Unravelling the role of signal transduction pathways in high-grade serous carcinogenesis

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The road towards personalized treatment of ovarian cancer?

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For reasons of consistency in this thesis, some terms and abbreviations have been standardised throughout the text and might therefore slightly differ from the original publications.

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Promotor

Prof. dr. R.L.M. Bekkers

Copromotoren

Dr. J.M.J. Piek (Catharina Ziekenhuis)
Dr. A. van de Stolpe (Drug Companion Diagnostics Company - Therapeutics)
Dr. S. Lambrechts

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Drs. I.P.W. Bekkers
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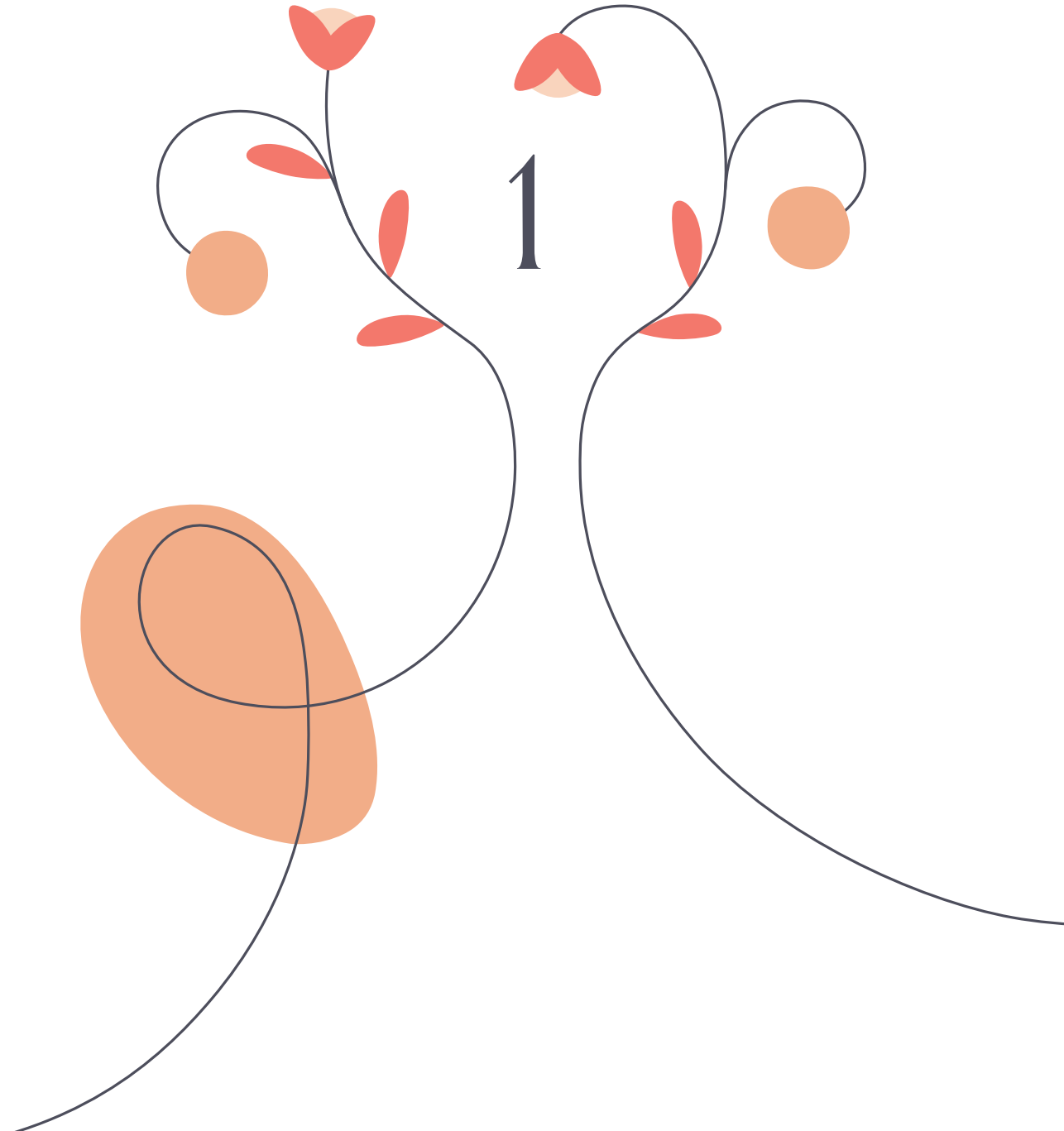
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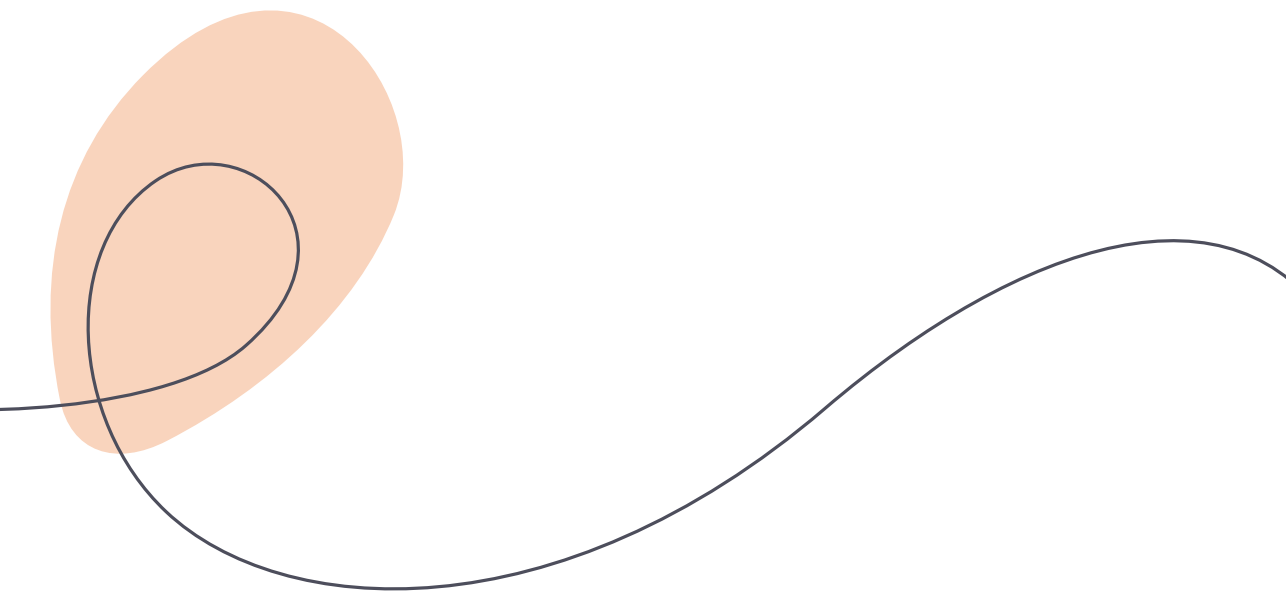
CHAPTER 1

General introduction and outline of this thesis



Ovarian cancer

Ovarian cancer represents a significant health problem. The disease typically presents at an advanced stage due to nonspecific early warning symptoms and a lack of effective screening options.[1] Consequently, ovarian cancer is the fifth leading cause of cancer death amongst women in the Western world.[2] The vast majority of ovarian cancers are of epithelial origin.[3] Histologically, epithelial ovarian cancers are classified into five main subtypes: high-grade serous, low-grade serous, endometrioid, mucinous and clear cell carcinomas.[3] The high-grade serous subtype accounts for approximately 70% of ovarian carcinomas, followed by endometrioid (10%), mucinous (10%), low-grade serous (<5%) and clear cell (<5%).[4] These subtypes differ in cellular origin, genomic alterations, chemosensitivity and prognosis resulting in a highly heterogeneous disease with major differences in molecular biology.[4] Traditionally, these differences have not been adequately recognized in clinical trials, resulting in a common therapeutic regime for advanced stage ovarian cancer, regardless of histological subtype, consisting of a combination of platinum and paclitaxel containing chemotherapy and cytoreductive surgery.[5]

High-grade serous ovarian carcinoma

In this thesis, we focus predominantly on high-grade serous ovarian carcinoma (HGSC) which covers ovarian, Fallopian tube and primary peritoneal carcinomas.[5] Generally, patients diagnosed with HGSC respond well to platinum-based chemotherapy at first-line treatment.[6] Unfortunately, despite an initial response rate of 70-80%, current standard treatment remains insufficient to acquire long-term survival in the majority of the patients.[7] Relapsed disease almost invariably occurs and becomes platinum-resistant, resulting in a five-year overall survival ranging between 26-42% for advanced stage disease.[8]

Despite the poor prognosis some patients do survive without recurrence for more than five-years, indicating a variability in biological behaviour.[9] So far, the molecular mechanisms behind rapid recurrence and platinum-resistance have only been partially elucidated. Studies show that amplification of *CCNE1*, a gene involved in cell cycle regulation, is associated with platinum-resistance at initial treatment, and several mechanisms related to deoxyribonucleic acid (DNA) repair processes are found to be associated with acquired platinum-resistance.[10-12] Moreover, the tumour micro-environment of platinum-resistant ovarian cancer is characterized by reduced infiltration of effector immune cells and increased infiltration of immune suppressive cells.[13] Although these results have broadened our understanding of response to standard platinum-based therapy, survival rates have not significantly improved over the last decades.[14] Thus, there remains a high need to unravel the molecular characteristics of HGSC to constitute new therapeutic approaches.

The high-grade serous subtype is characterised by a high frequency of mutations in the *TP53* tumour suppressor gene, a gene involved in DNA repair processes.[4] Although the name suggests an ovarian tissue of origin, until today there is no convincing evidence of precursor lesions of HGSC in the ovary.[15,16] In 2001, the discovery of atypical lesions, also known as serous tubal intraepithelial carcinoma (STIC), in the distal ends of prophylactically removed Fallopian tubes of women with a hereditary high risk to develop HGSC resulted in a paradigm shift in the prevailing theory of the cell-type of origin.[17] Similar to the nearly universal characteristics of HGSC, STIC were found to have mutations in the *TP53* gene.[18] Subsequent studies identified earlier lesions preceding STIC in the fimbrial epithelium, termed 'p53 signatures'.[19,20] These benign single-cell layer lesions lack the histological features of STIC but harbour the same *TP53* mutations, indicating aberrant p53 signalling as a critical early event in the initiation of STIC and HGSC. According to this hypothesis, precursor cells shed from the distal Fallopian tube and then spread to the ovary and/or peritoneal cavity to progress into HGSC. Currently, it is accepted that STIC play an important role in the pathogenesis of HGSC and the Fallopian tube epithelium is now recognized as the predominant site of origin of HGSC.[21]

Carcinogenesis and signal transduction pathways

The process of tumour development is characterized by abnormal cell proliferation, resistance to apoptosis, limitless potential for replication, sustained angiogenesis and the invasion of adjacent tissue and metastasis.[22] This complex and dynamic mechanism of action, also termed carcinogenesis, is mostly induced by genomic instability.[23] Genomic changes including mutations, amplifications and deletions can orchestrate these capabilities in tumour cells and thereby, drive abnormal cell proliferation either by activation of tumour-promoting signalling or by loss-of-function of tumour-suppressive factors.[23] Aberrant gene expression may also be triggered by nonmutational changes acquired by epigenetic mechanisms, for example DNA methylation and histone modification. Furthermore, the tumour microenvironment adds an additional dimension of complexity. For instance, infiltrating inflammatory immune cells could have both tumour-promoting and tumour-antagonizing actions.[24] Moreover, various tumour stroma cells such as fibroblasts and endothelial cells contribute to the secretion of growth factors to induce inflammation and tumour-associated angiogenesis.[25] As a result, the tumour cell phenotype encompasses not only genomic alterations, but also epigenetic changes and interactions with the tumour microenvironment.

Most of the abovementioned characteristics of carcinogenesis result from dysregulation of cellular activity of signal transduction pathways (STPs), in which genomic aberrations frequently trigger aberrant activity.[26] Naturally, STPs are tightly controlled cellular mechanisms of protein interactions and are core regulators of

important physiological processes.[27] STPs are typically activated by binding of an appropriate ligand to a membrane or intracellular receptor (**Figure 1.1**).

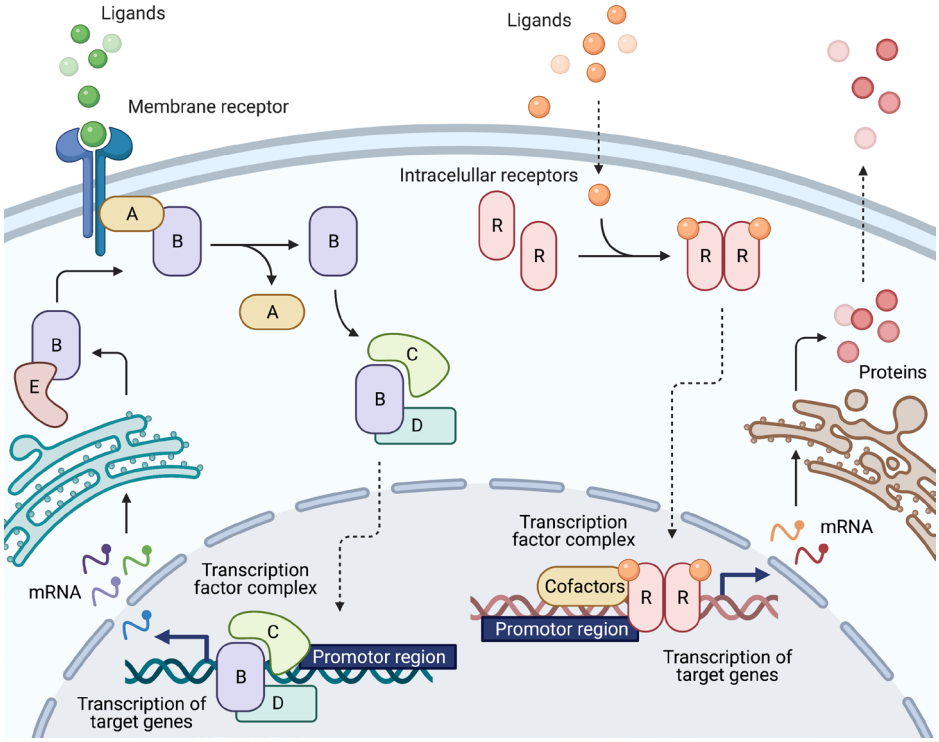


Figure 1.1 Schematic overview of the activation of signal transduction pathways. Left: extracellular binding of a ligand to a membrane receptor initiates an intracellular response resulting in the activation of protein B by the release of protein A. This allows binding of protein C and D to protein B. The formed transcription factor complex translocates to the nucleus where it binds the DNA promotor region and activates transcription of target genes. Right: Upon intracellular ligand-binding, the nuclear receptors undergo a conformational change and form a complex which translocates to the nucleus to bind the promotor region and recruit cofactors. Both sides: activation of gene transcription induces the synthesis of messenger RNA (mRNA), which is translated in the cytosol by the ribosome to produce proteins for intra- or extracellular actions (this figure is created with BioRender.com).

Ligand-binding initiates a conformational change which allows activation of several downstream signalling components and mediates the formation of a transcription factor complex. Subsequently, the transcription factor complex differentiates into the cell nucleus and binds to the promotor region of the DNA response element to activate transcription of pathway-specific target genes. Once messenger ribonucleic acid (mRNA) is produced by transcription, the nucleotide sequence is transported from the nucleus into the cytosol. Here, mRNA is translated by the ribosome into

proteins which either will be transported outside the cell or exert an intracellular function. By this communication mechanism, cells convert extracellular stimuli into specific cellular responses.

STPs can be classified as hormone driven pathways (e.g. androgen receptor (AR) and oestrogen receptor (ER)), growth factor pathways (e.g. phosphoinositide 3-kinase (PI3K)), and developmental pathways (e.g. Hedgehog (HH), transforming growth factor beta (TGF-β) and canonical wingless-type MMTV integration site (Wnt)).[28-33] STPs control important physiological processes in the female genital tract, such as regulation of the menstrual cycle, ovarian function and embryonic development.[34-37] However, in tumour cells, the strictly controlled balance of STP activity is disturbed, resulting in dysregulated cell proliferation, differentiation and migration. Tumour growth can be either driven by disruption of a single STP or by disruption of crosstalk between multiple STPs. For HGSC, concurrent dysregulation of several STPs appears to be more common as gene expression research showed intrinsic heterogeneity of genomic alterations and failed to identify a distinct prognostic gene expression pattern associated to survival.[38]

Signal transduction pathways and targeted treatment strategies

Over the past decades, increasing knowledge of the complex mechanisms of action of STPs and advances in whole genome sequencing technologies contributed to the introduction of personalized treatment strategies based on molecular characteristics. The identification of potentially actionable genomic alterations stimulated the development of numerous targeted drugs aiming to inhibit aberrantly activated STPs which drive tumour growth. In principle, this treatment strategy compromises a personalized approach involving drugs that target receptors or specific proteins to block hyperactivity of STPs in tumour cells. In contrast to cytotoxic chemotherapeutic agents that inhibit cell proliferation and cause apoptosis, targeted drugs interfere with the activity of STPs to alter the cellular response. The main rationale of personalized medicine is to allow for a tailored approach for individual patients aiming for a long-term response. For this treatment strategy to be successful, prediction of treatment response by identification of functional activity of the tumour-promoting STP is of most importance to enable accurate patient selection.

In clinical practice, genomic alterations are often used as a surrogate marker to determine the tumour-driving STP. Over the past years, this resulted in a few therapeutic successes for ovarian cancer patients.[39] For instance, somatic and germline pathologic variant mutations in *BRCA1/2* tumour-suppressor genes were observed in 22% of patients with HGSC.[40] These genes encode for proteins essential in DNA damage repair by homologous-recombination and alterations in these genes induce deficiencies to resolve spontaneous DNA damage.[41] In this landscape,

treatment strategies with poly(ADP-ribose) polymerase (PARP) inhibitors were introduced in germline *BRCA1/2*-mutated HGSC patients.[42,43] The inhibition of PARP results in DNA breaks which are usually repaired by the homologous-recombination repair pathway. However, due to loss-of-function of this pathway by *BRCA1/2* gene mutations, PARP inhibition results in cell death by synthetic lethality. Eventually, early clinical trials indicated a remarkable prolongation of progression-free survival and enabled the first successful approval of PARP inhibitors for standard treatment of HGSC patients in 2014.[44]

Besides mutations in *BRCA1/2* genes, molecular profiling studies indicated other potentially actionable genomic alterations in ovarian cancer patients.[45,46] Unfortunately, only a modest subset of the patients treated with mutation based targeted therapies yielded clinical benefit.[47] Although genomic aberrations frequently cause abnormal STP activity, the clinical relevance of genomic aberrations often remains difficult to establish. Unsatisfactory results may be related to the assumption that presence of genomic alterations provides information on the functional activation status of STPs. However, emerging evidence suggests that genomic alterations do not always translate into transcriptional activation, and therefore are insufficient predictors of therapy response.[48,49]

In addition to genomic alterations, immunohistochemical staining of signalling proteins remains a widely used technique to aid patient selection for targeted therapy. Immunohistochemistry involves the detection of expression levels of signalling proteins by labelled antibodies. For ovarian cancer, ER protein expression is often considered a biomarker for sensitivity to anti-oestrogen targeted drugs.[50] However, in line with genomic alterations, protein expression as detected by immunohistochemistry may not always be equivalent to transcriptional activation of a STP. With regard to the ER signalling pathway, nuclear presence of the receptor is required but availability of oestrogen and binding of cofactors remains essential for transcriptional activation of the pathway.[51]

In summary, identification of functional activity of tumour-driving STPs remains a challenge with current diagnostics and successful stratification of ovarian cancer patients for targeted therapy based on their molecular signature has been limited so far. Therefore, there remains a high unmet need for reliable diagnostics to predict targeted therapy response for the implementation of personalized medicine in the treatment of ovarian cancer patients.

Signal transduction pathway activity assays

In recognition of the importance of the tumour cell phenotype, Verhaegh *et al.* developed an alternative approach to identify functional STP activity while taking the

tumour microenvironment into consideration.[52,53] This approach provides information on gene transcription regulation by mRNA measurements of direct pathway-specific target genes. At the basis of these assays is a computational knowledge-based Bayesian network which uses mRNA measurements as input to quantitatively measure STP activity.[52] As shown in **Figure 1.2**, the Bayesian network structure is comprised of three layers and describes the causal relation between activation of a transcription factor complex and the measured mRNA levels of pathway-specific target genes. The first layer corresponds to the transcription factor complex associated with a specific STP, the second layer indicates direct target genes of the transcription factor complex and the third layer represents the measured mRNA expression levels. The pathway-specific target genes were selected based on validated literature. In contrast to traditional biomarkers where STP activity is derived from a single molecular trait (e.g. protein expression or gene mutation), the pathway model infers the odds of an active STP from the expression levels of several corresponding target genes and is therefore thought to be a more specific way to quantify STP activity.

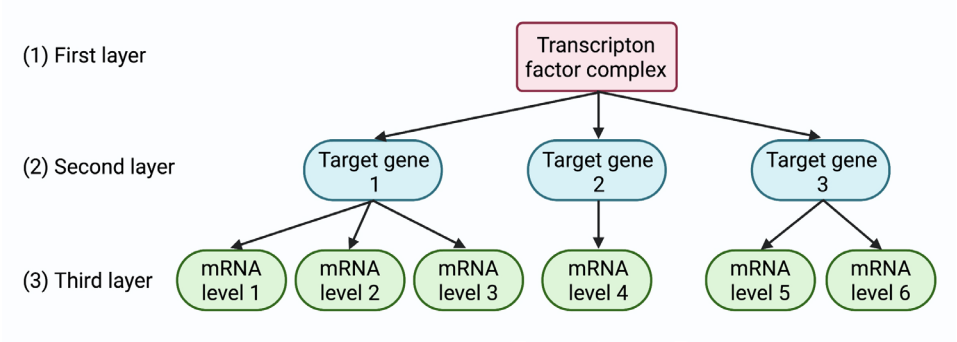


Figure 1.2 Schematic overview of the network structure of the pathway models describing the odds of the transcription factor complex being active or inactive being inferred backwards from the mRNA expression levels of pathway-specific target genes (this figure is created with BioRender.com).

Initially, the STP activity assays were developed using Affymetrix expression microarray data which facilitated the calibration and validation of the models by the use of publicly available datasets containing data of samples with a 'ground truth' pathway activity.[49,52-55] For instance, the Wnt pathway in known to be activated in colon carcinoma, while inactive in normal colon epithelium.[52] Furthermore, cell lines experiments in which a specific STP is activated by adding a ligand, or conversely, is inhibited by adding a blocking drug provided evidence that the models can discriminate between an active and inactive state.[55] Adaptation of the assays by a selection of the most informative target genes further enabled the use of mRNA

measurements from formalin-fixed paraffin-embedded tissue samples by real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis.[56-59] Until now, STP activity assays have been developed to assess the functional activity of the AR, ER, PI3K, HH, TGF-β, Wnt, Notch and mitogen-activated protein kinase (MAPK) pathways.

Aims and outline of this thesis

In order to improve the implementation of targeted treatment strategies and constitute new therapeutic approaches for patients with HGSC, further stratification with appreciation of molecular profiles is essential. In this context, we need to increase our understanding of HGSC behaviour and, more importantly, we need to improve patient stratification methods to allocate patients to effective therapies. Therefore, this thesis aims to unravel the role of important cellular processes that drive HGSC development and progression by the characterization of both normal STP activity in healthy Fallopian tube epithelium and aberrant STP activity in HGSC and precursor lesions. We explore several possible targeted treatment options for HGSC patients and investigate the therapeutic value of current biomarkers (e.g. immunohistochemical protein expression and genomic alterations) in the selection of patients for treatment with targeted drugs. Ultimately, we elaborate on the future role of a novel method for stratification of ovarian cancer patients in order to direct treatment to more effective personalized therapies.

In **Chapter 2**, we report on the activity of key STPs in morphologically normal fimbrial epithelium, the tissue of origin of most HGSC. We assess the range of normal STP activity and investigate the influence of the hormonal cycle on STP activity in the Fallopian tube. In order to identify early aberrations in STP activity that may contribute to high-grade serous carcinogenesis, we compare STP activity in STIC with concurrent HGSC in relation to STP activity in normal fimbrial epithelium in **Chapter 3**. In this chapter we elaborate on the possible roles of the ER, PI3K and HH signalling pathways in the processes related to transformation of normal fimbrial epithelium into STIC. **Chapter 4** describes a retrospective cohort study in which STP activity is compared in patients diagnosed with advanced stage HGSC with a short and long disease-free survival. In this chapter we investigate STP activity in relation to disease recurrence and report on the role of the ER signalling pathway in postmenopausal HGSC. In **Chapter 5**, we conduct a systematic review of the literature regarding the clinical benefit of anti-oestrogen therapy in HGSC patients. Furthermore, we explore the available evidence of a correlation between ER protein expression and therapy response to determine whether immunohistochemical ER protein expression is a predictive biomarker for treatment sensitivity. In the search for a biomarker for anti-oestrogen therapy, we study in **Chapter 6** whether ER protein expression could be considered indicative of a functionally active ER signalling pathway in HGSC.

Subsequently, in **Chapter 7** we perform a meta-analysis of the clinical benefit of PI3K/AKT/mTOR pathway inhibitors in ovarian cancer. Here, we describe the effectiveness of selection of patients based on current PI3K/AKT/mTOR biomarkers. Eventually, we propose a multicenter prospective, parallel group cohort study for the implementation of STP activity assays in selecting patients for matched targeted therapy in **Chapter 8**. Furthermore, **Chapter 9** presents the main findings of this thesis in a larger perspective and discusses the implications for clinical practice. After that, the results and conclusions are summarized in **Chapter 10**. Finally, **Chapter 11** reflects on the scientific relevance and societal impact of this thesis.

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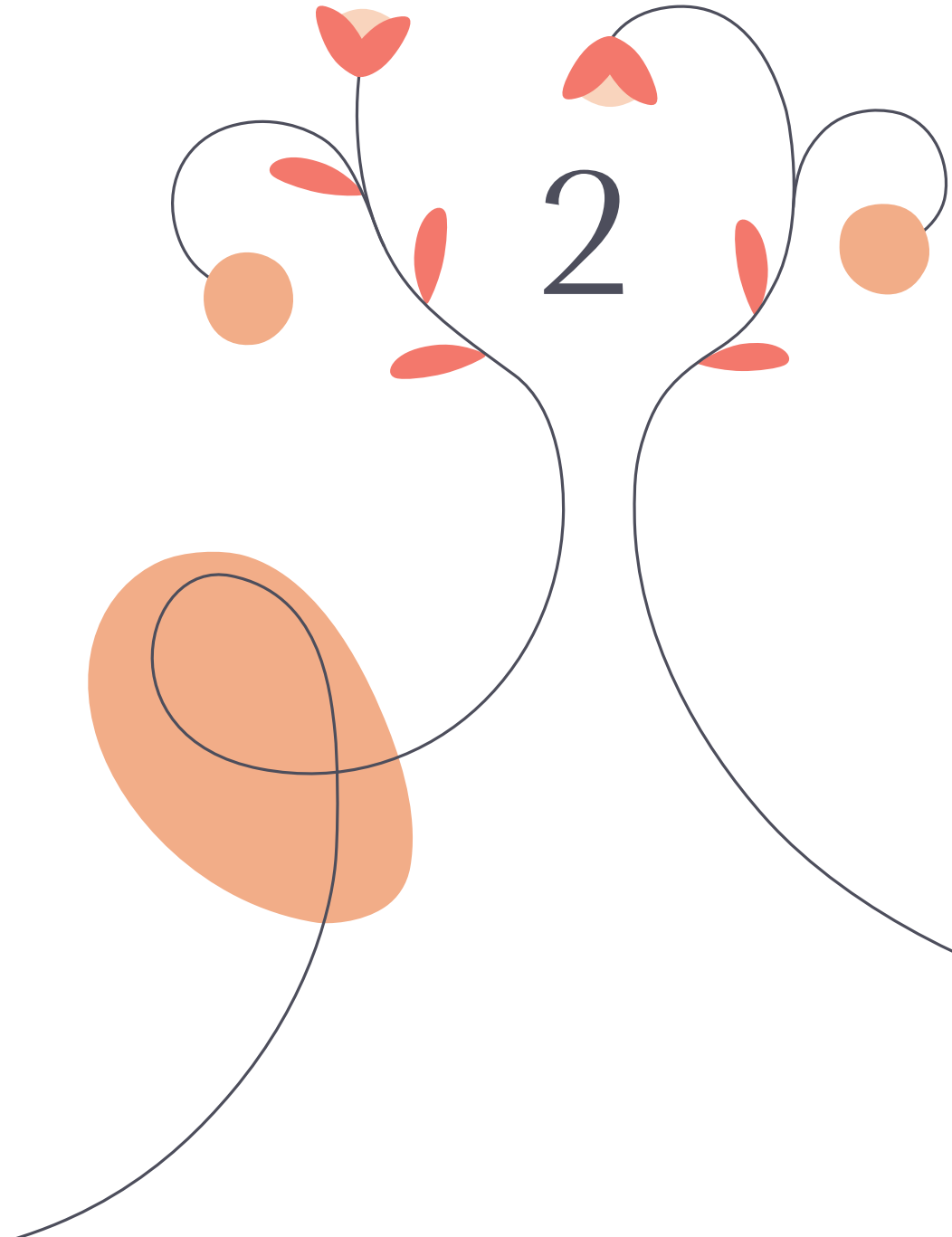
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CHAPTER 2

Cyclic activity of signal transduction pathways in fimbrial epithelium of the human Fallopian tube

Phyllis van der Ploeg, Aniek Uittenboogaard, Karlijn M.M. Bucks, Marjolein H.F.M. Lentjes-Beer, Steven L. Bosch, Minouche M.E. van Rumste, M. Caroline Vos, Paul J. van Diest, Sandrina Lambrechts, Anja van de Stolpe, Ruud L.M. Bekkers, Jurgen M.J. Piek

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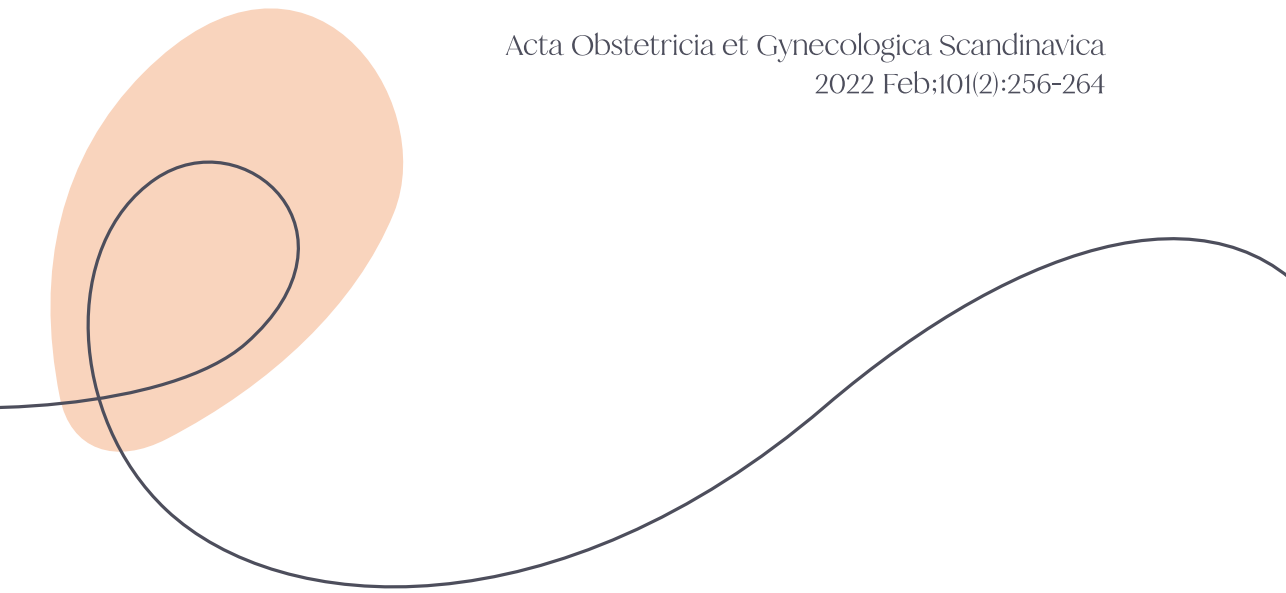

Abstract

Introduction

The local environment of the Fallopian tube represents the optimal conditions for reproductive processes. To maintain tissue homeostasis, signal transduction pathways (STPs) are thought to play a pivotal role. Enhancing our understanding of functional STP activity is important to be able to clarify the role of aberrant STP activity leading to female subfertility and other tubal diseases. Therefore, in this study we investigate the influence of the hormonal cycle on the activity of key STPs in the fimbrial epithelium of morphologically normal Fallopian tubes.

Material and methods

We included healthy pre- (n=17) and postmenopausal (n=8) patients who had surgical interventions for benign gynaecological conditions. Histological sections of the Fallopian tubes were reviewed by two pathologists and, for the premenopausal patients, hormone serum levels and sections of the endometrium were examined to determine the hormonal phase (early follicular (n=4), late follicular (n=3), early luteal (n=5), late luteal (n=5)). After laser capture microdissection, total mRNA was extracted from the fimbrial epithelium and real-time quantitative reverse transcription-PCR was performed to determine functional STP activity of the androgen receptor (AR), oestrogen receptor (ER), phosphoinositide 3-kinase (PI3K), Hedgehog (HH), transforming growth factor-beta (TGF-β) and canonical wingless-type MMTV integration site (Wnt) pathways.

Results

The early luteal phase demonstrated high AR and ER pathway activity compared to the late luteal phase (P=0.016 and P=0.032, respectively) and low PI3K activity compared to the late follicular phase (P=0.036), while the late luteal phase showed low activity of HH and Wnt compared to the early follicular phase (both P=0.016). STP activity in fimbrial epithelium from postmenopausal patients was most similar to the early follicular and/or late luteal phase with regard to the AR, ER and PI3K pathways. Wnt pathway activity in postmenopausal patients was comparable to the late follicular and early luteal phase. We observed no differences in HH and TGF-β pathway activity between pre- and postmenopausal samples. The cyclic changes in STP activity suggest a stage-specific function which may affect the morphology and physiology of the human Fallopian tube.

Conclusion

We demonstrated cyclic changes in activity of the AR, ER, PI3K, HH and Wnt pathways throughout the hormonal cycle.

Introduction

The Fallopian tube has essential roles in human reproduction as it facilitates ovum and semen transport, fertilization and early embryonic development.[1] Due to the close proximity, the Fallopian tube epithelium (FTE) is regularly exposed to changing hormone levels produced by the ovary.[2] At the time of ovulation, when the oocyte is expelled from the dominant follicle, the fimbrial epithelium is embedded in pro-inflammatory follicular fluid.[2] There is evidence that exposure to follicular fluid induces tissue injury and altered gene expression in the fimbrial epithelium.[3] As a result, the FTE must be able to restore tissue homeostasis to prevent irreversible damage.

Cascades of protein interactions, named signal transduction pathways (STPs), are known to regulate many cellular processes including cell proliferation, differentiation and survival.[4] During the hormonal cycle, physiological changes in the morphology and function of the FTE are thought to be regulated by STP activity. Previous studies indicated a role for the androgen (AR) and oestrogen receptor (ER)[5] and Hedgehog (HH)[6] pathways in normal epithelial homeostasis, while aberrant activity of the phosphoinositide 3-kinase (PI3K)[7], transforming growth factor-beta (TGF-β)[8] and canonical wingless-type MMTV integration site (Wnt)[9] pathways was associated with processes leading to female subfertility.

Interest in the FTE is growing as the local environment of the Fallopian tube represents the optimal conditions for the reproductive process. Establishing knowledge on normal STP activity in the FTE is pivotal in the understanding of reproductive aspects, as well as aberrant STP activity in tubal diseases. However, the molecular processes that affect gene expression in the Fallopian tube during the hormonal cycle remain poorly understood. To measure functional STP activity, Verhaegh *et al.*[10] developed a technique to quantitatively measure activity of the abovementioned STPs based on mRNA levels of pathway-specific target genes. In this study we investigate the activity of key STPs in fimbrial epithelium of morphologically normal Fallopian tubes from pre- and postmenopausal patients.

Materials and methods

Study population

We included healthy premenopausal patients (n=17) who participated in the HYSTUB randomized controlled trial in which patients were randomized to either undergo hysterectomy with or without concurrent salpingectomy (NCT02281487).[11] Surgical indications included heavy menstrual bleeding (n=6), uterine leiomyomas (n=5), cervical dysplasia (n=3) and abdominal pain (n=3). In addition, we included healthy

Fallopian tubes from postmenopausal patients (aged >55 years, n=8) obtained during surgical interventions for benign gynaecological conditions identified by the Dutch national pathology archive (PALGA) between 2009 and 2018. These patients had salpingectomy with or without hysterectomy for a benign ovarian mass (n=5), postmenopausal blood loss without (pre)malignancy (n=2) and uterine descensus (n=1). We excluded patients with a history of gynaecological cancer prior to surgery (except for cervical dysplasia), known pathogenic *BRCA1/2* germline mutations and patients with a positive family history with any type of hereditary cancer. Premenopausal patients did not use any hormones three weeks prior to surgery as this was an exclusion criterion for participation in the HYSTUB trial. None of the patients with uterine leiomyomas were treated with gonadotropin-releasing hormone agonists. For postmenopausal patients, we screened medication history for the use of hormones prior to surgery in order to prevent exogenous hormonal influences. Demographic data including age at surgery, parity, body mass index (BMI) and data of a history of subfertility, ectopic pregnancy, tubal sterilization, endometriosis and adenomyosis were extracted from medical records.

Determination of hormonal cycle phase

Histological sections of the endometrium of premenopausal patients were reviewed by two independent gynaecological pathologists (MHFML-B and SLB) according to predefined characteristics to determine the hormonal cycle phase.[12] As part of the HYSTUB trial, premenopausal patients had been subjected to blood sample collection to measure follicle-stimulating hormone (FSH), luteinizing hormone (LH) and oestradiol serum levels either one day before or the day of surgery.[11] Hormone concentrations and endometrial characteristics were matched to determine hormonal cycle phase. For the premenopausal patients, we decided to characterize the hormonal cycle into four phases (either early/late follicular or early/late luteal) to be able to determine subtle differences in STP activity.

Laser capture microdissection

Histological sections of the Fallopian tubes were reviewed by a pathologist (MHFML-B or SLB) and morphologically normal fimbriae were annotated. Five μm formalin-fixed paraffin-embedded sections were cut on PEN membrane slides (article number 415190-9041-000, Carl Zeiss B.V., Germany) with a microtome (RM2255, Leica Biosystems, Germany). Slides were manually haematoxylin stained. To avoid stromal contamination FTE was harvested using a laser microdissector (PALM MicroBeam 4, Carl Zeiss B.V., Germany).

mRNA extraction and real-time PCR analysis

After laser capture microdissection, total mRNA was extracted according to the manufacturer's protocol (VERSANT® Tissue Preparation Reagents kit, Siemens, Germany). Real-time quantitative reverse transcription-PCR (RT-qPCR) was performed using the SuperScript™ III Platinum™ One-Step qRT-PCR kit (Invitrogen, Thermo Fisher Scientific, USA). Commercially available OncoSignal 96-well PCR plates (Philips MPDx, the Netherlands) were processed with a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Internal quality control of reference genes confirmed sufficient input for pathway analysis.

Signal transduction pathway activity assays

With the use of commercially available RT-qPCR-based OncoSignal pathway assays (Philips MPDx, the Netherlands), STP activity of the following pathways was measured: the AR, ER, PI3K (indirectly, as it is inversely proportional to forkhead box protein O (FOXO) transcription factor activity; on the premise that that no cellular oxidative stress is present, as described before[13]), HH, TGF-β and Wnt pathway. The assays quantitatively measure activity of these signalling pathways using Bayesian network computational models which infer activity of the corresponding transcription factor complex from the expression of pathway-specific target genes. The approach has been described in detail before.[10] Originally, the models were developed and validated on multiple cell types using Affymetrix expression microarray data and included approximately 25 to 35 target genes per pathway.[13-15] The selected target genes corresponding to the transcription complex of the ER and Wnt pathways[10], AR, HH and TGF-β pathways[14] and PI3K pathway[13] have been described in detail previously. To facilitate clinical application, the models were adapted based on a selection of the best performing target genes (around 12 target genes) to enable the use of RT-qPCR mRNA measurements from formalin-fixed paraffin-embedded material.[16-18] A major advantage of the assays is the reliable readout of direct target genes of the respective pathways-associated transcription factor because increased expression levels are direct evidence of pathway activation. Conventional methods such as immunohistochemistry or immunoblotting can identify signalling proteins or transcription factor proteins but do not provide reliable and quantitative information on the functional activity state of the proteins, and therefore are not suitable to infer associated STP activity. The assays present functional pathway activity scores on a normalized 0-100 scale, where 0 theoretically corresponds to the lowest and 100 corresponds to the highest odds in favour of an active pathway; however, the actual activity range may be restricted to a certain part of this 0-100 scale, depending on the specific STP and the cell type in which it is measured, as explained before.[14] Given the cell type specific STP range, direct comparison of the activity scores of the different STPs is not possible. However, once

the STP range has been defined, the scores of every new sample can be interpreted against the defined range to classify normal or aberrant activity.

Statistical analysis

Patient characteristics between pre- and postmenopausal groups were compared using independent T-tests or Wilcoxon rank sum tests. Differences in STP activity between hormonal phase groups were tested with pairwise comparison using Wilcoxon rank sum exact tests as the analyses were considered exploratory. Correlations were assessed using Spearman's rank correlation coefficient. *P*-values <0.050 were considered statistically significant. Statistical analysis and data visualization was conducted using R (version 1.1.463).

Ethical approval

The study was approved by the Medical research Ethics Committees United (MEC-U, study number W18.134, 29 August 2018). All patients gave written informed consent and tissues were studied in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands.

Results

Study characteristics

Median age at surgery was 44 years (interquartile range (IQR) 41 – 47) and 62 years (IQR 59 – 67) for the pre- and postmenopausal groups (*P*<0.001), respectively (**Table 2.1**). We observed no statistically significant differences between the pre- and postmenopausal groups regarding BMI, parity, subfertility, ectopic pregnancy, tubal sterilization, endometriosis and adenomyosis. Evaluation of hormone serum levels and histological sections of the endometrium resulted in four early follicular, three late follicular, five early luteal and five late luteal samples. Representative histological images of each hormonal phase are presented in **Supplementary Figure S2.1**. Hormone serum levels per phase of the hormonal cycle are presented in **Figure 2.1** and show that the late follicular phase was characterized by high FSH and LH concentrations and the early luteal phase demonstrated lower oestradiol concentrations compared to the late follicular phase, as described previously.[19] In the early follicular phase, we noticed one patient with a markedly higher oestradiol serum level in relation to the other premenopausal patients. This patient was characterized by a relatively high BMI, which may have resulted in elevated oestradiol production by adipose tissue. However, we observed lower oestradiol levels in patients with higher BMI. Despite that information in the patients' medical record and medication history did not provide an explanation for the elevated oestradiol level, we decided to include this patient in our cohort given our small

sample size. Though, we marked the sample of this patient in the following analysis. Additional clinical details and pathological findings are described in **Supplementary Table S2.1**.

Table 2.1 Patient characteristics of the pre- and postmenopausal groups.

Variables	Premenopausal (n=17)	Postmenopausal (n=8)	*P*-value
Age at surgery (years)			<0.001
Median (IQR)	44 (41 – 47)	62 (59 – 67)	
BMI (kg/m^2)			0.457
Mean (SD)	26.4 (3.4)	27.5 (3.8)	
Parity (number)			0.109
Median (IQR)	2 (1 – 3)	2 (2 – 4)	
Subfertility			1.000
Yes (%)	2 (12%)	0 (0%)	
No (%)	15 (88%)	8 (100%)	
Ectopic pregnancy			1.000
No (%)	17 (100%)	8 (100%)	
Tubal sterilization			1.000
Yes (%)	4 (24%)	1 (12%)	
No (%)	13 (76%)	7 (88%)	
Endometriosis			0.527
Yes (%)	3 (18%)	0 (0%)	
No (%)	14 (82%)	8 (100%)	
Adenomyosis			0.411
Yes (%)	10 (59%)	3 (37%)	
No (%)	7 (41%)	5 (63%)	
Hormonal cycle phase			
Early follicular	4 (24%)	-	-
Late follicular	3 (18%)	-	-
Early luteal	5 (29%)	-	-
Late luteal	5 (29%)	-	-

Abbreviations: BMI, body mass index; IQR, interquartile range; SD, standard deviation.

Signal transduction pathway activity in the human Fallopian tube during the hormonal cycle

Our results demonstrate gradual differences in STP activity of the AR, ER, PI3K, HH and Wnt pathways in FTE during the hormonal cycle (**Figure 2.2**).

Throughout the follicular phase, we observed minimal changes in STP activity without statistically significant differences, possibly due to small sample number. The early follicular phase was distinguished by higher HH, TGF-β and Wnt pathway activity followed by a slight decrease towards the late follicular phase. In addition, a slight increase in AR and PI3K pathway activity was observed towards the late follicular phase.

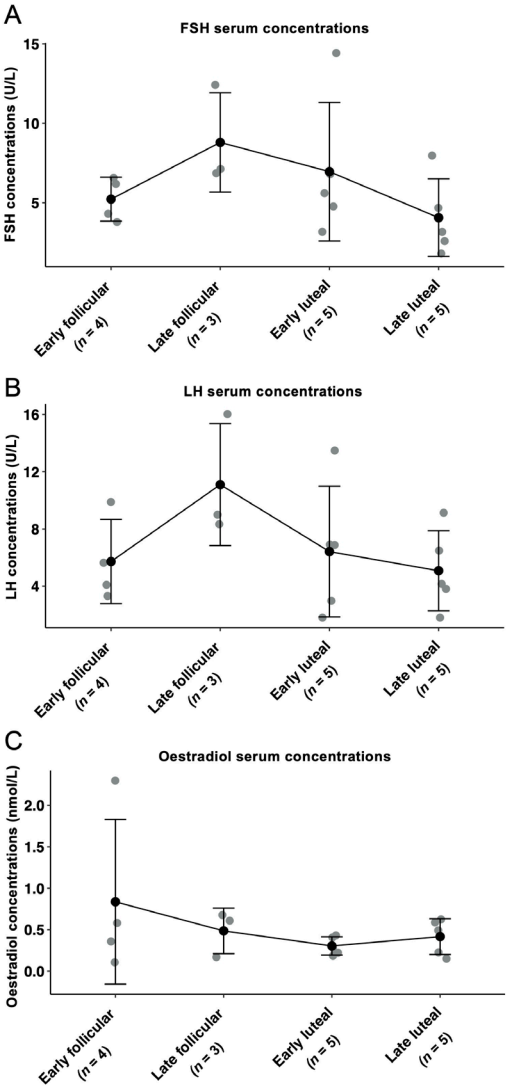

Figure 2.1 Line graphs displaying hormone serum concentrations in premenopausal patients during the menstrual cycle. **A.** Follicle-stimulating hormone (FSH) serum concentrations (U/L). **B.** Luteinizing hormone (LH) serum concentrations (U/L). **C.** Oestradiol serum concentrations (nmol/L). Black dots with error bars represent mean concentrations with standard deviations. Gray dots indicate individual patients. Note that in the early follicular phase there is one patient with markedly higher oestradiol concentrations which strongly influenced the mean concentration during this phase. Repeated analysis after exclusion of this patient demonstrated higher oestradiol concentrations during the late follicular and late luteal phase when compared to the early follicular and early luteal phase.

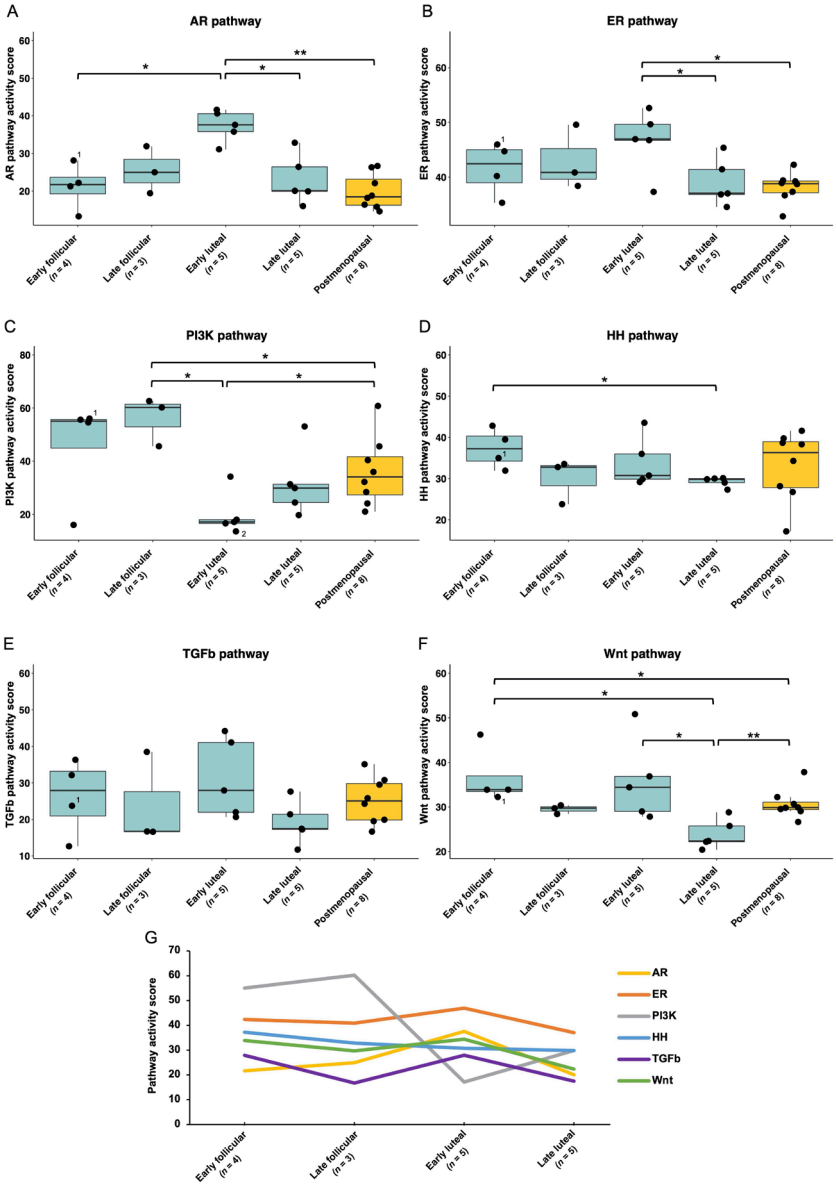

Figure 2.2 Signal transduction pathway (STP) activity in fimbrial epithelium of Fallopian tubes from pre- and postmenopausal patients. **A.** Androgen receptor (AR). **B.** Oestrogen receptor (ER). **C.** Phosphoinositide 3-kinase (PI3K). **D.** Hedgehog (HH). **E.** Transforming growth factor beta (TGFb). **F.** Canonical wingless-type MMTV integration site (Wnt). **G.** Simplified overview of median STP activity scores measured during the menstrual cycle. Direct comparison of the activity scores of the different STPs is not possible as every STP has his own cell type specific range of STP activity. *P<0.050; **P<0.010; [1] Patient with a relatively high oestradiol serum concentration. Repeated analysis after exclusion of this patient demonstrated minimal changes in significance levels without influencing our conclusions. [2] Sample with evidence for oxidative stress.

The early luteal phase was characterized by peak activities in AR and ER pathway activity and low PI3K pathway activity. AR pathway activity was significantly higher during the early luteal phase compared to the early follicular phase (*P*=0.016), while PI3K pathway activity was significantly lower during the early luteal phase compared to the late follicular phase (*P*=0.036). In one of the early luteal samples, we observed high FOXO activity in conjunction with high superoxide dismutase 2 (SOD2) expression. The *SOD2* gene codes for a protein that protects against oxidative damage.[13] This may indicate cellular oxidative stress as an alternative cause of FOXO activity. Therefore, in this sample PI3K pathway activity should be interpreted with caution. Repeated analysis after exclusion of this patient still demonstrated the lowest PI3K pathway activity in the early luteal phase but with borderline significance compared to the late follicular phase (*P*=0.057). ER pathway activity during the early luteal phase was not significantly different from the follicular phase, which was likely to be influenced by the outlier with low ER pathway activity in the early luteal phase. Consequently, exclusion of this sample showed a significant difference between the early follicular and early luteal phase (*P*=0.029).

Towards the late luteal phase, both the AR and ER pathway demonstrated a distinct decrease (*P*=0.016 and *P*=0.032, respectively). The decrease in TGF-*β* pathway activity between the early luteal phase and late luteal phase reached borderline significance (*P*=0.056). Activity of the Wnt pathway was significantly lower during the late luteal phase compared to the early luteal phase (*P*=0.016), while both the HH and Wnt pathway demonstrated significantly lower activity during the late luteal phase compared to the early follicular phase (both *P*=0.016).

The STP activity in FTE from postmenopausal patients showed most similarities with the early follicular and/or late luteal phase regarding the AR, ER and PI3K pathways. We observed significant differences in AR and ER pathway activity when comparing the postmenopausal samples to the early luteal phase (*P*=0.002 and *P*=0.040, respectively), while PI3K activity in postmenopausal samples differed from the late follicular phase (*P*=0.048) and early luteal phase (*P*=0.019). Wnt pathway activity in postmenopausal samples was similar to the late follicular and early luteal phase, as the activity clearly differed from the early follicular (*P*=0.028) and late luteal phase (*P*=0.003). For the HH and TGF-*β* pathways, we observed no differences in STP activity between the pre- and postmenopausal samples.

Subsequently, we examined whether oestradiol serum levels were associated to functional activity of the hormonal pathways. For both ER and AR pathway activity, we observed no statistically significant correlation with oestradiol levels (Spearman R=0.150, *P*=0.550 and Spearman R=0.037, *P*=0.890, respectively). However, a positive

correlation was found between and ER and AR pathway activity (Spearman R=0.620, *P*=0.001).

Discussion

This is the first study to investigate functional STP activity in the human FTE. Our results demonstrate that STP activity in FTE changes during the hormonal cycle. Specifically, the early luteal phase showed high AR and ER pathway activity compared to the late luteal phase and low PI3K activity compared to the late follicular phase, while the late luteal phase showed low activity of the HH and Wnt pathway compared to the early follicular phase (**Figure 2.2**). The STP activity in FTE from postmenopausal patients was most similar to the early follicular and/or late luteal phase with regard to the AR, ER and PI3K pathways. Wnt pathway activity in postmenopausal patients was comparable to the late follicular and early luteal phase.

Previously, presence of the androgen and oestrogen hormone receptors has been demonstrated by immunohistochemistry in FTE of both pre- and postmenopausal patients, suggesting hormonal responsiveness.[5] We observed a positive correlation and comparable pattern of gradual differences in AR and ER pathway activity in FTE during the course of the hormonal cycle (**Figure 2.2A-B**). However, the pattern of AR and ER pathway activity did not resemble the measured fluctuations in oestradiol serum levels (**Figure 2.1C**). In line with previous evidence indicating decreased oestradiol serum levels after ovulation, we observed lower oestradiol levels during the early luteal phase compared to the late follicular phase.[19] Interestingly, in FTE the highest ER and AR pathway activity was observed during the early luteal phase. With no significant correlation between oestradiol serum levels and ER or AR pathway activity, our findings show that pathway activity in FTE is not sufficiently reflected by serum ligand availability. In addition, it is more likely that the FTE is influenced by the local paracrine function of sex steroid hormones. After ovulation, the fimbriae are exposed to follicular fluid which is released from the dominant follicle. Follicular fluid is mainly composed of steroid hormones, growth factors, cytokines and reactive oxygen species.[20] In relation to serum levels, extremely high levels of oestradiol have been measured in follicular fluid.[21] Therefore, after ovulation the fimbriae are embedded in an environment enriched with steroid hormones, which may contribute to the prolonged high AR and ER pathway activity after ovulation.

Surprisingly, we found preserved AR and ER pathway activity in postmenopausal samples while the ovaries ceases to produce hormones after menopause, suggesting a role for hormonal signalling irrespective of circulating serum levels.[22] Although in postmenopausal patients residual hormones are produced by extragonadal conversion in peripheral tissues, for instances in vascular endothelium, brain, bone

and adipose tissue, these hormones predominantly act on local tissues.[23] Alternatively, our data may suggest that the Fallopian tube epithelial cells have the ability to synthesis hormones and act as an intracrine factor to maintain intra-cellular hormone metabolism. Thereby the epithelial cells may actively modify their hormonal signalling without being dependent on endocrine or paracrine concentrations. On this basis, it is reasonable to expect that FTE would express aromatase activity to facilitate local oestrogen biosynthesis from circulating precursors, as is the case in peripheral tissues.[24] Although aromatase activity has been reported in oviductal epithelium of mammals[25], a study with human Fallopian tubes of premenopausal patients failed to identify aromatase expression.[26] However, there is supportive evidence for intracrine biosynthesis via alternative enzymes for example conversion of the precursor estrone sulfate using steroid sulfatase.[27] So far, studies investigating intracrinology of the Fallopian tube have been underrepresented in previous literature, suggesting further studies may focus on this area of investigation.

Another finding of our study was high PI3K pathway activity during the follicular phase compared to the luteal phase (**Figure 2.2C**). Considering that the PI3K pathway is central to the control of cell proliferation, metabolism and survival, this might suggest a role in regulation of morphologic changes of the epithelium. In the endometrium, the downstream effectors of PI3K, such as AKT, have shown to influence cell motility.[28] Moreover, endometrial decidualization, which occurs during the luteal phase under influence of progesterone, is characterized by decreased activity of the PI3K/AKT pathway.[29] Progesterone is able to negatively influence PI3K/AKT signalling in endometrial cells, as progesterone receptor signalling induced FOXO transcription factor activity, and thus, caused decreased PI3K activity.[30] These findings are in line with our results as we observed a distinct decrease in PI3K pathway activity in FTE in the luteal phase.

