SEROLOGICAL AND MOLECULAR EVALUATION OF MRP14 IN THYROIDITIS AND ITS ROLE IN PRO-INFLAMMATORY CHEMOKINES ACTIVATION

Aya M. Hussein **Rasha Al Sahlanee**
Researcher **Lecturer**
Dept. of Biotechnology, Coll. of Sci. University of Baghdad, Iraq.
ayamohamed1996327@gmail.com **rasha.abdullah@sc.uobaghdad.edu.iq**

ABSTRACT

This study was aimed to investigate the involvement of Myeloid Related Protein 14 (MRP14) in the production of CXC Motif Chemokine Ligand 9 (CXCL9) in thyroiditis patients. The first group composed of 27 hypothyroidism (HO) patients (12 men and 15 women), age average about 46 years old. The second group had 43 patients (8 men and 35 women) with hyperthyroidism (HR), their mean of age was 42, while 30 healthy persons act as control group (HC) had an average age of 33.47 of men (13) and women (17). The three groups were testing for triiodothyronine (T3), thyroxine (T4) and thyroid stimulating hormone (TSH) furthermore, testing the thyroglobulin (TG), anti-thyroperoxidase (a-TPO), anti-TSH receptor (TSHR) and serum level of MRP14 by Enzyme-Linked Immunosorbent Assay (ELISA). The results showed a high significant (P< 0.0001) increase in MRP14 levels in hypothyroid group *vs* hyperthyroidism 123.1 ± 22.39 and 109.8 ± 4.479 ng/ml respectively. MRP14 showed a positive correlation with T4 and a negative correlation with anti-TSHR in HR patients. The gene expression of MRP14 and CXCL9 were assayed to all groups by RT-PCR, results showed a high expression of MRP14 as well as of CXCL9 in the patient groups which showed a high expression of CXCL9 in HO patients. Therefore, the results obtained from the study indicate that the higher concentration of MRP14 expression in Thyroiditis patients might play an important role in the development of the disease mediated by stimulating the secretion of pro-inflammatory chemokines.

Key words: S100 A9, autoimmune thyroiditis, hypothyroidism, hyperthyroidism, CXCL9.

التحريات المصلية والجزيئية للبروتين المرتبط بالنخاع في مرض التهاب الغدة الدرقية ودوره في تنشيط الكيموكينات الالتهابية

اية محمد حسين **رشا السهلاني**
باحث **مدرس**
قسم التقنيات الاحيائية –كلية العلوم – جامعة بغداد – العراق

المستخلص

هدفت الدراسة الى التحري عن مستوى بروتين MRP14 في مرضى التهاب الغدة الدرقية. المجموعة الاولى تكونت من 27 مريضًا بقصور الغدة الدرقية لرجال (12) ونساء (15) متوسط اعمارهم 45 والمجموعة الثانية 43 مريضًا بفرط نشاط الغدة الدرقية لرجال (8) ونساء (35) متوسط اعمارهم 42. اما مجموعة السيطرة (30 شخصًا سليمًا) لمتوسط اعمار 33.47 من الرجال (13) والنساء (17). اختبرت المجموعات الثلاث لقياس ثلاثي يودوثيرونين (T3)، هرمون الغدة الدرقية (T4)، وهرمون (TSH) وقياس هرمون الثايروغلوبيولين (TG) واختبار الأجسام المضادة لإنزيم بيروكسيداز الدرقي (a−TPO) وفحص الأجسام المضادة لمستقبلات الهرمون المحفّز للغدة الدرقية (TSHR) وMRP14بواسطة ELISA. أظهرت النتائج زيادة معنوية عالية (P <0.0001) في مستويات MRP14في مرضى قصور الغدة قياسا بذوي فرط نشاطها 123.1±22.39 و109.8±4.479 نانوغرام/مل على التوالي. أظهر MRP14ارتباطًا إيجابيًا مع T4 وعلاقة سلبية مع مضادات TSHR في مرضى فرط نشاط الغدة. أظهرت نتائج التعبير الجيني بواسطة تقنية تفاعل البوليمراز المتسلسل للنسخ العكسي ارتفاعا في التعبير عن MRP14 وكذلك في CXCL9 في مجموعات المرضى والتي أظهرت ارتفاعًا كبيرًا في التعبير عن CXCL9 في مجموعة مرضى القصور (HO) .لذلك، تشير النتائج المستحصلة من الدراسة إلى أن التركيز العالي في التعبير عن MRP14 في مرضى التهاب الغدة الدرقية قد يلعب دورًا مهمًا في تطور المرض بوساطة تحفيز إفراز الكيماويات المؤيدة للالتهابات.

الكلمات المفتاحية: التهاب الغدة الدرقية المناعي الذاتي، كيموكين CXCL9، قصور الغدة الدرقية، فرط نشاط الغدة الدرقية.

