

# Serum levels of soluble HLA-G correlate with disease activity in pediatric patients with Crohn's disease

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## Abstract

**Background:** Human leukocyte antigen (HLA)-G, a member of the HLA family, is crucial for fetomaternal tolerance. Transmembrane or circulating/soluble HLA-G (sHLA-G) is elevated in autoimmune conditions and the tumor microenvironment. Circulating sHLA-G levels and their association with disease activity have not yet been assessed in pediatric patients with inflammatory bowel disease (IBD). This study aimed to quantify the serum sHLA-G levels of pediatric patients with IBD and assess the association of serum sHLA-G with disease activity.

**Methods:** We enrolled 24 pediatric IBD patients Crohn's disease (CD) and ulcerative colitis (UC), n = 12 each] and 24 healthy controls. Based on the disease activity index, five and seven of the CD patients had mild and moderate/severe disease, respectively, whereas six of the UC patients were in remission and six had mild disease. Serum was collected and sHLA-G levels were determined by enzyme-linked immunosorbent assay (ELISA).

**Results:** Pediatric patients with CD had significantly higher sHLA-G levels compared with patients with UC and healthy controls. Notably, serum sHLA-G levels were significantly higher in patients with moderate/severe CD than in those with mild CD.

**Conclusions:** Serum sHLA-G levels correlate with disease activity in pediatric patients with CD and are higher in CD patients than in UC patients. Thus, sHLA-G is a potential biomarker for disease activity in CD.

**Keywords:** Crohn's disease, HLA-G antigens, ulcerative colitis

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## INTRODUCTION


The human leukocyte antigen (HLA)-G, a heterodimeric protein composed of an alpha (or heavy) chain and beta-2 microglobulin, is a member of the nonclassical HLA-Ib molecule family.<sup>[1,2]</sup> Unlike classical HLA molecules, HLA-G and other nonclassical HLAs show minimal polymorphism

and may serve as ligands for receptors other than the T-cell receptor (TCR).<sup>[2]</sup> HLA-G has seven isoforms: HLA-G1–HLA-G7.<sup>[1,3]</sup> The soluble HLA-G (sHLA-G) molecules G5, G6, and G7 contain intron 4, which enables the splicing of the transmembrane portion of the heavy chain to create the soluble form. Despite its transmembrane isoform

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structure, metalloproteinases can cleave HLA-G1 and shed the protein to create an additional soluble form, HLA-G,<sup>[1]</sup> which was initially found to be expressed by trophoblasts and likely plays a crucial role in fetomaternal tolerance.<sup>[4]</sup> Indeed, studies have suggested a correlation between lower sHLA-G levels in cord blood and recurrent spontaneous abortion, gestational diabetes mellitus, and preeclampsia.<sup>[5,6]</sup> Moreover, HLA-G is expressed by the thymus medulla, pancreatic islets, and cornea in the steady state; during stress, cancer, or inflammation, HLA-G expression is triggered in several tissues.<sup>[1,7]</sup>

Findings from several lines of research indicate that HLA-G is an important suppressor of immune responses in chronic inflammatory conditions, including inflammatory bowel disease (IBD), multiple sclerosis, psoriasis, asthma, rheumatoid arthritis, idiopathic juvenile arthritis, systemic lupus erythematosus, and cancer.<sup>[1,8-17]</sup> In addition, HLA-G expression in serum or graft tissues is associated with better prognosis in patients who have received kidney, heart, lung, or liver transplantation.<sup>[18-21]</sup>

Inflammatory bowel disease involves chronic inflammation of the gastrointestinal tract due to genetic, environmental, and microbial factors and manifests in two major forms: ulcerative colitis and Crohn's disease (CD). Several noninvasive biomarkers in serum, fecal matter, or urine, such as calprotectin, lactoferrin, and S100 family proteins, can help determine the severity of IBD.<sup>[22,23]</sup> Although the serum sHLA-G is elevated in adult IBD patients<sup>[24]</sup> and the expression of the membrane-bound sHLA-G is increased in the intestinal tissue of patients with ulcerative colitis,<sup>[25-27]</sup> no study has evaluated the correlation of the disease activity index (DAI) with the serum sHLA-G levels. More importantly, serum sHLA-G levels in pediatric patients with IBD have not been investigated.

