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Effects of continuous high-dose G-CSF administration on hematopoietic stem cell mobilization and telomere length in patients with amyotrophic lateral sclerosis – a pilot study

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease of complex and still poorly understood etiology. Loss of upper and lower motoneurons results in death within few years after diagnosis. Recent studies have proposed neuroprotective and disease-slowing effects of granulocyte-colony stimulating factor (G-CSF) treatment in ALS mouse models as well as humans. In this study, six ALS patients were monitored up to 3.5 years during continuous high-dose G-CSF administration. Repetitive analyses were performed including blood count parameters, $CD34^+$ hematopoietic stem and progenitor cell (HSPC) and colony forming cell (CFC) counts, serum cytokine levels and leukocyte telomere length. We demonstrate that continuous G-CSF therapy was well tolerated and safe resulting in only mild adverse events during the observation period. However, no mobilization of $CD34^+$ HSPC was detected as compared to baseline values. CFC mobilization was equally low and even a decrease of myeloid precursors was observed in some patients. Assessment of telomere length within ALS patients' leukocytes revealed that G-CSF did not significantly shorten telomeres, while those of ALS patients were shorter compared to age-matched healthy controls, irrespective of G-CSF treatment. During G-CSF stimulation, TNF-alpha, CRP, IL-16, sVCAM-1, sICAM-1, Tie-2 and VEGF were significantly increased in serum whereas MCP-1 levels decreased. In conclusion, our data show that continuous G-CSF treatment fails to increase circulating $CD34^+$ HSPC in ALS patients. Cytokine profiles revealed G-CSF-mediated immunomodulatory and proteolytic effects. Interestingly, despite intense G-CSF stimulation, telomere length was not significantly shortened.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by a progressive loss of upper and lower motoneurons with no effective treatment so far [1]. Due to paresis, muscle atrophy and respiratory and/or cardiac failure, patients die within one to four years [2] whereby the only approved drug for treatment (riluzole) prolongs the life span for approximately three months [3]. In recent years, the hematopoietic growth factor granulocyte-colony stimulating factor (G-CSF) has emerged as a potential option to treat ALS. Several studies revealed neuroprotective, anti-inflammatory and anti-apoptotic effects of G-CSF as well as a delayed disease progression in different ALS animal models ([4], reviewed in [5]). Trans-differentiation and co-localization of mobilized $CD34^+$ hematopoietic stem and progenitor cells (HSPC) in neuronal tissues have been discussed as possible mechanisms [6,7]. Other studies investigated therapeutic effects of G-CSF in regard to cytokine levels [8–10], since inflammation was shown to be involved in ALS pathogenesis and progression [11].

G-CSF is used to treat severe neutropenia [12] and in the context of hematopoietic stem cell transplantation (HSCT) [13]. Administration of G-CSF leads to an expansion of myeloid progenitors [14] and an egress of $CD34^+$ HSPC from the bone marrow (BM) into the peripheral blood

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(PB) [15]. Mobilization is mediated by several mechanisms, including disruption of the CXCL12-CXCR4 and VLA-4-VCAM-1 axis, which play crucial roles in maintaining HSC homeostasis within the BM niche [16]. G-CSF induces a proteolytic environment and down-regulation of homing molecules in the BM, thereby leading to enhanced circulation of HSPC [17,18]. A common G-CSF dose regime used for HSCT consists of injections of five to 10 µg per kg bodyweight for five consecutive days [19]. In a previous study, we could show that repetitive administration of G-CSF in a five-day dose regime for up to three years is feasible and safe in ALS patients [20]. In the present work, we now sought to analyze the effect of continuous high-dose G-CSF application for up to 3.5 years on mature peripheral blood cells as well as $CD34^+$ HSPC and colony forming cell (CFC) counts. To further evaluate G-CSF effects, we monitored serum cytokines throughout the study period. In addition, as continuous G-CSF stimulation might accelerate exhaustion of the hematopoietic system, telomere lengths of ALS patients' lymphocytes and granulocytes were measured during G-CSF treatment. Our data regarding safety and tolerability of long-term G-CSF administration might serve as a basis for future investigations.

2. Patients and methods

2.1. Patients and G-CSF treatment schedule

Six ALS patients provided written informed consent for an off-label G-CSF treatment on a named patient basis. Therapy was performed in accordance with the Declaration of Helsinki and data analysis was approved by the local ethics committee of the University of Regensburg (number 15-101-0106). Additionally, six ALS patients not receiving G-CSF provided informed consent for venipuncture (number 14-101-0011). Recombinant human G-CSF (Filgrastim) was obtained from HEXAL® (HEXAL AG, Holzkirchen, Germany) and was injected subcutaneously every second day. Dose was adapted individually for each patient at the start of treatment as well as during treatment course and ranged from 30×10^6 IU to 144×10^6 IU every second day. Patients were monitored regularly including treatment related adverse events (AE), infections and spleen size. Patient characteristics are summarized in Table 1.

2.2. Analysis of peripheral blood samples

PB samples from ALS patients were obtained on a monthly basis and analyzed for white blood cell (WBC), platelet, red blood cell (RBC) count and hemoglobin levels using an automated cell counter (Sysmex, Kobe, Japan). Serum samples were centrifuged, aliquoted and stored at −20 °C until further use.

2.3. Flow cytometric analysis of $CD34^+$ HSPC

For analysis of circulating HSPC in the PB, one ml of blood was processed as described earlier [20]. In brief, donor blood samples were lysed in NH_4Cl lysis buffer and washed twice with phosphate buffered saline (PBS, Sigma-Aldrich, Darmstadt, Germany) containing 2% fetal bovine serum (FBS, Sigma-Aldrich). Then cells were stained for 30 min at 4 °C with combinations of anti-CD45-FITC, anti-CD34-APC and anti-CD38-PE monoclonal mouse anti-human antibodies (all from Biolegend, San Diego, CA, USA). Analysis was performed monthly using a Becton Dickinson CALIBUR flow cytometer (BD, East Rutherford, NJ, US). At least 5×10^5 events were analyzed and total $CD34^+$ HSPC were determined according to the ISHAGE guidelines [21]. Additionally, $CD34^+CD38^-$ cells were gated excluding the corresponding isotype control (< 98%).