Received:22/4/2021, Accepted:27/7/2021

INTRODUCTION

Thyroiditis refers to a set of disorders characterized by inflammation, fibrosis, or lymphocytic infiltration. The best classified of thyroiditis diseases is according to the beginning of signs or symptoms, period of cellular infiltration or inflammation and thyroid pathology persistence (11). Autoimmune thyroiditis (AT), also referred to as chronic lymphocytic and Hashimoto's thyroiditis (HT), is a condition that is characterized by the generation of autoantibodies against the thyroid-specific antigens thyroperoxidase, thyroglobulin as well as inflammatory cell infiltration of the thyroid gland (33). Thyroid hormones affect practically every nucleated cell in the body and are necessary for normal growth and energy metabolism (2, 17). Thyroid dysfunction is prevalent, easily detectable, and treatable, but it can have serious implications if left misdiagnosed or untreated (15). Hypothyroidism is one of the thyroid diseases that can develop from thyroid gland damage or disorder, in which the thyroid gland fails to generate T3 and T4, resulting in a high level of TSH due to a negative feedback process. (12), while the thyroid hormone production and/or secretion are excessively high in hyperthyroidism (34). The formation of autoantibodies against particular thyroid antigens result from inflammatory cells infiltrate the thyroid gland, these conditions effects on fibrous replacement of thyroid follicular cells this will lead to hypothyroidism (13). Studies have shown that thyroid autoantibodies in the blood are linked to the intensity of histopathological thyroiditis and the presence of lymphocytic infiltration (9, 29). local cells in lesions of inflammatory release the chemokines CXCL9, CXCL10, and CXCL11, which can attract Th-1 cells; thus, CXCR3 and its ligand play the major role in inflammatory cell function (19). Attracted Th-1 lymphocytes, enhance production of tumor necrosis factor (TNF)-α and IFN-γ which activate multiple cells to release CXCL9 in the inflamed area, resulting in an establishment of feedback mechanism (5, 38). Various investigations have revealed an elevation in serum CXCL9 and/or tissue expressions in distinct organs related to autoimmune disorders. This has been discovered in a number of autoimmune disorders, including autoimmune thyroiditis (6, 7, 8). The EF-hand domain of all (MRP) proteins has the same structural features that provide them biological activity (41). These proteins have a crucial role in cell proliferation and differentiation, in addition to apoptosis and inflammatory cytokine production (22). S100A8 and S100A9 have been shown in preliminary research to be pro-inflammatory mediators that cause acute inflammation (18). S100A9 can contribute to inflammation by increasing the production of chemokines, This, in turn, promotes and facilitates immune cell recruitment and migration to the site of inflammation (16). As well MRP14 (myeloid-related protein 14) plays a major role function in inflammatory processes (30). This study aimed to confirm the participation role of MRP14 in thyroiditis pathogenesis and secretion of CXCL9 an inflammatory chemokine in molecular detection.

MATERIALS AND METHODS

Patients and Control: Prior to the study, the local ethics commission gave their approval. The research was initiated when obtained the agreement of all patients. Seventy patients with thyroid disorders were included in this study where they separated into two sets, the first group represented in 27 of hypothyroid patients distributed as (15) females and (12) males; and the second group contained (43) hyperthyroid patients distributed as (35) females (8) males, furthermore the third group consists of (30) healthy individuals who serve as the control group, with (17) females and (13) males. The blood samples were collected from December 2020 and April 2021, the study was conducted at AL-Amal Hospital and Teaching Laboratories in Baghdad's Medical City. Several demographic data of patient and control were recorded such as age, gender, and other data.

Blood collection

Five ml of samples were taken from each patients and control and 2ml of the collected fresh blood put in TRIzol^{TM} Reagent containing tube for RT-qPCR analysis. The other 3 ml of blood were centrifuged 5 min at 1107g for sera collection. All samples were kept at -20 $°C$ for further analysis.

Serum MRP14 detection: Serum MRP14 level was estimated used an ELISA detection kit (Human S100 Calcium Binding Protein A9 ELISA Kit (E1571Hu) Bioassay technology laboratory -china) according to the manufacturer's recommendations A microplate reader was used to measure optical density in 96 wells while measuring absorbance at 450 nm.

Thyroid function tests: As indicators of thyroid functions, hormones of T3, FT3, T4, FT4 and TSH levels were estimated using MiniVidas kits from Biomerieux (France). Other immunological tests for the investigation of Autoantibodies: Thyroglobulin (Anti-Tg AccuBind ELISA Kit (1025-300) Monobind Inc USA) and Thyroid Peroxidase (AESKULISA a-TPO ELISA Kit (3401)- AESKU Germany) and estimation of Human Thyroid Stimulating Hormone Receptor antibody (TSHR-Ab) (ELISA (SL2169Hu) Sunlong biotech china)

