# Malignant effusions in advanced ovarian carcinoma – Chemotherapy resistance and surgical treatment of stage IV disease

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To Thor

Marte & Torjus

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# Preface

Most women diagnosed with ovarian carcinoma are presenting with an advanced stage disease, and consequently have a less favorable prognosis. Additionally, at time of diagnosis, these women frequently present with effusions in the peritoneal and/or the pleural cavities containing carcinoma cells, or they develop malignant effusions along tumor progression. How to treat women with advanced ovarian carcinoma, both surgically and with chemotherapy, has been an issue under debate.

Primary surgery followed by chemotherapy is the gold standard, but chemotherapy preceding delayed primary surgery has evolved as an alternative treatment in patients expected to be initially inoperable. A major prognostic factor in these women is residual tumor after surgery, and the question is how this best is achieved.

Whereas solid tumors can be removed surgically, effusions can be eradicated with chemotherapy. Failure in their eradication is one of the main causes of treatment failure in advanced ovarian carcinoma. In clinical practice, relapses and remissions, and eventually progressive disease are often the clinical course of ovarian carcinoma, and tumors develop a broad cross-resistance to the multiple chemotherapeutic agents received. Resistance to chemotherapy is a central issue and obstacle in treatment of ovarian carcinomas.

Optimal surgical treatment of women with ovarian carcinoma stage IV, and the mechanisms behind chemotherapy resistance in women with advanced stage disease and malignant effusions are questions which will be discussed in this thesis, and which are of practical importance to the physicians and to the women suffering from this disease.
These aspects have been my main motivation for performing and completing the work in the present thesis.

## List of publications

**Paper I:**

Elstrand MB, Kleinberg L, Kohn EC, Tropé CG, Davidson B. **Expression and clinical role of antiapoptotic proteins of the Bag, Heat shock, and Bcl-2 families in effusions, primary tumors, and solid metastases in ovarian carcinoma.** Int J Gynecol Pathol 2009;28:211-221.

**Paper II:**

Bunkholt Elstrand M, Dong HP, Ødegaard E, Holth A, Elloul S, Reich R, Tropé CG, Davidson B. **Mammalian target of rapamycin is a biomarker of poor survival in metastatic serous ovarian carcinoma.** Hum Pathol 2010;41:794-804.

**Paper III:**

Elstrand MB, Stavnes HT, Tropé CG, Davidson B. **Heat shock protein 90 is a putative therapeutic target in patients with recurrent advanced-stage ovarian carcinoma with serous effusions.** Hum Pathol 2011;http//dx.doi.org/10.1016/j.humpath.2011.05.022

**Paper IV:**

Elstrand MB, Sandstad B, Oksefjell H, Davidson B, Tropé CG. **Prognostic significance of residual tumor in patients with epithelial ovarian carcinoma stage IV in a 20-year perspective.** Acta Obstet Gynecol Scand 2011;http//dx.doi: 10.1111/j.1600-0412.2011.01316.x.

**Paper V:**

Tropé CG, Elstrand MB, Sandstad B, Davidson B, Oksefjell H. **Neoadjuvant chemotherapy, interval debulking surgery or primary surgery in ovarian carcinoma FIGO stage IV?**

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# Abbreviations

| | |
|---|---|
| ADP | adenosine diphosphate |
| AHA 1 | activator of heat shock protein 90 ATPase homolog 1 |
| AIF | apoptosis inducing factor |
| AKT | V-Akt murine thymoma viral oncogene homolog |
| APAF1 | apoptotic protease-activating factor-1 |
| ATP | adenosine triphosphate |
| AUC | Area Under Curve |
| BAD | Bcl-2-associated death promoter homolog |
| Bag-1 | Bcl-2-associated anthanogene 1 |
| Bag-4/SODD | Bcl-2-associated anthanogene 4 / silencer of death domain |
| BAK | Bcl-2 antagonist/killer |
| BAX | Bcl-2-associated X-protein |
| Bcl-2 | B-cell chronic lymphocytic leukemia/lymphoma 2 protein |
| Bcl-w | B-cell chronic lymphocytic leukemia/lymphoma-w protein |
| Bcl-$X_L$ | Bcl-2-like 1 protein (long form) |
| Bcr-Abl | Philadelphia chromosome |
| BD | Bag domain |
| BH | Bcl-2 homology |
| BID | BH3 interacting domain death agonist |
| BIK | Bcl-2 interacting killer |
| BIM | Bcl-2 like 11 |
| BRAF | v-Raf murine sarcoma viral oncogene homolog B1 |
| BRCA1 / 2 | Breast Cancer Susceptibility Gene 1 / 2 |
| CA125 | Cancer Antigen 125 |
| Cdc37 | HSP90 co-chaperone |
| CDK | cyclin-dependent kinase |
| cDNA | complementary DNA |
| cFLIP | Caspase-8 and FADD-like apoptosis regulator |
| CHIP | C terminus of HSC70-Interacting Protein |
| CRM11 | Chromosome maintenance protein 1 |
| CT | Computer Tomography |
| DD | death domain |
| DISC | death-inducing signaling complex |
| DMSO | dimethylsulfoxide |
| DNA | deoxyribonucleic acid |
| DPS | delayed primary surgery |
| DR | death receptor |
| EGFR | epithelial growth factor receptor |
| eIF4E | eukaryotic initiation factor 4E |
| EMT | epithelial mesenchymal transition |
| ERK | Extracellular signal-regulated kinase |

| | |
|---|---|
| Fas | TNF receptor superfamily, member 6 |
| FCS | Fetal calf serum |
| FIGO | International Federation of Gynaecology and Obstetrics |
| FKBP12 | Peptidyl-prolyl cis-trans isomerase enzyme |
| FOXO | forkhead family of transcription factors |
| GAP | GTPase-activating protein |
| GEP | Granulin-epithelin precursor |
| GPCR | G-protein coupled receptors |
| HER-2 | Human epidermal growth factor receptor 2, synonymous to Erb-B2 |
| HGF | hepatocyte growth factor |
| HIF-1 | hypoxia-inducible factor-1 |
| HIP | HSC70 interacting protein |
| HIPEC | hyperthermic intraperitoneal chemotherapy |
| HOP | HSP70/HSP90 organizing protein |
| HSF | heat shock factor |
| HSP | heat shock protein |
| IAP | inhibitor of apoptosis protein |
| IDS | interval debulking surgery |
| IGFR | Insulin growth factor receptor |
| IHC | immunohistochemistry |
| IL-8 | interleukin-8 |
| I.P. | intraperitoneal |
| ITRT | individualized tumor response testing |
| Iκ-Bα | Inhibitory protein of NF-κB |
| JNK | c-jun N-terminal kinase |
| KRAS | Kirsten rat sarcoma 2 viral oncogene homolog |
| LMP | low malignant potential |
| MAPK | Mitogen-activated protein kinase |
| MCL-1 | Myeloid cell leukemia sequence 1 |
| MDR | Multi Drug Resistance |
| mLST8 | mTOR complex subunit LST8 protein |
| MMP-2 | matrix metalloproteinase 2 |
| MOMP | mitochondrial outer membrane permeabilization |
| mRNA | messenger RiboNucleic Acid |
| mTOR | mammalian Target of Rapamycin |
| mTORC1 | mTOR complex 1 (raptor) |
| mTORC2 | mTOR complex 2 (rictor) |
| NAC | neoadjuvant chemotherapy |
| NES | Nuclear export signals |
| NF-κB | Nuclear Factor κB |
| NLS | Nuclear localization signals |
| NRH | Norwegian Radium Hospital |
| OC | ovarian carcinoma |
| OS | overall survival |

| | |
|---|---|
| p27$^{Kip1}$ | Cyclin-dependent kinase inhibitor 1B |
| p75 | low-affinity nerve growth factor (NGF) receptor |
| Pak1 | p21-activated kinase 1 |
| PDGF | platelet derived growth factor |
| PDGFR | platelet derived growth factor receptor |
| PDK1 | 3-phosphoinositide-dependent kinase 1 |
| PDS | Primary debulking surgery |
| PFS | progression free survival |
| PH | pleckstrin homology |
| PI3-K | Phosphatidylinositol-3 kinase |
| PIP2 | phosphatidylinositol-4,5-bisphosphate |
| PIP3 | phosphatidylinositol-3,4,5-bisphosphate |
| PKB | protein kinase B family |
| PKC | protein kinase c |
| PRAS40 | Proline-rich AKT1 substrate protein |
| PTEN | phosphatase and tensin homolog protein encoded by the *PTEN* gene |
| PUMA | p53 upregulated modulator of apoptosis |
| qRT-PCR | quantitative Reverse Transcriptase Polymerase Chain Reaction |
| Raf-1 | v-Raf-1 murine leukemia viral oncogene homolog 1 |
| Ras | Rat sarcoma superfamily |
| RECIST | Response Evaluation Criteria in Solid Tumors |
| Rheb | Ras homolog enriched in brain (GTP-binding protein) |
| RIP | receptor interacting protein |
| RMI | Risk of Malignancy Index |
| Rsf-1 | remodeling and spacing factor 1 |
| RTK | receptor tyrosine kinase |
| S6K1 | Ribosomal protein S6 kinase beta-1 |
| SCID | Severe Combined Immunodeficiency |
| SIP1 | Smad interacting protein 1 |
| Smac/DIABLO | Second mitochondria-derived activator of caspases/Direct IAP-binding protein |
| tBID | truncated BID (the active form) |
| TIMP-2 | Tissue inhibitor of matrix metalloproteinase 2 |
| TMA | Tissue microarray |
| TNF | Tumor necrosis factor |
| TNFR | Tumor necrosis factor receptor |
| TRADD | TNFR-related death domain |
| TRAILR | TNF-related apoptosis-inducing ligand receptor |
| TRAP-1 | tumor necrosis factor receptor-associated protein 1 |
| TrkA | high-affinity nerve growth factor (NGF) receptor |
| TSC 1 | tuberous sclerosis complex 1 |
| TSC 2 | tuberous sclerosis complex 2 |
| UBL | Ubiquitin-like |
| UV | ultra violet |

| | |
|---|---|
| VEGF | vascular endothelial growth factor |
| WHO | World Health Organization |
| XIAP | X-linked inhibitor of apoptosis |
| 17-AAG | Tanepimycin (HSP90 inhibitor) |
| 4EBP1 | eukaryotic initiation factor 4E-binding protein 1 |

# Introduction

## 1. Development of cancer

### 1.1. Epidemiology

Cancer is a growing cause of mortality and morbidity in the industrialized world. In 2009, the Norwegian Cancer Registry reported on 27,520 new cancer cases, and 9,834 cancer deaths were registered. Since the mid-1950s the 5-year survival rate for all cancer sites has increased, and from 2005-2009 it was 66% in men and 68% in women (1).

### 1.2. Cellular homeostasis

Normal cells orchestrate a finely-tuned balance between cell proliferation and cell death which is essential for maintaining structure and function in normal tissues and organs (2). Cellular homeostasis in normal cells is genetically regulated. In principle, cancer is a genetic disease and results from mutations in somatic cells giving rise to tumor cells. Cancer occurs less frequently than mutations in somatic cells implying that it takes more than a single mutation to turn a normal cell into a cancer cell. These mutations do not occur simultaneously, but sequentially, and cancer usually develops over a period of many years (3). In general, alterations in three types of genes are responsible for carcinogenesis: proto-oncogenes, tumor-suppressor genes and stability genes (4). The proto-oncogene is a normal gene which has a mutant corresponding form called an oncogene which makes the affected gene product hyperactive, causing excessive cell survival and proliferation. The tumor-suppressor gene has defending qualities on normal cells, and mutations inactivate this quality, allowing for increased proliferation and cancer cell survival (3). Loss of capacity to repair genetic errors leads to an increased mutation rate (genomic instability), accelerating a cascade of changes contributing to the cancer phenotype. Moreover, development of cancer was originally characterized by six essential alterations in cell physiology that together stimulate malignant growth: (i) self-sufficiency in growth signals, (ii) insensitivity to anti-growth signals, (iii) tissue invasion and metastasis, (iv) limitless replicative potential, (v) sustained angiogenesis and (vi) evading apoptosis (5). Recently two additional hallmarks of cancer were proposed to be involved in the pathogenesis of cancer; deregulating cellular energetics and avoiding immune destruction. Furthermore two characteristics of cancer enable tumor progression; genetic instability and tumor-promoting inflammation (6). The core and emerging hallmarks of cancer, and the enabling characteristics illustrate that development of cancer as an ingenious multi-step process.

### 1.3. Proliferation and cell cycle regulation

Cell growth (proliferation) is used in the terms of cell development and cell division (reproduction) and is performed in an orderly sequence in which the cell duplicates its content and divides into two identical daughter cells – a cycle of events known as the cell cycle. The cell cycle is divided into four phases: G1, S, G2 and M (**Figure 1**). In S-phase (S for DNA synthesis) chromosome duplication occurs. The M-phase (M for mitosis) comprises two major events: nuclear division (mitosis) and cytoplasmic division where the cell divides into two daughter cells (2). The G1-phase is incorporated between the M-phase and S-phase, and the G2-phase separates the S-phase and M-phase. In particular the G1-phase is important for providing the cell with control before it either commits itself to S-phase or enters a specialized resting state known as G0 (zero) (7). Proliferation is down-regulated in the G0-phase where the main task is cellular differentiation. The process by which cells make the transition from proliferative to non-proliferative status is genetically controlled (8).

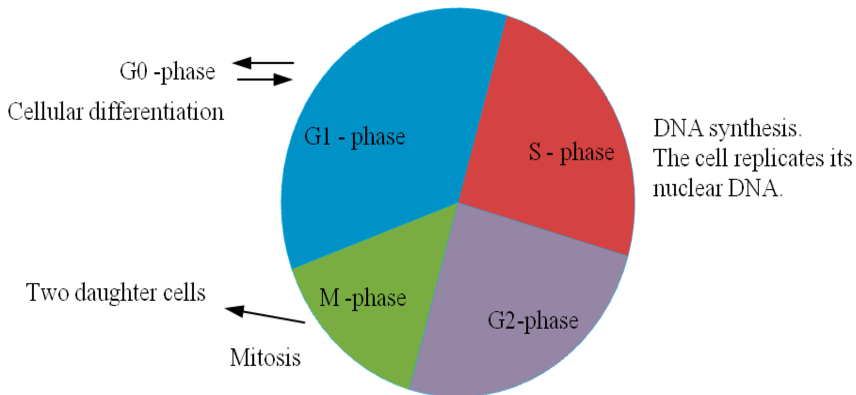


**Figure 1.** The cell cycle

Proper progression through the cell cycle is monitored by checkpoints, and central components of the control system are members of a family of protein kinases known as cyclin-dependent kinases (CDKs) (2). Activation of CDK induces cell cycle arrest at checkpoints in the G1- and G2-phase allowing for cells to properly repair defects, and thus preventing their transmission to the resulting daughter cells (9).

Cancer cells proliferate and undergo the same phases in the cell cycle as normal cells, but control mechanisms are altered or deregulated (7). Tumor growth results from an increased proliferation and a contemporary down-regulated cell death resulting in an imbalanced cellular homeostasis (2). Cancer cells also arrest temporarily in the quiescent G0-phase, a situation termed cellular dormancy. Since most chemotherapeutics directly or indirectly exert their effect on proliferating cells, cellular dormancy in cancer cells is, at least in part, assumed to reflect chemotherapy resistance (10). To balance growth, normal cells are also subjected to death. The well regulated multi-step process of self-destruction of cells is called apoptosis, or programmed cell death, and will be discussed in a later section (11).

# 2. Ovarian cancer

## 2.1. Epidemiology, etiology and risk factors

Ovarian cancer is the eighth most common cancer type in women worldwide and the second most lethal gynecological malignancy (12). In Norway approximately 440 new cases are diagnosed annually. From 2005-2009, the accumulated risk for developing ovarian cancer at age 75 was 1.2%, and the median age-group at diagnosis was 60-64 years. The 5-year relative survival for all cases was 37% from 1985-1989, increasing to 44% from 2005-2009 (1) (**Figure 2**).

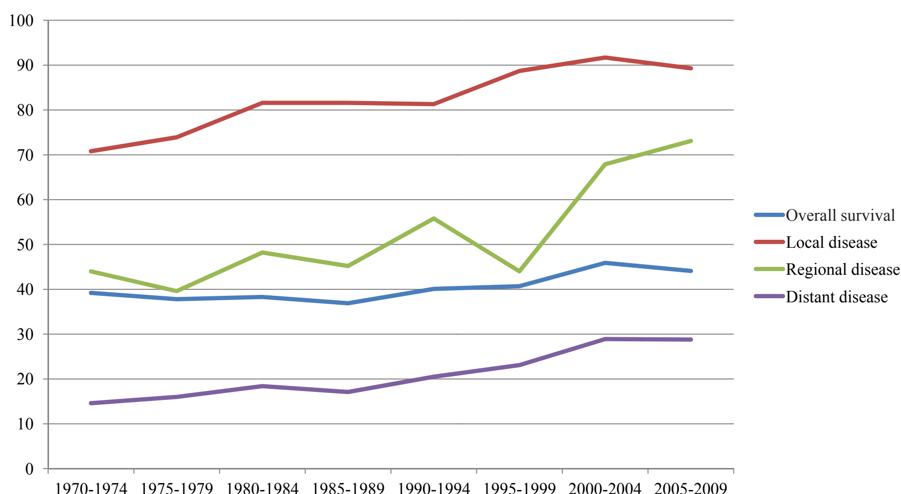


**Figure 2.** Ovarian cancer. 5-year relative survival 1970-2009.

The etiology of ovarian carcinoma (OC) remains poorly understood. Several hypotheses have been proposed to explain the underlying physiological processes which increase the risk of malignant transformation (13). The heterogeneity of OC can be roughly separated into two broad categories of carcinogenesis. Type I tumors are low-grade tumors which progress through a stepwise mutation process, grow more slowly, are less responsive to chemotherapy, and share molecular characteristics with low malignant potential (LMP) neoplasms. These tumors carry mutations in KRAS, BRAF and PTEN. Type II tumors are high-grade carcinomas which demonstrate greater genetic instability, are rapidly metastatic, relatively chemosensitive and without a clear precursor lesion. In these tumors EGFR, AKT2 and HER2 are often overexpressed, and p53 inactivity is frequent (13-15) (**Figure 3**).

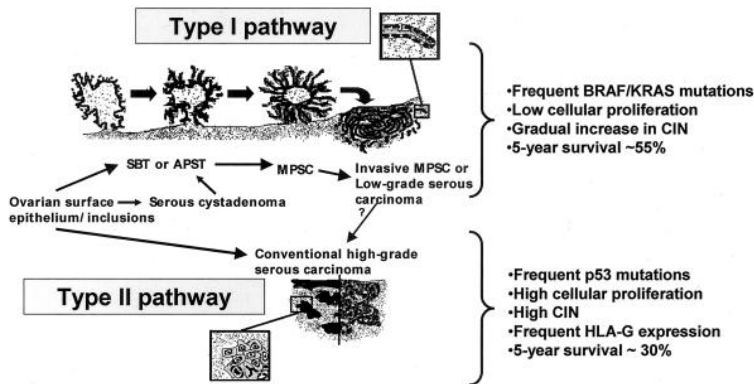


**Figure 3.** Type I and type II tumors. Reprinted from ref. (15) © (2004), with permission from Elsevier.

Incessant ovulations damage the ovarian surface epithelium and repair makes cells susceptible to mutations (16). The best evidence for this proposed mechanism is that early menarche, late menopause and nulliparity increase the risk of OC (17), while pregnancy, lactation and oral contraceptives decrease the risk (17-18). Environmental and genetic risk factors have also been identified. Hereditary OC accounts for approximately 10-15% of all OCs, and more than 90% of these result from germline mutations in BRCA1 or BRCA2. The lifetime risk of developing OC is 30-60% in women with BRCA1 mutation and 15-30% in women with BRCA2 mutation (14,19).

## 2.2. Clinical symptoms and examination

Contributing to the poor survival of OC is that most patients (>60%) present with advanced disease at the time of diagnosis (20). OC has traditionally been characterized as presenting without appreciable signs or symptoms until the late stages, and often these are ignored by the physician and the woman herself. Frequent symptoms are bloating, abdominal discomfort, early satiety, fatigue, fecal changes, urinary urgency, abdominal and pelvic pressure and pain (21). However, these symptoms are not specific for pelvic masses, but are also reported for other diseases. Recent studies have reported that symptoms are common, and awareness from doctors and the women themselves might improve the outcome (22-23). The diagnosis is based upon clinical examination, ultrasound, CT-scanning and serum parameters in order to distinguish a malignant pelvic mass from a benign one. Since its discovery, the serum cancer antigen, CA-125, has become a widely used tumor marker, and is now standard of care (24). Although CA-125 is elevated in more than 80% of women with OC at the time of diagnosis, it has proved poor in sensitivity and specificity for the detection of early stage disease (25). The combination of CA125, ultrasound and menopausal status of the woman has been used to calculate a risk of malignancy index (RMI) which improved the specificity and positive predictive value for the identification of a malignant pelvic mass (26). Identification of women at high risk of malignancy is crucial for the triage of these patients to appropriate cancer centers since treatment by multidisciplinary teams specialized in the management of OC have shown improved survival (27).

## 2.3. Staging

The present staging system for ovarian cancer was revised in 1988 by FIGO (The International Federation of Gynecology and Obstetrics) (28), and is detailed in **Table 1**. The staging system gives a detailed description of tumor spread inside and outside of the abdominal cavity. Extra-peritoneal tumor spread should be verified by biopsy or cytology preoperatively. Thorough initial staging is important to avoid “upstaging” from lower to higher stage in a later event (29). Malignant peritoneal effusions (ascites) can be present in patients at all stages (28), and occur in 2/3 of patients with advanced OC (30). The pleural space is the most common site for distant metastases outside the abdominal cavity (31), and malignant pleural effusions define OC stage IV even in the absence of solid metastases (28). Lymphatic spread to the pelvic and paraaortic lymph nodes is common in advanced stages (32), and lymphatic dissemination can also be seen above the diaphragm.

**Table 1 Carcinoma of the ovary: FIGO nomenclature (Rio de Janeiro 1988; ref.28).**

| Stage | Substage | Description |
|---|---|---|
| **Stage I** | | Growth limited to the ovaries |
| | Ia | Growth limited to one ovary; no ascites present containing malignant cells. No tumor on the external surface; capsule intact |
| | Ib | Growth limited to both ovaries; no ascites present containing malignant cells. No tumor on the external surfaces; capsules intact |
| | Ic [a] | Tumor either Stage Ia or Ib, but with tumor on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings |
| **Stage II** | | Growth involving one or both ovaries with pelvic extension |
| | IIa | Extension and/or metastases to the uterus and/or tubes |
| | IIb | Extension to other pelvic tissues |
| | IIc [a] | Tumor either Stage IIa or IIb, but with tumor on surface of one or both ovaries, or with capsule(s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings |
| **Stage III** | | Tumor involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equal Stage III. Tumor is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum. |
| | IIIa | Tumor grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologic proven extension to small bowel or mesentery |
| | IIIb | Tumor of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative |
| | IIIc | Peritoneal metastasis beyond the pelvis >2 cm in diameter and/or positive retroperitoneal or inguinal nodes |
| **Stage IV** | | Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV |

[a] In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage Ic or IIc, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings, or ascites.

Metastatic spread to the pelvic and/or paraaortal lymph nodes is of clinical importance for correct staging of disease, and for survival in certain subgroups of patients (29). Retroperitoneal lymphadenectomy unravels metastatic spread to lymph nodes in most patients with advanced stages of OC, and less frequently in patients with early stage disease. In the latter group, detection of retroperitoneal lymph node metastases will lead to upstaging from stage I to IIIC, and survival in this subgroup of patients is improved compared to patients with OC stage IIIC and intraperitoneal carcinomatosis (33). Whether patients with metastatic retroperitoneal lymph nodes without intraperitoneal carcinomatosis should

constitute a separate subcategory of stage IIIC is under debate (34). Hematogenous spread at the time of diagnosis is uncommon, but distant metastases to parenchymal organs, the central nervous system and the skeleton ultimately occurred during the course of the disease in 38% of patients whose disease was originally intraperitoneal (35). Hematogenous micrometastases are common in most epithelial malignancies, and in patients with OC metastatic cells in the bone marrow and peripheral blood were detected in 21% and 12% respectively (36). The presence of micrometastases might predict shorter disease-free survival, but the clinical significance of this finding needs to be further explored (37).

### 2.4. Histological classification and differentiation

Tumors of the ovary constitute three diverse pathological entities originating from the three cell types that make up the normal ovary; the epithelium covering the surface of the ovary or the inside of ovarian cysts, the germ cells and the sex cord/stromal cells. More than 60% of ovarian tumors originate from the surface epithelium, and they can be either benign, have low malignant potential or be malignant depending on the degree of proliferation, atypia and stromal invasion. The malignant forms account for almost 90% of ovarian cancers and are classified as ovarian carcinomas (OC). Germ cell and sex cord/stromal cell cancers collectively are responsible for fewer than 10% of malignant tumors of the ovary (38). Classification of OC is performed according to the cell subtype, the degree of differentiation, the pattern of growth and the presence or absence of a stromal component. The World Health Organization (WHO) recognizes eight major histological cell types, and the OCs are subclassified accordingly (39). The serous histological type represents approximately 75% to 80% of all epithelial cancer. Less common types are mucinous (10%), endometrioid (10%), clear cell, transitional type and squamous. According to their architectural features, carcinomas are differentiated into three grades corresponding to how much cancer cells differ from cells in normal tissue. Grade 1 corresponds to well differentiated, grade 2 to moderately differentiated and grade 3 to poorly differentiated tumors.

