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FROM THE EDITOR

Dear colleagues - readers of the *Haematopoiesis Immunology* and participants of the conference of the same name!

This year we return to the main idea of our journal, i.e. to publish abstracts of large completed works such as PhD, MD or DSc theses, processed into journal articles. This journal issue contains materials from Ludmila Grivtsova's doctoral thesis on subpopulations of mobilized hemopoietic stem cells in oncology. The main conclusion of this work is establishment of the important role of immature stem cell compartments in hemopoiesis recovery. This is a major step towards understanding of the need in transplantation of stem cell subsets.

The journal also contains abstracts of the 13th *Haematopoiesis Immunology* International Conference, Budapest, June 3–4, 2016. The conference is named *Bone marrow immunology in minimal residual cancer*. Three main aspects of the problem were presented, i.e. minimal residual disease in oncohematology, minimal cancer and immunotherapy of MRD. Bone marrow immune system in cancer patients is a new, yet poorly studied problem, and few scientists make research in this field. This may be explained by difficulty of bone marrow study, lack in appropriately trained immunologists and insufficient understanding of this problem among oncologists. However, it is bone marrow where tumor cells persist for many years or even decades and local immunity plays an important role in maintenance of their latency. Since subsets of disseminating and circulating tumor cells have features of tumor stem cells and are characterized by chemoradioresistance, it is of primary importance to make all effort to eradicate them. The conference participants including leading scientists will discuss whether Her2/neu receptor and molecule CD20 may be used as targets for this purpose.

N.N. Tupitsyn

Editor in Chief
Honored Scientist of Russian Federation
Professor

СПИСОК СОКРАЩЕНИЙ

АГ – антиген
аллоТГК – аллотрансплантация гематопоэтических клеток
Ауто-, алло-ТКМ – аутологичная или аллогенная трансплантация клеток костного мозга или мобилизованных стволовых клеток периферической крови
БРВ – безрецидивная выживаемость
БСВ – бессобытийная выживаемость
β-ХЛЛ – В-клеточный хронический лимфолейкоз
ИГ – иммуноглобулин
ИФА – иммуноферментный анализ
ИФТ – иммунофенотипирование
ЛАФ – лейкоз-ассоциированные фенотипы
ЛДГ – лактатдегидрогеназа
ЛСК – лейкозные стволовые клетки
МДС – миелодиспластические синдромы
МК – моноклональный компонент
МКА(Т) – моноклональные антитела
ММ – множественная миелома
МНК – мононуклеарные клетки
МОБ – минимальная остаточная болезнь
МПО – миелопероксидазы
МПЦ – мультипараметрическая проточная цитометрия
МРБ – минимальная резидуальная болезнь
НХЛ – неходжкинская лимфома
НЭ – α-нафтил-ацетат-эстеразы
ОМЛ – острый миелолейкоз
РА – рефрактерная анемия
РБ – резидуальная болезнь
РМЖ – рак молочный железы
РЛ – разнице логарифмов
СКПК – стволовые клетки периферической крови
кПЦР – количественная полимеразная цепная реакция
КМ – костный мозг
РИФ – реакция иммунофлуоресценции
ОЛЛ – острый лимфобластный лейкоз
ПК – плазматические клетки
ПР – полная ремиссия
ПЦ – проточная цитометрия
ПЦР – полимеразная цепная реакция
СОЭ – скорость оседания эритроцитов
СРБ – С-реактивный белок
ТПЛ – тумор-пенетрирующие лимфоциты
ФТС – фетальная телячья сыворотка
ЦТЛ – цитотоксические Т-лимфоциты
ЧР – частичная ремиссия

ABBREVIATIONS

Ag, antigen.
AML, acute myeloid leukemia
ALL, acute lymphoblastic leukemia
Auto-/allo-BMT, autologous or allogeneic transplantation of bone marrow cells or mobilized peripheral stem cells.
B-CLL, B-cell lymphocytic leukemia.
BSA, bovine serum albumin.
BC, breast cancer
CD (claster of differentiation) – кластер лейкоцитарных дифференцировочных антигенов
CR – complete remission
CRP, C-reactive protein
EIA, enzyme immunoassay
ESR, erythrocyte sedimentation rate
ESFT, ewing's sarcoma family of tumors
FC, flow cytometry
FISH, fluorescent hybridization *in situ* (флюоресцентная гибридизация *in situ*)
HMW-MAA, high molecular weight–melanoma-associated antigen
IG, immunoglobulin
IFR, immunofluorescence reaction
IPP, изопентенил пирофосфат
IPT, immunophenotyping
LAPs – leukemia-associated phenotypes
LDH, lactate dehydrogenase
LSC – leukemic stem cells
MDS, myelodysplastic syndromes
MC, monoclonal component
Mab, monoclonal antibodies.
MFC – multiparameter flow cytometry
MHC, major histocompatibility complex
MNC, mononuclear cells
MRD, minimal residual disease
NK-клетки, натуральные киллеры
NHL, Non-Hodgkin's lymphomas
QPCR – quantitative polymerase chain reaction
RFS – relapse-free survival
PNAS (Proceedings of National Academy of Sciences of the USA)
RD, residual disease
PBS, phosphate buffered saline
PC, plasma cells
PCR, polymerase chain reaction
SSC – боковое светорассеяние
VCAM-1, vascular cell adhesion molecule (молекула адгезии сосудистых клеток)

L.Yu. Grivtsova, N.N. Tupitsyn

MOBILIZED HEMOPOIETIC STEM CELLS: AUTOLOGOUS AND ALLOGENEIC TRANSPLANTATION IN ONCOLOGY PRACTICE

N.N. Blokhin Russian Cancer Research Center, Russian Health Ministry

Key words: mobilized hemopoietic stem cells, CD34, subsets, autologous and allogeneic transplantation, hematology malignancies, hemopoiesis recovery.

Running title: Stem cell subsets in hemopoiesis recovery

Abstract

Peripheral hemopoietic stem cells (HSC) that are transplanted to cancer patients to reduce critical pancytopenia vary in subset composition and include early polypotent precursors ($CD38^-$ and/or HLA-DR^-, $CD90^+$, CD45-negative), lymphoid precursors ($CD10^+$, $CD7^+$, $CD2^+$, $CD19^+$, $CD56^+$), megakaryocyte-($CD61^+$) and myeloid-committed precursors ($CD117^+$, $CD13^+$, $CD33^+$). These subsets of early and committed HSC are found in different proportions in cancer patients and normal donors. HSC from healthy donors include a marked proportion of early ($CD38^-$HLA-DR^-, $CD90^+$, CD45-negative) cells ($p < 0.045$), lymphoid ($CD7^+$, $CD2^+$, $CD19^+$, $CD56^+$) cells ($p = 0.01$) and megakaryocyte-committed precursors ($p = 0.006$). Autologous transplantation of subsets of early polypotent HSC (CD45-negative, $p < 0.04$; Thy-1-positive, $p < 0.05$) ensures highly effective hemopoiesis recovery and reduces period of critical cytopenia (up to 10 days for leukocytes and 14 days for platelets) in cancer patients irrespective of $CD34^+$ total count transplanted. Transplantation of $CD38^-$ HSC (less than 65%) contributes to effective leukocyte recovery after autologous transplantation in children irrespective of $CD34^+$ cell dosage. Proportion of early $CD45^-CD34^+$ precursors is prognostically significant for recovery of basic blood parameters after allogeneic transplantation of mobilized HSC in children with hematology malignancies. Marked proportion of myeloid-committed precursors ($CD13^+$, $CD117^+$) has a negative effect on recovery of hemopoietic parameters in both autologous and allogeneic transplantations of mobilized HSC.

Introduction

Autologous and allogeneic hemopoietic stem cells (HSC) ensure replacement and/or recovery of compromised, pathological hemopoiesis and are widely used in clinical oncology in patients receiving high-dose cytostatic therapy and/or irradiation. Efficacy of HSC transplantation is demonstrated in a broad range of diseases including hematology malignancies and some solid tumors as

well as in inherited or acquired bone marrow (BM) diseases. Peripheral HSC mobilized by exposure to growth factors are used in most cases (86% of autologous transplantations in Europe) for this purpose. Speed and completeness of compromised hemopoiesis recovery depend upon HSC content in the graft [1–5].

HSC is a small but heterogeneous cell population combining several types (subsets) of cells different in levels of differentiation and ability to proliferate. It includes both undifferentiated, practically not dividing stem cells (SC) and committed (obliged to follow an individual differentiation course) precursor cells [6–8].

SC concentration in peripheral blood (PB) in the steady state hemopoiesis is low, i.e. less than 0.01% which renders difficult their study even with most sensitive assays.

Discovery of the HSC mobilization phenomenon allowed blood SC to be used to recover hemopoiesis after high-dose cytostatic therapy and marked the advent of a new era in the treatment of patient cohorts requiring significant escalation of chemotherapy dosage [2].

Blood SC preserve their principal properties such as a considerable proliferation potential and the ability to differentiate into mature blood cells after mobilization [9–11].

During the mobilization HSC concentration rises to levels sufficient for transplantation. Detailed study of membrane markers of these cells is made by immunological techniques in particular by flow cytometry using multiparametric immunofluorescence and HSC identification basing on CD34 expression.

PB as well as BM and umbilical cord blood may be used as transplant sources. Choice of HSC source depends on conditions of the specific health center since each of the approaches has advantages and disadvantages of its own [12, 13].

Use of donors' SC in clinical practice allows a detailed study of immunophenotypic characteristics of healthy hemopoietic precursor cells.

Hemopoietic precursor cells arising in patient's blood during recovery of hemopoiesis that has been compromised due to cytostatic therapy may be identified by CD34 expression and the ability to form colonies. CD34-positive cells compose the total HSC pool that is heterogeneous because of the presence of multiple colony-forming units (CFU). The $CD34^+$ pool is composed of CFU-GEMM, CFU-GM, CFU-E, cluster-forming cells and stem bi- and unipotent precursor cells. The $CD34^+$ HSC pool includes minor subsets of non-proliferating and non-differentiating cells and the earliest non-committed precursor cells possessing a considerable self-maintenance potential.

The HSC pool is extremely heterogeneous and includes early pluripotent and polypotent precursors able to give rise to all hemopoiesis lineages, as well as bi- and unipotent precursors responsible for maturation of specific hemopoietic lineages [9, 14, 15].

HSC heterogeneity or, in other words, subset composition reflects their immunophenotyping profile, i.e. the totality of expressions of membrane and intracellular proteins that can also be detected by immunological techniques using multicolor flow cytometry [12, 16–18].

Improvement in techniques for cell identification and characterization basing on expression on cell surface of specific proteins, or multiparametric flow cytometry [18–22] has made possible a more detailed characterization of various HSC subsets. Immunophenotyping profiles of the earliest non-committed HSC and precursor cells of all hemopoiesis lineages with various differentiation levels were determined experimentally by estimation of expression levels of human leukocyte antigens within $CD34^+$ cell population, that is within the entire HSC compartment (fig. 1) [22]. The identification of the whole HSC antigen mosaic by multicolor flow cytometry is a key aspect in the understanding of SC biology.

Cell membrane phenotype is changing during the process of HSC differentiation from truly stem type to lineage-restricted unipotent cells. $CD34^+$ immunological phenotype is a very exact reflection of maturity degree of the SC or more accurately of the precursor cell. Basing on expression of certain antigens on $CD34^+$ membrane one may evaluate HSC subset composition, i.e. the presence of polypotent and lineage-committed cells.

Cell immunological phenotype implies the total range of antigens and receptor molecules that are expressed, i.e. present on cell membrane and/or in cytoplasm. CD34-expressing cells with no expression of non-lineage restricted antigens such as HLA-DR and CD38 are the earliest non-lineage restricted (not acquiring signs of any differentiation lineage) stem cells. As demonstrated by some authors [23–25], cells at this level may show weak expression of Thy-1 whose most isotypes may be expressed on non-hemopoietic cells. Therefore the immunophenotype $CD34^+CD90^{\pm}HLA\text{-}DR^-CD38^-$ may be associated with the earliest pluripotent HSC.

HSC just entering the cycle of differentiation, initiating growth of long-term culture (LTC-IC) and forming splenic colonies in mice may be associated with several populations in terms of immunological phenotype.

CD34 expression on these cells is high ($CD34^{++}$). HSC different in CD38 and HLA-DR expression are identified among the given HSC subsets. Some cells express CD90, and many publications confirm expression of pan-myeoid antigens CD13 and CD33 to be likely. However, most of the LTC-IC cells are $CD13^-CD33^-$, and one may expect that cells are making a choice between lymphoid or myeloid ways of development at this differentiation stage [26].

Expression of transferrin receptor molecule CD71 at this differentiation stage is equivocal. This cell type is traditionally attributed to erythroid precursor cells [27], however, expression of the antigen may be seen on actively proliferating hemopoietic cells at early differentiation stages.

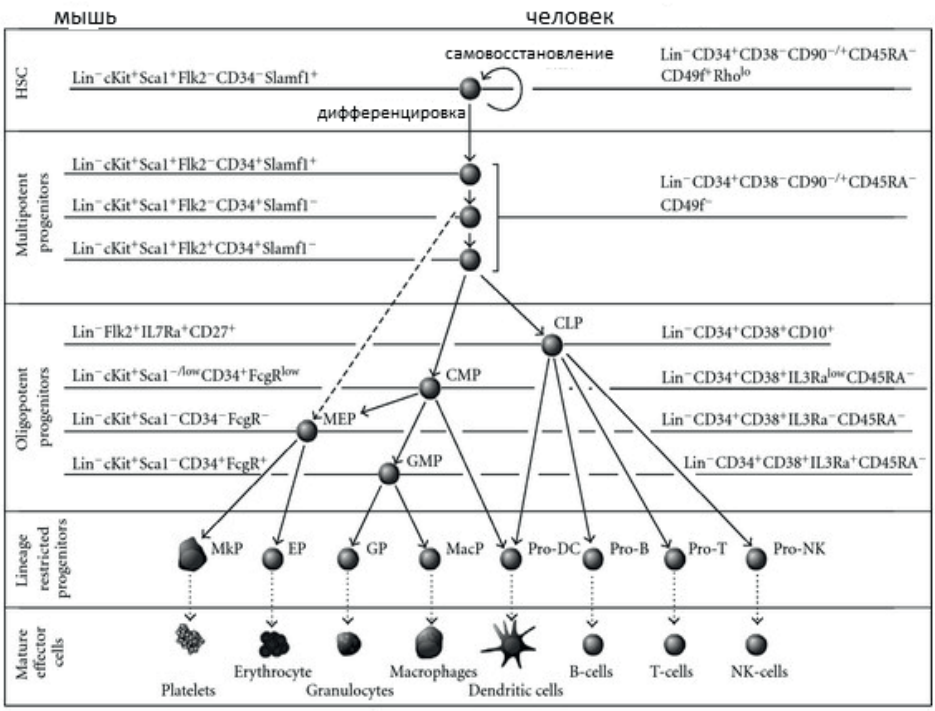


Figure 1. Hemopoiesis hierarchical structure [22].
The figure shows membrane antigens that are characteristic of human and murine HSC of various differentiation degrees. In mice multipotent precursors form bipotent erythroid-megakaryocyteic precursors (MEP, broken line) directly, avoiding the myeloid precursor stage, while in man generation of bipotent (erythroid-megakaryocyte) precursors follows the stage of formation of oligopotent myeloid precursors (CMP). CLP, common lymphoid precursor; DC, dendritic cells; EP, erythrocyte precursor; GMP, common granulocyte-macrophage precursor; GP, granulocyte precursor; HSC, pluripotent hemopoietic stem cells; MacP, macrophage precursor; MEP, common erythrocyte and megakaryocyte precursor; MkP, megakaryocyte precursor; NK, natural killers; Lin, lineage-restricted markers.

It is LTC-IC cells together with truly stem HSC that can maintain compromised hemopoiesis for a long time. There can be an earliest common lymphoid precursor characterized by CD10 expression on $CD34^+$ cells and no expression of other lineage-committed molecules [6, 28, 29].

The next differentiation stage is committed precursor cells. They include polypotent HSC that form large mixed colonies of granulocytes-macrophages-megakaryocytes-erythrocytes (CFU-GEMM), and burst-forming cells (BFC) with the polypotent ability to form myelomonocyte and erythroid (predominating) lineages.

Cells that form such colonies, beside marked expression of stem-cell antigen CD34, are positive with respect to nonlineage restricted antigens HLA-DR and CD38 and also show clear-cut signs of myeloid differentiation ($CD13^+CD33^+$). Subsets different in expression of pan-myeloid molecules may be present. A part of this HSC pool acquire signs of megakaryocyte differentiation ($CD61^+$) and are likely to differentiate towards erythroid lineage ($CD71^+CD236a^+$) [6, 12, 14, 30].

The committed HSC population is completed by bipotent (CFU-GM) and unipotent (CFU-M, CFU-G) myeloid-committed precursor cells different by level of expression of pan-myeloid antigens CD13 and CD33. There is naturally a unipotent lymphoid-committed lineage too. However, cells from these subsets practically fail to form short-term colonies, and study of HSC from this pool is possible by flow cytometry only.

It is the pool of lineage-committed cells (CFU-GEMM, CFU-GM, CFU-M, CFU-G) that is responsible for rather fast recovery of both leukocytes (mainly neutrophils) and platelets (polypotent CFU-GEMM population) after hemopoietic engraftment.

Detailed information about SC membrane immunophenotype and accordingly about HSC subset composition is of much importance both from the clinical (prognosis for time to recovery for each hemopoietic component) and fundamental (understanding of differentiation specificity of normal blood cells at different hemopoiesis stages) points of view.

The principal reference point for evaluation of efficacy of mobilized HSC transplantation, i.e. hemopoiesis recovery, is the total count of $CD34^+$ cells. Minimal concentration needed to recover hemopoiesis is 2 million per kg of recipient's body weight. Recent protocols for some hematology malignancies (diffuse large B-cell lymphoma, Hodgkin's lymphoma, multiple myeloma) recommend a transplantation dose of 4 million $CD34^+$ cells per kg patient's body weight [31–33].

Although $CD34^+$ cell subsets including the earliest cells from BM, umbilical cord blood and stimulated PB [34, 35] are characterized rather well, there is a certain ambiguity of data concerning contribution of each specific HSC subset [34, 36–44].

A part of publications analyze early multilineage post-transplantation recovery of hemopoiesis both in autologous and allogeneic HSC transplantation. While others consider the effect of different $CD34^+$ subsets on sustained long-term hemopoiesis recovery within 3 months or more from transplantation.

In patients with hematology malignancies receiving transplantation of mobilized peripheral HSC complete and sustaned recovery of platelts correlated with proportion of transplanted $CD34^+CD117^-HLA\text{-}DR^-$ cells ($p = 0.04$) and proportion of HSC free from CD133 expression ($CD34^+CD133^-$, $p = 0.027$). Proportion of $CD34^+CD38^-HLA\text{-}DR^-$ cells in leukapheretic product (LP) directly

correlates with recovery of total count of leukocytes ($p = 0.025$) and neutrophils ($p = 0.023$) within 3 to 6 months after transplantation. Long-term sustained recovery of leukocytes and neutrophils is also associated with doses of tranplanted $CD34^+CD133^-$ cells ($p = 0.03$), while the number of transplanted $CD34^+CD123^+HLA\text{-}DR^-$ cells is directly associated with sustained recovery of neutrophils only ($p = 0.038$). As demonstrated by multivariate analysis, sustained platelet recovery after BM HSC autotransplantation was associated with proportion of $CD34^+CD117^-HLA\text{-}DR^-$ cells ($p = 0.039$). While after autotransplantation of HSC from PB proportion of $CD34^+CD38^+HLA\text{-}DR^-$ cells was significantly associated with effective platelet recovery (by multivariate analysis) within the first 3 months following transplantation ($p = 0.011$), and this subset was also associated with sustained long-term erythroid recovery ($p = 0.023$). It was also shown that absolute count of transplanted (per kg bodyweight) $CD34^+CD117^-HLA\text{-}DR^-$, $CD34^+CD38^+HLA\text{-}DR^-$ and $CD34^+CD123^+HLA\text{-}DR^-$ cells was significantly lower in patients with delayed or no hemopiesis recovery within 3 to 6 months from transplantation. Patients receiving higher doses of the above-mentioned HSC subsets demonstrated complete sustained but delayed recovery of platelets and neutrophils [34].

The above-described findings mainly cencern populations of earliest HSC including (by literature data) $CD34^+CD38^+$ cells as mentioned above [38]. There are only 3 foreign publications on the role of this subset with rather equivocal results. For instance, the earliest study demonstrated the impact of mobilized autologous $CD34^+CD38^-$ dose on platelet recovery after autologous HSC transplantation in patients receiving high-dose chemotherapy (HCT), with higher dose being associated with delay in platelet recovery [39].

On the contrary, more recent studies demonstrated that a high $CD34^+CD38^-$ HSC dose (5×10^4 per kg bodyweight) was associated with better hemopoietic engraftment and faster three-lineage hemopoiesis recovery. Allogeneic transplantation of a higher absolute count of $CD34^+CD38^-$ HSC was associated with faster recovery of neutrophils up to 500 to 1000 cells per mcl and platelets up to 20 000–50 000 cells per mcl [38, 45].

The clinical role of Thy-1^+ HSC population was not avoided either. Most studies on the effect of Thy-1^+ HSC subsets focus on evaluation of late post-transplantation hemopoiesis recovery and hemopoietic engraftment. As found, higher doses of $CD34^+CD90^+$ HSC (more than 80×10^4 per kg bodyweight) correlated with better recovery of platelets ($p < 0.023$) within 6 to 12 months after transplantation and HCT [46, 47].

It should be noted that most authors analyze the impact of absolute cell number of each HSC subset (cell number per kg bodyweight) when evaluating the transplantation effect on hemopiesis recovery.

However this parameter may be vulnerable to the total dose of $CD34^+$ cells tranplanted which also plays a significant role in hemopoiesis recovery in the above-mentioned publications.

Therefore there is an unmet need in a more detailed evaluation of the role of specific subsets in mobilized HSC transplantation.

Materials and methods

Study of HSC subsets was performed in 569 specimens of hemopoietic tissue (blood cells and LP cells) from 167 adult cancer patients and on 557 specimens of hemopoietic tissue from 263 pediatric cancer patients. Sixty one LP specimens from 50 adult donors of allogeneic hemopoietic tissue for 47 recipients (children with hematology malignancies) were included in the study. All patients were managed at bone marrow transplantation units of hematology malignancy and oncology department of N.N. Blokhin Cancer Research Center from 1996 to 2014.

Characteristics of adult patients

We analyzed 179 PB specimens and 390 blood cell mononuclear fraction (LP) specimens isolated by PB separation using Baxter (CobeSpectra) blood separators.

Mean age of adult patients was 33 years (median 32 years, range 16 to 64 years), patients weight varied from 43 to 113 kg. The patients were mainly males (113 males and 54 [32.3%] females).

There was a predominance of patients with hematology malignancies (158, 94.7%) in the adult group, including Hodgkin's lymphoma (58, 34.7%), NHL (diffuse large B-cell lymphoma and pre-B-cell lymphoma, Burkitt's lymphoma [2 patients]) (57, 34.1%). The study group also included 40 (23.9%) patients with multiple myeloma and 3 patients with AML. There was a minor proportion of patients with non-hematology malignancies (9, 5.3%) including breast cancer (3 patients), small-cell lung cancer (2), non-germ-cell testicular tumor (1) and Ewing's sarcoma (2)

Granulocyte colony stimulating factor (G-CSF) was used to mobilize HSC in all adult patients. Chemotherapy regimens given prior to growth factors depended on diagnosis. Patients with Hodgkin's lymphoma and some patients with NHL received DEXA-BEAM and BEACOPP regimens. Some patients with B-cell lymphoma received rituximab-containing scedules (R-CHOP). CHOP was also used in some cases with Hodgkin's lymphoma. VAD was administered before growth factors in multiple myeloma and CAF in patients with breast cancer.

Characteristics of pediatric patients

HSC characterization was made in 557 specimens of stimulated hemopoietic tissue (115 PB, 442 LP) from 263 children (123 girls and 140 boys). 48 patients received treatment for recurrent or previous CT-resistant

neuroblastoma, 41 had other soft-tissue sarcoma types (rhabdomyosarcoma 14, sinovial sarcoma 7, high-risk retinoblastoma (20), the remaining tumor types included primitive neuroendocrine tumors (PNET, 24), high-risk Ewing's sarcoma (63), high-risk AML (22), Hodgkin's lymphoma (16), B-NHL (6), CNS tumors (glyoma, atypic teratoid rhabdoid tumor [ATRT], ependymoma, (16), medulloblastoma (28), metastatic osteosarcoma (10), germ-cell tumors (3). Mean age was 7.88±0.28 years (median 7 years, range 0.6 to 19 years), mean weight was 29.3±0.98 (median 23.0; range 7.0 to 87.0) kg.

95% of leukaphereses (420/442) were made after mobilization regimens consisting of chemotherapy with a single myelocytokin or a combination of myelocytokins to follow. 259 patients receivied G-CSF and 4 patients received GM-CSF.

At the leukapheresis start all patient achieved complete or partial response of the primary and/or metastatic lesions, there were no BM metastases either.

Mean number of CT cycles before the first collection was 3 (0 to 12) per patient. HSC collection was made after surgery or radiotherapy without prior CT in 9 patients.

Mobilization regimen in neuroblastoma cases included cyclophosphamide 3,000 mg/m^2/day on day 1 with mandatory CSF administration to follow.

Treatment protocol for Ewing's sarcoma consisted of 2 CT cycles and HSC harvesting after detection of no microscopic metastases in BM.

That is why HSC in patients with this disease type were mobilized with ifosfamide 2,400 mg/m^2/day on days 1 to 5 in combination with vepeside, 100 mg/m^2/day on days 1 to 5 (Ifo-VP); or CAV including adriamycin 70 mg/m^2 by a 48-h infusion, cyclophosphamide 2,100 mg/m^2/day on days 1 to 2, vincristine 1.5 mg/m^2/day, days 1, 8, 15.

Other CT regimens containing cisplatin, ifosfamide, vepeside, cyclophosphamide, adriamycin in various combinations were administered before leukapheresis in patients with solid tumors or hematology malignancies.

GM-CSF was given at an average dose 9.8 (5 to 12) mcg/kg/day, G-CSF was administered at the hemopoiesis recovery stage after prior CT at a mean dose 4.8 (3.4 to 12.8) mcg/kg/day beginning on day 18 (8–41) from CT start. In 7% of cases (21 patient) HSC were mobilized using G-CSF or GM-CSF without prior CT. In these cases CSF were given after stable recovery of all 3 hempoiesis lineages.

The first HSC mobilization failed in 14 patients (5.3%) who underwent second hemopoiesis stimulation and HSC harvesting at a 1 month interval. G-CSF was substituted for GM-CSF in 3 patients and double second HSC moblilization was made in 1 case. If the HSC harvest was less than the recommended dose (2×10^6 per kg bodyweight), BM samples were taken in parralel with continuing hemopoiesis stimulation (14 cases).

In patients with stable hemopoiesis without prior CT mean G-CSF dose was 5.7 (4.8–11.9) mcg/kg/day, G-CSF was given twice daily for 1 to 3 days with the dose doubled on day 4.

The collection was started on detection of a rapid rise in leukocyte count to more than 1.0×10^9/l with a stable platelet level above 50×10^9/l or a rapid platelet elevation at the recovery stage after CT. The platelet level of 50×10^9/l was not a strict determination factor.

Enumeration of $CD34^+$ cells in PB was made before HSC harvesting in patients free from marked hemopoiesis aplasia.

HSC were isolated using a Baxter CS-3000 plus blood separator (Special 1 and 2 programs) or a CobeSpectra continuous flow cytometer.

