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SSA autoantibodies associated with severely decreased free thyroxine levels in systemic autoimmune mixed connective tissue disease

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ABSTRACT

Background: Mixed connective tissue disease (MCTD) is a systemic autoimmune disease with variable symptoms and autoantibodies. Antinuclear antibody positivity is more common in autoimmune thyroid diseases with higher levels of antibodies to thyroid peroxidase (TPO) or thyroglobulin (Tg) leading to elevated TSH levels. Intracellular SSA autoantibodies have a direct role in tissue damage with a prevalence of 33% in MCTD.

Aims: To investigate the role of SSA autoantibodies in thyroid autoimmunity in MCTD patients. The effect of SSA autoantibodies on thyroid hormone levels was investigated in the presence and absence of thyroid autoimmunity.

Methods: Thirty-three patients with MCTD [41±10 years, 32 females and 1 male] and 34 healthy controls [33±14 years, 30 females and 4 males] were studied. Thyroid hormones (TSH, FT_4 and FT_3) were measured by luminescence immunoassay. Enzyme-linked immunosorbent assay was used for the detection of anti-TPO and anti-Tg autoantibodies. Biochemical data are presented as geometric mean with 95% confidence interval except for age and FT_3/FT_4 ratio, which are presented as mean±SD.

Results: Significant differences in age and serum FT_4 levels were observed between MCTD patients and controls (41±10 vs. 33±14 years, $p<0.0089$ and 8.83(3.55-22) vs. 10.82(7.33-15.96) pmol/l, $p<0.0229$, respectively). The difference in serum TSH and FT_4 levels was significant between SSA autoantibody positive MCTD patients and controls [2.43(0.42-13.96) vs. 1.63(0.75-3.53) mIU/ml, $p<0.0405$ for TSH, 6.59(2.22-19.53) vs. 10.82(7.33-15.96) pmol/l, $p<0.0001$ for FT_4]. The greater decrease in serum FT_4 levels could be demonstrated by the presence of SSA in combination with anti-Tg [3.74(1.37-10.18) vs. 11.05(8.57-14.24) pmol/l, $p<0.001$] or anti-TPO autoantibodies [4.68(1.65-13.28) vs. 11.75(6.05-22.8) pmol/l, $p<0.0001$] compared to those thyroid antibodies alone.

Conclusions: Our results showed that SSA autoantibodies together with anti-TPO and/or anti-Tg autoantibodies resulted in a greater decrease in serum FT_4 levels than SSA autoantibodies alone. The effect of SSA autoantibodies on FT_4 levels may be manifested in the absence of thyroid autoimmunity. These results highlight the importance of screening for thyroid hormone and autoantibody levels in MCTD patients regardless of thyroid autoimmunity.

Keywords: thyroid autoimmunity; mixed connective tissue disease; SSA autoantibodies; antibodies against thyroid peroxidase and thyroglobulin; decreased FT_4 serum levels

Introduction

Mixed connective tissue disease (MCTD) is a systemic autoimmune disease with multiple symptoms and autoantibodies. MCTD is a chronic autoimmune disease characterized by Raynaud's phenomenon, polyarthritis, polymyositis, sclerodactyly, swollen hands, esophageal dysmotility, pulmonary hypertension and interstitial lung fibrosis[1]. The above non-specific symptoms of MCTD overlap with those of other systemic autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), dermato-polymyositis (DM-PM) and scleroderma (SSc)[2]. Anti-U1-RNP autoantibodies are considered to be the most specific autoantibodies for MCTD, although several types of antinuclear autoantibodies (ANA) against double-stranded(ds) DNA, Sm, SSA/SSB, Jo1, Scl70 and phospholipids can be detected serologically[3].

