Preclinical evidence of progesterone as a new pharmacological strategy in human adrenocortical carcinoma cell lines

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Abstract

Background and Purpose: Adrenocortical cancer (ACC) is a rare malignant neoplasm with a dismal prognosis. The treatment includes mitotane and EDP chemotherapy (etoposide, doxorubicin, cisplatin). However, new therapeutic approaches for advanced ACC are needed, particularly targeting the metastatic process. Here, we deepen the role of progesterone as a new potential drug useful in ACC, in line with other studies that demonstrated its antitumoral effect in other cancers. Experimental Approach: NCI-H295R, MUC-1, and TVBF-7 ACC cell lines were used. Cell apoptosis and cell cycle were analyzed by flow cytometry. Cell migration and invasiveness were studied using transwell assays, and metalloprotease 2 activity by zymography. Cell xenografts in zebrafish embryos were performed by measuring both the tumor areas and the number of embryos with metastasis. Key Results: Progesterone exerted a long-lasting cytotoxic effect in metastatic cell lines, MUC-1 and TVBF-7. Progesterone caused apoptosis in NCI-H295R and MUC-1 cells, inducing changes in the cell cycle distribution, while autophagy was predominantly activated in TVBF-7 cells. In the zebrafish embryos, progesterone significantly reduced each ACC cell line's xenograft tumor area and metastasis formation in embryos injected with metastatic cells. These results were confirmed in vitro, where the reduction of invasion was mediated, at least in part, by the decrease in MMP2 levels. Conclusion and Implications: Our results give support to the role of progesterone in ACC. The involvement of its analogous (megestrol acetate) in reducing ACC progression in ACC patients undergoing EDP-M therapy is now under investigation in the PESETA phase II clinical study.

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A short-running title

Progesterone as a new pharmacological strategy in adrenocortical carcinoma

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Experimental Approach: NCI-H295R, MUC-1, and TVBF-7 ACC cell lines were used. Cell apoptosis and cell cycle were analyzed by flow cytometry. Cell migration and invasiveness were studied using transwell assays, and metalloprotease 2 activity by zymography. Cell xenografts in zebrafish embryos were performed by measuring both the tumor areas and the number of embryos with metastasis.

Key Results: Progesterone exerted a long-lasting cytotoxic effect in metastatic cell lines, MUC-1 and TVBF-7. Progesterone caused apoptosis in NCI-H295R and MUC-1 cells, inducing changes in the cell cycle distribution, while autophagy was predominantly activated in TVBF-7 cells. In the zebrafish embryos, progesterone significantly reduced each ACC cell line's xenograft tumor area and metastasis formation in embryos injected with metastatic cells. These results were confirmed in vitro, where the reduction of invasion was mediated, at least in part, by the decrease in MMP2 levels.

Conclusion and Implications: Our results give support to the role of progesterone in ACC. The involvement of its analogous (megestrol acetate) in reducing ACC progression in ACC patients undergoing EDP-M therapy is now under investigation in the PESETA phase II clinical study.

Keywords: adrenocortical carcinoma, ACC cell lines, progesterone, zebrafish model, migration, invasion.

Main text

Introduction

AdrenoCortical Carcinoma (ACC) is a rare and aggressive malignancy, with an annual incidence of 0.7-2.0 new patients per million population (Fassnacht et al., 2020). While surgery represents the cornerstone of the treatment of localized ACC, metastatic disease is hard to treat. Standard systemic therapy in the management of metastatic ACC is based on mitotane (M) administered alone (Fassnacht et al., 2020) or in association with the EDP regimen (etoposide, doxorubicin and cisplatin) (Berruti et al., 2005; Fassnacht et al., 2012). Unfortunately, the progression of advanced disease occurs almost invariably after less than 18 months and there are no defined lines of treatment (Fassnacht et al., 2012; Laganà et al., 2020) since neither molecular target agents nor immunotherapy has been shown to obtain substantial clinical benefit (Grisanti et al., 2019; Grisanti et al., 2020). New therapeutic strategies are therefore needed, and research is currently focused on finding new drugs and improving the efficacy of existing ones (Cremaschi et al., 2022).

Progesterone (Pg) is a lipophilic hormone that plays a fundamental role in normal developmental and reproductive functions and disease processes (Rossouw et al., 2002). Despite Pg being dysregulated in different

types of cancer and associated with cancer progression (Lieberman & Curtis, 2017), several studies demonstrated its antitumoral effect by regulating various cancer cell phenotypes, including proliferation, apoptosis, angiogenesis and autophagy, in addition to migration and invasion (Núñez et al., 2016). Pg inhibits the proliferation of breast cancer and osteosarcoma cells (Motamed et al., 2020) and contributes to the decreased progression of colorectal cancer (Lin et al., 2011; Schürmann et al., 2004; Simon et al., 2012) inhibiting cell proliferation (Motylewska & Mełeń-Mucha, 2009). Pg promotes as well apoptosis in endometrial cancer (Kong et al., 2020) and HeLa cells, arresting the progression from the G1 phase to the S phase (Liu et al., 2017) and it could induce autophagy in astrocytes (Hong et al., 2018; Kim et al., 2012; Kim et al., 2013). Moreover, increasing data suggest that Pg inhibits migration and invasion, in breast (Godbole et al., 2017), ovarian (Lima et al., 2020), and endometrial cancer cells, thereby reducing their invasive potential (Waheed et al., 2017).