Leukapheresis was made on a daily basis till a minimal total number of more than 2.0×10^6/kg or an optimal level of 5.0×10^6/kg or more $CD34^+$ cells in the transplant was obtained. Patients received cell growth factors during the entire leukapheresis period including the last harvest day. Growth factors were administered within 10 h before separation.

Characteristics of donors
for allogeneic transplantation to pediatric patients

We analyzed $CD34^+$ immunophenotype in 61 leukapheresis products (LP) (enriched fraction of mobilized PB SC) from 50 donors for allogeneic transplantation to 47 pediatric patients.

Before harvesting the donors underwent HSC mobilization by a standard stimulation protocol consisitng of growth factor administration without CT (G-CSF on days 1 to 3 at 5.0 mcg/kg bodyweight 2 times and 10.0 mcg/kg bodyweight 2 times on day 4).

The commonest transplantation types were from mother to daughter (19), from mother to son (14), from farther to son (9) and from farther to daughter (5). Choice of relative donors was mainly based on compatibility, potential donor performance status and age.

With otherwise equal characteristics the choice between farther and mother was made in favor of mother due to fetomaternal chimerism.

The donors' mean age was 34 (17 to 48) years, there were more females (36) than males (14) among the donors.

The recipients' (47 patients) mean age was 9 (1 to 18) years, the group was composed almost equally of boys and girls (23 and 24 respectively).

The recipients' group was heterogeneous in respect of disease type with most (33) cases having hematology malignancies (acute leukemia, non-Hodgkin's lymphoma, chronic myelomonocytic leukemia), solid tumors (14) including stage IV neuroblastoma, PNEO, Ewing's sarcoma and melanoma (1 case). A total of 51 transplantations were performed including 31 gaplo-

(partial compatibility) and 20 allogeneic relative transplantations. Collection of sufficient amount of cells for adequate transplantation (at least 2.0×10^6 $CD34^+$ cells per kg recipient's body weight) was made during a single leukapheresis.

Sufficient material for adequate transplantation was harvested by 2 leukaphereses in 8 and by 3 leukaphereses in 1 case. In 7 cases (14.9%) the number of transplanted $CD34^+$ cells was less than 2.0×10^6 per kg recipient's bodyweight (0.1 to 1.93), however the tranplantations were done.

Immunological characterization of transplant quality

SC enumeration and subset characterization was made in PB specimens on the day before cytapheresis, and directly in LP specimens. In most adult and in a part of pediatric patients enumeration of HSC from PB was made the day before or on the day of the first leukapheresis.

Transplant quality was assessed immunologically by direct immunofluorescence with flow cytometry findings taken into consideration.

Enumeration of $CD34^+$ and a part of mature T-cells ($CD3^+$) was made immediately after leukapheresis, enumeration of T-lymphocyte (CD8, CD4) subsets and NK-cells (CD56) was made in some donors.

Enumeration of stem $CD34^+$ was performed in accordance with the ISHAGE protocol by double immunofluorescence with direct conjugates of monoclonal antibodies to SC antigen CD34 and common leukocyte antigen CD45. Table 1 demonstrates flourochrome labels and antibody clones.

This protocol was modified to enumerate $CD34^+$ cells including those not expressing common leukocyte antigen. Percentage of $CD34^+$ cells was calculated in the whole cell population of the specimen, the resulting $CD34^+$ percentage was then recalculated with respect to leukocytes ($CD45^+$ cells) depending on their content in the specimen. HSC subset composition was assessed within $CD34^+$ pool using triple fluorescent labeling.

Proportion of early precursors was calculated by expression of non-lineage restricted antigens (HLA-DR, CD38, CD71), early differentiation stage markers (CD117, CD90) and cell adhesion molecules (CD50, CD56), CD45, proportion of lineage-committed HSC, such as myeloid (antigens CD13, CD33), lymphoid (CD7, CD19), megakaryocytic (CD61) and erythroid precursors by expression of red cell antigen glycophorin (CD236a).

Antigen analysis allows rather accurate characterization of differentiation course and enumeration of polypotent and bipotent (myeloid $CD13^+$, $CD33^+$) or lymphoid-committed (B-lineage: $CD19^+CD34^+$, T-lineage: $CD7^+CD33^+$) HSC, as well as of SC with megakaryocyte differentiation ($CD61^+$).

Population of erythroid precursors ($CD71^+CD236a^+$) in specimens from this group was small, and this subset was not analyzed.

Table 1

Characteristics of hemopoietic stem cell subsets. Antigens and monoclonal antibodies

Antigen (differentiation cluster)	Clone/class	Manufacturer
Gp105–120 (CD34)	HPCA-2/8G12/ IgG_1 κ	Becton Diskinson
Leukocyte common antigen (CD45)	Hl30/IgG_1 κ	Becton Diskinson
ICAM-3 (CD50)	TU41/IgG^2b κ	Immunotech, Coulter
HLA-DR	L243IgG_{2a}, κ	Coulter, Becton Diskinson
CD38	HLT2, HB7/IgG_1 κ	Becton Diskinson
Thy-1 (CD90)	5E10/ IgG_1 κ	Immunotech, Coulter
c-kit (CD117)	YB5.B8/ $IgG_{1,}$ κ	Becton Diskinson
N-CAM (CD56)	B159 /IgG_1 κ	Becton Diskinson
Transferrin receptor CD71	M-A712 /IgG_{2a}, κ	Coulter Becton Diskinson
CD13	L138/IgG_1, κ	Becton Diskinson
CD33	P67.6 IgG_1, κ	Becton Diskinson Coulter
Integrinβ_3 chain (CD61)	RUU-PL7F12/ IgG	Becton Diskinson
CD19	4G7	Coulter
CD7	M-T701/ IgG_1 κ	Immunotech, Coulter
CD57	HNK-1/ IgM	Becton Diskinson
LFA-2	RPA-2.10/IgG_1	Becton Diskinson
CD10	HI10a/ IgG_{2a}	Becton Diskinson
Glycophorin A (CD236a)	GA-R2 (HIR2)/IgG_{2b}, κ	Immunotech, Coulter

Statistical analysis of the findings was made using Windows SPSS version 17 software.

Data processing was made using frequency function to describe sequential variable series, means, medians, mean standard errors and intervals of variate values; function of bivariate correlations to determine correlation factor for two independent variables, correlation was significant at $p \leq 0.05$, comparison function of means of dependent and independent variables, 95% or higher confidence interval; function of variable coding with respect to intervals of values for the chosen variable.

Results

1. General immunophenotype characterization of mobilized hemopoietic stem cells from cancer patients and healthy donors

There was a marked proportion of $CD7^+CD34^+$ HSC among SC in LP from adults. There were HSC specimens with minimal proportions of myeloid-committed $CD34^+$ cells ($CD13^+$, $CD33^+$) and $CD38^+$ HSC. Proportion of $Thy\text{-}1^+CD34^+$ cells was higher in LP than in PB specimens as harvested the day before leukapheresis ($p = 0.040$).

As a whole, the total of $CD34^+$ leukocytes in LP from adults was inversely proportional to the number of $CD34^+CD33^+$ cells ($p = 0.049$) and directly proportional to the number of $CD34^+CD45^+$ ($p = 0.028$) and $CD117^+$ HSC ($p = 0.037$). The number of $CD34^+CD117^+$ HSC was inversely proportional to the number of megakaryocyte precursors $CD34^+CD61^+$ ($R = –0.579$, $p = 0.012$). HSC with megakaryocyte differentiation were inversely proportional to $CD34^+CD90^+$ ($R = –0.357$, $p = 0.013$). Myeloid-committed precursors $CD13^+CD34^+$ and $CD33^+CD34^+$ were very likely to occur in LP $CD71^+CD34^+$ pool ($R = 0.382$, $p = 0.002$ and $R = 0.285$, $p = 0.032$, respectively).

The content of $CD13^+$ HSC was inversely proportional to that of $CD34^+CD7^+$ cells ($R = –0.449$, $p = 0.002$). The proportion of $CD34^+CD7^+$ cells was significantly related to $CD34^+CD45^{+low}$ subset ($R = 0.471$, $p = 0.001$), population $CD34^+CD45^{+low}CD61^+$ was detected quite frequently ($R = 0.362$, $p = 0.05$).

There was a statistically significant correlation between $CD34^+CD33^+$ and $CD38^+$ HSC ($R = 0.323$, $p = 0.017$) as well as between $CD34^+CD7^+$ and $CD34^+CD19^+$ cells ($R = 0.473$, $p = 0.001$), and between $CD19^+$ and $CD34^+CD56^+$ HSC ($R = 0.396$, $p = 0.03$).

Comparison of $CD34^+$ subsets in PB and LP of the same harvest failed to demonstrate significant differences in proportions of any of the studied populations in adults.

HSC in LP from children demonstrated monomorphous CD34 expression in most cases.

As concerns light scattering, populations of smaller (FSC^{low}) and larger (FSC^{high}, fig. 2) cells were detected among HSC in both PB and LP from cancer patients and donors.

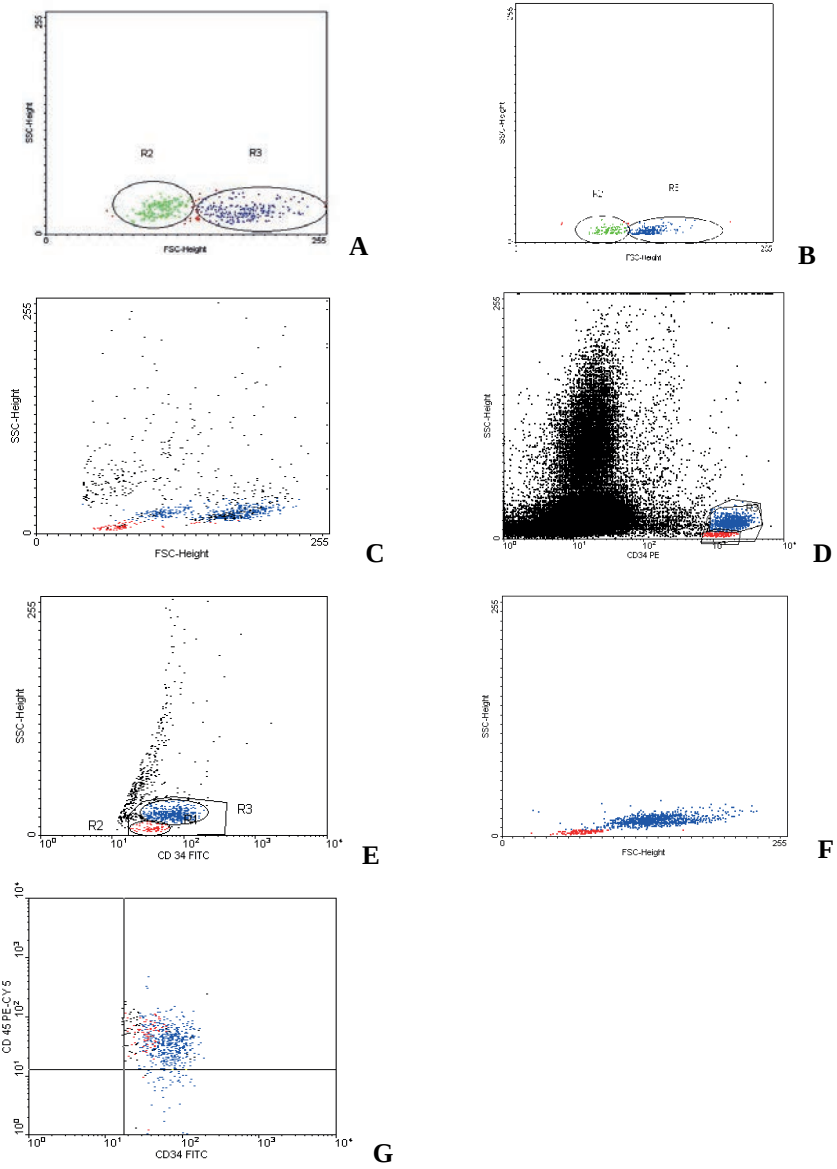


Figure 2. Light scattering characteristics of mobilized CD34$^+$ cells.
The figure shows LP specimens from 4 different patients (A, patient 1; B, patient 2; C and D, patient 3; E and G, patient 4) with specific light scattering characteristics of mobilized HSC.

Figs. A, B, D, E demonstrate variability in $CD34^+$ pool size [forward light scattering (FSC) on the χ axis and side light scattering (SSC) on the γ axis]. Cells with low and intermediate FSC levels, i.e. smaller HSC populations (morphologically corresponding to small lymphocytes) are colored green and red, cells with intermediate and high FSC levels, i.e. larger cells are colored blue.
Figs. C to F (patients 3 and 4) demonstrate $CD34^+$ variability by SSC levels. C and E show $CD34^+$ cells (χ-axis) against side scattering (γ-axis). Both patients had clear-cut minor $CD34^+SSC^{low}$ populations (red in all further cytograms); a part of this HSC subset expressed stem-cell antigen weaker than SSC^{med} population (blue cells). The $CD34^+SSC^{med}$ mainly consisted of very small cells (FSC^{low}) as compared to the $CD34^+SSC^{med}$ cells (D and F).
Fig. G characterizes $CD34^+SSC^{low}$ population ($CD34^+$ cells on the χ-axis, $CD34^+SSC^{low}$ cells are shown in red) by expression of common leukocyte antigen CD45 (γ-axis); as seen most cells are $CD34^+CD45^+$ with CD45 expression typical for most HSC.

Small cells (FSC^{low} $63.7 \pm 1.9\%$; FSC^{high} $39.5 \pm 1.9\%$) were predominating among HSC in children. Beside size differences, childhood $CD34^+$ contained subsets different in side scattering as SSC^{low} and SSC^{med} (Fig. 2, C, F). Together with variable levels of side light scattering these HSC subsets differed by expression of common leukocyte antigen CD45, and practically all $CD34^+SSC^{low}$ cells were CD45-positive (fig. 2, G).

In children LP HSC demonstrated lower content of $CD34^+CD33^+$ and greater quantity of $CD34^+Thy\text{-}1^+$ cells as compared with PB specimens. The difference by $Thy\text{-}1^+$ HSC was statistically significant ($p = 0.023$).

In both adults and children there were HSC specimens with minimal contents of myeloid-restricted $CD34^+$ ($CD13^+$, $CD33^+$) and $CD38^+$ HSC. However there were just few such specimens and the trend to predominance of myeloid-restricted precursors was preserved.

Comparison of PB and LP from children demonstrated a correlation between $CD34^+$ leukocytes from PB and LP ($R = 0.526$; $p < 0.0001$, $n = 94$). While subsets failed to demonstrate clear-cut correlations between PB and LP.

There was a certain relationship between individual HSC subsets in LP from both children and adults. For instance, similarly to PB, smaller cells were predominating in the $CD34^+CD45^+$ as well as in the $CD34^+Thy\text{-}1^+$ HSC populations. While there were larger cells in the $CD34^+CD45^{neg}$ populations.

Coexpression of HLA-DR and CD38, CD38 and CD33, CD13 and CD33 was often seen on a single $CD34^+$ cell, as well as that of both myeloid antigens and Thy-1 (CD90). Coexpression of CD90 and transferrin receptor CD71 within a single HSC was likely. Specimens with marked $CD34^+CD19^+$ and $CD34^+CD7^+$ populations also contained sufficient numbers of $CD2^+$ HSC.

Comparison of HSC subsets in LP of the first day harvest demonstrated the same trend as in the analysis of the whole group with the exception of no correlation between the numbers of $CD34^+CD2^+$ cells and of cells in HSC subsets

different by CD45 expression, and larger HSC and $CD34^+CD90^+$ cells. There were also significant correlations between the number of C-kit receptor$^+$ (CD117) and $CD71^+$ HSC ($p < 0.0001$).

There were no significant differences in subset composition of LP of the first and second harvests. While there was a significant ($p = 0.009$) preponderance (7.55% vs. 1.74%) of $CD34^+CD45^-$ cells in the second vs. third day harvests.

There were no significant differences in subset composition between LP of the first and second HSC harvests in children.

Donors' SC subset composition was more heterogeneous. A considerable proportion (more than 70.0%) of $CD34^+$ cells expressed common leukocyte antigen CD45. Donors' HSC were monomorphous with respect to ICAM-3 (antigen CD50), class II HLA (HLA-DR) and myeloid antigen CD13.

Similarly to adult patients, more than 70% of donors' $CD34^+$ cells were $CD117^+$, and similarly to children, most donors' HSC were $CD33^+$. The number of $CD71^+CD34^+$ cells in donors was larger as compared to HSC from cancer patients.

Donors' $CD34^+$ cells demonstrated a considerable heterogeneity as concerns expression of non-lineage-restricted antigens CD38 and Thy-1 (CD90).

The number of SC with expression of both pan-B (CD19) and pan-T (CD7) lymphocyte antigens, NK-cell antigen (CD56) and megakaryocyte precursors ($CD61^+$ cells) was higher than in cancer patients irrespective of age.

Comparison of proportions of various subsets in donors' transplantation tissue also demonstrated certain significant relationships. For instance, the content of B-precursors was significantly related with Thy-1^+ SC (subset $CD34^+CD90^+$). The presence of precursors that were polypotent with respect to myeloid lineage ($CD34^+CD13^+CD33^+$) and T-lymphoid cells ($CD34^+CD7^+$) was unlikely: there was a negative correlation for both pan-myeloid antigens.

Coexpression of pan-T-cell antigen on $CD34^+$HLA-DR^+ HSC from donors was also unlikely, different from common precursor of T/NK-cells among donors' SC with a quite possible chance to be present (CD7 and CD56). Proportion of megakaryocyte precursors ($CD61^+CD34^+$) was correlated with that of $CD38^+$ SC. It was likely to find coexpression of both pan-myeloid antigens and transferrin receptor CD71 on $CD34^+$HLA-DR^+ cells. Besides, coexpression of antigen CD13 on $CD34^+CD71^+$ could be found with a high probability.

We failed to find interrelationship of $CD90^+$ and $CD33^+$ HSC (common for cancer patients) in donors.

Comparison of subsets in specimens from children, adult cancer patients and donors discovered certain significant differences (table 2). There were less $CD34^+CD117^+$ HSC ($p = 0.003$) and $CD34^+CD38^+$ HSC ($p = 0.001$) in children than in adult patients and donors. Allogeneic HSC as compared to autologous HSC demonstrated a predominance of early Thy-1^+CD34^+ subsets ($p = 0.02$) and

transferrin receptor-expressing cells ($p = 0.0001$), a significantly greater proportion of HSC with expression of lymphoid antigens CD56 ($p < 0.03$), CD19 ($p < 0.01$) and a greater proportion of HSC with megakaryocyte restriction ($CD61^+$, $p < 0.006$). $CD34^+CD45^+$ HSC subset was smaller as compared to cancer patients ($p = 0.049$). Adult patients had a smaller proportion of myeloid-committed precursors $CD13^+$ and $CD33^+$ ($p < 0.009$).

Our analysis demonstrated mobilization of several heterogeneous HSC subsets into PB including myeloid-committed HSC with specific expression of myeloid antigens $CD34^+CD38^+CD33^+CD71^+$ and HSC with immunological phenotype $CD34^+CD13^+CD71^+$. Detection of $CD7^+$ HSC was unlikely within this population ($p = 0.002$).

Proportion of both T- and B-lymphoid precursors and probably of NK-precursors in adult patients was increasing simultaneously, i.e. the higher was proportion of $CD34^+CD7^+$ cells, the higher were proportions of $CD19^+CD34^+$ cells and $CD34^+CD56^+$ cells in LP ($p < 0.03$ for all lymphoid subsets).

In children proportion of $CD10^+$, $CD2^+$, $CD7^+$ HSC was increasing in parallel with $CD19^+$ HSC ($p < 0.017$).

As a whole, $CD34^+$ cells demonstrated a greater variability by size with a ratio of larger to smaller cells 0.7:1 in PB and 0.5:1 in LP specimens. Majority of larger HSC in PB and LP specimens demonstrated a weak or no expression of common leukocyte antigen CD45 ($p = 0.001$), while smaller HSC did express $CD45^+$ and most probably CD90 ($p = 0.029$).

Population of $CD34^+$ cells with CD90 (Thy-1) expression was not homogeneous and contained a small proportion of megakaryocyte precursors ($CD61^+$) ($p = 0.012$). The $CD34^+$Thy-1^+ HSC pool might contain early $CD34^+$CD90-HLA-DR^+ pluripotent cells ($p = 0.013$).

HSC from LP contained discrete subsets $CD34^+CD45^{low}$, subsets with megakaryocyte differentiation trend $CD34^+CD45^{low}CD61^+$ ($p = 0.05$) with expression of c-kit receptor, Thy-1 antigen and molecule HLA-DR ($p < 0.013$ for the above mentioned molecules), and development in T-lineage differentiation ($CD34^+CD45^{low}CD7^+$, $p = 0.001$) being unlikely.

Mobilized HSC from stimulated PB harvested the day before or on the day of leukapheresis, like in LP from children, demonstrated predominance of myeloid-committed precursors with immunophenotype $CD34^+CD50^+CD33^+CD38^+CD13^+$HLA-$DR^+CD71^{\pm}$ and commonly present Thy-1^+ HSC ($p = 0.012$). The earliest $CD90^+CD71^+CD117^+CD45^{neg}$ cells were found in some of LP. Specimens with a marked proportion of Thy-1^+ cells demonstrated coexpression of CD90 and CD33 on the same SC ($p = 0.012$).

The significant positive correlations between lymphoid antigens of different lineages suggest the presence of a common lymphoid precursor among stimulated.

Table 2

Subsets of $CD34^+$ cells in leukapheretic product (proportion of antigen-positive cells within the total HSC pool)

Antigen	Group	Mean± SE	Median	Range	n	p
CD45	children	79.2±1.2	88.5	1.1–100.0	392	
	adults	74.1±1.3	83.0	1.2–100.0	390	
	donors	70.4±3.4	77.9	1.5–100.0	54	0.049
HLA-DR	children	93.8±0.51	95.5	75.7–100.0	128	
	adults	93.7±0.6	95.4	80.7–100.0	77	0.087
	donors	92.9±1.1	94.0	74.4–99.9	29	
CD38	children	58.1±2.63	54.2	0.5–100.0	113	
	adults	62.9±3.2	66.5	5.1–99.3	71	0.001
	donors	65.0±4.6	71.5	22.2–97.6	32	
CD33	children	64.3±3.05	73.2	0.9–99.8	101	
	adults	43.7±4.4	34.4	0.4–99.6	63	0.0001
	donors	67.6±4.7	75.5	7.1–99.0	24	
CD13	children	85.7±1.97	92.95	1.0–100.0	104	
	adults	80.0±2.9	91.7	1.6–99.4	69	0.009
	donors	90.3±1.9	92.4	50.0–99.5	28	
CD71	children	49.1±2.11	45.7	8.8–99.2	121	
	adults	35.7±1.9	36.2	4.0–74.7	78	0.0001
	donors	78.7±4.9	88.9	9.8–98.4	25	
CD117	children	69.0±3.35	75.4	13.1–97.5	48	
	adults	83.1±3.1	89.8	37.7–99.1	25	0.003
	donors	79.6±3.9	82.4	48.4–97.5	16	
CD90 (Thy-1)	children	26.4±3.26	10.4	0.1–99.8	91	
	adults	21.8±2.8	16.5	0.0–85.6	54	
	donors	41.9±6.8	28.4	0.4–92.4	26	0.02
CD50	children	98.1±0.34	99.0	84.9–100.0	63	
	adults	98.9±0.3	99.4	89.2–100.0	36	0.2
	donors	98.1±0.6	98.9	92.4–100.0	18	
CD56	children	3.24±1.09	1.2	0.0–60.0	60	
	adults	0.8±0.3	0.0	0.0–10.2	37	
	donors	28.7±12.8	5.4	0–92.6	9	0.03
CD19	children	3.01±0.56	1.4	0.0–36.5	89	
	adults	2.1±0.4	0.0	0.0–16.0	60	
	donors	15.4±4.4	6.3	0–86.1	26	0.01
CD61	children	2.7±0.3	1.8	0.0–10.7	56	

Antigen	Group	Mean± SE	Median	Range	n	p
	adults	3.9±0.7	2.4	0.0–15.8	29	
	donors	10.6±2.3	6.4	2.7–28.0	13	0.006
CD7	children	3.5±0.4	2.3	0.0–31.0	109	
	adults	4.1±0.8	2.5	0.0–41.4	57	
	donors	11.2±3.8	4.5	0–98.1	28	0.06
CD10	children	2.11±0.46	1.1	0.0–13.1	34	
	adults	0.5±0.1	0.4	0.1–1.4	9	
CD2	children	3.24±0.93	1.7	0.4–32.4	34	0.01
	adults	1.4±0.4	1.1	0.4–3.6	7	

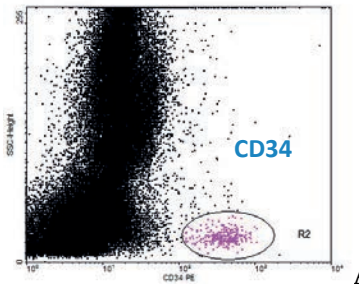
HSC in children. However, these findings need to be confirmed in further study and are evidence of just a simultaneous increase in these HSC subsets during mobilization.

A detailed immunological analysis of principal HSC subsets is given further in the text.

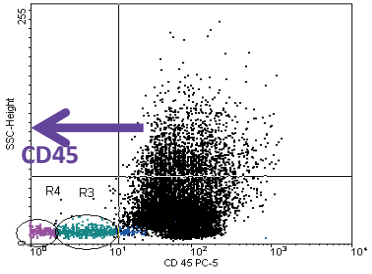
2. Non-lineage-restricted (CD45, CD50, HLA-DR, CD38, CD71, CD90, CD117) HSC antigens

Mobilized HSC and antigen CD45

Population of $CD34^{+}CD45^{neg}$ cells was found in most (more than 89%) mobilized HSC specimens from cancer patients and donors (fig. 3). This pool ($CD34^{+}CD45^{neg}$) is not homogeneous and contains $CD34^{+}CD45^{low}$ and $CD34^{+}CD45^{-}$ HSC (fig. 3, B, C). The $CD34^{+}CD45^{low}$ and $CD34^{+}CD45^{-}$ are two discrete ($p < 0.0001$) HSC populations that are heterogeneous by size. $CD34^{+}CD45^{-}$ demonstrated a more marked expression of SC antigen CD34 (fig. 3, C).



A



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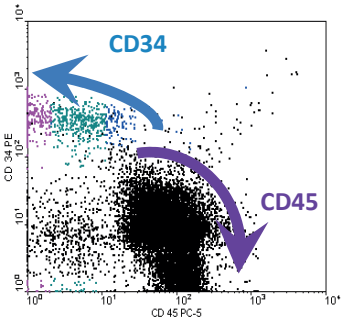


C

Figure 3. CD34 heterogeneity by expression of antigen CD45
A, $CD34^+$ gate; C, CD45 expression (χ-axis) on $CD34^+$ cells in comparison with granulocyte lineage with $CD34^+CD45^+$ (R2, blue), $CD34^+CD45^{low}$ (R3, green) and $CD34^+CD45^-$ HSC (R4, pink) also present. Most HSC are $CD45^{neg}$ (84.6%), $CD34^+CD45^-$ population (R4) is 35.4% of all $CD34^+$ cells. Median expression of CD34 within R4 gate was higher than in R3 and R2 gates (C).

$CD45^{neg}$ cells were more than 20.0% of mobilized HSC in all groups analyzed. They composed a large (29.7% ± 3.4%, median 22.1%, range 0.0% to 98.6%) proportion in donors. Effective mobilization of $CD34^+CD45^{neg}$ HSC was achieved in cases with hematology malignancies (more than 23% of antigen-positive cells, $0 < 0.04$ as compared to other diseases), and among them in children with HL and AML ($p < 0.02$ in comparison with NHL and solid tumors) (fig. 4).