Thyroid dysfunction is very common in systemic autoimmune diseases: 42% of patients with rheumatoid arthritis, 46% of patients with systemic lupus erythematosus, 27% of patients with scleroderma, 50% and 43% of patients with Sjögren's syndrome (SS) and MCTD, respectively[4]. Hashimoto's thyroiditis (HT) could be considered as a dominant autoimmune thyroid disease besides Graves' disease (GD). Hashimoto's thyroiditis was more common than Graves' disease in MCTD (21%), Sjögren's syndrome (7%) and rheumatoid arthritis (6%); Graves' disease was detected in 2.5%, 3% and 1.6%, respectively[5]. Systemic autoimmune diseases were associated with 51% of Hashimoto's thyroiditis and 16% of Graves' disease. The association between MCTD and Hashimoto's thyroiditis may be based on immune complex-mediated damage to thyroid follicular cells, resulting in subclinical or manifest hypothyroidism[6]. However, some report has shown a high prevalence of hypothyroidism in systemic lupus erythematosus and polymyositis without underlying autoimmune disease[7,8].

Anti-SSA/SSB autoantibodies are intracellular antibodies to a cytoplasmic complex of small ribonucleic acid (RNA) nucleotides. They are most commonly associated with Sjögren's syndrome (70-100%) and systemic lupus erythematosus (40-90%). The prevalence of SSA antibodies in MCTD was 33% in the work of Setty NY[9]. Two Ro-ribonucleoproteins (RNP, Ro52 and Ro-60) form the SSA antigens, which are localized in different cell compartments. SSA autoantibodies [SSA/Ro60 (the target for classical detection) and SSA/Ro52] play a direct role in tissue damage and are involved in proteasomal degradation[10]. SSB (La) is also part of the Ro heterogeneous antigen complex, which is an extractable nuclear complex. SSB autoantibodies are mainly associated with primary Sjögren's syndrome. Tests for SSA autoantibodies generally provide a common target for Ro60 and Ro52. The separate detection of autoantibodies against Ro52 and Ro60 highlights their clinical relevance in autoimmune diseases. Ro52 autoantibodies are mainly associated with inflammatory myositis. Ro60 autoantibodies are often associated with the presence of antiphospholipid antibodies[11]. Antinuclear antibody positivity is more common in autoimmune thyroid disease than in controls (45% vs. 14%)[12]. Antinuclear antibody positivity was associated with higher levels of anti-thyroid peroxidase (TPO) autoantibodies or elevated TSH levels[13,14].

The role of SSA autoantibodies was investigated in relation to thyroid autoimmunity in MCTD patients. The effect of SSA autoantibodies on thyroid hormone levels was investigated in the presence and absence of thyroid autoimmunity.

Patients and Methods

PATIENTS

Thirty-three patients with MCTD [41±10 years, 32 females and 1 male] and 34 healthy controls [33±14 years, 30 females and 4 males] were studied. The patients with MCTD were diagnosed and followed-up at the special clinic of the Department of Immunology, University of Debrecen. All clinical and immunological parameters were investigated at the University, except thyroid hormones and anti-TPO and anti-Tg autoantibodies. These parameters were measured from the patients' sera at the Kenezy Hospital in 2007.The diagnosis of MCTD was based on the classification criteria of the American College of Rheumatology[15]. Autoimmune thyroid diseases in 12 patients [10 cases of Hashimoto's thyroiditis and 2 cases of Graves' disease] were associated with MCTD. The diagnosis of Hashimoto's thyroiditis was based on the presence of anti-TPO and/or anti-Tg autoantibodies. The presence of anti-TPO antibodies in 17 cases and anti-Tg antibodies in 7 cases was detected in 33 patients with MCTD. Diffuse goitre with hyperthyroidism at onset was characterized by Graves' disease (none of the patients had ophthalmopathy). The non-specific symptoms were as follows: arthralgia in 26 cases, hand swelling in 16 cases, skin lesions in 7 cases, arthritis in 9 cases, myositis in 16 cases (confirmed by electromyography (EMG) and biopsy), Raynaud's phenomenon in 20 cases, vasculitis in 9 cases. The presence of SSA autoantibodies was demonstrated in 12 cases and cardiac disease (all heart failure) in 12 cases. All patients with MCTD had anti-U1-RNP autoantibodies. None of the patients had SSB autoantibodies. Disease duration at the time of the study was 9±4 years.

