Gene Expression of *NLRP3* Inflammasome in Celiac Disease of Iraqi Children

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Abstract

Celiac disease (CD) is an autoimmune disorder characterized by chronic inflammation that essentially affects the small intestine and is caused by eating gluten-containing foods. This study sought to determine gene expression of *NLRP3* Inflammasome in peripheral blood of Iraqi CD children using quantitative real-time PCR (qRT-PCR) assay. Thirty children with CD (12 males and 18 females) were enrolled in the study and their age range was 3-15 years. The diagnosis of the disease was confirmed by serological examinations and intestinal endoscopy. A control sample of 20 age-matched healthy children was also included. The children were stratified for age, gender, body max index (BMI), histological findings, and marsh classification. Further, the sera were examined for IgA anti-tissue transglutaminase (tTG) antibody, IgA anti-gliadin antibody, and interleukin-1 beta (IL-1β). Based on Marsh classification, the results revealed that the majority of patients (70%) had partial villous atrophy (Marsh Ⅲ 3A), while children with subtotal and total villous atrophy (Marsh III: 3B/3C) were presented with a lower frequency (30.0%). Neither Marsh I nor Marsh II has been observed among the patients studied. Serum levels of anti-tTG and anti-gliadin IgA antibodies were significantly higher in CD children than in control children (73.8 and 31.8 vs. 0.8 U//ml, respectively; $p < 0.001$). Conversely, IL-1β serum level was decreased in CD children but the difference was not significant (35.5vs. 53.4 pg/ml; p = 0.285). In the case of *NLRP3* inflammasome, the Relative Fold Change method ($2^{-\Delta\Delta Ct}$) was used to assess the gene expression. The results revealed that the expression of *NLRP3* inflammasome was decreased by 0.594 fold in CD children. In conclusion, the *NLRP3* inflammasome was down-regulated in the present sample of CD children, and it was accompanied by a decreased serum level of IL-1β.

Keywords :Celiac disease; *NLRP3* Inflammasome, IL-1β; Gene expression.

1. Introduction

Celiac disease (CD) is an autoimmune disorder that occurs in genetically predisposed individuals who develop an immune reaction to the ingestion of gluten-containing foods (wheat, barley, and rye) [1]. It is also characterized by chronic inflammation that primarily affects the small intestine and can lead to malabsorption of nutrients, chronic or intermittent diarrhea, growth failure or short stature, weight loss, iron deficiency, and osteopenia [2]. The global prevalence of CD is 1.4%, and it is more common in children than in adults. Further, the prevalence of CD varies with age, gender, and geographical location [3]. In Iraq, the prevalence was found to be 0.25% among healthy blood donors [4]. Human autoimmune

diseases affect 3-5% of the population and arise from complex interactions between genetic and environmental factors, which consequence in immune dysregulation and immune attack to target tissues [5]. The innate immune system works as the first line of defense in protection from pathogenic microbes and host-derived signals of cellular distress. One way in which these "danger" signals trigger inflammation is through activation of inflammasomes, which are multi-protein complexes that assemble in the cytosol after exposure to pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) [6]. They result in activation of caspase-1and subsequent cleavage of the pro-inflammatory cytokines IL-1β and IL-18, and this may be destructive to tissues; however, the specific regulatory mechanisms of *NLRP3* inflammasome activation remain unclear [7]. A role for the *NLRP3* inflammasome in recurrent and chronic inflammation was initially described in a group of rare auto-inflammatory conditions, such as rheumatoid arthritis, multiple sclerosis, and celiac disease [8]. Further, the *NLRP3* inflammasome has been implicated in many common diseases, including cancer, gout, and diabetes [9]. Being able to analyze gene expression patterns is essential for understanding protein function, biological pathways and cellular responses to external and internal stimuli [10]. To elucidate the role of the *NLRP3* inflammasome in celiac disease, this study examined the gene expression of *NLRP3* inflammasome in peripheral blood within the downstream regulatory region of the *NLRP3* gene for association with the expression of *NLRP3* in addition to the pro-inflammatory marker IL-1β.

