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Immunology of bone marrow CD34 subsets and clonal hematopoiesis of indeterminate potential in amyotrophic lateral sclerosis

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SUMMARY

Introduction Recent advances in pathogenesis of neurodegenerative diseases have shown that inflammation is a key factor of progression.

Methods The amyotrophic lateral sclerosis (ALS) group included 10 patients [mean age 55.1 ± 3.1 years (CI 95%: 48–62.2)]. Whole-exome sequencing and immunophenotyping of CD34+ subsets in bone marrow (BM) cells were performed before the start of therapy (baseline) and six months later (follow-up). The control group included 10 healthy donors, mean age was 39.9 ± 3.9 years (CI 95%: 31.2–48.6).

Results The peripheral blood stem cells (PBSCs) were collected after four-day granulocyte colony-stimulating factor administration. The total mean number of collected CD34+ cells was $290.4 \pm 53.5 \times 10^6$ (CI 95%: 177.6–403.3). Patients received fludarabine 25 mg/m^2/day, on days one and two. To induce hematopoietic stem cell transdifferentiation the PBSCs were incubated with human placenta double-stranded DNA fragments *ex vivo* and reinfused 48 hours post fludarabine. Clonal hematopoiesis of indeterminate potential (CHIP) was detected in three cases (30%) before therapy. A significant increase CD34+CD13+ and CD34+CD123+ hematopoietic stem cells (HSCs) in BM was detected. The CD34+CD44+ level significantly decreased. Levels of CD34+CD7+, CD34+CD2+ and CD34+CD56+ showed a trend toward increased mean value after treatment. In two cases CHIP disappeared, in one case a decrease in the allelic variant frequency has been shown. The mean amyotrophic lateral sclerosis functional rating scale-revised score did not change [39 ± 0.6 points (CI 95%: 37.6–40.4) *vs*. 40.4 ± 0.7 (CI 95%: 38.8–42)].

Conclusion Our study is the first attempt to characterize the subsets of BM HSCs in ALS. They demonstrate that BM is able to respond to immune-mediated neuroinflammation. Preliminary results indicate a possible link between CHIP and ALS and point the way to eliminating aberrant clones.

Keywords: CD34 subsets; bone marrow; amyotrophic lateral sclerosis; clonal hematopoiesis of indeterminate potential; fludarabine

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting primarily the motor system leading to paralysis and death within 3–5 years after the onset of symptoms. Most cases of classic ALS (~90%) are identified as sporadic, with little genetic contribution [1]. The identification of *C9orf72* mutations in patients without a family history and disease discordance in monozygotic twins challenge the traditional binary classification of the disease into familial and sporadic forms and suggest a strong contribution of epigenetic factors in ALS [2]. Sporadic ALS is clinically indistinguishable from familial forms, raising the possibility that the disease is caused by the interaction of several genes and/or epigenetic dysregulation of their function. Evidence is provided by the fact that the major epigenetic differences arise in lymphocytes, skeletal muscle and fat during the lifetime of monozygotic twins [3]. Somatic mosaicism, that is, the presence of several genetically different cell clones in the same tissue, is an inevitable consequence of human aging [4].

Somatic mutations also occur in hematopoietic stem cells (HSCs), the offsprings of which participate in immunopoiesis and give rise to some neuroglial cells. The expanded blood cell clones with mutations in driver genes and/or genetic alterations in chromosomes have been identified. This phenomenon is particularly prevalent in the elderly and the greatest known risk factor for ALS is aging [2, 4, 5, 6]. Some recent works have attempted to trace the connection between ALS and clonal hematopoiesis of indeterminate potential (CHIP) [7].

Recent advances in pathogenesis of neurodegenerative diseases have shown that inflammation is not only a result of neurodegeneration, but also a key factor in this process. Protein aggregates, which are a very common pathologic phenomenon in neurodegeneration, are now perceived rather as a consequence of the immune system's dysfunction in maintaining

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the genetic homeostasis of the body then as the cause of the disease. Increasing evidence suggests that common risk factors for neurodegenerative diseases may trigger an inflammatory response, initiating and exacerbating disease progression. In addition, many genetic risk factors for neurodegenerative diseases are associated with immunity. The interleukin (IL) family, especially IL-1, IL-6 and IL-17, plays a critical role in the pathogenesis of these diseases. It has been shown that patients with ALS have a significant decrease in Treg and FoxP3 protein expression. [7]. Moreover, the CD3, CD4, CD8 and CD3+CD56+ T cells, natural killer (NK) cells, monocytes and neutrophils were found to be increased in ALS patients and also associated with disease progression. While higher levels of effector CD4 T cells in both blood and cerebrospinal fluid (CSF) were associated with decreased survival, an increased frequency of activated regulatory T cells (Treg) in blood was associated with improved survival [8].

It can be assumed that clonal disorders of hematopoiesis at the level of a bone marrow (BM) stem cell that has acquired a somatic mutation or chromosomal aberration play an important role in the pathogenesis of neurodegenerative diseases, and ALS in particular. The progeny of such as HSCs acquires a clonal advantage, leading to their clonal expansion, development of chronic immune-mediated inflammation and disruption of the innate immune system. The degree of nervous system damage, the variety of clinical manifestations, the time of disease onset, and some other phenomena may vary depending on the repertoire of tissue somatic/germline mutations as well as the genes of HSCs involved in the somatic mutation, their differentiation, secreted cytokines and activated signaling pathways. The role of BM hematopoietic cells in the development and maintenance of immune-mediated inflammation and demyelination was recently demonstrated in patients with multiple sclerosis (MS) [9].

