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ORIGINAL RESEARCH

Dual agonist–antagonist effect of ulipristal acetate in human endometrium and myometrium

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ABSTRACT

The aim of this study was to assess the molecular effect of ulipristal acetate (UPA) on gene expression in myometrium and endometrium of patients with symptomatic fibroids. Tissues isolated from four women treated preoperatively with UPA (5 mg) were compared to those from untreated controls using NanoString platform to assess the expression of 75 candidate genes modulated by UPA and ovarian steroids. Deregulated genes were then validated by real-time PCR. In myometrium, UPA exerted an antagonistic effect similar to that observed in fibroids. In UPA-treated endometrium, six genes were identified as highly and significantly upregulated, including matricellular genes *CCN1* (54-fold, $P = 0.0018$) and *CCN2* (11-fold, $P = 0.00044$), Krüppel-like factor 4 (>3-fold, $P = 0.0036$), and mast cell markers including tryptases *TPSAB1/TPSB2* (31-fold, $P = 0.023$) and carboxypeptidase A (*CPA3*, 17-fold, $P = 0.05$). In endometrium, UPA induced the expression of genes involved in fibrogenesis and mast cell function—some of them being widely involved in hepatic injury, which could explain the marked fibrosis and inflammatory cell infiltration observed in explanted livers from patients under UPA treatment.

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1. Introduction

Uterine leiomyoma (UL), also called fibroid or myoma, is the most commonly diagnosed tumor of the female genital tract with an incidence of 40% at the age of 35 and nearly 70–80% around the age of 50 [1]. Severe symptoms develop in 15–30% of patients with menorrhagia or excessively heavy menses being the most common. Although the direct cause of development of these tumors is unknown, accumulating evidence suggests that genetic and epigenetic mechanisms are involved in fibroids initiation and growth [2,3]. Alteration of these (epi)genetic mechanisms could be triggered by intrinsic abnormalities of the myometrium, abnormal expression of estrogen receptors, hormonal changes, or altered responses to ischemic damage during the menstrual cycle [2]. In addition, dependency of sexual steroid hormones, especially progesterone (P4), is accepted as determinant for tumor initiation and growth [4]. For this reason, selective progesterone receptor modulators (SPRMs) have been developed as a promising pharmacological therapy for uterine fibroids.

Ulipristal acetate (UPA) is an SPRM that shows high affinity and selectivity for progesterone receptor (PGR) and upon receptor binding exerts an agonist or antagonist effect according to the cellular context. At clinical level, UPA modulates activity of PGR without suppressing estrogen (E2), avoiding hot flashes and bone loss frequently observed with hypoestrogenism. Importantly, through inhibition of ovulation and direct action on the endometrium, UPA effectively controls bleeding with a success rate of 90% while reducing fibroid size [5]. Although the benefits of UPA for symptomatic myoma management seem clear, sporadic cases of hepatic failure have been reported. Thus, among 900,000 patients who have received UPA as therapy for symptomatic fibroids since 2012, some sporadic cases reported liver injury and hepatic failure, with five of them ending in liver transplantation [6,7]. Accordingly, the European Medicines Agency recommended suspension of UPA for uterine fibroids due to drug-induced liver injury.

Comparative transcriptional analysis of tissues can reveal the cellular responses following exposure to a candidate drug. Hybridization and amplification are two different methods widely used to detect mRNA molecules. The NanoString nCounter gene expression platform has become widely used within various clinical and research applications due to its ability to directly measure mRNA expression levels without the bias introduced by cDNA synthesis and amplification steps [8]. This platform shows high sensitivity, reproducibility, and technical robustness, making it an ideal platform for clinical application [9]. Most of the studies on gene expression changes induced by UPA have been conducted in uterine

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Supplemental data for this article can be accessed here

leiomyoma, where UPA seems to affect different physiological mechanisms altered in these tumors, including extracellular matrix (ECM) synthesis and resorption, angiogenesis, proliferation, and apoptosis [10]. Therefore, we conducted extensive searches in PubMed to identify candidate genes affected by UPA in leiomyomas to extend their analysis to myometrium and endometrium. Since UPA may act as agonist or antagonist of PGR, progesterone-regulated genes were also considered. Finally, given the presence of mast cells (MCs) in endometrium and myometrium, its role in angiogenesis and ECM remodeling, and the positive effect of female hormones in MCs maturation and degranulation, we also analyzed MC-specific markers. According to this, we designed a probe set containing 75 target and 5 housekeeping (HK) genes to assess the effect of UPA in the myometrium and the endometrium from patients treated with 5 mg of UPA during 12 weeks and compared with tissues obtained from untreated patients. Significantly deregulated genes detected by hybridization using NanoString nCounter platform were then analyzed using real-time quantitative PCR (qPCR).

