Thymus Activity, Vitamin D, and Respiratory Infections in Adolescent Swimmers

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ABSTRACT:

Background: Several studies have identified associations between low vitamin D concentrations and risk of upper respiratory infections (URI). T lymphocytes have a major anti-viral role, are affected by vitamin D metabolism, and may mediate the link between vitamin D and URIs. Competitive swimmers have a relatively high rate of URIs, alongside a high prevalence of low vitamin D concentration.

Objectives: To examine the associations linking T cell receptor excision circles (TREC, markers of thymus activity), circulating 25(OH)D concentrations and the effect of vitamin D supplementation, and URI symptoms in young competitive swimmers.

Methods: We tested 82 adolescent swimmers for serum 25(OH)D and TREC concentrations and found that 55 had vitamin D insufficiency. Randomized supplementation of either vitamin D3 or placebo was given for 12 winter weeks. URI symptoms were recorded weekly. The associations between TREC copy numbers, vitamin D and URI burden were examined.

Results: TREC concentrations decreased with the participants' age ($r = -0.346$, $P = 0.003$), with no significant between-gender difference. TREC concentrations did not materially differ among subjects with normal, insufficient or deficient vitamin D status, and were not affected by vitamin D supplementation. No significant correlations were found between TREC levels or their changes during the study period, and mean URI severity or duration.

Conclusions: Thymus activity, represented by higher TREC levels, was not related to vitamin D concentrations or status, and was not affected by vitamin D supplementation in adolescent swimmers. TREC concentrations were not associated with URI severity or duration in this population.

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KEY WORDS: upper respiratory tract infections (URIs), lymphocytes, competitive swimming, vitamin D, adolescents

Upper respiratory tract infections (URIs) are among the most common causes of disease worldwide [1]. Lymphocytes are the main arm of the immune system that protects against viral respiratory tract infections [2]. Vitamin D plays an important role in many physiological processes, including various aspects of the immune system: namely, it has been shown to induce maturation of macrophages, natural killer cells and cytotoxic T lymphocytes [3] and affect many components of both the innate and the acquired immune system [4]. These effects have clinical implications for several types of infections, mainly those of the respiratory tract.

Several observational clinical studies have shown an association between low vitamin D status and a higher rate of URIs, while interventional studies of vitamin D supplementation demonstrated conflicting results [5]. It was therefore recommended that additional studies be performed, and in populations with a high prevalence of vitamin D deficiency [5].

Athletes training indoors, such as swimmers, have a high prevalence of both vitamin D insufficiency and deficiency (serum 25(OH)D < 20 ng/ml) [6]. In addition, competitive swimmers have an increased risk of URIs [7], and up to 74% reported nasal obstruction, rhinorrhea, sneezing and nasal itching [8]. We recently reported that a decrease in serum 25(OH)D concentrations in adolescent swimmers with vitamin D insufficiency was associated with increased duration and severity of URIs [9]. The mechanism by which low levels of vitamin D might increase URI burden remains unknown. Nevertheless, its effect on different components of the immune system is widely accepted [10].

A possible link between vitamin D and respiratory infections are the T lymphocyte cells, given that vitamin D affects lymphocyte activity [11] and that these cells have an important role in fighting the viruses of the respiratory tract [2]. It is well documented that T lymphocytes are direct target cells for vitamin D. They express the vitamin D receptor after immune activation, T cell proliferation is inhibited by vitamin D, the expression of chemokine receptors is affected by vitamin D,

production of Th1 and Th17 cytokines is decreased while Th2 cytokine production is stimulated, and T regulatory (Treg) cell development and production is favored by vitamin D [11-15]. The direct effect of vitamin D on the thymus gland, which is the main organ for T cell lymphocyte development and maturation, is still unknown.

During the T cell development process, T cell receptor excision circles (TREC) are produced. These are specific circular DNA byproducts, formed during the random rearrangements of T cell receptors. TRECs are only present in cells exported from the thymus and are not found in peripheral blood-replicating cells [16]. Arellano et al. [17] showed that TRECs are reliable markers to measure thymic output, since peripheral and intra-thymic TREC values correlated. Dion and co-researchers [18] demonstrated the accuracy of quantitatively assessing TREC levels as a non-invasive measure of thymic function. In infants with severe combined immunodeficiency, TREC levels were found to be undetectable or significantly low at birth, and this measure now serves as a screening tool for this medical condition in several countries, including Israel [19]. Therefore, TREC allows for quantifying the production of T lymphocytes at a young age, when the thymus is still functional, and hence is a marker for thymic activity. It is currently unknown whether thymic activity is affected by vitamin D concentrations, and whether thymic activity is associated with URI burden in children and youth.

