

Endothelial dysfunction in Graves' disease

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ABSTRACT

Purpose: Graves' disease (GD) is an organ-specific autoimmune thyroid disease, characterized by hyperthyroidism due to excessive production of thyroid hormone induced by thyrotropin receptor-specific stimulatory autoantibodies. In this study, we determined serum levels of the soluble forms of ICAM-1, VCAM-1, vWF, IL-6, IL-12, IL-18, fibrinogen and CRP in patients with subclinical (SH) and overt hyperthyroidism (OH) caused by GD to elucidate a possible role of those parameters as markers of endothelium dysfunction (ED).

Material/Methods: The study included 96 patients: 52 with GD and 44 euthyroid controls, divided into 3 groups according to their thyroid function tests: SH, OH and controls (CG).

Results: The values of IL-6, IL-12 and IL-18 were significantly higher in GD than in CG patients ($p < 0.0001$, $p < 0.0001$; $p < 0.00001$, respectively). Significant difference of sVCAM-1 values were found in the patients with GD compared to CG ($p < 0.0001$). Patients with GD had significantly higher levels of PAI-1 ($p < 0.00001$), vWF ($p < 0.0001$), fibrinogen ($p < 0.0001$) in comparison to CG. In patients with OH, we observed statistically higher values of fibrinogen compared to SH group ($p < 0.05$). There were no significant differences in serum concentration of other study parameters in patients with SH compared to the OH.

Conclusions: ED occurs during subclinical and overt hyperthyroidism causing decreased fibrinolytic activity, hypercoagulability and increased levels of IL-6, IL-12 and IL-18. These results support the notion that serum cytokines could be used as a marker of GD activity. Results of this study support the opinion that GD might require treatment as early as in the phase of SH.

Key words: hyperthyroidism, endothelium, Graves' disease

INTRODUCTION

Graves' disease (GD) is an organ-specific autoimmune thyroid disease, characterized by hyperthyroidism due to excessive production of thyroid hormone induced by thyrotropin receptor-specific stimulatory autoantibodies. In the recent years, it was underlined that altered balance of pro- and anti-inflammatory cytokines plays an important role in the pathogenesis of GD. The etiology of GD may be multifactorial, involving complex interactions between

genetic, environmental, endogenous and local factors [1]. However, immune response plays a central role in the pathogenesis of GD. Cytokines, a large group of non-enzymatic proteins, participate in the induction and effector phases of all inflammatory and immune responses, and are therefore likely to play a critical role in the development of autoimmune diseases [2]. It is also commonly known, that hyperthyroidism increases plasma levels of the biologically most active mediators [3], closely involved in hemostasis, fibrinolysis, growth factor synthesis and the regulation of

vessel tone and permeability. Thyroid hormones can activate vascular endothelium and may slow down the metabolism of adhesion molecules, and thereby cause an elevation of circulating adhesion molecules. The up-regulation of adhesion molecules on vascular cells appears to play a major role in the recruitment and targeting of the inflammatory response to certain tissues. The soluble forms of these adhesion molecules were demonstrated in various diseases, but their clinical significance is still undefined [4].

Endothelial dysfunction (ED), is characterized by an imbalance between relaxing and vasoconstricting factors, procoagulant and anticoagulant substances, and between pro-inflammatory mediators. Detection of ED is based on assessment of circulating markers of endothelial function (endothelin-1, von Willebrand factor, tissue plasminogen activator, plasminogen activator inhibitor, adhesion molecules) [5,6]. Previous studies found associations of subclinical hyperthyroidism (SH) with endothelial dysfunction [4,5]. However, data regarding the function of endothelium in SH caused by GD are insufficient. In the present study, we determined serum levels of the soluble forms of ICAM-1 (Inter Cellular Adhesion Molecule-1), VCAM-1 (Vascular cell Adhesion Molecule-1), von Willebrand factor (vWF), IL-6, IL-12, IL-18 (interferon-gamma-inducing factor), fibrinogen and CRP in patients with subclinical and overt hyperthyroidism (OH) caused by GD to elucidate a possible role of those parameters as markers of endothelium dysfunction.

MATERIAL AND METHODS

The study included 96 patients: 52 (40F/12M) with Graves' disease, free of Graves' ophthalmopathy and 44 (34F / 10M) euthyroid healthy controls of similar age and sex distribution. Based on the plasma levels of TSH and thyroid hormones, the patients were divided into 3 groups according to their thyroid function tests:

1. Subclinical hyperthyroidism (23 patients, 15F / 8M; TSH < 0.10 uIU/ml with normal FT4, FT3)
2. Overt hyperthyroidism (29 patients; 18F / 11M)
3. Control group (44 patients; 34F / 10M)

None of the participants received antithyroid therapy.

