The Journal of Clinical Endocrinology & Metabolism, 2020, Vol. 105, No. 12, e4767–e4777
doi:10.1210/clinem/dgaa588
Clinical Research Article

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Vitamin D Supplementation Modulates ICOS$^+$ and ICOS– Regulatory T Cell in Siblings of Children With Type 1 Diabetes

Silvia Savastio,[1] Francesco Cadario,[1,2] Sandra D'Alfonso,[3] Marta Stracuzzi,[1] Erica Pozzi,[1] Silvia Raviolo,[1] Stefano Rizzollo,[1] Luca Gigliotti,[3] Elena Boggio,[3] Giorgio Bellomo,[3] Chiara Basagni,[3] Gianni Bona,[3] Ivana Rabbone,[1,3] Umberto Dianzani,[2,3,4] and Flavia Prodam[1,2,3]

[1]SCDU of Pediatrics, University Hospital Maggiore della Carità, Novara, Italy; [2]Interdisciplinary Research Center of Autoimmune Diseases, Università del Piemonte Orientale, Novara, Italy; [3]Department of Health Sciences, Università del Piemonte Orientale, Novara, Italy; and [4]SCDU of Clinical Biochemistry, University Hospital Maggiore della Carità, Novara, Italy

ORCiD number: 0000-0001-9660-5335 (F. Prodam).

Received: 21 April 2020; Accepted: 21 August 2020; First Published Online: 26 August 2020; Corrected and Typeset: 14 October 2020.

Abstract

Objectives: Vitamin D plays an immunoregulatory activity. The aim of this study was to assess the correlation between blood serum 25(OH)D levels and Th17 and Treg circulating subsets, mainly Treg/inducible costimulatory-positive (ICOS$^+$), which seems to have a protective role in autoimmunity, in children with type 1 diabetes mellitus (T1D) and their healthy siblings (S). The secondary aim was to evaluate the impact of vitamin D supplementation on these subsets.

Patients and Methods: 22 T1D and 33 S were enrolled. Glucose, hemoglobin A1c, 25 OH vitamin D (25[OH]D), T helper type 17 (Th17; CD4$^+$CCR6$^+$), regulatory T cells (Treg; CD4$^+$CD25$^+$Foxp3$^+$), and Treg/ICOS$^+$ cells were evaluated. According to human leukocyte antigen (HLA) haplotypes, subjects were classified as "at risk" (HLA$^+$), "protective haplotypes" (HLA$^-$; "nested controls"), and "undetermined" (HLA^{UND}). T1D and S subjects were supplemented with cholecalciferol 1000 IU/die and evaluated after 6 months.

Results: Vitamin D insufficiency (74.4%) and deficiency (43%) were frequent. S subjects with 25(OH)D levels <25 nmol/L had Th17, Treg ($p < 0.01$), and Treg/ICOS$^+$ ($P < 0.05$) percentages higher than subjects with 25(OH)D >75 nmol/L. Treg/ICOS$^+$ percentages ($P < 0.05$) were higher in HLA$^-$ S subjects compared to percentages observed in S with T1D. At baseline, in S subjects, a decreasing trend in Th17 and Treg/ICOS$^+$ values ($P < 0.05$) from vitamin D deficiency to sufficiency was observed; 25(OH)D levels were negative predictors of Treg/ICOS$^+$ ($R^2 = 0.301$) and Th17 percentages ($R^2 = 0.138$). After 6 months, supplemented S subjects showed higher 25(OH)D levels ($P < 0.0001$), and lower Th17

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

($P < 0.0001$) and Treg/ICOS$^+$ ($P < 0.05$) percentages than at baseline; supplemented T1D patients only had a decrease in Th17 levels ($P < 0.05$).
Conclusion: Serum 25(OH)D levels seem to affect Th17 and Treg cell subsets in S subjects, consistent with its immunomodulating role. HLA role should be investigated in a larger population.

Key Words: Type 1 diabetes, Treg, Th 17, vitamin D, immunomodulation

Type 1 diabetes (T1D) is a multifactorial autoimmune disease involving both polygenic inherited and environmental risk factors (1,2). In recent years, low plasma levels of 25 OH vitamin D (25[OH]D) have been associated with increased risk of T1D (3-7) and other autoimmune diseases (8-11). Animal studies showed that vitamin D supplementation may prevent or blunt several autoimmune diseases including T1D, but human clinical trials are still inconsistent (3,11-15). Vitamin D metabolites may exert their immunoregulatory effects on both the innate and adaptive immune responses by interacting with vitamin D receptor (VDR) expressed in most immune cells (15-19).

A complex relationship connects vitamin D and T1D at multiple levels, including polymorphisms of the VDR gene (20) and other genes involved on vitamin D metabolism, such as *CYP27B1* and *CYP2R1* genes encoding the main enzyme for vitamin D activation (21-23), and *GC* gene encoding VDBP, the main circulating carrier for vitamin D metabolites (24, 25). The genetic complexity of T1D is well known. More than 60 genetic loci are involved in the risk of the disease development. By far, the strongest of them are the loci in the human leukocyte antigen (HLA) class II region where combinations of HLA-DRB1, -DQA1, and -DQB1 alleles can significantly increase or decrease the risk of T1D (26, 27).

