Dopamine receptors D1 and D2 show prognostic significance and potential therapeutic applications for endometrial cancer patients

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HIGHLIGHTS

- ADRβ2 expression was neither associated with clinico-pathological parameters nor prognostic value in the analyzed patients.
- DRD1 expression showed an inverse association with tumor size and stage in our endometrial cancer patient cohort.
- DRD2 expression showed significant positive association with non-endometrioid endometrial cancer, high grade and tumor size.
- High expression of DRD2 is an independent significant prognostic marker for predicting OS and DFS in the patient's cohort.
- DRD1 agonism and DRD2 antagonism combination reduced cellular viability and could have a potential therapeutic benefit.

ARTICLE INFO

ABSTRACT

Objective. Catecholaminergic signaling has been a target for therapy in different type of cancers. In this work, we characterized the ADRβ2, DRD1 and DRD2 expression in healthy tissue and endometrial tumors to evaluate their prognostic significance in endometrial cancer (EC), unraveling their possible application as an antitumor therapy.

Methods. 109 EC patients were included. The expression of the ADRβ2, DRD1 and DRD2 proteins was evaluated by immunohistochemistry and univariate and multivariate analysis to assess their association with clinic-pathological and outcome variables. Finally, HEC1A and AN3CA EC cell lines were exposed to different concentrations of selective dopaminergic agents alone or in combination to study their effects on cellular viability.

Results. ADRβ2 protein expression was not associated with clinico-pathological parameters or prognosis. DRD1 protein expression was reduced in tumors samples but showed a significant inverse association with tumor size and stage. DRD2 protein expression was significantly associated with non-endometrioid EC, high grade tumors, tumor size, worse disease-free survival (HR = 3.47 (95%CI:1.35–8.88)) and overall survival (HR = 2.98 (95%CI:1.40–6.34)). The DRD1 agonist fenoldopam showed a reduction of cellular viability in HEC1A and AN3CA cells. The exposure to domperidone, a DRD2 antagonist, significantly reduced cell viability compared to the control. Finally, DRD1 agonism and DRD2 antagonism combination induced a significant

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reduction in cell viability of the AN3CA cells compared to monotherapy, close to being an additive response than a synergistic effect (CI of 1.1 at 0.5% Fa).

Conclusion. DRD1 and DRD2 expression levels showed a significant association with clinico-pathological parameters. Both the combined activation of DRD1 and blockage of DRD2 may form an innovative strategy to inhibit tumor growth in EC.

1. Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy in developed countries and the fourth most frequent cancer in women [1]. Its incidence has increased over the last few years due to the increment in obesity prevalence together with improved life expectancy [2]. Most patients are diagnosed in the early stages and have favorable prognoses, ranging around 90% for 5-year overall survival [3]. However, between 10% and 20% of patients may present recurrences or be diagnosed in the more advanced stages [4].

EC treatment consists of surgery followed by radiotherapy and/or chemotherapy based on prognostic risk groups, as recommended in the ESMO-ESGO-ESTRO guidelines [5]. Those patients who present an advanced stage malignancy or that suffer a disease recurrence are offered to follow immunotherapy or targeted therapy, but unfortunately, only low treatment responses have been achieved at the moment [6]. The identification of new prognostic markers may contribute to a more accurate classification of endometrial cancer subtypes and may provide new therapeutic targets for treating the most aggressive forms of the disease.

Catecholamines, that include noradrenaline (NA), adrenaline (A) and dopamine (DA), are mediators in response to physical or emotional stress, and have emerged as key players in the relationship between chronic stress and cancer progression [7]. Evidence suggests that tumor cells use neurotransmitters and their receptors to activate mechanisms for tumor growth and metastasis via neuroendocrine pathways, or through nerve fibers that are part of the so-called tumor microenvironment (TME) [8].

The effects of NA and A are mediated through Beta-2 adrenergic receptors (ADRβ2). ADRβ2 are highly expressed in various tumor tissues, and this expression has been closely related to poor prognoses in several cancers [9]. Activation of the ADRβ2 pathway is involved in carcinogenesis, cell proliferation, immune regulation, invasion, and angiogenic processes, which are all related to the clinical prognosis and treatment resistance in various aggressive tumors, such as breast, prostate, melanoma and pancreatic cancers [10,11]. Moreover, ADRβ2 expression is influenced by hormonal regulation, including estrogen and progesterone [12] and is described in hormone-dependent breast cancer [13]. This makes this receptor an interesting target for investigation in endometrial cancer, since up to now there has been no information of the prognostic relevance of ADRβ2 in endometrial cancer.

Also participating in the catecholaminergic system, DRD1 and DRD2 dopamine receptors may be upregulated in the most aggressive tumors and show different effects depending on the type of tumor [14]. The DRD2 family of receptors are the most studied in cancer and are overexpressed in stress-sensitive tumors [7]. DRD2 is involved in both the activation of the HIF1α pathway that mediates tumor progression [15] and VEGF-induced angiogenesis, becoming an optimal candidate for therapy in oncology [16,17]. In this context, several antagonists of DRD2 have been evaluated in preclinical and clinical trials in the most prevalent cancers (NCT02250781, NCT02324621) [18]. On the contrary, the expression of DRD1 is variable among tumors [19,20] displaying differential drug responses [20]. In others female-associated cancers like breast cancer, DRD1 correlates with tumor progression and

the formation of metastases [21]. Moreover, in endometrial cancer, DRD2 is highly expressed in serous subtypes and demonstrates a positive association with the grade, the stage, worse progression and with lower overall survival [22].

Given the limited information regarding the clinical significance of ADRβ2 and DRD1, and to validate the prognostic value of DRD2 in our patient's cohort, this work aims at evaluating both the prognostic significance and the possible therapeutic value of ADRβ2, DRD1 and DRD2 in endometrial cancer. For that purpose, we immunohistochemically characterized the expression of these receptors in endometrial tumors from a patient series and studied their association with clinico-pathological and survival variables. Additionally, the effect of neuromodulation on EC cellular viability was evaluated in vitro.

2. Methods

2.1. Patient cohort and samples

This study was approved by the Clinical Research Ethics Committee (Reference IIBSP-CEN-2020-14) from *Hospital de la Santa Creu i Sant Pau (HSCSP)*. Data and material were registered in accordance with Declaration of Helsinki [23].

