Predictive and prognostic factors of epithelial ovarian cancer and pseudomyxoma peritonei

KATHRINE BJERSAND
Abstract


The overall aim of my thesis was to investigate potential prognostic and predictive factors associated with the tumor cells of epithelial ovarian cancer (EOC) and the gastrointestinal tumor pseudomyxoma peritonei (PMP) to improve and individualize cancer therapy. Both PMP and EOC can develop into peritoneal carcinomatosis (PC), which is characterized by widespread metastasis of cancer tumors in the peritoneal cavity. Major improvements in the management of PC, such as cytoreductive surgery in combination with chemotherapy, have dramatically changed the prognosis.

To further optimize and tailor treatment, increased knowledge on tumor biology and pathogenesis is needed. Today's choice of treatment is mainly based on clinical trials and standard protocols that have not taken individual differences in drug sensitivity into consideration. With ex vivo testing of tumor drug sensitivity, individuals at risk of side effects only (and no treatment benefit) could potentially be identified prior to treatment.

Napsin A is an anti-apoptotic protein that promotes platinum resistance by degradation of the cell cycle regulator and tumor suppressor TP53. Immunohistochemical stainings of 131 early EOC tumors in study I showed that expression of Napsin A was associated with expression of the apoptosis regulators p21 and p53 and with histological subtype. Positivity of Napsin A in an epithelial ovarian tumor strengthens the morphological diagnosis of clear cell carcinoma and should be useful in diagnostics. In study II, the relevance of the proteins HRNPM and SLC1A5 as prognostic factors for recurrent disease, survival and impact on clinical or pathological features was evaluated in 123 patients with early EOC. Our results support concomitant positivity of HRNPM and PUMA/p21 in ovarian cancer and indicate that HRNPM may trigger activity in systems of cell cycle regulation and apoptosis. In subgroup analyses of tumors from patients with non-serous EOC histology, expression of SLC1A5 was shown to be a prognostic factor in terms of prolonged disease-free survival. In studies III and VI, we investigated the ex vivo drug sensitivity of tumor cells from EOC and PMP with the 72-h cell viability assay fluorometric microculture cytotoxicity assay (FMCA). The two studies confirm that drug sensitivity varies considerably between tumor samples from patients within the same diagnostic group. In ovarian cancer, ex vivo results show that type I tumors were generally less sensitive to cytotoxic agents than type II tumors. Samples from patients previously exposed to cytotoxic drugs generally tended to be more resistant to most drugs than samples from unexposed patients in both EOC and PMP. This observation is in line with clinical experience and findings supporting that exposure to cytotoxic treatments contribute to development of chemo-resistance mechanisms. In ovarian cancer, resistance to the kinase inhibitors after exposure varied but was less pronounced than that for standard cytotoxic drugs. In PMP patients, ex vivo drug sensitivity provided prognostic information for progression-free survival, and this is in line with earlier findings.

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In every walk with nature one receives far more than he seeks.

John Muir
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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## Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ASA</td>
<td>American Society of Anesthesiologists</td>
</tr>
<tr>
<td>ASCT2</td>
<td>Alanine, serine, cysteine-preferring transporter 2, also called SLC1A2</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>BRAF</td>
<td>V-RAF Murine sarcoma viral oncogene homolog B-1</td>
</tr>
<tr>
<td>BRCA</td>
<td>Breast cancer gene</td>
</tr>
<tr>
<td>Ca-125</td>
<td>Cancer antigen 125</td>
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<tr>
<td>CC</td>
<td>Completeness of cytoreduction</td>
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<tr>
<td>CCC</td>
<td>Clear cell carcinomas</td>
</tr>
<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>CRS</td>
<td>Cytoreductive surgery</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td>DPAM</td>
<td>Disseminated peritoneal adenomucinosis</td>
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<tr>
<td>EOC</td>
<td>Epithelial ovarian cancer</td>
</tr>
<tr>
<td>EORTC</td>
<td>European Organisation for Research and Treatment of Cancer</td>
</tr>
<tr>
<td>EDR</td>
<td>Extreme drug resistance</td>
</tr>
<tr>
<td>FDA</td>
<td>Fluorescein diacetate</td>
</tr>
<tr>
<td>FIGO</td>
<td>International Federation of Gynecology and Obstetrics</td>
</tr>
<tr>
<td>FMCA</td>
<td>Fluorometric microculture cytotoxicity assay</td>
</tr>
<tr>
<td>G</td>
<td>Grade</td>
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</table>
IDR     Intermediate drug resistance
HIPEC   Hyperthermic intraperitoneal chemotherapy
HRNPM   Heterogeneous nuclear ribonucleoprotein M also called HnRNP M
IHC     Immunohistochemistry
IP      Intraperitoneal
IPC     Intraperitoneal chemotherapy
KRAS    Kirsten murine sarcoma virus 2
LDR     Low drug resistance
OC      Ovarian cancer
OS      Overall survival
PARP    Poly ADP ribose polymerase
PC      Peritoneal carcinomatosis
PCI     Peritoneal cancer index
PD-1    Programmed cell death protein 1
PD-L1   Programmed death-ligand 1
PI3K    Phosphatidylinositol 3-kinase
PMCA    Peritoneal mucinous carcinomatosis
PMP     Pseudomyxoma peritonei
PFS     Progression-free survival
PTEN    Phosphatase and tensin homolog
PUMA    TP53 upregulated modulator of apoptosis
ROC     Receiver operating characteristic
SLC1A5  Solute carrier 1A5 also called ASCT2
STIC    Serous tubal intraepithelial carcinoma
TP53    Tumor suppressor 53
VEGF-R2 Vascular endothelial growth factor receptor 2
WHO     World Health Organization
Introduction

Epithelial ovarian cancer

Ovarian cancer is the most lethal of the gynecological malignancies, with 150,917 deaths globally in 2012. The disease is most common in Northern Europe, with incidences of approximately 15–20/100,000. By comparison, the incidence in some parts of Africa is around 2/100,000 [1]. In Sweden, 625 women were diagnosed with ovarian cancer in 2011, corresponding to an incidence of 13.2/100,000. During the same year, 563 women died from the disease. Woman in all ages can be affected, but ovarian cancer is uncommon before the age of 30 [2]. Ovarian cancer is often diagnosed in advanced stages (60%), and the disease presents with diffuse symptoms such as constipation and increase in abdominal girth. The most common form of ovarian cancer is epithelial ovarian cancer (EOC).

Multiple pregnancies, breastfeeding and contraceptive pills are considered preventive factors of disease, whereas incessant ovulation is considered to elevate the risk [3]. Observations suggest that repeated stimulation of the epithelium of the ovarian surface, which occurs as a result of ovulations, predisposes the epithelium to malignant transformation. More recently, salpingectomy and sterilization have also proved to be protective factors for EOC, and the high prevalence of tubal carcinoma or precursors in tissue prophylactically resected from high-risk patients suggests that the fimbria might be the site of origin of most high-grade serous tumors [4, 5]. The findings of identical TP53 mutations in serous tubal intraepithelial carcinoma (STIC) and in concomitant ovarian carcinoma indicate a clonal relationship between them and argue for a tubal origin of epithelial ovarian cancer [6].

A family history of ovarian cancer confers an increased risk of disease, and epidemiological studies suggest a relative risk of approximately 5% for woman with a first-degree relative diagnosed with ovarian cancer before the age of 55. In women with two first-degree relatives, the lifetime risk increases to 7.2% [7]. At least 10% of all EOC is hereditary, and approximately 90% of the cases can be explained by mutations in BRCA 1 and 2 [8]. The origin and pathogenesis of ovarian cancer has long been poorly understood. It is now clear that EOC is not a single disease but a heterogeneous group of tumors that can be classified based on histological and genetic
properties. Kurman and colleagues suggested a dualistic model in which EOC was grouped into two broad categories of tumors, type I and type II tumors, based on the two main pathways of tumorigenesis [9], Table 1. This model has been shown to be useful in understanding the biology of EOC, but in the clinical setting, classification of ovarian tumors is still being done according to the WHO classification of female reproductive organs from 2014 [10].

Type I tumors consist of low-grade serous (G1), low-grade endometroid (G1+G2), mucinous and clear cell carcinomas, and often present at an early stage. Type I tumors are associated with corresponding benign ovarian cystic neoplasms, often through an intermediate (borderline) step. Borderline and type I tumors share histopathological features and genetic mutations. Type I tumors have distinct morphological and genetic features. Kirsten murine sarcoma virus 2 (KRAS) and V-RAF murine sarcoma viral oncogene homolog B-1 (BRAF) mutations are often present, whereas tumor protein (TP) 53 mutations are rare in type I tumors [11, 12].

Type II tumors include high-grade serous (G2+G3), high-grade endometroid (G3) and carcinosarcoma. Morphologic differences within type II tumors are sometimes subtle. The tumors are genetically unstable; high-grade serous tumors, which are the most common of type II tumors, are characterized by TP53 mutations in more than 80% of the cases. Type II tumors are highly aggressive, almost always present in advanced stages, and account for 75% of EOC and the majority of EOC mortality [6, 9].

Table 1. EOC classification.

<table>
<thead>
<tr>
<th>Epithelial ovarian cancer</th>
<th>WHO classification (FIGO grading)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td>Low grade serous (G1)</td>
<td>BRAF, KRAS, NRAS</td>
</tr>
<tr>
<td></td>
<td>Endometroid (G1, G2)</td>
<td>PI3K, PTEN</td>
</tr>
<tr>
<td></td>
<td>Mucinous</td>
<td>KRAS</td>
</tr>
<tr>
<td></td>
<td>Clear cell</td>
<td>PI3K, PTEN</td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td>High grade serous (G2+G3)</td>
<td>TP53, BRCA1, 2</td>
</tr>
<tr>
<td></td>
<td>Endometroid (G3)</td>
<td>PI3K, PTEN</td>
</tr>
<tr>
<td></td>
<td>Carcinosarcoma</td>
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Hallmarks and tumor biology of ovarian cancer

DNA is constantly being damaged due to errors in replication and external factors, and this may cause mutations. Left unrepaired, mutations may result in unstable chromosomes, affect cell signaling and lead to cancer development. Genes that code for proliferative signaling or prevent apoptosis are termed oncogenes and may be constantly turned on due to point mutations, chromosome translocation, or by extra copies of DNA (gene amplification). Genes that code for the control of normal and abnormal growth are termed tumor suppressor genes.

