C–C motif ligand 11 reduction in CLL patients serum after vitamin D supplementation

Marcin Kubeczko[1,2], Elżbieta Nowara[1], Dobromiła Karwasiecka[2], Grażyna Siewior[2], Paulina Czajka-Francuz[2], Jerzy Chudek[2,3], Jerzy Wojnar[2]

[1]Clinical and Experimental Oncology Department, Maria Skłodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Gliwice 44-400, Poland, [2]Department of Internal Medicine and Oncological Chemotherapy, School of Medicine in Katowice, Medical University of Silesia, Katowice 40-027, Poland, [3]Department of Pathophysiology, School of Medicine in Katowice, Medical University of Silesia, Katowice 40-752, Poland

Background: Vitamin D (VD) deficiency results in a worse prognosis in patients with chronic lymphocytic leukemia (CLL) and may affect the production of cytokines. Nonetheless, there is the lack of studies dealing with VD supplementation and its impact on chemokines in CLL patients.
Aim: The primary endpoint of our interventional study was to evaluate the effect of cholecalciferol supplementation on serum chemokines levels in CLL patients.
Materials and methods: Eighteen subjects with CLL were enrolled for the study. Six-month-long cholecalciferol supplementation was performed in CLL patients with serum 25-OH-D_3 levels below 30 ng/ml. Cytokines levels were assessed at the beginning of the study and after 6 months. Baseline measurements of cytokines were compared to those in apparently healthy controls.
Results: Increased levels of CCL2, CCL3, CCL4, CXCL8, CXCL10, TNFα, bFGF, G-CSF, and VEGF were found in CLL patients in comparison with the healthy controls. In the course of the VD supplementation a decrease in serum levels of chemokines CCL11, CCL3, and cytokine PDGF-BB was observed. The decrease of CCL11 was found in CLL patients on VD supplementation solely, whereas the decrease of CCL3 and PDGF-BB was observed in CLL subjects on both chemotherapy and VD supplementation.
Conclusion: The VD supplementation may exert beneficial effect on chemokines levels in CLL patients with VD deficiency.

Keywords: Chronic lymphocytic leukemia, Vitamin D, Chemokine

Introduction

Chronic lymphocytic leukemia (CLL) is the most common form of adult leukemia in the Western world, characterized by the accumulation of mature monoclonal CD5+/CD23+ B lymphocytes in blood, bone marrow, and secondary lymphoid tissues.[1] Microenvironment and various cytokines stimulating growth, proliferation and inhibiting apoptosis of CLL cells play a significant role in CLL.[2] Microenvironment may also cause resistance to such drugs as fludarabine, cyclophosphamide, and dexamethasone.

CLL3 (C–C motif ligand 3, also known as MIP-1α) and CCL4 (C–C motif ligand 4, also known as MIP-1β) are chemokines produced by CLL cells, which recruit cells from the monocyte/macrophage lineage and T cells to bone marrow microenvironmental sites.[3] High levels of these chemokines are produced after co-culture with nurse-like cells (NLC) or after BCR (B cell receptor) triggering.[3] Higher plasma levels of CCL3 are associated with an inferior clinical outcome in CLL patients.[4] CCL3 is also correlated with higher risk of cytogenetic abnormalities.[5]

CCL11 is another CC chemokine, also known as eotaxin-1, acting exclusively through CC chemokine receptor-3 (CCR3). CCL11–CCR3 interactions promote survival and proliferation of anaplastic large cell lymphoma cells via ERK1/2 activation.[6] Serum CCL11 levels in cutaneous T-cell lymphoma patients at advanced stages of the disease were significantly higher than in healthy individuals.[7] CCL11 is overexpressed in Hodgkin lymphoma tissues.[8] CCL11 secreted by CLL cells in serum-free cultures was the only one in 174 cytokines which was significantly increased, compared to the levels in normal B-cell cultures.[9] Co-culture of CLL cells and mesenchymal stromal cells induced a particular increase in

Correspondence to: Marcin Kubeczko, Department of Internal Medicine and Oncological Chemotherapy, School of Medicine in Katowice, Medical University of Silesia, Reymonta 8, Katowice 40-027, Poland.
Email: marcin.kubeczko@gmail.com

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CXCL8, CXCL10, CCL4, and CCL11.[10] CCL11 and CXCL10 are involved in CLL cells survival and growth.[10]

