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Assessment of Serum Complement Components C3 and C4 as Immunological Markers in Patients with Autoimmune Thyroid Disorders: A Cross-Sectional Comparative Study

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Abstract Autoimmune Thyroid Disorders (AITDs) are characterized by immune dysregulation that may influence activation of the complement system. Complement components C3 and C4 play important roles in innate and adaptive immunity and may reflect immunological alterations associated with thyroid dysfunction. This study aimed to evaluate serum complement C3 and C4 levels in Iraqi patients with autoimmune thyroid disorders. **Methods:** This cross-sectional comparative study included 90 participants: 30 healthy controls, 30 patients with hyperthyroidism and 30 patients with hypothyroidism. Blood samples were collected from Baqubah Teaching Hospital and Al-Batoul Teaching Hospital for Women and Children in Diyala Governorate between October 2020 and January 2021. Serum C3 and C4 concentrations were measured using enzyme-linked immunosorbent assay (ELISA). Data were analyzed using One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, with $p<0.05$ considered statistically significant. **Results:** Serum C3 levels were significantly lower in patients with hypothyroidism (1.32±0.084 mg/mL) than in healthy controls (2.12±0.78 mg/mL), whereas patients with hyperthyroidism showed significantly higher C3 levels (4.97±1.61 mg/mL; $p = 0.032$). Serum C4 levels were lower in the hypothyroidism group (0.06±0.003 mg/mL) and slightly higher in the hyperthyroidism group (0.11±0.02 mg/mL) compared with controls (0.09±0.03 mg/mL); however, these differences were not statistically significant. **Conclusion:** Serum complement C3 levels were significantly associated with thyroid dysfunction, whereas serum C4 levels showed no significant differences among the study groups. These findings suggest that C3 may be a more sensitive indicator of immunological alterations in autoimmune thyroid disorders. However, larger studies incorporating thyroid autoantibody profiling are required to confirm these observations.

Key Words Autoimmune Thyroid Disease; Complement C3; Complement C4; Hyperthyroidism; Hypothyroidism; Enzyme-Linked Immunosorbent Assay (ELISA)

INTRODUCTION

Autoimmune Thyroid Diseases (AITDs) are among the most prevalent organ-specific autoimmune disorders and represent a major cause of thyroid dysfunction worldwide. The two principal forms of AITDs are Hashimoto's Thyroiditis (HT), which is characterized by progressive destruction of thyroid tissue resulting in hypothyroidism and Graves' Disease (GD), which is characterized by excessive production of thyroid hormones leading to hyperthyroidism. Although these disorders present with different clinical manifestations, both share common immunopathological mechanisms involving the breakdown of immune tolerance against thyroid autoantigens and the development of chronic autoimmune inflammation. Recent evidence suggests that the global incidence of autoimmune thyroid diseases has increased over the past decade, highlighting the need for a better understanding of the underlying immunological mechanisms and the identification of reliable biomarkers that may improve disease evaluation and monitoring [1-3].

The pathogenesis of autoimmune thyroid diseases is multifactorial and involves complex interactions between genetic susceptibility, environmental exposures, hormonal influences and immune dysregulation. Genetic factors, including polymorphisms in immune-regulatory genes such as HLA, CTLA-4, PTPN22 and FOXP3, increase susceptibility to thyroid autoimmunity. Environmental

factors including excessive iodine intake, smoking, infections, psychological stress, selenium deficiency, vitamin D deficiency and several medications may trigger autoimmune responses in genetically predisposed individuals. These factors contribute to the activation of autoreactive T and B lymphocytes, production of thyroid autoantibodies against thyroid peroxidase (TPO), thyroglobulin (Tg) and thyroid-stimulating hormone receptor (TSHR) and subsequent inflammatory damage to thyroid tissue [2-5].

The immune response in autoimmune thyroid diseases is mediated by both innate and adaptive immunity. During disease development, immune cells infiltrate thyroid tissue and release numerous cytokines and inflammatory mediators that amplify local inflammation and contribute to progressive thyroid dysfunction. Increasing evidence indicates that components of innate immunity not only initiate inflammatory responses but also regulate adaptive immune mechanisms through interactions with antigen-presenting cells, T lymphocytes and B lymphocytes. Consequently, dysregulation of innate immune pathways has become an important area of investigation in autoimmune endocrine diseases [4-6].

