Early Diagnosis of Epithelial Ovarian Cancer

Analysis of Novel Biomarkers

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Cover illustration:

Ultrasound image of **"Serous Ovarian Epithelial Cancer"**

by permission of my dear friend Dr. Berit Gull

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"To be or not to be?…

That is the question"

William Shakespeare-Hamlet 1602

To the memory of my parents

Kristján G. Jónasson and Þorgerður Ragnarsdóttir

ABSTRACT

Early Diagnosis of Epithelial Ovarian Cancer - Analysis of Novel Biomarkers

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Background: Majority of epithelial ovarian cancer (EOC) is detected in advanced stage with bad prognosis and high mortality. Reliable diagnostic markers are lacking, pre-cancerous lesions in the more aggressive tumors are not clearly defined, vague or unspecific early symptoms, and the localization of the ovaries, deep in the pelvis contributes to late diagnose. Heterogeneity, not only different type of histology, but also different intrinsic biology and behavior characterizes ovarian cancer. Invasive surgery with histological examination is needed to confirm the diagnosis. Less than 25% EOC are diagnosed early, when there is great possibility to cure and 5-year survival >90%, in contrast to 20-30% 5-year survival in late stage EOC. Thus, early detection is of utmost importance. Proximal fluids, like ovarian cyst fluid, are promising in the search for early markers. Cancer antigen 125 (CA125), the most used biomarker since 30 years, and a promising marker human epididymis 4 (HE4) have recently been approved by FDA to be used in the prediction of malignancy in women with a pelvic mass.

Aims: To explore ovarian cyst fluid as a source mining for new diagnostic biomarkers for EOC, and to validate the markers found together with CA125 *(Paper I-III);* and to evaluate the diagnostic performance of HE4 and CA125, to distinguish between benign cysts and EOC, and EOC divided into slow growing type I and the aggressive type II EOC *(Paper IV-V).*

Method: Cross sectional, observational, explorative, and diagnostic clinical studies, with prospective and consecutive collection of cystic fluid, blood and tumor tissue at the time of operation and retrospective analysis. Women with suspicious malignant pelvic cysts, already scheduled for operation at our clinic for tumor surgery were included. High throughput proteomic analyses were used for searching for novel markers, and selected proteins were validated with ELISA or immunoblot. *Paper I:* The cyst fluid proteome was mined with surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) mass spectrometry (MS) (n=192). *Paper II:* Enrichment of a selection of known cancer antigens to overcome high abundant proteins, and with focus on inflammation, was followed by Immunoprecipitation MS (n=38). Significantly

differently expressed chemokines were validated (n=256). *Paper III:* Serous cystadenoma (n=5) and serous adenocarcinoma (n=10) of different stages were analyzed with isobaric tag for relative and absolute quantification (iTRAQ), followed by immunoblot validation (n=68). *Paper IV-V*: HE4 and CA125 levels in plasma were analyzed with ELISA and Risk of Ovarian Malignancy Algorithm (ROMA) was calculated (n=393). Significant differences, receiver operator characteristics (ROC) area under the curve (AUC), cut-off levels, sensitivity and specificity were estimated with regard to malignancy, grade, stage histologic subtype and type I and Type II.

Results: *Paper I:* Combination of Apolipoprotein CIII and Protein C inhibitor had the best AUC (0.91) in cyst fluid, and improved by CA125 (0.94). Abundant proteins were a problem in the cyst fluid analyses. *Paper II:* Interleukin-8 and Chemoattractant Protein-I were highly significantly increased expressed in cyst fluid. Increased inflammatory response was present in early tumor development and earlier than in blood. *Paper III:* Two of 87 differentially expressed proteins in cyst fluid, with high significance and fold change, Serum Amyloid A-4 (SAA4) and astacin-like metalloendopeptidase (ASTL) were validated, and SAA4 was significantly increased in cyst fluid, but not in blood. *Paper IV***:** HE4 complemented CA125 in the diagnosis of ovarian cysts, especially in the premenopausal women. Sensitivity for ROMA at set specificity of 75% was highest in the postmenopausal cohort (87%). *Paper V:* HE4 and CA125 diagnosed the aggressive type II EOC most correctly (AUC 0.93), but the results were not acceptable in early stage type II (AUC 0.85) or in type I EOC (AUC 0.79) respective early type I AUC 0.73).

Conclusion: Ovarian cyst fluid is an excellent source for the search of novel biomarkers for early diagnosis of EOC. Early events are found near the tumor in the early phase, like the inflammatory response and later on in the peripheral circulation. HE4 complements CA125 in predicting malignancy in cystic ovarian tumors. The result from this thesis support, that EOC should be looked upon as several different diseases. Finding early markers that are specific for each histology subgroup will be the future challenge. Combination of such markers in a panel could improve the early diagnosis of EOC.

Keywords: EOC; ovarian adenocarcinoma; ovarian cyst fluid; pelvic mass; tumor biomarker; mass spectrometry; SELDI-TOF MS; iTRAQ;

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I. Ovarian cyst fluid is a rich proteome resource for detection of new tumor biomarkers
Kristjansdottir B, Partheen K, Fung ET, Marcickiewicz J, Yip C, Brännström M, Sundfeldt K
Clinical Proteomics, 2012 Dec 27; 9(1):1
doi:10.1186/1559-0275-9-14

II. Early inflammatory response in epithelial ovarian tumor cyst fluid
Kristjansdottir B, Partheen K, Fung ET, Yip C, Levan K, Sundfeldt K
Manuscript

III. Potential tumor biomarkers identified in ovarian cyst fluid by quantitative proteomic analysis, iTRAQ
Kristjansdottir B, Levan K, Partheen K, Carlsohn E, Sundfeldt K
Clinical Proteomics, 2013 Apr 4; 10(1):4
doi: 10.1186/1559-0275-10-4

IV. Evaluation of ovarian cancer biomarkers HE4 and CA125 in women presenting with a suspicious cystic ovarian mass
Partheen K**,** Kristjansdottir B, Sundfeldt K
Journal of Gynecologic Oncology, 2011 Dec 22; 4:244-252
doi: 10.3802/jgo.2011.22.4.244.

V. Diagnostic performance of the biomarkers HE4 and CA125 in type I and type II epithelial ovarian cancer
Kristjansdottir B, Partheen K, Levan K, Sundfeldt K
Gynecologic Oncology 2013 Jul 25; 131:52-58
doi:pii: 10.1016/j.ygyno.2013.07.094

ABBREVIATIONS

ARID1A	AT-rich interactive domain-containing protein 1 A
ASTL	astacin-like metalloendopeptidase
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BRCA1/BRCA2	breast cancer type 1/2 susceptibility protein
CA125	cancer antigen 125
COX2	cyclooxygenase-2
CT	computed tomography
CTNNB1	catenin-interacting protein 1
EOC	epithelial ovarian cancer
ERBB2 (HER2)	Avian erythroblastic leukemia viral homolog 2
FIGO	International Federation of Gynecology and Obstetrics
GAPDH	glyceraldehyde-3phosphate dehydrogenase
GROα	growth regulated alpha protein
GRP78	78 kDa glucose regulated protein
HE4	human epididymis protein 4
HIF	hypoxia-inducible factor
HNPCC	hereditary nonpolyposis colorectal cancer
HOX	homeobox (DNA sequence)
IDHC	isocitrate dehydrogenase 1
IL-8	interleucin-8
iTRAQ	isobaric tags for relative and absolute quantification
LC	liquid chromatography
LTQ	linear trap quadrupole
MAPK	mitogen-activated protein kinase
MCP-1	macrophage chemoattractant protein 1

MS	mass spectrometry
NFκB	nuclear factor kappa B
PAX	paired box gene
PCI	protein C inhibitor
PIKC3CA	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
RAF	rapidly accelerated fibrosarcoma
RAS/KRAS	rat sarcoma/ kirsten rat sarcoma viral oncogene homolog
S100 A8/A9	S100 calcium binding proteins A8/A9 or calgranulins A and B
SAA4	serum amyloid A4
SCX	strong cation exchange chromatography
SELDI-TOF	surface-enhanced laser desorption/ionization time of flight
SPARCL1	secreted protein, acidic and rich in cysteine-like 1
STAT3	signal transducer and activator of transcription 3
TMT	tandem mass tag
TP53	tumor protein p53
TPI1	triosephosphate isomerase1
TTR	transthyretin
VEGF	vascular endothelial growth factor
YWHAZ	triosine 3-monooxygenase/tryptophan 5-mono-oxygenase activation protein, zeta polypeptideh factor

INTRODUCTION

HISTORICAL PERSPECTIVES

About 400 BC the Greek physician Hippocrates, introduced the term carcinoma from the Greek word karcinos, he described cancer as crablike in its spread through the body and in its persistence. About AD 200 the Greco-Roman physician Galen of Pergamum attributed the development of cancer to inflammation, but it was not until the end of the 18th century systematic studies of cancer started. A report in the year 1745, suggested that hereditary factors are involved in the causation of cancer, and 1761, the English physician John Hill, was the first to point out that substances found in the environment are related to cancer development, in the relationship between tobacco snuff and nasal cancer [1].

In the early 19th century the German physiologist Johannes Peter Müller (1801–58) and the pathologist Rudolf Virchow (1821–1902) could with the help form the microscope show that cancerous tissue was made up of cells [1], and 1863 Rudolf Virchow realized that inflammation is an important factor in initiation of cancer, he noted leucocytes in neoplastic tissues and made a connection between inflammation and cancer [2].

The knowledge about cancer genesis has culminated since 1960. Cancer is a disease in which normal cells change to dysplastic cells, that grow uncontrollably, and form a mass of cells called a tumor. Cells become cancer cells because of damage to DNA. In 1988, Professor Vogelstein at John Hopkins University in USA, a pioneer within genetics, proposed that “cancer is caused by sequential mutations of specific oncogenes and suppressor genes” [3]. People can inherit damaged DNA, but most DNA damage is caused by mistakes that happen while the normal cell is reproducing or by some factors in the

environment [4]. Complicated networks are interacting in cancer, including the genome, transcriptome, proteome, metabolome and inflammation.

The discovery of one of the oldest biomarkers carbohydrate antigen 125, or cancer antigen (CA125) was made 1981by Robert C Bast, JR and his colleagues at MD Anderson Cancer Center in Texas USA, by using an antibody that recognizes CA125 in ovarian cancer [5]. A huge number of novel biomarkers have been presented as promising in the diagnosis of ovarian cancer, but CA125 is still in the top and the currently most used in clinical practice.

EPITHELIAL OVARIAN ADENOCARCINOMA

Epithelial ovarian adenocarcinoma (EOC) is the most deadly of the gynecologic tumors. Although there have been advances in surgery and chemotherapy, the survival rate for this disease remains low [6]. The majority of cases diagnosed with EOC have already spread to the upper abdomen with omental cake and peritoneal metastasis. Noninvasive diagnostic procedures are lacking, therefore invasive surgery is needed followed by pathologic anatomic diagnosis (PAD) to confirm the definite diagnosis. The principle cause of the poor survival rate for the patients is diagnosis at a late stage, when a radical surgery is not possible or unsuccessful [7]. If diagnosed in stage I, when the cancer is confined to the ovaries, the 5-year survival is over 90% after optimal surgery, this in contrast to less than 30% when the cancer had spread to the abdomen or outside the abdomen (stage III or IV). Unfortunately less than 25% of cases are diagnosed early, and as a consequence the overall 5-year survival in EOC is less than 50%. Early diagnosis means possibility to cure with surgery and continuous life without sequel from expensive cytotoxic drugs and extensive operations [8, 9]. Correctly diagnosed EOC followed by treatment at the right level of care will improve the survival of women and decrease the number of unnecessary

extensive operations in the benign cases, which leads to improved quality of life for all women with ovarian tumors [10].

BIOLOGY

During embryonic development, the mesoderm build up normal ovary and the urinary tract, and the Müllerian duct forms the fallopian tubes, uterus, cervix and two thirds of the proximal vagina. Three major cell types build up the ovary, germ cells, sexcord-stroma cells and epithelial cells. About three % of malignant tumors develops from germ cells derived from the endoderm and differentiate into oocytes; seven % arise from sexcord-stroma cells with endocrine and interstitial cells that produce estrogen and progesterone; and approximately 90% of primary ovarian cancer is classified as epithelial. Germ cells tumors include dysgerminoma, yolk sac tumor, embryonal carcinoma, choriocarcinoma and teratoma. Sex cord-stomal tumors consist of granulose and theca cells-, stromal fibroblasts- and steroid cells tumors. Germ cells tumors are diagnosed more often in the first two decades of life, whereas sex cord/stromal tumors are more common in adult women [11]. Until recently, EOC has been considered to arise from the monolayer of epithelial cells covering the ovarian surface (OSE), invaginations and subserosal ovarian cysts [12-15]. Now there is growing evidence that EOC may also arise from Müllerian derivatives including the distal fallopian tube and the uterus, and the peritoneal tumors of ovarian type are classified as ovarian primaries [16-18].

EPIDEMIOLOGY

The incidence of EOC varies around the world, is highest in Northern, Central and Eastern Europe, followed by Western Europe and Northern America, and

lowest in Africa and parts of Asia [19]. About 10-15 % of cases have hereditary susceptibility and the vast majority due to *BRCA1* and *BRCA2* germline mutations, and the rest are supposed to be sporadic [20, 21]. Ovarian cancer accounts for about three percent of all cancer cases in women and is the fifth leading cause of cancer death in women, with nearly 225 000 new cases and 140 200 deaths worldwide in 2008 [6].

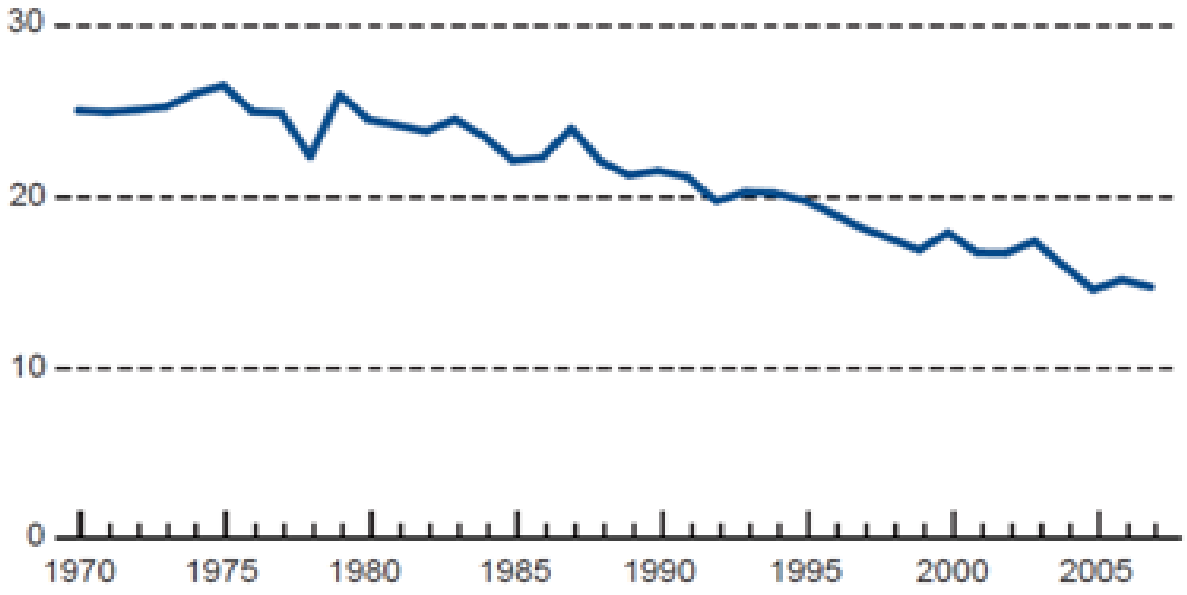

Figure 1. Incidence for ovarian cancer. Number of cases per 100 000 women each year, 1970- 2007, National Board of Health and Welfare Sweden.

The incidence of EOC increases with age and peaks at a rate of 61.5 per 100 000 women aged 75 to 79 years. The overall risk of a primary ovarian neoplasm being malignant increases from 13% in premenopausal women to 45 % following menopause [22]. The median age of diagnosis is 63 years of age. Lifetime risk of getting ovarian cancer is 1 in 72 and the risk to die in the disease is 1 in 95. Women with *BRCA1* mutation have estimated live-time risk of 40-50% of getting EOC at a mean age of 50-55 years, and if *BRCA2* positive

the risk is 10-20% at a mean age of 55-65 [20]. The age-standardized incidence rate has decreased about two percent each year under the last 20 years [23] (Figure 1).