CXCL10 (C–X–C motif chemokine 10), also known as interferon gamma-induced protein 10 (IP-10), binds with CXCR3, which is constitutively expressed on CLL cells and is involved in B cells migration.[11] CXCL10 is produced by mesenchymal stromal cells when co-cultured with leukemic cells, which may suggest its important role in CLL progression.[10] TNFα (tumor necrosis factor alpha) is produced by CLL cells, as well as CLL-associated monocytes,[12] and is involved in CLL progression.[13] Interferon gamma (IFNγ) is associated with advanced stages of CLL and reduces spontaneous apoptosis of leukemic cells, even with the addition of glucocorticoids.[14]

Angiogenesis has been reported as a prognostic factor in a range of hematological malignancies and contributes to the development and progression of CLL.[15] Basic fibroblast growth factor (bFGF) is involved in hematopoiesis and apoptosis, which correlates with a CLL stage and resistance to fludarabine.[16] Serum levels of VEGF are correlated with a risk of CLL progression.[17] Antiangiogenic factor, bevacizumab, displayed proapoptotic activity against CLL cells.[18] Mesenchymal stromal cells can be induced to secrete VEGF by PDGF (platelet derived growth factor).[19] These interactions may contribute to the induction of an angiogenic switch, which is known to be permissive for CLL progression.[19] PDGF-B is more strongly expressed in CLL than PDGF-A, and contributes to clonal proliferation of malignant pre-B cell lines.[20] IL-8, a member of the chemokine family, also known as CXCL8 (C-X-C motif ligand 8) or monocyte-derived neutrophil chemotactic factor, is involved in the recruitment and trafficking of leukocytes,[21] and in the process of pathological angiogenesis.[22] CXCL8 is associated with a poor prognosis in elderly CLL patients[23] and prolongs the survival of leukemic cells in autocrine fashion.[24]

1,25-$(OH)_2$-D_3, both *in vitro* and *in vivo* inhibits angiogenesis,[25] and *in vitro* inhibits secretion of a various range of cytokines, such as PDGF,[26] CCL11,[27] RANTES (regulated on activation, normal T-cell expressed and secreted, also known as C–C motif ligand 5, CCL5) and CXCL10.[28] In addition, *in vitro* 1,25-$(OH)_2$-D_3 suppresses, through the inhibition of NF-κB pathway, expression of IL-8 at both mRNA and protein levels.[29] Furthermore, 1,25-$(OH)_2$-D inhibits proliferation of T cells and production of IFN-γ.[30] It should be emphasized, that VD has been recently found to be a prognostic factor in CLL,[31] and its deficiency is associated with a worse prognosis, shorter overall survival and time to first treatment.[32] Vitamin D deficiency was also found to be prognostic factor in follicular lymphoma[33] and diffuse large B-cell lymphoma.[34] However, there is the lack of studies concerning prognostic role of the vitamin D deficiency in patients with Hodgkin lymphoma.

The aim of this prospective study was to assess the impact of VD repletion on serum cytokines levels in CLL patients.

Materials and methods

Patients and samples

Sixteen out of eighteen untreated in the previous year CLL patients had VD deficiency and were enrolled (November 2013–July 2015). A control group comprised 10 age-matched subjects that fulfilled similar inclusion and exclusion criteria as the study group, mostly infections over a period of 4 weeks. The protocol was approved by the Local Bioethics Committee and written informed consent was obtained from each participant. All laboratory tests were repeated in the study group after 6 months of the VD supplementation. Patients were assigned to chemotherapy or continued 'watchful waiting', according to the guidelines.[35]

25-OH-D_3 and serum cytokines measurements

The serum 25-OH-D_3 levels were measured on Cobas E422 Roche by electrochemiluminescent immunoassay (ECLIA) with the inter-assay variability below 10.3%.

