Graves' disease: epidemiology, genetic and environmental risk factors and viruses.

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Abstract

Graves' disease (GD) is the most common cause of hyperthyroidism in developed Countries. It is more common between 30-60 years; 5-10 times more frequent in women. The genetic predisposition accounts for 79% of the risk for GD, while environmental factors for 21%. About 70% of genes associated with autoimmune thyroid disorders (AITD) are implicated in T-cell function. Among GD endogenous factors, estrogens, X-inactivation and microchimerism are important. Among environmental risk factors, smoking, iodine excess, selenium and vitamin D deficiency, and the occupational exposure to Agent Orange have been associated with GD. Many studies showed that HCV is associated with thyroid autoimmunity and hypothyroidism, in patients with chronic HCV hepatitis (CHC); a significant link has been shown also between HCV-related mixed cryoglobulinemia and risk for GD. Moreover, IFN-α-treated CHC patients develop GD more frequently.

Novel studies are needed about possible risk factors to reduce the occurence of GD in West Countries.

Keywords: Graves' disease; epidemiology; risk factors; viruses; Hepatitis C Virus; Th1 chemokines.

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1. Introduction

Graves' disease (GD) is an organ-specific autoimmune disorder leading to the overproduction of thyroid hormones (hyperthyroidism). Although many disorders result in hyperthyroidism, GD is the most frequent cause in West Countries with an annual incidence of 20 cases/100,000 persons (1,2).

GD is one of the main autoimmune thyroid disorders (AITD), that are characterised by the breakdown of immune tolerance against thyroid antigens (3,4). GD is clinically characterised by thyrotoxicosis, and by the presence of serum anti-thyroid antibodies (ATA) and of autoreactive lymphocytes in the gland (5). The thyroid-stimulating hormone (TSH) receptor (TSH-R), thyroid peroxidase (TPO), and thyroglobulin (Tg) have unusual properties ("immunogenicity") contributing to the breakdown of tolerance (4).

Thyroid hormones affect several different body systems, and for this reason, signs and symptoms associated with GD can vary strongly and significantly influence the general well-being. Common symptoms are: tremor, heat sensitivity and warm, weight loss, even if with normal eating habits, anxiety and irritability, enlargement of the thyroid gland (goiter), alterations in menstrual cycles, erectile dysfunction or decreased libido, fatigue, frequent bowel movements, palpitations, and others (6).

As already stated above, the onset of GD implicates a breakdown of immune tolerance towards the thyroid, through an autoimmune multifactorial process, involving environmental and endogenous factors in genetically predisposed subjects (7).

In GD, autoimmune reaction causes the production of anti-TSH-R autoantibodies (TRAb) by B-cell clones, that infiltrate the gland. Depending on their respective actions on the TSH-R, TRAb antibodies can be classified as: thyroid stimulating antibodies (TSAb); thyroid blocking antibodies (TBAb); neutral antibodies (8). TRAb antibodies are implicated in GD pathogenesis and its extrathyroidal manifestations, i.e. GO and pretibial myxedema

(PTM)/Graves' dermopathy. Hyperthyroidism is associated with TSAb (9-12).

TSAb lead to similar downstream effects as the binding of TSH to TSH-R, inducing thyrocytes proliferation, thyroid growth, and secretion of thyroid hormones (T4 and T3) (8). The role of TBAb and neutral antibodies is less understood in thyroid autoimmune pathophysiology (8). TBAb can bind to the A subunit of the TSH-R and block the TSH action and its effects on the follicular cells, whilst the neutral antibodies bind to the receptor with no impact on cAMP generation or TSH binding (8).

Antithyroid drugs are the first-line therapy for GD. Ablative therapy, either from radioactive iodine or surgical thyroidectomy, can cause hypothyroidism and leads to lifelong thyroid hormone replacement (2). High dose iv immunoglobulins (13), or corticosteroids (CS) reduce inflammation and orbital congestion in patients with active Graves' ophthalmopathy (GO; a GD extrathyroidal manifestation).

2. Epidemiology

Although GD may affect anyone, it is more common among women, between 30 and 60 years of age. The risk of GD is 3% for women and 0.5% for men. The annual incidence of GO is 16 cases/100,000 women and 3 cases/100,00 men, and the age of appearance is between 30 and 60 years (2).

In particular, a study from Minnesota showed a peak age-specific incidence in patients with 20-39 years of age (14). Among 1,572 hyperthyroid patients in France, in a study published in 2016, 73.3% had GD, 85% of whom were females, of 43 years of age at first onset, and 44 years at recurrence (15). The risk of GD in Sweden is 1.7%, about 5–6 times higher in females than males, and at the diagnosis the mean age is approximately 48 years (16). Moreover, GD is more frequent in the Asian population and less in Sub-Saharan African people (17,18).

An increased risk of thyroid cancer in GD has been observed in many studies, particularly when patients with GD have thyroid nodules. Tall Cell Variant of papillary thyroid cancer (a more aggressive form of cancer) was significantly more common in patients with GD (19-21).

3. Risk factors

3.1 Genetic factors

A family history of GD is considered a risk factor. The concordance rate of GD in monozygotic twins in different studies is varying from 0.29 to 0.36, while in the same studies the concordance rate in dizygotic twins was between 0.00 and 0.04. Using structural equation modeling, it was found that the risk for GD development can be attributed to heritability for about 79% (22), while environmental factors can explain 21% of the risk (23). The genetic susceptibility might elucidate the ethnic differences in the prevalence of GD (24).

The predisposition of GD seems to be polygenic (7). Recently, bioinformatics and next-generation sequencing (NGS) permitted to conduct pangenomic analyses, identifying many predisposing genes, some regarding different autoimmune diseases, others regarding AITD, and others implicated in GD or Hashimoto thyroiditis (HT) (7).

GD is considered a HLA class II-associated disorder (25). The DR3 haplotype (i.e. DQB1*02, DQA1*0501, DRB1*03) predisposes to GD, because of the elevated degree of linkage disequilibrium among the DQA1, DQB1, and DRB1 loci, whereas the DR7 haplotype (i.e., DQA1*0201, DQB1*0302, DRB1*07 or DQA1*0201, DQB1*02, DRB1*07) appears to be protective (26). In presence of DRB1*07 and DRB1*03, the first hampers the susceptibility to GD given by the last (27). The following genes take part into the pathogenesis of GD: cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), TSH-R, Tg, CD40, protein tyrosine phosphatase-22 (PTPN 22), human leukocyte antigens (HLA), and CD25 (28). Considering the genes correlated with the risk of AITD, about 70% of those whose effects are clear, are

implicated in T cell function, indicating the importance of T lymphocytes in AITD pathogenesis (27).

