Early stages of thyroid autoimmunity: follow-up studies in the Amsterdam AITD cohort
Effraimidis, G.

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Chapter 5

Vitamin D deficiency is not associated with early stages of thyroid autoimmunity.

Effraimidis G
Badenhoop K
Tijssen JGP
Wiersinga WM

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Context: Vitamin D deficiency has been identified as a risk factor for a number of autoimmune diseases including type 1 diabetes and multiple sclerosis.

Objective: We hypothesized that low levels of vitamin D are related to the early stages of autoimmune thyroid disease (AITD).

Design: Two case-control studies were performed. In the cross-sectional study A, euthyroid subjects with genetic susceptibility for AITD but without thyroid antibodies were compared to controls. Cases were subjects from the Amsterdam AITD cohort (euthyroid women who had 1st or 2nd degree relatives with overt AITD) who at baseline had normal TSH and no thyroid antibodies; controls were healthy women examined at the same time period. In the longitudinal study B, subjects who developed de novo TPO-Ab were compared with those who did not. Cases and controls were subjects from the Amsterdam AITD cohort who at baseline had normal TSH and no thyroid antibodies and during follow up developed TPO-Ab (cases) or remained without thyroid antibodies (controls). Controls in both studies were matched for age, BMI, smoking status, estrogen use, month of blood sampling and in study B for the duration of follow up.

Results: Serum 25(OH)D levels in ng/ml were as follow: Study A: 21.0±7.9 vs. 18.0±6.4 ng/ml (78 cases vs 78 controls, p=0.01). Study B: baseline, 22.6±10.3 vs. 23.4±9.1; follow-up 21.6±9.2 vs. 21.2±9.3 (67 cases vs. 67 controls, NS).

Conclusions: Early stages of thyroid autoimmunity (in study A genetic susceptibility and in study B development of TPO-Ab) are not associated with low vitamin D levels.
INTRODUCTION

The recent description that many tissues and cells in the body express the vitamin D receptor and 1α-hydroxylase resulted in a growing interest in the role of vitamin D in extra-skeletal conditions such as common cancers, cardiovascular disease and autoimmune diseases [1-4]. Lower 25-hydroxy vitamin D levels have been reported in several autoimmune diseases. Low vitamin D intake and vitamin D deficiency have been identified as a risk factor for type 1 diabetes, multiple sclerosis, Crohn’s disease and rheumatoid arthritis [5-10] e.g. in a prospective case-control study, the risk of multiple sclerosis significantly decreased with increasing levels of 25(OH)D [8]. Recent studies indicate that 1,25(OH)₂D, the biologically active form of vitamin D, is a modulator of both the innate and adaptive immune system. Immune cells such monocytes, macrophages, dendritic cells and T-lymphocytes and B-lymphocytes are targets for the active vitamin D. Moreover, 1,25(OH)₂D seems to play an autocrine and paracrine role in the immune system as immune cells are capable to activate vitamin D [4]. However it is not known which vitamin D levels are sufficient to improve the immune regulatory function and lead to an effective immune response. Very few studies in the past have reported on a putative association between thyroid autoimmunity and vitamin D with inconclusive results [11-13]. In the present study we aimed to assess whether vitamin D deficiency is related to the early stages of autoimmune thyroid disease (AITD) [14]. We hypothesized that vitamin D levels are already low in subjects with genetic susceptibility for AITD, at a time when thyroid antibodies are absent and thyroid function is still normal and also lower in euthyroid subjects who will develop thyroid antibodies de novo. Thus, we compared family members of AITD patients who were euthyroid without thyroid antibodies with healthy controls (study A), allowing to evaluate whether genetic susceptibility for AITD by itself is already associated with lower vitamin D levels. We further evaluated vitamin D levels in euthyroid family members of AITD patients, comparing those who developed TPO-Ab during follow-up (seroconverters) and those who did not (study B), allowing to assess whether the occurrence of thyroid antibodies is associated with lower vitamin D levels.

