT protocol was developed in an international effort to enumerate $CD34^+$ cells accurately and to diminish inter-laboratory variation (McNiece I *et al.*, 1998, Dzik W *et al.*, 1999). The ProCOUNT protocol was used to analyse $CD34^+$ cells in the beginning of present study according to the instructions of the manufacturer. When ProCOUNT reagents became unavailable on the market, the dual platform ISHAGE protocol (Sutherland DR *et al.*, 1996) to enumerate $CD34^+$ cells was adopted from March 2001 in the Finnish Cord Blood Bank. The differences in the results obtained with two different methods were taken into account in the analyses (I, III, IV).

Compared to the present study, similar median $CD34^+$ cell total numbers in collected cord blood have been reported (2.2-2.3 $*10^6$/unit, range 0.007-28.0) (Armitage S *et al.*, 1999b, Ballen KK *et al.*, 2001). $CD34^+$ cell concentrations have been documented less often. Surbek and collaborators reported a median $CD34^+$ cell concentration of 34 /μl (range 7-121) in preterm deliveries of 33-36 weeks gestational age (Surbek DV *et al.*, 2000b), which accords with the median concentration of 24.9 /μl (range 2.3-180) in term infants of the present study (I). Despite interlaboratory differences, the described results reflect similar levels of $CD34^+$ cells in cord blood.

Parallel total $CD34^+$ cell numbers in ex utero collections both after vaginal (2.96 ± 2.25 $*10^6$/unit) and caesarean section delivery (3.08 ± 1.97 $*10^6$/unit) have been reported (Solves P *et al.*, 2003c). Likewise, in the present study, the concentrations between the median $CD34^+$ cell concentration in vaginal (33.2 / μl) and caesarean section deliveries (34.1 /μl, P=0.81) did not differ (IV).

In our studies, the correlation between pre- and post-volume-reduction cord blood $CD34^+$ cell concentrations was shown to be extremely good, suggesting a uniform and reliable process of enriching collected cord blood $CD34^+$ cells into the final transplant (I). $CD34^+$ cell concentrations, both pre- and post-volume-reduction, were also shown to correlate well with the CFU colony concentration in collected cord blood, the correlation being clearly better than that between nucleated cells and CFU colonies. Similar results, the correlation between cord blood $CD34^+$ cell and colony forming cell concentration ($r=0.62$) being better than between cord blood nucleated cell and colony forming cell concentration ($r=0.50$), have also been reported earlier (Lim F *et al.*, 1999). Thus, as CFU colony content of a cord blood transplant has been reported to be a good predictor of its haematopoietic potential (Migliaccio AR *et al.*, 2000), a high concentration of $CD34^+$ cells may be considered a marker of rapid haematopoietic progenitor and stem cell production, or production in high numbers.

$CD34^+$ cell content of a cord blood transplant has also been studied as a predictor of the haematopoietic potential in clinical setting (Wagner JE *et al.*, 2002). However, lack of international cord blood $CD34^+$ cell standard has prevented the standardisation of $CD34^+$ cell enumeration methods (Barnett D *et al.*, 1998). Therefore, widespred use of $CD34^+$ cell content as a criterion for selecting cord blood collections for clinical transplants can not be promoted. Still, cord blood $CD34^+$ cell content can be used in internal evaluation and comparisons of the haematopoietic potential of transplants in an individual bank.

9.3 CFU

Only few studies have reported cord blood colony forming unit concentrations. The median CFU-TOT concentration in the present study, 22.4 /µl (range 4.3-136) (Table 10), compares favourably with the median haematopoietic progenitor cell concentration of 15.4 /µl (range 0.7-87.0) reported by Lim and collaborators (Lim F *et al.*, 1999).

