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VITAMIN D RECEPTOR GENE POLYMORPHISMS/HAPLOTYPES AND SERUM 25(OH)D_3 LEVELS IN HASHIMOTO'S THYROIDITIS

Dottorando: *Salvatore* GIOVINAZZO

Tutor: *Ch.mo Prof. Salvatore* BENVENGA

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INDEX

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-mediate 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 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 influence 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].

Vitamin D

Vitamin D (D represents D2, D3, or both; Figure 1) is a secosterol produced endogenously in the skin from sun exposure or obtained from foods that naturally contain vitamin D, including cod liver oil and fatty fish (eg, salmon, mackerel, and tuna); UV-irradiated mushrooms; foods fortified with vitamin D; and supplements [25, 26]. During exposure to sunlight, 7-dehydrocholesterol (7-DHC) in the skin is converted to previtamin D3. The 7-DHC is present in all the layers of human skin [26-

28]. Approximately 65% of 7-DHC is found in the epidermis, and greater than 95% of the previtamin D3 that is produced is in the viable epidermis and, therefore, cannot be removed from the skin when it is washed [28]. Once previtamin D3 is synthesized in the skin, it can undergo either a photoconversion to lumisterol, tachysterol, and 7-DHC or a heat-induced membrane enhancedisomerization to vitamin D3 (Figure 1) [26, 27]. The cutaneous production of previtamin D3 is regulated. Solar photoproducts (tachysterol and lumisterol) inactive on calcium metabolism are produced at times of prolonged exposure to solar UVB radiation, thus preventing sun-induced vitamin D intoxication [26, 27]. Vitamin D3 is also sensitive to solar irradiation and is, thereby, inactivated to suprasterol 1 and 2 and to 5,6-trans-vitamin D3 [26]. Cutaneous vitamin D3 production is influenced by skin pigmentation, sunscreen use, time of day, season, latitude, altitude, and air pollution [26, 27, 29].

Once formed, vitamin D3 is ejected out of the keratinocyte plasma membrane and is drawn into the dermal capillary bed by the vitamin D binding protein (DBP).7,8 Vitamin D that is ingested is incorporated into chylomicrons, which are released into the lymphatic system, and enters the venous blood,2,7 where it binds to DBP and lipoproteins transported to the liver.1,2,7 Vitamin D2 and vitamin D3 are 25-hydroxylated by the liver vitamin D-25-hydroxylase (CYP2R1) to produce the major circulating vitamin D metabolite, 25(OH)D, which is used to determine a patient’s

vitamin D status [25, 26, 29]. This metabolite undergoes further hydroxylation by the 25(OH)D-1α-hydroxylase (CYP27B1) in the kidneys to form the secosteroid hormone 1α,25-dihydroxyvitamin D [1,25$(OH)_2$D] (Figure 1) [25, 26]. The 25(OH)D bound to DBP is filtered in the kidneys and is reabsorbed in the proximal renal tubules by megalin cubilin receptors.6,12 The renal 1α-hydroxylation is closely regulated, being enhanced by parathyroid hormone (PTH), hypocalcemia, and hypophosphatemia and inhibited by hyperphosphatemia, fibroblast growth factor-23, and 1,25(OH)2D itself [26].

Vitamin D receptor

The 25$(OH)D_3$ performs many of its biologic functions by regulating gene transcription through a nuclear high-affinity vitamin D receptor (VDR). [30]

The VDR is a transcription factor, member of the steroid hormone nuclear receptor family contains two overlapping ligand binding sites, a genomic pocket and an alternative pocket, that respectively bind a bowl-like ligand configuration (gene transcription) or a planar-like ligand shape (nongenomic rapid responses). (Figure 2)

<u>Genomic actions</u>

VDR is comprised of three domains: the N-terminal DNA binding domain with two zinc fingers that bind to the grooves of the DNA at discrete sites (VDREs), the C-terminal ligand binding domain, and the hinge region binding these two domains together. The ligand binding domain structure is comprised of 12 helices. The terminal helix serves as a gating mechanism closing around the incorporated ligand and forming an interface for coactivators as well as facilitating the interaction of VDR with its heterodimer partner, generally RXR. Although there is substantial variability in the sequence of VDREs, most of those with the highest affinity for VDR are direct repeats of hexanucleotides with a spacing of 3 nt between the half sites, a motif called a DR3. VDR binding to its VDRE then recruits coregulatory complexes required for its genomic activity. These complexes can be both gene and cell specific, enabling the selectivity of 25(OH)D_3 action from cell type to cell type. These complexes include a subunit that directly binds to the VDR generally through an LXXLL motif along with a number of subunits that contain enzyme activity such as histone acetyl transferases (coactivators such as the SRC family) or deacetylases (corepressors such as SMRT and NCoR), methyl transferases and demethylases, ATPasecontaining nucleosomal-remodeling activity (SWI/SNF), and links to RNA polymerase II (Mediator complex). The newer techniques of microarray, ChIP-chip, and ChIP-seq have markedly expanded our understanding of vitamin D mechanism of action at

the genomic level. The profile of VDR binding sites and genes activated varies from cell to cell with some albeit far from total overlap especially when comparing results with different time courses of 25(OH)D_3 exposure [31]. Moreover, these VDR binding sites can be anywhere in the genome, often many thousands of base pairs away from the gene being regulated. These sites are generally found associated with binding sites for other transcription factors. In osteoblasts, these include RUNX2, C/EBPα, and C/EBPβ, among others [32, 33]. These sites often demonstrate a distinct epigenetic histone signature involving methylation and/or acetylation of lysines within H3 and H4 [34]. In their recent review, Pike and Meyer [35] enunciated six principles of VDR/RXR action on target genomes: "1) the number of VDR binding sites on the genome is cell type-specific; 2) the active transcription unit is predominantly, but not exclusively, the VDR/RXR heterodimer; 3) VDR binding sites are predominantly, but not exclusively, classic hexamer half-sites separated by 3 base pairs; 4) enhancers are located promoter-proximal (near), promoter distal (far) or a combination thereof, relative to transcriptional start sites: many enhancers are located in clusters hundreds of kilobases from their target genes; 5) enhancers are modular in nature, containing binding sites for a number of different transcription factors; 6) enhancers that populate a genome are cell type-unique and highly dynamic."