### 2.5. Surgical treatment of OC

Surgery is the cornerstone in treatment of OC. All patients who are fit for surgery should be considered for a full staging laparotomy for accurate information on diseases stage. Histology from preoperative and surgically removed tissue is fundamental for correct staging and choice of chemotherapy. Primary surgery followed by adjuvant chemotherapy is

the gold standard in all stages (40), but in advanced disease alternative treatment strategies are under debate.

### 2.5.1. Surgical treatment of early stage OC

Radical surgery is crucial for prognosis in early stage OC. Standard surgical procedure involves a total hysterectomy, bilateral salpingo-oophorectomy, omentectomy, pelvic- and paraaortic lymphadenectomy, random peritoneal biopsies and tapping of ascites or peritoneal washing in the absence of ascites (41). The performance of an exact staging procedure with adequate lymphadenectomy will, in many cases, lead to stage migration (upstaging) from presumptive lower stage disease to a higher stage with microscopic spread outside the ovaries (29,32,42). All patients with early stage ovarian cancer should be considered for adjuvant chemotherapy after removal of all visible evidence of tumor at laparotomy, and risk factors should be evaluated individually. Current opinion is that no adjuvant chemotherapy should be given to low risk early stage OC (i.e. FIGO Ia or Ib, diploid tumors, grade 1or 2, and not clear cell subtype) provided patients undergo proper radical surgery and surgical staging (43).

### 2.5.2. Surgical treatment of advanced stage OC (FIGO IIIC-IV)

As for early stage OC, surgery is also the cornerstone in treatment of advanced stages, but radical treatment is far more challenging in these patients. In addition to surgical procedures similar to those in the early stage, it is recommended to perform maximal debulking of tumor lesions in the abdominal cavity and on all peritoneal surfaces (41). The principal of maximum cytoreductive surgery in advanced ovarian carcinoma was first recognized by Meig in 1934, and later documented by Aure et al. (44) and Griffiths (45). These two papers were the basis for the currently accepted opinion that prognosis depends not only on FIGO stage and histological subtype, but also the extent of surgery and the resulting postoperative residual tumor. Residual disease after initial surgery is a strong independent prognostic factor for both overall survival (OS) and progression-free survival (PFS) in patients with advanced OC (31,46-54). Cut-off values for residual tumor have varied in the different reports, but cytoreductive surgery benefits all patients as each 10% increase in maximal cytoreduction appears to be associated with a 5.5% increase in median survival time (49). The addition of extensive surgery in order to achieve optimal or complete cytoreduction improves survival in patients with widespread disease in the upper abdominal cavity affecting the liver, spleen, diaphragm, omentum and the bowel (55-56). Surgical treatment

has become increasingly complex and extensive in all stages of OC, and it is suggested that patients with advanced OC will improve their short-term survival if surgery is performed by physicians trained in gynecological oncology (57). However, after comprehensive primary surgery, no residual disease was only achieved in 23% of patients with OC stage IIIC (50), in 11-30% of patients with OC stages IIIC and IV (52,55) and in 6-13% of patients with OC IV (51,54,58-59).

The concepts of neoadjuvant chemotherapy (NAC) before delayed primary surgery (DPS), or a repeated attempt of interval debulking surgery (IDS) following an initial suboptimal cytoreduction and several cycles of chemotherapy, evolved with the recognition of no residual disease as an important prognostic factor for survival in patients with advanced OC. The principle of chemotherapy prior to surgery is to reduce tumor size and thereby the opportunity to perform less extensive surgery. Based on a meta-analysis, more patients achieved optimal surgery after NAC compared to primary surgery (60), and lately this was confirmed in a prospective randomized study (61). The latter report found similar OS for the two treatment strategies, but NAC showed less morbidity. As for IDS, two prospective randomized studies have demonstrated opposite results as for clinical outcome (62-63), while a systematic review found IDS not to have an appreciable impact on survival outcome (64).

### 2.6. Chemotherapy in OC

Ovarian cancer was one of the first solid malignant tumors to be treated with chemotherapy after introduction of cytotoxic drugs in the twentieth century. Chemotherapy can be administered either as the only treatment, as adjuvant chemotherapy following surgery, or as neoadjuvant chemotherapy preceding surgery. Chemotherapy is also recognized as palliative treatment in patients with incurable cancer. In general, in OC, cytotoxic drugs are given as an intra-venous (i.v.) infusion, but can also be administered as an intraperitoneal (I.P.) treatment or as hyperthermic intraperitoneal chemotherapy (HIPEC) (65-66).
In early stage OC, two randomized clinical trials, ICON1 and ACTION, have compared surgery and adjuvant chemotherapy to observation following surgery (67). The combined report on 925 patients described a significant benefit in 5-year OS of 8% for adjuvant chemotherapy compared to the observational group. However, when sub-fractioning the patient groups, comprehensive surgical staging appeared to show similar survival in both arms while adjuvant chemotherapy produced significantly better results when the surgical

staging was inadequate. Still, adjuvant chemotherapy is state of the art in high-risk early stage OC (68). The 5-year relative survival for local disease was 89% from 2005-2009 in Norway (1).

Standard chemotherapy for patients with advanced OC has developed based on the results of a series of randomized trials (40). Cisplatin was introduced in the late 1970s, and soon became first line treatment in OC often in combination or triple combination with other chemotherapeutics (69). The taxane era began in the mid-1990s, and two randomized trials, the GOG111 and the OVAR10, compared cisplatin with either cyclophosphamide or paclitaxel. Both PFS and OS were significantly longer in the cisplatin-paclitaxel group compared to the cisplatin-cyclophosphamide group, but side-effects were reported more frequently in the former group (70-71). A combination regimen consisting of carboplatin-paclitaxel was not inferior when compared to cisplatin-paclitaxel, and showed less non-hematologic toxicity, improved quality of life and was easier to administer (72-73). From previous studies it is suggested that in terms of survival, platinum-based treatment was better than non-platinum regimens, platinum in combination was better than single agent platinum when used in the same dose, and carboplatin and cisplatin were equally effective. The combination regimen with carboplatin and paclitaxel every three weeks for a total of six courses is considered standard current therapy in most parts of the world (74). The addition of a third chemotherapeutic agent has not improved survival in primary treatment, and has added toxicity. In recent years, new targeted therapy has developed as supplement to the traditional agents, and one such target has been the angiogenesis and inhibition of vascular endothelial growth factor (VEGF). Recently, a publication from the ICON7 trial reported on improved PFS in women who had achieved standard combination regimen with carboplatin and paclitaxel plus the VEGF inhibitor bevacizumab, and the benefit was even greater among those at high risk for disease progression. However, bevacizumab was associated with more toxic effects (75).

Treatment compliance for six cycles is approximately 85% (72-73), and clinical response rates vary from 65-70% (73,76). Median PFS is only 16-21 months, as most patients with advanced OC eventually will relapse (72-73,76). Evaluation of chemotherapy response should be performed thoroughly to avoid prolonged unintended treatment with potential cytotoxic side-effects to the patient, and to objectively measure the treatment efficacy. The original definitions of response from World Health Organization were retained in the

response evaluation criteria in solid tumors (RECIST), and have later been revisited (RECIST1.1). The categories are complete response, partial response, stable disease and progressive disease. Each category refers to residual tumor size (target lesions) prior to chemotherapy compared to a repeated measurement after six courses (77). CA125 has also been incorporated in the RECIST1.1 criteria (78). In clinical trials there are strict practices on following these criteria, but in the context of evaluation outside of protocols, other response criteria might be used: pelvic examination, vaginal ultrasound or chest x-ray.

### 2.7. Treatment of recurrent OC

Most patients with advanced OC will eventually suffer from recurrent disease. Few patients will be found eligible for secondary cytoreduction at first relapse (79), and currently there is no available curative chemotherapy for patients with disseminated recurrent disease. Depending on the time from completed first-line treatment until histologically verified relapse or tumor progression, the tumors are classified as platinum-resistant (≤6 months) or platinum-sensitive (>6 months) (80). Patients with platinum-resistant disease have a response rate of 10% for platinum, and should receive alternative chemotherapeutics. In patients with platinum sensitive tumor, carboplatin-paclitaxel improved PFS and OS (81), and superior to this standard regimen was the combination of carboplatin-pegylated liposomal doxorubicin (82). Recognition of platinum-resistant or -sensitive disease is a prerequisite for correct choice of chemotherapeutic drugs, and for subsequent treatment success in recurrent OC. A large number of new drugs have reached clinical trials in recurrent disease giving prospects of improved survival also in these women (83).

## 3. Chemotherapy resistance

### 3.1. Principles and problems in chemotherapy

The main goal in chemotherapy is to selectively eradicate tumor cells. More traditional chemotherapeutics exert cytotoxicity in different phases of the cell cycle, causing DNA damage and eventually cell death. The prerequisite for their mechanism of action is that cancer cells are in a proliferative state. However, a fundamental problem in cancer treatment is that the biological mechanisms are basically the same in all cells, including tumor cells, and thus the therapeutic index (the ratio between toxic and therapeutic dose) becomes low. Toxicity becomes a critical issue and implies side effects to the patients which are most

often the limiting factor in treatment situations. In addition, most chemotherapeutics are not tumor specific, and thus have potentials in many types of cancers. Finally, the chemotherapy efficacy is, among various factors, dependent on tumor growth rate, tumor load, dormancy, metabolism and vascularization (10,84-86).

### 3.2. Primary and acquired resistance to chemotherapy in OC

First-line treatment with optimal surgery and combination chemotherapy yields 40-60% complete response in advanced OC (87). Refractory disease, defined as failure in response to primary treatment, is observed in approximately 5% of patients due to inherently resistance to Platinum-Taxane based chemotherapy (88). The clinical response to Platinum-based chemotherapy, and the duration of response after completed first-line treatment until histologically verified relapse or tumor progression, defines Platinum sensitivity; the tumors are classified as Platinum-resistant (≤6 months) or Platinum-sensitive (>6 months) (80). Approximately 17% of patients with advanced OC experience progressive or recurrent disease during the first 6 months after completion of first-line treatment (88). However, despite optimal treatment and initial response, most patients with advanced OC later relapse, only to experience a diminished sensitivity to various cytotoxic drugs. These patients have acquired resistance to chemotherapy which is suggested to be an adaptive response of cancer cells.

### 3.3. Mechanisms of chemotherapy resistance

Chemotherapy resistance can develop from multiple mechanisms which can act individually or synergistically, leading to multidrug resistance (MDR) (89). The multiple mechanisms can be classified as pharmacokinetic or pharmacodynamic (90), or can be explained by chemotherapy-mediated response in cancer cells (89). Independent of classification, the different pathways and mechanisms are integrated in one chemotherapy resistant phenotype. Pharmacokinetic resistance to chemotherapy involves extracellular drug distribution, passage across the plasma membrane, drug metabolism and drug excretion (90). The pharmacokinetic resistance also involves the relationship between tumor mass and drug distribution (86). Pharmacodynamic resistance to chemotherapy involves the biochemical effects of chemotherapy on cancer cells (90). Most chemotherapeutic agents primarily target proliferating cells, and secondarily, most cytotoxic agents induce apoptosis. However, perturbations in the apoptotic pathways reduce sensitivity to chemotherapeutic agents and the tumor cells become resistant (91-94). Aberrant expression of proteins involved in

apoptosis and in adjacent signaling pathways is observed in most cancer types including OC (87,95-96). Generally, and in contrast to normal tissue, large tumor loads are associated with reduced vascularization, generating an acidic and hypoxic microenvironment within tumor. In addition, the local microenvironment encompasses quiescent cells, and in different manners, these three elements modulate tumor cells to become resistant to chemotherapy (90,97). Finally, in recent years, the discovery of cancer stem cells in solid tumors, and their proposed role as a source of chemotherapy resistance, has elicited new prospects for targeted cancer therapy (98) **(Figure 4)**.

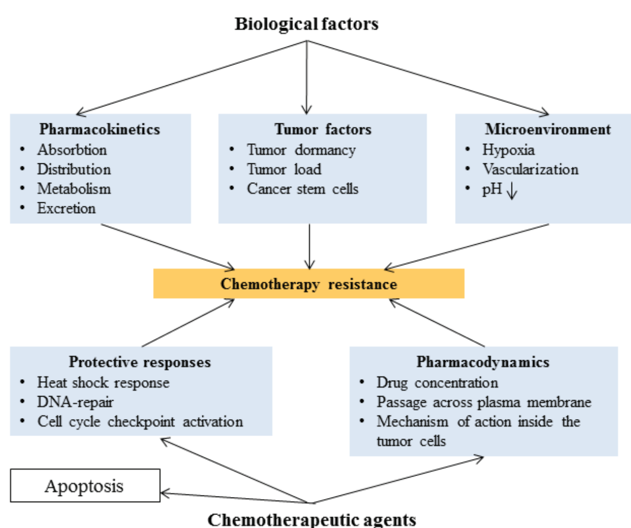


**Figure 4.** Factors involved in chemotherapy resistance.

### 3.4. Sensitivity to chemotherapy

Traditionally, the chemotherapy regimen used in advanced OC is evidence-based from clinical trials on large patient groups, and in many cases the choice of regimen is based on tumor histology. In recent years, a more individualized approach has been demanded, but an obstacle to patient-tailored chemotherapy has been cellular heterogeneity within tumor which impact processes like efficacy of chemotherapy (99). Individualized tumor response testing (ITRT) methods may predict tumor cell sensitivities to cytotoxic drugs, and may be used to individualize patient treatment. Different tissue *in vitro* assay techniques have been tested for evaluation of drug response (100), and in a prospective trial chemosensitivity

testing showed a trend towards improved response and PFS in patients with assay-directed treatment (101). Molecular methods for ITRT are based on single molecule markers of putative significance, and immunohistochemistry (IHC) can be a valuable tool in pilot studies. Ancillary methods include quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) for quantification of genes involved in therapy resistance in patients with OC (102-103), and oncogramme, with no published studies on OC yet (104).

## 4. Solid metastases and effusions

### 4.1. Invasion and metastases

Metastases, rather than the primary tumor are responsible for most cancer-associated deaths. It is suggested that metastatic dissemination of cancer cells from the primary tumor to various metastatic sites follows an orderly sequence of steps (105). The inception of the process is an uncontrolled cell proliferation in the primary tumor, leading to the invasion of the surrounding tissue. Increased primary tumor growth requires nutrition, and angiogenesis is the next central step of action. Once the microvasculature is established, the cancer cells can enter the blood and lymph vessels (intravasation), and provided that they survive in the bloodstream (micrometastases), cancer cells translocate to distant anatomic sites where they exit the microvessels and enter the distant tissues (extravasation). Finally, cancer cells adapt to their new microenvironment and proliferate in their new distant sites (colonization) (105-106), illustrated in **Figure 5**. The result of this multistep-process is metastases, the clinical sign of disseminated and advanced stage disease. The mechanisms behind metastasizing are complex and not fully elucidated. The tumor-stroma relationship is important for tumor vascularization, and stromal cells have been shown to be involved in this process (107-108). An important feature called 'epithelial-mesenchymal transition' (EMT) is the process where epithelial cells convert into mesenchymal cells and adapt properties that empower them to disseminate from a primary tumor (109). The metastatic cascade depends on the tumor cell genotypic and phenotypic diversity, the unique biological microenvironment at both the primary and the metastatic sites, and the interplay between these factors (110). Each step in the metastatic process is executed through a myriad of biochemical and genetic alterations in the cancer cells (111-112).

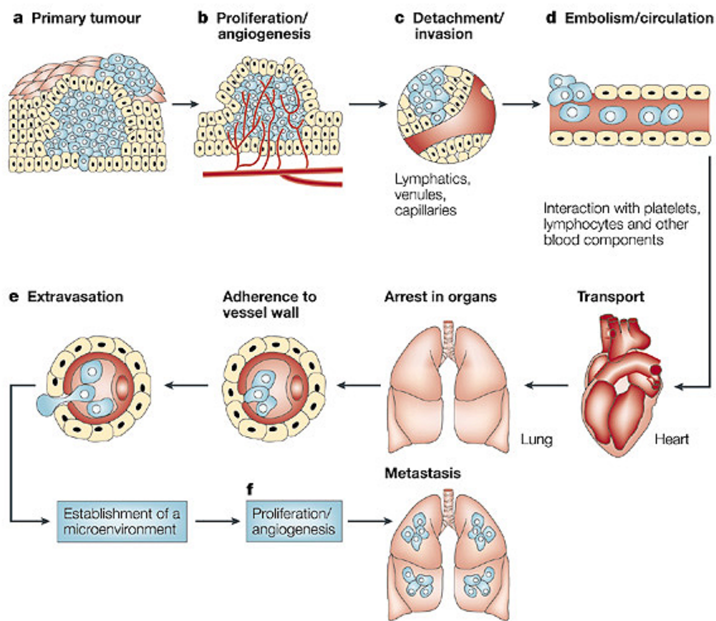


**Figure 5.** The metastatic cascade. Reprinted by permission from Macmillan Publishers Ltd: ref. (105), © (2003).

However, the recognition of intratumoral heterogeneity is fundamental since some cancer cells are primed to become metastatic, and some will stay anchored in their primary site. In addition, genetic changes develop along tumor growth and metastatic dissemination gives rise to numerous phenotypes which are modulated by their microenvironment, and different site-specific protein expression is observed in primary tumor and solid metastases, reflecting the heterogeneity in carcinoma cells along tumor progression (113-114).

### 4.2. Effusions in OC

The serosal cavities in the body are lined with a membrane consisting of mesothelial cells which have the ability to produce and secrete serous fluid. Excessive fluid in serosal cavities can affect the pleural, peritoneal (ascites) and, less often, the pericardial space. Ascitic accumulation is the combined result of lymphatic obstruction by tumor cells and thereby increased serous fluid (115-116), and vascular invasion and increased permeability due to secretion of VEGF and cytokines (117-118). Fluid is actively secreted into the serosal cavity, and increased volume is related to increased clinical symptoms. The clinical manifestations of excessive fluid in the pleural- and peritoneal cavities include abdominal swelling, heaviness and discomfort, and shortness of breath. Different medical conditions herald these clinical manifestations, and the appearance of malignant cells in effusions is a

frequent event in the clinical setting of cancer (119). More than any other malignant neoplasm, OC is associated with the accumulation of fluid in the peritoneal cavity. In OC, ascites is found in 75% of patients with advanced stages (30), and malignant pleural effusion defines stage IV disease even in the absence of solid metastases, and is present in approximately 40% of patients (28,31). Carcinoma cells spread primarily by exfoliation of malignant cells, and the close anatomical relationship between the surface epithelium of the ovaries and the serous membrane covering the peritoneal cavity facilitate implantation and give rise to malignant peritoneal effusion (30). Malignant cells in the pleural cavity have the same pathogenesis as in the peritoneal cavity, and results from hematogenous spread to the pleura, lymphatic obstruction and increased vascular and pleural membrane permeability (120). Direct shedding of cancer cells into the peritoneal cavity does not affect the pleural cavity, but a 'porous diaphragm syndrome' is proposed to enable substances to pass from the peritoneal to the pleural cavity through defects in the diaphragm (121).
Malignant effusions consist of malignant cells, reactive mesothelial cells and leukocytes, the latter consisting predominantly of macrophages and tumor-infiltrating T-cells, which may support the growth of cancer cells rather than limit it (122). In particular, mesothelial cells have cross-talk with tumor cells, and are able to synthesize many of the proteins that regulate tumor growth in malignant effusions (123). OC cells in effusions can proliferate and metastasize despite their hypoxic environment, and lack the ability to induce angiogenesis (124). Furthermore, the malignant cells in serous fluids have lost their tissue anchorage, but still have the capacity to overcome anoikis, the cell death that occur when cell-matrix interaction is insufficient (125). Cancer cell survival in this hypoxic and nutrient deficient microenvironment is a result of their versatile nature (124). Unlike solid tumors, carcinoma cells in effusions are not amenable to surgical eradication, and development of chemotherapy resistance along tumor progression is one of the main reasons for treatment failure in OC (87,123).

### 4.3. Anatomic site-related alterations of cancer-associated molecules in OC

Our group has previously observed extensive variations in protein expression patterns in the primary tumor and corresponding effusions from patients with OC, but few differences were seen in expression between pleural and peritoneal effusions (126). The genetic disparity between the primary tumor and its metastases will eventually lead to different protein expression patterns of cancer-associated molecules in these anatomic sites, and our group has extensively investigated this issue in patients with OC (119,123-124,127-128).

E-Cadherin and catenins, both involved in cell adhesion, have shown increased protein expression in effusions compared to primary tumor and solid metastases in patients with OC (129). Other molecules with up-regulated protein expression in effusions are β1-integrin (130), p75 (131), MMP-2 (132), Rsf-1 (133), Claudin-1, -3 and -7 (134) and XIAP (135).

Molecules which are down-regulated in OC effusions include; TrkA protein (131), TIMP-2 mRNA (132), cytoplasmic protein expression of Survivin (135), VEGF and IL-8 mRNA (118), GEP protein (136) and protein expression of nuclear factor-κB (NF-κB) and inhibitor-κBα (Iκ-Bα) (137).

### 4.4. Molecular alterations in effusions along tumor progression in OC

In addition to being differently expressed in effusions compared to primary tumor and solid metastases, several cancer-associated molecules are differently expressed in effusions taken at primary diagnosis (pre-chemotherapy effusions) compared to effusions obtained at disease recurrence (post-chemotherapy effusions). These differences may associate with patient survival, a finding related to disease progression. Even if there is no significant difference in expression between pre- and post-chemotherapy effusions from patients with OC, the studied molecules may still have prognostic value in the two groups (123-124). Significantly increased expression of SIP1 mRNA in pre-chemotherapy effusions, and increased expression of DJ-1 mRNA in post-chemotherapy effusions had no impact on survival (138-139).

In pre-chemotherapy effusions, increased protein expression of p21-activated kinase 1 (Pak1) and nuclear Survivin are prognostic factors for improved PFS (135,140), while increased Claudin-7 expression is associated with poor OS in univariate survival analysis, but was not a prognostic factor in Cox multivariate analysis (134).

In post-chemotherapy effusions, increased protein expression of Pak1 reduces OS significantly in Cox analysis (140). Increased Claudin-1 and -3 expressions were associated with poor OS in survival analysis, but only Claudin-3 was a prognosticator for poor OS in Cox analysis (134). Increased cleaved Caspase-3 in post-chemotherapy effusions improved PFS and OS in survival analysis, but was a prognostic factor only for improved PFS (137). In survival analysis, poor PFS and OS was associated with increased expression of Annexin-V protein, and poor OS was associated with increased Rsf-1 protein expression, but none of the molecules were prognostic factors for PFS or OS (133,141).

# 5. Apoptosis

## 5.1. Apoptosis – programmed cell death.

To balance growth, normal cells are subjected to death. The well-regulated multi-step process of self-destruction of cells is called apoptosis, or programmed cell death (11). Apoptosis is genetically programmed and is a vital component of various processes in the normal cells including cellular homeostasis, proper development and functioning of the immune system, hormone-dependent atrophy and embryogenesis (142). The morphological characteristics of apoptosis include cellular detachment, cell shrinkage, chromatin condensation, membrane blebbing and nuclear and chromosomal fragmentation (93). There are two main pathways to apoptosis-associated death; the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway (142). (**Figure 6**).

The extrinsic pathway is initiated by ligation of extracellular death ligands to transmembrane death receptors including tumor necrosis factor receptor (TNFR), Fas/CD95 and TRAILR. The intracellular receptor domain, the death domain (DD), attracts adaptor proteins which in turn recruit caspases (caspase-8 and -10) to the cellular membrane, and together they make up the death-inducing signaling complex (DISC). Activation of caspase-8 cleaves and activates caspase-3 and -7 which further initiates caspase activation events that culminate in substrate proteolysis and cell death (91,93).

The intrinsic pathway is initiated in response to a myriad of insults including DNA damage, growth-factor deprivation, oncogene activation, viral infections, hypoxia, UV and stress, which all can activate BID and BIM (143). Activated BID and BIM induce activation of the pro-apoptotic Bcl-2 family members BAK and BAX (144). The activated BAK-BAX-oligomers participate in the formation of pores in the outer mitochondrial membrane which facilitates the mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome *c*, Smac/DIABLO and other pro-apoptotic proteins into the cytosol (93,145). Cytochrome *c* release is the essential "point of no return" step in the intrinsic pathway of apoptosis, and is controlled and mediated by pro- and anti-apoptotic proteins of the Bcl-2 family (145-146).