Colony forming cells are reported in cord blood in approximately similar concentrations as $CD34^+$ cells, suggesting that both methodologies cover at least partially overlapping haematopoietic cell types. The correlation between CFU and $CD34^+$ cell concentration in the present study was good ($r=0.81$) (I). However, as the cells are allowed to grow for two weeks, CFU cultures cannot be used in selecting cord blood collections for cord blood banking process. Although standardisation of CFU culture methodology is still not adequate (Clarke E *et al.*, 2002 [abstract]), cord blood banks may use CFU cultures in internal comparisons.

Correlations between nucleated cell, $CD34^+$ cell and colony forming cell content of cord blood have been demonstrated. However, until more specific methods to enumerate stem cells are developed, best estimation of the haematopoietic potential of a given graft is obtained with laying more emphasis on the simultaneous analysis of all existing information on the haematopoietic potential of a single cord blood unit.

9.4 Birth weight

Normal birth weight varies widely depending both on gender and gestational age, and thus a specific measure to account for both of these factors is needed. Thus, relative birth weight, or birth weight z-score, which represents birth weight corrected for gestational age and gender, is used in obstetrics. Birth weight z-scores from a sample are interpreted as if they arose from a normally distributed population (Pihkala J *et al.*, 1989, Oken E *et al.*, 2003).

Positive correlations of birth weight with collected cord blood volume and thus with higher total cell counts have been reported (Ballen KK *et al.*, 2001, Jones J *et al.*, 2003). However, in these reports separating the individual effect of cord blood cell content from the volume of collected cord blood was not reported. We put forward the hypothesis (III) that a high concentration of haematopoietic progenitor and stem cells would reflect intrauterine growth, and on this basis we analysed the relationship of cord blood cell concentrations ($CD34^+$ cells, nucleated cells, and colony forming cells) with birth weight in term pregnancies.

$CD34^+$ cell concentration was almost double in infants belonging to the 95.0^{th} centile of relative birth weight compared with those belonging to the 5.0^{th} centile (III). Also, the infants in the top 50.0^{th} centile (above median) of relative birth weight had higher median $CD34^+$ cell concentration than infants in the low 50.0^{th} centile (below median) in a subgroup, where all $CD34^+$ analyses were performed using a single platform protocol. The positive correlation between birth weight and $CD34^+$ cell concentration remained statistically clearly significant even after adjusting for other variables in multivariate regression analysis. Ballen and collaborators have reported a positive correlation between birth weight and total $CD34^+$ cell content of cord blood in their study of 1240 collections (Ballen KK *et al.*, 2001). To our knowledge, a positive correlation between cord blood $CD34^+$ cell concentration - not confounded by collected blood volume - and birth weight has not been reported earlier.

Cord blood progenitor and stem cells may have a more central role in intrauterine growth and development than has been reported earlier. Higher observed haematopoietic progenitor cell concentration may also reflect differences in other progenitor and stem cell lineages. Interestingly, multi-potent mesenchymal cells have been isolated also from cord blood (Lee OK *et al.*, 2004, Lee MW *et al.*, 2004).

9.5 Gender

As gender was not used to select cord blood collections for processing, 47% of cord blood donors were female in this study. Geographically, cord blood was collected from donors resident in the capital area, which is considered well representative of the general Finnish population.

In study III, we demonstrated an association between higher cord blood $CD34^+$ cell concentration and higher infant birth weight. We thus formed the hypothesis that cord blood haematopoietic progenitor and stem cell concentration, measured as $CD34^+$ and colony forming colonies, would not only associate with birth weight but also with infant gender.

Indeed, male infants were observed to have higher cord blood $CD34^+$ cells concentration than females (IV). The difference between male and female median $CD34^+$ cell concentrations was, although small, still statistically significant (5.3%, $P=0.03$). In a recent report, where 572 samples from cord blood bank collections were analysed, the difference of 11% between male and female median $CD34^+$ cell concentrations (30 vs. 27 /μl, respectively) did not reach statistical significance ($P=0.36$) (Nakagawa R *et al.*, 2004).