In “Hallmarks of cancer” Hanahan and Weinberg review biological principles in the development of cancer, and these principles will together with Banerjees “New strategies in the treatment of ovarian cancer” be used to illustrate aspects of ovarian cancer tumor biology and potential targets for treatment (Figure 1) [13, 14].

![Figure 1](image.png)

*Figure 1.* Free after Hanahan and Weinberg “Hallmarks of cancer”. Enabling tumor characteristic in red, hallmarks of cancer presented in the green wheel, cancer mutations in blue, targeted treatment green text, and the aims of this thesis in circles.
Genomic instability and mutations enables tumor development
Cells of mutant genotypes are selected for growth advantage and subjected to further stepwise alterations, which can lead to tumor development. The meticulous system for the detection of defects and repair of DNA makes spontaneous mutations rare during each cell generation, and to orchestrate tumor development, several mutations are needed. Once initiated, the mutational accumulation is accelerated through enhanced sensitivity to mutagenic agents, through a breakdown of parts of the mutagenic repair system, or both [13].

Ovarian cancers in general and high-grade serous tumors in particular are considered sensitive to treatment with chemotherapy. High-grade serous tumors are genetically instable tumors, and traditional cytotoxic drugs often strike on pathways of DNA repair to kill cancer cells. Defects in the DNA repair systems foster tumor development but may also be used in anticancer treatment. Platinum-based drugs bind to DNA and are frequently used in EOC. Platinum-DNA complexes are recognized as DNA damage and trigger apoptosis [15]. In ovarian cancer treatment, it is also possible to take advantage of a specific DNA repair system (homologous recombination) that is defective in the hereditary forms of EOC. BRCA1 and 2 are tumor suppressor genes coding for proteins involved in homologous recombination and repair of DNA breaks. Individuals with the BRCA mutation have a (germinal or somatic) heterozygous mutation in the BRCA gene. As each cell contains two copies of a gene, additional events leading to harm of the second copy, loss of heterozygosity (LOH), need to take place in the tumor cell. Cells with a defect BRCA gene will have difficulties with DNA repair and need to use alternative pathways. Yet another protein involved in DNA repair is poly ADP ribose polymerase (PARP), and PARP pathways are important in cells lacking normal BRCA function [16]. PARP inhibitors block PARP function, and, in combination with BRCA mutation, this leads to selective cell death from irreversible DNA damage [16].

Sustaining proliferative signaling
Normal cells carefully control the progress of the cell through the cell cycle to maintain normal structure and function of the tissue. In the process toward a neoplastic state, cancer cells can stepwise deregulate this signal system and become masters of their own development, with the ability to sustain chronic proliferation. Signals of proliferation are typically mediated by growth factors that bind to cell surface receptors containing intracellular tyrosine kinases. These tyrosine kinases activate intracellular signal cascades for growth as well as progression through the cell cycle. Cancer cells can enhance growth factor signaling through production of growth factor ligands themselves, or by sending signals to surrounding normal cells to do so. Other options are to
elevate the levels of growth factor receptors on the cell surface or to activate the intracellular signaling system downstream of the growth factor receptors [13]. Somatic mutations in the gene encoding the BRAF protein in low-grade serous cancer and phosphoinositide 3-kinase (PI3-kinase) in endometroid ovarian cancer are both examples of downstream activation of systems usually triggered by growth factors [14, 17]. The cell has various systems to check and modulate proliferative hyperactivation, and mutations in this “negative feedback system” may lead to enhanced proliferative signaling. Neuroblastoma RAS viral oncogene (NRAS) and KRAS mutations in low-grade serous and mucinous ovarian cancer and tumor suppressor phosphatase and tensin homolog (PTEN) in clear cell cancers all lead to changes in intracellular negative signaling and sustained proliferation [17]. Tyrosine kinase inhibitors (TKI), like vemurafenib, sorafenib and nintendanib, seem promising in the treatment of mutation carriers, but surprisingly, responders often lack typical mutations, high-lightening the need for additional methods for patient selection [18, 19].

**Evading growth suppressors**

In addition to speeding up proliferation, cancer cells must circumvent programs that efficiently suppress growth; many of these programs depend on tumor suppressor genes. Among the most explored tumor suppressors is the TP53 gene, also known as p53. The TP53 protein detects signs of damage to the genome, enhanced proliferative signals, or altered metabolism. Thus, an activated TP53 system may stop further growth and division and thereby lead to cell senescence. Progression through the cell cycle may again be permitted if conditions are normalized, but if conditions remain abnormal, the TP53 will induce programmed cell death, apoptosis [13, 14].

**Resisting cell death**

Cell cycle control mediated by tumor suppressors like TP53 is a central process for prevention of cancer as it induces cell cycle arrest and apoptosis in damaged tissue [20]. Apoptosis may be triggered in response to various stresses like signaling imbalance, DNA damage, or anticancer therapy. The apoptosis may be mediated by extracellular (extrinsic/ death receptor) and intracellular (intrinsic) pathways. When DNA damage triggers intrinsic apoptosis, signals are captured by TP53, leading to elevated pro-apoptotic signals and cell death [13]. Tumor cells develop strategies to avoid this, one of the most common being the loss of the TP53 tumor suppressor gene. As mentioned, high-grade serous ovarian cancer is characterized by TP53 gene abnormalities in more than 80% of the cases [21]. One example of a TP53-regulating protein is Napsin A, an anti-apoptotic protein found to promote resistance to cisplatin by degradation of TP53 [22]. We have investigated Napsin A as a marker for CCC and its relation to TP53 in this thesis. Napsin A is located on chromosome 19q and our group recently showed that loss of
heterozygosity on chromosome 19q in early stage serous ovarian cancer is associated with increased risk of recurrence [23]. HRNPM and SLC1A5 are proteins expressed in EOC [24], and encoded by this region, and were therefore chosen as candidates for further research in this thesis.

Autophagy is a program that enables cells to break down cellular components like mitochondria and liposomes so that they can be recycled and used for biosynthesis and energy metabolism. Autophagy is taking place to some extent under normal circumstances but can be up-regulated in states of cellular stress. Phosphatidylinositol 3-kinase (PI3K) is stimulated by survival signals to block autophagy as well as apoptosis. Activation of the PI3K pathway occurs in approximately 30% of clear cell and endometroid tumors and in 5% of high-grade serous ovarian cancer [14].

**Enabling replicative immortality**

Most cells in the body are capable of only a limited number of cell-growth and division cycles. In cell culture, the regulation can be observed and involves first senescence, an irreversible entrance to viable but non-replicative state, and then crisis, i.e., cell death [13]. On rare occasions, cells emerge from crisis and go into a state of unlimited replications, so called immortalization. Telomere shortening is a central regulator of this process, because telomeres are protecting the ends of chromosomes. They are shortened successively every cell cycle, and when largely eroded, they can no longer protect the cell from crisis. Cancer cells express elevated levels of telomerase [13], which adds length to the telomeres and contributes to resistance to senescence and crisis/apoptosis.

**Inducing angiogenesis**

All tissues require oxygen and nutrients and must evacuate metabolites to survive. To be able to meet the increasing metabolism in the growing tumor, an induction of new blood vessel growth (angiogenesis) takes place early during tumor progression [13]. Angiogenesis is strictly regulated by factors that either enhance or suppress the sprouting of new vessels, and these factors can originate from the tumor cells themselves, stroma cells in the microenvironment, or inflammatory cells. One of the most well-known and potent inducer of angiogenesis is the vascular endothelial growth factor-A (VEGF-A). VEGF signals via receptor tyrosine kinases (VEGFR 1-2) and can be up-regulated via hypoxia or oncogene signaling [25]. Many genetic alterations associated with malignant transformation, involving TP53 and RAS, are associated with increased VEGF expression [26, 27]. New drugs such as the VEGF pathway inhibitor bevacizumab have been shown to prolong progression-free survival in ovarian cancer patients and are used in selected patients [13, 14, 27-29].
Activating invasion and metastasis
Carcinomas that proceed to a higher degree of malignancy develop alterations in shape and attachment to other cells, leading to invasion, and later on, distant metastases. The invasion and metastatic cascade begins with local invasion, subsequent intravasation of nearby blood and lymphatic vessels, extravasation of cancer cells to distant tissues, and finally the forming of new micro- and macroscopic tumors. The epithelial-mesenchymal transition program (EMT) describes the cellular changes necessary to invade and migrate into neighboring tissues. EMT-inducing transcription factors can drive most of the steps of invasion and metastasis [30]. An important early step is loss of cell-to-cell adhesion molecules, cadherins [13]. Again, signaling can originate from the cancer cell or from interactions with tumor-associated stromal cells and inflammatory cells. The formation of macroscopic tumors from micro-metastases is a complicated process because the tumor cells are likely to be poorly adapted to the microenvironment of the tissue in which they have landed. Further, cancer cells may not only escape to distant tissues, they can even return home, and this may explain progression within primary tumors and heterogenic tumor structure [13].

Cancer cells and the immune system
The presence of inflammatory cells in tumors has long been recognized by pathologists, and historically, this was thought to reflect the immune system’s attempt to destroy the tumor. It is now well known that inflammatory cells can enhance tumor development and progression, but also prevent tumor occurrence and growth [13]. Inflammation can supply the tumor with necessary substances such as growth factors for sustained proliferative signaling and molecules that limit cell death and facilitate angiogenesis and invasion.