Despite the knowledge about the effect of Pg in ACC is still limited, data published in the last years by our group demonstrated the role of this hormone as an antitumoral drug. Indeed, by investigating the therapeutic use of abiraterone acetate in preclinical models of ACC, our group demonstrated that its antiproliferative effect is due to the increased production of Pg. This hormone, through its receptors (PgRs), was able to reduce cell viability in NCI-H295R cells and primary secreting ACC cultures, in a concentration-dependent manner (Fiorentini et al., 2016), involving both genomic and non-genomic pathways (Fragni et al., 2019). In addition, we showed that the reduction of β-catenin nuclear translocation may contribute to the Pg cytotoxic effect and that Pg combined with other drugs such as mitotane (Fragni et al., 2019) or the CDK4/6 inhibitor ribociclib (Abate et al., 2022) enhances their antineoplastic activity. More recently, we showed that the metastasis-derived cell models, namely MUC-1 and TVBF-7 cells (formerly ACC115m primary cells) were less sensitive to the Pg effect, due to weaker PgR expression compared to NCI-H295R cells, strengthening the role of PgR in mediating the effect of Pg in reducing both cell proliferation and cell viability (Rossini et al., 2021b).

Here, we deepened the molecular mechanism underlying the cytotoxic effect of Pg, with a particular interest in metastatic cell lines. Moreover, we investigated whether Pg could influence ACC cell invasiveness and metastasis formation in both *in vitro* and*in vivo* models. This study aims to provide preclinical evidence for an association between Pg treatment and recurrence reduction in ACC patients.

Material and methods

Cell Lines. The human NCI-H295R cell line, derived from a primary ACC in a female patient (Rainey et al., 2004), was obtained from the American Type Culture Collection (RRID:CVCL_0458) and cultured as indicated. MUC-1 cell line, established from a neck metastasis of an EDP-M-treated male patient, was kindly given by Dr. Hantel and cultured as suggested (Hantel et al., 2016). Additionally, the new ACC cell line TVBF-7 (Sigala et al. 2022 a) was established from a primary culture derived from a perirenal lymph-node metastasis of a male ACC patient who underwent progression after EDP-M. A detailed description of these three cell lines can be found in Sigala et al. (Sigala et al. 2022 b). All three cell lines were periodically tested for mycoplasmas and authenticated by genetic profiling using polymorphic short tandem repeat loci with the PowerPlex Fusion system (Promega, BMR Genomics Cell Profile service).

Cell Treatments. Cells were treated for 4 days using their respective Pg IC_{50} values (H295R: 25 μM; MUC-1: 67,58 μM; TVBF-7: 51,56 μM) (Fragni et al., 2019; Rossini et al., 2021b). Pg (Merk) was dissolved in DMSO in a stock solution of 100mM, aliquoted and stored at -20°C. All treatments were conducted in charcoal-dextran-treated serum.

Measurement of cell apoptosis . Pacific BlueTM Annexin V/ SYOXTM AADVancedTM apoptosis kit (Invitrogen) was used to investigate Pg-induced cell death. ACC cells (5 x 10^5 cells/well) were seeded in a 6-well plate in a complete medium, 24 hours (h) later cells were treated for 24, 48, 72, or 96h using their Pg IC_{50} values. Cells were collected, washed with ice-cold PBS, resuspended in the binding buffer, and stained with Pacific BlueTM Annexin V/SYOXTM AADVancedTM, according to the manufacturer's instructions. Cells were then analyzed using a MACSQuant10 cytometer (Miltenyi), using unlabeled cells as a negative

control. Quantification of apoptosis was determined by FlowJo v10.6.2 software.

Cell cycle analysis . Flow cytometric cell cycle analysis was performed as described (Rossini et al., 2021 a), with minor modifications. Briefly, untreated and Pg-treated cells were fixed, treated with Rnase A (12.5 μg/mL) (Thermo Fisher Scientific), stained with propidium iodide (40 μg/mL) (Invitrogen), and analyzed by flow cytometry using a MACS Quant Analyzer (Miltenyi Biotec GmbH) for cell cycle status. Data were analyzed using FlowJo software (TreeStar).

Drug withdrawal experiments. ACC cells were plated in 24-well plates and treated with their IC_{50} value of Pg for 4 days. At the end of the treatment, the drug-containing medium was replaced by a fresh complete medium without the drug and cell viability was evaluated at different times, up to 10 days (about twice the doubling time). Cells were analyzed for cell viability by 3-(4,5-Dimethyl-2- thiazol)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay according to the manufacturer protocol (Merck).

