

Role of heat shock proteins and autoantibodies as biomarkers in advanced grade celiac disease

Hiba Ali Abdulsattar ^{1*}, Yasir M. Abdulateef ², Ahmad Sami Farhan ¹

¹Biology Department, College of Science, University of Anbar, Anbar, Iraq

²Medical College, University of Anbar, Anbar, Iraq

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ABSTRACT

Celiac disease is an autoimmune-mediated disorder triggered by gluten consumption in genetically predisposed individuals, leading to villous atrophy and malabsorption. Although duodenal biopsy remains a key diagnostic tool, the role of heat shock proteins (HSPs) in mucosal injury and recovery has not been fully clarified. This study aimed to examine the relationship between celiac disease-specific autoantibodies and the expression levels of heat shock proteins (HSP70, HSP72, and HSP75) in patients with celiac disease and to correlate these findings with histopathological grades (Marsh 3A–3C). This case-control study included 64 newly diagnosed patients with celiac disease and 26 non-celiac controls from Al-Anbar Governorate, Iraq. Serum anti-tissue transglutaminase antibodies (tTG-IgA and tTG-IgG), anti-gliadin antibodies (IgA and IgG), and anti-deamidated gliadin peptide antibodies (DGP-IgA and DGP-IgG) were measured by ELISA using the Chorus TRIO system. Serum HSP70 and HSP72 levels were measured by ELISA, whereas HSP70, HSP72, and HSP75 expression in duodenal biopsies was assessed by immunohistochemistry (IHC) according to the Marsh–Oberhuber classification. Statistical analysis was performed using SPSS version 29.0, with significance set at $p < 0.05$. All measured autoantibodies and serum HSP markers were significantly higher in patients with celiac disease than in controls ($p < 0.05$). The severity of mucosal damage was associated with increasing antibody titers, with the highest levels observed in Marsh 3C cases. Serum HSP70 and HSP72 levels were generally increased in patients with celiac disease; however, they did not differ significantly among the Marsh subgroups. IHC analysis showed that HSP70 and HSP72 expression decreased with increasing villous atrophy, while HSP75 expression showed no significant trend. Celiac disease-specific autoantibodies were strongly associated with histological severity, supporting their use as practical non-invasive biomarkers. Changes in HSP70 and HSP72 expression suggest their involvement in mucosal stress responses and disease progression, indicating their potential value as future therapeutic or predictive biomarkers in celiac disease.

Corresponding author

Hiba Ali Abdulsattar
hib21s1017@uoanbar.edu.iq

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1 INTRODUCTION

Celiac disease (CD) is a chronic inflammatory, gluten-sensitive enteropathy triggered by gliadin and related prolamins in wheat in genetically susceptible individuals, resulting in flattening of the intestinal mucosa [1]. Histologically, CD is characterized by varying degrees

of intestinal villous atrophy, crypt cell hyperplasia, and lymphocytic infiltration [2]. Although the progression and regression phases of the disease cannot be predicted solely from intestinal mucosal histology, reduced mucosal integrity and surface area interfere with nutrient absorption, leading to clinical symptoms of malabsorption [2,3].

The incidence of CD is progressively increasing because of both improved diagnostic accuracy and a genuine rise in disease occurrence [4]. Currently, the only available treatment is strict adherence to a gluten-free diet (GFD), which aims to achieve complete mucosal recovery and ensure a favorable prognosis [5].

Gluten-derived peptides can activate a harmful immune response by stressing intestinal epithelial cells. Heat shock protein (HSP)-dependent complexes appear in the extracellular space and bind to specific surface molecules on antigen-presenting cells, which internalize these complexes through receptor-mediated endocytosis [6]. This process leads to antigen presentation by major histocompatibility complex (MHC) molecules to lymphocytes, thereby activating the T-cell pathway. HSPs stimulate the production of inflammatory cytokines, including interleukin (IL)-15, IL-18, and IL-21, which are involved in CD pathogenesis. Consequently, HSPs act as danger signals for the immune system at sites of tissue injury [7].

Although the incidence and prevalence of CD are increasing, its pathogenesis and progression remain incompletely understood [8]. Clinical and serological correlations are required in addition to duodenal biopsy, which should be performed regardless of positive CD serology, especially in cases with high clinical suspicion, to establish a definitive diagnosis. Histological diagnosis is established in the presence of Marsh 2 lesions, whereas Marsh 1 is insufficient for diagnosing CD; therefore, this study focused on the Marsh 3 category [9].