3.2 Endogenous factors

GD has a female predominance, and estrogen receptor ESR2 polymorphisms are frequent in GD, indicating the importance of estrogen in its pathophysiology. During pregnancy, the menstrual cycle, and menopause, estrogen variation can clarify disease fluctuation (29). Estrogen α receptor expression is present on orbital fibroblasts, and glucocorticoids can modulate it (30).

In females, estrogens and X-inactivation alter the immune system and can contribute to the predominance of GD. Female tissues are mosaics of paternal and maternal cells with activated X chromosome (31). The asymmetric X-inactivation (reported as the inactivation of the same X chromosome in 80% of cells) (32) accounts for female predominance in AITD, and this has been confirmed in the onset of GD in females by a recent meta-analysis [odds ratio (OR), 2.54; 95% confidence interval (CI): 1.58–4.10] (33).

At the basis of GD, and AITD in general, also microchimerism is considered an important endogenous factor (23,34).

3.3 Environmental factors

Different environmental factors (i.e. viruses, smoking, stress, radiation, drugs, iodine, etc.) can trigger AITD in susceptible subjects (5,35).

The minimum recommended daily dose required for thyroid hormone synthesis is 150 microgram. Sudden exposure to excess serum iodide inhibits organification of iodide, thereby diminishing hormone biosynthesis by an intrinsic mechanisms that maintain normal thyroid function (this phenomenon is called the Wolff-Chaikoff effect). Abudant amounts of iodide

are present in different drugs (amiodarone, etc), antiseptics, food preservatives and contrast media (36). In regions of iodine deficiency there is a higher incidence of iodine-induced hyperthyroidism because of underlying areas of autonomy within the thyroid gland (the Jod-Basedow phenomenon). Furthermore, in regions of iodine deficiency many GD patients are clinically euthyroid, but they develop overt hyperthyroidism when more iodine become available. Similarly, GD euthyroid patients who have reached remission after therapy with antithyroid drugs can develop iodine-induced hyperthyroidism and iodine load in hyperthyroid GD patients may blunt the response to antithyroid drugs (36).

Smoking can affect the immune status of the thyroid gland, increasing the risk and severity of AITD, especially GD. The influence of tobacco smoking is mainly mediated by nicotine and also by toxins, such as thiocyanate. Previous studies have clearly demonstrated the association between smoking and the occurrence and severity of GO and the response to orbital radiation. Smoke-induced increased generation of reactive oxygen species may be involved. In fact a study demonstrated that GO fibroblasts have exaggerated response to cigarette smoke extract challenge along with increased oxidative stress, and fibrosis-related genes expression (37). However, smoke reduces the risk of hypothyroidism (38,39).

Selenium is an essential micronutrient that is required for the synthesis of selenocysteine-containing selenoproteins. It is known that the thyroid is one of the tissues that contain more selenoproteins. Selenium has been evaluated for its role in preventing immune mediated thyroid disorders. The onset of thyroid autoimmunity can be associated with the lack of selenium, whilst its supplementation protects from AITD (40), and also vitamin D deficiency is a potential risk factor (41). Several Authors support the idea of a potential efficacy of selenium (mainly selenomethionine) supplementation in reducing AbTPO antibody levels. To date, there are no compelling evidences regarding the routine use of selenium in the treatment of Graves' hyperthyroidism. Results of meta-analyses, including ten randomised controlled

trials, suggest that selenium supplementation can help to restore biochemical euthyroidism, but important limitations exist and relevant durable clinical outcomes (i.e. remission rate after antithyroid drug treatment), besides surrogate markers (i.e. hormone or autoantibody concentrations), are still needed. However, according to a single study, selenium supplementation is recommended in patients with mild and short-term orbitopathy since it may reduce inflammation and eye specific symptoms while also achieving a strong amelioration in disease specific quality of life (QoL). The impact of selenium supplementation on moderate-to-severe orbitopathy is still unknown (42).

Pesticides and halogenated organochlorines can alter thyroidal function. Polychlorinated biphenyls and their metabolites, and polybrominated diethyl ethers bind to thyroid transport proteins (i.e. transthyretin), impairing thyroidal function (35).

During the Vietnam war, the herbicide Agent Orange [a mixture of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid, contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)] was used for military purpose. Vietnam veterans exposed to this agent were more frequently affected by diabetes mellitus, and different disorders of the thyroid and the pituitary gland, with an approximately 3-fold higher prevalence of the diagnosis of GD, with respect to controls (43,44).

Vitamin D deficiency has been proposed to have a role in the development and course of GD. Muscle weakness and QoL impairments are shared features of GD and vitamin D deficiency. A study aimed to investigate whether vitamin D supplementation would improve restoration of muscle performance and thyroid-related QoL in GD. On the basis of the results, the Authors suggested that in patients with newly diagnosed GD, high-dose vitamin D supplementation should not be recommended to improve muscle function (45).

The postpartum period is associated with a raised risk of AITD (46). Furthermore, intestinal dysbiosis is associated with AITD (47).

4. Infections and Viruses

The development of AITD may be triggered by infectious agents (48). An increased prevalence of non-secretors (subjects who are unable to secrete the watersoluble glycoprotein form of the ABO blood group antigens into saliva) is present in GD patients. Since non-secretors have a raised susceptibility to infection (49), this leads to hypothesize that an infective agent can be implicated in the pathogenesis of GD. Furthermore, Valtonen et al. found evidence for a recent bacterial or viral infection in the sera of 36% of patients with newly diagnosed GD and in only 10% of control subjects (50). Moreover, an elevated frequency of antibodies to the influenza B virus has been demonstrated in patients with thyrotoxicosis (51).

Different viruses have been investigated for their role in the pathogenesis of GD.