Planning this study, we hypothesized that since BM is the central organ of hematopoiesis (both lympho- and myelopoiesis), it may be possible to restart and correct the abnormal immune response in ALS using minimal immunomodulation and *ex vivo* transdifferentiated autologous HSCs.

METHODS

Patients

The ALS group included 10 patients with confirmed disease (four men, six women), with a mean disease duration from the time of diagnosis 26.5 ± 4 months (CI 95%: 17.5–35.6). The median age was 53.9 ± 9.9 years (CI 95%: 28–47). The median ALS functional rating scale-revised (ALSFRS-R) score was 39 ± 0.6 points (CI 95%: 37.6–40.4). The median Karnofsky score at the time of inclusion in the study was 53 ± 3 (CI 95%: 46–60). All patients underwent BM puncture prior to therapy start in protocol. Whole-exome sequencing and immunophenotyping of CD34+ subsets in BM cells were performed at the baseline in "a steady state" before the administration of granulocyte colony-stimulating factor (G-CSF) (baseline) and during the first six months of follow-up, but not before three months (follow-up). The control group included 10 BM donors (seven men, three women), the median age of the donors was 39.9 ± 3.9 years (CI 95%: 31.2–48.6, p = 0.07). Toxicity of therapy was assessed by the National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0 (NCI CTCAE v5.0 scale).

Targeted gene panel sequencing

Targeted paired-end sequencing was performed on an MGISEQ-G400 instrument (MGI Tech Co. Ltd., Shenzhen, China) utilizing either Roche KAPA HyperExome (Roche Holding AG, Basel, Switzerland) or Nanodigmbio NEXome Plus Panel v1.0 (Nanodigmbio Pte. Ltd., Nanjing, China) whole-exome panels to a median sequencing depth of ~100 × across sites. For the germline and somatic analyses pipeline, the following software tools were employed: BWA2 v2.2.1 (Intel Corp., Santa Clara, CA, USA) for alignment to the human reference genome (*GRch37*), Streammd v4.3.0 (QIMR Berghofer Medical Research Institute, Herston, Australia) for marking and removal of polymerase chain reaction (PCR) duplicates, Sambamba v1.0.1 (Free Software Foundation, Inc., Boston, MA, USA) for filtering mapped variants.

For the germline analysis pipeline, the following software tools were employed: BWA2 v2.2.1 (Intel Corp.) for alignment to the human reference genome (*GRch37*), Streammd v4.3.0 (QIMR Berghofer Medical Research Institute) for marking and removal of PCR duplicates, Sambamba v1.0.1 (Free Software Foundation, Inc.) for filtering mapped variants, Genome Analysis Toolkit (GATK) Picard for quality control and mapping metrics assessment, Google Deepvariant v1.6.1 for nucleotide substitution detection, Bcftools v1.20 (Free Software Foundation, Inc.) for variant filtering, and Ensembl Variant Effect Predictor release 112 (Ensembl, Cambridge, UK) for variant annotation.

The somatic analysis pipeline utilized several shared tools including BWA2 v2.2.1 (Intel Corp.) for genome alignment, Streammd v4.3.0 (QIMR Berghofer Medical Research Institute) for PCR duplicate processing, and Sambamba v1.0.1 (Free Software Foundation, Inc.) for variant filtering. However, it diverged in using GATK v4.4.0.0 for quality control and mapping metrics, GATK Mutect 2 for nucleotide substitution identification, and FINGS v1.7.2 for variant filtering, while maintaining Ensembl Variant Effect Predictor release 112 (Ensembl) for annotation.

The analysis focused on a panel of genes implicated in relevant pathways, including *ASXL1*, *ASXL2*, *BRCC3*, *CBL*, *DNMT3A*, *ETNK1*, *GNAS*, *GNB1*, *IDH1*, *IDH2*, *JAK2*, *KRAS*, *NRAS*, *PPM1D*, *SETBP1*, *SF3B1*, *SRSF2*, *TET2*, *TP53*, *U2AF1*, *PTPN11*, *BCOR*, *BCORL1*, *UBA1*, *CTCF*, *DNMT1*, *DNMT3b*, *AKT1*, *MYD88*, *NLRP3*, *MTOR*, *NANOG*, *OCT4*, *PTEN*, and *SOX2*.

BM immunophenotyping by flow cytometry

The analysis of CD34 subset was performed using multicolor 5–6-parameter flow cytometry on a FACS CANTO II flow cytometer (BD, Franklin Lakes, NJ, USA) as described previously [10]. From 1,000,000 to 2,000,000 events (all cells in the sample) were recorded from each sample during flow cytometric analysis. Flow cytometry data were evaluated using Kaluza Analysis software, version 3.1 (Beckman Coulter, Brea, CA, USA). Pluripotent cell populations were characterized based on the combined expression on CD34+ cells of the antigens CD45, CD44, CD33, CD13, CD41, CD123, CD133, CD117, CD61, CD10, CD19, CD2, CD7 and CD56.

The gating sequence for CD34 subpopulation analysis was as follows: nucleated cells were identified based on SYTO16 expression in the side-angle scatter (SSC) *vs*. SYTO16 parameters. Within SYTO-positive cells, a gate was determined for CD34+ cells in the SSC *vs*. CD34 parameters. Next, expression of the antigens of interest was assessed within nucleated SYTO+CD34+ cells. Internal positive controls served as positivity controls. Each population was assessed separately in the parameters – target antigen (ordinate axis) *vs*. CD34 (abscissa axis).