As mentioned, tight regulation of (in)activation of STP activity may be important to support morphologic changes of the epithelium. Such transformations include increased mitotic activity and height of ciliated and secretory cells during the follicular phase stimulated by oestrogens to facilitate successful ovum and semen transport, while high levels of progesterone are associated with deciliation and atrophy during the luteal phase.[31] After menopause, the percentage of ciliated cells significantly decreases with loss of secretory activity.[32] There is supportive evidence that primary ciliary expression helps coordinate several signalling pathways, including TGF-β, HH and Wnt signalling, as mediators by receiving extracellular signals.[33-35] Besides that, the formation of cilia was also found to be regulated by signalling activity.[33] A study investigating primary cilia on secretory cells of the Fallopian tube demonstrated that primary cilia were responsive to HH signalling,

suggesting this pathway is involved in ciliary function.[6] Moreover, murine experiments confirmed a role for Wnt signalling in oviductal epithelial homeostasis as the addition of a Wnt inhibitor to an oviduct culture model resulted in a decreased embryo transport distance.[36] Others confirmed that conditional overactivation of *β*-catenin, an important signalling member of the Wnt pathway, resulted in expansion of secretory cells, while ablation of *β*-catenin reduced the proportion of secretory cells.[37] Our findings showed an identical pattern in HH and Wnt pathway activity, wherein the early follicular phase showed higher activity than the late luteal phase (**Figure 2.2D and F**). Unfortunately, our results reflect pathway activity in both ciliated and secretory cells, making it difficult to uncover a cell type specific function during the hormonal cycle. Nevertheless, detailed characterization of these pathways might help explain diseases associated with ciliary dysfunction, and consequently, female subfertility.

The major strength of this study is the quantitative measurement of functional activity of several key STPs. Conventional methods, such as immunohistochemical staining, are limited by the identification of individual proteins, and the presence of a protein does not automatically imply that the complete STP is activated. By the use of the pathway assays based on mRNA levels of pathway-specific target genes we were able to measure functional STP activity. A limitation of our study is the retrospective nature, as we were depended on the available data on hormone serum concentrations that lacked information on progesterone levels in both groups and oestradiol levels in the postmenopausal group to validate hormonal cycle phase. Moreover, the study is limited by the small sample size and the unequal distribution of inclusions in the different groups of the hormonal cycle as this study was designed as an exploratory study. It is possible that subtle changes in STP activity which lacked statistical significance due to small sample number, for example during the follicular phase, still be of biological importance. Though, we observed notable patterns in STP activity, which justifies future studies with expanded sample size to define the Fallopian tube-specific range of STP activity during the hormonal cycle.

Future research should compare STP activity in the different anatomical regions as we only investigated the fimbrial epithelium, and study the cell type specific STP activity of both ciliated and secretory cells. While the pathway assays have specifically been developed to measure functional STP activity, it would be interesting to supplement the results with information on the respective function of the activated pathway in terms of changes in tubal morphology. Cellular changes ultimately depend on the final product of an activated STP, namely the synthesized proteins. Unfortunately, mRNA expression levels are unreliable indicators of corresponding protein expression due to an unequal distribution of production and turnover. Therefore, the use of protein expression analysis (i.e. immunohistochemistry or

immunoblotting) might be considered. To further assess the functional relevance of an activated pathway, we suggest the use of ex vivo oviduct cultures or organoids, which allow experimental conditions to investigate the effect of pathway inhibitors and activators on protein expression and determine morphological changes of the tubal epithelium. Such experiments not only have the potential to improve our understanding of the molecular processes occurring in the Fallopian tube but are also crucial to identify possible therapeutic targets in case of tubal diseases, for example to improve tubal patency in case of obstruction or to treat tubal carcinogenesis.

Conclusion

We observed cyclic changes in STP activity in human FTE during the hormonal cycle, the early luteal phase was characterized by high AR and ER pathway activity and low PI3K pathway activity, while the late luteal phase showed low HH and Wnt pathway activity. AR, ER and PI3K pathway activity in FTE from postmenopausal patients was most comparable to the activity measured during the early follicular and/or late luteal phase. Wnt pathway activity in postmenopausal patients was comparable to the late follicular and early luteal phase. The cyclic changes in STP activity suggest a stage-specific function which may affect the morphology and physiology of the human Fallopian tube.

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Supplementary information

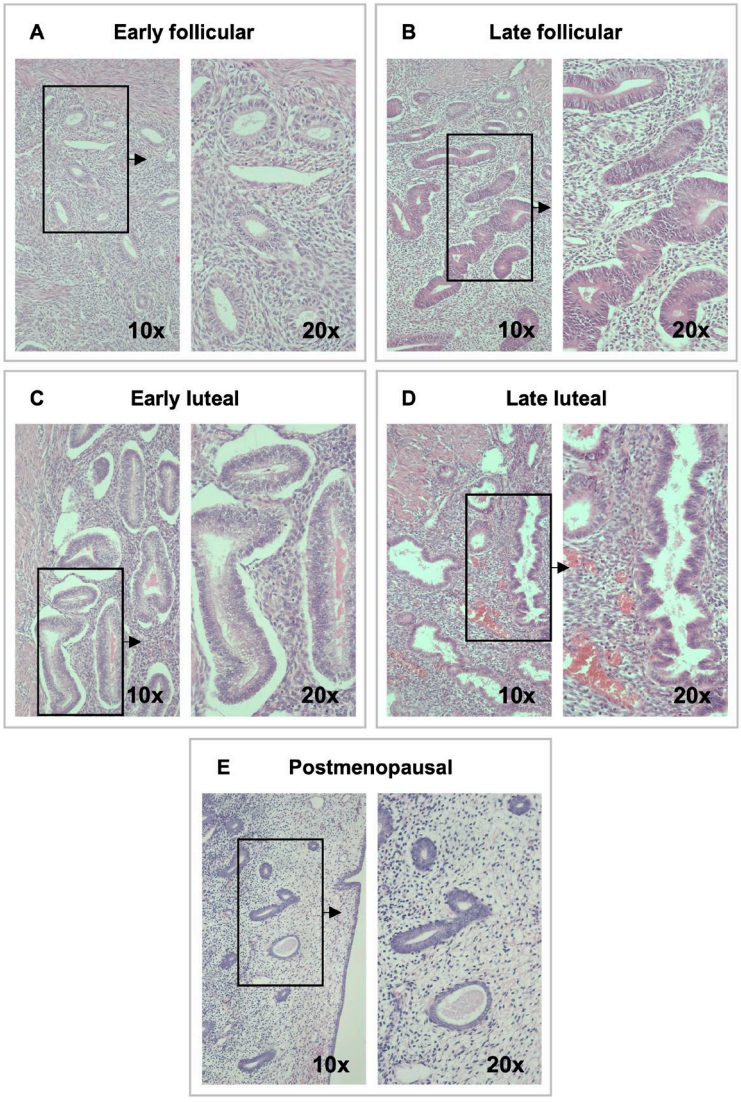
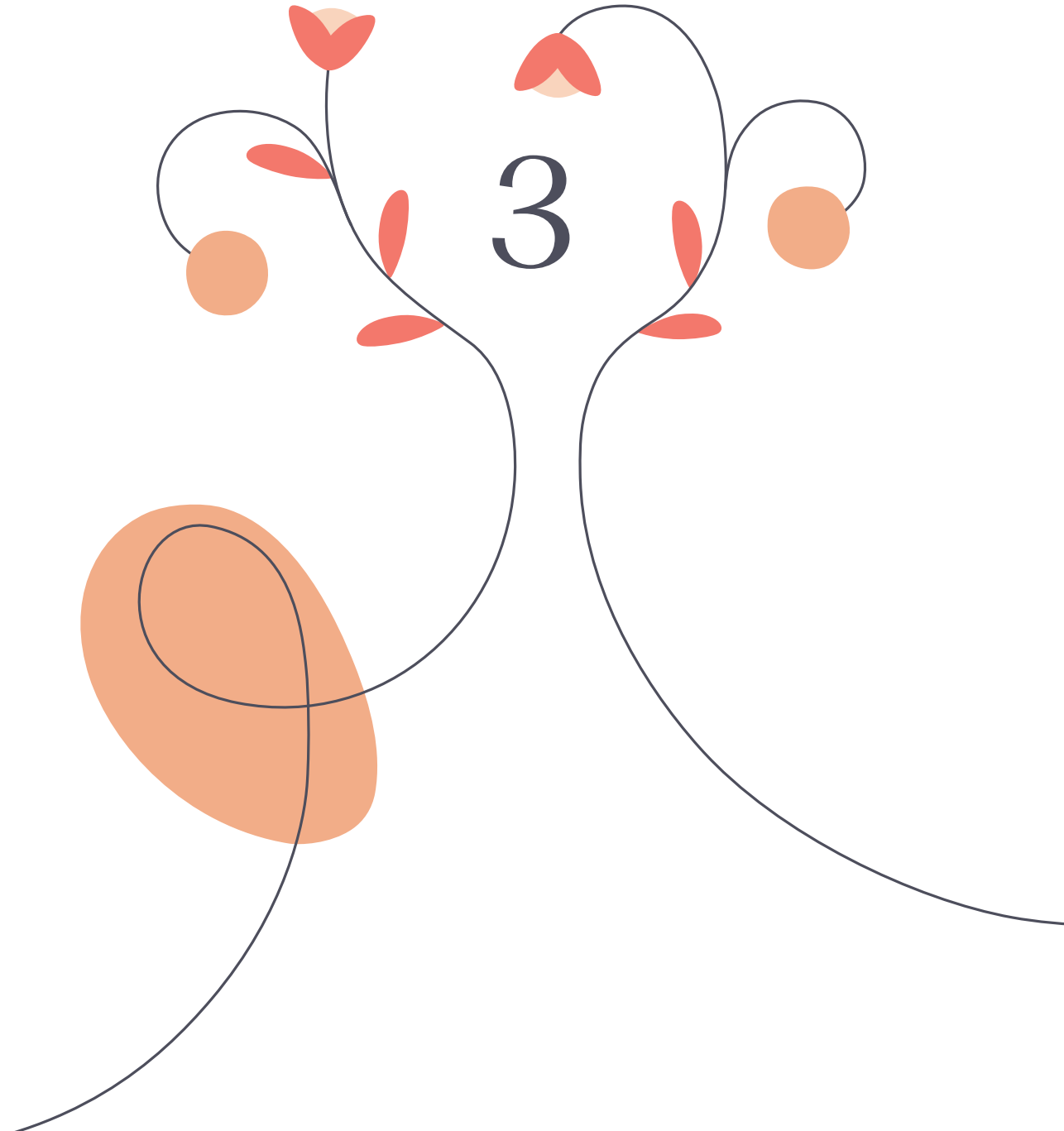


Figure S2.1 Representative histological images of immunohistochemical haematoxylin and eosin stained slides of the endometrium during the hormonal cycle. **A.** Early follicular phase with simple narrow tubular glands and compact stroma. **B.** Late follicular phase showing tortuosity of the glands and pseudostratification of the columnar epithelium with increasing prominence of nucleoli. **C.** Early luteal phase characterized by vacuoles and tortuous distended glands with small amounts of intraluminal secretion. **D.** Late luteal phase demonstrating collapsed involuting glands with saw-tooth appearance and spiral artrioles. **E.** Postmenopausal atrophic endometrium showing cuboidal inactive glandular epithelium.

Table S2.1 Clinicopathological characteristics of the pre- and postmenopausal groups.

Study number	Age (years)	Parity (number)	BMI (kg/m^2)	Surgery indication	Hormonal profile			Definitive hormonal cycle phase	Clinical details and pathological findings
					FSH (U/L)	LH (U/L)	Oestradiol (nmol/L)		
Premenopausal group									
1	47	1	28.3	Abdominal pain	3.8	9.9	2.296	Early follicular	Adenomyosis
2	48	4	22.0	Uterine leiomyomas	5.6	6.9	0.405	Early luteal	Leiomyoma
3	44	0	25.0	Cervical dysplasia	4.8	1.8	0.223	Early luteal	CIN I, adenomyosis and leiomyoma
4	47	2	23.8	Uterine leiomyomas	6.6	4.1	0.581	Early follicular	Leiomyoma
5	40	0	27.1	Cervical dysplasia	4.3	3.3	0.108	Early follicular	NA
6	44	3	22.8	Cervical dysplasia	14.4	13.5	0.184	Early luteal	Adenomyosis
7	41	2	24.6	Heavy menstrual bleeding	3.2	3.0	0.432	Early luteal	NA
8	48	1	27.7	Heavy menstrual bleeding	6.2	5.6	0.359	Early follicular	Novasure treatment, adenomyosis
9	46	2	26.4	Uterine leiomyomas	6.8	6.9	0.280	Early luteal	Leiomyoma, endometriosis around tuba
10	44	0	32.0	Uterine leiomyomas	7.1	8.3	0.608	Late follicular	Adenomyosis, endometrial polyp and leiomyoma
11	42	1	24.4	Heavy menstrual bleeding	8.0	6.5	0.227	Late luteal	Endometrial polyp, adenomyosis
12	44	2	21.3	Uterine leiomyomas	12.4	9.0	0.171	Late follicular	Leiomyoma, adenomyosis
13	32	2	32.1	Heavy menstrual bleeding	1.8	4.2	0.588	Late luteal	Adenomyosis
14	41	3	27.3	Abdominal pain	3.2	1.8	0.150	Late luteal	NA
15	43	0	24.9	Abdominal pain	6.9	16	0.678	Late follicular	NA
16	43	1	32.4	Heavy menstrual bleeding	4.7	3.8	0.626	Late luteal	Novasure treatment, leiomyoma
17	40	3	26.9	Heavy menstrual bleeding	2.6	9.1	0.492	Late luteal	Novasure treatment, adenomyosis
Postmenopausal group									
18	59	3	28.0	Postmenopausal blood loss	NA	NA	NA	Postmenopausal	Endometrial polyp, adenomyosis and multiple benign ovarian cysts
19	59	2	25.4	Uterine descensus	NA	NA	NA	Postmenopausal	Endocervical polyp, adenomyosis and ovarian fibroma
23	67	*Missing*	27.0	Postmenopausal blood loss and benign adnexal mass	NA	NA	NA	Postmenopausal	Endometrial polyp, adenomyosis, leiomyoma and multiple benign ovarian inclusion cysts
25	57	2	31.6	Benign adnexal mass	NA	NA	NA	Postmenopausal	Tubal cysts
27	74	8	22.6	Benign adnexal mass	NA	NA	NA	Postmenopausal	Ovarian fibroma
28	67	*Missing*	23.6	Benign adnexal mass	NA	NA	NA	Postmenopausal	Ovarian fibroma
29	61	2	28.5	Benign adnexal mass	NA	NA	NA	Postmenopausal	Tubal cyst
30	62	2	33.6	Benign adnexal mass	NA	NA	NA	Postmenopausal	NA

Abbreviations: BMI, body mass index; FSH, follicle-stimulating hormone; LH, luteinizing hormone; CIN, cervical intraepithelial neoplasia; NA, not applicable.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CHAPTER 3

Signal transduction pathway activity in high-grade serous carcinoma, its precursors and Fallopian tube epithelium

Phyllis van der Ploeg, Aniek Uittenboogaard, Steven L. Bosch, Paul J. van Diest, Yvonne J.W. Wesseling-Rozendaal, Anja van de Stolpe, Sandrina Lambrechts, Ruud L.M. Bekkers, Jurgen M.J. Piek

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Abstract

Objective

To determine the activity of key signal transduction pathways in serous tubal intraepithelial carcinoma (STIC) and concurrent high-grade serous carcinoma (HGSC) and compare this to pathway activity in normal Fallopian tube epithelium (FTE).

Methods

We assessed mRNA expression levels of pathway-specific target genes with RT-qPCR in STIC and concurrent HGSC (n=8) and normal FTE (n=8). Subsequently, signal transduction pathway assays were used to assess functional activity of the androgen (AR) and oestrogen receptor (ER), phosphoinositide 3-kinase (PI3K), Hedgehog (HH), transforming growth factor beta (TGF-β) and canonical wingless-type MMTV integration site (Wnt) pathways.

Results

There were no statistically significant differences in pathway activity between STIC and HGSC, but STIC and HGSC demonstrated significantly lower ER and higher PI3K and HH pathway activity in comparison to normal FTE, suggesting these pathways as putative early drivers. In addition, we determined FOXO3a protein expression by immunohistochemistry and found loss of FOXO3a protein expression in STIC and HGSC compared to normal FTE. This observation confirmed that activation of PI3K signalling by loss of FOXO is an early hallmark of serous carcinogenesis. Furthermore, HGSC demonstrated significant loss of AR and Wnt pathway activity in relation to FTE, suggesting these pathways contribute to disease progression.

Conclusion

Our observations, together with the previously described associations between p53 signalling and both PI3K and HH pathway activity, provide evidence that increased PI3K and HH pathway activity and loss of ER pathway activity may be underlying events contributing to neoplastic transformation of FTE into STIC.

Introduction

Nonuterine high-grade serous carcinoma (HGSC) refers to ovarian, Fallopian tube and primary peritoneal cancers.[1] HGSC typically present at an advanced stage, requiring treatment consisting of platinum-based chemotherapy and debulking surgery. Unfortunately, current treatment is insufficient to acquire long-term survival, as in more than 50% of the patients relapse of disease occurs within two years and becomes platinum-resistant.[2,3] Therefore, there is an urgent need to elucidate early cellular processes leading to HGSC in order to identify new leads for treatment options.

Traditionally, the ovarian surface epithelium was proposed as the cell of origin of HGSC.[4] Two decades ago, another hypothesis evolved as preneoplastic changes were observed in the epithelium of the fimbriae of prophylactically removed Fallopian tubes of women with a hereditary high risk to develop HGSC, but not in the ovarian surface epithelium.[5,6] These non-invasive dysplastic precursor lesions, later named serous tubal intraepithelial carcinoma (STIC), were also observed in Fallopian tubes of women with non-hereditary HGSC.[7] Proof of a clonal relationship between STIC and HGSC was given by the observation of identical mutations in the tumour suppressor gene *TP53* in paired cases of STIC and HGSC.[8] Furthermore, transformation of Fallopian tube secretory cells in genetically modified mice resulted in the development of lesions mimicking STIC and HGSC.[9] Additionally, xenografted tumours in mice derived from transformed human tubal stem cells showed similar histological and molecular characteristics as HGSC, suggesting a causal relation.[10] Finally, the tubal hypothesis received additional support from molecular profiling studies as STIC were found to have genetic features closely resembling HGSC.[11,12]

Despite accumulating evidence supporting Fallopian tube epithelium (FTE) as the cell of origin of most HGSC, the initial molecular processes underlying the transformation of FTE into STIC and HGSC remain poorly understood. Previously, loss of forkhead box protein O3a (FOXO3a) was suggested to be an early event in STIC.[13] FOXO3a is a tumour suppressive transcription factor of the forkhead family and is negatively regulated by the phosphoinositide 3-kinase (PI3K) growth factor signalling pathway.[14] The association between loss of FOXO3a and poor prognosis further supported the clinical relevance of PI3K-FOXO signalling in HGSC.[15,16] In addition to the PI3K pathway, heterogenous activity of other key signal transduction pathways (STPs) has been observed amongst HGSC samples, for instance the androgen (AR) and oestrogen receptor (ER), Hedgehog (HH), transforming growth factor beta (TGF-β) and canonical wingless-type MMTV integration site (Wnt) pathways.[17] The wide variety in STP activity suggests unique tumour activation patterns.[17] Determining whether these STPs are also activated in STIC could improve our understanding of

aberrant STP activity, and subsequently enable the characterization of early events of high-grade serous carcinogenesis.

Therefore, we investigated in this study the activity of the AR, ER, PI3K, HH, TGF-*β* and Wnt pathways in STIC and concurrent HGSC in relation to the STP activity in morphologically normal FTE. We aim to identify early aberrations in STP activity that may contribute to the processes related to neoplastic transformation of FTE into HGSC.

Materials and methods

Study design and population

We searched the pathology archive of the Catharina Hospital in Eindhoven, the Netherlands for morphologically normal Fallopian tubes obtained from healthy postmenopausal women who had surgery for benign gynaecological conditions between 2009 and 2018. Women were excluded in case of a history of gynaecological cancer, a known pathogenic germline mutation of the *BRCA1/2* genes or other personal or family hereditary risks of cancer. In addition, we searched for women diagnosed with STIC with concurrent HGSC diagnosis between 2004 and 2020. Medical records were screened for demographic and pathological data including, age at the time of surgery, contraceptive use, menopausal status, body mass index (BMI), parity, FIGO stage and type of treatment.

The study was approved by the Medical research Ethics Committees United (MEC-U, study number W16.108 and W18.134) and was conducted in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands. Written informed consent was obtained from women with benign diagnosis. The MEC-U waived the need for informed consent of women diagnosed with HGSC as the majority of the women would have succumbed from disease at the time of inclusion due to the aggressive nature of the disease. However, medical records were checked for any signs of objection to the use of residual tissue for research purposes.

Sample preparation

Archived sections were revised by an expert gynaecological pathologist (SLB) to confirm the presence of morphologically normal fimbriae, STIC and HGSC. Representative areas were annotated for analysis. Examples of STIC and HGSC are shown in **Supplementary Figure S3.1A-D**. HGSC samples with less than 40% tumour cell nuclei were excluded to minimize stromal contamination. Five μm formalin-fixed paraffin-embedded (FFPE) sections were cut from the selected tissue blocks with a microtome (RM2255, Leica Biosystems, Germany) and mounted onto PEM-

membrane coated slides (normal FTE and STIC samples) or glass slides (HGSC samples). The last section was haematoxylin and eosin (H&E) stained and evaluated for the presence of the annotated area of interest. For normal FTE and STIC samples, intermediate sections were manually haematoxylin stained for visualization of the selected areas. Subsequently, to eliminate stromal contamination, fimbrial epithelial cells and STIC were isolated by laser capture microdissection (PALM MicroBeam 4, Carl Zeiss B.V., Germany). Intermediate sections with HGSC areas were manually scraped for the collection of tumour cells using the last annotated H&E slide as a reference.

mRNA extraction, RT-qPCR analysis and signal transduction pathway activity assays

To evaluate STP activity, mRNA was isolated from the normal FTE, STIC and HGSC samples using the VERSANT® Tissue Preparation Reagents kit (Siemens, Germany). Quantitative activity of the AR, ER, PI3K (indirectly, as it is inversely proportional to FOXO transcription factor activity under the condition of absence of oxidative stress[18]), HH, TGF-β and Wnt pathways and the Ki-67 target gene expression were assessed with the use of commercially available OncoSignal pathway assays (Philips MPDx, the Netherlands).[19] In brief, expression levels of pathway-specific target genes were measured with real-time quantitative reverse transcription-PCR (RT-qPCR) analysis using the SuperScript™ III Platinum™ One-Step qRT-PCR kit (Invitrogen, Thermo Fisher Scientific, MA, USA) and commercially available OncoSignal 96-well PCR plates (Philips MPDx, the Netherlands). Sufficient mRNA input for pathway analysis was confirmed by an internal quality control of reference genes consisting of a set of common housekeeping genes. Subsequently, functional STP activity scores were calculated and provided by Philips MPDx (the Netherlands). The general Philips MPDx technology to measure STP activity has been described in detail before.[19,20] In brief, for each STP mRNA levels of target genes of the STP-associated transcription factor are measured, and from these mRNA expression data the STP activity is inferred using Bayesian network models. The models consist of three layers corresponding to (1) the transcription factor complex associated to the STP, (2) the direct target genes of the transcription factor complex and (3) the measured mRNA levels. As a result, the models describe the probabilistic activity of the STP transcription complex using the measured mRNA levels as input. Initially, the models were developed and validated using Affymetrix expression microarray datasets enabling the validation on independent data of various cell and tissue types. Pathway-specific target genes were selected based on elaborate literature study and each pathway model included approximately 25 to 35 target genes. Lists of the selected target genes in the original models of the AR, HH and TGF-β pathways[21], ER and Wnt pathways[19] and PI3K pathway[18] have been described previously. Adaptation of the original models to RT-qPCR models enabled the use of FFPE tissue samples

and included the best performing target genes, around 12 target genes per pathway. The RT-qPCR models have since been validated on various cell types, as described previously.[20,22,23] Functional STP activity is expressed on a normalized scale from 0 to 100, where 0 indicates the lowest probability of an active pathway, and inversely, 100 the highest probability of an active pathway.

Immunohistochemical staining of FOXO3a protein

To validate our PI3K pathway activity data, we determined FOXO3a protein expression by immunohistochemistry in normal FTE, STIC and HGSC samples using a Ventana Benchmark XT Autostainer (Ventana Medical Systems, AZ, USA). Four μm FFPE sections of the identical tissue blocks were cut and deparaffinized. After antigen retrieval and blocking of endogenous peroxidase activity, the sections were incubated with the primary anti-human FOXO3a mouse monoclonal antibody (diluted 1:150, CF809449, ThermoFischer Scientific, USA) for one hour at 36° Celsius. Detection of the primary antibody was achieved after incubation with horseradish peroxidase and visualization with hydrogen peroxidase/3,3'-diaminobenzidine. The sections were counterstained with haematoxylin. Normal pancreas and colon tissue were used as a positive control. FOXO3a stained sections were visually reviewed in a blinded manner by two independent pathologists (SLB and PJvD) without knowledge of each other's assessment and STP activity assay results. Positively stained tumour cell nuclei were reviewed by a semi-quantitative assessment of staining intensity (negative, weak, moderate or strong) and the percentage of positive tumour cells. FOXO3a histoscores were calculated by the sum of (1 x % weak) + (2 x % moderate) + (3 x % strong), resulting in scores ranging between 0 and 300. For further analysis we used mean FOXO3a histoscores derived from the assessments of both pathologists.

Statistical analysis

Clinical characteristics of the women with normal FTE were compared with the characteristics of the women diagnosed with STIC and concurrent HGSC using Wilcoxon rank sum tests for continuous variables with a skewed distribution and presented as median with interquartile range (IQR). Fisher's exact tests were used to compare categorical variables between the groups and presented as frequency and percentages. Differences in STP activity scores and FOXO3a histoscores between groups were tested with Wilcoxon rank sum and Kruskal-Wallis tests. Paired analyses were performed using paired Wilcoxon signed-rank tests. Overall concordance between FOXO3a histoscore assessments of both pathologists was evaluated with the intra-class correlation coefficient using a two-way mixed model. Furthermore, Spearman's rank correlation coefficient was used to determine the correlation between FOXO3a histoscores and PI3K pathway activity. Statistical analysis and data visualization were conducted using R (RStudio, Inc. version 1.1.463).

Results

Study population

We identified 13 postmenopausal women with morphologically normal fimbriae and 12 women with STIC and concurrent HGSC. After laser capture microdissection, we excluded five normal FTE and four paired STIC and HGSC samples due to insufficient mRNA concentrations. For one woman, the concurrent HGSC sample was not suitable for further analysis as it contained less than 40% tumour cell nuclei. Thus, eight normal FTE, eight STIC samples and seven concurrent HGSC samples were available for the analysis of STP activity. Clinicopathological characteristics of the study population are presented in **Table 3.1** and **Supplementary Table S3.1**. Women with STIC and concurrent HGSC had a lower BMI (*P*=0.007) as compared to women in the normal FTE group. There were no differences between the two groups in age at the time of surgery, menopausal status and parity. Women with paired STIC and HGSC were diagnosed with advanced stage disease (FIGO stage IIIC (n=6) or IV (n=2)). These women had primary debulking surgery followed by adjuvant chemotherapy (n=3) or neoadjuvant chemotherapy and interval debulking surgery (n=5). Consequently, for the latter group STIC and concurrent HGSC samples were taken after the start of chemotherapy. Women in the normal FTE group had had salpingectomy with or without hysterectomy for the following gynaecological conditions; benign adnexal mass (n=5), postmenopausal blood loss (n=2) and uterine descensus (n=1). Pathological revision showed no evidence of dysplasia or malignancy.

Comparable signalling pathway activity in STIC and HGSC samples

We assessed quantitative activity of the AR, ER, PI3K, HH, TGF-β and Wnt pathways and Ki-67 target gene expression using mRNA measurements of pathway-specific target genes.[19] Overall, we observed no differences in median STP activity between grouped STIC (n=8) and HGSC (n=7) samples (**Figure 3.1A-G**). Subsequent, analysis of paired STIC and HGSC samples (n=7) also revealed no statistically significant differences in AR, ER, PI3K, HH, TGF-β and Wnt pathway activity and Ki-67 activity scores (**Figure 3.1A-G**). However, the majority of the STIC samples were characterized by slightly higher AR, ER, PI3K and Wnt pathway activity and lower HH and TGF-β pathway activity as compared to their HGSC counterpart (**Supplementary Figure S3.2A-G**). For five patients, the samples were taken during interval debulking surgery. In STIC and HGSC samples taken after the start of chemotherapy, we measured higher AR pathway activity and lower HH pathway activity compared to samples taken prior to start of chemotherapy (*P*=0.018 and *P*=0.012, respectively) (**Supplementary Figure S3.3A and D**). One woman with STIC and concurrent HGSC diagnosis was premenopausal and used a levonorgestrel intrauterine device at the

time of surgery. Paired samples of this woman are marked in yellow in **Figure 3.1A-G** and show relatively low AR and high ER pathway activity as compared to the other samples.

Table 3.1 Summary of clinicopathological characteristics of the included women with normal Fallopian tube epithelium (FTE) and serous tubal intraepithelial carcinoma (STIC) with concurrent high-grade serous carcinoma (HGSC).

	Normal FTE (n=8)	**STIC with concurrent HGSC (n=8)**	***P*-value***
Age at surgery (years)			0.289
Median (IQR)	62 (59-67)	67 (64-71)	
Menopausal status			1.000
Premenopausal (%)	0 (0)	1 (12)	
Postmenopausal (%)	8 (100)	7 (88)	
BMI (kg/m^2)			0.007
Median (IQR)	27.5 (24.9-29.3)	22.6 (20.6-23.6)	
Parity (number)			1.000
Median (IQR)	2 (2-3)	2 (2-3)	
Missing (n)	2	0	
FIGO disease stage			-
IIIC (%)	-	6 (75)	
IV (%)	-	2 (25)	
Type of treatment			-
Salpingectomy with or without hysterectomy	8 (100)	-	
PDS + ACT	-	3 (37)	
NAC + IDS	-	5 (63)	

* For continuous variables with skewed distribution, *P*-values were obtained from Wilcoxon rank sum tests. Differences in categorical variables were tested with Fisher's exact tests.
Abbreviations: ACT, adjuvant chemotherapy; BMI, body mass index; IDS, Interval debulking surgery; IQR, interquartile range; NAC, Neoadjuvant chemotherapy; PDS, Primary debulking surgery.

STIC and HGSC samples were characterized by decreased ER and increased PI3K and HH pathway activity in relation to normal FTE samples

Next, we compared the STP activity data from STIC and HGSC samples to STP activity in normal FTE samples. As shown in **Figure 3.1B-D**, we observed significantly lower ER pathway activity as well as higher PI3K and HH pathway activity in both STIC and HGSC samples compared to normal FTE samples. For the AR and Wnt pathways, comparable activity was found in normal FTE and STIC samples, while the HGSC samples demonstrated significantly lower AR and Wnt pathway activity as compared to normal FTE samples (**Figure 3.1A and F**). Although TGF-β pathway activity did not differ between the groups, we observed a broad range in activity scores in the HGSC samples (**Figure 3.1E**). Assessment of Ki-67 activity demonstrated significantly higher activity scores in STIC and HGSC samples in comparison to normal FTE samples, indicating increased cell proliferation (**Figure 3.1G**).

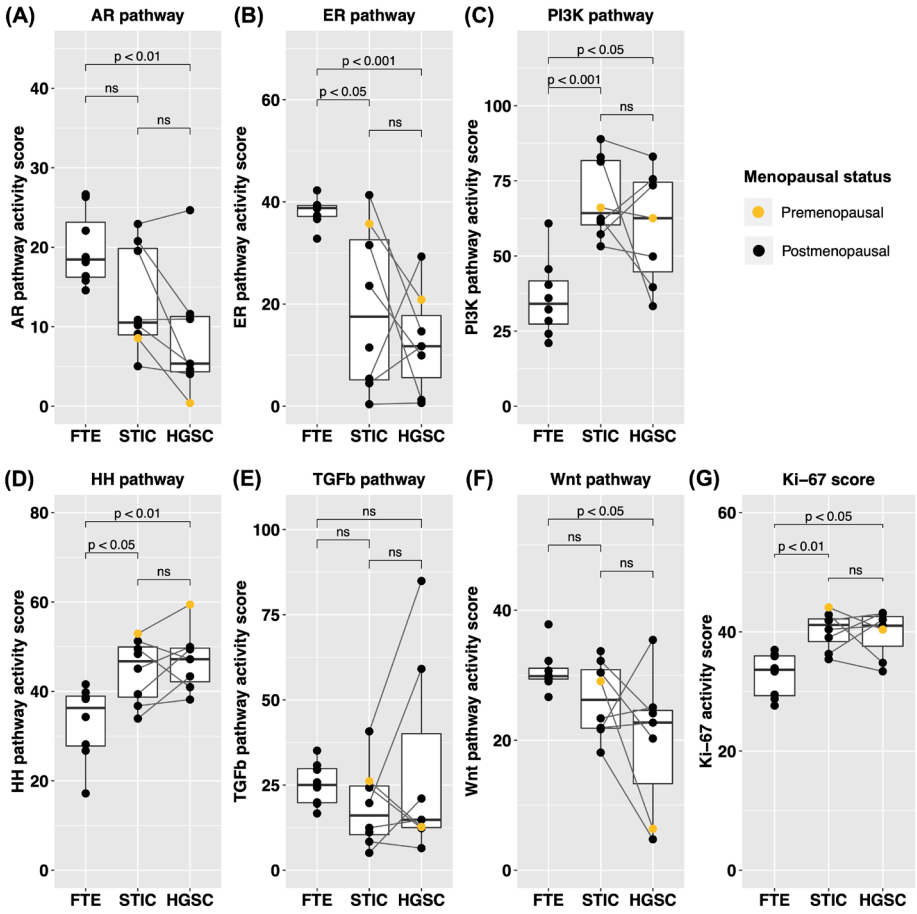

Figure 3.1 Boxplots displaying differences in signal transduction pathway activity between Fallopian tube epithelium (FTE) (n=8), serous tubal intraepithelial carcinoma (STIC) (n=8) and high-grade serous carcinoma (HGSC) samples (n=7). Yellow dots indicate a premenopausal woman with an intrauterine device *in situ* at the time of surgery. *P*-values were obtained from unpaired Wilcoxon rank sum tests. In addition, paired Wilcoxon signed-rank tests were used to analyse paired STIC and HGSC samples (n=7, indicated with grey lines). **A.** Androgen receptor (AR). **B.** Oestrogen receptor (ER). **C.** Phosphoinositide 3-kinase (PI3K). **D.** Hedgehog (HH). **E.** Transforming growth factor beta (TGFb). **F.** Canonical wingless-type MMTV integration site (Wnt). **G.** Ki-67 activity score. ns, not significant.

Loss of FOXO3a protein expression in STIC and HGSC samples in comparison to normal FTE samples

We subsequently assessed FOXO3a protein expression by immunohistochemistry in normal FTE, STIC and HGSC samples. Some samples were lost as the areas of interest were no longer present in the consecutive slides. In total, eight normal FTE, six STIC

and five HGSC samples were available for FOXO3a protein expression assessment. Representative images of FOXO3a immunohistochemically stained slides are presented in **Figure 3.2A-C**. Interobserver agreement for the FOXO3a histoscore assessment was excellent with an intra-class correlation coefficient based on absolute agreement of 0.916 (95% confidence interval 0.785-0.967). We determined median FOXO3a histoscores of 275 (IQR 270-286) in normal FTE, 238 (IQR 199-258) in STIC and 215 (IQR 155-220) in HGSC samples. STIC and HGSC samples had significantly lower FOXO3a histoscores in comparison to FTE samples (*P*=0.002 and *P*=0.033, respectively) (**Figure 3.2D**). The negative correlation between FOXO3a histoscores and PI3K pathway activity validated the inverse relation between FOXO and PI3K (*P*=0.020) (**Figure 3.2E**).

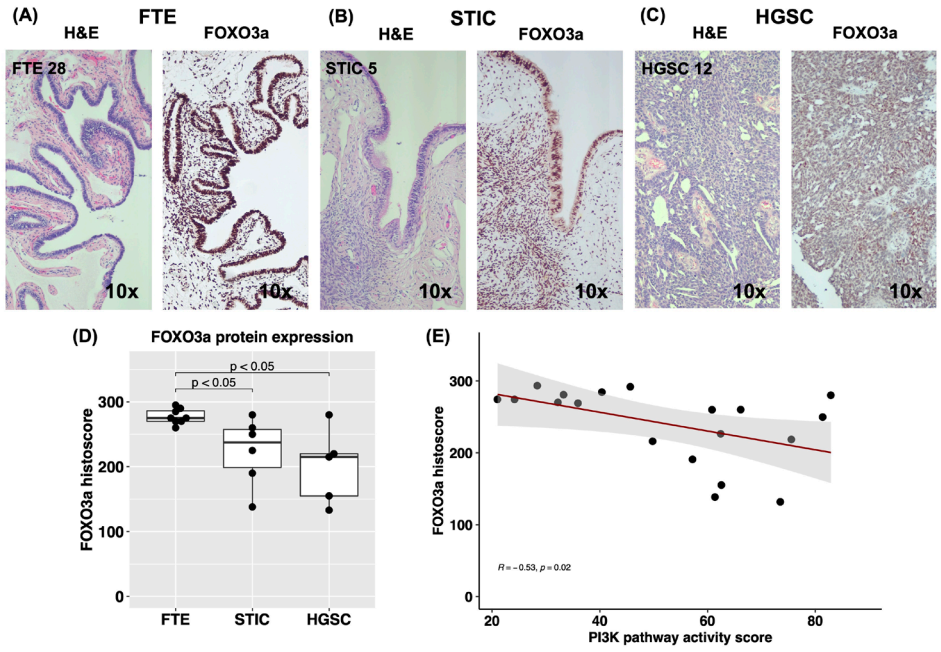

Figure 3.2 Forkhead box protein O3a (FOXO3a) protein expression. Representative images of haematoxylin and eosin (H&E) and FOXO3a immunohistochemically stained slides of **A.** Normal Fallopian tube epithelium (FTE). **B.** Serous tubal intraepithelial carcinoma (STIC). **C.** High-grade serous carcinoma (HGSC). **D.** Boxplots displaying differences in FOXO3a histoscores between the groups (FTE n=8, STIC n=6 and HGSC n=5). *P*-values were obtained from Wilcoxon rank sum tests. **E.** FOXO3a histoscores were negatively correlated to phosphoinositide 3-kinase (PI3K) pathway activity scores. The correlation was determined by Spearman's rank correlation coefficient.

Discussion

In this study, we assessed the activity of key STPs in normal FTE, STIC and HGSC samples in order to identify early aberrations in STP activity that may drive the development of FTE precursors and progression into HGSC. Among several signalling pathways that await exploration, we focused on the AR, ER, PI3K, HH, TGF-β and Wnt pathways as these pathways were previously associated to ovarian carcinogenesis.[24-29] Our data provide evidence that loss of ER pathway activity and increased PI3K and HH pathway activity may be underlying events contributing to neoplastic transformation of FTE into STIC, whereas loss of AR and Wnt pathway activity are more likely to be contributors of HGSC progression. With the use of Bayesian models integrating mRNA expression levels of direct pathway-specific target genes, our analysis enabled a unique assessment of the functional pathway activity profile.

We observed comparable Ki-67 activity scores in STIC and concurrent HGSC samples, but significantly higher Ki-67 activity scores compared to normal FTE samples, suggesting a marked increase in proliferative activity in STIC and HGSC samples (**Figure 3.1G**). These observations were in line with previous data on Ki-67 expression by immunohistochemistry in a similar cohort and confirmed that our cohort was representative of STIC and HGSC samples.[30] Overall, we observed similar STP activity across the STIC and HGSC samples, which might also suggest a causal relation with a common biological origin. We were unable to identify any statistically significant relations in STP activity between paired STIC and HGSC samples. However, we noticed subtle patterns in pathway activity up- or downregulation as the majority of the STIC samples had slightly higher AR, ER, PI3K and Wnt pathway activity and lower HH and TGF-β compared to their HGSC counterpart, but small sample sizes may have contributed to lack of statistical significance. Subsequent comparison to normal FTE samples from women who had surgery for benign diagnosis, provided a unique opportunity to assess normal pathway activity and revealed more distinct differences in ER, PI3K and HH pathway activity in STIC and HGSC samples.

Previously, others have implicated a role for PI3K signalling in HGSC development based on loss of FOXO3a protein expression.[15,16,31] Although nuclear FOXO3a protein expression is insufficient to describe functional activity of the PI3K pathway, it provides complementary information and enabled us to compare our results with previously published findings.[13] Levanon *et al.* investigated the transcriptional profile of normal FTE and HGSC samples and concluded that FOXO3a loss is involved in the pathogenesis of HGSC.[13] In line with results of Levanon *et al.*, we observed significant loss of FOXO3a protein expression in STIC and HGSC samples in comparison to normal FTE samples (**Figure 3.2D**).[13] The FOXO transcription factor complex is an important downstream component of the PI3K/AKT signalling pathway as

phosphorylation by AKT inhibits activity of the FOXO transcription factor and thereby promotes cell proliferation.[32] Thus, in the absence of activated PI3K/AKT, the FOXO transcription factor acts as a tumour suppressor with apoptotic effects. Our data further highlights the significance of PI3K signalling as we found significantly higher PI3K pathway activity in STIC and HGSC samples compared to normal FTE samples (**Figure 3.1C**). Others also found activation of PI3K signalling in STIC based on positive immunohistochemical staining of Stathmin 1 (STMN1), which is considered a surrogate marker for PI3K pathway activity.[33,34] Interestingly, the authors observed negative STMN1 expression in "p53 signatures", known as the morphologically normal putative precursor lesion identified by p53 protein accumulation, but positive STMN1 expression in "proliferative p53 signatures", which are characterized by both p53 and Ki-67 protein expression.[33] The presence of STMN1 expression as early as in these transitional lesions (i.e. proliferative p53 signatures) strongly suggests an association with tumour initiation rather than progression. In addition, mutant p53 in ovarian cancer cells mediated increased phosphorylation of AKT, this suggests an association between p53 and PI3K/AKT signalling.[35] In normal FTE samples from premenopausal woman, our group previously observed a cyclic pattern of PI3K activation during the proliferative phase of the menstrual cycle.[36] Given the tight regulation of PI3K (in)activation in normal FTE, it is likely that aberrations in PI3K signalling disrupt tissue homeostasis and confers a selective advantage to maintain a proliferative state. However, despite the fact that genomic alterations related to the PI3K pathway occur in HGSC, for instance *PIK3CA* amplifications or mutations (2-20%)[37-40], *PTEN* deletions or mutations (7%)[41] and *AKT2* amplifications (18%)[39], these are more frequently found in the endometrioid and clear cell subtypes. These findings, together with our current findings, suggest deregulated FOXO-PI3K signalling as the predominant mechanism during the early stages of serous carcinogenesis rather than genomic changes in the PI3K pathway.

Neoplastic transformation of FTE is likely to require abnormal functioning of several signalling pathways rather than a single pathway, as for instance is the case in the development of colorectal and lung cancer.[42] Besides increased activity of the PI3K pathway, our findings further associated decreased ER pathway activity with STIC and HGSC samples (**Figure 3.1B**). Although the association between oestrogen replacement therapy and ovarian cancer risk and the frequent expression of the ER protein suggest hormone sensitivity, the function of oestrogen signalling in terms of tumour initiation and progression is still not understood.[43] On the contrary, the use of anti-oestrogen therapy has been associated with tubal dysplasia.[44] Our results show that normal ER pathway activity, which is necessary to preserve normal function of the Fallopian tube, is obviously decreased in STIC samples and even lost in HGSC samples (**Figure 3.1B**). It must be noted that the difference in ER pathway activity may be influenced by the amount of oestrogen producing adipose tissue as

women in the normal FTE group had a higher BMI when compared to women in the STIC and HGSC group (*P*=0.007). Further validation on the exact role of ER signalling is needed to identify whether loss of ER pathway activity in particular triggers cellular differentiation and proliferation or, alternatively, is caused by the tumour-driving effects of other signalling pathways, such as aberrant PI3K signalling. Nevertheless, loss of functional activity of the ER pathway would at least explain the limited response to anti-oestrogen receptor targeted drugs in patients diagnosed with HGSC.[45]

Another finding of our analysis is hyperactivation of the HH pathway with limited variation between paired STIC and HGSC samples (**Figure 3.1D**). Given the tumour-promoting role of HH signalling in a wide variety of cancers, including ovarian carcinoma, the involvement of HH pathway activity in STIC is not surprising.[46,47] Previous studies revealed a collaboration between p53 and HH signalling and found that p53 negatively regulated GLI1 expression, a downstream effector of HH signalling.[48,49] Thereby, the high incidence of *TP53* mutations in STIC and HGSC could explain the increase in HH activity by loss of the inhibitory effect of p53 signalling on GLI1. Moreover, in ovarian cancer cells, an interplay between the PI3K and HH pathway has been described as PI3K/AKT signalling was found to enhance GLI1 activity, a downstream effector of HH signalling.[50,51] The induced activity of PI3K and HH signalling upon progression to STIC and HGSC suggests synergistic activation. However, the exact role of PI3K and HH crosstalk in STIC is currently unknown and requires future work. To further support the causality between aberrant pathway activity and early serous carcinogenesis, future studies might benefit from ex vivo oviduct cultures or organoids, which allow experimental conditions to investigate the effect of pathway activators and inhibitors.

Our study was limited by small sample size and technical challenges to acquire sufficient epithelial cells of small areas with STIC in consecutive slides. Unfortunately, we had to exclude some samples due to low input amounts and lost samples in the analysis of FOXO3a protein expression. Moreover, cytotoxic treatment affects STP activity and therefore has influenced the STP results in samples taken after the start of chemotherapy. In addition, it should be considered that some STIC may have represented metastases of HGSC onto the tubal epithelium and mimic de novo STIC, as described previously.[11] To rule out these confounding factors and further investigate the role of STP activity in serous carcinogenesis, a prospective study should be performed focusing on the presence of “p53 signatures” and incidental STIC in the Fallopian tubes of patients with benign diagnosis.

A major strength of the study is the quantification of STP activity from transcription factor-specific target gene mRNA levels enabling functional activity scores. More

importantly, as we determined the activity of key signalling pathways with clinical target in normal tissue, the pathway-specific range may be used as benchmark to define aberrant activity in individual HGSC samples. Therefore, once the range of normal STP activity has been validated on a larger set of samples, this approach could help guide the selection of patients for targeted therapy based on abnormal STP activity.

Taken together, our findings implicate a distinct role for hyperactivation of the PI3K signalling pathway as a putative early driver in the neoplastic transformation of FTE. Moreover, we show low ER and high HH signalling pathway activity in STIC and concurrent HGSC samples compared to FTE samples, suggesting these STPs are associated with the transition to neoplasia, whereas advanced disease is further characterized by loss of AR and Wnt pathway activity. Although much remains to be discovered on the exact role of these pathways during the early events of HGSC initiation and progression, our results open new perspectives on the molecular processes contributing to the pathogenesis of HGSC.

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Supplementary information

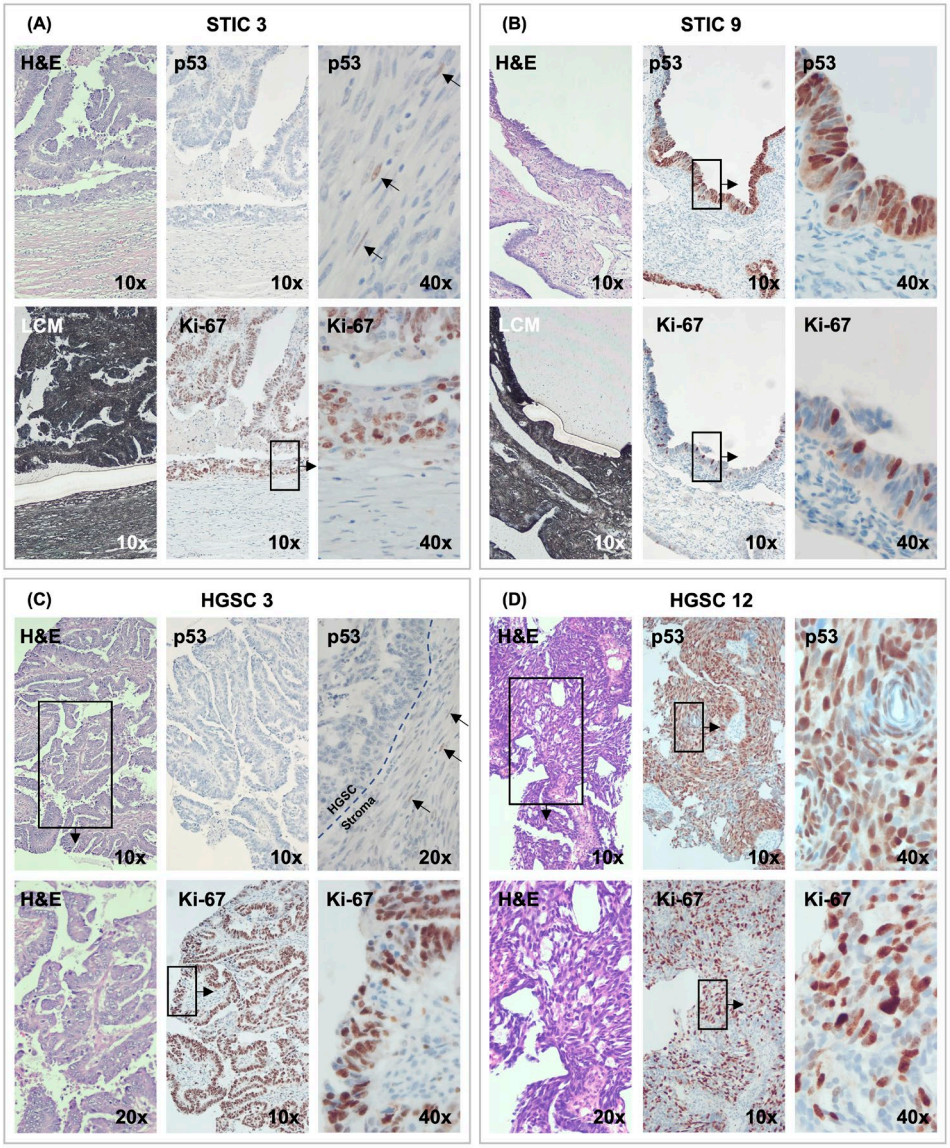


Figure S3.1 Representative images of haematoxylin and eosin (H&E), p53 and Ki-67 immunohistochemically stained slides of A and B. serous tubal intraepithelial carcinoma (STIC) and C and D. high-grade serous carcinoma (HGSC) samples. For STIC samples, laser capture microdissected (LCM) areas are indicated in the bottom left panels. The magnification is presented in the bottom right corner. Note that the samples of Patient 3 (A and C) show negative p53 staining caused by a *TP53* null mutation. Arrows in p53 stained images indicate weak positive stromal cells serving as an interval positive control.

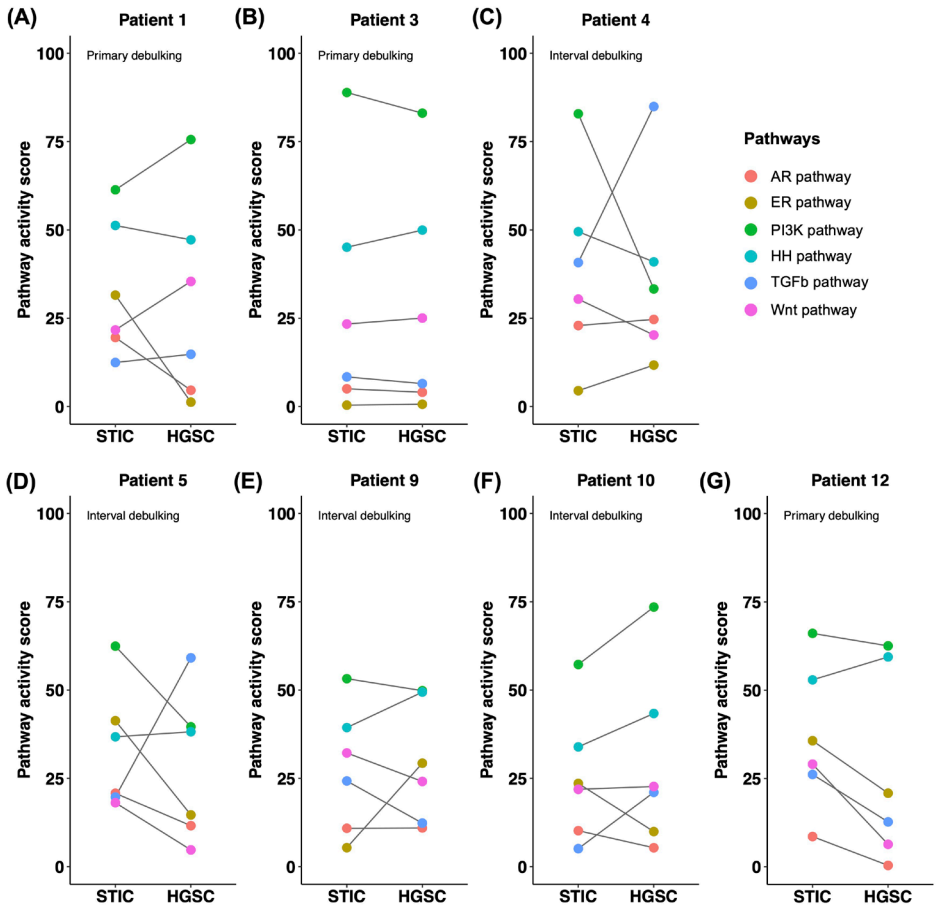


Figure S3.2 Scatterplots displaying paired signal transduction pathway activity measurements in serous tubal intraepithelial carcinoma (STIC) and concurrent high-grade serous carcinoma (HGSC) samples of individual patients. AR, androgen receptor; ER, oestrogen receptor; PI3K, phosphoinositide 3-kinase; HH, Hedgehog; TGFb, transforming growth factor beta; Wnt, Canonical wingless-type MMTV integration site.

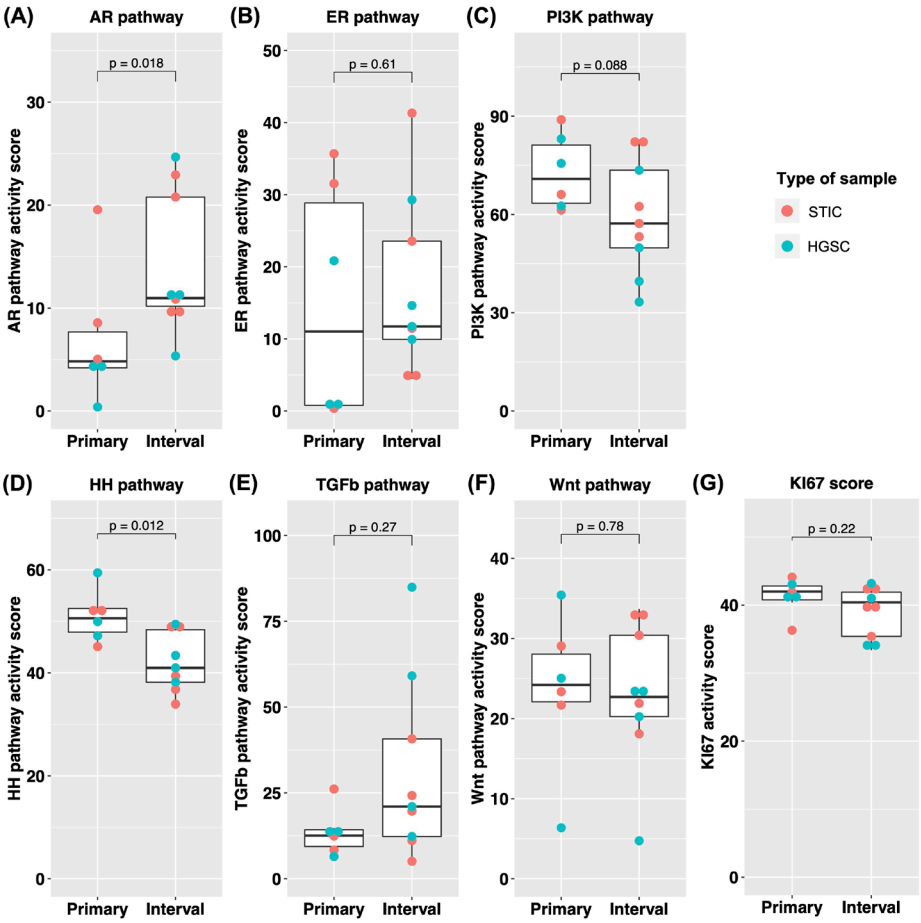
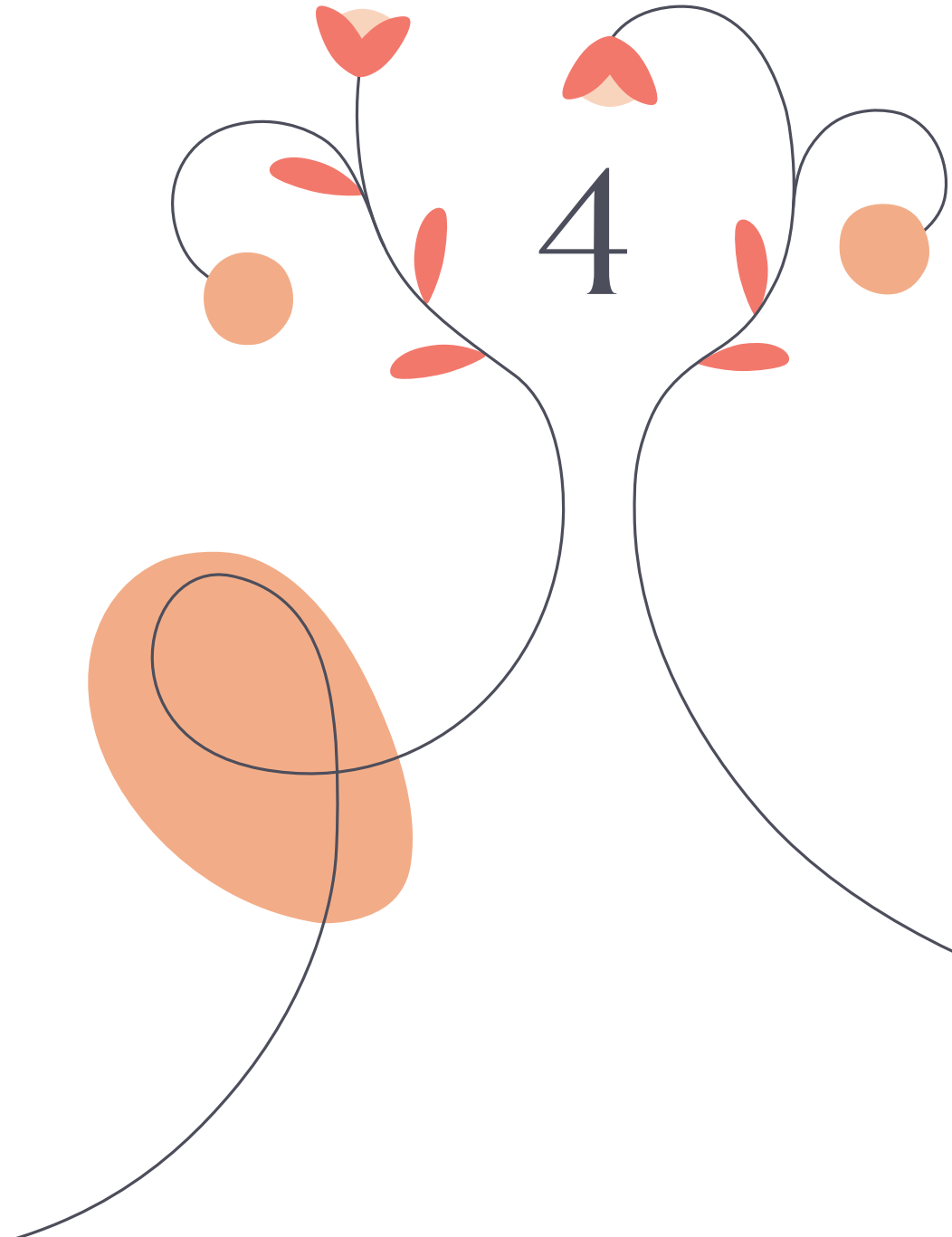


Figure S3.3 Boxplots displaying differences in signal transduction pathway activity between samples taken during primary debulking surgery (chemo-naïve) and interval debulking surgery (after chemotherapy). **A.** Androgen receptor (AR). **B.** Oestrogen receptor (ER). **C.** Phosphoinositide 3-kinase (PI3K). **D.** Hedgehog (HH). **E.** Transforming growth factor beta (TGFb). **F.** Canonical wingless-type MMTV integration site (Wnt). **G.** Ki-67 activity score. STIC, serous tubal intraepithelial carcinoma; HGSC, high-grade serous carcinoma.

