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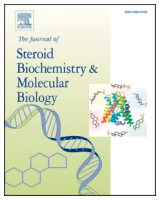

Spatiotemporal expression pattern of Progesterone Receptor Component (PGRMC) 1 in endometrium from patients with or without endometriosis or adenomyosis

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ABSTRACT

The endometrium plays a crucial role in reproduction and, in humans, is cyclically remodeled under hormonal control. Estradiol favors tissue proliferation whereas progesterone inhibits tissue growth and induces morphological changes. Endometriosis is often associated with fertility issues and with exacerbated estrogen and reduced progesterone concentration or response in the eutopic endometrium. However, underlying mechanisms remain unclear. Progesterone Receptor Membrane Component (PGRMC) 1 is a protein able to modulate progesterone response and its murine knockout reduced fertility. However, the precise spatiotemporal pattern of PGRMC1 expression in the human endometrium is still poorly characterized. We investigated variations of eutopic endometrial PGRMC1 expression by combining RT-qPCR, immunofluorescence and *in situ* hybridization. We found that PGRMC1 expression progressively increases during the proliferative phase and decreases during the secretory phase. However, immunolabeling and identification of mRNA-containing cells were regularly heterogeneous in samples, according to tissue depth, with a gradient extending from the surface epithelium towards the basalis. There was no significant difference in PGRMC1 mRNA amounts between patients with or without endometriosis or adenomyosis, for any phase of the menstrual cycle, but cells with strong or moderate PGRMC1 immunolabeling were reduced during the proliferative phase in endometriotic patients. In conclusion, although the cyclical variation of PGRMC1 expression globally follows fluctuation of ovarian steroids, further work is required to precisely characterize hormonal control and identify the additional levels of regulation responsible for local adjustment of PGRMC1 concentration. This is particularly important in the light of recent studies emphasizing the correlation between adequate PGRMC1 amounts and fertility.

1. Introduction

The endometrium is the uterus mucosa composed of epithelial (glandular or luminal) cells, and a rich stroma. Histologically, the basal layer, in contact with the myometrium, can be distinguished from the functional layer, extending towards the uterine cavity. The endometrium is a dynamic tissue, continuously subjected to tissue breakdown, regeneration and maturation throughout the menstrual cycle. These events are globally orchestrated by circulating ovarian steroids in order to prepare for potential blastocyst implantation [1]. The molecular mechanisms responsible for response to estradiol and progesterone therefore need to be finely regulated, both spatially and temporally, to ensure the major function of the endometrium, fertility [2].

Endometriosis and adenomyosis are gynecological pathologies characterized by the growth of endometrial tissue either outside the uterus (ectopic lesion) or within the myometrium respectively. Endometriosis affects approximately 1 out of 10 women worldwide and is the cause of severe pelvic pain and reduced fertility. In many aspects, adenomyosis shows similarities with endometriosis, particularly deep endometriosis [3]. In addition to common histological features,

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Table 1
Endometrial samples analyzed by immunofluorescence and/or *in situ* hybridization.

Sample #	Age	Menstrual Phase	Successful pregnancy (ies)	Group	Pathology (ies)	Experiment (s)
1	45	EP	Yes	CTL	Menometrorrhagia	IF-ISH
2	49	EP	No	CTL	Leiomyoma	IF
3	49	MP	Yes	CTL	Myoma, menorrhagia	IF-ISH
4	45	LP	Yes	CTL	/	IF-ISH
5	41	ES	Yes	CTL	Leiomyoma, fibroma, atheromatosis	IF-ISH
6	44	MS	Yes	CTL	Uterine tube cyst, hydrosalpinx	IF-ISH
7	44	MS	Yes	CTL	Leiomyoma, menometrorrhagia, atheromatosis, cervicitis	IF-ISH
8	37	MS	Yes	CTL	Prolapsus	IF-ISH
9	45	MS	Yes	CTL	/	IF
10	38	LS	No	CTL	/	IF
11	44	LP	Yes	A	Adenomyosis	IF
12	43	MS	Yes	A	Adenomyosis	IF
13	48	EP	Yes	E	Endometriosis	IF
14	44	MP	Yes	E	Peritoneal endometriosis, leiomyoma	IF
15	41	MP	Yes	E	Peritoneal endometriosis, dysmenorrhea, menorrhagia	IF
16	44	ES	Yes	E	Cervical endometriosis, salpingitis, hydrosalpinx, uterine tube cyst	IF
17	46	MS	No	E	Cervical endometriosis, fibroma, cervicitis	IF

Abbreviations: EP/MP/LP, Early/mid/late proliferative phase; ES/MS/LS, Early/mid/late secretory phase; /, with no identified gynecologic pathology; IF, immunofluorescence; ISH, *in situ* hybridization.

adenomyosis and endometriosis seem to share symptoms, etiological hypotheses, diagnostic methods, therapeutic molecules,. [4] so much so that some authors have suggested that they are simply different manifestations of a single disease [5].

Endometriotic lesions typically present an imbalance in their capacity for hormonal response which results in over-stimulation of estrogenic signaling pathways (estradiol dominance) and, conversely, in a decrease in their capacity for progestational response (progesterone resistance). Consequently, treatments which are mainly based on the prescription of progestogen compounds (progestins) [6,7] only succeed in limiting estradiol-primed cell proliferation. However, they do not eliminate the lesions of endometriosis [8] and are even ineffective in some patients [9,10]. The origin of progesterone resistance is multifactorial and not fully understood [11]. A decrease in the synthesis and/or biological activity of the nuclear progesterone receptor (nPR) has already been observed, in particular in endometriosis lesions, but not systematically. Moreover, the results are often contradictory in the eutopic endometrium from a patient with endometriosis and therefore cannot explain the infertility encountered in 50% of patients [12]. On the other hand, there are other receptors/proteins capable of transmitting or modulating a progesterone response that were poorly considered by comparison with nPR so far. A decrease in the expression and protein abundance of the membrane progesterone receptors α and β (also named PAQR7 and PAQR8 respectively) as well as of the protein PGRMC1 (Progesterone Receptor Membrane Component 1) has been observed in the eutopic endometrium [13,14].

