Increase of interferon-γ inducible α chemokine (C-X-C motif) ligand (CXCL)9 and CXCL11 serum levels in patients with active Graves' disease, and modulation by methimazole therapy.

Alessandro Antonelli, MD[1], Silvia Martina Ferrari, MSc[1], Alda Corrado, MSc[1], Ele Ferrannini, MD[1], Poupak Fallahi, MD[1].

[1] Department of Clinical and Experimental Medicine, University of Pisa, Via Savi 10, 56126, Pisa, Italy

Alessandro Antonelli: alessandro.antonelli@med.unipi.it
Silvia Martina Ferrari: sm.ferrari@int.med.unipi.it
Alda Corrado: corradoalda@gmail.com
Ele Ferrannini: ferranni@ifc.cnr.it
Poupak Fallahi: poupak@int.med.unipi.it

***Running title*:** Serum CXCL9 and CXCL11 in Graves' disease.

***Keywords*:** CXCL9, CXCL11, CXCL10, Graves' disease, hyperthyroidism, autoimmunity, chemokines.

Abstract

Background: Chemokine (C-X-C motif) ligand (CXCL)9 and CXCL11 play an important role in the initial phases of autoimmune thyroiditis (AT); however their serum levels in patients with Graves' disease (GD) have never been evaluated in relation to thyroid function and treatment.

Methods: To evaluate CXCL9 and CXCL11 serum levels in GD, to relate these parameters to the clinical phenotype, we measured CXCL9 and CXCL11 serum levels in 91 GD patients, 91 AT, 34 non-toxic multinodular goiters (MNG), 31 toxic nodular goiters (TNG) and 91 healthy controls (age- and sex-matched).

Results: Mean CXCL9, or CXCL11, levels were higher in GD, in comparison with controls, or euthyroid AT, or MNG, or TNG (*$p < 0.05$, ANOVA; CXCL9: 274±265, *76±33, *132±78, *87±48, *112±56 pg/mL; CXCL11: 140±92, *64±20, 108±48, *76±33, *91±41 pg/mL; respectively). Hyperthyroid GD had significantly higher CXCL9 or CXCL11 than euthyroid or hypothyroid GD. GD with untreated hyperthyroidism had higher CXCL9 or CXCL11 than hyperthyroid or euthyroid GD under methimazole (MMI) treatment. Comparable CXCL9 and CXCL11 levels were observed in newly diagnosed untreated hyperthyroid GD vs. untreated patients with relapse of hyperthyroidism after a previous MMI course.

Conclusions: Serum CXCL9, and CXCL11, levels are associated with the active phase of GD both in newly diagnosed and relapsing hyperthyroid patients. The reduction of serum CXCL9 and CXCL11 levels in treated patients with GD may be related to the immunomodulatory effects of MMI.

Introduction

The impressive complexity of the immune system involved in autoimmune disorders has been partially clarified in the last years. Briefly, the production of interleukin (IL)-12 promotes the development of T helper (Th)1 cells producing interferon (IFN)-γ, IL-2, and TNF-α, which activate macrophages and are responsible for cell-mediated immunity and phagocyte-dependent protective responses. By contrast, the production of IL-4 favors the development of Th2 cells producing IL-4, IL-5, and IL-13, which are responsible for strong antibody production, eosinophil activation, and inhibition of several macrophage functions, thus providing phagocyte-independent protective responses.

Th1 cells tend to produce the proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses, whereas Th2 cells are associated with the promotion of humoral immunity and of IgE and eosinophilic responses. The production of transforming growth factor (TGF)-β and IL-6 promotes the development of Th17 cells, a distinct type of effector T cell that induces tissues damage. Once Th17 cells are established, IL-23 also participates in their maintenance. Treg cells, which inhibit autoimmunity and protect against tissue injury, are induced by TGF-β in the absence of IL-6. Thus, TGF-β functions as a regulator of tissue-damaging Th17 cells when collaborating with IL-6 and as an activator of anti-inflammatory Treg cells when acting without IL-6 (1).

Chemokines are defined as small (8–15 kDa) proteins that induce chemotaxis and in some instances, modulate the functional properties of different leucocytes during inflammation. Chemokines are grouped into four distinct families according to the number and spacing of two conserved N-terminal cysteine residues. Two chemokine families have multiple members: the CXC (two N-terminal cysteines separated by a single amino acid) and the CC (two N-terminal cysteines adjacent) family. The remaining CX3C and the C families each

contain a single member only, named CX3CL1 (fractalkine) and XCL1 (lymphotactin), respectively. The CXC chemokines are additionally subdivided into those that contain a glutamic acid-leucine-arginine (ELR) motif near their N-terminus (e.g. CXCL1 and CXCL8), and those that do not contain this motif. The non-ELR CXC chemokines can be further subgrouped based on their structure and target receptor. Three structurally related chemokines comprise the IFN-inducible non-ELR CXC chemokine subgroup: chemokine C-X-C motif ligand (CXCL)9, CXCL10 and CXCL11 (1-5).

A number of properties distinguish CXCL9, 10 and 11 from the other non-ELR CXC chemokines and show that these chemokines are closely related. First, these molecules exhibit significant structural homology being more similar to each other than to any of the other non-ELR CXC chemokines (2). Second, the genes for these chemokines are all highly inducible by IFN-γ. Third, all three chemokines share the ability (albeit to varying degrees) to promote the directional migration of activated and memory, but not naive T cells. Finally, CXCL9, 10 and 11 all bind to a common receptor named CXCR3. Thus, these chemokines are considered appropriately as a distinct subfamily (3-5).

Two distinct domains that contributed to CXCR3 internalization were identified. The carboxyl-terminal domain and beta-arrestin1 were predominantly required by CXCL9 and CXCL10, and the third intracellular loop was predominantly required by CXCL11 (6).

CXCR3 chemokines play an important role in the initial phases of Graves' disease (GD) and autoimmune thyroiditis (AT) (1).

The CXC α chemokines inducible by IFN-γ, CXCL9, CXCL10, CXCL11, are associated with Th1-mediated immune responses, and among them CXCL10 is a prototype and its serum levels are increased in several endocrine autoimmune conditions (7-10).

Recent experimental evidences have demonstrated that CXC chemokines and particularly CXCL10 play an important physiopathological role in the initial phases of autoimmune thyroid disorders (AITD) (8, 11, 12).

Expression of CXCL10 and CXCL9 was poor or absent in normal thyroid tissue, while both the chemokines and their receptor were present in most thyroid glands of patients affected by GD. CXCL10 and CXCL9 localized to infiltrating lymphocytes and macrophages, as well as to resident epithelial follicular cells. Of note, maximal expression of CXCL10 and CXCL9 was found in the thyroid gland of patients with recent-onset GD and correlated with IFN-γ (8, 13). At the same time, it was shown that human thyrocytes in primary culture produce large amounts of CXCL10 when stimulated by IFN-γ (11).

