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1.Introduction

Hashimoto's thyroiditis is an organ-specific autoimmune disease characterized by diffuse goiter with lymphocytic infiltration and the presence of thyroid-specific autoantibodies, Hakaru Hashimoto,who first described the disease in 1912, He presented four patients with a chronic thyroid disorder, which he termed struma lymphomatosa, characterized by diffuse lymphocytic infiltration with germinal centers,fibrosis, parenchymal atrophy and eosinophilic change in some thyroid follicular cells. Today, it is one of the most widespread thyroid disorders and a cause of hypothyroidism in areas of the world where dietary iodine is sufficient. The incidence is 0.3–1.5 cases per 1000 population per year.And is most prevalent between 45 and 65 years of age (Paknys *et al.*, 2009).

Clinically the patients with Hashimoto's thyroiditis are usually asymptomatic and some patients develop goiters with or without hypothyroid (Adims J *et al*.,2011).

Grossly the thyroid is often diffusely enlarged although more localized enlargement may be seen in some cases the capsule is intact and the glands is well demarcated from adjacent structure (Rumonek J *et al*.,2010).

Hashimoto's thyroiditis is caused by a breakdown in self tolerance to thyroid autoantigens. Thus, circulating autoantibodies against thyroid antigens are present in the vast majority of patients, who demonstrate progressive depletion of thyroid epithelial cells (thyrocytes) and their replacement by mononuclear cell infiltration and fibrosis. The inciting events leading to breakdown in self-tolerance have not been fully elucidated,

but multiple immunologic mechanisms that may contribute to thyrocyte damage have been identified, including :

• CD8+ cytotoxic T cell–mediated cell death: CD8+ cytotoxic T cells may cause thyrocyte destruction.

• Cytokine-mediated cell death: Excessive T cell activation leads to the production of inflammatory cytokines such as interferon-γ in the thyroid gland, with resultant recruitment and activation of macrophages and damage to follicles.

• Binding of antithyroid antibodies (antithyroglobulin, and antithyroid peroxidase antibodies), followed by antibody-dependent cell–mediated cytotoxicity. A significant genetic component to the disease pathogenesis is supported by the concordance of disease in as many as 40% of monozygotic twins, as well as the presence of circulating antithyroid antibodies in approximately 50% of asymptomatic siblings of affected patients.

In a study done by (Gherardo Mazziotti *et al*., 2003) to investigate the type-1 T-cell response separately for the two lymphocyte sub-populations,they found that the euthyroid patients (18 cases) showed more expression of IL-4 in CD4+ lymphocytes than the control subjects, without any significant modification of IFN-g expression in CD4+ and CD8+ lymphocytes. Moreover, the expression of IL-4 in CD4+ cells from hypothyroid patients was significantly lower than that seen in the euthyroid cases and comparable to that found in the control subjects.

Moreover,a study done by Ceyla Konca Degertekin *et al.* they observed that IL-17 and IL-23 levels were higher in euthyroid HT patients compared to controls. The distinctive feature is that the hypothyroid group had lower levels of cytokines compared to euthyroid HT(Ceyla Konca

Degertekin *et al*, Shi *et al*. suggested that Th17 cells can play a central role in pathogenesis of HD rather than Th1 cells (Shi *et al*.,2010).

Where, in a study done by (Julieta Gerenova *et al*.,2012) 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 findings suggest that antagonists to these cytokines may have a potential therapeutic role against Hashimoto's thyroiditis.

1.1.Aim of study

Thus, according to such controversy the aim of the present study is to invistigate the role of Th17 and Th2 cells in the pathogenesis of Hashimoto's thyroiditis indirectly by measuring the local expression level of IL17 & IL4 respectively in association with disease clinical pathological feature by using immunohistochemistry techniques.

2. Materials and Methods

2.1. Patients and Sampling

Across sectional study was conducted on the following study groups during the period between March 2016 and September 2016, the first group composed of (46) patients sample with Hashimoto's thyroiditis ,(2) of them are males and (44) females, while the second group represents the control group which composed of (49) subjects ,(3) of them are males and (46) females with non inflamatory multinodular goiter **,** who were confirmed histopathologicaly by Dr.Attaa A.Altamimi in Al kindy hospital/ Baghdad. Information about patients and controls were obtained from previous medical reports. (a copy of the data sheet is provided in appendix).

Paraffin embedded blocks of were retrieved along with the histopathological report of each patient from histopathological laboratory and thyroid function tests. In addition, Adequate thin paraffin embedded sections (4µm thick) of Hashimoto's thyroiditis and multinodular goiter was prepared for immunohistochimistrey Techniques.

2.2. Immunohistochemistry for Detection of IL-17, and IL-4 cellular expression in Paraffin Embedded Sections

2.2.1. Principle of test

A primary antibody reacts with an antigen. A biotinylated secondary antibody then reacts with the primary antibody. This is followed by the attachment of an enzyme-conjugated-HRP (horseradish peroxidase) to the biotins on the secondary antibody. The enzyme converts a substrate to a colored reaction product. High levels of signal amplification are achieved

due to the binding of multiple units of secondary antibody to each primary antibody, the binding of multiple enzyme-conjugated streptavidin molecules to each secondary antibody, and the enzymatic conversion of the substrate. Diaminobenzidine (DAB) offers the greatest sensitivity of the colorimetric substrates for peroxidase enzyme. The intense, brown reaction product is insoluble in water, alcohol, and xylene. The DAB substrate is compatible with permanent mounting media (Clive *et al*.,2013).

2.2.2. Materials

1. Dilution/Blocking buffer (ready to use), (Dakocytometion, USA).

2. Antigen retrieval solution 10x, (Dakocytometion, USA).

3. Washing Buffer. 10x, (Dakocytometion, USA).

4. Myre's hematoxyline, (Dakocytometion, USA).

5. LSAB System-HRP staining kit (Dakocytometion, USA): consist of the following:

a. Peroxidase blocking reagent (ready to use).

b. Biotinylated secondary antibody, which is biotinylated goat anti-mouse immunoglobulin (ready to use).

b. Strep-Avidin-HRP (ready to use).

c. Diaminobenzidine (DAB) substrate (Chromogen).

d. DAB substrate buffer.

6. Monocolnal Antibodies:

- Mouse anti- human IL-17 protein (Satacruz, USA): Isotype : IgG1 to a native human IL-17 expressed in human cell line.

- -Mouse anti-human IL-4 protein (Abcam, USA): Clone ID-2: Isotype: IgG2b to a recombinant human IL-4 expressed in human cell line.

7. De-ionized distilled water.

8. Xylene (AnalaR, England).

9. Absolute ethanol (Merck, Germany).

10. DPX mounting medium, which is a mixture of distyrene (apolystyrene), a plasticizer (tricresyl phosphate), and xylene (BDH, England).

11. Positively charged microscope slides, Fisherbrand Superfrost/Plus(Fisher Scientific, USA).

12. Stirrer (Electrothermal, England).

13. Eppendorf pipette 2-20 µl and tips (Eppendorf, Germany).

14. Eppendorf pipette 50-200 µl and tips (Eppendorf, Germany).

15. Eppendorf pipette 100-1000 µl and tips (Eppendorf, Germany).

16. Washing bottles.

17. Eppendorf tubes.

18. Pasteur pipettes (10 ml).

19. Graduated cylinders.

20. Glass staining jars.

21. Slide holders (10-slides each).

22. Hot air oven (Gallenkamp oven BS, England).

23. Timer with alarms (Junghans, Germany).

24. Tissue paper.

25. Incubator (Memmert, Germany).

26. Gloves.

28. Coverslips.

29. Binocular light microscopy (Olympus, Japan).

2.2.3. Preparation of tissue sections and reagents

1. Paraffin embedded sections were cut 4 µm thickness, placed on Fisherbrand Superfrost/Plus slides and left overnight at room temperature to dry.

2. PBS-Tween or PBS-0.1% Tween 20 was prepared by adding 0.1 ml Tween 20 to 100 ml 1X PBS.

3. 100 ml of 10X-concentrated detergent wash buffer was diluted into 1000 ml with distilled water. The resulting 1X Detergent wash buffer is ready to use and stored at room temperature.

4. 100 ml of 10X-concentrated Antigen retrievel solution were diluted into 1000 ml with distilled water. The resulting 1X solution is ready to use and stored at 4°c.

5. DAB solution was prepared by mixing one drop of DAB chromogen with 1 ml of DAB buffer.

6. Primary antibody was diluted in 1X dilution/blocking buffer:

(a) 1:50 for IL17.

(b) 1:50 for IL4.

7. Absolute ethanol was diluted in distilled water to prepare 95% and 70% concentrations of alcohol.

8. Negative controls were included for each run of immunohistochemistry. The negative control was obtained by replacing the primary antibody with PBS buffer.

2.2.4. Immunohistochemistry procedure

1. Dewaxing: paraffin embedded sections were placed inside a hot air oven at 65°C overnight, then dipped in xylene and ethanol containing jars as following order:

a. Xylene : 5 minutes.

b. Fresh xylene : 5 minutes.

c. Absolute ethanol : 5 minutes.

d. Fresh absolute ethanol : 5 minutes.

e. Ethanol (95%) : 5 minutes.

f. Ethanol (70%) : 5 minutes.

2. Slides were washed in distlled water for 5 minutes then drained and blotted gently.

3- Antigen unmasking (Antigen retrievel): Certain antigenic determinants are masked by formalin fixation and paraffin embedding and may be exposed by heat method:

- Heat treatment (recommended method): slides were Placed in a coplin jar and covered with antigen retrievel solution (10 mM sodium citrate buffer, pH 6.0) with 0.01% EDTA . After that the slides undergo for heating at 95º C for 5 minutes. The slides were allowed to cool in the buffer for approximately 20 minutes at room temperature then washed

in deionized H2O three times for 2 minutes each. excess liquid was aspirate from slides.

3. One hundred µl of a peroxidase-blocking reagent was placed onto the section and incubated for 10 minutes in a humid chamber at room temperature. Then slides were drained and blotted gently.
4. One hundred µl of diluted primary antibody was placed onto the section and incubated for 1 hour at 37°C in a humid chamber. After incubation, the slides were drained and blotted gently.
5. Slides were rinsed with washing buffer for 5 minutes, then drained and blotted gently.
6. One hundred µl of secondary antibody was placed onto the section and incubated for 30 minutes at 37°C in humid chamber. Slides were drained and blotted gently.
7. Slides were rinsed with washing buffer for 5 minutes, then drained and blotted gently.
8. One hundred µl of streptavidin-HRP conjugate was placed onto the section and incubated for 20 minutes at 37°C in humid chamber. Slides were drained and blotted gently.
9. Slides were rinsed with washing buffer for 5 minutes, then drained and blotted gently.
10. One hundred microliter of the DAB substrate solution was placed onto the section and incubated for 10 minutes at room temperature.
11. Slides were washed in running water for 5 minutes and then drained and blotted gently.

12. One hundred µl of counterstain (Myer's hematoxyline) was placed onto the section and incubated for 1 minute at room temperature. Slides were drained and blotted gently.

13. Slides were washed in distilled water then dehydrated by placing them in ethanol and xylene as following :

a. 70% ethanol for 3 minutes.

b. 95% ethanol for 3 minutes.

c. Absolute ethanol for 5 minutes.

d. Xylene for 5 minutes.

e. Fresh xylene for 5 minutes.

14. A drop of mounting medium (DPX) was placed onto the xylene-wet section by using a xylene-moist cotton swab and the section was quickly covered with a cover slip. Slides were let to dry.

15. Slides were examined by pathologist by light microscope at 400X magnification. Immunostaining was scored by counting the mean of positive cells per 4 different fields.

2.2.5. Evaluation of the Immunostaining

Evaluation of the immunostaining positivity by the following equation: % of Positivity for Slide = $\frac{\text{Number of Positive Cells in 10 Field}}{10}$

2.2.6. Statistical analysis

Data was presented,summarised and analyzed using statistical package for social science (SPSS) version 23 and Micresoft office Excel 2013.

Numeric variable were expressed as mean and standard deviation,while nominal variable were expressed as number and percentage.

Chi-squared test was used to study association between categorical variables.while independent samples,T-test was used to study differences in mean between any two groups.

P-value was considered significant in equale or less than 0.05 (Kleinbaum *et al* ., 2007).

2.Literature Review

2.1.Hashimoto's thyroiditis

2.1.1.Defunition

Hashimoto’s thyroiditis (HT) is an autoimmune disorder of the thyroid gland,Which characterized by the progressive loss of follicular cells and attendant replacement of the thyroid tissue by lymphoid infiltrates and fibrosis (Weetman A.P., 2003). The disruption of the thyroid architecture and the loss of functionality complicate the course of HT by the development of progressive hypothyroidism. The mechanism behind this disrupted self-immune response seems to be quite complex.T helper type 1 (Th1) and T helper type 2 (Th2) cell imbalance has been accused in the pathogenesis of HT and this model has long been used to explain the mechanism of autoimmune thyroiditis as well as various other autoimmune diseases. However, there were certain phenomena that could not be explained solely by the Th1/Th2 hypothesis (Steinman L., 2007).

2.1.2.Background

Hashimoto’s thyroiditis was first described in 1912 by Dr. Hakuru Hashimoto. Based on the histological findings, Hashimoto originally used the term “Struma Lymphomatosa.” Over the years, this disease has been called by several names including lymphocytic thyroiditis,autoimmune thyroiditis, chronic thyroiditis, and lymph adenoid goiter. The debate about the relationship between Hashimoto’s thyroiditis and Graves’ disease has been ongoing for many decades as they differ in clinical and immunological presentation. However,Hashimoto’s thyroiditis and Graves’ disease, which depict the two extremes of the clinical spectrum, are now included in a

common entity called autoimmune thyroid disease. It is now believed that they share a common autoimmune pathology and are believed to be triggered by multiple genetic and environmental factors. Hashimoto's thyroiditis was initially perceived as an uncommon disease and most cases were incidentally diagnosed through histopathological examination of the thyroid gland after thyroidectomy. The advent of newer diagnostic modalities with increased diagnostic sensitivity made it possible to unveil more cases of Hashimoto's thyroiditis. With the increasing number of cases, the association of Hashimoto's thyroiditis with other autoimmune diseases is being studied extensively. Type 1 diabetes, multiple sclerosis, rheumatoid arthritis, celiac disease, vitiligo,and chronic urticaria have all been reported to be frequently associated with Hashimoto'sthyroiditis (Arvin Parvathaneni *et al*.,2012).

2.1.3.Epidemiology

Hashimoto's thyroiditis is about 15-20 times more common in women than in men and frequently involves people between the ages of 30 and 50 years of age. Determining the exact incidence and prevalence rates for Hashimoto's thyroiditis has been difficult due to variable expression of this disease. Some studies estimate that the current prevalence rate in the United States ranges between 0.3%-1.2% (Staii *et al*., 2010). Other studies estimate the prevalence among the general population to be approximately 2% (Arvin Parvathaneni *et al*.,2012). When attempts have been made to characterize the prevalence prospectively, with the aid of organized programs of ultrasound guided biopsy, the prevalence described has been at least 5%. It should be noted that studies employing the diagnostic modality of ultrasound guided

biopsy have recorded prevalence rates higher than studies using other investigative modalities (Staii *et al*., 2010). The National Health and Nutrition Evaluation Study-3 (NHANES-3) study has shown the prevalence of subclinical and clinical hypothyroidism to be 4.6% and 0.3%, respectively, in the United States (Hollowell *et al*., 2002). The Whickham survey, an epidemiological study conducted in the United States, has revealed the prevalence of hypothyroidism to be 1.5% in females and less than 0.1% in males .During the past few decades there has been a reported increase in the incidence of Hashimoto's thyroiditis, which could be attributed to newer diagnostic modalities such as needle biopsies and serological tests, and their increased sensitivity when compared to the older methods(Arvin Parvathaneni *et al*.,2012). Studies about age-specific incidence rates of Hashimoto's thyroiditis indicate the existence of a random distribution in both men and women and have shown an initial lag in the first few years of their life followed by a constant rate after this . A few studies have suggested a slight increase in the prevalence of autoimmune thyroiditis in adolescent girls following use of iodized food products ingested to prevent iodine deficiency (Zois *et al*., 2003).

