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Short title: UPA and the endometrium

Absence of predictable long-term molecular impact of ulipristal acetate (UPA) on the endometrium

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ABSTRACT

Research Question: To describe the effects of ulipristal acetate (UPA) on the expression of endometrial proliferation and maturation markers.

Design: A total of 45 endometrium-containing blocks of hysterectomy samples from non-menopausal women with a diagnosis of moderate to severe symptoms of uterine fibroids: 14 women operated on at the end of a three-month course of UPA; 4 women who had discontinued UPA treatment 1-12 months before surgery; 27 control unexposed samples (14 in the proliferative and 13 in the secretory phase). Immunohistochemical staining of Ki67, vascular endothelial growth factor-receptor 2 (VEGFR2), estradiol receptor (ER), PR, interleukin-15 (IL-15), indoleamin-2,3-dioxygenase (IDO) and C-C motif chemokine ligand-2 (CCL2) markers were analysed in both endometrial compartments and layers.

Results: Under UPA, ER and PR expression is similar to the proliferative phase in both layers, although with a decrease in cell proliferation. IL-15, IDO and CCL2 expressions are similar to the proliferative phase suggesting a progesterone-antagonist effect of UPA. VEGFR2 staining suggests a trend to a mixed agonist-antagonist effect. No significant difference is observed in the post-UPA proliferative phase group as compared to the control group in both layers of the endometrium.

Conclusion: The effect of three-month UPA treatment is mostly progesterone receptor (PR) antagonist-like. After treatment discontinuation there are no signs of any long-term effects of this molecule on both endometrial proliferation and maturation. Therefore UPA may be administered to women willing to conceive in the short term without consequences for further implantation.

KEYWORDS: Endometrium, ulipristal acetate, basalis, functionalis, maturation

ABBREVIATIONS:

CCL2 – C-C motif chemokine ligand-2

ER – estradiol receptor

IDO – indoleamin-2,3-dioxygenase

IL-15 – interleukin-15

PAEC – progesterone receptor modulator associated endometrial changes

PR – progesterone receptor

60 SPRM – selective progesterone receptor modulator

61 UPA – ulipristal acetate

62 VEGFR2 – vascular endothelial growth factor-receptor 2

63

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74

INTRODUCTION

Recently, the use of ulipristal acetate (UPA), a selective progesterone receptor modulator (SPRM) has been approved in women of reproductive age as a treatment for symptomatic myomas before surgical intervention or as alternative to surgery, using a long-term sequential regimen (Donnez et al., 2012). Currently, the use of SPRMs is being evaluated in women who wish to conceive subsequently (Donnez and Dolmans, 2016). SPRMs act as progesterone receptor ligands able to induce agonistic or antagonistic activities according to the target cell (Bouchard et al., 2011).

During UPA treatment the hormonal profile is modified, with blunted progesterone levels, while estradiol levels remain in the early follicular phase range (Chabbert-Buffet et al., 2007). In addition 95% of patients experience amenorrhea (Donnez et al., 2012). Therefore, the menstrual cycle phase cannot be defined *stricto sensu* under UPA.

Endometrial morphology during UPA treatment has been described as non-physiological secretory (Mutter et al., 2008; Williams et al., 2012) due to the combined progesterone receptor agonist antagonist activity of the molecule. The effects of UPA on morphology and gene expression in the superficial layer of the endometrium have been described in one study, consistent with UPA acting as a PR antagonist, although without any proliferative effects (Whitaker et al., 2017). However, the superficial layer undergoes a monthly shedding, regenerates from the basal layer, which acts as “the memory” of the endometrium, and finally, during the secretory phase, undergoes decidualization, a crucial step to allow embryo implantation (Gellersen et al., 2007; Perrier D’hauterive et al., 2002). Immune cells play a critical role in the regulation of cellular proliferation, tissue remodelling and angiogenesis in a direct and an indirect response to steroid hormones (Croxatto et al., 2014; Szekeres-Bartho et al., 2009). Their proliferation and activation are regulated under the action of progesterone by locally produced molecules, such as interleukin 15 (IL-15) (Okada et al., 2000; Wilkens et al., 2013), C-C motif ligand 2 (CCL2) (Szekeres-Bartho et al., 2009), and indoleamine 2,3-dioxygenase (IDO) (Kudo et al., 2001; Munn et al., 1999). Progesterone stimulates the expression of IL-15 (Kitaya et al., 2000) and VEGFR2 (Lai et al., 2015) and the secretion of CCL2 (Caballero-Campo et al., 2002) in the human endometrium. In an *in vitro* model of endometrial culture progesterone increases IL-15 secretion (Okada et al., 2000) and decreases IDO expression (Kudo et al., 2004). The angiogenesis of endometrial tissue is also regulated by immune cells-produced angiogenic factors,

104 such as vascular endothelial growth factor A (VEGFA) which interacts with its receptor VEGFR-2
105 (Lash et al., 2016).