2. Materials Method
2.1. Children studied
A total of 30 Iraqi children (12 males and 18 females; mean age ± SD: 9.60 ± 0.55 years) with CD attending the consultant clinic at the Central Pediatrics Hospital, Private Nursing Hospital, and Specialist Center for Endocrinology and Diabetes in Iraq-Baghdad during the period August 2018 - March 2019 were enrolled in this study. They were clinically examined and evaluated by the consultant medical staff at these hospitals, and the diagnosis was confirmed through serological and histological tests [11]. Serological diagnosis of CD was based on the presence of IgA antibodies against gliadin and tissue transglutaminase (tTG). This was followed by an examination of an intestinal biopsy. Cases seropositive for the two antibodies but their intestinal biopsy did not confirm the disease were excluded from the study. Children with giardiasis were also excluded. A control sample of 20 healthy children (12 male and 8 females; 9.99 ± 0.77 years) was included. They were randomly selected from healthcare units in Baghdad and had no clinical evidence or family history of CD or other autoimmune diseases.

2.2. Immunological parameters
Serum levels of anti-gliadin and anti-tissue transglutaminase test (anti-tTG) antibodies and IL-1β were measured using ELISA kits (Aeskulisa and Demeditec, respectively; Germany) and instructions of the manufacturer were followed.

2.3. Gene expression of *NLRP3* inflammasome
Gene expression of *NLRP3* inflammasome was determined by the reverse transcription-

quantitative polymerase chain reaction (RT-qPCR) method. Total RNA was isolated from peripheral using ready-to-use reagent (TRIzolTMLS Reagent; Thermo Fischer Scientific; USA). The isolated RNA was reverse-transcribed and the gene expression of NLRP3 inflammasome was assessed using GoTaq®1-Step RT-qPCR System kit (Promega, USA), and instructions of the manufacturer were followed. Forward and reverse primers for NLRP3 inflammasome (5'-AACAGCAGATGGAGAGTGGC-3' and 5'-GGTCGGACTCCTCAAACAGG-3', respectively) and the housekeeping gene GAPDH (5'-AGAAGGCTGGGGCTCATTTG-3' and 5'-AGGGGCCATCCACAGTCTTC-3', respectively) were according to published sequences. The primers were designed using primer design web-based service and validated using the Primer-BLAST-NCBI database. To determine gene expression of NLRP3 inflammasome (expression fold change), the $2^{-\Delta\Delta Ct}$ was calculated (Relative Fold Change). Thus, the expression was expressed as a fold change in the level of the target gene expression, which was normalized to the expression level of the housekeeping gene GAPDH (endogenous control) and relative to the target gene in control subjects (calibrator) [12].

2.4. Statistical analysis

The data was statistically analyzed using the statistical package for social sciences (SPSS version 19.0). Pearson Chi-square test or two-tailed Fisher exact test was used to analyzing categorical variables. For continuous variables and depending on the distribution of data, parametric variables were given as mean ± standard deviation (SD), and significant differences between means were assessed using the Student t-test. For non-parametric variables, median and interquartile range (IQR: 25-75%) were given and a significant difference between medians was assessed using either Mann–Whitney U test (to compare to groups) or Kruskal–Wallis test (to compare more than two groups). Receiver operating characteristic (ROC) analysis was employed to estimate the area under the curve (AUC) and optimum cut-off point that predicts CD. A probability (p) value ≤ 0.05 was considered statistically significant.

3. Results and Discussion

As shown in **Table (1)**, the highest incidence of CD was noticed in children aged 8-12 years (56.7%). This may indicate that the disease is more common in this age range. There is no specific explanation for such age-dependency, but delayed diagnosis and lack of awareness of disease due to the absence of any symptoms may account for underestimation of disease prevalence at a younger age [13]. Delayed onset of CD may also account for the development of disease later in life [14]. Thus, the majority of CD patients are diagnosed during adulthood [15]. The results revealed that female patients outnumbered male patients (60 vs. 40%) but the difference was not significant compared to controls (p = 0.248) **Table (1)**. It is generally agreed that CD is a female-predominant disease, but males had a shorter duration of illness before diagnosis and more severe manifestations of malabsorption [16]. The higher prevalence of CD among females could be explained by multiple factors such as hormones and genetics, the male Y chromosome might have a protective role [17]. Reasons for female predominance have not been well defined, but it has been proposed that females are at greater risk to develop immune-mediated diseases [18]. Regarding growth criteria, most of CD patients were of normal weight (5th to <85th; 63.3%) as shown in **Table (1)**. A study in Indian patients indicated that the BMI was significantly lower in CD patients compared to healthy children (17.18 vs. 21.2 kg/cm2; p <0.001), and there was no significant difference between