In a study made by(Hussain Aouda *et al*.,2014) at the Hilla teaching general Hospitals on 175 patients underwent thyroid surgery in order to study the percentages of Hashimoto's thyroiditis to the total cases which were taken and also to know the correlation between thyroid function and histopathological status in those patients whom diagnosed as Hashimotos thyroiditis,were 18 cases diagnosed as hashimotos thyroiditis, the incidence of hashiomato thyroiditis (13.4%).

The data on the incidence of thyroid autonomy (HT and Grave's disease) after increase iodization are scarce. While some reports indicate

short term increase (Lindi P *et al*; 2002**)**,others observed decrease in the first year after iodization (Zaletel K *et al***.,** 2011**)**. In Iraq, iodization was practiced during 1990s years i.e. during sanctions, with no re ference about the dose of iodization (Ahmed Mehdi Al-Hashimi,2014). Literature show that autoimmune thyroid disorders are an important cause of goiter in post-iodization phase (Das S *et al***.,**2011**)**.Iodization in Iraq might affect the prevalence of HT.

2.1.4.Risk Factors

For several decades a strong genetic predisposition to autoimmune thyroid disease has been recognised, predominantly on the basis of the family and twin studies. Nearly 50 years ago, soon after the discovery of TAbs, the presence of TAbs was reported in 56% of siblings of patients with autoimmune thyroid disease (Zaletel K, Gaberšček S,2011). This familial clustering of autoimmune thyroid disease and the presence of TAbs in up to 60% of first-degree relatives of patients has been later confirmed by several studies (Marwaha RK *et al.*,2003) . When both parents were affected, the prevalence of TPOAbs and TgAbs was 42% in daughters and 33% in sons, compared with 28.9% and 16.7%, respectively, when only one parent was TAb-positive. Among first-degree relatives of children with HT, 34% were diagnosed TPOAbs positive compared to only 13% first-degree relatives of children without autoimmune thyroid disease (Marwaha RK *et al.*,2003). The sibling risk ratio for HT, calculated on the basis of the data from the NHANES III study, was 28, thus confirming the highly significant contribution of genetic factors to the disease development (Villanueva R *et al.*,2003).

Recent data from Germany also indicate 32-fold increased risk for developing HT in children and 21-fold increased risk in siblings of

patients with HT, with females being significantly more often affected than males (Dittmar M *et al.*,2011). Twin studies provided further valuable data on the genetic contribution to thyroid autoimmunity. In healthy twin siblings of patients with overt autoimmune thyroid disease, positive TPOAbs and TgAbs in monozygotic twins were determined in 53% and 47%, respectively, in dizygotic twins in 22% and 13%, respectively, while in healthy control population only in 9% and 7%, respectively (Brix TH *et al.*,2004). The concordance rates for TPOAbs were 64% in monozygotic twins compared with 35% in dizygotic twins, while concordance rates for TgAbs were 74% and 32%, respectively (Phillips DI *et al*.,2002). The concordant rate for overt Hashimoto's hypothyroidism was 55% in monozygotic twins and 0% in dizygotic twins (Brix TH *et al*.,2000),indicating the importance of non-genetic influences on the disease development. As assessed by a study of Danish twins, 73% of the susceptibility to the development of TAbs seems to be attributable to the genetic factors (Hansen PS *et al*.,2006). Moreover, a recent twin study indicated that the liability to the production of antibodies directed against immunodominant region A of TPO is genetically determined (Brix TH *et al*.,2011).

2.1.4.1.Female sex

As indicated by numerous epidemiological studies, females present with positive thyroid autoantibodies (TAbs) up to three times more often than males. The largest NHANES III study has shown that females were positive for TPOAbs and TgAbs in 17% and 15.2%, respectively,while males only in 8.7% and 7.6%, respectively. According to the estimation provided by the study of Danish twins, the genetic contribution to TPOAb

and TgAb susceptibility in females was 72% and 75%, respectively, while in males it was only 61% and 39%, respectively. The possible explanation for high female predominance in thyroid autoimmunity might be associated with the X chromosome containing a number of sex and immune-related genes which are of key importance in the preservation of immune tolerance. Increased immunoreactivity might therefore be related to genetic defects of the X chromosome, such as structural abnormalities or monosomy.

Accordingly, a higher incidence of thyroid autoimmunity was reported in patients with a higher rate of X chromosome monosomy in peripheral white blood cells or in patients with Turner's syndrome. Another potential mechanism of impaired immunotolerance in females is skewed X-chromosome inactivation (XCI) leading to the escape of X-linked self-antigens from presentation in thymus with subsequent loss of T-cell tolerance. Skewed XCI was associated with a higher risk of developing autoimmune thyroid diseases.

Recently reported frequencies of skewed XCI in HT were 31%, 34.3%, 25.6% and 20%, respectively,which is significantly higher than in healthy controls, where the prevalences were only 8%, 8%, 8.6% and 11.2%, respectively. Furthermore, a study of Danish twins demonstrated a significant association of skewed XCI with TPOAb serum concentrations in dizygotic but not in monozygotic twin pairs, indicating that shared genetic determinants of XCI pattern and TPOAb production are more likely than causal relationship (Tao Yang and Xiaoyun Liu,2014).

2.1.4.2.Pregnancy and postpartum period

The tolerance of the fetal semi-allograft during pregnancy is enabled by the state of immunosuppression which is a result of hormonal changes and trophoblast expression of key immunomodulatory molecules. The pivotal players in regulation of the immune response are Tregs,which rapidly increase during pregnancy. Consequently, both cell-mediated and humoral immune responses are attenuated with a shift towards humoral immune response, resulting in immune tolerance of the conceptus tissues and suppression of autoimmunity. Accordingly,the decrease of both TPOAb and TgAb concentrations during pregnancy has been reported,reaching the lowest values in the third trimester. Postpartum rapid decrease of Tregs and reestablishment of the immune response to the pre-pregnancy state may lead to the occurrence or aggravation of the autoimmune thyroid disease. The increase of TPOAb concentrations occurred as soon as 6 weeks after delivery, reaching the baseline level at approximately 12 weeks and the maximum level at about 20 weeks after delivery. In up to 50% of females with positive TPOAbs in the early pregnancy, thyroid autoimmunity in the postpartum period exacerbates in the form of postpartum thyroiditis. It may occur within the first year after delivery, usually clinically presented with transient thyrotoxicosis and/or transient hypothyroidism,while in about a third of females permanent hypothyroidism may even develop (Tao Yang and Xiaoyun Liu,2014).

2.1.4.3.Fetal microchimerism

The term fetal microchimerism is defined by the presence of fetal cells in maternal tissues which are transferred in the maternal circulation during pregnancy. Several years after the delivery,the chimeric male cells

can be detected in the maternal peripheral blood as well as in maternal tissues, such as thyroid, lung, skin, or lymph nodes. The fetal immune cells, settled in the maternal thyroid gland, may become activated in the postpartum period when the immunotolerance ceases, representing a possible trigger that may initiate or exaggerate the autoimmune thyroid disease. In HT, fetal microchimeric cells were detected in thyroid in 28% to 83% which means that their occurrence is significantly higher than in the absence of autoimmune thyroid disease. Furthermore, a recent study of twins supported the putative role of microchimerism in triggering thyroid autoimmunity, showing a significantly higher prevalence of TAbs in opposite sex twins compared to monozygotic twins. Additionally, euthyroid females having been pregnant presented significantly more often with positive TPOAb compared to females with no history of being pregnant. However, the relation between parity and autoimmune thyroid disease was not confirmed by large population-based studies, advocating 10 Thyroid Disorders - Focus on Hyperthyroidism against the essential contribution of fetal microchimerism to the pathogenesis of autoimmune thyroid disease (Tao Yang and Xiaoyun Liu,2014).

2.1.5. Etiology

2.1.5.1.Genetic Triggers

The etiology of Hashimoto's thyroiditis is considered to be multifactorial, involving the interplay of various environmental and genetic factors. Studies conducted on the genetic associations of Hashimoto's thyroiditis have shown that the human leukocyte antigen (HLA*)* region, which plays a major role in other autoimmune disorders, is associated with development of Hashimoto's thyroiditis (Fisher *et al*.,

2000). The association of Hashimoto's thyroiditis with various other autoimmune diseases has further reinforced the probable involvement of genetic factors in the etiology. The major histocompatability complex(MHC), cytotoxic T-lymphocyte association (CTLA-4) and the human leukocyte antigen(HLA) are the genetic factors which are purported to play a major role in the pathogenesis.The selection of thyroid cells in the thymus and presentation of antigens in the periphery are modulated by, the human MHC analog, HLA. The sensitivity and specificity of the affinity to bind the peptides and recognize T-cells is determined largely by the genetic polymorphisms exhibited by the MHC molecule. The possibl polymorphisms within the MHC molecules play a pivotal role in the predisposition to autoimmune disease (Gebe *et al*., 2002). The association between the genetics of Hashimoto's thyroiditis and HLA gene loci has been investigated by serotyping the HLA, and deoxyribonucleic acid (DNA) typing the sequence- specific oligonucleotides. Different subsets of HLA genes have been found to show varying degree of associations with Hashimoto's thyroiditis in different races. The HLA class 1 and class 2 genes both showed association with Hashimoto's thyroiditis in Asian populations, while only HLA class 1 demonstrated the association in Caucasians (Katja Zaletel and Simona Gaberšček ,2011). No significant associations have been found between Hashimoto's thyroiditis and HLA class 3 or non-HLA genes of the HLA region (Hunt *et al*., 2001). An association between CTLA-4 and Hashimoto's thyroiditis has been noted in significant number of cases (Einarsdottir *et al*., 2003). CTLA-4 plays a vital role in upholding immunological self tolerance in the body and its down regulation is believed to be the initiating step for the pathogenesis of Hashimoto's thyroiditis as well as other autoimmune disorders such as Graves' disease (Chistiakov & Turakulov, 2003) .In addition to the

genetic factors numerous external factors also play a vital role in the etiology of the disease, preferentially affecting genetically predisposed individuals. (Arvin Parvathaneni *et al*.,2012).

2.1.5.2. Environmental Triggers

- **Iodine Intake**

Excessive iodine intake is well-established environmental factor for triggering thyroid autoimmunity. Several large population-based studies demonstrated higher prevalence of TAbs in the areas with higher iodine supply since the estimated prevalence was approximately 13% in iodine deficiency (Katja Zaletel and Simona Gaberšček , 2011), 18% in circumstances of sufficient iodine intake (Hollowell *et al*., 2002***)*** and about 25% in areas with excessive iodine intake (Kasagi *et al.*,2009*)*. Moreover, up to four-fold increase in prevalence of TAbs was demonstrated after the exposure to higher iodine intake due to the improvement of iodine prophylaxis in previously iodine deficient areas (Heydarian *et al.*,2007;and Fountoulakis *et al.*,2007). Valuable evidence was also provided by using experimental animal models of autoimmune thyroiditis, where the prevalence and severity of thyroid autoimmunity significantly increased when the dietary iodine was added (Rose *et al.*, 2002) .Several putative mechanisms by which iodine may promote thyroid autoimmunity have been proposed. Firstly,iodine exposure leads to higher iodination of Tg and thusincreases its immunogenicity by creating novel iodinecontaining epitopes or exposing cryptic epitopes. This may facilitate presentation by APC and enhance the binding affinity of the T-cell receptor which may lead to specific Tcell activation (Rose *et al.,* 2002). Secondly, iodine exposure has been shown to increase the level of reactive oxygen species in the thyrocyte which is generated during TPO oxidation of excessive amounts of iodine. They enhance the

expression of the intracellular adhesion molecule-1 (ICAM-1) on the thyroid follicular cells which could attract the immunocompetent cells into the thyroid gland (Burek *et al*.,2009).Thirdly, iodine toxicity to thyrocytes has been reported,since highly reactive oxygen species may bind to membrane lipids and proteins, causing thyrocyte damage and release of autoantigens (Fountoulakis *et al.*,2007). Fourthly, iodine excess has been shown to promote follicular cell apoptosis by inducing an abnormal expression of tumor necrosis factor-related apoptosisinducing ligand (TRAIL) and its death receptor (DR)-5 in thyroid (Yu *et al*.,2011). Fifthly, *in vitro* evidence also suggests an enhancing influence of iodine on the cells of the immune system, including augmented maturation of dendritic cells,increased number of T cells and stimulated B-cell immunoglobulin production (Fountoulakis *et al*.,2007).

- **Drugs**

Furthermore, certain drugs were reported to trigger or exacerbate thyroid autoimmunity in susceptible individuals.Interferon α (IFN-α) is extensively used to treat chronic hepatitis and is frequently associated with thyroid autoimmunity since TAbs were observed in up to 40% and clinical disease in 5-10% of patients treated with IFN α. Presumably, IFN-α has both thyroid toxic effects with consequent autoantigen presentation and immune effects,such as switching to Th1 immune response, suppression of Treg function, activation of immune cells, stimulation of cytokine release and expression of MHC class I on thyroid cells (Tomer& Y., 2010). Similarly, IL-2 treatment, used for melanoma and renal carcinoma, seems to act *via* immune and toxic mechanisms, leading to both TAb positivity and hypothyroidism (Barbesino& G .,2010). In patients with known autoimmune thyroid disease lithium may

increase the risk of hypothyroidism. According to some studies, treatment with lithium has also been shown to increase TAb titres and the prevalence of thyroid autoimmunity, although this observation has not yet been confirmed by other reports (Barbesino& G 2010; Fer *et al*.,2005). Among putative mechanisms direct toxicity of lithium on thyroid or toxicity of increased intrathyroidal iodine resulting from lithium treatment were discussed (Barbesino& G .,2010).Similarly, amiodarone alone as well as its high iodine content may act cytotoxically which may lead to thyroid autoantigen presentation and provoke thyroid autoimmunity (Martino *et al*.,2001).

- **Infections**

Not only the IFN-α treatment but also hepatitis C infection itself has been reportedly associated with thyroid autoimmunity and hypothyroidism. Among possible mechanisms, the molecular mimicry between viral and selfantigens has been suggested, whereas the release of proinflammatory mediators caused by viral infection may lead to activation of autoreactive T-cells (Tomer& Y. ,2010). Besides, in HT several other putative triggering viruses have been implicated such as parvovirus, rubella, herpes simplex virus,Epstein Barr virus, and human T-lymphotropic virus type 1 (Desailloud *et al*.,2009). A recent study of sera in pregnant women has also indicated an association between a prior infection with Toxoplasma gondii and an increase of TPOAbs (Wasserman *et al*.,2009). Nevertheless, the evidences are scarce and further studies are required in order to confirm the role of infections as causative agents.

- **Chemicals**

The exposure to environmental toxicants such as polyaromatic hydrocarbons or polyhalogenated biphenyls, both commonly used in a variety of industrial applications, has been shown to provoke thyroid autoimmunity not only in experimental animals but also in humans (Burek *et al*.,2009) . Recently, a significantly higher prevalence of HT and TAb (9.3% and 17.6%, respectively) has been demonstrated in residents living in the area of petrochemical complex of Sao Paolo compared to the control area (3.9% and 10.3%, respectively) (de Freitas *et al.*,2010). In Slovakia, the exposure to polychlorinated biphenyls was associated with TAb and hypothyroidism (Langer *et al.*,2007).Although there is strong evidence attesting the contribution of chemicals to thyroid autoimmunity, the exact mechanisms of their action are yet to be established.