A total of 118 white ethnicity patients with histologically confirmed EC were included in this retrospective study. Surgeries consisting of hysterectomy and bilateral oophorectomy added to lymph node assessment were performed at HSCSP from 2009 to 2018. None of the patients were treated with chemotherapy or radiotherapy prior to surgery, and adjuvant treatment indication was decided in the Gynecology Oncology Committee following the institutional guidelines recommendations. During the selection, four samples were excluded due to insufficient clinical data, and five of them were eliminated due to poorly conserved material for molecular assessments. As control samples, a group of 10 non-pathologic endometrial patient samples were also examined and compared to EC samples.

Clinical and pathological characteristics of our patient's cohort are depicted in Supplementary table 1. They include age, histological type, tumor grade, tumor size, stage at diagnosis, overall survival (OS) and disease-free survival (DFS).

2.1.1. Immunohistochemistry

The analysis of ADRβ2, DRD1 and DRD2 protein expression was assessed by immunohistochemical (IHC) staining. Fixed and paraffin-embedded tumor samples were obtained from the Department of Pathology from our hospital. Immunostaining was performed using the Autostainer Link 48 automated system (Dako, Agilent technologies, Santa Clara, CA, US), using EnVision kit reagents Flex, High pH (Dako). Tissue sections (4 μm) were initially deparaffinized and rehydrated. Antigen retrieval was performed using EDTA pH 9.0 buffer at 120 °C, and endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 for 10 min and washed with PBS-T prior the incubation with the respective primary antibody (Anti-ADRβ2 at dilution of 1:200, Abbiotech San Diego 251,604, CA, US; Anti-DRD1 at dilution of 1:200, R&D system, MAB8276, Minneapolis, MN, US; Anti-DRD2 at dilution of 1:200, Santa Cruz Biotechnology sc-5303, INC, Dallas, TX, US) for 30 min. The specificity of the antibodies have been validated previously

by other authors [24–26], and also in our study in control and tumor samples ($N = 5$) prior to use in the entire cohort. Following this, the slices were incubated with HRP-conjugated secondary antibody for 30 min at RT before chromogenic detection with DAB (DAKO). Sections were counterstained with hematoxylin, dehydrated and mounted using DPX medium.

Two representative images per sample were acquired using the Olympus model BX51 microscope and were analyzed digitally using Image J (v 1.50i). The analysis was carried out by a specialized pathologist who was unaware of the clinical data of the patients. Signal intensity was calculated by multiplying the count value of each image by the mean value, and finally, the H-Score was calculated by multiplying the staining area by the intensity.

2.1.2. Statistical analysis

The statistical analysis of ADRβ2, DRD1 or DRD2 expression between non-pathological and tumor tissue, and their association with the clinico-pathological factors were evaluated using the statistical program R (R Version 4.1.3 (2022), Copyright © 2022 The R Foundation for Statistical Computing) with the default packages *base, stats, graphics, utilities, datasets, and methods*, associated with the *ggplot2* packages, *rcompanion*, and *vtable*. Robust statistical methods based on the Wilcox WRS functions, using the WRS2 package [27] were applied. Each receptor expression was represented as the median, corresponding measure of dispersion or interquartile range (IQR).

The Cox proportional hazards regression model (using the *survival and survimer packages*) was applied to conduct a multivariable analysis of those prognostic variables identified to be significant in the univariate analysis. The *p*-value calculated by the Likelihood ratio method was used to assess the goodness of fit of the model. The results have been expressed as HR (Hazard Ratio or risk rate), with 95% confidence interval and the p-value. Overall survival (OS) was recorded as the time from tumor resection to death from any cause, while Disease-free survival (DFS) was calculated as the time between tumor resection and the first episode of disease progression confirmed by the pathological analysis.

Quantification of the receptor expression in the IHC assessment were evaluated as continuous variables and, in the additional analysis, the optimal cut-off point was detected to categorize them, using the *surv_cutpoint* function from the *survminer* package. *P* value <0.05 was considered statistically significant.

2.2. In vitro experiments

2.2.1. Endometrial tumor cell lines

Endometrial HEC1A (HTB-112™, ATCC) and AN3CA (HTB-111™, ATCC) tumor cell lines were selected for the in vitro experiments. HEC1A was considered as type 2 EC cell line, and AN3CA as type 1 EC cell line. Cells were grown in DMEM 1 g/L glucose (HEC1A) and DMEM 1 g/L glucose and F12 (50v:50v) (AN3CA), supplemented with 10% FBS (gibco; 10270–106), 1% (Penicillin/Streptomycin) (gibco; 15140–122), 1% Glutamine (gibco; 25030–024) and 0.2% Amphotericin (gibco; 15290–026). Cells were maintained within a humidified 5% O2/CO2 atmosphere at 37 °C in the incubator.

2.2.2. Gene expression of dopamine receptors

The extraction of RNA from the paraffin samples was performed with the RNeasy kit (ThermoFisher Sci. Waltham, MA, US) and stored at −80 °C until use. The ADNc synthesis was carried out from 1200 ng of RNA using the High Sensitivity cDNA Synthesis Kit (ThermoFisher Sci.). Gene expression analysis of HEC1A and AN3CA cells was performed by Real-time RT-qPCR (ThermoFisher Sci.) Specific TaqMan probes were used for each gene family and the procedure was performed following manufacturer's instructions. The expression of each gene was normalized by the value of the constitutive endogenous expression of the gene GADPH using the ΔCt method.

2.2.3. Cell viability assay

To perform the cell viability assay, cells were counted and seeded onto a 96-well plate at a final concentration of 5000 cells/ml. After 24 h, HEC1A and AN3CA cells were exposed to different concentrations (0–100 μM) of agonists: Fenoldopam (FE, for DRD1) and Pergolide (PE, for DRD2) and antagonists: LE 300 (LE, for DRD1) and Domperidone (DOM, for DRD2), for 48 h. Agonists and antagonists were prepared at 10 mM in water (FE, PE, DOM) and/or DMSO (LE) and stored at −20 °C until use.

Cell viability was assayed with the Cell Proliferation Kit II (XTT, 11465007001 Roche), as per manufacturer's instructions. Briefly, 50 μl of XTT reagent, prepared by combining XTT labeling reagent and the "Electron Coupling Reagent" in a ratio of 5:0.1 ml respectively, was added per well. After that, the plate was maintained in the 37 °C incubator for 4 h, and then the absorbance was read at 450 nm in a plate reader (Infinite M200 PRO, TECAN).

