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Cancer stem cells and homologous recombination deficiency in serous ovarian and endometrial carcinoma

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Cancer stem cells and homologous recombination deficiency in serous ovarian and endometrial carcinoma

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Cancer stem cells and homologous recombination deficiency in serous ovarian and endometrial carcinoma

Maria Bååth



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DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.

To be defended on June 11th, 2021, 13.00

Faculty opponent

Professor Camilla Krakstad

Haukeland University Hospital and University of Bergen, Norway

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Title and subtitle: Cancer stem cells and homologous recombination deficiency in serous ovarian and endometrial carcinoma		
Abstract <p>Pelvic high-grade serous carcinoma (HGSC) is an aggressive disease that kills approximately 50% of all women diagnosed. Chemotherapy treatments are effective, yet most women relapse within a few years. PARP inhibitors, which target the poor DNA repair system found in these tumors, are showing good results with longer survival, however, relapse rates are still high. A subpopulation of cancer stem cells has been suggested to be a likely driver of relapse, as these cells have mechanisms to evade chemotherapy and strong self-renewal capacity. I evaluated the prognostic values of the stem cell marker SOX2 and the growth factor receptor MET. I also investigated the level of malfunction in the DNA repair system homologous recombination (HR) in both HGSC and serous endometrial carcinoma (SEC) and compared this to clinical variables, as well as to specific genetic events, such as loss of important DNA repair genes. I found that the occurrence of SOX2 positive cells was associated with shorter survival in patients with tumor tissue remaining after surgery. Further, in the subgroup of tumors lacking SOX2 expression, MET expression was associated with shorter survival. Investigating serous endometrial carcinomas, I found that HR deficiency was common, and genetic loss and/or loss of heterozygosity in the BRCA1/2 and RAD51C genes was associated with a higher HRD score. The strong impact of SOX2 expression found in this thesis warrants further studies into the stem cell mechanism in HGSC. The estimated level of cancer stem cells should be included in clinical trials evaluating drug response. As MET was found prognostic in tumors lacking stem cell factor expression, MET inhibitors currently indicated in other tumor forms should be further evaluated also in HGSC, paying special attention to the level of cancer stem cells. Finally, I conclude that as HGSC and SEC have multiple overlapping genetic features, including poor DNA repair, patients diagnosed with SEC could potentially also be eligible for PARP inhibitor treatment.</p>		
Key words: Ovarian Cancer, HGSC, Endometrial cancer, SEC, Cancer stem cells, SOX2, MET, HRD, PARP inhibitor		
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List of original studies

Studies included in the thesis

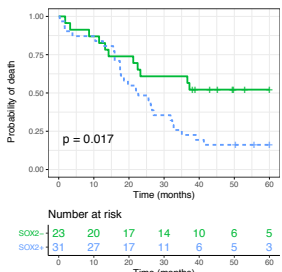

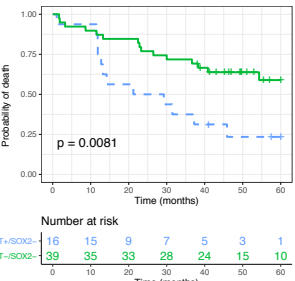
- I. **Maria Bååth**, Sofia Westbom-Fremer, Laura Martin de la Fuente, Anna Ebesson, Juliette Davis, Susanne Malander, Anna Måsbäck, Päivi Kannisto and Ingrid Hedenfalk (2020). **SOX2 is a promising predictor of relapse and death in advanced stage high-grade serous ovarian cancer patients with residual disease after debulking surgery** *Molecular and Cellular Oncology*, 7(6) 1805094.
- II. Jenny-Maria Jönsson, **Maria Bååth**, Ida Björnheden, Irem Durmaz Sahin and Ingrid Hedenfalk (2021). **Homologous recombination repair mechanisms in serous endometrial cancer** *Cancers*, 13, 254.
- III. **Maria Bååth**, Jenny-Maria Jönsson, Sofia Westbom Fremer, Laura Martín de la Fuente, Lena Tran, Susanne Malander, Päivi Kannisto, Anna Måsbäck, Gabriella Honeth and Ingrid Hedenfalk. **MET expression and cancer stem cell networks impact outcome in high-grade serous ovarian cancer**
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Study not included in the thesis

Ida Lindgren †, **Maria Bååth** †, Tina Uvebrant, Annika Dejmek, Louise Kjaer, Emir Henic, Mona Bungum, Leif Bungum, Corrado Cilio, Irene Leijonhufvud, Sven Skouby, Claus Yding Andersen and Yvonne Lundberg Givwerzman (2016). **Combined assessment of polymorphisms in the LHCGR and FSHR genes predict chance of pregnancy after *in vitro* fertilization** *Human Reproduction (Oxford, England)*, 31(3) 672–683

† These authors contributed equally to this work

Overview of the projects

Study	Questions	Methods	Results and Conclusion																
I	Can the stem cell factor SOX2 be used as a prognostic tool in HGSC?	Immunohistochemical staining of a tissue microarray.	<p>SOX2 is predictive of survival in the subgroup of patients with suboptimal debulking surgery.</p>  <p>Number at risk</p> <table><tr><td>SOX2-</td><td>23</td><td>20</td><td>17</td><td>14</td><td>10</td><td>6</td><td>5</td></tr><tr><td>SOX2+</td><td>31</td><td>27</td><td>17</td><td>11</td><td>6</td><td>5</td><td>3</td></tr></table>	SOX2-	23	20	17	14	10	6	5	SOX2+	31	27	17	11	6	5	3
SOX2-	23	20	17	14	10	6	5												
SOX2+	31	27	17	11	6	5	3												
II	<p>Is HRD a common feature in SEC?</p> <p>Is HRD score associated with copy number of any specific HRD associated genes?</p>	<p>Copy number analysis of SEC tumors.</p> <p>HRD was calculated from the copy number data.</p> <p>Analysis of HRD associated genes in relation to HRD.</p>	<p>HR deficiency is a common feature of SEC and these patients might also benefit from PARP inhibitor treatment, as is indicated in HGSC.</p> <p>The HRD score was associated with a higher level of genomic loss in key HR genes.</p> 																
III	Does the presence of cancer stem cells in the tumor affect the prognostic value of MET in HGSC?	Immunohistochemical staining of a tissue microarray. Exploration of mRNA expression in cell lines and a HGSC TCGA cohort.	<p>MET expression is prognostic in itself and the impact is even more pronounced in SOX2 negative tumors. This indicates that tumors with a weak stem cell network depend more on MET signaling.</p>  <p>Number at risk</p> <table><tr><td>MET+/SOX2-</td><td>16</td><td>15</td><td>9</td><td>7</td><td>5</td><td>3</td><td>1</td></tr><tr><td>MET-/SOX2-</td><td>39</td><td>35</td><td>33</td><td>28</td><td>24</td><td>15</td><td>10</td></tr></table>	MET+/SOX2-	16	15	9	7	5	3	1	MET-/SOX2-	39	35	33	28	24	15	10
MET+/SOX2-	16	15	9	7	5	3	1												
MET-/SOX2-	39	35	33	28	24	15	10												

Populärvetenskaplig sammanfattning

Äggstockscancer är en relativt ovanlig sjukdom som utgör endast 3% av alla cancerfall hos kvinnor, men då den är svår att upptäcka får kvinnan ofta inte någon diagnos förrän canceren har spridit sig i stora delar av buken och ibland till andra organ. Detta gör att tumören är svår att operera bort helt. Trots att man efter operation ger kvinnorna två olika cellgifter som är mycket effektiva och man på efterkontroller oftast ser full återgång av tumören, så kommer canceren tillbaka i de flesta av fallen. Man tror att dessa återfall kan bero på att det finns en mindre andel celler i tumören som kallas för cancerstamceller. Detta är en celltyp som har liknande egenskaper som vanliga stamceller, det vill säga att de kan kopiera sig själva utan att det finns andra celler intill, att de är anpassningsbara och kan utvecklas till celler med varierande egenskaper och växer långsammare än andra celler. Eftersom cellgifter dödar celler i delningsfas så har cancerstamceller alltså en möjlighet att komma undan cellgifterna. Det mesta av cancervävnaden däremot skadas och dör av cellgifterna, varför det ser ut som att tumören har försvunnit.

Studie I

Det finns ett protein som kallas SOX2, som man upptäckt att det finns mer av i cancerstamceller än i övriga tumörceller. Vi har räknat antalet celler som hade detta protein i tumörer från 125 kvinnor med hög-gradig serös tubal-ovarialcancer (HGSC), som är den vanligaste typen av äggstockscancer. Vi undersökte därefter om kvinnor som hade detta protein i några av sina tumörceller hade större risk att dö av sin sjukdom. Kvinnor som inte hade något SOX2 protein i tumörcellerna levde lite längre, men framför allt spelade närvaron av SOX2-celler roll för de patienter där man inte lyckats få bort all tumörvävnad vid operation. För dem gjorde en avsaknad av SOX2-celler att de levde lika länge som patienter som haft optimala operationer. SOX2 var alltså kopplat till en sämre prognos ifall det finns synlig tumörvävnad kvar efter operation, vilket vi tror beror på att de celler som har SOX2 protein troligen är cancerstamceller, som alltså har större förmåga att starta en ny tumörtillväxt än vanliga tumörceller. Eftersom de utgör en så liten del av tumören så kan en optimal operation med efterföljande cellgiftsbehandling göra att kvinnan slipper återfall, men om det finns tumörvävnad kvar så utgör närvaron av cancerstamceller en stor risk för återfall.

Studie II

Serös endometriecancer (SEC) är en ovanlig form av livmodercancer. Även om den växer i livmodern så är den på mikroskopisk och genetisk nivå väldigt lik HGSC, som vi undersökte i studie I. Det är därför troligt att dessa två tumörtyper skulle kunna behandlas på liknande sätt. I en normal cell finns det 2 kopior av varje gen, men i tumörceller så har det blivit små fel i vissa gener, till exempel mutationer, och ibland innebär det att gener som används till DNA reparationsmekanismer förstörs. När DNA-reparationen inte fungerar så leder det till fler fel i DNA:t varje gång cellen delar sig. Ibland kan en gen, eller stora delar av en kromosom, av misstag kopieras flera gånger, alternativt glömmas bort i kopieringen och försvinna bort. Både SEC och HGSC är tumörtyper som ofta har väldigt röriga genuppsättningar, med många kopior av vissa gener och få eller inga av andra. Det beror delvis på att många av dessa tumörer har fel i ett DNA reparationssystem som heter homolog rekombination (HR). Hur väl HR fungerar kan utvärderas genom att räkna fram ett HRD score. Vi undersökte genuppsättningarna för 19 SEC tumörer och räknade ut vilka HRD score de hade. Övriga hade en hög score och vi kunde se att SEC ofta hade fler genkopior av vissa gener som är vanliga att ha många av i HGSC. Det finns en behandling som specifikt dödar celler med problem i sitt DNA reparationssystem som redan används med goda resultat i HGSC, så kallade PARP hämmare. Vi tror att denna behandling även skulle kunna vara effektiv i SEC.

Studie III

I cellmembranet på vissa celler finns det en receptor som heter MET. Denna kan binda ett sorts tillväxtprotein som finns utanför cellen och på sått aktiveras och sätta igång olika processer inne i cellen. Man tror att denna receptor bland annat kan göra att det produceras mycket SOX2 protein och andra proteiner som i sin tur kan göra cellen mer lik en stamcell. Detta skulle eventuellt kunna öka risken för metastaser eller återfall. Vi räknade antalet celler med MET protein i samma 130 tumörer som vi undersökte i studie I och undersökte hur överlevnaden var hos kvinnor som hade olika kombinationer av MET och SOX2 proteinerna i sina tumörceller. Det vi fann var att kvinnor levde längre ifall de inte hade så mycket MET i sina celler. Vi kunde också se att den grupp av kvinnor som inte hade SOX2 i sina tumörer hade betydligt sämre prognos ifall de hade MET i cellerna, jämfört med inget av proteinerna. Vi tror detta kan betyda att tumörer som inte innehåller cancerstamceller är mer beroende av MET för att upprätthålla tillväxten.

Study contributions

Study I

I stained and scored the TMA in collaboration with Anna Ebbesson. I designed the cell experiments and performed them in collaboration with Juliette Davis. I performed all data analysis and was responsible for writing and revising the manuscript.

Study II

I was responsible for the data analysis and participated in the manuscript writing and revision.

Study III

I was responsible for the study design and performed all cell experiments, scored the TMA in collaboration with Lena Tran and performed all data analyzes. I was also responsible for writing the manuscript.

Aims of the thesis

Cancer stem cells and MET

One of the aims of this thesis was to evaluate the prognostic values of the transcription factor SOX2, a marker for cancer stem cells, and the receptor tyrosine kinase MET, an oncogene connected to cancer stem cell networks, in HGSC. I also wanted to investigate the connection between these two proteins and their relation to DNA repair mechanisms.

Homologous recombination deficiency

I also aimed to elucidate the mechanisms underlying homologous recombination deficiency (HRD) in SEC and HGSC, by relating HRD scores to clinical features and copy number aberrations in specific HR-associated genes.

List of abbreviations

BER	Base excision repair
CSC	Cancer stem cell
COCC	Clear-cell ovarian carcinoma
DSB	Double-strand break
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
HGF	Hepatocyte growth factor
HGSC	Pelvic high-grade serous carcinoma
HR	Homologous recombination
HRD	Homologous recombination deficiency
IHC	Immunohistochemistry
Klf4	Kruppel-like factor 4
LOH	Loss of heterozygosity
LST	Large-scale transitions
MMR	Mismatch repair
MOC	Mucinous ovarian carcinoma
MYC	MYC Proto-Oncogene
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
OCT4	Octamer-binding transcription factor 4
OS	Overall survival

LIST OF ABBREVIATIONS

OSE	Ovarian surface epithelium
PARP	Poly-(ADP-ribose)-polymerase
PFS	Progression-free survival
POU5F1	POU Class 5 Homeobox 1
PR	Progesterone receptor
RT-qPCR	Quantitative reverse transcription PCR
RTK	Receptor tyrosine kinase
SCM	Stem cell marker
SEC	Serous endometrial carcinoma
SNP	Single-nucleotide polymorphism
SOX2	SRY (sex determining region Y)-box 2
SSB	Single-strand break
STIC	Serous tubal intraepithelial carcinoma
TAI	Telomeric allelic imbalances
TCGA	The cancer genome atlas
TMA	Tissue microarray
WB	Western blot analysis
WT1	Wilms' tumor protein

Introduction

Cancer

A brief history

The first written record of the disease we now know as cancer can be found in the Edwin Smith Papyrus, written approximately 3000 BC¹. It describes eight cases of tumor or ulcer of the breast and the conclusion was that “there is no treatment”. Though this is the first documented reference of the disease, it can be assumed that cancer has been around for as long as life itself. The terms “carcinoma” and “cancer” were coined by Hippocrates (460-370 BC) and are derived from the Greek word for “crab”. It is generally assumed that Hippocrates thought that tumors resembled the shape of a crawling crab. It was however another Greek physician, Galen (130-200 AD), who introduced the word eventually used to describe the research field of cancer, “onco”, which is derived from the Greek word for “swelling”.

In 1665, the British researcher Robert Hooke first described the cellular structures in plants, which he examined through microscopy, and coined the word “cell”. From this point and on, only minor advances were made categorizing tumors, mostly based on noting the shape and consistency of the tissue. By the early 1800s, physicians had started to focus their attention on pathological examination of the morphology of tumor cells, rather than physical examination and palpation of the tumor mass, as they noticed that the histological appearance was distinct from normal tissue. In 1892, a connection between chromosomal abnormalities and cancer was first discovered; however, it was not until the ground-breaking discovery of DNA in the mid-1950s², that the research on genetic alterations and cancer really took off³.

Basic cancer theory

The simplest way to explain cancer is that it all starts with a mutation. Something goes wrong during DNA replication, and the daughter cells now possess an altered gene. This happens frequently during cell division and in most cases either nothing happens, or the problem is taken care of by apoptosis. In rare cases, however, the mutation causes

the inactivation of a gene that is responsible for keeping the proliferation in check: a so-called tumor suppressor gene. In the absence of this gene, given that apoptosis for some reason is not activated, the cell starts to divide more frequently, increasing the risk of more mutations occurring and manifesting, and the lesion could in the worst case transform into a malignant state. In other rare cases the initial mutation does not disable a gene, but rather enhances its effect. If this happens in a gene responsible for driving proliferation, a so-called proto-oncogene, this could also result in uncontrollable proliferation, additional mutations and subsequent tumor formation³.

Cancer is known as a disease of the elderly, and only 1.7% of cancer-related deaths occur in people below the age of 40⁴. There is a popular saying: “If you live long enough, you will eventually get cancer”, for which the reasoning is that during the course of life, mutations accumulate in our cells and it is statistically unlikely that none of these mutations would persist and initiate a tumor. From an evolutionary perspective, it is also reasonable that cancer would be more prone to arise once the reproductive years are concluded.

“The hallmarks of cancer” is a paradigm first presented in 2000 by Hanahan & Weinberg⁵, and it was subsequently updated in 2011⁶. It proposes a theory that all tumors depend on a certain set of capabilities to form a neoplastic disease. This theory provides an essential understanding of tumorigenesis, expanding far beyond the predominant model that tumors are driven by mutations in oncogenes and tumor suppressor genes. Initially, six defining qualities were included.

