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ORIGINAL ARTICLE

Vitamin D receptor gene polymorphisms/haplotypes and serum 25(OH)D_3 levels in Hashimoto's thyroiditis

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Abstract Vitamin D deficiency and/or reduced function, as per certain polymorphisms of the vitamin D receptor (VDR) gene, have been related to several autoimmune disorders. The present study was aimed to investigate the association of Hashimoto's thyroiditis with vitamin D status and functional polymorphisms (SNPs) of the VDR gene. In this case–control study, 200 euthyroid subjects were enrolled: 100 newly diagnosed HT patients (87 F, 13 M; mean age ± SD 42 ± 15 year) and 100 healthy individuals, matched for age, sex, BMI, and month of blood sampling. Serum 25(OH)D_3 was measured by HPLC. The VDR SNPs BsmI, ApaI, and TaqI, in strong linkage disequilibrium with each other, were detected by restriction fragment length polymorphism-PCR. The prevalence of vitamin D deficiency in HT patients was significantly higher than that in the control group (70 vs 18.2 %; $p < 0.0001$), and median serum 25(OH)D_3 level was significantly lower in HT patients than controls (median value: 16.2 vs 37.4 ng/ml; $p = 0.026$). Moreover, there was a significant inverse correlation between serum 25(OH)D_3 and TPOAb concentration ($r = -0.669$; $p = 0.034$). Contrarily, the genotype distribution of the studied SNPs was not different in the two groups (BsmI $p = 0.783$; ApaI $p = 0.512$; TaqI $p = 0.471$), as was the allelic frequency [f(B) $p = 0.776$, f(b) $p = 0.887$; f(A) $p = 0.999$, f(a) $p = 0.999$; f(T) $p = 0.617$; f(t) $p = 0.617$]. The present study first investigates newly diagnosed untreated HT and suggests that vitamin D deficiency may contribute to HT development and/or progression, acting as an environmental trigger, while the VDR locus does not appear to be involved in conditioning the genetic susceptibility to the disease, at least in Caucasians.

Keywords Hashimoto's thyroiditis · Vitamin D · Vitamin D receptor gene (VDR) · Single nucleotide polymorphisms · Autoimmunity

Introduction

Vitamin D has been shown to exert various anti-inflammatory and immune-modulatory effects, along with its major role in bone mineral homeostasis [1]. Vitamin D directly acts on immune cells, by promoting monocyte differentiation and by inhibiting lymphocyte proliferation and production of immunoglobulins and cytokines, such as IL2, INF-γ, and IL12 [1, 2]. It also inhibits dendritic cells differentiation and maturation [1], and reduces the expression of major histocompatibility complex (MHC) class II molecules on both immune and non-immune cells [1, 3]. As a consequence, both cell-mediated immune response and B cells proliferation and auto-antibodies production would directly down-regulated by vitamin D [1–3]. Such actions result in an overall protective effect of vitamin D against immune-mediated diseases. In animal

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models, vitamin D supplementation prevents and/or ameliorates experimental autoimmune diseases such as type 1 diabetes [4], thyroiditis [5], and encephalitis [6]. In humans, vitamin D status has been associated with susceptibility to several immune-mediated disorders, including chronic infections (tuberculosis) and autoimmune diseases [7–14], and administration of vitamin D supplements has been reported to reduce the risk to develop such diseases [1, 8, 13, 15]. The pleiotropic effects of vitamin D are exerted via its nuclear receptor (VDR), which belongs to the steroid receptor super-family and is widely expressed in many cell types, including lymphocytes, macrophages, and several endocrine cells [16]. The VDR gene, located on chromosome 12q12–14, shows an extensive polymorphism that influences its function. Four major single nucleotide polymorphisms (SNPs) have been described, namely FokI in exon 2, BsmI and ApaI in intron 8, and TaqI in exon 9, clustered in several haplotype blocks of extensive linkage disequilibrium. BsmI (rs1544410), ApaI (rs7975232), and TaqI (rs731236) SNPs are in strong linkage disequilibrium with each other, while no significant linkage disequilibrium with the FokI site was observed [16, 17].

Certain SNPs of the VDR gene may result in a reduced vitamin D function and have been associated to several diseases, including autoimmune disorders such as type 1 diabetes [18] and Addison disease [19]. Concerning autoimmune thyroid disorders, conflicting data are available from the literature [20–24].

The present study was aimed to assess the vitamin D status, by means of measurement of serum 25(OH)D_3, in Hashimoto's thyroiditis (HT) patients compared to healthy controls, in order to investigate the possible relationship between vitamin D deficiency and thyroid autoimmunity. In the same cohort, we studied the distribution of three VDR SNPs, the BsmI, ApaI, and TaqI SNPs which are in strong linkage disequilibrium with each other, to evaluate if VDR gene polymorphisms may contribute to the genetic susceptibility to HT.

