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HLA-G is upregulated in advanced endometriosis

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ABSTRACT

Objective: To assess whether the HLA-G immunomodulatory protein is potentially involved in the pathophysiology of endometriosis or disease progression.
Study design: Cross-sectional observational study of 227 women who underwent laparoscopy, being 146 for endometriosis excision and 81 for elective tubal ligation (control group). Soluble HLA-G (sHLA-G) levels in the serum and peritoneal fluid (PF), as well as the HLA-G protein expression in matched eutopic and ectopic endometrium of women with and without endometriosis were evaluated by ELISA and immunohistochemistry assays, respectively. Women with endometriosis were separated into groups according to the initial (I/II, n = 60) and advanced (III/IV, n = 86) stages of disease. sHLA-G measurement was performed only in women with matched serum and PF samples in both the control (CTRL; n = 77) and endometriosis (EDT; I–II, n = 60; III–IV, n = 83) groups. HLA-G protein expression was evaluated in 26 women with deep endometriosis (I–II, n = 12; III–IV, n = 14) and 22 controls.
Results: Higher concentrations of sHLA-G ($P = 0.013$) in the serum but not in the PF were observed in women with advanced endometriosis compared to the control group. *In situ* expression of HLA-G protein was also higher in ectopic ($P = 0.018$) but not in eutopic endometrium of women with advanced endometriosis compared to control group.
Conclusion: Our findings suggest that HLA-G upregulation in advanced stages may contribute to the state of immunosuppression in endometriosis as disease progresses.

Introduction

Endometriosis is a chronic inflammatory disease characterized by the presence of endometrial-like tissue (gland and stroma) outside the uterus and associated with chronic pelvic pain, dysmenorrhea, and infertility [1]. Endometriosis typically behaves as a neoplastic process by spreading into the adjacent stroma and being associated with distant lesions [2]. The ectopic implantation is thought to be mostly promoted by retrograde menstruation [3]. However, there must be other predisposing factors that facilitate the implantation, growth, and maintenance of ectopic endometrial cells, considering that retrograde menstruation is a physiological phenomenon in healthy women [4]. Thus, potential factors involved in the development of endometriosis have been investigated and implicated in underlying pathophysiological mechanisms, including the local and systemic immune responses.

In this regard, immune cells of peripheral blood (PB) and peritoneal fluid (PF) have shown decreased cytotoxicity in women with endometriosis [5–7], and local and systemic cytokine profiles display a shift towards a Th2 immune response [8]. Together, these data suggest that impaired effector immune responses generate an immunosuppressive environment likely to be involved in the defective immunological surveillance in endometriosis. Likewise, the influence of the immunomodulatory

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molecule, human leukocyte antigen G (HLA-G), has been discussed to play a role in the pathogenesis of endometriosis [9], but this is still unclear and it has not been investigated in relation to disease progression.

HLA-G mediates the inhibition of immune cell functions and has been identified as an important mechanism used in tumor escape from immune surveillance. HLA-G has been physiologically identified mostly on trophoblasts, but can also be neo-expressed on cells suffering stress or damage, such as in cases of tumors, allografts, and inflammatory and autoimmune diseases [10]. HLA-G is transcribed into seven isoforms generated by an alternative splicing encoding four membrane-bound proteins (HLA-G1 to HLA-G4) and three soluble molecules (HLA-G5 to HLA-G7). In addition, soluble HLA-G1 molecules, which are structurally similar to the HLA-G5 isoform, can be found following proteolytic cleavage of the HLA-G1 membrane-bound from the cell surface [11]. HLA-G proteins interact with specific inhibitory receptors on the surface of the different immune cell lineages, impairing the effector immune response. The soluble HLA-G (sHLA-G) isoforms can also inhibit NK and cytotoxic T cell function by inducing apoptosis through CD8 ligation or by enhancing the expression of Fas ligand on cytotoxic effectors and triggering Fas/Fas ligand mediated cell death [12,13]. Increased circulating sHLA-G levels have been found in patients with different solid tumors and blood cancers; sHLA-G is secreted by monocytes activated by tumor cells rather than by tumor cells themselves, dampens tumor immune responses and correlated with relapse [14–16].

