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The Impact of sHLA-G as a Biomarker on the Diagnosis and Severity of Celiac Disease

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ABSTRACT

Celiac disease (CD) is one type of autoimmune disorder that results in immune regulation and tolerance. The present study was carried out to detect the efficiency of soluble human leukocyte antigen-G (sHLA-G) in diagnosis and to determine CD severity. The study included 150 CD patients' blood samples; 75 of them were collected from newly diagnosed (ND) patients. DNA was extracted and sera were separated; the rest of the patients' blood samples were collected after four months of gluten-free diet (GFD) treatment for the serological study. Fifty healthy people donated blood, which was classified as the control group. DNA was extracted, and its purity and concentration were determined using the nanodrop technique. Real-time polymerase chain reaction (RT-PCR) was utilized to detect the DQ2 and DQ8 alleles of the ND group, while sHLA-G was assessed by the enzyme-linked immunosorbent assay (ELISA) technique. A graph-prism program was applied to analyze the results. The obtained results demonstrated that sHLA-G levels significantly dropped after four months of GFD. However, no significant alteration was shown according to gender. As a result of RTPCR, patients were categorized in accordance with the DQ2 and DQ8 alleles as homozygote, heterozygote and negative. According to the DQ2 gene, both groups ND and GFD recorded significant variations in sHLA-G values between heterozygote and negative groups. However, the grouping based on DQ8 did not reveal significant differences. Also, the results showed that sHLA-G had a high sensitivity for diagnosing CD. This study came to the conclusion that CD severity diagnosis and prognosis were significantly influenced by sHLA-G.

Key words: Soluble HLA-G, celiac disease, DQ2, DQ8

INTRODUCTION

CD is a chronic immune-mediated gastroenteropathy that affects people with specific genetic predispositions (Valitutti *et al.*, 2019). It is characterized by the consumption of gluten-containing foods or beverages (Iversen *et al.*, 2020). The Human Leukocyte Antigen HLA genes, particularly class II HLA, have been implicated in numerous autoimmune disorders (Lokki and Paakkanen, 2019). Nearly all CD patients carry the HLA-DQA1 and HLA-DQB1 genes, which code for the HLA-DQ2 and HLA-DQ8 molecules (Sciurti *et al.*, 2018). The presence of the glycoprotein DQ2 doubles the capacity of HLA to bind with gliadin-specific peptides and activate lymphocytes, thereby significantly influencing the risk of CD (Federica *et al.*, 2021).

HLA-G is a non-classical major histocompatibility complex (MHC) molecule belonging to class I (Zaborek-Lyczba *et al.*, 2021). It consists of beta-2 microglobulin and an alpha chain (Morandi *et al.*, 2016). In contrast to classical HLA molecules, non-classical HLA, including HLA-G, displays limited polymorphism and can bind to receptors other than the T-cell receptor (TCR; Jasinski-Bergner *et al.*, 2022). There are seven distinct forms of HLA-G, referred to as HLA-G1 through HLA-G7 (Morandi *et al.*, 2016). Some of the isoforms have intron 4, which makes it easier to splice the heavy chain's transmembrane region and produce soluble versions (G5, G6 and G7). Metalloproteinases can degrade HLA-G1, releasing the protein and generating a second soluble version (Morandi *et al.*, 2016).

Different HLA-G isoforms' kinetics and structure are directly related to their function as immunological check point molecules. Numerous pathologies, including cancer, transplantation, viral infections, autoimmune diseases and inflammatory disorders, have been linked to increased HLA-G expression (Zaborek-Lyczba *et al.*, 2021).

A study by Catamo *et al.* (2015) found a connection between HLA-G gene polymorphisms and susceptibility to CD development,

suggesting a potential role for HLA-G in the disease's etiology (Catamo *et al.*, 2015). Due to its ability to inhibit both T cell-mediated cytolysis and the cytotoxic activity of natural killer (NK) cells, HLA-G is vital in the control of the immune system (CTL) (Cross-Najafi *et al.*, 2022).

The objective of this research was to examine the involvement of sHLA-G in both diagnosing CD and monitoring its progression. Additionally, the study aimed at investigating the relationship between sHLA-G and the severity of the disease, considering its role as an immune system regulator.

