Aus der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe
Klinik der Universität München
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Signaling pathways and their receptors as prognostic factors and targets in ovarian cancer

Dissertation
zum Erwerb des Doktorgrades der Medizin
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von

Eileen Deuster

aus Mexiko Stadt
2022

Mit Genehmigung der Medizinischen Fakultät
der Universität München

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Contents

1. List of publications

1.1 Publications included in this thesis

Deuster E, Hysenaj I, Kahaly M, Schmoeckel E, Mayr D, Beyer S, Kolben T, Hester A, Kraus F, Chelariu-Raicu A, Burges A, Mahner S, Jeschke U, Trillsch F, Czogalla B. *The Platelet-Activating Factor Receptor's Association with the Outcome of Ovarian Cancer Patients and Its Experimental Inhibition by Rupatadine.* **Cells.** September 2021. 10(9):2337.

Czogalla, B., **Deuster, E**., Liao, Y., Mayr, D., Schmoeckel, E., Sattler, C., Kolben, T., Hester, A., Fürst, S., Burges, A., Mahner, S., Jeschke, U., & Trillsch, F. *Cytoplasmic VDR expression as an independent risk factor for ovarian cancer*. **Histochemistry and Cell Biology**. June 2020. 154 (4):421-429.

Deuster, E, Mayr, D, Hester, A., Kolben, T., Zeder-Göß, C., Burges, A., Mahner, S., Jeschke, U., Trillsch, F., & Czogalla, B. *Correlation of the Aryl Hydrocarbon Receptor with FSHR in Ovarian Cancer Patients.* **International Journal of Molecular Sciences.** June 2019. 20(12):2862.

1.2 Other publications

Liao Y, Badmann S, Kaltofen T, Mayr D, Schmoeckel E, **Deuster E**, Mannewitz M, Landgrebe S, Kolben T, Hester A, Beyer S, Burges A, Mahner S, Jeschke U, Trillsch F, Czogalla B. *Platelet-Activating Factor Acetylhydrolase Expression in BRCA1 Mutant Ovarian Cancer as a Protective Factor and Potential Negative Regulator of the Wnt Signaling Pathway*. **Biomedicines.** June 2021. 9(7):706.

Ye, Y., Peng, L., Vattai, A., **Deuster, E.,** Kuhn, C., Dannecker, C., Mahner, S., Jeschke, U., von Schönfeldt, V., & Heidegger, H. H. *Prostaglandin E2 receptor 3 (EP3) signaling promotes migration of cervical cancer via urokinase-type plasminogen activator receptor (uPAR).* **Journal of Cancer Research and Clinical Oncology**. May 2020. 146:2189–2203

Deuster, E, Jeschke, U., Ye, Y., Mahner, S., & Czogalla, B. *Vitamin D and VDR in Gynecological Cancers-A Systematic Review***. International Journal of Molecular Sciences.** October 2017. 18(11):2328

1.3 Poster presentation

Deuster, E & Kahaly, M & Kuhn, C & Trillsch, F & Burges, A & Kolben, Theresa & Mahner, S & Jeschke, Udo & Czogalla, Bastian. (2018). *AhR is a prognostic marker of survival in ovarian cancer patients.* Geburtshilfe Frauenheilkd. 78. 10.1055/s-0038-1671366. In Oct 2018 at 62. Kongress der Deutschen Gesellschaft für Gynäkologie und Geburtshilfe – DGGG'18

2. Introduction

2.1 Ovarian cancer

2.1.1 Epidemiology

Ovarian cancer is one of the most lethal cancer types among women. It ranks second in gynecological cancer deaths, accounting for 5 486 deaths in Germany in 2016 (1, 2). A women's risk of getting ovarian cancer is 1 in 75, a risk that gradually increases until the age of 85 (Figure 1). The diagnosis of ovarian cancer is often late due to a lack of effective screening methods and unspecific symptoms such as bloating, abdominal discomfort and an increase in micturition frequency (3). 75 % of ovarian cancer patients are diagnosed at a late stage, contributing to the very poor 5-year survival rate of 43 % (1, 2).

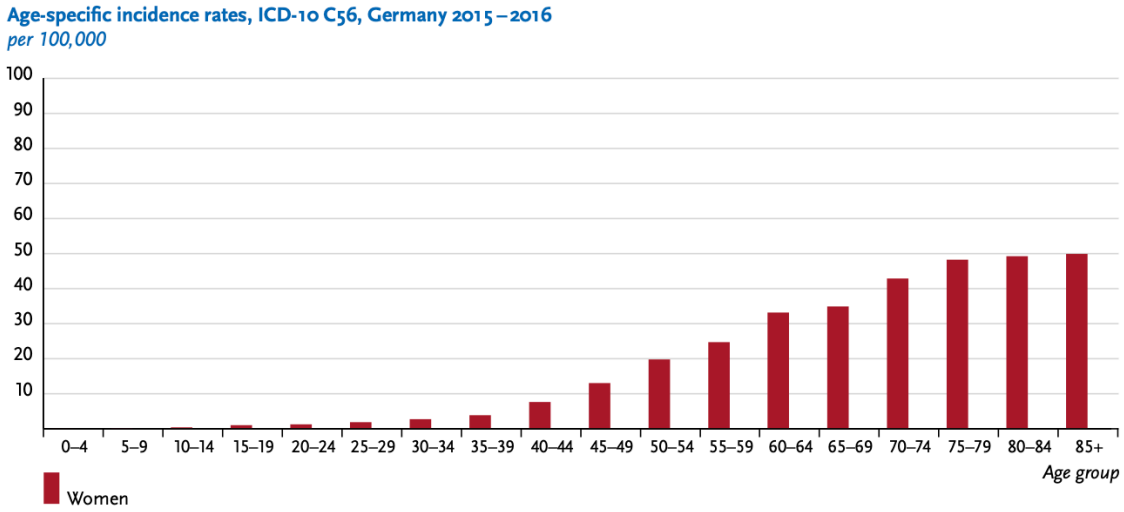

Figure 1: Incidence rates of ovarian cancer per 100,000 (2)

2.1.2 Pathology and etiopathogenesis

Ovarian cancer comprises a heterogeneous group of tumors in which epithelial ovarian cancer is the most prevalent one, with over 90 % of cases (4). Non-epithelial ovarian tumors include germ cell tumors, ovarian sarcomas, and sex cord-stromal tumors (5).

The five main histological subtypes of epithelial ovarian cancer differ in etiology, proliferation pattern, molecular pathogenesis, and survival and are divided into high grade serous, low grade serous, endometrioid, clear cell and, mucinous (3-6).

