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Hashimoto's Disease - Involvement of Cytokine Network and Role of Oxidative Stress in the Severity of Hashimoto's Thyroiditis

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1. Introduction

Autoimmune thyroid diseases (AITDs) such as Hashimoto's disease (HD) and Graves'disease (GD) are archetypes of organ-specific autoimmune disease (Davies et al., 1988; Volpe, 1995). Hashimoto's disease (HD), Hashimoto's thyroiditis (HT) or chronic autoimmune lymphocytic thyroiditis was first described by H. Hashimoto in 1912 as struma lymphomatosa (Hashimoto H.,1912). Histological and cytological features of HT include a dense thyroidal accumulation of lymphocytes, plasma cells and occasional multinuclear giant cells. The epithelial cells are enlarged, with a distinctive eosinophilic cytoplasm, owing to increased number of mitochondria. HD is characterized by the presence of thyroid autoantibodies to thyroglobulin (Tg) and to thyroid peroxidase (TPO). The autoantibodies present in this disorder were identified in 1956 by Roitt et al. (Roitt et al., 1956).

HT is the most common underlying cause for hypothyroidism. It has been estimated that about 3–4% of the population suffers from HT. This disorder is most commonly found in middle-aged and elderly females, but it also occurs in other age groups (Canaris et al. 2000). HT is distributed throughout the world without racial and ethnic restriction.

The severity of HT vary among patients. Most patients with HD maintain a lifetime euthyroid state without any medical treatment, whereas others become hypothyroid. The immunological differences that underlie differences in severity remain unclear. Various cytokines may play role in this process; thyroid autoantibodies are independently involved in the severity of HD (Ito et al., 2006). The increased oxidative stress and a deficiency of cellular antioxidative defense in HT patients may be related to the processes of development of hypothyroidism.

In this view, to clarify the role of serum cytokines and antioxidant enzyme activities in different stages of disease we investigated three sub-groups of patients with autoimmune thyroiditis according to the thyroid function: group I —euthyroid subjects; group II— hypothyroid subjects; and group III—subjects treated with Levothyroxine and healthy controls.

2. Genetic and environmental factors

The interaction between internal (genetic) and external (environmental and endogenous) factors is required to initiate Hashimoto's disease. Environmental triggers of HT include high iodine intake, selenium deficiency, pollution, stress, bacterial and viral infections, cytokine therapy (Noel et al., 2002; Tomer & Davies, 1993; Bartalena et al., 2007). Probably puberty, pregnancy and menopause are factors contributing to disease. The role of dietary iodine is well defined in epidemiological studies and in animal models and seems to be the most significant environmental factor to induce thyroiditis. Environmental factors (particularly, iodine intake and infection) could cause insult of the thyrocyte followed by abnormal expression of major histocompatibility complex (MHC) class I and class II molecules, as well as changes to genes or gene products (such as MHC class III and costimulatory molecules) needed for the thyrocyte to become an antigen-presenting cell (APC). In this stage, a modulating role of sequence variants of human leukocyte antigen (HLA) class II molecules could become pivotal in binding and presenting thyroid antigenic peptides derived from Tg, TPO and TSHR (thyroid-stimulating hormone receptor) (Weetman, 2003). Selenium is other micronutrient involved in thyroid hormone metabolism, which exert various effects, while maintaining the cell reduction-oxidation balance (Beckett & Arthur, 2004; Duntas, 2009). Genetic variations in Tg, and probably in TSHR and other thyroid-specific genes, might be responsible for generating an autoimmune response. Genetic factors predominate, accounting for approximately 80% of the likelihood of developing AITDs, whereas at least 20% is due to environmental factors.

3. Pathogenesis

Several antibody and cell-mediated mechanisms contribute to thyroid injury in autoimmune hypothyroidism. In general, in case of Hashimoto's thyroiditis, the expressions of death receptors such as CD95 and death receptor ligands such as CD95L and TRAIL in the thyroid tissue appear to be much higher compared to normal subjects. Also, the expression of positive effectors of apoptosis such as caspase 3 and 8, as well as Bax and Bak appear to be relatively high in thyroiditis samples as compared to controls. This expression pattern clearly supports enhanced apoptosis as the mechanism underlying the loss of thyrocytes in Hashimoto's thyroiditis. There is significant expression of Fas/CD95 and its ligand in the thyrocytes who undergo apoptosis in Hashimoto's thyroiditis. Cytokines appear to play a crucial role in the pathology of the disease by enhancing the expression of caspases and there by sensitizing cells to FAS mediated apoptosis (Weetman, 2004).

3.1 B Cell response

Three principal thyroid autoantigens mentioned above are involved in AITDs. These are TPO, Tg and the TSH receptor. TPO Abs appear involved in the tissue destructive processes associated with the hypothyroidism observed in Hashimoto's and atrophic thyroiditis. The appearance of TPO Abs usually precedes the development of thyroid dysfunction. Some studies suggest that TPO Abs may be cytotoxic to the thyroid (Chiovato et al., 1993; Guo et al., 1997). The pathologic role of Tg Abs remains unclear. TPO Abs and/or Tg Abs are frequently present in the sera of patients with AITDs (Doullay et all., 1991). However, occasionally patients with AITDs have negative thyroid autoantibody test results.

Longitudinal studies suggest that TPO Abs may be a risk factor for future thyroid dysfunction; changes in autoantibody concentrations often reflect a change in disease activity.

TPO is a 110 kD membrane bound hemo-glycoprotein with a large extracellular domain, and a short transmembrane and intracellular domain. TPO is involved in thyroid hormone synthesis at the apical pole of the follicular cell. Several isoforms related to differential splicing of TPO RNA have been described. TPO molecules may also differ with respect to their three-dimensional structure, extent of glycosylation and heme binding. Most of the TPO molecules do not reach the apical membrane and are degraded intracellularly. TPO autoantibodies were initially described as anti-microsomal autoantibodies (AMA) since they were found to react with crude preparations of thyroid cell membranes. The microsomal antigen was later identified as TPO (Czarnocka et al., 1985). TPO Abs is the most sensitive test for detecting autoimmune thyroid disease (Mariotti et al., 1990). TPO Abs are typically the first abnormality to appear in the course of developing hypothyroidism secondary to Hashimoto's thyroiditis. In fact, when TPO Abs are measured by a sensitive immunoassay, over 95% of subjects with Hashimoto's thyroiditis have detectable levels of TPO Abs.

Tg - the prothyroid globulin, is a high molecular weight (660 kDa) soluble glycoprotein made up of two identical subunits. Tg is present with a high degree of heterogeneity due to differences in post-translational modifications (glycosylation, iodination, sulfation etc). During the process of thyroid hormone synthesis and release, Tg is polymerized and degraded. Consequently, the immunologic structure of Tg is extremely complex. The heterogeneity of Tg Abs are restricted in patients with AITDs compared with other thyroid disorders. Tg Abs measurements do not appear to be a useful diagnostic test for AITDs in areas of iodide sufficiency (Ericsson et al., 1985; Nordyk et al., 1993). Tg Abs are found in less than 60% of patients with lymphocytic thyroiditis.

Tg and TPO antibodies occur in very high concentration in patients with Hashimoto's thyroiditis and primary myxedema. Both of the antibodies show partial restriction to the IgG1 and IgG4 subclass. Tg antibodies usually mediate Antibody mediated cytotoxicity (ADCC), where as TPO antibodies form terminal complement complexes within the thyroid gland. Cell mediated injury may be necessary for TPO antibodies to gain access to their antigen and become pathogenic.

Thyroid stimulating antibodies (TS Abs) occurs in 10% to 20% of patients with autoimmune hypothyroidism (AH) but their effects are obscured by TSH-R-blocking antibodies and destructive processes.

Karanikas at al. demonstrate that high TPO Abs titres correlate with increased frequencies of T cells producing cytokines, enhancing cellular cytotoxic immunity, e.g. Interferon Gamma (IFN-γ) and Tumor necrosis factor -alpha (TNF-α), reflecting high disease activity. The role of thyroid autoantibodies in different stage of Hashimoto's disease remains unclear. (Karanikas at al., 2005).

To clarify the prevalence of TPO Abs in different stages in Hashimoto's thyroiditis we measured serum levels of TPO Abs in 128 out-patients with autoimmune thyroiditis from the Department of Internal Medicine, Stara Zagora University Hospital (Bulgaria), with HT and in 52 healthy controls. In all patients diagnosis had been made by enlarged thyroid

glands, elevated TPO Abs and/or typical hypoechogenicity of the thyroid in high-resolution sonography. In negative TPO Abs patients fine needle aspiration biopsy (FNAB) was performed and typical cytological features of autoimmune thyroiditis were found. Serum levels of TSH, free thyroxin (fT4) were estimated. Fasting samples of venous blood were collected in the morning between 8.00 and 10.00 h. Serum samples were routinely collected and stored frozen at -20 C until assayed. At the time of sampling, neither of the patients and control subjects had clinical signs or symptoms of intercurrent illness. TPO Abs were measured by ELISA, using commercially available kits (The Binding Site LTD, England). FT4 was measured by competitive immunoassay on the ACS180 (Chiron Diagnostics USA). TSH was measured by a third generation two-site chemiluminometric assay on the ACS180. The reference range was 11.5-22.7 pmol/l for fT4 and 0.35-5.5 μIU/ml for TSH. Patients were divided into tree subgroups according to the thyroid function. Group I (n=40) involved subjects with normal thyroid function (TSH and fT4 within the normal range). Group II (n=17) included patients with hypothyroidism (high levels of TSH and low or normal serum levels of fT4). In group III (n=71) were enrolled subjects with hypothyroidism treated with Levothyroxine (LT4) in a dosage to maintain TSH and fT4 within the normal range. The medication of Levothyroxine (range: 50-200 μg) was given in the fasting state. Fifty two healthy subjects were included as controls. Informed consent was obtained from all participants in the study according to the ethical guidelines of the Helsinki Declaration. The relevant clinical and biochemical data of all of patients studied and controls are summarized in Table 1.