We have previously shown that CXCL10 is associated with the active phase of GD both in newly diagnosed and relapsing hyperthyroid patients, and that the reduction of circulating CXCL10 in treated patients with GD may be related to the immunomodulatory effects of methimazole (MMI) (14, 15).

Increased expression of CXCL10 and CXCL9 was also observed in thyroid tissue specimens obtained from subjects affected by AT by immunohistochemistry (11), and high levels of CXCL9 and CXCL11 have been recently shown in patients with AT, in particular in the presence of hypothyroidism (16-18). Furthermore, we have recently shown that IFN-γ and TNF-α are able to induce the secretion of the CXCL9 and CXCL11 chemokines in thyrocytes and fibroblasts of patients with GD and ophthalmopathy (19-21).

Briefly, it has been hypothesized that Th1 cells secreting these chemokines (CXCL9, 10, 11) were presumably originally attracted to the thyroid gland because of the thyroid autoantigens. In the thyroid, Th1 cells produce cytokines (such as IFN-γ and TNF-α) that can modulate the autoimmune response inducing the production of CXCL9, CXCL10 and CXCL11 chemokines not only by lymphocytes, but also from thyrocytes. These chemokines induce the

migration of other Th1 lymphocytes into the thyroid, which in turn, secrete more IFN-γ and TNF-α, stimulating further the chemokine production by the target cells, thus initiating and perpetuating the autoimmune cascade.

To our knowledge, no study has evaluated systematically the IFN-γ inducible CXCL9 and CXCL11 chemokines in patients with GD in relation to thyroid function and treatment. The aim of the present study therefore was to measure serum CXCL9 and CXCL11 levels in patients with GD and to relate the findings to the clinical phenotype, in order to assess the potential benefit of routine assessment of these chemokines in the clinical management of such patients.

Materials and Methods

Patients

From the outpatient clinic, we prospectively studied 91 consecutive Caucasian patients with GD, without clinical signs or symptoms of Graves' ophthalmopathy (**Table 1**). The patients were referred to us by general practitioners or other hospitals because of the presence of hyperthyroidism or of circulating thyroid autoantibodies, or clinical suspicion of a thyroid disorder. The diagnosis of GD (14, 15) was established from the clinical presentation (presence of a diffuse goiter, varying in size from normal to very large), thyroid hormones and thyroid autoantibodies measurements [presence of antithyrotropin-receptor autoantibodies (TRAb), and/or thyroid ultrasonography (decreased, dyshomogeneous echogenicity, and diffuse goiter)]. The majority of these patients had goiter (61%), the others showed a normal thyroid volume. A minority of patients (7%) were submitted to fine-needle aspiration (FNA) of thyroid nodules to exclude the presence of thyroid cancer or lymphoma; in these cases, cytology excluded the presence of a malignancy.

Among the GD patients, 31 were untreated hyperthyroid patients (11 of them had a relapse of hyperthyroidism after a MMI course of 8-31 months), 50 were in treatment with MMI (2-34 months duration), while the other 10 were euthyroid and in remission after a previous course of MMI therapy of 1-36 months duration.

In terms of thyroid function, 48 were hyperthyroid [low TSH associated with high levels of free T_3 (FT_3) and/or free T_4 (FT_4)], 34 were euthyroid (normal TSH, FT_3 and FT_4), and 9 were hypothyroid (high TSH, with normal or low levels of FT_4 and/or FT_3) while being treated with MMI.

Controls

We used two different controls to compare the features of GD not associated with hyperthyroidism (Comparison 1), or associated with hyperthyroidism (Comparison 2). The necessity to use two different comparisons was due to the fact that the mean age of GD patients was 41 years (**Table 1**), while the mean age of toxic nodular goiter (TNG) collected in the same period (used as control group of hyperthyroid GD) was 55 years; as serum CXCL10 levels are higher in older subjects (9), the Comparison 2 group was made excluding patients younger than 45 years in controls, with thyroiditis and GD, and with a matched age.

Comparison 1

Three control groups were used (**Table 1**). The first control group (controls I, n = 91) consisted of a random sample of the general population (matched by sex and age ± 2 years, with GD patients) from the same geographic area in whom a complete thyroid work-up [history, physical examination, TSH, FT_3, FT_4, antithyroglobulin (TgAb) and antithyroperoxidase (TPOAb) antibodies measurements, and ultrasonography] was available, and excluded the presence of thyroid disorders.

A second control group was made by 91 patients with euthyroid chronic AT (matched by sex and age ± 2 years, with GD patients) (**Table 1**). The diagnosis of AT (22) was established

from the clinical presentation (presence of a firm goiter, varying in size from small to very large, with a lobulated surface), thyroid hormones and thyroid autoantibodies measurements, and/or thyroid ultrasonography (decreased, dyshomogeneous echogenicity).

A third control group comprised 34 patients with non-toxic multinodular goiter (MNG) extracted from the same random sample of the general population (matched by sex and age ± 2 years, with GD patients). The majority of these patients had a normal thyroid volume, some showed goiter (41%). All these patients were submitted to FNA to exclude the presence of thyroid cancer; cytology confirmed the absence of a malignancy.

Comparison 2

In the same period we collected the clinical history and the blood samples of 31 patients affected by TNG (diagnosed by thyroid scintigraphy) (**Table 2**). All patients were hyperthyroid, and the majority of them had a goiter (69%). All these patients were submitted to FNA to exclude the presence of thyroid cancer; cytology confirmed the absence of a malignancy. Owing to the fact that the mean age of the patients with TNG was 55 years and that serum CXCL9 levels are higher in older subjects the comparison 2 was made by matching TNG patients by age (± 3 years) and sex with controls, i.e. hyperthyroid patients with **GD, or thyroiditis** (**Table 2**).

In all patients and controls, a blood sample was collected in the morning, after overnight fasting, and serum was kept frozen until thyroid hormones, TSH, thyroid autoantibodies, and CXCL9 and CXCL11 measurement.

All study subjects gave their informed consent to participate in the study, which was approved by the local Ethical Committee.

Ultrasonography of the neck and FNA

Neck ultrasonography was performed by the same operator, who was unaware of the results of thyroid hormones, autoantibodies and CXCL10 measurements (Esaote, AU5 with a

sectorial 7.5 MHz transducer). Thyroid volume was calculated using the ellipsoid formula, as described (14, 15). The presence of hypoechoic and dyshomogeneous echogenicity was arbitrarily rated at three levels (0 = normal echogenicity; 1 = slightly hypoechoic and dyshomogeneous; 2 = severely hypoechoic and dyshomogeneous) in order to evaluate structural abnormalities of thyroid tissue associated with thyroid autoimmunity (14, 15). The presence of thyroid nodules was recorded, and nodules with a diameter >10 mm were submitted to ultrasonography-guided FNA, which was performed by the same operator, using a free-hand method as already described (14, 15).