Celiac disease is characterized by continuous mucosal injury, and no validated non-biopsy method is currently available to confirm the status of the duodenal mucosa in newly diagnosed CD. Therefore, biopsy remains the gold standard for diagnosis and for grading the mucosal changes accompanying CD. These changes may be defined by a villous-to-crypt ratio of 3:1 or higher, shortened or absent villi, hyperplastic crypts, and an increased number of intraepithelial lymphocytes (IELs). These features represent the basic components of the Marsh classification system, a histological grading system used to diagnose and assess the severity of mucosal damage in the small intestine, particularly in CD [10, 11]. However, duodenal mucosal histology is not routinely used for follow-up during the course of therapy. Most sources rely on clinical data as the only marker of mucosal status to avoid repeated duodenoscopy, although the reliability of this approach in predicting mucosal recovery has not been fully evaluated [2, 10].

The histological features of CD occur along a continuum, ranging from nearly normal villous architecture at one end of the spectrum to flat mucosa with atrophied villi at the other [12]. Pathologists' awareness of the expected mucosal findings in follow-up biopsies of CD is important for patient management, as persistent mucosal injury is common in CD and may occur with discordant villous and IEL normalization [13].

Symptoms may occur at any point along this histological continuum. Patients usually progress through earlier stages of the spectrum and then regress through these lesions during healing after GFD restriction, which is essential for symptom remission and intestinal recovery [14]. However, limited knowledge about potential gluten-containing ingredients on food labels and the risk of cross-contamination make strict adherence difficult, especially when eating outside the home [15].

A patient who has normal Marsh 0 or only mildly enteropathic Marsh 1 duodenal biopsies but remains at risk of developing CD, as indicated by serological positivity and compatible human leukocyte antigen (HLA) status, is described as having potential celiac disease (PCD) [16]. Currently, CD rarely presents with severe malabsorption and advanced histology with complete villous flattening. At the same time, mild enteropathy is often overlooked and considered nonspecific rather than being further investigated [12].

Celiac disease is primarily diagnosed using serological antibody tests, including anti-tissue transglutaminase (tTG) antibodies, antigliadin antibodies, and anti-deamidated gliadin peptide antibodies [17]. However, existing antibody tests do not always provide sufficient sensitivity and specificity; therefore, the characteristic histopathological changes observed in duodenal biopsy remain the diagnostic gold standard for the primary diagnosis of CD [18].

Gliadin can induce oxidative stress responses. Increased inducible nitric oxide synthase and nitric oxide levels contribute to subsequent mucosal damage, leading to villous atrophy in CD. HSPs are involved in this process through several mechanisms [19]. Oxygen free radicals, such as superoxide, can induce HSP expression and participate in cellular defense against oxidative stress. HSPs protect small intestinal mucosal cells from H₂O₂-induced injury by enhancing the cytoprotective function of small intestinal epithelial cells [20, 21].

When exposed to stress, viable cells undergo cytoskeletal disruption and rapidly activate protective mechanisms known as the "stress response." These cellular

mechanisms enable repair and support survival after exposure to various stressors [22]. The main effectors belong to a highly conserved family of heat shock proteins. HSPs are categorized into several families, and their names are based on molecular weight. Among the best-known and most extensively investigated HSPs is the HSP70 family [23]. Members of the HSP70 family play crucial roles in targeting misfolded proteins for proteasomal degradation, facilitating the renaturation of damaged proteins, and providing cells with a natural mechanism of protection against environmental stressors [24].

Upregulation of HSP70 family members, including HSP70, HSP72, and HSP75, in the duodenal mucosa has been closely associated with post-translational repair processes [25, 26]. Their vital cytoprotective capacity is mediated by their function as molecular chaperones, and altered expression in CD specimens has been previously reported [25, 27].

HSP70 proteins are central components of the cellular network of molecular chaperones and folding catalysts [24]. HSP72 is localized mainly in villous enterocytes and immune cells of the lamina propria [28]. HSP72 has antiapoptotic effects and plays an important role in defense against gliadin-mediated cytotoxicity, thereby promoting epithelial cell survival. As a danger signal for immune cells, HSP72 may contribute to protection against tissue damage. In CD, HSP72 may modify the immune response against gluten peptides and alter intestinal barrier integrity. HSP72 levels are lower in the duodenal mucosa of treated CD patients than in untreated CD patients, although they remain higher than those in controls [29, 30]. HSP75 is a chaperone with ATPase activity that is involved in maintaining mitochondrial function. It is also considered a negative regulator of mitochondrial respiration and can modulate the balance between oxidative phosphorylation and aerobic glycolysis [31].

This study was designed to evaluate the role of autoantibodies and HSPs in newly diagnosed CD and to correlate these findings with duodenal biopsy grading and HSP expression.