A recognized dualistic model of ovarian cancer's etiopathogenesis has given way for a new histopathological grading of ovarian carcinoma (7). This model distinguishes between two mechanisms of origin and classifies ovarian cancer into type I carcinomas (low-grade serous (<5%), mucinous (3%), endometrioid (10%), and clear cell (10%)) and type II carcinomas (high-grade carcinoma (70%)) (6, 7). Type I carcinomas are slow growing, developing via precursor lesions, and are mostly genetically stable (8). In these types of cancers, mutations in the KRAS and BRAF genes are often detected, whereas p53 mutations are rare. Low-grade serous cancers develop slowly from serous borderline tumors to non-invasive and low-grade serous ovarian cancers.

In contrast, type II carcinomas (the high-grade serous) mostly develop de novo. Accounting for more than half of ovarian cancer cases, high-grade serous ovarian cancer represents the most common histological subtype. This subtype is aggressive and often diagnosed in an advanced stage. It exhibits a high frequency of p53 mutations and is most commonly associated with BRCA 1/ 2 (see chapter 2.1.3: risk factors) mutations (3, 9).

2.1.3 Risk factors

Several different risk factors have been discussed for ovarian cancer. It has been well established that the hormonal system plays a significant role in ovarian cancer. Peri-and post-menopausal women taking hormonal therapy have a significantly increased risk of developing ovarian cancer (8-10). Furthermore, a large meta-analysis could show that an increased Body-Mass-Index in adult women raised their risk for epithelial ovarian cancer (EOC) (11).

It is noteworthy that hereditary syndromes such as hereditary breast-ovarian cancer syndrome (HBOC) and hereditary non-polyposis colorectal carcinoma syndrome (HNPCC) are important risk factors in the pathogenesis of ovarian cancer (12, 13). 10-15 % of ovarian cancer can be attributed to the HBOC syndrome, in which the genetic mutations are mostly located in BRCA 1/ 2 genes. BRCA 1/ 2 genes are essential caretakers in normal epithelial cells that regulate transcription and act as tumor suppressors (14). A genetic mutation in the BRCA1 gene will increase the risk of developing ovarian cancer to about 40-60 %, leading to a familial accumulation of ovarian and breast cancer (15).

2.1.4 Staging

The clinical staging of ovarian cancer is based on the FIGO classification (Table 1). It holds an essential role in providing a standard terminology and allows patients to be allocated to groups determining their therapeutic approach and prognosis. The FIGO classification is a significant independent prognostic factor.

Table 1: TNM and FIGO classifications for ovarian cancer (16). (Table with courtesy of the Journal of Gynecologic Oncology.)

Stage I. Tumor confined to ovaries or fallopian tube(s)
T1-N0-M0
IA: tumor limited to one ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
T1a-N0-M0
IB: tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
T1b-N0-M0
IC: tumor limited to one or both ovaries or fallopian tubes, with any of the following:
IC1: surgical spill
T1c1-N0-M0
IC2: capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
T1c2-N0-M0
IC3: malignant cells in the ascites or peritoneal washings
T1c3-N0-M0
Stage II. Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer
T2-N0-M0
IIA: extension and/or implants on uterus and/or fallopian tubes and/or ovaries
T2a-N0-M0
IIB: extension to other pelvic intraperitoneal tissues
T2b-N0-M0
Stage III. Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes
T1/T2-N1-M0
IIIA1: positive retroperitoneal lymph nodes only (cytologically or histologically proven):
IIIA1(i) Metastasis up to 10 mm in greatest dimension
IIIA1(ii) Metastasis more than 10 mm in greatest dimension
IIIA2: microscopic extrapelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes
T3a2-N0/N1-M0
IIIB: macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
T3b-N0/N1-M0
IIIC: macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes (includes extension of tumor to capsule of liver and spleen without parenchymal involvement of either organ)
T3c-N0/N1-M0
Stage IV. Distant metastasis excluding peritoneal metastases
Stage IVA: pleural effusion with positive cytology
Stage IVB: parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)
Any T, any N, M1

2.1.5 Therapy

The recommended treatment for ovarian cancer is adapted according to the stage and consists of surgical and systemic therapy options.

Surgery with the aim of removing all macroscopic tumor tissue is a necessary treatment for most ovarian carcinomas and the most important prognostic factor for OC patients. Currently, standard systemic therapy for advanced ovarian cancer consists of a combination of Carboplatin and Paclitaxel (17). In advanced tumor stages and if appropriate anti-vascular endothelial growth factor therapy (Bevacizumab) is additionally employed (3).

Further treatment options are the ADP-ribose-polymerase inhibitors (PARP inhibitors). In recent years, these have and still are radically transforming the therapy of ovarian cancer patients. First data on the PARP inhibitors as first-line therapy was provided by the SOLO-1 trial published in

2018 (18). It found that Olaparib as maintenance therapy significantly improved progression-free survival of women with advanced ovarian cancer and a BRCA1/2 mutation. In comparison to the placebo group, ovarian cancer patients receiving Olaparib had a reduced risk of disease progression or death by 70 % (HR 0.30; 95 % CI 0.23-0.41; p<0.001). Based on these remarkable results, Olaparib has been approved as maintenance therapy in first-line therapy for BRCA-mutated ovarian cancer patients. Since then, several trials on the use of PARP inhibitors have been published, in which the groups of patients that benefit from the monoclonal antibodies have continuously been redefined (19). Recently, a PARP inhibitor was approved for advanced ovarian cancer regardless of mutation status if the cancer had responded to platinum-containing chemotherapy. Furthermore, combination therapy of a PARP inhibitor with Bevacizumab showed a significant benefit in terms of progression-free survival compared to Bevacizumab monotherapy in BRCA-mutated patients (20). These results led to an extension of the approval of a PARP inhibitor and illustrate how PARP inhibitors are currently significantly challenging existing guidelines.

Multimodal treatment strategies are also offered to patients with recurrent ovarian cancer; however, as the disease can no longer be considered curable, the therapeutic intentions are adjusted. Recent data from phase III trials have shown that selected patients with disease relapse benefit from a secondary cytoreductive surgery (21). Concerning the systemic therapy, the response to the last platinum-containing chemotherapy is carefully taken into consideration. For platinum-sensitive patients who relapsed after six months of receiving initial treatment, platinum-containing combination therapy can be employed. PARP inhibitors can subsequently be applied as maintenance therapy, regardless of the BRCA mutation status (22). For platinum-resistant patients, the therapeutic alternatives are much more limited. In this case, monotherapy with a non-platinum drug should be considered.

However, it is essential to underline that even though ovarian cancer's therapeutic regime is extensive, patients' long-term survival remains very poor (Figure 2). Thus, in the era of personalized cancer medicine, it is imperative to find new therapeutic options considering the heterogeneity of ovarian cancer and target specific subtypes.