A member of the MAPRs (Membrane Associated Progesterone Receptors) protein family, PGRMC1 has been the subject of several studies aimed at establishing its role in various gynecological pathologies and in particular in endometrial, ovarian and breast cancer where it appears to be overexpressed [15–18]. Thus, PGRMC1 was reported to modulate cell proliferation and apoptosis [17,19–21], promote cell migration and invasion [22], regulate the expression of enzymes involved in cholesterol metabolism, participate in the metabolism of therapeutic molecules [15,23] and interfere in some of their target interactions [24]. Additionally, its implication in fertility has already been suggested. First, a conditional Pgrmc1 knockout in the murine endometrium induced the development of endometrial cysts and a decrease in the offspring [25]. In humans, PGRMC1 expression was decreased in endometrium of patients suffering from recurrent miscarriage [16,26]. Moreover, PGRMC1 was reported to participate in the decidualization of primary endometrial stromal cells, a differentiation that is essential for embryo implantation [27,28]. Two studies, based on microarray [29] or proteomic [30] analyses, determined that PGRMC1 is abundantly expressed during the proliferative phase and that it decreased in secretory phase, especially in the stroma. However, the precise spatiotemporal pattern of PGRMC1 expression in the human endometrium is still poorly characterized.

In this study, we investigated the spatiotemporal variations of PGRMC1 expression in the human endometrium by combining quantification and localization of both its mRNA and the protein. Endometria from control patients were compared to endometria from patients suffering from endometriosis or from adenomyosis.

2. Materials and methods

2.1. Tissue collection

The study was approved by the Ethical Committee of the Université Catholique de Louvain (2017/10JUL/362). Tissue samples were provided by the Biobank of Saint-Luc University Clinics as surgical leftovers from patients operated for clinical reasons independent from the study. Biomaterial was obtained by biopsy during laparoscopy or following hysterectomy from patients with spontaneous menstrual cycle (<50 years old), without progestin treatment for the last 3 months and without uterine cancer or endometritis. Other gynecological pathology (ies) for samples used for immunofluorescence and/or *in situ* hybridization are detailed in Table 1. Samples of eutopic endometrium were separated in three groups: patients with endometriosis (E), patients with adenomyosis (A) and control patients without endometriosis or adenomyosis (CTL).

For each sample, the cycle phase was determined by an expert anatomo-pathologist on the basis of histological criteria. In a classical menstrual cycle of 28 days, the menstrual phase was considered to extend from day 1–5, the early, mid and late proliferative phases respectively from day 6–8, from day 9–11 and from day 12–14, and the early, mid and late secretory phases respectively extend from day 15–18, from day 19–22 and from day 23–28.

Tissue for histological examination was fixed in 4% paraformaldehyde and embedded in paraffin whereas tissue for RNA analysis was placed into RNA lysis buffer (SV total RNA isolation system; Promega, Madison, WT, USA) at − 80 °C until RNA extraction.

2.2. RNA extraction and quantitative real-time PCR

Total RNA was isolated from tissue samples using the SV total RNA isolation system according to the manufacturer's recommendations (Promega).

For all samples, the RNA concentrations were evaluated using a Nanodrop ND-8000 spectrophotometer. 500 ng total RNA were used for reverse transcription using SuperScript III Reverse Transcriptase kit

Table 2
Primary antibodies used for immunolabeling.

	Isotype	Company	Commercial reference	Dilution
PGRMC1 (D6M5M)	Rabbit mAb	Cell Signaling	13,856	1/250
E-cadherin (Clone 36)	Mouse IgG2a mAb	BD Transduction Laboratories	610,182	1/1000

(Invitrogen, Waltham, MA, USA) according to manufacturer's recommendations.

Primers for quantitative amplification of PGRMC1 cDNA were previously published [14] and amplification efficiency was checked before use. Real-time PCR amplification was performed with the KAPA SYBR FAST qPCR Master Mix (2X), 0.25 μM primers (forward and reverse) and 15 ng cDNA. Two housekeeping genes (HKGs), RPL13a and β-actin, were selected based on their stability of expression and the mean of their Ct values was used for normalization in each RT-qPCR experiment.

2.3. In situ hybridization

In situ hybridization analysis was performed on a selection of paraffin-embedded tissue samples cut in serial sections of 5 μm (Table 1). After dewaxing in xylene, antigen retrieval, detection of the *PGRMC1* mRNA and signal amplification were performed with the RNAscope kit (RNAscope 2.5 HD Reagent kit-RED; Bio Techne Ltd., Mineapolis, MN, USA) according to the manufacturer's recommendations [31]. The PGRMC1 specific probe (RNAscope Probe-HS-PGRMC1-C1) was especially designed for our laboratory and produced by the manufacturer. A negative control probe (RNAscope Negative Control Probe - DapB-C2) was systematically used to evaluate the background noise of each endometrium sample. At the end of the RNAscope protocol, the slides were immediately incubated in the blocking solution to continue with protein detection by immunofluorescence.

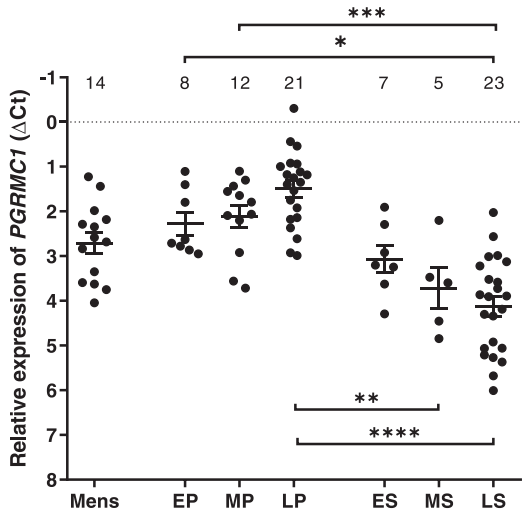

Fig. 1. *PGRMC1* mRNA levels vary during the menstrual cycle in the human endometrium. Relative concentrations of *PGRMC1* mRNA in functional layer of endometrium collected during the menstrual (Mens), early/mid/late proliferative (EP/MP/LP) or early/mid/late secretory phase (ES/MS/LS) were measured by RT-qPCR, normalized according to HKGs and are presented as dots for individual values (ΔCt), and as means ± SEM. The number of samples is indicated at the top of the graph. Statistical test: Kruskal-Wallis with Dunn's post-hoc, only statistically significant differences are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