Isolation of RNA and quantitative real-time polymerase chain reaction: Two ml of fresh blood were taken for RNA extraction to each group of patients used TRIzol. Quantifying total RNA by Qubit 4. cDNA was synthesized used total RNA. RT-PCR was achieved by SyberGreen fluorescence power, used the expression of β-actin gene as a control.Polymerase chain reaction were done for primer sequences of h*CXCL9* as F-CCACCGAGATCCTTATCGAA, R-CTAACCGACTTGGCTGCTTC; and primer sequence for h*MRP14* gene were: F-CACCAGCTCTTTGAATTCCCC, R-CCTCGGCTTTGACAGAGTG; *β-actin*: F-ATGCTTCTAGACGGACTGCG, R-GTTTCAGGAGGCTGGCATGA. Reactions were carried out in the total volume of 20µl and gene expression was quantified using Luna Universal qPCR MasterMix (M3003S) NEB -UK.) according to the guidelines provided by the manufacturer. Reactions started with the 60sec hot activation of the syber polymerase at 95°C, followed by 40-45 amplification cycles in three steps (denaturation at 95°C for 15sec, annealing at 60 °C for 30sec, and extension at 72 °C for 30sec). The ΔC_T values obtained by substructing the C_T control (actin) from the C_T target gene, measured in the same RNA preparation, were used to calculate relative levels of mRNA expression. The comparative C_T method ($\Delta\Delta C_T$ Method) was then used, were the mean ΔC_T values for pateints groups were compared to the mean ΔC_T values for healthy control group ($\Delta\Delta C_T = \Delta C_T$ pateints - ΔC_T control). The final result for each analysed gene was expressed, as previously described (36), as the stimulation factor (The amount of target (normalized to an endogenous reference and relative to a calibrator) is calculated using the formula of $2^{-\Delta\Delta CT}$ (1).

Statistical analysis: Graph Pad prism Software 6 was used for all statistical studies. Data are shown as mean±standard deviation (SD). One-way analysis (ANOVA) of variance was used to examine the differences between the three groups. The pearson correlation analysis approach was used to assess the correlations between datasets. Statistical significance was defined as a P value of less than 0.05.

RESULTS AND DISCUSSION

Investigated serum level of thyroid functions: A total of 100 subjects (43 HR, 27 HO, and 30 healthy control) were inducted in this study. Table 1 lists clinical and demographic characteristics. In terms of gender or age, there were non-significant differences between the three groups. TSH level was significantly higher in hypothyroid than in hyperthyroid patients (46.48± 5.79 vs 0.03± 0.01 IU/ml). Otherwise, non-statistically significant differences ($P > 0.01$) were identified in T3 levels between hypothyroid patients and the control group (1.29± 0.22 vs 1.64± 0.09 nmol/l). The T3 level was highly significant ($P<0.01$) in hyperthyroidism patients than in hypothyroid patients (2.54±0.4 vs 1.29 ± 0.22 nmol/l) when compared to the control group (2.54±0.4 vs 1.64 ± 0.09 nmol/l). High Significant differences of T4 level were found between hyperthyroid and hypothyroid patients ($P < 0.01$). The mean was (90.23±15.55 vs 0.43± 0.09 nmol/l), while there were no significant differences between hyperthyroid patients and the control group (90.23±15.55 vs 86.12±3.06 nmol/l).

Table 1. The demographic and clinical parameters of patients with Hyperthyroiditis and Hypothyroiditis and healthy controls

Demographic and clinical parameters	HR	HO	HC	P
NO.	43	27	30	-
Gender (M/F)	8/35	12/15	13/17	-
Age (years)	42±2.727	45±3.691	33.47±2.065	-
TSH (µIU/ml)	0.03±0.01	46.48±5.79	2.66±0.25	0.0001
T3 (n mol/l)	2.54±0.4	1.29±0.22	1.64±0.09	0.001
T4 (n mol/l)	90.23±15.55	0.43±0.09	86.12±3.06	0.0001
TG (IU/ml)	364.2± 46.96	257.5 ± 28.91	173.0 ± 9.870	<0.0001
TPO (IU/ml)	-26.79 ± 95.55	137.3 ± 147.6	-637.1 ±26.74	< 0.0001
TSH-R (pg/ml)	1486 ± 615.3	657.0 ±124.9	961.1 ± 155.7	0.45

According to the distribution, data are expressed as mean±standard deviation
F, female; M, male. HR, Hyperthyroiditis; HO, Hypothyroiditis; HC,healthy controls. P <0.05 was regarded as statistically significant; (-), referred to non-performed experements.

According to these results, the most common thyroid diseases are hypothyroidism, which could be overt or subclinical and characterized by high levels of TSH with decreased production of T3 and T4 hormone, and hyperthyroidism, which can be lowered levels of TSH with overt or subclinical and is characterized by excess T3 and T4 hormones production (42). Anti-TG antibody results revealed significant differences (P<0.0001) between the patients and control groups. Anti-TG levels are significantly higher in hyperthyroid patients (364.2± 46.96 vs 173.0 ±9.870IU/ml) when compared to the control group, as well as hypothyroid patients (257.5 ± 28.91 vs 173.0 ± 9.870IU/ml). Therefore, the level of anti-TG in hyperthyroid patients is much greater than in hypothyroid patients (364.2 ± 46.96 vs 257.5 ± 28.91 IU/ml). The results agreed with the study of (20), which demonstrated the level of anti-TG antibodies in hyperthyroidism patients was highly significant (P < 0.01) than in a control group, and also in the hypothyroidism patients group. Thyroid autoimmunity may play the role in the clinical symptoms of HT, as seen by elevated TgAb level in Hashimoto's Thyroiditis patients (10). In terms of anti-TPO levels, there have been significant differences (P< 0.0001) between patient groups and controls. The current study found elevated levels of anti-TPO in hypothyroid patients when compared to controls (137.3 ± 147.6 vs -637.1 ± 26.74 IU/ml), as well as increased levels of anti-TPO in hyperthyroid patients when compared to controls (-26.79 ± 95.55 vs -637.1 ± 26.74 IU/ml. However, no significant differences in anti-TPO levels were observed between hypothyroid and hyperthyroid patients (137.3 ± 147.6 vs -26.79 ± 95.55IU/ml). This result was agreed with (23). They were proved through a study conducted in Duhok province that demonstrated the high significant difference (p< 0.0001) when a mean anti-TPO level of patients and controls were compared. Anti TSH-R antibodies were shown to have no significant differences between hypothyroid and hyperthyroid patients (1486 ± 615.3 vs 657.0 ±124.9 pg/ml). Anti TSH-R Abs. were found to be higher in the hyperthyroid group (1486 ± 615.3 vs 961.1± 155.7 pg/ml), while there was no significant difference between the two types than in the control group (1486 ± 615.3, 657.0 ±124.9 vs 961.1 ± 155.7 pg/ml). TSH-R-Abs can either mimic or prevent TSH's action, or they can be functionally inactive. TSH-R-Abs are particular biomarkers for Graves' disease (GD), and they are responsible for several of the disease's clinical signs (25). The current results are corroborated by a study by (21) who discovered that Graves' patients had a higher level of TSH-R than Hashimoto's patients. Antibodies against the TSH receptor (TSHR-Ab) are closely involved in the pathophysiology of autoimmune thyroid disorders (AITD) (26).