The ability to:

- Sustain proliferative signaling
- Evade growth suppressors
- Activate invasion and metastasis
- Enable replicative immortality
- Induce angiogenesis
- Resist cell death

In the theory’s updated form⁶, four additional factors were taken into consideration:

- Avoiding immune destruction
- Deregulating cellular energetics
- Tumor-promoting inflammation
- Genome instability and mutation

Ovarian cancer

Ovarian cancer is a relatively rare disease, constituting only 3.4% of the cancer cases reported in women worldwide in 2020⁷. Unfortunately, given the location of the tumors and the diffuse symptom reported from patients (pain, nausea, and often swollen abdomen), the disease is often found at an advanced stage with metastases already occurring in the peritoneum (epithelial wall lining the abdominal cavity), lymph nodes or even distant organs. In this advanced stage, the tumors are difficult to resect with margin and despite radical surgery and postoperative chemotherapy, a majority of patients relapse⁸. Death from ovarian cancers constitutes approximately 4.7% of all cancer-related deaths in women⁷.

One location- several diseases

The tumors found in the ovaries and fallopian tubes have a wide variety of histologies and characteristics which need to be distinguished in order to understand how the tumors operate and how to plan the most optimal treatment. Practically all ovarian tumors arise from one of three cell types: stromal cells, germ cells, or epithelial cells (Figure 1). Epithelial tumors are by far the most common and can be further divided into high-grade or low-grade serous, endometrioid, mucinous, and clear cell types/tumors. Out of these, the high-grade serous subtype is the most common, and also constitutes the majority of ovarian cancer-related deaths. It has in later years been discovered that these high-grade serous tumors do not actually arise in the ovary itself⁹, but in the fallopian tube fimbriae. In patients with high-grade tumors in the ovaries, premalignant or malignant lesions are also commonly found in the tubas, making it difficult to pinpoint the location of the primary tumor.

These high-grade tumors are collectively referred to as pelvic high-grade serous carcinomas (HGSC), and this term comprises tumors located in the fallopian tubes, ovaries, or peritoneum, but not the uterus or cervix¹⁰. The practice of grouping these pelvic high-grade tumors under a common classification is relatively new, and much of the literature on the subject instead group the tumors according to their assumed primary site. For this reason, pelvic high-grade serous tumors are still normally categorized under ovarian tumors, and the term high-grade serous ovarian carcinoma is also commonly used to define the disease. The broader terms ovarian cancer, or ovarian carcinoma, are used in this thesis when studies that have not specified the cancer subtype are referenced, and when describing the different tumor types located in the ovaries.

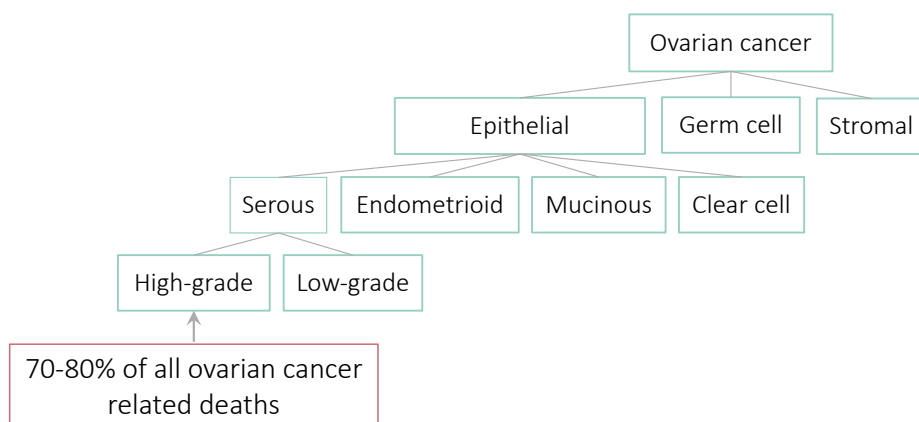


Figure 1. The most common ovarian cancer diagnoses.

HGSC - cell of origin

Traditionally, epithelial ovarian carcinomas (comprising 80-90% of all malignant ovarian neoplasms^{11,12}) have all been thought to have their origin in the ovarian surface epithelium (OSE), which is a part of the mesothelium. The problem with this view is that none of the morphologically distinct subtypes actually bears much resemblance with the OSE cell type, but rather resembles other epithelial cell types found in the abdominal and pelvic organs^{9,11}. Unlike the ovaries, the cervix, uterus, and fallopian tubes all originate from the müllerian ducts formed early in fetal development. Considering the phenotype of serous, endometrioid, and clear cell cancers, these would rather be considered to originate from müllerian-type tissue rather than from mesothelial tissue.

In the last couple of decades, there has been much discussion regarding the origin of different ovarian/pelvic cancers. After reports on the findings of *TP53* mutations in the fimbriae or fallopian tubes of *BRCA1* mutation carriers were published¹³⁻¹⁶ the discussion regarding the cell of origin for HGSC really took off. A somatic *TP53* mutation is found in virtually all patients diagnosed with HGSC, indicating that it is a very early event in the tumorigenesis. The proposed mechanism of forming this precursor lesion is that a *TP53* mutation occurs in the epithelial cells of the tubal fimbriae. These mutated cells proliferate quickly and form a p53 signature that develops into a serous tubal intraepithelial carcinoma (STIC) lesion. These cells are then shed onto the ovarian surface epithelial, or along the peritoneum lining, to manifest there. Alternatively, the cells are translocated to the ovary through cortical inclusion cysts^{9,11,17-22}.

Subtyping of epithelial ovarian carcinomas

There are several tools for categorizing tumors. The visual appearance of the cells is examined microscopically to determine the histologic type and the mutational profile can be determined either by DNA sequencing (targeted, whole exome, or whole genome) or proxy through the expression profile determined by immunohistochemistry (IHC). Gene expression can be investigated through IHC (protein) or gene expression data (mRNA). Copy number profile can be obtained through either DNA microarrays or DNA sequencing. Cost-effective tools for exploring mutation, gene expression, and copy number profiles are emerging quickly, enabling these methods in a clinical setting.

Determining the stage of a tumor is normally done during upfront debulking surgery, and the diagnosis is then confirmed or adjusted through pathological evaluation. The surgeon determines the size of the tumor as well as the extent of its spread within the abdominal cavity. Stage I is defined as a tumor that is confined to the ovaries or fallopian tubes, stage II is a tumor involving one or both ovaries or fallopian tubes and extending into the pelvic, or a primary peritoneal cancer. Stage III is the same as II but also requires confirmed spread to peritoneum outside the pelvis and/or metastasis to lymph nodes and stage IV requires distant metastasis, pleural effusion, or metastasis to extra-abdominal organs²³. Finding the distinction between subtypes is mainly performed through histologic examination combined with immunohistochemistry with markers more or less specific for different tumor entities. Representative images of the five most common histologic subtypes are depicted in Figure 2, and features characterizing the five tumor subtypes are summarized in Table 1.

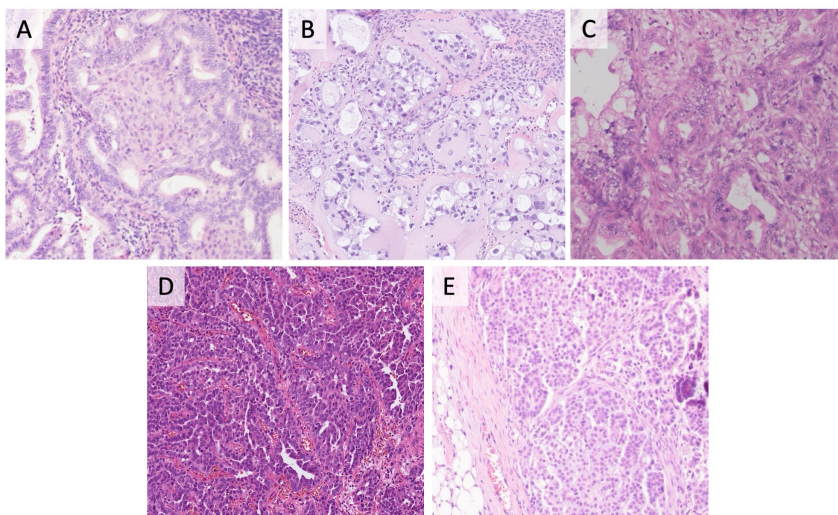


Figure 2. The most common epithelial cancer morphologies in the pelvis.

Endometrioid (A), clear-cell (B), mucinous (c), high-grade serous (D), and low-grade serous (E). Images courtesy of Sofia Westbom-Fremer, Lund University.

Histologic subtypes

The serous histology accounts for roughly 80-85% of all ovarian carcinomas^{12,24}. As serous is such a common histology there is a risk of bias during pathologic examination which historically has led to an overdiagnosis of this subtype¹². Most of the HGSC are diagnosed in an advanced stage (III-IV) and express WT1²⁵. Also, expression of the progesterone receptor (PR) and estrogen receptor (ER) is common. Practically all HGSCs have overexpression and mutation of *TP53*, and many display *BRCA1* or *BRCA2* mutations and loss of *BRCA1/2* expression. Also, the PAX8 marker has been reported to be highly expressed in HGSC, supporting the theory of HGSC origin in the fallopian tubes, as PAX8 expression is a known marker for müllerian-derived tissue²⁵. HGSC commonly co-exist with tubal intraepithelial carcinoma.

While *TP53* is the only defining mutation for HGSC, low-grade serous ovarian carcinomas (LGSC) display more variation in their mutational profile. *BRAF*, *KRAS* and *ERBB2* (HER2) are commonly mutated, but the *TP53* gene is normally wildtype⁹. Some low-grade tumors are also mixed with serous borderline tumor and, while the origin of HGSC is most likely STIC lesions, LGSC is thought to develop from borderline tumors and comprises <5% of all ovarian carcinomas²⁶.

The morphology of the endometrioid carcinoma resembles the endometrium. They are often associated with endometriosis and endometrioid borderline tumors. These tumors have often been misdiagnosed as serous, as their morphologies sometimes overlap. However, the immunophenotype differs in that endometrioid tumors mainly lack WT1 expression and p53 overexpression. These tumors have also been associated with a high degree of microsatellite instability, nuclear β -catenin expression, and *CTNNB1* (β -catenin) mutation^{27,28}. Mutations in *ARID1A* and *PIK3CA* are common²⁹, and the endometrioid histology accounts for 10% of ovarian carcinomas²³.

The clear cell ovarian carcinoma (CCOC) accounts for approximately 10% of ovarian carcinomas²³, and more common among Asian women compared to European women^{30,31}. These tumors are mostly diagnosed in stage I or II. These tumor cells usually have a very characteristic clear cytoplasm. This is a result of intracytoplasmic glycogen, which is cleared during tissue fixation, leaving clear, unstained areas on the tissue slide. The cell nuclei are generally large and atypical, with a large nucleolus. CCOC is strongly associated with endometriosis. Regarding the immunophenotype, tumors normally lack PR and ER and WT1 expression³², but are positive for PAX8²⁵. Napsin A expression is common and can be considered a marker specific for the clear cell tumor type³³. Some tumors also display p53 overexpression, and targeted sequencing has found that activating mutations in *PIK3CA* are common³⁴. In later years mutations in *ARID1A* and overexpression of HNF1B have been strongly associated with CCOC³⁵.

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The primary mucinous ovarian cancer (MOC) is also a rare subtype, comprising 3% of all ovarian carcinoma cases^{23,24}, but it is reported to be more common in the subgroup of women under the age of 40 years³⁶. The morphology closely resembles that of other mucinous carcinomas, such as the colorectal, and most mucinous cancers found in the ovaries are in fact metastases from other sites. MOC is generally a very heterogeneous tumor and can contain parts that are benign and parts that are borderline tumor. *TP53* mutations are found in one out of three tumors³⁶.

There are also a few rare morphologies that do not fit in either of the described subtypes, e.g., seromucinous, Brenner tumors, and undifferentiated carcinoma²⁹. Some tumors also have mixed morphology, meaning that two distinct histological types are present, and each account for at least 10% of the tumor bulk¹².

Table 1. Overview of the most common ovarian epithelial carcinomas.

	High-grade Serous	Low-grade Serous	Endometrioid	Mucinous	Clear cell
<i>Common FIGO stage</i>	Advanced (III or IV)	Early (I or II)	Early (I or II)	Early (I or II)	Early (I or II)
<i>Morphologies</i>	Papillary Glandular Solid	Papillary Glandular Solid	Papillary Glandular Solid	Glandular	Papillary Glandular Solid
<i>IHC markers</i>	WT1+ ER+ PR+ p16+ BRCA1- PAX8	WT1+ ER+ PR+	WT1- ER+ PR+ β-catenin nuclear expression MSI**	WT1- ER- p16- MSI**	ER- PR- WT1- Napsin A+ PAX8+
<i>Mutations</i>	<i>TP53</i> <i>BRCA1/2</i>	<i>BRAF</i> <i>KRAS</i> <i>ERBB2</i>	<i>CTNNB1</i> <i>PTEN</i> <i>PI3KCA</i> <i>ARID1A</i>	<i>KRAS</i> <i>ERBB2</i>	<i>ARID1A</i> <i>PIK3CA</i>
<i>Fraction of OC diagnosis</i>	75%-85%	<5%	10%	3%	5-20%*

*Lower frequency in Europe, higher in Asia.

**MSI= microsatellite instability, as determined by mutations in mismatch repair (MMR) genes and/or specific histopathological features

The dualistic model

In 2004, Shih et al.²⁸ published a review proposing a new way of categorizing pelvic carcinomas based on their different pathways of pathogenesis. This would be called the dualistic model and comprises two entities: type I and type II tumors. Type I tumors include low-grade serous, mucinous, endometrioid, and clear-cell carcinomas, while

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type II tumors include high-grade serous, high-grade endometrioid, and the more rare undifferentiated carcinomas, and carcinosarcomas (Table 2). Type I is more slow-growing and normally develops from borderline tumors or endometriosis. This type has a well-differentiated phenotype, and the tumors are usually chromosomally stable. Common mutations include *BRAF*, *KRAS*, *ERBB2*, *PTEN*, *CTNNB1*, and *PIK3CA*^{9,28}. Type II tumors, on the other hand, are more aggressive, and until recent years these tumors were partly characterized by having unrecognized precursor lesions.

Table 2. Features of type I and type II epithelial ovarian carcinomas

	Type I	Type II
<i>Defining features</i>	Low-grade Slow-growing	High-grade Evolve rapidly Metastasize early
<i>Morphologies</i>	Low-grade serous Endometrioid Mucinous Clear-cell	High-grade serous High-grade endometrioid
<i>Origin</i>	Borderline tumors Cystadenoma Endometriosis	Serous tubal intraepithelial carcinoma
<i>Proliferation</i>	Ki67 low	Ki67 high
<i>Mutations</i>	<i>BRAF</i> ⁺ <i>KRAS</i> <i>ERBB2</i> <i>PTEN</i> <i>CTNNB1</i> <i>PIK3CA</i> <i>ARID1A</i>	<i>TP53</i>
<i>Genomic stability</i>	Generally stable genome Microsatellite instability in certain tumors	Unstable Amplified and deleted genes or regions are common.
<i>Common FIGO stage</i>	Stage I-II	Stage III-IV
<i>Response to chemotherapy</i>	Poor	Good

It is now considered proven that HGSC tumors arise from lesions in tubal fimbriae epithelium, in cells initially presenting with a benign p53 signature. This signature develops into a STIC lesion which then either initiates a tubal cancer or sheds cells onto the ovary and develops into an invasive ovarian cancer^{14,17}. Type II tumors are poorly differentiated and possess few common mutations, except for the very frequent *TP53* mutation. These tumors also have a more unstable genome^{9,21}, often with large regions

of deletion or amplification, with amplifications predominantly in the chromosome 3q-arm, harboring e.g. *MECOM*, *PIK3CA*, and *SOX2*. High-level gain or amplification is also common in chromosome 8, specifically at the site of the *MYC* gene, and in chromosome 19, where *CCNE1* is encoded^{37–39}.

The dualistic model, where tumors are defined by their path of tumorigenesis rather than histological features, is now widely spread and an important tool in distinguishing ovarian tumors^{11,22}. It was implemented in the 2014 WHO classification of tumors along with the theory of origin for most if not all HGSC²⁶. It should be noted that this is a very simplified model, and approaches to more personalized treatments are underway, primarily focused on molecular features.

Molecular subtypes

As a complement to histological subtypes, there have been efforts to find arrays of molecular markers which could define different subgroups of ovarian cancer, primarily within the large group of HGSC. In 2008, Tothill et al. published a paper consisting of 285 ovarian tumors of serous or endometrioid histology, where they performed K-means clustering on gene expression data as a tool to split the tumors into subgroups⁴⁰. 251 of these tumors were assigned to one of 6 subgroups. They then compared clinical features characterizing the groups and identified differentially expressed genes between groups. This resulted in 6 distinct clusters/groups, four of which could be identified as being of high-grade serous or endometrioid histology, in advanced stages (C1, C2, C4, C5). Further, identifying differentially expressed genes, they could conclude that C1 was associated with a high stromal response, C2 with a high immune signature, C4 with a low stromal response, and C5 with a mesenchymal expression pattern. One group was identified as tumors of low-grade serous subtype with low-malignancy potential (C3), and one group consisted of low-grade, early-stage endometrioid tumors (C6).

A few years later, Bell et al. at the cancer genome atlas (TCGA) program performed a study including 489 HGSC tumors, again attempting to subgroup these tumors in distinct molecular entities³⁷. They clustered the tumors into four main groups, based on gene expression profiles, and inspired by Tothill et al., these were defined as “immunoreactive”, “differentiated”, “proliferative”, and “mesenchymal”. This subgroup classification was validated in 2014 by a study that confirmed the existence of the four subtypes, and their prognostic differences⁴¹, and in 2013 by a study that expanded the subtyping model by adding more predictors to render a clinically applicable tool⁴².

