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ABSTRACT

Tubo-ovarian high-grade serous carcinoma (HGSC) is the most common and lethal subtype of ovarian cancer, typically diagnosed at an advanced stage due to absence of specific symptoms and specific clinical biomarkers. Standard treatment has remained relatively unchanged for decades, consisting of primary debulking surgery and platinum-taxane chemotherapy. Usually, the initial treatment response is good, but relapse is common, leading to chemoresistance and death. Recent advances, especially the addition of PARP inhibitors to patients, whose tumors are homologous recombination (HR) deficient, have brought some benefit to patients. Yet, better understanding of the disease and translation of the extensive research into clinical practice is needed. Specifically, prognostic and predictive biomarkers that enable personalized treatment approaches are required to improve outcome of HGSC.

Therapy resistance in HGSC is multifactorial, with cancer stem cells (CSCs) purposed as key contributors. This thesis aimed to identify compounds targeting HGSC cancer cells and specifically CSCs by high-throughput drug screening of patient-derived cell cultures. In further studies, we focused on one of the potential drug compounds, a Wee1 inhibitor and studied its mode of action in more detail. A further objective was to evaluate the expression of four selected known CSC markers in patient tumors and their potential in tissue-based diagnostics by immunohistochemical and *in situ* -hybridization analysis.

Transcriptome analysis of cultured HGSC cells identified eight CSC markers that are associated with stemness features, and in clinical samples with poor prognosis. In the drug screen, the Wee1 inhibitor adavosertib emerged as an effective compound across most tested cell cultures, including those with CSC features. Functional *in vitro* assays revealed that adavosertib exerted broad cytotoxic effects. These effects were observed irrespective of the homologous recombination (HR) signature or functional HR status of the cells. We carried out further analysis of four CSC marker candidates: ALDH1A1, SOX2, MYC, and BMI1, in a tissue micro array to assess their potential as a diagnostic biomarker panel for HGSC. Notably, BMI1 and MYC emerged as individual markers independently associated with reduced platinum free interval and overall survival. Recent studies have indicated that BMI1 is involved in the control of HR which makes it as a potential target for novel therapeutic approaches.

KEYWORDS: HGSC, stemness, CSC, biomarker, HR, BMI1, Wee1, adavosertib

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TIIVISTELMÄ

Korkea-asteinen seroosi munasarjasyöpä (HGSC) on yleisin ja tappavin munasarjasyövän alatyyppi. Epäspesifien oireista ja kliinisten biomarkkereiden puutteen vuoksi tauti diagnosoidaan tavallisesti vasta, kun se on jo lähettänyt etäpesäkkeitä. Perushoitoon kuuluu leikkaus, jota täydennetään platina- ja taksaanisolunsalpaajien yhdistelmähoidolla. Hoitovaste on aluksi hyvä, mutta suurimmalla osalla potilaista tauti uusii ja kehittyy vastustuskykyiseksi annetuille lääkkeille. Huolimatta viimeaikaisista edistysaskeleista, kuten PARP-estäjien lisäämisestä uusiutuneen taudin hoitoon, on hoito säilynyt pääosin muuttumattomana jo vuosikymmeniä. Tarve tehokkaille, spesifeille kliinisille biomarkkereille ja kohdennetuille lääkehoidoille on suuri tämän heterogeenisen ja tappavan taudin hallitsemiseksi. Yhtenä keskeisenä mekanismina hoitoresistenssin kehittymisessä pidetään syövän kantasolujen olemassaoloa.

Tämän väitöskirjatutkimuksen tarkoituksena oli tunnistaa lääkeaineita, jotka kohdentuvat HGSC-soluihin ja erityisesti syövän kantasoluihin. Tutkimuksessa hyödynnettiin lääkeaineiden tehoseulontatekniikkaa ja potilaslähtöisiä HGSC-soluviljelmiä. Lisäksi tavoitteena oli arvioida kymmenen syövän kantasolumarkkerin biomarkkeripotentiaalia potilaskudoksissa ja avoimien tietokantojen *mRNA*-aineistoissa, ja tutkia neljän syövän kantasolumarkkerin (ALDH1A1, SOX2, MYC ja BMI1) ilmentymistä HGSC-monikudosblokeissa suhteessa kliiniseen potilastietoon.

mRNA-analyysien avulla tunnistimme kahdeksan syövän kantasolumarkkeria, joilla oli yhteys huonoon hoitovasteeseen. Näistä neljää (ALDH1A1, SOX2, MYC ja BMI1) tutkittiin proteiinitasolla monikudosblokkitutkimuksessa, ja havaittiin, että BMI1 ja MYC olivat itsenäisinä markkereina ennusteellisesti merkittäviä. Korkea BMI1- ja MYC-ilmentyminen ennustivat lyhyttä elossaoloaikaa. Lääkeaineiden tehoseulontatekniikalla tunnistimme Wee1-inhibiittori adavosertibin tehokkaaksi sekä syövän kantasolutyyppisissä (3D) että perinteisissä (2D) soluviljelmissä. Funktionaalisissa *in vitro* -kokeissa adavosertibillä osoitettiin olevan laajoja sytotoksisia vaikutuksia, ja oli tehokas HGSC-soluissa niiden homologisen rekombinaation statuksesta riippumatta.

AVAINSANAT: HGSC, syövän kantasolu, biomarkkeri, HR, BMI1, Wee1

Table of Contents

Abbreviations

ABCB1	ATP binding cassette subfamily member 1
AEC	3-amino-9-ethylcarbazole
ALDH1A1	aldehyde dehydrogenase isoform 1
AP	alkaline phosphate
APC/C	anaphase-promoting complex/cyclosome
ARF	ADP-ribosylation factor
ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia and Rad3-related
BARD1	BRA1-associated RING domain protein 1
BER	base excision pathway
BMI1	B-cell-specific Moloney murine leukemia virus integration region 1
PCGF4	polycomb group finger 4
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
BRIP1	BRCA interacting protein 1
CA-125	Cancer Antigen 125
CAN	copy number alteration
CBX	chromodomain protein
CCNE1	cyclin E1
CDK	cyclin dependent kinase
CIC	cortical inclusion cyst
CIN	chromosomal instability
CISH	chromogenic in situ hybridization
CK	cytokeratine
CKI	CDK inhibitor
CNA	copy number alteration
CNV	copy number variation
CPH-I	Copenhagen index
CTC	circulating tumor cell
ctDNA	ctDNA
CSC	cancer stem cell
Dab	3,3'-diaminobenzidine

DDR	DNA damage response
DSB	doble-strand break
DSS	drug sensitivity score
EGFR	epidermal growth factor receptor
EGR1	Early growth response protein 1
EMA	European Medical Agency
ENL	transcription elongation factor
EMT	epithelial-to-mesenchymal transition
EOC	epithelial ovarian cancer
FACS	fluorescence-activated cell sorting
FDA	US Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FIGO	International Federation of Gynecology and Obstetrics
FIMM	Institute for Molecular Medicine Finland
FISH	fluorescent in situ hybridization
FITC	fluorescence isothiocyanate
HE	hematoxylin-eosin
HE4	Human Epidydymis Protein 4
HER2	epidermal growth factor receptor 2
HER3	epidermal growth factor receptor 3
HGSC	high-grade serous carcinoma
HR	homologous recombination
HRD	homologous recombination deficiency
HRP	homologous recombination proficient
HRP	horseradish peroxidase
HRR	HR-repair
HTS	high-throughput screening
HUS	Helsinki University Hospital
IDS	interval debulking surgery
IF	immunofluorescence
IGF-2	Insulin-like growth factor 2
IHC	immunohistochemistry
ISH	in situ hybridization
Ki-67	marker of proliferation Kiel 67
KM	Kaplan-Meyer
lncRNA	long non-coding RNA
LOH	loss of heterozygosity
LST	large-scale state transition
MAD2	mitotic arrest deficient 2
MDM2	mouse double minute 2 homolog
MDR	multi drug resistance

MDR1	multi drug resistance protein; P-glycoprotein (P-gp)
MMR	mismatch repair
MYT1	membrane associated tyrosine- and threonine-specific cdc2-inhibitory kinase
NACT	neoadjuvant chemotherapy
NCI	National Cancer Institute
NER	nucleotide excision repair
NGS	next generation sequencing
NHEJ	non-homologous end-joining
OC	ovarian cancer
OSE	ovarian surface epithelium
OS	overall survival
PALB2	partner and localizer of BRCA2
PARP	poly (ADP ribose) polymerase
PARPi	PARP inhibitor
PAX8	paired gene box 8
PcG	polycomb group protein family
PCGF	polycomb group finger
PCR	polymerase chain reaction
PDGRA	plateled-derived growth factor receptor alpha
PDS	primary debulking surgery
PFI	platinum free interval
PFS	progression free survival
P-gp	P-glycoprotein
PIKK	phosphatidyl inositol 3-kinase-like family of serine/threonine protein kinases
PRC	polycomb repressive complex
PRC1	polycomb repressive complex 1
PRC2	polycomb repressive complex 2
PTEN	phosphatase and tensin homolog
Rb	retinoblastoma
RMI	risk of malignancy index
ROCA	risk of ovarian cancer algorithm
RNA-ISH	RNA in situ -hybridization
RPA	replication protein A
sDSS	specific drug sensitivity score
SOX2	(sex determining region Y)-box 2
SSB	single-strand break
ssDNA	single stranded DNA
STIC	serous tubular intra-epithelial carcinoma
TAI	telomeric allelic imbalance

TCGA	The Cancer Genome Atlas
TIS	therapy-induced senescence
TLR4	Toll-like receptor 4
TMA	tumor micro array
Twist	Twist-related protein 1
Valvira	The Finnish National Supervisory Authority for Welfare and Health
VEGF-A	vascular endothelial growth factor A
Wee1	Wee1 G2 checkpoint kinase
Wee1i	Wee1 inhibitor
WES	whole exome sequencing
WHO	World Health Organization
WT	wild type
WT1	Wilms tumor suppressor protein 1
53PB1	p53-binding protein 1

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Katja Kaipio, Ping Chen, **Pia Roering**, Kaisa Huhtinen, Piia Mikkonen, Päivi Östling, Laura Lehtinen, Naziha Mansuri, Taina Korpela, Swapnil Potdar, Johanna Hynninen, Annika Auranen, Seija Grénman, Krister Wennerberg, Sampsa Hautaniemi, Olli Carpén. ALDH1A1-related stemness in high-grade serous ovarian cancer is a negative prognostic indicator but potentially targetable by EGFR/mTOR-PI3K/aurora kinase inhibitors. *The Journal of Pathology*, 2019; 2: 159-169.

II **Pia Roering**, Arafat Siddiqui, Vanina D. Heuser, Swapnil Potdar, Piia Mikkonen, Jaana Oikkonen, Yilin Li, Sanna Pikkusaari, Krister Wennerberg, Johanna Hynninen, Seija Grénman, Kaisa Huhtinen, Annika Auranen, Olli Carpén and Katja Kaipio. Effects of Wee1 inhibitor adavosertib on patient-derived high-grade serous ovarian cancer cells are multiple and independent of homologous recombination status. *Frontiers in Oncology*, 2022; 2.

III **Pia Roering***, Lilla Csellar*, Sami Blom, Iona Raineva, Kia Colangelo, Anna Laury, Vanina D. Heuser, Olli Carpén. High expression of the multifunctional proto-oncogene BMI1 predicts poor outcome in high-grade serous carcinoma. *Manuscript,* 2025.

*Equal contribution

1 Introduction

Tubo-ovarian high-grade serous carcinoma (HGSC) is the most common and lethal subtype of ovarian cancer (Siegel et al., 2023). It is typically diagnosed at an advanced stage due to absence of specific symptoms. Standard treatment consists of primary debulking surgery (PDS) and platinum-taxane chemotherapy. If the disease is primarily inoperable, neoadjuvant chemotherapy (NACT) is given before interval debulking surgery (IDS). Usually, initial treatment response is good, but relapse and eventual chemoresistance is common (Bast, 2011; Hennessy et al., 2009).

HGSC is characterized by universal loss of function mutation in *P53* gene, cell cycle checkpoint G1/S inactivation, loss of DNA-damage response (DDR) and genomic instability (Bowtell, 2010). These features cause intratumoral heterogeneity and foster the cancer cells` adaptability to chemotherapy leading to drug resistance (Patch et al., 2015).

Introduction of poly-adenosine diphosphate ribose polymerase (PARP) inhibitors to relapsed patients with homologous recombination deficiency (HRD) of which 80% are caused by mutations in BRCA1/2 and other HRD genes (i.e. Fanconi Anemia pathway and mis-match repair) or epigenetic changes has brought therapeutic opportunities to HRD patients (Berek et al., 2021; Franzese et al., 2019; Konstantinopoulos et al., 2015). Targeting the DDR-pathways and the cell cycle checkpoints in combination with traditional chemotherapy agents increases the intrinsic genomic instability in the cancer cells leading to cell death. Despite recent advantages, the need for effective biomarkers and targeted therapies to enable personalized treatment approaches remains critical to improve outcomes in this heterogeneous and aggressive disease.

This thesis aimed to identify compounds targeting HGSC cells and to identify cancer stem cell (CSC) features in HGSC. Therapy resistance in HGSC is multifactorial, with CSCs proposed as key contributors, positioning them as promising targets for novel therapeutic strategies. Potential therapeutic drug compounds for HGSC were identified through high-throughput drug screening of patient-derived cell cultures to evaluate individualized drug responses. The most promising candidates were further evaluated using functional *in vitro* assays, including analysis of cell proliferation, invasion, and migration, as well as assessments of nuclear morphology and DNA integrity. Additionally, we compared

cytotoxic responses between homologous recombination proficient (HRP) and HRD cell cultures to explore potential stratification of HGSC subgroups for personalized treatment. A parallel screening approach was employed to specifically identify drug compounds effective against cell cultures exhibiting stemness phenotype. To achieve this, we used a specialized culture medium designed to sustain stemness features in the cells.

In addition to identify novel drug compounds for HGSC, this thesis aimed to evaluate the expression of CSC markers in patient tissues to assess the potential of HGSC associated stemness markers for clinical decision making and their potential as drug targets. For this, a tissue micro array (TMA) constructed from archived HGSC tissue samples was utilized. Expression of four potential candidate CSC markers was investigated and analyzed with clinical information of the patients to evaluate the clinical significance of these markers.

2 Review of the Literature

2.1 Ovarian cancer

Tubo-ovarian cancer is the most common cause of death among cancers of the female reproductive tract. Tubo-ovarian cancer compromises malignant epithelial and non-epithelial tumors originating from both the ovary and the fallopian tube (Soslow et al., 2025). Due to historical and epidemiological reasons, the term "ovarian cancer" (OC) is commonly used. The incidence of OC varies depending on ethnicity, geographical location, and level of economic development (Lisio et al., 2019). Globally, OC was the eight most common cancer in women in 2020 (Webb & Jordan, 2024). In Finland, the incidence of OC was 19.5 per 100000 women, with a total of 550 new cases diagnosed in 2023 (Finnish Cancer Statistics - Suomen Syöpärekisteri, 2025). Among the female cancers in the United States, it was the fifth leading cause of cancer-related deaths in 2023 (Siegel et al., 2023).

2.1.1 Anatomy and histology of adnexa

The female reproductive organs consist of ovaries, fallopian tubes, uterus, vagina, and vulva (Rosner et al., 2024) (Figure 1). The ovaries and fallopian tubes are surrounded by a thick peritoneal layer that is linked to the pelvic sidewalls. Ovaries consist of the outermost cortex and the inner medulla. The covering layer of simple cuboidal germinal epithelial cells surrounds the ovary and builds the ovarian surface epithelium (OSE) and a dense tunica albuginea beneath the OSE supports the structure (Gibson & Mahdy, 2023). The cortex consists of oocytes in various stages of development and the central medulla is filled with loose connective tissue with blood vessels, nerves, and lymphatics (S.-X. Zhang, 1999). Histologically, the fallopian tube is composed of three segments: mesosalpinx; the serosal layer with mesothelial cells, myosalpinx; the smooth muscle layer, and endosalpinx; the mucosal inner layer that consists of ciliated cells and secretory peg -cells. Anatomically, it can be divided into three parts: istmus is near the uterus, ampulla as the middle segment, and infundibulum that consists mostly of branching structures called fimbriae that surround the ovary (Han & Sadiq, 2023) (Figure 1). The ovaries and the fallopian tubes together with the supporting ligaments are called the adnexa.

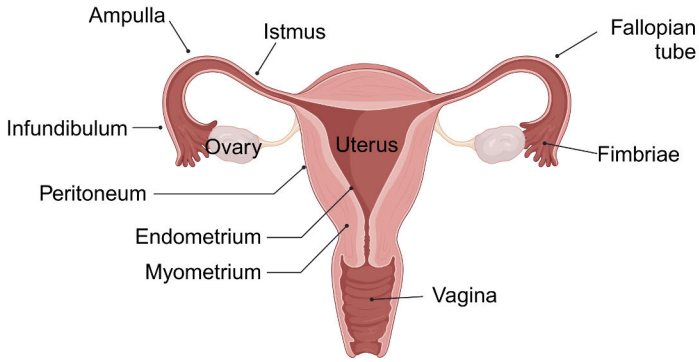


Figure 1: Anatomy of the female reproductive organs. Created with BioRender.

2.1.2 Classification

The classification of OC has undergone modifications during the recent years. According to the World Health Organization (WHO), ovarian tumors can be divided into six classes: epithelial, mesenchymal, mixed, sex cord stromal, germ cell, and miscellaneous (Soslow et al., 2025).

Epithelial ovarian cancers (EOCs) are the most common type of ovarian tumors consisting of approximately 80% of ovarian cancers. EOCs are further divided into serous-, mucinous-, endometrioid-, clear cell-, seromucinous-, and Brenner-tumors along with the other carcinomas (Figure 2). (Berek et al., 2021)

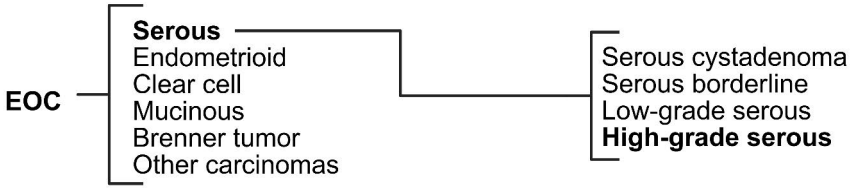


Figure 2: Classification of epithelial ovarian cancer subtypes and serous subtypes.

2.1.3 Staging and grading

Ovarian cancer staging was revised in 2014 when the FIGO (International Federation of Gynecology and Obstetrics) system was updated to incorporate ovarian, fallopian tube, and peritoneal cancers under a unified staging (Prat, 2014). This system uses three characterizing factors of the disease: 1) the size, location, and extent of the primary tumor in the ovaries, 2) spread of the cancer to lymph nodes, and 3) spread of the cancer to other organs (Figure 3). A clinical examination is performed prior to surgery, but the final staging is determined through surgical sampling of multiple sites within the pelvis and beyond. In addition to biopsies, cytological samples are collected

from peritoneal washings. The confirmation of the staging is done by a pathologist who histologically confirms the staging. FIGO staging divides EOCs into four main groups, stage I to IV (Renz et al., 2025). Low grade EOCs are stage I and II cancers (Figure 3). High grade cancers are spread outside the pelvic region in the whole abdomen to the peritoneum or peritoneal lymph nodes (stage III) or beyond the abdomen in distant parts of the body, outside the peritoneum (stage IV) (Figure 3).

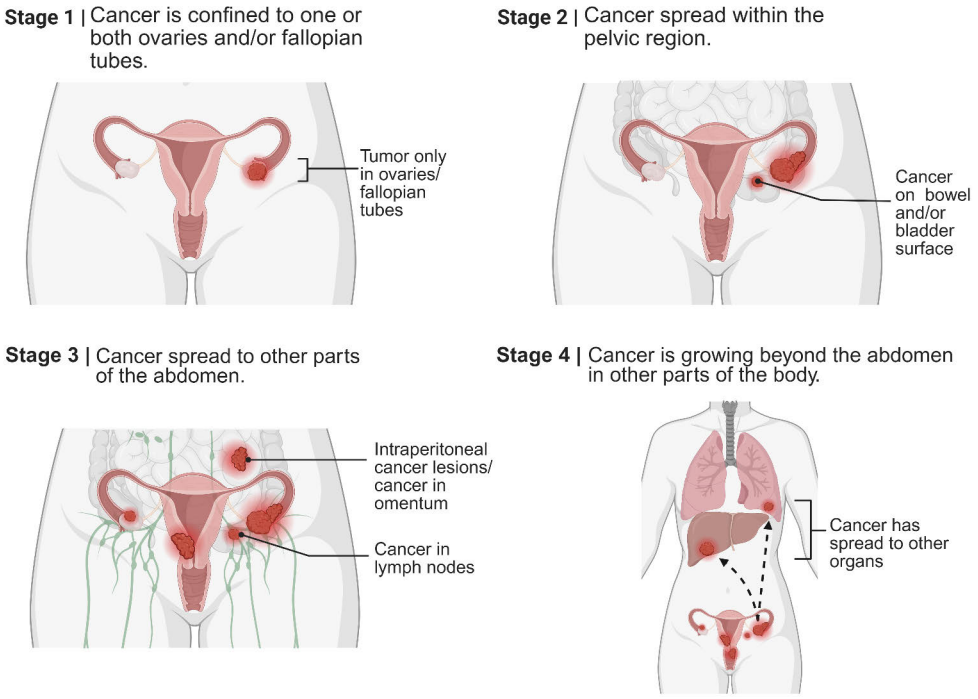


Figure 3: Stages of ovarian cancer. Created with BioRender.

2.2 High-grade serous carcinoma

Based on their morphology and molecular properties serous epithelial ovarian cancers (EOCs) are classified into four subtypes (Figure 2). High-grade serous carcinoma (HGSC) is the most common, aggressive, and lethal and constitutes about 70% of the malignant serous tumors (Kurman & Shih, 2016). Other serous tumors are serous cystadenoma, serous borderline tumor, and low-grade serous carcinomas (Figure 2)(Soslow et al., 2025). HGSC tumors can be further classified into molecular subtypes, as characterized in the Cancer Genome Atlas (TCGA) study (Bell et al., 2011), which exhibits distinct patterns of treatment resistance and prognostic outcomes. These four molecular subtypes are: immunoreactive, proliferative, differentiated, and mesenchymal (McMullen et al., 2020; Prat et al., 2018; Vang et

al., 2016). However, this molecular classification is not in clinical use. To date, clinically applicable histological, immunohistochemical, or other molecular methods have not been established to reliably distinguish between these molecular subtypes of HGSC.