Thyroid blood flow (TBF)

TBF by color-flow doppler (CFD) was studied in all patients (14, 15). The CFD pattern was defined as normal (or type 0): TBF limited to peripheral thyroid arteries; type I: TBF mildly increased; type II: TBF clearly increased; type III: TBF markedly increased (14, 15).

Laboratory evaluation

Thyroid function and thyroid autoantibodies were measured as previously described (22). Circulating FT_3 and FT_4 were measured by commercial RIA kits (AMERLEX-MAB FT3/ FT4 Kit; Amersham, UK). Serum TSH (DiaSorin, USA), TPOAb and TgAb (ICN Pharmaceuticals, USA) were evaluated by immunoradiometric assay (IRMA) methods. TRAb autoantibodies were measured with the use of a radioreceptor assay (Radim, Italy) (normal range 0-1 IU/mL). For TgAb, TPOAb, positivity was set at > 50, and > 10 IU/mL, respectively.

Serum CXCL9, CXCL11, IFN-γ and CCL2 levels by ELISA

Serum CXCL9 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems, Minneapolis, MN, USA), with a sensitivity ranging from 9-15.5 pg/mL and a mean minimum detectable dose of 5.6 pg/mL. The intra- and inter-assay coefficients of variation were 4.7% and 5.8%.

Serum CXCL11 levels were assayed by a quantitative sandwich immunoassay using a commercially available kit (R&D Systems), with a sensitivity ranging from 2.1-4.5 pg/mL and a mean minimum detectable dose of 12.1 pg/mL. The intra- and inter-assay coefficients of variation were 4.9% and 6.8%.

IFN-γ (Th1 cytokine) and CCL2 (Th2 chemokine) concentrations were also measured in serum using commercially available kits (R&D Systems). The mean minimum detectable level was 2.5 pg/mL for IFN-γ and 4.6 pg/mL for CCL2; the intra- and inter-assay coefficients of variation were 3.1% and 5.9% for IFN-γ, 4.3% and 5.2% for CCL2.

Data analysis

Values are given as mean ± SD for normally distributed variables, otherwise as median and interquartile range. Mean group values were compared by ANOVA for normally distributed variables, otherwise by the Mann-Whitney *U* or Kruskal-Wallis test. Proportions were compared by the χ^2 test. *Post-hoc* comparisons of normally distributed variables were carried out using the Bonferroni-Dunn test. Multivariate analysis was performed by multiple linear regression analysis using CXCL9 or CXCL11 as dependent variable and age, TSH, FT_3, as covariates.

Results

The demographic and clinical features of GD patients and controls are reported in **Table 1**. The mean CXCL9 levels were significantly higher in patients with GD, than in controls, or in patients with euthyroid AT or multinodular goiter (**Fig. 1**).

In GD patients, serum CXCL9 levels were significantly higher in patients older than 50 years ($p = 0.043$, ANOVA), in GD patients with a hypoechoic pattern (55%) ($p = 0.012$, ANOVA), and in those with hypervascularity (68%) ($p = 0.015$, ANOVA) (**Table 3**), while no

significant difference was observed in relation to the presence of goiter, TPOAb, TgAb, or TRAb positivity. In a multiple linear regression model including age, TSH, and FT_3, only age and FT_3 were slightly but significantly related to serum CXCL9 levels (**Table 4**).

Patients with GD and hyperthyroidism had significantly higher CXCL9 levels than euthyroid or hypothyroid GD patients ($p = 0.01$, ANOVA) (**Fig. 2A**), lower TSH ($p < 0.001$), and higher FT_4 ($p < 0.001$), FT_3 ($p < 0.001$), TRAb levels ($p < 0.001$), and higher degrees of hypervascularity ($p = 0.001$) (**Table 5**), while there was no significant difference in thyroid volume, echogenicity, TgAb and TPOAb titers.

GD patients with untreated hyperthyroidism had higher CXCL9 levels than hyperthyroid patients treated with MMI, or euthyroid patients treated with MMI ($p = 0.001$, ANOVA) (**Fig. 3A**). CXCL9 levels were not significantly different in newly diagnosed untreated hyperthyroid patients in comparison with untreated patients with relapse of hyperthyroidism after a previous MMI course (**Table 3**).

MMI-treated patients who were euthyroid had higher CXCL9 levels than patients in remission of hyperthyroidism without treatment ($p = 0.001$, ANOVA) (**Table 3**).

By defining a high CXCL9 level as a value of at least 2 SD above the mean value of the control group (> 142 pg/mL), 54% of patients with GD, 27% of AT, 3% of controls, and one of the MNG patients had high CXCL9 levels ($p < 0.001$, χ^2) (**Table 1**). No relationship was observed between CXCL9 and disease duration.

Patients with GD had higher levels of CXCL9 than patients with euthyroid AT, TNG, or age- and sex-matched controls (**Table 2**).

The mean CXCL11 levels were significantly higher in patients with GD, than in controls or multinodular goiter patients (**Table 1**), and it was not significantly different from patients with euthyroid AT (**Fig. 1B**).

In GD patients, serum CXCL11 levels were significantly higher in GD patients with hypervascularity (p = 0.041, ANOVA) (**Table 3**), while no significant difference was observed in relation to the presence of goiter, TPOAb, TgAb, or TRAb positivity.

Patients with GD and hyperthyroidism had significantly higher CXCL11 levels than euthyroid or hypothyroid GD patients (ANOVA, p = 0.03 respectively) (**Fig. 2B**).

In a multiple linear regression model including TSH and FT_3, they were not significantly related to serum CXCL11 levels.

Patients with GD had significantly higher levels of CXCL11 than age- and sex-matched controls, while CXCL11 levels were higher, although not significantly, compared to euthyroid AT, or TNG (**Table 2**).

GD patients with untreated hyperthyroidism had higher CXCL11 levels than hyperthyroid patients treated with MMI, or euthyroid patients treated with MMI (p = 0.006, ANOVA) (**Fig. 3B**). CXCL11 levels were not significantly different in newly diagnosed untreated hyperthyroid patients compared to untreated patients with relapse of hyperthyroidism after a previous MMI course (**Table 3**).

Patients who were euthyroid while being treated with MMI or during remission of hyperthyroidism without treatment showed similar CXCL11 levels (**Table 3**).

By defining a high CXCL11 level as a value of at least 2 SD above the mean value of the control group (> 104 pg/mL), 32 % of patients with GD, 27% of AT, 2% of controls, and one of the multinodular goiter patients had high CXCL11 levels ($p < 0.001$, χ^2) (**Table 1**). No relationship was observed between CXCL11 and the GD disease duration.

No significant relationship was observed between CXCL9 and CXCL11 serum levels in patients with GD, by simple regression.