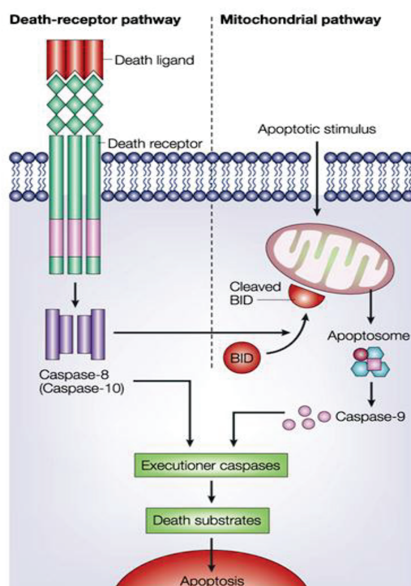


**Figure 6.** Apoptosis. The death-receptor pathway and the mitochondrial pathway. Reprinted by permission from Macmillan Publishers Ltd: ref. (91), © (2002).

In the cytosol, cytochrome *c* binds apoptotic protease activating factor-1 (APAF1), ATP and the inactive pro-caspase-9, forming a complex called the apoptosome, and at the apoptosome caspase-9 is activated. Caspase-9 activates effector caspases, most notably caspase-3. Both pathways converge at the level of caspase-3, an effector caspase which triggers the execution pathway through activation and cleavage of a cascade of caspases that eventually cleave cellular substrates, leading to the biochemical and morphological changes that define apoptosis (91,142). Apoptosis is not restricted to either one pathway, and considerable crosstalk exists between the two main pathways (93,147).

**5.2. Regulation and dysregulation of apoptosis**

The apoptotic machinery is tightly regulated at different levels by pro- and anti-apoptotic proteins, and defects in control mechanisms are essential features in cancer initiation, development, progression and chemotherapy resistance (91-92,94,148-150). A small selection of an increasing list of regulators includes the tumor-suppressor protein p53, which, when down-regulated, allows incipient cancer cells to avoid apoptosis. Up-regulation

of p53 can activate death receptors and pro-apoptotic Bcl-2 family members, and repress anti-apoptotic Bcl-2 family members (148-149). The dual roles of the Bcl-2 family members as either pro-apoptotic or anti-apoptotic proteins confirm their major role in regulating and modulating MOMP (145-146). Other main regulatory proteins include the inhibitor of apoptosis (IAP) proteins which bind to and inhibit caspases in the executioner pathway (151). Additional regulators include cFLIP which binds to the DISC to inhibit activation of caspase-8, NF-κB and Smac/DIABLO (91). Finally, members of the PI3-K/AKT-pathway (152) and heat-shock proteins (HSP) (153) also regulate apoptosis.

## 6. Anti-apoptotic molecules investigated in this thesis

### 6.1. Bcl-2 protein family

The BCL2 gene was initially identified from the breakpoint of the t(14;18) chromosomal translocation found in the vast majority of follicular lymphomas (154-155), and this initiated a new class of oncogenes, the Bcl-2 protein family. The Bcl-2 protein family share sequences homology in one to four domains known as Bcl-2 homology (BH) domains named BH1, BH2, BH3 and BH4. Depending on structure and function, the Bcl-2 protein family has classically been grouped into three classes; the anti-apoptotic Bcl-2 proteins, the pro-apoptotic Bcl-2 proteins and the BH3-only proteins (145). Among the 25 Bcl-2 protein family members detected so far, the core family members are multidomain proteins conserving three to four BH-domains, and are designated as either anti-apoptotic or pro-apoptotic proteins. The main anti-apoptotic proteins are Bcl-2 proper, Bcl-$X_L$, Bcl-w and MCL-1, and the main pro-apoptotic proteins are BAK and BAX. (146). A subset of pro-apoptotic molecules share sequence homology only in the BH3 domain and are referred to as BH3-only proteins, and these in turn can be divided into BH3-only activators (BID, BIM and PUMA) and BH3-only sensitizers (BAD, BIK and NOXA) (145-146). The ability of Bcl-2 proteins to selectively bind each other is integral to their function and finely balances pro-apoptotic and anti-apoptotic activity in the cell (146).

#### 6.1.1. The anti-apoptotic proteins Bcl-2 proper and Bcl-$X_L$

The bcl-X gene function as a Bcl-2 independent regulator of apoptosis, and due to alternative splicing it has shown to encode for Bcl-$X_L$, an apoptosis suppressor (156). Both the Bcl-2 and Bcl-$X_L$ proteins are embedded in the nuclear membrane, endoplasmic

reticulum and the outer mitochondrial membrane (157). In addition, Bcl-$X_L$ exists partly in the cytosol and translocates from the cytosol to the mitochondria during apoptosis (158). The Bcl-2 and Bcl-$X_L$ proteins share four domains of homology (BH1, BH2, BH3 and BH4) where BH1–BH3 forms a hydrophobic cleft further stabilized by the BH4 domain which is required for the function of anti-apoptotic Bcl-2 proteins (159). Bcl-2 and Bcl-$X_L$ proteins oppose apoptotic activity and thereby enhance cell survival, a function linking them to carcinogenesis, tumor progression and chemotherapy resistance (91,146,160). However, lymphoid cell cultures transfected with Bcl-2 and Bcl-$X_L$ have shown to differently block chemotherapy-induced cell death (161). The anti-apoptotic proteins Bcl-2 and Bcl-$X_L$ are capable of binding directly to BAK and BAX, and inhibit their oligomerization and thus initiation of MOMP. However, their most important mechanism of anti-apoptotic activity is binding and sequestration of BH3-only activators before they interact with BAK and BAX (162). To balance Bcl-2- and Bcl-$X_L$ anti-apoptotic activity, a subset of BH3-only sensitizers, which lack the ability to induce BAK and BAX activation directly, compete for binding to the anti-apoptotic proteins and thereby making them unable to sequester BH3-only activators. Then BH3-only sensitizers behave as antagonists to anti-apoptotic proteins and thereby enhance apoptosis. Sensitizer BH3-only proteins lower the threshold of apoptosis by occupying the binding pocket of anti-apoptotic molecules, and allowing activator BH3-only proteins to engage BAX/BAK to induce MOMP (146). There is also evidence that each individual anti-apoptotic protein is selective for interaction only with a subset of BH3-only proteins (163-164).

#### 6.1.2. Bcl-2 and Bcl-XL in OC

Bcl-2 expression is found in the surface epithelium in the majority of normal ovaries (79%), benign tumors (100%) and borderline tumors (78%; 165). The clinical role of Bcl-2 and Bcl-$X_L$ in OC has shown inconsistency in different studies (166-182). Two clinical studies confer Bcl-2 and Bcl-$X_L$ no prognostic significance by itself, but when Bcl-2 was co-expressed with selected apoptotic proteins they represented a significant prognostic factor for improved survival in OC stage III (166-167). Consistently, three other studies reported on similar results in patients with OC stage I-IV (168-170). However, one report showed that increased Bcl-2 expression itself was associated with improved survival in patients with OC FIGO stage II-IV (171). In addition, there are opposite results concerning tumor differentiation and Bcl-2 protein expression (172-173).

In chemotherapy resistant OC cell lines, Bcl-2 and Bcl-$X_L$ are overexpressed, and chemotherapy sensitive cell lines transfected with either Bcl-2 or Bcl-$X_L$ have increased resistance to chemotherapy (171,174-175). Contradictory to this, in one study both chemosensitive and insensitive cell lines showed high basal expression of Bcl-$X_L$, and when incubated with cisplatinum, the Bcl-$X_L$ protein was down-regulated in the chemosensitive cell line, and was associated with apoptosis and absence of recurrence. The chemoinsensitive cell line showed opposite results (176).
In analysis of paired patient tumors obtained at primary disease and at recurrence followed by cisplatinum-based regimens, Bcl-$X_L$ protein expression was increased in the latter group (175). In patients with both measurable and evaluable disease after surgery, Bcl-2 protein expression was decreased in patients who had complete response to chemotherapy. Additionally, in advanced stage OC, patients with Bcl-2-positive serous carcinomas had poorer survival (177). At the mRNA level, Bcl-$X_L$ expression did not differ between carcinomas, benign tumors and normal ovaries. Bcl-2 mRNA expression in ovarian cancers was lower than in benign tumors and normal ovaries, and no association was found between expression levels and survival (178). The above findings suggested a role for Bcl-2 and Bcl-$X_L$ in carcinogenesis and chemotherapy resistance (160).

**6.2. Bag protein family**

In search of Bcl-2 interactors, a novel protein was first discovered when complimentary DNA (cDNA) encoding proteins binding to Bcl-2 were identified. The gene encoding this protein was termed Bag-1, for Bcl-2-associated athanogene 1, and constituted a novel protein family. Bag-1 shares no similarity with Bcl-2 or its homologue proteins, but elevated levels cooperate with Bcl-2 in suppression of apoptosis (183). The Bag family consists of six family members, Bag-1 to Bag-6, which reportedly can regulate versatile biochemical processes including protein kinase activity, receptor signaling and transcription factor activity, that are important for cell stress responses, apoptosis, proliferation, neuronal differentiation, cell migration and hormone action (184-185). Homologues of the Bag family share a common conserved region located near the C-terminus, termed the Bag domain (BD), with the exception of Bag-5, which has four domains. Bag proteins also contain diverse N-terminal regions that target cellular locations and interact with other proteins involved in numerous cellular processes (184). Amongst these are Bcl-2 and Bcl-$X_L$, which bind directly to the Bag-1 protein (186). The BD in Bag proteins mediates direct interaction with the ATPase domain of HSP70, thus regulating its chaperone activity (185).

The co-chaperone activity of Bag proteins is essential to their function as bridges between different molecules bound to the N-terminal, and HSP70 bound to the BD, and also might explain why Bag proteins influence diverse activities (187).

### 6.2.1. Bag-1

Bag-1 is the most studied protein among the Bag family members, and is expressed in most normal human tissues. Four isoforms have been identified and designated Bag-1L, Bag-1M, Bag-1S and p29, of which Bag-1L is targeted to the nucleus, Bag-1M to the cytosol with a capacity for translocation to the nucleus, Bag-1S is the most abundantly expressed and p29 hardly detected in cells (188). Bag-1 exerts co-chaperone activity on the Bcl-2 protein and the HSP70 chaperone in an ATPase-dependent manner (183,189). In addition to the BD, all Bag-1 isoforms contain a ubiquitin-like (UBL) domain linking HSP70 to the proteasome, suggesting involvement in proteasome-mediated protein degradation (190). Bag-1 is a potent regulator of cell signaling molecules, and represents a link between anti-apoptotic mechanisms and growth factor receptors. Bag-1 binds to the serine/threonine kinase Raf-1 and stimulates its kinase activity, resulting in a cascade of phosphorylation events in the MAPK/ERK signal transduction pathway, ultimately controlling cell growth (191). Bag-1 has stronger affinity for Raf-1 than HSP70, but under stressful conditions with increased expression of HSP70, Bag-1/Raf-1 complexes are replaced by Bag-1/HSP70 complexes and the kinase activity of Raf-1 is inhibited (192). Bag-1 is also suggested to associate with platelet derived growth factor receptor (PDGFR) and the hepatocyte growth factor (HGF) receptor Met, promoting cell growth (193). Regulation of several nuclear hormone receptors is performed in an isoform-specific manner. Bag-1L, but not Bag-1M or Bag-1S, increases androgen receptor function (194), and in contrast, Bag-1M, but not Bag-1L and Bag-1S, inhibits glucocorticoid receptor activity (195). Furthermore, different Bag-1 isoforms have been shown to possess distinct anti-apoptotic functions in breast cancer cells in vitro (196).

### 6.2.2. Bag-4

Bag-4 was identified when searching for proteins regulating HSP70 (197), and contains a BD that is shorter than in any other family member and represents the minimal functional fragment that is capable of binding to HSP70 (198). Bag-4, also known as the silencer of death domain (SODD) protein, interacts with the intracellular DD of TNFR1 and death receptor 3 (DR3), maintaining their monomeric inactive state (199). It is suggested that HSP70 co-operate in regulating this interaction (200). SODD is released from the DD upon

ligand binding of TNFR1, allowing for receptor aggregation and recruitment of adaptor proteins to the membrane to form the TNFR1 signaling complex which again initiates apoptosis and activation of NF-κB (199-200). In a similar fashion to TNFR1, SODD interacts with DR3, but does not interact with TNFR2, Fas, DR4 or DR5 of the tumor necrosis factor receptor superfamily. Furthermore, SODD and TRADD cannot simultaneously interact with the intracellular DD, and consequently SODD overexpression inhibits TNF-induced cell death (199).

### 6.2.3. The clinical role of Bag-1 and Bag-4

The clinical role of Bag-1 and Bag-4 in OC has been less studied than that of Bcl-2 family proteins. Increased cytoplasmic Bag-4 expression was associated with improved PFS and OS in newly diagnosed OC, while no such association was found for Bag-1 (172). Conversely, increased Bag-4/SODD expression suppressed apoptosis and correlated with aggressive disease in pancreatic cancer (201). Overexpression of Bag-1 is connected to aggressive disease in colon and prostate carcinoma (202-203), while in non-small cell lung carcinoma it has proved to be an independent prognostic factor for improved survival (204). Different functions for different Bag-1 isoforms have been evaluated, and overexpression of Bag-1 p29 isoform by itself did not protect OC cells from drug-induced apoptosis in an in vitro study, but overexpression of Bag-1 p29 isoform in the presence of EGF enhanced resistance to drug-induced apoptosis in an OC cell line (205). In a Bag-1 transfected cervical carcinoma cell line treated with various cytotoxic drugs, some Bag-1 isoforms showed enhanced resistance to apoptosis (206). Bag-1 may have a role in chemotherapy resistance, and in an in vitro study a paclitaxel resistant OC cell line showed increased Bag-1 expression (207). Bag-1 expression has also previously been described in endometrial carcinoma (208).

## 6.3. Heat shock protein family

Heat shock proteins are a class of functionally related proteins present in virtually all eukaryotic cells, and their synthesis is tightly regulated at the transcriptional level by heat shock factors (HSF) (209). HSPs have been classified into five families according to their molecular weight: HSP110, HSP90, HSP70, HSP60 and the family of small HSPs including HSP27 (210). Additionally, each HSP family has isoforms which are targeted to different sub-cellular compartments (211). HSP27, HSP70 and HSP90 are mainly located in the cytosol and the nucleus, and HSP70 and HSP90 are also found in the endoplasmic reticulum

and the mitochondrion (209). HSP70 is hardly expressed while HSP90 is abundant at the basal level in non-stressed cells (212), and HSP27 is differently expressed from nearly undetectable and low levels to abundant depending on cell type (213). However, in cancer cells HSP27, HSP70 and HSP90 are abundantly expressed. The high molecular weight HSP70 and HSP90 are ATP-dependent chaperones and undergo a conformational change upon ATP binding (211). In contrast, the small molecule HSP27 acts in an ATP-independent fashion to achieve chaperone activity and forms oligomers regulated through phosphorylation (213). Furthermore, HSP90 and HSP70 chaperone activity is regulated by co-chaperones that increase or decrease their affinity for client substrates through stabilization of the ADP-ATP bound state, thus functioning in large complexes known as the chaperone machinery (211,214). A partial list of co-chaperones includes HSP40, Bag-1, CHIP, HIP and HOP for HSP70, and AHA 1, p23, HOP and Cdc37 for HSP90, while HSP27 has no co-chaperones (214-215). HSPs have been characterized as intracellular chaperones which have in common the property of modifying the structures and interactions of client proteins, and are required for 'housekeeping' functions such as protein folding during nascent polypeptide-chain synthesis, preventing protein aggregation, intracellular disposition and translocation of proteins across membranes, and protein degradation. Under physiological conditions, their expression must be low to allow for cellular activities to proceed, but in response to a wide variety of physiological and environmental stress conditions (heat, hypoxia, toxins, infection etc.) the intracellular expression of HSPs increases in an attempt to restore the normal protein-folding environment. The cytoprotective function of HSP activity and increased expression in response to stress insults and stimuli can largely be explained by HSPs' anti-apoptotic properties as they have been demonstrated to interact with molecules in the apoptotic pathway and thereby enhance cell survival, **figure 7**. HSPs are overexpressed in a wide range of human cancers and are implicated in tumor cell proliferation, differentiation, invasion, metastasis, death and recognition by the immune system (211-212,214,216-217). In addition, HSP70 and HSP90 are able to translocate to the extracellular matrix where they mediate tumor cell invasiveness and immunological functions, thus playing an important role to form the basis of anticancer vaccines (212,218-219).

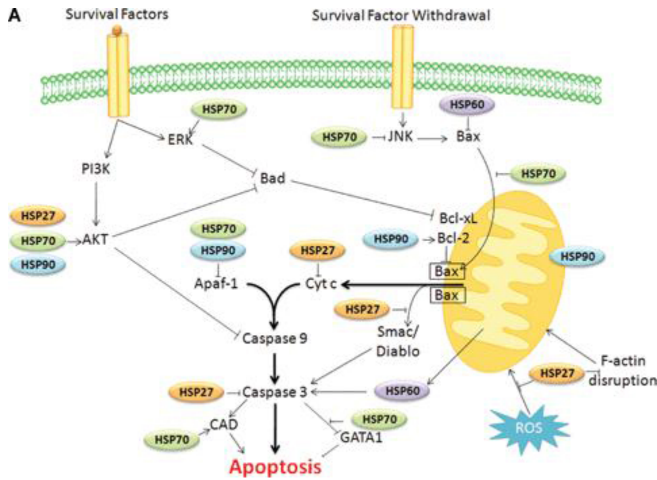


**Figure 7.** Heat shock proteins in apoptosis and cell survival. Reprinted from ref. (211) © (2008), with permission from John Wiley and sons.

### 6.3.1. HSP27 in apoptosis

HSP27 is able to regulate apoptosis at different stages; upstream, downstream and at the mitochondria, illustrated in **figure 7**. In stressed cells, HSP27 is suggested to regulate apoptosis through interaction and activation of AKT, and thereby indirectly inhibits BAX activation, BAD activation and caspase-9 cleavage (211).

HSP27 also binds directly to cytochrome *c* in the cytosol, thus inhibiting the formation of the apoptosome complex (220), and also suppresses procaspase-3 activation (221). Under stressful conditions induced by various stimuli, overexpression of HSP27 favors degradation of I-κBα, leading to amplification and enhanced activity of NF-κB in the nucleus, thus mediating negative regulation of apoptosis (222). In addition, HSP27 prevents translocation of pro-apoptotic factors like activated Bid (tBid) onto the mitochondrial membrane, and as shown as in multiple myeloma cells, inhibits the release of the mitochondrial protein Smac/Diablo (211).

### 6.3.2. HSP70 in apoptosis

HSP70 has also shown to inhibit the apoptotic pathway at different levels, **figure 7**. At the pre-mitochondrial level, HSP70 stabilizes and modifies AKT kinase and thus suppresses apoptotic signaling (223). In hyperosmolar cells, HSP70 modulates c-Jun N-terminal kinase (JNK), leading to inhibition of BAD pro-apoptotic activity. HSP70 has also shown to inhibit pro-apoptotic BID activation and downstream events. Together with co-chaperone HSP40 it hinders BAX translocation to the outer mitochondrial membrane, and thereby prevents permeabilization and release of cytochrome *c* and AIF (210-212). HSP70 is able to interact with Apaf-1 and thereby inhibit recruitment of procaspase-9 to the apoptosome and consequently caspase-3 activation (224). In Bcr-Abl expressing cells, HSP70 binds to the death receptors DR4 and DR5, thereby inhibiting the activity of the DISC in the external apoptotic pathway (225).

### 6.3.3. HSP90 in apoptosis

The chaperone role of HSP90 affects the activity and stability of many client proteins, **figure 7**. In relation to apoptosis, HSP90 regulates many kinases and transcription factors, such as AKT, p53, NF-κB, JNK and TNFR (216). HSP90 directly interacts with AKT by inhibiting its dephosphorylation and thus maintains its phosphorylated active state (226). Indirectly, through phosphorylated AKT, HSP90 is able to inactivate the pro-apoptotic protein BAD and caspase-9 (152,227). Activated AKT can also phosphorylate I-κB which promotes NF-κB-mediated cell survival. HSP90 is essential for TNF-mediated NF-κB activation through stabilization of receptor interacting protein (RIP) (228). In addition, it negatively regulates oligomerization of Apaf-1 and thus prevents further recruitment of procaspase-9 and the formation of an active apoptosome complex (229). The isoform HSP90β is able to associate with Bcl-2 in mast cells, preventing the release of cytochrome *c* and subsequent activation of caspase-3, and the HSP90 homologue tumor necrosis factor receptor-associated protein 1 (TRAP-1) is localized to mitochondria in tumor cells, regulating mitochondrial membrane permeabilization and cytochrome *c* release (211-212). Furthermore, TRAP1 is more abundant in tumor cells than in normal tissues, and is subjected to selective inhibition in malignant conditions (215).

### 6.3.4. Heat shock proteins in OC

Higher protein expression of HSP27 and HSP70 was associated with more aggressive disease in ovarian cancer (230-231). In addition, the expression of mRNA coding for HSP90

was increased in advanced stages of OC (232). In serological screening, HSP90 antibodies were frequently found in late stage ovarian cancer (233), and antibodies to HSP27 were elevated in 50% of patients with OC (234). Data regarding the clinical outcome of HSP27 protein expression is conflicting as one study found increased HSP27 to be associated with shorter OS (230) while another study found decreased HSP27 to correlate with increased stage and to be a prognostic factor for reduced survival in women with OC (235). Moreover, in advanced stage OC the absence of HSP27 was associated with increased survival, but HSP27 was not an independent factor (236). Finally, one study found increased expression of HSP27 to be a prognostic factor for reduced survival in multivariate analysis, while HSP70 and HSP90 were not associated with survival (237). Decreased expression of HSP70 was associated with increased stage of disease, and in endometrioid tumors, increased expression of HSP70 was associated with high grade OC (172). HSP27 protein expression in tumors taken at primary disease compared to tumors at recurrent disease did not predict chemotherapy resistance (238). HSP27 and HSP90 mRNA expression was found to be up-regulated in a resistant OC cell line, and the sensitive cell line became more resistant when transfected with HSP27 (239). HSP90 inhibitors have entered clinical trials, and the first HSP90 inhibitor, Tanepimycin (17-AAG), is extensively studied (240-241). Carboplatinum in combination with 17-AAG has shown additive growth inhibitory effects in OC cell lines (242). Furthermore, 17-AAG may sensitize a subset of OCs to paclitaxel, particularly in those tumors in which resistance is driven by ERBB2 and/or p-AKT (243).

## 7. PI3-K/AKT - signaling pathway

The complex communication between cells and between substances within the cells frequently involves extracellular signal reception and intracellular conversion of signals from one form to another, and finally altering the cell behavior. This process of extracellular signaling altering cellular behavior is known as cell signaling. The different signaling pathways govern basic cellular activities such as cell division, cell cycle arrest and apoptosis, and are essential to maintain normal cellular homeostasis. Conversely, errors in signaling pathways are often seen in malignant diseases (244).

The PI3-K/AKT-signaling pathway regulates cell metabolism, cell growth, cell survival, protein synthesis, migration and apoptosis. Activation of receptor tyrosine kinases (RTKs)

such as PDGFR, EGFR and insulin-like growth factor receptor (IGFR) is the most widely studied mechanism of PI3-K/AKT activation, **figure 8**.

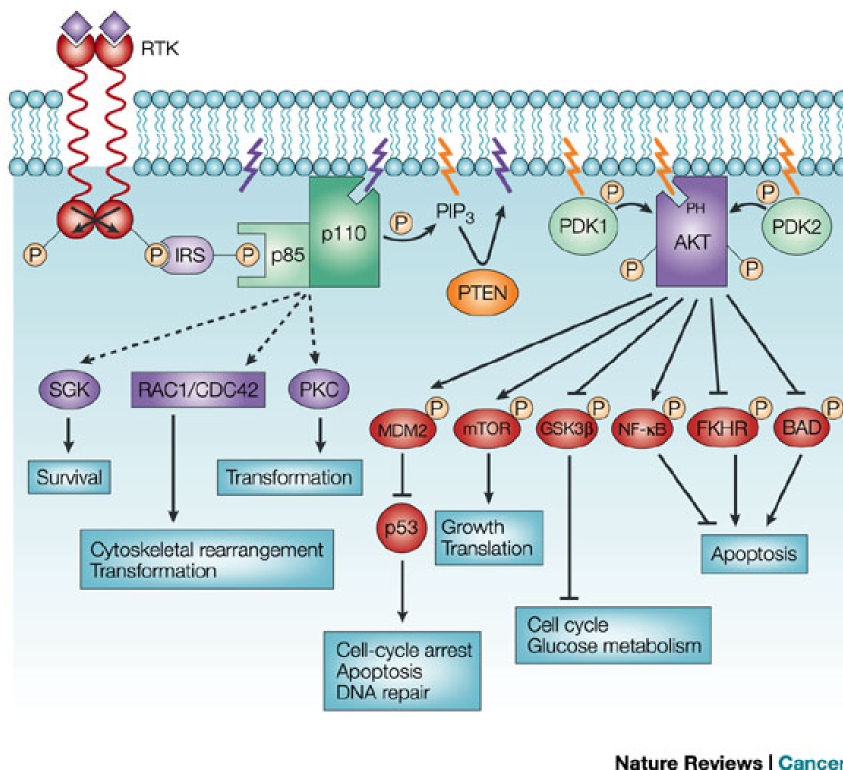


**Figure 8.** The PI3-K/AKT pathway. Reprinted by permission from Macmillan Publishers Ltd: ref. (251), © (2002).

In addition to RTKs, the PI3-K/AKT-pathway is activated by integrins and G-protein coupled receptors (GPCRs), and by intracellular proteins such as protein kinase c (PKC), Ras and steroid receptors (245-249). Activation of RTKs triggers phosphorylation of the cytoplasmic domain of the receptors followed by recruitment of phosphatidylinositol 3-kinase (PI3-K) to the membrane. The PI3-Ks are lipid kinases grouped into three classes (I, II and III) according to their structure, regulation and substrate selectivity (250-251). The class $I_A$ PI3-K is widely linked to RTK signaling, and it catalyzes the phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-bisphosphate (PIP3) (245). Phosphatase with tensin homology (PTEN) is a frequently mutated tumor suppressor gene in human cancer (248) and reverses the phosphorylation of

PIP3. The regulation of PTEN is unclear, but DJ-1 has proved to be a suppressor of the PTEN function, and is expressed in different cancers (252). DJ-1 mRNA has also shown to be frequently expressed in advanced OC (139), and in proteomic analysis DJ-1 was identified as a marker of cisplatin resistance in non-small cell lung cancer (253). However, PIP3 serves as a docking-site for proteins containing pleckstrin homology (PH) domains, and promote their translocation to the cell membrane. Among proteins recruited to the cell membrane are the serine-threonine kinase AKT and the 3-phosphoinositide-dependent kinase 1 (PDK1) (246-249).