Data analysis

Data on CDs were analyzed using the inverse variance of the as the mean ± SD, 95% confidence intervals (CI) and p values. The Wilcoxon signed-rank for ALS baseline *vs*. follow-up and t-test for donors *vs*. ALS baseline were used. Significance was set at $p \leq 0.05$ and a CI 95%. The data in the figures are presented as medians and degrees of dispersion. Student's t-test was used to compare continuous variables. Statistical analyses were based on a database snapshot taken on February 28, 2025, and performed using SPSS for Windows, version 22.0 (IBM Corp., Armonk, NY, USA). The primary endpoint was the change in the expression of the surface molecules in the CD34+ cell subpopulation between baseline and follow-up. Secondary endpoints included feasibility, changes in the CHIP allelic variant frequency (VAF) (if available) and the ALSFRS-R score.

Ethics: All patients signed informed consent to participate in the study. The protocol was approved by Ethics Committee at Far Eastern Federal University (№204-2023).

RESULTS

The patients received granulocyte colony-stimulating factor (5 μg/kg/day) subcutaneously for four days. The peripheral blood mononuclear cells (PBMCs) were collected on a Spectra Optia continuous flow separator (Terumo BCT Inc., Lakewood, CO, USA). Patients received fludarabine 25 mg/m^2/day, on days one and two (SD = 50 mg/m^2). The dose of fludarabine was selected to ensure lymphodepletion and effects on memory T cells while minimizing side effects. Previously harvested CD34+ cells were reinfused intravenously 48 hours after immunomodulatory therapy after *ex vivo*. incubation with human placenta double-stranded DNA fragments (Panagen®, Panagen LLC, Millersville, PA, USA) to induce HSC proliferation. Details of the clinical study protocol of Panagen® and its mechanism of action on a hematopoietic stem cell can be found in [11].

Two patients (20%) experienced transient increasing sensation of weakness and difficulty breathing during filgrastim administration. None of the patients experienced > grade 1 NCI CTCAE v5.0 toxicity during fludarabine therapy. The mean reinfusion dose was $2 \pm 0.3 \times 10^6$ CD34+/kg (CI 95%: 1.3–2.6). Median follow-up from the start of the therapy to control workup was 5 ± 1 months (CI 95%: 4.6–6). The mean ALSFRS-R score at the time of control workup was 40.4 ± 0.7 (CI 95%: 38.8–42), $p > 0.05$.

Somatic mutations were detected in three patients (30%) before therapy. After therapy, somatic mutations were not detected in two patients. A decrease in VAF was observed in one case (Table 1). No somatic mutations were detected in the donors.

No significant differences were found between CD34-subsets in healthy donors and ALS patients at baseline examination (Table 2).

Despite the fact that the number of CD34+ HSCs in the BM of ALS patients before and after therapy was not

Table 1. Dynamics of somatic mutations in patients with amyotrophic lateral sclerosis during therapy

Patients	Age (years)	Somatic mutation in bone marrow (gene, dbSNP, VAF (%))	
		Before therapy (baseline)	After therapy (follow-up)
#1	66.6	*DNMT3A*, rs151168784, 6.6%	*DNMT3A*, rs151168784, 3.9%
#2	45.5	*PTEN*, rs35632884, 6.4%	Not detected
#3	54.6	*ASXL1*, rs2011586997, 3% *ASXL1*, rs2145387839, 2.4% *CBL*, rs886041500, 3%	Not detected

dbSNP – database of single nucleotide polymorphisms; VAF – variant allele frequency

Table 2. Subsets of CD34+ cells in bone marrow of amyotrophic lateral sclerosis (ALS) patients before treatment compared to healthy donors

Subsets	Healthy donors, % (mean ± SD)	ALS, baseline, % (mean ± SD)	p
CD34+	0.9 ± 0.09	0.78 ± 0.11	> 0.05
CD34+CD38+	81.9 ± 4.4	67.6 ± 6.7	
CD34+CD13+	71.8 ± 4.2	63.7 ± 3.7	
CD34+CD33+	48.1 ± 8.6	47.7 ± 7.7	
CD34+CD2+	5.9 ± 0.9	5.2 ± 1.3	
CD34+CD7+	2.7 ± 1.1	3.2 ± 1.2	
CD34+CD10+	21.3 ± 3	10.9 ± 3.9	
CD34+CD19+	17.6 ± 3.3	13.5 ± 4.1	
CD34+CD90+	29.5 ± 4.7	28.0 ± 5.7	
CD34+CD56+	2.8 ± 1.8	3.3 ± 1.1	
CD34+CD123+	22.9 ± 8.6	6.4 ± 0.95	
CD34+CD133+	66.0 ± 3	60.2 ± 5.2	
CD34+CD41+	6.4 ± 0.7	5.6 ± 1.7	
CD34+CD44+	52.9 ± 9.2	62.6 ± 7.6	
CD34+CD61+	5.5 ± 1.5	4.3 ± 1	
CD34+CD117+	76.6 ± 1.8	80.0 ± 3.2	
CD34+HLA-DR	90.5 ± 3.7	81.7 ± 3	
CD34+CD45low	88.6 ± 2.3	91.4 ± 1.6	