106 The impact of UPA in the basal layer of the endometrium and its potential post-treatment impact is
107 currently poorly understood, and may impair the whole endometrial maturation process in post
108 treatment cycles.

109 Since previous data do not provide information on the impact the molecule has, either on the basal
110 layer or on the post-treatment endometrium, the aim of this study was to describe the possible effects
111 of UPA on proliferation and maturation markers in both layers of the endometrium, under the action of
112 UPA as well as after the end of treatment.

MATERIAL AND METHODS

Patients and samples

Adequate endometrium-containing blocks of hysterectomy samples from non-menopausal women aged 40–52 years, with a diagnosis of moderate to severe symptoms of multiple uterine myoma with a combination of subserous and/or intramural and/or submucous fibroids requiring hysterectomy, were retrospectively collected in three university hospital surgical gynaecology units (Department of Gynaecology Obstetrics and Reproductive Medicine, Tenon Hospital; Department of Gynaecology Obstetrics, Bicêtre University Hospital and Department of Gynaecology Obstetrics, Bichat University Hospital). Four groups of patients were included in the study. The control groups, not exposed to hormonal treatments or UPA, included samples of superficial and basal layers of endometrium obtained in the proliferative (No-UPA proliferative phase group, $n = 14$) and secretory phases (No-UPA secretory phase group, $n = 13$), classified on the basis of histological examination (Bouchard et al., 1991; Noyes et al., 1975). The UPA group ($n = 14$) included women operated on at the end of a three-month course of UPA (Esmya, Gedeon Richter, Paris, France, 5mg daily). The post-UPA proliferative phase group ($n = 4$) was composed of women who had been treated with UPA for at least three months, and who had discontinued treatment 1–12 months before surgery with a proliferative phase histological examination classification.

The mean ages for the No-UPA proliferative phase, No-UPA secretory phase, UPA and post-UPA groups were, respectively, 47.2 ± 2.8 years, 46.9 ± 3.0 years, 46.9 ± 2.6 years and 45.8 ± 1.5 years, $p = 0.796$. BMI was $27.2 \text{ kg/m}^2 \pm 5.5$, $26.7 \text{ kg/m}^2 \pm 5.3$, $27.7 \text{ kg/m}^2 \pm 6.8$ and $26.3 \text{ kg/m}^2 \pm 2.0$, $p = 0.995$ for the No-UPA proliferative phase, No-UPA secretory phase, UPA and post-UPA groups, respectively, and parity was 1.8 ± 1.0 versus 1.5 ± 1.3 versus 1.7 ± 1.1 versus 0.0 ± 0.0 , $p = 0.200$, respectively. Among evaluated samples from the UPA group only two included focal progesterone receptor modulator associated endometrial changes (PAEC) lesions.

The Institutional Review Board allowed retrospective sample analysis and approved the study. All women gave written informed consent.

Histological analysis and endometrial dating

Paraffin-embedded tissue sample sections of 10µm were processed and stained with haematoxylin and eosin by standard methods. All evaluations were performed under blinded conditions by one expert pathologist (CB). Endometrial differentiation was assessed by histological dating (Noyes et al., 1975) and evaluation of ER and PR expressions (Bouchard et al., 1991). Presence of progesterone receptor modulator associated endometrial changes (PAEC) was noted.

Immunohistochemistry

We selected proliferation (Ki67), angiogenesis (vascular endothelial growth factor-receptor 2 [VEGFR2]), immune pathways (interleukin-15 [IL-15], indoleamin-2,3-dioxygenase [IDO], C-C motif chemokine ligand-2 [CCL-2]) and endocrine maturation (ER, PR) markers on the basis of their major functions in human endometrial homeostasis (Ban et al., 2013; Dimitriadis et al., 2005; Jones et al., 1998; Kayisli et al., 2002; Kitaya et al., 2000; Lai et al., 2015; Munn et al., 1999; Sedlmayr et al., 2002).