2. Patients and methods

2.1. Patients and tissue sampling

This study was approved by the Institutional Review Board of the Committee for Clinical Research Ethics of the University Hospital of the Canary Islands, Spain (GIM-ULI-2014-01). Informed consent was obtained from the patients before the collection of any samples. All experiments involving human tissues were performed in accordance with Tenets of the Declaration of Helsinki.

The study included 37 pre-menopausal Caucasian patients (35–50) with heavy uterine bleeding caused by fibroids assessed by pictorial blood loss assessment chart (PBAC) >100. Patients received 5 mg of daily oral ulipristal acetate during 12 weeks. Participants were recruited between April 2015 and December 2016 in the hospital's obstetrics and gynecology unit. Fourteen patients did not complete the treatment period (lost to follow-up or protocol violation). The patients that completed the study (23) experienced amenorrhea during the first month of treatment. At the end of the treatment, four patients refused to undergo surgery because of successful control of the bleeding, four underwent myomectomy, and one woman underwent trans-hysteroscopic resection. From the remaining patients, 10 underwent surgery ≥2 weeks after treatment cessation and hysterectomy was performed only in four patients 3–9 days after the end of the treatment. Tissue histopathology from surgical samples from the four patients showed endometrium in the proliferative phase. Therefore, four Caucasian patients (age 39–47) who underwent hysterectomy for fibroid-associated menorrhagia (PBAC >100) without any previous treatment with endometrium in proliferative phase were included as control group. Histological analysis showed endometrium with tubular glands lined by pseudostratified columnar epithelium with infrequent mitotic figures in all cases. Hyperplastic endometrium was observed in one patient from the control group and one patient from the treatment group. Myometrium analysis showed normal histology without atypia and occasional mitotic figures. Adenomyosis and myometrial hyperplasia were found in two different patients from the treatment group.

2.2. RNA isolation

Tissue sections were stabilized in RNAlater after surgery and kept at −80°C until processed. Fifty milligrams of tissue were homogenized using lysing matrix D tubes (MP Biomedicals, UK) and RNA was isolated using the Direct-zol RNA Microprep according to the manufacturer's instructions (Zymo Research, Orange, CA). Residual genomic DNA was removed by incubating the RNA samples with RNase free DNase I and RNasin according to the manufacturer's instructions (Promega Corporation, Madison, WI, USA).

2.3. NanoString targeted gene expression

This analysis was performed by an external company using the NanoString nCounter system (IMIBIC, Córdoba, Spain). The platform counts hundreds of unique mRNA transcripts in a single reaction without the need for reverse transcription or amplification. A custom code set with 75 genes and 5 reference genes was applied (Supplementary Table S1). Briefly, RNA samples' concentration and integrity were determined using Agilent Bioanalyzer 2100. Probe set–target RNA hybridization reactions were performed according to the manufacturer's protocol using 100 ng of total RNA. Purified probe set–target RNA complexes from each reaction were processed, immobilized on nCounter Cartridges, and imaged in a Digital analyzer (NanoString Technologies).

2.4. NanoString data processing

Data from the project were processed and analyzed according to gene expression analysis guidelines from NanoString Technologies as follows: nCounter. RCC files for each sample were imported into nSolver analysis software 4.0 for review of quality control metrics (all passed). Background correction was performed using the eight negative controls included within each sample. Genes with counts below a threshold of 2σ of the mean background signal were excluded from subsequent analysis. Data normalization was performed on background-subtracted samples using internal positive controls and selected housekeeping genes that were identified with the GeNorm algorithm implemented in Genex 6.1.1.550 data analysis software (MultiD Analyses AB).

Differential gene expression analysis were performed using nSolver, which calculates the ratio of difference in the means of the log-transformed normalized data to the square root of the sum of the variances of samples in the two groups (Control and UPA) to assist in determining whether the fold change calculated is statistically significant. nSolver performs a two-tailed *t*-test on the log-transformed normalized data that assumes unequal variance. The distribution of the *t*-statistic was calculated using the Welch–Satterthwaite equation for the degrees of freedom in the estimation of the 95% confidence limits for observed differential expression between the two groups. The significance threshold was set at $P \leq 0.05$.