CD4$^+$ and CD8$^+$ T cells are involved in the autoimmune attack of the pancreatic islets in T1D. Proinflammatory T helper type 1 (Th1) and T helper type 17 (Th17) cells are key mediators of autoreactivity against beta cells (28,29), while CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells (Treg) are essential for maintenance of peripheral tolerance (30). Nonobese diabetic mice spontaneously develop T1D, and disease is worsened in Foxp3–/– nonobese diabetic (NOD) mice, displaying defective Treg function (31-33). In T1D patients, Th17 cells are increased in the peripheral blood and lymph nodes (34,35), while Treg cells are functionally impaired and numerically reduced (36-38). However, these findings are still debated (39-44).

In animal models, 1,25(OH)2D—that is, the active form of vitamin D acting on VDR—seems to enhance the activity of Treg cells and inhibits Th17 and Th1 cells (45, 46).

An immunological effect of vitamin D in T1D subjects has been suggested by some studies. Gabbay et al (47) showed that 2000 U/die of vitamin D for 18 months determined a significant increase in Treg percentages and a significant decrease in hemoglobin A1c (HbA1c) levels and glutamic acid decarboxylase (GAD65) titers in new-onset T1D supplemented than not supplemented ones. Similarly, Bogdanou et al (48) demonstrated that T lymphocyte profiles increased in male adults with T1D after 4000 IU/die of cholecalciferol for 3 months. Moreover, 70 IU/Kg body weight/day of cholecalciferol determined a significant increase in Treg suppressive capacity in T1D subjects as suggested by Treiber et al (49).

However, results on the effect of vitamin D on T cells in patients with T1D are scarce and inconsistent (36,39-44).

Inducible costimulator (ICOS) is a costimulatory receptor expressed on activated effector T cells and binds to ICOSL expressed by several cell types of both hemopoietic and non-hemopoietic origin. The ICOS/ICOSL interaction supports the activation of several T helper cell subsets, and particularly Th17 and Treg cells (50-54). ICOS seems to have a role in supporting Treg cell functions against autoimmunity activation, also in T1D. ICOS/ICOS-L stimulation can raise interleukin (IL)-2, inducing better survival, and suppressive activity of Treg cells in the islets. Moreover, pancreatic Treg cells upregulate CXCR3 chemokines to increase the recruitment of Treg cells into the inflamed islets. The expression of CXCR3 ligands results higher in ICOS$^+$ Treg cells compared to ICOS– Treg cells. In the islets, ICOS$^+$ Treg cells find interferon-gamma (IFN-γ) produced by Treg suppress effector cells and upregulate CXCR3 to migrate to the pancreas, inducing an auto-regulation of homing by the ICOS$^+$ Treg subset (32) Administration of monoclonal antibodies against ICOS disrupts the Treg/Tref suppress effector cells balance, resulting in rapid conversion of early insulitis into diabetes in NOD mice (55).

Furthermore, a faster T1D progression has been shown in NOD.BDC2.5 mice in which the ICOS pathway is abolished (56).

The aim of this study was to investigate the correlation between serum 25(OH)D levels and the peripheral blood distribution of T cells subsets modulated by ICOS activity (Th17, total Treg, Treg/ICOS$^+$) in children with T1D and their siblings (S), stratified for HLA genotypes. Several reports show that the S often display a silent anti-beta cells

autoimmune response and may be informative subjects to identify early markers of disease onset and/or progression (57,58).

Materials and Methods

Subjects and study design

Twenty-two children and adolescents with T1D and 33 related first-degree relatives S were recruited. All T1D patients were enrolled at least after 6 months from the onset of the disease. The study was carried out at the Division of Pediatrics, University of Piemonte Orientale (Novara, Italy) from December 1, 2014 to June 31, 2017. The protocol was approved by the Local Ethical Committee (CE 138/14) of the Novara Hospital, and a written consent was required at the time of enrollment (T0).

Subjects with acute illnesses, anorexia nervosa or other eating disorders, genetic diseases, or under treatments interfering with vitamin D metabolism or immune function (corticosteroids, anticonvulsants, immunosuppressive drugs) were excluded. Nobody had micro- or macrovascular complications at the enrollment, as expected. The diagnosis of T1D was performed according to the American Diabetes Association criteria (2). Height was measured by the Harpenden stadiometer, and body weight, by using an electronic scale. Body mass index (BMI) was calculated by dividing body weight (in kilograms) by height (in meters squared). Height, weight, and body mass index-z score were stratified according to growth charts (59).

At baseline (T0), all subjects underwent anamnestic evaluation, physical examination, and assessment of metabolic parameters. Deoxyribonucleic acid (DNA) samples were obtained to evaluate their HLA genotyping. Both T1D patients and S were characterized by a questionnaire on ethnic background, birth weight, birth order, duration of breastfeeding or consumption of milk formula, time of weaning and gluten first administration, oral supplementation with vitamin D3 (cholecalciferol), and intake of vitamin D-enriched foods in the first year of life. All S had no antibodies predisposing to diabetes.

At T0, children with T1D and first-degree relatives with hypovitaminosis D (25[OH]D values <75 nmol/L [30 ng/mL]) were supplemented with cholecalciferol, given once a week (7000 U/week). Because the authors report an immunomodulatory role of vitamin D system for 25(OH)D levels of ≥100 nmol/L (≥40 ng/mL) (60), subjects with serum 25(OH)D levels ranging between 75 and 100 nmol/L (30–40 ng/mL) were also supplemented (3,61,62). Clinical and metabolic parameters were revaluated after 6 months (T1). Adherence to the treatment was monitored with monthly phone calls.