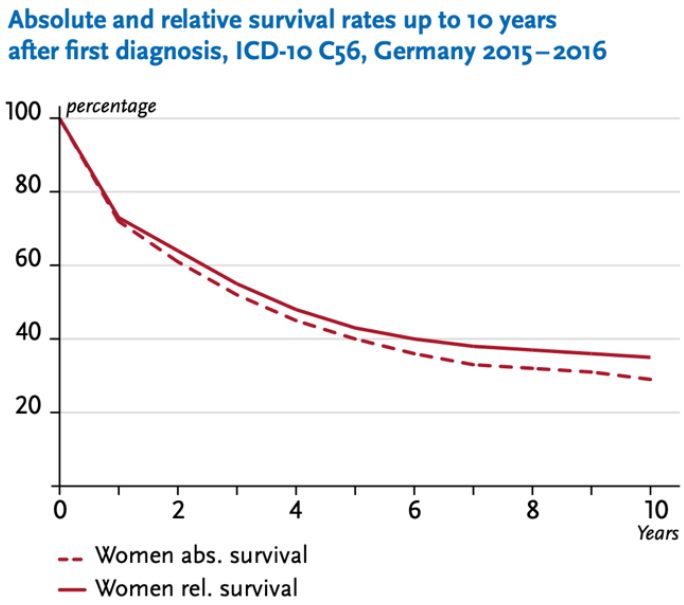

Figure 2. Absolute and relative survival rates of ovarian cancer patients (2)

2.1.6 Prognostic factors

Up till now, the most reliable prognostic factors are clinical and pathological parameters such as the International Federation of Gynecology and Obstetrics (FIGO) stage, residual disease after initial cytoreductive surgery, patients age, the volume of ascites, and histological subtype (23-25). However, there are no clinically used biomarkers as prognostic factors (3, 26-28). Testing of CA-125 (cancer antigen 125) is employed in ovarian cancer patients to monitor responses to chemotherapy and disease progression. Due to the unspecific elevation of CA-125 in patients who have other diseases than ovarian cancer, CA-125 has not been found useful as a screening biomarker (29). Therefore, new biomarkers need to be identified, which can be put into clinical practice and help improve the therapy of ovarian cancer patients.

3. Aim of this thesis

The lack of prognostic biomarkers for ovarian cancer patients and their low chances of survival make it imperative to develop new prognostic and therapeutic strategies. This thesis studied three different putative biological biomarkers by analyzing their association with the survival of ovarian cancer patients and exploring their molecular signaling pathways to be then able to inhibit them specifically.

1. The first aim of this thesis was to examine the immunohistochemical expression of three different receptors, namely the Aryl hydrocarbon receptor (AhR), the Vitamin D receptor (VDR), and, lastly, the Platelet-activating-factor receptor (PAFR) in the most common histological subtypes of epithelial ovarian cancer. For this, ovarian cancer samples from 156 patients who underwent surgery for epithelial ovarian cancer at the Department of Obstetrics and Gynecology at the Ludwig-Maximilians-University from 1990-2002 were examined. The correlation between clinicopathological data, overall survival, and expression levels was determined for the three receptors, considering the histological subtypes of EOC. (Paper 1,2,3)
2. Subsequently, after analyzing PAFR's association with long-term survival of ovarian cancer patients and determining it to be an independent risk factor, the aim was to further explore the PAFR's role in ovarian cancer. For this, functional assays were performed in vitro. (Paper 3)
3. The third aim of this thesis was to inhibit the PAFR on a molecular level specifically. A PAFR antagonist called Rupatadine was employed in a drug repurposing approach to evaluate its impact on the proliferation and migration of ovarian cancer cells, thereby exploring its use as a potential ovarian cancer treatment. (Paper 3)

3.1.1 The Aryl hydrocarbon receptor's role in different ovarian subtypes and its cross-link with the hormonal system (Paper 1)

The Aryl hydrocarbon receptor, a ligand-activated transcription factor, was initially discovered as the receptor that binds the potent environmental toxic and carcinogenic compound 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) (30, 31). The receptor's various ligands bind to it in the cytoplasm, and the new AhR-ligand complex translocates into the nucleus. After it heterodimerizes with the AhR nuclear translocator (ARNT), the complex binds to the dioxin response elements (DRE) and activates many downstream genes. AhR mediates a wide range of cellular processes and holds significance, amongst others, for epithelial barrier function, cell cycle, and cell migration (31).

Several studies have examined the function of the receptor in the ovary. It has been shown that it plays a vital role in normal ovarian growth and function, regulating ovarian follicle growth and ovulation (32). AhR-deficient mice were shown to have fewer antral follicles, corpora lutea, and a reduced number of ovulations than the wild-type mice (33, 34). The close link between the receptor and the hormonal system has been studied in the last years (33, 34). It was found that AhR enhances the responsiveness of the follicle-stimulating hormones (FSH) by positively affecting transcription of the follicle-stimulating hormone receptor (FSHR) through an E-box binding site (35, 36).

Furthermore, the AhR plays a part in cancer development, showing pro-oncogenic and anti-tumorigenic effects depending on the tumor type (31). In different cancer entities, the AhR has been revealed to promote tumor formation and progression. Its raised expression has been characterized in a variety of carcinomas from different organs. Immunohistochemical analysis of prostate, breast, and gastric tumors revealed high expression levels indicating its chronic activation.

However, until now, very little is known about the role of AhR in ovarian cancer. The first paper included in this thesis investigated for the first time the expression levels of AhR in the different histological subtypes of EOC and their correlation with the long-term survival of patients. We could show that all histological subtypes (serous, clear cell, endometrioid, and mucinous) displayed AhR expression, most notably the clear cell carcinoma. Paper 1 demonstrated that ovarian cancer patients with a low cytoplasmic AhR expression showed a better overall survival (median 183.46 vs. 85.07 months; $p = 0.021$) (37). The cytoplasmic AhR expression correlated positively with clinicopathological factors such as histology, tumor size, FIGO, and grading (except in low-grade-serous cancer). In accordance with these findings, other studies have found elevated AhR levels in different carcinomas to correlate with a poor outcome (38).

Bearing in mind the cross-link between AhR and FSHR in normal ovarian function, the aim was to analyze the correlation of the two receptors in ovarian cancer. Interestingly, ovarian cancer patients with high cytoplasmic AhR expression had a different outcome depending on their FSHR levels. High levels of AhR and FSHR decreased the outcome of ovarian cancer patients significantly. This analysis reinforces the cross-link between AhR and FSHR and provides evidence for both receptors to affect ovarian cancer prognosis.

