Short Thesis for the degree of doctor of philosophy (Ph.D.)

THE EXAMINATION OF VITAMIN D IN UNDIFFERENTIATED CONNECTIVE TISSUE DISEASE

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INTRODUCTION

The systemic autoimmune diseases are chronic inflammatory disorders involving connective tissues of the body's that are characterized by an undulating course of exacerbations and remissions. In fact, the evolution of diseases with immunopathogenetical background is usually slow and progressive. The appearance of characteristic symptoms, pathomorphology and immunological abnormalities is affected by various factors. The clinical onset of connective tissue disease is undifferentiated and some clinical manifestations and immunological abnormalities are suggestive of any systemic autoimmune disease (systemic lupus erthematosus-SLE, rheumatoid arthritis-RA, mixed connective tissue disease-MCTD, systemic sclerosis-SSc, Sjögren syndrome- Ss, polymyositis/dermatomyositis-PM/DM) but not sufficient enough to fulfil the diagnostic criteria of an established CTD. This condition has been defined as undifferentiated connective tissue disease (UCTD).

UCTD has no specific signs and symptoms. It has been reported that the most frequent clinical manifestation of UCTD are arthralgy, arthritis, morning stiffness, Raynaud's phenomenon, myalgia/myositis, skin symptoms, photosensitive rash, serositis (pleuritis/pericarditis), xerostomia and xerophtalmia, central and peripheral nervous system involvement (convulsion, headache, trigeminus neuralgia), functional and morphological lung disorders, subfebrility, fever or lymphoglandular enlargement, hepatomegaly. The most common laboratory and serological variance in patient with UCTD are quantitative and qualitative aberration of serum proteins, elevated acute phase proteins (CRP), antibodies against nuclear and cytoplasmic componens (anti-native DNA, anti-ENA, anti-Sm, anti-RNP, anti-SSA, anti-SSB, anti-Scl-70, anti-centromer, anti-Jo1, anti-PM1, anti-phospholipid antibodies), immuncomplexes, and aberration of complement system (decreased levels of

CH_{50}- and AP_{50}). Another time, there are low levels of leukocytes and thrombocytes, anemia, proteinuria, high levels of rheuma factor.

Whereas, UCTD seems to be dynamic, its progression is indicated by the changes in clinical symptomps. With the progression of the disease, new organ manifestation can appear and the existing clinical and immunological abnormalities can increase in intensity or even become permanent. About 30-40% of patients with UCTD will evolve to defined CTD during years of follow-up. Evolution to SLE and the other systemic autoimmune diseases has also been described. The highest probability of the evolution occurred within the fist 2 years during follow-up.

The pathomechanism of systemic autoimmune disease is various and now it is not known the provoke factors of the evolution to CTD. The presence of autoantibodies to nuclear or cytoplasmic components and more organ involvement indicate that the patients with UCTD have complex immunoregulatory abnormalities.

Environmental factors (such as viral and bacterial agents, drugs, sunlight) may important role in the development and progression of systemic autoimmune disease, along with susceptible genetic and hormonal background. It has been recently suggested that the vitamin D is an environmental factor, which by shaping the immune system affects the prevalence of autoimmune diseases; respectively vitamin D deficiency may have a role in the pathogenesis or progression of systemic autoimmune diseases.

The vitamin D is a well-known essential nutrient. The main supply of vitamin D is obtained through UV-mediated synthesis in the skin. Exposure of skin to ultraviolet-B light catalyzed the conversion 7-dehydrocholesterol, which is followed by isomerization into cholecalciferol. The cholecalciferol is first hydroxylated in the liver into 25-hydroxyvitamin D3 (calcitriol) and subsequently in the kidney into biological active 1,25-dihydroxyvitamin D3 (calcitriol). The classic and well-known function of vitamin D is the regulate mineral

