Exploring the heterogeneity of the hematopoietic stem and progenitor cell pool in cord blood

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For my amazing children; Mårten, Douglas and Lykke

"The hardest thing of all is to find a black cat in a dark room, especially if there is no cat"

Confucius

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ABSTRACT

Hematopoietic stem cell transplantation (HSCT) is a curative treatment for a wide range of malignant and hereditary disorders. It is yet the only clinically established stem cell treatment. Hematopoietic stem and progenitor cells (HSPC) can be harvested from bone marrow (BM), stimulated peripheral blood (PBSC) or umbilical cord blood (CB) collected from the placenta after clamping of the cord. A critical factor for the success of HSCT is the dose of functioning HSPC the recipient receives. The National Swedish Cord Blood Bank (NSCBB) was founded in 2005. We compiled the achievements of the NSCBB and investigated the impact of a change of practices from early to delayed clamping on CB collection volume and nucleated cell number. We developed novel methods using flow cytometry for measurement of functional HSPC in CB, firstly for the simultaneous definition of the Hoechst Side Population (SP), Aldehyde Dehydrogenase activity (ALDH) and the expression of the surface protein CD34 and secondly for the definition of viable and apoptotic cells in the ALDH and CD34 positive populations respectively. Finally, we screened for biomarkers in CB plasma that may predict the HSPC content in the corresponding CB collection using a multiplex immunoassay. The NSCBB stands up well in international comparison and the implementation of delayed clamping had no major effect on collection efficiency. There was no overlap between the SP and the ALDH populations, suggesting that they define HSPC pools with different properties. Few apoptotic cells were identified in the ALDH population compared to the viable CD34 positive population, indicating that the ALDH assay intrinsically excludes apoptotic cells. We identified the CDCP-1 protein as a possible biomarker for HSPC content in CB.

Keywords: Cord blood, Cord blood bank, Cord clamping, Hematopoietic stem cell transplantation, Hematopoietic stem and progenitor cells, CD34, Side Population, Aldehyde Dehydrogenase, Apoptosis, CDCP1

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SAMMANFATTNING PÅ SVENSKA

Hematopoetisk stamcellstransplantation (HSCT), i dagligt tal benmärgs-transplantation, är en botande behandling för ett brett spektrum av elakartade och ärftliga sjukdomar och den enda kliniskt etablerade stamcellsterapin. Blodstamceller, s.k. hematopoetiska stamceller (HSPC) kan hämtas från benmärgen, från perifert blod efter stimulering med läkemedel eller från överblivet navelsträngsblod som uppsamlas från placenta och navelsträng efter avnavling av det nyfödda barnet. Stamceller från navelsträngsblod förvaras nedfrysta i s.k. navelsträngsblodbanker tills de efterfrågas för transplantation. En kritisk faktor för en lyckad transplantationsbehandling är att man ger en tillräckligt stor dos HSPC till mottagaren. I 2005 fick Sverige sin egen navelsträngsblodbank, svenska nationella navelsträngsblodbanken (NSCBB), som ligger på Sahlgrenska Universitetssjukhuset i Göteborg. Just nu finns ungefär 5000 infrysta navelsträngsenheter i den svenska banken, och verksamheten håller hög kvalitet internationellt sett. Under 2012 ändrades avnavlingsrutinerna vid de flesta svenska förlossningsavdelningar. Från att ha avnavlat barnet direkt efter framfödandet väntar man nu minst en minut innan navelsträngen klipps. NSCBB kunde även efter denna praxisförändring samla in navelsträngsenheter med tillräcklig mängd HSPC. Som ett led i bankens forskningsverksamhet har vi utvecklat nya effektivare metoder att mäta mängden HSPC i navelsträngsblod. Genom att på olika sätt kombinera analyserna Hoechst Side population (SP), Aldehyd dehydrogenas aktivitet (ALDH) och Annexin V kunde vi definiera HSPC med olika mognadsgrad i navelsträngsblodet och också få en bättre uppfattning om deras funktion med en analystid på endast ett fåtal timmar. Vår förhoppning är att dessa nya metoder skall kunna ersätta de mycket tidskrävande och dyra stamcellsodlingsmetoder, colony-forming unit assays (CFU), som är standard för att bedöma kvaliteten på navelsträngsenheter idag. I ytterligare ett projekt undersökte vi blodplasma från navelsträngsblod och fann att koncentrationen av proteinet CDCP-1 var hög i blodplasma från navelsträngsblod som innehöll en hög koncentration av HSPC. Vi identifierade således CDCP-1-proteinet som en möjlig biomarkör för HSPC innehåll i navelsträngsblod.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **High quality cord blood banking is feasible with delayed clamping practices. The eight-year experience and current status of the national Swedish Cord Blood Bank.**
Frändberg S, Waldner B, Konar J, Rydberg L, Fasth A, Holgersson J
Cell Tissue Bank. 2016 Sep; 17(3):439-48

II. **Exploring the heterogeneity of the hematopoietic stem and progenitor cell pool in cord blood: simultaneous staining for side population, aldehyde dehydrogenase activity, and CD34 expression.**
Frändberg S, Boreström C, Li S, Fogelstrand L, Palmqvist L,
Transfusion. 2015 Jun; 55(6):1283-9

III. **The aldehyde dehydrogenase cord potency assay excludes early apoptotic cells.**
Frändberg S, Li S, Boreström C, Holgersson J, Palmqvist L,
Submitted

IV. **Concentration of the CDCP1 protein in human cord plasma may serve as a predictor of hematopoietic stem and progenitor cell content.**
Frändberg S, Asp J, Waldner B, Holgersson J, Palmqvist L,
Submitted

CONTENT

ABBREVIATIONS

7-AAD	7-aminoactinomycin D
AGM	Aorta gonad mesonephros region
ALDH	Aldehyde dehydrogenase (enzyme)
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
Auto-HSCT	Autologous hematopoietic stem cell transplantation
BFU-E	Burst forming unit erythrocyte
BM	Bone marrow
CB	Cord blood
CBB	Cord blood bank
CBBC	Cord blood buffy coat
CBT	Cord blood transplantation
CBU	Cord blood unit
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CDCP1	CUB domain containing protein 1
C/EBP-α	CCAAT/enhancer binding protein alfa
CFU	Colony forming unit
CFU-GEMM	Colony forming unit granulocyte erythrocyte monocyte megakaryocyte

CFU-GM	Colony forming unit granulocyte monocyte
C-Kit	Tyrosin protein kinase Kit (CD117)
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CPD	Citrate phosphate dextrose solution
CXCL	C-X-C motif chemokine ligand
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C motif chemokine receptor 4
DCBT	Double unit cord blood transplantation
DMSO	Dimethyl sulfoxide
EPCR	Endothelial protein C receptor
EPO	Erythropoietin
ES	Embryonic stem cells
Flt3	Fms related tyrosine kinase 3
Flt3L	Fms related tyrosine kinase 3 ligand
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GMP	Granulocyte monocyte progenitor
GvHD	Graft versus host disease
GvL	Graft versus leukemia

HES	Hydroxy-ethyl starch
HLA	Human leukocyte antigen
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSPC	Hematopoietic stem and progenitor cells
IL	Interleukin
IPA	Inherited paternal antigens
ISHAGE	International Society of Hematotherapy and Graft Engineering
KIR	Killer cell immunoglobulin like receptor
LTC-IC	Long term culture initiating cells
M-CSF	Macrophage colony-stimulating factor
MEP	Megakaryocyte erythroid progenitor cell
MSC	Mesenchymal stem cells
NC	Nucleated cells
NIMA	Non-inherited maternal antigens
NK	Natural killer cell
NPX	Normalized protein expression
NRBC	Nucleated red blood cells
NSCBB	The national Swedish cord blood bank
PB	Peripheral blood

PBSC	Peripheral blood stem cells
PCA	Principal component analysis
PLA	Proximity ligation assay
SBT	Sequence based typing
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
SDF-1	Stromal cell derived factor 1 (CXCL12)
SP	Side population
TCR-β	T-cell receptor beta
TGF-β	Transforming growth factor beta
TNC	Total nucleated cells
TNF-α	Tumor necrosis factor alfa
Tregs	Regulatory T cells
USSC	Unrestricted somatic stem cells
VCAM-1	Vascular cell adhesion molecule 1
VEGFR-2	Vascular endothelial growth factor receptor 2
VLA-4	Very late antigen 4

DEFINITIONS IN SHORT

Cell source	HSPC cell source, the source of HSPC used in HSCT; i.e. BM, PBSC or CB.
DFS	Disease free survival after HSCT, the length of time from HSCT that recipients are alive and free of disease.
Graft	Cellular material that contains HSPC and is infused to the recipient in HSCT.
TRM	Transplant related mortality in HSCT, the probability of dying without recurrence of disease.
OS	Overall survival, the length of time from HSCT that recipients are alive

1 INTRODUCTION

1.1 The stem cell concept

Stem cells are undifferentiated cells that have the capacity to divide indefinitely, self-renew and generate a functional progeny of differentiated specialized cells.

1.1.1 Types of stem cells

Mammalian life begins with the zygote; the totipotent stem cell that is formed when an egg and a sperm fuses. The zygote is the only stem cell capable of forming all fetal and adult cells and tissues including the placenta. As the zygote begins to divide embryonal stem cells (ES) are formed within the blastocyst; pluripotent cells capable of differentiation into all types of tissues but unable to form a fetus. Further development leads to establishment of the tissue specific multipotent stem cells, responsible for homeostasis and repair of the respective tissue (Apperley, Carreras, Gluckman, & Masszi, 2012).

1.1.2 Asymmetrical division and self-renewal

Stem cells, as opposed to differentiated somatic cells, can divide unsymmetrically. This leads to the formation of two daughter cells with different fates, one with stem cell properties (self-renewal) and one cell that differentiates and forms mature progeny. Recently it has been proposed that stem cells can alternate between asymmetrical and symmetrical division, reverting to symmetrical division to replenish stem cell pools depleted by injury or disease (Morrison & Kimble, 2006).

1.1.3 The stem cell niche

Due to their extensive capacities for proliferation and differentiation multipotent stem cells must be closely regulated. This is accomplished through the local environment surrounding the cell, the stem cell niche. Decisions on stem cell fate are made by presenting that cell with specific repertoires of soluble and immobilized extracellular factors through adjacent cells and extracellular matrix (Conway & Schaffer, 2012).

1.2 Hematopoiesis in mice and men

Hematopoiesis is preserved between vertebrate species and much of the understanding of the human hematopoietic system is based on studies in mice and other vertebrates.