This study was conducted with an aim to determine whether the serum sHLA-G levels correlate with disease severity in patients with UC or CD.

## PATIENTS AND METHODS

### Participants

Serum samples were obtained from peripheral blood samples of pediatric patients with CD (n = 12) or UC (n = 12) who visited the gastroenterology outpatient clinic, as well as from healthy pediatric controls (n = 24). The study was approved by the local ethics committee (approval no.: 2017/655), and all study procedures were undertaken in conformance with the relevant guidelines and regulations. Informed parental consent, age-appropriate patient

consent, or assent for study participation was obtained prior to enrolment. The DAI for pediatric patients with CD was calculated according to the method specified by Hyams *et al.*<sup>[28]</sup> (score range: 0–115, with 115 indicating the highest DAI; scores 0–10, 11–30, and  $\geq 31$  indicate inactive, mild, and moderate/severe disease, respectively), which is based on several items, including history, patient functioning and general well-being, laboratory findings, and findings on physical examination.<sup>[28]</sup> Similarly, the DAI for pediatric patients with ulcerative colitis was calculated according to the method described by Turner *et al.*<sup>[29]</sup> (score range: 0–85, with 85 indicating the highest DAI; scores 0–9, 10–34, 35–64, and  $\geq 65$  indicate remission, mild disease, moderate disease, and severe disease, respectively). In addition, the patients were classified according to the Paris Classification.<sup>[30]</sup>

### sHLA-G measurement

Serum from patients and healthy controls were collected and stored at  $-80^{\circ}\text{C}$  until all patient samples were obtained. None of the patients were receiving any medication at the time of sample collection. The sHLA-G levels were measured by a specific sandwich enzyme-linked immunosorbent assay (ELISA; ELISA Kit [detection limit: 0.6 U/mL], BioVendor (Palackeho, Brno, Czech Republic) in accordance with the manufacturer's protocol.

### Statistical analysis

Graph Pad Prism 6 software was used for all statistical analyses and for obtaining the receiver operating characteristics (ROC) curve and correlation graphs. ANOVA and/or nonparametric multiple comparison (Kruskal–Wallis) tests were used where appropriate. The association between the sHLA-G level and the DAI was evaluated by Spearman's rank correlation coefficient ( $r$ ) for nonparametric correlations.  $P < 0.05$  was accepted as indicative of statistical significance.

## RESULTS

### Participants and disease activity index

Twelve pediatric patients each with CD and UC (n = 12 each) and 24 pediatric healthy controls (n = 24) were enrolled, and the patient characteristics are summarized in Table 1. The CD, UC, and control groups included patients with a mean (range) age of 13.24 (7–16), 16 (9–19), and 14.70 (7–19) years and included 6 female: 6 male, 9 female: 3 male, and 15 female: 9 male participants, respectively. Based on Hyams *et al.*'s<sup>[28]</sup> DAI calculation for CD patients, five patients had mild disease (score range: 15–20), whereas seven patients had moderate/severe disease (range: 35–55). The classification of UC patients based on Turner *et al.*'s<sup>[29]</sup>