The aims of this study were to examine the association between TREC levels and circulating 25(OH)D concentrations, to examine the effect of vitamin D supplementation on TREC levels, and to examine the relationship between TREC levels and upper respiratory symptoms in adolescent swimmers.

SUBJECTS AND METHODS

Eighty-two adolescent competitive swimmers from four swimming teams in Israel were screened for serum 25(OH)D levels. Individuals were excluded if they refused to undergo any or all of the testing procedures, if they had a history of chronic health conditions, or if they were taking any chronic medications or dietary supplements, including multivitamins. The study was approved by the Institutional Review Board of Sheba Medical Center, Tel Hashomer, Israel, conducted according to the Declaration of Helsinki, and registered in a clinical trials registry prior to participant enrollment (clinicaltrials.gov, NCT01215682). Each participant and at least one parent signed an informed consent form.

DATA COLLECTION, RANDOMIZATION AND SUPPLEMENTATION
- **Blood sampling**

Blood samples were drawn from each of the study participants before a scheduled practice session at their usual training facility. For 25(OH)D analysis, sampled blood was drawn into gel-

containing test tubes, transferred to the endocrine laboratory at the Sheba Medical Center, centrifuged and stored at 4°C for analysis. Serum 25(OH)D was measured by radioimmunoassay (Diasorin, Stillwater, Minnesota, USA. Intra-assay CV 12%, Inter-assay CV 10%). Vitamin D insufficiency was defined as serum 25(OH)D concentrations < 30 ng/ml, and vitamin D deficiency was defined as serum 25(OH)D concentrations < 20 ng/ml [20].

- **Real-time quantitative PCR assays**

Analysis was performed using DNA extracted from the participants' peripheral blood mononuclear cells. Amplification reactions (25 μl) contained 0.5 μg of genomic DNA, 12.5 μl of TaqMan universal polymerase chain reaction (PCR) master mix (Perkin Elmer Applied Biosystem, Foster City, CA, USA), and the appropriate primers and probes. PCR conditions including primers and probe have been described previously [21]. Reactions were carried out in an ABI PRISM 7900 Sequence Detector TaqMan system (Applied Biosystems, Rotkreuz, Switzerland). The number of TRECs in a given sample was estimated by comparing the CT value with a standard curve obtained from PCRs performed with tenfold serial dilutions of an internal standard, kindly provided by Dr. Daniel Douek (Vaccine Research Center, National Institute of Allergy and Infectious Diseases, Bethesda, MD). The dilutions contained 10^2–10^6 copies of TREC, in triplicate. Amplification of RNAseP (Applied Biosystems) was used to verify the quantity and presence of genomic DNA. TREC values were adjusted for total DNA content.

- **Vitamin D3 supplementation**

Fifty-five participants (67%) had vitamin D insufficiency and comprised an interventional study group, where subjects were randomized to receive supplementation of either vitamin D3 (2000 IU/day) in liquid drops (CTS Chemical Industries Ltd, Israel), or a placebo identical in taste and appearance. Subjects who were vitamin D sufficient did not receive any supplementation or treatment. At the end of the 12 week supplementation period, blood samples were collected and analyzed for serum 25(OH)D and TREC as described but only among subjects with vitamin D insufficiency (the interventional study arm).

- **Upper respiratory tract infections**

URI data were collected for 12 weeks, starting 1 month after supplementation began (i.e., December 2010 to February 2011) and ending 1 month after supplementation ended, only from the 55 vitamin D-insufficient subjects who took part in the interventional part of the study. Since vitamin D is fat soluble, we allowed serum vitamin D concentrations to rise before starting to record URI events. During the data collection period, subjects filled out a respiratory symptoms questionnaire based on the Wisconsin Upper Respiratory Symptom

Table 1. Clinical and training data of the study participants

	No intervention group (n=27)	Vitamin D group (n=28)	Placebo group (n=27)
Age (years)	14 (13–16)	14 (13–15)	13 (12–16)
Males, n (%)	18 (67%)	17 (61%)	18 (67%)
Years competing	4 (3–4.5)	5 (3-6)	4 (3–5)
Amount of swimming (hours/week)	12 (11.5–12)	13 (13–13)	18 (12–18)
Amount of gym training (hours/week)	4 (3–4)	4 (4–4)	3 (3–5)

Data presented as median (interquartile range) or number and percentage, as appropriate

Survey (WURSS) [22]. A URI event was defined as having at least one URI symptom for at least 1 day and at least 3 days apart from a prior event. The duration of each URI event was the number of days it occurred. The severity of URI events was calculated as the average score of symptom severity on a scale of 0 to 7, as recorded on the questionnaire sheets by the subject.