Variables	Controls	HT	Range
N	52	128	
Gender (M/F)	9/43	9/119	
Age (years)	44.6±1.8	47.0±1.2	
TSH (mIU/l)	1.3±0.8 [1]	14.4±4.3 [1]	0.35-5.5
fT4 (pmol/l)	15.8±0.6 [2]	12.8±2.8 [2]	12-22
TPO Abs (U/ml)	12,7±1,9 [3]	528±39,2 [3]	< 150
TPO Abs Neg/Pos(%)	52/0 (0%) [4]	35/93 (73%) [4]	

Statistical significance: 1, 2, 3, 4 : $p<0.05$

Table 1. Clinical features of controls and Hashimoto's thyroiditis patients included in the study (mean±SEM).

Statistical analysis was carried out using the Statistica 5.5 for Windows. The results were reported as means ± SD (SE). Student's *t*-test or non-parametric Mann Whitney U test were used to determine whether differences between means were significant. Correlations between the different parameters were calculated by linear regression analysis. $P \leq 0.05$ was considered statistically significant.

The clinical and biochemical data of subgroups of Hashimoto's thyroiditis patients are presents in Table 2.

Characteristics	Group I	Group II	Group III	Range
N	40	17	71	
Gender (M/F)	0/40	1/16	8/63	
Age (years)	48.8±1.8	47.6±3.5	46.2±1.7	
TSH (mIU/l)	2.4±1.3 [1]	18.6±4.8 [1,2]	3.7±2.6 [2]	0.35-5.5
fT4 (pmol/l)	14.6±2.2 [3]	10.3±3.1 [3,4]	17.1±4.4 [4]	12-22
TPO Abs (U/ml)	397±57 [5,6]	691±95 [5]	574±58 [6]	<150
TPO Abs Neg/Pos(%)	16/24 (60%)[7]	1/16 (94%)[7]	18/53 (75%)	

Statistical significance: 1, 2, 3, 4, 5, 6, 7 : $p<0.05$

Table 2. Baseline characteristics and TPO Abs levels in Hashimoto's thyroiditis patients – euthyroid group (Group I), hypothyroid group (Group II) and group of patients treated with Levothyroxine (Group III) (mean±SEM).

We found statistical significant differences of serum TPO Abs levels in sub-groups of patients with autoimmune thyroiditis compared to controls. Concentrations of TPO Abs in euthyroid group (397±57 U/ml) are lower in comparison with both hypothyroid group (691±95 U/ml) and group of patients treated with levothyroxine (574±58 U/ml) ($p=0.02$; $p=0.03$ respectively). After treatment with thyroid hormones serum levels of TPO Abs declined without reached statistical significance. We found significantly higher TPO Abs (+) patients in hypothyroid group in comparison with euthyroid Hashimoto's group ($x2 =6.63$, $p=0.01$). Ito at al. also found significantly higher titers of TPO Abs in HT patients with overt hypothyroidism than in those with euthyroidism (Ito at al., 2006). The same study showed the significant association of IFN-γ gene polymorphisms with the severity of autoimmune thyroid diseases (Ito et al., 2006). In work of Schmidt et al. serum TPO Abs levels also declined on HT patients, but after a mean 50 months of treatment with levothyroxine (Schmidt et al., 2008). We may conclude that patients with euthyroid HT differ immunologically from patients with hypothyroid HT.

3.2 T Cell response

3.2.1 Cytokines and Th1/Th2 balance

Cytokines are small glycoprotein chemical structures that act in paracrine and endocrine fashion as soluble signals between cells and play a pivotal role in the immune response. They are the hormonal messengers responsible for most of the biological effects in the immune system, such as cell-mediated immunity and allergic type responses. T lymphocytes are a major source of cytokines. A wide array of cytokines including interleukin (IL)-2, IFN-γ, TNF-α, IL-4, IL-6, IL-10, IL-12, IL-13 and IL-15 are produced by the lymphocytes with some variation between patients (Nilsson et al., 1998). There are two main subsets of T lymphocytes, distinguished by the presence of cell surface molecules known as CD4+ and cytotoxic T cells (CD8+). T lymphocytes expressing CD4+ are also known as helper T cells, and these are regarded as being the most prolific cytokine producers. CD4+ T

helper lymphocytes play a key role in the pathogenesis of inflammatory and autoimmune diseases via the production of distinctive sets of cytokines (Druet et al., 1996). On the basis of their pattern of cytokine synthesis, CD4+ Th (helper) cells were originally classified into Th1 and Th2 lymphocytes, which are involved in cellular and humoral immune responses, respectively (Tato et al., 2006). The cytokines produced are known as Th1-type cytokines and Th2-type cytokines. Type 1 helper T cells are characterized by the production of pro-inflammatory cytokines like IFN-γ, IL-2, IL-12, IL-15, IL-18 and TNF-beta. Th1 cells are involved in cell-mediated immunity. The cytokines produced by Th1 cells stimulate the phagocytosis and destruction of microbial pathogens. Type 2 helper T cells are characterized by the production of IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13. Th2 cells are thought to play a role in allergy responses and facilitate humoral immune responses. Cytokines like IL-4 generally stimulate the production of antibodies. Improved understanding of Th1 and Th2 differentiation will improve our overall understanding of the immune system. Th1 and Th2 lymphocyte subpopulations are with different in some cases even contradictory functions (Ajjan et al., 1996; Sterzl 1999). A third subset of CD4+ cells (Th3 lymphocytes) mainly synthesize transforming growth factor - beta (TGF-β) and are considered as regulatory cells (Shevach, 2000).

Disturbed mechanism of innate immunity, resulting from macrophage activation through innate immunity receptors may be the basis of pathologically high levels of cytokine production and activation (Boraschi D. & Dinarello C.A., 2006). Classical macrophage activation in response to microbial products has long been recognised and gives rise to potent effector macrophages (M1), which kill microorganisms and tumour cells and produce proinflammatory cytokines and chemokines (including IL-12, TNF-α, IL-1, IL-6, IL-8). More recently, it has been shown that anti-inflammatory molecules, such as glucocorticoid hormones, IL-4, IL-13 and IL-10, are more than simple inhibitors of macrophage activation, in that they induce a distinct activation pathway (alternatively activated macrophages) (Gordon, 2003; Mosser, 2003). Alternative macrophage activation with IL-4 and IL-13 induces M2 macrophages, which can regulate inflammatory responses and adaptive Th1 immunity (Mantovani et al., 2002; Mantovani et al., 2004). Classically and alternatively activated (polarised) macrophages have been referred to as M1 and M2, in analogy with the Th1/Th2 dichotomy in T cell responses. M1 or M2 polarised macrophages differ in terms of receptor expression, cytokine and chemokine production, and effector function. Differential cytokine production characterises polarised macrophages. The M1 phenotype includes IL-12 and TNF-α, while M2 macrophages typically produce IL-10, the IL-1 receptor antagonist (IL-1Ra) and the type II IL-1 receptor (IL-1RII). Differential production of chemokines, which attract Th1 *versus* Th2 or T regulatory cells, integrates M1 and M2 macrophages in circuits of amplification and regulation of polarised T cell responses. The microenvironment thus influences macrophage activation and their subsequent functions. In this light, genetic and environmental conditions that promote M1/Th1 polarisation and inhibit M2/Th2 regulatory activity may contribute to the establishment of a chronic inflammatory condition. This may develop into autoimmunity following triggering events (e.g., an infection or trauma) that would induce an autoimmune adaptive response, through mechanisms of molecular mimicry.

IL-12 is a proinflammatory cytokine mainly produced by activated macrophages, dendritic cells, and granulocytes (Hsieh et al., 1993; Macatonia et al., 1993; Heufler et al., 1996). It acts

upon T, B, and natural killer (NK) lymphocytes, although it is best known for inducing the differentiation of CD4+ T lymphocytes from a Th0 to a Th1 phenotype (Trinchieri, 1993). IL-12 is a heterodimer (p70) composed of a p40 subunit that is expressed predominantly on antigen-presenting cells and a p35 subunit that is present constitutively in numerous cells. Both subunits, have to be secreted by the same cell for production of a bioactive molecule. IL-12 binds to a specific plasma membrane receptor, ultimately resulting in transcription of the genes involved in prototypic Th1 responses, such as IFN-γ. In light of its ability to stimulate Th1 responses, IL-12 has been invoked as a key cytokine in the pathogenesis of organ-specific autoimmune diseases, which are often mediated by cellular immunity (Trinchieri, 2003).

IL-18 is related to the IL-1 family in terms of its structure, receptor family and signal transduction pathways (Fantuzzi & Dinarello, 1999). The production of bioactive IL-18 is a multistep process involving synthesis of the precursor, synthesis and activation of the cleaving enzyme caspase-1, maturation and extracellular transport (Dinarello, 1999; Gracie et al., 2003). Mainly produced by macrophages and APC (Okamura H et al. 1995), IL-18 acts in synergy with IL-12 for Th1 differentiation (Kohno et al.,1997; Robinson et al., 1997; Micallef et al., 1996). It also exerts pro-inflammatory properties by inducing the production of IL-1beta, TNF-α, chemokines, nitric oxide and prostaglandins (Puren et al., 1998; Olee et al., 1999). The pleiotropic activities of IL-18 suggest an important role of this cytokine in the triggering and polarization of the immune response (Liew FY., 2003). Activated macrophages and Kupffer cells were first described to produce high levels of IL-18 (Okamura H. et al., 1995). IL-18 is also produced by dendritic and Langerhans cells (Brossart et al., 1998) and APC represent the major source of IL-18 production (Akita et al., 1997). Like IL-1beta, IL-18 exerts proinflammatory properties, but this cytokine is also related to IL-12 in view of its capacity to induce the production of Th1 cytokines and to enhance cell-mediated immune cytotoxicity. Indeed, enhanced production and activity of IL-18 appears to be at a fundamental level in autoimmune pathologies.