2.4. Colony forming cell (CFC) assay

To evaluate the colony forming ability of cells within the blood of G-CSF treated ALS patients, PB was processed as described (2.3) every third month. Burst-forming and colony-forming units erythrocyte (BFU-E, CFU-E), CFU granulocyte-macrophage (CFU-GM) and CFU granulocyte-erythrocyte-monocyte-macrophage (CFU-GEMM) were assayed as described before [20]. Briefly, defined cell numbers were plated in Human Methylcellulose Complete Medium (R&D Systems, Minneapolis, MN, US) and colonies were scored according to number and morphology after 14 days using an inverted microscope (Axiovert 200 M MAT and AxioVision Rel. 4.8 software; Carl Zeiss GmbH, Jena, Germany).

2.5. Telomere length measurements

Lymphocytes and granulocytes from frozen mononuclear cell aliquots of ALS patients #1, #2, #4 and #5 and also from six untreated ALS patients (all male; mean age: 61 years) were used for telomere length assessment. Telomere length was measured using flow-FISH as described earlier [22–24]. Cell samples were stained with FITC-(C3TA2) PNA or Alexa488-(C3TA2) PNA (Panagene, Daejeon, South Korea). Cow thymocytes of known telomere length were added for later telomere length calculation. Patients' lymphocytes and granulocytes as well as cow thymocytes were identified according to their forward scatter and LDS 751 binding to the double stranded DNA. Using the known lengths of cow thymocytes, patients' telomere lengths were calculated in kilobases. For each ALS patient, individual telomere length was compared to the median of age-matched healthy persons from the telomere data base [22,23].

2.6. Analysis of cytokines in serum of ALS patients

To monitor continuous G-CSF treatment, a predetermined panel of cytokines was analyzed in serum at different time points. Frozen serum samples were thawed and analyzed using the V-PLEX Human Biomarker 40-Plex Kit (Meso Scale Discovery®, Rockville, MD, US) according to the manufacturer's instructions and as described earlier [25]. Serum samples (25 µl) were assayed in duplicate in customized 96-well plates and cytokine amounts were determined via electrochemiluminescence using the MESO QuickPlex SQ 120 multiplexing

Table 1
Patient characteristics, adverse events and G-CSF treatment scheme.

Patient	Sex [m/f]	Age [years]	Max. spleen size [cm]	Adverse events/infections	Max. dose per cycle [Mio IU (range)]	Treatment duration [months]
#1	m	33	12.0 × 5.7	Bone pain, fatigue, pneumonia (antibiosis)	1440 (450–1440)	42
#2	m	48	17.3 × 6.8	Bone pain, back pain	1170 (450–1170)	42
#3	m	41	17.7 × 5.5	Bone pain	1440 (450–1440)	21
#4	m	70	12.5 × 8.0	Bone pain, fatigue, hot flushes, sleep problems	2160 (450–2160)	18
#5	m	36	15.1 × 5.0	bone pain, back pain, cold, mild cutaneous mycosis	1440 (720–1440)	25
#6	m	63	12.1 × 4.4	fatigue, weakness	720 (720)	11

Six ALS patients were treated with different doses of G-CSF every second day for up to 3.5 years and routinely monitored for adverse events and infections.

imager (Meso Scale Discovery®). The V-PLEX Human Biomarker 40-Plex Kit included the following cytokines: IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, TNF-alpha, GM-CSF, IL-1α, IL-5, IL-7, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF, VEGF-C, VEGF-D, Tie-2, Flt-1, PIGF, bFGF, Eotaxin, MIP-1β, Eotaxin-3, TARC, IP-10, MIP-1α, IL-8, MCP-1, MDC, MCP-4, SAA, CRP, VCAM-1 and ICAM-1. TNF-alpha concentration was determined in freshly isolated serum.

2.7. Statistical analysis

Data are presented as median (blood cell count, telomere length) or mean ± standard error of the mean (SEM). Statistical significance was determined using student's *t*-test, one-sample *t*-test, Wilcoxon signed rank test or ANOVA including Tukey's multiple comparisons test with a p-value < 0.05. Analyses and graphs were made using FlowJo (Treestar, Ashland, OR, US) and Prism 6 (GraphPad, San Diego, CA, US) statistical software.

3. Results

3.1. Patient characteristics

Six male ALS patients with an average age of 48.5 years were monitored up to 42 months. Patient #3, #4 and #6 died during the study period due to ALS progression. All patients continuously received G-CSF every second day with individual dosing per injection (30×10^6–144×10^6 IU per injection). As shown in Table 1, the overall G-CSF dose ranged from 450 to 2160×10^6 IU per month (cycle), only patient #6 was consistently treated with 720×10^6 IU per month. Except for patients #3 and #4, all patients had received G-CSF treatment before, specifically, patients #1, #2, #5 and #6 were treated with G-CSF 45, 10, three and nine months in a standard dose regime of five G-CSF injections per month or 10 injections distributed over two weeks per month (dose range: 30–48 $\times 10^6$ IU per injection; labeled with # in Fig. 2). Individual dosing and dose adjustments are displayed in Fig. 2 for each patient. Spleen size was in a normal range for patient #1 and #6 [26]. Patients #2 to #5 showed a moderate spleen enlargement (12.5 × 8.0 and 17.3 × 6.8 cm, respectively). The most common AE were bone and back pain or fatigue. In addition, patient #5 suffered from a mild cutaneous fungal infection and a cold during treatment and patient #1 developed a pneumonia which was successfully treated with antibiotics.

3.2. Effects of continuous G-CSF treatment on blood cell count

Basic blood cell counts were analyzed on a monthly basis. As shown in Fig. 1A, G-CSF induced an increase in WBC numbers in most cases, as expected. Baseline WBC counts ranged from 4.8×10^3 to 8.9×10^3 WBC/μl (Fig. 2). After G-CSF stimulation median WBC counts were between 44.6×10^3 (patient #1; n = 35) and 68.3×10^3 cells/μl (patient #4; n = 16). However, individual WBC counts were highly heterogeneous, with single WBC numbers within the physiological WBC range despite continuous G-CSF stimulation. Patient #4 had the highest G-CSF dose resulting in the highest WBC count, which was statistically significant when compared to patient #1, #2 and #5 ($p < 0.05$). To determine possible changes in WBC response over time, *t*-test was used to analyze significant differences between the beginning and the end of the monitoring period. Patient #2 showed a significantly increased WBC count ($p = 0.016$) during the last three cycles compared to the first three cycles due to G-CSF dose adjustment (data not shown). For all other patients, no significant differences were observed.