Nongenomic Actions

$25(OH)D_3$ also exerts effects that are too rapid to involve a genomic action. The first of these that was identified involved the rapid stimulation of intestinal calcium transport in a vitamin D replete chick, called transcaltachia [36]. Analogs of $25(OH)D_3$ with little genomic activity were comparable in function to $25(OH)D_3$ with respect to transcaltachia. Other examples emerged including effects on the chondrocytes in the growth plate [36] and keratinocytes in the skin [37]. Identification of the receptor for $25(OH)D_3$ has focused on the VDR itself albeit in a different configuration to enable binding by nongenomic VDR agonists [36, 38] and membrane-associated rapid response steroid binding protein (MARRS), also known as ERp57/GRp58/ERp60 [39]. These receptors are located in the membrane within caveolae/lipid rafts [40] where they are poised to activate kinases, phosphatases, and ion channels. The crystallographic form of VDR would indicate that it can accommodate only agonists with a 6-s-trans configuration, yet those agonists specific for the rapid responses are in a 6-s-cis configuration. However, a model of the VDR has been proposed with an alternative ligand pocket that can accommodate the 6-s-cis analogs [36, 38]. Crystallographic evidence for this configuration has not been obtained. In the three examples mentioned above, both the MARRS and VDRs have been implicated, and in the skin, both receptors

were found to be involved in the same study examining photoprotection [37].

Thus, the panoply of pathways now known to be regulated by 25(OH)D_3 opens up a large selection of targets for clinical application, with the proviso that functional selectivity can be achieved to match what the cell-specific genomic selectivity would seem to promise. This realization has spawned great interest in developing 25(OH)D_3 analogs to do just that.

Hashimoto's thyroiditis (HT)

HT is the most prevalent autoimmune disease, as well as the most common endocrine disorder, affecting about 2% of the general population, predominantly women [41]. It was initially described in 1912, but only rarely reported until the early 1950s. HT have an autoimmune etiology, that develops as a result of interactions between genetic susceptibility and further affecting environmental factors. Among the genes predisposing to the disease, the best confirmed are Human Leukocyte Antigen region genes (HLA), cytotoxic T lymphocyte antigen-4 (CTLA-4), protein tyrosine phosphatase nonreceptor-type 22 (PTPN22) and thyroglobulin (Tg) genes. (Figure 3) Recently, the significance of polymorphisms in cytokine gene sequences was also investigated [42, 43, 44]. According to twin studies, genetic factors are responsible for the disease's development to a degree of about 75% [45]. Environmental factors that may impact HT development

are viral and bacterial infections, medications (e.g. interferon α, amiodarone), iodine excess and also an inappropriate supply of other trace elements or vitamins [46].

Lately, the relationship between vitamin D insufficiency and the diseases of autoimmune origin has become the focus of interest.

AIM

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 (Figure 4), 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 yr) and 100 age- and sex-matched healthy individuals (12 men and 88 women, aged 19-69 yr) 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) [47]. 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 and thyroid autoantibodies 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 1450xg 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, 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; 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 [48], 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 7.5MHz 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'CAACCAAGACTACAAGTACCGCG3', Reverse 5'AACCAGCGGGAAGAGGTCAAGGG3'; Forward 5'CAGAGCATGGACAGGGAGCAA3', Reverse 5'GCAACTCCTCATGGCTGAGGTCTC3' for both ApaI e TaqI. The PCR conditions were 5 min at 94°C for initial denaturation, 30 sec at 94°C, 40 sec 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, TaqI (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions, and electrophoresed on 2% agarose gel stained with GelRed (Biotium, Hayward, CA). (Figure 5) 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 α=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) (Figure 6). In HT patients, $25(OH)D_3$ levels were significantly correlated with serum TPOAb (r = -0.669; p=0.034) (Figure 7, 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 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 (Figure 7, 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 [49, 50]. The number of environmental triggers potentially involved in the 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 [49, 51, 52].

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 recognised worldwide [13, 53]. 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 and co-workers [52]. The Authors of the review also provided personal data on the issue and showed no differences in vitamin D levels 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 [52]. 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, 54-59], and such a relationship with thyroid autoimmunity clearly emerged in a recent meta-analysis of the pertinent literature by Wang et al [60]. Vitamin D deficiency was significantly related to anti-thyroid antibodies [54, 55, 59] and to thyroid dysfunction [9, 10, 54, 56, 58]. Recently, Ma et al 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 risk for developing AITD will be [58]. 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, 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 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 [49, 52]. The sole longitudinal study, conducted by Effraimidis et al. 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-yr duration follow-up, concluding that very early stages of thyroid autoimmunity are not associated with low vitamin D levels [61] More recently, a prospective population-based study, including 12,555 individuals from Denmark with a median follow-up time of 10.8 yr, demonstrated a statistically significant inverse association between vitamin D status and appearance of any autoimmune disease [62]. Although not focused on thyroid diseases, this study clearly suggest a primary role of vitamin D in contributing to the development of autoimmune disorders [62].

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/o replacement therapy were avoided. Prior of us, only Bozkurt and co-workers 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 [57]. 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 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 [57]. More recently, Mazokopakis et al 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 [59].

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 reinforce findings from the recent literature, and suggest, 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 LT4, 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, 52]. 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, 52]. 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 [52], 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.

CONCLUSIONS

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 the variation in vitamin D levels is more likely to be a risk factor for HT.

REFERENCES

1. Baeke, F., Takiishi, T., Korf, H., Gysemans, C., Mathieu, C.: Vitamin D: modulator of the immune system. Curr. Opin. Pharmacol. 10, 482–496 (2010).
2. Willheim, M., Thien, R., Schrattbauer, K., Bajna, E., Holub, M., Gruber, R., Baier, K., Pietschmann, P., Reinisch, W., Scheiner, O., Peterlik, M.: 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. Tokuda, N., Mano, T., Levy, R.B.: 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. Takiishi, T., Ding, L., Baeke, F., Spagnuolo, I., Sebastiani, G., Laureys, J., Verstuyf, A., Carmeliet, G., Dotta, F., Van Belle, T.L., Gysemans, C.A., Mathieu, C.: 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. Fournier, C., Gepner, P., Sadouk, M., Charreire, J.: 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. Lemire, J.M., Archer, D.C.: 1,25-Dihydroxyvitamin D3 prevents the in vivo induction of murine experimental autoimmune encephalomyelitis. J. Clin. Invest. 87, 1103–1107 (1991).
7. Sutaria, N., Liu, C.T., Chen, T.C.: 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. Adorini, L., Penna, G.: Control of autoimmune diseases by the vitamin D endocrine system. Nat. Clin. Pract. Rheumatol. 4, 404-412 (2008).
9. Tamer, G., Arik, S., Tamer, I., Coksert, D.: Relative vitamin D insufficiency in Hashimoto's thyroiditis. Thyroid. 21, 891–896 (2011).
10. Yasuda, T., Okamoto, Y., Hamada, N., Miyashita, K., Takahara, M., Sakamoto, F., Miyatsuka, T., Kitamura, T., Katakami, N., Kawamori, D., Otsuki, M., Matsuoka, T.A., Kaneto, H., Shimomura, I.: Serum vitamin D levels are decreased in patients without remission of Graves' disease. Endocrine. 43, 230-232 (2013).
11. Muscogiuri, G., Palomba, S., Caggiano, M., Tafuri, D., Colao, A., Orio, F.: Low 25 (OH) vitamin D levels are associated with

autoimmune thyroid disease in polycystic ovary syndrome. Endocrine. 1-5 (2015).