Fish and Embryos Maintenance. Zebrafish were maintained and used according to EU Directive 2010/63/EU for animal use following protocols approved by the local committee (OPBA) and authorized by the Ministry of Health (Authorization Number 393/2017). Adult transgenic line Tg (kdrl:EGFP) and wild-type zebrafish lines were maintained as described in (Basnet et al., 2021). Breeding of adult male and female zebrafish was carried out by natural crosses, and embryos were collected and raised in fish water with incubation at 28.5 °C until the experiments. Embryos at 24h post-fertilization (hpf) were treated with 0.003% 1-phenyl-2-thiourea (PTU) to prevent pigmentation. After the conclusion of the experiments, the zebrafish embryos were euthanized with 400 mg/L tricaine (ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich, Milan, Italy)

Tumor Xenograft. To evaluate the toxic effect of Pg on the zebrafish model, 48 hpf wild-type embryos (AB) were divided into different groups as indicated and maintained in PTU/fish water to which solvent (DMSO) or increasing concentrations of Pg (10, 25, 50, and 100 μM) were added. After 3 days (T3), effects of Pg were observed. To evaluate the effect of Pg on tumor growth, Tg (kdrl:EGFP) zebrafish embryos at 48 hpf were dechorionated, anesthetized with 0.042 mg/mL tricaine, and microinjected with the labeled tumor NCI-H295R, MUC-1, and TVBF-7 cells (CellTrackerTM CM-Dil Dye, Thermo Fisher Scientific) into the subperidermal space of the yolk sac. Microinjections were performed with a FemtoJet electronic microinjector coupled with an InjectMan N12 manipulator (Eppendorf Italia, Milan, Italy). Approximately 250 cells/4 nL were injected into each embryo (about 25 embryos/group); embryos were maintained in PTU/fish water in a 32°C incubator to allow tumor cell growth. Pictures of injected embryos were acquired using Zeiss Axiozoom V13 (Zeiss, Jena, Germany) fluorescence microscope, equipped with Zen pro software, 2h after cell injection (T0). 6.25, 12.5 μM Pg or solvent (DMSO) were directly added to the PTU/fish water in respectively Pg-treated and untreated experimental groups. After 3 days (T3), the effects of the drug on cancer growth were scored by taking pictures, as previously described, to measure the tumor areas of each group at T0 and T3 using Zen 2.3 Black software from ZEISS. Embryos with metastases (that is the presence of at least one fluorescence dot outside the site of injection) were counted and some representative xenografted embryos were fixed, embedded in low melting agarose, and acquired with LSM 510 confocal laser microscope equipped with Achropla 10x/0.25 objective.

Migration and invasion assay. The motility and invasive capability of untreated and Pg-treated ACC cells were explored using the Transwell assays. For migration assay, QCMTM Chemotaxis 24-Well Cell Migration Assay with an 8 μm pore size (Merk) was used. The in vitro invasiveness of cells was evaluated using ECMatrix Cell Invasion Assay with an 8 μm pore size (Merck). Untreated or treated cells were added into the upper chamber of migration or invasion inserts according to manufacturers' protocols. The chemo-attractant gradient was created using a medium enriched with 10% of fetal bovine serum. After incubation in a humidified tissue culture incubator for 22h (for migration assay) or 72h (for invasion assay), the cells in the interior of the chamber that did not penetrate the membrane have been cleaned up. Penetrated cells were stained and after washing, inserts were air-dried. Images were acquired using an Olympus IX51 optical microscope (Olympus) equipped with a 10x objective. Subsequently, staining was eluted, and absorbance was detected using an EnSight Multimode Plate Reader (PerkinElmer) at 560nm.

Wound healing assay. To evaluate the wound healing assay, cells were cultured in a 6 wells plate until reaching 90%–100% confluency at which point a scratch was created using a p200 pipette tip. Media was removed and cells were washed in PBS before adding the medium conditioned with vehicle or Pg. Five different areas along the scratches of each well were analyzed by optical microscopy after 0 and 24h following the induced damage. The distance between each edge of the scratch was monitored under an Olympus IX51 microscope and was measured using the software NIH ImageJ Software.

Western Blot. Cells were homogenized in cold RIPA buffer, and total protein concentrations were determined by Bio-Rad Protein Assay (Bio-Rad Laboratories). Equal amounts of proteins (30 μg) were run in 10% polyacrylamide gels and transferred onto a polyvinylidene fluoride (PVDF) membrane. Anti-LC3 primary antibody (Sigma-Aldrich Cat# L8918, RRID: AB_1079382) and secondary HRP-labeled anti-rabbit antibody (Promega Cat# W4011, RRID: AB_430833) were used. Densitometric analysis of the bands was performed by using NIH ImageJ Software. To evaluate secreted MMP2 levels, the conditioned medium was obtained as described (Fiorentini et al., 2014). Briefly, equal amounts of untreated and Pg-treated cells were seeds in a free-serum medium (1x 106 cells/ 1 ml medium). After 2h, conditioned media were collected, stored at -80°C for at least 24h, and subsequently freeze-dried. The residues were resuspended in the same volume of PBS. Next, proteins were separated by electrophoresis on a 4–12% NuPAGEbis-tris gel system (Life Technologies, Italy), electroblotted to a PVDF membrane, and finally detected using an anti-MMP2 primary antibody (Proteintech Cat# 10373-2-AP, RRID: AB_2250823).