2.2. Laboratory tests

Elevated anti-TPO or anti-Tg antibody titers are the most specific laboratory findings to establish the diagnosis of autoimmune thyroid disease (AITD) or HT, typically making biopsy unnecessary. The 24-hours thyroid radioactive iodine-123 or-131 (^{123}I or ^{131}I) uptake (RIU) is also helpful to distinguish Hashitoxicosis from Graves' disease (GD); the RIU is low in patients with Hashitoxicosis, whereas it is elevated in those with GD. ^{123}I is preferred than ^{131}I because it has a shorter half-life (13 hours for ^{123}I, 8 days for ^{131}I) allowing quicker dissipation of background radiation. Since radioactive iodine is secreted in breast milk, and 123I has a short half-life, it is recommended for diagnostic thyroid studies in nursing mothers. Breast milk must be pumped and discarded for 2 days

after the intake of 123I either used for thyroid uptake or for thyroid scanning. Scintigraphy reveals in-homogeneous activity throughout the gland in 50% and a pattern suggestive of either hot or cold nodules or a combination of both in 30% of patients. Twenty percent of patient with HT have normal findings at the scintigraphic thyroid imaging (Tao Yang and Xiaoyun Liu,2014).

2.3. Pathology

The thyroid gland is pale and rubbery to firm. The characteristic histopathologic changes of HT include diffuse lymphoplasmacytic infiltration, lymphoid follicle formation with germinal centers, a varying degree of fibrosis, parenchymal atrophy and the presence of large follicular cells with abundant granular eosinophilic cytoplasm, so-called Hürthle, oxyphilic or Askanazy cells (Figure 1.1&1.2) (A kamizu T *et al*.,2012). Foreign body giant cells and granulomas are not features of HT. Electromicroscopy shows deposits of immunoglobulin (Ig) G and Tg along the basement membranes of follicular cells. Both B and T cells infiltrate the thyroid. Most infiltrating T cells have alpha/beta T cell receptors.Increased expression of Th1-related cytokines such as IFNg, interleukin-2 and CD25 reportedly occurs in intrathyroidal T cells from patients with HT. The impairment of hormone synthesis is due to apoptotic destruction of thyroid cells (Yuji Hiromatsu *et al*.,2013). Increased expression of Fas on thyroid follicular cells by the surrounding cytokines and Fas ligands on T cells may induce this apoptosis, although other cell death pathways, such as complement-fixing cytotoxicity and thyroglobulin specific T cell-mediated cytotoxicity, may also be involved (Zaletel K & Gaberšček S 2011; and Yuji Hiromatsu *et al*.,2013).

Figure 1.1: Histopathologic features of struma lymphomatosa as depicted in the original paper. a: Lymphoid follicle; b: Degenerated thyroid follicle; c: Giant cells; d: Hyperplastic interstitium with prominent round cell infiltration.

Figure 1.2 : Hashimoto thyroiditis. The thyroid parenchyma contains a dense lymphocytic infiltrate with germinal centers. Residual thyroid follicles lined by deeply eosinophilic Hürthle cells also are seen.

2.4. Clinical Features

Hashimoto thyroiditis comes to clinical attention as painless enlargement of the thyroid, usually associated with some degree of hypothyroidism, in a middle-aged woman. The enlargement of the gland usually is symmetric and diffuse, but in some cases it may be sufficiently localized to raise suspicion for neoplasm. In the usual clinical course, hypothyroidism develops gradually. In some cases, however, it may be preceded by transient thyrotoxicosis caused by disruption of thyroid follicles, with secondary release of thyroid hormones (hashitoxicosis). During this phase, free T4 and T3 concentrations are elevated, TSH is diminished, and radioactive iodine uptake is decreased. As hypothyroidism supervenes, T4 and T3 levels progressively fall, accompanied by a compensatory increase in TSH. Patients with Hashimoto thyroiditis often have other autoimmune diseases and are at increased risk for the development of B cell non-Hodgkin lymphomas ,

which typically arise within the thyroid gland. The relationship between Hashimoto disease and thyroid epithelial cancers remains controversial, with some morphologic and molecular studies suggesting a predisposition to papillary carcinomas (Vinay Kumar *et al*.,2013).

2.5.Pathogenesis

It is well known that HT results from a multistep process, requiring several genetic and environmental abnormalities to converge before disease development. Thus, thyroid follicle damage may be provoked by self-antigen presentation by antigen presenting cells and specific T lymphocyte activation. On the other hand, toxic destruction of thyroid cells possibly through the generation of oxygen radicals may participate in eclosion of autoimmunity (Noura Bougacha-Elleuch *et al*.,2012). Both proliferation and apoptosis are involved in the pathogenesis of HT. Analysis of the mechanisms by which such autoimmune pathology arises has been facilitated by the use of animal models. These include the Obese Strain (OS) chicken and the BioBreeding (BB) and Buffalo rats as spontaneous models of HT. HT can also be experimentally induced by specific immunization protocols with target auto antigens or elevation of dietary iodine.

2.5.1.Autoimmunity in HT

HT is considered to be a Th1-mediated disease leading to aberrant infiltration of lymphoid cells and destruction of thyroid follicles (figure 3). The final outcome is fibrosis replacing normal thyroid parenchyma and hypothyroidism resulting of thyroid cell destruction (Parish & Cooke, 2004). Indeed, a central phase of HT is characterized by an apparent

uncontrolled production of auto reactive CD4+ T cells, CD8+ cytotoxic T cells and immunoglobulin G auto antibodies. This immunological synapse is defined by the interface between antigen presenting cells and T-cells that is formed during T-cell activation (Chistiakov, 2005). On the other hand, existence of naturally existing CD4+ CD25+ foxp3+ T regulatory cells influencing thyroiditis development in naïve susceptible mice was recently demonstrated. Moreover, it has been shown that naturally T regulatory cells are required for induction of antigen specific tolerance, indicating that induced Murine experimental autoimmune thyroiditis tolerance is a result of activation of naturally existing T regulatory cells rather than de novo generation of induced T regulatory cells (Morris *et al*., 2009).

Interestingly, several of the AITDs susceptibility genes participate in the immunological synapse, suggesting that abnormalities in antigen presentation are important mechanisms leading to AITDs (Tomer, 2010).Initially, the production of self-reactive cells and auto antibodies occurs in the draining lymph nodes. Later, the lymphoid tissue often develops directly in the thyroid gland itself. This tissue is generally very well-organized, with cords of anti-Tg-antibody- producing plasma cells in the periphery (Chistiakov, 2005). In a final, destructive step of HT, the auto reactive T cells diffusely accumulate in large numbers and infiltrate thyroid parenchyma. This phenomenon will determine clinical phenotype of the disease. In the BB-DP rat model, Th1-mediated mechanisms involving production of IL-12, tumor necrosis factor-α (TNF-α) and interferon- γ play a major role in the destruction of thyrocytes (Sharma R *et al*.,2009; Wang SH *et al*.,2002).Furthermore, it has been recently shown that pro-IL18 is constitutively expressed in thyroid cells and IL18 up regulation by INF-γ is an immunological feature of HT patients with

an important role in promoting the local immune response (Liu *et al*., 2010).

2.5.2. Apoptosis in HT

Apoptosis appears to play a major role in the final stage of the disease (figure1.3 & 1.4). In fact, apoptotic molecules such as Fas and Fas ligand (FasL) expression was higher in rats with lympholytic thyroiditis indicating a possible role in thyrocyte death(Noura Bougacha-Elleuch *et al*.,2012).Theses molecules are expressed at low level by normal thyroid cells compared to patients with HT with an increasing number of apoptotic cells (Kaczmarek *et al*., 2011). The mechanism and regulation of apoptosis in thyroid gland are still little known. The most studied receptor mediated apoptic pathway is the Fas/Fas ligand system. Fas is substantially expressed on lymphocytes. Fas-Fas ligand interaction could lead to the thyrocyte cell death (Kaczmarek *et al*., 2011). Thyroid cells express constitutively Fas but these latters are normally unaffected by Fas-mediated apoptosis. In contrast, they can be sensitised to Fas-induced destruction under certain pathologic conditions such as the release of IFN- γ, TNF-α and IL-1β, by infiltrating immune cells (Giordano *et al*., 2001). Over the past few years, many reports have shown that mobilisation of the Fas/Fas ligand apoptotic pathway by proinflamatory cytokines plays a pivotal role in the devastation of thyroid follicular cells in HT leading to hypothyroidism.(Kaczmarek *et al*.,2011). Therefore, the Fas pathway is the most important mechanism of T lymphocyte mediated apoptosis. It is just possible that this process plays an essential role in the pathogenesis of Hashimoto thyroiditis, because cytotoxic T lymphocytes are fully present in the thyroid in places where apoptosis is located (Fountoulakis *et al*.,2008; Chen *et al* ., 2004; and Bretz.,2002).Mechanisms of regulation of this pathway include probably

changes in Fas expression level,and the expression of molecules that promote survival, including the Bcl-2 gene family(Noura Bougacha-Elleuch *et al*.,2012). This latter antiapoptotic protein and sFas system,which normally protect thyroid cells from apoptosis, are decreased in the thyroid cells of patients with HT, creating a proapoptotic phenotype (Fountoulakis & Tsatsoulis, 2004).Thus, the rate of thyrocyte apoptosis dictates the clinical outcome of thyroid autoimmunity. Though rare in normal thyroid, it markedly increases during HT, but not in GD with a divergent phenotype. Therefore, regulation of thyrocyte survival is a crucial pathogenic determinant via the balance between Th2 and Th1 response (Chistiakov, 2005). Despite the "crucial" role played by these apoptotic molecules, they are poorly investigated in HT pathogenesis at the genetic level. Therefore, arguments of their "real" implication in HT are still missing.

At the onset of disease, (HLA) class II-positive Antigen-presenting cells (APC), present thyroid-specific autoantigens to the naïve T cells, leading to the maturation of autoreactive T cells. Interaction with auto antigen leads to the production of different cytokines inducing T-helper type 1 (Th1)-mediated cell immune response. The stimulation of the Fas/Fas ligand apoptotic pathway by pro-inflammatory cytokines is the most important mechanism of T lymphocyte mediated apoptosis. The caspase cascade ultimately induces enzymes that progressively destroy the cell, leading to thyroid cell death and hypothyroidism.

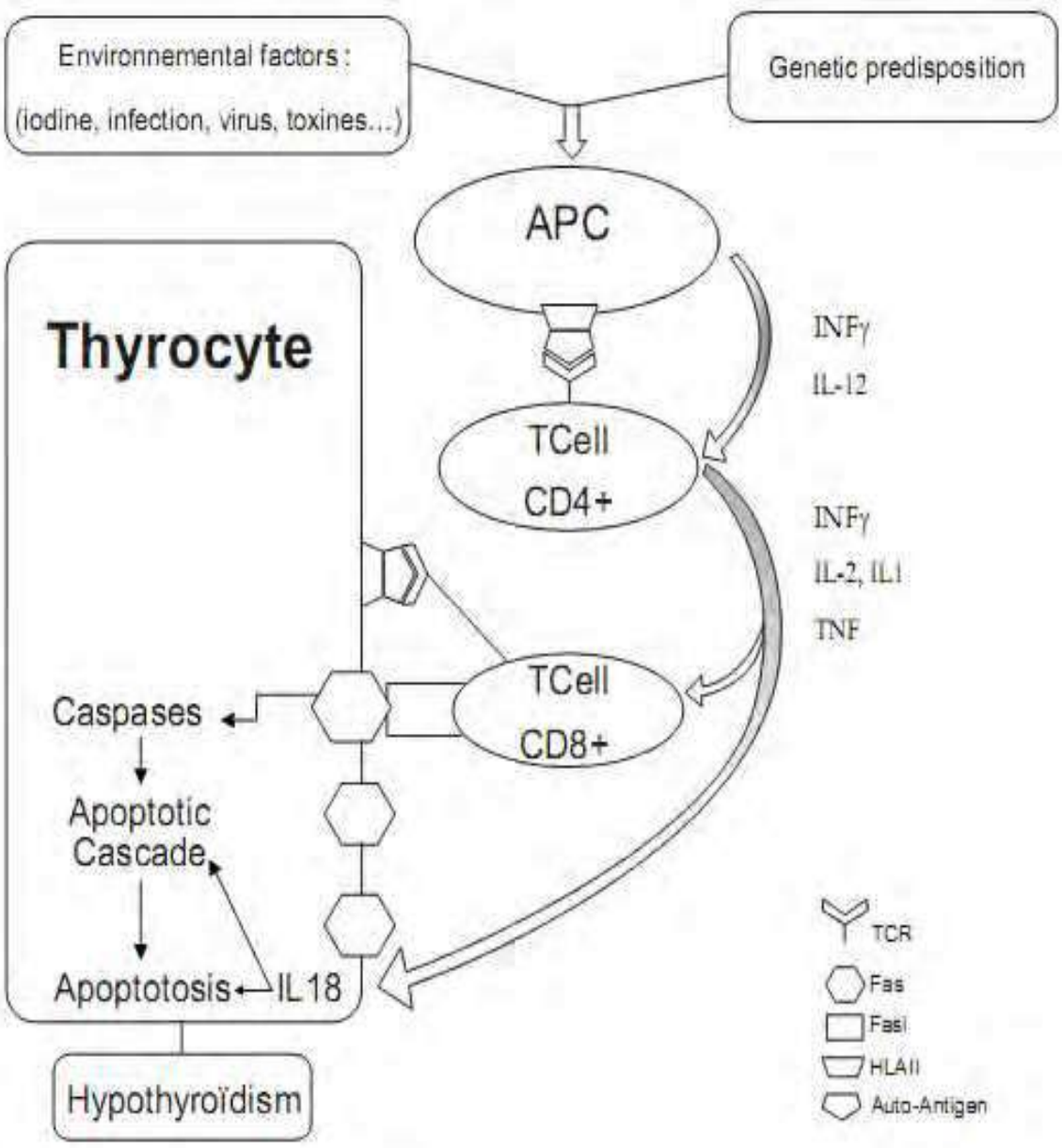

Figure 1.3 : Autoimmune events in Hashimoto's thyroiditis.

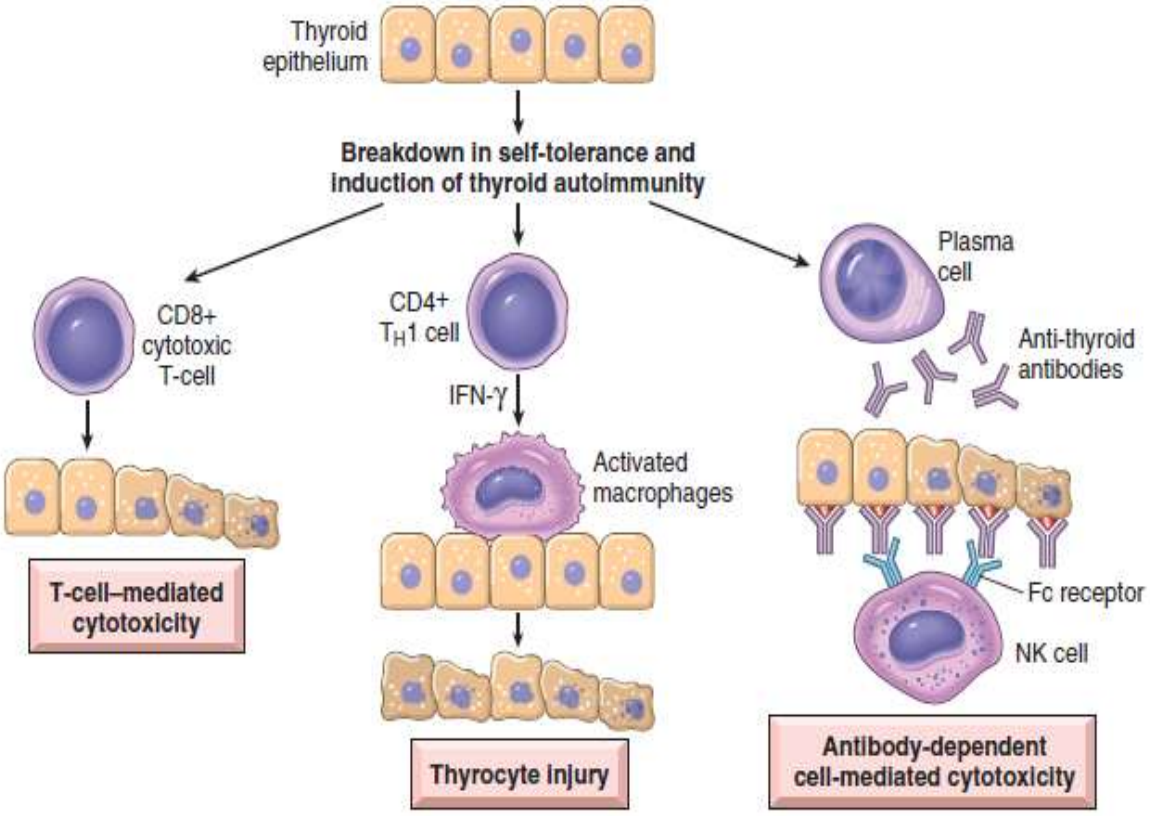

Figure 1.4 :Pathogenesis of Hashimoto thyroiditis. Breakdown of immune tolerance to thyroid autoantigens results in progressive autoimmune destruction of thyrocytes by infiltrating cytotoxic T cells, locally released cytokines, or antibody-dependent cytotoxicity (Vinay Kumar *et al*.,2013).