In Sweden 835 new cases were diagnosed in the year 2001, when we started collecting our samples, compared to 758 and 676 new cases 2010 respective 2011. In the Vest region of Sweden there were 147 compared to 108 new cases respective years. The mortality is still high and 645 respective 563 women died from the disease 2010 and 2011 in our country. The estimated world age-standardized incidence rate (ASR) for the more developed countries was nine per 100,000 and five per 100,000 women for the less developed countries in 2008 [6].The widespread use of oral contraceptives [24], and the high number operations done on benign ovarian tumors diagnosed with vaginal ultrasound, and hysterectomies for benign indication as well, may contribute to the decrease of new cases in the more educated countries. However, early childbearing, multiparty and long-lasting breastfeeding periods, common in developing countries may prevent women from EOC [25-27].

HISTOLOGY - GRADE

Ovarian tumors of epithelial origin are heterogeneous group of neoplasm [28]. According to the World Health Organization (WHO), EOC is classified due to cell type into the four major histotypes, serous, endometrioid, mucinous and clear cell, and the more uncommon transitional cell, squamous cancer and undifferentiated tumors. The malignant tumors are further divided into three grades (G1-3) well (G1), moderately (G2) and poorly differentiated (G3) according to the architectural features, set up by the International Federation of Gynecology and Obstetrics (FIGO) [29]. However, based on histopathology, immunohistochemistry and molecular genetic alterations, EOC is at least five

diseases, the most common high-grade serous (70%), the more uncommon low-grade serous (<5%), endometrioid (10%), clear cells (10%) and mucinous carcinomas (3%) [28]. In Sweden about 10-20% of all epithelial malignant tumors are classified as borderline, with serous (55%) and mucinous (40%) as the most common histology. Borderline tumors are more often seen in women of younger age, with 55 years as a median comparing to 63 years in invasive EOC [30]. Borderline tumors have higher epithelial proliferation and nuclear atypia than the benign tumors. However, in contrast to carcinomas, borderline tumors does not have any stromal invasion and are more like a pre-stage of EOC [31]. The macroscopic as well as ultrasound features of borderline tumors overlap with both the benign and invasive tumors and these tumors can evolve to cancer [32, 33]. Regular follow-up is essential for early detection of recurrence or development of invasive disease if ovarian sparing operation has been performed [34].

STAGING

Cancer staging is a description of the extent of the cancer. Cancer stages are defined by the growth of the primary tumor and its spread to other parts of the body. Clinical staging is based on tests done before surgery and pathologic staging on tests of tissue removed during the staging operation. An appropriate systematic surgical staging performed at the initial surgery is of great importance to find out how widespread the cancer is for planning further therapy. Microscopic examination, with assessment of specific histology type, grade and extent of disease is critical for predicting tumor behavior and for deciding the best therapeutic approach [35]. EOC is staged I-IV according to FIGO (Figure 2, Box 1). EOC confined to one or both ovaries is classified as stage I, spread to the uterus or other nearby organs in pelvis stage II, spread to the lymph nodes or abdominal lining is stage III, and spread to distant organs

such as the lung or liver is classified as stage IV EOC. Liver capsule metastasis is stage III, metastasis of liver parenchyma stage IV, and pleural effusion must have positive cytology to be classified as stage IV [29].

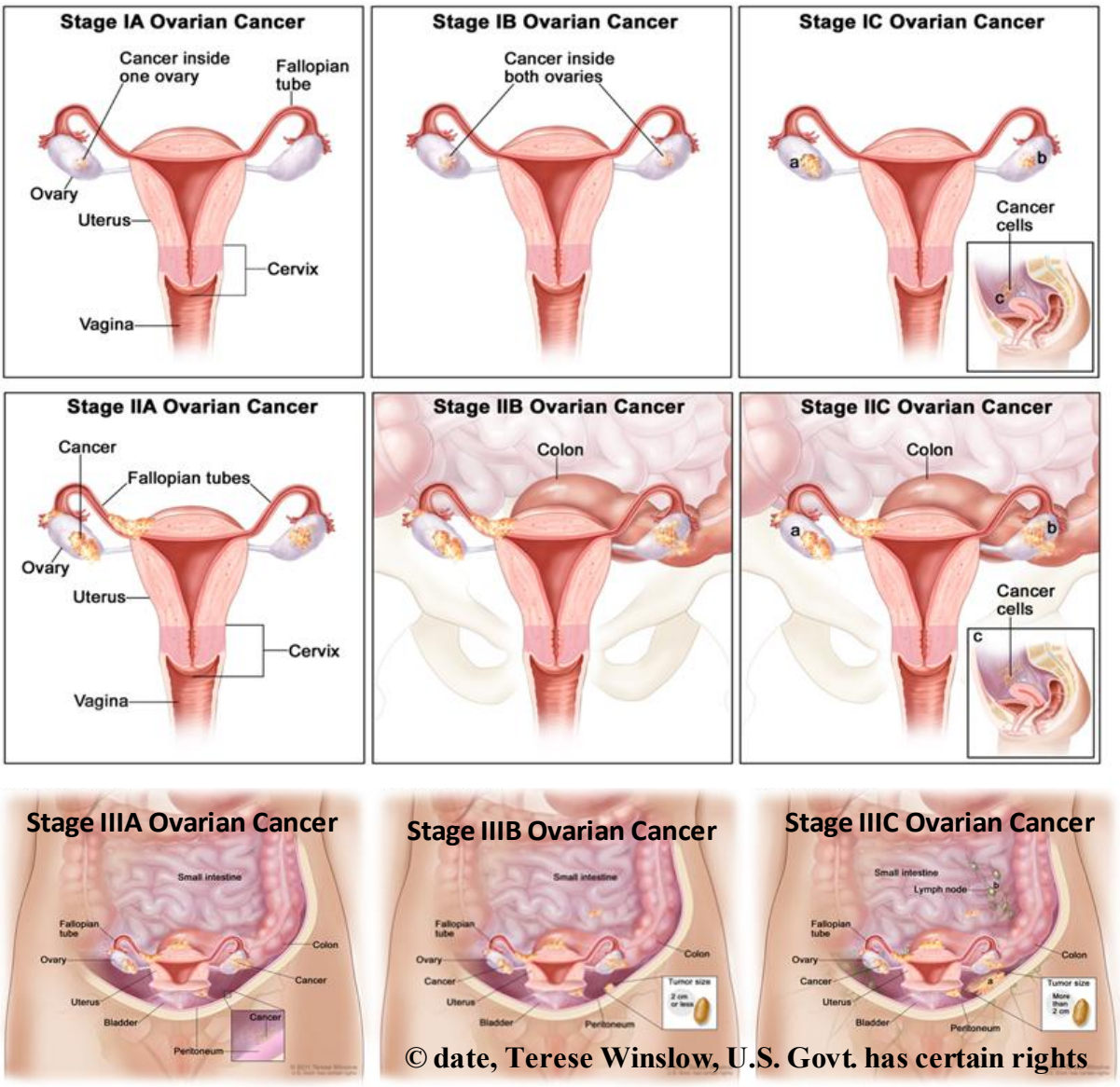

Figure 2. Staging of ovarian cancer according to FIGO (with permission from Terese Winslow)

Ovarian cancer spreads by direct contact with other tissues in the pelvis, by exfoliated tumor cells transported through the fluid in the abdominal cavity and

pleura, by invading lymph channels to spread through lymph nodes, and more seldom through the blood vessels to give metastases in other organs [36].

Box 1. Staging of EOC according to FIGO.

STAGING OF OVARIAN CANCER

Stage I — limited to one or both ovaries
- IA involves one ovary; capsule intact; no tumor on ovarian surface; no malignant cells in ascites or peritoneal washings
- IB involves both ovaries; capsule intact; no tumor on ovarian surface; negative washings
- IC tumor limited to ovaries with any of the following: capsule ruptured, tumor on ovarian surface, positive washings

Stage II — pelvic extension or implants
- IIA extension or implants onto uterus or fallopian tube; negative washings
- IIB extension or implants onto other pelvic structures; negative washings
- IIC pelvic extension or implants with positive peritoneal washings

Stage III — peritoneal implants outside of the pelvis; or limited to the pelvis with extension to the small bowel or omentum
- IIIA microscopic peritoneal metastases beyond pelvis
- IIIB macroscopic peritoneal metastases beyond pelvis less than 2 cm in size
- IIIC peritoneal metastases beyond pelvis > 2 cm or lymph node metastases

Stage IV — distant metastases to the liver or outside the peritoneal cavity

The biological behavior of EOC is unique in its early dissemination of detached cancer cells that are transported physically by peritoneal fluid. The tumor implants invade the mesothelial cell layers lining the abdominal cavity, and at the surface of bowel, liver and other organs in the abdomen, but interestingly rarely invade deep into the peritoneum [37].

ETIOLOGY

The etiology of EOC is complex and not clearly defined. The genome encodes proteins that control the function, growth, and division of cells. DNA damage and mechanisms to repair exist in order to decrease the likelihood of genetic mutation and cell transformation. In addition, the immune system is designed to recognize early changes in carcinogenesis and destroy cancerous cells to keep the balance in cell proliferation and cell death. Accumulation of disruptions in these homeostatic control mechanisms can lead to uncontrolled proliferation and cancer [4]. The six hallmarks of cancer introduced by Hanahan et al. include sustaining proliferative signaling, evading growth suppressors, resisting cell death, unlimited replication capability, inducing angiogenesis, and activating invasion and metastasis [38]. Cancer related inflammation is postulated to be the seventh hallmark, with smoldering inflammation in the tumor environment, that promotes genetic instability and accumulation of genetically altered cancer cells [39]. The interplay between milieu and genes is a fundamental mechanism in cancer, with epigenetic events like DNA methylation and histone modification as a link [40]. Ovarian carcinogenesis, as in most cancers, involves multiple genetic alterations and molecular changes, with important key pathways related to chronic inflammation. The crosstalk and signaling interactions between cancer cells and their supporting stroma evolves during the tumor development [26, 41, 42].

INFLAMMATION AND EPITHELIAL OVARIAN CANCER

Chronic inflammation underlies the progression of ovarian cancer [25, 39]. Pathways that link inflammation and cancer have been identified, an intrinsic pathway driven by genetic events (*RAS*, *MYS*, and *TP53* mutations) and an

extrinsic inflammatory driven pathway. The mitochondria, the organelle that supply energy to the cells, have an important role in coordinating life and death signaling in the convergence of these pathways, promoting inflammation by producing free radicals and activating transcriptions factors, such as nuclear factor kappa B (NFκB), signal transducer and activator of transcription 3 (STAT3) and hypoxia-inducible factor (HIF) to orchestrate the production of inflammatory mediators and generate cancer related inflammation [43]. Inflammatory responses, with communication between stromal microenvironment and the epithelial compartment, play a role at all stages of cancer development, including initiation by generating genotoxic stress, promotion by inducing cellular proliferation, malignant conversion, and metastasis by enhancing angiogenesis and invasion [44-46]. The immune system has a dual function which is called cancer immunoediting; both protecting the host against tumor development and as well as promoting the tumor to grow [47]. The response from the body to cancer is not an unique mechanism, actually there are many parallels with inflammation and wound healing, "a wound that does not heal " [2]. Use of anti-inflammatory drugs like aspirin has been related to reduction in the long-term risk of several cancers and the risk of distant metastasis, which further supports the relation between inflammation and cancer [48]. Key features of cancer-related inflammation include leukocyte infiltration, prominent tumor associated macrophages (TAMs), vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX2), cytokines (small cell signalling molecules) such as tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), interleukin-1 (IL-1), IL-6 (CXCL6) and chemokines (chemotactic cytokines) like IL-8 (CXCL8), growth regulated alpha protein (GROα; CXCL1) and Monocyte chemoattractant protein-1 (MCP-1; CCL2). MCP-1 attract TAMs, which are the major players in the cancer related inflammation, and enhances angiogenesis and tissue remodelling [49]. The interaction of the cytokines is strongly regulated with positive and negative

feedback aiming to maintain balance in the immune control. Ability of malignant cells to interact with and influence their environment is critical for the development of cancer, and chronic inflammation coordinate a cancer supporting microenvironment [50, 51].

Incessant ovulation and the gonadotropin theory, with exposure to follicle stimulating hormone (FSH) and luteinizing hormone (LH), have been considered to play a major role in ovarian cancer development [25, 52]. Ovulation is an inflammatory process which involves repeated minor trauma of the ovarian surface and exposure to estrogen rich follicular fluid, cytokine release, influx of inflammatory cells to the ovarian stroma [53]. Every month the epithelial cells are affected by increased oxidative stress, production of reactive oxygen and nitrogen species (ROS and RNS), cell damage, elevations of cytokines, proteases and prostaglandins, and the subsequent repair mechanisms with epithelial to mesencymal transition (EMT) that place the cells at increased risk of developing mutations, and this repeated activity may initiate oncogenesis by causing DNA damage in adjacent cells [50, 54]. Even the fimbria of the fallopian tube is exposed to iron induced oxidative stress, by floating in the bloody peritoneal fluid, derived from retrograde menstruation [42]. Many of the inflammatory cytokines and chemokines activated during ovulation have been found to exhibit overlap with that described in EOC [55-57]. The TGFβ- family of multifunctional cytokines acts as tumor suppressors by inhibition of cell proliferation in normal tissue and in early tumorigenesis, but during oncogenesis switches its role to promote progression by interfering with EMT [54, 58]. IL-8 and anti-IL-8 antibody are present in serum from EOC patients [59], and increased secretion of VEGF, IL-6, GROα and IL-8 promoting cancer growth have been noted in the oncogenic RAS-signaling, present in one third of human cancers [60]. IL-6 is a growth promoting and anti-apoptotic factor, found with high plasma levels in advanced stage EOCs and is known to correlate with poor prognosis [61]. The epidermal growth factor receptor (EGFR; HER1), a member

of erythroblastic leukemia viral oncogene family (ERBB-family), (HER 1-4) of receptor tyrosine kinase, have a key role in the development of a normal follicle [62], and is also involved in activation of multiple signaling cascades, that cause growth and invasion of tumor cells, and has been related to poor outcome, among other factors via increased co-expression of IL-6 and plasminogen activator inhibitor-1(PAI-1) [63].

Age is in general a risk factor for cancer, with changes in redox status and oxidative stress induced inflammatory reactions that lead to overall deregulation or acquired dysfunctional immunity, called immunosenescence [64, 65]. A typical feature of aging is a chronic, low-grade inflammatory status, an inflammatory aging with changes in the cytokine profile towards a pro-inflammatory condition, with increase of some chemokines; RANTES (CCL5), macrophage inflammatory protein 1 (MIP-1; CCL-3), IL-8, and MCP-1. GROα has been evaluated in this process and is supposed to be a cellular signal activated by extracellular oncogenic signals in aged epithelial cells, and may be a novel diagnostic marker for age-related pathology, including cancer [66]. Endometriosis and pelvic inflammatory disease are related to both acute and chronic inflammation [26, 67]. Obesity is as well associated with a chronic state of low-grade inflammation [68]. Increased concentrations of fatty acids, inflammatory cytokines and an influx of immune cells together with adipokines (cytokines secreted by adipocytes, which are regulators of metabolism and immunity, produced by the white adipose tissue contributes to the local inflammatory milieu in adipose tissue [69, 70]. The coagulation pathway is involved in cancer related inflammation, with IL-8 as the linking point. Coagulations factors promote not only formation of blood clots but also tumor cell proliferation, angiogenesis, invasion and metastasis [71]. Fibrin formation can be inhibited by membrane associated endothelial protein C receptor (EPCR) via activated protein C (APC) in ascites and promote fluid expansion. In blood

soluble EPCR can cause a hyper-coagulation state associated with malignancy [72].