2.2.1 Epidemiology, incidence and risk factors

HGSC is usually detected at the advanced stage with pelvic and extrapelvic metastases. It occurs usually in postmenopausal women and in the US the median of diagnosis is 63 years (National Cancer Institute NIH, 2024). The 5-year survival rate is 30-50% depending on the stage at diagnosis. Early detection and advances in genetic testing, targeted therapy and personalized medicine have improved management, but long-term survival remains challenging. HGSC accounts for approximately 70% of all OC deaths making it the most lethal form of OCs (J. Kim et al., 2018).

While most HGSC cases are sporadic, family history of ovarian or breast cancer, caused by genetic susceptibility is the most significant risk factor. Genetic mutations occur in DNA-repair pathway genes, most commonly in homologous recombination (HR) associated molecules (e.g., *BRCA1*, *BRCA2*, *RAD51C*, *BRIP1* and *PALB2*) (Kulkarni et al., 2025; Suszynska et al., 2020). Other risk factors are less clearly established. Protective factors include parity, hormonal contraceptive use, breastfeeding, and older age at pregnancy. The common cancer associated lifestyle risk factors like smoking, high body mass index or diet are not significant in HGSC (Webb & Jordan, 2024).

2.2.2 Origin

Earlier, it was thought that HGSC originates from ovarian surface epithelium (OSE) or so-called cortical inclusion cysts (CICs) (Ahmed et al., 2013; Lisio et al., 2019). However, current view is that a great majority, if not all HGSC tumors originate from the distal region of fallopian tubes. Early evidence dates back almost two decades (Crum et al., 2007; Kurman & Shih, 2011, 2010). Small dysplastic lesions like HGSC or the so called STICs (serous tubular intra-epithelial carcinomas) were found in the patients with inherited *BRCA*-mutations that underwent risk-reducing salpingo-oophorectomy (Kuhn et al., 2012; Kurman & Shih, 2016; Piek et al., 2001). STICs were first found only in women with inherited *BRCA* mutation, but later also in patients without these mutations in the *BRCA* genes (Berek et al., 2021). Along with this initial discovery of the lesions, molecular profiling studies indicate that the main origin of HGSC is the distal fallopian tube fimbria (Kindelberger et al., 2007; Kuhn et al., 2012; National Cancer Institute, 2017; Piek et al., 2001, 2004). Today the

understanding of the origin of HGSC is based on these findings and the STICs are considered as precursors (Bowtell, 2010; Prat et al., 2018) (Figure 4).

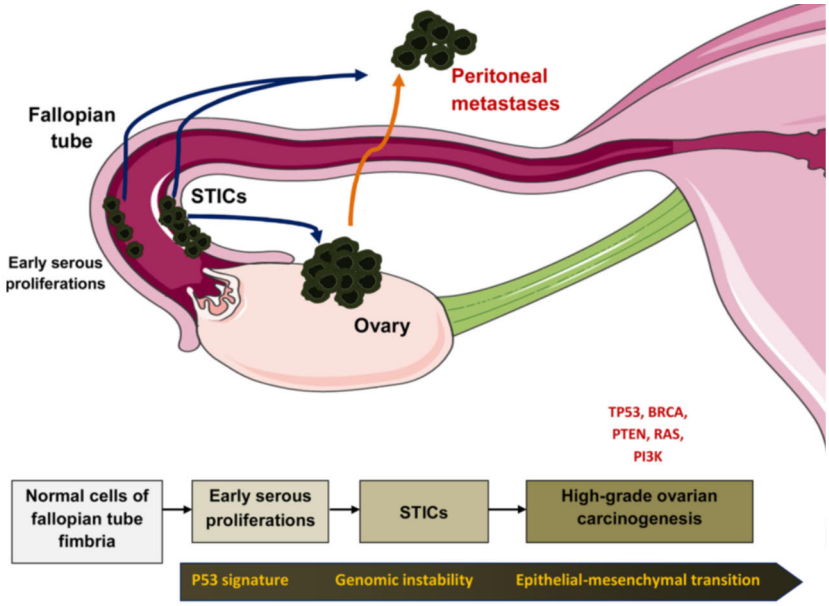


Figure 4: Formation of serous tubular intra-epithelial carcinomas and high-grade serous carcinoma. Modified from (El Bairi et al., 2021).

2.2.3 Clinical features

The symptoms of HGSC are often nonspecific and can resemble gastrointestinal or urinal discomfort. Patients frequently report symptoms of abdominal pressure, constipation, bloating, and lack of appetite. By the time the patient seeks medical help, the disease is at an advanced stage (III/IV) in most cases (Berek et al., 2021). Usually, a pelvic mass and ascites fluid is present in a physical exam in the abdomen. Diagnosis is done by imaging by transvaginal ultrasound and computer tomography scanning/magnetic resonance imaging (CT/MRI) to evaluate the pelvic and ovarian masses. Commonly used cancer biomarkers measured from blood are Cancer Antigen 125 (CA-125) and Human Epididymis Protein 4 (HE4). These markers lack specificity for HGSC cells but are well suited for monitoring treatment response and relapse.

2.2.4 Histopathology

The histopathology of HGSC is characterized by heterogeneity within the tumor. Architecture of the HGSC tissue can be solid, papillary, glandular, labyrinth- or epithelial-like (Figure 5). Cytology is marked by atypic nuclei with high mitotic

activity. Compared to the volume of the cell cytoplasm, the size of the nucleus is often large with prominent nucleoli. Necrotic areas in the tissue are common as well as multinuclear cells. (Kumar et al., 2021; Mäkinen et al., 2024; Soslow et al., 2025) Examples of HGSC histopathology are in Figure 5.

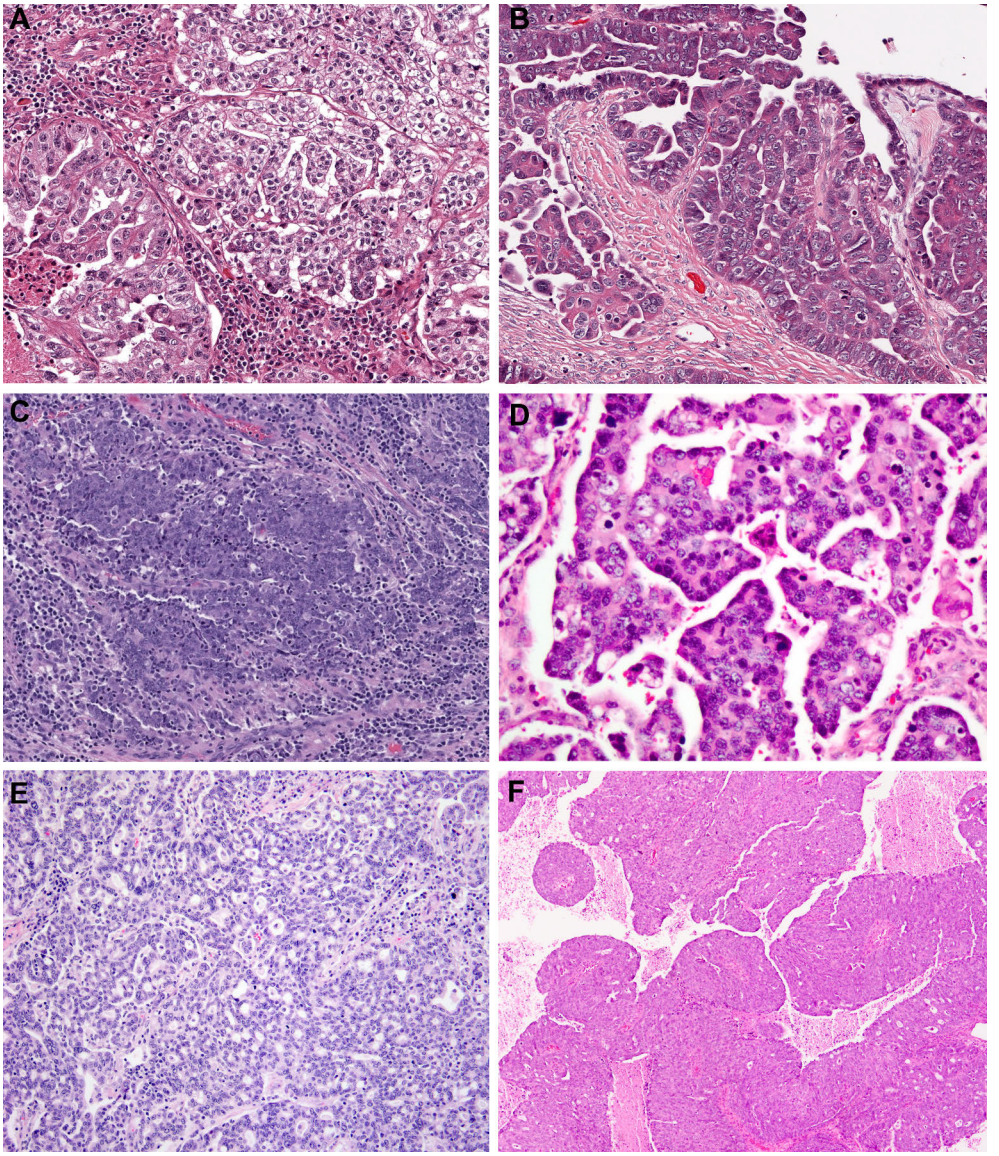


Figure 5: Hematoxylin-eosin stained HGSC tumor tissues heterogeneity. A) Prominent clear cell change, B) Papillary architecture, C) Solid architecture, with lymphocytes, D) Papillary structures, with cells containing conspicuous mitotic activity, E) Glandular architecture, F) Solid architecture, with endometrial and transitional cell-like architecture. Adopted and modified from (Soslow et al., 2025)

2.3 Treatment and surveillance of HGSC

The standard treatment for HGSC has remained similar for several decades and consist of surgical resection and chemotherapy. In the 1980s, platinum agents were introduced as a standard chemotherapy (Dasari & Bernard Tchounwou, 2014; Ghosh, 2019; Kelland, 2007). The addition of cisplatin showed improvement in treatment response over non-platinum agents (Aabo et al., 1998). In the 1990s, carboplatin became widely used. It is a derivate from cisplatin and is more tolerable for patients. During the same decade, the standard combination therapy of carboplatin and paclitaxel was established and several large-scale clinical trials on advanced OC were conducted that showed increased overall and progression free survival. (Martin, 1993) In the 2000s, weekly paclitaxel and intraperitoneal chemotherapy was introduced for advanced stage patients (Thomas & Rosenberg, 2002).

2.3.1 Standard treatment

There are two standard treatment strategies for HGSC in Finland that both consist of a cytoreductive surgery and intravenous chemotherapy (Figure 6). If the cancer is initially operable the treatment starts with a primary debulking surgery (PDS) where all visible tumor mass is removed. The surgery is followed by several cycles (usually six) of combination adjuvant chemotherapy. Paclitaxel and carboplatin are administered in cycles every three weeks (Karam et al., 2017). If the disease is not primarily operable, neoadjuvant chemotherapy (NACT) of three to four rounds is implemented to reduce the tumor load. After NACT an interval debulking surgery (IDS) is performed. Total tumor debulking is the objective, and the amount of residual disease is the most important prognostic factor (Berek et al., 2021). If the tumor burden is big and/or residual disease after surgery is left, additional rounds of standard chemotherapy are considered.

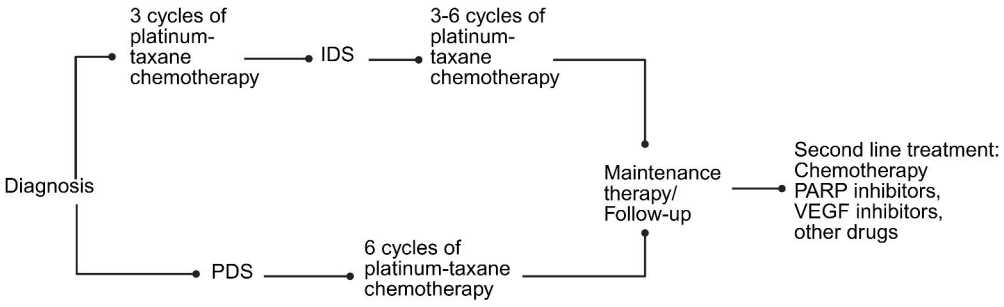


Figure 6: Treatment strategy of ovarian cancer in Finland.

2.3.2 Targeted therapies

Bevacizumab, an anti- vascular endothelial growth factor A (VEGF-A) antibody is approved for primary and recurrent platinum-resistant HGSC. Bevacizumab act as an anti-angiogenic drug and is shown to extend progression-free survival (PFS). However, the impact on overall survival (OS) is modest. (Dinkins et al., 2024; Konstantinopoulos et al., 2015; Lliberos et al., 2024)

For HGSC patients with mutations in BRCA genes PARP inhibitors (PARPi) can be used as targeted therapy. They have shown a promise of prolonging progression free survival. Three PARPis, olaparib, niraparib, and rucaparib, are US Food and Drug Administration (FDA) approved and in clinical use for recurrent HGSC also in Finland. All of them have shown consistent improvement in PFS in clinical trials (Kulkarni et al., 2025). The first European Medical Agency (EMA) approved PARPi was olaparib in 2014 for patients with recurrent, platinum sensitive disease and BRCA1/2 mutations (Arora et al., 2021). Two years later rucaparib and three years later niraparib were approved both by the FDA and EMA (Balasubramaniam et al., 2017; Ison et al., 2018). Molecular and genetic features associated to PARP inhibitors are described in more detail in section 2.4.4.1.

Emerging therapies targeting the cell cycle, its control, and DNA damage control are under investigation and in clinical trials in solid cancers including HGSC. Wee1 inhibitor adavosertib is one of the compounds that has given promising results, both *in vitro* and in clinical trials (Thangaretnam et al., 2025). Targeting Wee1 in HGSC and its biological function in cell cycle are described in section 2.4.6.

2.3.3 Surveillance

The most important routine clinical blood-based biomarker in HGSC is CA-125, a glycoprotein encoded by the gene *MUC16* (Felder et al., 2014). It is produced by several types of cells, including those lining the peritoneum, endometrium, fallopian tubes, pleura, and pericardium. The CA-125 level is elevated in 90% of serum samples of women with advanced HGSC. However, CA-125 is not specific for HGSC or other OC and can be elevated also in non-malignant situations like menstruation, endometriosis, or pregnancy (Charkhchi et al., 2020). Still, CA-125 is a good indicator of response to chemotherapy and in follow-up of disease recurrency. This makes it a biomarker that is routinely monitored in OC patients. Of the early-stage OC patients, only 50% show increased CA-125 (Bates, Mohamed, et al., 2024), which reduces its potential as a screening tool for early detection.

HE4 is another glycoprotein that is sometimes measured in monitoring of HGSC recurrence and progression. It is encoded by *WFDC2* gene. High preoperative levels of HE4 are associated with advanced disease and poor prognosis. (Bates, Mohamed, et al., 2024)

Today, numerous liquid biopsy biomarkers, such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), exosomes, microRNAs (small non-coding RNAs), and long non-coding RNAs (lncRNAs), are under intense investigation as biomarkers for various cancer types, including HGSC. (Bates, Mohamed, et al., 2024)

2.4 Genetics and molecular features of HGSC

HGSC is characterized by mutations in *TP53*, inactivation of homologous recombination (HR) pathway and extensive copy number alterations. In large-scale genomic analyses of ovarian, fallopian tube, and peritoneal high-grade serous carcinomas, *TP53* was mutated in 96%, while *BRCA1* or *BRCA2* mutations were found in 22% of the cases (Bell et al., 2011). Overall, the HR-pathway is altered in around 50% of the tumors due to mutations or epigenetic changes (Konstantinopoulos et al., 2015).

An additional characteristic genomic feature is high genomic instability. In contrast, oncogenic driver mutations, common in many other cancer types, are rare. (Bowtell et al., 2015; Konecny et al., 2014; Lliberos et al., 2024; Verhaak et al., 2013)

Aneuploidy, aberration of whole chromosomes, or gains and losses of chromosome arms has been investigated as mechanisms contributing to cancer initiation in different solid cancer types (Ben-David & Amon, 2019). Chromosomal instabilities (CINs), including amplifications, deletions, and rearrangements, are widespread in solid tumors, including HGSC. DNA copy number variations (CNV) are substantial in HGSC (Bell et al., 2011), and are associated with poor outcomes in many cancers including ovarian cancer (Macintyre et al., 2018; P. Smith et al., 2023).

Accurate characterization of the subtype-specific molecular features and epigenetic alterations is essential for the integration into clinical decision-making. Although, molecular classification of the HGSC tumors has not been yet adopted to clinical practice, emerging evidence show that, for example, the immunoreactive subtype might respond to immunotherapy (Berry et al., 2024).

2.4.1 Cell cycle

HGSC is often driven by dysregulation of the cell cycle due to aberrant control of cell cycle check points. The propagation of one cell cycle phase to the next is strictly guarded by specific cell cycle checkpoints: G1/S-, G2/M-, and M/G1-checkpoint (Alberts et al., 2008) (Figure 7). In each of these checkpoints the cell ensures that the previous phase is completed without errors, and only thereafter can be transferred to the next phase. Among the important drivers and regulators of these checkpoints are the cyclin dependent kinases (CDKs) that are activated by specific cyclins. CDK1 is the only CDK that is essential for cell cycle progression (Alberts et al., 2008). The main cyclin classes are D, E, A, and B. Of these, cyclin B is located in the cytoplasm

while the others are mainly located in the nucleus. (Satyanarayana & Kaldis, 2009) CDK levels are somewhat stable throughout the cell cycle, in contrast to the cyclin levels that are fluctuating and therefore cyclically activate CDKs. Cyclin E-CDK2 activity drives the cell to S phase (Alberts et al., 2008). Cyclin A- CDK1/CDK2 activity further drives the cell cycle towards mitosis, and cyclin B-CDK1 activity is responsible for mitosis initiation and continuation (Leal-Esteban & Fajas, 2020) (Figure 7).

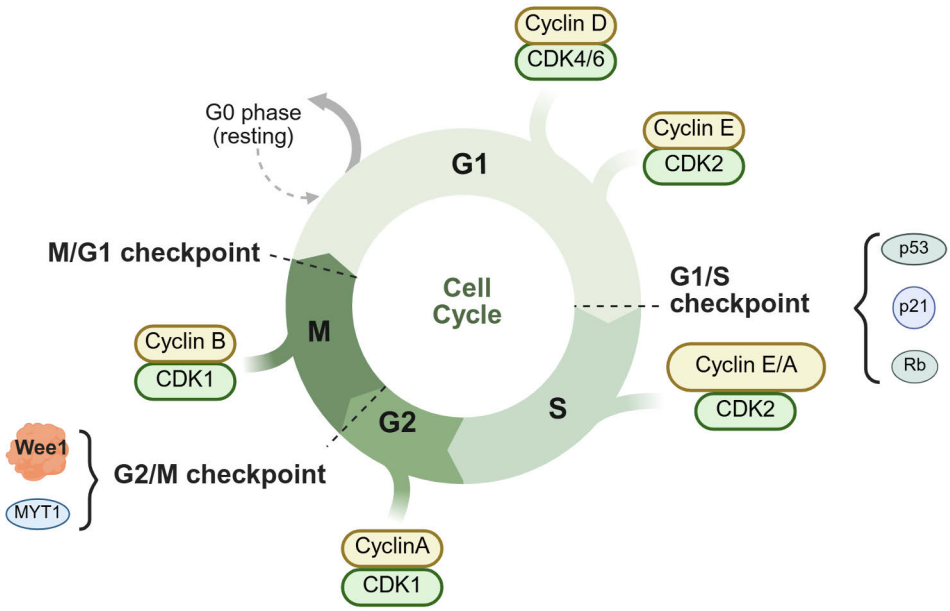


Figure 7: Cell cycle checkpoints and cell cycle regulating cyclins and kinases. Created with BioRender.

2.4.1.1 G1/S checkpoint

G1/S checkpoint is regulated by a tumor suppressor, retinoblastoma (Rb) protein. Mitogenic stimulation causes the G1 cyclin-CDK-complex (cyclin D-CDK4/6) to accumulate and inactivate Rb. This event releases transcription factors E2F, that are responsible for coding for proteins needed in DNA replication in the S phase (for example cyclin E). Another G1/S checkpoint regulatory protein is p21, a CDK inhibitor (CKI) and p53 target protein. This CKI is binding to and keeping the cyclin E-CDK2 complexes inactive if there is growth inhibitory signaling via p53 in the G1/S checkpoint (Otto & Sicinski, 2017). When growth inhibitory signaling ceases, the cyclin E-CDK2 complex is active by sequestering p21 by cyclin D-CDK4/6 complexes. The active cyclin E-CDK2 complexes are further phosphorylating Rb that

becomes inactive leads to S phase entry. (Leal-Esteban & Fajas, 2020; Alberts et al., 2008; Otto & Sicinski, 2017)

Cyclin E/A-CDK2-complex is active in initiating the replication firing in the replication origins in S-phase. Replication initiation is co-controlled by several checkpoint kinases, such as ATR, Wee1, and CHK1 (Otto & Sicinski, 2017). These kinases inhibit the CDKs and keep the origin firing at a controlled level. Inhibition of these kinases leads to uncontrolled initiation of replication and can cause severe problems during replication. CDK1 binds to and is activated by cyclin A1 and cyclin B (Otto & Sicinski, 2017). The cyclin A-CDK1 activity builds up during G2 phase, and cyclin A is degraded upon entry into mitosis. (Ghelli Luserna Di Rorà et al., 2020; Otto & Sicinski, 2017).

As the ATR/CHK1/Wee1 axis has potential as a target due to the uncontrolled cell cycle progression in several cancers it is of interest for combinatorial treatments. A few ATR and Wee1 inhibitors have shown promising results in clinical trials, whereas the CHK inhibitors have shown disappointing results with severe side effects (Hillmann et al., 2025).