Materials and methods

Patients

A total of 200 unrelated euthyroid subjects were enrolled in the study: 100 newly diagnosed HT patients (13 men and 87 women, aged 19–79 year) and 100 age- and sex-matched healthy individuals (12 men and 88 women, aged 19–69 year) from the same geographic area, as controls. Subjects were recruited over a seven-month period, from October 2013 to May 2014, that is from autumn to spring. Since seasonal variations of serum 25(OH)D_3 levels are well known, patient and controls were also matched for month of blood sampling, in order to minimize selection bias.

Each subject received a careful medical evaluation, including recording of past personal and family medical history, and physical examination. HT was diagnosed by the currently accepted laboratory and ultrasonographic criteria (serum anti-thyroid antibodies positivity and/or heterogeneous echo-structure with diffuse or patchy hypoechogenicity at ultrasonography) [25]. All HT patients were clinically and biochemically euthyroid and were not taking levo-thyroxine (L-T4) therapy at the time of sampling. All control subjects had no evidence of thyroid disease, as determined by clinical examination, thyroid function, thyroid auto-antibodies testing, and neck ultrasonography. Subjects with diabetes mellitus or kidney failure, history of neoplastic disease, and any comorbid autoimmune diseases were excluded. Also excluded were individuals taking calcium or vitamin D supplements 3 months before blood sampling.

Informed consent was obtained, and the study was approved by our local Ethics Committee.

Biochemical analysis

Peripheral blood samples were collected after overnight fasting from all the recruited patients and controls. Venous blood was centrifuged at 1450×g at 4 °C for 10 min. All samples were processed centrally in the laboratory of our University Hospital of Messina.

Calcium, glucose, insulin, and lipids [total cholesterol, high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol, and triglycerides] were immediately measured using commercial kits on routine methods; appropriate aliquots for other assays were stored at −20 °C.

Serum TSH, free thyroxine (FT4), and free tri-iodothyronine (FT3) concentrations, as well as anti-thyroglobulin (TgAb) and anti-thyroperoxidase (TPOAb) antibodies, were measured by electrochemiluminescence immunoassay (ECLIA), using commercial kits for Elecsys 1010/2010 e modular analytics E170 supplied by Roche Diagnostics. Reference values were TSH, 0.27–4.2 mIU/l; FT3, 2.0–4.4 pg/ml; FT4, 10.3–22.0 pmol/l; TgAb, 0–4 IU/ml; and TPOAb, 0–10 IU/ml. For all assays, the intra or the inter-assay CV were <5 and <10 %, respectively.

Serum 25(OH)D_3 levels were used to evaluate the vitamin D status. 25(OH)D_3 was measured by HPLC (Bio-Rad Laboratories S.r.l., Milano, Italy). Based on the Endocrine Society guidelines [26], 25(OH)D_3 status was defined as deficient (<20 ng/ml), insufficient (20–30 ng/ml), and sufficient (>30 ng/ml).

Thyroid imaging

Thyroid ultrasonography (US) was performed using a real-time 2D apparatus with a 7.5 MHz linear transducer (General Electric Healthcare, USA). An abnormal sonographic appearance of the thyroid, characterized by diffuse areas of decreased echogenicity, was considered an important criterion for the sonographic diagnosis of HT. The volume of thyroid lobes was calculated with the ellipsoid formula ($\pi/6 \times$ height $\times$ width $\times$ depth, each diameter being expressed in cm).

Genotyping

The VDR gene SNPs were investigated by restriction fragment length polymorphism (RFLP)-PCR using the restriction enzymes BsmI, ApaI, and TaqI.

Germline DNA was isolated from blood leukocytes of each subject with the GeneMatrix Quick Blood DNA Purification Kit (EURx Ltd, Poland), according to the manufacturer's instructions. DNA (200 ng) was amplified by thermal cycling, using the 5 Prime kit Master Mix (Eppendorf, Italy), and the following primers (Sigma-Aldrich, Italy): for BsmI, Forward 5′CAACCAAGACTAC AAGTACCGCG3′, Reverse 5′AACCAGCGGGAAGAG GTCAAGGG3′; Forward 5′CAGAGCATGGACAGGG AGCAA3′, Reverse 5′GCAACTCCTCATGGCTGAG GTCTC3′ for both ApaI e TaqI. The PCR conditions were 5 min at 94 °C for initial denaturation, 30 s at 94 °C, 40 s at 69 °C for BsmI, 65 °C for ApaI and TaqI, 1 min at 72 °C, 35 cycles, followed by 5 min at 72 °C for final extension. PCR products were digested with the restriction enzymes BsmI, ApaI, and TaqI (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions, and electrophoresed on 2 % agarose gel stained with GelRed (Biotium, Hayward, CA). Genotypes were determined according to the presence or absence of an appropriate restriction site. The lowercase allele represents the presence of the restriction site (b, a, or t) and the uppercase allele represents the absence of the restriction site (B, A, or T). BsmI digestion determines the genotypes BB (825 bp), Bb (825,649,176 bp), or bb (649,176). ApaI digestion reveals genotypes AA (740 bp), Aa (740, 530, and 210 bp), or aa (530 and 210 bp) and TaqI genotypes TT (495 and 245 bp), Tt (495, 290, 245, and 205 bp), or tt (290, 245, and 205 bp). In order to confirm that the amplified products represent genuine VDR regions, direct sequencing of some heterozygous or mutant homozygous PCR products was performed using an automatic ABI310 sequencer (Applied Biosystems) and the above reported primers. The cDNA of the human VDR sequence (GenBank Accession Number J03258) was used for comparison.