ORIGIN AND PATHOGENESIS

There is uncertainty surrounding the site of origin of EOC. Surface epithelium of the ovary (OSE), epithelial invaginations, inclusion cysts inside the ovaries, and dysplastic lesions from the fallopian tube and the uterus has been suggested to be the origin of EOC [13, 15, 31]. Auersperg state that both ovarian epithelium and the oviduct originate in the embryonic pleuripotential mesothelial coelomic epithelium and are therefore able to produce similar tumors [14]. Dysplasia of OSE differentiates into epithelia resembling Müllerian duct derivates, serous tumors will be like the fallopian tube epithelium, endometrioid tumors similar to endometrium in the uterus, and mucinous tumors like epithelium of endocervix [12, 13]. Homebox (HOX) genes are strongly expressed in ovarian cancer, and not in normal epithelium. These genes contain transcription factors that determine cellular identity, and play a key role in the embryonic development, were the *HOXA9* becomes expressed in the fallopian tubes, *HOXA10* in the developing uterus, *HOXA11* in the lower uterine segment and cervix and *HOXA13* in the upper vagina. It is thought that appropriate expression of these genes is an early step in neoplasia of the ovarian epithelium, as they induce aberrant epithelial differentiation [73].

During the past 10 years more evidence has led to a paradigm shift in the process of etiology and pathogenesis in the framework of different origins that may develop after distinct pathways, from the ovary, tube, peritoneum and endometrium [31]. Immunohistochemical, morphologic and molecular genetic analysis proposes that EOC is more like metastases [16, 17, 74-80]. Serous fallopian tube carcinoma was found more often in women harboring BRCA1

and BRCA2 mutation than in sporadic cases [81]. A suggested candidate precursor lesion for EOC, called serous tubal intra epithelial carcinoma (STIC), is present in the non-ciliated epithelium of the distal fimbria of the fallopian tube. STIC is then supposed to implant onto the ovarian and /or peritoneal surfaces [79, 82], and after an occult period will develop into fast growing high-grade serous cancer. P53 signature, an early alteration in p53 function, is proposed to occur before STIC [16, 83-85]. Different gene alterations have been discovered in the oviduct, as secretory cell outgrowths (SCOUT), increased in frequency as a function of older age and serous cancer [86]. The p53 signature and its malignant counterpart STIC have proposed the link between the fallopian tube, peritoneal and ovarian serous carcinomas. Supporting this theory is that the pared box gene 8 (PAX8), a marker of Müllerian-type epithelium was found expressed in high-grade serous cancer, but not in OSE, whereas calretinin, a marker of mesothelioma and OSE was not detected in EOC or in the tube [18]. Complexity of regulation on a genomic level with DNA repair mechanisms, as well as NOTCH pathways (an evolutionarily conserved pathway that regulates cell-fate determination during development and maintains adult tissue homeostasis) and the regulatory network of the transcription factor FOXM1 (forkhead box protein M1) - signaling are involved in the high-grade serous cancers [87].

The low-grade serous cancers are distinct tumors that might develop from more clearly defined lesions, such as cystadenom, adenofibroma and borderline tumors, in more indolent stepwise manner [4, 13, 18]. These tumors evolve from OSE, invaginations and inclusion cyst inside the ovaries via alteration in the RAS-RAF signaling pathway, which is responsible for normal cell growth, differentiation and survival, due to mutation in *KRAS* (GTPases, molecular switches for a variety of cellular signaling events) and *BRAF* (a kinase cascade, that send a signal from the surface of the cell to the DNA in the nucleus) [13].

The recent literature suggests that ovarian low-grade serous tumors and their non-invasive implants, ovarian epithelial inclusion glands and endosalpingiosis (fallopian tube-like epithelium is found outside of the fallopian tube) might arise from the fallopian tube rather than through Müllerian metaplasia of the OSE [88]. Low-grade endometrioid and clear cell cancer may arise from endometriosis via retrograde menstruation, with multi-factorial etiology including genetic, hormonal and immunological factors [18, 89, 90]. AT-rich interactive domain-containing protein 1 A (*ARID1A),* tumor- suppressor gene mutation frequently found in these lesions can be an early event in the transformation into cancer [91]. Mutation of the catenin-interacting protein 1 (*CTNNB1*) the gene that encodes β-catenin (regulating cell-cell adhesion and gene transcription), and phosphatase and tensin homolog (*PTEN*) a tumor suppressor gene (involved in the regulation of the cell cycle, preventing cells from growing and dividing too rapidly) are found primarily in low-grade endometrioid, whereas phosphatidylinositol 3-kinase (*PIKC3CA*) oncogene mutations characterize clear cell cancer [92]. Approximately 80% of all mucinous tumors are benign, and most of the remainder borderline. Mucinous tumors harbor high frequency of *KRAS* mutations; these tumors often show gastrointestinal differentiation and have also been related to the endocervix. Mucinous- and transitional cells tumors (Brenner) were recently reported to develop from transitional epithelial cells located near the tubo-peritoneal junction [93]. However, the majority of invasive mucinous tumors are metastases to the ovary, often from the gastrointestinal tract including colon, appendix or stomach, if appropriate examined only 3-4% are left as ovarian carcinomas [28].

DUALISTIC MODEL - TYPE I AND TYPE II

Kurman et al. proposed a novel tumor origination and progression model, based on morphological and molecular genetics, dividing EOC into type I and type II tumors [18, 31, 75, 94, 95]. This simplistic approach indicates that the two tumor types develop via two different pathways, slow-growing type I and rapid-growing highly aggressive type II tumors (Table 1). Low-grade serous, low-grade endometrioid, all clear cell, mucinous, and transitional (Brenner) carcinomas are classified as type I, were each histological type has a distinct molecular profile. Type II tumors are the most common, and include high-grade serous, high-grade endometrioid, undifferentiated carcinoma and malignant mixed mesodermal tumors or carcinosarcomas [18].

Table 1. Pathogenesis of slow-growing Type I and aggressive Type II EOC.

EOC	%	Precursor lesion	Gene mutation	Genom	Tempo
Type I	**25**	Ovary; Cystadenoma → → → → Borderline → LGSC	KRAS, BRAF, ERBB2,		
		Tube; Endosalpingiosis → LGSC			Slow Step-wise
		Uterus; Endometriosis →→ Clear Cell and LG-Endometrioid	PIKC3CA, PTEN, ARIDA1A β-catenin, PTEN, ARID1A	Stable	
		Cervix, G-I, Ovary, Tube; → → Borderline → Mucinous	KRAS		
Type II	**75**	Tubal fimbria/ovarium; STIC →HGSC	TP53, BRCA1-2	Chaotic	Fast
		? → HG-Endometrioid	TP53		

LGSC, low-grade serous carcinoma; HGSC, high-grade serous carcinoma; G-I, gastro-intestinal; STIC, serous tubal intraepithelial carcinoma

Low-grade type I carcinomas exhibit low-grade nuclei with infrequent mitotic figures. They evolve in a slow stepwise process from defined benign or borderline lesions to invasive cancer. These tumors harbor frequent somatic

mutations, encoding mismatch repair proteins and signaling proteins governing cell proliferation, such as *BRAF, KRAS, β-catenin*, *PTEN* or *ERBB2 (HER2)* genes, but lack *TP53* mutations [96].

Type I tumors are in general larger, in earlier stages and in younger women when diagnosed compared to type II EOC, and consequently type I EOC have a better prognosis [97, 98]. Type II tumors are more aggressive and genetically highly instable with frequent mitotic high grade nucleus, with increased expression of Ki-67 (a cellular marker of proliferation), and estrogen receptor (ER) is expressed in circa 75%. Majority of the tumors have *TP53* mutation, and almost half of the cases have mutation or dysfunction of *BRCA1/2* (10-20% have mutation of *BRCA1/2* and 10-40% hypermetylation or dysfunction of BRCA1) [87]. These aggressive tumors account for 75% of all EOC, and are responsible for 90% of death in the disease [99].

RISK FACTORS

Multiple endogenous and exogenous risk factors have been shown to influence ovarian cancer development [26]. Advancing age is one of the major risk factors and accumulated genetic damage is likely involved [100]. Cellular senescence (CS) could have a role in aging and age-related diseases [64]. Hereditary factors are involved in about 10-15% of cases, with history of earlier breast cancer, hereditary breast and ovarian cancer (HBOC), resulting from a *BRCA1* or *BRCA2* gene mutation and hereditary nonpolyposis colorectal cancer (HNPCC) gene, or *TP53* mutation. Carriers of *BCRA1*or *BRCA2* mutation have increased lifetime risk of ovarian cancer up to 50-60% respective 25%, and are estimated to cause 65-85% of all heredity cases [101, 102]. HNPCC or germline mismatch repair (MMR) gene mutations *(MLH1, MLH2, MSH6)* linked to Lynch syndrome accounts for 10-15% of heredity cases and have an increased lifetime

risk of 8% for EOC, and highest for those with *MLH2*, *MSH6* mutation. These women tend to be at younger age and with non-serous tumor presenting in an early stage [102]. “Incessant ovulation theory”, introduced by Fathalla 1971, more ovulations over a lifetime increases the risk of getting EOC by creating an unfavorable micro-environment. Poor reproductive history with long duration, low parity, early menarche, late menopause, and infertility, has been associated to increased risk of EOC [25-27, 103]. Endometriosis defined as endometrial implants outside the uterus, transported via retrograde menstruation, usually present on ovaries and peritoneum in the pelvis. Acute and chronic inflammation in combination with immune dysfunction is acting in endometriosis. Several characteristics are shared with invasive endometrioid and clear cell cancer; both harbor similar cytokines and genetic defects, and have a capacity to spread distantly [18, 26, 89, 90].

The observation that the incidence of ovarian cancer increases after menopause, and the increase in gonadotropin levels at the same time generated the “Gonadotropin theory” 1975 by Stadel [104]. Hormonal effect with increased estrogenic stimulation of the OSE as a result of excessive gonadotropin (FSH, LH) secretion, related to menopause, ovulation, or infertility therapy and hormone replacement therapy (HRT) has been implicated as possible risk factor for EOC [52]. Higher levels of androgens, which are increased in menopausal or obese women and seen among women with polycystic ovarian syndrome (PCOS), were associated with an increased risk of ovarian cancer, whereas progesterone had protective effect [52, 105]. In a populations study (n=29 000) in Sweden, obesity was related to significant excess risk for endometrial- (standard incidence ratio (SIR) 2.9), cervix- (1.4) and ovarian cancer (1.2) [106]. Pelvic inflammatory disease (PID) was coupled to increased risk in a study of 200 000 women in Taiwan [67]. Local inflammation like asbestosis and talc exposure has also been related to EOC [26].

Risk factors are found to have different effects in the different histological types of EOC. With longer use of oral contraceptives the risk decreased with 20% for each 5 years, and after 15 years the risk was halved, and the protective effect was on all types of EOC except for mucinous cancer [24]. In a recent study (n=849 EOC/n=169 391 healthy women under mean 5.1 years), HRT was associated to increased risk of serous and endometrioid EOC (RR=1.3), but to decrease in risk for the mucinous type (RR=0.37). In the same study, obesity (BMI >30) was found to be related to increased risk in endometrioid EOC (RR=1.67), similar to endometrial cancer of the uterus, but decreased risk was found for serous, mucinous and clear cell cancer [107]. Infertility itself is a risk factor for EOC, and it is still debated if fertility drugs will increase the risk for EOC or not [108]. Physical activity [109], smoking [110] dietary fat [111], and other life style factors may also affect the risk. Prevention of ovulation have been considered as protective against ovarian cancer; oral contraceptives, multiparty and long lactation periods, as well as obliteration of the tubes by tubal ligation, prophylactic oophorectomy and hysterectomy [26, 27].

DIAGNOSIS

Adnexal masses represent a spectrum of conditions from gynecologic and non-gynecologic sources, and may be benign, borderline or even highly malignant. The success of treatment for EOC depends on early diagnosis and the extent and quality of the primary surgery [7]. Therefore, initial detection and evaluation of an adnexal mass requires a high index of suspicion. Approximately 20% of women will be detected with an adnexal mass, 5 - 10% of women will have surgery for an ovarian neoplasm and only 13 - 21% of these masses will be malignant [112]. The localization of the ovaries deep in the pelvis, gives the tumor a possibility to grow without specific symptoms until spread to the upper

abdomen, with metastases to the omentum, peritoneal carcinomatosis, and ascites [113]. Common early symptoms are not specific for ovarian cancer and therefore often ignored. However, symptoms that are new and occur frequently may distinguish cancer cases from healthy women. Goff et al. introduced the "symptom index" (SI), including pelvic/abdominal pain, urinary urgency/frequency, increased abdominal size/bloating, and difficulty eating/feeling full. The SI was considered positive if one or more of the symptoms were currently present for less than one year and occurred more than twelve days per month, and the index could be of help in finding women at risk of EOC. Majority (80%) of the women diagnosed with advanced disease, and more than half (57%) of the woman diagnosed with early stage tumor had symptoms before diagnosis [114]. Combining CA125 to the SI has been reported to improve the detection of early stage EOC, and adding HE4 to the combination further improved the diagnosis with a sensitivity of 84% and a specificity of 98.5%, if any two of the variables were positive [115, 116]. Patient and doctors delay is a problem. Acute abdominal pain, abrupt distension of the abdomen and difficulties of breathing is too often a cause of first visit to the hospital, then already in late stage EOC [117, 118].

Not only early diagnosis of pelvic cysts, but also an accurate diagnosis prior to surgery and the type of surgery preformed is of importance to optimize the prognosis for the women with EOC [7]. Usually, a combination of a patient´s medical history, clinical examination, imaging data such as ultrasound, thorax-abdominal computed tomography (CT) and magnetic resonance imaging MRI results, and to some extent tumor marker profile are used to differentiate between benign and malignant ovarian tumor. Gastro-intestinal (GI) evaluation is done if clinically indicated. Appropriate anamnesis with special focus on symptoms, and risk factors like age, menopause status, reproductive history (parity, contraceptives), ovarian or breast cancer heredity and earlier history of breast cancer is of value [26]. For the younger patients (< 30 year), tumor

markers such as human gonadotropin (hCG), and alpha-fetoprotein (AFP) should be controlled regarding germ cell tumors, even though the markers are not specific in the pediatric population [119].

Adnexal masses with complex or solid morphology seen on ultrasound are more significantly related to malignancy, and unilocular ovarian cysts or cysts with thin septa but without solid component are most often benign [120]. Pattern recognition on ultrasound by an experienced examiner is reported superior to serum CA125 for discrimination between benign and malignant adnexal masses [32, 33]. Ultrasound and CT are good instruments to use in the evaluation and planning of therapy, before surgery or chemotherapy, when a pelvic mass is already a factum [33, 120]. We need other tools to detect preclinical early lesions in the high-grade EOC. Specific tumor markers, that could indicate the presence of early disease preferably before symptoms are urgently needed [121].

TREATMENT AND PROGNOSTIC FACTORS

The golden standard in treatment of EOC today is surgical resection or debulking surgery, followed by platinum-based chemotherapy [35]. To treat the patients at a right level of care can be crucial for their future live [7]. The benign tumors can be treated with surgery or observation at the local hospital, and the patients with EOC should be referred to tertiary center for evaluation and expertise treatment to enhance prognosis. [7, 10, 35, 122]. Optimal staging and primary surgery aiming for complete resection of all visible tumors is the goal. The basis of cytoreductive surgery is considered to be hysterectomy, bilateral salpingo-oophorectomy and infracolic omentectomy and can be supplemented with extirpation of other organs that are affected by tumor, such as peritoneum, bowel, lymphatic nodes, gallbladder, liver and spleen if necessary to remove all visible tumors [123]. However careful evaluation of patients with stage III C or

IV EOC should be done before deciding if primary surgery is feasible, neoadjuvant chemotherapy is maybe a better option in some cases [124]. The age of the patients, performance status and other co-morbidities are of importance as well as the disease burden, location of metastatic sites, and stage in the assessment if surgery will improve the patients prognosis or the quality of live [124]. Resistance to platinum is a major treatment challenge and high proportion of the patients have relapse [7]. Tumor stem cells or tumor initiating cells (TIC) are regarded as a major cause of relapse. High levels of circulating TICs have been reported in malignant ascites. Two distinct populations of TICs were present, a less invasive sphere (S), and monolayer (M) forming cells with more invasive marker profile with high levels of stem cells markers and cancer associated fibroblasts (CAFs). However, the S cells are thought to represent a chemoresistant population [125]. The area of targeted treatment is evolving with focus on new molecular targets to deal with these problems. Alternative molecular pathways are investigated to find the critical steps in the tumorigenesis of EOC that can be targeted. Heat chock protein 60 (HSP60) is implicated in mitochondrial protein import and macromolecular assembly, and play an essential role in survival of malignant cells. High expression of HSP60 may identify groups of advanced EOC with poor prognosis that may need alternative therapy [126]. Ongoing studies are testing multiple novel drugs, and hopefully some effective drugs and without harmful side effects will soon be implemented in the clinical praxis [92].

