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Original Article

Markers of Both Autoimmune and Apoptotic Processes in Initiation and Progression of Hashimoto's Thyroiditis

Popławska-Kita A[1*], Wawrusewicz-Kurylonek N[1], Kościuszko M[1], Popławski L[2], Siewko K[1], Milewski R[3], Szumowski P[4], Szelachowska M[1] and Krętowski AJ[1]

[1]Department of Endocrinology, Diabetology and Internal Medicine, Medical University of Bialystok, Poland

[2]Department of Radiology, Medical University of Bialystok, Poland

[3]Department of Statistics and Medical Informatics, Medical University of Bialystok, Poland

[4]Department of Nuclear Medicine, Medical University of Bialystok, Poland

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2. Key words

Hashimoto's thyroiditis; Autoimmune; Apoptosis; Chemokines

1. Abstract

1.1. Purpose: Hashimoto's thyroiditis (HT) is part of a spectrum of thyroid autoimmune conditions and the most frequent cause of hypothyroidism. Despite considerable progress achieved in identifying factors responsible for the development of HT, its pathogenesis remains unclear.

1.2. Methods: Following a literature analysis, we undertook a study aimed at a comprehensive evaluation of etiopathogenetic factors in patients with HT who remained in euthyreosis and control group (CG). We evaluated the markers of both autoimmune and apoptotic processes, i.e. TGFβ1, TNF-α, TRAIL and FAS Ligand as well as chemoactive factors: CXCL9/I-TAC, CXCL11/I-TAC, CXCL10/IP-10 in order to explain the pathogenetic role of many potential factors in the initiation and progression of HT.

1.3. Results: Serum TNF-α levels were markedly higher in HT in comparison with CG (p=0.01). HT patients also had significantly higher (p=0.049) TNF-α mRNA expression on the surface of thyroid follicular cells (0.029 AU (0.016-0.03) than controls (0.015 (0.002-0.03). In the HT group, a positive correlation between TNF-α mRNA expression and TPOAb concentration (R=0.389, p=0.003) was observed. In subjects with HT, we established a negative correlation between thyroid volume and levothyroxine doses (R=-0.34, p=0.005), and a borderline correlation between thyroid volume and treatment period (R=- 140 0.24, p<0.05). TGFβ, CXCL9, CXCL10, CXCL11, FAS Ligand, TRAIL values did not differ markedly between the two groups. Serum levels of CXCL10, and CXCL11 revealed a correlation with levels of TRAIL and FAS Ligand; CXCL9 with TPOAb titer.. Serum TRAIL levels were markedly higher in HT group with disease duration above two years (p=0.04). TGFβ, CXCL9, CXCL10, CXCL11, FAS Ligand, concentrations did not differ markedly according to disease duration.

1.4. Conclusion: It seems probable that increased TNF-α expression is stimulated by high antibody titer directed against thyroid peroxidase, which leads to greater severity of inflammatory processes in the thyroid gland. TRAIL concentration decreases significantly with disease duration, which, together with FasL, leads to an increased number of proinflammatory cytokines (CXCL10 and CXCL11) that activate thyrocyte destruction processes typical of HT. Increased concentration of CXCL9 seems to play a significant role in the initiation of HT while elevated levels of CXCL10 and CXCL11 impact on disease progression.

3. Introduction

The last one hundred years have brought a number of studies on chronic Hashimoto thyroiditis (aslo referred to as chronic lymphocytic thyroiditis, chronic autoimmune thyroiditis) which is an autoimmune disease. HT development involves the interplay of genetic predisposition, immunological dysfunction, and environmental factors [1-3]). Despite considerable progress made in identifying factors responsible for the development of autoimmune inflammation, the pathogenesis of HT has not yet been fully elucidated.

***Corresponding Author (s):** Anna Popławska-Kita, Department of Endocrinology, Diabetology and Internal Medicine, Medical University of Bialystok, ul. Sklodowskiej-Curie 24A, 15-276 Bialystok, Tel: 48 604 425 324; Fax: 48 85 7461909, E-mail: annapoplawskakita@op.pl*

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Both cellular and humoral immunity play a role in HT pathogenesis. Defects in T regulatory cells and increased activation of follicular helper T cells may play a part in disease initiation. An autoimmune attack on the thyroid results in infiltration of the gland by T and B lymphocytes, associated with the synthesis of thyroid peroxidase antibodies (TPOAb), thyroglobulin antibodies and thyroid stimulating hormone receptor antibodies (TRAb) [4]. Infiltrating lymphocytes can be directly cytotoxic to thyroid follicular cells (TFCs) or may impact cell viability/function indirectly through cytokine production, which alters TFCs integrity and modulates their metabolic and immune function [3-4].