IFN-γ was detectable in the serum of 5% of controls, 6% of MNG, 45% of GD, and 37% of AT ($p < 0.0001$, χ^2). IFN-γ levels were similar in GD (12 [5.2-25.4] pg/mL, median and

[interquartile range]), and in AT (10.1 [4.1-22.5] pg/mL) (p = ns).

No significant relationship was observed between CXCL9, or CXCL11, or IFN-γ serum levels in patients with GD, by simple regression.

CCL2 levels were similar in GD (403 [131-734] pg/mL, median and [interquartile range]), in AT (354 [154-673] pg/mL), MNG (339 [127-801] pg/mL) and controls (371 [143-724] pg/mL) (p = ns).

Discussion

The results of the present study confirm that CXCL9 and CXCL11 serum levels are increased in newly diagnosed patients with GD, and demonstrate a strong association with the hyperthyroid phase of the disease, with a decrease of both chemokines with MMI therapy. Furthermore, high levels of CXCL9 and CXCL11 were strongly associated with hypervascularity. The relapse of hyperthyroidism was characterized by CXCL9 and CXCL11 serum levels similar to those observed in newly diagnosed hyperthyroid patients.

Other studies suggest that a prevalent Th1 immune response is involved in AT, while a predominant Th2 response is associated with GD (23-26).

Our results are in agreement with some studies that have shown a prevalent Th1 immune response in the initial phase of GD (8, 15). IFN-γ serum levels were higher in GD patients than in controls, confirming a Th1 involvement in GD and the results of previous studies (27-29).

The increase of CXCL9 in hyperthyroid patients with GD is in agreement with previous studies showing the involvement of IFN-γ (27), TNF-α (30) and Th1 cytokines in GD (27, 31-35). Furthermore, the increase of serum CXCL9 in hyperthyroid patients with GD is in agreement with the results of another study that found that GD patients who relapsed or went

into remission had significantly different levels of CXCL9 (36).

Moreover, it has been recently shown that IFN-γ and TNF-α are able to induce the secretion of the CXCL9 and CXCL11 chemokines in thyrocytes of patients with GD (11, 19-21).

In our series, the increase of CXCL9 and CXCL11 seemed not associated with hyperthyroidism "*per se*"; in fact, the serum levels of these chemokines were higher in hyperthyroid Graves' patients than in toxic nodular goiter. Therefore, the reported reduction of circulating CXCL9 and CXCL11 levels under MMI therapy could be reasonably ascribed to the well known immunomodulatory effect of antithyroid drugs (37, 38). This is also in agreement with the results observed for CXCL10 in GD patients treated with MMI (14, 15, 34).

Recently, it has been shown that MMI inhibits CXCL10 secretion in human thyrocytes. MMI decreased cytokine-induced CXCL10 secretion by reducing TNF-α-induced upregulation of the IFN-γ receptor (39).

The site of production of CXCL9, CXCL10 and CXCL11 remains to be clarified. Cytokine production has been variably interpreted as sustained by thyroid follicular cells (TFC) (40), by intrathyroidal lymphocytes (27), or from the activation of humoral reactions in sites other than the thyroid (41, 42). However, the MMI-induced reduction of CXCL9 and CXCL11 levels in our GD patients suggests that both intrathyroidal lymphocytes and TFC could be responsible for CXCL9 and CXCL11 production. These findings are in agreement with the observed reduction of CXCL10 levels after ^{131}I treatment, or thyroidectomy in GD patients, that suggests that the thyroid gland itself is the main source of circulating CXCL10 (43, 44).

Patients with GD in remission after a previous course of MMI therapy show serum CXCL9 and CXCL11 levels similar to normal controls or euthyroid multinodular goiters, but lower than patients with euthyroid AT. These data are in agreement with previous reports showing that CXCL10 expression was comparable to controls in patients with long-standing GD (8)

and suggest that CXCL9, CXCL10 and CXCL11 are transiently involved in the active phase of GD, when an active inflammatory process is present, and the Th1-mediated immune response is prevalent, while it is no more significantly present when remission of the disease is achieved. This finding may be regarded as a result of the negative feedback of Th2 cytokines on IFN-γ production. This switch from a Th1 to a Th2 phenotype already reported in other long standing autoimmune diseases appears to be present also in GD, in line with a previous report showing that lymphocytes obtained from orbital and thyroid tissue of patients affected by Graves' ophthalmopathy had a predominant Th1 profile, whereas patients with remote onset of hyperthyroidism had a large majority of Th2 lymphocytes (33). However, during relapse of hyperthyroidism, a new increase of CXCL10 is demonstrable, in line with a novel activation of the Th1-mediated immune response.

The increase of CXCL9, CXCL10 and CXCL11 in the active phase of GD is in agreement with findings arisen from previous reports in which these chemokines have been contemporarily assessed in the serum and cerebrospinal fluid of multiple sclerosis patients (MS), showing significant modification in relation to the clinical phase of disease. Specifically, CXCL10 was higher in acute MS and lower in stable disease, suggesting a pathogenetic role for the chemokine in mediating clinical reexacerbation of MS (45). In addition, the previously reported inverse correlation between CXCL10 levels and time from last clinical relapse, together with the finding that CXCL9, CXCL10 and CXCL11 are upregulated during relapse in MS (46-48), strongly supports this hypothesis.

The increase of CXCL9 and CXCL11 in patients with relapse of hyperthyroidism suggests that CXCL9 and CXCL11 could be used as prognostic markers in patients with GD after the remission of the hyperthyroidism with MMI treatment. Currently, this is typically addressed by the determination of TRAb, which represent the most useful addition to the clinical armamentarium and a low-cost assay in treatment planning; the major hurdle consists in

increasing the sensitivity of the available assays for TRAb in order to be applied successfully to a greater proportion of patients with GD (49). We have failed to show a relationship between the differences of CXCL9 or CXCL11 concentrations and the presence of circulating TRAb, TPOAb, or TgAb suggesting that the activation of the CXCL9 and CXCL11 system may be independent of autoantibody reactions in the thyroid.

Interestingly, circulating levels of CXCL9 in GD patients were higher than those of CXCL11. This finding is in agreement with the results of previous studies that have shown that in primary cultures of thyrocytes, obtained from GD patients, the treatment with TNF-α plus IFN-γ has a significantly higher synergistic effect on CXCL9 secretion than on CXCL11 release, and reinforces the hypothesis that the thyroid gland itself is the main source of these chemokines (19, 20).

In conclusion, IFN-γ inducible chemokines CXCL9 and CXCL11 are associated with the active phase of GD both in newly diagnosed and in relapsing hyperthyroid patients. The reduction of circulating CXCL9 and CXCL11 levels in patients with GD treated with MMI may be related to the immunomodulatory effect of MMI. Future longitudinal studies in patients with GD will be necessary to assess the possible use of CXCL9 and CXCL11 serum levels as prognostic markers both in patients treated with MMI or after achievement of remission and as a possible addition to the TRAb assay.