One of the most important components of innate immunity is the complement system, a highly regulated cascade of plasma and membrane-bound proteins that plays a central role in host defense and immune homeostasis. Besides protecting against microbial infections, the complement system contributes to immune complex clearance, opsonization of pathogens, regulation of inflammatory responses and removal of apoptotic cells. Complement activation occurs through the classical, lectin and alternative pathways, all of which converge at complement component C3. Activation of C3 subsequently initiates downstream complement reactions that promote inflammation and immune cell recruitment, whereas complement component C4 mainly participates in the classical and lectin pathways and is closely associated with antibody-mediated immune responses [6-8].

LITERATURE REVIEW

Recent studies have highlighted the complement system as an important mediator of chronic inflammation and immune regulation in autoimmune diseases. Beyond its traditional role in innate immunity, complement activation has been shown to influence adaptive immune responses by regulating T-cell activation, B-cell maturation, cytokine production and immune tolerance. Dysregulation of complement pathways has been associated with several autoimmune disorders, including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and autoimmune thyroid diseases. These findings suggest that complement proteins may contribute not only to host defense but also to immune-mediated tissue injury and disease progression [9-11].

Among complement proteins, C3 is considered the central component of the complement cascade because it participates in all activation pathways. Changes in serum C3 concentration may reflect ongoing immune activation and inflammatory responses. In contrast, C4 is mainly involved in the classical and lectin pathways and has been investigated as an indicator of antibody-mediated complement activation. Alterations in circulating levels of C3 and C4 have therefore attracted increasing interest as potential biomarkers of autoimmune activity in several endocrine and systemic autoimmune disorders [10-12].

Previous investigations evaluating serum complement proteins in autoimmune thyroid diseases have reported inconsistent findings. Some studies demonstrated significantly increased serum C3 concentrations in patients with hyperthyroidism, suggesting enhanced inflammatory activity and complement activation. Conversely, other studies reported reduced C3 levels in hypothyroid patients, possibly reflecting increased complement consumption during chronic autoimmune inflammation. Similar discrepancies have also been reported for serum C4 concentrations, with some studies demonstrating elevated levels whereas others observed no significant differences compared with healthy individuals. These conflicting findings may be explained by differences in disease subtype, disease duration, treatment status, sample size, laboratory methodology and ethnic background [11-14].

Recent advances in immunology have improved understanding of the interaction between thyroid autoimmunity and the complement cascade. Experimental and clinical evidence suggests that complement activation may amplify thyroid tissue injury through immune-complex formation, recruitment of inflammatory cells and enhancement of cytokine-mediated inflammatory responses. Furthermore, complement proteins may interact with thyroid autoantibodies, particularly anti-thyroid peroxidase antibodies, thereby contributing to disease severity and progression. Consequently, complement components have gained attention as potential indicators of immune activity in autoimmune thyroid disorders rather than merely inflammatory markers [13-16].

Despite these advances, evidence regarding complement proteins in autoimmune thyroid disorders remains limited, particularly in Middle Eastern populations. Most published studies have focused primarily on thyroid hormones or thyroid autoantibodies, while relatively few have simultaneously evaluated serum complement C3 and C4 concentrations in patients with hypothyroidism, hyperthyroidism and healthy controls using standardized immunological methods. In Iraq, studies investigating complement proteins in autoimmune thyroid disease are scarce, leaving an important gap in understanding the immunological profile of affected patients. Therefore, further investigations are required to clarify the relationship between complement activation and thyroid dysfunction and to determine whether complement proteins may provide additional information regarding immune alterations associated with autoimmune thyroid diseases.

Study Objective

The present study aimed to evaluate serum complement components C3 and C4 in Iraqi patients with autoimmune thyroid disorders and to compare their concentrations among patients with hypothyroidism, patients with hyperthyroidism and healthy controls. In addition, the study sought to determine whether alterations in these complement proteins are associated with immune dysregulation accompanying thyroid dysfunction.

Study Hypothesis

It was hypothesized that patients with autoimmune thyroid disorders exhibit significant alterations in serum complement components C3 and C4 compared with healthy individuals, reflecting activation of immune-mediated inflammatory pathways associated with thyroid dysfunction.