Sample sections of 5µm were subjected to deparaffinisation and antigen retrieval (Supplemental Table I). All sections were subjected to quenching of endogenous peroxidase activity by incubation with 3% hydrogen peroxide (GPR Rectapur VWR Chemicals, Radnor, PA, USA), and non-specific activity was blocked with appropriate serum before incubation with antibodies specific to IDO, CCL2, IL-15 and VEGFR2 (Table A.1). Appropriate matched IgG was applied as a negative control.

The primary antibodies were detected using an avidin-biotin peroxidase technique with anti-rabbit or anti-mouse secondary antibodies as appropriate and Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). The reaction was developed with 3,3'-diaminobenzidine (DAB; Vector Laboratories, Inc.). Sections were counterstained with Mayer's haematoxylin and mounted with Immuno Mount (Thermo Scientific Shandon, Kalamazoo, MI, USA). Positive and negative controls (replacement of the primary antibody by appropriate non-immune serum) were included in each staining run.

The immunostaining of PR, ER and Ki67 was performed using the automated method Automate BenchMark XT, Roche Ventana®, (Ventana Medical Systems, Inc., Rotkreuz, Switzerland) (Table A.1). The demasking was done by heating at pH 8.8. The slides were incubated over 24h with pre-diluted anti-PR and anti-ER antibodies and over 32h with anti-Ki67 antibody (dilution 1/150). Then the slides were incubated with anti-rabbit and anti-mouse antibodies and revealed by UltraView Universal DAB

(ref. 760-500, Ventana Medical Systems, Inc.). Representative images were captured using a Nikon Eclipse 90i microscope equipped with a Nikon DSFi1 camera (Nikon Ltd, UK).

Semi-quantitative analysis of immunostaining

Blinded interpretation of immunostained sections was performed by an expert pathologist (JV). The percentage of stained cells was evaluated at 200x magnification. The stromal cells and glandular epithelium were evaluated separately in each layer of the endometrium. The percentage of immunostained cells was noted. For Ki67 a score from 1–3 was used, where 1 corresponded to <5% of stained cells, 2 to 5–50% of stained cells and 3 to >50% of stained cells.

To determine the protein expression profile under UPA treatment, immunostaining was compared between the UPA group and the No-UPA proliferative and secretory phase groups. In order to analyse the possible persistent endometrial effect of UPA, the post-UPA proliferative phase group was compared to the control No-UPA proliferative phase group. In addition, the effects of UPA were evaluated in both endometrial layers and in both cellular compartments, i.e. stromal cells and the glandular epithelium.

Statistical analysis

The Kruskal-Wallis test was performed to compare the continuous variables concerning study group characteristics. The qualitative variables were analysed using the chi-square test. The semi-quantitative evaluation of the immunostaining for each cellular type in each layer of the endometrium was expressed as a percentage and was compared using the Mann-Whitney test.

All reported p values are two-sided and $p < 0.05$ was considered statistically significant. All analyses were performed using the GraphPad Prism 7.

RESULTS

UPA treatment induces epithelial ER and PR expression in both layers of the endometrium

ER and PR are well known markers of progesterone-induced endometrial epithelium maturation. Their expression was decreased in the No-UPA secretory phase as compared to the No-UPA proliferative phase in the glandular epithelium in both layers ($65.8\% \pm 29.4$ versus $100.0\% \pm 0.0$, $p < 0.0001$ and $37.1\% \pm 38.8$ versus $100.0\% \pm 0.0$, $p < 0.0001$ for ER and PR, respectively in the superficial layer and $88.3\% \pm 21.2$ versus $100.0\% \pm 0.0$, $p = 0.03$ and $73.3\% \pm 22.3$ versus $100.0\% \pm 0.0$, $p < 0.0001$ for ER and PR, respectively in the basal layer) as previously described in women without any uterine disorder (Bouchard et al., 1991) (Figure 1). However, ER and PR staining in the No-UPA secretory phase was significantly lower in the superficial layer when it was compared with the basal layer ($65.8\% \pm 29.4$ versus $88.3\% \pm 21.3$, $p = 0.04$ and $37.1\% \pm 38.8$ versus $73.3\% \pm 22.3$, $p = 0.02$, respectively) (Figure A.1).

