STUDY OF A POLYMORPHISM IN THE PROMOTER REGION OF THE *SEPS1* GENE AND RISK OF AUTOIMMUNE THYROID DISEASES

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"You are never given a wish without also being given the power to make it come true. You may have to work for it, however." (Richard Bach)

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LIST OF ABBREVIATIONS

AITD – Autoimmune thyroid diseases

ER - Endoplasmic reticulum

GD - Graves' disease

HT - Hashimoto's thyroiditis

IL-1β -Interleukin 1 beta

IL-6-Interleukin 6

LATS- Long acting thyroid stimulator antibodies

OR - Odds ratio

CRP – c reactive protein

QTLs - Quantitative trait loci

NFκB – Nuclear Kappa-B factor

SEPS1 - Selenoprotein S gene

SNP- Single nucleotide polymorphism

TNF-α- Tumour necrosis factor alpha

Tg- Thyroglobulin

TPO – Thyroid peroxidase

Trab - TSH-receptor antibodies

TSH- Thyroid stimulating hormone

T3 – Hormone triiodothyronine

T4 -Thyroxine

RESUMO

Introdução & Objetivos: A doença de Graves (GD) e a Tiroidite de Hashimoto (HT) representam os dois extremos de uma doença que é caracterizada pela resposta imune contra a glândula tiroide levando, respetivamente, a uma estimulação autoimune do recetor da hormona estimuladora da tiroide e/ou à destruição das células foliculares. O gene da Selenoproteina S (*SEPS1*) está envolvido na regulação da resposta inflamatória e proteção do dano oxidativo. A associação entre o selénio e a inflamação sugere um papel das selenoproteinas na patogénese das doenças autoimunes da tiróide (AITD). O objetivo deste estudo é determinar o papel da variação genética polimórfica no gene *SEPS1* e o risco de desenvolvimento de doenças autoimunes da tiroide.

Material e Métodos: Foi efetuado um estudo caso-controlo (n=1111 indivíduos compreendendo 512 HT, 83 GD e 516 controlos não relacionados de Portugal) para estudar a associação entre o SNP- *SEPS1* (rs28665122) e AITD. A variação genética foi discriminada por TaqMan-PCR.

Resultados: A variação na região promotora *SEPS1* foi detetada significativamente mais frequente em doentes com AITD relativamente à população controlo. O genótipo *SEPS1* GA está associado com um risco quase 3 vezes superior para desenvolver GD (OR=2.81, CI=1.69-4.68, *p-value* $=6.9\text{x}10^{-5}$).Nos doentes com HT o genótipo *SEPS1* GA representa um risco 2,26 vezes superior (OR=2.26, CI=2.68-3.05, *p-value* $<1\text{x}10^{-6}$) e o alelo A encontrou-se numa proporção estatisticamente superior aos controlos (CI=1.69-2.99; *p-value* $<1\text{x}10^{-6}$).

Conclusão: Os nossos resultados evidenciam uma possível ligação entre as variantes genéticas da selenoproteina S e o risco de AITD.

Palavras-chave: Doenças autoimunes da tiróide, Tiroidite de Hashimoto, Doença de Graves, susceptibilidade, selenoproteinas

ABSTRACT

Background & Aims: Graves' disease (GD) and Hashimoto's thyroiditis (HT) represent the two ends of a wide spectrum of a disease where an immune response directed against the thyroid gland leads to an autoimmune stimulation of the thyroid stimulating hormone receptor or to the follicular cells destruction, respectively. Selenoprotein S (*SEPS1*) is a gene involved in the regulation of inflammatory response and protection from oxidative damage. The association between selenium and inflammation have directed attention to the role of selenoproteins in the pathogenesis of autoimmune thyroid diseases (AITD). The aim of this study was to determine the role of this genetic variation in the risk for autoimmune thyroid diseases.

Methods: We used the case-control design population (n=1111 individuals comprising 512 Hashimoto's thyroiditis, 83 Graves' disease patients and 516 unrelated controls from Portugal) to study the association of the *SEPS1* rs28665122 single nucleotide polymorphism with AITD. Genetic variants were discriminated by PCR using TaqMan SNP genotyping assays.

Results: *SEPS1* promoter variant was detected more frequently in patients with AITD than in control individuals The *SEPS1* GA genotype is associated with almost 3 fold increased risk to GD (OR=2.81, CI=1.69-4.68, *p-value* =$6.9x10^{-5}$). In the HT patients *SEPS1* GA represents a 2.26 increased risk (OR=2.26, CI=2.68-3.05, *p-value* <$1x10^{-6}$) and of the A allele *carrier* were found in higher statistically significant proportion when compared with control population (CI=1.69-2.99; *p-value* <$1x10^{-6}$).

Conclusions: Our findings highlight potential link between selenoprotein S genetic variation and AITD risk.

Key words: Autoimmune thyroid disease, Hashimoto thyroiditis, Graves' disease, susceptibility, Selenoproteins

INTRODUCTION

In 1956, Adams and Purves discovered long acting thyroid stimulator (LATS) in serum of patients with Graves' disease. This was the first description of thyroid stimulating autoantibodies [1]. In the same year, Roitt and Doniach described for the first time antibodies against thyroglobulin in serum of Hashimoto disease patients [2]. Also in this year, Rose and Witebsky created an animal model of hypothyroidism by immunization of rabbits with thyroid homogenate [3,4]. The concept of autoimmune thyroid disease (AITD) was born.

