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

Имунологија ЦД34 субпопулација у коштаној сржи и клонска хематопоеза са неодређеним потенцијалом у амиотрофичној латералној склерози

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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 ALS group included 10 patients (mean age 55.1 ± 3.1 years (95% CI, 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 6 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 PBSCs were collected after four-day G-CSF administration. The total mean number of collected CD34+ cells was 290.4 ± 53.5x10^6 (95% CI, 177.6-403.3). Patients received fludarabine 25 mg/m^2/day, on days 1 and 2. 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. CHIP was detected in 3 cases (30%) before therapy. A significant increase CD34+CD13+ and CD34+CD123+ 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 VAF has been shown. The mean ALSFRS-R score did not change (39.0 ± 0.6 points (CI 95%, 37.6-40.4) vs. 40.4 ± 0.7 (CI 95%, 38.8-42.0).

Conclusion Our study is the first attempt to characterize the subsets of bone marrow 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

САЖЕТАК

Увод Истраживање неуродегенеративних болести показало је да је упала главн покретач прогресије.

Методе Група са АЛС обухватала је 10 пацијената (просечна старост 55,1 ± 3,1 година (95% CI 48–62,2)). Секвенцирање целог ексома и имунофенотипизација CD34+ подскупова у коштаној сржи обављени су пре терапије (Baseline) и након шест месеци (Follow-up). Контролну групу је чинило 10 здравих донора.

Резултати ПБЦ су добијени 4 дана након примене G-CSF. Просечан број добијених CD34+ ћелија био је -290,4 ± 53,5 × 10^6 (95% CI 177,6–403,3). Пацијенти су примали флударабин у дози од 25 мг/$м^2$/дан 1. и 2. дана. За трансдиференцијацију, хематопоетске матичне ћелије (ХМЋ) су инкубиране са фрагментима дволанчане људске плацентарне ДНК ex vivo и реинфузиране 48 сати након флударабина. CHIP је детектован у 3 случаја (30%) пре терапије. Значајно повећање CD34+ CD13+ и CD34+CD123+ ХСК је детектовано у коштаној сржи. Нивои CD34+CD44+ су значајно смањени. Нивои CD34+CD7+, CD34+CD2+ и CD34+CD56+ показали су тренд ка повећању средњих вредности након третмана. Чип је нестао у 2 случаја, а смањење Vaf је примећено у једном случају. Просечан ALSFRS-R резултат је остао непромењен (39,0 ± 0,6 поена (95% CI, 37,6–40,4) наспрам 40,4 ± 0,7 (95% CI, 38,8–42,0).

Закључак Наша студија је први покушај карактеризације субпопулација ХМЋ коштане сржи код АЛС. Резултати показују способност коштане сржи да реагује на имунолошки посредовану неуроинфламацију. Прелиминарни резултати указују на могућу везу између CHIP и АЛС и указују на пут ка елиминацији аберантних клонова.

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

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting primarily the motor system leading to paralysis and death within 3 to 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, 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, 4-6]. 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 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 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 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 hematopoietic stem cells 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 hematopoietic stem cells involved in the somatic mutation, their differentiation, secreted cytokines and activated signaling pathways. The role of bone marrow hematopoietic cells in the development and maintenance of immune-mediated inflammation and demyelination was recently demonstrated in patients with multiple sclerosis [9].

Planning this study, we hypothesized that since bone marrow 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 hematopoietic stem cells (HSCs).

METHODS

Patients

The ALS group included 10 patients with confirmed disease (men 4, women 6), with a mean disease duration from the time of diagnosis 26.5 ± 4.0 months (CI 95%, 17.5-35.6). The median age was 53.9 ± 9.9 years (95% CI, 28-47). The median ALSFRS-R score was 39.0 ± 0.6 points (CI 95%, 37.6-40.4). The median Karnofsky score at the time of inclusion in the study was 53 ± 3 (95% CI, 46-60). All patients underwent bone marrow puncture prior to therapy start in protocol. Whole-exome sequencing and immunophenotyping of CD34+ subsets in bone marrow cells were performed at the baseline in a" steady state" before the administration of G-CSF (Baseline) and during the first 6 months of follow-up, but not before 3 months (Follow-up). The control group included 10 bone marrow donors (men 7, women 3), 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 utilizing either Roche KAPA HyperExome or Nanodigmbio NEXome Plus Panel v1.0 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 for alignment to the human reference genome (*GRch37*), Streammd v4.3.0 for marking and removal of polymerase chain reaction (PCR) duplicates, Sambamba v1.0.1 for filtering mapped variants.