Following antigen-specific lymphocyte activation by pro-inflammatory chemokines, the antigen-specific effector T cells are drawn to the inflammatory focus. Simultaneously, regulatory cells are recruited and the balance between effector and regulatory cells determines the result of local inflammation. Th1 cells secrete inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 and tumour necrosis factor alpha (TNF-α) which enhance the inflammatory response with nitric oxide and prostaglandins. Th2 cells secrete inflammatory cytokines IL-4 and IL-5, necessary for the antibody-mediated immune response. Cytokines in the thyroid gland also play a role in regulating antigen presentation and lymphocyte trafficking by enhancing the expression of HLA class II and adhesion molecules on TFCs [5].

TNF-α is a pro-inflammatory pleiotropic cytokine that regulates cell differentiation and growth. TNF-α increases the cytotoxic activity of lymphocytes which infiltrate the thyroid gland and is involved in the process of apoptosis-mediated death. TNF-α regulates the expression of proinflammatory cytokines and apoptosis in the thyroid [6,7]. However, there is insufficient data on whether TNF-α mRNA expression in the thyroid gland correlates with serum TNF-α concentration, and with markers of both autoimmune and apoptotic processes in the initiation and progression of HT.

Literature reports suggest that chemokine production induced by INF-γ may play a vital role in the development of HT. Human thyrocytes in primary cultures produce a large number of chemokines: CXCL9 (Chemokine (C-X-C motif) ligand 9, monokine induced by human IFN-γ (Mig)/), CXCL10 (IFN-inducible 10-kDa protein (IP-10) and CXCL11 (IFN-inducible T cell α chemoattractant (I-TAC). Chemokines are low-molecular weight proteins belonging to the cytokine superfamily which have the ability to induce migration or chemotaxis of many types of cells. Inflammatory chemokines show expression in inflamed tissues and their release is initiated by recognition of certain epitopes on the vascular endothelium and on inflamed cells. These chemokines are the first to activate effector cells that participate in the natural immune response [8,9].

A promising area of this research is immunodependent apoptosis of TFCs. Apoptosis is a highly regulated mechanism of cell death which differs from necrosis and plays an important role in normal tissue development, homeostasis and immune regulation. Defective apoptosis can cause systemic autoimmunity by enabling the survival of autoreactive lymphocytes. A defect in CD4+CD25+ T regulatory cells breaks the immunological tolerance of the host and induces abnormal cytokine production, which facilitates the initiation of apoptosis. The induction of apoptosis in HT results in the destruction of thyrocytes [10]. Thyroid cell destruction characteristic of autoimmune thyroiditis is the result of disturbed expression of Fas or TNF-related apoptosis-inducing ligand (TRAIL) death pathway molecules, and down-regulation of apoptosis controlling protein Bcl-2, which can be induced by cytokines released locally by infiltrating lymphocytes. Apoptosis activation in thyrocytes occurs in two ways: extrinsic and intrinsic. The former results from the binding of proapoptotic Fas Ligand (FasL) to Fas receptor on the cell surface. A small number of surface forms, both Fas and FasL, are always present in the thyrocyte cellular membrane, but this does not lead to the activation of the apoptotic pathway. Fas triggering by its ligand induces programmed cell death by activating the caspase cascade [11]. It has been suggested that activation of the Fas/FasL apoptotic pathway by proinflammatory cytokines in HT may play a role in the destruction of TFCs, which leads to hypothyroidism [12-14]. A crucial factor that participates in apoptosis is TRAIL. TRAIL expression is strongest after affecting INF γ cells and binding to TNF-α and IL-1β. It appears to play a more significant role in thyroid destruction than Fas [15]. Expression of FasL and TRAIL has been assessed in cell cultures or tissue samples collected during surgical procedures, which makes the tests difficult to conduct in everyday clinical practice. No evaluation of apoptotic markers in the serum of HT patients has been performed to date.

Following a literature review, we undertook a study aimed at a comprehensive evaluation of etiopathogenetic factors in HT patients who remained in clinical and hormonal euthyreosis. We evaluated markers of both autoimmune and apoptotic processes, i.e. TGFβ1, TNF-α, TRAIL and FAS Ligand as well as chemoactive factors: CXCL9, CXCL10 and CXCL11 in order to explain the pathogenetic role of many potential factors in the initiation and progression of HT.