boys and girls with CD [19]. It has been found that BMI can be increased significantly after a gluten-free diet [20]. Generally, withdrawal of gluten from the diet leads to rapid catch-up growth of body weight within 6-12 months, whereas the height catches up is more gradually [21]. A Swedish study showed that the BMI median was slightly lower among the children with screening-detected CD compared to their healthy children, but most of the CD cases had a normal BMI [22]. In the case of March classification, the majority of patients had partial villous atrophy (Marsh ШA; 70%), while children with subtotal and total villous atrophy (Marsh III B and III C) were less frequent (30%) **Table (1).** In a previous Iraqi study, different frequencies were found (24% Marsh IIIA, 47.8% Marsh IIIB, and 28.2% Marsh IIIC respectively) [23]. The reason behind the increased frequency of Marsh III A might be related to an early diagnosis of the studied samples. Comparable frequencies have also been presented; 5.3% Marsh III C, 24.2%, 55.3% Marsh III-A, and 15.1% Marsh III B and Marsh III C [24]. In this study, serum concentrations of anti-tTG and anti-gliadin IgA antibodies were significantly elevated in CD children compared to healthy children (73.8 and 31.8 vs. 0.8 U//ml, respectively; $p < 0.001$) **Table (1)**. Consistent with these findings, [25] indicated that an anti-tTG is the marker of choice for CD mass screening and helpful in identifying patients who can benefit from a gluten-free diet and follow-up. Although the gold standard for detecting CD is a duodenal biopsy, it has been shown that anti-tTG antibody together with an anti-gliadin antibody is a sensitive marker for CD [26, 27]. The findings of this study and other studies indicate the diagnostic potential of both antibodies in CD when conducting screening surveys for the disease in children and adolescents [28, 29]. In a study of CD children 5 years old and younger, it has been reached that anti-gliadin IgA is suitable to test for diagnosing the disease, because anti-tTG antibodies may only appear in the elderly [30]. The study also pointed to the role of IL-1β in the pathogenesis of CD. The serum level of this cytokine was decreased in CD children compared to healthy children (35.5 vs. 53.4 pg/mL respectively); however, the difference did not attend a statistical significance (p = 0.285) **Table (1)**. Consistent with these findings, it has been demonstrated that serum levels of IL-1β and other cytokines (TNF-α, IL-2, IL-4, and IL-8) showed no significant differences between 3 years old CD children and matched children. The authors also concluded that a gluten-free diet may also influence the serum level of some cytokines [31]. In **Table (1)**, the gene expression of NLRP3 inflammasome was also given as a relative fold changing ($2^{-\Delta\Delta CT}$). The expression was down-regulated by 0.594 in CD children and this may suggest a role for the NLRP3 inflammasome in the immunopathogenesis of CD. To the best knowledge of investigators, this study was the first in Iraqi CD patients. It has been demonstrated that inflammasomes play a significant role in the pathogenesis of autoimmune diseases including CD [8].

Table 1. Anthropometric and laboratory data of celiac disease patients and controls.

Characteristic*	CD patients (N = 30)	Control (N = 20)	p

	Age (year)	9.6 ± 3.0	10.0 ± 3.4	0.666
Age groups (year)	< 8	8 (26.7)	5 (25.0)	0.768
	8 – 12	17 (56.7)	10 (50.0)	
	13 - 15	5 (16.7)	5 (25.0)	
Gender	Male	12 (40.0)	12 (60.0)	0.248
	Female	18 (60.0)	8 (40.0)	
BMI percentiles	< 5th (underweight)	8 (26.7)	1 (5.0)	0.121
	5th to < 85th (normal weight)	19 (63.3)	15 (75.0)	
	≥ 85th (overweight/obese)	3 (10.0)	4 (20.0)	
Marsh classification	Marsh III: 3A	21 (70.0)	NA	
	Marsh III: 3B/3C	9 (30.0)	NA	
IgA anti-tTG antibody (U/mL)		73.8 (26.7-526.1)	0.8 (0.5-1.5)	< 0.001
IgA and anti-gliadin antibody (U/mL)		31.8 (18.0-94.6)	0.8 (0.5-1.2)	< 0.001
IL-1β (pg/mL)		35.5 (28 -47.7)	53.4 (25.7-132)	0.285
NLRP3 gene expression (2$^{-\Delta\Delta Ct}$)		0.594 (0.120-2.018)	NA	

