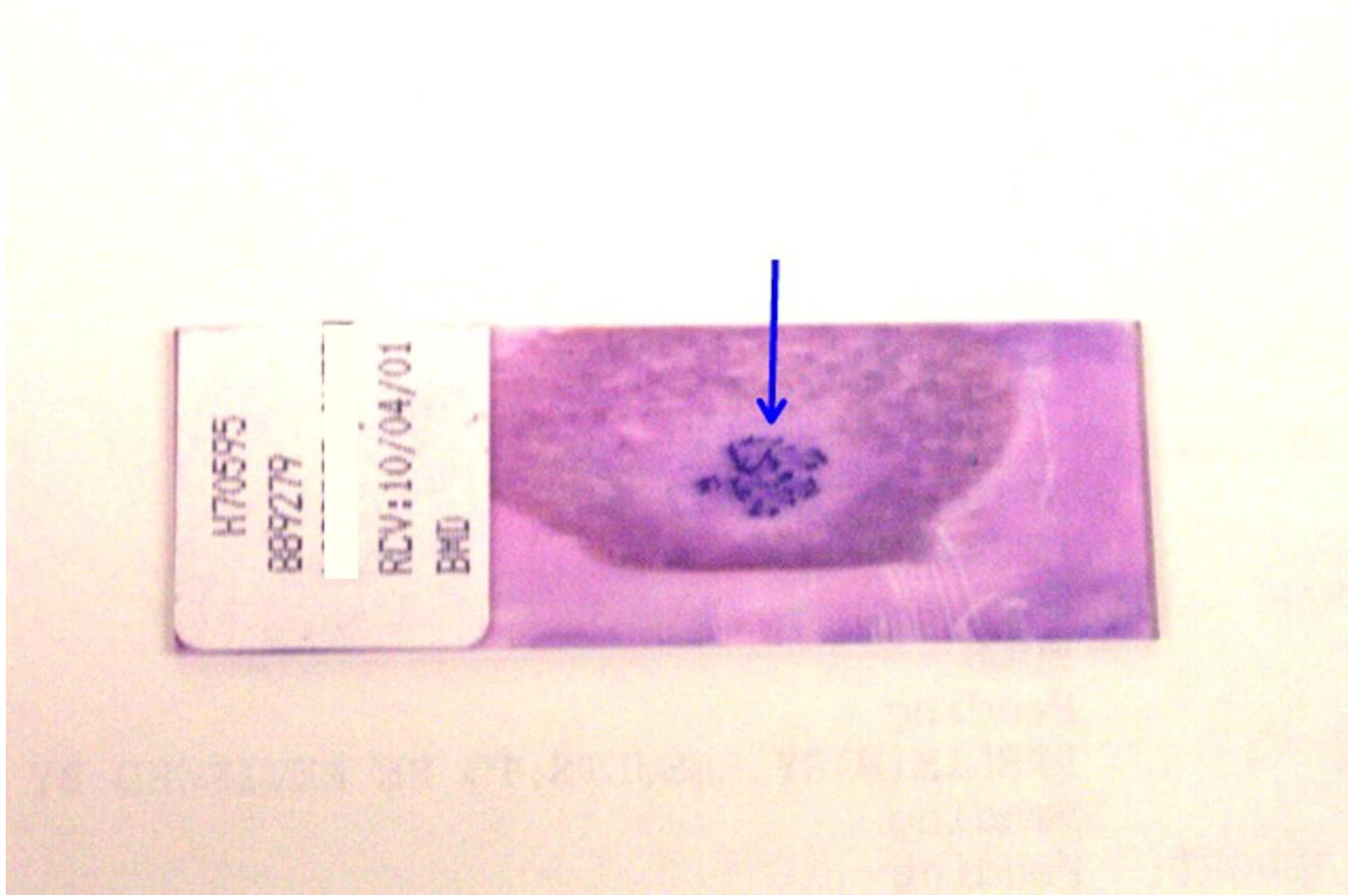
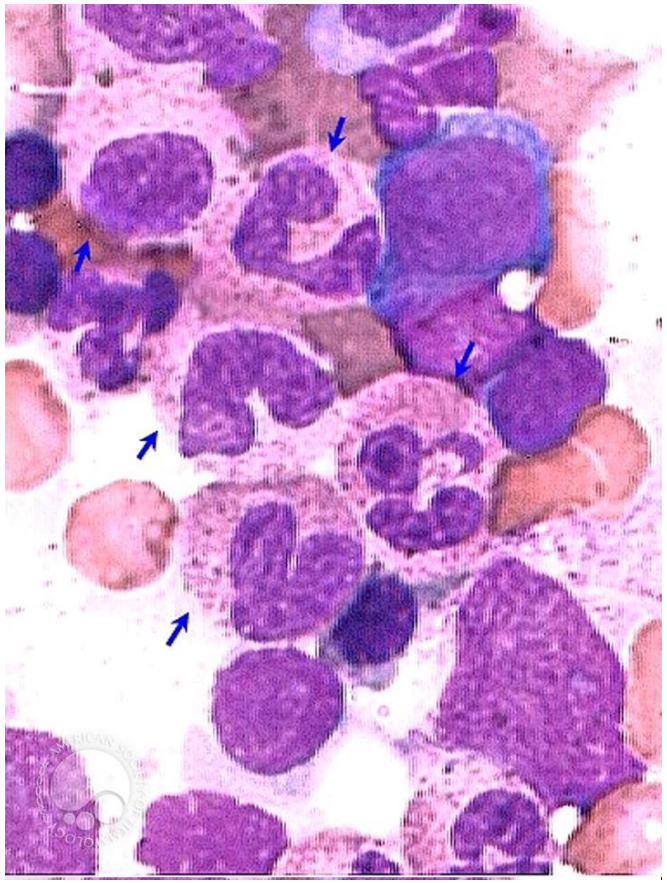
12. Studio delle cellule staminali

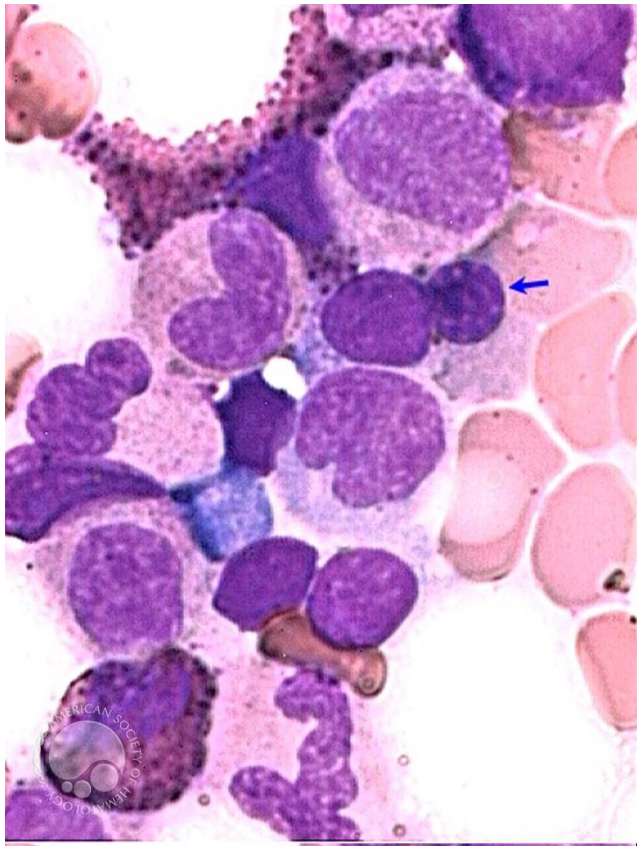
Prof. Gian Matteo Rigolin
Ematologia
Azienda Ospedaliero Universitaria
Arcispedale S. Anna Ferrara