The clinical impact of the immune system on tumors has been the subject of intense investigation, and infiltration of various immune cells has been shown to correlate positively or negatively with clinical outcome in ovarian cancer [31]. Recently, drugs modulating the tumor immune response have had great success in certain indications. For instance, PD-1 blocking antibodies have been successful in malignant melanoma [32]. Whole tumor infiltrating lymphocytes (TILs) in ovarian cancers are associated with sensitivity to platinum-based therapy and increase overall survival [31]. TILs express PD-1, i.e., the receptor for PD-L1 ligand that is expressed by tumor and inflammatory cells. PD-L1 acts as a brake on the immune cells and will help the tumor cell to evade the immune system. Nivolumab blocks binding of PD-L1 to PD-1 and thus boosts the immune system in its attack on the tumor. In a phase II study, it was shown that nivolumab had effect in some EOC patients, but the overall response rate was low [33].
Reprogramming energy metabolism
The uncontrolled proliferation in the growing tumor requires energy to maintain the expanding tissue. Normal cells generate energy via glycolysis in the cytosol. Under aerobic conditions, remaining pyruvate is metabolized in mitochondria, whereas under anaerobic conditions, pyruvate is reduced to lactate. Neoplastic cells reprogram their glucose metabolism to mainly glycolysis even in the presence of oxygen, termed “aerobic glycolysis” [13]. This glucose fueling has been associated with the TP53 and RAS mutations that are common in ovarian cancer [13, 14]. The remodeling of energy metabolism makes cancer cells well adapted to hypoxic conditions, and increased glycolysis facilitates proliferation by the release of building blocks. Within a tumor there may be two different subpopulations, one with glucose-dependent cells and one with cells that import and use lactate from their neighbors as their main fuel [34]. This heterogeneity of the neoplasia gives it an advantage compared to normal tissue. When cancer cells elevate their glucose uptake, it can be visualized by positron emission tomography (PET) diagnostics [35]. At present, PET is considered too costly for first-line diagnostics and treatment of ovarian cancer, but it is useful when localizing biochemical and clinical recurrences.

Cancer cells and cancer stem cells
The theoretic “cancer stem cell” (CSC) is a matter of debate [36]. In humans, a cell would be termed a CSC if it on its own can seed tumors in a recipient host mouse. This function is crucial since it gives the cell ability to form new tumors by itself and is thought to cause relapse and metastases in patients with complete remission after first-line treatment [36]. The origin of stem cells in solid tumors is not fully clarified and may differ between malignancies. CSC may rise from normal stem cells or from other tissue-specific cells that assume more stem-like characteristics after mutations [13]. In ovarian cancer, side population cells, expressing surface biomarkers typical for stem-like cells, have been isolated by different groups [37]. These cells are resistant to commonly used chemotherapeutic agents, and treatments that shrink the tumor load fail to kill the cancer stem cell. Treatment targeting specific mutations in CSC is a promising approach for new anticancer treatment.

Treatment of ovarian cancer
Over the past 40 years, the survival of patients with advanced ovarian cancer has improved due to the introduction of more advanced maximal cytoreductive surgery in combination with platinum and paclitaxel-based chemotherapy as standard first-line treatment [38]. Despite all this effort, it is still the
fourth commonest cause of death from cancer in women in the developed world [26].

In the early 1990s, Hoskins and colleagues conducted studies to evaluate the relationship between maximal diameter of residual disease after primary cytoreductive surgery and survival in patients with advanced ovarian cancer. Their results suggested that survival of patients improved as the diameter of the largest residual disease decreased [39]. Since then, it has been concluded that patients with minimal residual disease have better survival than those with a visible tumor load at the end of surgery. Furthermore, the maximal diameter of residual disease was found to be an independent predictor of overall survival [40]. Consequently, the designation of optimal surgical cytoreduction has evolved from residual disease less than 1 cm to no residual disease [40, 41].

Platinum-based therapy has been used as in the treatment of advanced EOC since the early 1980s, and in the 1990s, the combination of carboplatin and paclitaxel became standard treatment [42]. However the current chemotherapy regimens do not consider histopathological subgroups, even though response rates differ; for instance, high-grade serous tumors are generally sensitive to platinum-based first-line treatment, while the response rate in clear cell carcinomas (CCC) is usually low [43-45]. Chemotherapy is used postoperatively in patients with early-stage disease at high risk of relapse. Besides being used postoperatively in advanced stages, selected patients may receive preoperative treatment, followed by surgery after three cycles, and additional chemotherapy after surgery [46]. In advanced stages, addition of the angiogenesis inhibitor bevacizumab has been shown to prolong progression-free survival, and this treatment has now become standard treatment for selected patients [28].

Ovarian cancer is generally chemosensitive at the time of the initial diagnosis, and unlike most other tumors, it frequently displays chemosensitivity to multiple lines of chemotherapy. Although ovarian cancer responds well initially, advanced disease tends to relapse. In 1993, Markman and colleagues noticed that response to second-line treatment depended on time from last given chemotherapy to relapse [47]. Today relapse more than 6 months after treatment is termed platinum-sensitive disease. The standard therapy for patients with relapse of platinum-sensitive ovarian cancer is platinum in combination with paclitaxel, gemcitabine or pegylated liposomal doxorubicin (PLD) [48-50]. The PARP inhibitor olaparib prolongs progression-free survival (PFS) in patients with platinum-sensitive relapse and is used in the treatment of women diagnosed with BRCA mutations [51]. However, after multiple lines of therapy, most patients develop platinum-resistant disease. Whether patients with recurrence can benefit from cytore-
ductive surgery is not clear; several randomized multicenter trials have started, but this far no conclusive evidence has emerged [52].

**Drug resistance**

Resistance to cytotoxic drugs is usually categorized as intrinsic or acquired, although the distinction between these two mechanisms can be difficult. Intrinsic drug resistance is described as the ability of the cancer cell to survive the first anticancer treatment; acquired resistance is the evolution of cancer cells due to exposure to treatment that enables them to survive and grow in the presence of cytotoxic drugs [15, 53]. Intrinsic resistance can be mediated by drug efflux pumps, detoxifying agents, or changes in microenvironment like vascularization. Acquired resistance is developed by stepwise modulation of the expression of genes, often involved in DNA repair or apoptosis. Thus, acquired chemoresistance may in reality be the result of a selection of a few cells with intrinsic drug resistance that escape a given treatment. This selection may affect pathways used by more than one drug, resulting in resistance to drugs that have not yet been introduced or multidrug resistance. Chemo-resistant high-grade serous ovarian cancer overexpresses factors of the epithelial-mesenchymal transition program (EMT) of invasion and metastasis. Subpopulations of cancer stem cells are also identified in these tumor samples, which supports the connection between factors of EMT, cancer stem cells and chemo resistance [53]. Mechanisms of drug resistance in subtypes of EOC are not fully understood.

**Stage at diagnosis and screening**

Despite efficient treatment, the most important prognostic factor for ovarian cancer is the stage at time of diagnosis. The disease is staged surgically according to the International Federation of Gynecology and Obstetrics (FIGO) staging system, originally from 1998 but revised in 2013 [54]. For some women with advanced ovarian cancer, the FIGO staging system is insufficient to describe the extent of the disease prior to surgery and additional systems to quantify the disease have been proposed. Sugarbaker’s Peritoneal Cancer Index (PCI) was originally developed to evaluate the extent and localization of carcinomatosis from gastrointestinal cancer, but is now used in advanced cases of EOC to tailor surgery and exclude patients who will not benefit from extensive treatment due to high morbidity [55, 56].

Since ovarian cancer is often present in advanced stages, major efforts have been made to develop methods for screening or early detection. In a recently published large randomized controlled multicenter trial, more than 200,000 women in UK were randomized to multimodal screening with cancer antigen 125 (Ca-125) interpreted with use of the risk of ovarian cancer algorithm, annual transvaginal ultrasound, or no screening. The results suggested a trend in relative mortality reduction, 15% in the multimodal screening group
and 11% in the ultrasound group, but the results were not significant [57]. However, although Ca-125 alone is not sufficient for screening, it is valuable for patient follow-up and detection of recurrence [58].

Pseudomyxoma peritonei

Pseudomyxoma peritonei (PMP) is a rare disease with an incidence of approximately one per million per year [59]. It is characterized by disseminated mucus and mucinous tumor tissue implants on the peritoneal surfaces, and is thought to originate from a ruptured mucocoele of the appendix [60, 61]. PMP was earlier thought to be more common in women than in men, but later publications suggest that the incidence is similar between the sexes [62]. PMP is an important differential diagnosis in the case of peritoneal and ovarian lesions from ovarian cancer, as it often involves the ovaries as well as the appendix [63]. Clinically, PMP is a slowly progressive disease, which presents with distention of the abdomen, increased abdominal girth often in combination with paradoxical weight loss, symptoms of appendicitis, or newly onset hernia. In women, the most common presentation is ovarian mass [64]. Left untreated and without surgical intervention, the patients will suffer from bowel and bile obstruction, leading to death by cachexia and liver dysfunction.

Histopathological classification according to Ronnet [61] or Bradley is commonly used. In Ronnet’s three-graded classification, PMP consists of disseminated peritoneal adenomucinosis (DPAM), peritoneal mucinous carcinomatosis (PMCA) and an intermediate grade PMCA-I. DPAM is the most common of the three, found in approximately 60% of the cases, and is characterized by abundant proliferative mucinous epithelium with mild atypia and little mitotic activity. Features of PMCA are abundant mucinous epithelium with the histologic characteristics of carcinoma, and 27% of the cases will have PMCA histology. PMCA-I is seen as a highly differentiated mucinous adenocarcinoma. DPAM, PMCA and PMCA-I have 5-year survival rates of 84%, 7%, and 38%, respectively. Different treatments have been evaluated, leaving surgery, often in combination with intraperitoneal chemotherapy, as the best strategy. The traditional surgical treatment for PMP patients has been debulking surgery, where part of the large tumor load is removed, leaving behind tumor tissue difficult to resect. Such strategies are associated with 5-year overall survival of 30–40% [65]. Sugarbaker developed new strategies for surgery of PMP patients, and in 1995 he described radical cytoreductive surgery (CRS) that included peritoneal surgery and intraperitoneal (IP) chemotherapy with 10-year overall survival close to 80% [66].
Intraperitoneal (IP) administration of chemotherapy is considered preferable in PMP, as pharmacokinetic studies have shown a dose advantage for IP versus intravenous (IV) chemotherapy administration [67]. With hyperthermic intraperitoneal chemotherapy (HIPEC), the chemotherapy is delivered intraoperatively and heated to 40–43 °C to facilitate penetration of the drug. Typical drugs used for HIPEC include mitomycin C as single drug, or in combination with cisplatin [68, 69]. Although HIPEC nowadays is established treatment of PMP patients, no prior study has investigated the drug sensitivity in vitro of tumor cells in relation to clinical outcome.

Cancer markers such as CEA, Ca-125 and Ca19-9 have been investigated as markers for prediction of successful surgery [70], and it is possible that Ca19-9 can give some prognostic information in patients with DPAM histology [71]. Still, evidence is lacking to inform guidelines for clinical use.