IFN-γ, also called immune or type II interferon is a pleiotropic cytokine involved in the regulation of nearly all phases of immune and inflammatory responses, including the activation, growth and differentiation of T-cells, B-cells, macrophages, NK cells and other cell types such as endothelial cells and fibroblasts. It enhances MHC expression on antigen-presenting and IFN--γ production is characteristic of Th1 differentiation (Romagnani, 1997). IFN-γ enhances the cytotoxic activity of T cells, macrophages and natural killer cells and thus has antiproliferative effects. It also increases the production of antibodies in response to antigens administered simultaneously with alpha-interferon, possible by enhancing the antigen-presenting function of macrophages (Mitcham, 2005). IFN-γ is produced by Th0-cells, activated Th1-cells (CD4+) and cytotoxic T cells (CD8+), and by NK cells. Practically any antigen can cause the secretion of IFN-γ in one or other way, and it is enhanced by IL-2 and IL-12 which induce the NK-cells, and Th cells to form IFN- γ. B-lymphocytes need IL-1 to produce IFN-γ. During its secretion, IFN-γ influences on the secreting cells as well as on the cells around through IFN- γ - receptors. The first necessary step in the functioning of the IFN-γ pathway is its interaction with receptors located on the surface of the cells. IFN-γ stimulates the expression of class I and class II MHC molecules and co-stimulatory molecules on antigen presenting cells; promotes the differentiation of naive helper T cells

into Th1 cells; activates polymorphonuclear leukocytes (PMN) and cytotoxic T cells and increases the cytotoxicity of NK cells and suppresses humoral immunity.

IL-6 is a multi-functional cytokine, produced by lymphoid and non-lymphoid cells of the body, having regulatory effects on the immune and the hemopoietic system and causing an acute-phase reaction (Heinrich et al., 1990). IL-6 together with IL-2, TNF-α and other cytokines is an essential part of the accessory signal which is needed for the activation and the proliferation of antigen-stimulated T-lymphocytes. Interleukin-6 is especially important in the early stages of T-cell differentiation. In this phase, it reinforces the effect of IL-2 and promotes the differentiation of CD4 cells into T helper 2 cells (Janeway et al., 2001). It controls the growth and proliferation of early progenitor cells in the thymus and bone marrow and is later important in both T-cell and NK cell activation (Lee et al.; 1989). The molecular form of IL-6 responsible for T-cell activation is released by monocytes. It augments the early events of activation. (Heinrich et al., 1990; Van Snick, 1990) IL-6 also functions as the required second signal in both antigen- or mitogen-activated T-cells. (Clark & Shu, 1990) This protein holds a very important role in the life of NK cells. IL-6 provides support for continued development throughout the life of a natural killer cell (Van Snick, 1990). Interleukin-6 is very important in the stimulation of differentiation and proliferation of B-cells. Its most noted effect is found in the induction of permanent differentiation of B-cells into plasma cells, antibody producing cells (Nawata et al., 1989). IL-6 enhances the release of antibodies by acting as a growth factor for already differentiated plasma cells. It stimulates mostly the release of IgG and IgA antibodies from these cells. IL-6 induces increased production of antibodies in B-cells (Nawata et al., 1989).

IL-10 is a small protein known as a cytokine that functions as an important regulator of the immune system. Although IL-10 is known to have many different roles in the immune system, its two major activities include inhibition of cytokine production by macrophages and inhibition of the accessory functions of macrophages during T cell activation (Abbas et al., 1994). The effects of these actions cause IL-10 to play mainly an anti-inflammatory role in the immune system IL-10 is mainly produced by the Th2 subset of $CD4^+$ helper cells. However, it is also produced by some activated B cells, some Th1 cells (in humans), activated macrophages, and some other cells. Kinetics studies demonstrate that IL-10 is synthesized later than other immunoregulatory cytokines by activated T cells or monocytes. This data may reveal the regulatory role of IL-10 in later phases of the immune response (Delves et al., 1998). Studies have shown IL-10 to be an immunosuppressive agent that inhibits the synthesis of several monocyte and Th1 cell-derived cytokines (IL-1, -2, -6, -8, -12, TNF-beta, INF-γ). Moreover IL-10 has been demonstrated to down-regulate class II MHC expression thereby inhibiting the antigen-presenting capacity of monocytes. Like other cytokines interleukin-10 has many effects upon the functions of cells such as lymphocytes, monocytes, natural killer cells, and dendritic cells. Specifically, IL-10 is a cytokine that regulates immune-mediated inflammation. It appears to have two major functions: (1) to inhibit cytokine (i.e., TNF, IL-1, chemokine, and IL-12) production by macrophages and (2) to inhibit the accessory functions of macrophages in T cell activation. IL-10 accomplishes the latter function through the reduced expression of MHC class II molecules and certain co-stimulators (e.g., B7). The cumulative effect of these functions acts to inhibit T cell-mediated immune inflammation. IL-10 also has stimulatory actions on B cells and may

function as a switching factor for the production of IgG4 in humans (homologous to IgG1 in mice) (Delves et al., 1998)

TNF- α is a pleiotropic inflammatory cytokine. This cytokine possesses both growth stimulating properties and growth inhibitory processes, and it appears to have self regulatory properties as well. TNF-α is produced by neutrophils, macrophages, activated T- and B-lymphocytes, NK-cells, lymphokine-activated killer cell, astrocytes, endothelial cells, smooth and muscle cells. TNF-α has been implicated in the mediation of a number of diseases including septic shock syndrome, cachexia, AIDS and in pathogenesis of certain autoimmune diseases. TNF-α production may play a key kinetic role by amplifying release of cytokines IL-1α, IL-1β, and IL-6 and thereby affecting the severity of a response (Amiot et al., 1997). TNF-α participates in both inflammatory disorders of inflammatory and non inflammatory origin (Strieter al., 1993). TNF-α is an acute phase protein which initiates a cascade of cytokines and increases vascular permeability, thereby recruiting macrophage and neutrophils to a site of infection.

IL-15 shares many activities exerted by IL-2 including the stimulation and expansion of T cells thymocytes, B cells and natural killer cells owing to the fact that the receptors for IL-2 and IL-15 share the same β and γ subunits. At least in murine models, IL-15 unlike IL-2 fails to significantly activate the apoptotic pathways when stimulating T cells as well as other responding lineages, suggesting that IL-15 promotes the establishment of long-term memory T cells. IL-15 is produced by a variety of tissues, including skeletal muscle, kidney, placenta, hematopoietic stromal cells etc. IL-15 is expressed by a wide range of human tissues and cell lines, including skeletal muscle, placenta, and epithelial cell cultures (Tagaya et al., 1996). This cytokine induces T and B cell proliferation and it is essential for cell survival and for maintenance of long-lived memory cells (Perera et al., 1999; Oh et al., 2008). IL-15 is known to have noninflammatory actions because it can be detected in normal muscle tissue, where it is thought to have an anabolic activity and can modulate adipose tissue deposition (Quinn et al., 2009).

TGF-ß$_1$ belong to a family of multifunctional polypeptides and is produced by a wide variety of lymphoid and nonlymphoid cells. The TGF-ßs are involved in a variety of different biological processes, including tumorigenesis, fibrosis, hemopoiesis, and immunoregulation (Prud'homme & Piccirillo, 2000; Grande, 1997). Lymphoid cells mostly produce TGF-ß$_1$, an isoform that is also found in large amounts in bones and platelets and in the serum (Letterio & Roberts 1998). The activation of TGF-ß$_1$ may be mediated by macrophages in inflammatory sites (Wallick et al., 1990). Regarding the immunoregulating role of TGF-ß$_1$, this is an immunosuppressive cytokine, as it inhibits T and B cell proliferation, natural killer cell cytotoxic activity, and the generation of T cell cytotoxicity (Rook et al., 1986; Lee et al. 1987). Furthermore, TGF-ß$_1$ is able to inhibit both T helper type 1 and T helper type 2 cytokine production and decreases the interferon- γ -induced expression of HLA class II antigens (Czarniecki et al., 1988).