Zymography. Novex 10% Zymogram Plus (Gelatin) gels (Thermo Fisher Scientific) were used to measure secreted MMP2 activity in conditioned media. Different aliquots of the same conditioned medium samples were used for both western blot and zymography. The proteases were run under denaturing conditions and visualized as clear bands against a dark background using a renaturing, developing, and staining protocol (Thermo Fisher Scientific).

Gene expression.

Preliminary experiments to evaluate the gene expression of different metalloproteases (MMPs) in NCI-H295R, MUC-1 and TVBF-7 cell lines were performed using the Human Tumor Metastasis RT2 Profiler PCR Array (Qiagen). The RNA isolation, the treatment with DNase, and the RNA purification were performed using the Qiagen kit (Qiagen). For cDNA synthesis, the RT2 First Strand Kit (Qiagen) was used. The PCR was performed with VIIA7 (Applied Biosystems) using RT2 SYBR Green qPCR Mastermix (Qiagen) as fluorochrome.

For the other gene expression analysis total RNA was extracted from cells using the RNeasy kit (Qiagen) and 1 μg was transcribed into cDNA, using murine leukemia virus reverse transcriptase (Promega). Gene expression was evaluated by q-RT-PCR (ViiA7, Applied Biosystems) using SYBR Green as fluorochrome. Specific primers for MMP2, TIMP1 and TIMP2 were as follows: MMP2 (F: 5'-ACGACCGCGACAAGAAGTAT -3' and R: 5'- ATTTGTTGCCCAGGAAAGTG -3'), TIMP1 (F: 5'- GGGACACCAGAAGTCAACCA -3' and R: 5'- GGCTTGGAACCCTTTATACATC -3'), and TIMP2 (F: 5'- AAGCGGTCAGTGAGAAGGAA-3' and R: 5'- TCTCAGGCCCTTTGAACATC-3'). Expression levels were normalized to the β-actin mRNA level of each sample, obtained from parallel assays.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism software (version 5.02, GraphPad Software, La Jolla, CA, USA). One-way ANOVA with Bonferroni's correction was used for multiple comparisons. Where appropriate, the unpaired t-test was used. Unless otherwise specified, data are expressed as mean ± Standard Error of mean (S.E.M.) of at least three experiments run in triplicate. P values < 0.05 were considered statistically significant.

Results

Pg exerted cytotoxic effects on ACC cells through autophagy-related apoptosis. Results obtained with the acridine orange staining suggest that apoptosis could be induced by Pg in NCI-H295R cells (Fragni et al., 2019). Here, we conducted a time course of Pg exposure (24, 48, 72, or 96h) at its IC_{50}, using Annexin

V/PI staining to detect apoptotic cell death induced by Pg in the experimental ACC cell models used in the present study. Figure 1 and Table 1 show that a significant increase in apoptotic cells emerged, depending on the cell model examined. In NCI-H295R cells (Figure 1A and Table 1), exposure to Pg confirmed a significant increase in apoptotic cells after 48h of treatment, confirming published results obtained with the acridine orange staining (Fragni et al., 2019). In MUC-1 cells (Figure 1B and Table 1), Pg treatment induced a significant decrease in viable cells and a significant increase in apoptotic cell ratio after 72 and 96h of treatment (72 h viable cells: untreated: 96.13% ± 0.72%, Pg-treated cells: 79.25% ± 5.39%); (96h viable cells: untreated: 92.91% ± 1.11%, Pg-treated: 78.58% ± 0.48%). In MUC-1 cells, no significant increase in necrotic cells was observed. In the TVBF-7 cell line (Figure 1C and Table 1), no significant differences were observed, except for an increased percentage of viable cells after 96h of treatment (untreated viable cells: 83.69% ± 3.85%, Pg-treated viable cells: 95.90% ± 1.54%). To elucidate the death mechanism underlying the cytotoxic effect of Pg on TVBF-7 cells, we analyzed the expression of microtubule-associated protein light chain 3 (LC3) with particular attention to LC3-II, the maturation form of LC3-I, which can be used as a biomarker of autophagosome formation. Figure 2C shows that Pg induced autophagy on TVBF-7 cells with a significant increase in both LC3-I and LC3-II levels in a time-dependent manner. In NCI-H295R (Figure 2A) and MUC-1 (Figure 2B) cell lines, where apoptosis cell death was observed, a slight rise in LC3-I and LC3-II levels can be appreciated, although not significant and with a trend tending to decline. These observations confirm that, in a complex interplay between autophagy and apoptosis pathways, apoptosis is promoted when autophagy is inhibited (González-Polo et al., 2005). Taken together these results suggest that autophagy and apoptosis occur hierarchically or independently to contribute to Pg-induced ACC cell death.