2.4.1.2 G2/M checkpoint

Cyclin B-CDK1 complex is the main promoter of mitotic entry. The cyclin B levels rise gradually during S- and G2 phases due to CDK activity on several transcription factors (Ghelli Luserna Di Rorà et al., 2020). The transition regulation from S phase to G2 is not fully understood. Some studies (Eykelenboom et al., 2013; Katsuno et al., 2009; Saldivar et al., 2018) suggest that the S- to G2 phase is regulated by Ataxia-telangiectasia and Rad3-related (ATR). ATR checkpoint kinase is activated by DNA damage and thereby arrests the cells to S-phase by inactivating the CDK-pathway by inactivating CDC25. (Lindqvist et al., 2009) The combined transcription and protein degradation of cyclin B ensures the high cyclin B expression is limited to G2 and early steps in the mitosis. An active cyclin B degradation is regulated by the anaphase-promoting complex/cyclosome (APC/C), which is active in late mitosis. This degradation by poly-ubiquitinylation of mitogenic regulators occurs simultaneously with the transcription (Ghelli Luserna Di Rorà et al., 2020; Otto & Sicinski, 2017). CDK1 activity is inhibited by kinases membrane associated tyrosine- and threonine-specific cdc2- inhibitory kinase (MYT1) and Wee1 (Otto & Sicinski, 2017). This phosphorylation is relieved by CDC25 phosphatases. Wee1 function in cell cycle is discussed in more detail in chapter 2.4.6.1.

2.4.2 *TP53*

Aberrantly functioning of tumor suppressor p53 is strongly associated with increased cancer risk and is a hallmark of HGSC (Bowtell, 2010). It is essential for the Rb-

pathway and cell cycle checkpoint G1/S (Figure 7). Mutations in the p53 encoding gene *TP53* are typically missense mutations and produce a dysfunctional protein that can have a dominant negative effect or gain-of-function properties that lead to loss of tumor suppressor activity, genomic instability, and resistance to apoptosis (Bowtell, 2010). Functional domains of p53 are N-terminal transactivation domains, the central sequence specific DNA-binding domain, and the C-terminal regulatory domain (Vousden & Lane, 2007). Mutations are usually found in the central DNA-binding domain, and they are typically a sign of aggressive high-grade disease and can result in accumulation of several other mutations or abnormal genomic structures (Vousden & Lane, 2007).

The best described cellular response functions of p53 are related to regulation of expression of genes mediating cell-cycle arrest, senescence, and apoptosis. During normal development p53 acts as an important inhibitor of cell growth. The activity of p53 is regulated by several different mechanisms. The regulatory proteins include ubiquitin ligases that control the stability, enzymes controlling the post-translational modifications (kinases and acetylases), as well as transcriptional coactivators. (Harris & Levine, 2005; Hernández Borrero & El-Deiry, 2021; Vousden & Lane, 2007)

Several stress signals activate the p53-pathway, including genotoxic stress, oncogene activation, telomere erosion, hypoxia, and nutrient deprivation (Harris & Levine, 2005). The type of stress dictates the affected pathway and signaling molecules that activate p53. ADP-ribosylation factor (ARF) is a protein that inhibits Mouse double minute 2 homolog (MDM2) and participates in the response to some oncogenes, while it is not needed in DNA-damage stress response activation of p53 (D'Souza-Schorey & Chavrier, 2006; Hernández Borrero & El-Deiry, 2021). MDM2 is an important ubiquitin ligase that degrades the p53 and limits the level of p53 in normal cells. (Vousden & Lane, 2007)

2.4.3 *BRCA1/2*

Breast cancer gene 1 (*BRCA1*) and Breast cancer gene 2 (*BRCA2*) are tumor suppressor genes that encode proteins involved in the repair of damaged DNA by homologous recombination (HR) (Walsh, 2015). Mutations in *BRCA1* and/or *BRCA2* are associated and contribute to around 15-20% of the HGSC cases. *BRCA1* is mutated in 12.5% and *BRCA2* in 11.5% of the patients. These mutations can be either hereditary (around 80-90% of the mutations) or somatic (Bell et al., 2011). These genes are referred to as care takers of DNA and are essential in maintaining genomic stability by contributing to HR-machinery. The HR-repair (HRR) system gets impaired when one or both BRCA genes are mutated (Koi et al., 2024; Walsh, 2015). As a result, the genomic instability increases, which can result in cancer initiation and tumorigenesis.

2.4.4 Homologous recombination

Homologous recombination (HR) is the most prevalent pathway to repair DNA double-strand breaks (DSBs). HR deficiency (HRD) is present in around half of the HGSC patients (Bowtell et al., 2015). Mutations in the HR pathways genes increase genomic instability and make the cells more sensitive to DNA binding and crosslinking chemotherapy agents (e.g., platinum). Single strand breaks (SSBs) are usually caused by oxidative stress whereas the DSBs are often caused by chemical and physical agents. (Lau et al., 2022)

Cells rely on several DNA damage response (DDR) pathways of which homologous recombination (HR) is crucial for the correction of the DSBs in the DNA and preventing uncontrolled proliferation of cells. Other repair pathways include, for example, base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), and non-homologous end-joining (NHEJ). However, these pathways are not all similarly effective; HRR uses the sister chromatid as a template for repair and is therefore error free, while for example NHEJ uses single strand annealing without a template making it relatively error prone. (Lau et al., 2022; Mekonnen et al., 2022).

HR always requires a DNA-template and a functional BRCA-protein to repair the DSBs. HR functions as the predominating DNA-repair pathway during S and G2 phases of cell cycle. NHEJ is active throughout the cell cycle. The detection of the DSB within the cell triggers the normal HR process involving BRCA1 and BRCA2. The normal HR process involves several kinases and other proteins that are essential in the cascade that eventually leads to repaired DNA.

The double strand breaks are initially recognized by Ataxia-telangiectasia mutated (ATM) and Rad3-related (ATR) phosphatidyl inositol 3-kinase-like family of serine/threonine protein kinases (PIKKs) (Bian et al., 2019) (Figure 8). The downstream targets of these kinases, CHEK2, p53, BRCA1, and H2AX, are phosphorylated. BRCA1 with assistance of BRCA1-associated RING domain protein 1 (BARD1) and BRCA interacting protein 1 (BRIP1) organizes the repair proteins on the site of the DSB (Walsh, 2015). Next, the MRN-complex (including MRE11, RAD50, and NBS1) resects the DNA and produces single stranded DNA (ssDNA) 3′-overhangs that are bound by replication protein A (RPA) (Figure 8). At this point BRCA2 is recruited together with Partner and localizer of BRCA2 (PALB2) to load strand-exchange protein RAD51 on the RPA-coated ssDNA overhangs (Figure 8). RAD51B, RAD51C, and RAD51D are assisting when RAD51-ssDNA nucleoprotein filament is invading the homologous DNA strand in the strand invasion process. The sister chromatid acts as template for the error free repair process. (Krejci et al., 2012; Lin et al., 2015; Walsh, 2015)

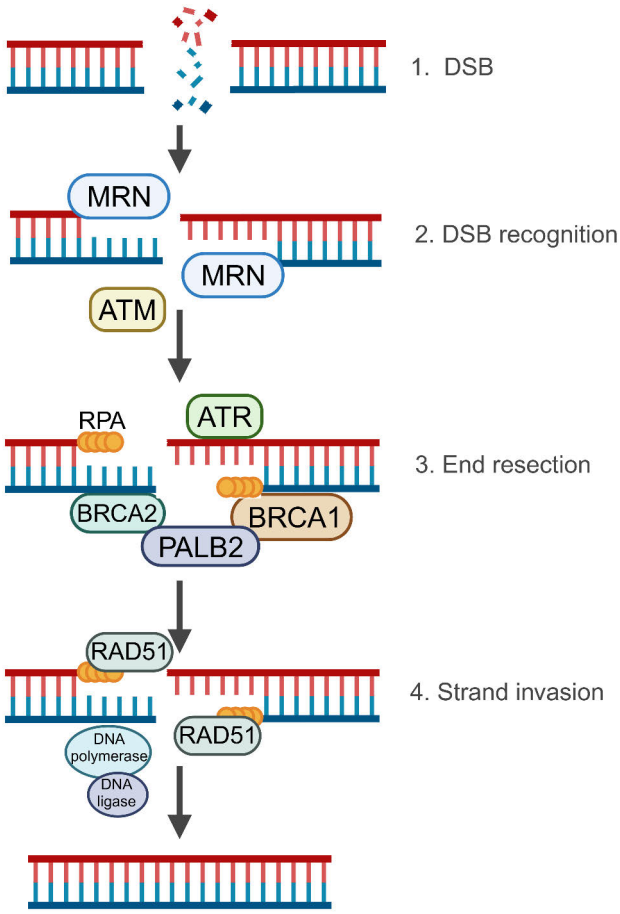


Figure 8: The main components and initiation of homologous recombination repair. Modified from (Ovejero-Sánchez et al., 2023; Walsh, 2015), created with BioRender.

The defects in HR repair capability are not always due to *BRCA1/2* mutations. Mutations in other HR machinery genes such as, *ATM*, *ATR*, *PTEN*, *RAD51*, *PALB2*, *RAD50*, *MRE11*, *NBS1*, *BLM*, *MLH1*, *MSH2* and *MSH6,* and Fanconi anemia, has been associated with HR deficiency in HGSC (Bates, Mohamed, et al., 2024; Bell et al., 2011; Loveday et al., 2011; Meindl et al., 2010). These alterations are described with a broader term BRCAness phenotype (Testa et al., 2018).

In addition, epigenetic changes such as, aberrant phosphorylation or histone acetylation may cause disruption in the HR repair (Bell et al., 2011). In around 11% of the defective HR pathway cases BRCA1 was silenced by hypermethylation (Bell et al., 2011), while other epigenetic changes can also affect the HR pathway (Konstantinopoulos et al., 2015).

2.4.4.1 PARP and their inhibitors

Poly (ADP ribose) polymerases (PARP) are a family of enzymes that are involved in the SSB and DSB repair. The most important and well-studied member of the family is PARP1 (Curtin & Szabo, 2020). Two decades ago, the concept of synthetic lethality was brought into the field of cancer research when two groups published their works on *BRCA1*-mutation and its effect on the DDR-pathway (Bryant et al., 2005; Farmer et al., 2005). The synthetic lethality concept applies in BRCA-mutated cells where HRR is defective because of a mutation in one allele of the gene and when the other allele is made dysfunctional with the use of PARP inhibitors (PARPi). In patients with mutations in *BRCA1/2* genes, inhibition of PARP induces synthetic lethal interactions where DSBs and dysfunctional DNA-repair mechanisms accumulate. These, in turn, can lead to chromosomal instability, cell cycle arrest, and apoptosis. (Farmer et al., 2005) Patients having pathogenic variants of the *BRCA1/2* or other HR genes, respond better to platinum-based chemotherapy than the patients with wild-type (WT) variants of the genes (McCarthy, 2005), and have a better survival probability.

The first clinical biomarker for PARPi use was platinum sensitivity along with germline mutated *BRCA1/2*. These patients had the best response to Olaparib (Kulkarni et al., 2025). Platinum sensitivity is not always the result of mutated *BRCA* but may also be due to something else impaired in the DDR pathway. In addition, the cause can also be a secondary mutation and restoration of BRCA1/2 function that leads to PARPi resistance instead of platinum resistance. (Boussios et al., 2022)

Diagnostic tests for germline *BRCA1/2* mutation identify variants and large rearrangements, but not small deletions and insertions in the genes or transcript processing errors. In addition, these signature tests do not detect the reverted HR function. At least one test creates a genomic scarring score that identifies both germline and somatic *BRCA1/2* mutations and HRD. This score summarizes the results of loss of heterozygosity (LOH), telomeric allelic imbalance (TAI), and large-scale state transitions (LST) as an estimate for PARPi sensitivity. (Boussios et al., 2022) RAD51 is yet another biomarker for PARPi sensitivity that is rather easy to test with a simple immunofluorescence assay. However, further optimization is required before this assay can be translated into clinical use. (Boussios et al., 2022; Konstantinopoulos et al., 2015; Walsh, 2015)

2.4.5 BMI1

The polycomb repressive complexes (PRCs) function as transcriptional repressors in the protective pathways of aberrant cell cycle or apoptosis by regulating chromatin structure and DNA replication. They are involved in the embryonic and adult stem cell maintenance, as well as in cancer development. (Chan & Morey, 2019) BMI1 (B-cell-specific Moloney murine leukemia virus integration region 1; PCGF4, Polycomb

group finger 4) is the best studied homolog of polycomb group finger (PCGF) proteins in PRC1 complex. BMI1s role in breast cancer is quite well studied, while other cancers are less explored (Chan & Morey, 2019). This chapter focuses on BMI1's role in DDR and cell cycle checkpoint pathways. The cancer stem cell-related topics are covered in more detail in chapter 2.5.2.4.

BMI1 belongs to a family of polycomb group proteins (PcG) that form multiprotein complexes: Polycomb repressive complex 1 (PRC1) and Polycomb repressive complex 2 (PRC2). These complexes function as chromatin modifiers that target histones H2A and H3. Several post-translational modifications, such as, ubiquitylation, phosphorylation, methylation, and sumoylation, are linked to PcG-complexes with BMI1 (Ismail et al., 2010; Xu et al., 2022). BMI1 is a subunit of the PRC1 complex that consists of several chromodomain proteins (CBX) and the BMI1-binding catalytic subunit RING2. Together, BMI1 and RING2 form the E3 ubiquiting ligase subunit. (Chan & Morey, 2019; Gao et al., 2012; Lin et al., 2015)

BMI1 regulates the DSB repair pathway choice between HR and NHEJ (Fitieh et al., 2021) (Figure 8). Within the PRC1-complex, BMI1 interacts with Ring1B, which possesses E3 ligase activity to monoubiquitinate histone H2A at lysine 119. In this transcription repression function, ATM phosphorylates the transcription elongation factor (ENL) that interacts with BMI1. This leads to PRC1 accumulation at the site, monoubiquitylation of the H2A lys119, and transcription repression (Lin et al., 2015). The chromatin is made inaccessible for HR-factors like BRCA1. At the same time, the tumor suppressor p53-binding protein 1 (53PB1) is recruited to the DSB and phosphorylated by ATM. This inhibits DNA end resection by stabilizing the DNA-ends for Ku70/80. The involvement of 53PB1 is vital for NHEJ. (Daley & Sung, 2014; Fitieh et al., 2021; Ginjala et al., 2011)

The PRC1-compex with BMI1 also affects other gene promoters by compacting the chromatin (Figure 9). One quite well-studied promoter is INK4/ARF locus, which is upstream of p53- and Rb-pathways (Jacobs et al., 1999). INK4/ARF encodes $p16^{INK4a}$ and $p14^{ARF}$. $p16^{INK4a}$ inhibits the activation of CDK4/6 at the G1/S checkpoint, thereby arresting the cell cycle by preventing cyclin D binding (Figure 9). This inhibition keeps the Rb-protein in its active phosphorylated state, repressing E2F1 transcription activity. $p14^{ARF}$ binds to MDM2 that prevents it to induce p53 degradation. This stabilizes p53 and inhibits the cell cycle by inducing $p21^{Cip1}$ expression, which leads to the promotion of apoptosis. (Fitieh et al., 2021; B. R. Kim et al., 2017) By inhibiting these pathways through chromosomal modification and repression of transcription, BMI1 promotes cell cycle progression and tumorigenesis during DNA damage.

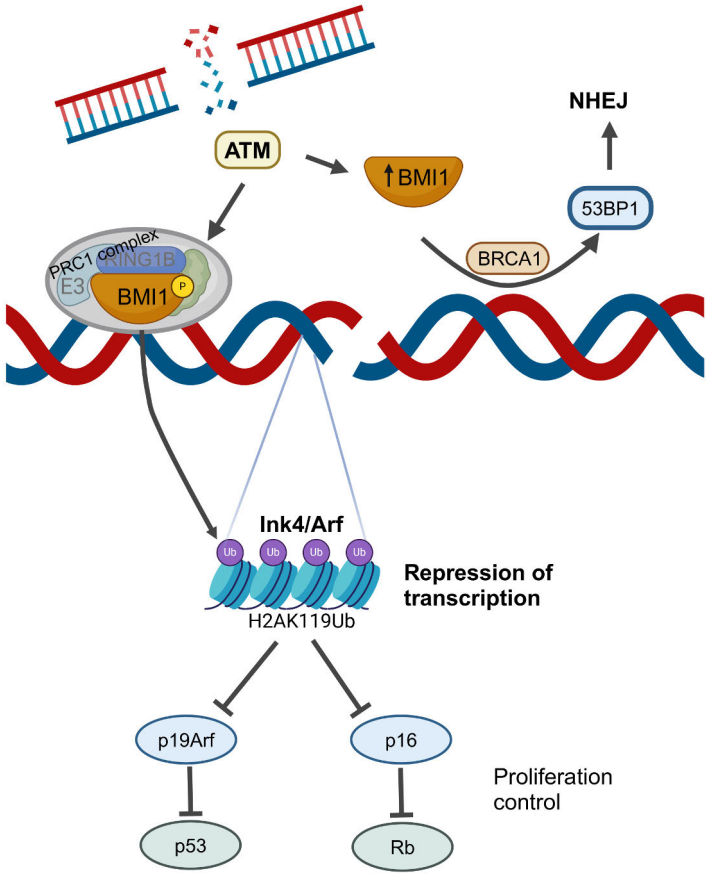


Figure 9: Double strand break in presence of BMI1. When BMI1 level is high the homologous recombination pathway is suppressed by chromatin condensing and repressing BRCA1. PRC1 complex binds to DNA near the double strand break and condenses it which causes repression of target genes thereby having impact on the cell proliferation/checkpoint control. The condensed chromatin also has effect on the DNA-damage response pathway selection. Created with BioRender.

2.4.6 Wee1

Wee1 (Wee1 G2 Checkpoint kinase) is one of the three members in the Wee1 kinase family of serine/threonine kinases (Ghelli Luserna Di Rorà et al., 2020). Wee1 acts as a tumor suppressor in nonmalignant cells and participates in the DDR pathways in the nucleus.

2.4.6.1 Wee1 function in normal cell cycle

Wee1 is one of the key regulators of the cell cycle, especially in G2/M checkpoint regulating the entry to mitosis and, to some extent, also in G1/S checkpoint (Matheson, Backos, et al., 2016; Matheson, Venkataraman, et al., 2016; Smith et al.,

2020). In G2/M, Wee1 ensures the proper timing of mitosis by controlling the DNA replication and repair process and by regulating the cell cycle in response to DNA-damage at the G2/M checkpoint. In G1/S checkpoint, Wee1 regulates the replication stress by stalling the replication forks. In normal cells, G1/S checkpoint is governed by p53-p21-pathway. Wee1's role is minor at this checkpoint in the DNA damage control. However, in HGSC where the p53-p21-pathway is dysfunctional, Wee1 is thought to have a minor role in G1/S checkpoint. (Ghelli Luserna Di Rorà et al., 2020).

The ATR- and ATM-pathways, upstream of Wee1, have overlapped activities and crosstalk (Weber & Ryan, 2015). In general ATM-CHK2-p53 pathway controls the G1/S checkpoint and acts via CDK2/cyclin A/E. In turn, ATR-CHK1-Wee1 pathway controls the G2/M checkpoint and acts via CDK1/cyclin B (Smith et al., 2020) (Figure 10).

ATR-pathway is activated by genotoxic stresses resulting from SSBs and inhibits the activity of its main downstream target CDK1-cyclinB -complex (Figure 10). Active ATR activates the CHK1, its major effector via phosphorylation. The active CHK1 phosphorylates Wee1 and increases its kinase activity, which protects it from degradation and prevents its export from the nucleus. This increased kinase activity of Wee1 inhibits CDK1 by phosphorylating it on tyrosine 15 (Tyr15), which prevents it from forming an active complex with cyclin B. In addition to Wee1, the active CHK1 also inhibits the CDC25C phosphatase by phosphorylating and thereby inactivating it. In normal conditions, when no DNA-damage is present, active CDK1 suppresses Wee1 and activates CDC25, and the CDK1-cyclin B -complex activates it, which leads to the cell entering mitosis. (Matheson, Backos, et al., 2016; Matheson, Venkataraman, et al., 2016)

ATM-pathway is activated by genotoxic stresses (i.e., ionizing radiation or agents that cause DSBs) resulting from DSBs and inhibits the activity of its two main downstream targets p53/p21 and CDK2-cyclinA/E -complex (Figure 10). ATM pathway acts via CHK2 kinase. (Wang et al., 2025)

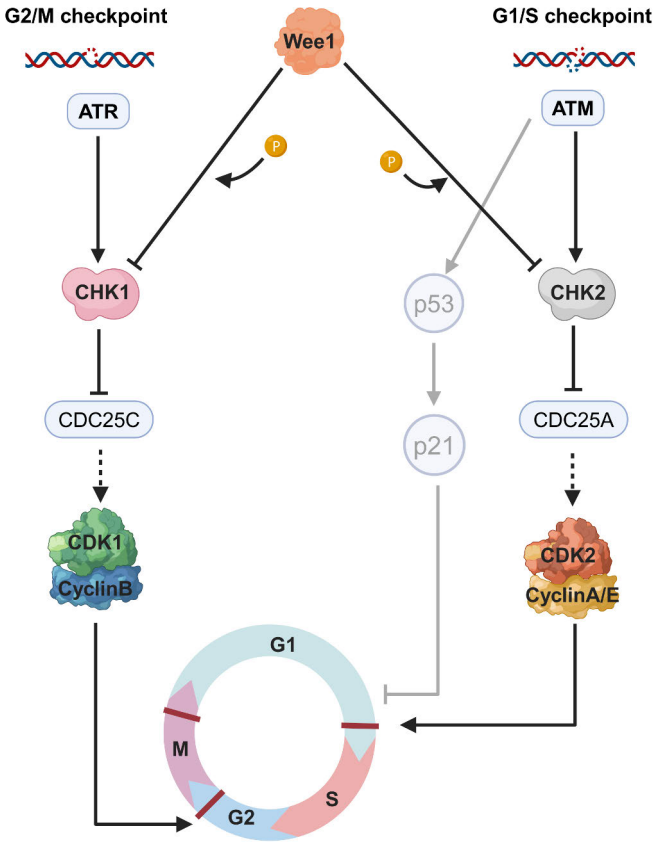


Figure 10: Cell cycle checkpoints G1/S and G2/M and Wee1´s role in checkpoint activation during ssDNA damage and dsDNA damage. Created with BioRender.

