Al-Rafidain J Med Sci. 2025;9(2):284-289.
DOI: https://doi.org/10.54133/ajms.v9i2.2551

AJMS

Online ISSN (2789-3219)

Research Article

Comparative Role of Heat Shock Protein 70 and tTG Autoantibodies as Biomarkers in Diagnosis and Follow-up of Marsh 3 Celiac Disease

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Received: 5 October 2025; Revised: 20 November 2025; Accepted: 23 November 2025

Abstract

Background: Celiac disease (CD) is an autoimmune disorder characterized by an abnormal immune response to gluten, leading to intestinal mucosal damage. Anti-tissue transglutaminase (anti-tTG) antibodies are well-known ways to diagnose CD. However, it is still not clear what role heat shock protein 70 (HSP70) plays in the disease and protecting the mucosa. ***Objective***: To evaluate serum and tissue levels of anti-tTG antibodies and HSP70 in patients with celiac disease and assess their potential as biomarkers for disease diagnosis and progression. ***Methods***: This case-control study included 64 CD patients and 26 healthy controls from Al-Anbar province. Anti-tTG IgA and IgG serum levels were analyzed, and HSP70 tissue expression was assessed in samples from a duodenal biopsy. Histopathological findings were graded according to the Marsh classification. ***Results***: CD was more prevalent in females (56.5%) than males (37.5%). Serum anti-tTG IgA and IgG levels were significantly elevated in CD patients compared to controls and correlated with the degree of intestinal damage. Both antibodies showed a progressive increase from Marsh 3A to 3C, with Marsh 3C exhibiting the highest levels (tTG-IgA: 19.12 µg/mL; tTG-IgG: 13.44 µg/mL). In contrast, HSP70 tissue expression was low or absent in 53.2% of CD patients, particularly in those with severe mucosal injury (Marsh 3C). ***Conclusions***: Anti-tTG antibodies remain useful for confirming CD diagnosis but are limited in predicting disease progression. HSP70 expression, which declines with increasing mucosal damage, may serve as a complementary biomarker reflecting cellular stress, mucosal integrity, and early disease activity.

Keywords: Anti-tTG, Celiac disease serology, Heat shock protein-70, Marsh classification.

الدور المقارن لبروتين صدمة الحرارة 70 والأجسام المضادة الذاتية tTG كمؤشرات حيوية في تشخيص ومتابعة مرض السيلياك في مارش 3

الخلاصة

الخلفية: مرض السيلياك (CD) هو اضطراب مناعي ذاتي يتميز باستجابة مناعية غير طبيعية للجلوتين، مما يؤدي إلى تلف الغشاء المخاطي للأمعاء. الأجسام المضادة للنسيج الترانسجلوتاميناز (Anti-tG) هي طرق معروفة لتشخيص المرض. ومع ذلك، لا يزال من غير الواضح ما هو الدور الذي يلعبه بروتين الصدمة الحرارية 70 (HSP70) في المرض وحماية الغشاء المخاطي. **الهدف**: تقييم مستويات الأجسام المضادة المضادة ل tGG و HSP70 في المصل والأنسجة لدى مرضى السيلياك وتقييم إمكاناتها كمؤشرات حيوية لتشخيص المرض وتطوره. **الطرائق**: شملت هذه الدراسة حالة وشاهد 64 مريضا من مرضى CD و26 ضابطا صحيا من محافظة الأنبار. تم تحليل مستويات مصل مضاد tTG IgA وIgG، وتم تقييم تعبير أنسجة HSP70 في عينات من خزعة الاثني عشر. تم تصنيف النتائج النسيجية المرضية وفقا لتصنيف مارش. **النتائج**: كان مرض السيلياك أكثر انتشارا بين الإناث (56.5٪) مقارنة بالذكور (37.5٪). كانت مستويات IgA وIgG المضادة ل tG في المصل مرتفعة بشكل ملحوظ لدى مرضى CD مقارنة بالشواهد، وكانت مرتبطة بدرجة تلف الأمعاء. أظهرت كلا الجسيمات المضادة زيادة تدريجية من مارش 3A إلى 3C، مع أعلى مستويات من مارش 3C (tTG-IgA: 19.12 ميكروغرام/مل؛ tTG-IgG: 13.44 ميكروغرام/مل). في المقابل، كان تعبير أنسجة HSP70 منخفضا أو غائبا في 53.2٪ من مرضى CD، خاصة في الحالات التي عانت من إصابة مخاطية شديدة (مارش 3C). **الاستنتاجات**: تظل الأجسام المضادة المضادة ل tTG مفيدة لتأكيد تشخيص CD لكنها محدودة في التنبؤ بتقدم المرض. قد يعمل تعبير HSP70، الذي يتراجع مع زيادة تلف الغشاء المخاطي، كمؤشر حيوي مكمل يعكس الإجهاد الخلوي، وسلامة المخاط، والنشاط المبكر للمرض.