Serum level of myloid related Protein 14 (MRP14: The sera results revealed the high significant increase (P< 0.0001) in the MRP14 levels in patients in compared to the control group (113.5 ±6.957 vs 71.39 ± 4.513 ng/ml). Figure (1-A) shown the level of MRP14 was high in hypothyroid comparison with the control group (123.1 ±22.39 vs 71.39 ± 4.513 ng/ml) and hyperthyroid (109.8 ±4.479 vs 71.39 ± 4.513 ng/ml). Therefore, hypothyroid patients have a higher level of MRP14 protein.

This study agreed with (28), which proved that patients with autoimmune thyroid disease had a high significant of S100A8/S100A9. MRP14 plays a key role in neutrophil-induced inflammation by releasing secondary granules and MRP14 activates neutrophils. This means MRP14 proteins are found not only in the neutrophil cytosol but also in secondary and tertiary granules (37). Also, found a higher level of calprotectin in Graves' orbitopathy disease than in control subjects, demonstrating calprotectin's potential as a GO biomarker (27). The level of calprotectin was compared between two stages of subacute thyroiditis when it found higher level during the acute inflammatory phase than in recovery phase (14).

Correlation between MRP14 and thyroid hormones: MRP14 and T4 levels manifest a significant positive correlation (r= 0.68) in the hyperthyroid group figure 1-B, this result was demonstrated that MRP8 and MRP14 are thought to be released from leukocytes during inflammatory events due to the tight relationship between serum levels and disease activity (35). S100A9 are pro-inflammatory endogenous toll-like receptor (TLR)4 ligands produced by activated monocytes and granulocytes. During both local and systemic inflammatory processes, S100 serum concentrations correlate with disease activity (24).