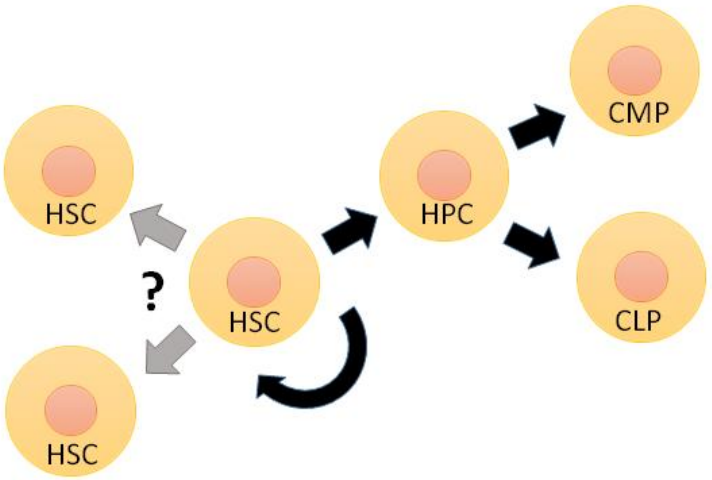
1.2.1 Fetal hematopoiesis

Multipotent hematopoietic stem cells (HSC) are derived from the ventral mesoderm. Fetal hematopoiesis begins in the yolk sac and in the aorta-gonad-mesonephros (AGM) region of the embryo in the first weeks of gestation. The placenta has also been shown to host cells with hematopoietic capacity. The relative contribution of these respective early sites to the final HSC pool in the adult is largely unknown. The fetal liver, spleen and thymus are subsequently colonized before final hematopoiesis is established in bone marrow following formation of the long bones in the last trimester. Blood cell formation can be detected in the spleen and liver until the first postnatal week (Orkin & Zon, 2008; Tavian, Biasch, Sinka, Vallet, & Peault, 2010).

1.2.2 Adult hematopoiesis

In adulthood hematopoiesis occurs in bone marrow (BM). Mature hematopoietic cells are short lived and must continually be replaced by HSC derived precursors dedicated to the specific hematopoietic lineages. The production of new cells is balanced to demand through extrinsic and intrinsic mechanisms in the stem cell niche regulating HSC quiescence, self-renewal and expansion. According to the classical hierarchical model of hematopoiesis, the HSC divides asymmetrically giving rise to a new HSC (self-renewal) and a hematopoietic progenitor cell (HPC). The HPC differentiates to either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). The CLP progenitor gives rise to T- and B- lymphocytes and NK-cells. The CMP follows the path to either a granulocyte-monocyte progenitor (GMP) giving rise to granulocytes and monocytes or a megakaryocyte-erythroid progenitor (MEP) that matures to erythrocytes or platelets (Iwasaki & Akashi, 2007). The existence of oligopotent CLP: s and CMP: s have been questioned in adult hematopoiesis favoring formation of unipotent precursors dedicated to one hematopoietic lineage, directly from HPC: s. However, cells with oligopotent characteristics could be isolated from fetal liver (Notta et al., 2016). Recently, through new methodology allowing tagging of single cells, the fate of embryonic HSC: s introduced into murine embryos and adult mice revealed that most HSC give rise to multi- or oligo-lineage clones and revealed a basic split between CMP and CLP development, lending support to the traditional tree-like model of the hematopoietic system (Pei et al., 2017).

Figure 1. Hematopoietic stem cells (HSC) divide asymmetrically and give rise to a new HSC (self-renewal) and a hematopoietic progenitor cell (HPC) that proliferates



and differentiates to a common myeloid progenitor cell (CML) or a common lymphoid progenitor cell (CLP) and subsequently mature blood cells. It has also been suggested that HSC can divide symmetrically to replenish the stem cell pool.

1.2.3 The hematopoietic stem cell niche

In BM HSC:s are found in the trabecular endosteum in close vicinity to osteoblastic cells which have been shown to be important for maintaining HSC properties such as quiescence and self-renewal (Wilson & Trumpp, 2006). Intrinsic mechanisms that regulate stem cell fate include lineage specific transcription factors such as C/EBP-α, PU.1, and GATA-1 and epigenetic regulators (Nakajima, 2011). The extrinsic mechanisms relate to the microenvironment trough stromal and osteoblastic cells. HSC: s adhere to and are modulated by niche cells through adhesion molecules such as integrins and cadherins. Membrane bound or secreted cytokines initiate specific signaling pathways within the HSC, for example, stem cell factor (SCF), flt3 ligand (flt3L), angiopoietin, thrombopoietin, IL-3, interferons, TNF-α, TGF-β, IL-6,

G-CSF and M-CSF, notch ligands and wnt ligands (Apperley et al., 2012; Zhao & Baltimore, 2015).

1.3 Methods to identify hematopoietic stem and progenitor cells

Under the microscope hematopoietic stem cells are medium sized mononuclear cells with prominent nucleoli, a high nucleus to cytoplasmic ratio and basophilic cytoplasm with no granules. Their visual appearance is however not enough to classify them as HSC: s. Human hematopoietic stem cells are also at present less well defined than murine, due to an extremely low concentration in bone marrow ($\leq$ 0.1% of all cells in BM) and absence of a specific HSC phenotype (Wognum, Eaves, & Thomas, 2003).

1.3.1 Total nucleated cells

The nucleated cell (NC) count, including immature nucleated red blood cells (NRBC), can be used to approximate hematopoietic stem and progenitor cell (HSPC) content in cell sources used for hematopoietic stem cell transplantation (HSCT). The number of NC the recipient receives correlates with outcome after transplantation (P. S. Martin et al., 2016; Remberger et al., 2015). NC are usually measured using automated hematology analyzers but must under certain circumstances be counted manually under the microscope, for instance in bone marrow (BM) harvests that commonly contain fat-particles and bone derived debris that interfere with automated analyzers. Total nucleated cells (TNC) stands for the total administered dose of nucleated cells.

1.3.2 Flow cytometry: immunophenotype

A multitude of surface determinants have been studied to define human HSPC: s, but their precise immunophenotype remains to be elucidated as opposed to the phenotype of murine HSPC: s. Murine HSPC: s are reliably defined as lacking surface determinants of mature cells *i.e.* they are devoid of lineage markers (lin-) but express the receptor c-Kit (CD117, c-Kit +) and the stem cell antigen (sca1+) (Osawa, Hanada, Hamada, & Nakauchi, 1996). Human HSPC are lin- and predominantly express the surface marker CD34 (CD34+). They are negative for the CD38 surface antigen (CD38-) and HLA-DR (HLA-DR-) but the population is heterogeneous and human HSPC can also express c-Kit, flt3, CD133 and CD90. Other determinants have also been suggested as HSPC markers such as the endothelial protein C receptor (EPCR) and the cub domain containing protein 1 (CDCP1) (Apperley et al., 2012; Beksac & Preffer, 2012; Buhring et al., 2004; Conze et al., 2003; Majeti, Park, & Weissman, 2007; G.

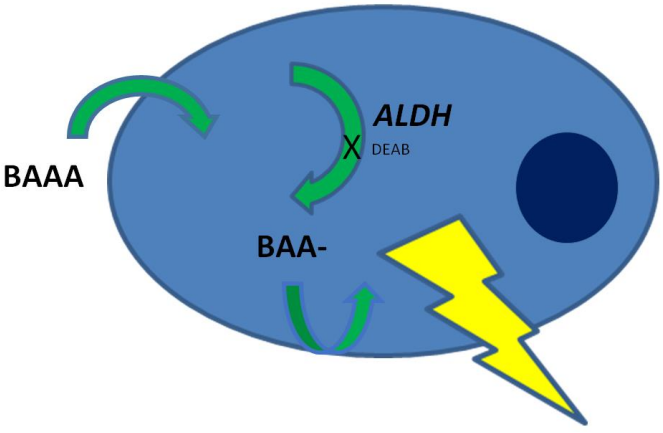
H. Martin & Park, 2017; Zubair et al., 2006). The integrin α6 antigen (CD49f) has been suggested to differentiate between HSC and HPC, where HPC: s are defined as lacking CD49f expression (CD49f-) (Notta et al., 2011). The CD34 surface antigen is however not expressed by all human HSPC: s and CD34- HSPC: s or HSPC: s with reversible expression of CD34 have been shown to exist (Kimura et al., 2007; Zanjani, Almeida-Porada, Livingston, Zeng, & Ogawa, 2003), CD34- HSPC may become CD34+ in *ex vivo* cell culture (Nakamura et al., 1999).The surface expression of CD34 can vary due to *ex-vivo* manipulation of cells for example cryopreservation (Sato, Laver, & Ogawa, 1999). The total CD34+ cell pool in cord blood (CB) or BM is also very heterogeneous, and besides primitive HSPC:s it also includes CMPs, GMPs, MEPs, CLPs, T-cell progenitors, NK-cell progenitors and pro-B cells. The CD34 antigen is also expressed on mature endothelial cells (Arber et al., 2011; Beksac & Preffer, 2012). The CD34 determinant is a highly glycosylated transmembrane protein with a molecular weight of 115 kDa. The function of CD34 remains to be fully understood, but has been implicated in HSPC adhesion and migration (Nielsen & McNagny, 2009). In the clinical setting, besides TNC, the number of CD34+ cells is used to approximate HSPC content in cell products intended for HSCT and as for TNC results correlate with outcome after transplantation (Purtill et al., 2014; Remberger et al., 2015). The most established protocol for CD34+ cells determination by flow-cytometry are the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines, where only mononuclear cells weakly co-expressing the leukocyte determinant CD45 (CD45dim), a marker of mature hematopoietic cells, qualifies as CD34+ HSPC, thus increasing the specificity of the assay for immature cells (Sutherland, Anderson, Keeney, Nayar, & Chin-Yee, 1996).

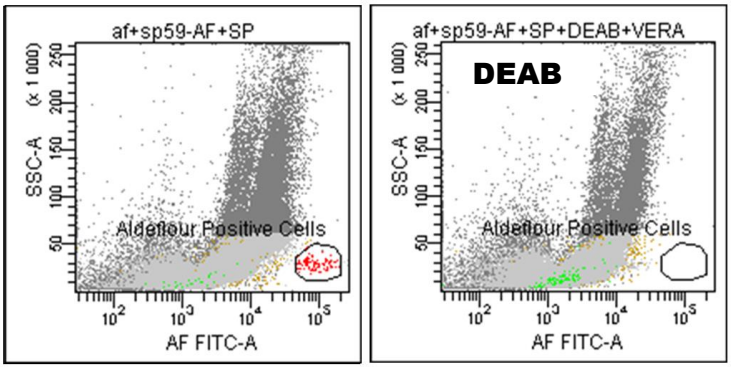
1.3.3 Flow cytometry: functional assays

Aldehyde dehydrogenase assay

Another approach to identify HSPC is to focus on the activity of intracellular enzymes that are involved in cellular differentiation (Chute et al., 2006). The activity of aldehyde dehydrogenase (ALDH) is elevated in the cytosol of primitive hematopoietic cells. Isolation of HSPC based on ALDH activity was first described by Storms et al in 1999 using a synthetic fluorescent substrate of the enzyme and has been confirmed in further studies (Christ et al., 2007; Storms et al., 2005; Storms et al., 1999). In 2016 Shoulars et al published data showing that the number of ALDH+ cells in frozen thawed CB correlated better with the results of short term HSPC cultivation protocols than the number of CD34+ cells (Shoulars et al., 2016).