For the germline analysis pipeline, the following software tools were employed: BWA2 v2.2.1 for alignment to the human reference genome (GRch37), Streammd v4.3.0 for marking and

removal of PCR duplicates, Sambamba v1.0.1 for filtering mapped variants, GATK Picard for quality control and mapping metrics assessment, Google Deepvariant v1.6.1 for nucleotide substitution detection, Bcftools v1.20 for variant filtering, and Ensembl Variant Effect Predictor release 112 for variant annotation.

The somatic analysis pipeline utilized several shared tools including BWA2 v2.2.1 for genome alignment, Streammd v4.3.0 for PCR duplicate processing, and Sambamba v1.0.1 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 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.

Bone marrow 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 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. 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 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) versus 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 versus follow-up and t-test for donors versus ALS baseline were used. Significance was set at $P \leq 0.05$ and a 95% CI. 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 (SPSS Inc., Chicago, Illinois). 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 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

Patients received granulocyte colony-stimulating factor (5 μg/kg/day) subcutaneously for 4 days. The peripheral blood mononuclear cells (PBMCs) were collected on a Spectra Optia

continuous flow separator (Terumo BCT, Lakewood, CO, USA). Patients received fludarabine 25 mg/m^2/day, on days 1 and 2 (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®) 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 in breathing during filgrastim administration. None of the patients experienced > grade 1 NCI CTCAE v5.0 toxicity during fludarabine therapy. The mean reinfusion dose was 2.0 ± 0.3x10^6 CD34+/kg (CI 95%, 1.3-2.6). Median follow-up from the start of the therapy to control workup was 5.0 ± 1.0 months (CI 95%, 4.6-6.0). The mean ALSFRS-R score at the time of control workup was 40.4 ± 0.7 (CI 95%, 38.8-42.0), p>0.05).

Somatic mutations were detected in 3 patients (30%) before therapy. After therapy, somatic mutations were not detected in 2 patients. A decrease in the allelic variant frequency (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 bone marrow of ALS patients before and after therapy was not significantly different (0.9% ± 0.09% (CI 95%. 0.7–1.1) and 0.78% ± 0.11% (CI 95%. 0.53–1.0), respectively), the level of CD34+ cells after therapy (Follow-up) approached the values of healthy donors (Fig. 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% 55.3-72.0) 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 bone marrow significantly decreased from 62.6 ± 7.6 (CI 95% 45.4-79.7) to 34.0 ± 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 (HLA) molecules on CD34+ in ALS patients before the start of therapy with its subsequent recovery (81.7 ± 3.0 (CI 95% 75.0-88.4) vs. 89.5 ± 8.4 (CI 95% 83.5-95.5) at Baseline and Follow-up, respectively) (Fig. 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 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) (Fig. 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) (Fig. 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 (80.0 ± 3.2 (CI 95% 72.6-87.1) vs. 70.8 ± 8.1 (CI 95% 52.6-89.1), 28.0 ± 5.7 (CI 95% 15.1-41.0) vs. 21.8 ± 3.0 (CI 95% 15.0-28.6), 60.2 ± 5.2 (CI 95% 48.4-72.0) 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 ± 5.0 (CI 95% 61.6-84.6) at Baseline and Follow-up, respectively) (Fig. 3K-3O).

DISCUSSION

Uncontrolled or prolonged neuroinflammation is potentially harmful resulting in cellular damage and exacerbates the severity of neurodegenerative diseases such as Parkinson's disease, multiple sclerosis 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, natural killer (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), interleukins (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 bone marrow as a contributing factor in the spread of neuroinflammation and autoimmune damage [9, 17]. In our study, we found no significant differences in bone marrow 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 bone marrow (Fig. 1B-1D).

In bone marrow, 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 multiple sclerosis (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 bone marrow 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, bone marrow cells showed no increase in expression of CD117, CD90 and CD133 as well as CD33 and CD38 (Fig. 3H-3O).

The adhesion molecule CD44 and its major ligand, hyaluronic acid (HA), 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 bone marrow 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 bone marrow 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 bone marrow adhesion molecules (e.g., CD44) due to the fact that the follow-up was carried out for at least 5 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. revealed the causal role for CHIP in reducing AD dementia risk [27].