Serum cytokines levels were measured by Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad Laboratories, Hercules, CA, USA). All procedures were followed according to manufacturer's manual. Limits of detection (LOD) for cytokines were as follows: CXCL8 – 1.0 pg/ml CCL11 – 2.5 pg/ml, basic FGF – 1.9 pg/ml, G-CSF – 1.7 pg/ml, GM-CSF – 2.2 pg/ml, IFNγ – 6.4 pg/ml, CXCL10 – 6.1 pg/ml, CCL2 – 1.1 pg/ml, CCL3 – 1.6 pg/ml, CCL4 – 2.4 pg/ml, PDGF-BB – 2.9 pg/ml, RANTES (CCL5) – 1.8 pg/ml, TNFα – 6.0 pg/ml, VEGF – 3.1 pg/ml. The Bio-Plex Manager™ software presents data as median fluorescent intensity (MFI) and concentration in pg/ml.

Intervention

Serum 25-OH-D_3 level <30 ng/ml was considered as VD deficiency, according to the current recommendations.[36] Patients with VD deficiency received adequate cholecalciferol (6000 IU/d when 25-OH-D_3 level <10 ng/ml, 4000 IU/d when 25-OH-D_3 level 10–19.9 ng/ml, 2000 IU/d when 25-OH-D_3 level 20–30 ng/ml).

CLL subjects, which required systemic treatment (group 2), received chemotherapy, comprising rituximab, fludarabine, cyclophosphamide, cladribine, vincristine, prednisone, bendamustine, idelalisib, according to schedules prepared on the basis of a given individual's clinical conditions.

The study design is shown in Fig. 1.

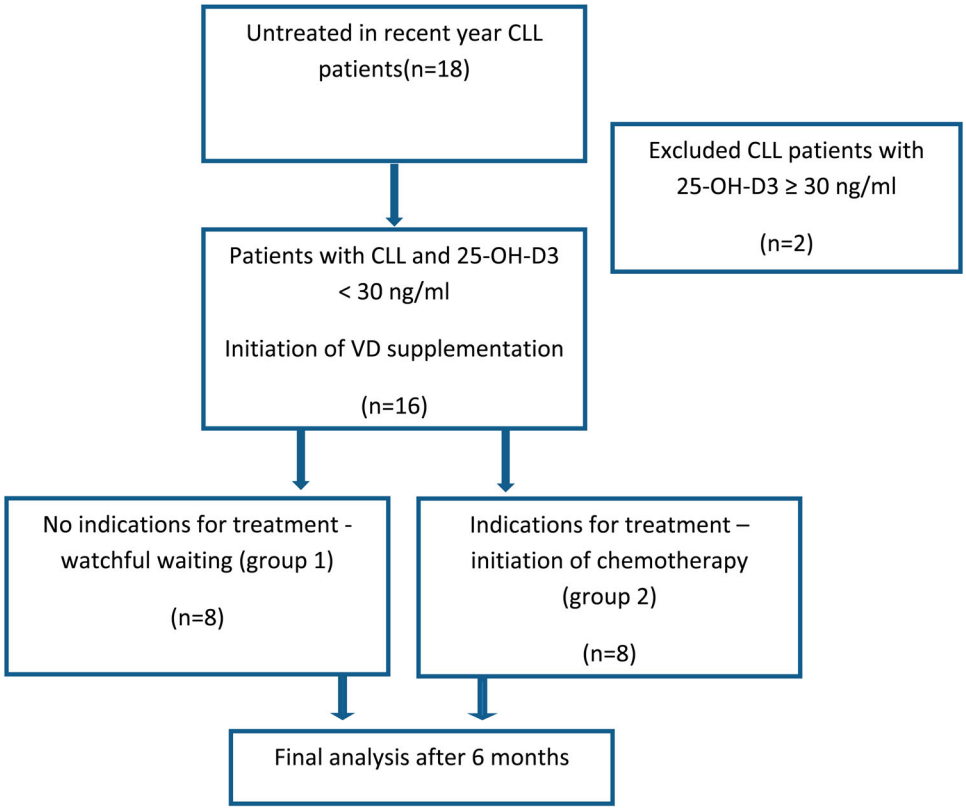

Figure 1. Flow diagram displaying design of the study.

Statistical analysis

The Mann–Whitney U test was used to assess differences in variables between groups at the beginning of the study and 6 months into the cholecalciferol supplementation. The Wilcoxon signed-rank test for paired data was used to assess changes in variables after intervention. The Spearman's rank correlation coefficient was used to assess the correlation between variables. Value of limit of detection (LOD) was used when cytokines measurements were lower than LOD. Statistica, version 10 (StatSoft, Inc.) was used to perform all analyses. $P < 0.05$ was considered significant.