METHODS

Subjects: Blood samples were taken from thyroiditis patients and healthy individuals from Baqubah Teaching Hospital and Al-Batoul Hospital Education Feminine and Pediatric in the Diyala Governorate between the beginning of October 2020 and the end of January 2021.

Participants were divided into three groups based on clinical evaluation and laboratory assessment of thyroid function, including serum Thyroid-Stimulating Hormone (TSH), triiodothyronine (T3) and thyroxine (T4) levels. The diagnosis of hypothyroidism and hyperthyroidism was confirmed by a specialist physician. Written informed consent was obtained from all participants before sample collection.

The control group consisted of 30 healthy individuals (3 males and 27 females) aged 18-45 years. The hyperthyroidism group included 30 patients (5 males and 25 females) aged 23-60 years, whereas the hypothyroidism group included 30 female patients aged 21-60 years.

Because thyroid autoantibodies (anti-thyroid peroxidase, anti-thyroglobulin and TSH receptor antibodies) were not available for all participants, classification was based on clinical findings and thyroid function tests. Future studies are recommended to include these autoantibodies for more accurate classification of autoimmune thyroid disease subtypes.

Collection of Blood Samples

A total of 5 mL of venous blood was collected from each participant using sterile disposable syringes after disinfecting the venipuncture site with 70% ethanol. Blood samples were transferred into plain tubes and allowed to clot at room temperature for 15 minutes. Serum was separated by centrifugation at 3000 rpm for 5 minutes.

The separated serum was aliquoted into 250 µL sterile Eppendorf tubes and stored at -20°C until analysis. Each aliquot was thawed only once to avoid repeated freeze-thaw cycles.

Assay

Commercially available human Complement C3 and C4 ELISA kits were used to quantify serum levels of C3 and C4 in accordance with the manufacturer's instructions. An ELISA microplate reader was used to measure the optical density at 450 nm. Standard curves created for every experiment were used to calculate concentrations (Figure 1-2).

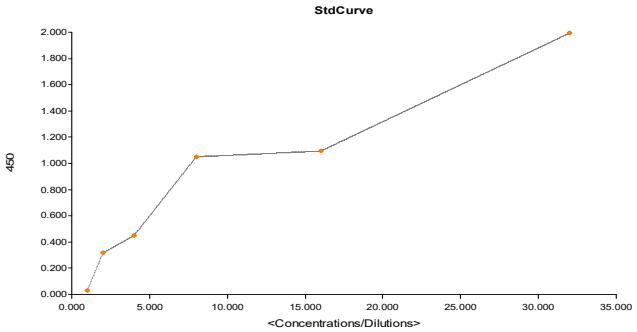

Figure 1: Serum Complement C3 Levels in Control, Hypothyroidism and Hyperthyroidism Groups

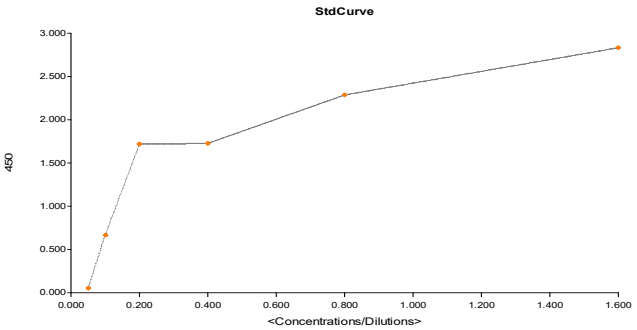

Figure 2: Serum Complement C4 Levels in Control, Hypothyroidism and Hyperthyroidism Groups

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics version 26.0. Data were expressed as Mean±Standard Error (SE). The normality of data distribution was assessed using the Shapiro-Wilk test. Comparisons among the three study groups were performed using One-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. A p-value less than 0.05 was considered statistically significant.

RESULTS

Measurement of Complement protein (C3)

Serum complement C3 concentrations differed significantly among the study groups. Patients with hyperthyroidism showed the highest mean serum C3 concentration (4.97±1.61 mg/mL), whereas patients with hypothyroidism exhibited the lowest concentration (1.32±0.084 mg/mL). Healthy controls had an intermediate mean serum C3 concentration (2.12±0.78 mg/mL). One-way ANOVA demonstrated a statistically significant difference among the three groups ($p = 0.032$). Tukey's post-hoc test indicated that serum C3 levels were significantly higher in patients with hyperthyroidism and significantly lower in patients with hypothyroidism compared with healthy controls (Table 1).