**Table 1: Patient information**

Patient	Gender	Age	Biopsy Diagnosis	Serum sHLA-G levels	Disease Activity Index	Stage	CRP	ESR	Paris Classification
P1	F	16	CD	1,7	15	Mild	28	30	A1b, L1, B1, G0
P2	F	12	CD	3,3	25	Mild	35	40	A1b, L3, B1, G0
P3	M	16	CD	2	15	Mild	25	20	A1b, L3, B1, G0
P4	M	13	CD	2,05	20	Mild	28	30	A1b, L1, B1, G0
P5	M	17	CD	2,6	20	Mild	27	45	A2, L1, B1, G0
P6	F	16	CD	6,35	35	Moderate/Severe	48	40	A2, L4b, B1, G0
P7	F	13	CD	6,85	35	Moderate/Severe	52	40	A1b, L4b, B1, G0
P8	F	7	CD	8,45	45	Moderate/Severe	61	50	A1a, L4b, B1, G0
P9	F	15	CD	10,3	55	Moderate/Severe	66	60	A1b, L4b, B1, G0
P10	M	13	CD	7,95	45	Moderate/Severe	58	70	A1b, L3, B1, G0
P11	M	12	CD	9,85	50	Moderate/Severe	68	75	A1b, L4b, B1, G0
P12	M	11	CD	6,75	40	Moderate/Severe	42	52	A1b, L3, B1, G0
CD Patient Aqe Average: 13.42; Median: 16; SD: 2.69; SEM: 0.811									
P13	F	18	UC	0,65	5	Remission	3	5	E1, S0
P14	F	19	UC	0,5	5	Remission	2	4	E1, S0
P15	F	16	UC	0,5	5	Remission	1	5	E1, S0
P16	F	17	UC	0,55	5	Remission	1	5	E2, S0
P17	F	18	UC	0,6	5	Remission	1	4	E4, S1
P18	F	15	UC	0,7	5	Remission	0,8	5	E4, S1
P19	F	9	UC	1,2	10	Mild	1	7	E1, S0
P20	F	10	UC	1	10	Mild	1,1	10	E1, S0
P21	F	16	UC	2,05	15	Mild	10	20	E3, S1
P22	M	19	UC	2	20	Mild	3	4	E2, S1
P23	M	19	UC	1,85	20	Mild	9	10	E2, S1
P24	M	16	UC	1	10	Mild	5	5	E4, S1

UC Patient Aae Average: 16; Median: 16.5;SD: 3.18; Sem: 0.961

pediatric DAI scoring system showed that six patients were in remission (all with total scores <5) and six had mild disease activity (range: 10–20). None of the patients had perianal disease or strictures.

### Positive correlation of serum sHLA-G levels with DAI in CD and UC

Among CD patients, serum sHLA-G levels were significantly higher in the moderate/severe disease group compared to the mild disease group or healthy controls [Figure 1a-c]. Furthermore, there were no sex-based differences in the upregulation of sHLA-G expression [Figure 1b and c]. UC patients with mild DAI had higher serum sHLA-G levels than patients in the remission group but not with regard to healthy controls [Figure 1d]. A sex-stratified analysis in the UC cohort was unfeasible due to the inclusion of fewer male patients. Furthermore, sHLA-G levels in moderate/severe CD patients were much higher than those in patients with mild UC [Figure 1a and d], whereas the sHLA-G levels were comparable in patients with mild UC and mild CD [Figure 1a and d].

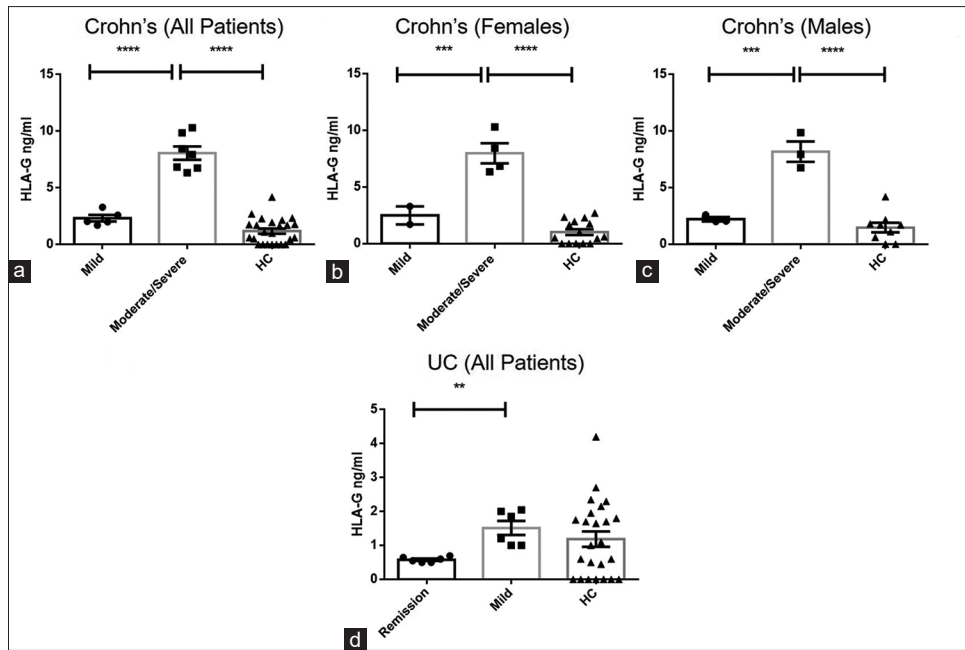
There was a significant correlation between serum sHLA-G levels and DAI in both CD and UC patients [ $r = 0.9824$  and  $r = 0.9080$ ;  $P < 0.0001$  for both; Figure 2a and b]. There was no correlation between age and DAI in the CD or UC groups [Figure 2c and d].