The most important prognostic factors for EOC are stage and grade of the tumor at diagnosis and the patient's age in combination with health status [35]. In stage I EOC five-year survival is more than 90% and only 20-30 % in stage III-IV [29]. Unfortunately only 25% of the patients are diagnosed in an early stage [8]. Outcomes are improved when primary operations are performed by gynecologic oncologist surgeons, and the size of the residual tumor after radical surgery, with no residual tumor left in the end of surgery seems to be of most importance for

survival in late stage disease [35]. Histological type is of importance with worse prognosis in the mucinous and clear cells types if diagnosed in late stage disease, because of their resistance to chemotherapy. Tumor grade has also been related to prognosis, with worse outcome in the higher grade, although tumor histo-type seems to be of more relevance [127].

BIOMARKERS - TUMOR MARKERS

Biomarkers are biological substances that are used to predict different biological conditions. Tumor markers are produced by the tumor, direct by the tumor cells or the tumor stroma, and they indicate the likely presence of cancer or information about its behavior, like a fingerprint of the specific tumor. Tumor markers are of great help in diagnostics, evaluating prognosis, planning of therapy and follow up of the malignant tumors. Diagnostic, screening and prognostic marker discovery has been suggested to follow the proposed "Prospective-specimen-collection, Retrospective-Blinded-Evaluation" (PRBE) design, with nested case-control analysis, were clinical data and samples are collected without knowledge of the results, and the analyze of cases and controls are blinded [128]. Biomarker discovery is only the first step in the process of biomarkers development. The validity of the newly detected biomarker, to correctly classify between i.e. cancer or not, has to be thoroughly evaluated before usage in medical decisions. Intern and extern validation of each individual study is necessary, and multiple studies from different groups have to be involved. The population that is supposed to be the target has to be tested rigorously in prospective manner before implementation in the clinic can be accepted. A biomarker has to be reliable, measurable, specific and predictive. Biomarkers now play a key role in the routine management of patients with cancer, and guide drug development in the age of targeted therapy. An

unreliable biomarker can do more harm than advantage, and validation is a crucial step in the biomarker development. The clinical situation is always in focus, and a reliable biomarker is an instrument that can add information in the assessment [129].

DIAGNOSTIC BIOMARKERS

CA125 is the most used tumor marker since 30 years and is still in the top; both in diagnosis and for follow up of EOC patients. The discovery of OC125, an antibody that recognizes CA125 *(MUC16),* was made by Robert Bast and his colleagues in 1981[5]. CA125 is a heterogeneous glycoprotein complex, found present in several isoforms, with a robust N-glycosylation, and of high molecular mass (110kD >2 millionD), and the major activity has been detected with 200kD when measured in serum with electrophoresis and imunoblot [130], however, CA125 mass measured with MS/MS in ascites was 2,359,682D [131]. CA125 is expressed as a membrane-bound protein in epithelial cancer cells lining the surface of the ovaries, peritoneum, the Müllerian-type epithelium, or is released in soluble form in bodily fluids. CA125 in blood is used in follow up, monitoring therapy, relapse and disease progression. A high level of CA125 in ovarian cyst fluid has been found in EOC and it was related to poor survival, but was not an independent factor [132]. However, CA125 have limitations when used in diagnosis, it is negative in 20% of EOC, and more than half of early cases, and it is often elevated (CA125 >35 U/ml), in different benign gynecological conditions such as endometriosis, pregnancy and pelvic inflammatory disease. CA125 is also elevated in some common medical conditions, like congestive heart failure, cirrhosis, peritonitis, and in several other types of carcinomas. CA-125 levels are higher in premenopausal women than after menopause, increasing the likelihood of false-positives in the younger

population, which is frustrating [133-135]. In the past decade, Human epididymis protein 4 (HE4) has emerged as a promising biomarker in EOC. HE4 has 4 stable disulfide core domains and is also known as Whey Acidic Protein 4-disulfide core domain 2 *(WFDC2).* HE4 was discovered by Kirchhoff et al in 1991 as a proteinase inhibitor with a possible function in sperm maturation [136]. In RNA expression arrays, HE4 was found overexpressed in ovarian cancer in 1999 [137]. Hellström and coworkers first reported HE4 as a biomarker for EOC 2003, with equally detection performance as CA125 but with a better capacity to differentiate healthy women and women with benign disease from cancer [138]. HE4 is a secreted glycoprotein (N-glycosylation) with low molecular weight, 25 kD, found with high expression (93%) in high-grade serous and (100%) in endometrioid EOC but only 50% in clear cell and 0% in mucinous EOC [139]. HE4 is like CA125 not specific for EOC. Other cancer types such as lung cancer and endometrial cancer of the uterus have increased levels of HE4 [140], and breast and pancreatic cancer [141]. Normal glandular epithelium of the breast, female genital tract, epididymis, vas deferens, distal renal tubules, respiratory epithelium, colonic mucosa, and salivary glands have also shown increased levels of HE4, with the highest expression in the trachea and salivary glands [141]. Both CA125 and HE4 have elevated levels in EOC; the levels are related to stage with highest expression in advanced tumor. HE4 complements CA125, has been found expressed by 32% of ovarian cancers without CA125 expression (22% of EOC), and is therefore interesting to use in diagnostic biomarker panels together with CA125 [142]. HE4 opposite to CA125, significantly increases with age, and are less frequently elevated in benign gynecologic conditions, has lower levels in pregnancy (49.7 picomole (pM) a 95th percentile upper limit compared to 118.9 pM in non-pregnant premenopausal woman) and normal levels in endometriosis [134, 135]. Renal failure was the most common course of increased levels of HE4 in patients with

benign disease and increased levels of HE4 has been found in both men and women with renal failure and without any cancer [143, 144].

CA125 and HE4 are both glycoprotein that promote EOC although the mechanism of their action is not clearly defined [145]. Novel evidence suggests a role for CA125 in immunological tolerance, by inhibiting cytotolytic responses of human natural killer cells and as a consequence suppress anticancer activity [146]. It is also speculated that CA125 promotes invasion and metastasis, and that mesothelin, a glycoprotein normally expressed by the mesothelial cells lining the peritoneum, may help ovarian tumor implants to bind to the mesothelial cells lining the peritoneal cavity [147]. HE4 suppresses the activity of multiple proteases, including serine proteases and matrix metalloproteinase's (MMPs), and specifically inhibits their capacity to degrade type I collagen. HE4 has been associated with cancer cell adhesion, migration and tumor growth, through its effects on the EGFR - MAPK (HER 1 -mitogen-activated protein kinases) signaling pathway [145].

The application and improvement of genomic and proteomic technologies has resulted in explosion of biomarker discovery. The genes encode proteins, signal transduction molecules, transcription factors, and other proteins that regulate processes in the cell cycle, cytoskeletal organization and epigenetic modifications [87]. Since Petricoin et al. 2002 demonstrated that serum proteomic pattern from low-resolution MS data could completely distinguish ovarian cancer from nonmalignant lesions on the ovaries [148], multiple studies using proteomics have been performed in the search for early cancer markers but not with the same success. The first results from 2002 were not reproducible either. Enormous number of serum biomarkers or panels of markers mostly on blood, urine [149] and ascites [150] have been reported as tumor markers for EOC, and some of them with better detection performance than CA125 [98, 151, 152]. Of these potential markers only few have been approved to be used in clinical practice. The OVA1 test, including apolipoprotein A1 (APOA1),

transtyretin (TTR), hepcidin (HEPC), β2-microglobulin (β2M), transferring (TRFR), biomarkers detected by SELDI-TOF MS, and CA125, [153] was approved by the US Food Drug and Administration (FDA) in September 2009 [153], and CA125 in combination to HE4 in September 2011. These tests should add help in the clinical assessment of women with a pelvic mass [154].

Box 2. Algorithms for calculation of RMI and ROMA risk scores

RMI = U x M x serum CA125 level

U (ultrasound) 0 = imaging score 0
1 = imaging score 1
2 = imaging score 2-5

M (menopause) 1 = premenopausal
3 = postmenopausal

RMI > 200 increased risk of EOC

(Jacobs I et al. *B J Obstetetrics and Gynecology 1990*)

ROMA Algorithms and thresholds of HE4 and CA125 and menopause status according to the manufacturer's insert

ROMA Predictive index (PI)

Pre-menopausal: PI= –12.0 + 2.38 x LN(HE4) + 0.0626 x LN(CA125)

Post-menopausal: PI = –8.09 + 1.04 x LN(HE4) + 0.732 x LN(CA125)

Predicted probability: ROMA value % = exp(PI) / (1+exp(PI) x 100

(Moore et al. *Gynecological Oncology 2009)*

In the last decades different algorithms and triage protocols have been developed, aiming improve the diagnosis of EOC. Various important variables are incorporated into these probability tests; ultrasound pattern [32, 120], biomarkers, menopause status [154, 155] symptom-index [115] and high-risk,

heredity [156]. The Risk of Malignancy Index (RMI) developed 1990 by Jacobs et al. has improved the diagnostic ability of EOC. This risk stratification algorithm includes CA125 together with ultrasound and menopause status, and RMI >200 means high risk of malignancy [155]. The Risk of Ovarian Malignancy Algorithm (ROMA), introduced 2009 by Moore et al., includes the dual marker combination, CA125 and HE4 in an algorithm with menopause status, but without ultrasound, was reported as superior to RMI in predicting the probability of EOC (Box 2) [154].

SCREENING TEST

Screening is used in an asymptomatic population with the intention to identify individuals having specific cancer or pre-cancer with the intention to refer them promptly for further diagnosis and treatment. An ideal test should be reproducible, non-invasive, inexpensive and able to distinguish perfectively between a healthy woman and a patient with early stage disease. Screening test for EOC needs very high specificity 99.6% (negative test and negative case) to avoid false positives, and a sensitivity (positive test, positive case) of 75% (25% false negative), because of the relative low prevalence (1 per 2500 women of age over 50 year) in the population. The high false positive rate in ovarian cancer screening is problematic, and is doing harm due to the frequent use of invasive surgery including oophorectomy and in many cases more radical surgery. General screening for ovarian cancer is currently not recommended, because it does not help in finding early lesions or reduce mortality. The tests available are not successful [157] . Women with heredity for ovarian cancer are recommended to undergo surgery with prophylactic laparoscopic excision of the ovaries and fallopian tubes after age 35-40 if childbearing is completed, aiming to decrease the risk of cancer. These women are also told to do a regular

check-up, even though evidence show limited or no benefit of early detection [158, 159]. Continuous efforts to find adequately sensitive and specific tumor markers for EOC screening are ongoing [8, 160].

Longitudinal randomized controlled screening trial, the Risk of Ovarian Cancer Algorithm (ROCA) in UK, uses rising levels of CA125 in serial annual measurements to select women for imaging [161] reported better detection performance in the multimodal analysis (sensitivity of 89.5%, specificity of 99.8% and positive predictive value (PPV) of 35%) compared to TVU screening alone (85%, 98% and 2.8% respectively) but not any reduction in mortality [162]. New results from the same study the Prostate Lung Colon and Ovary (PLCO) longitudinal screening trial in USA, TVU and CA125 annual screening, have not either demonstrated a mortality reduction, but 2.6% higher surgical rates in the TVU positive group compared to CA125 only [163]. Recent report showed a better result with earlier diagnosis when parametric empirical Bayes (PEB) algorithm was used, taking into account the screening history and diversity of patients characteristics that can affect the biomarkers [164]. Adding PEB to serial measurements of HE4 could be of value as well [165]. Diversity in tumor biology, the relative low prevalence of EOC in the population, the various intrinsic behaviors of the different types of EOC, and invisible early lesions, makes the early cancer diagnosis very challenging [31, 121].

DIAGNOSTIC TEST

Diagnostic tests are used in symptomatic women, as in our studies women with suspicious malignant pelvic cyst, with the intention to classify the women at risk of having malignant or benign cyst. Good quality diagnostic tests that are fit for purpose and provide accurate results are therefore of great importance in reducing the burden of the disease [166]. Tumor markers are present in tumor

tissue as well as body fluids and comprise a variety of different molecules including secreted proteins, cell surface receptors and transcription factors. Specific tumor markers may have the potential to reduce cancer mortality by facilitating early cancer diagnosis and by helping to individualize treatments. The power of a diagnostic test to correctly predict status is commonly measured as the sensitivity, specificity or a receiver operated characteristic (ROC) and the area under the curve (AUC). Sensitivity is the probability of a positive test given that the person have the disease (P (T+/D+)), and specificity is the probability of a negative test given that the person is without the disease (P (T-/D-)). It has been suggested that specificity of 75% is acceptable for a diagnostic test, 25% with positive test do not have cancer – (false positive), if the sensitivity of the test is 80% or more, a risk of missing up to 20% of the cases – (false negative) in the diagnosis of a pelvic mass [167]. ROC curve provides the sensitivity of a test as a function of 1-specificity. Greater ROC AUC means more powerful test. The clinical utility of a diagnostic test is often expressed by positive predictive value (PPV), the percentage of people who test positives that are true positives, and negative predictive value (NPV), the percentage of test negative that are actually negative. A diagnostic test must achieve a PPV that balances the benefits of early diagnosis against the risk and cost of unnecessary operations and anxiety of the patients associated with false positives. However, these values are dependent on the prevalence of the disease in the population studied [8]. A number of studies have been performed on multiple panels of biomarkers for EOC, and some of them will be mentioned later in the discussion of our results.

WHY OVARIAN CYST FLUID?

Ovarian cyst fluid contains huge amount of potential biomarkers, and is a source of special interest searching for early ovarian cancer markers. Different body fluids i.e. blood, urine [149], ascites [150], and pancreas cyst fluid [168] have been useful in biomarker research. We know, from earlier studies in our department, that ovarian cyst fluid contains a large amount of proteins even more than in the blood [169-171]. The ovarian cyst fluid is in closeness to the tumor, actually in the center of tumor activity. Proximal fluids are promising in searching for more tumor specific markers [172]. New produced tumor cells and products secreted direct form the ovarian tumor cells or stroma cells are most likely present in the ovarian cyst fluid before it will show up in the blood and also in a higher concentration. Tumor specific markers could be more easily detected in ovarian cyst fluid and then looked upon in blood or urine. The specific marker of interest labeled with antibody or nucleotide and detected by some imaging technique. Positron emission tomography (PET) could eventually be used to find chosen biologically active molecules[173].

Our hypothesis is that ovarian cyst fluid harbor early ovarian cancer biomarkers because of its closeness to the tumor, and in this thesis we investigated ovarian cyst fluid as a source for biomarker detection.

AIMS OF THE STUDY

The general goal of this thesis was to investigate benign and malignant ovarian cyst fluid and blood form women assigned for operation with suspicious malignant cystic pelvic tumor; searching for novel tumor biomarkers in the ovarian cyst fluid that could improve the early detection of EOC, and evaluate the diagnostic ability of a new promising tumor marker HE4 individually and together with the currently used tumor marker CA125 in blood for predicting risk of EOC in women with suspicious malignant cystic pelvic tumor.

The specific aims were:

1. Study the ovarian cyst fluid as a source for discovering early biomarkers for use in the diagnosis of EOC (Paper I-III).
    - Explore the whole ovarian cyst proteome (Paper I)
    - Explore the ovarian cyst inflammatory proteome (Paper II)
    - Explore the serous ovarian cyst proteome (Paper III)
2. Validate potential markers, found in the primary analysis, with conventional methods to investigate their capacity differing between benign, borderline tumors, EOC and early and late stages EOC (Paper I-III)
3. Evaluate the performance of HE4, CA125 biomarkers and ROMA risk score from women already qualified for operation because of suspicious cystic pelvic mass, predicting the risk of having EOC, and the different performance in early and late stage and in pre- and post-menopause (Paper IV)
4. Study the performance of HE4 and CA125 in predicting the risk of having EOC in the proposed slow growing type I and aggressive type II EOC, and in early and late stage and in pre- and post-menopause (Paper V)

MATERIAL AND METHODS

STUDY DESIGN AND ETHICS

The study is prospective, observational, cross-sectional, explorative and diagnostic clinical study, the samples taken prospectively before diagnosis, at the time of surgery, and the analysis were performed retrospectively. The local ethics committee at Gothenburg University approved the study protocol and samples were collected from all patients who signed a written informed consent.