Table 1: Serum Complement C3 Concentrations in Healthy Controls, Hypothyroidism and Hyperthyroidism Groups

Groups	Gender	Mean±SE. (mg/mL)
Control	Male	1.13±0.08
	Female	2.23±0.86
	Total	2.12±0.78(AB)
Hypothyroidism	Female	1.32±0.084
	Total	1.32±0.084(B)
Hyperthyroidism	Male	11.55±6.31
	Female	3.66±1.42
	Total	4.97±1.61(A)

Values are expressed as Mean±SE, Different letters indicate statistically significant differences according to Tukey's post-hoc test ($p<0.05$), whereas identical letters indicate no statistically significant difference

Table 2: Serum Complement C4 Concentrations in Healthy Controls, Hypothyroidism and Hyperthyroidism Groups

Groups	Gender	Mean±SE(mg/mL)
Control	Male	0.05±0.001
	Female	0.10±0.03
	Total	0.09±0.03 (A)
Hypothyroidism	Female	0.06±0.003
	Total	0.06±0.003(A)
Hyperthyroidism	Male	0.17±0.08
	Female	0.09±0.02
	Total	0.11±0.02(A)

Values are expressed as Mean±SE, Different letters indicate statistically significant differences according to Tukey's post-hoc test ($p<0.05$), whereas identical letters indicate no statistically significant difference

Measurement of Complement protein (C4)

The mean serum C4 concentration was 0.09±0.03 mg/mL in healthy controls, 0.11±0.02 mg/mL in patients with hyperthyroidism and 0.06±0.003 mg/mL in patients with hypothyroidism. Although patients with hyperthyroidism tended to have slightly higher serum C4 concentrations and patients with hypothyroidism exhibited lower concentrations than healthy controls, these differences were not statistically significant. One-way ANOVA showed no statistically significant difference among the three study groups ($p = 0.568$) (Table 2).

DISCUSSION

The present study demonstrated significant differences in serum complement C3 concentrations among the studied groups. Patients with hyperthyroidism exhibited significantly higher serum C3 levels than healthy controls, whereas patients with hypothyroidism showed significantly lower C3 concentrations. These findings suggest that alterations in serum C3 are associated with immune dysregulation accompanying autoimmune thyroid disorders.

The observed increase in serum C3 among patients with hyperthyroidism is consistent with recent evidence indicating that complement activation contributes to immune dysregulation and inflammatory processes in autoimmune thyroid diseases [13,14]. Increased C3 levels may reflect activation of the complement system secondary to enhanced inflammatory and immune responses associated with autoimmune thyroid disease. Recent studies have shown that complement activation contributes to the amplification of inflammatory signaling and recruitment of immune cells within thyroid tissue, thereby influencing disease progression.

In contrast, the decreased serum C3 levels observed in patients with hypothyroidism differ from the findings reported by Blanchin *et al.* [4], who demonstrated increased complement activation in Hashimoto's thyroiditis. This discrepancy may be attributed to differences in disease stage, disease duration, treatment status, sample size, laboratory methods and genetic or ethnic variations among the studied populations. Similar variations have also been reported in previous investigations evaluating complement proteins in autoimmune thyroid disorders [15].

Complement component C3 is the central molecule of the complement cascade and plays an essential role in both innate and adaptive immunity. Activation of C3 promotes opsonization, immune complex clearance, recruitment of inflammatory cells and regulation of immune responses. Persistent activation or dysregulation of the complement system has been implicated in the pathogenesis of several autoimmune diseases, including autoimmune thyroid disorders [16,17].

The present findings support the hypothesis that serum C3 is associated with immunological alterations in autoimmune thyroid disorders. However, because complement activity is influenced by multiple clinical and immunological factors, larger multicenter studies incorporating thyroid autoantibody profiles and additional inflammatory biomarkers are recommended to further clarify the clinical significance of serum C3 in patients with autoimmune thyroid disease.