MATERIALS AND METHODS

A total of 200 blood samples were collected in this study during the period from July 2021 until October 2022 in Raparin Children's Hospital and Rizgary Teaching Hospital in Erbil city, Iraq. Seventy-five of them were collected from ND of CD patients after they were diagnosed clinically and serologically, their age ranging from 2 to 56 years. DNA was extracted and sera were separated. It was kept at -80 and -20°C until used, respectively. After four months of GFD treatment, 75 blood samples were gathered from the same patients in addition to the control group, which contained 50 healthy donors. The study proposal was confirmed by the ethics committee of Erbil Technical Health and Medical College, Erbil Polytechnic University, Iraq, according to the research ethics committee (REC.2).

DNA extractions were done according to Wizbio solutions gDNA Mini kit-Korea procedure. Nanodrop technique was used to determine DNA purity and concentration by Thermo Fisher Scientific–USA.

RT-PCR (Roto Gene Australia) was performed to detect DQ2 and DQ8 alleles using Genmark Saglik Turkey that detected DQ2 and DQ8 alleles (DQA1*05, DQB1*02 and DQA1*03, DQB1*0302). sHLA-G were measured by enzyme linked immunosorbent assay (ELISA) technique using MyBioSource USA kit.

GraphPad Prism 8 was used to conduct all statistical analyses. Kruskal-Wallis, Mann-Whitney tests, and the receiver operating characteristics (ROC) curve were applied to analyze the results. An area under the curve (AUC) was frequently interpreted in relation to thresholds, with good models defined at 0.7, 0.8 or excellent models define at 0.9 (White *et al.*, 2023). A P-value of less than 0.05 was regarded as statistically significant.

RESULTS AND DISCUSSION

The level of sHLA-G significantly dropped after four months of GFD treatment as compared with the ND patients but remained higher than its level in healthy controls (27.72 IU/ml ±16.99, 43.55 IU/ml ±19.30 *vs.* 5.478 IU/ml ±3.016, respectively (Table 1 and Fig. 1).

Table 1. Comparison of sHLA-G mean for ND, GFD and control by Kruskal Wallis test

Groups (number)	Mean (IU/ml) ±SD	Range	P-Value
ND=75	43.55±19.30	10.54-98.33	<0.0001
GFD=75	27.72±16.99	6.400-74.30	
Control=50	5.478±3.016	1.400-12.53	

ND: Newly diagnosed, GFD: Gluten free diet and SD: Standard deviation.

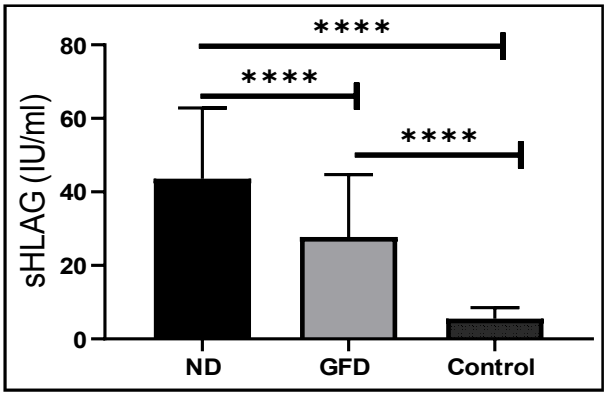

Fig. 1. sHLA-G mean for ND, GFD and control. ****Significant at P = 0.001.

CD, an autoimmune disease triggered by gluten ingestion, caused an immune response targeting the small bowel and elicited a serological response (Calado and Machado, 2022). In contrast to other autoimmune disorders, the specific antigen that set off the immunological reaction in CD was identified and thoroughly investigated (Sollid *et al.*, 2020). The removal of these antigens through GFD was the effective treatment for CD (Machado, 2023). CD was recognized by an impairment of immune tolerance, where T regulatory cells (Treg) were unable to effectively suppress the activity of effectors T cells (Cook *et al.*, 2017). The crucial role of HLA-G was in the onset and advancement of various autoimmune conditions including those affecting the

