VILNIUS UNIVERSITY

Jelena Rascon

CHIMERISM ANALYSIS IN ISOLATED CELL POPULATIONS AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION IN CHILDREN

Summary of doctoral thesis

Biomedical sciences, medicine (07 B)

Vilnius, 2009

The doctoral thesis was initiated in 2002 – 2006 at Humboldt University, Berlin.

Supervisor: Prof. Dr. Habil. Gerhard Gaedicke (Humboldt University, Berlin, biomedical sciences, medicine – 07 B).

The doctoral thesis was completed during 2007 – 2009 at Vilnius University.

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VILNIAUS UNIVERSITETAS

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CHIMERIZMO ANALIZĖ ATSKIROSE LĄSTELIŲ POPULIACIJOSE PO ALOGENINĖS KRAUJODAROS KAMIENINIŲ LĄSTELIŲ TRANSPLANTACIJOS VAIKAMS

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ABBREVIATIONS

ALD	adrenoleukodystrophy
ALL	acute lymphoblastic leukemia
ATG	antithymocyte globulin
Bu	busulfan
CD3	T lymphocytes
CD19	B lymphocytes
CD34	hematopoietic stem cells
CI	confidence interval
CR	complete remission
DC	donor chimerism
DNA	deoxyribonucleic acid
EFS	event-free survival
FA	Fanconi anemia
Flu	fludarabin
GvHD	graft-versus-host disease
HSCT	hematopoietic stem cell transplantation
ICP	isolated cell populations
MC	mixed chimerism
MMRD	mismatched related donor
MRD	matched related donor
MUD	matched unrelated donor
OKT3	muromonab
OS	overall survival
PCR	polymerase chain reaction
RFS	relapse-free survival
SD	standard deviation
STR	short tandem repeats
TBI	total body irradiation
TRM	transplant related mortality
WBC	whole blood cells

CONTENTS

1. INTRODUCTION

1.1. BACKGROUND

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative treatment option for several otherwise untreatable disorders. Success of this potentially harmful procedure depends on various issues such as graft-versus-host disease (GvHD), transplant related mortality (TRM) leukemic relapse and others. The interference of these events determines long-term survival after HSCT or treatment failure. Close surveillance of post-transplant course and early diagnostics of potential complications is very important to ensure favorable transplant outcome.

Monitoring of chimerism status provides a sensitive tool to assess quality of engraftment, the risk of GvHD, imminent malignant recurrence or transplant rejection. Sensitivity and reliability of chimerism measurements depend significantly on the methodic used. Nowadays PCR based analysis of DNA polymorphism (short tandem repeats, STR) is used for practical purpose by most of laboratories. Sensitivity of this approach reaches $1–5\times10^{-2}$. Lineage-specific chimerism analysis in isolated cell populations (ICP) may increase the sensitivity up to $10^{-3}–10^{-4}$.

There are a lot of publications dealing with chimerism analysis. Most of them evaluate chimerism in patients with malignant disorders. Clinical studies demonstrate possible predictive value of chimerism analysis in ICP for early diagnostic of leukemic relapse and assessment of the risk of GvHD. However most of the reported recipients suffered from different types of leukemia, treated with different conditioning regimens. Moreover, usually only one cell population was investigated. There was no comparative analysis of chimeric state in whole blood cells (WBC) and ICP. As a consequence it remains unclear whether lineage-specific analysis in leukemic patient brings real benefits for early relapse diagnostic or eventual prevention of malignant recurrence. In addition there is limited number of studies analyzing pediatric patients.

Significant number of children receiving allogeneic graft suffer from non-malignant disorders that usually present with early onset and progressive course. Due to some pathogenetic peculiarities successful HSCT results in this setting were obtained relatively recent. Most of publications reporting transplant outcome usually focus on optimisation of conditioning, donor selection, evaluating classical events such as GvHD,

TRM or survival. Chimerism studies in non-malignant setting are rather infrequent. In most cases chimerism monitoring is limited to WBC. Relevance of lineage-specific chimerism analysis has not been studied in these patients.

The purpose of this doctoral thesis was to evaluate chimerism analysis in WBC and ICP after allogeneic HSCT in pediatric patients suffered from three different diseases: acute lymphoblastic leukemia (ALL), Fanconi anemia (FA) and adrenoleukodystrophy (ALD). The analysis aimed to evaluate in a comparative way the benefit of lineage-specific chimerism analysis versus conventional WBC monitoring. The diagnoses were chosen as examples of three different pathologies, requiring different transplant approaches. The study was initiated in Pediatric Bone Marrow Transplant Service of Children's Hospital of the Charité Medical Faculty of the Humboldt University, Berlin. (http://www.ebmt.org/ebmt/members/search4b.htm). This is one of the most important pediatric transplant centers in Europe that specializes on grafting the above mentioned disturbances.

The doctoral thesis was accomplished at the Vilnius University. The aim of the continuation of the study was evaluation of the own experience in chimerism analysis. Chimerism kinetics in WBC of pediatric patients transplanted at Vilnius University Children's Hospital was reviewed. Isolation of different cell populations at the study moment was not available in the clinical routine. Therefore practical value of lineage-specific chimerism assessment based on the experience of Berlin clinic was supposed to be helpful to evaluate the need of implementation of this method in Lithuania.

1.2. AIM AND OBJECTIVES

Aim of the study:

To improve diagnostic facilities for prevention of potentially life-threatening complications after allogeneic HSCT by means of evaluation of the role of ICP separation in clinical practice.

Objectives:

1. To evaluate the frequency of full donor chimerism (DC) and mixed chimerism (MC) in each disease group after specific conditioning.
2. To compare chimerism kinetics in WBC and ICP.
3. To determine the role of chimerism in WBC and ICP for leukemic relapse and transplant rejection.
4. To assess the impact of pre-transplant variables on chimerism.
5. To assess the influence of chimerism on survival after HSCT.
6. To analyse chimerism kinetics after allogeneic HSCT in the pediatric patients transplanted in Lithuania.

1.3. SCIENTIFIC NOVELTY

- Otherwise the other studies reporting chimerism monitoring in malignant diseases we performed a long-term parallel chimerism observation in WBC and ICP in a very homogenous cohort of patients – children with ALL following myeloablative conditioning. For the first time chimerism analysis in CD34 subset of peripheral blood was evaluated.
- This is the first larger study focused on chimerism monitoring in ICP in FA patients. Other study groups reported chimerism changes in FA recipients mostly as a secondary proof of successful engraftment without giving to chimerism kinetics any relevant attention.
- Until now there are no published data on chimerism monitoring in ALD recipients. This study provides additional data not only about chimerism but also about transplant course in this particularly rare inheritant disorder.
- For the first time we performed chimerism analysis in Lithuanian children following allogeneic HSCT.

2. PATIENTS AND METHODS

2.1. PATIENTS

Overall 105 patients were included into the study. Children from 0 to 18 year old at the time of allogeneic HSCT were eligible for analysis. Exclusion criteria were as follows: reduced intensity conditioning for patients with ALL, haploidentical donor, T-lymphocyte depletion prior to transplantation, early TRM, insufficient number of chimerism analysis.