Statistical analysis

A power analysis was performed to establish the sample size. We calculated the minimum sample size required to accept the outcome of a statistical test with the confidence level $\alpha = 0.050$ and a power level of 0.85. Indeed, we stated that 100 subjects per group needed to ensure this specific power level.

The numerical data are expressed as median and range and the categorical variables as number and percentage. Examined variables did not present normal distribution as verified by *Kolmogorov–Smirnov test*; consequently the non-parametric approach has been used. We performed statistical comparisons between case and controls using *Chi-Square test* for categorical variables and *Mann–Whitney test* for numerical parameters. To compare numerical parameters of the three SNPs (APA, TAQ, and BSM), the Kruskall Wallis test was estimated. Conditioned to the obtained significance, we performed the two-by-two comparison between groups by means of *Mann–Whitney test* (using the Bonferroni correction for multiplicity control). To compare the categorical variables, between the groups and the two-by-two comparison, the means of *Chi-Square test* (using the Bonferroni correction for multiplicity control) were performed.

Linkage disequilibrium between markers and haplotype associations analyses were performed by means of the Haploview 4.2 (Broad Institute, Cambridge, MA, USA).

Statistical analyses were performed using SPSS 17.0 for Window package. $p < 0.050$ two sided was considered to be statistically significant.

Results

Hormonal data

The clinical and biochemical characteristics of our study population are given in Table 1. All subjects were euthyroid at the time of sampling. Serum levels of $25(OH)D_3$ were significantly lower in HT patients (21.2 ± 12.9 ng/ml, median 16.2 ng/ml) than in controls (35.7 ± 16.71 ng/ml, median 37.4 ng/ml; $p = 0.026$), and a status of vitamin D deficiency, defined as serum $25(OH)D_3 < 20$ ng/ml, was found in 70 % of HT patients compared to 18.2 % controls ($p < 0.0001$) (Fig. 1). In HT patients, $25(OH)D_3$ levels were significantly correlated with serum TPOAb ($r = -0.669$; $p = 0.034$) (Fig. 2, panel A), while no significant relationship was found between serum $25(OH)D_3$ and TSH ($p = 0.646$), FT3 ($p = 0.239$) and FT4 ($p = 0.330$) levels, or with thyroid volume ($p = 0.464$).

As shown in Table 1, the two groups of HT patients and age- and sex-matched healthy controls did not differ

Table 1 Demographic, clinical, and biochemical characteristics of our study population

	HT Patients	Healthy controls	*p*
Total no. of patient	100	100	
Sex			
Male	13	12	0.831
Female	87	88	
Age			
Mean ± SD	42 ± 15 (19–79)	40 ± 13 (19–69)	0.566
BMI (kg/m^2)[#]	27 ± 6 (17–39)	26 ± 5 (18–38)	0.157
Fasting glucose (mg/dl)	88 ± 12.9 (70–143)	88.3 ± 6.4 (71–98)	0.189
Basal fasting insulin (μIU/l)	11.2 ± 9.6 (2–42)	8.4 ± 6.4 (1.5–36.1)	0.533
HOMA index[§]	2.1 ± 2 (0.3–9)	1.9 ± 1.9 (0.2–11)	0.898
Total cholesterol (mg/dl)	186.2 ± 35.3 (127–292)	183.4 ± 34.3 (117–247)	0.891
HDL cholesterol (mg/dl)	63.8 ± 17.6 (31–112)	65 ± 15.9 (31–106)	0.575
Triglycerides (mg/dl)	88.2 ± 52.9 (32.6–268)	75.3 ± 34.1 (36–184)	0.298
Calcium (mg/dl)	9.0 ± 0.4 (8.3–9.9)	9.1 ± 0.5 (8.3–10)	0.994
TSH (mIU/l)	1.9 ± 1.7 (0.45–4.2)	1.5 ± 1.3 (0.8–3.8)	0.383
FT3 (pg/ml)	3.2 ± 1.3 (2.1–4.2)	3.1 ± 0.5 (2.2–4.3)	0.894
FT4 (pmol/l)	14.3 ± 2.9 (10.6–20.5)	13.4 ± 2.7 (10.3–20.8)	0.067
TgAb (IU/l)	104.8 (10–1895)	Absent	**<0.05**
TPOAb (IU/l)	133.5 (20–956)	Absent	**<0.05**
$25(OH)D_3$ (ng/ml)	21.2 ± 12.9 (8.8–57.3)	35.7 ± 16.7 (10.4–72.5)	**0.026**
Thyroid volume (ml)[^]	13.2 ± 4.6 (6.0–16.8)	14.2 ± 6.1 (6.0–17.6)	0.260

Data are mean ± SD and in parenthesis range, except TgAb and TPOAb which are median and, in parenthesis, range. Normal values are specified under "Materials and methods." *p* values typed in bold are significant ($p \leq 0.05$)

[#] The body mass index (BMI) was calculated by dividing the body weight (kg) with the square of height in meters

[§] Insulin resistance was estimated by the homeostatic model assessment index (HOMA)

[^] Thyroid volume was evaluated by US as specified under "Materials and methods"

significantly regarding the main anthropometric and metabolic parameters. In the whole study population, we also found that serum $25(OH)D_3$ levels were inversely correlated to fasting insulin levels and directly with serum HDL cholesterol, in a significant manner (Fig. 2, panel B and C, respectively), irrespective of thyroid autoimmunity and function.