In an independent experiment, the combined effect of FE and DOM on cell viability, compared to its respective single treatments, was evaluated in the AN3CA tumor cell line. The fraction affected (F_a) by the different treatments were calculated as 1-T/C*100. The combination index (CI) to determine the additive, synergistic or antagonistic dose-effect relationship of fenoldopam+domperidone treatment was estimated based on the median-effect principle by Chou T.C. and Talalay P [28,29], and using the free CompuSyn software (http://www.combosyn.com/) [30]. These results were further validated using the CISNE program (Code for the identification of synergism numerically efficient; https://cisnecode.github.io/) [31].

For that purpose, treatments were administered at 25, 50, 75 uM as a single fenoldopam or domperidone treatment, or its combination relation 1:1). Derived parameters from the median-effect plot, such as the "m" (slope), "Dm" (interception point), and the linear regression coefficient "r" was calculated to describe the dose-effect relationship following the treatments. Moreover, the C versus Fa plot was generated and CI values were calculated using the same softwares.

2.2.4. Statistical analysis

The analysis was carried out evaluating the sample normality using the Shapiro-Wilk test, and then, the non-parametric Kruskal-Wallis test and the Mann-Whitney *U* test (pair-wise) were applied. *P value* < 0.05 was considered statistically significant.

3. Results

3.1. Clinical characteristics of the patient's cohort

A cohort of 109 EC patients was included in the study. Baseline demographic characteristics are shown in Supplementary Table 1. In our series, histological distribution presented 80 endometrioid carcinomas with grading distributed as 44.9% for grade 1 and 2, and 55.1% for grade 3. In addition, 29 non-endometrioid tumors were identified, composed of 14 serous, 7 clear cell and 8 mixed cell carcinomas. In reference to pathological staging, 85 were staged initial (I-II) and 24 advanced (III-IV). The median follow-up of the series was 48 ± 30 months. A total of 22.9% of patients (25 cases) presented recurrence and 33.9% of patients (37 cases) died during the follow-up period.

The univariate survival analysis for disease-free survival (DFS) revealed the histological type, the tumor grade and the stage are significant prognostic factors in our patient cohort. The same study for overall survival (OS) demonstrated that age, histological type, tumor grade, tumor size, stage and recurrence presented as significant factors for mortality (Supplementary Table 2).

3.2. ADRβ2 expression is reduced in endometrial tumors and has no association with clinico-pathological variables

For immunohistochemical analysis of ADRβ2 in endometrial tumors, the majority of analyzed samples showed a predominant plasma

membrane expression pattern, although cytoplasmic staining was also identified in approximately 5% of samples (Supplementary Fig. 1A). The comparative analysis of ADRβ2 expression levels between non-pathologic and tumor tissue showed statistically significant differences both in the Intensity (3304.0 vs 1682.4 p value 0.048) and H-Score (1080.4 vs 121.0 p value 0.021) in favor of a reduction of expression in tumor tissue versus non-pathologic endometrial tissue (Supplementary Fig. 1B, C).

The analysis of the relationship between ADRβ2 expression and the most relevant clinico-pathological variables did not show statistically significant differences as presented in Table 1. In addition, a univariate statistical analysis was performed to determine whether ADRβ2 expression was associated with DFS or OS in the analyzed cohort; non-statistically significant differences were identified among both variables.

3.3. Low DRD1 expression in endometrial tumors is associated with higher tumor size and stage

DRD1 immunostaining showed both a plasma membrane and a cytoplasmic pattern (Fig. 1A) in all analyzed samples. The immunohistochemical analysis of DRD1 in endometrial tumors showed a reduced expression in the tumors compared to non-pathologic endometrial tissues, both in Intensity (2299.1 vs 42,459.3 p value 0.001 Intensity) and H-score (253.2 vs 526,378.5 p value 0.001 H-score) parameters as shown in Fig. 1B, C. Moreover, DRD1 protein expression showed a significant inverse relationship with tumor size when stratified in 2 cm (p value 0.0412 Intensity; p value 0.028 H-Score) and also in the stage variable sub analysis comparing stage IV versus stage I (p value 0.002 Intensity; p value 0.001 H-Score) (Fig. 1 D, E, Supplementary Table 3).

We further studied the expression of DRD1 influence in survival parameters and we observed that DRD1 was not associated with the survival of our patient cohort (Supplementary Fig. 2).

3.4. DRD2 expression is associated with poor prognosis in our patient cohort

As shown in Fig. 2A, the DRD2 immunostaining showed a marked plasma membrane pattern in the totality of the analyzed tumors. This expression did not present significant differences when comparing tumor and non-pathologic endometrial samples (6529.3 vs 5504.1 p value 0.610 Intensity; 5839.7 vs 1873.4 p value 0.127 H-Score) as depicted in Supplementary Fig. 3. The univariate analysis showed that the high DRD2 expression was significantly associated with non-endometrioid endometrial cancer (p value 0.048 H-Score), high grade tumors (p value 0.038 H-Score), and tumor size (p value 0.0126 Intensity) (Fig. 2 B, C, D, Supplementary Table 4).

We further studied the expression of DRD2 influence in survival parameters. Kaplan-Meier survival curves for patients divided by respective cut-off point are shown in Fig. 3. Regarding DRD2, significant differences were established in H-Score in the DFS analysis, showing an increased risk of recurrence with higher DRD2 expressions (p value 0.001) as shown in Fig. 3 A, C. The OS analysis showed a significant association between high DRD2 expression and mortality in Intensity values (p value 0.025), and also the H-Score parameter (p value 0.001) (Fig. 3 A, D, E).

Next, we designed a multivariable model presented in Table 2, in which we included all the conforming variables that showed significant differences in the univariate analysis in addition to the DRD2 expression values. In the multivariate analysis, the expression of DRD2 in endometrial tumors resulted in an independent significant prognostic marker for predicting OS in our patient cohort (p value 0.043).

3.5. The combined activation of DRD1 and blockage of DRD2 reduced cellular viability in EC tumor cells

Finally, we studied the effect of dopaminergic system neuromodulation in endometrial cancer. We used two representative endometrial cell lines such as HEC1A and AN3CA. The cells were exposed to different concentrations of selective dopaminergic agents alone or in combination evaluating their responses on cellular viability. Prior to that, we confirmed the expression of dopamine receptors in HEC1A and AN3CA cells by RT-PCR. Expression of mRNA levels corresponding to both DR families were detected in HEC1A ($1.2\ 10^{-4}$ and $1.3\ 10^{-5,}$ respectively) and AN3CA cells ($2.1\ 10^{-4}$ and $9.0\ 10^{-6}$, respectively).