The thyroid gland is the organ most commonly affected by autoimmune disease [4,5]. Graves' disease and Hashimoto's thyroiditis represent the two ends of a disease with a wide spectrum [4,7,8]. In AITD, the immune system produces autoantibodies that attack the thyroid gland either stimulating thyroid cells to produce an excess of thyroid hormone or destroying thyroid hormone producing cells, respectively, in Graves' disease – hyperthyroidism (GD) and Hashimoto's thyroiditis (HT) – hypothyroidism [9,10].

In GD, when hyperthyroidism emerges, the patient complains of loss of energy, severe weight loss, excessive perspiration, palpitations, and accelerated bowel activity with diarrhea, goiter, and agitation. Extrathyroidal manifestations of GD include Graves ophthalmopathy (GO) and dermatopathy (pretibial myxedema) with little understanding of the cause of these disease components. In the other hand, in HT associated hypothyroidism, patient complains of hoarsening of voice, loss of energy, loss of hair, weight gain, dry and thick skin, and intolerance for low temperature.

Clinically, overt AITD affect 1-2% of the world population and, as the great majority of autoimmune diseases, the prevalence of AITD is 6 to 8 times higher in females than in males [8,11]. AITD has a tendency to cluster within families but both, Graves' and Hashimoto's diseases may occur in the same family [12]. Relatives of affected individuals have a higher risk of developing AITD than the general population [13]. Twin studies suggest that genetic factors account for about 70% of the risk to develop AITD and consequently the

leaving 30% are responsibility of environmental factors [14, 15]. Studies using animal models have concluded that AITD should be regarded as a polygenic disease that is strongly influenced by environmental factors [16].

With respect to a genetic contribution to the inflammatory response seen in AITD, attention has been focused on polymorphisms in the proinflammatory cytokine genes [17,18]. *IL1*β, IL6, TNFα cytokines and INFγR1 have proinflammatory activity and are directly involved in the immunologic process seen in AITD [18].

Curran *et al* reported that genetic variation in the newly discovered Selenoprotein S gene (*SEPS1*) was strongly associated with circulating levels of cytokines. Selenoprotein S (*SEPS1*, also known as *SelS, SELENOS, VIMP*) is a novel selenoprotein located in the endoplasmic reticulum (ER) and the plasma membrane [19]. The human gene *SEPS1* is located on chromosome 15q26.3. This region is shown to contain QTLs influencing inflammatory disorders [20-23].

The gene encodes a protein involved in the control of the inflammatory response in the ER by retro-translocation of misfolded proteins from the ER lumen to cytosol for degradation through the proteasome [24]. *SEPS1* protects cells from oxidative damage and apoptosis, and is widely expressed in a variety of tissues, inducing the expression of a number of genes, which leads to activation of the transcription factor NFκB. Activated NFκB then translocates to the nucleus where it activates the transcription of several genes namely those that encode proinflammatory cytokines [25]. The -105G/A promoter polymorphism of *SEPS1* was shown to be strongly associated with plasma levels of pro-inflammatory cytokines, such as interleukin 1 beta (IL-1β), interleukin 6 (IL-6) and tumour necrosis factor alpha (TNF-α) [26].

Although, these data point to the important role of selenoproteins S mediating inflammation, there are no studies on the impact of *SEPS1* gene locus on the risk of AITD. In an attempt to understand the pathogenic process that underpin AITD we conducted a case-

control study to analyse the association between SEPS -105G/A polymorphism and risk to develop AITD in the Portuguese population. In fact, the general aim of this thesis was to investigate whether or not it is possible to delineate genetic, environmental and inflammatory risk factors for AITD in order to predict future development of AITD.

MATERIALS & METHODS

Patients and controls: We tested the association between -105G/A *SEPS1* promoter polymorphism and the risk for AITD using a case-control study. A total of 1111 Portuguese subjects were evaluated. Patients with AITD were identified at the Endocrinology Department of Hospital de S. João. The patients group comprised 595 individuals, 83 diagnosed with GD and 512 diagnosed with HT, and the control group included 516 unrelated individuals.

The clinicopathological data from the patients enrolled in this study between 2007 and 2010 are summarized in table 1. The diagnosis was made by medical and biochemical assessments of patients. The diagnosis of GD was established on the basis of clinical findings decreased serum thyroid stimulating hormone (TSH) (<0.35IU/ml), elevated serum free thyroxine (FT4) (>1.48ng/dl) and/or free triidothyrodine (FT3) (>3.71pg/ml), positive serum antibodies to TSH-receptor (TRAb) (>1.8IU/l), and typical ultrasound signs (hypoechogenicity and high perfusion). Diagnosis of HT was also obtained based on clinical findings positive serum antibodies to thyroid peroxidase (TPOAb) and/or thyroglobulin (TgAb) (according to the method applied before or after March 2009 in the Department of Clinical Pathology of Hospital S. João), and characteristic ultrasound signs (hypoechogenicity and non-homogeneous texture). Demographic (gender, age) and clinic-pathologic parameters (TSH, T3, T4, TRABs, TPOAb, TgAb) were obtained and correlated with the *SEPS1* genotypes.