Proportion of $CD34^+CD45^{neg}$ cells correlates with the HSC total: the lower percentage of $CD34^+$ leukocytes in LP specimens from adult patients, the greater proportion of $CD34^+CD45^{neg}/CD45^{low}$ ($R = -0.108$, $p = 0.034$; $R = -0.122$, $p = 0.016$, $n = 390$, respectively).

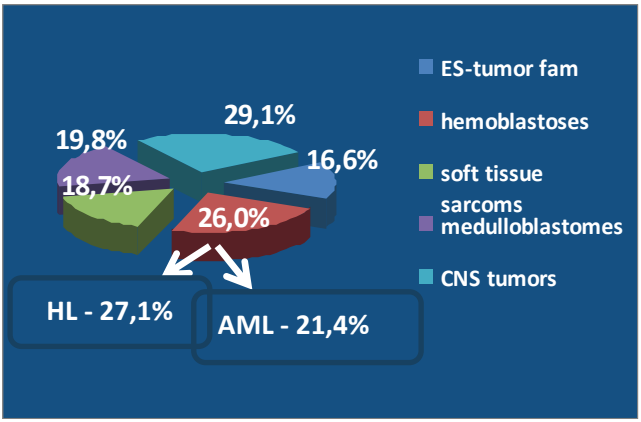
The analysis discovered that in cancer patients $CD34^+CD45^{low}$ cells might be represented by 2 subsets as T-lineage ($CD34^+CD45^{low}CD7^+HLA\text{-}DR^-CD13^-$, $p = 0.001$) or megakaryocyte-committed ($CD34^+CD45^{low}CD61^+HLA\text{-}DR^-CD13^-$, $p < 0.05$ for children and adults) precursors. Populations $CD45^-$ and $CD45^{low}$ demonstrated a significant difference with respect to HLA-DR expression: $CD34^+CD45^-$ cells were HLA-DR-positive ($p = 0.044$). There might be a considerable proportion of c-kit$^+$ ($CD117^+$) and $CD13^+$ HSC within the $CD34^+CD45^-$ pool ($p = 0.022$) (fig. 5)

In cancer patients the number of $CD33^+$ HSC was increasing in parallel with $CD34^+CD45^{neg}$ HSC ($p = 0.0001$), which might confirm hemopoietic nature of $CD34^+CD45^{neg}$ SC.

In children the number of B-lineage precursors and $CD38^+$ HSC was increasing in parallel with increase in $CD34^+CD45^{neg}/CD45^{low}$ populations ($p = 0.028$ and $p = 0.047$, respectively), while the number of $CD13^+$ myeloid-committed ($p = 0.042$) and Thy-1^+ ($p < 0.001$) HSC was decreasing.

In donors the increase in $CD45^{low}$ HSC was associated with a marked proportion of $CD34^+CD33^+$ and $CD34^+CD7^+$ HSC ($p < 0.01$ for both) and with the number of megakaryocyte precursors ($CD61^+$, $p = 0.029$).

A. Pediatric patients



B. Adult patients

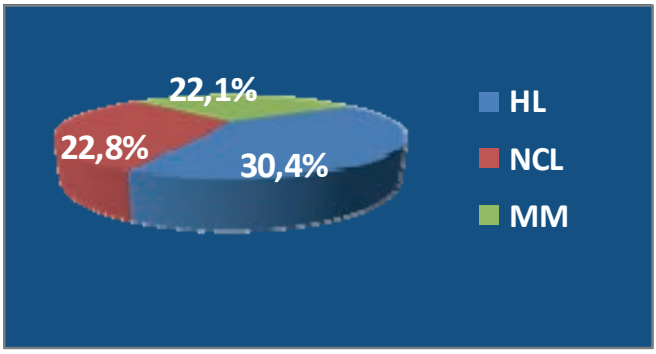


Figure 4. Detection frequency of CD45-negative subsets in cancer patients.

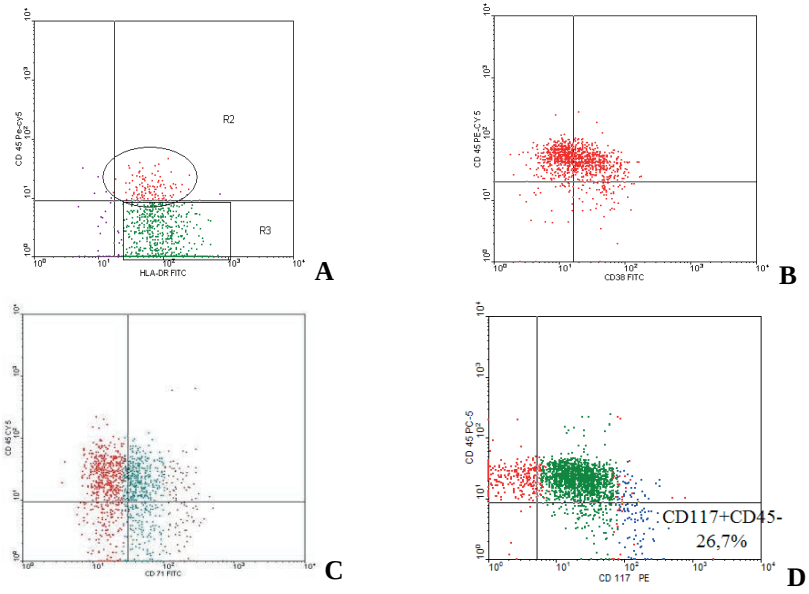
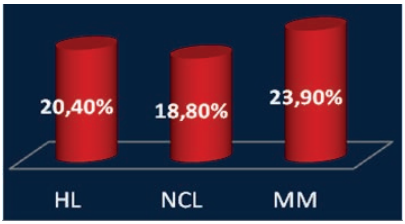


Figure 5. Heterogeneity of CD45-negative HSC subsets with respect to HLA-DR, CD38, CD71 and CD117.

The figure shows 4 specimens from different cancer patients. All cytograms demonstrate $CD34^+$ cells only, on the γ-axis: expression of common leukocyte antigen CD45. A, more marked expression of HLA-DR (χ-axis, $HLA\text{-}DR^+CD45^-$ subsets shown in green) on CD45-negative HSC. Fig. B demonstrates increase in CD38 expression (χ-axis) in parallel with decrease in CD45 expression on $CD34^+$ cells. C shows variable CD71 expression (χ-axis) within CD45-negative HSC. D demonstrates a more marked expression of c-kit receptor CD117 (χ-axis, $CD34^+CD117^{++}$ population is shown in blue) on $CD34^+CD45^-$ cells.

Thy-1^+ subset of mobilized HSC

A subset of early Thy-1^+ cells among mobilized HSC was detected with a high frequency in pediatric cancer patients (more than 21%) and donors (41.0%) (fig 6).



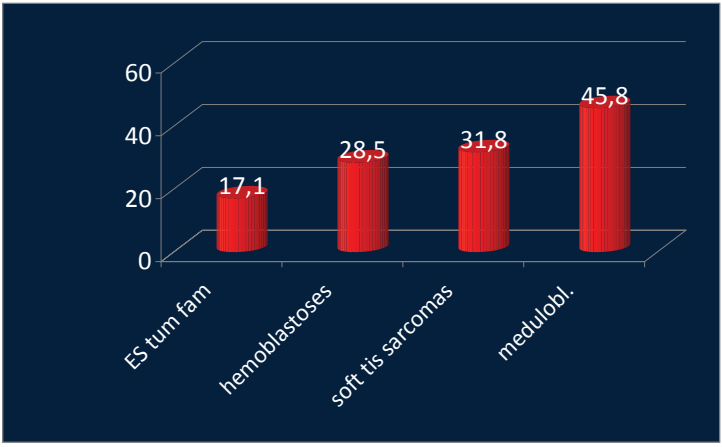
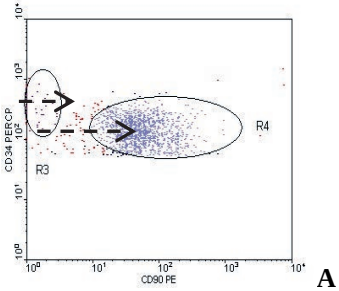


Figure 6. Detection frequency (%% of the $CD34^+$ total) of Thy-1^+ mobilized hemopoietic stem cells in cancer patients with respect to disease type.

The highest proportion of $CD34^+CD90^+$ cells was found among donors' HSC ($p = 0.019$) and in children with medulloblastoma and neuroblastoma ($p < 0.005$ in comparison to other diseases).

Thy-1^+ cells are a discrete population of smaller HSC with SSC^{med} and a weaker CD34 expression in some specimens (fig. 7, A). There were populations $CD71^+$ and $CD38^+$ among $CD34^+CD90^+$ cells.



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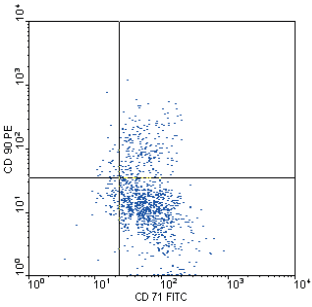

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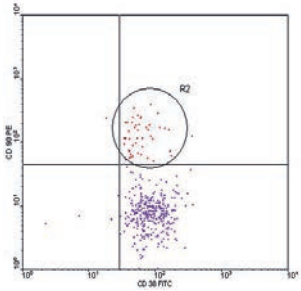

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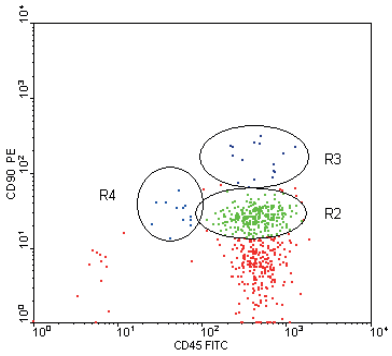
Figure 7. Thy-1$^+$ (CD90$^+$) HSC heterogeneity with respect to CD34, CD71 and CD38.

The figure shows 3 leukoconcentrate specimens, all cytograms demonstrate CD34$^+$ cells only. A, marked proportion of CD34$^+$CD90$^+$ HSC (χ-axis: CD90$^+$ cells, R4), with a weaker CD34 expression (γ-axis). B, the presence of CD90$^+$CD71$^+$ cells (the right upper quadrant) among HSC. C show CD38 coexpression (χ-axis) on CD90$^+$ cells (γ-axis), CD90$^+$CD38$^+$ HSC are in the right upper quadrant, R2.

Thy-1$^+$ HSC showed variable expression of common leukocyte antigen (fig. 8). In specimens with a marked proportion of CD34$^+$CD90$^-$ cells from both adults and children there were Thy-1 HSC subsets different by CD45 expression, such as CD90$^+$CD45^{low} (R2, fig. 8B; R4, fig. 7A), CD90$^+$CD45$^-$ (R3, fig. 8B), CD90$^+$CD45$^+$ (R3 and R2, fig. 8A).



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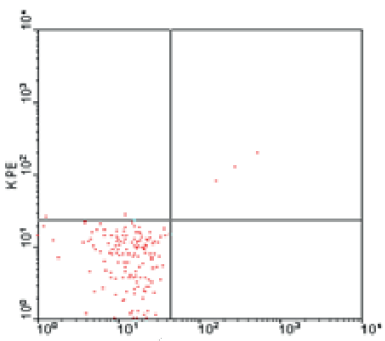

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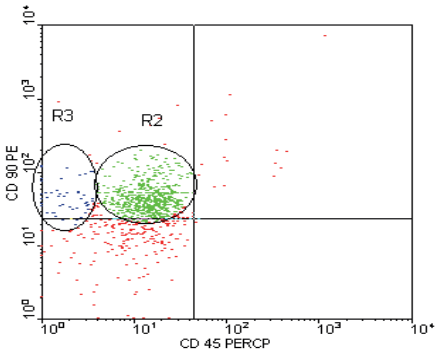


Figure 8. Thy-1$^+$ HSC and CD45.
The figure shows specimens of mobilized peripheral stem cells, all cytograms show CD34$^+$ cells only, γ-axis: CD90 expression, χ-axis: common leukocyte antigen expression, A, B, antigen-positive CD34$^+$CD90$^+$ cells are shown in regions R2, R3 and R4. In A, most CD34$^+$ cells demonstrate a weak CD90 expression and are CD45$^+$ (R2, green), there are CD45$^+$CD90^{++} HSC (R3) and CD34$^+$CD90$^+$CD45^{low} cells (R4). In B, more than 80% of HSC show a weak CD90 expression and are CD45-negative, there are also CD90$^+$CD45^{low} (R2) and CD34$^+$CD90$^+$CD45$^-$ HSC (R3). The markers and positive/negative cells regions are demarcated with respect to isotypic controls (C).

Analysis of coexpression of Thy-1 and CD7 on HSC (38 specimens) discovered possible their expression on a single HSC (fig. 9A,B). There were both CD90$^+$CD7$^+$ and CD90$^+$CD7^{++} HSC. Specimens from children and adults contained small populations of CD34$^+$CD7$^+$CD90$^-$ cells (fig. 9A). The discovery of the antigen coexpression confirms the correlation demonstrated earlier in this text between CD34$^+$CD7$^+$ and CD34$^+$CD90$^+$ HSC in specimens with minimal contents of CD34$^+$CD45^{neg} cells.

In one donor CD90$^+$CD7$^+$ cells were more than 70% of the CD34$^+$ total (fig. 9C). It is quite possible that most cells in this population are myeloid-committed precursors with CD7 coexpression, although it may not be excluded that a part of these HSC are the earliest HSC containing a common precursor of both myeloid and lymphoid restrictions.

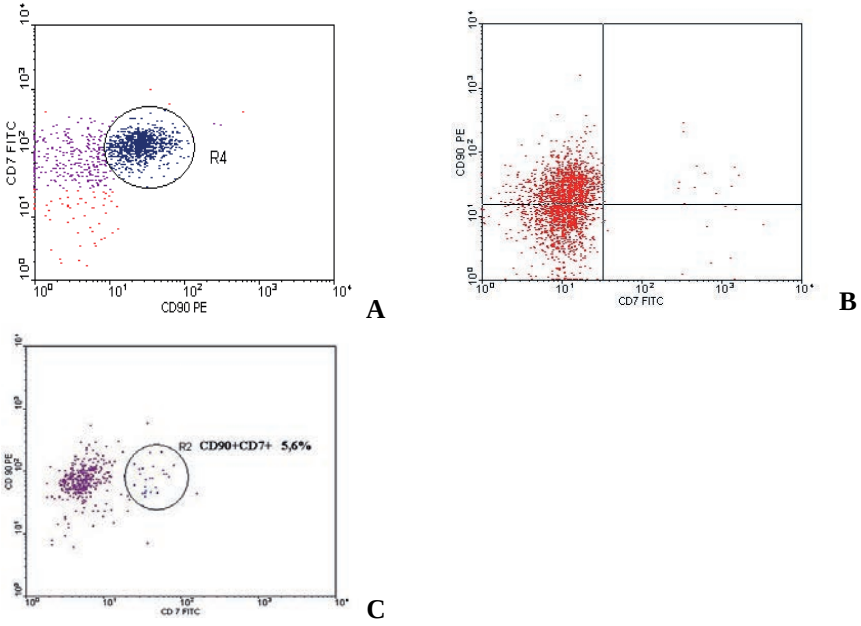


Figure 9. CD90 and CD7 coexpression on mobilized hemopoietic stem cells.
The figure shows possible coexpression of CD90 and CD7 on HSC (A, B, children: χ-axis: CD7 expression, γ-axis: CD90 expression). B, donors' HSC; χ-axis: CD90 expression, γ-axis: CD7 expression). All cytograms show $CD34^+$ cells only. The figure confirms the possibility of $CD90^+CD7^+$ presence (R2, R4 in B, C and right upper quadrant of A) irrespective of CD7 expression level and strength.

Increase in $CD90^+$ HSC was associated with a lower content of megakaryocyte precursors in adult patients ($p = 0.02$) and a higher percentage of $CD34^+CD33^+$ HSC in both pediatric and adult patients ($p = 0.005$).

Proportion of $CD117^+$ HSC was increased in specimens with increased Thy-1^+ content in children and donors ($p < 0.04$). In donors the increase in Thy-1^+ HSC proportion was associated with lower $CD34^+CD45^{neg}$ fraction ($p = 0.034$).

Detection frequency of $CD34^+HLA\text{-}DR^-CD38^-$ cells among mobilized HSC was not high and comparable with that in cancer patients (16% in children, 15.0% in adults). In children increase in $HLA\text{-}DR^-CD38^-$ cells was associated with increase in lymphoid precursors $CD56^+$, $CD19^+$, $CD2^+$ ($p < 0.015$).

Donors' HSC were characterized by a marked $HLA\text{-}DR^-CD38^-$ content, with higher $CD34^+CD38^-HLA\text{-}DR^-$ proportion associated with low proportion of $CD13^+$, $CD33^+$, $CD71^-$ HSC ($p < 0.039$) and a marked proportion of $CD7^+CD34^+$

cells ($p = 0.016$). HSC demonstrated a monomorphous expression of CD50 and HLA-DR. Subsets $CD50^+$ and $HLA\text{-}DR^+$ were heterogeneous with respect to size and expression of other antigens studied. Some specimens contained $CD34^+HLA\text{-}DR^-$ HSC with more effective mobilization in adult patients with MM and in children with medulloblastoma ($p < 0.01$).

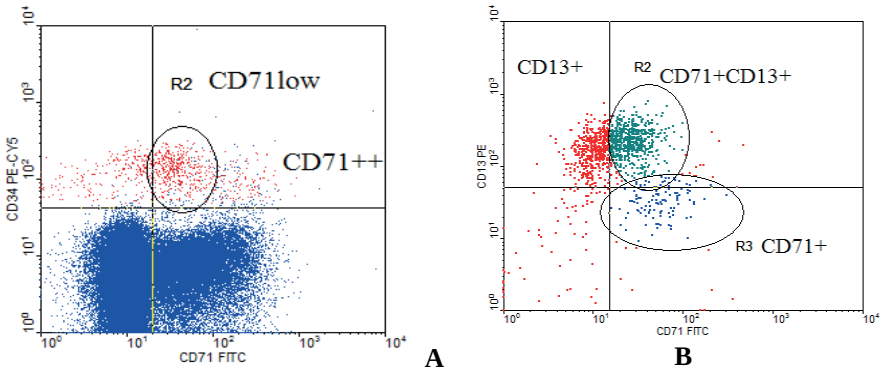
Specimens with $CD45^{neg}$ HSC also contained $CD34^+CD50^+CD45^{neg}$ and $CD34^+HLA\text{-}DR^+CD45^{neg}$ HSC, and CD45-negative HSC expressed HLA-DR more actively as compared to $CD34^+CD45^+$ cells (see fig. 4).

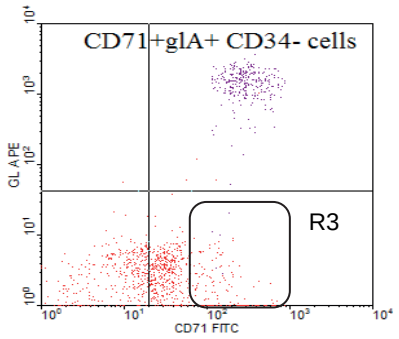
Mobilized HSC and transferrin receptor CD71

Expression of transferrin receptor CD71 on mobilized peripheral HSC was variable with populations $CD71^{low}$ and $CD71^{++}$ seen. CD71 expression level in population $CD34^+CD71^{++}$ was comparable with that on erythrokaryocytes. There were no cells with glycophorin A expression among $CD71^{low}$ cells. Expression levels of SC antigen on $CD71^{low}$ HSC were higher, expression levels of common leukocyte antigen were decreasing with increase in CD71 expression.

Donors' specimens demonstrated the highest content of $CD71^+$ HSC.

Both $CD71^{low}$ and $CD71^{++}$ HSC could coexpress CD117 and CD7 ($p < 0.049$ for both). The presence of $CD34^+CD13^+$ HSC among $CD71^{++}$ cells in cancer patients was unlikely ($p < 0.26$, fig. 10C).





C

Figure 10. Transferrin receptor CD71 and mobilized hemopoietic stem cells.
The figure demonstrates HSC heterogeneity by expression of transferring receptor CD71: 3 specimens of mobilized autologous HSC are shown. All the specimens contained $CD34^+$ cells with a weak expression of $CD34^+CD71^+$ (R2, A [adult patient], B, C (pediatric patients), R2 and population with a marked CD71 expression ($CD71^{++}$, R3, B, C).
$CD34^+CD71^+$ HSC population expressed transferrin receptor weaker than activated mononuclears (R2, A, $CD34^+CD71^+$ cells are shown in red, mononuclears are in blue) $CD34^+CD71^{++}$ population (R3, B) expressed CD71 practically at the level of nucleated erythroid cells $CD71^+glA^+CD34^-$ (lilac).
Cytogram C demonstrates HSC heterogeneity with respect to CD71 (χ-axis) and pan-myeloid antigen CD13 (γ-axis). Region R2: CD71+CD13+ cells (green), R3: $CD71^+/^{++}CD13^-$ subpopulations (blue).

Population $CD34^+CD71^{low}$ in children as a rule contained megakaryocyte-committed precursors ($CD61^+$, $p = 0.041$).

Mobilized HSC and c-kit receptor CD117

Specimens from donors and adult patients demonstrated the highest contents of c-kit receptor$^+$ HSC ($CD117^+$) with monomorphous expression.

Mobilization of $CD117^+$ HSC was more effective in patients with hematology malignancies ($p < 0.01$, especially in children with AML) and in children with medulloblastoma as compared with other solid tumors ($p < 0.007$, fig. 11).

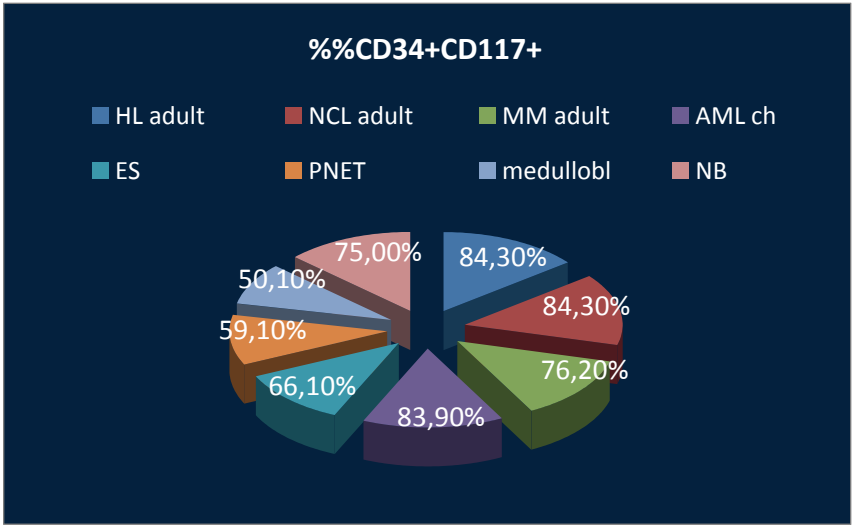


Figure 11. Detection frequency of c-kit$^+$ subsets of mobilized hemopoietic stem cells.

Expression of c-kit receptor CD117 on HSC was not even: there were CD117$^+$ and CD117^{++} cells different by size (fig. 12, A). CD117^{++} HSC expressed CD45 weaker, up to negative levels as compared to CD117$^+$ cells (fig. 12, B). CD117$^+$ HSC demonstrated variable expression of CD19, CD7 and CD2, and contained Ag$^+$ and Ag$^-$ cells (fig. 12C,D).

Increase in CD117$^+$ HSC content in children was associated with increase in CD71^{++} and Thy-1$^+$ proportion ($p = 0.015$ and $p = 0.06$, respectively). In donors the variable antigen expression was associated with a more marked proportion of CD34$^+$HLA-DR$^-$CD38$^-$ HSC and lower CD45^{low} HSC content ($p = 0.06$). In adults, specimens with a higher CD117$^+$ HSC proportion contained less CD61$^+$, CD34$^+$HLA-DR$^-$, CD19$^+$ HSC ($p < 0.038$), though CD34$^+$ content in this group was higher, like in children ($p = 0.036$).

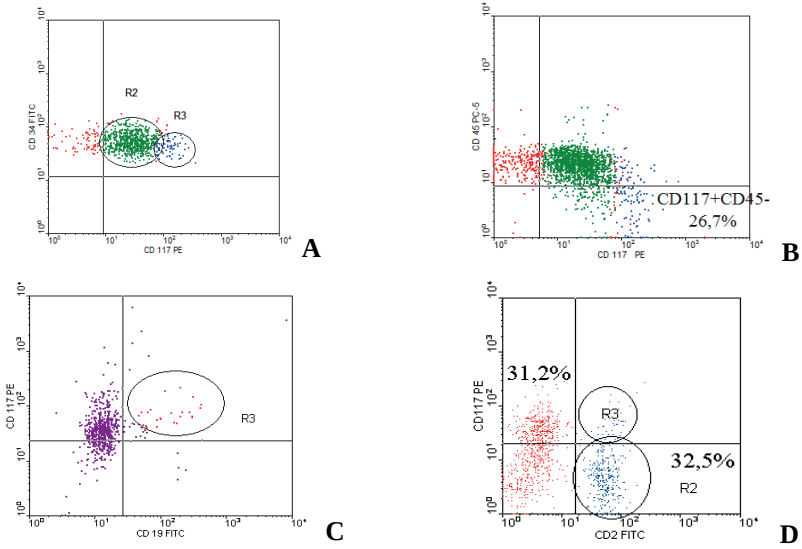


Figure 12. C-kit receptor CD117 and mobilized hemopoietic stem cells.
All cytograms demonstrate $CD34^+$ cells only. A, the presence of $CD34^+$ subsets (γ-axis) with different expression of CD117 (χ-axis), R2: $CD117^+$ subsets; R3: $CD117^{++}$ stem cells. B, marked proportion of $CD117^+$ HSC with 26.7% of $CD117^{++}CD45^{neg}$ HSC (right lower quadrant). C, HSC with variable expression of CD117(γ-axis) and CD19 (χ-axis) and possible mobilization of $CD117^+CD19^+$ HSC (R3). D, HSC with variable expression of CD117 (γ-axis) and CD2 (χ-axis), and possible mobilization of both $CD117^+CD2^+$ HSC (R3) and $CD117^-CD2^+$ HSC (R2).

3. Lineage-associated [myeloid (myel, CD13, CD33), megakaryocyte (CD61) and lymphoid (lymph, CD19, CD10, CD7, CD2, CD56)] antigens:

Myeloid-committed precursor cells

CD13 expression was seen on a higher proportion of HSC as compared with CD33 in all groups analyzed. Low content of $CD13^+$ HSC was found rarely and expression of CD13 on HSC was brighter than that of CD33 in most cases (fig. 13A). A somewhat brighter expression of CD33 as compared to CD13 was seen in some specimens from children. More effective mobilization of $CD33^+$ HSC was found in children with AML ($p < 0.04$ as compared to the remaining groups analyzed).

CD13 and CD33 HSC demonstrated variable expression of CD45 with both $CD45^+$ and $CD45^{neg}$ cells detected (fig. 13C—F). $CD13^+$ and $CD33^+$ HSC

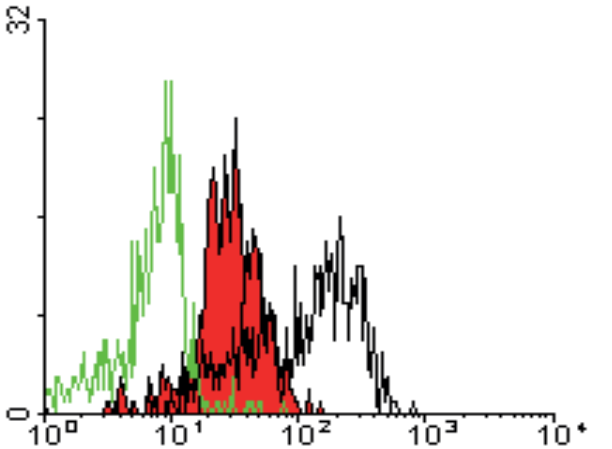
coexpressed CD7 and CD2 in most cases. $CD13^+$ HSC demonstrated variable expression of CD61.