12. Mansournia, N., Mansournia, M.A., Saeedi, S., Dehghan, J.: The association between serum 25OHD levels and hypothyroid Hashimoto's thyroiditis. J. Endocrinol. Invest. 37, 473-476 (2014).
13. Muscogiuri, G., Mitri, J., Mathieu, C., Badenhoop, K., Tamer, G., Orio, F., Mezza, T., Vieth, R., Colao, A., Pittas, A.: Mechanisms in endocrinology: vitamin D as a potential contributor in endocrine health and disease. Eur. J. Endocrinol. 171, 101-110 (2014).
14. Bellastella, G., Maiorino, M.I., Petrizzo, M., De Bellis, A., Capuano, A., Esposito, K., Giugliano, D.: 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. Pike, J.W.: Vitamin D3 receptors: structure and function in transcription. Annu. Rev. Nutr. 11, 189–216 (1991).
17. Pani, M.A., Knapp, M., Donner, H., Braun, J., Baur, M.P., Usadel, K.H., Badenhoop, K.: Vitamin D receptor allele combinations

influence genetic susceptibility to type 1 diabetes in Germans. Diabetes. 49, 504-507 (2000).

18. Frederiksen, B.N., Kroehl, M., Fingerlin, T.E., Wong, R., Steck, A.K., Rewers, M., Norris, J.M.: 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. Pani, M.A., Seissler, J., Usadel, K.H., Badenhoop, K.: Vitamin D receptor genotype is associated with Addison's disease. Eur. J. Endocrinol. 147, 635-640 (2002).
20. Ban, Y., Taniyama, M., Ban, Y.: Vitamin D receptor gene polymorphism is associated with Graves' disease in the Japanese population. J. Clin. Endocrinol. Metab. 85, 4639–4643 (2000).
21. Collins, J.E., Heward, J.M., Nithiyananthan, R., Nejentsev, S., Todd, J.A., Franklyn, J.A., Gough, S.C.: Lack of association of the vitamin D receptor gene with Graves' disease in UK Caucasians. Clin. Endocrinol. 60, 618–624 (2004).
22. Ramos-Lopez, E., Kurylowicz, A., Bednarczuk, T., Paunkovic, J., Seidl, C., Badenhoop, K.: Vitamin D receptor polymorphisms are associated with Graves' disease in German and Polish but not in Serbian patients. Thyroid. 15, 1125-1130 (2005).

23. Lin, W.Y., Wan, L., Tsai, C.H., Chen, R.H., Lee, C.C., Tsai, F.J.: 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. Feng, M., Li, H., Chen, S.F., Li, W.F., Zhang, F.B.: Polymorphisms in the vitamin D receptor gene and risk of autoimmune thyroid diseases: a meta-analysis. Endocrine. 43, 318–326 (2013).
25. Hossein-Nezhad, A., Holick, M.F.: Optimize dietary intake of vitamin D: an epigenetic perspective. Curr. Opin. Clin. Nutr. Metab. Care. 6, 567–579 (2012).
26. Holick, M.F.: Vitamin D deficiency. N. Engl. J. Med. 3, 266–281 (2007).
27. Holick, M.F.: Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am. J. Clin. Nutr. 6, 1678S–1688S (2004).
28. Holick, M.F., MacLaughlin, J.A., Clark, M.B., Holick, S.A., Potts, J.T. Jr., Anderson, R.R., Blank, I.H., Parrish, J.A., Elias, P.: Photosynthesis of previtamin D3 in human skin and the physiologic consequences. Science. 4466, 203–205 (1980).
29. Holick, M.F.: Vitamin D: extraskeletal health. Rheum. Dis. Clin. North. Am. 1:141–160 (2012).

30. Nagpal, S., Na, S., Rathnachalam, R.: Noncalcemic actions of vitamin D receptor ligands. Endocr. Rev. 5, 662–687 (2005).

31. Carlberg, C., Seuter, S., Heikkinen, S.: The first genome-wide view of vitamin D receptor locations and their mechanistic implications. Anticancer. Res. 32, 271–282 (2012).

32. Zella, L.A., Meyer, M.B., Nerenz, R.D., Lee, S.M., Martowicz, M.L., Pike, J.W.: Multifunctional enhancers regulate mouse and human vitamin D receptor gene transcription. Mol. Endocrinol.24, 128–147 (2010).

33. Meyer, M.B., Goetsch, P.D., Pike, J.W.: VDR/RXR and TCF4/β-catenin cistromes in colonic cells of colorectal tumor origin: impact on c-FOS and c-MYC gene expression. Mol. Endocrinol. 26, 37–51 (2012).

34. Ernst, J., Kheradpour, P., Mikkelsen, T.S., Shoresh, N., Ward, L.D., Epstein, C.B., Zhang, X., Wang, L., Issner, R., Coyne, M., Ku, M., Durham, T., Kellis, M., Bernstein, B.E.: Mapping and analysis of chromatin state dynamics in nine human cell types. Nature. 473, 43–49 (2011).

35. Meyer, M.B., Goetsch, P.D., Pike, J.W.: Genome-wide analysis of the VDR/RXR cistrome in osteoblast cells provides new mechanistic insight into the actions of the vitamin D hormone. J. Steroid. Biochem. Mol. Biol. 121, 136–141 (2010).