Genotyping analysis

For all DNA datasets, genotype frequencies were in Hardy–Weinberg equilibrium in both groups. The genotype analysis revealed similar frequencies of the three studied VDR SNPs in HT patients compared to healthy controls, as reported in Table 2. Therefore, no statistical difference emerged in both the genotype distribution and the allelic frequencies between the two groups (Table 2). Furthermore, we constructed a complete three markers haplotype analysis of all analyzed RFLPs. A complete list of haplotypes and the relative frequencies was generated (Table 3). The two most common BsmI–ApaI–TaqI haplotypes were *BAt and baT*. Comparing the distribution of these two haplotypes in HT patients and controls, no difference emerged once again (Table 3).

In addition, no statistically significant differences in serum $25(OH)D_3$ levels were found between the genotype variants of the three examined SNPs (BsmI $p = 0.412$; $p =$ ApaI 0.08; TaqI $p = 0.672$).

Discussion

HT is well-known to be a multifactorial disease which develops in genetically susceptible individuals triggered by various environmental factors [27, 28]. The number of environmental triggers potentially involved in the

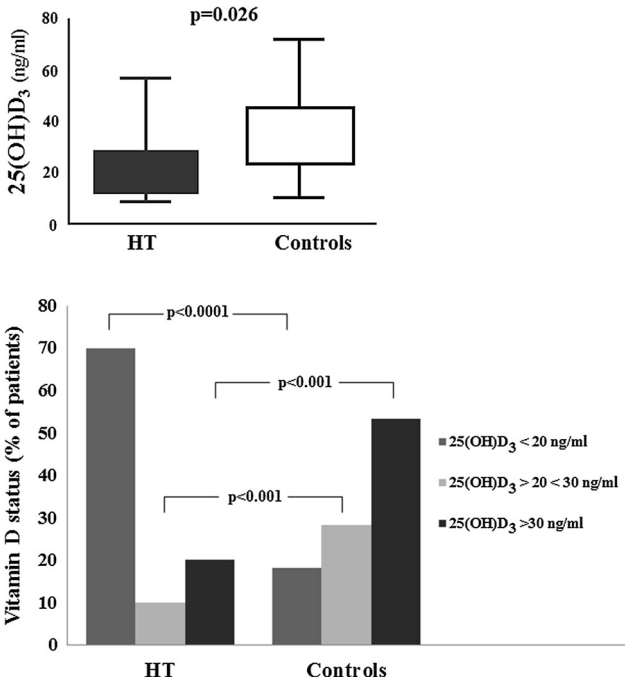

Fig. 1 Serum $25(OH)D_3$ levels in Hashimoto's thyroiditis patients compared to healthy controls (*top*), and percentages of subjects with vitamin D deficiency in patients and controls groups (*bottom*)

development and progression of the disease has increased over the years, including changes in life style (the "hygiene hypothesis," stress,…), pollutants, novel drugs (i.e., tyrosine kinase inhibitors), and nutrients other than iodine, such as selenium and vitamin D, whose role in autoimmunity is intensely debated at present [27, 29, 30].

In particular, interest in vitamin D has risen in recent years, as it has grown the appreciation of its anti-inflammatory and immunomodulatory effects, and the magnitude of hypovitaminosis D in the general health population has been better recognized worldwide [13, 31]. Several studies have been conducted in different population settings to investigate vitamin D status in subjects suffering from various autoimmune disorders (such as rheumatoid arthritis, systemic sclerosis, type 1 diabetes mellitus, multiple sclerosis, inflammatory bowel diseases, and autoimmune gastritis), and most of them reported decreased levels of vitamin D in patients compared to healthy subjects [8, 13]. A protective role of vitamin D against autoimmune disorders has been also supported by intervention studies [13, 15] and experimental data [4–6].