2.4.6.2 Wee1 as a target in HGSC

As G1/S checkpoint is dysfunctional in HGSC due to aberrant p53, the G2/M checkpoint functions as the sole control point for suppressing the cell cycle propagation in case of DNA damage (Jackson & Bartek, 2009; Smith et al., 2020). In addition, the main target for Wee1 kinase activity is CDK1 (not CDK2) which is essential in the G2/M checkpoint. The G2/M checkpoint is primarily regulated by Wee1 kinase that acts as an oncogene in many cancer cells (Ghelli Luserna Di Rorà et al., 2020). Thus, in HGSC (or other *TP53* mutated tumors) the G2/M checkpoint encounters heightened pressure and shows potential as a therapeutic target (Wang et al., 2025). Wee1 inhibition increases genomic instability and sensitizes cells to DNA-damaging agents. Some phase II clinical trials in OC have shown the Wee1 inhibitor (Wee1i) adavosertib (AZD1775) to be effective as a single agent or in combination with other drugs (Embaby et al., 2023; Leijen, Van Geel, Sonke, et al., 2016; Lheureux et al., 2021).

2.4.7 Other molecular changes in HGSC

Amplifications are often found in genes involved in cell cycle control or survival pathways. Amplification of *CCNE1* (cyclin E1) is frequently found in HGSC but is not exclusive to this subtype. (Etemadmoghadam et al., 2009). In the clinical setting, *CCNE1* amplifications are usually associated with poor response to platinum therapy and are a potential marker of treatment resistance (Etemadmoghadam et al., 2009) (Nakayama et al., 2010). Usually, amplification of *CCNE1* and mutations in *BRCA1/2* do not coincide. (Etemadmoghadam et al., 2009; Nakayama et al., 2010; Patch et al., 2015)

Moreover, there are known alterations in the cell signaling pathways PI3K/AKT/mTOR and RAS/MAPK that are sometimes found in the HGSC patients' tumors (Bast, 2011; Bowtell et al., 2015; Patch et al., 2015). These pathways are active, for example, in cell survival, proliferation, differentiation, and metabolism.

2.4.8 Chain of events leading to HGSC

In summary, the events leading to HGSC tumorigenesis are described in Figure 11. The initial event is *TP53* mutation resulting in loss of function, which leads to genomic instability, loss of cell cycle control, and failure to undergo apoptosis. A second common event is HRR inactivation, leading to chromosomal instability and accumulation of additional genomic abnormalities. Molecular subtypes emerge that may have malfunction in different signaling pathways and affect different endpoints. (Bowtell, 2010)

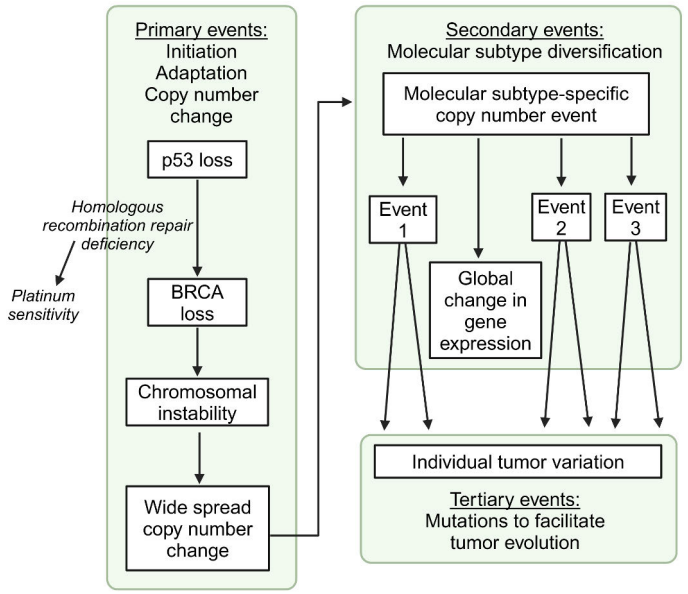


Figure 11: Molecular events leading to high-grade serous carcinoma. Modified from (Bowtell, 2010).

2.5 Chemoresistance in HGSC

Chemoresistance in HGSC is common, and a vast majority of patients encounter it during their treatment. In general, chemoresistance in solid tumors is a result of several different alterations in the cells. Some can be due to altered drug effects, efflux pumps may be overexpressed, drugs can be inactivated by tumor cell enzyme activity or drug targets may be mutated or altered to be less susceptible. Furthermore, tumor cell DDR mechanisms may be enhanced, and apoptotic pathways may be dysregulated. (Ghosh, 2019) In addition, the tumor microenvironment can influence drug effectivity via for example hypoxia, stromal cells or change in pH. (Shibue & Weinberg, 2017).

Primary drug resistance to first-line chemotherapy occurs in approximately 15-25% of the HGSC patients and is associated with HR proficiency. Two of the four molecular subtypes of HGSC are characterized as primary chemoresistant: the proliferative and the mesenchymal subtypes. (Freimund et al., 2018) Gene amplification in *CCNE1* in HGSC patients most likely brings resistance to first-line chemotherapy (Nakayama et al., 2010; Z. Wang, Liu, et al., 2025).

Most of the chemo-resistance in HGSC is acquired therapy resistance caused by the cancer therapy drugs. There are several different mechanisms known, and yet new ones are emerging. Platinum resistance usually results in PARPi resistance when the HR pathway is restored due to secondary mutations in *BRCA1/2*, an epigenetic change during relapse, or a decrease in BRCA1 promoter methylation. Similarly, reverse mutations in other HR pathway genes have been associated with PARPi resistance. (Bukłaho et al., 2023)

The resistance to taxane is thought to be a result of the changes in apoptosis control pathways, either increased expression of antiapoptotic proteins or downregulation of proapoptotic proteins. Multi-drug resistance (MDR), in other words, hyperactivity of the membrane-bound transport channel pumps (ATP binding cassette transporters) that can transfer substrates out from the cells, is a well-studied mechanism associated with paclitaxel resistance. The most studied channel pumps are multi-drug resistance protein 1 (MDR1 or P-glycoprotein, P-gp) and ATP Binding Cassette Subfamily B Member 1 (ABCB1). (Bukłaho et al., 2023; Freimund et al., 2018)

2.5.1 Cancer stem cells in OC

There is controversy and debate about the existence of human stem cells in the adult ovary. Traditionally, it is thought that women are born with an oocyte reserve and constitute the primordial follicles that in adulthood are matured one at a time monthly. This indicates that there is no need for germline stem cells in the adult ovarian tissue. This theory also explains the menopause. However, the ovulation process, where the

ovarian surface is cyclically ruptured and then again regenerated, gives hints from a regeneration process that might have a demand for stem cells (Testa et al., 2018). A recent study has identified a putative stem cell niche of the ovarian epithelium. These cells present stem cell features and are in the junction area between the ovarian tubal-peritoneal junction, where the epithelium of the fimbriated fallopian tube is connected to the mesothelial layer of the peritoneum (Flesken-Nikitin et al., 2013). The STICs are located within this area, and it is considered the primary initiation point for HGSC (Schmoeckel et al., 2017; Testa et al., 2018).

Tumor heterogeneity presents a major challenge in HGSC, contributing to variability in disease progression and complicating the development of effective treatments. One proposed driver for the heterogeneity is cancer stem cells (CSCs), estimated to comprise about 1% of tumor cells (Horowitz et al., 2020). CSCs in OC have been studied as an explanation for chemoresistance and recurrence of the disease. CSCs or tumor-initiating cells have been intensively studied during the last decades. These cells contribute to chemoresistance and drive tumor growth and are responsible for the tumor relapses (Barczynski et al., 2023; Capp, 2019; Phi et al., 2018). Their source, origin, and cellular markers have been studied in many types of solid cancers. There are several possibilities for where they are derived: adult stem cells, adult progenitor cells or differentiated cancer cells that have undergone mutations and obtained CSC-like properties (Phi et al., 2018).

CSC-dependent mechanisms of chemotherapy resistance include self-renewal, quiescence, epigenetic modifications, resistance to DNA-damage induced cell death, epithelial-to-mesenchymal transition (EMT), etc. (Phi et al., 2018). Also, DNA-damage inducing therapies are known to trigger cancer cells to senescence. This phenomenon is called therapy-induced senescence (TIS), and it can be a mechanism to later gain CSC features and a reason for relapses (Walcher et al., 2020). Because of the great plasticity of the CSCs, it has been suggested that they should be targeted with combinatorial therapy, together with the traditional therapy (Bajaj et al., 2019; Barczynski et al., 2023).

The first evidence of CSCs in solid cancers was found in 2007 in breast cancer. The first study to isolate and culture epithelial ovarian CSCs was published in 2005 (Bapat et al., 2005). This study showed that patient-derived ascites had CSCs (or cancer-initiating cells) that could produce a disease reminiscent of the advanced-stage disease in the patient. Another study in 2008 identified a population of OC initiating cells (Zhang et al., 2008).

2.5.2 Stemness markers in OC

Several studies on various serous ovarian cancer cell populations (primary tumors, tumor cell lines, and xenografts) have been performed with various methods to advance the field and find out what the OC specific (or subtype-specific) CSC markers

are (Testa et al., 2018). Steg and colleagues demonstrated immunohistochemistry and gene expression analysis that primary ovarian specimens had low densities of CSC markers ALDH1A1, CD44, and CD133, whereas specimens collected right after the first recurrence had more dense expression of these markers (Steg et al., 2012). Despite, several studies have attempted to identify markers that could mark CSCs in HGSC (Testa et al., 2018), no marker has been found that could identify all CSCs HGSC (Bates, Mohamed, et al., 2024).

2.5.2.1 ALDH1A1

Aldehyde dehydrogenase isoform 1A1 (ALDH1A1) is among the most studied CSC markers in HGSC. It has many functions, ranging from protection against free radicals to regulating stem cell proliferation and differentiation (I˙ Zycka et al., 2023). ALDH1A1 has been used to define CSCs in many cancer types and has been suggested to be involved in drug resistance and is therefore an interesting therapeutic target (Al-Alem et al., 2018; Wei et al., 2022). Several studies have shown that ALDH1A1 is associated with poor prognosis and stemness in many cancers (Deng et al., 2010; Landen et al., 2010; Y. Li et al., 2018) including OC (Chang et al., 2009a; Deng et al., 2010). Some controversial results indicating that ALDH1A1 expression correlates with favorable prognosis (Al-Alem et al., 2018; Chang et al., 2009b; Huang et al., 2015).

2.5.2.2 SOX2

SOX2 (sex determining region Y)-box 2, is a transcription factor that is essential during developmental processes and in adult stem cell populations (Niharika et al., 2024). Its aberrant function has been associated with many cancer types, and it has been shown to mediate resistance to cancer therapies (Novak et al., 2020). Amplification of the gene and increased protein expression are associated with cancer progression and the effect seems to vary between cancer types (Novak et al., 2020). In OC SOX2 has been associated with poor prognosis and CSC phenotype (Wen et al., 2017).

2.5.2.3 MYC

MYC (c-MYC) oncogene is a transcription factor that has a role in cell cycle progression, apoptosis, and cellular transformation. MYC is associated with reprogramming human somatic cells to pluripotent stem cells, but also with stemness properties in cancer cells. (Dhanasekaran et al., 2022; Whitfield & Soucek, 2025) Several studies have reported that MYC overexpression can be tumorigenic. In OC, MYC amplifications and CNV have been reported but the clinical significance has not

been clarified (Reyes-González & Vivas-Mejía, 2021). Jung et al (2018) hypothesized that different subgroups of EOC could have different relevance of MYC oncogene family members (Jung et al., 2018).

2.5.2.4 BMI1

BMI1 has an important role in cancer and normal stem cell maintenance, tumor initiation, and therapy resistance. BMI1 is involved in regulating the cell cycle checkpoint in the p53- and apoptosis in the Rb pathway through suppression of the Ink4a locus that results in repression of the tumor suppressor proteins $p16^{Ink4a}$ and $p19^{Arf}$ (Herzog et al., 2023; Min-Cong Wang et al., 2015). The role of BMI1 in regulating DNA repair and apoptosis as well as cell cycle checkpoints, were described in chapter 2.4.5 in the context of HR.

BMI1 is involved in the regulation of many transcription factors. Co-operation between BMI1 and MYC has been reported to downregulate p16Ink4a and $p19^{Arf}$. MYC has been reported to be a transcriptional activator of BMI1. (Lin et al., 2015; Parreno et al., 2022; Siddique & Saleem, 2012; Yoshida, 2018).

BMI1 is also regulating EMT through repressing the tumor suppressor Phosphatase and tensin homolog (PTEN), which is a regulator of PI3K/Akt pathway. This pathway activation results in increased expression of VEGF (Xu et al., 2022). Another EMT-regulating protein, Twist-related protein 1 (Twist), is also linked to BMI1. Both promote EMT and are associated with an aggressive and migratory phenotype of tumor cells (Herzog et al., 2023; Min-Cong et al., 2015). In addition, BMI1 participates in many other signaling pathways that are related to developmental or stem cell pathways, such as Wnt-signaling (Min-Cong et al., 2015; Siddique & Saleem, 2012).

2.6 Tissue based markers in HGSC

2.6.1 Methods used in the clinical pathology laboratory

In the clinic, cancer diagnosis is typically based on the pathologist`s evaluation of hematoxylin and eosin (HE) stained formalin-fixed paraffin-embedded (FFPE) sections of patient tissues. The morphologic analysis is often complemented with immunohistochemistry (IHC). Most diagnoses are based on these two methods. RNA *in situ* -hybridization (RNA-ISH) or DNA *in situ* -hybridization (DNA-ISH) can provide additional molecular information, including presence of genomic alterations or presence of viral genetic material, but these are not used in clinical diagnostics of HGSC. Genetic testing of patient tissue samples is increasingly utilized in clinical

pathology in collaboration with geneticists. Mutations are investigated by polymerase chain reaction (PCR) and DNA samples are sequenced for known mutations.

2.6.1.1 Immunohistochemical methods

IHC is a tissue-based immunoassay to detect specific proteins (or other tissue antigens) in a FFPE tissue section obtained from a patient. The diagnostic IHC was developed in the 1960s- and -70s. It is used in addition to HE and other histochemical stainings to diagnose the origin of the tumor. IHC is commonly used for tumor classification and differential diagnostics and accessing predictive markers for cancer treatment. (Mäkinen et al., 2024; Suvarna et al., 2018)

In addition to diagnostic IHC stainings, some markers can be used as predictive markers guiding treatment or predict survival. The HER2 test is the first biomarker used for development of targeted therapies and guiding treatment. This so-called companion diagnostics is an emerging field of developing diagnostics and treatment opportunities of cancer (Dabbs, 2022).

IHC method detects and visualizes the proteins in the tissue section with antibodies and the detection method can be based on either fluorescent (IF) or enzymatic. In IF, a small fluorescent molecule (a fluorophore) is conjugated to the antibody. The fluorophore absorbs light at specific wavelength, which raises its electron to a higher state and then emits light with a longer wavelength when the fluorophore returns to its normal state. The emitted light is examined in the fluorescent microscope with an appropriate wavelength light that is dependent on the selected fluorophore. For example, fluorescence isothiocyanate (FITC) is a commonly used fluorophore that is excited at 488 nm and emits light at 519 nm. The fluorescent reaction is short-lived and requires samples to be examined quite fast. (Kohale et al., 2023)

In the enzymatic IHC an enzyme is linked to the antibody and the reaction can be made visible with the help of a chromogen. The most used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP). The most used chromogens with HRP are 3,3'-diaminobenzidine (Dab) and 3-amino-9-ethylcarbazole (AEC), and with AP Fast Red or Fast Blue. The products of the enzymatic IHC reaction are long-lived and stays for decades. (Kohale et al., 2023)

IHC is a semi-quantitative method, because the antibodies have different affinities to the antigens and, because the enzyme reaction is not completely linear. Factors that are affecting the enzyme-substrate reaction are the local concentration of the target antigen, together with the activity and availability of the enzyme. The reaction time also has an impact and the diffusion of the substrate. The diffusion can be quite different between the samples depending on several factors, for example size and rate of fixation. The preanalytical procedures i.e. the quality of the sample tissue and its

fixation is essential for high quality pathological diagnostics. (Dabbs, 2022; Mäkinen et al., 2024)

2.6.1.2 *In situ* -hybridization

With the *in-situ* hybridization (ISH) technique nucleic acids are detected from the FFPE tissue sections. This method can be used to detect specific DNA or RNA (DNA-ISH or RNA-ISH, respectively) sequences and is used to visualize gene expression or alterations in the genome, and the method has high sensitivity and specificity (Suvarna et al., 2018).

Either chromogenic or fluorescent-labelled probes can be utilized for ISH. With fluorescent-ISH (FISH), specific areas in the genome are typically investigated. This method detects point mutations, chromosomal abnormalities (i.e., CNVs), amplifications, deletions, translocations, and gene fusions. When a chromogenic probe is used instead of a fluorescent probe the method is called chromogenic-ISH (CISH). (Suvarna et al., 2018)

RNA-ISH method detects specific *mRNA*-molecules or non-coding RNA transcripts and detects transcription activity of the target gene. It is mostly used in the clinics to assess diagnostic or predictive gene amplifications or translocations in some cancers and to indicate certain virus infections (Epstein-Barr, Human papillomavirus). (Coleman & Tsongalis, 2024).

2.6.1.3 Molecular genetic methods

Molecular genetic methods for cancer diagnostics include nucleic acid-based tests with gene panels, and whole exome (WES), and whole genome sequencing (WGS). In addition to the ISH-methods polymerase chain reaction (PCR) -based methods for investigating alterations in the genome are routinely used in clinical pathology laboratories. Reverse transcriptase PCR is used for detecting fusion transcripts. Next-generation sequencing (NGS) is used for targeted panels to detect cancer-related mutations, and traditional Sanger sequencing is used more for small panels for confirmation testing and during surveillance. (Mäkinen et al., 2024; Suvarna et al., 2018)

2.6.1.4 Artificial intelligence

Artificial intelligence (AI) tools are developed for clinical pathology laboratory use for more reproducible, accurate, and standardizable methodologies to analyze digitized tissue slides (Bera et al., 2019). These tools are for example quantifying IHC staining or RNA-ISH signals in the tissue slides or counting mitotic figures. Only few models are recently approved by authorities to be used in the diagnostic workflow.

Recently applications for automatic scoring or grading of i.e. prostate cancer and breast cancer have been taken into clinical use. Several AI tools to assess IHC biomarkers such as quantification of nuclei/cells, pattern of distribution, Ki67, and PD-L1 have been taken into use and gotten clinical approval (Shafi & Parwani, 2023). No AI tools for HGSC are yet available for clinical use, but research is ongoing (Joshua et al., 2025).

2.6.2 Diagnostic markers for HGSC

Currently, HGSC diagnosis is primarily set by morphological analysis of HE-stained tissue-slides. If the morphological pattern is ambiguous, a pattern of tissue markers can be used to distinguish HGSC from other epithelial OCs or other carcinomas. These markers include i.e. p53, WT1, PAX8, keratins CK7 and CK20, Napsin A, and HNF-1β. (Bates, Mohamed, et al., 2024; Dabbs, 2022; Kossaï et al., 2018). Typical staining pattern used for diagnostics for HGSC is in Table 1. Despite the extensive research contributions to the field, there are currently no prognostic and predictive tissue biomarkers in clinical use.

Table 1: Immunohistochemical staining pattern for typical HGSC.

Marker	Typical staining pattern in HGSC	Localization
p53	Aberrant	Nuclear/cytoplasmic
WT1	Positive	Nuclear
PAX8	Positive	Nuclear
CK7	Positive	Cytoplasmic/Membranous
CK20	Negative	
CDX2	Negative	
Napsin A	Negative	
HNF-1β	Negative	

2.6.2.1 P53 and WT1

In HGSC, the aberrant p53 is the characteristic hallmark. The mutations in *TP53* cause gain-of-function mutations and overamplification or non-functional p53 protein. These mutations can either evade apoptosis or promote tumorigenesis, invasion, and metastasis (Bates, Mohamed, et al., 2024). Addressing the IHC aberrant staining pattern of p53 protein is the key to diagnosing HGSC (Dabbs, 2022). Three aberrant p53 IHC-patterns exist: overexpression is the most common, loss of staining (negative), and the rare cytoplasmic localization (Table 1).

Wilms´ tumor suppressor protein 1 (WT1) is a protein used as a confirmation marker for p53 in serous-type ovarian cancers (Dabbs, 2022). WT1 regulates, for example, EGR1, IGF-2, PDGRA, EGFR, and p53 expression.

2.6.2.2 PAX8, cytokeratins, and CDX2

Some markers are used in the differential diagnosis between the different subtypes of OC or other carcinomas. Paired gene box 8 (PAX8) is a transcription factor highly expressed in most tubo-ovarian carcinomas but not in the mucinous carcinoma or gastrointestinal carcinomas (Dabss et al). It is often used for small samples and metastatic samples to evaluate the origin of the tumor.

Cytokeratins (CKs) form a protein family constituting the major components of the intermediate filaments of the epithelial cell cytoskeleton. The expression pattern of keratins can be used to identify the origin of the epithelial cells or the cell types in the tumor samples. Gastrointestinal and mucinous carcinomas are often CK20 positive whereas HGSC and non-mucinous ovarian carcinomas are usually CK7 positive (Dabbs et al).

CDX genes are encoding homeobox nuclear transcription factors of intestinal epithelial cells. CDX2 can be used for differential diagnostics from colorectal adenocarcinomas and mucinous ovarian tumors (Dabbs, 2022).

2.6.2.3 Napsin A and HNF-1β

Hepatocyte nuclear factor 1β (HNF-1β) have been used to differentiate HGSC from ovarian clear cell carcinoma, but the superior specificity of Napsin A has replaced it (Dabbs, 2022).

2.6.3 Emerging tissue markers in HGSC

Several prognostic and predictive tissue biomarkers are being investigated to guide personalized treatment, but none is yet in clinical routine use. Methods for identifying HRD status of the tumors based on RAD51 location in tumor cells are being developed (Pikkusaari et al., 2023).

Paclitaxel is an established ligand for Toll-like receptor 4 (TLR4). Thus, TLR4-MyD88 signaling pathway has been under investigation for being a biomarker and therapeutic target for EOC patients. High staining intensity of TLR4 has been shown to be associated with reduced survival in HGSC (Bates, Mohamed, et al., 2024; Bates, Mullen, et al., 2024; Zhu et al., 2012).

