RESEARCH ARTICLE

Down-regulation of the auto-aggressive processes in patients with hypothyroid Hashimoto's thyroiditis following substitutive treatment with L-thyroxine

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ABSTRACT. ***Background.*** **Hashimoto's thyroiditis is a chronic, organ-specific autoimmune disease. It is the most common cause of primary hypothyroidism during the adolescent period, *via* autoimmune thyroid tissue destruction, affecting 2% of the population. The pathogenesis of Hashimoto's thyroiditis involves a complex interaction between predisposing genetic and environmental factors.** ***Objective.*** **In this study, we wanted to investigate the role of cytokines such as IL-2, IL-4, IL-12 and IFN-γ in the pathogenesis of the disease, and the changes to cytokine levels brought about by treatment with L-thyroxine.** ***Methods.*** **Sixty five female patients, aged 18-73 years with Hashimoto's thyroiditis, referred to the Celal Bayar University Medical Faculty Endocrinology out-patients clinic, were included in this study. After a 10-12 week period of L-thyroxine treatment, all patients were restored to the euthyroid state. At the beginning and end of the treatment period, serum-free triiodothyronine (FT3), free thyroxine (FT4), thyroid-stimulating hormone (TSH), autoantibodies against thyroid peroxidase (anti-TPO), autoantibodies against thyroglobulin (anti-Tg) levels were measured using a chemiluminecent, immunometric method, and cytokine levels were measured using ELISA.** ***Results.*** **There was a statistically significant decrease in the serum levels of TSH ($p < 0.0001$) and a concomitant increase in FT4 serum levels ($p < 0.0001$). Also, during the post-treatment period, serum levels of anti-Tg ($p < 0.01$) and anti-TPO ($p < 0.001$) were significantly lower than during the pre-treatment period. A statistically significant decrease was shown for interleukin (IL)-12 serum levels during the post-treatment period ($p < 0.001$). However, the decrease in interferon (IFN)-γ serum levels was not statistically significant ($p = 0.276$). On the other hand, no change was demonstrated in serum IL-2 and IL-4 levels ($p = 0.953$ and $p = 0.313$, respectively) after treatment with L-thyroxine.** ***Conclusion.*** **Considering that our study involved a 10-12 week period of treatment, the statistically significant decrease in serum IL-12 levels, and the statistically non-significant decrease in IFN-γ levels, might indicate that a T helper type 1 inflammatory process had been halted or slowed down.**

Keywords: hashimoto's thyroiditis, L-thyroxine, cytokine, serum, Th1, Th2

All types of thyroiditis are heterogeneous inflammatory diseases of the thyroid gland that have different etiologies and which present with a variety of clinical pictures [1]. Hashimoto's thyroiditis is a chronic, organ-specific, autoimmune disease and is the most common cause of hypothyroidism [1, 2].

Although autoimmune diseases are caused by a variety mechanisms, it is accepted that the major process is the disruption of immunological tolerance mechanisms [3]. Recently, it has been shown that autoimmunities are caused by the deficiency of regulatory T (T reg) cells, which are control peripheral tolerance [4]. Functional deficiency of T reg cells causes T helper (Th)1- or Th2-driven autoimmune diseases [5, 6]. In animal and human studies, it has been shown that Hashimoto's thyroiditis is a prototypic autoimmunity that is caused by a Th1-driven, cell-mediated, cellular hypersensitivity reaction [7-13]. However, there are insufficient studies concerning the immunological effects of conventional hormone replacement (L-thyroxine) therapy on patients with Hashimoto's thyroiditis who develop hypothyroidism. Therefore, in this study, interleukin (IL)-2, being produced by all T cells, and other cytokines that are specific to Th1 and Th2 cells, were evaluated in patients who had developed hypothyroidism due to Hashimoto's thyroiditis, before and after substitutive treatment with L-thyroxine. The role of these cells and the cytokines they produce, in the pathogenesis of the disease, was also studied.