**Toward individual cancer treatment**

Decades of research have resulted in a better understanding of cancer biology and potential targets for tailored treatment [13]. However, today’s choice of chemotherapy treatment is usually based on clinical trials, where results are based on group survival. Standard treatment protocols in use do not take into consideration differences in drug sensitivity between histopathological groups or differences in tumor cell sensitivity between individual patients with the same histopathological diagnosis. As a result of this, individuals are at risk of major side effects while the tumor may be unresponsive to therapy [72].

As clinicopathological parameters in both EOC and PMP are insufficient for prediction of prognosis, as well as response to chemotherapy, additional methods are needed to individualize treatment. In order to tailor cytotoxic treatment, tumor drug sensitivity may be tested ex vivo in assays to predict cytotoxic effects of anticancer drugs. There are several assays available for testing tumor sensitivity to drugs ex vivo. Among the cell-based drug sensitivity tests, clonogenic assays are based on the ability of tumor cells to form colonies in the presence of cytotoxic drugs; colonies are counted after 2–3 weeks. Fluorometric microculture cytotoxicity assay (FMCA) is a cell esterase activity assay that measures fluorescence generated from cellular hydrolysis of non-fluorescent diacetate (FDA) to fluorescein by viable cells in microtiter plates [72]. Cytotoxicity assays may provide information about the extent and type of drug resistance and indicate what pathways are needed to investigate further prior to treatment. Potentially, in the future, cytotoxicity assays may be one tool in guiding the clinician to the best treatment for the patient. In this thesis, the FMCA was used to explore histopathological dif-
ferences in PMP and EOC, and also to evaluate the development of cytotoxic resistance in patients exposed to neoadjuvant chemotherapy.

Predictive and prognostic factors
The terms “prognostic” and “predictive” are often used interchangeably, but have different meanings. A pure prognostic factor is a clinical or biologic characteristic factor that is measurable and provides information on the likely outcome for the patient in an untreated individual. Such prognostic markers are helpful for identifying individuals that are at high risk of relapse and may therefore be useful in the selection of patients for (any kind of) adjuvant treatment. A prognostic factor does not, however, provide information about what drug or treatment would optimally improve the outcome. In contrast, a predictive factor is a factor that provides information on the likely benefit of a specific treatment in terms of decreased tumor size or prolonged survival [73]. Tumor histopathology would be a prognostic and in some cases even a predictive factor, and the impotence of careful histopathological review by pathologist with interest in tumor group cannot be stressed enough. Difficulties may occur in morphological classification of rare tumors like PMP as well as subgroups of EOC, and tumors may also be heterogenic. Immunohistochemical stainings are crucial in diagnostics, and specific markers are needed. In EOC, clear cell components may be found in endometroid and high-grade serous tumors, and careful review should be undertaken because important prognostic and predictive information may be missed. [74].
Aims

The overall aim of my thesis was to investigate potential prognostic and predictive factors of tumor cells of epithelial ovarian cancer and pseudomyxoma peritonei to improve cancer therapy.

The specific aims of the studies were:

I  To investigate the role of the protein Napsin A in early epithelial ovarian cancer.

II To investigate the roles of heterogeneous nuclear ribonucleoprotein M (HRNPM) and solute carrier 1A5 (SLC1A5) in early epithelial ovarian cancer with respect to cell cycle control, apoptosis and angiogenesis.

III To investigate the drug sensitivity to standard drugs and kinase inhibitors ex vivo of tumor cells from epithelial ovarian cancer in relation to histopathological subgroups as a basis for future individualized drug selection.

IV To investigate the drug sensitivity ex vivo of tumor cells from pseudomyxoma peritonei in relation to clinical outcome as a basis for future individualized drug selection.
Materials & Methods

Study population

Studies I and II
A total of 140 consecutive patients with FIGO stage I–II epithelial ovarian cancer, who underwent primary surgery and post-surgical chemotherapy in the Uppsala-Örebro Medical Region during a 5-year period from January 1, 2000 to December 31, 2004, were included in the study. All samples were collected with the patients’ informed consent, in compliance with the Helsinki Declaration [19], and used in accordance with the Swedish Biobank Legislation and Ethical Review Act (approval by Uppsala Ethical Review Board, decision ref. UPS-03-477).

In study I, 131 of the 140 patients were included. Of these, 131 tumors were available for analysis of p53 and p27, 129 tumors for analysis of p21, and 124 tumors were available for analysis of Napsin A. In study II, 123 of the 140 patients were included; 123 tumors were available for analysis of HRNPM and 121 tumors for analysis of SLC1A5, respectively.

The primary surgery was performed at nine different surgical gynecological departments, and the staging procedure was done at the time of primary surgery. Modified surgical staging [75] according to the European organisation for research and treatment of cancer (EORTC)-was undertaken in 34 (26%) of the 131 cases in study I, and in 34 (28%) of the 123 cases in study II. In the remaining 97 (74%) cases in study I, and 89 (72%) in study II, staging was regarded as minimal or inadequate. All patients received chemotherapy 4–6 weeks after primary surgery. In study I, 105 of the 131 patients and in study II 98 of 123 received paclitaxel 175 mg/m² and carboplatin (area under the curve (AUC) = 5) at 3-week intervals usually in four courses. The remaining 26 patients (same fraction in both studies) were treated with single-drug carboplatin in 4–6 courses. No patients were lost from clinical follow-up, and the mean follow-up time was 65 months (range 5–110 months). Survival was defined as date of confirmed histological diagnosis after primary surgery to date of recurrence, death, or last visit.
Study III
A total of 128 patients scheduled for ovarian cancer surgery at the Uppsala University Hospital, Örebro University Hospital, Falun hospital, and the private Uppsala Cancer Clinic were included in the study between May 2006 and December 2016. A successful chemotherapy sensitivity assay was obtained in 120 patients, and these were included in further analysis. Of these, 93 patients were scheduled for curative cytoreductive surgery, 18 underwent laparotomy but were found to have disease not accessible for surgery. Surgery was performed by gynecological surgeons, and tumor burden was assessed according to the Peritoneal Cancer Index (PCI) at start of surgery [76]. Residual disease after surgery was quantified according to the completeness of cytoreduction score (CC), where CC scores 0 (no macroscopic tumor left) and 1 (residual tumor < 0.25 cm) were considered as complete cytoreduction [77, 78]. Preoperative performance status was classified according to the American Society of Anesthesiologists (ASA) Physical Status Classification System [79]. Tumor samples were collected during surgery and were immediately sent for assessment of ex vivo drug activity.

Tumor histopathology was classified as type I (low grade serous G1, low grade endometroid G1/G2, mucinous or clear cell) or type II (high grade serous G2/G3, high grade endometroid G3 or carcinosarcoma) tumors [9]. Following surgery, patients started chemotherapy within four to six weeks, most commonly with paclitaxel 175 mg/m² and carboplatin (AUC = 5). Patients were monitored with computed tomography (CT) scans after completed treatment, clinical examination, transvaginal ultrasound, Ca-125 every three months for two years, every six months for another three years, and every 12 months up to 10 years. Findings at the clinical examination and/or increased levels of the tumor marker would trigger a CT scan for verification of relapse [58].

Information on histopathological subtype, clinical characteristics, chemotherapy, surgery, disease status and survival was obtained from the medical records at Uppsala University Hospital, Uppsala, Sweden, and the participating centers. Among patients with complete cytoreduction (n = 74), data for progression-free survival were collected until February 2017. All tumor sampling and data collection was performed following informed consent, and the study was approved by the Regional Ethical Committee in Uppsala (Dnr 2007/237).

Study IV
A total of 133 patients scheduled for cytoreductive surgery and HIPEC for PMP at the Department of Surgery, Uppsala University Hospital, Uppsala, Sweden, between May 2006 and December 2011, and from whom a tumor
sample for ex vivo assessment of drug activity was obtained, formed the basis for the study. Tumor sampling was performed intraoperatively prior to HIPEC, which consisted of 30–35 mg/m$^2$ of mitomycin C, 100 mg/m$^2$ of cisplatin combined with 15 mg/m$^2$ of doxorubicin or 360 mg/m$^2$ of both irinotecan and oxaliplatin [80]. None of the patients had adjuvant systemic chemotherapy following CRS and HIPEC. Tumor histopathology was classified as DPAM, PMCA, or PMCA-I [61]. Tumor load was assessed as PCI at the time of surgery [76]. Residual disease after surgery was assessed as in study III [78]. Patients with complete cytoreduction were monitored for progression-free survival by assessment of serum tumor markers (CEA, CA19-9, Ca-125, and CA 72.3) every 3 months and with CT scan of abdomen and thorax every 6 months for 3 years and then every 12 months, for another 2 years. An increase in a tumor marker $>25\%$ triggered a CT scan for verification of new lesions consistent with PMP relapse. PFS and overall survival (OS) were assessed from registry data up to February 2014. Tumor sampling and data collection were based on patient informed consent and approved by the Regional Ethical Review Board in Uppsala (Dnr 2007/237).
Tissue microarray, immunohistochemistry and interpretation

Studies I and II

The specimens were obtained from paraffin blocks containing the embedded tissue removed from the tumor at primary surgery. Two tissue core specimens (diameter 0.6 mm) from all 131 ovarian carcinomas were arranged in three recipient paraffin blocks. The presence of tumor tissue on the arrayed samples was verified by hematoxylin and eosin-stained sections by a single pathologist. Five µm thick sections were cut from each multi-tissue block and were put on coated slides. Details on the immunohistochemistry procedures are found in the respective papers. The following primary antibodies were used in studies I and II, Table 2.

Table 2. Primary antibodies used in IHC, papers I and II.