Under UPA, the ER immunostaining level was very intense in stromal and epithelial cells, in both endometrial layers (Figure 1) and the percentage of stained cells was generally increased as compared to the No-UPA secretory phase, except for the stromal compartment of the basal layer ($90.0\% \pm 17.5$ versus $67.5\% \pm 31.7$, $p = 0.04$ and $98.6\% \pm 5.3$ versus $65.8\% \pm 29.4$, $p = 0.0004$ for the stromal and epithelial compartments, respectively in the superficial layer and $92.9\% \pm 15.4$ versus $77.5\% \pm 24.2$, $p = 0.06$ and $100.0\% \pm 0.0$ versus $88.3\% \pm 21.3$, $p = 0.03$ for the stromal and epithelial compartments, respectively, in the basal layer) (Figure 1).

The percentage of PR labelled cells in the UPA group was significantly increased in the epithelial cells of both layers as compared to the No-UPA secretory phase ($100.0\% \pm 0.0$ versus $37.1\% \pm 38.8$, $p < 0.0001$ and $100.0\% \pm 0.0$ versus $73.3\% \pm 22.3$, $p < 0.0001$ for the superficial and basal layers, respectively) (Figure 1). This progesterone-antagonist pattern was not observed in the stromal compartment of either layer.

ER and PR expressions in the UPA and post-UPA groups were comparable to the control No-UPA proliferative phase group in both cellular populations of both layers (Figures 1 and A.1).

There was no layer-specific regulation of ER and PR under UPA (Figure A.1).

UPA treatment has no impact on cell proliferation in the basal layer

In the superficial layer in the UPA group, the percentage of Ki67 marked epithelial cells was significantly increased in comparison to the No-UPA secretory phase group and significantly decreased as compared to the No-UPA proliferative phase group (1.5 ± 0.5 versus 1.2 ± 0.6 , $p = 0.03$ and 1.5 ± 0.5 versus 2.1 ± 0.9 , $p = 0.047$, respectively). In the stroma, Ki67 staining was decreased in comparison to the No-UPA proliferative phase (1.1 ± 0.4 versus 1.8 ± 0.8 , $p = 0.02$) (Figure 2).

In contrast, in the basal layer, Ki67 staining in the study groups remained unchanged in both cellular compartments.

Ki67 staining score in the epithelial cells in the No-UPA proliferative phase and UPA groups was lower in the basal layer as compared to the superficial layer (2.1 ± 0.9 versus 1.4 ± 0.6 , $p = 0.0204$ and 1.5 ± 0.5 versus 1.1 ± 0.3 , $p = 0.0329$ for the No-UPA proliferative phase and UPA groups, respectively) (Figure 3).

UPA has a mixed agonist and antagonist effect on angiogenesis marker in both endometrial layers

We observed a layer-specific pattern of progesterone-regulated VEGFR2 expression. In the glandular compartment VEGFR2 was down regulated in the No-UPA secretory phase group as compared to the No-UPA proliferative phase group in the superficial layer ($61.5\% \pm 50.6$ versus $95.0\% \pm 18.7$, $p = 0.03$), but this pattern was not observed in the basal layer. In contrast, VEGFR2 was regulated in the stromal compartment of the basal layer but not in the superficial layer ($27.9\% \pm 36.0$ versus $4.6\% \pm 11.3$, $p = 0.047$ for the No-UPA proliferative and secretory phases, respectively, in the stromal compartment of the basal layer) (Figure 2).

In the UPA group the regulation of VEGFR2 expression was compartment- and layer-specific. In the superficial layer there was a trend to a proliferative phase-like profile in the glandular epithelium, while in the stromal compartment the trend was towards a secretory phase-like profile, although these results did not reach significance. In the post-UPA proliferative phase group VEGFR2 immunostaining was similar to that observed in the No-UPA proliferative control group in both layers of the endometrium with the exception of the basal layer stromal compartment (Figure 2).