### 7.1. The AKT kinase

AKT is classified as a family of three closely-related, highly conserved homologues; AKT1, AKT2 and AKT3. The encoded proteins are serine/threonine kinases that belong to the protein kinase B (PKB) family. Each AKT family member contains a PH domain with an N-terminal, a central kinase domain and a hydrophobic carboxyl-terminal regulatory motif (254). Activation of AKT is a multistep process where PDK1, also recruited to the membrane by PIP3, phosphorylates AKT at the Thr308 residue (T308), which is absolutely required for AKT activation (247). Subsequent phosphorylation by the rapamycin-insensitive mTOR complex - rictor (mTORC2) at the Ser473 residue (S473) potentiates AKT activity (255).

AKT exerts effect on substrates with diverse cellular roles including cell survival or cell death, growth, protein synthesis, proliferation, angiogenesis, metabolism and migration. Some AKT substrates have shown to control more than one cellular function, and each physiological response downstream of AKT appears to be mediated by multiple targets (246).

Among major AKT substrates are:

**1**. Regulators of apoptosis, such as BAD, caspase-9 and I-κB. AKT enhances cell survival by directly phosphorylate and inhibit the pro-apoptotic protein BAD (152), and AKT-mediated phosphorylation of pro-caspase 9 correlates with decreased caspase activity in vitro (227). AKT phosphorylates the forkhead (FOXO) family of transcription factors, and promotes cell survival by blocking FOXO-mediated transcription of pro-apoptotic protein BIM (249).

**2**. A major outcome of AKT activation is promoting protein synthesis and cell growth, and the predominant mechanism is through regulation of mammalian target of rapamycin (mTOR). AKT indirectly activates mTOR through phosphorylation of the tuberous sclerosis complex 2 (TSC2) in the TSC1-TSC2 complex. The phosphorylated TSC1-TSC2 complex inhibits the GTPase-activating protein (GAP) activity towards Rheb, and accumulation of GTP-bound Rheb activates the rapamycin-sensitive mTOR complex – raptor (mTORC1). Downstream effectors of mTORC1 regulate protein synthesis and growth through phosphorylation of regulators such as 4EBP1 and S6K1 (246,249,256).

**3**. AKT activation can stimulate proliferation through targets affecting cell cycle regulation. AKT phosphorylation of FOXO transcription factors inhibits FOXO-mediated transcription of cell cycle inhibitors, such as $p27^{Kip1}$ (246).

**4**. AKT also regulates angiogenesis, as AKT signaling leads to increased production of hypoxia-inducible factor-1 (HIF-1) transcription factor through mTORC1-dependent translation (246).
Positive and negative regulation of AKT activation by secondary binding proteins includes oncogenes, adaptor proteins and co-chaperones like the HSP90/Cdc37 molecular complex (257).

### 7.2. The mammalian target of rapamycin; mTOR kinase

The macrolide antibiotic rapamycin was found to be an anti-fungal agent, a potent suppressor of the immune system, and to have a unique action as a highly specific inhibitor of the serine/threonine kinase target of rapamycin (TOR). The mechanism of mTOR regulation is through activation of the AKT-signaling pathway, but also AKT-independent pathways are involved (258). The mTOR protein is ubiquitously expressed within cells (259). mTOR activities are mediated by its binding to different proteins to produce two notable complexes; the rapamycin sensitive mTOR complex - raptor (mTORC1), and the rapamycin insensitive mTOR complex - rictor (mTORC2); illustrated in **figure 9**. The mTORC1 complex consists of mTOR with its intracellular receptor FKBP12, raptor, PRAS40 and mLST8, where FKBP12 is the binding site for rapamycin. PRAS40 is directly phosphorylated by AKT, and is a negative regulator of mTORC1. mTORC1 phosphorylates its downstream target, eukaryotic initiation factor 4E-binding protein 1 (4EBP1), which further inhibits 4E-BP binding to eukaryotic initiation factor 4E (eIF4E), thus activating

translation (260). eIF4E selectively translates mRNAs which encodes for proteins such as cyclin D1, Bcl-2, Bcl-$X_L$ and VEGF, which enhance cell proliferation, cell survival and angiogenesis (261). Phosphorylation of S6 kinase 1 (S6K1), another downstream target, promotes cell growth (260,262). The mTORC2 complex contains mTOR, rictor and LST8, and phosphorylates AKT at S473, increasing the degree of AKT activation and thereby cell survival (255).

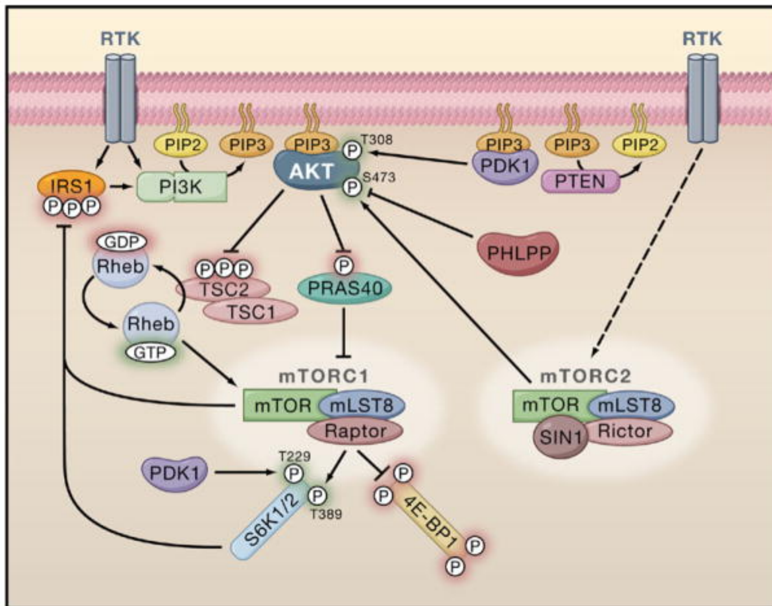


**Figure 9.** AKT-mTOR signaling pathway. Reprinted from ref. (246) © (2007), with permission from Elsevier.

The mechanism for activation of mTORC2 complex through RTKs is poorly understood, but mTORC2 has a role as regulator for PI3-K and its downstream effector AKT. The mTORC2 complex was originally believed to be rapamycin-insensitive, but in some cell lines prolonged rapamycin treatment inhibits mTORC2 assembly (263).

### 7.3. AKT and mTOR in OC

AKT is frequently activated, and significantly associated with phosphorylation of mTOR in OC tissue microarray (264), but their clinical role is not fully established. Protein expression of p-AKT is frequent in OC, but was not associated with OS (265-266). Another study found increased protein expression of p-AKT in more aggressive disease, and p-AKT was

negatively associated with survival, but was not a prognostic factor (267). Cell lines with either constitutive AKT1 activity or AKT2 gene amplification were more resistant to paclitaxel compared to cells with low AKT levels (268). AKT2-transfected OC cell lines had up-regulated β1-integrins leading to increased adhesion and invasion in vitro, and PTEN blocked invasion, indicating PI3-K-dependent AKT2 activity. In addition, transfected cells had increased metastatic potential in vivo (269). PTEN and p-AKT were shown to be inversely expressed in OC (270). Constitutive AKT activity in serum-deprived SKOV3 cells diminished upon treatment with PI3-K inhibitors, and pretreatment with PI3-K inhibitor in SKOV3 cells augmented apoptosis induced by cisplatinum (264). OC cell lines transfection with an active catalytic subunit of PI3-K conferred resistance to paclitaxel, which was reversed by PI3-K inhibitors. Malignant ascites in women with OC strongly inhibits TRAIL- and Fas-induced apoptosis through inhibition of Bid expression by AKT (271). Inhibition is abrogated by PI3-K inhibitor, demonstrating that malignant ascites protect against apoptosis through the PI3-K/AKT-signaling pathway. It further emphasizes the important role of the microenvironment and its contribution to chemotherapy resistance (272). Combination therapy with PI3-K inhibitor and paclitaxel reduced tumor growth and ascites production in vivo (273), and paclitaxel in combination with PI3-K inhibitor enhance apoptosis in OC cell lines (268). Novel therapeutics includes Perifosine, which selectively prevents AKT recruitment to the cell membrane and blocks activation of downstream effectors (275).

p-mTOR protein expression was frequent in primary OC compared to borderline tumors, and was related to the serous histological type. Increased expression was associated with improved survival and treatment with mTOR inhibitor RAD001 (everolimus) in OC cell lines reduced p-mTOR and elevated p-AKT expression (276). RAD001 (everolimus) inhibited cell proliferation and enhanced cisplatinum-induced apoptosis in OC cell lines with high AKT activity compared to cell lines with low activity. In addition RAD001 has shown to inhibit tumor growth, angiogenesis, peritoneal carcinomatosis and ascites production, and increased the efficacy of cisplatinum, leading to prolonged survival in transgenic mice (277). Strategies to down-regulate Bcl-2 and mTOR inhibitors may be a powerful combination in resistant OC cell lines in vitro (278). Combination therapy with Bevacizumab (vascular endothelial growth factor A; VEGF-A inhibitor) and rapamycin (mTOR inhibitor) reduced intraperitoneal tumor burden and ascites production in SCID mice (279).

# Aims of the present thesis

The present thesis is part of a larger research program performed by the Ovarian Cancer Research Group at the Norwegian Radium Hospital, Oslo University Hospital focusing on molecular markers, apoptotic mechanisms and resistance to chemotherapy in OC.
At the time of diagnosis, women with advanced OC frequently present with effusions containing carcinoma cells, or they develop malignant effusions along tumor progression. Malignant effusion in the pleural cavity at diagnosis also defines OC FIGO stage IV. Compared to the wide knowledge about solid ovarian tumors, cancer cells in effusions are yet to be investigated concerning mechanisms of apoptosis, cell survival and consequently chemotherapy resistance in this specific anatomic site. Our understanding of the biological differences between cancer cells and normal cells provides the basis for our understanding of chemotherapy resistance and development of new therapeutic strategies.

In general, this specific project aimed to detect anti-apoptotic molecular markers enhancing cancer cell survival and thus chemotherapy resistance in effusions tapped from women with advanced OC, and to evaluate surgery in women with FIGO stage IV.

**Papers I, II and III:**
Recognition of tumor cross-resistance comes from the widely accepted hypothesis that resistance to chemotherapy, and consequently cancer cell survival, is the result of alterations in the apoptotic cascade and the signaling pathways influencing apoptosis.
In order to approach this hypothesis we investigated the anti-apoptotic molecules of the Bcl-2 family (Bcl-2 proper and Bcl-XL), the Bag-family (Bag-1 and Bag-4) and the heat shock proteins (HSP27 and HSP70) in effusions, primary tumors and solid metastases in women with advanced OC (paper I). HSP90 protein expression was evaluated only in effusions from women with serous OC (paper III).
In paper II we evaluated the clinical relevance of p-AKT and p-mTOR protein expression in effusions, primary tumor and solid metastases in women with advanced serous OC. Additionally we investigated the relationship between protein expression of p-AKT, p-mTOR and DJ-1 in the PI3-K/AKT signaling pathway in effusions from women with advanced serous OC.

**Papers IV and V:**

In paper IV we retrospectively evaluated surgical treatment and chemotherapy in women with OC stage IV who were diagnosed from 1985-2005 and were submitted to the Norwegian Radium Hospital (NRH).

In paper V we further explored surgical timing and the extent of surgery in women with OC stage IV diagnosed from 1996-2005.

# Materials and methods

## 1. Ethics

The Regional Committee for Medical Research Ethics in Norway approved the research on the patient material in papers I-III (S-04300).
The office of the hospital's privacy protection supervisor gave approval to review patient data in papers IV and V.

## 2. Patients and material

### 2.1. Patient material. Papers I-III

The patient material studied in papers I-III was obtained from 265 patients with OC and serosal effusions referred to the Department of Gynecological Oncology, Oslo University Hospital, during 1998-2005. Paper I included 157 patients, paper II included 134 patients and paper III included 265 patients from the source cohort. More than one effusion was collected from 26 patients in paper I (21 patients had 2 effusions and 5 patients had 3 effusions) and 25 patients in paper II. The consequence of double and in 5 cases triple effusion samples from the same patient will be discussed later. Effusions were submitted to the Division of Pathology for routine diagnostic purposes immediately after tapping. Upon arrival, effusions were centrifuged at 2000 rpm in 10 minutes. The supernatants were decanted and fresh-frozen for the purpose of future research. Two Diff-Quik-stained and two PAP-stained smears were prepared for diagnostic purposes. Specimens were considered adequate when degenerated cells were not seen and viable tumor cells were present. The pellets were further divided in two. One half was fixed in 4% buffered formalin overnight before paraffin-embedded cell blocks were prepared using the thrombin clot method. The other half was fresh-frozen at
-70°C in RPMI 1640 medium supplemented with 50% fetal calf serum (FCS) and 20% dimethylsulfoxide (DMSO) at a ratio of 1:1. All effusions were evaluated by Prof. Ben Davidson. Diagnoses were established based on evaluation of smear and cell block morphology. Immunohistochemistry was applied in the cases where tumor origin had not been previously established by biopsy, morphology was equivocal with respect to histological type, or the differential diagnosis with reactive mesothelial cells and malignant mesothelioma was deemed relevant, as well as when patients had been operated at other hospitals.

In papers I and II, corresponding solid primary carcinomas and/or metastatic tumors from patients with serosal effusions were studied. Solid tumors were surgically removed and fixed in 4% buffered formalin overnight before paraffin-embedded tissue microarray (TMA) blocks were prepared containing 2 mm cores from primary carcinomas and/or solid metastatic tumors. Additionally in paper II, solid tumors from 17 OC stage I, 9 serous borderline tumors and 10 serous cystadenomas were studied following a request from one of the reviewers. All specimens to be included in the project were reviewed by Prof. Davidson with respect to diagnosis, histology and tumor grade.

**2.2. Clinical patient data. Papers I-III**

Clinical data in papers I-III were collected retrospectively from patient records kept in the archives of the NRH. Registered clinical and pathological data included patient age, date of diagnosis, FIGO stage, histological type, tumor grade, surgical treatment, residual disease, type of chemotherapy and evaluation of response to first-line treatment, date of relapse, second-line chemotherapy, evaluation of response to second-line treatment, and status at last updating of the database. Evaluation of response was performed according to the WHO criteria in paper I (280), and to the RECIST criteria and CA125 in papers II and III (281-282).

**2.3. Clinical data and patient selection. Papers IV-V**

Papers IV and V are population based retrospective observational studies of patients with OC stage IV treated at the NRH during 1985-2005 (paper IV) and 1996-2005 (paper V). Patients were referred from local hospitals in the South-East health region in Norway which covers approximately 60% of the Norwegian population. Patient lists were collected from the NRH code registry for diagnosis and surgery, and for the whole period a total of 793 patients had been registered with the diagnosis OC FIGO stage IV. Patient journals were collected from the hospital's archives, and all 793 patients had their data registered and evaluated. Dr. Oksefjell, one of the co-authors of publications IV and V, registered 47 variables with basic clinical data from patients who had their diagnoses from 1985-2000. The data file was converted into SPSS, 5 more years (2001-2005) were included in the study and 54 variables were additionally registered. These variables mainly concerned disease site defining stage IV, surgical treatment, complications from surgery and adverse effects of chemotherapy. Previous data were double-checked, and the additional clinical and

pathological registrations were performed by the PhD student except for one variable which was registered by Prof. Tropé; namely the 'extensive carcinomatosis' in paper V.

Inclusion criteria for the whole cohort:

1) Histology and/or cytology verifying OC FIGO stage IV.
2) Patients diagnosed with OC stage IV from 1985-2005.
3) OC stage IV according to the 1988 revised FIGO classification system.
4) Patients who underwent at least one surgical procedure other than diagnostic or explorative surgery.
4) Patients who had their first course of primary chemotherapy initiated at the NRH from 1985-2005.
5) Patients who received platinum-based chemotherapy during first-line treatment.

Efforts to minimize the heterogeneity in the patient cohort and statistical considerations finally settled the exclusion criteria which are outlined in **Figure 10**. A total of 394/793 (49.7%) patients were found eligible to be included in the statistical analyses of publication IV. The patient cohort was further divided in two, depending on whether the diagnosis was established from 1985-1995 or from 1996-2005. The first decade included 156/394 (39.6%) patients, and the second decade included 238/394 (60.4%) patients.
Of note, we included patients operated both at the NRH and at local hospitals in the South-East health region of Norway. For the whole period, 234/394 (59%) patients had their final surgery at the NRH and 160/394 (41%) were operated only at local hospitals. There was no difference in survival between patients operated at the NRH and at local hospitals in any of the two decades, and thus the two groups were merged in papers IV and V. In publication IV, the two time periods were evaluated for prognostic factors and their contribution to clinical outcome in two adjacent decades. In publication V, we included only 238 patients from the last decade. In this paper we aimed to evaluate how surgical timing and the extent of surgery influenced clinical outcome. The patient records from the first decade were lacking essential information on surgical treatment and preoperative diagnostic assessment, and consistently we included only patients from the last decade.

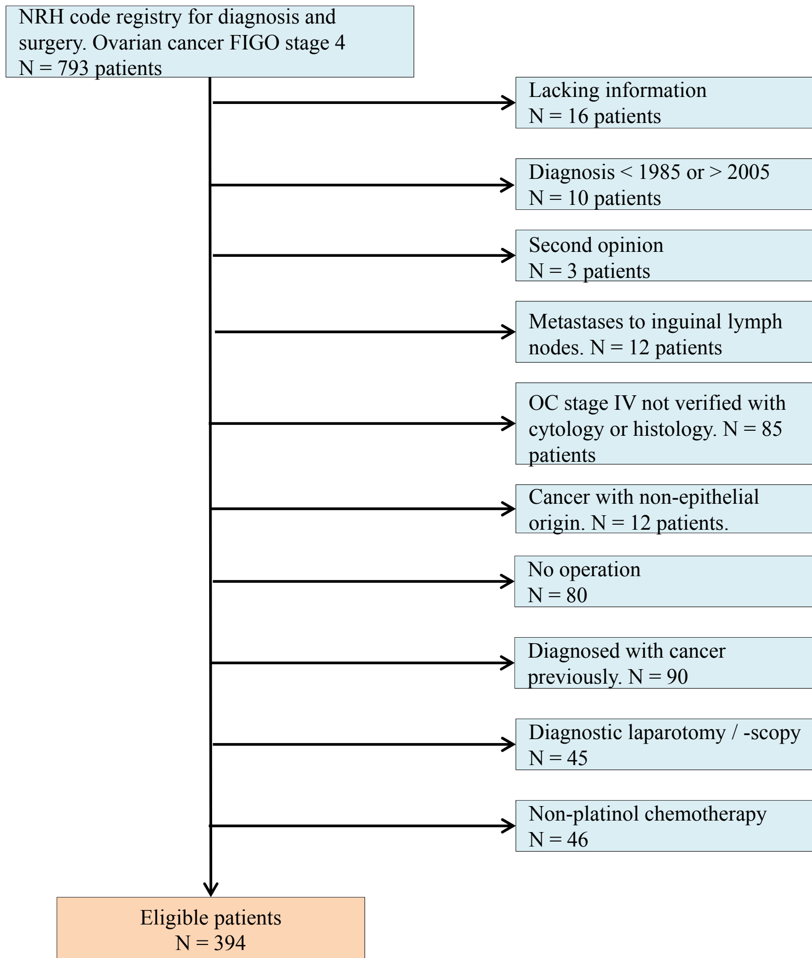


**Figure 10.** Flow chart of the exclusion criteria.

## 3. Laboratory methods

### 3.1. Immunohistochemistry (IHC)

In papers I-III we performed IHC. Formalin-fixed paraffin-embedded blocks were cut, sections were mounted on coated slides, and immunostaining was performed manually using the DAKO EnVision$^{TM}$ + system-HRP (Dako, Glostrup, Denmark). Slides were deparaffinized and rehydrated and then heat induced in adequate buffer in the microwave to retrieve the epitopes. Sections were then treated with Peroxidase Block (DAKO EnVision™

+ system-HRP) for five minutes to inhibit endogenous peroxidase. The sections were incubated for 30 minutes at room temperature with the respectively primary mono- or polyclonal mouse or rabbit antibodies studied, followed by additional 30 minutes of incubation with the corresponding peroxidase-labeled anti-mouse or anti-rabbit polymer. The binding sites were then stained for 10 minutes at room temperature, with liquid DAB substrate-chromogen solution, and thereafter counterstained with hematoxylin and ammonia as a bluing agent. The sections were dehydrated and mounted. All series included positive controls consisting of a primary OC that previously had demonstrated immunoreactivity for the studied antigens. Negative controls consisted of sections incubated for 30 minutes with a non-relevant antibody of the same concentration as the corresponding primary antibody isotype.

**IHC scoring system**

In papers I-III the scoring system for IHC was based on the staining extent in the cytoplasm and the nucleus, and the percentage of malignant cells with staining was scored on a scale of 0 to 4 as follows: 0 = no staining, 1 = 1-5%, 2 = 6-25%, 3 = 26-75% and 4 = 76-100%. The scoring system has been utilized by Prof. Davidson in all his OC studies involving the IHC method in the previous 10 years. Upon request from one of the referees in paper II, the scoring system for p-AKT deviates from our standard system. Cytoplasmic and nuclear p-AKT staining was combined into a 0 to 8 score, and for survival analyses the categories were grouped as low (0-4) and high (5-8).

### 3.2. Flow cytometry (FCM)

In publication II, FCM was performed by Hiep Phuc Dong, one of the co-authors, with assistance from the PhD student.

#### 3.2.1. FCM setup and optimization

Immunophenotyping by FCM was undertaken using the FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with a 15 mW Argon-ion laser (488 nm) and 12 mW red diode laser (635 nm). The filter configurations were fluoroscein isothiocynate (FITC, FL1, BP 530/30 nm), phycoerythrin (PE, FL2, BP 585/42 nm), peridinin chlorophyll protein (PerCP, FL3, LP 670 nm) and allophycocyanin (APC, FL4, BP 661/16). Forward light scatter channel (FSC) and side angle light scatter channel (SSC) parameters were defined in linear amplification mode, all fluorescence parameters (FL1, FL2, FL3 and FL4)

were defined in logarithmic amplification mode. For each measurement, data from at least 10,000 events were collected.
Control of instrument performance and time delay calibration were performed using FACSComp software version 4.1, Calibrite™ 3 beads and Calibrite™ APC beads (Becton-Dickinson, San Jose, CA) for four-color flow cytometer setup. Threshold was based on FSC as a primary parameter and compensation settings were determined as previously described (283).

### 3.2.2. Preparation

Fresh-frozen pellets were thawed and 10 ml RPMI 1640 with 10% FCS was added before centrifugation at 1200rpm in 5 minutes. The supernatants were decanted and 2 ml incubation buffer was added in each sample. The cell suspensions were mixed gently with a pipette, filtered through a 70 μm BD Falcon™ cell strainer and centrifuged again at 1200rpm in 5 minutes. Additionally 2 ml incubation buffer was added and cells were blocked for non-specific binding in incubation buffer for 10 minutes at room temperature with subsequently division of 100 μl of cell suspension ($1 \times 10^6$ cells) into respective tubes for staining. The OC cell line OVCAR-3 was used as positive control.

### 3.2.3. Staining procedure

Each effusion specimen and controls required 5 tubes; 1 for each of the antibodies to be studied and 2 controls of overall background staining and background staining of the secondary antibody respectively. The isotype surface marker mouse IgG1 was added to tube one, and the primary monoclonal antibodies (Ber-Ep4, EpCAM and CD45) for surface staining were added to the remaining four tubes. Cells were vortexed and incubated in the dark at room temperature for 25 minutes. Each tube was washed twice with 2 ml incubation buffer followed by centrifugation 1200 rpm in 5 minutes and decantation of the supernatant. 100 μl of medium A (FIX & PERM reagents, Caltag Laboratories, Invitrogen, Carlsbad, CA) was added to each tube and incubated for 15 minutes at room temperature in the dark. Cells were then washed twice with 2 ml phosphate buffer saline (PBS) followed by centrifugation 1200 rpm in 5 minutes and decantation of the supernatant. 100 μl of medium B (FIX & PERM reagents, Caltag Laboratories) was added to each tube and incubated for 20 minutes at room temperature in the dark. Cells were then washed twice with 2 ml PBS followed by centrifugation 1200 rpm in 5 minutes and decantation of the supernatant. 100 μl

of incubation buffer was added in each tube following incubation for 10 minutes at room temperature in the dark.

