

From the DEPARTMENT OF ONCOLOGY-PATHOLOGY  
Karolinska Institutet, Stockholm, Sweden

**MOLECULAR STEPS TOWARDS IMPROVING  
PROGNOSIS IN OVARIAN CANCER**

Elisabet Hjerpe



**Karolinska  
Institutet**

Stockholm 2014

Published and printed by Karolinska University Press  
Box 200, SE-171 77 Stockholm, Sweden  
Cover by Ingrid Wallmark Hjerpe  
© Elisabet Hjerpe, 2014  
ISBN 978-91-7549-496-8

Till Pappa

*Problems are for solving!*



## ABSTRACT

Epithelial ovarian cancer (EOC) is the most lethal of gynecological cancers, and cure rates have improved very little in the last 30 years. The most common histological subtypes are serous, endometrioid, clear cell and mucinous EOC. To date, all EOC have been treated as one entity. However, based on epidemiological and molecular studies it is now clear that the different subtypes should be considered as different diseases. Also, low-grade serous and high-grade serous EOC (HGSOC) has distinctive molecular characteristics. The majority of EOCs are HGSOCs characterized by genetic instability, advanced stage at presentation and acquired chemoresistance. There is an urgent need to identify new targets in order to improve prognosis for these tumors.

A deregulated energy metabolism is a hallmark of malignant disease that offers possible future targets for treatment. Its major features are an increased aerobic glycolysis and alterations in mitochondrial bioenergetics. This thesis aims at identifying prognostic and treatment predictive markers in advanced HGSOC. We specifically explore the expression of metabolic enzymes and heat shock protein 60 (HSP60) and test the chemo-potentiating effect of glycolysis inhibitor 2-deoxy-D-glucose (2-DG) *in vitro*.

We found a platinum-potentiating effect of 2-DG in two EOC cell lines and 17 freshly isolated ascites EOC samples. We also found the ability of the mitochondrial  $\beta$ -F1-ATPase:HSP60 ratio to predict sensitivity to such combination treatment.

We prospectively collected fresh tumor samples from 123 patients undergoing primary surgery for advanced EOC. Of these, 56 met the eligibility criteria with adequate sample RNA yield. Ninety-three percent were high-grade tumors. We performed real-time PCR and immunohistochemistry to study the expression of HSP60, glyceraldehyde-3P-dehydrogenase (GAPDH), pyruvate kinase M2 (PKM2), mitochondrial  $\beta$ -F1-ATPase (ATP5B) and the bioenergetic cellular (BEC)-index. We used Cox proportional hazards models to estimate overall survival (OS) and platinum-free interval (PFI). A high HSP60 mRNA was associated with shorter OS (HR, 3.4 95% CI 1.3-8.5) and PFI (HR, 3.3; 95% CI 1.5-7.2). At the protein level, HSP60 was also of independent prognostic value, with a median survival difference of 24 months between high- and low expressing groups. All patients with low tumor HSP60 protein expression responded to primary chemotherapy. High GAPDH mRNA levels (HR 2.1, 95% CI 1.0-4.5) and low BEC-index mRNA (HR 0.47, 95% CI 0.23-0.95) were both independently associated with shorter PFI.

We also compared the mRNA expression of metabolic markers and HSP60 in a series of 25 matched serous solid tumors and corresponding detached tumor cells in ascites. GAPDH, PKM2, ATP5B and HSP60 did not significantly differ in these respective cell states, indicating that further reprogramming of glycolysis or oxidative phosphorylation is not a prerequisite for serous cancer cell survival after detachment.

This thesis validates targeting glucose metabolism for increasing treatment efficacy in EOC. Our findings also indicate that HSP60, GAPDH and BEC-index may, within the seemingly homogenous group of advanced HGSOCs, identify patients with different prognosis.

## LIST OF PUBLICATIONS

- I. Hernlund E, Hjerpe E, Åvall-Lundqvist E, Shoshan M.  
**Ovarian carcinoma cells with low levels of  $\beta$ -F1-ATPase are sensitive to combined platinum and 2-deoxy-D-glucose treatment.**  
*Mol Cancer Ther* 2009; 8(7): 1916-1923.
- II. Hjerpe E, Egyhazi S, Carlson J, Frostvik Stolt M, Schedvins K, Johansson H, Shoshan M, Åvall-Lundqvist E.  
**HSP60 predicts survival in advanced serous ovarian cancer.**  
*Int J Gynecol Cancer* 2013; 23(3): 448-455.
- III. Hjerpe E, Egyhazi Brage S, Carlson J, Frostvik Stolt M, Schedvins K, Johansson H, Shoshan M, Åvall-Lundqvist E.  
**Metabolic markers GAPDH, PKM2, ATP5B and BEC-index in advanced serous ovarian cancer.**  
*BMC Clin Pathology* 2013; 13:30.
- IV. Hjerpe E, Egyhazi Brage S, Frostvik Stolt M, Johansson H, Shoshan M, Åvall-Lundqvist E.  
**Metabolic markers and HSP60 in chemo-naïve serous solid ovarian cancer versus ascites.**  
*Manuscript.*

# TABLE OF CONTENTS

1	EPITHELIAL OVARIAN CANCER.....	1
1.1	INTRODUCTION.....	1
1.2	EPIDEMIOLOGY.....	2
1.3	PATHOGENESIS.....	4
1.3.1	High- and low-grade serous carcinomas – HGSOC and LGSOC..	4
1.3.2	Endometrioid carcinomas.....	5
1.3.3	Clear cell carcinomas.....	6
1.3.4	Mucinous carcinomas.....	6
1.4	RISK FACTORS AND PROTECTIVE FACTORS.....	6
1.5	CLINICAL PRESENTATION.....	12
1.6	TREATMENT.....	15
1.6.1	Primary treatment.....	15
1.6.2	Treatment for platinum sensitive recurrent disease.....	16
1.6.3	Treatment for platinum resistant disease.....	16
1.6.4	Targeting angiogenesis.....	17
1.7	RESPONSE TO TREATMENT AND PROGNOSIS – SHORT SUMMARY.....	18
1.8	PROGNOSTIC AND TREATMENT PREDICTIVE MARKERS.....	18
1.8.1	HGSOC.....	20
2	ENERGY METABOLISM IN CANCER.....	22
2.1.1	Glycolysis.....	23
2.1.2	Oxidative phosphorylation.....	25
2.1.3	Metabolic markers.....	26
3	AIMS OF THE THESIS.....	28
4	PATIENTS AND METHODS.....	29
4.1	PATIENTS AND MATERIALS.....	29
4.1.1	PAPER I.....	29
4.1.2	PAPERS II AND III.....	29
4.1.3	PAPER IV.....	32
4.2	METHODS.....	32
4.2.1	PAPER I.....	32
4.2.2	PAPERS II, III and IV.....	33
4.3	STATISTICS.....	34
4.3.1	PAPER I.....	34
4.3.2	PAPER II AND III.....	34
4.3.3	PAPER IV.....	35
5	RESULTS, DISCUSSION AND CONCLUSIONS.....	36
5.1	PAPER I.....	36
5.1.1	Cell lines.....	36
5.1.2	Tumor cells from ascites.....	36
5.1.3	Discussion and conclusions.....	37
5.2	PAPERS II AND III.....	38
5.2.1	HSP60.....	38
5.2.2	GAPDH.....	39
5.2.3	PKM2.....	39

5.2.4	ATP5B .....	40
5.2.5	BEC-index.....	40
5.2.6	Discussion and conclusions .....	40
5.3	PAPER IV .....	41
5.3.1	Solid tumor versus ascites .....	41
5.3.2	Discussion and conclusion.....	41
5.4	CONSIDERATIONS IN CHOICE OF METHODS .....	42
5.5	STATISTICAL PITFALLS.....	43
6	GENERAL CONCLUSIONS .....	44
7	FUTURE PERSPECTIVES .....	45
8	ACKNOWLEDGEMENTS .....	46
9	REFERENCES .....	48



## LIST OF ABBREVIATIONS

ARID1A	AT-rich interactive domain 1A gene
ASCO	American Society of Clinical Oncology
ATP	adenosine 5'-triphosphate
$\beta$ -F1-ATPase	catalytic subunit of the H <sup>+</sup> -ATP synthase (ATP5B)
BEC-index	bioenergetic cellular index
BMI	body mass index
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA	breast cancer, early onset gene
CA125	cancer antigen 125
CCNE1	gene encoding cell cycle regulator cyclin E1
CI	confidence interval
c-Met/MET	gene encoding the hepatocyte growth factor receptor
CTNNB1	gene encoding cell adhesion protein $\beta$ -catenin
2-DG	2-deoxy- $\Delta$ -glucose
EOC	epithelial ovarian cancer
EMSY	gene encoding protein Emsy of the BRCA pathway
ER	estrogen receptor
ERBB2	gene encoding receptor tyrosine kinase erbB-2 (HER2)
ESGO	European Society of Gynecologic Oncology
FDG	<sup>18</sup> fluoro-deoxyglucose
FGF	fibroblast growth factor
FIGO	Federation Internationale de Gynecologie et d'Obstetrique
GAPDH	glyceraldehyde 3-P dehydrogenase
GCIG	Gynecologic Cancer InterGroup
GLOBOCAN	WHO global cancer database, <a href="http://www-dep.iarc.fr">www-dep.iarc.fr</a>
GOG	Gynecologic Oncology Group
HGSOC	high-grade serous ovarian cancer
HIF-1 $\alpha$	hypoxia-inducible factor 1 $\alpha$
HK	hexokinase
HR	hazard ratio
HR	homologous recombination
HRT	hormone replacement therapy

HSP60	heat shock protein 60
IC <sub>50</sub>	half maximal inhibitory concentration
IHC	immunohistochemistry
IVF	in vitro fertilization
JAK	janus kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LGSOC	low-grade serous ovarian cancer
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase (MAPK) kinase
mTOR	mammalian target of rapamycin
NA	not applicable
NAD <sup>+</sup>	nicotinamide adenine dinucleotide
NF-κB	nuclear factor κB
NORDCAN	WHO Nordic cancer database, <a href="http://www-dep.iarc.fr">www-dep.iarc.fr</a>
OR	odds ratio
OS	overall survival
OXPPOS	oxidative phosphorylation
p53	tumor protein/suppressor p53
PARP	poly ADP ribose polymerase
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PFI	platinum-free interval
PFS	progression-free survival
PGR	progesterone receptor
PI3K	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol-4,5-bis-P 3-kinase, catalytic subunit alpha
PKM2	pyruvate kinase, isoenzyme M2 (muscle)
PLD	pegylated liposomal doxorubicin
PTEN	phosphatase and tensin homolog
RAD51	gene encoding DNA repair protein RAD51 homolog 1
RB	retinoblastoma
RECIST	response evaluation criteria in solid tumors
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute (cell medium)

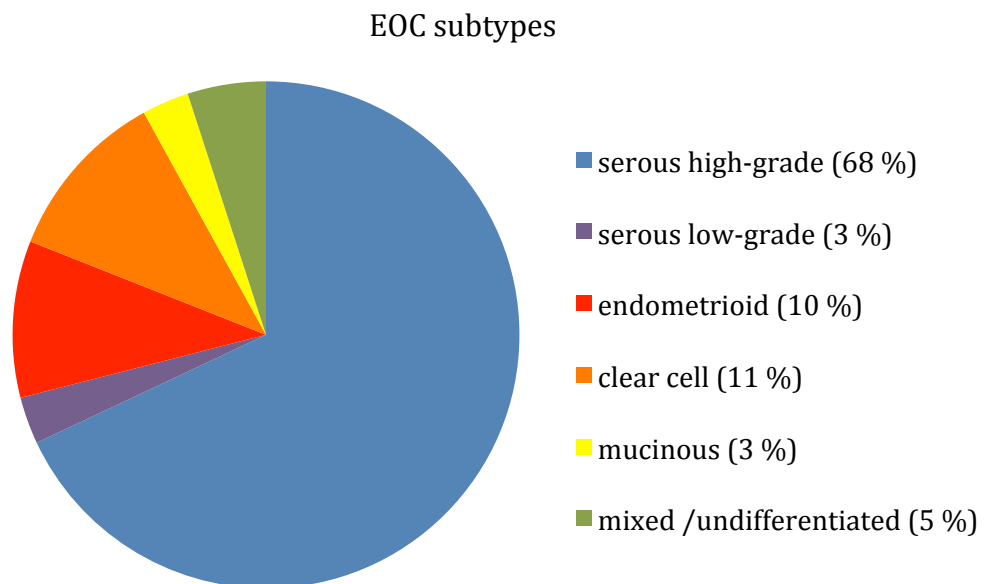
RR	relative risk
SNP	single nucleotide polymorphism
TCA	tricarboxylic acid cycle (citric acid cycle)
TKI	tyrosine kinase inhibitor
TP53	gene encoding tumor protein 53 (p53)
STAT	signal transducer and activator of transcription
STIC	serous tubal intraepithelial carcinoma
VEGF	vascular endothelial growth factor



# 1 EPITHELIAL OVARIAN CANCER

## 1.1 INTRODUCTION

Ovarian tumors are a heterogeneous mixture of benign, borderline and malignant lesions thought to arise in the ovary. Ninety percent of malignant tumors are of epithelial origin, but transformation can occur also in the ovarian stroma or germ cells. The epithelial ovarian carcinomas (EOCs) are classified according to the WHO histopathological standards into different morphological subtypes, essentially including serous, endometrioid, clear cell, mucinous and undifferentiated tumors [1, 2]. The grade of differentiation has traditionally been denoted as low, intermediate or high [3]. Below chart, based on two large reviewed North American case series, shows the approximate relative frequencies of EOC subtypes [4, 5].



The various subtypes of EOC are etiologically and molecularly distinct diseases, with different clinical presentation, prognosis and response to treatment. The high-grade serous carcinomas (HGSOs) comprise the majority of cases and will be the focus of this thesis. This subtype often responds well to initial chemotherapy, but since it is usually diagnosed at an advanced stage, with a high risk of recurrence, most patients eventually develop treatment resistance and die of their disease. The other subtypes are more frequently found at an earlier stage, when prognosis is better [4, 5]. Today there is interest in development of subtype-stratified strategies for prognostication and treatment of EOC, but despite progress in the understanding of the subtype-specific underlying mechanisms of disease, prognosis is still poor. For the purpose of this thesis, the term ovarian cancer/EOC encompasses also the prognostically and clinicopathologically indistinguishable high-grade serous cases originating in the fallopian tube or peritoneum.

A reprogrammed energy metabolism has recently been recognized as a hallmark of cancer [6] offering possible future targets for treatment. However, when searching the literature we found no publication investigating metabolic enzymes as potential prognostic or treatment predictive markers in ovarian cancer. Considering the genetic instability of high-grade serous ovarian tumors, we hypothesized glycolytic and oxidative enzymes to be altered. This thesis presents the results of our work, and is to our knowledge the first publication on this topic.

## 1.2 EPIDEMIOLOGY

Ovarian cancer is the seventh most common malignancy in women worldwide, with an incidence in 2012 of 240,000 cases causing 152,000 deaths [7]. International statistics often include all invasive ovarian tumors, of which the epithelial carcinomas constitute the majority. Northern Europe has the highest incidence with 10 cases per 100,000 women, which for Sweden translates into approximately 750 cases a year [8]. The Swedish ovarian cancer incidence has been decreasing, over the last decade by 2 % per year. This positive trend should be largely attributed the protective effect of oral contraception and reduced use of menopausal hormone replacement therapy.

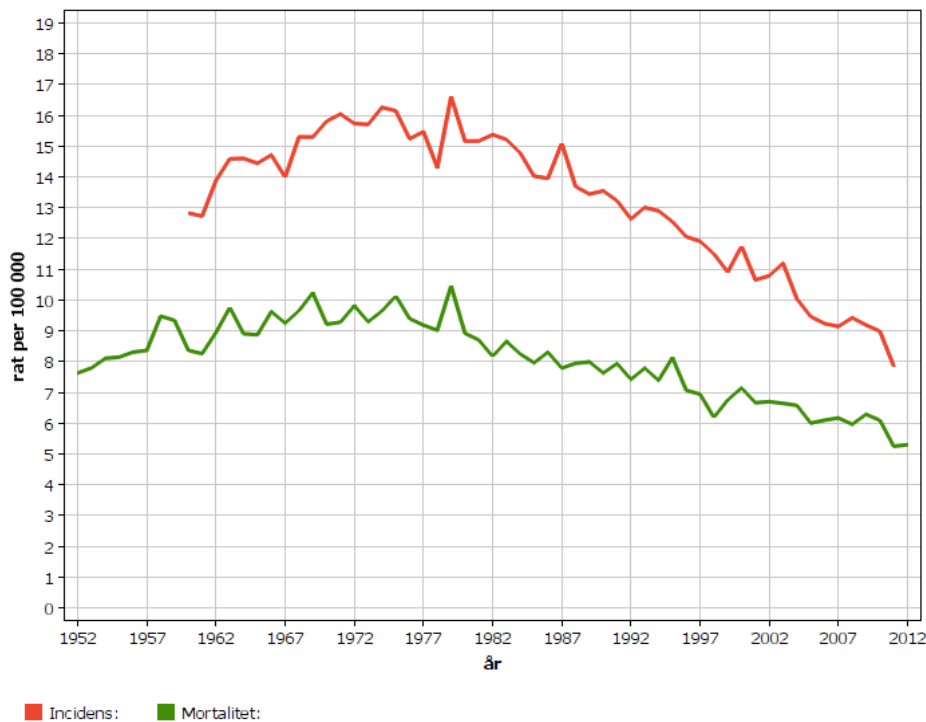


Figure 1. EOC incidence and mortality, Sweden 1980-2011 (NORDCAN database, [www-dep.iarc.fr](http://www-dep.iarc.fr)).

Ovarian cancer can affect women of all ages, but is rare before the age of 30. After that, the incidence gradually increases to peak at 43 per 100,000 Swedish women 65-74 years old (Figure 2).

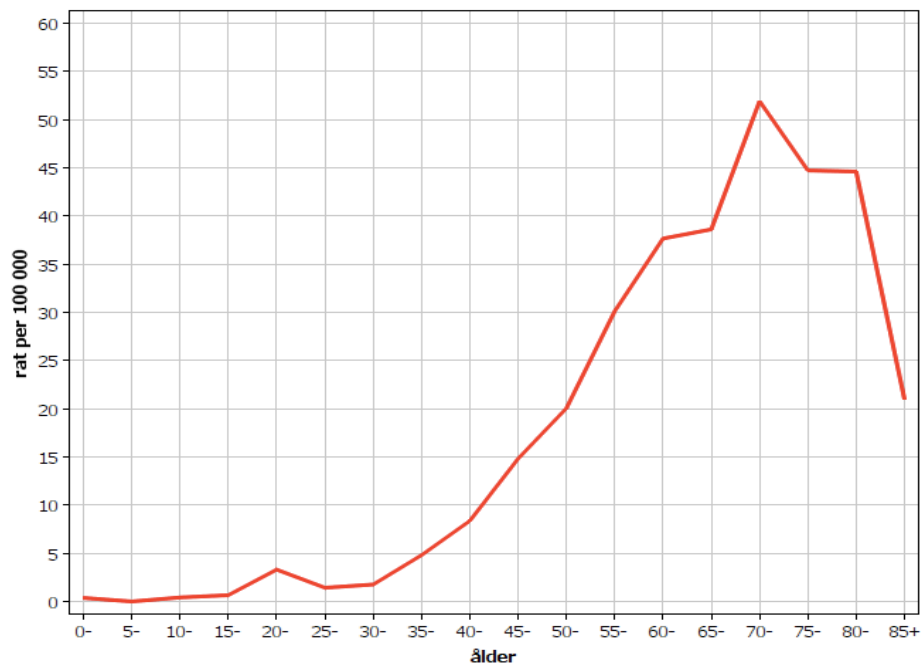


Figure 2. EOC age-specific incidence (Sweden 2010, NORDCAN database).

EOC is the deadliest of gynecological malignancies, with for Sweden a 5-year disease-specific survival rate of 52 %. Figure 3 shows the 5-year survival rates for the Nordic countries 1964-2011. The high mortality rate reflects the dismal fact that the majority of patients present with advanced, FIGO stage II-IV disease.

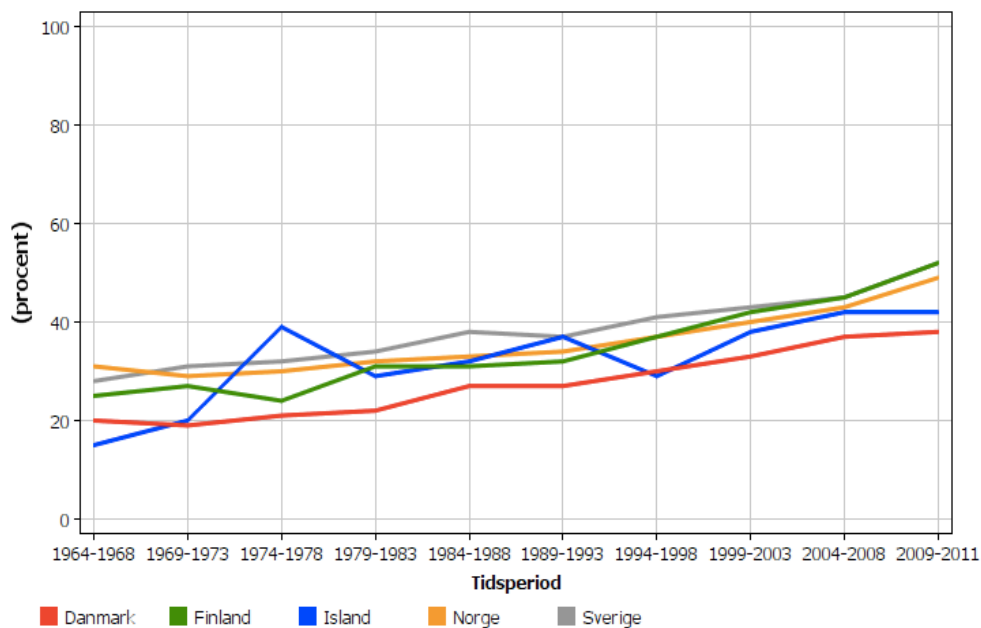


Figure 3. EOC relative survival, Nordic countries 1964-2011 (NORDCAN database).