Author Disclosure Statement

The authors have no conflicts of interest to disclose.

No competing financial interests exist.

Corresponding author and person to whom reprint requests should be addressed:
Alessandro Antonelli, MD
Department of Clinical and Experimental Medicine
University of Pisa
Via Savi, 10, 56126, Pisa, Italy
Phone: +39-050-992318
Fax: +39-050-553235
e-mail: alessandro.antonelli@med.unipi.it

References

1. Rotondi M, Chiovato L, Romagnani S, Serio M, Romagnani P 2007 Role of chemokines in endocrine autoimmune diseases. Endocr Rev 28:492-520.

2. Clark-Lewis I, Mattioli I, Gong JH, Loetscher P 2003 Structure-function relationship between the human chemokine receptor CXCR3 and its ligands. J Biol Chem 278:289-295.

3. Luster AD, Ravetch JV 1987 Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). J Exp Med 166:1084-1097.

4. Farber JM 1997 Mig and IP-10: CXC chemokines that target lymphocytes. J Leukoc Biol 61:246-257.

5. Cole KE, Strick CA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, Lin W, Boyd JG, Moser B, Wood DE, Sahagan BG, Neote K 1998 Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. J Exp Med 187:2009-2021.

6. Colvin RA, Campanella GS, Sun J, Luster AD 2004 Intracellular domains of CXCR3 that mediate CXCL9, CXCL10, and CXCL11 function. J Biol Chem 279:30219-30227.

7. Shimada A, Morimoto J, Kodama K, Suzuki R, Oikawa Y, Funae O, Kasuga A, Saruta T, Narumi S 2001 Elevated serum IP-10 levels observed in type 1 diabetes. Diabetes Care 24: 510-515.

8. Romagnani P, Rotondi M, Lazzeri E, Lasagni L, Francalanci M, Buonamano A, Milani S, Vitti P, Chiovato L, Tonacchera M, Bellastella A, Serio M 2002 Expression of IP-10/CXCL10 and Mig/CXCL9 in the thyroid and increased serum levels of IP-10/CXCL10 in the serum of patients with recent onset Graves'disease. Am J Pathol 161:195-206.

9. Antonelli A, Rotondi M, Fallahi P, Romagnani P, Ferrari SM, Buonamano A, Ferrannini E, Serio M 2004 High levels of circulating CXCL10 are associated with chronic autoimmune thyroiditis and hypothyroidism. J Clin Endocrinol Metab 89:5496-5499.

10. Antonelli A, Rotondi M, Fallahi P, Romagnani P, Ferrari SM, Paolicchi A, Ferrannini E, Serio M 2005 Increase of interferon-gamma inducible alpha chemokine CXCL10 but not beta chemokine CCL2 serum levels in chronic autoimmune thyroiditis. Eur J Endocrinol 152:171-177.

11. García-López MA, Sancho D, Sánchez-Madrid F, Marazuela M 2001 Thyrocytes from autoimmune thyroid disorders produce the chemokines IP-10 and Mig and attract CXCR3+ lymphocytes. J Clin Endocrinol Metab 86:5008-5016.

12. Kemp EH, Metcalfe RA, Smith KA, Woodroofe MN, Watson PF, Weetman AP 2003 Detection and localization of chemokine gene expression in autoimmune thyroid disease. Clin Endocrinol (Oxf) 59:207-213.

13. Romagnani P, Annunziato F, Lasagni L, Lazzeri E, Beltrame C, Francalanci M, Uguccioni M, Galli G, Cosmi L, Maurenzig L, Baggiolini M, Maggi E, Romagnani S, Serio M 2001 Cell cycle dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. J Clin Invest 107:53-63.

14. Antonelli A, Fallahi P, Rotondi M, Ferrari SM, Romagnani P, Grosso M, Ferrannini E, Serio M 2006 Increased serum CXCL10 in Graves' disease or autoimmune thyroiditis is not associated with hyper- or hypothyroidism per se, but is specifically sustained by the autoimmune, inflammatory process. Eur J Endocrinol 154:651-658.

15. Antonelli A, Rotondi M, Fallahi P, Romagnani P, Ferrari SM, Barani L, Ferrannini E, Serio M 2006 Increase of interferon-gamma-inducible CXC chemokine CXCL10 serum levels in patients with active Graves' disease, and modulation by methimazole therapy. Clin Endocrinol (Oxf) 64:189-195.

16. Antonelli A, Ferrari SM, Frascerra S, Galetta F, Franzoni F, Corrado A, Miccoli M, Benvenga S, Paolicchi A, Ferrannini E, Fallahi P 2011 Circulating chemokine (CXC motif) ligand (CXCL)9 is increased in aggressive chronic autoimmune thyroiditis, in association with CXCL10. Cytokine 55:288-293.

17. Antonelli A, Ferrari SM, Frascerra S, Di Domenicantonio A, Nicolini A, Ferrari P, Ferrannini E, Fallahi P 2011 Increase of circulating CXCL9 and CXCL11 associated with euthyroid or subclinically hypothyroid autoimmune thyroiditis. J Clin Endocrinol Metab 96:1859-1863.

18. Antonelli A, Ferri C, Ferrari SM, Frascerra S, Ruffilli I, Caponi L, Ulisse S, Miccoli M, Miccoli P, Fallahi P 2012 High Levels of Circulating Chemokine (C-X-C motif) Ligand 11 Are Associated with Euthyroid or Subclinically Hypothyroid Autoimmune Thyroiditis and with Chemokine (C-X-C Motif) Ligand 10. J Interferon Cytokine Res 32:74-80.

19. Antonelli A, Ferrari SM, Frascerra S, Pupilli C, Mancusi C, Metelli MR, Orlando C, Ferrannini E, Fallahi P 2010 CXCL9 and CXCL11 chemokines modulation by peroxisome proliferator-activated receptor-alpha agonists secretion in Graves' and normal thyrocytes. J Clin Endocrinol Metab 95:E413-E420.

20. Antonelli A, Ferrari SM, Fallahi P, Frascerra S, Santini E, Franceschini SS, Ferrannini E 2009 Monokine induced by interferon gamma (IFNgamma) (CXCL9) and IFNgamma inducible T-cell alpha-chemoattractant (CXCL11) involvement in Graves' disease and ophthalmopathy: modulation by peroxisome proliferator-activated receptor-gamma agonists. J Clin Endocrinol Metab 94:1803-1809.

21. Antonelli A, Ferrari SM, Frascerra S, Ruffilli I, Gelmini S, Minuto M, Pupilli C, Miccoli P, Sellari-Franceschini S, Ferrannini E, Fallahi P 2012 Peroxisome proliferator-activated receptor-α agonists modulate CXCL9 and CXCL11 chemokines in Graves' ophthalmopathy fibroblasts and preadipocytes. Mol Cell Endocrinol 349:255-261.