2.6.Progression of Autoimmune Process

Once the tolerance to thyroid antigens is broken, endothelial cells of regional postcapillary venulae are activated, allowing the extravasation of blood leucocytes attracted by chemokines. The recruitment and arrest of lymphocytes in the thyroid is only partially understood. Immune cells adhere to the endothelium, then migrate across it, then through the

interstitium and move toward thyroid follicular cells, sometimes organizing into germinal centers,resembling lymph node germinal centers. In autoimmune thyroid diseases there is an enhanced expression of adhesion molecules (selectins and integrins) on lymphocytes and endothelial cells (lymphocyte function associated antigen 1-LFA1, interstitial cell adhesion molecule 1-ICAM 1, very late antigen 4-VLA4) as well as selectin ligands.CXC chemokines are secreted by thyrocytes stimulated by TNFα, IFNγ and IL12; CXCL10,CXCL9 are responsible for recruitment of activated lymphocytes in the thyroid. CXCL10 promotes differentiation of Th0 cells into Th1 cells, while CCL2- the differentiation of Th0 cells into Th2 cells. CXCL10 binding to CXCR3A generates an angiostatic effect, while binding to CXCR3B exerts chemotactic and immune effects. CXCR3 is expressed on immune cells (T cells, especially Th1,B cells, NK cells), vascular pericytes, microvascular endothelial cells. Immune cells expressing CXCR3 are attracted in the inflamed thyroid.Th1 cells secrete IFNγ, which stimulates production of chemokines by thyroid follicular cells, thus maintaining and expanding the autoimmune process (Rotondi *et al*.,2007).

Predominance of Th1 a immune response promotes thyrocytes apoptosis (Corona *et al*.,2008) mediated by Fas and TRAIL (TNF – related apoptosis inducing ligand),leading to Hashimoto thyroiditis. Predominance of a Th2 immune response induces antigen-specific B cells to produce antithyroid antibodies; stimulatory antiTSH receptor antibodies are responsible for Graves' disease and blocking antiTSH receptor antibodies are responsible for atrophic thyroiditis. Thus, the clinical expression of thyroid autoimmunity depends on the Th1-Th2 balance (Tsatsoulis *et al*,2006).In Hashimoto thyroiditis hypothyroidism is not only the result of thyrocytes destruction, but also of the thyroid

function impairment induced by Th1 cytokines. IL-1 and IFNγ down regulate the expression of TG, TPO, Na/I symporter and H2O2 generating enzymes (DUOX), effects partially mediated by nitric oxide and antagonized by increased TSH concentrations. IL-4, a Th2 cytokine, blocks Th1 induced alterations in DUOX, TPO and Tg secretion. Th3 cytokines-TGFβ and IL-10-repress thyrocytes function but their effects can also be overturned by Th2 cytokines (Mclachlan *et al*.,2007).

2.7.Thyroid Dysfunction & The Immune System

Thyroid hormones are not essential for the development of the immune system but are involved in the maintenance of immune homeostasis.Hypothyroidism decreases thymic activity,causes spleen and lymph nodes involution and thus,represses both cell mediated and humoral immune responses. In hyperthyroidism both stimulatory and suppressing effects on the immune system have been described.It was demonstrated that thyroid hormones modulate lymphocytic activity through protein kinase C signaling pathway (Klecha *et al*.,2008).

2.8.Role of T Lymphocytes

Excessively stimulated T cells CD4+ are known to play the main role in the pathogenesis of HT (Figure 1.5). T cells perform two functions in the pathogenesis of HT. T helper type 2 Th2 cells lead to an excessive stimulation and production of B cells and plasmatic cells which produce antibodies against thyroid antigens leading to thyroiditis (Aleksandra Pyzik *et al*.,2015). T helper type 1 (Th1) and Th2 cells produce interferon- (IFN-) gamma, and interleukin- (IL-) 4, respectively. Nanba et

al. reported that IFN-gamma and IL-4 gene polymorphisms, which are related to higher IFN-gamma and lower IL-4 production, respectively, are more frequent in patients with severe HT than in those with mild HT (Nanba T. *et al* ., 2012). Th1 cells activate cytotoxic lymphocytes and macrophages, which directly affect thyroid tissue by destroying thyroid follicular cells. In the tissues of the thyroid in patients with HT Th1 are the predominant cells. Histopathological studies have shown that more T cells have been observed in HT both in the parenchyma and in the lymphatic infiltrations. In HT, damaged thyroid follicles with apoptotic thyrocytes (pyknotic nuclei, condensed cytoplasm with enlarged mitochondria and endoplasmic reticulum cisterns) were visible in this area. A number of CD4+ T cells in the thyroid infiltrates in HT were significantly decreased in the interstitium. Observations under a light microscope revealed that T suppressor/cytotoxic cells were accumulated at the sites of destruction of thyroid follicles. These sites were surrounded by connective tissue fibers and fibroblasts (Ben-Skowronek I. *et al*,.2013).

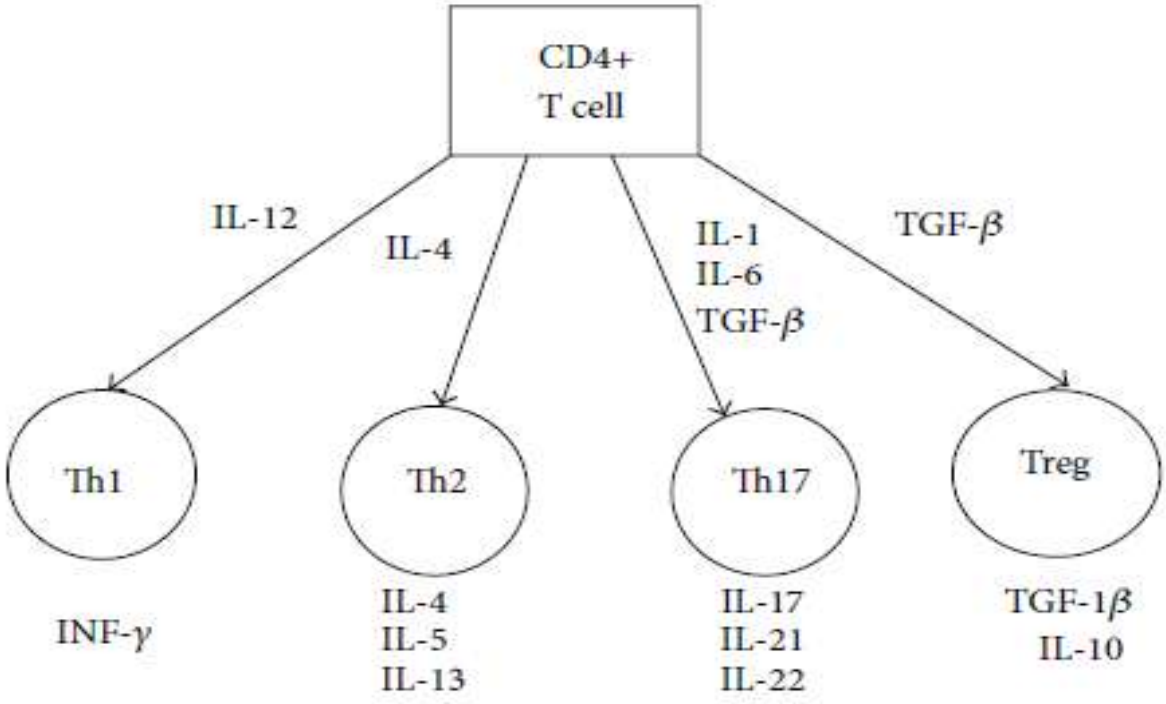

Figure 1.5: T CD4+ cell differentiation.

2.8.1.Role of IL-4

IL-4 is an important member of the class I cytokine family with four a helix structures that is secreted predominantly by activated Th2 cells (Leung *et al*.,2004; Lee *et al*.,2008). It induces Th2-mediated immune responses by upregulating the expression of MHC class II, CD23, and IL-4R on B cells (Risma *et al*.,2002; Lee *et al*.,2008). It has an anti-inflammatory effect owing to its efficient inhibition of the production of proinflammatory cytokines,such as TNF-a, IL-1a, IL-1b, IL-6, and IL-8. It also plays a crucial role in mediating allergic responses. In this process, IL-4 stimulates the proliferation of mast cells with IL-13 and promotes Ig class switching to IgE that binds to high-affinity Fcε

receptors on the surfaces of mast cells, basophils, and eosinophils, leading to the degranulation and production of inflammatory mediators when it is cross-linked by an Ag (Cortes *et al*.,2007; Li *et al*.,2007; Hoffjan *et al*.,2002; Kips & J. C. 2001;and Solymar *et al*.,2002). IL-4 exerts its biological functions by interacting with IL-4R (Kips & J. C. 2001; Ozaki *et al*.,2002). The latter is a heterodimer composed of a specific a-chain (IL-4Ra or IL-4BP) and a common γ-chain (γc). The γc also is coutilized by IL-2, IL-7, IL-9,IL-13, IL-15, and IL-21 for the activation of signaling pathways (Lv-yun Zhu *et al*.,2012) . As a class I cytokine receptor, IL-4Ra has specific and high affinities (Kd = 20–300 pM) in the recognition and binding to IL-4 (Lee *et al*.,2008). It could be detected on a number of hematopoietic or nonhematopoietic cell surfaces, including endothelial, epithelial,muscle, fibroblast, hepatocyte, and brain cells (Shore, S. A. ,2004).

CD4+ cells, which belong to subpopulation Th2 stimulate immunological response of B lymphocytes. There are some studies evaluating cytokines in these diseases, demonstrating the production of IL-4 and TNF- by infiltrating T cells and macrophages. However, the specific role of these molecules in the pathogenesis of autoimmune thyroid diseases (AITD) is still debated (Artur Bossowski *et al*.,2011). They presume that an altered balance of pro- and anti-inflammatory cytokines may play an important role in the pathogenesis of autoimmune thyroiditis.T-helper 1 (Th1) cell-mediated inflammatory responses predominate in the early pathogenesis of GD,whereas Th2 cell-mediated immunity may play a role in later stages. Th1 cells produce IFN-gamma and Th2 cells produce IL-4. Nanba T *et al*. reported that IFNgamma and IL-4 gene polymorphisms, which are related to higher IFN-gamma and lower IL-4 production,respectively, are more frequent in patients with

severe HT than in those mild HT. They investigated the proportion of peripheral Th1 and Th2 cells in patients with AITD and concluded that the peripheral Th1/Th2 cell ratio is related to the severity of HT and is related to the intractability of GD. They hypothesize that these patterns of peripheral Th cell subsets may be expressed within the thyroid (Nanba T *et al*,.2012).

2.8.2.Th17 cell and it's role in the pathogenesis of Hashimoto's thyroiditis

Th17 lymphocytes serve as a pathogenic factor in the development of various diseases. These could be autoimmune diseases like psoriasis, multiple sclerosis, rheumatoid arthritis or inflammatory bowel disease or neoplasms, allergies, engraftment, or transplant rejection. However, their role in AITD is still debatable. Th17 cells account for approximately 1% of CD4+ lymphocytes in blood serum and take part in the immune response against intercellular antigens. They are characterized by expression markers such as CCR6 (CD196), IL-23R, IL-12R-beta2, CD49, and CD161 and produce proinflammatory cytokines mainly: IL-17A, IL-17F, IL-21, IL-9, IL-22, and TNFA. They develop from T helper cells under the influence of various factors of differentiation, growth, and stabilization such as TGFB plus IL-6, IL-21, and IL-23 and transcription factors like STAT3, RORγ, and RORα (Miossec P. and Kolls J. K.,2012; Zambrano-Zaragoza J. F. *et al*.,2014) . Liu *et al*. and Qin *et al*. found that HT patients had a significantly increased serum concentration of IL-6 and IL-23 in comparison with healthy controls (Liu Y. *et al*.,2014; Qin Q. *et al*.,2012),whereas Kimura and Kishimoto showed that IL-6 induces Th17 differentiation together with TGFβ (Kimura A. and Kishimoto T.,2010). Bossowski *et al*. demonstrated an elevated level of Th17 cells in children with untreated Hashimoto’s disease, which suggests the participation of

these cells in the induction and development of the disease. However, they did not demonstrate such a relation in Graves' disease (Bossowski *et al*.,2012). Similarly, in the research by Li *et al*. a significantly higher concentration of IL-17 was visible in HT compared to in the thyroid cancer, in the nodular goiter, or in the studied group (Li *et al*., 2013).

Further research by Li *et al*. suggested a negative relationship between the level of IL-17 and the stage of hypothyroidism among patients with Hashimoto's disease. Histopathological examinations have shown a strong relationship between the concentration of IL-17 and the stromal fibrosis in the gland, which points to the fact that the presence of IL-17 increases local inflammation and leads to the fibrosis and atrophy of thyrocytes. Additionally, it was concluded that the impact of sodium iodide concentrations on the development of Th17 and Th1 lymphocytes, which can serve as inhibitors of regulatory T cells, might be various, whereas the research by (Shi *et al*.,2010) demonstrated that mRNAs of IL17 and transcription factor were significantly increased in PBMC (peripheral blood mononuclear cell) from patients with HT. Wang et al. tried to answer the question of why the number of Th17 rises in HT. The authors observed an elevated concentration of proinflammatory leptin in blood of the patients and concluded that this cytokine could induce the proliferation of T lymphocytes and promote immune response in the direction of Th17 (Wang S. *et al*.,2013).

The proportion of peripheral Th17 cells in patients with AITD was higher than in control subjects and it was dependent on disease activity and severity (Figueroa-Vega *et al*.,2010; Kim *et al*.,2012; and Nanba *et al*.,2012). However, (Zheng *et al*.,2013) reported increased expression of

IL-17 mRNA also in euthyroid patients with Graves' disease and it was upregulated after stimulation with IL-23. (Bossowski *et al*.,2012) examined a group of children with AITD and they found increased percentage of CD4+IL-17+ cells in children with untreated Hashimoto's disease, but not in children with Graves' disease. Additionally, percentage of CD4+IL-17+ cells positively correlated with titers of anti-thyroid peroxidase immunoglobulins. *Shi et* al. suggested that Th17 cells can play a central role in pathogenesis of Hashimoto's disease rather than Th1 cells. These conclusions were based on confirmed higher expression of IL-17 mRNA than IFN-γ mRNA in peripheral blood cells.Unfortunately, authors did not include any data about duration of disease what in combination with age of patients ranged 23–60 leading to conclusions that patients were rather not in active phase of lymphocytic thyroiditis.This could explain low activity of Th1 response and relative predominance of IL-17 producing cells (Shi *et al*.,2010).