3.1.2 Role of Vitamin D receptor in ovarian cancer and its use as a prognostic biomarker (Paper 2)

Vitamin D and its receptor have attained considerable importance in the last two decades. Formerly known for its role in calcium homeostasis and metabolism, it is nowadays well established that vitamin D and the vitamin D receptor (VDR) play a crucial role in medical illnesses such as diabetes, cardiovascular disease, and cancer. The Vitamin D receptor is a nuclear receptor that binds the activated fat-soluble Vitamin D, also known as Calcitriol or 1α,25(OH)2D3. This metabolite is synthesized from cholesterol in multiple steps (39).

A vast number of epidemiological and preclinical studies have analyzed the role Vitamin D plays in cancer. The hormone's high blood levels have been associated with a reduced risk of developing certain cancer types (colorectal, gastric, hematological, kidney, lung, skin, prostate, ovarian, and breast cancer). In vitro and vivo Vitamin D inhibits proliferation and induces differentiation of cancer cells (40-42).

In the review ''Vitamin D and VDR in Gynecological Cancers-A Systematic Review of vitamin D and its receptor", we systematically analyzed the evidence on vitamin D and its receptor in gynecological cancers (Figure 3) (39). Low levels of the prehormone of Vitamin D, 25-hydroxyvitamin D, were associated with increased ovarian cancer incidence in several studies (43-47).

The VDR is expressed in healthy ovaries as well as in ovarian cancer. In the ovary, it affects estrogen biosynthesis and alters aromatase gene expression (48). VDR- null mice were shown to suffer from low aromatase levels and gonadal insufficiency, indicating its importance for full ovarian function. In EOC, previous studies found VDR's expression to be elevated (49-51). It was postulated that the receptor promotes cancer cell proliferation by interacting with growth-stimulating factors such as the androgen receptor (52).

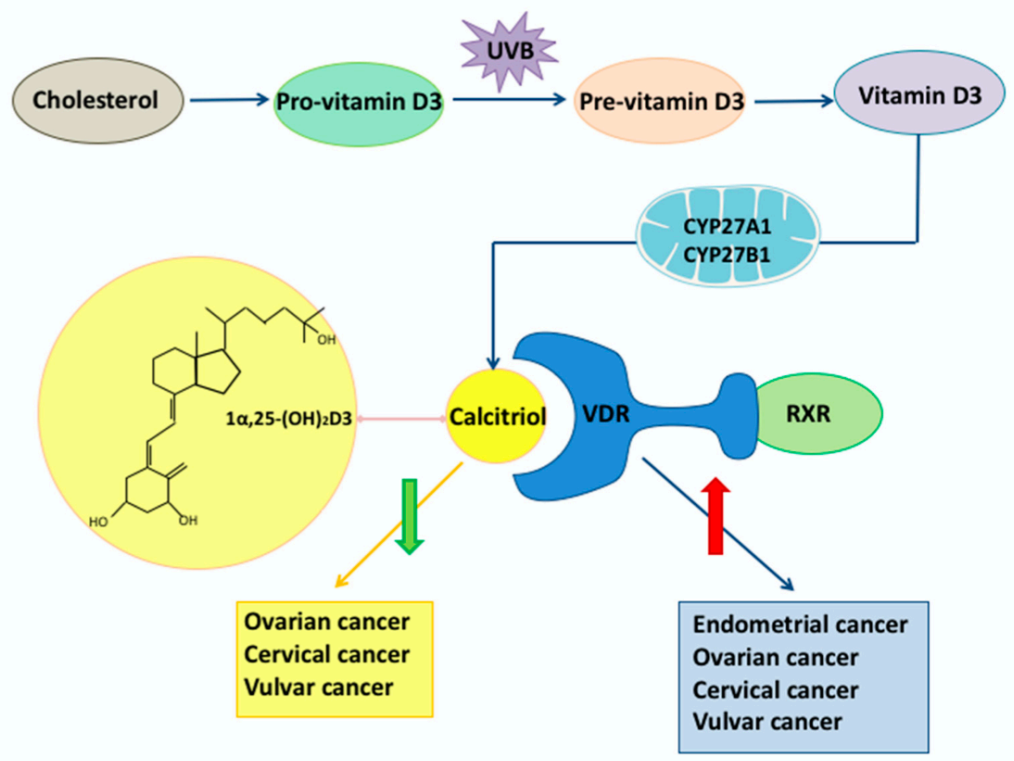

Figure 1. The role of vitamin D and vitamin D receptor (VDR) in gynecological cancers: Endogenous synthesis of vitamin D begins with the oxidation of cholesterol, resulting in pro-vitamin D3. In the skin, ultraviolet B (UVB) radiation transforms pro-vitamin D3 to pre-vitamin D3. Pre-vitamin D3 isomerizes to vitamin D3, also named as cholecalciferol. Two hydroxylations by the enzymes vitamin D 25-hydroxylases (CYP27A1) and renal mitochondrial 1-hydroxylase (CYP27B1) are necessary to transform vitamin D3 into the active 1α,25(OH)2D3. Different tissues, as well as gynecological cancer tissue, can synthesize calcitriol. 1α,25(OH)2D3 binds to the vitamin D receptor which belongs to the family of nuclear receptors and forms a complex with retinoid X receptor (RXR) to regulate gene expression. Both vitamin D and its receptor have a protective role in gynecological cancers. Low levels of vitamin D are found in ovarian, cervical and vulvar cancer. As a response to cancer, the expression of the vitamin D receptor is upregulated in endometrial, ovarian, cervical and vulvar cancer.

Figure 3: The role of vitamin D and the vitamin D receptor (VDR) in gynecological cancers (39). (Picture with courtesy of MDPI)

Paper 2 included in this thesis aimed to investigate the VDR expression in different histological subtypes of EOC. Similar to previous studies, we found a heightened immunohistochemical expression of the receptor in all histological subtypes. In 2010 Silvagno et al., examined the correlation between VDR and clinicopathological factors, finding no significant association (53). We wanted to reevaluate these findings using a larger sample size. The ovarian cancer patients included in Paper 2 demonstrated to have a significant positive correlation between cytoplasmic VDR expression and clinical prognostic factors such as FIGO stage, positive lymph node status, and high-grade serous histology. Furthermore, high cytoplasmic VDR expression was defined as

an independent risk factor impairing overall survival for ovarian cancer patients. Thus Paper 2 included in this thesis provided further evidence on the receptor as a biomarker (54).