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METHODS AND MATERIALS

The study included 65 randomly-selected subjects from patients who had been diagnosed with hypothyroidism due to Hashimoto's thyroiditis in the Celal Bayar University Department of Endocrinology and Metabolism between May 2007 and March 2008. All of the patients were female, and the mean age was 40.32 ± 13.5 (18-73) years. Inclusion criteria that were used to select patients were identical to those previously described [14-16] and were as follows:

– serum thyroid-stimulating hormone (TSH) levels above normal, in thyroid function tests (> 7 μIU/mL);
– high levels of autoantibodies against thyroglobulin (anti-Tg) and autoantibodies against thyroid peroxidase (anti-TPO) (> 115 IU/mL and >34 IU/mL; respectively);
– decreased echogenicity in parenchyma or heterogeneous appearance of the parenchyma in ultrasonographic thyroid examination.

The exclusion criteria of our study were as follows:

– previous treatment for Hashimoto's thyroiditis and/or for any other thyroid disease;
– treatment with L-thyroxine for nodular goitre or any other thyroid disease;
– having any other autoimmune disease;
– corticosteroid or immunosuppressive treatment in the two months prior to the study;
– having an additional endocrinological disease (Cushing syndrome, Addison's, etc.);
– signs of disease such as acute coronary disease that might be a contraindication for L-thyroxine treatment;
– taking medication that would decrease the efficacy of L-thyroxine (cholestyramin, furosemide, coumadin, proton pump inhibitors).

Informed consent for the described procedures was obtained from all patients. Approval for the study was given by the ethics committee of our hospital.

Blood was drawn from patients diagnosed with hypothyroidism due to Hashimoto's thyroiditis before treatment, to evaluate serum levels of TSH, free triiodothyronine (FT3), free thyroxine (FT4), anti-Tg, anti-TPO, as well as serum levels of IL-2, IL-4, IL-12, interferon (IFN)-γ. After serum extraction from blood, these samples were stored at -30°C. Thyroid hormone replacement (L-thyroxine) treatment was initiated in the same patient group, 20-30 minutes before breakfast, in one dose. The dose was titrated every 2-3 weeks. Use of other medications that could influence treatment was monitored during check-up visits. After euthyroidism (serum TSH = 1-2 μIU/mL) was achieved, peripheral blood samples were taken again and serum was extracted for evaluation of cytokine levels.

Concentrations of serum FT3, FT4, TSH, anti-Tg, anti-TPO were measured using the chemiluminecent immunometric method, in a DPC Immulite 2000 automatic analyser (Los Angeles, CA, USA), using the Immulite 2000 commercial kit (Siemens Medical Solutions Diagnostics Los Angeles, CA, USA). Serum IL-2, IL-4, IL-12, IFN-γ levels were measured with the ELISA method using human commercial kits (BioSource Europe S.A., Nivelles, Belgium). Levels of these mediators below the sensitivity of the assay were 1 pg/mL for IL-2, 2 pg/mL for IL-4, 1.5 pg/mL for IL-12 and 4 pg/mL for IFN-γ.

The results were analysed with SPSS for Windows version 10.0 for personal computer with the Windows XP system. After calculating the average pre- and post-treatment values for serum hormones, anti-Tg, anti-TPO and cytokines and the mean ± standard error of mean (SEM), the paired Student's *t*-test with 95% confidence interval was used for the statistical analysis. Moreover, percentage changes were calculated using the pre-treatment level – post-treatment/pre-treatment level x 100 formula for all parameters. Correlations between the percentage changes in serum hormones and autoantibodies levels and percentage changes in serum cytokine levels were evaluated by the Pearson correlation test. P values less than 0.05 were accepted as statistically significant (2-tailed).