Comparative analysis of chimerism kinetics in WBC and ICP was performed in children who received allogeneic graft at the Bone Marrow Transplant Service of the Clinic of General Pediatrics, Children's Hospital, Charité Medical Faculty of the Humboldt University, Berlin. The HSCT was performed between July 1999 and January 2006. Overall 86 children were included into the study. Patients' numbers according to underlying diseases are represented in Table 1. Chimerism kinetics was monitored prospectively. Data evaluation was completed on 1 May 2006.

A cohort of Lithuanian patients comprised 19 patients. The same inclusion and exclusion criteria were applied. Chimerism kinetics was monitored only in WBC. Chimerism assays and medical records were reviewed retrospectively. The data evaluation was completed on May 2007. The study was approved by the Lithuanian Bioethics Committee.

In all patients cell kinetics pattern was analysed parallel to clinical course. Patients included into the study were divided in two groups based on their chimeric state: those with DC and those with MC. MC was defined as 1% or more recipient cells in two consecutive analyses. One hundred percent of donor-derived cells were defined as full DC. Pre-transplant variables were proven for their impact to the chimeric state. Finally main outcome parameters were compared in the both patients' groups.

Table 1. Studied patients.

	Number of patients	Year of age at HSCT (mean ± SD)	Sex		
			Boys	Girls	Sex ratio
ALL	43	11.2 ± 4.9	24	19	1.3:1
FA	22	9.9 ± 4.9	15	7	2.1:1
ALD	21	15.3 ± 8.3	21	0	21:0
Lithuanian patients	19	9.8 ± 5.4	8	11	1:1.4
Total	105	10.1 ± 4.7	71	34	2.1:1

2.2. CHIMERISM ANALYSIS

Prior to HSCT blood samples were collected from donor and recipient for the determination of the pre-transplant STR profile consisted of nine autosomal loci and the Amelogenin gene. In each donor-recipient pair at least one informative allele was identified. After HSCT approximately 2.7-8 ml freshly obtained peripheral blood was collected for chimerism analysis. Blood samples were taken weekly up to day 50, following once every two weeks until day 100, thereafter once a month during the first year.

Chimerism was analysed in WBC as well as in CD3, CD19 and CD34 cell populations. Cell subsets were isolated with antibody-coated immunomagnetic beads (Dynabeads® M-450, Deutsche Dynal GmbH, Hamburg, Germany) according to the manufacturer.

Genomic DNA was extracted from WBC and ICP using chaotropic reagents for cell lysis, binding of the DNA to silica-coated magnetic particles followed by elution of DNA (Qiamp Blood Kit, Qiagen, Hilden, Germany). All DNA-processing steps were performed automatically on the GenoM^{TM}-48 Robotic Workstation (GenoVision, Vienna, Austria). Prior to STR amplification all DNA samples were quantified using ultraviolet spectroscopy (GeneQuant-Spectrophotometer, Amersham Pharmacia Biotech, Freiburg, Germany). Thereafter the quantified samples were diluted to the optimal working concentration.

STR polymorphisms were analysed on ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Darmstadt, Germany) after co-amplification of nine autosomal STR loci (D3S1358/vWA/FGA, D8S1179/D21S11/D18S51 and D5S818/D13S317/D7S820) plus the Amelogenin gene with Amp*Fl*STR Profiler Plus^{TM} PCR Amplification Kit (PE Applied Biosystems, Darmstadt, Germany). The ratio of donor and recipient was determined by calculating the proportion of the peak areas of donor and recipient signals. Samples were excluded from the analysis when no unequivocal donor or recipient profile was detected.

2.3. STATISTICAL ANALYSIS

The data were analysed using the statistical software package SPSS 13.0 and STATISTIKA. A significance level of 0.05 was assumed for all statistical tests. Categorical variables in the DC- and MC-groups were compared using Fisher's exact test, Monte-Carlo test for small samples or chi-square test for trend. Quantitative variables of the two independent groups were compared using the Mann-Whitney test. The survival estimations in both groups were calculated using the Kaplan-Meier product limit method. The differences between two groups were compared with the log-rank test.

3. RESULTS

3.1. ACUTE LYMPHOBLASTIC LEUKEMIA

Between April 1999 and January 2006 allogeneic HSCT was performed to 48 children with ALL. Five of them were removed from the analysis according to the exclusion criteria (see p. 10). Thus overall 43 patients were analysed. All the recipients received a myeloablative conditioning: in most of them (93.0%, 40/43) 12 Gy of total body irradiation was delivered. The remaining three children (7%) were conditioned with chemotherapy alone.

Following this conditioning WBC analysis revealed 100% DC in 74.4% (32/43) of children. However recipient DNA was still present in ICP: autologous CD3 cells were detectable in 19.4%, CD19 – in 9.4% and CD34 – in 70.0% of patients. Host T and B lymphocytes were present only during the first two months after transplantation. The amount of recipient DNA in these cell fractions did not exceed 17%. However the percentage of recipient stem cells ranged from 3 to 80%. They circulated in peripheral blood up to two years following HSCT (Fig. 1).

Mixed chimerism in WBC was found in 25.6% (11/43) of patients. Five of these recipients showed increasing MC, four – low-level and two – decreasing MC. In 4 of 5 patients increasing MC was followed by leukemic relapse. Median time to bone marrow relapse was 204 days (ranged from 48 to 831 days). Recovery of the malignant clone led

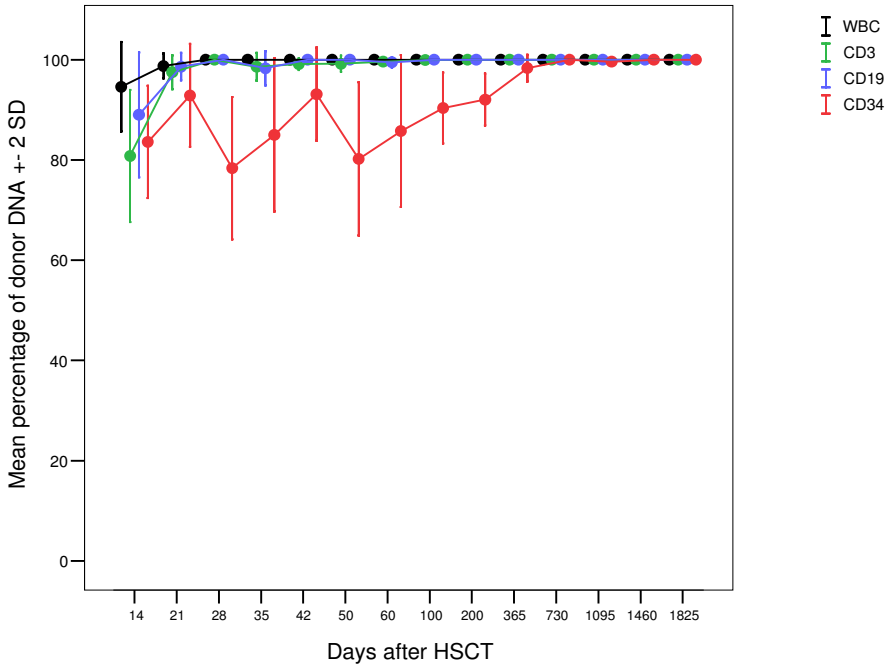


Figure 1. ALL: chimerism kinetics in ICP and WBC (n = 32)

to abrupt and concurrent decrease in donor compartment in WBC as well as in all analysed ICP (Fig. 2). In one patient increasing MC was associated with recovery of host hemopoiesis without leukemic recurrence.