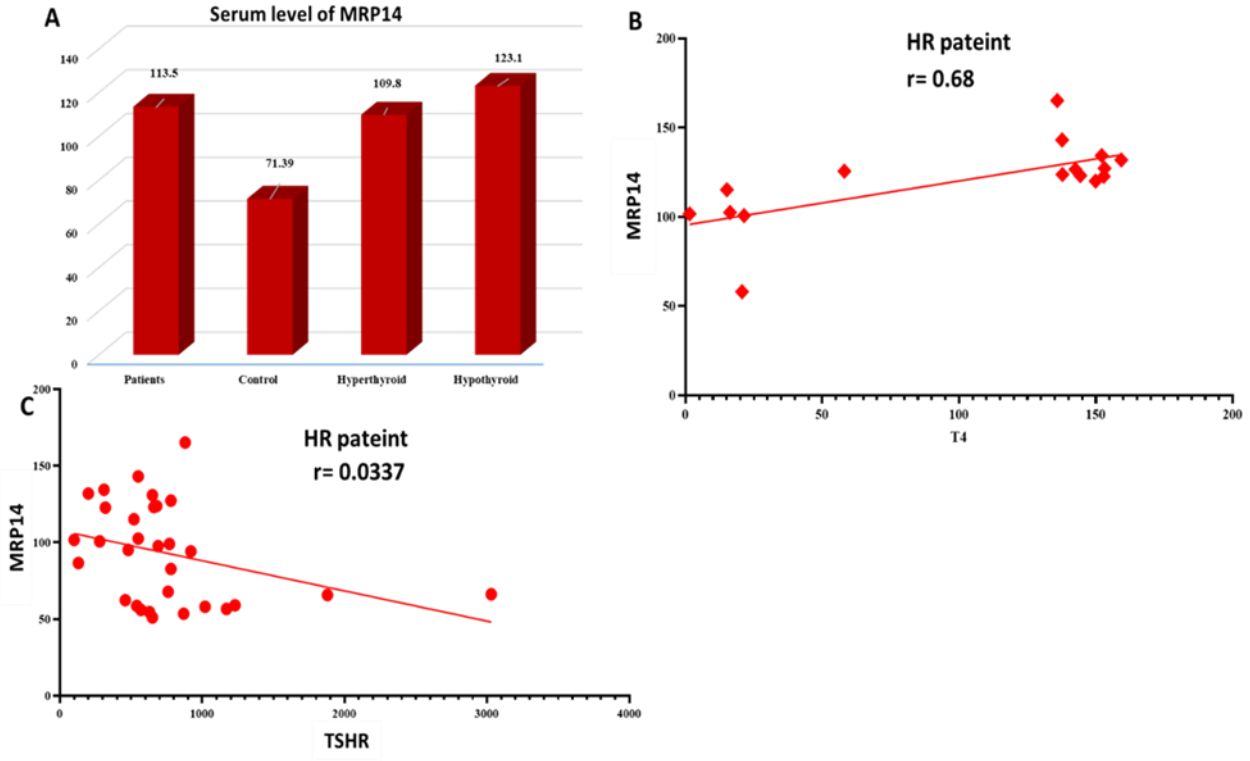

Figure 1. Serum level of MRP14 in thyroid patients.

(A) levels of MRP14 in thyroid patients (B) significant positive correlation between MRP14 and T4 levels in the hyperthyroid group, (C) negative correlation significant between the Anti-TSH-R level and the concentration of MRP14 protein in hyperthyroid group. As demonstrated in figure (1-C), there was the negative correlation significant between the Anti-TSH-R level and the concentration of MRP14 protein in hyperthyroid individuals (r = -0.337). The results by (32) show a positive correlation between serum MRP14 and TPO. On the other hand, they found no significant with TG antibody. TSH-R antibodies were known to be the best indicator for distinguishing patients with GD from those with AIT (40).

MRP14 gene expression and its effect on the expression of pro-inflammatory chemokine: With the aim to clarify if MRP14 participate in the development of thyroiditis pathogenesis or not, we first analyzed the expression profiles of MRP14 gene of blood samples. The mean Ct value of MRP14 amplification was (22.12) in hyperthyroid patient group and (23.60) in hypothyroid. While the mean Ct healthy group

was (22.25) as represented in figure (2-A). The outcomes revealed the ΔCt value (0.55) for hyperthyroid and (1.86) for hypothyroid patient compared to control ΔCt (1.2). The calculation of gene expression fold change was made using relative quantification (3). The mean of 2- ΔΔCt values of hyperthyroid was (1.5691-fold) while (0.6328-fold) in hypothyroid patient group. The fold expression in thyroid was higher ($p<0.0001$) compared to control group (Figure 2-A), furthermore it was found to be higher in hyperthyroid more than hypothyroid patient. In second time, we studied the expression profiles of a pro-inflammatory chemokine's gene CXCL9, the transcriptomic analysis showed in hypothyroidism and hyperthyroidism patients compared to group control. The mean Ct value of CXCL9 amplification was (22.60) in hyperthyroid patient group and (25.18) in hypothyroid. While the mean Ct healthy group was (23.13). The outcomes revealed the ΔCt value (1.03) for hyperthyroid and (3.44) for hypothyroid patient compared to control ΔCt (2.08). The mean of 2- ΔΔCt values of hyperthyroid was (2.0705-fold) while (0.3895-fold) in hypothyroid patient group. In figure (2-B), the fold expression was higher ($p<0.0001$) in thyroid patient vs control group, and was founded to be high in hyperthyroid than in hypothyroid patient.