The isotype rabbit IgG, used as control for intracellular staining, was added to tube 1, tube 2 was left without primary antibody, and the primary antibodies mTOR, p-Akt1/2/3 (Thr 308) and DJ-1 for intracellular staining were added to tubes 3-5. All tubes were incubated for 45 minutes at room temperature in the dark. Cells were washed twice with 2 ml incubation buffer and centrifuged for 5 minutes at 1200 rpm. The supernatant was decanted and the secondary antibody for intracellular staining, secondary donkey anti-rabbit PE, was added to tubes 2-5. Tube 1 was left without the secondary antibody. All tubes were incubated for 25 minutes at room temperature in the dark. At the end of incubation the washing step was repeated twice with 2 ml incubation buffer following addition of 200 µl FacsFlow sheath fluid (Becton- Dickinson) to each tube. The samples were kept on ice until analysis.

### 3.2.4. Evaluation of FCM immunophenotyping

Analysis of the FCM results was undertaken in a standardized manner by using the FlowJo analysis software (version 8.7.3; Tree Star Inc., Ashland, OR). A gating procedure was generated by combining side angle light scatter channel (SSC) versus CD45 PerCP fluorescence and a region was drawn around clear-cut populations having negative CD45 PerCP fluorescence. Cells in this region were again viewed by generating a cytogram combining SSC versus FSC, and a gating procedure was used in order to exclude cell debris, by including only cells with relatively high SSC and FSC values. Quadrant cursors were set by using isotypic negative controls. A quadrant setting was undertaken so that in negative controls 99% of the cells were localized in the left lower quadrant. The percentage of carcinoma cells expressing mTOR, DJ-1 and p-Akt1/2/3 was scored. Expression in <1% of cells was scored as negative.

## 3.3. Western blotting

In paper II we performed Western blot to detect the total AKT protein and the phospho-specific residues Thr308 and Ser473 in the AKT protein. By studying both the total protein amount and the phosphorylated fraction, we obtained a true measurement of AKT activation in the effusion samples. Antibodies were commercially available from a company with long experience in designing phospho-antibodies (Biosource). Antibody against α-tubulin was used as housekeeping protein. Protein expression of p-AKT (Thr308), p-AKT (Ser473) and pan-AKT was measured in relation to the expression of α-tubulin to reflect the true target

protein amount. Protein levels are evaluated as of how intense and large the band was using densitometry. Densitometer analysis of the Western blotting bands was performed using a computerized image analysis program, and the values for p-AKT were divided by the α-tubulin band size to yield a final expression value. A ratio of less than 0.05 was considered negative.

## 4. Statistical methods

In papers I-III, continuous variables were analyzed using the non-parametric Mann-Whitney U test due to skewed distributions. In papers I and II, comparative analyses of patient-matched site-related specimens were performed using the Wilcoxon Signed Ranks Test. In paper II, the association between studied protein values by FCM was analyzed using a paired-sample *t* test.
OS and PFS were estimated using the Kaplan-Meier method and groups were compared with the Log-Rank test. Variables with $p < 0.2$ in survival analysis were entered into the multivariate analyses. In multivariate analyses, the Cox Proportional Hazard regression with forward stepwise elimination was performed.
In the statistical analyses, the clinical and pathological parameters were grouped as follows: age ≤60 versus age >60 years, tumor grade 1 and 2 versus grade 3, FIGO stage III versus stage IV, and response to first-line and second-line chemotherapy was complete versus partial response/stable disease/progressive disease.
In survival analyses the staining categories were clustered as follows; staining extent 0-2 was designated low, and staining extent 3-4 was designated high. In paper II, p-AKT staining extent was clustered as 0-4 which was low versus 5-8 which was high.

In papers IV-V, descriptive analyses were performed and the association between categorical variables was assessed using the two-sided Pearson Chi-square test or the Fisher's Exact Test as appropriate. The Kaplan-Meier method was performed to estimate OS, and groups were compared with the Log-Rank test. Parameters with $p<0.15$ in univariate survival analysis were included in the Cox Proportional Hazard regression with backward stepwise elimination.

The statistical analyses of all papers were performed using the SPSS-PC package version 13.0 (paper I), version 15.0 (paper II), version 16.0 (papers III and IV) and version 18.0 (paper V). In all papers the level of significance was set at $p<0.05$.

## Summary of results

**Paper I:**

**Expression and clinical role of anti-apoptotic proteins of the Bag, Heat shock, and Bcl-2 families in effusions, primary tumors, and solid metastases in ovarian carcinoma.**

In this study we analyzed the protein expression of the anti-apoptotic co-chaperones Bag-1 and Bag-4 and their molecular partners Bcl-2, Bcl-$X_L$, HSP27 and HSP70 in 188 effusions, in 43 patient-matched primary tumors and 81 corresponding solid metastases from a total of 157 patients.

Bag-1, Bag-4 and HSP70 showed protein expression in both the nucleus and the cytoplasm, whereas HSP27, Bcl-2 and Bcl-$X_L$ were exclusively expressed in the cytoplasm. In effusions, more than 85% of cases expressed the Bag proteins in both the nucleus and the cytoplasm while HSP70 was more frequently found in the cytoplasmic subcellular localization. HSP27 and Bcl-$X_L$ were expressed in the majority of specimens, whereas Bcl-2 was detected in 46% of effusions. Cytoplasmic and nuclear Bag-1 expression was higher in pleural compared to peritoneal effusions, whereas Bag-4 expression was higher in peritoneal effusions. In 55 patients with malignant effusions, matched primary tumor and/or solid metastases were available for comparative analyses. The six proteins studied were expressed in most solid tumors. However, none of them were differentially expressed in primary carcinomas compared to solid metastases, although they were differently expressed in effusions compared to solid tumors.

In analyses of the association between the anti-apoptotic molecules and the clinical and pathological parameters there was a significant relationship between HSP27 expression and higher histological grade (p=0.01) and between higher Bcl-$X_L$ expression and poor response to chemotherapy (p=0.02). In separate analyses of pre-chemotherapy effusions there was a significant relationship between higher histological grade and higher HSP27 expression (p=0.009), and between FIGO stage IV and higher nuclear Bag-1 expression (p=0.004) and lower cytoplasmic Bag-4 expression (p=0.02). In post-chemotherapy effusions, lower nuclear Bag-1 (p=0.03) and higher Bcl-2 (p=0.02) expression were found in patients with FIGO stage IV compared to stage III disease, and higher Bcl-$X_L$ and lower HSP27 expression were found in patients who responded poorly to chemotherapy (p=0.04 for both). In the analysis of primary carcinomas and clinical and pathological parameters, there was a positive association between higher Bcl-$X_L$ and older age (p=0.04) and between higher cytoplasmic HSP70 expression and higher histological grade (p=0.02). Increased

cytoplasmic HSP70 expression in effusions correlated with poor OS for the entire cohort ($p=0.01$), and may be a prognostic marker. A similar role was seen for Bcl-2 in primary carcinomas as it correlated with worse OS ($p=0.04$) and PFS ($p=0.02$).

**Paper II:**

**Mammalian target of rapamycin is a biomarker of poor survival in metastatic serous ovarian carcinoma.**

Three molecules in the PI3K-AKT signaling pathway, namely p-AKT, p-mTOR and DJ-1, were analyzed in effusions, primary carcinomas and solid metastases from women with OC. The serine-threonine kinase AKT was more frequently phosphorylated at Th308 compared to Ser473 in 33 effusions using Western blot. In FCM analysis, OC cells were found in all effusions (n=33), and quantitatively the expression was as follows: p-AKT (median=45%), mTOR (median=28%) and DJ-1 (median=24%). There was a significant association between the expression levels of the three proteins. The protein expression of p-AKT Thr308 and p-mTOR Ser2448 in effusions, primary carcinomas and solid metastases were frequently expressed at all anatomical sites using IHC. In 52 patient-matched specimens, more cells expressed p-AKT Thr308 in solid metastases than in effusions ($p<0.001$), but no differences were found between primary carcinomas and effusions, or primary carcinomas and solid metastases. No anatomical site-related differences were found for p-mTOR Ser2448. Higher p-AKT expression was shown in effusions ($p=0.013$) and solid metastases ($p=0.008$) originating from grade 3 tumors, whereas p-mTOR Ser2448 expression was higher in grade 1 and 2 primary tumors ($p=0.001$). In addition, p-mTOR Ser2448 expression was higher in primary carcinomas from patients with FIGO stage IV disease ($p=0.017$), and in pre-chemotherapy compared to post-chemotherapy effusions ($p=0.028$). By Western blotting, the level of p-AKT Thr308 and the p-AKT Thr308/pan-AKT ratio were higher in pre-chemotherapy compared to post-chemotherapy effusions ($p=0.008$ and $p=0.035$, respectively). Higher p-AKT Thr308/pan-AKT ratio was additionally associated with more advanced FIGO stage ($p=0.018$).

In survival analysis, significant association was found between higher p-mTOR Ser2448 expression in post-chemotherapy effusions and poor PFS ($p=0.005$). In Cox analysis, p-mTOR Ser2448 expression, FIGO stage and response to chemotherapy at primary disease and at disease recurrence were prognostic factors of poor PFS.

**Paper III:**

**Heat shock protein 90 is a putative therapeutic target in patients with recurrent advanced-stage ovarian carcinoma with serous effusions.**

Heat shock protein 90 (HSP90) modulates anti-apoptotic activity in malignant cells and was explored in 265 effusions from patients with advanced serous OC using IHC. Protein expression of HSP90 was analyzed for association with clinical and pathological characteristics, and for association with previously studied anti-apoptotic proteins, including proteins investigated in paper I and II. HSP90 was expressed in the cytoplasm and nucleus in 97% and 18% of specimens, respectively. Nuclear expression was higher in post-chemotherapy compared to pre-chemotherapy effusions (p=0.005), and was significantly related to previous treatment with both platinum (p=0.016) and paclitaxel (p=0.007). Cytoplasmic HSP90 expression was higher in effusions from patients with complete compared to incomplete or no response after second-line chemotherapy (p=0.016). Additionally, cytoplasmic expression was significantly associated with Bcl-2 expression in pre-chemotherapy effusions (p=0.04), and marginally associated with cytoplasmic Survivin expression in post-chemotherapy effusions (p=0.05). HSP90 expression was unrelated to survival in OC effusions. We found increased expression of HSP90 in post-chemotherapy effusions already exposed to platinum and paclitaxel which might indicate a potential role for HSP90 inhibitors as treatment in patients with recurrent advanced OC.

**Paper IV:**

**Prognostic significance of residual tumor in patients with epithelial ovarian carcinoma stage IV in a 20-year perspective.**

This is an epidemiological population-based study of prognostic factors impacting survival in patients with OC stage IV treated at the NRH from 1985-2005. The cohort consisted of 394 patients with histologically verified OC stage IV who underwent at least one surgical procedure other than diagnostic surgery, and received platinum-based chemotherapy as first-line treatment. The cohort was divided in two depending on whether date of diagnosis was from 1985-1995 or 1996-2005. Clinical and pathological characteristics were registered and compared between the two decades. More patients had macroscopic radical surgery (28%-11%), underwent delayed primary surgery (29%-3%) and received Platinum-Taxane combination therapy (80%-1%) in the second decade compared to the first decade. Median OS improved from 1985-1995 to 1996-2005 (1.3-2.1 years). In survival analyses of the

whole cohort and of the two subgroups, WHO, histology and residual tumor were significant in both decades and for the whole period. In addition, disease site defining OC stage IV was significant for survival in the first decade, surgical approach was significant in the second decade and chemotherapy was significant in both the last decade and the whole period. In multivariate analyses WHO, histology and residual tumor were prognostic factors in all three periods. Chemotherapy was a prognostic factor for the whole period, while surgical approach was not a prognostic factor in any period.
Treatment algorithm has been under debate in patients with OC stage IV. In this study we found more patients achieving macroscopic radical surgery after delayed primary surgery, but surgical approach was not a prognostic factor for OS while residual tumor was in all three periods.

**Paper V:**
**Neoadjuvant chemotherapy, interval debulking surgery or primary surgery in ovarian carcinoma FIGO stage IV?**
This is an epidemiological population-based study investigating the impact of timing (i.e. primary debulking, interval debulking and delayed primary surgery) and extent of surgery on OS during first-line treatment. The extent of surgery was classified as radical, standard or suboptimal surgery. Additionally, chemotherapy was explored in each surgical group, and all patients received Platinum-based compounds. Clinical and pathological information was collected retrospectively from 238 patients with OC stage IV treated at the NRH from 1996-2005.
The surgical approach was not significant for OS, but the extent of surgery was significant in the whole cohort ($p<0.001$), and in the primary and the interval debulking surgery group ($p=0.01$ and $p=0.05$, respectively). The radical and standard surgery groups had significantly longer survival compared to the suboptimal surgery group, with median survival of 2.6, 2.1 and 1.6 years respectively ($p=0.001$). No residual tumor was achieved in 66 patients with primary debulking, interval debulking or delayed primary surgery, but there was no difference in survival between these groups. More patients with radical surgery achieved no residual tumor, but survival was not superior to patients who achieved no residual tumor after standard surgery. Whenever cytoreductive surgery is performed the objective remains to achieve no residual tumor. Whether this is achieved after primary debulking, interval debulking or primary delayed surgery is not important, and radical

surgery and standard surgery have similar survival in patients with no residual tumor. However, chemotherapy tended to improve survival in patients with no residual tumor, and was a prognostic factor for OS in the primary debulking surgery group. Prognostic factors for OS in the whole cohort were residual tumor, performance status and histology.

# Discussion

## 1. Methodological considerations

### 1.1. Material and methods. Papers I-III.

The patients constituting the studied cohort in papers I-III were selected from the pool of women with advanced OC and serosal effusions referred to the Department of Gynecological Oncology, the NRH, from 1998-2005, based on the availability of cytological material. However, not all patients with advanced OC develop malignant effusions, and our results are only applicable to patients with malignant effusions. Whether these patients represent a genetic subgroup of women with advanced OC is yet to be evaluated.

The inter-observer variations from the registrations of the original patient records are limited in papers I-III since most of the registrations were performed by one skilled person (284). In addition, the effusions were submitted to the Division of Pathology for routine diagnostic purposes, and all histological and cytological evaluations were performed by one pathologist (Prof. Davidson), thus increasing the accuracy of the diagnosis.

In contrast, the accuracy of the patient data is threatened by multiple clinicians being responsible for diagnostic assessment, surgical treatment, chemotherapy and clinical evaluation. Even though state-of-the-art treatment and evaluation criteria were followed, we believe arbitrary divergences of measurements (in either direction) occur (285). To some extent, these random errors are compensated for by the large cohort studied.

In paper I, patients with different histological types of OC were included in the study, while in paper II and III inclusion was restricted to the serous type. The patient groups with histology other than serous carcinomas were small, and were not separately analyzed in the statistical analyses in paper I, but were clustered into one group. Furthermore, the majority of non-serous cases were either mixed tumors with a serous component or tumors registered as undifferentiated that recently were shown to be serous carcinomas by Prof. Davidson using immunostaining for WT-1 (data not shown). This problem was omitted in papers II and III where only patients with serous histological type were included.

There was a large sample size in papers I-III, but the number of patients in papers I and II do not correspond to an equivalent number of effusions. In paper I, 188 effusions were obtained from 157 patients, which results in a surplus of 31 effusion samples, or a 19.7% increase of cases. In paper II, 159 effusions were collected from 134 patients which is a surplus of 25 samples, or an 18.6% increase of cases. In the statistical analyses, these

additional effusions were included in descriptive analyses and in analyses of associations between staining extent and clinicopathologic parameters. The reason for doing so was the fact that several effusions from the same patient did not always have the same expression level, and were therefore regarded as separate entities. However, excess effusions were not included in comparative, survival and multivariate analyses, in which only a single event per patient is allowed, in papers I and II. In paper III, only one effusion per patient was included, thereby eliminating this potential difficulty.
In papers I-III, we performed multiple statistical analyses which increased the number of p-values and consequently the risk of a false positive result. The Bonferroni correction is one way to reduce the risk of a false positive result (286), but was not performed in any of the studies. We also performed statistical tests in subgroups of the whole cohort which also increased the number of p-values and the risk of a false positive result. On the other hand, when generating too small groups by dividing the cohort into subgroups, the probability of detecting significance between the groups will be low, and thus might give a false negative result. One way to omit the problem with too small groups would have been to perform sample size calculations. However, we did not perform sample size calculations in any of the papers I-III. This suggests that additional data from other reaearch groups with respect to these molecules may be contributory in the future.

### 1.2. The problem with pre- and post-chemotherapy effusions, solid tumors and metastases

In the last decade our ovarian cancer research group has focused on molecular alterations in primary tumors, solid metastases and malignant effusions from patients with OC, and also on the different protein expression pattern in pre-chemotherapy and post-chemotherapy effusions. Malignant cells in pre-chemotherapy effusions and tissues are unexposed to chemotherapy and aberrant protein expression thus represents the real genetic variations and/or dysregulation of the apoptotic pathway. The altered protein expression often detected in post-chemotherapy effusions and tissue may be attributed to an increasing genetic instability along tumor progression and exposure to chemotherapy and its resulting post-translational modifications of cancer-associated molecules.

In papers I-III, both patients with pre- and post-chemotherapy effusions were included in the study, as they have been in previous studies by our group (123,134-135,137-138,141). In

papers I and II corresponding pre- and post-chemotherapy solid tumors were also included. All patients included in the post-chemotherapy group have received platinum-based chemotherapy, and most patients had combination therapy with platinum and paclitaxel. Previous studies have shown that treatment compliance for six cycles is approximately 85% (72-73), and accordingly there is reason to believe that not all patients in the post-chemotherapy group completed six cycles of chemotherapy for reasons like allergic drug reactions, medical limitations or progressive disease. Alternative treatment in these patients is not registered. However, since the large majority of patients did receive the full six cycles of platinum-based therapy, we do not regard this as a major weakness.

Another point worth considering regarding these studies is that the majority of pre-chemotherapy and post-chemotherapy effusions were not coupled specimens. The statistical analyses are based on comparing a patient cohort with pre-chemotherapy effusions to a group of patients with post-chemotherapy effusions, and are not evaluating alterations in protein expression along tumor progression in the individual patients. On the other hand, this is an analysis of a large series of pre- and post-chemotherapy specimens, which we regard as useful for understanding chemotherapy-induced changes. Patient-matched pre- and post-chemotherapy specimens are hard to come by, and, in the literature, have been predominantly obtained by matching primary carcinomas operated at diagnosis with solid metastases resected in exploratory laparotomy at disease recurrence, this constituting a different scenario than the one studied by us.

A similar problem exists concerning solid tumors and metastases studied in paper I. Tissue samples were obtained from patients both before and after administration of chemotherapy, and in patient-matched comparative analyses between effusions, primary tumors and solid metastases these two groups were not analyzed separately. Nevertheless, the majority of solid specimens were from primary operation, obtained prior to chemotherapy, and changes between the primary tumors and the matched effusions are regarded as reflecting tumor progression or the effusion microenvironment.

### 1.3. Protein detection

In papers I-III we performed IHC to detect the expression of proteins of interest in isolated malignant cells from effusions, and in tissue preparations from patients diagnosed with OC. Additionally in paper II, we used Western blot to analyze the phosphorylation pattern of p-AKT in effusions. In paper II we also performed FCM in a smaller series of effusions.

### 1.3.1. IHC

The IHC method determines whether a selected molecule, mainly protein, is present or not in the studied cells and tissues, and also gives information about its subcellular localization. Together this provides more specific information about functional changes in malignant cells and tissues. IHC is a complex method when it comes to standardization and interpretation (287). Counting for its advantages is that IHC is a simple, sensitive and well-known method widely used in most pathology departments, allowing others to reproduce the results. The quality of the method relies on the specificity and avidity of the antibodies for a single epitope on the target molecule. Major factors influencing antibody-antigen binding include the methods used for fixation, tissue processing and staining which all can distort the morphology and organization of the tissues and cells examined (288). False-negative findings occur when failure during fixation or tissue processing disguise the epitopes of the antigen, and false-positive findings can appear due to non-specific antibody binding to tissue components other than the antigen of interest.

In papers I-III, we used only commercially available antibodies, and in order to reduce the risk for erroneous conclusions the antibody dilution, pretreatment and incubation condition for retrieval of the antigen was optimized for each antibody by qualified personnel. In paper I the antibodies investigated had already been validated in a previous study (172). In paper II Western blot was performed as a validation method for phosphorylation of AKT and mTOR in OC effusions. All series included positive control samples consisting of tissue already demonstrating immunoreactivity for the studied antigens. Negative controls included substitution of the primary antibody with a non-relevant antibody of the same subclass and concentrations as the mono- and polyclonal antibodies used. Both positive and negative controls gave satisfactory results in all series. Presently the IHC method lacks standardization and is referred to as semi-quantitative, making it less reliable and accurate than more quantitative methods (289).

### 1.3.2. Interpretation of protein detection in IHC

Interpretations of results from IHC are known to be highly subjective, and the scoring system is not subjected to any standardization.

The scoring system used in papers I-III has been applied by Prof. Davidson in all his OC studies involving the IHC method in the previous 10 years on which he was the principal investigator. The system is based on the staining extent in the membrane, cytoplasm or nucleus, and not on staining intensity since this can be biased by the fixation and other

technical parameters related to the staining procedures. In papers I-III, the percentage of malignant cells with protein staining was scored on a scale of 0 to 4 as follows: 0 = no staining, 1 = 1-5%, 2 = 6-25%, 3 = 26-75% and 4 = 76-100%. In this thesis we refer to other studies which have different cut-off values from ours, thus making the results less comparable. Lack of standardized scoring methods also makes studies utilizing IHC methods less reproducible (290-291). However, studies utilizing IHC still have relevance as pilot studies for further investigation of select proteins.

### 1.3.3. FCM

In a cell population there are individual differences between cells, and seemingly identical cells are recognized as heterogeneous partly due to variations in enzyme activity (292). In paper II we performed FCM to detect the expression of proteins in individual cells in effusions from patients with OC. FCM is a highly valuable armamentarium for investigating single-cell assays of protein-enzyme activity, and was suitable for analysis of the proteins p-AKT, p-mTOR and DJ-1. The possibility of measuring multiple markers simultaneously makes the procedure fast. However, the pitfalls with this method are the sample preparation (washing) and the staining procedure, conditions which can alter the epitopes. Incomplete re-suspension with light vortexing might lead to aggregation of cells. The correct choice of fluorochromes partly depends on the density of the molecules studied. Preparation and staining procedures have previously been described by our group, and have achieved recognition as a method with high reproducibility (293).

## 1.4. Patients and material. Papers IV-V.

Papers IV and V are observational studies based on a cohort of women with histologically verified OC stage IV who had their diagnosis from 1985-2005. The cohort was selected from the population of the South-East health region of Norway and referred to the NRH for treatment. Surgery was performed either at the local hospitals, the NRH, or at both places. Patient data were collected retrospectively from files in the hospital's archives, and variables registered. Inclusion and exclusion criteria are described previously in 'Material and Methods'.

The infrastructure of the health care services in Norway enabled a population-based cohort study covering about 60% of the population. However, there is reason to believe that due to high age, poor performance status and increased morbidity, not all women with OC stage IV were referred from the local hospitals to the NRH, and we did not confirm this number of

patients with the Norwegian Cancer Registry. It is questionable whether these patients would have met the inclusion criteria, or would have been excluded, and thus we suppose the studied cohort is representative for the intended measurements which was surgical approach and extent of surgery. In papers IV and V, approximately 50% of the initial cohort was excluded, but still the remaining 50% represents a large cohort of women with OC stage IV (n=394 in paper IV and n=238 in paper V).

The advantage of retrospective cohort studies is the possibility to study multiple exposures in a cheap and time efficient manner, and that it in many cases allows complete information on the subjects' exposures (294). Typically for a study with retrospective design is that the subjects are identified, and the baseline for exposure is already set for all subjects eligible for the study. Patient data in our cohort were also collected after the events had taken place. Subsequent disease course was studied during the observation period, and in most cases the outcome variables were settled even before our studies were initiated. Moreover, no patient was lost to follow-up in our cohort, and at end of follow-up, 14 patients were alive in paper IV, and 13 in paper V.

In retrospective cohort studies the term "hypothesis screening" has been proposed, and is in many respects more applicable to our studies (295). In paper IV our intention was to study how surgery and chemotherapy associated with the outcome in patients with OC stage IV in a 20 year period, and was not restricted to an a priori hypothesis but rather was a search among multiple exposures.

### 1.4.1. Validity. Papers IV-V.

Among other things, observational studies are performed to investigate causal factors or exposures having an effect on response or outcome in a given population. In studies of causation, such as observational studies, internal validity refers to accurate measurements of effects apart from random variation, and internal validity is a prerequisite for external validity (296). Systematic errors are the variability of measurements which differs in either one direction from the true value, and are independent of sample size. This affects the internal validity as the study will be biased and the estimates of association will be either larger or smaller than the true association. Systematic errors are commonly referred to as biases, and the internal validity can be threatened by all sources of systematic errors such as selection bias, information bias and confounding factors (296).