In 2017, Wang et al. collected data from 14 publicly available mRNA expression datasets (two of which were from the Tothill and TCGA studies). Only untreated HGSC tumors were included (n=2,000)⁴³. In large, this study confirmed the results of Tothill and TCGA, in defining tumors “immunoreactive”, “proliferative” and “mesenchymal”

as distinct subtypes; however, they proposed further dividing the group previously described as “differentiated” into two distinct groups: “differentiated” and “anti-mesenchymal”.

A study by Chen et al.⁴⁴ questions the reproducibility of the published studies which uses subtype classifiers, and proposes a consensus model that includes several classifiers, that produces a confidence score for the group affiliation, and that accepts that some tumors not be given a clear affiliation.. In a recent work from 2020, Schwede et al. also raise concerns about reproducibility and argue that the effect of stroma admixture should be taken into consideration in subtyping models and that microdissection or single-cell analysis, separating tumor and stroma cells, might be a better tool for tumor classification⁴⁵.

Copy number signatures

In another study, aiming to identify markers for subgrouping tumors to evaluate treatment options and prognosis, Macintyre et al.⁴⁶ introduced so-called copy number signatures. They defined 36 features in copy number data, which they then sorted into seven so-called copy number signatures. Allele-specific copy numbers from each tumor could then be assigned a certain exposure for each signature. With this method tumors are not divided into groups, as is the case with the methods of Tothill, Bell, and Wang^{37,40,43}, but instead are defined by a range of features. Each signature was further evaluated for associations with mutational and clinical features. For example, one signature was found to be associated with a *BRCA1/2* related HRD, while another was associated with a non-*BRCA1/2* related HRD. Associations were found between signatures and prognosis, and specific underlying mechanisms likely responsible for the genomic instability.

DNA repair systems

To maintain a stable genome, the cell needs an array of different DNA repair mechanisms that corrects mistakes made during DNA replication or breaks caused by external DNA-damaging factors. The DNA is built of a double-strand helix², and damage can occur either in one of the strands, which is referred to as a single-strand break (SSB), or in both strands, referred to as double-strand break (DSB). These issues are tackled by different systems. SSBs are repaired by mismatch repair (MMR), base excision repair (BER) or nucleotide excision repair (NER) while double-strand breaks, which are much more severe, are repaired through either homologous recombination (HR) or non-homologous end-joining (NHEJ)⁴⁷.

Homologous recombination

HR is considered the superior method of DNA repair, as it provides an accurate reading frame, modeled after an intact sister chromatid. Bases on the 5'-strands of the blunt ends are resected to form strings of single-strand DNA on either side of the DSB (Figure 3A). RAD51, in collaboration with BRCA2, then uses one of the 3'-single-strand ends as a template to search for the matching homologous DNA string in the sister chromatid. Once this is located, a so-called D-loop intermediate is formed that gives room for the loose 3'-end to anneal to its corresponding DNA strand. The DNA string is elongated by a DNA polymerase, removed from the model DNA, and is now ready to anneal with the free 3'-string on the other side of the DSB. The remaining regions of single-strand DNA are repaired through DNA synthesis⁴⁷⁻⁴⁹. There is a large group of proteins involved in this process. When a signal of DSB occurs, ATM, ATR, and CHEK2 respond by phosphorylating BRCA1⁴⁷. This causes a cell cycle arrest to allow time for the damage to be repaired. BRCA1 forms a complex with BARD1, stabilizing the protein, and then binds the DSB end. BRCA2 participates in relocating RAD51 into the nucleus and modifies its function to form a RAD51 filament on the loose 3' end, which then guides the DNA to a corresponding DNA template^{47,50}. The significance of RAD51 was highlighted by Lee et al.⁵¹ who found that an overexpression of RAD51 restored HR in 50% of BRCA2-depleted mouse cells. It appeared that an abundance of RAD51 led to a higher nuclear concentration of the protein, which rendered the BRCA2 function less important.

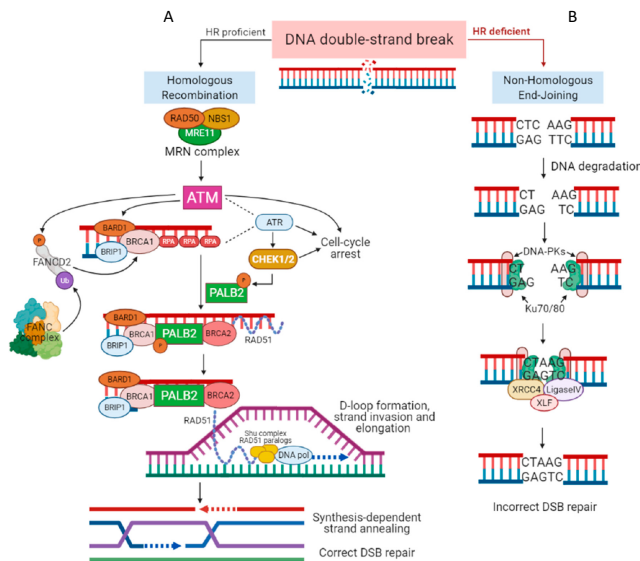


Figure 3. The two main systems of DSB repair. HR (A) and NHEJ (B). Figure adapted from Cortesi et al.52

Non-homologous end joining

While the HR system is good at producing accurate DNA strings, it cannot be used in all phases of the cell cycle, only in the S or G2 phase, that is, during or after DNA synthesis but before mitosis. This is when the sister chromatid, which is needed as a template, is the most accessible. The NHEJ system on the other hand can function in all phases of the cycle. Also, in cases of mutations in genes essential for HR, the NHEJ system is activated. As this system is more error-prone, it can result in large chromosomal defects⁵³. The proteins Ku70/80 recognize and bind the ends of the open DNA strands and then recruit and form a complex with DNA-PKcs (Figure 3B). If needed, an end processing is then performed by e.g. the Artemis protein to remove mismatched bases, and the gaps are filled by polymerases Pol μ and Pol λ . Finally, XLF and the XRCC4/DNA ligase IV complex ligate the DNA strings⁵⁴.

Upon DNA damage in the cell, the NHEJ system is used more often than the HR system and, though error-prone, the majority of the DNA breaks are repaired accurately. Depending on the number of missing bases in the gap, small or large genomic deletions can however occur, as the system has no template to model the DNA string after. Also, in the case of several DSB occurring simultaneously, wrong pairings of DNA strings may occur, leading to chromosomal rearrangements and the formation of fusion genes⁵⁴.

Homologous recombination deficiency

Defects in DNA repair systems is a common feature in several cancer types, and defects in the HR system are especially common in HGSC. When genes required for the HR system are mutated, extensive and irreversible damage can be done to the genome. Typically, this damage consists of global loss of heterozygosity (LOH), telomeric allelic imbalances (TAI), and large-scale transitions (LST). The extent of these genomic defects can be calculated, and the sum of the three calculations is referred to as a homologous recombination deficiency (HRD) score⁵⁵. Determining the HRD status is used clinically to evaluate the possible benefit of inhibitors⁵⁶. The HR function can also be assessed through the HRDetect method. HRDetect is a powerful tool originally developed to detect *BRCA* mutations in breast cancer tumors from whole-genome sequencing data, but it can also detect non-*BRCA* related HR deficiency⁵⁷. Functional assays can also be used to estimate HR function, by counting RAD51 and γ H2ax foci either in cultured cells⁵⁸ or in primary tumor samples using the RECAP test⁵⁹.

PARP function

The Poly-(ADP-ribose)-polymerase (PARP) enzyme was first discovered in 1963⁶⁰, and in 1980 it was found that when inducing single-strand DNA breakage in cultured cells, synthesis of the PARP enzyme could be induced⁶¹. This indicated that PARP has an

important role in DNA repair. It has later been found that the enzyme has a role in the system of base excision repair (BER)⁴⁷. The enzyme is activated upon DNA damage signals. It binds the DNA using its two zinc fingers, and then phosphorylates itself as well as histone glutamate residues. This loosens the DNA's attachment to the histone and allows for DNA repair enzymes to access the damaged DNA⁶².

Heredity in HGSC

Hereditary breast-ovarian cancer syndrome

There is a clear hereditary component in the risk of developing HGSC, thought to account for 20% of the cases. The most well-known risk factors are the *BRCA1* and *BRCA2* genes, which account for 75% of the heritability in ovarian carcinomas^{47,63}. Approximately 10-15% of all patients with ovarian carcinomas harbor a germline *BRCA1/2* mutation⁶⁴⁻⁶⁷. Mutation carriers have an earlier onset of disease (especially in the case of *BRCA1*), and many women carrying these mutations also have a family history of ovarian or breast cancer⁶⁷. A large meta-study from 2003, including over 8,000 patients, investigated the cumulative cancer risk associated with *BRCA1/2* germline mutations and found that *BRCA1* and *BRCA2* mutation carriers had a 39% and 11% lifetime risk of developing ovarian cancer, respectively⁶⁸. Similar results were found in later studies^{69,70}. In cases where no *BRCA* mutation is found, an estimated 10-15% of patients have a germline mutation in another gene involved in double-strand repairs, such as *RAD51C*, *RAD51D*, *CHEK*, *BRIP1*, or *PALB2*⁴⁷.

Lynch syndrome

10-15% of the heredity in ovarian carcinomas can be ascribed to mutations in mismatch repair (MMR) genes, which are involved in a mechanism of repairing DNA single-strand breaks⁴⁷. Commonly mutated genes include *MSH2*, *MLH1*, *MSH6*, *PMS2*, and *EPCAM*. The hereditary condition of carrying these mutations is referred to as Lynch syndrome and formally known as non-polyposis colorectal cancer (HNPCC), as the syndrome is associated with a strong predisposition to developing colorectal cancer. Also, other cancer forms are markedly more common in people with Lynch syndrome, most predominantly endometrial and ovarian, but also stomach, small bowel, and hepatobiliary tract, to name a few^{47,71,72}.

Li-Fraumeni syndrome

Among ovarian cancer patients with documented heritability, approximately 3% carry a germline mutation in the *TP53* gene, residing in chromosome 17⁴⁷, and the condition is referred to as Li-Fraumeni syndrome⁷³. People with this condition are more likely to develop multiple tumors early in life^{47,72}, though they rarely develop ovarian tumors.

The *TP53* gene is referred to as “the guardian of the genome” and acquired somatic mutations in this gene are extremely common in many cancer types. In HGSC, *TP53* mutation is found in practically all tumors, but germline mutations are rare.

Non-hereditary HGSC

There has been extensive research conducted on women with hereditary ovarian cancer, but it is important to remember that 80% of patients have no known predisposition and are considered to have sporadic disease⁴⁷. Given the fact that *BRCA1/2* carriers have been reported to respond better to chemotherapy⁵⁰ it should be emphasized that better treatment options are needed for the large group of non-*BRCA* mutation carriers.

Treating HGSC

Surgery

The most common treatment for HGSC is upfront surgery, during which the surgeon performs tumor staging, removes ascites from the abdomen, and resects as much of the tumor bulk as possible. In stage I disease, fertility-preserving surgery can be discussed; however, in advanced stages, upfront radical surgery is normally performed. This means that the uterus, fallopian tubes, ovaries, and omentum are removed, as well as other tissues affected by the tumor. An alternative to upfront surgery can in certain cases be neoadjuvant chemotherapy, aiming to shrink the tumor mass before surgery, followed by interval surgery. The importance of successful debulking, aiming at complete macroscopic removal, is emphasized in several reports and is a strong prognostic factor^{74,75}.

Chemotherapy

Chemotherapy is recommended as post-operative treatment in HGSC. The most commonly used regimen is platinum (carboplatin) in combination with a taxane (paclitaxel). In the case of advanced disease, an anti-angiogenic drug (bevacizumab) can also be administered. The use of platinum has a long history, dating back to the landmark report of Rosenberg et al. from 1965, in which it was discovered that proliferation in *E. coli* could be inhibited by exposing the cells to an electrical field generated by platinum electrodes⁷⁶. Searching for the mediator of this effect the authors concluded that cisplatin had a strong anti-proliferative effect in these cells. These findings led to experiments in *in vivo* models, proving cisplatin to also have a strong anti-tumor effect. It has later been found that the anti-tumor effect can be ascribed to the compound's ability to form covalent bonds to DNA and thereby stop proliferation. In addition, recent studies have indicated that also molecules other than DNA could potentially act as targets for platinum, and reports an increased immunogenic response to the treatment⁷⁷. In 1978, The U.S. Food and Drug Administration (FDA) approved cisplatin

for use in metastatic testicular, bladder, and ovarian cancer⁷⁸. In 1989, the second-generation platinum drug carboplatin, which had been proven to be less toxic and to have fewer side-effects, was approved for patients with advanced ovarian cancer.

The use of taxanes as a standard treatment for HGSC also has a long history. In 1962, an effort to find naturally occurring compounds exhibiting anti-tumor effects was undertaken in the US. Thousands of plant species were investigated, and the bark of the Pacific yew tree, *Taxus brevifolia*, was found to possess cytotoxic effects. In the years that followed, researchers managed to isolate the active ingredient, and the drug would be referred to as Taxol⁷⁹. During the 1970s, Taxol was further investigated. By 1984, the first phase I clinical trial was initiated and in 1992 the FDA approved the compound for the treatment of ovarian cancers⁸⁰.

The effect of taxanes (the class of drugs in which Taxol is included) is mediated by a stabilization of the microtubule which causes cell arrest in metaphase, with chromatids lined up but unable to divide⁸¹. Reports on low-dose Taxol experiments have also shown that when concentrations are too low to induce complete cell arrest the partial stabilization of the microtubule disturbs proper polarization of chromosomes during metaphase of the cell cycle. This results in multipolar cell division, sometimes with three daughter cells, with the wrong number of chromosomes^{82,83}. A clear survival benefit is accredited to the drug, however, it is unclear whether this is due to the effect of mitosis and cell death, seen in high-dose *in vitro* experiments, or due to multipolar division and subsequent cell death⁸³.

PARP inhibition

Drugs targeting the PARP enzymes are a class of inhibitors that have been developed more recently than standard chemotherapy. As PARP is an important part of the repair of SSBs, a malfunction in this enzyme can lead to an accumulation of DSB. This is not a big problem in cells with a properly functioning HR system; however, in tumors where the HR is impaired, due to mutations in *BRCA* or other important HR genes, a poor SSB repair system can cause irreparable damage and eventually lead to cell death. Though defects in either the SSB or DSB repair individually do not pose an immediate threat to the cell, impaired function in both systems simultaneously is lethal. This phenomenon, referred to as synthetic lethality, can be utilized by administering drugs binding the PARP enzymes, namely a PARP inhibitor, effectively keeping it from assisting in the SSB repair (Figure 4). An accumulation of DSB will follow, which eventually leads to cell death. PARP inhibition is a quickly emerging tool in the field of HGSC and has recently been incorporated as a part of the standard treatment for this disease^{84,85}.

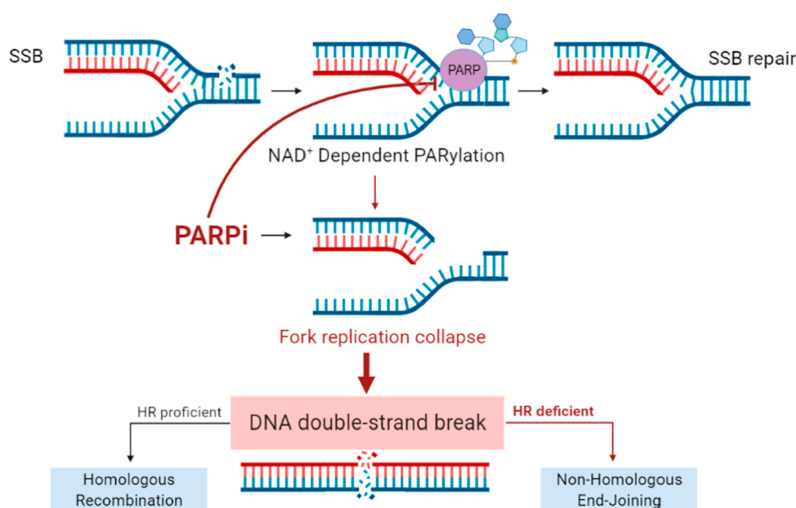


Figure 4. PARP inhibitor function. When a SSB occurs, the PARP enzyme is recruited to the site, and activates BER to repair the DNA. If the PARP enzyme binds a PARP inhibitor, the DNA repair activation will be stopped and the stalled replication fork will collapse, causing a DSB. If the cell is HR deficient, NHEJ will attempt to repair the damage, but this DNA repair system will not be sufficient to save the cell. Figure adapted from Cortesi et al.⁵²

In 2014, the first PARP inhibitor was granted FDA-approval for use in advanced stage HGSC patients with germline *BRCA* mutations and ≥ 3 prior lines of chemotherapy⁸⁶. Several clinical trials were conducted during the following years and the indications for PARP inhibitor treatment have broadened, as benefits have been clear, and side-effects have been manageable⁸⁷. In Sweden, PARP inhibitors are currently indicated as a maintenance treatment for advanced-stage HGSC patients with a *BRCA* mutation, who had at least partial response to 1st line platinum-containing chemotherapy⁸⁸. In a relapse setting, the drug can be used regardless of *BRCA* mutational status, provided that the tumor still responds to platinum^{87,89}. Patients with platinum-resistant disease could potentially also benefit from PARP inhibitors, however to a lesser extent. In a study including 50 HGSC patients with *BRCA1/2* mutations, the complete response rate was 41.7% in patients with resistant tumors and 61.5% in patients with platinum-sensitive tumors⁹⁰.