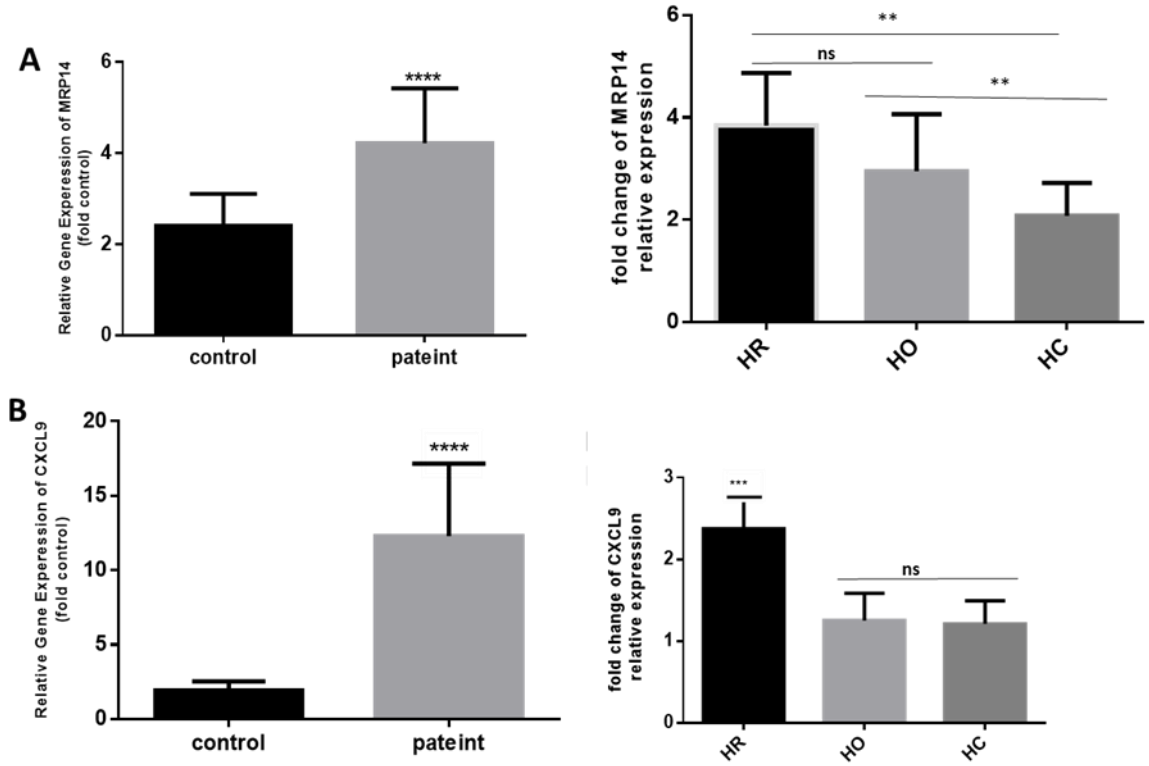

Figure 2. Relative levels of expression of MRP14 and CXCL9 in thyroiditis patients and healthy control to each group.

(A) level of fold expression in MRP14 gene (B) level of fold expression in CXCL9 gene. The qPCR analysis calculated using the B actin as normalised gene and expressed as 1/d CT. the Mann and Whitney test was used for statistical analysis.abbreviation: ctrl = healthy control , **** $p < 0.1\%$.

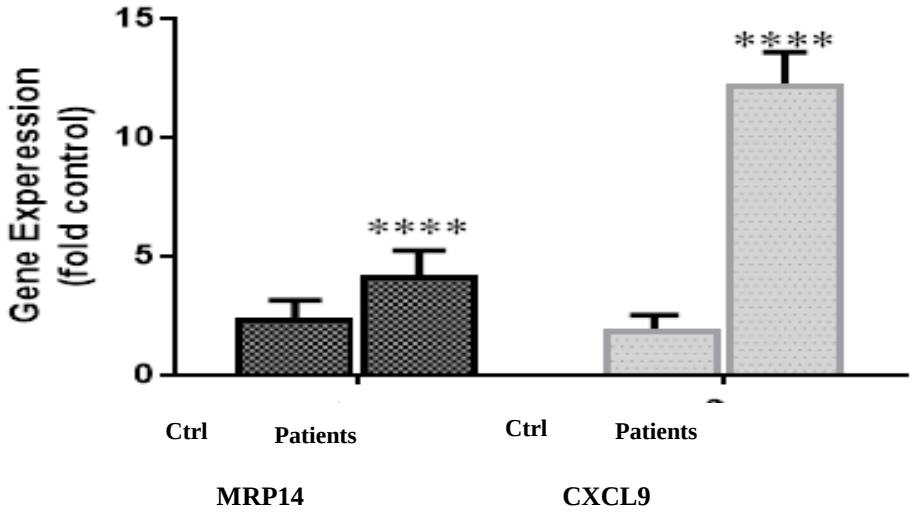

Figure 3. Relative levels of expression of MRP14 and CXCL9 in thyroiditis patients and healthy control to each group

The qPCR analysis calculated using the B actin as normalised gene and expressed as 1/d CT. the Mann and Whitney test was used for statistical analysis.abbreviation: ctrl = healthy control , **** p < 0.1%. Recent evidence suggests that cytokines and chemokines play a role in the pathophysiology of autoimmune thyroiditis. (43). The CXCL9 and other chemokines were shown to be overexpressed in autoimmune thyroid disease tissues when the IL-1 pathway was activated, according to a previous study (4). The study of (30) reported that the homodimer expression level of MRP14 was increased not only in the serum, as well as in the HT patients' thyroid tissues, but not a heterodimer of MRP-8/MRP-14, and an aberrant MRP-14 expression suggests which MRP-14, as an inflammatory biomarker's, plays a role in HT pathogenesis (39). A study by (30) provided evidence which IL-1β stimulates a synthesis of chemokine by up-regulating expression of MRP-14 in the MAPK / NF-κB pathway-dependent manner, suggesting that controlling the chemokines expression in TFCs could be the potential mechanism of HT pathogenesis. At regions of inflammation, the S100 proteins S100A8, S100A9, and S100A12 are released by activated phagocytes (31). According to study of (32), MRP-8 / MRP-14 and MRP-6 expression in thyroid blood and tissue of people with AITD is greater than in healthy people.

