Vitamin D in Hashimoto's thyroiditis and its relationship with thyroid function and inflammatory status

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Abstract. Several studies have shown the correlation between vitamin D [25(OH)D] deficiency and thyroid autoimmunity and reducing of thyroid autoantibodies in patients with normal levels of vitamin D combining with thyroid hormone replacement. However, other authors not agree with this association. It is still unclear whether the low 25(OH)D levels are the result of HT disease or a part of its cause. We studied 88 patients with HT regarding vitamin D status and thyroid autoimmunity markers as well as the relationship with cytokines produced by Th1, Th2, and Th17 cells compared with a control group of 71 euthyroid healthy subjects. The present study demonstrated that vitamin D concentrations were similar in patients HT and the control group. The reduction of free T4 levels was a predictor of vitamin D insufficiency for Hashimoto's thyroiditis, but not for the control group. Lower concentrations of TNF- α was a predictor of lower levels of vitamin D. Differences in the association between HT and vitamin D insufficiency remain unresolved in the literature. The thyroid hormone status would play a role in the maintenance of vitamin D sufficiency, and its immunomodulatory role would influence the presence of autoimmune thyroid disease. The positive correlation between free T4 and vitamin D concentrations suggests that adequate levothyroxine replacement in HT would be an essential factor in maintaining vitamin D at sufficient levels.

Key words: Hashimoto's thyroiditis, Cytokines, Vitamin D, Autoimmunity

HASHIMOTO'S THYROIDITIS (HT) is one of the autoimmune thyroid diseases (AITDs), also called chronic lymphocytic thyroiditis. Autoimmune attack on the thyroid plays with infiltration of the gland by T and B lymphocytes associated with thyroid peroxidase antibodies (TPO-Ab) and thyroglobulin antibodies (TG-Ab) production [1-5].

In HT occurs a genetic defect in T cell suppressor (Treg) function and CD4⁺ T cells are not deleted when they are free to promote activation of B-lymphocytes. Concomitantly, the Th cells produce cytokines that

induce thyrocytes to express surface antigens HLA-DR making them susceptible to immune attack. There is an interaction between susceptibility genes and environmental factors associated with autoimmune dysfunction [1-3, 6].

Th1 cells secrete inflammatory cytokines such as interferon- γ (IFN- γ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF- α), essential for the cell-mediated immune response. Th2 cells secrete the inflammatory cytokines IL-4 and IL-5, necessary for the antibody-mediated immune response. Th-17, another Th cell subtype composed of CD4⁺ T lymphocytes shows involvement in the pathophysiology of autoimmune diseases, especially in HT, and the production of interleukins such as IL-17 and IL-23 [2, 7-9].

HT manifests clinically in goiter or non-goiter (or atrophic) forms. The goiter form is related to the predominance of cellular immunity through activation of

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Th1 promoting apoptosis of the thyroid follicular cells leading to the dysfunction. The non-goiter form is related to the predominance of humoral immunity, *via* activation of Th2 that induces antigen-specific B lymphocytes to produce anti-TSH receptor antibodies (TRAb) stimulus blockers thus causing the disease [3, 10].

Several studies have shown the correlation between vitamin D deficiency and thyroid autoimmunity. Vitamin D3 (cholecalciferol) is produced in the skin when in the presence of ultraviolet-B radiation (UVB) and can also be obtained through supplementation and diet. There is a hydroxylation in the liver and an action of 1 α -hydroxylase in the kidney for product its active form calcitriol (1,25 (OH)₂VitD), that binds to the nuclear vitamin D receptor (VDR), which is expressed in various immune cells such as monocytes, macrophages, dendritic cells, B and T lymphocytes, promoting immunomodulatory actions. Low concentrations of vitamin D are associated with predisposition to various autoimmune diseases such as type 1 diabetes mellitus, rheumatoid arthritis, multiple sclerosis and autoimmune thyroid diseases (AITDs). There are reports on the association between low vitamin D levels and the presence of TPO-Ab as well as the association of polymorphisms in the VDR gene in patients with AITDs. Calcitriol inhibits proliferation of Th1 cells and production of cytokines as well as induces B cell apoptosis [3, 11-21].