As shown in Fig. 1B, average neutrophil percentage was between 85.0 ± 1.6% (patient #2; n = 37) and 95.4 ± 0.3% (patient #4; n = 16) who obtained the highest G-CSF dose. As expected, G-CSF treatment resulted in a relative decrease of lymphocytes, monocytes, eosinophils and basophils.

Median platelet numbers (Fig. 1C) ranged from 167×10^3 cells/μl (patient #5; n = 24) to 257×10^3 cells/μl (patient #6; n = 10). Patient #5 had significantly lower platelet counts as compared to the other patients that were partially below the minimal value of healthy individuals. However, long-term analysis showed no difference in platelet response within the patient group between the start and the end of the treatment period (data not shown).

RBC counts and hemoglobin levels are shown in Fig. 1D and E. Erythrocyte counts ranged from 4.5×10^6 (patient #6; n = 10) to 5.6×10^6 (patient #2; n = 37) cells/μl blood and were in normal range for all patients but patient #2 that had a significant higher cell count. This is in accordance with measured hemoglobin levels. For patients #3, #5 and #6 no change of RBC and hemoglobin levels could be observed over time, whereas patients #1 and #2 had significantly decreased RBC numbers and hemoglobin levels ($p < 0.05$) at the end of the monitoring period when compared to the beginning of treatment (data not shown). In contrast, a significant increase of RBC and hemoglobin ($p < 0.05$) was detected in patient #4.

3.3. Effects of continuous G-CSF stimulation on mobilization of $CD34^+$ HSPC

Flow cytometry was used to analyze circulating $CD34^+$ and $CD34^+CD38^-$ HSPC within the PB as described earlier [20]. As shown in Fig. 2, baseline values before start of G-CSF treatment for each cell population (bar graph) ranged from 1555 to 3711 $CD34^+$ cells/ml, 15 to 82 $CD34^+CD38^-$ cells/ml and 4.8×10^3 to 8.9×10^3 WBC/μl. Response to G-CSF stimulation was heterogeneous within individual patients and also inter-individually. Patient #6 had the lowest average $CD34^+$ cell count with 1625 ± 111 cells/ml (n = 10) which is in line with the lowest G-CSF dose applied during the observation period. Patient #1 mobilized the highest number of $CD34^+$ cells (average 4656 ± 192 cells/ml; n = 35). However, patient #4 did not mobilize $CD34^+$ HSPC efficiently with an average number of 1968 ± 412 cells/ml (n = 16) ranging only slightly above baseline, although receiving the highest G-CSF dosage. Mobilization of $CD34^+CD38^-$ HSC was low, average amounts ranging from 11 ± 3 cells/ml (patient #2; n = 37) to 119 ± 12 cells/ml (patient #1; n = 35). In general, mobilization efficiency of $CD34^+$ HSPC was rather low and dose enhancement did not consistently increase HSPC mobilization.

At the end of the monitoring period, patient #1, #2, #4 and #5 changed from continuous G-CSF application to an alternative regime with G-CSF injections every second day for one week followed by one week off stimulation (labeled with **). As a result, a slight increase in mobilized $CD34^+$ HSPC and $CD34^+CD38^-$ cells could be observed in patient #1, #2 and #4. As indicated in Fig. 2, patient #5 returned to G-CSF dosing every second day without pause which did not result in elevated mobilization of $CD34^+$ HSPC.

3.4. Effects of continuous G-CSF stimulation on mobilization of CFC

CFC and subtypes were analyzed every three months. As demonstrated in Fig. 3A, the lowest CFC number in PB was observed in patient #6 with 1.47 ± 0.4 × 10^2 cells/ml (n = 3). In contrast, patient #5 had the highest CFC counts with 5.3 ± 1.1 × 10^2 cells/ml; (n = 8). In general, CFC numbers mirrored $CD34^+$ HSPC numbers at the indicated time points as demonstrated in Fig. 2. CFC subtype distribution (Fig. 3B) was similar between patient #1, #2 and #5, although patient #5 tended to mobilize less CFU-GM at the end of the monitoring period. Further, patients #3 and #6 had a considerable decrease in CFU-GM which is in line with low $CD34^+$ mobilization and patient #4 additionally displayed a total loss of CFU-GM, CFU-GEMM and BFU-E at the end of the monitoring period (cycle 16).