Biopsia midollare: vetrino



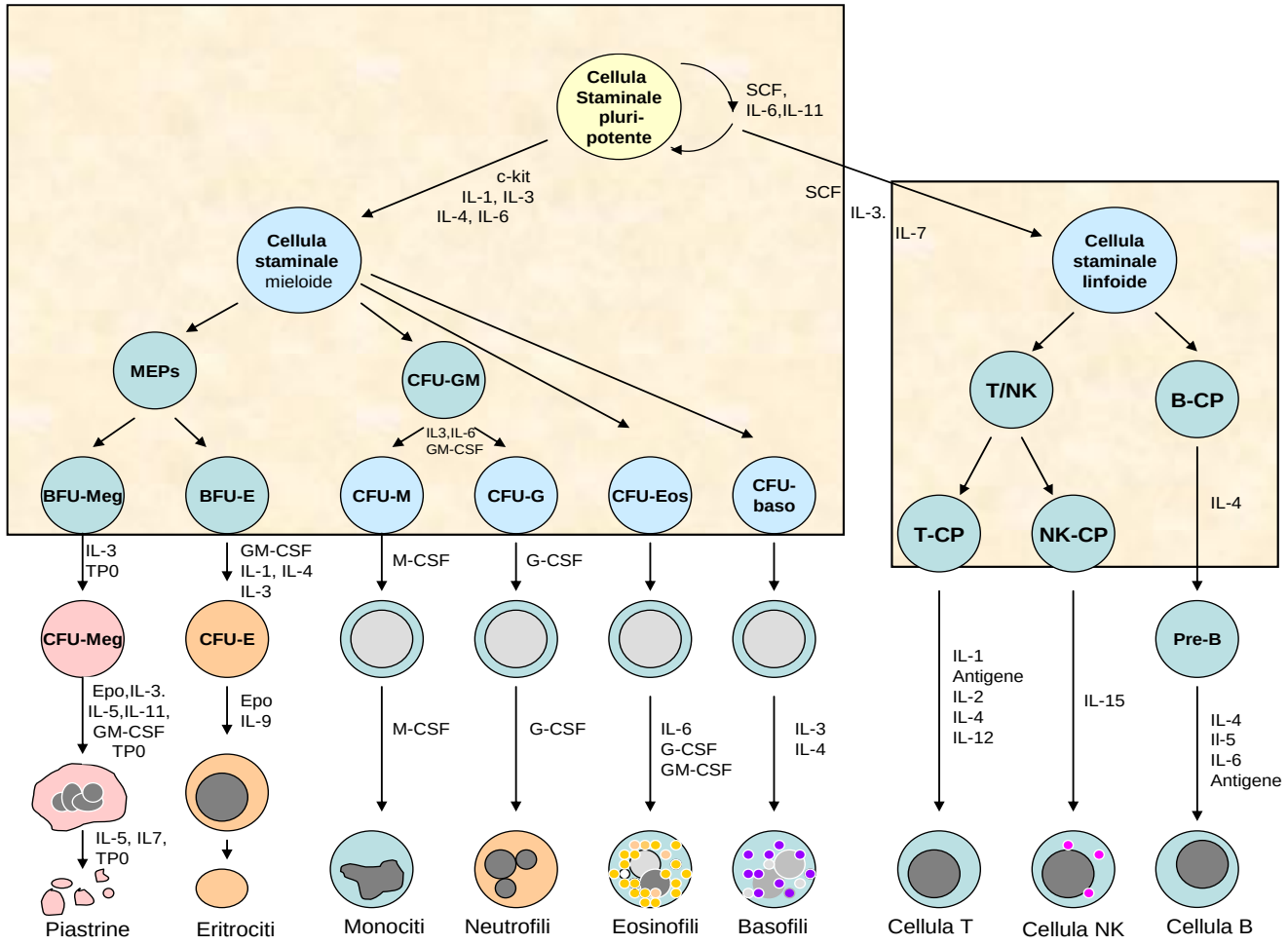
Normal bone marrow





ASH image bank

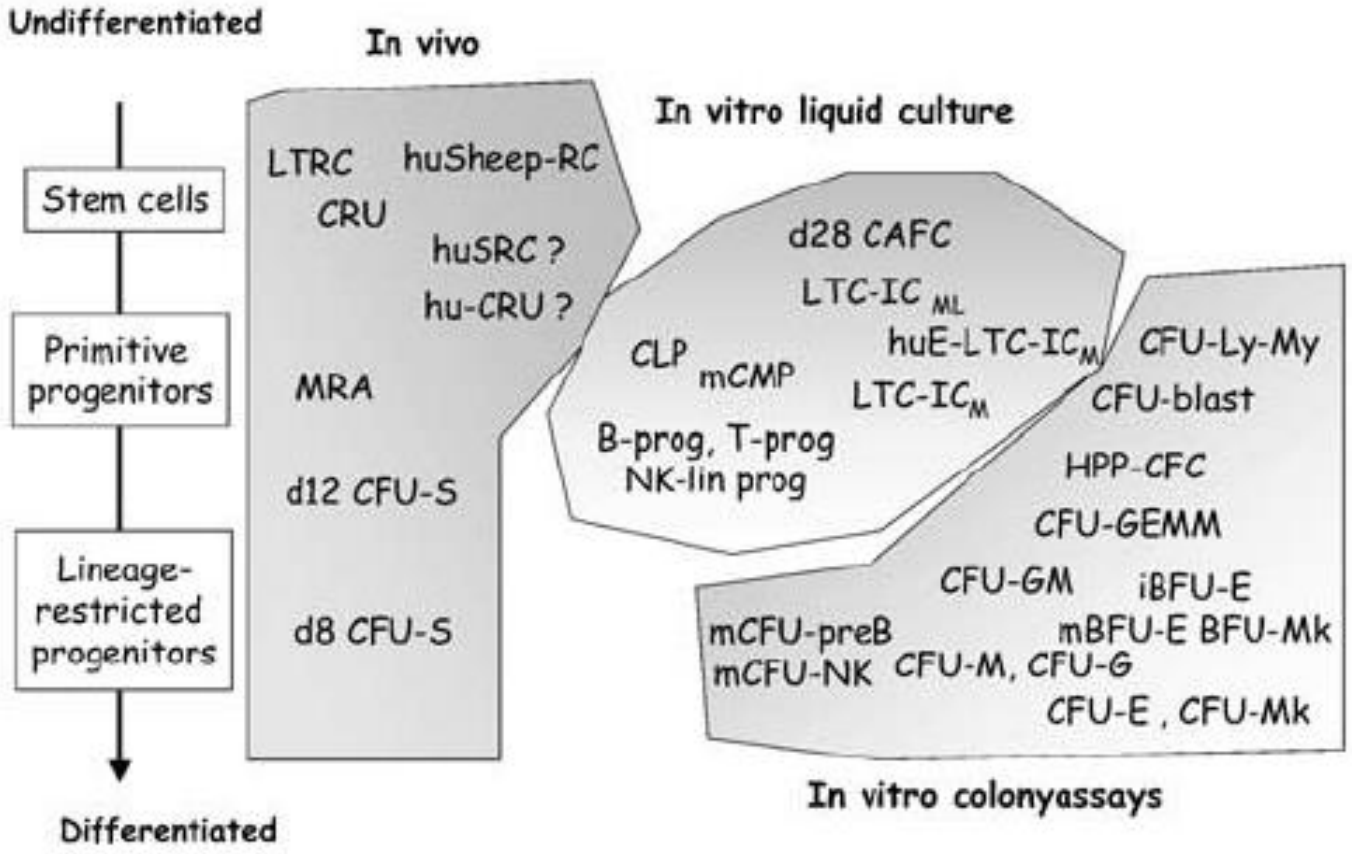
Hemopoiesis



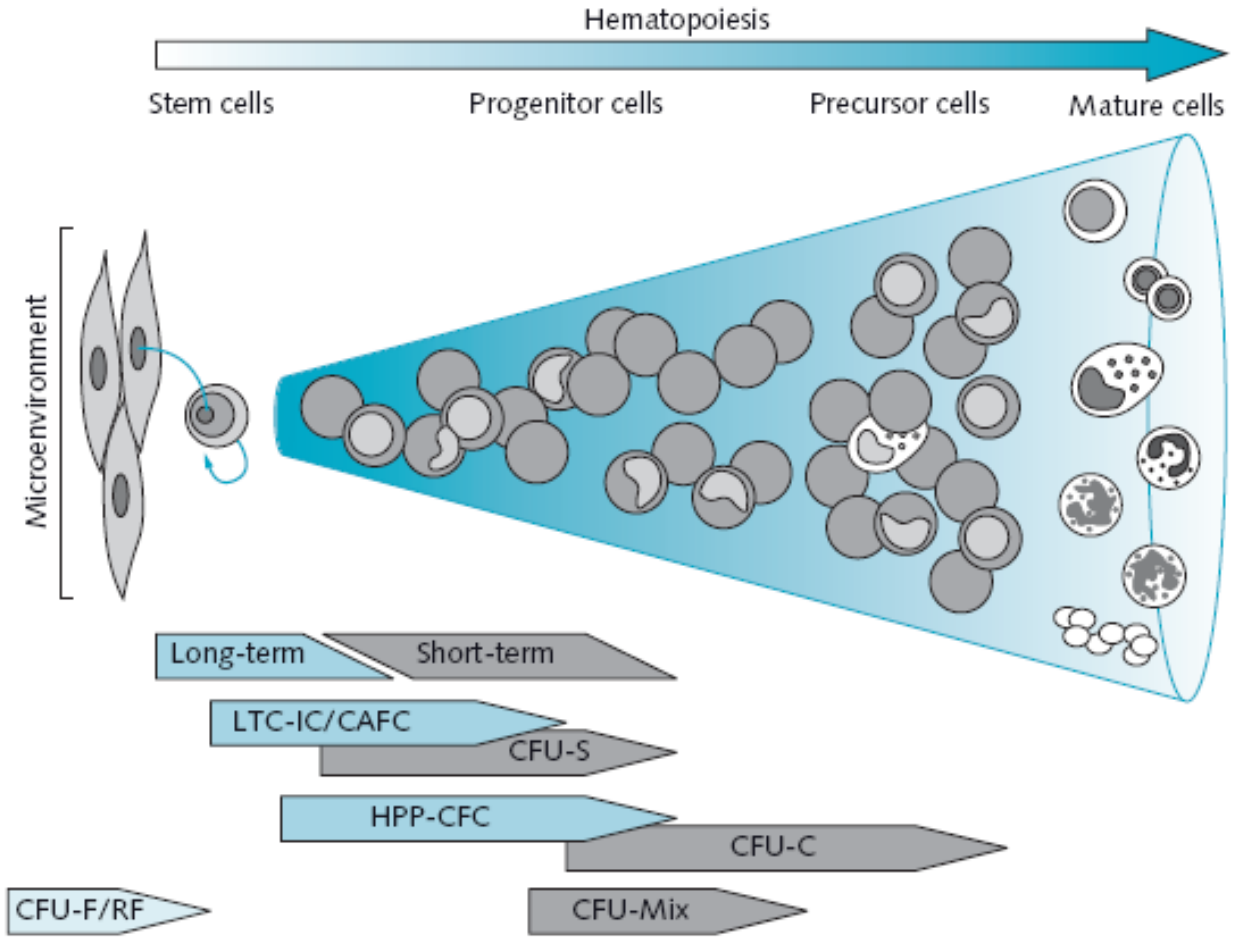
Functional analysis of HSCs

- Functional assays for HSCs **do not actually measure the activity of HSCs** but instead assess more differentiated progeny, such as progenitor and precursor cells.
  - *in vitro* assays measure mature populations,
  - *in vivo* assays detect the activity of primitive cells capable of homing and engrafting in the proper microenvironment to produce functional hematopoietic progeny.

Functional analysis of HSCs



Schematic view of hematopoiesis



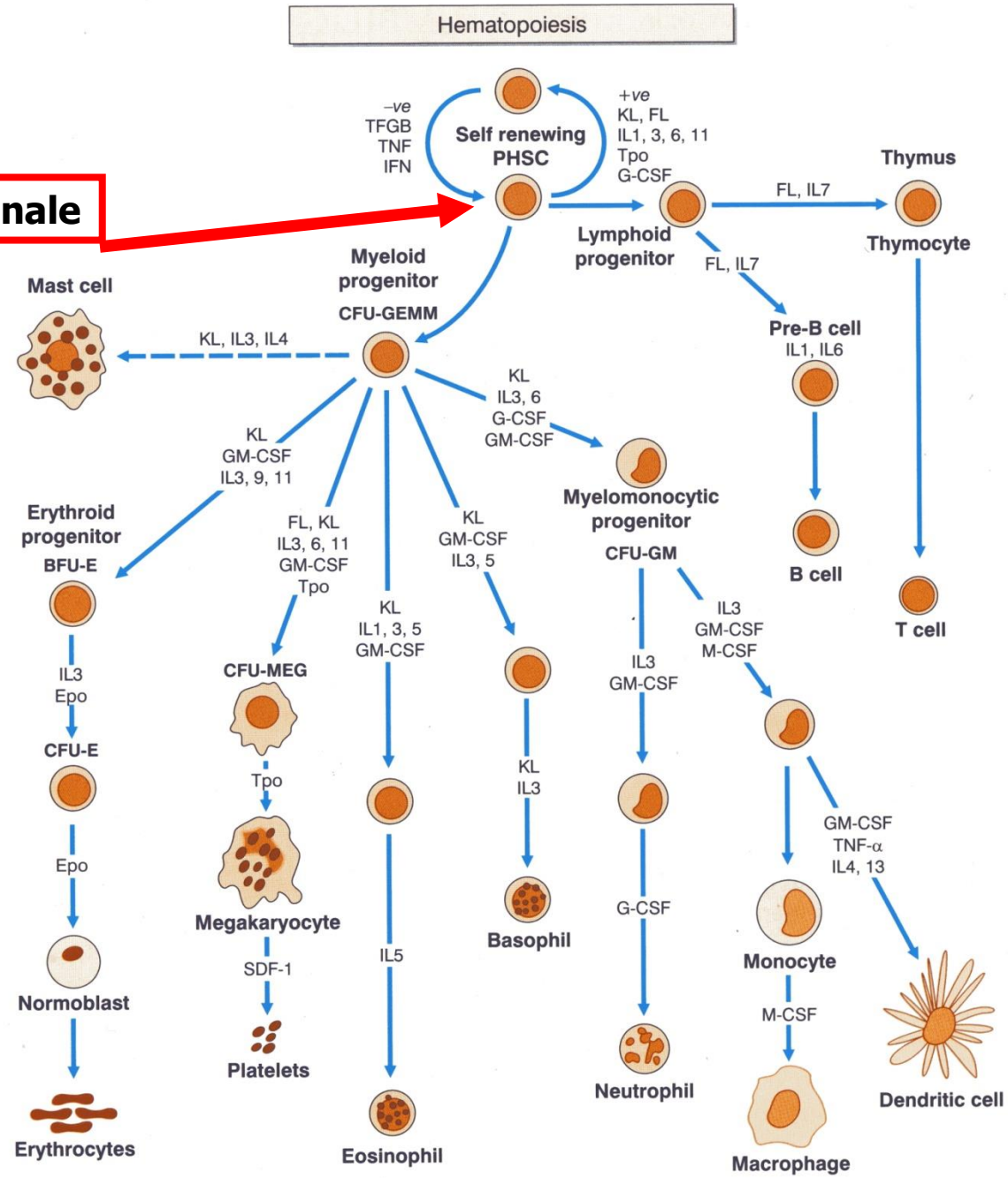
long - term culture – initiating cells (LTC - ICs)
cobblestone area- forming cells (CAFCs)
colony- forming units, spleen (CFU - S)
colony -forming cells (CFCs)

In vitro *assays*

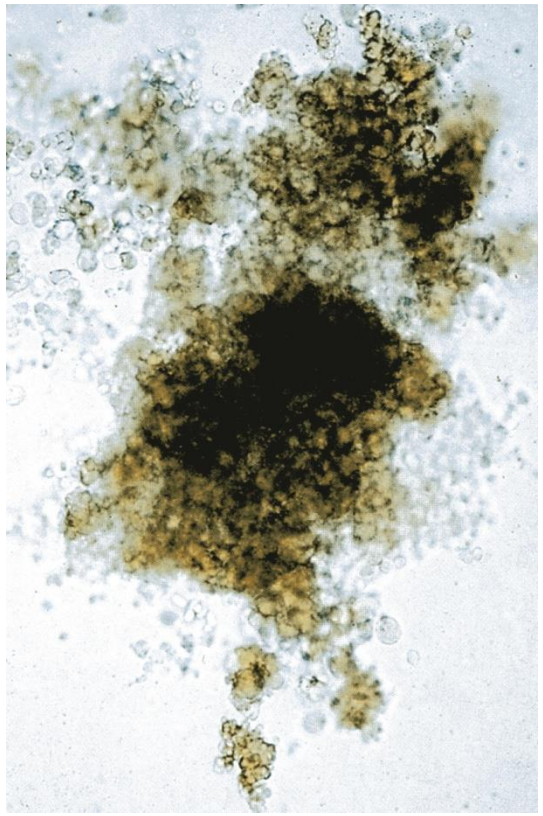
- The **CFU- C** measures hematopoietic progenitor function and is performed by plating cells in semisolid media containing methylcellulose and one or more cytokines.
  - After 5 – 14 days, colonies comprising mature cell populations committed to either myeloid or lymphoid lineages may be observed.
  - While most colonies obtained using this assay are composed of cells of a single lineage, less frequently multipotent progenitors can yield colonies containing multiple lineages.