PATIENTS

Patients were consecutively and prospectively included when admitted to operation for a clinically suspicious malignant ovarian cystic mass at the section of gynecologic oncology surgery, Sahlgrenska University Hospital, Gothenburg, Sweden, from March 2001 to February 2010. Generally, the criteria used for this selection was clinical examination and assessment with tumor complexity at ultrasound and computed tomography (size, multiple cysts, thick walls, and solid parts), fixed or bilateral nature of the tumors, pre- or post-menopausal status, prior family or personal history of ovarian or breast cancer. Patients with a solid tumor but without any cyst were not invited to participate, and patients who did not want to were not included. The surgery included peritoneal washing for cytology, hysterectomy, bilateral salphingo-oophorectomy, omentum resection, appendectomy in most cases of mucinous cysts, and with intention to radical operation, if fertility was not to be saved. Patient was post-operatively excluded if the final histology of malignancy was other than EOC. PAD was checked for all patients, confirming the postoperative diagnosis and stage of the tumor, as well as age and menopause status.

SAMPLE COLLECTION AND PROCESSING

According to our protocol, blood samples were taken after anesthesia but before surgery, and ovarian cyst fluids were collected after removal of the cyst from the abdomen. All samples were immediately put in 4°C for 15-30 minutes, centrifuged, and aliquoted into Eppendorf tubes. The fluids were transferred to −80°C, within 30–60 minutes after collection. The samples that were used in this study had one freeze-thaw cycle. Handling and processing of the samples were standardized for all patients included. All tumors were examined by an experienced pathologist for diagnosis, histology, and grade. The tumors were staged (I-IV) according to FIGO standards [29], and in Paper V with regard to the gene and histology-unifying model into type I and type II EOC [18] (Table 2).

Paper I

Of the 218 women that were originally included, from March 2001 to September 2006, 192 were eligible for the analysis (26 excluded; 14 metastases, 3 granulosa cell cancer, 9 not able to analyze). Validation was done in 40 cyst fluid samples from the original cohort and in 40 new cyst fluid samples; 20 benign and 20 EOC in respective cohort.

Paper II

38 cyst fluid samples, 22 benign and 16 EOC were selected from the 192 eligible in Paper I. Validation of potential markers was done in cyst fluid and serum from 256 patients that were eligible for analysis from March 2001 to September 2007; 156 benign, 22 borderline tumors and 74 EOC, 3 granulosa cell cancer and 1 malignant teratoma.

Paper III

A total of 15 cyst fluid samples were selected from samples collected from March 2001 to April 2008, all with serous histology; 5 benign, 5 stage I and 5 in stage III EOC. Cyst fluid and plasma from 68 patients included from March

2001 to February 2010; 32 benign and 36 EOC; totally 136 samples with mixed histology, were used in verification and validation of selected significant proteins from the primary analysis.

Paper IV & V

Under the period March 2001 to February 2010 we included totally 393 patients. 374 and 373 women were eligible for analysis in Paper IV and V; 215 benign, 45 borderline tumors and 113 EOC (19 respective 20 were excluded; 3 granulosa cell cancer, and 16 metastases in Paper IV, additionally 1 malignant teratoma was excluded in Paper V) Table 2.

Table 2. Illustrating the patient distribution between the different studies performed.

Paper	Method	Included	Benign	Borderline	EOC	Granulosa	Dermoid	Metastases	Excluded	Eligible
I	SELDI	218	**129**	16	**47**			14	26	**192**
	ELISA	80	**40**		**40**					**80**
II	IM-MS	38	**22**		**16**					**38**
	ELISA	291	**156**	22	**74**	3	1	15	36	**256**
III	iTRAQ	15	**5**		**10**					**15**
	Immunoblot	68	**32**		**36**					**68**
IV	ELISA	393	**215**	45	**113**	3	1	16	19	**374**
V	ELISA	393	**215**	45	**113**	3	1	16	20	**373**

PROTEOMICS

In our study we used three different high throughput proteomic analytic tools to mine the ovarian cyst fluid with the intention to detect novel tumor markers for early EOC. Verification and validation of potential markers were done with ELISA and immunoblot.

Surface Enhanced Laser Desorption / Ionization Time of Flight Mass Spectrometry SELDI-TOF MS

In Paper I the whole ovarian cyst fluid proteome was explored in the search for novel biomarkers. We analyzed 192 ovarian cyst fluid samples, from benign (n=129), borderline (n=16) and malignant ovarian cysts (n=47).

SELDI-TOF MS facilitate protein profiling and detection of proteins in complex biologic mixtures and combines two powerful processes in one, chromatography with modified target and MS. Depending on the type of chromatographic matrix used, CM10 (weak-positive ion exchange), H50 (hydrophobic surface), IMAC30 (metal-binding surface), or Q10 (strong anion exchanger), a subset of proteins in the sample bind to the surface of the chip.
A portion of the cyst fluid samples were unfractionated and another portion fractionated by anion exchange chromatography. Standard profiling was done, and Tandem Equalizer beads (EB) and Mercapto ethyl pyridine (MEP) beads were used, meant to reduce the concentration of high-abundant proteins with wide dynamic range and to purify of recombinant proteins from host cell impurities. A small amount of fluid was robotically applied on the surface modified with chemical functionality biochips. Some proteins bind to the different targets, while others were washed off. The proteins bound to the SELDI surface were analyzed with TOF MS. A laser-ionized peptides from crystals of the sample and matrix mixture were then accelerated through an electric field and down into a flight tube. When the ions reached the end of the tube a detector measured the mass-to-charge (m/z) of the ions. Ratio of each ion can then be determined from the length of the tube, time it takes to travel through the tube and the kinetic energy given to ions by the electric field. Spectra of polypeptides in the samples were generated on a Ciphergen PBSII mass spectrometer with mass accuracy of +/- 0.15%. Data software from Ciphergen, Biomarker Wissard was used and analyzed the MS spectra for

protein identification (Figure 3). This MS profiling is effective and fast processing and could be able to pick up complete spectrum of very low abundance proteins in a short period of time.

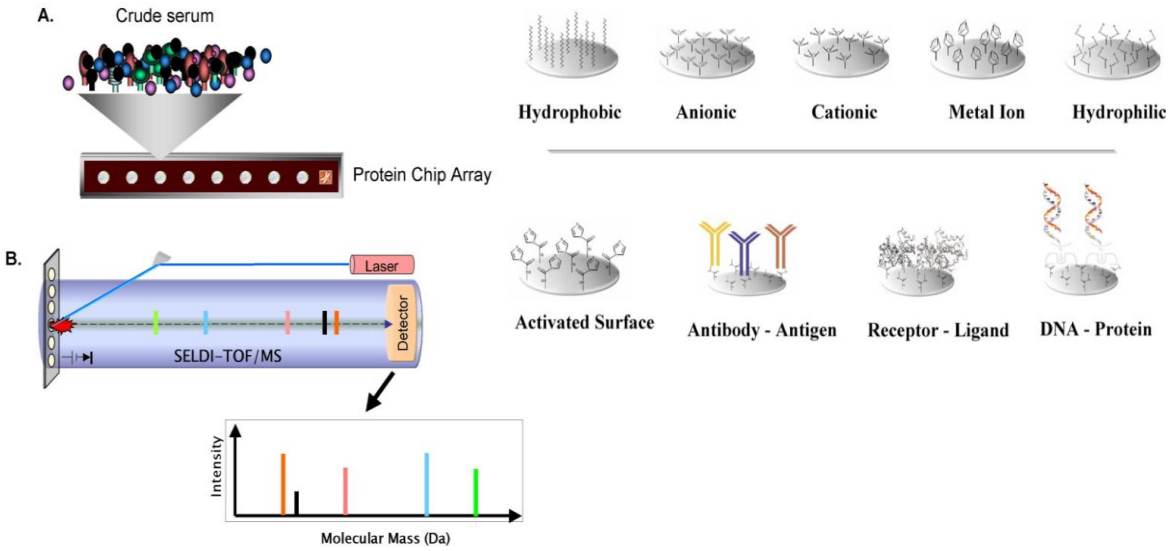

Figure 3. Proteins are extracted from the ProteinChip Array by Laser Desorption/ionization. Different target modifiers are applied on the chromatographic surface with a property to target specific proteins from complex solution. Ionized proteins and their mass accuracy are determined by TOF-MS, and the spectra is generated by a spectrometer. Protein peaks are identified as mass/charge (m/z), and protein identification is done by software-database. SELDI-TOF MS, Ciphergen 2007.

Proteins are often in clusters and in different modifications, pre- (allelic variants, slice variants and RNA editing forms) and post-translational (glycosylation, phosphorylation, lipidation, oxidation, methylation, cystinylation, sulphonation and acetylation) conditions. Protein identification need to be done with other methods if the specific spectra generated are not earlier defined, and reproducibility issues need to be addressed. The practices with MS require technical training, and the handlings of samples are of importance, therefore

strict protocols need to be followed [174]. SELDI technology employing Protein Chip arrays from Ciphergen Biosystems Inc. (Fremont, California, USA), and experts in the field (Eric T Fung and Christine Yip at the American company Vermillion inc., before Ciphergen Biosystems inc.) performed the analysis. More detailed description of the procedure used is found in Paper I.

Immunoprecipitation Method - Mass Spectrometry

To focus on the inflammatory profile of ovarian cysts we enriched our material by using selected inflammatory proteins known to be involved in the immune response in cancer, and in the same time overcome the problem with abundant proteins. The immunoproteome was explored in 38 ovarian cyst fluid samples, benign (n=22) and EOC (n=16) in Paper II.

Table 3. Inflammatory markers selected for the Immunoprecipitation.

Accession no	Protein symbol	Protein name	Expected m/z kDa
Ab Mix I			
P01584	IL-1β	Interleucin-1β	17,375
P10145	CXCL8 = IL-8	Interleucin-8	8,376/8,920
P13500	CCL2 =MCP-1	Monocyte chemoattractant protein 1	8,664
P10147	CCL3 =MIP-1α	Macrophage inflammatory protein 1-α	7,441/7,712
P13501	CCL5 =RANTES	C-C motif chemokine 5	7,550/7,847
P09341	CXCL1 = GROα	Growth-regulated α protein	7,862
P48061	CXCL12=SDF-1α	Stromal cell-derived factor 1	7606/8297/8520
Ab Mix II			
P05231	IL-6	Interleucin-6	27
P48061	IL-12	Interleucin-12	75
P01137	TGF-β	Transforming growth factor β	13
P01375	TNF-α	Tumor necrosis factor	17,34
P15692	VEGF	Vascular endothelial growth factor	27/39
P09919	CSF3 = GCSF	Granulocyte colony-stimulating factor	19
P04141	CSF2 = GMCSF	Granulocyte macrophage colony-stimulating factor	16
P09038	FGF2 = HBGF-2	Heparin-binding growth factor 2	18/24

Immunoprecipitation is a direct targeting technique, using specific antibodies; in our case selected monoclonal antibodies. The antibody mixture I and II used are presented in Table 3. The inflammatory markers in the ovarian cyst fluid were specially targeted by the antibodies or captured onto the beads and become immunoprecipitated. SELDI-based enriching immunoassay by tandem antibody libraries bead was used and experts (Eric T Fung and Christine Yip) in the lab of the company, Vermillion inc. in USA performed the analyses.

Isobaric Tags for Relative and Absolute Quantification Mass Spectrometry - iTRAQ MS

In Paper III we explored the serous ovarian cyst fluid proteome. For more homogeneity and to increase our chances of finding a true novel biomarker we choose only patients with serous histology; benign (n=5), stage I (n=5) and stage III EOC (n=5).

To further come near the deep proteome of ovarian cyst fluid we used selected depletion of albumin and immunoglobulin G (IgG) before the specific proteomic analysis. iTRAQ is a specific MS-based quantitative proteomics, a non-gel based technique that enables both identifying proteins and studying changes in protein abundance in biological samples. The proteins are separated according to size and detected with MS. The protein differences between groups are more easily noted because the proteins in the solution are labeled with isobaric tags. The samples were not pooled in order to see individual differences, and protein selection was based on significance and high fold change between the benign and malignant cysts. Protein identification from the five sets was done with help of database search by Mascot search engine. The ratios of iTRAQ reporter ion intensities in MS/MS spectra from the raw data sets, derived by Proteome Discoverer version 1.1, were used to calculate fold changes between samples. The method has a low variance between runs and can take up to seven samples

together with a reference sample under identical conditions. Analyses were performed at the Proteomic Core Facility at the University of Gothenburg of the experts in the field, and the experimental design is described in detail in Paper III.

iTRAQ – technique is high qualitative method, that enables direct peptide-protein identification, and direct quantification of the proteins and with high reproducibility [175]. This in contrast to SELDI-TOF MS, which generates peak spectra (mass/z) were direct protein identification for unknown proteins is not possible, and variance between runs is more common as well [174]. Both methods have potential to measure thousands of proteins in a small sample that reflect the different expression of proteins in cells, tissue and body fluids.

Immunoblot

Verification and validation of significant selected proteins, SAA4 and ASTL, were done with immunblot in 132 samples from 68 patients in ovarian cyst fluid and plasma with mixed histology. The samples were chosen from our "cyst fluid bank"; benign (n=32), EOC (n=36) in stage I (n=18), stage III-IV (n=18) (Paper III).

Immunoblot is a method used to identify specific proteins with help of specific antibodies directed to the protein of interest, and one of the most powerful methods to use in a complex protein mixture. Total amount of protein from a cell can be extracted, or fractionated in cytoplasm, cell membrane and nuclear extracts, depending on the detergent used. The proteins are separated by gel-electrophoresis (SDS-PAGE) and then transferred to a micromembrane which is immunoblotted with a specific antibody and incubated over night. Then the immunoblotted membranes are exposed and optical density of individual bands quantified from the membrane images by densitometry, relative quantification. More detailed description of the procedure is presented in Paper III.

Enzyme-Linked Immunosorbent Assays – ELISA

ELISA was used to verify expression of and to validate specific selected proteins; ApoC-III and PCI in ovarian cyst fluid (Paper I); GROα, IL-8 and MCP-1 in ovarian cyst fluid and serum (Paper II); HE4 in plasma (Paper IV-V); CA125 in serum and plasma (Paper –I-II and IV-V); (Table 4).

ELISA is a technique that uses antibodies and color change to identify a substance or a protein. Solid-phase enzyme immunoassay (EIA) is used to detect the presence of a substance, usually an antigen, in a liquid sample or wet sample. A specific monoclonal antibody for the protein of interest has been pre-coated onto a microplate. Standards that are used in the quantification step and samples are pipetted into the wells and any antigen present binds to capture antibody, proteins bound to the immobilized antibody and any unbound substances are washed away. An enzyme-linked polyclonal antibody specific for the protein measured is then added to the well and binds to the antigen. Following a wash to remove the unbound antibody-enzyme reagent, a substrate solution is added and a color develops in proportion to the amount of the protein bound in the initial step, and the color intensity is then measured.

Figure 4. Summary of the ELISA kits that were used to perform the validation experiments in the different papers presented.

ELISA-CA125 Cisbio Bioassays, France	**CA125**	**Paper I-II**
ApoC-III (human) ELISA kit (KA0465, Abnova Taiwan)	**ApoC-III**	**Paper I**
PCI Actibind ELISA Reagent kit (TC16100, Technoclone, Austria)	**PCI**	**Paper I**
Quantikine®, a solid phase ELISA; Human CXCL1/GROα immunoassay	**GROα**	**Paper II**
Quantikine®, a solid phase ELISA; Human CXCL8/IL-8 immunoassay	**IL-8**	**Paper II**
Quantikine®, a solid phase ELISA; Human CCL2/MCP-1 immunoassay	**MCP-1**	**Paper II**
Architect CA-125 II (Abbott Diagnostics), Illinois, USA	**CA125**	**Paper IV-V**
HE4 EIA assay (Fujirebio Diagnostics, Gothenburg, Sweden)	**HE4**	**Paper IV-V**

If samples generated higher values then the highest standard, we diluted the samples and repeated the assay. The cyst fluids were more often needed to be diluted, because of higher protein concentration in the ovarian cyst fluid than the serum.