Results

Characteristics of study subjects

The median age of CLL patients was 67 years (interquartile range 63–73) and was not significantly different from the age in the control group (median 62.5, interquartile range 57–66, $P = 0.13$). Males comprised 50% of the subjects in the CLL group and 40% in the control group. Seven patients had Binet stage A, eight patients Binet stage B and three patients Binet stage C. The median time from diagnosis was 2 years (range 0–7 years). Seven patients (39%) were previously treated with chemotherapy.

The CLL patients were characterized by significantly increased levels of CXCL8, CXCL10, CCL2, CCL3, CCL4, TNFα, bFGF, G-CSF, and VEGF (Fig. 2). Serum levels of CCL11, IFNγ, and PDGF-BB in CLL patients did not differ from control subjects ($P = 0.2$, $P = 0.2$, and $P = 0.3$, respectively). Serum levels of GM-CSF were below the detection range in nine control group subjects and 13 CLL patients, whereas RANTES in nine control subjects and 16 CLL patients.

CLL patients with B symptoms ($n = 6$) had higher serum levels of IFNγ ($P = 0.03$), CCL3 ($P = 0.01$), CCL4 ($P = 0.004$), and a tendency toward higher G-CSF serum levels ($P = 0.05$) than patients without B symptoms ($n = 12$).

Patients with a deletion of the long arm of chromosome 13 (del13q) in CLL cells ($n = 9$) had lower serum levels of CXCL10 ($P = 0.014$) and of CCL4 ($P = 0.014$), whereas subjects with a deletion of the long arm on chromosome 11 (del 11q) in CLL cells ($n = 4$) had higher serum levels of VEGF ($P = 0.04$) and a tendency toward higher serum levels of CCL4 ($P = 0.08$), than patients without these deletions ($n = 9$ and $n = 14$, respectively).

Serum levels of CCL11 were found to be positively correlated with CXCL8 ($r = 0.54$, $P = 0.02$), TNFα ($r = 0.47$, $P = 0.048$), and GM-CSF ($r = 0.65$, $P = 0.003$).

Serum level of β2-microglobulin was found to be correlated with CCL4 ($r = 0.54$, $P = 0.02$) and CXCL10 ($r = 0.57$, $P = 0.01$). In addition, the number of lymph node groups involved, was proportional to the levels of CCL2 ($r = 0.48$, $P = 0.04$), CCL3 ($r = 0.55$, $P = 0.02$), CXCL8 ($r = 0.49$, $P = 0.04$), and CXCL10 ($r = 0.47$, $P = 0.05$), whereas