The DA exposure at the tested concentration of 100 μM induced a statistically significant reduction of 20% in the number of HEC1A cells, and about a 40% (p value ≤0.001) in AN3CA cells compared to the control without neurotransmitter (Fig. 4 A).
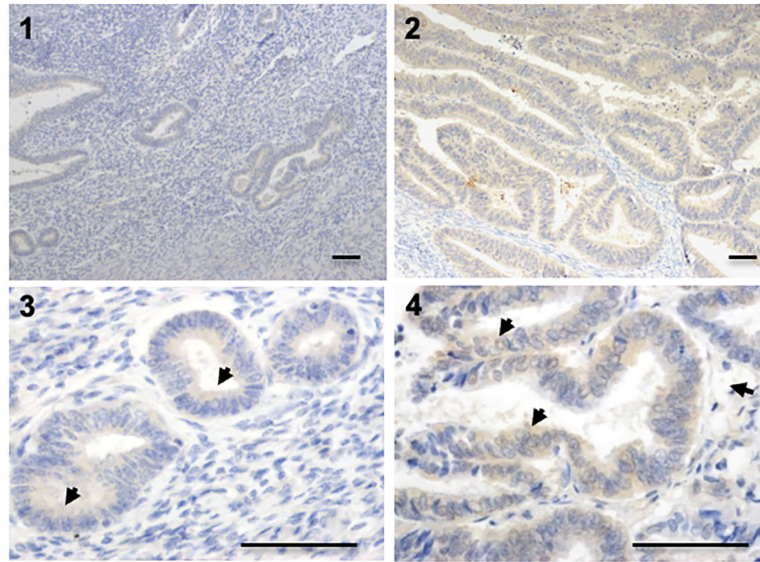
In addition, dopaminergic selective neuromodulation in HEC1A cells showed that FE, an agonist of DRD1-like family of receptors, significantly reduced cell viability (30% p value <0.05) at 100 μM. In contrast, there was no evidence of variation in cellular viability using the agonist for DRD2, PER or the antagonist for DRD1, LE. Similar results were observed using AN3CA cells; the exposure to FE, at a concentration of 100 μM, significantly reduced by 40% (p value ≤0.001) the number of cells with respect to the control and the lowest concentrations tested. In addition, a remarkable variation in HEC1A was observed with the exposure to DOM, a selective antagonist of DRD2-like family, which significantly reduced by 70% the cell viability compared to the control (p value ≤0.001). A similar reduction effect (90%) was observed in AN3CA cells (p value ≤0.001) (Fig. 4 A, B).

To determine the possible combined therapeutic effect of DRD1 agonism and DRD2 antagonism, a separate assay was performed in AN3CA cells, which resulted in the highest responder cells to dopaminergic signaling. The reduction in cell viability induced by DRD1 agonism or DRD2 antagonism in monotherapy, showed a dose-dependent response, being this effect significantly greater at concentrations of 50 μM than at 25 μM, up to 20% vs 1% p < 0.05 with FE and 40% vs 20% (p < 0.05) with DOM, respectively. Moreover, the response was higher after exposure to DOM compared to FE at 50 μM (up to 40% vs 20% p value <0.05).
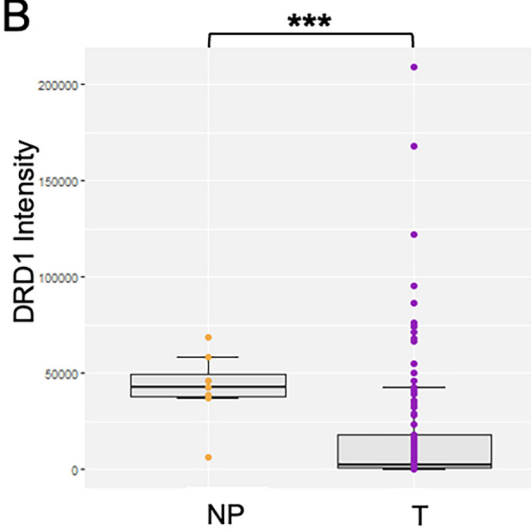
The combined treatment of cells with FE and DOM, assayed at either 25 or 50 μM (reduction up to 40% or 50%, respectively per dose), significantly reduced the number of AN3CA cells in comparison with its effect in monotherapy (p value ≤0.01) (Fig. 4 D).

Moreover, we used the Chou-Talalay method for drug combination that is based on the median-effect principle [28] to describe the dose-effect relationship of the combined treatment. This allow quantitative determination of drug interactions using the CompuSyn software, where Combination index (CI) <1,=1, and > 1 indicates synergistic, additive, and antagonistic effects, respectively. This analysis resulted in a CI value of 1.1 at the 50 % of affected fraction (0.5% Fa) for the
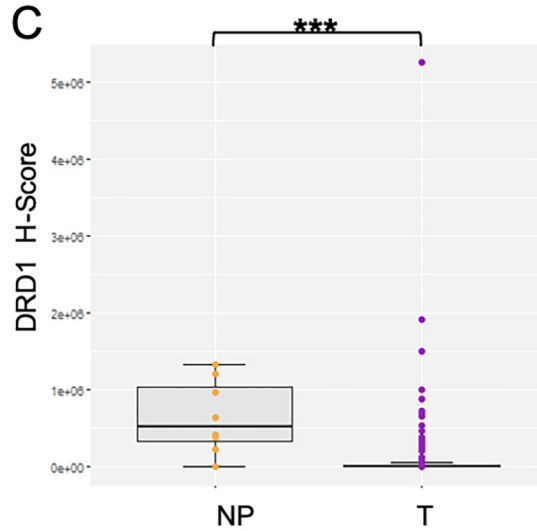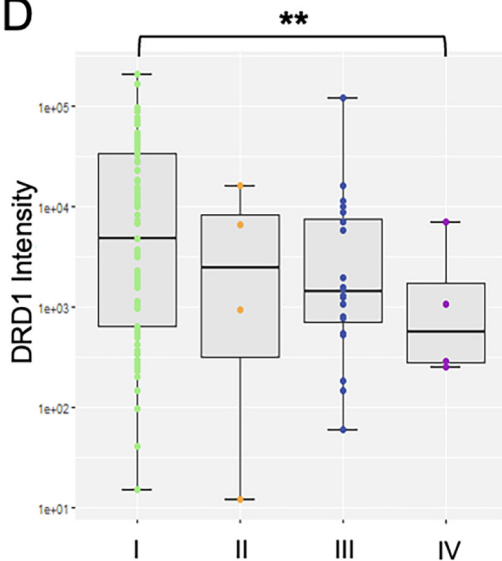
Table 1
Association between the clinicopathological variables and ADRβ2 expression levels.