### 1.3 PATHOGENESIS

Over 90 % of ovarian tumors are of epithelial origin. The ovarian surface epithelium is a single layer of phenotypically uncommitted mesothelial cells that, at the hilus, is continuous with the peritoneal mesothelium. During transformation, the ovarian surface epithelial cells can take on characteristics of Müllerian duct derived tissues, such as the fallopian tube, endometrium and cervix. Tumors can arise directly at the ovarian surface or within inclusion cysts, which are invaginations of surface epithelium into the ovarian stroma. In addition, a large part of the high-grade serous tumors originate in the distal fallopian tube (see below). Ovulation traumatizes the ovarian surface, requiring cyclic tissue reconstruction. This repeated process of epithelial damage, local inflammatory response and cellular proliferation imposes a risk of oncogenic errors during DNA replication. Also, the possible exposure of the fallopian tubes and ovaries to retrograde infections or exogenous carcinogens could further contribute to the risk of transformation.

#### 1.3.1 High- and low-grade serous carcinomas – HGSOC and LGSOC

The serous carcinomas are today classified by a two-tier system into low- and high-grade lesions [9-11]. These two subtypes are clinically and molecularly distinct diseases [12, 13], arising via either of two different routes, type I or type II [11, 14]. Type I tumors are thought to originate in inclusion cysts and to progress via a slow stepwise transformation of a borderline lesion into a low-grade invasive carcinoma (LGSOC). Type II tumors are high-grade lesions arising rapidly without a well-defined clinically detectable precursor. These high-grade serous carcinomas (HGSOC) can originate in the ovarian epithelium, but also in the epithelia of the distal fallopian tube or in the peritoneum [15, 16]. The relative contributions of ovarian, fallopian and peritoneal sites to the genesis of high-grade serous malignancies is unclear at present, but several studies point at the distal part of the fallopian tube as the origin in the majority of cases [15-17].

#### ***HGSOC***

The high-grade serous carcinomas account for almost 70 % of EOC cases. *TP53* mutations are the earliest event associated with HGSOC transformation, and p53 dysfunction is ubiquitous for this subtype. Mutations in the *TP53* gene have been shown in 96 % of these tumors, and in the remaining cases p53 dysfunction is caused by post-translational mechanisms [18, 19]. Foci of intensely p53-positive, but benign linear stretches of secretory cells in the tubal mucosa have been identified in approximately 30 % of both BRCA-positive women and controls [16]. These putative precursor lesions, termed p53-signatures, are significantly more frequent in tubes also containing a serous tubal intraepithelial carcinoma (STIC), and transitions between the two have been documented indicating the distal tubes as a major site of the initial high-grade serous carcinogenic sequence [15]. Supporting this, identical *TP53* mutations have been described in synchronous p53-signatures, STICs and HGSOCs [16, 20].



The second hallmark of HGSOC is BRCA pathway dysfunction, which by the Cancer Genome Atlas Research Network has been shown to affect 51 % of cases [19]. *BRCA1/2* germline mutations were in this study of 489 HGSOCs seen in 17 % and *BRCA* somatic mutations in 3 %. In another 11 %, *BRCA1* was epigenetically silenced. Other BRCA pathway alterations were seen in *EMSY* (amplified in 8 %), *RAD51C* (hypermethylated in 3 %) and *PTEN* (deleted in 7 %). The term BRCAness has gained acceptance as descriptive of the defective homologous recombination DNA repair system that sporadic high-grade serous cancers share with tumors occurring in *BRCA* mutation carriers [21].

Other pathways often altered in HGSOC are the retinoblastoma (RB), PI3K/Akt and Notch signaling cascades, which exhibit oncogenic changes in approximately 67, 45 and 22 % of cases, respectively [19]. Amplification or copy number gain of the cell cycle regulator cyclin E1 gene (*CCNE1*) has been reported in more than 50 % of BRCA wild-type HGSOC [19, 22], and has been suggested to be an early event in the transformation of the secretory tubal mucosa [22].

The early TP53, BRCA and cyclin E1/RB pathway defects cause genomic instability, and HGSOC thus exhibit a pronounced degree of DNA copy number changes [12, 19]. The resulting gene expression alterations are many, and HGSOC is a genetically highly heterogeneous disease [19, 23, 24]. Thus, a model has been proposed, in which HGSOC evolve as a consequence of initial disruption of DNA-repair with subsequent chromosomal instability and segregation into molecular subtypes [12, 25].

### ***LGSO***

The low-grade serous carcinomas represent only 5-8 % of serous tumors [4, 9]. This subtype share molecular features with serous borderline tumors and 60 % of LGSOCs also contain areas of borderline malignant potential [10, 26]. Like their borderline counterparts, LGSOCs often harbor mutually exclusive mutations of the *KRAS*, *BRAF* or *ERBB2* genes, with approximately two thirds of cases having a mutation in either [11, 17, 27]. All three genes are upstream regulators of the mitogen-activated protein kinase (MAPK), and these mutations result in consecutive activation of MAPK signaling and enhanced cell proliferation. *TP53* mutations are uncommon in LGSOC, and the level of chromosomal instability is low [11, 17].

### **1.3.2 Endometrioid carcinomas**

Recent studies show the endometrioid tumors to comprise approximately 10 % of EOCs [1, 5]. This apparent reduction in proportion is due to improved morphological and immunocytochemical diagnostics, leading to the recognition that many tumors that would previously have been classified as high-grade endometrioid are, in fact, serous in type [1]. Above change in subtyping is also supported by global gene expression studies, which have not been able to separate high-grade endometrioid and serous tumors [1, 28].

Most of the endometrioid ovarian carcinomas are thought to arise via a stepwise, type I transformation of an endometriotic lesion [1, 17, 29, 30]. Known molecular features of

these tumors include frequent *CTNNB1* (encoding  $\beta$ -catenin), *PTEN*, *ARID1A*, *KRAS* and *PIK3CA* mutations and microsatellite instability [1, 17, 28, 31, 32]. Low-grade endometrioid tumors lack *TP53* mutations [17].

### 1.3.3 Clear cell carcinomas

Ovarian clear cell carcinomas occur with about the same frequency as the endometrioid subtype, thus accounting for 10-12 % of EOCs [1, 4, 5]. The prevalence in Japan is higher, 15-25 % [33]. Like the endometrioid tumors, the majority of clear cell cases originate from endometriosis [17]. Almost 50 % of clear cell EOCs exhibit mutated *ARID1A* [32, 34], and *PIK3CA* mutations have been found in up to 40 % of cases [33, 35]. *C-MET* amplifications have also been described [36]. Clear cell EOCs are almost invariably *TP53* wild-type and have a low level of chromosomal instability [33].

### 1.3.4 Mucinous carcinomas

Primary mucinous carcinomas of the ovary account for only 3 % of EOCs [1, 4, 5]. This figure is lower than previously thought, which can be ascribed better radiological, biochemical, morphological and immunocytochemical diagnostics of metastatic gastrointestinal carcinomas [37]. Mucinous carcinomas arise via the stepwise type I model through transformation of a mucinous borderline lesion. *KRAS* mutations are common and suggested to be an early event in the evolution of these tumors [1, 38]. *ERBB2* overexpression or amplification is seen in 15-20 % [28, 39].

## 1.4 RISK FACTORS AND PROTECTIVE FACTORS

The strongest known risk factors for EOC are age and certain hereditary mutations, but reproductive history, gynecological conditions and treatments as well as life style factors also influence the risk of developing this disease.

### **Risk factors**

#### *Family history and genetic factors*

Having a single affected first-degree relative is associated with a 2- to 3-fold increased risk of ovarian cancer [40]. Inherited disorders account for approximately 10 % of EOCs, and 90 % of these are germline mutations in *BRCA1* or *BRCA2*. Women with *BRCA1* mutations have a 30-40 % risk of developing EOC before the age of 70 [41-43]. The corresponding risk for *BRCA2* mutation carriers is 10-20 %. There are many different mutations described for both genes, and the individual risk is influenced by which specific *BRCA*-mutation the family carries. *BRCA*-mutations particularly increase the risk of high-grade serous carcinomas. Lynch syndrome with mutations in the hereditary non-polyposis colorectal cancer DNA mismatch repair genes (*MSH2*, *MLH1*, *PMS1* and *PMS2*) account for 10 % of hereditary cases, mostly of the endometrioid or clear cell subtypes [1, 44]. Carriers of this syndrome have a 12 % risk of EOC [14, 45].

Other high penetrance, functionally deleterious mutations have been described in the BRCA pathway *RAD51C* and *D* genes in families with ovarian and breast cancer [40]. Also, hypermethylation of *RAD51C* has been described in 3 % of HGSOc [19]. Using high throughput technologies, genome-wide association studies of single-nucleotide polymorphisms (SNPs) have found several mild penetrance gene loci conferring susceptibility for ovarian cancer [40, 46]. These loci all confer modest effects, with per SNP relative risks ranging from approximately 0.8 to 1.2 [40, 46]. For example, in a study of 15,604 EOC cases and 23,235 controls, a SNP in the immune modulatory gene *IL1A* (coding for interleukin 1 $\alpha$  with activity in the NF- $\kappa$ B pathway) was found associated with decreased risk of clear cell ovarian cancer (OR 0.84, 95% CI 0.76-0.93) [47].

Women with *BRCA1* or *BRCA2* mutations or Lynch syndrome are recommended prophylactic surgical removal of ovaries and fallopian tubes. Prophylactic surgery reduces the risk for EOC, but there remains a 5 % risk of developing a primary peritoneal carcinoma. Screening programs for women with a family history of EOC have not yet been proven effective for downstaging of disease [43]. This can be ascribed the fact of the majority of BRCA-positive cases being HGSOcs, which usually, when diagnosable, have already disseminated. However, screening may be of value in picking up serous carcinomas when the tumor burden is less and might also identify other morphological subtypes before spread of disease.

#### *Endometriosis*

Endometriosis increases the risk of developing endometrioid or clear cell ovarian cancer, which often arise within endometriotic lesions [1, 48, 49]. Thus, in a meta-analysis of case-control and cohort studies including 444,225 women, endometriosis was associated with early-stage, low-grade disease and endometrioid or clear cell subtypes [50]. In another pooled analysis of 13 case-control studies comprising 7,911 women with invasive ovarian cancer, 1,907 borderline cases and 13,226 controls, self-reported endometriosis was associated with an increased risk of clear cell (OR 3.05, 95% CI 2.43-3.84) and endometrioid (OR 2.04, 95% CI 1.67-2.48) subtypes. No association was noted between endometriosis and mucinous cancers, HGSOcs or borderline lesions [49].

#### *Infertility and its treatment*

Multiple studies have addressed the issue of whether ovary-stimulating drugs imposes a risk of EOC, but results have been divergent. Thus, the risk of ovarian cancer in women treated with infertility drugs was recently assessed in a Cochrane review of case-control and cohort studies including 182,972 women [51]. In conclusion, the authors found no convincing evidence of an increased risk of invasive ovarian cancer with fertility drug treatment. However, subfertile women treated with IVF might carry an increased risk of borderline ovarian tumors. The few studies showing an increase in risk of EOC had a high risk of bias; due to retrospective design, lack of accounting for potential confounders and estimates based on small number of cases.

### *Hormone replacement therapy (HRT)*

Menopausal estrogen treatment, with or without the addition of progestin, increases the risk of ovarian cancer. In a Danish nationwide prospective cohort study of 909,946 women, current HRT users had an EOC incidence rate ratio of 1.44 (95% CI 1.30-1.58) compared to never users [52]. The risk declined with time since last use, with a risk of 0.63 (95% CI 0.41-0.96) after 6 years off treatment. The observed risk increase was not significantly affected by duration of hormonal treatment, estrogen only or combination treatment or route of administration.

After the 2002 Women's Health Initiative report on HRT increasing the risk of ovarian cancer, there has been a reduction in HRT use. Although not proving a causal role of hormones, the US ovarian cancer incidence has since then declined by 2.4 % per year, with the largest change for the endometrioid subtype [53]. In a cohort of 169,391 women participating in the NIH-AARP Diet and Health Study, of which 849 developed EOC, ever use of HRT was associated with increased risk of ovarian cancer (RR 1.33, 95% CI 1.16-1.53). When assessing the risk for respective histological subtype, an increased risk was observed for all EOC subtypes except for mucinous carcinomas, where instead a decreased risk was seen (RR 0.37, 95% CI 0.18-0.80) [54].

### *Perineal talc exposure*

Perineal use of talc powder (containing possible carcinogenic particles) has been reported to increase the risk of EOC. Accordingly, in a pooled analysis of altogether 8,525 ovarian cancer cases and 9,859 controls, ever use of genital powder was associated with increased risk of EOC (OR 1.24, 95% CI 1.15-1.33) [55]. The risk increase was approximately the same for the serous, endometrioid and clear cell subtypes, while no significant association was found for development of mucinous tumors.

### *Diet and body size*

To address the issue of whether women's height and weight impacts the risk of developing ovarian cancer, the Collaborative Group on Epidemiological Studies of Ovarian Cancer recently performed a meta-analysis of data from 47 studies comprising 25,157 women with ovarian cancer and 81,311 women without this diagnose [56]. The relative risk of ovarian cancer was increased with both height and body mass index (BMI). The adjusted risk per additional 5 cm in height was 1.07 (95% CI 1.05-1.09). For BMI, the relative risk for ovarian cancer differed according to HRT use. Thus, the relative risk of ovarian cancer per 5 kg/m<sup>2</sup> increase in BMI was 1.10 (95% CI 1.07-1.13) in never users and 0.95 (95% CI 0.92-0.99) in ever users of HRT. The findings suggest height to be a risk factor for EOC and that increased BMI among HRT never users also imposes risk of developing this disease.

Another pooled analysis, performed by the Ovarian Cancer Association Consortium, evaluated the association between BMI and risk of EOC by histological subtype [57]. This study included data from 13,548 cases and 17,913 controls, and a high BMI was found associated with increased risk of serous borderline tumors, LGSOC, endometrioid and mucinous carcinomas. However, the risk of HGSOC was not

affected, indicating that a reduction in population BMI would not have a major impact on the number of EOC deaths.

The possible relevance of the quality of fat intake has also been investigated. In a New England study of 1,872 cases and 1,978 controls, the findings were suggestive of a protective effect of omega-3 for all EOC subtypes (high intake: OR 0.79, 95% CI 0.76-0.96), whereas greater consumption of trans-fat instead increased the risk (OR 1.30, 95% CI 1.08-1.57) [58].

In a recent systematic review of 24 prospective cohort studies, each including > 200 cases, the authors suggest no dietary factors to be consistently associated with risk of ovarian cancer. Thus, no significant associations were demonstrated for red meat, fiber, fruit, vitamin A, vitamin E,  $\beta$ -carotene or folate, but tea and vegetables were suggested as possibly protective [59].

### *Smoking*

In 2009, the International Agency for Research on Cancer added mucinous ovarian tumors to their list of tobacco-related cancers. A later comprehensive meta-analysis by the Collaborative Group on Epidemiological Studies of Ovarian Cancer has further explored the association between smoking and EOC subtypes [60]. For current vs never smokers, the overall relative risk of ovarian cancer was only slightly increased (RR 1.06, 95% CI 1.01-1.11). The impact of smoking on the relative risk for mucinous tumors was found to be more prominent for borderline tumors (RR 2.25, 95% CI 1.91-2.65) than for mucinous carcinomas (RR 1.49, 95% CI 1.28-1.73). The risk of endometrioid (RR 0.81, 95% CI 0.72-0.92) and clear cell cancer (RR 0.80, 95% CI 0.65-0.97) was reduced, and there was no association between smoking and HGSOC.

### **Protective factors**

#### *Parity*

One full-term pregnancy lowers the risk of ovarian cancer by up to one third, and additional pregnancies reduce the risk further [45]. Also, pregnancy at a later age is more protective than pregnancy early in life [45]. In the NIH-AARP Diet and Health Study cohort of 169,391 women, parity was inversely associated with risk of all EOC subtypes (RR=0.71, 95% CI 0.61-0.85) [54]. The risk reduction was greatest for the clear cell subtype (RR=0.28, 95% CI 0.13-0.62). Compared to nulliparous women, parous women had a slightly reduced risk of developing a serous carcinoma (RR 0.83, 95% CI 0.65-1.06). Similarly, in a case-control study of 1,571 women diagnosed with EOC and 2,100 population-based controls, having one child was protective of high-grade carcinomas with an OR of 0.64 (95% CI 0.49-0.83) [61].

#### *Breastfeeding*

The majority of published studies suggest breastfeeding to lower the risk of ovarian cancer [45]. In a study of 881 cases and 1,345 controls, women who had ever breastfed had a 22 % reduction in risk of ovarian cancer compared to women who had never breastfed (OR 0.78, 95% CI 0.64-0.96) [62]. The risk reduction was greater with longer duration of feeding, with an 18 months average duration conferring an OR of 0.56 (95% CI 0.32-0.98). The overall protective effect appeared greatest for the

endometrioid and clear cell subtypes, for which an average of at least 6 months of breastfeeding per child resulted in OR 0.48 (95% CI 0.27-0.87). The number of breastfed children was not found to significantly affect EOC risk.

#### *Oral contraception (chemoprevention)*

The protective effect of the contraceptive pill increases with duration of use, with an approximate 6 % risk reduction for each year [63]. In a collaborative analysis of 23,257 ovarian cancer cases and 87,303 controls, the relative risk for ever- versus never users was 0.73 (95% CI 0.70-0.76). This risk reduction was found to persist for more than 30 years after ceased use, although attenuated over time [64]. Thus, the proportional risk reduction per 5 years of use was 29 % (95% CI 23-34%) for use that had ceased within 10 years, and 15 % (95% CI 9-21%) for use that had ceased 20-29 years previously.

The authors calculate that oral contraception has already prevented some 200,000 ovarian cancers and 100,000 deaths from this disease, and that the number of prevented cancers over the next few decades will rise to at least 30,000 per year.

In the comprehensive NIH-AARP Diet and Health Study, the protective effect of oral contraception was restricted to the serous subtype (RR 0.69, 95% CI 0.55-0.85) [54].

Accordingly, oral contraception can be used as chemoprevention for *BRCA1/2* mutation carriers, with odds ratios for ever- compared to never users in studies ranging from approximately 0.4 to 0.6 [65]. As for non-mutation carriers, the protection increases with duration of use.

#### *Progestins*

The progestin component of oral contraception and HRT has been suggested to confer protection against ovarian cancer [45]. In a systematic review of fourteen case-control and cohort studies, the risk imposed by menopausal estrogen-only therapy was compared to that of combination estrogen-progestin treatment [66]. The EOC risk after 5 years of use was increased for both HRT alternatives, but significantly more so for the estrogen-only (RR 1.22, 95% CI 1.18-1.27) than for the progestin-combination treatment (RR 1.10, 95% CI 1.04-1.16). The authors conclude that menopausal estrogens increase the risk of ovarian cancer in a duration-dependent manner, and that the addition of progestin partially blocks this effect.

Interestingly, the hen has a high prevalence of ovarian cancer and treatment of hens with progestin only has been shown to reduce their risk of ovarian cancer by as much as 91% (RR 0.09, 95% CI 0.01-0.70) [67]. The egg production was also significantly reduced by treatment.

#### *Surgery*

Tubal ligation and hysterectomy each reduces the risk of ovarian cancer by approximately one third [45, 68]. A recent collaborative analysis of pooled data from 10,157 cases and 13,904 controls assessed the impact of tubal ligation on the risk for development of the different EOC subtypes [69]. Tubal ligation was found to significantly reduce the risk of all subtypes, with the greatest effect on risk for endometrioid (OR 0.48, 95% CI 0.40-0.59) and clear cell cancers (OR 0.52, 95% CI

0.40-0.67). The odds ratio, after a tubal ligation, for invasive serous cancer was 0.81 (95% CI 0.74-0.89).

As a consequence of acknowledging the tubal fimbriae as a major contributor to the HGSOE cases, prophylactic salpingectomy is increasingly being discussed as an alternative preventive strategy for young women at high risk for ovarian cancer. However, there is yet no prospective data on the efficacy of bilateral salpingectomy in preventing EOC [70].

## **Indetermined**

### *Physical activity*

There is no firm relationship between exercise and ovarian cancer risk. A cohort study from the Netherlands, including 62,573 women aged 55-69 years at baseline, found women who spent more than 2 hours a week on recreational biking or walking to have a reduced risk of ovarian cancer (RR 0.65, 95% CI 0.41-1.01) compared to women who never participated in such activity [71]. However, in the NIH-AARP Diet and Health Study of 148,892 women aged 50-71 years, neither physical activity nor sedentary behavior was associated with EOC risk, with similar findings for serous and non-serous subtypes [72].