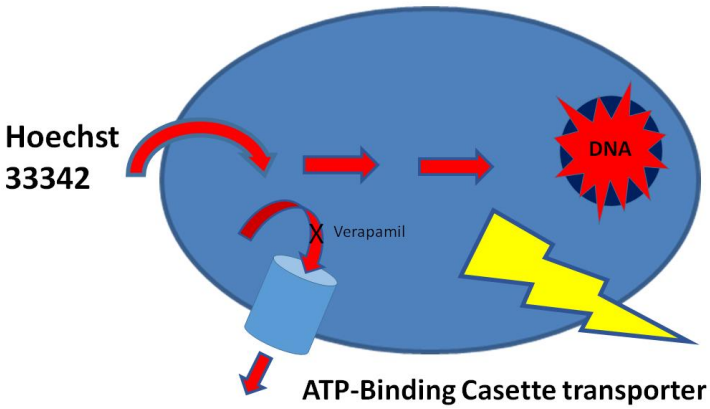
Figure 2. The aldehyde dehydrogenase (ALDH) assay. The florescent substrate (BAAA) readily permeates the cell membrane and is converted to the polar product (BAA-) that is retained in cells with an intact cell membrane. The cleaved

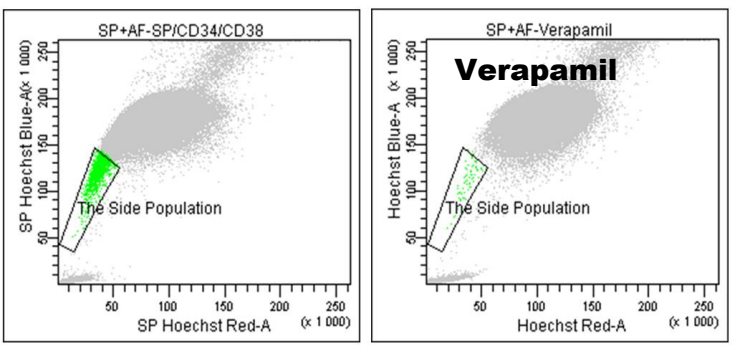




substrate is excited with a blue laser and emits in the FITC channel (515-545 nm). An ALDH inhibitor (diethylaminobenzaldehyde, DEAB), added to a separate tube, is used as negative control to define the population with high enzyme activity, i.e. the ALDH+ cells. Panels below depict flow-cytometry plots of the ALDH assay performed on CB.

Figure 3. The Side population (SP) assay. The DNA binding dye Hoechst 33342 is cell-permeable and binds to double stranded DNA in the nucleus or is effectively eliminated from the cytoplasm by the membrane efflux pumps of the ATP-binding cassette transporter superfamily. The drug transporter inhibitor Verapamil, added to





a separate tube, is used as negative control. Hoechst can be excited with a 375nm near-UV laser; unbound dye has a maximum emission in the 510-540 range (Hoechst red) and bound dye around 461 nm (Hoechst blue) Panels below depict flow-cytometry plots of the SP assay performed on CB, displaying the SP in a tail below and to the left of the main population of cells.

Side Population assay

Human HSC express the drug transporter proteins of the ATP-binding cassette transporter superfamily, inferring the ability to actively efflux dyes such as the DNA binding dye Hoechst 33342 and the mitochondrial binding dye Rhodamine 123 (Apperley et al., 2012). The Hoechst dye efflux assay was first described by Goodell and colleagues (Goodell, Brose, Paradis, Conner, & Mulligan, 1996). When investigating murine bone marrow the group identified a group of cells with a complex fluorescence pattern when Hoechst fluorescence was displayed simultaneously in two wavelengths (Hoechst red and blue). The cells showed an overall low fluorescence level and a dissimilar blue/red emission ratio, placing the cells in a tail to the left of the main population of cells, hence the "side population" (SP). SP cells have since then been shown to express high levels of stemcell-like genes and possess multipotent differentiation potential (Challen & Little, 2006; Rossi, Challen, Sirin, Lin, & Goodell, 2011). Studies combining the SP and ALDH assay have indicated that the SP defines the multipotent HSC pool whereas the ALDH+ population is dominated by committed progenitor cells (Alt et al., 2009; Challen & Little, 2006; Christ et al., 2007).

1.3.4 Cell cultivation and transplantation assays

Cell cultivation and murine transplantation assays aim to mimic human hematopoiesis *in vivo*. The most primitive hematopoietic cells can only be appreciated using serial transplant models in immunodeficient mice which present a cellular environment most similar to the human hematopoietic niche. Examples of such mice include the non-obese diabetic/severe combined immunodeficiency (NOD/SCID) strain or more recently the Rag2-/-γc-/- mice (Ito, Takahashi, Katano, & Ito, 2012). The *ex-vivo* long term cultivation assays (LTC-IC) are also able to identify immature HSPC, using co-culture with stromal cells for 5-8 weeks (Weaver, Ryder, & Testa, 1997; Verfaillie, 1994). In the clinical setting, when evaluating grafts intended for HSCT, HSPC content is approximated using short-term culture systems primarily detecting committed progenitors, the so-called colony-forming unit assay (CFU). According to this protocol cells are cultured in cytokine-supplemented semisolid media for 14 days. Growing colonies (clones) are counted and according to their appearance and size under the microscope as either erythroid (BFU-E), granulocyte-monocyte (CFU-GM) or granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM). Compared to the TNC and CD34+ cells assays the functional CFU assay shows the best correlation with outcome after HSCT (Page et al., 2011; Yoo et al., 2007), however it´s labor intensive and hard to standardize between laboratories (Lumley et al., 1999).

Figure 4. The CFU assay. Viable mononuclear cells are cultured in cytokine-supplemented semisolid media for 14 days. Growing colonies (clones) are counted and classified according to their appearance and size under the microscope. Representative image of a granulocyte-monocyte colony (CFU-GM).

1.3.5 Viability and apoptosis

Cryopreservation procedures inflict both apoptotic and necrotic cell death, resulting in subsequent loss of cellular viability and function after thawing (Bissoyi, Nayak, Pramanik, & Sarangi, 2014). Apoptosis, programmed cell death, is a general mechanism for removal of unwanted or damaged cells and includes chromatin condensation, DNA cleavage and membrane asymmetry with exposure of phosphatidylserine on the cell surface. Membrane asymmetry can be appreciated using flow cytometry through the Annexin V assay, which measures Annexin V bound to phosphatidylserine exposed on the cell surface (Koopman et al., 1994; Krysko, Vanden Berghe, D'Herde, & Vandenabeele, 2008). Cells in later stages of apoptosis and necrotic cells are identified by cytolytic or membrane leakage assays, such as Trypan blue, Propidium iodide or 7-AAD. Numerous studies have shown loss of nucleated and CD34+ cells after cryopreservation and thawing of cellular products intended for HSCT and post-thaw measurements of viable nucleated and CD34+ cells correlated better with outcome after transplantation than pre-freeze values (D'Rozario, Parisotto, Stapleton, Gidley, & Owen, 2014; Winter et al., 2014). Also, Kim et

al found that in median 5.3% ± 4.1% of viable CD34+ cells in frozen thawed CB were Annexin V+, *i.e.* apoptotic (Kim, Huh, Hong, & Kang, 2015). Duggleby et al (Duggleby et al., 2012) showed that exclusion of apoptotic CD34+ cells from the CD34+ population improved correlation with results from the CFU assay.

1.4 Hematopoietic stem cells in cord blood

1.4.1 Biology of cord blood hematopoietic stem cells

In humans hematopoietic stem and progenitor cells are only present in bone marrow (BM) and at birth in umbilical cord blood (CB). The latter was discovered by Knudtzon in 1974 when he cultivated mononuclear cells from umbilical cord blood collected at birth and found that the number of CFU cells were comparable to the number found in bone marrow (Knudtzon, 1974). Broxmeyer et al confirmed his findings in the late 80´s and concluded that the amount of HSPC in CB collections would be sufficient for autologous and human leukocyte antigen (HLA) matched allogeneic hematopoietic reconstitution (Broxmeyer et al., 1989). The biology and properties of cord blood HSPC compared to their bone marrow counterparts have since then been studied. The overall frequency of immature HSPC in BM and CB is similar, around 1 in 10^4-10^5 nucleated cells (Ratajczak, 2008). Arber et al (Arber et al., 2011) investigated HSPC (CD34+Lin-) cells from BM and CB, and found a higher proportion of CD34+Lin- cells to be CD38-, i.e. of a more immature phenotype, in CB compared to BM. Also CB contained the highest proportion of GMP and T/NK cell progenitors. Whereas BM contained the largest number of Pro-B cells. MEP and CLP contents were not significantly different between CB and BM cell sources. Bhatia et al found that the concentration of cells capable of restoring hematopoiesis in SCID-mice was at 1 per 600 CD34+ CD38- cells in CB, a frequency greater than that found in adult BM (Bhatia, Wang, Kapp, Bonnet, & Dick, 1997). CD34+ cells from CB exhibited a higher proliferative capacity than BM CD34+ cells when cultured *in vitro* (Broxmeyer et al., 1992; Traycoff et al., 1994). There are indications that CB HSPC: s (CD34+Flt3+Lin-) are less responsive to stromal cell derived factor-1 (SDF-1) also known as CXCL12 and engraft better injected directly into the bone marrow cavity in murine models (Castello et al., 2004; Kimura et al., 2007).

1.4.2 Factors that influence hematopoietic stem cells numbers in cord blood

NC and NRBC numbers correlate positively with CD34+ cell concentration in CB (Pope, Hokin, & Grant, 2014). Aroviita et al reported the median NC and

CD34+ cells concentration without anticoagulant from 1368 full term CB collections to be $10.2*10^8$/L (range 4.3-36.5) and 33 cells/µl (range 1.9-663), respectively (Aroviita, Teramo, Hiilesmaa, Westman, & Kekomaki, 2004). Similar levels and the same wide range in results have been reported by others (Mousavi, Abroun, Zarrabi, & Ahmadipanah, 2017). Numerous maternal and neonatal parameters have been studied to elucidate how they influence NC and CD34+ cell counts in CB. Maternal weight, maternal age and maternal smoking do not seem to affect results (Mousavi et al., 2017). CB CD34+ cells concentration correlates negatively with non-Caucasian ethnicity and maternal iron status (Akyurekli, Chan, Elmoazzen, Tay, & Allan, 2014; Pope et al., 2014). CB CD34+ cell numbers correlates positively with birth weight, male sex, vaginal delivery and fetal distress in prolonged first and secondary stage of labor with lower CB venous pH (Cairo et al., 2005; Lim et al., 2000; Lim, van Winsen, Willemze, Kanhai, & Falkenburg, 1994; Pope et al., 2014). CB collected from children born to mothers with preeclampsia show lower numbers of CD34+ cells compared to children born to unaffected mothers (Surbek et al., 2001; Wahid et al., 2012). Wisgrill et al (Wisgrill et al., 2014) compared CB harvested from pretem infants born in weeks 24-32 and compared the HSPC content with CB from term infants born weeks 38-42. Term CB displayed a higher concentration of NC, but pretem CB exhibited a higher concentration of CD34+ cells and a higher proportion of CD34+ CD38- HSPC, *i.e.* HSCP of a more primitive phenotype. Isolated preterm CB CD34+CD133+ cells and ALDH+ cells showed a higher proliferative capacity compared to the same cells from term CB. Each extra gestational week decreases the number of CD34+ cells and CFU-GM concentration in the corresponding CB with 9 % and 11% respectively (K. K. Ballen et al., 2001). CD34+ cells concentration in peripheral neonatal blood drops rapidly after birth, in median 25% in the first 3 h and reaches low adult levels in 60 h. The decline correlates with the concentration of erythropoietin (EPO) in cord and neonatal plasma (Gonzalez et al., 2009).