REFERENCES

1. Al-jumaily, A. K. T., and A. M, Turkie. 2018. Molecular investigation of gene expression of beta-lactamases enzymes gen for *Pseudomonas Aeruginosa bacter*. The Iraqi Journal of Agricultural Sciences, 49(5), 803

2. Al-Sa'ady, A. J. R., and M. H, Hilal. 2020. The effect of vitamin d in kidney function and thyroid gland. The Iraqi Journal of Agricultural Sciences, 51(3), 924-929

3. Alyasin, S.; Karimi, M.; Amin, R.; Babaei, M.; Darougar, S. 2013. Interleukin-17 Gene Expression and Serum Levels in Children with Severe Asthma, Iranian Journal of Immunology, 10 (3):177-185

4. Antonelli, A., S. M., Ferrari, A., Corrado, A., Di Domenicantonio, and P, Fallahi. 2015. Autoimmune thyroid disorders. Autoimmunity reviews, 14(2), 174-180

5. Antonelli, A., S. M, Ferrari, D, Giuggioli, E, Ferrannini., C, Ferri., and P, Fallahi. 2014. Chemokine (C–X–C motif) ligand (CXCL) 10 in autoimmune diseases. Autoimmunity reviews, 13(3), 272-280

6. Antonelli, A., S. M, Ferrari., S, Frascerra., F, Galetta., F, Franzoni., A, Corrado., ... and P, Fallahi. 2011. Circulating chemokine (CXC motif) ligand (CXCL) 9 is increased in aggressive chronic autoimmune thyroiditis, in association with CXCL10. Cytokine, 55(2), 288-293

7. Antonelli, A., S. M, Ferrari., S, Frascerra., A, Di Domenicantonio., A, Nicolini., P, Ferrari., ... and P, Fallahi. 2011. Increase of

circulating CXCL9 and CXCL11 associated with euthyroid or subclinically hypothyroid autoimmune thyroiditis. The Journal of Clinical Endocrinology and Metabolism, 96(6), 1859-1863

8. Antonelli, A., S. M, Ferrari., P, Fallahi., E, Ghiri., C, Crescioli., P, Romagnani., ... and E, Ferrannini. 2010. Interferon-alpha, -beta and-gamma induce CXCL9 and CXCL10 secretion by human thyrocytes: modulation by peroxisome proliferator-activated receptor-gamma agonists. Cytokine, 50(3), 260-267

9. Arai, T., C, Kurashima., M, Utsuyama., M, Sawabe., and H, Ito. 2000. Measurement of anti-thyroglobulin and anti-thyroid peroxidase antibodies using highly sensitive radioimmunoassay an effective method for detecting asymptomatic focal lymphocytic thyroiditis in the elderly. Endocrine journal, 47(5), 575-582

10. Barić, A., L, Brčić., S, Gračan., V, Škrabić., M, Brekalo., M, Šimunac., ... and V, Boraska Perica. 2019. Thyroglobulin antibodies are associated with symptom burden in patients with Hashimoto's thyroiditis: a cross-sectional study. Immunological investigations, 48(2), 198-209

11. Begum, A., M. S, Bari., K. F. M, Ayaz., R, Yasmin., N. C, Rajib., M. A, Rashid, and F. M, Siddiqui. 2006. Thyroiditis â A Review. Journal of Medicine, 7(2), 58-63

12. Bello, F. and A. G, Bakarai. 2012. Hypothyroidism in adults: A review and recent advances in management. Journal of Diabetes and Endocrinology, 3(5):57-69

13. Burek, C. L., and M. V, Talor. 2009. Environmental triggers of autoimmune thyroiditis. Journal of Autoimmunity, 33(3-4), 183-189

14. Cengiz, H., T, Demirci., C, Varim., and E, Gönüllü. 2021. The relationship between serum calprotectin levels and disease activity in patients with subacute thyroiditis. European Review for Medical and Pharmacological Sciences, 25(10), 3745-3751

15. Chaker, L., A. C, Bianco., J, Jonklaas and R. P, Peeters. 2017 Hypothyroidism. Lancet, doi:10.1016/s0140-6736(17)30703-1

16. Chernov, A. V., J, Dolkas., K, Hoang., M, Angert., G, Srikrishna., T, Vogl., S, Baranovskaya., A.Y, Strongin and V.I, Shubayev. 2015. The calciumbinding proteins S100a8 and S100a9 initiate the early inflammatory program in injured peripheral nerves. J. Biol. Chem. 290: 11771–11784

17. Dumont, J., R, Opitz., D, Christophe., G, Vassart., P. P, Roger., and C, Maenhaut. 2011. Ontogeny, anatomy, metabolism and physiology of the thyroid. Thyroid Dis. Manag, 1-102

18. El Gazzar, M. 2015. Immunobiology of S100A8 and S100A9 proteins and their role in acute inflammation and sepsis. Int J Immunol Immunother, 2(2).

19. Elia, G., and G, Guglielmi. 2018. CXCL9 chemokine in ulcerative colitis. La Clinica Terapeutica, 169(5), e235-e241

20. Fadhil, M. F., S. R, Ibraheem., and A. A. K. A, Al-Kazaz. 2019. Study the association between IL-17 level and autoimmune antibodies in hypo and hyper thyroidisms patients. Iraqi Journal of Science, 1967-1976

21. Figueroa-Vega, N.; M, Alfonso-Pérez.; I, Benedicto.; F, Sánchez-Madrid.; R, González-Amaro and M, Marazuela. 2010. Increased circulating pro-inflammatory cytokines and Th17 lymphocytes in Hashimoto's thyroiditis. J Clin Endocrinol Metab, 95:953-62

22. Fritz, G., H. M, Botelho., L. A, Morozova- Roche., and C. M, Gomes 2010. Natural and amyloid self- assembly of S100 proteins: structural basis of functional diversity. The FEBS Journal, 277(22), 4578-4590