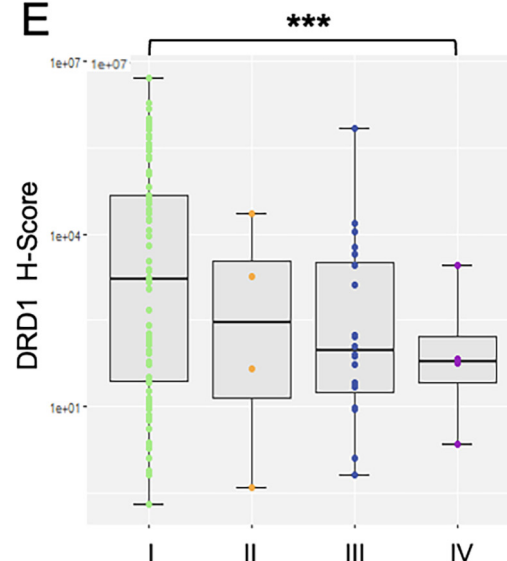| ADRβ2	Intensity			H-Score		
Variables	Median	IQR	p-value	Median	IQR	p-value
Histological type	1835.8	2713.3	0.434	204.6	582.4	0.358
Endometrioid	1596.8	2611.3		88.7	690.6	
Non Endometrioid						
Grade	2012.1	2313.2	0.463	217.1	408.5	0.365
Grade 1–2	1621.0	3212.3		92.3	737.8	
Grade 3						
Tumor size	2505.9	3478.3	0.343	369.9	995.6	0.391
≤2 cm	1645.2	2523.4		107.9	380.4	
>2 cm						
FIGO stage	1730.5	2655.1	0.783	192.1	662.1	0.475
Stage I-II	1638.1	2649.8		93.2	577.9	
Stage III-IV						

IQR: Interquartile range.

Fig. 1. (A) Microscopic representative imaging of moderate [1,3] and high [2,4] DRD1 IHC expression in CE tumors. Bar: 100 μm. Arrows: Cellular pattern of DRD1 expression. (B–C) Values of Intensity (B) and H-Score (C) of DRD1 expression in non-pathological and tumor tissue. (D, E) Intensity (D) and H-Score (E) distribution of DRD1 expression levels in tumors by stage. NP: non-pathological sample. T: tumor sample. (**): $p \leq 0.01$; (***): $p \leq 0.001$.
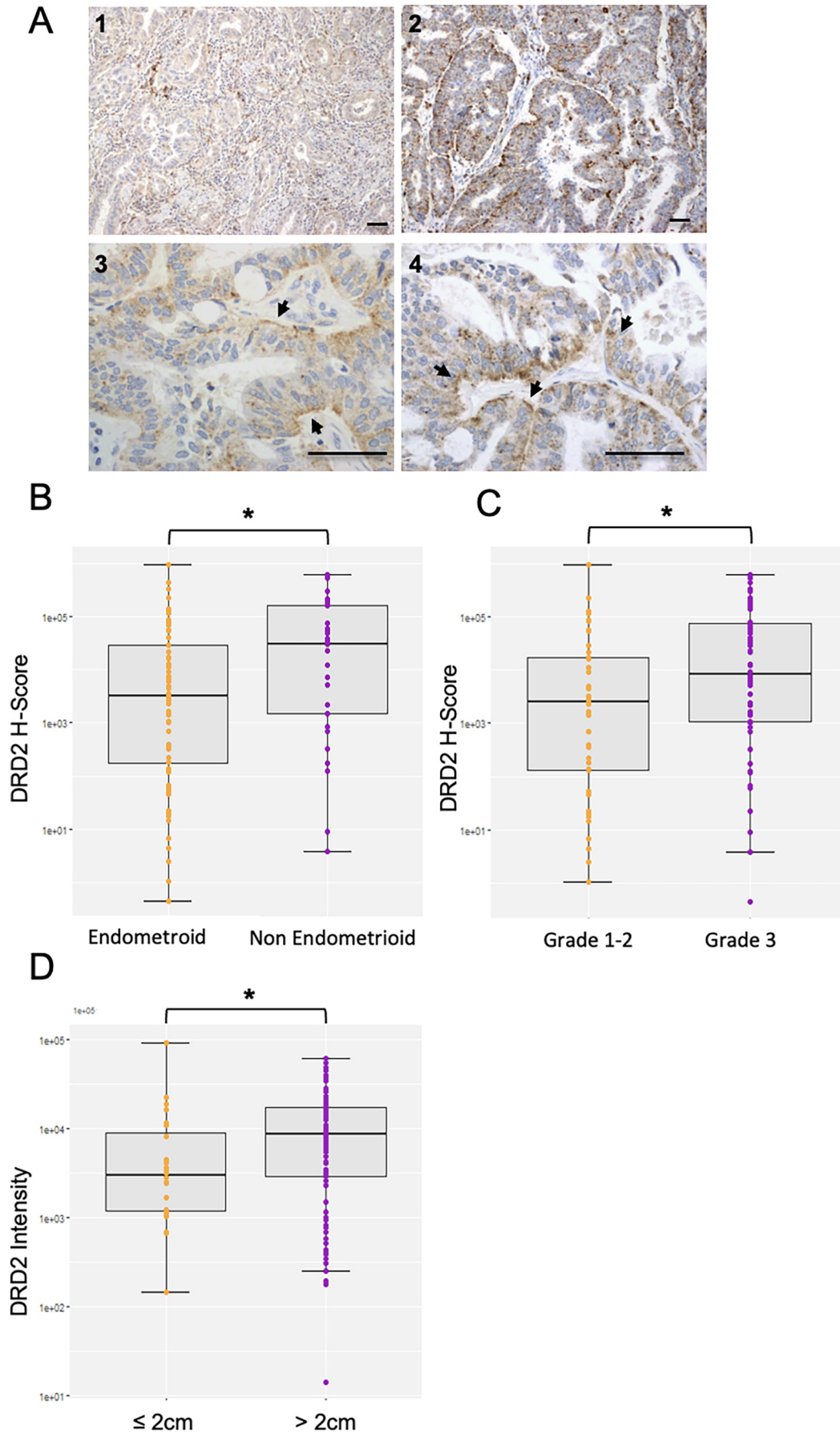
Fig. 2. (A) Microscopic representative imaging of moderate [1,3] and high [2,4] DRD2 IHC expression in CE tumors. Bar: 100 μm. Arrows: Pattern of DRD2 expression. B–D) Graphical association between DRD2 expression and clinicopathological variables, demonstrated statistically significant positive association with histology (B), grade (C) and tumor size (D).
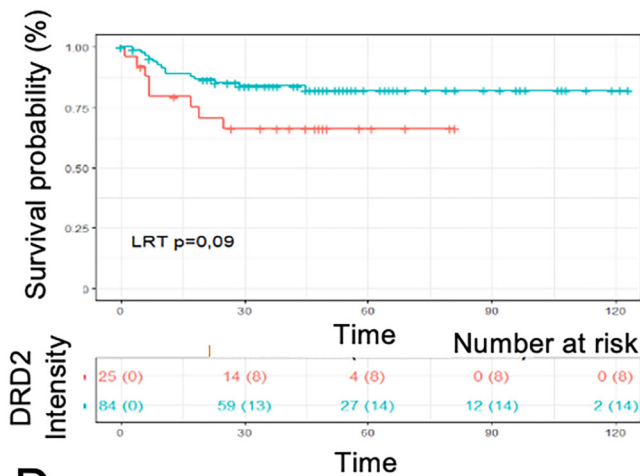
A