STATISTICS

Statistical differences in protein levels between groups were evaluated using the Mann-Whitney U test or the corresponding Kruskal-Wallis one-way analysis of variance for 3 or more groups. Correlation between peak levels and protein levels in ovarian cyst fluid and serum samples was evaluated using bivariate Spearman correlation. Correlation of age between groups was evaluated with bivariate Pearson correlation coefficient. The natural log of protein levels was included as independent variables in logistic regression analysis. The predicted probabilities for each model were used to construct ROC curves, and AUC was calculated. Sensitivity, specificity were calculated for individual markers and their combinations (Paper I-V) and PPV and NPV (Paper IV-V). Threshold values for HE4 and ROMA were calculated at a specificity of 75% in Paper IV. For all statistical comparisons a value of $p < 0.05$ was considered significant. In Paper III significant results presenting proteins with $p<0.05$ and at least a 1.8 fold change were generated, and immunoblotting was evaluated with bivariate correlation using Spearman correlation coefficient.

Statistical analyses were performed in; SPSS for Windows (version 17, Inc., Chicago, IL, USA) in all papers, CiphergenExpress (Ciphergen Biosystem, Fremont, CA, USA) and Prism 5.0 (GraphPad Software, San Diego, CA, USA) in Paper I-II, and Stata 12.1 (Stata Corp., Texas, USA) in Paper V.

RESULTS AND COMMENTS

This thesis is based on five papers. For the first three different proteomic techniques were used to evaluate the ovarian cyst fluid as a potential source to find novel biomarkers for early diagnosis of EOC. After exploring the whole ovarian cyst fluid proteome we continued to search for more specific markers in the deep proteome. The focus was on the early response in inflammation, and the deep proteome of serous tumor histology. Paper IV and V have a more clinical approach. We evaluated the diagnostic performance of the newly approved dual marker HE4 and CA125 to predict the risk of EOC in women presenting with a suspicious malignant ovarian cyst. In Paper V the evaluation were done according to the dualistic model in pathogenesis of EOC, slow growing type I and fast growing, aggressive type II EOC. Ovarian cyst fluid and blood used in our analyses was collected prospectively at the time of operation in women with a suspicious malignant ovarian cyst, and analyzed retrospectively.

Proteomic profiling of the ovarian cyst fluid proteome – SELDI-TOF MS – Paper I

In order to explore the whole cyst fluid proteome in a total of 192 ovarian cyst fluid samples were analyzed; 129 benign, 16 borderline tumors and 47 malignant (46 EOC, 1 malignant dermoid).

Half of the malignant tumors were in early stage EOC (FIGO I-II). The age of the study population was between 16-86 years old, representing a relative high median age in the borderline (56 years), and the benign cohort (60 years), which was almost the same as for the malignant (61 years). A total of 1180 protein peaks were resolved by SELDI-TOF MS. Seventeen of the 221 peaks differently expressed (p<0.0001) between benign and malignant ovarian cyst fluid samples

revealed ROC AUC values >0.70. Five proteins in different isoforms were detected among these 17 peaks (Table 5). Apolipoprotein C-III (ApoC-III) was identified in five peaks, ApoC-I in three peaks, transthyretin (TTR) in two peaks, serum amyloid A4 (SAA4) in two peaks and protein C inhibitor (PCI; SerpinA5, PAI III) in one peak.

Table 5. Proteins with significantly (p<0.001)different mass peaks m/z between benign and malignant cyst fluid samples, AUC >70 and specificity at fix sensitivity of 81.8%

Protein - ID	Peak m/z value	Mean intensity Benign/ Malignant	ROC AUC	Specificity % (CI)	
PCI	3902	30.23 / 7.00	0.79	67.7	(58.9-75.6)
ApoC-III	9743	3.60 / 15.09	0.82	68.5	(59.7-76.3)
ApoC-III	9448	5.29 / 22.00	0.80	60.8	(51.8-69.2)
ApoC-III	9751	4.65 / 24.2	0.80	60.8	(51.8-69.2)
ApoC-III	9777.5	3.20 / 14.7	0.79	63.1	(54.2-71.4)
ApoC-III	9453	7.30 / 33.20	0.78	50	(41.1-58.9)
ApoC-I	6647	37.53 / 121.14	0.78	57.7	(48.7-66.3)
ApoC-I - truncated	6448	68.91 / 184.53	0.76	53.1	(44.1-61.9)
ApoC-I - truncated	6489	24.49 / 52.75	0.76	53.1	(44.1-61.9)
SAA4	12886	1.35 / 2.42	0.76	58.5	(49.5-67.0)
SAA4	12863	1.53 / 2.72	0.74	59.2	(50.3-67.8)
TTR	13900	7.80 / 23.16	0.77	59.2	(50.3-67.8)
TTR	13925	5.36 / 14.27	0.75	57.7	(48.7-66.3)
Hb beta	8037	2.61 / 4.64	0.75	65.4	(56.5-73.5)
Albumin	54622	0.73 / 0.30	0.76	61.5	(52.6-69.9)
Albumin	54426	1.72 / 0.25	0.75	60.8	(51.8-69.2)
Albumin ion	44754	1.06 / 0.50	0.76	57.7	(48.7-66.3)

These protein peaks have all been identified earlier in serum and have prominent mass peaks in SELDI and matrix assisted laser desorption ionization (MALDI) profiles that characterize each protein [174]. These proteins are mainly representing highly abundant proteins and fragments hereof (Table 5).

To find the marker with best predicative probability in cyst fluid or panel of markers for diagnosis of EOC a multiple logistic regressions analysis was

performed for the differentially expressed proteins, individually and together in different combinations with and without CA125 in corresponding serum. ApoC-III (AUC 0.82) and PCI (AUC 0.79) were independent factors in predicting malignancy (p<0.0001 and p= 0.001 respectively), and these two in combination reached the same ROC AUC (0.91) as the five cyst fluid proteins together in a panel. Adding CA125 to the dual combination ApoC-III and PCI generated the highest AUC 0.94 (CI 0.89-0.98). However, no marker alone had higher AUC than serum CA125 0.87 (CI 0.80-0.94). The specificity of the three-marker panel (ApoC-III, PCI and CA125) was 88.4% compared to 68.2% for CA125. CA125 with cut-off value of 35U/ml had a sensitivity of 81.8%, accordingly specificity for the novel markers were calculated at the same sensitivity as CA125. ApoC-III and PCI had specificity similar to CA125 of 68.5% and 67.7% respectively.

In order to verify the proteins, ApoC-III and PCI, and their expression levels in ovarian cyst fluid, 40 cyst fluid samples were selected from samples used in the SELDI analysis. In addition to these previous analyzed samples we added a set of 40 consecutively collected new cyst fluid samples for validation. The two different methods SELDI-TOF MS and ELISA correlated for ApoC-III (Spearman´s rho=0.328; p=0.04). We verified a significant increase in expression for ApoC-III (p< 0.05) in the malignant samples from SELDI, using ELISA, and the validation of ApoC-III showed also significant increase in expression levels (p=0.001) in the new malignant samples. For PCI a contradictory result was found, instead of lower expression in the malignant samples (SELDI profiling) we identified an increased expression in the malignant samples (ELISA), even though it was not significant. Further validation in another set of samples showed increase of PCI levels in the malignant samples but not significant (p=0.26). PCI with m/z 3902 Da detected in the SELDI profiling is a part of the entire PCI protein, (C-terminal fragment (SwissProt#05154), and the antibody used in the verification/validation step

picked up the active part of the PCI. Interestingly, high-abundant proteins were present in high amounts in the cyst fluid similar to blood, which was causing a problem in detecting the interesting low abundant proteins, which are thought to be more usable in the clinical work as tumor specific markers.

Despite the drawbacks of the method used in this study we strengthen our hypothesis that ovarian cyst fluid is a promising source for detection of early biomarkers.

Proteomic profiling of the ovarian cyst fluid immunoproteome with Immunoprecipitation -MS – Paper II

We explored the immunoproteome, enriched our material with known cancer inflammatory proteins; in addition, we used a direct targeting method and scrutinized the deep proteome. For the immunoprecipitation method 38 ovarian cyst fluid samples 22 benign and 16 EOC were selected from the original material in Paper I. Validation was done in cyst fluid and serum from 256 patients; 156 benign, 22 borderline tumors and 74 EOC.

We detected 150 high quality peaks (signal/noise ratio of 3:1 and present in 20% of the spectra) with significant expression ($p<001$) between benign and malignant cysts. Of the proteins that were identified, MCP-1 and IL-8 showed highest significance, with AUC at 0.82 and 0.80 respectively, and a seven fold difference in expression (Figure. 4).

MCP-1 and IL-8 were further validated with ELISA together with GROα, another promising chemokine, in a bigger set (n=256); both in ovarian cyst fluid and corresponding serum. Evaluation of these markers was done together with CA125 in serum to investigate if the selected biomarkers improved the diagnostic ability of CA125. The age in our benign patient population was relative old, with almost equal mean age in the benign and malignant cohorts, 60 compared to 61 years, and women with borderline tumor were 10 years younger,

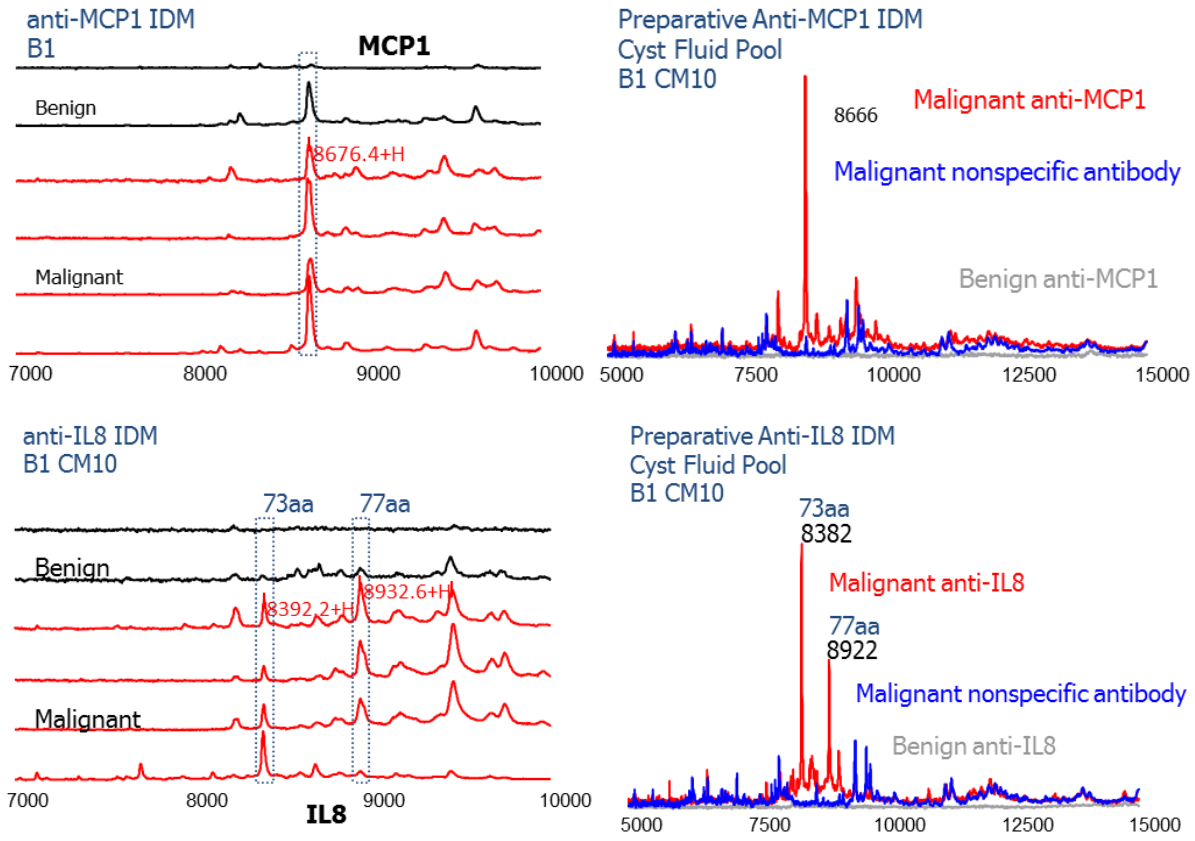

Figure 4. Representative mass spectra of MCP-1 and IL-8 in Immunoprecipitation MS.

51 years. High proportion of EOC (50%; n=39/74) were in early stage EOC (stage I-II FIGO).

Higher expression (p>0.001) of inflammatory proteins was found in the ovarian cyst fluid than in blood, and the difference was even noticed in the benign samples. The most interesting finding was the increase in cytokines response in early tumorigenesis. Significant (p<0.001) increase in cytokine expression (GROα, IL-8 and MCP-1) was found in borderline tumors (n=22) in the cyst fluid when compared to the benign samples (n=156). Combination of the cyst fluid markers and CA125 resulted in the highest AUC 0.86, with MCP-1 as an independent marker (*p*=0.004). In serum, CA125 was the only marker with significance (*p*<001), and revealed the same AUC (0.78) as all markers analyzed in serum together. In the comparison between benign tumors and stage I

malignant (n=32) IL-8, GROα and MCp-1 in cyst fluid showed significantly higher expression in early stage I; IL-8 ($p < 0.001$), GROα ($p = 0.003$) and MCP-1 ($p = 0.003$). Beyond CA125 ($p < 0.001$) IL-8 was the only cytokine, which was significant ($p = 0.006$) in serum stage I tumors. The same result was achieved including stage II tumors (n=7) as an "early stage" group (n=39).

When comparing cyst fluid samples from patients with a benign cyst to all malignant cysts significant difference were found for all markers (GROα, IL-8 ($p<0.001$ both) and MCP-1 ($p=0.006$). In serum CA125, GROα and IL-8 were significant ($p<0.001$ all), but MCP-1 was not (p=0.99). In multiple regression analyze, CA125 ($p<0.001$) and GROα ($p=0.005$) were significant. ROC AUC for the marker combination in cyst fluid together with CA125, was almost equal (AUC 0.87) to the marker combination in serum (AUC 0.88), the same as for CA125 alone. CA125, IL-8 and GROα were independent markers in serum ($p < 0.001$, $p = 0.009$, $p = 0.009$, respectively). IL-8 had best AUC of the cytokines tested individually both in serum and cyst fluid with AUC 0.76 and 0.73 respectively.

Cytokines that are involved in the early inflammatory response are still confined to the ovarian cyst fluid in borderline and early stage EOC, but in a later stage the inflammatory proteins have secreted to the blood. Inflammatory proteins, although not tumor specific, may serve as tumor biomarkers.

Proteomic profiling of the serous ovarian cyst fluid proteome – Paper III

We further concentrated on the deep proteome, now in a selection of serous ovarian cystic tumors. Only patients with serous histology, five benign, five stage I EOC and five stage III EOC were included, and depletion of abundant proteins (albumin/Immunoglobulin G) were done before analysis with a high qualitative and quantitative method to scrutinize the deep proteome of the most common type of EOC. Validation using immunoblot was done in samples of mixed histology from 68 patients; 32 benign and 36 EOC.