Mitotic arrest deficient 2 (MAD2) is a protein involved in the spindle assembly checkpoint during the cell cycle. In OC cells the silencing of MAD2 induces resistance

to paclitaxel. In addition, its low expression has been associated with worse prognosis. (Byrne et al., 2017; Park et al., 2013)

The Epidermal growth factor receptor (EGFR) family receptors HER2 and HER3 activate several pathways that contribute to tumor aggressiveness. OC studies have shown mixed results, but several studies have shown that high expression of HER2 is associated with a negative prognosis. High expression of HER3 is seen in primary tumors and CSCs of EOC and is associated with metastasis, chemoresistance and reduced survival. (Bates, Mohamed, et al., 2024) HER3 is an attractive target for personalized therapy due to its role in metastasis. Its prognostic potential in EOC has been limited, because of the difficulties in standardizing and interpreting the staining patterns (Bates, Mohamed, et al., 2024; K. Wang et al., 2017).

3 Aims

The aims of this thesis were to:

1. Characterize the stemness features in patient-derived HGSC cells and study associations of the markers with clinical outcome.
2. Examine patient-derived HGSC cells' sensitivity and resistance for 306 drugs by carrying out a high-throughput drug screen in conventional 2D and stemness-promoting 3D conditions.
3. Evaluate the expression of four suggested cancer stem cell markers in HGSC tissues and correlate the results with patient clinical data.
4. Dissect the inhibitory activities of the Wee1 inhibitor adavosertib on conventional and patient-derived HGSC cells.

4 Materials and Methods

A summary of the materials used in this thesis project and the main methods are presented. A complete list of methods is in appendix 1. The detailed information can be found in the original studies or their supplementary data.

4.1 Approvals of the patient materials

4.1.1 Mupet/Hercules cohort (I, II)

For the prospective ovarian cancer study MUPET/HERCULES (ClinicalTrials.gov ID: NCT01276574), patients at the Turku University Central Hospital were recruited. The cohort is known as Mupet study (ETMK: 53/180/2009) and Mupet phase 2/Hercules study (ETMK: 145/1801/2015). The Ethics Committee of the Hospital District of Southwest Finland and the Finnish National Supervisory Authority for Welfare and Health in Finland (Valvira) have approved the study protocol and use of all materials. The patients/participants provided their written informed consent to participate.

4.1.2 Biobank cohort (III)

The study cohort consisted of patients from the Helsinki biobank collection diagnosed with HGSC and treated at Helsinki University Hospital (HUS) between 2007 and 2013. The implementation of the study protocol and all materials was approved by the local ethical committee and permission was granted by the responsible hospital authority (permission numbers HUS/244/2021, HUS/430/2021 and HUS/124/2023). Specific consent from patients for the use of remnants of diagnostic tissue samples for research purposes is not required for retrospective studies according to the Finnish Biobank Act (688/2012).

4.2 Patients and samples

4.2.1 Mupet/Hercules cohort and sampling (I, II)

Treatment-naive ascites samples were collected during diagnostic laparoscopy or primary debulking surgery (PDS). From neoadjuvant chemotherapy (NACT) patients, new samples were taken during interval debulking surgery (IDS). Patient-derived cell cultures were obtained from the fresh ascites or tumor samples. In addition, freshly frozen tumor samples were processed into RNA samples and snap-frozen (-80ºC) for later use. During treatment and follow-up, several tissue, ascites, and blood samples were collected, until the progression of the disease. Full clinical data were collected from the patient records during the treatment and follow-up period.

For study I, tumor and ascites samples, and full clinical data were collected from forty patients with stage III/IV HGSC. In total, thirty-six treatment-naïve and 18 NACT samples were collected, of which ten were paired. Of these samples twenty-five cell cultures were established (19 treatment naïve, six NACT), of which three were paired (Figure 12). Longitudinal clinical data included stage, residual disease information, treatment, and survival data (Table 2). Detailed clinical characteristics of the patients are in Study I and II.

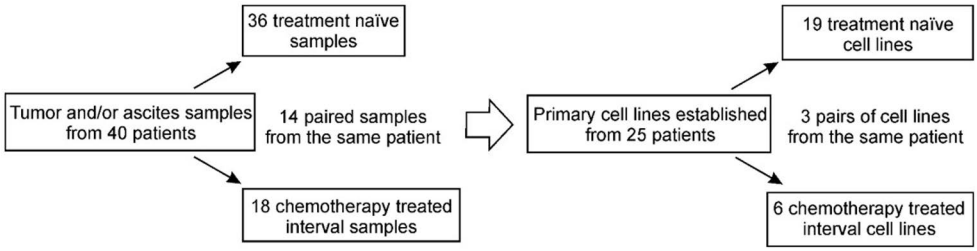


Figure 12: Patient-derived material from study I. The workflow of generating tumor and ascites-derived cell cultures and tissue material for qPCR analyses. Adopted from study I.

Table 2: Study I patient characteristics.

Variable	Average	Median	Range	Count (%)
Total number of patients				40 (100)
Survival				
PFS	14	12	2–46	37 (93)
OS	35	35	4–86	34 (85)
Age (years)	66	65	51–80	40 (100)
Residual tumor (mm)				
0				3 (8)
>10				2 (5)
1-10				16 (40)
Missing values				*19 (48)*
Surgery type				
PDS				16 (40)
IDS				24 (60)
Number of primary chemotherapy cycles				
3				4 (10)
6				19 (48)
7				2 (5)
8				3 (8)
9				9 (23)
≥10				3 (8)

4.2.2 Biobank cohort (III)

The tissue micro array (TMA) from 95 HGSC Helsinki biobank patients was used in study III. The TMA was built from three replicate 1 mm cores from adnexal and extrapelvic tumors from each patient. The inclusion criteria for the patients included morphologically confirmed stage III/IV HGSC diagnosis, PDS, platinum-based chemotherapy (≥6 cycles), and availability of tissue blocks. The diagnosis was reconfirmed with p53 and WT1 IHC staining (aberrant and positive, respectively). In addition, complete longitudinal clinical data was collected. The patient characteristics are in Table 3.

Table 3: Study III patient characteristics. Adopted from study III.

Variable	Average	Median	Range	Count (%)
Total number of patients				95 (100)
Follow-up time (months)	65	55	11–117	
Survival				
PFI	16	8	0–117	82 (86,3)
OS	50	44	11–159	71 (74,7)
Age (years)	62	63	40–82	95 (100)
Body Mass Index (kg/m2)	25,8	24,8	17,1-41,3	93 (97,9)
missing value				*2 (2,1)*
CA-125 (u/ml) *	1612	563	9-49100	95 (100)
Residual tumor				
R0				20 (21,1)
R1				8 (8,4)
R2				67 (70,5)
ASA status				
1				8 (8,4)
2				39 (41)
3				40 (42,1)
4				3 (3,2)
missing value				5 (5,3)
FIGO stage				
IIIa1				10 (10,5)
IIIb				11 (11,6)
IIIc				61 (64,2)
Iva				2 (2,1)
IVb				11 (11,6)

*At diagnosis

4.3 Cells (I, II)

Both commonly available cell lines and patient-derived cell cultures were utilized. All the cell cultures were maintained in a standard culture environment at 37ºC in a humidified 5% CO^2 atmosphere. Detailed cell culture protocols are described in the study I and II.

4.3.1 Cell line cultures

The commonly available cell lines (Table 4) were cultured in varying media depending on the purpose of the experiment. In general, the cultures were in the

adherent (2D) media recommended by the supplier: DMEM or RPMI supplemented with 5-10% FBS, 100 µg/ml penicillin/streptomycin and 2 mM ultraglutamine. In addition, for the high-throughput screening of a panel of 306 drugs, spheroidal medium (3D, DMEM-F12 without FBS) was used in addition to 2D-medium.

Table 4: Commonly available cell lines in study I and II.

Cell line	Tissue of origin	Supplier	Used in
CAOV3	ovary adenocarcinoma	ATCC*	I, II
CAOV4	fallopian tube adenocarcinoma	ATCC*	I
OVCAR4	ascites high grade ovarian serous adenocarcinoma	NCI**	I
OVCAR8	ovary high grade serous adenocarcinoma	NCI**	I, II
TYK-nuCP-r	ovary undifferentiated carcinoma	JCRB***	I
TYK-nu	ovary undifferentiated carcinoma	JCRB***	I

*American Type Culture Collection
**National Cancer Institute
***Health Science Research Resources Bank

4.3.2 Patient-derived cell cultures

The patient-derived cell cultures (Table 5) were established from patient ascites fluid or from tumor tissue, either from treatment naïve tissues collected during DBS, or after NACT during IDS. The tumor-derived cultures were established from primary tumors and extrapelvic tumor tissues. The OC origin of the cultures was confirmed with PAX8 and WT1 (study I) antibodies and/or characterized by DNA sequencing (study II).

In general, the patient-derived cells were cultured in the DMEM-F12-based spheroidal (3D) media. To sustain adherent (2D) cultures for the functional studies for IncuCyte experiments, in Study II, a modified OCMI medium (Ince et al., 2015) was used (study II).

Table 5: Patient-derived cell cultures and patient-related clinical information (Mupet/Hercules cohort, study I and II).

Cell code	Patient code	Stage	Diagnosis	PFI (days)	PFS (months)	OS (months)	NACT	IDS	Used in
M022	M022	IIIC	HGSC	45	3.1	4.0	yes	no	I
M022i	M022	IIIC	HGSC	45	3.1	4.0	yes	yes	I, II
M019i	M019	IVB	HGSC	30	2.4	34.3	yes	yes	I
M068i	M068	IVA	HGSC	31	9.3	11.2	yes	yes	I
M048i	M048	IVB	HGSC	121	9.4	35.8	yes	yes	II
OC002	OC002	IIIC	HGSC	135	10.1	12.0	yes	no	II

i in cell code: sample collected during interval debulking surgery (IDS) after neoadjuvant treatment (NACT)

4.4 Datasets from other studies (I)

To study the co-expression of stemness markers two existing patient-derived expression datasets were used. The primarily dataset from 2009 by Crijns et al was with 144 treatment-naïve primary tumor samples with clinical data from stage III-IV HGSC patient (Crijns et al., 2009). For validation, a smaller dataset from 2017 by Kreuzinger et al with RNA sequencing data from primary tumors of 66 HGSC patients were used (Kreuzinger et al., 2017). To enable the comparison of the datasets, eight stemness markers were included in both platforms.

4.5 High-throughput screening (I, II)

Drug testing was conducted using a panel of 306 oncology compounds through high-throughput screening (HTS), in patient-derived HGSC cell cultures and conventional cell lines. The drug panel consisted of compounds in clinical use (worldwide) and in clinical trials. The screens were conducted in collaboration with FIMM (Institute for Molecular Medicine Finland) High Throughput Biomedicine Unit at their facilities at the University of Helsinki.

The testing was performed under two growth conditions, i.e., conventional adherent (2D) and stemness features inducing spheroid (3D) conditions that mimic the environment of malignant cells in ascitic fluid. Five different drug concentrations spanning a 100000-fold concentration range were used. Cell viability was measured using CellTiter-Glo (Promega) according to the manufacturer's instructions after 72h exposure to the drugs.

The raw data from the cell viability testing was analyzed with a quantitative scoring approach at FIMM using the BREEZE tool, which quantifies and produces a drug sensitivity score (DSS) for each drug (Potdar et al., 2020). The gained DSS was compared to human healthy bone marrow-averaged controls to evaluate a specific selective DSS (sDSS).

The first set of screened (study I) cell lines included patient-derived M019i, M068i and M022i (Table 3). In study II an additional set of cell lines was screened: commonly available cell lines CAOV3, OVCAR8, and patient-derived cells OC002, and M048i (Table 4 and 5).

4.6 Flow cytometry (I, II)

In study I fluorescence-activated cell sorting (FACS) was used for sorting the ALDH1A1 positive and negative cells using (FACS Calibur and Aria instruments, BD Biosciences). Data were analyzed with FlowJo software (BD Biosciences). FACS was performed after staining cells with Aldehyde Dehydrogenase Based Cell Detection Kit (Stemcell Technologies).

Apoptosis and cell cycle were studied using BD LSRFortessa (BD Biosciences) flow cytometer in study II. The data was analyzed with Flowing Software 2.5.1. For apoptosis detection, Annexin V-FITC Apoptosis Detection Kit (ab14085, Abcam) was used. For cell cycle phase analysis, Click-iT EdU Flow Cytometry Assay Kit Pacific Blue (C10425, Invitrogen), was applied, both according to manufacturer's instructions.

4.7 Cell viability (I, II)

To validate the results of high-throughput screening (HTS) of drugs and determine the IC50-values of Wee1 inhibitor (Wee1i) adavosertib (Selleckchem) for each cell culture, a luminescence CellTiter-Glo (Promega) method was used. The same method was used for measuring the sensitivity of the cell cultures to cisplatin. Cells were plated on 96-well plates and treated with five different concentrations of adavosertib and cisplatin (1-10000 nM and 0.01-100 µM, respectively). After 72h, the cell viability was measured using Victor2 luminometer (Wallac). The IC50 values were calculated and 500 nM adavosertib was selected for functional cell studies.

The effects of 500 nM adavosertib (Selleckchem) on cell proliferation were measured in IncuCyte S3 high-content imager (Essen BioScience). The monolayer cell cultures were scanned every 2h for 72h. The proliferation was measured by confluence area with IncuCyte software (Essen BioScience).

4.8 Wound healing assays (II)

In study II, the effects of adavosertib (500 nM), on invasion and migration were investigated in adherent cell cultures using wound healing assay. An initial scratch was created with WoundMaker Tool (Essen Biosciences) on a confluent monolayer of cells in the IncuCyte ImageLock 96-well plates (Essen Biosciences). Wound closure was monitored every 2h for 72h in IncuCyte S3 high-content imager (Essen BioScience). Wound healing capacity was quantified by measuring relative wound density compared to untreated cells. Geltrex (Gibco) was used as precoating to attach the cells in the migration experiments, and Matrigel (Corning) in the invasion experiments.

4.9 Immunofluorescence (II, III)

In study II, DNA damage and nuclear abnormalities induced by adavosertib treatment (500 nM, 72h) of the cells were studied with immunofluorescence (IF) staining. The cells were cultured on Geltrex-coated coverslips to attach the cells to the surface in modified OCMI or RPMI medium. Cells were stained to visualize the nuclei, and DSB in the DNA caused by the adavosertib treatment. Mounting medium with DAPI (ProLong Gold Antifade Mountant with DAPI, Thermo Fisher) was used to stain the nuclei. Primary antibodies mouse monoclonal anti-α-Tubulin (B-5-1-2, Sigma), for actin filament visualization, and anti-γ-H2AX (phosphor S139, Abcam), for DNA damage detection, were used together with secondary antibodies, Alexa Fluor 555 Donkey anti-Mouse (Invitrogen), and Alexa Fluor 488-conjugated phalloidin (Invitrogen). The images were taken with Nikon Eclipse Ni fluorescence microscope, and ImageJ v1.53a software was used to merge the channels.

In study III, the TMAs were stained with the primary antibodies Mouse anti-ALDH (Clone 44/ALDH, BD Transduction Laboratories) and Goat anti-SOX2 (polyclonal, AF2018, R&D Systems). Secondary antibodies, Donkey anti-Mouse, Alexa Fluor Plus 647 (Invitrogen), and Donkey anti-Goat, Alexa Fluor Plus 555 (Invitrogen) were used before mounting with ProLong Gold (Invitrogen). The slides were scanned using Zeiss Axioscan Z.1 and Zen Blue 3.4. (Zeiss) Software was used for visualization.

4.10 Immunohistochemistry (III)

The TMAs were stained with ALDH1A1, SOX2, MYC, and BMI1 antibodies to visualize the expression of these markers in the tumor tissues. Two chromogenic immunohistochemistry (IHC) detection methods were used: horseradish peroxidase (HRP), and alkaline phosphatase (AP).

An HRP conjugated polymer kit (EnVision Detection Systems Peroxidase/Dab, Rabbit/Mouse, HRP. Rabbit/Mouse (DAB+), Agilent) was used for the detection of ALDH1A1, MYC, and BMI1. The primary antibodies were incubated overnight at +4ºC: Mouse anti-ALDH Clone 44/ALDH (BD Transduction Laboratories), anti-c-MYC phospho S62 (ab51156, Abcam), and Rabbit anti-BMI1 (D20B7, XP Rabbit mAB, Cell Signaling Technologies). Following primary antibody incubation, anti-mouse/rabbit reagents containing HRP-conjugated secondary antibodies were applied for detection, followed by counterstaining with hematoxylin.

For the detection of SOX2, the alkaline phosphatase polymer kit (ImmPRESS AP REAGENT KIT, Anti-Goat IgG, MP-5405, Vector Laboratories) was used. The primary antibody Goat anti-SOX2 (polyclonal Goat, AF2018, R&D Systems) was incubated overnight at +4ºC. Next, polymer reagent containing a secondary Goat IgG antibody conjugated with alkaline phosphatase (AP) was used for detection and followed by counterstaining with hematoxylin.

The stained TMA-slides were scanned using a 3DHISTECH Pannoramic 250 FLASH III digital slide scanner (3DHistech Ltd.) at 40x at the Helsinki Biobank and the scoring was conducted with SlideViewer (3DHistech Ltd.) software.

Semiquantitative IHC analysis was performed on the stained slides. Nuclear (SOX2, MYC, and BMI1) and cytoplasmic (ALDH1A1) immunoreactivity were evaluated. The extent (by %, intervals of 5) and intensity: 0, 1, 2, and 3 (negative, weak, moderate, and strong, respectively) were assessed. An intensity score (i.e., histoscore, H-score, 0-300 was calculated with a weighted algorithm (Kirkegaard et al., 2006) to address the heterogeneity of the immunoreactivity within the tumors.

4.11 *In situ* hybridization (III)

RNAscope 2.5 Assay-Brown detection kit (Bio-Techne) was used for the RNA *in situ* hybridization (RNA-ISH) to detect BMI1 mRNA in the patient tissues and was performed by manufacturer's instructions. The TMA slides were treated with hydrogen peroxide and treated with Protease Plus for 15 minutes at 40C. The BMI1 probes (591931-C1, Hs-PPIB 313901, and DapB 310043) were hybridized for 2 hours at 40C. TMA sections were treated with 50% hematoxylin, dipped into ammonia water, and dehydrated before mounting. The slides were scanned with 3DHISTECH Panoramic 250 FLASH III digital scanner, with 40x magnification and with 7 focus layers.

The scoring was performed manually and with an automated quantification tool. The manual scoring of RNA-ISH signals was performed semi-quantitatively by ACD scoring guidelines (ACD Biotecne, 2024). An automated quantification tool was developed on Aiforia`s cloud-based platform (Aiforia Technologies).

4.12 Statistical analysis (I, II, III)

For all statistical analyses *p*-values <0.05 were considered statistically significant. Statistical analyses were performed with MS Excel, GraphPad Prism 10.1.2 (www.graphpad.com), and VassarStats: Website for Statistical Computation (www.vassarstats.net).

4.12.1 Cell experiments

The IC50 values for the adavosertib cytotoxicity validation were acquired using a sigmoidal dose-response curve. Differences in proliferation, wound healing, cell cycle distribution, apoptosis, frequency of nuclear abnormalities, and protein expression levels between vehicle- and adavosertib-treated cells were compared using the two-sided t-test.

4.12.2 Clinical data-related experiments

Association of the markers and clinical characteristics was analyzed with Spearman´s correlation for the linear relationships and Fisher´s exact test for categorical variables. Paired t-test and Wilcoxon Signed-Rank were used to compare the difference between the adnexal and extrapelvic results.

Survival analyses were performed with the Kaplan-Meier (log-rank) method. The cut-off values for survival analyses were determined using the "auto select best cut off" feature available at Kaplan-Meier Plotter (Lánczky & Győrffy, 2021). Cox proportional hazards regression model was used for multivariate analysis.

4.13 Experimental procedures and methods

The full list of the experimental procedures and methods used in all studies is in Appendix 1. The procedures are represented in detail in each of the original publications and their supplementary data.

5 Results

5.1 Stemness features in patient-derived HGSC cells (I)

Cancer stem cells (CSCs) are key contributors to disease relapse and treatment resistance, as they are capable of remaining dormant and surviving conventional therapies. In study I, we established a patient-derived cell culture model to investigate the CSC phenotype in HGSC. By integrating existing expression datasets with our own patient cohort, we demonstrated that stemness-associated markers are enriched following neoadjuvant chemotherapy (NACT). Furthermore, in treatment naïve tumors, the stemness markers were associated with poor survival.

5.1.1 Identification of stemness marker-enriched subset of patients

The mRNA expression of ten stemness markers was investigated in a microarray expression dataset, which was built from 144 treatment-naïve samples from HGSC patients with stage III-IV disease (Crijns et al., 2009). Eight out of the ten stemness markers (*ALDH1A1*, *CIP2A*, *MYC*, *LIN28A*, *NANOG*, *SOX2*, *PROM1* and *BMI1*) stratified the patient samples into two clusters according to their mRNA expression. The enriched stemness cluster showed significantly higher stemness marker expression compared to the baseline cluster (Figure 13). The enriched stemness cluster included 32% of all cases. In addition, the same division of patients into two clusters was evident in a smaller validation dataset (Kreuzinger et al., 2017).

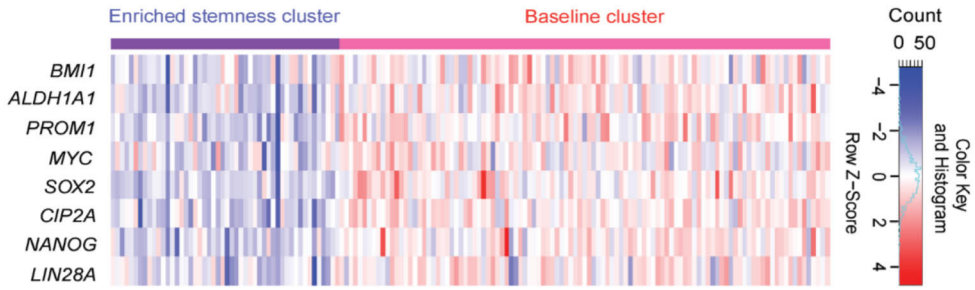


Figure 13: Expression heatmap of eight stemness markers in 144 treatment-naive high-grade serous carcinoma tumors in a microarray dataset (Crijns et al., 2009). Tumor samples were clustered by k-means clustering (k = 2). Adopted from study I.