<table>
<thead>
<tr>
<th>Protein of interest</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Napsin A</td>
<td>NCL-L, Mouse monoclonal ab, Novocastra, Newcastle, UK</td>
<td>1:400</td>
</tr>
<tr>
<td>p53</td>
<td>DO-7, Dako, Glostrup, Denmark</td>
<td>1:1000</td>
</tr>
<tr>
<td>HNRNPM</td>
<td>LS-B4384, Mouse monoclonal ab, LifeSpan BioScience</td>
<td>1:50</td>
</tr>
<tr>
<td>SLC1A5</td>
<td>LS-A9042, Rabbit polyclonal ab, LifeSpan BioScience</td>
<td>1:150</td>
</tr>
<tr>
<td>p21</td>
<td>P21 protein, Dako, Glostrup, Denmark</td>
<td>1:50</td>
</tr>
<tr>
<td>p27</td>
<td>NCL- p27, Vision Biosystems Novocastra, Newcastle, UK</td>
<td>1:40</td>
</tr>
<tr>
<td>PUMA</td>
<td>PUMA-α, Abcam, Cambridge Science, Cambridge, UK</td>
<td>1:50</td>
</tr>
<tr>
<td>PTEN</td>
<td>PTEN/MMAC1 Ab-4, Clone 17.A, Lab Vision Neomarkers, Fremont, CA, USA</td>
<td>1:50</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>Flk-1, <em>polyclonal</em> mouse ab, Santa Cruz Biotechnology, Santa Cruz, CA, USA</td>
<td></td>
</tr>
</tbody>
</table>
The immunohistochemical (IHC) stainings were interpreted by two of the authors (IS and TS), using a semi-quantitative analysis [81]. Details on the grading of staining for the different proteins are given in each manuscript. The tissue microarray construction was done at the Department of Pathology, the University Hospital MAS in Malmö, Sweden, but the immunohistochemical analyses and interpretation were performed at the Department of Pathology, Halmstad Medical Central Hospital, Halmstad, Sweden.

The fluorometric microculture cytotoxic assay (FMCA)

*Studies III and IV*

The fluorometric microculture cytotoxic assay is a semi-automated nonclonogenic microplate-based assay to measure living cell density after 2–4 days of incubation [82]. FMCA measures esterase activity of cells with intact plasma membranes when a non-fluorescent probe (fluorescein diacetate (FDA)) is hydrolyzed [83]. The method requires a high fraction of tumor cells, as it cannot differ normal viable cells from tumor cells. A tumor cell content of at least 70% is determined by morphological examination of May-Grüwald-Giemsa-stained cytocentrifugate preparations prior to incubation. The tumor cells are incubated for 72 h in the presence of small volumes of relevant anticancer drugs, normally including a duplicate or triplicate for each drug/ concentration. In our studies, the PMP tumor specimen was kept in buffer at 6 °C and the EOC specimens in transport medium culture at room temperature until preparation, which usually started within 3 h from tumor sampling. Tumor cells were prepared by collagenase digestion as described [84]. The cells obtained were mostly single cells or small cell clusters with ≥90% viability and with <30% contaminating nonmalignant cells.

The cytotoxic drugs tested are described in detail in Table 2 in paper III, and Table 3 in paper IV. The cytotoxic drugs were tested at three or five ten or three-fold dilutions from the maximal concentration (µM). All drugs were from commercially available clinical preparations or obtained from Selleck Chemicals LLC. The drug concentrations used ex vivo were chosen empirically to produce concentration-response curves allowing for extraction of 50% inhibitory concentrations (IC50), i.e., the drug concentration producing a cell survival of 50% compared with an unexposed control. 384-well microplates (Nunc) were prepared with 5-µl drug solution at 10× the final drug concentration using the pipetting robot BioMek 2000 (Beckman Coulter). The plates were then stored at -70 °C until further use. FMCA, described above, was used to assess drug sensitivity [82]. Briefly, tumor cells from patient samples (5000 cells/well in 45 µl culture medium RPMI 1640 supplemented with 10% fetal calf serum, glutamine and antibiotics) were seeded
in the drug-prepared 384-well plates using the pipetting robot Precision 2000 (Bio-Tek Instruments Inc., Winooski, VT, USA). From mid 2013, drug was added immediately after cell seeding using the liquid handling system ECHO® 550 (Labcyte Inc., Sunnyvale, CA, USA). This allows for fast transfer of volumes ≥2.5 nL from source plates into destination wells. In ECHO® experiments, source plates were prepared with appropriate concentrations of drugs in dimethyl sulfoxide and stored in the oxygen and moisture free MiniPod™ system (Roylan Developments Ltd, Surrey, UK) until further use. The method for drug addition does not affect assay results. Three columns without drugs served as controls and one column with medium only served as blank. The culture plates were incubated at 37 °C in humidified atmosphere containing 95% air and 5% CO₂. After 72 h incubation, the culture medium was washed away, and 50 µl/well of a physiological buffer containing 10 µg/ml of FDA were added to control, experimental, and blank wells. After incubation for 30–45 min at 37 °C, the fluorescence from each well was read in a FluoroScan 2 (Labsystems OY, Helsinki, Finland). Quality criteria for a successful assay were: a fluorescence signal in control cultures of ≥5 x mean blank values, and a coefficient of variation of cell survival in control cultures of ≤30 %. The results obtained by the viability indicator FDA are calculated as survival index (SI), defined as the fluorescence of the test expressed as a percentage of control cultures, with blank values subtracted.

Statistics

General
The Pearson’s chi-square test was used for testing proportional differences in univariate analyses. Logistic regression models were used for both crude and multivariable analyses with different endpoints, depending on study. The survival curves were generated by use of the Kaplan–Meier technique, and differences between these curves were tested by the log-rank test. Multivariable Cox regression models were used with overall survival or disease-free survival (DFS) as endpoints, while at the same time adjusting for relevant covariates. All tests were two-sided and the level of statistical significance was p < 0.05. Data are presented as mean ± SD unless otherwise stated. The Statistica11.0 (StatSoftTM) or SPSS 23.0 (IBM) statistical package for personal computers was used for the analyses.

Studies III and IV
Drug IC₅₀ was calculated using non-linear regression to a standard sigmoidal dose–response model in GraphPad Prism version 5.0 for Mac (GraphPad Software, San Diego, CA, USA). Sample sensitivity was categorized as low
drug resistance (LDR): \( IC_{50} \) below the median, intermediate drug resistance (IDR): \( IC_{50} \) between the median and median plus two standard deviations (SDs) or extreme drug resistance (EDR): \( IC_{50} \) above median plus two SDs based on all samples investigated ex vivo [82, 85]. Drug sensitivity correlations for assessment of cross-resistance were calculated at the drug concentration where the tumor samples showed the greatest scatter of SI values.

\( IC_{50} \) values were compared between histopathological subtypes and between those who had or had not received preoperative cytotoxic drug treatment by Mann–Whitney U-test or ANOVA.
Results

Study I

*Napsin A as a marker of clear cell ovarian carcinoma.*

The study population included 40 type I tumors (30.5%), 75 type II tumors (57.3%) and 16 clear cell carcinomas (12.2%), and primary cure was achieved in all 131 patients. Recurrent disease was significantly associated with FIGO sub-stages, FIGO grade, surgical staging and residual disease.

Positivity for Napsin A was detected in 12 (80%) of the 15 clear cell tumors available for analysis compared with four (4%) of the type I and II tumors. Differences in p21 status, p53 status, and p21 + p53- status were striking when clear cell tumors were compared to the other groups (types I and II). and p21 + p53-status was associated to positive staining of Napsin A and clear cell histology. In two separate multivariate logistic regression analyses with Napsin A as endpoint, both clear cell carcinoma (Table 3) and p21 + p53-status were independent predictive factors (Table 5, original paper).

Table 3. Predictive factors for positivity of Napsin A.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Multivariate OR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.97 (0.92–1.04)</td>
<td>0.4</td>
</tr>
<tr>
<td>Stage (I/II)</td>
<td>3.20 (0.28–37.05)</td>
<td>0.4</td>
</tr>
<tr>
<td>Grade(^a)</td>
<td>0.94 (0.09–9.91)</td>
<td>1.0</td>
</tr>
<tr>
<td>Clear cell(^b)</td>
<td>153 (21.0–1107)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

\(^a\) Grade (G1 vs G2 + G3)

\(^b\) Clear cell vs Type I and Type II tumors

The predictive value of the marker Napsin A for CCC was evaluated by ROC curve, and as demonstrated in Figure 2, the AUC for Napsin A was 0.882.
Figure 2. ROC curve “Napsin A phenotype”.

Study II

The clinical and prognostic correlation of HRNPM and SLC1A5 in pathogenesis and prognosis in EOC.

The study population included 58% type I tumors and 42% type II tumors, and 84% of the patients had a stage I disease. Primary cure was achieved in all 123 patients, the total number of recurrences was 32 out of 123 (26%), and 22 of these patients (68%) died due to disease during the follow-up. Recurrent disease was significantly associated with FIGO sub-stages (IA-IB/IC/II) (p = 0.0002), FIGO-grade (p = 0.023), residual disease (p = 0.001), and type of tumor (I/II) (p=0.023).

HRNPM positivity was detected in 85 (61%) out of the 123 tumors. Positivity of HRNPM was more frequently found in tumors positive for PUMA (p = 0.04) and VEGF-R2 (p = 0.003). HRNPM status was not associated with recurrent disease or survival.

Positive staining for SLC1A5 was detected in 92 (86%) of the 121 available tumors; an example of staining is given in Figure 3.
SLC1A5 staining was associated with p27 positivity, but not with the p21 status of tumors. Furthermore, SLC1A5 positive tumors usually had concomitant positivity for PTEN (p = 0.03), PUMA (p = 0.04) and VEGF-R2 (p = 0.04).
In the subgroup of non-serous tumors (n = 72), the SLC1A5 status was significantly associated with recurrent disease (p = 0.02). Among the 53 patients with SLC1A5 positivity of non-serous tumors, eight (15%) patients had recurrent disease, whereas the corresponding number in women with SLC1A5 negative tumors was eight (42%). The 5-year disease-free survival for the subgroup of patients with SLC1A5 positivity of tumors was 92% compared with the 5-year disease-free survival of 66% for the subgroup of patients with SLC1A5 negativity of tumors (log-rank = 15.343; p < 0.01), Figure 4.

![Cumulative Proportion Surviving](image)

**Figure 4.** Non-serous tumors. 5-year DFS for patients with SLC1A5 positivity of tumors was 92% compared with 66% for patients with SLC1A5 negativity of tumors.