In order to a better characterization of the study population, C-reactive protein, a pro-inflammatory molecule which has been implicated in various inflammatory diseases and body mass index (BMI) a measure for human body shape based on individual's weight and height, were also obtained.

The control group included 516 (480 females/36males) samples obtained from unrelated Portuguese Caucasian healthy blood donors (mean age 44 years; range 18-83). This

group consisted of permanent residents in the area of Hospital of S. João (Porto, Portugal), selected during the assembling of the EpiPorto cohort [27].

To eliminate possible confounding factors participants with reported history of thyroid cancer disease, medication such as levothyroxine or methimazole and/or previous thyroid surgery were excluded from the study.

Enrolment of participants was performed under ethical approval of Hospital of S. João (Porto, Portugal) ethic committee and included informed consent for data and DNA usage. The use of spare sections from the paraffin blocks of the sporadic tumours was in accordance with national and institutional guidelines.

SNP genotyping: Patients and controls genomic DNA was isolated from blood using standard proteinase K digestion with phenol/chloroform extraction. To screen for *SEPS1* polymorphism the SNP *SEPS1* (rs28665122) was genotyped using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, USA). PCR amplification and allelic discrimination were performed according to product specifications with the ABI 7500 Fast real-time PCR system (Applied Biosystems, Carlsbad, USA). Optimization of each TaqMan Assay was performed using controls of known genotype selected through DNA sequencing.

Statistical analysis: The statistical analysis of the results, namely genotype frequencies for SNP, was performed using SPSS v 19.0. The Hardy Weinberg equilibrium was evaluated at the level of the control population using a χ^2 test. Comparison of genotype frequencies between groups defined by *status* (HT and GD patients *vs* controls) was assessed by logistic regression using SPSS 19.0. Odds ratios (OR) with respective confidence intervals (95% CI) for association of candidate *loci* with both GD and HT were calculated for the genotypic

model of inheritance. To adjust for multiple testing, a Bonferroni correction was applied (Bonferroni-corrected P <0.0125) [28].

Comparison of genotype frequencies between groups defined by status (HT patients and GD patients *vs* controls) was assessed by logistic regression using SPSS 19.0. Odds ratios (OR) with respective confidence intervals (95% CI) for association of candidate *loci* with both GD and HT were calculated for the recessive and dominant models of inheritance. Independence of the variables gender, age, BMI, CRP and polymorphisms (*1IL1β* and *SEPS1*) was established using a X^2 test.

RESULTS

Characteristics of the subjects

The clinicopathological characteristics of patients with Graves' disease and Hashimoto's thyroiditis are summarized in Table 1. AITD affects people of all ages with peak occurrence in women of 40-50 years. In our study there is no significant difference in age onset when we compare GD with HT (*p-value*=0.426). But there is a trend to develop hyperthyroidism earlier that HT (Table 1). This is in accordance with surveys in the general population, all reporting a higher prevalence of hypothyroidism with advancing age.

As expected, AITD in Portuguese population is more prevalent in females. In our study the female: male ratio for Graves' disease and Hashimoto thyroiditis was 15.4:1 and 10.6:1 respectively. All data is reported in table 1.

The comparison of thyroid function, in GD patients, to gender-matched HT patients is shown in table 2. There was a statistically difference (p=0.020) in free T3 (GD: 3.41±2.02; HT: 4.49±20.94), but not in free T4 (GD:1.38 ±1.33; HT:1.99 ±8.01) between the two types of diseases (p>0.05). As expected, in terms of TSH (GD: 3.87±15.95; HT: 2.15±2.89) there was significantly differences when the two diseases were compared (p<0,0001).

According to the values shown in the Table 3, no significant correlation was found between the baseline serum levels of C reactive protein (CRP) between GD and HT patients (p>0.05). However the tendency in our study is to a higher CRP in HT (HT: 0.67 ± 5.80 vs GD: 0.44±0.67). All reported in table 3 and figure 1.Body-mass index (kg/m2) were also evaluated and there were no difference between GD and HT (p>0.05). As expected there is trend to an elevated BMI in HT patients when compared to GD (table 3)

In HT, TPO is the major autoantigen, and autoantibodies to TPO are closely associated with disease activity. Here we found anti-TPO in 67.2% of the subjects. Although there was no difference regarding anti-TPO and anti-TG when comparing HT and GD, we found there

was a statistically significance difference in anti-TRab between HT and GD (p=0.000057). The large majority of the TRab antibody positive persons were found in GD subset (48.8%).

Distribution of the SEPS1 genotypes

The distribution of *SEPS1* -105 promoter genotypes in healthy controls and in patients with AITD is shown in Table 4. This polymorphism was typed in 1111 subjects. Among controls, the distribution was as follows: 71.9% were GG, 24.6% GA and 3.5% AA. In the control group the genotype frequencies of *SEPS1* -105G/A polymorphism did not deviate significantly from those expected under Hardy-Weinberg equilibrium (p=0.45). Among cases the distribution of genotypes was as follows: 53.8% were GG 40.5% were GA and 5.7% were AA. We can observe that there was a significant difference regarding the genotype distribution for this locus between the control group and AITD patients either for HT and for Graves' disease.