5.1.2 Prognostic significance of stemness-enriched subset

The ability of the ten stemness markers to classify tumor subsets and predict survival was first studied in the same mRNA microarray expression dataset (Crijns et al., 2009). In a Kaplan–Meier (KM) survival analysis, patients belonging to the enriched stemness cluster had shorter OS than baseline cluster patients (median OS 20 months *versus* 33 months, log-rank test $p = 0.047$) (Figure 14). A similar grouping pattern was also found in the smaller validation data set of sixty-six treatment-naïve samples (Kreuzinger et al., 2017). However, in the validation dataset, the difference in OS between the two groups was not significant.

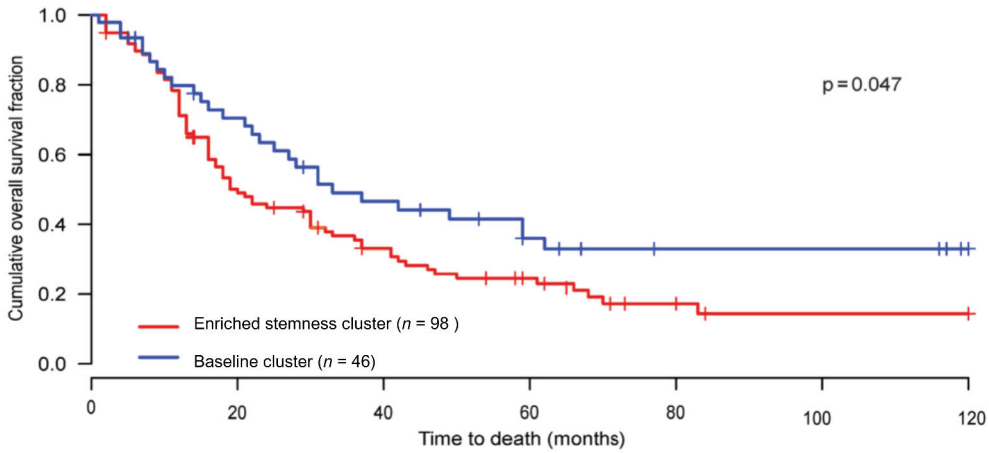


Figure 14: Kaplan–Meier survival curves of the different clusters. Enriched stemness cluster patients are associated with shorter overall survival than baseline cluster patients (Median 20 months versus 33 months, log-rank test p = 0.047). Modified from study I.

5.1.3 Stemness is increased in samples after NACT

To investigate whether platinum-taxane chemotherapy induces stemness features in HGSC samples, we performed a pairwise analysis of twenty-one treatment naïve-interval surgery samples in an RT-qPCR-based mRNA-expression dataset from the tumors. Several stemness markers showed increased expression in the interval samples compared to the treatment-naïve samples. ALDH1A1 level in interval tissue was significantly higher than in the treatment naïve samples ($p<$ 0,0001). The ALDH1A1 expression was increased in the interval sample in 71.4% (15/21) of the cases, and in 12.8% (3/21) the expression remained stable. Expression was decreased in the remaining 12.8% (3/21) of the interval samples. In the paired analysis, ALDH1A1 increased in interval samples and correlated with elevated MYC and BMI1 levels. On the other hand, an inverse correlation was observed between ALDH1A1 and the expression of markers such as SOX2. ALDH1A1, MYC, BMI1, and SOX2 expression was further investigated in a TMA in study III (results in section 5.2).

A gene ontology analysis was performed on the twenty-one paired treatment-naïve and interval samples. Potential differences in enrichment of stemness-related pathways were especially investigated. Significant enrichment was found in pathways associated with cell number maintenance, and stemness features in the interval samples. The most pronounced increase in interval samples was observed in somatic stem cell population maintenance pathway, with over 100-fold enrichment (GO:0035019, FDR-corrected p-value 1.2×10^{-44}). Similarly, pathways related to maintenance of cell number (GO:0098727, FDR-corrected p-value 1.7×10^{-66}) and stem cell population maintenance (GO:0019827, FDR-corrected p-value 2.1×10^{-66}) showed significant enriched in the NACT treated (interval) samples.

5.1.4 Stemness-enriched subset exhibits chemoresistance

Next, we explored the cell cultures' sensitivity towards the platinum-taxane standard treatment drugs, cisplatin, and paclitaxel. Three commonly available HGSC cell lines and a patient-derived paired set (treatment-naïve and interval=after NACT) cell cultures were examined in adherent and spheroidal cell culture conditions. We hypothesized that spheroidal culture medium promotes increase of stemness marker expression compared to adherent culture medium in HGSC cell cultures and that spheroidal cultures are more resistant to cisplatin and paclitaxel.

The cells cultured in spheroidal (3D) conditions were more resistant to both drugs (cisplatin, p=0.0141 and paclitaxel, p=0.0188), than cells cultured in adherent (2D) medium. Five (ALDH1A1, p=0.042; CIP2A, p=0.005; POU5F1, p=0.021; SOX2, p=0.019; and BMI1, p=0.003) of the ten selected stemness markers were

upregulated in cells cultured in spheroidal culture media when compared to the cells cultured in adherent media.

5.2 Expression and clinical significance of a putative stem cell marker panel in HGSC (III)

The dysregulated stem cell pathways are thought to be one reason for HGSC chemoresistance and reduced survival, but stemness markers for this disease are not well specified at tissue level. In study III, the main objective was to study the concomitant expression of ALDH1A1, SOX2, MYC, and BMI1, in a TMA of a cohort of treatment-naïve samples of ninety-five patients. The two specific questions were: 1) can these markers as a panel be used to identify CSCs in HGSC tissues, and 2) does expression of these markers correlate with clinical features and is there association with outcome. The protein expression was evaluated by immunohistochemistry, both adnexal and extrapelvic (typically omental) samples and scored using a weighted algorithm (H-score) to capture the heterogeneity of the immunoreactivity within the tumors. For BMI1, also RNA-ISH analysis was performed and the results quantified both by visual and automated scoring.

5.2.1 Clinicopathological characteristics

The cohort consisted of 95 HGSC patients from the Helsinki biobank collection. The inclusion criteria and detailed clinical characteristics that include age, body mass index, ASA physical performance status, blood CA-125 level at diagnosis, residual tumor in PDS, FIGO stage, PFI and OS, are presented in the section 4.2. No progression of disease was evident in thirteen out of 95 (14%) of the patients during follow-up (11-171 months, median 55 months).

5.2.2 Immunohistochemistry results for ALDH1A1, MYC, SOX2, and BMI1

Immunohistochemistry (IHC) was performed on the 95-patient TMA to study the co-expression of ALDH1A1, SOX2, MYC, and BMI1 proteins. SOX2, MYC, and BMI1 immunoreactivity was detected in the nuclei, whereas ALDH1A1was detected in the cytoplasm of tumor epithelial cells (Table 6). Additional staining was noted in the stromal fibroblast, except SOX2. Examples of the immunoreactions for each marker are in Figure 15.

Table 6: IHC expression results

Marker	ALDH1A1		SOX2		MYC		BMI1	
Cellular localization	cytoplasmic		nuclear (± cytoplasmic)		nuclear		nuclear (± cytoplasmic)	
Sample / patient size *n*	525	95	518	95	525	95	509	95
Positive staining *n* *(%)*	313 (59.7 %)	90 (94.7 %)	96 (18.5 %)	36 (37.9 %)	511 (97.3 %)	94 (98.9 %)	508 (99.8 %)	95 (100%)
Intensity score range (cut-off value)	0-300	0-280 (≥30)	0-285	0-255 (>0)	0-300	0-291 (≥195)	0-300	30-298 (≥172)
High expression *n* *(%)*		26 (27.4 %)		36 (37.9 %)		29 (30.5 %)		47 (49.5 %)

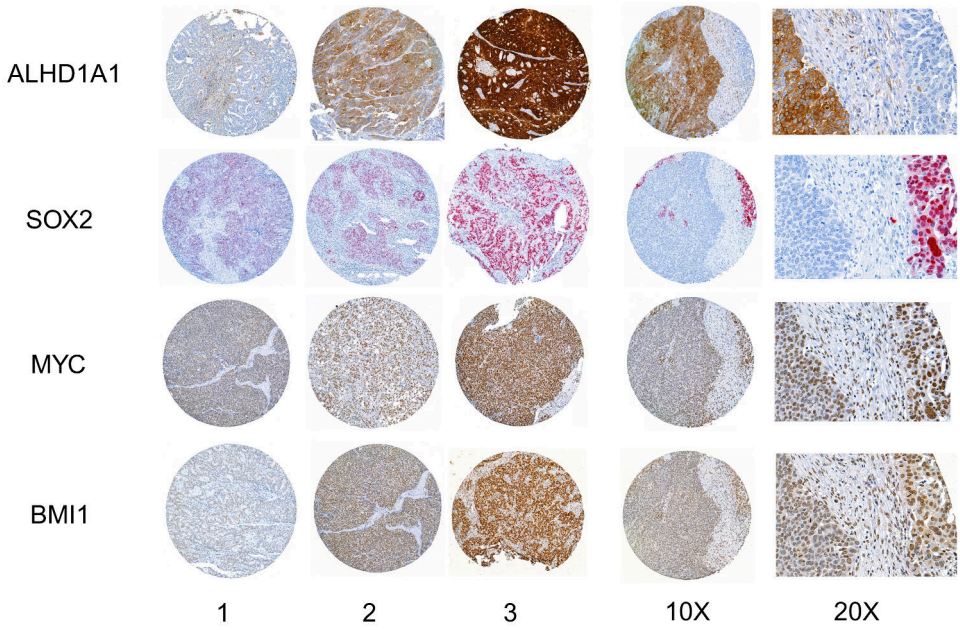


Figure 15: Examples of immunohistochemistry stainings of ALDH1A1, MYC, SOX2 and BMI1 in HGSC: 1 (weak), 2 (moderate), 3 (strong). Modified from study III.

Interestingly, ALDH1A1 and SOX2 immunoreactivity patterns were mutually exclusive at the cellular level (Figure 15). This was also evident in the multicolor immunofluorescence (Figure 16). The expression of MYC, BMI1, and ALDH1A1 throughout the tumor tissues indicates that these markers do not identify the CSCs in the HGSC tumor tissue. In addition, the mutually exclusive staining pattern of

ALDH1A1 and SOX2 strengthens the observation that this panel of markers does not specify the small subpopulation of HGSC CSCs in the tumors.

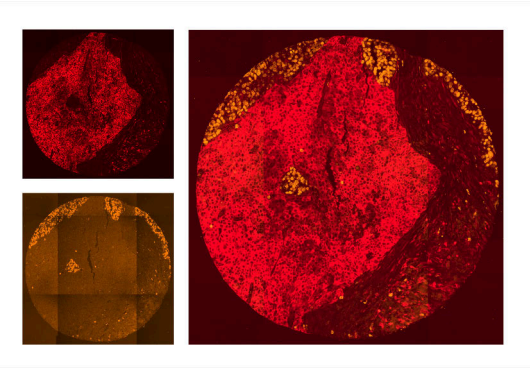


Figure 16: Example of immunofluorescence staining of ALDH1A1 (red) and SOX2 (orange): proteins are reciprocally expressed. Modified from study III.

Staining intensities of all the markers were heterogenous within the tumors. Therefore, we utilized histoscore to calculate an intensity score for each sample as described in section 4.9. Weighted patient-specific intensity scores were calculated. For further analysis with patient's clinical information, tumors were categorized into low and high intensity score at patient level. The intensity score range, cut-off for the groups, and number of positive staining (sample and patient level) are in Table 6.

The patient-level intensity scores of MYC and BMI1 correlated ($p<0.001$), whereas correlation was not evident between the other markers. A comparison of the protein intensity scores was done between the adnexal and extrapelvic tissues. The average MYC and BMI1 staining intensity score was higher in extrapelvic tissues than in adnexal ($p\leq0.001$ and $p=0.036$, respectively), but no difference was observed for ALDH1A1 and SOX2.

In summary, these results do not indicate that IHC analysis of ALDH1A1, SOX2, MYC, and BMI1 is a viable method for identifying CSCs in HGSC tissues.

5.2.3 Associations with clinical features and outcome

We next explored whether the individual markers were associated with any of the clinical parameters or survival. For all four markers, a prediction analysis for outcome (PFI and OS) was performed, using Kaplan-Meyer (KM) analysis. ALDH1A1 and SOX2 did not show association with outcome. High BMI1 intensity score was associated with reduced survivals, for both PFI (HR:1.86; $p=0.004$) and OS (HR:2.18; $p=0.0006$) (Figure 17). The average PFI for the low staining intensity

group was 38 months versus 16 months in the high intensity group, and the OS was 78 months versus 44 months, respectively. In addition, high MYC protein expression showed significant association with poor PFI (HR:1.62; p=0.033) and OS (HR:1.72; p=0.023) compared to low MYC expression.

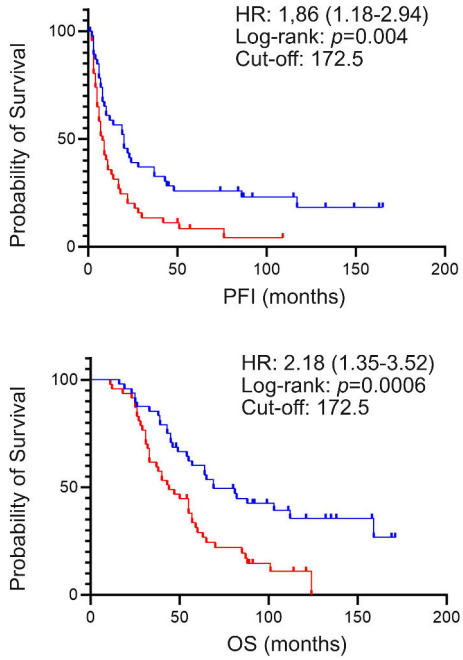


Figure 17: Kaplan-Meier survival analysis for patient level BMI1 immunohistochemistry intensity scores (low intensity score = blue, n=47; high intensity score = red, n=48). Platinum-free survival is 22 months and overall survival 34 months shorter in high intensity score BMI1 patients compared to low intensity score patients. Modified from study III.

The association of clinical features presented in Table 3 was compared with the intensity scores (high vs. low groups) of each of the four markers. When the clinical parameters were compared with the intensity scores (high vs. low groups), no association with body mass index, ASA, surgery outcome, or preoperative CA-125 was detected for any of the markers. Age (>63 years) was associated with high BMI1 intensity score (p=0.014) and higher FIGO stage (III vs. IV) with high MYC intensity score (p=0.019). In the multivariable analysis of the four proteins, their IHC intensity scores were correlated with survival (PFI and OS). High intensity score of BMI1 expression remained an independent prognosticator of shorter PFI (p=0.044) and OS (p=0.005). This analysis also showed that the presence of residual tumor after PDS (R1 or R2) was associated with worse PFI (p=0.006) and OS (p<0.001).

In summary, these findings demonstrate that high expression of BMI1 and MYC is associated with poor outcome and may serve as potential prognostic markers for HGSC.

5.2.4 Verification of BMI1 result with RNA-ISH

The survival analysis results based on BMI1 IHC was validated using RNA *in situ* hybridization (RNA-ISH) to confirm the finding at RNA-level. Even though protein and RNA expression are not necessarily identical, their co-existence support the interpretation that these genes and proteins are actively expressed.

In the KM-survival analysis of the RNA expression, a significant difference was observed in OS between the low and high expression groups. In the multivariate analysis, the RNA expression alone was an independent prognostic factor for OS, but the association was not as strong as with IHC results. When comparing the RNA expression between two tumor sites (adnexal and extrapelvic) the extrapelvic signal was higher, similar to that with IHC, but not statistically significant.

In conclusion, the RNA-ISH results supported the finding of BMI1 IHC being an independent potential prognosticator for the outcome in HGSC. In addition, the BMI1 expression is higher in the extrapelvic tumor tissues than in adnexal.

5.3 High-throughput drug testing (I, II)

High-throughput drug testing was performed on HGSC patient-derived cell cultures and on commonly available HGSC cell lines in two studies. In both study I and II, the main goal was to find effective state-of-the-art drug compounds using patient-derived HGSC cell cultures.

In study I, the objective was to perform the drug sensitivity testing on patient-derived cultures, as such studies were scarce for HGSC at the time. In addition, another aim was to perform the testing in two different cell culture conditions, spheroidal (3D) and adherent (2D), to investigate the potential effect of different culture conditions on drug sensitivity. Spheroidal culture condition was used to study the CSC phenotype and compare it to the conventional adherent culture phenotype drug activities. In study II, the objective was to use commonly available cell lines and compare their drug sensitivity to that of the patient-derived cells. Another rationale was to screen additional patient-derived cell cultures. In both studies I and II, our aim was to identify effective novel drug compounds for treating HGSC cells, and in further studies, to investigate their effects on cells and uncover novel targets for HGSC therapy.

The drug screens yielded selective drug sensitivity scores (sDSS) for each individual drug and cell line and growth condition. The comparison of selective DSS

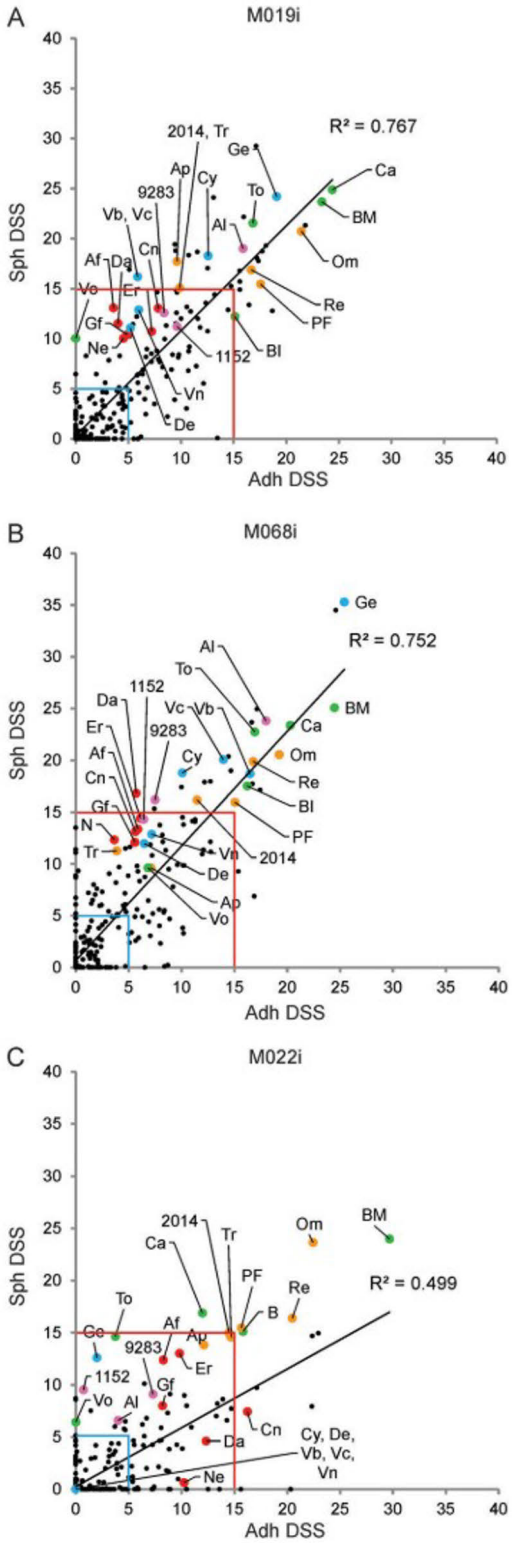
(sDSS) values between the screened cells revealed significant differences between individual cell cultures and between the culture conditions. The number of effective (sDSS>5) drugs from the screen of 306 drug compounds varied between screened cells and was partially dependent on growth conditions.

The results demonstrate that compounds target cancer cells from individual patients differently, possibly reflecting the variability of individual patients´ response to chemotherapy/targeted therapy.

5.3.1 Results from study I

In this first HTS, patient-derived cell cultures from interval (after NACT) DBS ascites fluid were utilized. Of the 306 compounds tested in the three patient-derived cell cultures, 33 to 14% of the drugs were effective (sDSS>5), depending on the cultured cells and the culture condition (adherent or spheroidal). The details of the cell lines used are in Table 5.

Five drug compounds were highly effective against all three cell cultures regardless of the culture media. When only the culture conditions were compared, thirteen compounds were more effective in the spheroidal cultured cells than in the adherent cultures. These thirteen drugs target EGFR, PI3K-mTOR, and aurora kinase activity. (Figure 18)


A
M019i
R² = 0.767
Sph DSS
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2014
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◄ **Figure 18:** Response of spheroid and adherent primary high-grade serous carcinoma cells to oncology compounds. Three correlation plots are presented on patient-derived cell lines: A) M019i, B) M068i, and C) M022i. Correlation plots represent sDSS of spheroidal (y-axis) and adherent (x-axis) cells. Drugs with a sDSS over five are considered effective and sDSS over fifteen represent highly effective drugs. The dots on the correlation plots represent drugs: BM, BMS-754807; Om, omipalisib; Re, refametinib; BI, BIIB021; PF, PF-04691502; Cn, canertinib; Da, dacomitininb; Ne, neratinib; Er, erlotinib; Af, afatinib; Gf, gefitinib; Tr, trametinib; 2014, AZD2014; Ap, apitolisib; Ca, camptothecin; To, topotecan; Ge, gemcitabine; 9283, AT9283; Al, alisertib; 1152, AZD1152-HQPA; De, decitabine; Vn, vinorelbine; Vb, vinblastine; Vc, vincristine; Cy, cytarabine; Vo, volasertib. Red=EGFR inhibitor (six); pink = aurora kinase inhibitor (three); orange=mTOR/PI3K inhibitor/MEK inhibitor (six); green= IGFR-/HSP90-/topoisomerase-/polo-like kinase inhibitor (five); blue = classic/nucleoside analogue (six). Adopted from study I.

In summary, the patient-derived cell cultures have different affinities for the drugs, M022i cells being more resistant than the other two cell cultures. Furthermore, the culture conditions can alter the effect of the drug compounds. The thirteen drugs that were effective only in spheroidal cultured HGSC cells might have been overlooked in previous screens performed solely on conventionally adherent cultured cells. Third, the HGSC cell cultures with stemness features can be targeted with drugs that are already in clinical use or trials.