During the whole study period, 40% of the women included in papers IV and V had their only surgery at their local hospitals. The reason for their inclusion was that survival was equal in both decades for patients operated at the NRH and at the local hospitals which may indicate that surgery did not differ substantially between hospitals in this period. One must have in mind that this is a retrospective study evaluating surgery and chemotherapy in patients with OC stage IV treated over a period of 20 years from 1985-2005. During this period, the concept of more extensive surgery and no residual tumor gradually evolved to become the gold standard it is today. While primary surgery was performed at both local hospitals and at the NRH, delayed primary surgery and interval debulking surgery were performed at the NRH exclusively. The differences and similarities between the three surgical groups may be conditioned by whether surgery was performed locally or at the NRH, and thus this may represent a selection bias in papers IV and V. However, the surgical approach was not significant for OS in either paper IV or V, and whether this would have been the result if primary surgery was solely performed at the NRH, remains to be answered.

Neoadjuvant chemotherapy is self-selective for delayed primary surgery since not all patients intended to treat were eligible for surgical treatment after 3-4 cycles with chemotherapy, and consequently, patients who underwent delayed primary surgery were systematically and positively biased into the studied cohort in both papers IV and V. The magnitude of this bias may be measured since all patients were registered.

During the 20-year observation period many clinicians with different working experiences and from various hospitals were involved in the diagnostic assessment, surgical treatment, evaluation of residual tumor and response to chemotherapy. Consequently, with the increased number of clinicians involved, there will be a suspected increase in errors for the whole cohort. In particular, residual disease has not been restricted to any standard notification postoperatively, and is entirely dependent on the surgeons' individual judgments.
Whether this interobserver variability should be characterized as random or systematic measurement errors is an issue for discussion. The interobserver variability is a known phenomenon in clinical studies, and one study has found that more surgeons underestimated than overestimated tumor size intraoperatively (285). From this we assume that in our cohort the interobserver variability of residual disease may be subjected to information bias.

Our cohort was collected retrospectively and eventually all patients underwent surgery, and thus misclassifications concerning staging and histology do not represent a problem in the registered data. However, preoperative misclassification of staging and histology may proceed to an erroneous decision concerning surgical approach. Lack of representative biopsies has been a problem in patients who received neoadjuvant chemotherapy, since histology is not unrelated to treatment and prognosis (297-298). Operated patients are less susceptible to misclassifications than non-operated ones. The impact of misclassification of histology on surgical approach is unknown in our cohort, but in general, the risk of an incorrect decision concerning surgery should be taken into consideration when patients are subjected to neoadjuvant chemotherapy.

Multiple exposures recognize a cohort study, and in general there are many associations between exposure variables and outcomes, but associations do not necessarily imply causality. Confounding is a challenge to cohort studies in general, and thus there is reason to believe that the measurements of associations in papers IV and V may be confounded. A potential confounder is performance status which has shown to be a prognostic factor for OS in both papers. However, performance status often influences the choice of surgical approach, and patients with a poor performance status are more likely to receive chemotherapy prior to surgery than patients with a better performance status. Thus performance status may be a condounding factor in statistical analyses between associations of surgical approach and overall survival.

## 2. Discussion of the main findings in papers I-V

### 2.1. Paper I:

#### 2.1.1. The clinical role of the Heat Shock Proteins HSP27 and HSP70 in advanced OC

Under normal cellular conditions heat shock proteins possess housekeeping functions, but in response to stress conditions the protein level increases in order to restore the intracellular physiological environment. The cytoprotective functions of heat shock proteins can be explained by their chaperone activity which enables them to interact with anti-apoptotic molecules and thereby inhibit apoptosis and thus enhance cell survival (210,214). In response to the stress conditions caused by chemotherapy, elevated expressions of heat shock proteins have been demonstrated. In this context we have focused on investigating the role of heat shock proteins in apoptosis and their relevance for resistance to chemotherapy.

The protein expression of HSP27 and HSP70 in ovarian cancer has previously been investigated in several studies both in vivo and in vitro (172,230-231,235-239). In paper I we evaluated the role of these heat shock proteins in malignant effusions and corresponding primary tumors and solid metastases from women with advanced OC.

We found HSP70 immunostaining in both the nucleus and the cytoplasm, while HSP27 had cytoplasmic subcellular localization exclusively. In effusions, HSP70 immunostaining was more often localized to the cytoplasm, whereas the nuclear subcellular localization was absent in most patients. In the primary tumor and solid metastases both the nuclear and cytoplasmic expression of HSP70 was abundant. HSP27 was expressed in the majority of cancer cells in effusions, primary tumor and in solid metastases. Notably, in our study more patients expressed HSP70 and HSP27 than in previous studies of OC (231,235-238), and this difference might reflect heterogeneity in the patient cohort or differences in the methods utilized, as discussed in methodological considerations.

Predilection towards metastases within the serosal cavities and accumulation of malignant effusions in these specific anatomic sites is often the clinical course of OC (123-124,127). Therapy directed towards this hypoxic and nutrient deficient microenvironment is crucial in the clinical setting of metastatic OC, and thus the biology of cancer cells needs to be explored in this anatomic site. Previously we have detected multiple molecules that are differently expressed in OC cells in effusions, the primary tumor and solid metastases, and that have different prognostic roles along tumor progression (118,129-137).

In paper I both cytoplasmic and nuclear HSP70 protein expression was more highly expressed in primary tumors and solid metastases than in effusions, and this is in accordance with previous studies by our group (118,131-132,135-137). However, HSP70 and HSP27 were not differently expressed in primary tumor compared to solid metastases, and in pre-chemotherapy compared to post-chemotherapy effusions, as we have seen for other molecules studied by our group (123-124,134-135,138-140).

We found poorly differentiated primary tumors to associate with increased cytoplasmic HSP70, but this finding was not significant during tumor progression. One previous report has demonstrated nuclear HSP70 expression to be associated with high grade primary tumor (172). The mechanism behind nuclear and cytoplasmic subcellular localization of HSP70 has been investigated, and under normal conditions HSP70 is believed to shuttle between the nucleus and the cytoplasm. Stress has demonstrated to trigger the accumulation of

HSP70 in the nucleus and inhibit the shuttling, a process that is reversible when normal physiological condition is restored (299). This diverges from our findings, but supporting the association between cytoplasmic HSP70 and aggressive disease is that increased protein expression in effusions from these patients has shown to be associated with poor OS in the whole cohort and in the post-chemotherapy subgroup. Furthermore, in effusions from patients with poorly differentiated tumors, increased protein expression of HSP27 was observed in the whole cohort as well as in the pre-chemotherapy subgroup. In a clinical setting, malignant effusion represents a unique type of metastasis and is a manifestation of more aggressive disease, and thus one may expect increased HSP27 in effusions to be associated with tumor progression. In contrast to the association between aggressive disease and increased expression of HSP27, in post-chemotherapy effusions decreased protein expression was observed in patients with poor response to primary chemotherapy, which in a clinical setting is synonymous with progressive disease. The equivocal significance of increased and decreased HSP27 protein expression is also reflected in previous reports which are inconclusive concerning the clinical role of HSP27 in OC (172,230,235-238).
In vitro, HSP27 was elevated in a cisplatin resistant OC cell line, and was associated with cisplatin resistance (239). An inhibitor of HSP27 (OGX-427) has already reached phase I clinical trial in ovarian cancer (www.clinicaltrials.gov), and has shown to inhibit proliferation, induce apoptosis and enhance Gemcitabine chemosensitivity in pancreatic cancer (300). No current clinical trial is registered for inhibitors of HSP70 and ovarian cancer, but efforts are put into development of new targeted therapy (301). Even more interesting are the prospects for cancer vaccines generated from extracellular HSP70 which interact with receptors on antigen presenting cells, which further enable presentation of tumor antigens to T-lymphocytes (212,219).
In paper I we found frequent expression of HSP27 in effusions, the primary carcinoma and solid metastases, and frequent HSP70 expression in the two latter anatomic sites. In addition our results were indicating an association between these proteins and aggressive disease and poor OS. Thus we believe that patients with advanced OC and malignant effusions in the serosal cavities may be good candidates for therapy targeting these heat shock proteins.

### 2.1.2. The clinical role of anti-apoptotic proteins from the Bcl-2 and the Bag families in OC

Defects in apoptosis have been demonstrated to play a major role in cancer cell survival and chemotherapy resistance (87,91,95-96). Inappropriate regulation of apoptosis involves multiple molecules, and understanding their interplay is essential to how multidrug resistance develop (90,150). In this aspect, in paper I, we evaluated the anti-apoptotic proteins Bcl-2 and Bcl-$X_L$ from the Bcl-2 family, and Bag-1 and Bag-4/SODD from the Bag family in the same clinical setting as we did with HSP27 and HSP70.

We found Bag proteins expressed in both the nucleus and the cytoplasm, whereas Bcl-2 and Bcl-$X_L$ had cytoplasmic subcellular localization exclusively. Nuclear and cytoplasmic Bag-1 and Bag-4/SODD, and Bcl-$X_L$ were expressed in most effusions and in most solid tumors, both primary carcinomas and solid metastases. Bcl-2 showed immunostaining in less than half of the effusions, but was expressed in most solid tumors.

In paper I, the heat shock proteins were analyzed together with the Bcl-2, Bcl-$X_L$ and the Bag proteins. All six proteins are known to have anti-apoptotic activity, and additionally, HSP70 and the Bag proteins have chaperone and co-chaperone properties, respectively (146,153,185). HSP70 is recruited to the death receptor by Bag-4/SODD in order to inactivate the DD of the receptor (200). Bag-1 is a co-chaperone of HSP70, and its overexpression down-regulates HSP70 chaperone activity (187). Bag-1 also enhances anti-apoptotic activity of the Bcl-2 protein (183), and moreover, Bcl-2 together with the Bcl-$X_L$ protein is guarding the MOMP and thereby the release of cytochrome *c*, which is the ultimate step in the intrinsic apoptotic pathway (146). Finally, HSP27 and HSP70 regulate apoptosis by inhibiting stress-inducing signals, preventing MOMP and thus the release of cytochrome *c* and recruitment of molecules to the apoptosome, and consequently caspase cleavage (210). The tight bonds between the six investigated proteins may give the impression that they exert their anti-apoptotic activity synergistically. However, the proteins were not analyzed for co-expression, although expression of the closely-related proteins Bcl-2, HSP70 and cytoplasmic Bag-1 was increased in solid tumors compared to effusions. The relationship has previously been reported in vitro (183,186,302). In contrast, Bcl-$X_L$, nuclear Bag-1, cytoplasmic and nuclear Bag-4/SODD expression was significantly higher in effusions compared to solid tumors.

In two in vitro studies, Bag-1 has been shown to enhance resistance to drug-induced apoptosis (205,207). However, the clinical role for Bag proteins in OC is not yet established, and we found variable associations between their protein expression and clinical parameters. In particular Bag-1 and Bag-4/SODD were not associated with chemotherapy response, and were equivocal with respect to disease aggressiveness.

Bcl-2 and Bcl-$X_L$ were associated with more aggressive disease, and increased Bcl-2 protein expression in solid tumors was associated with poor OS and PFS in our study. We found Bcl-$X_L$ to be frequently overexpressed in OC, which is in concordance with previous studies (167,174-175,179), but in our study Bcl-$X_L$ was not associated with survival. We found, however, increased Bcl-$X_L$ protein expression in effusions to be associated with reduced response to chemotherapy, and this corresponds to four previous reports in which Bcl-$X_L$ has been shown to be related to chemotherapy resistance in OC cells in vitro (174-176,303). Previous reports have not been conclusive regarding the clinical role of the Bcl-2 protein in OC (166,169-172,177-178). The reason for this could be the molecular interactions of members of the Bcl-2 family during apoptosis, and the variation in activation of the pro-apoptotic partners like BAK and BAX, and that these molecules may be more important in determining resistance to chemotherapy in OC than Bcl-2 itself.
Bcl-2 antagonists have already reached clinical trials, also in OC, whereas Bag antagonists have not (www.clinicaltrials.gov/2011). Bcl-2 antagonists are predominantly BH3-only protein mimetics generated naturally or synthetically (304), and their function has yet to be explored in detail (94,305). The most extensively studied Bcl-2 antagonist is oblimersen, an antisense Bcl-2, which has been explored in multiple cancer types, but no study is yet published on OC (306).
Since inconclusive reports on the clinical role of Bcl-2 in OC may imply mechanisms of chemotherapy resistance which are not entirely dependent on Bcl-2 protein expression alone, but rather on the interaction of anti- and pro-apoptotic molecules of the Bcl-2 family, we suggest a role for BH3-only mimetics as future therapeutics in OC.

### 2.2. Paper II:

### AKT and mTOR in clinical settings

Impaired apoptosis is not the only cause of chemotherapy resistance in malignant tumors. Errors in signaling pathways are also a common feature, and, together with apoptosis, affect

resistance and cancer cell survival in several human malignancies (150). The PI3K-AKT signaling pathway constitutes one of the main pathways that are dysregulated in malignant tumors (251,254).
Previous reports have documented p-AKT and p-mTOR protein expression in primary OC (264-267,270,276), but have not investigated their expression along disease progression. In paper II we evaluated p-AKT and p-mTOR protein expression by IHC in effusions, primary carcinoma and solid metastases from women with advanced OC. In addition, we analyzed the phosphorylation pattern of AKT by western blotting, and analyzed the expression level of p-AKT, mTOR and DJ-1 in effusions using FCM.

p-AKT was expressed in both the cytoplasm and nucleus, whereas p-mTOR was expressed in the nucleus exclusively. Both proteins were frequently expressed in effusions, primary carcinomas and solid metastases from women with advanced OC. In addition, frequent p-mTOR protein expression was detected in cystadenomas, borderline tumors and stage I OC, and thus analyses from advanced stages need to be interpreted carefully in our study. The high basal expression of this protein in benign cysts, borderline tumors and OC stage I is unclear in view of the high proportion of malignant cells that express p-mTOR in the advanced stage OC. Additionally, p-mTOR was equally expressed in effusions, primary carcinoma and solid metastases. These findings suggest a high basal p-mTOR expression independent of stage and localization.
AKT is activated through phosphorylation, and Thr308 is the first amino acid residue to be phosphorylated, followed by phosphorylation at the Ser473 residue (255). In concordance with previous studies, we confirmed with western blotting that the AKT is frequently phosphorylated at Thr308, and does not always reach a state of fully activation. The AKT immunostaining was performed with an anti-AKT 1/2/3 Thr308 antibody, and this may explain the frequent p-AKT protein expression in our study. In concordance with several previous studies from our group demonstrating different protein expression along tumor progression, we observed increased p-AKT expression in solid metastases compared to effusions and primary carcinomas, which did not differ significantly (118,131-132,135-137).

The close relationship between the proteins involved in the PI3K-AKT pathway was demonstrated for p-AKT, mTOR and DJ-1 as they were co-expressed in effusions. Using data from paper I, we also analyzed for associations between the anti-apoptotic proteins of

the Bag, Heat shock and Bcl-2 families, and p-AKT and mTOR in the PI3K-AKT signaling pathway, and found Bag-1 and HSP70 to be co-expressed with p-AKT and mTOR. Co-expression of proteins is valuable information regarding chemotherapy resistance and future targeted therapy. Cancer cell survival is not dependent upon one single molecule but rather is a result of dysregulation in multiple molecules affecting programmed cell death (150). The interaction between the AKT and mTOR proteins and how they relate to chemotherapy resistance is demonstrated in previous reports (264,277). High levels of AKT activity result in hypersensitivity to mTOR inhibitors (307), and treatment with the mTOR inhibitor RAD001 enhanced cisplatin-induced apoptosis and inhibited cell proliferation in OC cell lines with high p-AKT/mTOR activity (277). Inhibition of mTOR leads to the blocking of the cell cycle in late G1-phase, and, to further highlight the interconnection between signaling pathways and apoptosis, OC cell lines resistant to the mTOR inhibitors have been shown to express Bcl-2 (278).

Both p-AKT and p-mTOR showed different protein expression in pre-chemotherapy compared to post-chemotherapy effusions, a difference which is in accordance with previous observations by our group (308).

Protein expression of p-mTOR is frequent in effusions, and the increased expression seen in pre-chemotherapy compared to post-chemotherapy effusions may rather be interpreted as down-regulation of p-mTOR in the latter. Since p-mTOR is equally expressed along tumor progression, the difference between pre- and post-chemotherapy effusions may be explained by exposure to chemotherapy and variation in protein expression in response to chemotherapy treatment.

AKT expression was associated with more aggressive disease in effusions, and p-mTOR protein expression in patients with post-chemotherapy effusions was associated with poor PFS. In the latter cohort, the response to chemotherapy at primary and recurrent disease was associated with PFS too, and remained prognostic in multivariate analyses together with p-mTOR expression. When considering the above arguments as to the co-expression of p-AKT and p-mTOR in targeted therapy, our results with respect to the increased p-AKT in aggressive disease support the use of mTOR inhibitors in patients with advanced OC and malignant effusions.

In vitro studies have shown the improved treatment effect of mTOR inhibitors in combination with platinum or taxane cytotoxic drugs in ovarian cancer cell lines (277,309-310). Promising clinical data and extensive research on mTOR inhibitors during the last decade have generated several analogs of rapamycin which are currently being evaluated in

phase I and II clinical trials (260,262,www.clinicaltrials.gov). One recent phase II clinical trial on Temsirolimus in persistent and recurrent epithelial ovarian cancer showed modest activity, but was only monitored as single therapy (311). As for renal clear cell carcinomas, mTOR inhibitors are currently the established treatment, and have also shown promising results in clear cell carcinomas of the ovary (312).
We suggest that mTOR inhibitors may provide a potential clinical benefit in combination therapy with a platinum agent in the treatment of recurrent disease in patients with advanced OC.

### 2.3. Paper III:
### HSP90 – the clinical relevance of subcellular localization

In paper I we found interest in HSP70 as a putative therapeutic target in OC, and postulated that other family members might have similar qualities.
HSP90 is a molecular chaperone required for cellular homeostasis, and increases its expression in response to various stimuli. The chaperone activity is regulated through the dynamic complex known as the HSP90 chaperone machinery, which consists of the HSP70 chaperone in addition to various co-chaperones (215-216). HSP90 exerts chaperone activity on numerous oncoproteins, and thus is an interesting target in cancer therapy.
Hence, in paper III we evaluated HSP90 in effusions from women with advanced OC.
We found that most cancer cells expressed HSP90 in the cytoplasm, and only 18% showed nuclear staining. The nuclear HSP90 expression was significantly higher in post-chemotherapy compared to pre-chemotherapy effusions, and cytoplasmic HSP90 expression was significantly higher in patients with complete response after second-line chemotherapy. These results may indicate a more aggressive disease in patients with increased nuclear HSP90 expression in carcinoma cells.
In addition to the nuclear and cytoplasmic subcellular localization, organelle specific HSP90 has been found in the mitochondrion (TRAP1) and endoplasmatic reticulum (Grp94) (215,313). TRAP1 protects cells from apoptosis by antagonizing mitochondrial cell death (314), and is proposed as a target for ovarian cancer therapy (315).

Intracellular transport mechanism is controlled by very specific amino acid sequences contained within each protein, and these signaling sequences govern import into the nucleus (nuclear localization signal; NLS), export out of the nucleus (nuclear export signals; NES),

and transport in and out of the mitochondria, endoplasmic reticulum, peroxisomes and lysosomes. NLS has not yet been recognized in HSP90, and translocation from the cytoplasm to the nucleus may be facilitated by co-transport with client proteins of which steroid hormone receptors are best described (316). There is also evidence that under heat shock conditions the HSP90 co-chaperones assist in nucleo-cytoplasmic shuttling as has been shown *in vitro* for the co-chaperone HSP70/HSP90 organizing protein (HOP) (317). The transcription factor heat shock factor 1 (HSF1) is a client of HSP90, and is held in an inactive complex under normal conditions. In response to environmental stimuli, HSF1 dissociates from HSP90 and translocates to the nucleus, where it starts transcription of heat shock proteins including HSP90. Thus, HSP90 has a role in regulating its own transcription (318). However, increased transcription of HSP90 eventually necessitates translocation from the nucleus to the cytoplasm. This export of proteins requires assistance of nuclear-cytoplasmic transport receptors which recognize the presence of NES in the amino acid sequence of HSP90. The chromosome maintenance protein 1 (CRM1) is one of the main mediators of NES-dependent protein transport, and exports both proteins and RNA. Whether CRM1 assists HSP90 in translocation from the nucleus to the cytoplasm is unclear, but in general, nuclear export of tumor suppressors, cyclin-dependent kinases and drug targets can result in chemotherapy resistance (319-320). CRM1 has been shown to be highly expressed in aggressive and advanced stage OC, and correlated with poor patient outcome (321).

The clinical relevance of subcellular localization of the HSP90 has not been described in OC previously, and we postulate that increased nuclear HSP90 protein expression in post-chemotherapy effusions is associated with more aggressive disease. Supporting this view is a study which found nuclear HSP90 to be associated with more advanced TNM stage in breast carcinoma (322). One deduces from these results that the blocking of nuclear HSP90 may be a more direct target in patients with advanced OC suffering from effusions in the serosal cavities. The traditional HSP90 inhibitor, Tanespimycin, has so far shown additive growth inhibitory effects in OC cell lines in vitro (242).

### 2.4. Paper IV:

### OC stage IV in a 20-year perspective

In papers I-III we examined malignant effusions from the serosal cavities in women with advanced OC, and proposed the mechanisms of chemotherapy resistance in this specific

disease site. In paper IV, our object was to evaluate surgical treatment in women with OC stage IV. These patients frequently present with malignant ascites, and cancer cells in pleural effusions are the most frequent extra-peritoneal metastatic site at the time of diagnosis in these women.

Paper IV is a population-based retrospective observational study of women with OC stage IV in the South-East health region of Norway who had their diagnosis verified from 1985-2005, and who were referred to the NRH for treatment. The limitations and possible biases of the study have been discussed previously.

In paper IV we found improved survival from 1985-1995 to 1996-2005, and in many ways this is an expected consequence of more radical surgery and improved chemotherapy in the same period. The question is rather how surgery has improved survival in the 20-year period.
We found the residual tumor to be a prognostic factor for survival in both decades and in the whole period, and this is in line with previous multiple studies (31,45-49,51-54,323). There has been a gradual decrease in cut-off values of residual tumor from 2 cm in the earlier studies (46-48) until it presently has reached zero or no evidence of macroscopic disease (51,54). In one specialized gynecological oncology center a reported paradigm shift in surgical efforts to minimize residual disease improved PFS and OS significantly from 1996-1999 to 2001-2004 (52). Our study is spanning a period of 20 years from 1985-2005, and covers the dramatic change in surgical approach to OC stage IV. During data collection we found in our cohort that in most patient files the cut-off value of 2 cm was used to describe the amount of residual tumor after surgery, as was considered the standard in its time (46-48). In recent files more detailed surgery records provided precise measurements and localization of the residual tumor, but to avoid small subgroups in the cohort, the cut-off value remained unchanged also in the last decade. However, the surgeons' evaluation of their own operative assessment and residual disease is highly objective as shown in a prospective study of patients with advanced OC and cytoreduction to ≤ 1 cm. The association between self-reported residual disease and postoperative CT scan evaluation coincided in 52% of cases (324). In a follow-up study of the same cohort 67 patients were eligible for continuous evaluation, and in these patients 57% of reported residual tumor was concordant with CT scan evaluation. No difference in PFS and OS was seen between concordant and disconcordant findings, but the number of patients was small (325).

In our study, more patients achieved complete cytoreduction after neoadjuvant chemotherapy and delayed primary surgery than after primary surgery, and there was no difference in survival between the two groups, which has also been demonstrated in previous studies (61,326). One study showed improved survival after neoadjuvant chemotherapy, and less morbidity and need for aggressive surgery in patients with OC stage IV (327).

The lack of survival benefit in patients with neoadjuvant chemotherapy may be explained by the increased tumor load in the primary disease and the development of chemotherapy resistance during the initial courses with chemotherapeutics (86). Large tumors are also more susceptible to tumor necrosis, which indicates decreased vascularization, and thus reduced drug transport to these areas (85). The indirect evidence of tumor load implication on chemotherapy resistance is that increased response to chemotherapy, less platinum resistance and improved survival was observed in patients who had maximal cytoreduction by primary debulking surgery (59). These findings also suggest that the number of treatment cycles and the timing of delayed primary surgery are important factors to success when neoadjuvant chemotherapy is administered in patients with advanced OC. In contrast, the time-interval from primary surgery until initial course with chemotherapy had no significant impact on short-term survival (328-329).

The gold standard is still primary surgery, but there is evidence that a subgroup of patients will benefit from neoadjuvant chemotherapy (330). Effort to triage patients with advanced OC to either primary debulking surgery or neoadjuvant chemotherapy is on the agenda, and in clinical practice this means diagnostic assessment of patients and tumors, and the judgment of tumor resectability. In our study, we found the performance status to be a prognostic factor for survival in the whole period and in the separate decades. The performance status does not discriminate between actual co-morbidity and associated diseases due to OC stage IV, and thus performance status is a suspected confounding factor for patient selection to surgery. One randomized study confirms the performance status and quality of life to be an independent prognostic factor for PFS and OS in patients with advanced OC (331).