Pg-induced changes in the cell cycle distribution. To evaluate the effect of Pg administration on the cell cycle profile, the cell cycle distribution in ACC cell lines after Pg treatment for 72h and 96h was studied. Figure 3 A-B showed that, in NCI-H295R cells, treatment with Pg increased the proportion of cells in the G1 phase both after 72h and 96h. (72h: +10.65 ± 4.1%, 96h: + 3.08 ± 0.05%). An increase in the G2 phase fraction was observed in MUC-1 cells at the same time points (72h: +11.1 ± 0.05%, 96h: +8.18 ± 1.42%) and a decrease of cells in the S phase after 96h of treatment (-7.2 ± 1.02%). No changes in the cell cycle distribution were observed in Pg-treated TVBF-7 cells compared to controls.

Progesterone exerted a cytotoxic effect that was maintained after the drug is withdrawn. To evaluate whether the cytotoxic insult of Pg was permanent after the suspension of the treatment, cells were treated with the respective IC_{50} values for 4 days and thereafter kept in culture for up to 10 days in a complete, drug-free medium. Cell viability was evaluated at the end of treatment and at different times after discontinuation, as shown in Figure 4. The effect of Pg on NCI-H295R cells lasted up to 2 days after withdrawal. Cells then recover from the cytotoxic insult and the cell viability after 10 days of withdrawal was similar to untreated cells (Figure 4A). In MUC-1 cells, Pg induced cell damage, leading to cell death, with an effect that was maintained after drug discontinuation (Figure 4B). Interestingly, cell viability significantly continued to decrease up to 10 days after drug withdrawal (-83.2 +- 1.5 % compared to untreated cells). Figure 4C shows the effect of the drug withdrawn in TVBF-7 cells. Even if the cells seemed to slowly recover, the reduction of the cell viability remained significant (- 39.4 +- 2.07 % compared to untreated cells).

Pg inhibited tumor growth and metastasis formation in the zebrafish/ tumor xenograft model. The Pg cytotoxic effect observed in vitro was then evaluated *in vivo* , through ACC cell xenografted in kdrl-GFP zebrafish embryos. Preliminary experiments were conducted to evaluate the Pg toxicity on wild-type (AB) strain zebrafish embryos. Doses higher than 50 µM caused 100% mortality. Exposure to 25 µM Pg was not lethal, but nearly 50% of embryos developed pericardial edema and yolk sac edema (Supplementary Figure 1). while 10 µM Pg was a safe dose. Based on these results, the concentrations of 6.25 µM and 12,5 µM were chosen to evaluate the effects of Pg on ACC cell growth in the xenografted zebrafish model. Figure 5 (A-B) shows results obtained by exposing embryos to 6.25 µM Pg and demonstrates that Pg was able to significantly reduce the tumor mass in each cell line (NCI-H295R: -30.15% ± 7.22%, $p<0.05$; MUC-1: -41.5% ±10.47%; TVBF-7: -34.03% ±4.28%). When embryos were treated with 12.5 µM, we observed a high mortality rate in embryos xenografted with MUC-1 cell line, that did not allow us to calculate the Pg effect

on xenograft, while a significant reduction of tumor area in those injected with NCI-H295R (-46.48% ± 4.82%) and TVBF-7 (-37.53% ±2.15%) cell lines (Supplementary Figure 2). According to their metastatic origin, MUC-1 and TVBF-7 cells were found to be able to metastasize, though with some differences in terms of rate and metastasis localization (Figure 6). No metastasis was observed in embryos xenografted with NCI-H295R cells, in line with their origin from a primary ACC. Pg was able to reduce the rate of embryos with MUC-1 cells migrating to the caudal region from 62,5 ± 9,6% to 10,8 ± 0,85% ($p<0.05$). TVBF-7 cells were able to form metastasis at a lower rate, compared with MUC-1 cells (metastasis-positive embryos:12.07% ± 7.31%), and metastasis was mostly localized in the pericardial zone. No embryos with metastasis were found in the Pg-treated group. Collectively, these data indicated that Pg suppressed ACC cell growth and metastasis formation in *in vivo* model.