Multiple factors influence the synthesis of vitamin D through the skin such as duration of sun exposure, latitude, season, time of day, pigmentation of the skin, use of sunscreen, behavioral habits and diet. The reference values for vitamin D concentrations in our setting were recently reviewed, being considered as vitamin D insufficiency values below 20 ng/mL [22-25].

Many studies have shown reducing of thyroid autoantibodies in patients with normal levels of vitamin D combining with thyroid hormone replacement. However, other authors not agree with this association. It is still unclear whether the low 25(OH)D levels are the result of HT disease or a part of its cause [26-28].

Considering the possible role of vitamin D in thyroid autoimmunity, we aimed to study the relationship of vitamin D with thyroid function and inflammatory *status* in patients with HT.

Methods

Study design

We studied patients with HT regarding vitamin D sta-

tus and thyroid autoimmunity markers as well as the relationship with cytokines produced by Th1, Th2 and Th17 cells compared with a control group of euthyroid healthy subjects.

An interim analysis was conducted to evaluate the statistical power of correlation between thyroid hormone, vitamin D and thyroid autoimmunity markers resulting in the minimum of 88 patients. Then, we included 88 patients with HT followed in our university hospital and 71 individuals without AITD, all aged 18 to 65 years. Blood samples were collected from the two groups for measurements of serum total 25OH vitamin D, thyrotropin (TSH), free thyroxine (FT4), calcium, phosphorus, parathormone (PTH), TPOAb, TGAb, and TRAb. Cytokines produced by Th1 cells (IL-2, IFN- γ , TNF- α), Th2 (IL-4, IL-5) and Th17 (IL-17) were measured in all participants.

Ultrasound estimated thyroid volume in patients. Data on weight, height, body mass index, parity and time since direct interview collected diagnosis. Written informed consent was obtained from each patient or subject after full explanation of the purpose and nature of all procedures. The University Ethics in Research Committee approved the study.

Inclusion criteria

Only patients with the diagnosis of HT were included in the study.

The diagnosis of thyroid dysfunction was based on high concentrations of TSH and low FT4. At the time of collection, all patients with HT were on replacement therapy with levothyroxine. Only patients with high levels of antithyroid antibodies confirming the etiology of AITD were included.

All subjects in the control group were clinical and laboratory euthyroid, TPOAb, TGAb and TRAb undetectable and were selected from patient's companions or hospital staff.

Exclusion criteria

Exclusion criteria were: previous history of thyroidectomy, acutely ill patients, active malignant or inflammatory disease, use of amiodarone, steroids, calcium and/or vitamin D, use of iodinated contrast less than 3 months before the start of the study, heart failure (class III or IV NYHA), severe liver disease (reduced albumin or increased INR), advanced kidney disease (stage 4 or 5), patients under hemodialysis, seropositive for HIV or hepatitis C, and pregnant.

Laboratory evaluation of thyroid and vitamin D status

For evaluation of thyroid status, TSH was measured by electrochemiluminescence (Roche Cobas Elecsys—reference values RV 0.41 to 4.5 μ UI/mL) and FT4 dosed by competitive chemiluminescence immunoassay Elecsys FT4 II (RV 0.9 and 1.8 ng/mL). The intra-assay coefficient of variation (CV) was 5%; measuring range 0.01–100 μ UI/mL, analytical sensitivity 0.01 pg/mL and functional sensitivity 0.014 μ UI/mL with inter-assay CV of 20%. For FT4 measurement interval was used between 0.02 to 7.76 ng/dL, intra-assay CVs of 5% analytical sensitivity 0.023 ng/mL and functional 0.39 ng/mL and inter-assay CV 20%. TPOAb and TGAb were measured by chemiluminescent immunometric assay Elecsys (RV up to 34 UI/mL and up to 115 IU/mL, respectively). For TPOAb, measurement interval was 5.0 to 600.0 IU/mL, CV 5% analytical sensitivity 5.0 IU/mL Functional 34 IU/mL. For TGAb measurement interval was between 10.0 to 4,000.0 IU/mL (5% CV); analytical sensitivity 10 IU/mL and functional sensitivity 34 IU/mL. TRAb was measured by competitive electrochemiluminescence immunoassay using Elecsys TRAb TSH receptors (RV up to 1.22 IU/L); measurement interval range from 0.3 to 40.0 IU/L, CV 5%; analytical sensitivity of 0.3 IU/L and functional sensitivity of 0.9 IU/L. Total vitamin D (25OHVitD) was measured by the test LIAISON® 25 OH Vitamin D TOTAL using chemiluminescent immunoassay technology (CLIA) for the quantitative determination of 25-hydroxyvitamin D and other hydroxylated metabolites of vitamin D in human serum, plasma or EDTA plasma with lithium heparin using the evaluation of the amount of vitamin D using the family LIAISON® analyzers.