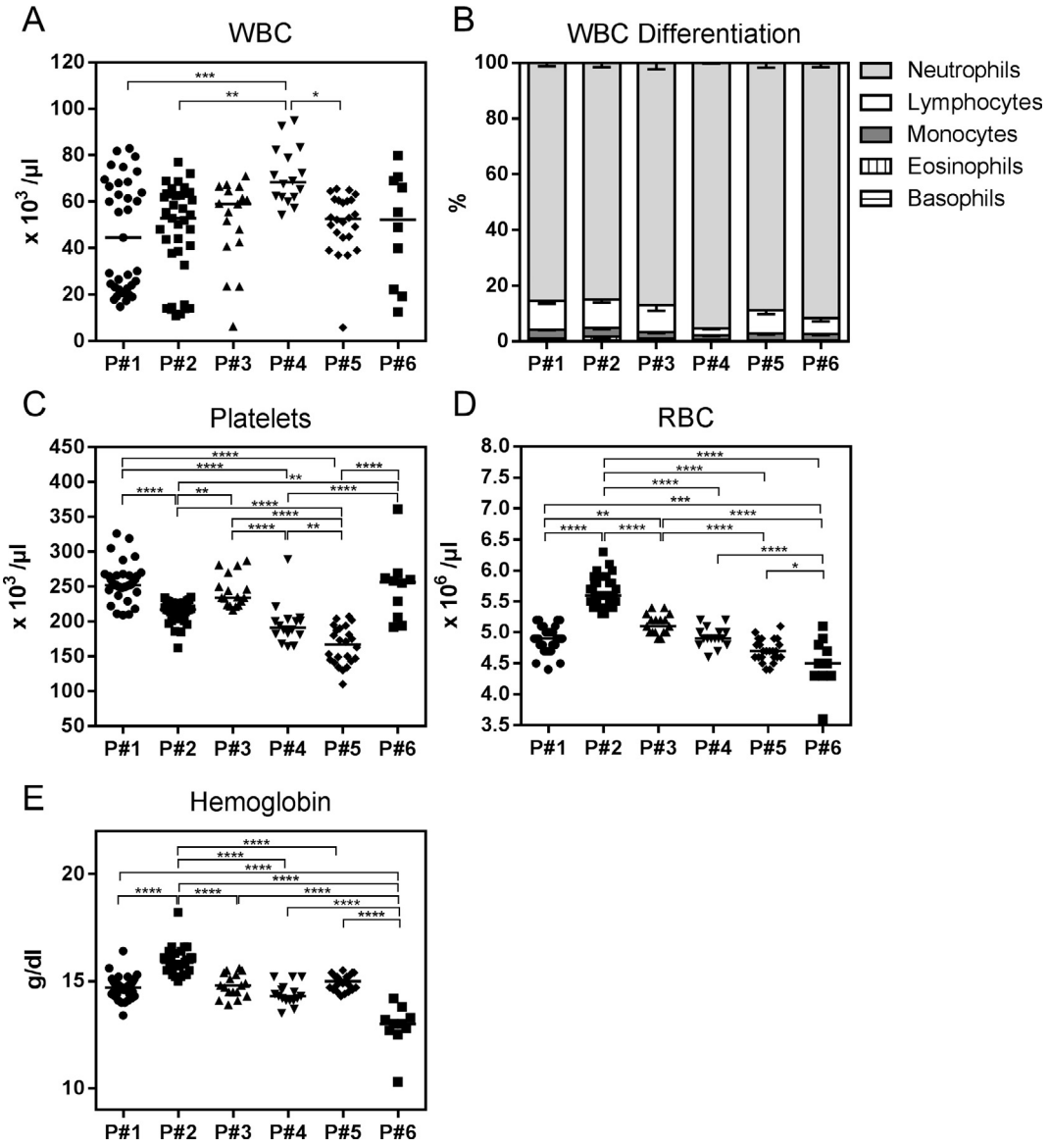

Fig. 1. Continuous G-CSF treatment results in a marked increase of neutrophils and slightly decreases platelet counts. White blood cell (WBC; A), red blood cell (RBC; D), platelet (C) and hemoglobin counts (E) were determined on a monthly basis using an automated cell counter. Data are presented as dot plots with median, where every dot represents one measurement. Significant differences between patients' blood cell counts are indicated ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; Tukey's multiple comparisons test). In addition, WBC differentiation (B) was analyzed. Bar charts depict average percentage of WBC subtypes (neutrophils, lymphocytes, monocytes, eosinophils and basophils) - SEM (n = 10–37).

3.5. Effects of continuous G-CSF on telomere length

Lymphocytes and granulocytes of G-CSF treated patients #1, #2, #4 and #5 were analyzed for their telomere length (at cycle 69, 34, 12 and 11, respectively) using flow-FISH and compared to those of healthy individuals and ALS controls. Lymphocyte telomeres of all ALS patients were significantly shorter than those of healthy age-matched controls from the telomere database (Fig. 4A; median shortening of 0.97 kb, $p = 0.007$) regardless whether they were treated with G-CSF or not. Lymphocyte telomere length of ALS controls was 0.69 kb shorter (median; range −0.21 to −1.26 kb) than those of healthy age-matched controls ($p < 0.05$) while granulocyte telomeres were 0.16 kb shorter (Fig. 4B; median; range +0.86 to −1.13 kb, not significant). Regarding G-CSF treated ALS patients, lymphocyte telomeres were 1.08 kb shorter (median; range −0.68 to −2.10 kb, $p = 0.125$) than those of healthy age-matched controls and granulocyte telomeres were reduced by 0.62 kb (median; range +2.70 to −1.39 kb, not significant) but did not differ significantly from those of ALS controls.

3.6. Effects of continuous G-CSF stimulation on serum cytokine profile

A panel of 40 cytokines that were found to be associated with G-CSF stimulation, including pro-inflammatory cytokines and chemokines, adhesion molecules, kinase receptors and growth factors, was measured within the serum. Only those that changed significantly are displayed in Fig. 5. TNF-alpha was significantly increased in all patients when compared to baseline (except patient #6: $p = 0.06$). The pro-inflammatory protein CRP was elevated in all but one patient (#3) mostly at the end of the monitoring period (all $p < 0.05$). MCP-1 was significantly lower in all patients as compared to baseline, IL-16 concentrations were significantly higher. Soluble VCAM-1 significantly increased in all patients, while sICAM-1 was significantly elevated in three patients (#3, #5 and #6) and unchanged in the other patients. Serum Tie-2 increased, with the exception of patient #2 who had significantly lower Tie-2 levels in comparison to baseline value ($p = 0.02$). VEGF was found to be significantly higher in serum of patients #1, #2 and #5 ($p < 0.05$) but was unchanged in the other patients.