Comparison of pre-transplant variables such as remission before HSCT, conditioning, donor type, stem cell source did not reveal any significant difference between patients with DC or MC in WBC (Table 2). Analysis of adverse events showed

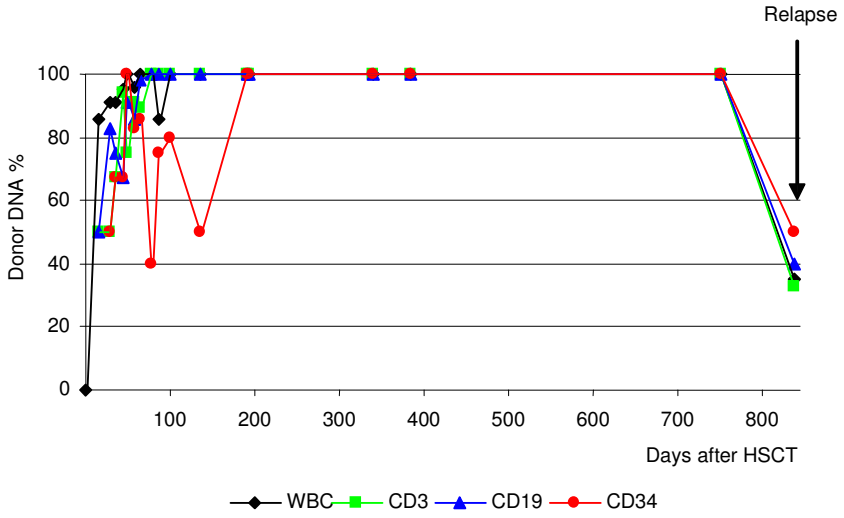


Figure 2. Mixed chimerism due to leukemic relapse.

Table 2. ALL: comparison of clinical parameters according to chimerism in WBC (n = 43).

	DC (n = 32)	**MC (n = 11)**	***p* value**
Frequency (%)	74.4	25.6	0.001[a]
Remission			
1-2 CR	28	9	0.637[a]
> 1-2 CR	4	2	
Donor			
MRD	12	5	0.728[a]
MUD	20	6	
Stem cell source			
bone marrow	21	9	0.456[a]
peripheral blood	11	2	
Conditioning			
TBI	31	9	0.156[a]
non-TBI	1	2	
Transplant related mortality			
alive/other causes of death	26	9	0.156[b]
dead	6	2	
Leukemic relapse			0.004[a, 1)]
remission	29	7	
bone marrow	0*	4*	*0.003[b]
extramedullary	3	0	
Continuous complete remission			
alive in remission	26	5#	0.091[b]
dead	6	5	
Follow-up (days after HSCT) Median [min - max] Median range [95% CI]	1057 [63-2203] 23.91[c] [747-1220]	328 [71-2275] 16.45[c] [178-1070]	0. 092[c]

[a]Chi-square test; [b]comparative criterion for probabilities; [c]Mann-Whitney test.

[1)]$\chi^2 = 6.93$; df = 2 (df – degree of freedom).

- one patient is alive in relapse

that the percentage of patients deceased because of TRM was similar in both groups: 15.6% (3/32) of patients with DC and 18.2% (2/11) of those with MC (p = 0.156). However leukemic relapse occurred significantly more frequent in case of MC was found in WBC (p = 0.004), especially bone marrow relapse (p = 0.003). Three patients developed isolated extramedullary relapse. At the time of data evaluation 81.3% (26/32) of patients with DC and 45.5% (5/11) of those with MC were in continuous complete remission (p = 0.091) by median follow-up of 1057 (63-2203) and 328 (71-2275) days after HSCT respectively in DC and MC groups (p = 0.092).

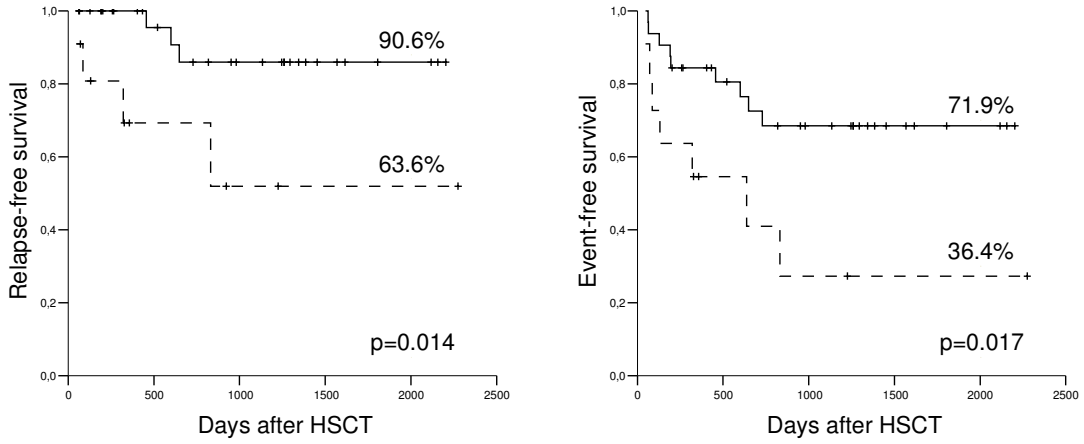


Figure 3. ALL: survival parameters according to chimerism in WBC: —— DC, – – – MC.

Survival analysis revealed that DC in WBC is associated with significantly better relapse-free (90.6% for DC vs 63.6% for MC, p = 0.014) and event-free survival (71.9% vs 36.4% respectively, p = 0.017; adverse event was defined as bone marrow or extramedullary relapse, TRM and autologous regeneration, Fig. 3). However chimerism in WBC did not affect overall survival – 3-year overall survival was 81.3% for patients with DC comparing with 54.5% for those with MC (p = 0.086). Assessment of the same three survival parameters according to chimeric state in ICP did not reveal any significant difference between DC and MC groups (Table 3).

Table 3. ALL: Impact of chimerism in WBC and ICP on survival after HSCT.

	WBC			**CD3**			**CD19**			**CD34**		
	DC	**MC**	***p****	**DC**	**MC**	***p****	**DC**	**MC**	***p****	**DC**	**MC**	***P****
RFS (%)	90.6	63.6	0.014	92.0	75.0	0.087	90.0	77.8	0.257	88.9	85.7	0.803
EFS (%)	71.9	36.4	0.017	76.0	50.0	0.069	66.7	66.7	0.850	66.7	71.7	0.846
OS (%)	81.3	54.5	0.086	88.0	62.5	0.073	76.7	88.9	0.447	77.8	85.7	0.582

*** -** Log-Rank test

3.2. FANCONI ANEMIA

Between April 1999 and January 2006 allogeneic HSCT was performed to 24 children with FA. Two of them were removed from the analysis because of early TRM according to the exclusion criteria (see p. 10). Thus, overall 22 patients were analysed. All the recipients received irradiation-free reduced intensity conditioning according to GEFA-protocol consisted from fludarabin 180 mg/m^2 and busulfan 2 mg/kg. Prevention of graft rejection and GvHD was achieved using antithymocyte globulin (ATG, Fresenius®) and muromonab (OKT3®). In addition standard dose of cyclosporine A was prescribed.