We observed a statistically significant association between the *SEPS1* GA genotype and GD (OR=2.81, CI=1.69-4.68, *p-value* =$6.9x10^{-5}$). We did not observe any statistically significant difference in the frequency of AA genotype between GD and controls (Table 4). The A *carriers* present an increased risk of 2.65 (CI=1.62-4.34; p=$1.1x10^{-4}$) for GD. In the HT patients a statistically significant higher proportion of the *SEPS1* GA genotype (OR=2.26, CI=2.68-3.05, *p-value* <$1x10^{-6}$). The frequency of AA genotype was also higher in HT (OR=2.14, CI=1.12 -4.11, *p-value* =0.0217). The A allele *carriers* present an increased risk of 2.25 (CI=1.69-2.99; *p-value* <$1x10^{-6}$) for HT. All values are reported in Table 4.

When we consider AITD globally there were also significant differences. We found a significant increase in the frequency of the GA genotype in patients than in the controls. The individuals with GA genotype present a 2.34 fold increased risk of developing AITD than

individuals with GG genotype. (OR=2.34, CI=1.76-3.12), *p-value* $<1x10^{-6}$). When grouping the population that should produce higher levels of *SEPS1*, that is the A allele *carrier* (GA/AA), we can see a 2.3 fold increased risk for development of AITD (OR=2.31, CI= 1.76-3.03, $p<1x10^{6}$) in patients carrying this allele.

When we stratified the analysis regarding gender, there were significant differences too. Although, there were only 42 HT males and 5 GD males, the A *carrier* was significantly increased in male patients with HT representing a 6 times increased risk (*p-value* =0.000376). But in GD this association was not verified (Table 5).

Association of SEPS1 genetic variants with inflammatory markers

We carried out marginal association analysis by comparing HT to GD subsets. In our study, we assessed the effect of minor allele of the SNP on serum levels of C-Reactive Protein (clinical marker of inflammation) before normalization of thyroid function and we verified that the risk genotypes are not associated with CRP serum levels ($p>0.05$) not only in HT but also in GD.

In our study, either HT and GD cases carrying the minor allele of SEPS does not completely explain the clinical differences between the diseases. This polymorphism was not associated with BMI categories ($p>0.05$). A similar result was evident with thyroid hormones levels: FT3, FT4 and TSH, $p>0.05$. There was no difference between gender and FT3, FT4, TSH and CRP. Serum thyrotropin (TSH) is the best screening test for primary thyroid dysfunction for the vast majority of outpatient clinical situations. Because it is poor specificy it is normal that there isn´t such great differences.

Interaction between 1IL1β -511 and SEPS -105

It is known that *SEPS1* polymorphism exhibits epistasis with -511 polymorphism of *IL1β*, greatly increasing the risk of rheumatoid arthritis in individuals with both polymorphisms, although there was no correlation of polymorphisms with rheumatoid arthritis alone. [29] The genotype frequencies for different combinations of *IL1β*-511 and SEPS-105 for AITD cases and controls are shown in table 6 and 7. No association of *IL1 β* – 511 genotype and AITD susceptibility was previously detected by our group (In GD – CT genotype OR=0.81, *p-value*=0.539; T *carrier* OR=1.10, *p-value* =0.699; in HT – CT genotype OR=1.14, *p-value* =0.528; T *carrier* OR=1.12, *p-value*=0.421) [30].

The presence of a significant interaction was detected using a stratified analysis. Regarding HT, the OR comparing individuals with AA/GA genotype to the GG genotype at the *SEPS1* -105 locus in participants who were CC at the *IL1 β* – 511 locus was around 2.4 (95%CI 1.51-3.77, *p-value* =0.0002). When we compare T *carrier* (*IL1 β* – 511 *locus*) to A *carrier* (*SEPS1* -105 locus) the risk increased to 3.02 (*p-value* $<1x10^{-7}$, CI: 1.99-4.59) (Table 6).

Taking into consideration Graves disease: stratification of *SEPS1 -105* genotypes by *1IL1B -511* genotypes showed that the disease risk (comparing CC to A *carrier* at SEPS -105 locus) was around 5 times higher in HT patients (OR=4.65, CI: 2.17-9.96, *p-value* =0.000079). When we compare T *carrier* to A *carrier* the risk was 3,76 (p=0.000555, CI: 1,77-7,99). This considerably exceeds the Bonferroni threshold providing evidence for a statistical interaction between the *SEPS1 -105* and *1IL1B -511 loci* in AITD (Table 7).

DISCUSSION

Biosynthesis of selenoproteins is mediated by a conserved machinery of *trans*-acting and *cis*-acting factors that requires a specific 3' UTR based mechanism for selenocisteine incorporation. Therefore, it can be expected that single nucleotide polymorphisms within the selenoprotein gene regions and variations in selenium compounds concentration can affect selenoprotein synthesis [31, 32].

Genetic variation in *SEPS1* gene have been associated with increased risk in chronic inflammatory diseases such as coronary heart disease and ischemic stroke [33], gastric cancer [34] , colon cancer [35], osteoarthritis [36] and preeclampsia [37]. However other authors do not found any associated risk in patients presenting *SEPS1/SelS* polymorphisms and type 1 diabetes, rheumatoid arthritis or inflammatory bowel diseases [38].