UPA treatment has an anti-progesterone effect on immunological markers

IL-15 expression was not statistically differentially expressed in the No-UPA proliferative and secretory phases in either compartment or layer, although a trend to a decreased expression in the No-UPA secretory phase was observed (Figure 4, Figure A.2). IDO expression was down regulated by progesterone in the glandular epithelium of both layers (Figure 4, Figure A.2). UPA administration resulted in a proliferative phase-like expression profile of IL-15 and IDO in both layers (Figure 4, Figure A.2). In the post-UPA proliferative phase group their expression was similar to the No-UPA control group in all analysed compartments.

CCL2 expression profile was similar in both endometrial layers, showing an increased staining in the stromal compartment during the No-UPA secretory phase. In the UPA and post-UPA proliferative phase groups, a proliferative phase-like CCL2 expression pattern was observed in both endometrial layers (Figure 4, Figure A.2).

DISCUSSION

Our results show that under UPA, ER and PR expression mirrors the proliferative phase in both layers, although with a decrease in cell proliferation. In fact, in the UPA group the ER immunostaining was generally increased as compared to the No-UPA secretory phase, except for the stromal compartment of the basal layer. The percentage of PR labelled cells in the UPA group was significantly increased in the epithelial cells of both layers as compared to the No-UPA secretory phase.

IL-15, IDO and CCL2 expressions mirror the proliferative phase, as a proliferative phase-like expression profile of IL-15, IDO and CCL2 was observed in both layers suggesting a progesterone-antagonist effect of UPA. In contrast VEGFR2 staining suggests a trend to a mixed agonist-antagonist effect, since an intermediary aspect was observed.

No significant difference is observed in the post-UPA proliferative phase group as compared to the proliferative phase control group in both layers of the endometrium.

This is the first report evaluating the effect of UPA on both layers of the human endometrium. In addition, samples obtained after treatment discontinuation were also evaluated.

The long-term impact of SPRM administration on the endometrium is debatable, as morphologically reversible benign alterations – PAEC (Mutter et al., 2008; Williams et al., 2012) – are observed in 11–26% of patients treated by UPA, based on endometrial biopsy evaluation (Donnez et al., 2012). In our study we observed PAECs in 14% of patients treated with UPA. The significance and impact of PAECs on endometrial functions is still incompletely understood since they are also observed in approximately 10% of women with myomas before any treatment with UPA (Williams et al., 2012). Long-term daily administration of UPA results in blunted progesterone levels, while oestradiol levels are constantly in the early follicular phase range (Chabbert-Buffet et al., 2007), which has raised the question of a potential proliferative effect.

Endometrial proliferation (Ki67) and maturation markers (ER, PR, AR, HAND2, FOXO1, HOXA 10, PTEN) have recently been described in endometrial biopsies of women under UPA treatment (Whitaker et al., 2017). Of note, although the short-term effects of mifepristone have been widely studied (for example, in the review by Danielsson *et al* (Danielsson et al., 2003)), its long-term and post-treatment effects are likewise imperfectly understood, while continuous administration of

asoprisnil, another SPRM, results in an original pattern of expression of endometrial maturation markers (Wilkens et al., 2013).

In the previous report of UPA effects in the superficial layer (Whitaker et al., 2017) the authors reported that ER expression mirrored the proliferative phase in stromal and epithelial cells. In the present study, a similar impact of UPA on ER expression profile was observed suggesting an anti-progestin effect of UPA. No effect on ER expression profile was found in the post-UPA proliferative group as compared to the control proliferative group in either endometrial layer, which suggests that UPA impact on ER expression does not persist after treatment discontinuation.

Dose- and molecule-specific SPRM effects on PR expression have been described previously (Danielsson et al., 2003; Wilkens et al., 2013). Under UPA, Whitaker *et al.* (Whitaker et al., 2017) reported that PR expression does not phenocopy either the secretory or proliferative endometrium with a compartment-specific profile in the superficial layer, however in the glandular epithelium PR expression was modified towards a proliferative phase profile. In the present study, under UPA PR expression is proliferative phase-like in both layers. No specific effect was seen after treatment discontinuation. This suggests that UPA has a reversible anti-progestin effect on PR expression.