2.4. Immunolabeling

Immunofluorescence was performed on paraffin-embedded tissue samples (Table 2) cut in serial sections of 6 μm (or 5 μM if after RNAscope *in situ* hybridization). After dewaxing in Histosafe, antigen retrieval was performed in citrate Buffer for 75 min at 100 °C and tissue sections were permeabilized for 15 min in phosphate-buffered saline (PBS), 0.3% Triton X-100. Nonspecific binding sites were blocked for 1 h at RT with PBS, 0.3% Triton X-100, 10% bovine serum albumin (BSA) and 3% milk before incubating the slides overnight at 4 °C with the primary antibody (Table 2). The next day, tissue samples were washed 3 times for 5 min in PBS, 0.1% Triton X-100, and incubated with 1/1000 secondary antibody (adequate combination of Goat anti-rabbit IgG (Alexa 488) for PGRMC1, Goat anti-mouse IgG2a (Alexa 568) or Goat anti-mouse IgG2a (Alexa 647) for E-cadherin; Life Technologies, Carlsbad, CA, USA) for 60 min at RT. Nuclei were counterstained with Hoechst (BisBenzimide H33342, 1 μg/mL, Sigma, St Louis, MO, USA) during incubation with the secondary antibody.

Because the antigen retrieval solution of the RNAscope kit was stronger than the one used for single immunolabeling, the concentration of primary and secondary antibodies was multiplied by 2 for immunolabeling performed after the RNAscope *in situ* hybridization.

In accordance with the recommendations of Hewitt et al. [32], the specificity of the anti-PGRMC1 antibody was previously validated *in vitro* by our laboratory [33]. To do this, the expression of PGRMC1 was specifically repressed by siRNA-PGRMC1 in two endometrial cell lines, one stromal (T-HESC) and one epithelial (HEC-1A), and confirmed by RT-qPCR, immunocytofluorescence and Western blotting. Additionally, a negative control without primary antibody was used in each experiment to correct background noise.

2.5. Microscopy and signal quantification

Protein and mRNA were detected with a Cell Observer Spinning Disk (COSD) confocal microscope (Zeiss, Jena, Germany) and with a Pannoramic P250 Flash III slide scanner (3DHistech, Budapest, Hungary).

The signals were analyzed and quantified with the HALO software, an image analysis platform (Indica Labs, Albuquerque, NM, USA). This software allowed to (i) count the cells positive for each signal, (ii) define their epithelium-stroma tissue distribution, and (iii) distinguish 3 progressive thresholds of PGRMC1 protein signal intensity.

The analysis procedure is schematized in Fig. S1. Each sample was virtually divided into 500 μm thick sections from the surface epithelium down to the basal layer (Fig. S1a). The cells were then identified by nucleus detection (Hoechst signal) followed by modelization by the artificial intelligence of the Halo software (Fig. S1b). For each modelized cell, the average of the pixel intensities present in the cell was calculated and a color was assigned according to the 3 intensity thresholds. The cells expressing E-cadherin marker were considered as epithelial cells. These modelized cells were then spatially plotted (Fig. S1c, partial images presenting representative tissue areas) and quantified slice by slice (Fig. S1d, the whole tissue area) to estimate their distribution according to the cell type and the depth of the endometrium.

The background noise was corrected with the appropriate negative control for each slide. Background variability from sample to sample was negligible for immunolabeling. Therefore, intensity thresholds were kept identical for all analysed samples. Conversely, background intensity between samples was extremely variable for *in situ* hybridization. Consequently, assignment of signal intensity based on fixed intensity thresholds was not recommended and was not used for *in situ* hybridization. Cells are either positive or negative for the presence of *PGRMC1* mRNA.

2.6. Statistical analysis

All statistical analyses were performed with GraphPad Prism 8.4.3.