Table S3.1 Clinicopathological characteristics of the included women with normal Fallopian tube epithelium (FTE) and serous tubal intraepithelial carcinoma (STIC) with concurrent high-grade serous carcinoma (HGSC).

Patient number	Age at time of surgery	Menopausal status	BMI	Parity	FIGO stage	Treatment	Histology	Tumour cells (%)	Comment
Women with normal FTE									
18	59	Postmenopausal	28.0	3	N.A.	Salpingectomy with hysterectomy	Endometrial polyp, adenomyosis and multiple benign ovarian cysts	N.A.	Postmenopausal blood loss
19	59	Postmenopausal	25.4	2	N.A.	Salpingectomy with hysterectomy	Endocervical polyp, adenomyosis and ovarian fibroma	N.A.	Uterine descensus
23	67	Postmenopausal	27.0	*Missing*	N.A.	Salpingectomy with hysterectomy	Endometrial polyp, adenomyosis, leiomyoma and multiple benign ovarian inclusion cysts	N.A.	Postmenopausal blood loss and benign adnexal mass
25	57	Postmenopausal	31.6	2	N.A.	Salpingectomy	Tubal cysts	N.A.	Benign adnexal mass
27	74	Postmenopausal	22.6	8	N.A.	Salpingectomy	Ovarian fibroma	N.A.	Benign adnexal mass
28	67	Postmenopausal	23.6	*Missing*	N.A.	Salpingectomy	Ovarian fibroma	N.A.	Benign adnexal mass
29	61	Postmenopausal	28.5	2	N.A.	Salpingectomy	Tubal cyst	N.A.	Benign adnexal mass
30	62	Postmenopausal	33.6	2	N.A.	Salpingectomy	No abnormalities	N.A.	Benign adnexal mass
Women with STIC and concurrent HGSC									
1	61	Postmenopausal	24.4	2	IIIC	PDS + ACT	STIC + HGSC + ovarian carcinosarcoma	40	
3	70	Postmenopausal	19.5	3	IIIC	PDS + ACT	STIC + HGSC	60	
4	67	Postmenopausal	20.8	2	IV	NAC + IDS	STIC + HGSC	40	
5	73	Postmenopausal	23.0	4	IIIC	NAC + IDS	STIC + HGSC	50	Extraovarian carcinoma.
7	65	Postmenopausal	20.0	2	IIIC	NAC + IDS	STIC + HGSC	<40	Simultaneous diagnosis of breast and ovarian cancer.
9	67	Postmenopausal	22.1	2	IIIC	NAC + IDS	STIC + HGSC	80	
10	79	Postmenopausal	23.6	3	IV	NAC + IDS	STIC + HGSC	80	
12	42	Premenopausal	23.7	2	IIIC	PDS + ACT	STIC + HGSC	90	Levonorgestrel IUD *in situ* at the time of surgery.

Abbreviations: ACT, adjuvant chemotherapy; BMI, Body mass index; IDS, Interval debulking surgery; IUD, intra-uterine device; N.A., Not applicable; NAC, Neoadjuvant chemotherapy; PDS, Primary debulking surgery.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CHAPTER 4

Survival is related to oestrogen signal transduction pathway activity in post-menopausal women diagnosed with high-grade serous ovarian carcinoma

Laura van Lieshout*, Phyllis van der Ploeg*, Yvonne Wesseling-Rozendaal, Anja van de Stolpe, Steven Bosch, Marjolein Lentjes-Beer, Meggy Ottenheijm, Annelen Meriaan, Caroline Vos, Joanne de Hullu, Leon Massuger, Ruud Bekkers, Jurgen Piek
*Dual first author

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Abstract

High-grade serous ovarian carcinoma (HGSC), the most common subtype of ovarian cancer, has a high mortality rate. Although there are some factors associated with survival, such as stage of disease, there are remarkable differences in survival among women diagnosed with advanced stage disease. In this study, we investigate possible relations between survival and signal transduction pathway (STP) activity. We assessed the functional activity of the androgen receptor (AR), oestrogen receptor (ER), phosphoinositide 3-kinase (PI3K), Hedgehog (HH), transforming growth factor beta (TGF-β) and canonical wingless-type MMTV integration site (Wnt) pathway in 85 primary tumour samples of patients with FIGO stage IIIC to IVB HGSC and disease-free survival (DFS) below 12 (n=52) or over 24 months (n=33). There were no significant differences in median pathway activity between patients with a short and long DFS. In univariate Cox proportional hazards analysis, ER pathway activity was related to a favourable DFS and overall survival (OS) in postmenopausal women (P=0.033 and P=0.041, respectively), but not in premenopausal women. We divided the postmenopausal group into subgroups based on ER pathway activity quartiles. Survival analysis revealed that postmenopausal women in the lowest ER quartile had a shorter DFS and OS (log-rank P=0.006 and P<0.001, respectively). Furthermore, we were able to form subgroups of patients based on an inverse relation between ER and PI3K pathway activity. In conclusion, in postmenopausal patients with advanced stage HGSC, a poorer survival outcome was associated with low functional ER pathway activity.

Introduction

Ovarian cancer is the most lethal gynaecological malignancy and the fifth leading cause of cancer related death in women.[1] High-grade serous ovarian carcinoma (HGSC) is the most common histotype of ovarian cancer and is often detected at an advanced stage of disease (i.e., FIGO stage IIIC-IVB).[2,3] At this point, the overall five-year survival rate is less than 40%, even after successful first-line treatment with debulking surgery and chemotherapy.[2] Despite the generally poor prognosis, the range in both disease-free (DFS) and overall survival (OS) in patients diagnosed with advanced stage HGSC is remarkable, with some patients remaining disease-free for over a decade.[4]

Several clinicopathological factors associated with improved survival have been established like stage of disease, CA125 concentration at diagnosis and after treatment, the possibility of primary debulking surgery and residual disease after surgery. Furthermore, immune factors such as tumour infiltrating lymphocytes have been identified as prognostic factors.[5,6] However, even in patients with poor prognostic factors, long-term survival is not uncommon.[7] Furthermore, in long-term survivors with recurrent disease, both short and long DFS times are seen; some patients recur swiftly but respond well to therapy while others have a long DFS with a short survival time after recurrence.[5] Despite well-established prognostic factors, a proportion of tumours intrinsically behaves more or less aggressively. Unfortunately, the assessment of tumour specific characteristics associated with survival in ovarian carcinoma is complicated by a large heterogeneity in genomic mutations. For many cancer types, a single identifying trait is found in a substantial number of patients, such as HER2 amplification which is present in 20 to 25% of breast cancer patients.[8,9] However, research aimed at the analysis of gene expression profiles and patterns to reveal a relation to survival, could not confirm this for ovarian carcinoma.[7] In addition, a focus on specific gene alterations in the genotype of cancer cells disregards the functional phenotype of cancer cells, while it is becoming increasingly clear that the functional phenotype is influenced by other factors, such as the tumour microenvironment.

In 2010, Verhaegh *et al.* developed a technique to quantitatively measure functional signal transduction pathway (STP) activity, and therefore the functional phenotype, of cancer cells. With the assays, mRNA levels of target genes of major oncogenic STPs are used as input for knowledge-based Bayesian network models.[10-13] In previous studies in breast and colon cancer, the accuracy of the assays in determining aberrant STP activity has been validated. For breast and endometrial cancer, ER pathway activity was related to prognosis and in breast cancer the assay was

superior to traditional immunohistochemical staining in the prediction of response to tamoxifen treatment.[10,14,15]

In this exploratory study, we use these pathway assays in patients diagnosed with advanced stage HGSC who achieved complete remission after treatment with debulking surgery and (neo-)adjuvant chemotherapy. To investigate disease recurrence and survival in relation to STP activity, we compare STP activity in patients with a short and long DFS as well as define interrelations between different pathways with regard to menopausal status and survival. We aim to explain the differences in survival and ultimately provide new leads for accurate selection of patients for targeted therapies.

Materials and methods

Study design and population

We retrospectively searched the Netherlands Cancer Registration (NCR) database for patients diagnosed with FIGO stage IIIC and IV HGSC between January 2000 and December 2016 in three Dutch hospitals (Catharina Hospital Eindhoven, Elisabeth-TweeSteden Hospital Tilburg and Radboud University Nijmegen Medical Center). Patients were eligible for inclusion if histology of the primary tumour was taken prior to start of chemotherapy and available for analysis and if treatment with primary or interval debulking surgery combined with (neo-)adjuvant chemotherapy with carboplatin and paclitaxel resulted in complete remission. Complete remission was chosen as inclusion criterium as we aimed to compare DFS, which requires patients to achieve complete remission first. Patients were excluded if they objected to further use of pathology samples or if they were diagnosed with any other malignancy, either prior to or following HGSC diagnosis, with the exception of basal cell carcinoma as cases where this affects life expectancy are exceedingly rare. Based on reported median DFS for advanced stage HGSC of 16 and 19 months, we decided to exclude patients with a DFS between 12 and 24 months from our analysis to form two clearly defined groups.[16,17] As such, we hypothesized to be able to clearly identify possible differences in STP activity between short-term and long-term disease free survivors. Patients with a DFS below 12 months were defined as the ‘short DFS group’ and patients with a DFS above 24 months as the ‘long DFS group’.

Data collection

The following data were retrieved from the patients’ medical records: parity, menopausal status at diagnosis, age at diagnosis, CA125 concentrations at the time of diagnosis and at the end of treatment, number of chemotherapy cycles and response, type of debulking surgery, debulking outcome, number of recurrences,

type of treatment for the recurrences, vital status at the end of follow-up, DFS and OS. DFS was defined as time between final day of primary treatment until histological confirmation of recurrent disease or start of second-line therapy. Patients were censored if they had no recurrence by the 1 May 2020, or if they were deceased prior to the end of follow-up without evidence of a recurrence. For OS, death was recorded as an event while patients were censored at the end of follow-up (1 May 2020) or on the date of last contact if they chose to continue follow-up in a different hospital. Debulking outcome was classified as either 'complete' (i.e., no macroscopic disease), 'optimal' (i.e., macroscopic residue <1 cm) or 'incomplete' (i.e., macroscopic disease >1 cm).[18] Data on menopausal status was lacking for ten patients below the age of 55. For these patients, endometrial sections were reviewed by an expert gynaecological pathologist (SLB) and menopausal status was determined based on atrophy of the endometrium.

Sample preparation, mRNA extraction and RT-qPCR analysis

Original histological sections of HGSC samples were reviewed by one of two expert gynaecological pathologists (SLB and MHFML-B). Representative sections with sufficient tumour cells were annotated and HGSC samples containing <40% tumour cells were excluded from analysis to minimize stromal contamination. Five-micrometer formalin-fixed paraffin-embedded (FFPE) sections of primary HGSC samples were cut with a microtome (RM2255, Leica Biosystems, Germany). Depending on total annotated tumour area, multiple sections were scraped manually resulting in at least 20 mm^2 tumour surfaces. Total mRNA was isolated following the manufacturer's protocol (VERSANT® Tissue Preparation Reagents kit, Siemens, Erlangen, Germany) and mRNA concentrations were measured using the Qubit® RNA HS Assay Kit and Qubit® Fluorometer (Invitrogen, Thermo Fisher Scientific, Waltham, USA). Pathway-specific target gene expression levels were measured by RT-qPCR using the SuperScriptTM III PlatinumTM One-Step qRT-PCR kit (Invitrogen, Thermo Fisher Scientific, USA), PCR plates (OncoSignal, Philips MPDx, Eindhoven, The Netherlands) and a CFX96 Real-Time PCR Detection System (BioRad, Hercules, USA).

OncoSignal pathway assays

Anonymized RT-qPCR data were provided to Philips Research to determine functional STP activity using previously described knowledge-based probabilistic Bayesian computational pathway models.[10,12,13] An important and unique advantage of the commercially available pathway activity assays is that, in principle, they can be performed on every cell or tissue type. Alternative approaches often require fresh or fresh frozen tissue samples and may be limited by the requirement of a large amount of tissue.[19] OncoSignal pathway assays are developed and validated using Affymetrix microarray expression data.[10] The assays infer activity of the

corresponding transcription factor complex from the expressions of pathway-specific target genes. To facilitate the use of RT-qPCR data obtained from FFPE samples, the assays were adapted based on a selection of the most informative pathway-specific target genes. STP activity of the following pathways was determined: androgen receptor (AR), oestrogen receptor (ER), phosphoinositide 3-kinase (PI3K), Hedgehog (HH), transforming growth factor beta (TGF-β) and the canonical wingless-type MMTV integration site (Wnt) pathway. The selected target genes included in the original assays have been described in detail previously: the ER and Wnt pathways[10]; the AR, HH and TGF-β pathways[13] and the PI3K-FOXO pathway.[13,20] Activity scores represent the likelihood of a certain pathway being active, where 0 corresponds to the probability of an inactive pathway and 100 to the probability of an active pathway, as described previously.[14] For PI3K, the pathway activity is determined based on forkhead box protein O (FOXO) transcription factor activity as they are directly inversely related in the absence of cellular oxidative stress.[13] To assess cellular oxidative stress, mRNA expression levels of superoxide dismutase 2 (SOD2), a FOXO target gene, were used. There are a few important considerations for the interpretation of the generated pathway activity scores: 1. the pathway activity score range (minimum-maximum activity) on the normalized scale is unique for each cell or tissue type. Once the range has been defined using samples with known pathway activity, the absolute value for every new sample can be directly interpreted against that reference. If the range has not been defined, differences in pathway activity scores between samples can be interpreted; 2. in the same sample the pathway activity scores of different signalling pathways cannot be compared, since each of the signalling pathways has its own range of activity scores; and 3. pathway activity scores are highly quantitative, and even small differences can be reproducible and meaningful. In addition to pathway-specific target genes, we determined the expression levels of SOD2 and KI-67 as a marker for cellular oxidative stress and cell proliferation, respectively.

Statistical analysis

For clinicopathological characteristics, normally distributed continuous variables are presented as mean values with standard deviation (SD) and compared with a T-test. Skewed continuous variables are presented as the median with interquartile range (IQR) and compared with a Mann-Whitney U test or Kruskal-Wallis test. Categorical variables are presented as frequencies with percentage and compared with a Fisher's exact test. STP activity per survival group is presented as boxplots displaying the median and IQR with overlying dot plots representing individual patient samples. Univariate Cox proportional hazards regression analysis was used to assess possible correlations between pathway activity and DFS and OS. Given the differences in hormonal status, we performed separate analyses for premenopausal and postmenopausal women. Subgroups were formed based on ER pathway activity,

dividing the sample set in quartiles. Boxplots displaying pathway activity per ER subgroup were generated. DFS and OS of the newly formed subgroups were visualized in Kaplan-Meier curves and log-rank tests were used to test for differences. Subsequently, samples were divided over three subgroups containing: 1. samples with ER pathway activity in the lowest quartile and PI3K pathway activity in the highest quartile; 2. samples with ER pathway activity in the highest quartile and PI3K pathway activity in the lowest quartile; and 3. the remaining samples. Boxplots were created to visualize pathway activity per subgroup. *P*-values <0.050 were considered statistically significant. Basic statistical analyses were performed using SPSS (IBM SPSS Statistics, version 26) and data visualization was conducted using Rstudio (Rstudio, Inc. version 1.1.463).

Ethical approval

Due to the retrospective nature of the study, the Medical Research Involving Human Subjects Act (Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen) does not apply, which was confirmed by the Medical research Ethics Committees United (MEC-U, study number W16.108). Given that a majority of patients had passed away by the time of inclusion and that our analysis would not yield any outcome of interest to either the patient or their families, patient approval was waived by local hospital committees under the condition, that prior to inclusion, medical files were checked for any signs that a patient would disapprove of the use of residual bodily tissue.

Results

Study population

We identified 580 patients with advanced stage HGSC in the NCR database treated in our region, 157 of which met our eligibility criteria. Thirty-five patients with a DFS between 12 and 24 months were excluded. For the remaining 122 patients, histological sections of the primary tumour were retrieved. Review of the histological sections resulted in the exclusion of 36 women as the samples contained less than 40% tumour cells. Thus, 86 primary tumour samples were available for analysis of STP activity. Internal quality control resulted in the exclusion of one more patient due to insufficient mRNA concentration. STP activity results of 85 patients were included in our analysis, of which 52 were in the short DFS group and 33 in the long DFS group. There were no differences between the two groups in age at diagnosis, parity, menopausal status at diagnosis or FIGO stage. In the long DFS group, we found lower CA125 concentrations at diagnosis (*P*=0.003) and after treatment (*P*=0.027), as well as a higher number of primary debulking surgeries (*P*=0.007) and complete debulking outcomes (*P*=0.033). Furthermore, the number of recurrences was lower in the long

DFS group (*P*<0.001). An overview of clinicopathological characteristics per group is presented in **Table 4.1**.

Table 4.1 Clinicopathological characteristics of the included women diagnosed with advanced stage high-grade serous ovarian carcinoma. Women were divided into two groups based on short (<12 months) and long (>24 months) disease-free survival (DFS).

Variable	Short DFS n=52 (%)	Long DFS n=33 (%)	*P*-value*
Age at diagnosis			0.856
Mean (SD)	62 (12)	61 (12)	
Parity			0.477
0	7 (13)	7 (21)	
1-2	20 (38)	16 (48)	
≥ 3	17 (33)	8 (24)	
Missing	8 (15)	2 (6)	
Menopausal status			1.000
Premenopausal	10 (19)	6 (18)	
Postmenopausal	40 (77)	27 (82)	
Missing	2 (4)	0 (0)	
FIGO stage			0.758
IIIC	43 (83)	29 (88)	
IV	9 (17)	4 (12)	
CA125 concentration at diagnosis			0.003
Median (IQR)	657 (258 – 2125)	244 (120 – 415)	
Missing	2	2	
CA125 concentration after treatment			0.027
Median (IQR)	13 (9 – 17)	10 (6 – 14)	
Missing	12	3	
Debulking type			0.007
Primary	21 (40)	24 (73)	
Interval	26 (50)	9 (27)	
Other**	5 (10)	0 (0)	
Debulking outcome			0.033
Complete (no macroscopic residue)	30 (58)	27 (82)	
Optimal (residue <1cm)	9 (17)	5 (15)	
Incomplete (residue >1cm)	12 (23)	1 (3)	
Missing	1 (2)	0 (0)	
Number of recurrences			<0.001
No recurrence	0 (0)	16 (49)	
1	40 (76)	14 (42)	
2	6 (12)	1 (3)	
≥3	6 (12)	2 (6)	
Disease-free survival (days)			<0.001
Median (IQR)	195 (128 – 297)	1192 (952 – 2210)	
Overall survival (days)			<0.001
Median (IQR)	704 (425 – 991)	2058 (1618 – 2804)	

* Differences in continuous variables were tested with a T-test (normal distribution) or Mann–Whitney U test (skewed distribution). For categorical variables, *P*-values were obtained from a Fisher's exact test.

** Incomplete primary debulking followed by neo-adjuvant chemotherapy and afterwards a secondary interval debulking with adjuvant chemotherapy. Abbreviations: SD, standard deviation; IQR, interquartile range.

Signal transduction pathway activity in the short and long DFS groups

For 85 samples, we determined activity of the AR, ER, PI3K, HH, TGF-β and Wnt pathways. For two samples there were high SOD2 levels which provided evidence of cellular oxidative stress, indicating that the PI3K pathway activity may be underrepresented and thus should be interpreted with caution. These samples are clearly marked in the figures. When comparing the short- and long DFS groups, no significant differences between median STP activity of the abovementioned pathways were found (**Figure 4.1**). In both survival groups, we observed a wide variety in STP activity among individual samples, mainly for the PI3K, TGF-β and Wnt pathways.

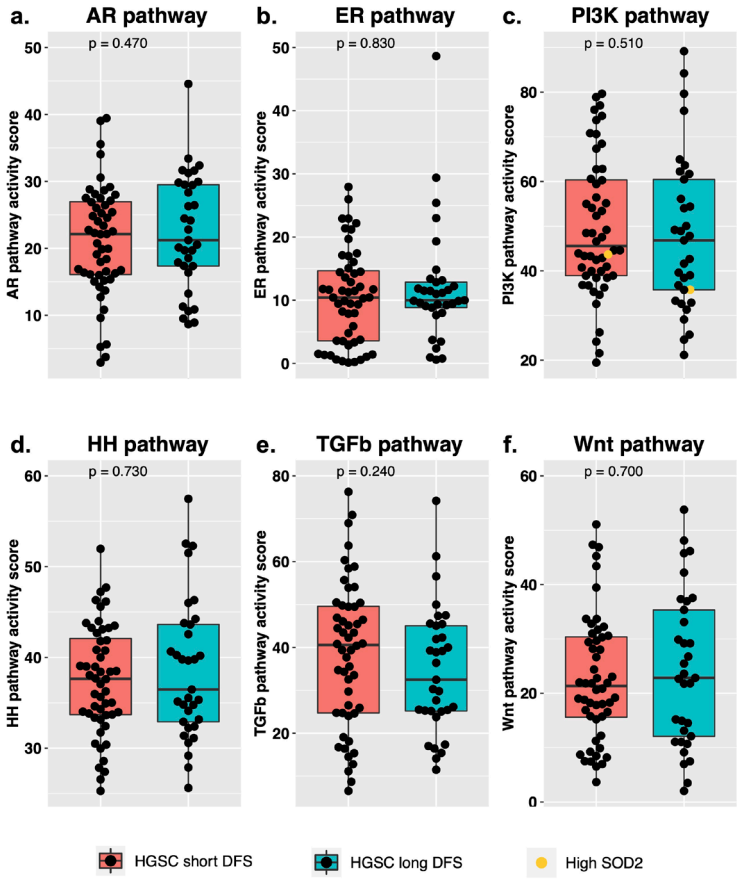
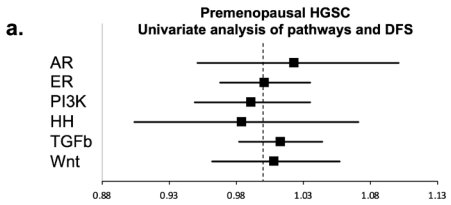


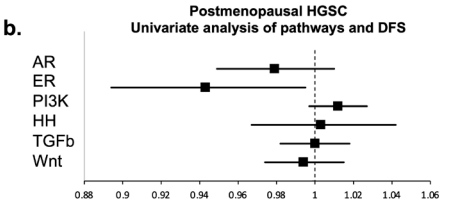
Figure 4.1 Signal transduction pathway activity measured in high-grade serous ovarian carcinoma (HGSC) samples in the short and long disease-free survival (DFS) groups, for the **A.** androgen receptor (AR), **B.** oestrogen receptor (ER), **C.** phosphoinositide 3-kinase (PI3K), **D.** Hedgehog (HH), **E.** transforming growth factor beta (TGF-β) and **F.** canonical wingless-type MMTV integration site (Wnt) pathway. *P*-values were obtained from a Mann-Whitney U test. The samples with high SOD2 levels are marked in yellow. "HGSC short DFS" refers to a DFS below 12 months and "HGSC long DFS" refers to a DFS over 24 months.

Our cohort included both pre- and postmenopausal women. As menopausal status may affect the availability of androgens and oestrogens and therefore AR and ER pathway activity, univariate Cox proportional hazards regression analysis were used to assess the effect of menopausal status on the relation between pathway activity and survival. In premenopausal women (n=16), none of the pathways were significantly related to OS or DFS. In postmenopausal women (n=67), ER pathway activity was associated with favourable DFS (Hazard Ratio (HR)=0.943; 95% confidence interval (95% CI) 0.894 to 0.995; *P*=0.033) and OS (HR=0.930; 95% CI 0.868 to 0.997; *P*=0.041). Results are visualized in **Figure 4.2**.



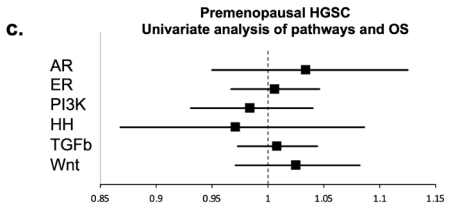
DFS (univariate)

Variable	Number	HR	Lower	Upper	*p*-value
AR	16	1.023	0.951	1.101	0.540
ER	16	1.001	0.968	1.035	0.954
PI3K	16	0.991	0.949	1.035	0.684
HH	16	0.984	0.904	1.071	0.707
TGFb	16	1.013	0.982	1.044	0.422
Wnt	16	1.008	0.962	1.057	0.739



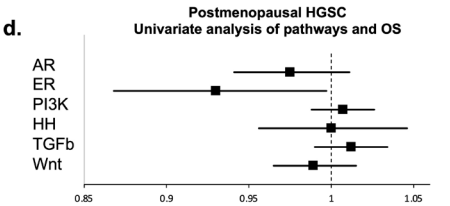
DFS (univariate)

Variable	Number	HR	Lower	Upper	*p*-value
AR	67	0.979	0.949	1.010	0.176
ER	67	0.943	0.894	0.995	0.033
PI3K	67	1.012	0.997	1.027	0.122
HH	67	1.003	0.967	1.042	0.859
TGFb	67	1.000	0.982	1.018	0.991
Wnt	67	0.994	0.974	1.015	0.588



OS (univariate)

Variable	Number	HR	Lower	Upper	*p*-value
AR	16	1.034	0.950	1.125	0.438
ER	16	1.006	0.967	1.046	0.764
PI3K	16	0.984	0.931	1.040	0.563
HH	16	0.971	0.868	1.086	0.606
TGFb	16	1.008	0.973	1.044	0.660
Wnt	16	1.025	0.971	1.082	0.368



OS (univariate)

Variable	Number	HR	Lower	Upper	*p*-value
AR	67	0.975	0.941	1.011	0.166
ER	67	0.930	0.868	0.997	0.041
PI3K	67	1.007	0.988	1.026	0.488
HH	67	1.000	0.956	1.046	0.989
TGFb	67	1.012	0.990	1.034	0.282
Wnt	67	0.989	0.965	1.015	0.407

Figure 4.2 Forest plots of univariate Cox proportional hazards regression analysis of all pathways and survival. Hazard ratios (HR) with 95% confidence intervals are described for disease-free survival (DFS) in **A.** premenopausal and **B.** postmenopausal women, and overall survival (OS) in **C.** premenopausal and **D.** postmenopausal women.

To investigate the relation between survival and ER pathway activity in postmenopausal women, this subgroup was divided into quartiles based on ER

pathway activity. Samples in quartile 1 had an ER pathway activity score ranging from 0.12 to 4.80 (median 1.33), for quartile 2 ER scores ranged from 5.88 to 9.87 (median 9.23), for quartile 3 from 9.90 to 12.14 (median 11.46) and for quartile 4 from 12.23 to 27.94 (median 14.80). Survival analysis revealed a difference in both DFS and OS among the quartiles (log-rank P=0.006 and P<0.001, respectively) with the shortest DFS and OS for patients with ER pathway activity in the lowest quartile. Kaplan-Meier curves of DFS and OS per quartile are shown in **Figure 4.3**. There were no significant differences in baseline characteristics among the quartile groups that may influence the difference in survival such as CA125 concentration at diagnosis, debulking outcome or CA125 concentration after complete treatment. An overview of clinicopathological characteristics of the ER subgroups is provided in **Supplementary Table S4.1**. We hypothesized that the association between ER pathway activity and survival might have been influenced by the activity of other pathways. **Figure 4.4** provides an overview of pathway activity per ER subgroup. Comparing median STP activity of the remaining pathways did not reveal significant differences. However, the subgroup with the lowest ER pathway activity scores was characterized by higher PI3K pathway activity when compared to the other subgroups. Inversely, the subgroup containing samples with the highest ER pathway activity scores was characterized by lower PI3K pathway activity.

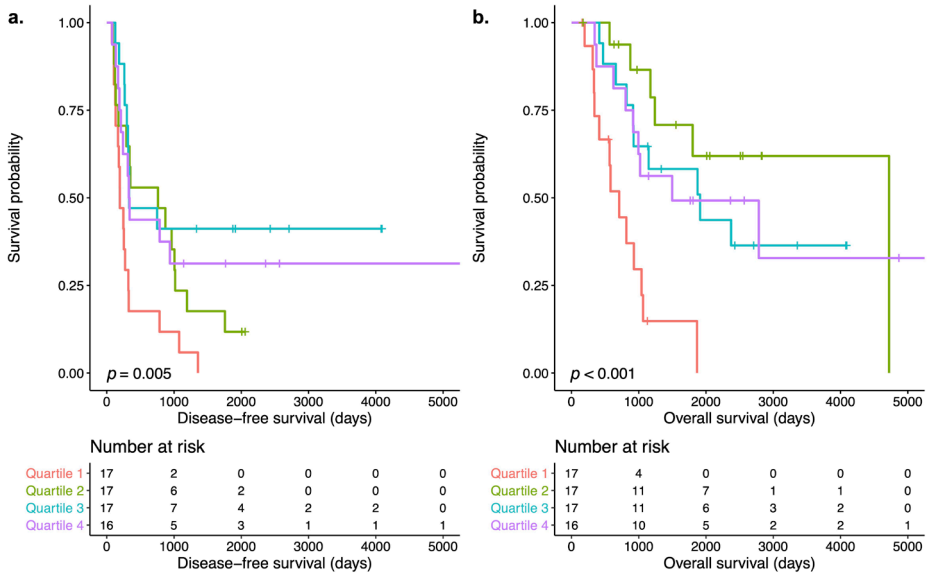


Figure 4.3 Kaplan-Meier survival analysis with log-rank tests and number at risk tables of subgroups based on quartiles of ER pathway activity in postmenopausal high-grade serous ovarian carcinoma. **A.** Disease-free survival curves; **B.** Overall survival curves.

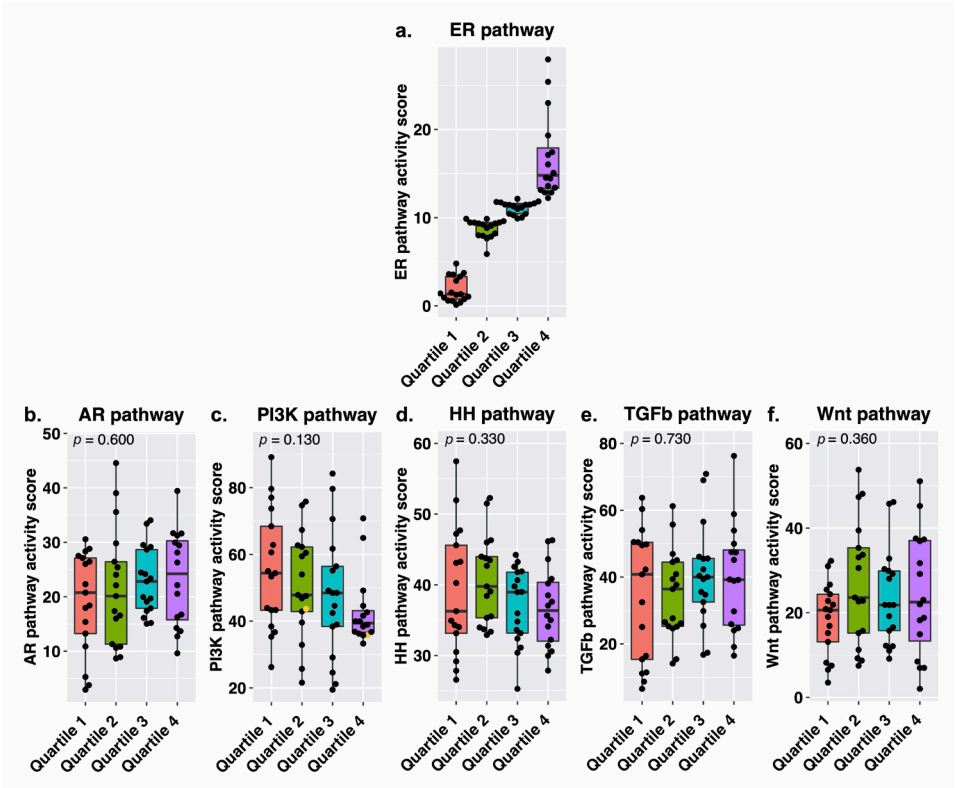


Figure 4.4 Signal transduction pathway activity measured in high-grade serous carcinoma samples of postmenopausal patients, for the **A.** oestrogen receptor (ER); **B.** androgen receptor (AR); **C.** phosphoinositide 3-kinase (PI3K); **D.** Hedgehog (HH); **E.** transforming growth factor beta (TGF-β) and **F.** canonical wingless-type MMTV integration site (Wnt) pathway. Subgroups were created by dividing ER pathway activity into quartiles (Q1, Q2 and Q3 n=17, Q4 n=16). Median pathway activity was compared among the ER quartiles, *P*-values were derived using a Kruskal-Wallis test. The samples with high SOD2 levels are marked in yellow.

To further investigate the inverse relation between ER and PI3K pathway activity, we divided samples of postmenopausal women in groups based on ER and PI3K pathway activity. Subgroup 1 contained all samples with ER pathway activity in the lowest quartile and PI3K pathway activity in the highest quartile (n=6), subgroup 2 contained all samples with ER pathway activity in the highest quartile and PI3K pathway activity in the lowest quartile (n=6) and subgroup 3 contains all remaining samples (n=55). For subgroup 1, ER pathway activity scores ranged from 0.36 to 4.80 (median 0.91) and PI3K pathway activity scores ranged from 62.83 to 89.17 (median 75.39). For subgroup 2, ER scores ranged from 12.86 to 27.94 (median 18.22) and PI3K scores from 33.28 to 36.82 (median 36.01) and for subgroup 3, ER scores ranged from 0.12 to 25.40 (median 9.87) and PI3K scores ranged from 19.44 to 84.23

(median 44.59). **Figure 4.5** provides an overview of pathway activity per subgroup. When comparing pathway activity among the subgroups, there was a difference in AR (*P*=0.009) and TGF-*β* pathway activity (*P*=0.041). Subgroup 1 was associated with low AR and TGF-*β* pathway activity compared to the other subgroups, while subgroup 2 was characterized by higher TGF-*β* pathway activity. There was no statistically significant difference in expression levels of the KI-67 proliferation marker among the subgroups, neither were there differences in DFS or OS.

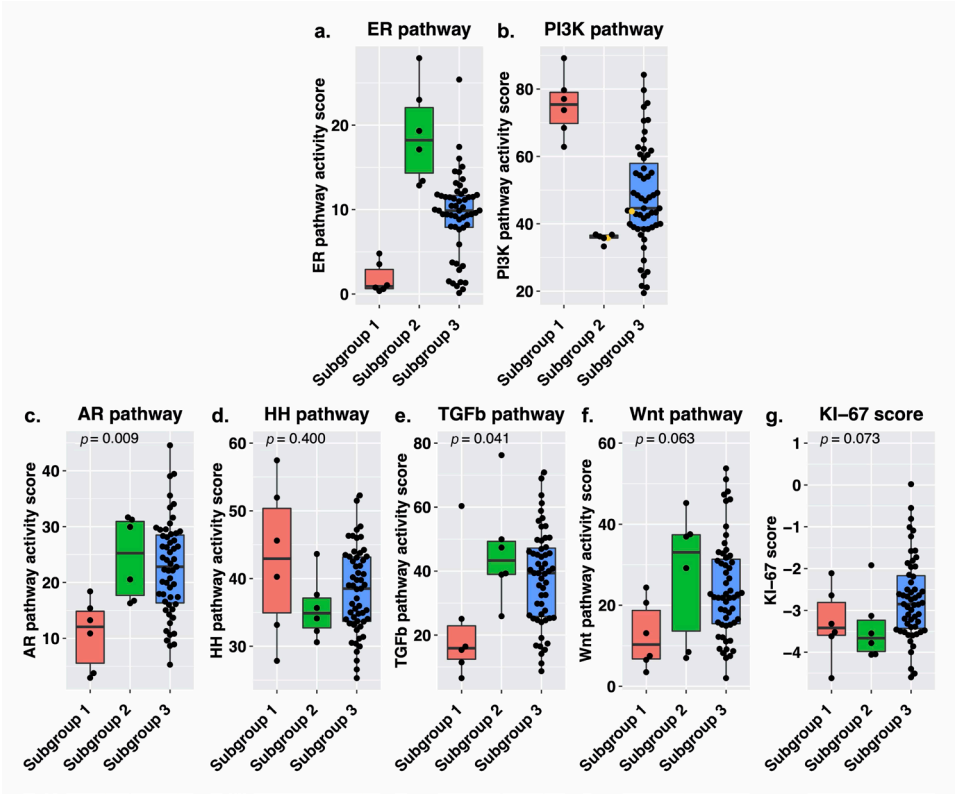


Figure 4.5 Signal transduction pathway activity measured in the different subgroups containing high-grade serous carcinoma samples of postmenopausal women, for the **A.** oestrogen receptor (ER); **B.** phosphoinositide 3-kinase (PI3K); **C.** androgen receptor (AR); **D.** Hedgehog (HH); **E.** transforming growth factor beta (TGF-*β*); **F.** canonical wingless-type MMTV integration site (Wnt) pathway and **G.** KI-67 expression levels. Subgroups were based on ER and PI3K pathway activity. Subgroup 1 (n=6) contains samples with low ER pathway activity (quartile 1) and high PI3K pathway activity (quartile 4). Subgroup 2 (n=6) contains samples with high ER pathway activity (quartile 4) and low PI3K pathway activity (quartile 1). Subgroup 3 (n=55) contains the remaining samples. Median pathway activity was compared among the subgroups, *P*-values were derived using a Kruskal-Wallis test. The samples with high SOD2 levels are marked in yellow.

Subsequently we repeated the analysis including both pre- and postmenopausal women. Again, samples were divided based on low ER and high PI3K pathway activity

(subgroup A, n=9), high ER and low PI3K pathway activity (subgroup B, n=6) and the remaining samples (subgroup C, n=70). For subgroup A, ER pathway activity scores ranged from 0.22 to 4.80 (median 1.05) and PI3K pathway activity scores ranged from 60.61 to 89.17 (median 73.74). For subgroup B, ER scores ranged from 14.82 to 27.94 (median 21.16) and PI3K scores from 31.31 to 36.82 (median 34.51) and for subgroup C, ER scores ranged from 0.12 to 48.64 (median 10.72) and PI3K scores ranged from 19.44 to 84.23 (median 44.59). **Figure 4.6** shows an overview of pathway activity per subgroup. The difference in AR (*P*=0.001) and TGF-*β* (*P*=0.001) was retained; furthermore, there was a difference in Wnt (*P*=0.018) pathway activity between the subgroups.

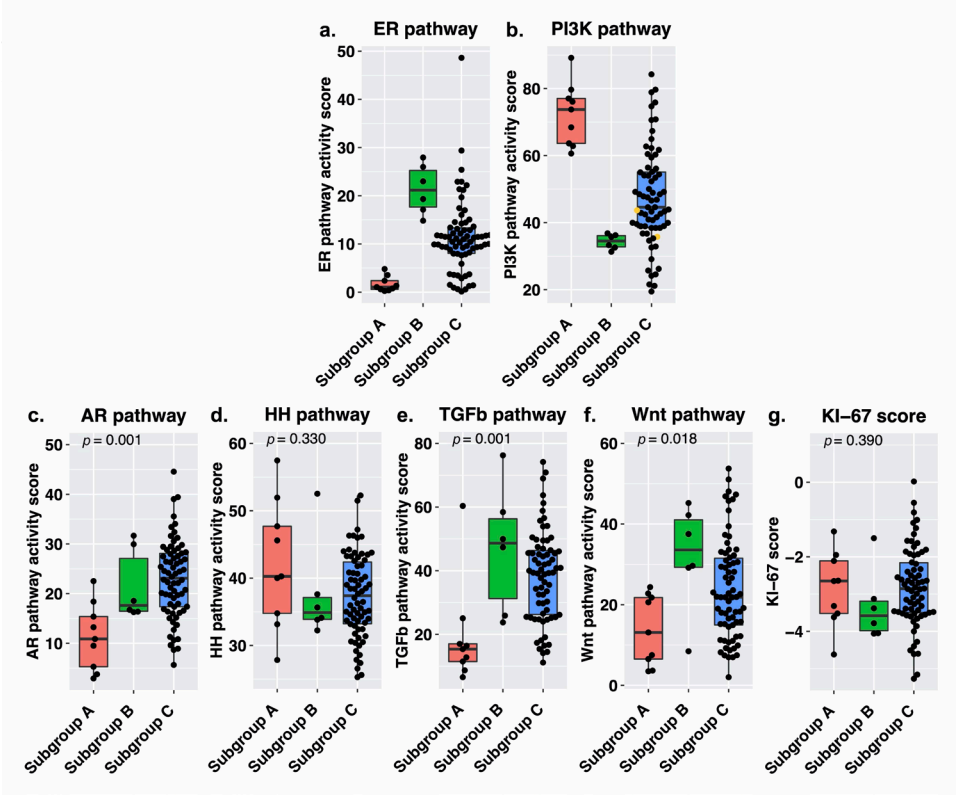


Figure 4.6 Signal transduction pathway activity measured in the newly formed subgroups containing high-grade serous carcinoma samples of both pre- and postmenopausal women, for the **A.** oestrogen receptor (ER); **B.** phosphoinositide 3-kinase (PI3K); **C.** androgen receptor (AR); **D.** Hedgehog (HH); **E.** transforming growth factor beta (TGF-*β*); **F.** canonical wingless-type MMTV integration site (Wnt) pathway and **G.** KI-67 expression levels. Subgroups were based on ER and PI3K pathway activity. Subgroup A (n=9) contains samples with low ER pathway activity (quartile 1) and high PI3K pathway activity (quartile 4). Subgroup B (n=6) contains samples with high ER pathway activity (quartile 4) and low PI3K pathway activity (quartile 1). Subgroup C (n=70) contains the remaining samples. Median pathway activity was compared among the subgroups, *P*-values were derived using a Kruskal-Wallis test. The samples with high SOD2 levels are marked in yellow.

Discussion

In this exploratory study, we assessed whether STP activity can explain differences in survival in HGSC patients. We analysed STP activity in 85 primary tumour samples of patients diagnosed with advanced stage HGSC who achieved complete remission after treatment and a DFS below 12 months (short DFS) or over 24 months (long DFS). There were no differences between these two groups in median AR, ER, PI3K, HH, TGF-β and Wnt pathway activity. Since we observed a wide variety in activity of several STPs in both short and long DFS groups, our division of HGSC in two groups may have precluded the discovery of more subtle interactions between pathway activity and survival. The wide variety of STP activity may also indicate the existence of more specific subgroups. In univariate Cox proportional hazards analysis, stratification for menopausal status revealed a positive correlation between ER pathway activity and both DFS and OS in postmenopausal women. Moreover, Kaplan-Meier survival analysis demonstrated a difference in both DFS and OS among subgroups based on ER pathway activity quartiles in postmenopausal women. The difference is mostly due to the lowest quartile compared to the others as it was characterized by the shortest DFS and OS. Within each of the other quartiles, there were large differences in DFS and OS, for example in quartile 2 which had the second shortest DFS but the longest OS. The differences between DFS and OS within the clusters are illustrative of differences in intrinsic behaviour. Alternatively, the low ER pathway activity in quartile 1, while there was no evident difference in survival among the second, third and fourth quartiles, may also suggest that an inactive pathway in particular is negatively related to survival. The comparability in survival among the higher quartiles may result from a lack of samples with a particularly active ER pathway or may indicate that whether or not the ER pathway is active is more important than the actual level of activity. While these samples may be active compared to other HGSC samples, ER pathway activity is still low to moderate when compared to healthy Fallopian tube tissue.[21] Thus, normal ER pathway activity, which is necessary for differentiated cell functions in healthy cells, is lost in HGSC. Alternatively, the differences in survival may result from preferential activity of the ER-α transcription factor over the ER-β transcription factor.[22,23] ER-β mediated signalling is tumour suppressing while ER-α mediated signalling results in increased proliferation and thus acts as a tumour promotor. Preferential signalling may thereby contribute to a tumour-driving role of the ER pathway. A slight upregulation of ER-α in HGSC samples has been described previously.[22] It should be noted that the number of included women is small and diminishes over time; results should therefore be interpreted with caution.

Although individual studies on the prognostic role of ER protein expression have previously resulted in conflicting outcomes, a recent meta-analysis concluded that OS was unrelated to ER protein expression in serous ovarian carcinoma (HR 0.90;

95% CI 0.75 to 1.08).[24-27] Unfortunately, due to a lack of suitable immunohistochemical antibodies, there is no reliable distinction between ER-α and ER-β expression. Furthermore, a direct comparison to our results is hindered as ER protein expression does not necessarily reflect an active ER signalling pathway.[28] None of the studies differentiate between pre- and postmenopausal women, while our findings suggest that hormonal status (e.g., pre- and postmenopausal) could influence tumour behaviour. Climacteric changes in steroid hormone metabolism may alter the effect of hormone receptor pathway signalling on ovarian carcinogenesis. In premenopausal women, endocrine oestrogen synthesis by the ovaries results in fluctuating levels of circulating oestradiol (E2).[29] In postmenopausal women, estrone (E1) is most abundant due to depletion of the ovarian function.[30] However, active E2 is synthesized from E1 in peripheral tissue such as adipocytes or by intracellular formation in oestrogen-dependent tumour cells.[31] Our results suggest that, in a subgroup of postmenopausal women, the tumour is either insensitive to residual levels of oestrogens or is unable to produce oestrogens itself. As a consequence, inactivity of the ER signalling pathway may promote tumour progression to a more aggressive phenotype, resulting in poorer survival outcomes. Thus, this subset of postmenopausal women may benefit from high dosed oestrogen replacement therapy under the condition that ER-β is the dominant receptor type. Alternatively, selective ER-β agonists might be required.[22,23]

When forming new subgroups based on ER and PI3K pathway activity, we found that subgroup 1 was also characterized by the lowest AR pathway activity, which further supports the loss of normal pathway activity in HGSC which is required for normal differentiated cell functions. An interplay between these pathways has been reported in ovarian cancer previously. In the study of Martins *et al.*, immunohistochemical ER, phosphatase and tensin homologue (PTEN), and AR protein expression was found in 3244 HGSC samples.[32] PTEN is a tumour suppressor of the PI3K pathway and loss of PTEN is associated with hyperactivation of the PI3K pathway activity. In line with our findings, positive PTEN protein expression was strongly correlated to both ER and AR protein expression.[32] Furthermore, a study in PTEN-deficient prostate cancer showed that AR and PI3K pathway activity were inversely related. Inhibition of AR signalling resulted in an upregulation of PI3K signalling and vice versa.[33] In our subgroups based on ER and PI3K pathway activity, we see an inverse relation between AR and PI3K pathway activity (**Figure 4.5 and Figure 4.6**). Hill *et al.* studied AR and PI3K in ovarian cancer and, although they reported some level of interaction, it was not reciprocal as is the case in prostate cancer.[34] This outcome seems typical for ovarian cancer research and may result from the inclusion of several histological subtypes of ovarian cancer or from intra-tumoral heterogeneity in which a single tumour constitutes of several cell populations with their own features and specific behaviours.[35] In their study, Hill *et al.* reported that the relation between the two

pathways requires further studies as the outcome may also result from their choice of AR activity marker or the use of Metformin as PI3K inhibitor.[34]

Another finding in our subgroups based on the inverse relation between ER and PI3K pathway activity is the low TGF-β pathway activity of subgroup 1. A crosstalk between the PI3K and TGF-β pathway has been described previously, in which the anti-proliferative effect of TGF-β signalling is decreased by PI3K pathway activation or even reversed to tumour promoting depending on the concomitant presence of an active MAPK-AP1 pathway.[20] Analysis of pre- and postmenopausal women also resulted in a difference in Wnt pathway activity among the subgroups. In subgroup A, we found low TGF-β and Wnt pathway activity, while in subgroup B both pathways appear to be active. An intricate cooperation of the TGF-β and Wnt pathways acts as a tumour-promotor, as described previously.[36] In contrast, the combination of Wnt pathway activity and FOXO transcription factor activity (i.e., an inactive PI3K pathway) acts as tumour suppressive in prostate cancer cells.[37]

In a previously published study we used STP assays on a publicly available dataset of clinically annotated HGSC samples by Tothill *et al*.[38,39] We applied a similar analysis to the Tothill dataset, with subgroups based on low ER and high PI3K pathway activity and high ER and low PI3K pathway activity compared to the remaining samples. Although the dataset contained ample clinical details, there were no data on menopausal status and thus we were unable to perform a subgroup analysis of postmenopausal women. Instead, we included all HGSC patients with a DFS below 12 or above 24 months. This analysis revealed comparable results for AR pathway activity (P=0.002); the subgroup with low ER pathway activity and high PI3K pathway activity had low AR pathway activity and the subgroup with high ER pathway activity and low PI3K pathway activity had high AR pathway activity.

A major strength of our study is the clearly defined patient population. Ovarian carcinoma is a heterogeneous disease and a generally used term for several histotypes, each with their own distinct characteristics, course of disease and optimal treatment. To limit heterogeneity and treatment effects on our main outcome, DFS, we formulated concise in- and exclusion criteria. Although this approach has its limitations, as it results in the exclusion of a substantial number of patients, we feel it is justified and even necessary to answer our research question. As a result, the number of included patients is moderate to small, especially for the subgroup analysis. Future studies including larger groups of patients should be conducted to establish the translational value of our results. The effect of FIGO stage and treatment modalities are profound and thus may conceal the effects of differences in STP signalling. Another strength is the translation of RT-qPCR results to pathway activity scores with potential clinical target and, in contrast to other tools to

determine pathway activity, the STP assays used here can be used for individual patient samples and thus are suitable for use in daily clinical practice. This more personalized approach to determine tumour specific characteristics may also be beneficial for the selection of targeted therapies.

A limitation of our study is the retrospective nature, as we were dependent on the quality and tumour percentage of readily available samples and on medical files for patient characteristics. The hospitals from which patients were included were large referral centers for gynaecologic oncology. This generally means that patients are in care of a nearby hospital and are only referred after a first round of diagnostics indicated an ovarian malignancy, lowering the availability of primary tumour samples. Patients may also choose to return to the referring hospital for adjuvant treatment and follow-up, limiting the availability of follow-up data for a small number of patients. In addition, we chose to exclude women with a DFS between 12 and 24 months to maximize possible differences between short- and long-term survivors. Although the number of excluded patients was limited (n=35), and it is unknown for how many of these patients' primary tumour samples were available for RT-qPCR analysis, this may have concealed subtler differences. Furthermore, the use of achieving complete remission as inclusion criterium has resulted in a selection bias in which patients with the poorest outcome were excluded.

Conclusions

While we have found a relation between survival and ER signalling pathway activity in advanced stage postmenopausal HGSC, much remains to be elucidated when it comes to STP activity in HGSC. Identification of patients with high risk of recurrence and poor survival could be particularly useful in stratification of patients to (maintenance) targeted therapies. In our assessment of STP activity of short- and long-term disease-free survivors of HGSC, we were unable to identify a single pathway responsible for the differences in survival. However, we were able to identify subgroups of patients which were characterized by high ER and AR pathway activity and low PI3K pathway activity and conversely low ER and AR pathway activity and high PI3K pathway activity.

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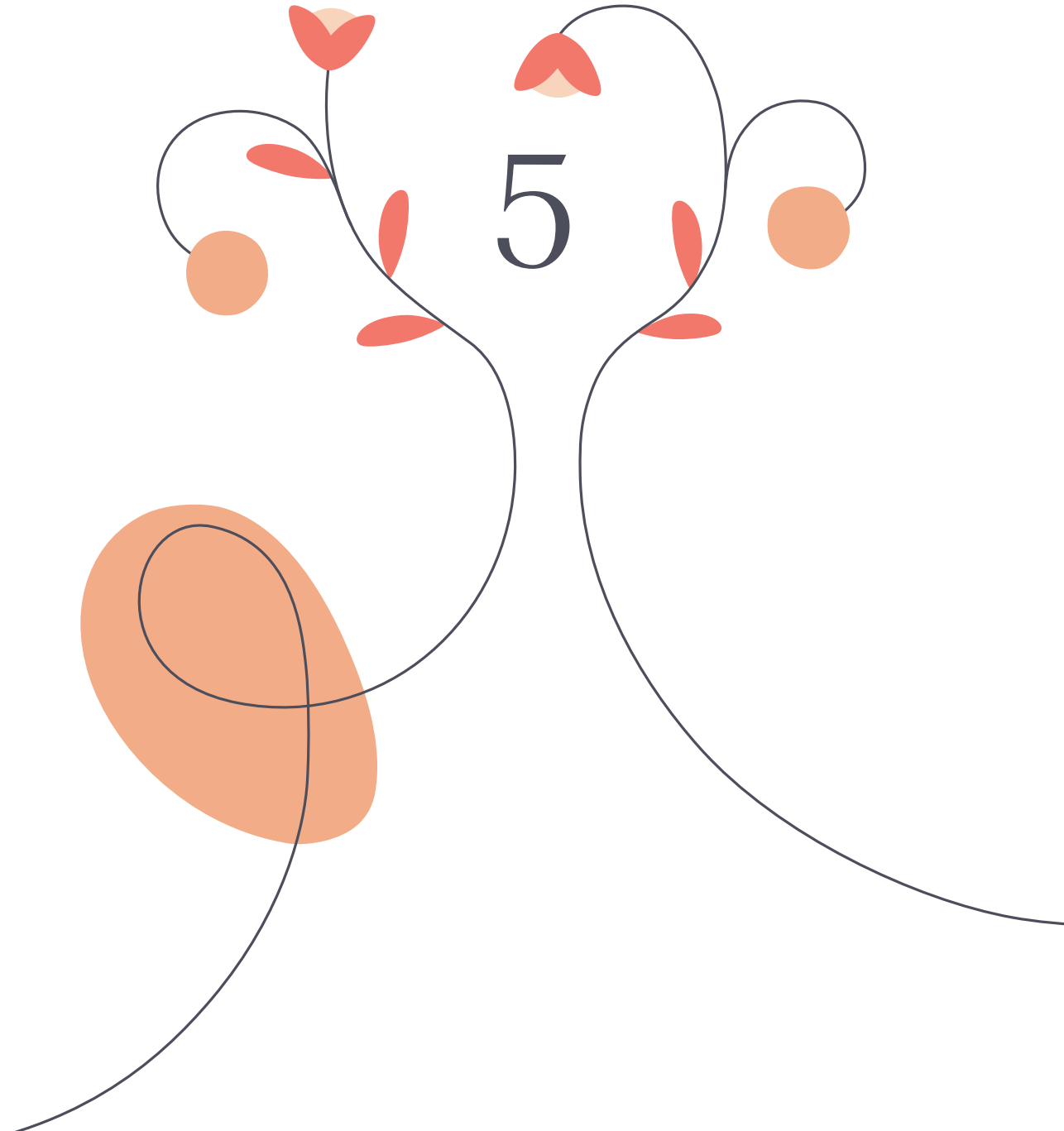
Supplementary information

Table S4.1 Clinicopathological characteristics of the subgroups based on ER pathway activity in postmenopausal HGSC patients.