SD – standard deviation; HLA – human leukocyte antigen

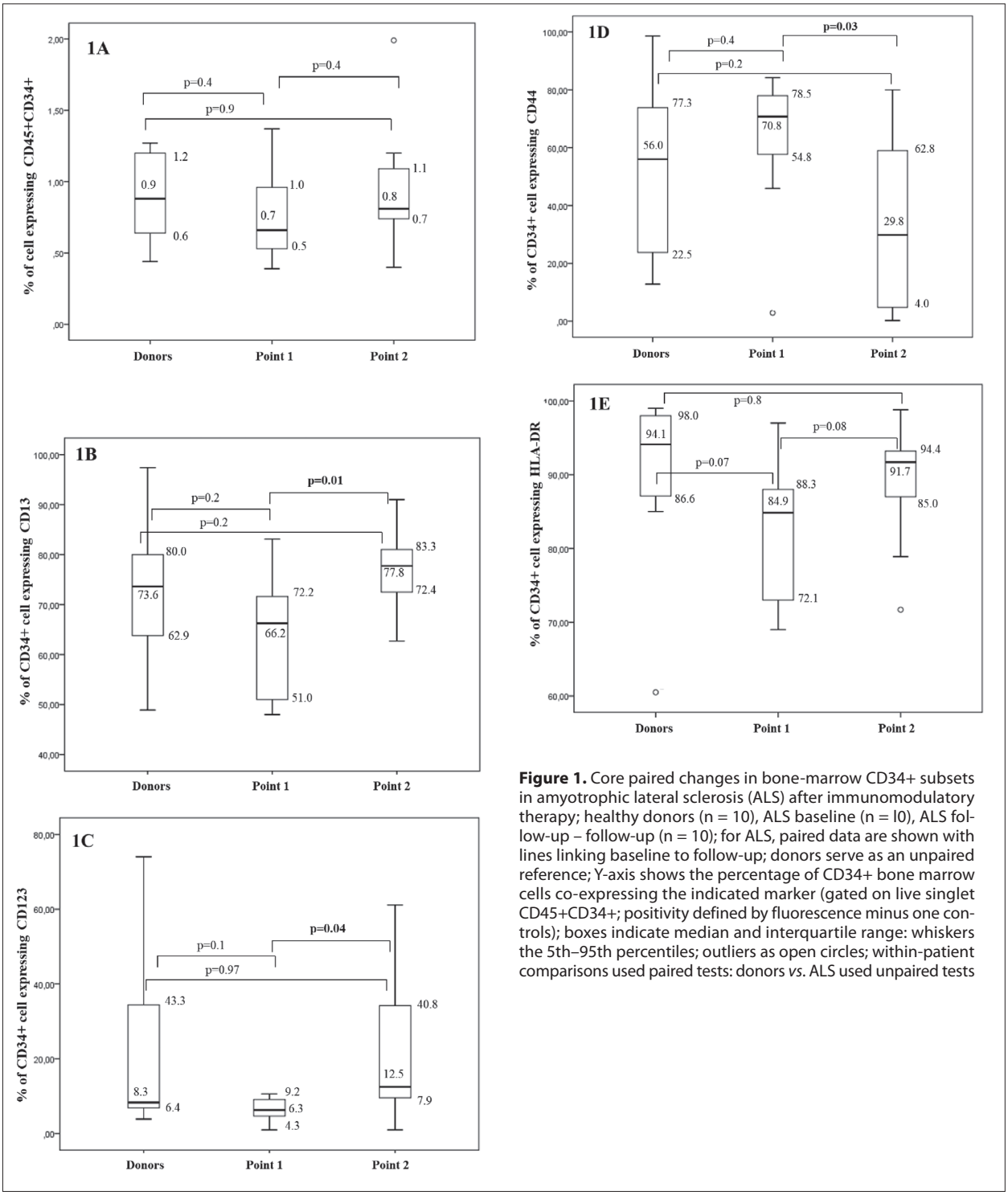

Figure 1. Core paired changes in bone-marrow CD34+ subsets in amyotrophic lateral sclerosis (ALS) after immunomodulatory therapy; healthy donors (n = 10), ALS baseline (n = l0), ALS follow-up – follow-up (n = 10); for ALS, paired data are shown with lines linking baseline to follow-up; donors serve as an unpaired reference; Y-axis shows the percentage of CD34+ bone marrow cells co-expressing the indicated marker (gated on live singlet CD45+CD34+; positivity defined by fluorescence minus one controls); boxes indicate median and interquartile range: whiskers the 5th–95th percentiles; outliers as open circles; within-patient comparisons used paired tests: donors *vs*. ALS used unpaired tests

significantly different (0.9% ± 0.09% (CI 95%: 0.7–1.1) and 0.78% ± 0.11% (CI 95%: 0.53–1), respectively), the level of CD34+ cells after therapy (follow-up) approached the values of healthy donors (Figure 1A). A significant increase in the number of CD34+CD13+ and CD34+CD123+ HSCs was detected after the reinfusion of transdifferentiated CD34+ HSCs [63.7 ± 0.9 (CI 95%: 55.3–72) *vs.* 78.1 ± 2.6 (CI 95%: 72.2–84.1) and 6.4 ± 0.9 (CI 95%: 4.2–8.5) *vs.* 23.4 ± 7.1 (CI 95%: 7.4–39.4) at baseline and follow-up, respectively]. The level of CD34+CD44+ in BM significantly decreased from [62.6 ± 7.6 (CI 95%: 45.4–79.7) to 34 ± 9.5 (CI 95%: 12.4–55.5) at baseline and follow-up, respectively]. There was a trend towards a decrease in surface expression level of class II (human leukocyte antigen) molecules on CD34+ in ALS patients before the start of therapy with its subsequent recovery [81.7 ± 3 (CI 95%: 75.0–88.4) *vs.* 89.5 ± 8.4 (CI 95%: 83.5–95.5) at baseline and follow-up, respectively] (Figure 1B–1E). Levels of lymphoid-leaning CD34+CD2+ and CD34+CD56+, but not CD34+CD7+, showed a trend toward increased mean value and widened confidence intervals compared with the values before treatment (baseline), although they did not reach statistical