2 MATERIALS AND METHODS

2.1 Study design and participants

This case-control study was conducted between January and December 2024 at Al-Ramadi Teaching Hospital, Al-Fallujah Teaching Hospital, and selected private diagnostic centers in Al-Anbar Governorate, Iraq. The study included 90 participants: 64 patients diagnosed

with celiac disease (CD) and 26 control individuals.

Patients were enrolled based on gastrointestinal symptoms suggestive of malabsorption and positive celiac-specific serology for anti-tissue transglutaminase (anti-tTG) IgA/IgG or anti-deamidated gliadin peptide (DGP) IgA/IgG antibodies. The diagnosis was confirmed by duodenal biopsy and histopathological examination according to the Marsh-Oberhuber classification. Only patients with Marsh 3 lesions were included in the immunohistochemical evaluation of HSP70, HSP72, and HSP75.

The control group consisted of 26 age- and sex-matched individuals divided into two subgroups. The serum control group (n = 14) included healthy volunteers without autoimmune, infectious, hepatic, or metabolic disorders. The histological control group (n = 12) included patients who underwent upper gastrointestinal endoscopy for non-celiac dyspepsia or malabsorption and showed normal duodenal histology.

Exclusion criteria included autoimmune comorbidities, such as type 1 diabetes or autoimmune thyroiditis, chronic infections, liver disease, inflammatory bowel disease, and use of immunosuppressive drugs. Ethical approval was granted by the Ethics Committee of the University of Anbar (Ref. 174, dated 2 August 2024). Written informed consent was obtained from all adult participants and from the legal guardians of children.

2.2 Blood sample collection and handling

From each participant, 5 mL of venous blood was collected into plain tubes using sterile techniques. Samples were allowed to clot at room temperature for 15 min and then centrifuged at 1,500 rpm for 10 min to separate the serum.

The serum was divided into two aliquots. The first aliquot was used immediately for the detection of celiac-related autoantibodies, including anti-tTG, DGP, and anti-gliadin antibodies (AGA), using the Chorus TRIO ELISA system (Diasorin Diagnostica Senese, Italy). The second aliquot was stored at -20 °C for a maximum of 3 months, with no more than one freeze-thaw cycle, before analysis of HSP70 and HSP72 by ELISA.

2.3 Immunohistochemical analysis of HSP70, HSP72, and HSP75

Immunohistochemistry (IHC) was performed on formalin-fixed, paraffin-embedded duodenal tissue sections (4 µm thick). Sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval

(HIER) using 10 mM citrate buffer (pH 6.0) at 95 °C for 20 min in a water bath. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min, followed by incubation with 5% normal goat serum to prevent nonspecific binding.

Slides were incubated overnight at 4 °C with rabbit polyclonal antibodies specific for each heat shock protein (Elabscience, China). The antibody details were as follows:

HSP70: Catalog No. E-AB-12349, dilution 1:200, retrieved with citrate buffer (pH 6.0).

HSP72: Catalog No. E-AB-20041, dilution 1:150, retrieved with EDTA buffer (pH 8.0).

HSP75: Catalog No. E-AB-15110, dilution 1:100, retrieved with citrate buffer (pH 6.0).

After washing, a polymer-based HRP detection system (EnVision+ System, Dako, Denmark) was applied, and staining was visualized using 3,3'-diaminobenzidine (DAB) as the chromogen. Slides were counterstained with hematoxylin, dehydrated, and mounted for microscopic examination.

Positive control tissues included normal gastric mucosa for HSP70, tonsil for HSP72, and liver for HSP75. Negative controls were prepared by omitting the primary antibody. All slides were independently reviewed by two blinded pathologists, and interobserver agreement was assessed using Cohen's kappa coefficient ($\kappa > 0.8$) to ensure consistency.

2.4 Sample size determination

The sample size was calculated using the following formula for case-control studies:

$$n = \frac{(Z_{1-\alpha/2}/2\sigma^2)}{d^2}$$

where $Z_{1-\alpha/2} = 1.96$ (for a 95% confidence level, $\alpha = 0.05$), and $Z_\beta = 0.84$ (corresponding to a study power of 80%). The parameters σ and d represent the estimated standard deviation and the allowable margin of error, respectively. Based on these parameters and expected variations in HSP positivity between patients with CD and controls, the calculated minimum required sample size was 60 participants (30 per group). To enhance reliability and compensate for possible data loss, the final sample included 64 patients and 26 controls.