respiratory tract, gastrointestinal tract, rheumatoid diseases and type 1 diabetes mellitus. HLA-G was a vital molecule involved in promoting natural tolerance, which was extensively studied in the context of pathology. Despite the increasing studies on HLA-G role, there were new aspects, interactions and mechanisms continued to be discovered about its complex functioning as immune regulator which served as a protective barrier against immune attack (Zaborek-Lyczba *et al.*, 2021). The regulatory role of sHLA-G was supported by its elevated levels in cancerous diseases, viral infections and autoimmune diseases (Xu *et al.*, 2020). Research findings of Morandi *et al.* (2016) suggested that HLA-G played a crucial role in suppressing immune reactions in chronic inflammatory conditions such as multiple sclerosis, psoriasis, inflammatory bowel disease (IBD), rheumatoid arthritis, asthma, systemic lupus erythematosus, idiopathic juvenile illness and cancer.

In the context of CD, the present study focused on the importance of sHLA-G in CD patients follow up after GFD treatment and predicting disease prognosis. This concept emerged from the pivotal role of s-HLAG in immune response regulation as a predictive biomarker in individuals with cancer or autoimmune disorders including CD (Morandi *et al.*, 2016).

The results of the current study indicated that sHLAG levels decreased after four months of GFD but were still higher than in healthy donors. This finding supported the regulatory role of sHLAG. Similarly, juvenile CD patients had considerably greater sHLA-G levels in comparison to patients with ulcerative colitis and healthy controls. Among CD patients, individuals with moderate to severe CD exhibited greater sHLA-G levels than those with mild CD (Cansever *et al.*, 2022).

The upregulation in sHLA-G levels may serve as a homeostatic reaction to counteract the ongoing inflammatory process in IBD, which can be triggered by dysbiosis or gut inflammation (Cansever *et al.*, 2022). Consistent with the present study, previous research has shown that CD patients experienced triggered intestinal mucosa and inflammatory responses with a normal diet, while adopting a GFD suppresses these reactions, reduced the production of proinflammatory biomolecules and mitigating tissue lesions (Saborido *et al.*, 2018).

With respect to the gender, there were no statistically significant variations between male and female patients of ND as well as GFD group in sHLA-G mean levels (37.45±10.58 and 46.59±21.89; 20.63±8.721and 31.27±18.97, respectively; (Table 2 and Fig. 2).

Table 2. Comparison of sHLA-G mean for ND and GFD patients according to gender using Mann-Whitney test

Groups	Male=25		Female=50		P-Value
	Mean (IU/ml) ± SD	Range	Mean (IU/ml) ± SD	Range	
ND	37.45±10.58	10.54-56.30	46.59±21.89	20.19-98.33	0.2617
GFD	20.63±8.721	6.400-39.40	31.27±18.97	11.80-74.30	0.0597

ND: Newley diagnosed, GFD: Gluten free diet and SD: Standard deviation.

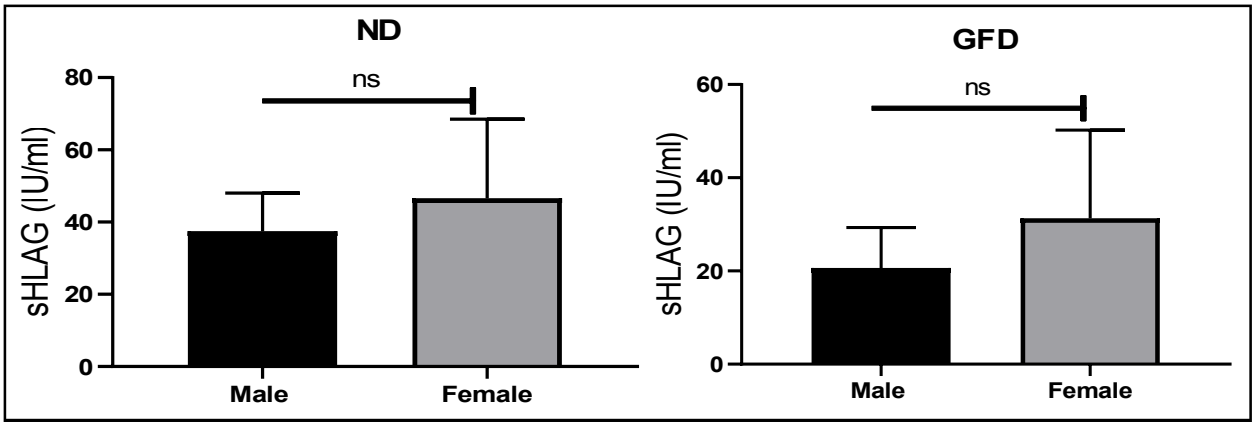

Fig. 2. sHLA-G mean for ND and GFD patients according to gender. NS = Non-significant at P = 0.05.