Patients with GD met the following criteria: free triiodothyronine (FT3) > 3.48 pg/ml, free thyroxine (FT4) > 1.85 ng/dl, thyroid-stimulating hormone (TSH) < 0.01 uIU/ml, clinical hyperthyroidism (agitation, heat intolerance, sweating, diarrhea, palpitations and restlessness), elevated levels of anti-thyroid antibodies, the first onset of the disease and no clinically apparent ophthalmopathy.

The blood samples were collected from antecubital vein without the use of tourniquet, between 7:30 and 8:30h, after an overnight fast to avoid the differences of diurnal variation.

Samples after clotting were processed for centrifugation, and sera were stored at -80°C until analysis.

All subjects underwent a comprehensive assessment including documentation of medical history, physical examination, and measurement of laboratory parameters. Physical examination included systolic and diastolic blood pressure, body weight and height. All patients and controls gave informed consent to participate in the study before enrolment. The protocol was approved by the local ethics committee.

TSH concentration (reference range 0.3 - 4.01 uIU/ml) was measured by means of a third-generation enzyme immunoassay, free thyroxine (reference range 0.71 - 1.85 ng/dl) and free triiodothyronine reference range 1.45 - 3.48 pg/ml) were determined using an enzyme immunoassay (MEIA, Abbott Park, USA). Serum concentrations for sICAM-1, sVCAM-1 and E-selectin were determined by enzyme-linked immunoassays (Sandwich Enzyme Immunoassay, R&D Systems, Minneapolis, USA). Serum levels of TPO antibodies and TG antibodies were routinely determined by immunoassays (Microparticle Enzyme Immunoassay-MEIA, Abbott AxSYM). Activity of vWF and PAI-1 were determined by immunoassays (Asserachrom, Diagnostica Stago, France). CRP was measured by means of Sandwich Enzyme Immunoassay (DSL-10-42100 Active, DSL, Webster, Texas, USA).

Statistical analysis

Statistica 7.1 was used for the statistical analysis. The normally distributed data were analysed by Student t-test. Mann-Whitney test was used to compare the differences between non-normally distributed data. Correlations between variables were assessed by Spearman rank order test. P values lower than 0.05 were considered statistically significant.

RESULTS

The main characteristics and laboratory parameters of the study population are reported in *Tab. 1*. Comparison of data of hyperthyroid patients with GD and controls are presented in *Tab. 2*. The correlations in the GD group are presented in *Tab. 3*.

The values of IL-6, IL-12 and IL-18 were significantly higher in GD (33.0 ± 3.0 ng/ml, 48.0 ± 6.4 pg/ml, 292.0 ± 131.4 pg/ml) than in CG patients (1.5 ± 1.3 ng/ml, 1.2 ± 1.2 pg/ml, 223.0 ± 53.4 pg/ml) ($p < 0.0001$, $p < 0.0001$, $p < 0.00001$, respectively). The highest values of IL-6 were observed in SH group (3.7 ± 3.3 ng/ml) compared to OH patients (3.2 ± 2.8 ng/ml) ($p < 0.01$). Significant difference of sVCAM-1 values were found in the patients with GD (362.3 ± 96.6 ng/ml) compared to CG (835.4 ± 302.6 ng/ml) ($p < 0.0001$). Patients with GD had significantly higher levels of PAI-1 (67.0 ± 38.6 ng/ml;

Table 1. Characteristics and laboratory parameters of study and control groups.

| Parameters | Graves' disease (n=52) Mean ± SD | Control group (n=44) Mean ± SD |
|---------------------------|--|--------------------------------------|
| Sex F/M | 40/12 | 34/10 |
| Age (years) | 49.2 ± 12.9 | 47.9 ± 12.1 |
| BMI (kg/m) | 24.6 ± 3.5 | 25.4 ± 3.4 |
| HR (bpm) | 89.4 ± 11.7 ¹ | 73.02 ± 7.04 |
| Systolic pressure (mmHg) | 136.3 ± 13.3 ¹ | 126.8 ± 9.4 |
| Diastolic pressure (mmHg) | 84.2 ± 8.6 ² | 80.1 ± 7.1 |
| TSH (uIU/ml) | 0.025 ± 0.052 ¹ | 1.150 ± 0.593 |
| FT3 (pg/ml) | 12.16 ± 21.77 | - |
| FT4 (ng/dl) | 6.80 ± 20.30 | - |