36. Norman, A.W., Okamura, W.H., Hammond, M.W., Bishop, J.E., Dormanen, M.C., Bouillon, R., van Baelen, H., Ridall, A.L., Daane, E., Khoury, R., Farach-Carson, M.C.: Comparison of 6-s-cis- and 6-s-trans-locked analogs of 1alpha,25-dihydroxyvitamin D3 indicates that the 6-s-cis conformation is preferred for rapid nongenomic biological responses and that neither 6-scis- nor 6-s-trans-locked analogs are preferred for genomic biological responses. Mol. Endocrinol. 11, 1518–1531 (1997).
37. Sequeira, V.B., Rybchyn, M.S., Tongkao-On, W., Gordon-Thomson, C., Malloy, P.J., Nemere, I., Norman, A.W., Reeve, V.E., Halliday, G.M., Feldman, D., Mason, R.S.: The role of the vitamin D receptor and ERp57 in photoprotection by 1a,25-dihydroxyvitamin D3. Mol. Endocrinol. 26, 574–582 (2012).
38. Mizwicki, M.T., Norman, A.W.: The vitamin D sterol-vitamin D receptor ensemble model offers unique insights into both genomic and rapid-response signaling. Sci. Signal. 2, re4 (2009).
39. Nemere, I., Farach-Carson, M.C., Rohe, B., Sterling, T.M., Norman, A.W., Boyan, B.D., Safford, S.E.: Ribozyme knockdown functionally links a 1,25(OH)2D3 membrane binding protein (1,25D3-MARRS) and phosphate uptake in intestinal cells. Proc. Natl. Acad. Sci. USA.101, 7392–7397 (2004).

40. Huhtakangas, J.A., Olivera, C.J., Bishop, J.E., Zanello, L.P., Norman, A.W.: The vitamin D receptor is present in caveolae-enriched plasma membranes and binds 1 alpha,25(OH)2-vitamin D3 in vivo and in vitro. Mol. Endocrinol. 18, 2660–2671 (2004).
41. Tunbridge, W.M., Vanderpump, M.P.: Population screening for autoimmune thyroid disease. Endocrinol. Metab. Clin. North. Am. 29, 239–253 (2000).
42. Zaletel, K., Gaberšček, S.: Hashimoto's Thyroiditis: From Genes to the Disease. Curr. Genomics. 12,576–588 (2011).
43. Ruggeri, R.M., Saitta, S., Cristani, M., Giovinazzo, S., Tigano, V., Trimarchi, F., Benvenga, S., Gangemi, S.: Serum interleukin-23 (IL-23) is increased in Hashimoto's thyroiditis. Endocr. J. 4, 359-63 (2014).
44. Ruggeri, R.M., Minciullo, P., Saitta, S., Giovinazzo, S., Certo, R., Campennì, A., Trimarchi, F., Gangemi, S., Benvenga, S.: Serum interleukin-22 (IL-22) is increased in the early stage of Hashimoto's thyroiditis compared to non-autoimmune thyroid disease and healthy controls. Hormones (Athens). 3, 338-344 (2014).
45. Brix, T.H., Hegedüs, L.: Twins as a tool for evaluating the influence of genetic susceptibility in thyroid autoimmunity. Ann, Endocrinol, (Paris), 72,103–107 (2011).

46. Eschler, D.C., Hasham, A., Tomer, Y.: Cutting edge: the etiology of autoimmune thyroid diseases. Clin, Rev. Allergy. Immunol. 41, 190–197 (2011).

47. Pearce, E.N., Farwell, A.P., Braverman, L.E.: Thyroiditis. N. Eng. J. Med. 348, 2646–2655 (2003).

48. Holick, M.F., Binkley, N.C., Bischoff-Ferrari, H.A., Gordon, C.M., Hanley, D.A., Heaney, R.P., Murad, M.H., Weaver, C.M., Endocrine Society: Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J. Clin. Endocrinol. Metab. 96, 1911–1930 (2011).

49. Ajjan, R.A., Weetman, A.P.: Pathogenesis of Hashimoto's Thyroiditis: Further Development in our Undestanding. Horm. Metab. Res 47, 702–710 (2015).

50. Trimarchi, F.: Clinical Endocrinology in the near future: a post-modern challenge. J. Endocrinol. Invest. 38, 95-99 (2015).

51. Duntas, L.H., Benvenga, S.: Selenium: an element for life. Endocrine. 48, 756-775 (2015).

52. D'Aurizio, F., Villalta, D., Metus, P., Doretto, P., Tozzoli, R.: Is vitamin D a player or not in the pathophysiology of autoimmune thyroid diseases? Autoimmun. Rev. 14, 363-369 (2015).

53. Cashman, K.D., Kiely, M.: Tackling inadequate vitamin D intakes within the population: fortification of dairy products with vitamin D may not be enough. Endocrine. 51, 38-46 (2016).

54. Kivity, S., Agmon-Levin, N., Zisappl, M., Shapira, Y., Nagy, E.V., Dankó, K., Szekanecz, Z., Langevitz, P., Shoenfeld, Y.: Vitamin D and autoimmune thyroid diseases. Cell. Mol. Immunol. 8, 243–247 (2011).

55. Camurdan, O.M., Döğer, E., Bideci, A., Celik, N., Cinaz, P.: Vitamin D status in children with Hashimoto thyroiditis. J. Pediatr. Endocrinol. Metab. 25, 467–470 (2012).

56. Mackawy, A.M., Al-Ayed, B.M., Al-Rashidi, B.M.: Vitamin D deficiency and its association with thyroid disease. Int. J. Health. Sci. (Qassim). 7, 267–275 (2013).

57. Bozkurt, N.C., Karbek, B., Ucan, B., Sahin, M., Cakal, E., Ozbek, M., Delibasi, T.: The association between severity of vitamin D deficiency and Hashimoto's thyroiditis. Endocr. Pract. 19, 479–484 (2013).

58. Ma, J., Wu, D., Li, C., Fan, C., Chao, N., Liu, J., Li, Y., Wang, R., Miao, W., Guan, H., Shan, Z., Teng, W. Lower Serum 25-Hydroxyvitamin D Level is Associated With 3 Types of Autoimmune Thyroid Diseases. Medicine (Baltimore). 94: e1639 (2015).

59. Mazokopakis, E.E., Papadomanolaki, M.G., Tsekouras, K.C., Evangelopoulos, A.D., Kotsiris, D.A., Tzortzinis, A.A. Is vitamin D related to pathogenesis and treatment of Hashimoto's thyroiditis? Hell J Nucl Med. 18:222-227 (2015)..
60. Wang, J., Lv, S., Chen, G., Gao, C., He, J., Zhong, H., Xu, Y. Meta-analysis of the association between vitamin D and aut oimmune thyroid disease. Nutrients. 7:2485-98 (2015).
61. Effraimidis G, Badenhoop K, Tijssen JGP, Wiersinga WM. Vitamin D deficiency is not associated with early stages of thyroid autoimmunity. Eur J Endocrinol 2012; 167:43–48.
62. Skaaby, T., Husemoen, L.L., Thuesen, B.H., Linneberg, A.: Prospective population-based study of the association between vitamin D status and incidence of autoimmune disease. Endocrine. 50, 231-238 (2015).