We have hypothesized that the continuous stress taking place in the peritoneal cavity in endometriosis [17], including the production of various cytokines, induces upregulation of HLA-G expression in ectopic endometrial cells and also in immune cells. Higher levels and cellular expression of HLA-G protein, in turn, would contribute to the failure in the clearance of ectopic endometrium, and to generating an overall immunologic context with excessive immunoregulation and impaired effector activity. HLA-G would, thus, participate in the pathophysiology of endometriosis.

To test our hypothesis, we investigated whether HLA-G is differentially expressed in endometriosis according to the disease stages, by evaluating the HLA-G protein expression in eutopic and ectopic endometrium, as well as the levels of soluble HLA-G (sHLA-G) in serum and PF of women with endometriosis, compared with healthy women.

Material and methods

Study design

This cross-sectional observational study, approved by the *Hospital das Clínicas* of the University of Sao Paulo Institutional Review Board (CAPPesq # 0386/11) and conducted between August 2011 and August 2016, enrolled a total of 227 women with monthly menstrual periods who underwent laparoscopy for endometriosis excision (n = 146) or elective tubal ligation (n = 81). Women included in endometriosis (EDT) group had superficial (n = 18), ovarian (n = 9) or deep (n = 119) endometriosis. Endometriosis was classified during laparoscopy based on the stage of the disease (I to IV), according to the 1996 revised American Society for Reproductive Medicine (rASRM) criteria [18]. For statistical analysis purposes, stages I and II were grouped as initial stages (n = 60), while stages III and IV were grouped as advanced stages (n = 86) of the disease. Healthy women who underwent tubal ligation with no signs of endometriosis at laparoscopy were included in the control (CTRL) group. Women from both groups had the time point in the menstrual cycle determined from the date of onset of the last menstrual period, by histopathological examination of eutopic endometrium, confirming the menstrual cycle phase-menstrual (days 1–4), proliferative (days 5–14) or secretory (days 15–28).

Exclusion criteria were the use of hormonal medication during the three months prior to surgery and the existence of other inflammatory, autoimmune or malignant disease. Women with inflammatory signs detected in the histopathological analysis of the eutopic endometrium were also excluded from the study.

Soluble HLA-G (sHLA-G) measurement was performed by enzyme-linked immunosorbent assay (ELISA) in matched serum and PF samples collected from both the control (CTRL; n = 77) and endometriosis (EDT; I–II, n = 60; III–IV, n = 83) groups.

Tissue HLA-G protein expression was evaluated by immunohistochemistry in eutopic endometrium specimens from 22 women of the CTRL group and in matched eutopic and ectopic endometrium from 26 women with deep endometriosis (DE) who had rectosigmoid (15) or retrocervical (11) lesions excised. Clinical features of women evaluated for sHLA-G and tissue HLA-G protein expression in control and endometriosis groups are shown in Table 1.

Table 1
Distribution of women evaluated for soluble HLA-G and tissue HLA-G protein expression in control and endometriosis groups, according to the menstrual cycle phase and stage of endometriosis.

	Soluble HLA-G protein		Tissue HLA-G protein	
	Control (n = 77)	Endometriosis (*n* = 143)	Control (*n* = 22)[a]	Endometriosis (n = 26)[a]
Age (Years)				
Mean (SD)		35.0 (5.8)	32.8 (5.9)	34.7 (6.3)
Menstrual cycle phase, *n* (%)				
Menstrual	4 (5)	6 (4)	2 (9)	5 (19)
Proliferative	33 (43)	39 (27)	15 (68)	6 (23)
Secretory	31 (40)	94 (66)	5 (23)	15 (58)
Unknown[b]	9 (12)	4 (3)		
rASRM stage, *n* (%)				
I–II (Initial)		60 (42)		12 (46)
III–IV (Advanced)		83 (58)		14 (54)

[a] Four women in CTRL and 3 women in EDT group included in tissue HLA-G protein evaluation were not among those evaluated for soluble HLA-G protein.
[b] Dating of endometrial specimen not possible.