2.Literatures Review

2.1.Hashimoto's thyroiditis

2.1.1.Definition

Hashimoto’s thyroiditis (HT) is an autoimmune disorder of the thyroid gland,which characterized by the progressive loss of follicular cells and attendant replacement of the thyroid tissue by lymphoid infiltrates and fibrosis (Weetman., 2003). The disruption of the thyroid architecture and the loss of functionality complicate the course of HT by the development of progressive hypothyroidism. The mechanism behind this disrupted self-immune response seems to be quite complex.T helper type 1 (Th1) and T helper type 2 (Th2) cell imbalance has been accused in the pathogenesis of HT and this model has long been used to explain the mechanism of autoimmune thyroiditis as well as various other autoimmune diseases (Steinman ., 2007).

2.1.2.Epidemiology

Hashimoto’s thyroiditis is about 15-20 times more common in women than in men and frequently involves people between the ages of 30 and 50 years of age. Determining the exact incidence and prevalence rates for Hashimoto’s thyroiditis has been difficult due to variable expression of this disease. Some studies estimate that the current prevalence rate in the United States ranges between 0.3%-1.2% (Staii *et al*., 2010). Other studies estimate the prevalence among the general population to be approximately 2% (Arvin *et al*.,2012). When attempts have been made to characterize the prevalence prospectively, with the aid of organized programs of ultrasound guided biopsy, the prevalence described has been at least 5%. It should be noted that studies employing the diagnostic modality of ultrasound guided biopsy have

recorded prevalence rates higher than studies using other investigative modalities (Staii *et al*., 2010). The National Health and Nutrition Evaluation Study-3 (NHANES-3) study has shown the prevalence of subclinical and clinical hypothyroidism to be 4.6% and 0.3%, respectively, in the United States (Hollowell *et al*., 2002). The Whickham survey, an epidemiological study conducted in the United States, has revealed the prevalence of hypothyroidism to be 1.5% in females and less than 0.1% in males .During the past few decades there has been a reported increase in the incidence of Hashimoto's thyroiditis, which could be attributed to newer diagnostic modalities such as needle biopsies and serological tests, and their increased sensitivity when compared to the older methods (Arvin *et al*.,2012). Studies about age-specific incidence rates of Hashimoto's thyroiditis indicate the existence of a random distribution in both men and women and have shown an initial lag in the first few years of their life followed by a constant rate after this . A few studies have suggested a slight increase in the prevalence of autoimmune thyroiditis in adolescent girls following use of iodized food products ingested to prevent iodine deficiency (Zois *et al*., 2003).

In a study made by (Hussain *et al*.,2014) at the Hilla teaching general Hospitals on 175 patients underwent thyroid surgery in order to study the percentages of Hashimoto's thyroiditis to the total cases which were taken and also to know the correlation between thyroid function and histopathological status in those patients whom diagnosed as Hashimotos thyroiditis,were 18 cases diagnosed as hashimotos thyroiditis, the incidence of Hashiomato thyroiditis (13.4%).

Also, in a retrospective study done by (Nasheiti.,2005) to study the childhood hypothyroidism in Iraq, the results showes that forty-five patients were diagnosed as cases of primary hypothyroidism, 5 (11.1%) had Hashimoto thyroiditis.

The data on the incidence of thyroid autonomy (HT and Grave's disease) after increase iodization are scarce. While some reports indicate short term increase (Lindi *et al*; 2002**)**, others observed decrease in the first year after iodization (Zaletel *et al***.,** 2011**)**. In Iraq, iodization was practiced during 1990s years i.e. during sanctions, with no reference about the dose of iodization (Al-Hashimi, 2014). Literature show that autoimmune thyroid disorders are an important cause of goiter in post-iodization phase (Das *et al***.,**2011**)**. Iodization in Iraq might affect the prevalence of HT.

2.1.3.Risk Factors

For several decades a strong genetic predisposition to autoimmune thyroid disease has been recognised, predominantly on the basis of the family and twin studies. Nearly 50 years ago, soon after the discovery of TAbs, the presence of TAbs was reported in 56% of siblings of patients with autoimmune thyroid disease (Zaletel *et al*; 2011). This familial clustering of autoimmune thyroid disease and the presence of TAbs in up to 60% of first-degree relatives of patients has been later confirmed by several studies (Marwaha *et al*., 2003) . When both parents were affected, the prevalence of TPOAbs and TgAbs was 42% in daughters and 33% in sons, compared with 28.9% and 16.7%, respectively, when only one parent was TAb-positive. Among first-degree relatives of children with HT, 34% were diagnosed TPOAbs positive compared to only 13% first-degree relatives of children

without autoimmune thyroid disease (Marwaha *et al.*,2003). The sibling risk ratio for HT, calculated on the basis of the data from the NHANES III study, was 28, thus confirming the highly significant contribution of genetic factors to the disease development (Villanueva *et al.*,2003).

Recent data from Germany also indicate 32-fold increased risk for developing HT in children and 21-fold increased risk in siblings of patients with HT, with females being significantly more often affected than males (Dittmar *et al.*,2011). Twin studies provided further valuable data on the genetic contribution to thyroid autoimmunity. In healthy twin siblings of patients with overt autoimmune thyroid disease, positive TPOAbs and TgAbs in monozygotic twins were determined in 53% and 47%, respectively, in dizygotic twins in 22% and 13%, respectively, while in healthy control population only in 9% and 7%, respectively (Brix *et al.*,2004). The concordance rates for TPOAbs were 64% in monozygotic twins compared with 35% in dizygotic twins, while concordance rates for TgAbs were 74% and 32%, respectively (Phillips *et al*.,2002).

The concordant rate for overt Hashimoto's hypothyroidism was 55% in monozygotic twins and 0% in dizygotic twins (Brix *et al*.,2000),indicating the importance of non-genetic influences on the disease development. As assessed by a study of Danish twins, 73% of the susceptibility to the development of TAbs seems to be attributable to the genetic factors (Hansen *et al*.,2006). Moreover, a twin study indicated that the liability to the production of antibodies directed against immunodominant region A of TPO is genetically determined (Brix *et al*.,2011).

2.1.3.1.Pregnancy and postpartum period

The tolerance of the fetal semi-allograft during pregnancy is enabled by the state of immunosuppression which is a result of hormonal changes and trophoblast expression of key immunomodulatory molecules. The pivotal players in regulation of the immune response are Tregs,which rapidly increase during pregnancy. Consequently, both cell-mediated and humoral immune responses are attenuated with a shift towards humoral immune response, resulting in immune tolerance of the conceptus tissues and suppression of autoimmunity.Postpartum rapid decrease of Tregs and reestablishment of the immune response to the pre-pregnancy state may lead to the occurrence or aggravation of the autoimmune thyroid disease. The increase of TPOAb concentrations occurred as soon as 6 weeks after delivery, reaching the baseline level at approximately 12 weeks and the maximum level at about 20 weeks after delivery. In up to 50% of females with positive TPOAbs in the early pregnancy, thyroid autoimmunity in the postpartum period exacerbates in the form of postpartum thyroiditis. It may occur within the first year after delivery, usually clinically presented with transient thyrotoxicosis and/or transient hypothyroidism,while in about a third of females permanent hypothyroidism may even develop (Tao Yang and Xiaoyun Liu,2014).

2.1.3.2.Fetal microchimerism

The term fetal microchimerism is defined by the presence of fetal cells in maternal tissues which are transferred in the maternal circulation during pregnancy. Several years after the delivery,the chimeric male cells can be detected in the maternal peripheral blood as well as in maternal

tissues, such as thyroid, lung, skin, or lymph nodes. The fetal immune cells, settled in the maternal thyroid gland, may become activated in the postpartum period when the immunotolerance ceases, representing a possible trigger that may initiate or exaggerate the autoimmune thyroid disease. In HT, fetal microchimeric cells were detected in thyroid in 28% to 83% which means that their occurrence is significantly higher than in the absence of autoimmune thyroid disease.

Furthermore, a recent study of twins supported the putative role of microchimerism in triggering thyroid autoimmunity, showing a significantly higher prevalence of TAbs in opposite sex twins compared to monozygotic twins. Additionally, euthyroid females having been pregnant presented significantly more often with positive TPOAb compared to females with no history of being pregnant. However, the relation between parity and autoimmune thyroid disease was not confirmed by large population-based studies, advocating 10 Thyroid Disorders - Focus on Hyperthyroidism against the essential contribution of fetal microchimerism to the pathogenesis of autoimmune thyroid disease (Tao Yang and Xiaoyun Liu,2014).

2.1.4. Etiology

2.1.4.1.Genetic Triggers

The etiology of Hashimoto's thyroiditis is considered to be multifactorial, involving the interplay of various environmental and genetic factors. Studies conducted on the genetic associations of Hashimoto's thyroiditis have shown that the human leukocyte antigen (HLA) region, which plays a major role in other autoimmune disorders, is associated with

development of Hashimoto's thyroiditis (Fisher *et al*., 2000). The association of Hashimoto's thyroiditis with various other autoimmune diseases has further reinforced the probable involvement of genetic factors in the etiology. The major histocompatability complex(MHC), cytotoxic T-lymphocyte association (CTLA-4) and the human leukocyte antigen(HLA) are the genetic factors which are purported to play a major role in the pathogenesis.The selection of thyroid cells in the thymus and presentation of antigens in the periphery are modulated by, the human MHC analog, HLA. The sensitivity and specificity of the affinity to bind the peptides and recognize T-cells is determined largely by the genetic polymorphisms exhibited by the MHC molecule. The possibl polymorphisms within the MHC molecules play a pivotal role in the predisposition to autoimmune disease (Gebe *et al*., 2002).

The association between the genetics of Hashimoto's thyroiditis and HLA gene loci has been investigated by serotyping the HLA, and deoxyribonucleic acid (DNA) typing the sequence- specific oligonucleotides. Different subsets of HLA genes have been found to show varying degree of associations with Hashimoto's thyroiditis in different races. The HLA class 1 and class 2 genes both showed association with Hashimoto's thyroiditis in Asian populations, while only HLA class 1 demonstrated the association in Caucasians (Hunt *et al*., 2001). An association between CTLA-4 and Hashimoto's thyroiditis has been noted in significant number of cases (Einarsdottir *et al*., 2003). CTLA-4 plays a vital role in upholding immunological self tolerance in the body and its down regulation is believed to be the initiating step for the pathogenesis of Hashimoto's thyroiditis as well as other autoimmune disorders such as

Graves' disease (Chistiakov & Turakulov, 2003) .In addition to the genetic factors numerous external factors also play a vital role in the etiology of the disease, preferentially affecting genetically predisposed individuals. (Arvin *et al*.,2012).

2.1.4.2. Environmental Triggers

- **Iodine Intake**

Excessive iodine intake is well-established environmental factor for triggering thyroid autoimmunity. Several large population-based studies demonstrated higher prevalence of TAbs in the areas with higher iodine supply since the estimated prevalence was approximately 13% in iodine deficiency (Zaletel *et al*., 2011), 18% in circumstances of sufficient iodine intake (Hollowell *et al*., 2002***)*** and about 25% in areas with excessive iodine intake (Kasagi *et al*.,2009*)*. Moreover, up to four-fold increase in prevalence of TAbs was demonstrated after the exposure to higher iodine intake due to the improvement of iodine prophylaxis in previously iodine deficient areas (Heydarian *et al*.,2007*;*and Fountoulakis *et al*.,2007). Valuable evidence was also provided by using experimental animal models of autoimmune thyroiditis, where the prevalence and severity of thyroid autoimmunity significantly increased when the dietary iodine was added (Rose *et al.*, 2002) .Several putative mechanisms by which iodine may promote thyroid autoimmunity have been proposed. Firstly,iodine exposure leads to higher iodination of Tg and thus increases its immunogenicity by creating novel iodine containing epitopes or exposing cryptic epitopes. This may facilitate presentation by APC and enhance the binding affinity of the T-cell receptor which may lead to specific Tcell activation (Rose *et al.*, 2002). Secondly,

iodine exposure has been shown to increase the level of reactive oxygen species in the thyrocyte which is generated during TPO oxidation of excessive amounts of iodine. They enhance the expression of the intracellular adhesion molecule-1 (ICAM-1) on the thyroid follicular cells which could attract the immunocompetent cells into the thyroid gland (Burek *et al*.,2009).Thirdly, iodine toxicity to thyrocytes has been reported,since highly reactive oxygen species may bind to membrane lipids and proteins, causing thyrocyte damage and release of autoantigens (Fountoulakis *et al*.,2007). Fourthly, iodine excess has been shown to promote follicular cell apoptosis by inducing an abnormal expression of tumor necrosis factor-related apoptosis inducing ligand (TRAIL) and its death receptor (DR)-5 in thyroid (Yu *et al*.,2011). Fifthly, *in vitro* evidence also suggests an enhancing influence of iodine on the cells of the immune system, including augmented maturation of dendritic cells,increased number of T cells and stimulated B-cell immunoglobulin production (Fountoulakis *et al*.,2007).

- **Drugs**

Furthermore, certain drugs were reported to trigger or exacerbate thyroid autoimmunity in susceptible individuals.Interferon α (IFN-α) is extensively used to treat chronic hepatitis and is frequently associated with thyroid autoimmunity since TAbs were observed in up to 40% and clinical disease in 5-10% of patients treated with IFN α. Presumably, IFN-α has both thyroid toxic effects with consequent autoantigen presentation and immune effects,such as switching to Th1 immune response, suppression of Treg function, activation of immune cells, stimulation of cytokine release and

expression of MHC class I on thyroid cells (Tomer *et al*., 2010). Similarly, IL-2 treatment, used for melanoma and renal carcinoma, seems to act *via* immune and toxic mechanisms, leading to both TAb positivity and hypothyroidism (Barbesino *et al*., 2010). In patients with known autoimmune thyroid disease lithium may increase the risk of hypothyroidism. According to some studies, treatment with lithium has also been shown to increase TAb titres and the prevalence of thyroid autoimmunity, although this observation has not yet been confirmed by other reports (Barbesino *et al*., 2010; Fer *et al*.,2005). Among putative mechanisms direct toxicity of lithium on thyroid or toxicity of increased intrathyroidal iodine resulting from lithium treatment were discussed (Barbesino *et al*.,2010).Similarly, amiodarone alone as well as its high iodine content may act cytotoxically which may lead to thyroid autoantigen presentation and provoke thyroid autoimmunity (Martino *et al*.,2001).

- **Infections**

Not only the IFN-α treatment but also hepatitis C infection itself has been reportedly associated with thyroid autoimmunity and hypothyroidism. Among possible mechanisms, the molecular mimicry between viral and selfantigens has been suggested, whereas the release of proinflammatory mediators caused by viral infection may lead to activation of autoreactive T-cells (Tomer *et al*., 2010). Besides, in HT several other putative triggering viruses have been implicated such as parvovirus, rubella, herpes simplex virus,Epstein Barr virus, and human T-lymphotropic virus type 1 (Desailloud *et al*.,2009). A recent study of sera in pregnant women has also indicated an association between a prior infection with *Toxoplasma gondii*

and an increase of TPOAbs. Nevertheless, the evidences are scarce and further studies are required in order to confirm the role of infections as causative agents (Wasserman *et al.*,2009).