2. Subjects and Methods

2.1. Study Group

The study comprised 81 patients, including 53 with a diagnosed HT in clinical and hormonal euthyerosis and 28 healthy individuals, negative for antithyroid autoantibodies, who had never been treated for autoimmune thyroid diseases and were selected in terms of gender, age, multivitamin preparation intake and the use of hormonal anticonception. The diagnosis of HT was based on

commonly accepted clinical and laboratory criteria. The patients with HT were treated with levothyroxine for 6 months – 13 years, whereas control subjects did not receive any treatment which might influence thyroid function. Written informed consent was obtained from all participants before enrolment, and the protocol was approved by the local ethics committee (Medical University of Bialystok)

2.2. Analytical Methods

Blood samples were collected from the antecubital vein between 7:30 and 8:30 am, after an overnight fast, in order to avoid diurnal variations. Serum TSH concentration was measured using an enzyme-linked immunoassay (DiaSource, Louvain-la-Neuve, Belgium). TPOAb and TNF-α concentrations were also determined by commercial immunoassays (Euroimmun, Lubeck, Germany, and R&D Systems, Minneapolis, USA). The concentration of TRAb was measured by a commercial radioimmunoassay (TRAK HUMAN, B-R-A-H-M-S Berlin, Germany). The following chemokines and apoptotic markers were also measured: chemokines CXCL9/I-TAC, CXCL10/IP-10, and CXCL11/I-TAC, and Human Fas Ligand/TNFSF6, Human TRAIL/TNFSF10, Human TGF-β1 (Human Quantikine ELISA R&D Systems, Minneapolis, USA).

All participants underwent thyroid ultrasonography, (Aloka SSD 1100) in order to calculate thyroid volume. Then all subjects who gave the informed consent (39 patients with HT and 15 controls) underwent an ultrasound guided fine-needle aspiration biopsy (FNAB) of the thyroid. Each aspirate was smeared for conventional cytology, while the remaining part was immediately washed out of the needle with 350 ml of RLT buffer with β-mercaptoethanol (1 ml RLT/10 ml of β-mercaptoethanol) and the obtained cell material was frozen in -80°C until assayed. RNA extraction from FNAB and cDNA synthesis. Total RNA was isolated using the RNase Mini Kit (Qiagen, Germany), following the manufacturer's protocol. Total RNA concentration was determined using Nano\ Drop ND-1000 spectrophotometer (NanoDrop 1000, THERMO Scientific, USA). cDNA synthesis was performed by High Capacity cDNA Reverse Transcription Kit (Life Technologies, USA) in the MJ Research Thermal Cycler (Model PTC-200, USA). Quantitative real-time RT-PCR. RT-PCR was performed with TaqMan Low-Density Array chemistry (TLDA, Life Technologies, USA). The reaction mixture consisted of 5 μl cDNA and 10 μl of Gene 114 Expression Master Mix (TaqMan Gene Expression Master Mix, Life Technologies, USA). Each sample was transferred to the fill port of a TaqMan Array Micro Fluidic Card and run on the 7900HT Fast Real Time PCR System (Applied Biosystems, USA). The thermal cycling conditions included an initial activation step at 95°C for 15min, followed by 40 cycles of denaturation, annealing and amplification (95°C for 30s, 55°C for 15s and 72°C for 30s). The levels of TNF-α transcripts were calculated after normalization of the 6 products to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, using the 2ΔCt formula, where Ct (threshold cycle) was define as the intersection between an amplification curve and a threshold line

2.3. Statistical Analysis

STATISTICA 10.0 for Windows (StatSoft. Inc., USA) and IBM SPSS Statistics 21.0 (Predictive Solutions, USA) Software were used for the statistical analysis. Prior to the analysis, data were tested for normality of distribution using Kołmogorow-Smirnow test with Lilliefors correction and Shapiro-Wilk test. Differences between the groups were compared by Mann-Whitney U test. P value lower than 0.05 was considered statistically significant.