1.4.3 Other types of stem cells and immune cells in cord blood

Small populations of other types of stem cells, such as mesenchymal stem cells (MSC) (Goodwin et al., 2001), unrestricted somatic stem cells (USSC) (Kogler et al., 2004) and endothelial progenitor cells (CD34+ VEGFR-2+ CD133+ cells) have also been defined in CB (Peichev et al., 2000). Taken together these different cell types have been shown to be able to differentiate into epithelial cells, osteoblasts, chondroblasts, adipocytes and neural cell types including astrocytes and neurons, suggesting that they may be clinically useful in regenerative medicine applications (Broxmeyer, 2005). Immune cells in CB

have been compared to the corresponding cells cells in adult peripheral blood (PB); CB and adult PB have parallel percentages of CD4+ and CD8+ T cells, but the majority of T cells in UCB have a naive phenotype (CD45RA+) (Brown & Boussiotis, 2008; Harris et al., 1992). The T-cell repertoire in CB is polyclonal and naive but with a complete repertoire, with the ability to expand T-cell receptor beta (TCR-β) subfamilies upon stimulation (Garderet et al., 1998). T lymphocytes from CB have similar proliferation rates compared to T-cells from adult PB, but show reduced allo-antigenic cytotoxicity (Kaminski et al., 2003). CB holds higher numbers of regulatory T cells (Tregs) (Xu et al., 2014) and CB-derived B lymphocytes show a reduced capability to produce immunoglobulins upon stimulation compared to their counterparts in adult PB (Lucchini, Perales, & Veys, 2015; Roncarolo, Bigler, Ciuti, Martino, & Tovo, 1994; Wu, Blanco, Cooper, & Lawton, 1976). The lymphocyte populations in CB have also been shown to be affected by delivery mode. Cairo et al found that collections after Cesarean section held lower numbers of CD4+ T-cells, CD8+ T-cells and B-cells but higher numbers of NK-cells compared to vaginal deliveries (Cairo et al., 2005).

1.5 Hematopoietic stem cell transplantation

The first steps in hematopoietic stem cell transplantation (HSCT) were taken in the late 1950`s when the first human BM infusions gave proof of concept that they could restore hematopoiesis in irradiated patients with acute leukemia (Thomas, Lochte, Lu, & Ferrebee, 1957). But success was limited until the discovery of the HLA system a decade later (van Rood, 1968). Subsequent improvements in donor procurement and pre- and post-transplant treatments have since then established HSCT as a routine clinical procedure. HSCT is still the only stem cell therapy that is broadly implemented in healthcare worldwide. Major treatment indications are hematological disorders, solid tumors, immune disorders and inborn errors of metabolism (Apperley et al., 2012).

1.5.1 Allogeneic and autologous HSCT

Indications, choice of donors and HSCT cell source

In autologous HSCT (auto-HSCT) the patient´s own HSPC are used and in allogeneic HSCT (allo-HSCT) cells from related or unrelated volunteer donors are used for treatment. HSPC can be harvested from several cell sources. BM cells are aspirated from the posterior iliac crest under general anesthesia in volumes of about 10-20 ml per kilogram donor weight. HSCT can be mobilized from BM to peripheral blood trough treatment with growth factors such as

granulocyte colony stimulation factor (G-CSF), reversible inhibitors of SDF-1/CXCL12 binding to CXCR4 and in the autologous setting following myelosuppressive chemotherapy. The mobilized peripheral blood stem cells (PBSC) are harvested through peripheral venous access and so-called apheresis procedures. CB is the most recent cell source and CB HSCT will be discussed in full detail in the following chapter. The choice between allogeneic versus autologous HSCT is based on the underlying condition and indication for HSCT whereas the choice of cell source depends on whether the recipient is an adult or a child, the underlying disease, the availability of related or unrelated donors, the urgency of the procedure, and institutional preference (K. K. Ballen, 2015; Eapen, O'Donnell, et al., 2014; Kekre & Antin, 2014). In Europe, according to the European group for blood and marrow transplantation (EBMT) survey in 2009, 31322 HSCT were performed that year, 41% of transplantations were allogeneic and 59% autologous. The main indication for allogeneic HSCT was leukemia and for autologous HSCT lymphoproliferative disorders. The dominating cell source was PBSC (71%) followed by BM (12%) and CB (7%) Among the allogeneic donors, 42% were a HLA identical sibling, 46% an unrelated volunteer donor and 7% CB (Baldomero et al., 2011). In allogeneic HSCT HLA match plays a central role in choosing the donor. The best donor is a HLA identical sibling identified by family typing, but since there is only a 25% chance that siblings are HLA identical, this is not an option for all patients. When no sibling donor is available, HLA matched unrelated donors are searched in worldwide registries for adult volunteer donors. In this case the donor must generally be matched for the HLA loci, HLA A, B, C, DRB1, and DQB1 on the allele level, a so called 10/10 match. For European Caucasoid recipients the chance of identifying 10/10 donor is approximately 40-50% (Tiercy et al., 2007). For patients from other ethnic backgrounds or with rare HLA alleles, the chance is significantly smaller. In this circumstance so called “alternative” HSCT sources are used; CB, haplotype-identical relatives or partially matched unrelated donors.

Treatment and complications associated with HSCT

Conditioning, *i.e*. the preparing of the recipient for the stem cell transplant, is the first step in HSCT and is performed to eradicate tumor cells if the indication is a malignant disorder, to eradicate immune memory, to achieve immunosuppression to prevent HSPC rejection and to create space in the BM (Vriesendorp, 2003). The latter objective is somewhat controversial, and based on the concept that HSPC occupy distinct stem cell niches in the BM. Recipient HSPC must hence be eradicated to create space for donor HSPC. Experimental support for this hypothesis comes from murine models in which very few HSPC engraft in non-myeloablated mice (Stewart, Crittenden, Lowry, Pearson-White, & Quesenberry, 1993). Various combinations of

chemotherapy, irradiation and anti-T-cell therapies are used to accomplish conditioning depending on the underlying disease. The graft, *i.e*. the HSPC containing product is usually transfused intravenously, although intra-bone administration has been tried in cord blood transplantation (CBT) (Frassoni et al., 2008). Post-transplant immunosuppression is a major feature of allo-HSCT and is required to prevent graft versus host disease (GvHD). GvHD arises when the immune system of the donor is activated against the recipient´s tissues because of an interaction between recipient antigen presenting cells and donor T-cells. However the level of immunosuppression must be kept sufficiently low to retain the major curative effect of allo-HSCT *i.e*. the graft versus tumor/leukemia (GvL) effect. The post-transplant period is characterized by severe cytopenia and peripheral cell counts are monitored to detect when the transplanted HSPC start to reproduce in the recipient´s BM. Myeloid engraftment is defined as the first of three consecutive days with an absolute neutrophil count above $0.5*10^9$/L and platelet engraftment as the first of three consecutive days with a platelet count above a defined level usually between $30\text{-}50*10^9$/L (Rihn, Cilley, Naik, Pedicano, & Mehta, 2004). Graft failure, *i.e.* when donor HSCT fail to engraft and reproduce, is primarily caused by immunological mechanisms mediated by recipient T and possibly also NK-cells. Other mechanisms such as drug toxicity, septicemia and virus infections may also contribute to graft failure (Olsson et al., 2013). Major post-HSCT complications are infections and GvHD. The infectious complications post-transplant reflect the different stages of immune system reconstitution. Bacterial and fungal infections dominate during the cytopenic period when monocytes and granulocytes are scarce, while viral infections appear later due to deficiencies in cellular immunity, primarily in the CD4+ T-cells and B-cells compartments. Post-transplant immune deficiency generally lasts for more than one year after allo-HSCT. GvHD is classified as either acute or chronic depending on when it appears after HSCT, before or after 100 days post-HSCT respectively. The severity of the disease is graded from I-IV for acute GvHD and from 1-3 for chronic GvHD, based on how many of the recipient´s organs are involved and the level of involvement. Acute GvHD involves skin, liver and gut with rashes, diarrhea and elevated transaminases whereas chronic GvHD mimics autoimmune disorders such as scleroderma, chronic biliary cirrhosis, immune cytopenia and chronic immunosuppression. Complications and mortality after HSCT are classified trough a number of established definitions; transplant related mortality (TRM), disease-free survival (DFS) and overall survival (OS) which are used to compare results after HSCT from different cell sources and treatment regimens.

1.6 Cord blood hematopoietic stem cell transplantation and cord blood banking

1.6.1 Cord blood collection, processing and public banking

Donor selection and collection

CB is collected in full-term vaginal or cesarean deliveries shortly after clamping of the cord, through sterile cannulation of the umbilical vein, with placenta in-utero, ex-utero or a combination of both. CB is gathered into a sterile bag set containing an anticoagulant solution. The collection is performed by birth attendants or dedicated midwifes employed by the cord blood bank (CBB) (Aroviita, Teramo, Westman, Hiilesmaa, & Kekomaki, 2003; Frandberg et al., 2016; Vanegas et al., 2017). A thorough medical history is taken to exclude donations from parents with a history of inherited metabolic or hematopoietic disorders, previous malignant diseases and infectious diseases that may be transmitted to the neonate. Individual banks may recruit CB collections from donors among minority groups with non-Caucasian or mixed ethnic backgrounds to achieve greater HLA diversity in available cord blood units (CBU) (Frandberg et al., 2016; Jefferies, Albertus, Morgan, & Moolten, 1999; Kurtzberg et al., 2005).