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Article citation: Abdulsattar HA, Abdulateef YM, Farhan AS. Comparative Role of Heat Shock Protein 70 and tTG Autoantibodies as Biomarkers in Diagnosis and Follow-up of Marsh 3 Celiac Disease**.** *Al-Rafidain J Med Sci.* 2025;9(2):284-289. doi: https://doi.org/10.54133/ajms.v9i2.2551

INTRODUCTION

Celiac disease (CD) is a complex immune-mediated enteropathic disorder that occurs due to lack of tolerance to indigestible gluten peptides in genetically susceptible individuals with specific HLA genes. Gluten peptides hurt the gut lining by setting off an incorrect immune response. This leads to long-lasting inflammation, different levels of damage to the gut lining, and the production of certain autoantibodies [1]. It affects about 1% of people worldwide, with a recent increase in its incidence as a result of greater awareness and testing [2]. The development of celiac enteropathy and its clinical manifestation happen together with the emergence of serum antibodies with special diagnostic concerns of the IgA and IgG classes against tissue transglutaminase (anti-tTG) [3]. The sure diagnosis of celiac disease is based on duodenal biopsies that show the particular diagnostic triad of histological abnormalities in addition to its characteristic autoantibodies. Histological mucosal damage degree and autoantibodies can both be utilized as indicators for celiac disease diagnosis and monitoring [4]. HSP70 is a member of a highly conserved chaperone family of

HSPs that play several biological functions in both health and disease. HSP 70 is a vital cytoprotective protein that protects the intestinal mucosa from the damaging effects of numerous stressors. HSP70 preserves the tight junction integrity of the intestinal barrier to function under stressful conditions. [5]. HSP70's compartmentalization and biological functions are antagonistic; eHSP70 encourages the destruction of cells and tissues, while iHSP70 plays protective roles when an organism is under stress, triggering and mediating the necessary physiological changes to maintain homeostasis and ensure survival [6]. The fact that HSP70 functions as an endogenous stress sensor and can be found in the extracellular (eHSP70) area further complicates this topic [7]. HSP70 has an immunomodulatory function; it can increase the production of pro-inflammatory cytokines. Therefore, in response to cellular stresses like infection or inflammation, its serum level and its tissue expression are modulated and changed [8]. The aim of the present study is to evaluate HSP70's role in modulating immune responses, which are critical in the context of celiac disease pathophysiology, and its probable useful usage as a biomarker for CD diagnosis and follow-up compared to traditional autoantibodies (anti-tissue transglutaminase) through serological and histological evaluation.

METHODS

Study design and setting

This case-control study was conducted from January 1 to July 1, 2024, at the Internal Medicine and Endoscopy Unit of Ramadi Teaching Hospital. It included 85 individuals presenting with malabsorption symptoms suggestive of celiac disease (CD), such as chronic diarrhea, abdominal pain, weight loss, anemia, and distension. ELISA and CHORUS TRIO techniques were used to test all of the participants for anti-tTG IgA, anti-tTG IgG, and HSP70. None had elevated total IgE or IgG4 levels.

Inclusion criteria

All patients with gastrointestinal manifestations suggesting features of malabsorption, with special concern for celiac disease, are included.

Exclusion criteria

All patients with documented autoimmune disease, intestinal infections, and immunological disorders, including IgA deficiency, high levels of total IgE, and IgG4 that may interfere with serological tests, are excluded.

Sample size calculation

Based on previously reported data (average 73 CD cases/year across two local hospitals), a sample of 64 CD patients was calculated to achieve a 95% confidence level with a 5% margin of error.

Outcome measurements

Patients with confirmed CD based on serology and duodenal biopsy (classified by Marsh-Oberhuber criteria) also underwent immunohistochemistry (IHC) on formalin-fixed, paraffin-embedded biopsy samples to assess HSP70 expression in the duodenal mucosa. The IHC results were scored as negative, low, moderate, or high based on how intense the staining was and where it was found in the epithelium and lamina propria. A 5 mL venous blood sample was collected from each participant and healthy volunteer, allowed to clot at room temperature, and processed for serological assays.