Lastly, ROC curve analyses revealed a significant area under the curve [Figure 3a and b] for patients with CD (0.9236,  $P < 0.0001$ ), but not for patients with UC (0.5208;  $P = 0.8404$ ), which suggests that the serum sHLA-G level may be used as an indicator of disease severity for CD patients.

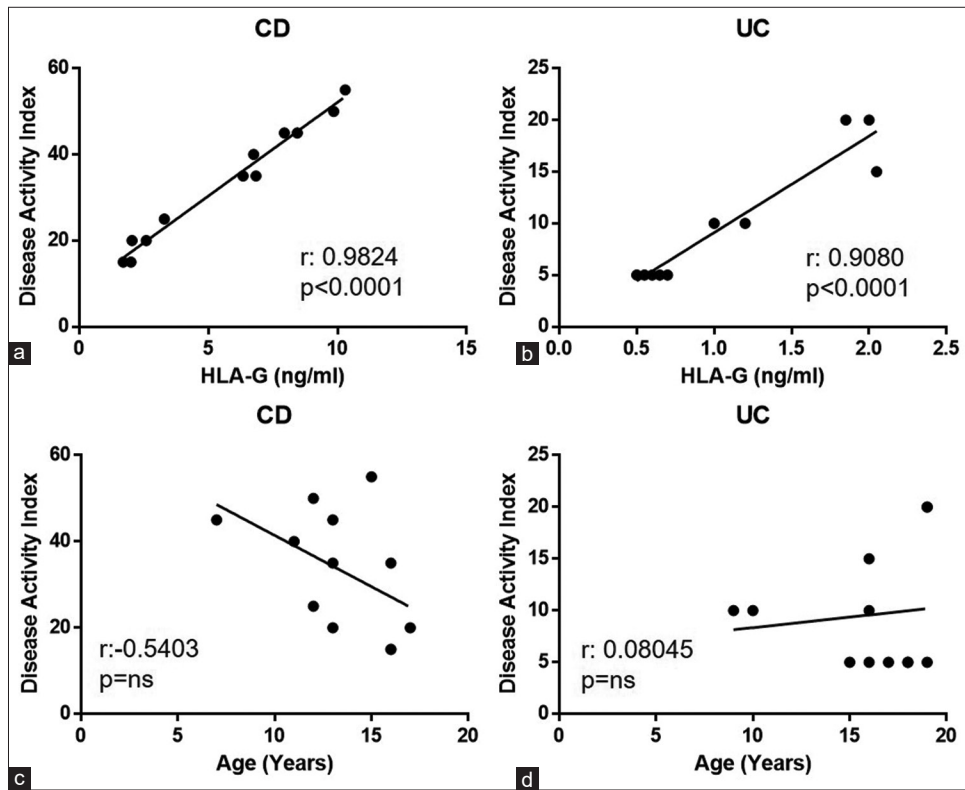
The correlation between sHLA-G levels and the parameters used for the Paris Classification showed that the sHLA-G levels positively correlated with location criteria ( $r = 0.7824$   $P < 0.0039$ ) but not with diagnosis, age, growth, or behavior [Supplemental Figure 1a-d], in CD patients. In contrast, the HLA-G levels did not correlate with disease extent ( $r = -0.2, 5$ ;  $P = 0.41$ ) or severity ( $r = 0.55, P = 0.07$ ) in UC patients [Supplemental Figure 1e and f]. Furthermore, C-reactive protein (CRP) ( $r = -0.97, P < 0.0001$ ) and erythrocyte sedimentation rate (ESR) ( $r = 0.86, P = 0.0005$ ) values showed a strong and positive significant correlation with sHLA-G levels in CD patients [Supplemental Figure 2]. However, CRP and ESR levels moderately correlated nonsignificantly with the sHLA-G levels in UC patients.