Chimerism analysis in this cohort of patients showed that after the above mentioned reduced intensity conditioning 45.5% (10/22) of patients developed full DC in WBC. In contrast to the ALL-group there no trace of autologous hemopoiesis was detected in any of the analysed ICP.

Mixed chimerism in WBC was found in 54.5% (12/22) of recipients. Evaluation of chimerism kinetics revealed two kinetic patterns: increasing MC in five children and stable long-lasting MC in seven patients. Increasing MC was associated with graft rejection. In all cases decrease in donor DNA appeared first of all in ICP, thereafter – in WBC (Fig. 4). In one of five recipients impending graft lost was prevented with additional dose of OKT3®.

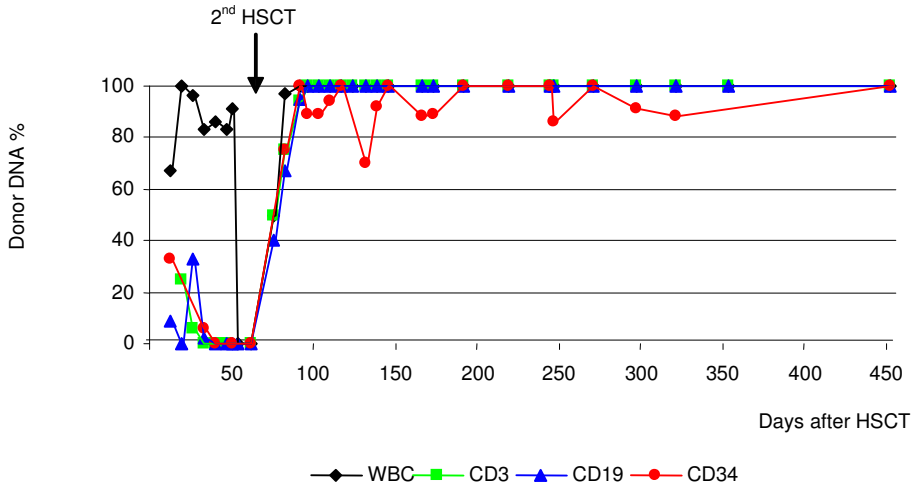


Figure 4. FA: mixed chimerism due to graft rejection.

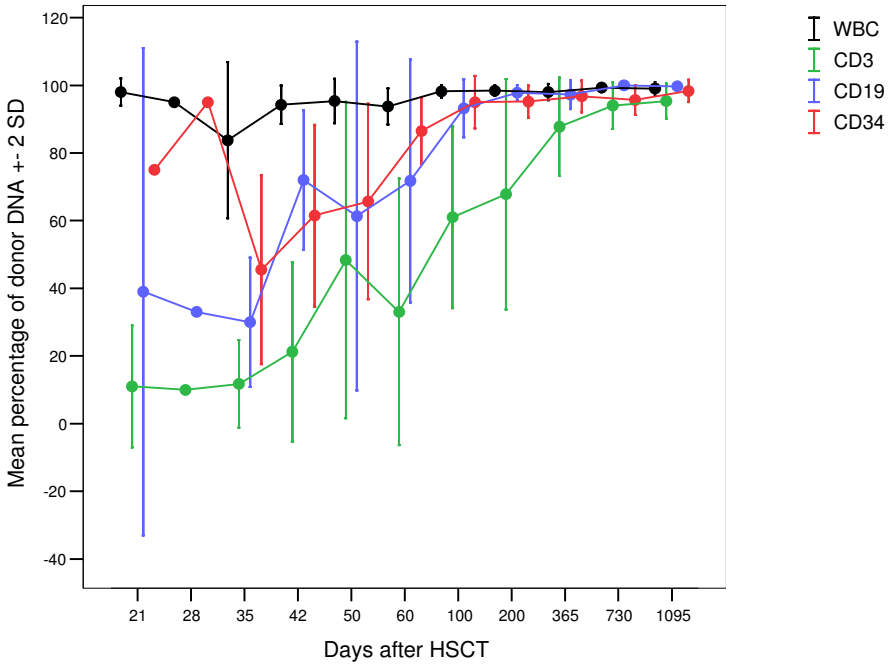


Figure 5. FA: stable long-lasting mixed chimerism (n = 7)

Stable long-lasting mixed chimerism was observed in seven recipients. In all of them there was a dissociation between the percentage of DNA detected in WBC and ICP (Fig. 5): in unfractionated blood the recipient compartment did not exceed 15%, meanwhile in ICP it fluctuated from 0 to 100%. Residual host hemopoiesis diminished gradually, however autologous cells, although in a very small proportion, were still detectable following several years after transplantation.

Comparison of pre-transplant variables showed that the both patients' groups differed significantly according to the donor and stem cell source used (Table 4). An intermediate correlation between these two variables was found (r = 0.295) however it was not significant (p = 0.163) Logistic regression analysis revealed that the odds ratio for development of MC after infusion of bone marrow was 20.0 with 95% CI (2.29-175.04; p = 0.007).

Chimerism kinetics appeared to be significantly associated with graft rejection: all four cases of graft loss were preceded by increasing MC (Table 5). In contrast none of the patient who had full DC or stable long-lasting MC lost their graft (p = 0.001). GvHD-associated morbidity was the main cause of death in the DC-group. In the MC-group less TRM was counterbalanced by graft rejection and one case of secondary carcinoma. Consequently the event-free survival did not differ significantly between

Table 4. FA: comparison of clinical parameters according to chimerism in WBC (n = 22).

	DC (n = 10)	**MC (n = 12)**	***p* value**
Frequency (%)	45.5	54.5	0.670[a]
Donor			0.058[a, 1)]
MRD	1	5	
MUD	9*	5*	0.026*, [b]
MMRD	0	2	
Stem cell source			
bone marrow	2	10	0.008[b]
peripheral blood	8	2	
Conditioning	10 – Flu/Bu/ATG/OKT3	3 – Flu/ATG/OKT3 9 – Flu/Bu/ATG/OKT3	0.221[c]
Transplant related mortality			
alive	4	9	0.192[b]
dead	6	3	
Follow-up (days after HSCT) Median [min - max] Median range [95% CI]	469 [47-2376] 10.8[c] [1607-2376]	1027 [115-2243] 12.08[c] [216-2243]	0.674[c]

[a]Chi-square test; [b]comparative criterion for probabilities; [c]Mann-Whitney test.

[1)]$\chi^{2} =$ 5,675; df = 2 (df – degree of freedom).

Table 5. FA: comparison of outcome parameters according to chimerism kinetics (n = 22).

Outcome parameter	**Full DC (n = 10)**		**Increasing MC (n = 5)**		**Stable MC (n = 7)**		***p* value**
	n	**%**	**n**	**%**	**n**	**%**	
Graft rejection	0	0	4	80	0	0	0.001[a, 1)]
Stable engraftment	10	100	1	20	7	100	
Alive	4	40	3	60	6	86	0.214[a, 2)]
Dead	6	60	2	40	1	14	

[a]Exact Test (Monte-Carlo-Simulation)

[1)] $\chi^{2} =$ 16.622; df = 2; [2)]$\chi^{2} =$ 3.562; df = 2 (df –degree of freedom).