SUBJECTS & METHODS

Participants
The present study was carried out among the 803 subjects from the Amsterdam AITD Cohort. The cohort has previously been described in detail [15]. In short, the cohort consisted of women between 18 and 65 years of age in self-proclaimed good health without a history of thyroid disease, who had at least one 1st or 2nd degree relative with documented autoimmune hyper- or hypothyroidism. Results of thyroid function tests at study entrance revealed overt hypothyroidism in 10 subjects and overt hyperthyroidism in 3 subjects, leaving 790 subjects to be included in the present study. Subjects were followed for five years, or shorter when overt hyper- or hypothyroidism had occurred (defined as TSH <0.4 mU/L in combination with fT₄ >20.1 pmol/L, or TSH >5.7 mU/L
in combination with fT4 <9.3 pmol/L respectively). At each annual visit to our institution blood samples were collected to measure TSH, fT4, TPO-Ab, Tg-Ab and TBII. Plasma and serum samples were stored at –20° C until assay. We performed two case-control studies A and B. In both studies, for each case one control was selected. A case-control approach was chosen due to cost-effectiveness. Subjects using drugs affecting vitamin D levels were excluded in both studies.

All subjects gave informed written consent and the Medical Ethics committee of the Academic Medical Center in Amsterdam approved the study.

**Study A: Vitamin D and genetic susceptibility for AITD.**

Cases were selected from the Amsterdam AITD cohort at inception: euthyroid subjects without serological signs of thyroid autoimmunity at baseline (i.e. normal TSH, normal free T4, negative TPO-Ab, negative Tg-Ab and negative TBII) were included. As a control group, we used female subjects between 20 and 69 years of age, who were recruited through advertisements in local newspapers, to participate in an ongoing program within our institution for delineating reference values of endocrine function tests. They were also in self-proclaimed good health, had no family or personal history of thyroid disease and had normal TSH and no thyroid antibodies. Blood samples were collected over the same period of time as those of the Amsterdam AITD cohort, and processed in the same manner. Because the number of controls satisfying these criteria was limited to 78, we selected also 78 cases matched for known factors to affect 25(OH)D status: age, BMI, smoking status, estrogen use and month of blood sampling accepting a difference of 1 month. We defined seasons as winter (December to February), spring (March to May), summer (June to August), and autumn (September to November).

**Study B: Vitamin D and de novo development of TPO-Ab.**

We selected participants from the inception cohort of the 790 euthyroid subjects as follows: first we excluded 241 who had thyroid antibodies at baseline (i.e. serum concentrations of either TPO-Ab of 100 kU/L or greater, Tg-Ab of 100 kU/L or greater, or TBII of 12U/L or greater). From the remaining 549 euthyroid subjects without thyroid antibodies we subsequently excluded those who had subclinical hyper- or hypothyroidism and those who had no follow-up. Consequently, 521 euthyroid participants without any serological sign of AITD at baseline were thus enrolled. A subject was recruited as a case when she remained euthyroid but had developed TPO-Ab during follow-up. The end-point for a case was the time at which she had become positive for the first time for TPO-Ab without developing abnormal TSH. This happened in 67 subjects.