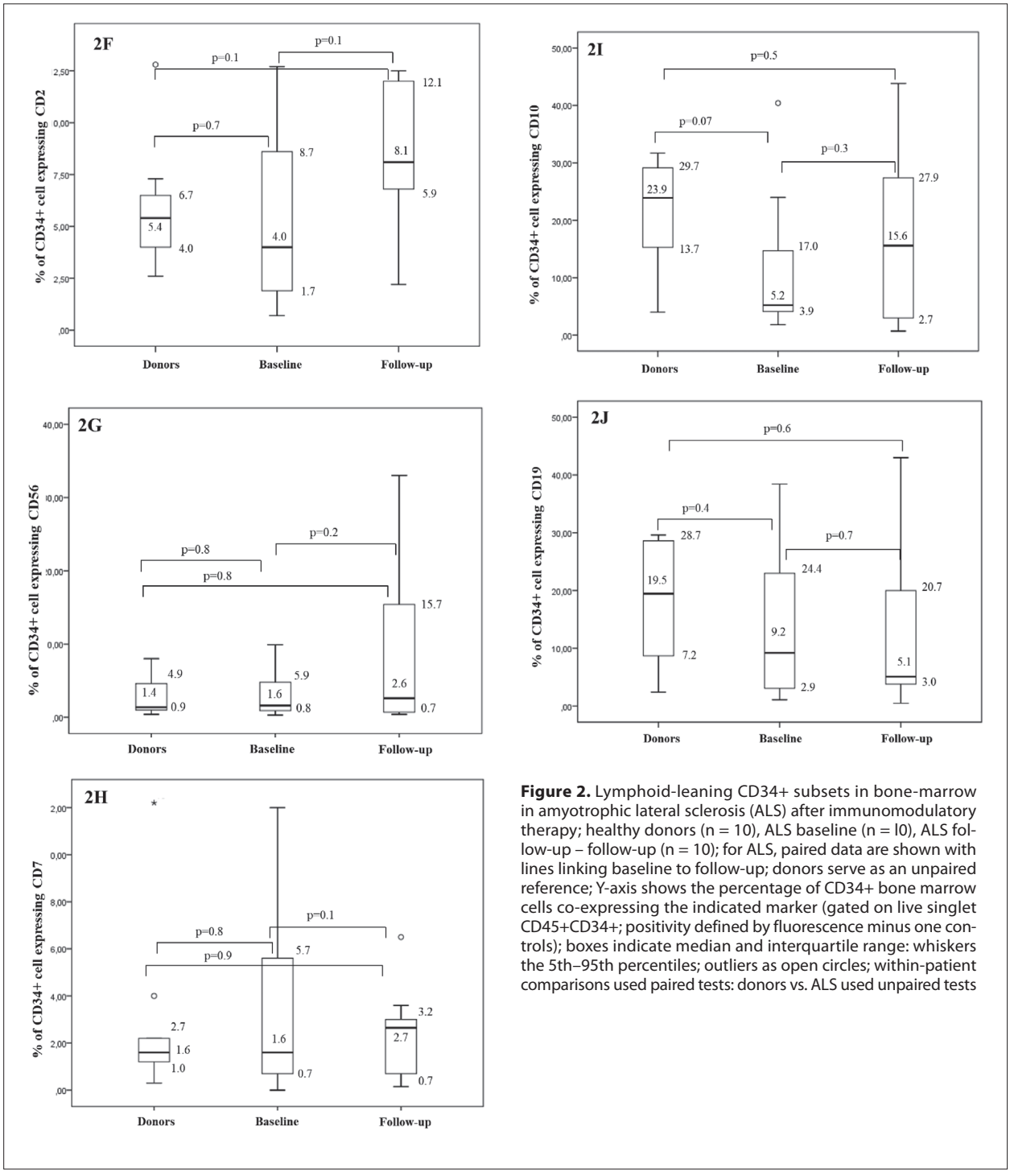

Figure 2. Lymphoid-leaning CD34+ subsets in bone-marrow in amyotrophic lateral sclerosis (ALS) after immunomodulatory therapy; healthy donors (n = 10), ALS baseline (n = l0), ALS follow-up – follow-up (n = 10); for ALS, paired data are shown with lines linking baseline to follow-up; donors serve as an unpaired reference; Y-axis shows the percentage of CD34+ bone marrow cells co-expressing the indicated marker (gated on live singlet CD45+CD34+; positivity defined by fluorescence minus one controls); boxes indicate median and interquartile range: whiskers the 5th–95th percentiles; outliers as open circles; within-patient comparisons used paired tests: donors vs. ALS used unpaired tests