STATISTICAL ANALYSIS

Continuous variables are presented as median and interquartile range (IQR) due to a non-normal distribution of TREC concentrations and were analyzed accordingly. Continuous variables were compared using the Mann-Whitney U test when comparing two independent samples and the Kruskal-Wallis one-way analysis of variance when three independent samples were compared. The Wilcoxon signed ranks test was used to compare within-group pre- and post-supplementation changes. The relationships between two quantitative variables were determined using Spearman's correlation coefficient. Statistical significance was defined as $P < 0.05$.

RESULTS

Clinical and training data of the 82 study participants are presented in Table 1. Two-thirds were males. Participants were swimming competitively for over 4 years on average, with 17–18 hours of overall training per week. Twenty-two swimmers reported having URI symptoms during the study period, 11 from each supplementation group (vitamin D or placebo; the "no intervention" group was not required to record URIs). There was no significant difference in URI duration or severity between the study groups [Table 2], as we previously reported in depth [9].

Throughout the study period, serum 25(OH)D levels increased significantly in the vitamin D supplementation group, but decreased significantly in both the no-intervention and placebo groups [Table 2].

TREC concentrations at the start and end of the study are presented in Table 3. Baseline TREC concentrations decreased with age in the whole study population (r = -0.346, P = 0.003),

Table 2. Serum 25(OH)D concentrations at study beginning and end, and URI severity and duration scores by study group

Characteristic	No intervention group (n=27)	Vitamin D group (n=28)	Placebo group (n=27)	P value**
URI severity score	–	5.0 (4.2–5.8)	5.0 (3.0–5.7)	0.70
URI duration (days)	–	4.0 (3.0–5.2)	5.0 (4.0–7.0)	0.24
Baseline serum 25(OH)D (ng/ml)	32.5 (31.5–35.6)*	24.7 (20.4–28.6)	25.0 (21.4–28.3)	‹ 0.001
Final serum 25(OH)D (ng/ml)	27.6 (24.0–31.5)	29.7 (24.0–35.3)	20.6 (18.3–23.1)*	‹ 0.001
Serum 25(OH)D change (ng/ml)	-6.2 (-1.5–(-)9.6)	6.2 (0.0–8.8)*	-4.1 (-2.3–(-)6.6)	‹ 0.001

Data presented as median (interquartile range)
*Significant difference from the two other values in the same row (Mann-Whitney U test).
**Obtained by Wilcoxon signed ranks test
URI = upper respiratory tract infection

Table 3. TREC concentrations at study beginning and end by study group, after adjustment for total DNA content

Characteristic	No intervention group (n=27)	Vitamin D group (n=28)	Placebo group (n=27)	P value
Baseline TREC (copies)	2681 (1517–4929)	3352 (1770–5018)	2524 (1441–4013)	0.34
Final TREC (copies)	–	3615 (2414–4986)	2902 (1910–4023)	0.15
TREC copies (change from baseline)	–	-475 (-1150–960)	552 (-1038–1874)	0.30

TREC data presented as median (interquartile range)
TREC = T cell receptor excision circles

Table 4. Spearman's correlation coefficients between TREC concentrations at baseline and URI severity/ duration and 25(OH)D concentrations in all study participants

Characteristic	Correlation coefficient	P value
URI severity	-0.083	0.73
URI duration	-0.274	0.24
Baseline serum 25(OH)D (ng/ml)	0.148	0.22
Final serum 25(OH)D (ng/ml)	0.183	0.16
Serum 25(OH)D change (ng/ml)	0.043	0.74

URI = upper respiratory tract infection

and did not differ significantly between males and females (P = 0.88) or between the three study groups at baseline [Table 3]. Baseline TREC levels also did not differ significantly between subjects with vitamin D deficiency (n=11, 1677, IQR 990-4669) copies) or vitamin D insufficiency (n=44, 3220, IQR 1697-4637) copies), P = 0.15. Table 4 presents Spearman's correlation coefficients between TREC concentrations at baseline, URI severity and duration, and 25(OH)D concentrations; none were found significant. The changes in TREC concentrations throughout the study period, regardless of participant grouping, were also unrelated to URI severity (r = -0.14, P = 0.58) or duration (r = -0.02, P = 0.94).