23. Hasan, R. I., and A. H, Raziq 2019. Studying the frequency of autoimmune thyroid diseases in duhok province. Science Journal of University of Zakho, 7(2), 45-49

24. Holzinger, D., D, Foell., and C, Kessel 2018. The role of S100 proteins in the pathogenesis and monitoring of autoinflammatory diseases. Molecular and cellular pediatrics, 5(1), 1-5

25. Kahaly, G. J., T, Diana., and P. D, Olivo 2020. TSH receptor antibodies: relevance and utility. Endocrine Practice, 26(1), 97-106

26. Kahaly, G. J. and T, Diana 2017. TSH receptor antibody functionality and nomenclature. Front Endocrinol (Lausanne), 8:28

27. Kim, J. W., J, Ko., J, Kim., and J. S, Yoon 2020. Proinflammatory effects of calprotectin

in Graves' orbitopathy. Ocular Immunology and Inflammation, 28(1), 156-163

28. Korkmaz, H., S, Tabur., E, Savaş., M, Özkaya., N, Aksoy., Ş. N, Aksoy., and E, Akarsu 2016. Evaluation of serum S100A8/S100A9 levels in patients with autoimmune thyroid diseases. Balkan Medical Journal, 33(5), 547-551

29. Lindberg, B., J, Svensson., U. B, Ericsson., P, Nilsson., E, Svenonius., and S. A, Ivarsson 2001. Comparison of some different methods for analysis of thyroid autoantibodies: importance of thyroglobulin autoantibodies. Thyroid, 11(3), 265-269

30. Luo, X., T, Zheng., C, Mao., X, Dong., X, Mou., C, Xu., ... and Y, Xiao. 2018. Aberrant MRP14 expression in thyroid follicular cells mediates chemokine secretion through the IL-1β/MAPK pathway in Hashimoto's thyroiditis. Endocrine Connections, 7(6), 850-858

31. Nacken, W., J, Roth., C, Sorg., and C, Kerkhoff 2003. S100A9/S100A8: Myeloid representatives of the S100 protein family as prominent players in innate immunity. Microscopy research and technique, 60(6), 569-580

32. Peng, S., X, Sun., X, Wang., H, Wang., Z, Shan., W, Teng., and C, Li 2018. Myeloid related proteins are up-regulated in Autoimmune Thyroid Diseases and activate toll-like receptor 4 and pro-inflammatory cytokines in vitro. International immunopharmacology, 59, 217-226

33. Pezzano, M. 2019. MIG in autoimmune thyroiditis: review of the literature. *La Clinica Terapeutica*, *170*(4), e295-e300

34. Ross, D. S., H. B, Burch., D. S, Cooper., M. C, Greenlee., P, Laurberg., A. L, Maia., ... and M. A, Walter 2016. 2016 American Thyroid Association guidelines for diagnosis and management of hyperthyroidism and other causes of thyrotoxicosis. Thyroid, 26(10), 1343-1421

35. Schäfer, B. W., and C. W, Heizmann 1996. The S100 family of EF-hand calcium-binding proteins: functions and pathology. Trends in biochemical sciences, 21(4), 134-140

36. Schmittgen, T. D., and K. J, Livak. 2008. Analyzing real-time PCR data by the comparative CT method. Nature Protocols, 3(6), 1101-1108

37. Stroncek, D. F., R. A, Shankar., and K. M, Skubitz. 2005. The subcellular distribution of myeloid-related protein 8 (MRP8) and MRP14 in human neutrophils. Journal of Translational Medicine, 3(1), 1-9

38. Touma, M. M., H. S, Jassim., S. M, Hyyawi., H. J, Nayyef., and A. H, Abbas. 2021. The role of ifn-γ and tnf-α in experimental mastitis. The Iraqi Journal of Agricultural Sciences,52 (1):121-128

39. Vogl, T., K, Tenbrock., S, Ludwig., N, Leukert., C, Ehrhardt., M. A, Van Zoelen., and J, Roth. 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nature Medicine, 13(9), 1042-1049

40. Wu, Z., Y, Zhu., M, Zhang., C, Wang., L, Zhou., W, Liu., ... and L, Ji. 2021. Serum ratio of free triiodothyronine to thyroid-stimulating hormone: a novel index for distinguishing graves' disease from autoimmune thyroiditis. Frontiers in Endocrinology, 11, 1087

41. Yan, W. X., C, Armishaw., J, Goyette., Z, Yang., H, Cai., P, Alewood., and C. L, Geczy. 2008. Mast cell and monocyte recruitment by S100A12 and its hinge domain. Journal of Biological Chemistry, 283(19), 13035-13043

42. Zhai, X., L, Zhang., L, Chen., X, Lian., C, Liu., B, Shi., ... and W, Teng. 2018. An age-specific serum thyrotropin reference range for the diagnosis of thyroid diseases in older adults: a cross-sectional survey in China. Thyroid, 28(12), 1571-1579

43. Zheng, T., C, Xu., C, Mao., X, Mou., F, Wu., X, Wang., ... and Y, Xiao. 2018. Increased interleukin-23 in Hashimoto's thyroiditis disease induces autophagy suppression and reactive oxygen species accumulation. Frontiers in immunology, 9, 96.