RESULTS

Euthyroidism was achieved clinically and confirmed by laboratory values in all the patients diagnosed with hypothyroidism due to Hashimoto's thyroiditis in our study. Mean serum levels of TSH in our patients before treatment were 11.68 ± 2.04 μIU/mL, while levels after treatment were 1.903 ± 0.1338 μIU /mL. This decrease was statistically significant ($p < 0.0001$) *(figure 1A)*. Mean serum levels of FT3 were 2.91 ± 0.0838 pg/mL before treatment, while they were 2.928 ± 0.1061 pg/mL after treatment; comparison of these values did not reveal a statistically significant difference ($p = 0.958$) *(figure 1B)*. A significant increase in the mean serum FT4 levels after treatment was detected (1.059 ± 0.0356 ng/dL *versus* 1.388 ± 0.0317 ng/dL; $p < 0.0001$) *(figure 1C)*. When thyroid antigen-specific antibodies were evaluated before and after treatment, there was a significant decrease in serum anti-Tg and anti-TPO levels (397.8 ± 92.46 IU/mL *versus* 287.6 ± 79.81 IU/mL for anti-Tg; $p < 0.01$, 225.6 ± 31.80 IU/mL *versus* 119.7 ± 23.31 IU/mL for anti-TPO; $p < 0.001$) *(figure 2A, B)*.

It was seen that serum IL-12 levels decreased significantly after treatment (120.0 ± 6.714 pg/mL *versus* 92.99 ± 6.501 pg/mL; $p < 0.0001$). On the other hand, although serum IFN-γ levels decreased after treatment, the decrease was not found to be statistically significant (7.095 ± 0.7572 pg/mL *versus* 6.296 ± 0.3724 pg/mL; $p = 0.276$). In our study, evaluation of IL-2 and IL-4 levels revealed that their values did not change following treatment (6.567 ± 0.9083 pg/mL *versus* 6.602 ± 0.7464 pg/mL; $p = 0.953$, 0.7464 ± 0.0788 pg/mL *versus* 1.569 ± 0.1099 pg/mL; $p = 0.313$; respectively). Data for serum levels of cytokines are shown in *figure 3A, B, C, D*. When all the percentage change values, calculated for all parameters, were correlated, no correlation was observed between levels of serum hormones or thyroid-specific autoantibodies and serum cytokines.

DISCUSSION

Hashimoto's thyroiditis is the most common autoimmune disease of the thyroid gland. Hypothyroidism is com-