4.1 Foamy Viruses

Foamy viruses (FV), or Spumaviruses, include one of the 7 genera of Retroviridae. Since their discovery in 1971, FV have been involved in various human diseases (52). A French study in GD patients reported the presence of FV DNA by PCR in 19/29 patients (53). In another study (54), DNA was isolated from peripheral blood lymphocytes from 24 GD patients and 23 healthy blood donors. However, in the most recently published review (52) about FV in GD, it was shown that most of the studies were not able to find any association between GD and FV.

4.2 Parvovirus-B19

Erythrovirus B19 (EVB19, or Parvovirus B19) is a non-enveloped, small, ubiquitous human virus. EVB19 is suspected to be involved in HT, but no evidence is available about its role in

GD.

A paper evaluated serum and thyroidectomy specimens for EVB19 DNA by q-PCR, and for EVB19 thyrocyte infection by immunochemistry, in 20 patients with GD and 44 with non-autoimmune multinodular thyroid (55). Patients were negative for EVB19 IgM and q-PCR, and no cases of acute or chronic systemic EVB19 infection were reported. Controls were positive for EVB19 serology more frequently than GD patients (88% vs. 45%, $P<0.0001$). Positive EVB19 serology did not correlate with TSH-R antibodies or TPO antibodies in GD patients (55).

Another study was conducted in 32 subjects undergoing thyroidectomy, in whom prior to surgery anti-EVB19 antibodies and EVB19 itself were assessed (56). No cases of acute EVB19 infection were present, since no anti-EVB19 IgM antibodies were assessed. A viral load was reported in 2 serum samples and 6 thyroid biopsies. Three subjects had both a positive qPCR assay and a positive immunohistochemical assay for the thyroid tissue, demonstrating the presence of EVB19 in the gland (56).

According to the literature of the last 20 years, EVB19 is able to infect and persist in normal thyrocytes with low-level expression and for this reason it could be implicated in the induction or the pathogenesis of AITD (57). In particular, EVB19 seems to be involved in HT. Nevertheless, at present, no study has demonstrated that EVB19 takes part directly in the induction or pathogenesis of AITD, or GD (57).

4.3 Epstein-Barr virus

Epstein-Barr virus (EBV) is a ubiquitous virus that infects most of humans during their lifetime and, after the acute phase, persists for the rest of the life. EBV reactivation can occur in a subset of individuals with different types of cancers, autoimmune diseases, the autoimmune-like disease, and others (58).

It has been supposed that as EBV persists in B cells and is reactivated from time to time, it could contribute to TRAb production in GD via the stimulation of the TRAbs-producing B cells (59). Cultured or non-cultured peripheral blood mononuclear cells (PBMCs) from 13 GD patients and 11 healthy subjects were analyzed. Cultured PBMCs from 8 GD had TRAbs(+) EBV(+) double positive cells. TRAbs(+) cells PBMCs in culture were significantly more frequent in patients than in controls (P=0.021). These data suggested that the presence of EBV-infected B lymphocytes with TRAbs on their surface could be involved in the production of excessive TRAbs (59).

Then, it was evaluated whether TRAb(+)EBV(+) cells produce TRAbs during persistent EBV reactivation (60). PBMCs from 12 GD patients and 12 healthy subjects were cultured with cyclosporine A to amplify the EBV-infected cells, and subsequently TRAb levels were compared after EBV reactivation (conducted in culture at 33 °C) or nonreactivation (conducted in culture at 37 °C). TRAb were significantly higher in supernatants after reactivation than from healthy subjects. These data indicated the TRAb production from TRAb(+)EBV(+) cells in response to reactivation (60).

In another study circulating IgG and IgM TRAb levels in 34 GD patients and 15 controls were quantified to clarify the mechanism at the basis of EBV-related antibodies production (61). GD patients had higher TRAb-IgG and TRAb-IgM levels than controls; even if total IgG levels were higher than total IgM levels, TRAb-IgM were significantly higher than TRAb-IgG. Patients with EBV reactivation had frequently an increased production of TRAb-IgM, in agreement with higher percentage of autoreactive IgM B cells than that of autoreactive IgG B cells. These data supported the EBV-related polyclonal B cell activation (61).

Moreover, as GD is more frequent in women, the involvement with estrogen has been suggested. PBMCs from GD patients and healthy subjects were cultured with estradiol (0, 1, and 100 nM, corresponding respectively to control, midluteal, and pregnancy levels). Total-

IgG, total-IgM, and TRAb levels, during EBV reactivation, were analyzed (62). TRAb levels increased in presence of 1 nM estradiol, while they decreased slightly with 100 nM estradiol both in patients and controls. In GD patients, IgM production at 0 nM estradiol was significantly higher than that at 100 nM estradiol (P=0.028). Estradiol raised the ratio of IgG production to IgG/IgG+IgM, suggesting an increase in class switch recombination during the Ig production induced by EBV reactivation. Furthermore, TRAb production was induced by midluteal levels of estradiol and was inhibited by pregnancy levels of it in controls and patients, consistently with the premenstrual worsening and maternity amelioration of autoimmune diseases, such as GD (62).

IgG4-related disease (IgG4-RD) is a recently described systemic fibroinflammatory disorder with characteristic histological findings and elevated serum IgG4 levels. A study (63) evaluated whether EBV reactivation induced IgG4 production, and the presence of EBV-positive B cells or IgG4-positive plasma cells in thyroidal tissues of GD patients with lymphoplasmacytic infiltration. Immunohistochemistry of 7 cases with moderate lymphoid cell infiltration demonstrated the presence of EBV-encoded small RNA1 (EBER1)-positive cells and IgG4-positive plasma cells in the area of lymphoid cell infiltration. In GD patients, the IgG4/IgG percentage was higher than that in normal serum. The Authors hypothesized that IgG4 could be stimulated by EBV reactivation in GD with lymphocytic infiltration as a thyroid-specific IgG4 thyroiditis. As IgG4 production induced by EBV reactivation was shown *in vitro* in PBMCs, it can occur both in serum and in local thyroid tissues in patients with lymphoplasmacytic infiltration with EBV reactivation (63).