Our findings were confirmed using multivariate linear regression analysis, where male gender was associated with higher $CD34^+$ cell concentration ($P<0.05$) after adjusting for mode of delivery and relative birth weight. Thus, gender may affect the characteristics of cord blood haematopoietic progenitor cell transplants.

Although cord blood from male infants was observed to have 5% higher $CD34^+$ cell concentration than that from female infants, the difference does not justify collections from male donors only. Despite intensive research, the complex mechanisms underlying gender differences, including multiple genetic factors in addition to sex hormones, are only beginning to unravel (Dennis C, 2004).

9.6 Effect of the mode of delivery

CFU-Mixed concentration distributions of male and female infants differed in caesarean section deliveries compared with those in vaginal deliveries, although the total CFU concentrations according to the mode of delivery did not differ. In caesarean section deliveries, male infants had significantly higher CFU-Mixed concentrations than female infants, while in vaginal deliveries difference in CFU-Mixed concentrations between male and female infants was not statistically significant (IV). This dissimilarity observed in the present study between CFU-Mixed concentrations of male and female infants in caesarean section deliveries compared with those in vaginal deliveries could not be explained by any technical factor in the study setting. Notably, when CFU-Mixed concentrations from all caesarean section and all vaginal deliveries were compared, a central tendency in the concentrations from caesarean section deliveries was observed. While the range of cord blood CFU-Mixed concentrations was comparable between cesarean section and vaginal deliveries, in the latter the 25^{th}-75^{th} percentile concentration range was wider. This probably reflects activating factors during labor, which might cause either a stimulating or depressing effect on the regulation of progenitor cells. Accordingly, associations of various stress factors during labor with cord blood CFU content has been demonstrated (Lim FT *et al.*, 2000, Aufderhaar U *et al.*, 2003).

Study III also revealed a correlation between CFU-TOT concentration and birth weight in caesarean section deliveries but not in vaginal deliveries. Thus, cord blood collections from caesarean section deliveries may represent better sampling from the in vivo situation, and may facilitate evaluations of physiologic phenomena during gestation. The possible effect of the delivery mode on transplant outcome remains to be studied.

9.7 HLA and blood groups

HLA population data derived from clinically oriented studies often remain small due to the expenses involved in HLA typing. Valuable HLA distribution data have been derived from volunteer bone marrow donor registries (Siren MK *et al.*, 1996, Schipper RF *et al.*, 1997, Mori M *et al.*, 1997, Brown J *et al.*, 2000), revealing considerable ethnic variance.

Cord blood donors represent a population of healthy infants. As cord blood donors are HLA typed the data of the most common HLA types may be used for analysing the association of HLA with infant physiological phenomena. In the present study, comparisons of HLA allele frequencies between the bone marrow donor registry and the cord blood bank were not possible because of the changing typing accuracy of serological and molecular techniques over time.

In study II we were able to explore associations between cord blood bank and HLA DRB1 data because DRB1 typing was performed by a DNA method. To our knowledge this possibility has not been utilised earlier. Although the number of infants in the study (N=1263) was large, the low prevalence of many alleles of the polymorphic loci of the HLA system rendered all but preliminary analyses impossible.

HLA DRB1*13 was shown to be over-represented in infants with the highest birth weights, suggesting a possible growth advantage for HLA molecules or some unknown factor linked to the HLA DRB1 region of chromosome 6 in normal intrauterine growth and development (II). As DRB1*13 has been associated with protection from infectious diseases (Hill AV *et al.*, 1991, Apple RJ *et al.*, 1994, Thursz MR *et al.*, 1995), the mechanism could be through molecular host responses. An association like this presents challenges for further research on the molecular level.

Recently, longer thrombocytopenia periods have been documented after major ABO-incompatible peripheral blood progenitor cell transplantation (Canals C *et al.*, 2004). Data on the possible association between HLA match and slower platelet engraftment in transplantations using cord blood as compared with cells from adult sources is scarce. Thus, it is not possible to exclude the potential effect of HLA matching on the speed of platelet engraftment after cord blood transplantation.