3. Results

The clinical and biochemical characteristics of the groups studied are shown in (Table 1). As expected, the patients with HT had significantly higher concentrations of TPOAb (p=0.00001) and TRAb (p=0.00001), as well as higher BMI (p=0.02) and smaller median thyroid volume (p=0.001) than the healthy individuals, whereas TSH values did not differ markedly between the two groups. Serum TNF-α levels were also markedly higher in the HT group in comparison with the healthy controls (p=0.01) (Table 2). The patients with HT also had significantly higher (p=0.049) TNF-α mRNA expression on the surface of TFCs (0.029 AU (0.016-0.03) than the controls (0.015 (0.002-0.03) (data are shown as medians (interquartile range). In the HT group, a positive correlation between TNF-α mRNA expression and TPOAb concentration (R=0.389, p=0.003) was observed.

In the subjects with HT, we established a negative correlation between thyroid volume and levothyroxine doses (R=-0.34, p=0.005), and a borderline correlation between the former and the treatment period (R=- 140 0.24, p<0.05). As shown in Table 2, TGFβ, CXCL9, CXCL10, CXCL11, FAS Ligand, TRAIL values did not differ markedly between the two groups. To summarise, this results demonstrated that increased TNF-α mRNA expression on thyroid follicle cells coexisted with increased TNF-α and TPOAb concentrations in the sera of patients with HT in clinical and hormonal euthyreosis due to hormonal replacement therapy when compared to the healthy controls.

The concentration of proinflammatory cytokines and apoptotic markers in the HT patients depended on disease duration, as demonstrated in Table 3. Serum TRAIL levels were markedly higher in the HT group with disease duration above two years (p=0.04). TNFα, TGFβ, CXCL9, CXCL10, CXCL11, FAS Ligand, concentrations did not differ markedly according to disease duration, as shown in (Table 3).

We observed the presence of positive correlations between serum TPOAb concentration and CXCL9, also between serum FasL lev-

els and CXCL10 and CXCL11 concentrations (R=0.30, p=0.03; R=0.22, p<0.05; respectively) (Table 4). Serum TRAIL concentration positively correlated with chemokine concentration: CXCL10 (R=0.473, p=0.0001), CXCL11 (R=0.339, p=0.01) and with thyroid volume in HT patients (R=0.38, p=0.005) as shown in Table 4.

Table 1: Clinical and biochemical characteristics of studied groups

	Control group n=28	Hashimoto thyroiditis n=53	p value
Age (years)	38.5 (25.5-54.0)	50.0 (33.5-56.5)	ns
BMI (kg/m2)	22.1 (20.7-27.1)	27.2 (23.9-32.1)	0.02
Thyroid volume (ml)	18.5 (12.5-19.5)	12.2 (7.9-15.3)	0,001
TSH (uIU/ml)	1.13 (0.92-1.37)	1.48 (1.0-1.86)	ns
TPOAb (U/ml)	6.14 (4.91-10.17)	169.7 (27.23-413.27)	0.00001
TRAb (U/l)	0.30 (0.30-0.53)	1.16 (0.82-1.35)	0.00001
Levothyroxine dose (μg/day)	-	50.0(0-88.0)	
Treatment period (years)	-	4 (2-8)	

Data are shown as medians (interquartile range), differences between groups were tested by U Mann-Whitney test.

Table 2: Proinflammatory cytokines and apoptotic markers in patients with HT and control group

	HT n=53		Control group n=28	P value
TGF β1(pg/ml)	324944	(289801-360941)	32293 (29017-38791)	ns
TNFα (pg/ml)	6.1	(5.3-7.6)	5.3 (4.1-6.4)	0.01
TRAIL (pg/ml)	109.1	(89.6-129.6)	107.2 (87.3-129.6)	ns
FAS Ligand (pg/ml)	89.25	(76.6-106.7)	87.7 (68.4-111.7)	ns
CXCL9 (pg/ml)	79.77	(53,5-105.8)	62.7 (51.3-92.5)	ns
CXCL10 (pg/ml)	95.34	(80.2-125.6)	96.0 (96.3-129.6)	ns
CXXL11 (pg/ml)	56.16	(24.7-70.4)	56.2 (37.2-110.0)	ns

Data are shown as medians (interquartile range), differences between groups were tested by Mann-Whitney U test. p- between HT and control group.

Table 3: Proinflammatory cytokines and apoptotic markers in the HT patients depends on duration of the disease.