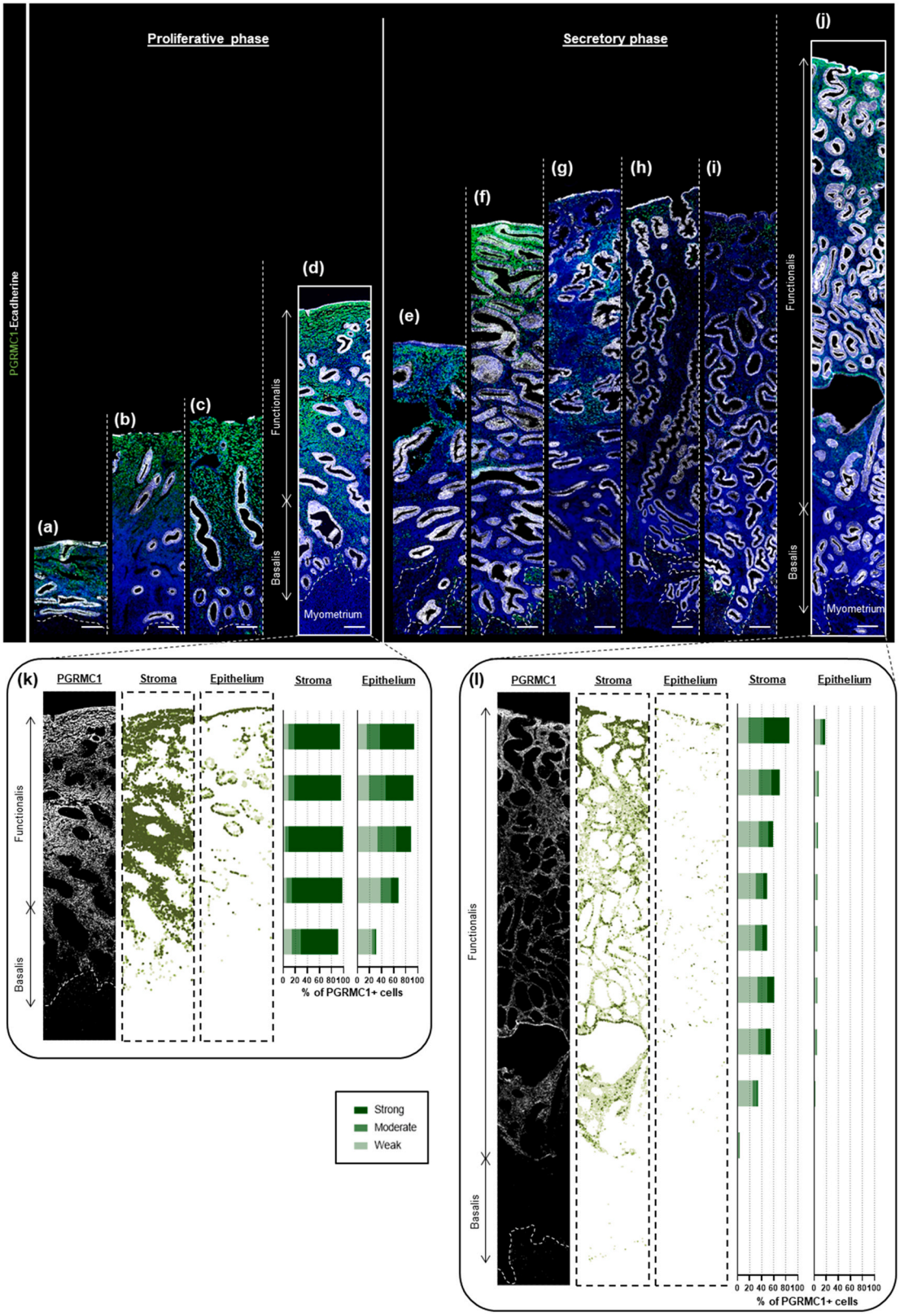

Fig. 2. PGRMC1 immunolabeling in the human endometrium along the menstrual cycle. PGRMC1 was immunolocalized in endometrium collected during the proliferative (a-d, k) or secretory (e-j, l) phase. (a-j) Immunofluorescence of PGRMC1 (in green) and epithelial marker E-cadherin (in white). The nuclei were stained in blue. Scale bar = 200 μm. (k,l) Original PGRMC1 immunolabeling (in white), corresponding spatial plots (in green) and quantification after modelization of whole tissue sections and virtual cutting of the tissue into 500-μm-thick slices. Each point of spatial plot represents a modelized cell and the level of green reflects the intensity of the PGRMC1 signal for each cell. PGRMC1 signal was quantified according to the intensity threshold (low, medium and high), the cell type and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice. Panels k and l are presented as representative images and correspond to panels d and j, respectively.

Table 3
Quantification of PGRMC1 immunolabeling.

% of cells (mean ± SEM)	Proliferative phase		Secretory phase	
	Stroma	Epithelium	Stroma	Epithelium
Positive (total)	94.3 ± 2.1 ****	91.5 ± 4.8 ****	38.7 ± 4.6	20.6 ± 3.4
Strong	31.9 ± 9.2 **	13.8 ± 4.6	7.7 ± 2.7	0.9 ± 0.4
Moderate	25.3 ± 5.0	23.2 ± 3.6 **	10.6 ± 2.2	4.1 ± 1.4
Weak	37.1 ± 7.4 *	54.5 ± 7.0 ****	20.4 ± 3.0	15.6 ± 3.0

Significant differences between cycle phases for the same cell compartment are indicated by * and significant differences between cell compartments within the same phase are indicated by #. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown. * $p < 0.05$, ** $p < 0.01$, ***/### $p < 0.001$, **** $p < 0.0001$.

Analyses of RT-qPCR data were performed on ΔCt values and the outliers were identified and excluded using the Grubbs' test.

For the quantification of PGRMC1 immunolabeling, statistical comparisons were performed using values for virtual slices rather than for the whole endometria to account for differences in tissue thickness between endometrial samples (essentially due to cycle phases).

3. Results

3.1. PGRMC1 mRNA concentrations vary during the menstrual cycle in the human endometrium

PGRMC1 expression was measured by RT-qPCR, in samples of the functional layer of human endometria covering the three phases of the menstrual cycle and collected from hysterectomy specimens from control patients (without endometriosis or adenomyosis).

The proliferative and secretory phases were subdivided into early, mid and late subphases to appreciate the progressive variation in the mRNA expression. Indeed, a gradual increase in endometrial *PGRMC1* mRNA concentration was observed from the menstrual phase until the end of the proliferative phase, reaching 2.3x between the means of these two phases. Values then decreased approximately 6.3x between averages of the late proliferative and the late secretory phases. Values increased again from the late secretory to the menstrual phase (Fig. 1).

3.2. PGRMC1 immunostaining varies with the menstrual cycle and the tissue depth in the human endometrium

The tissue localization of PGRMC1 was identified by immunofluorescence in 4 proliferative and 6 secretory human endometrial samples (Fig. 2). The distinction between epithelium and stroma was achieved by co-labeling epithelial structures with E-cadherin.

Immunofluorescence images show that PGRMC1 is present in the stroma and epithelium and suggest a more intense immunostaining of PGRMC1 during the proliferative phase (Fig. 2a-d) than during the secretory phase (Fig. 2e-j), in agreement with the mRNA variations measured by RT-qPCR (Fig. 1). Moreover, we suspected an intensity gradient of this signal as a function of tissue depth.

Modelization (illustrative spatial plots in Fig. 2k and l) combined with a virtual cutting of the tissue into 500-µm-thick slices, and followed by a quantification of the whole tissue section (see Fig. S1 and corresponding text in Material and Methods) allowed to objectify this suspicion. More specifically, this analysis (Figs. 2k-l and S2) allowed to consider separately the percentage of cells immunolabeled for PGRMC1 and the intensity of the PGRMC1 signal per positive cell (strong, moderate or weak, see levels of green in Figs. 2k-l and S2). We found that the proportion of cells immunostained for PGRMC1 is indeed significantly higher in the proliferative endometrium (Figs. 2k, S2a-c and i-k, and Table 3) than in the secretory endometrium (Figs. 2l, S2d-h and l-p, and