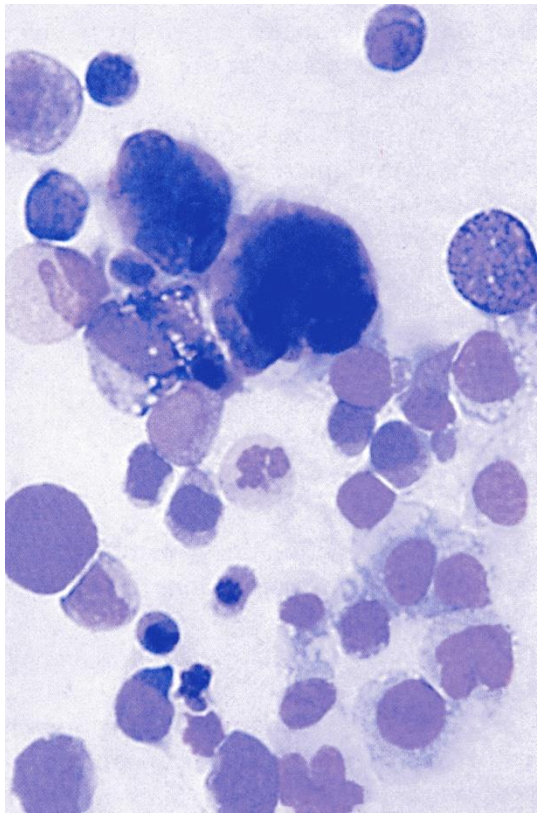
Cellula staminale

EMOPOIESI

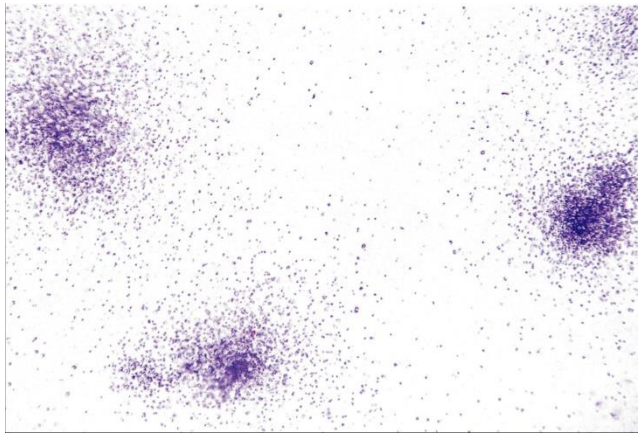


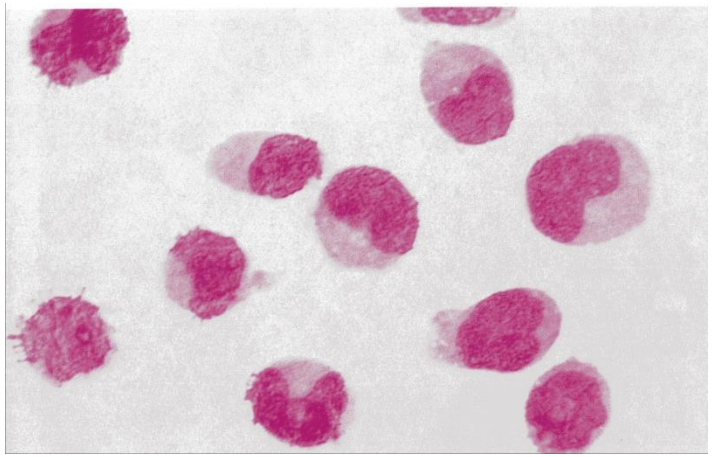
Colture cellulari: CFU-GEMM

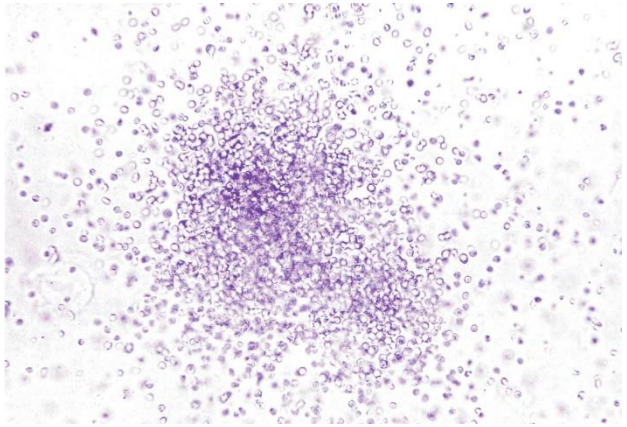


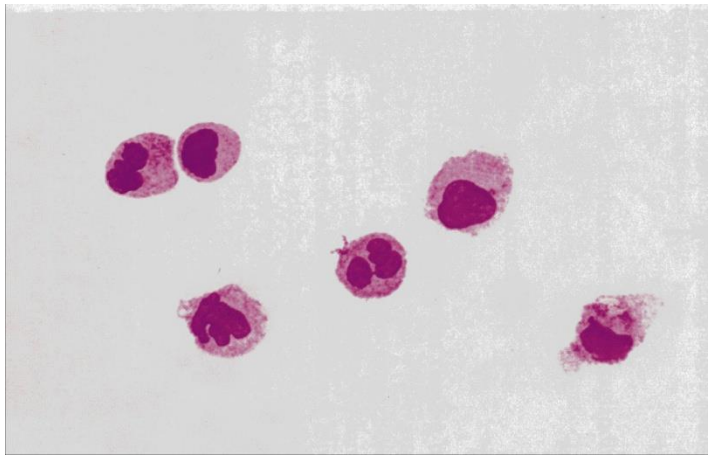


Colture cellulari: CFU-GM

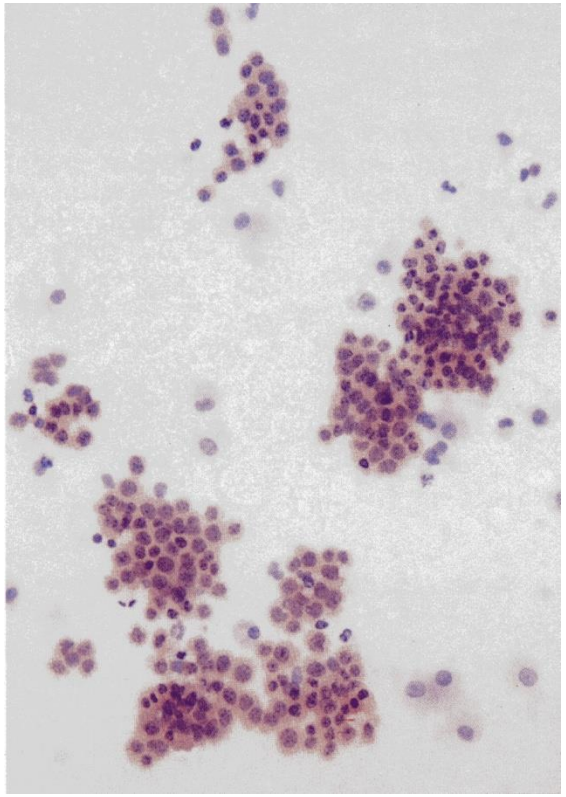


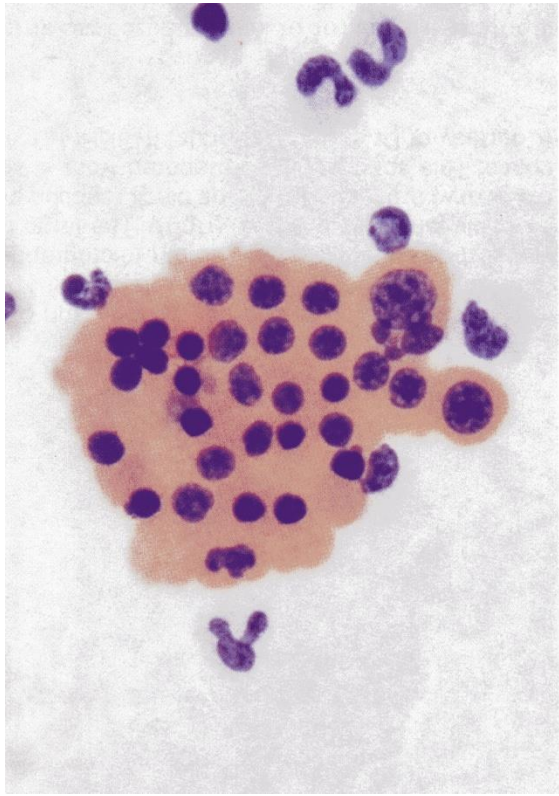




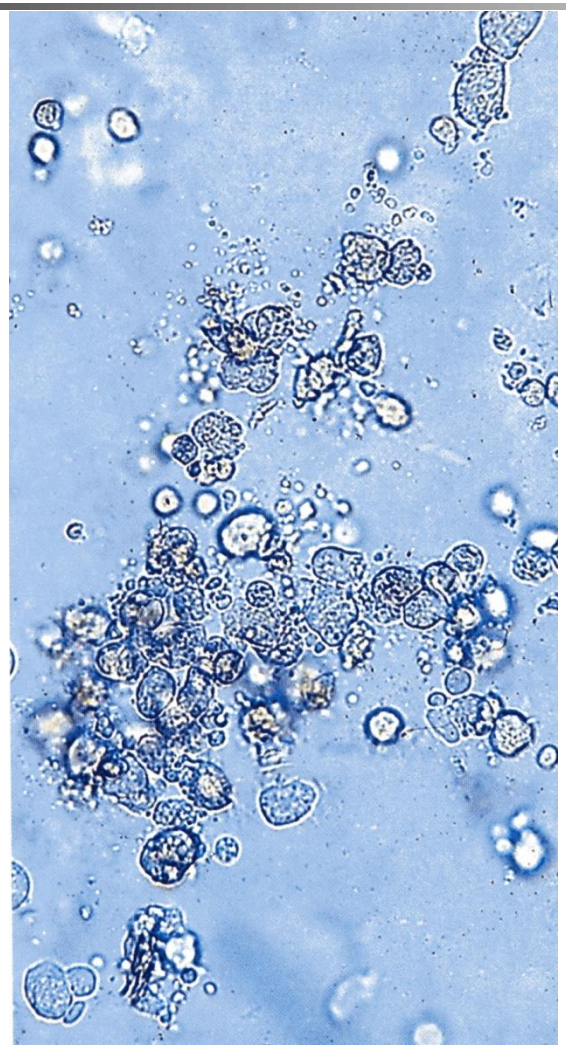


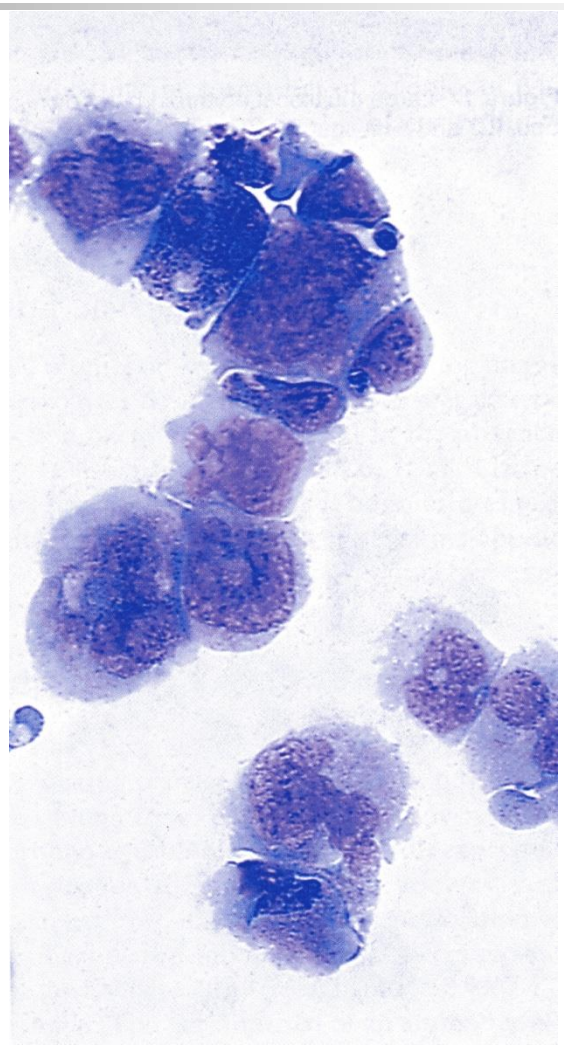
Colture cellulari: CFU-E





Colture cellulari: CFU-Meg





LTIC assay

- **The LTC-IC assay correlates more closely to HSCs.**
  - HSC are plated on top of stromal cell lines or irradiated primary BM stroma.
  - Primitive HSCs are able to initiate growth and to generate progeny *in vitro* for up to 12 weeks.
    - Progenitor cells and mature myeloid cells are removed weekly to prevent overgrowth.

- HSCs, characterized by high proliferative and self–renewal capabilities, are able to sustain long-term culture and may be enumerated at the conclusion of the assay.

CAFC assay: cobblestone area-forming cells (CAFCs)