### DISCUSSION

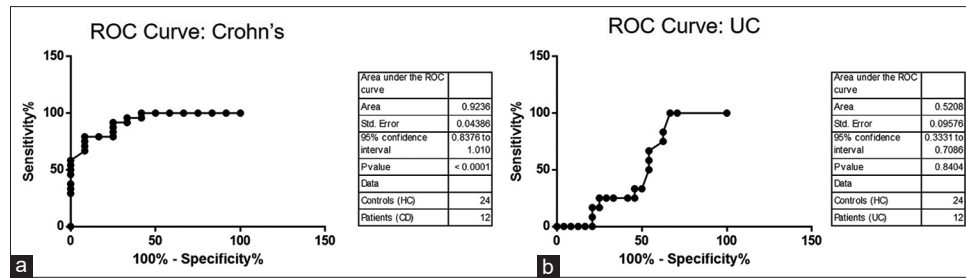
Membrane-bound HLA-G or sHLA-G in serum or tissue is a potential prognostic biomarker in adult patients with cancer or autoimmune conditions, including CD.<sup>[1,3]</sup> There are few studies of sHLA-G levels in pediatric patients with IBD. Tissue-specific HLA-G levels in IBD were first



**Figure 1:** Serum sHLA-G levels are elevated in active pediatric IBD patients. (a) Serum SHLA-G levels in mild (n = 5) and moderate/severe (n = 7) pediatric CD patients and healthy controls (n = 24) female and male patients combined; (b) Only female patients in “A” graphed; mild (n = 2) and moderate/severe (n = 4) pediatric CD patients and healthy controls (n = 15); (c) only male patients in “A” graphed; mild (n = 3) and moderate/severe (n = 3) pediatric CD patients and healthy controls (n = 9). (d) Serum SHLA-G levels in remission (n = 6), mild (n = 6) pediatric UC patients, and healthy controls (n = 24) female and male patients combined



**Figure 2:** Serum sHLA-G levels correlate with disease activity index in pediatric IBD patients. Spearman’s correlation test was applied to evaluate the correlation between sHLA-G levels and CD DAI (a), or UC DAI (b) or between age and CD DAI (c), or UC DAI (d). CD, n = 12; UC, n = 12; HC, n = 24



**Figure 3:** Receiver operating curve analyses for sHLA-G levels and DAI in pediatric IBD. (a) ROC analyses for CD (b) ROC analyses for UC

studied by Torres *et al.*,<sup>[26]</sup> although the age information of the study cohort was not specified. However, the participants appeared to be adult patients. Interestingly, the authors showed dramatic HLA-G staining in apical intestinal epithelial cells (IEC) of the biopsy specimen obtained exclusively from UC, but not CD, patients. Moreover, HLA-G expression was observed in the ascending, transverse, and descending colon as well as the ileum in UC patients. Notably serum sHLA-G levels were not assessed in this study. Rizzo *et al.*<sup>[13]</sup> first investigated sHLA-G levels in the context of IBD, but the study differed from ours in two major aspects. First, the authors measured sHLA-G levels in the supernatant of peripheral blood mononuclear cells (PBMC) cultured *ex vivo* and not in the serum of IBD patients. A subsequent letter by the same authors revealed that serum sHLA-G levels correlated with the sHLA-G level in the PBMC supernatant.<sup>[25]</sup> Second, the study cohort included both CD and UC adult patients (mean age: 54 and 51 years in the former,<sup>[13]</sup> and 55 and 50 years in the latter<sup>[25]</sup>) but not pediatric patients. More recently, Zhu *et al.*<sup>[24]</sup> studied serum sHLA-G levels in colorectal cancer, adenoma, hyperplastic polyps, and IBD patients; in spite of the lack of an exclusive focus on IBD, their results revealed significantly higher sHLA-G levels in adult patients with IBD (mean age: 59 years), but sHLA-G levels were not analyzed separately for UC or CD patients. Zidi *et al.*<sup>[31]</sup> investigated specific insertion/deletion polymorphisms in the HLA-G locus in relation to the serum sHLA-G levels of adult patients with CD and found higher sHLA-G levels compared with that of controls, and that sHLA-G levels were modulated by the following polymorphisms: 14-bp Del/Del, Del/Ins and the 14-bp Ins/Ins that generate a low HLA-G-producing and low HLA-G-producing phenotype, respectively. Importantly, the results obtained by Zidi *et al.*<sup>[31]</sup> suggested that serum HLA-G dimers may be associated with disease severity in adult patients with CD. However, data in this regard in the pediatric population with IBD are unavailable. Therefore, this report presents findings from the first investigation of serum sHLA-G levels in a pediatric population with IBD. Moreover, we assessed the association of serum sHLA-G