- **Chemicals**

The exposure to environmental toxicants such as polyaromatic hydrocarbons or polyhalogenated biphenyls, both commonly used in a variety of industrial applications, has been shown to provoke thyroid autoimmunity not only in experimental animals but also in humans (Burek *et al*.,2009) . Recently, a significantly higher prevalence of HT and TAb (9.3% and 17.6%, respectively) has been demonstrated in residents living in the area of petrochemical complex of Sao Paolo compared to the control area (3.9% and 10.3%, respectively) (Freitas *et al.*,2010). In Slovakia, the exposure to polychlorinated biphenyls was associated with TAb and hypothyroidism (Langer *et al.*,2007). Although there is strong evidence attesting the contribution of chemicals to thyroid autoimmunity, the exact mechanisms of their action are yet to be established.

2.2. Laboratory tests

Elevated anti-TPO or anti-Tg antibody titers are the most specific laboratory findings to establish the diagnosis of autoimmune thyroid disease (AITD) or HT, typically making biopsy unnecessary. The 24-hours thyroid radioactive iodine-123 or-131 (^{123}I or ^{131}I) uptake (RIU) is also helpful to distinguish Hashitoxicosis from Graves' disease (GD); the RIU is low in patients with Hashitoxicosis, whereas it is elevated in those with GD. ^{123}I is

preferred than ^{131}I because it has a shorter half-life (13 hours for ^{123}I, 8 days for ^{131}I) allowing quicker dissipation of background radiation. Since radioactive iodine is secreted in breast milk, and 123 I has a short half-life, it is recommended for diagnostic thyroid studies in nursing mothers. Breast milk must be pumped and discarded for 2 days after the intake of 123 I either used for thyroid uptake or for thyroid scanning. Scintigraphy reveals in-homogeneous activity throughout the gland in 50% and a pattern suggestive of either hot or cold nodules or a combination of both in 30% of patients. Twenty percent of patient with HT have normal findings at the scintigraphic thyroid imaging (Tao Yang and Xiaoyun Liu,2014).

2.3. Pathology

The thyroid gland is pale and rubbery to firm. The characteristic histopathologic changes of HT include diffuse lymphoplasmacytic infiltration, lymphoid follicle formation with germinal centers, a varying degree of fibrosis, parenchymal atrophy and the presence of large follicular cells with abundant granular eosinophilic cytoplasm, so-called Hürthle, oxyphilic or Askanazy cells (Figure 1.1&1.2) (kamizu *et al*.,2012). Foreign body giant cells and granulomas are not features of HT. Electromicroscopy shows deposits of immunoglobulin (Ig) G and Tg along the basement membranes of follicular cells. Both B and T cells infiltrate the thyroid. Most infiltrating T cells have alpha/beta T cell receptors.Increased expression of Th1-related cytokines such as IFNg, interleukin-2 and CD25 reportedly occurs in intrathyroidal T cells from patients with HT. The impairment of hormone synthesis is due to apoptotic destruction of thyroid cells (Hiromatsu *et al*.,2013). Increased expression of Fas on thyroid follicular

cells by the surrounding cytokines and Fas ligands on T cells may induce this apoptosis, although other cell death pathways, such as complement-fixing cytotoxicity and thyroglobulin specific T cell-mediated cytotoxicity, may also be involved (Zaletel *et al*., 2011; and Hiromatsu *et al*.,2013).

Figure 2.1: Histopathologic features of struma lymphomatosa as depicted in the original paper. a: Lymphoid follicle; b: Degenerated thyroid follicle; c: Giant cells; d: Hyperplastic interstitium with prominent round cell infiltration (Hiromatsu *et al*.,2013).

Figure 2.2 : Hashimoto thyroiditis. The thyroid parenchyma contains a dense lymphocytic infiltrate with germinal centers. Residual thyroid follicles lined by deeply eosinophilic Hürthle cells also are seen (Kumar *et al*.,2013).

2.4. Clinical Features

Hashimoto thyroiditis comes to clinical attention as painless enlargement of the thyroid, usually associated with some degree of hypothyroidism, in a middle-aged woman. The enlargement of the gland usually is symmetric and diffuse, but in some cases it may be sufficiently localized to raise suspicion for neoplasm. In the usual clinical course, hypothyroidism develops gradually. In some cases, however, it may be preceded by transient thyrotoxicosis caused by disruption of thyroid

follicles, with secondary release of thyroid hormones (hashitoxicosis). During this phase, free T4 and T3 concentrations are elevated, TSH is diminished, and radioactive iodine uptake is decreased. As hypothyroidism supervenes, T4 and T3 levels progressively fall, accompanied by a compensatory increase in TSH. Patients with Hashimoto thyroiditis often have other autoimmune diseases and are at increased risk for the development of B cell non-Hodgkin lymphomas , which typically arise within the thyroid gland. The relationship between Hashimoto disease and thyroid epithelial cancers remains controversial, with some morphologic and molecular studies suggesting a predisposition to papillary carcinomas (Kumar *et al*.,2013).

2.5.Pathogenesis

It is well known that HT results from a multistep process, requiring several genetic and environmental abnormalities to converge before disease development. Thus, thyroid follicle damage may be provoked by self-antigen presentation by antigen presenting cells and specific T lymphocyte activation. On the other hand, toxic destruction of thyroid cells possibly through the generation of oxygen radicals may participate in eclosion of autoimmunity (Noura *et al*.,2012). Both proliferation and apoptosis are involved in the pathogenesis of HT. Analysis of the mechanisms by which such autoimmune pathology arises has been facilitated by the use of animal models. These include the Obese Strain (OS) chicken and the BioBreeding (BB) and Buffalo rats as spontaneous models of HT. HT can also be

experimentally induced by specific immunization protocols with target auto antigens or elevation of dietary iodine.

2.5.1.Autoimmunity in HT

Hashimoto's thyroiditis is considered to be a Th1-mediated disease leading to aberrant infiltration of lymphoid cells and destruction of thyroid follicles (figure 3). The final outcome is fibrosis replacing normal thyroid parenchyma and hypothyroidism resulting of thyroid cell destruction (Parish *et al*., 2004). Indeed, a central phase of HT is characterized by an apparent uncontrolled production of auto reactive CD4+ T cells, CD8+ cytotoxic T cells and immunoglobulin G auto antibodies. This immunological synapse is defined by the interface between antigen presenting cells and T-cells that is formed during T-cell activation (Chistiakov, 2005). On the other hand, existence of naturally existing CD4+ CD25+ foxp3+ T regulatory cells influencing thyroiditis development in naïve susceptible mice was recently demonstrated. Moreover, it has been shown that naturally T regulatory cells are required for induction of antigen specific tolerance, indicating that induced Murine experimental autoimmune thyroiditis tolerance is a result of activation of naturally existing T regulatory cells rather than de novo generation of induced T regulatory cells (Morris *et al*., 2009).

Interestingly, several of the AITDs susceptibility genes participate in the immunological synapse, suggesting that abnormalities in antigen presentation are important mechanisms leading to AITDs (Tomer, 2010). Initially, the production of self-reactive cells and auto antibodies occurs in

the draining lymph nodes. Later, the lymphoid tissue often develops directly in the thyroid gland itself. This tissue is generally very well-organized, with cords of anti-Tg-antibody- producing plasma cells in the periphery (Chistiakov, 2005). In a final, destructive step of HT, the auto reactive T cells diffusely accumulate in large numbers and infiltrate thyroid parenchyma. This phenomenon will determine clinical phenotype of the disease. In the BB-DP rat model, Th1-mediated mechanisms involving production of IL-12, tumor necrosis factor-α (TNF-α) and interferon- γ play a major role in the destruction of thyrocytes (Sharma *et al*.,2009; Wang *et al*.,2002).Furthermore, it has been recently shown that pro-IL18 is constitutively expressed in thyroid cells and IL18 up regulation by INF-γ is an immunological feature of HT patients with an important role in promoting the local immune response (Liu *et al*., 2010).

2.5.2. Apoptosis in HT

Apoptosis appears to play a major role in the final stage of the disease (figure1.3 & 1.4). In fact, apoptotic molecules such as Fas and Fas ligand (FasL) expression was higher in rats with lympholytic thyroiditis indicating a possible role in thyrocyte death (Noura *et al*.,2012).Theses molecules are expressed at low level by normal thyroid cells compared to patients with HT with an increasing number of apoptotic cells (Kaczmarek *et al*., 2011). The mechanism and regulation of apoptosis in thyroid gland are still little known. The most studied receptor mediated apoptic pathway is the Fas/Fas ligand system. Fas is substantially expressed on lymphocytes. Fas-Fas ligand interaction could lead to the thyrocyte cell death (Kaczmarek *et al*., 2011). Thyroid cells express constitutively Fas but these latters are normally

unaffected by Fas-mediated apoptosis. In contrast, they can be sensitised to Fas-induced destruction under certain pathologic conditions such as the release of IFN- γ, TNF-α and IL-1β, by infiltrating immune cells (Giordano *et al*., 2001). Over the past few years, many reports have shown that mobilisation of the Fas/Fas ligand apoptotic pathway by proinflamatory cytokines plays a pivotal role in the devastation of thyroid follicular cells in HT leading to hypothyroidism.(Kaczmarek *et al*.,2011). Therefore, the Fas pathway is the most important mechanism of T lymphocyte mediated apoptosis. It is just possible that this process plays an essential role in the pathogenesis of Hashimoto thyroiditis, because cytotoxic T lymphocytes are fully present in the thyroid in places where apoptosis is located (Bretz.,2002 ; Chen *et al* ., 2004;and Fountoulakis *et al*.,2008;).Mechanisms of regulation of this pathway include probably changes in Fas expression level,and the expression of molecules that promote survival, including the Bcl-2 gene family (Noura *et al*.,2012).

This latter antiapoptotic protein and sFas system,which normally protect thyroid cells from apoptosis, are decreased in the thyroid cells of patients with HT, creating a proapoptotic phenotype (Fountoulakis *et al*., 2004). Thus, the rate of thyrocyte apoptosis dictates the clinical outcome of thyroid autoimmunity. Though rare in normal thyroid, it markedly increases during HT, but not in GD with a divergent phenotype. Therefore, regulation of thyrocyte survival is a crucial pathogenic determinant via the balance between Th2 and Th1 response (Chistiakov, 2005).

At the onset of disease, (HLA) class II-positive Antigen-presenting cells (APC), present thyroid-specific autoantigens to the naïve T cells, leading to the maturation of autoreactive T cells. Interaction with auto antigen leads to the production of different cytokines inducing T-helper type 1 (Th1)-mediated cell immune response. The stimulation of the Fas/Fas ligand apoptotic pathway by pro-inflammatory cytokines is the most important mechanism of T lymphocyte mediated apoptosis. The caspase cascade ultimately induces enzymes that progressively destroy the cell, leading to thyroid cell death and hypothyroidism (Noura *et al*.,2012).

Figure 2.3 : Autoimmune events in Hashimoto's thyroiditis (Noura *et al*.,2012).

Figure 2.4 :Pathogenesis of Hashimoto thyroiditis. Breakdown of immune tolerance to thyroid autoantigens results in progressive autoimmune destruction of thyrocytes by infiltrating cytotoxic T cells, locally released cytokines, or antibody-dependent cytotoxicity (Kumar *et al*.,2013).

2.6.Progression of Autoimmune Process

Once the tolerance to thyroid antigens is broken, endothelial cells of regional postcapillary venulae are activated, allowing the extravasation of blood leucocytes attracted by chemokines. The recruitment and arrest of lymphocytes in the thyroid is only partially understood. Immune cells adhere to the endothelium, then migrate across it, then through the interstitium and

move toward thyroid follicular cells, sometimes organizing into germinal centers,resembling lymph node germinal centers. In autoimmune thyroid diseases there is an enhanced expression of adhesion molecules (selectins

and integrins) on lymphocytes and endothelial cells (lymphocyte function associated antigen 1-LFA1, interstitial cell adhesion molecule 1-ICAM 1, very late antigen 4-VLA4) as well as selectin ligands.CXC chemokines are secreted by thyrocytes stimulated by TNFα, IFNγ and IL12; CXCL10,CXCL9 are responsible for recruitment of activated lymphocytes in the thyroid. CXCL10 promotes differentiation of Th0 cells into Th1 cells, while CCL2- the differentiation of Th0 cells into Th2 cells. CXCL10 binding to CXCR3A generates an angiostatic effect, while binding to CXCR3B exerts chemotactic and immune effects. CXCR3 is expressed on immune cells (T cells, especially Th1,B cells, NK cells), vascular pericytes, microvascular endothelial cells. Immune cells expressing CXCR3 are attracted in the inflamed thyroid.Th1 cells secrete IFNγ, which stimulates production of chemokines by thyroid follicular cells, thus maintaining and expanding the autoimmune process (Rotondi *et al*.,2007).

Predominance of Th1 a immune response promotes thyrocytes apoptosis (Corona *et al*.,2008) mediated by Fas and TRAIL (TNF – related apoptosis inducing ligand),leading to Hashimoto thyroiditis. Predominance of a Th2 immune response induces antigen-specific B cells to produce antithyroid antibodies; stimulatory antiTSH receptor antibodies are responsible for Graves' disease and blocking antiTSH receptor antibodies are responsible for atrophic thyroiditis. Thus, the clinical expression of thyroid autoimmunity depends on the Th1-Th2 balance (Tsatsoulis *et al*,2006).In Hashimoto thyroiditis hypothyroidism is not only the result of

thyrocytes destruction, but also of the thyroid function impairment induced by Th1 cytokines. IL-1 and IFNγ down regulate the expression of TG, TPO, Na/I symporter and H2O2 generating enzymes (DUOX), effects partially

mediated by nitric oxide and antagonized by increased TSH concentrations. IL-4, a Th2 cytokine, blocks Th1 induced alterations in DUOX, TPO and Tg secretion. Th3 cytokines-TGFβ and IL-10-repress thyrocytes function but their effects can also be overturned by Th2 cytokines (Mclachlan *et al*.,2007).

2.7.Thyroid Dysfunction & The Immune System

Thyroid hormones are not essential for the development of the immune system but are involved in the maintenance of immune homeostasis.Hypothyroidism decreases thymic activity,causes spleen and lymph nodes involution and thus,represses both cell mediated and humoral immune responses. In hyperthyroidism both stimulatory and suppressing effects on the immune system have been described.It was demonstrated that thyroid hormones modulate lymphocytic activity through protein kinase C signaling pathway (Klecha *et al*.,2008).

2.8.Role of T Lymphocytes

Excessively stimulated T cells CD4+ are known to play the main role in the pathogenesis of HT (Figure 1.5). T cells perform two functions in the pathogenesis of HT. T helper type 2 Th2 cells lead to an excessive stimulation and production of B cells and plasmatic cells which produce antibodies against thyroid antigens leading to thyroiditis (Pyzik *et al*.,2015).

T helper type 1 (Th1) and Th2 cells produce interferon- (IFN-) gamma, and interleukin- (IL-) 4, respectively. Nanba *et al*. reported thatm IFN-gamma and IL-4 gene polymorphisms, which are related to higher IFN-gamma and lower IL-4 production, respectively, are more frequent in patients with

severe HT than in those with mild HT (Nanba *et al* ., 2012). Th1 cells activate cytotoxic lymphocytes and macrophages, which directly affect thyroid tissue by destroying thyroid follicular cells. In the tissues of the thyroid in patients with HT Th1 are the predominant cells. Histopathological studies have shown that more T cells have been observed in HT both in the parenchyma and in the lymphatic infiltrations. In HT, damaged thyroid follicles with apoptotic thyrocytes (pyknotic nuclei, condensed cytoplasm with enlarged mitochondria and endoplasmic reticulum cisterns) were visible in this area. A number of CD4+ T cells in the thyroid infiltrates in HT were significantly decreased in the interstitium. Observations under a light microscope revealed that T suppressor/cytotoxic cells were accumulated at the sites of destruction of thyroid follicles. These sites were surrounded by connective tissue fibers and fibroblasts (Ben-Skowronek *et al*,.2013).