Ethical considerations

The Ethics Committees of the University of Anbar and Anbar Health Directorate approved the study protocol.

Statistical analysis

Data were analyzed using GraphPad Prism v8. Normality was assessed, and appropriate tests (t-test, Mann–Whitney, one-way ANOVA, Wilcoxon, and Spearman's correlation) were applied. Results are expressed as mean ± SD, with statistical significance set at $p < 0.05$.

RESULTS

Out of the 85 people who were first tested, 64 were confirmed to have celiac disease (CD) based on a positive anti-tTG IgA/IgG test and the typical histopathological triad found on a duodenal biopsy. The remaining 21 patients, who lacked histological evidence of CD, served as disease controls for histological comparisons. Additionally, 26 healthy volunteers were included as serological controls. Among the 64 CD patients, females predominated (40 cases, 62.5%) compared to males (24 cases, 37.5%). The most affected age group was 10 to 19 years (20 patients, 31.3%), then 20 to 29 years (17 patients, 26.6%), and finally 30 to 39 years (15 patients, 23.4%). The mean age of CD patients was 23.3 ± 11.5 years (range: 2–47), with no statistically significant difference in age distribution between CD patients and healthy controls. The serological level of autoantibodies was significantly elevated in celiac disease patients compared to the healthy control group. The celiac patients had much higher tTG-IgA levels (9.188 ± 8.193 AU/mL) and a median of 6.20, compared to the healthy control group, which had a mean of 4.531 ± 0.875 and a median of 4.50. Similarly, tTG-IgG levels were significantly elevated in patients with celiac disease (mean: 8.038 ± 5.051; median: 6.70) with a range of 3.30–25.10 compared to controls (mean: 3.931 ± 0.802; median: 3.60), with a range of 2.80–5.40 for the non-celiac healthy control group. While HSP70 concentrations (2.892 ± 1.170 ng/ml) were significantly higher than those of the non-celiac group (1.770 ± 0.430 ng/ml) (Table 1).

Table 1: Characteristic levels of celiac disease tTG antibodies and HSP 70 in sera of patients and control

Variable	Celiac	Healthy control	*p-value*
TTG-IgA (AU/ml)	9.188±8.193 (3.24-39.00) [6.20]	4.531±0.875 (3.10-6.20) [4.50]	0.005
TTG-IgG (AU/ml)	8.038±5.051 (3.30-25.10) [6.70]	3.931±0.802 (2.80-5.40) [3.60]	0.0001
Heat Shock Proteins HSP 70 (ng/ml)	2.892±1.170 (1.186-5.538) [2.743]	1.770±0.430 (0.991-2.510) [1.650]	0.0001

Data were presented as Mean±SD (Range) [Median]

The serology results show a significant association of all serological biomarkers in the celiac disease group. The mean tTG-IgA concentration was 6.025 ± 2.106 µg/mL in patients with Marsh 3A and 7.442 ± 3.938 µg/mL in patients with Marsh 3B. On the other hand, those with Marsh 3C showed a significant and disproportionate rise in mean tTG-IgA levels (19.12 ±14.09 µg/mL). A similar pattern seen with anti-tTG IgG showed Marsh 3A had mean values of 5.940 ± 2.028 µg/mL, Marsh 3B had mean concentrations of 7.324 ± 3.687 µg/mL, and Marsh 3C reached 13.44 ± 7.705 µg/mL (Table 2).

Table 2: The correlation of age, Marsh and IHC HSP70 with serum level of autoantibodies and HSP70