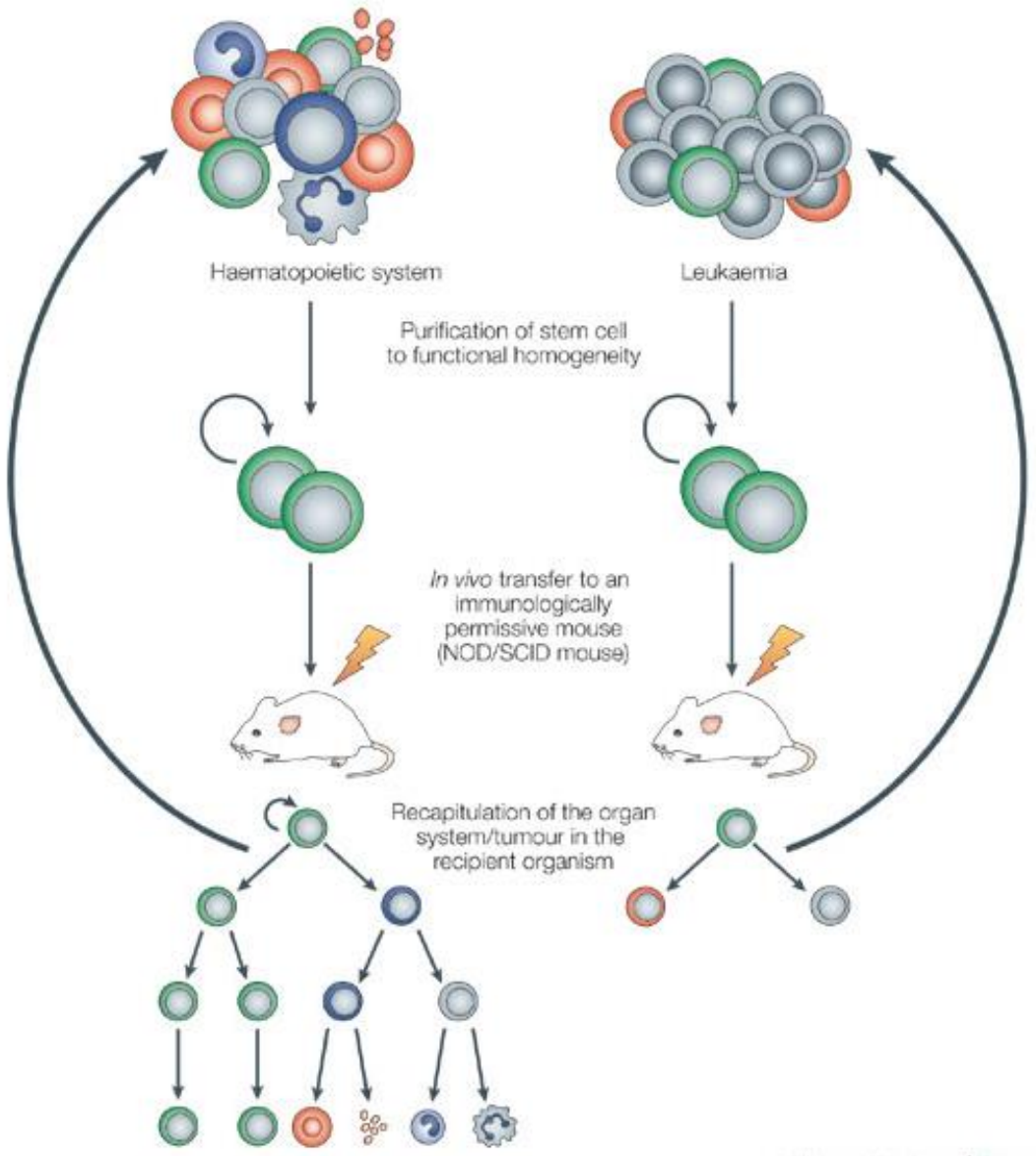
- It is a type of LTC-IC that similarly measures the ability of cells to initiate growth and generate progeny *in vitro* for up to 12 weeks.
  - HC are plated at limiting dilution on top of a monolayer consisting of irradiated BM stroma or a stromal cell line.
  - The growth of colonies consisting of at least 5 small non-refractile cells reminiscent of cobblestones, found underneath the stromal layer, are counted.
  - Such cultures are maintained using weekly half-media changes until up to 5 weeks after seeding.

CAFC assay:
cobblestone area- forming cells (CAFCs)

- In this assay,more primitive cells appear later, and day-35 CAFCs represent a close correlate of a cell with *in vivo* long-term multilineage repopulating potential.

- LTC-ICs may be enumerated after day 35 by completely removing the CAFC medium, overlaying methylcellulose and counting the number of colonies produced after 8–10 days.

Functional assays for normal and malignant stem cells



Huntly BJP & Gilliland DG, Nat Rev Cancer 5;311-321:2005.

In vivo *assays:* CFU-S

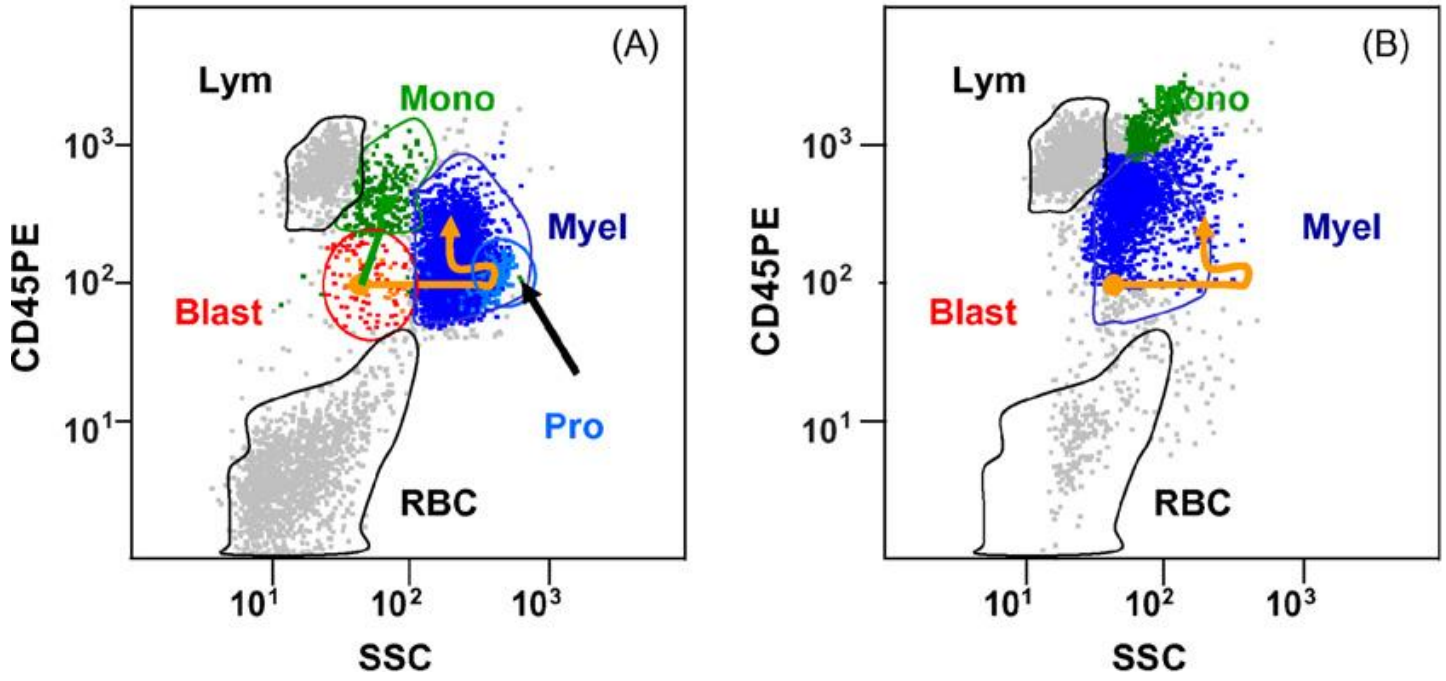
- In the CFU-S assay, BM or spleen cells are transplanted to irradiated recipients and animals are killed after 8 or 12 days for analysis of spleen colonies, termed CFU-S8 and CFU-S12.
  - Cells that give rise to CFU-S8 are **predominantly unipotential** and produce erythroid colonies.
  - CFU-S12 colonies consist of several types of myeloid cells, including erythrocytes, megakaryocytes, macrophages and granulocytes.
- Cells giving rise to CFU-S12 **represent a more primitive population of multipotent cells** than those that result in CFU-S8.

In vivo *assays*

- The **long-term repopulation assay is a more accurate measure of HSC activity**.
- Whole collections of hematopoietic cells or fractionated subpopulations are transplanted to lethally irradiated syngeneic mice, typically by tail vein injection.
- Recipients are screened for ongoing hematopoiesis 8 – 10 weeks after transplantation.
- By this time, hematopoiesis is firmly established and donor-derived blood is produced by transplanted HSCs.

- This assay requires that cells fulfill the two central features of HSCs:
  - multilineage reconstitution, consistent with multipotentiality,
  - indefinite hematopoiesis, indicative of self - renewal.

FC CD45 gating



normal

mds

Leukemia Research 32 (2008) 5–17

Table 1. MoAbs recognizing stem cell populations[19,20]

Ag	*Other names*	*Differentiated cell expression*	*Stem/progenitor expression*
CD9	P24, DRAP-1, MRP1	Leukocytes, endothelial, epithelial	NSC
CD34	8G12, 581, QBEnd10	Endothelial	HSC, EPC
CD73	Ecto-5′ nucleotidase	Leukocyte subsets, endothelial, epithelial	MSC
CD81	TAPA-1	Lymphocytes, endothelial, epithelial	NSC
CD90	Thy1	Endothelial	MSC, HSC
CD105	Endoglin, SH2	Endothelial, macrophage/ monocyte	MSC with CD73+, CD16+, CD90+ CD29+ CD34−, CD45−
CD117	c-kit, steel, SCF	Breast epithelial, renal tubule, melanocytes	Myeloid, HSC
CD133	AC133, Prominin-1	Endothelium, epithelial	HSC, EPC, NSC
CD135	FMS-like kinase-3 (FLT3) or STK-1, or Flk-2	Macrophage/ monocytes	Human marrow CD34+ and dendritic precursors, murine short-term HSC
CD146	MUC-18, Mel-CAM, MCAM	Activated T cells, endothelial	EPC
CD150	SLAMf1; used with CD244 and CD48	T, B, dendritic, endothelial	Distinguishes HSC from multipotent and B-cell progenitors
Aldefluor			HSC/MSC
Side population			HSC

Abbreviations: EPC = endothelial progenitor cells; HSC = hematopoietic stem cells; NSC = neural stem cells.

Table 3. Flow cytometric markers used to define the most frequently observed stem cell subpopulations[12,26,32,35]

	Cell type	*Lin − subpopulation immunophenotype*
HSC	Hematopoietic stem cell	CD34+/CD38−
CMP	Common myeloid progenitor	CD34+/CD38+/IL3Rα low/CD45RA
GMP	Granulocyte-macrophage progenitor	CD38+/IL3Rα low/ CD45RA+
MEP	Megakaryocyte–erythroid progenitor	CD38+/IL3Rα−/CD45RA−
CLP	Common lymphoid progenitor	CD10+/CD19−
	Pro-B cells	CD10+CD19+
	T and NK progenitors	CD10−/CD7+/CD45RA+
MSC	Mesenchymal stem cell	CD90+/CD105+/ALDH+/ CD45−/CD34−/CD133−/ CD38−/HLA−DR−
STRC-M	Early myeloid short-term repopulating	CD34+/CD38+/CD45−/ ALDH−/CD133−/CD90+
	Late myeloid HSC	CD34+/CD38−/ CD45dim+/ALDH+[a]/ CD133+/CD90+

Abbreviations: ALDH = Aldehyde dehydrogenase; STRC-M = short term repopulating cells-myeloid. [a]Bright positive cells have the highest repopulation capacity compared with dim positive cells.

Beksac M, BMT (2011) 1- 6

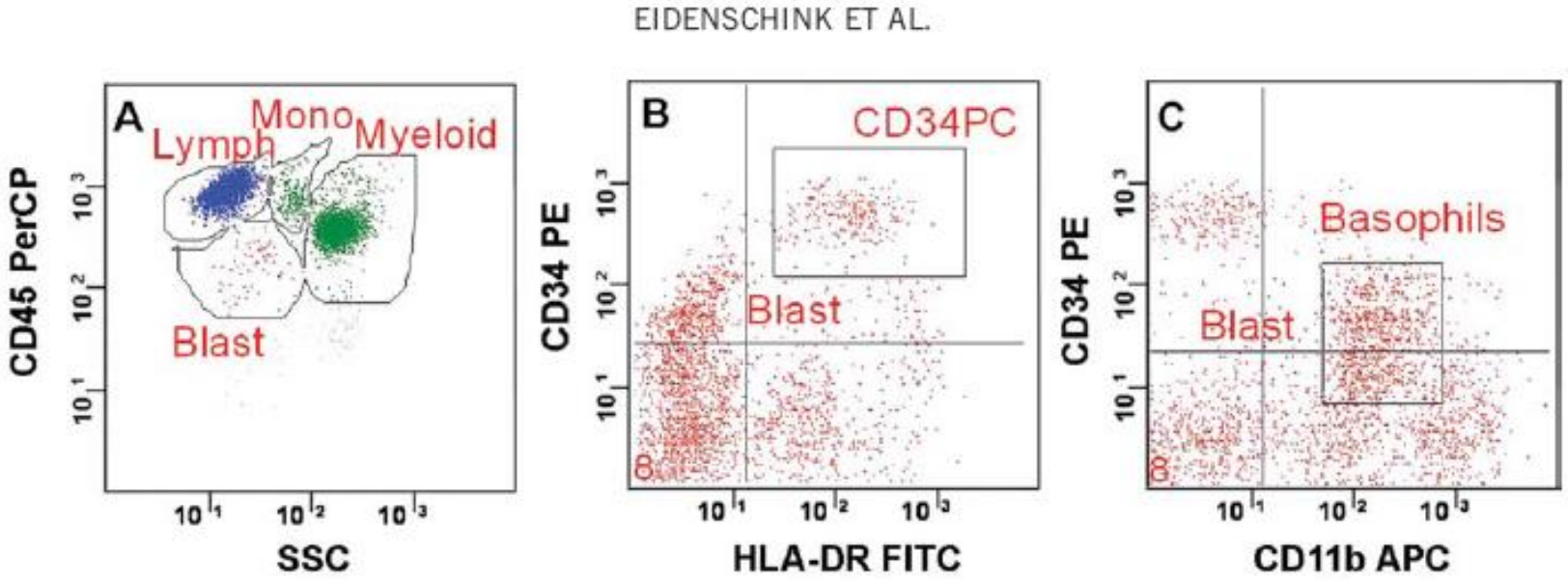
Frequency and engraftment potential of HSC subpopulations

Cell type	*Frequency in*	*Cell dose*	*PNL engraftment*	*Plt engraftment*
Total CD34+[5,7,10]	1/200 (of MNC, BM)	2–8 × 10^6/kg	Yes	Yes (month 3,6,9)
CD34+38−[22-24]	1/40	Unknown	Yes (month 6,9)	Yes
	1/1000 (of MNC, BM)			No
CD34+90+[22,25,26]	1/1000	25–80 × 10^4/kg	Yes	Yes (month 3,6,9,12[a])
			no	
CD34+CD110+[27]				Yes
CD34−/low[24]	1/24000	Unknown	Unknown	Unknown
CD133+[28]	Same as CD34	Same as CD34+	Similar to CD34	Similar to CD34
CD34+133+[22]	Not reported	Not reported	Yes (month 3)	Yes(month 3,6,9)
SSC low/ALDH bright[29-31]	1.2% (BM)	5.4 × 10^6/kg (PNL)	Yes	Yes
		7.22 × 10^6/kg (Plt)	Yes	Yes
			No	No
Lin−CD34+CD38−IL3Ra low CD45RA- (CMPs)[32]	73.20 (17.8–86.2) % (PBSC)	> 4.4 × 10^6/kg	Similar to CD34	Similar to CD34
	35.2 (20.4–42.3) % (BM)	(PNL, Plt and erythroid)		
	3.2 (1.3–16.1) % (CB)			

Abbreviations: CMP = common myeloid progenitors; MNC = mononuclear cell; PNL = polymorphonuclear cells; SSC = side light scatter. [a]Also enhanced erythroid engraftment on month 12.