Disease-specific therapies included methylprednisolone (2-12 mg/day), methotrexate (10-25 mg/week) and cyclophosphamide (500-1000 mg/3 weeks), as well as adalimumab, chloroquine and immunoglobulin therapies. Non-specific therapies included pentoxifylline, NSAIDs with proton pump inhibitors, acenocoumarol and low molecular weight (LMW) heparin. Patients with Hashimoto's thyroiditis were treated with levothyroxine (50-75 ug/day), but all patients with Graves' disease were euthyroid at the time of the study and did not require treatment.

Methods

DETERMINATION OF SERUM THYROID HORMONE LEVELS

Thyroid hormones (TSH, FT_4 and FT_3) were measured by fully automated luminescence immunoassay (LIA-MAT, Byk Sangtec, Germany). Normal values were as follows: 0.3-3 mIU/l for TSH, 7.72-23.18 pmol/l for FT_4 and 2.5-4.5 pg/ml for FT_3. FT_3 values were converted from pg/ml to pmol/l before calculation of the FT_3/FT_4 ratio.

Anti-TPO and anti-Tg antibodies were detected by enzyme-linked immunosorbent assay (ELISA) (SIGMA,

USA) at Kenézy Hospital. Autoantibodies to SSA/SSB (including both Ro60 and Ro52 proteins/La protein) and U1-RNP were detected by ELISA (Cogent Diagnostics, UK) at the University[16, 17]. The results were expressed as positive and negative antibody forms for SSA, anti-TPO and anti-Tg antibodies. The calculation gave a ratio of optical density for MCTD patient sera to mean optical density + 2 SD for controls. Ratios greater than 0.64 for anti-Tg antibodies and greater than 0.73 for anti-TPO antibodies were considered positive.

STATISTICS

Biochemical data are presented as geometric mean with 95% confidence interval except for age and FT_3/FT_4 ratio, which are presented as mean±SD. The presence of autoantibodies against TPO, Tg and SSA was considered as positive and negative. Thyroid hormones, such as serum levels of TSH, FT_4 and FT_3 were skewed, so their logarithms were used, which showed an approximately normal distribution. Chi-squared tests were used to compare categorical data. Two-way and three-way ANOVA were used to show the effect of SSA autoantibodies together with anti-TPO and/or anti-Tg autoantibodies on the changes in logFT_4 levels, which were presented as mean with 95% confidence interval after conversion from logarithm. The general linear model was used to estimate the relationship between FT_4 levels as the dependent variable and SSA, amti-TPO and anti-Tg autoantibodies as independent variables, as well as between their interactions. P values less than 0.05 were considered significant. Statistical analyses were performed using Medcalc 17.9.7. and SPSS 26.0.0. softwares.

Results

THYROID HORMONE STATUS IN PATIENTS WITH MIXED CONNECTIVE TISSUE DISEASE AND CONTROLS

The difference in age and serum FT_4 levels between patients with MCTD and controls was significant (41±10 vs. 33±14 years, $p<0.0089$ and 8.83(3.55-22) vs. 10.82(7.33-15.96) pmol/l, $p<0.0229$, respectively (Table 1). There was no significant difference in serum TSH and FT_3 levels or FT_3/FT_4 ratio. The presence of SSA autoantibodies was detected in 12 out of 33 MCTD patients. Only 4 out of 12 patients with thyroid autoimmunity showed SSA autoantibodies. The presence of anti-TPO antibodies in 12 cases and anti-Tg antibodies in 6 cases was not associated with autoimmune thyroid diseases. Six cases with anti-TPO antibody positivity and 8 cases with anti-Tg antibody positivity showed SSA autoantibodies in patients with MCTD. The cut-off value of serum FT_4 levels was based on the categories of SSA autoantibody positivity and negativity using receiver operating characteristic (ROC) curve analysis. The cut-off value was given at <7.91 pmol/l for FT_4, Younden index: 0.6905, $p<0.0013$ for the area under the ROC curve (AUC).

The results showed an association between serum FT_4 levels and SSA autoantibodies, the main presence of which affected FT_4 levels independently of thyroid autoimmunity in MCTD.

Table 1: Parameters studied in patients with mixed connective tissue disease (MCTD) and controls.