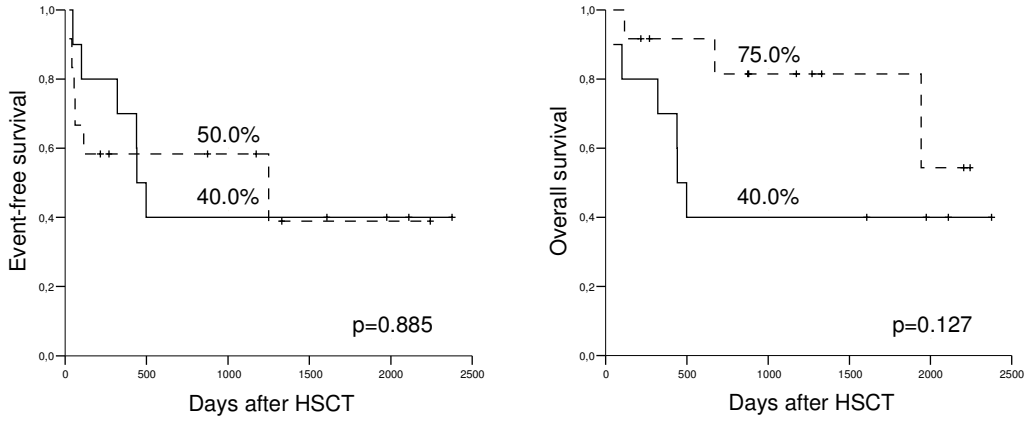


Figure 6. FA: survival parameters according to chimerism in WBC: —— DC, – – – MC.

the both groups (p = 0.192; Fig. 6). Assessment of overall survival showed a trend for longer life estimation for patients who had MC in WBC: 75.0% versus 40.0% for patients with DC. Although these results look encouraging the difference did not reach statistical significance (p = 0.127; Fig. 6). Taking into account chimerism kinetics (Table 5) revealed that at the time point of data evaluation 6 from 7 (86%) of patients with stable MC were alive in contrast to 4 from 10 (40%) of those with full DC or 2 from 5 (60%) of those with increasing MC (p = 0.214). Assessment of event-free and overall survival according to chimeric state in ICP failed to determine any significant difference between patients with full donor and mixed chimerism in investigated cell subsets (Table 6).

Table 6. FA: Impact of chimerism in WBC and ICP on survival after HSCT.

	WBC			**CD3**			**CD19**			**CD34**		
	DC	**MC**	***p****	**DC**	**MC**	***p****	**DC**	**MC**	***p****	**DC**	**MC**	***p****
EFS (%)	40.0	50.0	0.885	57.1	50.0	0.390	50.0	71.4	0.888	33.3	55.6	0.937
OS (%)	40.0	75.0	0.127	57.1	75.0	0.598	50.0	100	0.114	50.0	77.8	0.288

***** - Log-Rank test

3.3. ADRENOLEUKODYSTROPHY

Between April 1999 and January 2006 allogeneic HSCT was performed to 22 boys affected by ALD. One of them was excluded from the analysis because of haploidentical donor according to the exclusion criteria (see p. 10). Thus overall 21 patients were analysed. All the recipients received myeloablative conditioning consisted from busulfan 16 mg/kg and cyclophosphamide 200 mg/kg. Prevention of graft rejection and GvHD was achieved using antithymocyte globulin (ATG Merieux® and Fresenius®) and muromonab (OKT3®). In addition standard doses of cyclosporine A and methotrexate were prescribed.

Chimerism analysis in these patients showed that after myeloablative conditioning 66.7% (14/21) of recipients developed full DC in WBC. Similar to the patients with ALL, chimerism evaluation in cell subsets revealed persistence of autologous hemopoiesis. In CD34 fraction it was determined in up to 71.4% of recipients (Fig. 7). The amount of host DNA fluctuated from 55 to 98%. As in leukemic patients autologous compartment disappeared gradually following two years after HSCT. Other ICP were positive for recipient signals in a significantly lower proportion of children (CD3 in 7.4% and CD19 in 15.4%). Recipient DNA in these cell populations was detectable only

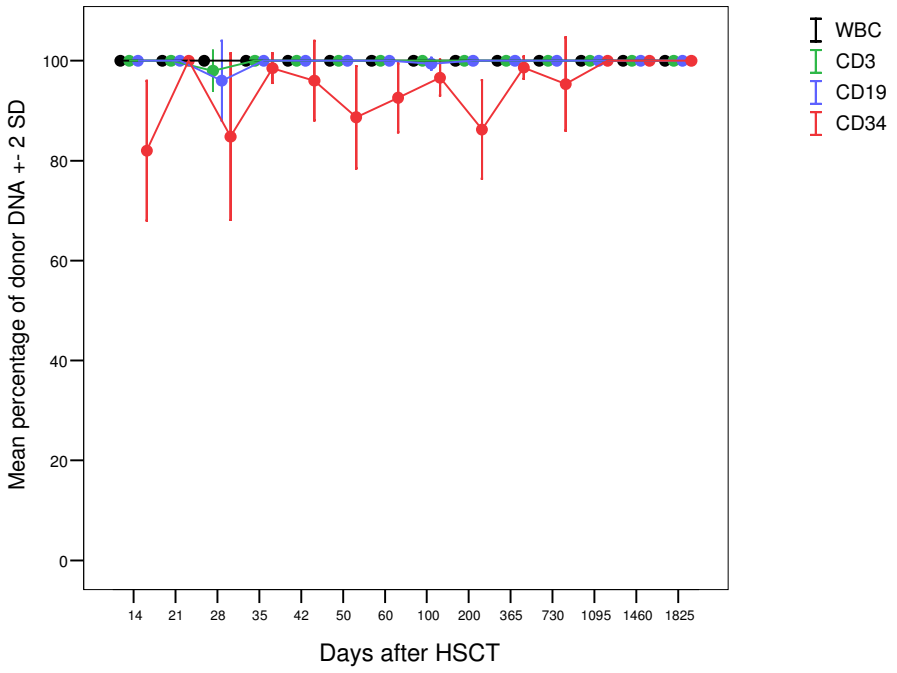


Figure 7. ALD: chimerism kinetics in ICP and WBC (n = 14)

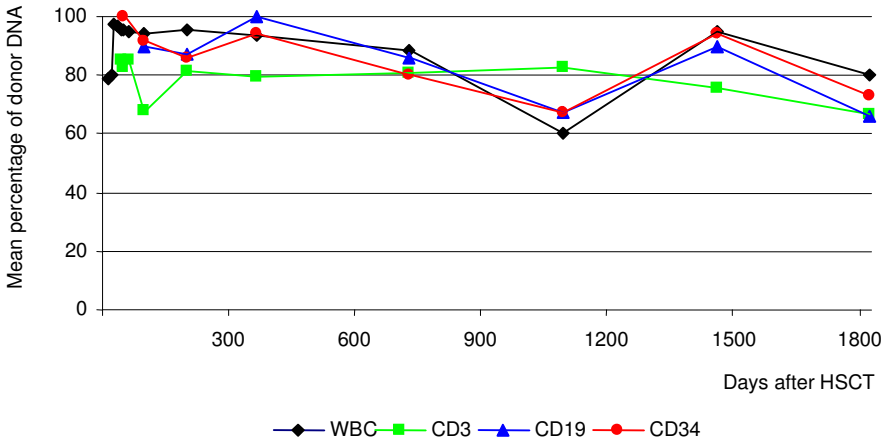


Figure 8. ALD: stable long-lasting MC (n = 5).

during the first two months after HSCT.