2.5 Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 29.0 and GraphPad Prism version

8.0. Data were first tested for normality using the Shapiro-Wilk test, which showed a non-normal distribution for several parameters due to small subgroup sizes, particularly in the Marsh 3C category ($n = 12$). Consequently, non-parametric tests were primarily applied. Comparisons between the CD and non-celiac control groups were performed using the Mann-Whitney U test, whereas comparisons among Marsh 3 subcategories (3A, 3B, and 3C) were analyzed using the Kruskal-Wallis test, followed by Dunn's post hoc correction for multiple comparisons. For categorical immunohistochemical data, group differences were evaluated using Pearson's chi-square (χ^2) test. Data are presented as mean $\pm$ standard deviation (SD). Effect sizes were calculated as $r = Z/\sqrt{N}$ for Mann-Whitney U tests and η^2 for Kruskal-Wallis analyses to estimate the magnitude of group differences. Statistical significance was set at $p < 0.05$ (two-tailed), with 95% confidence intervals applied throughout.

3 RESULTS

Of the 100 cases examined, 64 were confirmed as celiac disease (CD) cases within the Marsh 3 category, and 26 were classified as non-celiac controls. All tested parameters showed a significant association between CD and the presence of specific autoantibodies, as well as elevated HSP levels, compared with the control group, as shown in Table 1.

Table 1 summarizes the comparison of mean serum levels (mean $\pm$ SD), ranges, and median values of CD-related autoantibodies, including tTG-IgA, tTG-IgG, anti-gliadin IgA, anti-gliadin IgG, anti-deamidated gliadin peptide IgA, and anti-deamidated gliadin peptide IgG, as well as HSP70 and HSP72, between patients with CD and non-celiac controls. Patients with CD showed significantly higher mean concentrations of all tested antibodies and HSPs compared with the non-celiac group. Specifically, the mean tTG-IgA and tTG-IgG levels were 9.188 ± 8.193 U/mL and 8.038 ± 5.051 U/mL, respectively, compared with 4.531 ± 0.875 U/mL and 3.931 ± 0.802 U/mL in controls ($p = 0.005$ and $p = 0.0001$, respectively). Similarly, anti-gliadin IgA, anti-gliadin IgG, DGP-IgA, DGP-IgG, HSP70, and HSP72 concentrations were significantly elevated among patients with CD ($p < 0.05$ for all parameters).

Statistical analysis using Student's t-test confirmed that these differences between independent means were significant at the 0.05 level, indicating a strong relationship between CD and increased expression of both

Table 1 Characteristic levels of specific antibodies and heat shock proteins in patients with and without celiac disease

Parameter	Celiac disease	Non-celiac disease patients	P value
TTG-IgA	9.188 ± 8.193 (3.24–39.00)	4.531 ± 0.875 (3.10–6.20)	0.005#
TTG-IgG	8.038 ± 5.051 (3.30–25.10)	3.931 ± 0.802 (2.80–5.40)	0.0001#
ANTI-GLIA IgA	8.138 ± 4.679 (3.20–24.60)	4.265 ± 1.026 (3.00–6.20)	0.0001#
ANTI-GLIA IgG	6.877 ± 4.510 (3.10–27.40)	4.262 ± 0.797 (3.10–5.60)	0.004#
ADG-IgA	7.467 ± 4.415 (3.1–23.0)	4.138 ± 0.705 (3.1–5.3)	0.0001#
ADG-IgG	7.166 ± 4.988 (3.10–27.50)	4.127 ± 0.658 (3.10–5.30)	0.003#
Heat shock protein HSP70 (ng/mL)	2.892 ± 1.170 (1.186–5.538)	1.770 ± 0.430 (0.991–2.510)	0.0001#
Heat shock protein HSP72 (ng/mL)	5.378 ± 2.480 (2.345–15.245)	4.155 ± 1.142 (1.993–5.987)	0.018#

Note. Data are presented as mean ± SD (range).

A significant difference between the two independent means, as determined using Student's *t*-test at a significance level of 0.05.

Abbreviations: ADG, anti-deamidated gliadin; HSP, heat shock protein; IgA, immunoglobulin A; IgG, immunoglobulin G; SD, standard deviation; TTG, tissue transglutaminase.

disease-specific autoantibodies and HSPs.

The results showed a significant association between serum levels of CD-specific autoantibodies and the histopathological severity of mucosal damage, as classified by the Marsh 3 subcategories (3A, 3B, and 3C). Patients in the Marsh 3C category, characterized by total villous atrophy, exhibited markedly higher mean serum levels of all tested autoantibodies compared with those in Marsh 3A and Marsh 3B, as presented in Table 2.