Subjects from the Amsterdam AITD cohort qualified to act as controls if they remained euthyroid and seronegative for TPO-Ab up to the time at which the case they were matched to, had received her end-point. Controls were matched by age, BMI, smoking status, estrogen use and month of blood sampling at study entrance and by duration of follow up. 25(OH)D status and 1,25(OH)_{2}D status at baseline and at the time of the seroconversion to TPO-Ab were compared between cases and controls.
Methods
Serum TSH and fT4 were measured using time-resolved fluoroimmunoassay (Delphia, Turku, Finland). Reference values are for TSH 0.4-5.7 mU/L and for fT4 9.3-20.1 pmol/L. Thyroid peroxidase (TPO) antibodies and thyroglobulin (Tg) antibodies were measured by chemiluminescence immunoassays (LUMI-test anti-TPO and LUMI-test anti-Tg respectively, Brahms, Berlin, Germany). Improved versions of both assays became available during follow-up: detection limits of these new assays were for TPO-Ab 30 kU/L and for Tg-Ab 20 kU/L. TPO-Ab concentrations obtained with the old assay were multiplied by a factor 0.72 to obtain comparative values in the new assay. TPO-Ab and Tg-Ab concentrations were considered to be positive at values ≥100 kU/L. TSH receptor antibodies were determined as TSH binding inhibitory immunoglobulins (TBII) using the TRAK assay (Brahms, Berlin, Germany); detection limits in the 1st and 2nd generation TRAK assays were 5 and 1 IU/L respectively, and values above 12 and 1.5 U/L respectively were considered as positive.

25(OH)D and 1,25(OH)_{2}D were measured by radioimmunoassay (I125 Radioimmunoassay IA Kit; DiaSorin, Stillwater, MN). Plasma 25(OH)D concentrations of <20 ng/mL was defined as 25(OH)D deficiency and <30 ng/mL as 25(OH)D deficiency and insufficiency [16]. A range of 20–67 pg/ml of 1,25(OH)_{2}D was considered normal according to the manufacturer.

Statistical analysis
Differences between cases and controls were evaluated by Student’s t-test for age, BMI, duration of follow up and 25(OH)D and 1,25(OH)_{2}D status, by Mann-Whitney U-test for TSH, fT4, TPO-Ab and Tg-Ab. Seasonal variation in 25(OH)D levels was assessed by ANOVA. A p-value of <0.05 was considered to indicate significant differences.

Results
Study A: Vitamin D and genetic susceptibility for AITD.
The 78 cases and 78 controls were comparable in age, BMI, proportion of smokers and estrogen users, month of blood sampling, TSH, fT4, TPO-Ab and Tg-Ab levels (Table 1). Seasonal variation in 25(OH)D was observed in cases and controls. Cases had a higher serum 25(OH)D concentration than controls (mean±SD: 21.0±7.9 ng/ml vs. 18.0±6.4 ng/ml, p=0.01). The prevalence of 25(OH)D deficiency (<20 ng/ml) was lower in cases than in controls (48.7% vs. 64.1% respectively, p=0.05); the same was true for the prevalent rates of 25(OH)D deficiency and insufficiency (<30 ng/ml) (83.3% vs. 94.9%, p=0.02).

Study B: Vitamin D and de novo development of TPO-Ab.
During the 5 year follow up period 67 subjects developed TPO-Ab while their TSH remained normal (Table 2). The mean±SD age at baseline of the converters to TPO-Ab was 35.5±11.5 years and the mean±SD follow up was 2.8±1.3 years. Controls mean age and mean follow up did not differ from cases. The same was true for BMI, proportion of smokers and estrogen users, month of bleed, TSH, fT4, TPO-Ab and Tg-Ab levels at baseline. Seasonal variation in 25(OH)D and 1,25(OH)_{2}D was observed in cases and controls.
Table 1. Characteristics and 25(OH)D status of healthy euthyroid thyroid antibodies-negative female relatives of AITD patients and healthy controls (Study A).