22. Antonelli A, Fallahi P, Nesti C, Pupilli C, Marchetti P, Takasawa S, Okamoto H, Ferrannini E 2001 Anti-CD38 autoimmunity in patients with chronic autoimmune thyroiditis or Graves' disease. Clin Exp Immunol 126:426-431.

23. Qin Q, Liu P, Liu L, Wang R, Yan N, Yang J, Wang X, Pandey M, Zhang JA 2012 The increased but non-predominant expression of Th17- and Th1-specific cytokines in Hashimoto's thyroiditis but not in Graves' disease. Braz J Med Biol Res 45:1202-1208.

24. Nanba T, Watanabe M, Inoue N, Iwatani Y 2009 Increases of the Th1/Th2 cell ratio in severe Hashimoto's disease and in the proportion of Th17 cells in intractable Graves' disease. Thyroid 19:495-501.

25. McLachlan SM, Nagayama Y, Rapoport B 2005 Insight into Graves' hyperthyroidism from animal models. Endocr Rev 26:800-832.

26. Phenekos C, Vryonidou A, Gritzapis AD, Baxevanis CN, Goula M, Papamichail M 2004 Th1 and Th2 serum cytokine profiles characterize patients with Hashimoto's thyroiditis (Th1) and Graves' disease (Th2). Neuroimmunomodulation 11:209-213.

27. Watson PF, Pickerill AP, Davies R, Weetman AP 1994 Analysis of cytokine gene expression in Graves' disease and multinodular goiter. J Clin Endocrinol Metab 79:355-360.

28. Antonelli A, Rotondi M, Ferrari SM, Fallahi P, Romagnani P, Franceschini SS, Serio M, Ferrannini E 2006 Interferon-gamma-inducible alpha-chemokine CXCL10 involvement in Graves' ophthalmopathy: modulation by peroxisome proliferator-activated receptor-gamma agonists. J Clin Endocrinol Metab 91:614-620.

29. Matsubayashi S, Kasuga Y, Sakatsume Y, Akasu F, Volpe R 1990 Serum interferon gamma levels in autoimmune thyroid disease. Clin Invest Med 13:271-274.

30. Pichler R, Maschek W, Hatzl-Griesenhofer M, Huber H, Crespillo-Gómez C, Berg J 2003 Soluble tumour necrosis factor-alpha receptor I and interleukin-6 as markers of activity in thyrotoxic Graves' disease. Horm Metab Res 35:427-433.

31. Miyauchi S, Matsuura B, Onji M 2000 Increased levels of serum interleukin-18 in Graves' disease. Thyroid 10:815-819.

32. Siddiqi A, Monson JP, Wood DF, Besser GM, Burrin JM 1999 Serum cytokines in thyrotoxicosis. J Clin Endocrinol Metab 84:435-439.

33. Mariotti S, Del Prete GF, Mastromauro C, De Carli M, Romagnani S, Ricci M, Pinchera A 1991 The autoimmune infiltrate of Basedow's disease: analysis of clonal level and comparison with Hashimoto's thyroiditis. Exp Clin Endocrinol 97:139-146.

34. De Carli M, D'Elios MM, Mariotti S, Marcocci C, Pinchera A, Ricci M, Romagnani S, Del Prete G 1993 Cytolytic T cells with Th1-like cytokine profile predominate in retroorbital lymphocytic infiltrates of Graves' ophthalmopathy. J Clin Endocrinol Metab 77:1120-1124.

35. Aniszewski JP, Valyasevi RW, Bahn RS 2000 Relationship between disease duration and predominant orbital T cell subset in Graves' ophthalmopathy. J Clin Endocrinol Metab 85:776-780.

36. Domberg J, Liu C, Papewalis C, Pfleger C, Xu K, Willenberg HS, Hermsen D, Scherbaum WA, Schloot NC, Schott M 2008 Circulating chemokines in patients with autoimmune thyroid diseases. Horm Metab Res 40:416-421.

37. Cooper DS 1984 Antithyroid drugs. N Engl J Med 311:1353-1362.

38. Weetman AP, McGregor AM, Hall R 1984 Evidence for an effect of antithyroid drugs on the natural history of Graves' disease. Clin Endocrinol (Oxf) 21:163-172.

39. Crescioli C, Cosmi L, Borgogni E, Santarlasci V, Gelmini S, Sottili M, Sarchielli E, Mazzinghi B, Francalanci M, Pezzatini A, Perigli G, Vannelli GB, Annunziato F, Serio M 2007 Methimazole inhibits CXC chemokine ligand 10 secretion in human thyrocytes. J Endocrinol 195:145-155.

40. Díez JJ, Hernanz A, Medina S, Bayón C, Iglesias P 2002 Serum concentrations of tumour necrosis factor-alpha (TNF-alpha) and soluble TNF-alpha receptor p55 in patients with

hypothyroidism and hyperthyroidism before and after normalization of thyroid function. Clin Endocrinol (Oxf) 57:515-521.

41. Salvi M, Pedrazzoni M, Girasole G, Giuliani N, Minelli R, Wall JR, Roti E 2000 Serum concentrations of proinflammatory cytokines in Graves' disease: effect of treatment, thyroid function, ophthalmopathy and cigarette smoking. Eur J Endocrinol 143:197-202.

42. Senturk T, Kozaci LD, Kok F, Kadikoylu G, Bolaman Z 2003 Proinflammatory cytokine levels in hyperthyroidism. Clin Invest Med 26:58-63.

43. Antonelli A, Rotondi M, Fallahi P, Grosso M, Boni G, Ferrari SM, Romagnani P, Serio M, Mariani G, Ferrannini E 2007 Iodine-131 given for therapeutic purposes modulates differently interferon-gamma-inducible alpha-chemokine CXCL10 serum levels in patients with active Graves' disease or toxic nodular goiter. J Clin Endocrinol Metab 92:1485-1490.

44. Antonelli A, Fallahi P, Rotondi M, Ferrari SM, Serio M, Miccoli P 2006 Serum levels of the interferon-gamma-inducible alpha chemokine CXCL10 in patients with active Graves' disease, and modulation by methimazole therapy and thyroidectomy. Br J Surg 93:1226-1231.

45. Sørensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, Qin S, Rottman J, Sellebjerg F, Strieter RM, Frederiksen JL, Ransohoff RM 1999 Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. J Clin Invest 103:807-815.

46. Mahad DJ, Howell SJ, Woodroofe MN 2002 Expression of chemokines in the CSF and correlation with clinical disease activity in patients with multiple sclerosis. J Neurol Neurosurg Psychiatry 72:498-502.