Because this was an exploratory experiment, the *P* values from the *t*-test were not adjusted for multiple comparisons to avoid losing candidate genes. Statistically significant deregulated genes with a fold change greater or lower than 1.6 (myometrium) or 2-fold (endometrium) were then analyzed using qPCR.

2.5. Gene expression validation using qPCR

Genes differentially regulated using nSolver platform were subsequently analyzed by quantitative PCR as previously described [11]. Plate data were imported into the GenEx 6.1.1.550 data analysis software (MultiD Analyses AB). Data were represented as individual triplicate runs and as average triplicates (with outliers excluded). The relative expression of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method normalized to expression levels of the three reference genes most stably expressed and to the mean of the control group. Log2 transformation of fold change was used for statistical analysis.

2.6. qPCR statistical analysis

GraphPad Prism v. 5.0 (GraphPad Software, La Jolla, CA, USA) was used for the statistical analysis. qPCR data were not normally distributed and were thus analyzed using nonparametric one-tailed Mann–Whitney U test. In all analyses, $P \leq 0.05$ was considered to be statistically significant.

4. Results

4.1. Housekeeping validation

GeNorm analysis using raw count data from the 80 genes present in the probe set and across the 8 samples of each group (myometrium and endometrium) showed that the five HK genes selected to normalize gene expression in myometrium samples were among the most stably expressed genes (data not shown), confirming our previous study [12]. *XIAP* emerged as the most stably expressed gene in myometrium, while *GNB2L1* showed the greatest variation among HK genes and hence it was excluded from the analysis. For this reason, normalization of these samples was achieved using *XIAP, GUSB, PUM1, RPL32*, and *ALAS1* in NanoString platform and the first three HK genes were used for qPCR normalization. In addition to *XIAP, VEGFB* appeared as the second most stably expressed gene in endometrium samples. For this reason, *RPL32, VEGFB, PUM1, XIAP*, and *ALAS1* were used to normalize endometrium samples in NanoString platform and *RPL32, PUM1*, and *XIAP* were used for qPCR normalization.

4.2. Expression changes associated with UPA in myometrium

Sixteen different genes were significantly downregulated in UPA-treated compared to non-treated myometrium using NanoString platform (Supplementary Table S2). Cyclin D1 (*CCND1*, $P = 0.008$) and the inhibitor of the matrix metalloproteinases 3 (*TIMP3*, $P = 0.008$) showed greater than 2-fold decreased expression while the matrix metalloproteinase 11 (*MMP11*, $P = 0.006$) showed increased expression of almost 5-fold in the UPA-treated group. It is noteworthy that the decreased expression of growth factors and their associated receptors are known to be important in UL pathogenesis, such as *PDGFA/ PDGFRA* ($P = 0.003$, $P = 0.04$), *TGFβ3/ TGFBR2* ($P = 0.033$, $P = 0.011$) and *FGFR1* ($P = 0.04$) (Supplementary Table S2). In addition, *ESR1* expression was upregulated in UPA-treated myometrium ($P = 0.03$). qPCR analysis confirmed significant deregulation of 7 out of 8 genes analyzed (Table 1). *KDR, IGFBP6, FGF2*, and *PGR* (transcripts encoding isoforms B and D) were close to significance in NanoString platform (Supplementary Table S2) but qPCR analysis did not show any significant deregulation in UPA-treated myometrium compared to controls (data not shown).

4.3. Expression changes associated with UPA in Endometrium

Contrary to the general transcriptional repression exerted by UPA on myometrium, most of the genes in endometrium were positively regulated and 13 genes were significantly upregulated in UPA-treated endometrium compared to controls using NanoString platform (Table 2, Supplementary Table S3). High expression levels of matricellular genes, such as *CCN1* (>50-fold, $P = 0.0018$), *CCN2* (>11-fold, $P = 0.00044$), the angiogenic marker *ADM* (5-fold, $P = 0.013$), and genes related to mast cell metabolism, such as carboxypeptidase A (*CPA3*, 17-fold, $P = 0.05$) and tryptases alpha and beta (*TPSAB1/TPSB2*, >31-fold, $P = 0.023$). Other significantly upregulated genes included estrogen receptor-β (*ESR2*, >4-fold, $P = 0.016$), Krüpple-like transcription factor 4 (*KLF4*, 3.2-fold, $P = 0.0036$), the apoptosis regulator *BCL2* (>3-fold, $P = 0.025$), and the epidermal growth factor receptor (*EGFR*, 2-fold, $P = 0.00018$) are noteworthy. Genes upregulated greater

Table 1. Significantly deregulated genes in UPA-treated myometrium compared to control group. Genes with fold changes greater or lower than 1.6-fold in NanoString platform were then analyzed by qPCR.