Regarding HLA, children with DRB1*03-DQA1*05:01-DQB1*02:01 (DR3/DQ2) and/or DRB1*04-DQA1*03:01-DQB1*03:02 (DR4/DQ8, with any DRB1*04 allele different from DRB1*04:03) T1D risk haplotypes were categorized as "at risk" (HLA^+). Conversely, children carrying at least 1 T1D protective DRB1*15:01 or DRB1*15:02 or DRB1*04:03 allele or DRB1*07-DQA1*02:01-DQB1*02:01 haplotype were categorized as "protective haplotypes" (HLA^-). Individuals carrying other different HLA haplotypes were considered as "undetermined" (HLA^{UND}). HLA^- S were considered as nested controls.

Subjects were also divided according to their serum 25(OH)D levels graded as insufficient <75 nmol/L (<30 ng/mL), deficient <50 nmol/L (<20 ng/mL), and severely deficient <25 nmol/L (<10 ng/mL) according to criteria of Endocrine Society (63).

Biochemical and immune evaluations

Fasting glucose, glycated hemoglobin (HbA1c), serum 25(OH)D levels, fasting c-peptide, islet autoantibodies GAD65, insulin (insulin autoantibodies), and protein tyrosine phosphatase islet antigen 2 antibodies were evaluated at the enrollment. Biochemical evaluations were performed in the Hospital Biochemical Laboratory according to certificate standard techniques.

Plasma glucose levels (mg/dL) were measured by the gluco-oxidase colorimetric method (GLUCOFIX, Menarini Diagnostici, Florence, Italy). HbA1c levels were measured by the high-performance liquid chromatography, using a Variant machine (Biorad, Hercules, CA); intra- and interassay coefficients of variation are respectively lower than 0.6% and 1.6%, respectively. Linearity is excellent from 3.2% (11 mmol/mol) to 18.3% (177 mmol/mol). 25(OH)D serum levels (nmol/L) were measured with a direct competitive chemiluminescent immunoassay (Liaison Test 25OHD total, DiaSorin Inc, Stillwater MN, USA). Coefficients of variation for interassay analyses was 10%. The presence and the antibody titer of GAD65, islet antigen 2 and insulin autoantibodies, expressed in IU/mL, were carried out by immunoradiometric assay with analytical variability coefficient of 13%, 8.4%, and 13%, respectively.

Flow cytometry

The circulating peripheral levels of total $CD4^+CCR6^+$ T cells (Th17 cells), total $CD4^+CD25^+FoxP^{3+}$ regulatory T cells (Treg), and $CD4^+CD25^+FoxP^{3+}$ Treg expressing ICOS (Treg/ $ICOS^+$) were evaluated at each time point by flow cytometry. The following monoclonal antibodies (mAb) were used: Percp-Cy5-conjugated anti-CD4 mAb (peridinin chlorophyll protein complex-cyanine 5), PE-conjugated

anti-CCR6 mAb; PE-conjugated anti-CD25 mAb, FITC-conjugated anti-ICOS mAb (fluorescein isothiocyanate), APC-conjugated (allophycocyanin) anti-Foxp3 mAb. For Foxp3 intracytoplasmic staining, the surface-stained cells were fixed with an appropriate volume of fixing solution provided by the FIX & PERM Cell Permeabilization Reagents (Life Technologies) kit for a further 20 min at a temperature of 4°C and then incubated with the anti-FoxP3 mAb. Samples were analyzed with a FACSCalibur instrument (BD Biosciences) and the CellQuest Pro software (BD Biosciences).

HLA haplotypes genotyping

Genomic DNA was extracted from whole blood samples using the kit ReliaPrep™ Blood gDNA Miniprep System (Promega).

The presence of T1D risk or protective HLA alleles/haplotypes were identified according to the following strategy. First, individuals were genotyped using the "Celiac disease with mix for the investigation of homozygosity DQB1*02" kit (Nuclear Laser Medicine), based on polymerase chain reaction (PCR) with sequence specific primers. This kit identifies the following HLA haplotypes:

- HLA-DRB1 * 03, DQA1 * 05:01, DQB1 * 02:01
- HLA-DRB1 * 04, DQA1 * 03:01, DQB1 * 03:02
- HLA-DRB1 * 07, DQA1 * 02:01, DQB1 * 02:02
- HLA-DRB1 * 11, DQA1 * 05:05, DQB1 * 03:01

Second, to further characterize the different DRB1*04 alleles, exon 2 of the DRB1 gene has been amplified with an allele-specific PCR allowing to amplify only DRB1*04 alleles. The amplified exon 2 has been sequenced using BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) on an automated 3130 XL DNA analyzer (Applied Biosystems, Foster City, CA, USA),which identifies the specific DRB1 * 04 alleles (DRB1*04: 01, DRB1*04: 02, DRB1*04: 03, DRB1*04:04, and DRB1* 04: 05)

Finally, to genotype the DRB1*15 alleles (DRB1*15:01 and DRB1*15:02), allele-specific PCRs were used amplifying the exon 2 of the DRB1 gene. The allele-specific PCR methods for DRB1*04 and DRB1*15 genotyping do not distinguish between homozygous or heterozygous subjects for the allele. Primer and PCR conditions are available upon request.