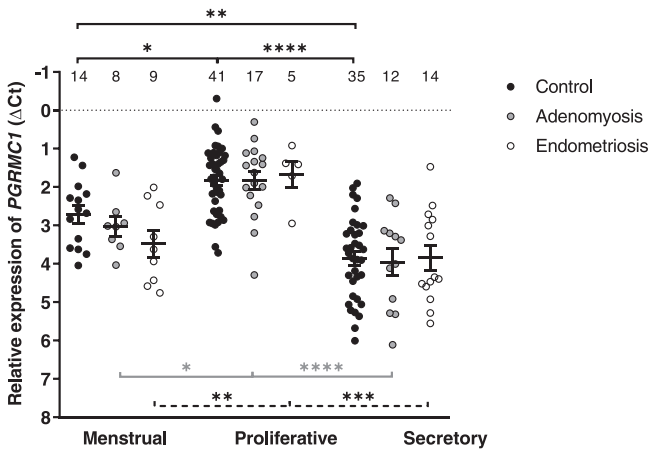

Fig. 3. *PGRMC1* mRNA levels vary during the menstrual cycle in the endometrium of patients with endometriosis/adenomyosis. Relative concentrations of *PGRMC1* mRNA in the functional layer of endometrial samples from patients with endometriosis (in white) or adenomyosis (in gray) collected during menstrual, proliferative or secretory phase were measured by RT-qPCR and normalized according to HKGs. Values are presented as dots for individual values (ΔCt), and as means ± SEM and compared to samples from control patients (values from Fig. 1, in black). The number of samples is indicated at the top of the graph. Statistical test: Anova two-way and post-hoc Tukey. Tests were performed to compare phases in the same pathology group or to compare pathology groups in the same phase. Only statistically significant differences are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. No statistical difference was found between pathology groups from identical phases.

Table 3).

Moreover, the proportion of cells showing a strong or moderate signal was significantly higher during the proliferative than the secretory phase, both in the stroma and the epithelium. Increased PGRMC1 labeling in upper endometrial layers during the proliferative phase is essentially due to stronger labeling per positive cell rather than larger percentage of positive cells (Fig. S2a-c and i-k). This gradient is less frequent in secretory endometrium (see Fig. S2d, e, l and m) since global PGRMC1 expression is decreased during this phase.

3.3. PGRMC1 mRNA concentrations also vary with tissue depth in the human endometrium

To gain further insight in PGRMC1 expression and determine whether spatiotemporal distribution of *PGRMC1* mRNA concentrations reflects protein immunolabeling, *in situ* hybridization analysis was performed using the RNAscope technique in parallel with immunostaining of the PGRMC1 protein. Given the variability observed between the expression profiles of the different samples, the 7 analyzed samples are presented (Fig. S3).

In immunofluorescence images, *PGRMC1* mRNA labeling (in red in Fig. S3) appears punctiform and cytoplasmic. Enlarged images (Fig. S3c and g) suggest that *PGRMC1* mRNA localization is frequently correlated with a protein signal (in green) within the same cell, especially in areas with a high percentage of immunolabeled cells.

Analysis by modelization (Fig. S3), identification of virtual tissue slices and signal quantification in whole tissue sections (Fig. S4) was applied simultaneously to mRNA and protein.

Although quantification of *PGRMC1* mRNA did not take into account the intensity of the signal per cell, the values are in good agreement with immunolabeling and support both stronger expression during the proliferative phase and increased expression of PGRMC1 in upper layers of the endometrium. However, in the basalis, the percentage of cells positive for *PGRMC1* mRNA was systematically higher in the epithelium than in the stroma during both cycle phases.