	NANOSTRING						qPCR	
GENE NAME	TREATMENT MEAN	TREATMENT StDev	CONTROL MEAN	CONTROL StDev	**FOLD CHANGE**	*P* value	**FOLD CHANGE**	*P* value
CCND1	1167.87	352.34	2842.65	238.93	**−2.43**	**0.008**	**−3**	**0.02**
TIMP3	19,329.68	6021.25	46,686.11	4602.15	**−2.42**	**0.008**	**−2.8**	**0.01**
PDGFRA	2222.8	665.06	3917.8	1105.56	**−1.76**	**0.04**	**−1.73**	**0.05**
PDGFA	154.32	24.98	259	41.65	**−1.68**	**0.003**	**−1.7**	**0.02**
FGFR1	3069.51	1056.86	5148.28	661.81	**−1.68**	**0.04**	**−1.6**	**0.02**
TPSB2	6132.25	1339.18	3676.39	533.18	**1.67**	**0.009**	1.47	0.17
ESR1	6876.15	1599.27	4272.02	1060.93	**1.61**	**0.03**	**2.35**	**0.01**
MMP11	881.68	475.66	188.27	104.85	**4.68**	**0.006**	**5.5**	**0.02**

Table 2. **Significantly upregulated genes in UPA-treated endometrium compared to controls**. Genes with fold changes greater than 2-fold in NanoString platform were then analyzed by qPCR.

	NANOSTRING						qPCR	
GENE NAME	TREATMENT MEAN	TREATMENT StDev	CONTROL MEAN	CONTROL StDev	**FOLD CHANGE**	*P* value	**FOLD CHANGE**	*P* value
CCN1	28,959.03	15,159	538.23	991.42	**53.8**	**0.0018**	**51**	**0.01**
TPSAB1/TPSB2	1850.93	5463.28	58.12	85.47	**31.85**	**0.023**	**29**	**0.02**
CPA3	583.58	1871.68	34.14	17.16	**17.09**	**0.05**	**41**	**0.01**
CCN2	25,766.16	16,224.61	2222.38	845.58	**11.59**	**0.00044**	**12**	**0.01**
ADM	538.82	392.02	100.37	49.22	**5.37**	**0.013**	3.5	0.1
ESR2	282.41	190.13	60.07	34.63	**4.7**	**0.016**	4.3	0.1
BCL2	2003.26	828.32	589.77	417.42	**3.4**	**0.025**	2.2	0.1
KLF4	2112.31	634.83	651.59	306.81	**3.24**	**0.0036**	**4**	**0.01**
KIT	225.72	216.09	73.58	37.58	3.07	0.07	**4**	**0.01**
EGFR	3801.99	455.91	1855.86	225.99	**2.05**	**0.00018**	1.37	0.1
TIMP2	7044.22	1294.54	3922.52	1019.1	**1.8**	**0.008**		
TGFB3	621.75	158.5	357.82	129.01	**1.74**	**0.037**		
IGF1R	4952.41	282.39	3735.14	622.22	**1.33**	**0.028**		
NFAT5	1339.49	71.68	1075.43	139.29	**1.25**	**0.039**		

than 2-fold were analyzed by qPCR confirming the increased expression in six of them (Table 2). We also included *KIT* and the vascular endothelial growth factor receptor 1 (*FLT1*) in the qPCR analysis because they were very close to significant upregulation with NanoString platform ($P = 0.051$ and $P = 0.07$, respectively, Supplementary Table S3) and increased expression 3- and 2.25-fold, respectively. Analysis of qPCR data confirmed upregulation only for *KIT* ($P = 0.01$, Table 2).

5. Discussion

Depending on the location, the presence of co-activators or co-inhibitors of gene expression, and the serum levels of progesterone, UPA may act as an agonist or antagonist of progesterone. In light of the present data, we propose that UPA exerts antagonistic and agonistic effects in myometrium and endometrium, respectively.