We found in our study that clinical outcome was more dependent on performance status than age which is in accordance with two previous studies (51,54). This finding may be interpreted as if surgery is a safe procedure in elderly women as long as they have a good performance status. The problem with the treatment in the elderly is rather chemotherapy, and one study reports on the standard dose platinum combination therapy being used in 28%

of women ≥80 years old, whereas completion of six cycles or more was seen in only 57% (332). In our study we did not perform analyses on age subgroups, and neither did we analyze chemotherapy specifically in the different age groups.
The results in paper IV are in accordance with previous studies confirming residual disease as an important prognostic factor for survival. How the least residual tumor was achieved did not have an impact on survival in our study, but in the literature this is equivocal. Primary surgery is still the gold standard treatment, but neoadjuvant chemotherapy should be considered in subgroups of patients. The selection criteria for surgical approach should include performance status and histology type. Delayed primary surgery encompasses less morbidity than primary surgery which leaves the patients with more bowel stomas. We suggest neoadjuvant chemotherapy followed by delayed primary surgery as a viable alternative in a subset of patients where primary surgery is unlikely to be successful.

### 2.5. Paper V:
### The value of comprehensive surgery in OC stage IV

In paper IV, the residual tumor was a significant factor for survival in patients with OC stage IV, while the surgical approach was not. Therefore, in paper V our object was to more profoundly investigate the relationship and impact of surgical timing, the extent of surgery and chemotherapy on overall survival in women with OC stage IV who had their diagnosis verified from 1996-2005, and who were referred from local hospitals in the South-East Health Region of Norway to the NRH for treatment. Possible biases of the study have been discussed previously.

In paper V the level of surgery was categorized as radical surgery, standard surgery and suboptimal surgery, and they were equally distributed with respect to primary surgery, interval debulking surgery and delayed primary surgery. This is not in line with previous studies which demonstrated less aggressive surgery and thus less morbidity in patients who had neoadjuvant chemotherapy (61,326-327).
In the whole cohort more patients had radical surgery, and this group had significantly improved survival compared to those who had suboptimal surgery. The significance of level of surgery also appears in patients who had primary and interval debulking surgery, but interestingly enough, radical surgery did not significantly improve the survival in patients

who received neoadjuvant chemotherapy. This may indicate a need for less aggressive surgery in the latter group, as demonstrated by Hou et al. (327).
Patients who underwent radical surgery significantly more often had no residual disease postoperatively compared to those who had standard and suboptimal surgery, but there was no difference in survival compared to patients who achieved no residual disease after standard surgery. This is in line with two previous studies (55-56), and emphasizes again that as long as no tumor residuum is achieved, the manner by which surgery is performed is less relevant. Recently, Chi et al. reported on improved survival in patients who had primary surgery compared to those who had neoadjuvant chemotherapy, but the latter group accounted for only 10% of the total number of patients in the study (330).
In accordance with three previous studies, no residual tumor was achieved more often in patients who had delayed primary and interval debulking surgery than in patients who had primary surgery (61,326-327). However, only Hou et al. found significantly improved survival in the subgroup of patients with extraperitoneal disease and neoadjuvant chemotherapy (327).
In our study, we found that general carcinomatosis in the abdomen did not impact survival in patients with OC stage IV when compared to patients with no general carcinomatosis and similar extraperitoneal site-specific metastases. This may indicate that extraperitoneal metastases are more suitable as predictors of survival in stage IV disease than general carcinomatosis. The benefit of radical surgery was significant for patients with pleural effusions with or without general carcinomatosis.
Whether it is the radical surgery in itself which is beneficial to these patients, or whether the benefit of radical surgery results from the fact that more patients achieve no residual tumor postoperatively remains to be answered. It could be that we are actually analyzing the residual tumor which then must be regarded as a confounding factor for this specific analysis. Underscoring the argument that residual disease is a confounding factor in radical surgery are two previous studies which reported that patients with malignant pleural effusions have a survival benefit compared to those with distant solid metastases only if they are completely cytoreduced in the abdomen (333-334). We also found a tendency of longer survival after radical surgery in patients with parenchymal liver metastases, but following the same argument as described for pleural effusions, this may be due to the residual disease status and not to the radical surgery itself. We found that more patients with radical surgery achieved no residual disease postoperatively in our study, but survival was not improved compared to patients who achieved no residual tumor after standard surgery,

implicating that residual disease was an important prognostic factor, whilst the level of surgery was not. In agreement with this, two reports have shown no survival difference in patients with radical surgery compared to standard surgery as long as no residual tumor was achieved postoperatively (55-56), but extensive surgery in the upper abdomen will benefit patients as long as no tumor residuum is within reach (55).

In our study we found more postoperative complications in patients who underwent primary surgery, but no analysis was performed on complications after the different surgical levels. The calculation of risk factors for surgery, both the surgical approach and the extent of surgery, should be taken into consideration when deciding on treatment (58,335). The increased postoperative complications may delay time to chemotherapy, but time to initial course is not a significant factor for survival in patients with advanced OC, and should not be used as an argument for not performing extensive surgery (328-329).

In paper V we found that maximal intraabdominal cytoreductive surgery was the cornerstone in the treatment of patients with OC stage IV irrespective of the site of the metastases. How this was achieved was less relevant since survival was equal in patients with radical surgery and standard surgery as long as no residual disease was the end result. However, radical surgery should be performed whenever complete cytoreduction in the abdomen is within reach. A definite prerequisite to surgery is a thorough preoperative and peroperative evaluation of surgical possibilities and limitations, which is invaluable to the patients. Surgical training of gynecological oncologists is necessary, and the surgical procedures should be performed in specialized medical centers (57). We believe that dedication and a systematic approach to the task are important issues in order to further improve survival in women with OC stage IV (52,336).

# Conclusions

- OC cells show different expression of anti-apoptotic proteins of the Bag (Bag-1 and Bag-4/SODD), heat shock (HSP27 and HSP70) and Bcl-2 (Bcl-2 and Bcl-$X_L$) families in effusions compared to primary tumors and solid metastases.

- High p-AKT expression in OC cells in effusions is associated with more aggressive disease in women with serous histology and advanced disease.

- High cytoplasmic p-mTOR expression in OC cells in post-chemotherapy effusions is associated with poor PFS in women with serous histology and advanced disease.

- Protein expression of HSP90 is more frequent in the cytoplasm than in the nucleus of OC cells in effusions. However, nuclear HSP90 expression is higher in post-chemotherapy compared to pre-chemotherapy effusions, which may indicate an upregulation in the nucleus along tumor progression. Nuclear HSP90 in post-chemotherapy effusions also associated with previous treatment with platinol and paclitaxel.

- Residual tumor is a strong prognostic factor for survival in patients with OC stage IV. How minimal residual tumor volume is achieved in terms of both the surgical timing (primary surgery and delayed primary surgery) and the level of surgery (radical surgery and standard surgery) does not impact survival.

- Patients with OC stage IV benefit from maximal intraabdominal cytoreduction irrespective of the site of distant metastasis defining the disease stage.

## Future perspectives

The versatile mechanisms leading to chemotherapy resistance in women with advanced OC have not yet been fully explored or explained, but dysregulation of apoptosis has proved to be involved in cancer cell survival. The constantly increasing list of new chemotherapeutic or biological agents aiming at inducing apoptosis or modifying pro-survival signaling reflects the effort towards overcoming resistance to chemotherapy.

In the present thesis, we have highlighted the clinical relevance of aberrant expression of anti-apoptotic proteins of the Bag, heat shock and Bcl-2 families, and of the cell survival-related molecules p-AKT and p-mTOR in advanced OC. Interesting molecules for further investigation include HSP70, HSP90 and mTOR, and probably also Bcl-2 family members other than Bcl-2 itself, even though they were not studied specifically in this thesis.

Targeted therapy is presently the hot issue in battling chemotherapy resistance, but development of resistance is multifactorial. Treatment failure in women with OC is also affected by tumor load and its relation to chemotherapy resistance. Surgery is still the cornerstone in treatment of ovarian cancer, and in stage IV disease, criteria for patient triage to the correct surgical approach are a prerequisite for success.

In search of future biological targets, chemotherapeutic agents and treatment strategies in women with OC, some clinical and biological factors should be emphasized. Since most women frequently present with malignant effusions in the serosal cavities and with advanced stage metastatic disease at time of diagnosis, our focus should be directed at research in these anatomical sites. In this context, I.P. chemotherapy in the form of HIPEC may prove to be an important therapeutic approach in the future. Also, there may be evidence for different mechanisms causing chemotherapy resistance in primary tumors, solid metastases and malignant effusions, and thus our research should continue to focus on elucidating the differences along tumor progression. Since targeted therapy still focuses on inhibiting activation events and has not reached the point of replacing lost tumor suppressor function, there are good reasons to continue our evaluation of dysregulated apoptotic molecules in tumor cells from women with advanced OC. However, new knowledge should also be considered as research areas in order to overcome chemotherapy resistance.

These areas include mechanisms of forcing cancer cells from a G0-phase into a state of proliferation, in which they become more vulnerable to traditional chemotherapeutic agents. Optional to this is to explore mechanisms of forcing cancer cells to undergo programmed cell death apart from apoptosis, i.e. anoikis. Cancer cells in effusions are devoid of surrounding extracellular matrix, and thus they are potential candidates for research in this field.

The plasma membrane of cancer cells represents an impediment to the efficacy of chemotherapeutic agents, and nanomedicinal strategies have been proven to facilitate transport across the cell membrane and thus improve drug efficacy. We believe that transport strategies for chemotherapeutic agents will be an important area of research in the future. This may provide the possibility of directly targeting cancer cells in their local environment, and not systemically as we do today. If so, this would be an interesting thought in treatment of carcinoma cells in effusions. Finally, the prospect of anti-cancer vaccines in OC is within reach. Among others, HSP70 and HSP90 are able to translocate to the extracellular matrix, where they mediate immunological functions and thus form the basis for vaccines.

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# II

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# Neoadjuvant chemotherapy, interval debulking surgery or primary surgery in ovarian carcinoma FIGO stage IV?

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## ABSTRACT

**Objectives** The aim of this study was to investigate the impact of surgical approach, the extent of surgery and chemotherapy on overall survival in patients with ovarian carcinoma (OC) stage IV.

**Methods** We retrospectively collected population-based data from the Norwegian Radium Hospital code registry on the diagnosis and surgery of 238 patients diagnosed with OC stage IV from 1996-2005. All patients received platinum-based chemotherapy. Surgical approach was registered as primary debulking surgery (PDS), interval debulking surgery (IDS) and delayed primary surgery (DPS). Surgery level was classified as radical surgery (RS), standard surgery (SS) or suboptimal surgery (SUBS). Univariate and multivariate analyses identified prognostic factors in PDS, IDS and DPS groups and subgroups.

**Results** There were no differences in overall survival between the PDS, IDS and DPS groups. Surgery level was significantly associated with overall survival in the whole cohort ($p<0.001$), the PDS and IDS groups, but not in the DPS group. More patients with RS achieved no residual tumor (RT), but overall survival was not superior compared to no RT in the SS group. In 66 patients with no RT there were no differences in overall survival between those who underwent PDS, IDS and DPS. Chemotherapy with platinum/paclitaxel tended to improve survival. RT, WHO performance status and histology were prognostic factors for overall survival in the whole cohort.

**Conclusion** No RT remains the objective, whether PDS, IDS or DPS is performed, and no differences in overall survival were found in the three treatment groups.



**Keywords** Ovarian carcinoma, surgery, chemotherapy, survival, population based analysis

## INTRODUCTION

Ovarian carcinoma (OC) stage IV occurs in 16% of women diagnosed with the disease [1,2] and is characterized by extra-peritoneal and/or parenchymal metastases, and an overall 5-year survival of only 16-20% [3].

Residual tumor (RT) after primary debulking surgery (PDS) is the most frequently reported independent prognostic factor for overall survival [4-12], and two recent retrospective studies found that no RT compared to any postoperative RT improved overall survival in women with OC stage IV [11-12]. However, after PDS no RT was achieved in only 6-13% of women [11-13]. Patients with OC stage IV present with extra-peritoneal and/or parenchymal metastases, and the potential for no RT may be limited. Additionally, as a result of their advanced malignancy, some patients have associated co-morbidities which may impact the extent of surgery. Consequently, the role of PDS in these patients has been debated [14-16]. Neoadjuvant chemotherapy (NAC) before delayed primary surgery (DPS), or repeated interval debulking surgery (IDS) following an initial suboptimal surgery (SUBS) and several cycles of chemotherapy, have been suggested as alternatives to PDS. The NAC theory has been evaluated in both a meta-analysis and a prospective randomized trial [17,18]. Two prospective studies on IDS have shown opposite results [15,19], while two systematic reviews found that IDS had no major impact on survival [20,21]. In a meta-analysis of 835 patients, PDS was feasible and significantly improved overall survival compared to NAC in advanced OC [22]. Therefore, the recommended standard treatment has been PDS followed by platinum/paclitaxel combination chemotherapy (PP) in most gynecologic oncology centers [22,23].

This population-based retrospective study aimed to investigate the impact of surgical approach (PDS, IDS and DPS), surgery level (radical surgery (RS), standard surgery (SS) or SUBS), and chemotherapy (platinum single (P), platinum/non-paclitaxel combination

chemotherapy (NP) and PP) on the clinical course and outcome of patients with OC stage IV referred to the Norwegian Radium Hospital (NRH; approximately 60% of the Norwegian population) from 1996-2005.

## PATIENTS AND METHODS

Approval to review patient data was obtained from the office of the NRH's privacy protection supervisor.

Our cohort consisted of 238 patients with histologically-verified OC stage IV according to the 1988 FIGO classification, who underwent at least one surgical procedure and platinum-based chemotherapy. Patients with parenchymal spleen metastasis were included. Registered clinical and pathological data included WHO performance status, age, histology, tumor grade, stage IV disease site, ascites, measurement of metastatic tumors in the upper abdomen, surgical approach, RT and chemotherapy (Table 1).

The diagnostic tools used to triage and evaluate resectability were gynecologic examination, ultrasound or CT of the pelvis and abdomen, and biopsy or fine needle aspiration of primary tumor and/or metastases. Selection criteria for PDS were WHO performance status 0 or 1, and possibility to perform RS. However, following a publication by Vergote et al. (24), primary chemotherapy and subsequent DPS were increasingly performed in patients with WHO performance status 2-3 and initial inoperable tumor during the study period. Patients with initial SUBS underwent IDS if clinical examination and CT after three cycles of chemotherapy showed treatment response. RT was recorded postoperatively for intra-abdominal disease in three categories: 0 cm, 0.1-2.0 cm, and >2.0 cm. Surgeons were consultants in gynecology with experience in debulking surgery. Most patients received chemotherapy (P, NP or PP) either as 6-9 courses after PDS, 3-4 NAC courses before DPS, or 2-3 courses after SUBS before IDS. Patients with tumor nodules involving the majority of the

bowel surface, including the parietal peritoneum of the upper abdomen and pelvis, were considered to have extensive carcinomatosis (Table 1). If total hysterectomy, bilateral salpingo-oophorectomy, omentectomy and tumor extirpation was performed, patients were classified as undergoing SS. If any bowel resection, splenectomy, pelvic or abdominal lymph node resection, liver resection or extensive pelvic and/or abdominal peritoneal stripping together with standard abdominal surgery and tumor extirpation en block was performed, the patients were classified as undergoing RS. All other types of surgery were classified as SUBS. The feasibility of RS and SS was not influenced by parenchymal liver or lung metastases. Peri- and postoperative deaths and major complications occurring within 28 days of surgery were also registered. Overall survival was our primary endpoint and was calculated from date of diagnosis to date of death, or date of last follow-up (end of follow-up 31 December 2009). No patients were lost to follow-up.

Descriptive analyses of clinical and pathological parameters in the whole cohort and in subgroups were performed. Associations between the different categorical variables were assessed using Chi-square tests. Overall survival was estimated using the Kaplan-Meier method, and groups were compared with log-rank tests.

Cox proportional hazard regression with backward stepwise elimination was performed to find prognostic variables associated with survival for the whole cohort and for the PDS, IDS, and DPS groups separately. Factors with $p<0.20$ in log-rank tests were entered into the analysis. P-values $<0.05$ were regarded as significant. Data analysis was performed using SPSS-PC (version 18.0, Chicago, Illinois, USA).

**RESULTS**

Of 238 patients, 127 underwent PDS and postoperative chemotherapy, 42 underwent IDS, and 69 received NAC prior to DPS. Patient characteristics were well-balanced between the

three treatment groups, save the presence of more patients with WHO performance status 2-3 in the DPS group (p=0.01), more patients with non-extensive carcinomatosis in the PDS and DPS groups (p=0.04), and more patients who achieved no RT in the IDS (45%) and DPS (44%) groups compared to the PDS group (14%) (p<0.001). RS was performed in 108 patients (45%), SS in 77 (32%) and SUBS in 53 (22%) (Table 1).

Univariate analysis did not show any differences in overall survival between the three treatment groups. Median survival time (MST) for PDS was 2.1 years, for IDS 2.6 years, and for DPS 1.9 years (Figure 1). The 5-year overall survival for PDS, IDS and DPS was 12%, 21%, and 19%, respectively, and for all 238 patients it was 16% (Table 2). Age, stage IV disease-site, ascites and extensive carcinomatosis were not significantly associated with survival in any of the three treatment groups. WHO performance status, histology, chemotherapy, RT and surgery level had a significant impact on survival in the PDS group. WHO performance status, histology, tumor grade and surgery level were also of importance for survival in IDS. Only tumor grade had a significant impact on survival in the DPS group (Table 3).

Seventy-eight percent of the patients in the PDS group received PP compared to 81% and 84% in the IDS and DPS group, respectively. There were no differences between the groups receiving P and NP (henceforth called P). In the PDS, but not the IDS and DPS groups, PP was associated with significantly longer overall survival than P, with a MST of 2.5 vs. 1.1 years (Table 3). Survival analysis for the RS, SS and SUBS groups in relation to PP and P showed that PP in the three surgical groups conferred a significantly longer overall survival compared to P (data not shown). Five-year overall survival for PP was 17% compared to 10% for P (Table 2).

MST in the PDS group was: 2.7 years for RS, 1.8 for SS and 1.5 for SUBS. Corresponding values in the IDS group were 3.2, 2.9 and 1.7 years. No significant difference

was observed in the DPS group (Table 3). In the whole cohort there was a significant difference in MST between the three surgery levels (RS: 2.6, SS: 2.1, and SUBS: 1.6 years. p<0.001) (Figure 2, Table 4). Five-year overall survival for RS, SS and SUBS was 20%, 18%, and 4%, respectively (Table 2). No RT was achieved in 46/108 (43%) patients after RS compared to 19/75 (25%) after SS and 1/52 (2%) after SUBS (p<0.001). No difference in MST was observed between RS and SS in patients with no RT (data not shown). MST of all patients with no RT vs. RT >2.0 cm was 3.2 and 1.7 years, respectively (p<0.001) (Figure 3, Table 4).

The effect of surgery level on overall survival in 19 patients with parenchymal liver metastases was compared to patients with other stage IV disease sites. A tendency of longer survival was registered after RS in the former group, with an MST of 3.7 years compared to 1.4 years for the other subgroups (p=0.08) (data not shown).

Among 91 patients in the cohort with no pleural exudates, 69 had solid extra-abdominal metastases and 22 had both solid extra-abdominal metastases and general carcinomatosis. There was no difference in overall survival between these two groups. Twenty-six of 54 patients (48%) with solid extra-abdominal metastases achieved no RT after RS compared to 4/19 (21%) after SS, with an MST of 3.1 and 1.9 years, respectively (p=0.03). In the RS group, PP was associated with significantly better overall survival than P (MST 2.9 compared to 1.3 years, respectively, p=0.02) (data not shown).

One-hundred and forty-seven patients (62%) had only positive pleural effusion to define OC stage IV. Forty-four of these patients (30%) also had extensive carcinomatosis, but there was no difference in overall survival between these two groups. MST in the RS group was 2.6 years compared to 2.3 and 1.5 years in the SS and SUBS groups, respectively (p=0.001) (data not shown).

In 66 patients with no RT, there were no differences in overall survival between PDS, IDS and DPS. Patients who achieved no RT after RS had an overall survival similar to that of patients who achieved no RT without aggressive procedures. The MST for the bowel resection and the splenectomy group was 2.1 and 3.5 years, respectively. In the RS group, 40 patients underwent bowel resection with end-to-end anastomosis; 11 underwent stoma operation.

Perioperative mortality and major postoperative complications were registered in 73 patients (31%). There was only one perioperative death out of 238 operations (PDS). In addition, three patients in the PDS group underwent reoperation due to ileus. Manageable complications like severe cardiac arrhythmia, hemorrhages requiring >4 units of blood, severe infections and abscesses were more common in the PDS group.

The prognostic factors for prolonged survival for the whole cohort were WHO performance status, histology, and RT (Table 4). For PDS patients, RT and chemotherapy were included in the final model. For IDS patients, tumor grade, WHO performance status and surgery level were included, and for DPS patients, tumor grade and RT were prognostic factors. Histology was not included in the last three analyses due to the limited number of patients in the different subgroups.

## DISCUSSION

The strength of this population-based study lies in the large number of patients, none of whom were lost to follow-up. The weakness lies in is its retrospective design and subjectivity (non-randomized) of treatment decision (whether up-front treatment would be PDS, IDS or DPS), and also in the interobserver variability in estimating RT. The number of hospitals and surgeons performing the surgery is also a weakness of the study.

RT after PDS is one of the most powerful determinants of survival in patients with OC stage IV [11,12,22]. In the whole cohort the hazard rate was 1.7 and 2.2 for RT 0.1-2.0 cm and RT >2.0 cm, respectively, compared to no RT. However, it should be noted that no RT was achieved in only 14% of the PDS group. A prospective randomized study has shown that PDS was not superior to NAC as a treatment option for patients with OC stage IIIC or IV, and concluded that RS of all macroscopic disease and no postoperative RT remains the gold standard, regardless of how cytoreductive surgery is performed [18]. This is consistent with the conclusions of a meta-analysis [17], and our present study, which showed no differences in survival between the PDS, IDS and DPS groups. We expected the DPS group to have better overall survival than the PDS group, but the increased rates of no RT in the DPS group did not fully translate into improved overall survival. This could be explained by development of resistance to chemotherapy when NAC is started for bulky disease. It is unclear whether the DPS strategy is useful for gynecologic oncologist surgeons who achieve extremely high rates of no RT, or if patients with a low risk of SUBS will benefit from DPS. Patients who underwent DPS had a higher WHO performance status (2-3); although this was not significant for overall survival in the DPS group, it was for the whole cohort.

Patients with malignant pleural effusion and extensive carcinomatosis showed improved overall survival if no RT was achieved and PP was given postoperatively in the RS and SS groups. This is in agreement with Wimberger et al. [12], who found macroscopically complete resection in patients with OC stage IV to be an important prognostic factor for overall survival, irrespective of the site of metastasis, and Bristow et al. [9], who advocated as much intra-peritoneal tumor reduction as possible even in patients with unresectable parenchymal liver metastases. However, in our cohort extensive carcinomatosis and metastatic tumor size in the upper abdomen was not a prognostic factor for overall survival. Patients who achieved no RT by RS had the same survival advantage as patients who achieved no RT without

aggressive surgery, which is in agreement with Munkarah et al. [5]. In addition, eight patients with peritoneal and parenchymal spleen involvement underwent splenectomy, and cytoreduction to no RT was achieved in seven of them, corresponding to an impressive MST of 3.5 years. Therefore splenectomy, and also bowel surgery should be performed when no RT is within reach.

The complication rate was acceptable. Our data, like Winter et al. [11], show that variations in the size of RT were not associated with an increased risk of severe complications. This suggests that there is no increased level of morbidity or mortality when cytoreduction to no RT is performed by an experienced gynecologic oncologist surgeon.

In this study, the majority of patients in the PDS and DPS group were treated with PP. Interestingly, the significance of chemotherapy was greater for patients who achieved no RT, which is consistent with Eisenhauer et al., who found maximal cytoreduction to be associated with improved initial chemotherapy response [13].

Currently, preoperative identification of patients most likely to achieve no RT is limited to radiologic imaging and laboratory testing [10,11,25,26]. Vergote et al. have suggested criteria for NAC in OC stage IIIC and IV [27,28].

In conclusion, our findings concur with previous reports that maximal intra-abdominal cytoreductive surgery is the cornerstone of treatment for patients with OC stage IV, irrespective of site of metastasis [5,9,11,12]. RS should be performed if no RT is within reach, and the complication rate is acceptable when surgery is done by an experienced gynecologic oncologist surgeon. DPS represents a viable alternative strategy for patients in the group deemed initially unresectable. In our cohort we found that the survival outcome of the DPS group was not inferior to that of the PDS group, which is consistent with a current prospective study and a retrospective study [18,29].

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## FIGURE LEGENDS

**Figure 1: Survival of 238 patients with ovarian cancer stage IV in relation to primary debulking surgery (PDS), interval debulking surgery (IDS) and delayed primary surgery (DPS)**

P=0.2.