Beksac M, BMT (2011) 1- 6

Basal Levels of CD34 Positive Cells in Peripheral Blood



Gating strategy used to identify CD34 positive progenitor cells. Progenitor Cells are identified in the Blast gate by CD45/SSC (A).
Cells that are positive for CD34 and HLA-DR (B) excluding the cells that are positive for CD11b (C) are used for the total CD34 count in a specimen.

Cytometry Part B (Clinical Cytometry) 82B:18–25 (2012)

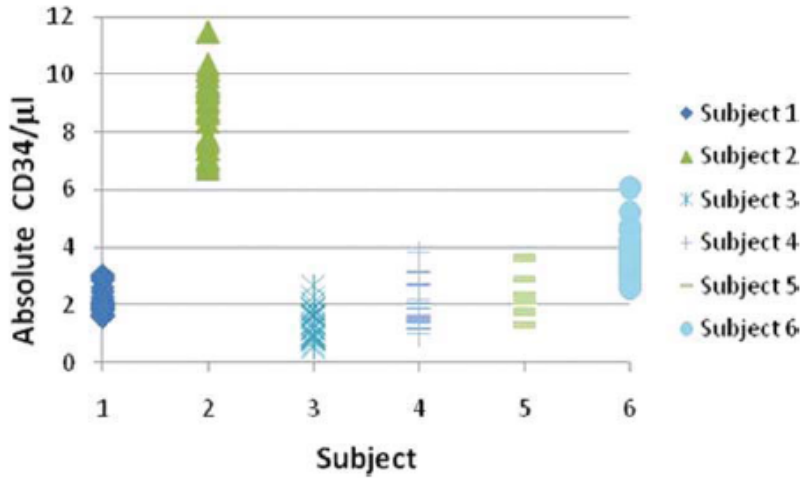


FIG. 2. Absolute CD34/µl values for individual subjects assayed 26 times in the 6 day study. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

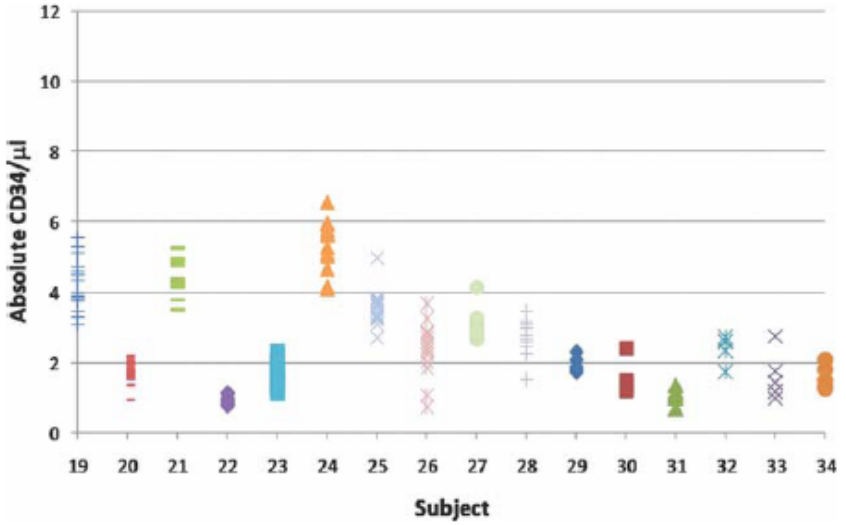


FIG. 4. Absolute CD34/µl values for individual subjects assayed 14 separate times during a 10 week study (subjects 19–28) and absolute CD34/µl values for individual subjects assayed six different times over an 18 month period (subjects 29–34).

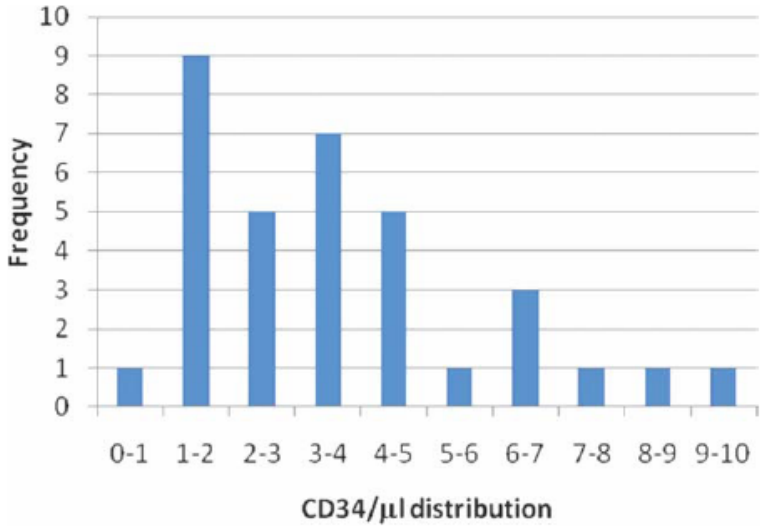


FIG. 5. A Normal range distribution of CD34 µl absolute amounts for subjects in all three studies.

Cytometry Part B (Clinical Cytometry) 82B:18–25 (2012)

STEM CELL: mobilization

- Mobilization is the iatrogenic augmentation of HSC recirculation that occurs at low levels in steady state.

***Blood*. 2011;118(17):4530-4540**

G-CSF–based mobilization protocols

- G-CSF (Granulocyte Colony Stimulating Factor) with or without myelosuppressive chemotherapy is been the most commonly used mobilization protocols.

- When used alone G-CSF is given at 10 μg/kg/day sc with apheresis beginning on the 5th day until the yield target is reached.

- Combining G-CSF with chemotherapy achieves the twin aims of mobilization and antitumor activity and has been shown to result in a higher CD34 cell yield than G-CSF alone.

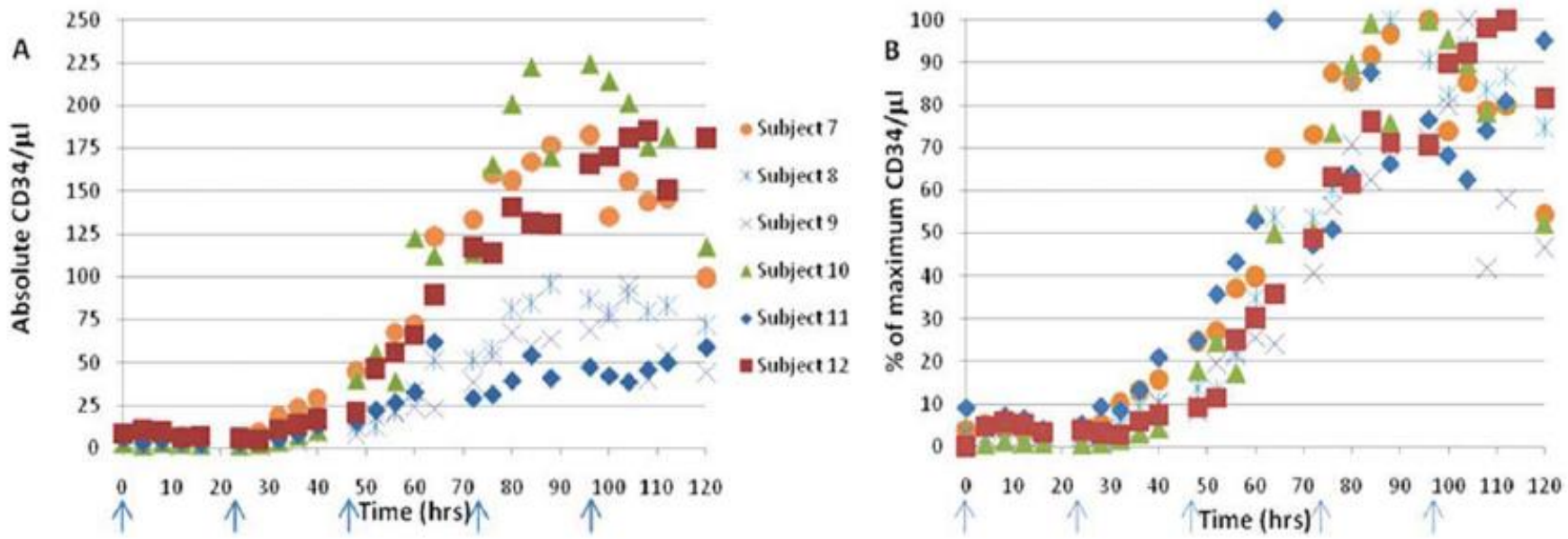
***Blood*. 2011;118(17):4530-4540**

Plerixafor

- Plerixafor a **CXCR4 antagonist**, reduces the binding and chemotaxis of HSCs to the BM stroma.
- It is used at 0.24 mg/Kg subcutaneously the evening before the scheduled apheresis because it generates peak CD34 levels 6-9 hours after administration.
- It synergizes with G-CSF and chemotherapy.

***Blood*. 2011;118(17):4530-4540**

Absolute CD34 counts in response to G-CSF treatment



Arrows indicate time points when G-CSF was administered.

Cytometry Part B (Clinical Cytometry) 82B:18–25 (2012)

Risk factors, mechanisms, and strategies to optimize collection in predicted poor mobilizer patients

Risk factor	Postulated mechanism	Mobilization strategy
Low steady-state platelet counts and PB CD34$^+$ level	Reflects overall HSC reserve	Regimen promoting HSC proliferation, eg, SCF, cyclophosphamide
Low steady-state TNF-α level	May reflect niche dysfunction, including the macrophage response to G-CSF	Regimen bypassing the macrophage-dependent pathways, eg, plerixafor-containing regimen
Increasing age	Reduced HSC reserve because of the following: Age-related HSC senescence Age-related loss or dysfunction of HSC niche Age-related bone loss or altered bone metabolism	Regimen promoting HSC proliferation, eg, SCF, cyclophosphamide Add risk-adapted plerixafor to augment niche response to G-CSF Bisphosphonate treatment continued throughout collection PTH of interest in experimental models
Underlying disease	Paraneoplastic niche dysfunction Loss of niche to mass effect of tumor	Aim to clear BM of disease before collection
Prior extensive radiotherapy (RT) to red marrow	Direct HSC toxicity Toxicity to HSC niche	Rainy day collection before extensive RT when possible Risk-adapted plerixafor Regimen promoting HSC proliferation, eg, SCF, cyclophosphamide
Prior chemotherapy		
Melphalan	Direct HSC toxicity	Avoid melphalan until autologous cells collected
Fludarabine	Direct HSC toxicity, niche damage	Collect HSCs early, after < 4 cycles of fludarabine
Intensive chemotherapy (eg, hyper-CVAD)	Dose-dense cycles may cause niche damage, and HSCs forced into cell cycle may not engraft as well	Use SCF or preemptive risk-adapted plerixafor for fludarabine-exposed and heavily pretreated patients
Prior lenalidomide	Possible effects on HSC motility Possible dysregulated HSC niche because of antiangiogenic effects	Collect HSC early, after < 4 cycles of treatment Temporarily withhold lenalidomide during collection.