Mixed chimerism in WBC was found in 33.3% (7/21) of studied patients. Evaluation of chimerism kinetics revealed two kinetic patterns: stable long-lasting MC in five patients and decreasing MC in five children. Stable long-lasting chimerism was detectable equally in WBC and all other cell subsets (Fig. 8). Proportion of autologous DNA remained approximately at the same level over the years after HSCT. None of these patients lost their graft without any modulation of immune therapy. Decreasing MC in two boys was attributable to prolonged engraftment that converted into full DC following cessation of immune suppression.

Comparison of pre-transplant variables showed that the degree of demyelinisation before HSCT assessed according to Loes score did not differ significantly between recipients who developed DC or MC after HSCT ($p = 0.287$). Other parameters such as donor type, stem cell source, antithymocyte globulin delivered prior to graft infusion did not differ between the two groups either (Table 7). ALD progress and TRM were the main adverse events that affected equally boys with DC and MC ($p = 0.151$ and $p = 1.0$ respectively). Only one patient of the entire cohort died due to infectious-toxic complications. The other three deceases occurred due to progression of the underlying disorder. Thus the event free survival was 76.9% for patients who were found to have full DC in WBC in comparison with 42.9% for those with MC in WBC ($p = 0.087$, Fig. 9). Progressive demyelinisation was not always associated with fatal outcome. Therefore

Table 7. ALD: comparison of clinical parameters according to chimerism in WBC (n = 21).

	DC (n = 14)	**MC (n = 7)**	***p* value**
Frequency (%)	66.7	33.3	0.127[a]
Loes score before HSCT (mean rank)	9.93	13.14	0.287[c]
Donor			
MRD	4	2	1.0[b]
MUD	10	5	
Stem cell source			
bone marrow	6	5	0.098[a, 1)]
peripheral blood	8	1	
cord blood	0	1	
Antithymocyte globulin			
Merieux®	2	4	0.172[a, 2)]
Fresenius®	10	3	
none	2	0	
ALD progress			
no	10	3	0.151[b]
yes	3	4	
not applicable	1#	0	
Transplant related mortality			
alive	13	7	1.0[b]
dead	1	0	
Overall survival			
alive	12	5	0.574[b]
dead	2	2	
Follow-up (days after HSCT) Median [min - max] Median range [95% CI]	968 [251-2426] 11.14[c] [802-1607]	1600 [117-1991] 1071[c] [435-1991]	0.913[c]

[a]Chi-square test; [b]comparative criterion for probabilities; [c]Mann-Whitney test.

[1)] $\chi^2 = 4.727$; df = 2; [2)] $\chi^2 = 4.615$; df = 2 (df – degree of freedom).

- the patient died on day 251 after HSCT

overall survival appeared to be superior to the one of event-free in the both groups (Fig. 9): 85.7% (12/14) of patients with DC versus 71.4% (5/7) of those with MC (p = 0.490) remained alive at the time point of data evaluation by comparable follow-up (p = 0.913). Assessment of event-free and overall survival according to chimeric state in ICP failed to determine any significant difference between patients with full donor and mixed chimerism in investigated cell subsets (Table 8). Summarizing, chimerism appeared no

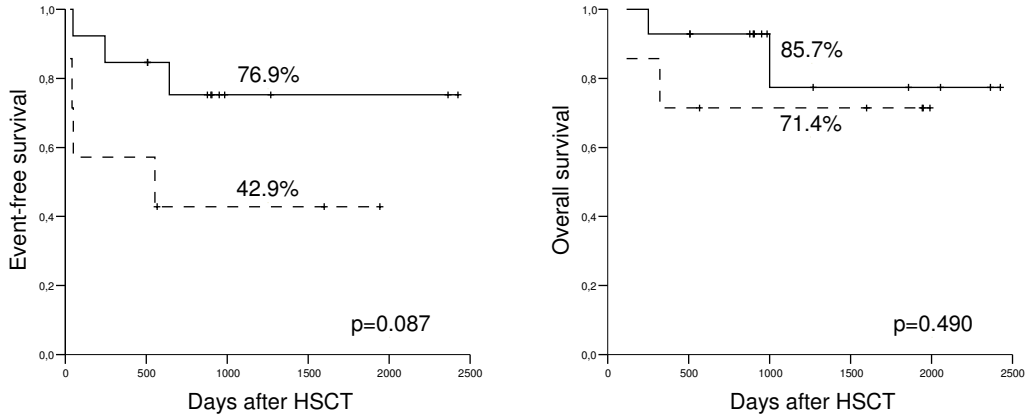


Figure 9. ALD: survival parameters according to chimerism in WBC: —— DC, – – – MC.

to have any significant impact on transplant results in ALD recipients.

Table 8. ALD: impact of chimerism in WBC and ICP on survival after HSCT.

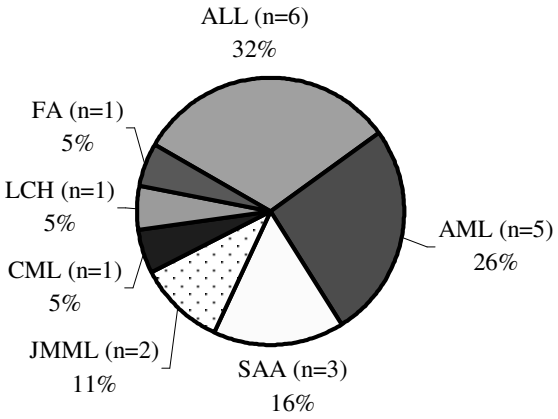
	WBC			**CD3**			**CD19**			**CD34**		
	DC	**MC**	***P****	**DC**	**MC**	***p****	**DC**	**MC**	***P****	**DC**	**MC**	***p****
EFS (%)	76.9	42.9	0.087	75.0	50.0	0.209	84.6	33.3	0.302	80.0	60.0	0.498
OS (%)	85.7	71.4	0.490	84.6	75.0	0.605	85.7	66.7	0.522	81.0	75.0	0.199

***** – Log-Rank test

3.4. LITHUANIAN PATIENTS

Between February 2002 and May 2007 overall 21 children underwent an allogeneic HSCT in Vilnius University Children's Hospital. Two patients were excluded from the review according to the exclusion criteria (see p.10): one because of early toxic death on day 16 after HSCT before the engraftment occurred, the second one because of insufficient follow up of less than one week. Thus, totally 19 patients were included in the study.

Peculiarity of this patients' cohort was heterogeneity of the diagnoses (Fig. 10). The majority of the children (73.7%, 14/19) were transplanted because of different type of leukemia. ALL and AML were predominant underlying malignancies (31.6% and 26.3% respectively). Five of nineteen patients (26.3%) had non-malignant disorders.



Abbreviations: ALL – acute lymphoblastic leukemia; AML – acute myeloblastic leukemia; CML – chronic myeloid leukemia; FA – Fanconi anemia; JMML – juvenile myelomonocytic leukemia; LCH – Langerhans cell histiocytosis; SAA –severe aplastic anemia.