Statistical analyses

Data were expressed as mean ± standard error of the mean or percentages, as appropriate. Sample size of 15 subjects for each group was calculated on a previous report about stimulations with multipotent stem cells of Treg ICOS+ derived from blood of children with T1D (64). For continuous variables, differences were compared using nonparametric Mann-Whitney U test and $\chi 2$ test for categorical variables. Variations from T0 to T1 were analyzed with analysis of variance for repeated measures. Correlations of serum 25(OH)D levels with continuous variables (clinical, hormonal, and T cells) were examined using Pearson correlation coefficients. Logistic regression analysis was used to analyze trends among 25(OH)D categories. A stepwise multivariate regression test was carried out to assess the influence of serum 25(OH)D levels and HLA haplotypes on Treg/ICOS$^+$ and Th17 cells (dependent variables). Statistical significance was defined according to a P value < 0.05. All statistical analyses were performed using SPSS for Windows version 22.0 (IBM SPSS Statistics for Windows, Armonk, NY, USA).

Results

Patients

A total of 22 children with T1D and 33 S were recruited. Table 1 presents data at baseline (T0) and after 6 months (T1) of vitamin D supplementation. T1D patients and S had an age (mean ± standard error of the mean) of 10.0 ± 0.6 and 9.8 ± 1.0 years, respectively. The disease duration in

Table 1. Auxologic, Metabolic, and Immune Parameters in T1D and S

	T1D	S	P
T0			
Number	22	33	
Age (years)	10 ± 0.6	9.8 ± 1	0.521
Weight (Kg)	36.9 ± 3	31.9 ± 3	0.141
Height (cm)	141.8 ± 4.1	131.8 ± 4.8	0.286
Serum 25(OH)D (nmol/L)	20 ± 2.3	22.7 ± 1.9	0.888
Neutrophils (x 103/µl)	4.77 ± 2.2	3.5 ± 1.0	<0.05
Th17 (%)	3.6 ± 0.5	3.9 ± 0.3	0.395
Treg (%)	1.2 ± 0.2	1.2 ± 0.2	0.782
Treg/ ICOS+ (%)	2.5 ± 0.6	3.5 ± 0.8	0.545
T1			
Number	16	22	
Age (years)	9.9 ± 0.9	10.5 ± 1.1	1.000
Serum 25(OH)D (nmol/L)	31.8 ± 2.2	31.3 ± 2.3	0.758
Neutrophils (× 103/µL)	2.3 ± 0.3	3.1 ± 0.7	0.218
Th17 (%)	2.4 ± 0.2	2.7 ± 0.3	0.735
Treg (%)	0.8 ± 0.1	1.1 ± 0.1	0.184
Treg/ ICOS+ (%)	1.5 ± 0.3	1.4 ± 0.1	0.982

Data, expressed as mean ± standard error of the mean, are given at the enrollment (T0) and after 6 months (T1) of vitamin D supplementation (cholecalciferol 7000 IU/week).

T1D subjects was 2.6 ± 0.8 years. During the first year of life, 49.8% of subjects had received vitamin D supplementation and vitamin D-enriched foods with an average daily intake of about 400 IU. No differences were shown in the past medical history between T1D and S subjects, apart from the candidate disease diagnosis. All S were negative for antibodies predisposing to diabetes, while all T1D were positive. Patients with T1D had higher neutrophils ($P < 0.05$), glucose and HbA1c levels than S. No other significant differences were found, including lymphocyte subsets.

Lymphocyte subsets according to serum 25(OH)D levels and HLA genotyping

Regarding vitamin D levels, the T1D group showed insufficiency, deficiency, and severe deficiency in 36.4%, 31.8%, and 9.1% respectively, whereas in the S group, the results were in 30.3%, 34.0%, and 8.2%, respectively, without statistically significant differences.

S subjects showing serum 25(OH)D levels <25 nmol/L had Th17 (5.39 ± 0.47 *vs* 2.66 ± 0.39%; $P < 0.03$), Treg (1.98 ± 0.36 *vs* 0.64 ± 0.16%; $P < 0.01$), and Treg/ICOS$^+$ (8.87 ± 3.63 *vs* 1.72 ± 0.26% $P < 0.03$), which are statistically higher than those with serum 25(OH)D >75 nmol/L (Table 2). Furthermore, in S, an increasing trend in Th17 and Treg/ICOS$^+$ values ($P < 0.03$) was found from sufficiency to severe deficiency of 25(OH)D status. By contrast, a similar trend was only observed in the subset of Th17 cells in T1D patients ($P < 0.05$) (Table 2).

Most of T1D subjects (20/22, 91%) carried T1D risk HLA$^+$ haplotypes (HLA$^+$), and only 2 (9%) were HLA^{UND}. In S, the frequencies of HLA$^+$, HLA$^-$ (carrying T1D protective alleles), and HLA^{UND} individuals were 57.6% (19/33), 18.2% (6/33), and 24.2% (8/33), respectively. Vitamin D levels were 62.4 ± 19.0 and 36.7.7 ± 18.0 nmol/L in T1D HLA$^+$ and HLA^{UND}, respectively; S presented mean vitamin D values of 57.4 ± 19, 56.4 ± 15 and 47.4 ± 23 nmol/L in HLA$^+$, HLA$^-$, and HLA^{UND}, respectively. No significant differences in vitamin D levels were present according to HLA typing in each group. Analysis of Treg/ICOS+ lymphocyte subset showed that S HLA$^-$ subjects displayed percentages of Treg/ICOS$^+$ ($P < 0.05$) higher than T1D (Fig. 1). No differences in Treg/ICOS$^+$ were present between S HLA$^+$ ($P = 0.729$) or HLA^{UND} ($P = 0.872$) and T1D.