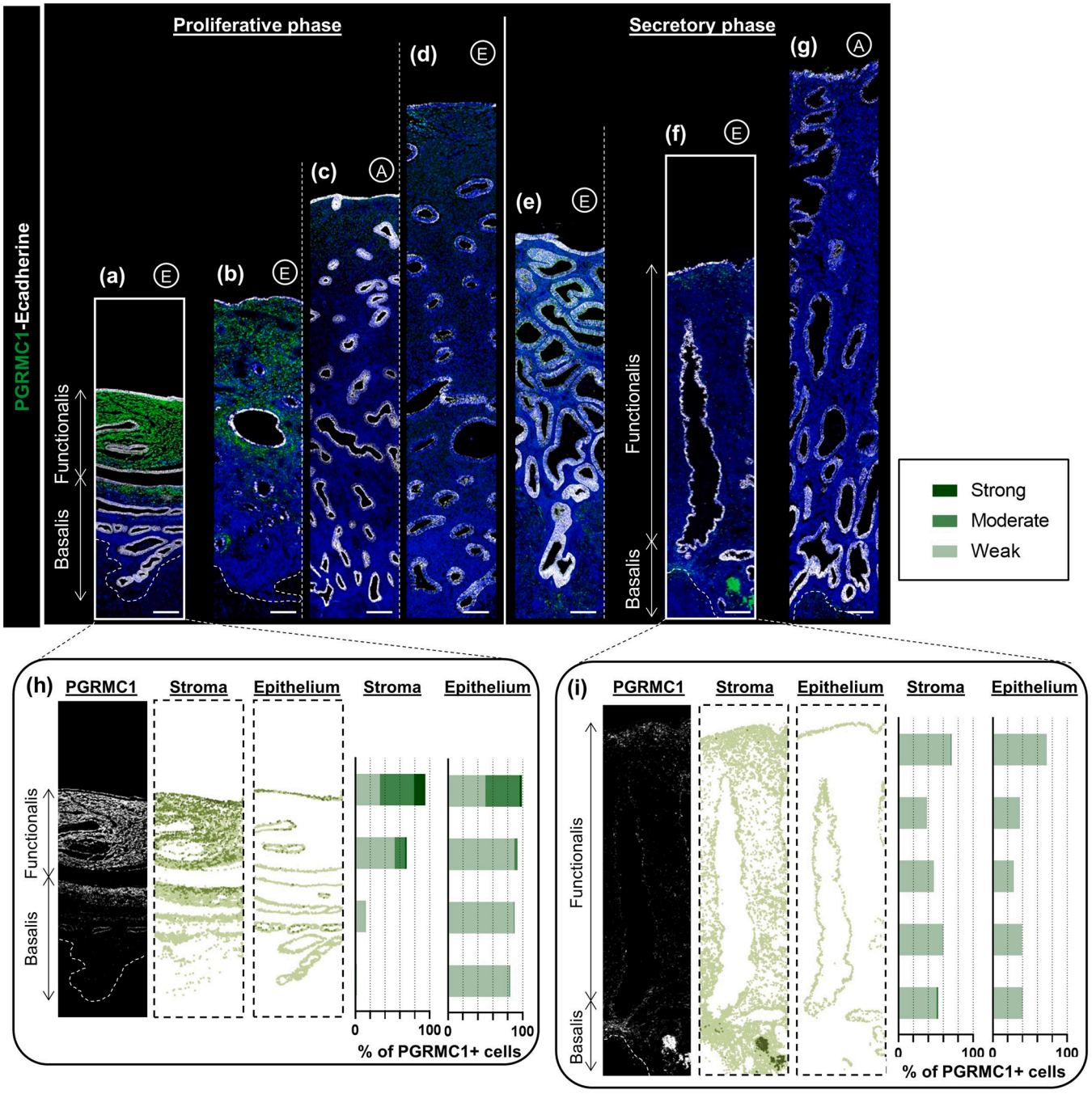

Fig. 4. PGRMC1 immunolabeling in the human endometrium of patients with endometriosis/adenomyosis. PGRMC1 was immunolocalized in endometrium of patients with endometriosis (E) or adenomyosis (A), collected during the proliferative (a-d, h) or secretory (e-g, i) phase. (a-g) Immunofluorescence of PGRMC1 (in green) and epithelial marker E-cadherin (in white). The nuclei were stained in blue. Scale bar = 200 μm. (h and i) Original PGRMC1 immunolabeling (in white), corresponding spatial plots (in green) and quantification after modelization of whole tissue sections and virtual cutting of the tissue into 500-μm-thick slices. Each point of spatial plot represents a modelized cell and the level of green reflects the intensity of the PGRMC1 signal for each cell. PGRMC1 signal was quantified according to the intensity threshold (low, medium and high), the cell type and the depth in the endometrium. Values are presented as a percentage of the total number of stromal or epithelial cells within each virtual tissue slice. Panels h and i are presented as representative images and correspond to panels a and f, respectively).

3.4. Cyclical variation of PGRMC1 mRNA levels is similar in the endometrium of patients with endometriosis/adenomyosis and control patients

We extended our study to analyze the expression of PGRMC1 in the functional layer of endometrium samples from patients with endometriosis or adenomyosis. Quantification of *PGRMC1* mRNA by RT-qPCR shows cyclical variations identical to those measured in patients not affected by these pathologies: an increase during the proliferative phase and a decrease during the secretory phase (Fig. 3). Interestingly, no significant difference in the expression of *PGRMC1* was observed between the pathological groups and the control group, for any of the three phases of the menstrual cycle.

Table 4
Quantification of PGRMC1 immunolabeling in the human endometrium of patients with endometriosis/adenomyosis.

% of cells (mean ± SEM)	Proliferative phase		Secretory phase	
	Stroma	Epithelium	Stroma	Epithelium
Positive (total)	59.2 ± 6.9 **	61.6 ± 6.1 ****	34.7 ± 6.1	31.3 ± 4.7
Strong	1.1 ± 0.7	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
Moderate	4.3 ± 2.4	3.1 ± 2.2	2.3 ± 1.0	0.4 ± 0.2
Weak	53.8 ± 6.4 **	58.5 ± 5.7 ****	32.3 ± 5.5	30.9 ± 4.7

Differences between cycle phases for the same cell compartment. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown.
** $p < 0.01$.
**** $p < 0.0001$.

3.5. PGRMC1 immunolabeling is decreased in the endometrium of patients with endometriosis/adenomyosis

The pattern of PGRMC1 immunostaining in the endometrium of patients with adenomyosis and/or endometriosis (Fig. 4 and S5 and Table 4) are similar to those of patients in the control group (Figs. 2 and S2 and Table 3): the PGRMC1 signal (i) is mainly stromal, (ii) is broadly more abundant during the proliferative phase than during the secretory phase, and (iii) decreases from the uppermost layer of the endometrium to the basal layer. However, comparison of PGRMC1 immunolabeling quantification between patients with or without endometriosis/adenomyosis showed that the percentage of cells positive for PGRMC1 labeling during the proliferative phase was significantly reduced in the endometrium of patients with endometriosis/adenomyosis compared to control samples (Fig. 5a). This was essentially due to a reduction in the proportion of cells with strong (Fig. 5b) and moderate (Fig. 5c) signal. During the secretory phase, the global percentage of positive cells was similar in both patient groups (Fig. 5a) but we noticed a trend for a (not significant) decrease in the percentage of cells with strong and moderate signal in samples of patients with endometriosis/adenomyosis (Fig. 5b and c), and consequently a significant increase in the percentage of cells with weak signal (Fig. 5d).

PGRMC1 labeling appeared essentially stromal in immunofluorescence images of control and endometriosis/adenomyosis patients (Figs. 2 and 4). However, paired comparison between corresponding stroma and epithelium within tissues slices did not reach significance neither for the percentage of positive cells nor for signal intensity, except during the secretory phase for the percentage of positive cells in control patients. This suggests that visual predominance of stromal PGRMC1 signal essentially reflects the abundance of stromal cells in the tissue, rather than a specific PGRMC1 enrichment in these cells.

4. Discussion and conclusion

In this study, we investigated the spatiotemporal profile of PGRMC1 expression in the human endometrium along the menstrual cycle and compared endometria from patients without or with endometriosis/adenomyosis. In summary, we identify temporal and spatial variations. PGRMC1 expression progressively increases during the proliferative phase and decreases during the secretory phase. In addition, expression in endometria often displays a gradient from the surface to the basal layer, mostly during the proliferative phase.

Quantitative RT-PCR was used to monitor changes in global concentration of *PGRMC1* mRNA in the functional layer. Our results are in agreement with and extend microarray and qPCR data [14,29,34] reporting increased levels (by 2–3 fold in average) of *PGRMC1* mRNA during the proliferative phase by comparison with the secretory phase in the endometrium of patients with no detected endometriosis/adenomyosis. The larger number of samples in our study allowed us to distinguish subphases (early/mid/late) at reveal that: (1) mean *PGRMC1* mRNA concentration is minimal during the late secretory phase and then (2) progressively increases during the menstrual and proliferative phase, (3) to peak during the late proliferative phase and (4) to progressively decrease during the secretory phase. Moreover, a similar cyclical variation was measured in the functional layer of the eutopic endometrium from patient with endometriosis or adenomyosis, with no significant difference when comparing the 3 patient groups (control/endometriosis/adenomyosis) for each of the 3 cycle phases. To the best of our knowledge, this is the first analysis of PGRMC1 expression in the endometrium of patients with adenomyosis. Expression in endometriosis was addressed in microarray data ([34] and Gene Expression Omnibus entry GSE6364). These showed no difference in *PGRMC1* mRNA quantification between control and endometriosis patients during the proliferative phase (19,841+/- 2061 *vs* 19,555+/- 1614 arbitrary units, respectively) or the secretory phase (9926 +/- 4198 *vs* 8298 +/- 1970 arbitrary units, respectively). In support of our data, other teams using macaque experimental models reproducing the menstrual cycle and endometriosis concluded that *PGRMC1* mRNA levels during the [estradiol and progesterone]-primed pseudo-secretory phase were lower than during the [estradiol]-primed pseudo-proliferative phase [35,36] but were not significantly different between animals without or with artificial endometriosis [37].