Studied parameters	Patient groups		*P*
	Patients with MCTD n=33	Controls n=34	
Age (yr)	41 ± 10	33 ± 14	0.0089
Gender (woman/male)	32/1	34/4	
*TSH (mIU/ml)	2.58(1.42-4.68)	2.93(1.82-4.72)	0.0624
*FT_4 (pmol/l)	8.83(3.55-22)	10.82(7.33-15.96)	0.0229
*FT_3 (pg/ml	2.17(0.4-11.75)	1.63(0.75-3.53)	0.0843
Ratio of FT_3/FT_4	0.36 ± 0.32	0.29 ± 0.1	0.2425
Thyroid autoimmunity (n)	12	0	
Anti-Tg antibody positive/negative	7/26	0	
Anti-TPO antibody positive/negative	17/16	0	
SSA antibody positive/negative	12/21	0	

* Geometric mean with 95% confidence interval

THE EFFECT OF SSA AUTOANTIBODIES ON THYROID HORMONE LEVELS IN PATIENTS WITH MIXED CONNECTIVE TISSUE DISEASE

Serum thyroid hormone levels (TSH, FT_4, FT_3) and the ratio of FT_3/FT_4 between patients with MCTD and controls in relation to the presence of SSA autoantibodies are shown in Figure 1. The difference in serum TSH and FT_4 levels, as well as the ratio of FT_3/FT_4, was significant between SSA autoantibody positive patients with MCTD and controls [2.43(0.42-13.96) vs. 1.63(0.75-3.53) mIU/ml for TSH, $p<0.0405$, 6.59(2.22-19.53) vs. 10.82(7.33-15.96) pmol/l for FT_4, $p<0.0001$ and 0.55±0.47 vs. 0.29±0.1 for the ratio of FT_3/FT_4, $p<0.0033$]. The difference in serum FT_3 levels was significantly lower in

SSA autoantibody negative patients compared to controls [2.42(1.36-4.29) vs. 4.79(1.71-13.48) pg/ml, $p<0.0106$]. The ratio of FT_3/FT_4 was significantly higher in MCTD patients with SSA autoantibody positivity than negativity [vs. 0.25±0.1, $p<0.0073$].