Variables		n	tTG-IgA	tTG-IgG	HSP 70 (ng/ml)
Age (year)	<10	7	8.500±4.330 [7.20]	9.271±7.148 [7.10]	3.345±1.291 [3.340]
	10–19	20	8.847±9.638 [6.30]	7.827±5.327 [6.75]	3.045±1.209 [2.967]
	20–29	17	11.41±9.647 [7.70]	7.535±4.450 [6.40]	2.720±1.220 [2.740]
	30–39	15	7.587±6.909 [4.40]	8.040±5.245 [6.40]	2.669±1.016 [2.417]
	≥ 40	5	8.780±4.061 [8.40]	8.860±3.167 [7.70]	2.901±1.328 [2.696]
	***p*-value**		0.763	0.949	0.693
Marsh	3A	20	6.025±2.106 [5.35]	5.940±2.028 [6.40]	2.890±1.456 [2.340]
	3B	32	7.442±3.938 [6.55]	7.324±3.687 [6.75]	3.006±1.028 [2.920]
	3C	12	19.12±14.09 [15.5]	13.44±7.705 [12.95]	2.590±1.031 [2.364]
	***p*-value**		0.0001	0.0001	0.582
HSP70 Expression IHC	Negative	8	19.24±16.23 [18.15]	12.68±6.098 [12.95]	2.452±0.827 [2.298]
	Low	26	8.417±6.613 [5.70]	8.226±5.690 [6.85]	3.261±1.201 [3.032]
	Moderate	23	7.496±3.700 [6.40]	6.596±3.151 [6.40]	2.565±1.210 [2.174]
	High	7	6.129±2.445 [5.40]	6.771±3.791 [4.80]	3.099±0.943 [2.820]
	***p*-value**		0.001	0.024	0.123

Data were presented as mean±SD [Median].

Percentile analysis showed a consistent rightward shift in HSP70 levels among celiac disease patients compared to controls. The median HSP70 level in CD patients (2.743 ng/mL) was more than double that of healthy individuals (1.650 ng/mL). Notably, even the lowest 25% of CD patients (25th percentile: 1.965 ng/mL) had higher HSP70 levels than the middle value in the control group. This shows that HSP70 elevation is a common feature in all CD patients (Table 3).

Table 3: Serum levels of HSP70 in celiac and non-celiac groups

Variable		Celiac	Non-Celiac
Heat Shock Proteins HSP 70 (ng/ml)	Mean±SD	2.892±1.17	1.770±0.43
	Range	1.186-5.538	0.991-2.51
	Mode	2.92	1.575
	Percentile 5th	1.292	1.027
	25th	1.965	1.482
	50th (Median)	2.743	1.65
	75th	3.578	2.172
	95th	4.953	2.477
	99th	5.538	2.51
	p-value	0.0001	

Immunohistochemical analysis of duodenal biopsies from people with celiac disease (CD) and people who did not have CD showed that there was no statistically significant difference in the expression of HSP70 in the tissues (p= 0.772). Based on the amount of staining and where it was found in the epithelium and lamina propria, HSP70 immunoreactivity was scored as negative, low, moderate, or high. (Figures 1-3). Among the 64 CD patients, histological staging revealed 31.3% (n=20) with Marsh 3A, 50.0% (n=32) with Marsh 3B, and 18.7% (n=12) with Marsh 3C. Notably, 53.2% of CD cases (34 patients) exhibited negative or low HSP70 expression, particularly in those with severe mucosal damage (Marsh 3C), suggesting a downregulation of this cytoprotective protein as epithelial injury progresses This inverse relationship between mucosal damage severity and HSP70 expression highlights its potential role as a marker of cellular stress and tissue integrity in CD (Table 4).

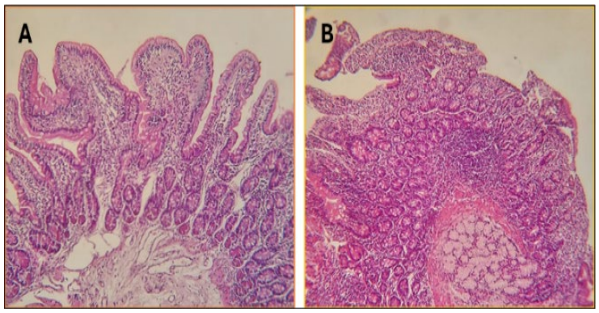

Figure 1: Celiac disease: **A**) Marsh 3A, H&E Stain, X10; **B**) Marsh 3C., H&E Stain, X10.

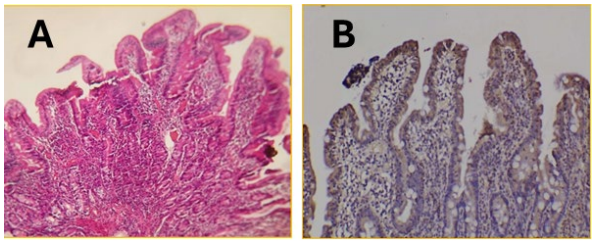

Figure 2: **A**) Celiac disease, Marsh 3B, H&E Stain, X25; **B**) Celiac disease, Marsh 3B, IHC-HSP70, X25.