### *Alcohol*

A few studies have explored the relevance of alcohol intake for risk of EOC. In an American analysis of 1,910 ovarian cancer cases and 1,989 controls, alcohol appeared to have a protective effect. Compared to women with no alcohol intake, women with any intake had a 17 % lower risk of developing ovarian cancer, but this protective effect was attenuated after adjustment for education and race (OR 0.89, 95% CI 0.77-1.03) [73]. In a later pooled analysis of 5,342 cases and 10,358 controls, recent alcohol consumption did not significantly affect the risk of ovarian cancer [74].

### *NSAIDs*

Women who are consistent users of non-steroid anti-inflammatory drugs (NSAIDs) have been reported to have a reduced risk for developing EOC [75, 76]. However, a systematic Danish review of fourteen case-control and seven cohort studies, found the risk of invasive ovarian cancer to be only slightly reduced with use of aspirin (RR 0.88, 95% CI 0.79-0.98), and not significantly lowered with use of non-aspirin NSAIDs (RR 0.94, 95% CI 0.84-1.06) [77].

## 1.5 CLINICAL PRESENTATION

The majority of ovarian cancers are diagnosed at a late stage (stage III-IV). This is due to the high proportion HGSOCs, which arise rapidly with typically early dissemination to the peritoneum and/or pelvic and paraaortic lymph nodes. The other subtypes are more commonly found before spread of disease and therefore carry a better prognosis. Figure 4 schematically shows the EOC pattern of spread and Table 1 the 2013 FIGO staging of ovarian cancer [78].

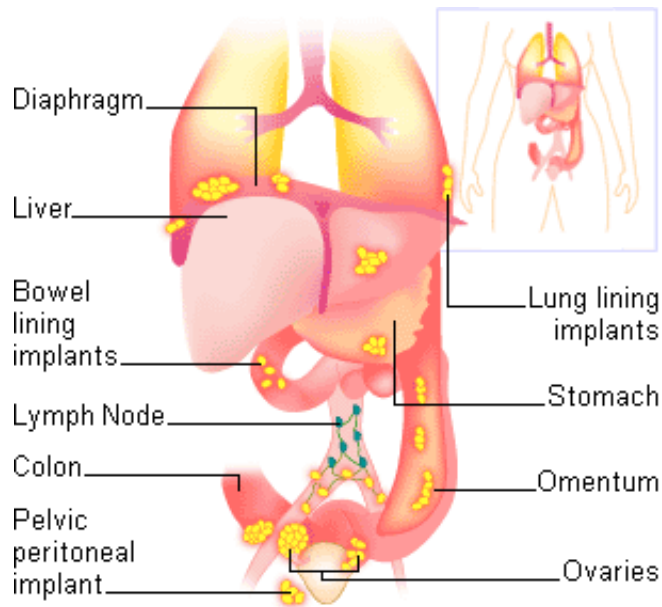


Figure 4. EOC pattern of spread (from <http://cancer-the-dangerous-disease.blogspot.se>).

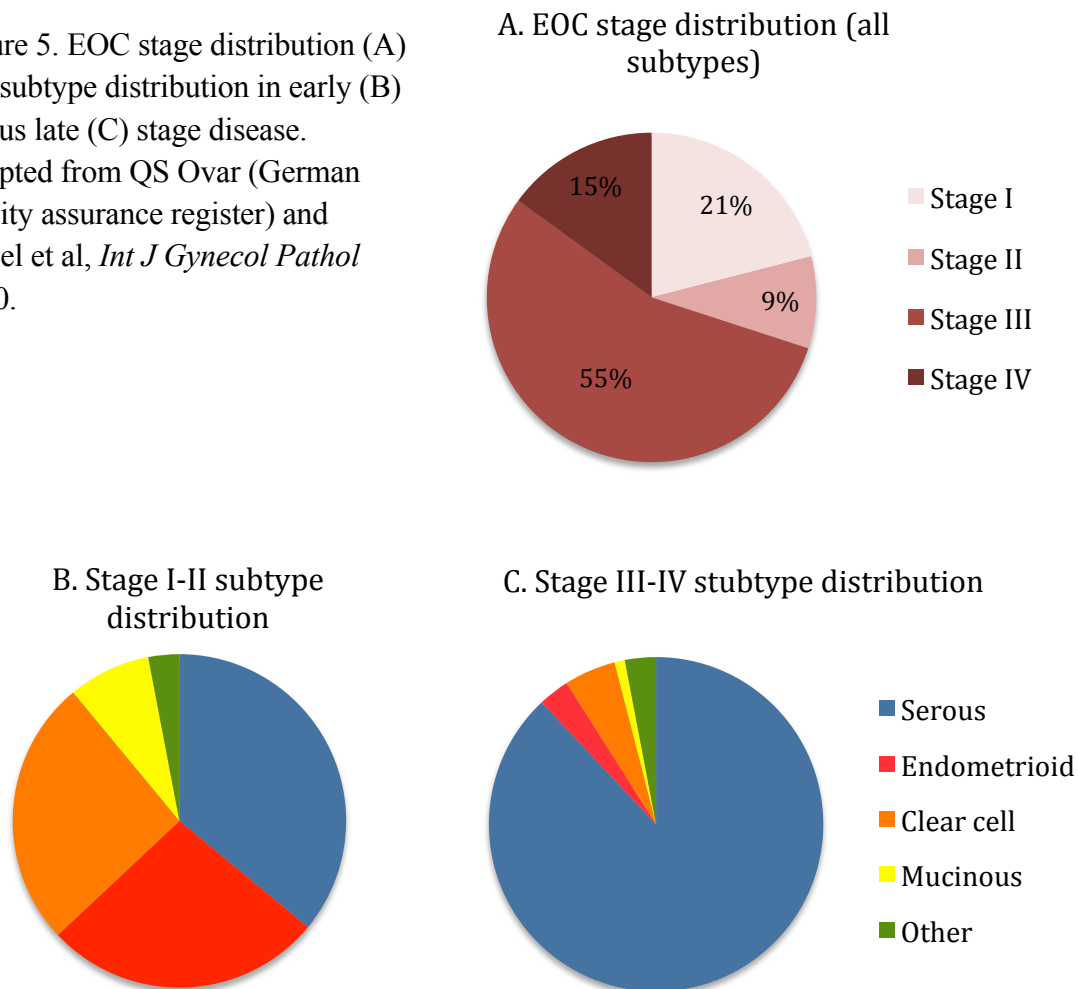


Table 1. 2013 FIGO staging of ovarian cancer.

<b>Stage I</b>	Tumor confined to ovaries or fallopian tubes
<b>IA</b>	Tumor limited to one ovary/fallopian tube (capsule intact), no tumor on ovarian/fallopian tube surface, no malignant cells in ascites/peritoneal washings
<b>IB</b>	Tumor limited to both ovaries/fallopian tubes (capsules intact), no tumor on ovarian/fallopian tube surface, no malignant cells in ascites/peritoneal washings
<b>IC</b>	Tumor limited to one or both ovaries/fallopian tubes with any of the following:
IC1	Surgical spill
IC2	Capsule ruptured before surgery or tumor on ovarian/fallopian tube surface
IC3	Malignant cells in ascites or peritoneal washings
<b>Stage II</b>	Tumor involving one or both ovaries/fallopian tubes with pelvic extension below the pelvic brim or primary peritoneal cancer confined to the pelvis
<b>IIA</b>	Extension to, and/or implants on, the uterus/fallopian tubes/ovaries
<b>IIB</b>	Extension to other pelvic intraperitoneal tissues or primary peritoneal cancer
<b>Stage III</b>	Tumor involving one or both ovaries/fallopian tubes or primary peritoneal cancer with cytologically/histologically confirmed spread to the peritoneum outside the pelvis and/or retroperitoneal lymph node metastases
<b>IIIA</b>	Positive retroperitoneal lymph nodes and/or microscopic extrapelvic peritoneal metastases
IIIA1	Positive retroperitoneal lymph nodes only (cytologically/histologically proven)
IIIA1(i)	Lymph node metastases $\leq 10$ mm in greatest dimension
IIIA1(ii)	Lymph node metastases $> 10$ mm in greatest dimension
IIIA2	Microscopic extrapelvic peritoneal involvement with/without positive retroperitoneal lymph nodes
<b>IIIB</b>	Macroscopic extrapelvic peritoneal metastases $\leq 2$ cm in greatest dimension, with/without positive retroperitoneal lymph nodes
<b>IIIC</b>	Extrapelvic peritoneal metastases $> 2$ cm in greatest dimension, with/without positive retroperitoneal lymph nodes
<b>Stage IV</b>	Distant metastasis (excluding peritoneal metastases)
<b>IVA</b>	Pleural effusion, cytologically verified
<b>IVB</b>	Parenchymal metastases and metastases to extraabdominal organs (including inguinal lymph nodes and lymph nodes outside the abdomen)

The approximate stage distribution of cases and the contribution of respective subtype to early (I-II) versus late (III-IV) stage disease is shown in Figure 5. It can be seen that serous, endometrioid and clear cell carcinomas are approximately equally represented among stage I-II cases, and that nearly all mucinous tumors are early stage. In late stage disease, the vast majority of cases are of the serous subtype [4].

Figure 5. EOC stage distribution (A) and subtype distribution in early (B) versus late (C) stage disease. Adapted from QS Ovar (German quality assurance register) and Köbel et al, *Int J Gynecol Pathol* 2010.



Most women diagnosed with EOC have had signs of disease for some time, but these are often unspecific and erroneously attributed to benign, age-related conditions. Symptoms can be abdominal swelling, changes in bowel-function, urinary dysfunction, loss of appetite, fatigue and pain. When present, malignant ascites or pleural effusion is usually caused by the high-grade serous subtype. Stage I carcinomas are not uncommonly found unexpectedly at, or after, surgery for a presumed benign ovarian cyst.

## 1.6 TREATMENT

### 1.6.1 Primary treatment

Standard treatment for EOC consists of surgery followed by adjuvant platinum-based combination chemotherapy. The surgical procedure typically includes removal of the uterus, tubes, ovaries and omentum and excision of all visible tumor lesions. The staging-operation for apparent early stage disease also comprises paraaortic and pelvic lymphadenectomy. Surgery for advanced stage disease often requires additional skill-demanding procedures, such as multiple bowel incisions, splenectomy and stripping of the peritoneal surfaces including the diaphragm. For patients with very advanced stage IIC-IV disease, adequate upfront cytoreduction can be technically unfeasible. In these cases, 2-4 neoadjuvant courses of chemotherapy can be given to increase the possibility of successful surgery [79].

Patients who have undergone adequate staging surgery with proven stage IA-B, grade 1 disease have an excellent prognosis and require no further treatment. Unfortunately, this applies to only a small proportion of cases, whereas the great majority will receive chemotherapy. The current standard platinum-taxane combination treatment is based on two randomized controlled multicenter trials showing the cisplatin-paclitaxel combination to be superior to the previous standard cisplatin and cyclophosphamide. Thus, in the 1996 GOG 111 trial, the cisplatin-paclitaxel combination conferred a gain in progression-free survival (PFS) of 5 months (18 vs 13 months, HR 0.7, 95% CI 0.5-0.8) and in overall survival (OS) of 14 months (38 vs 24 months, HR 0.6, 95% CI 0.5-0.8) [80]. These findings were confirmed in the OV10 trial, which in 2000 reported a corresponding gain in PFS and OS of 4 and 10 months [81]. Subsequent studies have proven carboplatin to be equally effective, but less toxic than cisplatin, wherefore this drug has replaced the latter as standard together with paclitaxel [82, 83].

Standard adjuvant chemotherapy consists of 6 courses of carboplatin-paclitaxel given intravenously at 3-week intervals. Possibly, the administration of 3-weekly carboplatin together with weekly paclitaxel can increase treatment efficacy. This dose dense regimen has in a Japanese phase III study of patients with stage II-IV EOC been shown to substantially increase PFS (median 28.2 vs 17.5 months, HR 0.71, 95% CI 0.58-0.88) and OS (median 100.5 vs 62.2 months) when compared to the standard 3-weekly combination treatment [84]. The subsequent American GOG 262 trial could not verify this finding, but did show a 4 months gain in PFS for the dose dense regimen in the subgroup of patients not receiving the addition of bevacizumab [85].

Chemotherapy can also be administered intraperitoneally. This route facilitates higher drug concentrations at the site of disease, but the penetration through tumor tissue is limited and toxicity is high. Adjuvant intraperitoneal chemotherapy can thus be considered only for advanced stage cases in good performance status with postoperative minimal residual disease. For this group of patients, several studies have shown the intraperitoneal (i p) administration of drug to confer a better survival than standard intravenous (i v) treatment [86, 87]. In the GOG 172 trial, 429 patients were randomized to either i v paclitaxel plus i p cisplatin followed by i p paclitaxel on day 8

(i p arm) or i v paclitaxel-cisplatin only (i v arm) [86]. The median survival was significantly longer in the i p arm (65.5 vs 49.7 months, HR 0.75, 95% CI 0.58-0.97), but dosing was not comparable and toxicity was high, with only 42 % of cases being able to complete the prescribed 6 courses of treatment. Because of its pronounced toxicity and heterogeneity of studies, this route of administration is not widely used in Sweden [88] or other European countries.

At present, the decision of adjuvant chemotherapy for EOC is mainly dependent upon tumor stage and grade rather than subtype, but this practice is likely to change with the application of subtype-specific clinical trials and development of targeted therapies.

### 1.6.2 Treatment for platinum sensitive recurrent disease

Approximately 75 % of advanced EOC cases will relapse within 3 years [89]. Recurrent ovarian cancer is not curable, but treatment of a late relapse can prolong life. The time interval between the last course of platinum-based chemotherapy and recurrence is termed the platinum-free interval (PFI), and this time span guides the choice of further treatment. A platinum sensitive recurrence is defined as relapse at least 6 months after last given platinum-based treatment. These patients often respond to platinum compounds also in second line, with response rates increasing with longer PFI.

Compared to single agent treatment, the combination of carboplatin with either paclitaxel, pegylated liposomal doxorubicin (PLD) or gemcitabine has been shown to increase at least PFS. Thus, in the ICON 4 trial, the increase in PFS for the addition of paclitaxel was 3 months (12 vs 9 months, HR 0.76, 95% CI 0.66-0.89) [90]. This study also reported a 5 months longer OS for the platinum-paclitaxel combination (29 vs 24 months, HR 0.82, 95% CI 0.69-0.97). Similarly, in the AGO-OVAR 2.5 trial, patients receiving the carboplatin-gemcitabine combination had a 2.8 months longer time to progression than patients given single agent carboplatin (8.6 vs 5.8 months, HR 0.72, 95% CI 0.58-0.90) [91]. The CALYPSO study, comparing the carboplatin-PLD and carboplatin-paclitaxel combinations, showed the two treatment alternatives to be as effective, but to exhibit distinctly different toxicity profiles [92]. Thus, patients receiving the PLD-combination experience less alopecia and neuropathy, but instead more thrombocytopenia, mucositis and hand-foot syndrome. An updated assessment of this trial has confirmed a similar median OS for the two treatment arms (33.0 months for the paclitaxel-combination, 30.7 months for the PLD-combination) [93]. Above three combination alternatives are all possible treatment options for late relapsing disease. For women who for different reasons cannot receive platinum the PLD-trabectedin combination can be an alternative [94]. In clinical practice, the choice of specific combination becomes dependent on the individual patient's previously experienced toxicities and expected side effects of respective combination.

### 1.6.3 Treatment for platinum resistant disease

Platinum resistant disease is defined as tumor progressing on platinum-based chemotherapy or recurring less than 6 months after the last administered cycle of such

treatment. An uncommon extreme is tumor not even initially responding to primary platinum-based treatment. These cases, usually with subtypes other than HGSOE, are termed platinum refractory.

Chemotherapy in the platinum-resistant situation aims at palliation, wherefore quality of life becomes a major consideration when choosing treatment. No randomized trial has in this situation been able to prove polychemotherapy superior to monochemotherapy [89]. There are several single agent alternatives available, for example PLD, paclitaxel, topotecan, trabectedin and cyclophosphamide. Hormonal treatment can also be an option. Response rates in the platinum resistant situation are low, for monotherapies usually 10-15 % [89]. However, in the recent AURELIA trial, evaluating the addition of bevacizumab to physician's choice of standard chemotherapy, the results showed an absolute 3.3 months improvement in PFS (6.7 vs 3.4 months, HR 0.48, 95% CI 0.38-0.60) and objective response rate (27.3% vs 11.8%) with the addition of the antibody [95].

#### 1.6.4 Targeting angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing ones, is a prerequisite for tumor growth beyond 1-2 mm. Over the last years, several drugs targeting this process have been developed, and some of them are used as adjuvants in standard treatment of different malignancies.

The angiogenic process promotes metastatic spread within the peritoneum and is associated with the formation of malignant ascites [96-98]. Its pathways are complex. Tumor cells release different isoforms of pro-angiogenic vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and fibroblast growth factor (FGF), which, through activation of endothelial cells, leads to new blood vessel formation ("sprouting"). Angiogenic growth factors may also induce growth of tumor cells *per se*. VEGF, PDGF and FGF exert their effects through their tyrosine kinase receptor counterparts, VEGFR, PDGFR and FGFR, on the surface of endothelial (or tumor) cells. The ligand-receptor interaction leads to receptor dimerization and subsequent intracellular signaling. The angiogenic growth factors thus mediate activation of the PI3K/Akt, JAK/STAT and MAPK signaling cascades, with resulting cell survival and proliferation [96, 97]. Similarly, the angiotensin pathway, with angiotensins binding to Tie receptors, also activates PI3K/Akt signaling.

Bevacizumab, a monoclonal antibody targeting VEGF, has been shown to moderately increase PFS when given in first line EOC treatment in combination with carboplatin and paclitaxel. In the GOG 218 trial, the bevacizumab-chemotherapy combination followed by 15 months bevacizumab maintenance resulted in a PFS-increase of 3.8 months (14.1 vs 10.3 months, HR 0.72, 95% CI 0.62-0.82) [99]. Similarly, the ICON 7 trial showed a 2.4 months longer PFS for the addition of the antibody and subsequent 12 months maintenance (19.8 vs 17.4 months, HR 0.87, 95% CI 0.77-0.99) [100]. The benefit in this setting seems to be greatest for advanced stage patients with residual tumor after primary surgery. Accordingly, a subgroup analysis of the ICON 7 trial did

show an improvement in overall survival of 7.8 months for these most advanced cases (36.6 vs 28.8 months, HR 0.64, 95% CI 0.48-0.85).

Bevacizumab has also been shown to increase PFS when given together with carboplatin and gemcitabine in the platinum-sensitive recurrent setting. Thus, the OCEANS trial reported a 4 months longer PFS for the bevacizumab-combination (12.4 vs 8.4 months, HR 0.48, 95% CI 0.39-0.60) [101]. In addition, as discussed in section 1.6.3, this antibody has been reported to increase PFS and response rates when added to standard chemotherapy in the treatment of platinum-resistant disease [95].

Overall, bevacizumab has a confirmed place in the treatment of EOC. However, because of lack of predictors of response, toxicity and quality of life related issues, there is currently no international consensus regarding the dosing and timing of this drug.

With the exception of bevacizumab, there are yet no angiogenesis-targeting drugs in routine management of ovarian cancer, but several agents have been tested and there are many ongoing trials. For example, the AGO-OVAR 12 trial recently reported the addition of nintedanib, an inhibitor of angiokinase receptors VEGFR, PDGFR and FGFR, to somewhat increase PFS when given concomitantly in first line with subsequent maintenance (17.3 vs 16.6 months, HR 0.84, 95% CI 0.72-0.98) [102]. Pazopanib, another multi-targeted tyrosine kinase inhibitor, has also been reported to improve PFS when given as maintenance after completed primary treatment. Thus, in the AGO-OVAR 16 trial, the median PFS was 5.6 months longer in the pazopanib-arm (17.9 vs 12.3 months, HR 0.77, 95% CI 0.64-0.91) [103].

## **1.7 RESPONSE TO TREATMENT AND PROGNOSIS – SHORT SUMMARY**

The most important predictor of survival from ovarian cancer is stage at diagnosis. The five-year overall survival after diagnosed EOC ranges from at least 90 % for stage IA cases to less than 20 % in stage IV [104]. Treatment response rates differ greatly between subtypes, with the majority of HGSOEs responding to standard primary chemotherapy. In contrast, the response rates for advanced clear cell, low-grade serous and mucinous carcinomas are low. For example, in one study of advanced LGSOCs only four percent reached a complete remission [105].

Despite the initial high responsiveness of HGSOEs, most patients recur. Approximately 75 % of advanced EOC cases relapse within 3 years. Along with disease recurrence and subsequent treatment, the tumor chemosensitivity gradually diminishes. The expected OS in the platinum resistant situation is less than 12 months, and only 10-20 % of these progressed cases respond to the drugs in use today [89].

## **1.8 PROGNOSTIC AND TREATMENT PREDICTIVE MARKERS**

The word prognosis stems from the Greek *pro* = before and *gnosis* = knowledge, meaning the ability to foretell the course of events, e.g. after diagnose of a specific malignancy. The individual prognosis at any given time point is influenced by many

things, for example age, concomitant diseases, spread of disease and previous response to treatment. A prognostic marker can thus be any biologic indicator of outcome. A treatment predictive marker is a prognostic marker with the ability to aid in assessing the probability of response to a specific treatment.