levels with disease severity, and similar to Rizzo *et al.*'s<sup>[13]</sup> findings from the tissue culture supernatants obtained from PBMCs of IBD patients, we found that pediatric patients with CD had higher serum sHLA-G levels than pediatric patients with UC and healthy controls. Of note, all pediatric UC patients had mild disease; therefore, it is conceivable that pediatric patients with moderate/severe UC might have higher sHLA-G levels than those with mild UC. Nonetheless, further study is needed to ascertain whether patients in the moderate/severe category in both CD and UC have similar serum sHLA-G levels. HLA-G has various immunoregulatory effects, including inhibition of cytotoxic T lymphocyte production, natural killer (NK) lysis, induction of apoptosis in T lymphocytes and NK cells, inhibition of T-cell chemotaxis, and modulation of pro-angiogenic factor release from NK cells.<sup>[9]</sup> Therefore, the upregulation of serum HLA-G, an inhibitory molecule, may be a homeostatic response to counter the active inflammatory process in IBD, which may be induced by inflammation itself or dysbiosis/altered microbial profile in the gut. However, these predictions require investigation in more detailed immunopathogenesis studies.

Our study has some limitations. The sample size for both CD and UC was small, thus this research needs to be repeated in a larger study sample, which could improve the correlation between UC and sHLA-G levels. Moreover, since our UC cohort only comprised patients with mild disease the inclusion of patients with moderate/severe UC will enable a better assessment.

## CONCLUSION

In summary, our results reveal that serum sHLA-G levels in IBD patients are positively correlated with the disease severity and can serve as an indicator or biomarker of the DAI. sHLA-G has greater significance as a biomarker in Crohn's disease than in ulcerative colitis.

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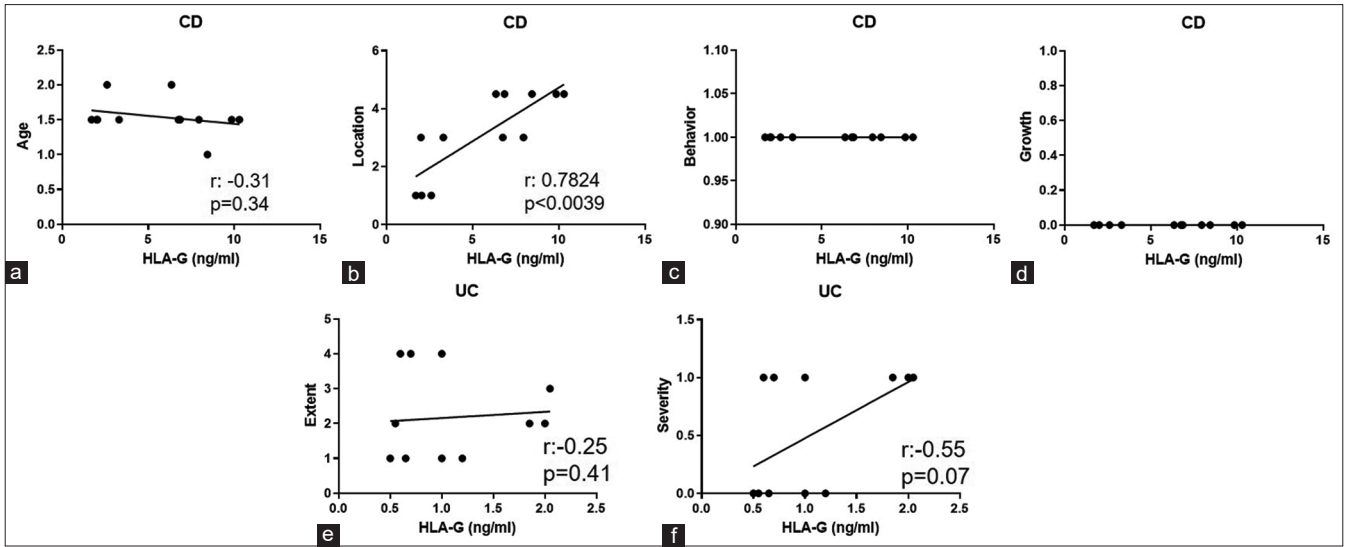
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### Conflicts of interest