	Group I n=23 <2 years		Group II n=30 >2 years		P value
TGF β1(pg/ml)	32293.7	(30051.7-35796.7)	33284.4	(28980.1-36779.4)	ns
TNFα (pg/ml)	5.9	(5.3-6.4)	6.1	(4.98-76.6)	ns
TRAIL (pg/ml)	112.5	(97.3-134.8)	99.0	(83.8-116.3)	0.04
FAS Ligand (pg/ml)	88.5	(70.9-95.0)	95.8	(78.95-109.4)	ns
CXCL9 (pg/ml)	74.1	(53.5-116.4)	86.1	(59.9-103.7)	ns
CXCL10 (pg/ml)	93.1	(80.6-128.1)	101.9	(79.7-125.6)	ns
CXXL11 (pg/ml)	52.5	(24.7-63.4)	59.8	(33.1-90.7)	ns

Dare are shown as medians (interquartile range), difference between groups were tested by Mann-Whitney U test.

4. Discussion

In the study, we evaluated the expression of TNF-α mRNA on TFCs together with their soluble forms in the sera of HT patients in order to assess whether the expression of these molecules coexisted with serum TNF-α concentration. Our results demonstrated that TNF-α mRNA expression is up-regulated in the TFCs in HT patients. The patients with HT also had significantly higher serum TNF-α concentrations than the controls. TNF-α concentrations did not differ markedly depending on disease duration. These results are similar to the observations of other authors and confirm direct TNF-α participation in HT development [7]. Similar to our results, Díez et al. [7] confirmed the relevance of activation of the TNF-α system in patients with thyroid dysfunction, and observed no reduction in elevated levels of TNF-α and TNF-α receptors after levothyroxine replacement therapy. Concurrently, we observed a significant correlation between TNF-α expression levels and TPOAb titer. We should mention that higher TNF-α mRNA expression in TFCs and increased serum TNF-α concentrations in HT patients, as well as a positive correlation between TNF-α and TPOAb levels might confirm the direct contribution of this proinflammatory cytokine to the initiation and progression of HT throughout disease duration. The main limitations of our study were the facts that only mRNA, but not protein expression, was analysed in FNAB aspirates and that HT patients were treated with levothyroxine. However, since only the subjects with clinical and hormonal euthyreosis were included in the study, a potential impact of excessive TSH stimulation was avoided.

Table 4: Spearman's correlation coefficients of proinflammatory and proapoptotic markers in patients with Hashimoto's Thyroiditis.

Variable (concentrations)	R Sperman	P value
TRAIL and CXCL 10	0.47	0.0005
TRAIL and CXCL 11	0.34	0.01
TRAIL and thyroid volume	0.38	0.05
FasL and CXCL 10	0.3	0.03
FasL and CXCL 11	0.22	0.05
TPOAb and CXCL9	0.35	0.01
TGF b1 and CXCL 11	0.24	0.03
TSH and TPOAb	0.35	0.01

Experimental studies of numerous researchers have shown that chemokines, particularly CXCL9 and CXCL10, play a vital pathophysiological role in the early HT phase [16]. Human thyrocytes produce large quantities of CXCL9, CXCL10 and CXCL11 which are stimulated by IFN-γ. Increased expression of CXCL9 and CXCL10 was also observed in samples of thyroid tissue collected from patients with HT compared to healthy thyroid tissue. According to our study, no statistically significant differences in serum concentration of CXCL9, CXCL10 and CXCL11 chemokines in HT patients were observed compared to CG. Antonelli et al. revealed significantly higher levels of CXCL10 in HT patients but the greatest differences were present in the group with hypothyroidism [8-9]. In our study comprising a diagnosis-related group all patients were in clinical and hormonal euthyreosis, which suggests that the increase in chemokine concentration observed by other researchers may have resulted from a deficiency in thyroid hormones and not directly from autoimmune processes. Concurrently, we observed no significant correlations between CXCL9, CXCL10 and CXCL11 concentrations and TSH levels in the studied group but it

should be emphasised that in this study TSH was within the normal range in all patients. It is possible that chemokine production could be stimulated by hypothyroidism.