homeostasis and thus bone formation and resorption. On the other hand, less-traditional function of vitamin D has bend demonstrated, including substantial effects on the regulation of cell proliferation and differentiation and on the immune system. Most of the known biological effects of vitamin D are mediated through the vitamin D3 recepor (VDR), which is principally located in the nuclei of cells. The binding of calcitriol to the VDR allows the VDR to act as a transcription factor that modulates the gene expression of proteins. The active vitamin D has been shown to inhibit the differentiation and maturation of myeloid dentritic cells by a VDR dependent mechanism. Vitamin D has significantly reduced the expression of MHC II, costimulatory molecules (CD80, CD86, CD4) and the maturation proteins (CD1a, CD83). In addition, antiten-presenting capacity of macrophages and dentritic cells is suppressed and the immunostimulatory IL-12 is inhibited by active vitamin D. Th1 and Th2 cells are direct targets of active vitamin D. *In vitro*, the vitamin D inhibits the proliferation of T helper1 cells moreover lowered the production of IL-2, IFN-γ, and TNF-α of Th1 cells and had an anti-proliferative effect. The active vitamin D decreases the differentiation of Th17 and promotes the number and function of regulative T cells (Treg), and function of Th2 cells. The vitamin D modifies the balance T cells, and has tolerogenic properties.

As yet, there has been no data on the plasma levels and seasonal variance of vitamin D in UCTD patients. It is not known it is possible effect the supplementation of vitamin D on immunregulative factors in UCTD patients.

AIMS:

The undifferentiated connective tissue disease is important disorder in research of pathomechanism of systemic autoimmune disease. Identifying trigger factors and the mode of action, that is how they can influence the immune system, are currently the subject of intensive research

1. We asssessed the plasma levels and seasonal variance of vitamin D in UCTD patients.
2. We determined a possible connection between the low levels of vitamin D and the clinical and serological manifestation of the disease.
3. In addition, we determined the prevalence of the 25(OH)D_3 levels in patients with UCTD and assessed its probable pathogenic role in the progression toward a well-defined CTD
4. Further aim was to determine whether a 5-week treatment with the vitamin D analogue alfacalcidol, administered in a daily dose of 0.5 μg, could modify the number and function of nTregs and the IL-17-producing capacity of Th17, as well as the rate of nTreg and Th17 cell production, respectively.
5. Our aim was to investigate the serum concentrations of proinflammatory Th1 cytokines IL-12 and IFN-g, as well as IL-23, IL-17, and IL-6, and also the key regulatory cytokine IL-10 before and after 5 weeks of alfacalcidol treatment.
6. We intended to measure whether 5 weeks of alfacalcidol treatment could increase 25-hydroxyvitamin D [25(OH)D] levels

PATIENTS AND METHODS

Patients

The study population involved 161 patients (154 women and 7 men) with UCTD followed up with and treated at the Division of Clinical Immunology, 3rd Department of Internal Medicine, Medical and Health Science Center, University of Debrecen (Debrecen, Hungary). The second part of our study, the study population involved 25 UCTD patients (all female) with UCTD followed up and treated at the Division of Clinical Immunology, 3rd Department of Internal

Medicine, Medical and Health Science Centre, University of Debrecen (Debrecen, Hungary). In case of these patients at the time of screening, the inclusion criteria were as follows: 25(OH)D < 30 ng/mL,

No patients had received corticosteroids or immune-suppressive or cytotoxic drugs, and had normal calcium homeostasis, normal bone mineral density (BMD), and normal renal function.

All patients with UCTD were enrolled based on the following criteria: (a) symptoms and signs suggestive of a CTD not fitting the accepted classification criteria for any of the defined CTDs, (b) disease duration of at least 1 year, and (c) the presence of at least one non-organ-specific autoantibody.

Firstly, age- and gender-matched 58 healthy individuals served as controls with no autoimmune/endocrine or malignant neoplastic diseases (residents and health care workers)(mean age 43,9 ± 15,01 years). Secondly, Twenty-one age-matched healthy female volunteers (mean age 55.3 ± 7.9 years) constituted the control group (health-care workers).