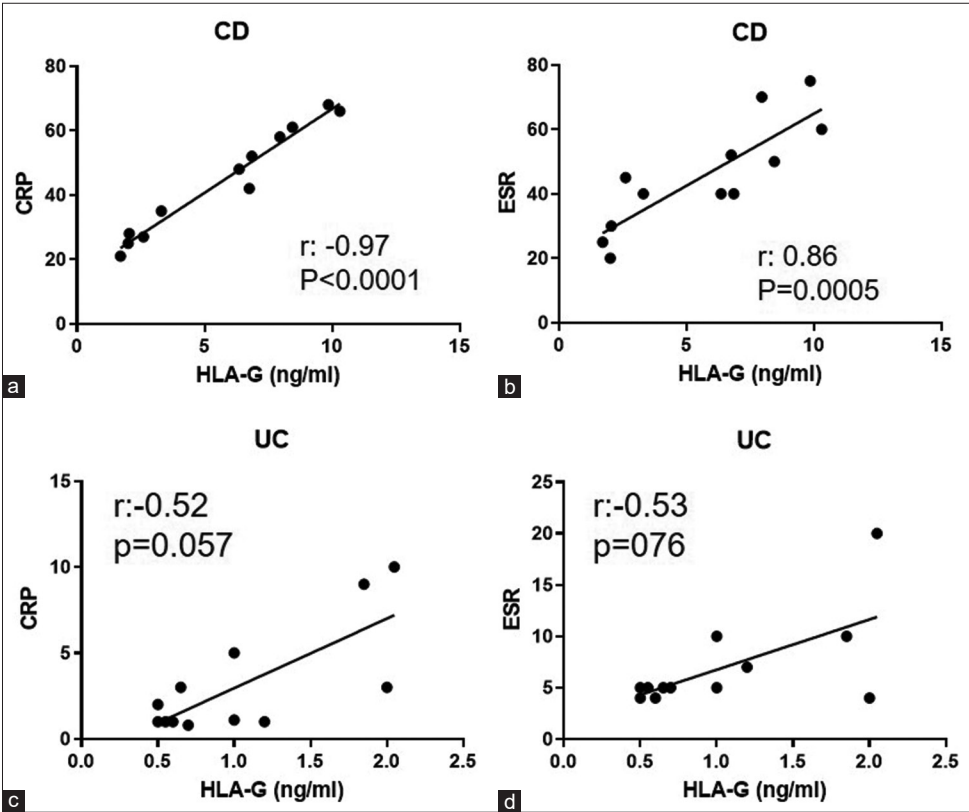
There are no conflicts of interest.

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**Supplemental Figure 1:** Correlation of Serum sHLA-G levels with Paris Classification Parameters—Age, Location, Behavior, Growth, Extent, and Severity—in pediatric UC and CD patients. Spearman’s correlation test was applied to evaluate the correlation between sHLA-G levels and Age (a), Location (b), Behavior (c), and Growth (d) for CD patients, n = 10; Extent (e) and Severity (f) for UC patients, n = 11



**Supplemental Figure 2:** Correlation of Serum sHLA-G levels with CRP and ESR in pediatric CD and UC patients. Spearman’s correlation test was applied to evaluate the correlation between sHLA-G levels and CRP (a) and ESR (b) for CD patients or UC patients (c and d) CD, n = 12; UC, n = 12