3.2.2 Cytokines and the thyroid

Both CD4+ and CD8+ T-Cells occur in thyroid lymphocytic infiltrate with a preponderance of CD4+ cells. There is an increase in activated T Cells expressing markers like HLA-DR. Thyroid cells express MHC class-II molecules as well as other immunologically important

molecules and behaves as an APC. Expression of ICAM-1, LFA-3 and MHC class I molecules by thyrocytes is enhanced by IL-1, TNF-α and IFN-γ (Weetman, 2004). This response increases the ability of cytotoxic T cells to mediate lysis. Thyroid cell destruction is mediated by Fas dependent mechanisms (Weetman, 2004; Wu et al., 1994). Cytokines and other toxic molecules such as nitric oxide and reactive oxygen metabolites probably also contribute directly to cell mediated tissue injury (Weetman, 2011). (Fig. 1)

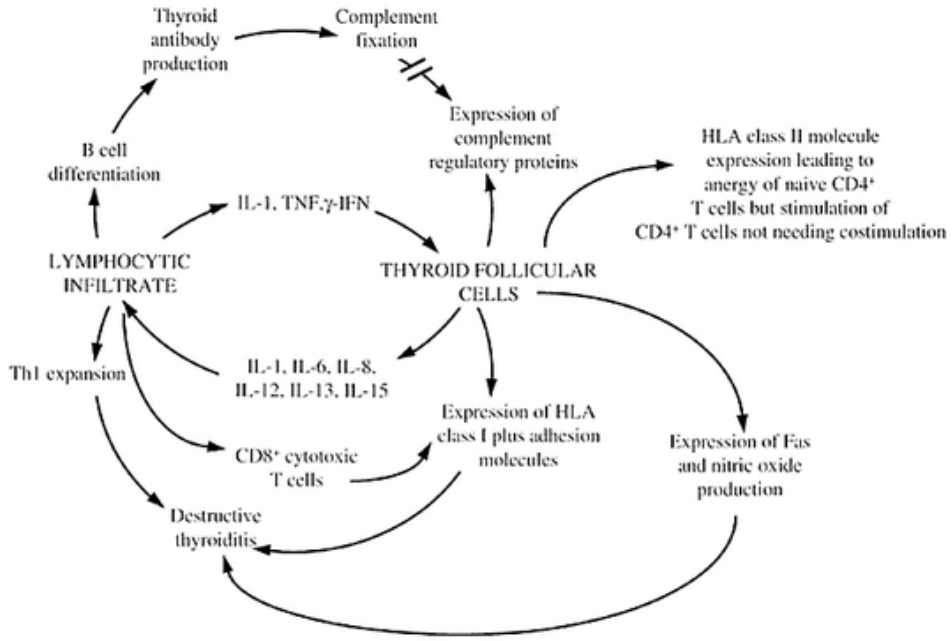

Fig. 1. Interaction between thyroid cells and the immune system via cytokines.

Humoral immunity exacerbates cell-mediated damage in a secondary fashion, both by direct complement fixations (TPO antibodies) and by ADCC (Chiovato et al., 1993). Complement attack initiated via the classic or alternative pathway, impairs the metabolic function of thyroid cells and induces them to secrete IL-1, IL-6, reactive oxygen metabolites and prostaglandin. All of these enhance the autoimmune response.

As well as T and B cell, dendritic cells and monocyte/ macrophages accumulate in the thyroid. Presumably they play a major role as APC and capable of providing co-stimulatory signals. Thyroid cell-derived monocyte chemoattractant-I, produced after TNF-α, IFN-γ, or IL-1 stimulation, is likely to be responsible for the accumulation of monocytes, which are important source of cytokines (Simons, 1998). Many cytokines are now known to be produced by thyroid cells especially after stimulation with IL-1, including IL-1, IL-6, IL-8, IL-12, IL-13 and IL-15 (Weetman et al. 1990; Weetman et al. 1992; Ajjan et al. 1997).

Gene expression of IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, IL-14, IL-15, IL-16, IFN-γ, TNF-α and a number of chemokines have been shown in both GD and HT tissue samples

(Ajjan et al. 1996; Azmi et al. 1997; Ashhab et al., 1999). Generally, a mixed Th1 and Th2 pattern has been found in the samples analysed, although some studies, using quantitative techniques, have shown a Th1 response in HT and a Th2 response in GD (Heuer et all. 1996). However, the analysis of mRNA can be misleading, as gene expression does not necessarily correlate with protein production. To address this issue, immunohistochemical methods have been applied which demonstrated cytokine protein production by a variety of cells in AITDs tissue in vivo. IFN-γ was found in infiltrating lymphocytes, IL-1 in endothelial cells, whereas IL-1, IL-6 and TNF-α were produced by thyroid follicular cells (TFC) (Ajjan et al. 1996).

Cytokine production by TFC in vitro can be stimulated by IL-1, IFN-γ and TNF-α, and this may be important in increasing the size and the activity of the infiltrate in vivo. Cytokines enhance the expression of adhesion molecules on TFC and can stimulate nitric oxide and prostaglandin production by these cells, which may further have a role in localising and augmenting the inflammatory reaction (Ajjan et al. 1996).

MHC class I expression is upregulated on TFC by IFN-γ and TNF-α treatment in vitro, which may play a role in tissue destruction through T-cell-mediated cytotoxicity (Ajjan et all. 1996). IFN-γ also enhances MHC class II expression on TFC in vitro, which may have a role in enhancing the proliferation of non-B-7-dependent T cells, but may also have a protective role as detailed below.

Apoptosis seems to play a role in AITDs, in particular HT (Palazzo et al. 2000). Cytokines can upregulate proapoptotic genes and downregulate antiapoptotic genes on TFC predosposing these cells to apoptosis.

TFC are resistant to complement-mediated cell lysis, an effect mediated in part by the expression of several protective proteins, which can be upregulated by cytokines (Ajjan et al. 1996). IFN-γ and TNF-α treatment of TFC in vitro renders these cells resistant to cell-mediated cytotoxicity, whereas TGF-β_1 inhibits T cell proliferation and thyroid autoantigen recognition (Ajjan et al. 1996).

IL-1 and IL-6 enhance TFC proliferation in culture but can also have inhibitory effects if cells are stimulated with TSH, emphasising the complex interaction of these molecules in vivo. IFN-γ and TNF-α inhibit TFC growth and proliferation, without affecting cell viability (Ajjan et al. 1996). TPO gene expression and Tg production are decreased by cytokine treatment of TFC in vitro, which may affect iodine organification in vivo (Rasmussen et all. 1994). IFN-γ can also downregulate TSHR gene expression (Nishikawa et al., 1993).

Levels of IL-5 are increased in GD and HT sera (Hidaka et all., 1998), whereas IL-6, IL-8 and IL-12 concentration is increased in GD sera compared with controls (Tamaru et al., 1999; Salvi et al., 2000). Increased serum levels of IFN-γ, IL-4, IL-6, IL-10 and TNF-α were found in GD compared with controls indicating a mixed Th1/Th2 response in this disease (Al-Humaidi, 2000). In recent study Sieminska at al. found increased production of IL-6 in postmenopausal women with Hashimoto's thyroiditis (Sieminska et al., 2010). On balance, the concept of a predominance of a Th1 and Th2 response, in HT and GD respectively, is almost certainly an over-simplification as features of cell-mediated and humoral immunity can be found in both diseases. Some authors considered HT as Th1 disease (Phenekos et al., 2004; Colin et al., 2004), others found a mixed Th1 and Th2 pattern (Heuer et al., 1996;

Weetman A.P, 2004). Th1-associated cytokines have antagonistic and counterregulatory effects on the functions of Th2 type cells and vice versa (Mosmann & Sad, 1996; Elenkov & Chrousos, 1999). Cytokines can modulate Th1/Th2 cell differentiation via chromatin remodeling of Th cell loci (Murphy & Reiner, 2002, Morinobi et al., 2004; Spilianakis & Flavell, 2004).

The role of cytokines in different stages of Hashimoto's thyroiditis is not well established and their participation in processes leading to hypothyroidism remains contradictory. Relatively few studies are available on the role of IL-12 and IL-18 in autoimmune lymphocytic thyroiditis, either in patients with Hashimoto's thyroiditis or in mice. IL-15 mRNA was detected in the majority of thyroid tissue samples from patients with multinodular goiter, GD, and HT (Ajjan et al. 1996). Furthermore the expression of IL-15 was increased after stimulation of TFC cells with TSH, IL-1 or IFN-γ, suggesting that these cells are a source of IL-15 in the thyroid.

To provide the involvement of Th1 and Th2 lymphocyte subpopulations and to clarify the role of some cytokines in different stages of Hashimoto's disease we investigated 128 out-patients from the Department of Internal Medicine, Stara Zagora University Hospital (Bulgaria), with autoimmune thyroiditis. Fifty two healthy subjects were included as controls. In all patients diagnosis had been made by enlarged thyroid glands, elevated TPO Abs and/or typical hypoechogenicity of the thyroid in high-resolution sonography. In negative TPO Abs patients FNAB was performed and typical cytological features of autoimmune thyroiditis were found. Serum levels of TSH, free thyroxin (fT4) were estimated. Fasting samples of venous blood were collected in the morning between 8.00 and 10.00 h. Serum samples were routinely collected and stored frozen at -20 C until assayed. At the time of sampling, neither of the patients and control subjects had clinical signs or symptoms of intercurrent illness. Concentrations of IL-12, IL-18, IFN-γ, IL-10, IL-6, TNF-α, IL-15 and TGF- ß$_1$ in the serum samples of patients and controls were evaluated by ELISA, using commercially available kits (R&D Systems, Minneapolis, USA). Patients were divided into tree subgroups according to the thyroid function. Group I (n=40) involved subjects with normal thyroid function (TSH and fT4 within the normal range). Group II (n=17) included patients with hypothyroidism (high levels of TSH and low or normal serum levels of fT4). In group III (n=71) were enrolled subjects with hypothyroidism treated with Levothyroxine (LT4) in a dosage to maintain TSH and fT4 within the normal range. The medication of Levothyroxine (range: 50-200 μg) was given in the fasting state. Informed consent was obtained from all participants in the study according to the ethical guidelines of the Helsinki Declaration.

The relevant clinical and biochemical data of all of patients studied and controls are summarized in Table 1. and Table 2.

The results obtained for cytokine levels in blood serum are shown in Table 3.

IL-18 was significantly higher in all HT patients (p=0.02) and both group II (p=0.03) and group III (p=0.01) in comparison with controls. The mean serum IL-12 levels in patients with HT were significantly higher than those in control subjects (p=0.014). (Fig.2) The serum IL-12 levels were also higher in sub-groups in patients, statistically significant in group I and group III (p=0.015 and p=0.036 respectively) in comparison with controls. (Fig.3) Concentrations of IL-10 in sub-groups of patients - I and II tended to be lower in comparison

with controls. IL-15 was significantly lower in group II in comparison with controls ($p<0.05$). Serum concentrations of TGF- ß$_1$ were not statistically different in sub-groups of patients and controls, but in all sub-groups of HT patients levels tended to be lower than in controls.