*Values are mean ± SD (standard deviation), number followed by percentage in parentheses or median with the interquartile (IQR) range (25% - 75%); CD: Celiac disease; BMI: Body mass index; tTG: Tissue transglutaminase; NLRP3: Nucleotide-binding oligomerization domain leucine-rich repeat and pyrin domain-containing protein 3; NA: Not applicable; p: Probability; Significant p is bold-marked.

To make a further understanding of the investigated immunological and molecular parameters in the pathogenesis of CD, the patients were distributed into subgroups according to some anthropometric and clinical characteristics **Table (2)**. No significant variations between the medians of anti-tTG and anti-gliadin IgA antibodies, IL-1β, and NLRP3 gene expression between subgroups of CD children were distributed according to age, gender, BMI, and March classification. Two exceptions were encountered. In the first, the decreased level of IL-1β was more pronounced in male patients compared to female patients and the difference was significant (24.7 vs. 37.6 pg/ml; *p* = 0.050). Although CD patients were children, sex hormones might have a role in deviating serum levels of IL-1β in males and females [32]. The second exception involved a significantly increased concentration of anti-tTG IgA antibody in CD children with Marsh III: 3B/3C classification compared to Marsh III: 3A children (501.6 vs. 36.8 IU; *p* = 0.022). This may suggest a correlation between anti-tTG IgA antibody and Marsh classification; however, the low sample size of patients may limit such suggestion.

Table 2. IgA anti-tTG antibody, anti-gliadin antibody, IL-1β, and *NLRP3* gene expression (2$^{-\Delta\Delta Ct}$) distributed according to anthropometric and clinical characteristics of celiac disease patients.

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Characteristic	Median with the interquartile (IQR) range (25% - 75%)			
	IgA anti-tTG antibody (U/mL)	IgA and anti-gliadin antibody (U/mL)	IL-1β (pg/mL)	NLRP3 gene expression ($2^{-\Delta\Delta Ct}$)
Age groups (year)				
< 8	7.0 (35.4 - 70.8)	35.4 (7.0 - 70.8)	36.9 (31.4 - 49.6)	1.214 (0.305 - 3.807)
8 – 12	29.0 (56.0 - 147.4)	56.0 (29.0 - 147.4)	36.2 (31.3 - 40.9)	0.300 (0.182 - 1.774)
13 - 15	17.2 (19.4 - 22.4)	19.4 (17.2 - 22.4)	24.4 (15.1 - 25.0)	0.051 (0.029 - 2.954)
p-value	0.537	0.088	0.155	0.353
Gender				
Male	39.2 (24.5 - 73.4)	22.4 (14.3 - 38.8)	24.7 (19.5 - 36.3)	0.191 (0.058 - 3.043)
Female	220.6 (29.9 - 529.2)	51.8 (25.9 - 123.7)	37.6 (31.4 - 49.6)	0.725 (0.246 - 1.657)
p-value	0.204	0.086	0.050	0.703
BMI percentiles				
< 5th (underweight)	28.3 (1.5 - 377.9)	29.6 (22.4 - 244.5)	37.6 (28.7 - 40.9)	0.422 (0.246 - 1.207)
5th to < 85th (normal weight)	183.7 (41.5 - 801.8)	31.0 (15.0 - 93.0)	32.6 (24.1 - 37.4)	0.508 (0.065 - 3.132)
≥ 85th (overweight/obese)	15.5 (14.5 - 16.4)	73.8 (47.5 - 100.0)	38.4 (29.0 - 47.8)	1.214 (0.887 - 1.540)
p-value	0.061	0.562	0.591	0.562
Marsh classification				
Marsh III: 3A	36.8 (18.1 - 226.5)	28.8 (15.0 - 48.6)	36.3 (29.0 - 40.9)	0.592 (0.176 - 2.010)
Marsh III: 3B/3C	501.6 (152.8 - 992.0)	93.4 (56.0 - 95.5)	33.1 (24.1 - 40.9)	0.775 (0.120 - 1.774)
p-value	**0.022**	0.110	0.635	0.839