According to available literature reports, Fas and FasL proteins undergo expression in normal thyrocytes but their concentrations are insufficient to initiate apoptosis. Bossowski et al. [13] described a significantly higher percentage of the expression of apoptotic molecules Fas/FasL on the surface of TFCs and a significant expression of proapoptotic proteins Fas/FasL in thyroid cells. Based on our results, serum FasL concentration positively correlated with thyroid volume. Some authors have observed a significantly lower percentage of lymphocytes incoming to the thyroid gland, which demonstrated the expression of these molecules. Changes in the apoptotic molecule expression on T lymphocyte surface and TFCs suggest their vital role in the pathogenesis of HT [13]. This leads to the destruction of TFCs, either in apoptosis or as a result of T cells activity, subsequently resulting in the development of hypothyroidism [17-19]. Our results confirm these findings. In the subjects with HT, we established a negative correlation between thyroid volume and levothyroxine doses, which is used in the treatment of hypothyreosis. Contrary to the findings of other researchers [13], our study did not reveal any significant differences in serum FasL concentrations between HT patients and CG, even after the division of the studied group depending on disease duration. Bona et al. [14] observed a direct correlation between TPOAb concentration in blood serum and the resistance of T cells to Fas-induced apoptosis in a group of untreated individuals. This relationship was not observed in a group of patients who received replacement therapy. However, a lack of FasL expression by the same cells may prevent their elimination by apoptotic death. We observed the presence of positive correlations between serum FasL levels, and CXCL10 and CXCL11 concentrations. Therefore, the intrathyroidal interaction between FasL and chemokines may play a role in promoting a local immune response, which contributes to thyroid destruction seen in HT.

A crucial factor which participates in intra-thyroid apoptosis is TRAIL [15]. It seems that it plays a more significant role in thyroid destruction than Fas/FasL. It participates in thyrocyte death while Fas has a more profound impact on fiobroblasts and smooth muscle cells. Lymphocytes with TRAIL expression infiltrate the thyroid and in particular conditions may either induce TRAIL dependent apoptosis or 'become the victim' of this process. The role of TRAIL in HT pathogenesis remains unclear. In clinical practice, assessment of the expression of these proteins on thyrocytes is difficult and cannot be used as a simple apoptotic marker. Therefore, we determined serum TRAIL concentrations but no significant differences between HT patients and CG were observed. Serum TRAIL levels were positively correlated with thyroid volume in HT patients. Our study demonstrated that TRAIL concentration significantly decreased with disease duration. However, some patients in the present study had suffered from the disease for a number of years and had been undergoing treatment with levothyroxine, which could justify the low concentrations of TRAIL, usually present in the initial phase of the disease. Moreover, it is known that the main factors which impact on TRAIL expression are proinflammatory cytokines that are excessively present in the thyroids of patients with chronic lymphocytic inflammation. In the current study, a statistically significant increase in serum TNF-α concentrations and higher TNF-α mRNA expression in TFCs in HT patients were observed compared to CG. The hypothesis that lymphocytes showing TRAIL expression participate in thyrocyte destruction is confirmed by the results obtained in our study. Serum TRAIL concentration was positively correlated with the concentrations of CXCL10 and CXCL11. The outcomes of our study confirm the observations of Antonelli et al. [8-9] that increased serum concentrations of CXCL9, CXCL10 and CXCL11 are directly associated with inflammatory processes in HT. Nevertheless, our study revealed an increase in CXCL9, CXCL10 and CXCL11 concentrations concomitant with disease duration, but it was no statistically significant.

To summarise, we should highlight the fact that TPOAb titer correlated with CXCL9 serum concentration which seems to play an significant role in the initiation of HT. Additionally, the observed correlations between CXCL10 and CXCL11 and the levels of proapoptotic proteins, both TRAIL and FasL, confirm the role of these chemokines in thyrocyte destruction.

Difficulties in interpreting the obtained data are associated with the fact that some findings may relate to the current state of disease and may be the consequence, rather than the cause, of the condition.

5. Conclusions

It seems probable that increased TNF-α expression is stimulated by high antibody titer directed against thyroid peroxidase, which leads to greater severity of inflammatory processes in the thyroid gland. TRAIL concentration decreases significantly with disease duration, which together with FasL, leads to an increased number of proinflammatory cytokines (CXCL10 and CXCL11) that activate thyrocyte destruction processes typical of HT. Increased concentration of CXCL9 seems to play a significant role in the initiation of HT while elevated levels of CXCL10 and CXCL11 impact on disease progression.

7. Acknowledgment

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8. Authors' Contribution

Anna Popławska-Kita and Robert Milewski performed statistical analyses. Anna Popławska-Kita wrote the paper. Anna

Popławska-Kita, Natalia Wawrusewicz-Kurylonek, Katarzyna Siewko, Piotr Szumowski, Łukasz Popławski and, Małgorzata Szelachowska, processed the samples and analysed and prepared the data. Anna Popławska-Kita, designed the experiments and interpreted the data. Adam Jacek Krętowski contributed to the interpretation of the data and revised the paper. All the authors approved the final version of the paper.

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