Vitamin D3 supplementation

At enrollment, all T1D and S subjects had serum 25(OH)D values <100 nmol/L and then were supplemented with cholecalciferol 1000 IU/day. After 6 months (T1), 16 T1D and 26 S resulted adherent to the supplementation, while 7/33 S (21.0%) and 6/22 (27.0%) T1D subjects did not regularly take cholecalciferol due to poor compliance. Before the supplementation, in S, 26.9% of children (7/26) had 25(OH)D insufficiency and 46.2% (12/26), vitamin D deficiency, whereas 26.9% (7/26) had 25(OH)D levels ranging between 75 and 100 nmol/L. In T1D, 35.0% had vitamin D insufficiency and 45.0%, vitamin D deficiency, whereas 20.0% had 25(OH)D levels ranging between 75 and 100 nmol/L.

Table 2. Lymphocyte populations (Th17, Th17/IL17$^+$, Treg, Treg/ICOS$^+$) across Vitamin D levels [25(OH)D] in S and T1D Subjects

	25(OH) D > 75 nmol/L (S)	25(OH)D 50–75 nmol/L (I)	25(OH) D < 50 nmol/L (D)	25(OH) D < 25 nmol/L (SD)	$P^{\text{for trend}}$
S					
Number	9	10	8	6	
Serum25(OH)D (nmol/L)	85.3 ± 6.4	62.8 ± 2.3	38.6 ± 2.5	17.9 ± 4.2	*P < 0.0001*
Th17 (%)	2.66 ± 0.39	3.98 ± 0.74	4.24 ± 0.76	5.39 ± 0.47	*P = 0.03*
Treg (%)	0.64 ± 0.16	1.44 ± 0.59	0.97 ± 0.33	1.98 ± 0.36	ns
Treg/ ICOS$^+$ (%)	1.72 ± 0.26	2.08 ± 0.53	3.5 ± 0.81	8.87 ± 3.63	*P = 0.03*
T1D					
Number	5	8	7	2	
Serum25(OH)D (nmol/L)	88.1 ± 2.5	62.6 ± 2.6	39.9 ± 2.4	17.4 ± 1.4	*P < 0.0001*
Th17 (%)	3.49 ± 0.41	2.79 ± 0.32	3.41 ± 0.37	8.5 ± 5	*P < 0.05*
Treg (%)	1.07 ± 0.34	0.96 ± 0.22	1.16 ± 0.49	3.09 ± 1.6	ns
Treg/ICOS$^+$ (%)	1.55 ± 0.49	2.3 ± 0.98	3.46 ± 1.60	2.44 ± 0.43	ns

Data, expressed as mean ± standard error of the mean, are given at the enrollment (T0) for S and T1D. Data are vitamin D sufficiency (S), insufficiency (I), deficiency (D), severe deficiency (SD). *P < 0.05.

Abbreviations: S, sufficiency; I, insufficiency; D, deficiency; HLA$^+$, at-risk T1D; HLA, protective haplotypes T1D; HLA^{UND}, undetermined T1D risk;. SD, severe deficiency.

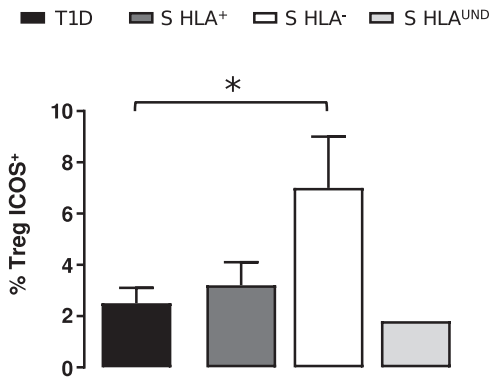

Figure 1. Percentages of ICOS costimulatory molecule (Treg/ ICOS$^+$) in T1D and S according to the HLA typing.

In both T1D (57.4 ± 23.2 *vs* 79.3 ± 20.7%; $P < 0.05$) and S (55.1 ± 24.9 *vs* 71.6 ± 29.2%; $P < 0.01$) children mean serum 25(OH)D levels increased after 6-month supplementation.

Siblings. The frequencies of HLA$^+$, HLA$^-$ and HLA^{UND} supplemented cases were 46.2% (12/26), 23.1% (6/26), and 30.8% (8/26), respectively. After Vitamin D3 supplementation, 23.1% and 19.2% of S subjects were still serum 25(OH)D insufficient and deficient, respectively. Nobody presented serum 25(OH)D levels <25 nmol/L or higher than 125 nmol/L. S subjects who still displayed deficient 25(OH)D levels (<50 nmol/L) had Treg percentages higher than subjects with a sufficient 25(OH)D status (>75 nmol/L; 1.8 ± 0.45 *vs* 1.1 ± 0.27%; $P < 0.05$). Like baseline, increasing trends in Th17 and Treg/ICOS$^+$ values were found from 25(OH)D sufficiency to deficiency, but no more significant (Fig. 2). After supplementation, all supplemented S showed higher serum 25(OH)D levels (21.5 ± 2.1 *vs* 31.3 ± 2.3 nmol/L; $P < 0.0001$), lower Th17 (3.8 ± 0.3 *vs* 2.7 ± 0.3%; $P < 0.0001$), and Treg/ICOS$^+$ (3.8 ± 1.0 *vs* 1.4 ± 0.2%; $P < 0.05$) percentages compared to baseline, regardless of HLA. Treg percentages were not modified by vitamin D treatment (1.17 ± 1.0 *vs* 1.18 ± 1.0%; $P = 0.987$) (Fig. 3). S not regularly supplemented showed no change in these cell subsets.