Differences between Graves' disease and control group:
¹p<0.01, ²p<0.05

33.0 ± 8.5 ng/ml; p < 0.00001), vWF (129.6 ± 25.8 ng/ml; 92.6 ± 32.9 ng/ml; p < 0.0001), fibrinogen (337.4 ± 208.4 ng/ml; 250.2 ± 83.1; p < 0.0001) in comparison to CG. In patients with OH we observed statistically higher values of fibrinogen compared with SH group (345.6 ± 128.7 ng/ml; 323.9 ± 301.3; p < 0.05). There were no significant differences in serum

Table 3. Spearman's rank correlation coefficients in the Graves' disease group (n=52).

| | Spearman's R | Statistical significance p < 0.05 |
|----------------------|--------------|--------------------------------------|
| TSH / vWF | -0.412 | <0.00001 |
| FT4 / sVCAM-1 | 0.363 | <0.05 |
| TPO / sVCAM-1 | 0.310 | <0.05 |
| sICAM-1 / IL-6 | 0.401 | <0.005 |
| sICAM-1 / E-selectin | 0.425 | <0.005 |
| vWF / Fibrinogen | 0.399 | <0.005 |
| IL-6 / E-selectin | 0.445 | <0.005 |
| PAI-1 / CRP | 0.394 | <0.01 |

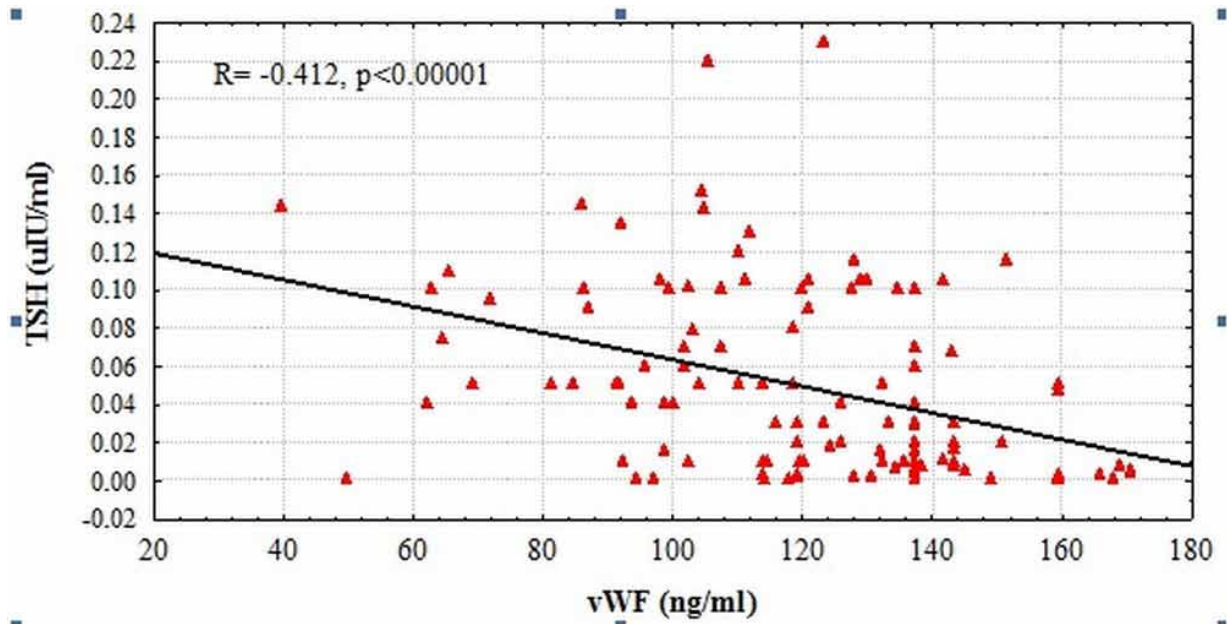
Table 2. Comparison of data in subclinical and overt hyperthyroid patients with Graves' disease and controls.