Discrete CD13 and CD33 populations were found in specimens from adult patients with less than 70.0% of $CD13^+$ HSC ($p = 0.012$).

Specimens from children with monomorphous (more than 90.0%) expression of CD13 on HSC demonstrated a lower proportion of $CD34^+$ leukocytes, a low proportion of $CD45^{low}$, $CD7^+$ and $CD61^+$ HSC ($p < 0.05$) and a high content of $CD33^+$, $CD71^+$, $CD90^+$ HSC ($p < 0.006$).

In donors $CD13^+$ HSC predominance was associated with lower proportions of $HLA\text{-}DR^-$ and $HLA\text{-}DR^+CD45^-$ HSC ($p < 0.006$).

Adult specimens containing less than 65.0% of $CD34^+CD33^+$ HSC demonstrated higher proportions of $CD38^-$, $CD38^-HLA\text{-}DR^+$ ($p < 0.045$) and $CD34^+$ HSC ($p = 0.029$). However, such specimens from children contained lower proportions of Thy-1^+, $CD38^+$, $HLA\text{-}DR^+CD45^-$ and $CD34^+CD45^-$ HSC ($p < 0.041$) while in donors the content of Thy-1^+ HSC was greater, as well as the proportion of $CD19^+$ HSC ($p = 0.06$).



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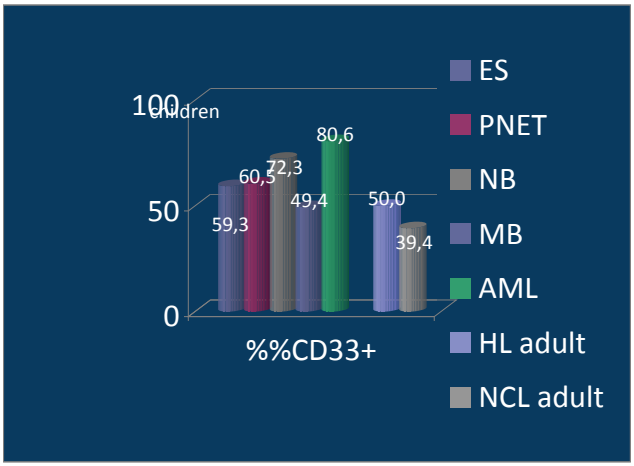

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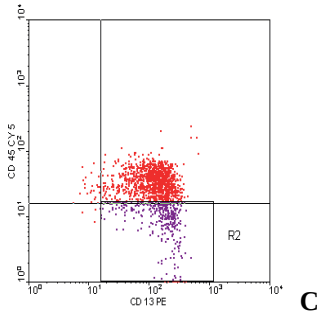

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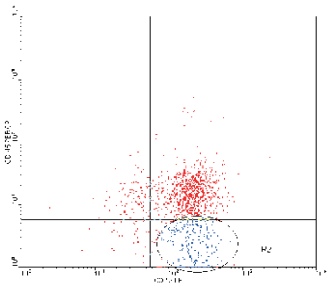

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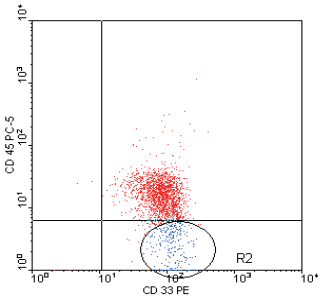

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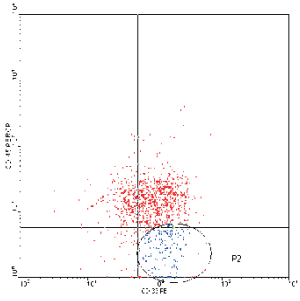


F

Figure 13. Pan-myeloid antigens CD13 and CD33 and mobilized hemopoietic stem cells.

The figure demonstrates expression of pan-myeloid antigens CD13 and CD33 on mobilized peripheral hemopoietic stem cells (A, C—E) and detection frequency of $CD33^+$ subsets of mobilized HSC in cancer patients. A, donors' HSC show the most frequent situation for both donors and patients with a brighter expression of CD13 (clear peak with a black outline) in comparison with CD33 (red peak), green peak is isotopic control.
C, (donor), D (adult patient) demonstrate possible mobilization of $CD34^+CD45^{neg}CD13^+$ HSC (R2, right lower quadrant). E (child), F (adult patient) show CD33 expression on $CD34^+CD45^{neg}$ HSC (R2, blue cells).

Megakaryocyte-committed precursor cells $CD61^+CD34^+$ are a clear-cut discrete population with the largest proportion in donors (fig. 14A,B). The highest effect of $CD34^+CD61^+$ HSC mobilization was reported in children with medulloblastoma ($p < 0.04$ as compared to the other malignancies in children). There were $CD45^+$ and $CD45^{neg}$ subsets among $CD61^+$ HSC (fig. 14C).

$CD34^+CD61^+$ HSC from cancer patients could very likely contain $CD45^{low}$ cells ($p = 0.038$ in children, $n = 56$; $p = 0.05$ in adults , $n = 29$). The pool of megakaryocyte precursors in adult patients could very likely contain $CD34^+CD38^-$ cells ($p = 0.05$) while the presence of $CD34^+CD117^+$ and $CD34^+CD90^+$ HSC ($p < 0.013$) was hardly possible. The megakaryocyte-committed populations contained both $CD61^+CD13^+$ and $CD61^+CD13^-$ subsets (fig. 14C,D).

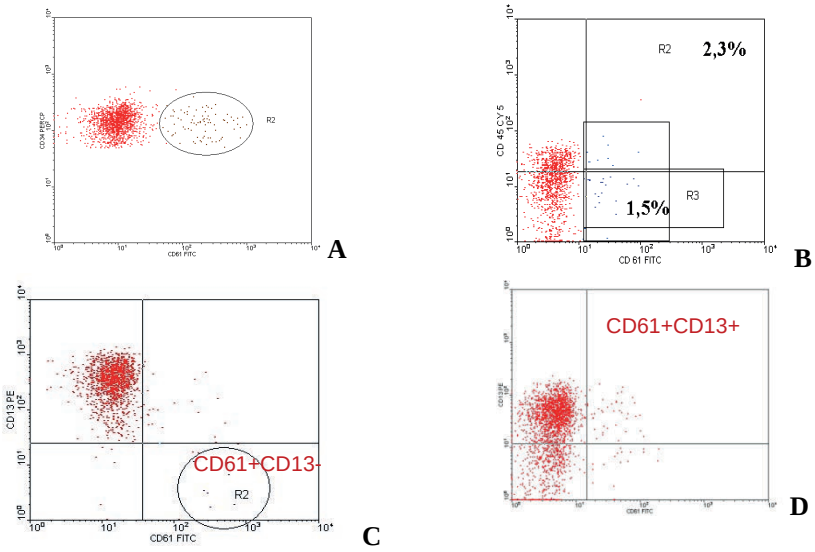


Figure 14. Megakaryocyte-committed $CD61^+$ mobilized hemopoietic stem cells.

The figure demonstrates subsets of megakaryocyte-committed HSC. Most $CD34^+CD61^+$ cells are a rather clear-cut discrete subset of HSC in this case reaching 12.1% of the $CD34^+$ total in auto HSC of a child (A, γ-axis: CD34 expression, χ-axis: CD61). B (auto HSC, adult patients), $CD61^+$ HSC variability by CD45 with both $CD61^+CD45^+$ populations (right upper quadrant, the total content of megakaryocyte precursors shown) and $CD61^+CD45^-$ HSC (R3) that are 65% of the megakaryocyte-committed precursor total).
C and D (auto HSC from a child), expression of pan-myeloid antigen CD13 and megakaryocyte antigen CD61 on HSC (only $CD34^+$ cells are shown). CD61 expression is shown on the χ-axes (FITC label), pan-myeloid antigen CD13 expression on the γ-axes (PE label). There is a $CD13^+CD61^+$ population (right upper quadrant) that is 50% or more of the $CD61^+CD34^+$ cell total. There are $CD61^+CD13^-$ HSC (C, region R2, D, right lower quadrant) together with $CD34^+CD61^+$ cells.

Lymphoid-committed precursor cells

The content of HSC with lymphoid antigen expression was not high. Some specimens from children and donors demonstrated a marked proportion of $CD19^+$, $CD7^+$, $CD2^+$, $CD56^+$ HSC (fig 15, 16). As a whole a significantly more successful mobilization with respect to lymphoid antigens was achieved in donors ($p < 0.03$).

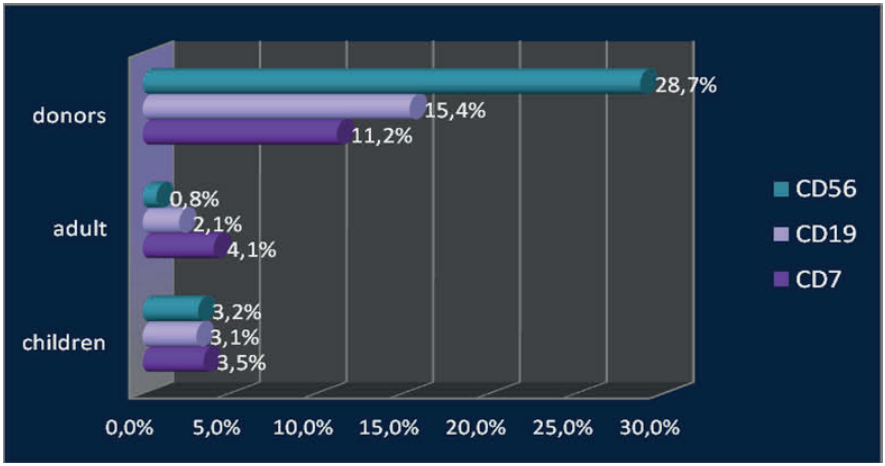


Figure 15. Mobilization efficiency of HSC with lymphoid antigen expression.

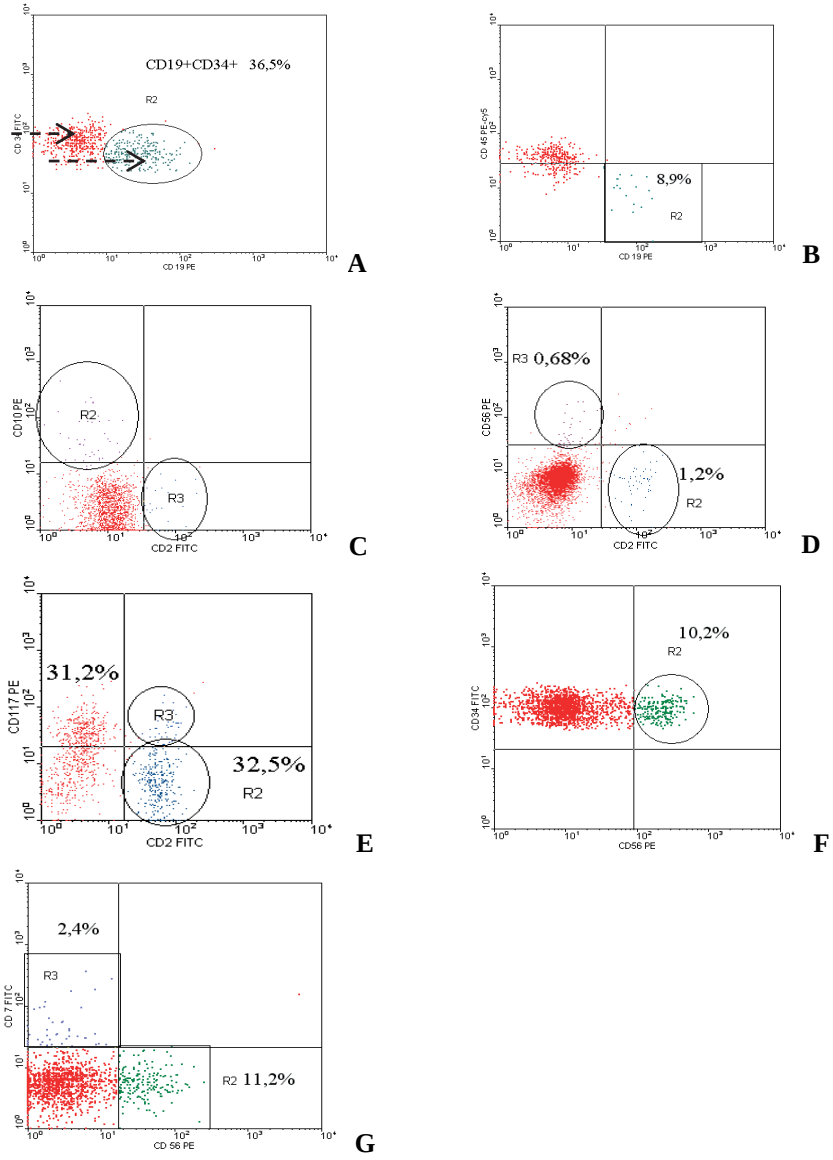


Figure 16. Lymphoid antigens CD19, CD10, CD2, CD56 and mobilized HSC.

Six specimens of auto HSC (A — E, G) and 1 specimen of donor's HSC (F) demonstrate the presence of subsets with lymphoid antigen expression. Specimens A and B show mobilization of $CD19^+$ HSC (χ-axis, R2, green), CD34 expression on $CD19^+$ cells was weaker as compared to CD19-negative populations, $CD19^+$ HSC were CD45-negative (B, R2, right lower quadrant) in most cases.
Specimens C — E demonstrate mobilization of $CD2^+$ HSC subsets (C, R3 and D, E, R2) that are discrete from $CD10^+$ HSC (C, R2), $CD56^+$ HSC (D, R3) and show practically no coexpression of CD117 (E, R2). F, G show possible mobilization of a marked proportion of $CD56^+$ HSC (R2, green).

Increase in proportion of HSC with expression of one lymphoid antigen was as a rule associated with increase in $CD34^+$ cells showing expression of the remaining lymphoid antigens. A parallel increase in content of $CD34^+HLA\text{-}DR^-CD38^-$ HSC was seen in children.

The highest mobilization effect of $CD19^+$ HSC was discovered in adult patients with myeloma and children with medulloblastoma and PNET as compared to other malignancies ($p < 0.03$). There were no $CD19^+$ or $CD10^+$ HSC in any of AML cases.

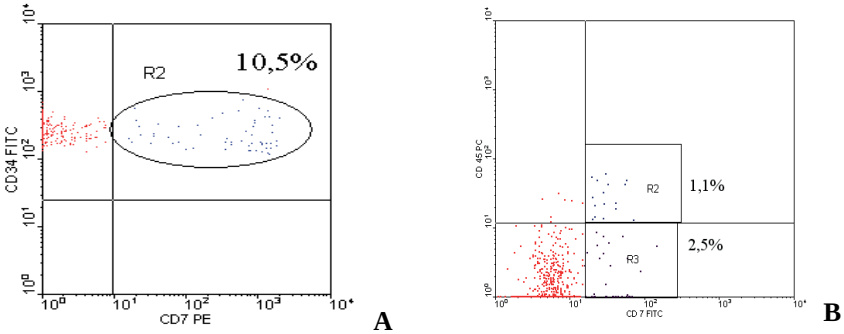
$CD19^+CD34^+$ HSC were smaller cells. In children this population coexpressed CD10, was discrete with respect to $CD45^+$ HSC and negative with respect to CD90 and myeloid antigens.

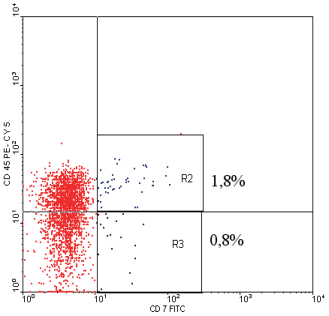
Donors' HSC with expression of lymphoid antigens CD19 and CD7 were Thy-1-positive.

$CD19^+$ and $CD10^+$ as well as $CD2^+$ and $CD10^+$ in adults, unlike children, formed separate HSC subsets (see fig. 16C).

$CD2^+$ HSC content was the highest in children; this situation was associated with low proportion of HSC with myeloid restriction (see fig. 16E).

CD7 expression on HSC was not even, with both $CD34^+CD7^+$ and $CD34^+CD7^{++}$ HSC seen in specimens (fig. 17).





C

Figure 17. CD7 expression on mobilized HSC.
The figure demonstrates $CD7^+$ HSC mobilization in 3 auto-HSC specimens. Only $CD34^+$ cells are shown. CD7 expression is on the χ-axis. CD7 expression levels varied from weak to marked (A), and a part of $CD7^+$ HSC were CD45-negative (B, C, R3).
There was one case of mobilization of a marked proportion of lymphoid precursors with $CD34^+HLA\text{-}DR^+CD7^+CD38^-CD19^-CD71^-myel^-CD50^+$ phenotype among adult patients and one case of discrete $CD34^+CD13^+CD7^-$ and $CD34^+CD13^-CD7^+$ HSC in children.
Marked proportions of $CD34^+CD7^+$ and $CD34^+CD56^+$ cells were seen simultaneously among donors' HSC. Mobilization of both $CD7^+$ and $CD56^+$ HSC was most effective in children with neuroblastoma ($p < 0.02$ for all comparison groups).

4. Subsets of mobilized peripheral HSC and efficiency of autologous hemopoietic engrafting

Adult patients with hematology malignancies

Stimulation effect by $CD34^+$ leukocyte content was better in patients with MM and NHL as compared to HL. Specimens with the highest $CD34^+$ leukocyte content contained smaller proportions of $CD34^+CD38^+$, $CD34^+CD33^+$ and $CD34^+CD7^+$ HSC.

Cells from PB only were used for transplantation in 65 adult patients (60 patients had first transplantations and 5 second transplantations; doses harvested for a single LP cycle were sufficient for the second transplantation in MM patients in accordance with the clinical protocol). Nine patients underwent transplantation of both PB and BM.

As a whole, in the group undergoing PB transplantation only mean time of platelet recovery to 20 000 cells/mcl was 14.26 ± 1.14 days, median 12 days, range 9 to 59 days. Mean time of neutrophil recovery to 500 cells/mcl was 12.2 ± 0.3, median 12 days, range 7 to 24 days.

The recovery period in cases with transplantation of peripheral HSC was shorter than for combined transplantation of pHSC+BM (leukocytes 12 days

[pHSC] vs. 14 days (pHSC+BM); platelets 14 days (pHSC) vs. 18 days (pHSC+BM). The differences were not statistically significant most likely due to difference in the number of transplantation (60 pHSC vs. 9 PB+BM transplantations).

Neutrophil recovery was significantly faster after transplantation of the full dose harvested as compared to transplantation of a part of HSC harvest in spite of the lower HSC dose transplanted (3.5×10^6/kg vs. 6.2×10^6/kg, $p = 0.001$).

Shorter periods of critical thrombopenia were associated with higher doses of $CD34^+CD71^{++}$ and $CD34^+CD13^+$ HSC ($p < 0.03$), a shorter time of critical neutropenia was observed in cases with higher proportions of $CD34^+CD71^{++}$ and $CD34^+CD19^+$ HSC ($p < 0.035$). Shorter duration of thrombopenia was due to small content of $CD34^+CD61^+$ HSC in the first day harvest.

The dose 3.0 to 5.0×10^6 of $CD34^+$cells containing more than 1.0×10^6/kg of $CD34^+CD45^{low}$ HSC was the optimal total transplantation HSC dose ensuring neutrophil and platelet recovery within 11 days post-transplantation in adult patients. The presence of a 15% or greater $CD34^+CD45^-$ subset in the HSC total ensured neutrophil recovery within 10 days (table 3).

Table 3

Time to recovery of blood components is associated with proportions of transplanted populations in the first day LP HSC harvest in adult cancer patients

Subset	Threshold level	Mean time to recovery (days)	No. of specimens compared	p Value
HSC absolute number ($\times10^6$/kg)				
	neutrophils to 500 cells/mcl			
$CD34^+$	5.0 or more	13.1±0.7	26	0.023
	Less than 5.0	11.6±0.4	25	
$CD34^+CD45^+$	3.0 or more	13.6±0.7	19	0.015
	Less than 3.0	11.3±0.4	23	
platelets to 20 000 cells/mcl				
$CD34^+CD45^{low}$	1.0 or more	11.3±0.6	13	0.04
	Less than 1.0	15.3±1.8	29	
HSC proportion (%%)				
	neutrophils to 500 cells/mcl			
$CD34^+CD45^-$	15.0% or	10.9±0.4	10	0.049

Subset	Threshold level	Mean time to recovery (days)	No. of specimens compared	p Value
	more			
	Less than 15.0%	12.1±0.4	48	
$CD34^+CD33^+$	70.0% or more	13.1±0.9	7	0.05
	Less than 70.0%	10.8±0.6	5	
	platelets to 20 000 cells/mcl			
$CD34^+CD90^+$	35.0% or more	10.3±0.8	5	0.05
	Less than 35.0%	13.0±0.6	12	
$CD34^+CD7^+$	5.0% or more	10.0±0.1	4	0.0001
	Less than 5.0%	12.7±0.6	12	

Content of myeloid-restricted $CD33^+$ HSC is of critical importance with smaller proportions associated with a shorter time to neutrophil recovery. Reduction to 10 days in critical thrombocytopenia could be achieved with a more than 35.0% proportion of $CD34^+Thy\text{-}1^+$ and a more than 5.0% proportion of $CD34^+CD7^+$ HSC in the transplant.

Pediatric patients with hematology malignancies

Higher percentage of $CD34^+$ leukocytes in LP of the first day harvest was seen in the group of ESFT ($p = 0.03$) and soft tissue tumors ($p = 0.035$) as compared to the group of hematology malignancies.

Detailed analysis of PNET, ES, medulloblastoma and neuroblastoma, AML and HL discovered that the best mobilization effect was achieved in children with neuroblastoma and ES. The lowest $CD34^+$ leukocyte proportions (mean 0.6%) were found in patients with HL, AML and medulloblastoma. Significant differences were detected for HL against all other groups ($p < 0.03$), AML and neuroblastoma, medulloblastoma and neuroblastoma ($p = 0.05$ in both).

Mobilization of $CD45^{neg}$ subsets was most effective in the group of hematology malignancies (HL and AML). HSC from AML patients demonstrated higher proportions of $CD34^+CD117^+$ and $CD34^+CD33^+$ HSC ($p < 0.04$ vs. medulloblastoma and PNET) and complete absence of B-precursors ($CD34^+CD19^+$

and $CD34^+CD10^+$).

HSC from medulloblastoma patients contained the lowest content of $CD34^+CD38^-$ HSC ($p < 0.035$ in all cases). Specimens from medulloblastoma patients had the highest contents of $CD34^+HLA\text{-}DR^-$ cells ($p < 0.041$), B-lineage precursors ($CD34^+CD19^+$ ($p < 0.01$ vs. ES, AML and neuroblastoma) and megakaryocyte precursors as compared to ES and neuroblastoma ($p < 0.04$).

The PNET group was characterized by the highest content of $CD34^+CD19^+$ HSC as compared to other solid tumors ($p < 0.003$ for ES and neuroblastoma).

HSC from neuroblastoma patients demonstrated a more marked proportion of $CD7^+CD34^+CD56^+CD34^+$ cells ($p < 0.02$ for both subsets vs. PNET and ES).

HSC mobilization was effective and ensured HSC threshold transplantation dose for a single LP in 50% of patients.

Comparison of LP with 2.0 or more (group 1) versus 2.0×10^6 (group 2) $CD34^+$HSC per kg bodyweight discovered significant differences in certain subsets (table 4).

Table 4

Comparison of cell subsets in groups containing 2.0×10^6 or more vs. 2.0×10^6 or less CD34 HSC

Subset	Mean: Group 1 / Group 2	No. of specimens	p
Subset absolute number $\times10^6$/kg			
$CD34^+CD45^{neg}$	0.66±0.12	48	0.041
	0.21±0.06	17	
$CD45^{low}CD34^+$	0.47±0.09	48	0.044
	0.14±0.03	17	
$CD45^+CD34^+$	4.89±0.7	47	0.001
	0.95±0.09	17	
$HLA\text{-}DR^+CD34^+$	5.2±0.75	37	0.001
	1.90±0.1	16	
$CD38^+CD34^+$	3.24±0.54	32	0.009
	0.62±0.16	11	
$CD13^+CD34^+$	5.83±0.96	30	0.024
	1.05±0.19	7	
$CD71^+CD34^+$	3.12±0.5	33	0.002
	0.36±0.09	12	
$CD90^+CD34^+$	2.47±0.78	21	0.006

	Mean:		
	0.08±0.01	7	
$CD50^+CD34^+$	7.8±1.49	14	0.023
	1.74±0.7	4	
$CD7^+CD34^+$	0.14±0.027	30	0.013
	0.026±0.007	12	
Subset proportion (%)			
$CD45^{low}CD34^+$	12.2±1.1	155	0.008
	16.8±1.3	148	
$CD34^+$ $HLA\text{-}DR^-CD38^-$	2.3±0.44	32	0.05
	4.3±1.1	11	
$CD34^+13^+$	89.7±1.49	88	0.004
	75.9±6.7	23	
$CD34^+CD71^+$	50.3±2.79	75	0.044
	40.7±3.39	35	

Most of the differences were in absolute cell number of HSC individual subsets per kg which was most likely due to higher total dose of HSC transplanted. As concerns subset proportions, higher doses of $CD34^+$ HSC in a single LP were associated with lower contents of $CD34^+CD45^{low}$ and $CD34^+HLA\text{-}DR^-CD38^-$ subsets and higher contents of $CD34^+CD13^+$ and $CD34^+CD71^+$ HSC (table 4).

Specimens with more than 3.0×10^6/kg $CD34^+$ HSC demonstrated a predominance of larger HSC ($CD34^+FSC^{++}$, $p = 0.004$), $CD34^+CD45^{low}$ HSC ($p = 0.0001$) and $CD34^+HLA\text{-}DR^-CD38^-$ ($p = 0.025$) populations.

LP with optimal content of $CD34^+$ HSC per kg bodyweight (2.0 to 4.99×10^6/kg of $CD34^+$ HSC) in comparison with a group of higher doses (5 or more $\times10^6$/kg of $CD34^+$ HSC) showed a preponderance of $CD34^+CD45^{low}$ ($p = 0.024$), $CD34^+HLA\text{-}DR^-$ HSC ($p = 0.037$) and HSC with bright expression of transferrin receptor ($p = 0.053$) and $CD34^+CD45^-$ HSC ($p = 0.039$). These specimens contained a significantly higher proportion of $CD13^+$ and $CD71^+$ HSC ($p < 0.02$ in all cases) than specimens with less than the threshold dose of $CD34^+$ HSC.

In the whole group the harvest contained $12.08 \pm 0.9\times10^7$ $CD34^+$ HSC per the entire volume (median 8.0, range 0.1 to 73.0×10^7, n = 185) and $4.94 \pm 0.32\times10^6$ CD34 HSC per kg bodyweight (median 3.76, range 0.05 to 30.0×10^6, n = 208).

Transplantation was made in 107 patients. Mean time to leukocyte recovery to 1000 cells was 10.9 days (median 10, range 8 to 32 days), mean time for platelet recovery to 20 000 cells was 17.3 days (8 to 51 days, median 14.5 days) and for

platelet recovery to 40 000 cells was 24.7 days (8 to 80, median 20 days). Patients with platelets failing to recover to 40 000 cells per mcl before discharge were excluded from the analysis.

The HSC transplant consisted of mobilized peripheral SC in most cases (96 transplantations), in 11 cases transplantation was made using HSC from both stimulated PB and BM.