T1D. All supplemented T1D subjects (16/16) had the high-risk HLA$^+$ haplotype. In T1D patients, after regular supplementation, subjects showed higher serum 25(OH)D levels (58.9 ± 8.4 vs 76.3 ± 6.4 nmol/L; ns) and lower Th17 percentages (3.2 ± 0.3 *vs* 2.4 ± 0.3%; $P < 0.05$) than at T0, but no significant differences of lymphocyte subsets were detected across the serum 25(OH)D levels.

Correlations

At T0, in S, serum 25(OH)D levels showed an inverse correlation with Treg/ICOS$^+$ (r: –0.490, $P < 0.01$) and Th17 (r: –0.410, $P < 0.01$). These correlations were no more present after vitamin D supplementation. No correlations were present in subjects with T1D.

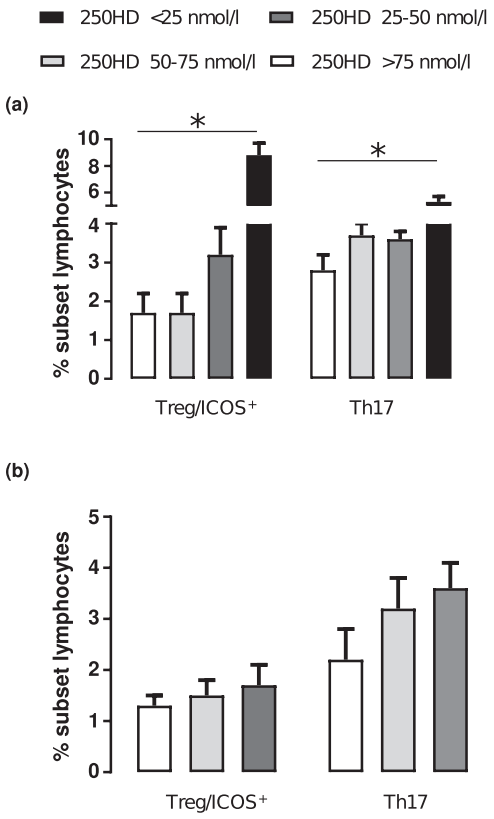

Figure 2. Percentages of subset lymphocytes (Treg/ICOS$^+$ and Th17) across vitamin D levels [25(OH)D] in regularly supplemented Siblings (n 26) at baseline (a) and after 6 months of Vitamin D 3 supplementation (b). Data are expressed as mean ± standard error of the mean. *P trend < 0.05.

A stepwise regression analysis was performed in S to evaluate the role of serum 25(OH)D levels and HLA typing in determining lymphocyte subsets. Only serum 25(OH)D levels ($R^2 = 0.301$; β = –0.540; $P < 0.01$) were negative predictors of Treg/ICOS$^+$ ($R^2 = 0.301$; β = –0.540; $P < 0.01$) and Th17 percentages ($R^2 = 0.138$; β = –0.406; $P < 0.01$) at baseline. The same model was not statistically significant in individuals with T1D.

Discussion

T1D is a multifactorial disease characterized by immune tolerance breakdown and development of a pathological autoimmune response against beta cells mainly driven by Th1 and Th17 cells and impaired Treg activity. The S of T1D patients have high likelihood of sharing the same genetic and environmental predisposing factors as the patients and often display signs of an ongoing autoimmune reaction, such as serum T1D autoantibodies. This may or not result in subsequent development of overt T1D, depending on the