| Parameters Mean ± SD median (min-max) C ₂₅ -C ₇₅ | Graves' disease (n = 52) | Graves' disease Overt hyperthyroidism (n = 29) | Graves' disease Subclinical hyperthyroidism (n = 23) | Control group (n = 44) |
|--|--|---|---|---|
| IL-6 (ng/ml) | 3.3 ± 3.0 2.3 (0.7- 11.3) ^a 1.3-3.9 | 3.2 ± 2.8** 2.1 (0.7-11.3) 1.2-4.4 | 3.7 ± 3.3 2.7 (0.7-10.2) 1.3-3.8 | 1.5 ± 1.3 1.2 (0.7-8.7) 0.7- 1.8 |
| IL-12 (pg/ml) | 4.8 ± 6.4 3.3 (0.5-39.9) ^a 1.6-6.4 | 4.9 ± 5.7 3.1 (0.5 39.9) 1.7-5.7 | 3.0 ± 3.0 1.8 (0.5- 15.6) 0.65-4.6 | 1.2 ± 1.2 0.5 (0.5-6.8) 0.5-1.4 |
| IL-18 (pg/ml) | 292.0 ± 131.4 287.2 (122.4 -605. 5) ^b 180.5-368.9 | 288.2 ± 124.0 276.3 (109.3- 620.5) 186.0-368.5 | 259.4 ± 104.9 212.8 (94.9-605.5) 161.0-300.2 | 223.0 ± 53.4 232.8 (117.1-376.6) 181.1-257.8 |
| PAI-1 (ng/ml) | 67.0 ± 38.6 58.5 (13.6-162.4) ^a 42.4-92.5 | 72.5 ± 40.3 58.3 (13.6-162.4) 44.9-98.8 | 60.0 ± 35.6 58.5 (15.6-137.8) 29.9-83.6 | 33.0 ± 8.5 31.5 (6.6-72.1) 18.2-47.0 |
| vWF (ng/ml) | 129.6 ± 25.8 133.3 (39.8-170.5) ^a 118.7-141.8 | 134.1 ± 23.8 137.5 (86.4-169.0) 119.4-159.6 | 122.3 ± 28.0 128.2 (39.8-170.5) 110.8-137.5 | 92.6 ± 32.9 94.2 (25.6-150.8) 73.0-116.3 |
| E-selectin (ng/ml) | 61.4 ± 31.2 53.0 (18.0-166.0) 42.0-80.0 | 65.3 ± 34.6 61.0 (18.0-166.0) 42.0-86.0 | 54.9 ± 24.3 52.0 (24.0-115.0) 39.0-69.0 | 58.0 ± 17.5 58.0 (22.0-90) 40.0-68.0 |
| sICAM-1 (ng/ ml) | 362.3 ± 96.6 341.0 (212.0-622.0) 300.0-418.0 | 361.3 ± 89.8 347.0 (212.0-584.0) 300.0-434.0 | 363.9 ± 109.9 325.0 (240.0-622.0) 300.0-393.0 | 333.7 ± 81.9 314.0 (208.0-532.0) 278.0-404.0 |
| sVCAM-1 (ng/ml) | 1369.0 ± 595.4 1210.0 (560.0-2980.0) ^b 950.0-1666.0 | 1420.9 ± 654.2 1210.6 (560.0-2980.0) 950.0-1666.0 | 1284.6 ± 493.4 1160.9 (715.0-2465.0) 933.0-1527.5 | 835.4 ± 302.6 792.0 (435.0-1770.0) 607.0-1010.0 |
| CRP (ng/ml) | 5.0 ± 4.6 3.4 (2.2-26.2) 2.9-4.7 | 5.7 ± 5.5 3.4 (2.2-26.2) 3.1-4.7 | 3.5 ± 1.1 3.5 (2.2-6.1) 2.6-4.1 | 4.4 ± 1.3 3.2 (1.6-16.1) 2.7-4.3 |
| Fibrinogen (ng/ml) | 337.4 ± 208.4 303.5 (82.0-1280.0) 212.0-368.0 | 345.6 ± 128.7* 326.0 (92.0-589.0) 295.0-446.0 | 323.9 ± 301.3 271.0 (82.0-1280.0) 171.5-293.0 | 250.2 ± 83.1 244.0 (67.0-374.0) 185.0-325.0 |

Differences between GD and control group at: ^ap<0.0001, ^bp<0.000001.

Differences between SH and OH at: *p<0.05, **p<0.01.

Figure 1. Correlation between TSH and vWF in the Graves' disease group (n=52).



concentration of other study parameters in patients with SH compared to the OH (Tab. 2).

We observed significantly negative correlation between TSH and vWF ($R = -0.412$, $p < 0.00001$) (Tab.3, Fig.1), but we have also found no correlation between the levels of vWF and thyroid hormones in GD group.