In primary EBV infection, some infected cells become lytic. During infectious mononucleosis (IM), in the acute phases, defined as the symptomatic EBV primary infection, different autoantibodies are present in the serum of patients (64). EBV primary infection and lytic infection induce the production of antibodies. If antibodies are produced in subclinical EBV

infection, TRAb(+) EBV(+) cells or serum TRAb can be assessed in healthy children at the EBV primary infection. A paper (64) evaluated TRAb production during EBV primary infection or lytic reactivation even in absence of symptoms, to investigate the time-dependent expansion of TRAb(+) cells, EBV(+) cells, or TRAb(+) EBV(+) cells in PBMCs in 29 normal or subclinical children without GD and one cord blood. Children without symptoms of IM had low levels of TRAb in EBV primary infection and lytic reactivation. Moreover, during the primary infection, TRAb(+) cells, EBV(+) cells, and TRAb(+) EBV(+) cells were small, but they expanded during EBV lytic reactivation. EBV infection reactivation might be associated with B lymphocytes expansion, and GD recurrence in Japanese patients (64).

The above mentioned paper suggests that EBV reactivation might be associated with GD in Japanese patients. However, it has to be underlined that there are marked geographical differences in the epidemiology of EBV-associated diseases, in fact epidemic Burkitt lymphoma is present in Africa and Middle East, and nasopharynx cancer in East Africa and East Asia. So, the importance of EBV in the pathogenesis of GD in Caucasian patients has not yet been demonstrated.

4.4 Chronic Hepatitis C virus (HCV) infection and AITD

Hepatitis C Virus (HCV) is responsible for both hepatic and extrahepatic diseases (HCV-related extrahepatic diseases = HCV-EHDs). The last ones include organ- and non-organ-specific immunological disorders and malignancies (65). Type 2 diabetes mellitus (T2D) and thyroid disorders are the most frequent and clinically important endocrine HCV-EHDs.

In patients with chronic HCV hepatitis (CHC) and receiving low doses of interferon (IFN)-α, thyroid dysfunction (TDs) are frequent. In 1992, Pateron et al. (66) evaluated if the TD observed during treatment with IFN-α for CHC could also derive from the aggravation of latent autoimmune thyroiditis. The Authors suggested that IFN-α-induced TD could be due to

the exacerbation of a previous latent autoimmune thyroiditis and that patients with ATA are at high risk of overt TD during IFN-α therapy (66).

The association between CHC and presence of ATA has been confirmed by another study (67), in which ATA were measured in 72 patients with CHC (43 men and 29 women) before IFN-α therapy, and in 60 chronic HBsAg-positive patients (34 men and 26 women), as controls. In CHC patients, no men had ATA, while 9/29 women had ATA, whereas in controls only 1 man had thyroid microsomal antibody (TMA), at a very low titer. Among CHC, 6 (20.7%) had elevated titers of ATA, and 2 were hypothyroid. In all the 9 CHC women, HCV viremia was detected. One year later, titers of ATA had increased in 1 CHC patient, 3 progressed to hypothyroidism, and 4/29 patients (13.8%) showed HT (67).

The prevalence of HCV antibodies and viraemia has been studied also in the serum of 39 patients with thyroid autoimmunity, of whom 18 with GD. Four GD patients were positive for HCV at confirmatory ELISA test, and HCV was detected in the serum of 3/4 subjects, all of whom had GD (68). This study suggested a possible association between GD and CHC.

In another study 215 HCV seropositive patients were screened for TD, and AbTPO and AbTg antibodies (69). Eighteen patients (8%) had ATA, 12 with AbTPO (5.6%) and 10 with AbTg (4.7%). Five patients (2.3%) had hyperthyroidism, and among them 3 had GD. The Authors concluded that the prevalence of AbTPO and AbTg antibodies was similar to the prevalence usually observed in the general population and did not suggest a pathogenic role of HCV in AITD (69).

In another study, serum TMA and TSH levels were measured in 130 patients with CHC, 130 age/sex (+/- 2 years) matched patients with chronic hepatitis B virus (HBV) infection, and 260 matched healthy subjects (70). The prevalence of ATA in male patients with CHC was <2%. Female patients with CHC had a significantly higher prevalence of TMA (<1:400) than HBV patients (22.1 vs. 1.6%; $P<0.001$), and higher but not significantly vs. controls (13.5%).

Among the 23 HCV patients seropositive for ATA, 7 had HT, 2 had GD and 3 had received subtotal thyroidectomy. During follow-up, 4/15 female patients had a 14-16-fold increase in TMA titre, and hyperthyroidism occurred in one case. These data suggested a quite elevated prevalence of thyroid autoimmunity in CHC patients (70).

The largest study about the association of HCV infection and thyroid disorders was published in 2004, and it investigated the prevalence of thyroid disorders in 630 patients with CHC, 389 control subjects from an iodine-deficient area, 268 from an iodine-sufficient area, and 86 patients with chronic HBV (71). Mean TSH levels were higher, and free T3 (FT3) and free T4 (FT4) were lower, in patients with CHC than in the other groups. Patients with CHC were more likely to have hypothyroidism, AbTg, and AbTPO than the other groups of patients, suggesting that hypothyroidism and thyroid autoimmunity are more common in patients with CHC than in normal controls or those with chronic HBV infection (71).

A very recent meta-analysis (published in 2016) evaluated the controversial topic about the involvement of thyroid autoimmunity and dysfunction in patients with CHC before IFN-α therapy (72). Twelve studies were included, involving 1,735 HCV-infected and 1,868 non-HCV infected subjects. The pooled OR for AbTg was 2.40 (95% CI 1.85-3.13), for AbTPO was 1.96 (95% CI 1.19-3.23), for hypothyroidism was 3.10 (95% CI 2.19-4.40), for hyperthyroidism was 1.10 (95% CI 0.71-1.57), suggesting that CHC may be an independent risk factor for AITD and hypothyroidism but not for GD (72).

To summarize, the frequency of elevated levels of ATA in HCV-patients varies strongly, from 8% to 48%. Thyroid disorders and serum ATA were generally more prevalent in HCV-patients than in those with type B hepatitis or uninfected subjects. HCV-patients were more inclined to develop hypothyroidism (13%), AbTg (17%) and AbTPO (21%), than subjects in the control groups (71).

Clinical and subclinical hypothyroidism were reported in 5–13% of HCV-patients. Data from

literature agree that females have a higher risk to develop AITD, whilst major risk factors for the onset of hypothyroidism are the female sex and the presence of AbTPO (73). Patients with HCV-related MC have also a higher prevalence of GD than controls. Differences in genetic variability within the studied populations, and environmental factors (i.e. iodine intake) could play an important role in AITD development.