**Figure 2: Survival of 238 patients with ovarian cancer stage IV in relation to different surgery levels**

P<0.001. RS=radical surgery, SS=standard surgery, SUBS=suboptimal surgery

**Figure 3**: **Survival of 234 patients with ovarian cancer stage IV in relation to residual tumor**

P<0.001.

Table

**Table 1. Clinical characteristics of 238 patients with ovarian cancer stage IV by surgical approach**

| Characteristics | Patients N (%) | PDS N (%) | IDS N (%) | DPS N (%) | p-value |
|---|---|---|---|---|---|
| WHO performance status | 238 | 127 | 42 | 69 | 0.01 |
| 0 | 110 (46) | 64 (50) | 20 (47) | 26 (38) | |
| 1 | 88 (37) | 48 (38) | 18 (43) | 22 (32) | |
| 2-3 | 40 (17) | 15 (12) | 4 (10) | 21 (30) | |
| Age (years) | 238 | 127 | 42 | 69 | 0.33 |
| <50 | 36 (15) | 19 (15) | 9 (21) | 8 (12) | |
| 50-59 | 79 (33) | 35 (28) | 14 (34) | 30 (43) | |
| 60-69 | 62 (26) | 37 (29) | 10 (24) | 15 (22) | |
| >70 | 61 (26) | 36 (28) | 9 (21) | 16 (23) | |
| Histology | 238 | 127 | 42 | 69 | 0.33 |
| Serous | 208 (87) | 108 (85) | 39 (93) | 61 (89) | |
| Endometrioid | 5 (2) | 5 (4) | 0 (0) | 0 (0) | |
| Mucinous or clear cell | 6 (3) | 2 (2) | 1 (2) | 3 (4) | |
| Mixed or unclassified | 19 (8) | 12 (9) | 2 (5) | 5 (7) | |
| Tumor grade[1] | 238 | 127 | 42 | 69 | 0.17 |
| Low | 51 (21) | 35 (27) | 5 (12) | 11 (16) | |
| High | 173 (73) | 85 (67) | 34 (81) | 54 (78) | |
| Not graded | 14 (6) | 7 (6) | 3 (7) | 4 (6) | |
| Stage IV disease site | 238 | 127 | 42 | 69 | 0.52 |
| Pleural effusion | 147 (61) | 75 (59) | 30 (72) | 42 (61) | |
| Pulmonary metastasis | 7 (3) | 4 (3) | 0 (0) | 3 (4) | |
| Parenchymal liver metastasis | 19 (8) | 14 (11) | 2 (5) | 3 (4) | |
| Supra-diaphragmatic lymph node | 33 (14) | 18 (14) | 3 (7) | 12 (18) | |
| Subcutaneous | 18 (8) | 9 (7) | 3 (7) | 6 (9) | |
| Other[2] | 14 (6) | 7 (6) | 4 (9) | 3 (4) | |
| Ascites | 144 | 75 | 26 | 43 | 0.11 |
| Positive cytology | 130 (90) | 64 (85) | 25 (96) | 41 (95) | |
| Negative cytology | 14 (10) | 11 (15) | 1 (4) | 2 (5) | |
| Extensive carcinomatosis | 238 | 127 | 42 | 69 | 0.04 |
| Yes | 66 (28) | 27 (21) | 17 (41) | 22 (32) | |
| No | 172 (72) | 100 (79) | 25 (59) | 47 (68) | |
| Largest metastatic tumor in the upper abdomen | 151 | 80 | 30 | 41 | 0.35 |
| No tumor described | 40 (26) | 25 (31) | 6 (20) | 9 (22) | |
| <2 cm | 16 (11) | 7 (9) | 2 (7) | 7 (17) | |
| ≥2 cm | 95 (63) | 48 (60) | 22 (73) | 25 (61) | |
| Chemotherapy | 238 | 127 | 42 | 69 | 0.51 |
| Platinum/paclitaxel | 191 (80) | 99 (78) | 34 (81) | 58 (84) | |
| Platinum single | 37 (16) | 24 (19) | 5 (12) | 8 (12) | |
| Platinum/non-paclitaxel | 10 (4) | 4 (3) | 3 (7) | 3 (4) | |
| Surgery level | 238 | 127 | 42 | 69 | 0.30 |
| Radical surgery[3] | 108 (45) | 55 (43) | 19 (45) | 34 (49) | |
| Standard surgery[4] | 77 (33) | 37 (29) | 15 (36) | 25 (36) | |

| | | | | | |
|---|---|---|---|---|---|
| Suboptimal surgery[5] | 53 (22) | 35 (28) | 8 (19) | 10 (15) | |
| | | | | | |
| Residual tumor | 234 | 123 | 42 | 69 | <0.001 |
| 0 cm | 66 (28) | 17 (14) | 19 (45) | 30 (43) | |
| 0.1-2.0 cm | 76 (33) | 44 (36) | 10 (24) | 22 (32) | |
| >2.0 cm | 92 (39) | 62 (50) | 13 (31) | 17 (25) | |
| | | | | | |
| Peritoneal excision/ablation | | | | | |
| Liver | 1 | 1 | 0 | 0 | |
| Spleen | 8 | 3 | 3 | 2 | |
| Upper peritoneum | 4 | 3 | 0 | 1 | |
| Lower peritoneum | 33 | 18 | 5 | 12 | |
| Peritoneal biopsies | 18 | 7 | 6 | 5 | |
| Appendectomy | 31 | 19 | 5 | 7 | |
| | | | | | |
| Bowel surgery | | | | | |
| Colon/rectum | 31 | 18 | 6 | 7 | |
| Small bowel | 3 | 1 | 1 | 1 | |
| Colon and small bowel | 3 | 3 | 0 | 0 | |

[1]Low grade=grades 1 and 2; high grade=grade 3. [2]Includes parenchymal spleen metastases. [3]Standard surgery plus bowel resection, splenectomy, pelvic or abdominal lymph node resection, liver resection or extensive pelvic abdominal stripping. [4]Hysterectomy plus bilateral salpingo-oophorectomy, omentectomy and tumor extirpation. [5]Less than standard surgery.

PDS=primary debulking surgery, IDS=interval debulking surgery, DPS=delayed primary surgery.

**Table 2. 1-, 3-, and 5-year overall survival for different surgical approaches, surgery levels and chemotherapy in 238 patients with ovarian cancer stage IV**

| | Overall survival | | |
|---|---|---|---|
| **Variable** | **1-year (%)** | **3-year (%)** | **5-year (%)** |
| Primary debulking surgery | 80 | 27 | 12 |
| Interval debulking surgery | 93 | 38 | 21 |
| Delayed primary surgery | 84 | 38 | 19 |
| Radical surgery[1] | 92 | 38 | 20 |
| Standard surgery[2] | 83 | 31 | 18 |
| Suboptimal surgery[3] | 66 | 21 | 4 |
| Platinum/paclitaxel | 86 | 35 | 17 |
| Platinum single | 70 | 21 | 10 |
| All patients 238 | 83 | 32 | 16 |

[1]Standard surgery plus bowel resection, splenectomy, pelvic or abdominal lymph node resection, liver resection or extensive pelvic abdominal stripping. [2]Hysterectomy plus bilateral salpingo-oophorectomy, omentectomy and tumor extirpation. [3]Less than standard surgery.

**Table 3. Survival analysis in 238 patients with ovarian cancer stage IV for different clinical, pathological, surgical and chemotherapy variables by different surgical approaches**

| | | Primary debulking surgery | | | | | | Interval debulking surgery | | | | | | Delayed primary debulking surgery | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Variable | Total | N | Median survival (years) | p-value log rank test | HR | 95% CI | p-value multi-variate | N | Median survival (years) | p-value log rank test | HR | 95% CI | p-value multi-variate | N | Median survival (years) | p-value log rank test | HR | 95% CI | p-value multi-variate |
| WHO performance status | 238 | 127 | | 0.03 | | | NS | 42 | | 0.02 | | | 0.05 | 69 | | 0.14 | | | NS |
| 0 | | 64 | 2.6 | | | | | 20 | 3.7 | | 1 | | | 26 | 2.9 | | | | |
| 1 | | 48 | 1.9 | | | | | 18 | 1.9 | | 2.9 | 1.2-6.8 | 0.02 | 22 | 1.8 | | | | |
| 2-3 | | 15 | 1.4 | | | | | 4 | 1.7 | | 1.9 | 0.6-6.1 | 0.3 | 21 | 1.4 | | | | |
| Age (years) | 238 | 127 | | 0.28 | | | | 42 | | 0.70 | | | | 69 | | 0.20 | | | |
| <50 | | 19 | 1.9 | | | | | 9 | 2.3 | | | | | 8 | 2.1 | | | | |
| 50-59 | | 35 | 2.4 | | | | | 14 | 2.1 | | | | | 30 | 1.5 | | | | |
| 60-69 | | 37 | 2.1 | | | | | 10 | 2.9 | | | | | 15 | 2.5 | | | | |
| >70 | | 36 | 1.6 | | | | | 9 | 2.6 | | | | | 16 | 2.9 | | | | |
| Histology[1] | 238 | 127 | | < 0.001 | | | | 42 | | < 0.001 | | | | 69 | | 0.46 | | | |
| Serous | | 108 | 1.9 | | | | | 39 | 2.7 | | | | | 61 | 1.9 | | | | |
| Endometrioid | | 5 | 3.9 | | | | | 0 | | | | | | 0 | | | | | |
| Mucinous or clear cell | | 2 | 0.2 | | | | | 1 | 0.3 | | | | | 3 | 1.4 | | | | |
| Mixed or unclassified | | 12 | 2.8 | | | | | 2 | 2.2 | | | | | 5 | 1.4 | | | | |
| Tumor grade[2] | 238 | 127 | | 0.09 | | | NS | 42 | | < 0.001 | | | 0.004 | 69 | | 0.03 | | | 0.007 |
| Low | | 35 | 2.4 | | | | | 5 | 2.0 | | 1 | | | 11 | 3.5 | | 1 | | |
| High | | 85 | 2.1 | | | | | 34 | 2.9 | | 0.46 | 0.1-1.4 | 0.19 | 54 | 1.7 | | 2.7 | 1.3-5.6 | 0.01 |
| Not graded | | 7 | 1.5 | | | | | 3 | 1.1 | | 11.0 | 1.3-92.6 | 0.03 | 4 | 3.3 | | 1.1 | 0.3-3.7 | 0.87 |
| Chemotherapy | 238 | 127 | | <0.001 | | | <0.001 | 42 | | 0.34 | | | | 69 | | 0.72 | | | |
| Platinum/paclitaxel | | 99 | 2.5 | | 1 | | | 34 | 2.7 | | | | | 58 | 1.9 | | | | |
| Platinum single | | 28 | 1.1 | | 2.3 | 1.5-3.7 | | 8 | 2.2 | | | | | 11 | 1.3 | | | | |
| Residual tumor | 234 | 123 | | < 0.001 | | | 0.001 | 42 | | 0.33 | | | | 69 | | 0.17 | | | 0.06 |
| 0 cm | | 17 | 4.0 | | 1 | | | 19 | 2.9 | | | | | 30 | 3.0 | | 1 | | |
| 0.1-2.0 cm | | 44 | 2.1 | | 2.2 | 1.1-4.1 | 0.02 | 10 | 2.2 | | | | | 22 | 1.5 | | 1.1 | 0.6-2.0 | 0.7 |
| >2.0 cm | | 62 | 1.7 | | 2.9 | 1.5-5.4 | 0.001 | 13 | 1.8 | | | | | 17 | 1.6 | | 2.2 | 1.2-4.2 | 0.02 |
| Stage IV disease site[1] | 238 | 127 | | 0.38 | | | | 42 | | 0.32 | | | | 69 | | 0.46 | | | |
| Positive pleural effusion | | 75 | 2.1 | | | | | 30 | 2.6 | | | | | 42 | 1.8 | | | | |
| Pulmonary metastasis | | 4 | 1.3 | | | | | 0 | | | | | | 3 | 1.9 | | | | |
| Parenchymal liver metastasis | | 14 | 1.7 | | | | | 2 | 1.4 | | | | | 3 | 1.4 | | | | |
| Supra-diaphragmatic lymph node | | 18 | 2.1 | | | | | 3 | 3.9 | | | | | 12 | 1.5 | | | | |
| Subcutaneous | | 9 | 1.8 | | | | | 3 | 2.8 | | | | | 6 | 3.4 | | | | |
| Other | | 7 | 2.4 | | | | | 4 | 2.2 | | | | | 3 | 2.8 | | | | |
| Ascites[1] | 144 | 75 | | 0.17 | | | | 26 | | 0.94 | | | | 43 | | 0.47 | | | |
| Positive cytology | | 64 | 1.9 | | | | | 25 | 2.4 | | | | | 41 | 2.5 | | | | |
| Negative cytology | | 11 | 2.1 | | | | | 1 | 2.9 | | | | | 2 | 1.5 | | | | |

| | | Primary debulking surgery | | | | | | Interval debulking surgery | | | | | | Delayed primary debulking surgery | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Total** | **N** | **Median survival (years)** | **p-value log rank test** | **HR** | **95% CI** | **p-value multi-variate** | **N** | **Median survival (years)** | **p-value log rank test** | **HR** | **95% CI** | **p-value multi-variate** | **N** | **Median survival (years)** | **p-value log rank test** | **HR** | **95% CI** | **p-value multi-variate** |
| Extensive carcinomatosis[1] | 238 | 127 | | 0.61 | | | | 42 | | 0.55 | | | | 69 | | 0.92 | | | |
| No | | 100 | 1.9 | | | | | 25 | 2.4 | | | | | 47 | 1.8 | | | | |
| Yes | | 27 | 2.7 | | | | | 17 | 2.8 | | | | | 22 | 2.5 | | | | |
| Surgery level | 238 | 127 | | 0.01 | | | NS | 42 | | 0.05 | | | 0.04 | 69 | | 0.59 | | | |
| Radical surgery[3] | | 55 | 2.7 | | | | | 19 | 3.2 | | 1 | | | 34 | 1.8 | | | | |
| Standard surgery[4] | | 37 | 1.8 | | | | | 15 | 2.9 | | 1.7 | 0.8-4.0 | 0.2 | 25 | 2.1 | | | | |
| Suboptimal surgery[5] | | 35 | 1.5 | | | | | 8 | 1.7 | | 3.4 | 1.3-8.5 | 0.01 | 10 | 1.4 | | | | |
| Patients with solid metastases[1] | 91 | 52 | | 0.36 | | | | 12 | | 0.03 | | | | 27 | | 0.45 | | | |
| Radical surgery | | 32 | 2.4 | | | | | 10 | 2.8 | | | | | 13 | 2.9 | | | | |
| Standard surgery | | 10 | 1.4 | | | | | 1 | 2.2 | | | | | 8 | 1.4 | | | | |
| Suboptimal surgery | | 10 | 1.9 | | | | | 1 | 0.6 | | | | | 6 | 1.4 | | | | |

[1]Histology, stage IV carcinomatosis, and solid metastasis were not included in the multivariate analysis. [2]Low grade=grades 1 and 2; high grade=grade 3. [3]Standard surgery plus bowel resection, splenectomy, pelvic or abdominal lymph node resection, liver resection or extensive pelvic abdominal stripping. [4]Hysterectomy plus bilateral salpingo-oophorectomy, omentectomy and tumor extirpation. [5]Less than standard surgery.

HR=hazard rate, CI=confidence interval.

**Table 4. Survival analysis in 238 patients with ovarian cancer stage IV for different clinical, pathological, surgical and chemotherapy variables**

| Variable | N | Median survival (years) | p-value log rank test | HR | 95% CI | p-value multi-variate |
|---|---|---|---|---|---|---|
| WHO performance status | 238 | | 0.001 | | | 0.001 |
| 0 | 110 | 2.7 | | 1 | | |
| 1 | 88 | 1.9 | | 1.5 | 1.1-2.1 | 0.005 |
| 2-3 | 40 | 1.4 | | 1.9 | 1.3-2.8 | 0.001 |
| | | | | | | |
| Age (years) | 238 | | 0.68 | | | |
| <50 | 36 | 2.1 | | | | |
| 50-59 | 79 | 1.9 | | | | |
| 60-69 | 62 | 2.5 | | | | |
| >70 | 61 | 2.1 | | | | |
| | | | | | | |
| Histology | 238 | | 0.001 | | | 0.002 |
| Serous | 208 | 2.1 | | 1 | | |
| Endometrioid | 5 | 3.9 | | 0.6 | 0.2-1.8 | 0.40 |
| Mucinous + clear cell | 6 | 0.5 | | 4.3 | 1.9-10.0 | 0.001 |
| Mixed + unclassified | 19 | 2.8 | | 0.6 | 0.3-0.9 | 0.03 |
| | | | | | | |
| Tumor grade[1] | 238 | | 0.17 | | | NS |
| Low | 51 | 2.6 | | | | |
| High | 173 | 2.1 | | | | |
| Not graded | 14 | 1.4 | | | | |
| | | | | | | |
| Chemotherapy | 238 | | 0.01 | | | NS |
| Platinum/paclitaxel | 191 | 2.4 | | | | |
| Platinum single | 47 | 1.4 | | | | |
| | | | | | | |
| Residual tumor | 234 | | <0.001 | | | <0.001 |
| 0 cm | 66 | 3.2 | | 1 | | |
| 0.1-2.0 cm | 76 | 2.1 | | 1.7 | 1.2-2.4 | 0.01 |
| >2.0 cm | 92 | 1.7 | | 2.2 | 1.5-3.0 | <0.001 |
| | | | | | | |
| Stage IV disease site | 238 | | 0.41 | | | |
| Positive pleural effusion | 147 | 2.1 | | | | |
| Pulmonary metastasis | 7 | 1.9 | | | | |
| Parenchymal liver metastasis | 19 | 1.7 | | | | |
| Supra-diaphragmatic lymph node | 33 | 2.1 | | | | |
| Subcutaneous | 18 | 2.8 | | | | |
| Other | 14 | 2.4 | | | | |
| | | | | | | |
| Extensive carcinomatosis | 238 | | 0.45 | | | |
| No | 172 | 1.9 | | | | |
| Yes | 66 | 2.7 | | | | |

| Variable | N | Median survival (years) | p-value log rank test | HR | 95% CI | p-value multi-variate |
|---|---|---|---|---|---|---|
| Surgery level | 238 | | 0.001 | | | NS |
| Radical surgery[2] | 108 | 2.6 | | | | |
| Standard surgery[3] | 77 | 2.1 | | | | |
| Suboptimal surgery[4] | 53 | 1.6 | | | | |
| Surgical approach | 238 | | 0.20 | | | |
| Primary debulking surgery | 127 | 2.1 | | | | |
| Interval debulking surgery | 42 | 2.6 | | | | |
| Delayed primary surgery | 69 | 1.9 | | | | |

[1]Low grade=grades 1 and 2; high grade=grade 3. [2]Standard surgery plus bowel resection, splenectomy, pelvic or abdominal lymph node resection, liver resection or extensive pelvic abdominal stripping. [3]Hysterectomy plus bilateral salpingo-oophorectomy, omentectomy and tumor extirpation. [4]Less than standard surgery.

HR=hazard rate, CI=confidence interval.

Figure 1

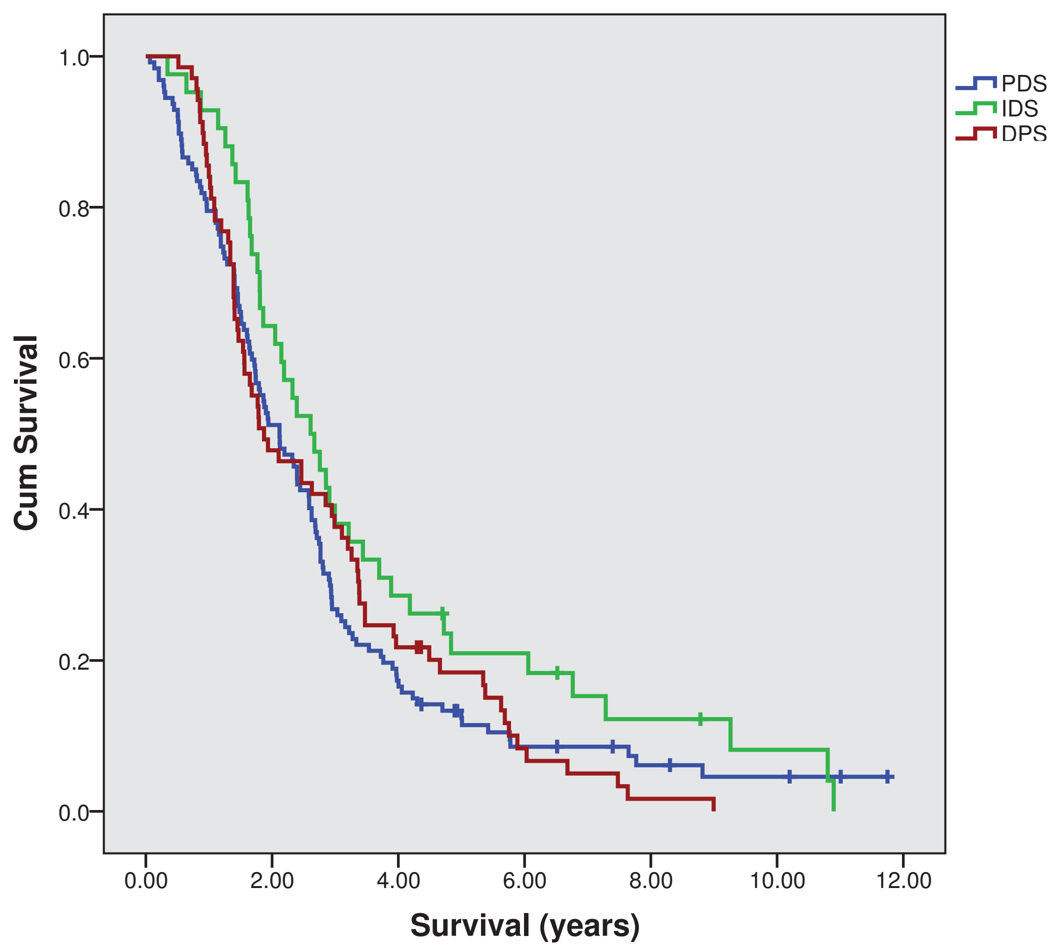

**Figure 2**

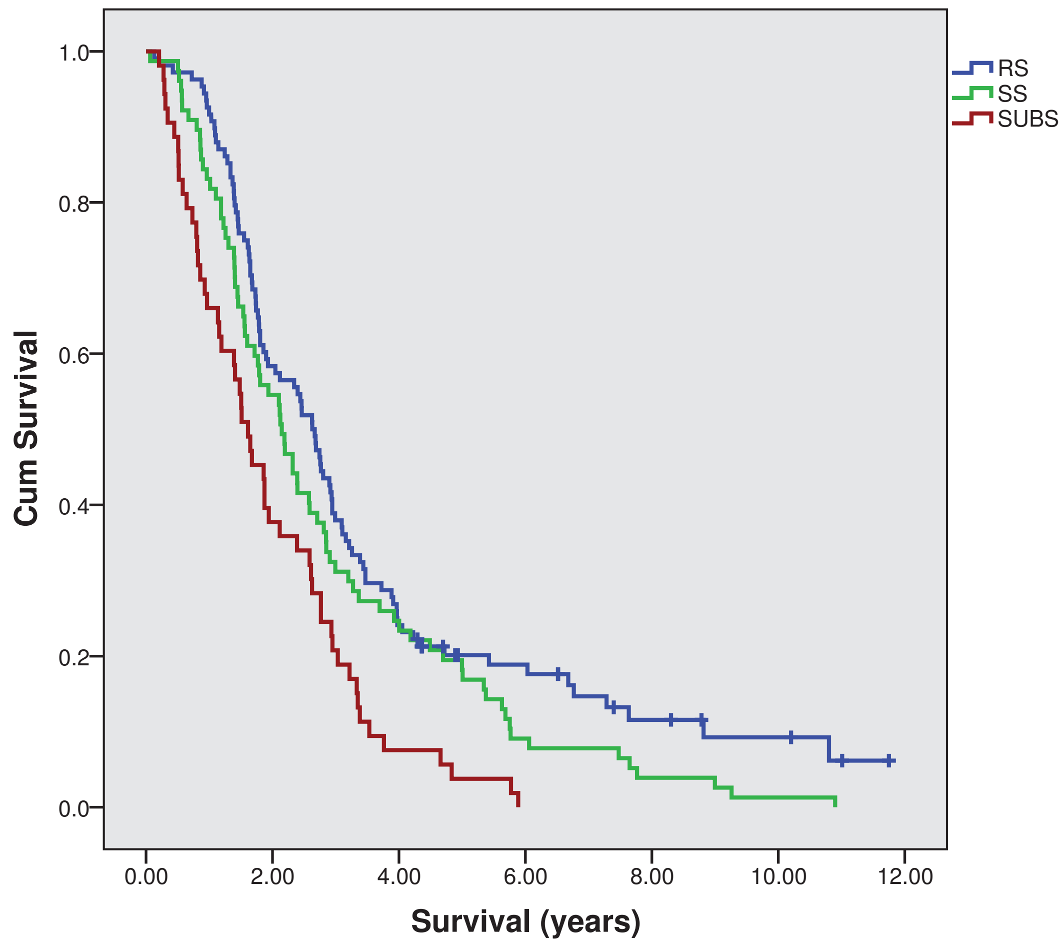

**Figure 3**