Univariate survival analysis for DRD2 expression

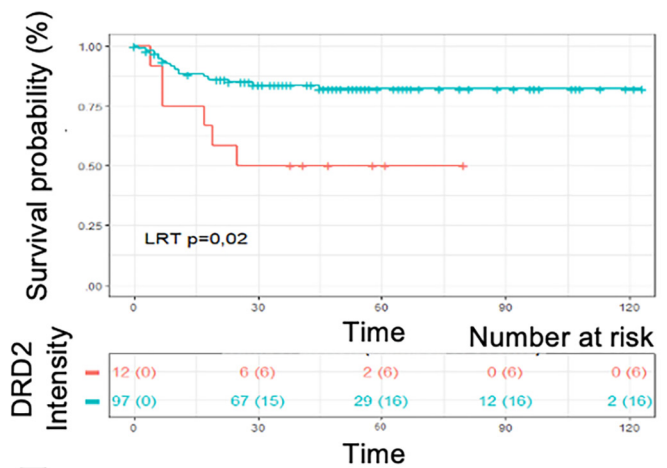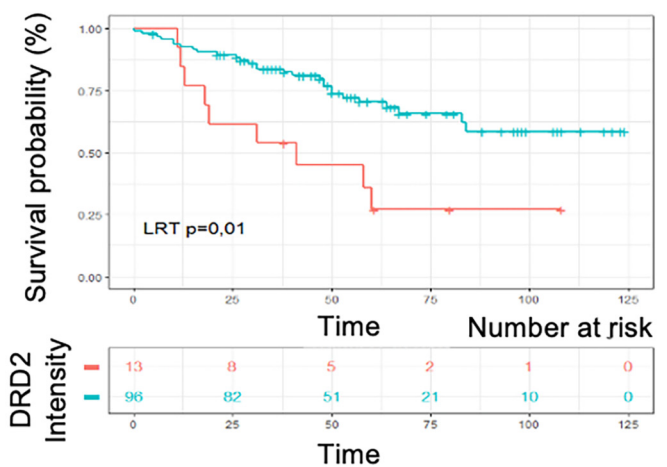|	Disease-free survival			Overall survival		
	Optimal Cut-Point value	HR (IC 95%)	p-value	Optimal Cut-Point value	HR (IC 95%)	p-value
DRD2 Intensity	Low vs High expression	2.21 (0.92 5.28)	0.073	Low vs High expression	2.29 (1.10-4.77)	0.025*
DRD2 H-Score	Low vs High expression	3.47 (1.35-8.88)	0.009*	Low vs High expression	2.98 (1.40-6.34)	0.004*
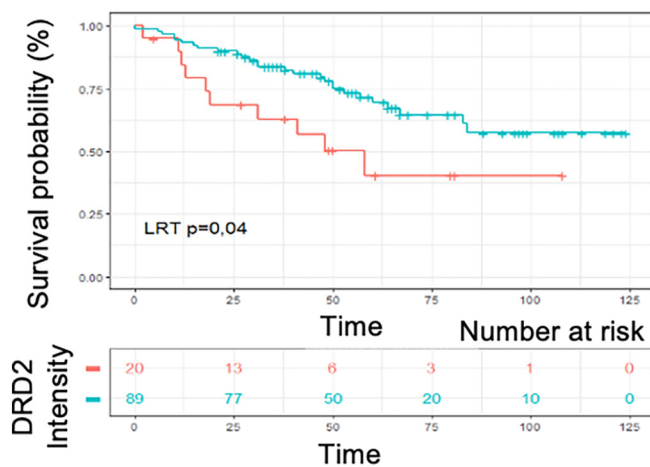
Fig. 3. Survival analysis of the patient's cohort related to DRD2 expression. (A) Univariate analysis of prognostic value for DRD2 immunohistochemical expression in DFS and OS. (B, C) Disease-free survival curves of DRD2 expression quantified by both, Intensity (B) and H-Score (C). (D, E) Overall survival curves of DRD2 expression quantified by both, Intensity (D) and H-Score (E). Red line: high expression, blue line: low expression. P value obtained using the Likelihood ratio test. Abbreviations: DFS, disease free survival; OS, overall survival.

Table 2
Multivariate analysis of the predictors of disease free survival and overall survival in the patient's cohort.

Variables	Disease-free survival		Overall survival	
	HR (IC 95%)	p-value	HR (IC 95%)	p-value
Age	1.29 (0.45–3.65)	0.624	3.21 (1.40–7.33)	0.005*
≤ 73 years old				
>73 years old				
Histological type	2.03 (0.66–6.17)	0.191	1.88 (0.77–4.63)	0.164
Endometrioid				
Non Endometrioid				
Grade	2.00 (0.00–0.00)	0.997	1.34 (0.32–5.55)	0.677
Grade 1–2				
Grade 3				
Tumor size	2.2 (0.88–5.64)	0.086	2.70 (1.20–6.09)	0.016*
≤2 cm				
>2 cm				
FIGO stage	8.70 (2.35–32.19)	0.001*	3.86 (1.06–14.04)	0.040*
Stage I-II				
Stage III-IV				
Relapse	na	na	3.92 (1.75–8.78)	0.000*
DRD2	2.37 (0.80–6.98)	0.114	1.84 (1.1–4.43)	0.043*
Low expression				
High expression				

HR: Hazard ratio, 95%; CI: 95% confidence interval; na: Not applicable.
(*) Statistically significant as indicated.

combination of fenoldopam and domperidone, close to being an additive response than a synergistic effect (Fig. 4 E).