Few EOC biomarkers have been established, probably in part due to the extreme heterogeneity of the disease, but also because subtype-specific associations might be obscured in studies including all subtypes. Today, there is no routine marker guiding the choice of therapy for EOC.

### *CA125*

Cancer antigen 125 (CA125) is a tumor marker widely used for detection and monitoring of EOC. This protein (also known as MUC16) is a surface, transmembrane mucin which extracellular domain can be released by proteolytic cleavage. The resulting plasma biomarker is unspecific in that its elevation can be caused also by benign conditions, such as endometriosis, inflammatory disease and postoperatively. An elevated serum CA125 is seen in approximately 80 % of women with advanced EOC [106]. HGSOC cases almost invariably exhibit increased CA125 levels, but mucinous cases less often do [107].

CA125 is routinely used for monitoring the efficacy of chemotherapy and, for patients in remission, for detection of recurrent disease. When monitoring EOC during chemotherapy, CA125 has treatment predictive value. A rapid, steep decrease in CA125 implicates a better chance of reaching a complete remission. In contrast, an only slowly decreasing or stable CA125 is indicative of upcoming chemoresistance [108, 109]. The postoperative, pre-chemotherapy CA125 level has also been shown to correlate with PFS with, for the serous subtype, a 1-fold increase in CA125 being associated with a 7 % increase in risk of disease progression [107].

### *Hormone receptors*

In a study performed by the Ovarian Cancer Tumor Tissue Analysis consortium, the expression of estrogen (ER) and progesterone (PGR) receptors were analyzed immunohistochemically in 2,933 EOC cases and correlated with subtype-specific survival [110]. A positive (weak or strong) ER or PGR expression was found associated with improved survival in endometrioid carcinoma (HR 0.33, 95% CI 0.21-0.51), and a strong PGR expression was also associated with improved survival from HGSOC (HR 0.71, 95% CI 0.55-0.91). No significant associations were found for the clear cell, mucinous or LGSOC subtypes.

### *Markers for targeted pathways*

Overexpression of VEGF and PDGF has in EOC been associated with worse outcome [111-113]. A recent meta-analysis of 16 studies including 1,111 ovarian cancer patients, found an elevated serum VEGF to be associated with poor PFS (HR 2.46, 95% CI 1.84-3.29) [113]. Also, a subgroup analysis of studies with predominantly early stage cases found tumor VEGF overexpression to significantly impact PFS (HR 5.34, 95% CI 1.95-14.59) and OS (HR 6.13, 95% CI 2.47-15.26). However, this association was not seen in studies with mostly advanced stage patients, in which tumor VEGF

expression did not notably influence outcome. Unfortunately, studies have not been able to show any of the angiogenic markers to reliably predict response to anti-VEGF treatment [114].

As outlined in section 1.3.1, LGSOCs often harbor mutations in the Ras/Raf/MEK pathway. Studies on MEK-inhibitors in the treatment of this subtype have reached phase III. As regards treatment prediction, results from a phase II trial of a MEK-inhibitor in LGSOC reported the mutation status of *KRAS* or *BRAF* not to be correlated with response [115].

### 1.8.1 HGSOC

Patients with high-grade serous ovarian cancers can, despite having histopathologically indistinguishable tumors, have very different outcome. Accordingly, three comprehensive gene expression analyses have indicated that these tumors can be further subdivided into groups with distinct expression profiles predictive of prognosis [19, 23, 24]. In the study reported by Verhaak et al, a collaborative group used the dataset derived from 489 HGSOCs included in the previously reported Cancer Genome Atlas Research Network study [19] to develop subtype- (differentiated / immunoreactive / mesenchymal / proliferative) and survival (good / poor) gene expression signatures with the ability to predict survival [24]. The signatures were validated in an independent HGSOC dataset. The combination of the survival-, immunoreactive and mesenchymal signatures could further enhance the prognostic ability, with the worst outcome group (accounting for 23 % of cases) having a median survival of 23 months and a platinum-resistance rate of 63 % compared to a median survival of 46 months and a platinum-resistance rate of 23 % in other cases. Taken together, all three above analyses show HGSOCs with overexpression of immune-response genes to have a more favorable prognosis. In contrast, tumors with a mesenchymal gene expression pattern seem to have the worst outcome. Groups characterized by high expression of either genes of proliferation or differentiation have also been described, the latter profile with a positive prognostic impact [19, 24].

#### *BRCA mutations*

As outlined in 1.3.1, approximately 50 % of the HGSOCs exhibit BRCA pathway defects [19]. Both *BRCA1* and *BRCA2* germline mutation carriers have been shown to have higher response rates to chemotherapy and improved survival compared to non-carriers [116]. In a study including 316 HGSOCs, of which 29 were *BRCA2* mutated (somatic or germline), *BRCA2* mutation was found to predict a higher response rate (100 % versus 82 %) and longer OS (5-year survival rate 61% for *BRCA2* mutated and 25% for wild-type cases, HR 0.33, 95% CI 0.16-0.69) compared to *BRCA1* mutated or wild-type cases [117]. This study did not find any association between *BRCA1* mutation status and survival.

*BRCA1* and *BRCA2* take part in the homologous recombination (HR) DNA-repair process. *BRCA* dysfunction thus leads to deficient repair of DNA double-strand breaks, and the cell becomes dependent on alternative ways to cope with DNA damage, e.g.



base excision repair. Poly-ADP-ribose polymerase (PARP) is an enzyme participating in this repair process. Inhibition of PARP consequently renders BRCA-deficient, HR-dysfunctional cells especially vulnerable to DNA-damaging chemotherapy. This concept of targeting one of the genes in a synthetic lethal pair in which the other is defective is termed “synthetic lethality”. Given that half of the high-grade serous tumors are BRCA pathway defective, PARP seems an attractive target for treatment of these patients, and results from phase II trials investigating PARP-inhibitors in the recurrent setting have been encouraging [114]. Notably, in a randomized, double-blind, placebo-controlled phase II study of olaparib as maintenance following response to platinum-based treatment of recurrent disease, the median PFS was significantly longer in the olaparib arm (median 8.4 vs 4.8 months, HR 0.35, 95% CI 0.25-0.49) [118]. Existing data suggest the *BRCA* mutation status to be predictive of response to PARP-inhibitors, with the highest probability of response in *BRCA* germline mutation carriers [114, 118]. Also, immunohistochemical staining for BRCA has been shown to correlate well with *BRCA* genetic events, and could be an approach to identify patients amenable for such treatment [119].

## 2 ENERGY METABOLISM IN CANCER

Normal cells produce more than 90 % of their adenosine 5'-triphosphate (ATP) via oxidative phosphorylation in the mitochondria, but tumor cells utilize their metabolic pathways differently. Even under normoxic conditions, cancer cells often exhibit increased glucose consumption and lactate production alongside a decreased oxidative phosphorylation. This altered metabolic phenotype of malignancy was described by Otto Warburg already in the 1920s, and has been termed “the Warburg effect”. The glucose avidity of cancer has long been exploited for positron emission tomography (PET) imaging, where tumors are detected using the radiolabeled glucose analog <sup>18</sup>F-deoxyglucose (FDG).

In recent years, the understanding of the malignant cell's complex use of fuel has gradually become more detailed, and this reprogrammed energy metabolism has been proposed as an eighth hallmark of cancer [6]. Its major features are an increased aerobic glycolysis, tumor- and nutrient dependent alterations in mitochondrial bioenergetics and increased fat-metabolism (Figure 6). The mitochondrial changes include a truncated TCA cycle with an increased use of glutamine/serine as substrate and a reduced oxidative phosphorylation [120, 121]. These metabolic pathway alterations offer several possible targets for future cancer treatment, but they are believed to be dependent upon tumor type-specific activation of oncogenes and local oxygen- and nutrient availability and thus variable along with tumor progression [121, 122].

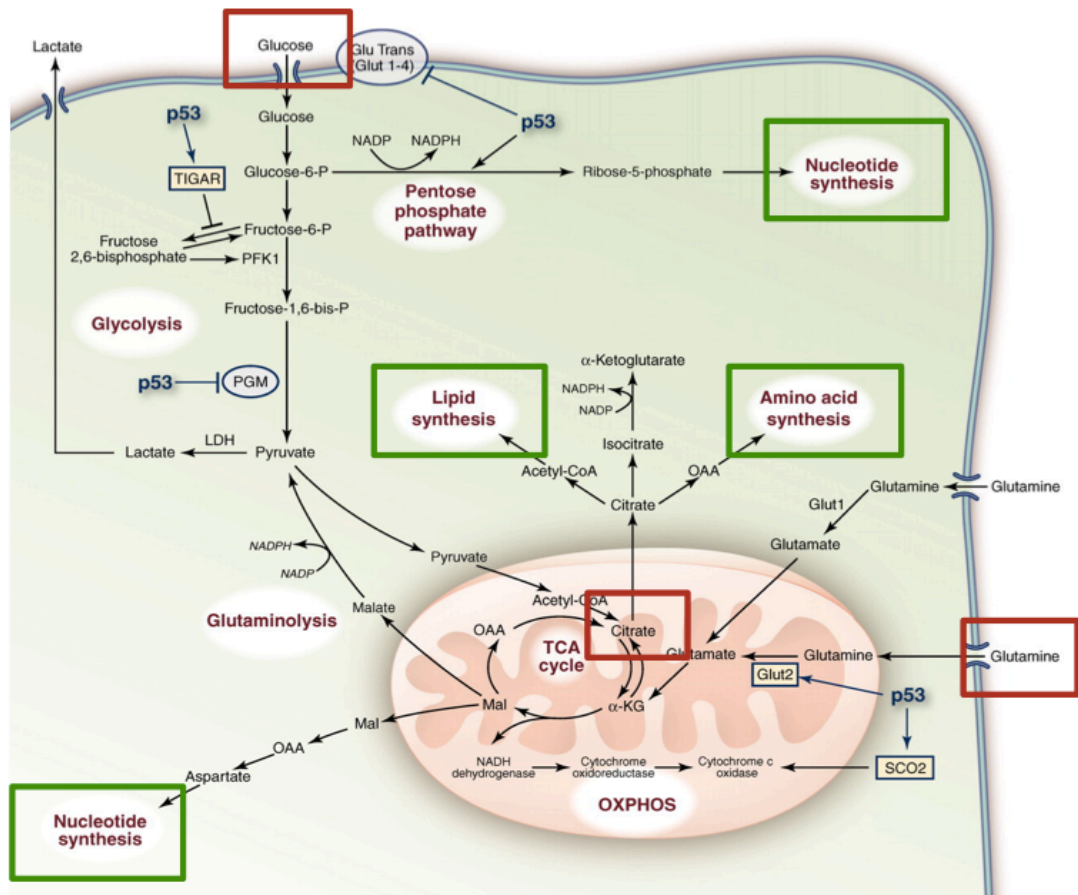


Figure 6. Metabolic features of the malignant cell. Important substrates in red boxes, anabolic end products in green (adapted from AJ Levine et al, *Science* 2010, reprinted with permission).

### 2.1.1 Glycolysis

Glycolysis is the cytosolic stepwise enzymatic process of converting glucose to pyruvate and ATP (Figure 7). It does not require or consume oxygen. The terms “aerobic” and “anaerobic” glycolysis refers only to whether this degradation of glucose occurs in the presence or absence of oxygen. Metabolizing one glucose molecule through glycolysis generates only two ATP molecules, whereas its complete oxidation through oxidative phosphorylation renders 36 ATP. Why do cancer cells, with an obvious great need for energy, use such a wasteful form of metabolism? Although the ATP yield per consumed glucose is low, the glycolytic flux in malignant cells can be so high that the amount ATP produced exceeds that from oxidative phosphorylation [120]. Also, proliferating cells have important metabolic requirements extending beyond the production of ATP. By redirecting glycolytic intermediates to either the pentose phosphate pathway for nucleotide production or to synthesis of amino acids and phospholipids, the tumor cell can sustain proliferation. The cancer cell thus diverts about 10 % of its glucose uptake upstream of pyruvate to generate biomass [123].

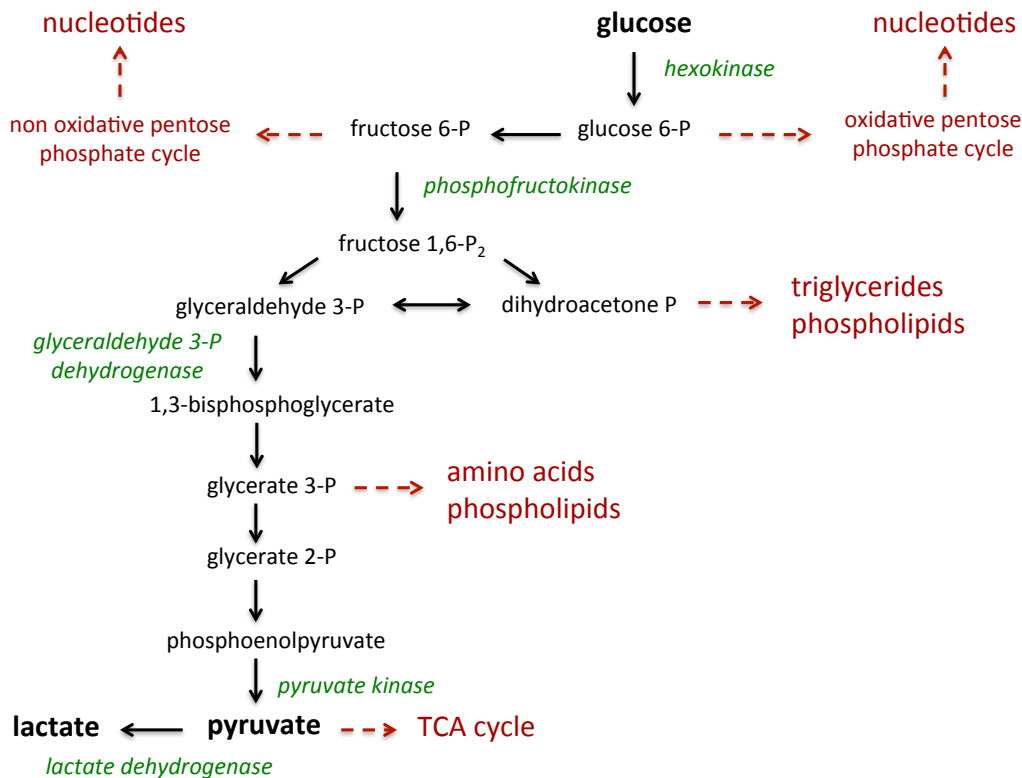


Figure 7. Glycolysis. Important regulated enzymes in green.

### *Hexokinase*

Hexokinase (HK) facilitates the first step of glycolysis, using ATP to phosphorylate glucose. Inhibitors of HK, i.e. 2-deoxyglucose (2-DG), 3-bromopyruvate and lonidamine, have in preclinical studies been shown to have chemo-potentiating effects [124-126]. 2-DG is a competitive inhibitor of HK, blocking access of glucose to the enzyme. HK thus phosphorylates 2-DG, which then cannot be further metabolized and becomes trapped within the cell. This leads to accumulation of 2-DG-P and cellular depletion of ATP.

### *Glyceraldehyde 3-P dehydrogenase*

Glyceraldehyde 3-P dehydrogenase (GAPDH) performs its catalytic reaction in the presence of nicotinamide adenine dinucleotide (NAD<sup>+</sup>) and inorganic phosphate, and mediates formation of NADH, contributing to the cellular redox balance. GAPDH thus plays an important role in protecting the cell from free radical or ROS-mediated damage [127]. Its glycolytic activity is repressed by oxidative stress, which thus increases flux through the pentose phosphate pathway generating NADPH, the cells reducing power to protect from oxidative damage. GAPDH was once considered a simple “housekeeping” protein, but recent insight into its functions reveals a multifaceted molecule participating in various cellular processes, also within the nucleus [128, 129].

### *Pyruvate kinase*

Pyruvate kinase (PK) catalyzes the final step of glycolysis and is one of its key regulators. There are four tissue-specific isoforms of this enzyme of which PKM2 is the one predominately expressed in proliferating cells [130]. PKM2 form dimers and tetramers, of which the tetramers are the more glycolytically active, favoring pyruvate-production. The dimeric form is less active, causing accumulation of upstream glycolytic intermediates. Thus, in cancer cells, the ratio between the tetrameric and dimeric forms of PKM2 determines whether glucose is used for energy or anabolic precursors. This tetramer:dimer ratio is regulated by nutrient availability, different oncoproteins and reactive oxygen species [130, 131]. Like GAPDH, PKM2 also has additional, non-glycolytic functions. In its dimeric form, it can translocate to the nucleus, where it acts as a protein kinase supporting cell proliferation [130].

### *Malignant regulation of glycolysis*

In malignant cells, transcription of glycolytic enzymes is initiated by transcription factors Ras, c-Myc and hypoxia inducible factor (HIF)-1 $\alpha$  [120, 122, 132]. Hypoxia in the tumor microenvironment stabilizes HIF-1 $\alpha$ , further promoting glycolytic activity. Another major stimulator of glycolysis is the PI3K-Akt-mTOR pathway, which enhances glucose uptake and up-regulates glycolytic enzymes; both by direct transcriptional activation and via induction of HIF-1 $\alpha$  and c-Myc [120, 130, 132]. Akt also activates glycolytic enzymes by phosphorylation [132].

The tumor-suppressor p53 has an inhibitory effect on glycolysis and instead up-regulates cytochrome *c* oxidase 2 of the electron transport chain favoring oxidative phosphorylation [132]. Loss of p53 thus shifts metabolism from mitochondrial respiration towards glycolysis.

## 2.1.2 Oxidative phosphorylation

Oxidative phosphorylation is an electrochemically driven process taking place over the inner mitochondrial membrane (Figure 8). Its key enzyme is H<sup>+</sup>-ATP synthase, which functions as a rotary engine to produce large amounts of ATP.  $\beta$ -F1-ATPase (ATP5B) is the catalytic  $\beta$ -subunit of H<sup>+</sup>-ATP synthase, and rate-limiting component for ATP production. ATP5B is both pre- and post-transcriptionally regulated [133]. Thus, transcriptional repression by hypermethylation of the *ATP5B* gene has been described. However, the major regulation of ATP5B is thought to occur post-transcriptionally, with the Ras-GAP SH3-binding protein 1 (G3BP1) binding the ATP5B mRNA transcript, thus hindering its translation and resulting in cytosolic sequestration of the transcripts.

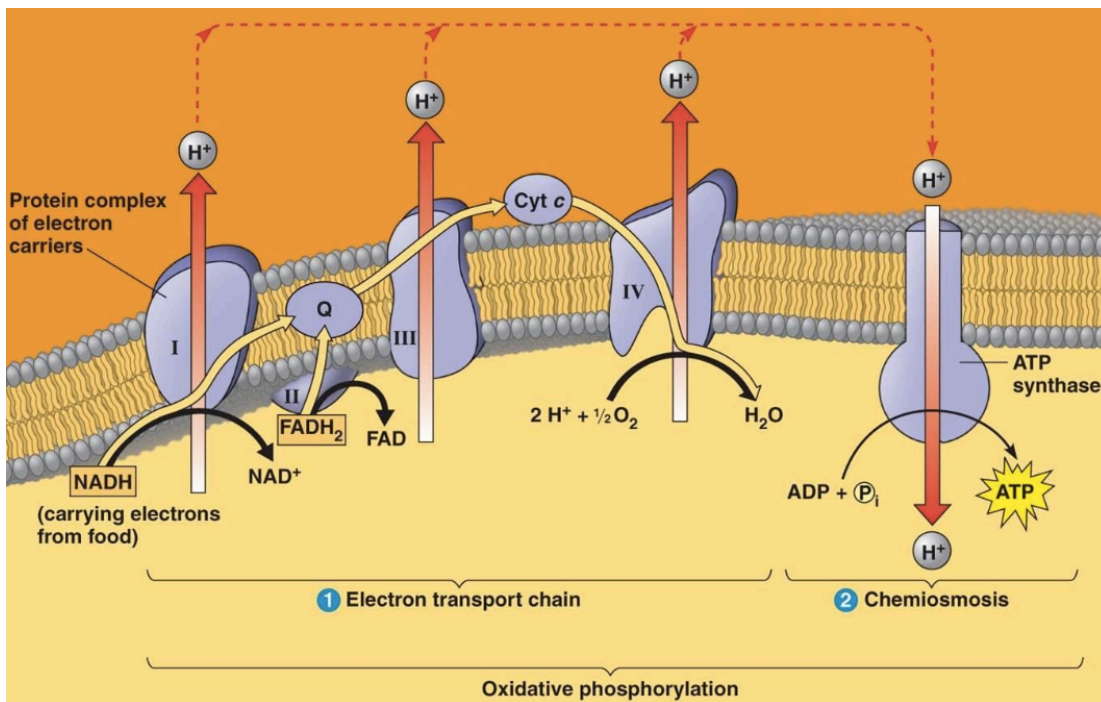


Figure 8. Oxidative phosphorylation over the inner mitochondrial membrane (from <http://edu.docdat.com>).

### 2.1.3 Metabolic markers

#### 2.1.3.1 GAPDH

The glycolytic enzymes are generally upregulated in cancer [122, 134]. As a prognostic marker, GAPDH has not been extensively studied. A high GAPDH has, however, been associated with relapse and poor survival in breast cancer [135-137]. In a study of 404 breast cancer patients, enhanced GAPDH mRNA expression was inversely correlated to estrogen and progesterone receptor status, young age and grading [137]. Thus, GAPDH seemingly reflects breast tumor aggressiveness. Likewise, in a study of 82 non small cell lung cancer (NSCLC) patients, a high GAPDH mRNA expression was found to predict shorter survival [138]. The authors validated their findings using six microarray datasets including 1,250 NSCLC patients, and were able to confirm the prognostic impact of GAPDH in this disease.