Bivariate and multivariable Cox analyses with DFS as endpoint are shown in Table 4. In this analysis, both FIGO stage and SLC1A5 status were significant and independent prognostic factors.
Table 4. Cox analysis (bi- and multivariable) with DFS as endpoint in patients with non-serous tumors (n = 72).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Bivariate HR (95% CI)</th>
<th>Multivariable HR (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>1.01 (0.97–1.05)</td>
<td>1.01 (0.97–1.05)</td>
<td>0.7</td>
</tr>
<tr>
<td>Stage (I/II)</td>
<td>3.32 (1.61–6.86)</td>
<td>4.53 (1.57–13.07)</td>
<td><strong>0.005</strong></td>
</tr>
<tr>
<td>Type (I/II)</td>
<td>2.94 (1.06–2.94)</td>
<td>1.88 (0.55–6.49)</td>
<td>0.3</td>
</tr>
<tr>
<td>HRNPM pos</td>
<td>0.62 (0.23–1.68)</td>
<td>0.45 (0.14–1.37)</td>
<td>0.1</td>
</tr>
<tr>
<td>SLC1A5 pos</td>
<td>0.27 (0.10–0.73)</td>
<td>0.28 (0.09–0.85)</td>
<td><strong>0.024</strong></td>
</tr>
</tbody>
</table>
Study III

A successful ex vivo assay was obtained in 120 out of 128 samples (93%). Ninety-nine patients had type II tumors, of which 93 had high-grade serous histology. The majority of patients, 105 (87.5%) were in stage III and stage IV. Among patients with type I tumors (n = 21), low-grade serous histology was the most common type. Fifty-two patients (43%) had received chemotherapy prior to surgery, 50 of these paclitaxel and carboplatin.

Cytotoxic drug sensitivity varied considerably between patient samples as indicated by the high standard deviations (SDs) in the IC\textsubscript{50} values for the tested drugs, Table 5. Tumors previously exposed to chemotherapy were less sensitive, i.e., had higher IC\textsubscript{50}, to all cytotoxic drugs, and for three out of the nine kinase inhibitors, reaching statistical significance for 5-FU, irinotecan, dasatinib and nintendanib. Interestingly, for cisplatin the difference in sensitivity with respect to treatment status was very small.

Compared with type I tumors, type II tumors were more sensitive to all drugs, reaching statistical significance for cisplatin, Table 5. The pattern was similar for most of the kinase inhibitors, with type II tumors being more sensitive, but statistical significance was only reached for dasatinib.

Cross-resistance between cisplatin and some selected cytotoxic drugs and kinase inhibitors was modest, yet statistically significant in most cases, see table 4 in the original manuscript.
Table 5. IC50 values for cytotoxic drugs and kinase inhibitors in ovarian cancer samples (n = 120), according to preoperative cytotoxic drug treatment and histopathological subtype

<table>
<thead>
<tr>
<th>Preoperative cytotoxic drug treatment</th>
<th>Yes</th>
<th>No</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n = 52</td>
<td>n = 68</td>
</tr>
<tr>
<td></td>
<td>mean ± SD</td>
<td>mean ± SD</td>
<td></td>
</tr>
<tr>
<td>5-FU, µM</td>
<td>119</td>
<td>309 ± 328</td>
<td>171 ± 181</td>
</tr>
<tr>
<td>Oxaliplatin, µM</td>
<td>118</td>
<td>32.9 ± 32.1</td>
<td>22.8 ± 24.2</td>
</tr>
<tr>
<td>Cisplatin, µM</td>
<td>106</td>
<td>11.9 ± 15.4</td>
<td>10.0 ± 14.2</td>
</tr>
<tr>
<td>Docetaxel, µM</td>
<td>105</td>
<td>45.9 ± 46.7</td>
<td>42.0 ± 38.0</td>
</tr>
<tr>
<td>Irinotecan, µM</td>
<td>119</td>
<td>90.8 ± 79.9</td>
<td>66.7 ± 62.2</td>
</tr>
<tr>
<td>Crizotinib, µM</td>
<td>69</td>
<td>16.7 ± 23.6</td>
<td>9.44 ± 16.1</td>
</tr>
<tr>
<td>Dasatinib, µM</td>
<td>67</td>
<td>11.3 ± 11.2</td>
<td>6.64 ± 9.04</td>
</tr>
<tr>
<td>Nintendanib, µM</td>
<td>44</td>
<td>23.8 ± 29.5</td>
<td>11.5 ± 21.7</td>
</tr>
<tr>
<td>Regorafenib, µM</td>
<td>71</td>
<td>15.4 ± 7.91</td>
<td>12.4 ± 9.05</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histopathological subtype</th>
<th>Type I</th>
<th>Type II</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 21</td>
<td>n = 99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>mean ± SD</td>
<td>mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Oxaliplatin, µM</td>
<td>118</td>
<td>35.3 ± 37.0</td>
<td>25.4 ± 25.9</td>
</tr>
<tr>
<td>Cisplatin, µM</td>
<td>106</td>
<td>16.5 ± 22.5</td>
<td>9.81 ± 12.6</td>
</tr>
<tr>
<td>Docetaxel, µM</td>
<td>105</td>
<td>65.7 ± 66.5</td>
<td>39.2 ± 34.6</td>
</tr>
<tr>
<td>Crizotinib, µM</td>
<td>69</td>
<td>20.2 ± 27.1</td>
<td>11.0 ± 18.0</td>
</tr>
<tr>
<td>Dasatinib, µM</td>
<td>67</td>
<td>18.3 ± 13.6</td>
<td>6.71 ± 8.35</td>
</tr>
<tr>
<td>Erlotinib, µM</td>
<td>92</td>
<td>57.3 ± 37.0</td>
<td>62.6 ± 36.0</td>
</tr>
<tr>
<td>Sorafenib, µM</td>
<td>99</td>
<td>12.5 ± 7.44</td>
<td>15.1 ± 16.3</td>
</tr>
</tbody>
</table>

Comparisons made by Mann–Whitney U-test. P-values <0.05 are indicated in bold type.
To remove the great prognostic importance of tumor type, analysis of progression-free survival was performed separately for patients with type II tumors with complete cytoreduction (n = 61). With all drugs, intermediate and/or extreme drug resistance was associated with higher risk of progression compared with low drug resistance, reaching statistical significance for the kinase inhibitors crizotinib, dasatinib, erlotinib, regorafenib and sorafenib, Figure 5.

*Figure 5.* PFS of patients with type II epithelial ovarian cancer and complete cytoreductive surgery (n = 61) based on ex vivo activity of the kinase inhibitors indicated and found to provide statistically significant prognostic information. Drug activity was classified into low drug resistance (LDR), intermediate drug resistance (IDR) and extreme drug resistance (EDR) as detailed in the methods section. All samples were not investigated for all drugs and therefore, the data points do not necessarily add up to 61 in each panel.
Study IV

A successful ex vivo assay fulfilling the quality criteria was obtained from 92 tumor samples (69%), and data from these patients were included for analysis in the study. The majority of patients had a histopathology of DPAM (n = 57), whereas 24 had PMCA, and 11 patients had a PMCA intermediate histology.

Drug sensitivity varied considerably between patient samples, and tumor samples obtained from patients previously exposed to cytotoxic drugs were generally more resistant to drugs.

Because of the strong prognostic value of complete cytoreductive surgery, analyses of the prognostic impact of ex vivo drug sensitivity were performed in patients with complete cytoreduction (n = 61), with PFS as the clinical endpoint. Following adjustment for performance status, PCI score and histopathologic subtype, a strong trend toward longer PFS was observed for individuals with tumors sensitive to mitomycin C and cisplatin.

As very high concentrations of cytotoxic drugs are obtained locally when subjects are treated with IPC, additional analyses on drug sensitivity in relation to PFS were conducted based on the drug activity, categorized into LDR, IDR and EDR, at the highest drug concentration used ex vivo [86]. There was a stepwise increase in risk for disease progression from LDR to IDR to EDR ex vivo sensitivity scores for cisplatin, 5FU and mitomycin C (Table 6).

Table 6. Bivariate and multivariable Cox regression for PFS according to drug sensitivity at the highest cytotoxic drug concentration used ex vivo in patients with PMP with complete cytoreduction (n = 61).

<table>
<thead>
<tr>
<th>Drug</th>
<th>LDR n</th>
<th>Bivariate HR</th>
<th>P</th>
<th>Multivariablea HR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitomycin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDR</td>
<td>35</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IDR</td>
<td>22</td>
<td>2.32</td>
<td>0.2</td>
<td>3.38</td>
<td>0.05</td>
</tr>
<tr>
<td>EDR</td>
<td>4</td>
<td>5.19</td>
<td>0.05</td>
<td>6.00</td>
<td>0.05</td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDR</td>
<td>35</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IDR</td>
<td>20</td>
<td>1.86</td>
<td>0.3</td>
<td>3.00</td>
<td>0.064</td>
</tr>
<tr>
<td>EDR</td>
<td>4</td>
<td>5.16</td>
<td>0.05</td>
<td>14.35</td>
<td>0.001</td>
</tr>
<tr>
<td>5 FU</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDR</td>
<td>30</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>IDR</td>
<td>26</td>
<td>0.52</td>
<td>0.3</td>
<td>0.55</td>
<td>0.4</td>
</tr>
<tr>
<td>EDR</td>
<td>4</td>
<td>3.38</td>
<td>0.05</td>
<td>4.91</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a Adjusted for histopathological subtype, PCI score, and WHO performance status
The stepwise decrease in PFS related to drug resistance is illustrated in Figure 6.

*Figure 6. Progression-free survival in patients with complete cytoreduction according to ex vivo sensitivity to mitomycin C categorized to LDR, IDR and EDR at the highest drug concentration.*
Discussion

Methodological considerations

Tumor staging
Tumor stage has strong prognostic value for disease-free and progression-free survival. In studies III–IV, tumor staging was performed according to today’s standard, and/or tumor burden was assessed according to the peritoneal cancer index. In studies I and II, which recruited patients at an earlier time-point, staging was not performed in accordance with the same standard. Modified staging according to the EORTC surgical staging, Table 7 [75], was undertaken in 34 (26%) of the 131 cases in study I and in 34 (28%) of 123 cases in study II. Even so, staging according to the EORTC does not meet contemporary standards, and the modified staging was undertaken in less than one-third of the patients. Thus, the risk of misclassification, i.e., that more advanced stages were missed is high, but prognostic results remain unchanged when information on lymph node surgery was incorporated into the multivariable model. Needless to say, staging problems are only of relevance for the prognostic parts of papers I–II, not for the diagnostic and tumor biology aims.
Table 7. Requirements for surgical staging following bilateral salpingo-oophorectomy and total abdominal hysterectomy. Patients with Ia stage who wished to preserve fertility were permitted to have only salpingo-oophorectomy.