Variable	Quartile 1 n=17 (%)	Quartile 2 n=17 (%)	Quartile 3 n=17 (%)	Quartile 4 n=16 (%)	*P*-value*
Age at diagnosis					0.828
Median (IQR)	65 (63 – 72)	65 (61 – 71)	67 (59 – 73)	64 (58 – 72)	
FIGO stage					1.000
IIIC	15 (88)	15 (88)	15 (88)	14 (87)	
IV	2 (12)	2 (12)	2 (12)	8 (13)	
CA125 concentration at diagnosis					0.220
Median (IQR)	340 (68 – 737)	690 (242 – 2057)	245 (59 – 603)	288 (163 – 1032)	
Missing (n)	-	1 (6)	1 (6)	-	
CA125 concentration after treatment					0.826
Median (IQR)	13 (9 – 16)	12 (8 – 16)	11 (7 – 13)	12 (6 – 14)	
Missing (n)	4 (24)	3 (18)	4 (24)	2 (13)	
Debulking type					0.990
Primary	9 (53)	10 (59)	9 (53)	8 (50)	
Interval	7 (41)	6 (35)	8 (47)	7 (44)	
Other**	1 (6)	1 (6)	0 (0)	1 (6)	
Debulking outcome					0.910
Complete	13 (76)	11 (65)	13 (76)	11 (69)	
Optimal	3 (18)	4 (23)	2 (12)	2 (12)	
Incomplete	1 (6)	2 (12)	2 (12)	3 (19)	

* Differences in continuous variables were tested with a Mann-Whitney U test (skewed distribution). For categorical variables, *P*-values were obtained from a Fisher's exact test; ** Incomplete primary debulking followed by neo-adjuvant chemotherapy and afterwards a secondary interval debulking with adjuvant chemotherapy.
Abbreviation: IQR, interquartile range.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CHAPTER 5

Efficacy of anti-oestrogen therapy in oestrogen receptor positive high-grade serous ovarian carcinoma: a systematic review

Phyllis van der Ploeg, Meggy P.M. Ottenheijm, Laura A.M. van Lieshout, Anja van de Stolpe, Steven L. Bosch, Anna M.J. Thijs, Ruud L.M. Bekkers, Jurgen M.J. Piek

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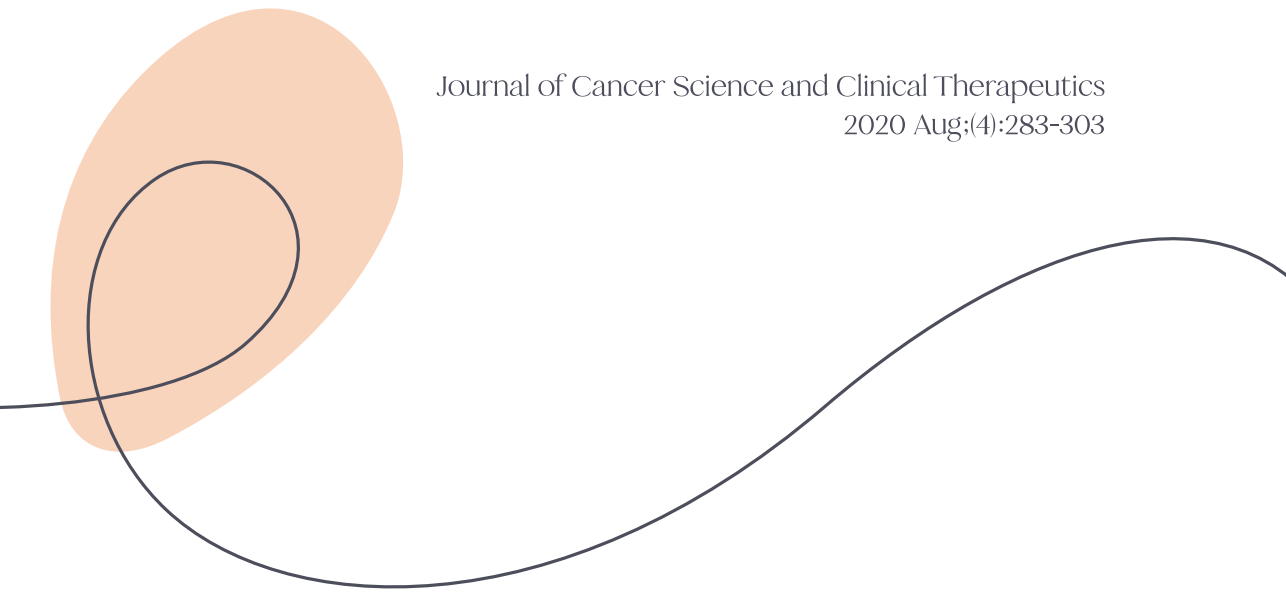

Abstract

Therapy targeting the oestrogen receptor (ER) pathway is being explored as a treatment option in ovarian carcinoma. However, studies on the efficacy of anti-oestrogen therapy include a broad range of histological subtypes and/or do not select patients based on ER status. This systematic review provides an analysis of the literature on the clinical benefit rate (CBR) of anti-oestrogen therapy in ER positive high-grade serous carcinoma (HGSC) and on the correlation between ER expression by immunohistochemistry and clinical response. We did not find studies with populations consisting solely of ER positive HGSC. However, we included six studies reporting on 407 evaluable patients of whom 376 were HGSC (92%) and 302 were confirmed ER positive (80%). Anti-oestrogen therapy resulted in a CBR of 27-65% and an overall response rate of 0-16%. No correlation was found between ER expression and clinical response. Therefore, ER protein expression alone is not a specific predictor of response. This may result from the incorrect assumption that ER expression equals ER pathway activity, since in the absence of ER activating mutations, ER pathway activity depends on availability of the oestradiol ligand. In order to apply effective ER targeted therapy, it is important to develop better predictors to identify (non)-responders.

Introduction

Ovarian carcinoma reflects a heterogenous disease compromised of five histological subtypes, namely high-grade serous, low-grade serous, endometrioid, clear cell and mucinous, that all differ in their cell-of-origin, pathogenesis and prognosis.[1] High-grade serous carcinoma (HGSC) is the most common subtype (70%) of ovarian carcinoma, of which 80% of the patients is diagnosed with advanced stage disease due to asymptomatic and rapid tumour progression.[2,3] Current treatment consisting of debulking surgery and (neo)adjuvant chemotherapy with carboplatin and paclitaxel frequently results in complete remission.[4] However, most patients will experience relapse of disease, which is often complicated by resistance to platinum containing chemotherapy. As only few options then remain, research focusses on the use of alternative therapies, such as targeting the oestrogen receptor (ER) signalling pathway.[5]

The ER signalling pathway can be initiated through direct or indirect oestradiol signalling.[6] As a steroid hormone, oestradiol can directly enter the cytoplasm where it can bind nuclear ER monomers (ER-α and ER-β) and induces receptor dimerization. The transcription factor complex translocates to the nucleus where it binds to ER response elements in gene promoter regions and activates transcription of ER target genes. Alternatively, oestradiol can bind a G-protein coupled receptor on the plasma membrane and hereby activate intracellular second messengers. In this manner oestradiol can indirectly influence activation of other signalling pathways, such as the phosphoinositide 3-kinase (PI3K) signalling pathway.[6]

ER signalling pathway activity can be inhibited by selective oestrogen receptor modulators (SERMs; e.g. tamoxifen), selective oestrogen receptor downregulators (SERDs; e.g. fulvestrant) or aromatase inhibitors (e.g. anastrozole, letrozole or exemestane).[7] Tamoxifen is able to act both as a partial agonist and antagonist.[8] Like oestradiol, it can bind to ER but the induced transcriptional activation of ER is lower. Tamoxifen competes with oestradiol for ER binding and antagonizes the effect of oestradiol, but in the absence of oestradiol it will act as a partial agonist.[8-10] Fulvestrant has a pure antagonistic effect as it binds reversibly to ER monomers, which prevents receptor dimerization and thereby stimulates degradation of ER.[11] Aromatase is an essential molecule in the formation of oestradiol and inhibition by anastrozole or letrozole leads to blockage of the final step in the steroid biosynthetic pathway to generate oestradiol.[12] As a result, aromatase inhibitors are able to almost completely block oestradiol production by aromatase-expressing cells.

Ovarian carcinoma is considered a hormone-dependent disease as oestrogens caused proliferation of ovarian cancer cells *in vivo* and *in vitro*.[13,14] However, the exact

mechanism of action of oestrogens in ovarian carcinoma is not fully understood. The use of anti-oestrogen therapy is a well-established treatment for hormone-dependent breast cancer.[15] Oral administration and low toxicity makes anti-oestrogens an attractive therapy option, which has also been studied in ovarian carcinoma during the past decades.[16,17] A meta-analysis concluded that anti-oestrogen therapy in ovarian cancer was associated with a modest clinical benefit rate (CBR) of 41% (95% confidence interval (CI) 34-48).[17] CBR was defined as the total proportion of patients who had achieved complete response (CR), partial response (PR) and stable disease (SD). The selected trials in this meta-analysis included women with a broad range of ovarian cancer histological subtypes and multiple studies did not select patients based on ER status, resulting in a heterogenic population.

Immunohistochemical (IHC) ER protein staining of formalin-fixed paraffin-embedded sections is widely used to identify ER protein expression. A study from the Ovarian Tissue Analysis Consortium investigated ER positivity based on ≥50% stained tumour cell nuclei in 2,933 ovarian carcinomas.[18] They found strong ER expression in 60% of the HGSC. However, in contrast to breast cancer, the predictive value of ER status on anti-oestrogen response has not been well-established for HGSC. Additional ER scoring methods such as the histoscore method, which takes intensity and percentage of stained tumour cells into account, have been developed.[19] The first phase II trial with ovarian cancer patients treated with letrozole found a significant correlation between response and increasing ER histoscores (P<0.001).[20] Another phase II trial with letrozole also reported a significant correlation (P=0.028), suggesting that patients with high ER histoscores respond better to anti-oestrogen therapy.[21] However, this correlation has not been found in other studies.[22,23] The conflicting findings may result from the inclusion of ER negative patients in these studies, as these patients may have obscured the relation between ER expression and response to anti-oestrogen therapy.

In this systematic review, we aim to analyse the literature on the CBR of anti-oestrogen therapy in a homogenic population of ER positive metastatic or recurrent HGSC. Additionally, we aim to correlate ER expression based histoscores to clinical response.

Methods

Search strategy

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA).[24] Studies were identified by performing a literature search in the electronic databases PubMed, the Cochrane

Library and ClinicalTrails.gov. The search query combined synonyms and Mesh terms for 'ovarian carcinoma' and 'anti-oestrogen therapy'. The full search is shown in **Supplementary Table S5.1**. The last search was conducted on December 13th, 2019.

Inclusion criteria

Studies were eligible for inclusion when reporting clinical response rates of anti-oestrogen therapy in ER positive metastatic or recurrent HGSC patients. Anti-oestrogen therapy included the following drugs: tamoxifen, anastrozole, letrozole, exemestane and fulvestrant. In order to review the latest clinical results, only studies published during the last 10 years were included. Language was restricted to English and full study results had to be available. All clinical studies were included with the exception of case reports. Reviews and meta-analysis were not eligible for inclusion, but reference lists were carefully screened for any additional inclusions.

Data extraction and quality assessment

Eligibility assessment was performed independently by two reviewers (PvdP and MPMO) and any disagreements were resolved by discussion with a third reviewer (JMJP). Data was extracted using pre-designed standardized data collection forms which included publication details, study design, sample size, study population, histologic subtype, type of treatment, method of response measurement, clinical outcomes, type of ER measurement, ER status and measured correlation between ER status and therapy response.

Two reviewers (PvdP and MPMO) independently assessed risk of bias using the ROBINS-I tool for non-randomized studies.[25] Risk of bias was scored as high, low or unclear risk and assessed for the following domains: confounding, selection of participants, co-interventions, missing data, measurement of outcome, selective reporting and other sources of bias. To use the ROBINS-I tool, a hypothetical 'target' trial is necessary to assess bias. We defined this trial as a phase II or III clinical trial of anti-oestrogen therapy in ER positive metastatic or recurrent HGSC. Criteria that this trial should meet are as follows: 1. baseline information should include tumour histology, number of prior lines of (chemo)therapy and ER status, 2. detailed information on the intervention is required and no co-interventions would be allowed during the treatment period and 3. CBR should be measured after a minimum of three months by GCIG and/or RECIST criteria.

Statistical analysis

The primary outcome measure was CBR, defined as the percentages of patients that achieved CR, PR and SD. Secondary outcome measures were overall response rate (ORR), defined as the percentages of patients with CR and PR, median progression-

free survival (PFS) and median duration of response. For CBR and ORR 95% CI were calculated using the modified Wald method.[26]

Results

Search results

We identified a total of 461 references from PubMed, the Cochrane Library and ClinicalTrials.gov, of which 434 remained after removal of duplicates. After title and abstract screening 386 articles were excluded. Full-text screening of the remaining 48 articles resulted in the exclusion of an additional 41 articles as they did not meet the inclusion criteria, mostly because they lacked data on ER status. There were no clinical studies available with a population consisting entirely of ER positive HGSC. However, we identified seven articles describing six individual clinical studies reporting on anti-oestrogen therapy in ovarian cancer populations partially consisting of ER positive HGSC. The screening and selection process and reasons for exclusion are illustrated in a PRISMA flowchart (**Figure 5.1**).

Description of included studies

The sample size of included studies ranged from 19 to 164 evaluable patients, resulting in a total of 407 included ovarian cancer patients, 376 of whom were diagnosed with HGSC (92%). ER positivity was confirmed in 302 out of 376 HGSC (80%). Study characteristics are summarized in **Table 5.1** and a detailed description of the included studies is given below.

Argenta *et al.*[27,28] reported two studies on one phase II clinical trial of fulvestrant treatment in 31 (26 evaluable) ER positive recurrent ovarian carcinoma patients, 16 (62%) of which were HGSC. Treatment regimen consisted of 500 mg intramuscular (IM) on day 1, followed by 250 mg IM on day 15, day 29 and every 28 days thereafter. Primary endpoint was CBR at 90 days based on modified Rustin and RECIST criteria. ER positivity was based on >10% stained tumour cell nuclei in primary tumour tissue and ER histoscores were obtained in 24 patients (92%) using the archived paraffin-embedded blocks.

Bonaventura *et al.*[29] performed a phase II clinical trial of 53 (49 evaluable) platinum-resistant recurrent ovarian carcinoma treated with anastrozole (1.0 mg daily). Most patients were HGSC, but the exact percentage was not specified. CBR was determined by GCIG or RECIST 1.1 criteria every three months of treatment. All patients were ER positive based on >10% stained tumour cell nuclei and tumour tissue blocks for ER histoscore assessment were available in 34 patients (69%) with clinical response data.

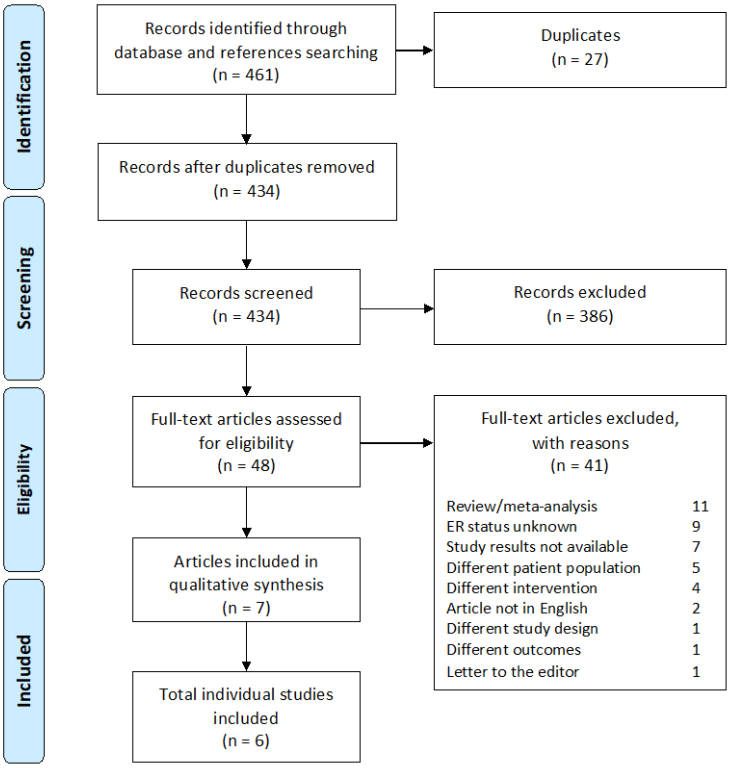

Figure 5.1 PRISMA flowchart of systematic literature search and selection of studies. ER, oestrogen receptor.

Colon-Otero *et al.*[30] presented a phase II clinical trial enrolling 20 patients with platinum-resistant or -sensitive relapsed ovarian cancers treated with a combination of letrozole (2.5 mg per oral (PO) daily) and the mammalian target of rapamycin (mTOR) inhibitor everolimus (10 mg PO daily). Nineteen patients were evaluable for response evaluation, 17 of which were HGSC (89%). Primary endpoint was PFS at 12 weeks based on CA125 tumour marker concentrations or radiological assessments by RECIST 1.1. All patients were defined ER positive, however no information was given about the threshold for ER positivity or whether this was based on primary or recurrent tumour tissue.

Table 5.1 Patient and treatment characteristics of studies including high-grade serous carcinoma treated with anti-oestrogen therapy.

Author and year	Number of patients	Study population	Histologic subtype (n)	Prior lines of chemotherapy	Type of anti-oestrogen treatment	Method of response measurement
Phase II clinical trials						
Argenta *et al.* 2009 and 2013[27,28]	31 (26 evaluable)	Multiple recurrent disease	High-grade serous (16) Endometrioid (4) Clear cell (1) Other (5)	Median 5.0	Fulvestrant Day 1 500 mg Day 15 250 mg Day 29 250 mg Every 28 days till progression 250 mg	CA125 by modified Rustin criteria and modified RECIST
Bonaventura *et al.* 2017[29]	53 (49 evaluable)	Platinum-resistant recurrent disease	Most high-grade serous	Median 2.0	Anastrozole 1.0 mg daily	CA125 by GCIG criteria and/or radiological assessment by RECIST 1.1
Colon-Otero *et al.* 2017[30]	20 (19 evaluable)	Platinum-resistant or -sensitive relapsed disease	High-grade serous (17) Carcinosarcoma (1) Transitional cell carcinoma (1)	Median 3.0	Letrozole 2.5 mg and everolimus 10 mg daily	CA125 and/or radiological assessment by RECIST 1.1
Kok *et al.* 2019[31]	54 (52 evaluable)	Asymptomatic recurrent disease	High-grade serous (40) Low-grade serous (5) Serous carcinoma, unknown grade (4) Endometrioid (4) Clear cell (1)	Median 1.0	Anastrozole 1.0 mg daily	CA125 by GCIG criteria and/or radiological assessment by RECIST 1.1
Retrospective cohort studies						
George *et al.* 2017[32]	97	Platinum-resistant or -sensitive relapsed disease	High-grade serous (90) High-grade endometrioid (5) Clear cell (2)	Median 3.0	Tamoxifen 20 mg (n=36) 40 mg (n=7) Letrozole 2.5 mg (n=54)	CA125 by GCIG criteria and radiological assessment by RECIST 1.1
Stanley *et al.* 2019[33]	269 (164 evaluable)	Platinum-resistant or -sensitive relapsed disease	High-grade serous (164)	Median 1.0	Letrozole (n=128) Tamoxifen (n=36)	CA125 by modified GCIG criteria

Kok *et al.*[31] reported a phase II clinical trial of anastrozole (1.0 mg PO daily) in asymptomatic ER positive recurrent ovarian cancer patients based on CA125 progression. These patients normally await (chemo)therapy until symptoms occur. The study population consisted of 52 evaluable patients of whom 40 were HGSC (77%). Primary endpoint was CBR at three months based on GCIG or RECIST 1.1 criteria. ER positivity was based on >10% stained tumour cell nuclei and histoscores were calculated retrospectively in 28 patients (54%) on available archival tissue blocks.

The study of George *et al.*[32] was a retrospective cohort study in 97 platinum-resistant or -sensitive relapsed ovarian carcinoma patients, 90 of which were HGSC (93%). Patients were treated with tamoxifen 20 or 40 mg PO daily (44%) or letrozole 2.5 mg PO daily (56%). CBR was measured after three months based on GCIG or RECIST 1.1 criteria. Fifty-two percent of the population was classified ER positive, although a threshold of percentage positive ER stained tumour cell nuclei was not mentioned. In 47% ER status was unknown and 1% was ER negative. The authors did not specify if IHC staining was conducted on primary or recurrent material and ER histoscores were not calculated.

Stanley *et al.*[33] conducted a retrospective cohort study in 267 (164 evaluable for CA125 response) platinum-resistant or -sensitive relapsed HGSC patients treated with letrozole (78%) or tamoxifen (22%). Patients received at least four weeks of treatment. Response after 12 weeks was based on modified GCIG criteria due to variable frequency of CA125 measurements. ER histoscores were calculated in 225 patients (148 evaluable for CA125 response) of which the majority (85%) was based on primary tumour tissue prior to chemotherapy.

Risk of bias in included studies

Studies were subjected to a comprehensive quality assessment for the risk of bias on seven predefined domains and reviewers' judgements of each domain were summarized in **Table 5.2**.

Bias due to confounding

We considered four studies to be at low risk of bias due to confounding related to the detailed description of the patient characteristics.[27-31] Populations consisted of recurrent or metastatic ER positive disease with at least one prior line of chemotherapy. In two studies a proportion of the population had unknown ER status and therefore these studies were considered to be at high risk of bias.[32,33]

Bias in selection of participants

Five studies we rated at low risk of bias due to selection of participants as the inclusion- and exclusion criteria and selection process were descripted in detail and reasons for exclusion were mentioned.[27-31,33] One study did not sufficiently describe the exclusion criteria and selection process and thus was rated as at unclear risk of bias.[32]

Bias in classification of intervention

We rated three studies as at low risk of bias due to classification of intervention as the intervention was described in detail and no co-interventions were allowed.[27,28,30,32] Three studies did not sufficiently describe the intervention and/or did not describe if the use of co-interventions during the study period was excluded. These studies were considered to be at unclear risk of bias.[29,31,33]

Bias due to deviations from intended intervention

We judged two studies to be at low risk of bias due to deviations from intended intervention as treatment delay did not occur and reasons for dose modifications were specified.[27,28,30] Three studies did not describe if deviations from intended intervention, dose modifications or treatment delay occurred and were rated as at unclear risk of bias.[29,31,33] One study was considered to be at high risk of bias as duration of anti-oestrogen therapy use was not defined and a substantial proportion (18%) of the population received both tamoxifen and letrozole as a single agent during the study period.[32]

Bias due to missing or incomplete data

Four studies sufficiently described number of included and evaluable patients and were judged as low risk of bias due to missing or incomplete data.[27-31] One study was considered as unclear risk of bias as they did not specify the number of exclusions based on missing response data.[32] One study was considered to be at high risk of bias as only 64% of the patients were evaluable for CA125 response.[33]

Bias in measurement of outcome

We judged four studies as at low risk of bias due to measurement of outcome related to a detailed description of criteria for response evaluation.[27-31] Two retrospective studies were rated as at high risk of bias as timepoints of response measurements were not standardized.[32,33]

Bias in selection of reported results

One study reported the outcomes according to the published protocol and thus we considered it at low risk of bias in selection of reported results.[27,28] Four studies were rated at unclear risk of reporting bias: two studies referred to the same protocol[29,31] and for the other studies we were not able to find a published protocol.[32,33] One study was rated to be at high risk of reporting bias as the primary objective in the study protocol stated to compare the PFS of combination therapy with letrozole and everolimus with results from a previously reported phase II study with letrozole monotherapy.[30] However, results were not compared as the article does not refer to a previously conducted phase II trial.

Other sources of bias

We identified no other sources of bias.

Table 5.2 Risk of bias of included studies by reviewers' judgement.

Author and year	Bias due to confounding	Bias in selection of participants	Bias in classification of intervention	Bias due to deviations from intended intervention	Bias due to missing or incomplete outcome data	Bias in measurement of outcome	Bias in selection of reported results	Other sources of bias
Argenta *et al.* 2009 and 2013 [27,28]	✓	✓	✓	✓	✓	✓	✓	✓
Bonaventura *et al.* 2017 [29]	✓	✓	?	?	✓	✓	?	✓
Colon-Otero *et al.* 2017 [30]	✓	✓	✓	✓	✓	✓	–	✓
Kok *et al.* 2019 [31]	✓	✓	?	?	✓	✓	?	✓
George *et al.* 2017 [32]	–	?	✓	–	?	–	?	✓
Stanley *et al.* 2019 [33]	–	✓	?	?	–	–	?	✓

✓ Low, ? Unclear, – High

Clinical outcome of anti-oestrogen therapy

The included studies in this systematic review reported a CBR ranging between 27-65% and an ORR ranging between 0-16% after approximately three months of anti-oestrogen therapy (**Table 5.3 and Figure 5.2**). The median PFS ranged between 2.0-3.9 months and the mean duration of response ranged between 2.8-6.5 months. Argenta *et al.* investigated the effect of fulvestrant injections in patients with multiple recurrent disease and observed a CBR of 50% (95% CI 32-68) based on computer tomography evaluation.[27,28] Both Bonaventura *et al.* and Kok *et al.* reported on anastrozole therapy and observed a CBR of 27% (95% CI 16-40) and 35% (95% CI 23-48), respectively.[29,31] The study population of Bonaventura *et al.* included platinum-resistant recurrent patients compared to asymptomatic recurrent patients in the study of Kok *et al*.[29,31] The combination of the aromatase inhibitor letrozole with the mTOR inhibitor everolimus resulted in a CBR of 53% (95% CI 32-73) in platinum-resistant or -sensitive relapsed patients in the study of Colon-Otero *et al*.[30] Two of the included trials reported results from both tamoxifen and letrozole therapy in platinum-resistant or -sensitive relapsed patients. George *et al.* reported an overall CBR of 60% (95% CI 50-69).[32] They found a CBR of 65% (95% CI 50-78) with tamoxifen therapy, which was not statistically higher than the 56% CBR (95% CI 42-68) of letrozole therapy (*P*=0.140). Though, the median duration of response in the group with partial responders was longer with letrozole therapy compared to tamoxifen therapy (26.0 versus 11.5 months, respectively, *P*=0.030). Stanley *et al.* reported an overall CBR of 40% (95% CI 32-47) but did not find a significant difference between tamoxifen (33%, 95% CI 20-50) or letrozole (41%, 95% CI 33-50) CBR (*P*=0.495).[33] They noticed a longer median duration of letrozole therapy compared to tamoxifen (126 versus 98 days, respectively, *P*=0.006 in univariable analysis, *P*=0.255 in multivariable analysis).

Table 5.3 Oestrogen receptor (ER) targeted therapy response and ER status in high-grade serous carcinoma (HGSC).

Author and year	Number of HGSC (%)	Type of anti-oestrogen	CR (%)	PR (%)	SD (%)	ORR (%)	CBR (%)	Median PFS	Median duration of response	Type of ER measurement or scoring	ER status (n)	Correlation between ER status and response
Phase II clinical trials												
Argenta *et al.* 2009 and 2013 [27,28]	16 (62)	Fulvestrant	0	0	50	0	50	2.0 months (62 days)	-	IHC ≥ 10% ER histoscores	Positive (26) 0-100 (12) 101-200 (11) 201-300 (1)	No correlation, but significant difference between group with or without response (*P*=0.020)
Bonaventura *et al.* 2017 [29]	49 (100)	Anastrozole	0	0	27	0	27	2.7 months	2.8 months	IHC > 10% ER histoscores	Positive (49) 0-100 (13) 101-200 (11) 201-300 (10)	No correlation
Colon-Otero *et al.* 2017 [30]	17 (89)	Letrozole and Everolimus	0	16	37	16	53	3.9 months	-	Unknown	Positive (19)	-
Kok *et al.* 2019 [31]	40 (77)	Anastrozole	0	4	31	4	35	2.7 months	6.5 months	IHC > 10% ER histoscores	Positive (52) 0-100 (8) 101-200 (12) 201-300 (8)	No correlation
Retrospective cohort studies												
George *et al.* 2017 [32]	90 (93)	Tamoxifen	0	14	51	14	65	-	-	Unknown	Positive (50)	-
		Letrozole	0	15	41	15	56		-		Negative (1) Unknown (46)	
Stanley *et al.* 2019 [33]	164 (100)	Letrozole	3	5	34	8	41	-	-	ER histoscores	Unknown (27) 0–150 (35)	No correlation, but significant difference
		Tamoxifen	3	8	22	11	33				151–200 (35) 201–250 (44) 251–300 (34)	between group with 0-150 and 251-300 ER histoscores (*P*=0.040)

Abbreviations: CR, complete remission; PR, partial response; SD, stable disease; ORR, overall response rate; CBR, clinical benefit rate; PFS, progression-free survival; IHC, immunohistochemistry.

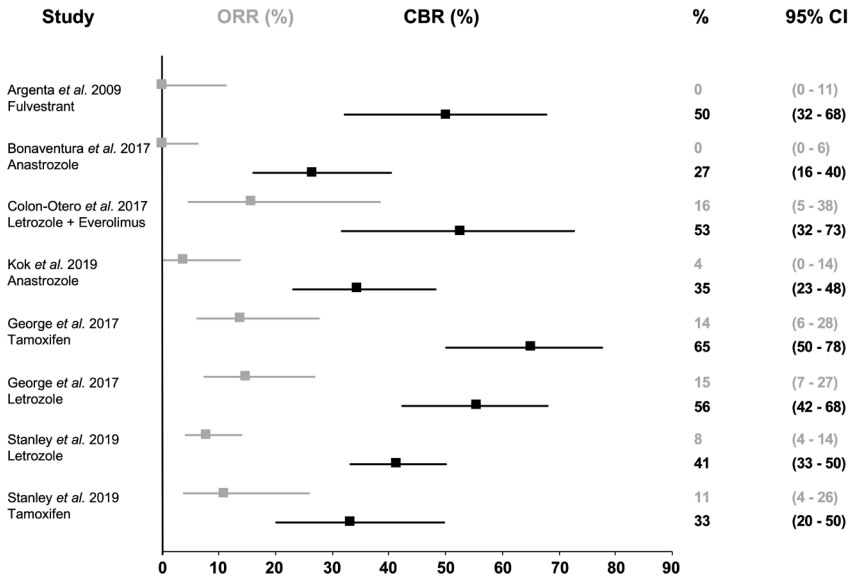

Figure 5.2 Overall response rate (ORR) and clinical benefit rate (CBR) of anti-oestrogen therapy. ORR and CBR are shown with 95% confidence intervals (CI). ORR is defined as the proportion (%) of patients with complete or partial response. CBR is defined as the proportion (%) of patients with complete response, partial response or stable disease.

Correlation between ER histoscores and therapy response

Four of the included studies obtained ER histoscores and correlated this to anti-oestrogen therapy response (**Table 5.3**).[28,29,31,33] In total, ER histoscores of 234 ovarian cancer patients were reported, which in most cases was assessed on archived primary tumour tissue instead of a tissue sample of recurrent disease. Argenta *et al.* demonstrated significantly higher ER histoscores in subjects responding to fulvestrant therapy compared to non-responders (P=0.020).[28] Bonaventura *et al.* clustered histoscores into groups of ER histoscores <100 (n=13), 100 to 200 (n=11) and >200 (n=10) and correlated this to a CBR of 31%, 18% and 50%, respectively.[29] The highest response rates were seen in patients with the highest histoscores although a statistically significant difference could not be objectified. A paradoxical difference was found in the lowest histoscore group were the CBR was superior to the middle-range histoscore group, which might indicate that an increasing ER histoscore is not directly proportional to therapy response. Kok *et al.* were also unable to find a significant correlation, as they found a CBR of 25% in patients with ER histoscores of 0-100 (n=8), 50% in the group with ER histoscores of 101-200 (n=12) and 25% in the group with ER histoscores of 201-300 (n=8).[31] In the retrospective

cohort of Stanley *et al.* a positive trend in response was seen with increasing ER histoscores.[33] They reported a CBR of 34% in patients with ER histoscores of 0-150 (n=12), 40% in the group with ER histoscores of 151-200 (n=14), 48% in the group with ER histoscores of 201-250 (n=21) and 47% in the group with ER histoscores of 251-300 (n=16) (*P*=0.404). The authors included an additional analysis in which they also included patients with a delayed stable disease (patients whose CA125 tumour maker rose and afterwards stabilized) to the responding group. Then, the group with the highest ER histoscores (251-300, n=22) had a significant higher CBR of 65% compared to 37% in the lowest ER histoscores group (0-150, n=13) (*P*=0.040).

Discussion

Clinical benefit of anti-oestrogen therapy in HGSC

This systematic review aims to analyse studies reporting on the clinical benefit of anti-oestrogen therapy in ER positive metastatic or recurrent HGSC. Although there were no studies including solely ER positive HGSC patients, we were able to identify six individual clinical studies reporting on anti-oestrogen therapy in ovarian cancer populations partially consisting of ER positive metastatic or recurrent HGSC. The included studies reported a CBR ranging between 27-65% and an ORR ranging between 0-16% in a population consisting of 92% recurrent or metastatic HGSC, 80% of whom were confirmed ER positive.

Predicting anti-oestrogen therapy response by ER expression by immunohistochemistry

We analysed the literature to find a correlation between ER expression and clinical response in order to identify the specificity of this marker as a predictor of anti-oestrogen therapy response. Of the four studies reporting ER histoscores, two studies were not able to find a significant correlation.[29,31] The other two studies did report significant differences between groups but were unable to provide strong evidence for a correlation between increasing ER histoscores and improved clinical response.[27,28,33] Argenta *et al.* found a significant difference in mean ER histoscore between the responding and non-responding group.[27,28] Furthermore, Stanley *et al.* reported a statistical significant difference in CBR between two ER histoscore groups, but they did not report a significant correlation between increasing histoscores and better clinical response.[33] Additionally, this difference was only significant when patients with a delayed stable disease were included to the response data. These results do suggest that clinical response is more likely in HGSC with the highest levels of ER histoscores (>250), but lower histoscores seem to be inconclusive in predicting therapy response. To conclude, we had insufficient data to identify ER expression by

IHC as a specific predictor for anti-oestrogen therapy response, which could be caused by the relatively small number of patients with available ER histoscores.

Another possible explanation for the lack of support for a correlation between ER expression and therapy response might be the use of primary tumour samples for ER expression assessment, while anti-oestrogen therapy was administered to metastatic or recurrent disease after (multiple lines of) chemotherapy. Both the metastatic process and cytotoxic regimes may result in a change in tumour driving signalling pathways in recurrent disease compared to the primary tumour. ER expression discordance between untreated primary tumour and tumour tissue taken after chemotherapy treatment has been demonstrated in HGSC by Van Kruchten *et al.*[34] They found differing ER expression in tumour tissue taken at diagnosis compared to tumour obtained at debulking surgery after neo-adjuvant chemotherapy. In two patients IHC ER staining appeared to be negative at diagnosis, while the subsequent tumour sample taken after chemotherapy showed positive ER expression. Furthermore, ER expression instability has been reported in 35% of paired primary and recurrent HGSC patients, of which 14% showed loss and 21% gain of ER expression.[35] In addition, discrepancies in ER expression between primary tumour and corresponding distant metastases have been found. Several studies investigated primary breast cancers and corresponding distant metastases and reported changes in ER status in 15-40% of the patients.[36] Visualization of ER expression by a fluorescent oestradiol tracer on positron emission tomography (PET) imaging in metastatic breast cancer patients revealed heterogenous ER expression between primary tumour and metastases in up to 45% of the patients.[37] ER expression discordance may have been caused by tumour evolution, although heterogeneity in ER expression within a tumour as an alternative cause cannot be excluded. Despite this uncertainty as to the cause, results support the importance of ER status re-assessment in the actual tumour to be treated, preferably by taking multiple histological samples, to improve identification of patients sensitive to anti-oestrogen therapy.

Moreover, it could be questioned if receptor staining is the most adequate predictor of signalling pathway activity. In absence of ER the direct ER signalling pathway is inactive. However, positive nuclear ER staining does not automatically imply that the ER signalling pathway is transcriptionally active, since pathway activity depends on availability of the oestradiol ligand[38,39], or alternatively (but rare) an activating ER mutation.[40] The potential clinical relevance of the discrepancy between positive ER staining and actual ER pathway activity has recently been investigated using a mRNA based ER pathway activity assay which provides an ER pathway activity score based on computational interpretation of the expression values of the ER target genes.[41-44] In three ER positive breast cancer cohorts, low ER pathway activity scores were found

in breast cancer patients who failed to respond to aromatase inhibitors and showed progressive disease.[42] The ER pathway activity assay was also used to investigate the correlation between ER IHC staining and actual ER signalling pathway activity in a metastatic breast cancer cohort.[43] While low ER expression was always associated with very low ER pathway activity scores, indicating an inactive ER pathway, cases with high ER expression appeared to have a wide variation in ER pathway scores. These results were confirmed in another retrospective breast cancer cohort in which metastatic disease was treated with tamoxifen.[44] Although all primary tumour samples were ER positive, 41% of the patients had high ER pathway activity scores which was associated with longer time to progression of metastases (*P*=0.005). No correlation was observed between ER expression and ER pathway activity (*P*=0.400). These results provide evidence that positive ER expression not necessarily means an activated ER signalling pathway and that ER expression alone is not a sufficiently specific predictor of anti-oestrogen therapy response.

Mechanism of action and clinical response of anti-oestrogen agents

Our results do not demonstrate distinct superiority of one type of anti-oestrogen therapy over another. The CBR of tamoxifen and letrozole treatment were comparable.[32,33] However, letrozole in general was associated with longer median duration of response in two studies investigating both agents.[32,33] This might be explained by the partial agonistic effect of tamoxifen, which activates the ER pathway to some extent and carries a risk for tumour progression.[9,10]

Within the group of aromatase inhibitors, it is suggested that letrozole is more effective than anastrozole.[45,46] A pharmacodynamic study in breast cancer patients reported letrozole to be more effective in inhibiting aromatase activity and circulating oestradiol levels compared to anastrozole.[47] Whether this also translated in improved survival rates was recently studied in a phase II randomized trial designed to compare the efficacy of letrozole and anastrozole in breast cancer patients.[48] No statistically significant difference in disease-free and overall survival between letrozole and anastrozole therapy was observed. In our review, comparison of letrozole and anastrozole therapy in HGSC shows a CBR of 41-56% compared to 27-35%, respectively.[29,31-33] However, these results are based on indirect comparison of studies with heterogeneity in study design (retrospective cohorts versus phase II clinical trials) and patient population (platinum-resistant or -sensitive versus asymptomatic recurrent disease). Therefore, no conclusion can be drawn about the superiority of one aromatase inhibitor over another in HGSC.

One of the included studies investigated the use of fulvestrant; an ER downregulator which stimulates degradation of ER.[27,28] Fulvestrant resulted in a CBR of 50% according to RECIST criteria.[27,28] Fulvestrant can be effective in case of activating

mutations in the *ESR1* gene encoding for ER-*α*.[49,50] Mutations in *ESR1* confer ligand-independent transcriptional activity of the ER transcription factor. *ESR1* mutations can arise *de novo* prior to anti-oestrogen therapy.[40] However, acquired *ESR1* mutations frequently occur as a resistance mechanism to aromatase inhibitor therapy, as seen in metastatic breast cancer patients, were mutations were found in approximately 20% of the patients.[51-53] In contrast to breast cancer, little is known about *ESR1* mutations in ovarian cancer. A recent study identified *ESR1* alterations in 2.1% of the studied ovarian cancers (n=5,594).[40] As the majority of the investigated samples might be from primary disease, further research is needed to explore the prevalence of *ESR1* mutations in metastatic ovarian cancers.

Taken together, the different mechanisms of actions of anti-oestrogen agents emphasize the importance of knowledge on functionality of the ER signalling pathway in order to select the appropriate anti-oestrogen agents. In case of positive ER expression in the absence of oestradiol, tamoxifen could act as a partial agonist and might stimulate tumour progression. In case of *ESR1* mutations, treatment with aromatase inhibitors probably results in non-responding patients as ER activation is independent from the oestradiol ligand.

Anti-oestrogen therapy in combination with a mTOR inhibitor

In the study of Colon-Otero *et al.*, letrozole was combined with the mTOR inhibitor everolimus.[30] mTOR is a member of the PI3K/AKT/mTOR signalling pathway which plays a critical role in cell growth and proliferation and is frequently activated in ovarian cancer.[54-56] The rationale for combined therapy is that PI3K/AKT/mTOR pathway activation could diminish the effect of anti-oestrogen therapy, as the PI3K/AKT/mTOR pathway may activate oestradiol-independent transcription of ER.[57] This second tumour-driving pathway may cause primary resistance to anti-oestrogens. A phase III clinical trial in advanced breast cancer patients treated with exemestane in combination with everolimus after progression on aromatase inhibitors resulted in a significant prolonged PFS by more than twofold compared to exemestane alone.[58] The combination of letrozole and everolimus in the study of Colon-Otero *et al.* resulted in a relative promising CBR of 53%, but it must be noted that this study had a smaller sample size compared to the other included studies in this review.[30] A lower CBR has been reported in a phase I clinical trial with 50 ER positive advanced gynaecologic and breast malignancies treated with anastrozole and everolimus.[59] Two of the six serous ovarian carcinoma patients (33%) had SD for ≥6 months. However, this study was not included in this review as it could not be confirmed that the serous carcinomas were high-grade. The molecular alterations and crosstalk between the PI3K/AKT/mTOR and ER signalling pathways suggest a promising role for combinations of these inhibitors, which should be further explored in clinical trials focusing on selecting optimal patient populations.

Relation between ER expression and tumor differentiation

Although the majority of the patient populations of the included studies in this review represent HGSC, small numbers of endometrioid, low-grade serous (LGSC), clear cell, transitional cell carcinoma, carcinosarcoma and granulosa cell tumours were included. The Ovarian Tissue Analysis Consortium reported significant differences in ER expression between histologic subtypes.[18] Strong ER expression was found in 71% of LGSC and in 60% of HGSC and endometrioid carcinoma in contrast to a mere 14% in clear cell carcinoma. Apart from ER expression, these subtypes differ clearly in cell type of origin and clinical behaviour.[60,61] Noticeably, relatively high response rates were observed in better-differentiated carcinomas. A phase II study treating patients with ER positive recurrent or metastatic LGSC (89%) and serous borderline tumours (11%) with anastrozole found a CBR of 64% for ≥6 months.[62] In addition, a retrospective cohort study with a homogenous population of 64 patients diagnosed with recurrent LGSC treated with anti-oestrogen therapy reported a CBR of 71%.[63] These improved response rates may be related to the inverse relationship between ER expression and tumour grade, in which low-grade tumours have high ER expression, which may reflect higher ER pathway activity. In line with this, studies in breast cancer patients reported loss of ER expression by increasing histologic grade.[64-67] The inverse association between ER expression and tumour grade was also found in two independent endometrial carcinoma cohorts, in which ER pathway activity was also measured, using the aforementioned ER pathway activity assay.[68] In this study, higher ER expression as measured with ER IHC staining was associated with higher ER pathway activity scores. These results suggest that indeed the inverse correlation between ER staining and grade extends to an inverse relation between actual ER pathway activity and tumour grade. This provides a logical explanation for the better response to anti-oestrogen therapy in lower grade ovarian cancer types with higher ER expression: the ER pathway is probably more active providing an effective therapy target.

Efficacy of anti-oestrogen therapy in ER negative patients

Although we focused on studies with ER positive populations in this review, interesting results have been reported in studies that included ER negative patients as well. Del Carmen *et al.* compared time to disease progression after anastrozole therapy between patients with ER positive (n=32) and negative (n=13) asymptomatic recurrent or persistent ovarian carcinoma.[69] They found a median time to progression of 72 days in the ER positive group compared to 125 days in the ER negative group. Although these results suggest a superior response in ER negative patients, survival analysis showed no differences in time to progression between the ER positive and negative group. In line with this, a retrospective cohort of Stasenko *et al.* also was not able to find a significant improved PFS in ER positive patients

compared to ER negative patients. They reported a median time to progression of 4.0 months in the ER positive group (n=44) compared to 2.0 months in the negative group (n=19) (*P*=0.360).[23] In addition, a meta-analysis including a heterogenic ovarian cancer population with ER positive and negative patients reported subgroup analysis for hormone receptor status.[17] They found a CBR of 46% in ER positive and/or progesterone receptor positive patients, 44% in exclusively ER positive patients and 37% in patients with unknown receptor status, which was not statistically significant (*P*=0.540).[17] The fact that these studies did not report a significant superior therapy response in ER positive patients compared to ER negative patients, cautions against deciding on anti-oestrogen therapy based on ER expression assessment on a single tissue sample from the primary tumour. It is important to acknowledge that tumour heterogeneity could lead to sampling errors and that tumour tissue from primary diagnosis is not representative for the recurrent tumour.

Recommendations

Further research on the identification of responding patients for anti-oestrogen therapy should focus on the use of mRNA levels of target genes of the ER transcription factor. We mentioned an ER pathway activity assay based on computational interpretation of the expression levels of several ER target genes.[41] In our opinion, measuring a panel of ER specific target genes would be a more appropriate approach to predict ER signalling pathway activity.

Furthermore, we would like to address the lack of large prospective trials comparing anti-oestrogen therapy with standard next-line treatment. There has been only one phase III randomized controlled trial comparing tamoxifen to chemotherapy treatment in platinum-resistance ovarian carcinoma patients.[70] Unfortunately, determination of ER status was not incorporated in the study design, resulting in exclusion of this study from our analysis. Although, the authors report a better PFS after chemotherapy treatment compared to tamoxifen treatment, there was no difference in overall survival between the treatment groups.[70] In addition, tamoxifen treatment was associated with less toxicity and better quality of life. These results suggest a role for anti-oestrogens in ovarian carcinoma treatment but emphasize the need for further prospective trials comparing standard treatment to anti-oestrogen therapy in selected ovarian carcinoma patients with a functionally active ER pathway.

Conclusion

In our systematic review we included six clinical studies and found a clinical benefit to anti-oestrogen therapy in 27-65% of the population consisting for 92% of recurrent or metastatic HGSC, of which 80% was confirmed ER positive. Complete and partial response rates are low, as we found an ORR of 0-16%. No correlation was found

between ER expression and clinical response. Therefore, ER protein expression alone is not a specific predictor of response. Treatment with anti-oestrogen therapy is probably only effective when the ER pathway is functionally active, which depends on availability of the oestradiol ligand or alternatively (but rare) on an activating ER mutation. The currently used IHC ER staining is insufficiently specific for identification of transcriptionally active ER in HGSC, probably resulting in treatment of a population of non-responder patients. Even worse, in the absence of oestradiol, tamoxifen may exert its partial agonistic action and may potentially stimulate tumour progression. As ER expression remains an unreliable response predictor, it is of great importance to measure actual ER pathway activity in order to improve therapy response.

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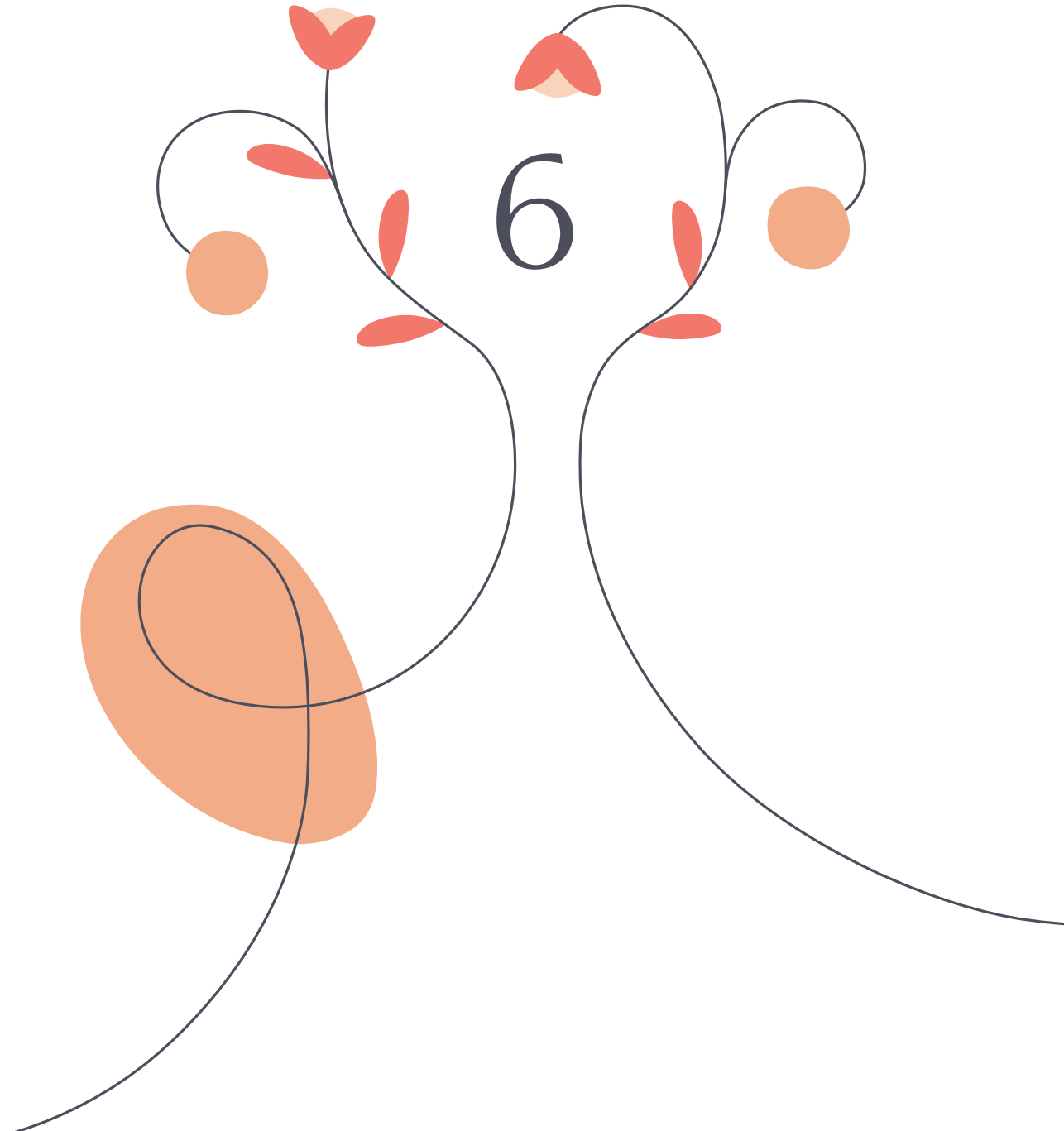
Supplementary information

Table S5.1 Search strategy

PubMed	Search ((((((((((((((((((((((((("ovarian cancer"[TIAB]) OR "ovarian cancers"[TIAB]) OR "ovary cancer"[TIAB]) OR "ovary cancers"[TIAB]) OR "ovarian carcinoma"[TIAB]) OR "ovarian carcinomas"[TIAB]) OR "ovary carcinoma"[TIAB]) OR "ovary carcinomas"[TIAB]) OR "ovarian tumor"[TIAB]) OR "ovarian tumors"[TIAB]) OR "ovary tumor"[TIAB]) OR "ovary tumors"[TIAB]) OR "ovarian tumour"[TIAB]) OR "ovarian tumours"[TIAB]) OR "ovary tumour"[TIAB]) OR "ovary tumours"[TIAB]) OR "ovarian neoplasm"[TIAB]) OR "ovarian neoplasms"[TIAB]) OR "ovary neoplasm"[TIAB]) OR "ovary neoplasms"[TIAB]) OR "cancer of ovary"[TIAB]) OR "cancer of ovarian"[TIAB]) OR "cancer of the ovary"[TIAB]) OR "cancer of the ovarian"[TIAB]) OR ((((("Ovarian Neoplasms"[Mesh]) OR ("Hereditary Breast and Ovarian Cancer Syndrome"[Mesh])) OR "Ovarian epithelial cancer"[Supplementary Concept])))))) AND ((((((((((((((((((((((((("Antihormonal therapy"[TIAB]) OR "Anti-hormonal therapy"[TIAB]) OR "Antihormonal treatment"[TIAB]) OR "Anti-hormonal treatment"[TIAB]) OR "Antiestrogen therapy"[TIAB]) OR "Anti-estrogen therapy"[TIAB]) OR "Antiestrogen treatment"[TIAB]) OR "Anti-estrogen treatment"[TIAB]) OR "Estrogen receptor therapy"[TIAB]) OR "Estrogen receptor treatment"[TIAB]) OR "Estrogen receptor downregulator"[TIAB]) OR "Estrogen receptor inhibitor"[TIAB]) OR "Estrogen receptor antagonist"[TIAB]) OR "Estrogen antagonist"[TIAB]) OR "Selective Estrogen Receptor Modulator"[TIAB]) OR "Estrogen receptor modulator"[TIAB]) OR "Aromatase inhibitor"[TIAB]) OR "Tamoxifen"[TIAB]) OR "Anastrozole"[TIAB]) OR "Letrozole"[TIAB]) OR "Fulvestrant"[TIAB]) OR "Antineoplastic Agents, Hormonal"[Mesh]) OR "Selective Estrogen Receptor Modulators"[Mesh]) OR "Aromatase Inhibitors"[Mesh]) OR "Estrogen Antagonists"[Mesh]) OR "Estrogen Receptor Modulators"[Mesh]) OR "Tamoxifen"[Mesh]) OR "Anastrozole"[Mesh]) OR "Letrozole"[Mesh]) OR "Fulvestrant"[Mesh])))) OR "exemestane"[TIAB]) OR "exemestane"[Supplementary Concept])
The Cochrane Library	("ovarian carcinoma"):ti,ab,kw OR ("ovarian cancer"):ti,ab,kw OR ("ovarian tumor"):ti,ab,kw OR ("ovarian tumour"):ti,ab,kw OR ("ovarian neoplasm"):ti,ab,kw OR MeSH descriptor: [Ovarian Neoplasms] explode all trees OR MeSH descriptor: [Carcinoma, Ovarian Epithelial] explode all trees AND ("Antihormonal therapy"):ti,ab,kw OR ("Anti-hormonal therapy"):ti,ab,kw OR ("Antihormonal treatment"):ti,ab,kw OR ("Anti-hormonal treatment"):ti,ab,kw OR ("Antiestrogen therapy"):ti,ab,kw OR ("Anti-estrogen therapy"):ti,ab,kw OR ("Antiestrogen treatment"):ti,ab,kw OR ("Anti-estrogen treatment"):ti,ab,kw OR ("Estrogen receptor downregulator"):ti,ab,kw OR ("Estrogen receptor antagonist"):ti,ab,kw OR ("Estrogen antagonist"):ti,ab,kw OR ("Selective Estrogen Receptor Modulator"):ti,ab,kw OR ("Estrogen receptor modulator"):ti,ab,kw OR ("Aromatase inhibitor"):ti,ab,kw OR ("Tamoxifen"):ti,ab,kw OR ("Anastrozole"):ti,ab,kw OR ("Letrozole"):ti,ab,kw OR ("Fulvestrant"):ti,ab,kw OR ("Exemestane"):ti,ab,kw OR MeSH descriptor: [Estrogen Antagonists] explode all trees OR MeSH descriptor: [Antineoplastic Agents, Hormonal] explode all trees OR MeSH descriptor: [Selective Estrogen Receptor Modulators] explode all trees OR MeSH descriptor: [Estrogen Receptor Modulators] explode all trees OR MeSH descriptor: [Aromatase Inhibitors] explode all trees OR MeSH descriptor: [Tamoxifen] explode all trees OR MeSH descriptor: [Anastrozole] explode all trees OR MeSH descriptor: [Letrozole] explode all trees OR MeSH descriptor: [Fulvestrant] explode all trees

Table S5.1 (continued)

ClinicalTrials.gov	(Ovarian Cancer [DISEASE] OR Ovarian Neoplasm [DISEASE] OR Ovarian Carcinoma [DISEASE] OR Ovarian Tumor [DISEASE] OR Ovarian Tumour [DISEASE]) AND (Estrogen Antagonists [TREATMENT] OR Estrogen receptor antagonist [TREATMENT] OR Selective Estrogen Receptor Modulator [TREATMENT] OR Aromatase inhibitor [TREATMENT] OR Tamoxifen [TREATMENT] OR Anastrozole [TREATMENT] OR Letrozole [TREATMENT] OR Fulvestrant [TREATMENT] OR Exemestane [TREATMENT])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CHAPTER 6

Functional oestrogen receptor signalling pathway activity in high-grade serous ovarian carcinoma as compared to oestrogen receptor protein expression by immunohistochemistry

Phyllis van der Ploeg, Laura A.M. van Lieshout, Anja van de Stolpe, Steven L. Bosch, Marjolein H.F.M. Lentjes-Beer, Ruud L.M. Bekkers, Jurgen M.J. Piek

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Abstract

Purpose

Anti-oestrogen therapy may be used as a palliative treatment option in high-grade serous ovarian carcinomas (HGSC). However, clinical implementation is limited as the use of oestrogen receptor (ER) protein expression by immunohistochemistry remains insufficient in predicting therapy response. To determine the accuracy of ER protein expression as a marker for ER signalling pathway activity, we aim to correlate ER protein expression to functional ER signalling pathway activity in HGSC.

Methods

Immunohistochemical ER protein expression was visually scored using total percentage of stained tumour cells and histoscores. Subsequently, mRNA was extracted, and RT-qPCR analysis was performed. Functional ER pathway activity was assessed by a computational Bayesian model inferring ER signalling pathway activity from mRNA levels of ER-specific target genes.

Results

Our analysis of 29 HGSC shows that neither total percentage of ER protein expression, nor ER histoscores are significantly correlated to ER signalling pathway activity (*P*=0.473 and *P*=0.606, respectively). Classification of HGSC into three groups based on ER histoscores 0-100 (n=6), 101-200 (n=15) and 201-300 (n=8) resulted in comparable mean ER signalling pathway activity among the groups (*P*=0.356). Several samples in the higher ER histoscore groups had low ER signalling pathway activity, indicating that nuclear ER protein expression is not sufficient to describe transcriptional ER activation.

Conclusion

Positive immunohistochemical ER staining is not always indicative of an active ER signalling pathway and is therefore a poor predictor of anti-oestrogen response. Further research is needed to prove the predictive value of ER signalling pathway activity regarding anti-oestrogen sensitivity in HGSC patients.

Introduction

Anti-oestrogen targeted therapy has been studied extensively in recurrent and metastatic ovarian carcinoma during the past decades.[1] Although anti-oestrogen therapy generally has an excellent tolerability, best therapeutic response is often disease stabilization, yielding a clinical benefit in 27-65% of high-grade serous ovarian carcinomas (HGSC) and 64-71% of low-grade serous ovarian carcinomas (LGSC).[2-4] The implementation of anti-oestrogen therapy in the clinical setting is limited as reliable predictive biomarkers for the identification of sensitive ovarian carcinomas are lacking.