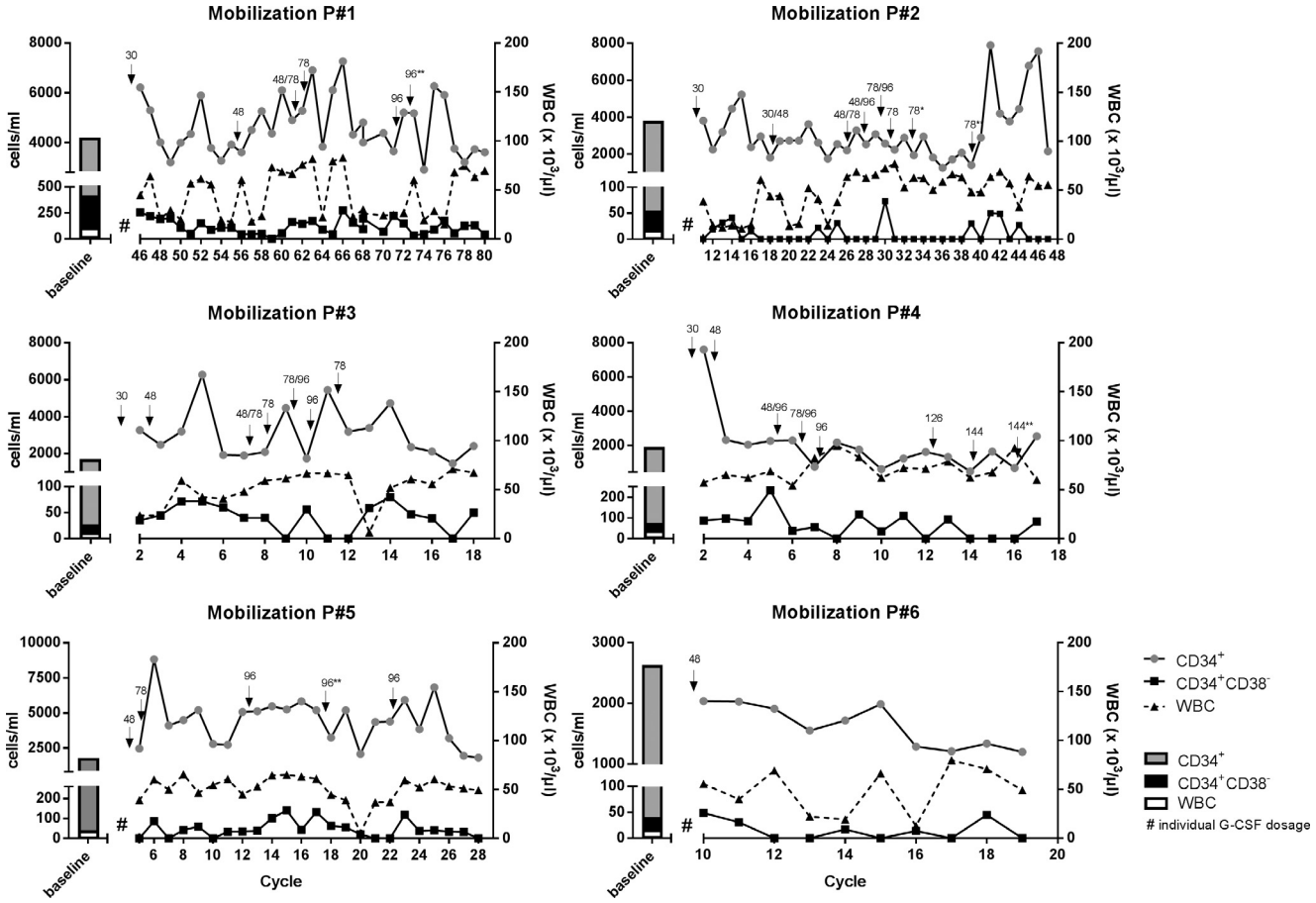

Fig. 2. Continuous G-CSF treatment does not induce substantial mobilization of $CD34^+$ stem and progenitor cells (HSPC). To determine the mobilization efficiency of continuous G-CSF application flow cytometry analyses of peripheral blood samples were performed on a monthly basis and total circulating $CD34^+$ HSPC as well as $CD34^+CD38^-$ cells were measured. Total $CD34^+$ and $CD34^+CD38^-$ cell numbers per ml blood (mean of two measurements) were plotted against cycle for individual patients. Numbers with arrows within the graphs indicate individual G-CSF dose ($\times 10^6$ IU) and the time point, when G-CSF dose was changed. In addition, corresponding WBC counts (triangles) are plotted over time. The baseline amount of all measured cells before G-CSF treatment is shown as bar graph on the left including WBC, $CD34^+$ and $CD34^+CD38^-$ cells (white bar: WBC $\times 10^6$/ml, grey bar: $CD34^+$ cells/ml, black bar: $CD34^+CD38^-$ cells/ml). *G-CSF was applied every second and third day; **G-CSF was applied every second day followed by one week without G-CSF in an alternating manner.

4. Discussion

In this report, we present for the first time hematological and telomere data on ALS patients that were continuously stimulated with varying doses of G-CSF every second day throughout an observation period of 3.5 years. In general, WBC counts differed among patients which mirrors different G-CSF doses, individual pre-treatment, patients' age and probably non-compliance. Compared to our previous study with a five-day treatment regime [20], WBC counts were significantly higher this time. This could explain the splenomegaly observed in some patients, which was absent in our earlier study [20]. As previously shown, G-CSF resulted in neutrophil expansion [27], an effect that is clinically used to reduce infections in patients with neutropenia [28]. Since only two of our ALS patients had an infection during the G-CSF treatment course, elevated neutrophil numbers might have reduced infectious complications [29]. A common side effect of G-CSF is thrombocytopenia that is often mild and normalizes spontaneously [30]. In our study platelet counts were rather low but never below the normal range except in one patient (#5). This low platelet number was most likely attributed to an antidepressant therapy with amitriptyline as platelets recovered after discontinuation. RBC and hemoglobin levels were low in one patient and a significant decrease could be seen in two patients at the end of the treatment period. A decrease of hemoglobin levels was already described after G-CSF application [30]. However, patient #4 receiving the highest G-CSF dose showed significantly increased RBC counts and hemoglobin level in the last treatment cycles. Hence, this observation is possibly a patient characteristic independent of G-CSF treatment.

To our knowledge, this is the first study exploring high-dose and long-term G-CSF application every alternate day in patients without BM impairment. Several studies have been performed using G-CSF for treatment of ALS patients, however, those were only short-term and with low G-CSF dosages [5]. In contrast to our previous study using a five-day monthly mobilization regime [20], we observed a rather low mobilization efficiency of HSPC. In particular, $CD34^+$ cells hardly reached a 2-fold increase versus baseline, whereas $CD34^+$ cells were increased about 18-fold in our previous work [20]. Patient #6 received the lowest G-CSF dose in our patient group and did not exceed baseline $CD34^+$ cell count whereas patient #4 injected the highest G-CSF dose up to 2160×10^6 IU per month and mobilized only slightly more $CD34^+$ cells than at baseline. Both were the oldest patients within our study which might add to the observed mobilization failure [31]. Interestingly, dose escalation did not substantially augment HSPC mobilization. This observation is probably due to an impairment of the BM environment during intense G-CSF therapy. As G-CSF induces a proteolytic environment in the BM by induction of metalloproteases [32], several homing related molecules are elevated in plasma, e.g. sVCAM-1, sICAM-1 [33] which was also true in our patient group. With regard to sVCAM-1 which mediates retention of HSC within the BM via binding to VLA-4 [34], it is assumed that cleaved VCAM-1 can still bind to HSC