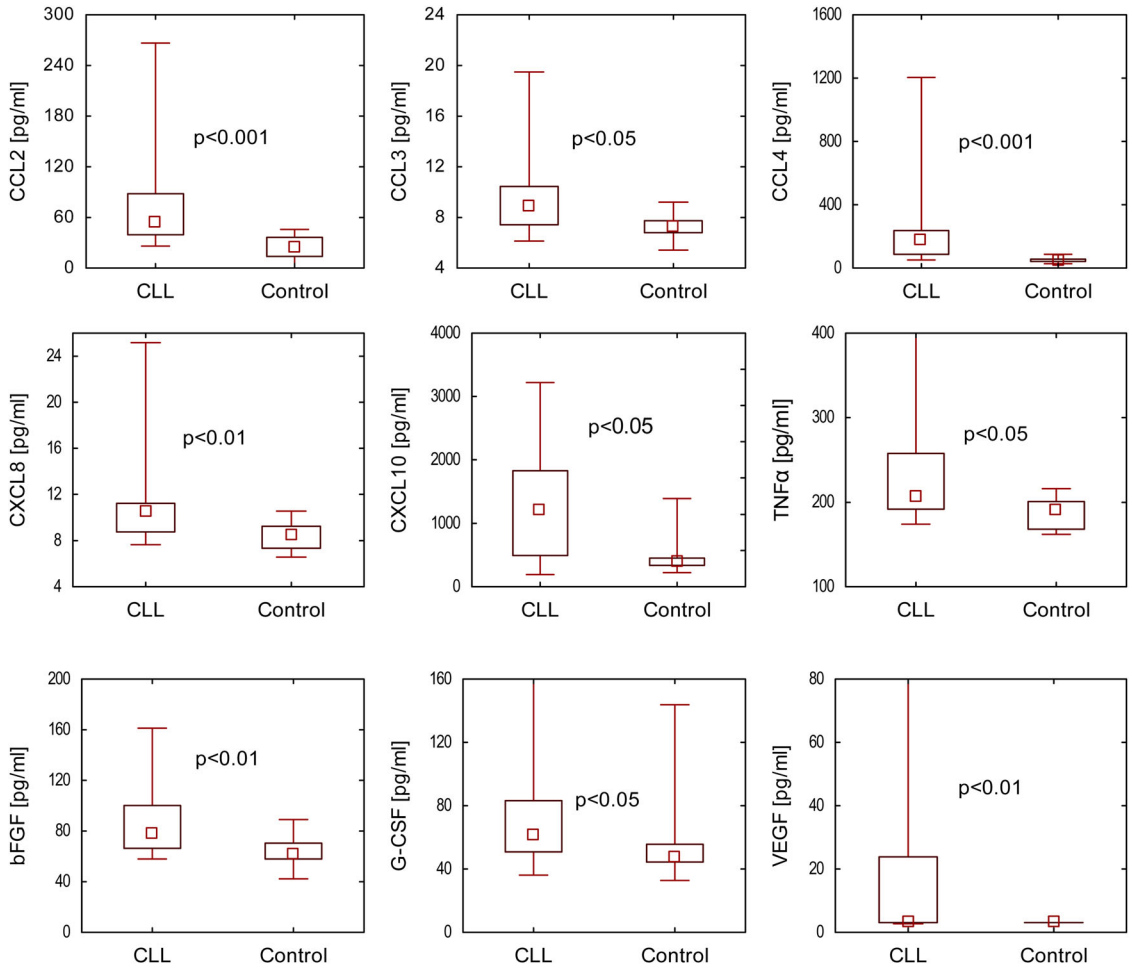

Figure 2. Cytokines serum levels in patients with CLL and subjects in the control group, presented as box plots (medians, interquartile range (1–3 Q) and range).

size of the spleen to the levels of CCL3 ($r = 0.55$, $P = 0.02$), CXCL10 ($r = 0.49$, $P = 0.04$), and G-CSF ($r = 0.51$, $P = 0.03$).

Changes of serum cytokines levels after intervention

16 CLL patients in 18 had VD deficiency and received supplementation. After 6-month supplementation 11 subjects achieved optimal VD levels.

Eight patients (group 2) initiated chemotherapy, resulting in PR (partial remission) in five, CR (complete remission) in three, and stable disease (SD) in one. In group 1 the disease remained stable (SD) in all subjects.

During VD supplementation a significant decreases in serum levels of CCL11, CCL3, and PDGF-BB were found (Fig. 3). The decrease of CCL11 was observed in CLL patients on VD supplementation solely. The decrease of PDGF-BB and CCL3 was found in CLL patients on CTH and VD supplementation. The decrease of TNFα was neither observed in group 1 ($P = 0.1$) nor group 2 ($P = 0.2$), however it was found in the combined CLL groups ($P = 0.05$). Serum levels of CXCL8, CXCL10, CCL2, CCL4, IFNγ, bFGF, G-CSF, and VEGF have not changed significantly, nonetheless, a tendency toward lowering CXCL8, CCL4, IFNγ, bFGF, and VEGF was observed, particularly in the group receiving only cholecalciferol ($P = 0.1$ for each cytokine).

Discussion

In the studied group of CLL patients the suppressive effect of VD on cytokines release can be attributed only to CCL11. In the case of CCL3 and PDGF-BB the decrease was obtained rather as result of chemotherapy.