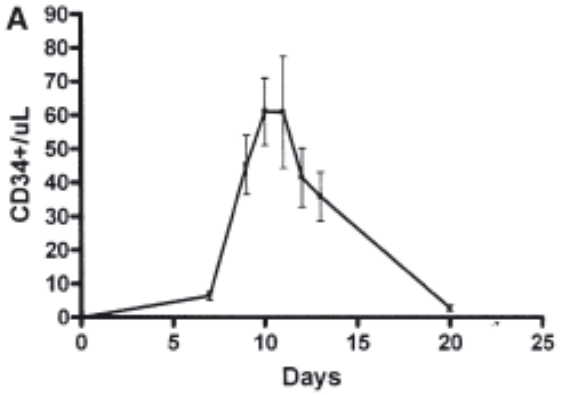
***Blood*. 2011;118(17):4530-4540**

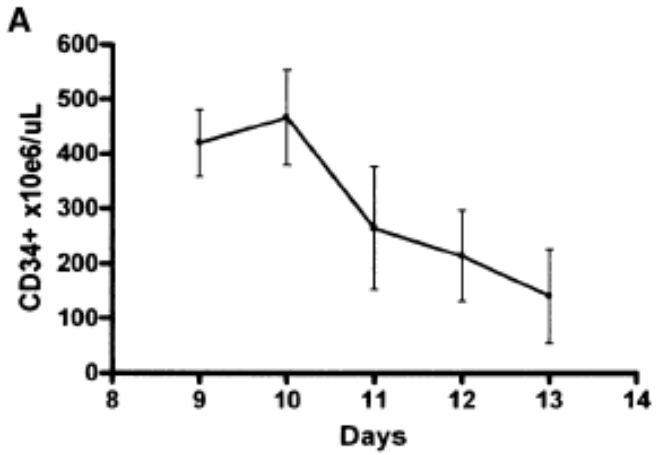
constitutive poor mobilizers

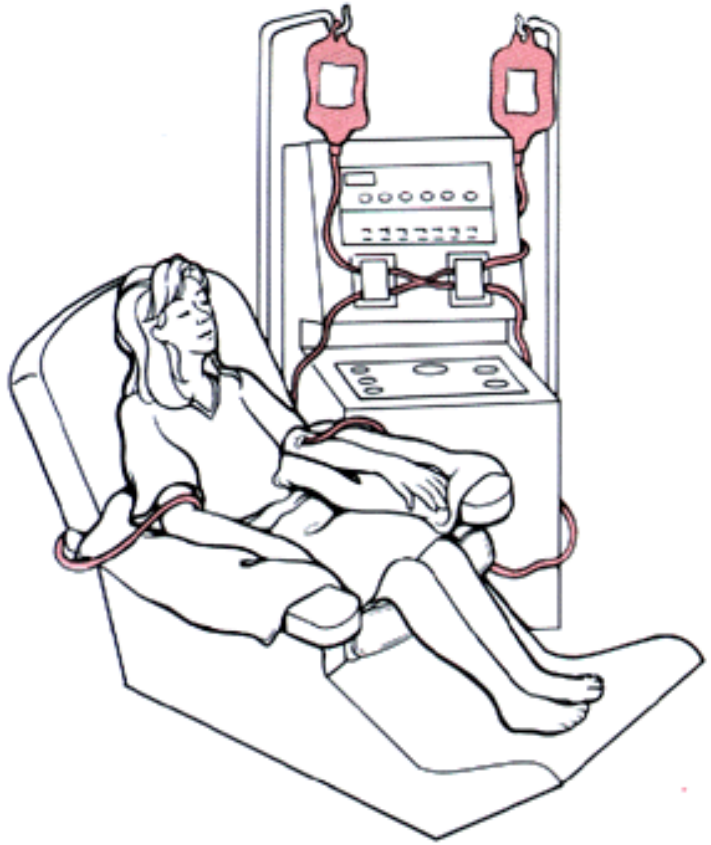
- Up to 5% of healthy donors fail to mobilize with conventional regimens, and some patients with no obvious risk factors will also.
- The mechanistic understanding of these constitutive poor mobilizers" is complex and incomplete.

***Blood*. 2011;118(17):4530-4540**

CD34+ cell mobilization: chemio + G-CSF







Mauro et al EJH 78 (374–380) [a] 2007

Enumeration of CD34+ Hematopoietic Stem and Progenitor Cells

- The Basic Protocol is a modified version of the protocol developed by Sutherland et al. (1994) that was subsequently incorporated into a set of clinical guidelines for the International Society for Hematotherapy and Graft Engineering (ISHAGE; Sutherland et al., 1996).
  - The counterstaining of CD34 by the CD45 MAb, allowing the identification of leukocytes (CD45+)
  - The verification of “true” CD34+ cells as being dim for CD45 fluorescence and having low side scatter (CD45dim, SSlow).
- CD34+ cell enumeration can be applied to the PB, apheresis products, bone marrow, and cord blood.

Enumeration of CD34+ Hematopoietic Stem and Progenitor Cells

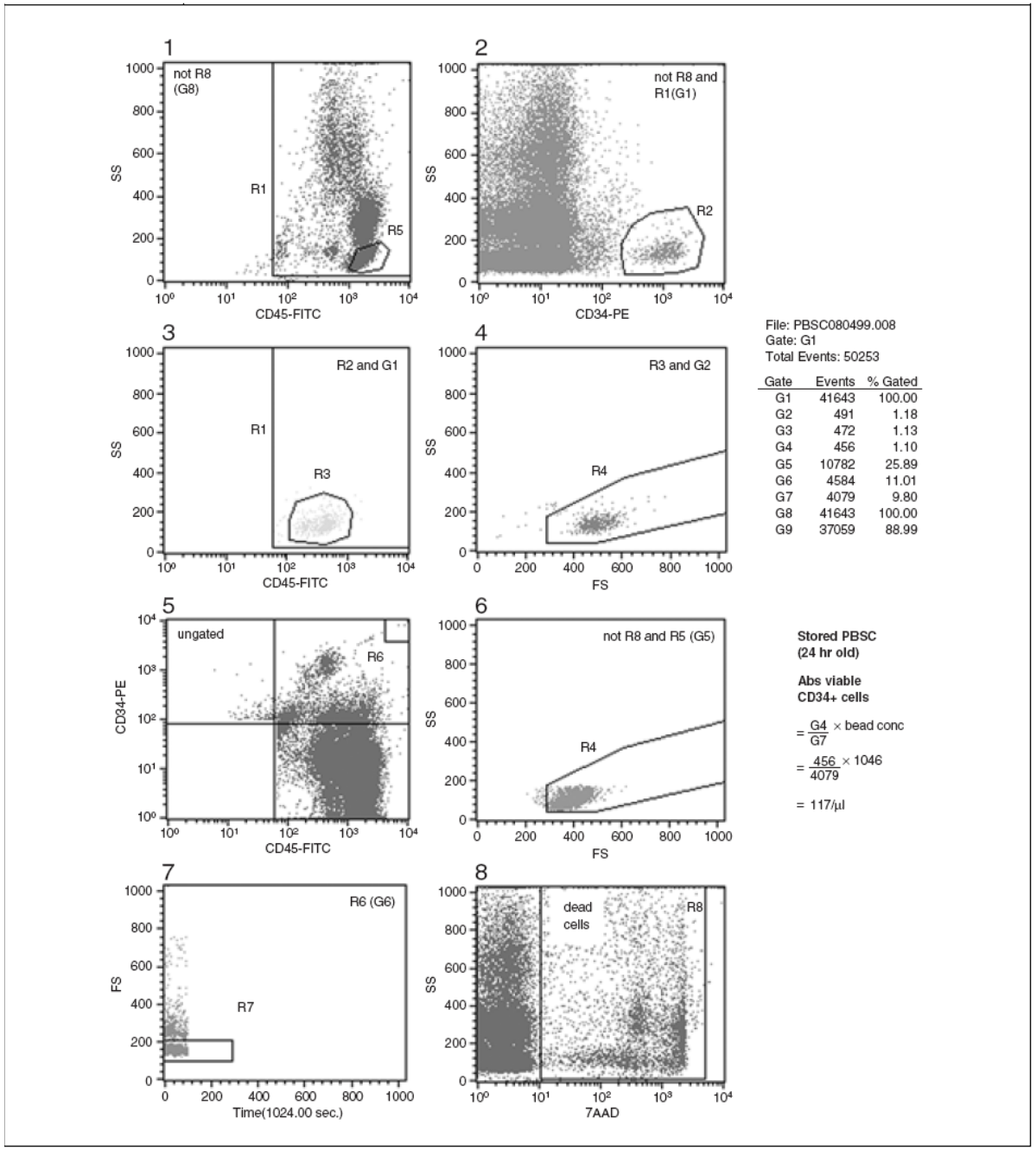
- The addition of predefined numbers of **counting beads** to the cell suspension yields the concentration of CD34+ cells per unit of sample volume (i.e., the absolute CD34+ cell count) from a single flow-cytometric assessment (**single-platform technique**).

- Dead cells can be excluded from analysis using the DNA stain 7-aminoactinomycin D (7-AAD).
  - 7-AAD is excited at 488 nm and has maximum emission at 660 nm.
  - The dye cannot be used in single-laser systems with other fluorochromes that emit at >600 nm, such as PerCP or PE-Cy5.

Table 6.4.1 Summary of Instrument-Specific Logical Gates Using Dead Cell Exclusion

Histogram	Beckman-Coulter XL	BD Biosciences FACS
1	Gated on J	G8 = not R8
2	Gated on AJ	G1 = (not R8) and R1
3	Gated on ABJ	G2 = R2 and G1
4	Gated on ABCJ	G3 = R3 and G2
5	Ungated	Ungated
6	Gated on EJ	G5 = (not R8) and R5
7	Gated on H	G6 = R6
8	Ungated	Ungated

Current Protocols in Cytometry **(2003) 6.4.1-6.4.23**



Example of the Basic Protocol combined with optional dead-cell exclusion for a BD Biosciences FACScan flow cytometer. Data were acquired and analyzed using CellQuest version 3.3 software.

Data are shown from apheresis collection.

Sample contained 11% dead cells (i.e., 7-AAD+ gated in R8) and 117 viable CD34+ cells/µl. In this example, 456 viable CD34+ cells were identified in gate 4 and 4079 singlet beads were enumerated in gate 7.

The assayed bead concentration was 1046/µl.

Current Protocols in Cytometry **(2003) 6.4.1-6.4.23**

External quality assessment

Table 3
Multivariate Analysis of Factors with Systematic Effects (bias) on Absolute CD34+ Cell Count Results

Variable (overall *P*)	Category	*P*-value
Gating strategy (<0.01)	Milan	<0.01
	Bender	n.s.
	ISHAGE	n.s.
	SIHON	n.s.
	ProCOUNT™	n.s.
	Stem-KIT™	<0.01
Laboratory (<0.01)	n.a.	
Labeling CD34 mAb (0.01)	FITC	<0.01
	PE	n.s.
Flow cytometer (0.02)	FACScan™	n.s.
	FACScalibur™	0.03
	FACStar™	n.s.
	Epics XL™	n.s.
	Cytoron™	0.04
Sample preparation (0.03)	LW	n.s.
	LNW	0.05
	NLNW	n.s.
	MNC	n.s.

Only categorical variables with significant effects are shown.
n.a., not applicable; n.s., not significant.

Table 4
Multivariate Analysis of Factors Influencing Variability of Absolute CD34+ Cell Count Results

Variable (overall *P*)	Category	*P*-value
Platform methodology (<0.01)	Single platform	n.s.
	Dual platform	0.01
Laboratory (<0.01)	n.a.	

Only categorical variables with significant effects are shown.
n.a., not applicable; n.s., not significant.

the variability in results of CD34+ cell enumeration has declined with time; in particular, after a practical workshop in which participants were trained to use the "single platform ISHAGE protocol."

Between-laboratory variation in CD34+ cell enumeration can be reduced by standardization of methodologies between centres

Cytometry Part B (Clinical Cytometry) 72B:178–188 (2007)

Recovery of viable CD34+ cells from cryopreserved hemopoietic progenitor cell products

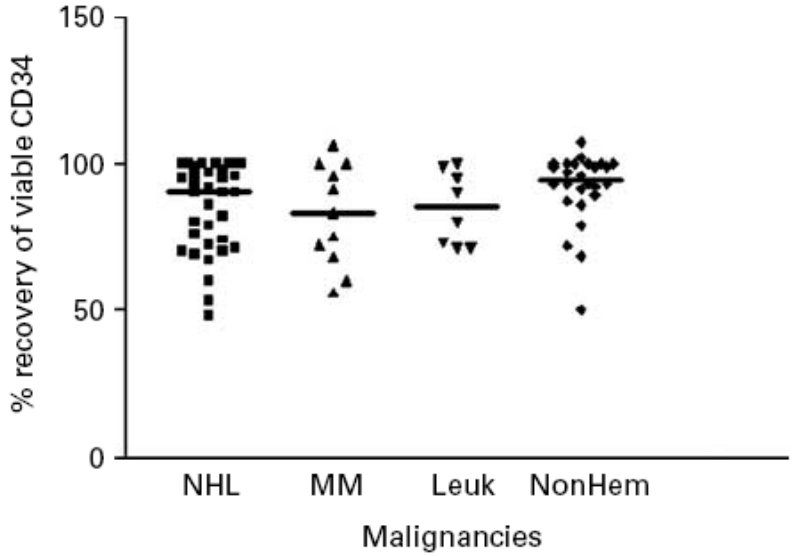


Figure 4 Comparison of the median recovery of viable CD34$^+$ cells within four groups of malignancies. NHL: median recovery of viable CD34$^+$ cells 90% (range 48–100%, $n=34$). MM: 83% (range 56–106%, $n=11$). Acute leukaemias (Leuk): 85% (range 71–100%, $n=7$). Non-hematological malignancies (Non-Hem): 94.5% (range 50–107%, $n=18$). There was no significant difference in the recovery of viable CD34$^+$ cells for all groups $P>0.17$.

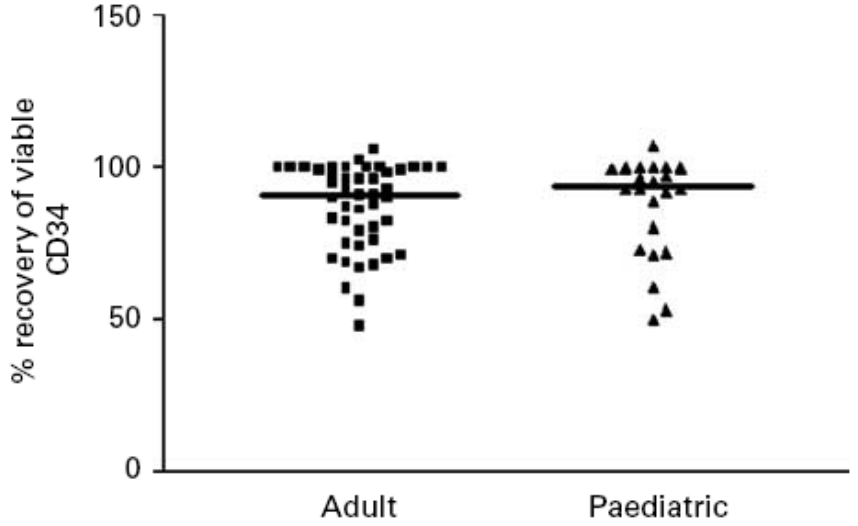


Figure 5 Comparison of the median recovery of viable CD34$^+$ cells between adult and pediatric HPC products. Adult HPC recovered a median 91% viable CD34$^+$ cells (range 48–106%, $n=51$), compared to 94% (range 50–107%, $n=28$) for pediatric HPC products, $P=1.06$).