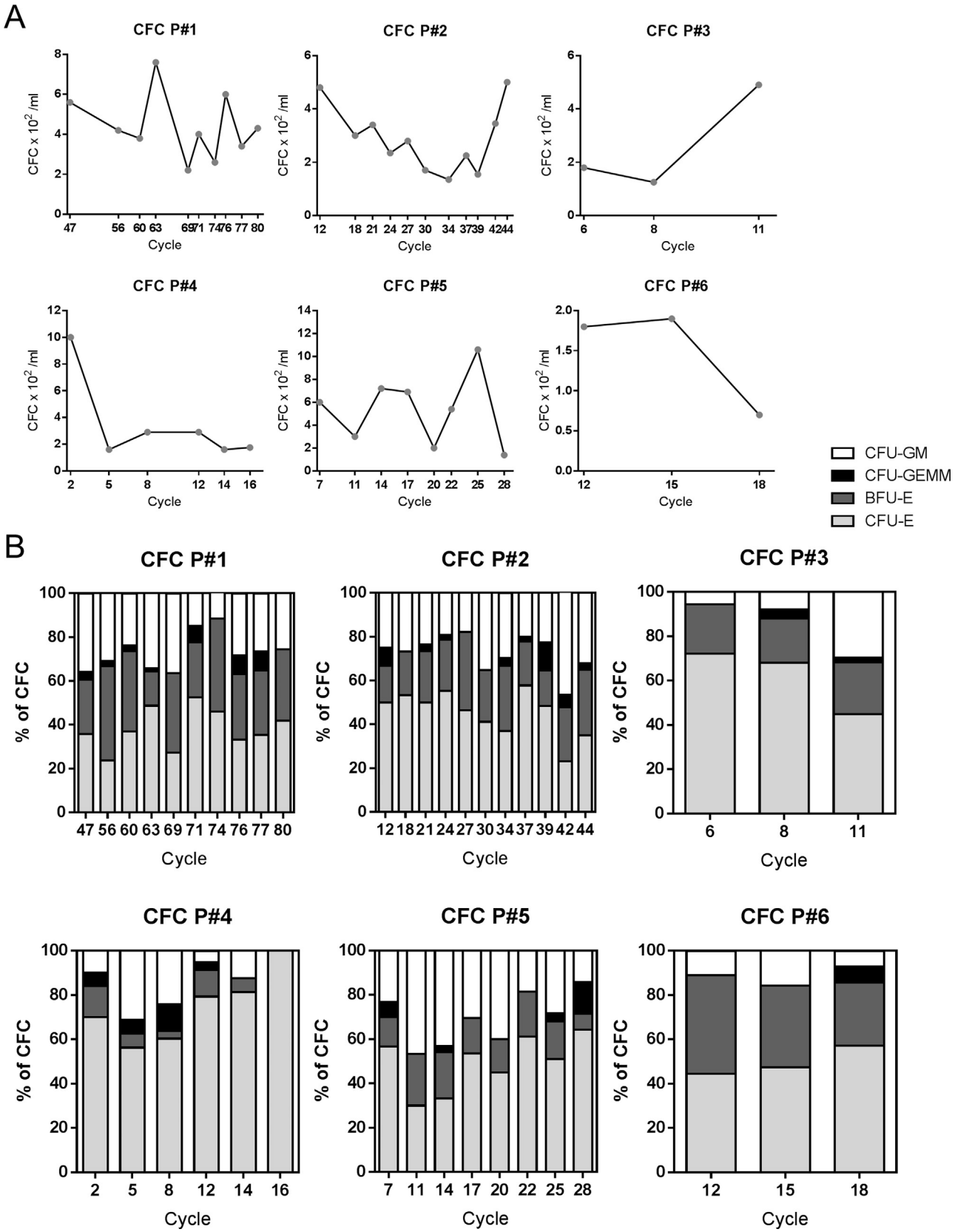

Fig. 3. Mobilization of colony forming cells (CFC) during continuous G-CSF treatment. Colony forming cells were determined every three months after 14 days of culture in semisolid medium using an inverted microscope. (A) The total amount of CFC × 10^2 per ml blood is shown for each patient during the G-CSF treatment period as mean of two measurements for each cycle. (B) Individual CFC subtypes (colony forming units-erythrocyte [CFU-E], burst forming units-erythrocyte [BFU-E], colony forming units-granulocyte erythrocyte monocyte macrophage [CFU-GEMM] and colony forming units-granulocyte macrophage [CFU-GM]) are plotted as percentage of total CFC amount for individual time points.

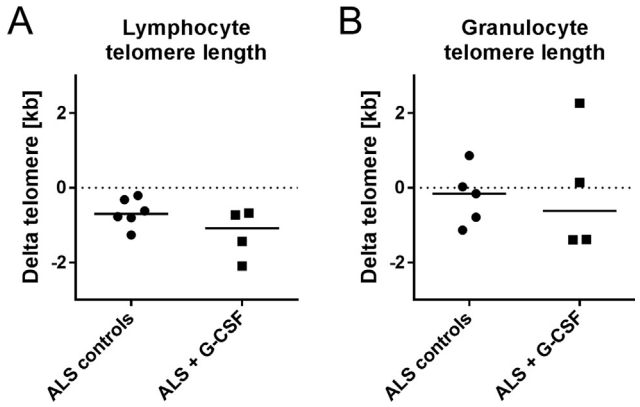

Fig. 4. Continuous G-CSF treatment does not induce telomere attrition. Telomere lengths of ALS peripheral blood lymphocytes and granulocytes were determined using Flow-FISH. Data are presented as dot plots with median. Telomere length was then compared to the median of healthy age-matched controls (dotted line) to calculate the difference in length in kilo bases. Changes in telomere lengths of ALS controls (n = 6) were compared to ALS patients who received G-CSF treatment (n = 4, not significant). (A) ALS patients had significantly shorter lymphocyte telomeres compared to healthy age-matched controls regardless of G-CSF treatment. (B) Granulocyte telomere attrition was not significant.

thereby reducing adhesiveness to endothelial cells and BM stroma [35]. In addition, it has been shown that G-CSF down-regulates key homing receptors like VLA-4 and CXCR4 [16]. G-CSF further induces several mediators like TNF-alpha as demonstrated in the present work and by others [36] by up-regulating the activity of TNF-alpha converting enzyme (TACE). Besides, it has been reported that osteoblasts are also negatively affected by G-CSF [37], further underpinning the hypothesis of BM impairment and a decrease of the HSPC pool. However, mature blood counts were not drastically impaired in our patient group and, in this context, we observed a slight increase in $CD34^+$ HSPC mobilization when continuous G-CSF treatment was interrupted for one week, which might be due to a recovery of the BM environment.