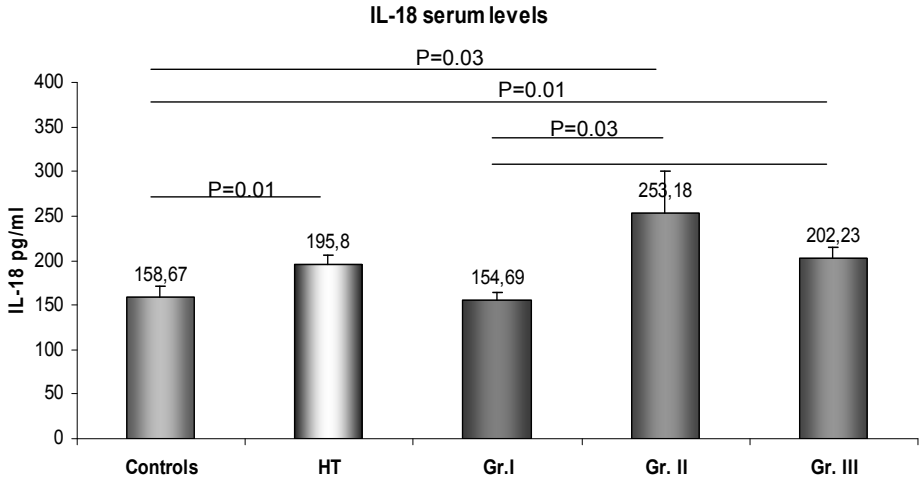

Fig. 2. IL-18 serum levels in controls, in all patients with Hashimoto's thyroiditis (HT), in euthyroid group (Gr. I), hypothyroid group (Gr. II) and group of patients treated with Levothyroxine (Gr. III). Data are presented as mean ± SEM.

The relevant clinical and biochemical data of all of patients studied and controls are summarized in Table 1. and Table 2. The results obtained for cytokine levels in blood serum are shown in Table 3.

Variables	Controls	HT	Group I	Group II	Group III
IL-10	0.77 ± 0.23	1.34±0.89	0.41±0.17	0.51±0.23	1.85±1.38
IL-15	2.06±0.24 [1]	1.48±0.11	1.67±0.21 [2]	0.62±0.40 [1,2]	1.56±0.12
IL-18	158.67±13.14 [3,4,5]	195.80±10.64 [4]	154.69±9.84 [6,7]	253.18±46.51[3,6]	202.23±13.26 [5,7]
TGF-β_1	25874,1±2894	18742±737	18967±1151	19830±2516	18305±1011
TNF-α	10.99±0.60	10.27±0.45	10.54±0.53	11.18±0.97	9.35±0.85
IL-12	67.99±9.85 [8,9,10]	93.48±7.05 [8]	103.73±14.409 [9]	83.41±14.0	90.48±8.19 [10]
IFN-γ	1.79±0.11 [11]	1.61±0.72	1.68±0.12	1.56±0.29 [11]	1.58±0.09
IL-6	1.47±0.29	2.22±0.36	1.79±0.50	3.96±1.30	1.71±0.29

Statistical significance: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11: $p<0.05$

Table 3. Cytokine concentrations (pg/ml) in all Hashimoto's thyroiditis patients and in particular groups of patients: euthyroid group (Group I), hypothyroid group (Group II) and group of patients treated with Levothyroxine (Group III) and controls (mean±SEM).

To further analyze the balance of immune regulation (Th1/Th2 balance) in individuals, the ratio between IL-12 and IL-6 was calculated. In comparison to control subjects, a clear bias towards Th1-dominated immune reactivity was found in group I - euthyroid Hashimoto's patients (p=0.018).

We found statistically significant lower serum levels of TGF-ß$_1$ in TPO Abs (+) patients (17802±876 pg/ml) in comparison with TPO Abs (-) patients (21200±1291 pg/ml) (p=0.018, Mann Whitney U test).

An advantage of investigating serum cytokine levels in ATD is the ability to analyse cytokine profile early in the disease process, when the immune response is presumably more specific. However, serum cytokine levels may not reflect the intrathyroidal cytokine profile as levels of some cytokines may be very low in the periphery (falling below the detection sensitivity of the assay), despite high intrathyroidal concentrations.

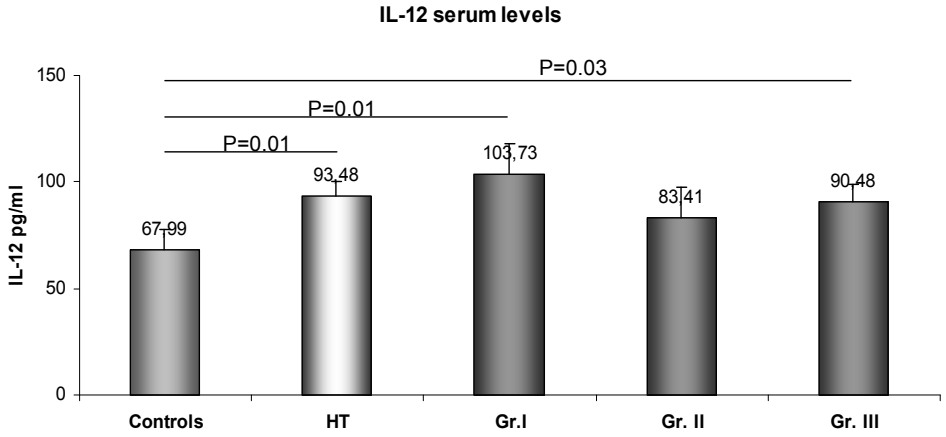

Fig. 3. IL-12 serum levels in controls, in all patients with Hashimoto's thyroiditis (HT), in euthyroid group (Gr. I), hypothyroid group (Gr. II) and group of patients treated with Levothyroxine (Gr. III). Data are presented as mean ± SEM.

Our results demonstrate that serum IL-12 levels were increased in all sub-groups in patients independently of functional state of the disease. The ratio between IL-12 and IL-6 demonstrate a clear bias towards Th1-dominated immune reactivity in group of euthyroid Hashimoto's patients in comparison to control subjects. In our previous work we found significantly increased serum levels of IL-12 in patients suffering from both GD and HT, which suggests that IL-12 might affect the immune response in both GD and HT (Halacheva et al., 2005). The results shown in the existing research carried out so far on IL-12 serum levels in GD and HT are contradictory. Phenekos et al. have shown that IL-12 serum concentrations were significantly higher than those in normal controls in HT patients but were not increased in patients suffering from GD (Phenekos et al., 2004). During iodine-induced autoimmune (lymphocytic) thyroiditis in NOD mouse IL-12 is produced in the thyroid gland early and throughout the course of the disease (Bonita et al., 2003). Kimura at

al. have shown that the local production of IL-12 in the thyroid enhances the expression of sodium-iodide symporter and inhibits thyroid hormonogenesis downstream of the organification, thus inducing primary hypothyroidism; and the disease promoting effect of IL-12 was independent of of interferon-γ (Kimura et al., 2005). On the basis of our results and data from experimental mouse models we may conclude that the effect of IL-12 on the initiation and regulation of immune responses and also on thyroid function is crucial in Hashimoto's thyroiditis.

We found that serum levels of IL-18 were significantly higher in HT; particularly in patients with severe disease and no change significantly after treatment with Levothyroxine in comparison with controls. Concentrations of IL-10 in sub-groups of patients tended to be lower in comparison with controls. Phenekos et al. demonstrated significantly increased IL-18 serum levels in HT patients than those in normal controls and in patients suffering from GD and toxic nodular goiter (Phenekos et al., 2004). IL-18 expression was increased in the thyroid tissues of HT compared with control thyroid tissues in canine model and in humans (Choi et al., 2006, Liu et al., 2010). IL-18 acts in synergy with IL-12 for Th1 differentiation induce the production of Th1 cytokines and inhance cell-mediated cytotoxicity (Lebel-Binay et al., 2000).; Increased serum levels of IL-18 particularly in patients with severe disease and no change significantly after treatment with Levothyroxine suggest an altered immunological status in severe hypothyroid stage of the disease and during Levothyroxine replacement remained unchanged. Mazziotti et al. found different expression of IL-4 in CD4+ in hypothyroid and euthyroid patients with HT (Mazziotti et al., 2003). Taking together our and these results suggest a different immunological status for euthyroid and hypothyroid HT patients and the interaction between IL-12 and IL-18 may be related to development of thyroid destruction.

Our results indicate lower serum levels of IL-15 in hypothyroid HT patients. Treatment with Levothyroxine increases serum levels of IL-15. In study of Ajjan at al. IL-15 was detected in all HT samples, and the expression was increased after stimulation of thyroid follicular cells with TSH, but all patients had been treated with Levothyroxine before surgery (Ajjan et al., 1997). IL-15 expression has been detected in numerous tissues, many of which are not sites of immune responses (Fehniger & Caligiuri, 2008). Accordingly we may suppose that decreased serum levels of IL-15 in hypothyroid HT may be related to the process of thyroid apoptosis and hypometabolism of all tissues.