47. Comini-Frota ER, Teixeira AL, Angelo JP, Andrade MV, Brum DG, Kaimen-Maciel DR, Foss NT, Donadi EA 2011 Evaluation of serum levels of chemokines during interferon-≤

treatment in multiple sclerosis patients: a 1-year, observational cohort study. CNS Drugs 25:971-981.

48. SzczuciDski A, Kalinowska A, Losy J 2007 CXCL11 (Interferon-inducible T-cell alpha chemoattractant) and interleukin-18 in relapsing-remitting multiple sclerosis patients treated with methylprednisolone. Eur Neurol 58:228-232.

49. Davies TF, Roti E, Braverman LE, DeGroot LJ 1998 Thyroid controversy--stimulating antibodies. J Clin Endocrinol Metab 83:3777-3785.

Table 1. Thyroid status of control subjects, or patients with autoimmune thyroiditis, or euthyroid multinodular goiter, or Graves' disease.

	controls I	*thyroiditis*	*multinodular goiter*	*Graves' disease*	*p*
n	91	91	34	91	
Age (years)	43 ± 13	42 ± 12	43 ± 10	41 ± 11	ns
Sex (M/F)%	23	23	24	23	ns
Thyroid volume (mL)	10 ± 11	12 ± 13	19 ± 14	23 ± 16*	< 0.001
Hypoechoic (%)	0	65	0	55	< 0.001
Hypervascular (%)	0	41	0	68	< 0.001
Serum TSH (μU/mL)	1.4 ± 0.7	1.5 ± 1.1	1.0 ± 0.9	0.6 ± 1.7*	0.006
TPOAb (IU/mL)	8 ± 11	456 ± 532°	10 ±6	342 ± 297°	< 0.001
TgAb (IU/mL)	7 ± 9	212 ± 284°	9 ± 11	164 ± 351°	< 0.001
TRAb (IU/mL)	0	0	0	25 ± 27§	< 0.001
TPOAb positivity (%)	0	78	0	42	< 0.001
TgAb positivity (%)	0	72	0	35	< 0.001
CXCL9 (pg/mL)	76 ± 33	132 ± 78°	87 ± 48	274 ± 265§	< 0.001
CXCL9 (> 142 pg/mL)	3%	27%	3%	54%	< 0.001
CXCL11 (pg/mL)	64 ± 20	108 ± 48^	76 ± 33	140 ± 92°	0.009
CXCL11 (> 104 pg/mL)	2%	27%	3%	32%	< 0.001

Antithyroperoxidase antibody=TPOAb; Antithyroglobulin antibody=TgAb; Antithyrotropin-receptor antibody=TRAb. Mean group values were compared by ANOVA for normally distributed variables, otherwise by Kruskal-Wallis test. Proportions were compared by the χ^2 test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test.

* $p < 0.05$ or less vs. controls or vs. autoimmune thyroiditis.

° $p < 0.05$ or less vs. controls and vs. multinodular goiters.

^ $p < 0.05$ or less vs. controls.

§ $p < 0.05$ or less vs. controls, vs. autoimumune thyroiditis and vs. multinodular goiters.

Table 2. Thyroid status of control subjects and patients with euthyroid autoimmune thyroiditis, or toxic nodular goiter, or Graves' disease.

	controls	*thyroiditis*	*toxic nodular goiter*	*Graves' disease*	*p*
n	31	31	31	31	
Age (years)	57 ± 6	56 ± 10	55 ± 9	53 ± 10	ns
Sex (M/F)%	29	29	29	29	ns
Thyroid volume (mL)	11 ± 13	18 ± 16	31 ± 32°	27 ± 21°	< 0.001
Serum TSH (μU/mL)	1.7 ± 0.9	1.8 ± 1.2	0.03 ± 0.09°	0.02 ± 0.06°	< 0.001
TPOAb (IU/mL)	11 ± 7	453 ± 356*	9 ± 7	241 ± 276*	< 0.001
TgAb (IU/mL)	12 ± 9	542 ± 371*	10 ± 8	213 ± 275*	< 0.001
TRAb (IU/mL)	0	0	0	21 ± 19§	< 0.001
CXCL9 (pg/mL)	88 ± 41	147 ± 91^	112 ± 56	261 ± 283§	< 0.001
CXCL11 (pg/mL)	67 ± 23	105 ± 52^	91 ± 41	135 ± 79 ^	0.012

Antithyroperoxidase antibody=TPOAb; Antithyroglobulin antibody=TgAb; Antithyrotropin-receptor antibody=TRAb. Mean group values were compared by ANOVA for normally distributed variables, otherwise by Kruskal-Wallis test. Proportions were compared by the χ^2 test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test.

° $p < 0.05$ or less vs. controls or vs. autoimmune thyroiditis.

*$p < 0.05$ or less vs. controls and vs. toxic nodular goiter.

^ $p < 0.05$ or less vs. controls.

§ $p < 0.05$ or less vs. controls, vs. autoimumune thyroiditis and vs. toxic nodular goiter.

Table 3. Serum CXCL9 and CXCL11 levels in relation to various parameters in patients with Graves' disease.

CXCL9		
Age		
> 50 years	< 50 years	*p*
309 ± 243 pg/mL	216 ± 267 pg/mL	0.043
Hypoechoic pattern		
No	Yes	*p*
209 ± 241 pg/mL	328 ± 284 pg/mL	0.012
Hypervascularity		
No	Yes	*p*
234 ± 231 pg/mL	314 ± 301 pg/mL	0.015
Euthyroidism under MMI	In remission of hyperthyroidism	*p*
191 ± 235 pg/mL	122 ± 83 pg/mL	0.001
Newly diagnosed untreated hyperthyroidism	Untreated in relapse of hyperthyroidism	*p*
302 ± 295 pg/mL	295 ± 308 pg/mL	ns
CXCL11		
Hypervascularity		
No	Yes	*p*
111 ± 84 pg/mL	160 ± 98 pg/mL	0.041
Euthyroidism under MMI	In remission of hyperthyroidism	*p*
101 ± 61 pg/mL	90 ± 44 pg/mL	ns
Newly diagnosed untreated hyperthyroidism	Untreated in relapse of hyperthyroidism	*p*
168 ± 103 pg/mL	154 ± 87 pg/mL	ns

Table 4. Multiple linear regression of CXCL9 vs. age, TSH, and FT_3.

	standardized coefficient (β)	*regression coefficient (r.c.)*	*CI (r.c.) 95% lower*	*CI (r.c.) 95% upper*	*p*
Age (years)	0.19	1.5	0.1	2.7	0.032
TSH (ln[μU/mL])	-0.11	-1.2	-3.1	2.3	0.514
FT_3 (ng/L)	0.24	2.9	0.3	7.4	0.033

Free T_3=FT_3; Confidence Interval=CI.

Table 5. CXCL9 serum levels in relation to thyroid status of patients with Graves' disease.