The ratio of female to male CD disease patients included in this study was 2:1, which aligned with the findings of Jansson-Knodell *et al.* (2019) who reported that the frequency of CD was higher in women, with a female-to-male ratio between 2:1 and 2.5:1. However, serological examinations carried out by Ditah *et al.* (2015) indicated that female-to-male ratio was 1.5:1. Similarly, Tolone *et al.* (2021) conducted a study supporting these findings, which indicated a lower percentage of male patients and a reciprocal proportional prevalence of sHLA expression. IBS, a functional bowel abnormality, affected approximately 10 to 20% of the US population, with a higher occurrence in women than in men, as stated by Kim and Kim (2018).The prevalence of autoimmune illnesses was 2 to 1 higher in women than in men, demonstrating a clear sex bias. Various autoimmune diseases predominantly affected women as a result of hormonal changes or pregnancy (Angum *et al.*, 2020). Additionally, the chromosome X contained a higher abundance of genes related to the immune system and immune regulation, contributing to immunological reactions in the body (Schurz *et al.*, 2019) . The greater number of genes on the chromosome X increased the likelihood of mutations, thus placing women at a higher risk of autoimmune disorders development due to their possession of two chromosomes X compared to men's single X chromosome. This resulted in a "double dose" of genes located on the chromosome X and made females more prone to autoimmunity. Autoimmune disorders predominantly impacted women, making up approximately 80% of all affected individuals, which could be attributed to variations in sex chromosomes and hormonal changes (Angum *et al.*, 2020).

Although there was a double ratio of CD epidemics in women, there was no statistically significant alteration in sHLA-G concentrations according to gender of ND or even patients on a diet without gluten in the current study. The study conducted by Abdul-Hussein *et al.* (2020) supported the current findings, as they found no significant variation in sHLA-G concentrations between men and women with CD, even among patients who followed a GFD. However, in the case of UC, male patients exhibited significantly higher mean levels of sHLA-G antigens when compared with female patients.

Based on the results obtained from RTPCR for DQ2 and DQ8 alleles, patients were categorized into three groups, the homozygote group included 22 patients encoded for DQA1*05 only, whereas DQB1*02 was not detected as homozygosity for DQ2 in the studied patients. Likewise, the homozygosity for DQ8 was represented by a detecting DQA1*03 allele only in 19 patients, but DQB1*0302 was not recorded. The heterozygote group included 41 and 48 patients who gave positive RTPCR, a negative group was used to describe 12 and 8 patients when both alleles were not coded for DQ2 and DQ8, respectively.

sHLA-G levels were also found to be comparable according to DQ2 alleles polymorphism. Statistically non-significant differences were recorded of sHLA-G level in homozygote group when compared with heterozygote and negative groups of CD patients (40.05±15.09 *vs.* 48.56± 21.74 and 32.81±10.35, respectively), whereas significant elevation was found in heterozygote group in comparison with negative group (Table 3 and Fig. 3). The same result was obtained after GFD treatment where sHLA-G mean concentration of DQ2 heterozygote group significantly elevated in comparison with the negative group (31.99±18.56 *vs.* 18.42±5.951, respectively) in contrast with the sHLA-G level of the DQ2 homozygote group, which did not record significant alteration with other groups as pointed out in Table 3 and Fig. 3.

Categorizing patients according to DQ8 gene positivity, whether it was homozygote or heterozygote, and gene negativity, sera collected from ND and GFD patients did not record significant alteration of sHLA-G among the mentioned groups, as pointed out in Table 4 and Fig. 4.