In the present study, no association between nucleated or $CD34^+$ cell concentrations and ABO, Rh or Kell blood group frequencies were observed. To our knowledge, this has not been reported earlier. At the time of the study, the frequencies of ABO and Rh blood groups in the Finnish Cord Blood Bank were similar to those in the healthy adult Finnish population. In the future, as infants are being selected for cord blood collection to increase cell yields, cord blood bank data will not represent normal distributions of tissue types, blood groups and haematopoietic progenitor and stem cell concentrations in healthy infants.

9.8 Unrelated cord blood as a haematopoietic transplant

Lack of accurately HLA-matched haematopoietic stem cell donors has justified the search of alternative stem cell sources. Transplantation of unrelated bone marrow or peripheral blood stem cells has become an established alternative

(Thomas ED *et al.*, 1975a, Davies SM *et al.*, 2000). Cord blood has proven a promising option in children, and lately also in adult patients.

Even though the required HLA match may be more relaxed for a cord blood transplant than for a transplant from adult sources, better matching may also improve cord blood transplantation results (Rubinstein P *et al.*, 1998). One HLA mismatch has been suggested to be equivalent to a dose of $3*10^7$ cord blood nucleated cells per kilogram of patient weight (Rubinstein P *et al.*, 2000 [abstract]). Transplantation results using HLA matched (6/6) cord blood units are still scarce, however.

Increasing the infused cord blood nucleated cell dose in general has also been reported to yield improved outcomes. Thresholds of 2-4 $*10^7$ nucleated cells per kilogram of patient weight have been suggested for cord blood transplantation (Gluckman E, 2000, Gluckman E, 2001). Cord blood has been succesfully transplanted to adult patients provided that the cell dose has been adequate (see above) or a double transplantation has been given (Barker JN *et al.*, 2005).

Cord blood is collected after normal pregnancy and delivery from a healthy term infant with no known associated risk factors for disease. Cord blood collection poses no risk to the donor as it is always performed after clamping of the cord. Moreover, cord blood carries a low risk of transmitting infectious diseases (Rubinstein P *et al.*, 1993). Collections from both vaginal and caesarean section deliveries have proven successful, yielding high collected blood volumes with predictable pre- and intra-delivery obstetric and infant-related factors, such as gender, affecting the cord blood quality.

As shown in this study, a well-standardised cord blood banking process may yield tranplants with highly predictable haematopoietic progenitor and stem cell contents. However, as cord blood banks currently select units with highest cell counts for cryopreservation, the possible contents of the present-day transplants may not be in line with the reported ones and may thus warrant further studies. The volume reduction method (Rubinstein P *et al.*, 1995) adapted in use during this study has been shown to produce clinically effective transplants . Also, as the processing methods are developed further, the real benefit of change must be validated to result in at least as good clinical outcome as the classical processing method.

Time required for cord blood unit acquisition can be kept short for patients in urgent need of transplantation (Dalle JH *et al.*, 2004). Once identified through an international bank search, a cord blood unit is made available after confirmation of its identity through repeat HLA typing of a bag segment, and preferably also its viability with colony forming culture (Goodwin HS *et al.*, 2003). The unit is then transferred to the transplant centre before the patient's conditioning treatment begins, thus leading to reduced time from diagnosis to transplant. A wide variety of archived samples is available in case of further requested analyses.

Long term outcome after cord blood transplantation has been comparable with bone marrow transplantation although engraftment after cord blood transplantation is delayed. Recently, comparable long-term outcome results of unrelated cord blood transplantation and allogeneic-related haematopoietic stem cell transplantation have even been reported (Jacobsohn DA *et al.*, 2004).

Cord blood transplants do not contain ABO isoagglutinins. Cord blood has also been reported to yield low incidence of severe acute GVHD (Rocha V *et al.*,

2001). Evidence is growing that cord blood transplants carry a GVL effect similar to that of haematopoietic stem cell transplants from adult sources (Jacobsohn DA *et al.*, 2004).