As studies have shown that also patients without *BRCA* mutation can have a poorly functioning HR⁵⁵, it is becoming clear that PARP inhibitors could be useful also in these patients. Clinical studies have shown promising results in this patient group^{89,91}, and the phase III study PRIMA even reports survival benefits in patients with a seemingly functioning HR^{92,93}. An interesting finding by Patel et al.⁵⁵ is that the HRD appears to be relatively stable throughout the disease. They compared primary and recurrent tumors, and found that the HRD score was comparable in the two, indicating that

a HRD scoring performed on tissue from the primary tumor does not need to be redone in tissue from the recurrence. It should be noted that this only means that the *genetic scarring* from a dysfunctional HR system is stable throughout the disease. A reversal mutation can still occur in, for example, a mutated *BRCA* gene, which will restore its function⁹⁴. The tumor will then regain a better functioning HR system, despite the high HRD score⁵⁵.

Treatment resistance

Though some tumors have intrinsic resistance to platinum, most HGSC tumors initially respond well to treatment and the complete response rate is reported around 60-80%⁹⁵. Unfortunately, tumors are commonly sensitized to treatment, and most patients relapse one or several times⁸. Resistance to treatment can have many causes, and the most relevant mechanisms of intrinsic resistance are thought to be drug efflux, low drug uptake, and a good defense against apoptosis. In cases of acquired resistance, alternations in copy numbers and methylation status of certain genes can occur in response to the pressure induced by the DNA damaging agents⁹⁵. Another possible mechanism of resistance is the enrichment of cells already resistant to chemotherapy. It is thought that a subpopulation of the cells in tumor bulk can possess features that resemble those found in normal stem cells, so-called cancer stem cells (CSC)⁹⁶. These cells are more likely to be in a dormant state, are less proliferative, have increased capability of epithelial-mesenchymal transition (EMT), and display self-renewal capacity. They have also been reported to have a more pronounced level of drug transporters and higher functioning DNA repair systems^{97,98}. As chemotherapeutic drugs specifically target actively dividing cells, these CSCs have a higher chance of surviving the treatment. Steg et al.⁹⁹ measured the level of CSCs in high-grade serous or endometrioid carcinomas in primary tumors, and tissue collected after surgery and platinum/taxane treatment, and saw a clear enrichment of cells expressing certain stem cell markers. The features of self-renewal and increased EMT further indicate that this subpopulation of cells could have an important role in metastasis and relapse.

Cancer stem cells

Simplistically, a tumor, similar to normal tissue, consists of three distinct cell populations: stem cells, transit-amplifying cells, and terminally differentiated cells⁹⁶. The fraction of CSCs in a tumor is estimated to be in the range of 0.01-1%¹⁰⁰. What characterizes these cells is their capacity to divide either in a symmetrical manner, resulting in two CSCs, or an asymmetrical manner, producing one CSC and one cell that will develop into a more differentiated state¹⁰¹. They also have the capability of evading chemotherapy and to initiate an entire new tumor bulk when transplanted into an animal model.

In the 1970s, Pierce and Wallance described a particularly aggressive undifferentiated type of cell, capable of generating squamous cell carcinoma *in vivo*¹⁰², and in a study from 1997, a subpopulation of tumor initiating (TIL) cells was identified in acute myeloid leukemia which possessed stem-like properties¹⁰³. Following these pivotal studies, CSCs have been identified in a wide range of cancer forms^{98,104}, and intensive work is put into finding stable markers for stemness so that subpopulations of CSCs in tumors can be clearly identified.

Turdo et al. recently defined seven characteristics that they referred to as the hallmarks of CSCs⁹⁸, namely: DNA repair, immune evasion, ABC transporters, EMT, metabolism, quiescence and detoxifying enzymes. Several of these are also commonly described in relation to multidrug resistance¹⁰⁵, supporting the CSCs association with chemotherapy resistance, such as a high expression of efflux drug transporters which can effectively remove drugs from the cell¹⁰⁶.

Nobel Prize awarded to stem cell research

In 2012, the Nobel prize in physiology or medicine was awarded to John Gurdon (UK) and Shinya Yamanaka (Japan) with the motivation: "For the discovery that mature cells can be reprogrammed to become pluripotent". Gurdon laid the foundation for this in 1962 when he challenged the current dogma, that cells can never go back from a differentiated to a pluripotent state, by inserting the genome of a differentiated cell from a tadpole, into a frog egg¹⁰⁷. The egg developed into a fully functional, cloned frog. This meant that the nuclear content was still capable of creating all the cell types needed for an entire organism.

The other award winner, Yamanaka, performed studies 40 years later, where he discovered that by use of only a small number of transcription factors, a differentiated cell can be reprogrammed into a pluripotent state¹⁰⁸. The transcription factors indicated are SRY (sex determining region Y)-box 2 (SOX2), Kruppel-like factor 4 (Klf4), Octamer-binding transcription factor 4 (OCT4), and MYC Proto-Oncogene (MYC), and these are therefore referred to as "Yamanaka factors".

SOX2

After being described as one of the Yamanaka factors, research involving SOX2 accelerated in the last decade and its role in CSCs is continuously being investigated. SOX2 is one of the SOX genes (SRY-related HMG-box genes) which encodes a family of transcription factors, all containing a high mobility group (HMG). In the case of SOX2, this group binds its target genes in a sequence-specific manner in the minor groove of the DNA and forces a 90° bend of the double-helix making the DNA more accessible and improving affinity¹⁰⁹. The SOX2 protein, which was first characterized in 1994¹¹⁰, has a crucial role in early embryonic development. The SOX2 gene has also

been reported to be amplified and/or overexpressed in several cancer types^{111,112} and is commonly described as a predictor of poor prognosis^{113–115}. Expression of SOX2 has also been reported in the non-malignant tissue of the fallopian tube from HGSC patients and in tubal tissue from *BRCA1/2* mutation carriers who underwent prophylactic salpingo-oophorectomy¹¹⁶. This indicates that SOX2-expressing CSCs could have a role in early development of the disease and might serve as an early biomarker for HGSC in a premalignant stage before the establishment of STICs.

A study following up on the discovery of the Yamanaka factors reported that, at least in certain settings, only SOX2 and OCT4 are actually needed for the induction of pluripotency¹¹⁷, further focusing the attention on these factors. Cells expressing SOX2 and other stem cell factors have also been reported to be enriched in HGSC cells growing in sphere formation^{118,119}, and when overexpressing SOX2 in a cell line normally negative for the protein (CAOV3), the sphere-forming capacity increased markedly, supporting the connection between SOX2 and self-renewal¹¹⁸.

In a recent study, Robinson et al.¹¹⁹ injected mice with HGSC cells (ACI23 cell line), and once tumors had formed they found that the levels of the stem cell marker SOX2, but not the markers OCT4 and Nanog, were significantly higher in tumors developing rapidly compared to more slow-growing tumors, indicating that SOX2 could be a more important factor in HGSC regrowth than OCT4 and Nanog.

Other markers of stemness

Tools for identifying CSCs in tumor tissue have mainly focused on stem cell surface markers and nuclear transcription factors. Some of the surface markers most often described and investigated in the context of ovarian cancers are CD133, CD117, CD44, and ALDH^{98,99,119,120} and these are commonly used in flow cytometry cell sorting. Among the nuclear stem cell markers most often described, the three transcription factors SOX2, OCT4, and Nanog are particularly interesting. They have been shown to constitute a gene-regulatory network essential for maintaining pluripotency^{121,122}. The OCT4 protein is encoded by the POU Class 5 Homeobox 1 (*POU5F1*) gene and contains two DNA binding domains. The SOX2 and OCT4 proteins can act independently on certain targets, but commonly form dimers that can bind to specific gene targets crucial in cell reprogramming¹⁰⁹. The dimers also bind the *SOX2* and *POU5F1* genes, as well as the gene encoding the stem cell factor Nanog, which further increases the level of transcription factors in the cell. Kalmar et al. reported that the levels of Nanog in embryonic stem cells fluctuated in a heterogeneous manner and in states of low Nanog expression the cells were more prone to differentiate. They concluded that the stem cell factors SOX2, OCT4, and Nanog forms a dynamic stem cell network that interferes with differentiation signals¹²¹. Much like SOX2, OCT4 expression has also been implicated in poor prognosis in several cancer forms^{123–125}.

Different signaling pathways have also been studied in the context of CSCs. The activation of the Wnt/ β -catenin pathway has a well-established role in normal stem cells¹²⁶, and studies have also implicated its connection to DNA repair¹²⁷. The expression of various components in this pathway has also been associated with multiple cancer forms¹⁰². Other pathways associated with CSC and SOX2 include the PI3K/AKT, Hedgehog/GLI, and Notch pathway¹¹¹. When overexpressing SOX2 in laryngeal squamous cell carcinoma cells, activation of the PI3K/AKT pathway accompanied by an increase in migration and invasion was detected. Also, levels of PI3K, Akt, and p-Akt have been found to decrease following SOX2 gene silencing in HGSC cell lines¹²⁸. GLI1/2 has been found to bind the SOX2 promoter, and it has been reported that SOX2 is required for the growth-inducing effect of the hedgehog signaling pathway in melanoma cells¹²⁹. In ovarian cancer cell lines, activation of Notch (through hypoxia or overexpression of Notch1) led to an increase in stemness and SOX2 expression¹³⁰.

Cancer stem cells and DNA repair

The subpopulation of CSC has repeatedly been associated with a capacity to evade DNA damaging therapy^{127,131}. It has been speculated that CSC might have a better functioning DNA repair, but most studies conclude that the effect is most likely due to the slow growth of these cells, allowing more time to repair DNA damage in between cell cycle checkpoint^{98,106}. A study by Ropolo et al.¹³¹ compared DNA damage response in CD133+ and CD133- glioma cells and found that the CD133+ cells (which in this model would be considered the CSC population) did not display more markers associated with either SSB or DSB repair, and concluded that the increased resistance to radiation and DNA damaging drugs was due to longer cell cycles. Also, higher levels of the cell cycle checkpoint kinases CHK1/2 was found in CD133+ cells, which are proteins that have been shown to increase the cell's tolerance for DNA damage and thereby help the cell to evade apoptosis¹³².

MET and tumorigenesis

The receptor tyrosine kinase (RTK) MET is a proto-oncogene which when overexpressed or amplified has been associated with poor prognosis in a number of cancer types, including glioblastoma¹³³, non-small cell lung cancer¹³⁴, and also advanced stage ovarian/peritoneal carcinomas¹³⁵. MET expression and MET amplification is a common feature in several cancer types¹³⁶.

MET is activated upon binding its ligand, the hepatocyte growth factor (HGF). The connection between MET and cancer growth was first reported in 1994 when the expression of these two proteins was shown to promote metastasis *in vivo*¹³⁷. The expression of MET and its ligand HGF have both been shown to increase as a result of focal

hypoxia in tumors¹³⁸, indicating that MET signaling most likely plays a role in tumor progression and maintenance, rather than in tumor transformation. This was confirmed by Corso et al.¹³⁹ who found that silencing the *MET* gene led to a regression of established primary and metastatic tumors *in vivo*. This discovery means that drugs targeting MET could be effective even in late-stage disease.

Yamamoto et al. investigated the prevalence of MET expression in different ovarian cancer entities through IHC and found that the protein was markedly more common in clear-cell tumors compared to other histologies, and the staining intensity was also stronger¹⁴⁰. Using a cut-off of 10% cells with cytoplasmic and/or membranous staining, 66% of the clear-cell tumors were MET positive, compared to 16% of tumors with a serous histology. Further, copy number analysis revealed that gain or amplification of *MET* was also strongly associated with the clear-cell histology, compared to serous. That study does not specify if the serous tumors were high-grade or low-grade, however, in a study by Battista et al., a comparison between type I and II ovarian cancers showed that MET was predominantly expressed in type I tumors¹⁴¹.

Studies in lung cancer and gastric cancer cell lines have also found that *MET* amplification was a good predictor of response to MET inhibitors¹⁴², and a strong connection between *MET* amplification and EGFR inhibitor resistance^{143,144}. Engelman et al. further reports that MET drives the resistance to EGFR inhibitors (such as gefitinib and erlotinib), through the MET protein's ability to activate ERBB2-dependant PI3K-signalling, helping the cells to find new ways of survival. When MET was inhibited in these cells, the sensitivity to EGFR inhibitors was restored.

MET expression has also been proposed to play an important role in CSC, with higher levels of MET found in subpopulations of glioblastoma cells with increased clonogenic and tumorigenic potential. These cells were also shown to be more resistant to radiation¹⁴⁵. MET inhibition of these cells *in vitro* and *in vivo* resulted in disrupted tumor growth and invasiveness, indicating that MET could be a common feature in CSC and an interesting target for treatment.

Endometrial cancer

Uterine cancers are more common than pelvic cancers but far less deadly. There were 417,000 cases reported worldwide in 2020, and 97,000 deaths⁷. Incidences vary across populations and are higher in Europe and countries populated to a large degree of people of European descent and are lower in most African regions and South-central Asian countries.

Tumor subtypes

Histological subtyping

Neoplasms of the uterus can be either epithelial (carcinoma), or mesenchymal (sarcoma). The epithelial subgroup is commonly referred to as endometrial cancers as these tumors arise in the epithelial that is lining the inner wall of the uterine cavity, i.e., the endometrium. Endometrial cancers can be roughly divided into one of two pathogenic types, as proposed by Bokhman et al. in 1983¹⁴⁶. They defined tumors with endometrioid histology, often found in women displaying metabolic and endocrine disturbances, as type I, and all non-endometrioid malignancies, more common in women not displaying these disturbances, as type II (Table 3 & Figure 5).

Table 3. Features of the Type and II endometrial carcinomas^{146–148}

	Type I	Type II
<i>Defining characteristics</i>	Highly or moderately differentiated Good response to gestagen treatment	Poorly differentiated Poor response to gestagen treatment High frequency of metastasis
<i>Histological types</i>	Endometrioid adenocarcinomas (87-90%)	Non-endometrioid carcinomas: Serous (3-10%) Clear-cell (2-3%) Carcinosarcoma (<2%) Mucinous (0.6%) Squamous-cell (0.2%) Other (7%)
<i>Defining genetic aberrations</i>	KRAS PIK3CA mutation PTEN loss of function Nuclear accumulation of β-catenin CTNNB1 mutation ARID1A MSI*	TP53 mutation PIK3CA mutation/amplification HER2 overexpression/amplification p16 Loss of function
<i>5-year survival</i>	85%	43%

*MSI = microsatellite instability, as determined by mutations in mismatch repair (MMR) genes and/or specific histopathological features

Type II tumors are poorly differentiated, frequently metastasized, and have low hormone sensitivity. The prognosis for patients with these tumors is substantially worse than for patients with type I tumors. An overactivated PI3K/PTEN/AKT/mTOR pathway is common in both type I and II^{149,150}.

Serous endometrial carcinoma (SEC) is the most common subtype of the non-endometrioid tumors. This tumor type is histologically similar to HGSC, and prognosis is very poor. Recommended treatment regimen in these tumors is upfront radical surgery followed by combination treatment with carboplatin and paclitaxel, much like the standard treatment for HGSC.

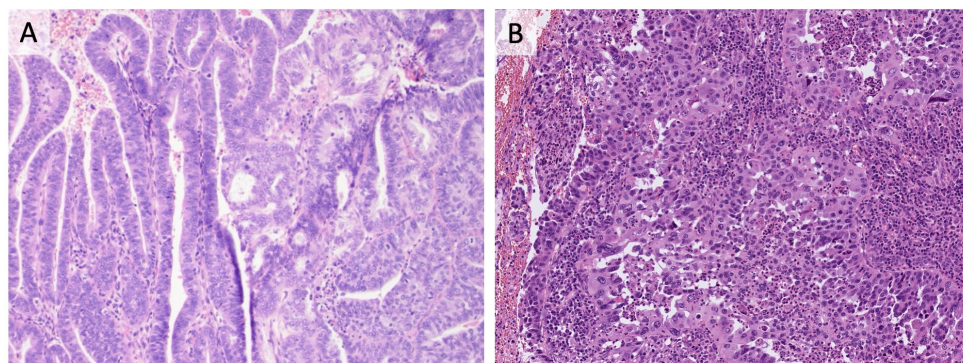


Figure 5. The most common histologic subtypes of endometrial carcinoma. Endometrioid (A), and serous (B). Images courtesy of Sofia Westbom-Fremer, Lund University.

Molecular subtypes

The TCGA organization published a paper in 2013¹⁴⁸ where they presented the result of an attempt to subgroup endometrial carcinomas into molecularly distinct tumor types. Using copy number array data from 363 endometrial cancers they performed unsupervised hierarchical clustering, which yielded four groups of tumors with varying genomic profiles (Figure 6). Adding mutational data from exome sequencing, focusing on single nucleotide polymorphism in certain genes and mutations in MMR genes, they named each sample cluster after factors that defined them. Cluster 1 was named “POLE (ultramutated)”, as these tumors had distinctly more mutations per megabase pair than the others and in addition, without exception, harbored a POLE mutation. Cluster 2 was named “MSI (hypermuted)”, as these were also very high in mutation rates, commonly displayed MSI and a silencing methylation in the MMR gene *MLH1*. Cluster 3 was named “Copy number low (endometrioid)” as it consisted of mostly microsatellite stable endometrioid tumors with a lower mutational frequency. Finally,

cluster 4 was assigned the name “copy number high”. This cluster contained all of the serous tumors, many tumors with mixed histology, and a few cases of endometrioid histology. The mutational load in these tumors was low, however, the occurrence of copy number alterations was distinctly higher than in the other clusters.