Pg suppressed the migration and invasion ability of ACC cells. To confirm and deepen the results obtained in the zebrafish model, we decided to further investigate the motility of ACC cells using *in vitro* approaches such as the transwell assay and wound healing assay. Figures 7 shows the effect of Pg on the invasion and migration ability of each ACC cell line with some representative images. Results obtained*in vitro* confirmed those obtained *in vivo* . MUC-1 cells displayed a high invasive capability, confirming results obtained in the zebrafish models. The invasion and migration ability of MUC-1 cells were strongly reduced by Pg (-49.42 ± 5,34% invasive cells, $p < 0.005$; -42.76 ± 1.17% migrated cells; cell separation distance: 421.7 µm ± 16.64 µm in Pg-treated cells vs 303.1 µm ± 18.61 µm in untreated cells). A similar anti-metastatic effect of Pg was observed in TVBF-7 cells, confirming also in vitro results obtained in vivo, showing a low invasion ability (-36.85% ± 7,83% invasive cells; -62.29% ± 16.28% migrated cells). The transwell migration assay revealed an even lower ability to migrate compared to the ability to invade. The purely technical reason may lie in the less incubation time used for migration assay (22h for migration assay and 72h for invasion assay, as indicated in the manufacturer's protocols). On the other side, the wounding assay proved that TVBF-7 cells could migrate and that Pg hindered the edges reconnection (cell separation distance: 606.7 µm ± 9.14 µm in Pg-treated cells vs 480.9 µm ± 14.13 µm in untreated cells).

In line with the previous observations, NCI-H295R showed a limited ability to migrate or to invade that was not significantly modified by Pg treatment.

Pg interfered with metalloprotease 2 (MMP-2) activity. The activity of metalloproteases (MMP) is crucial for the cell invasion process (Cabral-Pacheco et al., 2020). Among these, ACC tissues specifically express high levels of MMP-2 compared to normal adrenal tissues, representing an unfavorable prognostic factor (Volante et al., 2006). Our preliminary qRT-PCR analysis on MMPs expression (Supplementary Table 1) confirmed that on ACC cell lines the most expressed is MMP2, even if at different levels between NCI-H295R and the metastatic cell lines (Supplementary Table 1). The effect of Pg on MMP2 expression and secretion was thus studied. No significant differences were observed in MMP2 gene expression as well as in its inhibitors (TIMP1 and TIMP2) after treatment in each ACC cell line (Supplementary Table 2). Western blot analysis performed on conditioned media revealed that Pg significantly reduced the secreted MMP2 levels compared to untreated cells in metastatic cell lines (Figure 8A). Figure 8B shows the zymography results for the activity of MMP-2 equally impaired by Pg treatment (Pg-treated MUC-1 cells -27.82 ± 1.17% vs untreated; Pg-treated TVBF-7 cells -24.19 ± 4.43%). No significant differences were observed in MMP-2 levels and activity after treatment on NCI-H295R cells. The low MMP2 levels observed in NCI-H295R cells compared with metastatic cell lines, are in line with their primary origin and lack of capability to invade both *in vitro* and *in vivo* models. These results strengthen the involvement of MMP-2 in the progression and invasiveness of ACC.

Discussion

Identifying new molecular pathways druggable in the pharmacological armamentarium continues to be a major challenge in ACC therapy. PgRs are expressed at different intensities in both normal and neoplastic adrenal gland (de Cremoux et al., 2008; Rossini et al., 2021b) and mediate the cytotoxic effect of Pg in ACC cell models (Fragni et al., 2019; Rossini et al. 2021b), suggesting the possibility to evaluate another pharmacological tool over the usual systemic therapy. Indeed, our previously published results indicate that

Pg is effective in inducing a cytotoxic effect in several cell models of ACC, although at lower potency in metastatic ACC cell lines compared to the NCI-H295R cell line. This effect seems to be strictly related to the level of PgR expression since the lower potency could be due to the lower PgR expression in metastatic lines compared to H295R cells (Rossini et al., 2021 b). Thus, the evaluation of the PgR expression during the pathological staging could be of interest to stratify the patients in a view to personalized medicine.

In this study, we investigated the mechanism of action by which Pg, through its receptors, induces the cytotoxic effect, and whether this effect was maintained over time.

We found that Pg induced G1-phase arrest in the cell cycle only in the NCI-H295R cell line, and G2-phase arrest in MUC-1 cells, culminating in apoptotic cell death. While the TVBF-7 cell line did not show any inhibition and, although a cytotoxic effect was present, no increase in apoptotic or necrotic cells was observed. This discrepancy may be due to the different regulatory mechanisms of cell growth inhibition in specific ACC cell types that give support to the well know heterogeneity of ACC (Bothou et al., 2021). The mechanism underlying the cytotoxic effect of Pg on TVBF-7, seemed to be linked to an increase in LC3-II levels, representing the activation of autophagy. The role of autophagy in the ACC context is challenging because it plays a double-edged role as a survival response to chemotherapeutic drugs, resulting in treatment failure, but also as an important mechanism underlying tumor cell suicide (Huang et al., 2019; Sousa et al., 2022; Tompkins et al., 2019). The cytoprotective function of autophagy is activated in many circumstances by suppressing apoptosis and this evidence justifies the observation of an increase in the cell viability in TVBF-7 cells exposed to Pg. However, we would like to underline that interminable autophagy has been shown to enhance anticancer drug-induced cell death (Yang et al., 2011). Remarkably, this study demonstrated that Pg triggered cytotoxicity due to autophagy or apoptosis depending on the ACC cell type. Accordingly, Pg is able to induce both apoptosis (Atif et al., 2015; Syed & Ho, 2003) or autophagy (Hong et al., 2018; Kim et al., 2012; Kim et al., 2013) depending on cellular contexts; accordingly, it was already known that Pg induces cell cycle modifications in cell lines derived from human tumors, with sometimes opposite results that depend on the tumor origin (Fedotcheva et al., 2021; Saha et al., 2021). Further studies are needed to deepen the molecular features underlying this difference.