Sample collection and processing

Prior to the anesthetic procedures, 3–5 mL of PB was collected. After anesthesia induction, specimens of eutopic endometrium were obtained using a Pipelle curette. During laparoscopy, PF samples were collected from the anterior or posterior *cul-de-sac* by aspiration, using a laparoscopic cannula before surgical intervention to minimize blood contamination. Specimens of DE lesions surgically excised were also collected and confirmed by histological analysis. The PB and PF samples were placed in sterile tubes and centrifuged (10 min at 2500 *g*) to isolate cell-free supernatant. The serum and PF supernatants were aliquoted and stored at -80 °C until assayed. Samples of the endometrial tissues and the excised DE lesions were formalin-fixed, paraffin-embedded and stored until the tissues were sectioned at 3 μm and mounted on glass slides to perform immunohistochemistry.

ELISA

Serum and PF concentrations of sHLA-G were determined using a specific sandwich ELISA kit BioVendor-Exibo (catalog number RD194070100R) that includes the MEM-G/9 mouse monoclonal antibody which recognizes the most abundant soluble isoforms of HLA-G: shed sHLA-G1 and native sHLA-G5 [19]. Soluble HLA-G assay was performed according to the manufacturer's instructions using undiluted serum and PF samples. Concentrations of sHLA-G are presented in units per microliter (U/mL). The limit of detection was 0.6 U/mL.

Immunohistochemistry and scoring

The mouse IgG1 anti-HLA-G monoclonal antibody 4H84 Exibo (catalog number 1B-291-C100) was used at 1:100 to stain histological sections of eutopic endometrium and DE lesions. Immunostaining was performed using Vector Laboratories' ABC KIT Vectastain Elite Mouse IgG horseradish peroxidase system (catalog number PK-6102), which includes an anti-mouse IgG as the reporter antibody.

The stained tissues were blindly examined by a pathologist experienced in interpreting immunohistochemical staining in endometriosis tissues (K.P.), and were evaluated for both the extent and intensity of HLA-G protein, using a semi-quantitative scale, from 0 to 4 and 0 to 3, respectively, as previously described [20]. A final immunoreactivity score (IRS) was obtained by multiplying the distribution score of positive cells by the staining intensity score. The IRS was a value between 0 and 12, for both epithelial and stromal cells evaluation.

Statistical analysis

Continuous variables were tested for normality using the Kolmogorov-Smimov and Shapiro Wilk tests. The women's age was described by mean and standard deviation. Non-parametric data had values expressed as median and minimum-maximum. Medians were compared using the Mann–Whitney *U* test for two independent samples or Kruskal–Wallis test with a Müller-Dunn post-hoc test for three or more samples. *P*-values < 0.05 indicated statistical significance. Analyses were performed using GraphPad Prism 5.0 statistical package.

Results

sHLA-G concentrations (U/mL) were significantly higher in PF than in serum of women from both the CTRL and EDT groups, irrespective of the menstrual cycle phase ($P < 0.0001$). Considering the whole EDT group, sHLA-G concentration did not significantly differ from the CTRL group, either in the serum ($P = 0.090$) or in the PF ($P = 0.554$) (Fig. 1).