The most studied marker for anti-oestrogen therapy response is nuclear oestrogen receptor-*α* (ER) protein expression by immunohistochemistry.[5] The presence of nuclear ER varies considerably between histological subtypes, as 71% of LGSC and 60% of HGSC are indicated ER positive compared to 14% of clear cell carcinomas.[6] Currently, positive ER staining is considered indicative of an active ER signalling pathway, and endocrine sensitivity is suggested to correspond with ER status. However, evidence for a predictive correlation remains weak as multiple studies failed to discover a significant relation between increasing ER protein expression and improved response to anti-oestrogen therapy.[4,7-11] Therefore, we question the accuracy of ER protein expression as a marker for ER signalling pathway activity in ovarian carcinomas.

In the absence of an activating mutation, the substrate oestradiol is required to activate the ER and initiate transcription of ER target genes.[12] While immunohistochemical staining of nuclear ER demonstrates the presence of the receptor, it might be insufficient to indicate functional ER activation. In order to assess transcriptional ER signalling pathway activity, an ER pathway activity assay based on measurements of mRNA levels of ER-specific target genes has been developed.[13,14] Previous use of the ER pathway activity assay in breast cancer patients produced functional ER signalling pathway activity scores with better predictive value regarding anti-oestrogen therapy response than ER protein expression.[13,15,16]

As the ER signalling pathway is considered a potential target for therapy in a subset of HGSC, the most common histological subtype of ovarian cancer, reliable markers to identify this anti-oestrogen sensitive subset are needed. In this study we investigate whether ER protein expression correlates to functional ER signalling pathway activity in advanced stage HGSC in the search for an alternative predictive biomarker for anti-oestrogen therapy response.

Materials and methods

Patient population and data collection

We retrospectively selected patients diagnosed with advanced stage HGSC in the Catharina Hospital Eindhoven, the Netherlands. To prevent any interference of cytotoxic or anti-oestrogen therapy with ER signalling pathway activity measurements, patients were excluded if: 1. formalin-fixed paraffin-embedded (FFPE) tumour samples were obtained after the start of chemotherapy, 2. patients had a medical history of any other malignancy prior to HGSC diagnosis, with the exception of basal cell skin carcinoma and 3. medical records stated recent use of oral contraceptives or hormone replacement therapy. We retrieved the following data from medical records: age and menopausal status at diagnosis, FIGO stage and tumour origin. In case medical records lacked information on menopausal status and age at diagnosis was insufficient to confirm postmenopausal status, endometrial sections were reviewed to determine menopausal status.[17] Tumour histology was confirmed by an expert gynaecological pathologist (MHFML-B) and areas containing at least 30% tumour cell nuclei were annotated for further analysis.

ER protein expression by immunohistochemistry

FFPE sections of four μm were cut with a microtome and dried at 80° Celsius in a convection oven for 20 minutes. Fully automated immunohistochemical staining for the detection of ER-*α* was performed on a BOND III stainer (Leica Biosystems, Germany). Slides were incubated with rabbit monoclonal antibody (SP1) (1:60, Thermo Scientific, USA) for 30 minutes at 20° Celsius after heat-induced epitope retrieval with ER-2 (ethylenediaminetetraacetic acid-based buffer, pH 9, Leica Biosystems, Germany) for 30 minutes at maximum 100° Celsius. Detection of the primary antibody was performed using Bond Polymer Refine Detection (Leica Biosystems, Germany), including two incubation steps of 8 minutes at 20° Celsius. Positive cells were visualized after 10 minutes incubation with 3,3-diaminobenzidine/H_2O_2 (Leica Biosystems, Germany) at room temperature.

ER protein expression scoring methods

Two expert gynaecological pathologists (SLB and MHFML-B) independently determined ER protein expression in the annotated tumour areas. The pathologists were blinded for each other's assessment and the results of the ER pathway activity assay. ER protein expression was visually scored according to two methods. First, ER protein expression was estimated by the total percentage of positive stained tumour cell nuclei. Second, staining intensity was categorized in percentages of tumour cells with negative, weak, moderate or strong staining. Finally, ER histoscores were calculated using a weighted method from the sum of (1 x % weak cells) + (2 x %

moderate cells) + (3 x % strong cells), deriving histoscores between 0 and 300.[18] Mean scores for both ER protein expression scoring methods were calculated and used for further analysis.

mRNA extraction and RT-qPCR analysis of ER pathway-specific target genes

Consecutive FFPE sections of five μm with identical annotated tumour areas were manually scraped for the collection of tumour tissue. Depending on total annotated tumour area, multiple sections were macro-dissected resulting in at least 20 mm^2 tumour surface. Total mRNA was extracted according to the manufacturer's protocol (VERSANT® Tissue Preparation Reagents kit, Siemens, Germany). mRNA concentrations were measured with the Qubit® RNA HS Assay Kit and Qubit® Fluorometer (Invitrogen, Thermo Fisher Scientific, USA). Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis was performed using the SuperScript™ III Platinum™ One-Step qRT-PCR kit (Invitrogen, Thermo Fisher Scientific, USA). OncoSignal PCR plates (Philips MPDX, The Netherlands) were filled with one nanogram mRNA per well and processed with a CFX96 Real-Time PCR Detection System (BioRad, USA). Sufficient mRNA input was confirmed by an internal quality control of reference genes.

ER pathway activity assay

Functional ER signalling pathway activity was assessed using the OncoSignal pathway assay (OncoSignal, Philips MPDx, the Netherlands), which is based on mRNA expression levels of ER-specific target genes and has been described in detail previously.[13,14] In summary, an extensive literature search yielded in 27 ER pathway-specific target genes for the Affymetrix ER pathway activity assay.[13] The assay has been developed and validated on Affymetrix expression microarray data.[19] For the use on FFPE material, the assay was adapted based on a selection of the most informative ER target genes as has been described before.[15,20] The ER pathway activity assay is based on a model consisting of 3 nodes corresponding to the (i) ER transcription complex, (ii) ER target genes and (iii) measured probeset expression levels. The activity of the ER transcription complex (i) is inferred from the expression of ER target genes (ii and iii) by a computational Bayesian network. The ER pathway activity assay generates functional scores defined on a normalized scale from 0 to 100, where 0 corresponds to the lowest odds for an active ER signalling pathway and 100 corresponds to the highest odds for an active ER signalling pathway. However, the biological range of ER pathway activity scores will differ between various tissue types. All samples were analysed blinded for ER protein expression levels.

Statistical analysis

Intra-class correlation coefficients were determined with a two-way mixed model to test the overall concordance between total percentage ER protein expression and ER histoscore assessments of both pathologists. Differences in mean ER signalling pathway activity between ER histoscore groups were tested with a Kruskal-Wallis test. Spearman's rank-order correlation coefficient was used to assess correlations. Statistical testing results were considered significant if the *P*-value was below 0.05. All statistical analyses were conducted using SPSS (IBM SPSS Statistics, version 26, RRID:SCR_019096) and RStudio (RStudio, Inc. version 1.1.463, RRID:SCR_000432) was used for data visualization.

Results and discussion

In this study we included 29 patients diagnosed with advanced stage HGSC with a median age at diagnosis of 63 years (range 31-85 years). Four patients were premenopausal at the time of diagnosis, one patient was considered perimenopausal and 23 patients were postmenopausal. For one patient we were unable to determine menopausal status as endometrial sections were unavailable for revision and age at diagnosis was insufficient to confirm postmenopausal status. Patients were diagnosed with FIGO stage IIIC (72%) or IV (28%) disease and tumour origin was defined as ovarian (83%), Fallopian tube (7%) and extra-ovarian (10%) (**Table 6.1**).

Table 6.1 Clinicopathological characteristics of patients diagnosed with high-grade serous ovarian carcinoma.

	n=29 (%)
Age at diagnosis	
Median (range)	63 (31 – 85)
Menopausal status	
Premenopausal	4 (14)
Perimenopausal	1 (3)
Postmenopausal	23 (79)
Unknown	1 (3)
Tumour origin	
Ovarian	24 (83)
Fallopian tube	2 (7)
Extra-ovarian	3 (10)
FIGO stage	
IIIC	21 (72)
IV	8 (28)

Both gynaecological pathologists assessed ER protein expression according to two methods in 29 HGSC samples (**Supplementary Table S6.1**). **Figure 6.1** shows representative images of immunohistochemical ER staining intensities with corresponding total percentage ER protein expression and ER histoscores.

Interobserver agreement for both assessments was excellent with an intra-class correlation coefficient based on absolute agreement of 0.968 (95% Confidence Interval (CI) 0.927-0.985) for total percentage ER protein expression and 0.919 (95% CI 0.526-0.974) for ER histoscores. The majority of the HGSC samples demonstrated positive ER expression with mean total percentage ER protein expression of 74% (standard deviation (SD) 36%) and mean ER histoscore of 161 (SD 89) (**Table 6.2**). We classified HGSC samples in three groups based on ER histoscores 0-100 (n=6), 101-200 (n=15) and 201-300 (n=8).

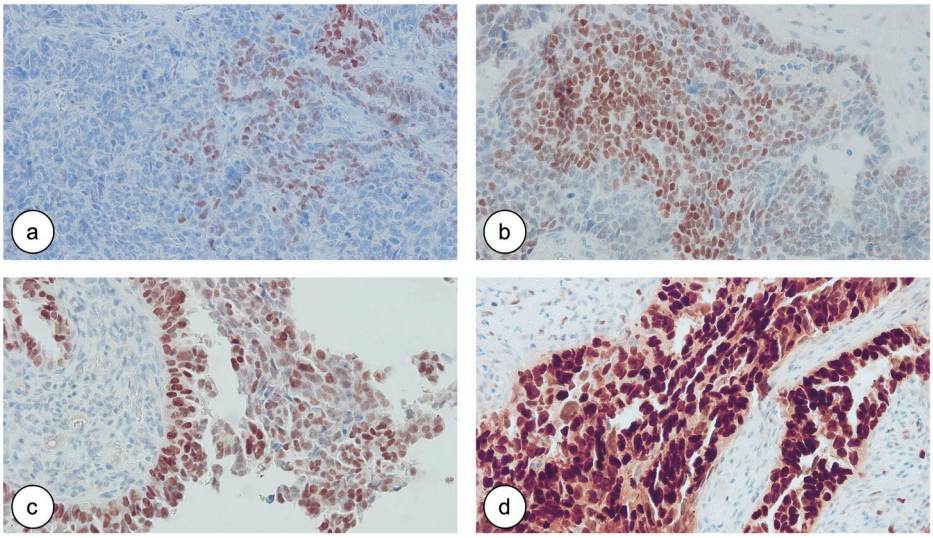

Figure 6.1 Oestrogen receptor (ER) nuclear protein expression by immunohistochemistry in high-grade serous ovarian carcinoma samples. Representative images (20x magnification) illustrate the following ER staining intensities: **A.** predominant negative (1% ER expression and ER histoscore of 1), **B.** predominant weak (85% ER expression and ER histoscore of 138), **C.** predominant moderate (95% ER expression and ER histoscore of 190) and **D.** predominant strong (100% ER expression and ER histoscore of 278).

Table 6.2 Descriptive statistics of total percentage oestrogen receptor (ER) protein expression, ER histoscores and ER signalling pathway activity in high-grade serous ovarian carcinoma samples.

Method	n	Mean	Standard deviation	Median	Range
Total percentage ER protein expression	29	74%	36%	90%	0 – 100%
ER histoscores	29	161	89	175	0 – 278
ER signalling pathway activity scores	29	12.09	6.40	11.30	0.22 – 27.94

Functional ER signalling pathway activity was determined in the same 29 HGSC samples. Mean ER signalling pathway activity was 12.09 (SD 6.40) (**Table 6.2**). When grouped according to ER histoscores (0-100, 101-200 and 201-300), we measured

mean ER signalling pathway activities of 9.97 (SD 7.36), 12.85 (SD 5.13) and 12.26 (SD 8.20), respectively (**Figure 6.2**). We observed a wide variation in ER signalling pathway activity within the three ER histoscore groups. Although the lower ER histoscore group demonstrated the lowest mean ER signalling pathway activity, this was not statistically different from the mean ER signalling pathway activity measured in the higher ER histoscore groups (**Figure 6.2**) (*P*=0.356). In these higher ER histoscore groups, we observed several HGSC samples with low ER signalling pathway activity indicating that presence of nuclear ER is required, but not sufficient to prove a transcriptionally active ER signalling pathway.

Next, we investigated the correlation between ER protein expression and functional ER signalling pathway activity. For both ER scoring methods, we observed no statistically significant correlation with ER signalling pathway activity (total percentage ER protein expression (R=0.139, *P*=0.473) and ER histoscores (R=0.100, *P*=0.606) (**Figure 6.3**)). These results address that ER protein expression is not equivalent to transcriptional ER signalling pathway activity in HGSC and might explain the insufficiency of ER protein expression as a predictive marker for anti-oestrogen therapy response.

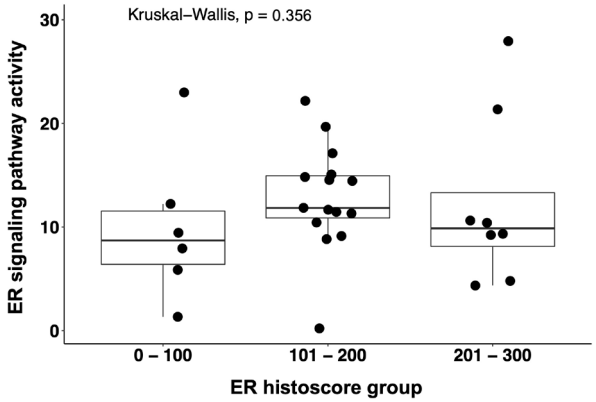

Figure 6.2 Oestrogen receptor (ER) signalling pathway activity per ER histoscore group determined in high-grade serous ovarian carcinoma samples (*P*=0.356).

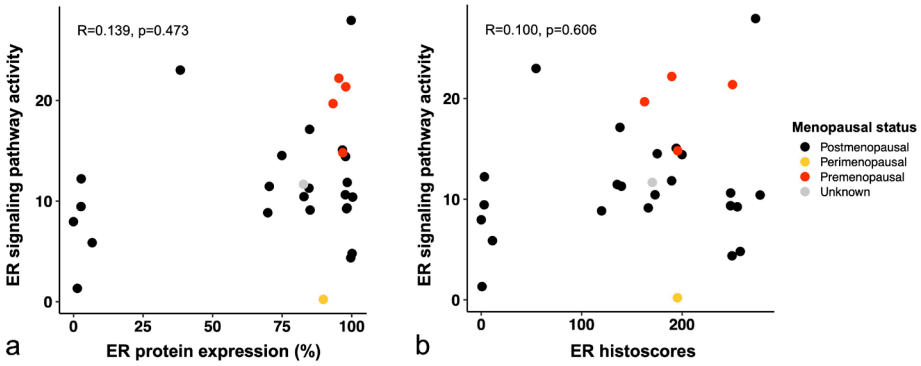

Figure 6.3 Relation between oestrogen receptor (ER) protein expression and ER signalling pathway activity in high-grade serous ovarian carcinoma samples. ER protein expression defined as: **A.** total percentage positive stained tumour cell nuclei (R=0.139, *P*=0.473) and **B.** ER histoscores (R=0.100, *P*=0.606).

In premenopausal HGSC (n=4) we measured significant higher ER signalling pathway activity compared to postmenopausal HGSC (n=23) (mean ER signalling pathway activity, respectively, 19.52 and 11.33, *P*=0.014). The association between ER signalling pathway activity and menopausal status indicates that the biological availability of oestradiol in premenopausal women might affect the tumour's sensitivity to hormones. Since in premenopausal women oestradiol is constantly produced by the ovaries, the tumour's signalling pathway activity could become dependent on the paracrine and endocrine availability of oestradiol. With increasing age, the depletion of ovarian follicles causes a steady decline in oestradiol production by the ovary.[21] After menopause, when the oestradiol production of the ovary has ceased, the formation of oestradiol depends on the availability of androgens and oestrogen precursors.[22] In postmenopausal women, tumour cells are thought to produce oestradiol by the aromatase and sulfatase pathways.[23] In the aromatase pathway, oestradiol is formed by intracellular conversion of androgens which is mediated by the enzyme aromatase. In the sulfatase pathway, oestradiol is mainly produced from the inactive precursor estrone sulphate. Therefore, in postmenopausal women, high ER signalling pathway activity in HGSC is likely to be caused by local oestradiol production of the tumour itself (autocrine production) or alternatively by the extragonadal production in liver, brain or adipose tissue.[22,23] Again, these results address that presence of the ER is a prerequisite, but transcriptional ER signalling pathway activity depends on availability of the ligand. We hypothesize that, despite positive ER protein expression, only high ER signalling pathway activity represent functionally active ER signalling in HGSC, and therefore only these HGSC patients are likely to benefit from anti-oestrogen targeted therapy.

Our findings are supported by a study investigating ER signalling pathway activity in 130 ER positive breast cancer patients using the ER pathway activity assay.[16] In this cohort, ER protein expression was also not significantly correlated with ER signalling pathway activity (*P*=0.400). In addition, the authors reported no correlation between *ESR1* levels, the gene coding for ER-*α*, and ER signalling pathway activity (*P*=0.510). Others studied ER signalling pathway activity in a cohort mainly consisting of endometrial cancer patients (n=83) using the ER pathway activity assay.[20] Here, significantly lower ER signalling pathway activity was observed in the group with 0-10% ER protein expression compared to the group with 51-100% ER protein expression (*P*<0.001). In line with our observations, a wide variation in ER signalling pathway activity was detected in the higher ER protein expression groups (11-50% and 51-100%), indicating that positive ER protein expression in endometrial cancer also did not automatically imply transcriptional activation of the ER signalling pathway.

Besides the limited number of included patients, our study is lacking anti-oestrogen response data as the included HGSC patients did not receive anti-oestrogen therapy. Therefore, we were unable to study the predictive value of the ER pathway activity assay regarding anti-oestrogen response. However, in multiple cohort studies the assay was able to select subsets of ER positive breast cancer patients with an active ER signalling pathway and significant better response to treatment with anti-oestrogen targeted therapy.[13,15,16] Our research group is currently investigating whether ER signalling pathway activity is associated with anti-oestrogen therapy response in LGSC. The search for a predictive marker remains warranted as clinical studies report subsets of ovarian carcinoma patients who benefit from anti-oestrogens with minimal side effects. However, in order to implement anti-oestrogen targeted therapy as an effective treatment strategy a more reliable marker is required as ER protein expression alone remains insufficient in predicting anti-oestrogen sensitivity.

Taken together, our study demonstrates that ER protein expression by immunohistochemistry in HGSC not always translates into active ER signalling pathway activity based on mRNA levels of ER-specific target genes. Further investigation is necessary to confirm ER signalling pathway activity as a predictive marker for response to anti-oestrogen therapy.

Acknowledgements

The authors gratefully acknowledge Dr. Judith Jeuken and Ms. Wendy Pellis-van Berkel for their contribution to the sample analysis and the laboratory staff of Stichting PAMM for their technical assistance. In addition, the authors would like to thank Ms. Eveline den Biezen-Timmermans, Mr. Diederick Keizer, Ms. Sieglinde

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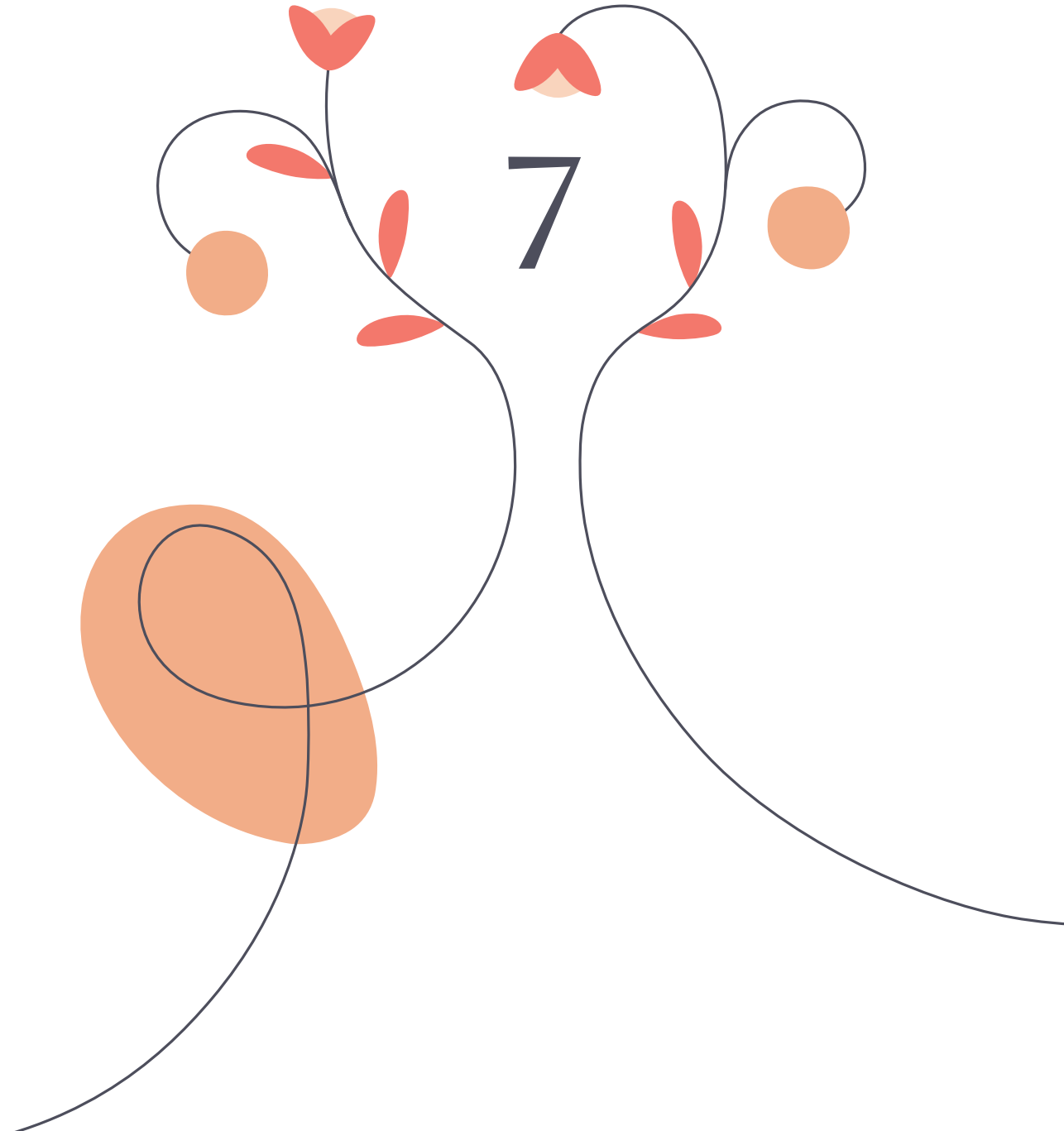
Table S6.1 Specification of high-grade serous ovarian carcinoma samples.

Study number	Age at diagnosis	Menopausal status	Tumour cell nuclei in annotated area (%)	ER positive stained tumour cell nuclei (%)*	ER histoscore*	ER signalling pathway activity
01219545	79	Postmenopausal	30	85	166	9.12
04314003	70	Postmenopausal	80	3	3	9.45
07100488	66	Postmenopausal	50	7	11	5.88
07566314	57	Postmenopausal	70	3	3	12.23
09150039	60	Postmenopausal	60	0	0	7.95
09269592	73	Postmenopausal	80	70	135	11.46
09458744	65	Postmenopausal	70	75	175	14.53
09599315	62	Postmenopausal	40	98	190	11.84
14480786	68	Postmenopausal	95	98	248	10.62
15070758	59	Postmenopausal	95	98	200	14.44
23151466	67	Postmenopausal	60	85	138	17.12
23698483	63	Postmenopausal	30	100	278	10.40
23811739	80	Postmenopausal	90	100	273	27.94
27162504	42	Premenopausal	50	97	196	14.82
32208416	55	Perimenopausal	90	90	195	0.22
42999755	60	Postmenopausal	40	85	140	11.30
46813066	72	Postmenopausal	70	100	250	4.36
47365415	63	Postmenopausal	90	98	255	9.23
55370021	49	Unknown	80	83	170	11.67
56304671	31	Premenopausal	70	93	163	19.69
64887591	65	Postmenopausal	80	70	120	8.83
66438258	64	Postmenopausal	60	83	173	10.44
69046268	69	Postmenopausal	90	100	258	4.80
69517641	45	Premenopausal	80	98	250	21.37
78415558	43	Premenopausal	40	95	190	22.19
83849892	71	Postmenopausal	95	97	194	15.07
92242302	58	Postmenopausal	80	98	248	9.34
93070915	85	Postmenopausal	95	1	1	1.33
99070404	63	Postmenopausal	95	38	55	23.00

Abbreviations: ER, Oestrogen receptor; HGSC, high-grade serous ovarian carcinoma.

* Scores were assessed by two independent expert gynaecological pathologists (SLB and MHFML-B) in annotated HGSC areas. Mean ER protein expression and ER histoscores are described.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CHAPTER 7

The effectiveness of monotherapy with PI3K/AKT/mTOR pathway inhibitors in ovarian cancer: a meta-analysis

Phyllis van der Ploeg*, Aniek Uittenboogaard*, Anna M.J. Thijs, Hans M. Westgeest, Ingrid A. Boere, Sandrina Lambrechts, Anja van de Stolpe, Ruud L.M. Bekkers, Jurgen M.J. Piek
* Dual first author

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Abstract

Objective

To determine the clinical benefit of monotherapy with PI3K/AKT/mTOR inhibitors in patients diagnosed with advanced or recurrent ovarian cancer and to investigate the predictive value of current PI3K/AKT/mTOR biomarkers on therapy response.

Methods

A systematic search was conducted in PubMed, Embase and the Cochrane Library for articles reporting on treatment with PI3K/AKT/mTOR inhibitors in ovarian cancer. The primary endpoint was defined as the clinical benefit rate (CBR), including the proportion of patients with complete (CR) and partial response (PR) and stable disease (SD). Secondary endpoints included the overall response rate (ORR, including CR and PR) and drug-related grade 3 and 4 adverse events.

Results

We included 233 patients from 19 studies and observed a pooled CBR of 32% (95% CI 20-44%) and ORR of 3% (95% CI 0-6%) in advanced or recurrent ovarian cancer patients treated with PI3K/AKT/mTOR inhibitors. Subgroup analysis tended to favour the studies who selected patients based on current PI3K/AKT/mTOR biomarker criteria (e.g. genomic alterations or loss of PTEN protein expression), but the difference in CBR was not statistically significant from studies with unselected populations (CBR of 42% (95% CI 23-62%) and 27% (95% CI 14-42%), respectively, *P*=0.217). To better reflect true patient benefit, we excluded SD <6 months as a beneficial outcome which resulted in a pooled CBR of 7% (95% CI 2-13%). The overall proportion of patients with drug-related grade 3 and 4 adverse events was 36%.

Conclusions

The efficacy of monotherapy with PI3K/AKT/mTOR inhibitors in advanced recurrent ovarian cancer patients is limited to a small subgroup and selection of patients with the use of current biomarkers did not improved the CBR significantly. Given the toxicity profile, we suggest that current treatment with PI3K/AKT/mTOR inhibitors should not be initiated unless in clinical trials. Furthermore, improved biomarkers to measure functional PI3K/AKT/mTOR pathway activity are needed to optimize patient selection.

Introduction

Ovarian cancer is the most lethal gynaecological malignancy and reflects a heterogenous disease.[1] Histologically ovarian cancer can be categorized in five subtypes, namely high-grade serous, low-grade serous, endometrioid, mucinous and clear cell carcinoma.[2] First-line treatment consists predominantly of platinum-based chemotherapy and debulking surgery.[1] Despite complete remission after initial treatment, 70-80% of the patients will develop relapse of disease, which eventually becomes platinum-resistant.[3] As a result, numerous trials have been conducted to identify alternative treatment strategies. One such strategy involves inhibition of the phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) growth factor signalling pathway, as this pathway is frequently activated in ovarian cancer by gain-of-function mutations and amplifications or by loss-of-function of tumour suppressor genes.[4]

The PI3K/AKT/mTOR pathway is a complex signalling network which plays an essential role in survival mechanisms of the cell.[5] The pathway has many loops and branches, starting with the activation of PI3K enzymes via extracellular growth factors (**Figure 7.1**). PI3Ks are lipid kinases including three subclasses, of which particularly class IA PI3Ks are of therapeutic importance due to frequent alterations.[6] Class IA PI3Ks consist of a regulatory (p85) and catalytic (p110) subunit.[7] The regulatory (p85) subunit of PI3K can bind and stabilize the catalytic (p110) subunit, and therefore, is functioning as an endogenous inhibitor of the pathway.[4] Isoforms of both subunits have been reported, of which the p110 isoforms result in the expression of three different genes, namely *PIK3CA*, *PIK3CB*, and *PIK3CD*.[4,5] A gain-of-function amplification or mutation in the *PIK3CA* gene, resulting in the p110α-isoform, is found in 2-20% of the high-grade serous carcinoma and 20-46% of the endometrioid, mucinous and clear cell carcinoma.[4,5]

Following activation, PI3K phosphorylates phosphatidylinositol (4, 5)-bisphosphate (PIP2) to generate the second messenger phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) (**Figure 7.1**).[7] Phosphatases and tensin homolog (PTEN) is able to dephosphorylate PIP3 and is therefore another endogenous inhibitor of the PI3K/AKT/mTOR pathway.[7] Loss-of-function mutations, deletions or silencing of the *PTEN* gene are found in 7% of the high-grade serous carcinoma and 21-45% in endometrioid and clear cell carcinoma.[4,7-9] Subsequently, PIP3 may activate AKT by specific phosphorylation to initiate several downstream effects, e.g. inhibition of apoptosis, protein synthesis, and cell growth and survival.[10] Eventually, activated AKT may directly and indirectly activate mTOR, which controls, among others, cell proliferation, metabolism, autophagy and angiogenesis.[5]

Many preclinical and clinical studies have been conducted to inhibit PI3K/AKT/mTOR pathway activity with the use of targeted agents, which are classified in four categories: PI3K inhibitors, AKT inhibitors, mTOR inhibitors and dual PI3K/mTOR inhibitors (**Figure 7.1**). Others have summarized response rates to PI3K/AKT/mTOR pathway inhibitors, either as monotherapy or in combination with other therapeutics, in advanced solid tumours.[8,11-13] However, no meta-analysis has been conducted focusing on treatment efficacy in solely ovarian cancer patients. In addition, study results have been inconclusive on the predictive value of PI3K/AKT/mTOR alterations, either defined by mutations, amplifications or deletions by sequencing analysis or by loss of PTEN protein expression by immunohistochemistry.[8,11,12] Therefore, in this meta-analysis, we will focus on the effects of monotherapy with PI3K/AKT/mTOR inhibitors and aim to determine the clinical benefit rate, defined as complete and partial response and stable disease, in patients diagnosed with advanced or recurrent ovarian cancer. Furthermore, we aim to investigate the predictive value of current PI3K/AKT/mTOR biomarkers on therapy response by subgroup analysis of clinical benefit in studies selecting patients based on PI3K/AKT/mTOR biomarker criteria and studies with unselected populations.

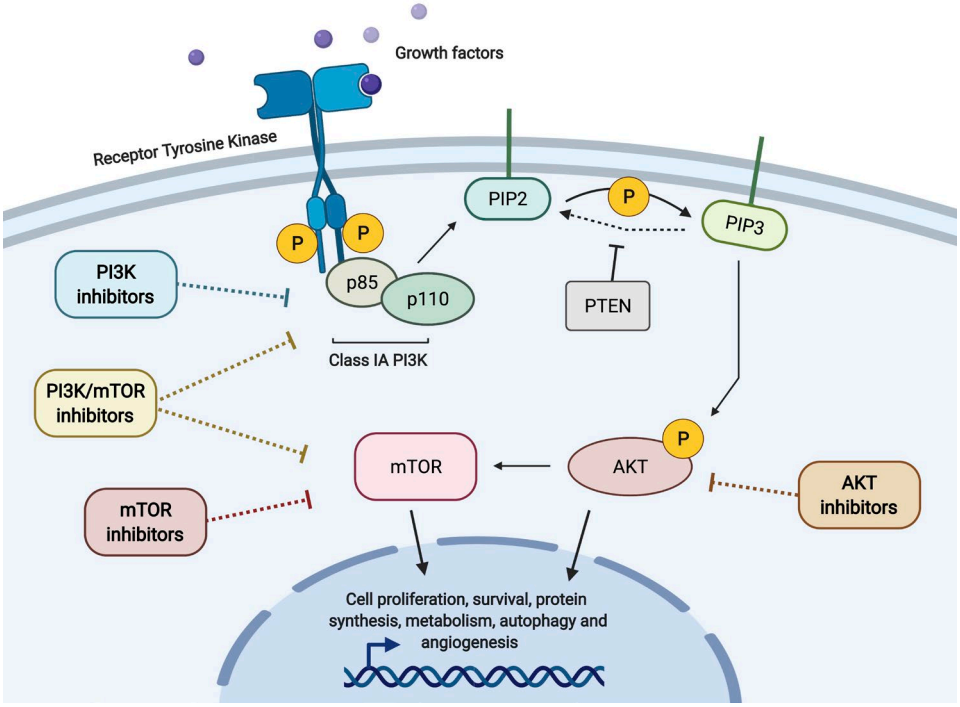

Figure 7.1 Schematic overview of the major components of the PI3K/AKT/mTOR signalling pathway with different strategies for inhibition (this figure is created with BioRender.com).

Methods

Protocol and registration

This meta-analysis was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-analysis (PRISMA) statement.[14] A study protocol was published at Prospero International prospective register of systematic reviews (CRD42020164469).[15]

Eligibility criteria

Studies were eligible for inclusion when reporting on treatment with monotherapy of PI3K/AKT/mTOR inhibitors in women with advanced or recurrent ovarian cancer. We focused on the use of drugs directly targeting the PI3K/AKT/mTOR components and excluded the use of human epidermal growth receptor 2 (HER2) inhibitors. Phase I, II and III clinical trials, randomized controlled trials, prospective and retrospective cohort studies and case series were eligible for inclusion. To be included in the meta-analysis, study populations should at least consist of five ovarian cancer patients. Systematic reviews and meta-analysis were carefully screened for additional inclusions of individual studies. A language restriction for English was applied.

Literature search

Studies were retrieved by a literature search in the electronic databases; PubMed, Embase via Ovid and the Cochrane Library. The search consisted of free terms (several synonyms) and Mesh terms for "ovarian cancer" and "PI3K/AKT/mTOR inhibitors" and "PI3K/AKT/mTOR proteins". An example of the full search strategy is provided in **Supplementary Material S7.1**. We restricted the literature search to studies published during the last 10 years (from 2010 to present). The databases were searched on January 10, 2020 and the last search was conducted on January 6, 2021.

Study selection

Titles and abstracts were independently screened by two reviewers (AU and PvdP) based on pre-defined exclusion criteria. Next, both reviewers (AU and PvdP) conducted eligibility assessment of the full-text articles. In case of disagreement, a third author was consulted (JMJP). Reasons for exclusion were documented. Authors were contacted to obtain additional data if clinical response data was not presented separately for ovarian cancer patients (for example if multiple types of cancers were included in the study).

Data extraction

For the included studies, study characteristics and outcome data were extracted according to a pre-defined data extraction template (**Supplementary Material S7.2**). The primary outcome of this meta-analysis was clinical benefit rate (CBR) defined as the proportion of patients with best overall response of complete response (CR), partial response (PR) or stable disease (SD), as defined by RECIST 1.1 or GCIG criteria.[16] We selected this primary outcome measure over progression-free survival to allow for the inclusion of phase I clinical trials, in which antitumour activity is often defined by response rates rather than progression-free survival. Additional outcomes were overall response rate (ORR) defined as the proportion of patients with CR or PR defined by RECIST 1.1 or GCIG criteria and the proportion of patients experiencing drug-related grade 4 and 5 adverse event according to the Common Terminology Criteria for Adverse Events (CTCAE).[17]

Bias screening

The risk of bias was assessed independently by two reviewers (AU and PvdP) via the ROBINS-I tool (Risk Of Bias in Non-randomised Studies of Interventions).[18] Any disagreements were resolved by consulting a third reviewer (JMJP). Risk of bias was judged as low, high or unclear risk for seven predefined domains of bias: confounding, selection of participants, description of intervention, deviation from intervention, missing data, measurements of outcome and selective reporting. The ROBINS-I tool requires the establishment of a 'hypothetical target trial', which we defined as a phase II or III clinical trial investigating PI3K/AKT/mTOR monotherapy in ovarian cancer patients either with or without evidence of PI3K/AKT/mTOR pathway dysregulation. The trial should meet the following requirements: 1. baseline information should include number of prior lines of treatment and histological subtype, 2. a detailed description of the selection process and the intervention, 3. deviations from intended intervention and missing data should concern ≤25% of the population and 4. therapy response should be measured by RECIST 1.1 or GCIG criteria at standardized time points. Finally, the overall risk of bias of the seven domains was considered low if none of the domains was judged as high risk of bias, moderate if one or two domains were judged as high risk of bias and high if three or more domains were judged as high risk of bias.

Statistical analysis

Meta-analysis and subgroup analysis were performed using a random-effect model with the DerSimonian-Laird estimator for between study variance τ^2 to estimate the pooled proportion of patients with clinical benefit with 95% confidence intervals (CI). To assess for heterogeneity across the included studies I^2 values of 25%, 50% and 75% were considered to indicate, respectively, low, moderate and high

heterogeneity. The Freeman-Tukey Double arcsine transformation was applied to stabilize the variance of the proportions of individual studies. Confidence intervals of individual studies were estimated using the Clopper-Pearson method and Jackson method was used for the confidence intervals of τ^2. Funnel plots with Egger's tests for asymmetry were created to assess for publication bias. Additionally, a leave-one-out sensitivity analysis was conducted to investigate the influence of outliers. Statistical analysis was conducted using the 'metaprop' command of the 'meta' package in R, version 3.5.2. (Rstudio Inc.).[19]

Results

Study selection

We identified 2538 records through database and reference searching, of which 164 duplicates were removed (**Figure 7.2**). Subsequent title and abstract screening resulted in the exclusion of 2117 records. After full-text screening of the remaining 257 records, we excluded an additional 238 records based on: the use of combination therapy with other targeted agents or chemotherapy (131 records); the inclusion of less than five ovarian cancer patients (58 records); the publication of a conference abstract without availability of a full-text article (17 records); the publication of a conference abstract of which the full-text article already was included (14 records); an ongoing clinical trial (four records); an unknown number of ovarian cancer patients treated with a PI3K/AKT/mTOR inhibitor (four records); the inclusion of an unknown number of ovarian cancer patients (two records); absence of the primary outcome measure (two records) and population without ovarian cancer patients (two records). In total, 23 studies complied with the inclusion criteria. However, several of the included studies did not specify therapy response for ovarian cancer patients, for which we contacted the authors to retrieve additional data. Eventually, we were unable to obtain sufficient response data of the ovarian cancer patients of four studies,[20-23] resulting in the inclusion of 19 studies to conduct the meta-analysis.[24-42]

Study characteristics

The included studies involved a total of 233 ovarian cancer patients. The sample size of individual studies ranged from five to 54 ovarian cancer patients treated with PI3K/AKT/mTOR inhibitors (**Table 7.1**).[24-42] Nine phase I and six phase II studies were included, as well as one phase I/IIA study. In addition, one case report on six clear cell carcinoma patients[37] and two studies aiming to implement molecular profiling were included.[30,38] Four studies assessed the effect of PI3K inhibitors in a total of 40 patients,[28,29,33,34] whereas five studies assessed the response to AKT inhibitors in a total of 61 patients.[26,27,31,41,42] The response to mTOR inhibitors was investigated in

five studies including 100 patients,[24,25,30,37,39] four studies assessed the effect of PI3K/mTOR inhibitors in a total of 24 patients,[32,35,36,40] and one study including 8 patients assessed both AKT and mTOR inhibitors based on their molecular profile.[38] Almost all studies assessed a different type of PI3K/AKT/mTOR inhibitor, with the exception of the mTOR inhibitors temsirolimus (used in three studies) and everolimus (used in two studies) and the AKT inhibitors MK-2206 and uprosertib (both used in two studies).

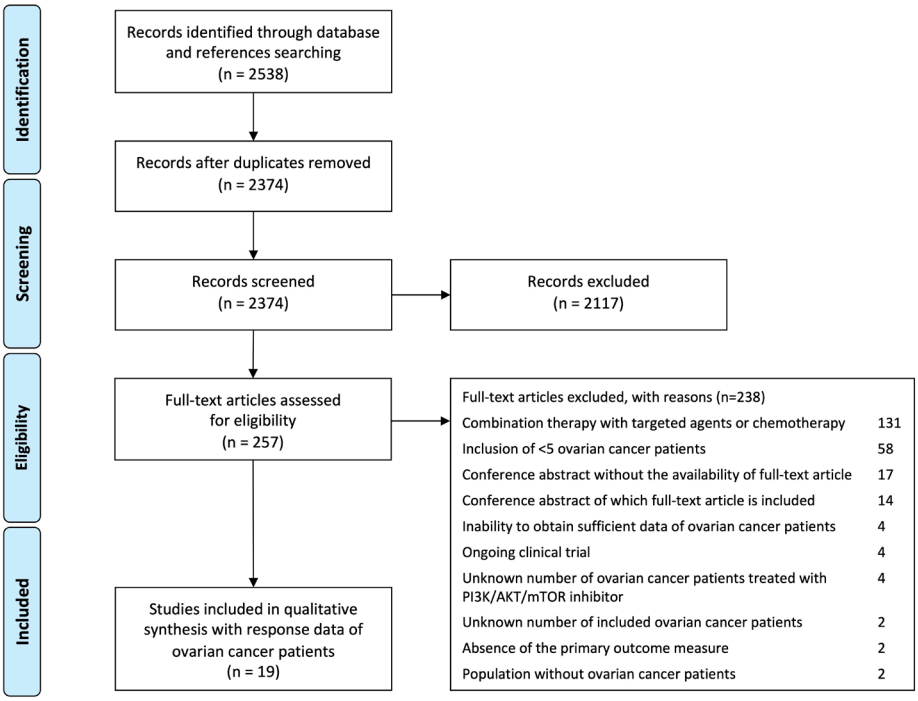

Figure 7.2 PRISMA flowchart of the identified studies and the selection procedure of the included studies.

Risk of bias assessment

Studies were subjected to a comprehensive quality assessment for the risk of bias on seven predefined domains. In case studies included populations consisting of different advanced solid malignancies, bias domains were applied to the study as a whole. A detailed description of the reviewers' judgements of each bias domain can be found in **Supplementary Material S7.3** and data is summarized in **Supplementary Table S7.1**. The combined risk of bias assessment of the seven predefined domains is reported in **Table 7.1**.

Table 7.1 Study characteristics, outcome data and overall risk of bias assessment of studies reporting on monotherapy with PI3K/AKT/mTOR inhibitors in ovarian cancer patients.

Study	Phase	OC patients treated (n)	Total patients treated (n)	Tumour histology	Type of treatment	CR (%)	PR (%)	SD <6 months (%)	SD ≥6 months (%)	Not evaluable OC patients (%)	ORR (%)	CBR (%)	Drug-related grade 3 and 4 adverse events according to CTCAE (total population)	Overall risk of bias assessment
PI3K inhibitors														
Piha-Paul *et al.* 2019 [34]	II	12	146	*N.A.*	Buparlisib	0	0	8	17	17	0	25	82% (all cause)	Moderate
Juric *et al.* 2018 [29]	IA	14	134	*N.A.*	Alpelisib	0	7	64	7	14	7	79	44%	High
Juric *et al.* 2017 [28]	I	9	71	*N.A.*	Serabelisib	0	0	22	22	*N.A.*	0	44	27%	Moderate
Mateo *et al.* 2017* [33]	I/IIA	5	65	*N.A.*	GSK2636771	0	0	40	0	0	0	40	23%	Moderate
AKT inhibitors														
Lee *et al.* 2020 [31]	II	6	6	6 HGSC (all platinum-resistant)	MK-2206	0	0	50	17	20	0	67	80%	Low
Aghajanian *et al.* 2018 [42]	I	9	77	*N.A.*	Uprosertib	0	11	0	0	11	11	11	n=22** (all cause)	Low
Hasegawa *et al.* 2017* [27]	II	21	71	7 serous, 11 clear cell, 1 endometrioid, 1 mucinous, 1 unknown	Perifosine	0	0	19	0	14	0	19	n=47** (all cause)	Low
Gungor *et al.* 2015 [26]	I	11	12	8 serous, 1 endometrioid, 1 clear cell, 1 unknown (all platinum-resistant)	Uprosertib	0	9	0	18	0	9	27	17%	Moderate
Yap *et al.* 2015* [41]	I	14	71	*N.A.*	MK-2206	0	0	0	0	*N.A.*	0	0	n=12**	Low
mTOR inhibitors														
Voss *et al.* 2020* [39]	I	9	198	*N.A.*	Sapanisertib	0	0	22	0	*N.A.*	0	22	44%	Moderate
Emons *et al.* 2016 [25]	II	21	44	17 serous, 4 unknown (all platinum-resistant)	Temsirolimus	0	5	33***		14	5	38	98% (all cause)	Low
Le Tourneau *et al.* 2015* [30]	II	10	46	10 adenocarcinoma unspecified	Everolimus	0	0	30	0	40	0	30	*N.A.*	Moderate

Table 7.1 (continued)

Study	Phase	OC patients treated (n)	Total patients treated (n)	Tumour histology	Type of treatment	CR (%)	PR (%)	SD <6 months (%)	SD ≥6 months (%)	Not evaluable OC patients (%)	ORR (%)	CBR (%)	Drug-related grade 3 and 4 adverse events according to CTCAE (total population)	Overall risk of bias assessment
mTOR inhibitors														
Behbakht *et al.* 2011 [24]	II	54	54	39 serous, 8 adenocarcinoma unspecified, 4 endometrioid, 3 clear cell	Temsirolimus	0	9	41***		11	9	50	n=44** (all cause)	Moderate
Takano *et al.* 2011 [37]	Case series	6	6	6 clear cell	Temsirolimus	0	17	17	0	0	17	33	0%	Moderate
PI3K/mTOR inhibitors														
Rodon *et al.* 2018* [35]	I/IB	8	183	*N.A.*	Dactolisib	0	0	0	0	0	0	0	44% (all cause)	Moderate
Wicki *et al.* 2018 [40]	I	6	28	*N.A.*	Bimiralisib	0	0	67	0	17	0	67	57%	Moderate
Shapiro *et al.* 2015* [36]	I	5	77	1 granulosa cell tumour, 1 endometrioid, 3 unknown	Gedatolisib	0	20	0	0	60	20	20	29%	Moderate
Mahadevan *et al.* 2012 [32]	I	5	44	5 adenocarcinoma unspecified	SF1126	0	0	40	20	*N.A.*	0	60	11%	Moderate
Combination study with AKT and mTOR inhibitors														
Varnier *et al.* 2017 [38]	Prospective cohort study	8	39	6 serous, 1 endometrioid, 1 germ cell tumour	LY2780301 or everolimus	0	25	0	0	0	25	25	*N.A.*	Moderate

Aberrations: OC, ovarian cancer; CR, complete response; PR, partial response; SD, stable disease; ORR, overall response rate; CBR, clinical benefit rate; CTCAE, Common Terminology Criteria for Adverse Events, PI3K, phosphatidylinositol-3-kinase; N.A., data not available; HGSC, high grade serous carcinoma; mTOR, mammalian target of rapamycin; MTD, maximum-tolerated dose.

* We received additional information from these authors on therapy response rates in ovarian cancer patients; ** Number of adverse events counted. Possibility that multiple adverse events occurred in the same patients and patients are therefore counted more than once; *** For these studies it was not possible to distinguish between SD < or ≥6 months. These studies results were excluded from the revised analysis of the pooled CBR (**Supplementary Figure 7.3**).

Effectiveness of PI3K/AKT/mTOR inhibitors in ovarian cancer

Our meta-analysis revealed that treatment with PI3K/AKT/mTOR inhibitors was associated with a pooled CBR of 32% (95% CI 20-44%) in ovarian cancer patients, with moderate to high between-study heterogeneity (I^2=64%) (**Figure 7.3**). Consistently low response rates were observed across all studies. Although several patients achieved a PR, none of the included studies reported on complete tumour regression in ovarian cancer patients. With regard to the ORR to PI3K/AKT/mTOR inhibitors, our meta-analysis revealed a pooled ORR of 3% (95% CI 0-6%), with low between-study heterogeneity (I^2=0%) (**Figure 7.4**).

Subgroup analysis by type of inhibitor showed that treatment with PI3K inhibitors was associated with the highest pooled CBR of 48% (95% CI 22-75%, I^2=61%), but this was not statistically significant from the results of other subgroups (*P*=0.331) (**Figure 7.3**). For mTOR inhibitors we observed a pooled CBR of 42% (95% CI 32-52%, I^2=0%), and treatment with dual PI3K/mTOR inhibitors resulted in a pooled CBR of 30% (95% CI 1-72%, I^2=72%). Treatment with AKT inhibitors resulted in the lowest pooled CBR of 18% (95% CI 3-41%, I^2=69%).

Analysis of ORR per type of inhibitor favoured mTOR inhibitors with a pooled ORR of 5% (95% CI 1-11%, I^2=0%), but this not statistically significant from the ORR of other subgroups (*P*=0.381) (**Figure 7.4**). For the other types of inhibitors consistent pooled ORRs were observed, namely 1% for PI3K inhibitors (95% CI 0-9%, I^2=0%), 1% for AKT inhibitors (95% CI 0-7%, I^2=0%) and 1% for PI3K/mTOR inhibitors (95% CI 0-14%, I^2=0%). The study of Varnier *et al.* assessed both AKT and mTOR inhibitors and observed two PRs (one for both type of inhibitor) resulting in an ORR of 25% (95% CI 3-65%).

Additionally, we assessed whether small studies with small response rates were missing in our analysis. Evaluation of funnel plots for the CBR and ORR meta-analysis and analysis with Egger's tests for asymmetry did not indicate obvious publication bias (**Supplementary Figure S7.1**). Finally, we performed a sensitivity analysis based on the leave-one-out method to detect if one of the studies distorted the pooled effect. The analysis indicated two studies with high contribution to the overall heterogeneity in the CBR meta-analysis; Juric *et al.* and Yap *et al*.[29,41] Recalculating the pooled CBR by omitting these studies resulted in a minimal pooled CBR of 28% (95% CI 18-40%, I^2=56%) by removing Juric *et al.* and a maximal pooled CBR of 35% (95% CI 25-46%, I^2=51%) by removing Yap *et al.* (**Supplementary Figure S7.2**).[29,41] The leave-one-out method resulted in minimal change in pooled ORR (minimal 2%, maximal 3%).

Our meta-analysis showed that best overall response was most often defined as SD. Therefore, in terms of the CBR, the effectiveness of PI3K/AKT/mTOR inhibitors can almost completely be attributed to disease stabilization. Although SD is a valuable outcome in the advanced and recurrent disease setting, the duration of disease stabilization is of particular importance to assess meaningful clinical benefit. Therefore, we categorized SD based on the duration of <6 months or ≥6 months. Within the group of patients with a best overall response evaluation of SD (n=71), progression of disease primarily occurred within 6 months after the start of therapy. To assess a better reflection of true patient benefit, we revised the pooled CBR with a longer-term measure of SD. Therefore, we excluded SD for <6 months as outcome measure from the CBR, resulting in the inclusion of PR and SD for ≥6 months. The revised analysis demonstrated a pooled CBR of 7% (95% CI 2-13%, I^2=21%) (**Supplementary Figure S7.3**).

Effectiveness of patient selection based on current PI3K/AKT/mTOR biomarker criteria

In seven of the 19 included studies, patients were selected based on evidence of dysregulation of the PI3K/AKT/mTOR pathway (n=60).[29-31,33,34,36,38] In these studies, enrolment depended on biomarker criteria of activating genomic PI3K/AKT/mTOR alterations (e.g. mutations, amplifications or deletions) and/or loss of PTEN expression by immunohistochemistry. In addition, six studies did not apply biomarker criteria for patient selection but did performed the abovementioned PI3K/AKT/mTOR molecular assessment during the study period, resulting in mixed populations with (n=20) and without (n=44) evidence of dysregulation of the PI3K/AKT/mTOR pathway.[26-28,35,40,42] Of the remaining six studies who did not apply biomarker criteria, four studies (n=82) conducted immunohistochemical biomarker analysis of other downstream PI3K/AKT/mTOR proteins for instance phosphorylated-AKT,[24,32,39,41] and two studies (n=27) did not conduct any PI3K/AKT/mTOR molecular assessments.[25,37] Overall, studies allowed the use of both archived tumour tissue and recently taken biopsies for PI3K/AKT/mTOR molecular assessments. An overview of the number of patients with evidence of dysregulation of the PI3K/AKT/mTOR pathway and the type of analysis used for molecular assessment is provided in **Supplementary Table S7.2**.

We performed subgroup analysis to assess the predictive value of current PI3K/AKT/mTOR biomarkers (e.g. mutations, amplifications or loss of PTEN function by immunohistochemistry) on the effectiveness of PI3K/AKT/mTOR inhibitors. We stratified the studies into two groups; A. studies selecting patients based on PI3K/AKT/mTOR biomarker criteria and therefore including solely patients with evidence of dysregulation of the PI3K/AKT/mTOR pathway (n=60), and B. studies who did not select patients based on biomarker criteria, resulting in mixed populations

with and without dysregulated PI3K/AKT/mTOR pathway activity (n=173). We observed a trend towards a better pooled CBR in studies selecting patients based on PI3K/AKT/mTOR biomarker criteria (CBR of 42%, 95% CI 23-62%, I^2=51%) compared to studies who did not apply biomarker criteria for patient selection (CBR of 27%, 95% CI 14-42%, I^2=69%), however, this difference was not significant (*P*=0.217) (**Figure 7.5**). For pooled ORR, there was no difference between the two groups (**Supplementary Figure S7.4**).

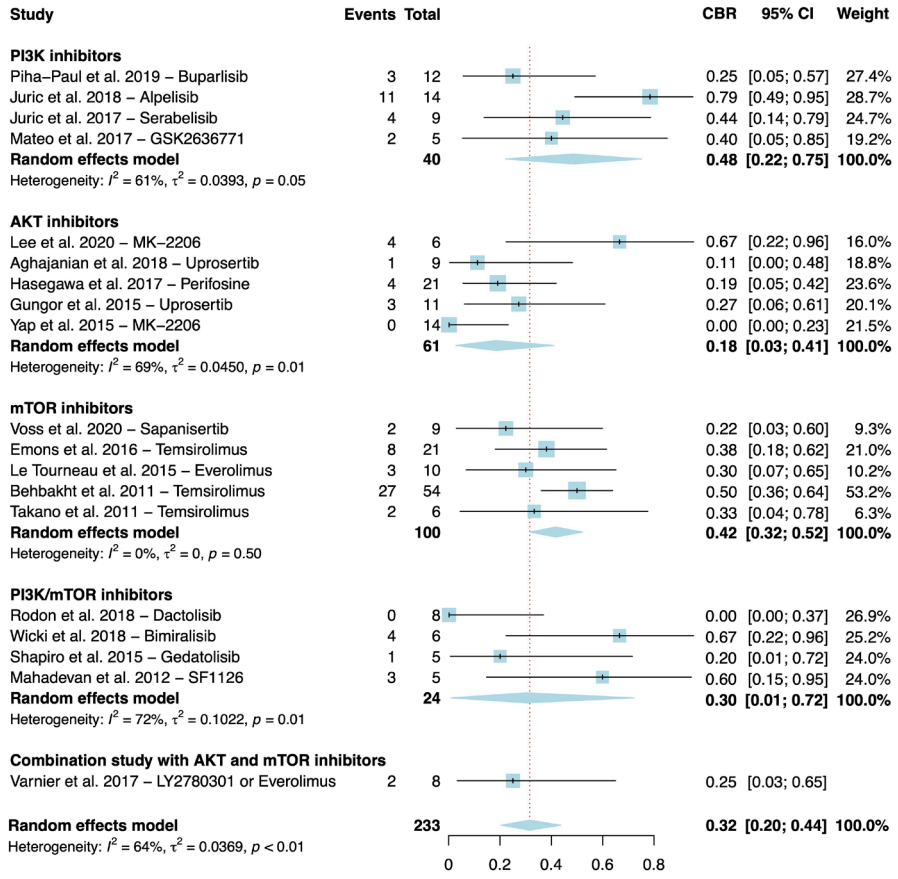

Figure 7.3 Forest plot of the association between treatment with PI3K/AKT/mTOR inhibitors and clinical benefit rate (CBR) in ovarian cancer patients. CBR is defined as the proportion of patients with best overall response of complete or partial response or stable disease (both <6 and ≥6 months). The blue squares and black bars represent the CBR with 95% confidence interval (CI) of individual studies. The pooled CBR with 95% CI by type of inhibitor is represented by the blue diamonds. The final blue diamond indicates the pooled CBR with 95% CI of all studies. PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin.