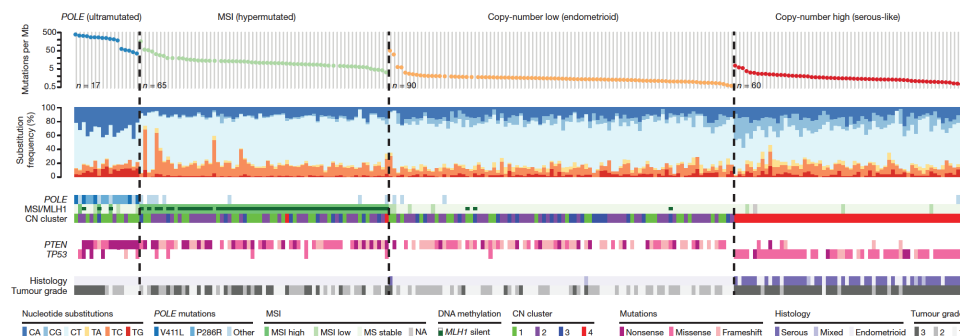


Figure 6. Summary of clustering results from TCGA study. Adapted from Getz et al. ¹⁴⁸

Based on the characteristics of the four different clusters presented by the TCGA, a clinically applicable method of classifying endometrial tumors was later developed and referred to as ProMisE^{151,152}. In a study by Stelloo et al. from 2015, a molecular-based tumor classification was proposed which largely confirmed the results of the TCGA study¹⁵³.

Comparing serous pelvic and serous endometrial carcinomas

The phenotyping of epithelial pelvic and endometrial cancers is in many aspects overlapping. Both cancers can be divided into entities of high-grade or low-grade malignancy (type I and II) and the same kind of histologies are found in both organs. Adding to the information provided by the TCGA study one also discovers that the SEC is not only extremely similar in histology to HGSC (Figure 7A&B) and share the same *TP53* inactivation (>90% of cases), clinical features, and poor prognosis, but also that the two tumor entities also display similar global mutational profiles and a high frequency of copy number alterations. This is a genetic profile that is also, to a large degree, shared with basal-like breast cancer¹⁴⁸ (Figure 7C). As HGSC patients generally respond well to PARP inhibitors, such as olaparib, it has been suggested that these drugs should also be properly evaluated in SEC¹⁵⁴. Studies in HER2 negative breast cancers with *BRCA* mutation or other HR deficiency have also shown promising results¹⁵⁵ and olaparib has

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been approved in this patient group as well as in HGSC. The striking similarity in copy number profiles for HGSC, SEC, and basal-like breast cancer was presented by the TCGA¹⁴⁸ and later confirmed by Ashley et al.¹⁵⁶. However, this latter study also emphasizes that the mutational load is lower in SEC than in HGSC, and response to platinum poorer, which might mean that SEC would respond differently to PARP inhibitors.

Regarding immunophenotypes, both SEC and HGSC display a high expression of the proliferation marker Ki67, but while the WT1 is a distinctive marker for HGSC, it is not always expressed in SEC. Up to 70% of these tumors display WT1 expression, however, staining is diffuse and heterogenous across the tumors. Another distinctive difference between these tumor types is that the hormone receptors ER and PR are commonly expressed in HGSC, but are rare in SEC¹⁵⁷.

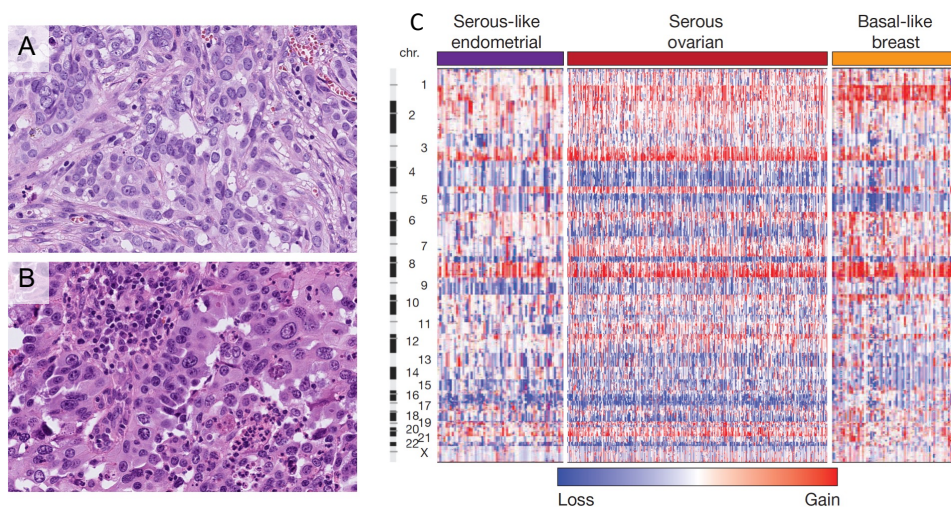


Figure 7. Comparing histologies and copy number profiles in different tumors entities. Histology of HGSC (A) and SEC (B). Similarities in copy number profiles in different tumor entities (C). Histologic images courtesy of Sofia Westbom-Fremer at Lund University. Copy number profiles adapted from Getz et al.¹⁴⁸

Materials and Methods

Tissue microarray (TMA) evaluation

Constructing a tissue microarray

Constructing a tissue microarray (TMA) is a very convenient approach when evaluating a large number of tissue samples for expression of multiple different proteins. A pathologist examines sections of formalin-fixed paraffin-embedded (FFPE) tumor tissue, then punches out thin cylinders of tissue (0.6-2mm in diameter) and collects these in a recipient block of paraffin. Several cores per patient can be used, and around 100-1000 cores can fit in a single block¹⁵⁸. Thin sections of a block (3-5µm) are then cut and placed on glass slides, ready to be used in a staining protocol (Figure 8). This method of preparation was introduced by Battifora et al. in 1986¹⁵⁹ and was then referred to as a multitumor tissue block (MTTB). The technique was later refined by Kononen et al., in 1998¹⁵⁸. Once you have gone through the trouble of recruiting patients, collecting clinical data, and preparing the blocks, you can use them for years and stain sections of the tissue with many different antibodies. Apart from protein expression analysis, the TMA can also be used for *in situ* hybridization (ISH) assays, for detection of specific DNA sequences (Fluorescence ISH or Chromogenic ISH), or mRNA (RNA ISH).

There are several advantages of working with TMAs compared to working with whole-tissue sections. Gathering many tumors together saves time in handling, requires less tissue, and reduces the volume of reagents and antibodies needed. Also, as the cores are stained simultaneously, the intraexperiment variation is minimized and samples can be compared. The small size of the tissue is, however, a disadvantage and so it is advisable to use multiple cores per patients to account for heterogeneity within a tumor.

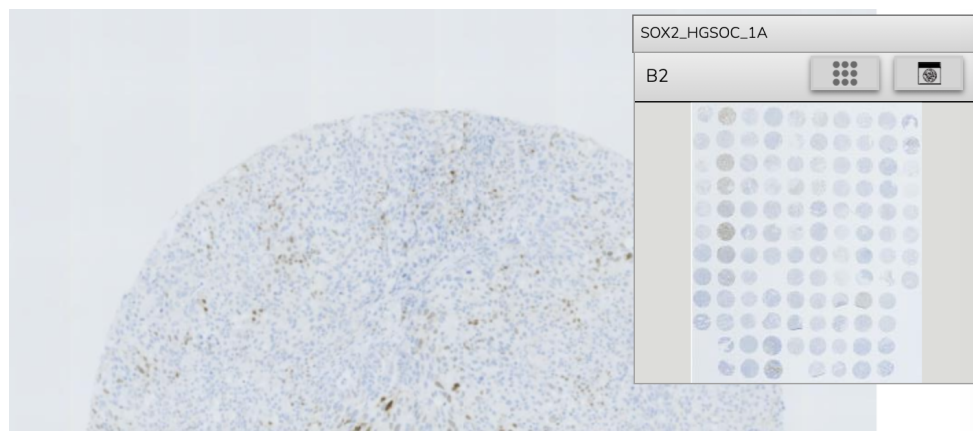


Figure 8. Image of the cores of a SOX2 IHC stained TMA slide with one of the cores magnified. Screenshot from the TMA scoring software PathXL.

In studies I and III, we used a TMA constructed by experienced gynecologic pathologists in our research group. It consisted of a clean and well-annotated HGSC cohort which included 141 patients of which 130 were diagnosed with advanced stage disease.

Immunohistochemistry

The method of double-staining tissue with hematoxylin and eosin to visualize microscopical structures was introduced by Wisoosky in 1877¹⁶⁰. The use of antibodies, conjugated with a fluorescent group, enabling the visualization of protein expression however, was first introduced by Coons et al. in 194¹⁶¹, and provided the basis for the field of immunohistochemistry (IHC), in which detection can either be performed using fluorescent antibodies, or a chromogen. In studies I and III, we used chromogenic IHC to stain sections of the described TMA.

Performing IHC involves a number of steps. Following deparaffination and hydration, the tissue is exposed to a blocking solution, then to a primary antibody specific for the protein of interest, and finally to a secondary antibody that can bind the primary antibody and that is conjugated with peroxidase. A detection chromogen (substrate) is then added, which is subsequently oxidized by the peroxidase and turns brown. Brown is the most common color, but the specific color depends on the substrate used. The brown color is visible in light microscopy and marks the location of the protein of interest.

We stained sections of the TMA with SOX2, a transcription factor that is located in the nucleus, and MET, a RTK located in the membrane. To evaluate the TMA, SOX2

stainings were scored by counting the fraction of tumor cells with nuclear stain and MET stainings were scored by counting the fraction of cells with membranous stain (Figure 9).

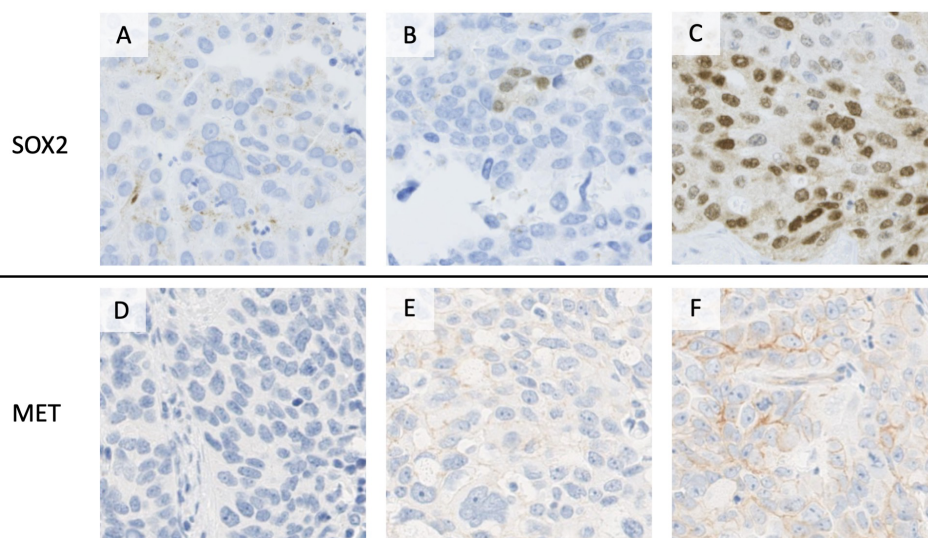


Figure 9. IHC stainings with antibodies for SOX2 and MET. SOX2 negative core (A), SOX2 positive cores (B-C), MET negative (D), MET positive cells with weak membranous staining (E) and strong membranous staining (F)

Utilizing publicly available data sets

Whole-genome DNA sequencing and RNA sequencing are relatively expensive procedures compared to the less extensive microarray methods available; however, as they are becoming cheaper, these techniques are getting more common. As a result, massive amounts of sequencing data is now being produced, and many journals encourage (or demand) that sequencing data is to be uploaded to a public database allowing other researchers to use it for further analyses.

The Cancer Genome Atlas (TCGA) data

In addition to datasets produced by individual groups, there is also an American governmental project with the goal of creating molecular maps of a range of different cancer types and to increase knowledge of the molecular processes defining and driving cancer. The Cancer Genomic Atlas (TCGA) is a joint project between The National Cancer Institute (NCI) and the National Human Genome Research Institute

(NHGRI), both institutes operating under the American National Institutes of Health (NIH). A first set of tumor data was uploaded in 2007 and in 2011 a landmark paper in ovarian cancer subtyping was published³⁷. To date, 33 different tumor types have been explored, encompassing over 11,000 patients. The data produced under the TCGA project is publicly available and many tools are available that facilitates downloading and analyzing the data¹⁶².

In study III, we retrieved data from the TCGA publication on HGSC and compared the mRNA levels of several genes to each other as well as to clinical outcome variables, also available in the TCGA database.

Copy number analysis

DNA microarray

The HGSC genome is characterized by large-scale alterations in its copy number profile. The copy number profile can be analyzed using a DNA microarray which is the most cost-effective method. To also include the global mutational profile exome sequencing or whole-genome sequencing can be used instead.

For the copy number analyses in studies II and III, we used the Affymetrix OncoScan CNV assay. This technique is also referred to as a single nucleotide polymorphism (SNP) array. In this specific array, approximately 220,000 different probes are used with sequences homologous to the target DNA. The probes and the DNA are hybridized overnight, and the product is split into two separate tubes (Figure 10). The DNA samples are incubated with either a mix of nucleotides A and T or nucleotides G and C. Depending on which base is found in the SNP, the complementary building block will only be available in one of the tubes, in which the gap of the probe will filled, creating a circular probe. The probe is then dissociated from the gDNA and all non-circular fragments in the tube are digested by exonucleases. The remaining circular fragments are now cleaved to produce linear fragments. These are PCR amplified and labeled with biotin and are then placed on two individual microchips. The signal intensity is measured, and the result of the two arrays provides both a total intensity measure (the sum of the two arrays, for each probe) and a ratio of the signals found on the A/T array and the C/G array.

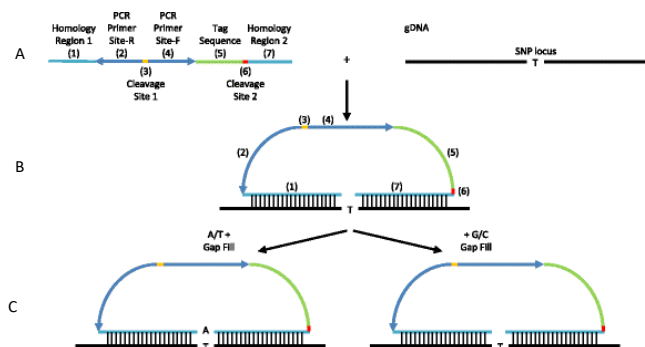


Figure 10. The OncoScan CNV analysis Probes have two regions that are homologous to a specific SNP locus (A). These are hybridized to the genomic DNA (B). The DNA is incubated with one of two nucleotide-mixes and the probe-gap is either filled, forming complete circular DNA, or is not filled, leading to subsequently degradation (C). Figure adapted from Jung et al.¹⁶³

The output generated from the microarray is the log-transformed ratio of the signal detected in both chips by the signal expected in the case when two DNA copies are present. This measure is referred to as a logR value¹⁶⁴. A logR=1 indicates a normal, diploid DNA, while higher or lower logR values indicate a gain or a loss of genomic material in the specific locus. The microarray output also provides the B-allele frequency (BAF) which is the ratio of copy numbers for one of the alleles divided by the total number of alleles in each locus.

ASCAT

To further analyze the logR and BAF data we used functionalities included in the ASCAT R package¹⁶⁵. This package includes a method to segment DNA microarray data, calculate the level of normal cell contamination, ploidy, and ultimately estimate the allele-specific number of copies of each gene in the tumor. These measures are useful when analyzing patient tissue, both if you have normal tissue controls or only the cancer tissue. The ASCAT package also provides an algorithm that uses the heterogeneity resulting from normal cell contamination to create a control. However, this algorithm is not optimized for cell line samples as these lack the heterogeneity normally seen in tumor tissue.

HRD score

There are a few different methods that can be used to determine HR deficiency status, one of them being the HRD score. For research purposes the HRD score can be calculated with an algorithm made publicly available^{166,167} and in a clinical setting, the Myriad myChoice® CDx test can be used. The Myriad test provides both the mutational status of *BRCA1/2* and the calculation of the HRD score. This is, again, determined

from the sum of LOH, TAI, and LST. The Myriad test is currently the only FDA-approved test in use for the indication of the PARP inhibitors olaparib or niraparib⁹².

There are also functional analyses developed to estimate HR deficiency and PARP inhibitor sensitivity in cell lines, which involves counting RAD51 and γ H2ax foci⁵⁸. RAD51 is a crucial component in the HR repair system and phosphorylation of the histone protein H2AX has been reported to be a good marker for DSB¹⁶⁸, as this phosphorylation creates γ H2ax which accumulates at DSB sites at an early phase in the induction of HR repair. The number of γ H2ax foci in a cell thereby correlates with the number of DSBs. Mukhopadhyay et al.⁵⁸ reported that all six cell lines tested displayed an increase in γ H2ax foci formation following PARP inhibition, while only four lines showed an increase in RAD51 foci. These four cell lines also displayed less cytotoxicity following treatment and were concluded to be HR proficient while the remaining two lines were considered HR deficient. Further investigation in primary ovarian cancer cells showed a strong correlation between treatment resistance and the number of RAD51 foci formed following PARP inhibitor treatment.

In studies II and III, we calculated HRD using the method described by Telli et al.¹⁶⁶, using a custom implementation in the R statistical environment.

Data analysis and statistical approaches

“If your experiment needs statistics, you ought to have done a better experiment”

Ernest Rutherford, physicist (1871-1937)

It is unfair that in physics, laws can be tested and proven. In biology on the other hand, even with a meticulously planned experiment, biological replicates never yield exactly the same results, albeit some experiments are more replicable than others. This is why statistics is needed in biology. Cellular processes are intricate and depend on many factors, both intrinsic and external, that always vary between experiments. Drawing conclusions from biological data with high variability is hard to do without relying on statistical analysis.