Table 2 summarizes the distribution of serum levels (mean ± SD) of anti-tissue transglutaminase antibodies (tTG-IgA and tTG-IgG), anti-gliadin antibodies (IgA and IgG), and anti-deamidated gliadin peptide antibodies (DGP-IgA and DGP-IgG) across the different Marsh 3 grades of CD. A significant upward trend was observed with increasing histological damage. Specifically, the mean tTG-IgA level increased from 6.025 ± 2.106 U/mL in Marsh 3A to 19.12 ± 14.09 U/mL in Marsh 3C ($p=0.0001$), while the mean tTG-IgG level increased from 5.94 ± 2.028 U/mL to 13.44 ± 7.705 U/mL ($p = 0.0001$). Similarly, anti-gliadin IgA and IgG concentrations showed progressive elevation with increasing disease severity ($p = 0.001$ and $p = 0.0001$, respectively). Although DGP-IgA did not show a statistically significant difference among the groups ($p = 0.194$), DGP-IgG demonstrated a significant increase ($p = 0.007$).

Analysis of variance (ANOVA) confirmed that these differences among the three independent means were statistically significant at the 0.05 level, indicating that serum autoantibody concentrations were positively correlated with the degree of mucosal atrophy.

Table 3 illustrates the relationship between serum HSP70 and HSP72 levels and the histopathological severity of CD, classified according to the Marsh 3 subcategories (3A, 3B, and 3C). Although minor fluctuations in mean HSP levels were observed across the three categories, these differences were not statistically significant, as indicated by $p = 0.582$ for HSP70 and $p = 0.153$ for HSP72. The mean serum HSP70 concentration was 2.890 ± 1.456 ng/mL in Marsh 3A, 3.006 ± 1.028 ng/mL in Marsh 3B, and 2.590 ± 1.031 ng/mL in Marsh 3C. Similarly, the mean serum HSP72 levels were 6.269 ± 3.693 ng/mL, 4.962 ± 1.504 ng/mL, and 5.002 ± 1.783 ng/mL in Marsh 3A, 3B, and 3C, respectively.

These results suggest that, within the Marsh 3 category, serum HSP70 and HSP72 concentrations do not differ significantly with increasing degrees of villous atrophy. Although HSP levels were generally elevated in patients with CD compared with non-celiac controls, as shown in Table 1, their levels appeared to stabilize across the subgrades of advanced mucosal damage. This finding may indicate that HSP expression reaches a plateau once severe immune-mediated mucosal injury has occurred.

Statistical analysis using one-way ANOVA confirmed that there were no significant differences among the three independent means at the 0.05 significance level.

Immunohistochemical expression of HSPs in duodenal biopsies was assessed in patients with Marsh 3 CD, stratified into subcategories 3A, 3B, and 3C. Expression levels of HSP70, HSP72, and HSP75 were categorized into four grades: negative, low, moderate, and high, based on tissue protein expression. For HSP70, high expression

Table 2 Association between CD-specific antibodies and Marsh 3 categories of celiac disease

Celiac disease–Marsh grade		No.	TTG-IgA (U/mL)	Positive cut-off ≥ 10 U/mL	TTG-IgG (U/mL)	Positive cut-off ≥ 8 U/mL
Marsh category	3A	20	6.025 $\pm$ 2.106	3/20 positive (15%)	5.940 $\pm$ 2.028	4/20 positive (20%)
	3B	32	7.442 $\pm$ 3.938	6/32 positive (18.8%)	7.324 $\pm$ 3.687	8/32 positive (25%)
	3C	12	19.12 $\pm$ 14.09	9/12 positive (75%)	13.44 $\pm$ 7.705	10/12 positive (83.3%)
	P value		0.0001 [^]		0.0001 [^]	
Celiac disease–Marsh grade		No.	ANTI-GLIA IgA (U/mL)	Positive cut-off ≥ 7 U/mL	ANTI-GLIA IgG (U/mL)	Positive cut-off ≥ 7 U/mL
Marsh category	3A	20	6.530 $\pm$ 3.309	5/20 positive (25%)	5.475 $\pm$ 2.048	4/20 positive (20%)
	3B	32	7.506 $\pm$ 3.199	10/32 positive (31.3%)	5.839 $\pm$ 1.665	6/32 positive (18.8%)
	3C	12	12.50 $\pm$ 7.14	8/12 positive (66.7%)	11.98 $\pm$ 8.144	9/12 positive (75%)
	P value		0.001 [^]		0.0001 [^]	
Celiac disease–Marsh grade		No.	ADG-IgA (U/mL)	Positive cut-off ≥ 7 U/mL	ADG-IgG (U/mL)	Positive cut-off ≥ 7 U/mL
Marsh category	3A	20	6.435 $\pm$ 3.637	4/20 positive (20%)	5.460 $\pm$ 2.144	5/20 positive (25%)
	3B	32	7.403 $\pm$ 3.792	12/32 positive (37.5%)	6.820 $\pm$ 4.176	14/32 positive (43.8%)
	3C	12	9.358 $\pm$ 6.514	8/12 positive (66.7%)	10.93 $\pm$ 8.017	9/12 positive (75%)
	P value		0.194		0.007 [^]	

Note. Data are presented as mean $\pm$ SD.