<table>
<thead>
<tr>
<th>Surgical staging category</th>
<th>Staging guidelines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal</td>
<td>Inspection and palpation of all peritoneal surfaces, biopsy of any suspect lesions for metastases, peritoneal washing, infracolic omentectomy, blind biopsies of right hemidiaphragm, of right and left paracolic gutter, of pelvic sidewalls, of ovarian fossa, of bladder peritoneum and of cul-de-sac, sampling of iliac and periaortic lymph nodes.</td>
</tr>
<tr>
<td>Modified</td>
<td>Everything between optimal and minimal staging.</td>
</tr>
<tr>
<td>Minimal</td>
<td>Inspection and palpation of all peritoneal surfaces and the retroperitoneal area, biopsies of any suspect lesions for metastases, peritoneal washing, infracolic omentectomy.</td>
</tr>
<tr>
<td>Inadequate</td>
<td>Less than minimal staging but at least careful inspection and palpation of all peritoneal surfaces and the retroperitoneal area, biopsies of any suspect lesions for metastases</td>
</tr>
</tbody>
</table>


Tissue sampling
Ovarian cancers are heterogeneous tumors, and different histopathological structures may be present within the same tumor. At sampling for tissue microarray, site of biopsy may not be representative for the histopathological
diagnosis or IHC. To circumvent these problems, two tissue core specimens from each ovarian carcinoma were used, and the presence of tumor tissue on the arrayed samples was verified by hematoxylin-eosin-stained sections by a pathologist.

For studies III and IV, fresh samples were used, and these were collected by the surgeon. Sampling for FMCA, or any sampling of fresh tumor tissue, cannot ever be done at the cost of the tumor tissue needed for histopathological diagnosis. Thus, in cases with a small tumor volume, FMCA samples were taken after tissue for diagnostics had been collected. Tumor samples were taken from exposed tissue with macroscopic tumor features, and may in some cases represent site of tumor origin, in others metastases. Obviously, this may contribute to the heterogeneity of tumor samples, and possibly affect the read-out in the ex vivo drug sensitivity analyses.

Further problems arose with tissue sampling in study IV. PMP tumors are characterized by disseminated mucus and mucinous tumor tissue, and samples may contain few tumor cells. The FMCA, on the other hand, requires quite a great number and high fraction of tumor cells, as it cannot differentiate normal viable cells from cancer cells, and the quality of the assay may be affected. To illustrate the problem with PMP, only 69% of PMP tumor samples fulfilled quality criteria for inclusion in the study, whereas the corresponding number for the EOC tumor samples was 93%.

**Transportation of samples**

Many different centers have participated in the sampling of ovarian tumor tissue for the studies in this thesis. In studies I–II, patients underwent surgery at nine different surgical centers, whereas the corresponding number in paper III was four centers. Obviously, while many centers are needed to obtain a sufficient number of ovarian cancer patients, surgical procedures and, as mentioned previously, staging procedures may be different. However, other problems may also arise.

Tumor samples require transportation from the surgical to the pathological department, and suboptimal conditions may cause tissue damage and difficulties in diagnosis and IHC diagnostics. Similarly, EOC tumor specimens for the ex vivo assessment of drug sensitivity needed to be transported from all participating centers. All EOC tumor specimens were kept in transport culture medium at room temperature until cell preparation, and for the majority sampled in Uppsala, cell preparation usually started within 3 h from tumor sampling. In contrast, only one surgical clinic collected the PMP tumor samples for study IV, which may be considered a strength for that specific aim.
**Immunohistochemistry**

The quality and specificity of the primary antibody is crucial, and choice of antibody may affect IHC-based methods in terms of outcome, reliability and interpretation. The monoclonal antibodies used for this thesis are likely to be specific in terms of antigen binding but require a high presence of antigen in order to be used for antigen detection and interpretation. The polyclonal antibodies used for this thesis may be less specific for antigen binding and produce “off target” and “background” binding affecting interpretation [81, 87].

Interpretation of immunostaining may vary between the same observer and between observers. In our studies, two authors who, at the time of evaluation, were blinded to the diagnosis and prognosis for the individual cases interpreted the IHC stains.

Finally, when studying a novel antigen, characteristics of positive staining may not be very well characterized and result in problems in interpretation.

**The fluorometric microculture cytotoxic assay (FMCA)**

FMCA samples contain a small number of cells other than tumor cells but the assay conditions do not provide an in vivo like “tumor microenvironment”, even if all cell types are represented. This probably limit the ability of the assay to correctly predict response to anticancer drugs. Furthermore, drug exposure and pharmacokinetics differ between the FMCA and the in vivo conditions and this will also limit the predictive accuracy of the ex vivo test.

Despite these limitations, the thesis produced a number of relevant findings highlighted in this discussion.

**Study I**

In study I, the role of Napsin A in patients with early epithelial ovarian cancer was investigated.

The immunohistochemical profile, based on staining for Napsin A, p21 and p53, was unique in clear cell carcinomas compared to other histological subtypes of ovarian cancer. Also, Napsin A was more frequently detected in clear cell carcinomas than in other histological subtypes, with a detection rate of 80% in the CCC. As a diagnostic measure, the ROC curves indicated good sensitivity and specificity, with an AUC for Napsin A alone of 0.88.
Importantly, these findings have been confirmed by several publications from 2013 and onwards. In the confirmatory studies, detection of Napsin A in CCC has reportedly been 100%, or close to 100% [88-91]. As difficulties may arise in the morphological diagnosis between CCC and high-grade serous ovarian carcinomas, Napsin A has become a useful tool in CCC diagnostics.

From a tumor biology perspective, our study also provided some insights into the effects of Napsin A. In our study, none of the 16 CCC samples stained positive for p53, a finding also confirmed by other groups and useful in the diagnostics of CCC [92]. Napsin A is known to be up-regulated in cisplatin-resistant cells, and knock-down of the corresponding gene NAPA sensitizes the tumor cells to cisplatin [93]. This effect is found only in cells with intact p53 function, and findings support that Napsin A acts by degrading p53 and by blocking p53-mediated apoptosis [22]. This may explain why the platinum response rate in clear cell carcinomas is low [43]. Further studies are needed to investigate whether up-regulated Napsin A may predict lack of platinum sensitivity.

Study II

In study II, the relevance of HRNPM and SLC1A5 as prognostic factors for recurrent disease, survival and impact on clinical or pathological features in patients with early epithelial ovarian cancer was evaluated. The expression of HRNPM and SLC1A5 in relation to the proteins of cell cycle regulation and apoptosis was also explored.

HRNPM positivity was detected in 61% of the tumors, and positivity for HRNPM was more frequently found in tumors positive for PUMA. HRNP (also called hn RNP) proteins are RNA binding proteins. They are altered in many types of cancer, and recent publications and reviews conclude that they are crucial players in cancer development [94, 95]. HRNPs are predominantly present in the nucleus, but can translocate into the cytoplasm. Many HRNPs, including HRNPM, are now also known to be involved in alternative splicing, in which alterations may affect cell signaling. In breast cancer, high HRNPM levels are thought to be a part of the EMT and to be associated with lymph node metastasis and shorter overall survival [96, 97]. Our results support concomitant positivity for HRNPM and PUMA and/or p21 in ovarian cancer and that the presence of HRNPM in the tumor cell triggers apoptosis and cell cycle arrest by PUMA and p21[98]. PUMA is normally expressed at a low level in tissues but is rapidly induced in response of a wide range of stimuli. PUMA-mediated apoptosis acts as a safeguard against neoplastic transformation [99].
Positivity for SLC1A5 was detected in 92 (86%) of 121 available tumors in our study. In the subgroup of non-serous tumors, it was possible to identify 53 patients with SLC1A5 positivity that had excellent disease-free survival, 92% at 5 years and 78% at 9 years; this subgroup could be considered as long-term survivors. The role of SLC transporters in chemo-resistance of human cancer is well described in the literature. SLCs typically mediate uptake and chemo-sensitivity for hydrophilic drugs, and regulation of uptake may mediate chemo-resistance [100, 101]. SLC1A5 is also known to be a glutamine transporter and to act as a donor of nitrogen for nucleotide and protein synthesis. Cancer cells exhibit increased needs of glutamine fueling, and glutamine has also been described as an activator of m TORC1, a protein translation, cell growth and autophagy regulator [102].

Several articles published simultaneously, or just after ours, have investigated the importance of SLC1A5 in cancer development. In gastric cancer cells, expression of SLC1A5 in tumors was found to correlate with malignant features like deeper invasion, lymph node metastasis and more advanced stage. Knock-down of SLC1A5, on the other hand, inhibited migration and invasion of gastric cancer cells and tumor growth in vivo in xenograft tumors [103]. In KRAS mutated colorectal cancers, SLC1A5 expression correlated with invasion depth and vascular invasion. SLC1A5 knockdown exhibited a suppressive effect on cell growth and migration in cell lines [104].

Even in breast cancer, SLC1A5 status seems to give prognostic information, and, in contrast to our findings in non-serous ovarian cancer, expression of SLC1A5 in breast cancer generally seems associated with poor prognosis. A recent study of 800 women treated for breast cancer reported that those with high tumor tissue expression of SLC1A5 had shorter recurrence-free survival [105]. Geldermans and colleagues demonstrated that ASCT2 (SLC1A5) transport is critical for cell growth and cell cycle progression in breast cancer cells, with the effect being subtype dependent and limited to triple negative (i.e., estrogen receptor-, progesterone receptor- and human epidermal growth factor receptor (HER)-negative) breast cancer cells. In addition, low ASCT2 tumor expression conferred a significant survival advantage [102].

Thus, in contrast to our findings, recent data suggest that elevated expression of SLC1A5 in breast and colorectal cancer constitutes a negative prognostic factor, and many authors suggest that SLC1A5 be used as a target for cancer therapy, as pharmaceutical blocking and knock out of SLC1A5 seem to suppress tumor growth. The results are only seen in specific histopathological subgroups, and some are at least partly from mouse models. In our study, SLC1A5 expression was an independent prognostic factor for disease-free survival in patients with non-serous epithelial ovarian cancer. Unlike results
from breast and gastrointestinal cancers, it seems that SLC1A5 protects from recurrent disease and prolongs disease-free survival. Clearly, further studies on the role of SLC1A5 in ovarian cancer are needed.