#### 2.1.3.2 PKM2

PKM2 overexpression has also been reported to indicate poor prognosis. In a study of 60 colorectal cancers, PKM2 was found associated with advanced stage [139]. Also, PKM2 overexpression by immunohistochemistry has been shown to independently predict shorter survival in esophageal squamous cell cancer [140] and cancer of the gallbladder [141].

#### 2.1.3.3 ATP5B

Studies on  $\beta$ -F1-ATPase (ATP5B) expression in cancer are few, and with apparently diverging results. Its down-regulation has been associated with shorter survival in colon cancer [142, 143]. However, in one study in breast cancer, a *high*  $\beta$ -F1-ATPase conferred worse survival [136].

#### 2.1.3.4 *BEC-index*

In 2002, Cuezva and coworkers proposed a bioenergetic cellular (BEC) index as prognostic in cancer [142]. This index is a ratio comparing an oxidative mitochondrial index with the cellular glycolytic potential. The mitochondrial part of the index (its numerator) consists of the ratio of the protein levels of  $\beta$ -F1-ATPase to mitochondrial chaperone heat shock protein 60 (HSP60). The cellular glycolytic potential (the BEC-index denominator) is represented by GAPDH protein expression.

$$\frac{\text{mitochondrial index} \quad \beta\text{-F1-ATPase / HSP60}}{\text{glycolytic potential} \quad \text{GAPDH}} = \text{BEC-index}$$

Thus reflecting a shift towards higher glycolytic dependence, a low BEC-value has been shown to predict shorter survival in colon, lung and breast carcinomas [136, 142, 144]. Neither the BEC-index, nor its metabolic component markers, has been previously studied as prognosticators in ovarian cancer.

### 3 AIMS OF THE THESIS

The overall aim of this thesis was to study metabolic markers in advanced serous ovarian cancer, with specific focus on their significance for prognosis, treatment prediction and tumor progression.

- I. To examine the chemopotentiating effect of glycolysis inhibitor 2-deoxy- $\Delta$ -glucose (2-DG) in ovarian carcinoma cell lines and ovarian cancer cells from ascites and to investigate potential treatment predictive markers for sensitivity to this strategy.
- II. To investigate the prognostic and treatment predictive value of HSP60 in advanced serous ovarian cancer.
- III. To investigate the prognostic value of glycolytic enzymes GAPDH and PKM2, mitochondrial  $\beta$ -F1-ATPase (ATP5B) and the bioenergetic cellular (BEC) index in advanced serous ovarian cancer.
- IV. To study potential differences in mRNA expression of glycolytic enzymes GAPDH and PKM2, mitochondrial ATP5B and HSP60 in solid tumor versus ascites.



## 4 PATIENTS AND METHODS

### 4.1 PATIENTS AND MATERIALS

#### 4.1.1 PAPER I

The study material in paper I consists of two ovarian cancer cell lines, SKOV-3 and CaOv-4, and freshly isolated ascites tumor cells from 17 EOC cases. The initial reports on SKOV-3 and CaOv-4 does not specify their respective subtype, but the former is regarded of clear cell origin (*PIK3CA*- and *ARID1A* mutated, *TP53* wild-type) and the latter is likely from a serous carcinoma (*TP53* mutated, *MYC* amplified) [145]. Clinical data were available for 15 of the 17 ascites cases, and is briefly presented in Table 2. Ascites samples were collected between September 2007 and July 2008, either at primary surgery or at later palliative laparocentesis.

Table 2. Characteristics of ascites cases (paper I).

Case no.	Stage at diagnosis	Subtype	Number of previous chemotherapy regimens
1	IV	HGSOC	0 (sampling cycle 1)
2	IIIC	HGSOC	0 (chemonaïve)
3	IIIC	HGSOC	5 (platinum resistant)
4	IIIC	HGSOC	0 (chemonaïve)
5	IIIC	LGSOC	0 (chemonaïve)
6	IIIC	HGSOC	0 (chemonaïve)
7	IIIC	HGSOC	0 (chemonaïve)
8	IIIC	HGSOC	0 (chemonaïve)
9	IC	HGSOC	0 (chemonaïve)
10	IIIC	HGSOC	0 (chemonaïve)
11	IC	HGSOC	7 (platinum resistant)
12	IV	HGSOC	3 (platinum resistant)
13	IIIC	HGSOC	0 (chemonaïve)
14	IIIC	HGSOC	0 (sampling cycle 1, later defined platinum refractory)
15	IIIC	HGSOC	0 (chemonaïve)

#### 4.1.2 PAPERS II AND III

We prospectively collected fresh tumor samples (solid tumor and, if present, ascites) from 123 patients undergoing primary surgery. Of these, 57 met the eligibility criteria; stage IIC-IV, serous or endometrioid subtype and specimens containing at least 50 % tumor cells. Patients also had to receive platinum-based chemotherapy. An adequate quantity ( $\geq 1.5 \mu\text{g}$ ) RNA could be extracted in all but one case. The resultant study population thus consists of 56 patients (dark blue in Figure 9).

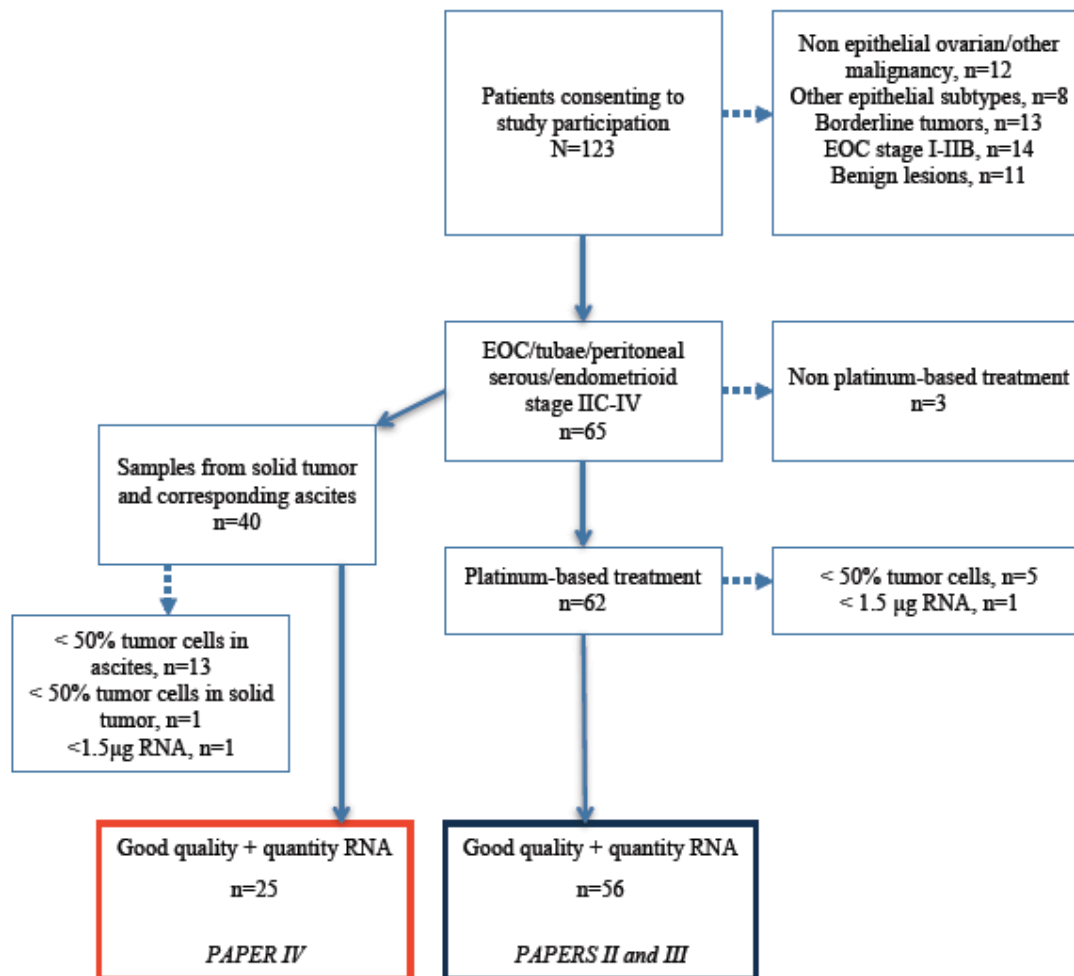


Figure 9. 123 patients consented to participate in the study. Study cohort for papers II and III in dark blue. Subgroup constituting the paper IV study cohort in red.

Patients were operated at the Karolinska University Hospital (Solna or Huddinge) between April 2003 and July 2008. Clinical data were prospectively collected in case report forms. Patient characteristics are summarized in Table 3. Of the included 56 overall poor prognosis cases, 89 % had stage IIC-IV disease. Eighty-six percent of tumors were diagnosed as serous, and 93 % were grade 2-3. In 70 %, the postoperative tumor residuals measured at least 10 mm. Patients with residual tumor at start of chemotherapy were considered evaluable for response, and response assessment was done according to modified RECIST- and GCIG criteria. Eighty-two percent responded to platinum-based primary treatment, but 45 % relapsed within six months with a median PFI of only 7.2 months and a median survival of 34.6 months. At study closure, 19 patients (34 %) were alive, with no evidence of disease in 9 cases (16 %). Median follow-up was 60 months.

Table 3. Patient characteristics.

<i>Characteristic</i>	<i>n</i>
<i>Age at diagnosis (years)</i>	
Median 64.5	NA
<i>Diagnosis</i>	
Epithelial ovarian	43 (77 %)
Fallopian tube	10 (18 %)
Peritoneal	3 (5 %)
<i>FIGO stage</i>	
IIC-IIIB	6 (11 %)
IIIC	43 (77 %)
IV	7 (12 %)
<i>Subtype</i>	
Serous	48 (86 %)
Endometrioid	8 (14 %)
<i>Grade of differentiation<sup>a</sup></i>	
High	4 (7 %)
Moderate	4 (7 %)
Poor	48 (86 %)
<i>Postoperative residual tumor size</i>	
0 mm	7 (12 %)
1-10 mm	10 (18 %)
> 10 mm	39 (70 %)
<i>1<sup>st</sup> line chemotherapy</i>	
Carboplatin + Paclitaxel	49 (88 %)
Other platinum based	7 (12 %)
<i>Response at end of treatment<sup>b</sup></i>	
CR	24 (49 %)
PR	16 (33 %)
SD	4 (8 %)
PD	5 (10 %)
<i>Time from EOT to recurrence/ progression</i>	
< 6 months	25 (45 %)
≥ 6 months	30 (54 %)
Unknown	1 (2 %)
<i>Survival</i>	
Alive, no evidence of disease	9 (16 %)
Alive, with disease	10 (18 %)
Death from disease	36 (64 %)
Death from other cause	1 (2 %)

Abbreviations: NA=not applicable, FIGO=federation Internationale de Gynecologie et d'Obstetrique, EOT=end of treatment, CR=complete response, PR=partial response, SD=stable disease, PD=progressive disease.

<sup>a</sup>Grade of differentiation according to WHO international standards.

<sup>b</sup>Response evaluation only in cases with residual tumor at start of chemotherapy (n=49).

### 4.1.3 PAPER IV

Within above described patient cohort, we prospectively collected tumor biopsies and corresponding ascites from 40 women undergoing primary surgery for suspected advanced ovarian cancer (Figure 9). Of these, 25 had stage III-IV disease of the serous (24) or endometrioid (1) subtype with solid and ascites samples containing at least 50 % tumor cells with adequate mRNA yield. These 25 patients thus constitute the paper IV study cohort (red in Figure 9). All but two patients (92 %) had type II disease.

The ethics committee at Karolinska Institutet, Stockholm, approved the respective study (paper I-IV).

## 4.2 METHODS

### 4.2.1 PAPER I

#### 4.2.1.1 *Cell separation and treatment*

Tumor cells from fresh ascites were pelleted, resuspended in PBS and then separated using the Lymphoprep™ (Axis-Shield, Oslo, Norway) density gradient method [146]. The separation was thus done over a 3-layer discontinuous gradient consisting of (from the bottom): Lymphoprep, Lymphoprep / Krebs HEPES Ringer solution 3:1 and Lymphoprep / Krebs HEPES Ringer solution 1:2. After centrifugation, tumor cells were collected at the interphase between the top and middle layers, washed with PBS and plated.

All cells were cultured in RPMI 1640 with supplements and kept at +37°C in 5% CO<sub>2</sub>. Cells were treated for 24 (apoptosis experiments) or 48 hours with either platinum (cis- or carboplatin), 2-DG (Sigma-Aldrich, Stockholm, Sweden) or a combination of the two. 2-DG competes with glucose for phosphorylation by hexokinase, the first enzyme of glycolysis, but is not further metabolized and becomes trapped within the cell. Drug doses for cis- and carboplatin were 1-20 and 16-160 μM, respectively, and for 2-DG 1-10 mM.

#### 4.2.1.2 *Assaying cell survival, apoptosis and metabolic characteristics*

We used the Sulforhodamine B-based TOX6® assay (SRB, Sigma-Aldrich, Stockholm, Sweden) to at different timepoints study cell survival, including regrowth capacity after treatment (48 h drug treatment followed by 72 h in drug-free medium). Briefly, cells were seeded in 96-well plates and, after over-night incubation, drugs were added in fresh medium. At above timepoints, adherent (still viable) cells were fixed in the wells by addition of trichloroacetic acid followed by 1h incubation at +4°C. Fixed cells were stained with sulforhodamine B dye before assessment at absorbance 565 nm minus background. Survival was thus measured as post-treatment remaining cellular protein and expressed as proportion of protein compared to control samples. Concentrations of

drugs causing a 50 % reduction in survival (half maximal inhibitory concentrations, IC<sub>50</sub>) after 48 h treatment were calculated.

Apoptosis was assessed after 24 hours using the M30 Apoptosense<sup>®</sup> assay (Peviva, Sundbyberg, Sweden), which specifically detects caspase-3/caspase-7 cleaved cytokeratin-30 fragments in total cell lysates. This method is based upon antibody-detection of a neopeptide formed by caspase-cleavage of cytokeratin 18. These fragments are stable, wherefore the assay in effect quantitates accumulated apoptosis. The protein expression of GAPDH,  $\beta$ -F1-ATPase and HSP60 was assayed by Western blotting and, based on densitometric analyses of the blots, BEC-index values were calculated as described in 2.1.3.4. Observed signals in SKOV-3 were used for normalization of the signals in ascites tumor cells. Cell line glucose consumption was assessed as <sup>18</sup>F-deoxyglucose uptake in the presence or absence of glucose and lactate levels were measured in supernatants.

## 4.2.2 PAPERS II, III and IV

### 4.2.2.1 *Tumor sample collection and processing*

At surgery, tumors were immediately transported on ice to the Unit for Pathology, where representative tumor wedge- or core biopsies were taken and imprints made before immersing the biopsies in RNAlater<sup>®</sup>. The specimens were then directly transported to the lab, where they were stored at 4° for up to 72 hours before RNA extraction. Ascites samples were collected in 2 x 50 ml tubes, and separated using Lymphoprep<sup>™</sup> (see section 4.2.1.1) upon arrival at the lab. All tumor handling data were prospectively collected using tissue sample worksheets.

Solid tumor imprints and ascites cytospin preparations were used for pathological evaluation of tumor cell proportion, and only samples with  $\geq 50$  % tumor cells were included in the study. We used the RNeasy<sup>®</sup> Midi Kit (Qiagen, Hilden, Germany) for RNA extraction and RNA quality and quantity was checked using Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). First-strand cDNA synthesis was performed by Superscript<sup>™</sup> III reverse transcriptase (Invitrogene AB, Stockholm, Sweden).

### 4.2.2.2 *Real-time PCR (papers II, III and IV)*

Amplification reactions were done on the ABI 7500 system (Applied Biosystems, Stockholm, Sweden). We chose SYBR Green<sup>™</sup> for detection of transcribed genes. Relative quantity expression values were calculated by the  $\Delta\Delta$ Ct method. The Ct value is the PCR cycle number at which the increase in fluorescence crosses a set threshold. The  $\Delta\Delta$ Ct value is the difference between the Ct value of the gene of interest and control, after correction for loading control (=reference genes). Samples were run in duplicates, and only reproducible amplification curves were further analyzed. HPRT1 (hypoxanthine guanine phosphoribosyltransferase 1) and B2-microglobulin served as reference genes, and we used Universal Human Reference RNA (Stratagene, Santa Clara, CA, from 10 pooled tumor cell lines) as positive control.

#### 4.2.2.3 Immunohistochemistry (papers II and III)

Formalin-fixed, paraffin embedded tissue blocks could be obtained in 54 of the 56 cases. Four  $\mu\text{m}$  tumor sections were deparaffinized and rehydrated in xylene and graded alcohols. We used the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) for staining. For antigen retrieval, sections were heated in a microwave oven in citrate buffer for 20 min. After addition of primary antibodies, slides were incubated overnight at 8°. Sections were then incubated with secondary antibody before addition of the avidin-biotinylated peroxidase complex. Slides were developed with diaminobenzidine chromogen and counterstained with Mayer's haematoxylin. We obtained negative controls by omission of the primary antibody. Positive controls were from breast- (HSP60, GAPDH and PKM2) and colon (ATP5B) carcinomas.

Three observers, blinded for clinical outcome, independently evaluated all slides by assessing the whole tumor area. The maximum staining intensity of tumor cells was scored 0-3+, and the proportion of cells thus stained estimated. Cases were dichotomized into low and high expressing groups with arbitrarily set cut-offs as follows; HSP60: high expression:  $\geq 30\%$  of tumor cells staining 3+, GAPDH: high expression:  $\geq 50\%$  staining 2+ or any proportion staining 3+, PKM2: high expression:  $\geq 20\%$  staining 3+, ATP5B: high expression: any proportion staining 3+. Inter-observer discrepancies were found for up to 15% of examined slides, in which cases consensus was reached on further review.

### 4.3 STATISTICS

#### 4.3.1 PAPER I

We used the Student's *t* test for determination of statistical significant differences in treatment effects and the Mann-Whitney *U* test for analysis of the treatment predictive value of the  $\beta\text{-F1-ATPase:HSP60}$  ratio and BEC-index for potentiation by 2-DG.

#### 4.3.2 PAPER II AND III

We estimated the platinum-free interval (PFI) and overall survival by Kaplan-Meier curves. Assessment of each marker's value as independent prognosticator was done by Cox proportional hazards regression models, adjusted for standard confounding risk factors age, stage, grade and postoperative residual tumor. Fisher's exact test was used for evaluating the impact of HSP60 on response.

For comparative analyses of quantitative real-time PCR results, we divided cases into three equally sized groups according to their relative quantity of mRNA expression. The choice of splitting the material into three groups was made because, in a small material with a lesser chance of significant findings, it enables evaluation of possible trends. Also, in our poor-prognosis patient cohort, one third of cases equalized the proportion alive at end of study.

For analyses of GAPDH, PKM2 and ATP5B mRNA data, the group with lowest expression (one third of patients) was compared to cases with higher expression. The merging of the two thirds of cases with higher mRNA was made because their expression seemingly did not differ. The BEC-index was calculated, at the mRNA level, as outlined in section 2.1.3.4. The third of cases with the highest BEC-index value was compared to cases with lower values.

Since IHC is a relatively insensitive, semi-quantitative method with risk of misclassification, we chose to stop at two groups for comparison of protein expression results. Thus, for all studied protein markers, statistical analyses were made after dichotomizing cases into low- and high expression groups.

#### 4.3.3 PAPER IV

In paper IV, the real-time PCR expression value for respective solid tumor was pairwise compared to the corresponding expression in ascites using the Wilcoxon matched pairs signed rank sum test. This non-parametric test is well suited for comparisons of paired observations when the sample size is small and when there is risk of a skewed distribution.

## 5 RESULTS, DISCUSSION AND CONCLUSIONS

### 5.1 PAPER I

#### Ovarian carcinoma cells with low levels of $\beta$ -F1-ATPase are sensitive to combined platinum and 2-deoxy- $\Delta$ -glucose treatment

##### 5.1.1 Cell lines

CaOv-4 cells were more resistant to platinum drugs than SKOV-3 cells (cisplatin  $IC_{50}$  of 35 and 12  $\mu$ M, respectively), but more sensitive to potentiation by 2-DG. The 2-DG-mediated decrease in  $IC_{50}$  was thus greatest for CaOv-4 cells, where co-treatment with 2-DG at 5 and 10 mM reduced the cisplatin  $IC_{50}$  from 35 to 14 and 5  $\mu$ M, respectively.

To investigate the regrowth capacity after low-dose platinum  $\pm$  2-DG, cells were first treated with drugs for 48 hours and then allowed to recuperate for 72 hours in fresh, drug-free medium. After release from drug, cells treated with either platinum or 2-DG resumed growth, but the combination-treated cells did not. Thus, the 2-DG-platinum combination seems to have irreversible antiproliferative effects, see Figure 10.