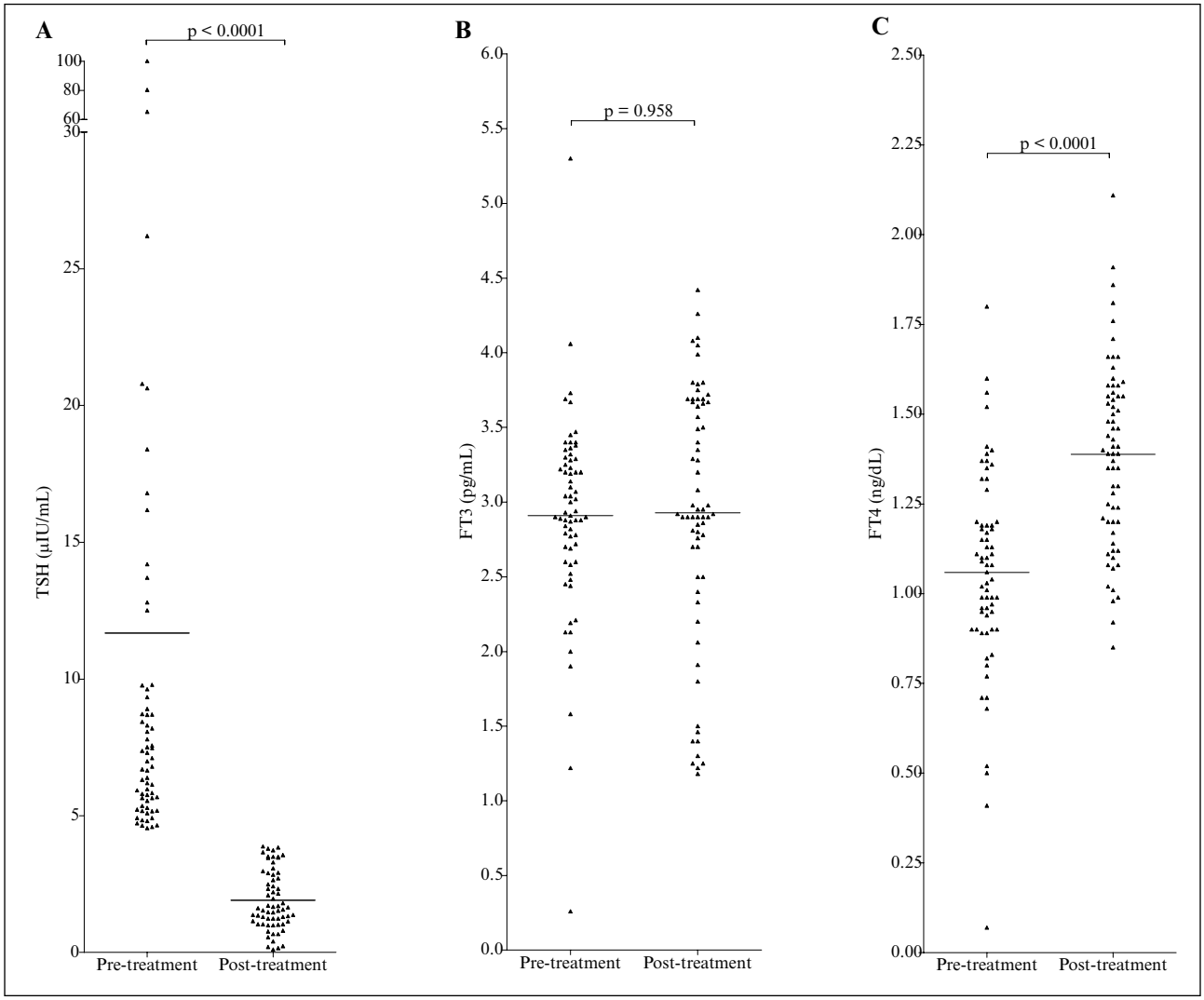

Figure 1
Serum thyroid-stimulating hormone (TSH) (**A**), triiodothyronine (FT3) (**B**) and free thyroxine (FT4) (**C**) levels in patients with Hashimoto's thyroiditis, before and after treatment.

monly encountered in these cases as a result of genetic, environmental and immunological factors [1, 2].

Euthyroidism was achieved in patients who were diagnosed with hypothyroidism secondary to Hashimoto's thyroiditis after receiving substitutive treatment with L-thyroxine. A significant decrease in serum TSH and a significant increase in serum FT4 were achieved with this treatment. This finding is an expected feature of L-thyroxine treatment in cases of hypothyroidism [17, 18]. When serum anti-Tg and anti-TPO levels were compared before and after treatment, both anti-Tg and anti-TPO levels were seen to decrease significantly as a result of treatment. Research that has been done in this field reports a significant decrease in both anti-thyroid antibodies as a result of L-thyroxine treatment [17, 18]. In an early study, the expression of autoantigens (such as TPO) in thyroid cells, was reported to be a dynamic phenomenon that is enhanced by TSH [19]. According to this phenomenon, supression of serum TSH levels via succesful L-thyroxine treatment causes a reduction in expression of thyroid cell autoantigens. Thus, the autoimmune response to thyroid cells is downregulated by inhibiton of autoantigenic expression. Another study has demonstrated that there was a significant decrease in these autoantibody levels in clinically euthyroid patient with Hashimoto's thyroiditis [20]. As a consequence of these data, the decrease in thyroid-specific autoantibody titres we obtained following L-thyroxine treatment was an expected and desired condition. In our study, the decrease in anti-TPO level was more significant than the decrease seen in the anti-Tg level. Our current knowledge indicates that anti-TPO levels are more important in the pathogenesis of the hypothyroidism seen in Hashimoto's thyroiditis. In patients positive for anti-TPO, a positive correlation with the development of hypothyroidism has been shown in previous research [21]. Moreover, another study has reported that the thyroid cell damage and/or death, in patients with Hashimoto's thyroiditis is due to anti-TPO antibodies as well as Th1 cytokines [22]. Another recent study has also emphasized the importance of anti-TPO in disease pathogenesis in patients with Hashimoto's thyroiditis [23]. These findings indicate that the decrease in anti-TPO antibody levels that we observed is much more important.