We hypothesized that genetic variations in *SEPS1* gene, can affect Selenoprotein S expression and modulate chronic inflammatory processes in thyroid. In fact our hypothesis has a proven physiological background. The thyroid is known to be among the organs with the highest selenium content *per* gram of tissue because it expresses several specific selenoproteins [32]. Among the well-characterized selenoproteins are the iodothyronine deiodinases, glutathione peroxidases and thioredoxin reductases, enzymes involved in thyroid hormone metabolism, regulation of redox state and protection from oxidative damage. Moreover, the physiological and pathological effects of selenoproteins are closely related to selenium status in selenium-sensitive tissues, however in the thyroid, these effects are largely independent from dietary selenium intake and thyroid selenoproteins are differently expressed [39].

Facing these facts in this large case-control association study, we have for the first time demonstrated association of a polymorphism in the recently discovered inflammatory response gene *SEPS1* with AITD. In fact, *SEPS1* -105G/A polymorphism is significantly more frequent in AITD patients than in healthy controls, putatively influencing the

proinflammatory cytokine profiles observed in AITD [19] The *SEPS1* GA and also AA genotype are associated with almost 2 fold increased risk to AITD. The promoter variant can down-regulate expression of SEPS, causing a build-up of misfolded proteins in the ER [24]. Stress to the ER can induce NF-*κ*B, which can up-regulate inflammatory cytokines (*IL1*β, IL6 and TNFα), and can also lead to apoptosis [19] (figure 2).

Cytokines are key regulators in inflammatory response. Various cytokines and respective polymorphisms have been reported to be associated with AITD. IL1β, IL6 and TNFα cytokines have a proinflammatory function acting as facilitators of the immunologic process involved in HT and GD. Taking all in consideration, *SEPS1* gene seems to be a good candidate gene involved in the autoimmune pathogenesis shared by HT and GD.

In our study we found that the *SEPS1* GA and AA genotype are associated with 2 fold increased risk to HT patients. And as expected the A allele *carriers* were found in higher statistically significant proportion when compared with control population. In HT several factors strengthen the hypothesis of *SEPS1* gene involvement in the process, that lead to thyrocytes death through thyroid-reactive T cells that have escaped tolerance and infiltrate the thyroid. Selenium deficiency impairs adequate thyroid hormones metabolism, leads to an increase thyroid volume and thyroid hypoechogenicity (a marker for lymphocytic infiltration) [40] and cell death through *Fas* and *FasL* [41, 42]. This was reversible in animal models where selenium supplementation leads to a significant reduction in antithyroglobulin antibodies associated with decreased lymphocyte infiltration of the thyroid [40, 43- 45]

Passing through the great limitation of this study (low number of GD patients, n=83) that might unmask the lack of association of AA genotype to an increased risk for GD, we also observed that in general the genotype and allele frequency of *SEPS1* observed in patients with GD was similar to that observed in HT. *SEPS1* GA genotype is associated with almost 3 fold increased risk to develop GD. In fact, the thyroid is particularly prone to oxidative status

leading to an increased reactive oxygen species (ROS) production. Recently it was proved that selenium concentrations in GD disease patients were positively correlated to TSH receptor antibody concentrations and a negative correlation was observed in the remission group [35]. The selenium administration in GD has a beneficial effect at least for treatment of moderate or mild orbitopathy [46,47].

Given that evidences exist for the affected expression and activity of some selenoproteins depending on sexual dimorphism in mouse and human [48,49], we decided to check for a gender specific association of -105G/A *SEPS1* polymorphism with autoimmune disorders under study and this ideia of gender-bias was evident in our results. But this should be confirmed by increasing the number of male patients especially in GD subtype. Our results suggest a 6 time increased risk for HT in males A *carriers* which is in agreement with Schomburg and collaborators, who suggest a special role of selenium according to the gender regulating the SEPS expression by modifying cytokine response patterns in serum and controlling the immune response using male animal models [48,50].

Curran and collaborators proved that genetic variation of *SEPS1* influences circulating levels of cytokines. Polymorphism at this site was specifically chosen as the presence of A allele at this site impairs the expression of SEPS, such that the genotypes AA, GA and GG are respectively high, medium and low producing of IL1β and TNF-α. The effect of the A allele was smaller on IL6 levels. The polymorphisms in these pro-inflammatory cytokines were previously investigated by our group and *IL1β* was the only one that shows no statistically significant differences in the frequencies of genotypes and alleles of the *IL1β*-511 C/T polymorphism between healthy subjects and patients with HT and GD.

Several studies have reported conflicting results regarding the possible role of *IL1β* -511polymorphism in AITD may be due to a huge number of factors including genetic and disease heterogeneity, gene-gene and gene-environment interactions. We proposed that a gene

with a weak effect on overall disease risk may be important in combination with other gene. So, the presence of epistasis of *SEPS1* with a functional cytokine variant was investigated. Our data demonstrate that of *IL1β* and *SEPS1* interactively increase the risk of developing HT and GD. Taking in consideration these observations and *SEPS1* genetic variation demonstrated by Curran *et all*, the increased frequency of the -105 A and -511 A alleles was expected. Our data suggest that in HT the T allele at *IL1*β-511 locus is a new candidate for AITD pathogenesis in individuals who are A *carrier* at *SEPS1* -105 locus. In GD we verified that there was a trend to decrease the risk when compared the A *carrier SEPS1* /CC *IL1*β to A *carrier* /T *carrier*. This may be in agreement with previous studies where authors suggested the TT genotype of *IL1*β acts as a protective variant [51]. This is just a pivot study based on small numbers that need to be confirmed but start to highlight the importance of epistatic interactions in the pathogenesis of complex disease.