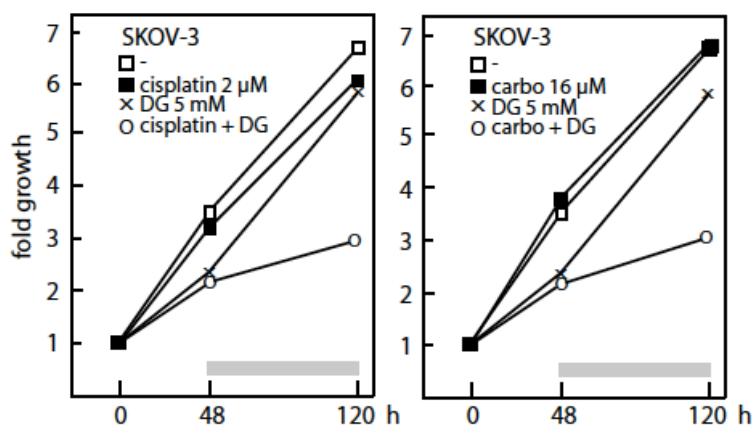


Figure 10. Regrowth capacity of SKOV-3 cells after treatment with low-dose cisplatin or carboplatin  $\pm$  2-DG. Cell survival assessed after 48 h of drug treatment and after a further 72 h in drug-free medium.

The cell lines' BEC-indices, based on digital scanning of Western blots, were 0.09 for SKOV-3 and 0.03 for CaOv-4, suggesting a higher glycolytic activity in CaOv-4. This was verified by assessments of glucose uptake and lactate production, which was approximately twice as high in CaOv-4 compared to SKOV-3 cells.

##### 5.1.2 Tumor cells from ascites

Treatment of ascites tumor cells with 2-DG alone caused a 0-50 % reduction in survival with an  $IC_{50}$  of < 20 mM in 6 of the 17 samples. The median cisplatin  $IC_{50}$  was 23  $\mu$ M. After elimination of five carboplatin-resistant outliers, the median  $IC_{50}$  was 282  $\mu$ M for this compound. Combination treatment with cisplatin and 5 mM 2-DG did in all



samples cause a reduction in cisplatin IC<sub>50</sub>, with a median decrease of 68 %. In contrast, 2-DG potentiated responses to carboplatin in only 7 of 17 samples.

Acute apoptosis was assessed after 24 hours of treatment. Carboplatin-induced apoptosis was generally not potentiated. For the cisplatin-2-DG combination, potentiation of apoptosis was seen in some samples, but not in all (despite all samples showing a 2-DG-mediated reduction in IC<sub>50</sub>). We therefore conclude that although 2-DG may increase cisplatin-induced apoptosis, this is not the only mode of potentiation.

To test the possible ability of the BEC-index to predict 2-DG-potentiation of cisplatin, the ascites cases were dichotomized according to the percentage decrease in cisplatin IC<sub>50</sub> induced by 2-DG. Samples with a greater than 50 % reduction were designated highly potentiated, and compared to less potentiated samples. The BEC-index values of the highly potentiated group were homogeneously low, but did not in this small material differ significantly from the less potentiated group. However, the BEC-index numerator ratio  $\beta$ -F1-ATPase:HSP60 was found to predict sensitivity to 2-DG-mediated potentiation of cisplatin ( $p=0.028$ ).

### 5.1.3 Discussion and conclusions

The 2-DG-mediated increased platinum-efficiency demonstrated in this study seems to be more pronounced for the cisplatin- compared to the carboplatin-combination. This might in part be due to the different molecular effects induced by the two drugs, where cisplatin is a more highly reactive molecule inducing a greater degree of acute apoptosis than carboplatin [147]. Cisplatin also bind directly to mitochondrial DNA and proteins, including the voltage-dependent anion channel protein, which couples metabolic processes between the cytosol and mitochondria [148]. A thus impaired oxidative phosphorylation could render the cell more dependent on glycolysis, and thereby particularly susceptible to glycolysis-inhibition. Also, one must consider that a proportion of the patients providing the ascites samples had been previously exposed to carboplatin-based chemotherapy, wherefore a specific resistance to this drug cannot be excluded.

The cell line experiments over longer time with low-dose combination treatment and subsequent regrowth in fresh medium did, however, show similar results for both platinum compounds. Thus, the total antiproliferative effect is different from the acute apoptotic response, with the addition of 2-DG also affecting subsequent recuperation capacity of surviving tumor cells.

Targeting malignant cell glycolysis is attracting interest as a novel therapeutic strategy in cancer [127]. Several small molecule agents inhibiting various glycolytic enzymes are being investigated. 2-DG is the most studied, and this agent has in preclinical studies of various malignancies been shown to significantly increase the efficacy of concomitant chemo- or radiotherapy [149]. For example, 2-DG has *in vivo* been shown to potentiate the effect of adriamycin and paclitaxel in osteosarcoma and non-small cell lung cancer [126] and to sensitize anaplastic thyroid carcinoma cells to cisplatin [150].

2-DG can be administered intravenously or orally with a blood half-life of approximately 90 minutes, and toxicity, mainly hypoglycemia-like symptoms, has in early clinical studies been transient [149]. Likely in part due to the unfavorable pharmacokinetics with rapid elimination of the drug, clinical trials have not been able to validate the promising preclinical findings [127, 149, 150].

In conclusion, this study shows a platinum-potentiating effect of glycolysis inhibitor 2-DG in ovarian cancer cell lines and tumor cells from ascites. Our results validate targeting glycolysis in serous EOC and also indicate the  $\beta$ -F1-ATPase:HSP60 ratio to be predictive of sensitivity to such combination treatment.

## **5.2 PAPERS II AND III**

### **HSP60 predicts survival in advanced serous ovarian cancer**

and

### **Metabolic markers GAPDH, PKM2, ATP5B and BEC-index in advanced serous ovarian cancer**

#### **5.2.1 HSP60**

At both mRNA and protein levels, PFI and survival were significantly shorter in the HSP60 high expression group. The median interval from end of treatment to relapse was 4, 6 and 13 months for the mRNA high, intermediate and low expression groups ( $p=0.010$ ), respectively, with corresponding 5 and 12 months for the groups with high and low protein expression ( $p=0.024$ ). The median survival for the mRNA high, intermediate and low expression groups were 25 months, 35 months versus not yet reached ( $p=0.037$ ). Accordingly, the median survival in the high and low protein expression groups were 31 and 55 months ( $p=0.016$ ). In multivariate analyses, HSP60 mRNA and protein expression were both verified to have independent impact on PFI and survival (Table 4). A subgroup analysis of the grade 3 serous tumors ( $n=40$ ) showed higher HRs when including only these (OS: HR 3.8, 95% CI 1.3-11.0, PFI: HR 4.8, 95% CI 1.9-12.0).

Table 4. Uni- and multivariate analyses of survival and platinum-free interval in relation to HSP60 mRNA and protein expression

Method	Endpoint	HSP60 expression	Multivariate analyses <sup>a</sup>	
			HR (95% CI)	<i>p</i> -value
Real time-PCR (N=55)	OS	<0.50 (n=18)	1	0.037
		0.50-1.31 (n=18)	2.2 (0.9-5.6)	
		>1.31 (n=19)	3.4 (1.3-8.5)	
	PFI	<0.50 (n=18)	1	
		0.50-1.31 (n=18)	2.0 (0.9-4.2)	
		>1.31 (n=19)	3.3 (1.5-7.2)	
IHC (N=54)	OS	<30% 3+ (n=17)	1	0.004
		≥30% 3+ (n=37)	3.2 (1.5-7.1)	
	PFI	<30% 3+ (n=17)	1	
		≥30% 3+ (n=37)	2.6 (1.3-5.3)	

Abbreviations: HR=hazard ratio, CI=confidence interval, OS=overall survival, PFI=platinum-free interval, IHC=immunohistochemistry.

<sup>a</sup>Multivariate model with adjustment for age, FIGO stage, grade and postoperative residual tumor size

All patients with low HSP60 levels responded to first line chemotherapy. Concordantly, the protein expression of this marker was shown significantly associated with treatment response ( $p=0.02$ ). At the mRNA level, though, we found only a borderline significant association ( $p=0.055$ ).

### 5.2.2 GAPDH

The median PFI was significantly shorter for patients with high GAPDH mRNA expression compared to cases with low expression (5.0 and 10.1 months, respectively,  $p=0.031$ ). In univariate analysis, a high GAPDH mRNA was also found associated with shorter OS ( $p=0.015$ ). However, in multivariate analysis, a high GAPDH remained significant only for shorter PFI (HR 2.1, 95% CI 1.0-4.5,  $p=0.043$ ).

Using IHC, we found no statistically significant differences in PFI or survival between groups with high or low GAPDH protein expression. GAPDH-reactivity was predominantly cytoplasmatic, but in 22 cases (41 %) we also found a nuclear localization of the enzyme.

### 5.2.3 PKM2

There were no statistically discernible differences in PFI or survival between groups with high or low PKM2 expression, neither at the mRNA-, nor at the protein level. The assessed PKM2-reactivity was cytoplasmatic, but in 11 cases (20 %) a nuclear localization of the protein was observed in mitotic cells.

#### 5.2.4 ATP5B

In univariate analysis, a high ATP5B mRNA expression predicted poor OS ( $p=0.025$ ), but this finding did not remain significant at the multivariate level (HR 2.3, 95% CI 1.0-5.3,  $p=0.062$ ). Similarly, in univariate analysis, a high ATP5B protein expression predicted short PFI ( $p=0.039$ ), but did not reach significance in the multivariate model (HR 1.7, 95% CI 1.0-3.2,  $p=0.075$ ).

#### 5.2.5 BEC-index

The median PFI was significantly longer in the group with high compared to the group with low BEC-index (9.8 and 5.3 months, respectively,  $p=0.028$ ). Accordingly, multivariate analysis showed a high BEC-index mRNA to independently predict longer PFI (HR 0.47, 95% CI 0.23-0.95,  $p=0.035$ ). Also, univariate analysis indicated a high BEC-index to predict longer survival ( $p=0.033$ ), but it did not remain significant in multivariate calculations (HR 0.49, 95% CI 0.22-1.31,  $p=0.088$ ).

#### 5.2.6 Discussion and conclusions

Our data suggest high tumor HSP60 expression, at both mRNA and protein levels, to be indicative of early relapse and poor survival of HGSOC patients. We also show high GAPDH as well as low BEC-index based on mRNA to be associated with early disease progression. In addition, low HSP60 protein expression may predict response to first-line platinum-based chemotherapy.

Heat shock proteins are induced by endogenous or exogenous stress to promote cell survival and homeostasis. They predominantly function as chaperones for other cellular proteins. By ensuring the right conformation of these “client” proteins, they take part in the regulation of their downstream targets [151, 152]. Rapidly dividing malignant cells thus rely on chaperones for correct folding of oncoproteins, and HSP60 has been shown to have anti-apoptotic and pro-survival features [153-155]. For example, HSP60 stabilizes apoptosis inhibitor survivin [156], inhibits mitochondrial permeability transition and caspase-dependent apoptosis [157], and has also been shown to play a role in the nuclear factor  $\kappa$ B survival pathway [155].

The specific mechanisms by which HSP60 mediate the observed poor prognosis and treatment-resistance in serous EOC remains unclear. However, it has previously been associated with platinum-resistance in ovarian carcinoma cell lines [158, 159]. Our results are also in accordance with findings in other malignancies, such as lung and prostate cancer [160-162].

To our knowledge, this is the first study of metabolic markers in ovarian cancer patients, and the first publication evaluating the BEC-index at the mRNA level. Despite the limited study size, our results on GAPDH and BEC-index are in line with findings in other malignancies, such as breast, colon and lung cancer [128, 136, 137, 142, 144]. The observed up-regulated GAPDH and low BEC-index in the early relapsing cases both reflect an increased dependence upon glycolysis in these tumors. These changes

are in accordance with the original Warburg hypothesis of a universal cancer cell glycolytic phenotype, and provide examples of the prognostic bearing of the metabolic alterations seen in malignancy. The fact that we did not, in this small patient cohort, find any independent impact on outcome for ATP5B- or PKM2 expression does not exclude these enzymes from playing a role in serous tumor aggressiveness. The data for all our included markers needs validation in a larger cohort before generalizing the results to all HGSOC.

GAPDH has been widely used as housekeeping reference gene/protein, also in studies of cancer. However, our data, as well as previous findings by others [128], suggest it unsuitable as such for studies in malignancy.

In conclusion, our results indicate that HSP60, GAPDH and BEC-index expression may be able to aid in identifying groups of advanced HGSOCs with different prognosis. In addition, HSP60 protein expression may be of value as predictive of response to first-line chemotherapy.

### **5.3 PAPER IV**

#### **Metabolic markers and HSP60 in chemo-naïve serous solid ovarian cancer versus ascites**

##### **5.3.1 Solid tumor versus ascites**

In contrast to our pre-study hypothesis, the mRNA expression of GAPDH, PKM2, ATP5B and HSP60 did not differ in pairwise comparison of serous solid tumor and corresponding malignant ascites. On the contrary, when examining the expression value distribution of solid tumor versus ascites tumor cells, there was not even a discernible trend towards an altered expression in detached cells. Thus, for all investigated metabolic markers, roughly half of the cases exhibited an equal or higher expression in ascites, with the other half accordingly showing a higher expression in solid tumor.

##### **5.3.2 Discussion and conclusion**

We are not aware of any other study having compared the expression of glycolytic and oxidative markers in a clinical material of solid tumors and corresponding effusions. Our results indicate that further reprogramming of glycolysis or oxidative phosphorylation is not a prerequisite for cancer cell survival after detachment.

This study is too small to rule out potential alterations in mRNA expression of analyzed markers, but our very similar findings in the respective cell state argue against there being any. Also, a similar expression at the transcriptional level does not necessarily translate into activity of respective enzyme. However, we suggest that the highly malignant, progressed cell state of the solid advanced serous carcinoma may already

exploit its glycolytic and oxidative pathways maximally, making further changes in expression along disease progression difficult to detect. The major metabolic reprogramming in serous EOC would thus occur at an earlier stage.

Above arguments might in principle also explain the similar HSP60 expression in compared adherent and detached cells. Supporting here presented findings, in a recent study of ascites cells spontaneously forming spheroids compared to single cells, we found no difference in HSP60 or ATP5B protein expression [163].

A similar transcription of glycolytic and oxidative genes does not exclude changes in other metabolic pathways. Cancer cells can, when glucose availability is low, compensate by increasing their use of glutamine or serine as energy substrates [120, 122, 164]. Whether ascites tumor cells enhance their use of alternative fuel remains unknown.

In conclusion, our results on GAPDH, PKM2, ATP5B and HSP60 mRNA in advanced serous ovarian cancer indicate there being no major difference in expression of either marker in solid tumor compared to corresponding malignant ascites.

#### **5.4 CONSIDERATIONS IN CHOICE OF METHODS**

In paper I, we used a sulforhodamine B-based assay to study cell survival *in vitro*. In contrast to the alternative tetrazolium assays, the SRB assay does not depend upon cell enzymatic activity, which confers an advantage when studying cellular metabolic alterations.

In paper II-III, we wanted to investigate the expression of a small group of markers and their association with outcome. Knowing that the expression of cellular molecules at the RNA and protein levels is not always concordant, we decided to study both. The quantitative real-time PCR method was well suited for the limited RNA analyses. We used SYBR Green™ for detection of PCR-products. This dye fluoresces when bound to all double-stranded DNA, which imposes a risk of signal also from contaminating DNA. However, when analyzing abundantly expressed genes, the proportion of signal from non-target DNA will be negligible. SYBR Green™ is thus not as specific as the alternative TaqMan-probe, but adequate when analyzing highly expressed genes (as in our studies). Also, our forward and reverse primers were localized in different exons, minimizing risk of transcription of contaminating DNA.

For validation at the protein level, we chose to do immunohistochemistry. This is a method used in everyday clinical routine and the cost is manageable. Another obvious advantage is the availability of tissue blocks. In addition, IHC provides the possibility to discern the subcellular localization(s) of assessed proteins. The drawbacks of this method are its semi-quantitative nature and the risk of subjectivity in evaluation of slides. It is also a fairly insensitive method, in which small differences in staining are not discernible. To in part overcome this, digitalized procedures have been developed,

but these are costly and time-consuming and yet not applicable in the clinic. Also, the problem of subjectivity still remains.

To reduce the error of subjectivity in our study, the evaluation of slides were done by three independent observers, blinded for clinical data.

## **5.5 STATISTICAL PITFALLS**

Our study population is limited by its small size, and we cannot rule out a risk of misrepresentation because of sampling from the targeted study base (bias due to non-participation). Results are therefore not directly generalizable to all HGSOE patients, but needs validation in a larger cohort. The close-to-perfect analysis would have included all patients undergoing surgery for advanced endometrioid or serous ovarian cancer during the study period. This scenario is difficult to achieve, however, since preoperative diagnostics are not always accurate and clinical logistics sometimes an obstacle.

The risk of misclassification, i.e. measuring errors, is obvious when using a semi-quantitative, possibly subjective method as IHC. In our studies, we have reduced this risk by setting up pre-defined measuring means and by being three independent observers, unaware of case records.

Misclassification could also apply to clinical assessments, e.g. histopathological diagnose of EOC subtype and evaluations of response to treatment and progression. To decrease these potential errors, a subspecialized gynae-pathologist classified all cases and pre-defined modified RECIST- and GCIG criteria were applied for clinical evaluations.

## 6 GENERAL CONCLUSIONS

- In ovarian cancer cells, glycolysis inhibitor 2-DG can potentiate the antiproliferative effects of concomitant platinum treatment, and the  $\beta$ -F1-ATPase:HSP60 ratio might be predictive of sensitivity to such combination treatment.
- HSP60 expression, at both mRNA and protein levels, may identify groups of advanced high-grade serous carcinomas with different prognosis. Also, HSP60 protein expression may aid in prediction of resistance to first-line platinum-based chemotherapy.
- High GAPDH as well as low BEC-index mRNA is indicative of early disease progression in high-grade serous EOC, and these metabolic markers may thus also be of value in distinguishing patients with different prognosis.
- Neither glycolytic GAPDH or PKM2, nor mitochondrial ATP5B or HSP60 mRNA expression differ between solid high-grade serous cancer and corresponding malignant cells in ascites. These findings indicate that further reprogramming of glycolysis or oxidative phosphorylation is not a prerequisite for serous cancer cell survival after detachment. Also, possible future drugs targeting glycolysis should have equal effect in both solid tumor lesions and malignant ascites.



## 7 FUTURE PERSPECTIVES

Epithelial ovarian cancer remains a disease usually detected after dissemination, and it still carries a poor prognosis. A more radical surgical approach has over the last decade somewhat improved survival, but more effective systemic treatment is badly needed. Since the various EOC subtypes are essentially different diseases, future developments in prognostication and treatment should focus on subgroup-specific phenotypic and molecular alterations.

Over the last years, many trials have investigated the addition of targeting substances (e.g. inhibitors of VEGF, tyrosine kinases and PARP, see sections 1.6.4 and 1.8.1) to routine chemotherapy, and some of these have shown positive results. In the future, the addition also of drugs targeting malignant metabolic pathway alterations could further improve treatment efficacy.

In the clinic, with a growing number of treatment options, it will be of vital importance to be able to better discriminate which patients who will gain from a certain toxic treatment, and who will not. Finding subgroup-specific prognostic and treatment predictive markers will make these decisions less difficult to make. Especially for the seemingly homogenous, large group of high-grade serous cancers, a growing number of studies indicate there being subgroups with very varying molecular alterations and prognosis [19, 23, 24].

In conclusion, our studies represent a few steps on the road towards better prognostication and personalized treatment of epithelial ovarian cancer. Targeting the various metabolic pathways could be a way of increasing treatment efficacy in this disease. Further exploration also of enzymes of glutaminolysis and combination treatment using inhibitors of more than one metabolic pathway thus provide additional options of study.

## 8 ACKNOWLEDGEMENTS

At last, when all this is finally over, I would like to thank

**Elisabeth Åvall-Lundqvist**, my supervisor, for taking a young, reluctant me under your competent wings and keeping me there, for expert guidance in scientific thinking and writing, for pushing me when needed, for believing in my capacity and for making me believe in myself.

**Suzanne Egyhazi Brage**, my co-supervisor, for enjoyable discussions, always friendly support and for sticking with me through the years.

**Mimmi Shoshan**, my co-supervisor, for introducing me to the field of metabolomics, for your catching enthusiasm and for at all times providing fast support and input.

**Joe Carlson**, my latest co-supervisor, for instructive and fun times at the microscope.

Co-authors **Emma Hernlund**, **My Björklund**, **Kjell Shedvins**, **Marianne Frostvik Stolt** and **Hemming Johansson** for stimulating discussions and guidance in the lab and methodology, well performed surgery, support at the PCR-machine and help in understanding the statistics.

**Inger Bodin** and **Liss Garberg** for friendly and skillful help with the immunohistochemistry.

**All my heroic patients** for showing me that the way to go through life is to put the right foot in front of the left, and for making me see that small victories are also victories.

**Anders Hjerpe** for showing me the worlds of science and medicine (at the age of four), for always giving immediate and knowledgeable help whenever I need it and for never ending enthusiasm and positive thinking (why didn't I inherit any of that?!).

**Alexandra Hofsjö** for the clinical discussions and laughs, the support during our mutual scientific first trembling steps and for all the *really* important talks in our room. Here's to teenage daughters and early morning debriefings!

**Margareta Bergdahl**, **Christina Hising** and **Bengt Tholander**, my mentors, for years of wise clinical guidance.

**Mariann Iristo** and **Alexandra** for, despite tough times, giving me the time to complete this work.

**Caroline**, **Catharina**, **Roxane**, **Ann-Cathrin**, **Kristina**, **Britta**, **Hanna**, **Rikard**, **Josefin**, **Daria** and **Eneida** for hanging in there and doing the business.