FIGURES and TABLES

Figure 1. Vitamin D

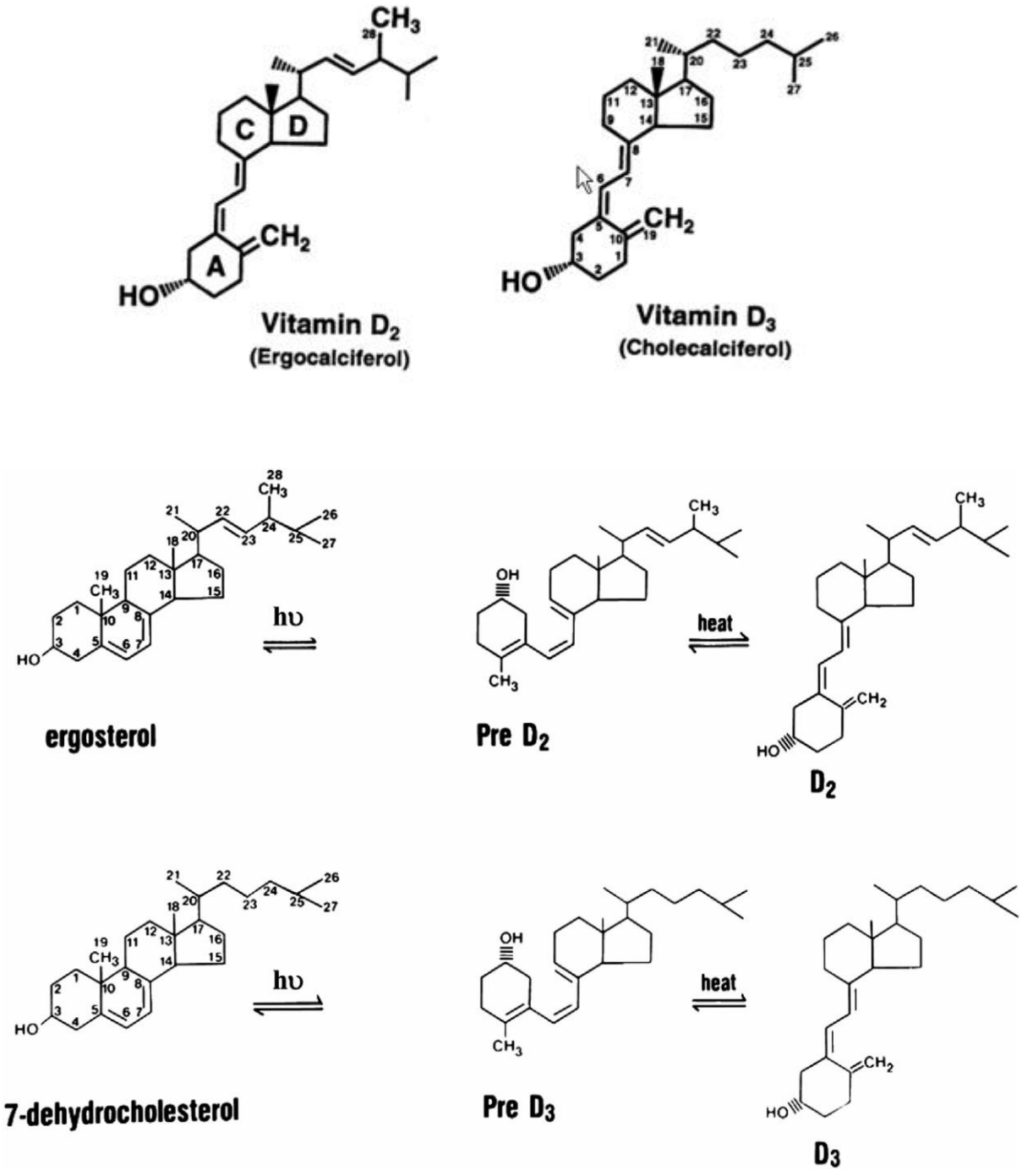


Figure 2. Vitamin D receptor

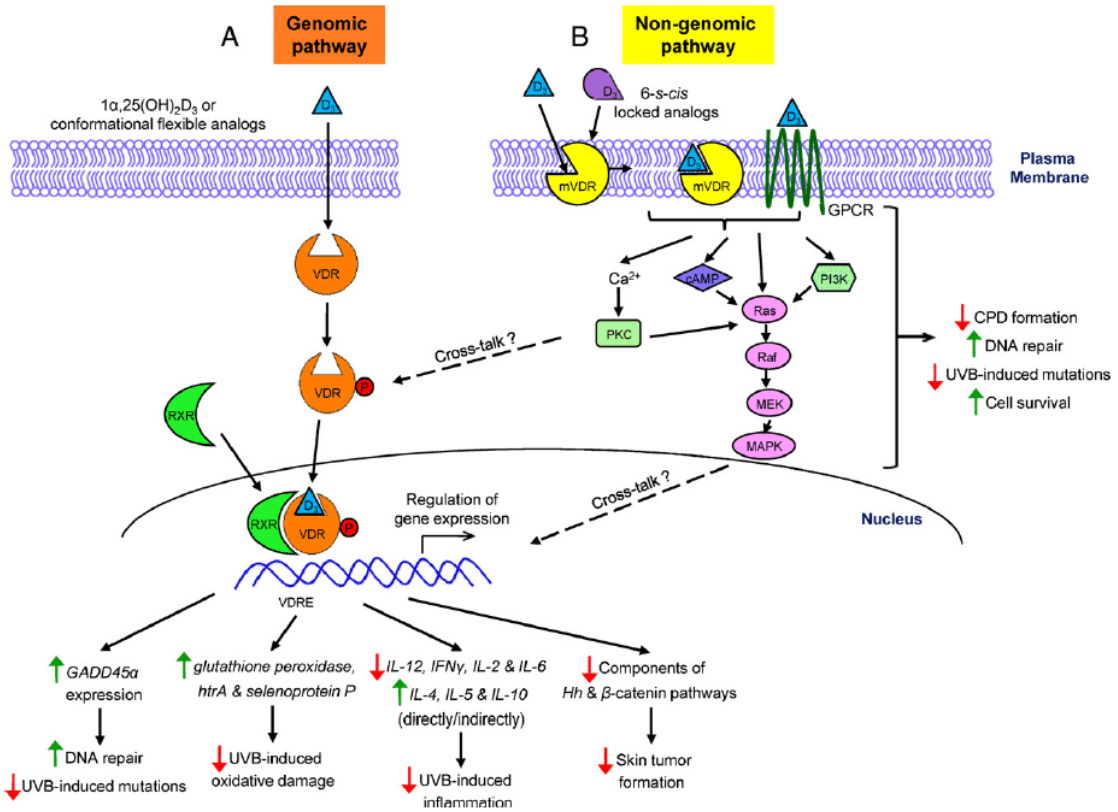


Figure 3. Schematic representation of the autoimmune events of HT