	Benign					Stage IA					Stage IIIC				
Symbol						HG	LG	HG	HG	LG	HG	HG	HG	HG	HG
ASTL	NA	1,16	2,30	2,05	NA	NA	-1,69	-1,56	-1,09	NA	NA	-0,86	-1,64	-0,64	NA
ALB	-0,74	0,86	1,79	0,69	0,78	-0,54	-1,51	-0,79	-0,43	-0,76	-1,09	-1,29	-0,49	-0,15	-0,14
C7	0,58	0,12	0,28	0,64	0,68	-0,64	-1,22	-0,47	-0,84	0,08	-0,25	-0,81	-0,40	0,01	0,24
AMY1A	NA	NA	-0,29	1,72	3,66	NA	NA	-1,03	-1,32	-1,25	NA	NA	-0,14	-1,84	-1,00
SPARCL-1	1,55	-0,81	-0,18	NA	1,86	-1,79	-1,15	-1,09	NA	-0,97	-0,89	-0,23	-0,71	NA	-0,30
PLTP	NA	-0,01	0,18	1,32	-0,14	0,11	-1,18	-0,56	-0,60	-0,62	-1,09	0,58	-0,38	-1,06	-1,00
TARSH	NA	0,19	0,41	NA	1,26	NA	-0,15	0,20	NA	-0,17	NA	-0,14	-0,42	NA	-0,69
CTSD	NA	0,93	-0,40	2,33	-0,36	NA	0,48	-0,97	-0,04	-1,12	NA	-1,36	-1,79	-1,25	-1,40
CHAF1A	-0,36	0,94	NA	0,75	-0,27	0,11	-1,03	NA	-1,18	-0,42	-1,15	-0,49	NA	0,03	-1,32
COL6A3	1,56	-0,10	1,40	NA	0,33	-0,97	-1,64	-0,54	NA	0,04	-0,22	0,67	-0,10	NA	-0,10
CRISP3	-0,84	-0,69	-0,71	0,59	2,83	-0,47	-2,32	-1,12	0,48	-0,92	-1,51	-1,94	-1,47	-1,22	-0,84
KIAA0196	-0,92	0,78	1,04	NA	-0,22	-0,56	-1,36	-0,84	NA	-0,12	-1,09	-0,69	-0,14	NA	-1,36
MSLN	-2,64	0,79	0,18	1,50	1,40	0,25	-2,94	-2,32	-0,84	-0,14	-1,06	-1,94	-0,79	-2,74	-0,97
OVGP1	-4,06	-0,22	-4,32	2,64	2,84	-2,94	-5,64	-4,06	-2,64	-1,60	-2,84	-4,32	-5,64	-2,40	-1,22
APOA1	0,44	-1,51	-1,51	-2,06	-1,74	-0,06	-0,71	-0,09	-0,09	0,16	0,15	0,32	0,43	0,74	0,26
APOB	-1,29	-1,43	-0,86	-1,74	-1,64	0,18	-0,69	1,16	1,10	-0,84	-0,97	-0,07	0,08	-0,92	-0,15
GRP78	NA	-1,56	-1,29	NA	-1,69	NA	1,61	-0,76	NA	0,08	NA	-0,32	-0,47	NA	-1,18
APOA4	1,28	-2,25	-2,94	-2,47	-1,56	0,36	-1,74	-0,17	-0,43	0,64	-0,14	0,64	0,61	0,58	1,01
IDHC	-3,47	-1,60	-2,64	NA	-3,64	-1,09	1,59	-2,12	NA	0,83	-1,69	-0,32	-1,89	NA	-1,29
ALDOA	-1,29	-1,69	-2,64	-0,62	-2,47	-0,25	0,54	-0,51	-1,06	1,89	-0,62	0,55	-0,69	-1,74	-1,18
TPI1	-2,64	-0,71	-1,32	0,03	-1,25	-0,23	1,21	-0,43	-0,56	0,67	-0,67	0,40	-1,00	-0,30	0,33
GAPDH	-3,18	-0,32	-2,64	-0,40	-2,18	-0,14	1,01	-0,69	-0,97	0,58	-1,22	0,21	-0,06	-1,94	0,38
C4BPA	-1,12	-0,67	-0,89	-1,03	-1,06	0,39	-1,06	0,99	0,96	-0,56	-1,06	0,04	0,19	-0,74	-0,27
CLTC	NA	-1,32	-0,79	-2,64	-2,18	NA	-0,92	0,95	1,18	-1,69	NA	-0,23	NA	-1,15	-0,60
APOC1	0,37	-1,22	-1,47	-0,56	-1,94	0,38	-1,69	0,21	0,14	-0,18	0,10	-0,07	0,29	0,30	-0,06
S100A8	-5,06	-2,56	-1,89	-2,06	-3,32	0,14	1,88	-0,40	-0,12	1,68	-2,40	-1,94	-1,25	-3,32	-2,84
SYT13	NA	-0,94	-1,22	NA	-0,60	NA	-0,69	0,89	NA	0,32	NA	-0,49	-0,17	NA	0,49
YWHAZ	-5,64	-0,86	-2,74	0,43	-3,32	-0,79	1,62	-1,22	-0,64	0,61	-0,84	-0,25	-1,36	-0,69	-1,47
APCS	-0,09	-2,40	-0,42	-2,64	0,01	0,32	-1,06	0,59	-0,43	-0,81	0,58	-0,03	0,07	0,24	0,32
SAA4	0,26	-1,60	-1,32	-1,32	-1,47	-0,89	-1,60	-0,74	-0,07	-0,38	1,06	0,23	0,36	0,44	0,34
PRDX	NA	-2,25	-4,06	-0,76	-3,18	NA	0,28	0,14	1,77	-0,56	NA	-0,25	-2,40	-1,00	-2,40
S100A9	-2,74	-1,74	-3,84	-1,79	-2,32	0,43	1,75	-0,23	-0,12	1,62	-2,56	-1,74	-0,79	-2,94	-2,47

Figure 5. Protein, n=32, detected with iTRAQ analysis in cyst fluid from serous ovarian tumors. Indicating the tumor type; HG, High grade, LG, low grade. The green color indicates lower and the red higher expression levels in the samples.

The protein concentration was significantly higher (p=0.02) in the malignant cysts compared to the benign, 837 proteins were identified, and 87 as differently expressed (p<0.05) between the groups. Proteins with only single or two-peptide identified and fold change <1.8 and a number of immunoglobulins were also excluded. Thirty two proteins left were significantly ($p < 0.05$) differently expressed between benign serous adenoma and serous EOC, and 59% (n=19) were expressed in all 5 sets (Figure 5). Serum amyloid A-4 (SAA4) and astacin-like metalloendopeptidase (ASTL) were selected for further validation by immunoblot in ovarian cyst fluid and plasma, 136 samples with mixed histology

from 68 patients. The protein selection was based on either, high significance and high fold change or abundant appearance and several peptide recognitions in the sample sets *(p* = 0.04, FC = 1.95) and (*p* < 0.001, FC = 8.48) for SAA4 and ASTL respectively. In the comparison between benign and stage I EOC ASTL expression was still significant (*p*=0.001). In the validation step done with immunoblot SAA4 was significantly (*p* = 0.001) expressed in the cyst fluid, and with higher expression in the malignant cysts. However results for ASTL were contradictory, had lower expression in EOC in the iTRAQ analysis, and significantly higher *(p*=0.003) expression in the malignant samples analyzed with immunoblot. However, there were no significant differences in expression levels between benign and EOC in plasma for either SAA4 or ASTL. Seven serous tumors included in iTRAQ were within the validation cohort (two benign and five EOC), and correlated for SAA4 (*p*=0.008), but ASTL (*p*=0.58) did not correlate within the two methods. The peptide recognition in SAA4 was based on five to eleven peptides in each set of five. ASTL had only one to three peptides detected in three of five sets, and that can indicate a more uncertain identity.

Interestingly some proteins had their highest expression in stage I EOC in the iTRAQ analysis (Figure 5). S100A8 and S100A9 had higher expression in all the five early EOC compared to late stage, and peroxiredoxin 2 (PRDX 2) were higher expressed in four samples. Moreover, two of the five stage I EOC serous tumors analyzed were low-grade serous and the other malignant tumors were all high-grade EOC. Several proteins (GRP78, IDHC, TPI1) had higher expression in the low-grade tumors in stage I than in stage III, and generally low expression in the high-grade tumors. The high-grade tumors had also some proteins (APOB, C4BPA, CLTC) with higher expression in stage I than in stage III EOC.

Fluid from ovarian cysts connected directly to the primary tumor harbor many possible new tumor specific biomarkers. To enhance the discovery of tumor

specific proteins that could represent novel biomarkers a depletion of highly abundant proteins that can mask the detection of proteins present in low concentrations can be performed.

Further studies will be continued on selected potential biomarkers, and their ability to differ between benign and early stage EOC will be tested.

HE4, CA125 and ROMA separate benign and malignant ovarian cysts –Paper IV

HE4, one of the most promising diagnostic markers in EOC was evaluated individually and together with the currently used marker CA125. Their diagnostic performance was assessed in differing benign ovarian cyst from malignant cyst, in patients presenting with a suspicious malignant cystic pelvic tumor. Additionally the newly introduced ROMA score was validated, and cut off values for HE4 was estimated for best performance in our study population; 373 were included in the analysis, 215 benign, 45 borderline tumors and 113 EOC.

Levels of HE4 significantly ($p<0.001$) differed between benign and stage I EOC as well as for all EOC, with increased levels in EOC. Borderline tumors were not significant, except in the postmenopausal women ($p<0.05$). The dual marker combination and ROMA were highly significant ($p<0.001$) in all comparisons, but with lower stringency ($p<0.05$) for benign vs. premenopausal stage I EOC. The best diagnostic prediction, differing between benign and malign cyst was presented by ROMA and CA125 (ROC AUC 0.87 respectively), followed by the combination of CA125 and HE4 (AUC 0.85), and HE4 alone (AUC 0.84). The estimated cut-off value for HE4 was 85pM calculated at a specificity of 75% according to Moore et al. [154], and the cut-off for positive diagnosis by CA125 was set at 35U/mL (specificity 80%), which resulted in a sensitivity of 78% and 82 % for HE4 and CA125 respectively. However, when using the cut-off value of 70pM for HE4, introduced by Moore, we achieved a sensitivity of 88% and

specificity of only 57%. Threshold value may vary depending upon the study population. Characteristics of the study population were different from the study performed by Moore, which as well included borderline tumors in the analysis. Our population included high proportion of postmenopausal women in the benign cohort (74% compared to 43%), high percent of women with EOC (42% vs. 27%) and high percent of early stage tumors (50% vs. 27%). Therefore, we calculated cut-off values for our study population and at 75% specificity, accepted as relevant [154]. In our study population, the threshold values for HE4 in the premenopausal cohort was 71.8 pM and in post menopause 85 pM, and for ROMA 17.3%, and ROMA 26.0% respectively.

Table 6. ROC AUC, specificity, sensitivity when using the calculated cut-off values of HE4 and ROMA, and commonly used 35U/mL for CA125, comparing benign and malignant EOC, pre-, postmenopausal (pre-MP, post-MP), stageI EOC and borderline tumors.

	ROC AUC (%) (CI 95%)	Specificity (%)	Sensitivity (%)
Malignant (n = 114)			
HE4 (85 pM)	84.4 (79.5-89.2)	75	78.1
CA125 (35 U/mL)	86.8 (82.3-91.4)	80	81.6
HE4 and CA125	84.8 (80.1-89.6)	66	88.6
Malignant pre-MP (n = 21)			
HE4 (71.8 pM)	82.3 (70.4-94.2)	75	80.9
CA125 (35 U/mL)	84.6 (75.1-94.2)	75	76.2
ROMA (17.3%)	83.1 (71.6-94.7)	75	81.0
Malignant post-MP (n = 93)			
HE4 (85 pM)	84.8 (79.5-90.1)	75	80.6
CA125 (35 U/mL)	87.0 (81.8-92.1)	83	82.8
ROMA (26%)	88.7 (84.2-93.2)	75	87.1
Stage I (n = 47)			
HE4 (85 pM)	72.2 (63.4-81.1)	75	59.6
CA125 (35 U/mL)	76.3 (68.1-84.4)	80	61.7
Borderline (n = 45)			
HE4 (85 pM)	58.2 (49.6-66.8)	75	35.6
CA125 (35 U/mL)	78.8 (71.8-85.7)	80	62.2

ROMA revealed best performance to diagnose (AUC 0.89) malignant cysts in postmenopausal women, followed by CA125 (AUC 0.87) and HE4 (AUC 0.85). ROMA had also the highest sensitivity 87%, compared to 83% and 81% for CA125 and HE4 respectively. CA125 had best diagnostic accuracy (AUC 0.85) in the premenopausal cohort, followed by HE4 (AUC 0.83) and ROMA (AUC 0.82). However, HE4 and ROMA had better sensitivity (81% both) than CA125 (76%) among the premenopausal cohort. CA125 had better capacity (AUC 0.76) than HE4 (AUC 0.72) in the diagnosis of stage I EOC (Table 6).

Cases with marker levels above cut-off levels were considered to have a positive test result. When used in combination the test was positive if one of the markers were positive, and negative if both of the markers were negative. The combination of HE4 and CA125 resulted in 13 false negative (FN) cases. The false positive (FP) benign cases were evenly distributed between the histology groups, except in endometriosis where CA125 had higher FP- rate than HE4 with five of eleven (45%) compared to three of eleven (27%) tested. Negative predictive value (NPV) was high (92%), and FN were all in early stage EOC.

CA125 showed generally better performance than HE4 in the differential diagnosis between benign or malignant cyst. However, HE4 complemented the total diagnostic picture, with higher sensitivity than CA125 (81% respectively 76% at a set specificity) in the premenopausal cohort. The high age in our study population especially within the benign cohort is in favor of CA125, which increases with increasing age in contrast to HE4.

HE4 and CA125 together showed good capacity to diagnose the aggressive type II EOC – Paper V

With regard to the gene and histology-unifying model of type I and type II EOC we evaluated the diagnostic performance of HE4 and CA125 in diagnosing these tumors in blood from 373 patients presenting with suspicious malignant pelvic cysts; 215 benign, 45 borderline tumors and 113 EOC; n=42 type I EOC, and n=71 type II EOC

Type I EOC included 42 tumors (37%); low-grade serous (n=18), low-grade endometrioid (n=6) mucinous (n=11) and clear cells cancer (n=7). Type II included 71 tumor (63%); high-grade serous (n=54), high-grade endometrioid (n=11), and undifferentiated carcinoma (n=6). Type I tumors were more often in early stage (69%; n=29) compared to type II (38%; n=27).

Most of the women with EOC were postmenopausal (81.4%); in type I and type II EOC 79% and 83% respectively. The benign cohort included as well high proportion (78%) of postmenopausal women.

Both biomarkers significantly ($p<0.001$) differed between benign and type I, and type II EOC respectively, type I vs. type II EOC, and borderline tumors vs. type II EOC. HE4 was significant ($p=0.026$) but CA125 ($p=1.000$) was not in comparison between borderline tumors and type I EOC. HE4 and CA125 generated high ROC AUC for type II EOC (0.92 HE4; 0.93 CA125; 0.93 HE4+CA125), and considerably lower AUC for type I EOC (0.72 HE4; 0.76 CA125; 0.79 HE4+CA125). The sensitivity for the markers in type II EOC was high individually (91.5% HE4, 93% CA125) at a 75% specificity, and improved further by using the marker combination (94.4%). Much lower sensitivity was found in type I EOC for the markers (54.8%, 71.4%, and 61.9% respectively). The highest ROC AUC for the combination HE4 and CA125 was in late stage type II EOC (AUC 0.99), and considerable lower in early stage (AUC 0.85).

Table 7. HE4 and CA125 levels according to histology, type, stage and menopause status; significant differences, ROC AUC and sensitivity at 75% specificity in benign vs. Type I and II.

	Benign n=215	**Type I EOC n=42**				**Type II EOC n=71**			
	Median (range)	Median (range)	p-value	ROC AUC (95%CI)	Sensitivity (%)	Median (range)	p-value	ROC AUC (95%CI)	Sensitivity (%)
CA125 35U/mL	16 (8-3250)	53	<0.001	0.76 (0.68-0.85)	71.4	395 (6-14880)	<0.001	0.93 (0.89-0.97)	93
Pre-M	23	40	0.076	0.78	55.6	731	<0.001	0.90	83.3
Post-M	14	64	<0.001	0.76	66.7	327	<0.001	0.93	96.6
Early stage*		36	<0.001	0.70	62.0	104	<0.001	0.85	81.5
Late stage*		194	<0.001	0,9	92.3	564	<0.001	0.98	100
HE4	66 (31-469)	93 (40-784)	<0.001	0.72 (0.63-0.81)	54.8	354 (39-7933)	<0.001	0.92 (0.87-0.96)	91.5
Pre-M **71.8pM**	57	73	1.0	0.71	55.6	239	<0.001	0.91	91.7
Post-M **85pM**	69	109	<0.001	0.73	60.6	412	<0.001	0.91	91.5
Early stage*		74	<0.001	0.66	45.0	132	<0.001	0.81	81.5
Late stage*		129	<0.001	0.86	76.9	474	<0.001	0.98	97.7
HE4 + CA125		No of samples	% of Type I	0.79 (0.72-0.86)	61.9	No of samples	% of Type II	0.93 (0.89-0.98)	94.4
Pre-M		n=9	-21%	0.80	44.4	n=12	-17%	0.92	83.3
Post-M		n=33	-79%	0.79	66.7	n=59	-83%	0.94	93.2
Early stage*		n=29		0.73	48.3	n=27		0.85	85.2
Late stage*		n= 13		0.93	92.3	N=44		0.99	1000

*According to FIGO Early stage=I+II, Late stage=III+IV; Pre-M/Post-M=pre/postmenopausal

In type I ROC AUC was also higher in late stage (0.93) compared to early stage disease (0.73). Type I EOC premenopausal was not significantly different from the benign tumors but other combinations were ($p< 0.001$).