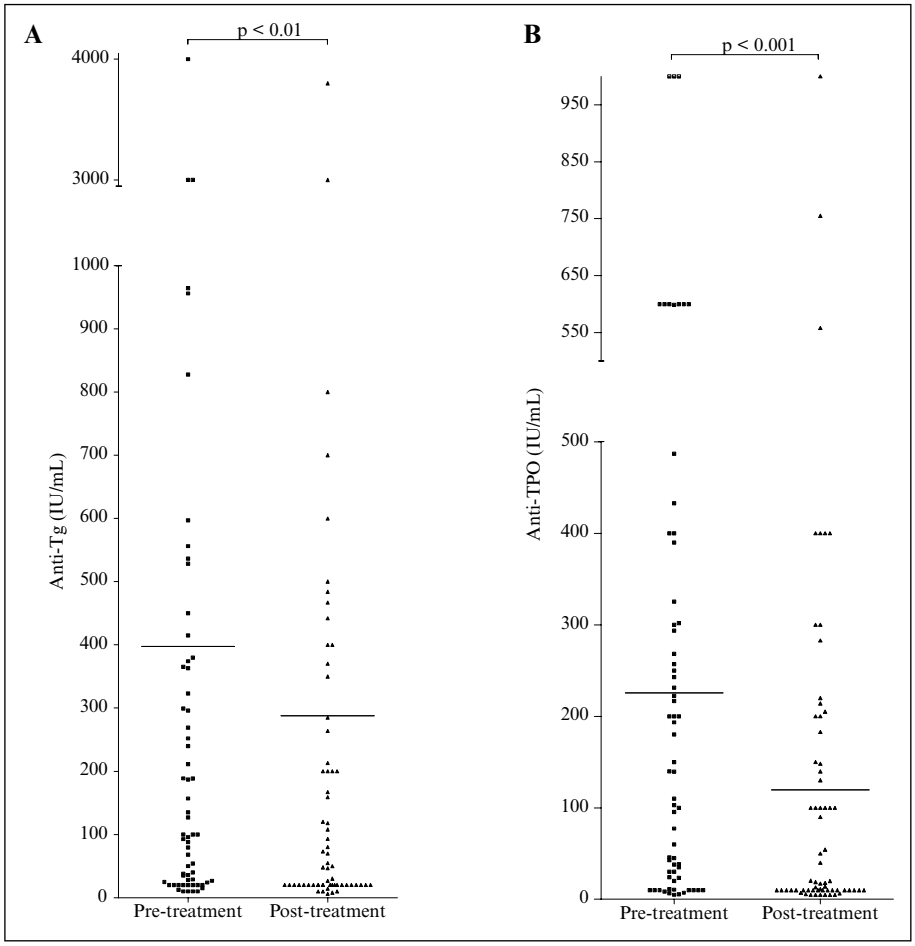

Figure 2
Serum autoantibodies against thyroglobulin (anti-Tg) (**A**) and autoantibodies against thyroid peroxidase (anti-TPO) (**B**) levels in patients with Hashimoto's thyroiditis, before and after treatment.