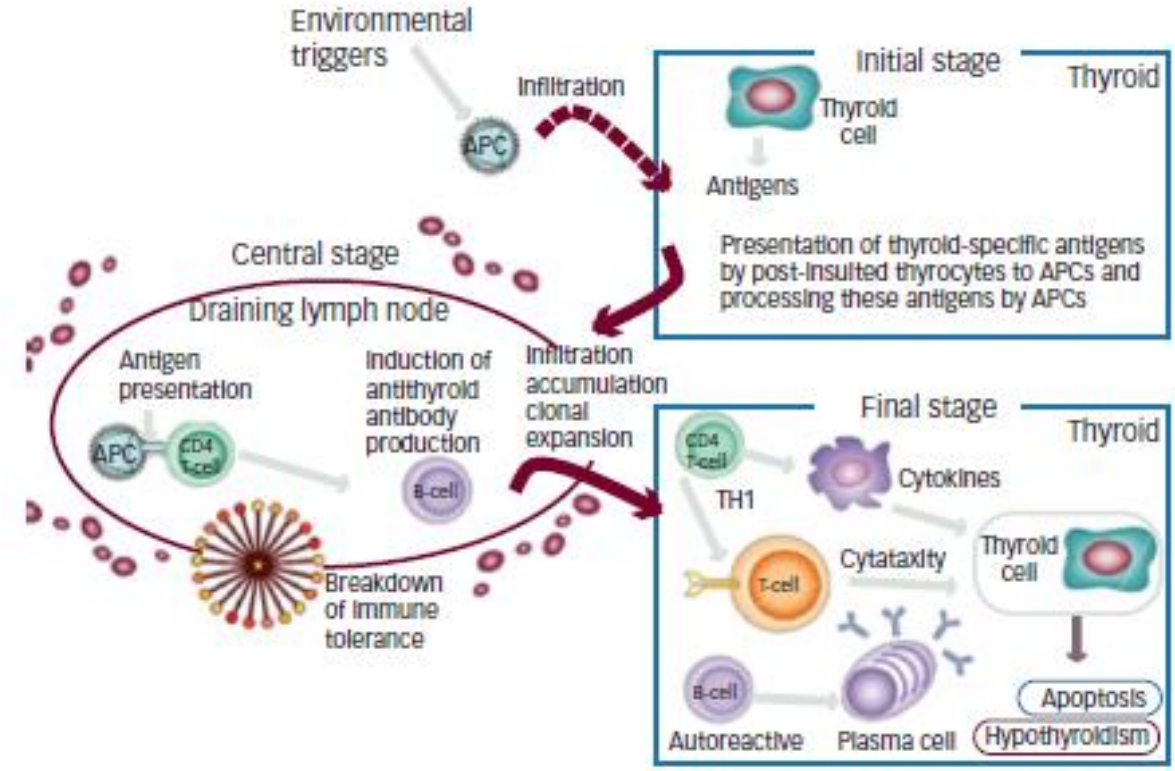


Figure 4. VDR gene polymorphisms

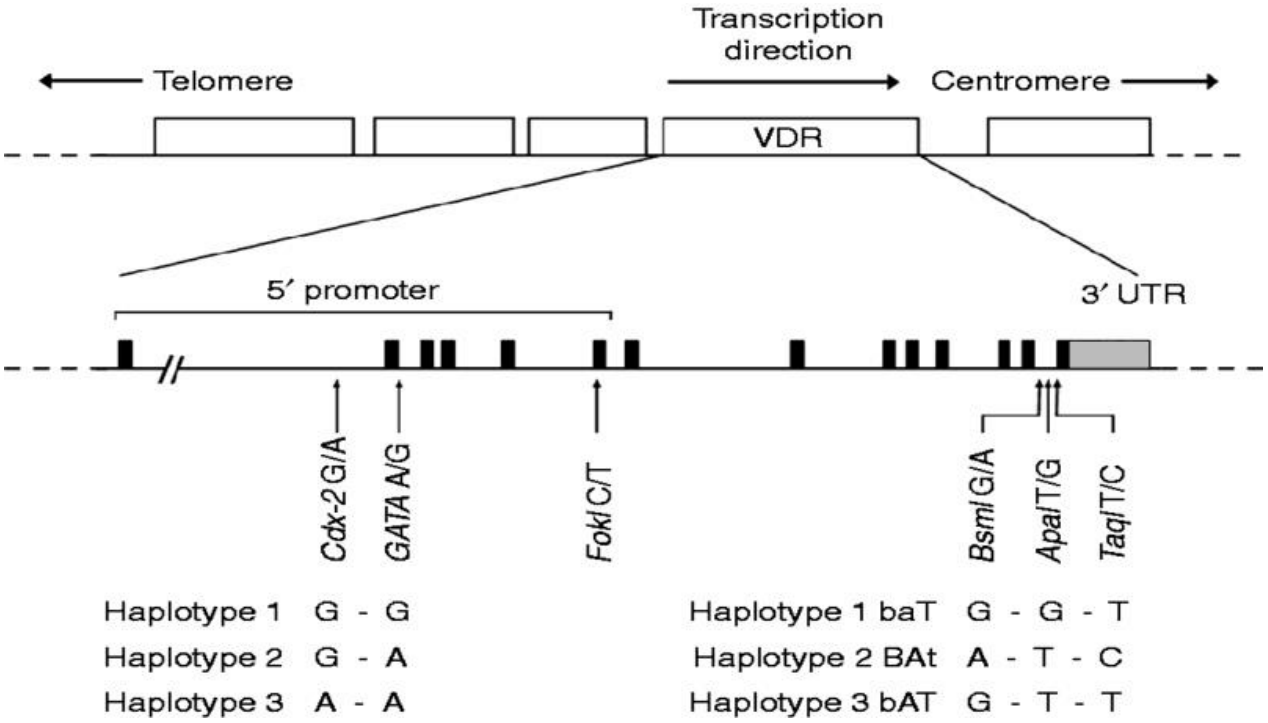
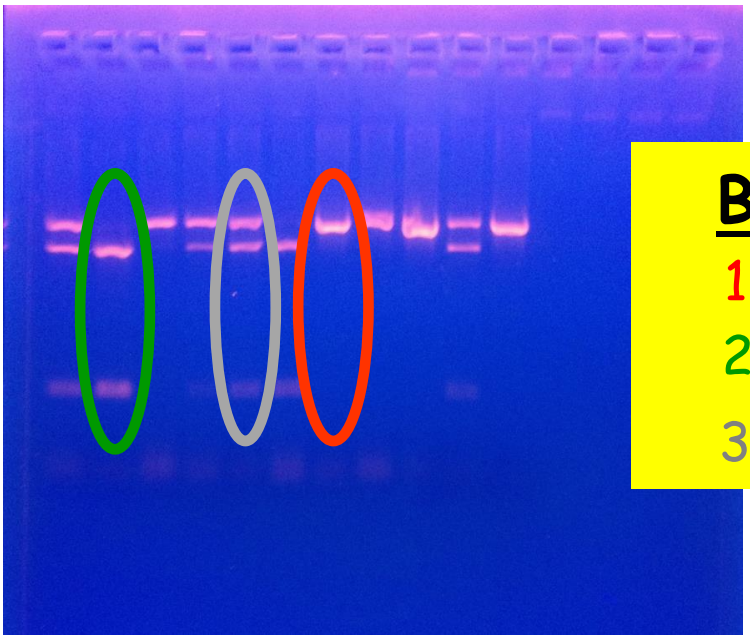
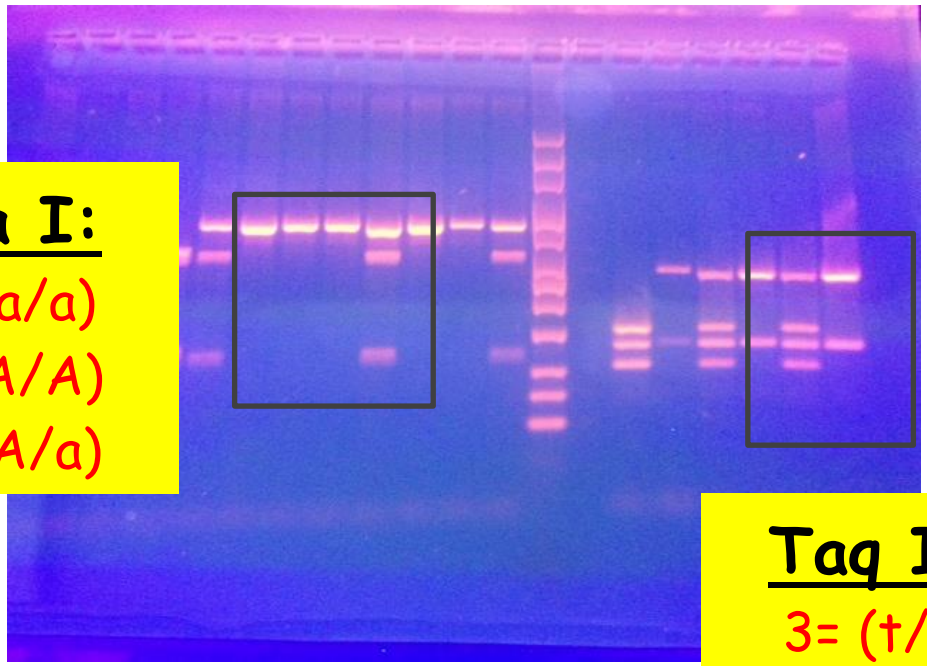


Figure 5. Gel Agarose 2%