Delayed cord clamping

During the past few years evidence has cumulated suggesting that delayed umbilical cord clamping, *i.e.* clamping at least 30-60 s after birth, increases post-birth hemoglobin levels and improves iron stores in the first months of life. (Andersson, Hellstrom-Westas, Andersson, & Domellof, 2011; Andersson et al., 2015; "Committee Opinion No. 684: Delayed Umbilical Cord Clamping After Birth," 2017; McDonald, Middleton, Dowswell, & Morris, 2013). This practice, implemented by maternity clinics worldwide, increases the volume of the placental-neonatal transfusion and consequently decreases the volume of collectable CB remaining in the placenta after clamping (Frandberg et al., 2016; Katheria, Lakshminrusimha, Rabe, McAdams, & Mercer, 2017). In previous publications the close relationship between collected CB volume and CBU cell content has been stressed (Allan et al., 2016; Naing et al., 2015; Nakagawa et al., 2004).

Processing, unit quality, HLA-typing and reasons for unit rejection

Nikiforow et al (Nikiforow et al., 2017) recently reviewed processing policies in 34 cord blood banks (CBB) worldwide. The acceptable time-lapse between

collection and processing was less than 48 h for 97% of investigated CBB. Eighty-eight percent of CBB performed a volume reduction and erythrocyte depletion step creating a cord blood buffy coat (CBBC) before freezing, hence only 12% held large volume erythrocyte replete units. Sixty-eight percent of banks utilized automated processing systems with addition of hydroxyl-ethyl starch (HES). All banks used controlled rate freezing after supplementation with DMSO and stored CBU in ether liquid or gaseous nitrogen. Reduction of CB volume saves storage space for the CBB, and reduces toxicity from infusion of larger volumes freezing medium containing dimethyl sulfoxide (DMSO) and disrupted erythrocytes, regardless of ABO status (Nagamura-Inoue et al., 2003). In a retrospective study of double CBT, Purtill et al (Purtill et al., 2014) found that CBU with volumes < 24.5 or > 26 ml were associated with reduced incidence of neutrophil engraftment. Several procedures can be used besides HES sedimentation, such as top and bottom separation and various filter systems. CD34+ cells recovery has been shown to be comparable between methodologies, but CFU recovery and erythrocyte depletion efficiency were superior with the HES-based systems (Rubinstein et al., 1994; Solves et al., 2005; Takahashi et al., 2006). The HSC content and quality of each CBU is routinely approximated on fresh material before freezing by assessing the total number of viable nucleated cells (TNC), CD34+ cells and CFU:s. The time to engraftment and graft failure incidence are reduced with higher TNC and CD34+ cell numbers. The number of CFU correlates best with time to engraftment (Barker, Scaradavou, & Stevens, 2010; Page et al., 2011; J. E. Wagner et al., 2002). However, pre-freeze estimations do not invariably reflect on cell numbers and quality post-storage and thaw and other studies have shown that only post-thaw estimations of TNC, CD34+ cells and CFU correlate with engraftment (McManus et al., 2012; Schuurhuis et al., 2001; Yoo et al., 2007). HLA matching is also imperative when searching for and choosing a CBU for patients in need of a HSCT. Traditionally, due to the higher permissibility for HLA mismatch in CBT, HLA typing of CBU:s was mainly based on low to intermediate resolution typing with antigen level HLA-matching of the HLA-A and B loci and allele-level matching of DRB1 (Barker, Scaradavou, et al., 2010; M. Delaney & Ballen, 2010; Oran et al., 2015). However, TRM is reduced and OS increased by allele-level matching of the HLA-A, B, C and DRB1 loci (Eapen, Klein, et al., 2014; Eapen et al., 2017; Oran et al., 2015). During pregnancy, the immune system of the mother and fetus are in close contact and this may sensitize, or induce tolerance in the fetal *i.e.* CB immune system. Hence, HLA permissiveness for the tolerogenic non-inherited maternal antigens (NIMA) and HLA sensitization with an increased GvL effect against inherited paternal antigens (IPA) trough fetal-maternal microchimerism have been proposed as complementing tools for HLA matching in CBT (Scaradavou, 2012; van den Boogaardt, van Rood, Roelen,

& Claas, 2006; Van der Zanden et al., 2014; van Rood, Scaradavou, & Stevens, 2012). The effect of KIR mismatching in CBT have shown conflicting results on OS and DFS (Brunstein, Wagner, et al., 2009; Rocha et al., 2016; Willemze et al., 2009). In recent CBU selection algorithms HLA match and TNC dose are considered in parallel. Increasing TNC doses can be used to trade off an HLA mismatch and conversely the better the HLA match the less important is the TNC dose. In 2010 Barker et al showed that the best transplantation outcomes were in recipients receiving CBU with 6/6 matching on the allele level for the HLA-A, -B and DRB1-loci regardless of TNC dose, but 6/6 matched units with TNC dose < $1.5*10^7$/kg are still not recommended. For 5/6 matched units a dose of ≥$2.5*10^7$/kg and for 4/6 matched units a dose of ≥ $5*10^7$/kg is proposed (Barker, Byam, & Scaradavou, 2011; Barker, Byam, et al., 2010; Barker, Rocha, & Scaradavou, 2009). Current British guidelines recommend ≥ $3*10^8$/kg for CBU: s with an 8/8 allele match at the HLA- A,-B, -C and DRB1-loci and ≥ $5*10^8$/kg for the 5-7/8 match situation (Hough et al., 2016). The TNC content of the CBU is hence a major quality criterion and most CBB have pre- and post-processing TNC cut-offs to increase the efficiency of their inventories; a post-processing cut-off level at ≥ $9*10^8$ TNC has been proposed by Querol et al. Units with TNC ≥ $12.5*10^8$ can be used for larger children and adults with a body weight of > 50 kg (Querol, Gomez, Pagliuca, Torrabadella, & Madrigal, 2010; Querol, Rubinstein, Marsh, Goldman, & Madrigal, 2009). Maternal and CB infectious disease testing is performed for CB collections, usually Hepatitis B and Hepatitis C, HIV, HTLV, CMV, Syphilis and in some instances also West Nile virus and Chagas disease. Bacterial and fungal cultures and screening for hereditary hemoglobinopathies are also performed (Barker et al., 2011). The discard rate and reasons for rejection of collected CB varies between CBB: s worldwide, and is largely attributable to differences in the TNC pre- and post-processing cut-off levels. A higher level means a higher rejection rate, Querol et al calculated the fraction of discarded units with a TNC cut- off at $5*10^8$ to 28%, at $9*10^8$ to 54% and at $12.5*10^8$ as high as 62% (Querol et al., 2009). Other major reasons for rejection a CBU are incomplete or erroneous documentation at the collection site, too long transportation or storage times, abnormal sterility testing and insufficient numbers of CD34+ cells or CFU:s (Jaime-Perez et al., 2012; Lauber, Latta, Kluter, & Muller-Steinhardt, 2010; Liu et al., 2012).

Cord blood banking worldwide and CBU search procedures

Public CBB, *i.e*. banks that collect CB for altruistic unrelated use, currently hold over 700,000 CBU: s available for transplantation worldwide, but few CBB exist outside Europe and North America (K. Ballen, 2017; K. K. Ballen, Gluckman, & Broxmeyer, 2013; Barker, Byam, et al., 2010). Cord blood banks

list validated units giving minimal data required for search procedures through national registries and Bone Marrow Donors Worldwide (BMDW). Data include HLA typing at different resolution levels, the cell dose by the number of NC and sometimes and CFU are also given. Once a suitable CBU is identified, the CBB can be contacted for further requests such as extended HLA typing, post-thaw CFU and additional infectious disease testing as regulated by national legislations (Apperley et al., 2012). In CBU search and selection it´s important to know the quality standards of the CBB hosting the selected CBU (Barker et al., 2009; McCullough, McKenna, Kadidlo, Schierman, & Wagner, 2005), and in order to organize the development of quality assured cord blood banking, international standards have been developed by the NetCord working group (www.netcord.org) of the World Marrow Donor Association (WMDA) and the Foundation for the Accreditation of Cellular Therapy (FACT). The standards give requirements for all phases of donor management, CB processing, CBU testing, storage and distribution to clinical programs. CBB: s that successfully document that they comply with the standards receive FACT accreditation after on-site inspection. Forty-nine public CBB are as of August 2017, FACT accredited organizations, whereof 80% are situated in Europe or North America (www.factwebsite.org) FACT-NetCord accreditation status correlates with neutrophil engraftment in CBT (Purtill et al., 2014).

1.6.2 Cord blood transplantation

Cord blood is today an established cell source in HSCT next to BM and PBSC and over 30,000 CBT have been performed worldwide (Welte, Foeken, Gluckman, & Navarrete, 2010). There are specific benefits of CB as HSPC source, such as the low collection related risks involved for the donor and the reduced likelihood of transmitting viral infections such as CMV. CBB: s store validated HLA typed and frozen CBU: s that can be shipped immediately if the transplantation is urgent or problems arise concerning adult donor health or availability (Barker et al., 2011; Barker et al., 2002). As reviewed above CB contains a higher fraction of Tregs as compared to adult blood, and in CBT this plays out as a higher tolerance for HLA mismatch (Eapen et al., 2007; J. E. Wagner et al., 2002; Van Besien, Liu, Jain, Stock, & Artz, 2013), and relatively lower risk for both acute and chronic GvHD compared to BM or PBSC (Locatelli et al., 2013; Newell et al., 2013; Ponce et al., 2013; Terakura et al., 2016; Wang et al., 2010). This is of particular importance for recipients of racial and ethnic minorities for whom it is difficult to find matched unrelated adult donors in international registries (Barker et al., 2009; Gragert et al., 2014). Studies have also reported low rates of malignant relapse after CBT compared to HSCT with stem cells from unrelated adult donors, proposing that

CB may become the firsthand choice for patients with high risk of relapse (Atsuta et al., 2012; Brunstein et al., 2010; Eapen et al., 2007). There are also major drawbacks with the use of CB for HSCT instead of BM and PBSC from adult donors. The most obvious is the limited available cell dose in each CBU, expressed as either TNC or CD34+ cells, and cell doses in CBT are in general one log lower per kilo recipient compared to HSCT from adult sources (Kurtzberg et al., 2008; Mehta, Dave, Bollard, & Shpall, 2017). The low cell dose in CBT combined with the relative immaturity of the CB immune system leads to slower engraftment with prolonged cytopenia and slower immune reconstitution post-transplant, resulting in higher rates of TRM caused by viral or opportunistic infections, graft rejection and graft failure compared to BM or PBSC (Baron et al., 2015; Eapen, Klein, et al., 2014; Ruggeri et al., 2014; J. E. Wagner et al., 2002). Multiple retrospective studies have confirmed that CBT can achieve comparable DFS to that of adult donor transplants in patients with hematologic malignancies, in both children and adults, but prospective randomized studies are lacking. In the pediatric setting, where HSCT is also performed for non-malignant disorders such as inherited metabolic disorders and primary immune deficiencies, CB is sometimes the preferred cell source (Atsuta et al., 2012; Brunstein et al., 2010; Eapen et al., 2007; Gragert et al., 2014; Konuma et al., 2016; Milano & Boelens, 2015; Ponce et al., 2011; Wang et al., 2010). In the last few years the number of performed CBT worldwide have declined in favor of HSCT with haploidentical related donors, especially in low-income countries, where the cost of a CBU and the post-transplant care may constitute a barrier to the use of CB (Berglund, Magalhaes, Gaballa, Vanherberghen, & Uhlin, 2017; Dahlberg & Milano, 2017). The few retrospective comparative studies performed show similar OS at one year for CB and haploidentical HSCT, with higher TRM for CB but balanced by lower relapse rates in CBT, and further randomized studies are ongoing (Brunstein et al., 2011; Dahlberg & Milano, 2017).