The main results of studies I and III focus on survival outcome in relation to protein markers and Kaplan-Meier plots are used for visualization. This is a well-known way of presenting survival differences between groups; however, it can only include one or, possibly, two variables before the groups are so many that the lines cannot be distinguished from each other. Cox regression can then be used as a tool to analyze the impact for several variables, many more than would be possible to include in a visualization. In a multivariable Cox analysis, many factors can be taken into consideration and the

effect of each variable can be estimated, with the assumption that all other variables are held constant¹⁶⁹. In both studies, we included interaction terms, which can be used to estimate the effect of two factors when they co-occur. We also reported the results of log-rank tests, even though these are less robust and provide less information than the Cox regression analysis; however, we included the log-rank tests because this is common practice, and because they provide a complement to the Cox-regression analysis.

In studies II and III, we investigated the connection between HRD scores and different genetic and clinical factors. The analysis is exploratory and focuses on visualization, and statistical tests were only used as a complement. For analysis of the pairwise association between two continuous variables we used Pearson's correlation and to compare the means of continuous variables between two groups, we used Welch's t-test.

All calculations and analyses were conducted using the R statistical environment, versions 3.6.3-4.0.5¹⁷⁰

In vitro cell line models

Cell lines

In the presented studies, a total of six cell lines were cultured and analyzed. In study I, the two HGSC lines OVCAR3 and COV362^{171,172} were used. These were chosen based on their high SOX2 expression, as determined by western blot analysis. The SOX2 levels were examined following chemotherapy treatment, and sensitivity to chemotherapy treatment was evaluated following SOX2 siRNA gene silencing.

In study III, four cell lines were used. Two of these were derived from tumor cells found in the ascites of the same patient, before and after her tumor was considered platinum-resistant¹⁷³. The cells retrieved from the platinum-sensitive tissue had a *BRCA2* mutation and in the resistant cells, this gene displayed a reversal mutation¹⁷⁴. The third cell line, UWB1.289, was derived from a woman with a germline *BRCA1* mutation, and the fourth cell line was derived from UWB1.289 cells in which a functional *BRCA1* had been introduced through plasmid transfection¹⁷⁵. Sensitivity to platinum and PARP inhibitor treatments was compared within each cell line pair and stem cell marker levels, and HR markers were evaluated on the protein or mRNA level in untreated cells. The purpose of the cell experiments was to compare drug sensitivity and expression profiles between the *BRCA* proficient lines and their deficient counterparts.

In examining the role of *BRCA* function, an alternative to these models could have been to use a gene-silencing technique, such as siRNA or shRNA, or permanent gene editing

using CRISPR/Cas9 techniques, in any *BRCA* proficient HGSC cell lines. Temporary gene silencing of the *BRCA1/2* genes has however been proven challenging^{176,177}, possibly due to their large size, encoding proteins of 220 and 384kDa, respectively¹⁷⁸.

siRNA knockdown

To evaluate the role of SOX2 in treatment response in study I, we used reverse transfection of small interfering RNA (siRNA) to silence the gene. The technique of using RNA strings to interfere with DNA and blocking transcription was first described in 1998 by Andrew Fire¹⁷⁹, who together with Craig C. Mello was awarded the 2006 Nobel Prize in Physiology or Medicine. Silencing genes by use of siRNA results in a temporary blocking of transcription. If a more stable silencing is needed, short-hairpin RNA can be used, and if a complete and permanent silencing is required, knock-down using CRISPR/Cas9 techniques can be implemented, editing the actual DNA sequence to produce a non-functional gene¹⁸⁰. Traditional gene knock-out in mice is performed by introducing a dysfunctional version of the gene into stem cells and then relying on the normal HR of the cell to incorporate the DNA into the right position. These stem cells can then be injected into an early mouse embryo (blastocyst)¹⁸¹.

Cytotoxicity assay

In studies I and III, we performed growth inhibition analyses, that is, we compared the proliferation between multi-well plate seeded cells cultured in different conditions. Conditions were typically changed by varying the drug concentration. This can be done in several ways. One common approach is metabolic assays which involve measuring the viability of the cells by use of a reagent added to the cell culture media. The cells are lysed and then produce a luminescence signal corresponding to the ATP content of the cells¹⁸². Another technique is a protein assay in which the cells that are attached to the culture plate are fixed and protein is stained with Sulforhodamine B (SRB)¹⁸³. Following the wash of excess dye, the plates are dried, the stain is dissolved, and the absorbance is measured. These are simple techniques that are commonly used; however, they only provide a snapshot of the drug response process. There are also systems available for live monitoring of cell viability, such as the Agilent xCELLigence real-time cell analysis¹⁸⁴. This system uses electrode plates in which the cells are seeded. Biosensors at the bottom of the plates register how much biomass has attached to the surface, corresponding to the viability of the cells in each well, and a value is produced that can be compared to the values of control wells. This technique provides another dimension to a cytotoxicity experiment; that you can analyze data from multiple time points of the treatment. The cells cannot, however, be visualized microscopically when they are seeded, which is a disadvantage.

Evaluating gene activity

Western blot analysis

The level of gene activity can be examined on either the mRNA or protein level. For sections of tissue, IHC is commonly used to evaluate protein expression, but western blot (WB) analysis on cell lysates is the most common technique used when working with cell lines. We used this technique in study I to see how SOX2 levels in cell lines were affected by chemotherapy, and in study III we used it to evaluate the difference in expression of certain proteins in two closely related cell lines (UWB1.289 & UWB1.289+BRCA1).

In short, WB analysis is performed by lysing a cell pellet and measuring the protein content by spectrophotometry. Equal amounts of protein from each sample are then loaded onto polyacrylamide gels, which are placed in an electric current causing the proteins to separate by order of molecular mass. The proteins are transferred to nitrocellulose membranes which are in turn exposed to blocking solution, primary antibody, and peroxidase-conjugated secondary antibody. Finally, the membranes are exposed to a chemiluminescent substrate and the luminescence signal is detected¹⁸⁵.

WB analysis originates from the work of Sir Edwin Southern, who originally developed a method for separating DNA fragments, referred to as Southern blot¹⁸⁶. When this was adjusted to be used for protein, RNA, and post-translational modification the techniques were named western, northern, and eastern blot¹⁸⁷. WB analysis is a labor-intensive technique with several manually performed steps, which means that there is a considerable risk of errors and intra-experimental variability. Artifacts in the gels or membranes are also common and the actual amount of protein can be hard to quantify when comparing samples. Nevertheless, WB is still the standard technique for the quantification of protein content in cultured cells.

With WB, the total amount of a specific protein in a cell sample is measured, but when examining the distribution of protein expression within the cell population, immunofluorescence is a common technique. This involves fixing the cells on the culture surface and staining them using an approach similar to the indirect labeling used in IHC and WB, but with the secondary antibody conjugated to a fluorescent probe, which can then be visualized in a fluorescence microscope¹⁸⁸.

RT-qPCR

One method to examine expression on the mRNA level is the reverse transcription-quantitative polymerase chain reaction (RT-qPCR), which gives an estimation of the number of mRNA copies of a specific gene transcript present in a given sample. When the expressions of many genes need to be analyzed, a microarray method or RNA sequencing can be used instead. In microarrays, the number of transcripts, corresponding

to at least one out of the hundreds of thousands of probes bound to the surface of a DNA chip, is measured. This provides a good estimate of the global gene expression. RNA sequencing has additional advantages, including possible detection of alternative splicing¹⁸⁹. We used RT-qPCR to investigate how the mRNA levels of *SOX2* were affected when treating *SOX2* expressing cell lines with chemotherapeutics in study I.

In this method, RNA is extracted from cell samples and translated into complementary DNA (cDNA) through a technique called reverse transcription. mRNA is mixed with short random primers, nucleotides, and a polymerase, and the transcription is induced using a heat cycle. For the next step, short primers of single-strand DNA are designed which are complementary to the start and end of the specific segment of the cDNA you are interested in. To perform the actual quantitative PCR, the cDNA sample is mixed with nucleotides, the specific primers, and a reagent containing both a polymerase and a fluorescent molecule. During repeated heat cycles in a PCR thermo-cycler, the polymerase copies the specific segment encompassed by the primers, and the fluorescent molecule binds all double-stranded DNA produced. Upon binding, these molecules send out a fluorescent signal detected by the thermo-cycler¹⁹⁰. The number of cycles needed to produce a fluorescent signal can be interpreted as a measure of the concentration of starting material in the cDNA sample. By comparing how many heat cycles are needed to produce the fluorescent signal in different samples, the mRNA expression relative to other samples can be calculated after the results have been normalized to total mRNA content, using the mRNA expression level of so-called house-keeping genes which are known to be more or less constant in cells and relatively unaffected by different treatments.

Results and Discussion

Cancer stem cells in HGSC

Most women diagnosed with HGSC respond well to treatment, yet a majority relapse within a few years⁸. The fact that seemingly cancer-free patients still develop new tumors could be the result of a subpopulation of CSCs in the tumor, which even at a very low cell count have the ability to initiate a new aggressive tumor^{96,191,192}. Several different proteins are currently being evaluated for their role in self-renewal, as well as their prognostic potential. Highlighting the significance of the CSC subpopulation and identifying CSC biomarkers could help guide the treatment towards drugs targeting stem cell signaling pathways, such as EGFR and PI3K/AKT¹¹¹. Understanding the role of cancer stem cells in angiogenesis and DNA repair could also help fine-tune the treatments with the currently available PARP and VEGF inhibitors.

In this thesis, I have primarily focused on the nuclear transcription factor SOX2, which is one of the most central stem cell markers^{111,119}.

Cancer stem cells and relapse

Studies I and III in this thesis were based on the same consecutive cohort of HGSC patients (n=141). Only patients with advanced-stage disease (n=130) were included in the analyses as the prognosis is substantially worse in this group (Figure 11).

In study I, we reported an interaction effect on survival between SOX2 expression and the presence of residual tumor tissue after primary debulking surgery. For these analyses, we excluded patients who did not undergo a debulking surgery (n=5) (Figure 11). Comparing survival in the whole cohort of SOX2 did not show a significant relation to survival outcome (Figure 12A). Subgroup analysis, however, revealed that when including only patients with tumor tissue remaining, SOX2 was prognostic of overall survival (Figure 12B). Statistical analysis was performed both using log-rank tests for subgroup analysis and Cox regression analysis using an interaction term for SOX2 and residual disease (OS: $\beta=1.1$, 95% CI: [0.21; 2.1], $p=0.017$). In the Cox regression analysis, all patients were included, making this result more robust than the log-rank test that was applied separately to each subgroup.

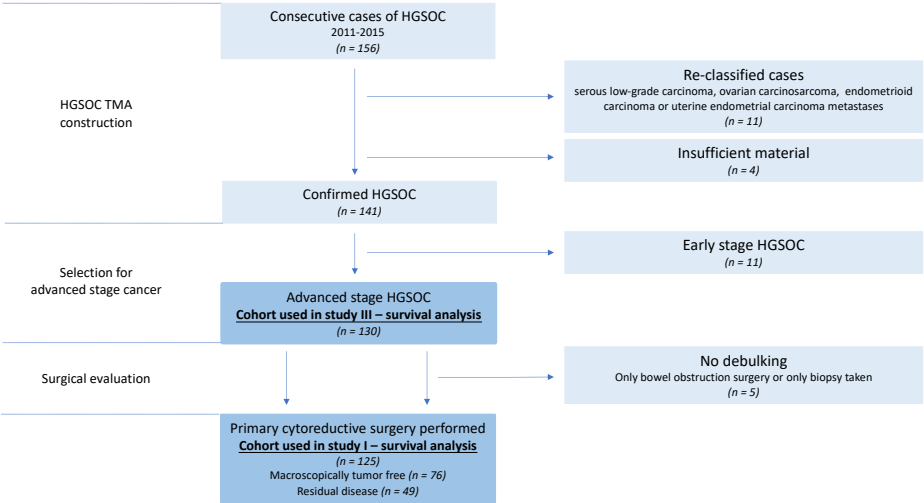


Figure 11. Inclusion chart for study I & III

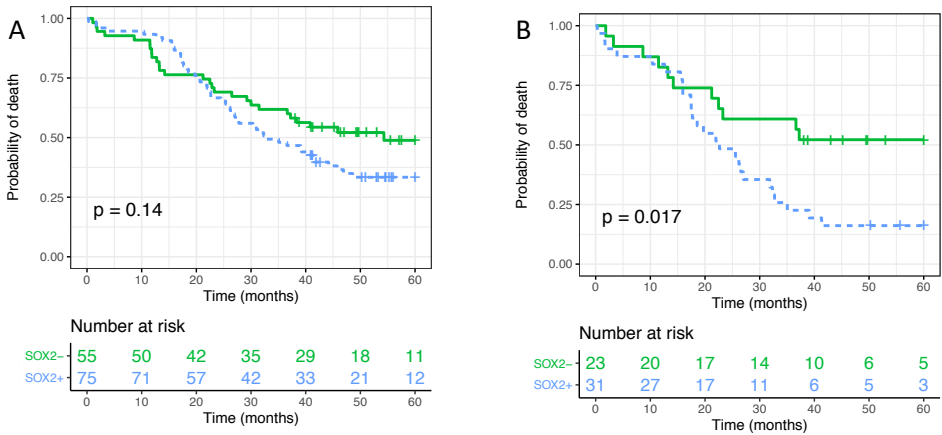


Figure 12. Kaplan-Meier estimates of SOX2 and overall survival. All patients (n=130) (A), and the subgroup of patients with residual tumor n=54 (B). The p-values are the result of log-rank tests.

The poor prognosis in women with tumors expressing SOX2 has been reported in several cancer forms¹¹¹ and this is likely connected to the self-renewal capacity connected to this transcription factor^{129,193}. SOX2 has also been reported as a prognostic factor in ovarian cancers previously. The results have; however, not been consistent, and cut-offs

for what is considered SOX2 positive have varied^{115,194,195}. Taking the expected size of a CSC subpopulation into consideration⁹⁶, we used the lowest possible cut-off, ≥ 1 cell, as we believe that the often sporadic expression of SOX2 we saw in the TMA is consistent with the expected fraction of CSCs in tumors. The composition of tumor types included in other ovarian cancer cohorts has also varied, while our cohort only included advanced stage HGSC tumors. I believe that evaluation of CSC levels in tumors should be included in the clinical IHC analysis routine for prognostic reasons, and possibly when evaluating treatment options. As DNA damaging agents do not target CSCs, alternative treatment regimens should be evaluated for these patients. Evaluating a range of stem cell markers (SCM) is preferable to only using SOX2, and further studies could evaluate which markers are most representative for CSCs.

MET and cancer stem cells

MET and stem cell factor expression

Comparing MET and stem cell factor mRNA expression levels

In study III, we performed correlation analyzes between the mRNA expression levels of *MET* and the four nuclear stem cell factors *SOX2*, *OCT4*, *Nanog*, and *Klf4*. In this thesis, I also include the transcription factor *MYC* and the stemness-associated proteins *ALDH1A3* and *PROM1*. *ALDH1A3* and *PROM1* have previously been linked to *MET* expression in triple-negative breast cancer¹⁹⁶. Out of the seven genes, only *PROM1* displayed a significant correlation with *MET* ($\rho=0.17$, $p<0.001$). A significant correlation was also seen for *POU5F1*, however, the correlation was fairly weak ($\rho=0.093$, $p=0.047$). In a study by Nozaki et al.¹⁹⁶, a correlation was found between *MET* and stem cell factors *PROM1*, *POU5F1*, and *ALDH1A1* in breast cancers. This study also reported a higher expression of *MET* in basal-like breast cancer compared to other breast cancers, with the histology most resembling HGSC and SEC^{148,156}.

Comparing copy number and mRNA expression levels

I also performed correlation analyzes to evaluate the connection between copy numbers and mRNA expression of the seven SCM genes. Four of these showed a strong correlation between copy number and gene expression: *POU5F1*, *KLF4*, *MYC*, and *PROM1* (encoding CD133), indicating that the expression of these genes is driven by gene amplification (Figure 13). *SOX2* was not one of the genes with a strong correlation, and an increase in mRNA expression was not found even in cases of high-level amplification. This indicates that *SOX2* expression is not dependent on gene amplification, but

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it could also mean that amplification only ensures the *possibility* of expression, but that this expression will only be induced in a small fraction of CSC and not in the tumor bulk. For this reason, the expression might not be detectable by bulk mRNA analysis.

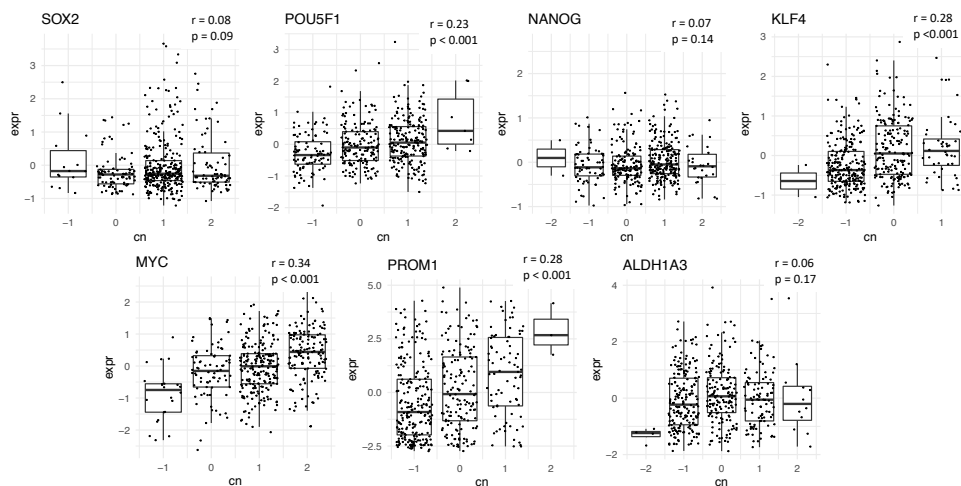


Figure 13. TCGA mRNA expression in relation to level of copy number. The y-axes (expr) show mRNA expression relative to the mean of the cohort (z-score), the x-axes (cn) show copy number level categorized using GISTIC analysis (-2: deep deletion, -1: shallow deletion, 0: diploid, 1: low-level amplification, 2: high-level amplification).