Figure 10. Distibution of diagnoses (n = 19).

Fifteen from nineteen patients (78.9%) underwent HSCT from their genotypic identical siblings meanwhile an unrelated transplant from matched unrelated donor was performed only in four of them (21.1%). Bone marrow was the principal source of stem cells. The graft was infused after the disease-specific conditioning. Standard GvHD prophylaxis with cyclosporin and methotrexate was applied with addition of antithymocyte globulin in unrelated transplants.

After the disease-specific conditioning 9 from 19 patients (47.4%) developed full DC, 10 from 19 patients (52.6%) showed MC. Chimerism was assessed only in WBC. Analysis of chimerism kinetics revealed stable and increasing patterns of MC.

Stable long-lasting MC was found in three patients with non-malignant disorders. This pattern of MC appeared to be associated with favourable prognosis resulting in a continuous complete remission without any sign of chronic GvHD. Donor compartment in the WBC fluctuated from 80 to 98% donor cells and did not require any modification of immune therapy. Two patients with non-malignant disorders showed increasing MC that caused rapid decrease of the donor compartment followed by the transplant rejection.

All five leukemic patients showed an increasing MC pattern. Following conditioning and stem cell infusion all these patients engrafted successfully with 100

Table 9. Comparison of clinical parameters according to chimerism in WBC (n = 19).

	DC (n = 9)	MC (n = 10)	*p* value
Frequency (%)	47.4	52.6	
Underlying disorder			
malignant	9	5	0.033[a]
non-malignant	0	5	
Conditioning			
myeloablative	9	5	0.033[a]
non-myeloablative	0	5	
Donor			
MRD	7	8	1.0[a]
MUD	2	2	
Stem cell source			
bone marrow	6	7	1.0[a]
peripheral blood	3	3	
CD34 x10^6/kg cells per recipient body weight (mean rank [95% CI])	9.67[1.497-6.832]	10.3[2.220-7.948]	0.842[b]
Transplant related mortality			
alive/died due to other causes	5	10	0.033[b]
dead	4	0	
Transplant rejection			
yes	0	2	0.474[a, b]
no	9	8	
Leukemic relapse			
remission	9	0	<0.001[b]
relapse	0	5	
not evaluable	0	5‡	
Continuous complete remission			
remission	5	4	0.656[b]
dead	4	6#	
Follow-up (days after HSCT) Median [min - max] Median range [95% CI]	146 [57-1154] 8.0[c] [75.13-747.09]	509 [174-1833] 11.8[c] [301.54-1024.86]	0.156[c]

[a]Chi-square test; [b]comparative criterion for probabilities; [c]Mann-Whitney test.
‡ - five patients suffered from non-malignant disorders
# - one patient was alive in relapse

percent DC documented by chimerism analysis. However recovery of the host malignant cells resulted in MC that led to the decrease of the donor fraction and subsequent BM relapse. Median time from the detection of MC and the relapse was 134 days ranging from 50 to 400 days after HSCT.

Pre-transplant characteristics of the patients according to their chimeric state and post-transplant events are summarized in Table 9. All five patients with non-malignant

disorders revealed MC ($p = 0.033$) that seemed to be attributable to the non-myeloablative conditioning ($p = 0.033$) applied to this cohort. All the children with malignant disorders received a myeloablative conditioning thereafter most of them (9/14) became full donor chimera. Donor type and stem cell source did not differ significantly in the both patients' group ($p = 1.0$ for the both variables). Median stem cell dose was higher in the MC-group ($4.83x10^6$ CD34/kg recipient body weight) than in the DC-one ($2.50x10^6$ CD34/kg recipient body weight) by the comparable use of bone marrow and peripheral blood stem cells. Mortality due to toxic-infectious complications was significantly associated with DC ($p = 0.033$) meanwhile occurence of transplant rejection did not differ significantly between two groups ($p = 0.474$). Leukemic relapse occured significantly more frequent in patients with MC ($p < 0.001$). As a consequece the relapse-free survival in leukemic patients was found to be unequivocally dependent on the chimeric state ($p = 0.002$, Fig. 11). Due to the fact that recurrence of leukemia was the main event in the MC-group meanwhile the DC-patients succumbed mostly to the toxic complications the event-free and overall survival did not differ significantly between the both groups ($p = 0.435$ and $p = 0.601$ respectively, Fig. 11).

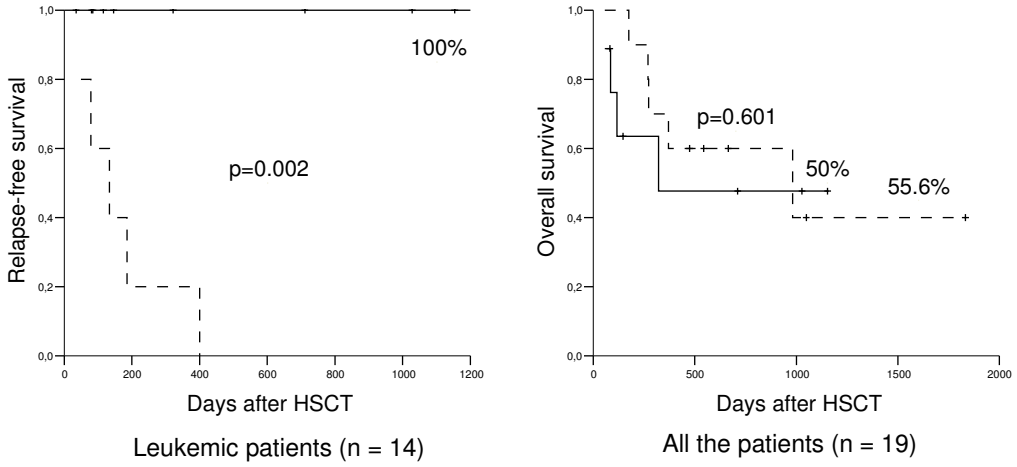


Figure 11. Survival parameters according to chimerism in WBC: —— DC, – – – MC.

4. CONCLUSIONS

1. Following myeloablative conditioning 74.4% of patients with ALL and 66.7% with ALD developed full DC. Despite stable DC in WBC analysis of ICP revealed persistent autologous hemopoiesis. In contrast 45.5% of patients with FA had no evidence of autologous DNA either in WBC, or in ICP after reduced intensity conditioning.
2. ALL and ALD patients who retained full DC in WBC had transient MC in CD3 and CD19 fractions meanwhile decreasing MC in CD34 cells was detectable up to two years following HSCT. FA and ALD recipients had stable long-lasting MC, persisting several years after HSCT in WBC and in all analysed ICP.
3. In case of ALL increasing MC in WBC was found to be strongly associated with leukemic relapse, especially with bone marrow relapse, in case of FA – with graft rejection.
4. In the FA group the development of MC was related to the infusion of bone marrow but not to one of peripheral blood stem cells.
5. In ALL patients full DC in WBC was a statistically significant prognostic factor for better relapse-free and event-free survival, however it had no impact on the overall survival. FA recipients with MC tended to have better overall survival than those with full DC in WBC. In case of ALD chimerism in WBC did not affect survival parameters. Chimerism in ICP had no influence on transplant results in any of the analysed patients‘ groups.
6. Chimerism analysis in Lithuanian patients revealed full DC in 47.4% of recipients and MC – in 52.6% of them. Patients transplanted due to non-malignant disorders developed increasing and stable MC associated with non-myeloablative conditioning, meanwhile leukemic patients showed increasing MC predictive for leukemic relapse. Chimerism did not affect survival after HSCT.