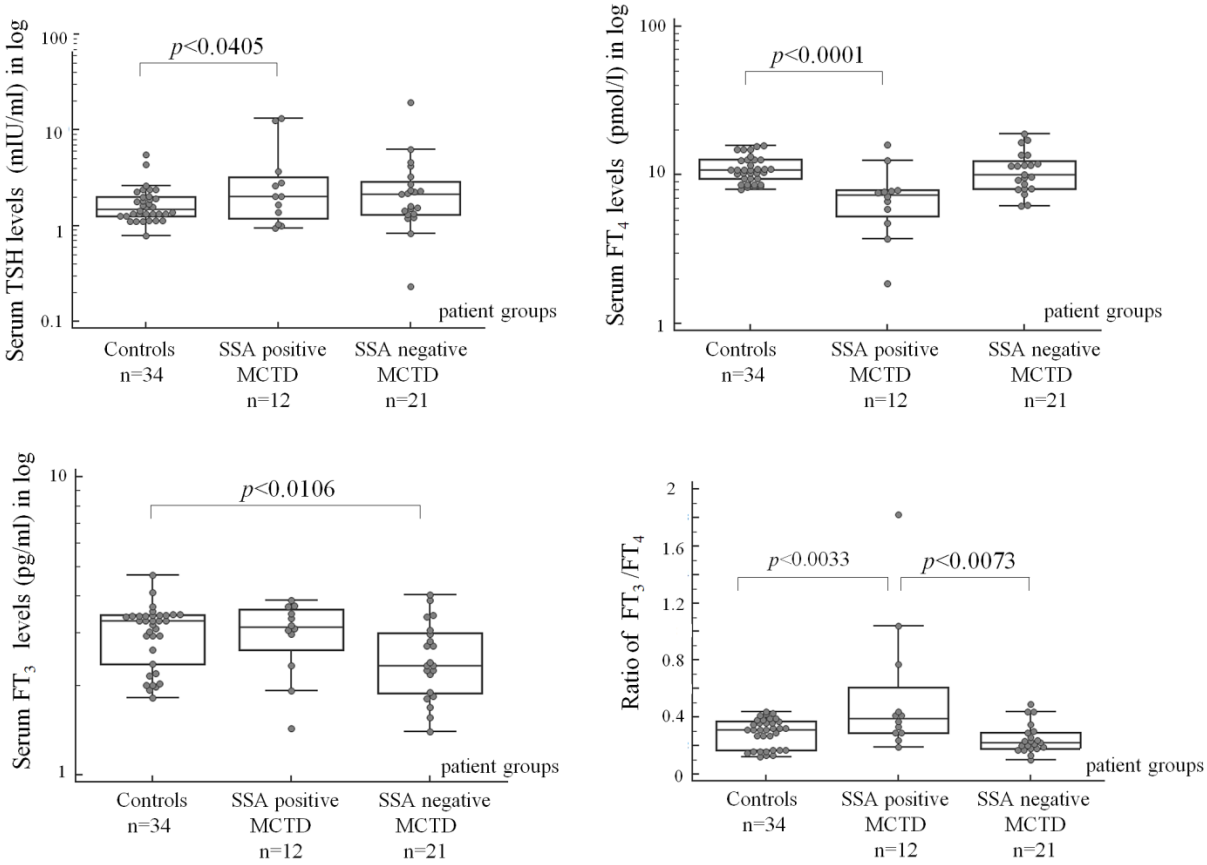


Figure 1: Thyroid hormone levels and the ratio of FT_3/FT_4 in SSA positive and negative MCTD patients and controls.

The patients with MCTD without autoimmune thyroid diseases, who were SSA autoantibody positive had slightly higher serum FT_4 levels than controls [11.19(5.78-21.64) vs 10.82(7.33-15.96) pmol/l] (Figure 2). In MCTD, the presence of SSA autoantibodies in patients without thyroid diseases was associated with lower serum FT_4 levels compared to patients with thyroid diseases who were SSA autoantibody negative [5.91(1.71-20.44) vs. 9.35(5.84-14.98) pmol/l, $p<0.0762$*]. The difference in FT_4 levels was significant between patients with SSA autoantibody positivity and thyroid disease negativity and controls [vs. 10.82(7.33-15.96) pmol/l, $p<0.0001$] or patients with SSA autoantibody negativity and thyroid disease negativity ($p<0.0069$). Patients who were both SSA autoantibody positive and thyroid disease positive had significantly lower FT_4 levels compared to controls ($p<0.0164$).

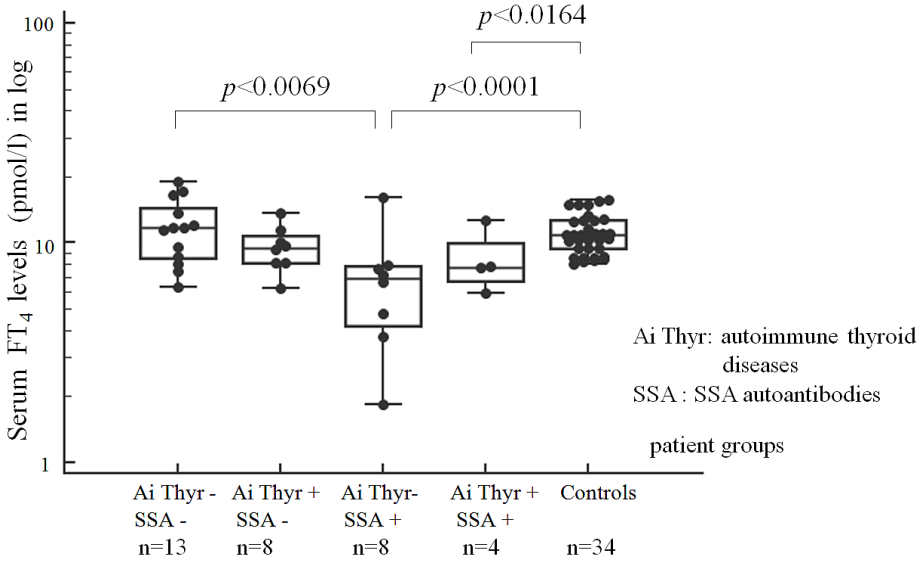


Figure 2: Changes in serum FT_4 levels according to the presence and absence of SSA autoantibodies and autoimmune thyroid diseases in MCTD patients and controls.