Previous studies demonstrated that UPA induced ECM remodeling by modifying the MMP/TIMP balance toward degradation in fibroids [5]. We extend this finding to myometrium, where we observed a significant upregulation of metalloproteinase *MMP11* and downregulation of *TIMP1, TIMP2,* and *TIMP3* in treated tissues *in vivo*. Additionally, UPA negatively regulated proliferation markers (*CCND1*/*CCND2*), growth factors and their receptors (TGF-β3/TGFBR2), as well as genes involved in angiogenesis (*VEGFB*/*KDR*). Therefore, UPA seems to exert an antagonistic effect in the myometrium, downregulating the expression of target genes known to be induced by progesterone, such as growth factors [11,13] and angiogenic markers [11,14,15], and upregulating MMP genes and the estrogen receptor alpha (*ESR1*), which are negatively regulated by progesterone [16–18]. Therefore, UPA effect on myometrium seems similar to that reported in fibroids and agrees with the physiological response observed in patients with tumor reduction and control of menstrual bleeding as the major outcome [5]. It should be noted that changes observed after UPA administration occurred in the myometrium from women in proliferative phase. Therefore, under low progesterone levels, UPA may preferentially bind to PGR, translocate to the nucleus, and regulate gene expression in the opposite way to that observed for its natural ligand. Persistently low levels of progesterone were observed in patients during 3-month treatment with UPA [19], suggesting that gene expression changes observed at the end of the treatment when progesterone levels are still low may occur throughout the treatment course.

In endometrium, genes from three different biological processes were significantly and highly upregulated in response to UPA compared to controls (Table 2). Cysteine-rich angiogenic inducer 61 *(CYR61)/CCN1* and connective tissue growth factor *(CTGF)/CCN2* are matricellular proteins, which have been detected in luminal and glandular epithelial cells and endothelial cells of human endometrium [20,21]. *CCN1* is an estrogen-regulated gene in human endometrium while progestin bond to PGR induced *CCN1* transcription through a progesterone response element [22]. In fact, both E2 and P4 induced the expression of *CCN2* mRNA in mouse and goat endometrium [23,24]. Both proteins play key roles in ECM production, angiogenesis, wound healing, and fibrosis [25,26]. Enhanced CCN1/CCN2 expression has been observed in different uterine pathologies such as endometriosis and intrauterine adhesions [27,28] and in the endometrium of women with polycystic ovarian syndrome and premalignant lesions [29]. Interestingly, CTGF plays a central role in the pathogenesis of hepatic fibrosis by triggering activation and transformation of quiescent hepatic stellate cells (HSCs) into myofibroblasts. Activated cells overproduce collagens, fibronectin, and laminin under stimulation with CTGF in an autocrine or paracrine mechanism [30–32]. In fact, upregulation of CTGF is a signal of HSCs activation and cirrhosis in rat models of hepatic disease [30,33–36]. In addition to HSC cells, bile duct epithelial cells also express CTGF and its mRNA levels increased sevenfold in a rat model of biliary fibrosis [33]. In humans, CTGF is considered a master fibrogenic marker involved in liver fibrosis related to a variety of liver diseases [30,37–39]. Similarly, increased expression of CCN1 was detected in a mouse model of induced liver fibrosis, in human hepatocellular carcinoma (HCC) tissue and was markedly increased in cancer-adjacent hepatic cirrhosis tissue compared to

healthy individuals [40]. Moreover, CCN1 affected the function of activated HSCs inducing fibrogenesis and angiogenesis [41]. On the contrary, *in vivo* transduction of recombinant adenovirus with CCN1 in a mouse model of induced liver fibrosis resulted in decreased expression of the fibrogenic collagen type I protein and regression of liver fibrosis through induction of cellular senescence in hepatic myofibroblast [42]. In the present study, we found that UPA highly increased *CCN1/CYR61* (>50-fold) and *CCN2/CTGF* (>11-fold) mRNA expression, suggesting an agonistic activity in the endometrium similar to that exerted by P4. In light of these data, we suggest that UPA may exert agonistic action in liver, increasing the expression of the matricellular proteins CCN1/CCN2. In this sense, the histological analysis of the explanted liver in patients under UPA treatment reveals cholestasis (impairment of bile flow) and marked fibrosis [6], compatible with the pathophysiological role triggered by CCN1/CCN2. *In vitro* testing of UPA effect on liver cells is necessary to confirm these findings. In addition, treatment of animal models with UPA would allow detection of matricellular proteins from serum, plasma, or urine samples. A positive correlation of protein levels and liver fibrosis assessed by histology would open the possibility to use them as biomarkers of liver injury during UPA therapy [43].