Detection of somatic mutations was not the main aim of our study. However, CHIP was detected in 3 of 10 ALS patients. After mild immunomodulatory therapy and reinfusion of transdifferentiated autologous CD34+ cells, the mutated clone in the bone marrow 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 x100 coverage was achieved. Nevertheless, our data give new look at the problem of controlling unwanted cell clones in bone marrow and peripheral blood to reduce chronic systemic inflammation. Nevertheless, our data provide new insights into the challenge of targeting unwanted cell clones in bone marrow 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 (x2000-fold) 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 bone marrow 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 bone marrow 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 N.I., Bryukhovetsky A.S collected, analysed and interpreted the clinical data. Shatalov P.A. contributed to sequencing data collection and carried out the mutation analysis. Grivtsova L.Y. contributed to immunophenotyping data collection, carried out the CD34+ subset analysis, and edited the manuscript. Dolgopolov I.S., Rykov M.Yu. performed the data analyses, wrote the manuscript, supervised and revised the manuscript for intellectual content.

Conflict of Interest: None declared.

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Table 1. Dynamics of somatic mutations in patients with amyotrophic lateral sclerosis on the 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, 3.0% *ASXL1*, rs2145387839, 2.4% *CBL*, rs886041500, 3.0%	Not detected

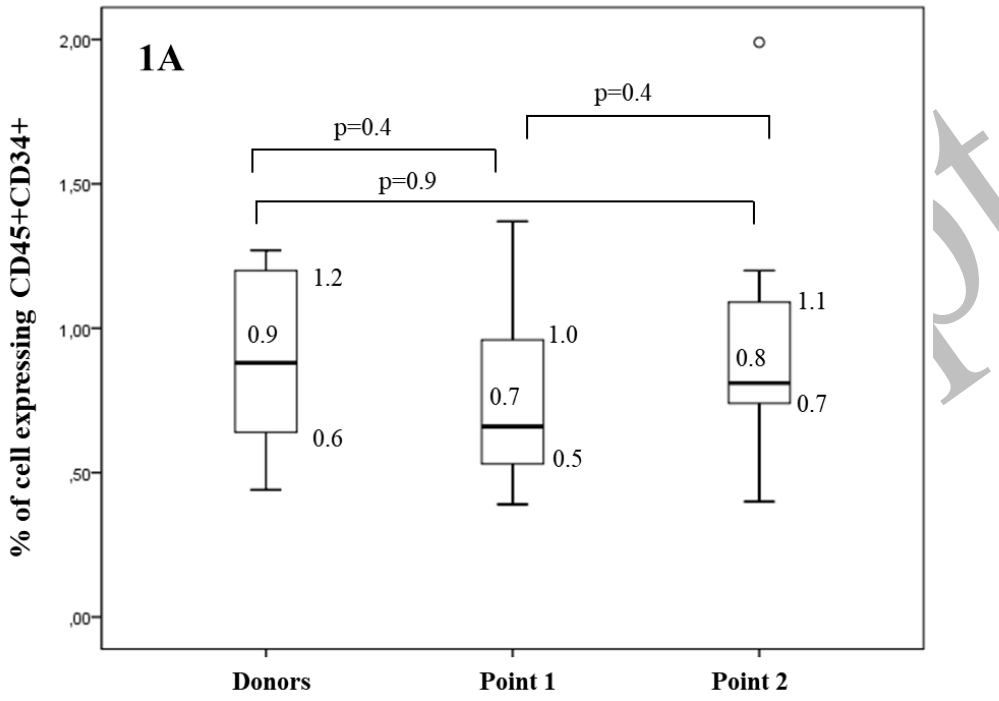
dbSNP – database of single nucleotide polymorphisms; VAF – variant allele frequency