<table>
<thead>
<tr>
<th></th>
<th>cases</th>
<th>controls</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>78</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>age yr</td>
<td>42.1±13.2</td>
<td>42.3±13.1</td>
<td>NS</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>23.5 (21.6-26.3)</td>
<td>23.8 (21.2-26.5)</td>
<td>NS</td>
</tr>
<tr>
<td>current smokers %</td>
<td>27%</td>
<td>27%</td>
<td>NS</td>
</tr>
<tr>
<td>current estrogen users %</td>
<td>13%</td>
<td>13%</td>
<td>NS</td>
</tr>
<tr>
<td>blood sampling %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(winter/spring/summer/autumn)</td>
<td>23/44/13/20 %</td>
<td>23/50/5/22 %</td>
<td>NS</td>
</tr>
<tr>
<td>TSH mU/l</td>
<td>1.60 (1.0-2.0)</td>
<td>1.58 (1.1-1.8)</td>
<td>NS</td>
</tr>
<tr>
<td>fT4 pmol/l</td>
<td>14.0 (12.3-15.4)</td>
<td>14.1 (12.2-15.3)</td>
<td>NS</td>
</tr>
<tr>
<td>TPO-Ab kU/l</td>
<td>&lt;30 (&lt;30-&lt;30)</td>
<td>&lt;30 (&lt;30-&lt;30)</td>
<td>NS</td>
</tr>
<tr>
<td>Tg-Ab kU/l</td>
<td>&lt;10 (&lt;10-21)</td>
<td>&lt;10 (&lt;10-&lt;10)</td>
<td>NS</td>
</tr>
<tr>
<td>25(OH)D ng/ml</td>
<td>21.0±7.9</td>
<td>18.0±6.4</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data are given as mean±SD or median with interquartile range between parenthesis.

BMI: Body mass index, TSH: thyroid stimulating hormone, fT4: free T4, TPO: thyroid peroxidase, Tg: thyroglobulin, 25(OH)D: 25-hydroxyvitamin D3.

At the time of seroconversion the TPO-Ab concentration had a median value of 140 kU/L (interquartile range 110-160 kU/L). Statistical analysis at baseline and at time of seroconversion revealed no significant differences in the 25(OH)D and in the 1,25(OH)₂D levels between cases and controls. The frequency of 25(OH)D deficiency (<20 ng/ml) at baseline was comparable in cases and in controls (49.2% vs. 34.3% respectively, p=0.05); the same was true for the prevalent rates of 25(OH)D deficiency and insufficiency (<30 ng/ml) (79.1% vs. 86.5%, p=0.25). Similar figures were obtained at the time of seroconversion (52.2% vs 55.2% and 88.1% vs 83.6% respectively, NS).

**Discussion**

The main finding of our study is that 25(OH)D levels in the cases were not lower than in the controls, neither in the subjects with genetic susceptibility for AITD in study A, nor in the seroconverters with de novo occurrence of TPO-Ab in study B. We therefore have to reject our hypothesis that the early stages of thyroid autoimmunity are associated with low 25(OH)D levels.

The present study is the first to evaluate the relationship between 25(OH)D and thyroid autoimmunity in a prospective manner. All previous studies have been cross-sectional
Table 2. Characteristics, 25(OH)D and 1,25(OH)₂D status of healthy euthyroid thyroid antibodies-negative women who developed TPO-Ab (cases) and who did not develop TPO-Ab (controls) at baseline and at the time of seroconversion, in a nested case-control study of euthyroid women with 1st or 2nd degree relatives with proven AITD derived from the Amsterdam AITD cohort (study B).