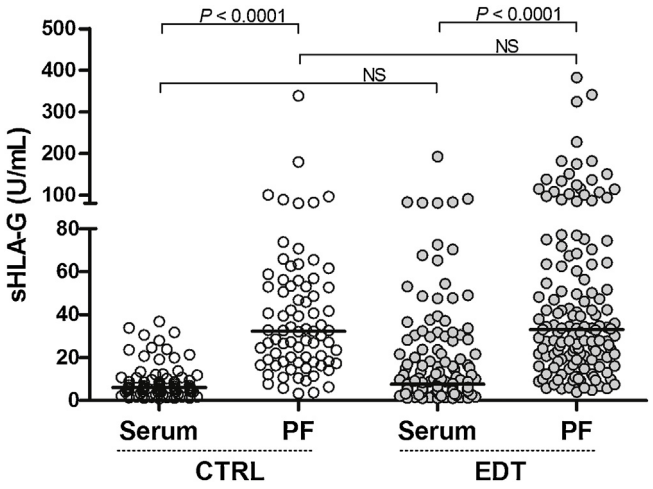

Fig. 1. Median and range values of sHLA-G concentration (U/mL) in serum and peritoneal fluid (PF) of control ($n = 77$) and endometriosis ($n = 143$) groups. CTRL: serum (6.1, 0.1–36.9), PF (32.3, 3.3–338.6). EDT: serum (7.7, 1.2–192.6), PF (33.1, 4.0–382.7). Horizontal bars represent the median values, whiskers, the minimum and the maximum; NS, not significant *p*-value; Mann–Whitney *U* test.

Considering the stages of endometriosis, sHLA-G levels (median; range) in serum of women with advanced endometriosis (EDT III–IV; median 9.3 U/mL; range 1.2–192.6) were significantly higher than those in the CTRL group (6.1; 1.0–36.9; $P = 0.013$). In constrast, serum sHLA-G levels of women with initial endometriosis (EDT I-II; 6.0; 1.2–83.4) did not significantly differ either from those of the CTRL group ($P = 0.872$) or with advanced EDT ($P = 0.076$) (Fig. 2A). Comparison of sHLA-G levels in the PF of women with either initial (33.8; 4.0–382.7; $P = 0.737$) or advanced (32.8; 4.9–324.2; $P = 0.240$) EDT did not significantly differ from the CTRL group (median, 32.3; range 3.3–338.6). Comparison of initial and advanced EDT showed no significant difference (P=0.210) of sHLA-G levels in PF (Fig. 2B).

The menstrual cycle phase did not impact the serum sHLA-G levels within the CTRL ($P = 0.701$) or EDT ($P = 0.166$) groups, as well as the PF sHLA-G levels within the CTRL ($P = 0.860$) group; but within the EDT group, higher levels of sHLA-G was found in PF of women in the menstrual phase compared to secretory phase ($P = 0.022$) (Table 2).

Immunostaining for HLA-G protein in DE lesions, as well as in eutopic endometria was positive in all samples of both the EDT and CTRL groups. The immunostaining pattern of rectosigmoid and retrocervical DE lesions was similar ($P = 0.36$; Mann–Whitney test; data not shown).

HLA-G protein expression was found mostly in the glandular epithelium and rarely in the stroma, in DE lesions and eutopic endometrium of both the EDT and CTRL groups (Fig. 3). Once Stromal IRS median values were zero for DE lesions and for eutopic endometrium of both CTRL and EDT groups (Table 3), only Epithelial IRS was considered for statistical comparisons between the groups (Table 4).

The IRS of HLA-G protein expression was also not impacted by the menstrual cycle phase in either CTRL ($P = 0.316$) or EDT ($P = 0.149$) endometrium and in DE lesion ($P = 0.126$), showing no statistically significant difference between proliferative and secretory phases (Table 3).

HLA-G expression in eutopic endometrium did not significantly differ between the CTRL and EDT groups, either considering the total specimens of EDT endometrium ($P = 0.268$; Table 4) or analyzing them according to the initial (I–II; $P = 0.912$) and