According to the above reported data, HCV is associated with thyroid autoimmunity and hypothyroidism, but not with GD.

4.4.1 HCV-related mixed cryoglobulinemia (MC)

MC is a systemic vasculitis, involving peripheral nerves, skin, certain internal organs, and joints. HCV is considered the etiologic agent for >90% of MC cases, and for autoimmune, lymphoproliferative, and neoplastic disorders. In humans HCV-related MC is considered a model of virus-triggered autoimmune/neoplastic diseases (74).

HCV is a hepatotropic and lymphotropic agent, responsible also for various disabling extrahepatic diseases and, especially, B-cell lymphoproliferative disorders (LPDs) (75). HCV-related LPD pathogenesis is a multifactorial and multistep process, in which it is determinant the cooperation between direct or indirect action of viral particles or proteins, and antiapoptotic mechanisms acting on B-cells, determining the expansion of B lymphocytes. The final evolution to MC could be caused by a predisposing genetic background and specific environmental factors. The progressive addition of genetic aberrations would lead to a frank neoplastic transformation, i.e. B-cell non-Hodgkin's lymphoma (NHL) (75).

The prevalence of thyroid disorders has been investigated in 93 patients with HCV-associated MC (HCV+MC), 93 patients with CHC (without MC) and 93 HCV-negative controls (76). It was shown that the percentage of patients with AITD was 35% in MC, 22% in CHC, 16% in controls, and hypothyroidism was in 11%, 7%, and 2%, respectively, indicating that the

prevalence of thyroid disorders is increased in patients with HCV-related MC. The percentage of patients with hyperthyroidism was higher in MC patients (10% vs. 4% in CHC or controls), even if not significantly (76).

A prospective study evaluated the prevalence of other autoimmune diseases in 3209 patients with GD (984 with GO), with respect to 1069 healthy control subjects, or 1069 AT patients, or 1069 with multinodular goiter (MNG) (77). GD, and MNG patients had a significantly larger thyroid volume. GD, and AT patients had a hypoechoeic and hypervascular thyroid. All GD patients were hyperthyroid, whilst 24% of AT patients were hypothyroid; MNG patients and controls had no TD. In GD, and AT, AbTPO (65% and 84%, respectively), or AbTg (54% and 75%, respectively) autoantibodies were shown. TRAb were present in 87% of GD patients. HCV-related MC was present in 14/3209 GD patients, in 6/1069 AT, in 0/1069 controls or MNG patients (P=0.0153) (77). This finding first suggested that HCV infection in MC is a risk factor for GD. To our knowledge, this is the first demonstration of an association between GD and a specific infection.

In conclusion, HCV-related MC is associated with thyroid autoimmunity and hypothyroidism (such as CHC), but MC patients have a higher risk for GD, too.

4.4.2 IFN-α, HCV and thyroid autoimmunity

Patients with hepatitis C treated with IFN-α develop TD in 2.5–20% of cases, mainly AITD. Females and subjects with AbTPO are at higher risk of developing TDs upon IFN-α treatment, including autoimmune primary hypothyroidism, Graves' hyperthyroidism, and destructive thyroiditis, with hypothyroidism being the most common side effect. In about 50% of cases, TDs resolve within 6 months after the end of IFN-α treatment (78).

A retrospective analysis was performed in CHC patients treated with IFN-α or pegylated IFN-α (PEG-IFN)±ribavirin (RBV). Among 511 patients, 45 cases with TD were reported (8.8%).

PEG-IFN was associated with higher rates of TD than IFN (P=0.0029) (79).

Furthermore, in chronic hepatitis B, the frequency of pre-treatment ATA and the induction of ATA and TD during IFN-α therapy, are all lower than in chronic hepatitis C. This suggests that the increase of TD in IFN-α treated HCV-patients could not only depend on the effect of the drug *per se*, but also on the effect of the IFN-α on a predisposed immunological background present in HCV-patients. Since IFN-α has a direct inhibitory effect on the synthesis and production of thyroid hormones, the increased half-life resulting from pegylation could enhance this effect (78).

Nevertheless, a meta-analysis compared PEG-IFN with standard IFN-α, and no differences in TD were found (80).

A retrospective study evaluated the incidence of TD in a UK cohort of patients with CHC treated with IFN-α and ribavirin combination therapy (IFN/RBV) (81). Among 260 patients, 22.3% developed TD during the therapy: a suppressed serum TSH in 10.4% of patients (0.8% GD, 9.6% transient thyroiditis), an elevated circulating TSH in 11.9%, with 1.5% becoming permanently hypothyroid and requiring levothyroxine treatment. In studies of adult subjects, the incidence of TD associated with IFN/RBV differs strongly between countries (the lowest incidence has been reported in Brazil and the highest in Poland) (81).

A more favorable course of GD in patients with CHC treated with IFN-α compared with GD occurring in patients without CHC was shown (82). One hundred-seventy five patients with CHC were considered because of the discovery of TD during treatment with IFN-α. These patients represented a cohort of 7.3% of 2400 CHC patients who were treated with IFN-α during the same period. From 1999 to 2000, patients were treated with recombinant IFN-α at a dose of 10.6 mg/kg per day. Then, they were treated with a recombinant PEG-IFN at a dose of 1-1.5 Kg/kg once a week together with RBV (10.6 mg/kg per day). Of these 175 IFN-treated patients, 23 were treated with IFN-α as monotherapy; 31, with IFN-α + RBV; and 121,

with PEG-IFN + RBV. After exclusion of patients with hyperfunctioning thyroid nodules or a reduced iodine uptake or recurrences of thyroid disease, 39 of these patients (group 1), who were found to be affected by GD, were considered for analysis and were matched with 43 subjects (group 2) with a spontaneous occurrence of GD, which required treatment. In group 1, 7 patients were treated with IFN-α as monotherapy; 10, with IFN-α + RBV; and 22, with PEG-IFN + RBV. No patients of this group discontinued IFN-α treatment. In both groups, treatment with methimazole (MMI) was started at an initial dose of 10-20 mg/d. Two patients of group 1 and 4 patients of group 2 were given an initial dose of 25 mg/d of MMI because of the severity of the clinical picture (82). No differences were found in TSH and free thyroid hormone levels or autoantibody concentrations. Thyroid volume as well as both 6- and 24-hour iodine uptake were comparable between the groups. As expected, serum levels of both AST and ALT were significantly higher in patients with CHC. The daily dose of MMI was found to be lower in group 1 vs. group 2 ($P<0.01$), as also the duration of MMI treatment ($P<0.01$). The remission rate from GD was higher in the patients of group 1 compared to the patients of group 2 ($P<0.005$). The reported data demonstrated that the course of GD is mild in patients treated with IFN-α (82).