With regard to the association between thyroid autoimmunity and vitamin D, however, data from the literature remain conflicting and inconclusive, as extensively depicted in a recent review by D'Aurizio et al. [30]. The Authors of the review also provided personal data on the issue and showed no differences in vitamin D levels

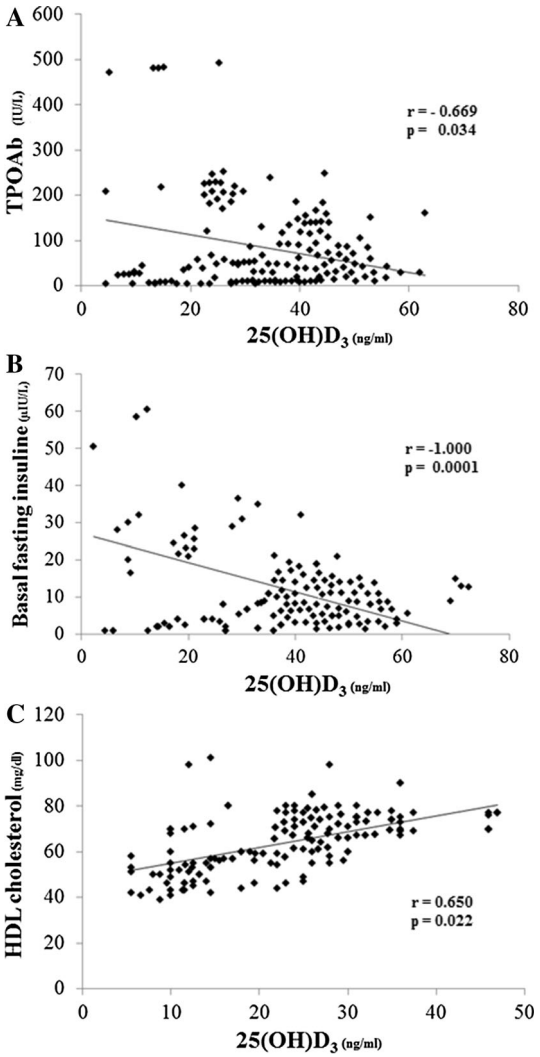

Fig. 2 Correlation between serum levels of $25(OH)D_3$ and serum levels of TPOAb (**a**), fasting insulin (**b**), and high-density lipoprotein (HDL) cholesterol (**c**)

between either HT or Graves' patients and healthy controls, in contrast to most of the studies described in the review, further underlying how a aetiopathogenetic link between vitamin D and thyroid autoimmunity is far from proven [30]. Anyway, most of the studies reported lower levels of vitamin D and higher rates of hypovitaminosis D in HT patients than controls, both in adulthood and childhood [9, 10, 32–37], and such a relationship with thyroid autoimmunity clearly emerged in a recent meta-analysis of the pertinent literature by Wang et al. [38]. Vitamin D deficiency was significantly related to anti-thyroid antibodies [32, 33, 37] and to thyroid dysfunction [9, 10, 32, 34, 36]. Recently, Ma et al. [36] first reported reduced vitamin levels also in post-partum thyroiditis, beside HT and Graves' disease, and concluded that the lower the vitamin D level is, not vitamin D deficiency per se, the higher the

Table 2 Genotype and allelic frequencies of the three SNPs under study in patients affected by Hashimoto's thyroiditis (HT) versus healthy controls

	HT patients ($n = 100$)	Healthy controls ($n = 100$)	p*
BsmI			
Genotype			
BB	37	34	0.768
Bb	40	41	0.999
bb	23	25	0.919
Allele			
B	114	109	0.776
b	86	91	0.887
ApaI			
Genotype			
AA	31	35	0.652
Aa	53	45	0.322
aa	16	20	0.581
Allele			
A	115	115	0.999
a	85	85	0.999
TaqI			
Genotype			
TT	38	30	0.296
Tt	42	49	0.394
tt	20	21	0.999
Allele			
T	118	109	0.617
t	82	91	0.617

* Comparison between proportions was made by means of z test

risk for developing AITD will be. However, results of these studies are not comparable, and therefore not conclusive, because of differences in the study populations background, thyroid functional status of patients, season of blood sampling, and criteria for definitions of vitamin D deficiency and vitamin D assays. Moreover, the cross-sectional design of most studies, including patients with hypothyroidism or under L-T4 therapy, do not allow to exclude that the vitamin D insufficiency may be the result of the metabolic changes of thyroid dysfunction *per se* rather than a primary event involved in the pathogenesis of the disease [27, 30]. The sole longitudinal study, conducted by Effraimidis et al. [39] on subjects with genetic susceptibility for autoimmune thyroid disease from the Amsterdam AIDT cohort, failed to demonstrate differences in vitamin D levels between subjects who developed TPOAb and those who did not during a 5-year duration follow-up, concluding that very early stages of thyroid autoimmunity are not associated with low vitamin D levels. More recently, a prospective population-based study, including 12,555 individuals from Denmark with a median follow-up time of 10.8 year, demonstrated a statistically significant inverse association between vitamin D status and appearance of any autoimmune disease [40]. Although not focused on thyroid diseases, this study clearly suggest a primary role of vitamin D in contributing to the development of autoimmune disorders [40].