Overcoming the limited cell dose and improving engraftment in CBT

The recommended TNC dose in CBT varies but is usually not lower than 2.5-3*10^7/kg for highly HLA matched CBU:s. Units with TNC ≥ 12.5*10^8 can be used for larger children and adults > 50 kg body weight, but such large dose CBU:s are rare in CBB inventories (Querol et al., 2010; Querol et al., 2009). The cell dose obstacle can be overcome by using two CBU:s that are infused together, a so called double unit cord blood transplantation (DCBT) Outcome data after DCBT is comparable to single unit CBT where a sufficiently large CBU could be acquired and no added benefits of DCBT have been shown (J. E. Wagner, Jr. et al., 2014). Also, co-transplantation of a matched CBU with HSPC from haploidentical related donors or unrelated mismatched adult

donors, haplo-cord, shortens the cytopenic period post-CBT through a transient engraftment of the mismatched cells before the CB HSCT establish long-time engraftment (Taskinen, Huttunen, Niittyvuopio, & Saarinen-Pihkala, 2014; van Besien & Childs, 2016). Another sought approach is to expand the number of HSPC in CB in *vitro* and a number of protocols have been proposed. Studies have used unselected, CD34+ or CD133+ selected cells from frozen thawed CBU, various types of culture media, cytokine cocktails frequently including SCF, co-culture with mesenchymal stromal cells and varying duration of culture periods (1-10 weeks) (Mehta et al., 2017). To avoid HSPC differentiation during expansion, various protocols have also been tried such as copper chelators (de Lima et al., 2008), continuous activation of Notch signaling (C. Delaney et al., 2010), aryl hydrocarbon receptor antagonists (Boitano et al., 2010), and most recently the vitamin B3 analogue nicotinamide (Horwitz et al., 2014). The expanded CBU has in most cases been co-transplanted with an unmanipulated unit. All studies achieved expansion of TNC and CD34+ cells and reduced time to neutrophil engraftment but in most cases the unmanipulated CBU provided long-time engraftment indicating that the protocols did not expand the most primitive HSPC: s (Mehta et al., 2017). As reviewed above pre-clinical studies indicate that CB HSPC are less responsive to SDF-1 gradients and engraft better injected directly into the bone marrow cavity in murine models (Castello et al., 2004; Kimura et al., 2007). Intra-bone marrow infusion into the posterior iliac crest has since then been tried in small clinical studies of CBT, but without conclusive evidence that time to engraftment is shortened using this approach (Brunstein, Barker, et al., 2009; Frassoni et al., 2010). Pre-transplant incubation of CBU: s with either prostaglandin E2 analogues or fucosyltransferases before infusion, oral treatment of recipients with dipepdidyl-peptidase-4 or hyperbaric oxygen treatment of the recipient are other investigated alternatives to improve homing. Lymphocyte populations from CB have also been used in adoptive cell therapies, including simple expansion of T-cells, virus specific T-cells, tumor specific lymphocytes and so called CAR-T cells (Berglund et al., 2017; Mehta et al., 2017).

1.7 Cord plasma proteomics

Proteomics is the study of proteomes, i.e. the full range of proteins produced in a biological system. Proteomics can be used to investigate proteins involved in a biological process and to identify biomarkers *i.e.* proteins that can act as indicators of a specific biological process, disease-associated or not. Commonly used methodologies include but are not limited to immunoassays and mass-spectrometry.

1.7.1 The proteomics of HSPC mobilization and homing

As discussed above, fetal hematopoiesis is in a constant and sequential state of migration between different hematopoietic sites during gestation. At birth CB holds similar levels of HSPC as BM, but the concentration declines rapidly during the first hours after birth (Gonzalez et al., 2009). The fetal biological processes governing these pre- and postnatal mobilizations and homing transitions are not well known. In the adult setting the process of mobilization and homing of HSPC is more researched. Close interaction between HSPC and stromal cells in the niche are mediated by membrane bound ligands and receptors such as the CXCR4 receptor expressed on HSPC with the CXCL12 (SDF-1) ligand on stromal cells and the VLA-4/VCAM-1 and CD44/hyaluronan/osteopontin interactions. HSPC mobilization, for instance following chemotherapy and as a result of G-CSF treatment, involves proteolytic degradation of these extracellular ligand-receptor pairs by proteases such as elastase and cathepsin G (Richter, Forssmann, & Henschler, 2017). Biomarkers of the mobilization process can be found in plasma. Szmigielska-Kaplon et al studied cytokines in plasma following mobilization with chemotherapy and G-CSF in hematological malignancies, and found significant increases in VCAM-1 that correlated with the concentration of CD34+ cells in peripheral blood, whereas SDF-1(CXCL12) levels decreased (Szmigielska-Kaplon et al., 2015). In patients with myeloma mobilization increased the levels of several cytokines, chemokines and growth factors such as CCL 2/3/4, CXCL 5/8/10/11, thrombopoietin, IL-4 and GM-CSF (Mosevoll et al., 2013). G-CSF treatment of healthy HSPC donors increased the concentration of several cytokines in plasma, including matrix metalloproteinase-9 (MMP-9) osteopontin, tumor necrosis factor α (TNF-α) and IL-6. Pre-mobilization levels of TNFα and IL-6 correlated with CD34+ cell mobilization efficacy (Lysak et al., 2011; Melve et al., 2016). In the fetal-neonatal setting, investigation of CB plasma from full term neonates found high levels of G-CSF, GM-CSF, flt3L, IL-11, SCF and EPO compared to adult plasma samples (Gonzalez et al., 2009; Laver et al., 1990). The level of EPO in neonatal plasma after birth correlated with the decline of CD34+ cells concentration in peripheral neonatal blood, suggesting that oxygenation played a part in the homing of HSPC to BM, also the fraction of CD34+ cells expressing CXCR4 declined in neonatal blood after birth consistent with an ongoing homing process (Gonzalez et al., 2009) The concentration of CCL 28 in cord plasma has been shown to correlate with the number of CD34+ cells in the corresponding CBU (Yoon et al., 2015).

AIM

The overall aim of this thesis was firstly to give an account of the achievements of the national Swedish cord blood bank from an international perspective and secondly to develop more efficient methods to define the functional hematopoietic stem and progenitor cell pool in human cord blood units intended for hematopoietic stem cell transplantation.

1.8 Specific aims

- To summarize and compare, from an international perspective, the experiences and current status of the National Swedish Cord Blood Bank with special focus on the impact of late versus early cord clamping on cord blood collection efficiency (paper I).
- To develop new methods using flow-cytometry for increased resolution of the hematopoietic stem and progenitor cell pool regarding functionality and differentiation, which correlate better with results from HSPC cultivation assays than standard methods currently in clinical use (Papers II and III).
- To screen for possible biomarkers in cord plasma that can predict the corresponding HSPC content in cord blood using a multiplex immunoassay (Paper IV)

2 PATIENTS AND METHODS

2.1 The National Swedish cord blood bank

2.1.1 Inception

The national Swedish cord blood bank (NSCBB) was founded in 2005 as part of a governmental decision. The aim was to create an altruistic CBB holding approximately 5000 CBU with focus on collections from ethnic minorities. The bank was awarded International Foundation for the Accreditation of Cellular Therapy (FACT) approval in 2013.

Figure 5. The official logo of the National Swedish cord blood bank.

2.1.2 Cord blood collection

Cord blood is collected at two obstetric wards in Sweden, Sahlgrenska University Hospital/Östra in Gothenburg and the Karolinska University Hospital/Huddinge in Stockholm. All pre-donation information material, medical questionnaires and consent forms are available in 12 different

languages. Written consent from both parents is obtained prior to collection if married or cohabitating. Collecting midwifes are employed by the NSCBB and dedicated to CB collection only. According to NSCBB policies the cord is clamped 60s after birth and collection is made with placenta in utero after sterile cannulation of the umbilical vein. CB is collected in a sterile bag containing 29 ml CPD solution (MacoPharma, Turcoing, France) Collections are kept in a controlled environment of 16-25 °C and no units older than 44h are processed. The ethnic background of both parents is specified according to the European marrow donor information system (EMDIS).

2.1.3 Donor selection

Collections are only made from normal simplex pregnancies past 37 full weeks. A detailed medical history is taken from both parents to exclude donations from families with a history of inherited metabolic, hematopoietic or immune system disorders. Parents with a history of malignant diseases or infectious disorders that may be transmitted to the child are deferred. Mothers with a history of infectious risk behavior are not accepted. All children are evaluated after birth, and CB is only collected after uncomplicated births with normal Apgar scores, normal birth weight and no apparent malformations.

2.1.4 Cord blood processing

A full blood count is performed on all collections before and after processing. (CellDyn Sapphire, Abbot Diagnostics, Chicago, USA). Since January 1 2014 further processing is only performed on units with a TNC, *i.e*. the sum of all NC including NRBC, above 15 $x10^8$ for donors of Caucasian origin and above 12.5 x 10^8 for donors with both parents of non-Caucasian origin. Post-processing limits are 12.5 $x10^8$ and 10 $x10^8$ respectively. A volume and erythrocyte reduction step is executed using the Sepax instrument (Biosafe, Eysins, Switzerland) after the addition of 20% hydroxy-ethyl starch (Grifols, Los Angeles, USA). The resulting cord blood buffy coat (CBBC) of 21 ml is supplemented with 10% DMSO (WAK-Chemie, Steinbach, Germany) before controlled rate freezing in the BioArchive (Thermogenesis, Rancho Cordoba, USA) Collections with volumes above 185 ml are divided and processed in two separate runs to increase recovery of NC (non-published validation data from the NSCBB)

2.1.5 CBU definition and quality

The total number of nucleated cells is given for each banked CBU, and the total number of viable cells expressing the surface markers CD34 and CD3 are quantified using flow cytometry (BD FACSCalibur, BD Trucount tubes, BD

Biosciences, SanJose CA, USA). Also, one ml aliquot of each CBBC is sampled to a separate vial just before freezing of the unit and subsequently thawed to determine post-thaw Trypan blue viability and CFU (StemCell Technologies, Vancouver, Canada). Until March 2017 all units were typed in-house at the intermediate resolution level using PCR-SSO (LABType, OneLambda, Woodland Hills, USA) for the HLA-A, HLA-B, HLA-C, DRB1 and DQB1 loci, and since then for the same loci using high-resolution HLA Sequence-based Typing (SBT) at the Histogenetics LLC laboratory (Ossining NY, USA) Sterility checks and screening for hereditary hemoglobinopathies using high performance liquid chromatography is also performed for each CBU. Serologic testing for Syphilis, HTLV, Hepatitis B, Hepatitis C, CMV and HIV is performed on maternal serum and CB plasma.