CD: Celiac disease; BMI: Body mass index; tTG: Tissue transglutaminase; NLRP3: Nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain-containing protein 3; p: Probability; Significant p is bold-marked.

Receiver operating characteristic (ROC) curve analysis was performed to determine whether there was an additional advantage of using the study parameters for predicting CD. Both antibodies (anti-gliadin and anti-tTG IgA antibodies) occupied a significant area under the curve (AUC). It was 0.999 for the former antibody, while it was lower in the latter antibody (AUC = 0.956) with sensitivity and specificity of 100 and 95% and 91.7 and 90%, respectively. Whereas, IL-1β and NLRP3 gene expression did not occupy a significant AUC (0.398 and 0.627, respectively); therefore, their diagnostic performance in CD was limited **Table (3)** and **Figure (1)**.
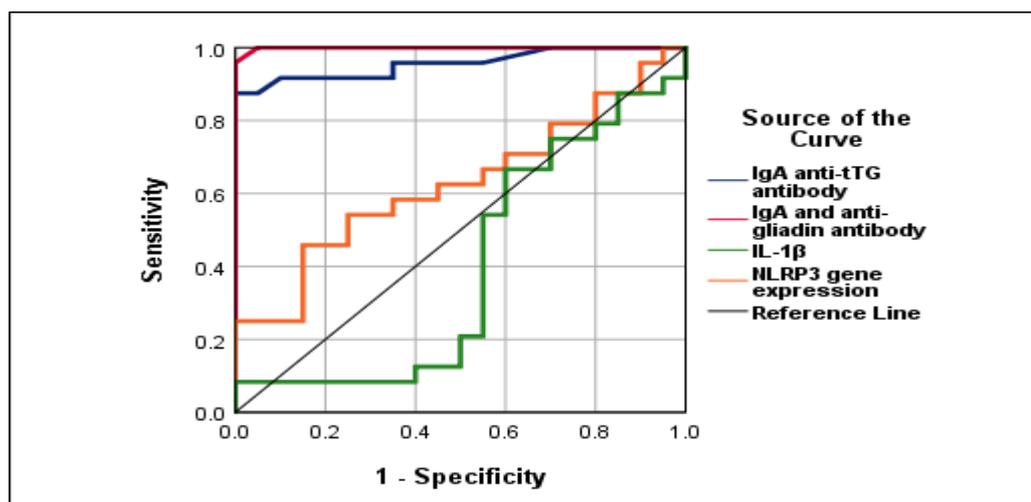
Table 3. ROC curve analysis of IgA anti-tTG, IgA antibody, anti-gliadin antibody, IL-1β and NLRP3 gene expression in celiac disease patients.

Variable	AUC	95% CI	*p*-value	Sensitivity (%)	Specificity (%)	Cut-off value
IgA and anti-gliadin antibody (U/mL)	0.999	0.995-1.000	< 0.001	100.0	95.0	1.7
IgA anti-tTG antibody (U/mL)	0.956	0.896-1.000	< 0.001	91.7	90.0	1.8
IL-1β (pg/mL)	0.398	0.219-0.576	0.248	55.0	50.0	56.4
NLRP3 gene expression (dCT)	0.627	0.461-0.793	0.150	65.0	58.0	0.800

Figure 2. ROC curve analysis of IgA anti-tTG, IgA antibody, anti-gliadin antibody, IL-1β and *NLRP3* gene expression in celiac disease patients showing area under the curve.

4. Conclusion

In conclusion, the *NLRP3* inflammasome was down-regulated in the present sample of CD children, and it was accompanied by a decreased serum level of IL-1β. The diagnostic significance of anti-tTG and anti-gliadin IgA antibodies was reinforced by the present study.

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