We have seen that the most dramatic response was the decrease in IL-12 levels when changes in cytokine levels were evaluated in our study before and after treatment. IL-12 is secreted by antigen-presenting cells, including B lymphocytes, monocytes/macrophages and dendritic cells. It is one of the most important cytokines responsible for Th1-type cytokine responses and is actually required for differentiation of Th0 or naïve cells into Th1 cells [24-26]. In a study performed by Kimura *et al*., IL-12 was supposed to be specifically responsible for the hypothyroidism that develops as a result of thyroiditis [27]. Our patients achieved the euthyroid state, both clinically and according to laboratory results, following L-thyroxine treatment. This condition may be explained by suppression of the inflammatory process by the L-thyroxine treatment. Therefore, the decrease in IL-12 cytokine levels, regarded as the major etiology of hypothyroidism, in our patients in whom euthyroidism was achieved, was not a surprise or a coincidence.

By the end of our study, it was observed that serum IFN-γ levels in those patients in whom the euthyroid state was achieved with L-thyroxine treatment, tended to decrease following treatment,. Although no statistically significant decrease was detected, this result might also be important. The known sources of IFN- γ are $CD4^+$ cells, particularly Th1 and $CD8^+$ T-cells and NK cells. Production of IFN-γ, which is produced by Th1 cells, is positively regulated by IL-12 [26, 28]. For this reason, decreasing IL-12 leads to inhibition of Th1-type differentiation and IFN-γ production by Th1 cells. In our results, the tendency towards decreasing IFN-γ levels was evaluated with the decrease in serum IL-12 levels, suggesting a suppression of the Th1-type inflammatory response. Nevertheless, in a study by Karanikas *et al*., IFN-γ was reported to be high, especially in patients with Hashimoto's thyroiditis accompanied by anti-TPO, and this was reported to have an important role in its pathogenesis [22]. According to this study, it can be hypothesised that IFN-γ may decrease in patients whom the inflammatory process is suppressed.

When serum IL-2 levels were evaluated in our study, it was seen that there was no significant change after the euthyroid state had been achieved with L-thyroxine in patients with hypothyroidism secondary to Hashimoto's thyroiditis. Previous literature has reported initial studies showing significantly high IL-2 levels in patients with hypothyroidism [29]. This condition can be regarded as an indicator of T lymphocyte-mediated inflammation in these patients. IL-2 is a proliferative factor for all T cells [26]. Although in our patients, a significant decrease in serum IL-12 levels and the tendency for IFN-γ levels to decrease after treatment, might point out a suppression of