We assessed the plasma 25(OH)D_3 levels of 161 UCTD patients and 58 control subjects during the summer (from June to October) and winter (from January to May) periods

than in the course of alfacalcidol treatment the blood samples from patients and controls were collected at the same period of the year (between July to September).

Determination of vitamin D levels

Plasma levels of 25(OH)D_3 vitamin of patients and controls were assessed at the Department of Clinical Biochemistry and Molecular Pathology Laboratory of the University of Debrecen Medical and Health Science Center. Samples were analyzed by a high-performance liquid chromatograph (HPLC) method using the Jasco HPLC system (Jasco, Inc., Easton, MD, USA) and a Bio-Rad reagents kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sample (500 μL of plasma from EDTA [ethylenediaminetetraacetic acid] anticoagulated blood) was purified from proteins, and 50 μL of the cleaned supernatant was injected into the instrument. Separation was achieved with a reverse-phase C18 Bio-Rad column (90 × 3.2 mm) (Bio-Rad Laboratories, Inc.). The mobile phase (methanol-water mixture) had a flow rate of 1.1 mL/minute. For quantitative determination of the separated compound, a diode array detector (set at 265 nm) was used. According to current recommendations, plasma 25(OH)D_3 levels of less than 30 and 10 ng/mL were defined as vitamin D insufficiency and vitamin D deficiency, respectively.

Immune serological analyses

Antinuclear antibodies were determined by indirect immunofluorescence on HEp-2 cells. Anti-U1-ribonucleoprotein (anti-U1-RNP), anti-Sm, anti-SSA, anti-SSB, anti-Jo1, anti-Scl70, and anti-cardiolipin (anti-CL) antibodies were analyzed in all patients by enzyme-

linked immunoabsorbent assay (ELISA) in accordance with the instructions of the manufacturers (Pharmacia & Upjohn Diagnostics GmbH, Freiburg, Germany, and Cogent Diagnostics Ltd, Edinburgh, UK). IgM rheumatoid factor (RF) was assessed by nephelometry, and values greater than 50 U/L were considered positive. Anti-cyclic citrullinated peptide (anti-CCP) levels were measured using a second-generation ELISA (Quanta Lite™, CCP ELISA; Inova Diagnostics, Inc., San Diego, CA, USA) and using synthetic citrullinated peptides bound to the surface of a microtiter plate as antigen. The test was performed in accordance with the manufacturer's instructions. Serum samples, collected immediately after the initial diagnosis of patients, were separated and stored at -70°C.

Isolation of CD4+CD25+ T cells

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood of UCTD patients and healthy controls by density gradient centrifugation over Ficoll/Hystopaque (Sigma Aldrich Corp, St Louis, MO, USA). CD4+CD25+ T cells were isolated from PBMCs by the Miltenyi Regulatory T Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer’s instructions.

Suppression assay

Magnetically isolated 1 □105 CD4+CD25+ and CD4+CD25– T cells were cultured alone and together in 200 □L of cRPMI 1640 in 96-well U-bottom plates for 72 h. CD4+CD25+ and CD4+CD25– cells were cultured in a 1:1 ratio in the mixed lymphocyte reaction. For polyclonal stimulation, cells were stimulated with anti- CD3/CD28 T-cell expander microbeads (Dynal Biotech, Invitrogen Corporation, Carlsbad, CA, USA) at 1 bead/

cell concentration. The proliferation was assessed by using a tetrazolium-based assay (EZ4U Proliferation Kit, Biomedica Inc). The substrate was added in the last

2.5 h of culture and finally the optical density (OD) values were detected at 450 nm by an enzyme-linked immunosorbent assay (ELISA) reader. OD values of the mixed lymphocyte reaction were corrected by OD values of the CD4+CD25+ T cells cultured alone. Suppression activity (proliferative index) was determined as the ratio of OD values of the mixed lymphocyte reactions (MLRs) and CD4+CD25+ T cell cultures (correction: ODMLRcorr = ODMLR – ODCD25+; proliferative index = ODCD25–/ODMLRcorr).