CFC mobilization was also reduced during continuous G-CSF application as compared to our previous study [20]. A substantial decrease of CFU-GM was detected in three patients which is a new finding. It has been described that G-CSF predominantly leads to an increase of myeloid but not erythrocyte progenitors [38]. As the G-CSF receptor itself is not expressed on HSC [39], but on myeloid progenitors [40], one might suggest that these were not efficiently mobilized during continuous G-CSF stimulation as observed for $CD34^+$ HSPC. However, as only three time points of CFC distribution were available for two patients, the observed effect needs further validation. Interestingly, a total loss of myeloid CFC (-GM and -GEMM) and BFU-E was seen in one patient (#4) after 16 months of treatment with the highest G-CSF dose, possibly indicating a disruption of BM function. As the patient died, further evaluation was not feasible.

To further assess the effects of continuous G-CSF stimulation on the hematopoietic system, we determined leukocyte telomere length in G-CSF treated and untreated ALS patients as well as healthy controls. Telomeres, the ends of chromosomes ensuring genomic stability, shorten by 25–40 base pairs per year in somatic cells [41]. In BM stem cells, the enzyme telomerase extends telomeres to maintain their replicative capacity [42]. Telomere shortening is characteristic of diseases such as dyskeratosis congenital [41] and has also been found in ALS [43]. In the present study, telomere lengths of all ALS patients' lymphocytes were significantly shorter than those of healthy controls with the median shortening corresponding to a premature aging of approximately 20 years. In addition, we observed similar results in ALS granulocyte telomeres that showed a yet not significant trend to shortened telomeres compared to healthy age-matched controls which is most likely due to the small sample size. Our observations confirm findings by De Felice et al., who discovered that telomeres of ALS patients' leukocytes are shorter than those of healthy age-matched controls and that telomerase activity of ALS leukocytes is dramatically lower [43]. Since telomerase consists of the ribosomal component TERC being critical for proper telomerase elongation, impaired ribosomal processing as proposed for ALS [44] would result in reduced telomerase activity sufficiently explaining the observed results. Of note, impaired telomerase activity would occur independently in lymphocytes as well as in hematopoietic stem cells indicating a systemic process not limited to one cellular compartment as proposed for ALS. Alternatively, ALS might predominantly develop in patients with premature shortened telomeres. Finally, our results of shortened telomeres might be due to an enhanced activity of the adaptive immune system in lymphocytes [45] combined with an increased proliferation due to G-CSF stimulation in granulocytes.

Notably, G-CSF did not significantly influence telomere length of granulocytes in our patients during follow-up. This is of importance since the influence of G-CSF on telomere length is being discussed controversially. Li et al. reported that patients receiving G-CSF due to neutropenia suffered from telomere shortening and acute myeloid leukemia after 10–25 years of continuous treatment [46]. In contrast, Aladdin et al. saw no change in telomere length of peripheral blood mononuclear cells of HIV patients receiving anti-retroviral treatment in combination with G-CSF [47]. This is in line with unpublished data [Beier F, personal communication] who did not observe an effect of long-term G-CSF treatment on telomere length in patients with autoimmune neutropenia. However, the question of whether or not long-term G-CSF treatment shortens granulocyte telomeres needs further exploration as well as greater sample sizes. Moreover, the individual treatment duration for each patient should be kept in mind when interpreting these results.

With regard to cytokine dynamics, we found increased amounts of TNF-alpha, CRP, IL-16, sVCAM-1, sICAM-1, Tie-2 and VEGF as well as decreased levels of the pro-inflammatory chemokine MCP-1 in ALS patients' sera. TNF-alpha was previously shown to be increased in healthy individuals undergoing G-CSF therapy [33,36]. However, Chio et al. did not observe this effect in a previous report on ALS patients with a limited time of G-CSF treatment [8]. TNF-alpha is basically assumed to exert positive as well as negative effects on ALS pathophysiology, depending on the type of receptor and the cellular context [48]. As there are several studies indicating either increased amounts of TNF-alpha [25,49] or decreased levels in serum of ALS patients, irrespective of G-CSF treatment [50,51], this finding needs further evaluation. The general pro-inflammatory marker CRP was also markedly increased in our patient group. Carstanjen and colleagues likewise observed this effect after a five-day G-CSF application in healthy controls [52]. In particular, the increase of CRP levels at the end of our observation period suggests a cumulative inflammatory process during ALS disease progression as observed by others [25]. In contrast, the pro-inflammatory chemokine MCP-1 was significantly reduced in our patient group as shown before [8]. MCP-1 has been reported to be augmented in ALS patients [25] and is associated with shortened survival, possibly due to neurotoxic effects [53]. Therefore, one might suggest that G-CSF-induced decrease of MCP-1 has a neuroprotective effect. A yet unreported finding is an increased IL-16 level in all patients during G-CSF stimulation. Expression of IL-16 was found in neutrophils and infiltrating granulocytes [54], which might explain increased IL-16 levels due to G-CSF-induced proliferation of neutrophils. In the context of immune system regulation, IL-16 has been shown to induce regulatory T cells (Treg) [55]. High numbers of circulating Treg have been associated with prolonged survival in ALS patients [56], thus increased IL-16 might be beneficial. Soluble VCAM-1 and ICAM-1 were elevated in serum of G-CSF treated ALS patients which is most likely due to G-CSF-induced proteolytic cleavage within the BM [32], as already described by others [33,35]. Nonetheless, elevated sVCAM-1 and sICAM-1 levels