4. Discussion

Endometrial cancer is a heterogenic and highly prevalent gynecological cancer. About 10–20% of affected women are diagnosed in more advanced stages or present recurrences [3]. The aim of this study is to characterize the expression of the adrenergic ADRβ2 and the dopaminergic receptors DRD1 and DRD2 in endometrial tumors, and to evaluate the prognostic value of these catecholaminergic receptors, exploring their potential role as antitumor therapy.

Adrenergic signaling, specifically ADRβ2, has been described as an important key player in the initiation and progression of solid tumors, suggesting a possible prognostic role [13,32–35]. Information for endometrial cancer is limited. In our study, the expression of ADRβ2 does not present an association with the analyzed clinical variables, unlike other tumor types previously cited. Subsequently, its association between DFS and OS was analyzed without obtaining significant data, so we can finally conclude that the ADRβ2 expression does not have a prognostic value in the series of patients evaluated. In terms of treatment, there are multiple β-blocker already validated for other pathologies, which have shown favorable effects on survival even in other gynecological cancers such as ovarian cancer [36]. In a retrospective study of 1964 patients diagnosed with EC, Roque et al., described that the use of β-blockers does not seem to have a protective effect [37].

In recent years, the dopamine system has shown a relevant role in cancer experimentation [18]. Contrary to the adrenergic system, the dopamine system has shown antiproliferative effects in several tumors, mostly mediated by an angiogenesis modification process [17,38]. DA acts differently in each type of tumor [39]. Our study offers a detailed expression pattern of dopaminergic receptors from a cohort of 109 patients affected by endometrial cancer, obtaining relevant prognostic information.

In our data, DRD1 showed a reduced expression in the tumor tissue compared to non-pathologic endometrial tissue. These results are consistent with other tumor types derived from a public database (TCGA) [39]. To our knowledge, this is the first study evaluating the correlation between DRD1 expression levels and the clinical significance of this receptor in EC. The results highlight that tumors larger than 2 cm presented a significantly lower expression of DRD1. In the same direction, the subanalysis carried out on stage classification showed a higher

expression of DRD1 in the first stage (I) of diagnoses, compared with stage IV.

Regarding the association between DRD2 expression and clinicopathological parameters, it showed a different relationship to that observed for DRD1. Pierce et al. [22] previously found that DRD2 expression correlated with clinical variables, and within the endometrial molecular subtypes, it was increased in the high copy number subtype. This last piece of information corroborates the association of DRD2 with more aggressive types of EC. Similarly, in our patient cohort, the obtained results identified and confirmed that DRD2 overexpression was significantly associated with the non-endometrioid type of endometrial cancer, the grade 3 group, and tumor size. In terms of survival, unlike DRD1, DRD2 showed an association with both DFS and OS. In the multivariate analysis, DRD2 was defined as an independent prognostic marker in OS. These results are consistent with the survival study performed by Prabhu et al. [40] in several tumor types, where elevated levels of DRD2 have a relatively lower overall survival, and standing out among them is EC. In the same line, Pierce et al. found that the overexpression of DRD2 had an association with DFS and OS in their 118 cohort patient [22].

Our DRD1 and DRD2 expression analyses in endometrial cancer samples can be considered the first phase of the biomarker pipeline, as recommended in the guidelines from REMARK [41] Subsequently, further verification and validations must be done. A new research line to be explored could be the analysis of DRD2 in serous endometrial intraepithelial carcinoma (SEIC) samples [42].

Similar to the adrenergic system, DR signaling has also been studied as a therapeutic target, with research being mainly focused on the serous-type carcinoma subtype. Experimental and clinical results have recently been published using the ONC201 and ONC206 molecules described as DRD2 antagonists, with promising results when they are administered as monotherapy or associated with other cytotoxic agents such as paclitaxel [22,40,43].

To neuromodulate dopaminergic signaling and evaluate its effect on endometrial tumor cell viability, we used a 2D-model. HEC1A and AN3CA cells are some of the most representative cell lines of endometrial cancer and are widely used in literature [44]. The expression levels of DRD1 and DRD2 have previously been reported [22]; we obtained similar expression results for HEC1A and for AN3CA cells, with AN3CA being the higher dopamine expressing cells.