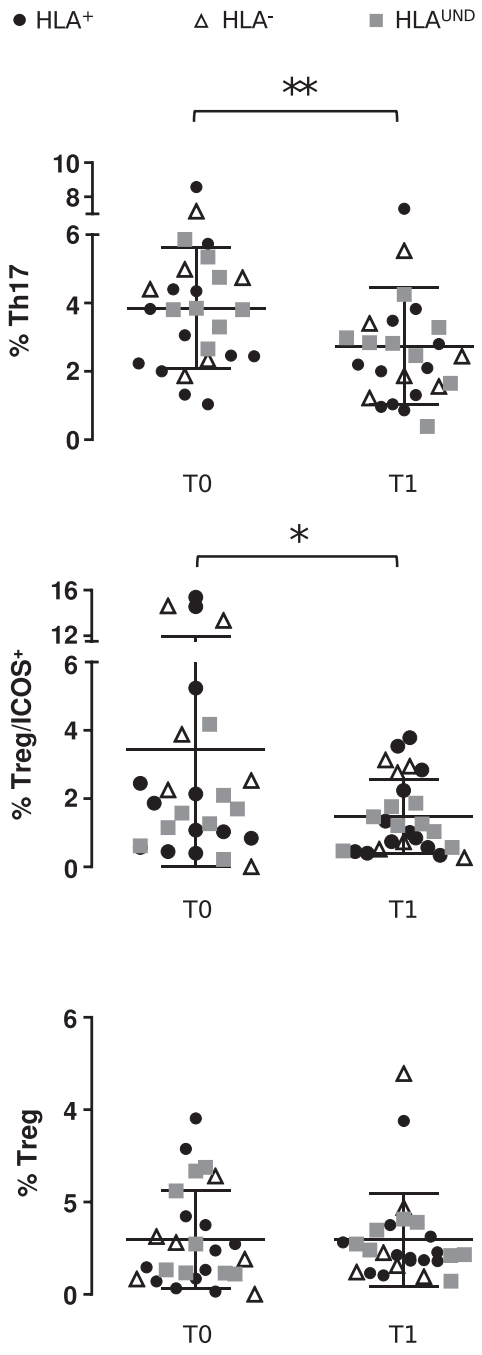

Figure 3. Lymphocyte populations (Th17, Treg, Treg/ICOS$^+$) at the enrollment (T0) and after Vitamin D3 supplementation in S according HLA haplotypes. Data are expressed as mean ± standard error of the mean. HLA$^+$: S with HLA at risk for T1D, n = 12; HLA$^-$: S with HLA not at risk for T1D, n = 6; HLA^{UND}: S with HLA at not determined risk for T1D, n = 8. *$P < 0.05$ T0 *vs* T1 for all HLA groups, **$P < 0.0001$ T0 *vs* T1 for all HLA groups.

outcome of the conflict between autoimmune effector and suppressor cells, such as Th17 and Treg cells, respectively.

The Vitamin D system seems to be involved in preventing autoimmune responses and vitamin D deficiency may favor development of several autoimmune diseases including T1D (3,65). However, a clear link between vitamin D, T1D, and lymphocyte subsets is still elusive. In this paper, we show that serum 25(OH)D levels and vitamin D supplementation may modulate the peripheral blood distribution of T cell subsets possibly involved in T1D development.

Vitamin D has several activities by acting on VDR expressed in most human tissues including immune and pancreatic β cells and modulating more than 200 genes, including genes involved in proliferation, differentiation, cells apoptosis, and angiogenesis (61). The vitamin D role in the immune system is predominantly immunoregulatory. In innate immunity, vitamin D mainly exerts a stimulatory effect by promoting the chemotactic and phagocytic activity of macrophages and stimulating the production of antimicrobial cytokines (66). In adaptive immunity, it mainly exerts inhibitory effects by inhibiting dendritic cells maturation, B/T lymphocyte functions, proinflammatory cytokine (IL-2 and IFN-γ) secretion and modulating Th cell type differentiation (6,19,67-69).

Our results show insufficient and deficient serum 25(OH)D levels in 74.4% of all our subjects without substantial differences between T1D and S. The high prevalence of hypovitaminosis D displayed by these children is widely reported in the pediatric population with or without T1D (70-73). Although vitamin D insufficiency is relatively common in T1D at onset (74), other recent studies did not show differences between T1D and their S in serum 25(OH)D values (75,76).

We focus on 2 T cell subsets, which strongly depend on the function of the ICOS costimulatory receptor (ie, Treg and Th17 cells), which would exert opposite effects on the autoimmune response.

Among Tregs, we further distinguished the Treg/ICOS$^+$, which exerts partly different functions than the ICOS$^-$ counterpart. A balance between Th17 and Treg cells is crucial for the immune homeostasis (77). In T1D subjects, Treg cells have been shown to be functionally impaired and numerically reduced (78), while Th17 cells may be upregulated in the peripheral blood and lymph nodes. However, other studies showed that Treg and Th17 cells are in normal amounts but display an overall abnormal suppressor activity (39,40).

In our study, the distribution of Treg, Treg/ICOS$^+$, and Th17 cells was similar in T1D patients and S subjects, but intriguing differences were detected by dissecting data according to serum 25(OH)D levels and HLA alleles.

In the S group, the levels of Treg/ICOS$^+$ cells, Treg and Th17 cells were higher in those with low serum 25(OH)D (<25 nmol/L) levels compared with those with high (>75 nmol/L) levels, suggesting an activation effect of the immune system in case of vitamin D failure. Besides, vitamin D showed an inverse association with Th17 and Treg/ICOS$^+$ to further support this statement. By contrast, in T1D, only

the levels of Th17 cells showed an increasing trend from sufficiency to severe deficiency of 25(OH)D status. Moreover, supplementation with vitamin D3 significantly increased the serum 25(OH)D level and decreases the level of both Th17 and Treg/ICOS$^+$ in S, and Th17 in the T1D group.

Increasing findings stress the protective effect conferred by vitamin D against autoimmunity. Vitamin D has an inhibitory effect on Th17 lymphocytes: the increase in Th17 and IL-17 mediates the destruction of tissues during inflammation, also inducing the expression of mediators of inflammation, nitric oxide, and metalloproteinases, which contribute to the establishment and progress of tissue damage (6,11). Our results suggest that low levels of 25(OH)D allow expansion of Th17 cells that may be counteracted by the increased Treg/ICOS$^+$ levels mainly in S. However, vitamin D seems unable to act when the disease is already established in T1D. The vitamin D effect is more evident in S than in T1D, indicating that the supplementation could be more useful in the prevention phase before the beta-cell destruction.

Moreover, the Treg/ICOS+ counteraction would be particularly effective in S carrying protective haplotypes, who show the highest levels of Treg/ICOS$^+$ cells without substantial differences in Th17 cells. By contrast, all T1D patients carried risk or UND HLA alleles and did not display the expansion of Treg/ICOS$^+$ cells in any 25(OH)D group.

Since supplementation with vitamin D3 can decrease Th17 cells, it might be useful especially in subjects carrying the risk alleles and having low Treg/ICOS+ cells.