3.1.3 The Platelet-activating-factor receptor as an inflammatory mediator influencing survival in ovarian cancer patients and its experimental inhibition by Rupatadine (Paper 3)

The importance of chronic inflammation in ovarian cancer pathogenesis has been well established (55, 56). The platelet-activating factor and its G protein-coupled receptor, PAFR, are key inflammatory mediators activating neutrophils, macrophages, platelet, and endothelial cells (57).

In different kinds of cancers, including the EOC, the PAF/PAFR axis has been investigated, finding tumorigenic effects, promoting angiogenesis, metastasis, and antiapoptosis (58). The PAFR increases downstream signaling via EGFR/Src/FAK/Paxillin (58). In breast cancer cells, it was demonstrated that in vitro stimulation with factors produced in the cancer microenvironment (vascular endothelial growth factor, basic fibroblast growth factor, hepatocyte growth factor, tumor necrosis factor, thrombin) induced the PAFR pathway (59).

Several tumors express PAFR. Its overexpression has been shown to help the development of tumors and metastatic processes (60). Interestingly, transgenic mice overexpressing PAFR were reported to develop melanocytic tumors spontaneously (61). Aponte et al. found increased levels of PAFR in non-mucinous ovarian cancer compared to benign tumors (58). Their studies found that when EGF binds to the EGF receptor, it transactivates PAFR, thereby promoting PAF production in ovarian cancer (62, 63). The combined targeting of both receptors was found to reduce tumor growth and progression in mice (64).

However, the significance of the PAFR on the long-term survival of ovarian cancer patients was not elucidated before. As part of the third paper, we therefore first analyzed the immunohistochemical expression of PAFR and correlated the expression levels with clinical and pathological markers. Similar to other studies, the serous, clear cell, endometrioid subtype displayed elevated expression levels compared to the mucinous subtype (58). We found that PAFR significantly correlates with clinical parameters, and most notably, an elevated expression of cytoplasmic PAFR was associated with poor overall survival of ovarian cancer patients. Paper 3 proved elevated PAFR expression to be an independent risk factor by a multivariate cox-analysis.

After finding PAFR to affect long-term overall and recurrence-free survival, Paper 3 aimed to investigate the receptor in vitro. The PAFR mRNA and protein expression was characterized in

four different ovarian cancer cells: OVCAR-3 (serous, BRCA WT), UWB1.289 (serous, BRCA1 negative), ES-2 (clear cell), and TOV 112D (endometrioid). In accordance with previous studies, we found that especially the serous ovarian cancer subtypes (OVCAR-3 and UWB1.289) displayed elevated protein and mRNA levels (58, 65). PAFR knockdown with siRNA was performed, and functional assays were included in the paper. The results confirmed PAFR's protumorigenic effect displaying a significantly decreased cancer cell proliferation.

The results obtained by immunohistochemistry and in vitro strongly reinforced the receptor's role in ovarian cancer pathogenesis. Thus, in a next step, we aimed to target and inhibit the platelet-activating-factor receptor. Other studies have previously used PAFR specific antagonists (WEB 2086 and Ginkgolide B) and assessed their antitumorigenic effect (65, 66). We used Rupatadine, a PAFR antagonist that is already clinically used and approved (67). Rupatadine is an antihistaminic drug employed for allergic disease with a good safety profile (68, 69). In cell proliferation and migration assays, we examined Rupatadines effect on four ovarian cancer cell lines We found that Rupatadine significantly inhibited cell proliferation and migration in vitro in all studied epithelial ovarian cancer subtypes.

4. Contribution to the papers

4.1 Contribution to paper 1

In the first study, I analyzed the specimens and collected the data. I carried out the statistical analysis with support from my doctoral supervisor, Prof. Jeschke. All the graphs, tables, and figures included in the paper were created by me. I wrote the first draft of the manuscript and revised and finalized it together with my supervisor, PD. Dr. Czogalla.

4.2 Contribution to paper 2

For the second paper included in this thesis, I worked as a co-author in the development and design of the project. I was involved in the statistical analysis and discussion of the results. I helped prepare the manuscript and thoroughly proofread it.

4.3 Contribution to paper 3

In the third publication, I analyzed the tissue samples, performed functional experiments in vitro and analyzed the data. The manuscript was drafted by myself, revised and finalized with my supervisor. I created and revised all the graphs, tables and figures published in the paper. I independently performed the statistical analysis. Co-authors participated in the conduction of experiments and discussion of results.

5. Paper I

Title:

Correlation of the Aryl Hydrocarbon Receptor with FSHR in Ovarian Cancer Patients

Authors:

Eileen Deuster, Doris Mayr, Anna Hester, Thomas Kolben, Christine Zeder-Göß, Alexander Burges, Sven Mahner, Udo Jeschke, Fabian Trillsch and Bastian Czogalla

Journal:

International Journal of Molecular Sciences

Abstract:

Expression of the aryl hydrocarbon receptor (AhR) has been described in various tumor entities from different organs. However, its role in ovarian cancer has not been thoroughly investigated. We aimed to elucidate the prognostic impact of AhR, its correlation with the follicle-stimulating hormone receptor (FSHR), and their functional role in ovarian cancer. By immunohistochemistry, AhR staining was analyzed in a subset of 156 samples of ovarian cancer patients. AhR staining was assessed in the nucleus and the cytoplasm using the semi-quantitative immunoreactive score (IRS), and the scores were grouped into high- and low-level expression. AhR expression was detected in all histological subtypes, with clear cell ovarian cancer displaying the highest staining intensity. Low cytoplasmic expression of AhR was associated with longer overall survival (median 183.46 vs. 85.07 months; $p = 0.021$). We found a positive correlation between AhR and FSHR ($p = 0.005$). Ovarian cancer patients with high cytoplasmic AhR and concurrent FSHR expression had the worst outcome (median 69.72 vs. 43.32 months; $p = 0.043$). Consequently, low cytoplasmic AhR expression seems to be associated with improved survival in ovarian cancer patients. Our data suggest that AhR and FSHR levels correlate with each other, and their concurrent expression was observed in ovarian cancer patients with the worst outcome. Further investigation of the interaction of both receptors and their functional role might better predict the impact of endocrine therapy in ovarian cancer.