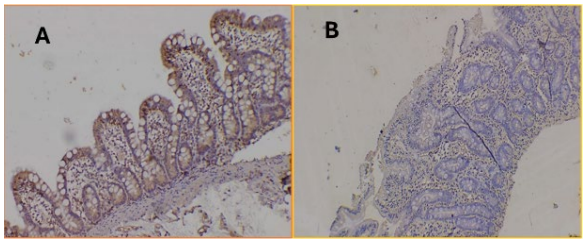

Figure 3: **A**) IHC-HSP70, CD; Marsh 3A; Positive X25. **B**) IHC-HSP70, A-CD; Marsh 3C, X25, Negative.

Table 4: IHC Expression of HSP70 intensity in celiac disease stages according to Marsh classification

Variable		Marsh			*p-value*
		3A	3B	3C	
HSP70 Expression IHC	Negative	1.0(5)	1.0(3.1)	6.0(60)	0.0001
	Low	3.0(15)	20(62.5)	3.0(25)	
	Moderate	11(55)	9.0(28.1)	3.0(25)	
	High	5.0(25)	2.0(6.3)	-	

Values are expressed as frequency and percentage. * Pearson Chi-square test at $p<0.05$.

DISCUSSION

Despite growing awareness of celiac disease (CD) and the role of gluten, diagnostic gaps persist; fewer than 20% of cases are accurately identified. The wide variability in serological results among CD patients reflects the heterogeneity of the humoral immune response to gluten, aligning with the disease's complex immunopathology. Table 1 showed highly significant differences in both tTG-IgA and tTG-IgG levels between celiac disease patients and controls, confirming their strong diagnostic value. tTG-IgA showed high discriminatory power, consistent with its established role, while tTG-IgG showed even greater statistical significance, highlighting its reliability, especially in IgA-deficient individuals who may yield false-negative tTG-IgA results. In line with recent research [9], these results support the use of both antibodies as strong serological biomarkers for screening, diagnosing, and keeping an eye on people's dietary compliance. The study demonstrates a clear, stage-dependent increase in tTG-IgA and IgG levels corresponding to worsening mucosal damage across Marsh 3 sub-stages—peaking in Marsh 3C—confirming that histological severity drives serological antibody burden, not random variation. [10]. This supports the view that humoral autoimmunity in celiac disease is a secondary response to prolonged gluten exposure and progressive mucosal injury, rather than an initial trigger. As expected from previous research, antibody levels are low or nonexistent in the early stages (Marsh 0–2) and rise significantly with advanced damage. This supports the use of tTG antibodies to measure how bad the disease is and their role in the first screening and diagnosis [11]. The development of antibodies for celiac disease requires structural damage to the gut mucosa, in contrast to traditional organ-specific autoimmune diseases where loss of self-tolerance occurs without an external trigger [12]. This makes it better classified as an antigen-driven, immune-mediated enteropathy rather than a primary autoimmune disorder. Some important pieces of evidence are that the damage can be fixed by cutting out gluten, that the disease is dependent on gluten, and that disease-specific antibodies are only made in the gut after mucosal injury, not before [13]. These points underscore gluten—not a breakdown in self-tolerance—as the central driver of immunopathology, contrasting with views that label celiac disease as a typical autoimmune condition [14]. These findings support a more complex disease model in which the production of antibodies functions as a biomarker of disease activity and the burden of mucosal damage rather than the primary pathogenic mechanism and suggest a celiac disease place in the area between food hypersensitivity and autoimmunity [15]. When gut cells are stressed but still alive, they make a protective protein called HSP70. This protein has an inverse relationship with antibody levels: tissue that didn't have any HSP70 had high levels of tTG-IgA (mean 19.24 U/mL) and tissue that did have high levels of HSP70 had lower levels (mean 6.13 U/mL). As the disease gets worse, this pattern probably shows as the damage to the mucosa gets worse, HSP70-producing cells die off through cell shedding and apoptosis, which lowers the amount of HSP70 that can be detected just as antibody levels peak [16]. The absence of HSP70 in intestinal tissue reflects a loss of viable cells capable of mounting a stress response, not a lack of stress itself, while ongoing antibody production may be driven by immunological memory and persistent gluten exposure. Although HSP70 levels showed significant association with celiac disease status in statistical analyses, suggesting potential as a biomarker, Table 2 results reveal high variability among patients (wider range and higher standard deviation). This indicates that the intensity of the cellular stress response differs individually, likely influenced by factors like disease duration, extent of gut damage, genetics, and environmental stressors. HSP70 expression was analyzed in duodenal biopsies across Marsh stages 3A to 3C, which reflect increasing mucosal damage in celiac disease. A highly significant link was found between rising HSP70 levels and more advanced Marsh stages (p= 0.0001), suggesting that HSP70 upregulation is biologically tied to worsening intestinal injury. In Table 4, HSP70 expression is highest in early mucosal damage (Marsh 3A), and 80% of patients show moderate to high levels. This shows that cells are responding strongly to stress. This declines significantly in more advanced stages: only 34.4% in Marsh 3B and 25% in Marsh 3C showed moderate