TGF-ß$_1$ regulate proliferation of follicule cells of the thyroid in many experiments *in vitro*. It has been proved that TGF- ß$_1$ is an immunosuppressive cytokine, as it inhibits T and B cell proliferation, natural killer cell cytotoxic activity, and the generation of T cell cytotoxicity (Prud'homme & Piccirillo, 2000). In our work serum levels of TGF-ß$_1$ are not statistically different in sub-groups of patients compared to controls, but in all HT patients they tended to be lower in comparison with controls. Our findings of significantly lower serum levels of TGF-ß$_1$ in TPO Abs (+) patients than TPO Abs (-) suggest that TGF-ß$_1$ may contribute to the severity of autoimmune thyroiditis. Akinci et al. found lower levels of TGF- ß$_1$ in hypothyroid HT patients when compared with control cases and their levels remained unchanged after Levothyroxine replacement (Akinci et al., 2008). Vural at al. also measured decreased plasma TGF-ß$_1$ concentrations in HT patients in comparison with controls (Vural at al., 2009). On the basis of our and these data we suppose that autoimmunity may have

been triggered as a result of decreased immunosuppressive effect induced by depressed TGF- ß$_1$ levels in patients with HT.

We may summarize that Th1 pattern of immune response characteristic of cellular immunity is dominant in HT and sub-groups of patients have a different immunological status which contribute to development of hypothyroidism.

3.3 Oxidative stress and antioxidant protection in Hashimoto's thyroiditis

3.3.1 Generation of reactive oxygen metabolites and antioxidant enzymes

Molecular oxygen gives rise to dangerous reactive metabolites upon reduction to water (Fridovich, 1978). The term "free radical" covers any atom or molecule that contains one or more unpaired electrons (Halliwell 1991). Some of the reactive oxygen species (ROS) are free radicals, such as superoxide, nitric oxide and hydroxyl radical, whereas hydrogen peroxide (H2O2) is reactive and important, but not a free radical. Therefore, it is appropriate to speak of reactive oxygen species. ROS are continuously formed in the mitochondrial respiratory chain, via the cyclo-oxygenase pathway and by cellular enzymes, such as xanthine oxidase, NADPH oxidase and cytochrome P450 oxidase (Gadjeva et al., 2000). The main intracellular source of ROS is mitochondria.

A typical feature of free radical reactions is that they proceed as chain reactions, amplifying the damage of the initial event. ROS cause cell injury by reacting with proteins, lipids, and DNA (De Zwart et al., 1999), but they are also an essential part of normal cellular physiology, such as signal transduction. Oxidative reactions occur in living organisms under physiological conditions. ROS and free radicals are essential for numerous metabolic processes. Under physiological conditions, there is a balance between the production and detoxification of ROS. However, any internal or external pathological factor may disrupt this balance, leading to conditions referred to as oxidative stress. Reactive oxygen species – O2-, OH-, H_2O_2 and NO when being in excess cause oxidative damage to molecules. Indeed, oxidative stress plays a significant role in the pathogenesis of several diseases. Excess H_2O_2 has been reported to induce oxidative damage to membrane lipids, proteins and DNA that may result in cell death by necrosis or apoptosis. The levels of lipid peroxidation products (malondialdehyde – MDA) in plasma are widely used in practice as an indicator of free radical damages.

Detoxification of ROS is one of the prerequisites of aerobic life, and hence an elaborate antioxidant system has evolved. Antioxidants are agents that scavenge ROS, prevent their formation, or repair the damage they cause (De Zwart et al., 1999). This complex system consists of antioxidant enzymes (superoxide dismutases, catalase, glutathione peroxidase) and other substrates. Of the antioxidant enzymes superoxide dismutases (SOD) catalyse the conversion of two superoxide molecules to hydrogen peroxide and oxygen, and hydrogen peroxide is mainly eliminated by catalase (CAT) and glutathione peroxidase (GPX).

SOD is an endogenously produced intracellular enzyme present in essentially every cell in the body. Cellular SOD is actually represented by a group of metalloenzymes with various prosthetic groups. The prevalent enzyme is cupro-zinc (CuZn) SOD, which is a stable dimeric protein.

Catalase is a protein enzyme present in most aerobic cells in animal tissues. Catalase is present in all body organs being especially, concentrated in the liver and erythrocytes. The brain, heart, skeletal muscle contains only low amounts.

Glutathione peroxidase is a selenium-dependent enzyme, which decomposes H_2O_2 and various hydro- and lipid peroxides. (Kinnula et al. 1995). The classical form of GPX is cellular and dispersed throughout the cytoplasm, but GPX activity is also found in mitochondria. GPX is considered more important in physiologic conditions (reviewed by Kinnula et al., 1995). Selenium is essential for the protein synthesis and enzymatic activity of GPX.

SOD is considered fundamental in the process of eliminating ROS by reducing (adding an electron to) superoxide to form H_2O_2. Catalase and the selenium-dependent glutathione peroxidase are responsible for reducing H_2O_2 to H_2O. Catalase and glutathione peroxidase seek out hydrogen peroxide and convert it to water and diatomic oxygen. An increase in the production of SOD without a subsequent elevation of catalase or glutathione peroxidase leads to the accumulation of hydrogen peroxide, which gets converted into the hydroxyl radical.

The respective enzymes that interact with superoxide and H_2O_2 are tightly regulated through a feedback system. Excessive superoxide inhibits glutathione peroxidase and catalase to modulate the equation from H_2O_2 to H_2O. Likewise, increased H_2O_2 slowly inactivates CuZn-SOD. Meanwhile, catalases and glutathione peroxidase, by reducing H_2O_2, conserve SOD; and SOD, by reducing superoxide, conserves catalases and glutathione peroxidase. Through this feedback system, steady low levels of SOD, glutathione peroxidase, and catalase, as well as superoxide and H_2O_2 are maintained, which keeps the entire system in a fully functioning state (Fig.4). (Gadjeva et al., 2000; Al-Gubory K et al.; 2010).

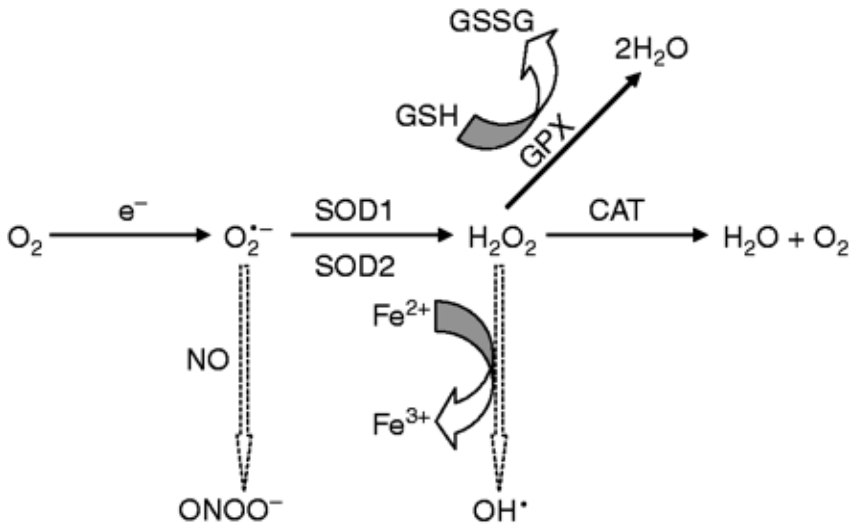

Fig. 4. Schematic representation of the pathways producing reactive oxygen species (ROS) and key cellular antioxidant enzymatic systems controlling ROS production.

3.3.2 Oxidative stress and antioxidant protection in Hashimoto's thyroiditis

In the thyroid hydrogene peroxide is necessary for thyroid hormonogenesis acting at different steps of the process. Excess H_2O_2 has been reported to induce oxidative damage to membrane lipids, proteins and DNA that may result in cell death by necrosis or apoptosis. The levels of lipid peroxidation products (malondialdehyde – MDA) in plasma are widely used in practice as an indicator of free radical damages. To prevent the damages caused by the ROS, multiple defense systems, collectively called antioxidants, are present in human serum, erythrocytes as well in the tissues. The presence of following antioxidative enzymes in the thyroid gland has been documented: superoxide dismutase, catalase and glutathione peroxidase. GPX and TR (thioredoxin reductase) are selenoenzymes capable of modifying cell function by acting as antioxidants.

In the thyroid, ROS and free radicals are constantly formed and participate in physiological and pathological processes in the gland. For example, H_2O_2 is necessary for thyroid hormonogenesis (Nunez & Pommier, 1982; Fayadat et al., 1999). But an *in vitro* experimental study H_2O_2 has been found to influence the process of cell death (Riou et al., 1998).

Cells have developed a comprehensive set of antioxidant defense mechanisms to limit the action of ROS. SOD catalyse the conversion of two superoxide molecules to H_2O_2 and oxygen. CAT and GPX mainly eliminate H_2O_2, as primary participants in the most important antioxidant enzyme pathways. The involvement of hyperthyroidism due to Graves' disease in lipid peroxidation and antioxidant enzyme activities has been studied (Komosinska-Vassev et al., 2000; Gerenova & Gadjeva, 1996). The papers concerninig the influence of hypothyroidism have shown that this condition results in complex effects such as the augmentation of SOD and GPX activities and significant decrease of the CAT activity in rat liver mitochondria in experimental work (Das & Chainy, 2001) and augmentation of oxidative stress and disturbance of antioxidant defense in humans (Erdamar et al.,2008). The complex regulation of ROS generation and free radical-scavenging systems activity in patients with Hashimoto's thyroiditis in different stages of disease activity has not been studied. The study of Hashimoto's thyroiditis is plagued by the difficulties in examining a disease that progresses over long periods of time (Davies & Amino, 1993; Dayan & Daniels, 1996).