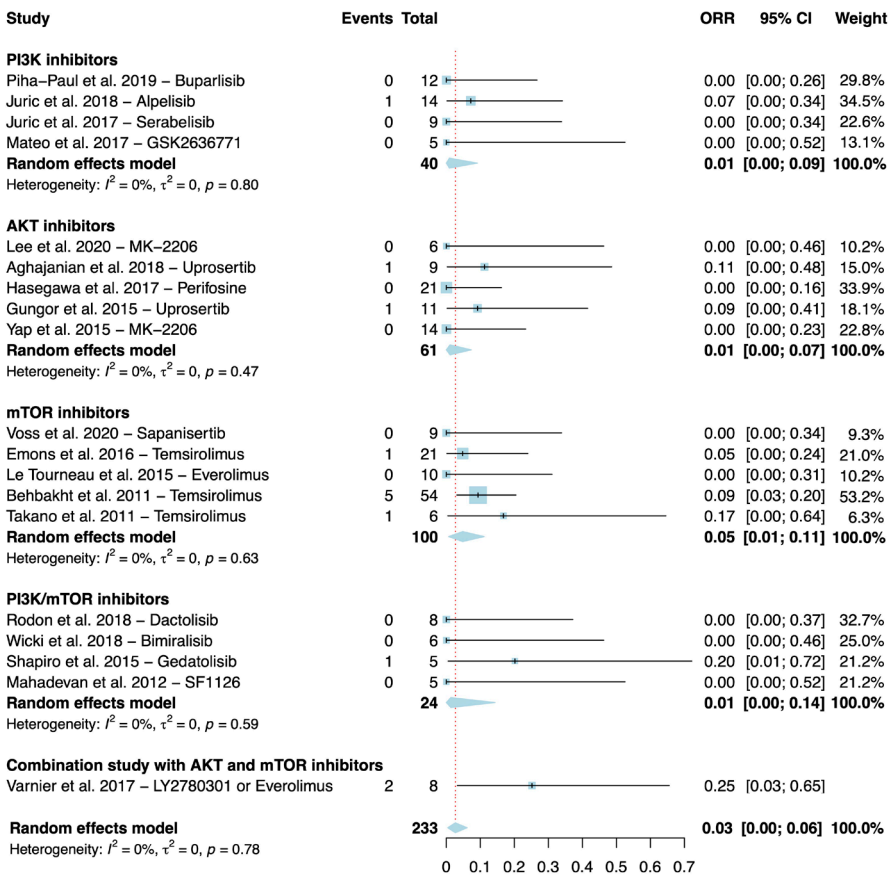

Figure 7.4 Forest plot of the association between treatment with PI3K/AKT/mTOR inhibitors and overall response rate (ORR) in ovarian cancer patients. ORR is defined as the proportion of patients with best overall response of complete or partial response. The blue squares and black bars represent the ORR with 95% confidence interval (CI) of individual studies. The pooled ORR with 95% CI by type of inhibitor is represented by the blue diamonds. The final blue diamond indicates the pooled ORR with 95% CI of all studies. PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin.

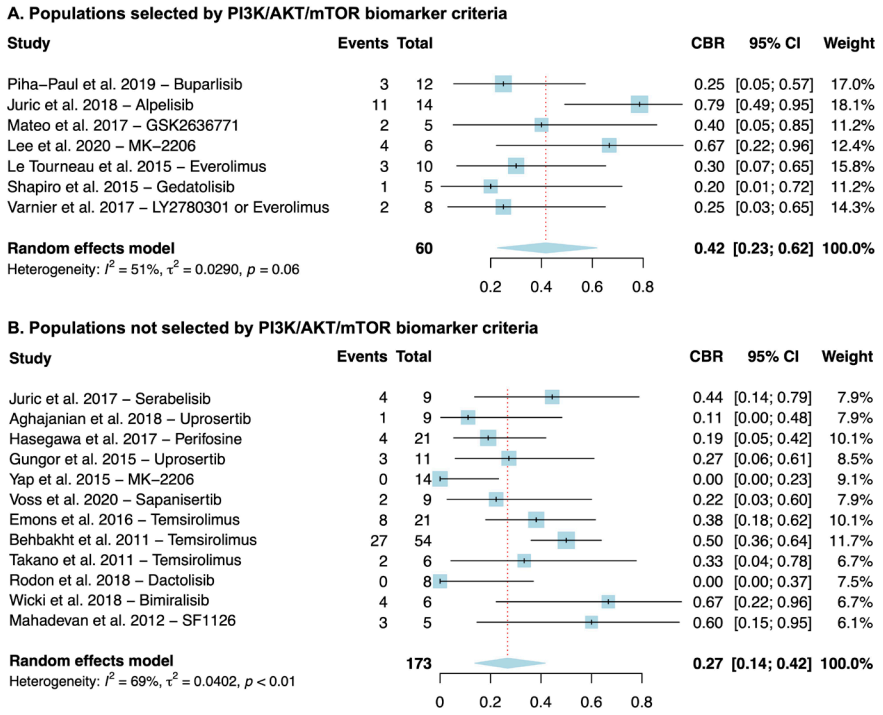

Figure 7.5 Forest plot of the association between PI3K/AKT/mTOR inhibitors and clinical benefit rate (CBR) by dysregulation of PI3K/AKT/mTOR pathway activity in ovarian cancer patients. CBR is defined as the proportion of patients with best overall response of complete or partial response or stable disease. The blue squares and black bars represent the CBR with 95% confidence interval (CI) of individual studies. The pooled CBR with 95% CI per group is represented by the blue diamonds. **A.** Populations selected by PI3K/AKT/mTOR biomarker criteria. **B.** Populations not selected by PI3K/AKT/mTOR biomarker criteria. PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin.

Grade 3 and 4 adverse events according to the Common Terminology Criteria for Adverse Events (CTCAE)

Unless otherwise stated, the proportion of patients with drug-related grade 3 and 4 adverse events is reported in **Table 7.1**. Most of the included studies reported on treatment with PI3K/AKT/mTOR inhibitors in populations including different advanced solid malignancies and did not specify incidence rates of adverse events per tumour type. Therefore, we reported the proportion of the total study population in **Table 7.1**. For several studies we reported the number of grade 3 or 4 adverse events counted as studies lacked information on the total proportion. In total, drug-related grade 3 or 4 adverse events occurred in 36% of the treated patients (229 of 641 patients (range 0-80%), excluding the studies reporting on all-cause adverse events and counted events). Whereby the case series of Takano *et al.* reported no

grade 3 and 4 adverse events in their ovarian cancer population.[37] It must be noted that, due the retrospective nature of the case series, reporting bias may have distorted the documentation of adverse events. **Supplementary Table S7.3** provides additional information on the proportion of patients with all cause grade 3 and 4 adverse events, as well as the most frequently reported grade 3 and 4 adverse events, number of dose-interruptions and -reductions and number of patients with treatment discontinuation due to adverse events. Most common grade 3 and 4 adverse events included hyperglycaemia, elevated liver enzymes and gastro-intestinal complaints (e.g. diarrhoea, nausea, vomiting and stomatitis). The high incidence of adverse events may have resulted in suboptimal dosing in a substantial proportion of the patients. Dose-interruptions and -reductions were required in 2-62% of the patients and in 2-25% adverse events lead to early discontinuation of treatment.

Discussion

Our meta-analysis includes 233 patients from 19 studies and indicates that targeted therapy with PI3K/AKT/mTOR inhibitors is associated with a pooled CBR of 32% (95% CI 20-44%) and a pooled ORR of 3% (95% CI 0-6%) in advanced or recurrent ovarian cancer patients. Dysregulation of the PI3K/AKT/mTOR pathway is considered to be one of the hallmarks of cancer development and alterations in genes associated with this pathway are commonly found in ovarian cancer. Alterations are assumed to mediate hyperactivation of the PI3K/AKT/mTOR pathway, supporting the hypothesis that targeting this signalling pathway might represent a useful treatment strategy. As a result, several PI3K/AKT/mTOR inhibitors have been developed for the treatment of cancer over the past years. To the best of our knowledge, this is the first meta-analysis investigating the effectiveness of these inhibitors solely in ovarian cancer patients.

The CBR may be criticized as outcome measure due to the inclusion of SD without any consideration of the duration of response. In order to better reflect patient benefit, we revised the pooled CBR by the exclusion of SD for <6 months as a beneficial outcome. This resulted in a pooled CBR of 7% (95% CI 2-13%, I^2=21%). Furthermore, in 36% of the patients (range 0-80%), treatment with PI3K/AKT/mTOR inhibitors was associated with drug-related grade 3 and 4 adverse events, including hyperglycaemia, gastro-intestinal complaints and elevated liver enzymes. The incidence of severe toxicities further contributes to unsatisfactory results, which in combination with the limited clinical benefit suggests that current treatment with PI3K/AKT/mTOR inhibitors should not be initiated unless in clinical trials aimed to identify those patients who benefit from this treatment.

Overall, we observed a wide variety in efficacy among the different types of inhibitors. Our subgroup analysis tended to favour the effectiveness of PI3K and mTOR inhibitors over AKT and dual PI3K/mTOR inhibitors, but this difference was not statistically significant. Within the mTOR subgroup, a substantial proportion of the effectiveness could be attributed to temsirolimus, as the inhibitor was investigated in three individual studies. Although the intravenous administration of temsirolimus could have improved bioavailability, the highest CBR was observed in the pooled group of orally administrated PI3K inhibitors. The PI3K component of the signalling pathway is most frequently altered, which might explain the slightly improved CBR as compared to the other types of inhibitors.[5,10] In comparison, limited clinical benefit was achieved with AKT inhibitors, the component in which mutations are rare.[5,10] Potentially, dual PI3K/mTOR inhibitors could exert more inhibitory effects over single PI3K or mTOR inhibitors.[43] In case tumours harbour both PI3K and mTOR alterations, dual inhibitors have the opportunity to suppress pathway activity at both levels. In addition, simultaneous inhibition of mTOR may overcome potential mechanisms of adaptive resistance to PI3K inhibitors.[44,45] However, our results do not confirm this hypothesis as dual PI3K/mTOR inhibitors were not found to have superior efficacy over the other types of inhibitors.

Furthermore, we assessed the potential role of patient selection by current PI3K/AKT/mTOR biomarkers based on sequencing or immunohistochemical analysis (e.g. mutations, amplifications or loss of PTEN function) as a marker for sensitivity to PI3K/AKT/mTOR inhibitors. Subgroup analysis showed a trend towards an improved CBR in studies including patients solely with evidence of PI3K/AKT/mTOR pathway dysregulation compared to studies who did not apply biomarker criteria for patient selection, but this difference was not significant (pooled CBR of 42% and 27%, P=0.217, respectively). The lack of support regarding the predictive value of current PI3K/AKT/mTOR biomarkers (most frequently *PIK3CA*, *PIK3R1* and *AKT2* genes and PTEN protein expression) to select responding patients might be explained by the heterogeneity of analysis techniques used, including next-generation sequencing and immunohistochemistry. A recent study by Sieuwerts *et al.* measured functional activity of the PI3K pathway based on mRNA expression levels of pathway-specific target genes, in addition to genomic mutation analysis, in ER positive breast cancer samples using a novel assay technology.[46] In contrast to genomic mutations, functional PI3K pathway activity was associated with shorter progression-free survival in metastatic patients treated with tamoxifen. In addition, their findings demonstrated that functional PI3K pathway activity did not correlate to *PIK3CA* mutation status, indicating that the activation state of a signalling pathway cannot simply be inferred from genomic alterations.[46] Moreover, in addition to genomic alterations, the functional phenotype of tumour cells is affected by epigenetic modifications and influenced by the tumour microenvironment. This is a possible

explanation for the lack of support for a relation between genomic alterations or loss of protein expression and activity of the corresponding pathway and the limited predictive value of current biomarkers on the efficacy of pathway inhibitors. In the search for an alternative biomarker, the focus on transcriptional activation of the PI3K/AKT/mTOR pathway may provide useful information to guide patient selection for targeted therapy. Furthermore, most studies used archived tumour tissue of the primary tumour or a previous recurrence for biomarker analysis rather than recently taken biopsies of the recurrent tumour. Treatment with PI3K/AKT/mTOR inhibitors was often preceded by prior treatment regimens for multiple recurrences. The use of archived material from the primary tumour or a previous recurrence may have precluded the detection of alterations that have emerged during tumour evolution. Both platinum-based chemotherapeutics and changes in the tumour associated with recurrence have been shown to increase genetic heterogeneity in ovarian cancer.[47,48] In addition, previous research in breast cancer patients revealed substantial discordance in PTEN protein expression and *PIK3CA* mutations between primary disease and metastases.[49] Therefore, assessment of alteration status in recently taken tumour tissue might provide more useful information for therapy selection.

The PI3K/AKT/mTOR signalling pathway does not exert its function independently, as crosstalk with other signalling pathways, such as the poly (ADP-ribose) polymerase (PARP) and mitogen-activated protein kinase (MAPK) pathways, has been described.[12,50] Preclinical work has shown that PI3K inhibitors can sensitize tumours to PARP inhibitors via downregulation of *BRCA1/2* genes, abrogation of intrinsic or acquired homologous recombination repair (HRR) proficiency and DNA damage.[51] In addition, previous clinical studies indicated potential mechanistic synergy of combined therapy. Konstantinopoulos *et al.* performed a phase IB trial in which PARP inhibitor olaparib was combined with p110*α*-isoform-specific PI3K inhibitor alpelisib in 30 patients with epithelial ovarian cancer.[51] Their preliminary results are promising, with 10 patients (36%) having a RECIST 1.1 PR and 14 (50%) SD, of which eight patients had SD lasting ≥6 months. The toxicity profile was acceptable and further follow-up of patients who completed treatment is still ongoing. Furthermore, a recent study by Bardia *et al.* combined MEK inhibitor binimetinib with pan-PI3K inhibitor buparlisib in a phase IB trial.[52] The expansion phase included different types of tumours, including 18 patients with *RAS*- or *BRAF*-mutant advanced ovarian cancer. The best responses were observed in this subgroup with a CBR of 61% (95% CI 36-83%), with six patients showing a PR. However, continuous dosing beyond the dose-limiting toxicity period with buparlisib was not feasible due to unacceptable toxicity. Similarly, a trial by Matulonis *et al.* in which olaparib and buparlisib were administered to recurrent ovarian and breast cancer patients, could not achieve meaningful dose-escalation of buparlisib due to unacceptable central nervous system toxicity and grade 3 transaminase elevation.[53] This indicates that treatment

with p110*α*-isoform-specific inhibitors such as alpelisib with a favourable toxicity profile might be preferable in combination strategies over pan-PI3K inhibitors such as buparlisib. On the other hand, combination regimens may benefit from lower or intermittent dosing schedules to improve long-term tolerability on the premise that optimal pathway inhibition is sustained. In the near future, new drugs targeting the PI3K/AKT/mTOR signalling pathway might be developed by using bioinformatic analysis using tools such as String database.[54]

The strength of this meta-analysis is the comprehensive review of the existing evidence on the effectiveness of PI3K/AKT/mTOR inhibitors in advanced or recurrent ovarian cancer, including subpopulations of larger studies with different advanced solid tumours. However, this resulted in a relatively small number of included ovarian cancer patients, which could have caused selection bias. Our meta-analysis is further limited by large heterogeneity in study designs. In comparison to the fixed drug dosage in phase II studies, phase I dose-escalation studies used a variety of dosages and schedules to obtain the maximum-tolerated dose, resulting in within-study bias. In addition, heterogeneity in histological subtype could have distorted therapy efficacy as genomic alterations are more common in endometrioid and clear cell carcinoma as compared to high-grade serous carcinoma.[8]

Taken together, our findings demonstrate limited to no efficacy of monotherapy with PI3K/AKT/mTOR inhibitors in advanced or recurrent ovarian cancer patients. Best overall response was often defined by disease stabilization lasting for a short period of time (<6 months). Although SD is a valuable outcome in this highly pretreated population, the short duration of response may be insufficient to qualify as true patient benefit. Moreover, clinical evidence that current biomarkers are properly predicting response to pathway inhibitors is lacking. Given the overall toxicity rate of 36% grade 3 or 4 adverse events, we suggest that PI3K/AKT/mTOR inhibitors should only be used within clinical studies, preferably in combination with other targeted drugs, in a highly selected population based on reliable biomarkers that measure functional activity of the PI3K/AKT/mTOR pathway.

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Supplementary information

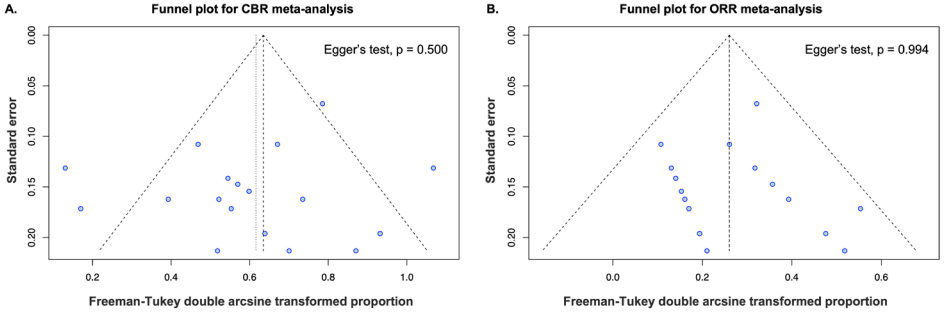

Figure S7.1 Funnel plots with Egger's test. **A.** Clinical benefit rate (CBR) **B.** Overall response rate (ORR).

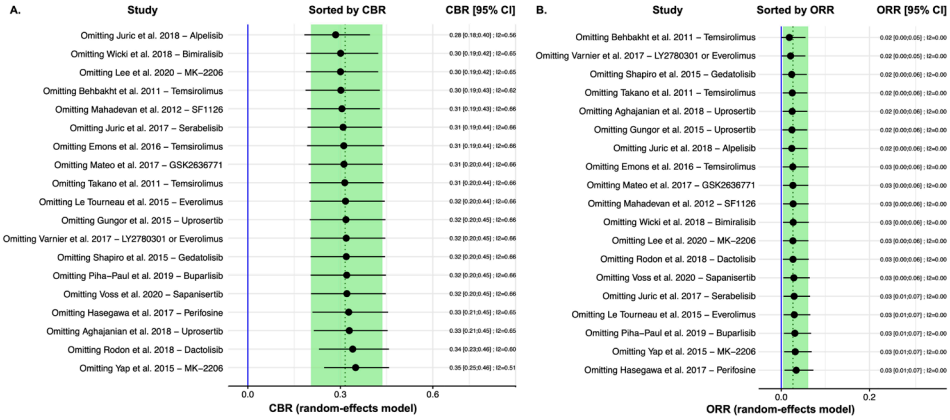

Figure S7.2 Sensitivity analysis leave-one-out. The black dots and bars represent the **A.** pooled clinical benefit rate (CBR) and **B.** pooled overall response rate (ORR) with 95% confidence interval (CI) of individual studies by omitting one study out of the analysis.

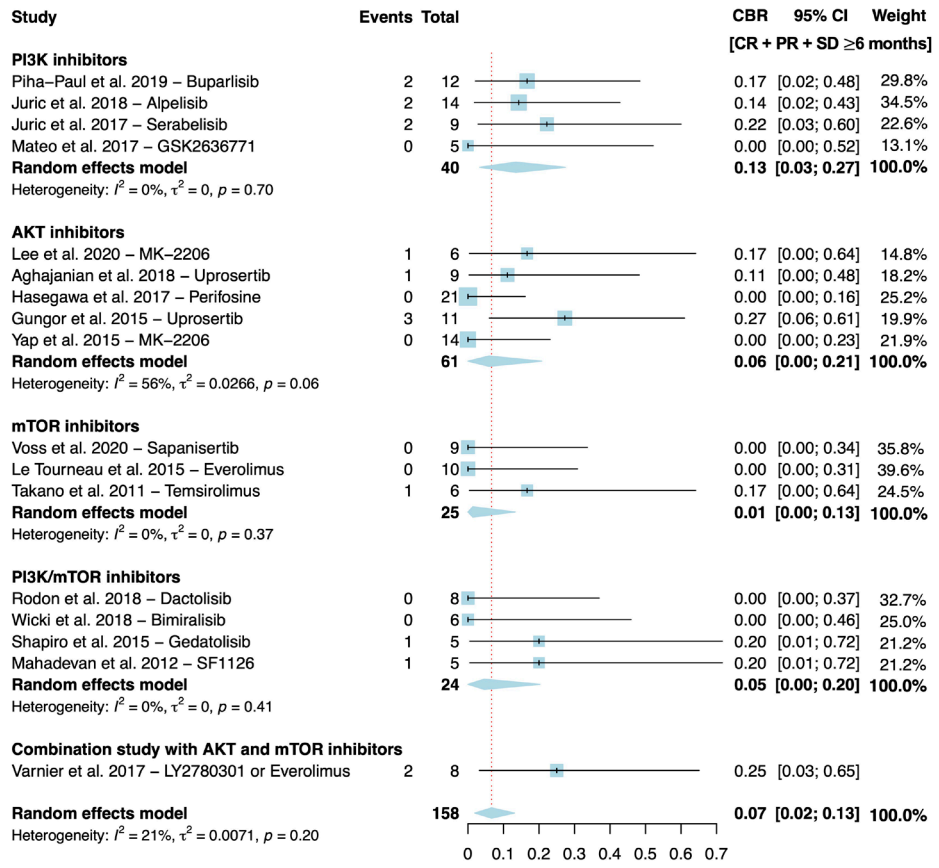

Figure S7.3 Revised forest plot of the association between treatment with PI3K/AKT/mTOR inhibitors and clinical benefit rate (CBR) in ovarian cancer patients. CBR is defined as the proportion of patients with best overall response of complete or partial response or stable disease ≥6 months. The blue squares and black bars represent the CBR with 95% confidence interval (CI) of individual studies. The pooled CBR with 95% CI by type of inhibitor is represented by the blue diamonds. The final blue diamond indicates the pooled CBR with 95% CI of all studies. PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin.

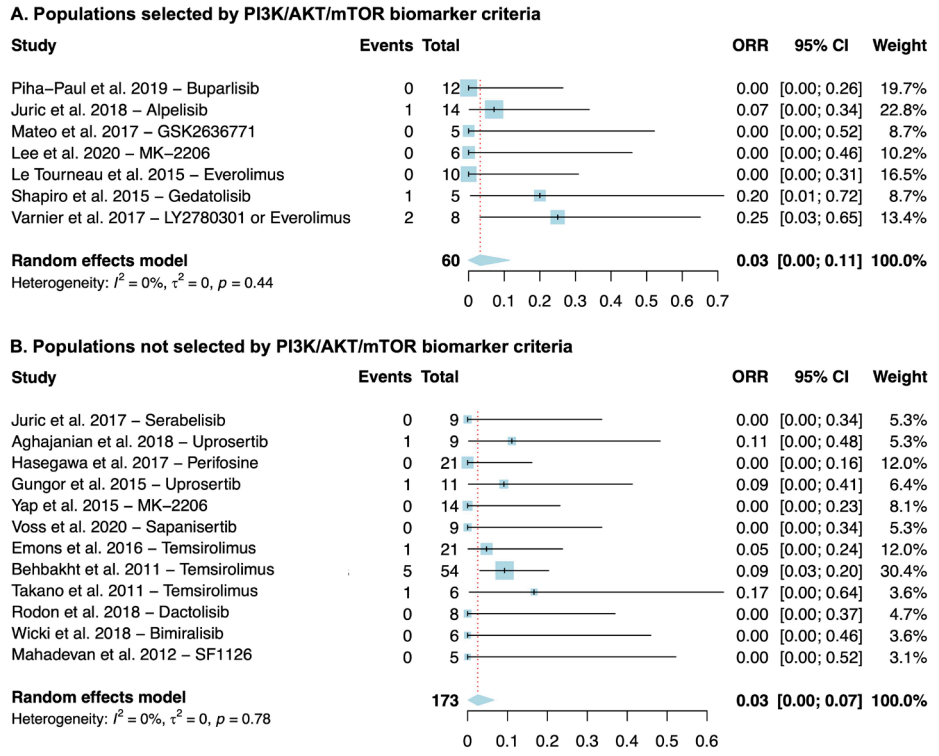

Figure S7.4 Forest plot of the association between PI3K/AKT/mTOR inhibitors and overall response rate (ORR) by dysregulation of PI3K/AKT/mTOR pathway activity in ovarian cancer patients. ORR is defined as the proportion of patients with best overall response of complete or partial response. The blue squares and black bars represent the ORR with 95% confidence interval (CI) of individual studies. The pooled ORR with 95% CI per group is represented by the blue diamonds. **A.** Populations selected by PI3K/AKT/mTOR biomarker criteria. **B.** Populations not selected by PI3K/AKT/mTOR biomarker criteria. PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin.

Table S7.1 Summary of the risk of bias assessment by reviewers' judgement for studies included in the meta-analysis.

Authors and year	Bias due to confounding	Bias in selection of participants	Bias in classification of intervention	Bias due to deviations from intended intervention	Bias due to missing or incomplete outcome data	Bias in measurement of outcomes	Bias in selection of reported results	Other sources of bias
PI3K inhibitors								
Piha-Paul *et al.* 2019 [34]	+	?	+	−	?	+	+	−
Juric *et al.* 2018 [29]	+	?	+	−	+	−	+	−
Juric *et al.* 2017 [28]	+	?	+	−	+	−	+	+
Mateo *et al.* 2017 [33]	+	?	+	+	?	−	+	−
AKT inhibitors								
Lee *et al.* 2020 [31]	+	+	+	?	?	+	+	?
Aghajanian *et al.* 2018 [42]	+	?	+	+	?	?	+	+
Hasegawa *et al.* 2017 [27]	+	+	+	+	+	+	+	+
Gungor *et al.* 2015 [26]	+	?	+	−	+	?	+	+
Yap *et al.* 2014 [41]	+	?	+	+	?	−	+	+
mTOR inhibitors								
Voss *et al.* 2020 [39]	+	?	+	−	?	−	+	+
Emons *et al.* 2016 [25]	+	?	+	?	?	+	+	+
Le Tourneau *et al.* 2015 [30]	+	+	+	−	+	+	+	+
Behbakht *et al.* 2011 [24]	+	?	+	−	+	+	+	+
Takano *et al.* 2011 [37]	+	−	+	+	+	?	?	+
PI3K/mTOR inhibitors								
Rodon *et al.* 2018 [35]	+	?	+	+	?	−	+	+
Wicki *et al.* 2018 [40]	+	?	+	+	+	−	+	+
Shapiro *et al.* 2015 [36]	+	?	+	+	+	−	+	−
Mahadevan *et al.* 2012 [32]	+	?	+	−	+	−	+	+
Combination PI3K/AKT/mTOR inhibitors								
Varnier *et al.* 2019 [38]	+	+	+	−	+	?	+	−

+ Low ? Unclear − High

Table S7.2 Study characteristics of PI3K/AKT/mTOR molecular assessments in the included studies.

	Number of patients with evidence of dysregulated pathway activity / total ovarian cancer patients	Type of analysis	Type of PI3K/AKT/mTOR molecular assessment	Type of tumour material	Relation with therapy response
Populations selected by PI3K/AKT/mTOR biomarker criteria					
Piha-Paul *et al.* 2019 [34]	12 / 12	NGS and IHC	Local analysis: PIK3CA, PIK3R1 and PTEN (all patients at least one alteration). Central analysis: PIK3CA (4 patients), PIK3R1 (no patient) and PTEN (1 patient).	Archived tissue	*N.A.*
Juric *et al.* 2018 [29]	14 / 14	Unknown (local analysis) and NGS (central analysis)	Local analysis: PIK3CA (all patients). Central analysis: confirmed PIK3CA alterations in 7 of these patients.	Archived tissue or recent biopsy	*N.A.*
Mateo *et al.* 2017 [33]	5 / 5	NGS and IHC	PIK3CB (no patient), PIK3CA and AKT2 (1 patient) and PTEN (all patients had PTEN loss). Biomarker analysis of phosphorylated-Ser473, -Thr246 PRAS40, -Ser235/236, and -Thr308.	Archived tissue or recent biopsy	*N.A.*
Lee *et al.* 2020 [31]	6 / 6	CLIA-certified assay and IHC	PIK3CA (no patient), AKT (no patient) and PTEN (all patients had PTEN loss)	Not described	*N.A.*
Le Tourneau *et al.* 2015 [30]	10 / 10	NGS and gene copy number alterations by Cytoscan HD	PIK3CA (2 patients), AKT2 (no patient), mTOR (no patient), RPTOR (1 patient), and PTEN (7 patients).	Recent biopsy	*N.A.*
Shapiro *et al.* 2015 [36]	2/5*	Unknown (genomic analysis), IHC and reverse phase protein microarrays	PIK3CA (1 patient) and PTEN (1 patient had PTEN loss). Biomarker analysis of phosphorylated-AKT, -S6, -S6K, -mTOR, -4EBP-1 and -PRAS40.	Archived tissue or recent biopsy	There was no clear relationship between PIK3CA gene alterations or PTEN protein status and clinical benefit.
Varnier *et al.* 2017 [38]	8 / 8	NGS	PIK3CA (7 patients) and AKT2 (1 patient).	Archived tissue or recent biopsy	*N.A.*

Table S7.2 (continued)

	Number of patients with evidence of dysregulated pathway activity / total ovarian cancer patients	Type of analysis	Type of PI3K/AKT/mTOR molecular assessment	Type of tumour material	Relation with therapy response
Populations not selected by PI3K/AKT/mTOR biomarker criteria					
Juric *et al.* 2017 [28]	4 / 9	Type of mutation analysis not specified	PIK3CA (4 patients).	Archived tissue	All four responding ovarian cancer patients harbored PIK3CA alterations.
Aghajanian *et al.* 2018 [42]	3 / 9	WAVE-Surveyor denaturing high-performance liquid chromatography (DHPLC) technology and IHC	PIK3CA (no patient) and PTEN (3 patients had PTEN loss).	Not describe	One ovarian cancer patient with PTEN loss had PR. No correlation between response and aberrations could be established.
Hasegawa *et al.* 2017 [27]	5 / 21	Mutation analysis by PIK3CA mutation test kit (QIAgen), NGS and IHC	PIK3CA (5 patients). Biomarker analysis of PTEN and phosphorylated-AKT.	Archived tissue	13% of wild-type patients responded compared to 40% of PIK3CA-mutated patients (p=0.22).
Gungor *et al.* 2015 [26]	5 / 11	Genotyping assay on the Illumina Golden Gate platform and hot spot mutation detection by a mass spectroscopy-based approach (Sequnom MassARRAY), IHC and reversed-phase protein array	PIK3CA (1 patients), PIK3R1 (1 patient), PIK3CA and PIK3R1 (2 patients) and PIK3CA, PIK3R1 and PIK3R4 (1 patient). Biomarker analysis of AKT and phosphorylated-AKT.	Archived tissue and recent biopsy	33% of wild-type patients responded compared to 20% of mutated patients.
Yap *et al.* 2015 [41]	Unknown / 14	Quantitative electrochemiluminescence assays (MSD) and ELISA assays (EnVision)	Biomarker analysis of phosphorylated-Ser473 AKT, -Ser9 GSK3beta, and -Thr246 PRAS40.	Recent biopsy	*N.A.*
Voss *et al.* 2020 [39]	Unknown / 9	IHC	Biomarker analysis of phosphorylated-4EBP1, -S6 and -NDRG1.	Archived tissue or recent biopsy	
Emons et al. 2016 [25]	No PI3K/AKT/mTOR molecular assessment performed.				
Behbakht *et al.* 2017 [24]	Unknown / 54	IHC	Biomarker analysis of phosphorylated-AKT (14 patients), -mTOR (33 patients), -p70-S6 (21 patients), -4E-BP1 (25 patients) and cyclin D1 (21 patients).	Archived tissue	*N.A.*

Table S7.2 (continued)

	Number of patients with evidence of dysregulated pathway activity / total ovarian cancer patients	Type of analysis	Type of PI3K/AKT/mTOR molecular assessment	Type of tumour material	Relation with therapy response
Populations not selected by PI3K/AKT/mTOR biomarker criteria					
Takano et al. 2011 [37]	No PI3K/AKT/mTOR molecular assessment performed.				
Rodon *et al.* 2018 [35]	2 / 8**	SNaPshot genotyping assay and IHC	PIK3CA (1 patient) and PTEN (1 patient had PTEN loss).	Recent biopsy	*N.A.*
Wicki *et al.* 2018 [40]	1 / 6	NGS	PIK3CA (1 patient).	Recent biopsy	The PIK3CA-altered patient achieved SD. Of the remaining 5 patients with unknown alteration status 60% responded with SD.
Mahadevan *et al.* 2012 [32]	Unknown / 5	IHC	Biomarker analysis of phosphorylated-Ser473 AKT and -Ser235/Ser236 S6K.	Recent biopsy	*N.A.*

Abbreviations: PI3K, phosphatidylinositol-3-kinase; mTOR, mammalian target of rapamycin; NGS, next-generation sequencing; IHC, immunohistochemistry; N.A., not applicable; PR, partial response; SD, stable disease.

* We received information on the alteration status of two of the five ovarian cancer patients; ** We received information on the alteration status of five of the eight ovarian cancer patients.

Table S7.3 Grade 3 and 4 adverse events according to the Common Terminology Criteria for Adverse Events (CTCAE) in the included studies. Numbers relate to the total study population.

Study	Type of treatment	OC patients / total patients (n)	Number of patients with all cause grade 3 and 4 adverse events according to CTCAE	Number of patients with drug-related grade 3 and 4 adverse events according to CTCAE	Top 3 most common grade 3 and 4 adverse events	Number of patients with all cause dose interruptions or reductions	Number of patients with treatment discontinuation due to all cause adverse events (including all grades)
PI3K inhibitors							
Piha-Paul *et al.* 2019 [34]	Buparlisib	12 / 146	n=120 (82%)	*N.A.*	Hyperglycaemia (n=20, 14%), fatigue (n=19, 13%) and elevated aspartate aminotransferase (n=12, 8%) / alanine aminotransferase (n=12, 8%)	n=57 (39%)	n=33 (23%)
Juric *et al.* 2018 [29]	Alpelisib	14 / 134	*N.A.*	n=59 (44%)	Hyperglycaemia (n=32, 24%), diarrhoea (n=4, 3%), fatigue (n=4, 3%)	Dose interruptions n=78 (58%) and dose reductions n=51 (38%)	n=18 (13%)
Juric *et al.* 2017 [28]	Serabelisib	9 / 71	n=38 (54%)	n=19 (27%)	Hyperglycaemia (n=6, 8%), elevated alanine aminotransferase (n=6, 8%) and elevated aspartate aminotransferase (n=5, 7%)	n=29 (41%)	n=9 (13%)
Mateo *et al.* 2017 [33]	GSK2636771	5 / 65	n=33 (51%)	n=15 (23%)	Hypophosphatemia (n=6, 9%), rash (n=3, 5%), fatigue (n=3, 5%)	n=4 (22%) (in recommended phase II dose cohort)	n=10 (15%)
AKT inhibitor							
Lee *et al.* 2020 [31]	MK-2206	6 / 6	*N.A.*	n=4 (80%)	Maculo-papular rash (n=4, 80%), no other grade 3 or 4 adverse events occurred	*N.A.*	n=1 (20%)
Aghajanian *et al.* 2018 [42]	Uprosertib	9 / 77	n=22*	*N.A.*	Hyperglycaemia (n=6, 7%), diarrhoea (n=3, 4%), abdominal pain (n=3, 4%)	n=11 (14%)	n=13 (17%)
Hasegawa *et al.* 2017 [27]	Perifosine	21 / 71	n=47*	*N.A.*	Anaemia (n=16, 23%), anorexia (n=8, 11%), tumour pain (n=7, 10%)	n=6 (8%)	n=5 (7%)
Gungor *et al.* 2015 [26]	Uprosertib	11 / 12	n=7 (58%)	n=2 (17%)	Nausea (n=1, 8%), vomiting (n=1, 8%), rash (n=1, 8%)	Dose interruptions n=9 (75%) and dose reductions n=2 (17%)	n=3 (25%)
Yap *et al.* 2015 [41]	MK-2206	14 / 71	*N.A.*	n=12*	Rash (n=11, 15%), dermatitis acneiform (n=1, 1%)	n=17 (24%)	n=3 (4%)

Table S7.3 (continued)

Study	Type of treatment	OC patients / total patients (n)	Number of patients with all cause grade 3 and 4 adverse events according to CTCAE	Number of patients with drug-related grade 3 and 4 adverse events according to CTCAE	Top 3 most common grade 3 and 4 adverse events	Number of patients with all cause dose interruptions or reductions	Number of patients with treatment discontinuation due to all cause adverse events (including all grades)
mTOR inhibitors							
Voss *et al.* 2020 [39]	Sapanisertib	9 / 198	n=134 (68%)	n=87 (44%)	Hyperglycaemia (n=29, 15%), fatigue (n=14, 7%), stomatitis (n=12, 6%)	n=123 (62%)	n=40 (20%)
Emons *et al.* 2016 [25]	Temsirolimus	21 / 44	n=43 (98%)	*N.A.*	Nausea (n=4, 9%), ascites (n=3, 7%), subileus (n=3, 7%)	*N.A.*	*N.A.*
Le Tourneau *et al.* 2015 [30]	Everolimus	10 / 46	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*
Behbahkt *et al.* 2011 [24]	Temsirolimus	54 / 54	n=44*	*N.A.*	Metabolic (n=8, 15%), pain (n=6, 11%), gastrointestinal (n=6, 11%)	n=27 (50%)	n=3 (6%)
Takano *et al.* 2011 [37]	Temsirolimus	6 / 6	n=0 (0%)	n=0 (0%)	*N.A.*	*N.A.*	*N.A.*
PI3K/mTOR inhibitors							
Rodon *et al.* 2018 [35]	Dactolisib	8 / 183	n=80 (44%)	*N.A.*	Fatigue (n=10, 5%), diarrhoea (n=9, 5%), anaemia (n=8, 4%)	n=18 (10%)	n=3 (2%)
Wicki *et al.* 2018 [40]	Bimiralisib	6 / 28	n=21 (75%)	n=16 (57%)	Hyperglycaemia (n=7, 25%), rash maculopapular (n=5, 18%), hypertension (n=4, 14%)	n=3 (11%)	n=5 (18%)
Shapiro *et al.* 2015 [36]	Gedatolisib	5 / 77	*N.A.*	n=22 (29%)	Mucosal inflammation and stomatitis (n=8, 5%), elevated alanine aminotransferase (n=3, 4%), hyperglycaemia (n=3, 4%)	n=12 (16%)	n=3 (4%)
Mahadevan *et al.* 2012 [32]	SF1126	5 / 44	*N.A.*	n=5 (11%)	Oedema, elevated alkaline phosphatase, diarrhoea, muscular weakness, hypoglycaemia, haemoglobin decreased, pruritus/urticaria, hypokalaemia, hypersensitivity (all n=1 (2%))	n=1 (2%)	n=1 (2%)
Combination study with AKT and mTOR inhibitors							
Varnier *et al.* 2017 [38]	LY2780301 or everolimus	8 / 39	*N.A.*	*N.A.*	*N.A.*	*N.A.*	*N.A.*

Abbreviations: OC, ovarian cancer; CTCAE, common terminology criteria for adverse events; NA, data not available; mTOR, mammalian target of rapamycin; MTD, maximum-tolerated dose.

* Number of adverse events counted. Possibility that multiple adverse events occurred in the same patients and patients are therefore counted more than once.

Supplementary Material S7.1 Embase via Ovid search query

01. ovary tumor/
02. uterine tube tumor/
03. (Ovarian Neoplasm*).ti,ab,kw
04. (Ovary Neoplasm*).ti,ab,kw
05. (Ovarian cancer*).ti,ab,kw
06. (Ovary cancer*).ti,ab,kw
07. (Cancer of Ovary).ti,ab,kw
08. (Cancer of the Ovary).ti,ab,kw
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10. (Fallopian Tube Cancer*).ti,ab,kw
11. (Cancer of the Fallopian Tube*).ti,ab,kw
12. (Solid malignancy).ti,ab,kw
13. (Gynaecologic carcinoma*).ti,ab,kw
14. (Gynecologic carcinoma*).ti,ab,kw
15. (Gynaecologic cancer*).ti,ab,kw
16. (Gynecologic cancer*).ti,ab,kw
17. (Advanced malignancies).ti,ab,kw
18. (Advanced malignancy).ti,ab,kw
19. (Solid tumor*).ti,ab,kw
20. (Solid tumour*).ti,ab,kw
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23. Pi3K/Akt signaling/
24. Akt signaling/
25. PI3K/Akt signaling/
26. Akt/mTOR signaling/
27. mTOR signaling/
28. transcription factor FOXO/
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30. (PI3Kinase).ti,ab,kw
31. (Phosphatidylinositol 3 Kinase).ti,ab,kw
32. (1 Phosphatidylinositol 3 Kinase).ti,ab,kw
33. (Phosphoinositide 3 Kinase).ti,ab,kw
34. (Fox Transcription Factors).ti,ab,kw
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36. (Forkhead Box Proteins).ti,ab,kw
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52. everolimus/
53. perifosine/
54. buparlisib/
55. temsirolimus/
56. mammalian target of rapamycin inhibitor/
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59 (SDZ RAD).ti,ab,kw
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61. (RAD001).ti,ab,kw
62. (Afinitor).ti,ab,kw
63. (Certican).ti,ab,kw
64. (mTOR inhibitor).ti,ab,kw
65. (Akt inhibitor).ti,ab,kw
66. (PI3K inhibitor).ti,ab,kw
67. (PI3K pathway inhibitor).ti,ab,kw
68. (PAMi inhibitor).ti,ab,kw
69. (D 21266).ti,ab,kw
70. (Class I Phosphatidylinositol 3 Kinase).ti,ab,kw
71. (BYL719).ti,ab,kw
72. (Piqray).ti,ab,kw
73. (buparlisib).ti,ab,kw
74. (BKM120).ti,ab,kw
75. (rapamycin).ti,ab,kw
76. (CCI 779).ti,ab,kw
77. (Torisel).ti,ab,kw
78 (Alpelisib).ti,ab,kw
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81. 51 or 52 or 53 or 54 or 55 or 56 or 57 or 58 or 59 or 60 or 61 or 62 or 63 or 64 or 65 or 66 or 67 or 68 or 69 or 70 or 71 or 72 or 73 or 74 or 75 or 76 or 77 or 78 or 79 or 80
82. 21 and 50 and 81

Key:
ti=title
ab=abstract
kw=key words

Supplementary Material S7.2 Data extraction template

The following study characteristics were extracted:
- Authors;
- Year of publication;
- Country;
- Setting;
- Inclusion and exclusion criteria;
- Study design;
- Accrual period (time to recruit target population);
- Follow-up duration.

The following subject characteristics were extracted:
- Total number of subjects;
- Age;
- Comorbidities;
- FIGO stage;
- Histological subtype;
- Type of inhibitor;
- Duration of treatment;
- Dose;
- Cycles of treatment;
- Route of administration.

Supplementary Material S7.3 Risk of bias of studies included in the meta-analysis

Studies were subjected to a comprehensive quality assessment for the risk of bias on seven predefined domains and reviewers' judgements of each domain were summarized in **Supplementary Table S7.1**. In case study populations consisted of different advanced solid malignancies, bias domains were applied to the study as a whole.

Bias due to confounding

We judged all studies to be at low risk of bias due to confounding related to the detailed description of the patient characteristics. Study populations consisted of

advanced solid malignancies, including ovarian cancer patients,[28-30,32-36,38-42] gynaecologic malignancies,[26,27] or solely ovarian cancer patients.[24,25,31,37] Almost all patients were treatment with at least one prior line of treatment.

Bias in selection of participants

We rated four studies as at low risk of bias due to selection of participants as the inclusion- and exclusion criteria and the selection process were described in detail.[27,30,31,38] In addition, the number of excluded patients and reasons for exclusion were mentioned. Fourteen studies did not (sufficiently) describe the selection process and the number of excluded patients and thus was considered as at unclear risk of bias.[24-26,28,29,32-36,39-42] One study was rated as at high risk of bias due to the lack of predefined in- and exclusion criteria based on the retrospective case series design.[37]

Bias in classification of intervention

All studies were considered to be at low risk of bias due to classification of intervention as a detailed description of the intervention was provided and, if applicable, the use of variable dosages during a dose-escalation phase was clearly described.[24-42] All studies investigated PI3K/AKT/mTOR monotherapy or included a subgroup of patients treated with PI3K/AKT/mTOR monotherapy.

Bias due to deviations from intended intervention

We considered eight studies to be at low risk of bias due to deviations from intended intervention as treatment delay, interruptions or dose modifications did not occur or involved a limited proportion (≤25%) of the total patient population.[27,33,35-37,40-42] Two studies did not describe if deviations from intended intervention occurred, therefore we were not able to assess the risk of bias and thus these studies were considered to be at unclear risk of bias.[25,31] Six studies were rated as at high risk of bias as treatment delay, interruptions or dose modifications occurred in a substantial proportion (>25%) of the population.[24,26,28,29,34,39] Two studies were considered as at high risk of bias as a substantial proportion (>25%) of patients did not received a single dose of the recommended treatment.[30,38] Another study was judged as at high risk of bias as the study protocol was amended during the trial period and the primary outcome measure (maximum-tolerated dose (MTD)) was not reached.[32]

Bias due to missing or incomplete data

Eleven studies were judged as low risk of bias due to missing or incomplete data.[24,26-30,32,36-38,40] These studies clearly described the number of evaluable patients and stated the reasons for missing response data. Furthermore, the proportion of missing response data was ≤25% of the included patients. We considered eight

studies to be at unclear risk of bias as the reasons for missing data were not specified.[25,31,33-35,39,41,42]

Bias in measurement of outcomes

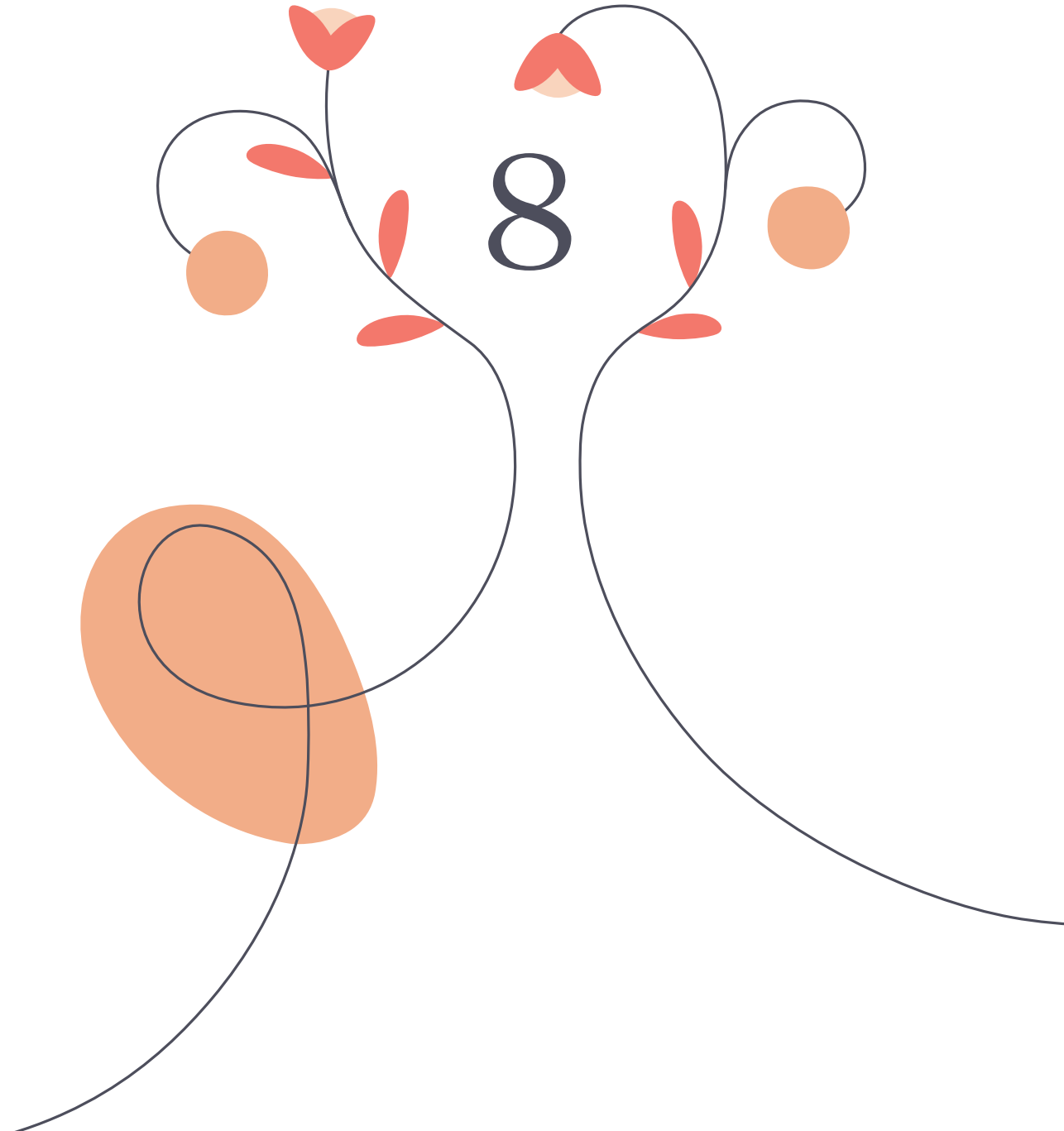
We considered six studies as at low risk of bias due to measurement of outcomes.[24,25,27,30,31,34] These studies provided a detailed description of response evaluation and apprehended standardized timepoints for response assessment. Four studies were rated as at unclear risk of bias as insufficient information was provided to confirm the use of standardized timepoints for response assessment.[26,37,38,42] Nine of the included studies used differing dosing schedules, dosing amounts and/or formulations of the study drug.[28,29,32,33,35,36,39-41] The variability could have led to biased tumour response between individual patients in a study, thus we judged these studies as at high risk of bias.

Bias in selection of reported results

Eighteen studies reported the outcomes according to a published protocol and no deviations were observed.[24-36,38-42] We therefore considered these studies as at low risk of bias. For one study a published protocol was unavailable due to the study design (case series) and thus was rated at unclear risk of bias.[37]

Other sources of bias

We identified no other sources of bias in 13 studies and thus considered them as at low risk of bias.[24-28,30,32,35,37,39-42] Several studies performed biomarker analysis for the selection of patient with evidence of dysregulation of the PI3K/AKT/mTOR pathway. These studies included patients based on activating genomic PI3K/AKT/mTOR alterations and/or loss of PTEN protein expression by immunohistochemistry. However, the use of archived tumour tissue may not provide an accurate assessment of genomic alterations or PTEN protein expression status at the time of progressive or recurrent disease. Therefore, PI3K/AKT/mTOR molecular assessments on archived tumour tissue may have caused bias. One study did not specify whether PI3K/AKT/mTOR molecular assessment was conducted on recently taken tumour tissue and thus was rated as at unclear risk of bias.[31] In five studies eligibility was based on genomic PI3K/AKT/mTOR alterations and/or loss of PTEN protein expression, which was determined on archived tumour tissue.[29,33,34,36,38] Therefore, these studies were considered as at high risk of bias.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CHAPTER 8

Phenotype-guided targeted therapy based on functional signal transduction pathway activity in recurrent ovarian cancer patients: the STAPOVER study protocol

Phyllis van der Ploeg*, Cynthia S.E. Hendrikse*, Anna M.J. Thijs, Hans M. Westgeest, Huberdina P.M. Smedts, M. Caroline Vos, Mathilde Jalving, Christianne A.R. Lok, Ingrid A. Boere, Maaike A.P.C. van Ham, Petronella B. Ottevanger, Anneke M. Westermann, Constantijne H. Mom, Roy I. Lalisang, Sandrina Lambrechts, Ruud L.M. Bekkers, Jurgen M.J. Piek

* Dual first author

Submitted

Abstract

Objective

Ovarian cancer is the fifth cause of cancer-related death among women. The benefit of targeted therapy for ovarian cancer patients is limited even if treatment is stratified by molecular signature. There remains a high unmet need for alternative diagnostics that better predict targeted therapy, as current diagnostics are generally inaccurate predictors. Quantitative assessment of functional signal transduction pathway (STP) activity from mRNA measurements of target genes is an alternative approach. Therefore, we aim to identify aberrantly activated STPs in tumour tissue of patients with recurrent ovarian cancer and start *phenotype*-guided targeted therapy to improve survival without compromising quality of life.

Study design

Patients with recurrent ovarian cancer and either 1) have platinum-resistant disease, 2) refrain from standard therapy or 3) are asymptomatic and not yet eligible for standard therapy will be included in this multi-centre prospective cohort study with multiple stepwise executed treatment arms. Targeted therapy will be available for patients with aberrantly high functional activity of the oestrogen receptor, androgen receptor, phosphoinositide 3-kinase or Hedgehog STP. The primary endpoint of this study is the progression-free survival (PFS) ratio (PFS2/PFS1 ratio) according to RECIST 1.1 determined by the PFS on matched targeted therapy (PFS2) compared to PFS on prior therapy (PFS1). Secondary endpoints include among others best overall response, overall survival, side effects, health-related quality of life and cost-effectiveness.

Ethics and dissemination

Ethical approval is obtained from the Medical research Ethics Committees United (MEC-U) and Central Committee on Human Research (CCMO). All participants will be informed about the study and will provide written informed consent before enrolment. Results will be disseminated through peer-reviewed journals and conferences.

Conclusion

The results of this study will show the clinical applicability of STP activity in selecting recurrent ovarian cancer patients for effective therapies.

Trial registration number

NL2020-005091-36 (EudraCT) and NCT03458221 (ClinicalTrials.gov).

Introduction

Ovarian cancer is the fifth leading cause of cancer death among women in the Western world.[1] The majority of the patients is diagnosed with advanced stage disease, which requires extensive cytoreductive surgery in combination with platinum and paclitaxel containing chemotherapy.[2] Despite this extensive treatment, recurrent disease almost invariably occurs resulting in a five-year survival rate ranging between 20-41%[3], which has not changed significantly over the last decades.[4] Recurrent ovarian cancer could be treated with second-/third-line chemotherapy and targeted therapies, such as poly(ADP-ribose) polymerase (PARP) inhibitors.[2] Although other targeted therapy options are rapidly expanding[5], response rates often fall short of expectations.[6] This can be the result of suboptimal selection based on inadequate molecular diagnostics. Therefore, there is an urgent need to improve patient stratification methods to allocate patients with ovarian cancer to effective targeted therapies and prevent unnecessary side effects of ineffective therapies.

Tumour cell proliferation, differentiation and migration is often driven by aberrant cellular activity of signal transduction pathways (STPs).[7] STPs can be categorized as nuclear receptor pathways (e.g. oestrogen receptor (ER) and androgen receptor (AR)), developmental pathways (e.g. Hedgehog (HH), transforming growth factor beta (TGF-β), Notch), and highly complex growth factor regulated pathways (e.g. phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK)). During the past decades, our knowledge on the complex mechanisms of action of these STPs has expanded and studies demonstrated associations between aberrant STP activity and ovarian carcinogenesis.[8-14]

The introduction of whole genome sequencing technologies enabled the identification of genomic alterations associated with these STPs in tumour samples. Unfortunately, the implementation of genomic-based targeted therapy has not been as successful as initially expected.[15] Although a proportion of patients in molecular profiling studies demonstrates clinical benefit, the majority of these carefully selected patients lack response to costly targeted drugs.[16,17] These findings suggest that we have overestimated the contribution of the cancers' *genotype* in predicting therapy response, but it could be questioned if the sole presence of gene alterations (e.g. gene mutations or amplifications) is sufficient to provide information on the functional activation status of STPs. Until, now the *functional phenotype* of tumour cells that is influenced by other factors, such as the tumour microenvironment is often disregarded.

In recognition of the significance of the cancer's *phenotype*, an alternative approach to assess STP activity was developed using Bayesian computational network models.[18-23] The models infer STP activity from mRNA measurements of well-validated direct target

genes of the transcription factor complex associated with the respective STP from paraffin-embedded tumour samples. Until now, models have been developed for the ER, AR, HH, TGF-β, Notch, PI3K, and MAPK pathways. While current approaches focus on a single molecular trait, these models use expression levels of several pathway-specific target genes and are therefore thought to be a more precise way to interpret functional STP activity. Previous studies including other tumour types, such as breast and salivary duct cancer, suggested the mRNA-based assay to be of value in the selection of patients for targeted therapy based on activated STPs.[24-26]

Our research group investigated STP activity in several subtypes of ovarian cancer to identify which STPs could be potential targets for personalized medicine.[27,28] Furthermore, we determined STP activity in morphologically normal Fallopian tube epithelium (FTE) of healthy women, the considered tissue of origin of most high-grade serous carcinoma (HGSC), to establish normal STP activity as a reference.[29] In the current study, we aim to determine actionable tumour-promoting STPs in tumour samples of recurrent ovarian cancer patients based on these results and implement *phenotype*-guided targeted therapy to improve survival without compromising quality of life. Therefore, we propose a prospective, parallel-group cohort study involving patients with recurrent ovarian cancer to be treated with approved targeted drugs if identified with an aberrantly activated STP.

Methods

Study design

The Signal TrAnsduction Pathway activity analysis for OVarian cancER treatment (STAPOVER) study is an investigator-initiated, multi-centre, prospective, parallel-group cohort study with stepwise executed treatment arms. Patients with platinum-resistant recurrent ovarian cancer, patients with recurrent ovarian cancer who refrain from standard therapy and patients with recurrent ovarian cancer who are not yet eligible for standard palliative chemotherapy due to asymptomatic disease, will be included to receive targeted therapy based on aberrantly active STPs, identified by the STP activity assay. In the individual treatment arms, a modified version (three-stage) of Simon's two-stage design is incorporated (**Figure 8.1**).[30]

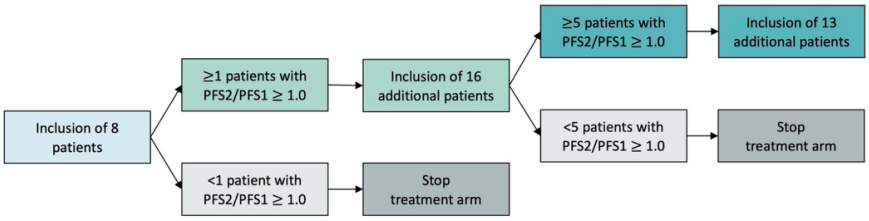

Figure 8.1 Flowchart displaying the three-stage design for inclusion of patients per treatment arm. PFS, progression-free survival.

Eligibility criteria

Adult women (age ≥18 years) diagnosed with recurrent ovarian cancer of any histological subtype who either 1. have platinum-resistant disease, defined as disease recurrence or progression within six months of last platinum-based chemotherapy (e.g. cisplatin or carboplatin) 2. refrain from standard therapy or 3. are asymptomatic and not yet eligible for standard therapy with an increased CA125 tumour marker of a nadir above 35 U/ml for at least 28 days, are eligible for enrolment when the following inclusion criteria are met:

- Progressive disease after at least one prior line of systemic treatment for recurrent disease.
- Radiologically evaluable disease according to RECIST 1.1 criteria.[31]
- Ability and willingness to provide a tumour biopsy after the last course of standard treatment and before start of the study.
- Ability and willingness to provide written and oral consent.
- Able to speak and understand the Dutch language.
- WHO performance status 0-II.
- Adequate renal and liver function to start matched targeted therapy (according to the local clinician).
- Adequate use of contraceptives in case of patients with childbearing potential.