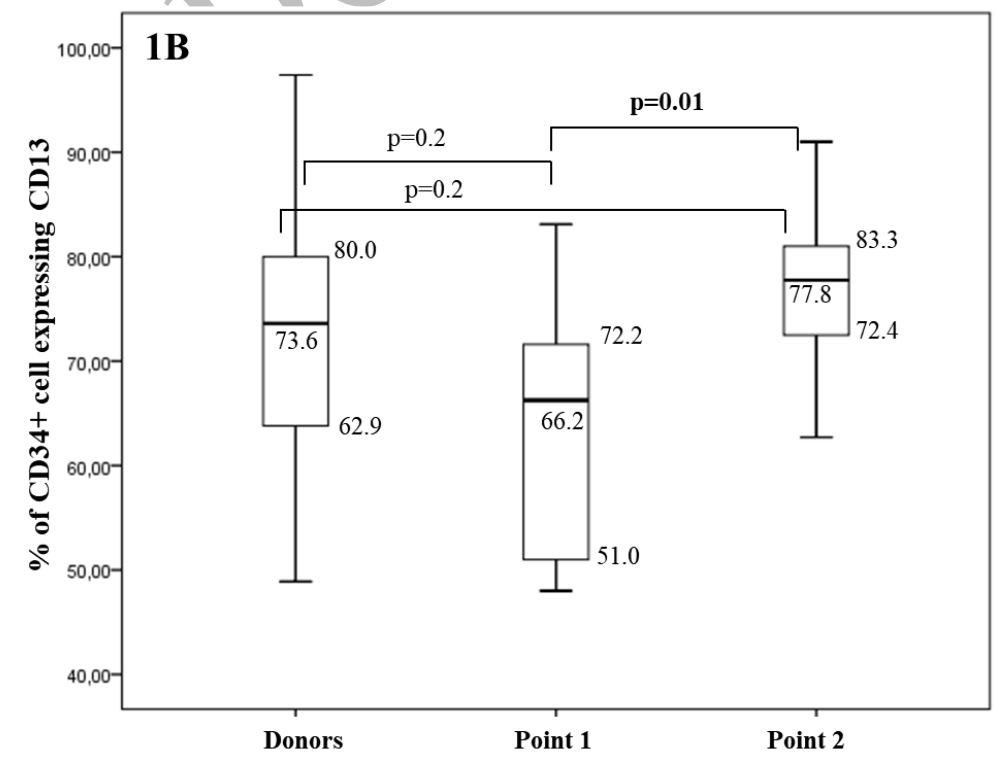
Table 2. Subsets of CD34+ cells in bone marrow of ALS patients before treatment compared to healthy donors

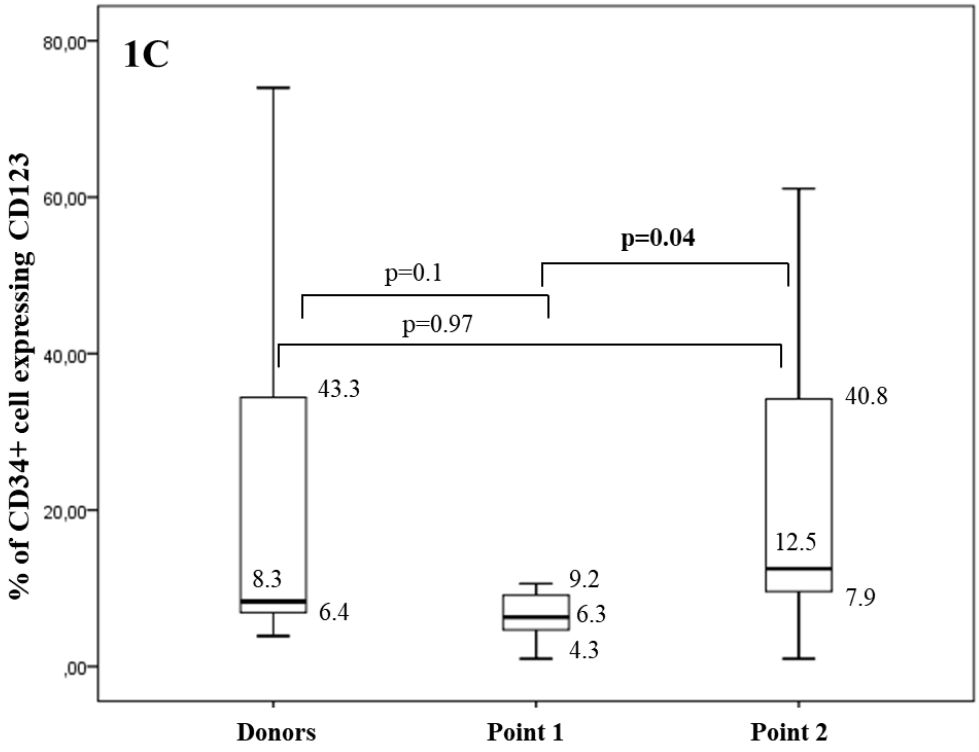
Subsets	Healthy donors, % (mean ± SD)	ALS, Baseline, % (mean ± SD)	p
CD34+	0.9 ± 0.09	0.78 ± 0.11	> 0.05
CD34+CD38+	81.9 ± 4.4	67.6 ± 6.7	
CD34+CD13+	71.8 ± 4.2	63.7 ± 3.7	
CD34+CD33+	48.1 ± 8.6	47.7 ± 7.7	
CD34+CD2+	5.9 ± 0.9	5.2 ± 1.3	
CD34+CD7+	2.7 ± 1.1	3.2 ± 1.2	
CD34+CD10+	21.3 ± 3	10.9 ± 3.9	
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	