[^] A significant difference among more than two independent means, as determined using the ANOVA test at a significance level of 0.05.

Abbreviations: ADG, anti-deamidated gliadin; ANOVA, analysis of variance; CD, celiac disease; IgA, immunoglobulin A; IgG, immunoglobulin G; SD, standard deviation; TTG, tissue transglutaminase.

was observed mainly in Marsh 3A (25%) and Marsh 3B (6.3%), whereas 50% of Marsh 3C cases showed negative expression. Moderate expression predominated in Marsh 3A (55%) and Marsh 3B (28.1%). Statistical analysis demonstrated a significant difference in HSP70 expression among the three subcategories ($p = 0.0001$, Pearson's chi-square test).

For HSP72, low expression was most frequent in Marsh 3B (62.5%) and Marsh 3C (50%), whereas negative expression was observed in 18.8% of Marsh 3B cases and 33.3% of Marsh 3C cases. Moderate expression was highest in Marsh 3A (60%). Differences in HSP72 expression across the Marsh subcategories were statistically significant ($p = 0.001$). In contrast, HSP75 expression showed less variability among the subcategories. Low expression predominated in Marsh 3A (65%), Marsh 3B (56.3%), and Marsh 3C (33.3%), whereas negative expression increased with severity, reaching 41.7% in Marsh 3C. These differences were not statistically significant ($p = 0.099$). Overall, these results indicate that HSP70 and HSP72 expression varied significantly with the severity of mucosal damage, whereas HSP75 showed no significant trend. This pattern suggests that certain HSPs may be more actively involved in the cellular stress response associated with progressive mucosal injury in

celiac disease.

Table 3 Association between heat shock protein levels and Marsh 3 categories of celiac disease

Marsh category		No.	HSP70 (ng/mL)	HSP72 (ng/mL)
Marsh	3A	20	2.890 $\pm$ 1.456	6.269 $\pm$ 3.693
	3B	32	3.006 $\pm$ 1.028	4.962 $\pm$ 1.504
	3C	12	2.590 $\pm$ 1.031	5.002 $\pm$ 1.783
	P value		0.582	0.153

Note. Data are presented as mean $\pm$ SD.

Abbreviations: HSP, heat shock protein; SD, standard deviation.

Histopathological analysis of duodenal biopsies from patients with CD showed a progressive pattern of mucosal damage across the Marsh 3 subcategories (3A–3C). As shown in Figures 1–3, hematoxylin and eosin (H&E)-stained sections revealed gradual villous atrophy, progressing from partial villous atrophy in Marsh 3A to subtotal villous atrophy in Marsh 3B and diffuse villous atrophy with complete mucosal flattening in Marsh 3C.

Immunohistochemical analysis showed that the expression levels of HSP70, HSP72, and HSP75 were closely associated with the extent of mucosal damage.

Table 4 Immunohistochemical expression of HSPs in duodenal biopsies across Marsh 3 categories of celiac disease

Celiac disease mucosal IHC	Expression	Marsh 3A		Marsh 3B		Marsh 3C		P value
		No.	%	No.	%	No.	%	
HSP70 IHC	Negative	1	5.0	1	3.1	6	50.0	0.0001*
	Low	3	15.0	20	62.5	3	25.0	
	Moderate	11	55.0	9	28.1	3	25.0	
	High	5	25.0	2	6.3	—	—	
HSP72 IHC	Negative	—	—	6	18.8	4	33.3	0.001*
	Low	5	25.0	20	62.5	6	50.0	
	Moderate	12	60.0	6	18.8	2	16.7	
	High	3	15.0	—	—	—	—	
HSP75 IHC	Negative	1	5.0	4	12.5	5	41.7	0.099
	Low	13	65.0	18	56.3	4	33.3	
	Moderate	5	25.0	10	31.3	3	25.0	
	High	1	5.0	—	—	—	—	

Note. *A significant difference between percentages was determined using Pearson's chi-square test (χ^2 test) at the 0.05 significance level.

Abbreviations: HSP, heat shock protein; IHC, immunohistochemistry.

Overall cytoplasmic expression showed moderate HSP70 and HSP72 expression and low HSP75 expression in Marsh 3A lesions. As mucosal damage advanced in Marsh 3B, HSP expression decreased. In the more severe Marsh 3C lesions, which showed marked villous epithelial atrophy and substantial epithelial cell loss, most samples demonstrated weak or absent immunoreactivity for all studied HSPs. These findings suggest that reduced HSP expression may be associated with the progression of mucosal damage in the later stages of CD.