significance [5.3 ± 1.3 (CI 95%: 2.3–8.1) *vs*. 8.2 ± 1.2 (CI 95%: 5.6–10.8), 3.3 ± 1.1 (CI 95%: 0.7–5.8) *vs*. 7.7 ± 3.4 (CI 95%: 0.03–15.4), 3.2 ± 1.2 (CI 95%: 0.5–5.9) *vs*. 2.5 ± 0.6 (CI 95%: 1.2–3.8) at baseline and follow-up, respectively] (Figure 2F–2H). A trend towards a decrease in the level of early B-lymphocyte precursors (CD34+CD10+) in ALS patients was revealed at baseline. At follow-up, there was a tendency to restore the level of CD34+CD10+ with a decrease in the level of later precursors (CD34+CD19+), which is probably associated with the effect of fludarabine on B-lymphocytes [10.9 ± 3.9 (CI 95%: 2.1–19.8) *vs*. 17.2 ± 4.5 (CI 95%: 7.0–27.4) and 13.5 ± 4.1 (CI 95%: 4.2–22.7) *vs*. 12.1 ± 4.3 (CI 95%: 2.5–21.8) at baseline and follow-up, respectively] (Figure 2I and 2J). The mean values of myeloid-leaning markers, particularly CD34+CD117+, CD34+CD90+ CD34+CD133+, CD34+CD33+ and CD34+CD38+ did not undergo significant changes and was defined as [80 ± 3.2 (CI 95%: 72.6–87.1) *vs*. 70.8 ± 8.1 (CI 95%: 52.6–89.1), 28 ± 5.7 (CI 95%: 15.1–41) *vs*. 21.8 ± 3.0 (CI 95%: 15–28.6), 60.2 ± 5.2 (CI 95%: 48.4–72) *vs*. 62.0 ± 4.2 (CI 95%: 52.6–71.5), 47.7 ± 7.7 (CI 95%: 30.2–65.1) *vs*. 53.1 ± 3.4 (CI 95%: 45.5–60.8) and 67.6 ± 6.7 (CI 95%: 52.2–82.6) *vs*. 73.0 ± 5 (CI 95%: 61.6–84.6) at baseline and follow-up, respectively] (Figure 3K–3O).