The recovery period in cases with transplantation of peripheral HSC only was shorter than for combined transplantation of pHSC+BM (10.5 days vs. 14.5 days for leukocyte recovery to 1000 cells per mcl [p = 0.0001] and 24.6 days vs. 37.5 days for platelet recovery to 40 000 cells per mcl [p = 0.01]). Comparison of leukocyte and platelet recovery periods in patients receiving 3.0×10^6 or more vs. less $CD34^+$ HSC per kg bodyweight discovered statistically significant differences in recovery of platelets both to 20 000 and 40 000 cells per mcl (16 vs. 22 days and 22 vs. 35 days, p = 0.0001, respectively). More rapid leukocyte recovery was associated with lower content of $CD34^+CD117^+$ (p = 0.012) and larger content of $CD34^+CD2^+$ (p = 0.047) cells. Shorter thrombopenia periods were associated with lower content of $CD34^+$HLA-DR^+ (p = 0.024), $CD34^+CD13^+$ (p = 0.049) and $CD34^+CD71^{++}$ (p = 0.035) HSC subsets.

Analysis of recovery of principal blood components with respect to proportions of HSC subsets in LP of the first day harvest (irrespective of the number of leukapheresis needed for the harvest) found that more rapid recovery of leukocytes to 1000 cells per mcl (within 10 days) was associated with more than 3.0% of $CD45^{low}$ and less than 65.0% of $CD38^-CD34^+$ HSC (table 5).

Table 5

Recovery time of blood components is associated with proportions of subsets in HSC first day harvest

Subset %%	Threshold level	Mean recovery time (days)	No. of specimens	p Value
	Leukocytes to 1000 cells/mcl			
$CD45^{low}$	3.0% or more	10.3±0.2	61	0.028
	Less than 3.0%	11.5±0.6	30	
$CD45^{neg}$	5.0% or more	10.3±0.2	56	0.027
	Less than 5.0%	11.5±0.6	35	
$CD38^-$	65.0% or more	12.5±1.8	8	0.044
	Less than 65%	10.1±0.2	25	
$CD13^+$	90.0% or more	11.0±0.7	22	0.043
	Less than 90.0%	9.4±0.3	8	
	Platelets to 20 000 cells/mcl			
$CD13^+$	90.0% or more	20.5±1.9	22	0.076

	Threshold level	Mean recovery time (days)	No. of specimens	p Value
	Less than 90.0%	13.3±3.4	8	
HLA-DR$^+$	95.0% or more	20.7±2.4	21	0.07
	Less than 95.0%	15.8±1.5	20	
	Platelets to 40 000 cells/mcl			
CD45^{low}	3.0% or more	23.1±1.4	52	0.045
	Less than 3.0%	29.3±3.1	27	
CD13$^+$	90.0% or more	28.8±2.8	22	0.07
	Less than 90.0%	19.0±4.1	8	

Shorter terms of critical thrombopenia (platelets to 20 000 cells per mcl within 13 days and leukocytes to 1000 cells/mcl within 9 to 14 days) were due to at least 3.90×10^6 HSC (per kg patient's bodyweight) with less than 90.0% of CD13$^+$CD34$^+$ cells in the transplant.

5. Donors' mobilized HSC subsets and efficiency of allogeneic stem cell transplantation in pediatric oncology

We assessed the effect of hemopoiesis recovery after 51 allogeneic transplantations in 47 pediatric cancer patients from 50 relative donors.

Donors' leukoconcentrates containing a large number of CD34$^+$ cells were characterized by a marked proportion of CD45^{low} ($p = 0.03$) and a lower proportion of CD33$^+$ HSC ($p = 0.023$).

Increase in CD34$^+$CD45^{neg} cells in the transplant led to increase in the absolute number of myeloid-committed CD13$^+$ and CD33$^+$ SC ($p < 0.002$), CD38-negative CD34$^+$ ($p < 0.0001$), HLA-DR$^+$ ($p = 0.001$), CD117$^+$, CD71$^+$ ($p = 0.004$) and CD50$^+$ ($p = 0.002$) cells among CD34$^+$ HSC.

Shorter periods to hemopoiesis recovery in children with hematology malignancies after allogeneic HSC transplantation were associated with higher proportions of CD13$^+$ ($p = 0.004$), CD38$^-$CD45$^+$ ($p = 0.011$), CD71$^+$ ($p = 0.022$) and HLA-DR$^+$ ($p = 0.042$) and lower proportions of CD38$^-$HLA-DR$^-$ ($p = 0.034$) and CD34$^+$CD7$^+$ ($p = 0.01$) HSC subsets.

Shorter terms of critical thrombocytopenia after donors' HSC transplantation were associated with lower proportions of myeloid-committed CD33$^+$/CD13$^+$ HSC ($p < 0.047$).

Analysis of HSC threshold levels associated with faster recovery of principal blood components in childhood recipients discovered the following (table 6).

Table 6

Recovery period for blood components in recipients (pediatric patients with hematology malignancies) is associated with proportions of subsets in donors' HSC

Subset %%	Threshold level	Recovery period (days)	No. of specimens	p Value
HSC absolute number ($\times 10^6$/ kg)				
	Platelets to 40 000 cells/mcl			
$CD34^+$	3.0 or more	14.3±1.4	32	0.049
	Less than 3.0	9.7±1.6	10	
HSC proportion (%%)				
	Leukocytes to 1000 cells/mcl			
$CD34^+CD45^{neg}$	25.0% or more	10.4±0.38	18	0.024
	Less than 25.0%	14.9±1.8	25	
$CD34^+CD7^+$	6.0% or more	12.8±0.8	8	0.022
	Less than 6.0%	10.5±0.4	12	
$CD34^+CD13^+$	90.0% or more	11.7±0.4	12	0.004
	Less than 90.0%	10.8±0.6	8	
	Platelets to 40 000 cells/mcl			
$CD34^+CD13^+$	90.0% or more	15.8±2.5	12	0.03
	Less than 90.0%	9.7±2.1	8	
$CD34^+CD71^+$	60.0% or more	14.5±1.9	15	0.004
	Less than 60.0%	29.1±13.0	5	

Platelet recovery within 10 days might be expected following transplantation of 3 million $CD34^+$ cells per recipient's kg bodyweight containing less than 90.0% of $CD34^+CD13^+$ HSC. Leukocyte recovery within 9 to 10 days might be expected following transplantation of donor's HSC containing more than 25% of $CD34^+CD45^{neg}$ HSC, less than 6.0% of $CD34^+CD7^+$ HSC and less than 90.0% of $CD34^+CD13^+$ HSC.

Analysis of a potential impact of subsets in allogeneic transplantation in children with hematology malignancies discovered that graft rejection after gaploidentical transplantation might be associated with concentration of $CD34^+CD90^+$ and $CD34^+CD50^+$ HSC in the transplant, while severity of GVHR might be related to proportion of $CD34^+CD38^+$ and $CD34^+CD90^+$ subsets in donors' HSC transplants.

Discussion

We analyzed 1187 specimens of blood mononuclear fraction (LP, leukoconcentrate) and stimulated PB to assess expression of membrane antigens

associated with HSC of various differentiation levels. The earliest HSC compartment was characterized basing on the absence of CD38 and HLA-DR on $CD34^+$ cells and as a result of discovery of Thy-1^+ ($CD90^+$) subsets within the $CD34^+$ pool.

Pluripotent populations were identified and characterized basing on coexpression of CD45, CD38, HLA-DR, CD117, CD90 and CD71 on CD34+ cells. Poly- and bipotent HSC were studied by expression of CD117, CD71, GlyA, CD13, CD33, CD61 (HSC of the CFU-GEMM, BFU-EMeg and CFU-GM levels), as well as lymphoid-committed precursor cells as identified by expression of CD10, CD19 (B-lineage restriction) and CD2, CD7, CD57 and CD56 (T- and NK-cell restriction).

Efficacy of HSC mobilization into PB and autologous HSC harvesting depends first of all on the number of previous chemotherapy cycles and irradiation load. Characteristics such as patient age, BM involvement and time from last chemotherapy are less important though still significant factors [1–5, 48]. However, there was practically no comparative study of HSC mobilization efficacy with respect to disease type in patients with similar pretreatment and mobilization. There was only a mention of significantly rarer cases of mobilization failure in NHL, MM and HL (effective mobilization in 93% of cases, $p = 0.0001$) as compared to acute leukemia with less effective mobilization (81%) basing on the $CD34^+$ total [49].

Our findings demonstrated that the administered chemotherapy schedules were adequate for effective mobilization. Minimal material quantity needed for transplantation (more than 2 million per kg recipient's bodyweight) was harvested for 2 to 3 leukaphereses in adult patients. While in most children (58%) minimal amount of autologous HSC was harvested for a single leukapheresis. Minimal number of donors' cells needed for allogeneic transplantation was also harvested for a single leukapheresis in most cases (more than 65% of LP).

$CD34^+$ proportion in a single LP harvest in adult patients was significantly higher than in donors and reached $1.12 \pm 0.07\%$, median 0.59% (range 0.01 to 8.8%, $n = 390$, $p = 0.02$). In children mean $CD34^+$ proportion in LP from a single leukapheresis was $0.82 \pm 0.05\%$ (median 0.45%, range 0.01 to 10.0, $n = 446$). Donors had the lowest $CD34^+$ proportion per leukoconcentrate $0.36 \pm 0.03\%$ (median 0.29%, range 0.02 to 1.23%, $n = 61$). However, analysis of absolute $CD34^+$ HSC number per kg recipient's bodyweight (taking into account smaller weight in children receiving allogeneic HSC transplantation: 28.1 ± 1.9 kg, median 27 kg) demonstrated that the SC number was the highest in allogeneic HSC (donors' HSC $4.82 \pm 0.67 \times 10^6$/kg, median 3.6×10^6/kg, range 0.1 to 35.5×10^6/kg, $n = 61$). Absolute $CD34^+$ HSC number per a single leukapheresis was the lowest in adult cancer patients ($3.11 \pm 0.38 \times 10^6$/kg, median 1.5, range 0.02 to 28.7×10^6/kg, $n = 285$) as compared to donors ($p = 0.027$).

Mobilization of the total HSC pool (%%$CD34^+$) was significantly higher in children with ES and neuroblastoma (1.03% and 1.19% of $CD34^+$ cells respectively, $p = 0.035$) while the effect was the poorest in hematology malignancy group, specifically in HL (mean 0.31% of $CD34^+$ cells in transplantation tissue, $p = 0.05$). Interestingly, mobilization effect was significantly poorer in medulloblastoma in spite of less heavier pretreatment as compared to children with soft tissue sarcoma ($0.66 \pm 0.12\%$, $n = 38$ vs. $1.05 \pm 0.12\%$, $n = 131$, $p = 0.025$) and children with ES ($0.66 \pm 0.12\%$, $n = 38$ vs. $1.03 \pm 0.12\%$, $n = 100$, $p = 0.04$).

The total amount of HSC for all leukaphereses was the highest in adult patients: $7.61 \pm 0.9 \times 10^6$/kg, median 4.7, range 0.55 to 28.0×10^6/kg, $n = 55$. Harvest for autologous transplantation from children was $4.97 \pm 0.32 \times 10^6$/kg, median 3.78 (0.05 to 30.0×10^6/kg, $n = 204$), donors SC were $5.74 \pm 0.77 \times 10^6$ $CD34^+$ cells per kg recipient's bodyweight (median 3.68×10^6/kg, range 0.1 to 35.5×10^6/kg, $n = 51$).

We studied in detail the role of HSC subset composition in transplantation tissue. Mobilization effect was assessed for pools of early, pluri-/polypotent cells, granulocyte-macrophage differentiation cells, megakaryocyte-committed HSC, erythroid precursor cells and lymphoid HSC.

As known, CD45 expression is increasing and CD34 expression is decreasing during SC differentiation from pluripotent to lineage-committed cells [22, 50].

Antigen CD45 has not been studied in clinical trials of hemopoietic tissue transplantation till now because the existing cytometry protocols recommend leukocyte gating basing on CD45, i.e. only $CD45^+$ cells are included in the analysis and SC proportion is assessed within this pool [51–53].

Our study has demonstrated that CD45 expression on HSC membrane can be very weak up to negative. Common leukocyte antigen CD45 showed a considerable variability of expression on $CD34^+$ SC from weak (like on granulocytes) to strictly negative in all groups analyzed. Among the mobilized HSC there were subsets with different expression of the common leukocyte antigen including CD45-positive cells ($CD34^+CD45^{+/low}$, denoted as $CD45^+$) with CD45 expression level similar to that on granulocytes, SC with very low CD45 or practically negative expression density ($CD34^+CD45^{-/low}$, denoted as $CD45^{low}$) and HSC that were strictly negative with respect to expression of the common leukocyte antigen ($CD34^+CD45^-$).

We studied in a greater detail the cell proportion with very weak or negative expression of the antigen which was the least mature HSC fraction according to the literature [54–56].

Decreased CD45 expression on cells is associated with a poorer mobilization effect and lower mobility of cells in response to the action of external factors [55, 57, 58]. This suggests that $CD34^+CD45^{neg/low}$ HSC are a less readily

mobilized subset composing a sort of body's reserve. These cells submit to mobilization only in patients with rather exhausted hemopoiesis or as a result of impairment in BM microenvironment.

CD45-negative HSC were a rather large subset (more than 20.0%) of the HSC total pool in all groups analyzed, and were more than 10% of the $CD34^+$ cell total in most patients (more than 60% of specimens). These cells were different by size, there was an inverse correlation between their content and SC total in transplantation tissue among autologous HSC from children and adult patients ($p = 0.045$ in children and $p = 0.016$ in adults). Expression of the SC antigen was higher in $CD34^+CD45^-$ HSC populations.

This subset was immunophenotypically heterogeneous with a trend to higher expression of antigens associated with polypotent pool (HLA-DR, CD38, CD117, CD71) on $CD45^{neg}$ HSC, this observation indirectly suggesting a part of these cells to belong to precursors of the CFU-GEMM level.

Correlation analysis demonstrated that $CD34^+CD45^{low}$ cells in adult cancer patients might be represented by 2 HSC subsets such as T-lineage ($CD34^+CD45^{low}CD7^+HLA\text{-}DR^-CD13^-$) or megakaryocyte-committed ($CD34^+CD45^{low}CD61^+HLA\text{-}DR^-CD13^-$) precursors. The $CD34^+CD45^-$ subset is still more heterogeneous with only $CD34^+HLA\text{-}DR^+$ cells surely present in this fraction. The discovered correlations might suggest the presence of a considerable proportion of SC expressing c-kit receptor ($CD117^+$) and $CD13^+$ HSC. $CD33^+$ HSC proportion was increasing with increase in the $CD34^+CD45^{low}$ fraction within HSC from adult patients. The fact of CD33 expression within the $CD34^+CD45^{neg}$ cells may be evidence of hemopoietic nature of these populations since molecule CD33 is identified on hemopoietic cells only [70, Peiper S.C. et al., 1995].

In leukoconcentrates from children increase in $CD34^+CD45^{neg}CD45^{low}$ populations was associated with increase in B-lineage precursors and $CD38^+$ HSC and with decrease in $CD13^+$ myeloid-committed HSC. $CD34^+CD33^+$ proportion was increasing and $Thy\text{-}1^+$ was decreasing in parallel with increase in $CD34^+CD45^-$ HSC.

In donors increase in $CD45^{low}$ HSC was associated with higher proportions of $CD34^+CD33^+$ and $CD34^+CD7^+$ HSC as well as with the number of megakaryocyte precursors ($CD61^+$). A lower $CD34^+CD45^-$ content among donors' HSC was associated with a greater $CD34^+CD13^+$ proportion and a lower content of $CD19^+$ B-lineage precursors.

Mobilized $CD34^+CD45^{neg}$ HSC from children and adult patients demonstrated certain immunophenotype peculiarities. In adults (both cancer patients and healthy donors) this subset demonstrated rather a myeloid restriction trend, while in children it was rather of a lymphoid type. These findings are indirectly confirmed by studies of HSC biological changes due to the body aging

as changes in HSC subset proportions with earlier forms and lymphoid precursors decreasing in parallel with increase in myeloid precursor cells [8, 59–62].

Content of CD45-negative populations within autologous HSC was different in different disease types. For instance, in children this subset was significantly larger in cases with CNS tumors and hematology malignancies and in HL within the latter group (more than 27% of the HSC total, $p < 0.04$).

In adults CD45-negative cell proportion was also larger (more than 30.0%) in HL with the highest concentrations found in first harvest leukoconcentrates.

Analysis of specimens with different contents of $CD34^+$ leukocytes demonstrated that CD45-negative cells made a significantly higher proportion ($p = 0.013$) in leukoconcentrates with lower SC contents from adults (except some specimens with very low $CD34^+$ content).

In children LP with low proportions of $CD34^+$ leukocytes also contained large quantities of CD45-negative SC, though differences with all $CD45^{neg}$ HSC subsets were not significant.

Our data confirm relationship between mobilization efficacy and $CD45^{neg}$ SC. Satisfactory mobilization was associated with inverse correlation between $CD34^+$ cells and $CD45^{neg}$ HSC.

This means that detection of a considerable content of CD45-negative HSC in harvests may be evidence of poor mobilization of the total HSC pool and inefficiency of further leukaphereses.

Given variability of SC with respect to CD45 we used 2 modes of HSC identification, i.e. basing on CD50 and nucleotropic dye SYTO 16. $CD34^+$ cells showed a more even expression of CD50 and always exceeded 98.0%. However, SYTO 16 was more informative because it clearly identified both nucleated and viable cells irrespective of their differentiation degree [63–65].

Our study demonstrated that all $CD34^+$ cells were SYTO $16^{+}/^{++}$, and therefore gating with SYTO 16 for HSC identification was more accurate in comparison with common leukocyte antigen CD45. So, this dye is the best tool for accurate identification of HSC proportion in transplantation tissue.

The absence of CD38 and HLA-DR expression on $CD34^+$ cells is the most typical and well described immunophenotype of the earliest pluripotent HSC; the proportion of such cells among peripheral HSC is very low [22, 50, 66–69]. Most of these studies, however, were made with separate $CD34^+HLA\text{-}DR^-$ and $CD34^+CD38^-$ subsets and demonstrated their considerable functional difference.

There are few studies of double negative $CD38^-HLA\text{-}DR^-$ HSC, and proportion of $CD34^+CD38^-HLA\text{-}DR^-$ SC was 0.015% to 0.023% among leukocytes from umbilical cord blood with a 0.2% HSC mean total content among leukocytes of [70].

In our study proportion of $CD34^+CD38^-HLA\text{-}DR^-$ cells in transplanted tissue was low and in accordance with findings of earlier foreign studies [69, 71].

This subset was the largest in donors. In cancer patients $CD34^+CD38^-HLA\text{-}DR^-$ subsets were similar in adults ($3.2 \pm 0.64\%$) and children ($2.96 \pm 0.42\%$). The $CD34^+CD38^-HLA\text{-}DR^-$ cells were in inverse correlation with myeloid-committed precursors in donors ($CD34^+CD13^+$, $CD34^+CD33^+$ and $CD34^+CD71^+$, $p < 0.039$, $n = 12$ for all subsets). While in children this subset was in direct correlation with lymphoid-committed precursors $CD34^+CD56^+$ ($R = 0.527$, $p = 0.002$, $n = 33$), $CD34^+CD19^+$ ($R = 0.486$, $p = 0.002$, $n = 37$) and $CD34^+CD2^+$ ($R = 0.479$, $p = 0.015$, $n = 25$).

According to the literature, Thy-1^+ cells also belong to the earliest HSC [72]. Some authors characterize true HSC as $CD34^+CD90^+CD117^+$ cells. This means that in most cases Thy-1 expression is considered together with c-kit receptor expression while individual roles of each of the molecules is not clear. These molecules are involved in cell-cell and cell-stroma interactions and may mediate HSC mobilization into PB [73–75]. It is HSC belonging to Thy-1-positive fraction that maintain growth of long-term BM cultures. A rather weak ability to accumulate rhodamine (Rh123) is an additional confirmation of $CD34^+$Thy-1^+ cells belonging to non-committed early SC. This fraction contains so called cobble-stone area forming cells and cells responsible for regulation of hemopoietic organs during transplantation in SCID-hu mice [25, 73].

SC with CD90 expression are shown to predominate in the early SC fraction ($CD34^+CD38^-HLA\text{-}DR^-$) [24]. These data confirm Thy-1 expression at SC early differentiation stages and allow $CD34^+CD90^+$ cells to be attributed to true SC able to ensure sustained and adequate hemopoiesis recovery if they make a considerable fraction in the transplant. $CD34^+$Thy-1^+ SC demonstrate a variable immunophenotype. Most $CD34^+CD90^+$ cells coexpressed HLA-DR, CD38, CD33, CD45 and CD54, while only few of them coexpressed CD56 and CD71 [77, 78].

The $CD34^+$Thy-1^+ HSC subset is transitory for PB and reveals itself at rather early stages of SC mobilization, therefore the peak of $CD34^+$Thy-1^+ cells fails to coincide by time with the mobilization peak of the total $CD34^+$ pool [78, 79]. It is this fact that may play a role in variability of data about the molecule expression on mobilized $CD34^+$ cells in PB.

In our study $CD34^+CD90^+$ cell proportion was significantly larger among donors' SC. Thy-1 subsets were represented by the smallest cells, and their number was inversely proportional to $HLA\text{-}DR^+$ and $CD61^+$ HSC in adults. In children $CD34^+CD90^+$ proportion was significantly related to myeloid-committed pool.

Mobilization of Thy-1 subsets was the most effective in donors and children with medulloblastoma and neuroblastoma ($p < 0.04$). Basing on the literature data about mobilization of $CD90^+$ HSC this observation may be explained by less intensive hemopoiesis stimulation in this case. The lowest proportion of such cells was characteristic of HSC from patients with hematology malignancies and CNS

tumors ($p = 0.03$). Taking into account a considerable difference in previous chemotherapy burden it may be stated that Thy-1^+ HSC mobilization is the most effective in cases with more preserved hemopoiesis at the moment of mobilization (donors and children, patients with medulloblastoma).

There is evidence that $CD34^+HLA\text{-}DR^-CD117^-$ subset predominates among cells initiating growth of long-term BM cultures and as such possessing functions of the earliest HSC. This subset appeared clinically significant. Proportion of cells with this immunophenotype in the transplant correlated with efficiency of sustained recovery of platelets (3 to 6 months) while $CD34^+CD38^+HLA\text{-}DR^-$ HSC played a role in recovery of neutrophil and erythrocyte components [34].

The main purpose of the transplantation is recovery of principal hemopoiesis components to prevent infectious, hemorrhagic and other complications associated with cytopenia as a result of HCT. That is why GM- and G-CSF are used among mobilization agents. These regimens ensure mobilization of SC enriched with myeloid precursors [80] that may contribute to more rapid neutrophil recovery.

Markers of myeloid HSC include both early $CD117^+$ precursors and cells with expression of pan-myeloid antigens CD13, CD33 that is seen at most stages of differentiation of myeloid-restricted precursors.

C-kit receptor CD117 is thought to dominate among myeloid-committed precursors of early differentiation stages [81, 82].

Mobilized HSC were characterized by a broad range of variability of $CD117^+$ cells significantly associated with the HSC total in cancer patients ($p = 0.03$). There were 2 HSC populations with high-density expression of c-kit receptor ($^{++}$) and $CD117^{+/low}$ HSC, that made two functionally different HSC subsets according to the literature [83].

$CD117^{++}$ HSC expressed common leukocyte antigen weaker (up to negative level) as compared to $CD117^+$ cells. Coexpression of CD117 and CD19 in children was unlikely, though was seen in some cases. $CD117^+$ HSC demonstrated variable CD7 and CD2 expression.

Increase in $CD117^+$ HSC in children was associated with increase in $CD71^{++}$ and Thy-1^+ cells as well as with increase in the total $CD34^+$ leukocyte proportion in specimens. In donors the variable expression of the antigen was accompanied by a larger proportion of $CD34^+HLA\text{-}DR^-CD38^-$ HSC and lower content of $CD45^{low}$ HSC. These findings confirm possible mobilization of the earliest clinically significant HSC with $CD34^+HLA\text{-}DR^-CD38^-CD117^{low}$ immunophenotype described previously [34]. In adults specimens with large proportions of $CD117^+$ HSC contained smaller proportions of $CD61^+$, $CD34^+HLA\text{-}DR^-$, $CD19^+$, $CD7^+$ HSC, while the total percentage of $CD34^+$ leukocytes was higher, like in children.

Our data demonstrated variability in mobilized HSC subsets with respect to CD117 in all groups, i.e. cancer patients (adults and children) and donors. For instance, in adult patients $CD117^+$ HSC were more likely to belong to myeloid-restriction cells, while in children they could rather be early subsets with CD90 coexpression. The earliest $CD34^+HLA\text{-}DR^-CD38^-$ HSC from donors could be CD117-negative.

Our data are somewhat contradictory to other findings demonstrating that decrease in the receptor expression on cell membrane accompanies HSC differentiation processes and that means that the more the cell is differentiated, the less is CD117 expression on its membrane, this phenomenon being mediated by receptor internalization during SCF incubation. However endocytosis is not the only and by no means the main factor regulating c-kit receptor expression on HSC. TGF_β (transforming growth factor) is another factor decreasing c-kit receptor expression on cell membrane and influencing the receptor stability [84, 85].

There is also evidence that HSC with long-term repopulation ability isolated from AMG (aorta-gonad-mesonephros) aorta of 9- to 13-day murine embryos included 6 SC populations different in CD45 and CD117 expression. $CD45^{low}CD117^+$ cells appeared multipotent and able to form colonies of hemopoietic cells, and this ability was decreasing in cells from 11-day embryos as compared to 9-day ones [86].

The conclusion may therefore be made that CD117 expression is a reflection of HSC functional differences, though is not stage-specific.

Analysis of the total $CD117^+$ pool demonstrated that the least mean content of c-kit receptor-positive HSC was found in children with PNET and medulloblastoma, this subset was mobilized the most readily in patients with hematology malignancies ($p < 0.01$). Given the range and variability of expression, the content of $CD117^+$ HSC was not significantly related with other subsets except $Thy\text{-}1^+$ HSC in children.

It is important to note that the content of $CD117^+$ HSC was directly proportional to the $CD34^+$ total in leukoconcentrates from cancer patients.

$CD13^+$ and $CD33^+$ made considerable proportions in mobilized HSC. However expression of CD13 was always more intensive than that of the second pan-myeloid antigen CD33 whose expression was more typical for myelo-monocytic lineage of differentiation [87].

Opinions concerning sequence of CD13 and CD33 expression at different stages of HSC differentiation are currently controversial. For instance, there is a description of $CD34^+CD13^+CD33^-$ HSC subset from BM and umbilical cord blood that expressed antigens CD90, CD117 and not expressed markers of lymphoid restriction. While in our study $CD34^+CD33^+CD13^-$ cells were $CD90^-$ and $CD117^-$, i.e. $CD13^+$ $CD33^-$ HSC were less mature than $CD34^+CD33^+CD13^+$ cells. Though this subset was rather small and made not more than 0.5% of BM mononuclear cells [26].

There is evidence of $CD33^+$ cells belonging to early precursors without immunophenotypical characteristics of a certain differentiation lineage [88, 89].

Taking into account degree of stimulating effect of HSC mobilizing stem factors, most mobilized peripheral $CD34^+$ cells are $CD13^+CD33^+$.

In our study CD13 expression was more marked as compared with CD33 on a greater percentage of HSC both in cancer patients and donors.

$CD33^+$ HSC proportion was the lowest in adult cancer patients and was associated with HSC total dose.

Both CD13 and CD33 HSC demonstrated variable CD45 expression. One and the same specimen might express both $CD45^+$ and $CD45^{neg}$ HSC with similar CD45 expression on $CD33^+$ and $CD13^+$ HSC. In majority of specimens $CD13^+$ and $CD33^+$ HSC expressed CD7 and CD2. And there were also $CD7^+CD13^-$ and $CD2^+CD33^-$ HSC subsets among the mobilized cells. $CD13^+$ HSC were also heterogeneous with respect to CD61 expression.