5. Immune-pathogenesis of AITD in chronic HCV infection, cryoglobulinemia, and IFN-α treatment

The pathogenesis of AITD has not yet been completely described. Among the involved triggers (i.e. iodine, medications, infections), a strong association of AITD with HCV infection and IFN-α has been reported. Furthermore, HCV and IFN appear to act in synergism to induce AITD. Actually, clinical or subclinical disease occurs in about 40% of HCV patients during IFN-α therapy. IFN-induced thyroiditis can be of a non-autoimmune type (such as destructive thyroiditis, or non-autoimmune hypothyroidism), or autoimmune

thyroiditis (with clinical features of GD or HT) (83).

A paper evaluated whether HCV envelope proteins could induce thyroidal inflammation and whether the major HCV receptor CD81 was expressed and functional on thyrocytes (84). To assess the ability of E2 protein to bind to thyrocytes surface, primary human thyrocytes or Huh7.5 cells (as control) were cultured in presence of recombinant E2 protein. Huh7.5 cells and primary human thyrocytes showed E2 binding of 45% and 27%, respectively. Moreover, primary human thyrocytes or Huh7.5 cells were cultured with 5 mg/ml of E2 for 48 h, and IL-8 levels were quantified. After E2 treatment, IL-8 increased significantly in both cell types: in Huh7.5 cells of 10%, while a stronger increase was shown in human primary thyroid cells. The above mentioned data indicate that E2 can bind to CD81 on thyrocytes and induce IL-8 release (84).

The hypothesized mechanism for the development of IFN-induced thyroiditis involves both autoimmune and non-autoimmune actions of IFN-α. The first activate the immune cells and lead to the switch to Th1 pathways, downregulate Treg cells, and induce cytokine secretion and MHC I expression. Direct toxic effects are the upregulation of thyroid-specific proteins [TSH-R, Tg, TPO, sodium/iodide symporter], the induction of heat shock proteins, and thyrocytes death. Combining both types of effects can lead to the release of thyroid autoantigens and their presentation to resident T-cells, initiating an autoimmune response (85).

A paper investigated the interactions between HCV and the thyroid at a cellular level, evaluating whether a human thyroid cell line (ML-1) could be infected productively *in vitro* with HCV (86). ML-1 cells expressed strongly CD81 on their surface. The incubation with an anti-CD81 antibody inhibited efficiently virions JFH1 infection of ML-1 cells, and JFH1 infection of Huh7.5 cells. Scavenger receptor class B type 1 (SR-B1/CLA1) plays an important role in HCV entry into hepatocytes. Anti-CLA1 antibody inhibited JFH1 infection,

though the inhibition was less pronounced in ML-1 cells vs. Huh7.5 cells (86). HCV entry into ML-1 cells depended on CD81 and SR-B1/CLA1, whereas IFN-α inhibited HCV replication in ML-1 cells dose-dependently. Supernatants from HCV-infected ML-1 cells could infect fresh ML-1 cells, indicating the possible *in vivo* transfer of infectious virions from infected to naive thyroid cells. Moreover, HCV infection of ML-1 cells led to an elevated expression of IL-8. These data suggested that HCV infection of thyroid cells may have a key role in the association between thyroid autoimmunity and chronic HCV infection (86).

The above reported data have been confirmed also by another study (87). Human ML-1 cells and human primary thyrocytes were cultured with HCV recombinant E2 protein, and the mRNA and protein levels of the major proinflammatory cytokines were measured. In human primary thyrocytes, a strong increase in IL-6 (about 5-fold) and IL-8 (about 4-fold) mRNA expression was observed. The expression of TNFα mRNA was increased by about 2-fold. The cytokine response to E2 protein was higher in primary thyrocytes than in ML-1, such as the increase in TNFα. In human primary thyrocytes, protein levels of cytokines had a similar response to that of mRNA expression. The release of TNFα, IL-8, and IL-6 in cells cultured with E2 protein, vs. those in absence of E2, increased rapidly and remained significantly higher for up to 7 days (87). These data showed that the interaction between E2 and CD81 on human thyrocytes, independently from HCV entry into the cells, modulated a cascade of intracellular signals, leading to the activation of pathways involved in immune responses to viral infection, and pathways that participate in thyroid autoimmunity. This supports a role for HCV in triggering autoimmune thyroiditis through bystander mechanisms (87).

Many studies correlated Th1 immune response with thyroid autoimmunity (1,88), HCV infection, or diabetes. A potential common immunological Th1 pattern could be the pathophysiological basis of the association of HCV-EHDs, with T2D and thyroid

autoimmunity (89).

The activation of cellular innate immune pathways depends on the recognition of foreign DNA, RNA, or protein motifs, known as pathogen associated molecular patterns (PAMPs). Specific PAMPs are recognized by innate pattern recognition receptors (PRRs) belonging to 1 among 3 families: Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), or Nod-like-receptors (NLRs). The interplay of these receptors and their downstream signaling pathways determines the resultant innate immune response (90). Following HCV infection in hepatocytes, the positive sense HCV RNA genome is recognized by 2 different PRRs within the hepatocyte: Toll-like receptor 3 (TLR3), and the retinoic acid inducible gene 1 (RIG-I). After the binding of PAMP, a conformational change of RIG-I occurs, that permits it to bind the mitochondrial antiviral-signaling protein (MAVS) signaling adaptor, while activated TLR3 binds the signaling adaptor TIR-domain-containing adapter-inducing IFN-β (TRIF). MAVS and TRIF activate different transcription factors [i.e. nuclear factor (NF)-κB, C/EBP-β, activator protein (AP)-1, and IFN regulatory factors (IRFs), whose binding sites are supposed to be in the CXCL10 promoter] that translocate into the nucleus inducing gene transcription (90).