Mast cells (MCs) are found in all layers of the human endometrium where they play an important role in angiogenesis and ECM remodeling, which are essential for embryo implantation and growth [44]. In this study, we found upregulation in MC-specific genes in UPA-endometrium, including two proteases, tryptase (*TPSAB1/TPSB2*) and carboxypeptidase A3 (*CPA3*) and the proto-oncogene *KIT* encoding a transmembrane tyrosine kinase receptor. Human, mouse, and rat MCs express estradiol and PGRs and female hormones seem to activate MCs [45]. E2 and P4 individually increased expression of tryptases and together exerted synergistic action inducing higher mRNA expression levels of tryptases in the uterus of mice treated with both hormones compared to control group [46]. Sexual steroids induce the maturation of MCs and directly cause degranulation in a dose-dependent manner [45]. Activated MCs release a plethora of factors that attract other inflammatory cells and cause collateral damage to surrounding tissues, which leads to scar formation and fibrosis [47]. Interestingly, a recent work has demonstrated a significant increase in MC number during hepatic injury, suggesting an important role of these cells in hepatic disease [48]. In fact, explanted liver in patients under UPA treatment reveals massive infiltration of inflammatory cells [6]. Altogether, our results suggest that UPA may act as an agonist in MCs, inducing the expression of *KIT* and proteases involved in angiogenesis and ECM remodeling. Alternatively, UPA may also attract MCs to the endometrium, which would explain the high expression of MCspecific markers. This is not surprising since combination of ovarian steroids can attract MCs to uterine cells *in vivo* [46].

Finally, UPA effect on MCs seems to be specific and independent of the tissue where they reside. Thus, in myometrium where UPA decreased the expression of most of the genes under study, the expression of MC-specific markers *TPSAB1/TPSB2, CPA3,* and *KIT*, increased when analyzed in NanoString platform although not significantly (Supplementary Table S2).

Krüppel-like factor 4 (KLF4) belongs to a family of zinc finger-containing transcription factors that are epithelial cell-enriched, where it acts as an inhibitor of cell proliferation. In human endometrial epithelial cells, P4 increased the expression of *KLF4* mRNA, followed by a decrease in cyclin D1 mRNA and inhibition of cell proliferation [49]. Similarly, we found that UPA induced upregulation of *KLF4* mRNA (>3-fold) while appeared to decrease cyclins *CCND1* and *CCND2* mRNA expression in endometrium (Supplementary Table S3), which is compatible with its role as tumor suppressor gene in endometrial tumors [50].

Limitations to the current study include the small number of samples analyzed and comparison of UPA-treated tissues with non-treated tissues from different patients, which may be a source of inter-individual variability. Gene expression changes were not confirmed at protein level. Although all samples analyzed were in proliferative phase, surgery was performed between days 1 and 9 after treatment. This may lead to fluctuations in the progesterone and estradiol levels that may account for intra- and inter-group variation on gene expression levels. Certainly, further studies with greater number of patients are required to confirm these findings.

6. Conclusions

Several studies have analyzed the molecular effect of UPA on fibroids obtained from hysterectomies after one or multiple 3-month treatment courses. This contrasts with the paucity of studies analyzing normal uterine myometrium and endometrium tissues, which could have been collected and processed at the same time as tumor tissue. The analysis of healthy tissues is essential not only to determine drug safety, but also to gain insight into the role of drugs in other target tissues. In this sense, we have detected a striking increase in genes actively involved in hepatic injury after UPA treatment.

Author contributions

TA Almeida: studied conception and design, analysis and interpretation of data, and revising the manuscript critically for intellectual content.

D Báez: secured funding, study conception and design, interpretation of data, and revising the manuscript critically for intellectual content.

A Salas: acquisition of data, analysis and interpretation of data, and manuscript drafting.

P Vázquez: acquisition of data, analysis and interpretation of data, and manuscript drafting.

AR Bello: acquisition of data, analysis and interpretation of data, and revising the manuscript critically for intellectual content.

All authors approved this version of the manuscript and agree to be accountable for all aspects of the work.

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Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from that disclosed.

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