We adopted the following recommendations for TSH levels: patients <60 years old 1.0–2.5 mU/L; between 60–70 years 3–4 mU/L and >70 years 4–6 mU/L [29].

Vitamin D levels between 30 and 60 ng/mL are recommended for at-risk groups as patients with autoimmune diseases and were adopted as adequate to the study population [22].

Cytokines evaluation

Cytokines were measured by the immunoassay technique of Milliplex Map based on the Luminex Xmap technology. Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets polystyrene microspheres or magnetic

microspheres can be created, each of which is coated with a specific capture antibody. The reference values of cytokines are described below: IL-2 RV 1.0–1.6 pg/mL; INF- γ RV 0.8–1.1 pg/mL; TNF- α RV 0.7–1.1 pg/mL; IL-4 RV 4.5–7.1 pg/mL; IL-5 RV 0.5–0.7 pg/mL; IL-17 RV 0.7–1.2 pg/mL.

Thyroid ultrasound evaluation

The total thyroid volume was determined in milliliters (mL) by the product of the longitudinal, transverse and anteroposterior measurements multiplied by the constant 0.52, adding up the volumes of the right and left lobes and isthmus. Values between 6 and 15 mL ($10-11 \pm 3-4$ mL) were considered normal for adults [30].

Statistical methods

Exploratory data analysis was performed through summary measures (frequency, percentage, mean, standard deviation, minimum, median and maximum). The groups were compared using Kruskal-Wallis, Chi-Square or Fisher's exact test. The correlation of Vitamin D and Interleukins with the other variables was evaluated using the Spearman coefficient or the Mann-Whitney test. Factors associated with vitamin D deficiency were assessed through logistic regression using the *stepwise* selection criteria. The significance level for statistical analysis was 5% [31–33].

Results

Descriptive analysis

The study included 159 participants, 88 patients with HT, of which 82 were female (93%). In the control group, there were 71 subjects, 61 female (85.9%). Mean-time of diagnosis of HT was ten years (range 1–47 years). Clinical and laboratory characteristics of patients and control groups are described in Table 1.

Vitamin D levels below 30 ng/dL were found in 59.1% ($n = 39$) of the control group and in 71.8% ($n = 61$) of HT group ($p = 0.1024$).

Comparative analysis

There was no significant difference between levels of vitamin D, calcium, phosphorus or parathormone when comparing the two groups. TSH concentrations were higher in patients compared to the control group as expected, with no difference in free T4. We did not find differences related to gender, alcohol consumption or cigarette smoking (Table 1).

Table 1 Demographic and laboratory characteristics of HT and control group [mean (min–max)*]

Characteristics	Patients HT (<i>n</i> = 88)	Control Group (<i>n</i> = 71)	<i>p</i>
Age (years)*	46.2 (20–66)	47.4 (19–77)	0.5346
Gender (F/M) (<i>n</i>)	82/06	61/09	0.1983
BMI (Kg/m ²)*	28.1 (16.8–45.5)	26.4 (17–42.6)	0.0516
Time of diagnosis (years)*	10.6 (1–47)	—	—
Alcohol consumption*	1 (1.2%)	0 (0%)	1.000
Smoking*	9 (11%)	12 (19%)	0.2148
FT4 (ng/dL)*	1.2 (0.5–1.8) Median: 1.2	1.2 (0.8–1.6) Median: 1.2	0.4510
TSH (μUI/mL)*	6.3 (0.3–57) Median: 3.3	2.0 (0.6–5.0) Median: 1.7	<0.0001
TPOAb (UI/mL)*	571.4 (30–3,000)	—	—
TGAb (UI/mL)*	677.3 (100–4,000)	—	—
TRAb (UI/L)*	1.9 (0.2–40)	—	—
Calcium (mg/dL)*	9.1 (7.6–10.4)	9.1 (8.5–10.1)	0.9194
Phosphor (mg/dL)*	3.3 (2.0–5.4)	3.4 (2.5–4.5)	0.1486
PTH (pg/mL)*	41.4 (8.4–207)	38.4 (11–127.1)	0.3779
25OHVitaminD (ng/mL)*	26.4 (7.6–48.2)	28.6 (13–51.2)	0.1917
Vitamin D Insufficiency	61 (71.8%)	39 (59.1%)	0.1024