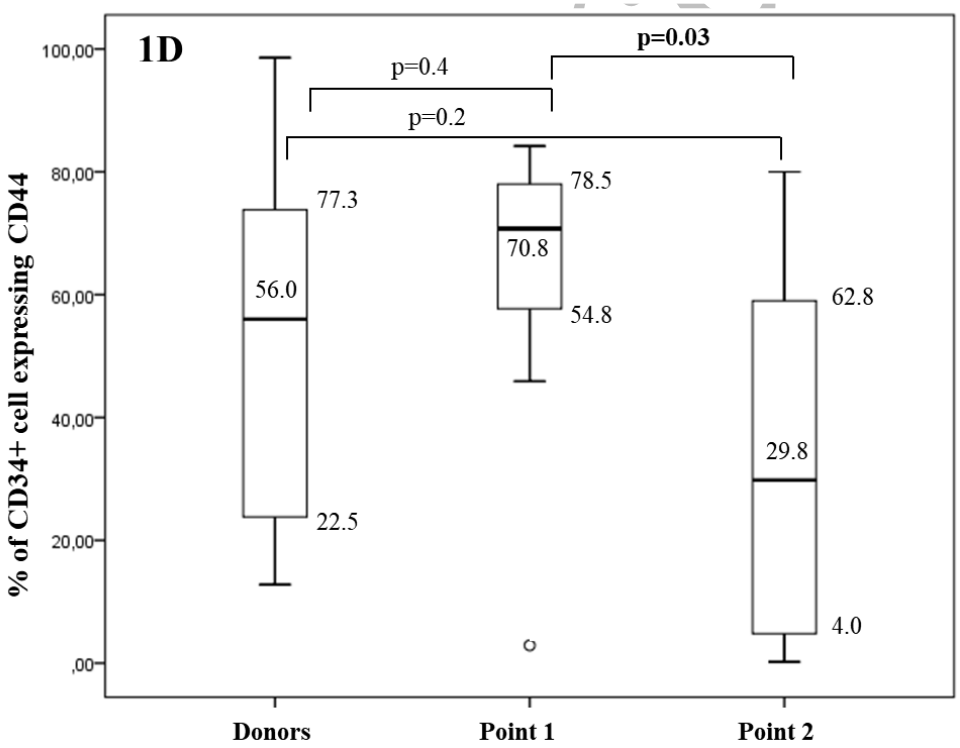
ALS – amyotrophic lateral sclerosis; SD – standard deviation