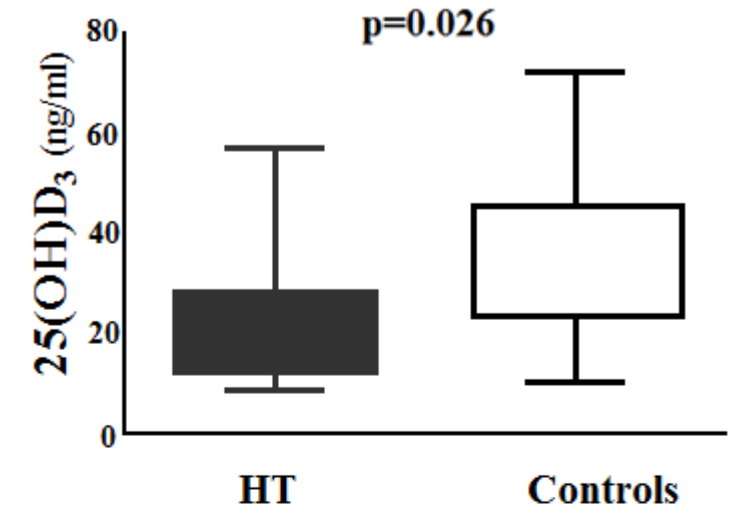
BSM I:
1= (B/B)
2= (b/b)
3= (B/b)



Apa I:
2= (a/a)
1= (A/A)
3= (A/a)

Taq I:
3= (t/t)
2= (T/T)
4= (T/t)

Figure 6. 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)