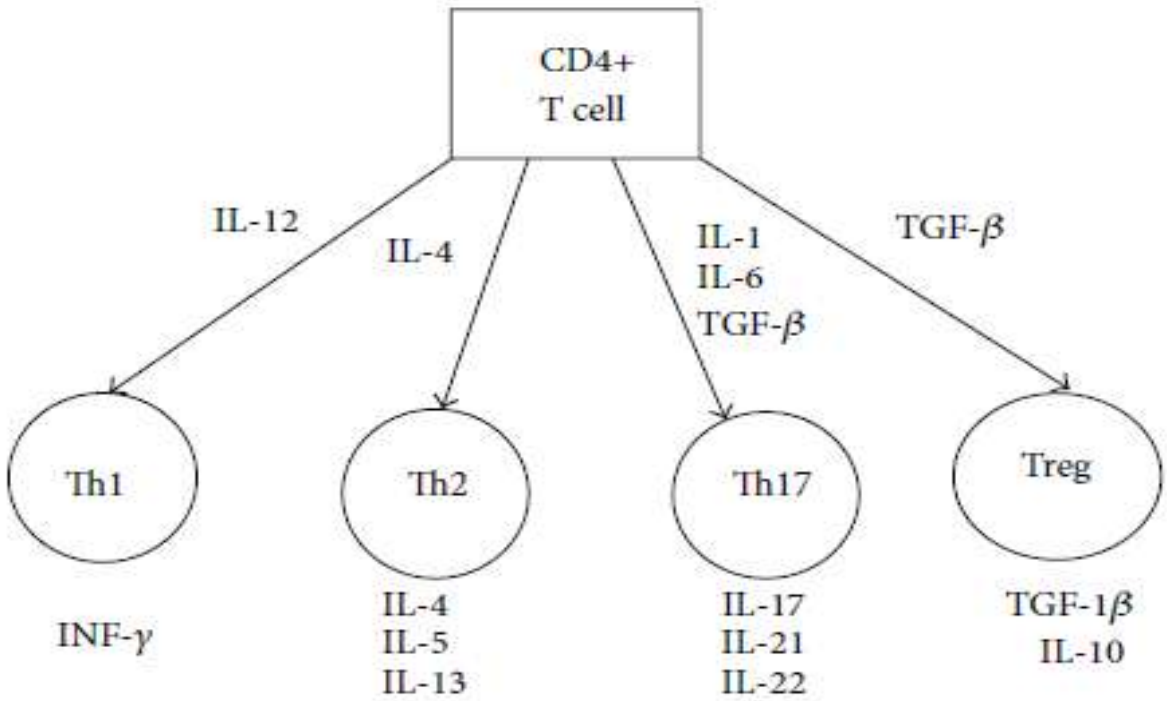

Figure 2.5: T CD4+ cell differentiation (Pyzik *et al*.,2015).

2.8.1.Role of IL-4

Interleukin 4 is an important member of the class I cytokine family with four a helix structures that is secreted predominantly by activated Th2 cells (Leung *et al*.,2004; Lee *et al*.,2008). It induces Th2-mediated immune responses by upregulating the expression of MHC class II, CD23, and IL-4R on B cells (Risma *et al*.,2002; Lee *et al*.,2008). It has an anti-inflammatory effect owing to its efficient inhibition of the production of proinflammatory cytokines,such as TNF-a, IL-1a, IL-1b, IL-6, and IL-8. It also plays a crucial role in mediating allergic responses. In this process, IL-4 stimulates the proliferation of mast cells with IL-13 and promotes Ig class switching to IgE that binds to high-affinity Fcε receptors on the surfaces of mast cells,

basophils, and eosinophils, leading to the degranulation and production of inflammatory mediators when it is cross-linked by an Ag (Kips *et al*., 2001;and Solymar *et al*.,2002; Hoffjan *et al*.,2002; Cortes *et al*.,2007; and Li *et al*.,2007). IL-4 exerts its biological functions by interacting with IL-4R (Kips *et al*., 2001; Ozaki *et al*.,2002). The latter is a heterodimer composed of a specific a-chain (IL-4Ra or IL-4BP) and a common γ-chain (γc). (Zhu *et al*.,2012) . As a class I cytokine receptor, IL-4Ra has specific and high affinities (Kd = 20–300 pM) in the recognition and binding to IL-4 (Lee *et al*.,2008). It could be detected on a number of hematopoietic or nonhematopoietic cell surfaces, including endothelial, epithelial,muscle, fibroblast, hepatocyte, and brain cells (Shore ,2004).

CD4+ cells, which belong to subpopulation Th2 stimulate immunological response of B lymphocytes. There are some studies evaluating cytokines in these diseases, demonstrating the production of IL-4 and TNF- by infiltrating T cells and macrophages. However, the specific role of these molecules in the pathogenesis of autoimmune thyroid diseases (AITD) is still debated (Artur Bossowski *et al*.,2011). They presume that an altered balance of pro- and anti-inflammatory cytokines may play an important role in the pathogenesis of autoimmune thyroiditis.T-helper 1 (Th1) cell-mediated inflammatory responses predominate in the early pathogenesis of GD,whereas Th2 cell-mediated immunity may play a role in later stages. Th1 cells produce IFN-gamma and Th2 cells produce IL-4. Nanba T *et al*. reported that IFNgamma and IL-4 gene polymorphisms, which are related to higher IFN-gamma and lower IL-4

production,respectively, are more frequent in patients with severe HT than in those mild HT. They investigated the proportion of peripheral Th1 and Th2 cells in patients with AITD and concluded that the peripheral Th1/Th2 cell ratio is related to the severity of HT and is related to the intractability of GD. They hypothesize that these patterns of peripheral Th cell subsets may be expressed within the thyroid (Nanba T *et al*,.2012).

2.8.2.Th17 cell and it's role in the pathogenesis of Hashimoto's thyroiditis

Th17 lymphocytes serve as a pathogenic factor in the development of various diseases. These could be autoimmune diseases like psoriasis, multiple sclerosis, rheumatoid arthritis or inflammatory bowel disease or neoplasms, allergies, engraftment, or transplant rejection. However, their

role in AITD is still debatable. Th17 cells account for approximately 1% of CD4+ lymphocytes in blood serum and take part in the immune response against intercellular antigens. They are characterized by expression markers such as CCR6 (CD196), IL-23R, IL-12R-beta2, CD49, and CD161 and produce proinflammatory cytokines mainly: IL-17A, IL-17F, IL-21, IL-9, IL-22, and TNFA. They develop from T helper cells under the influence of various factors of differentiation, growth, and stabilization such as TGFB plus IL-6, IL-21, and IL-23 and transcription factors like STAT3, RORγ, and RORα (Miossec and Kolls .,2012; Zaragoza *et al*.,2014) . Liu *et al*. and Qin *et al*. found that HT patients had a significantly increased serum concentration of IL-6 and IL-23 in comparison with healthy controls (Qin *et al*.,2012; Liu *et al*.,2014),whereas Kimura and Kishimoto showed that IL-6 induces Th17 differentiation together with

TGFβ (Kimura and Kishimoto., 2010). Bossowski *et al*. demonstrated an elevated level of Th17 cells in children with untreated Hashimoto's disease, which suggests the participation of these cells in the induction and development of the disease. However, they did not demonstrate such a relation in Graves' disease (Bossowski *et al*.,2012). Similarly, in the research by Li *et al*. a significantly higher concentration of IL-17 was visible in HT compared to in the thyroid cancer, in the nodular goiter, or in the studied group (Li *et al*., 2013).

Further research by Li *et al*. suggested a negative relationship between the level of IL-17 and the stage of hypothyroidism among patients with Hashimoto's disease. Histopathological examinations have shown a strong relationship between the concentration of IL-17 and the stromal fibrosis in the gland, which points to the fact that the presence of IL-17

increases local inflammation and leads to the fibrosis and atrophy of thyrocytes. Additionally, it was concluded that the impact of sodium iodide concentrations on the development of Th17 and Th1 lymphocytes, which can serve as inhibitors of regulatory T cells, might be various, whereas the research by (Shi *et al*.,2010) demonstrated that mRNAs of IL17 and transcription factor were significantly increased in PBMC (peripheral blood mononuclear cell) from patients with HT. Wang et al. tried to answer the question of why the number of Th17 rises in HT. The authors observed an elevated concentration of proinflammatory leptin in blood of the patients and concluded that this cytokine could induce the proliferation of T lymphocytes and promote immune response in the direction of Th17 (Wang *et al*.,2013).

The proportion of peripheral Th17 cells in patients with AITD was higher than in control subjects and it was dependent on disease activity and severity (Figueroa-Vega *et al*.,2010; Kim *et al*.,2012; and Nanba *et al*.,2012). However, (Zheng *et al*.,2013) reported increased expression of IL-17 mRNA also in euthyroid patients with Graves' disease and it was upregulated after stimulation with IL-23. (Bossowski *et al*.,2012) examined a group of children with AITD and they found increased percentage of CD4+IL-17+ cells in children with untreated Hashimoto's disease, but not in children with Graves' disease.

Additionally, percentage of CD4+IL-17+ cells positively correlated with titers of anti-thyroid peroxidase immunoglobulins. *Shi et* al. suggested that Th17 cells can play a central role in pathogenesis of Hashimoto's disease rather than Th1 cells. These conclusions were based on confirmed higher expression of IL-17 mRNA than IFN-γ mRNA in peripheral blood

cells.Unfortunately, authors did not include any data about duration of disease what in combination with age of patients ranged 23–60 leading to conclusions that patients were rather not in active phase of lymphocytic thyroiditis.This could explain low activity of Th1 response and relative predominance of IL-17 producing cells (Shi *et al*.,2010).

Results

3.1. Demographic characteristic of the study samples

Mean age of patients group was not significantly different from that of control group, 37.30 ±10.47 years versus 35.02 ±8.78 years ($P \leq 0.290$). Table 3-1 and 3-2 showed the distribution of patients and control subjects according to 10 years age intervals.

Table 3-1: Mean age and age range in patients and control groups

Group	**No.**	**Mean age ±SD (years)**	**Age Range (years)**	***P*-value**
Control	49	35.02 ±8.78	21 -51	0.290
HT	46	37.30 ±10.47	16 -57	

Table 3-2: Distribution of patients and control subjects according to age interval

Age interval	**Control n (%)**	**HT n (%)**
<20 y	0 (0.0)	2 (4.3)
20-29 y	15 (30.6)	10 (21.7)
30-39 y	17 (34.7)	10 (21.7)
40-49 y	14 (28.6)	18 (39.1)
≥ 50 y	3 (6.1)	6 (13.0)
Total	49 (100.0)	46 (100.0)

Majority of patients with Hashimoto's thyroiditis were female, and there was no significant difference in distribution of patients according to sex in both groups ($P \geq 1.000$), table 3-3.

Table 3-3: Distribution of patients and control subjects according to sex

Sex	Control group No. (%)	HT patient group No. (%)	χ^2	*P*-value
Male	3 (6.1)	2 (4.3)	0.000	≥ 1.000
Female	46 (93.9)	44 (95.7)		
Total	49 (100.0)	46 (100.0)		

Hashimoto's thyroiditis is a common form of chronic AITDs. The disorder affects from 2% up to 10% (Canaries *et al*., 2000) of the general population. It is more common in older women and ten times more frequent in women than in men (Tunbridge *et al*., 2000).

As indicated by numerous epidemiological studies, females present with positive thyroid autoantibodies (TAbs) up to three times more often than males. The largest NHANES III study has shown that females were positive for TPOAbs and TgAbs in 17% and 15.2%, respectively,while males only in 8.7% and 7.6%, respectively. According to the estimation provided by the study of Danish twins, the genetic contribution to TPOAb and TgAb susceptibility in females was 72% and 75%, respectively, while in males it was only 61% and 39%, respectively. The possible explanation for high female predominance in thyroid autoimmunity might be associated with the X chromosome containing a number of sex and immune-related genes which are of key importance in the preservation of immune tolerance. Increased immunoreactivity might therefore be related to genetic defects of the X

chromosome, such as structural abnormalities or monosomy (Tao and Xiaoyun, 2014).

Accordingly, a higher incidence of thyroid autoimmunity was reported in patients with a higher rate of X chromosome monosomy in peripheral white blood cells or in patients with Turner's syndrome. Another potential mechanism of impaired immunotolerance in females is skewed X-chromosome inactivation (XCI) leading to the escape of X-linked self-antigens from presentation in thymus with subsequent loss of T-cell tolerance. Skewed XCI was associated with a higher risk of developing autoimmune thyroid diseases.

Recently reported frequencies of skewed XCI in HT were 31%, 34.3%, 25.6% and 20%, which is significantly higher than in healthy controls, where the prevalences were only 8%, 8%, 8.6% and 11.2%. Furthermore, a study of Danish twins demonstrated a significant association of skewed XCI with TPOAb serum concentrations in dizygotic but not in monozygotic twin pairs, indicating that shared genetic determinants of XCI pattern and TPOAb production are more likely than causal relationship (Tao and Xiaoyun, 2014).

3.2. Comparison of mean serum hormone levels between patients and control groups

Mean serum of T3 was significantly higher in patients group ($P \leq 0.003$) when compared with control group,while mean serum T4 level shows no significant difference between the two groups ($P \geq 0.200$). In addition,mean serum of TSH was significantly higher in patients group than in control group ($P \leq 0.030$), table 3-4.

Table 3-4: Comparison of mean T3, T4 and TSH between patients and control groups by the Vidas technique, where the normal value for T3 was 0.9-2.3 (nmol/L), T4 between 60-120 (nmol/L), and for TSH was 0.25-5.0 (nuIUol/L).

Hormone	Control (n = 49)		HT (n = 46)		P-value
	Mean ±SD	Range	Mean ±SD	Range	
T3	1.71 ±0.31	1.22 -2.20	1.99 ±0.58	0.93 -4.00	0.003
T4	88.10 ±28.86	6.00 -150.30	84.27 ±26.88	9.40 -132.00	0.200
TSH	2.08 ±1.56	0.05 -5.30	4.62 ±7.93	0.05 -40.40	0.030

3.3. The mean of cytokine expression in both thyroiditis & control group

Mean of cytokine expression in patients with Hashimoto's thyroiditis was significantly higher than that of control group for both IL-17 and IL-4 (P<0.001, <0.001, respectively), table 3-5 and 3-6. Figure 3-1 A and 3-2 B, show the Immunohistochemichal staining for slide tissue samplesof both patients and controls.

A

Figure 3-1:A. Immunohistochemichal cytoplasmic staining of IL17 in Hashimoto's thyroiditis section stained by Diaminobenzidine (DAB) (brown stain) counterstained with Myer's hematoxyline (blue stain).
(A) IL17 cytoplasmic expression in Hashimoto's thyroiditis section.

(B) IL17 cytoplasmic expression in multinodular goiter section, magnification power (400X).

Figure 3-2:B. Immunohistochemichal cytoplasmic staining of IL4 in Hashimoto's thyroiditis section stained by Diaminobenzidine (DAB) (brown stain) counterstained with Myer's hematoxyline (blue stain).

(A) IL4 cytoplasmic expression in Hashimoto's thyroiditis section.

(B) IL4 cytoplasmic expression in multinodular goiter section, magnification power (400X).

Table 3-5: Comparison of mean IL-17 and IL-4 expression between patients and control groups

Cytokine %	Control (n = 49)		HT (n =46)		*P*-value
	Mean ±SD	Range	Mean ±SD	Range	
IL17 %	49.01 ±14.76	31.50 -86.25	61.25 ±15.53	33.50 -95.00	<0.001
IL4 %	44.89 ±16.91	27.00 -97.75	65.03 ±17.08	45.00 -92.00	<0.001

These changes in the pro- and anti-inflammatory cytokines is thought to play an important role in the pathogenesis of autoimmune thyroid diseases.Because disruption of thyroid self tolerance generates abnormal thyroid-immune interactions, implicating an array of cytokines and their receptors. Thyrocytes achieve antigen presenting cell properties, which stimulate effectors immune cells: Th1 and Th2, in the context of defective immunomodulatory T regulatory cells,resulting in thyroid lymphocytic infiltration and activation of B cells, with production of antibodies against thyroid antigens, thyroid destruction or stimulation,depending on the Th1-Th2 balance (Lichiardopol C and Moţa M,2009).