Link:

https://www.mdpi.com/1422-0067/20/12/2862

6. Paper 2

Title:

Cytoplasmic VDR expression as an independent risk factor for ovarian cancer

Authors:

Bastian Czogalla, **Eileen Deuster**, Yue Liao, Doris Mayr, Elisa Schmoeckel, Cornelia Sattler, Thomas Kolben, Anna Hester, Sophie Fürst, Alexander Burges, Sven Mahner, Udo Jeschke, Fabian Trillsch

Journal:

Histochemistry and Cell Biology

Abstract:

The vitamin D receptor (VDR), primarily known as a crucial mediator of calcium homeostasis and metabolism, has been shown to play a significant role in various cancer entities. Previous studies have focused on vitamin D and its receptor in gynecological cancers, noting that the receptor is upregulated in epithelial ovarian cancer (EOC). The aim of this study is to analyze the prognostic impact of VDR and its functional significance in ovarian cancer. Through immunohistochemistry, VDR staining was examined in 156 ovarian cancer samples. Evaluation of VDR staining was conducted in the nucleus and the cytoplasm using the semi-quantitative immunoreactive score, and the scores were classified into high- and low-level expressions. Expression levels were correlated with clinical and pathological parameters as well as with overall survival to assess for prognostic impact. Differences in cytoplasmic VDR expression were identified between the histological subtypes (p = 0.001). Serous, clear cell, and endometrioid subtypes showed the highest staining, while the mucinous subtype showed the lowest. Cytoplasmic VDR correlated with higher FIGO stage (p = 0.013; Cc = 0.203), positive lymph node status (p = 0.023; Cc = 0.236), high-grade serous histology (p = 0.000; Cc = 0.298) and grading from the distinct histological sub- types (p = 0.006; Cc = − 0.225). Nuclear VDR did not correlate with clinicopathological data. High cytoplasmic expression of VDR was associated with impaired overall survival (HR 2.218, 32.5 months vs. median not reached; p < 0.001) and was confirmed as a statistically independent prognostic factor in the Cox regression multivariate analysis. Additional knowledge of VDR as a biomarker

and its interactions within the mitogen-activated protein kinase (MAPK) signaling pathway could potentially improve the prognosis of therapeutic approaches for specific subgroups in EOC.

Link:

https://link.springer.com/article/10.1007/s00418-020-01894-6

7. Paper 3

Title:

The Platelet-Activating Factor Receptor's Association with the Outcome of Ovarian Cancer Patients and Its Experimental Inhibition by Rupatadine

Authors:

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Journal:

Cells

Abstract:

The platelet-activating factor receptor (PAFR) and its ligand (PAF) are important inflammatory mediators that are overexpressed in ovarian cancer. The receptor is an important player in ovarian cancer development. In this study, we aimed to evaluate the prognostic value of PAFR in epithelial ovarian cancer (EOC) and the potential use of its antagonist, rupatadine, as an experimental treatment. Tissue microarrays of ovarian cancer patients, most markedly those with a non-mucinous subtype, immunohistochemically overexpressed PAFR. Elevated cytoplasmic PAFR expression was found to significantly and independently impair patients' overall and recurrence-free survival (OS: median 83.48 vs. 155.03 months; $p = 0.022$; RFS: median 164.46 vs. 78.03 months; $p = 0.015$). In vitro, the serous ovarian cancer subtypes especially displayed an elevated PAFR gene and protein ex- pression. siRNA knockdown of PAFR decreased cell proliferation significantly, thus confirming the receptor's protumorigenic effect on ovarian cancer cells. The clinically approved PAFR antagonist rupatadine effectively inhibited in vitro cell proliferation and migration of ovarian cancer cells. PAFR is a prognostic marker in

ovarian cancer patients and its inhibition through rupatadine may have important therapeutic implications in the therapy of ovarian cancer patients.

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8. Summaries

8.1 Summary in English

Ovarian cancer is the second most common malignant tumor of the female genital tract in Germany (1, 2). The late diagnosis and lack of prognostic biomarkers contribute to ovarian cancer patients' poor survival. Thus, it is crucial for new prognostic and therapeutic strategies to be developed. This thesis analyzed three different putative prognostic biomarkers by studying their association with long term survival of ovarian cancer patients and by exploring their molecular signaling pathways to be then able to inhibit them (37, 54, 70). All three receptors analyzed in this thesis (Aryl hydrocarbon receptor, Vitamin D receptor and the Platelet-activating factor receptor) had been shown in prior studies to be involved in cancer formation and progression (31, 39, 52, 71, 72).

In the immunohistochemical analysis of the most common histological subtypes of EOC, all three receptors showed an elevated expression and correlated positively with clinical and pathological factors. For this analysis, ovarian cancer specimens from 156 patients who had surgery at the Department of Obstetrics and Gynecology at the Ludwig-Maximilians-University from 1990-2002, were included. This thesis could show that all three receptors were prognostic biomarkers significantly affecting the long-term survival of ovarian cancer patients (37, 54, 70) (Figure 4). Furthermore, we performed correlation analysis of the AhR with another important mediator of ovarian cancer (FSHR) and could elucidate a cross-link between those two receptors (37).

Having analyzed PAFR's association with overall and recurrence free survival of ovarian cancer patients and determined it to be an independent risk factor, we further explored the PAFR on a molecular level. The functional analysis of the receptor in different epithelial ovarian cancer cells, reinforced PAFR's protumorgenic role. We determined the PAFR to be highly expressed on a protein and mRNA level and the receptor's knockdown to reduce cancer cell proliferation significantly. Subsequently, this thesis aimed to target and inhibit the PAFR in vitro specifically. In a drug repurposing approach, Rupatadine, a PAFR

antagonist used for allergic disease, was employed. The proliferation and migration analysis performed indicated that Rupatadine inhibited cancer cell proliferation and migration in all histological subtypes (70).