In our results we did not find association between the *SEPS* polymorphism and thyroid hormones titres in the two disease subsets. But we can not exclude that selenium has a role here because it is an essential part of the iodothyronine deiodinase enzymes (DIO) and selenium deficiency is followed by a decreased conversion of thyroxine (T4) to the active hormonetriiodothyronine (T3) [52].

Thyroid hormones play an important role in regulating lipid metabolism, and thyroid dysfunction can result in lipid abnormalities which increase the risk of endothelial dysfunction, hypertension and cardiovascular disease. *SEPS1* may also have a role in lipid metabolism [32-33] and the relationship between thyroid hormones and lipids has long been studied Concentration of selenoprotein markers are known to be dependent on BMI and selenoprotein genotype [32].In our study we did not find significant differences between BMI and *SEPS1* genetic variants which doesn't mean that the clinical phenotype of the diseases are not directly linked with genetic variation in *SEPS1* gene.

In common with, probably, all autoimmune disorders, the interplay of genetic environmental and endogenous factors is required in the right combination to initiate thyroid autoimmunity. This was the first time that was demonstrated that a single gene polymorphism in selenoprotein S gene can increase the risk to develop AITD in areas with low selenium soil content such as in Europe. However functional studies are needed in order to clarify the recent discoveries [53].

Conclusion

Our awareness of key immunological parameters predisposing to AITD has been enabling the development of therapeutic approaches to harness the autoreactive response. In fact, the genetic aetiology of AITD is well documented but until now, no confirmed susceptibility gene was found and used in disease prediction or therapy.

What is known for sure is that AITD is a major world concern and the number of patients is increasing all over the world. Although AITD has a very low mortality, it is associated with significant morbidity, both physically and mentally, and leads to diminished quality of life [50]. Lifelong treatment and monitoring of serum hormone levels are the rule rather than the exception.

In conclusion, carrying an A allele at the *SEPS1* -105G>A polymorphism is a risk factor for AITD. The A allele may be contributing to the GD and HT by influencing the inflammatory response. Further studies in larger cohorts of HT and GD with clinical serological controls are needed to investigate the actual role of *SEPS1* and important implications of genetic alterations of selenoproteins in chronic inflammatory responses and autoimmunity.

Autoimmune disorders can cluster in individuals and their relatives. A family history of autoimmunity and screening for autoantibodies can identify at-risk subjects. Knowledge of

these disorders and their disease genetic associations can lead to earlier diagnosis and management, resulting in less morbidity and, in some cases, mortality. As known the ideal population for preventive intervention would be subjects at risk mainly relatives of AITD patients with A *carrier* genotype, in whom TSH is normal but TPO-Ab are already present. Where the intervention with selenium in early stages might be a very attractive option.

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APPENDIX

Table 1| Demographic data and clinical characteristics of patients with Graves' disease and Hashimoto's thyroiditis.

Groups	N	Gender (F:M)	Age of onset (y) [†]	FreeT3 (pg/ml)*	FreeT4 (ng/ml)*	TSH (UI/l)*	TgAb positive n (%)*[#]	TPOAb positive n (%)*[#]	TRAb positive n (%)*[#]
HT	512	10.6:1	46.1±15.95[†]	4,49± 20,94	1,99± 8,01	2,15± 2,89	182 (54.3)	225(67.2)	13(3.9)
GD	83	15.4:1	45.0±14.67[†]	3,41 ± 2,02	1,38± 1,33	3,87 ±15,947	37(45.1)	63(76.8)	40(48.8)

Data are expressed as mean ± standard deviation. *The number of patients to which we had access to the clinical parameters was different for each characteristic.
[†] Difference in age onset: GD/HT (*p-value*=0.426). # Among the antibodies; anti-TRab was statistically different between HT and GD (p=0.000057).