DISCUSSION

In this supplementation trial in adolescent swimmers we examined the associations between thymic activity, as represented by serum TREC concentrations, circulating 25(OH)D concentrations and URI symptoms, and the effect of vitamin D supplementation. TREC levels were not associated with 25(OH)D concentrations, were not significantly affected by vitamin D supplementation, and were not associated with URI symptoms. Our findings suggest that thymic activity and production of naïve T lymphocytes are not associated with, or affected by, vitamin D concentrations. TREC concentrations were not associated with URI severity and duration, and hence probably do not mediate the link between low serum 25(OH)D and URI burden in adolescent swimmers, which we reported previously [9].

There is currently only one published study in children that showed that URI risk was significantly lowered by vitamin D supplementation [23]. This study of children from Mongolia demonstrated a very low mean vitamin D concentration, 7 ng/ml. In the group treated with vitamin D supplementation the risk of developing URIs decreased significantly, suggesting that the very low vitamin D concentration is detrimental to the capacity of the immune system to combat URI. Several other studies of vitamin D supplementation, in various populations, failed to show a beneficial effect of supplementation on URI burden, yet baseline concentrations were much higher [5,9]. Given that vitamin D affects both innate and adaptive immune responses and enhances lymphocyte function [2,10,24], it seemed feasible to examine whether it affects circulating TREC levels as markers of thymic output. We found no significant correlation between vitamin D and TREC levels, either before or after supplementation, suggesting that the immunomodulatory effects of vitamin D are not exerted directly on the thymus gland. Indeed, several other mechanisms by which vitamin D may influence T cell function have been proposed, including direct endocrine effects on T cells mediated via systemic calcitriol, direct intracrine conversion of 25(OH)D to calcitriol by T cells, direct paracrine effects of calcitriol on T cells following conversion of 25(OH)D to calcitriol by monocytes or dendritic cells, and indirect effects on antigen presentation to T cells mediated via localized antigen-presenting cells affected by calcitriol [24]. Since this was the first study to examine the relationship between vitamin D and direct thymic activity, it is pertinent to pursue this aspect in future studies.

TREC is a biomarker of normal T cell development, and has been used in many clinical settings in which T cell immunity is involved, including diagnosing, understanding and monitoring T cell immunodeficiencies, human immunodeficiency virus infection, aging, autoimmune diseases, and immune reconstitution after bone marrow transplantation [16]. In addition, TREC levels may negatively correlate with the severity of the T cell immunodeficiency [21] and affect the intensity of the immune response to viral infections such as cytomegalovirus [25]. Thus, it was reasonable to assume that higher TREC levels may better protect individuals against URI, yet this was not found in our study.

We acknowledge that our study has several limitations. Our sample size was relatively small due to the number of participants who reported URI symptoms. Another limitation is that baseline vitamin D levels were not very low, and all study participants but one had a circulating 25(OH)D concentration of ≥ 20 ng/ml. We presume that a wider range of vitamin D concentrations could have increased the ability to identify a correlation with TREC levels. Another issue is the normalization of TREC levels among individuals. There are no specific guidelines as to how to normalize circulating TREC copies, and we chose to adjust to total DNA content in the sample. The wide range of TREC concentrations seen in these healthy individuals, together with the restricted sample size, prevented us from performing more in-depth regression analyses, which may have identified independent associations between the different variables tested. Finally, our study results cannot necessarily be extrapolated to other pediatric populations or other sport types.

In summary, this is the first study to our knowledge in which correlations between vitamin D, a marker of T cell production, and URI burden were sought. Although we did not find significant relationships linking these three parameters, there is a scientific basis to search further since the mechanism by which vitamin D is associated with URI burden probably involves lymphocytes and their activity. In addition, the importance of TREC as a surrogate marker for T cell production in healthy individuals with common viral infections should be determined in further studies.