**Lotta** (our family surgeon), **Sahar**, **Henrik**, **Ulrika**, **Angelos**, **Barbro** and **Katja** for having the strength and endurance, for the progress in the effort- and skill-demanding surgery for ovarian cancer and for all the heart-felt laughs.

**Anna-Maria**, **Karin P** and **Karin B** for all the intellectually challenging and philosophic discussions over many dinners and even more glasses of good wine.

**Ingrid** for being the best possible sister-in-law, for all the fun and for your stylish input to this thesis (and everything else).

**Olle** for big hugs and life-long support in all that matters.

**Mom** and **Dad** for being there, always.

**Hanna** – my talented daughter, you're the joy of my life! Always looking forward to watching you play;).

**Kerstin** for everything - without your stubborn endurance, loving, master-chef ground support and knowledge in computers I probably wouldn't have been able to do it. We'll keep travelling. Kerstin är bäst!

and

**Eriksdalsbadet** for at all times providing this neurotic body the opportunity to surf the endorphin wave...

### **Funding**

The studies in this thesis were supported by The Cancer Research Funds of Radiumhemmet, The Swedish Cancer Society and The Swedish State under the ALF agreement, Stockholm.

## 9 REFERENCES

- 1 McCluggage WG. Morphological subtypes of ovarian carcinoma: a review with emphasis on new developments and pathogenesis. *Pathology (Phila)*. 2011;43:420-32.
- 2 Tavassoéli F and Devilee P. World Health Organization Classification of Tumours. *Pathology and Genetics of Tumours of the Breast and Female Genital Organs*. Lyon: IARC Press; 2003.
- 3 Silverberg SG. Histopathologic Grading of Ovarian Carcinoma: A Review and Proposal. *Int. J. Gynecol. Pathol.* 2000;19:7-15.
- 4 Köbel M, Kalloger SE, Huntsman DG, Santos JL, Swenerton KD, Seidman JD *et al*. Differences in Tumor Type in Low-stage Versus High-stage Ovarian Carcinomas. *Int. J. Gynecol. Pathol.* 2010;29:203-11.
- 5 Seidman JD, Horkayne-Szakaly I, Haiba M, Boice CR, Kurman RJ and Ronnett BM. The Histologic Type and Stage Distribution of Ovarian Carcinomas of Surface Epithelial Origin. *Int. J. Gynecol. Pathol.* 2004;23:41-4.
- 6 Hanahan D and Weinberg Robert A. Hallmarks of Cancer: The Next Generation. *Cell* 2011;144:646-74.
- 7 GLOBOCAN database. WHO. [www-dep.iarc.fr](http://www-dep.iarc.fr).
- 8 NORDCAN database. WHO. [www-dep.iarc.fr](http://www-dep.iarc.fr).
- 9 Bodurka DC, Deavers MT, Tian C, Sun CC, Malpica A, Coleman RL *et al*. Reclassification of serous ovarian carcinoma by a 2-tier system. *Cancer* 2012;118:3087-94.
- 10 Malpica A, Deavers MT, Lu KH, Bodurka DC, Atkinson EN, Gershenson DM *et al*. Grading Ovarian Serous Carcinoma Using a Two-Tier System. *Am. J. Surg. Pathol.* 2004;28:496-504.
- 11 Vang R, Shih I-M and Kurman RJ. Ovarian Low-grade and High-grade Serous Carcinoma: Pathogenesis, Clinicopathologic and Molecular Biologic Features, and Diagnostic Problems. *Adv. Anat. Pathol.* 2009;16:2067-282.
- 12 Bowtell DDL. The genesis and evolution of high-grade serous ovarian cancer. *Nat. Rev. Cancer* 2010;10:803-8.
- 13 Gershenson DM, Sun CC, Lu KH, Coleman RL, Sood AK, Malpica A *et al*. Clinical Behaviour of Stage II-IV Low-Grade Serous Carcinoma of the Ovary. *Obstet. Gynecol.* 2006;108:361-8.
- 14 Landen CN, Birrer MJ and Sood AK. Early Events in the Pathogenesis of Epithelial Ovarian Cancer. *J. Clin. Oncol.* 2008;26:995-1005.
- 15 Jarboe E, Folkins A, Nucci MR, Kindelberger D, Drapkin R, Miron A *et al*. Serous Carcinogenesis in the Fallopian Tube: A Descriptive Classification. *Int. J. Gynecol. Pathol.* 2008;27:1-9.
- 16 Lee Y, Miron A, Drapkin R, Nucci MR, Medeiros F, Saleemuddin A *et al*. A candidate precursor to serous carcinoma that originates in the distal fallopian tube. *The Journal of Pathology* 2007;211:26-35.
- 17 Lim D and Oliva E. Precursors and pathogenesis of ovarian carcinoma. *Pathology (Phila)*. 2013;45:229-42.
- 18 Ahmed AA, Etemadmoghadam D, Temple J, Lynch AG, Riad M, Sharma R *et al*. Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. *The Journal of Pathology* 2010;221:49-56.
- 19 The Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature* 2011;474:609-15.
- 20 Carlson J, Roh MH, Chang MC and Crum CP. Recent advances in the understanding of the pathogenesis of serous carcinoma: the concept of low- and high-grade disease and the role of the fallopian tube. *Diagn. Histopathol.* 2008;14:352-65.
- 21 Turner N, Tutt A and Ashworth A. Hallmarks of 'BRCAness' in sporadic cancers. *Nat. Rev. Cancer* 2004;4:814-9.

- 22 Karst AM, Jones PM, Vena N, Ligon AH, Liu JF, Hirsch MS *et al.* Cyclin E1  
Deregulation Occurs Early in Secretory Cell Transformation to Promote  
Formation of Fallopian Tube–Derived High-Grade Serous Ovarian Cancers.  
*Cancer Res.* 2014;74:1141-52.
- 23 Tothill RW, Tinker AV, George J, Brown R, Fox SB, Lade S *et al.* Novel  
Molecular Subtypes of Serous and Endometrioid Ovarian Cancer Linked to  
Clinical Outcome. *Clin. Cancer Res.* 2008;14:5198-208.
- 24 Verhaak RGW, Tamayo P, Yang J-Y, Hubbard D, Zhang H, Creighton CJ *et al.*  
Prognostically relevant gene signatures of high-grade serous ovarian carcinoma.  
*J. Clin. Invest.* 2013;123:517-25.
- 25 Berns EMJJ and Bowtell DD. The Changing View of High-Grade Serous  
Ovarian Cancer. *Cancer Res.* 2012;72:2701-4.
- 26 Meinhold-Heerlein I, Bauerschlag D, Hilpert F, Dimitrov P, Sapinoso LM,  
Orlowska-Volk M *et al.* Molecular and prognostic distinction between serous  
ovarian carcinomas of varying grade and malignant potential. *Oncogene*  
2004;24:1053-65.
- 27 Singer G, Oldt R, Cohen Y, Wang BG, Sidransky D, Kurman RJ *et al.*  
Mutations in BRAF and KRAS Characterize the Development of Low-Grade  
Ovarian Serous Carcinoma. *J. Natl. Cancer Inst.* 2003;95:484-6.
- 28 Gilks CB. Molecular Abnormalities in Ovarian Cancer Subtypes Other than  
High-Grade Serous Carcinoma. *J. Oncol.* 2010;2010:7.
- 29 Bell KAMD and Kurman RJMD. A Clinicopathologic Analysis of Atypical  
Proliferative (Borderline) Tumors and Well-Differentiated Endometrioid  
Adenocarcinomas of the Ovary. *Am. J. Surg. Pathol.* 2000;24:1465-79.
- 30 Stern RCMD, Dash RMD, Bentley RCMD, Snyder MJMD, Haney AFMD and  
Robboy SJMD. Malignancy in Endometriosis: Frequency and Comparison of  
Ovarian and Extraovarian Types. *Int. J. Gynecol. Pathol.* 2001;20:133-9.
- 31 Catasús L, Bussaglia E, Rodríguez I, Gallardo A, Pons C, Irving JA *et al.*  
Molecular genetic alterations in endometrioid carcinomas of the ovary: Similar  
frequency of beta-catenin abnormalities but lower rate of microsatellite  
instability and PTEN alterations than in uterine endometrioid carcinomas. *Hum.*  
*Pathol.* 2004;35:1360-8.
- 32 Wiegand KC, Shah SP, Al-Agha OM, Zhao Y, Tse K, Zeng T *et al.* ARID1A  
Mutations in Endometriosis-Associated Ovarian Carcinomas. *N. Engl. J. Med.*  
2010;363:1532-43.
- 33 Anglesio MS, Carey MS, Köbel M, MacKay H and Huntsman DG. Clear cell  
carcinoma of the ovary: A report from the first Ovarian Clear Cell Symposium,  
June 24th, 2010. *Gynecol. Oncol.* 2011;121:407-15.
- 34 Jones S, Wang T-L, Shih I-M, Mao T-L, Nakayama K, Roden R *et al.* Frequent  
Mutations of Chromatin Remodeling Gene ARID1A in Ovarian Clear Cell  
Carcinoma. *Science* 2010;330:228-31.
- 35 Kuo K-T, Mao T-L, Jones S, Veras E, Ayhan A, Wang T-L *et al.* Frequent  
Activating Mutations of PIK3CA in Ovarian Clear Cell Carcinoma. *Am. J.*  
*Pathol.* 2009;174:1597-601.
- 36 Yamashita Y, Akatsuka S, Shinjo K, Yatabe Y, Kobayashi H, Seko H *et al.* Met  
Is the Most Frequently Amplified Gene in Endometriosis-Associated Ovarian  
Clear Cell Adenocarcinoma and Correlates with Worsened Prognosis. *PLoS*  
*ONE* 2013;8:e57724.
- 37 Lee KR and Young RH. The distinction between primary and metastatic  
mucinous carcinoma of the ovary: gross and histologic findings in 50 cases.  
*Am. J. Surg. Pathol.* 2003;27:281-92.
- 38 Gemignani ML, Schlaerth AC, Bogomolny F, Barakat RR, Lin O, Soslow R *et*  
*al.* Role of KRAS and BRAF gene mutations in mucinous ovarian carcinoma.  
*Gynecol. Oncol.* 2003;90:378-81.
- 39 Anglesio MS, Kommoss S, Tolcher MC, Clarke B, Galletta L, Porter H *et al.*  
Molecular characterization of mucinous ovarian tumours supports a stratified  
treatment approach with HER2 targeting in 19% of carcinomas. *J. Pathol.*  
2013;229:111-20.
- 40 Gayther SA. Inherited Risk of Ovarian Cancer and the Implications for  
Screening. *Int. J. Gynecol. Cancer* 2012;22:S12-S5.

- 41 Antoniou A, Pharoah PDP, Narod S, Risch HA, Eyfjord JE, Hopper JL *et al.*  
Average Risks of Breast and Ovarian Cancer Associated with BRCA1 or  
BRCA2 Mutations Detected in Case Series Unselected for Family History: A  
42 Combined Analysis of 22 Studies. *Am. J. Hum. Genetics* 2003;72:1117-30.
- 43 Ramus SJ and Gayther SA. The Contribution of BRCA1 and BRCA2 to  
Ovarian Cancer. *Mol. Oncol.* 2009;3:138-50.
- 44 Stan DL, Shuster LT, Wick MJ, Swanson CL, Pruthi S and Bakkum-Game JN.  
Challenging and Complex Decisions in the Management of the BRCA  
Mutation Carrier *J. Women's Health* 2013;22:825-34.
- 45 Fleming JS, Beaugié CR, Haviv I, Chenevix-Trench G and Tan OL. Incessant  
ovulation, inflammation and epithelial ovarian carcinogenesis: Revisiting old  
46 hypotheses. *Mol. Cell. Endocrinol.* 2006;247:4-21.
- 47 Hunn J and Rodriguez GC. Ovarian Cancer: Etiology, Risk Factors, and  
Epidemiology. *Clin. Obstet. Gynecol.* 2012;55:3-23.
- 48 Bolton KL, Ganda C, Berchuck A, Pharaoh PDP and Gayther SA. Role of  
common genetic variants in ovarian cancer susceptibility and outcome: progress  
to date from the ovarian cancer association consortium (OCAC). *J. Intern. Med.*  
2012;271:366-78.
- 49 Charbonneau B, Block MS, Bamlet WR, Vierkant RA, Kalli KR, Fogarty Z *et*  
*al.* Risk of Ovarian Cancer and the NF- $\kappa$ B Pathway: Genetic Association with  
IL1A and TNFSF10. *Cancer Res.* 2014;74:852-61.
- 50 Nezhat F, Datta MS, Hanson V, Pejovic T, Nezhat C and Nezhat C. The  
relationship of endometriosis and ovarian malignancy: a review. *Fertil. Steril.*  
2008;90:1559-70.
- 51 Pearce CL, Templeman C, Rossing MA, Lee A, Near AM, Webb PM *et al.*  
Association between endometriosis and risk of histological subtypes of ovarian  
cancer: a pooled analysis of case-control studies. *Lancet Oncol.* 2012;13:385-  
94.
- 52 Kim HS, Kim TH, Chung HH and Song YS. Risk and prognosis of ovarian  
cancer in women with endometriosis: a meta-analysis. *Br. J. Cancer* 2014.
- 53 Rizzuto I, Behrens RF and Smith LA. Risk of ovarian cancer in women treated  
with ovarian stimulating drugs for infertility. Vol. 2013(8). *Cochrane database*  
*of systematic reviews* 2013.
- 54 Mørch L, Løkkegaard E, Andreassen A, Krüger-Kjær S and Lidegaard Ø.  
Hormone therapy and ovarian cancer. *JAMA* 2009;302:298-305.
- 55 Yang HP, Anderson WF, Rosenberg PS, Trabert B, Gierach GL, Wentzensen N  
*et al.* Ovarian Cancer Incidence Trends in Relation to Changing Patterns of  
Menopausal Hormone Therapy Use in the United States. *J. Clin. Oncol.*  
2013;31:2146-51.
- 56 Yang HP, Trabert B, Murphy MA, Sherman ME, Sampson JN, Brinton LA *et*  
*al.* Ovarian cancer risk factors by histologic subtypes in the NIH-AARP diet  
and health study. *Int. J. Cancer* 2012;131:938-48.
- 57 Terry KL, Karageorgi S, Shvetsov YB, Merritt MA, Lurie G, Thompson PJ *et*  
*al.* Genital Powder Use and Risk of Ovarian Cancer: A Pooled Analysis of  
8,525 Cases and 9,859 Controls. *Cancer Prev. Res.* 2013;6:811-21.
- 58 Collaborative Group on Epidemiological Studies of Ovarian Cancer. Ovarian  
Cancer and Body Size: Individual Participant Meta-Analysis Including 25,157  
Women with Ovarian Cancer from 47 Epidemiological Studies. *PLoS Med*  
2012;9:e1001200.
- 59 Olsen CM, Nagle CM, Whiteman DC, Ness R, Pearce CL, Pike MC *et al.*  
Obesity and risk of ovarian cancer subtypes: evidence from the Ovarian Cancer  
Association Consortium. *Endocrine-Related Cancer* 2013;20:251-62.
- 60 Merritt MA, Cramer DW, Missmer SA, Vitonis AF, Titus LJ and Terry KL.  
Dietary fat intake and risk of epithelial ovarian cancer by tumour histology. *Br.*  
*J. Cancer* 2014;110:1392-401.
- 61 Crane TE, Khulpateea BR, Alberts DS, Basen-Engquist K and Thomson CA.  
Dietary Intake and Ovarian Cancer Risk: A Systematic Review. *Cancer*  
*Epidemiol. Biom. Prev.* 2014;23:255-73.
- 62 Collaborative Group on Epidemiological Studies of Ovarian Cancer. Ovarian  
cancer and smoking: individual participant meta-analysis including 28 114

- women with ovarian cancer from 51 epidemiological studies. *Lancet Oncol.* 2012;13:946-56.
- 61 Merritt MA, De Pari M, Vitonis AF, Titus LJ, Cramer DW and Terry KL. Reproductive characteristics in relation to ovarian cancer risk by histologic pathways. *Hum. Reprod.* 2013;28:1406-17.
- 62 Jordan SJ, Cushing-Haugen KL, Wicklund KG, Doherty JA and Rossing MA. Breast-feeding and risk of epithelial ovarian cancer. *Cancer Causes Control* 2012;23:919-27.
- 63 Purdie DM, Bain CJ, Siskind V, Webb PM and Green AC. Ovulation and risk of epithelial ovarian cancer. *Int. J. Cancer* 2003;104:228-32.
- 64 Collaborative Group on Epidemiological Studies of Ovarian Cancer. Ovarian cancer and oral contraceptives: collaborative reanalysis of data from 45 epidemiological studies including 23 257 women with ovarian cancer and 87 303 controls. *Lancet* 2008;371:303-14.
- 65 Gadducci A, Sergiampietri C and Tana R. Alternatives to risk-reducing surgery for ovarian cancer. *Ann. Oncol.* 2013;24:viii47-viii53.
- 66 Pearce CL, Chung K, Pike MC and Wu AH. Increased ovarian cancer risk associated with menopausal estrogen therapy is reduced by adding a progestin. *Cancer* 2009;115:531-9.
- 67 Treviño LS, Buckles EL and Johnson PA. Oral Contraceptives Decrease the Prevalence of Ovarian Cancer in the Hen. *Cancer Prev. Res.* 2012;5:343-9.
- 68 Sueblinvong T and Carney M. Current Understanding of Risk Factors for Ovarian Cancer. *Curr. Treat. Options in Oncol.* 2009;10:67-81.
- 69 Sieh W, Salvador S, McGuire V, Weber RP, Terry KL, Rossing MA *et al.* Tubal ligation and risk of ovarian cancer subtypes: a pooled analysis of case-control studies. *Int. J. Epidemiol.* 2013;42:579-89.
- 70 Schenberg T and Mitchell G. Prophylactic bilateral salpingectomy as a prevention strategy in women at high risk of ovarian cancer: a mini-review. *Frontiers in Oncology* 2014;4.
- 71 Biesma R, Schouten L, Dirx MM, Goldbohm RA and Brandt P. Physical Activity and Risk of Ovarian Cancer: Results from the Netherlands Cohort Study (The Netherlands). *Cancer Causes Control* 2006;17:109-15.
- 72 Xiao Q, Yang HP, Wentzensen N, Hollenbeck A and Matthews CE. Physical Activity in Different Periods of Life, Sedentary Behavior, and the Risk of Ovarian Cancer in the NIH-AARP Diet and Health Study. *Cancer Epidemiol. Biom. Prev.* 2013;22:2000-8.
- 73 Harris HR, Cramer DW, Vitonis AF, DePari M and Terry KL. Folate, vitamin B6, vitamin B12, methionine and alcohol intake in relation to ovarian cancer risk. *Int. J. Cancer* 2012;131:E518-E29.
- 74 Kelemen LE and Consortium OCA. Recent alcohol consumption and risk of incident ovarian carcinoma: a pooled analysis of 5,342 cases and 10,358 controls from the Ovarian Cancer Association Consortium. *BMC Cancer* 2013 13:28
- 75 Altinoz MA and Korkmaz R. NF-kappa beta, macrophage migration inhibitory factor and cyclooxygenase-inhibition as likely mechanisms behind the acetaminophen- and NSAID-prevention of ovarian cancer. *Neoplasma* 2004;51:239-47.
- 76 Wernli KJ, Newcomb PA, Hampton JM, Trentham-Dietz A and Egan KM. Inverse association of NSAID use and ovarian cancer in relation to oral contraceptive use and parity. *Br. J. Cancer* 2008;98:1781-3.
- 77 Baandrup L, Faber MT, Christensen J, Jensen A, Andersen KK, Friis S *et al.* Nonsteroidal anti-inflammatory drugs and risk of ovarian cancer: systematic review and meta-analysis of observational studies. *Acta Obstet. Gynecol. Scand.* 2013;92:245-55.
- 78 Prat J. Staging classification for cancer of the ovary, fallopian tube, and peritoneum. *Int. J. Gynecol. Obst.* 2014;124:1-5.
- 79 Vergote I, Tropé CG, Amant F, Kristensen GB, Ehlen T, Johnson N *et al.* Neoadjuvant Chemotherapy or Primary Surgery in Stage IIIC or IV Ovarian Cancer. *N. Engl. J. Med.* 2010;363:943-53.