Serum C4 concentrations showed only slight variations among the study groups and these differences were not statistically significant. Although patients with hyperthyroidism exhibited slightly higher mean C4 levels and patients with hypothyroidism exhibited lower levels than healthy controls, the observed differences did not reach statistical significance. These findings suggest that serum C4 may be less sensitive than C3 in reflecting complement-related immunological alterations associated with autoimmune thyroid disorders.

Unlike complement component C3, which occupies a central role in all complement activation pathways, C4 primarily participates in the classical and lectin pathways. Therefore, serum C4 concentrations may remain relatively stable unless there is substantial activation of antibody-mediated complement responses. The absence of significant changes in serum C4 observed in the present study may indicate that complement activation in autoimmune thyroid disorders is not uniformly reflected by circulating C4 concentrations or that serum C4 is less responsive to variations in thyroid function (Figure 3).

The current findings are consistent with previous reports indicating that serum C4 concentrations often remain unchanged in autoimmune thyroid disorders despite

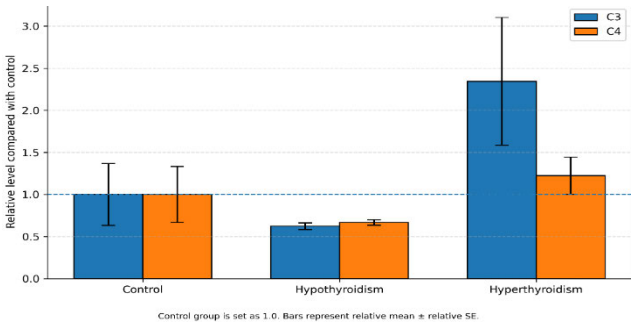

Figure 3: Relative Changes in Serum C3 and C4 Among Study Groups

evidence of complement activation, suggesting that C4 may be less sensitive than C3 in reflecting immune alterations associated with thyroid dysfunction [21]. However, the present results differ from those reported by Blanchin *et al.* [4], who observed increased complement activation in patients with Hashimoto's thyroiditis. These discrepancies may be attributed to differences in disease subtype, disease activity, treatment status, laboratory methodology, sample size and population characteristics.

Complement component C4 plays an important role in the initiation of the classical and lectin complement pathways through the formation of C3 and C5 convertases following its activation. Experimental studies have suggested that complement activation may contribute to autoimmune thyroid disease through interactions between thyroid autoantigens and complement proteins, thereby promoting inflammatory responses within thyroid tissue [22]. Nevertheless, circulating C4 concentrations may not accurately reflect local complement activation occurring within the thyroid gland, which may explain the absence of statistically significant differences in the present study.

The lack of statistical significance should be interpreted with caution because the relatively small sample size may have limited the ability to detect subtle differences in serum C4 concentrations. Therefore, further studies involving larger patient populations, assessment of thyroid autoantibodies and additional complement activation markers are recommended to better define the clinical significance of C4 in autoimmune thyroid disorders.

CONCLUSIONS

The present study demonstrated significant alterations in serum complement C3 levels among patients with thyroid dysfunction. Serum C3 concentrations were significantly increased in patients with hyperthyroidism and significantly decreased in patients with hypothyroidism compared with healthy controls, suggesting an association between C3 and the immunological alterations accompanying thyroid dysfunction. In contrast, serum C4 concentrations did not differ significantly among the study groups, indicating that C4 may be less sensitive than C3 in reflecting complement-related immune changes in autoimmune thyroid disorders. Although these findings support a potential association between complement activation and thyroid dysfunction, further multicenter studies with larger sample sizes, assessment of thyroid autoantibodies and additional inflammatory biomarkers are required to confirm these observations and better define the clinical significance of complement proteins in autoimmune thyroid disease.

Limitations

This study has several limitations that should be considered when interpreting the findings. First, the relatively small sample size may limit the generalizability of the results. Second, only female patients were included in the hypothyroidism group, resulting in an unequal sex distribution among the study groups. Third, thyroid autoantibodies, including anti-thyroid peroxidase (anti-TPO), anti-thyroglobulin (anti-Tg) and thyroid-stimulating hormone receptor antibodies (TRAb), were not measured, limiting the accurate immunological characterization of autoimmune thyroid disease. In addition, inflammatory cytokines and other complement activation markers were not evaluated. Future studies with larger, well-matched populations and comprehensive immunological assessments are recommended to further clarify the role of complement proteins in autoimmune thyroid disorders.

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