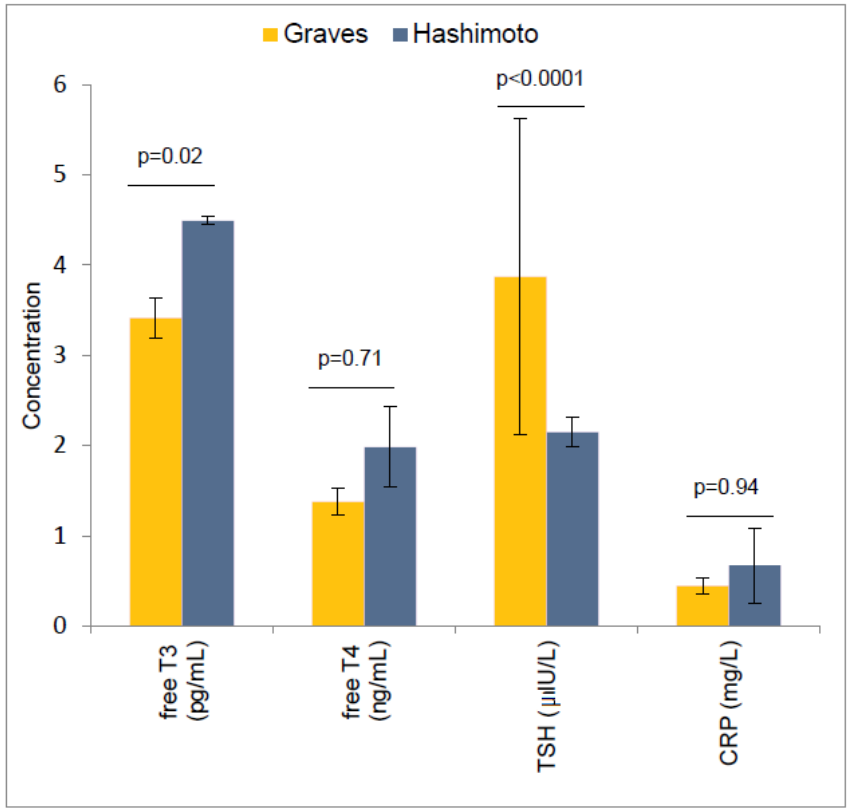

Figure 1| TH and CRP levels - comparison between both groups excluding subjects with previous thyroid disease, medication with levothyroxine and/or previous thyroid surgery.

Table 2| TH levels comparison between both groups excluding subjects with previous thyroid disease, medication with levothyroxine and/or previous thyroid surgery

	GD patients (n =83)	**HT patients (n =323)**	*p- value*
TSH (µIU/mL)	3,87 ±15,947	2,15± 2,89	**<0.0001**
Free T3 (pg/mL)	3,41 ± 2,02	4,49± 20,94	**0,02**
Free T4 (ng/dL)	1,38± 1,33	1,99± 8,01	0,71

Results are expressed as mean ± SD. Statistical analysis made with Mann-Whitney U test. TH, thyroid hormones.

Table 3| CRP levels and BMI comparison between both groups excluding subjects with previous thyroid disease, medication with levothyroxine and/or previous thyroid surgery

	GD patients (n =83)	**HT patients (n =323)**	*p- value*
CRP (mg/L)	0,44±0,67	0,67±5,80	0,941
BMI (Kg/m^2)	25,99±5,17	27,24±5,14	0,08

Results are expressed as mean ± SD. Statistical analysis made with Mann-Whitney U test. CRP – reactive C protein (mg/L); BMI – (Kg/m2)

Table 4| Distribution frequencies of *SEPS1* -105G/A genotypes among AITD patients (Graves' disease[a] and Hashimoto's thyroiditis[a])and controls, and risk analysis results for each genotype adjusted to age

Locus	Genotye	Controls[b] n (%)	GD n (%)	OR (95% CI)	p-value [d]	HT n(%)	OR (95% CI)	p-value [d]	AITD n (%)	OR (95% CI)	p-value [d]
SEPS1		n=516	n=83			n=512			n=595		
-105 G/A	GG	371 (71.9)	39 (47.0)	1[c]		281 (54.9)	1[c]		320 (53.8)	1[c]	
	GA	127 (24.6)	40 (48.2)	2.81 (1.69-4.68)	**$6.9x10^{-5}$**	201 (39.3)	2.26 (2.68-3.05)	**$<1x10^{-6}$**	241 (40.5)	2.34 (1.76-3.12)	**$<1x10^{-6}$**
	AA	18 (3.5)	4 (4.8)	1.65 (0.51-5.37)	0.4052	30 (5.9)	2.14 (1.124.11)	**0.0217**	34 (5.7)	2.07 (1.10-3.90)	**0.0242**
	GG vs A *carrier*	371/145 (71.9/28)	39/44 (47.0/53.0)	2.65 (1.62-4.34)	**$1.1x10^{-4}$**	281/231 (54.9/45.1)	2.25 (1.69-2.99)	**$<1x10^{-6}$**	320/241 (53.8/46.2)	2.31 (1.76-3.03)	**$<1x10^{-6}$**

[a]The number of cases and controls genotyped for each SNP differs according to their genotyping success. [b]Reference category.[c]Reference estimate. [d]Values were considered statistically significant when the *p-value* <0.05 and are highlighted in bold.

Table 5| Distribution frequencies of *SEPS1* -105G/A genotypes among AITD patients (Graves' disease[a] and Hashimoto's thyroiditis[a])and controls, and risk analysis results for each genotype adjusted to age in woman and man.

	Genotye	GD OR (95% CI)	p-value [d]	HT OR (95% CI)	p-value [d]	AITD OR (95% CI)	p-value [d]
Man							
	GG vs A carrier	1,8 (0,26-12,41**)**	0,551	6,08 (2,25-16,4)	**0,000376**	5,22 (2,01-13,59)	**,001**
Woman							
	GG vs A carrier	2,97 (1,83-4,84)	**0,000012**	2,02 (1,54-2,66)	**< $1x10^{-7}$**	2,142 (1,65-2,79)	**< $1x10^{-7}$**

[a]The number of cases and controls genotyped for each SNP differs according to their genotyping success. [b]Reference category.
[c]Reference estimate. [d]Values were considered statistically significant when the *p-value* <0.05 and are highlighted

Table 6| Stratification analysis according to *IL1β* – 511 locus *and SEPS1* genotypes in Hashimoto thyroiditis patients

	*IL1*β *– 511*			
	CC		**CT/TT**	
SEPS-105	OR(95%CI)	*p-value*	OR (95% CI)	*p-value*
GG	Ref=1		1.204(0.834-1.737)	0.322
AA/GA	2,39 (1,51-3,77)	**0.0002**	3,02 (1,99-4,59)	**<1 x10^{-7}**

The number of cases and controls genotyped for each SNP differs according to their genotyping success rates.
Values in bold are statistically significant with a Bonferroni-corrected *p-value* <0,0125.