The results showed that the presence of SSA autoantibodies primarily affected serum FT_4 levels, lowering them, while simultaneously increasing TSH levels and the FT_3/FT_4 ratio. The presence of SSA autoantibodies resulted in a greater decrease in serum FT_4 levels compared to those who were negative for SSA autoantibodies and negative for autoimmune thyroid diseases, and borderline significance in MCTD patients who were positive for thyroid diseases only.

THE EFFECT OF SSA AUTOANTIBODIES ALONE AND WITH ANTI-THYROID PREOXIDASE AND/OR ANTI-THYROGOBULIN AUTOANTIBODIES ON FT_4 LEVELS IN PATIENTS WITH MIXED CONNECTIVE TISSUE DISEASE

The effect of SSA autoantibodies alone and together with anti-Tg or anti-TPO antibodies on serum FT_4 levels in patients with MCTD was investigated using two-way ANOVA. The relationship between SSA and anti-Tg or anti-TPO autoantibodies is shown in Figure 3. The presence of SSA autoantibodies alone was associated with lower serum FT_4 levels compared to their absence [8.71(4.68-16.2) vs. 10.38(5.32-20.25) pmol/l]. Their association with anti-Tg antibodies represented the greater decrease in FT_4 levels compared to anti-Tg positive cases alone [3.74(1.37-10.18) vs. 11.05(8.57-14.24) pmol/l, $p<0.001$], and cases with SSA autoantibody positivity ($p<0.002$) or SSA autoantibody negativity ($p<0.0001$) (Figure 3A).

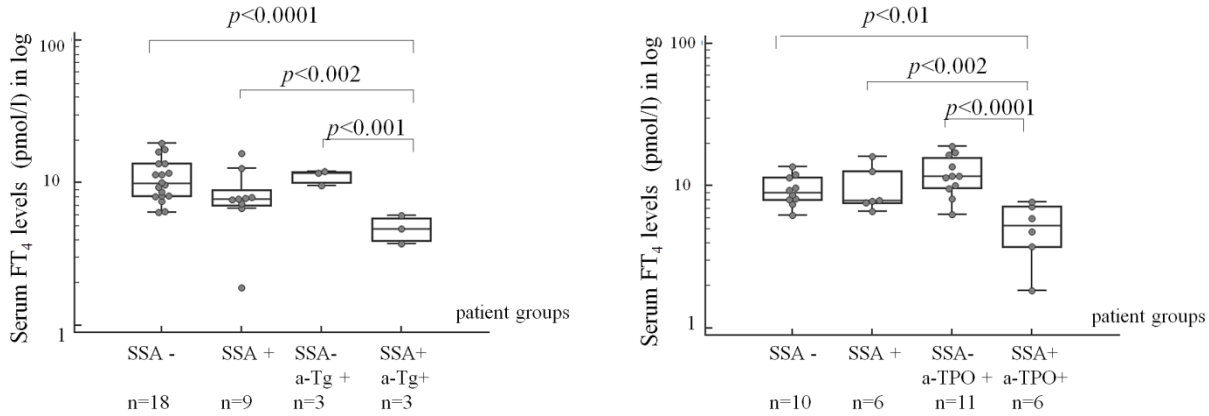


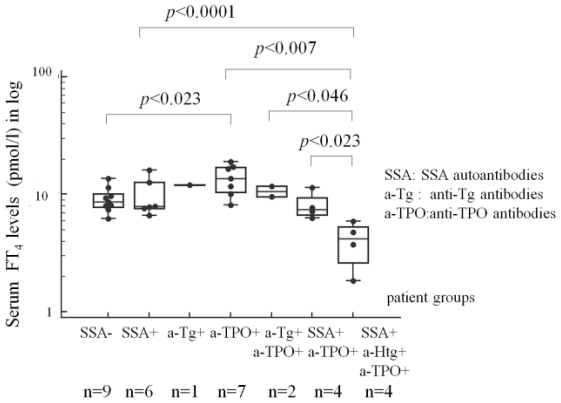
Figure 3: Effect of SSA autoantibodies alone and together with anti-Tg or anti-TPO antibody positivity on serum FT_4 levels using two-way ANOVA in MCTD.
SSA: SSA autoantibodies; a-Tg: anti-Tg antibodies; a-TPO: anti-TPO antibodies

The presence of SSA or anti-TPO autoantibody positivity alone did not significantly decrease serum FT_4 levels, but their association resulted in significantly lower FT_4 levels [9.23(4.64-18.35) and 11.75 (6.05-22.8) vs. 4.68(1.65-13.28) pmol/l, $p<0.014$ and $p<0.0001$, respectively] (Figure 3B).