Women are not eligible to participate in the study in case any of the following exclusion criteria are met:

- Patient is receiving any other anti-cancer therapy or is chemotherapy naïve. The required wash out period prior to start of matched targeted therapy is at least three weeks.
- Patient is diagnosed with or treated for a second primary tumour (except non-melanoma skin tumour) one year prior to study inclusion.
- Inability to obtain (sufficient) tumour material.
- Previous use of the selected targeted drug as anti-cancer agent.
- Pregnant or lactating women.
- Simultaneous participation in another treatment-related clinical trial.
- Patients with any other clinically significant medical condition which, in the opinion of the local clinician, makes it undesirable for the patient to participate in this study or which could jeopardize compliance with study requirements.

Study procedures

Informed consent will be obtained from the patients who are willing to participate and meeting the inclusion criteria. Subsequently, one histological biopsy will be collected to obtain tumour tissue for the STP activity assay. Patients are eligible for treatment with matched targeted therapy if an aberrantly activated STP is observed.

Women with high activity of the ER, AR, PI3K or HH pathway will be included for treatment with either letrozole (arm A), bicalutamide (arm B), or itraconazole (arm C and D) (**Figure 8.2**). These targeted drugs have been studied in ovarian cancer patients with acceptable side effects. Patients who are not eligible to participate in the study will continue to receive standard of care by choice of the treating clinician. These patients are asked for consent to remain on study to complete questionnaires at baseline, every 12 weeks during the next course of treatment and 12 weeks after the end of treatment. Questionnaires are used for secondary analyses, such as side effects, quality of life and cost-effectiveness.

Tumour sampling

Tumour tissue will be obtained from at least one tumour site through a biopsy guided by ultrasound or CT-scan, or through a (pre-planned) cytoreductive surgical procedure. In case of multiple tumour sites, a tumour site based on accessibility and safety risks will be selected. Successfully obtained tumour tissue will be formalin-fixed paraffin-embedded (FFPE) by the hospital's histopathological laboratory. In case biopsy fails, e.g. because of insufficient tumour tissue or no accessible tumour site, the patient will be excluded from the study.

Preparation of tumour samples

In addition to standard diagnostics, for the STAPOVER study at least 20 sections of 5 µm will be cut from the FFPE sample with a microtome. The last study slide will be haematoxylin and eosin (H&E) stained. The H&E-stained slide will be revised by a local pathologist to annotate a tumour area containing the highest amount of tumour cell nuclei (≥40%).

The coordinating centre will provide a study number for each individual patient to label the study slides before they will be transported to InnoSIGN for STP activity assay. The remainder of the FFPE samples and slides for standard diagnostics will be archived in the participating centre. If sufficient study slides have been obtained, several slides will be archived by the coordinating centre for optional analysis (such as immunohistochemical biomarker analysis and DNA/RNA sequencing).

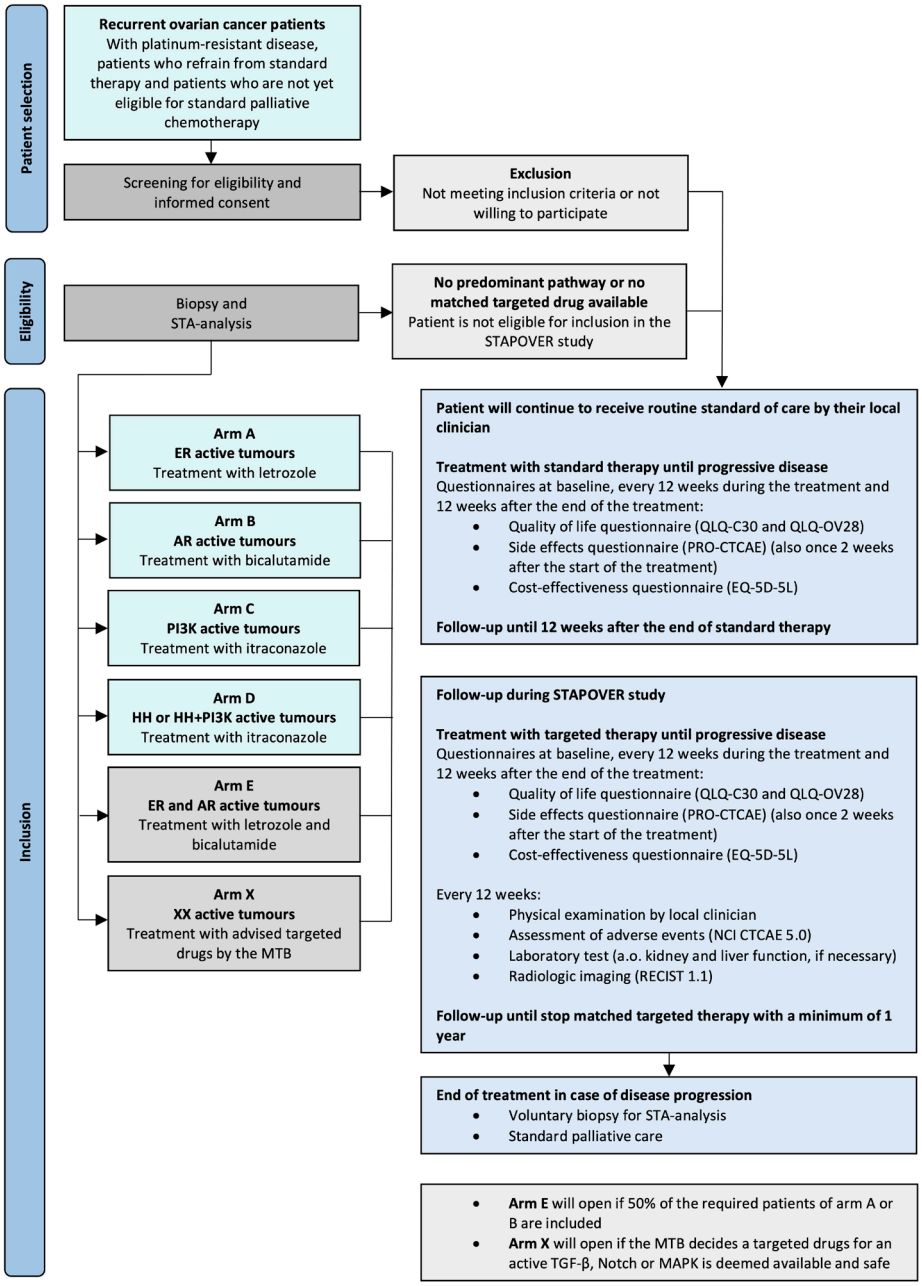

Figure 8.2 Flowchart of the study design. STP, signal transduction pathway; ER, oestrogen receptor; AR, androgen receptor; HH, Hedgehog; PI3K, phosphoinositide 3-kinase; TGF-β, transforming growth factor beta; MAPK, mitogen activated protein kinase.

STP activity assay at InnoSIGN laboratory

Using the annotated H&E slide as reference, tumour cells will be manually scraped off the study slides for the STP activity assay. Next, mRNA extraction will be performed according to Qiagen standard protocol. mRNA concentrations will be measured before real-time quantitative reverse transcription-PCR (RT-qPCR) is performed to confirm sufficient mRNA input for the assay.

STP activity assay provides a functional pathway activity score defined on a scale from 0 to 100, where the measured range of pathway activity may vary with the analysed tissue type. A score of 0 corresponds with the lowest odds of an active pathway and conversely 100 corresponds with the highest odds. Individual STP activity assay results will be presented and analysed in a graph as shown in **Figure 8.3**. This format contains the STP activity distributions measured in previous cohorts of normal FTE (blue curve), HGSC samples (red curve) and low-grade serous ovarian carcinoma (LGSC) samples (purple curve).[28,29,32] For each measured STP, data is displayed in a single graph. The black dotted line represents the cut-off value for high STP activity, which is defined as two standard deviations above the mean value of STP activity measured in FTE samples. Before the start of the study, cut-off values will be updated to values in which the most recent results of the STP activity of the normal FTE are included. The individual STP activity measured in the patient's tumour sample will be plotted in the format. In case the individual STP activity transcends the black dotted line, the STP will be classified as aberrantly activated. STP activity results will be sent to the principal investigators, who share the results with the multidisciplinary tumour board (MTB).

Multidisciplinary tumour board

The nationwide MTB will consist of gynaecological oncologists, medical oncologists and a translational researcher (trial manager). The MTB will decide on matched targeted therapy based on the STP activity assay results, their scientific experience and the available literature. STP activity results will be presented to the MTB in a standard format (**Figure 8.3**). To aid the decision in case of multiple aberrantly activated STPs, predefined questions need to be answered through a pre-developed selection form. If the MTB decides the patient is eligible for matched targeted therapy, treatment will be initiated by the local clinician within 28 days. In case no aberrantly activated pathway is identified, the patient will receive standard of care.

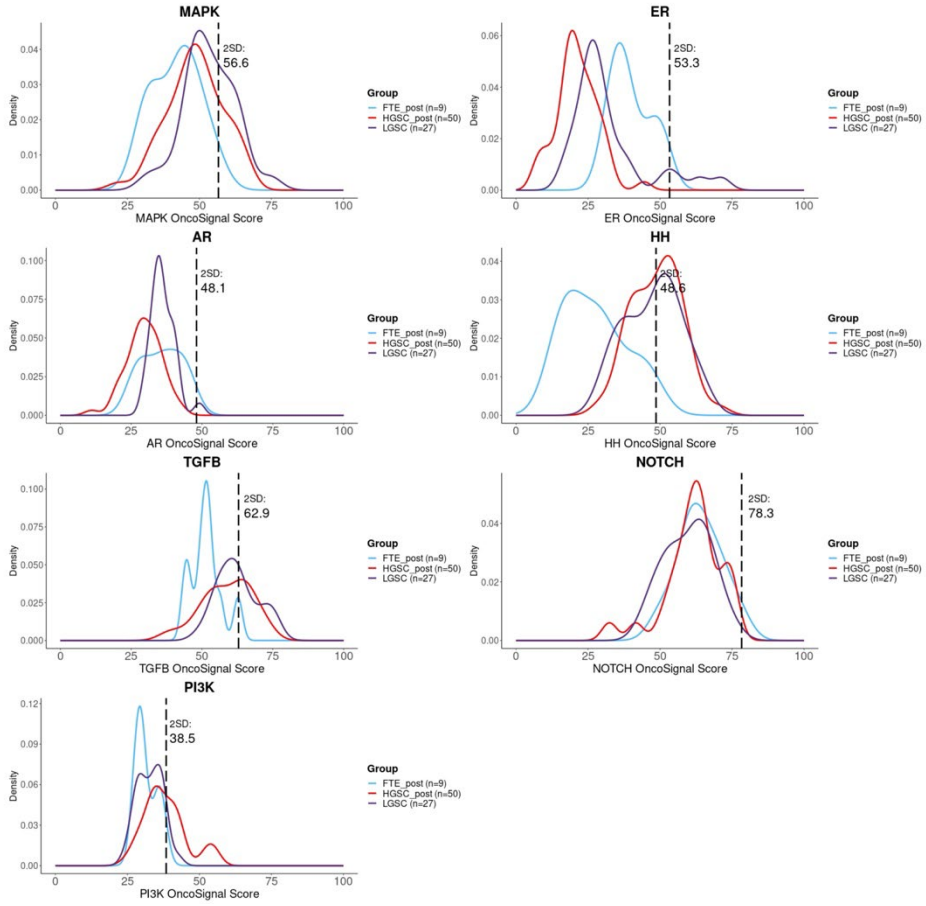

Figure 8.3 Example of the standard format for signal transduction pathway (STP) activity assay data presentation to the multidisciplinary tumour board. The figure shows curves of the STP activity of a cohort high-grade serous ovarian carcinoma (HGSC, n=50) in red, a cohort of low-grade serous ovarian carcinoma (LGSC, n=27) in purple and a cohort of Fallopian tube epithelium samples of healthy postmenopausal women (FTE_post, n=9) in blue, for each of the seven measured STPs. The STP specific thresholds are defined as two standard deviations above the mean value of the STP activity measured in Fallopian tube epithelium samples (n=9). MAPK, mitogen activated protein kinase; ER, oestrogen receptor; AR, androgen receptor; HH, Hedgehog; TGFB, transforming growth factor beta; PI3K, phosphoinositide 3-kinase.

Interventions

Women diagnosed with tumours with aberrantly high activity of the ER, AR, PI3K or HH STP, will receive the following treatment with the recommended dosage (**Figure 8.2**):

- Treatment arm A (ER active tumours) with aromatase-inhibitor letrozole 2.5 mg daily.
- Treatment arm B (AR active tumours) with anti-androgen bicalutamide 150 mg daily.
- Treatment arm C (PI3K active tumours) with anti-fungal agent itraconazole 300 mg twice daily.
- Treatment arm D (HH or concurrent HH and PI3K active tumours) with anti-fungal agent itraconazole 300 mg twice daily.

These targeted drugs are approved for clinical use and available in the Netherlands but will be used outside their therapeutic indications during this study. A combination of two drugs targeting the ER and AR pathway is allowed if advised by the MTB (arm E) (**Figure 8.2**).

Other STPs (TGF-β, Notch and MAPK) may be targeted during the study period by other available drugs, provided there is sufficient evidence for its use and safety. If deemed beneficial, the MTB could decide to open a new treatment arm (arm X) (**Figure 8.2**).

Pre-treatment evaluation before start of matched targeted therapy

Before the start of the recommended matched targeted therapy, patients will undergo a pre-treatment evaluation by their treating clinician. Baseline characteristics will be registered in the electronic case report form (eCRF). Furthermore, physical examination will be performed and the treating clinician will determine if any safety concerns exist. The treating clinician will assess if the patient has adequate renal and kidney function and checks for interactions between the matched targeted therapy and concomitant medication. Imaging of the tumour and evaluation by the RECIST 1.1 criteria of the tumour will be performed at baseline.[31]

Follow-up during matched targeted therapy

The treating clinician will be responsible for adequate follow-up during treatment with matched targeted therapy. Response will be evaluated at least every 12 weeks by imaging of the tumour according to the RECIST 1.1 criteria.[31] Additionally, physical examination and assessment of liver and kidney function are performed. Adverse events are evaluated according to the NCI CTCAE 5.0.

Both patients using matched targeted therapy, as well as patients who were not eligible to participate in the study and continued to receive standard of care, will be asked to complete the questionnaires to investigate health-related quality of life (QLQ-C30 and QLQ-OV28)[33], side effects (PRO-CTCAE) and cost-effectiveness (EQ-5D-5L)[34] at baseline, every 12 weeks during treatment and 12 weeks after the end of the treatment.

End of treatment

In case of disease progression, patients will be withdrawn from the study and treatment with matched targeted therapy will be ended. After disease progression, it is not possible to be included in the study for a second time; patients will receive standard (palliative) care. Patients will be asked to undergo a voluntary second biopsy to determine STP activity for research purposes after treatment has ended and before other systemic treatment has started.

Results

Outcome measures

The primary objective of this study is the progression-free survival (PFS) ratio (PFS2/PFS1 ratio) defined by the PFS on matched targeted therapy by STP activity assay (PFS2) in comparison to the PFS recorded on the therapy administered immediately prior to enrolment (PFS1).[31]

Secondary outcome measures include:

- Proportion of patients with an actionable active STP for which targeted therapy is recommended in relation to the number of patients who underwent a biopsy.
- Proportion of patients who receive matched targeted therapy in relation to the number of patients included in each study arm.
- Best overall response defined by RECIST 1.1 criteria based on radiological imaging.[31]
- One-year survival defined as the time from start matched targeted therapy till death or the end of the one-year follow-up period.
- Overall survival defined as the time from start matched targeted therapy till death.
- Predictive value of STP activity assay results (and if available, immunohistochemical biomarker analysis and DNA/RNA sequencing results) on matched targeted therapy response.
- Change in STP activity score after disease progression (if available) compared to STP activity score before start of matched targeted therapy.
- Side effects according to PRO-CTCAE™.

- Health-related quality of life scored using standardized questionnaires from the EORTC (QLQ-C30 and QLQ-OV28).[33]
- Cost-effectiveness will be calculated with health state utilities assessed in the standardized EuroQol 5D (EQ-5D-5L) questionnaire.[34]

Sample size

In this study, the PFS2/PFS1 ratio will be used as primary outcome measure, in which the correlation of two consecutive lines of treatment allows the patient to serve as her own control and compensates for heterogeneity in patient characteristics and tumour histology. Two prior prospective studies used the PFS2/PFS1 ratio to measure treatment response on targeted therapies based on molecular profiling in patients with advanced cancers.[17,35] Both studies deemed a PFS2/PFS1 ratio ≥1.3 to clinically beneficial. Their null hypothesis stated that ≤15% of the patient population would have a PFS2/PFS1 ratio of 1.3 on standard therapy. In order to reject the null hypothesis, the authors assumed that the true proportion of patients with a PFS2/PFS1 ratio ≥1.3 in the studies would be equal to 30%[35] and 24%.[17]

Based on these results, we calculated that 37 evaluable patients are required per treatment arm (total of 148 patients for treatment arm A, B, C and D) to reject the null hypothesis of an estimated 15% of patients achieving PFS2/PFS1 ratio ≥1.3 on standard palliative therapy with 80% power at a one-sided significance level of 0.10.

Per stepwise executed treatment arm, interim analyses will be performed after the inclusion of the first eight and 24 patients (**Figure 8.1**).[30] In case none of the first eight patients achieve PFS2/PFS1 ≥1.0, the treatment arm will be closed due to lack of efficacy. The treatment arm will be complemented by 16 additional patients if at least one of the first eight patients achieves PFS2/PFS1 ≥1.0. In case at least five of the enrolled 24 patients achieve PFS2/PFS1 ≥1.0, the treatment arm is considered a potentially successful treatment and may proceed enrolment until 37 patients have been included. If four or less patients achieve PFS2/PFS1 ≥1.0, the treatment arm will be closed due to insufficient efficacy. For this monitoring rule, we choose the threshold of 1.0 to represent sufficient patient benefit to continue patient accrual. After closing a treatment arm, it is allowed to open a new treatment arm targeting the same STP with a new targeted drug, if this is deemed beneficial by the MTB.

Patients who withdraw from the study before start of matched targeted therapy will be replaced until a total of 37 patients are available for response measurements. If a patient withdraws from the study after the start of matched targeted therapy, the patient will not be replaced and will be included in the analysis.

Our research group investigated STP activity in previous cohorts of women with HGSC and LGSC.[28,29,32] Based on our observations in these studies, we expect to be able to recruit sufficient number of patients for study arm A, B, C and D in five years, in a total of six participating centres in the Netherlands.

Plan of analysis

The proportion of patients who achieved a PFS2/PFS1 ratio ≥1.3 will be calculated from the total of patients that received matched targeted therapy. The primary endpoint will be tested by a one-sided exact test. Since histological subtype is a prognostic factor for PFS, subgroup analysis will be performed in case of adequate numbers.[36] Planned statistical analysis for the secondary outcome measures include: the Kaplan-Meier survival curves tested with log-rank tests and Cox proportional hazard models for survival analysis. Furthermore, the predictive value of STP activity assay will be determined by correlations between STP activity scores and PFS2 using the Pearson or Spearman correlation coefficient. If patients undergo a voluntary second post-treatment biopsy, change in STP activity score will be calculated for each respective STP compared to the STP scores from the pre-treatment biopsy.

Using the appropriate algorithms, the side effects and health-related quality of life questionnaires will be analysed and compared accordingly between the group of patients that received matched targeted therapy and patients that received standard therapy. Together with the PFS, these results will be used in a quality adjusted life year calculation in order to calculate the cost-effectiveness and cost-utility ratios of matched targeted therapy. In addition, subgroup analysis per treatment arm will be conducted.

Descriptive statistics will be used to describe patient characteristics. Categorical variables will be expressed as a number with the percentage of the total study group. The chi-squared test or Fisher's exact test will be used to analyse associations between categorical variables. Continuous variables will be presented as mean with standard deviation or as median with interquartile range, whenever appropriate. A T-test or Mann-Whitney U test will be used to analyse associations between continuous variables.

Ethics and dissemination

The study will be conducted according to the principles of the Declaration of Helsinki (amended by the 68th WMA General Assembly, Chicago, United States, October 2017). All researchers involved in the study will follow the Dutch Medical Research Involving Human Subjects Act and 'Code Goed Gebruik' (Federa 2002, amended in 2011). Ethical approval for the study is obtained from the Medical research Ethics

Committees United (MEC-U, study number R21.033) and Central Committee on Human Research (CCMO, study number NL77022.100.21). The study has been registered with the European Union Drug Regulating Authorities Clinical Trials (EudraCT) database under reference number NL2020-005091-36 and with ClinicalTrials.gov under reference number NCT03458221.

All participants will be informed on the purpose, procedure, duration and possible risks and benefits of the study. Participants need to provide written informed consent prior to study inclusion and, in case a matched targeted drug is available and recommended, prior to the start of therapy. Participants can withdraw consent to participate at any time for any reason and without any consequences.

All data will be handled strictly confidential and will be collected using an eCRF in the secured database Research Manager (https://my-researchmanager.com). Data and tumour tissues will be coded with a random study number and will be stored for at least 15 years after the end of the study.

Study results of individual cohorts or pooled data will be offered to international peer-reviewed journals for publication and presented at conferences. Publication will not disclose the identity of the participants.

Conclusion

This is the first study to evaluate the clinical implementation of *phenotype*-guided targeted therapy based on STP activity in tumour tissue of patients with recurrent ovarian cancer. Patients are treated with existing targeted drugs with tolerable toxicity profiles to investigate whether these drugs have therapeutic value beyond their approved indications. The unique study design allows for simultaneous screening for several promising treatment strategies and rapid incorporation of new cohorts based on other (off-label) targeted drugs or STPs. Intra-individual comparison of treatment response through the PFS ratio will limit confounders such as tumour histology. The results of this study will show the clinical applicability of STP activity to match recurrent ovarian cancer patients to targeted therapies.

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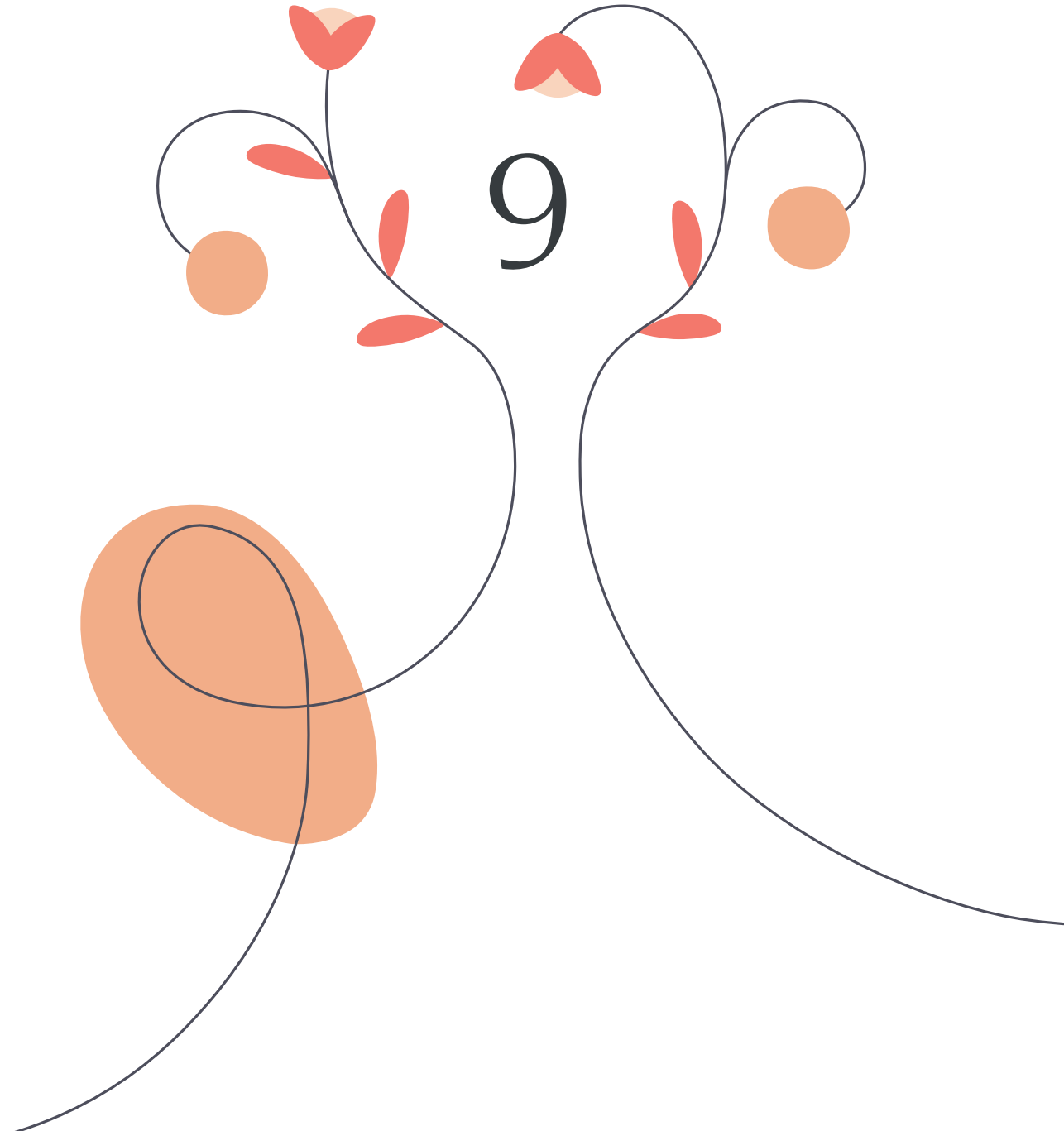
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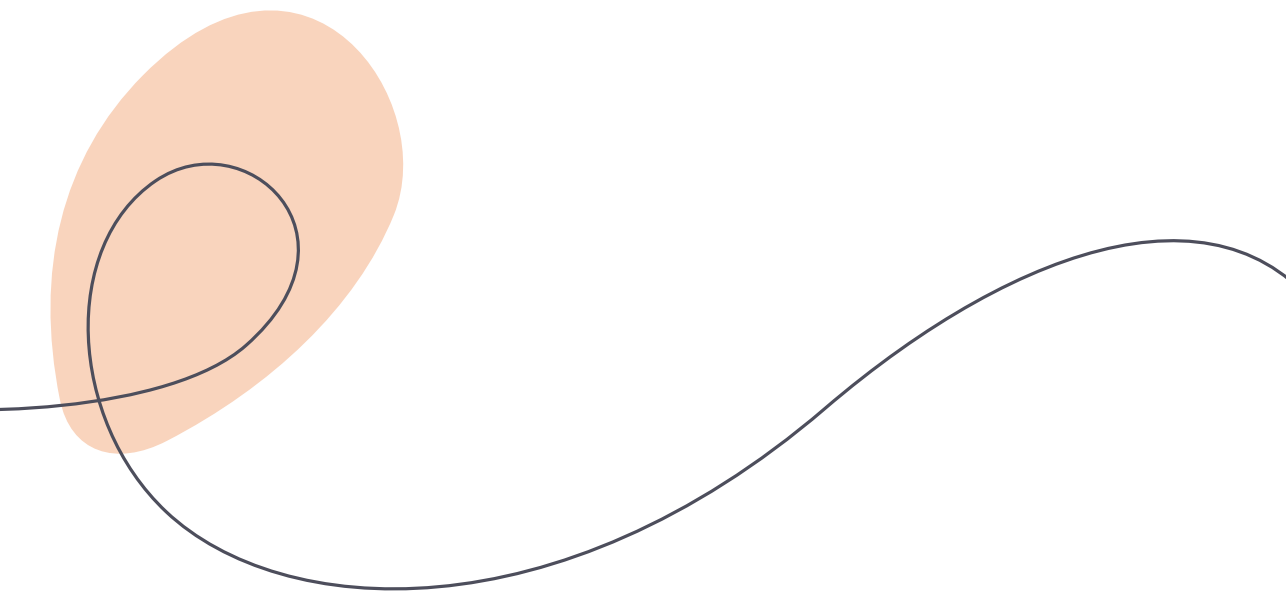
CHAPTER 9

General discussion and future perspectives



General discussion

Background

Approximately 314,000 women were diagnosed with ovarian cancer worldwide in 2020 alone.[1] Nearly 90% of ovarian cancers are of epithelial origin and among them high-grade serous carcinoma (HGSC) are by far the most common histological subtype.[2] Despite aggressive treatment with debulking surgery and chemotherapy, more than 80% of women will develop relapsed disease which eventually will become resistant to chemotherapy, resulting in a poor prognosis.[3] Unfortunately, survival rates have not significantly improved over the last decades, indicating the high need for new therapeutic approaches.[4]

The use of whole genome sequencing techniques has broadened our view of the molecular biology of ovarian cancer and has led to the introduction of personalized medicine.[5] This approach aims to provide a tailored treatment for individual patients based on tumour characteristics such as gene mutations. In general, gene mutations affect protein synthesis and regulation, and therefore might cause dysregulation of signalling transduction pathways (STPs).[6] Naturally, STPs are tightly controlled mechanisms involved in important functions of the body including the female genital tract.[7,8] Aberrant activity of STPs can result in abnormal cell proliferation, and therefore drive tumour growth and metastasis.

The shift towards therapeutic stratification by the tumour's genomic profile stimulated the development of numerous targeted drugs, designed to target specific receptors or proteins of a STP to alter pathway activity and inhibit tumour growth. For successful implementation of personalized medicine, reliable biomarkers are needed to determine the tumour-driving STP and enable accurate patient selection for treatment with a STP-specific drug. Currently, gene mutations and protein expression by immunohistochemistry are used to predict response to targeted drugs.[9] Unfortunately, results of genotype- and immunohistochemistry-guided treatments often fall short of expectations as only a subset of patients gain clinical benefit, suggesting that current diagnostics are not sufficiently reliable to allocate patients to effective therapies. These unsatisfactory results could be attributed to the fact that presence of gene mutations or signalling proteins may not always translate into transcriptional activation of a STP. Moreover, current approaches focus on a single molecular trait and disregard the functional phenotype of tumour cells, including interactions with the microenvironment.

To enable the measurement of functional STP activity, assays have been developed to determine transcriptional activation of a STP.[10-15] These assays use Bayesian

network models, which are probabilistic models calculating probabilities based on defined relationships between variables. The assays quantitatively assess the odds of an activated STP-specific transcription factor complex from mRNA levels of direct pathway-specific target genes. Functional STP activity is presented on a normalized scale from 0 to 100, where 0 indicates the lowest odds and 100 the highest odds of an active pathway (**Figure 1.2**). Depending on the STP and tissue type, the activity range may be restricted to a certain part of the scale. Given the assessment of functional STP activity, clinical application of these assays has the potential to determine actionable tumour-promoting STPs and enable the implementation of *phenotype*-guided targeted therapy for (ovarian) cancer patients.

The research presented in this thesis aims to unravel the role of important STPs that drive development and progression of HGSC, as this histological subtype represents the most common ovarian cancer type. We focus on the role of the hormone driven pathways androgen receptor (AR) and oestrogen receptor (ER), the growth factor pathway phosphoinositide 3-kinase (PI3K) and the developmental pathways Hedgehog (HH), transforming growth factor beta (TGF-β) and canonical wingless-type MMTV integration site (Wnt), as these STPs were previously associated with ovarian carcinogenesis.[16-21] Furthermore, we explore the clinical benefit of possible targeted treatment options and investigate the predictive value of current biomarkers on therapy response. Ultimately, we provide insight into the use of STP activity assays for stratification of HGSC patients in order to direct treatment to more effective personalized treatment strategies. This chapter presents the main findings of this thesis in a larger perspective and discusses the implications for clinical practice.

Classification of normal signal transduction pathway activity

The current prevalent theory on high-grade serous carcinogenesis points towards the tubal fimbrial epithelium as cell of origin of most HGSC.[22,23] Although the tubal origin has gained increasing support over the last two decades, the molecular biology of the Fallopian tube epithelium (FTE) and the implication for carcinogenesis remain poorly understood. Moreover, in comparison to the well-known influence of the hormonal cycle on the endometrium, less is known about cyclic changes in the fimbrial epithelium. In an effort to identify physiological fluctuation in STP activity during the hormonal cycle, we assessed STP activity in morphologically normal fimbrial epithelium of pre- (n=17) and postmenopausal (n=8) women who had surgical interventions for benign gynaecological conditions. In **Chapter 2**, we observed gradual differences in AR, ER, PI3K, HH and Wnt pathway activity, indicating that the STP activity in FTE is influenced by the hormonal cycle.

There is compelling evidence that steroid hormones mediate cyclic changes in the morphological structure of the Fallopian tube.[24-26] As described in **Chapter 2**, our

results showed simultaneous peak activities of the hormone driven AR and ER pathways during the early luteal phase, suggesting that the FTE is more likely to be stimulated by local paracrine hormone signalling caused by high hormone levels in follicular fluid released during oocyte expulsion than by endocrine signalling (**Figure 2.2**). Interestingly, despite the depletion of ovarian function, activity of the hormone driven pathways is preserved in postmenopausal women, indicating the importance of these pathways to sustain tissue homeostasis. Beyond menopause, hormonal signalling in FTE might be stimulated by extragonadal steroid biosynthesis in peripheral tissues.[27] However, extragonadal produced hormones are thought to be metabolized locally with limited systemic effects.[28] Alternatively, our findings might suggest that the FTE is able to actively maintain intracellular hormonal signalling by intracrine hormone production. Although distinct proof for this hypothesis is currently lacking, there is some evidence of alternative conversion of oestrogen precursors in FTE, indicating an interesting area for further investigation.[29]

Given the central role in controlling cell growth, survival and metabolism, activated PI3K signalling is commonly observed in human cancers.[30] In **Chapter 2** we found higher activity of the PI3K pathway in FTE during the follicular phase when compared to the luteal phase, resulting in a considerable range of normal PI3K activity (**Figure 2.2**). An important stage-specific function for PI3K signalling was previously reported in the ovary during follicle development.[8] In mice, overactivation of PI3K signalling resulted in excessive follicle activation and depletion, indicating this pathway as a critical regulator of ovarian function.[31] In line with the association between PI3K signalling and endometrial cell differentiation and motility, the distinct increase in PI3K signalling during the follicular phase and decrease during the luteal phase might have essential roles in the regulation of ciliary expression and deciliation.[32] Consequently, the tight regulation of (in)activation of PI3K signalling in FTE may also provide an opportunity for aberrations to maintain the proliferative state.

With regard to the HH, TGF-β and Wnt signalling pathways, the differences in STP activity during the hormonal cycle were less marked (**Figure 2.2**). However, even subtle fluctuations, which might have lacked statistical significance due to small sample number, might still be of biological importance. While the functional relevance of these STPs remains to be addressed, these pathways could play a role in tubal morphology changes. Both HH and Wnt signalling are involved in many developmental processes in embryonic and adult tissues, as well as regulation of stem cell proliferation and differentiation.[33,34] In addition, TGF-β signalling is implicated in a wide variety of cellular processes, including immune regulation.[35] TGF-β activity could even have opposite effects depending on the cell type and cellular context. Given the important roles of the HH, TGF-β and Wnt signalling pathways it is

likely to assume that the conserved activity of these pathways is essential to maintain homeostasis in the Fallopian tube.

To conclude, in **Chapter 2** we presented the range of normal activity for the AR, ER, PI3K, HH, TGF-β and Wnt pathways in morphologically normal Fallopian tubes. These data enable us to discriminate between normal and aberrant STP activity, as the absolute values determined in future samples could directly be interpreted against these reference values in relation to the hormonal cycle. Although the range of normal STP activity needs to be validated with a larger set of samples, the results create the opportunity to define abnormalities in signalling activity and thereby indicate tumour-promoting pathways for clinical application. However, when using the normal range as benchmark to characterize abnormal activity in diseased cells, the patient's hormonal status needs to be taken in consideration as our results demonstrated STP specific ranges for pre- and postmenopausal women.

Molecular mechanisms involved in high-grade serous carcinoma evolution

Besides information on the extent of abnormal signalling activity (e.g. up- or downregulation), insight into whether a pathway is likely to be tumour-driving is important to unravel carcinogenesis and to help guide the decision on targeted therapy. The identification of serous tubal intraepithelial carcinoma (STIC) as precursor lesions of most HGSC enables the study of HGSC evolution.[22,23] To distinguish between STPs that may be potential drivers of the initial development of HGSC precursor lesions and STPs involved in subsequent progression into HGSC, we determined STP activity in STIC (n=8) and concurrent HGSC (n=7) and compared this to STP activity in normal FTE from postmenopausal women (n=8). In **Chapter 3**, we concluded that decreased ER pathway activity and increased PI3K and HH pathway activity are aberrations that may contribute to the processes related to the transformation of FTE into STIC, because both STIC and concurrent HGSC demonstrate significant lower ER and higher PI3K and HH pathway activity in comparison to normal FTE.

We hypothesize that the cyclic activation of ER, PI3K and HH signalling observed in FTE during the menstrual cycle might affect the susceptibility to molecular changes, which could give cells selective advantage for continuous proliferation and dysplastic transformation. Although most HGSC patients are diagnosed after menopause with a mean age at diagnosis of 62 years,[36] previous studies concluded that the time between progression from STIC into HGSC may take up to seven years.[37,38] Moreover, it is estimated that the development of 'p53 signatures', the benign putative precursor lesions, into HGSC will take at least two decades.[38] Therefore, it is likely that the dysplastic transformation of normal FTE takes place during the premenopausal years.

Previously, the contribution of abnormalities in PI3K signalling in malignant transformation of FTE was confirmed in a mouse model.[39] Conditional deletion of the *BRCA1/2*, *TP53* and phosphatase and tensin homolog (*PTEN*) genes led to the development of precursor lesions and tumours with histologic and genomic features resembling STIC and HGSC.[39] PTEN is an important tumour suppressor which negatively regulates PI3K signalling (**Figure 7.1**). In line with these observations, another study reported 33% of the STIC to have complete loss of PTEN protein expression by immunohistochemistry, and in an additional 33% of the STIC heterogeneous PTEN protein expression was observed, while the remaining samples showed positive PTEN protein expression.[40] In addition, we observed loss of FOXO3a protein expression, another important tumour suppressor downstream of PI3K, in STIC and HGSC, which further supports the role of abnormal PI3K signalling in serous carcinogenesis (**Figure 3.2**).

Since mutations in the *TP53* gene cause accumulation of the p53 protein, which in turn is the hallmark of 'p53 signatures', *TP53* mutations are considered the earliest step in HGSC development.[37] In this context, our results raise the question whether downregulation of ER signalling and activation of PI3K and HH signalling are individual putative early drivers that coincident with *TP53* mutations or result from *TP53* gene mutations and occur in a stepwise manner during the emergence of STIC. The latter is more biologically plausible as a previous study indicated negative Stathmin 1 staining, a proposed immunohistochemical surrogate marker of PI3K signalling, in 'p53 signatures', but observed positive Stathmin 1 staining in 'proliferative p53 signatures', which are suggested to mark the transition from benign to proliferative lesions by both p53 and Ki-67 protein expression.[41] These findings suggest that activation of PI3K signalling initiates after loss of the *TP53* tumour suppressor function. Subsequently, crosstalk between PI3K and ER signalling might explain the decrease in ER pathway activity in STIC. An inverse interaction between PI3K and ER signalling has been observed in breast cancer, as activation of PI3K signalling resulted in reduced ER signalling.[42] Likewise, it is possible that abnormal PI3K signalling triggers increased HH pathway activity, because an interaction has been described in ovarian cancer cells previously.[43,44] Alternatively, studies revealed an interplay between p53 and HH signalling, in which p53 has an inhibitory effect on GLI1, a downstream effector of the HH pathway, and conversely, GLI1 was able to repress p53 signalling.[45,46] Taken together, aberrant ER, PI3K and HH pathway activity is likely to provide a selective advantage during the transformation of FTE into STIC. In **Figure 9.1** we show an overview of the STP activity during the emergence of STIC and HGSC from FTE and present potential relations between the STPs. However, the exact mechanisms and the interactions with p53 signalling remains to be elucidated in further research.

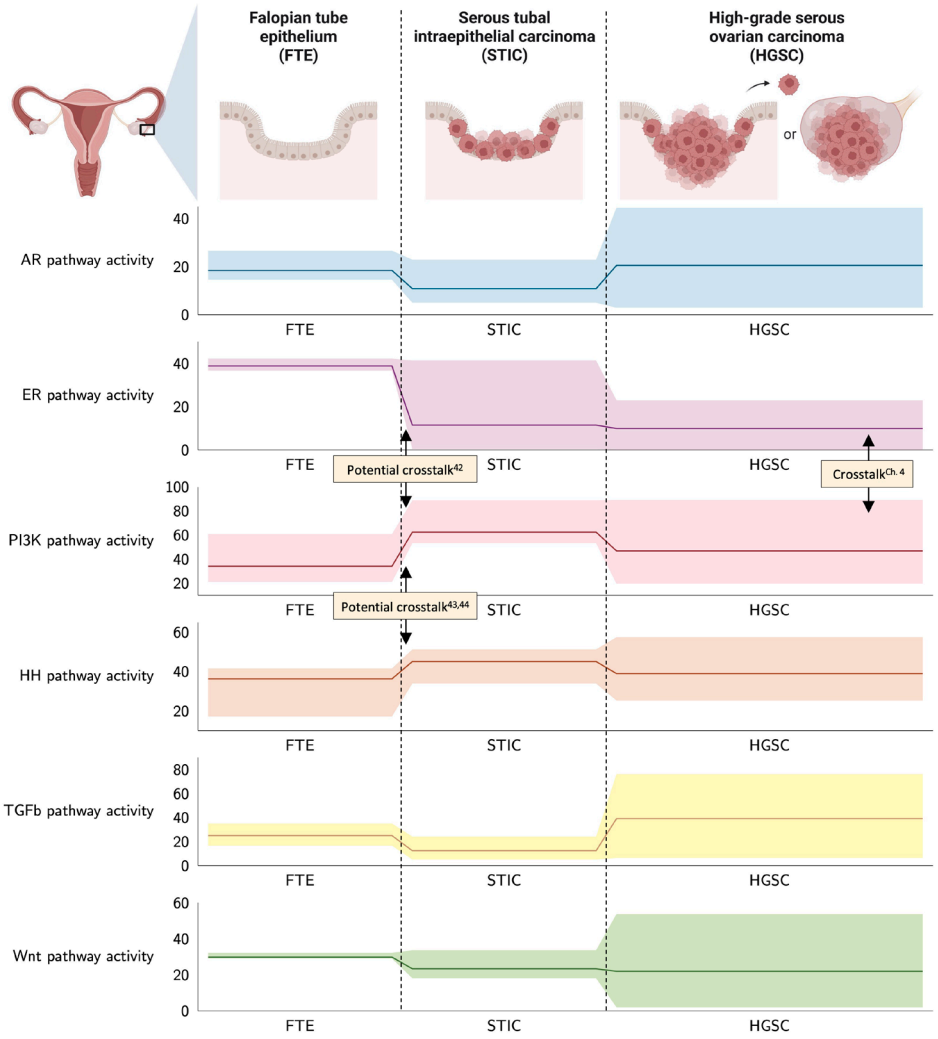


Figure 9.1 Overview of the changes in signal transduction pathway (STP) activity during the evolution of high-grade serous ovarian carcinoma (HGSC) starting in the Fallopian tube epithelium (FTE). The figure presents the STP activity measured in normal FTE from postmenopausal women (n=8), serous tubal intraepithelial carcinoma (STIC) from postmenopausal women (n=7) and HGSC from postmenopausal women (n=73) as described in **Chapter 2, 3 and 4** in this thesis. In each graph, the horizontal line presents the median STP activity per group. The coloured area indicates the upper and lower bound of the STP activity excluding the outliers. A sample is considered an outlier if the STP activity is more than 1.5 times the interquartile range from the first and third quartile. The potential interactions between signalling pathways are based on previous literature. Note that no conclusions can be drawn about the exact stepwise manner of dysregulation of pathway activity. AR; androgen receptor, ER; oestrogen receptor, PI3K; phosphoinositide 3-kinase, HH; hedgehog, TGF-*β;* transforming growth factor beta, Wnt; canonical wingless-type MMTV integration site (this figure is partially created with BioRender.com).

Furthermore, the results of **Chapter 3** suggested that loss of AR and Wnt pathway activity is more likely to be involved in subsequent neoplastic progression into HGSC rather than disease initiation, due to significantly lower AR and Wnt pathway activity in HGSC in comparison to FTE but relatively sustained AR and Wnt signalling in STIC (**Figure 3.1**). In **Figure 9.1**, we present the STP activity in STIC and a larger set of HGSC samples and noticed a subset of HGSC with higher AR and Wnt pathway activity than STIC. Interestingly, previous studies searched for associations between activation of the AR and Wnt pathways and HGSC progression, but inconsistent findings have been reported. *In vitro* studies investigating the role of androgens in ovarian cancer progression showed conflicting evidence.[47,48] Moreover, a large study including 2,603 HGSC samples demonstrated that a modest 36% of the samples showed positive AR protein expression (defined by AR protein expression in >1% tumour cell nuclei), suggesting a less prominent role for AR pathway activity in HGSC.[49] Regarding the Wnt pathway, several studies implicated a tumour-promoting role for this pathway in ovarian carcinogenesis.[50] For instance, hyperactivation of β-catenin and Wnt7a, both important signalling members of the Wnt pathway, was associated with HGSC progression.[51,52] However, results should be interpreted with caution as ovarian surface epithelium was used as comparative material. Contrary to these findings, a recent study demonstrated that the growth of HGSC organoids required a low-Wnt signalling environment, whereas Wnt pathway activation led to growth arrest.[53] Although the role of AR and Wnt pathway activity in HGSC progression may be more complex, our findings suggest that both loss and activation of AR and Wnt pathway activity may be tumour-promoting in HGSC.

Survival is related to oestrogen receptor pathway activity in postmenopausal women diagnosed with high-grade serous carcinoma

Most HGSC patients are diagnosed at advanced stage of disease, defined as tumour beyond the pelvis, often with distant metastases and/or pleural effusion.[54,55] Despite successful first-line treatment, more than 50% of the patients will develop relapse of disease within two years following diagnosis.[56] Nevertheless, a fraction of the patients experiences exceptional long-term survival, indicating a remarkable variability in biological behaviour.[57] We hypothesized that there might be possible relations between survival and STP activity, which could explain why some HGSC intrinsically behave more or less aggressive. Therefore, we assessed STP activity in women with advanced stage HGSC with a short (<12 months) and long (>24 months) disease-free survival in **Chapter 4.**

In total, we analysed STP activity in 85 primary tumour samples of advanced stage HGSC patients who achieved complete remission after first-line treatment with chemotherapy and debulking surgery. In this clearly defined patient population, we were unable to identify a single pathway responsible for the differences in survival

between patients with short- and long-term disease-free survival because there were no differences in median AR, ER, PI3K, HH, TGF-β and Wnt pathway activity between the groups (**Figure 4.1**). However, illustrative of the difference in intrinsic behaviour, we found a wide variety in STP activity among individual HGSC samples, which might indicate the existence of more specific subgroups, possibly in relation with other tumour-driving pathways. In a previously published study, analysis of STP activity in HGSC samples revealed two distinct groups with survival differences based on PI3K and nuclear factor-kappa B (NF-kB) pathway activity.[58] In this cohort, low PI3K and high NF-kB pathway activity was associated with a favourable prognosis.

In **Chapter 4**, stratification of advanced stage HGSC patients for menopausal status revealed a favourable relation between ER pathway activity and disease-free survival (hazard ratio=0.943, *P*=0.033) and overall survival (hazard ratio=0.930, *P*=0.041) in postmenopausal women, but not in premenopausal women (**Figure 4.2**). Further analysis showed that postmenopausal HGSC patients with the lowest ER pathway activity were associated with worse prognosis (**Figure 4.3**). The relation between low ER pathway activity and adverse clinical outcome was found in endometrial cancer previously.[59] Although no stratification for menopausal status was applied, endometrial cancer patients with the lowest ER pathway activity were associated with decreased disease-free survival. If we compare the range of ER pathway activity in our HGSC samples with ER pathway activity in normal FTE from postmenopausal women, as presented in **Chapter 2**, we can conclude that none of the HGSC samples show hyperactivation of the ER pathway (**Figure 9.1**). As such, these results support the conclusion of **Chapter 3**, indicating that decreased ER pathway activity either contributes to or results from neoplastic transition.

As previously mentioned, the preserved ER pathway activity in normal FTE from postmenopausal women indicates the importance of this pathway to sustain normal differentiated cell function. Interestingly, we reported in **Chapter 6** higher ER pathway activity in HGSC samples from premenopausal women in comparison to HGSC samples from postmenopausal women, suggesting that high-grade serous carcinogenesis proceeds differently in pre- and postmenopausal patients. Alternatively, the difference in ER pathway activity might indicate that in an oestradiol rich environment the tumour could be more likely to maintain normal ER signalling. Climacteric changes in hormone metabolism may alter ER signalling in tumour tissue and therefore influence ovarian carcinogenesis. Beyond the age of menopause, serum oestradiol levels are significantly lower than in premenopausal women.[60] Therefore, in tumours of postmenopausal patients, ER signalling might result from a high sensitivity to residual hormone levels resulting from extragonadal steroid biosynthesis in peripheral tissues or by intracrine production of oestrogens by the FTE or tumour itself. Tumour cells are thought to be able to produce oestradiol by

the conversion of androgens or inactive oestradiol precursors.[61] However, if the tumour is either insensitive to residual hormone levels or is unable to actively maintain intracellular ER signalling by intracrine oestrogen production, this may result in complete loss of normal ER function. Our results demonstrate that an inactive ER pathway in postmenopausal HGSC patients is in particular negatively related to survival, suggesting that complete loss of ER signalling contributes to an aggressive phenotype.

A recent study concluded that postmenopausal women with advanced stage HGSC who had used hormone replacement therapy prior to diagnosis have a better survival compared to women who never used menopausal hormone therapy.[62] The beneficial effect was restricted to the use of hormone replacement therapy for five years or more. Furthermore, the authors report that these patients had a lower likelihood of residual disease following primary debulking surgery, suggesting less disseminated disease.[62] Others investigated the effect of postoperative use of hormone replacement therapy in pre- and postmenopausal ovarian cancer patients and reported similar survival benefits.[63,64] Unfortunately, these studies did not provide subgroups analysis by hormonal status at diagnosis. However, a possible explanation for this favourable effect in postmenopausal women may be that long-term oestradiol substitution supports normal ER signalling or alternatively restores the loss of ER signalling and therefore promotes better tumour differentiation with less aggressive behaviour. On the other hand, the survival advantage for both pre- and postmenopausal women may be attributed to the influence of oestrogens on the immune response.[65] Hormone replacement therapy is thought to have anti-inflammatory properties based on a negative effect on several inflammatory biomarkers.[66] Consequently, this may promote a tumour suppressive environment.

As suggested in **Chapter 3**, we observed a relationship between PI3K and ER signalling in the advanced stage HGSC samples in **Chapter 4**. Further stratification of the HGSC patients based on the inverse crosstalk between ER and PI3K pathway activity revealed interrelations between different pathways, for instances the interplay with TGF-β pathway activity (**Figure 4.5** and **Figure 4.6**). In HGSC samples with high ER and low PI3K signalling we observed relatively high TGF-β signalling, which may support anti-inflammatory effects. However, this particular pathway profile was not associated with better survival, possibly influenced by small sample numbers in our subgroups.

Thus far, our analysis enabled the classification of the pathway-specific range in a larger set of HGSC samples and confirmed that HGSC is a strongly heterogenous disease with remarkable variability in STP activity. The unique tumour-promoting patterns may have clinical implications in the development of alternative treatment

options. Although much remains to be elucidated on the role of STP activity in HGSC behaviour and survival, we can conclude that in postmenopausal HGSC patients an inactive ER signalling pathway is associated with poor survival outcome.

Current advances in targeted therapy for recurrent high-grade serous carcinoma patients

During the last decades, developments in chemotherapeutic treatment strategies for HGSC patients have not significantly improved survival rates, emphasizing the need for more effective personalized treatment strategies.[4] The increasing knowledge on cancer behaviour supported the development of targeted therapeutics. Over the past years, a few successful targeted drugs have emerged for the treatment of HGSC patients.[67] Currently approved targeted strategies are directed towards inhibition of angiogenesis with bevacizumab and DNA repair with poly(ADP-ribose) polymerase (PARP) inhibitors.[68,69] Other extensively studied treatment strategies focussed among others on targeting the ER and PI3K signalling pathways but clinical implementation of these treatment strategies is limited.

Therapy with oestrogen receptor pathway inhibitors

In contrast to our previously mentioned association between decreased ER pathway activity and HGSC evolution, there is *in vitro* and *in vivo* evidence showing oestrogens to cause proliferation of ovarian cancer cells.[70,71] In addition, the small but increased risk to develop HGSC after postmenopausal use of hormone replacement therapy supported the assumption that HGSC could be a hormone-dependent disease and therefore inhibition of ER signalling was suggested a potential target for therapy.[72] In breast cancer, nuclear ER-*α* protein expression by immunohistochemistry is widely used in patient selection for anti-oestrogen therapy.[73] Likewise, for HGSC, positive ER staining was considered to be indicative of an active ER signalling pathway and endocrine sensitivity was suggested to correspond to ER status.

Chapter 5 presented a systematic review of recent literature on the efficacy of anti-oestrogen therapy in a relatively homogenic population with metastatic or recurrent HGSC with positive ER protein expression. We included six studies and found a clinical benefit rate (CBR), defined as the percentages of patients that achieved complete or partial response and stable disease, ranging between 27-65% after approximately three months of anti-oestrogen therapy (**Figure 5.2**). Treatment response was mostly attributed to disease stabilization as the overall response rate (ORR) varied between 0-16%, defined as the percentages of patients with complete or partial response (**Figure 5.2**). Despite the relatively favourable CBR, anti-oestrogen therapy was generally not supporting long-term effect considering the modest median progression-free survival ranging between 2.0-3.9 months (**Table 5.3**).

We studied the relation between anti-oestrogen therapy response and ER protein expression using ER histoscores, a weighted scoring method including intensity and percentages of stained tumour cells. Our analysis suggested treatment response to be more likely in HGSC patients with the highest levels of ER histoscores, but lower ER histoscores were inconclusive in predicting clinical response. With the available data we were unable to provide strong evidence for a positive correlation between anti-oestrogen therapy response and ER protein expression. Hence, we conclude that ER protein expression alone is an unreliable predictive biomarker for the identification of responders to anti-oestrogen therapy. A possible explanation for the lack of support for a relation between increasing ER histoscores and improved therapy response might be that ER protein expression is not equivalent to functional ER pathway activity in HGSC samples (**Figure 6.3**). Positive ER protein expression demonstrates presence of the receptor but, in the absence of an activating mutation, the substrate oestrogen is required to activate the receptor and initiate transcription of ER target genes. As shown in **Chapter 6**, our data confirmed that presence of nuclear ER is a prerequisite, but not sufficient to prove a transcriptionally active ER signalling pathway.

Although inhibition of ER signalling with anti-oestrogen therapy may have therapeutic value in a small subgroup of HGSC patients, these findings bring a reasonable concern as an inactive ER signalling pathway is associated with poor survival outcomes in postmenopausal women. Therefore, it is crucial to use a predictive biomarker which adequately presents functional ER pathway activity in order to apply effective ER targeted therapy. While almost all included HGSC samples in **Chapter 4** showed lower ER pathway activity in comparison to the normal range of ER pathway activity presented in **Chapter 2**, this does not necessarily mean that the ER pathway was inactive in all these HGSC samples (**Figure 9.1**). We hypothesize that the subset of HGSC patients with the highest ER signalling pathway activity still represent functionally active ER signalling and that, therefore, these HGSC patients might constitute the subgroup that is likely to benefit from anti-oestrogen targeted therapy.

Therapy with PI3K/AKT/mTOR pathway inhibitors

Considering the biological significance of PI3K signalling in HGSC, inhibition of the PI3K pathway is suggested to represent a beneficial treatment strategy. Following activation of PI3K, the signalling cascade involves AKT and its downstream effector mTOR and thereby forms a complex network associated with various functions including regulation of cell growth, survival and metabolism.[18] Alterations in genes associated with the PI3K/AKT/mTOR pathway are commonly found in ovarian carcinoma.[18,74] As a result, multiple clinical studies evaluated the therapeutic efficacy of PI3K/AKT/mTOR inhibitors.

The meta-analysis presented in **Chapter 7** reported the results of 19 studies on the effectiveness of PI3K/AKT/mTOR inhibitors in advanced and recurrent ovarian cancer patients. Our analysis demonstrated treatment with PI3K/AKT/mTOR inhibitors to be associated with a pooled CBR of 32% (95% confidence interval 20-44%) after approximately three months of therapy (**Figure 7.3**). The effectiveness was almost completely attributed to disease stabilization considering the pooled ORR of 3% (95% confidence interval 0-6%) (**Figure 7.4**). Although stable disease is a valuable outcome in the advanced and recurrent disease setting, the duration of response is of importance to assess a better reflection of true patient benefit. Therefore, we revised the pooled CBR by exclusion of stable disease for less than six months as a beneficial outcome, which decreased the pooled CBR to 7% (95% confidence interval 2-13%) (**Supplementary Figure 7.3**).

Additional subgroup analysis did not show a significantly improved CBR in studies including patients solely with evidence of PI3K/AKT/mTOR dysregulation based on DNA-sequencing or immunohistochemical analysis compared to studies who did not apply biomarker criteria for patient selection (**Figure 7.5**). Therefore, current PI3K/AKT/mTOR biomarkers have limited predictive value to enable accurate patient selection. Consequently, we concluded that the activation state of a signalling pathway cannot simply be inferred from genomic changes or changes in protein expression patterns.

Our results showed that single-agent therapy with PI3K/AKT/mTOR inhibitors may not always be sufficient to induce long-term response, probably caused by potential mechanisms of therapy resistance. Prolonged disease control with single-agent therapy might therefore be more likely to be achieved in patients who have no other aberrantly activated pathway besides the PI3K pathway, as only then it can be accurately assumed that the tumour-promoting pathway is targeted. Moreover, patients with concomitant aberrantly activated pathways could benefit from synergistic anti-tumour activity of combination strategies. Targeting multiple STPs simultaneously might also overcome therapy resistance by blocking tumour escape mechanisms and therefore enable long-term effective therapeutic strategies. Unfortunately, these combination strategies are often complicated by unacceptable toxicity resulting in early discontinuation of treatment. To improve the therapeutic potential, combination therapy may benefit from lower or intermittent dosing schedules, or alternatively, from sequential single-agent therapy.