Analysis of CD4+CD25^{high}Foxp3+ T cells by Flow Cytometry

Foxp3 staining was carried out according to the instructions of the manufacturer (eBioscience, San Diego, CA, USA). Briefly, PBMCs were separated by Ficoll/Hystopaque gradient centrifugation then 100 □l of prepared cells was added to each tube (1x10^6 cell/ml). After cell surface staining with CD4 and CD25 monoclonal antibodies, cells were washed in cold Flow cytometry staining buffer (eBioscience). Freshly prepared 1 ml Fixation/Permeabilization working solution was added to each sample. Cells were incubated at 4°C for 30-60 minutes in dark, then washed by adding 2ml 1X Permeabilization Buffer (eBioscience) and blocked with 2% (2□l) normal rat serum in 1X Permeabilization Buffer in 100 □l at 4°C for 15 minutes. After blocking 20 □l anti human Foxp3-PE antibody (eBioscience) was added and cells were incubated at 4°C for at least 30 minutes in dark. Finally, cells were washed with 2 ml 1X Permeabilization Buffer and resuspended in Flow Cytometry Staining Buffer (eBioscience).

Lymphocytes were gated on the basis of their forward and side scatter properties. Ten thousand gated events (lymphocytes) were collected in each sample on FACSCalibur

equipment (Becton Dickinson, Germany, Heidelberg). Data were analyzed using CellQuest software (Becton Dickinson). Foxp3 positivity of $CD4^{+}CD25^{high}$ suppressor T cells was verified with Foxp3 staining. The mean fluorescence intensity (MFI) of Foxp3 was significantly higher in $CD4^{+}CD25^{high}$ suppressor T cells compared to the $CD4^{+}CD25^{low}$ or $CD4^{+}CD25^{-}$ cells ($p<0.01$). The following reagents were used: Ficoll and CD4-FITC monoclonal antibody (Sigma Aldrich Corp, St. Louis, MO, USA), CD25-PC5 (Immunotech, Marseilles, France), Foxp3-PE and intracellular staining kit (eBioscience).

Evaluation of intracellular cytokines by flow cytometry

Briefly, 1ml heparinized blood was diluted twofold in RPMI 1640 (GIBRO-BRL, Invitrogen Corporation, Carlsbad, CA, USA). Cells were stimulated using 25ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich Corp), and 1 ng/ml ionomycin (Sigma Aldrich Corp) for 4 hours in the presence of 10 mg/ml brefeldin-A (BFA) (Sigma Aldrich Corp). The incubation was carried out at $37^{o}C$ in a 5% CO_2 environment. Unstimulated cells containing BFA served as controls. After stimulation cells were surface stained with anti-human CD4-PC5 or CD8-PC5 antibody (Immunotech) for 30 minutes at room temperature. Samples were washed, followed by fixation and permeabilization using IntraPrep™ Permeabilization Reagent according to the manufacturer's instruction (Beckmann Coulter, Miami, FL). Subsequently, samples were incubated for 30 minutes in dark with monoclonal antibodies, specific for intracytoplasmic antigen, anti-human IL-17-PE (R&D Systems Inc., Minneapolis, MN, USA), or their proper isotype controls (anti-mouse IgG1-FITC or IgG1-PE isotype antibodies). The cells were fixed using 1% paraformaldehyde and analyzed within 6 hours by a Coulter FC500 flow cytometer (Beckmann Coulter). At least ten thousand CD4+ or CD8+ cells were counted (only CD4+ cells for IL-17) and analyzed using the CXP Analysis Software (Beckmann Coulter, Fullerton, CA, USA).

ELISA detection of plasma IFN-γ, IL-12, IL-6, IL-17 IL-23, and IL-10

Plasma levels of IL-23, were measured by enzyme-linked immunosorbent assay (ELISA) following the manufacture's instructions (IL-23 ELISA kits, from Bender MedSystems, Burlingame, CA, USA; IL-10, IL-6, IL-17, IFN-γ and IL-12 (p40) ELISA kits both from R&D system, Minneapolis, MN, USA). The minimal detectable concentrations were 0.7 pg/ml for IL-6, 78 pg/ml for IL-23, 15 pg/ml for IL-17, 7.8 pg/ml for IL-10, and 5.6 pg/ml for IFN-γ. Intra-assay and inter-assay coefficients of variation for all ELISA were <5%, and <10%, respectively. All samples were measured in duplicate.