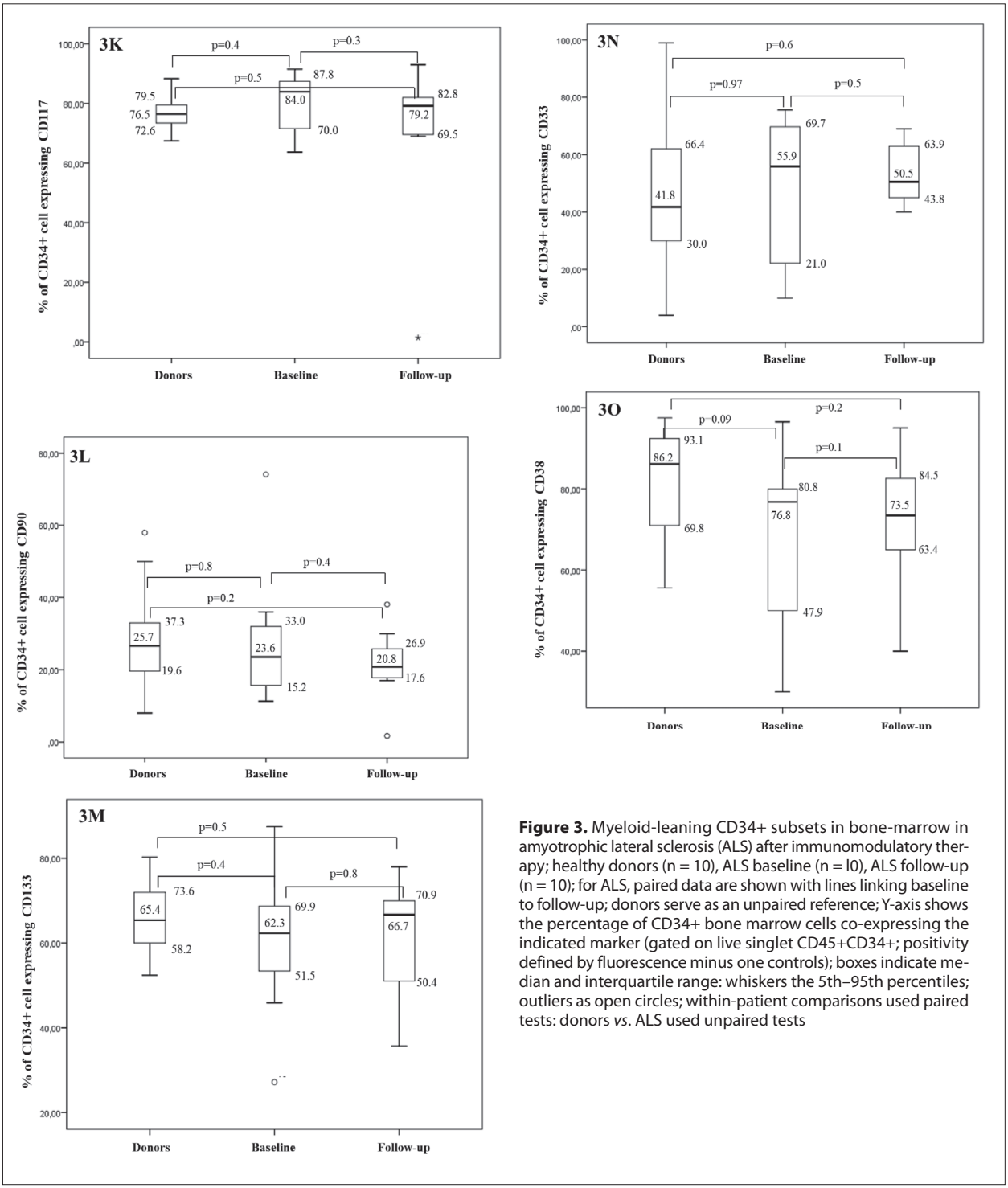

Figure 3. Myeloid-leaning CD34+ subsets in bone-marrow in amyotrophic lateral sclerosis (ALS) after immunomodulatory therapy; healthy donors (n = 10), ALS baseline (n = l0), ALS follow-up (n = 10); for ALS, paired data are shown with lines linking baseline to follow-up; donors serve as an unpaired reference; Y-axis shows the percentage of CD34+ bone marrow cells co-expressing the indicated marker (gated on live singlet CD45+CD34+; positivity defined by fluorescence minus one controls); boxes indicate median and interquartile range: whiskers the 5th–95th percentiles; outliers as open circles; within-patient comparisons used paired tests: donors *vs*. ALS used unpaired tests

DISCUSSION

Uncontrolled or prolonged neuroinflammation is potentially harmful resulting in cellular damage and exacerbates the severity of neurodegenerative diseases such as Parkinson's disease, MS and ALS [12, 13]. This is particularly relevant to neurodegenerative diseases, which are typified by evidence of microglial activation and pro-inflammatory cytokine's oversecreting [7, 14]. Growing evidence suggests that, in addition to microglia, several other subsets of innate immune cells, including macrophages, monocytes, neutrophils, NK cells, and T cells are involved in the pathogenesis of ALS [8, 15]. Evidence has been provided that the overexpression of inflammatory cytokines such as tumor necrosis factor α (TNF-α), IL-1β, IL-2, IL-6, and IL-8 in blood, CSF, and spinal cord tissues are elevated in ALS patients relative to healthy donors [16].

Only single publications have focused on the role of BM as a contributing factor in the spread of neuroinflammation and autoimmune damage [9, 17]. In our study, we found no significant differences in BM CD34+ cell subsets in ALS patients compared to donors. However,

after immunomodulatory therapy there was a significant increase in the CD34+CD13+ and CD34+CD123+ and a decrease in the cells expressing CD34+CD44+ in the patient's BM (Figure 1B–1D).

In BM, the expression of CD123, the alpha chain of IL-3 receptor, is limited to a sub-population of normal stem cells and few lymphoid progenitors. CD123-expressing progenitor cells have increased resistance to apoptosis and high proliferative activity [18]. In humans, IL-3 promotes the activation of plasmacytoid dendritic cells, which are involved in the maintenance of T-cell tolerance [19]. IL-3 suppresses Th17 differentiation and enhances differentiation towards Th2 lymphocytes. In addition, IL-3 promotes the differentiation of naive CD4+CD45RA+ T cells into CD3+CD4+CD25+CD127- regulatory T cells and promote the migration of regulatory T cells by changing in kinase phosphorylation and actin cytoskeleton structure [20]. In Alzheimer's disease (AD), IL3Rα expression in postmortem brain samples is elevated in frontal cortex tissues and correlates with both disease duration and β-amyloid (Aβ) levels. Exposure to IL-3 mediated by CD123+ induces transcriptional, morphological, and functional reprogramming of microglia, endowing them with an acute immune response program, increased motility and the ability to cluster and clear Aβ and tau aggregates [21]. On the other hand, in MS, CD123 is expressed mainly by microglia and recruited myeloid cells in the spinal cord, leading to infiltration by immune cells, increasing the severity of MS [22]. Thus, this discrepancy between AD and MS indicates a dual role of IL-3 in CNS inflammation, when the same mechanism (reprogramming of IL-3R+ myeloid cells) leads to two different consequences (favorable in AD and detrimental in MS).

The transmembrane aminopeptidase CD13 is highly expressed in myeloid lineage cells, hematopoietic progenitors, and stem cells. The CD34+CD13+ subset is found in donor BM and cord blood, reflecting an early stage of human myeloid cell differentiation. The CD13 expression on CD34+ cells precede CD33 expression and is associated with early hematopoietic cells, in the absence of lineage-associated markers [23]. In our observations, BM cells showed no increase in expression of CD117, CD90 and CD133 as well as CD33 and CD38 (Figure 3H–3O).

The adhesion molecule CD44 and its major ligand, hyaluronic acid, play an important role in the migration of normal CD34+ cells [23]. Interestingly that G-CSF-mediated mobilization of stem cells into the peripheral bloodstream results in increased expression of matrix metalloproteinases to which CD13 belongs. As a result, cleavage is activated and CD44 level on BM cells decreases [24, 25]. Our study also demonstrated a negative correlation between the levels of CD34+CD13+ and CD34+CD44+, which may indicate the mobilization of progenitor cells with a certain immunophenotype (probably not expressing CD13) into blood. The detected challenges in the BM may indicate that transfused transdifferentiated *ex vivo* CD34+ HSCs managed to startle out of "steady state" and to start the processes of linear differentiation. We believe that the use of fludarabine and G-CSF were not able to alter BM adhesion molecules (e.g., CD44) due to the fact that the follow-up was carried out for at least five months, which significantly exceeds the period of action of the above-mentioned drugs.

Little is known about the link between CHIP and the risk of neurodegenerative diseases. A recent study found an increased risk of neurodegenerative diseases in patients with *DNMT3A*-mutant CHIP*, ASXL1*-mutant CHIP*, or SRSF2*-mutant CHIP [26]. However, this association does not appear to be unidirectional. Bouzid et al. [27] revealed the causal role for CHIP in reducing AD dementia risk.