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









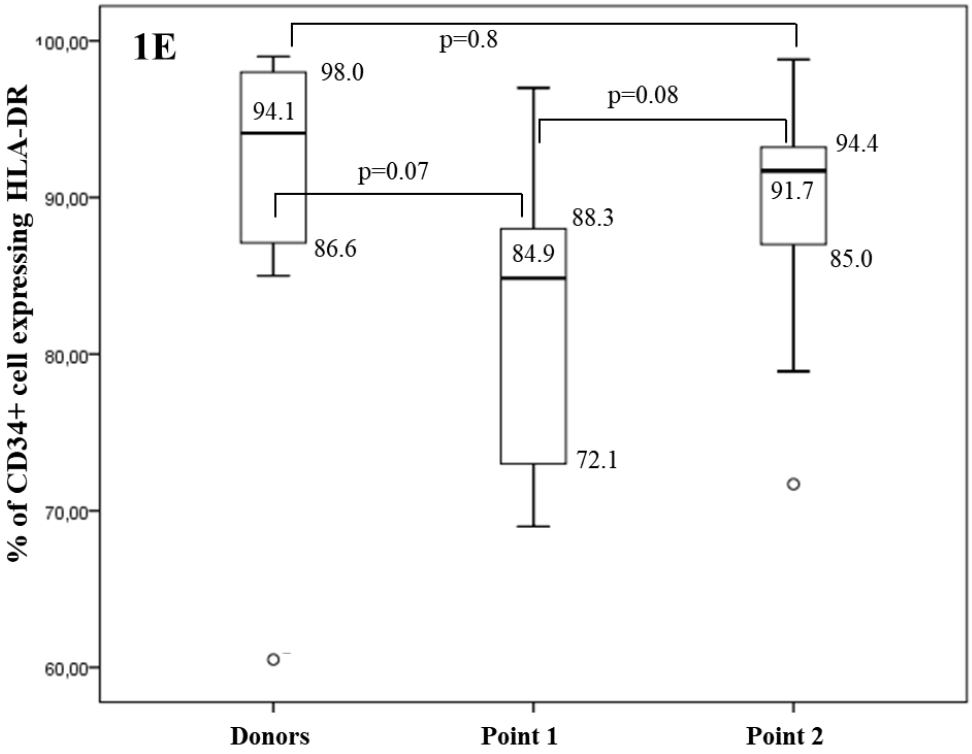
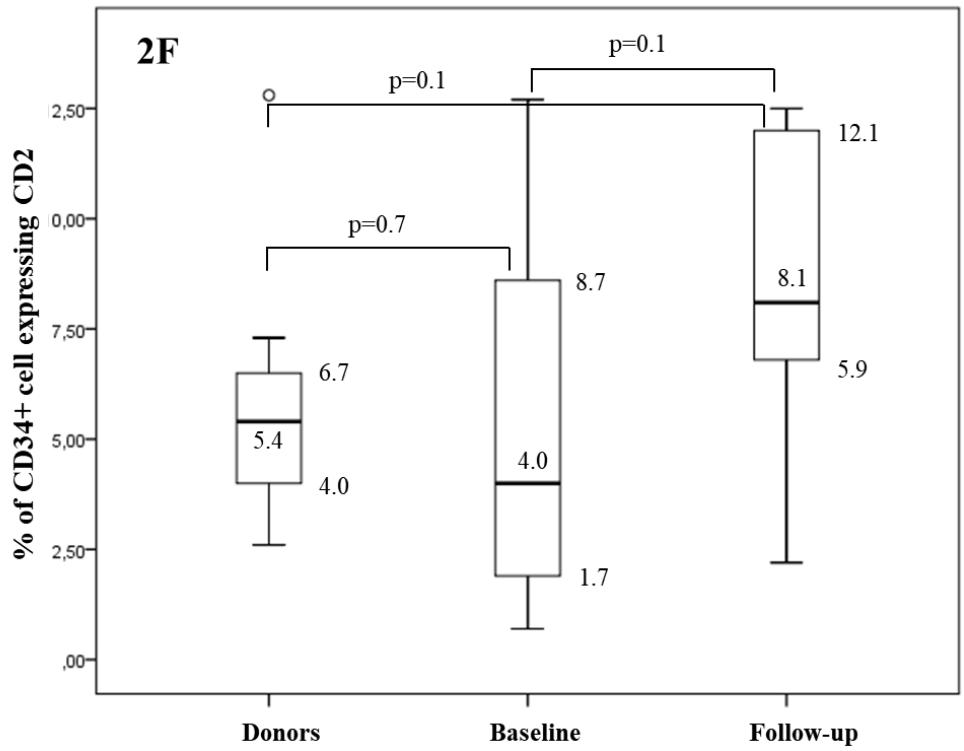