Stem cell factors and MET in relation to relapse

TMA data, protein expression

In study III, we used multivariable survival analysis to combine the stem cell factor SOX2 with another potential cancer driver gene, the RTK MET, previously reported to have a role in CSCs^{136,145,197}. We stained sections of the same TMA used in study I (Figure 11) with an antibody for MET and labeled tumors with $\geq 5\%$ positive membrane staining as being MET positive. We found MET to be an independent predictor of overall survival (Figure 14A). By combining the MET and SOX2 protein expression we found that the prognostic significance of MET was even more pronounced in the subgroup of patients with SOX2 negative tumors compared to the full cohort (Figure 14B).

Several studies have reported associations between MET and various stem cell markers. One study reported that the MET ligand HGF can activate Wnt/ β -catenin signaling in colon cancer cells and thereby support CSC clonogenicity¹⁹⁸, and another found correlations between MET and stem cell markers in breast cancer¹⁹⁶. Stem cell factors

have also been reported to be higher in FACS sorted MET positive glioma cells as compared to MET negative cells¹⁹⁹, and MET itself has been connected to the CSC phenotype, regardless of the expression of established CSC markers¹⁴⁵. Poor prognosis in patients with MET positive tumors has also been reported in ovarian cancers¹³⁵ as well as in other cancer types^{133,134,200}. This is why we set out to evaluate the interaction between SOX2 and MET in relation to survival. To explain the interaction we found, we hypothesize that tumors with a smaller fraction of CSCs are more dependent on MET signaling for survival and relapse, which is why the effect of MET was more prominent in the group with SOX2 negative tumors.

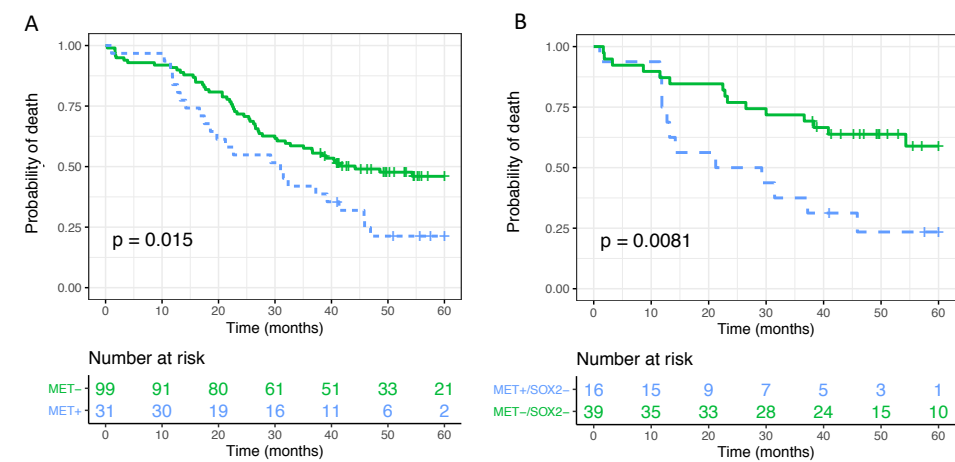


Figure 14. MET, SOX2, and overall survival in the TMA. Overall survival for MET positive/negative patients in the full cohort (A) and in the subgroup of patients with SOX2 negative tumors (B). The p-values are the result of log-rank tests.

Cut-offs, and rationale for interaction effects

To stratify SOX2 scoring in the TMA, we used a very low cut-off; ≥ 1 positive cells in any of the cores, where there were typically (median) six cores per patient. This resulted in 75 positive tumors and 55 negative tumors. To stratify MET we used a cut-off of $\geq 5\%$ cells on average in all cores, a cut-off commonly used in MET scoring²⁰¹, which resulted in 31 positive and 99 negative tumors. The rationale for choosing the different cut-offs was mainly for the purpose of creating groups of similar size, but we also consider it reasonable that for a stem cell marker like SOX2, with its known connection to self-renewal¹¹⁸, already very few cells would be enough to affect survival. Even though MET is also considered related to stemness features^{196,198}, it is unclear whether it is

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closely connected to self-renewal, and therefore a higher cut-off was chosen for this marker.

In analyzing survival and SOX2 expression in study I, we found that the prognostic value of SOX2 was only seen in the group of patients with suboptimal debulking. However, when analyzing MET in relation to survival we saw no difference between the groups with tumor tissue remaining and the group of patients with optimal debulking surgery. We reason that this is plausible considering that most SOX2 positive patients had a very low fraction of SOX2 positive cells (Figure 15), and a patient with macroscopically optimal surgery might not actually have enough (if any) of these CSCs left after surgery. However, if patients with tumors categorized as MET positive all have $\geq 5\%$ stained cells, it is fair to assume that, in the small number of cells remaining after macroscopically optimal debulking surgery, some of these might express MET.

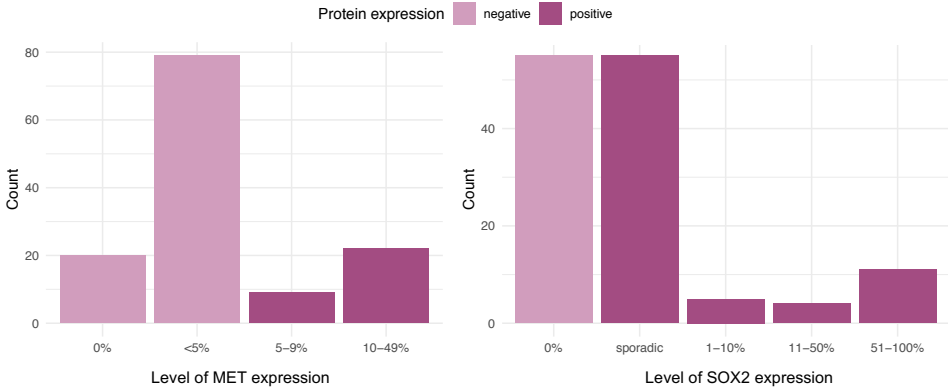


Figure 15. Distribution of protein expression levels in HGSC tumors

TCGA cohort, mRNA expression

Finding that MET protein expression was more predictive in tumors lacking SOX2 expression than in the full cohort was unexpected, as we had hypothesized that expressing both of these markers would lead to a worse prognosis than each marked individually. To further investigate this, we retrieved publicly available TCGA data³⁷ for 461 HGSC cases. For survival analysis of the TCGA data, protein expression data was not available. As a substitute, we used mRNA z-scores. This is a measure that compares each tumor's expression to the rest of the cohort, and a z-score of 0 is the mean expression of all samples. A z-score > 0 means that a tumor has an expression higher than the mean expression level in the cohort. This is the cut-off level we chose when stratifying mRNA expression of *MET* and stem cell factor genes. In most cases this method of categorization divided the samples into groups that were fairly equal in size. Using a

RESULTS AND DISCUSSION

cut-off that resulted in groups comparable in size was necessary when combining several different markers.

In the TCGA dataset, we saw no predictive value of *SOX2* or *MET* individually, or *MET* in the subgroup of patients with *SOX2* negative tumors. We then analyzed the predictive value of three additional nuclear stem cell factors, closely interacting with the *SOX2* protein; *OCT4* (encoded in *POU5F1*), *Nanog*, and *Klf4*. We analyzed the SCMs separately in relation to *MET* and survival and found that in the subgroup of patients with tumors lacking *SOX2*, no significant effect of *MET* was seen (Figure 16A), and this was also the case in tumors lacking *Nanog* or *Klf4*. In tumors lacking the *POU5F1* expression, however, the expression of *MET* was a strong predictor of overall survival (Figure 16B). We also calculated how many out of these four stem cell factors were expressed and analyzed the subgroup of tumors expressing only one or even none of the factors. In these tumors, where the cancer stem cell population could be considered to be smaller, *MET* expression was shown to be prognostic (Figure 16C).

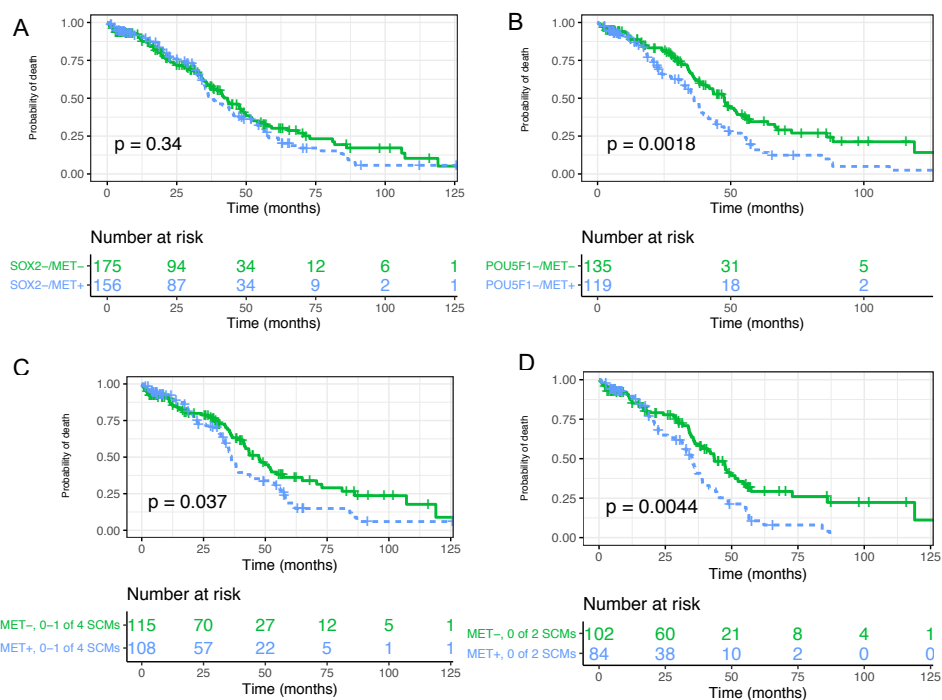


Figure 16. Stem cell markers, MET, and survival in TCGA data. Tumors negative for *SOX2* (A), *POU5F1* (B), stratified by number or negative stem cell markers (SCMs) out of four (*SOX2*, *POU5F1*, *Nanog* and *Klf4*) (C), and number of SCMs out of two (*SOX2* and *POU5F1*) (D).

In this thesis, I performed an analysis combining only the two most investigated and potentially most important markers, *SOX2* and *POU5F1*^{117,202}, and found that when expressing none of these, *MET* expression was highly prognostic (Figure 16D). Even though we could not replicate the interaction between *MET* and *SOX2*, I believe that the results from the TCGA cohort is an interesting complement that provides a broader picture of the interplay between cancer stem cell factors and *MET*. In this cohort, *POU5F1* (or the encoded protein OCT4) appears to be a more interesting prognostic factor, though earlier reports have found better correlations between *SOX2* and the CSC phenotype¹¹⁹. It has been reported that cancer stem cell factors fluctuate over time¹²¹, which is why we conducted the additional survival analyses with the collective non-expression of SCMs.

Comparing molecular features in HGSC and SEC

Copy number profiles

The typical histological appearances of the HGSC and SEC tumor entities are very similar (Figure 7). Examining frequency plots of amplified and deleted regions, we also found many overlapping regions in the copy number profile of these two tumor entities (Figure 17). This has also been previously reported in the TCGA project¹⁴⁸ (Figure 7).

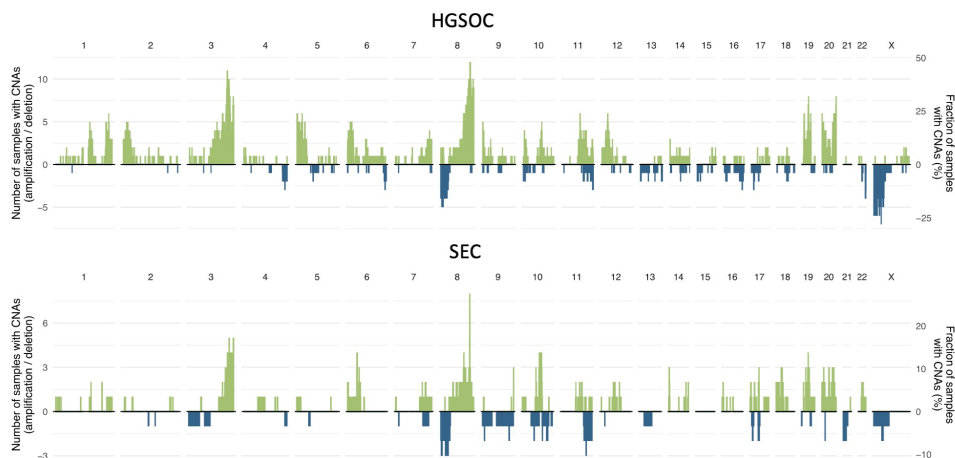


Figure 17. Frequency of copy number alterations (CNAs) in HGSC and SEC genomes. The count of tumors with amplifications or deletions, left side, and fraction with CNAs out of all tumors, right side. HGSOC (n=25), SEC (n=19).

Examining the frequency of amplifications, it was evident in both HGSC and SEC that *SOX2* amplification, located at the end of chromosome 3, was very common. However, *SOX2* was not the only amplified gene in this amplification peak, but the wide region of 3q26-29 and often even the whole q-arm of chromosome 3, was amplified (Figure 17). This region comprises several potential cancer driver genes, such as *PIK3CA*, an integral part of the PIK3-pathway and frequently mutated in cancer, *MECOM*, a transcription factor, *PRKCI*, a protein kinase, and *ECT2*, a guanine nucleotide exchange factor which in its mutated form is associated with breast cancer²⁰³. These are all potential reasons for the common amplification in this region, and perhaps the fact that so many potentially cancer-promoting genes are gathered here makes the region more prone to amplification. Some studies, however, argue that that *SOX2* is a likely driver of this large region amplification^{113,204}.

HRD scores

HRD and HR genes

The occurrence of HR deficiencies has been investigated repeatedly in HGSC^{55,205,206}, while reports on HR deficiencies in SEC are more rare^{154,156}. In studies II and III we investigated copy number data and HRD scores of 19 SEC and 25 HGSC. The scores in HGSC were high, as previously reported²⁰⁵. Also in SEC, we found surprisingly high HRD scores (Figure 18). It has been reported that the occurrence of *BRCA1/2* mutations are connected to higher HRD scores^{206,207}, but in our HGSC cohort we also found several *BRCA* mutations tumors in the lower range of scores. It should be emphasized that the patients included in this HGSC cohort are not representative of HGSC patients in general, but are selected for having suffered at least one relapse, and still having platinum-sensitive disease. This could explain the lack of a strong correlation between *BRCA* mutation and HRD score in our cohort.

The fact that HRD scores are high in both the HGSC and SEC cohorts is interesting and warrants further investigation into the HR system in SEC, as well as clinical studies on PARP inhibitors in this tumor entity. The HRD scores in HGSC are, however, notably higher than HRD scores in SEC. One reason for this could be the skewed HGSC cohort but there might be other explanations. SEC is usually detected in early-stage disease, thanks to the distinct and common symptom of postmenopausal bleeding, unlike HGSC which is typically diagnosed in advanced-stage disease, with more diffuse symptoms. For this reason, it is challenging to collect a cohort of late-stage SEC for HRD analysis, and it is possible that late-stage tumors would have scores more similar to the scores found in HGSC, which would further emphasize the similarity between these tumor types. It has, however, been reported that HRD scores do not change

RESULTS AND DISCUSSION

significantly over the course of the disease⁵⁵, and the HRD scores in SEC might actually be generally lower than HRD scores in HGSC.

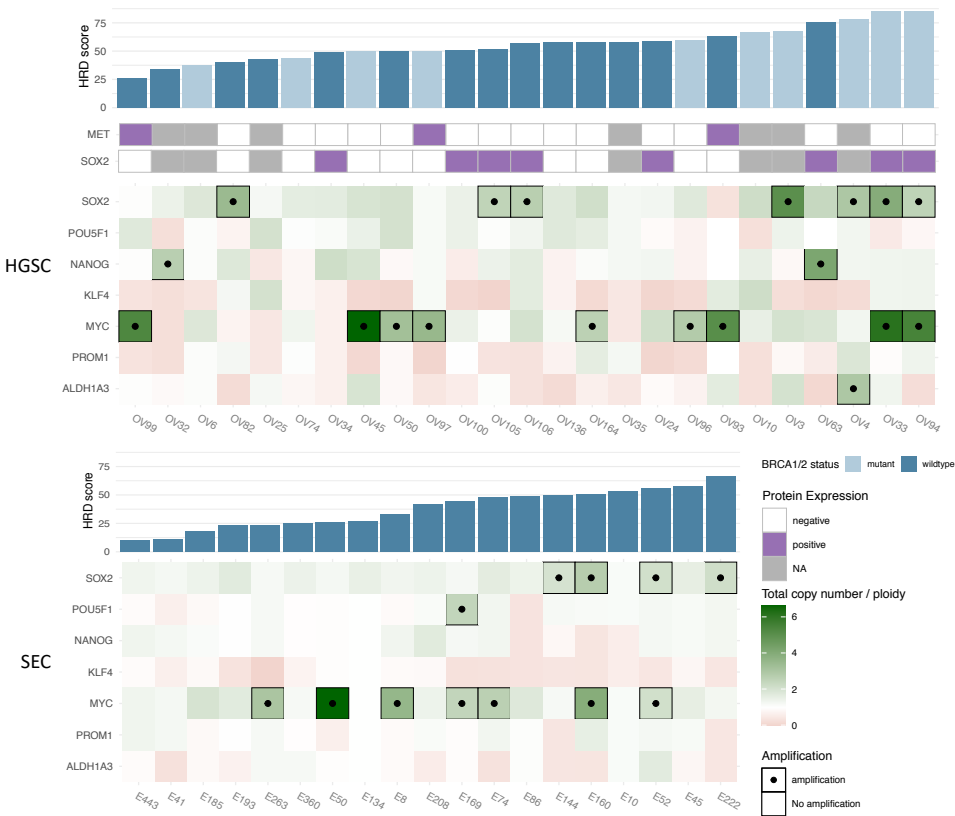


Figure 18. Relative copy numbers of stem cell factors in HGSC and SEC. Also included are HRD scores, BRCA mutations, and protein expressions of SOX2 and MET.