Our results on the impact of UPA on cell proliferation are different from those of Whitaker (Whitaker et al., 2017). In the superficial layer these authors reported a decrease in Ki67 expression under UPA as compared to the proliferative phase in stromal cells, while it was unchanged in the glandular epithelium. In the present study, Ki67 was down regulated compared to the proliferative phase in stromal cells as well, while in the glandular epithelium it was distinct from both the proliferative and secretory phases, suggesting a mixed progesterone agonist-antagonist effect. Altogether, these results suggest an anti-proliferative effect of UPA in the stromal compartment and a specific intermediate effect in the glandular epithelium of the superficial layer of the human endometrium. Importantly, non-specific regulation of Ki67 expression was observed in the basal layer during UPA treatment or after discontinuation, suggesting the absence of any long-term effect of UPA on cell proliferation.

The discrepancies between our results and those of Whitaker and colleagues may be due to clinical differences such as a distinct timing in the secretory phase of control samples. In addition, our assessment of Ki67 staining was not automated. However, its evaluation was reproducible and

performed blinded. VEGFR2 is a progesterone-regulated marker. While in the superficial layer VEGFR2 expression was down regulated in the glandular epithelium during the secretory phase, which is consistent with a previous report in the literature (Lai et al., 2015), in the basal layer a distinct and specific pattern of VEGFR2 was observed. VEGFR2 expression was unchanged in the glandular epithelium and down regulated in the stromal cells in the secretory phase.

Under UPA there was a trend to a proliferative phase-like profile in the glandular epithelium in the superficial layer. In the stromal compartment the course was towards a secretory phase-like profile in both layers. In the post-UPA proliferative phase group VEGFR2 expression was broadly similar to that observed in the proliferative control group. This is the first report on VEGFR2 staining under UPA. Clinically, UPA treatment results in the rapid control of abnormal uterine bleeding and amenorrhea is observed in 91% of patients (Donnez et al., 2012). The mechanism behind this effect is still incompletely understood. UPA impact on angiogenesis has been assessed by Ravet and team (Ravet et al., 2008). These authors reported a UPA effect similar to the progesterone action on vascular maturation and proliferation, that is, integrity of the collagen-rich fibrillar network and no change in the relative vascular area or localisation and level of VEGF-A expression.

IDO plays a major role in tryptophan degradation (Kudo et al., 2001; Munn et al., 1999), an important pathway in regulatory T lymphocyte generation and immunosuppression. IDO expression was down regulated in the secretory phase in both endometrial layers as reported previously in the superficial layer (Sedlmayr et al., 2002).

IL-15, which regulates proliferation and activation of immune cells, showed a trend to a decreased expression in the secretory phase, while CCL2, which stimulates immune cell migration, displayed a trend to an increased expression in the stromal compartment in the secretory phase. There was no layer-specific pattern of expression. In the literature these markers have been evaluated in miscellaneous situations. In women with either uterine leiomyomas or cervix cancer, IL-15 was detected in the proliferative phase, primarily in the glandular epithelium (Kitaya et al., 2000). Its production increases in stromal cells in the vicinity of spiral arteries during the secretory phase (Kitaya et al., 2000). In healthy women IL-15 mRNA expression was increased in total endometrial samples during the secretory phase (Wilkens et al., 2013). CCL2 expression was increased from the early proliferative phase in endometrial biopsies from unselected women (Jones et al., 1997). These two markers are specifically implicated in endometrial maturation, while ER and PR participate in

endometrial regeneration as well. The differences between our results and the literature regarding the superficial layer might be explained by the fact that in our study endometrial samples were obtained from hysterectomies of selected patients with symptomatic myomas, while different physiological and pathological conditions were included in other studies. It is known that uterine myomas can have a specific effect on expression of endometrial molecular markers (Ikkena and Bulun, 2018; Rackow and Taylor, 2010).

Asoprisnil and UPA administration have been reported to result in the reduction of IL15 mRNA (Whitaker et al., 2017; Wilkens et al., 2013). No data is available on the impact of SPRMs on IDO or CCL2 expression. In the present study, a proliferative phase-like effect of UPA on IL-15, IDO and CCL2 expression was found. In the post-UPA proliferative phase group a proliferative phase-like expression profile of these markers suggests a limited impact of UPA on the immunomodulatory processes of endometrial maturation. This is in accordance with data available on emergency contraception with UPA, showing that this molecule does not interfere with embryo implantation (Berger et al., 2015; Gemzell-Danielsson et al., 2014). A series of post-UPA treatment pregnancies has been described in women treated for myomas (Luyckx et al., 2014) and a case report of pregnancy during UPA treatment has recently been published (Hrgovic et al., 2018). UPA may therefore be administered to women willing to conceive in the short term without any consequences for further implantation. .