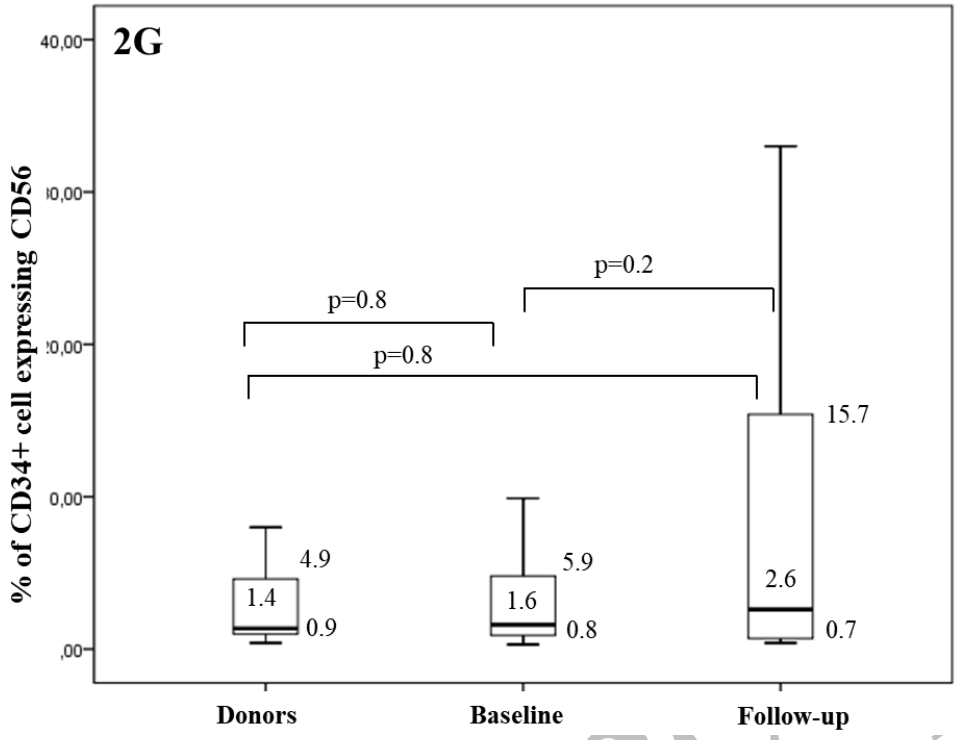
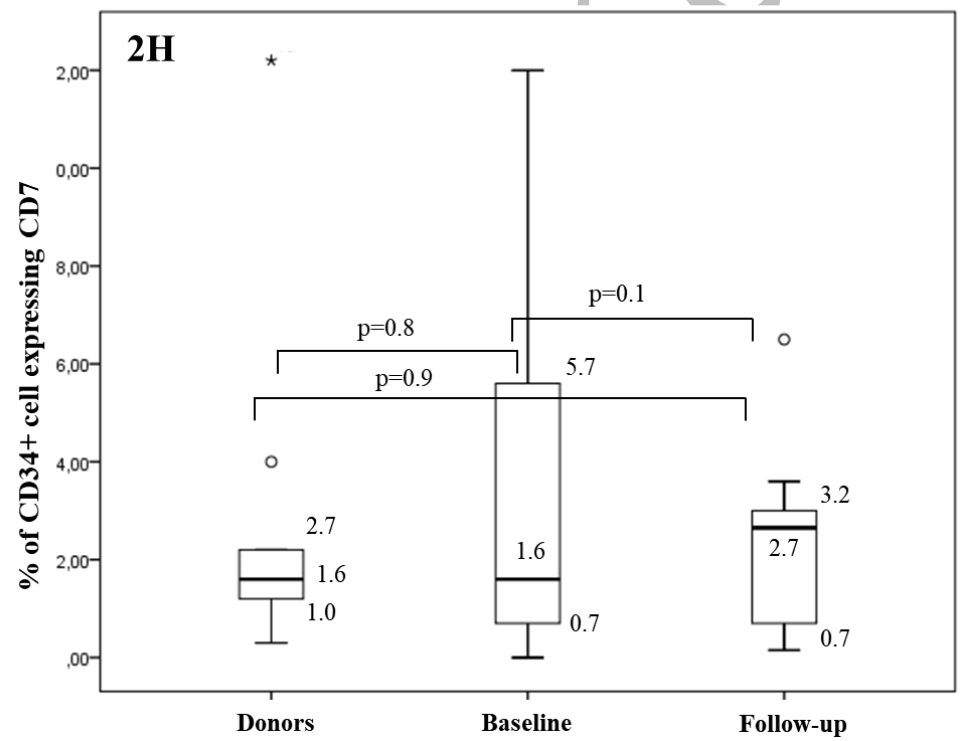
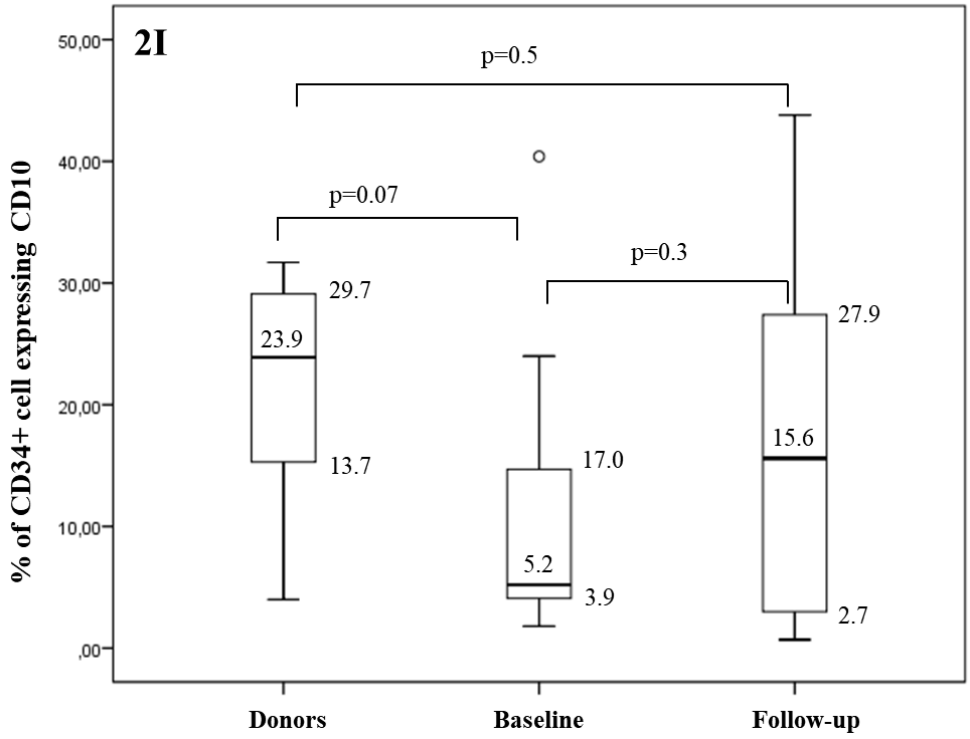