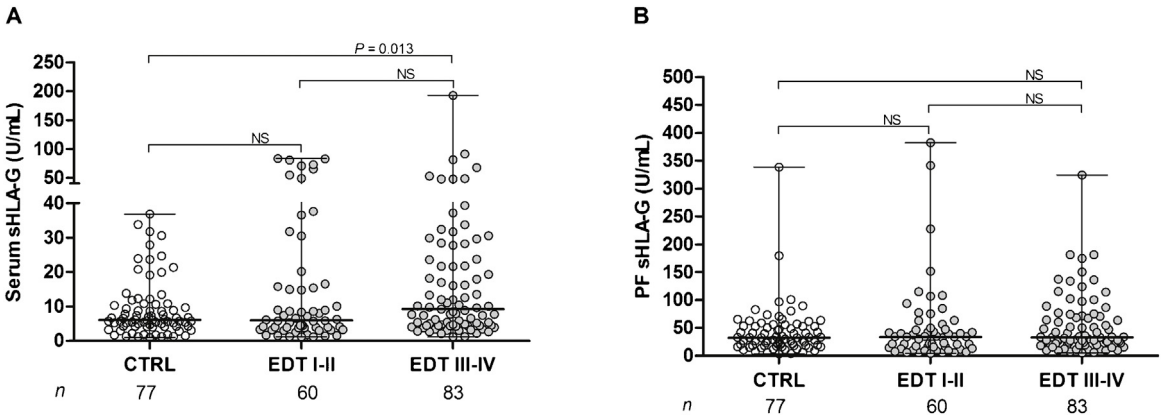

Fig. 2. Soluble HLA-G concentration (U/mL) in serum (A) and PF (B) of women from the Control (CTRL) and endometriosis (EDT) groups, according to the initial (I–II) and advanced (III–IV) stages of EDT. A. Significant difference ($P = 0.013$) between CTRL and advanced EDT (III–IV) were found in serum. B. No significant differences were found among the groups in PF. Horizontal bars represent the median values, whiskers, the minimum and the maximum; NS, not significant *p*-value; Mann-Whitney U test.

Table 2
Soluble HLA-G concentration[a] in serum and peritoneal fluid of women from control and endometriosis groups, according to the menstrual cycle phases.

	Serum		Peritoneal Fluid	
	CTRL	EDT	CTRL	EDT
Menstrual cycle phase				
Menstrual	9.2 (1.2–23.7)	5.9 (3.4–80.7)	34.5 (23.5–65.6)	125.9 (16.2–341.2)
Proliferative	5.7 (1.0–33.8)	11.0 (1.2–83.4)	32.1 (5.9–338.6)	35.2 (5.6–382.7)
Secretory	7.0 (1.2–36.9)	7.0 (1.2–192.6)	28.6 (3.3–100.9)	32.3 (4.0–324.2)
P-value	0.701	0.166	0.860	**0.022**[b]

[a] Values of soluble HLA-G concentration (U/mL) are given as median (minimun–maximum).
[b] Statistically significant difference between menstrual and secretory phases. Kruskal-Wallis test with Dunn's multiple comparison post-test.

advanced (III–IV; $P = 0.105$) stages of endometriosis (Table 4). In contrast, DE lesions in the advanced stages ($P = 0.018$; Table 4), but not the in the initial stages ($P = 0.386$; Table 4) showed higher HLA-G expression than the CTRL endometrium.

Comments

We here reported, for the first time, that HLA-G protein is upregulated both systemically and within the lesions only in the advanced stages of endometriosis. Considering the tumor-like phenotype of endometriosis and the HLA-G immunoregulatory activities, these findings suggest that HLA-G may be implicated in disease progression. Indeed, the direct contribution of HLA-G to tumor progression has already been shown *in vivo* [21]. Likewise, a significant association between increasing HLA-G protein staining and advanced stages of endometrial cancer has been reported [22], and HLA-G protein expression was also found in high grade ovarian tumors and very infrequently in low grade tumors [23].

Previous studies have reported immunosuppressive activity in both the serum [24] and PF [25] from women with endometriosis. Among immunosuppressive factors, such as various cytokines present in serum [26] and PF [27], we believe that HLA-G protein upregulation, both within endometriosis lesions and circulating, is likely to contribute to the immunosuppressive *status* of endometriosis, affecting effector immune cells, by binding to inhibitory receptors on their surface. Indeed, taken the higher number of immune cells in PF of women with endometriosis [28,29], we reason that the membrane-bound HLA-G expression on ectopic endometrial cells, and soluble HLA-G in excess, would locally favor the binding of HLA-G to inhibitory receptors on immune cells and, consequently, impair effector immune cell functions. This binding could explain why we did not find significant difference in soluble HLA-G available in PF between women with and without endometriosis.