Studies III and IV

Studies III and IV confirm that drug sensitivity may vary considerably between samples from patients within the same diagnostic group. In ovarian cancer, ex vivo results show that type I tumors were generally less sensitive to cytotoxic agents than the type II tumors, typically illustrated by the difference for cisplatin. This finding confirms clinical experience where type II ovarian tumors are characterized by initial sensitivity to cytotoxic agents that often strike on DNA repair pathways, whereas type I tumors show more indolent behavior and are less sensitive to established treatment [106, 107]. Similarly, tumors of PMCA histology were slightly more resistant than those with PMCA-I and DPAM histology.

Samples from patients previously exposed to cytotoxic drugs generally tended to be more resistant to most drugs than samples from unexposed patients, and this was true for both ovarian cancer and PMP samples. This observation is in line with clinical experience and findings supporting that exposure to cytotoxic treatments contribute to development of chemoresistance mechanisms [15].

In ovarian cancer, resistance to kinase inhibitors after cytotoxic drug exposure varied but was less pronounced than resistance to standard cytotoxic drugs. Sorafenib and sunitinib seemingly lack development of resistance after prior cytotoxic drug exposure and may be interesting drugs for further investigation in the treatment of resistant disease [108], although the limited clinical experience with these drugs is not very promising [19, 109-111]. Cross-resistance between cisplatin and docetaxel was modest to low and supports the suitability of clinical use of this combination.

In PMP patients and in patients with type II EOC, ex vivo drug sensitivity provided prognostic information for progression-free survival, and this is in line with other studies in which FMCA and similar assays have been shown to be useful in terms of providing prognostic information [112-114].

According to the American Society of Clinical Oncology’s (ASCO) report from 2004, treatment should not be based on chemosensitivity assays outside clinical trials, because of lack of randomized studies [115]. When in vitro sensitivity results in EOC are compared with clinical outcome, results are variable [112, 114, 116-125]. A randomized controlled trial with 180 patients suggested a trend toward better responses and longer progression-free
survival from assay-guided therapy, but no significant impact on overall survival could be demonstrated [122]. In another comparative, but non-randomized, trial in patients with EOC relapse, assay-guided treatment provided prolonged progression-free and overall survival in patients with platinum-sensitive disease [125].

Today’s choice of chemotherapy treatment is usually based on clinical trials, where results are based on group survival. Standard treatment protocols in use do not take into consideration differences in drug sensitivity between histopathological groups or differences in tumor cell sensitivity between individual patients with the same histopathological diagnosis. As a result of this, individuals are at risk of major side effects while the tumor may be unresponsive to therapy, and effective therapy delayed [72]. Treatment decisions are made on the basis of histopathology and immunohistochemical patterns and markers. These markers can sometimes, as for example regarding the estrogen receptor in breast cancer, also serve as prognostic and predictive factors for treatment [126]. A few treatments based on single genetic alterations like trastuzumab in HER-positive breast cancer or olaparib in BRCA-mutated ovarian cancer have been introduced [127, 128].

In an effort to personalize cancer treatment we have evaluated whether tumor sensitivity to anticancer drugs in vitro can be used to predict their effects in patients. In the age of tailored treatment, the perfect drug would be administrated after careful genetic testing of the tumor and hit specific mutated pathways [127, 128] and in 2017, the first treatment based on mutation and not tumor type was approved by the US Food and Drug Administration [126]. In reality, diagnosed mutations and tailored treatment are no guaranty for treatment response. In fact BRAF mutated malignant melanoma respond to vemurafenib which inhibit mutated BRAF, while colon cancers with the same mutation are resistant due to alternative pathways if proliferative signaling [129]. One option is genetic testing of the tumor, and as a second step, to use the assay to sort out ineffective drugs.

Published reports on clinical relevance of assays usually investigate retrospective correlations between drug activity in vitro and in vivo. In this setting, which is also used in the present thesis, choice of treatment is not based on the ex vivo results [83, 130]. The tests, thus, add prognostic information, but further studies evaluating the predictive value in prospective clinical trials are clearly needed [113].
Conclusions

Positivity of Napsin A in an epithelial ovarian tumor strengthens the morphological diagnosis of clear cell ovarian carcinoma.

SLC1A5 seems to be a prognostic factor for non-serous epithelial ovarian cancer.

Ex vivo assessment of drug activity based on total cell kill reveals that EOC type I and type II are differently sensitive to standard cytotoxic drugs.

Ex vivo assessment of tumor cell sensitivity to cytotoxic drugs provides prognostic information in PMP and may be useful in sparing the most resistant patients treatment expected to be futile.

Tumor samples from EOC and PMP patients previously exposed to cytotoxic drugs generally tended to be more resistant to most drugs than samples from unexposed patients. This supports that exposure to cytotoxic treatments contributes to development of chemoresistance.

Ex vivo reported tumor cell drug sensitivity is in line with clinical experience and outcome, pointing toward a role for such assays for optimization of drug therapy in EOC and PMP. Whether selection of drugs based on ex vivo assessment is predictive for a treatment effect, and thus could be used for treatment individualization, needs to be investigated in prospective clinical trials.

Pseudomyxoma peritonei (PMP) är en ovanlig cancer som utgår från blindtarmen och sedan sprider sig diffust i bukhålan. Den finns i tre olika varianter beroende på mikroskopisk undersökning, en mer godartad variant, disseminated peritoneal adenomucinosis (DPAM), en elakartad variant, peritoneal mucinous carcinomatosis (PMCA) och en mellanvariant, intermediate grade PMCA-I.

Både EOC och PMP kan sprida sig i bukhålan och sätta metastaser i bukhi nan sk. ”peritoneal carcinos” och de två sjukdomarna behandlas likartat med stor kirurgi som syftar att avlägsna all synlig tumör i bukhålan. Patienter med PMP får ofta behandling med varm cytostatika direkt i bukhålan.
(HIPEC) medan ovarial cancerpatienterna i alla fall inledningsvis svarar bra på intravenös cytostatika.

En prognostisk faktor är en faktor som ger information om hur det sannolikt kommer att gå för en patient utan behandling, en sådan faktor hjälper sjukvården att välja vilka patienter som bör få efterbehandling för att hindra återfall i sjukdom, men det ger ingen information om vilken behandling som fungerar bäst. En prediktiv faktor ger däremot information om sannolikheten att en individ kommer ha nytta av en viss behandling.

De övergripande målen med min avhandling är att undersöka potentiella prognostiska och prediktiva faktorer för tumörceller av EOC och PMP för att förbättra och individualisera cancerterapi. För att optimera och skräddarsy behandlingen krävs ökad kunskap om tumörbiologi och hur äggstockscancer uppkommer.

Dagens val av cytostatikabehandling baseras huvudsakligen på kliniska prövningar och standardprotokoll som inte beaktar individuella skillnader i läkemedelssvensnässlighet. Vid laboratorietestning av tumörcellernas känslighet för cytostatika kan personer som riskerar biverkningar men ingen behandlingseffekt förhoppningsvis identifieras före behandling.

**Delarbete I**


**Delarbete II**

I detta delarbete undersöktes vilken roll proteinererna HRNPM och SLC1A5 hade i äggstockscancer. Proverna var från samma patienter som i delarbete I, och även i denna studie användes mikroskopisk undersökning efter immunhistokemisk färgning. Resultaten talar för ett samband mellan HRNPM och proteinererna PUMA/p21 i äggstockscancer och för att HRNPM påverkar reglering cellcykeln och apoptos. I undersökningar av tumörer från patienter med icke-seröös EOC visade sig uttryck av SLC1A5 ha betydelse för patientens prognos. De patienter som uttryckte SLC1A5 hade längre sjukdomsfri överlevnad.
**Delarbete III och IV**

I delarbete III och VI undersökt läkemedelskänsligheten hos tumörceller från EOC och PMP. Vi använde oss av testet ”fluorometric microculture cytotoxic assay” (FMCA) där man undersöker tumörcellers känslighet för cytostatika i laboratoriet. Under operation hämtas tumörceller ut, skickas till laboratoriet och odlas sen i 72 timmar i små cellodlingsbrunnar i närvaro av olika cytostatika. Sen läser man av hur stor andel celler som överlevt i närvaro av cytostatika, och jämför med en brunn där samma typ av cell har odlats bara i närvaro odlingsmedium.

De två studierna bekräftar att cellernas läkemedelskänslighet varierar väsentligt mellan prover från patienter med samma typ av EOC eller PMP cancer. Försöken med äggstockscancer visar att typ I-tumörer i allmänhet är mindre känsliga för cytotoxiska medel än typ II-tumörerna och detta stämmer bra överens med hur det brukar vara i kliniskt arbete. Prov från patienter med PMP och EOC som tidigare fått behandling med cytostatika hade generellt en tendens att vara mer resistenta mot läkemedel än prov från patienter som aldrig fått cytostatika. Även detta stämmer med klinisk erfarenhet och stöder att cytostatikabehandling bidrar till utveckling av resistensmekanismer. Resultatet i FMCA testningen visade sig även ge information om patientens prognos, båda i EOC och PMP. De patienter vars tumörer var känsliga för cytostatika hade längre sjukdoms fri överlevnad.

**Slutsatser**

Om Napsin A färjar positivt i en EOC är det mycket sannolikt att det rör sig om en klarcellscancer, vilket är användbart diagnostiskt.

Vår studie visar att SLC1A5 är en prognostisk faktor för icke-serös epitelial ovariecancer.

FMCA analyser visar att EOC typ I och typ II skiljer sig åt vad gäller känslighet för cytostatika. Typ II tumörer är generellt mer känsliga för cytostatika.

Bedömning av tumörcellers cytostatikakänslighet i laboratoriet ger information om prognos vid båda PMP och typ II EOC. Testet skulle kunna spara vissa patienter från verkningslös behandling.

Prov från patienter med PMP och EOC som tidigare fått behandling med cytostatika hade generellt en tendens att vara mer resistenta mot läkemedel än prov från patienter som aldrig fått cytostatika. Detta stöder att cytostatikabehandling bidrar till utveckling av resistensmekanismer.
Acknowledgements

This thesis was carried out at the Department of Women’s and Children’s Health, Uppsala University, and was supported by grants from Uppsala-Örebro Regional Research Council.

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Mt Tamalpais, for always being there when I needed you.
References


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