Mann-Whitney; Chi-square; Fisher exact test

Table 2 Serum concentrations of interleukins Th1 (IL-2, TNF-α, IFN-γ), Th2 (IL-4, IL-5) and Th17 (IL-17) in HT and control group [mean (min–max)]

Interleukins	HT (<i>n</i> = 88)	Control Group (<i>n</i> = 71)	<i>p</i>
IL-2 (pg/mL)	0.7 (0.1–7.3)	0.6 (0.1–5.7)	0.9556
IL-4 (pg/mL)	50.4 (1–330.2)	48.0 (0.9–378.3)	0.2067
IL-5 (pg/mL)	1.6 (0.0–15.5)	1.6 (0.0–20.8)	0.3597
TNF-α (pg/mL)	13.3 (0.2–35)	11.8 (0.2–35.5)	0.0904
IFN-γ (pg/mL)	17.0 (0.1–228)	13.4 (0.1–152)	0.9165
IL-17 (pg/mL)	13.9 (0.1–176)	12.3 (0.1–177)	0.6157

Mann-Whitney test

We did not observe differences between the concentrations of interleukins (Table 2).

Correlations

Hashimoto's Thyroiditis

A positive correlation was observed between vitamin D and free T4, IL-17, TNF-α and IL-5 (Table 3). There was no significant correlation between vitamin D and

other immunological markers, phosphorus or TSH (data not shown). We also found no correlation between vitamin D and PTH ($r = -0.07044$; $p = 0.5373$).

TNF-α was positively correlated with thyroid volume. IFN-γ correlated positively with TPOAb and negatively with calcium. IL-5 and IL-17 correlated negatively with TRAb (Table 3). There was no correlation between interleukins and age, BMI, diagnostic time, parity, TSH, free T4, TPOAb, TGAb, phosphorus or PTH (data not shown).

Among these patients, 58 (66.7%) presented a non-goiter form ($mean = 8.3$ mL) and 29 (33.3%), the form with goiter ($mean = 24.62$ mL). In the subgroup with goiter there was a negative correlation of volume with IL-2 (Th1) concentrations ($r = -0.47330$; $p = 0.0146$). The volume did not correlate with other interleukins or vitamin D in both subgroups.

Control Group

In the control group, there was a negative correlation between vitamin D and age ($r = -0.31374$, $p = 0.013$) whereas there was no correlation between vitamin D and interleukins, thyroid hormone profile or phosphorus

Table 3 Correlations between interleukins, vitamin D and variables in HT. Correlation coefficients (Spearman's rho)

	Thyroid volume	Calcium	TRAb	TPOAb	Vitamin D
IFN- γ	<i>r</i>	−0.21906	−0.22278	0.24208	
	<i>p</i>	0.0415	0.0381	0.0265	
TNF- α	<i>r</i>	0.22508			0.37505
	<i>p</i>	0.0372			0.0004
IL-17	<i>r</i>		−0.26848		0.3505
	<i>p</i>		0.0119		0.0011
IL-5	<i>r</i>		−0.30565		0.2661
	<i>p</i>		0.004		0.0144
FT4	<i>r</i>				0.25199
	<i>p</i>				0.0224

r: correlation *p*: statistical significance level

(data not shown). Still, we did not find correlation between vitamin D and PTH in the controls ($r = -0.16112$; $p = 0.1998$).

Analysis of logistic regression

In HT group, simple logistic regression analysis showed FT4 (OR = 0.063; $p = 0.0066$, CL 95%: 0.009–0.464) and TNF- α (OR = 0.907, $p = 0.0130$, CL 95%: 0.840–0.980) at lower levels as predictive factors of reduction of vitamin D levels. There were no significant values between vitamin D insufficiency and the other variables. In the multiple logistic regression analysis, lower levels of free T4 was a risk factor for vitamin D insufficiency (OR = 0.076; 95% CI: 0.008–0.764, $p = 0.0286$).