Clinical application of signal transduction pathway activity assays in targeted therapy selection

Further stratification with appreciation of the functional phenotype of tumour cells is essential to improve the implementation of targeted treatment strategies. The use of

STP activity assays has the potential to allocate (ovarian) cancer patients to effective therapies based on transcriptional pathway activation. To determine whether or not a pathway could be considered a clinical target, we used the STP activity ranges measured in **Chapter 2** to define cut-off values to discriminate between normal and aberrant STP activity. We consider a STP as potentially tumour-promoting if the activity exceeds the cut-off value of two standard deviations above the mean value of STP activity in FTE from postmenopausal women. These cut-off values need to be studied in relation to response to targeted treatment to determine the therapeutic value in patient selection. Cut-off values may be too strict, as for example could be the case for the ER pathway considering the relatively low activity in HGSC as compared to normal FTE. Thus, more research is needed to define optimal cut-off values to distinguish (non-)responding patients with the highest predictive value.

In **Chapter 8**, we designed a multicenter prospective, parallel group cohort study to evaluate the clinical applicability of STP activity assays in selecting ovarian cancer patients for matched targeted therapy. The study, entitled the STAPOVER study, aims to detect an improvement in progression-free survival on matched targeted therapy by STP activity assays in recurrent ovarian cancer patients. Initially, women identified with a potentially tumour-promoting AR, ER, PI3K or HH signalling pathway will be eligible for treatment with selected commercially available targeted drugs, including bicalutamide, letrozole and itraconazole. If deemed beneficial, it is possible that the study is expanded with additional treatment arms including drugs targeting other pathways as well, such as the TGF-β, Notch, mitogen-activated protein kinase (MAPK) pathways. Targeted therapy recommendations will be made by a multidisciplinary tumour board based on the predefined cut-off values and, in case of more than one potentially tumour-promoting pathway, the board could propose combination therapy with two targeted drugs.

The study of anticancer agents in ovarian cancer patients bears several challenges. First, given the relatively low incidence of ovarian cancer and the wide variety in pathway activity profiles, it is difficult to reach sufficient patient numbers to preform conventional randomized controlled trials. Second, the heavily pretreated patients with advanced disease, for which the available standard therapeutic options have been exhausted, constitute a hard-to-treat population. Third, the selection of an appropriate efficacy outcome remains a key issue, considering targeted drugs are expected to cause delayed tumour progression by growth modulation rather than immediate shrinkage of the tumour. The STAPOVER study design is proposed to overcome these issues by simultaneous screening for several promising treatment strategies and enrolment into parallel treatment arms. Treatment response will be assessed by the progression-free survival (PFS2/PFS1) ratio, a correlation between the progression-free survival established by matched targeted therapy (PFS2) and the

progression-free survival recorded on the treatment administered prior to study enrolment (PFS1).[75] The correlation of two consecutive lines of treatment allows the patient to serve as her own control and compensates for heterogeneity in patient characteristics and tumour histology.[76,77] If the matched targeted treatment is ineffective, PFS2 will be shorter than PFS1 resulting in a ratio below 1.0. To define significant treatment efficacy, a ratio in excess of 1.3 will be used to indicate that the matched targeted treatment is successful. In addition to the potential to enable accurate patient selection based on functional STP activity, the STAPOVER study is designed to demonstrate that existing drugs with tolerable toxicity profiles may have therapeutic value beyond their approved indications. Thereby, drug rediscovery offers new treatment possibilities in a cost-effective manner with preservation of quality of life.

Future perspectives

Personalized medicine is a rapidly evolving field that has already influenced the treatment of ovarian cancer patients. However, as shown in this thesis, incorporation of personalized medicine into clinical practice continues to be a challenge as well-proven predictive biomarkers for targeted therapy response are lacking. From our perspective, there are several important considerations and upcoming priorities for the road ahead.

The cell type of origin should be considered in the development of therapeutic strategies as it offers the most reliable road map to normal cellular functions. Thereby, targeted therapy should not be limited to inhibitors that block activated molecular processes, but conversely, should also focus on activating downregulated processes to restore molecular homeostasis. Our observations of normal STP activity remain to be validated on a larger set of FTE samples to optimize the cut-off values for abnormally high and low STP activity, but they may eventually guide therapeutic decision making. Although in this thesis we focused on the HGSC subtype, future research should adapt the cut-off values per ovarian cancer histological subtype as they are thought to differ in cellular origin.

Evaluation of drug combinations will increase clinical efficacy as concurrent dysregulation of several STPs appears to be more common in ovarian cancer. However, to overcome therapy resistance, we need to determine frequent escape mechanisms of tumour cells, for example by studying STP activity in pre- and post-treatment biopsies. If we increase our knowledge on the biologic behaviour of ovarian cancer during treatment with a certain targeted agent, we will be able to suggest synergistic or sequential treatment strategies which have the potential to evade mechanisms of resistance. Given the heterogeneous nature of ovarian cancer,

research will benefit from more comprehensive molecular profiling tools. In this context, STP activity analysis could be used complementary to whole genome sequencing as information on gene mutations may facilitate the search for the tumour-driving pathway.

Lastly, molecular profiling results are often difficult to interpret. Physicians are faced with the complex task to translate potential actionability of signalling pathways into clinical decision-making. To facilitate the interpretation and maximize clinical utility, a formal entity such as multidisciplinary tumour boards, including both physicians and translational researchers, should exist in tertiary oncology centers and be accessible for all cancer patients. These boards are able to transfer interdisciplinary knowledge and eventually provide guidelines which will increase the scope of personalized medicine.

Conclusion

We have taken a novel approach to provide an overview of the influence of six important STPs in the development and progression of HGSC. Understanding cancer behaviour is critical for appropriate selection of STPs as potential therapeutic targets. Moreover, our results show that we need to develop better predictive biomarkers to achieve significant breakthrough in personalized medicine. Although cytoreductive surgery and chemotherapy will remain fundamental in the treatment of primary ovarian cancer, the use of targeted therapy as second-line treatment will continue to evolve. Further advances in drug combinations or sequential single-agent strategies, need to focus on the phenotypic tumour profile to contribute to more effective targeted treatment strategies.

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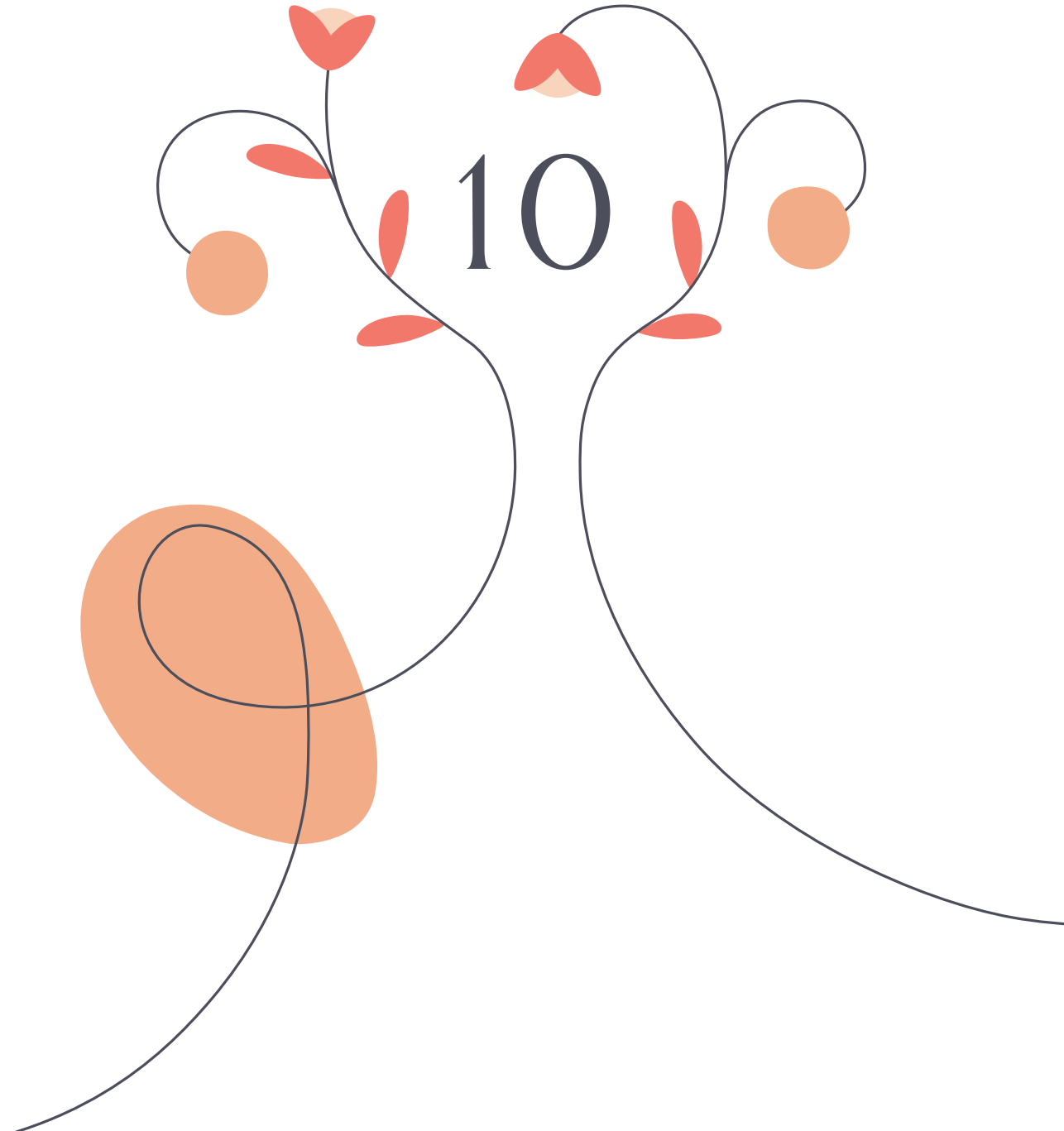
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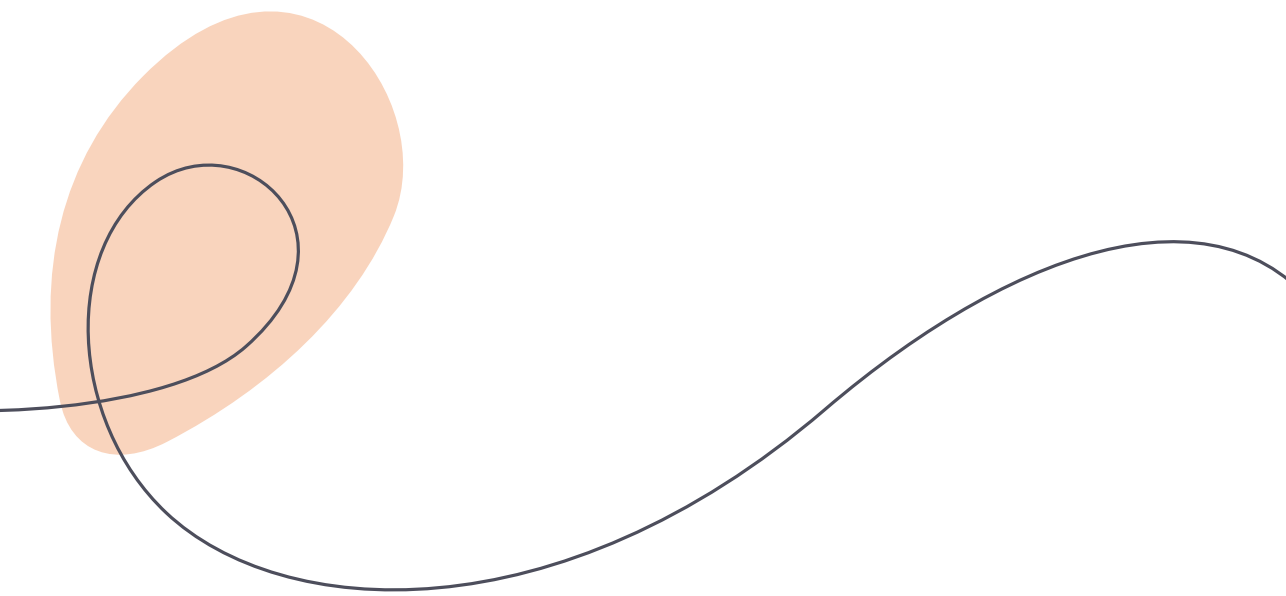
CHAPTER 10

Summary
Samenvatting



Summary

Ovarian cancer is characterized by poor prognosis. The disease often recurs despite aggressive treatment with cytoreductive surgery and chemotherapy. During the last decades, survival rates have not significantly improved, indicating the high need to unravel the molecular characteristics of ovarian cancer to constitute new therapeutic approaches. In this thesis, we aim to increase our understanding of ovarian cancer behaviour using a novel method measuring signal transduction pathway (STP) activity. Ultimately, we aim improve the implementation of targeted treatment strategies for ovarian cancer patients by stratification based on STP activity. As described in **Chapter 1**, carcinogenesis results from dysregulation of cellular activity of signalling pathways, which is frequently triggered by gene mutations. With regard to targeted treatment strategies, information on STP activity is used to provide a tailored treatment for cancer patients with specific targeted drugs. For this treatment strategy to be effective, identification of the tumour-driving STP is of most importance to enable accurate patient selection. However, current diagnostics for patient selection focus on a single molecular trait (e.g. immunohistochemical protein expression and genomic alterations) and disregard other factors, such as the tumour micro-environment. Therefore, in this thesis, we used an alternative approach to quantify functional STP activity with consideration of the tumour cell phenotype. The use of STP activity assays, enabled us to investigate six major signalling pathways, all of which were previously associated with ovarian carcinogenesis.

Identification of the tumour-driving STP requires knowledge on normal STP activity in healthy cells to determine whether a certain STP is aberrantly activated. The Fallopian tube epithelium (FTE) is recognized as the predominant cell type of origin of the most common type of ovarian cancer, the high-grade serous subtype. In **Chapter 2**, we investigated the influence of the hormonal cycle on STP activity in the fimbrial epithelium of morphologically normal Fallopian tubes. We included healthy pre- (n=17) and postmenopausal (n=8) women who had surgical interventions for benign gynaecological conditions. For the premenopausal women, hormone serum levels and histological sections of the endometrium were used to determine the hormonal phase (early follicular (n=4), late follicular (n=3), early luteal (n=5) and late luteal phase (n=5)). After laser capture microdissection, total messenger ribonucleic acid (mRNA) was extracted from the fimbrial epithelium and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis was performed. We used STP activity assays to assess functional activity of the hormone driven pathways androgen receptor (AR) and oestrogen receptor (ER), the growth factor pathway phosphoinositide 3-kinase (PI3K) and the developmental pathways Hedgehog (HH), transforming growth factor beta (TGF-β) and canonical wingless-type MMTV integration site (Wnt). The early luteal phase demonstrated high AR and ER

pathway activity compared to the late luteal phase (*P*=0.016 and *P*=0.032, respectively) and low PI3K activity compared to the late follicular phase (*P*=0.036), while the late luteal phase showed low activity of HH and Wnt compared to the early follicular phase (both *P*=0.016). In FTE from postmenopausal women, we observed differences in AR, ER, PI3K and Wnt pathway activity in comparison to the follicular and/or luteal phase in premenopausal women. In summary, we found cyclic changes in activity of the AR, ER, PI3K, HH and Wnt pathways, indicating that STP activity in FTE is influenced by the hormonal cycle.

To increase our understanding of tumour-driving mechanisms during high-grade serous carcinogenesis, we aimed to identify early aberrations in functional STP activity in precursor lesions of HGSC in **Chapter 3**. We searched the pathology archive for tissues from patients diagnosed with serous tubal intraepithelial carcinoma (STIC) and concurrent HGSC. Then, we performed mRNA extraction and RT-qPCR analysis on STIC (n=8) and HGSC (n=7) samples to assess STP activity and compared this to STP activity in normal FTE from postmenopausal women (n=8). We found no statistically significant differences in the activity of the AR, ER, PI3K, HH, TGF-β and Wnt pathways between STIC and concurrent HGSC. However, STIC and HGSC demonstrated significantly lower ER and higher PI3K and HH pathway activity in comparison to normal FTE, suggesting these pathways as putative early drivers in the neoplastic transformation of FTE. In addition, we determined forkhead box O protein 3a (FOXO3a) expression by immunohistochemistry and found loss of FOXO3a expression in STIC and HGSC compared to normal FTE. This observation confirmed that activation of PI3K signalling by loss of FOXO is an early hallmark of high-grade serous carcinogenesis. Furthermore, HGSC were characterized by significant loss of AR and Wnt pathway activity in relation to FTE, suggesting these pathways contribute to HGSC progression.

In **Chapter 4**, we explore the activity of the previously mentioned oncogenic STPs in HGSC in relation to survival. We assessed functional STP activity in 85 primary tumour samples of patients with advanced stage HGSC and a disease-free survival below 12 months (n=52) or above 24 months (n=33). There were no significant differences in STP activity between patients with short- and long-term disease-free survival. In univariate Cox proportional hazards analysis, stratification of HGSC patients for menopausal status revealed a favourable relation between ER pathway activity and disease-free survival (hazard ratio=0.943) and overall survival (hazard ratio=0.930) in postmenopausal women (*P*=0.033 and *P*=0.041, respectively), but not in premenopausal women. We divided the postmenopausal group into four subgroups based on ER pathway activity quartiles. Survival analysis revealed that postmenopausal women in the lowest ER quartile had a shorter disease-free and overall survival (log-rank *P*=0.006 and *P*<0.001, respectively). In conclusion, in

postmenopausal women with advanced stage HGSC, low functional ER pathway activity was associated with a poorer survival outcome.

Therapy targeting the ER signalling pathway may be used as a palliative treatment option in HGSC patients. Currently, positive ER protein expression by immunohistochemistry is considered a biomarker for sensitivity to anti-oestrogen therapy. In **Chapter 5**, we conducted a systematic review of the literature on the clinical benefit of anti-oestrogen therapy in a homogenic population of ER positive metastatic or recurrent HGSC and searched for a correlation between ER protein expression and clinical response. The primary outcome was the clinical benefit rate (CBR) defined as the proportion of patients with complete or partial response or stable disease. The secondary outcome was the overall response rate (ORR) defined as the proportion of patients with complete or partial response. There were no studies with populations consisting solely of ER positive HGSC. However, we included six studies reporting on 407 evaluable patients of whom 376 were HGSC (92%) and 302 were confirmed ER positive (80%). Anti-oestrogen therapy resulted in a CBR of 27-65% and an ORR of 0-16% after approximately three months of therapy. No correlation was found between ER expression and clinical response. Therefore, ER protein expression alone is not a reliable predictor of response. This may result from the incorrect assumption that ER protein expression equals functional ER pathway activity, since in the absence of ER activating mutations, the substrate oestrogen is required to activate the receptor and initiate transcription of ER target genes. We concluded that, to apply effective ER targeted therapy, it is important to develop better predictors to identify (non-)responders.

Subsequently in **Chapter 6**, we determined the accuracy of ER protein expression as a biomarker for functional ER pathway activity. In 29 HGSC samples, immunohistochemical ER protein expression was visually scored using total percentages of stained tumour cells and ER histoscores. In addition, functional ER pathway activity was determined using mRNA measurements of ER-specific target genes. Our analysis showed that neither total percentages of ER protein expression, nor ER histoscores were significantly correlated to ER pathway activity (P=0.473 and P=0.606, respectively). Classification of HGSC into three subgroups based on ER histoscores 0-100 (n=6), 101-200 (n=15) and 201-300 (n=8) resulted in comparable mean ER pathway activity among the subgroups (P=0.356). Several HGSC in the higher ER histoscore subgroups showed low ER pathway activity, indicating that nuclear ER protein expression is not sufficient to describe transcriptional ER activation. We recommended that further studies are necessary to prove the predictive value of functional ER pathway activity regarding anti-oestrogen sensitivity in HGSC patients.

The signalling pathway involving PI3K, AKT and mammalian target of rapamycin (mTOR) proteins is considered an attractive targeted treatment option as genomic alterations in one of the components of this pathway are frequently found in ovarian cancer patients. We performed a meta-analysis of the clinical benefit of PI3K/AKT/mTOR pathway inhibitors in ovarian cancer and investigated the predictive value of current biomarkers on therapy response in **Chapter 7**. The primary and secondary outcomes were CBR and ORR, respectively. We included 233 patients from 19 studies and observed a pooled CBR of 32% (95% confidence interval (CI) 20–44%) and ORR of 3% (95% CI 0–6%) in advanced or recurrent ovarian cancer patients treated with PI3K/AKT/mTOR inhibitors. Subgroup analysis tended to favor the studies who selected patients based on current PI3K/AKT/mTOR biomarker criteria (e.g. genomic alterations or loss of PTEN protein expression), but the difference in CBR was not statistically significant from studies with unselected populations (respectively, CBR of 42% (95% CI 23–62%) and 27% (95% CI 14–42%), *P*=0.217). To better reflect true patient benefit, we excluded stable disease below six months as a beneficial outcome which resulted in a pooled CBR of 7% (95% CI 2–13%). Thus, the efficacy of monotherapy with PI3K/AKT/mTOR inhibitors in ovarian cancer patients is limited to a small subgroup and selection of patients with the use of current biomarkers did not improved the CBR significantly. We found that the overall proportion of patients with drug-related severe or life-theathing adverse events was 36%. Given the toxicity profile, we suggested that current treatment with PI3K/AKT/mTOR inhibitors should not be initiated unless in clinical trials. Furthermore, we concluded that reliable biomarkers that measure functional activity of the PI3K/AKT/mTOR pathway are needed to optimize patient selection.

Taken together, successful implementation of targeted therapy for ovarian cancer patients based on stratification by their molecular signature has been limited so far. Moreover, there remains a high unmet need for reliable diagnostics to predict targeted therapy response as protein expression and gene alterations are insufficiently reliable. Therefore, we designed a multicenter prospective, parallel-group cohort study to evaluate the clinical applicability of STP activity assays in selecting ovarian cancer patients for matched targeted therapy. As described in **Chapter 8**, the STAPOVER study aims to identify aberrantly activated STPs in recurrent ovarian cancer patients and implement *phenotype*-guided targeted therapy to improve survival and maintain quality of life. We used the normal STP activity ranges measured in postmenopausal women to define cut-off values to discriminate between normal and aberrant STP activity. Patients will be selected for treatment with existing targeted drugs with tolerable toxicity profiles to investigate whether these drugs have therapeutic value beyond their approved indications. Treatment response will be assessed by the progression-free survival ratio, in which the correlation of two consecutive lines of treatment allows the patient to serve as her

own control and compensates for heterogeneity in patient characteristics and tumour histology. Initially, the study will include ovarian cancer patients identified with a potentially tumour-promoting AR, ER, PI3K or HH signalling pathway, but the adaptive study design enables expansion of the trial with additional treatment arms including drugs targeting other pathways as well.

Finally, the main findings of this thesis are presented in a larger perspective in **Chapter 9**. In this chapter we discussed the implications of our findings for clinical practice and provided future perspectives on the targeted treatment of ovarian cancer patients.

Samenvatting

Ovariumcarcinomen, oftewel eierstokkankers, worden gekenmerkt door een grote kans op recidiverende ziekte en een slechte prognose, ondanks een uitgebreide behandeling met cytoreductieve chirurgie en chemotherapie. In de laatste decennia zijn de overlevingskansen van patiënten met ovariumcarcinomen niet significant verbeterd. Om deze reden bestaat er een grote behoefte om de moleculaire kenmerken van ovariumcarcinomen in kaart te brengen, zodat nieuwe therapeutische benaderingen kunnen worden ontwikkeld. Dit proefschrift is gericht op het vergroten van onze kennis van het gedrag van ovariumcarcinomen met behulp van een nieuwe methode die de activiteit van de signaaltransductiepaden (STP) meet. Daarnaast richt dit proefschrift zich op het verbeteren van de implementatie van doelgerichte behandelingsstrategieën voor patiënten met ovariumcarcinomen door stratificatie op basis van STP-activiteit. Zoals beschreven in **Hoofdstuk 1** is carcinogenese het gevolg van ontregeling van cellulaire STP-activiteit, dat vaak wordt veroorzaakt door genetische mutaties. Informatie over de STP-activiteit wordt gebruikt om een doelgerichte medicijn te selecteren voor een persoonlijk behandelplan voor kankerpatiënten. Voor het toepassen van effectieve doelgerichte behandelingen is het belangrijk om het tumor-aansturende signaaltransductiepad te identificeren, zodat een nauwkeurige patiëntselectie gemaakt kan worden. Echter, de huidige methoden voor patiëntselectie (bijvoorbeeld immunohistochemische eiwitexpressie en genetische afwijkingen) zijn gericht op afzonderlijke moleculaire eigenschappen en houden geen rekening met andere factoren, zoals cellulaire interacties met de omgeving van de tumor. In dit proefschrift hebben we een methode gebruikt om functionele STP-activiteit te bepalen, waarbij we rekening houden met het fenotype van de tumorcellen. Met behulp van STP-activiteit analyse onderzochten we zes belangrijke STP welke in eerdere onderzoeken geassocieerd werden met de wijze waarop ovarium-carcinomen ontstaan, groeien of uitzaaien.

Kennis over normale STP-activiteit in gezonde cellen is van belang om afwijkend geactiveerde STP te identificeren als tumor-aansturende STP. De epitheelcellen van het fimbriële uiteinde van de tuba, oftewel de eileider, worden erkend als het celtype waaruit de meest voorkomende vorm van het ovariumcarcinoom ontstaat; het hooggradig sereus ovariumcarcinoom (HGSC). In **Hoofdstuk 2** onderzoeken we de invloed van de hormonale cyclus op STP-activiteit in fimbriële epitheelcellen van morfologisch normale tubae. In dit onderzoek includeerden we gezonde pre- (n=17) en postmenopauzale (n=8) vrouwen die chirurgische ingrepen ondergingen voor goedaardige gynaecologische aandoeningen. Hormoonspiegels en histologische coupes van het endometrium werden gebruikt om bij de premenopauzale vrouwen de hormonale fase te bepalen (vroeg folliculaire (n=4), laat folliculaire (n=3), vroeg

luteale (n=5) en laat luteale fase (n=5)). Epitheelcellen van de fimbriële uiteinden werden met behulp van laser microdissectie verkregen en vervolgens werd het *total messenger ribonucleic acid* (mRNA) geëxtraheerd en *real-time quantitative reverse transcription-polymerase chain reaction* (RT-qPCR) analyse uitgevoerd. Met behulp van STP-activiteit analyse werd de functionele activiteit bepaald van de hormonale STP androgeenreceptor (AR) en oestrogeenreceptor (ER), het groeifactor signaal-tranductiepad *phosphoinositide-3-kinase* (PI3K) en de ontwikkelings-STP *Hedgehog* (HH), *transforming growth factor beta* (TGF-*β*) en *canonical wingless-type MMTV integration site* (Wnt). De vroeg luteale fase toonde hoge AR en ER activiteit vergeleken met de laat luteale fase (respectievelijk, *P*=0,016 en *P*=0,032) en lage PI3K activiteit vergeleken met de laat folliculaire fase (*P*=0,036). Dit is in tegenstelling tot de laat luteale fase, welke werd gekarakteriseerd door lage activiteit van HH en Wnt in vergelijking met de vroeg folliculaire fase (beide *P*=0,016). In postmenopauzale vrouwen zagen we verschillen in de AR, ER, PI3K en Wnt activiteit in vergelijking met de folliculaire en/of luteale fase van premenopauzale vrouwen. Samenvattend vonden we cyclische veranderingen in de activiteit van de AR, ER, PI3K, HH en Wnt paden, wat aangeeft dat de STP-activiteit in tuba epitheelcellen wordt beïnvloed door de hormonale cyclus.

In **Hoofdstuk 3** richten we ons op het identificeren van vroege afwijkingen in functionele STP-activiteit in voorloperlaesies van HGSC, met het doel om onze kennis van tumor-aansturende mechanismen bij het ontstaan van HGSC te vergrootten. In dit hoofdstuk hebben we patiënten met een gelijktijdige diagnose van sereus tuba intra-epitheliaal carcinoom (STIC) en HGSC geïncludeerd. Vervolgens voerden we mRNA-extractie en RT-qPCR analyse uit op STIC (n=8) en HGSC (n=7) weefsels om STP-activiteit te bepalen en vergeleken dit met STP-activiteit in normale tuba epitheelcellen van postmenopauzale vrouwen (n=8). We vonden geen statistisch significante verschillen in de activiteit van de AR, ER, PI3K, HH, TGF-*β* en Wnt STP tussen STIC en HGSC. Echter, zowel STIC als HGSC lieten significant lagere ER en hogere PI3K en HH activiteit zien in vergelijking met normale tuba epitheelcellen. Deze bevindingen suggereren dat deze STP vermoedelijke vroege drijfveren zijn in de neoplastische transformatie van tuba epitheelcellen. Daarnaast onderzochten we immunohistochemische *forkhead box O protein 3a* (FOXO3a) expressie en vonden we een verlies van FOXO3a expressie in STIC en HGSC in vergelijking met normale tuba epitheelcellen. Deze resultaten bevestigden dat activering van PI3K-signalering door verlies van FOXO een vroeg kenmerk is van hooggradig sereuze carcinogenese. Bovendien werden HGSC gekenmerkt door significant lagere AR en Wnt activiteit in relatie tot normale tuba epitheelcellen, wat suggereert dat deze STP bijdragen aan progressie naar HGSC.

In **Hoofdstuk 4** analyseren we de activiteit van de eerdergenoemde oncogene STP in HGSC in relatie tot overleving. We bepaalden de STP-activiteit in primaire tumorweefsels van 85 patiënten met gevorderd stadium HGSC én een ziektevrije overleving van minder dan 12 maanden (n=52) of meer dan 24 maanden (n=33). Er waren geen significante verschillen in STP-activiteit tussen patiënten met korte en lange ziektevrije overleving. Na stratificatie van de patiënten op basis van hormonale status, toonde univariate Cox regressieanalyse aan dat ER activiteit gerelateerd was aan een langere ziektevrije overleving (hazard ratio=0,943) en totale overleving (hazard ratio=0,930) in postmenopauzale vrouwen (respectievelijk, *P*=0,033 en *P*=0,041). Vervolgens hebben we de postmenopauzale vrouwen in vier subgroepen verdeeld op basis van de ER activiteit kwartielen. Overlevingsanalyse liet zien dat postmenopauzale vrouwen met ER activiteit in het laagste kwartiel een kortere ziektevrije en totale overleving hadden (respectievelijk, log-rank *P*=0,006 en *P*<0,001). We concludeerden dat lage functionele ER activiteit geassocieerd was met slechtere overlevingsuitkomsten in postmenopauzale vrouwen met HGSC in een vergevorderd stadium.

Therapie gericht op het ER signaaltransductiepad kan worden gebruikt als een palliatieve behandelingsoptie bij patiënten met HGSC. Momenteel wordt positieve immunohistochemische ER-eiwitexpressie beschouwd als een biomarker voor gevoeligheid voor anti-oestrogeentherapie. In **Hoofdstuk 5** beschrijven we een systematische review van de literatuur over het effect van anti-oestrogeentherapie in een homogene populatie van patiënten met ER-positieve gemetastaseerde of gerecidiveerde HGSC. Daarnaast onderzochten we de correlatie tussen ER-eiwitexpressie en anti-oestrogeentherapie respons. De primaire uitkomstmaat was de *clinical benefit rate* (CBR), gedefinieerd als het percentage patiënten met volledige of gedeeltelijke respons of stabiele ziekte. De secundaire uitkomstmaat was de *overall response rate* (ORR), gedefinieerd als het percentage patiënten met een volledige of gedeeltelijke respons. Er waren geen studies met onderzoekspopulaties die uitsluitend uit patiënten met ER-positieve HGSC bestonden. In totaal hebben we zes studies geïncludeerd, waarvan de resultaten van 407 evalueerbare patiënten werden beschreven. Van de 376 patiënten gediagnosticeerd met HGSC (92%) waren 302 bevestigd ER-positief (80%). Behandeling met anti-oestrogeentherapie resulteerde in een CBR van 27-65% en een ORR van 0-16% na ongeveer drie maanden behandeling. Er werd geen correlatie gevonden tussen ER-eiwitexpressie en anti-oestrogeentherapie respons. Daarom veronderstellen we dat ER-eiwitexpressie op zichzelf geen betrouwbare voorspeller is van anti-oestrogeen-therapie respons. Dit kan het gevolg zijn van de onjuiste aanname dat ER-eiwitexpressie gelijk is aan functionele activiteit van het ER signaaltransductiepad, aangezien bij afwezigheid van ER-activerende mutaties het hormoon oestrogeen nodig is om de receptor te activeren en transcriptie van ER target genen te initiëren.

We concludeerden dat het belangrijk is om betere biomarkers te ontwikkelen om de hormoongevoelige patiënten te identificeren zodat effectieve ER-gerichte therapie toegepast kan worden.

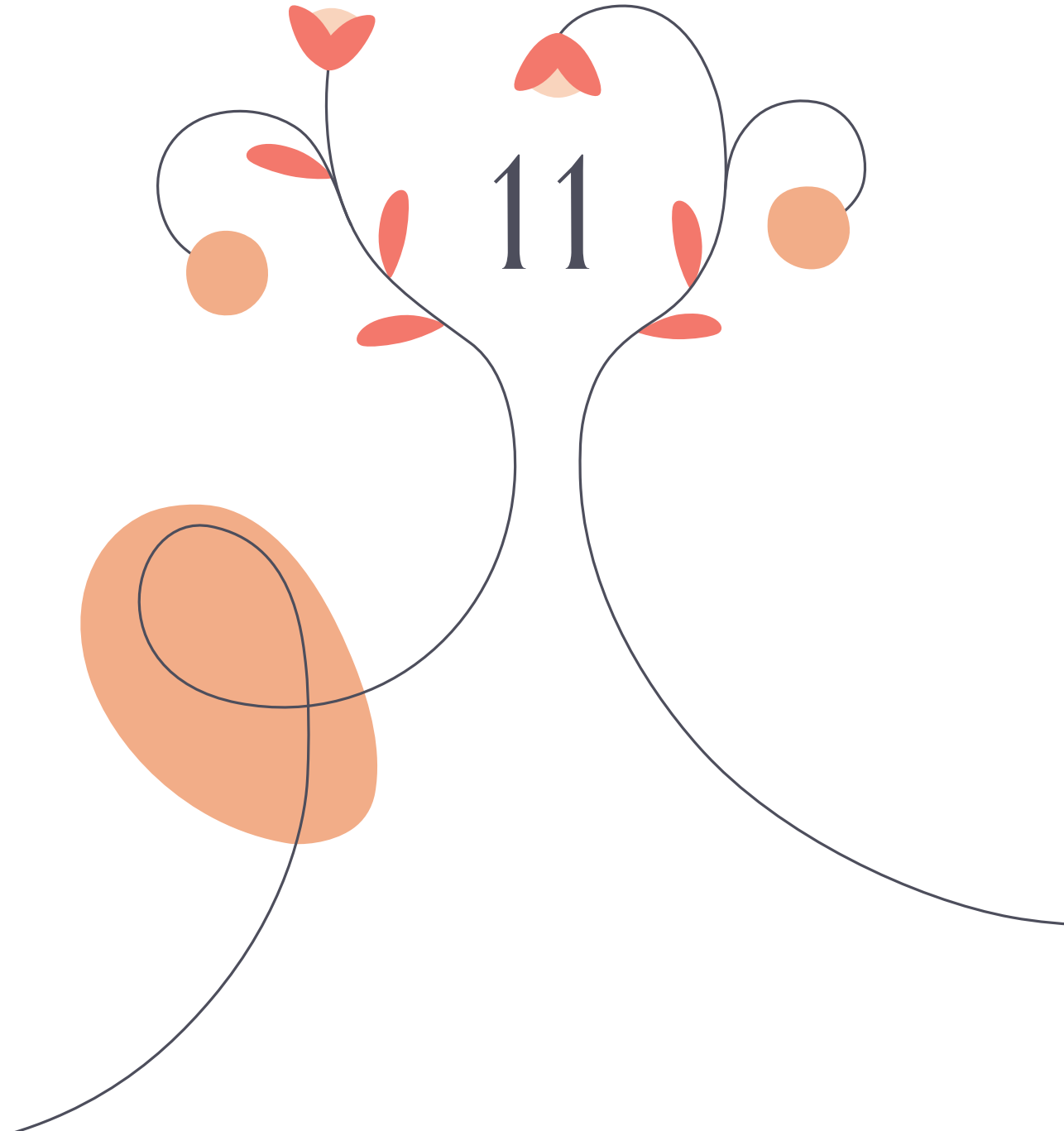
Vervolgens hebben we in **Hoofdstuk 6** de nauwkeurigheid van ER-eiwitexpressie bepaald als biomarker voor functionele activiteit van het ER signaaltransductiepad. We onderzochten de immunohistochemische ER-eiwitexpressie in 29 tumorweefsels van patiënten met HGSC en bepaalden het totale percentage positief gekleurde celkernen en ER-histoscores. Daarnaast werd de functionele activiteit van het ER signaaltransductiepad gemeten met behulp van mRNA-metingen van ER-specifieke target genen. Onze analyse liet zien dat noch het totale percentage van ER-eiwitexpressie, noch de ER-histoscores significant gecorreleerd waren met activiteit van het ER signaaltransductiepad (respectievelijk, *P*=0,473 en *P*=0,606). De gemiddelde activiteit van het ER signaaltransductiepad was vergelijkbaar in een subgroep analyse met drie gevormde subgroepen op basis van ER-histoscores 0-100 (n=6), 101-200 (n=15) en 201-300 (n=8) (*P*=0,356). Tevens toonden verschillende HGSC in de hogere ER histoscore-subgroepen een lage activiteit van het ER signaaltransductiepad, wat aangeeft dat nucleaire ER-eiwitexpressie niet voldoende is om transcriptionele ER-activering te beschrijven. Op basis van deze resultaten adviseerden we dat aanvullende studies nodig zijn om de voorspellende waarde van functionele activiteit van het ER signaaltransductiepad te bepalen voor de gevoeligheid voor anti-oestrogeentherapie.

Het signaaltransductiepad waarbij PI3K-, AKT- en *mammalian target of rapamycin* (mTOR) eiwitten betrokken zijn wordt beschouwd als een aantrekkelijke doelgerichte behandeloptie, omdat genetische veranderingen in een van de componenten van dit signaaltransductiepad vaak worden gevonden bij patiënten met ovariumcarcinomen. In **Hoofdstuk 7** beschrijven we een meta-analyse naar het effect van PI3K/AKT/mTOR doelgerichte geneesmiddelen in patiënten met ovariumcarcinomen en onderzoeken we de voorspellende waarde van huidige PI3K/AKT/mTOR biomarkers op therapierespons. De primaire en secundaire uitkomsten waren respectievelijk de CBR en ORR. We includeerden 233 patiënten met ovariumcarcinomen uit 19 studies en vonden een gepoold CBR van 32% (95% betrouwbaarheidsinterval (BI) 20–44%) en ORR van 3% (95% BI 0–6%) na PI3K/AKT/mTOR doelgerichte behandeling. Een subgroep analyse naar het effect van de huidige PI3K/AKT/mTOR biomarkers (bijvoorbeeld genetische veranderingen of verlies van PTEN-eiwitexpressie) toonden een hoger gepoold CBR in onderzoeken met geselecteerde populaties dan onderzoeken met niet-geselecteerde populaties, maar het verschil in CBR was niet statistisch significant (respectievelijk, 42% (95% BI 23–62%) en 27% (95% BI 14–42%), *P*=0,217). Om het werkelijke voordeel voor de patiënt beter weer te geven, hebben we stabiele ziekte voor minder dan zes maanden uitgesloten als positieve

uitkomstmaat, wat resulteerde in een gepoold CBR van 7% (95% BI 2-13%). Dit laat zien dat de werkzaamheid van monotherapie met PI3K/AKT/mTOR doelgerichte geneesmiddelen bij patiënten met ovariumcarcinomen beperkt is tot een kleine subgroep van patiënten en dat de CBR niet significant verbeterd na selectie van patiënten met behulp van de huidige PI3K/AKT/mTOR biomarkers. Bovendien werden ernstige geneesmiddel-gerelateerde bijwerkingen gezien in 36% van de patiënten. Op basis van dit bijwerkingsprofiel adviseerden we PI3K/AKT/mTOR doelgerichte behandeling alleen te gebruiken in klinisch onderzoek. Verder concludeerden we dat betrouwbare biomarkers die de functionele PI3K/AKT/mTOR activiteit meten nodig zijn om de selectie van patiënten voor PI3K/AKT/mTOR doelgerichte behandeling te optimaliseren.

Samenvattend is de implementatie van doelgerichte behandelingsstrategieën met stratificatie op basis van moleculaire kenmerken voor patiënten met ovariumcarcinomen tot nu toe beperkt gebleven. Daarnaast is er een blijvende behoefte aan betrouwbare diagnostiek om doelgerichte therapierespons te voorspellen, aangezien eiwitexpressie en genetische veranderingen onvoldoende betrouwbaar blijken te zijn. Om deze reden hebben we een multicenter prospectief cohortonderzoek met parallelle onderzoeksgroepen ontworpen om de klinische toepasbaarheid van STP-activiteit analyse te evalueren bij het selecteren van patiënten met ovariumcarcinomen voor doelgerichte therapie. Zoals beschreven in **Hoofdstuk 8**, heeft de STAPOVER-studie het doel om het afwijkend geactiveerde signaaltransductiepad te identificeren bij patiënten met een recidiverend ovariumcarcinoom. Op deze manier willen we doelgerichte therapie implementeren dat gericht is op het fenotype van de tumorcellen en willen we de overlevingskansen van patiënten verbeteren. We hebben de normale STP-activiteit range, gemeten bij postmenopauzale vrouwen, gebruikt om afkapwaarden te definiëren voor afwijkende STP-activiteit. Patiënten zullen worden geselecteerd voor behandeling met bestaande doelgerichte geneesmiddelen met verdraagbare bijwerkings-profielen. We zullen onderzoeken of deze geneesmiddelen therapeutische waarde hebben buiten hun goedgekeurde indicaties met behoud van kwaliteit van leven. Het effect van de behandeling zal worden beoordeeld aan de hand van de progressievrije overlevingsratio, een uitkomstmaat waarbij de patiënt dient als haar eigen controle door het vergelijken van de progressievrije overleving tijdens twee opeenvolgende behandelingen. Daarnaast compenseert deze uitkomstmaat voor de heterogeniteit in patiëntkenmerken en tumorhistologie. In eerste instantie zullen patiënten met ovariumcarcinomen met een afwijkend geactiveerd tumor-aansturend AR, ER, PI3K of HH signaaltransductiepad in aanmerking komen voor doelgerichte behandeling binnen de STAPOVER-studie. Echter, het adaptieve onderzoeksdesign maakt uitbreiding van de studie mogelijk met aanvullende behandelingsarmen, waaronder doelgerichte geneesmiddelen die zich op andere STP richten.

Ten slotte worden de belangrijkste bevindingen van dit proefschrift in een groter perspectief gepresenteerd in **Hoofdstuk 9**. In dit hoofdstuk bespreken we de implicaties van onze bevindingen voor de klinische praktijk en bieden we toekomstperspectieven voor de doelgerichte behandeling van patiënten met ovariumcarcinomen.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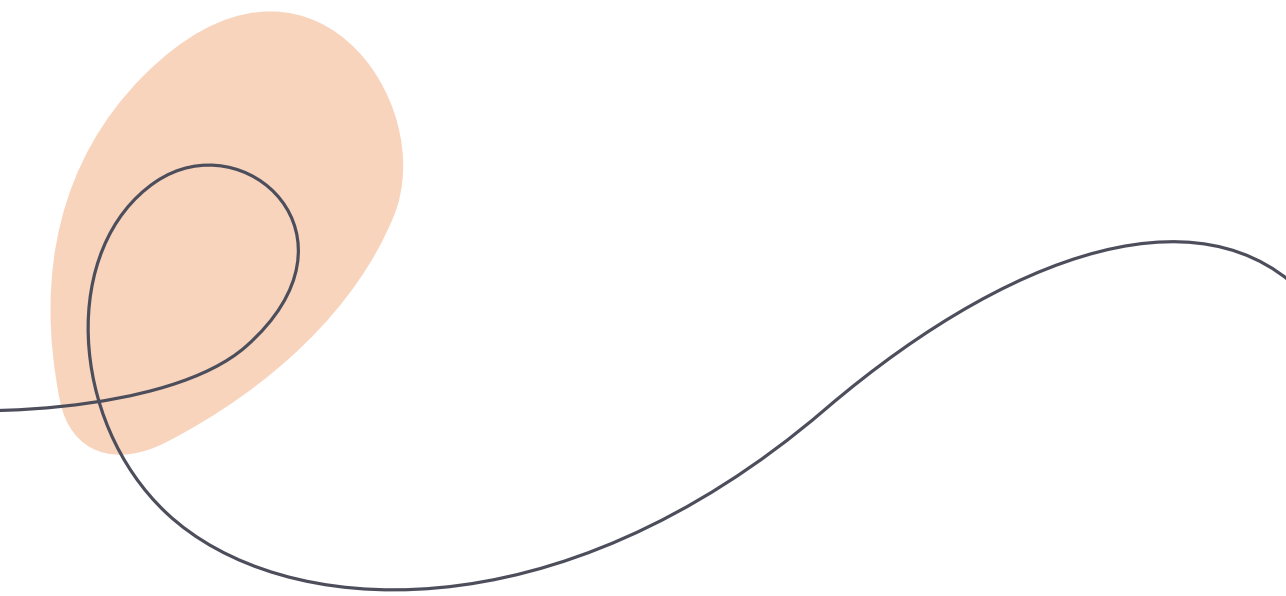
CHAPTER 11

Valorisation



Valorisation

Valorisation entails the process of creating value out of knowledge, by making knowledge suitable and available for economic or societal use.[1] New insight from scientific research might contribute to solutions for the societal challenges of today and tomorrow. With this thesis, we aim to improve the implementation of targeted treatment strategies for ovarian cancer patients by stratification based on a novel method measuring signal transduction pathway (STP) activity. This chapter describes the scientific and societal impact of the results and conclusions of this thesis.

From bench to bedside: translation of laboratory results to the clinic

The process of cancer development is characterized by complex and dynamic mechanisms of action.[2] One of the major challenges of today is to interpret the enormous amount of information on cancer biology and use it to develop successful treatment strategies. While the growing number of targeted drugs makes it challenging for physicians to choose the optimal treatment, as physicians are generally not trained to interpret molecular information. In this thesis, we demonstrate that results from translational research, in which we aim to understand the differences in STP activity between healthy and malignant cells, can be translated to the clinic as potential targets for therapeutic strategies. Moreover, with an alternative approach, we show that it is possible to map the pathway activity profile of a tumour sample and easily interpret the results to a 0-100 scale. Our cut-off values help to discriminate between normal and aberrant STP activity and could guide physicians to define potentially tumour-promoting STPs for treatment decision-making strategies. With our research, we demonstrate that collaboration between basic researchers, physicians and the industry could support the translation of laboratory findings into clinically applicable therapeutics.

The right treatment to the right patient

Cancer treatment is a major global burden and health spending on cancer keeps increasing each year. In the Netherlands, the total cost of cancer care was 10,163 million euros in 2018.[3] Especially for patients with recurrent or metastatic cancer, costly chemotherapeutics and targeted drugs are used as treatment options, often with limited effect on survival and quality of life.[4] To ensure affordable health care in the coming decades, we must focus on treatment optimization and improve diagnostic testing to help identify patients who do and do not respond well to particular treatments. With reliable biomarkers for response prediction, we could reduce the number of suboptimal therapies and thereby contain the health care cost. In this thesis, we investigate several possible targeted treatment options for ovarian cancer patients and demonstrate effectiveness in a small proportion of the

patients. Unfortunately, our results show that current biomarkers (e.g. immunohistochemical protein expression and evaluation of genomic alterations) have low predictive value on therapy response, indicating that the activation state of a signalling pathway cannot simply be inferred from changes in protein expression patterns or genomic alterations. With our research, we attempt to create scientific awareness for the functional phenotype of STP activity in tumour cells to enhance the search for more accurate predictive biomarkers. We expect that our results can motivate others to accomplish *phenotype*-guided profiling strategies and design innovative clinical trials to improve the biomarker-drug matching process for cancer patients.

Drug rediscovery: the use of old drugs for new therapeutic purposes

Drug development is a high-cost and time-consuming process. Currently, the time needed to develop a new drug for cancer treatment can take up to 15 years and only 7% of the drugs entering phase I clinical trials will ultimately receive approval.[5,6] Drug rediscovery is a strategy to identify effectiveness of existing drugs in new indications and offers rapid implementation of potential therapeutic strategies in a cost-effective manner.[7] Particularly, the use of generic drugs whose patents have expired could significantly reduce health care cost. The individual chapters of this thesis resulted in the development of the STAPOVER study, a study to match ovarian cancer patients to off-label treatment with existing targeted drugs. The study aims to alleviate the shortage of effective cancer drugs by applying functional STP activity as response prediction biomarker. Furthermore, the use of trusted drugs brings additional benefit to patients by the well-known toxicity profile, and thereby, minimisation of side effects will help maintain quality of life of cancer patients. Our hope is that the results of the STAPOVER study will stimulate health insurance companies, governmental agencies, and the industry to further support drug rediscovery initiatives in order to increase the societal impact and lower the burden of healthcare cost.

Conclusion

Health care faces increasingly complex challenges with the increasing rise of cancer incidence along with unsustainable cost. This thesis contributes to the implementation of targeted treatment strategies by elucidating ovarian cancer behaviour on a molecular level, investigating the therapeutic value of current biomarkers for targeted therapy, and improving patient stratification methods to allocate patients to effective therapies.

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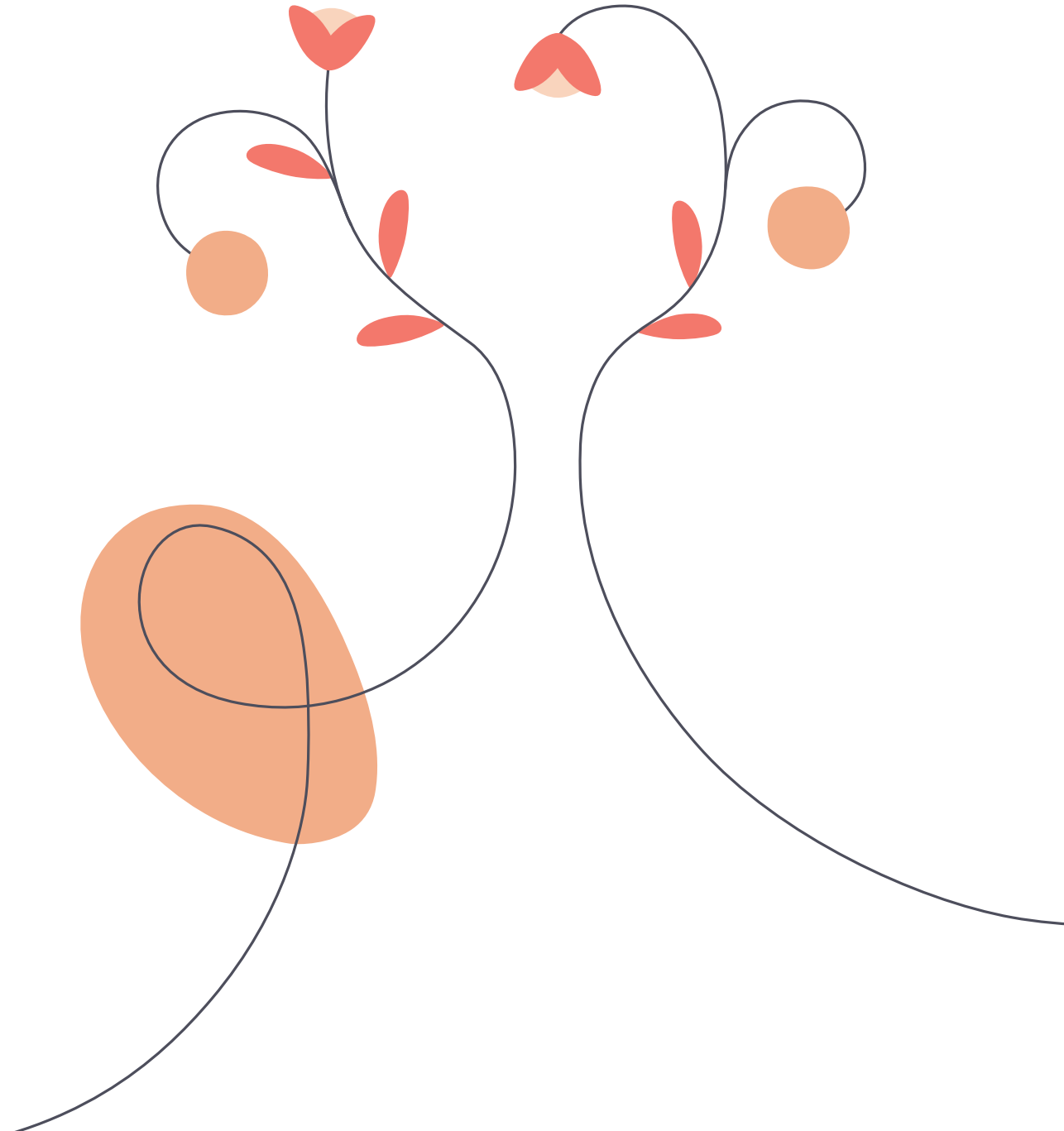

APPENDIX

Abbreviations
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Abbreviations

ACT	Adjuvant chemotherapy
AR	Androgen receptor
BMI	Body mass index
CBR	Clinical benefit rate
CI	Confidence interval
CIN	Cervical intraepithelial neoplasia
CR	Complete response
CTCAE	Common terminology criteria for adverse events
DFS	Disease-free survival
DNA	Deoxyribonucleic acid
ER	Oestrogen receptor
FFPE	Formalin-fixed paraffin-embedded
FOXO	Forkhead box protein O
FSH	Follicle-stimulating hormone
FTE	Fallopian tube epithelium
H&E	Haematoxylin and eosin
HER2	Human epidermal growth receptor 2
HGSC	High-grade serous ovarian carcinoma
HH	Hedgehog
HR	Hazard ratio
HRR	Homologous recombination repair
IDS	Interval debulking surgery
IHC	Immunohistochemistry
IM	Intramuscular
IQR	Interquartile range
IUD	Intra-uterine device
LCM	Laser capture microdissection
LGSC	Low-grade serous ovarian carcinoma
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
MEC-U	Medical research ethics committees united
mRNA	Messenger ribonucleic acid
MTD	Maximum-tolerated dose
mTOR	Mammalian target of rapamycin
NA	Not applicable
NAC	Neoadjuvant chemotherapy
NCR	Netherlands cancer registration
NF-kB	Nuclear factor-kappa B
NGS	Next-generation sequencing

NS	Not significant
ORR	Overall response rate
OS	Overall survival
PARP	Poly(ADP-ribose) polymerase
PET	Positron emission tomography
PDS	Primary debulking surgery
PFS	Progression-free survival
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4, 5)-bisphosphate
PIP3	Phosphatidylinositol (3, 4, 5)-triphosphate
PO	Per oral
PR	Partial response
PRISMA	Preferred reporting items for systematic reviews and meta-analyses
PTEN	Phosphatase and tensin homologue
ROBINS-I	Risk of bias in non-randomised studies of interventions
RT-qPCR	Real-time quantitative reverse transcription-polymerase chain reaction
SD	Standard deviation / Stable disease
SERDs	Selective oestrogen receptor downregulators
SERMs	Selective oestrogen receptor modulators
SOD2	Superoxide dismutase 2
STIC	Serous tubal intraepithelial carcinoma
STMN1	Stathmin 1
STPs	Signal transduction pathways
TGF-β	Transforming growth factor beta
Wnt	Canonical wingless-type MMTV integration site

About the author

Phyllis van der Ploeg werd op 19 oktober 1992 geboren in Nunspeet. Samen met haar ouders Edward en Joyce en zusje Shannon woonden zij vanaf 1997 in Hoogkarspel en verhuisden zij in 2003 naar Oosterhout. In 2011 behaalde Phyllis haar vwo-diploma aan het Monseigneur Frencken College en begon zij aan de opleiding Biomedische wetenschappen aan de Universiteit Maastricht. Na het afronden van deze bacheloropleiding doorliep zij met succes de selectieprocedure van de opleiding Arts-Klinisch Onderzoeker en startte zij in 2014 aan deze vierjarige master aan de Universiteit Maastricht. Het laatste jaar van haar opleiding sloot zij af met een semi-arts stage in het Catharina Ziekenhuis in Eindhoven waar zij kennis maakte met het onderzoek naar Hedgehog signaaltransductie activiteit in hooggradig sereus ovariumcarcinomen onder begeleiding van Dr. Jurgen Piek.

Na het behalen van haar artsendiploma in 2018 werkte zij als arts-onderzoeker verder aan het onderzoek naar de activiteit van signaaltransductiepaden in ovariumcarcinomen in het Catharina Ziekenhuis. Onder begeleiding van prof. Dr. Ruud Bekkers, dr. Jurgen Piek, dr. Anja van de Stolpe en dr. Sandrina Lambrechts groeide het onderzoek uit tot een promotietraject aan de Universiteit Maastricht. De intensieve samenwerking met Molecular Pathway Diagnostics, Philips Research (momenteel InnoSIGN) resulteerde in dit proefschrift.

Van september 2018 tot december 2019 wisselde zij haar onderzoeksactiviteiten af met klinische werkzaamheden als arts niet in opleiding tot specialist (ANIOS) op de afdeling gynaecologische oncologie in het Catharina Ziekenhuis. Per september 2021 woont zij samen met haar vriend Berend-Jan in Amsterdam en werkt zij als ANIOS op de afdeling obstetrie en gynaecologie in het Onze Lieve Vrouwe Gasthuis (OLVG) locatie West.

List of publications

Hendrikse CSE*, Theelen PMM*, **Van der Ploeg P**, Westgeest HM, Boere IA, Thijs AMJ, Ottevanger PB, Van de Stolpe A, Lambrechts S, Bekkers RLM, Piek JMJ. The potential of RAS/RAF/MEK/ERK (MAPK) signaling pathway inhibitors in ovarian cancer: a systematic review and meta-analysis. *Submitted*

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* Dual first author

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