In our previous study we investigated the possible induction of oxidative stress and changes in antioxidant enzyme activities in Hashimoto's thyroiditis and compared these parameters in different subgroups of patients (Gerenova & Gadjeva, 2007). For this purpose seventy-one patients with autoimmune thyroiditis and 30 healthy controls were studied. Patients were divided into tree subgroups according to the thyroid function: group I – euthyroid subjects; group II – hypothyroid subjects; group III – subjects with hypothyroidism treated with Levothyroxine (LT4) to maintain TSH and fT4 winthin the normal range. The levels of of lipid peroxidation products - MDA in the plasma, and the antioxidant defences such as SOD, CAT and GPX activities in erythrocytes were measured.

Between June 2003 and April 2005 seventy-one out-patients (4 males, 67 females, of mean age 45.9±13.1 years) from the Department of Internal Medicine, Stara Zagora University

Hospital (Bulgaria) with Hashimoto's thyroiditis were recruited and investigated in prospective study. From 74 patients selected, 71 agreed to participate in the study. In all patients diagnosis had been made by enlarged thyroid glands, elevated TPO Abs and/or Tg Abs as well as typical hypoechogenicity of the thyroid in high-resolution sonography. Serum levels of TSH, free thyroxin (fT4) and lipid profile [total cholesterol (TC), triglyceride (TGs), LDL- and HDL- cholesterol (LDL-C and HDL-C)] were estimated. Patients were divided into tree subgroups according to the thyroid function. Group I (n=19) involved subjects with normal thyroid function (TSH and fT4 within the normal range). Group II (n=20) included patients with hypothyroidism (high levels of TSH and low or normal serum levels of fT4). In group III (n=32) were enrolled subjects with hypothyroidism treated with Levothyroxine (LT4) in a dosage to maintain TSH and fT4 within the normal range. The medication of Levothyroxine was given in the fasting state, mean Levothyroxine doses were 83.2±27.7 μg daily (range: 50-200 μg).

Blood samples, obtained from 30 healthy individuals (4 males, 26 females, of mean age 43.8 ± 12.3 years) who had no family history of autoimmune disease were used as controls. To eliminate the factors, that might affect parameters of oxidative stress, we excluded from Hashimoto's thyroiditis patients and healthy controls, all smoking and alcohol drinking subjects, as well as individuals suffering from acute or chronic diseases. Informed consent was obtained from all participants in the study according to the ethical guidelines of the Helsinki Declaration. Fasting samples of venous blood were collected in the morning between 8.00 and 10.00 h. FT4 was measured by competitive immunoassay on the ACS180 (Chiron Diagnostics USA). TSH was measured by a third generation two-site chemiluminometric assay on the ACS180. The reference range was 11.5-22.7 pmol/l for FT4 and 0.35-5.5 μIU/ml for TSH. Serum TC, HDL-C and TG concentrations were determined enzymatically in the routine laboratory by automated procedures (Roche). Serum LDL-C was calculated according to the Friedewald formula.

Blood for determining the parameters of oxidative stress was collected in tubes containing ethylendiamine-tetraacetic acid (EDTA), centrifuged at 3000 rpm for 15 min and plasma was carefully separated. The erythrocyte pellets were washed three times with saline, and 0.5 ml of the cell suspension was diluted with 2 ml cold water to lyse the erythrocytes. To 0.2 ml lysat 1.8 ml water and ethanol/chloroform (3:5/v:v) were then added to precipitate hemoglobin. The tubes were shaken vigorously for 5 min. The supernatant was used for determination of enzyme activity.

Total amount of lipid peroxidation products in the plasma of healthy volunteers and patients was estimated using the thiobarbituric acid (TBA) method, which measures the malondialdehyde (MDA) reactive products (Plaser et al., 1996). The results were expressed as μM/l. Superoxide dismutase activity was determined as described by Sun et al., (Sun at al., 1988) with minor modifications. The results were expressed as U/gHb. Catalase activity in the erythrocyte lysats was assessed by the method described by Beers and Sizer (Beers & Sizer, 1952). The hemoglobin concentration of lysate was determined by the cyanmethemoglobin method (Mahoney et al., 1993). Glutathione peroxidase activity was measured by the method of Paglia et al (Paglia et al., 1967). Activity was given in units per g hemoglobin (U/g Hb).

Characteristics	**Group I**	**Group II**	**Group III**
N	N=19	N=20	N=32
Age (yr)	46.1±14.9	41.8±14.5	48.4±10.7
Sex F/M	17/2	1/19	1/31
BMI (kg/m^2)	27.6±3.2	28.4±5.9	28.8±5.8
TSH basal (mIU/l)	2.3±1.2 *	16.0±4.9 * #	3.9±4.8 #
Free T4 (pmol/l)	14.8±1.3 *	10.6±2.4 *#	16±2.4#
Total cholesterol (mmol/l)	6.0±1.1	6.3±1.3	5.6±0.8
LDL-C (mmol/l)	4.0±1.0	3.8±1.1	3.5±0.6
HDL-C (mmol/l)	1.4±0.5	1.6±0.3	1.4±0.3
Triglycerides (mmol/l)	1.6±0.6	1.6±0.9	1.4±1.1

* $p<0.05$; # $p<0.05$ are statistically significant (Student's t-test)

Table 4. Baseline characteristics of Hashimoto's thyroiditis patients - euthyroid group (Group I), hypothyroid group (Group II) and group of patients treated with Levothyroxine (Group III). The results are expressed as mean ± S.D

Statistical analysis was carried out using the Statistica 5.5 for Windows. The results were reported as means ± SD (SE). Student's *t*-test was used to determine whether differences between means were significant. Correlations between the different parameters were calculated by linear regression analysis. $P \leq 0.05$ was considered statistically significant. Clinical and biochemical data of subgroups of Hashimoto's thyroiditis patients are presented in Table 4. In hypothyroid Hashimoto's patients cholesterol levels tended to be higher compared to euthyroid patients and patients treated with LT4. Results of studied parameters of oxidative stress in controls and Hashimoto's patients are listed in Table 5.

There were no significant changes of plasma levels of MDA between the groups of patients (group I - 1.68±0.08; group II - 1.90±0.13; group III – 1.71±0.07 μmol/l; respectively) as well as between the patients and the controls (1.70±0.06 μmol/l). Only plasma levels of MDA in hypothyroid Hashimoto's patients (group II) tended to be higher. No significant differences in SOD activity in erythrocytes were observed between the groups of patients (group I - 2377±262; group II - 2602±190; group III – 2308±267 U/gHb; respectively) and the controls

(2597±156 U/gHb. Our results showed that CAT activity was significantly lower in hypothyroid patients (group II) in comparison with controls (16016±3875 vs 26855±2272 U/gHb, p=0.01) and in coparison with subjects treated with LT4 (group III) (16016±3875 vs 29250±3939 U/gHb, p=0.02). Erythrocyte CAT activity of patients in euthyroid stage (group I) was also found significantly decreased compared to the controls (18733±3188 vs 26855±2272 U/gHb, p=0.04). Activity of GPX in erythrocytes in hypothyroidism (group II) was higher compared to control group (8.3±0.6 vs. 6.4±0.4 U/gHb, p=0.02). In euthyroid Hashimoto's patients (group I) and in patients treated with LT4 (group III), the activity of GPX was found significantly decreased in comparison with hypothyroid patients (5.7±1.1 vs 8.3±0.6 and 5.2±1.1 vs 8.3±0.6 U/gHb; p=0.05, p=0.047, respectively). GPX activity in both groups I and III, also tended to be lower in comparison with controls, but not significantly (Fig. 5).

Groups Parameters	**Controls**	**Group I**	**Group II**	**Group III**
N	30	19	20	32
MDA (μmol/l)	1.70 ± 0.06	1.68 ± 0.08	1.90 ± 0.13	1.71 ± 0.07
SOD (U/gHb)	2597 ± 156	2377 ± 262	2602 ± 190	2308 ± 267
CAT (U/gHb)	26855 ± 2272 [a b]	18773 ± 3188 [b]	16016 ± 3875 [a c]	29250 ± 3939[c]
GPX (U/gHb)	6.4 ± 0.4 [a]	5.70 ± 1.1 [c]	8.3 ± 0.6 [a b c]	5.2 ± 1.1 [b]

The results are expressed as mean ± S.E.

Student's $_{t\text{-}test}$ was used to compute the statistical significance of values

[a]CAT $_p$<0.05; [b]CAT $_p$ <0.05; [c]CAT$_p$ < 0.05

[a]GPX: $_p$<0.05; [b]GPX $_p$ <0.05; [c]GPX $_p$ < 0.05

Table 5. Levels of lipid peroxidation products – malondialdehyde (MDA) (μmol/l) in the plasma and the activities of antioxidative enzymes superoxide dismutase (SOD) (U/gHb), catalase (CAT) (U/gHb), and glutathione peroxidase (GPX) (U/gHb) in erythrocytes of control group and Hashimoto's thyroiditis patients – euthyroid group (Group I), hypothyroid group (Group II) and group of patients treated with Levothyroxine (Group III).

Significant negative linear correlation was found between activities of GPX and CAT in control group (r=- 0.57, p<0.05). However, no correlation was noted between activities of GPX and CAT in patient's groups. Significant negative correlation was also observed between serum levels of TSH and CAT activity (r= -0.40, p<0.05) in both groups euthyroid (group I) and hypothyroid (group II) Hashimoto's patients.