- 80 McGuire WP, Hoskins WJ, Brady MF, Kucera PR, Partridge EE, Look KY *et al.* Cyclophosphamide and Cisplatin Compared with Paclitaxel and Cisplatin in Patients with Stage III and Stage IV Ovarian Cancer. *N. Engl. J. Med.* 1996;334:1-6.
- 81 Piccart MJ, Bertelsen K, James K, Cassidy J, Mangioni C, Simonsen E *et al.* Randomized Intergroup Trial of Cisplatin–Paclitaxel Versus Cisplatin–Cyclophosphamide in Women With Advanced Epithelial Ovarian Cancer: Three-Year Results. *J. Natl. Cancer Inst.* 2000;92:699-708.
- 82 Du Bois A, Lück H-J, Meier W, Adams H-P, Möbus V, Costa S *et al.* A Randomized Clinical Trial of Cisplatin/Paclitaxel Versus Carboplatin/Paclitaxel as First-Line Treatment of Ovarian Cancer. *J. Natl. Cancer Inst.* 2003;95:1320-9.
- 83 Ozols RF, Bundy BN, Greer BE, Fowler JM, Clarke-Pearson D, Burger RA *et al.* Phase III Trial of Carboplatin and Paclitaxel Compared With Cisplatin and Paclitaxel in Patients With Optimally Resected Stage III Ovarian Cancer: A Gynecologic Oncology Group Study. *J. Clin. Oncol.* 2003;21:3194-200.
- 84 Katsumata N, Yasuda M, Isonishi S, Takahashi F, Michimae H, Kimura E *et al.* Long-term results of dose-dense paclitaxel and carboplatin versus conventional paclitaxel and carboplatin for treatment of advanced epithelial ovarian, fallopian tube, or primary peritoneal cancer (JGOG 3016): a randomised, controlled, open-label trial. *Lancet Oncol.* 2013;14:1020-6.
- 85 Chan J, Brady M, Penson R, Monk B, Boente M, Walker J *et al.* Phase III trial of every-3 weeks paclitaxel vs. dose dense weekly paclitaxel with carboplatin +/- bevacizumab in epithelial ovarian, peritoneal, fallopian tube cancer: GOG 262 (NCT01167712). *Int. J. Gynecol. Cancer* 2013;23(8). Supplement 1. Abstracts from ESGO, 19-20 October 2013, Liverpool, UK.
- 86 Armstrong DK, Bundy B, Wenzel L, Huang HQ, Baergen R, Lele S *et al.* Intraperitoneal Cisplatin and Paclitaxel in Ovarian Cancer. *N. Engl. J. Med.* 2006;354:34-43.
- 87 Jaaback K, Johnson N and Lawrie TA. Intraperitoneal chemotherapy for the initial management of primary epithelial ovarian cancer. *Cochrane database of systematic reviews* 2011.
- 88 SBU kommenterar: Cytostatika i bukhålan (intraperitonealt) vid äggstockscancer (ovarialcancer). Statens beredning för medicinsk utvärdering; 2012-08-28 (SBU assessing health care interventions).
- 89 González-Martín A. Update on randomized trials on recurrent disease. *Ann. Oncol.* 2013;24:x48-x52.
- 90 ICON4. Paclitaxel plus platinum-based chemotherapy versus conventional platinum-based chemotherapy in women with relapsed ovarian cancer: the ICON4/AGO-OVAR-2.2 trial. *Lancet* 2003;361:2099-106.
- 91 Pfisterer J, Plante M, Vergote I, du Bois A, Hirte H, Lacave AJ *et al.* Gemcitabine Plus Carboplatin Compared With Carboplatin in Patients With Platinum-Sensitive Recurrent Ovarian Cancer: An Intergroup Trial of the AGO-OVAR, the NCIC CTG, and the EORTC GCG. *J. Clin. Oncol.* 2006;24:4699-707.
- 92 Pujade-Lauraine E, Wagner U, Aavall-Lundqvist E, Gebiski V, Heywood M, Vasey PA *et al.* Pegylated Liposomal Doxorubicin and Carboplatin Compared With Paclitaxel and Carboplatin for Patients With Platinum-Sensitive Ovarian Cancer in Late Relapse. *J. Clin. Oncol.* 2010;28:3323-9.
- 93 Wagner U, Marth C, Largillier R, Kaern J, Brown C, Heywood M *et al.* Final overall survival results of phase III GCIG CALYPSO trial of pegylated liposomal doxorubicin and carboplatin vs paclitaxel and carboplatin in platinum-sensitive ovarian cancer patients. *Br. J. Cancer* 2012;107:588-91.
- 94 Monk BJ, Herzog TJ, Kaye SB, Krasner CN, Vermorken JB, Muggia FM *et al.* Trabectedin Plus Pegylated Liposomal Doxorubicin in Recurrent Ovarian Cancer. *J. Clin. Oncol.* 2010;28:3107-14.
- 95 Pujade-Lauraine E, Hilpert F, Weber B, Reuss A, Poveda A, Kristensen G *et al.* Bevacizumab Combined With Chemotherapy for Platinum-Resistant Recurrent Ovarian Cancer: The AURELIA Open-Label Randomized Phase III Trial. *J. Clin. Oncol.* 2014, published online.



- 96 Bamias A, Pignata S and Pujade-Lauraine E. Angiogenesis: A promising therapeutic target for ovarian cancer. *Crit. Rev. Oncol. Hematol.* 2012;84:314-26.
- 97 Gavalas N, Lontos M, Trachana S-P, Bagratuni T, Arapinis C, Liacos C *et al.* Angiogenesis-Related Pathways in the Pathogenesis of Ovarian Cancer. *Int. J. Mol. Sc.* 2013;14:15885-909.
- 98 Naora H and Montell DJ. Ovarian Cancer Metastasis: Integrating insights from disparate model organisms. *Nat. Rev. Cancer* 2005;5:355-66.
- 99 Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H *et al.* Incorporation of Bevacizumab in the Primary Treatment of Ovarian Cancer. *N. Engl. J. Med.* 2011;365:2473-83.
- 100 Perren TJ, Swart AM, Pfisterer J, Ledermann JA, Pujade-Lauraine E, Kristensen G *et al.* A Phase 3 Trial of Bevacizumab in Ovarian Cancer. *N. Engl. J. Med.* 2011;365:2484-96.
- 101 Aghajanian C, Blank SV, Goff BA, Judson PL, Teneriello MG, Husain A *et al.* OCEANS: A Randomized, Double-Blind, Placebo-Controlled Phase III Trial of Chemotherapy With or Without Bevacizumab in Patients With Platinum-Sensitive Recurrent Epithelial Ovarian, Primary Peritoneal, or Fallopian Tube Cancer. *J. Clin. Oncol.* 2012;30:2039-45.
- 102 Du Bois A, Kristensen G, Ray-Coquard I, Reuss A, Pignata S, Colombo N *et al.* AGO-OVAR 12: A randomized placebo-controlled GCIG/ENGOT-Intergroup phase III trial of standard frontline chemotherapy +/- nintendanib for advanced ovarian cancer. *Int. J. Gynecol. Cancer* 2013;23(8). Supplement 1. Abstracts from ESGO, 19-22 October 2013, Liverpool, UK.
- 103 Du Bois A, Floquet A, Kim JW, Rau J, Del Campo JM, Friedlander M *et al.* Randomized, double-blind, phase III trial of pazopanib versus placebo in women who have not progressed after first-line chemotherapy for advanced epithelial ovarian, fallopian tube, or primary peritoneal cancer (AEOC): Results of an international Intergroup trial (AGO-OVAR16). *ASCO Meeting Abstracts* 2013;31:LBA5503.
- 104 Heintz APM, Odicino F, Maisonneuve P, Quinn MA, Benedet JL, Creasman WT *et al.* Carcinoma of the Ovary. *Int. J. Gynecol. Obst.* 2006;95, Supplement 1:S161-S92.
- 105 Schmeler KM, Sun CC, Bodurka DC, T. Deavers M, Malpica A, Coleman RL *et al.* Neoadjuvant chemotherapy for low-grade serous carcinoma of the ovary or peritoneum. *Gynecol. Oncol.* 2008;108:510-4.
- 106 Díaz-Padilla I, Razak A, Minig L, Bernardini M and Campo J. Prognostic and predictive value of CA-125 in the primary treatment of epithelial ovarian cancer: potentials and pitfalls. *Clin. Transl. Oncol.* 2012;14:15-20.
- 107 Zorn KK, Tian C, McGuire WP, Hoskins WJ, Markman M, Muggia FM *et al.* The prognostic value of pretreatment CA 125 in patients with advanced ovarian carcinoma. *Cancer* 2009;115:1028-35.
- 108 Gadducci A, Cosio S, Fanucchi A, Negri S, Cristofani R and Genazzani AR. The predictive and prognostic value of serum CA 125 half-life during paclitaxel/platinum-based chemotherapy in patients with advanced ovarian carcinoma. *Gynecol. Oncol.* 2004;93:131-6.
- 109 Rocconi RP, Matthews KS, Kemper MK, Hoskins KE, Huh WK and Straughn Jr JM. The timing of normalization of CA-125 levels during primary chemotherapy is predictive of survival in patients with epithelial ovarian cancer. *Gynecol. Oncol.* 2009;114:242-5.
- 110 Sieh W, Köbel M, Longacre TA, Bowtell DD, deFazio A, Goodman MT *et al.* Hormone-receptor expression and ovarian cancer survival: an Ovarian Tumor Tissue Analysis consortium study. *Lancet Oncol.* 2013;14:853-62.
- 111 Lassus H, Sihto H, Leminen A, Nordling S, Joensuu H, Nupponen NN *et al.* Genetic alterations and protein expression of KIT and PDGFRA in serous ovarian carcinoma. *Br. J. Cancer* 2004;91:2048-55.
- 112 Rudlowski C, Pickart A-K, Fuhljohn C, Friepoertner T, Schele B, Biesterfeld S *et al.* Prognostic significance of vascular endothelial growth factor expression in ovarian cancer patients: a long-term follow-up. *Int. J. Gynecol. Cancer* 2006;16:183-9.

- 113 Yu L, Deng L, Li J, Zhang Y and Hu L. The prognostic value of vascular  
endothelial growth factor in ovarian cancer: A systematic review and meta-  
analysis. *Gynecol. Oncol.* 2013;128:391-6.
- 114 Banerjee S and Kaye SB. New Strategies in the Treatment of Ovarian Cancer:  
Current Clinical Perspectives and Future Potential. *Clin. Cancer Res.*  
2013;19:961-8.
- 115 Farley J, Brady WE, Vathipadiekal V, Lankes HA, Coleman R, Morgan MA *et al.*  
Selumetinib in women with recurrent low-grade serous carcinoma of the  
ovary or peritoneum: an open-label, single-arm, phase 2 study. *Lancet Oncol.*  
2013;14:134-40.
- 116 Vencken PMLH, Kriege M, Hoogwerf D, Beugelink S, van der Burg MEL,  
Hooning MJ *et al.* Chemosensitivity and outcome of BRCA1- and BRCA2-  
associated ovarian cancer patients after first-line chemotherapy compared with  
sporadic ovarian cancer patients. *Ann. Oncol.* 2011;22:1346-52.
- 117 Yang D, Khan S, Sun Y and *et al.* Association of BRCA1 and BRCA2  
mutations with survival, chemotherapy sensitivity, and gene mutator phenotype  
in patients with ovarian cancer. *JAMA* 2011;306:1557-65.
- 118 Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G *et al.*  
Olaparib Maintenance Therapy in Platinum-Sensitive Relapsed Ovarian Cancer.  
*N. Engl. J. Med.* 2012;366:1382-92.
- 119 Garg K, Levine DA, Olvera N, Dao F, Bisogna M, Secord AA *et al.* BRCA1  
Immunohistochemistry in a Molecularly Characterized Cohort of Ovarian High-  
Grade Serous Carcinomas. *Am. J. Surg. Pathol.* 2013;37:138-46.
- 120 DeBerardinis RJ, Lum JJ, Hatzivassiliou G and Thompson CB. The Biology of  
Cancer: Metabolic Reprogramming Fuels Cell Growth and Proliferation. *Cell*  
*Metabol.* 2008;7:11-20.
- 121 Rossignol R. Bioenergetics of cancer. *Biochim. Biophys. Acta (BBA) Bioenerg.*  
2011;1807:533.
- 122 Smolková K, Plecítá-Hlavatá L, Bellance N, Benard G, Rossignol R and Ježek  
P. Waves of gene regulation suppress and then restore oxidative  
phosphorylation in cancer cells. *Int. J. Biochem. Cell Biol.* 2011;43:950-68.
- 123 Vander Heiden MG, Cantley LC and Thompson CB. Understanding the  
Warburg Effect: The Metabolic Requirements of Cell Proliferation. *Science*  
2009;324:1029-33.
- 124 Hernlund E, Ihlund LS, Khan O, Ates YO, Linder S, Panaretakis T *et al.*  
Potentiation of chemotherapeutic drugs by energy metabolism inhibitors 2-  
deoxyglucose and etomoxir. *Int. J. Cancer* 2008;123:476-83.
- 125 Ihlund LS, Hernlund E, Khan O and Shoshan MC. 3-Bromopyruvate as  
inhibitor of tumour cell energy metabolism and chemopotentiator of platinum  
drugs. *Mol. Oncol.* 2008;2:94-101.
- 126 Maschek G, Savaraj N, Priebe W, Braunschweiger P, Hamilton K, Tidmarsh  
GF *et al.* 2-Deoxy-d-glucose Increases the Efficacy of Adriamycin and  
Paclitaxel in Human Osteosarcoma and Non-Small Cell Lung Cancers In Vivo.  
*Cancer Res.* 2004;64:31-4.
- 127 Ganapathy-Kanniappan S and Geschwind J-FH. Tumor glycolysis as a target  
for cancer therapy: progress and prospects. *Mol. Cancer* 2013;12:152
- 128 Guo C, Liu S and Sun M-Z. Novel insight into the role of GAPDH playing in  
tumor. *Clin. Transl. Oncol.* 2013;15:167-72.
- 129 Tristan C, Shahani N, Sedlak TW and Sawa A. The diverse functions of  
GAPDH: Views from different subcellular compartments. *Cell. Signal.*  
2011;23:317-23.
- 130 Chaneton B and Gottlieb E. Rocking cell metabolism: revised functions of the  
key glycolytic regulator PKM2 in cancer. *Trends Biochem. Sci.* 2012;37:309-  
16.
- 131 Mazurek S, Boschek CB, Hugo F and Eigenbrodt E. Pyruvate kinase type M2  
and its role in tumor growth and spreading. *Semin. Cancer Biol.* 2005;15:300-8.
- 132 Jang M, Kim SS and Lee J. Cancer cell metabolism: implications for  
therapeutic targets. *Exp. Mol. Med.* 2013;45:e45.

- 133 Willers IM and Cuezva JM. Post-transcriptional regulation of the mitochondrial H<sup>+</sup>-ATP synthase: A key regulator of the metabolic phenotype in cancer. *Biochim. Biophys. Acta (BBA) Bioenerg.* 2011;1807:543-51.
- 134 Altenberg B and Greulich KO. Genes of glycolysis are ubiquitously overexpressed in 24 cancer classes. *Genomics* 2004;84:1014-20.
- 135 Descotes F, Jézéquel P, Spyratos F, Champion L, Grenot C, Lerebours F *et al.* Identification of potential prognostic biomarkers for node-negative breast tumours by proteomic analysis: A multicentric 2004 national PHRC study. *Int. J. Oncol.* 2012;41:92-104.
- 136 Isidoro A, Casado E, Redondo A, Acebo P, Espinosa E, Alonso AM *et al.* Breast carcinomas fulfill the Warburg hypothesis and provide metabolic markers of cancer prognosis. *Carcinogenesis* 2005;26:2095-104.
- 137 Révillion F, Pawlowski V, Hornez L and Peyrat JP. Glyceraldehyde-3-phosphate dehydrogenase gene expression in human breast cancer. *Eur. J. Cancer* 2000;36:1038-42.
- 138 Puzone R, Savarino G, Salvi S, Maria Giovanna Dal Bello MG, Barletta G, Genova C *et al.* Glyceraldehyde-3-phosphate dehydrogenase gene over expression correlates with poor prognosis in non small cell lung cancer patients. *Mol. Cancer* 2013;12:97.
- 139 Zhou C-F, Li X-B, Sun H, Zhang B, Han Y-S, Jiang Y *et al.* Pyruvate kinase type M2 is upregulated in colorectal cancer and promotes proliferation and migration of colon cancer cells. *IUBMB Life* 2012;64:775-82.
- 140 Zhan C, Shi Y, Lu C and Wang Q. Pyruvate kinase M2 is highly correlated with the differentiation and the prognosis of esophageal squamous cell cancer. *Dis. Esophagus* 2013;26:746-53.
- 141 Li J, Yang Z, Zou Q, Yuan Y, Li J, Liang L *et al.* PKM2 and ACVR 1C are prognostic markers for poor prognosis of gallbladder cancer. *Clin. Transl. Oncol.* 2014;16:200-7.
- 142 Cuezva JM, Krajewska M, de Heredia ML, Krajewski S, Santamaria G, Kim H *et al.* The Bioenergetic Signature of Cancer: A Marker of Tumor Progression. *Cancer Res.* 2002;62:6674-81.
- 143 Lin P-C, Lin J-K, Yang S-H, Wang H-S, Li A-Y and Chang S-C. Expression of  $\beta$ -F1-ATPase and mitochondrial transcription factor A and the change in mitochondrial DNA content in colorectal cancer: clinical data analysis and evidence from an in vitro study. *Int. J. Colorectal Dis.* 2008;23:1223-32.
- 144 Cuezva JM, Chen G, Alonso AM, Isidoro A, Misek DE, Hanash SM *et al.* The bioenergetic signature of lung adenocarcinomas is a molecular marker of cancer diagnosis and prognosis. *Carcinogenesis* 2004;25:1157-63.
- 145 Domcke S, Sinha R, Levine DA, Sander C and Schultz N. Evaluating cell lines as tumour models by comparison of genomic profiles. *Nat. Commun.* 2013;4.
- 146 Bjorge L, Hakulinen J, Vintermyr OK, Jarva H, Jensen TS, Iversen OE *et al.* Ascitic complement system in ovarian cancer. *Br. J. Cancer* 2005;92:895-905.
- 147 Goodisman J, Hagrman D, Tacka K and Souid A-K. Analysis of cytotoxicities of platinum compounds. *Cancer Chemother. Pharmacol.* 2006;57:257-67.
- 148 Yang Z, Schumaker LM, Egorin MJ, Zuhowski EG, Guo Z and Cullen KJ. Cisplatin Preferentially Binds Mitochondrial DNA and Voltage-Dependent Anion Channel Protein in the Mitochondrial Membrane of Head and Neck Squamous Cell Carcinoma: Possible Role in Apoptosis. *Clin. Cancer Res.* 2006;12:5817-25.
- 149 Dwarakanath BS and Jain V. Targeting glucose metabolism with 2-deoxy-D-glucose for improving cancer therapy. *Future Oncology* 2009;5:581-5.
- 150 Sandulache VC, Skinner HD, Wang Y, Chen Y, Dodge CT, Ow TJ *et al.* Glycolytic Inhibition Alters Anaplastic Thyroid Carcinoma Tumor Metabolism and Improves Response to Conventional Chemotherapy and Radiation. *Mol. Cancer Ther.* 2012;11:1373-80.
- 151 Almeida MB, do Nascimento JLM, Herculano AM and Crespo-López ME. Molecular chaperones: Toward new therapeutic tools. *Biomed. Pharmacother.* 2011;65:239-43.

- 152 Guttman DM and Koumenis C. The heat shock proteins as targets for radiosensitization and chemosensitization in cancer. *Cancer Biol. Ther.* 2011;12:1023-31.
- 153 Beere HM. Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *J. Clin. Invest.* 2005;115:2633-9.
- 154 Chaiwatanasirikul KA and Sala A. The tumour-suppressive function of CLU is explained by its localisation and interaction with HSP60. *Cell Death and Dis.* 2011;2:e219.
- 155 Chun JN, Choi B, Lee KW, Lee DJ, Kang DH, Lee JY *et al.* Cytosolic Hsp60 Is Involved in the NF- $\kappa$ B-Dependent Survival of Cancer Cells via IKK Regulation. *PLoS ONE* 2010;5:e9422.
- 156 Ghosh JC, Dohi T, Kang BH and Altieri DC. Hsp60 Regulation of Tumor Cell Apoptosis. *J. Biol. Chem.* 2008;283:5188-94.
- 157 Ghosh JC, Siegelin MD, Dohi T and Altieri DC. Heat Shock Protein 60 Regulation of the Mitochondrial Permeability Transition Pore in Tumor Cells. *Cancer Res.* 2010;70:8988-93.
- 158 Abu-hadid M, Wilkes JD, Elakawi Z, Pendyala L and Perez RP. Relationship between heat shock protein 60 (HSP60) mRNA expression and resistance to platinum analogues in human ovarian and bladder carcinoma cell lines. *Cancer Lett.* 1997;119:63-70.
- 159 Li S-L, Ye F, Cai W-J, Hu H-D, Hu P, Ren H *et al.* Quantitative proteome analysis of multidrug resistance in human ovarian cancer cell line. *J. Cell. Biochem.* 2010;109:625-33.
- 160 Castilla C, Congregado B, Conde JM, Medina R, Torrubia FJ, Japón MA *et al.* Immunohistochemical Expression of Hsp60 Correlates With Tumor Progression and Hormone Resistance in Prostate Cancer. *Urology* 2010;76:1017.e1-.e6.
- 161 Glaessgen A, Jonmarker S, Lindberg A, Nilsson BO, Lewensohn R, Ekman P *et al.* Heat shock proteins 27, 60 and 70 as prognostic markers of prostate cancer *APMIS* 2008;116:888-95.
- 162 Xu X, Wang W, Shao W, Yin W, Chen H, Qiu Y *et al.* Heat shock protein-60 expression was significantly correlated with the prognosis of lung adenocarcinoma. *J. Surg. Oncol.* 2011;104:598-603.
- 163 Wintzell M, Hjerpe E, Avall Lundqvist E and Shoshan M. Protein markers of cancer-associated fibroblasts and tumor-initiating cells reveal subpopulations in freshly isolated ovarian cancer ascites. *BMC Cancer* 2012;12:359.
- 164 Jose C, Bellance N and Rossignol R. Choosing between glycolysis and oxidative phosphorylation: A tumor's dilemma? *Biochim. Biophys. Acta (BBA) Bioenerg.* 2011;1807:552-61.