A major part of our work was dedicated to investigating spatial modulation of PGRMC1 expression, both at the mRNA and protein levels. For this purpose, we turned to high resolution whole slide scanning of sections representing the entire thickness of the tissue (from the luminal epithelium towards the interface between the basalis and the myometrium). Concomitant distinction between epithelial and stromal cells combined with cell modelization followed by virtual cutting of the sample in slices, and quantification of the fluorescent signals allowed us to provide a detailed atlas of PGRMC1 expression according to cell type, tissue depth and strength of the protein signal. The results of this study are however influenced by the inclusion and exclusion criteria of the patients. Variations could therefore be observed by broadening these criteria, in particular by analyzing samples from younger patients (age less than 40 years old).

Since the initial observation that endometrial PGMRC1 protein was more abundant during the mid-proliferative than during the mid-secretory phase [30], other teams have investigated and commented on PGRMC1 immunolabeling in the human endometrium but these studies relied on less or non-quantitative techniques and were essentially based on analysis of limited tissue areas. Our findings thus confirm these studies and provide more detailed spatiotemporal information.

First, PGRMC1 immunolabeling was reported to be more abundant in stromal cells compared with glandular and luminal epithelium [14]. Direct comparison of values for stromal and epithelial PGRMC1 immunolabeling in corresponding tissue compartments (Fig. S2 and Table 3) shows that the predominance of the stromal PGRMC1 signal essentially results from the surface covered by the stroma by comparison with epithelial structures, rather than from differences in the percentage of positive cells between the two tissue compartments.

Second, to the best of our knowledge, our study shows for the first time that the intensity of PGRMC1 immunolabeling is maximal, in most samples, within the superficial part of the functional layer, both in the stromal and in epithelial cells. PGRMC1 immunolabeling then progressively decreases with depth within the functional layer, and in the basalis, by reduction either of the total amount of positive cells and/or of the intensity of the signal. This gradient is much more pronounced in proliferative endometria than in secretory endometria in which immunolabeling globally decreases. Changes in percentage of cells positive for *PGRMC1* mRNA roughly follow a similar pattern. Our findings support observations in the macaque model showing that PGRMC1 mRNA and protein were more abundant in the functionalis than in the basalis [37]. We further show that expression is not homogenous throughout the whole thickness of the functionalis suggesting that spatial regulation of