In CHC, the HCV and intrahepatic production of IFN-γ cause an elevated CXCL10 expression by sinusoidal endothelium and hepatocytes, and recruit CXCR3-expressing T cells into the liver. Pretreatment CXCL10 levels can predict early virological response and sustained virological response (SVR) to IFN-α and RBV, and permit to evaluate candidates for treatment. The occurrence of single-nucleotide polymorphism (SNP) adjacent to IL-28B (rs12979860, rs12980275, and rs8099917), and CXCL10 levels below 150 pg/mL, independently predicted the early viral decline and rapid virological response, that independently predicted SVR (91).

Moreover, the presence of HCV in the thyroid tissue of HCV-patients has been demonstrated

(92,93).

A paper evaluated CXCL10 levels in patients with CHC in presence/absence of autoimmune thyroiditis (AT) (94). CXCL10 was significantly higher: in AT patients than controls without AT ($P<0.001$); in patients with CHC than controls without AT, and patients with AT ($P<0.001$); in patients with CHC and AT than controls without AT, and patients with AT ($P<0.001$), and hepatitis C ($P=0.004$). This suggests a stronger Th1 immune response in these patients (94).

Moreover, another paper showed higher serum CXCL10 levels in 60 patients with "mixed cryoglobulinemia and hepatitis C virus chronic infection" (MC) in the presence of AT, vs. 45 control subjects with AT ($P<0.0001$). CXCL10 levels were elevated also in patients with MC without AT, with values similar to those found in CHC without cirrhosis, suggesting that in MC patients without AT, CXCL10 serum levels are mainly sustained by the liver HCV chronic infection (95). Serum CXCL10 levels were significantly higher in MC patients in presence of AT vs. MC patients without AT; the presence of thyroiditis increases serum CXCL10 levels in MC, suggesting an evident predominance of Th1 immune response in the presence of that specific clinical association (95,96). According to the above reported data, it could be speculated that HCV thyroid infection may upregulate CXCL10 gene expression and secretion in thyroid cells (as demonstrated in human hepatocytes), which recruit Th1 lymphocytes, that secrete IFN-γ and TNF-α, which induce CXCL10 secretion by thyroid cells, reiterating the immune cascade, that may lead to the onset of AITD in genetically predisposed individuals (89).

We have recently demonstrated in primary human thyroid cells obtained from patients with CHC that the stimulation with IFN-γ plus TNF-α induced a more potent secretion of CXCL10 (about 3400±560 pg/ml) with respect to the same dose of IFN-γ plus TNF-α in primary thyrocytes not infected by HCV.

This finding reinforces the above mentioned data about the importance of Th1 chemokines in the pathogenesis of AITD induced by HCV.

To summarize, the etiopathogenesis of HCV syndrome involves both hepatic and extrahepatic diseases, deriving from a multifactorial process which involves chronic HCV infection, other environmental factors, genetical host predisposition (hormonal and/or metabolic factors, HLA alleles, etc.), and cellular and molecular alterations (97). Different pathogenetic mechanisms coexist: HCV-driven immune system alterations with "benign" lymphoproliferation and autoantibodies production, and oncogenetic alterations causing frank B-cell neoplasias and other malignancies (B-NHL, HCC, and papillary thyroid cancer). Various organ- and non-organ-specific autoimmune/neoplastic disorders can occur in the same HCV-infected patient, among which TD (97,98). The presence of HCV antigens (i.e. envelope E2, core, NS3, NS4, NS5A proteins) could exercise a chronic stimulus on the host immune system. Determinant pathogenetic processes involve the binding, with a high affinity, between HCV-E2 and CD81 and the following t(14;18) translocation with bcl-2 proto-oncogene activation, cross-reaction between certain HCV antigens and host autoantigens (molecular mimicry mechanism), and HCV direct cell infection causing neoplastic cell transformation. In patients with CHC, the benign B-cell proliferation with production of various autoantibodies (i.e. rheumatoid factor and cryo- and non-cryoprecipitable immune complexes) is frequently observed. In this setting different organ- and non-organ-specific autoimmune reactions may develop, including TD with/without MC syndrome or cryoglobulinemic vasculitis (97).

In particular, it has been hypothesized that HCV can enter into thyrocytes inducing the production of Th1 chemokines (CXCL9, CXCL19, and CXCL11) and other cytokines/chemokines (IL-8, etc.) that recruit Th1 lymphocytes into the thyroid inducing the appearance and the perpetuation of the autoimmune inflammation in the thyroid gland itself, leading to the appearance of circulating thyroid autoantibodies and of hypothyroidism (99-

102).

Moreover, in patients with MC, the presence of an expansion of B lymphocytes population is associated with the appearance of different types of autoantibodies (rheumatoid factor, cryoglobulins, etc.) and also with the appearance of TSAb, that leads to an increased prevalence of GD.

On the basis of the above mentioned studies, the most recent international diagnostic and therapeutic guidelines for patients with HCV-related extrahepatic disorders suggest that the assessment of thyroid function and thyroid autoantibodies are necessary in patients with CHC or MC, both basally or during/after the IFN treatment (103,104).

6. Conclusion

Graves' disease (GD) is an organ-specific autoimmune disorder associated with the presence of circulating TSH-R autoantibodies, and it is the most common cause of hyperthyroidism in developed countries, with an annual incidence of 20 cases/100,000 persons (1,2).

Although GD may affect anyone, it is more common between 30 and 60 years of age and is 5-10 times more frequent in women than in men. In fact, the lifetime risk is 3% for women and 0.5% for men. GD is associated with extrathyroidal manifestations, such as GO and dermopathy. The annual incidence of GO is about 16 cases/100,000 women and 3 cases/100,00 men, with the age of appearance between 40 and 60 years (2).

The GD risk factors are not completely clear, and they include genetic predisposition, and interactions between endogenous and environmental factors.

The genetic predisposition accounts for about 79% of the risk for GD, while environmental factors for about 21%.

Considering the genes correlated with the risk of AITD, about 70% of those whose effects are known, are implicated in T cell function, indicating the importance of T lymphocytes in

AITD pathogenesis.