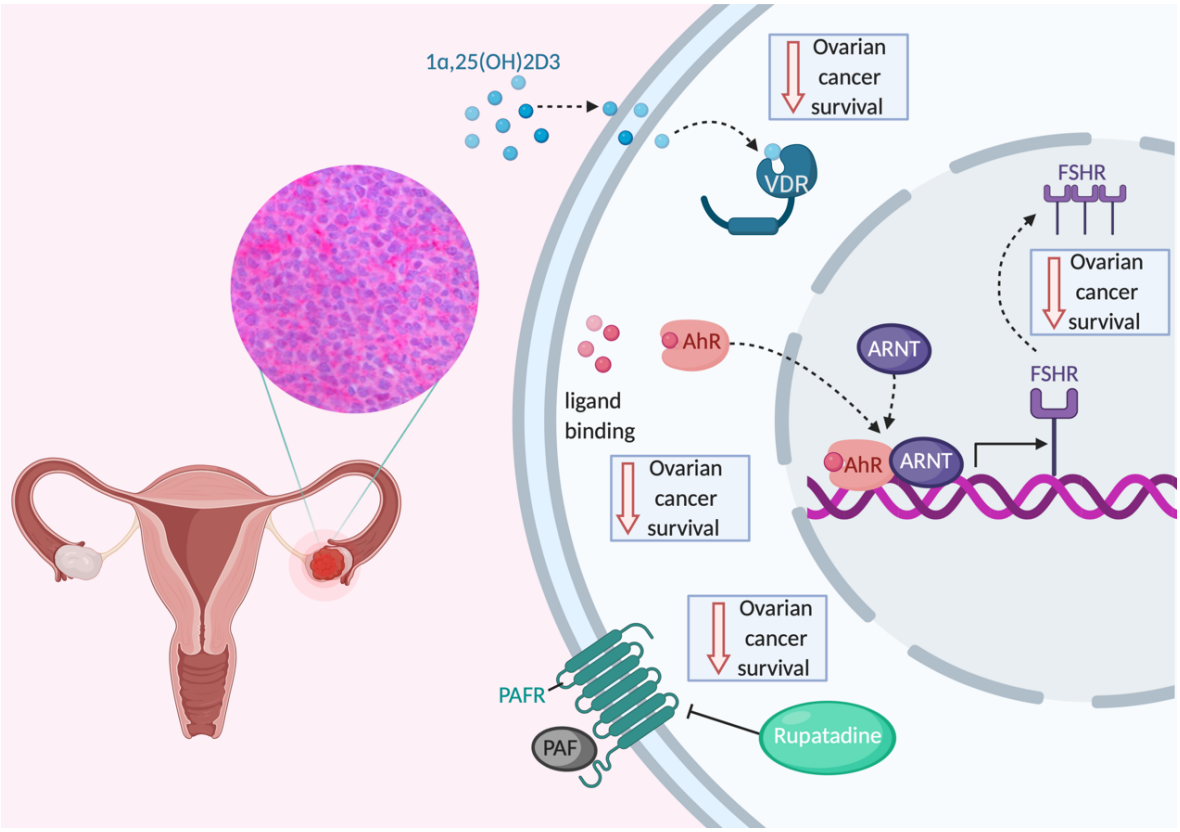

Figure 4: Summary of the findings of all three papers included in this thesis (37, 54, 70). The left part of the figure demonstrates ovarian cancer and exemplifies the immunohistochemical tissue analysis of the patients included in this thesis. The right part of the figure represents an ovarian cancer cell. On the top, the cytoplasmic VDR is depicted with its ligand, Calcitriol, stating its negative prognostic impact. As part of the second publication included in this thesis, we found that cytoplasmic AhR is associated with overall survival of ovarian cancer patients. After AhR translocates into the nucleus and binds to ARNT, the new complex promotes FSHR transcription. We found that patients with elevated AhR and FSHR levels had a significant worse outcome. This figure further depicts the PAFR, as a G protein coupled receptor that binds PAF. An elevated cytoplasmic PAFR expression was found to impair long-term survival. Our in vitro analysis showed that Rupatadine, a PAFR antagonist, successfully inhibits ovarian cancer cell development. (Figure created with BioRender.com)

In conclusion, this thesis found three different potential predictive biomarkers: AhR, VDR, and PAFR. PAFR's protumorgenic role in EOC was reaffirmed in vitro and pharmacological therapy with Rupatadine determined to successfully inhibit ovarian cancer cell proliferation. Further

studies are required to elucidate whether Rupatadine might be a potential treatment for ovarian cancer patients.

8.2 Zusammenfassung

Eierstockkrebs ist das zweit häufigste bösartige Malignom des weiblichen Genitaltrakts in Deutschland (1, 2). Die späte Diagnose und der Mangel an prognostischen Biomarkern tragen zum schlechten Überleben der Patientinnen mit Ovarialkarzinom bei. Daher ist es wichtig, neue prognostische und therapeutische Strategien zu entwickeln. In dieser Arbeit wurden drei verschiedene potenzielle prognostische Biomarker untersucht. Dafür wurde ihre Assoziation mit dem Langzeitüberleben von Eierstockkrebspatientinnen korreliert. Zudem wurden die molekularen Signalwege der Rezeptoren näher charakterisiert, mit dem Ziel die Rezeptoren therapeutisch zu hemmen (37, 54, 70). Für alle drei Rezeptoren, die in dieser Arbeit analysiert wurden (Aryl-Hydrocarbon-Rezeptor, Vitamin-D-Rezeptor und der Plättchen-aktivierende Faktor-Rezeptor), konnte in früheren Studien dargelegt werden, dass sie an der Krebsentstehung und -progression beteiligt sind (31, 39, 52, 71, 72).

In der immunhistochemischen Analyse der häufigsten histologischen Subtypen des epithelialen Ovarialkarzinoms zeigten alle drei Rezeptoren eine erhöhte Expression und korrelierten positiv mit klinischen und pathologischen Faktoren. Für diese Analyse wurden Ovarialkarzinom-Präparate von 156 Patientinnen, die von 1990-2002 an der Klinik für Geburtshilfe und Gynäkologie der Ludwig-Maximilians-Universität operiert wurden, einbezogen. Dabei konnte gezeigt werden, dass alle drei Rezeptoren prognostische Biomarker sind, die das Langzeitüberleben von Patientinnen mit Ovarialkarzinom signifikant beeinflussen (37, 54, 70) (Abbildung 4). Darüber hinaus führten wir eine Korrelationsanalyse des AhR mit einem weiteren wichtigen Mediator des Ovarialkarzinoms (FSHR) durch und konnten eine Verbindung zwischen diesen beiden Rezeptoren bestätigen (37).

Nachdem wir die Assoziation des PAFR mit dem Gesamt- und rezidivfreien Überleben von Patientinnen mit Ovarialkarzinom analysiert und ihn als unabhängigen Risikofaktor bestätigen konnten, untersuchten wir den PAFR weiter auf molekularer Ebene. Die funktionellen Analysen des Rezeptors in verschiedenen epithelialen Ovarialkarzinomzellen untermauerten die protumorgene Rolle des PAFR. Wir stellten zudem fest, dass der PAFR in Ovarialkarzinomzellen auf Protein- und mRNA-Ebene hoch exprimiert wird und dass der Knock-down des Rezeptors die Proliferation der Krebszellen signifikant reduzierte. Ein weiteres Ziel dieser Arbeit war es daher, spezifisch den PAFR in vitro zu hemmen. Dafür verwendeten wir Rupatadin, ein PAFR-Antagonist, der

bei allergischen Erkrankungen eingesetzt wird. Die durchgeführte Proliferations- und Migrationsanalysen konnten zeigen, dass Rupatadin die Proliferation und Migration von Krebszellen in allen histologischen Subtypen hemmte (70).