Three-way ANOVA was used to examine the relationship between SSA, anti-Tg and anti-TPO autoantibodies and FT_4 levels in patients with MCTD (Figure 4). The results are limited by the small number of patients. The strength of the relationship between FT_4 as the dependent variable and independent variables such as SSA, anti-Tg, and anti-TPO autoantibodies, and their interactions was evaluated using general linear model. The presence of all three autoantibodies resulted in a greater decrease in serum FT_4 levels compared to those with SSA autoantibodies alone, or with the presence of anti-TPO antibodies alone, or with the combination of SSA and anti-TPO autoantibodies, or with the combination of anti-TPO and anti-Tg antibodies [3.74 (1.37-10.18) vs. 9.23(4.64-18.35) pmol/l, $p<0.0001$ or 13.27(7.18-24.53) pmol/l, $p<0.007$ or 7.91(4.69-13.3) pmol/l, $p<0.023$ or 10.59(7.9-14.2) pmol/l, $p<0.046$, respectively] (Figure 4A).

The model was significant: $p<0.0001$, adjusted R squared: 0.489 (Figure 4B). In this model, the results showed that the combined effect of anti-TPO and anti-Tg antibodies was not sufficient to significantly reduce serum FT_4 levels. In contrast, SSA autoantibodies alone and in the combination with anti-TPO and anti-Tg antibodies showed a significant decrease in FT_4 levels in the model.

A



B

Effects between subjects

Dependent variable: logFT_4

Source	Type III Sum of squares	df	Mean square	F	Sig	Partial ETA squared
Corrected model	.766	6	.128	6.112	.000	.585
Intercept	12.576	1	12.576	602.312	.000	.959
CatSSA	0.2672	1	.262	12.527	.002	.325
CatTg and CatTPO	.116	3	.039	1.847	.163	.176
Cat SSA, CatTg and CatTPO	.223	2	.112	5.35	.011	.292
Error	.543	26	.021			
Total	30.864	33				
Corrected total	1.309	32				

Adjusted R squared = .489

Figure 4: Effect of SSA autoantibodies alone and together with anti-Tg and anti-TPO antibody positivities on serum FT_4 levels in MCTD using three-way ANOVA in general linear model.

The greater reduction in serum FT_4 levels in the presence of SSA alone, and in combination with anti-Tg or anti-TPO autoantibodies was demonstrated using two-way ANOVA. In a general linear model, in which all three autoantibodies were present as independent variables, thyroid autoantibodies may be potentiating factors for the effect of SSA autoantibodies on the decrease in serum FT_4 levels.

Discussion

The presence of anti-TPO and anti-Tg antibodies is more commonly associated with antinuclear antibodies and elevated IgG immunoglobulin levels independent of thyroid autoimmunity[18]. Sjögren's syndrome is frequently associated with autoimmune thyroid diseases (Hashimoto's thyroiditis in 17% and Graves' disease in 5%) highlighting the risk of developing lymphoma in both diseases. Genetic factors, infections and cytokine-mediated inflammation represent a multistep process for the induction of B cell activation and proliferation[19]. SSA autoantibodies have been detected in 30-60% of patients with Sjögren's syndrome[20]. The study of the adjuvant activity of U1-RNP demonstrated both B- and T-cell activation and dendritic cell maturation, suggesting that U1-RNP may play an endogenous adjuvant role[21]. Systemic sclerosis also shows a high association with autoimmune thyroid diseases (33%) resulting in hypothyroidism (50%)[22].