The vitamin D3 supplementation seems to decrease Th17 cells, mainly in subjects carrying the risk alleles and not expanding Treg/ICOS+ cells. The fact that vitamin D3 supplementation also compresses Treg/ICOS$^+$ would not be detrimental because of the parallel decrease of Th17 cells. These findings are supported by the negative correlations among serum 25(OH)D levels and Treg ICOS$^+$ and Th17 percentage in S subjects before vitamin D3 supplementation.

Our results suggest a preventive role of Treg/ICOS+ in S with protective haplotypes and fit well with Pruul et al that show a loss of ICOS expression in T1D peripheral blood vs healthy controls suggesting that Treg/ICOS+ cells could represent a key stabilizing factor for Treg cells in S, not at risk for diabetes (41-44). Accordingly, since in S total Tregs were unchanged after supplementation, vitamin D status could influence the immune system turning off the immune attack.

Viisanen et al showed an increase in ICOS$^+$ Treg cells in children with newly diagnosed T1D (<1 week after clinical diagnosis) and only in at-risk children positive for 2 or more biochemical autoantibodies, suggesting a Treg dysfunction associated with progression to clinical disease. These could occur as an attempt to counter the disease. These findings are partially in contrast with our results since we found similar Treg and Treg ICOS$^+$ distribution between T1D and S. However, our patients were not investigated in the early stage of the disease but after 6 months from the onset, and all S were negative for autoantibodies (79). Moreover, the same authors showed altered Treg cells in children with newly diagnosed T1D, but not in children at risk positive for autoantibodies, and they did not differentiate Treg subsets (ICOS$^+$ or ICOS$^-$) as we did in our paper. Similar results on long-term T1D in Treg ICOS$^+$ have been reported by other authors (64). The knowledge of a Treg dysfunction can be important for improving current immunotherapeutic drugs to treat or prevent T1D.

After 6 months of vitamin D3 supplementation in S and T1D, we found a reduction of 25(OH)D insufficiency and absence of severe 25(OH)D deficiency. Only those S regularly taking the cholecalciferol supplementation showed higher serum 25(OH)D levels and significantly lower Th17 and Treg/ICOS$^+$ percentages than at the enrollment, reinforcing our hypothesis that the vitamin D system influences the balance between these T cell subsets.

Recently, Dankers et al, using rheumatoid arthritis as a model of autoimmune disease, showed that 1,25(OH)2D3 supplementation inhibited pro-inflammatory cytokines (IL-17A, IL-17F, IL-22, and IFN-γ) in IL-17A-producing memory CCR6$^+$ Th cells both in healthy controls and in rheumatoid arthritis patients. Moreover, after 1,25(OH)2D3 supplementation, CCR6$^+$ Th cells presented an increase of anti-inflammatory factors as IL-10 and cytotoxic T-lymphocyte-associated protein 4 and suppressed proliferation of autologous CD3+ T cells like classical Tregs (80). This immunoregulatory effect of vitamin D supports our findings.

In addition, Bogdanou et al reported that cholecalciferol treatment (4000 IU/die for 3 months) was associated with a significant increase in Tregs percentage in males but not in females, along with a significant reduction in daily insulin requirements and HbA1c (48). Moreover, 70 IU/Kg body weight/day of cholecalciferol led to a significant increase in Treg suppressive capacity without changing their levels in the blood of young T1D subjects (49). In healthy control subjects, a high dose of cholecalciferol (140 000 IU/month) over 20 weeks was followed by an increase of peripheral blood Treg cells (81). Our results do not confirm these findings, but all these studies are different in timing of vitamin D administration and characteristics of study participants.

The vitamin D3 effect on the immune system seems more evident in S than in T1D, suggesting that the supplementation could be more useful in prevention phases before the beta cell destruction. In adult-onset latent autoimmune diabetes patients, Li et al found that the protective effect of vitamin D3 is detectable when the supplementation starts

in the earliest phase of the disease when beta cell function is still preserved (82).

This study had several limitations. First, we enrolled a limited number of subjects, both T1D and S. Second, we did not include healthy control subjects, although HLA$^-$ S were considered nested controls. Third, subjects with T1D were not enrolled at the onset. However, these data are preliminary to design further studies investigating the role of vitamin D supplementation in prevention of T1D.

In conclusion, our study suggests that there is a link between serum 25(OH)D levels, HLA background and Th17 and Treg/ICOS$^+$ ratio in subjects with T1D and their S. Dysregulated immune responses due to poor vitamin D status seem to occur, blunted by cholecalciferol supplementation in S. Trials on the preventive role of cholecalciferol supplementation in relatives of patients with T1D are warranted.

Acknowledgments

The authors would like to thank Dr. Emanuela Agosti for technical support and AGD Novara (Associazione per l'Aiuto ai Giovani Diabetici).

***Financial Support*:** This work was supported by Fondazione Cariplo (cod. 2017-0535).

Additional Information

Correspondence and Reprint Requests: Flavia Prodam, MD, PhD, Associate Professor in Clinical Nutrition, Department of Health Sciences, Università del Piemonte Orientale, V. Solaroli 17, 28100 Novara, Italy. E-mail: flavia.prodam@med.uniupo.it.

***Disclosure Summary*:** L.G., E.B., and U.D. are founder members of Novaicos srl. Other authors have no conflict of interest.

Data Availability: The data sets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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