expression, with no high expression in Marsh 3C, suggesting a loss of the gut's ability to mount a stress response as tissue damage worsens. These findings show that HSP70 is most strongly induced in early mucosal injury, when epithelial cells are stressed but still viable. As damage progresses to Marsh 3B and 3C, HSP70 expression in the gut tissue declines due to loss of functional enterocytes. This drop in mucosal HSP70 levels is different from rising serum levels. This is likely because HSP70 is released into the bloodstream when stressed cells die and break. This shows a major difference: tissue HSP70 shows active cellular defense, while serum HSP70 shows cumulative cell damage and death. The failure of local stress adaptation does not preclude systemic biomarker elevation. In fact, the elevated serum HSP70 likely reflects the cumulative burden of epithelial damage and immune activation, rather than a functional cytoprotective response that plays a major role in HSP70, as confirmed by another recent study [17]. On the other hand, long-term antigen stimulation might throw off the heat shock response pathway, making it harder for HSP70 to be activated even though tissue damage is still happening. So, mucosal HSP70 expression (IHC) is not a good way to tell the difference between celiac and non-celiac people but circulating HSP70 is a strong biomarker of damage to the lining of the intestines and disease activity. However, the declining trend in HSP70 levels with increasing histological severity implies that HSP70 upregulation in early Marsh stages represents an early adaptive response to cellular stress in celiac disease pathogenesis and not a hallmark of late-stage disease. The statistical analysis revealed a P-value of 0.0001, which is extremely significant and supports a frank biological difference related to the disease state's chronicity and activity. In individuals with active celiac disease, release of HSP70 as a damage-associated molecular pattern (DAMP) may worsen inflammation and aid in triggering the activation of the innate immune system [18]. The results of autoantibody and HSP70 may give a good picture of how celiac disease starts, with the immune system of the patient making strong antibodies against gliadin, the gluten antigen, and its deamidated form, as well as tissue transglutaminase, the autoantigen that has been changed by enzymes. The amount of damage to the mucosa is inversely related to the expression of HSP70. This is probably because cell death is more important than stress-induced induction. These findings point to a paradox in the heat shock response in advanced celiac disease, even though HSPs are typically produced under stress. This highlights how critical disease stage and sampling timing are when examining HSP profiles as markers of intestinal stress or immune activation. This shows how important it is to look at the structure and make-up of cells when looking at HSP70 expression profiles. This is especially true in chronic inflammatory diseases where structural breakdown can throw off measurements of molecular biomarkers, as opposed to chronic non-ulcerative inflammatory disorders where HSP70 expression stays high [5]. The difference between high levels of HSP70 in the blood and low levels of HSP70 IHC in the mucosa brings to light an important pathophysiological paradox (Figures 2 and 3). The distinction between blood and tissue HSP70 levels highlights a crucial concept in mucosal immunology: Systemic development of biomarkers is not ruled out when local stress response fails, and the raised blood HSP70 most likely represents the combined weight of immune activation and epithelium injury rather than being a positive cytoprotective response.

Conclusion

While mucosal HSP70 expression (as IHC) lacks diagnostic discriminative power, circulating HSP70 becomes a significant biomarker of intestinal epithelial damage and disease activity.

ACKNOWLEDGEMENT

The authors thank the Medical Research Ethics Unit at the University of Anbar and the Anbar Health Directorate for their follow-up and facilitation of completing this work.

Conflict of interests

The authors declared no conflict of interest.

Funding source

The authors did not receive any source of funds.

Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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