DISCUSSION

Cytokines are small molecules secreted by cells of the immune system that serve to regulate various other components of the immune system [7]. The role of cytokines is often confusing and is neither independent nor exclusive of other immune mediators. A regulatory cytokine may either favor induction of tolerance against thyroid autoimmune disease or activation and/or exacerbation of autoimmune responses. These apparently contradictory functions of a given cytokine are primarily influenced by the nature of co-signaling delivered by other cytokines [8]. Consequently, a thorough understanding of the role of a particular cytokine in the context of a specific immune response is essential for the development of appropriate strategies to modulate cytokine responses to maintain or restore health [9]. Cytokine production was studied in thyroid tissue from patients with GD, Hashimoto's thyroiditis and non-toxic goiter [7]. These results demonstrated that the lymphocytic infiltrate found in autoimmune and non-autoimmune thyroid disorders is associated with cytokine production. The cytokines produced by thyroid follicular cells may have an important role in stimulating autoantigen specific T cells in vivo as both interleukin-1 and interleukin-6 facilitate T cell activation. Interleukin-6 promotes changes peripheral thyroid hormone

metabolism, and IL-12 seems to be involved in the regulation of the central part of the hypothalamic-pituitary-thyroid axis during illness. IL-18 is a pro-inflammatory cytokine which shares important biological properties with IL-12, such as interferon gamma-inducing activity. In the present study, we assessed several markers of cell-mediated immune response in patients with GD and healthy subjects. In our study, Graves' disease has been associated with increased levels of IL-6, IL-12 and IL-18 suggesting a strong and systemic stimulation of the immune response in autoimmune thyroid disease. These results support the notion that serum cytokines could be used as a marker of GD activity.

In the present study, we found that the serum IL-6 levels of GD patients in subclinical hyperthyroidism were higher than that of the overt hyperthyroid patients. This suggests that IL-6 could play a role in the pathogenesis of GD. Measurements of IL6 in hyperthyroidism have shown conflicting results: these levels have been found normal [10,11] or increased [12-15]. In other study, serum IL-6 levels remained unchanged despite marked abnormalities in thyroid hormone levels, suggesting that the previously increased serum IL-6 levels reported in patients with Graves' disease were the result of the autoimmune process underlying this disorder [10]. On the other hand, no association of serum IL-6 concentrations with changes in thyroid function has been found [16] in contrast to our present results. Considering that in our present study the changes in IL-6 levels were observed in patients with subclinical hyperthyroidism suggests that thyroid hormones directly modulate circulating markers before clinical symptoms occur:

Adhesion molecules play a central role in cell-to-cell communications for the ultimate induction of an effective immune response. sICAM-1 and sVCAM-1 are co-expressed

on activated endothelium by inflammatory mediators [17]. Previously it was reported that patients with GD show a reduction in the physiological protective mechanisms against endothelial damage, probably induced by increased inflammation and oxidative stress. Thus, thyroid hormones could activate vascular endothelium at non-related sites and decrease the metabolism of adhesion molecules, and thereby cause an elevation of circulating adhesion molecules. To assess the potential role of adhesion molecules in the pathogenesis of thyroid disease, we examined the level of several of these adhesion molecules, including intercellular adhesion molecule-1, vascular cell adhesion molecule and E-selectin. In our study, the level of sVCAM-1 was higher in GD group than in controls patients, and the differences were statistically significant. Serum concentration of sVCAM-1 correlated closely to serum levels of fT4. Also, De Ciuceis C *et al.* [18], found that patients with GD had an increase in circulating levels of IL-18 and sVCAM-1. Jublanc C *et al.* [19], found that autoimmune-mediated dysthyroidism was associated with increased peripheral blood concentrations of sVCAM-1 and sICAM-1. In the present study, similar values for sICAM-1 and E-selectin were demonstrated in the groups of overt and subclinical hyperthyroid patients with GD as well as in the group of controls. In contrast to our results, Miyazaki *et al.* [20] in their study of the patients with GD, found that serum levels of sELAM-1 and sICAM-1 were elevated, while the expression of sELAM-1 and sVCAM-1 on thyroid perifollicular endothelial cells showed no statistical significance.