In the present case–control study, we assessed the vitamin D status in newly diagnosed, euthyroid HT patients in comparison with healthy subjects. None was under L-T4 therapy. Thus, even if it was a cross-sectional study, confounding factors related to thyroid dysfunction and/or replacement therapy were avoided. Prior of us, only Bozkurt et al. [35] had investigated vitamin D levels in euthyroid subjects with comparable values of TSH (FT4 values not provided), but half of HT patients were under L-T4 therapy, which is well-known to increase vitamin D metabolism. The Authors reported the lowest vitamin D levels in treated HT patients compared to both euthyroid untreated HT patients and healthy controls, but vitamin D did not seem to differ significantly between untreated HT

Table 3 Frequency of the BAt and baT haplotypes in our study population, as a whole and subdivided in Hashimoto's thyroiditis patient and healthy controls

Haplotypes	Study population	HT patients ($n = 100$)		Healthy controls ($n = 100$)		p
	Frequency	Ratio count	Frequency	Ratio count	Frequency	
BAt	0.370	73.0:127.0	0.365	74.9:125.1	0.374	0.8476
baT	0.341	68.5:131.5	0.342	67.9:132.1	0.339	0.951
BAT	0.117	26.6:173.4	0.133	20.0:180.0	0.100	0.3077
bAT	0.066	13.1:186.9	0.066	13.2:186.8	0.066	0.9803
BaT	0.044	9.8:190.2	0.049	7.8:192.2	0.039	0.6274
Bat	0.029	5.5:194.5	0.028	6.2:193.8	0.031	0.8395
bAt	0.023	2.2:197.8	0.011	6.8:193.2	0.034	0.1248
bat	0.011	1.2:198.8	0.006	3.1:196.9	0.015	0.3544

Haplotype associations analyses were performed by means of the Haploview 4.2, as specified under "Materials and methods"

patients and controls. Furthermore, the prevalence of hypovitaminosis was higher in either treated or untreated HT patients than controls, but it was very high in the whole population (94 % of the subjects under study had $25(OH)D_3$ <25 ng/ml), and this may represent another confounding factor [35]. More recently, Mazokopakis et al. [37] reported low vitamin D levels in a large cohort of Greek euthyroid HT patients. Noteworthy, the Authors re-evaluated thyroid auto-antibodies levels after a short course of cholecalciferol supplementation and found a significant reduction of TPOAb levels, without changes in serum TSH.

In our study population of two hundred euthyroid subjects matched for sex, age, BMI, and month of sampling, we found that serum levels of $25(OH)D_3$ were significantly lower and the prevalence of vitamin D deficiency significantly higher in HT patients than controls. Moreover, we found a statistically significant negative correlation between serum $25(OH)D_3$ and TPOAb levels in HT patients, while no correlation emerged with serum TSH and/or thyroid hormones. Therefore, vitamin D levels seem to be mainly related to the autoimmune inflammation rather than to variations of thyroid hormones levels, and a status of hypovitaminosis is demonstrated prior of the onset of any thyroid dysfunction. Our study further reinforces the findings from the recent literature and suggests, by speculative inference, that decreased vitamin D may play a pathogenic role in the development of HT, justified by its direct effects on immune cells responses and indirectly by its effects on non-immune cells and stromal components [1–3]. The present study first investigated newly diagnosed HT without treatment with L-T4 and would shed new light on such a controversial issue, already debated in the literature.

Vitamin D modulates immune response through its receptors VDR, and several genetic studies have demonstrated an association between thyroid autoimmunity susceptibility and functional VDR gene polymorphisms in different ethnic populations [20, 22–24, 30]. Such polymorphism of the VDR could lead to a reduction in vitamin D function and, in turn, a reduced inhibitory effect on various steps of the immune response. Of note, some papers did not confirm such a connection, mostly in Caucasians [21, 24, 30]. In our study population, composed of unrelated Caucasian individuals, we also investigated the genotype distribution of three VDR SNPs, BsmI, ApaI, and TaqI, which are in strong linkage disequilibrium with each other, to evaluate if such SNPs may contribute to confer susceptibility to the disease. In line with other studies in Caucasian populations [30], we failed to find out any difference in either genotype or allelic frequencies of any of the SNPs under study in HT patients compared to healthy individuals. Also the frequency of the two most common haplotypes, *BAt and baT,* was similar the two groups. Thus, such VDR polymorphisms cannot be considered determinants for susceptibility to the disease, at least in Caucasians.

In conclusion, vitamin D levels in euthyroid patients with newly diagnosed HT are decreased and significantly correlated with TPOAb levels, clearly indicating a link between vitamin D status and thyroid autoimmunity. No differences in genotype or allele frequencies were observed between HT cases and control subjects for any of the VDR SNPs studied. Our data do not support a role of VDR locus in genetic susceptibility to HT and suggest that the variation in vitamin D levels is more likely to be a risk factor for HT.

Statement of authorship Each Author gave a substantial contribute to the paper and approved the final version to be published.

Compliance with ethical standards

Conflict of interest There is no potential conflict of interest and the Authors have nothing to disclose.