Drug withdrawal experiments have equally shown peculiar results in the different ACC cell models. NCI-H295R cells were able to quickly recover after cytotoxic insult and restart to proliferate, while metastatic cell models, MUC-1 and TVBF-7 cells, kept a low proliferation rate, evident especially in MUC-1 cells. This in vitro result is especially relevant considering the possible clinical application of this treatment, in particular in patients with advanced ACC, in whom maintaining a cytotoxic effect with low proliferative rate cells, would represent a very important aspect.

Moreover, the *in vitro* cytotoxic effect of Pg was strengthened by results obtained in the *in vivo* model of ACC cell lines xenografted in zebrafish embryos. This result is of particular interest, as the Pg concentration that induces a significant reduction in the tumor mass area of each ACC cell line is remarkably lower compared to the *in vitro* effective concentration. Besides being a useful tool for *in vivo* first drug screening (Letrado et al., 2018), this animal model allowed us to demonstrate that Pg inhibits metastasis formation in zebrafish embryos xenografted with metastatic cell lines such as MUC-1 and TVBF-7. As the metastatic process is associated with collective cell migration and invasion, which are common phenotypes in epithelial cancers, we deepened this aspect. First, using different in vitro assays, we demonstrated that Pg reduces migration and invasion in ACC cell lines in line with previous reports demonstrating that Pg inhibits cell invasion and migration in other tumors (Godbole et al., 2017; Lima et al., 2020). Next, we investigate the molecular mechanism underlying the anti-metastatic effect of Pg, by studying whether this effect involved MMP-2.

Indeed, the dissemination of malignant neoplasms is assumed to require the degradation of different components of the matrix and basement membrane. MMPs are responsible for the degradation of several extracellular matrix (ECM) components. There are over 20 recognized MMPs, each with specific substrate requirements and structural domains. Among these, MMP-2 and MMP-9 are two highly associated with tumor dissemination and invasiveness (Webb et al., 2017). Among these, ACC tissues specifically express MMP-2 (as compared also to normal adrenal tissue), and the elevated MMP-2 expression in ACC is an

unfavorable prognostic factor (Volante et al., 2006). Based on this, we found that Pg impairs the secretion and the activity of MMP2, suggesting that it could mediate at least in part the effect of Pg in suppressing the invasion of metastatic ACC cells. Despite this, Pg has not influenced the gene expression of MMP2 and its inhibitors TIMP1 and TIMP2, letting us speculate that it may act at different levels on the process of MMP2 maturation and secretion. This mechanism is worth being deepened with future studies.

In conclusion, our results fit well above the published evidence regarding the role of Pg G in ACC. It is worth underlining that the Pg analog megestrol acetate is already part of cancer supportive care, thus allowing having another pharmacological tool with a well-known risk/benefit profile over the usual systemic therapy in reducing ACC progression in patients undergoing EDP-M therapy. This hypothesis is now under study in the ongoing randomized phase II clinical trial PESETA (EudraCT Number: 2020-004530-38).

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Tables

Table 1. Percentage of apoptotic cells observed in the Pg-treatment time-course in ACC cell lines

	Apoptotic cells	**Apoptotic cells**	**Apoptotic cells**	**Apoptotic cells**	**Apoptotic cells**	**Apoptotic cells**
	NCI-H295R	**NCI-H295R**	**MUC-1**	**MUC-1**	**TVBF-7**	**TVBF-7**
Pg	**-**	+	**-**	+	**-**	+
24h	14.66 ± 4.44	23.45 ± 1.52	2.16 ± 0.45	5.43 ± 1.19	16.07 ± 2.73	12.95 ± 1.18
48h	11.28 ± 4.76	**27.95 ± 3.02 §**	1.90 ± 0.14	3.68 ± 1.09	4.63 ± 1.14	3.73 ± 1.58
72h	17.98 ± 1.93	23.23 ± 0.13	3.42 ± 0.86	**15.47 ± 2.21 ***	8.07 ± 1.15	7.50 ± 2.48
96h	16.72 ± 2.84	22.57 ± 1.83	6.81 ± 1.13	**21.08 ± 0.64 ***	10.64 ± 3.55	3.51 ± 1.24

§ $p<0.05$ vs untreated cells; * $p < 0.001$ vs untreated cells

Figure legends

Figure 1 . Pg promoted apoptotic cell death in NCI-H295R (A) and MUC-1 (B) cell lines but not in TVBF-7 cells (C). Cells were treated for 24, 48, 72, or 96h using their Pg IC50 values, stained with Pacific BlueTM Annexin V, and analyzed by flow cytometry. Histograms representative of the mean ± SEM of three experiments were shown. *$p < 0.0001$, # $p < 0.001$ cells, §$p < 0.05$ vs untreated cells.