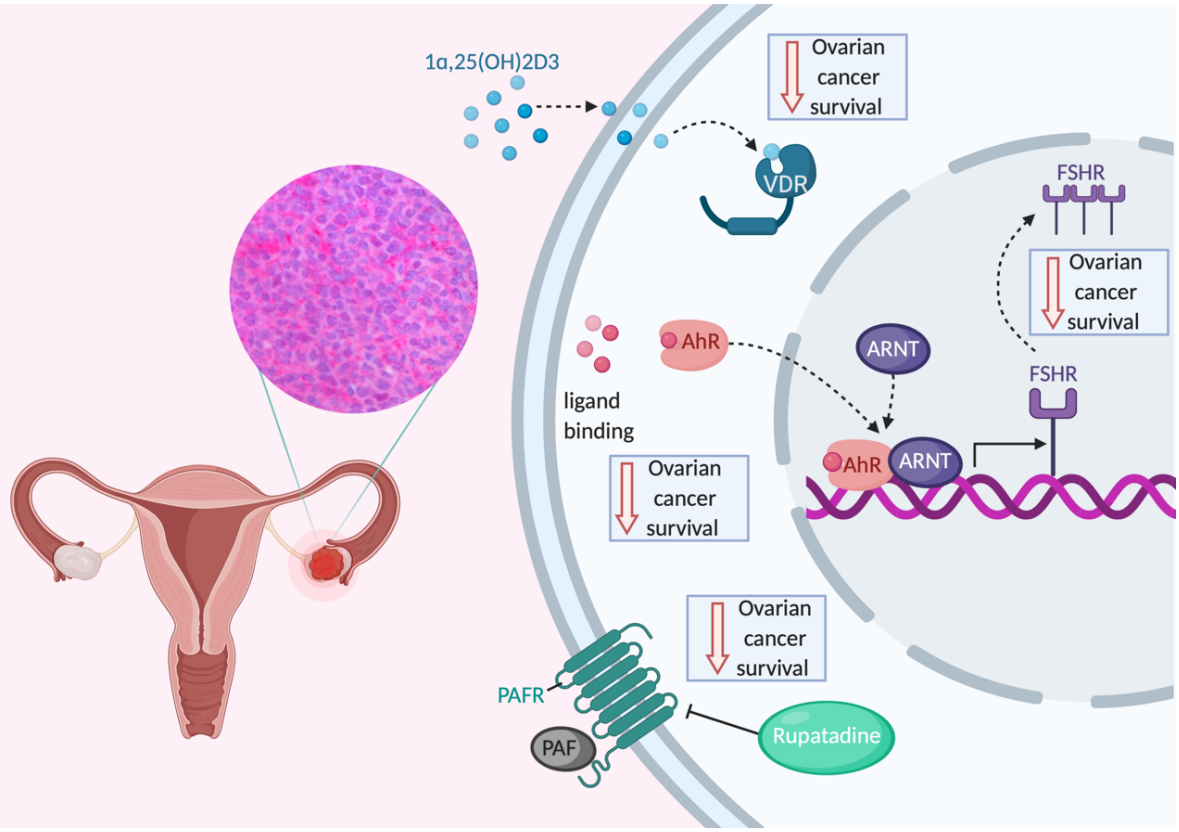

Abbildung 4: Zusammenfassung der Dissertationsergebnisse (37, 54, 70). Der linke Teil der Abbildung zeigt schematisch den weiblichen Reproduktionstrakt mit einem maligen Ovarialtumor und veranschaulicht die immunhistochemische Analyse der eingeschlossenen Patientinnen. Der rechte Teil der Abbildung stellt eine Ovarialkrebszelle dar. Oben ist der zytoplasmatische VDR mit seinem Liganden, Calcitriol, dargestellt. Dieser zeigte in unseren Untersuchungen eine negative prognostische Bedeutung. Im Rahmen der zweiten Publikation konnten wir belegen, dass der zytoplasmatische AhR mit dem Gesamtüberleben von Ovarialkarzinompatientinnen korreliert. Nachdem AhR in den Zellkern transloziert und an ARNT bindet, fördert der neue Komplex die FSHR-Transkription. Zudem ergab unsere Analyse, dass Patientinnen mit erhöhter AhR- und FSHR-Expression ein signifikant schlechteres Überleben zeigten. Diese Abbildung beinhaltend zudem den G-Protein-gekoppelten PAFR mit seinem Liganden PAF. Wir konnten zeigen, dass eine erhöhte zytoplasmatische PAFR-Expression das Langzeitüberleben von Patientinnen mit Ovarialkarzinom negativ beeinträchtigt. In weiterführenden in vitro-Analysen konnte Rupatadin, ein PAFR-Antagonist, die Entwicklung von Eierstockkrebszellen hemmen. (Die Abbildung wurde mit BioRender.com erstellt)

Zusammenfassend wurden in dieser Arbeit drei verschiedene potenzielle prädiktive Biomarker gefunden: AhR, VDR und PAFR. Die protumorgene Rolle des PAFR im epithelialen Ovarialkarzinom wurde in vitro bestätigt. Es konnte gezeigt werden, dass eine pharmakologische Therapie mit Rupatadin die Proliferation von Ovarialkarzinomzellen erfolgreich hemmt. Weitere Studien sind erforderlich, um zu klären, ob Rupatadin eine mögliche Behandlung für Patientinnen mit Ovarialkarzinom darstellen könnte.

9. Bibliography

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10. Abbreviations

AhR	Aryl hydrocarbon receptor
ARNT	AhR nuclear translocator
BRCA	breast cancer gene
EOC	epithelial ovarian cancer
FIGO	International Federation of Gynecology and Obstetrics
HBOC	hereditary breast ovarian cancer syndrome
HNPCC	hereditary non-polyposis colorectal carcinoma syndrome
IHC	immunohistochemistry
PAF	Platelet-activating factor
PAFR	Platelet-activating factor receptor
PARP	Poly [ADP-ribose] polymerase
VDR	Vitamin D receptor

11. Acknowledgements

Throughout this dissertation, I received a great deal of support and assistance.

I would like to express my thanks to Prof. Dr. rer. nat. Udo Jeschke for allowing me to undertake the research project, providing continuous support and helpful advice. His encouragement and insightful feedback pushed me to sharpen my thinking throughout these last years and improved my work.

I would like to particularly thank my supervisor, PD. Dr. med. Bastian Czogalla, for his exceptional support throughout this project. His competence was invaluable in formulating the research questions and methodology. His patient support and close supervision made it a pleasure to work with him. His motivation and drive are truly inspiring.

I would also like to thank Mrs. Martina Rahmeh and Mrs. Christina Kuhn for training me in the various scientific methods and their excellent technical support.

Most of all, I would like to thank my parents and partner, Julian, for their wise counsel and empathic ear. Their love and support have guided me throughout the way.