5. PUBLICATIONS

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6. SANTRAUKA

Chimerizmo tyrimas yra vienas pagrindinių alogeninės kraujodaros kamieninių ląstelių transplantacijos (KKLT) eigos vertinimo būdų, leidžiantis nustatyti persodintų kraujodaros kamieninių ląstelių (KKL) prigijimą, leukemijos recidyvo ar transplantato atmetimo riziką. Potransplantacinių komplikacijų savalaikei diagnostikai labai svarbu naudoti kuo jautresnę chimerizmo tyrimo metodiką. Šiuo metu chimerizmui tirti naudojami polimorfiniai DNR žymenys, kurių analizė periferinio kraujo leukocituose (PKL) užtikrina tyrimo jautrumą $1–5\times10^{-2}$. PKL suskaidymas į atskiras ląstelių populiacijas (ALP) padidina tyrimo jautrumą iki $10^{-3}–10^{-4}$.

Mokslinis darbas pradėtas Charité klinikose, vykdant doktorantūros studijas Humboldto universitete (Berlynas, Vokietija). Chimerizmo tyrinėjimams pasirinkti recipientai, sergantys ūmia limfoblastine leukemija (ŪLL), Fanconi anemija (FA) ir adrenoleukodistrofija (ALD). Sergant šiomis ligomis nuoseklus chimerizmo stebėjimas ALP, tame tarpe ir KKL, atliktas pirmą kartą. Darbas tęstas Vilniaus universitete, kuomet pirmą kartą buvo atlikti chimerizmo tyrinėjimai Lietuvoje transplantuotiems vaikams.

Darbo tikslas buvo pagerinti vaikų ištyrimą po alogeninės KKLT, siekiant pegerinti ankstyvą gyvybei pavojingų komplikacijų diagnostiką. Siekta nustatyti visiško donoro chimerizmo (DC) ir mišraus chimerizmo (MC) dažnį PKL ir ALP kiekvienoje ligos grupėje, palyginti chimerizmo kinetiką PKL ir ALP, nustatyti chimerizmo PKL ir ALP ryšį su ligos recidyvu ir transplantato atmetimu, išanalizuoti prieštransplantacinių veiksnių įtaką chimerizmui bei įvertinti chimerizmo PKL ir ALP įtaką išgyvenamumui po transplantacijos.

Tirti vaikai iki 18 metų po alogeninės KKLT: ŪLL (n = 43), FA (n = 22), ALD (n = 21) ir Lietuvoje transplantuoti vaikai (n = 19). Donoro ir recipiento ląstelėms atskirti naudoti polimorfiniai DNR žymenys, kurie tirti periferinio kraujo PKL ir ALP. Ląstelių frakcijoms atskirti naudotos imunomagnetinės dalelės. Išskirta DNR išmatuota ultravioletinių spindulių spektroskopijos metodu. DNR žymenys tirti daugine PGR, kurios metu gausintos devynios autosominės genetinės sritys ir amelogenino genas. PGR gausinimo produktų analizei naudota kapiliarinė elektroforezė. Išanalizavus atliktus tyrimus, apskaičiuotas recipiento ir donoro DNR procentinis santykis. Chimerizmo

tyrimai gretinti su klinikine eiga. Statistinė analizė atlikta programų paketais SPSS 13.0 ir STATISTIKA.

Atlikę chimerizmo analizę nustatėme, kad tiriant PKL 74,4 proc. ŪLL ir 66,7 proc. ALD recipientams po mieloabliacinio kondicionavimo išsivysto visiškas DC. Esant visiškam DC leukocituose visose kitose tirtose ALP išlieka autologinė kraujodara: T ir B limfocituose recipiento frakcija matoma trumpą laiką, o KKL populiacijoje mažėjantis MC, aptinkamas iki dvejų metų po transplantacijos. Sergant ŪLL, didėjantis MC leukocitų frakcijoje yra reikšmingai susijęs su leukemijos recidyvu. ŪLL grupėje visiškas DC bendroje leukocitų frakcijoje lemia reikšmingai geresnį išgyvenamumą be recidyvo ir be neigiamo įvykio, tačiau neturi įtakos bendram išgyvenamumui.

Po sumažinto intensyvumo kondicionavimo 45,5 proc. FA recipientų autologinės kraujodaros pėdsakų nerandama nei PKL, nei ALP. FA ir ALD ligoniams vystosi stabilus ilgalaikis MC, išliekantis iki kelerių metų po KKLT tiek leukocituose, tiek ALP. Didėjantis MC yra susijęs su transplantato atmetimu, o stabilus ilgalaikis MC – su ilgesniu išgyvenamumu po transplantacijos. Nustatėme, kad sergantiems FA, galimybė išsivystyti MC yra 20 kartų didesnė perpylus kaulų čiulpus, nei periferinio kraujo KKL. ALD recipientų išgyvenamumo rodikliai nepriklausė nuo chimerizmo PKL. Chimerizmas ALP neturėjo įtakos transplantacijos rezultatams nė vienoje tirtoje ligų grupėje.

Lietuvoje transplantuotiems vaikams chimerizmas tirtas tik PKL: visiškas DC rastas 47,4 proc. ligonių, o MC – 52,6 proc. vaikų. Ligoniams su nepiktybinėmis kraujo ligomis stebėtas didėjantis ir stabilus MC, susijęs su nemieloabliaciniu kondicionavimu, o sergantiems leukemija rastas didėjantis MC, susijęs su leukemijos recidyvu. Chimerizmas neturėjo įtakos išgyvenamumui po transplantacijos.

Remiantis tyrimo rezultatais, pasiūlėme tirti chimerizmą ALP sergant nepiktybinėmis kraujo ligomis, tuo tarpu sergant leukemija chimerizmo tyrimas nefrakcionuotuose leukocituose yra pakankamai informatyvus. Ruošiant transplantacijai FA recipientą rekomenduojame persodinti kaulų čiulpus.

7. BRIEF BIOGRAPHY

Jelena Rascon was born on 27 February 1972 in Vilnius. In 1995 she graduated the program of Pediatrics at the Faculty of Medicine at the Vilnius University. During 1995-1997 she completed clinical training in general pediatrics and gained the qualification of general pediatrician. In 1997-1998 she fulfilled the residency in pediatric oncology and hematology and gained the qualification of pediatric oncologist and hematologist. Since 1998 she has been employed as a pediatric oncologist and hematologist at the out-patient clinic of Vilnius University Children's Hospital. Since October 2000 she entered the in-patient department of pediatric oncology and hematology. Since the Bone marrow transplantation unit was founded in February 2002 she became a member of its stuff. Since January 2008 she became the Supervisor of the Bone marrow transplantation unit.

In 2001 and 2002-2003 Jelena Rascon was a clinical and research trainee at the Pediatric Bone Marrow Transplant Service of the Clinic of General Pediatrics in Children's Hospital of Charité-CVK (Faculty of Medicine of the Humboldt University, Berlin). She has published six scientific articles and over 20 abstracts printed in proceedings of international conferences. She is married and has got three children.

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