Table 7| Stratification analysis according to *IL1β* – 511 locus *and SEPS1* genotypes in Graves' disease patients

	*IL1*β *– 511*			
	CC		**CT/TT**	
SEPS-105	OR(95%CI)	*p-value*	OR (95% CI)	*p-value*
GG	Ref=1		1.68(0.82-3..41)	0.154
AA/GA	4.65 (2.17-9.96)	**0.000079**	3,76 (1,77- 7.99)	**0.000555**

The number of cases and controls genotyped for each SNP differs according to their genotyping success rates.Values in bold are statistically significant with a Bonferroni-corrected *p-value* <0,0125

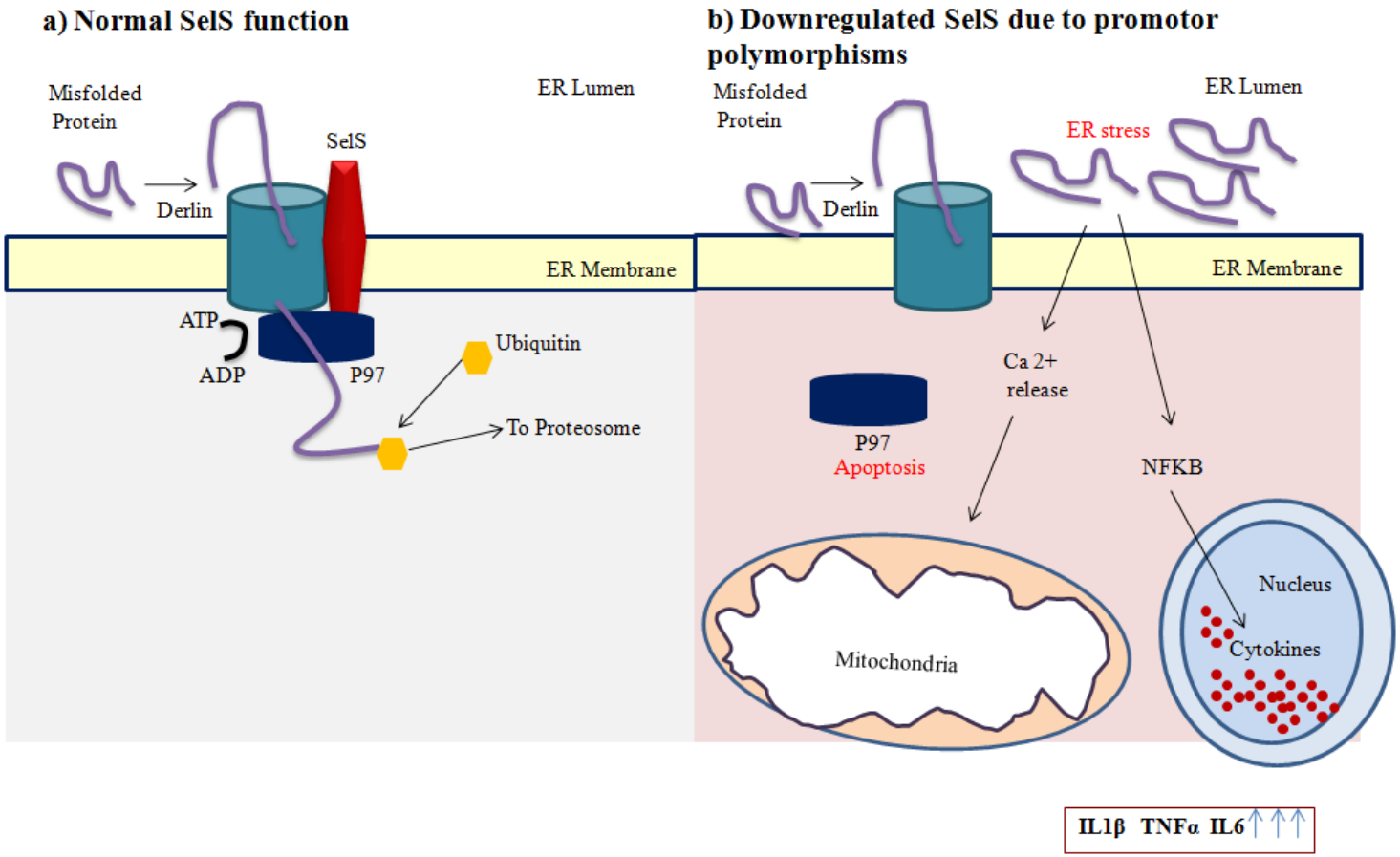

Figure 2| Role of SPES in removal of misfolded proteins from ER (**A**) SEPS is an ER membrane protein that interacts with the ER membrane protein derlin and the cytosolic ATPase p97 to transport misfolded proteins out of the ER. Once in the cytosol, the protein is tagged with ubiquitin via the E3 ubiquitin ligase and shuttled to the cell proteasome. (**B**) Promoter SEPS polymorphisms can down-regulate expression of SEPS, causing a build up of misfolded proteins in the ER. Stress to the ER can induce NFκB, which can up-regulate inflammatory cytokines, and can also lead to apoptosis.