Table 3. Comparison of HLA-G mean for ND and GFD patients according to DQ2 polymorphism by Kruskal Wallis test

Groups	Homozygote (n = 22)		Heterozygote (n = 41)		Negative (n = 12)		P-Value
	Mean (IU/ml) ±SD	Range	Mean (IU/ml) ±SD	Range	Mean (IU/ml) ±SD	Range	
ND	40.05±15.09	10.54-76.93	48.56±21.74*	20.35-98.33	32.81±10.35*	20.19-50.25	0.0370
GFD	24.84±15.76	6.400-64.50	31.99±18.56*	12.30-74.30	18.42±5.951*	12.50-28.30	0.0259

*Significantly different; ND: Newley diagnosed; GFD: Gluten free diet and SD: Standard deviation.

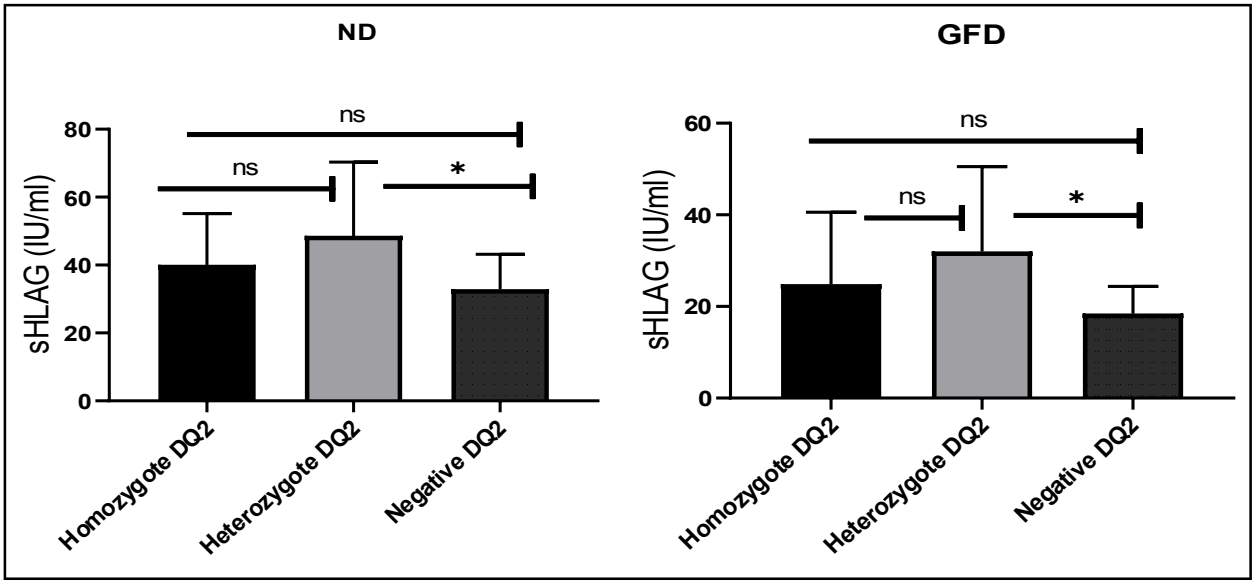

Fig. 3. sHLA-G mean for ND and GFD patients according to DQ2 alleles polymorphism. NS–Non- significant at P= 0.05 and *Significant at P = 0.05.

Table 4. Comparison of HLA-G mean for ND and GFD patients according to DQ8 polymorphism by Kruskal Wellis test

Groups	Homozygote = 19		Heterozygote = 48		Negative = 8		P-Value
	Mean (IU/ml) ±SD	Range	Mean (IU/ml) ±SD	Range	Mean (IU/ml) ±SD	Range	
ND	43.63±21.75	10.54- 76.93	42.69±16.07	20.35-98.33	48.45±30.89	20.19- 97.00	0.9116
GFD	28.04±20.58	6.400- 64.50	26.62±13.70	12.30-70.43	33.55±25.56	13.50- 74.30	0.5689

ND: Newly diagnosed; GFD: Gluten free diet and SD: Standare deviation.