Specimens from adult patients containing less than 70.0% of $CD13^+$ HSC might contain discrete CD13 and CD33 populations which confirmed the previous findings of potential expression of these antigens on different HSC subsets [90].

Leukoconcentrates from children with monomorphous (more than 90.0%) expression of CD13 HSC were characterized by a lower percentage of $CD34^+$ leukocytes, larger proportions of $CD45^{low}$, $CD7^+$ and $CD61^+$ HSC and a lower proportion of $CD33^+$ HSC. In donors the predominance of $CD13^+$ HSC was associated with lower percentages of $HLA\text{-}DR^-$ and $HLA\text{-}DR^-CD45^-$ HSC.

Leukoconcentrates from adults containing less than 65.0% of $CD34^+CD33^+$ HSC were characterized by greater proportions of $CD38^-$ and $CD38^-HLA\text{-}DR^+$ HSC belonging to the earliest HSC and a greater proportion of $CD34^+$ leukocytes. This marker may therefore be used clinically for prognosis of completeness and speed of hemopoiesis recovery after transplantation.

Similar (containing less than 65.0% of $CD34^+CD33^+$ HSC) specimens from children contained lower proportions of $Thy\text{-}1^+$, $CD38^+$, $HLA\text{-}DR^+CD45^-$ and $CD34^+CD45^-$ HSC while in donors the $Thy\text{-}1^+$ proportion was larger, similarly to $CD19^+$ HSC.

$CD33^+$ HSC percentage in children was proportional to that of $Thy\text{-}1^+$ cells, with coexpression of these antigens present at the level of a single SC which confirmed hemopoietic nature of $Thy\text{-}1^+$ HSC, since (as mentioned above) CD33 expression was characteristic of hemopoietic cells only.

HSC of myeloid restriction demonstrated coexpression of lymphoid antigens and variability with respect to transferrin receptor CD71. There were several subsets of HSC different by expression of CD71 and CD13. The finding of $CD13^+CD71^+$ and $CD71^+CD13^-$ HSC was of importance, since it confirmed transferrin receptor expression on precursors not belonging to cells of myeloid restriction.

Mobilization of myeloid $CD33^+$ HSC was most effective in patients with hematology malignancies ($p < 0.04$), i.e. most heavily pretreated patients.

Data on mobilization of myeloid precursors are opposite to findings about early HSC subsets (Thy-1^+) and commeasurable with mobilization effect of $CD34^+CD45^{neg}$ subsets: the best mobilization effect of early myeloid precursors and $CD33^+$ HSC was achieved in more pretreated patients (with hematology malignancies, in particular HL) with most exhausted hemopoiesis.

Platelet recovery is one of the main purposes of transplantation of SC-enriched hemopoietic tissue. It is reasonable therefore to assess proportion of megakaryocyte-committed precursors which was more marked in donors' cells ($p = 0.001$). $CD34^+CD61^+$ HSC from cancer patients (both children and adults) most likely contained $CD45^{low}$ cells (significant correlation between these subsets both in children [$p = 0.038$, $n = 56$] and adults [$p = 0.05$, $n = 29$]). In adults the presence of $CD34^+CD38^-$ was likely, while the presence of $CD34^+CD117^-$ and $CD34^+CD90^+$ HSC was hardly to be expected. Not all $CD61^+$ HSC expressed CD13.

Therefore, immunophenotype of HSC of megakaryocyte restriction in cancer patients may be described as $CD34^+CD61^+CD45^{low}CD38^-$. The presence of $CD38^-CD45^{low}$ cells allows them to be attributed to the compartment of the earliest HSC. More so since according to the literature, there is evidence of existence of an alternative way for HSC differentiation into megakaryocyte precursors, i.e. directly from a common multipotent precursor avoiding the BFU-EMeg stage [28, 91, 92].

The lowest proportion of megakaryocyte precursors was found among SC from children with neuroblastoma ($p = 0.04$) as compared to medulloblastoma and PNET.

Glycophorin A (GlyA) is the principal differentiation marker for erythroid cells. This marker arises at later stages of HSC differentiation towards erythroid cells, and the earliest erythroid precursors do not express GlyA. In our study $GlyA^+$ proportion was very low — less than 1.0% of the HSC total. Transferrin receptor is another marker associated with erythroid cells [27, 93]. Our data demonstrated a high association between $GlyA^+$ and $CD71^+$ cells, i.e. all $GlyA^+$ SC expressed CD71, though there was also a $CD71^+GlyA^-$ population. This confirms a considerable variability of $CD71^+$ HSC with respect to lineage and levels of differentiation, i.e. possible expression of transferrin receptor at earlier HSC differentiation stages and on lineages other than erythroid precursors.

Notwithstanding the fact that $CD71^+$ population is often attributed to erythroid precursors there is evidence of $CD71^+$ (including $CD34^+CD71^+$) cells belonging to actively proliferating cells, thus the antigen may be defined as rather a non-lineage restricted than a narrow lineage differentiation molecule. Transferrin receptor CD71 expression on mobilized peripheral HSC was variable with both

$CD71^{low}$ and $CD71^{++}$ cells present. CD71 expression level in $CD34^{+}CD71^{++}$ population was similar to that on erythrocytes. The $CD71^{low}$ population did not contain cells with GlyA expression. Stem cell antigen expression on $CD71^{low}$ HSC was brighter, common leukocyte antigen expression was decreasing as CD71 expression was increasing.

Maximal $CD71^{+}$ HSC proportions were found in donors.

Both $CD71^{low}$ and $CD71^{++}$ populations could contain $CD117^{+}$ and $CD7^{+}$ precursors. The presence of $CD34^{+}CD13^{+}$ HSC among $CD71^{++}$ cells was unlikely. $CD34^{+}CD71^{low}$ HSC in children could most likely contain megakaryocyte-committed CD61 precursors.

Cells with bright expression of transferrin receptor may therefore belong to erythroid precursors, while $CD34^{+}CD71^{low}$ linage is ambiguous and needs a more detailed study.

Increase in $CD71^{+}$ HSC both in adult patients and children was associated with low proportion of $CD45^{low}$ HSC. In adult patients increase in $CD71^{+}$ HSC occurred in parallel with increase in proportions of $CD13^{+}$ and $CD34^{+}CD38^{+}$HLA-DR^{-} HSC (early bipotent precursors of erythrocytes and megakaryocytes BFU-EMeg). In donors increase in $CD71^{+}$ HSC was associated with a larger population of $CD34^{+}CD38^{-}$HLA-DR^{-} HSC (the earliest pluripotent HSC). In children increase in $CD71^{+}$ HSC occurred in parallel with increase in $CD13^{+}$ and $CD117^{+}$ cells (polypotent myeloid HSC), and decrease in $CD38^{+}$HLA-$DR^{-}CD38^{-}$ cells.

These findings demonstrate differences in $CD71^{+}$ immunophenotype of mobilized HSC between cancer patients and donors with predominance of CD38-expressing myeloid restriction cells and no expression of class II main histocompatibility complex among $CD34^{+}CD71^{+}$ auto-HSC in cancer patients, while $CD34^{+}CD71^{+}$ HSC from donors could contain the earliest $CD34^{+}$HLA-$DR^{-}CD38^{+}$ cells.

Lymphoid lineage was studied basing on expression of B-cell marker CD19, pan-T-cell antigen CD7 and NK-cell antigens CD56 and CD57.

Mean proportion of HSC with expression of lymphoid antigens was not high, though some specimens from children and donors did contain marked proportions of $CD19^{+}$, $CD7^{+}$, $CD2^{+}$, $CD56^{+}$ HSC.

Increase in HSC with expression of one of the lymphoid antigens was as a rule associated with increase in proportions of the remaining ones, in children there was a parallel increase in $CD34^{+}$HLA-$DR^{-}CD38^{-}$ HSC.

Mobilization effect for all HSC lymphoid subsets was the best in donors ($p < 0.03$) similarly to the mobilization effect of the earliest fraction $CD34^{+}CD38^{-}$HLA-DR^{-} in this group. $CD7^{+}$ demonstrated variability in expression spectrum. In spite of the fact that this molecule is considered a T-lineage-

associated antigen and CD7 expression on T-cell precursors is very high, existence of myeloid precursors with weaker CD7 expression is also demonstrated [94]. This finding allows discrimination between T-lineage and myeloid-committed precursors and may be used to describe and differentiate HSC subsets. The presence of both $CD34^+CD7^{++}$ that may be considered T-lymphoid HSC and $CD34^+CD7^+$ cells most likely being myeloid-committed precursors was discovered among mobilized $CD34^+$ cells. The existence of $CD7^+CD13^+/CD33^+$ HSC is well documented [27, 95].

Analysis of leukoconcentrates from adult patients demonstrated a significant correlation between $CD34^+CD7^+$ and B-lineage-committed precursors ($R = 0.339$, $p = 0.024$, $n = 44$) and $CD34^+CD56^+$ cells ($R = 0.396$, $p = 0.03$, $n = 29$). This suggests a likely coexistence of B-lineage and T-restricted $CD34^+$ cells within one and the same specimen and possibility of mobilization of a common lymphoid precursor.

There were few cases of a marked presence of $CD34^+CD7^+$ cells (more than 50% of the HSC total) in cancer patients, whose immunophenotype ($CD34^+$HLA-DR^+, $CD38^-$, $CD7^+$, $CD13^-$, $CD33^-$, $CD19^-CD71^-CD50^+$) was defined as T-restricted HSC, and of $CD34^+CD45^{low}CD7^+CD13^-$ cells that were also T-restricted.

Among cancer patients $CD7^+$ HSC were mobilized significantly more readily in children with neuroblastoma and soft-tissue sarcoma and in adults with HL (more than 4.8% of the HSC total, $p < 0.02$). It is most likely that $CD7^+$ HSC in this case were early myeloid precursors since coexpression of myeloid antigens and CD7 was detected.

$CD19^+CD34^+$ HSC were smaller cells. In children this population might be $CD10^+$, was discrete with respect to CD45 and negative with respect to CD90 and myeloid antigens. Therefore the presence of $CD19^+CD10^+$ B-lymphoid precursor among the mobilized HSC raises no doubt.

In adult cancer patients $CD19^+$ and $CD10^+$ as well as $CD2^+$ and $CD10^+$ are discrete HSC subsets, i.e. mobilization of $CD19^-CD10^+$ HSC has been confirmed. According to the literature, this subset corresponds to the earliest pluripotent common lymphoid precursor (CLP) [6, 28, 29]. We confirmed the presence of $CD34^+CD10^+CD19^-$ cells among mobilized peripheral SC also in the absence of $CD19^+$ cells and by simultaneous assessment of CD10 and CD2 that were also discrete populations in adult cancer patients.

According to the literature, minor but very important subsets of lymphoid precursors ($CD10^+CD19^-$ [$0.14 \pm 0.09\%$ of the total $CD34^+CD38^+lin^-$ HSC pool] and $CD10^+CD19^+$ [$1.49 \pm 1.3\%$ of the total $CD34^+CD38^+lin^-$ HSC] were found among the G-CSF-mobilized population of peripheral $CD34^+CD38^+lin^-$ HSC in a large (more than 70%) proportion of cases.

This content of circulating lymphoid precursors is practically 10-fold higher than the number of such cells in BM both during calm hemopoiesis and under BM stimulation. These cell subsets demonstrated the ability to differentiate both to B- and NK-cells [96]. Differentiation of $CD34^+CD19^+CD10^+$ HSC into NK-cells seems unlikely, and it is $CD34^+CD38^+CD10^+lin^-$ population that may be a common lymphoid precursor in this case, which is mediated by CD10 expression in normal B-lymphocyte ontogenesis [97, 98].

Higher mobilization effect of $CD19^+CD34^+$ cells was seen in children with PNET ($p = 0.03$) and medulloblastoma ($p = 0.010$), while no $CD19^+$ HSC were found in AML cases.

In the study of HSC subsets from the clinical point of view it is of principal importance to assess contribution of each HSC subset into transplantation effect as a whole, i.e. time to and completeness of hemopoiesis recovery in recipients. This aspect seems the most meaningful since leukocyte recovery to 500 cells within 15 days from transplantation reflects transplant survival and determines a better prognosis in patients with hematology malignancies [99, 100].

Predominance of this or that individual subset may play a considerable role in both early and long-term hemopoiesis recovery and transplant survival.

We compared contributions of various HSC subsets and assessed times to granulocyte and platelet recovery with respect to proportions of early precursors and lineage-committed HSC.

Marked proportions of CD45-negative HSC (> 15.0%) were associated with faster neutrophil and platelet recovery in children and adult patients undergoing autologous HSC transplantation, and faster leukocyte recovery in children after allogeneic transplantation. As mentioned above, low CD45 expression is characteristic of less mature HSC subsets with proliferative potential decreasing as their differentiation is progressing. This property may determine the shorter time to and completeness of hemopoiesis recovery (both for neutrophils and platelets) following autologous and allogeneic hemopoietic tissue transplantation.

The earliest $CD34^+CD38^-HLA\text{-}DR^-$ cell population appeared informative in allogeneic transplantation of donors' HSC. It demonstrated direct relationship with time to leukocyte recovery: the more cells of this population were present in the transplant, the more was time to leukocyte recovery. Of note, this relationship was seen with donors' HSC and not found in autologous HSC transplantation in cancer patients. We believe that this observation might be explained by pretreatment of cancer patients that could affect proportion of immature SC sensitive to chemotherapy.

$CD34^+CD38^-HLA\text{-}DR^-$ subset is the earliest HSC that show predominance among cells initiating long-term BM cell cultures, this subset needs more time for proliferation and differentiation than committed precursors [22, 50, 101]. According to the literature, proportion of $CD34^+CD38^-HLA\text{-}DR^-$ cells in LP

directly correlates with recovery of the whole number of leukocytes ($p = 0.025$) and neutrophils within 3 to 6 months from transplantation [34].

In our study CD38-negative subset was informative in children undergoing transplantation of autologous HSC with proportions less than 65% associated with shorter period of critical leukopenia.

This population (according to the literature) may represent the earliest HSC forms [102, 103].

As demonstrated earlier, the dose of mobilized autologous $CD34^+CD38^-$ in the transplant has impact on platelet recovery in patients receiving HCT and HSC autotransplantation, with high doses mediating delay in platelet recovery [39].

On the other hand, there is evidence that high $CD34^+CD38^-$ HSC dose in autologous transplantation of peripheral HSC (5×10^4 per kg recipient's bodyweight) is associated with a better transplant survival and shorter time to three-lineage hemopoiesis recovery [38]. Allogeneic transplantation of a higher total number of $CD34^+CD38^-$ HSC determines earlier recovery of neutrophils up to 500–1000 cells per mcl and platelets to 20 000–50 000 cells per mcl [45].

Controversy in clinical significance of this subset may be explained by a considerable heterogeneity of the $CD34^+CD38^-$ HSC subset. $CD34^+CD38^-$ cells are functionally variable and include both short-living precursor cells and initiators of long-term cultures. These fractions differ by immunological parameters and are represented by $CD34^+CD33^-CD38^-Rho^{low}$ and $CD34^+CD33^-CD38^-Rho^{high}$ respectively [104]. Besides, it is the effect of absolute number of subsets that is assessed in most cases, so the results may depend on the total number of $CD34^+$ cells that was also significant in respect of hemopoiesis recovery in these studies.

The highest mobilization of $CD34^+CD38^-$ cells with properties of the earliest HSC was achieved after administration of plerixaforum in patients with poor effect of the first mobilization by G-CSF [105].

It should be noted that the modern leukemiology associates $CD34^+CD38^-$ subset with leukemia SC, and its proportion has a negative impact on disease prognosis [57, 106, 107].

Thy-1^+ HSC are the third clinically significant subset of immature cells in terms of transplantation with a higher dose also being associated with faster platelet recovery in our study.

Most studies of Thy-1^+ effect focus on assessment of completeness of late posttransplantation hemopoiesis recovery and transplant survival. It is found that a higher dose of $CD45^+CD90^+$ HSC (more than 80×10^4/kg patient's bodyweight) correlates with a better platelet recovery ($p < 0.023$) within 6–12 months from transplantation and HCT [108, 109].

Markers of immature cells CD45 and Thy-1 seem to determine early pluripotent HSC that are different from $CD34^+CD38^-$HLA-DR^- HSC by a greater

proliferation potential. The number of such cells in the transplant is associated with faster and more complete hemopoiesis recovery within a short term after transplantation, and their ability to maintain long-term cell cultures mediates sustained long-term hemopoiesis recovery after transplantation of these HSC subsets.

As concerns subsets of myeloid restriction, we studied proportions of $CD13^+$, $CD33^+$ and $CD117^+$ HSC.

Contribution of these HSC subsets was opposite to that of immature hemopoietic precursors.

For instance, higher $CD117^+$ HSC concentrations were associated with longer time to neutrophil recovery. These findings are consistent with data of a foreign study of the role of early HSC in posttransplantation hemopoiesis recovery, in particular as concerns $CD34^+CD117^+$ and $CD34^+CD117^-$ subsets. Absolute number of the transplanted (per kg patient's bodyweight) $CD34^+CD117^-HLA\text{-}DR^-$ cells was significantly lower in patients with delayed or failed hemopoiesis recovery within 3–6 months from transplantation [34]. Accordingly, high content of $CD117^+$ HSC in the transplant had a negative impact on transplantation effect.

Situation with pan-myeloid antigens, in particular CD13, was similar to that with CD117. The higher was the content of these cells in the transplant, the longer was the recovery period both for neutrophils and platelets. A marked proportion of $CD33^+$ HSC (70.0% or more) was associated with longer critical leukopenia in adult patients.

These findings are confirmed to a certain degree for megakaryocyte lineage (though the number of compared transplantations was not high): the higher was the proportion of transplanted $CD34^+CD61^+$ HSC, the longer was the thrombopenia period. However this finding needs a more detailed study, since it contradicts to data demonstrating inverse relationship between content of $CD34^+CD61^+$ subset and thrombopenia period after allogeneic and autologous transplantation of peripheral HSC. However, as mentioned above it was absolute HSC number that was taken into account. The association with period of platelet recovery was based only on correlation between the number of subsets transplanted and recovery time, while doses of $CD34^+CD61^+$ cells in groups with faster vs. slower hemopoiesis recovery were not significantly different in these studies [39, 45].

There is one exception from the common trend, i.e. transferrin receptor CD71 which is antigen with a very broad range of cell expression [11] and can by no means be associated with erythroid lineage only for which it has the highest specificity. Our findings are controversial due to lineage variability of cells expressing this receptor. For instance, there was an inverse relationship with platelet recovery period both after autologous and allogeneic transplantations, and direct correlation with duration of leukopenia. Further study of this important marker in the context of its lineage belonging is needed to more accurately

interpret CD71 findings. As concerns lymphoid antigens, significant associations similar to the trend for early pluripotent HSC (CD45-negative, Thy-1^+ HSC) were demonstrated for $CD19^+$ and $CD56^+$ HSC: higher concentrations of these subsets were associated with faster recovery of neutrophils.

Marker CD7 is considered a T-lineage associated antigen, while it is known (and confirmed in our study) to be expressed on myeloid precursors at rather early differentiation stages. Therefore study of this marker may be conducted in the context of both immature and committed HSC. We discovered that speed of leukocyte recovery was inversely proportional to $CD7^+$ content (similarly to the CD45-negative, Thy-1^+ pluripotent HSC pool) in allogeneic transplantation. On the other hand, in autologous transplantation this marker acted as a lineage-associated antigen, i.e. a greater proportion of $CD34^+CD7^+$ HSC in the transplant might be associated with delayed hemopoiesis recovery.

So, the pool of mobilized HSC is heterogeneous and represented by pluripotent precursors and committed HSC in different proportions that are in variable, rather sophisticated interrelations. Mobilization effect of SC individual subsets is related with disease type.

To achieve fast recovery of granulocyte lineages after HSC autologous or allogeneic transplantation one should not focus only on proportion of committed myeloid HSC: optimal HSC content to be transplanted should be in a certain balance between committed HSC and their earliest subsets. It is the determinant condition for optimal time to and completeness of hemopoiesis.

Both in autologous and allogeneic transplantation of mobilized HSC the effect of hemopoiesis recovery depends on proportion of granulocyte-($CD34^+CD13^+$, $CD34^+CD117^+$) or other lineage-committed HSC and the pool of immature cells defined as $CD34^+CD45^{neg}$Thy-1^+ and $CD34^+CD38^-CD45^{low}$ cells, rather than on the total $CD34^+$ HSC count.

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DISSEMINATED TUMOR CELL SUBPOPULATIONS: APPROACHES TO IDENTIFICATION AND CLINICAL SIGNIFICANCE

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Abstract

The problem of minimal cancer is a hot topic. There are different approaches to estimation (evaluation of CTCs in peripheral blood or DTCs in bone marrow) and many methods for diagnosis of minimal residual disease. We proved possible DTCs detection in bone marrow by flow cytometry. Moreover, at the Laboratory of Hemopoiesis Immunology, FGBI N.N. Blokhin Cancer Research Center, we identify subpopulations of DTCs, such as cancer stem cells, using a variety of antigens (CD45, Cytokeratines, CD326, CD44, CD24, CD133), and assess Her2/neu expression, HLA-I, HLA-DR and CD71 levels.

Key words: minimal residual cancer, DTCs, cancer stem cells, flow cytometry

Introduction

Over the last few years, there was an increasing scientific interest to the problem of minimal cancer as seen by the activity of Russian and foreign publications and a rising number of international congresses, symposia and conferences devoted to this subject, such as the 11th International Conference "Haematopoiesis Immunology" (Budapest, June 6–7, 2014), the 10th International Symposium for Minimal Residual Cancer (Hamburg, March 19–21, 2016).

There are two different approaches to estimation of minimal residual cancer: the first approach is based on evaluation of circulating tumor cells (CTCs) in peripheral blood and the second one involves detection of disseminated tumor cells (DTCs) in bone marrow (BM). The question which of the approaches is optimal is still discussed.

The presence of CTCs and DTCs is associated with a high risk of disease progression and death of cancer patients. It is reflected in TNM classification as index cM0(i+), which is rather characteristic of deposits of molecularly or microscopically detectable tumor cells not larger than 0.2 mm in circulating blood, BM or other non-regional nodal tissues, than clinical or radiographic evidence of distant metastasis in patients free from symptoms or signs of metastases [1].

It took more than a century from theoretical prediction of DTCs existence to their use in the clinical practice. The presence of circulating tumor cells (CTCs) was first described in 1869 by Thomas Ashworth, an Australian physician [2].

Twenty years later Steve Paget published in *The Lancet* first issue his “seed and soil hypothesis,” according to which “metastasis depends on cross talk between selected cancer cells (the seed) and specific organ microenvironment (the soil),” many years later I.J. Fidler [3] addressed this hypothesis again. Today the key role of DTCs in metastasis development is commonly accepted [4]. Several European groups of scientists have clearly demonstrated the clinical significance of DTCs or CTCs in patients with breast cancer, colorectal cancer, non-small cell lung cancer, prostate cancer and gastric cancer in many studies [5–9].

DTC detection methods

There are three groups of methods for DTCs detection: (1) molecular techniques, i.e. various modifications of polymerase chain reaction (PCR), (2) visualization technologies, such as CellSearch system (FDA approved), Ariol system and flow cytometry, and (3) assays for detection of specific proteins, e.g. EpiSpot system [10].

Multiple international studies resulted in the description of DTCs phenotype as $EpCam^{+}$/Cytokeratine $(CK)^{+}$ [11].

In our laboratory we developed a method for detection of DTCs by flow cytometry using an MCF-7 breast cancer model system. Pan-leukocyte antigen CD45 labeled with V450 (Beckton Dickenson, USA) in combination with antigen to epithelial molecule EpCam labeled with PE (Beckton Dickenson, USA) and antigens to cytokeratins type 7 and 8 labeled with FITC (Beckton Dickenson, USA) were used as negative control. This combination of fluorescent labels was chosen because of practically no significant overlap in their emission [12].

In a series of experiments DTCs location area was determined on a model system with MCF-7 breast cancer cells added to morphologically normal BM cells. There were practically no cells in this gate in morphologically normal BM.

In comparison experiments DTCs were detected in BM after immunomagnetic enrichment with anti-CD326 magnetic particles, according to the manufacturer’s instructions. The number of MCF-7 cells in BM after immunomagnetic enrichment was reduced which was evidence of cell loss during immunomagnetic separation [12].

We therefore proved possible DTCs detection in BM by flow cytometry [12].

Epithelial-mesenchymal transition and DTCs subpopulations

Detection of DTCs in cancer patients should address a key problem of epithelial-mesenchymal transition (EMT), i.e. loss of epithelial markers during malignant cell transition from the primary tumor into circulation and migration to BM. This transitory form has CK^{low}/$EpCam^{low}$/$Vimentin^{high-}$ phenotype [11]. Therefore, to make a more complete and accurate enumeration one has to find

additional markers that are more stable in the cell transformation process. Over recent years, there was a vast discussion on DTCs detailed phenotype and probable stem-cell origin. DTCs subpopulations with the $CD44^{+}/CD24^{-/low}/CK^{+}/EpCam^{+}$ phenotype relate to stem cells. Their role in oncogenesis is not fully clear. According to one of the existing theories, these cells have a metastasis potential and are indicative of progression of the primary tumor [11, 13]. However, these complex mechanisms require further clarification.

Another focus in the DTCs study is identification of additional therapeutic targets. This involves detection of estrogen (ER) and progesterone (PR) receptors, as well as evaluation of Her/2neu receptor expression in breast cancer patients, which is of a great prognostic value. The receptor status and Her2/neu expression are not always the same as those of the primary tumor. It remains unclear whether patients with asynchronous expression of Her/2neu on DTCs are eligible to anti-Her/2neu treatment [14].

At the laboratory of hematopoiesis immunology, N.N. Blokhin Cancer Research Center, we detect DTCs using pan-leukocyte antigen CD45, epithelial molecule EpCam (CD326), antigens to CKs type 7 and 8 (Cam5.2), and identify DTCs subpopulations or cancer stem cells by CD44, CD24, CD133 expression levels; we also analyze expression of Her2/neu, CD71 (transferrin receptor), CD33, HLA-I and HLA-II molecules. The DTCs analysis is made according to the EuroFlow approach, i.e. 8-color flow cytometry as implemented in the diagnosis of hematology malignancies. This approach allows DTCs subpopulations to be characterized in detail with a limited number of probes and ensures collection of a sufficient number of events for assessment.

Let us consider a clinical case. Patient L., a 50-year old female had BRCA1-associated primary multiple metachronous cancer of both breasts. In 2006 she was diagnosed with T2N1M0 — IIb, grade III infiltrative ductal carcinoma of the left breast. Immunohystochemichal analysis (IHC) of the primary tumor showed ER 25 H-points, PR 14 H-points, Her2/neu 1+, Ki-67–75%. The patient received multimodality treatment consisting of radical resection at the left breast, 6 courses of adjuvant chemotherapy (FAC), radiation therapy on the left breast and regional zones. In 2008 BRCA1 mutation was detected. In 2009 the patient was diagnosed with T1N3M0 — IIIc, grade II infiltrative lobular carcinoma of the right breast.

IHC of the primary tumor showed ER 7 points, PR 4 points, Her2/neu 0, Ki-67–15%; IHC of a lymph node gave ER 7 points, PR 6 point, Her2/neu 0, Ki-67–17%. The patient received multimodality treatment consisting of radical right mastectomy, prophylactic left mastectomy with simultaneous reconstruction of both breasts using thoracodorsal flaps and implants; and adjuvant chemotherapy with paclitaxel $70 mg/m^2$ + carboplatin-AUC-2 weekly, 15 injections. In March 2010, after chemotherapy completion the patient was given adjuvant hormone therapy with tamoxifen, 20 mg daily.