References

1. F. Baeke, T. Takiishi, H. Korf, C. Gysemans, C. Mathieu, Vitamin D: modulator of the immune system. Curr. Opin. Pharmacol. **10**, 482–496 (2010)
2. M. Willheim, R. Thien, K. Schrattbauer, E. Bajna, M. Holub, R. Gruber, K. Baier, P. Pietschmann, W. Reinisch, O. Scheiner, M. Peterlik, Regulatory effects of 1a,25-dihydroxyvitamin D3 on the cytokine production of human peripheral blood lymphocytes. J. Clin. Endocrinol. Metab. **84**, 3739–3744 (1999)
3. N. Tokuda, T. Mano, R.B. Levy, 1,25-Dihydroxyvitamin D3 antagonizes interferon-gamma-induced expression of class II major histocompatibility antigens on thyroid follicular and testicular Leydig cells. Endocrinology **127**, 1419–1427 (1990)
4. T. Takiishi, L. Ding, F. Baeke, I. Spagnuolo, G. Sebastiani, J. Laureys, A. Verstuyf, G. Carmeliet, F. Dotta, T.L. Van Belle, C.A. Gysemans, C. Mathieu, Dietary supplementation with high doses of regular vitamin D3 safely reduces diabetes incidence in nod mice when given early and long-term. Diabetes **63**, 2026–2036 (2014)
5. C. Fournier, P. Gepner, M. Sadouk, J. Charreire, In vivo beneficial effects of cyclosporin A and 1,25-dihydroxyvitamin D3 on the induction of experimental autoimmune thyroiditis. Clin. Immunol. Immunopathol. **54**, 53–63 (1990)
6. J.M. Lemire, D.C. Archer, 1,25-Dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. J. Clin. Invest. **87**, 1103–1107 (1991)
7. N. Sutaria, C.T. Liu, T.C. Chen, Vitamin D status, receptor gene polymorphisms, and supplementation on tuberculosis: a systematic review of case–control studies and randomized controlled trials. J. Clin. Transl. Endocrinol. **1**, 151–160 (2014)
8. L. Adorini, G. Penna, Control of autoimmune diseases by the vitamin D endocrine system. Nat. Clin. Pract. Rheumatol. **4**, 404–412 (2008)
9. G. Tamer, S. Arik, I. Tamer, D. Coksert, Relative vitamin D insufficiency in Hashimoto's thyroiditis. Thyroid **21**, 891–896 (2011)
10. T. Yasuda, Y. Okamoto, N. Hamada, K. Miyashita, M. Takahara, F. Sakamoto, T. Miyatsuka, T. Kitamura, N. Katakami, D. Kawamori, M. Otsuki, T.A. Matsuoka, H. Kaneto, I. Shimomura,