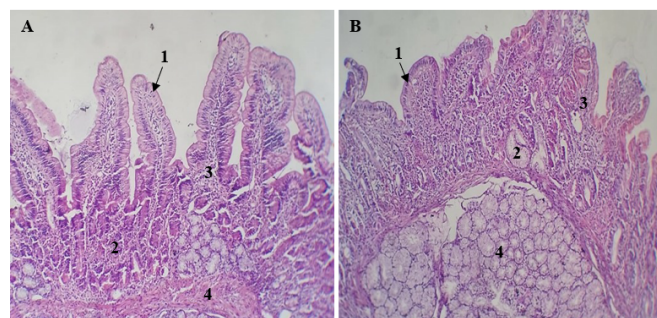


Fig. 1 Histopathological changes in duodenal mucosa of patients with Celiac Disease stained with Haematoxylin and Eosin (H&E) at 400× magnification. (A) Marsh 3A lesion showing partial villous atrophy with elongated crypts and increased intraepithelial lymphocytes. (B) Marsh 3B lesion showing subtotal villous atrophy and marked crypt hyperplasia. 1- Villous atrophy. 2- Crypt hyperplasia. 3- Mucosal lymphocytosis. 4- Brunner's gland.

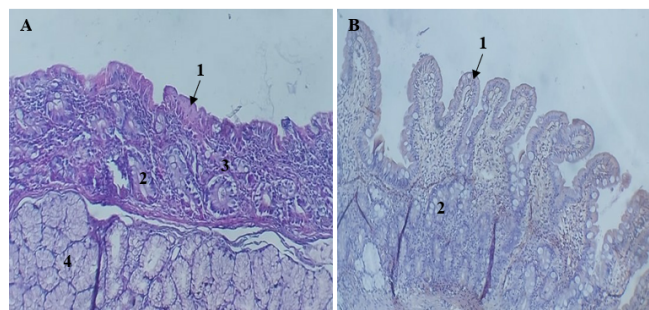


Fig. 2 Immunohistochemical (IHC) expression of HSP75 protein in duodenal biopsies of CD patients (400× magnification). (A) Marsh 3C lesion showing advanced mucosal damage with diffuse villous atrophy (H&E). (B) Marsh 3A lesion showing low cytoplasmic HSP75 expression in the epithelial lining. 1- Villous atrophy. 2- Crypt hyperplasia. 3- Mucosal lymphocytosis. 4- Brunner's gland.

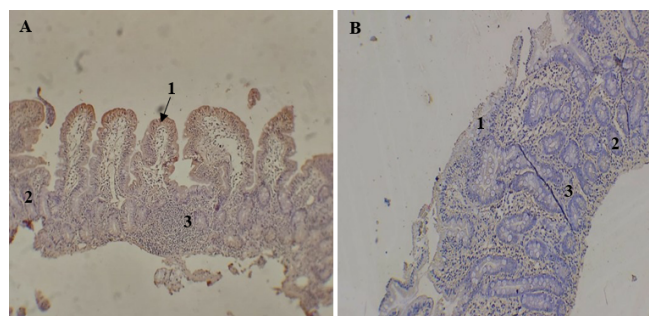


Fig. 3 IHC staining of duodenal mucosa for HSP70 and HSP72 proteins in Celiac Disease (10× magnification). (A) Marsh 3A lesion showing moderate positive cytoplasmic expression of HSP72. (B) Marsh 3C lesion with advanced villous atrophy showing negative HSP70 immunoreactivity. 1- Villous atrophy. 2- Crypt hyperplasia. 3- Mucosal lymphocytosis.

4 DISCUSSION

This study was conducted among patients with Marsh grade 3 celiac disease (CD), which represents advanced mucosal injury characterized by subtotal or complete villous atrophy. The primary objective was to determine the relationship between the degree of histopathological changes and the serological expression of disease-specific autoantibodies. The findings revealed that serum levels of anti-tissue transglutaminase antibodies (tTG-IgA and tTG-IgG), anti-gliadin antibodies (IgA and IgG), and anti-deamidated gliadin peptide antibodies (DGP-IgA and DGP-IgG) were significantly higher in patients with CD than in non-celiac controls, supporting their diagnostic value during active stages of the disease.

These findings are consistent with previous studies that verified the usefulness of these antibodies as biomarkers of immune-mediated mucosal damage in CD [4, 8]. Similarly, Abbasi et al. [1] and Voisine and Abadie [6] emphasized the central role of gluten-mediated immune responses and HLA-mediated mechanisms in autoantibody formation and subsequent villous atrophy. Brown et al. [2] and Patel et al. [13] also confirmed that the degree of mucosal damage, as assessed by the Marsh classification, is closely related to antibody titers, particularly tTG-IgA, which remains a key marker in serological diagnosis. The consistency of the current findings with these reports supports the relevance of the relationship between serological activity and histopathological damage in the pathogenesis and monitoring of CD.