Bone Marrow Transplantation (2005) 36, 199–204.

TRAPIANTO DI CELLULE STAMINALI EMOPOIETICHE (I)

- Definizione
  - Prelievo di cellule staminali da midollo, sangue periferico o cordonale, ad un donatore od al paziente, e reinfusione delle stesse dopo trattamento radio-chemioterapico mieloablativo

Nobel prize for stem-cell transplantation

Photograph of the Seattle team after announcement of the Nobel Prize in Medicine, which was awarded to E. D. Thomas in 1990. From left to right: Paul Neiman, Alexander Fefer, E. Donnall Thomas, C. Dean Buckner and Rainer Storb.

***Marie-Térèse Little & Rainer Storb Nature Reviews Cancer* 2, 231-238 (2002)**

TRAPIANTO DI CELLULE STAMINALI EMOPOIETICHE (II)

- Sorgente delle cellule staminali emopoietiche
  - Midollo osseo:
  - Sangue periferico:
  - Sangue cordonale: al momento del parto

haematopoietic stem-cell transplantation

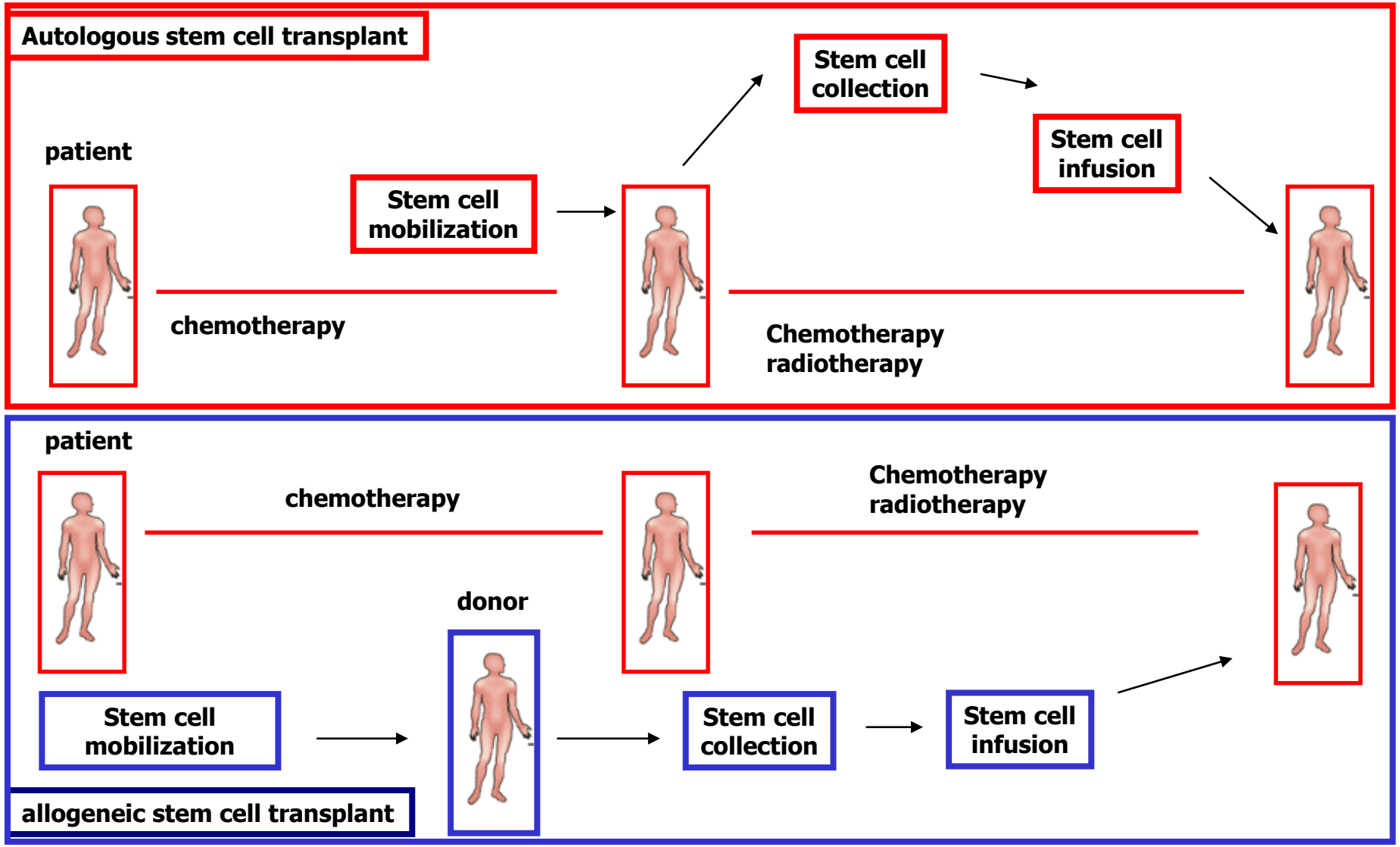
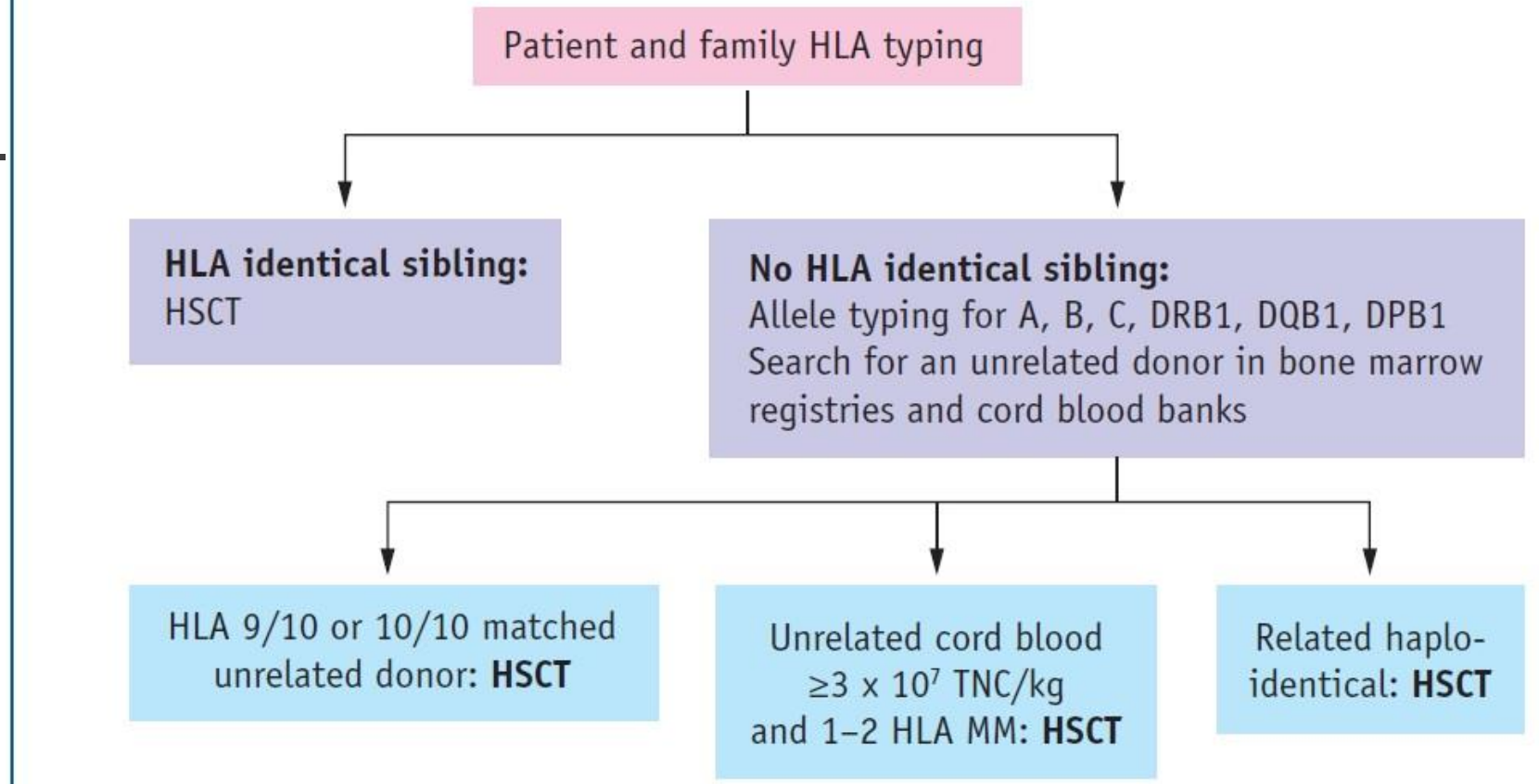
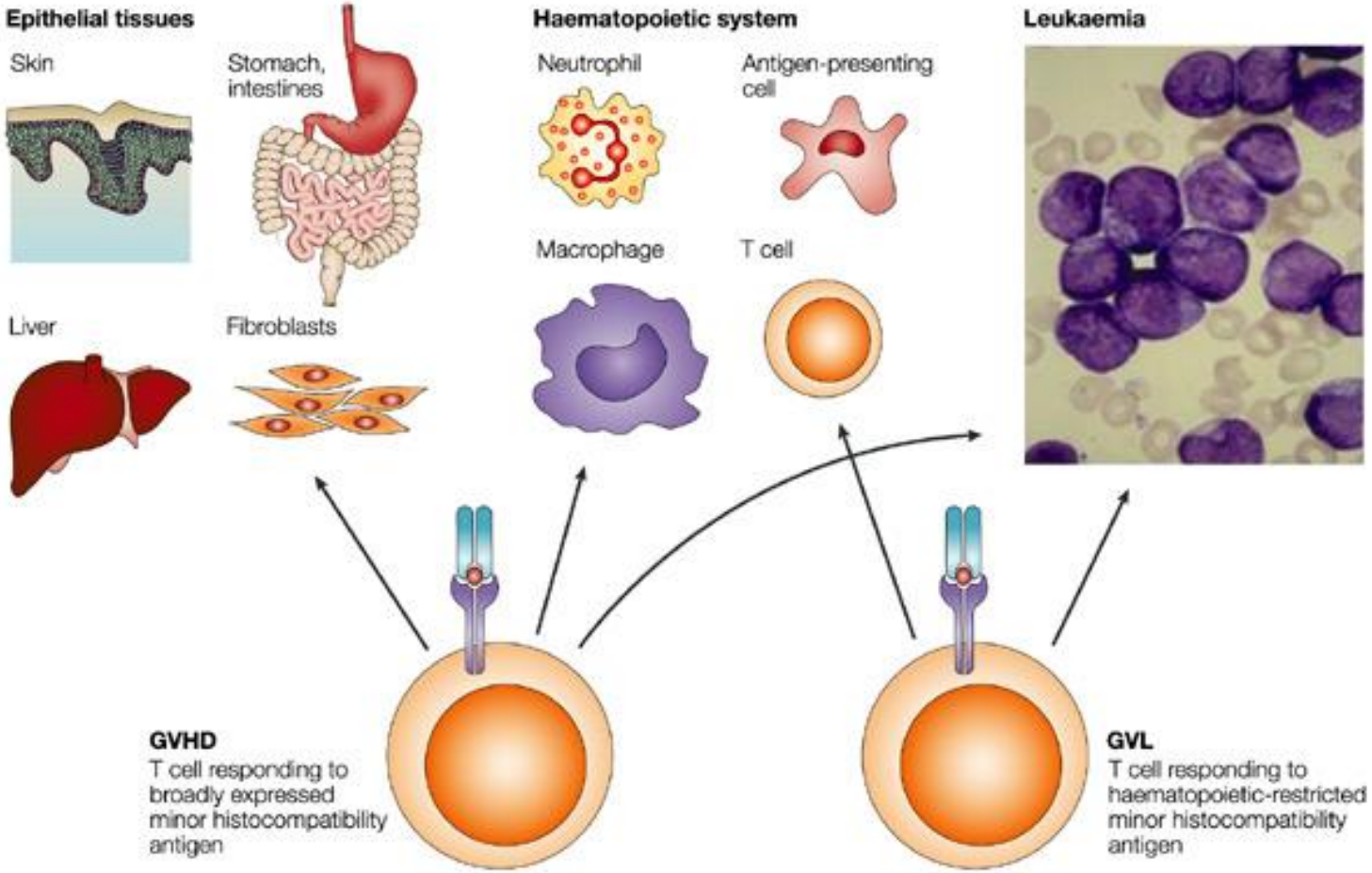


Figure 4: **Algorithm of donor search**



- If transplant urgent: prefer cord blood or related haploidentical transplant
- If not enough cells in a single cord blood unit: consider double cord
- Consider other factors: indication of the transplant, ABO, CMV, donor sex
- Expertise of the centres is very important for donor selection for HLA mismatched transplants