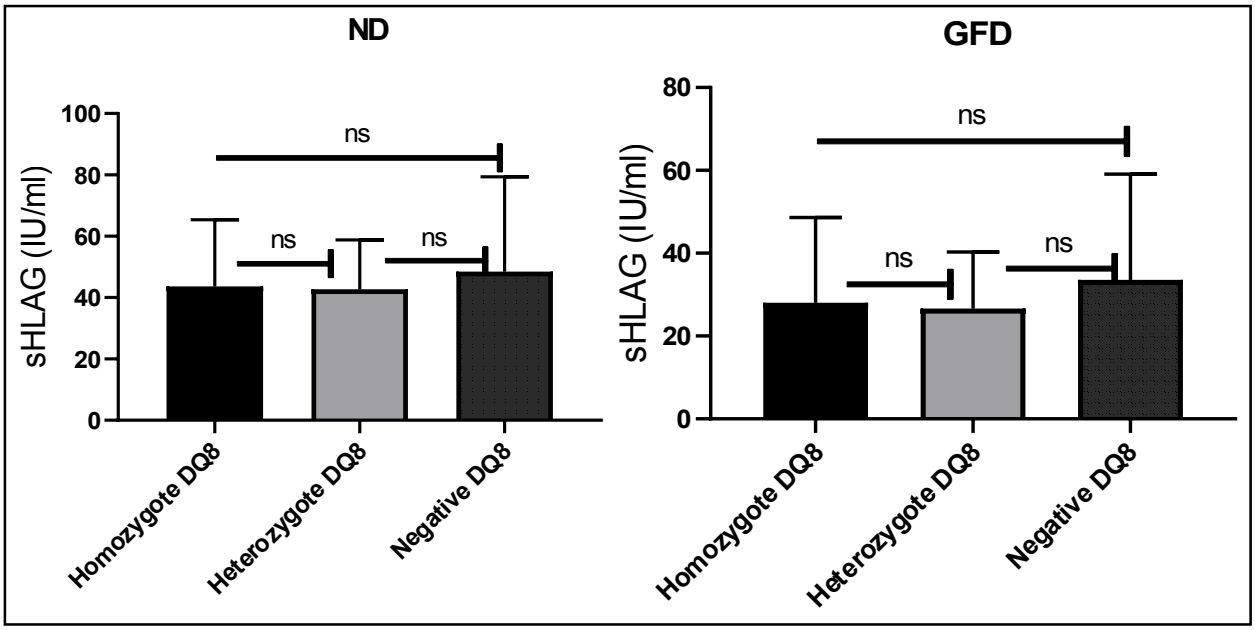

Fig. 4. sHLA-G mean for ND and GFD patients according to DQ8 allele polymorphism. NS: Non- significant at P= 0.05.

There was a lack of data on the levels of sHLA-G in CD patients according to polymorphisms in the DQ2 and DQ8 alleles. Consequently, the current study examined the allele variations of DQ2 and DQ8 to classify the study participants' patients based on allele

polymorphism. The sHLA-G concentration of ND and GFD patients that estimated in the DQ2 heterozygote group (whose genome consisted of DQA1*05 and DQB1*02 alleles) did not record a significant alteration compared to its concentration in the homozygote group (whose genome consisted of identical alleles of DQA1*05), whereas the DQ2 heterozygote group revealed a statistically elevation in the sHLA-G concentration comparing to group of patients who recorded the absence of the DQ2 gene otherwise classification of ND and GFD patients according to DQ8 gene did not reveal any statistically significant differences between homozygote (two copies of DQA1*03), heterozygote (DQA1*03 and DQB1*0302 alleles coded in patient) and DQ8 negative group.