Figure 2. Lymphoid-leaning CD34+ subsets in bone-marrow in amyotrophic lateral sclerosis (ALS) after immunomodulatory therapy; healthy donors (n = 10), ALS baseline (n = l0), ALS follow-up – follow-up (n = 10); for ALS, paired data are shown with lines linking baseline to follow-up; donors serve as an 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









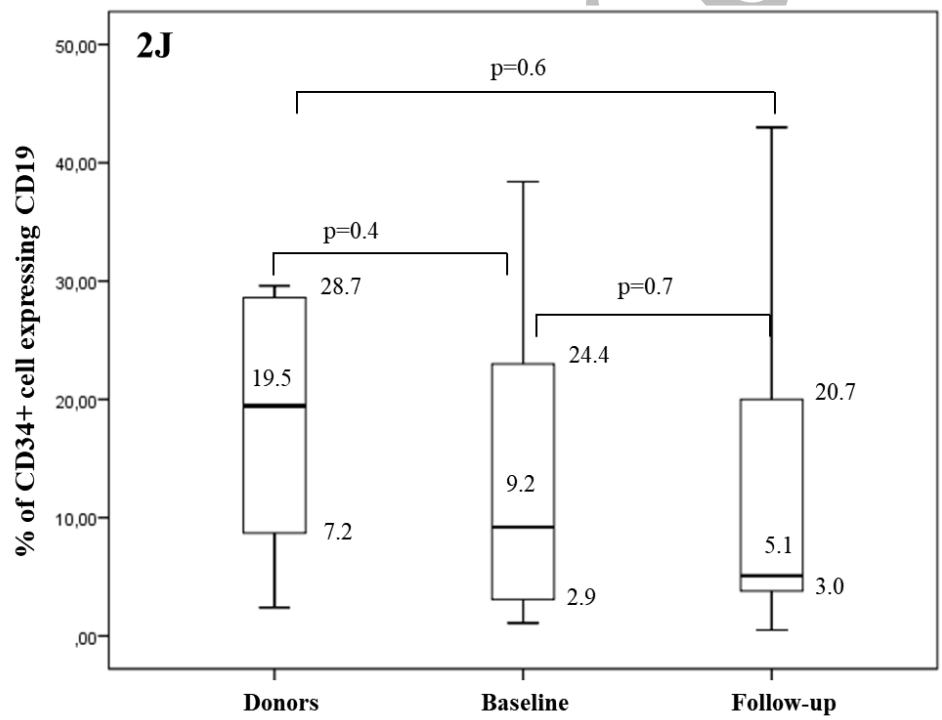


Figure 3. Myeloid-leaning CD34+ subsets in bone-marrow in amyotrophic lateral sclerosis (ALS) after immunomodulatory therapy; healthy donors (n = 10), ALS baseline (n = l0), ALS follow-up (n = 10); for ALS, paired data are shown with lines linking baseline to follow-up; donors serve as an unpaired reference; 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