Poor or delayed immune reconstitution is a common problem after haematopoietic progenitor and stem cell transplantation. Cord blood contains primitive haematopoietic progenitor and stem cells with proliferation potential at least comparable to adult bone marrow or peripheral blood cells (Haneline LS *et al.*, 1996, Carow CE *et al.*, 1991, Theilgaard-Monch K *et al.*, 1999, Pettengell R *et al.*, 1994). Although haematopoietic engraftment and immune reconstitution after cord blood tranplantation may be slower than after transplantation of a graft from an adult donor, the long-term regenerative potential of both immunologic and haematopoietic systems may be in favour of cord blood transplantation (Talvensaari K *et al.*, 2002, Frassoni F *et al.*, 2003).

The size and number of active cord blood banks are growing steadily, reflecting the encouraging results from clinical studies. Currently, up to 200 000 cord blood units is estimated to be in storage in public cord blood banks and, although exact statistics are not available, between 5000 and 6000 cord blood tranplantations have been performed worldwide (Sanz MA, 2004, Steinbrook R, 2004). Future studies will reveal whether development of spesific cell components (e.g. NK cells, cytotoxic T-lymphocytes) from cord blood is possible for clinical use (Ayello J *et al.*, 2004 [abstract], Satwani P *et al.*, 2004 [abstract]). Current information technology provides the means to explore the possibilities of transforming the vast amounts of data gathered in a cord blood banking process into valuable information for further development of cord blood transplants.

10 CONCLUSIONS

The validation of policies and procedures adopted in a cord blood bank were described in the present study. Cord blood bank data were prospectively collected in a spreadsheet application specifically developed for the purpose. After hypotheses were created, the data were explored using a variety of statistical approaches, including descriptive analysis, comparison of groups based on extreme high and low centiles of selected parameters, as well as multivariate linear regression analysis.

Highly significant internal associations of cord blood cell concentrations, which are considered to reflect the haematopoietic progenitor cell content, between collected cord blood and processed cord blood units were observed. These associations proved that the applied volume reduction process was predictable, and that the laboratory tests themselves produced highly repeatable results over a time period of four years. In addition, the excellent correlation observed between whole blood CFU concentration and cord blood unit $CD34^+$ cell concentration suggests that the $CD34^+$ cell determination can be used for evaluating the haematopoietic potential of cord blood transplants within a single laboratory.

Volume of a cord blood collection has traditionally been the main determinant of whether the collection was selected for processing or not. Several obstetric and infant related variables were analysed to help clarify those affecting the cord blood cell concentrations. Birth weight, especially relative birth weight, of term infants was shown to be the single most important variable predicting higher nucleated and $CD34^+$ cell concentrations. Thus, the results indicate that collection of cord blood from high birth weight infants with higher cell concentrations yields the highest total cell contents in the cord blood transplant. Also, cord blood from male infants was shown to have higher $CD34^+$ cell concentration and, in caesarean section deliveries, more abundant early series haematopoietic progenitors than cord blood from female infants. These new findings may suggest further gender-dependent differences in other progenitor and stem cell lineages.

A significant and previously unreported association between HLA DRB1*13 and higher relative birth weight could be derived from the molecular DRB1 typing routinely tested in all cord blood transplants prior to their release to international search registries. This association may be of particular importance, as previously described DRB1*13 associations have demonstrated protective characteristics against infectious diseases.

The evidence accumulated shows that important new predictive factors affecting cord blood transplant quality, as well as useful information about neonatal physiology, can be derived from cord blood bank data originating from a well-standardised process. As cord blood banks place more and more emphasis on selecting for long-term storage only those collections with highest cell contents, future data collections may no longer reflect physiological variation. However, what is urgently needed is the development of more specific means for evaluating each cord blood unit for its stem cell content and plasticity.

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