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References
1. Heikkinen T, Jarvinen A. The common cold. *Lancet* 2003; 361: 51-9.
2. Mosmann TR, Coffman RL. Th-1 and Th-2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7: 145-73.
3. Hewison M. Vitamin D and the immune system: new perspectives on an old theme. *Endocrinol Metab Clin North Am* 2010; 39: 365-79.
4. Russell WC. Vitamin D and The Magic Mountain: The anti-infectious role of the vitamin D. *J Pediatr* 2010; 156: 698-703.
5. Jolliffe DA, Griffiths CJ, Martineau AR. Vitamin D in the prevention of acute respiratory infection: systematic review of clinical studies. *J Steroid Biochem Mol Biol* 2013; 136: 321-9.

6. Constantini NW, Arieli R, Chodick G, et al. High prevalence of vitamin D insufficiency in athletes and dancers. *Clin J Sport Med* 2010; 20: 368-71.

7. Bougault V, Turmel J, Levesque B. The respiratory health of swimmers. *Sports Med* 2009; 39: 295-312.

8. Bougault V, Boulet LP. Airway dysfunction in swimmers. *Br J Sports Med* 2012; 46: 402-6.

9. Dubnov-Raz G, Rinat B, Hemilä H, Choleva L, Cohen A, Constantini N. Vitamin D supplementation and upper respiratory tract infections in adolescent swimmers: a randomized controlled trial. *Pediatr Exerc Sci* 2015; 27: 113-19.

10. Wolden-Kirk H, Gysemans C, Verstuyf A, Mathieu C. Extraskeletal effects of vitamin D. *Endocrinol Metab Clin North Am* 2012; 41: 471-594.

11. Maruotti N, Cantatore FP. Vitamin D and the immune system. *J Rheumatol* 2010; 37: 491-5.

12. Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1alpha,25-Dihydroxyvitamin D3 has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol* 2001; 167: 4974-80.

13. Imazeki I, Matsuzaki J, Tsuji K, Nishimura T. Immunomodulating effect of vitamin D3 derivatives on type-1 cellular immunity. *Biomed Res* 2006; 27: 1-9.

14. Matsuzaki J, Tsuji T, Zhang Y, et al. 1alpha,25-Dihydroxyvitamin D3 downmodulates the functional differentiation of Th1 cytokine-conditioned bone marrow-derived dendritic cells beneficial for cytotoxic T lymphocyte generation. Cancer Sci 2006; 97: 139-47.

15. Rigby WF, Noelle RJ, Krause K, Fanger MW. The effects of 1,25-dihydroxyvitamin D3 on human T lymphocyte activation and proliferation: a cell cycle analysis.

J Immunol 1985; 135: 2279-86.

16. Somech R. T-cell receptor excision circles in primary immunodeficiencies and other T-cell immune disorders. *Curr Opin Allergy Clin Immunol* 2011; 11: 517-24.

17. Arellano MV, Ordonez A, Ruiz-Mateos E, et al. Thymic function-related markers within the thymus and peripheral blood: are they comparable? *J Clin Immunol* 2006; 26: 96-100.

18. Dion ML, Sékaly RP, Cheynier R. Estimating thymic function through quantification of T-cell receptor excision circles. *Methods Mol Biol* 2007; 380: 197-213.

19. Somech R, Lev A, Simon AJ, et al. Newborn screening for severe T and B cell immunodeficiency in Israel: a pilot study. *IMAJ* 2013; 15: 404-9.

20. Weisman Y. Vitamin D deficiency and insufficiency. *IMAJ* 2013; 15: 377-8.

21. Amariglio N, Lev A, Simon A, et al. Molecular assessment of thymus capabilities in the evaluation of T-cell immunodeficiency. *Pediatr Res* 2010; 67: 211-16.

22. Barrett B, Brown R, Mundt M, et al. The Wisconsin Upper Respiratory Symptom Survey is responsive, reliable, and valid. *J Clin Epidemiol* 2005; 58: 609-17.

23. Camargo CA Jr, Ganmaa D, Frazier AL, et al. Randomized trial of vitamin D supplementation and risk of acute respiratory infection in Mongolia. *Pediatrics* 2012; 130: e561-7.

24. Hewison M. An update on vitamin D and human immunity. *Clin Endocrinol* (Oxf) 2012; 76: 315-25.

25. Alonso Arias R, Moro-García MA, Echeverría A, Solano-Jaurrieta JJ, Suárez-García FM, López-Larrea C. Intensity of the humoral response to cytomegalovirus is associated with the phenotypic and functional status of the immune system. *J Virol* 2013; 87: 4486-95.