A receiver operator characteristic (ROC) curve was built to discriminate the best threshold for concentrations of free T4 to discriminate insufficiency and sufficiency of vitamin D. We found a threshold of 1.18 ng/dL, with an accuracy of 70.8%, specificity of 82.6% and sensitivity of 55.9% (Fig. 1).

In the control group, after simple logistic regression analysis, it was verified that, age (OR = 1.051, $p = 0.0182$, CL 95%: 1.008–1.095) and IL-4 (OR = 1.013, $p = 0.0415$, CL 95%: 1.000–1.026) were predictive factors of vitamin D insufficiency. There were no significant values between lower levels of vitamin D and the other variables: sex, smoking, parity, calcium, phosphorus, PTH, TSH, free T4 and other interleukins studied. There were no risk factors in the multiple logistic regression analysis for the control group.

Discussion

The present study demonstrated that vitamin D concentrations were similar in patients HT and the control group. Similarly, D'Aurizio *et al.* revealed no differences in vitamin D deficiency and 25OHD levels between 100 AITDs patients (52 TH and 48 GD) and healthy controls. Goswami *et al.* reported no association of vitamin D deficiency and TPOAb positivity, but only a weak inverse correlation between serum 25OHD and TPOAb levels in 642 patients. Effraimidis *et al.* developed a longitudinal study of 803 AITDs subjects who concluded that vitamin D deficiency was not associated with the early stages of thyroid autoimmunity. Yasmeh *et al.* reported a higher prevalence of sufficient vitamin D levels in HT females relative to control, observed a significant positive correlation between vitamin D and TPOAb levels in HT males relative to control concluding that HT was not associated with vitamin D deficiency relative to the control group. Zhang *et al.* pointed out that Vitamin D status was not associated with positive thyroid autoantibodies in a cross-sectional study. Higher vitamin D levels were associated with lower TSH levels in males. On the other hand, Ma *et al.* (2015) evaluated the association between vitamin D concentrations and AITDs (GD, HT, and postpartum thyroiditis) in two independent case-control studies, observing a decrease in vitamin D levels in these patients when compared to controls. Meta-analysis of Wang (2015) and the Mazokopakis review (2014) also demonstrated this association. Thus, differences in the association between HT and vitamin D

Fig. 1 ROC (receiver operator characteristic) curve used to identify threshold values related to insufficiency of 25OHvitamin D (<30 ng/mL) in patients with Hashimoto's thyroiditis. For a concentration of free thyroxine of 1.18 ng/dL, sensitivity was 55.97%, specificity was 82.6%, and accuracy was 70.8%.

insufficiency remain unresolved in the literature [11, 17, 26-28, 34-36].

We emphasize that the reduction of free T4 levels was a predictor of vitamin D insufficiency for Hashimoto's thyroiditis, especially at concentrations below 1.18 ng/dL, but not for the euthyroid individuals of the control group, suggesting that the thyroid hormone status would play a role in the maintenance of vitamin D sufficiency, and its immunomodulatory role would influence the presence of autoimmune thyroid disease. Additionally, the positive correlation between free T4 and vitamin D concentrations suggests that adequate levothyroxine replacement in HT would be an essential factor in maintaining vitamin D at sufficient levels, similarly previous reports. Likewise, Bozkurt *et al.* (2013) demonstrated a direct relationship with vitamin D in patients with recent and long-standing HT and demonstrated that the severity of vitamin D deficiency correlated positively with dis-

ease time and higher concentrations of anti-thyroid antibodies. Ma *et al.* (2015), D'Aurizio *et al.* (2015) and Ke *et al.* (2017) found no association between free T4 and TSH with vitamin D insufficiency in GD and HT. Zhang *et al.* (2014) observed in a cross-sectional study that higher concentrations of vitamin D were associated with low TSH, independent of free T4 values in euthyroid men from middle age to the elderly. Mansournia *et al.* and Tamer *et al.* found that vitamin D level presented decreasing trend in hypothyroidism patients who failed to administer medication. Krysiak *et al.* (2017) finding that vitamin D administration decreased thyroid antibody titers, especially TPOAb in HT women already receiving levothyroxine treatment suggesting that vitamin D may potentiate the effect of levothyroxine on autoimmune thyroid control [12, 17, 27, 36-40].