<table>
<thead>
<tr>
<th></th>
<th>BASELINE</th>
<th>FOLLOW UP</th>
<th>p-value</th>
<th>BASELINE</th>
<th>FOLLOW UP</th>
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</tr>
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<tbody>
<tr>
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<td>NS</td>
<td>67</td>
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<td>NS</td>
</tr>
<tr>
<td>age yr</td>
<td>35.5±11.5</td>
<td>35.3±11.2</td>
<td>NS</td>
<td>38.3±11.5</td>
<td>38.1±11.3</td>
<td>NS</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>23.5 (21.6-26.3)</td>
<td>23.8 (21.2-26.5)</td>
<td>NS</td>
<td>23.0 (21.1-25.3)</td>
<td>24.2 (21.3-26.5)</td>
<td>NS</td>
</tr>
<tr>
<td>current smokers %</td>
<td>27%</td>
<td>27%</td>
<td>NS</td>
<td>28%</td>
<td>27%</td>
<td>NS</td>
</tr>
<tr>
<td>current estrogen use %</td>
<td>13%</td>
<td>13%</td>
<td>NS</td>
<td>28%</td>
<td>37%</td>
<td>NS</td>
</tr>
<tr>
<td>blood sampling %</td>
<td>21/21/28/30 %</td>
<td>21/22/31/26 %</td>
<td>NS</td>
<td>21/22/14/43 %</td>
<td>24/21/15/40 %</td>
<td>NS</td>
</tr>
<tr>
<td>(winter/spring/summer/autumn)</td>
<td>1.50 (1.20-2.20)</td>
<td>1.50 (1.00-1.90)</td>
<td>NS</td>
<td>1.60 (1.10-2.30)</td>
<td>1.50 (0.87-2.10)</td>
<td>NS</td>
</tr>
<tr>
<td>TSH mU/l</td>
<td>13.0 (11.3-14.4)</td>
<td>13.3 (12.0-14.9)</td>
<td>NS</td>
<td>12.9 (11.8-14.5)</td>
<td>13.3 (12.0-14.6)</td>
<td>NS</td>
</tr>
<tr>
<td>TPO Antibody kU/l</td>
<td>&lt;30 (&lt;30-&lt;30)</td>
<td>&lt;30 (&lt;30-&lt;30)</td>
<td>NS</td>
<td>140 (110-160)</td>
<td>&lt;30 (&lt;30-50)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tg Antibody kU/l</td>
<td>&lt;10 (&lt;10-25)</td>
<td>&lt;10 (&lt;10-15)</td>
<td>NS</td>
<td>18 (&lt;10-47)</td>
<td>&lt;10 (&lt;10-13)</td>
<td>NS</td>
</tr>
<tr>
<td>25(OH)D ng/ml</td>
<td>22.6±10.3</td>
<td>23.4±9.1</td>
<td>NS</td>
<td>21.6±9.2</td>
<td>21.2±9.3</td>
<td>NS</td>
</tr>
<tr>
<td>1,25(OH)₂D pg/ml</td>
<td>58.5±20.8</td>
<td>59.1±18.1</td>
<td>NS</td>
<td>56.7±18.3</td>
<td>59.3±19.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD or median with interquartile range between parenthesis.