Hypothyroidism is more common sign in the association between systemic and organ specific autoimmune thyroid diseases, especially by Hashimoto's thyroiditis. Thyroid autoantibodies were present in 6 patients out of 33 in MCTD without thyroid autoimmunity highlighting that thyroid autoantibodies can occur independently of thyroid autoimmunity. The presence of SSA autoantibodies was associated with significantly decreased serum FT_4 and concomitantly increased TSH levels in MCTD patients. Surprisingly, in the absence of SSA autoantibodies, serum FT_3 levels were significantly lower in MCTD patients compared to controls. This rules out the causative role of non-thyroidal illness in thyroid hormone changes by SSA autoantibody positivity[23]. The low T_3 levels characterize the non-thyroidal illness syndrome, are due to the inhibitory effect of cytokines on deiodinase enzymes (type 1 and type 2) as observed in critically ill patients[24]. Maternal SSA autoantibodies can be transferred to the foetus during pregnancy. The presence of maternal SSA/SSB autoantibodies can be transmitted a danger in utero by activating the type 1 interferon system, leading to congenital heart block[25]. A Chinese study showed a strong association between thyroid autoimmunity and the prevalence of antinuclear antibodies (ANA) in pregnancy[26]. They found that high ANA titres associating with high anti-TPO antibody levels (in 30%) were connected to the risk of neonatal damage in pregnancy. Congenital heart block (14-42%) has two main aetiologies: AV septal defects and anomalies of the great arteries[27]. Neuromyelitis optica spectrum disorder (NMOST) is an autoimmune inflammatory disease characterized by astrocytopathy leading to blindness[28]. Neuromyelitis optica spectrum disorder is most commonly associated with the systemic autoimmune diseases Sjögren's syndrome (35%) and systemic lupus erythematosus (51%). Estrogen plays an immune enhancing role. In these diseases, the SSA autoantibodies alone can be considered to be the cause of the tissue damage.

Our results showed that SSA autoantibodies in combination with anti-TPO and/or anti-Tg autoantibodies led to a greater decrease in serum FT_4 levels than SSA antibodies alone. In mixed connective tissue disease, hypothyroidism may develop in the absence of thyroid autoimmunity, so measurement of thyroid hormones and autoantibodies must be routine. Endothelial cell dysfunction is associated with MCTD and plays a role in atherosclerotic complications such as cardiovascular disease and pulmonary arterial hypertension[29]. In turn, hypothyroidism promotes the development of endothelial dysfunction[30]. Hypothyroidism is associated with decreased cardiac output, increased capillary permeability and vascular resistance, and decreased insulin sensitivity[31]. The above pathomechanisms are involved in the symptoms of MCTD. In euthyroid patients with Hashimoto's thyroiditis, non-specific rheumatic symptoms such as polyarthralgia and myalgia/fibromyalgia were observed in 62% [32].

The exact mechanism by which SSA autoantibodies can interfere with thyroxine (T_4) synthesis is not yet understood. Both proteins, SSA antigen and T4 synthesis, are located in the nucleolus, so the role of proteasomes may be a common factor[33]. Nuclear autoantigens and T_4 are degraded by proteasomal pathways. Proteasomal events play a role in systemic autoimmunity by antigen processing and influencing the activity of inflammatory processes[34,35].

Limitations of the study could be the small number of patients and categorizing the levels of SSA, anti-Tg and anti-TPO autoantibodies. The relationships between the effect of SSA autoantibodies and decreased serum FT_4 levels may represent a real clinical conditions using multiple statistical analyses.

Conclusions

The presence of SSA autoantibodies was associated with significantly lower serum FT_4 levels compared to controls in MCTD with concomitant elevated TSH levels. The effect of SSA autoantibodies on FT_4 levels can also be observed in the absence of thyroid autoimmunity. SSA autoantibody positive MCTD patients, together with anti-TPO and anti-Tg antibody positivities showed the greatest decrease in serum FT_4 levels. The results highlight the importance of screening for thyroid hormone and autoantibody levels in MCTD patients independently of thyroid autoimmunity, especially by the co-occurrence of SSA, anti-TPO and anti-Tg antibody positivity and pregnancy.

Conflicts of interest statement:

The authors declare no conflicts of interest.

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