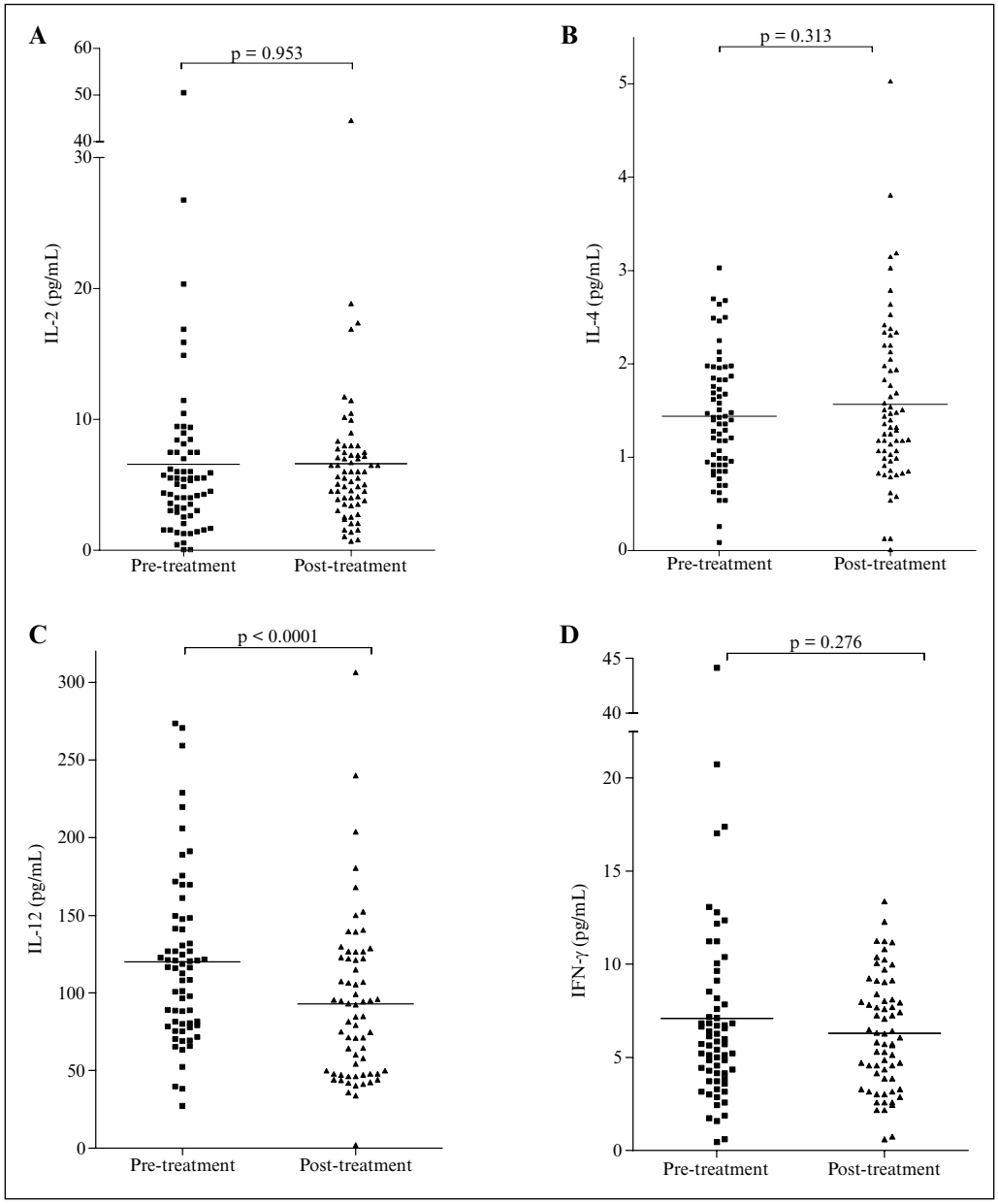

Figure 3
Serum interleukin (IL)-2 (**A**), IL-4 (**B**), IL-12 (**C**) and interferon (IFN)-γ (**D**) levels in patients with Hashimoto's thyroiditis, before and after treatment.

the Th1-type immune response, it does not mean that other T cells are inhibited too. It might be suggested that IL-2, which is also required for the proliferation of T cells other than Th1, did not decrease due to this.

In our study, IL-4 levels did not change significantly after L-thyroxine treatment. IL-4 is mainly a Th2 cytokine, which is involved in the antibody-mediated immune response. More commonly, it is responsible for IgE type antibodies, and it has an important role in allergic diseases and parasitic infestations [26]. There is no definitive study that has reported that IL-4 may be responsible for the pathogenesis of Hashimoto's thyroiditis in patients in whom the Th1-type immune response predominates. However, at the end of our study, it could be expected that the Th2-type immune response that is thought to increase relatively with suppression of the Th1-type inflammatory response, might be expected to demonstrate itself by an increase in IL-4. Although, we did not investigate cytokines that are indicators of regulatory T cells (such as IL-10 and transforming growth factor-β), the reason for suppression of inflammation after treatment with L-thyroxine might be the increase in the predominance of regulatory T cells. These cells are known to suppress particularly Th1- but also Th2-type immune responses [5, 30]. When this is considered, the absence of an increase in Th2 cell cytokines that oppose Th1 cells is not a surprise.

In conclusion, the suppression of inflammation that characterises the clinical and laboratory euthyroidism seen after L-thyroxine treatment in patients who develop hypothyroidism due to Hashimoto's thyroiditis, brings about a decrease in serum levels of Th1-induced IL-12.

This indicates that the Th1-type inflammatory response is important in pathogenesis of this condition as has been mentioned in earlier literature.

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