Statistical analysis

Statistical analyses were performed by using SPSS software, version 15.0 (SPSS Inc. Chicago, IL, USA). Data are presented as mean values ± standard deviation. After testing for normality, data were compared with Student's *t*-test (paired and unpaired) or Mann–Whitney *U* test and correlated with Spearman's rank correlation. Individual relative risk and 95% confidence intervals were calculated using separate logistic regression for variables found to be significant or approaching significance in the former analysis. *P* values less than 0.05 were considered statistically significant for all tests.

CONCLUSIONS

In the present study we first analyzed the serum levels and seasonal variance in the plasma levels of 25(OH)D in a large cohort of patients with UCTD.

During the follow-up period, 35 out of 161 UCTD patients (21.7%) developed an established CTD. The evolution to defined CTD was an average of 2.3 ± 1.2 years.

According to our studies in UCTD patients vitamin D levels significantly lower than the control group both the summer and winter periods (UCTD-summer: 33,0 ± 13,4 ng/ml, control summer: 39,5 ± 11,2 ng/ml; p< 0,01; UCTD-winter: 27,8± 12,48, control winter: 38,2± 12,1 ng/ml; p< 0,001).

Circulating levels of vitamin D fluctuate seasonally in UCTD patients, with low levels of 25(OH)D in the winter months and high levels during the summer months. The variance between the summer and winter level of vitamin D was significant (UCTD summer: 33,0± 13,4 ng/ml, UCTD winter: 27,8 ± 12,48 ng/ml, p< 0,001), but no in the control group (control summer: 39,5±11,2 ng/ml, control winter: 38,2± 12,1; ns).

Our data suggested that patients with UCTD have vitamin D insufficiency in 41% of cases in the summer months and **even more became vitamin D-deficient during the wintertime (58 %, 88 patients)**. There was only 18,4% (14 cases) in control with vitamin D insufficiency.

In UCTD patients with vitamin D insufficiency, the winter levels of vitamin D were significantly lower than the summer levels (UCTD [<30 ng/mL] summer: 21.9 ± 4.7 ng/mL and winter: 18.1 ± 5.9 ng/mL, $P = 0.03$).

Vitamin D deficiency was found in 5 of the UCTD cases (3.1%) compared with none in the control group.

In our results, **the probability to develop dermatological symptoms (photosensitivity, vasculitis, and erythema), oesophagus dysmotility and pleuritis correlated with vitamin D insufficiency** (<30 ng/ml). The **presence of anti-U1-RNP (p=0,024), anti-SSA(p=0,029), and anti-CCP (p=0,0001) occurred more frequently in these particular patients.**

Interestingly, the lowest levels of vitamin D (<30 ng/mL) were measured in UCTD patients who subsequently evolved to defined CTDs (evolution to well-established CTD**:** 14,7 ± 6,45 ng/ml, stable UCTD: 33,0 ± 13,4 ng/ml; p<0,0001). All patient with vitamin D deficiency diffentitated to any well-definted CTD. It raises the possibility that vitamin D deficiency may contribute to the progression into well-defined CTDs.

We firstly investigated the effect of vitamin D analogue alfacalcidol on the regulatory T cells and IL-17 expressing Th17 cells in patients with UCTD.

In the beginning of the follow-up period, 25(OH)D levels of UCTD patients were lower compared to healthy controls in summer months and all 25 UCTD patients had vitamin D insufficiency (< 30 ng/mL). Oral administration of alfacalcidol during 5 weeks (0.5 μg/ day) elevated the plasma 25(OH)D levels.