VD plays a role in both innate and adaptive immune response.[37] Influence of VD supplementation on markers of innate immunity such as CCL11, IL-8, CXCL10, CCL2, CCL4, and RANTES was studied in healthy postmenopausal women. Treatment with VD led to decrease in serum concentrations of majority of investigated markers (except CXCL10 and partially RANTES), indicating a decrease in the level of innate immune activity.[38]

CCL11 concentration in CLL and the control group was similar, which is consistent with the previously published data.[39] It was expected as both malignant and normal B cells expressed the same pattern of secretion of CCL11.[10] Since microenvironment

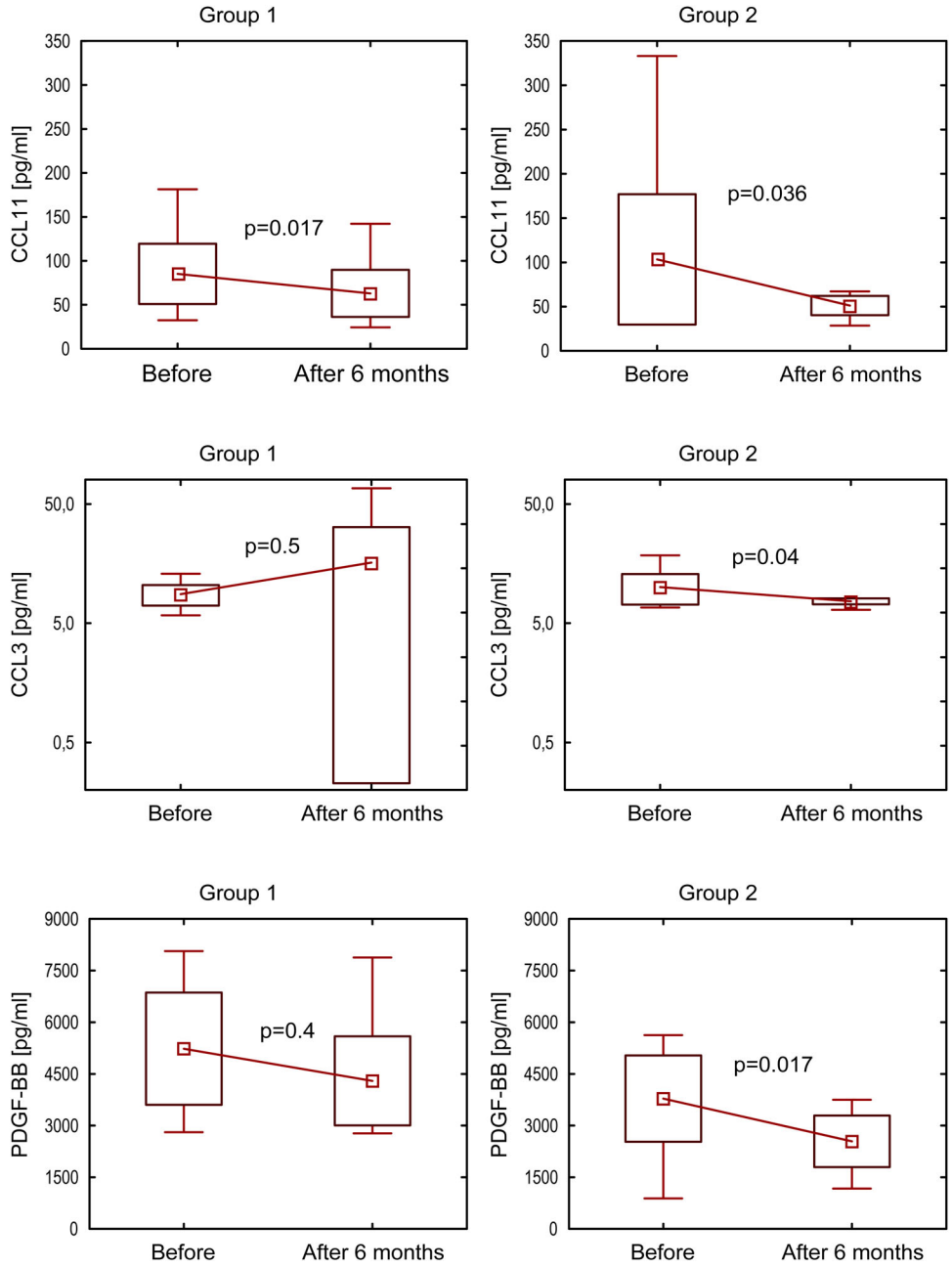

Figure 3. Serum levels of CCL11, CCL3, and PDGF-BB in CLL patients before and 6-month after the initiation of supplementation, presented as box plots for group 1 and group 2 (means, standard error and range).

protects CLL cells from drug-induced apoptosis,[40] lowering levels of microenvironment stimuli, such as CCL11, might render leukemic cells more susceptible to chemotherapy. The inhibitory effect of VD on CCL11 secretion has been recently reported *in vitro*.[27]