Even though we did not find significantly higher levels of sHLA-G in PF of women with endometriosis compared with healthy women, we found higher levels of sHLA-G in PF of women with endometriosis during menstrual phase compared to secretory phase (Table 2), maybe due to the exacerbate volume of retrograde menstruation in women with endometriosis [4], which, in turn, carries HLA-G-expressing endometrial fragments to the peritoneal cavity [30]. PF sHLA-G assessment in women with endometriosis has been previously performed, but in comparison to women with other gynecological diseases, and similar levels of sHLA-G were observed. [31]. On the other hand, the much higher sHLA-G levels we found in the PF than in PB sera of healthy women, also point to a physiological role of sHLA-G in keeping an immunomoregulatory environment within the peritoneal cavity, even in the absence of any inflammatory disease.

We raise that the higher levels of sHLA-G in serum of women with advanced endometriosis, may have been peripherally-induced by cytokines in circulating immune cells [16], or could also be remnant from a local production, by both ectopic endometrial cells or immune cells in the endometriosis microenvironment. In fact, HLA-G-inducing cytokines, such as IL-10 [32], IFN-β [16] or IFN-γ [33], were found upregulated both local and systemically in women with endometriosis [34–36].

Previous investigation has shown that sHLA-G could be accumulated in serum of women with DE compared with women with ovarian cancer [37]. We, in turn, compared the serum of women with endometriosis with healthy women. Therefore, we may say that the higher sHLA-G serum levels we found only in advanced endometriosis are distinct from the physiological state, pointing to a potential use of seric sHLA-G levels as a marker of endometriosis progression.

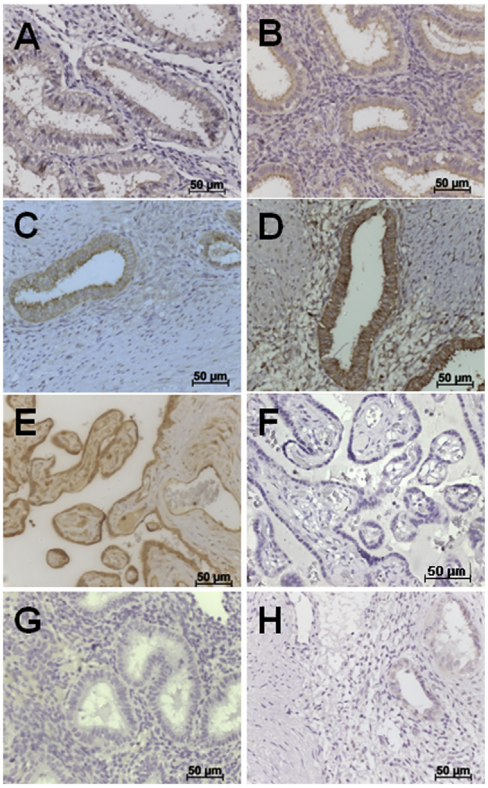

Fig. 3. Representative examples of immunostaining for HLA-G protein expression in eutopic endometrium and deep endometriosis (DE) lesion. (A) and (B), eutopic endometrium of women from control and endometriosis groups, respectively; (C) and (D), rectosigmoid DE lesion from women in the initial and advanced stages of endometriosis, respectively. **(**E), Human placenta, positive control; (F), (G), (H), negative controls (secondary antibody only) for human placenta, eutopic endometrium and rectosigmoid DE lesion, respectively. Original magnification ×40.