5.3.2 Results from study II

In the second HTS of 306 drug compounds, two additional HGSC patient-derived cell cultures and two conventional HGSC cell lines were screened. Detailed information about the cell lines is presented in Tables 4 and 5. Percentages of the effective drugs for each cell cultured in adherent (2D) conditions and spheroidal (3D) cultures are in Table 7.

Table 7: Percentages of effective drugs (sDSS>5) of all drug compounds tested.

Cell line	Adherent	Spheroidal
OC002	19.3	32.7
M022i	23.5	15.4
M048i	9.1	na*
OVCAR8	24.2	16.3
CAOV3	33.3	33.0

*Did not grow as spheroidal culture.

In summary, the effectivity of the drugs varies depending on the cultures from different patients, reflecting on the patient's response to therapy. There was variation

between the patients-derived cell cultures, but in general they were more resistant than the conventional cell lines, especially the spheroidal cultures.

5.3.2.1 Selection of drug compound for functional studies

We had a particular interest in drugs that are already approved for other cancers or have promising clinical trials ongoing or completed. With this rationale in mind, we sought ten clinically interesting drugs for further investigation according to two criteria: literature search and ongoing clinical studies in HGSC or other cancers. The selected ten compounds and their corresponding drug sensitivity score-based heatmap are in Figure 19.

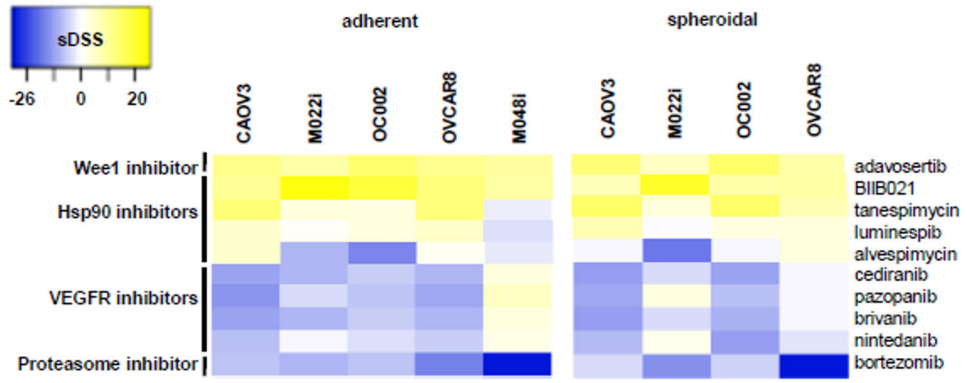

Figure 19: Heatmap of the selected drug compounds based on sDSS (selective drug sensitivity score) values tested in adherent and spheroidal conditions. Bright yellow = sensitive, dark blue = resistant; sDSS >5 effective, sDSS>10 highly effective. M048i spheroidal data not available. Adopted from study II.

Five of the ten drugs seen in Figure 17 were categorized as highly effective (sDSS>10) in most cell lines. The patient-derived M048i cells were relatively resistant to all ten drugs. Of these compounds, the Wee1 inhibitor, adavosertib, was chosen for further research due to its ongoing clinical trials and the validation results, which showed cytotoxic effects for all cells, with IC50s between 578-785 nM. The patient-derived M048i cells were an exception with rather strong resistance to adavosertib.

5.3.3 Summary of HTS results

In summary, the sensitivity of patient-derived cells to the screened drug compounds varied among the patients, reflecting the heterogeneity of clinical course and

response to treatment. In general, they were more resistant than the commonly available cell lines. The effect of the culture medium on sensitivity varied depending on the cell line. Additionally, there are available drug compounds that are effective on the HGSC cells with stemness features.

5.4 Effects of adavosertib on HGSC cell cultures (II)

Study II had two goals, to perform the HTS for drugs presented in 4.14.2 to help to choose a drug compound for further functional studies and to investigate the drug's effects on HGSC cells in the functional studies. With help of the drug screening, of literature search, and promising results from clinical studies we decided to select ten drug candidates for further analysis. In the drug screens, the Wee1 inhibitor, adavosertib (Wee1i, AZD1775), showed activity against most cell lines and was selected for further functional studies. These included the effect of the drug on cell survival, proliferation and migration and cell cycle progression, and effects on the nuclear morphology and DNA integrity.

5.4.1 Adavosertib inhibits proliferation and induces apoptosis

First, we investigated the effect of adavosertib on the HGSC cell proliferation and cell death. Proliferation was significantly reduced in all five cell cultures after adavosertib treatment (500 nM, $p<0.0001$, *t*-test), inhibition varying between 58.1% and 67.0% in the slowly proliferating patient-derived cells M022i and OC002, respectively. In the faster proliferating cultures M048i, CAOV3, and OVCAR8 the reduction was 55.2%, 38.0%, and 82.3%, respectively. Additionally, the proliferation marker PCNA (proliferating cell nuclear antigen) was detected by Western blotting to confirm the results. In all tested cell cultures, except OVCAR8, PCNA levels were reduced following Wee1 inhibition.

Cell mobility under and after adavosertib (500 nM) treatment was monitored using IncuCyte confluence area measurements in migration and invasion assays. Both migration and invasion were reduced in all tested cell cultures (Figure 20).

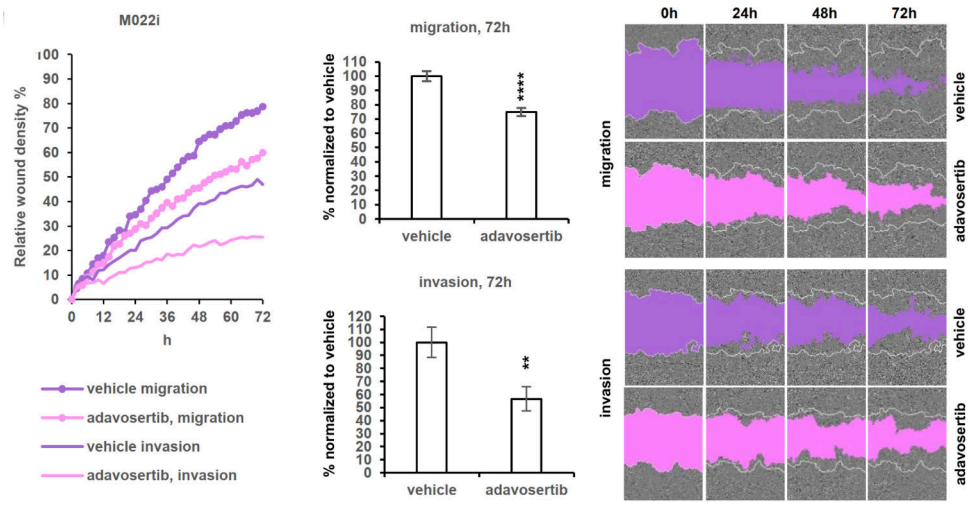

Figure 20: Example of wound healing assay. Patient-derived cell culture M022i was treated with adavosertib and monitored every 2h for 72h. Modified from study II.

Flow cytometry with Annexin V revealed that Wee1 inhibition by adavosertib induced apoptosis in all treated cell cultures, including the most resistant M048i cell cultures (Figure 21).

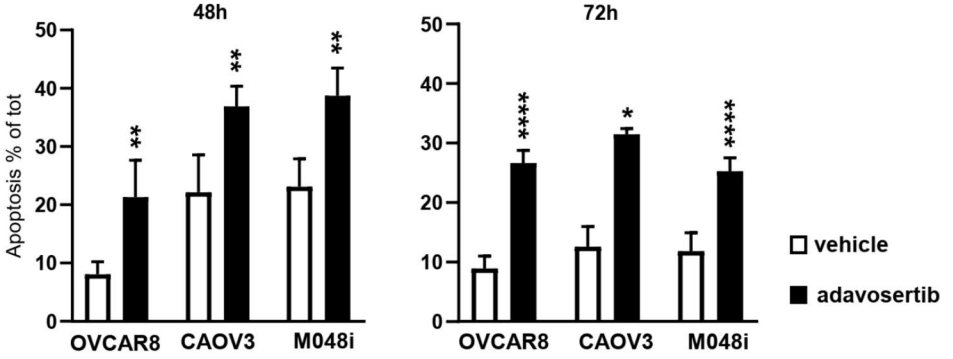

Figure 21: Apoptosis measured with Annexin V staining with Flow cytometry in three cell cultures. Apoptotic cell percentage was elevated significantly in all adavosertib-treated cultures after 72h treatment. Modified from study II.

5.4.2 Adavosertib causes cell cycle arrest

The effects of Wee1 inhibition on cell cycle progression were studied with flow cytometry. Compared to untreated cultures, all tested cell cultures showed a significant accumulation of G2/M phase and reduction in G1 phase of the cell cycle (Figure 22).

Wee1 inhibition diminished the proportion of G1 cells by 27-54%, depending on the cell line. Accumulation of G2 cells was observed in all tested cell cultures (Figure 18). The percentage (of the total cell number) of G2 cells in the treated cells and vehicle cells in OVCAR8, CAOV3, M048i and OC002 were: 58.3% and 14.4%, 45.3% and 16.0%; 54.0% and 23.9%; 57.2% and 24.8%, respectively. The trend towards reduced S phase was evident in all cell cultures but was significant only in CAOV3 and OC002.

Even in the most resistant patient-derived cell line, M048i, a drift from G1 to G2/M was observed. This, and the fact that adavosertib treatment increased apoptosis, suggest that this Wee1 inhibition has beneficial effects even on these patient-derived HGSC cells.

The changes in the cell cycle caused by Wee1 inhibition were further investigated with Western blot analysis. Cyclin E1 and cyclin B1, which regulate the G1/S and G2/M transition, respectively, were investigated. Cyclin B1 was reduced after adavosertib treatment, except in OVCAR8 where it remained unchanged. Cyclin E1 was reduced in CAOV3, OVCAR8, and M022i following adavosertib treatment. A key regulatory protein upstream of the Wee1-pathway, CHK1 was additionally studied. CHK1 expression was reduced in all tested cell lines after Wee1 inhibition. The amounts of these cyclins and CHK1 varied between the cell lines. M048i and OC002 expressed exceptionally low levels.

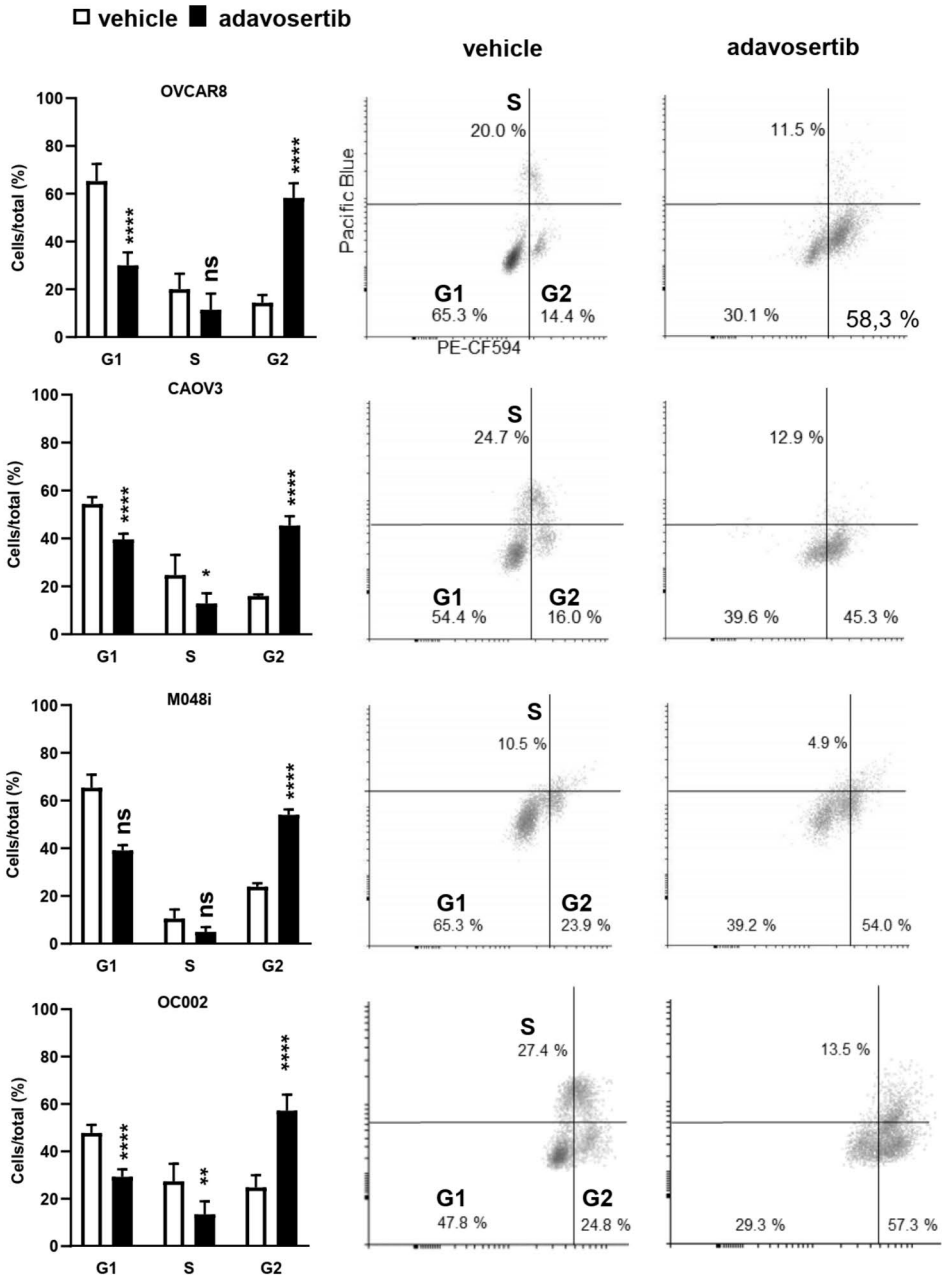

Figure 22: HGSC cells were labeled with 5-ethynyl-2-deoxyuridine (EdU), and cell-cycle phases were monitored with flow cytometry. The distribution of cells in the G1, S, and G2/M phases is shown after 72 h of treatment with adavosertib (500 nM) or vehicle. White bars = vehicle and black bars = adavosertib treated (average, ±SD). Dot blots present the distribution of cells with EdU staining. Adopted from study II.

5.4.3 Adavosertib induces DNA damage

In addition to cell cycle effects the impact of Wee1 inhibition on DNA damage and nuclear morphology was inspected. Adavosertib induced a significant increase in the number of aberrant nuclei in the cell lines, except in M022i. The DNA damage marker γH2AX (phospho S-139) was investigated with IF (Figure 23). It showed clear upregulation in all tested cells. This was confirmed with a Western blot.

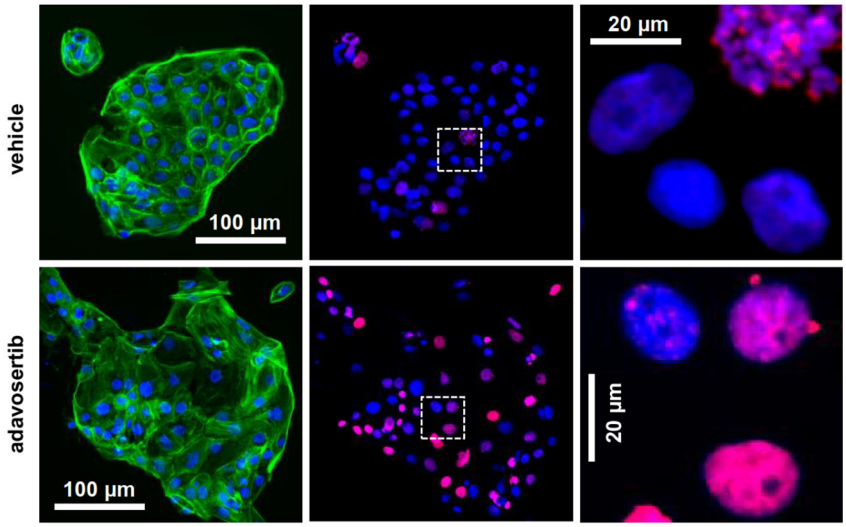

Figure 23: An example of DNA damage. M048i cells were cultured on coverslips and treated with 500 nM adavosertib for 72h. Cells were fixed and stained with gH2AX (phosphor S-139) for DNA damage, DAPI for cell nuclei, and phalloidin for the actin filament (green, actin; blue, nucleus; and red, DNA damage). Modified from study II.

5.4.4 HR-status does not influence the effect of adavosertib

The HGSC cell lines were evaluated for HR-capacity using genomic and functional testing. The efficacy of several HGSC drugs depends on HR DNA repair capacity of the cancer cells. Taken together, our analyses suggested that OC002 and OVCAR8 were HR-deficient (HRD) while M022i, M048i, and CAOV3 were HR-proficient (HRP). The detailed procedures and tables are in the original paper and supplementary data. The result suggests that the effects of adavosertib are independent of the HR status of the cells.

5.4.5 Wee1 inhibition in HGSC

In conclusion, study II shows that Wee1 inhibitor, adavosertib, affects the cell cycle checkpoints G1/S and G2/M regardless of the tumor cells' HR repair capacity status.

This, combined with a defective p53-pathway, makes the cells unable to halt the cell cycle for DNA repair in both cell cycle checkpoints resulting mitosis with the damaged DNA. This uncontrolled cell proliferation leads eventually to apoptosis due to very unstable DNA. (Figure 24).

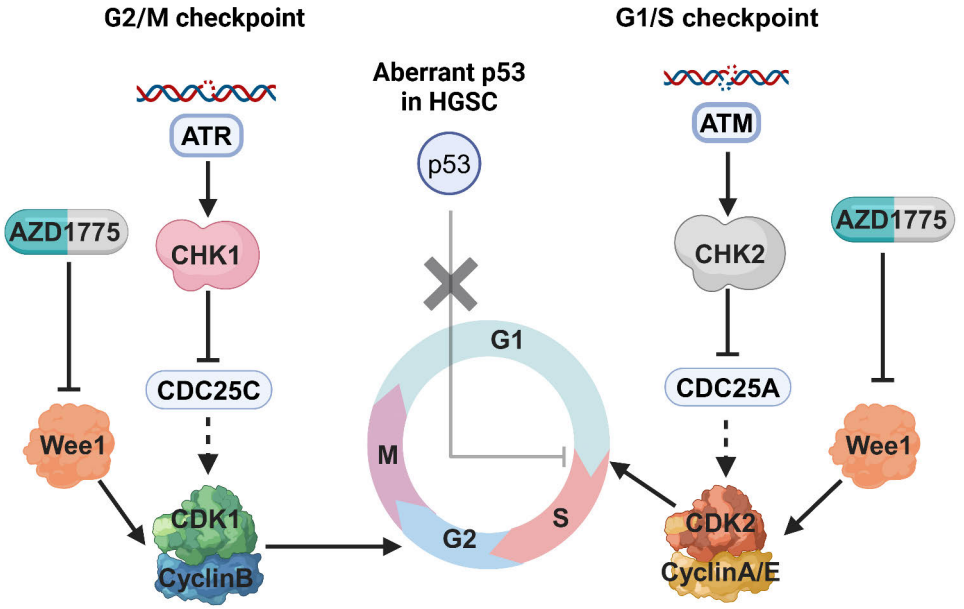

Figure 24: Wee1 inhibitor adavosertib (AZD1775) effect on HGSC cell cycle regulation in G1/S and G2/M checkpoints. By making both checkpoints dysfunctional the cells can freely proceed in cell cycle with damaged DNA and increased genomic instability. Modified from original study II (Roering et al., 2022) ,Created with BioRender.

6 Discussion

HGSC is heterogenous disease that has been managed with uniform treatment strategies for several decades. To improve therapeutic efficacy, there is critical need for well-characterized research models and to investigate subtype-specific characteristics that could enable the development of personalized targeted therapies. In this work, we established experimental stemness model of patient-derived cell cultures to study HGSC stemness markers *in vitro*. Thereafter, we analyzed the stemness markers expression using publicly available datasets as well as tissue samples and patient-derived cell cultures from our own patient cohorts.

Our results demonstrated a clear association of stemness marker expression with patient survival in treatment-naïve tumors. In the following study, we evaluated the prognostic value of CSC markers in a patient cohort TMA to bring the findings towards a more clinically applicable context. We identified the stemness-associated transcription factor BMI1 as being associated with poor OS and PFI, highlighting its potential as a therapeutic target in HGSC.

In a complementary approach, we performed a high-throughput drug screen to identify compounds effective against CSC-like HGSC cells. Using patient-derived cell cultures , we assessed drug resistance and sensitivity profiles and identified the Wee1 inhibitor adavosertib as highly effective in both HRD and HRP patient-derived cell culture models. Subsequent functional *in vitro* studies demonstrated that adavosertib impaired several critical cellular processes, including proliferation, cell cycle control, apoptosis and invasion, across all tested cell cultures, independent of the HR status. These findings suggest a potential therapeutic benefit of Wee1 inhibition for patients with HGSC.

6.1 *In vitro* -research models in HGSC (I, II)

HGSC tumors are heterogeneous. Over the past decade, the research community has increasingly recognized the importance and critical need for high-quality *in vitro* models to enable functional studies of HGSC (Bowtell et al., 2015; Domcke et al., 2013; Ince et al., 2015; Konstantinopoulos & Matulonis, 2013). Comprehensive genomic profiling has shown catalogues of somatic alterations that characterize the HGSC tumors (Bell et al., 2011; I˙ Zycka et al., 2023; Vang et al., 2016), prompting

closer scrutiny of the *in vitro* models used in experimental research. The profound characterization of the widely utilized OC cell lines has revealed that many of these cell lines fail to recapitulate the characteristics of the clinical tumors and accurately reflect the molecular and phenotypic features of HGSC (Domcke et al., 2013; Elias et al., 2015; Ince et al., 2015). To address this, Thu, and colleagues (2017) have made a great attempt to generate comprehensively characterized cancer cell lines, which are publicly available to study HGSC tumor biology (Thu et al., 2017).

In this study, we aimed to thoroughly characterize patient-derived HGSC cell cultures, with particular emphasis on preserving stemness features to better model tumorigenesis, metastasis, and therapy resistance progression of HGSC. In addition to various adherent cell culture models for patient-derived cultures, we employed a spheroidal growth medium designed to mimic the tumor microenvironment of HGSC cells more accurately within ascitic fluid. Most of our patient-derived cells (M019i, M068i, M022i, M022, OC002) were originally collected from ascitic fluid. This approach aimed to specifically support the spheroids and stemness features. The main difference of spheroidal culture compared to adherent cultures is the absence of stromal interactions (fibroblast, immune cells, endothelial cells, extracellular matrix, and vasculature) that are present in the tissues. In addition, spheroids in ascites are influenced by hypoxic and nutrient-deprived conditions.