Secretion of cytokines within the thyroid accounts for the accumulation and expansion of the intrathyroidal lymphocyte pool. Also, the thyroid cells themselves contribute to this secretion. The thyroid cells also produce a number of proinflammatory molecules which will tend to exacerbate the autoimmune process, eg. IFN- g and IL-4 (Weetman AP,2004).

Multiple research has demonstrated a prominent role of Th17 (CD4+IL-17+) or Treg lymphocytes (CD4+CD25+highFoxP3+) in the induction of autoimmune disorders (Korn *et al*.,2009).(Zha *et al*.,2014) observed a significant infiltration of lymphatic cells in the thyroid specimens, and they could not find lymphatic invasion in all the normal thyroid tissues. Zha *et al* .revealed that thyroid tissue in Hashimoto's disease was mainly infiltrated with B cellsCD20+. Immunohistochemical analysis showed ameaningful infiltration of lymphatic cells in the thyroid specimens from all HT patients, while no clear infiltration of lymphatic cells in the normal thyroid specimens was found . Astudy conducted by I. Be´n-Skowronek *et al*.,2007) found a significant difference in the profile analysis of lymphocyte subsets in Hashimoto's disease and the control group.

3.4. Variation in the expression level of IL17 & IL4 according to thyroid status

When patients classified according to T4 serum level into euthyroid and hypothyroid subgroups, the following results were obtained: mean IL-17 expression was significantly higher in euthyroid subgroup than control group (P<0.001), the same was applied to IL-4 (P<0.001); Moreover,Mean IL-17 shows no significant difference between hypothyroid patients and control group (P=0.892), and it was the same with respect to IL-4 ($P \geq 0.255$); in addition mean IL-17 expression was significantly higher in euthyoid patients than

hypothyroid patients (P=0.039) and it was similar in case of IL-4% (P<0.01), table 3-6 and figure 3-3.

Table 3-6: Comparison of mean IL-17 and IL-4 expression among patients according to thyroid status

Cytokine %	Control (n = 49)	Euthyroid (n = 40)	Hypothyroid (n = 6)	*P*1	*P*2	*P*3
IL17% (Mean±SD)	49.01±14.76	62.93±15.14	50.08 ±14.54	<0.001	0.892	0.039
IL4 % (Mean ±SD)	44.89±16.91	67.89±16.51	46.00 ±1.18	<0.001	0.255	<0.001

***P*1= Euthyroid vs Control, *P*2= Hypothyroid vs Control,**
***P*3= Euthyroid vs Hypothyroid.**

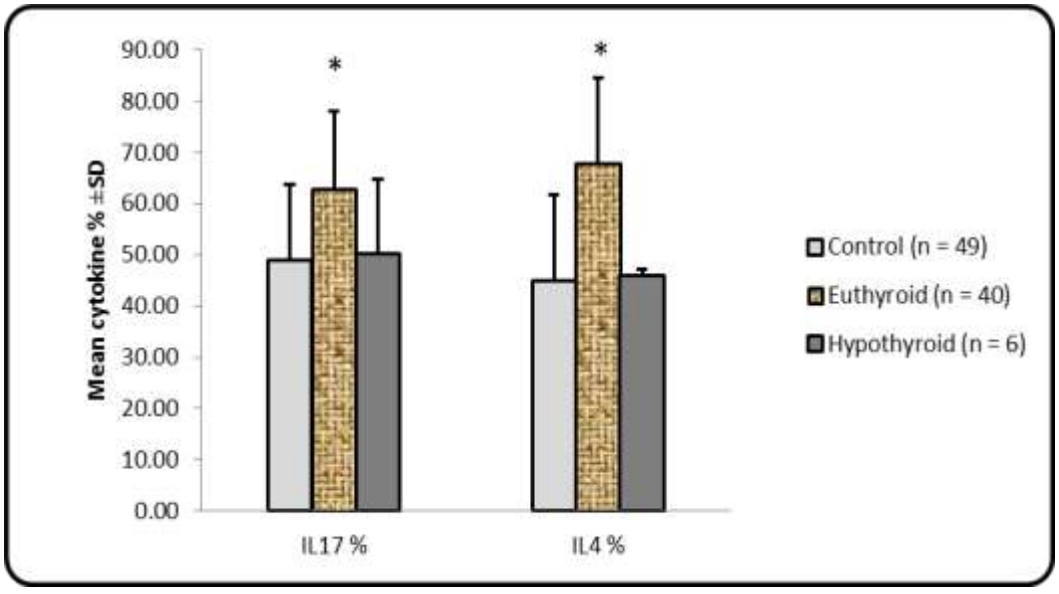

Figure 3-3: Comparison of mean IL-17 and IL-4 expression among patients according to thyroid status

In the present study, the IL17 and IL4 cytokines status is altered in a way that euthyroid HT is associated with higher IL-17 and IL-4 levels compared to hypothyroid HT and non-thyroiditis controls. Cytokines play a pivotal role in the pathogenesis of HT, although with different effects in relation to the phase of disease (Caturegli *et al*.,2000).

The results of this study agree with a study done by Ceyla Konca Degertekin *et al*. They observed that IL-17 and IL-23 levels were higher in euthyroid HT patients compared to controls. And mentioned that the hypothyroid group had lower levels of cytokines compared to euthyroid patients (Degertekin *et al*.,2016).

Hypothyroidism is known to be associated with depressed humoral and cell-mediated immunity (Klecha A.J. *et al*.,2000) and this data may explain hypothyroidism itself had a depressive effect on Th17 cytokine responses in the present study. Because the cytokine levels of the hypothyroid group in this study were lower comparable to euthyroid controls. These finding suggest that IL17 might have different functions in different stages of HT.

(Dapeng *et al*.,2013) showed that there was progressive decline in serum IL-17 levels with respect to the degree of hypothyroidism. Also There were a strong association between IL-17 expression and stromal fibrosis, rather than lymphocytic infiltration and eosinophilic changes of thyroid epithelial cells, suggesting that the proinflammatory effect of IL-17 drives the development of thyroid tissue toward HT specific fibrosis which distinguishes this disease from other benign thyroid diseases . (Dapeng *et al*.,2013) suggest that the Th17 response might have a pathological role in the earlier stages of HT leading to local inflammation and local stromal fibrosis.Thus, Th1 response, which has more cytotoxic effects than Th17 response might play a more dominant role in aggressive HT or in the later phase of HT. Similar shifts in dominant autoimmune response profile have been reported for other

diseases. For example, in a mouse model of experimental autoimmune uveoretinitis,Th17 response was more pronounced in early stages of disease whereas Th1 cells were more abundant at a later stage (Amadi-Obi *et al*.,2007). This shift throughout the course of the disease is attributed to the substantial plasticity of T-cell subtypes in their subtype differentiation (Bending *et al*.,2009) and potential instability of Th17 phenotype, which converts to Th1 cells (Nistala *et al*.,2010). Thus, a shift in the microenvironment and cytokine profiles due to several interfering factors might alter the dominant T-cell type in the thyroid gland and might affect the type and titer of the cytokine that we measure during the study.

(Wang *et al*.,2013) tried to answer the question of why the number of Th17 rises in HT. The authors observed an elevated concentration of proinflammatory leptin in blood of the patients and concluded that this cytokine could induce the proliferation of T lymphocytes and promote immune response in the direction of Th17.

In another study,the proportion of peripheral Th17 cells in patients with AITD was higher than in control subjects and it was dependent on disease activity and severity (Figueroa-Vega *et al*.,2010; Kim *et al*.,2012; and Nanba *et al*.,2009). However, (Zheng *et al*.,2013) reported increased expression of IL-17 mRNA also in euthyroid patients with Graves' disease and it was upregulated after stimulation with IL-23. (Bossowski *et al*.,2012) examined a group of children with AITD and they found increased percentage of CD4+IL-17+ cells in children with untreated Hashimoto's disease, but not in children with Graves' disease. Additionally, percentage of CD4+IL-17+ cells positively correlated with titers of anti-thyroid peroxidase immunoglobulins. (Shi *et al*.,2010) suggested that Th17 cells can play a central role in pathogenesis of Hashimoto's disease rather than Th1 cells. These conclusions

were based on confirmed higher expression of IL-17 mRNA than IFN-c mRNA in peripheral blood cells.

Regarding the IL4 expression level and it's correlation with disease progression, unfortunately there is no such previous study which demonstrate the role of such cytokines in the pathogenesis Hashimoto's thyroiditis,thus.The present study demonstrated a prominent role for IL4 in disease progression in association with IL17 and further studies are needed to clarify at which stage IL4 have more powerful effects.

However, another studies indirectly mentioned the role of Th1 versus Th2 in Hashimoto's thyroiditis. In a study done by Gherardo Mazziotti *et al*.They performed single-cell analysis of the intracellular cytokine expression in peripheral CD4+ and CD8+ lymphocytes from patients with Hashimoto's thyroiditis (HT) to investigate the type-1 T-cell response. However, the HT patients showed a higher number of CD4+IFN-g+, CD4+IL-4+ and CD8+IFN-g+ cells than the control subjects. (Gherardo Mazziotti *et al*., 2003) found that the euthyroid patients showed more expression of IL-4 in CD4+ lymphocytes than the control subjects (Which agree with this stydy). Moreover, the expression of IL-4 in CD4+ cells from hypothyroid patients was significantly lower than that seen in the euthyroid cases and comparable to that found in the control subjects. In addition they demonstrated that patients with HT show a response that involves both the peripheral CD4+ and CD8+ lymphocytes. Moreover, They observed that the cytokine expression in these lymphocyte sub-populations was also different in relation to the occurrence of thyroid dysfunction (Gherardo Mazziotti *et al*., 2003).

Th2 cells stimulate immunological response of B lymphocytes. However, the specific role of these molecules in the pathogenesis of autoimmune thyroid diseases (AITD) is still debated (Bossowski *et*

al.,2011). They presume that an altered balance of pro- and anti-inflammatory cytokines may play an important role in the pathogenesis of autoimmune thyroiditis.T-helper 1 (Th1) cell-mediated inflammatory responses predominate in the early pathogenesis of GD, whereas Th2 cell-mediated immunity may play a role in later stages. Th1 cells produce IFN-gamma and Th2 cells produce IL-4. Nanba T *et al*. reported that IFNgamma and IL-4 gene polymorphisms, which are related to higher IFN-gamma and lower IL-4 production, respectively, are more frequent in patients with severe HT than in those mild HT. They concluded that the peripheral Th1/Th2 cell ratio is related to the severity of HT and is related to the intractability of GD (Nanba *et al*.,2012).

3.5.The Correlations of IL17 & IL4 with thyroid hormones level

Correlations between cytokine IL-17 and IL-4 % and serum hormone levels (T3,T4 and TSH) were shown in table 3-7. Generally speaking, there was no significant correlation except TSH and IL17.

Table 3-7: Correlation between cytokines expression and serum hormone levels

Hormone	Correlation	IL17	IL4
T3	R	0.032	0.219
	P	0.830	0.143
T4	R	0.159	0.263
	P	0.291	0.078
TSH	R	0.290	0.201
	P	0.050	0.181

In the previous table there was asignificant positive correlation between TSH level and and IL17 ($P \leq 0.050$), This results disagree with a study done by (Ceyla Konca Degertekin *et al*.,2016),Since they where propose that hypothyroidism itself had adepressive effect on Th17 cytokine responses. The negative correlations of IL-17 with TSH levels in their study might support this inhibitory effect. These controversy suggest that Th17 system might have different functions in different stages of HT.

In addition,this study did not show any significant correlation between IL17 and both of T3 & T4. Most of the previous studies that focus on the role of IL17 in the pathogenesis of Hashimoto's thyroiditis,No one exclude the role of IL17 in the disease progression.

The possible explanation for the contrasted results between this study and a study done by (Ceyla Konca Degertekin *et al*.,2016) regarding that hypothyroidism in their study had adepressive effect on Th17 cytokine responses, may be due to the genetic polymorphisms. Increased susceptibility to Hashimoto thyroiditis is associated with polymorphisms in multiple immune regulation–associated genes, the most significant of which is the linkage to cytotoxic T lymphocyte–associated antigen-4 gene (CTLA4), which codes for a negative regulator of T cell function.

The possible explanation for the source of IL4 is the follicular helper T cells or TFH, largely termed on the basis of their localization in B cell follicles, where these antigen-experienced CD4+ T cells found in the periphery within B cell follicles of secondary lymphoid organs such as lymph nodes, spleens and Peyer's patches, which plays a crucial role in B cell response induction (Nicolas Fazilleau *et al*,.2009). TFH cells express CXCR5, and thus migrate toward CXCL13, which is made in follicular centers of secondary lymphoid organs. Upon cellular interaction and cross-signaling with their related follicular (Fo B) B cells, TFH cells trigger the formation and maintenance of germinal centers through the expression of CD40 ligand (CD40L) and the secretion of IL-21 and IL-4. TFH cells also migrate into these seeded germinal centers, mainly composed of rapidly dividing and mutating B cells. Within germinal centers, TFH cells play a critical role in mediating the selection and survival of B cells that go on to differentiate either into special plasma cells capable of producing high affinity antibodies against foreign antigen, and memory B cells capable of quick immune re-activation for the future if ever the same antigen is re-encountered (Zaretsky *et al*.,2009).

Unfortunately, there wsa no previous study mentioned the role of IL4 in the pathogenesis of Hashimoto’s thyroiditis.The absent of significant correlation in this study may be due to the small number of samples.Thus, another study based on larger sample size is needed to solidify present study.

While Nanba *et al*. reported that IFN gamma and IL-4 gene polymorphisms, which are related to highrer IFN gamma and lower IL-4 production, respectively, are more frequent in patients with severe HT than in those mild HT. They hypothesize that these patterns of peripheral Th cell subsets may be expressed within the thyroid (Nanba *et al*.,2012).

In conclusion, the prominent role for IL17 in pathogenesis of disease severity of Hashimoto's thyroiditis especially through it's correlation with TSH level and the degree of hypothyroidisim which reflect it's indirect role in the degenerative and fibrotic change in thyroid tissue.

Recommendations

- The possibility of specific targeting of IL-17 may be a good project for future research in order to validate its role in treatment.
- Larger multicentric study is recommended to validate results obtained in the present concerning the equivocal role of IL-4 in the pathogenesis of Hashimotos thyroiditis and hypothyroidism in general.
- The study of the role of gene polymorphisim in association with hypothyroid state in relation to cytokines (IL-17 and IL-4).
- Study the combined role of other cytokines, not included in the present study, like IL-12 and TNF-alpha in relation to hypothyroidism.

Conclusions

- An established role for IL-17 in relation to hypothyroid clinical state and pathologic fibrosis has been clarified in the present study. IL-4 has proved to have minor or at least no role in the pathogenesis of fibrosis and hypothyroid state in patients with hashimotos thyroiditis.
- This study demonstrated that upregulation of both IL17 and IL4 cytokines have asignificant role in the disease progression,When compared to control group.
- When patient group classified according to thyriod function test, both IL17 & IL4 cytokines shows ahigher expression in euthyroid group than hypothyroid group,These results may reflect the important role of these anti-inflammatory cytokine in the pathogenesis of Hashimoto's thyroiditis.
- When the expression level of IL17 & IL4 has been analyzed in association with hormonal level (T3,T4,and TSH), only IL17 demonstrates significant positive correlation with TSH.