Serum vitamin D levels are decreased in patients without remission of Graves' disease. Endocrine **43**, 230–232 (2013)
11. G. Muscogiuri, S. Palomba, M. Caggiano, D. Tafuri, A. Colao, F. Orio, Low 25 (OH) vitamin D levels are associated with autoimmune thyroid disease in polycystic ovary syndrome. Endocrine (2015). doi:10.1007/s12020-015-0745-0
12. N. Mansournia, M.A. Mansournia, S. Saeedi, J. Dehghan, The association between serum 25OHD levels and hypothyroid Hashimoto's thyroiditis. J. Endocrinol. Invest. **37**, 473–476 (2014)
13. G. Muscogiuri, J. Mitri, C. Mathieu, K. Badenhoop, G. Tamer, F. Orio, T. Mezza, R. Vieth, A. Colao, A. Pittas, Mechanisms in endocrinology: vitamin D as a potential contributor in endocrine health and disease. Eur. J. Endocrinol. **171**, 101–110 (2014)
14. G. Bellastella, M.I. Maiorino, M. Petrizzo, A. De Bellis, A. Capuano, K. Esposito, D. Giugliano, Vitamin D and autoimmunity: what happens in autoimmune polyendocrine syndromes? J. Endocrinol. Invest. **38**, 629–633 (2015)
15. EURODIAB Study Group, Vitamin D supplement in early childhood and risk for type I (insulin-dependent) diabetes mellitus The EURODIAB Substudy 2 Study Group. Diabetologia **42**, 51–54 (1999)
16. J.W. Pike, Vitamin D3 receptors: structure and function in transcription. Annu. Rev. Nutr. **11**, 189–216 (1991)
17. M.A. Pani, M. Knapp, H. Donner, J. Braun, M.P. Baur, K.H. Usadel, K. Badenhoop, Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. Diabetes **49**, 504–507 (2000)
18. B.N. Frederiksen, M. Kroehl, T.E. Fingerlin, R. Wong, A.K. Steck, M. Rewers, J.M. Norris, Association between vitamin D metabolism gene polymorphisms and risk of islet autoimmunity and progression to type 1 diabetes: the diabetes autoimmunity study in the young (DAISY). J. Clin. Endocrinol. Metab. **98**, 1845–1851 (2013)
19. M.A. Pani, J. Seissler, K.H. Usadel, K. Badenhoop, Vitamin D receptor genotype is associated with Addison's disease. Eur. J. Endocrinol. **147**, 635–640 (2002)
20. Y. Ban, M. Taniyama, Y. Ban, Vitamin D receptor gene polymorphism is associated with Graves' disease in the Japanese population. J. Clin. Endocrinol. Metab. **85**, 4639–4643 (2000)
21. J.E. Collins, J.M. Heward, R. Nithiyananthan, S. Nejentsev, J.A. Todd, J.A. Franklyn, S.C. Gough, Lack of association of the vitamin D receptor gene with Graves' disease in UK Caucasians. Clin. Endocrinol. **60**, 618–624 (2004)
22. E. Ramos-Lopez, A. Kurylowicz, T. Bednarczuk, J. Paunkovic, C. Seidl, K. Badenhoop, Vitamin D receptor polymorphisms are associated with Graves' disease in German and Polish but not in Serbian patients. Thyroid **15**, 1125–1130 (2005)
23. W.Y. Lin, L. Wan, C.H. Tsai, R.H. Chen, C.C. Lee, F.J. Tsai, Vitamin D receptor gene polymorphisms are associated with risk of Hashimoto's thyroiditis in Chinese patients in Taiwan. J. Clin. Lab. Anal. **20**, 109–112 (2006)
24. M. Feng, H. Li, S.F. Chen, W.F. Li, F.B. Zhang, Polymorphisms in the vitamin D receptor gene and risk of autoimmune thyroid diseases: a meta-analysis. Endocrine **43**, 318–326 (2013)
25. E.N. Pearce, A.P. Farwell, L.E. Braverman, Thyroiditis. N. Eng. J. Med. **348**, 2646–2655 (2003)
26. M.F. Holick, N.C. Binkley, H.A. Bischoff-Ferrari, C.M. Gordon, D.A. Hanley, R.P. Heaney, M.H. Murad, C.M. Weaver, Endocrine Society: evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J. Clin. Endocrinol. Metab. **96**, 1911–1930 (2011)
27. R.A. Ajjan, A.P. Weetman, Pathogenesis of Hashimoto's thyroiditis: further development in our undestanding. Horm. Metab. Res. **47**, 702–710 (2015)
28. F. Trimarchi, Clinical endocrinology in the near future: a postmodern challenge. J. Endocrinol. Invest. **38**, 95–99 (2015)
29. L.H. Duntas, S. Benvenga, Selenium: an element for life. Endocrine **48**, 756–775 (2015)
30. F. D'Aurizio, D. Villalta, P. Metus, P. Doretto, R. Tozzoli, Is vitamin D a player or not in the pathophysiology of autoimmune thyroid diseases? Autoimmun. Rev. **14**, 363–369 (2015)
31. K.D. Cashman, M. Kiely, Tackling inadequate vitamin D intakes within the population: fortification of dairy products with vitamin D may not be enough. Endocrine **51**, 38–46 (2016)
32. S. Kivity, N. Agmon-Levin, M. Zisappl, Y. Shapira, E.V. Nagy, K. Dankó, Z. Szekanecz, P. Langevitz, Y. Shoenfeld, Vitamin D and autoimmune thyroid diseases. Cell. Mol. Immunol. **8**, 243–247 (2011)
33. O.M. Camurdan, E. Döğer, A. Bideci, N. Celik, P. Cinaz, Vitamin D status in children with Hashimoto thyroiditis. J. Pediatr. Endocrinol. Metab. **25**, 467–470 (2012)
34. A.M. Mackawy, B.M. Al-Ayed, B.M. Al-Rashidi, Vitamin D deficiency and its association with thyroid disease. Int. J. Health. Sci. (Qassim) **7**, 267–275 (2013)
35. N.C. Bozkurt, B. Karbek, B. Ucan, M. Sahin, E. Cakal, M. Ozbek, T. Delibasi, The association between severity of vitamin D deficiency and Hashimoto's thyroiditis. Endocr. Pract. **19**, 479–484 (2013)
36. J. Ma, D. Wu, C. Li, C. Fan, N. Chao, J. Liu, Y. Li, R. Wang, W. Miao, H. Guan, Z. Shan, W. Teng, Lower serum 25-hydroxyvitamin D level is associated with 3 types of autoimmune thyroid diseases. Medicine (Baltimore) **94**, e1639 (2015)
37. E.E. Mazokopakis, M.G. Papadomanolaki, K.C. Tsekouras, A.D. Evangelopoulos, D.A. Kotsiris, A.A. Tzortzinis, Is vitamin D related to pathogenesis and treatment of Hashimoto's thyroiditis? Hell. J. Nucl. Med. **18**, 222–227 (2015)
38. J. Wang, S. Lv, G. Chen, C. Gao, J. He, H. Zhong, Y. Xu, Meta-analysis of the association between vitamin D and aut oimmune thyroid disease. Nutrients **7**, 2485–2498 (2015)
39. G. Effraimidis, K. Badenhoop, J.G.P. Tijssen, W.M. Wiersinga, Vitamin D deficiency is not associated with early stages of thyroid autoimmunity. Eur. J. Endocrinol. **167**, 43–48 (2012)
40. T. Skaaby, L.L. Husemoen, B.H. Thuesen, A. Linneberg, Prospective population-based study of the association between vitamin D status and incidence of autoimmune disease. Endocrine **50**, 231–238 (2015)