Figure 2. Pg triggered autophagy cell death in TVBF-7 cell lines. Representative Western Blot of LC3-I and LC3-II proteins levels in NCI-H295R (A), MUC-1 (B) and TVBF-7 (C) are shown. On the right side, quantification results are presented as a relative optical density means ± SEM of three independent experiments. *$p < 0.0001$, # $p < 0.001$, §$p < 0.05$ vs untreated cells.

Figure 3 . Pg-induced changes in the cell cycle distribution of ACC cell lines. Cells were treated for 72 h (A) and 96h (B) with respective IC_{50} values of Pg, stained with propidium iodide, and analyzed for DNA content by flow cytometry. Histograms representative of the mean ± SEM of three experiments were shown. # $p < 0.001$, §$p < 0.05$ vs untreated cells.

Figure 4. Effect of drug withdrawal on NCI-H295R (A), MUC-1 (B), and TVBF-7 (C) ACC cell lines. Cells were treated for 4 days with respective IC_{50} values of Pg, then the drug was withdrawn from the medium and cells were kept in culture for up to 10 days. Cell viability time course was measured by the MTT assay. Points represent the mean ± SEM of at least three experiments performed in triplicate. *$p < 0.0001$, # $p < 0.001$, §$p < 0.05$ vs untreated cells.

Figure 5. Pg induced a reduction of the tumor xenograft area of ACC cells. (A) Representative, lateral-view pictures of Tg(kdrl:EGFP) control and Pg-treated embryos at 120 hpf. Each ACC cell line was labeled with

a red fluorescent lipophilic dye while the embryos' endothelium was labeled with a green fluorescent protein reporter driven by the kdrl promoter. Images were acquired using a Zeiss LSM 510 META confocal laser-scanning microscope at 10x magnification. (B) Tumor areas of 48 hpf (T0 – the start of treatment) and 120 hpf (T3 – end of treatment) of drug-treated and vehicle-treated groups were measured using Zen 2.3 Black software from ZEISS. Data are shown as mean of independent experiments ± SEM. *$p < 0.0001$; §$p < 0.05$.

Figure 6. MUC-1 and TVBF-7 ACC cells induced metastasis formation in zebrafish embryos. (A) Enlargement of the embryo tail with metastasized MUC-1 cells. (B) A representative acquisition of the metastases of TVBF-7 cells in the pericardial area of the embryo is shown. Cells are labeled with a red fluorescent lipophilic dye while the embryos' endothelium is labeled with a green fluorescent protein reporter driven by the kdrl promoter. Images were acquired at 120 hpf using a Zeiss LSM 510 META confocal laser-scanning microscope at 10x magnification.

Figure 7. Pg suppresses ACC cell invasion (A) and migration (C) ability in transwell assays. (B, D) Quantification of the number of invasive and migrated cells was analyzed using the absorbance of the staining detected at 560nm. (E) Representative images of wound-healing assay used to detect migrated ACC cells. (F) The distance between each edge of the scratch was measured using NIH ImageJ Software. Images were acquired using an Olympus IX51 optical microscope equipped with a 10x objective. Data are shown as mean ± SEM. *$p < 0.0001$, #$p < 0.001$, §$p < 0.05$ vs untreated cells.

Figure 8. The secretion and the activity of MMP 2 were decreased after Pg treatment. (A) Representative western blot of MMP2 in the conditioned medium secreted by ACC cells. (C) Representative zymogram of MMP2 in the conditioned medium secreted by ACC cells. (B, D) Quantification of western blots and zymography. Results are presented as a relative optical density means ± SEM of three independent experiments. *$p < 0.0001$, §$p < 0.05$ vs untreated cells.

Bullet point summary:

What is already known:

Progesterone through its receptors exerts a cytotoxic effect on ACC cell lines.

Progesterone receptors expression in metastatic lines is lower compared to primary tumor cells.

What this study adds:

Progesterone induces apoptosis or autophagy depending on ACC cell types with long-lasting effect.

Progesterone reduces ACC cells invasiveness *in vitro* and in zebrafish model.

Clinical significance:

Pg and its derivative are already part of cancer-supporting care a well-known risk/benefit profile.

This study supports the association between progesterone-treatment and recurrence reduction in ACC patients.

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`Table 1.docx` available at https://authorea.com/users/563262/articles/610473-preclinical-evidence-of-progesterone-as-a-new-pharmacological-strategy-in-human-adrenocortical-carcinoma-cell-lines