The *in situ* investigation of HLA-G protein expression in endometriosis and adenomyosis has also been carried out before and showed conflicting results [30,38,39]. In our research, the similar expression of HLA-G in eutopic endometrium of healthy and endometriosis women, lead us to interpret that factors in the endometriosis microenvironment may have promoted the upregulation of HLA-G expression in the DE lesions. But, in contrast to other investigators [30], the HLA-G expression we found in the eutopic endometrium in the various phases of the menstrual cycle indicates that modulatory stimuli for HLA-G expression in the endometrium are physiologically activated even during non-inflammatory phases.

Some data in literature support the interpretation that HLA-G expression could be induced both in the endometriosis microenvironment and systemically, in advanced stages of endometriosis [40,41]. In the light of our findings and data in the literature, we reason that endometrial cells translocated to the peritoneal cavity may change their phenotype locally, favoring ectopic implantation and proliferation [42]. In addition, the continuous stress in the peritoneal cavity would stimulate mechanisms that promote increased HLA-G expression [9,17], as we noticed in DE lesions.

Therefore, we argue that HLA-G is likely to be involved in the pathophysiology of the disease, favoring the occurrence of a defective effector immune response, in the advanced stages, possibly contributing to endometriosis progression.

Conclusions

Our data support that the increased expression of HLA-G protein in women with advanced endometriosis could contribute to the context of immunosuppression, driven locally by the ectopic lesions and with systemic repercussion.

Conflicts of interest

The authors have no conflict of interest to declare.

Table 3
Epithelial and stromal immunoreactivity score (IRS)[a] of HLA-G protein expression in eutopic endometrium and deep endometriosis (DE) lesion in women from the control (CTRL) and endometriosis (EDT) groups, according to the menstrual cycle phase.

	CTRL endometrium		EDT endometrium		DE Lesion	
	Epithelial IRS	Stromal IRS	Epithelial IRS	Stromal IRS	Epithelial IRS	Stromal IRS
Menstrual cycle phase						
Menstrual	3 (3–3)	0 (0–0)	4 (2–12)	0 (0–1)	4 (3–8)	0 (0–1)
Proliferative	6 (1–12)	0 (0–0)	3 (2–12)	0 (0–1)	8 (3–12)	0 (0–0)
Secretory	4 (4–12)	0 (0–0)	8 (3–12)	0 (0–6)	8 (3–12)	0 (0–1)
P-value	0.194		0.287		0.142	

[a] IRS, immunoreactivity score represents a value ranging from 0 to 12 obtained by multiplying the distribution score (0–4) by the intensity score (0–3). Values of IRS given as median (min-max). Only Epithelial IRS was considered for statistical comparisons, once Stromal IRS median values were zero for the three tissue specimens (CTRL endometrium, EDT endometrium and DE Lesion). Kruskal–Wallis test with Dunn's multiple comparison post-test.

Table 4
Epithelial Immunoreactivity Score (IRS)[a] of HLA-G protein expression in eutopic endometrium and deep endometriosis (DE) lesion of women from the control (CTRL) and endometriosis (EDT) groups, according to the stages of disease.

	CTRL endometrium	EDT endometrium		DE lesion	
	Epithelial IRS	Epithelial IRS	*P-value*[b]	Epithelial IRS	*P-value*[b]
ASRM stage					
Initial (I–II)		4 (2–12)	0.912	6 (3–12)	0.386
Advanced (III–IV)		8 (2–12)	0.105	8 (3–12)	**0.018**
Total	4 (1–12)	6 (2–12)	0.268	8 (3–12)	**0.043**

[a] IRS, immunoreactivity score represents a value ranging from 0 to 12 obtained by multiplying the distribution score (0–4) by the intensity score (0–3) observed both in the epithelial and stromal cells of endometrium and endometriotic cells in DE lesions. Only epithelial IRS was considered for statistical comparisons, once stromal IRS median values were zero for the three tissue specimens (CTRL endometrium, EDT endometrium and DE Lesion). Values of epithelial IRS were given as median (min-max).
[b] *P*-value in comparison with control (CTRL) endometrium. Bold *P*-value highlight the statistically significant differences. Mann–Whitney *U* test.

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