We found in our study significantly higher levels of CCL3 and CCL4 in comparison to the control group, which is consistent with other reports.[39] A tendency toward lowering these cytokines in CLL patients, on VD supplementation exclusively, was observed, however, significant decrease was found only in CCL3 level in CLL patients receiving cholecalciferol and chemotherapy. We found a correlation between levels of CCL4 and β2-microglobulin, as well as between CXCL10 and β2-microglobulin. Previously, an association between CCL3 and β2-microglobulin was reported in multiple myeloma.[41] Furthermore, CCL3 plasma concentrations were strongly associated with clinical stage in CLL.[42] We found an association between CCL3 serum level and size of the spleen, as well as number of lymph node groups involved, which is in consistence with previous reports.

There are discrepancies between studies in terms of serum levels of IFNγ in CLL patients: both increased[39] and decreased[43] concentrations in relation to healthy controls were reported. In our study, there was no difference in IFNγ serum level between CLL and control subjects. Negative correlation between IFN-γ and 25-OH-D_3 was previously reported[44];

however, we were unable to prove it. In addition, we showed only a tendency toward lowering IFN-γ after VD repletion. A reduction of IFN-γ serum level after VD supplementation has already been reported in children with atopic dermatitis.[45]

Increased concentrations of bFGF and VEGF in peripheral blood of CLL patients, in comparison to control subjects and decrease of both cytokines after fludarabine-containing regimens has already been reported.[46] Increased levels of PDGF and VEGF in CLL patients plasma were positively associated with high-risk factors and more advanced stage of the disease.[19] We found that higher serum levels of VEGF were associated with 11q deletion, which is an established marker of poor prognosis in CLL. We observed decrease in PDGF-B concentration in patients receiving chemotherapy and cholecalciferol and in all CLL patients. Imatinib is a tyrosine kinase inhibitor for PDGF-Rs (PDGF-receptors)[47] and VD analogs were previously reported to potentate the antitumor effect of imatinib.[48]

TNFα concentration in non-survived CLL patients was significantly higher than in both survived subjects and control subjects, thus, TNFα could be potentially used as a survival prognostic factor in CLL patients.[49] In that study, TNFα level was not reduced after treatment. In our study, TNFα level was moderately increased in CLL subjects and decreased after VD repletion in the entire group of CLL patients. Such effect of VD supplementation was observed before,[50] however, in meta-analysis comprising obese patients, VD did not have a significant influence on changes in the concentration of TNFα.[51]

Targeting the microenvironmental interactions could disrupt their protective effect toward CLL cells. Non-malignant myeloid cells might serve as a potential target and a novel immunotherapeutical strategy for CLL. *In vivo*, myeloid cell depletion resulted in a significant control of CLL development, marked a repair of innate immune cell phenotypes and a reduce in CLL-associated T cell imbalance.[12] BCR- and NLC-dependent induction of CCL3/CCL4 is abolished by fosfamatinib, which is a Syk (spleen tyrosine kinase) inhibitor.[3] Therapeutic approaches of targeting the microenvironment are very promising. Further studies are needed to determine whether adding VD could have a synergistic effect.

The small number of enrolled patients was the main drawback of our study and could prevent finding association between cholecalciferol supplementation and changes in serum levels of other cytokines.

However, it should be emphasized that it is the first report of the effect of VD supplementation on the concentrations of chemokines in CLL patients. VD repletion may exert a favorable effect on cytokines levels in VD deficient patients. However, further studies with larger study groups are necessary to confirm the effect of the cholecalciferol supplementation on the CLL microenvironment.

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Disclaimer statements

Contributors M.K.: conceiving and designing the study, obtaining funding and ethics approval, collecting the data, analyzing the data, interpreting the data, writing the article. E.N.: writing the article, revising the article. D.K.: collecting the data, revising the article. G.S.: collecting the data, revising the article. P.C.-F.: collecting the data, revising the article. J.C.: conceiving and designing the study, analyzing the data, interpreting the data, writing the article, revising the article. J.W.: conceiving and designing the study, collecting the data, revising the article.

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Conflicts of interest The authors declare no conflicts of interest.

Ethics approval The protocol was approved by the Local Bioethics Committee.

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