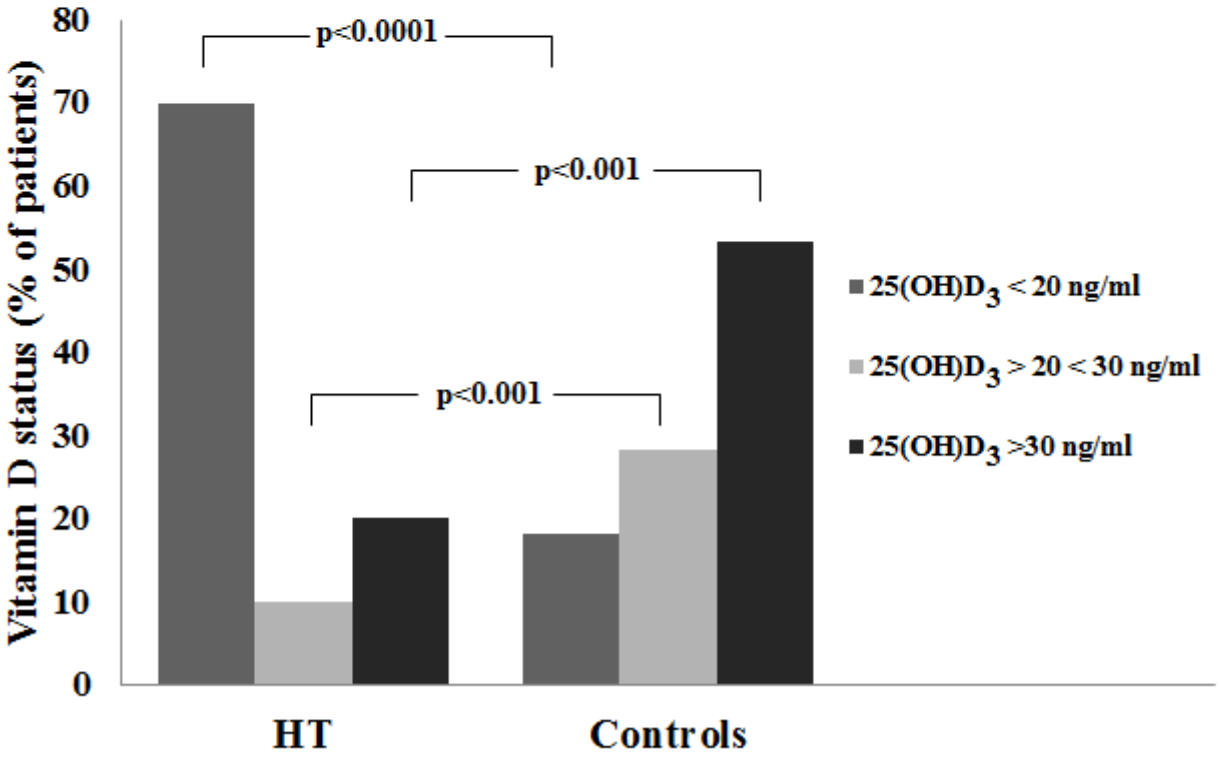


Figure 7. Correlation between serum levels of 25(OH)D_3 and serum levels of TPOAb (panel A), fasting insulin (panel B) and high density lipoprotein (HDL) cholesterol (panel C)

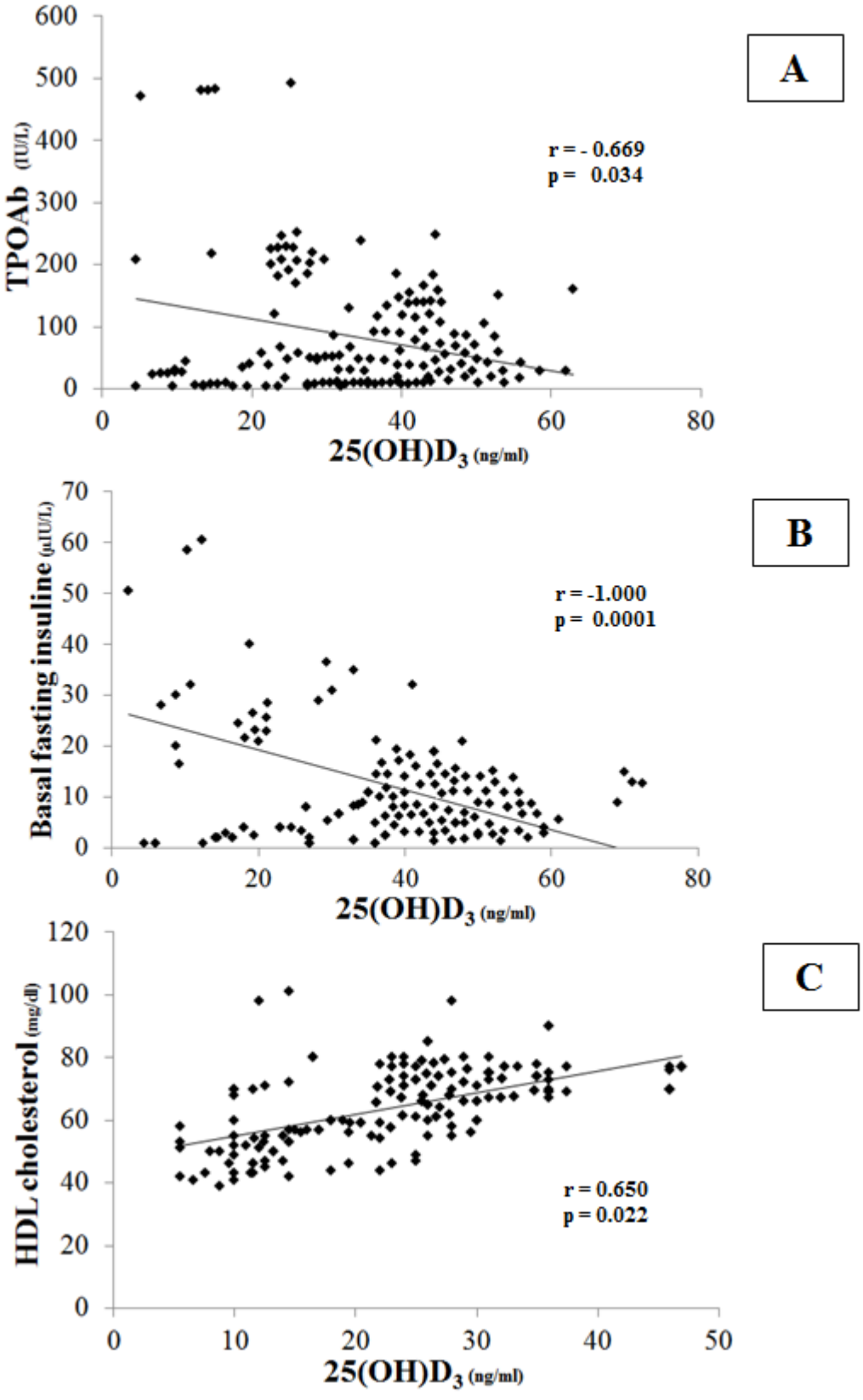


Table 1. Demographic, clinical and biochemical characteristics of our study population*

	HT PATIENTS	HEALTHY CONTROLS	P
Total no. of patient	100	100	
Sex Male Female	 13 87	 12 88	0.831
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 (2.2-4.7)	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.
[¶] WC, waist circumference, measured midway between lower costal margin and iliac crest at the end of normal expiration; WHR, waist hip ratio, calculated by the formula waist circumference (cm)/hip circumference (cm).
[§] Insulin resistance was estimated by the homeostatic model assessment index (HOMA).
[^] Thyroid volume was evaluated by US as specified under Material and Methods.

Table 2. Genotype and allelic frequencies of the three SNPs under study in patients affected by Hashimoto's thyroidits (HT) *vs* 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.

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 Material and Methods.