The main genetic risk factor identified thus far in CD was the presence of specific HLA heterodimer genes, as DQ2 (in a position either cis or trans) or DQ8 [DQA103-DQB10302/0305]. Homozygosity for DQA10501-DQB10201 or DQA10201-DQB102 indicated a heightened risk for the development of CD (Poddighe and Capittini, 2021). Sharp *et al.* (2020) identified CD patients with genome polymorphism DQ2.5/DQ2.5, DQ2.2/DQ2.5 and DQ2.5/DQ8 as the highest risk patients. CD patients with specific genome polymorphisms, such as DQ2.5/DQ2.5, DQ2.2/DQ2.5 and DQ2.5/DQ8 were classified as high-risk individuals. However, an investigation conducted by Sharp *et al.* (2020) indicated that the HLA-DQ genotype DQ8, which was usually linked to CD risk, had a smaller effect than other HLA genotypes. The study also revealed that having two copies of DQ2.5 or a combination of DQ2.5/DQ2.2 presented a very strong risk for CD. Additionally, a slightly elevated risk was linked to DQ7.5/DQ2.5. These findings suggested that the risk associated with DQA10501 was dominant and not a dose-dependent effect. However, the risk associated with DQB10201 appeared to be dose-dependent, providing further evidence for existing theories.

To find out if patients with moderate/severe forms of both UC and CD have comparable amounts of sHLA-G, additional research is required. HLA-G has a variety of immunoregulatory effects, including lowering the development of cytotoxic T cells, inhibiting T cell chemotaxis, raising the rate of death in both T cells and NK cells, and modulating the release of pro-angiogenic factors from NK cells (Tahan *et al.*, 2017). According to the findings of Cansever and others the disease activity index can be calculated using the correlation between sHLA-G concentrations and disease severity in IBD patients. Additionally, sHLA-G is more significant as a biomarker in Crohn's disease than UC. The suppressor molecule sHLA-G is upregulated in IBD, which may operate as a homeostatic response to combat the chronic inflammatory disease. This response can be triggered by inflammation or alterations in gut microbial composition and dysbiosis. However, these theories need to be further explored by in-depth research on the immunopathogenesis of IBD (Cansever *et al.*, 2022). Martin-Villa *et al.* (2022) study has demonstrated that HLA-G is essential for regulating autoimmunity, and both its levels and polymorphisms are associated with increased susceptibility and severity of various disorders. Modulating the expression of HLA-G in the affected tissues of patients with autoimmune diseases or oncology patients hold potential as a therapeutic approach for these conditions.

The result obtained from the ROC curve test for calculation of the area under the curve AUC value referred to test sensitivity and diagnostic efficiency indicated that sHLA-G recorded high sensitivity where AUC equlled to 0.998 (Fig. 5).

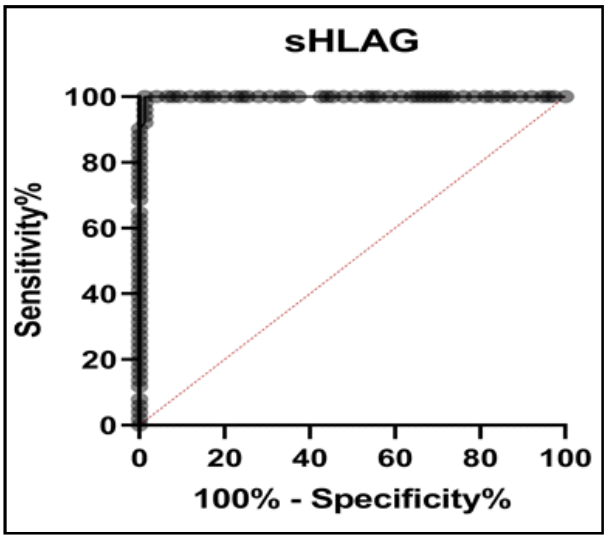

Fig. 5. ROC curve for sHLA-G.

The value of AUC obtained in present study demonstrated high efficiency of overall diagnostic performance of sHLA-G that measured by ELISA technique as a confirmation and prognostic test for CD

diagnosis (Abdul-Hussein *et al.*, 2020). Results analogue with present finding reported AUC value for sHLA-G antigens exhibiting AUC in IBD = 0.944, UC = 0.961 and CD = 0.928.

CONCLUSION

The result of the current study emphasized that sHLA-G had an important role in determining the severity of CD, which was pertinent to the characteristics of genes related to this disease. As well, the results revealed that sHLA-G reflected the sequelae of this disease after consuming gluten-free meals. Such findings provided updated information to guide future studies, with a larger sample size, for clarifying the relationship between sHLA-G and other inflammatory markers that were affected by autoimmune diseases.

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