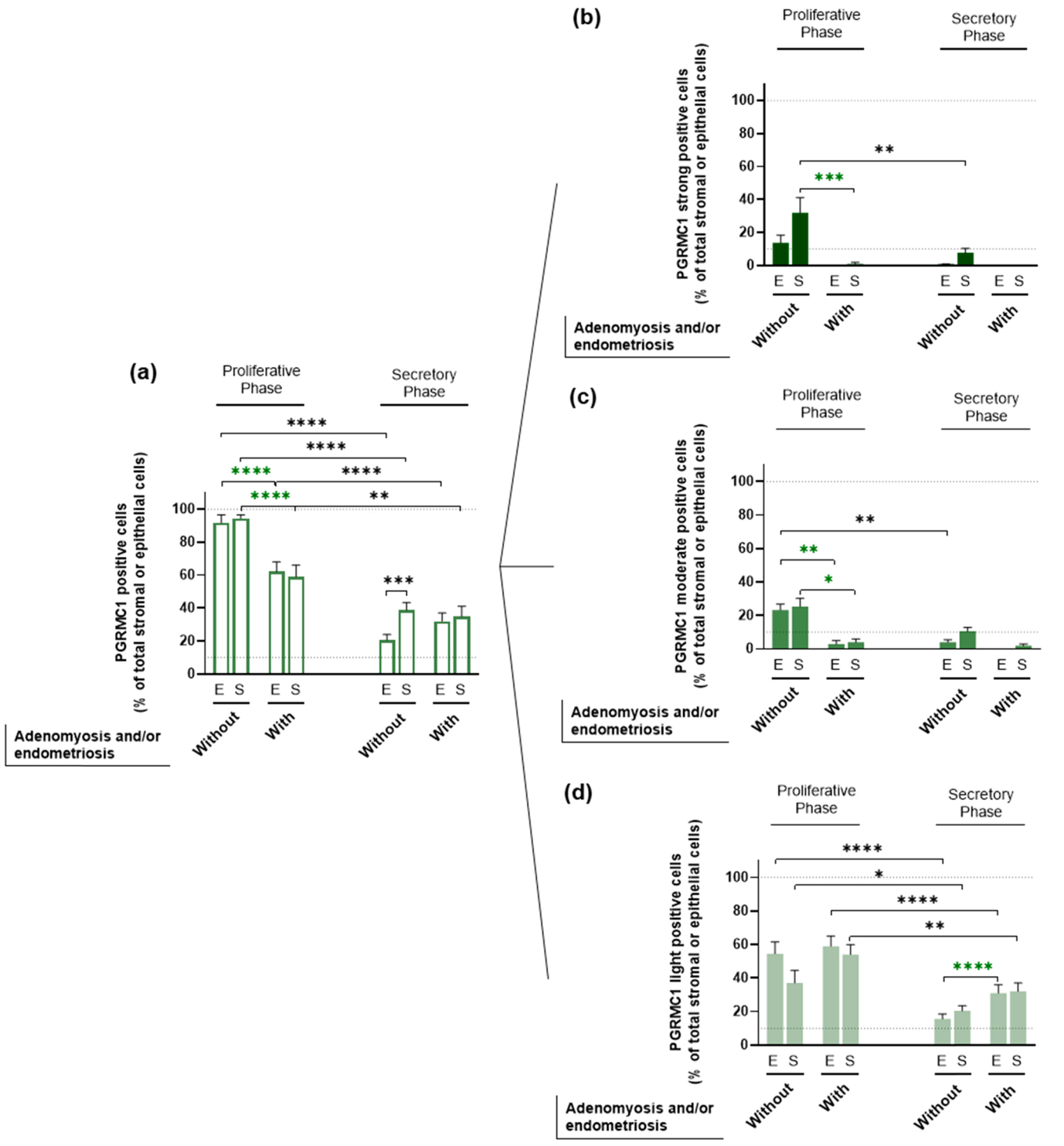

Fig. 5. Comparison of endometrial PGRMC1 immunolabeling between control patients and patients with endometriosis/adenomyosis. Quantification results of PGRMC1 immunolabeling in endometrium from patients with endometriosis and/or adenomyosis (values derived from Fig. S5 and Table 4) were compared to patients without endometriosis/adenomyosis (values derived from Fig. S2 and Table 3). The PGRMC1 signal was quantified in each virtual tissue slice according to the intensity threshold and the cell type (epithelial, E or stromal, S). Values are presented as mean ± SEM of the percentage of the total number of stromal or epithelial cells. In a, percentage of positive cells. In b-d, percentage of cells with strong, moderate or weak immunolabeling, respectively. Statistical test: Anova two-way and post-hoc Tukey. Only statistically significant differences are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

PGRMC1 expression is not limited to differences between the functionalis and the basalis.

Occasional divergences between RNA and protein data within a same virtual tissue slide could result from the use of two different techniques targeting two different molecular products. Such discrepancies are in agreement with a recent article stating that the concordance rate between results obtained by RNAscope and immunolabeling varies between 58.7% and 95.3% [38]. However, additional biological hypotheses can be formulated, such as a different half-life for the mRNA and the protein, or post-transcriptional regulatory mechanisms.

Variation of PGRMC1 protein and mRNA amounts along the menstrual cycle suggests that PGRMC1 expression is increased by estradiol and repressed by progesterone (combined with estradiol) in the human endometrium. This hypothesis is supported by fluctuations of endometrial PGRMC1 in *in vivo* models mimicking the menstrual cycle in the macaque [37] or using low-dose intrauterine administration of the progestin levonorgestrel in patients with abnormal uterine bleeding, resulting in substantial decrease in PGRMC1 expression after 6 months [21]. Be that as it may, fluctuating concentration of the steroid hormones may explain the temporal control of PGRMC1 expression, but additional levels of regulation responsible for spatial adjustment remain to be identified.

A previous study concluded that levels of *PGRMC1* mRNA and of PGRMC1 immunolabeling in stromal cells were reduced during the secretory phase in patients with endometriosis by comparison with control patients [14]. Such differences were not evidenced by our analysis. Although differences in technical approaches could be involved, it is important to note that we used the same primers for the quantitative PCR. The discrepancy could result from the heterogeneity between patients. Indeed, firstly, by using a larger panel of patients for our qPCR analysis, we noticed large interpatient variations within each phase of the cycle, both in patients with endometriosis/adenomyosis and in control patients. Secondly, interpatient variations were also evidenced by the immunolabeling, especially for the percentage of labeled cells during the secretory phase. Finally, we highlight differences in PGRMC1 expression according to tissue depth while the localization of the sections analyzed was not specified in the other study.

The major novelty of our work is to highlight the spatial adjustments of PGRMC1 expression, underscoring the importance to precisely consider local amounts of PGRMC1 when investigating its potential functions. Endometrial roles of PGRMC1 remain largely unknown but two major topics have been approached: proliferation and decidualization. Experiments involving xenografts containing Ishikawa endometrial adenocarcinoma cells suggest that PGRMC1 supports cell growth [39]. Indeed, PGRMC1 depletion resulted in reduced cell growth in the grafts. This is consistent with the spatiotemporal pattern of PGRMC1 expression evidenced in the present study, which could be related with cell proliferation. Decidualization (differentiation of endometrial stromal cells) and successful fertility seem to require appropriate amounts of PGRMC1. Indeed, reduced amounts of PGRMC1 during the secretory phase were required for receptivity [28] but insufficient amounts were linked to miscarriage [26]. The peak of PGRMC1 expression in the proliferative phase would support a role as a mediator of estrogenic rather than progestin activity. Moreover, its preferential localization around the surface of the functional layer could be consistent with a stimulatory function of PGRMC1 on endometrial cell proliferation. It could therefore be hypothesized that PGRMC1 influences endometrial receptivity by participating in the molecular mechanisms necessary to prepare the endometrium for embryonic implantation. Although a decrease in PGMRC1 protein was observed in the endometrium of patients with endometriosis, most of these patients had at least one successful pregnancy. Additional work is therefore needed to elucidate the precise requirement and contribution of PGRMC1 in fertility.

In conclusion, we show that endometrial PGRMC1 expression is controlled both in time, with maximal expression in the late proliferative phase as reported before, and in space, with stronger expression occurring in the outermost layer of the endometrium to progressively decrease towards the basalis. PGRMC1 is expressed quite similarly in the stroma and in the epithelial structures, but appears essentially stromal due to predominance of this tissue. Although ovarian steroids may be suspected to control the global temporal variation of PGRMC1 expression, their action is not straightforward and further work is required to identify the additional levels of regulation responsible for local adjustment. This is particularly important in the light of recent studies highlighting crucial roles of PGRMC1 and emphasizing the correlation between adequate PGRMC1 amounts and fertility [26,28].

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CRediT authorship contribution statement

Charlotte Thieffry: Conceptualization, Validation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Marie Van Wynendaele:** Conceptualization, Validation, Investigation, Writing – review & editing. **Lucie Samain:** Investigation, Writing – review & editing. **Donatienne Tyteca:** Conceptualization. **Christophe Pierreux:** Conceptualization, Writing – review & editing. **Etienne Marbaix:** Conceptualization, Supervision, Writing – review & editing. **Patrick Henriet:** Conceptualization, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Institutional review board statement

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Informed consent statement

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Conflicts of interest

The authors declare no conflict of interest.

Data Availability

Not applicable.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2022.106153.

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