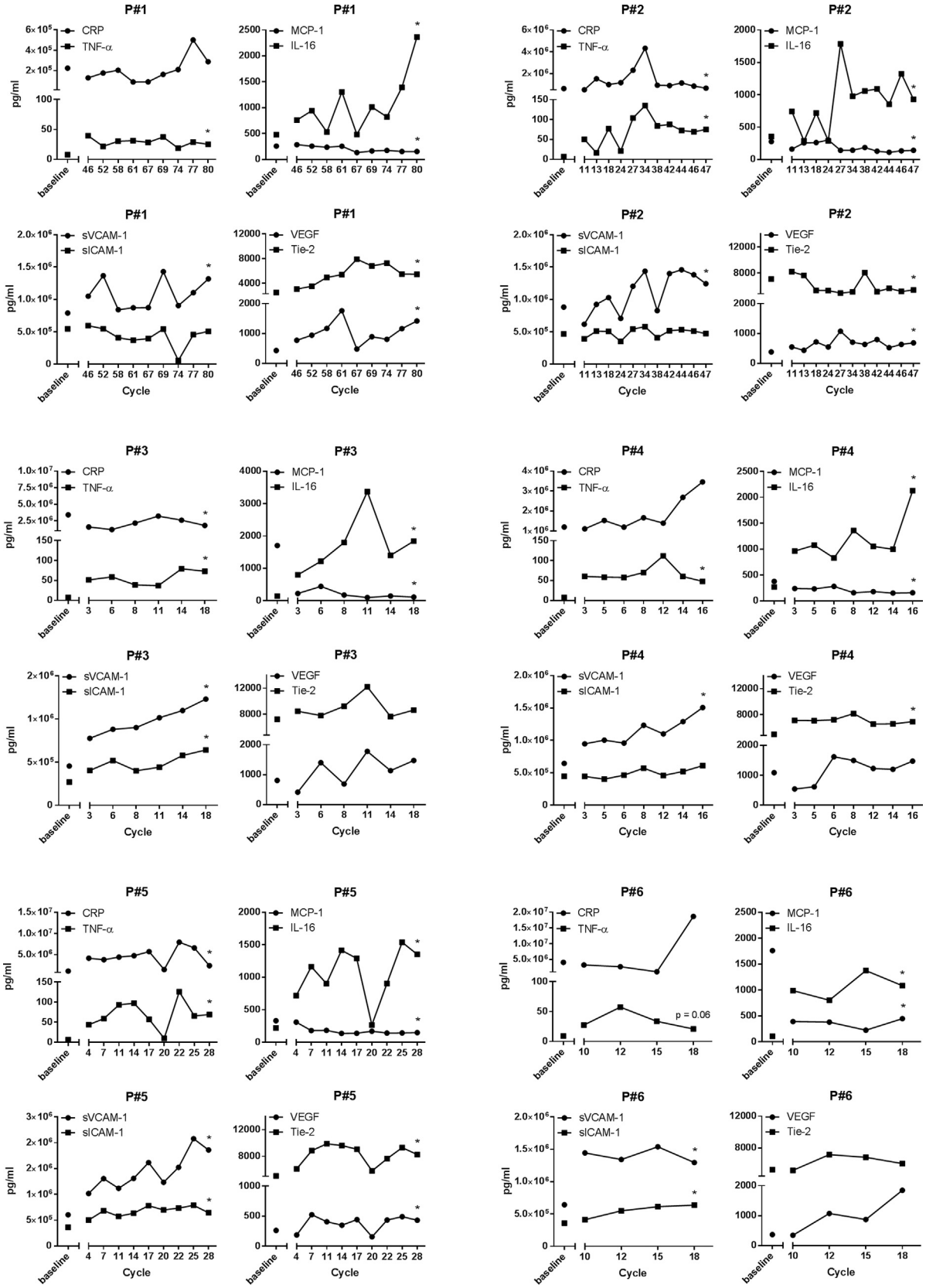

Fig. 5. Changes in serum cytokine profile during continuous G-CSF application. Serum cytokines of ALS patients were measured at different time points (once per cycle) throughout the G-CSF treatment. Measurements were performed in duplicates and data points are presented as mean in pg/ml. Statistical significance was determined using one sample *t*-test or Wilcoxon signed rank test with a p-value < 0.05. Significantly changed amounts of TNF-alpha, CRP, MCP-1, IL-16, sVCAM-1, sICAM-1, VEGF and Tie-2 in comparison to baseline concentration are labeled with asterisks (*).

have also been described in sera of untreated ALS patients [25], possibly indicating a general proteolytic environment and endothelial activation in ALS. Also, the tyrosine kinase receptor Tie-2 was enhanced, which is an unreported G-CSF effect so far. Tie-2 is expressed on vascular endothelial cells and HSC playing a major role for quiescence by binding to Ang-1 within the BM niche [57]. Possibly, long-term and high-dose G-CSF administration leads to proteolytic cleavage of Tie-2, too. Additionally, VEGF was increased in serum of our patients. VEGF has been demonstrated to be released from neutrophils after G-CSF treatment [58] and is supposed to be neuroprotective [59] and to positively correlate with ALS survival [53]. In general, we assume that our observed changes in cytokine levels during G-CSF therapy are most likely due to proteolytic induction within the BM as well as expansion of myeloid cells, particularly the neutrophil subset. This becomes obvious in patient #5, who showed a clear decrease of TNF-alpha, CRP, IL-16, sVCAM-1, Tie-2 and VEGF at cycle 20. At that time point, WBC count was low, indicating no actual G-CSF stimulation. Thus, cytokine changes are most likely reversible when G-CSF treatment is discontinued.

5. Conclusion

To our knowledge, this is the first study exploring high-dose and long-term G-CSF application every alternate day in ALS patients without BM impairment. Altogether, our data show that after continuous and high-dose G-CSF treatment BM function remained intact as assessed by mature blood count parameters. However, no sustained mobilization of $CD34^+$ HSPC from BM into PB was achieved despite high G-CSF dosing. In addition, CFC mobilization was low, suggesting an overall impairment of the BM microenvironment as changes in cytokine and vascular factor levels reflect immunomodulatory and proteolytic properties of G-CSF. Interestingly, G-CSF treatment did not significantly shorten telomeres in lymphocytes and granulocytes of ALS patients. Though, ALS patients in general and regardless of G-CSF application displayed significant telomere attrition within the lymphocyte compartment when compared to healthy controls, indicating an enhanced immune system activation. In summary, possible neuroprotective effects of G-CSF as postulated seem not to be mediated by enhanced circulating $CD34^+$ HSPC. In general, the applied G-CSF dosing was relatively safe and well tolerated. Therefore, our study may pose as a basis for further investigations.

Authorship/Acknowledgements/Disclosure

S. Iberl and A.-L. Meyer wrote the manuscript. U. Bogdahn, J. Grassinger and S. Johannesen were responsible for the treatment concept and performed the clinical work. S. Iberl, G. Müller, S. Peters, A.-L. Meyer, J. Grassinger and F. Beier planned and performed experiments. U. Bogdahn, S. Johannesen and I. Kobor provided patient material and reviewed the manuscript. J. Grassinger, F. Beier, T. H. Brümmendorf, C. Hart, R. Schelker and W. Herr reviewed the manuscript.

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