We conclude, that the antithyroid antibodies involved in the pathogenesis of Graves' disease cause the elevation of the concentration of adhesion molecules, which act as mediators of lymphocyte inflow and adhesion to the tissue of the thyroid gland. The evaluation of the serum level of soluble forms of adhesion molecules in hyperthyroidism allows control over the autoimmune process. Graninger *et al.* [21], reported that in contrast to other endothelial markers, which are elevated in nonimmunologically mediated hyperthyroidism, an elevation of sVCAM-1 could be specific for immunopathological effects of the vessel wall. In our study, the level of sVCAM-1 was higher in overt and subclinical patients compared to control group and the differences were statistically significant. We also found significantly negative correlation between serum levels of s-VCAM-1 and FT4. Based on the obtained results it may be concluded that elevated sVCAM-1 concentrations are dependent on auto immune factors, as well as on hyperthyroidism. We concluded that serum concentrations of sVCAM-1, but not sICAM-1 and E-selectin, could be useful as clinical marker for disease activity in addition to thyroid hormones and auto-antibodies.

The hemostatic balance is a complex system where the delicate equilibrium is regulated by several factors including hormones. Various abnormalities of coagulation and fibrinolysis occur in patients with thyroid diseases,

and may range from subclinical laboratory abnormalities to clinically significant disorders of coagulation and rarely, major hemorrhage or thromboembolism [22]. According to the recent literature, most of the coagulation or fibrinolytic abnormalities associated with thyroid dysfunction are the consequences of direct effects of thyroid hormones on the synthesis of various hemostatic parameters. Thyroid autoimmunity may also modify the processes of secondary hemostasis. Hyperthyroidism is generally associated with hypercoagulability and decreased fibrinolytic activity [23]. One of these factors which is synthesized in endothelial cells is vWF. It has been previously shown that increased vWF levels reflect ED and is increased in subclinical [4,5] and overt hyperthyroid group [24,25]. We found that compared with the euthyroid control group, vWF level was significantly increased in the hyperthyroid, but FT3, FT4 did not correlate with vWF. We observed negative significant correlation between TSH and vWF. In our study, hyperthyroidism was caused by GD and therefore it is impossible to discriminate the impact of autoimmunity, inflammation vs. thyroid hormone excess on ED. Plasma vWF was measured as our 'gold-standard' marker, alongside with fibrinogen which is well-known and widely described acute phase reactant. Its metabolites can cause endothelium damage and dysfunction. We observed higher fibrinogen level in overt hyperthyroidism group compared to the SH group. Similar results were obtained by Coban *et al.* [26]. The results suggest that subjects with subclinical and overt hyperthyroidism present a state of relative hypercoagulability, but it is yet to be clarified whether higher fibrinogen level is a primary cause or is secondary to endothelial dysfunction. This state could contribute to increased thromboembolic risk in subclinical overt hyperthyroidism.

The imbalance between thrombotic and antithrombotic factors leads to pathological thrombus formation. A defect in the fibrinolytic system is sufficient for extended fibrin deposition and enhanced procoagulation. Plasma plasminogen activator inhibitor-1 is the main inhibitor of the fibrinolytic system. Both, increased and decreased fibrinolytic activity, have been reported in patients with hyperthyroidism. Elevated levels of PAI-1 antigen have been found in hyperthyroid patients, in both SH and OH groups [14,27]. In the present study, PAI-1 level was statistically significantly higher in SH and OH hyperthyroid patients compared to controls. Li *et al.* [25] reported that serum thyroid hormone concentrations were strongly correlated with plasma PAI-1. Acinci *et al.* [28] found that decreased serum TSH was an independent predictor of elevated PAI-1 antigen levels. On the contrary, in our study there was no significant correlation between TSH and PAI-1 serum levels and we observed significant correlation between PAI-1 level and CRP concentration.

CRP plays a crucial role in low-grade inflammation of arterial wall, which is believed to be the first step of atherosclerotic plaque formation, just before structural

changes. However, the impact of hyperthyroidism on CRP is still controversial. In the current study, CRP levels were higher in patients with subclinical and overt hyperthyroidism compared to the healthy controls, but they did not differ significantly. Some authors did not find any significant differences in CRP levels according to the thyroid function status.

CONCLUSIONS

In summary, our results suggest that subjects with Graves' disease develop endothelial dysfunction, which depends on autoimmune factors and not on the degree of hyperthyroidism. Endothelial dysfunction occurs in the stage of subclinical and overt hyperthyroidism, causing decreased fibrinolytic activity, hypercoagulability and increased levels of IL-6, IL-12 and IL-18. These results support the notion that serum cytokines could be used as a marker of GD activity. Results of this study support the opinions that Graves' disease might require treatment as early as in the phase of subclinical hyperthyroidism.

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