In August 2014, on tamoxifen therapy the patient reported of pain in the sacrum. MRI discovered total metastatic involvement of pelvic, proximal femoral and lumbar vertebral BM. BM involvement was confirmed by trepan biopsy which discovered cancer metastases, by histology with findings not inconsistent with breast cancer, by morphology and cytology study detecting multiple complexes of metastatic cancer, and by flow cytometry identifying 1.95% of $CD45^-/EpCam^+$ cells (fig. 1). Tamoxifen was discontinued. In January 2015 first-line chemotherapy was started with carboplatin-AUC-5 + doxorubicin 50 μg/m^2 every three weeks + bisphosphonates (zoledronic acid, 4 μg/m^2 every 4 weeks).

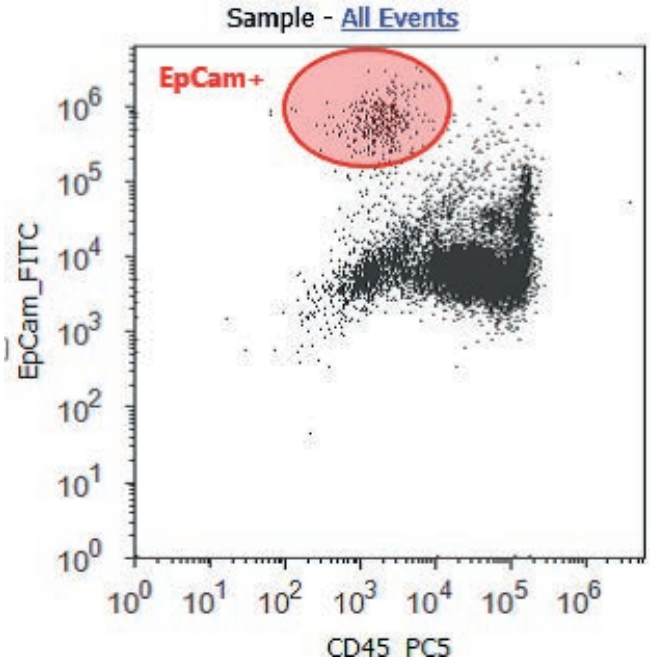

Figure. 1. Patient L. In BM before treatment there are 1.95% DTCs with the phenotype $CD45^-/EpCam^+$ (red zone).

Assessment of response to 2 cycles of first-line chemotherapy by MRI still discovered total metastatic involvement of pelvic, proximal femoral and lumbar vertebral BM; tumor response was difficult to assess by MRI.

The presence of metastases was confirmed morphologically and immunologically; flow cytometry found 1.377% of $CD45^-/EpCam^+$ cells. Clinically, the patient reported of improvement in condition and pain syndrome.

Chemotherapy was continued as usual with DTCs monitoring after the next cycle. After 3 cycles of first-line chemotherapy immunological analysis discovered the proportion of DTCs $CD45^{-}/EpCam^{+}$ to increase to 1.83% and the proportion of $CD45^{-}/Cam5.2^{+}$ cells to reach 2.16%, MRI showed increase in BM involvement, the response was defined as progressive disease.

In April 2015 the patient started second-line chemotherapy with vinorelbine 25 μg/m^2 on days 1 and 8, capecitabine 2 g/m^2/day on days 1 to 14 every 3 weeks, bisphosphonates were continued. After 4 cycles of second-line chemotherapy it was difficult to assess response by MRI, however clinically, the patient reported of improvement in condition, and administration of non-steroid anti-inflammatory drugs was decreased. After 6 chemotherapy cycles, response was assessed by MRI as positive. However, it was not possible to evaluate response by RECIST criteria, because BM disease was not measurable. Follow-up assessment after 10 second-line chemotherapy cycles (January 2016) found improvement in patient's condition with MRI confirming stable disease. BM metastases were morphologically undetectable. By flow cytometry, DTCs with $CD45^{-}/Cam5.2^{+}/EpCam^{+}$ phenotype were 0.0045% (Fig. 2). Moreover, 65% of the cells expressed Her2/neu, 9.14% were HLA-DR^{+} and 8.4% were $CD71^{+}$. The patient started maintenance chemotherapy with capecitabine 2 g/m^2/day, days 1 to 14 every 3 weeks.

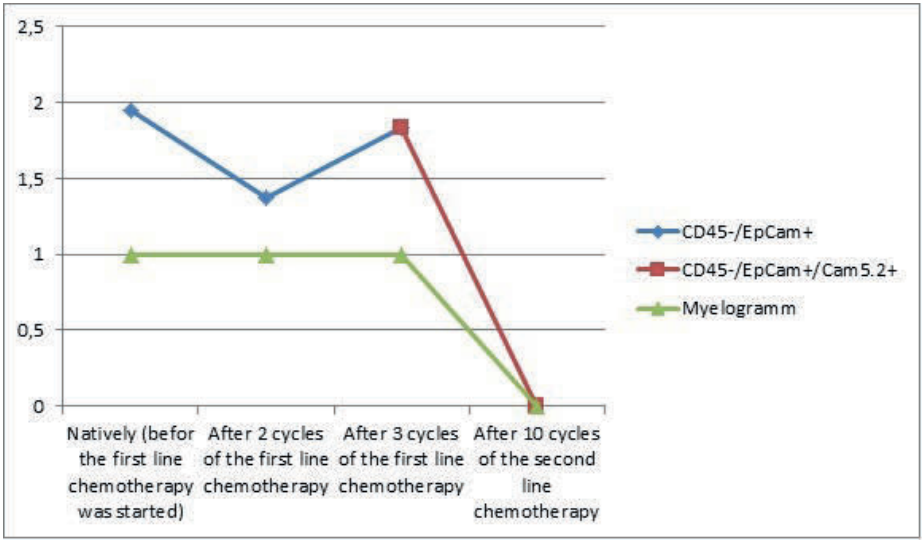

Fig. 2. Diagram shows the dynamic process of the BM involvement and DTCs detection by flow cytometry.

Conclusion

DTCs detection and subpopulation analysis is of much clinical value. We proved possible evaluation of DTCs and their subpopulations by flow cytometry which had certain advantages, such as high speed and the ability to analyze simultaneously up to 8 markers with a single probe. Flow cytometry allows detailed characterization of DTC subpopulations using a limited number of probes and ensures collection of a sufficient number of events for assessment.

Given the limitations of standard diagnostic methods, quantification of DTCs can be used to assess response to systemic therapy by changes in DTCs levels. However, impact of different modes of chemotherapy on the DTCs levels is yet unclear, and additional assessment of viability of these cells is needed. Our laboratory will continue this research.

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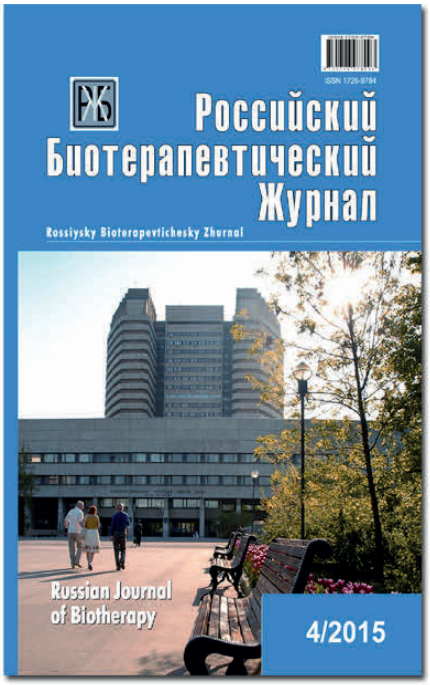

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STATE OF THE ART OF MINIMAL RESIDUAL DISEASE IN ACUTE MYELOID LEUKEMIA

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Minimal Residual Disease (MRD) monitoring is now often used in "risk-adapted" clinical protocols that are crucial for the identification of AML patients with a high risk of relapse. Several studies have demonstrated that MRD is a powerful and independent prognostic factor in AML. The level of minimal residual disease depends on the efficacy of the initial and consolidation treatments and its assessment is now extremely important to decide between post-induction therapeutic options (transplantation versus chemotherapy for instance) or to propose an early intervention in case of relapse.

Technological advances in the laboratory have produced highly sensitive techniques such as multiparametric flow cytometric (MFC) detection of aberrant immunophenotypes and real-time quantitative PCR (RQ-PCR) for leukemia-specific targets. The important improvement of techniques enhancing the sensitivity of MRD detection, e.g. by next-generation sequencing, will lead to a more reliable estimation of total tumor burden. However, the methods of MRD detection are not standardized and thresholds for defining MRD positivity vary depending upon MRD detection methods.

Modern Multiparametric Flow cytometry is an important tool for MRD measuring based on the high-throughput nature of the technique, rapidity and the possibility to well characterize the cells with several multicolor protocols.

The principle is to identify leukaemia-associated phenotypes (LAPs) when leukemic cells differ from the large majority of healthy hematopoietic bone marrow or blood cells. The surrogate immunophenotypes that can be used for LAPs identification can be divided into four different types: cross-lineage expression, when lymphoid cell markers are expressed on the myeloid blasts; overexpression or lack of expression of normally expressed antigens; and finally, asynchronous expression, when immature and mature myeloid markers are expressed together in an aberrant manner.

The evaluation of MRD is performed at different time-points during the AML disease course: at the time of treatment evaluation, pretransplantation and postconsolidation**.** The best method and timing of MRD detection are still discussed.

The majority of studies of MRD are performed in the bulk population. Recently, Schuurhuis et al. [1] have showed that is necessary to study the Leukemia Stem Cells which resides in the $CD34^{+}CD38^{-}$ population and are responsible for the relapse. This population could show aberrant phenotypes.

The drawback of MRD evaluation by MRD is the absence of the antigen imprints associated with LAPs.

New markers are being studying to better differentiate normal from leukemic blasts. The assessment of LSC-MRD can improved the detection of resistant residual cells.

The development of Real-time Quantitative Polymerase Chain Reaction (RQ-PCR) provided many technological advantages compared to other methodologies. RQ-PCR is a highly sensitive technique, integrated to clinical practice in routine laboratory, and with an accessible price. A good molecular target for MRD monitoring must be specific for the leukemic cells, stable to be predictive of relapse and useful for numerous patients. There is no universal molecular marker but several targets are commonly used. In AML, a wide majority of patients, but not all [2] can be monitored by RQ-PCR targeting fusion transcripts, mutated genes or overexpressed genes.

Evaluation of fusion transcripts levels such as BCR-ABL, PML-RARA, or CBFB-MYH11, when applicable, is now included in clinical practice and has proved its relevance. Nucleophosmin (NPM1) gene mutations can be seen in 25–30% of cases of AML and are a good reflect of the disease burden and significantly associated with prognosis. WT1 overexpression can be used for approximately 50% of AMLs and stays a validated and stable molecular target when there is no fusion transcript or NPM mutation.

The next generation sequencing (NGS) technology will certainly increase the number of potential molecular targets, as DNMT3A, CEBPA, and FLT3-ITD. This mutation FLT3-ITD was considered as non-informative for MRD monitoring because a low stability with classical techniques, but is highly predictive of relapse.

MFC and RQ-PCR comparison

Malagola et al [3] retrospectively analyzed 104 newly diagnosed AML patients, consecutively treated and monitored by quantitative polymerase chain reactions (Q-PCR on WT1 and by multiparametric flow cytometry (MFC) on leukemia-associated immunophenotypes (LAIPs) at baseline, after induction, after first consolidation and after first intensification. They showed the benefits of sequential

MRD monitoring with both Q-PCR and MFC. Miyazaki et. al. [4] recently compared the use of FC with quantitative PCR for WT1 and/or leukemia-specific fusion transcripts in 41 acute leukemia patients after allo-SCT [4]. In their analysis of 156 samples they demonstrated good concordance (71.8%) between FC and WT1 PCR based MRD detection, with most of the discordance from samples that were PCR positive for WT1 but negative by FC.

Conclusion

MFC and PCR are the most sensitive techniques methods to study the MRD detection. Several different trials are using MRD evaluation in AML clinical trials. MRD monitoring is important for the identification of AML patients at high risk of relapse, MFC and PCR are useful and complementary techniques.

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Thus CTCs can be developed into novel important type of biomarkers. The potential of using CTCs as biomarkers has been explored in several types of human cancers.

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ACUTE LEUKAEMIAS OF AMBIGUOUS LINEAGE: RARE TYPE OF LEUKAEMIAS

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Keywords. Acute leukaemias of ambiguous lineage, mixed phenotype acute leukaemias, NK-cell lymphoblastic leukemia/lymphoma.

Background. Acute leukaemias of ambiguous lineage (ALAL) are rare type of acute leukaemias. They form a separate chapter in the 2008 WHO classification. ALAL is a heterogeneous group of diseases that includes acute undifferentiated leukaemia (AUL), mixed phenotype acute leukaemias (MPAL), and natural killer (NK)-cell lymphoblastic leukaemia/lymphoma (NK-LL).

Aims. To determine clinical and laboratory features of ALAL.

Methods. In the Laboratory of Immunology Hematopoietic of N.N. Blokhin Russian Cancer Research Center from 2000 to 2014 ALAL was diagnosed in 17 patients of 2864, which amounted to 0.5%. The disease was diagnosed in accordance with the 2008 WHO classification.

Results. There were 13 pts with MPAL and 4 pts with NK-LL. There were no specific clinical predictors of MPAL. In all cases MPAL blast population was morphocytochemical heterogeneous. Among MPAL B/M-immunophenotype was diagnosed in 11 cases (84.6%), T/M-immunophenotype in 2 (15.4%). All pts were characterized by high frequency (92.3%) and the level of expression (75.2%) antigen CD34. In the structure of chromosome aberrations translocation t(9;22)(q34;q11.2) and/or its molecular analog chimeric gene *BCR-ABL* (88.9%) prevailed. Protein p210 and p190 determined with equal frequency. All pts NK-LL stated the total bone marrow blast metaplasia (> 70%), generalized extramedullary lesions. Cytochemical reaction blasts on myeloperoxidase, lipids and nonspecific esterase were negative. In all cases the blast cells were strong positive to CD56 (69.8–99.1%) and the T-associated antigen CD7 (66.2–92%), and negative to myeloid, T- and B-lymphoid antigens. PCR in one patient identified the lack of gene rearrangement chain T-cell receptor. Cytogenetic study was not performed.

Conclusion. There are no specific clinical predictors of ALAL. It is required a complex diagnostic approach to identify ALAL comprising morphocytochemistry, cytogenetic and molecular analyzes and immunophenotyping with a comprehensive panel of monoclonal antibodies. It is necessary to carry out a differential diagnosis NK-LL with other CD56-positive acute leukaemias: blastic plasmacytoid dendritic cell neoplasm, acute myeloid leukaemia with minimal differentiation, T lymphoblastic leukaemia/lymphoma.

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MOLECULAR CHARACTERIZATION OF SINGLE TUMOR CELLS BY NGS

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Distant metastases are the main course of cancer-related death. Disseminated tumor cells (DTCs) extracted from bone marrow and circulating tumor cells (CTCs) extracted from peripheral blood provide a unique source of tumor material. Molecular characterization of individual DTCs and CTCs may contribute to a better understanding of the metastatic spread in cancer patients, role of genetic heterogeneity in therapy resistance, and possible identification of therapy sensitive and resistant clones. Individual tumor cells can be investigated using a combination of whole genome amplification (WGA) and next generation sequencing (NGS). However effectiveness of currently available WGA technologies in combination with NGS and material preservation is still elusive.

Effectiveness of 3 WGA techniques for exome-Seq on 2 NGS platforms on single and pooled tumor cells with and without material fixation for long term preservation was tested. The proof-of-principal experiments were performed on CTCs from metastatic breast cancer patient.

More accurate SNP/mutation calling was demonstrated using single tumor cells obtained from EDTA-collected blood in respect to CellSave-preserved blood, whereas copy number aberration (CNA) analysis in our study was not detectably affected by fixation. Pooling of up to 10 single cells improved SNP/mutation and CNA analyses. Combination of PCR-based WGA with HiSeq2000 demonstrated the best concordance with results obtained from genomic DNA for SNP/mutation analysis. CNA profiles produced with combined MDA-PCR WGA on both HiSeq2000 and IonProton, independent of blood preservative, resembled unamplified DNA the most. Analyzed CTCs demonstrated different CNA profiles, suggesting genetic heterogeneity of this patient's disease. Mutation analysis revealed missense mutations annotated in COSMIC database and present in both CTCs.

We demonstrate the feasibility of single cell genotyping of differently preserved material, nevertheless, WGA and NGS approaches have to be chosen carefully depending on the study aims. Genomic analysis of single DTCs and CTCs could aid companion diagnostics in cancer patients.

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POSSIBILITIES OF OVERCOMING DRUG RESISTANCE OF BLAST CELLS IN CHILDREN WITH RELAPSE OF ACUTE LYMPHOBLASTIC LEUKEMIA

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Relapses of acute lymphoblastic leukemia (ALL) are the main causes of failures and develop in 10–12% of patients, and in 1–2% cases is not achieved remission with the use of modern protocols. Furthermore, not always possible to achieve remission using only chemotherapy. Therefore, search for new drugs overcoming drug resistance in children with relapsed ALL is a topical problem. One of the drugs, which can modify the sensitivity of tumor cells to chemotherapy is bortezomib (Velcade ®).

From June 2011 to December 2015 24 patients with relapsed ALL aged 2–21 years (8.5 years) were enrolled in this study. Boys were 18 (75,0%), girls — 6 (25,0 %). B-cell ALL was in 17 pts (70.9%), T-cell ALL was in 7 (29,1%). First relapse was in 16 (66.7%), initial refractory occurred in 7 cases (29.2%), one (4,1%) child had a second tumor (T-ALL). Isolated extramedullary relapse was in 6 (25,0%) cases, isolated BM in 9 (37.5 %) and combined in 9 (37.5%) children. Relapse localized in: isolated BM — 14 (58.3%), BM and CNS — 3 (12.5%), skin and testes — 1 (4.2%), isolated CNS — 1 (4.2%), CNS and testes — 1 (4.2%). Very early relapse revealed in 3 (12,5%), early in 5 (20,8%) children, late in 9 (37,5%) pts.

Chemotherapy consisted on induction (VCR 1.5 mg/m² N4, DNR 60 mg/m² N1, PEG-ASP 2,500 IU/ m² N4, PRED 40 mg/m² 1–28 days, and bortezomib 1.3 mg/m² N4); two courses of post induction chemo: 1) VP-16–100 mg/m² N5, CPM 440 mg/m² N5, MTX 5000 mg/m² N1, bortezomib 1.3 mg/m² N3 and 2) ARA-C 6000 mg/m² N4, ASP 6000 IU/m² N2. Response after each course estimated by BM results and minimal residual disease (MRD) by immunophenotyping.

Complete remission achieved 20 (83,3%) pts. Complete response (CR) after induction was in 17 (70,8%) children. After the second course CR was in 3 (12,5%) pts. Three (12,4%) pts didn't achieve CR, one (4.2%) died from sepsis on day 23. Evaluation of MRD after the first course of chemotherapy performed in 14 patients (58,3%), and the level of MRD was less than 0.001% in 11 pts (45,8%).

The long-term results: DFS in patients with late relapse was 33.3 +/– 27.2%, follow-up 22 months. In patients with early relapse good results were obtained. DFS in patients with isolated BM was 41,7 ± 30,0% (follow-up 22 months), in combined relapse was 31,3 ± 24,5% (follow-up 12 month).

At present 7 pts (29.2%) are alive in CR. 5 (20.9%) had late isolated BM relapse B-ALL, two pts with late relapse of T-lymphoblastic lymphoma. Four (16.6%) pts with BM relapse underwent a SCT, relapse developed in 2 (13.3%) children, both died.

Thus, the use of bortezomib in combination with standard chemotherapy allowed achieve CR in 83,3% pts. This therapy is more effective for late relapses ALL.

HOSPICELL, A SUBSET OF BONE MARROW STROMAL CELL REGULATE ATP-BINDING CASSETTE GENE EXPRESSION VIA INSULIN-LIKE GROWTH FACTOR-I IN A LEUKEMIA CELL LINE

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The importance of the insulin-like growth factor, IGF, as a signaling axis in cancer development, progression and metastasis is highlighted by its effects on cancer cells, notably proliferation and acquired resistance. The role of the micro-environment within which cancer cells evolve and which mediates this effect is far from clear. Here, the involvement of IGF-I in inducing multidrug resistance in a myeloid leukemia cell line, grown in the presence of bone marrow-derived stromal cells called “Hospicells” (BMH), is demonstrated. We found that i) drug sensitive as well as resistant leukemia cells express IGF-I and its receptor IGF-IR. However, the resistant cells were found to secrete high levels of IGF-I. ii) Presence of exogenous IGF-I promoted cell proliferation, which decreased when an inhibitor of IGF-IR (picropodophyllin, PPP) was added. iii) BMH and IGF-I are both involved in the regulation of genes of the ATP binding cassette (ABC) related to resistance development (MDR1, MRP1, MRP2, MRP3 and BCRP). iv) The levels of ABC gene expression by leukemia cells were found to increase in the presence of increasing numbers of BMH. However, these levels decreased when IGF-IR was inhibited by addition of PPP. v) Co-culture of the drug-sensitive leukemia cells with BMH induced protection against the action of daunorubicin. This chemoresistance was amplified by the presence of IGF-I whereas it decreased when IGF-IR was inhibited. Our results underline the role of microenvironment in concert with the IGF-1 pathway in conferring drug resistance to leukemia cells.

Keywords: IGF signaling pathway, ATP binding cassette, hospicells, chemoresistance, leukemia.

Carmen Aanei

FRENCH MYELOID DATABASE: RELEVANT TOOL FOR ANTICIPATION THE MOST FREQUENT RECURRENT GENETIC ABNORMALITIES AND TO IMPROVE THE ACUTE MYELOID LEUKAEMIA DIAGNOSTIC

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Introduction. Multiparameter flow cytometric immunophenotyping has become the technique of choice for mature lymphoid neoplasms diagnosis and monitoring. Despite the significant benefits provides by this technique that fulfils the requirements for high speed and broad applicability for diagnosis and follow-up of wide range of lymphoid disorders, for myeloid disorders there are still drawbacks related to lack of consensus for panels of antibodies with high specificity and sensitivity for diagnostic assessment and prognostic stratification. Alongside, standardization on sample preparation, instrument setup protocols, fluorescence compensations and data interpretation across institutions are needed.

Aims. The aim of this study is to evaluate the potential of an immunophenotypic myeloid database for identification of surrogate markers of genetic aberrations, which could improve the AML diagnostic.

Methods. The database is an archive of cell-surface antigen expression profiles representative for normal and malignant haematopoiesis. The database of normal haematopoiesis was constructed with twenty samples of normal bone marrows from healthy donors of allogeneic bone marrow transplants, without myeloid growth factor priming prior to bone marrow harvesting. Informed consent was obtained from all donors prior to the study. The data were acquired on three different centres from Rhône-Alpes Auvergne region under the standardised conditions set by France Flow Clinical Cytometry Group. Immunostaining was performed after erythrocyte lysis using the 8-color antibody panel according to the EuroFlow consortium's guideline (JJM van Dongen et al., Leukemia 2012). As already referred, the strategy of data analysis consist in separately analysis of the $CD34^{+/-}$ $CD117^{+}$immature committed precursors from more mature myeloid cells (S Matarraz et al., Leukemia 2008; Paula Laranjeira et al., Flow Cytometry — Recent Perspectives: InTech 2012). In addition, data from distinct subtypes of human acute myeloid leukemia (AML) was included in a database allowing to directly comparing antigens expression of leukemic cells with those of their closest normal counterpart. Furthermore, the comparison of patterns of expression was performed in bulk bone marrow of the three karyotypes of AML. The Compass database was constructed with five AML t(8;21), eight AML with t(15;17), six AML with inv(16)/t(16;16). Finally, a blind analysis of five previously acquired files and nine new cases were tested to determine the reliability of the Compass database for anticipation of the most frequent recurrent genetic abnormalities.

Results. Initial results showed that the $CD34^+$ and $CD34^-/CD117^+$ neutrophil precursors are the most useful for building a myeloid database, proving remarkable applicability and outstanding reproducibility for anticipation of the most frequent recurrent genetic abnormalities. No differences of reproducibility were observed between the two types of precursors and the most discriminative markers are: FSC, SSC, HLA-DR, CD117, CD13 and CD11b.

Conclusion. The Myeloid Database is a comprehensive visualization interface that can make it useful as a daily tool for pathologists and cancer researchers to expand repertoire of testing capability for diagnostic, prognostic and therapeutic applications of myeloid leukemic malignancies.

Alex Chenchik, Director of R&D, Cellecta, Inc.

CANCER TISSUE MICROENVIRONMENT BIOMARKER PROFILING ASSAY

Abstract

Robust methods for molecular characterizations of the immune mechanism and cell composition in the tumor microenvironment, lymph node and bone marrow are essential to meet the imminent need for diagnostic approaches. Toward this goal, we developed the TME 3000 assay based on multiplex RT-PCR amplification followed by Next-Generation Sequencing for profiling cellular composition and discovery of novel prognostic and predictive immune response biomarkers. The TME 3000 panel includes 1,000 key cancer genes, 500 immune mechanism genes and a comprehensive set of 1,700 genes specific for profiling in the tissue microenvironment of different types of activated immune cells of adaptive and innate immunity, cancer, stromal, fibroblast, endothelial, epithelial and adipose cell types. The TME 3000 assay quantitatively profiles the 5 orders of magnitude variation of expression levels of approximately 3,000 genes from 10–100ng of total RNA from as few as 100 cells from frozen biopsies, and PBMC. We will present profiling data from infiltrating immune cells and key intact and deficient immune mechanisms in the tumor microenvironment of breast cancer samples to demonstrate the performance and utility of the TME 3000 assay.

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COMPARATIVE ANALYSIS OF MULTICOLOR FLOW CYTOMETRY AND IMMUNOHISTOCHEMISTRY FOR THE DETECTION OF DISSEMINATED TUMOUR CELLS

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Tumour cell detection in bone marrow is important in staging of malignant diseases and also in therapy planning. Disseminating cells of a primary solid tumour may represent the origin of metastases and relapses. We compared the diagnostic efficacy of multicolour flow cytometry (MFC) and morphology/immunohistochemistry (IHC) in the detection of disseminated tumour cells (DTC) in the bone marrow and body fluids of patients with solid tumours and in pediatric neuroblastoma cases.

We investigated 72 samples retrospecively from 50 patients by MFC. Morphology/immunohistochemistry data were available in 48 cases. In the first cohort, 36 samples derived from 34 patients with various forms of suspected and proven solid tumours, in the second cohort, 36 samples of 16 children with neuroblastoma were analysed at diagnosis and/or during follow-up in a four-colour setting by MFC and results were compared to IHC. In the group of various solid tumours we found 91% concordance between IHC and MFC and it was 65% in the neuroblastoma group, and 77% overall. Detection of DTCs was found to be more effective by MFC in de novo neuroblastoma samples (100% vs. 86%). The advantage of MFC was even more pronounced when minimal residual disease (MRD) was evaluated (efficacy 92% vs. 68%). In contrast, efficacy of IHC was 100% in the group of various solid tumours while it was 91% for MFC.

We conclude that MFC and IHC are both essential tools for examining infiltration of bone marrow and body fluids by disseminating solid tumour cells. In the case of neuroblastoma however, MRD detection by MFC in a hypoplastic/aplastic bone marrow environment is more effective than IHC, since considerably more cells can be analysed.

G. Petrova, K. Melkova, T. Chernyavskaya

PRIMED BONE MARROW AS AN ALTERNATIVE SOURCE OF HEMATOPOIETIC STEM CELLS FOR AUTOLOGOUS TRANSPLANTATION IN PATIENTS WITH HODGKIN'S LYMPHOMA

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Background Bone marrow (BM) and peripheral blood stem cells (PBCS) as well as their combination are currently used for autologous transplantation of hematopoietic stem cells (AHSCT). Primed BM (pBM), which is used in some European and Russian clinics, is a relatively new source of hematopoietic stem cells (HSC). The aim of our study was to investigate the influence of the sources of HSC on engraftment and determine the long-term results of AHSCT in patients with Hodgkin's Lymphoma (HL).