	GD hyperthyroidism	GD euthyroidism	GD hypothyroidism	*p*
CXCL9 (pg/mL)	340 ± 285	182 ± 175	195 ± 170	0.015
TSH (μU/mL)	0.03 ± 0.07	0.65 ± 1.2	12.1 ± 17.4	< 0.001
FT_4 (pg/mL)	21.7 ± 12.4	9.3 ± 3.9	3.7 ± 2.5	< 0.001
FT_3 (μU/mL)	11.5 ± 7.4	3.9 ± 1.4	2.5 ± 0.8	< 0.001
TRAb (IU/mL)	36 ± 31	17 ± 15	14 ± 12	< 0.001
Degrees of hypervascularity (score units)	1.1 ± 0.2	0.7 ± 0.3	0.3±0.4	0.001

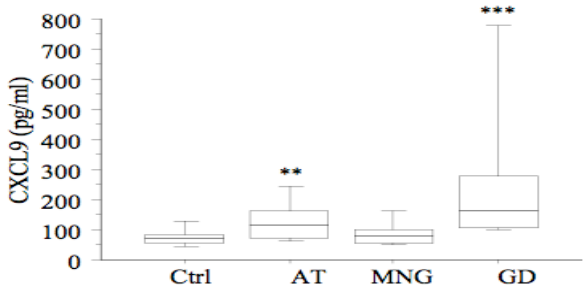
Legends to Figures

Figure 1. Distribution of serum CXCL9 (**A**), or CXCL11 (**B**), values in control subjects (Ctrl), in patients with autoimmune thyroiditis (AT), euthyroid multinodular goiter (MNG) and Graves' disease (GD). The box indicates the lower and upper quartiles and the central line is the median value; the horizontal lines at the end of the vertical lines are the 2.5% and 97.5% values (* $p < 0.05$ or less vs. controls; ** $p < 0.05$ or less vs. controls, or vs. MNG; *** $p < 0.05$ or less vs. controls, or vs. AT, or vs. MNG; by Bonferroni-Dunn).

Figure 2. Patients with Graves' disease and hyperthyroidism (Hyper) had significantly higher CXCL9 (**A**), or CXCL11 (**B**) levels than euthyroid (Eu) or hypothyroid (Hypo) GD patients. The box indicates the lower and upper quartiles and the central line is the median value; the horizontal lines at the end of the vertical lines are the 2.5% and 97.5% values (* $p < 0.05$ or less vs. Eu, or Hypo; by Bonferroni-Dunn).

Figure 3. Patients with Graves' disease with untreated hyperthyroidism (Hyper) had higher CXCL9 (**A**), CXCL11 (**B**) levels than hyperthyroid patients treated with MMI (Hyper+MMI), or euthyroid patients treated with MMI (Eu+MMI) (* $p < 0.05$, by Bonferroni-Dunn). The box indicates the lower and upper quartiles and the central line is the median value; the horizontal lines at the end of the vertical lines are the 2.5% and 97.5% values.

A



B

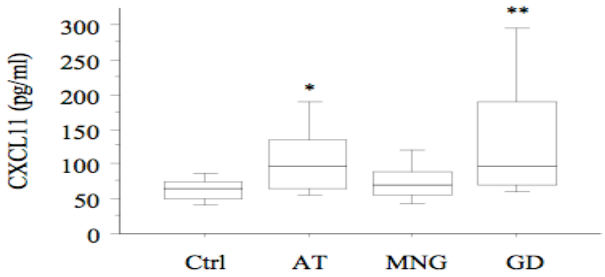


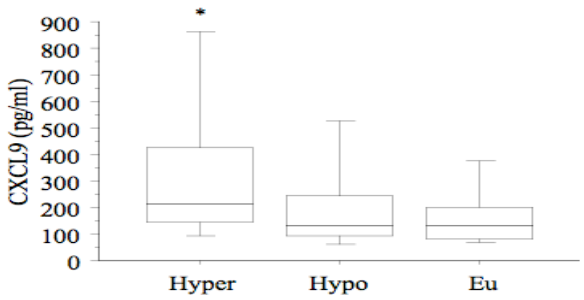
Figure 1

1

2

3

A



B

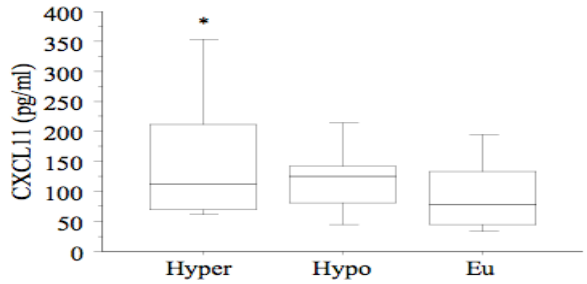


Figure 2

1

2

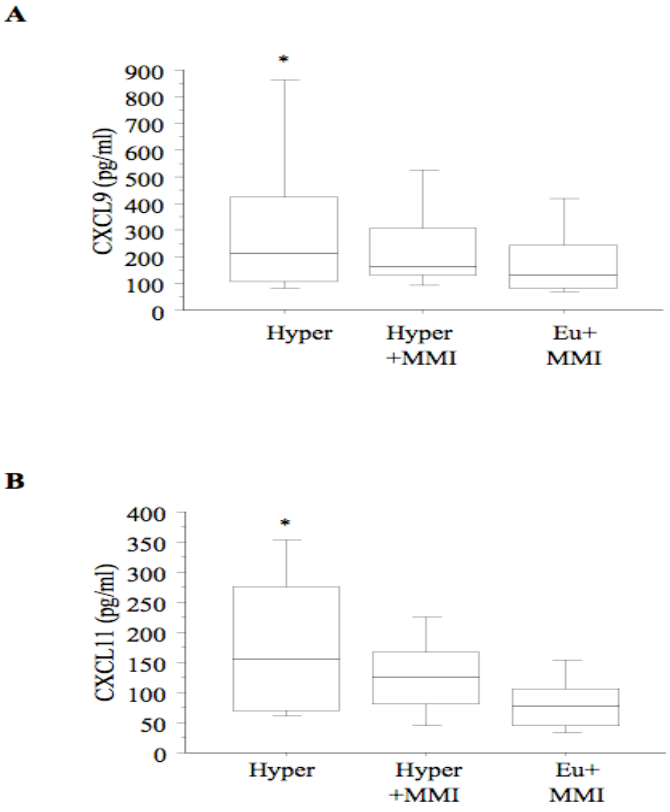

A
*
900
800
700
600
500
400
300
200
100
0
CXCL9 (pg/ml)
Hyper
Hyper +MMI
Eu+ MMI
B
*
400
350
300
250
200
150
100
50
0
CXCL11 (pg/ml)
Hyper
Hyper +MMI
Eu+ MMI

Figure 3