Detection of somatic mutations was not the main aim of our study. However, CHIP was detected in three out of 10 ALS patients. After mild immunomodulatory therapy and reinfusion of transdifferentiated autologous CD34+ cells, the mutated clone in the BM fell below detection at the applied coverage. This finding requires a larger patient cohort, a longer follow-up period and the use of NGS technology with high coverage and error correction.

We hypothesized that *ex vivo* manipulated quiescent HSCs may result in restoring a loss of clonal diversity in the entire blood system. It is possible that CHIP was not eliminated, but a reduction in VAF of somatic mutant clones below their detection level at × 100 coverage was achieved. Nevertheless, our data give new look at the problem of controlling unwanted cell clones in BM and peripheral blood to reduce chronic systemic inflammation. Nevertheless, our data provide new insights into the challenge of targeting unwanted cell clones in BM and peripheral blood to reduce chronic systemic inflammation. We have initiated a clinical protocol in ALS patients involving *ex vivo* reinfusion of manipulated autologous CD34+ cells after repeated courses of fludarabine and tocilizumab. The new targeted gene sequencing panel with a higher coverage (× 2000) will provide more information on CHIP dynamics under treatment.

Our hypothesis-generating study has limitations. First, this was a preliminary study investigating the relationship between the CD34+ subsets, CHIP, and the clinical course of ALS; therefore, a power calculation for the sample size was not performed. Consequently, the statistical power may have been insufficient to detect the influence of these factors on therapy outcomes and laboratory findings. Second, the small sample size did not allow us to exclude the random nature of the data obtained in the follow-up period. Third, immune cell subsets were not assessed simultaneously in peripheral blood and, for obvious reasons, in brain and spinal cord tissue.

CONCLUSION

Our study is the first attempt to characterize the subsets of BM HSCs in ALS patients and to reveal changes in their follow-up patterns under the immunomodulatory therapy. Our results have clinical significance, although they are limited and preliminary. First, they demonstrate that BM is one of the organs responding to immune-mediated neuroinflammation. Second, the issue of whether the abnormal

immune response leading to neurodegeneration can be restarted and corrected is raised. In addition, preliminary results indicate a possible link between CHIP and ALS and point the way to eliminating aberrant clones.

Author contributions: Kovalenko NI, Bryukhovetsky AS collected, analyzed and interpreted the clinical data; Shatalov PA contributed to sequencing data collection and carried out the mutation analysis; Grivtsova LY contributed to immunophenotyping data collection, carried out the CD34+ subset analysis, and edited the manuscript; Dolgopolov IS, Rykov MYu. performed the data analyses, wrote the manuscript, supervised and revised the manuscript for intellectual content.

Conflict of Interest: None declared.

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Имунологија *CD*34 субпопулација коштане сржи и клонална хематопоеза неодређеног потенцијала у амиотрофичној латералној склерози

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САЖЕТАК

Увод Савремена сазнања о патогенези неуродегенеративних болести указују на то да је инфламација кључни фактор њихове прогресије.

Методе Група са амиотрофичном латералном склерозом обухватила је 10 болесника [просечна старост 55,1 ± 3,1 година (*CI* 95%: 48–62,2)]. Секвенцирање целог егзома и имунофенотипизација *CD34+* субпопулација у коштаној сржи обављени су пре терапије и после шест месеци. Контролну групу је чинило 10 здравих донора, просечне старости 39,9 ± 3,9 година (*CI* 95%: 31,2–48,6).

Резултати Матичне ћелије периферне крви прикупљане су после четвородневне примене фактора стимулације колонија гранулоцита. Просечан број добијених *CD34+* ћелија био је 290,4 ± 53,5 × 10^6 (*CI* 95%: 177,6–403,3). Болесници су примали флударабин у дози од 25 *mg*/m^2 дневно, првог и другог дана. Ради индуковања трансдиференцијације хематопоетских матичних ћелија, матичне ћелије периферне крви су инкубиране *ex vivo* са фрагментима дволанчане ДНК људске плаценте и реинфундоване 48 сати после примене флударабина. Клонална хематопоеза неодређеног потенцијала детектована је у три случаја (30%) пре терапије. Забележено је значајно повећање *CD34+CD13+* и *CD34+CD123+* хематопоетских матичних ћелија у коштаној сржи. Нивои *CD34+CD44+* ћелија значајно су смањени. Нивои *CD34+CD7+*, *CD34+CD2+* и *CD34+CD56+* показали су тренд пораста просечних вредности после третмана. У два случаја, клонална хематопоеза неодређеног потенцијала је нестала, док је у једном случају примећено смањење учесталости варијантног алела. Просечан резултат Ревидиране функционалне скале за амиотрофичну латералну склерозу остао је непромењен [39 ± 0,6 поена (*CI* 95%: 37,6–40,4) наспрам 40,4 ± 0,7 (*CI* 95%: 38,8–42)].

Закључак Наша студија представља први покушај карактеризације субпопулација хематопоетских матичних ћелија коштане сржи код амиотрофичне латералне склерозе. Резултати показују способност коштане сржи да реагује на имунолошки посредовану неуроинфламацију. Прелиминарни резултати указују на могућу везу између клоналне хематопоезе неодређеног потенцијала и амиотрофичне латералне склерозе, отварајући пут ка елиминацији аберантних клонова.

Кључне речи: *CD34* субпопулације; коштана срж; амиотрофична латерална склероза; клонална хематопоеза неодређеног потенцијала; флударабин