In a recent review published in 2022 by Grant et al. [18], the authors proposed the mechanisms of action of different compounds targeting
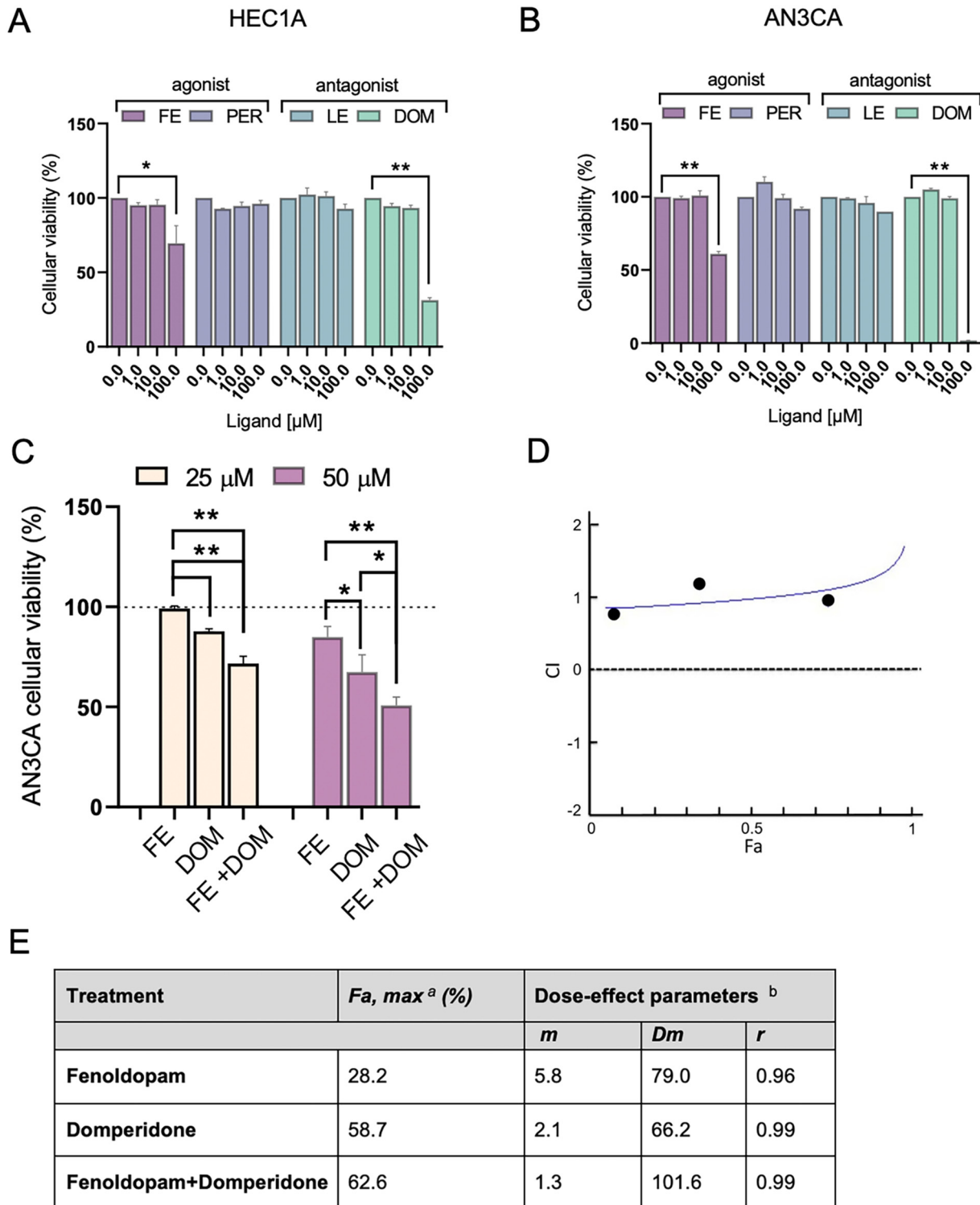
Fig. 4. Effect of dopaminergic neuromodulation in endometrial cells. (A) Quantitation of cellular viability in HEC1A after treatment with selective agonists or antagonists of dopamine D1-like and D2-like receptors. (B) Quantitation of the cellular viability in AN3CA after treatment with selective agonists or antagonists of dopamine D1-like and D2-like receptors. (C) Percentage of the effect on AN3CA cell viability following the treatment of FE (DRD1 agonist) and DOM (D2 antagonist) and their combination in AN3CA cell viability at 25 and 50 uM after 48 h of exposition (D) CI-Fa plot and resulted combination index (CI) values using the median-effect principle by Chou and Talalay [28]. (E) Median-effect parameters showing the dose-effect relationship following the treatments. Abbreviations and notes: FE, fenoldopam; PE, pergolide; LE, LE-300; DOM, domperidone. (*) $p < 0.05$; (**): $p \leq 0.01$; (***): $p \leq 0.001$; [a] *Fa*, max, maximum fraction affected or effect level defined as the change in cellular viability of each treated (T) and control (C) groups, *Fa*, max = (1-T/C)*100; [b] Derived parameters from the median-effect plot: [logFa/(1-Fa)] versus log (Dose), where *m* is the slope, *Dm* is the intercept of the plot and *r* is the linear regression coefficient.

DRD1 and DRD2 in preclinical models and clinical trials of different types of cancer. Although the article does not contain data referring to EC, it seems that cAMP/PI3K/AKT and MAPK/ERK pathways are involved in these effects. The associated complexity between tissue-dependent effects involving multiple targets or receptors, which at the same time are dynamic, requires more experimental studies to describe the role of dopamine modulation applicable in oncology therapy.

Interestingly, some studies have shown that DRD1 agonists or DRD2 antagonists can independently induce an anticancer effect in in vitro and in vivo models [45,46]. In our study, the most notable result using dopaminergic drugs comes from the DRD2 antagonist domperidone, which notably reduced the proliferation in both cell lines. Meanwhile, the DRD1 agonist fenoldopam also independently showed a reduction of cellular viability in HEC1A and AN3CA. The use of the combined DRD1 agonism and the DRD2 antagonism induced a significantly greater reduction in the cellular viability of AN3CA cells, compared to both monotherapies. It should be remarked that the benefit of combined therapy in this case is due to an additive effect. It is described that fenoldopam directly inhibits cell proliferation, reduces AKT/IGF-1 activation in cells tumors and, decisive for angiogenesis, abolishes the growth of endothelial smooth muscle cells induced by IGF- 1 in in vitro and in vivo models [45]. Moreover, domperidone has been also studied as a combination therapy with other agents in the induction of tumor cell death [46].

On the other hand, tumors treated with drugs directed to DRs seem to produce resistance, and secondary, a change in the expression pattern of the receptors [47]. This barrier can be addressed through the application of a combined therapy, that has been described as a tool to increase in drug efficacy [48,49]. And finally, due to the expensive and frequently unsuccessful new drug developments, the repurposing of the dopaminergic agents for cancer therapy could overcome this inconvenience to greatly benefit patients.

5. Conclusions

Information obtained from our study provides new knowledge about the role of dopaminergic receptors in endometrial cancer. In our patient cohort, ADRβ2 expression showed no prognostic value in endometrial cancer, while DRD1 expression was inversely related to tumor size and stage. Moreover, DRD2 was associated with non-endometrioid subtype, high grade tumors, tumor size and worse DFS and OS, becoming an independent significant prognostic marker to predict the overall survival. Combining these results, the DRD1 agonism and DRD2 antagonism could show a possible therapeutic benefit for endometrial cancer patients.

Its application as a prognostic and/or therapeutic tools may well contribute to improving the clinical management of affected patients. Further investigations should allow for a need to evaluate the therapeutic effectiveness of the combination of dopaminergic agents at a preclinical level and to validate the repositioning of these drugs for oncological treatment.

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CRediT authorship contribution statement

Pia Español: Conceptualization, Formal analysis, Writing – original draft, Project administration. **Ramón Rovira:** Supervision, Visualization. **Pablo Caruana:** Methodology, Resources, Investigation. **Rocío Luna-Guibourg:** Investigation, Data curation. **Cristina Soler:** Investigation, Data curation. **Natalia Teixeira:** Investigation, Data curation. **Francisco Rodríguez:** Methodology, Resources. **Maria Edwards:** Writing – review & editing. **Oriol Porta:** Writing – review & editing. **Maria Gámez:** Methodology, Resources, Investigation. **Olga Sánchez:** Validation. **Elisa Llurba:** Supervision, Writing – review & editing. **Jose Luis Corchero:** Funding acquisition, Writing – review & editing. **María Virtudes Céspedes:** Funding acquisition, Conceptualization, Formal analysis, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

Acknowledgments

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