(Before and after vitamin D treatment: 23,5±5,6 ng/ml vs. 34,5±7,4 ng/ml; p=0,059) Although the serum vitamin D level elevation was not significant, 5 weeks after alfacalcidol treatment only 28% (seven patients) of the UCTD patients still remained vitamin D insufficient. Our data suggest that continuously applied vitamin D supplementation is required, but future prospective studies are needed to determine adequate doses and treatment length in UCTD patients.

Our study firstly demonstrated the vitamin D analogue alfacalcidol influences favorably the immune-functions.

We found significant elevation of the proinflammatory cytokines IL-12 and IFN-*g*, as well as the Th17-cell associated cytokines IL-23, IL-17, and IL-6 in UCTD patients.

In this study we found that alfacalcidol treatment reduced IL-17 production and decreased the production of the proinflammatory cytokines IL-6 and IL-23, which synergistically induce its secretion, in addition to decreasing the serum concentration of Th1-related cytokines (IFN-*g* and IL-12). Thus, our data show that, in patients with UCTD, alfacalcidol inhibits both Th1 and Th17 cells, while shifting the response towards the regulatory cytokine, IL-10.

We found negative correlation between the baseline 25(OH)D levels and the serum levels of IL-23 and IL-6 (IL-23: -0.713, IL-6: -0.751).

Our study is the first clinical investigation in which the IL-17 expression of CD4+ T lymphocytes was assessed in patients with UCTD and we found that the IL-17 expression of CD4+T lymphocytes was significant higher in untreated patients, compared to UCTD patients, treated with alfacalcidol.

Moreover, the percentage and the absolute number of nTregs(CD4+CD25highFoxp3+) were significantly lower in untreated patients with UCTD than after vitamin D analogue supplementation.

Both the relative and absolute rate of nTreg/Th17 cells decreased after alfacalcidol treatment in UCTD patients, compared to the pretreatment values (CD4+CD25+^{high}Foxp3 / CD4+Th17+ before treatment: 0.00114 ± 0.0061, after treatment 0,00355 ± 0,00223, $p< 0,05$).

These findings we demonstrated that alfacalcidol treatment has an effect on CD4+CD25+ Treg cells, and this vitamin D analogue can increase the suppressive effect of Tregs on the proliferation of autologous, effector CD4+CD25– T cells. Data indicating that

alfacalcidol treatment enhanced the suppressor activity of CD4+CD25+ T cells in four out of five UCTD patients (Proliferative index: 0,638±0,1 vs. 0,415±0,15)

In our results, in UCTD patients the regulation of immune responses is defected and the suppressor activity towards autoimmune processes is diminished. The IL-17 expression of CD4+T lymphocytes was high in UCTD patients. We found significant elevation of the proinflammatory cytokines IL-12, IFN-γ, as well as the TH17 cell associated cytokines, namely IL-23, IL-17 and IL-6 in UCTD patients. The Th17/nTreg imbalance exists in patients with UCTD, suggesting a potential role for this immunoregulatory error in the progression from the UCTD stage towards well-established CTDs

Alfacalcidol treatment has an affect on CD4+CD25+ Treg cells, and the vitamin analogue can increase the suppressive effect of Tregs on the proliferation of autologous CD4+CD25- T cells.

Alfacalcidol treatment reduced IL-17 production and decreased the production of proinflammatory cytokines, IL-6, IL-17 and IL-23 that synergistically induce its secretion, and decreased the plasma concentration of Th1 related cytokines (IFN-γ and IL-12)

Alfacalcidol inhibits both Th1 and TH17 cells, while shifting the response towards the regulatory cytokine, IL-10. The supplementation of vitamin D can restore the immune-homeostatic disturbance and it may influence Th17/nTreg imbalance, therefore could be beneficial in the management of autoimmune diseases.

LIST OF PUBLICATION

Scientific publication of the thesis

1. **Zöld É**, Szodoray P, Nakken B, Baráth S, Kappelmayer J, Csáthy L, Hajas Á, Sipka S, Gyimesi E, Gaál J, Barta Z, Hallay J, Szegedi G, Bodolay E: Alfacalcidol treatment restores derailed immune-regulation in patients with undifferentiated connective tissue disease

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