The major strength of our study is the large number of patients from a homogeneous population of women with symptomatic myomas included in the analysis. The analysis of hysterectomy samples permitted the evaluation of both endometrial layers. The immunostaining was assessed by a blinded expert.

The limitations of this study include its retrospective design and the absence of hormonal assessment of the menstrual cycle phase. Control sample numbers were limited, as symptomatic myomas require hormonal treatment. The post-UPA group was composed of women in proliferative phase, because the majority of patients without hormonal treatment are operated on in the first phase of the menstrual cycle. Since all included patients were experiencing severe myoma-related symptoms, our data cannot be extrapolated to other clinical situations. Quantification of markers' expression was semi-quantitative since paraffin-embedded samples did not allow Western Blot analysis.

379

380 **CONCLUSION**

381 A three-month UPA course exerts a specific, compartment- and layer-dependent effect on the human
382 endometrium. Although in the superficial layer the effect of UPA is mostly PR antagonist-like, our
383 observations regarding the basal layer during treatment and after its discontinuation suggest the
384 absence of a long-term effect of this molecule on endometrial proliferation and maturation.

385

386 **APPENDICES**

387 **Table A.1.** Primary antibodies used in the study

Protein	Antibody reference	Species	Pre-treatment	Blocking	Dilution	Incubation time	Positive control	Isotype control
*PR SP1 ^a	790-4296	Monoclonal rabbit IgG	pH 8.8		Pre-diluted	24h	Human breast cancer	
*ER 1E2 ^a	790-4325	Monoclonal rabbit IgG	pH 8.8		Pre-diluted	24h	Human breast cancer	
IDO ^b	ab55305	Monoclonal mouse IgG2b	Citrate buffer pH 6.0 at 98°C	SNG 10% + BSA 1%	1/150	Overnight, 4°C	Human spleen	Mouse IgG2b
CCL2 ^b	ab9669	Polyclonal rabbit IgG	Citrate buffer pH 6.0 at 98°C	SNG 10% + BSA 1%	1/200	Overnight, 4°C	Human invasive ductal breast carcinoma	Rabbit IgG
IL-15 ^b	ab55276	Monoclonal mouse IgG2a	Citrate buffer pH 6.0 at 98°C	SNG 10% + BSA 1%	1/50	Overnight, 4°C	Human invasive ductal breast carcinoma	Mouse IgG2a
VEGFR2 ^c	sc-6251	Monoclonal mouse IgG1	EDTA buffer pH 8.0 at 98°C	SNG 10%	1/50	30 min, RT	Human colon carcinoma	Mouse IgG1
*Ki67 Mib1 ^d	M7240	Monoclonal mouse IgG	pH 8.8		1/150	32h	Tonsil	Mouse IgG

388 ^aVentana Medical Systems, Inc.

389 ^bAbcam, France

390 ^cSanta Cruz Biotechnology, USA

391 ^dDAKO

392 *Automate BenchMark XT, Roche Ventana®, (Ventana Medical Systems, Inc.)

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394 IL-15, interleukin-15; IDO, indoleamin-2,3-dioxygenase; CCL2, C-C motif chemokine ligand-2; VEGFR2, vascular endothelial growth factor-2; SNG, serum
 395 normal goat (Clinisciences); BSA, bovine serum albumin (Sigma-Aldrich); EDTA, ethylenediaminetetraacetic acid (Ivitrogen); RT, room temperature

Figure A.1. Oestrogen (ER) and progesterone (PR) receptor expression is layer dependent in the epithelial compartment.

The quantification of ER and PR immunostaining expressed as a percentage of marked cells in each endometrial layer. * $p < 0.05$. Error bars: mean $\pm$ SD.

Under UPA, the percentage of ER stained cells in the stroma was $90.0\% \pm 17.5$ in the superficial layer versus $92.9\% \pm 15.4$ in the basal layer ($p = 0.7186$) and in the epithelium $98.6\% \pm 5.3$ in the superficial versus $100.0\% \pm 0.0$ in the basal layers ($p = 1$). The percentage of PR stained cells in the stroma was $96.4\% \pm 9.3$ in the superficial layer versus $97.9\% \pm 8.0$ in the basal layer ($p = 1$) and in the epithelium $100.0\% \pm 0.0$ in the superficial versus $100.0\% \pm 0.0$ in the basal layers ($p = 1$).