GVL and GVHD



Marie Bleakley & Stanley R. Riddell Nature Reviews Cancer **4**, 371-380 (2004);

Manipulating hematopoietic stem cells for clinical use

Isolating stem cells for manipulation

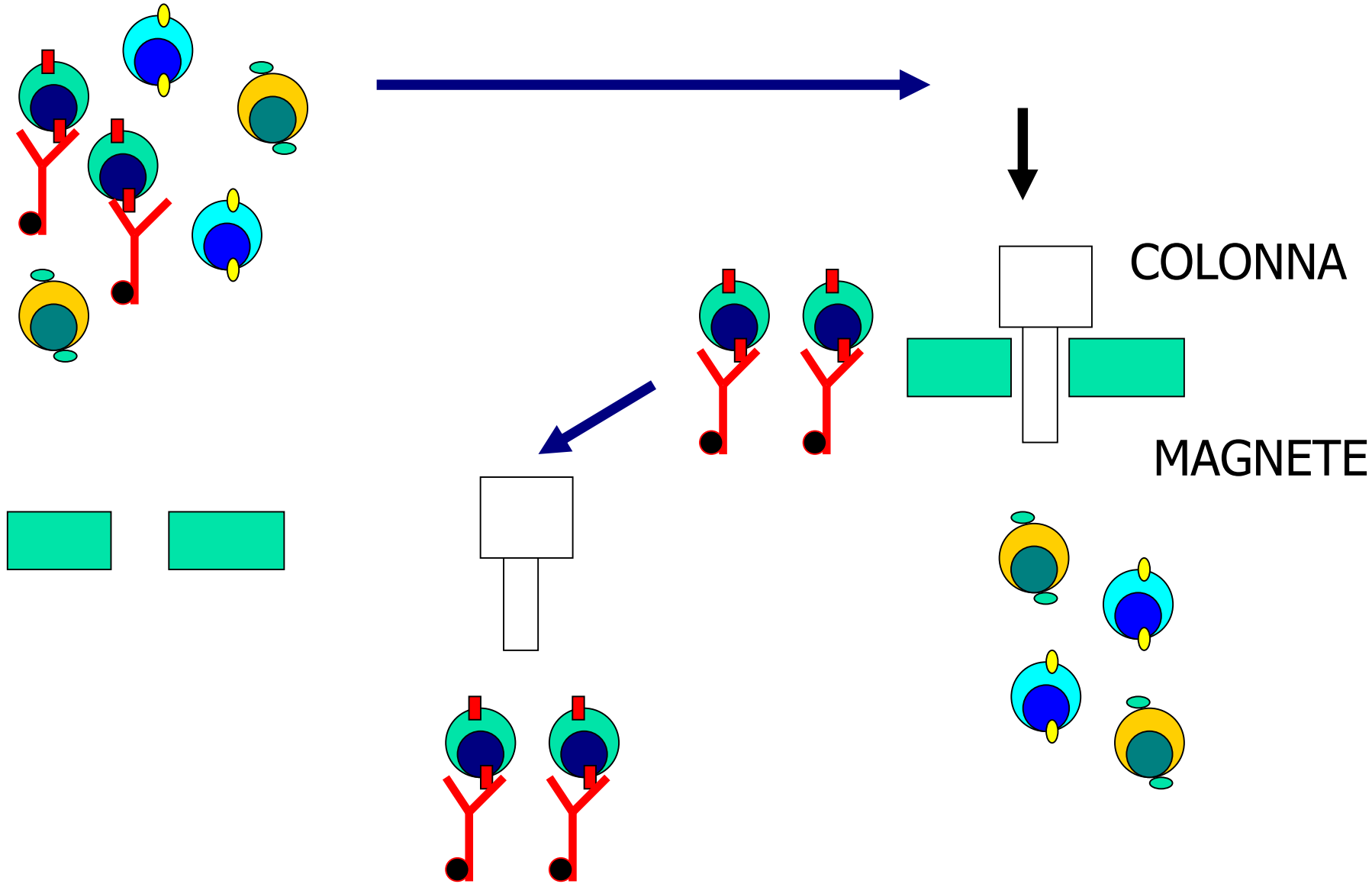
Frequency and engraftment potential of HSC subpopulations

Cell type	*Frequency in*	*Cell dose*	*PNL engraftment*	*Plt engraftment*
Total CD34+[5,7,10]	1/200 (of MNC, BM)	2-8 $\times$ 10^6/kg	Yes	Yes (month 3,6,9)
CD34+38−[22-24]	1/40	Unknown	Yes (month 6,9)	Yes
	1/1000 (of MNC, BM)			No
CD34+90+[22,25,26]	1/1000	25-80 $\times$ 10^4/kg	Yes	Yes (month 3,6,9,12[a])
			no	
CD34+CD110+[27]				Yes
CD34−/low[24]	1/24000	Unknown	Unknown	Unknown
CD133+[28]	Same as CD34	Same as CD34+	Similar to CD34	Similar to CD34
CD34+133+[22]	Not reported	Not reported	Yes (month 3)	Yes(month 3,6,9)
SSC low/ALDH bright[29-31]	1.2% (BM)	5.4 $\times$ 10^6/kg (PNL)	Yes	Yes
		7.22 $\times$ 10^6/kg (Plt)	Yes	Yes
			No	No
Lin-CD34+CD38−IL3Ra low	73.20 (17.8-86.2) % (PBSC)	> 4.4 $\times$ 10^6/kg	Similar to CD34	Similar to CD34
CD45RA- (CMPs)[32]	35.2 (20.4-42.3) % (BM)	(PNL, Plt and erythroid)		
	3.2 (1.3-16.1) % (CB)			

Abbreviations: CMP = common myeloid progenitors; MNC = mononuclear cell; PNL = polymorphonuclear cells; SSC = side light scatter. [a]Also enhanced erythroid engraftment on month 12.

Beksac M, BMT (2011) 1- 6

SEPARAZIONE IMMUNOMAGNETICA



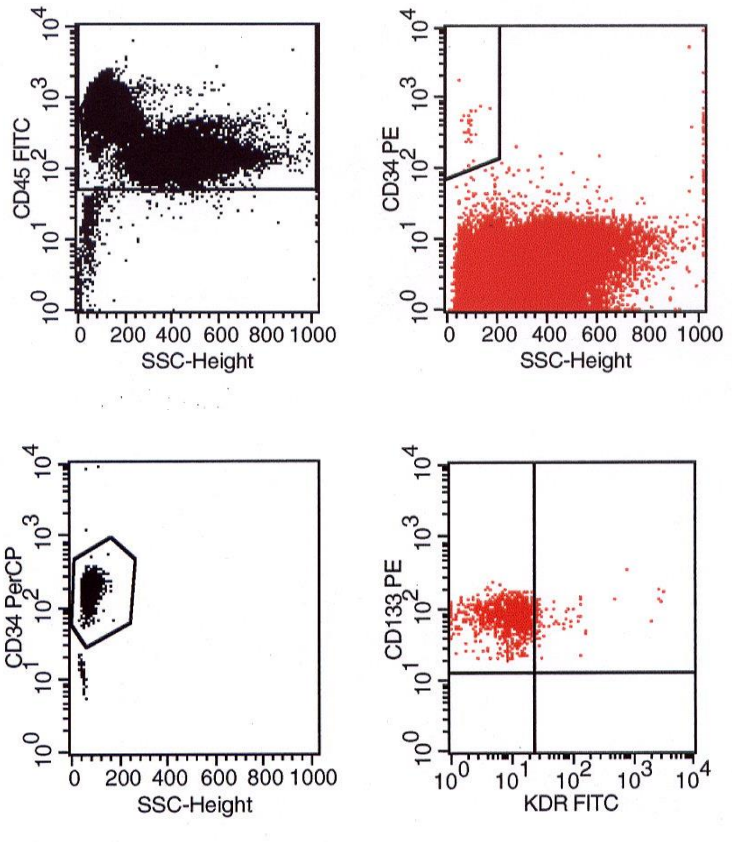
MACS® Microbeads Technology

MACS
Miltenyi Biotec

Separazione immunomagnetica di cellule staminali CD34+

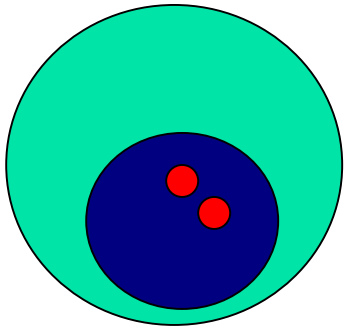
Prima della separazione

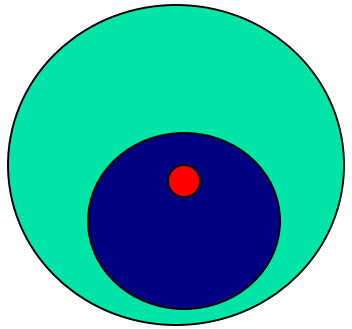
Dopo la separazione



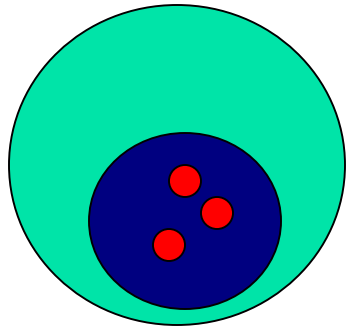
Cellula staminale

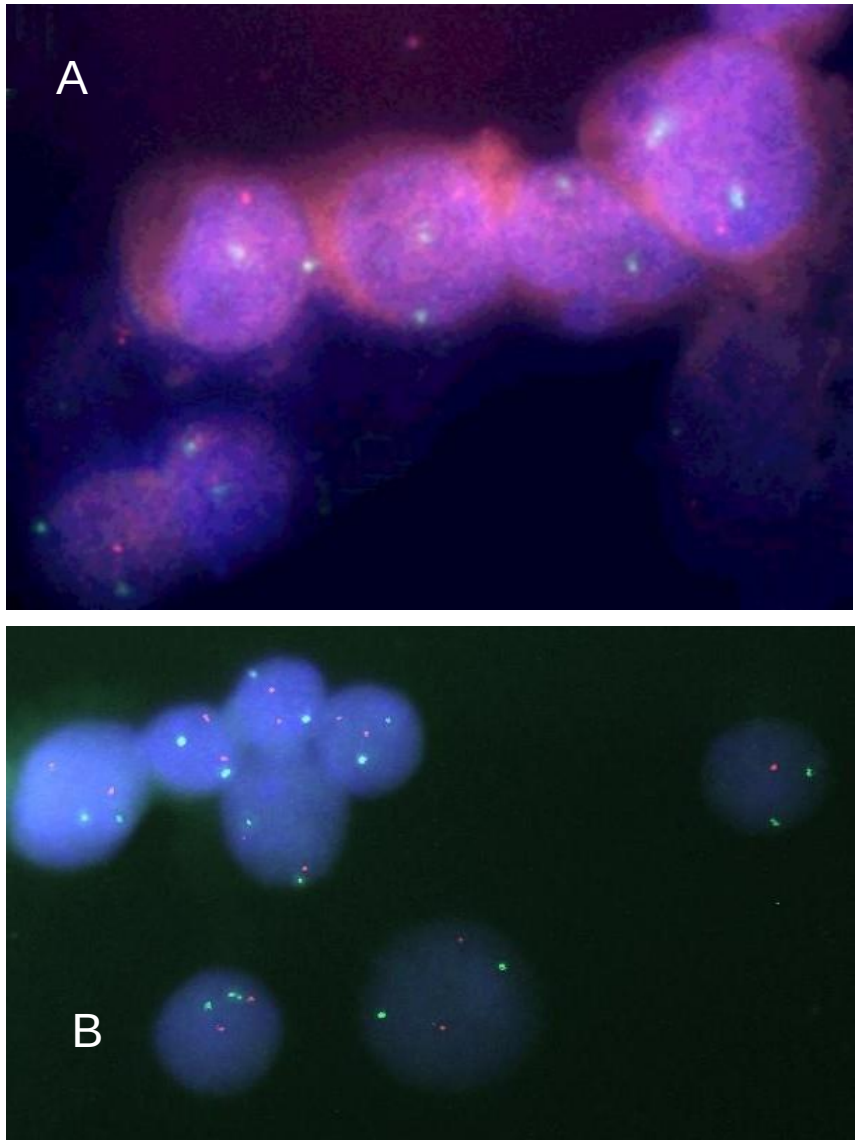
normale





patologica





EPO+G: MDS

Dual colour combined FISH analysis performed on immunomagnetically sorted CD34+ cells before (A) and after (B) treatment in a MDS patient with del(5q) responding to rHuEpo and G-CSF administration. In B, rHuEpo and G-CSF administration has produced a significant reduction of the proportion of CD34+ cells with the 5q deletion. Pathologic CD34+ cells are identified by the presence of only one red signal (DNA probe to the band 5q31). As control, the two green signals (DNA probe to the band 5p15.2 locus D5S23) indicate the presence of two chromosomes 5.

Rigolin et al, BJH 2004

Methods of isolation of HSCs: *FACS*

- The flow cytometer may physically sort cells of desired fluorescence or fluorescence pattern, size and granularity characteristics.
- Using a magnetic field, these cells may be diverted to a collection tube during analysis and later analyzed using techniques of molecular and cellular biology.
- FACS may be used to isolate HSCs using both positive and negative selection strategies with fluorescence - labeled antibodies directed against primitive hematopoietic cell antigens.