Among endogenous factors, GD predominance in females suggests the importance of estrogen in its pathophysiology. Estrogens and X-inactivation alter the immune system and can contribute to the predominance of GD, such as microchimerism.

Among environmental risk factors, an iodine excess is associated with the increased risk of GD. Selenium and vitamin D deficiency have been also associated with GD, and several studies have been conducted to evaluate the effect of selenium or vitamin D supplementation on the prevention or treatment of GD, with controversial results.

Smoking has multiple effects on the thyroid gland, and it is associated with the increased risk and severity of GD and ophthalmopathy.

Vietnam Veterans exposed to the Agent Orange were more frequently affected by GD.

Many studies have evaluated if GD development may be triggered by infectious agents [including Foamy Viruses, Parvovirus-B19, Epstein-Barr virus, Hepatitis C Virus (HCV)] even if with discordant results for FV, and Parvovirus-B19. EB-V reactivation seems to be associated with GD recurrence in Japanese patients; however, no evidence is present for Caucasian ones. Many studies have shown that HCV is associated with thyroid autoimmunity and hypothyroidism, in CHC patients; and most recently a significant link has been shown between HCV-related MC and the risk of GD. Moreover, patients with CHC treated with IFN-α develop TD and GD in 2.5–20% of cases. A more favorable course of GD in patients with CHC treated with IFN-α compared with GD occurring in patients without chronic HCV hepatitis was shown.

The Th1 immune response prevails in the immunopathogenesis of GD and GO, during which Th1 chemokines, and their (C-X-C)R3 receptor, play a key role.

In particular, it has been hypothesized that HCV can enter into thyrocytes inducing the production of Th1 chemokines (CXCL9, CXCL19, and CXCL11) and other

cytokines/chemokines (IL-8, etc.), that recruit Th1 lymphocytes into the thyroid, inducing the appearance and perpetuation of the autoimmune inflammation in the gland itself, leading to the appearance of circulating thyroid autoantibodies and of hypothyroidism.

Moreover, in patients with MC, the presence of an expansion of B lymphocytes population is associated with the appearance of different types of autoantibodies (rheumatoid factor, cryoglobulins, etc.) and also with the appearance of TSAb, that leads to an increased prevalence of GD.

Further researches are necessary to identify novel risk factors of GD, in order to reduce its occurence in West Countries.

Conflict of Interest

The Authors have nothing to declare.

Role of the funding source

The Authors have nothing to declare.

Summary

Graves' disease (GD) is an organ-specific autoimmune disorder, associated with the presence of circulating TSH-R autoantibodies, and it is the most common cause of hyperthyroidism in developed Countries.

The GD risk factors include genetic predisposition, and interactions between endogenous and environmental factors. About 70% of genes associated with autoimmune thyroid disorders (AITD) are implicated in T-cell function. Among GD endogenous factors, estrogens, X-inactivation and microchimerism are important. Among environmental risk factors, smoking, iodine excess, selenium and vitamin D deficiency, and the occupational exposure to Agent Orange have been associated with GD.

Among viruses, EB-V reactivation seems to be associated with GD recurrence in Japanese patients; however, no evidence is present for Caucasian ones. Many studies showed that HCV is associated with thyroid autoimmunity and hypothyroidism, in patients with chronic HCV hepatitis (CHC); a significant link has been shown also between HCV-related mixed cryoglobulinemia and risk for GD. Moreover, IFN-α-treated CHC patients develop GD more frequently.

The Th1 immune response prevails in the immunopathogenesis of GD and GO, during which Th1 chemokines, and their (C-X-C)R3 receptor, play a key role. In particular, it has been hypothesized that HCV can enter into thyrocytes inducing the production of Th1 chemokines (CXCL9, CXCL19, and CXCL11) and other cytokines/chemokines (IL-8, etc.), that recruit Th1 lymphocytes into the thyroid, inducing the appearance and perpetuation of the autoimmune inflammation in the gland itself, leading to the appearance of circulating thyroid autoantibodies and of hypothyroidism.

Further researches are necessary to identify novel risk factors of GD, and novel studies are needed about possible risk factors to reduce the occurence of GD in West Countries.

Practice Points

- Graves' disease (GD) is an organ-specific autoimmune disorder leading to the overproduction of thyroid hormones (hyperthyroidism).
- GD is one of the main autoimmune thyroid disorders (AITD), that are characterised by the breakdown of immune tolerance against thyroid antigens.
- The GD risk factors are not completely clear, and they include genetic predisposition, and interactions between endogenous and environmental factors.
- The genetic predisposition accounts for about 79% of the risk for GD, while environmental factors for about 21%.
- Among GD endogenous factors, estrogens, X-inactivation and microchimerism are important.
- Among environmental risk factors, smoking, iodine excess, selenium and vitamin D deficiency, and the occupational exposure to Agent Orange have been associated with GD.
- Many studies have evaluated if GD develpment may be triggered by infectious agents [including Foamy Viruses, Parvovirus-B19, Epstein-Barr virus, Hepatitis C Virus (HCV)] even if with discordant results for FV, and Parvovirus-B19. EB-V reactivation seems to be associated with GD recurrence in Japanese patients; however, no evidence is present for Caucasian ones. HCV is associated with thyroid autoimmunity and hypothyroidism, in CHC patients; and most recently a significant link has been shown between HCV-related MC and the risk of GD. Moreover, patients with CHC treated with IFN-α develop TD and GD in 2.5–20% of cases.
- It has been hypothesized that HCV can enter into thyrocytes inducing the production of Th1 chemokines (CXCL9, CXCL19, and CXCL11) and other cytokines/chemokines (IL-8, etc.), that recruit Th1 lymphocytes into the thyroid, inducing the appearance and perpetuation of the autoimmune inflammation in the gland itself, leading to the appearance

of circulating thyroid autoantibodies and of hypothyroidism. In patients with MC, the presence of an expansion of B lymphocytes population is associated with the appearance of different types of autoantibodies (rheumatoid factor, cryoglobulins, etc.) and also with the appearance of TSAb, that leads to an increased prevalence of GD.

Research agenda

- The mechanisms that induce the breaking of immune tolerance in GD are still unclear.
- Further researches are necessary to identify novel risk factors of GD, in order to reduce its occurence in West Countries.

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