2.1.6 The Tobias registry

The Tobias Registry (www.tobiasregistret.se) was founded in 1992 and is the Swedish registry for unrelated altruistic donation in hematopoietic stem cell transplantation. Today it holds approximately 80 000 registered adult donors. Also, all NSCBB CBU: s are searched and requested through the Tobias Registry.

2.2 Flow-cytometry

2.2.1 Basics

Flow-cytometry is a laser-based technology developed for definition of single cells in a heterogeneous solution of cells trough differences in expression of surface and intracellular molecules. The methodology measures fluorescence intensity emitted from a wide range of fluorochromes that accumulates intracellularly or binds surface molecules through fluorochrome-conjugated antibodies. A wide range of fluorochromes are available allowing multi-parameter definition of cells.

2.2.2 Technical details

Measurements in paper II and III were performed using a FACS Aria II instrument (FACS Aria II, BD Biosciences), equipped with a 375 nm near-UV laser, a blue 488 nm laser and a red 640 nm laser. Resulting data was analyzed using the BD FACSDiva software (FACSDiva, BD Biosciences). For all assays unspecific staining was investigated using isotype controls when appropriate.

2.2.3 Surface markers

In paper II and III HSPC were investigated trough measurement of surface expression of CD45 (expressed on all nucleated hematopoietic cells), CD 117 (expressed on HSPC and common myeloid progenitor cells), CD 38 (expressed on mature leukocytes), CD 235a (expressed on immature and mature erythrocytes) and CD 34 (primarily expressed on HSPC)

2.2.4 Side Population

The Side Population (SP) assay is also known as the Hoechst dye efflux assay (figure 3). It was originally described by Goodell and colleagues (Goodell et al., 1996) during a study of murine HSPC and is based on the DNA-binding vital dye Hoechst 33342 that effectively penetrates the cell membrane of living cells. The Hoechst dye is effectively eliminated from the cytoplasm by the membrane efflux pumps of the ATP-binding cassette transporter superfamily, which can be inhibited by pharmacological drug transporter inhibitors such as Verapamil and Fumitremorgin C (Zhou et al., 2001). The staining procedure was performed in paper II according to previous publications (Pearce & Bonnet, 2007; Pierre-Louis et al., 2009; Sandstedt et al., 2012). In short, nucleated cells were incubated in medium supplemented with Hoechst 33342 at 37 °C for 45 minutes (staining period), followed by resuspension in new medium and re-incubation for 45 minutes (efflux period). Verapamil was added to the control tube and stained in parallel. Hoechst fluorescence was detected with a 375 nm near-UV laser based on fluorescence in both Hoechst red and Hoechst blue channels.

2.2.5 Aldehyde dehydrogenase (ALDH) assay

Cytosolic aldehyde dehydrogenase activity is elevated in HSPC and can be used to identify these cells (Christ et al., 2007; Storms et al., 2005; Storms et al., 1999). Cells are incubated for 30 minutes at 37°C with a fluorescent substrate of the ALDH enzyme (Aldecount substrate, Stemcell Technologies, Vancouver, British Colombia, Canada), which readily permeates the cell membrane and becomes charged after cleavage resulting in accumulation in viable cells with an intact cell membrane. Thus, there is no need for separate viability staining. The cleaved substrate is excited with the blue laser and emits in the FITC channel (515-545 nm). An ALDH enzyme inhibitor (diethylaminobenzaldehyde, DEAB, Stemcell Technologies) is added in a separate tube and is used to define the population with increased enzyme activity (figure 2). The staining procedure was performed in paper II to define the HSPC population in CB and in paper III to investigate apoptosis in cells

with high ALDH activity in frozen and thawed CB. Cells high in ALDH activity are named ALDH+ cells herein and in papers II-III.

2.2.6 Viability and apoptosis

In papers II and III exclusion of non-viable cells in flow cytometry analysis was performed using the DNA binding dye 7-aminoactinomycin-D (7-AAD) 7-AAD stains non-vital cells because it can only enter the cytoplasm of cells with a damaged cell membrane. 7-AAD is excited with a 488 nm argon laser and emits in the far red range of the spectrum (650 nm long-pass filter). Apoptosis, programmed cell death, was investigated in paper III using Annexin V binding. In the early stages of apoptosis the cell exposes negatively charged phosphatidylserine on the outer cell membrane to which the Annexin V protein can bind through a calcium-ion bridge. In the Annexin V assay fluorochrome conjugated Annexin V is allowed to bind to the cell surface of apoptotic cells in a calcium supplemented buffer (BD Pharmingen, San Diego, USA)

2.3 Cell cultivation assays

2.3.1 CFU assay

The colony-forming-unit (CFU) assay is an in vitro functional assay used to study HSPC (figure 4). It is based on the ability of lineage committed hematopoietic progenitor cells, so called CFU: s, to proliferate, differentiate and form colonies of progeny in cytokine-supplemented semisolid media (H84435, StemCell Technologies). Colonies are classified according to lineage and counted manually under the microscope after 14 days of culture in an incubator (Pamphilon et al., 2013). In all papers the CFU assay was performed on frozen and thawed CBBC and the number of granulocyte-monocyte colonies (CFU-GM) and the total number of growing colonies, CFU-total, were given for each investigated CBU.

2.4 Cord plasma proteomics

2.4.1 Protein biomarker panel

The protein biomarker profile in cord plasma was investigated in paper IV trough simultaneous analysis of 92 unique protein biomarkers involved in apoptosis, cell adhesion and the cellular response to cytokine stimulus (Inflammation panel, Olink proteomics, Uppsala, Sweden)

2.4.2 Proximity ligation assay

The multiplex immunoassay rests on the so-called proximity ligation assay (PLA). Two primary antibodies with complementary single stranded DNA bind to the target protein and the DNA strands hybridize if in close proximity. Finally, the resulting double stranded DNA sequence is detected and quantified with subsequent real-time PCR. The assay yields semi-quantitative protein expression data on all biomarkers included in the panel, expressed as "normalized protein expression" NPX.

2.5 Statistical methods

2.5.1 Student's t-test

Student's t-test is used to compare the means of a numerical variable between two investigated groups whose numerical results are assumed to be normally distributed. In paper I Student's t-test was performed to compare results from early and delayed clamping procedures respectively. For the multiplex immunoassay on CB plasma in paper IV a Benjamini-Hochberg procedure (Klipper-Aurbach et al., 1995) was performed to compensate for multiple comparisons. The false discovery rate (FDR) was set to 0.15 and FDR adjusted p-values were calculated to evaluate significant differences in expression of individual proteins between CBU: s high or low in HSPC concentration.

2.5.2 Spearman's correlation

Spearmans's rank correlation coefficient also named Spearman's rho was used to investigate correlations between variables in paper II and III. Spearman correlation investigates statistical dependence between the rankings of results from two measured variables. Spearmans's rank correlation is the non-parametric equivalent of the Pearson correlation and is recommended in small datasets and if results are not normally distributed.

2.5.3 Principal component analysis

Principal component analysis (PCA) was used to find and visualize differences in protein expression between CBU: s high or low in HSPC concentration in paper IV. PCA can be explained as a statistical procedure that identifies the variables that best explain the variance in the dataset.

2.5.4 Ordinary multiple regression

A multiple regression model was built in paper IV to investigate how results for the most significantly different CB plasma protein in the array contributed

together with clinical data from collection, delivery and child related variables on resulting HSPC concentration in the CB collection. Regression analysis investigates how changes in independent variables (protein concentration and clinical data in paper IV) influences the results in the dependent variable (HSPC concentration in the collected CB in paper IV). The model was built using a so called stepwise approach were only significant parameters were kept in the final model.

3 RESULTS AND DISCUSSION

3.1 High quality cord blood banking is feasible with delayed clamping practices. The eight-year experience and current status of the national Swedish Cord Blood Bank (I)

The era of cord blood transplantation began in the early 1990s. CBT has since then become an established alternative to HSCT with HSPC from BM or PBSC and outcomes are comparable (Alfraih, Aljurf, Fitzhugh, & Kassim, 2016; Atsuta et al., 2012; Brunstein et al., 2010; Eapen et al., 2007; Gragert et al., 2014; Kanakry, de Lima, & Luznik, 2015; Konuma et al., 2016; Milano & Boelens, 2015; Ponce et al., 2011; Wang et al., 2010). CBB holding validated ready-for-use CBU have been founded worldwide. The National Swedish Cord Blood Bank (NSCBB) situated at Sahlgrenska University Hospital in Gothenburg was initialized in 2005 as a result of a governmental resolution. The NSCBB specifically aims to collect CB from ethnic minorities and immigrant groups, as patients with non-Caucasoid ethnic backgrounds find fewer HLA-matched donors in the worldwide adult donor registries. (Tiercy et al., 2007). New obstetric practices, *i.e*. delayed cord clamping might affect CB volume and cell dose collected (Allan et al., 2016; Naing et al., 2015; Nakagawa et al., 2004). We compiled the 8-year experience (2006-2014) and the status of the NSCBB in August 2014. Further, we investigated the impact of a change of practices from immediate to late cord clamping (60s) on collected cell volume and cell content.

By August 31st, 2014 the NSCBB held close to 5000 CBU, which is a small bank on an international scale (Howard et al., 2008; Song et al., 2014). With current TNC processing limits (donors of Caucasian origin; $15*10^8$ and non-Caucasian origin $12.5*10^8$) the discard rate was 64%, close to the 62% anticipated from an analysis of collections from the Barcelona CBB in 2009 with a theoretical TNC cut-off of $12.5*10^8$ (Querol et al., 2009). Considering that NS-CBB applied delayed clamping practices and a higher TNC cut-off level of $15*10^8$ for 80% of collections (Caucasian donors) this means a comparatively high CB collection efficiency. Still, the median TNC of the CBU released for transplantation from the NSCBB was 22.5 $*10^8$, indicating a demand for even larger units, a discrepancy previously reported by other CBB (Gutman et al., 2011; Lee et al., 2013; Querol et al., 2010). In 2014, 30% of stored CBU were from donors of non-Caucasian origins, and although this was far from the set aim of 50% it corresponded to the ethnicity of the CBU: s

requested for transplantation. Also, as reported by Haimila et al in 2013, the HLA haplotype diversity of the NSCBB inventory was greater compared to three CBB: s in Finland, Belgium and Japan (Haimila, Penttila, Arvola, Auvinen, & Korhonen, 2013). In the summer of 2012 most obstetric units in Sweden had responded to the findings from Andersson et al in 2011, indicating a slight increase in iron deficiency in children subjected to immediate clamping (Andersson et al., 2011), and implemented delayed cord clamping practices. In a retrospective study, consecutive CB collections performed before (n=146) and after (n=162) the change from immediate to delayed cord clamping practices (60 seconds after birth) were compared. We found that although the volume of the collections, slightly but significantly, decreased (mean volume early clamping 119 ml and delayed clamping 111 ml, p=0.02), the total TNC did not (p=0.1). This lends support to and promotes the notion that high quality cord blood banking is feasible with delayed clamping practices.