Methods We collected data of 90 AHSCT (75 initial and 15 repeated) which were performed between 2007 and 2014 in patients with HL. More than half (54%) of the participants were women. Median age was 27 (16–49) years. Among the used conditioning regimens were the following: BEAM (82%), MITO/L-PAM (10%), ICE (8%). The sources of HSC were PBSC (43%), pBM (29%) and their combination PBSC + pBM (28%). Exfusion of pBM was performed at the inefficient procurement of PBSC and a high risk of ineffective mobilization. The median number of transfused HSC for one transplantation was: at PBSC — CD34+cells 3 × 106/kg (2,09–14,43). The main characteristics of pBM were: NCS — 3,2 (2,09–4,8) × 108/kg, CD34+—cells — 2,04 (0,46–6,5) × 106/kg, CD3+—cells — 0,9 (0,2–2,43) × 107/kg, CD4+—cells — 0,38 (0,14–1,4)×107/kg, CFU–GM — 37,25 (29–45,5)×104/kg.

Results Successful sustained engraftment was noticed in all patients. In the whole group median days to neutrophil engraftment was 13 (8–30), platelet engraftment — 12 (7–39). The transplant-related mortality (TRM) was 3%. The 5-year overall survival (OS) rate was 48%. Median days to neutrophil engraftment in the PBSC, pBM and PBSC+ pBM groups were 12 (9–22), 18 (8–30) and 12 (8–30) days respectively. Median days to platelet engraftment in the PBSC, pBM and PBSC + pBM groups were 11 (7–33), 16 (9–39) and 12 (8–37) days respectively. The speed of neutrophil and platelet engraftment was significantly higher in PBSC transfusion ($p = 0,0002$ and $p = 0,00002$ respectively) than in pBM. The use of PBSC + pBM strongly correlated with the speed of neutrofil engraftment in PBSC transfusion ($p = 0,4$).

However, it took longer time for the platelet engraftment ($p = 0,01$). Rates of 5-year OS in the PBSC, pBM and PBSC + pBM groups were 40%, 54% and 58% respectively and were not statistically different between the groups.

Conclusion In comparison to PBSC outcomes of transplantation, pBM or a combination of PBSC + pBM could be acceptable alternative source of HSCs at ASCT in patients with HL.

Ekaterina S. Kolotova.

MECHANISMS
OF CHRONIC MYELOGENOUS LEUKEMIA CELL DEATH
INDUCED BY A NEW INHIBITOR OF BCR-ABL TYROSINE KINASE

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The genomic rearrangement resulted in the formation of the chimeric tyrosine kinase Bcr-Abl is considered a major mechanism in pathogenesis of chronic myelogenous leukemia (CML). Pharmacological inhibition of Bcr-Abl with Imatinib (Gleevec) has been proved as an exceptionally successful example of targeted chemotherapy. However, mutations in the kinase domain of Bcr-Abl hamper the efficacy of inhibitors. The group led by Dr. G. Chilov at Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences developed an inhibitor of wild type and mutant Bcr-Abl forms (compound PF-114) and demonstrated its therapeutic efficacy, safety and good pharmacokinetic properties in murine models (*Leukemia* 2015). PF-114 was potent against CML cells carrying T315I mutation; moreover, this compound showed more specific kinase inhibitory profile than FDA approved 3d generation drug ponatinib.

Studies of molecular mechanisms of CML cell death by PF-114 revealed its nanomolar potency whereas leukemia cells that lack the chimeric tyrosine kinase were substantially less susceptible. Phosphorylation of CrkL adaptor protein, a downstream Bcr-Abl substrate, was blocked by nanomolar concentrations of PF-114 within 2 h but was restored within 20 h after the drug was washed off. CML cell death was preceded by dephosphorylation of ERK1/2 and Akt and G1 arrest followed by (or concomitant with) a decrease of phospho-BAD, loss of mitochondrial transmembrane potential, increase of Annexin V reactivity, activation of effector caspases and PARP cleavage. These apoptotic events converged to internucleosomal DNA fragmentation. Interestingly, a decrease of phospho-STAT5, a known marker of various modes of cell death, was reciprocally associated with simultaneous increase of phospho-STAT3 in PF-114 treated cells. One may hypothesize that the latter phenomenon might reflect cell defense since STAT3 phosphorylation can trigger anti-apoptotic gene expression. Altogether, our studies demonstrated that PF-114, the newly developed inhibitor of wild type and panmutation Bcr-Abl is a potent inducer of apoptosis in CML cells. The cell type specific mode of cytotoxicity, that is, preferential kill of Bcr-Abl-positive cells, along with favorable preclinical characteristics make PF-114 a perspective candidate for clinical trials.

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PROGNOSTIC BIOMARKERS FOR ADVERSE EVENTS IN PATIENTS TREATED BY INHIBITORS OF IMMUNE CHECKPOINTS

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A major problem with cancer treatment by immune checkpoint inhibitors ipilimumab (anti-CTLA4 monoclonal antibody (mAb)) and nivolumab (anti-PD1 mAb) is developing severe autoimmune adverse events. Recognizing factors that lead to these reactions may help to reduce unfavorable autoimmune effects and improve effectiveness of these newly established therapies. The study aimed at evaluating immune parameters in patients treated by anti-CTLA4 and anti-PD1 mAbs to assess their predisposition to autoimmune processes.

Materials and methods. Pleural effusion of patients with melanoma and NSCLC receiving immune checkpoint mAb immunotherapy (n = 4) and that of non-treated patients (n = 3) was evaluated for IgG and IgM (ELISA (Hycult Biotech, The Netherlands). Immunophenotype of mononuclear cells (MNC) derived from peripheral blood and effusion by Ficoll-gradient technique were analyzed by flow cytometry (mAb: CD45, CD3, CD8, CD4, CD56, CD 25, CD16, CD38, CD14, HLA-DR (BD Biosiences, USA; Beckman Canto II).

Results. The elevated levels of IgG in blood (46–160 mg/ml) and effusion (58 mg/ml) were registered in patients treated by inhibitors of immune checkpoints (standard IgG 5.6–16.5 mg/ml), but not in non-treated patients. No significant alteration was seen in IgM levels. Increased number (up to 20%) and higher expression of NK cells ($CD16^+CD56^+$) was determined in MNCs of the treated patients as compared to those of non-treated (less than 0.8). No increase of Tregs ($CD4^+/CD25^+$) was noticed in treated patients (0–2.0%), while non-treated showed different numbers (0–12.9%).

Conclusion. Further research is necessary to reveal specific biomarkers that may predict development of severe immune-related adverse events in patients treated by inhibitors of immune checkpoints.

Prashant Kumar, Ph.D.

EXPANSION AND CHARACTERIZATION OF BREAST CIRCULATING CANCER CELLS PREDICTS RESPONSE TO ANTI-CANCER THERAPY

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Abstract

Circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) are recognized for their potential utility in disease monitoring and therapeutic targeting. It is one of the most promising areas of cancer research for guiding patient treatment and predicting cancer progression. However, little is known about the biology of these cells, mostly because as today no reliable method to enrich significant amounts of CTCs. The copious technical approaches developed for CTCs detection have thus far failed to retrieve an adequate number of viable cells for culture and characterization. CTCs identification and characterization require extremely sensitive and specific methods. Consequently, there is an urgent need for techniques that can successfully expand CTCs. We have devised a microwell-based culture method to assess breast cancer CTCs from patients undergoing neoadjuvant therapy without the need for prior enrichment. Our culture system enabled us to perform various characterizations with primary cells after a short-term culture of 14 days. Within the nucleated blood cell fraction, most cells and endothelial cells are progressively eliminated from the culture over time, allowing for the selective enrichment of CTCs, which go on to form proliferative clusters. Furthermore, the cluster formation by these cells may provide a unique and predictive method for treatment response and efficacy. This new technique provides an opportunity to analyze CTC clonal heterogeneity and adapt therapeutic modalities in refractory breast cancer patients which may help determine the efficacy of selected therapeutic regimes.

Gerrit J Schuurhuis

MINIMAL RESIDUAL DISEASE IN AML AND THE ROLE OF LEUKEMIA STEM CELLS HEREIN

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Minimal residual disease in acute myeloid leukaemia has been shown in many studies to be an independent post-diagnosis prognostic factor, also providing information in cytogenetically normal cases. Flow cytometry is mostly used since it allows to define aberrant immune-phenotypes (Leukaemia Associated Immune-Phenotypes, LAIP) in approximately 90% of the patients, whereas molecular methods are applicable in roughly about half of the patients. Independent prognostic information can be obtained both after induction therapy and consolidation therapy. MRD information obtained after induction therapy has started to be used in clinical studies to re-define, together with prognostic factors mainly assessed at diagnosis, risk for individual patients. Based on that, patients may be re-directed from one therapy to another, e.g. in the HOVON/SAKK consortium, patients with intermediate cytogenetic/molecular risk planned for autologous transplantation with high MRD will now undergo allogeneic transplantation. MRD is also increasingly used to monitor outcome in allogeneic transplantation, not only to define patients at high risk prior to transplantation and following transplantation, but also to plan immune-suppression and to guide the time point of donor lymphocyte infusions.

When using MRD at a fixed time point, one important pitfall in the prognostic impact is the occurrence of MRDlow/negative patients who nevertheless experience a relapse. This percentage may amount to 20% — 40% of the patients. Possible explanations are: 1) specificity of immune-phenotypic aberrancies and thereby of sensitivity of detection of the specific events is dependent on the type of aberrancies. For flowcytometry the maximally achieved sensitivity for specific events is hereby 1:10 000 to 1:100 000 occasionally, but in between 1:1 000 and 1:10 000 for most cases. MRD negative is defined as MRD below a certain threshold value that has, for reasons of easiness of clinical application, mostly been chosen to be the same for all aberrancies (often 0.1% of WBC). As a result, patients with low but clearly present MRD will not be identified as patients at risk of relapse. This is, however, not a problem specific for flow cytometry since molecular MRD with the highly sensitive and specific target NPM1 shows similar false negatives; 2) a problem identified already decades ago for flow cytometry, is the occurrence of “immune-phenotype shifts”, i.e. at relapse particular aberrancies defined at diagnosis and used to detect MRD at follow up, have disappeared, while, at the same time, new aberrancies may have emerged. Nowadays it is known that particular mutations like FLT3ITDs may come and go, and since such mutations can often be associated with particular immune-phenotypes, there maybe is a link between the immune-phenotype shifts and molecular/mutational shifts; 3) it should be realized that MRD assessment at a fixed time

point, e.g. after induction therapy ignores possible differences between patients in onset of MRD growth even when starting with similar MRD burden. To account for differences in kinetics that may explain false-negativity of MRD in part of the cases, multiple time points should preferably be applied.

Efforts have been and are made to circumvent these three problems. Ad1). As argued above, with cut-offs like 0.1%, the possible relevance of specific events below 0.1% is under-estimated. To circumvent this, an alternative is to define all specific events as MRD positive, irrespective their frequencies. This approach has not extensively been validated, however. Other approaches include to involve percentages malignancy on the primitive (CD34, CD133, CD117) compartment. For molecular MRD there are no real options to increase the already high sensitivity.

Ad2) originally, in the absence of information on diagnosis aberrancies, the so-called "different-from-normal" approach was applied: an un-biased assessment of aberrant differentiation patterns under MRD conditions. Such approach can also be applied to the LAIP approach: instead of studying LAIPs at diagnosis and searching for these at follow up, additional LAIPs not present at diagnosis may be searched for at follow up by using total MRD panels.

Ad3) Presently changes of therapy are restricted to changes from chemotherapy or autologous transplantation to allogeneic transplantation. To plan such, at time of diagnosis unforeseen, transplantation, it should be realized that, for logistic reasons (time necessary to plan an allogeneic transplantation), the last possible time point of MRD detection is after induction therapy. For that reason, alternative parameters additional to MRD, that possibly reflect differences in onset of MRD growth, and to be performed not later than after induction therapy, are warranted. The leukemia stem cell (LSC) frequency is such a parameter, that identifies part of MRDlow but nevertheless relapsing patients. Maybe in future, sequential MRD assessment beyond the point of end of induction therapy, will be opportune if new therapies have emerged that are applicable as (part of) consolidation treatment. In such cases it may also be of importance to consider sequential MRD assessment in peripheral blood, being less burden to patient and physician.

A pitfall additional to the problems with MRD false negative patients, is the continuous remission of patients with relatively high levels of MRD. Although not particularly described for immunophenotypic MRD, it is known that molecular aberrancies, such as t(8;21), as well as so-called founder mutations may persist for a long time, likely as part of pre-leukaemic clones, thereby contributing to false-positivity. Leukemia stem cells help to define relapsing patients in part of cases with MRD false-positivity, by identifying, within the group of MRD positive non-relapsing patients, a MRD^{high}/LSC^{high} sub-group of relapsing patients. Future perspectives include efforts are underway to use more objective analysis of flow cytometric patterns, using particular approaches like SPADE and viSNE. In addition, efforts are ongoing to investigate the possible future role in flow cytometric MRD of multi-dimensional CyTOF approach and in molecular MRD of next generation sequencing.

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INFLUENCE OF *C-MYC* HYPEREXPRESSION AND *RAS* GENES MUTATIONS ON THE DISEASE PROGNOSIS AND THERAPY RESPONSE IN MULTIPLE MYELOMA PATIENTS

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Multiple myeloma (MM) treatment is complicated due to the dissimilarity in therapy resistance among tumor clones even within patient. MM cells are characterized by the function defects in signaling pathway proteins such as KRAS, NRAS, and c-myc. The number of publications reveals the importance of these genes changes for tumor progression. However it remains unclear what specific changes might be involved in tumor therapy resistance. This study is conducted to define the influence of *RAS* genes mutations in the, and of *c-MYC* expression level on the therapy response and multiple myeloma prognoses.

Results: The total incidence of *c-MYC* hyperexpression in $CD138^+$ bone marrow cells in patients reached 75% of the samples analyzed (16 patients). *C-MYC* average value for MM patients is 55,014 ($2^{\Delta Ct}$) and for donors — 7,183 ($2^{\Delta Ct}$). The sequencing analysis of *RAS* genes showed that 10 MM patients got *n-RAS* mutation in and 4 patients got mutations for *k-RAS* genes out of 31 patients. Two mutations out of 10 in n-*RAS* were found in codon number 13, the rest is in 61 codon. *K-RAS* gene is mutated at the codons 13 and 61, two rare mutations were found in the codons 19 and 64. The combination of *c-MYC* overexpression and *RAS* mutations was investigated in 5 patients. The therapy of 4 patients resulted in antitumor response, one case was therapy resistant. The survival after therapy was 40% with *c-MYC* hyperexpression and *RAS* mutations cases versus 88,9% for the patients where mutations and hyperexpression were not found.

Conclusion: *c-MYC* level expression is higher in MM patients 138^+ cells than among healthy donors ($p = 0,011$). We suppose that simultaneous c-MYC activation and mutations in *RAS* gene are an adverse prognostic factor for patients with MM. However, due to the small sample size and limited observation period, these differences were statistically insignificant.

Shyam Sushama Jose

ROLE OF TELOMERASE MUTATION ON MYELODYSPLASTIC SYNDROME AND OTHER HAEMATOPOIETIC DISORDERS

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Telomeres are DNA-protein structures essential for maintenance of genomic integrity, characterised by TTAGGG repeats at the ends of chromosomes, protecting them from degradation and fusion.

Telomere length has been shown to gradually shorten over time as cells divide and enter the state of senescence. There are evidences of short telomere length association with cancer; specifically in Myelodysplastic Syndrome (MDS) but also for bladder, esophageal, gastric, and renal cancers. Genetic mutations in telomere length control result in disorders including altered haematopoiesis and reduced myeloid subset differentiation.

The exact cellular or molecular mechanism leading to this pathology remains unknown. Stem cells including induced pluripotent stem cells (iPSCs) have increased telomere lengths as high as 2000 kb/diploid genome compared to 400–500 kb/diploid genome, making them a suitable study platform for telomere biology. We have designed a Telomerase-loss of function model by silencing the telomerase RNA component (TERC), a mandatory subunit of human Telomerase in a line of human induced pluripotent stem cells (wild type iPSCs). A significant alteration in the TERC level upon silencing can be found by qPCR.

Both the silenced and wild type iPS cells are differentiated in vitro and their haematopoietic capacity measured as development of $CD34^{+}CD45^{+}$ is compared using flow cytometry. The HSCs are further induced to myeloid lineage to understand the influence of telomerase activity over immune cell differentiation and functionality. The TERC-silenced iPSCs are also checked for normalization of telomere length and hematopoietic potential by an overexpression vector of TERC, which gives an insight to serve as a reversible telomerase-loss of function model for MDS and also other tumours having characteristic telomere shortening. This iPSCs based disease model helps us to understand the biology behind the disease and provide a major tool for investigating future therapies for patients with bone marrow failure due to defined telomerase deficiencies.

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SELECTION Of DNA-APTAMERS To HUMAN INTERLEUKIN-6 (hIL6)

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Interleukin-6 belongs to the group of proteins which are related to cellular receptor gp130. They have rather pleoitropic effects on the cells including B cell differentiation and hepatocyte stimulation. In biomedical research hIl6 is consider to be a factor of aggression for the cancer, multiple myeloma; where human life span has a reverse correlation with blood concentration of hIL6. Therefore, it is very challenging to search for molecules able to bind hIL6 specifically.

The systematic evolution of ligands by exponential enrichment (SELEX) process is a combinatorial chemistry method for the isolation of nucleic acid ligands (aptamers) that bind to a desired target molecule with high affinity. Recombinant hIL6 was immobilized by CM5 chip. For four rounds of DNA combinatorial library selection to hIL6 we were isolated a pool of aptamers using surface plasmon resonance method, that was converted to double-strand DNA, amplified by PCR and subcloned into a pUC 19 with the SmaI restriction site. Individual clones were sequenced by the dideoxy method using standard protocols and method. Sequences were aligned relative to the consensus sequences. Some of them had high affinity to IL6. One of the aptamers turned out to be able binding hIL6 with apparent dissociation constant 0.31 ± 0.03 μM.

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IMMUNOCHEMOTHERAPY WITH RITUXIMAB FOR PEDIATRIC PATIENTS WITH 3–4 BURKITT LYMPHOMA RISK GROUP

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Progress in Burkitt lymphoma (BL) treatment was associated with not only chemotherapy intensification, supportive care improvement but also new drug use. One of such drugs — rituximab (anti-CD20 monoclonal antibody). We present rituximab experience for pediatric pts with advanced (3–4) BL risk groups.

From 2006 to 2016 forty-five primary pts with BL were enrolled. Median age was 8,6 years.

All pts had 3–4 risk groups by BFM stratification and treated by B-NHL-BFM 95protocol with rituximab. Dose of rituximab was 375 mg/m2 in day 0 of A and BB blocks. If complete response (CR) was achieved after two blocks (A+rituximab and BB+rituximab) the following treatment included CC, AA, BB. For pts who were not in CR after two blocks with rituximab the following treatment was by standard B-NHL-BFM 95 protocol with rituximab in day 0 of each block.

Forty-four (97,8%) pts achieved CR, one case of primary BL resistance was associated with atypical c-MYC rearrangement. 8-year event-free survival, relapse-free survival, and overall survival were 96,5+/–1,7%. Rituximab was well tolerated and helped reduce methotrexate dose in first chemotherapy block from 5000 mg/m2 to 1000 mg/m2 without decrease of survival level. Besides, total number of blocks were reduced from 6 to 5 for pts with 4 risk group and early (after 2 blocks) CR.

We conclude that rituximab+B-NHL-BFM 95 protocol is feasible and highly effective in the treatment of advanced (3–4) BL risk groups. It improved survival in unfavorable risk group of pts and reduced chemotherapy in early responders.

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ANTIGEN EXPRESSION HETEROGENICITY USED FOR MRD MONITORING IN B-LYMPHOBLASTIC LEUKEMIA

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Identification of minimal residual disease (MRD) consists of leukemic cells detection among the normal bone marrow precursors. In this study we analyzed CD34, CD20, CD10, CD58 antigens expression used for MRD monitoring on the B-lymphoblasts of 68 untreated patients with B-ALL. In the study results didn't present antigen expression in some samples; in positive expression samples some cells expressed antigens homogeneously; in some tests were revealed positive as well as negative blasts simultaneously (Table). During the carrying out of multiple staining with the other antigens (T-lymphoid, myeloid, activation) were observed different cell distribution in tumor population with positive expression CD10, CD34, CD58, CD20. There were revealed sub-populations with different phenotypes: bright/bright, bright/dim, dim/dim.

Table

Expression of antigens in patients with B-ALL

Antigen	Absence of expression (n)	$^-$expression		
		Presence of $^{+}/^{-}$ cells (n)	Homogeneous expression (n)	Distribution heterogenicity in double staining (%)
CD10	6	62		
		12	50	4
CD34	18	50		
		14	36	14
CD58	1	67		
		13	54	67
CD20	41	27		
		9	18	46

Analysis of antigen CD10, CD34, CD58, CD20 expression by tumor cells showed that in majority of samples in presence of homogeneous co-expression with the standard "anchor" diagnostic markers ($CD10^+/CD19^+$, $CD34^+/HLA\text{-}DR^+$), at least one antigen among CD10, CD34, CD58 or CD20 distributed heterogeneously in population of B-lymphoblasts. The achieved result allowed to conclude that population of B-lymphoid tumor cells at the disease beginning is heterogeneous; there are sub-populations with various physical and functional characteristics. This fact should be considered by MRD monitoring with multicolored antibody combinations, and control populations with nonstandard phenotype.

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FRIDAY JUNE 3RD

08.50 **Welcome address.**
Prof George Janossy, Prof Janos Kappelmayer, Prof Klaus Pantel, Prof Lydia Campos, Prof Jean-Francois Rossi, Prof Bruno Brando & Dr Catherine Alix-Panabières

09.10 Who should be Involved in harmonizing MRD detection on solid tumours in PB and BM. *Andy Rawstron, Leeds, United Kingdom*

09.50 MRD IN ONCOHAEMATOLOGY

Chairman: Prof Lydia Campos

09.50 State of the art in acute myeloid leukemia minimal residual disease assessment. *Prof Lydia Campos, Saint-Étienne, France*

10.10 NGS for upfront characterization and assessment of treatment response in AML and MDS. *Prof Christian Thiede, Dresden, Germany*

10.30 Minimal residual disease in AML and the role of leukemia stem cells herein. *Prof Gerrit Jan Schuurhuis, Amsterdam, The Netherlands*

10.50 Coffee break

11.20 French Myeloid Database: relevant tool for anticipation the most frequent recurrent genetic abnormalities and to improve the acute myeloid leukaemia diagnostic. *Dr Carmen Aanei, Saint-Étienne, France*

11.40 MINIMAL CANCER

Chairmen: Prof Klaus Pantel & Dr Catherine Alix-Panabières

11.40 Circulating and disseminated tumor cells: biology and clinical significance. *Prof Klaus Pantel, Hamburg, Germany*

12.10 Methods of CTC/DTC viability and function assessment. *Dr Catherine Alix-Panabières, Montpellier, France*

12.40 CytoQuest™ CR – Circulating rare cells positive enrichment and retrieval system. *Michaela Adamcová, Brno, Czech Republic*

12.50 Molecular characterization of single tumor cells by NGS. *Anna Babayan, Hamburg, Germany*

13.00 Comparative analysis of multicolor flow cytometry and immunohistochemistry for the detection of disseminated tumour cells. *Eszter Szánthó, Debrecen, Hungary*

13.10 Selected poster presentations:

13.10 Selection of DNA aptamers to human Interleukin-6 (hIL6). *Vera A. Spiridonova, Moscow, Russia*

13.15 Influence of c-MYC hyperexpression and RAS genes mutations on the disease prognosis and therapy response in multiple myeloma patients. *M. V. Nareyko, E. Y. Demidova, A. M. Sergeeva, V. L. Surin, L. P. Mendeleeva, Moscow, Russia*

13.20 Lunch

14.30 New data on DTC detection and monitoring by flow cytometry. *Olga Beznos, Moscow, Russia*

15.00 Flow Cytometry for the identification of rare cells. *Sara de Biasi, Modena, Italy*

15.30 IMMUNE THERAPY OF MRD

Chairmen: Prof Jean-Francois Rossi, Katherina Psarra & Prof Bruno Brando

15.30 Myeloid suppressor cells in hematological malignancies. *Katherina Psarra, Athens, Greece*

16.00 Myeloma and immune system: dangerous cross-roads. *Prof Bruno Brando, Legnano, Italy*

16.30 Coffee break

17.00 New frontiers in immunopathology: from dysimmune diseases to lymphoid malignancies. *Prof Jean-Francois Rossi, Montpellier, France*

17.30 Targets for immune therapy of minimal residual cancer. *Dr Alisa Petkevich, Moscow, Russia*

17.40 Mechanisms of chronic myelogenous leukemia cell death induced by a new inhibitor of Bcr-Abl tyrosine kinase. *Dr Ekaterina Kolotova, Moscow, Russia*

17.50 Cancer tissue microenvironment biomarker profiling assay. *Alex Chenchik, Cellecta*

20.00 Evening in Budapest Operetta

SATURDAY JUNE 4TH

09.00 ALLOGENEIC BONE MARROW IN CANCER TREATMENT

*Chairman: Prof Suleyman Dincer, Ankara, Turkey **

09.00 Primed bone marrow as an alternative source of hematopoietic stem cells for autologous transplantation in patients with Hodgkin's Lymphoma. *Galina Petrova, Moscow, Russia*

Round table

09.20 How to make accessible for practice new diagnostic approaches created by science. *WHO experts*

Seminars

09.30 *Chairman: Dr Yana I. Gurkovskaya & Dr Farkhad Shamilov, Petrovax*
Influence of polyoxidonium (immune adjuvant) on primary tumor and bone marrow in breast cancer patients.

10.00 *Chairman: Dr Maria.E. Ilatovskaya*
Perspectives of CTC and DTC HER2 detection for therapeutic targeting: questions and concerns.

10.30 Coffee break

11.00 *Chairman: Prof Nikolay N.Tupitsyn, Moscow, Russia & Lubov Kuprova, Bioline*
8-color Euroflow panels in MRD diagnosis in leukemias and lymphomas.

11.30 *Chairmen: Prof Piarvin A. Zeynalova, Prof Timur T. Valiev & Mobil Akhmedov*
CD20 in B-cell lymphomas: diagnosis and treatment of MRD.

** To be confirmed.*

11.50 YOUNG SCIENTIST SELECTED ORAL PRESENTATIONS

11.50 Immunochemotherapy with rituximab for pediatric patients with 3-4 Burkitt lymphoma risk group. *Timur Valiev, Moscow, Russia*

12.00 Antigen expression heterogenicity used for MRD monitoring in B-lymphoblastic leukemia. *Kateryna Vilchevska, Donetsk, Ukraine*

12.10 Possibilities of overcoming drug resistance of blast cells in children with relapse of acute lymphoblastic leukemia. *Natalia Batmanova, Moscow, Russia*

12.20 Hospicell, a subset of bone marrow stromal cell regulate ATP-binding cassette gene expression via insulin-like growth factor-I in a leukemia cell line. *Nadia Benabou, Paris, France*

12.30 Expansion and characterization of breast circulating cancer cells predicts response to anti-cancer therapy. *Prashant Kumar, India*

12.40 Role of telomerase mutation on myelodysplastic syndrome and other haematopoietic disorders. *Sushama Jose Shyam, Brno, Czech Republic*

12.50 Acute leukaemias of ambiguous lineage: rare type of leukaemias. *Alina Antipova, Russia*

13.00 **Take home message.**
End of the congress.

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HAEMATOPOIESIS IMMUNOLOGY