In study II, we reported that genetic loss and LOH in *BRCA1* and *RAD51C*, as well as genetic loss in *BRCA2*, were all associated with a higher HRD score in SEC. This correlation was not seen for the same genes in HGSC. Loss and LOH in *BRCA* genes and *RAD51C* were seen in most samples but was not connected to the HRD score. Considering the differences between tumor entities, in that *BRCA* mutations are less frequent¹⁴⁸ and the general mutational load is lower in SEC than HGSC¹⁵⁶, CNAs in important HR genes might be a more important factor in malfunctioning HR in SEC than in HGSC.

HRD and stem cell factors

Patient samples

Analyzing the relative copy numbers of stem cell factors in studies II and III (defined as the number of estimated copies of the genes normalized to the estimated ploidy of the sample), we found that the number of *SOX2* gene copies correlated with HRD scores in both HGSC and SEC (Figure 18). As described in an earlier section (*Comparing copy number and mRNA expression levels*), copy numbers for *SOX2* did not seem to correlate with *SOX2* mRNA expression in the TCGA cohort. When comparing the 18 patients overlapping the two HGSC cohorts (TMA cohort and CNA cohort), *SOX2* protein expression and relative copy number appear to be connected; however, not to a significant degree. These results indicate that *SOX2* protein expression is not dependent on *SOX2* amplification. However, in all but one of the five tumors with *SOX2* amplification, *SOX2* protein expression was also found. This is consistent with a study in lung squamous cell carcinoma where a higher level of *SOX2* protein expression was found in tumors with *SOX2* amplification than those lacking amplification¹¹³. Regarding the lack of correlation between copy numbers and mRNA expression in the TCGA data, I think it is important to keep in mind that mRNA expression in bulk tumor is a much less sensitive analysis than IHC in a TMA, where even sporadic positive cells can be counted. As *SOX2* is used as a marker for a generally small fraction of CSC in the tumor, analyzing bulk mRNA data is likely not as robust as analyzing IHC protein expression data.

In vitro

To further investigate the connection between HR function, MET and stem cell factors, we retrieved mRNA and copy number data for the pair cell lines PEO1 and PEO4 from the CCLE database²⁰⁸. PEO1 is a cancer cell line taken from a woman with platinum-sensitive HGSC and germline *BRCA2* mutation, and PEO4 is collected from the same woman once platinum-resistant recurrence occurred, and this cell line harbors a reversal mutation restoring *BRCA2* function. We evaluated these cell lines for platinum (carboplatin), PARP inhibitor (olaparib), and MET inhibitor (crizotinib) sensitivity (see appendix Study III) and found that while PEO4 was more resistant to carboplatin, as expected, it was more sensitive to the PARP inhibitor treatment than to its *BRCA2*-proficient counterpart PEO1. Examining the mRNA expression of 40 HR-associated genes (see appendix Study III), we discovered that PEO1 expressed higher levels of these genes than PEO4, which is exemplified by the *RAD51* expression in Figure 19A. Expression levels of the crizotinib targets *MET*, *ROS1* and *ALK* were comparable between the cell lines. This is in line with the shared level of sensitivity to crizotinib we also found. Levels of established stem cell markers were higher in PEO1 cells, indicating a connection between CSCs and DNA repair¹²⁷.

RESULTS AND DISCUSSION

We also investigated another paired cell line model, UWB1.289, which harbors a *BRCA1* mutation, and its *BRCA1* proficient counterpart UWB1.289+BRCA1. In the latter cell line, a vector containing a functional *BRCA1* gene was transfected into UWB1.289 cells. No data was available for the UWB1.289+BRCA1 line so to compare these two lines, we performed western blot analyses (Figure 19B). The expression of MET was comparable between cell lines, in line with the similar response to the crizotinib treatment that we found (see appendix Study III). The cell lines did have distinctly different responses to carboplatin and olaparib, with the *BRCA1* sufficient line displaying resistance to both. RAD51 expression was, however, comparable. This could indicate that the HR still functions to some extent even without the BRCA1 protein. SOX2 expression was considerably higher in the *BRCA1* proficient line, again, indicating that CSCs could have a connection to the HR function.

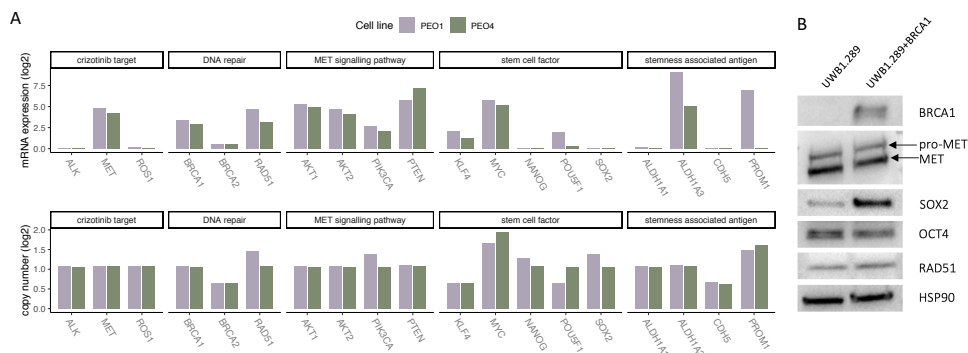


Figure 19. Comparing mRNA/protein expression and copy number levels within cell line pairs. mRNA expression and copy numbers for selected genes in the PEO1 and PEO4 cell lines (A), and protein expression levels for UWB1.289 and UWB1.289+BRCA1 cells (B). mRNA and copy number data were retrieved from DepMap (DepMap, Broad (2020): DepMap 21Q1 Public)²⁰⁸.

Chemotherapy and stem cell factors

The role of SOX2 in DNA damaging drug sensitivity

In study I, we used siRNA knockdown of *SOX2* in two *SOX2* expressing HGSC cell lines to determine if a lack of this protein would sensitize the cells to treatment. Results showed that the expression of *SOX2* did not affect the cell's sensitivity to carboplatin or paclitaxel (Figure 20). This could be explained by a compensating effect of other stem cell factors, or by that the treatment response is not dependent on *SOX2* expression. Wen et al. reported an increased platinum sensitivity in spheres developed from ovarian cancer cell lines with stable sh*SOX2* knockdown as compared to control, and a decreased sensitivity in cells overexpressing *SOX2*²⁰⁹. This contradicts our results;

RESULTS AND DISCUSSION

however, I would assume that spheres depend more on stem cell factor expression than monolayer cells and therefore are more affected by the gene silencing. Robinson et al.¹¹⁹ also report a decreased sensitivity to carboplatin following *SOX2* knockdown in HGSC cell lines, however, only in two out of the three lines tested. The clinical data from the HGSC cohort used in study I showed no difference in treatment response between patients with high *SOX2* protein expression (>50% positive cells) and *SOX2* negative tumors (0% positive cells). Complete response was reached in 70% and 74% of cases, respectively (see appendix, Study I). This result supports the notion that the *SOX2* expression in itself does not provide resistance to chemotherapy, which is in line with Bareiss et al.¹¹⁸ who found that, while *SOX2* overexpression could induce a CSC phenotype, it did not affect proliferation.

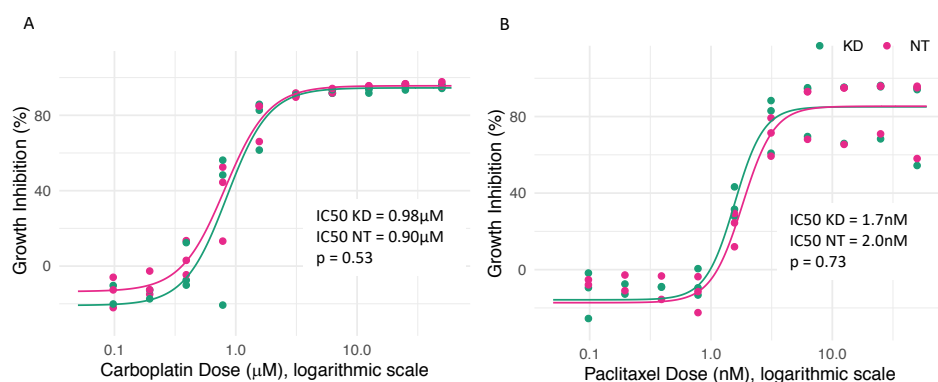


Figure 20. The effect of *SOX2* gene silencing on sensitivity to chemotherapy in OVCAR3, a HGSC cell line. Sensitivity to carboplatin (A) and paclitaxel (B).

Treatment effect on stem cell factor levels

In study I, we also treated the two HGSC cell lines with carboplatin and paclitaxel to see how mRNA and protein levels of *SOX2* were affected. Carboplatin caused a time and dose-dependent decrease in expression in both lines, while paclitaxel treatment resulted in a decrease in *SOX2* expression in one of the lines. A previous study has reported enrichment of stem cell factors following chemotherapy⁹⁹, which is what we also expected in this experiment. It is, however, important to distinguish between the direct, short-term drug effect on cells, as tested by us, and levels of *SOX2* in cells treated and then regrown without the treatment pressure, as was the case in the work of Steg et al.⁹⁹. Robinson et al.¹¹⁹ also evaluated the expression of stem cell factors following carboplatin treatment, but used a sequential treatment regimen. This resulted in a marked increase

in SOX2 expression and more modest increases of Nanog and OCT4. I would suggest that the downregulation we observed could be a temporary effect of the treatment, and SOX2 might rapidly increase again once treatment is ended, possibly to levels exceeding baseline levels, as indicated in Steg et al.⁹⁹ and Robinson et al.¹¹⁹.

Investigating stem cell factors in vitro

It is worth considering if *in vitro* models are suitable for examining the stem cell factor's effect on drug response. The cell lines used for the experiments, OVCAR3 and COV362, both had a naturally high expression of SOX2, and it could be risky trying to compare immortalized cells with such a large fraction of SOX2 expressing cells to tumors which, in most cases, express SOX2 in a very small fraction of their cells.

The CSCs in tumors are thought to be slow-growing, rendering them less sensitive to DNA damaging agents¹⁰⁶. Treatment of tumors containing stem cells would therefore lead to enrichment of these cells⁹⁹, which could lead to relapse. The function of CSCs in tumors might be difficult to mimic in immortalized cell lines, especially if they are growing in monolayer. Performing experiments using cell lines with a lower natural expression of CSC markers, cultured on ultra-low attachment plates or in hydrogels, would meet some of these concerns and would likely be a better model for examining stem cell factors than monolayer models with extreme SOX2 expression.

Perspectives on treatment

Targeting cancer stem cells

The expression of stem cell markers in CSCs appears to be fluctuating^{121,192}, and targeting specific CSC proteins is likely not an effective strategy to eliminate these cells. As SOX2 and CSCs seem to have a role in angiogenesis, as they express high levels of VEGF^{98,210,211}, targeting this system might have a good effect on CSC. Bevacizumab is an effective drug targeting VEGF-A, which is currently used in advanced stage HGSC²¹². Efforts on targeting the Notch, Wnt/ β -catenin, or Hedgehog pathways, have also been evaluated, but so far has not yielded any clear, promising results⁹⁸. Cytokines could also potentially serve as targets for treatment. The drug Reparixin, an inhibitor of the IL-8 receptor, has been evaluated in breast cancer with promising results on stem cell markers^{213,214}.

Targeting MET

Several drugs that inhibit the activity of MET are currently used in other cancer forms. Most target not only MET but also a broader range of receptors. The drug crizotinib, for example, also targets the RTKs ALK and ROS1²¹⁵. Crizotinib is currently used in non-small cell lung cancer cases where ALK or ROS1 is expressed. Cabozantinib, which is currently indicated in renal cell carcinoma, hepatocellular carcinoma, and thyroid cancer, has been reported to target also VEGF, AXL, RET, ROS1, TYRO3, MER, KIT, TRKB, FLT3, and TIE-2²¹⁶.

The effect of MET inhibitors could be dependent on co-administration with other drugs. Our group has previously reported that crizotinib and olaparib synergize in multiple HGSC cell lines and patients samples, and increase cell death compared to single drug treatment²¹⁷. As studies have shown that MET can phosphorylate PARP, making it less accessible to PARP inhibitors²¹⁸, it is thought that inhibition of MET could increase the effect of PARP inhibitors in treatment-resistant tumors, and this has also been shown in cell lines^{217,219,220}. Treating the non-cancer cell line MCF10A with crizotinib revealed drug sensitivity similar to what was found in the HGSC lines, showing that the inhibitor effect is not cancer cell specific²¹⁷. The synergistic effect with PARP inhibitors was, however, only seen in the HGSC cells. Another MET inhibitor, MK8033, has been reported to act synergistically with platinum and taxane²²¹.

Cabozantinib has recently been evaluated in clinical phase II trials including ovarian cancers but with varying results^{222–224}. Sunitinib is a broad-range RTK inhibitor which has also gone through clinical trials (Baumann et al., 2012). No MET inhibitor is, however, currently recommended in HGSC²⁰¹. The somewhat unclear results of these trials could be due to the sample group tested and it is possible that subgrouping could reveal which patients would benefit most from these treatments.

Conclusions and Future Perspectives

Pelvic high-grade serous carcinoma

HGSC is an aggressive disease, with a high risk of relapse even when patients initially have responded well to treatment, and a subpopulation of CSCs could potentially be the key player in the regrowth of aggressive tumors following treatment. Based on the fraction of SOX2 positive cells we observed, expression of this stem cell marker is, for the most part, restricted to a subpopulation of cancer stem cells. This finding is consistent with the theory of cancer stem cells and indicates that SOX2 could be a specific marker for stemness. We found a prognostic potential of SOX2 expression, however, using a wider panel of stem cell markers would likely provide a better picture of the stem cell network, and would be a better prognostic tool. Regarding clinical studies, I think it is important that long-term effects, such as overall survival, are the focus and not only short-term effects, such as response rate and time to recurrence.

Several studies have reported that CSCs could be connected to angiogenesis, and it might benefit clinical studies to take CSCs into account when evaluating VEGF inhibitors. It is plausible that tumors expressing stem cell markers could have more long-term benefits from these drugs. As the VEGF inhibitor Bevacizumab is already in clinical use it would be interesting to investigate if CSC-enriched tumors benefit more from this drug compared to tumors with low fractions of CSCs. Also, MET has been reported to be connected to angiogenesis, most likely through hypoxia-induced overexpression, and the use of MET inhibitors could be a complement or alternative to VEGF inhibitors.

MET inhibitors could also be a way of targeting the effect of CSCs. In our data, the significance of MET expression on survival was most evident in the subgroup of tumors lacking CSC marker expression, indicating that tumors harboring CSC were less dependent on MET signaling. A clear interaction was detected between the MET and SOX2 proteins, and this warrants further studies into the underlying mechanism. I believe that markers of CSC expression should be investigated in clinical trials evaluating MET inhibitors, as HGSC patients with SOX2-positive tumors could be more responsive to treatment than patients with SOX2-negative tumors.

As CSCs appear to have the ability to evade cell death from DNA damaging treatments, PARP inhibitors are not likely to target these cells. It is however plausible that long-term use of PARP could suppress the tumor bulk and thereby prolong the time to relapse. This would probably also work in CSC-containing tumors, however, there is a risk that the fraction of CSCs will increase after treatment. Our data suggest that SOX2 levels initially decrease with treatment, but we suspect that SOX2 levels increase again once treatment is concluded. I believe that PARP inhibitors are effective in prolonging survival, but they might not kill all cancer cells, and the expression of CSC markers should be taken into consideration when evaluating the benefit of PARP inhibitors as well as other treatments.

Serous endometrial carcinoma

Several studies, including study II in this thesis, have shown that the SEC phenotype highly resembles the HGSC. As SEC is rare, it is difficult to collect cohorts large enough for proper studies and so to apply the results of HGSC studies on SEC patients is tempting. PARP inhibitors have been proven effective in HGSC and there are many indications that these would be beneficial in SEC as well, a conclusion that is supported by the results presented in study II. Given the significance of CSC and SOX2 in several cancers, including HGSC as presented in Study I, it would be interesting to evaluate CSC markers in SEC as well. As we saw that *SOX2* was commonly amplified in these tumors, we would likely also see SOX2 protein expression in a SEC TMA. Evaluating MET expression would also be interesting given the interaction effect found between MET and CSCs in regard to survival in study III. The *MET* gene is overexpressed or amplified in several cancer types, however, in HGSC the expression is weak and rather uncommon. Amplification is rare both in HGSC and in SEC. For this reason, I would not expect to find a large group of MET-expressing tumors in SEC, yet, evaluating its relevance in prognosis would be interesting. If clinical trials evaluating MET inhibitors find subgroups of HGSC that respond better than others, then results could potentially be translated to the treatment of SEC patients as well.

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