3.2 Exploring the heterogeneity of the hematopoietic stem and progenitor cell pool in cord blood: simultaneous staining for side population, aldehyde dehydrogenase activity and CD34 expression (II)

In recent years focus in CBB has shifted from quantity to quality, following the insight that less than 1 in 10 stored CBU: s are ever requested for transplantation (Querol et al., 2010; Saccardi et al., 2016). To identify high quality units the individual CBB must command assays that can thoroughly appreciate the CBU HSPC content and function in a material-sparing, high-throughput and cost-effective manner. To address this issue we developed a flow-cytometry based protocol for simultaneous definition of several HSPC properties in viable immature mononuclear cells (*i.e*. weakly expressing the leucocyte CD45 antigen, CD45dim); the surface markers CD34 and CD117, the activity of the intracellular enzyme ALDH and the activity of the membrane efflux pumps of the ATP-binding cassette transporter superfamily through the Hoechst 33342 Side population (SP). In 4 hours and using only 200 µl CBBC from each CB collection (n=30), we found that the median size of the ALDH+ population was 3% (range 1-28%) and the SP 0.9% (0.1-5.3%) of viable CD45dim cells. There was no overlap between the ALDH+ and SP populations and they exhibited dissimilar surface marker expression. The ALDH cells were CD34+ and CD117+ whereas the SP population was negative for both markers.

Also, the fraction of ALDH+ cells correlated (r=0.4, p=0.03) with the number of CFU-GM colonies from the 14 day CFU assay in contrast to the SP (p=0.69). Results taken together, and also building on previously published data, this implies that the SP assay distinguishes a separate more immature HSPC pool and that the ALDH assay defines a more committed stem cell pool primarily of the myeloid lineage (Alt et al., 2009; Challen & Little, 2006; Christ et al., 2007; Goodell et al., 1997; Pearce & Bonnet, 2007; Storms et al., 2005). In summary, we have designed a functional assay able to appreciate the heterogeneity of the HSPC population in CB feasible for implementation in routine cord blood banking procedures.

3.3 The aldehyde dehydrogenase cord potency assay intrinsically excludes early apoptotic cells (III)

Engraftment, *i.e*. restoration of hematopoiesis, after HSCT relies on a sufficient infused dose of functional HSPC. In this context CBT stands out, since the HSPC cell dose is generally only 5-10% of the corresponding number in BM and PBSC HSCT (Kurtzberg et al., 2008; Mehta et al., 2017). Also, as opposed to BM and PBSC grafts, CBU: s are frozen, stored for years and thawed before use, manipulations that affect the functionality of HSPC (Ikeda, Toyama, Matsuno, Fujimoto, & Isoyama, 2013; Schuurhuis et al., 2001). Consequently, delayed engraftment and graft failure are more frequent in CBT (Alfraih et al., 2016; Kanakry et al., 2015). In 2012 Duggleby et al published results indicating that exclusion of apoptotic CD34+ cells improved correlation between results of the CD34+ cells and the CFU assay (Duggleby et al., 2012). We investigated whether the enzyme-activity based, and hence functional, ALDH HSPC assay, intrinsically *i.e*. by itself excluded not only viable but also apoptotic cells. Apoptotic cells were defined as exhibiting membrane bound Annexin V (Annexin V+), through exposure of the negatively charged phospholipid phosphatidylserine. CBBC from frozen and thawed randomly selected CBU: s (n=57) were analyzed for viability (7-AAD), CFU: s and surface expression of the CD45, CD34 and Annexin V markers. In our study, the ALDH+ population included a smaller fraction of apoptotic cells compared to the viable CD34+ population, in median 0.4% (range 0-4.9%) compared to 1.9% (range 0-15.8%). Also, the ALDH+ population correlated better (r=0.72, p<0.0001) with the number of CFU-GM colonies from the CFU assay than both the viable CD34+ and the viable non-apoptotic CD34+ population (r= 0.66 p<0.0001 and r=0.68 p<0.0001) Taken together, our results support our primary hypothesis, that the ALDH assay omits the need for separate viability and apoptosis staining and hence in itself excludes non-viable and apoptotic

cells. The reasons for this might be that the ALDH assay identifies a population of more immature HSPC:s that are more resilient to apoptosis compared to the CD34+ population or that the activity of the cytosolic ALDH enzyme is reduced in apoptosis. Notably, the ALDH assay has recently been shown to be implementable in large-scale pre-transplant quality testing of CBU: s (Shoulars et al., 2016). Taken together we suggest that the ALDH assay might replace the CFU assay for pre-transplant evaluation of CBU HSPC content.

3.4 Concentration of the CDCP1 protein in human cord plasma may serve as a predictor of hematopoietic stem cell content (IV)

The measurement of HSPC in CB relies on definition of cellular properties; which means that assays expend cellular material. CB processing leaves surplus cord plasma that may express biomarkers that correlate with the HSPC concentration in the corresponding CB collection. Several previous observations on perinatal physiology, CB HSPC properties and CB plasma composition lend support to this hypothesis (Castellano et al., 2017; Gonzalez et al., 2009; Laver et al., 1990; Majka et al., 2001; Yoon et al., 2015). In order to screen for such factors frozen surplus cord plasma samples from 95 CBU were selected and divided in two groups based on CD34+ cells concentration in the corresponding CB collection. Units with $\geq$ 50 cells/µl were considered as high (n=47) and units with $\leq$ 40 cells/µl as low (n=48) in HSPC content. Units with intermediate CD34+ cells concentration (40-50 cells/µl) were not included in the study. All CB plasma samples were investigated with a multiplex immunoassay based on the PLA technology covering 92 protein biomarkers involved in cellular responses to cytokine stimulus, cellular adhesion, extracellular matrix organization and apoptosis (Proseek Multiplex Inflammation Panel, Olink Proteomics Uppsala Sweden) Semi-quantitative expression data were acquired as NPX. Clinical data on CB collection volume, child gender, birth weight, placental weight, gestational age and mode of delivery was also available. Comparison between groups high and low in CD34+ cells concentration identified five proteins that significantly differed in concentration between groups, whereof CUB-domain containing protein 1 (CDCP1) was the most significant (FDR adjusted p=0.006) Also, the CDCP1 level correlated with the concentration of CD34+ cells and the number of CFU colonies in the corresponding CB collection (CD34+ r=0.54 p< 0.0001, CFU-total r=0.30 p=0.003) To further investigate the relative impact of clinical data and CB collection volume compared to CD34+ cells concentration on CDCP1

plasma concentration, we used ordinary multiple linear regression. In the final model only child gender and CD34+ cells concentration variables were significant ($p= 0.03$ and $p<0.0001$), but the model could only explain 25% of the total variation in CDCP1 concentration ($R_2=0.25$). CDCP1 is a cell surface transmembrane glycoprotein proposed to be involved in regulation of cellular differentiation and proliferation through interactions with extracellular matrix proteins. CDCP-1 is expressed on immature hematopoietic cells with repopulation capacity in NOD/SCID mice (Buhring et al., 2004; Conze et al., 2003). In addition to previous knowledge on the function and expression of CDCP1, our present results imply that CDCP1 may be a potential biomarker for HSPC content in CB and warrants further investigation.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

Allogeneic hematopoietic stem cell transplantation is the only curative option for many patients with leukemia, myelodysplasia, lymphoma and severe inherited disorders. Only about 30% of these will have a HLA-matched sibling donor. The rise of alternative HSPC sources; matched unrelated donors, umbilical cord blood units and related haplotype identical donors, means that *in 2017 there is virtually a donor for every patient* (Kindwall-Keller 2017).

The National Swedish cord blood bank has since its inception in 2005 become a quality certified unit that holds up well in international comparison (Guerra-Marquez et al., 2011; Lauber et al., 2010; Liu et al., 2012; Page et al., 2014). We are still able to collect high quality units after implementation of delayed clamping practices (for the safety of the CB donor) in 2012 and are closing up on our goal of 5000 stored CBU: s. The inherent logic of cord blood banking means that the best units go first, *i.e* our collection efforts must continue albeit at a slower pace to keep a high-quality inventory even after our objectives are met. Even so, the accelerated development of approaches to expand the HSPC pool, augment HSPC homing and engraftment might mean that the CBU cell dose becomes less critical in a few years (Berglund et al., 2017; Mehta et al., 2017).

Successful CBT rests on correct estimation of the HSPC dose, HSPC heterogeneity and functionality of the unit/units in question both pre-freeze and post-thaw. TNC and CD34+ cells are established surrogate markers for HSPC dose and results are through international efforts of societies such as ISHAGE comparable between transplant centers and CBB: s (Whitby et al., 2012). For the functional CFU assay the situation is altogether different and despite efforts standardization has been difficult (Pamphilon et al., 2013; Powell et al., 2016). Our combined SP/ALDH staining protocol, described herein, allow for fast, material-sparing and simultaneous determination of both HSPC heterogeneity and functionality based on ISHAGE guidelines. We have also confirmed the excellent correlation described by others between ALDH and CFU results in the CBB setting (Shoulars et al., 2016), and propose that this may be explained by the ALDH-assay`s capacity to exclude apoptotic cells. We suggest that time is ripe to retire the CFU assay for the benefit of the ALDH-assay.

The perinatal physiology behind the BM-like levels of HSPC in CB is intriguing and as yet mostly unknown (Gonzalez et al., 2009). One could try to extrapolate knowledge from the adult setting, where biomarkers of mobilization can be measured in plasma (Lysak et al., 2011; Melve et al., 2016; Mosevoll et al., 2013; Szmigielska-Kaplon et al., 2015). CB plasma is an available surplus material since most CBB: s use volume reduction steps to streamline storage space. Using a multiplex immunoassay we identified the CDCP1 protein as candidate biomarker for CD34+ cell content in the corresponding CB collection. Interestingly enough, CDCP1 is expressed on HSCT and its interactions with BM stroma commands intracellular signaling pathways controlling cellular differentiation (Buhring et al., 2004; Conze et al., 2003), supporting the notion that that CDCP1 may actually be involved in HSCT mobilization. We do not know if the full protein or only parts of it is present in CB plasma, so to refine our findings this must be investigated through further proteomic studies. Also, the CDCP1 concentration does only explain a fraction of the variability in CD34+ cells concentration in CB plasma, so other supporting factors must be identified before CB plasma biomarkers can be considered to replace cell-based HSPC surrogate assays.

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