An increasing trend in disease-specific autoantibody titers was observed across the Marsh 3 subgroups (3A–3C), indicating a strong relationship between serological activity and histopathological severity of mucosal injury. Patients classified as Marsh 3C, characterized by complete villous atrophy, had the highest levels of anti-tTG, anti-gliadin, and anti-DGP antibodies. This gradient supports the view that antibody production intensifies with progressive mucosal destruction, probably reflecting sustained immune activation in the intestinal mucosa. Similar trends were reported by [2, 13], who emphasized that high antibody levels, particularly tTG-IgA, are associated with higher histological grades of villous atrophy. In addition, persistent elevation of antibody levels has been reported in some studies despite adherence to a gluten-free diet (GFD), suggesting incomplete mucosal recovery and persistent subclinical inflammation [5, 9]. This is also supported by Lebwohl and Rubio-Tapia [4], who reported

that normalization of antibody levels does not necessarily correlate with complete histological healing, limiting their use as independent follow-up markers. Therefore, serological monitoring should not be interpreted independently of clinical and histological assessment, especially in patients with persistent symptoms despite dietary adherence.

Regarding HSPs, the present study showed significantly increased serum levels of HSP70 and HSP72 in patients with CD compared with non-celiac controls, suggesting increased cellular stress associated with sustained mucosal inflammation. These results are consistent with previous studies indicating that HSP induction acts as a compensatory mechanism aimed at maintaining intestinal epithelial integrity during inflammatory stress [23, 24, 28]. However, no statistically significant differences in HSP concentrations were observed among the Marsh 3 subgroups, suggesting that the expression of these chaperones may stabilize once mucosal injury reaches an advanced stage. This may reflect cellular exhaustion or reduced adaptive stress capacity of enterocytes after prolonged gluten-mediated immune activation. Similar findings were reported by Hoter and Naim [25] and Albakova et al. [31], who suggested that chronic inflammation can dysregulate HSP-mediated cytoprotective pathways and subsequently impair epithelial regeneration.

Immunohistochemical analysis further illustrated the spatial patterns of HSP expression in damaged duodenal mucosa. HSP70 and HSP72 showed moderate-to-high expression in Marsh 3A lesions, consistent with an active but still compensatory stress response. In contrast, their expression was reduced or absent in Marsh 3C lesions, which showed severe villous atrophy and enterocyte loss. This inverse relationship between HSP expression and the degree of mucosal damage supports the assumption that HSPs act as early protective chaperones that help stabilize epithelial junctions and regulate intestinal barrier permeability [6, 30]. Downregulation in advanced lesions is likely related to severe epithelial destruction, in which cellular repair mechanisms become insufficient. However, HSP75 expression did not show significant variation across the Marsh categories, suggesting that this isoform may be less responsive or more constitutively expressed during intestinal stress. Ghulam-Mohyuddin et al. [32] reached a similar conclusion, indicating that HSP70 contributes to maintaining tight junctions during inflammatory stress and that its loss is associated with epithelial hyperpermeability.

Together, these findings indicate complementary di-

agnostic and pathophysiological roles for serological and cellular stress biomarkers in CD. Active disease stages may be characterized by high antibody levels with variable or declining HSP expression, reflecting persistent immune activation and impaired cellular stress adaptation. These two biomarker profiles may be useful for disease monitoring by helping to assess immune activity and mucosal recovery in a less invasive manner. Such a combined approach could potentially reduce the need for repeated duodenal biopsies, which, although informative, are invasive and less feasible in pediatric populations [9, 17, 18].

5 CONCLUSION

Most patients with advanced CD, particularly Marsh 3B and Marsh 3C, showed increased autoantibody biomarkers, which may reflect persistent immune activity rather than disease occurrence alone. In contrast, patients with earlier lesions, such as Marsh 3A, may show lower or even negative antibody results, which could limit early diagnosis and accurate grading of mucosal damage. Early-grade disease, particularly Marsh 3A and Marsh 3B, was associated with increased heat shock protein expression, indicating an active cellular stress response. However, HSP expression decreased in advanced disease, especially Marsh 3C, possibly because of enterocyte exhaustion and severe epithelial loss. This pattern suggests that HSPs may contribute to mucosal defense and healing during the course of the disease.

For newly diagnosed patients, duodenal biopsy remains important for confirming diagnosis and grading mucosal injury. During follow-up, serological biomarkers and HSP levels may help monitor disease activity and mucosal recovery. However, biopsy remains necessary when malabsorption symptoms persist, when serological and clinical findings are discordant, or when complications are suspected.

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