UPA, ulipristal acetate

Figure A.2. VEGFR2, IL-15, IDO and CCL2 expression in both endometrial layers.

The quantification of IL-15, IDO and CCL2 immunostaining expressed as a percentage of marked cells in each endometrial layer. * $p < 0.05$, ** $p < 0.005$. Error bars: mean $\pm$ SD.

VEGFR2, vascular endothelial growth factor-receptor 2; IL-15, interleukin-15; IDO, indoleamin-2,3-dioxygenase; CCL2, C-C motif chemokine ligand-2

413 VITAE

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417 endometrial physiology and diseases, and endometrial impact of hormone treatments.

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419 KEY MESSAGE

420 Ulipristal acetate (UPA) effects on proliferation and maturation markers in both endometrial layers
421 during treatment and after discontinuation suggest the absence of long-term effect.

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- 519

FIGURE CAPTIONS

Figure 1. Ulipristal acetate (UPA) treatment modifies the expression of the oestrogen receptor (ER) and progesterone receptor (PR) in both layers of the endometrium.

Representative images showing immuno-localisation of ER and PR in the superficial (A-D, I-L) and basal (E-H, M-P) layers of the endometrium of women with symptomatic fibroids during No-UPA proliferative and secretory phases, at the end of the cycle of 3 months of UPA treatment and after discontinuation. (-) Negative control: human breast cancer without expression of PR and ER, respectively. Magnification x200 (scale bar = 100 μ m).

The quantification of ER (Q and S) and PR (R and T) immunostaining expressed as a percentage of marked cells in each endometrial layer. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Error bars: mean $\pm$ SD. SC, stromal cells; GE, glandular epithelium.

Figure 2. Ulipristal acetate (UPA) treatment has no impact on Ki67 expression in the basal layer and has a mixed agonist-antagonist effect on vascular endothelial growth factor-receptor 2 (VEGFR2).

Representative images showing immuno-localisation of Ki67 and VEGFR2 in the superficial (A-D, I-L) and basal (E-H, M-P) layers of the endometrium of women with symptomatic fibroids during No-UPA proliferative and secretory phases, at the end of the cycle of 3 months of UPA treatment and after discontinuation. (-) Negative control: Ki67 – non-specific antibody isotype control, VEGFR2 – human endometrium + non-specific antibody isotype control. Magnification x200 (scale bar = 100 μ m). The quantification of Ki67 (Q and S) and VEGFR2 (R and T) immunostaining expressed as a percentage of marked cells in each endometrial layer. * $p < 0.05$, ** $p < 0.005$. Error bars: mean $\pm$ SD.

SC, stromal cells; GE, glandular epithelium.

Figure 3. Ki67 expression is layer dependent.

The quantification of Ki67 immunostaining expressed as a percentage of marked cells in each endometrial layer. * $p < 0.05$, ** $p < 0.005$. Error bars: mean $\pm$ SD.

UPA, ulipristal acetate

Figure 4. Ulipristal acetate (UPA) treatment has an anti-progesterone effect on immunological markers.

Representative images showing immuno-localisation of IL-15, IDO and CCL2 in the superficial (A-D, I-L and Q-T) and basal (E-H, M-P and U-X) layers of the endometrium of women with symptomatic fibroids in the No-UPA proliferative phase, the secretory phase, the UPA and the post-UPA groups. (-) Negative control: human endometrium + non-specific antibody isotype control. Magnification x200 (scale bar = 100 μ m).

The quantification of IL-15 (Y and AB), IDO (Z and AC) and CCL2 (AA and AD) immunostaining expressed as a percentage of marked cells in each endometrial layer. * $p < 0.05$, ** $p < 0.005$. Error bars: mean $\pm$ SD.

SC, stromal cells; GE, glandular epithelium; IL-15, interleukin-15; IDO, indoleamin-2,3-dioxygenase; CCL2, C-C motif chemokine ligand-2.

564 **Figure 1.**

568 **Figure 2.**

Figure 3.

579 **Figure 4.**

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Figure A.1.

588 **Figure A.2.**

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591 **Author photo**
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