BMI: Body mass index, TSH: thyroid stimulating hormone, fT4: free T4, TPO: thyroid peroxidase, Tg: thyroglobulin, 25(OH)D: 25-hydroxyvitamin D3, 1,25(OH)₂D: 1,25 dihydroxyvitamin D3
in nature. A study from India [11] reported 25(OH)D deficiency (25(OH)D serum levels ≤ 10ng/ml) in 87% of 642 students and teachers aged 16-60 years; blood sampling was done in the winter months. Comparing subjects with ≤ 10 ng/ml or >10 ng/ml, there were no differences in the prevalence of TPO-Ab (21.3% vs. 18.1%), the concentration of TPO-Ab (58±4.9 vs. 46±10.4 IU/ml) and the prevalence of subjects with TPO-Ab and decreased TSH (13.2% vs. 12.0%). A study from Hungary [12] (with blood sampling in March) observed a higher prevalence of 25(OH)D deficiency (25(OH)D serum levels ≤ 10 ng/ml) in outpatients with thyroid disease than in age-matched healthy controls (63% vs. 30%, p<0.001). However, the prevalence of 25(OH)D deficiency was not different between AITD patients (both Hashimoto’s and Graves’ disease) and non AITD patients (72% vs. 52%, p=0.08); abnormal thyroid function (not specified any further) in these groups occurred in 44% and 15% respectively (p=0.003). This may constitute a bias since 1,25(OH)_{2}D levels tend to be low in hyperthyroidism as a result of the increased calcium release from bone; conversely 1,25(OH)_{2}D levels are slightly higher in hypothyroidism. The third and last study [13] on this topic comes from Turkey (performed between October 2008 and February 2009). Vitamin D levels were lower in patients with Hashimoto’s thyroiditis than in age and sex matched controls (25(OH)D 16.3±10.4 vs 29.6±25.5 ng/ml, p<0.0001), as was the prevalence of 25(OH)D deficiency and insufficiency (25(OH)D <30 ng/ml, 92% vs. 63%, p<0.0001). There were no significant differences in 25(OH)D levels between Hashimoto’s patients who were overtly hypothyroid, subclinically hypothyroid or euthyroid (15.4±9.3, 15.7±7.4 and 17.4±12.9 ng/ml respectively, p=0.87). Seasonal variation could still be a confounding factor in this study. The authors concluded there might be an association between vitamin D insufficiency and Hashimoto’s thyroiditis but not between vitamin D insufficiency and progress of thyroid damage in Hashimoto’s thyroiditis. They recommended further studies to evaluate whether vitamin D insufficiency is a causal factor in the pathogenesis of AITD. Our studies do not support the hypothesis that low 25(OH)D levels in adults are involved in the pathogenesis of AITD neither at the early stages in which there is nothing else except genetic susceptibility, nor in the preclinical stage in which thyroid antibodies develop but thyroid function is still normal. It cannot ruled out that 25(OH)D deficiency at even earlier time points (than those we studied) i.e. in early life, might effect the development of AITD in genetically high risk individuals. This view is supported by the finding that serum 25(OH)D levels of ≥ 100 nmol/L before the age of 20 years protect against the development of multiple sclerosis later in life [8]. Studies on type 1 diabetes have shown increased prevalence of type 1 diabetes when vitamin D deficiency was present in the first months of life [17]; also the onset of type 1 diabetes is accelerated in mice with vitamin D deficiency in early life [18]. We observed a high prevalence of 25(OH)D insufficiency in our study, in agreement with previous Dutch population-based studies [19]. One may argue that a high prevalence of 25(OH)D insufficiency may enhance the chance to miss a small difference in 25(OH)D levels between AITD patients and healthy controls. However, the finding of a difference in Turkey with also a high prevalence of 25(OH)D insufficiency, does not support this possibility. However we cannot exclude, that other environmental factors – such as infections – may interact with vitamin D deficiency and enhance the risk for thyroid autoimmunity by differential immune pathways only operative if these two factors coincide, possibly with additional immunogenetic susceptibility regulating this process. An interaction
of vitamin D deficiency with susceptibility to several viral and retroviral infections is documented [20-22]. Serum 25(OH)D and 1,25(OH)2D concentrations may also not always accurately reflect the hormonal effect in target tissues, which can be modified in multiple ways, e.g. by paracrine effects of locally synthesized 1,25(OH)2D and polymorphisms in the vitamin D receptor [23,24]. The finding that 25(OH)D levels in study A were higher in cases than controls might also be interpreted as to suggest that women with genetic susceptibility to AITD do not have thyroid autoantibodies because they are protected from developing antibodies through higher levels of 25(OH)D. This interpretation, however, does not change our conclusion that low 25(OH)D levels are not associated with early stages of AITD.

A weakness of our study is the limited number of subjects and a larger number of cases is required to more accurately outline the role of vitamin D. Another limitation is that we studied adults, as vitamin D levels earlier in life may be critical in conferring protection against autoimmune diseases. The strengths of our studies are their prospective nature and their well executed matching procedure for cases and controls. The large sample size of the Amsterdam AITD cohort allowed to match for a great number of variables known to influence 25(OH)D levels: age, BMI, smoking status, estrogen use and month of blood sampling. We did not inquire about recent holidays in the sun, but most likely variation in sunny holidays between cases and controls is effectively neutralized by our matching for seasonal variation in blood sampling. Thus, we feel rather confident on the validity of our conclusion: vitamin D deficiency in adults is not related with early stages of thyroid autoimmunity.

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