Additionally, lower concentrations of TNF- α was a predictor of lower levels of vitamin D, pointing to the

relationship between vitamin D and the immune system. This finding not corroborates data from the literature about the direct action of TNF- α /Th-1 cytokines on the pathophysiology of HT and its presence in higher concentrations in vitamin D insufficiency. However, the patients in the present study had several years of established disease and were under treatment with levothyroxine, which could justify the low concentrations of TNF- α , a cytokine usually present in the disease development phase. Also, serum cytokine levels may be different from the levels within the thyroid cells [7, 10, 12, 26, 39, 41].

There was also a positive correlation between vitamin D and TNF- α , IL-5, and IL-17. Low levels of TNF- α and IL-17 in detriment of low levels of vitamin D, different from the literature description, could be justified by the control of cytotoxicity arising from the treatment of the disease since the patients of this study present in mean ten years of diagnosis and are on levothyroxine replacement therapy. Regarding IL-5, the literature has demonstrated, as in the present study, a positive association with vitamin D levels. According to Cantorna *et al.*, vitamin D is associated with reduced production of cytokine group associated with cytotoxicity. Furthermore, a correlation was demonstrated between stimulation of auto-antibodies, as well as higher concentrations of CD8 T lymphocytes and specific cytokines in the peripheral blood of patients with intense disease activity. Marchiori *et al.* observed reduction of pro-inflammatory cytokines in hypothyroid patients under treatment. On the other hand, Ke *et al.* (2017) not found an association with vitamin D levels and serum cytokines IL-4, IL-17 and TNF- α in patients with HT and GD [38, 41, 42].

In addition, we observed no association of vitamin D and thyroid volume, different from Bizzaro *et al.*, 2015, who reported relation between low vitamin D and AITDs, anti-thyroid antibodies and higher thyroid volumes with higher levels of TSH, describing as a predisposing factor the VDR polymorphism present in cells of the immune system. Besides that, Pani *et al.* found polymorphisms in the vitamin D carrier protein (DBP) in GD but not in HT [43, 44].

Many researchers have been studied the single nucleotide polymorphisms (SNPs) in vitamin D receptor (VDR) to identify genes involved in the pathogenesis of autoimmune thyroid diseases (AITDs). Among the known polymorphisms related to the VDR locus are FokI, BsmI, ApaI, and TaqI. Ban *et al.* described the presence of VDR polymorphisms in both HT and GD besides the association between VDR-FokI polymorphism and risk

of osteoporosis in GD. On the other hand, Giovinnazzo *et al.* found no relationship between VDR polymorphisms and the presence of HT unlike Lin W *et al.* who described a correlation between VDR-FokI polymorphism and the risk of developing TH [20, 45-48].

Interestingly, the correlation between vitamin D and PTH did not reach significance in both patients and controls. This finding may be justified due to the mean values of vitamin D in both groups were above 25 ng/mL, not affecting calcium levels in general. Also, there was no correlation with vitamin D in both the patients' group and the control group. Although studies have shown that vitamin D levels below 30 ng/mL could lead to an increase in PTH, this did not happen in the present study [19, 49, 50].

We considered as limitations of this study the small number of patients and individuals in the control group. However, it was possible to obtain significant results and we reached the number recommended by the sample calculation. Blood samples were collected in the months between spring and summer, however, in our region, the seasons do not show significant differences for sun offered during the months.

In conclusion, our study verified that lower levels of vitamin D have not been associated with HT, however thyroxine levels were determined as a risk factor for vitamin D insufficiency. We emphasize the importance of maintaining adequate concentrations of free T4 for the adequate vitamin D status in patients with HT, a fact not verified in euthyroid individuals. The association between vitamin D and TNF- α , IL-5 and IL-17 in these patients pointed to the relevance to this relationship with autoimmunity in HT.

Additional studies are warranted to clarify the precise role of vitamin D in AITD.

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Disclosure

None of the authors has any potential conflicts of interest associated with this research.

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