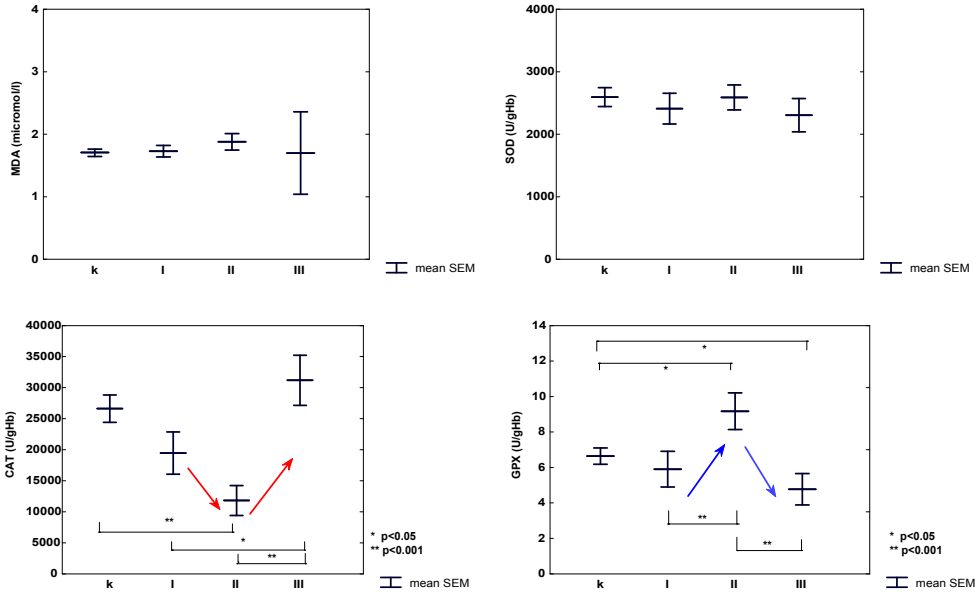

Fig. 5. Comparison of MDA levels in plasma and the activities of antioxidative enzymes superoxide dismutase (SOD) (U/gHb), catalase (CAT) (U/gHb), and glutathione peroxidase (GPX) (U/gHb) in erythrocytes of control group and Hashimoto's thyroiditis patients – euthyroid group (I), hypothyroid group (II) and group of patients treated with Levothyroxine (III). The results are expressed as mean ± S.E.

The thyroid function is normal in a great number of patients with Hashimoto's thyroiditis. Some of them, however, may progress to hypothyroidism over time. Although the highest titers of TPO antibodies are found in hypothyroid patients with Hashimoto's thyroiditis, they can also be found in euthyroid patients, that is, the correlation of titers with thyroid functional status is contradictory (Amino et al., 1976, Aksoy et al., 2005; Ito et al., 2006). Pathogenitic role of TPO Abs is not clearly established, but they are recognized as marker of aitoimmune thyroid diseases and the presence of TPO Abs indicates that, the processes of thyroid destruction started many years, before establishment of thyroid hypofunction (Kraiem, 1998). Thyroid cells undergoing apoptosis occur with high level of frequency in thyroids from patients with Hashimoto's thyroiditis (Kotani et al., 1995; Okayasu et al., 1995; Tanimoto et al., 1995). Many of the apoptotic cells in these glands are detected in areas of disrupted follicles in proximity to infiltrating lymphoid cells (Kotani et al., 1995; Hammond et al., 1997). This suggests that the thyroid destruction in this disease occurs through thyroid cell apoptosis.

In hypothyroid Hashimoto's patients both the level of MDA tended to be higher as well as the cholesterol levels compared to euthyroid patients and patients treated with LT4. This might lead to the development and progression of atherosclerosis and possibly contribute to enhanced atherosclerosis risk in this group.

Our data showed a lack of significant changes in activities of SOD in all groups of Hashimoto's patients compared to controls, but in contrast, the results concerninig GPX and

CAT activities differed significantly in studied groups. At high concentrations ROS have been reported to induce oxidative damage to membrane lipids, proteins and DNA, and that might result in cell death by necrosis or apoptosis (Gamaley & Klyubin, 1999; Hampton & Orrenius, 1997). Both GPX and CAT are major defense against harmful side effect of ROS in cells and in cultured thyrocytes both have a high capacity to degrade exogenious H_2O_2 (Björkman & Ekholm, 1995). Specifically, the observations indicate that GPX is involved in the degradation of fairly low H_2O_2 levels (100 μmol/l) whereas CAT is required to degrade H_2O_2 at mmol/l concentrations. In thyroid tissue GPX is active in the cytosol and conceivably degrade H_2O_2 produced at the apical plasma membrane as soon as it enters the cell. CAT on the other hand, is mainly enclosed in the peroxisomes and therefore not directly accessible at the side of H_2O_2 production. It is thus possible that the impaired GPX and CAT activities may lead to H_2O_2-induced apoptosis to thyroid cells in Hashimoto's thyroiditis patients.

Our study clearly demonstrates lower activities on both enzymes – CAT and GPX in euthyroid Hashimoto's patients. In hypothyroid stage, GPX production increased probably through hyperstimulation of TSH receptor by TSH (Beckett & Arthur, 2005), but CAT activity remained reduced. Significant negative linear correlation was found between activities of GPX and CAT in control group, but no correlation was noted between activities of GPX and CAT in patient's groups. In *in vitro* study Demelash et al. found that impaired capacity of GPX to degrade H_2O_2 in cultured thyroid pig cells aggravates the apoptic response (Demelash et al., 2004). This data and our results suggest the possibility that reduced GPX and CAT activities in euthyroid Hashimoto's patients might participate in the initiation of the autoimmune process and might lead to H_2O_2-induced damage of thyroid cells related to cytosolic oxidative stress. The compensatory increased activity of GPX in hypothyroid stage of disease is not sufficient to protect the thyrocytes from harmful effects of excess H_2O_2.

After restoring euthyroidism with LT4 medication MDA reached levels close to control group and CAT activity increased while GPX again tended to be lower. Under *in vitro* conditions thyroid hormones triidothyronine and thyroxine revealed the capacity to scavenge free radicals (Aziol et al., 2001). Erdamar et al. demonstrate an increased generation of reactive oxygen species and impairment of the antioxidant system in patients with hypothyroidism due to Hashimoto's thyroiditis.; after restoring euthyroidism the values normalize (Erdamar et al.,2008). These findings indicate that thyroid hormones have a strong impact on oxidative stress and the antioxidant system. Our results demonstrate that treatment with LT4 in Hashimoto's patients is useful, but insufficient to normalize all of parameters of oxidative stress. Aksoy et al. found significant decrease of TPO Abs and Tg Abs in euthyroid Hashimoto's thyroiditis after prophylactic thyroid hormone replacement, but increase of CD8+ cell counts (Askoy et al., 2005). We may suppose that thyroid hormones in small doses may be used in some groups of euthyroid Hashimoto's thyroiditis patients e.g. with subtle brain dysfunction or depression in view of their antioxidant properties.

Selenium supplementation produced a significant decline in TPO Abs in hypothyroid Hashimoto's patients (Gärtner et al., 2002; Duntas et al., 2003). In experimental study selenium increases cellular levels of GPX and CAT (Alvarado al., 2006). Our results indicate a deficiency of cellular antioxidative defense in Hashimoto's thyroiditis patients in all stages of disease and the observed imbalance may be connected to the processes of thyroid cell

apoptosis. We may speculate that the supplementation with antioxidants including selenium, from an early stage of the disease, in addition to thyroid hormone replacement may have positive benefit in Hashimoto's disease's treatment.

4. Conclusion

In the past decade, significant progress has been made in our understanding of the genetic and environmental triggers contributing to HD. Meanwhile, HT is also a heterogeneous disorder exhibiting various clinicopathological presentations and outcomes. The thyroid autoantibodies, cytokines and antioxidative cellular enzymes are involved in the severity of Hashimoto's thyroiditis and they may be influenced by ethnic differences and environmental factors. The determination of cytokines in peripheral blood provides information about the involvement of cytokine network in the severity of Hashimotos's thyroiditis and variations in their concentrations may be connected with different clinical course of disease in patients. Our results have shown that IL-12 and IL-18 play an influential role in inflammatory response; in the induction and perpetuation of chronic inflammation in autoimmune thyroiditis. These findigs suggest that antagonists to these cytokines may have a potential therapeutic role against Hashimoto's thyroiditis. We found a deficiency of cellular antioxidative defense in Hashimoto's thyroiditis patients in all stages of disease and the observed imbalance may be connected to the processes of thyroid cell apoptosis. Levothyroxine treatment and the supplementation with antioxidants mainly with selenium restore the imbalance, but what is needed is a new type of antioxidant, such as a CAT/GPX mimetic, that works continuously and may be a powerful new approach for correction of Hashimoto's thyroiditis-induced oxidative stress. Further studies are necessary to establish the exact mechanism of autoantibodies, cytokines and antioxidant enzymes interaction influencing the severity of Hashimoto's thyroiditis.

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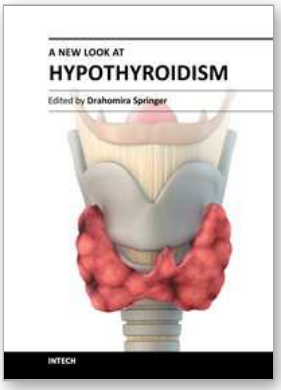

A New Look at Hypothyroidism

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Hypothyroidism is the most common thyroid disorder. It can cause a variety of changes in women's menstrual periods, reduce their chances of becoming pregnant, as well as affect both the course of pregnancy and the neuropsychological development of babies. During pregnancy there is a substantially increased need for thyroid hormones and a substantial risk that a previously unnoticed, subclinical or latent hypothyroidism will turn into overt hypothyroidism. The thyroid inflammation caused by the patient's own immune system may form autoimmune thyroiditis (Hashimoto's thyroiditis). Congenital hypothyroidism (CH) occurs in approximately 1:2,000 to 1:4,000 newborns. Nearly all of the developed world countries currently practice newborn screening to detect and treat congenital hypothyroidism in the first weeks of life. "A New Look at Hypothyroidism" contains many important specifications and innovations for endocrine practice.

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