In addition to the goal of sustaining the stemness features with help of spheroidal medium, we also aimed to compare differences between the cell cultures, to access potential differences between the tumors, and to contribute to the broader goal of developing physiologically relevant models, that better reflect the heterogeneity of HGSC and facilitate the advancement of personalized therapeutic strategies.

To address this, we performed HTS drug sensitivity and resistance testing using patient-derived cultures and commonly available cell lines cultured in adherent and spheroidal medium. This approach enabled us to evaluate the impact of the culture environment on drug responses. At the time, only few drug screening experiments had been conducted using patient-derived cells, and no studies had been conducted in the context of OC. Since then, the field has progressed significantly, with increasing recognition of the importance of maintaining the intrinsic biological characteristics of patient-derived material. A recent review by Zhang and colleagues (2024) summarized the current advances and challenges in the development of OC organoid models, highlighting the diversity of methods and emphasizing the need for standardization of culture protocols to facilitate the clinical translation of the cell-based research (M. Zhang et al., 2024).

Efforts have been made to develop more relevant research models for OC to better capture potential differences in drug responses under different culture conditions. Jabs and colleagues (2017) introduced an automated microscopy-based method to compare the drug responses between traditional 2D-monolayer and 3D-

organoid systems (Jabs et al., 2017). Their findings indicated that the type of culture system is a key determinant of cytostatic drug response efficacy, with organoid cultures exhibiting a stronger correlation with genomic alterations in the tumors than the 2D monolayer cultures. Another study has established OC organoids from patient-derived samples for drug sensitivity and resistance testing (Nanki et al., 2020). These organoids retained the histological subtype-specific character and mutational landscape of the original tumors, demonstrating their relevance for personalized therapy screening (Nanki et al., 2020). More recently, Senkowski and colleagues (2023) developed a robust protocol for establishing OC organoids that preserved both the genomic and phenotypic characteristics of the original tumors and further explored how different culture media influence the drug responses (Senkowski et al., 2023). In addition, several others have introduced standardized organoid protocols to improve reproducibility and clinical relevance in drug testing (Lliberos et al., 2024). Comparative studies have also emphasized the advantages of 3D-cultures over 2D-monolayers, particularly in drug discovery applications, as the limited penetration of compounds into spheroids can significantly affect therapeutic efficacy (Edmondson et al., 2014; Kapałczyńska et al., 2018).

In our drug screening studies (Kaipio et al., 2020; Roering et al., 2022), we focused primarily on the differences in drug efficacy between the culture conditions and the cell cultures. However, it would be valuable to investigate potential biological and genomic differences in more detail in the cells cultured in the 2D-medium versus spheroidal 3D-medium, beyond drug response alone.

Notably, HGSC is unique in its metastatic pattern, with tumor cell clusters disseminating and surviving freely in ascites fluid. Our study utilized a spheroidal culture medium, rather than fully developed organoid models, which may provide an attachment surface for the cells. A recent study has investigated dormancy and metastasis during disease evolution in spheroid and organoid models, reporting a dormancy-associated transcriptomic signature in spheroids, in contrast to an enhanced cell cycle regulation pattern in organoids, and speculates that EOC cells modify their biology between tumor and spheroid states (Tomas et al., 2025).

In summary, we found that cell culturing conditions affect the drug responses; however, the differences between the patient-derived cell cultures and cell lines had a greater impact on drug efficacy than the culture conditions. Notably, certain stemness features, for example, spheroid formation, and expression of stemness markers, were better maintained in spheroidal medium cultures compared to traditional 2D-cultures. Additionally, the standard chemotherapy agents, paclitaxel, and cisplatin, were more effective when cells were cultured in 2D-medium than those cultured in spheroidal medium.

In conclusion, the importance of *in vitro* cell culture for studying HGSC is evolving, and significant efforts are being made to better recapitulate the tumor

biology in research settings to enable more clinically relevant exploration of the disease. First, we need to study the alterations' biological significance in reliable *in vitro* settings to be able to translate the important findings *in vivo*. Only this way can the therapeutic potential be thoroughly investigated (M. Zhang et al., 2024). Second, we also need to know properly the cells we are using for the experiment. Thorough characterizing of patient-derived cell cultures to verify the origin, using methods such as sequencing and protein-based assays, is important.

6.2 Stemness features in HGSC cells (I)

The developing of the spheroidal medium culture condition and establishing patient-derived cell cultures gave us a tool to explore the HGSC stemness features. In HGSC the CSCs are thought to be critical in tumor initiation, chemoresistance, and metastasis. We aimed to identify a panel of known stemness-associated intracellular markers in HGSC. While stemness markers have been widely studied in different cancers, specific markers for HGSC are lacking. The heterogeneity of HGSC tumors adds challenges to the search for specific markers for the disease. An additional challenge in identifying CSCs lies in their highly dynamic nature, the phenotypic plasticity, and the suggested variable expression of stemness-associated markers (Saygin et al., 2019).

Our focus was on ten markers controlling the pathways for spheroidal stem cell-like phenotype: ALDH1A1, PROM1, MYC, LIN28A, NANOG, POU5F1B, POU51, SOX2, CIP2A, and BMI1. Our mRNA-analysis revealed that these markers were upregulated in treatment-naïve tumors from patients with poor survival outcomes. Notably, in cells cultured under spheroidal conditions, five markers: ALDH1A1, CIP2A, SOX2, POU5F1, and BMI1, showed increased mRNA-expression. Three additional markers: MYC, LIN28A, and NANOG, were co-clustered in the same tumor tissues alongside with the upregulated markers. Additionally, the cell experiment results indicate that ALDH1A1 may be one of the most prominent stemness markers that can be visualized in live cells and tumor tissue with Western blotting, IF, RNA-expression and IHC. This is in line with previous studies and has been reviewed recently (Barczynski et al., 2023; Lučić et al., 2023).

On the cellular level, we showed that these markers were upregulated in cells surviving chemotherapy and that cells cultured in spheroidal cell medium demonstrated sensitivity to a lesser amount of drug compounds. Reasons for resistance of the CSCs might lie in the phenomena of epithelial-to-mesenchymal transition (EMT) that is associated with stem cell features (Padilla et al., 2019; Shibue & Weinberg, 2017). Further investigation of the mechanisms involved in the program that activates the EMT of cancer cells to enter the CSC state are needed to better understand and target the CSCs. An additional mechanism for CSCs ability to

resist drugs lies in the efflux pumps that actively transport drug molecules out from the cell. These pumps are known to have significant role in multi drug resistance (MDR) phenomena and, for example, in platinum resistance. (Frezzini & Lonardi, 2024; Khan et al., 2021)

Our drug sensitivity and resistance HTS results identified several compounds that are associated or targeting CSC-related pathways. We found compounds targeting epidermal growth factor receptor (EGFR), phosphoinositide 3-kinase (PI3K) and aurora kinases to be effective on the stemness phenotype cells. Several studies and clinical trials have demonstrated efficacy and benefits of drug compounds targeting PI3K-mTOR and aurora kinase pathways in several cancers (Mou et al., 2021). Aurora kinase inhibitors have shown efficacy in cell cultures and benefit in clinical trial in OC, in addition to which Aurora kinases have been shown to have a role in CSC biology (Alcaraz-Sanabria et al., 2017; Matulonis et al., 2012). Compounds targeting EGFR and their effect on ovarian cancer is less studied with modest results in clinical settings (Glaysher et al., 2013; Wei et al., 2023).

6.3 Expression of stemness markers and BMI1s clinical significance in HGSC (III)

The standard treatment for HGSC has remained largely unchanged for decades and is limited in long term due to, the development of chemoresistance during therapy. Despite significant research efforts, no HGSC-specific biomarkers have yet been translated into clinical use for predicting treatment response, prognosis, or clinical decision-making. Currently, the only tissue-based IHC markers routinely used in clinical settings are p53 and WT1, which are used to support histopathological diagnosis. The serum biomarker, CA-125, though nonspecific and elevated in various benign and malignant conditions, remains widely used in the diagnosing and monitoring disease progression. Additional blood-based markers, such as, HE4 and CA19-9, may also be monitored during follow-up, but their specificity for HGSC is limited. However, the assessment of BRCA1/2 mutational status and HRD represent notable advancements in the field of HGSC biomarkers. These are used to guide targeted therapies of PARPi, in recurrent HGSC patients with HRD. Currently, BRCA and HRD status serve as key clinical biomarkers for treatment stratification, and p53 and WT1 immunostaining are used diagnostically. Serum biomarker CA-125 remains in use for disease progression and relapse surveillance. (McMullen et al., 2021)

To address the lack of clinical biomarkers for HGSC, we initially investigated a panel of CSC markers, which demonstrated promising results on the mRNA-expression level in functional *in vitro* assays and database analyses (Kaipio et al., 2020). We aimed to investigate the role of CSCs as biomarkers in HGSC at the tissue

level to bring the research closer to the clinical application and to correlate the findings with clinical data. We further studied four of these markers, ALDH1A1, SOX2, MYC, and BMI1, in a TMA of the HGSC cohort of ninety-five patients (Roering et al., 2025, manuscript). The wide expression of these markers across the tumor cells indicated that they are not specific for CSC cells in HGSC. This conclusion is further supported by the notion that the spatial expression of the marker panel is not overlapping, *i.e.,* that in individual tumors, ALDH1A1 and SOX2 staining patterns were mutually exclusive. However, this does not exclude the existence of CSCs in these tissues as the heterogeneity and the plasticity of the phenotype between cancer cells and CSC is elusive and continuously changing (da Silva-Diz et al., 2018; Lupia & Cavallaro, 2017). In addition, heterogenous staining patterns of the CSC markers, including BMI1, in OC tissues have previously been reported (Zeng et al., 2014).

In the present study, even though the four markers did not perform as a panel to identify the CSCs in HGSC tissue, we found that BMI1 and MYC are expressed in parallel, and a high expression level is associated with poor outcome. We showed that BMI1 is an independent prognosticator of PFI and OS and is a potential tissue biomarker in HGSC. This was evident in the IHC and RNA-ISH results. Regarding the BMI1 IHC, the PFI for the low expression patient group was 15 months longer and OS 34 months longer than for the BMI1 high expression patient group. The co-expression of these two markers is not surprising as they can regulate each other. In addition, the BMI1-MYC-axis may contribute to treatment resistance by promoting CSC phenotype. (Benetatos et al., 2013) In line with our study, four other studies have shown that high BMI1 expression is associated with poor outcome (El Hafez et al., 2014; Gui et al., 2014; Yang et al., 2010; Zeng et al., 2014). It is important to note, that these studies were performed using EOC cohorts that were not necessarily curated according to the molecular categorization standard available today, highlighting the need for additional studies focused specifically on HGSC subtype. It is known that EOC subtypes differ in their molecular biology, and clinical course (Lheureux et al., 2019). This need has been acknowledged by other groups as well, for example, one study reported BMI1 expression heterogeneity in endometriosis vs non-endometriosis OC (Lozneanu et al., 2021).

As we and others have acknowledged the potential of BMI1 as a biomarker in OC it is important to continue the research in the different EOC subtypes, especially in the most common and lethal HGSC.

BMI1 plays a multifaceted role in several CSC-associated pathways, including the cell cycle, survival, and senescence (Bhattacharya et al., 2015; Floyd et al., 2024; E. Wang et al., 2011; Xu et al., 2022). In addition, BMI1 is also implicated in the HR pathway (Fitieh et al., 2022; Ginjala et al., 2011; Ismail et al., 2010) and is recognized as a transcriptional and epigenetic regulator. Recent proteogenomic

analyses by Bateman and colleagues (2024) demonstrated elevated BMI1 expression in HRP HGSC tumors, but not in HRD tumors. Notably, increased BMI1 expression was correlated with worse outcome (Bateman et al., 2024). In addition, they showed that HRP cells are sensitive to BMI1 inhibition. Additional *in vivo* and *in vitro* studies have reported promising results with BMI1 inhibitors (BMI1i: PTC-209, PTC-596, and PTC-028) (Dey et al., 2016, 2018, 2022), and several of these compounds are currently being evaluated in clinical trials (Shapiro et al., 2021). Furthermore, at least one *in vitro* study demonstrated that microRNA-132-mediated suppression of BMI1 expression in OC sensitized cells to cisplatin (X. L. Zhang et al., 2019).

Collectively, the above-mentioned studies are consistent with our observations and, the known role of BMI1 in the regulation of HRR, supporting its potential role as a therapeutic target in HGSC. The combination of BMI1i with platinum-based therapy may represent a promising therapeutic approach for patients with HRP tumors. Conversely, in HRD-patients, combination therapy of BMI1i with PARPi may offer a synergistic benefit by targeting multiple DNA repair pathways, thereby advancing therapeutic efficacy in this subgroup of patients.

However, translating these findings into clinical use requires further research. In particular, the role of HR-status warrants deeper investigation, both in terms of underlying biological mechanisms and tissue-level characterization and extending to clinical evaluation. To robustly assess the potential of BMI1 IHC as a predictive biomarker for BMI1i therapy, studies utilizing larger, well-characterized patient cohorts with HGSC subtype, including HR-status, and appropriate validation cohorts, are essential. Moreover, comparing primary, metastatic, and, recurrent as well as NACT treated tumors, would provide valuable insights into BMI1 expression throughout disease progression.

6.4 Adavosertibs effects on HGSC cells and its clinical potential (II)

In addition to examining drug sensitivity and resistance in the HTS from the stemness perspective, we analyzed the results independently of the specific culture conditions, and we focused on compounds that are effective in both adherent and spheroid cultures. This approach was based on the hypothesis that such agents could serve as promising first-line therapies that could enhance the efficacy of the standard carboplatin-paclitaxel chemotherapy and reduce risk of relapse. To guide compound selection, we reviewed the existing literature and ongoing clinical trials, identifying ten candidate molecules from the HTS. Of these, five demonstrated activities in both adherent and spheroidal culture conditions, and two of which, Wee1i adavosertib and the Hsp90 inhibitor (Hsp90i) BIIB021, showed particularly high efficacy

(sDSS>10). The other three effective compounds, tanespimycin, luminespib, and alvespimycin, were all Hsp90 inhibitors. Based on further validation studies, adavosertib was selected for functional *in vitro* experiments to evaluate its effect and therapeutic potential in both patient-derived and commonly available HGSC cells.

In this study, we found that adavosertib treatment reduces proliferation, increases apoptosis, and increases replication stress, as well as impairs cell migration and invasion, in all tested HGSC cell cultures, both patient-derived and commonly available. Interestingly, the effects were observed even in the most resistant cell culture, M048i, despite its resistance in the conventional cytotoxicity assay. Several cell studies investigating Wee1 inhibition on the cell cycle have obtained similar results to ours: shortened S-phase and accumulation of cells in the G2/M phase (Chen et al., 2018; Heijink et al., 2015; H.-Y. Kim et al., 2016; F. Li et al., 2020). However, a comprehensive investigation of the mechanisms and validation of Wee1 inhibition has been lacking in HGSC. There are some studies in other cancers, even though the focus with Wee1 inhibition has been in OC.

Several clinical trials have demonstrated the promising cytotoxic activity of adavosertib, both as monotherapy and in combination with other agents, across various solid tumors (Cuneo et al., 2019; Do et al., 2015; Leijen, Van Geel, Pavlick, et al., 2016; Leijen, Van Geel, Sonke, et al., 2016; Lheureux et al., 2021; Mendez et al., 2018; Oza et al., 2015; Sanai et al., 2018). However, these combinations regimens have shown limited clinical benefit in HRP and platinum-resistant OC. In response, alternative combinatorial strategies have been explored. Notably, a clinical trial demonstrated the promising efficacy of adavosertib combined with gemcitabine in platinum-resistant OC patients (Leijen et al., 2016). This finding was further supported by *in vitro* data suggesting that Wee1 inhibition sensitizes cells to gemcitabine (Saini et al., 2015).

Importantly, our findings demonstrate that adavosertib effectively inhibits HGSC cell viability irrespective of HR-status. Several ongoing clinical trials are investigating the combination of adavosertib with PARPi (Olaparib) in treatment refractory tumors, including OC (W. Zhang et al., 2024). Previous studies in other malignancies have suggested that adavosertib can impair HR and enhance the efficacy of PARP inhibition (Chiappa et al., 2022; Garcia et al., 2017). These investigations primarily focus on the combinatorial potential of adavosertib and PARPi (Schutte et al., 2023), and emerging clinical data indicate that this approach may benefit in PARPi-resistant OC patients (Westin et al., 2021). Preliminary results from the same clinical trial also suggest that adavosertib monotherapy is beneficial for these patients (Westin et al., 2021). However, our *in vitro* results suggest that adavosertib may have therapeutic efficacy as a single agent therapy in HGSC, independent of HR-status, supporting its broader potential for the HRP patients that are beyond current combination strategies.

To facilitate the clinical translation of our findings regarding the role of Wee1 and the therapeutic potential of adavosertib in HGSC, additional in-depth functional *in vitro* studies are needed to study the mechanistic actions of adavosertib further. These studies should be conducted using carefully selected and well-characterized cell lines and patient-derived cell cultures, as well as a well-curated patient cohort with clinical data and tissue samples that accurately represent the molecular heterogeneity and subtype-specific features of HGSC. Furthermore, clinical trials stratified by tumor subtypes are essential to determine differential responses to Wee1 inhibition and to identify patient populations most likely to benefit from Wee1-targeted monotherapy or combination therapy. It is particularly important to assess the impact of HR status on therapeutic outcomes and whether it modulates the efficacy of Wee1 inhibitors. As adavosertib is associated with some cytotoxic effects, the clinical evaluation needs to be studied further. A study has been evaluating the potential of sequential therapy using PARP and Wee1 inhibitors to minimize the toxic effects (Fang et al., 2019). Recently, new Wee1 inhibitors have also been developed and studied, showing promising results with potentially less toxicity (Ma et al., 2024, 2025). One of these, azenosertib, has shown promising results in a clinical trial for cyclin-E1-positive platinum-resistant OC patients (NCT05128825). In addition to clinical trials, it is important to identify biomarkers that can predict which patients may benefit from Wee1i therapy. Alongside *in vitro* mechanistical studies, tissue-based analyses would be valuable to perform for clinical application.

6.5 Limitations and prospects of the study

This thesis research is largely based on the patient-derived tissue samples and on cell cultures established from tumor and ascites material. The clinical data of the patients play a central role in the research material. During the initial stages of sample collection and the establishing the patient-derived cell cultures, our knowledge developed gradually, and in retrospect it is possible to identify procedures that could have been performed differently with the expertise gained over the years. Similar challenges have been reported in other studies, particularly regarding the limited availability of reliable and physiologically relevant *in vitro* models for studying drug responses in HGSC, and the tumor microenvironments involvement in influencing the drug efficacy. Cancer research is challenging due to complex, interconnected signaling systems within cells and, at the tissue level, the more intricate networks of intra- and extracellular communication. A further complicating factor is the heterogenous nature of HGSC. Typically, *in vitro* cancer research begins with simple model systems, which are then upgraded into more complex more representative models with the targets that have shown promising results in the initial phase.

Although our approach to screening drug responses using ascites mimicking 3D cell culture is by no means inadequate, the promising results obtained with the Wee1 inhibitor adavosertib warrant further investigation. These studies should be extended to *in vitro* systems that more comprehensively recapitulate the tumor microenvironment to generate deeper insight into the underlying mechanisms and the complex, interconnected cellular pathways involved in the tumor tissues.

Another perspective of particular interest is the potential to translate these findings into clinical applications in more rapid manner. To this end, the role of Wee1 as a tissue biomarker, and as a prognostic or predictive marker, should be investigated in a well-characterized cohort of HGSC patients in order to assess its association with survival. To date, only a limited number of tissue-based studies in HGSC cohorts have been conducted, and the existing ones are often small in scale.

Moreover, during the years of this thesis project, treatment guidelines for HGSC patients have evolved. The introduction of PARP inhibitors into the treatment strategy for advanced HGSC has urged clinical testing of HR-status to guide treatment decisions. This information, however, has not been available for our patient cohorts. In the light of our findings –particularly BMI1 and Wee1– it would be important to further investigate these markers in patient cohorts and patient-derived cell cultures for which HR-status is known. A further limitation for the biobank cohort study with BMI1 marker is the absence of a validation dataset. Our findings should be confirmed with another dataset.

In conclusion, the establishment of well-curated HGSC patient cohorts is essential for advancing the study of the disease, the identification of potential tissue-level biomarkers, and the evaluation of drug targets. Equally important is to establish and use well-characterized patient-derived cell cultures for the cell-based studies. Comprehensive clinical data collected from these patients play a central role in enabling such studies. A major challenge, however, lies in the extended time required for the collection of patient cohort and clinical data.

7 Conclusions

This thesis work focused on searching novel biomarkers and drug molecules for HGSC. Based on this thesis, we found following:

Stemness features in HGSC cells and tissues (I)

1) The established spheroidal cell culture medium sustains stemness features in cultured HGSC cells.
2) In treatment-naïve tumors, a set of eight stemness markers identifies a patient group with reduced survival probability.
3) Stemness features are increased in NACT tumors compared to treatment-naïve tumors.

A putative stem cell marker panel for HGSC (III)

4) The panel of four stemness markers (ALDH1A1, SOX2, MYC, and BMI1) does not specify HGSC cancer stem cells in IHC analysis.

Stemness markers BMI1 and MYC in HGSC (III)

5) IHC-based high BMI1 and MYC protein expression in treatment-naïve HGSC tumors are independent predictors of poor outcome. Similarly, ISH-based BMI1 predicts outcome.
6) BMI1 is a potential therapy target in HGSC.

Drug testing on HGSC cells (I, II)

7) Drug responses of patient-derived cell cultures reflect the patient´s response to therapy. Patient-derived cell cultures are more resistant than conventional cell lines.
8) HGSC cells cultured in spheroidal medium are more resistant than cells cultured in conventional 2D medium.

Effects of adavosertib on HGSC cells (II)

9) Functional *in vitro* studies show that Wee1 inhibition with adavosertib affects HGSC cells on several levels independently of HR status.

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