There were thirteen FN all in early stage. Nine of them were type I EOC (n=5) low-grade serous, (n= 2) mucinous, (n=1) endometrioid and (n=1) clear cell cancer, and four were type II EOC, and all (n=4) high-grade serous stage I. Serous and endometrioid EOC were more easily diagnosed than clear cell and mucinous cancer. HE4 and CA125 showed significant difference ($p=0.0045$, $p=0.0002$) between benign serous and serous type I and serous type II EOC ($p= 0.0001$). CA125 but not HE4 were significantly different ($p=0.0001$ and 0.569) in the comparison of serous benign and serous borderline tumors. However, HE4 but not CA125 differed ($p=0.003$ and $p=1.0$) when comparing serous borderline tumors to serous type I, and HE4 but not CA125 were as expected significantly different ($p=0.0019$ and $p=0.1380$) comparing endometriosis and endometrioid EOC. No significant difference was found between benign, borderline and EOC according to mucinous histology.

HE4 and CA125 biomarker combination was much better diagnosing type II EOC than type I EOC and especially late stage type II EOC (AUC 0.99). Menopause status did not affect the diagnostic performance with regard to EOC type grouping.

DISCUSSION

Early diagnosis- Triage

Survival of women with EOC is high if diagnosed at an early stage, before its metastases to the pelvis and peritoneum, and methods that can improve the early detection seem to be a priority option [8]. Proficient radical primary surgery performed by experts in the field is another priority option to improve the survival of women with EOC [7, 10]. Early diagnostic tumor markers and a reliable risk score for triaging patients with EOC to a right level of care is of the utmost interest. Women with benign disease can be spared unnecessary radical operations and women with EOC will have the opportunity to get the most optimal available therapy. In this thesis the focus was on detection of early diagnostic markers, by exploring the ovarian cyst fluid, and to find women with risk of having EOC by evaluating the newly approved biomarkers HE4, CA125. Only women with malignant suspicious ovarian cysts were included in the study and evaluated. We state that ovarian cyst fluid is a rich proteome resource with huge number of potential early tumor markers that remain to be detected and validated [176, 177]. In the differential diagnosis of pelvic cysts HE4 is promising to add to the clinical assessment, especially in the premenopausal women [178]. HE4 and CA125 had excellent performance in diagnosing aggressive late stage type II EOC, but with insufficient ability to diagnose early stage type II and generally inferior in type I EOC. Markers specific for the different tumor types, according to behavior and histology may improve the diagnosis [179].

A clinical dilemma is that a lot of women will be evaluated because of cystic pelvic mass under their lifetime and just a few of these women actually have

malignant ovarian cyst, and preoperative discrimination between benign, borderline and malignant ovarian cyst is really very challenging [112, 120, 180]. The smaller cysts in combination with the solid tumors are more often poorly differentiated in contrast to the larger cysts [181]. The female anatomy poses a challenge as well, with the ovaries and tubes localized deep in the pelvis with difficulties in the clinical evaluation [113], and not easily assessable for tissue biopsy or cyst fluid sampling without risk of spill or spread of cancer cells [182]. However, fine needle biopsy diagnosis is found to be reliable, and is accepted to be used in the differential diagnosis of uncertain advanced pelvis tumor [183, 184]. Invasive surgical intervention is needed and confirmation of the definite diagnosis has to be done by microscopic and complex immunohistopathological examination[28]. General screening of the population, with low prevalence of the disease, using unreliable testing methods are not recommended. Screening with current tools does not help in identifying early cases [8]. Anxiety and unnecessary interventions, causing serious harm in women who do not have EOC will be highly expensive consequences. However, women with known heredity should be informed more thoroughly about the risks they have and the prophylactic surgery that could be performed to reduce the risk [185]. High suspicion and recognition of potential early symptoms for EOC in the primary care providers, with quick and appropriate referral for further assessment may improve the diagnostic process [186], and the presence of reliable tumor markers at this instance would facilitate early diagnosis.

Differential diagnosis and the accurate prediction of EOC in women presenting with a suspicious pelvic cyst is of great importance for the chance to get the best choice of therapy [10].

To avoid unnecessary radical operations sensitive tumor specific biomarkers and imaging tools are critical in the risk stratification between benign or malignant cases [154]. The ultrasound is continuously developing, together with simpler models to differ between benign, borderline and malignant tumors, which will

surely improve the diagnosis of EOC. Two logistic regressions models (LR1 and LR2) for ultrasound scanning included twelve respective six demographic and ultrasound variables are highly effective in the hands of experienced ultrasound examiner to distinguish between benign and malignant mass. LR1 includes 12 variables: 1) personal history of ovarian cancer, 2) current hormonal therapy, 3) the patients age, 4) maximum diameter of the lesion, 5) pain during examination, 6) ascites, 7) blood flow within a solid papillary projection, 8) a purely solid tumor, 9) the maximum diameter of the solid component, 10) irregular internal cyst walls, 11) acoustic shadows, and 12) color score. LR2 includes 6 variables: 1) age, 2) ascites, 3) blood flow within a solid papillary projection, 4) maximal diameter of the solid component, 5) irregular internal cyst walls, and 6) acoustic shadows [187]. Ultrasound examination by an expert in the field outperformed CA125, HE4, RMI and ROMA as a secondary test in a tertiary centre, in the prediction of cancer when dealing with a known pelvic mass [188]. However, ultrasound techniques will always be influenced by the skills of its operators and is often, subjective. Additionally experts in gynecology or in gynecologic ultrasound are not available in all hospitals or in large geographic areas on the countryside in many countries.

In a study performed in the same lab as our study (Paper I), and using the same technique as well, (SELDI-TOF MS) three biomarkers were discovered in serum, APOA1 (down-regulated in cancer), a truncated transthyretin (TTR) (down-regulated) consistent with our results in cyst fluid [176], and a cleavage fragment of inter-alpha-trypsin inhibitor heavy chain H4 (IATIH) (up-regulated) were reported to improve the detection of EOC (AUC 74) compared to CA125 alone (AUC 65 at 97% specificity) [151]. Few years later, five marker panel from the same research group, APOA1, TTR, hepcidin (HEPC), β2-microglobulin (β2M), transferring (TRFR) together with CA125 were included into the OVA1 risk score approved by the FDA in September 2009 to be used in

the differential diagnosis of pelvic mass in females [153]. However, when validated as an early detection marker in pre-diagnostically collected sera the OVA1 failed to improve the sensitivity for preclinical diagnosis of CA125 alone. CA125 was increased in (40 of 65) 61% of samples within 12 months prior to diagnosis and in (8 of 51) 16% more than one year prior to diagnosis [189]. This indicates that promising biomarkers should be tested thoroughly in an appropriate population before implementation into the clinic; biomarkers that aid in the differential diagnosis of a pelvic mass are not necessarily usable for screening or early detection.

The immune system is reacting early in the tumor development with increase in proinflammatory factors that could be of help in the early diagnostic of EOC (Paper II). In a study by Gorelik et al. the inflammatory proteins Interleukin (IL)-6, IL-8, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) in serum improved the performance of CA125 in diagnosis of early stage EOC (n=45) and resulted in 84% sensitivity at 95% specificity when a panel of 24 cytokines and chemokines was tested using a novel multiplexed immunobead-based cytokine profiling (LabMAP profiling technology) [152]. Palmer et al evaluated fourteen candidate blood markers from symptomatic women, both in pre-clinical and clinical samples, and reported CA125, HE4, mesothelin (MSLN) and metalloproteinase7 (MMP7) as promising diagnostic markers especially for the serous EOC, were CA125 and HE4 showed the best performance [98]. An increase in concentration of CA125, HE4 and MSLN was even detected one to three years before the clinical diagnosis of EOC [190]. These results are promising for future research. Intervention is needed prior to progression and early detection marker must have the ability to detect the cancer before symptomatic and pre-clinical samples can be used to search for novel early markers. Thorough validation of potential biomarkers on appropriate samples is

mandatory before clinical application [98, 190]. More sensitive and specific biomarkers are needed to find preclinical disease, the small volume tumors not detected by ultrasound. These tumor specific markers could be used with more accuracy in triage and referral of patient at risk, and in the choice of customized treatment to the individual patient [191].

During the last decade, mass spectrometry has obtained a key role in most of the proteomic analyses that are focused on identifying cancer biomarkers in human serum, making it possible to identify and characterize differently expressed proteins or peptides at a molecular level. A major problem confronting the early diagnostic has been defining the cell of origin and the early events in the etiology of the disease. The knowledge about the different genomic pathways that are acting in different types of cancer has culminated and evidently p53 mutation is present in nearly all the aggressive high-grade serous tumors. The genome is highly instable, with focal DNA copy aberrations and promoting epigenic changes are common [87]. Since the proteins are products of the genetic code, and they drive the processes of the cells, the combination of proteomic and genetic knowledge can be used to improve the biomarker detection [192].

Ovarian cyst fluid – A biomarker source of the tumor microenvironment

The majority of EOC (>85%) cases have combination of cystic and solid formations [181]. The cystic compartments are lined with cells derived from the ovarian surface, fallopian tube, mesothelial tubal junction and the endometrium [14, 18]. The local microenvironment of the tumor might be reflected in the ovarian cyst fluid inside the tumor which makes the ovarian cyst fluid an ideal medium to use in the early tumor marker research. In this thesis we used three

different high power proteomic methods searching for novel biomarkers in ovarian cyst fluid for early diagnosis in EOC. Our hypothesis is that early tumor markers that are shed and secreted by the tumor cells, ovarian stroma cells, inflammatory cells or other proteins involved in early carcinogenesis are more likely to be found in proximity to the tumor, in the ovarian cyst fluid in this case, rather than in the peripheral blood circulation [172]. We found a huge number of proteins, potential tumor markers, differently expressed in benign and malignant ovarian cyst fluid. Higher amount of protein were present in the cyst fluid compared to blood [176], which is in line with previous studies [169-171, 193]. Colleges from Bergen, Norway, state that access to fluid that reliably reflects the local microenvironment enables us to identify substances that can be used in early detection and monitoring of disease [194], and colleges from Kentucky conclude that ovarian cyst fluid is an "physiologic germane cache of differentially expressed ovarian tumor biomarkers" [195].

Ovarian cyst fluid proteome

Many proteins are bound to other carrier proteins, and exist in different posttranslational modifications, such as glycosylations or phosphorylations. High throughput proteomics, including SELDI, have an outstanding advantage to examine a huge number of proteins, and reflect their different post-translational modifications [172]. The two proposed markers APOC-III and PCI from our protein profiling resulted in high AUC (0.94) when combined to CA125. However, the identity of PCI, with just one peak identification was more uncertain after the validation step [176]. PCI harbor extreme heterogeneity, is presented in complex bindings together with various proteins, and PCI exhibit different glycosylations that will change the proteins appearance as well as functionality [196]. The diversity in modifications of proteins detected makes difficulties in the identification and quantification in the validation phase.

Thus an extremely well characterized, high-affinity antibody, that can detect the protein form of interest is needed in the validation [172]. Probably we used an antibody in the ELISA analysis which did not detect the specific posttranslational modification of the protein desired. The protein peaks suggested being PCI perhaps were another potential biomarker. This study was done in the early days of SELDI, when peak detection was in the developing phase, and without a sequence based identification [197]. Moreover, ovarian cyst fluid had not been studied with this technique before. However, our significant high abundant protein peaks detected in the ovarian cyst fluid were well known in blood and SELDI is capable of differing between diverse posttranslational forms [174]. PCI also called plasminogen activator inhibitor 3 (PAI-3), with recommended name plasma serine protease inhibitor (SerpinA5), is beyond coagulation, fertilization and some other regulation processes, involved in tumor defense. PCI inhibit tumor invasion by inhibiting urinary plasminogen activator (uPA), a mediator of tumor cell invasion, and has been found to be decreased in renal cancer [198]. It is the protease activity of PCI that inhibits uPA, and additionally suppresses tumor progression independently [199]. In a recent study, PCI (SerpinA5) expression in tumor tissue was significantly reduced in advanced stage serous EOC when compared with the early stage tumors, and lower expression of PCI in borderline tumors was associated to more aggressive borderline tumor [200]. Components of the uPA system are increased in many cancer types, including EOC, and increased uPA has been related to shorter survival of EOC [201]. Activated protein C (APC) promotes tumor invasion by endothelial protein C receptor (EPCR) - and plasminogen activator receptor-1 (PAR-1) mediated protease activity [199]. These studies are in line with our results from the SELDI profiling [176].

The body fluid proteome is complex and there are significant obstacles in proteomics biomarker research. Humans are genetically and environmentally diverse. The plasma proteome is known to harbor extreme dynamic range of

protein concentration, and high proportion of abundant proteins that take up 99% of the proteome, with only 1% left for the more interesting low abundant proteins [197, 202]. Higher protein concentration was present in the cyst fluid than in the blood; however we had similar problems as in plasma with presence of large high abundant proteins that may hide the small tissue specific proteins in cyst fluid [176]. The abundant proteins play an important role in the normal physiological processes in the body. Albumin, immunoglobulin and transferrin comprise more than 70% of the proteome. The other high abundant proteins are transport proteins like haptoglobin, transthyretin, lipoproteins; protease and protease inhibitors such as clotting factors and alpha-1-antitrypsin; and immune response proteins such as complement factors and C- reactive protein (CRP) [197]. Most of these abundant proteins are components of the host response generated against various pathophysiological conditions, and are often increased or decreased in different types of cancer, and are therefore not cancer specific. However, different cancer diseases trigger different acute phase response and the markers can be in different posttranslational forms even in their earliest stages, and can therefore be used as biomarkers in cancer [203]. Significantly ($p=0.001$) decreased expression of albumin were present in serous EOC compared to benign samples in the iTRAQ analysis in Paper III [177]. A low level of albumin is an ominous sign in ovarian cancer, associated with ascites, cachexia and malnutrition. Low levels are associated to short survival (<25 g/l median survival of 4.8 months (95% CI 0-13.1 months), whilst levels >35 g/l of 43.2 months (CI 11.6-20.9), and albumin can be used as a prognostic marker [204]. The capacity to heal after surgery is diminished, risk for anastomosis leak after colon resection is increased in patients with low levels of albumin, and protective colostomy or ileostomy is a better alternative for these patients [205]. A challenge to observe is that the proteins are not in a steady state. Individual differences are found between patients and their individual proteome profile is constantly changing depending on varying conditions. Genetics, environmental

and lifestyle factors, coherent diseases, sample collection, processing and storage are some examples, that could alter the protein expression [172]. Handling the samples should be done according to strict protocol to minimize changes of this factor. However, the optimal biomarker have to be robust to be able to use in the clinical work [174].

Despite of some drawbacks in the validation process and problems with high abundant proteins in the ovarian cyst fluid we are convinced that the ovarian cyst fluid is an excellent source searching for early tumor biomarkers because of the direct connection to the tumor pathology [176, 177].

Ovarian cyst immunoproteome

There is growing evidence that inflammation and the cross talk between the ovarian epithelium and ovarian stroma is involved in the initiation of cancer [44-46]. More ovulations over a lifetime increases the risk of getting EOC by creating an inflammatory microenvironment that favors malignification of cells [26]. EOC is related to inflammation with complex network of cytokines and chemokines that promote and modulate the tumor progress [71]. For mining the inflammatory proteome of the ovarian cyst fluid and aiming to overcome the abundant proteins we enriched our selected proteins by using known tumor inflammatory antibodies and a direct targeting method in Paper II. We found significantly higher presence of pro-inflammatory cytokines, which are low abundant proteins, in borderline tumors and early stage EOC in the ovarian cyst fluid, than in serum. These findings confirm that inflammation is an early event in EOC, and support that ovarian cyst fluid is an excellent source in the search for early biomarkers. An interesting finding was the upregulation of MCP-1 in early tumorigenesis. Significantly increased levels of MCP-1 were found already in the borderline tumors. MCP-1 is one of the key elements of the immunological response to malignant growth, mainly via attraction and

activation of tumor-associated macrophages (TAMs). TAMs are the major players and modulators in cancer related inflammation [49]. Significantly higher levels of MCP-1 in serum from ovarian cancer patients have been reported [206], but our validation did not confirm any difference in MCP-1 in serum. In serum, IL-8 was the only significantly increased chemokine in early stage EOC. Other studies have shown increased levels of IL-8 in ovarian cyst fluid, ascites, serum and tumor tissue [59, 169, 193, 207, 208]. IL-8 is a multifunctional chemokine that interact in all steps in carcinogenesis, inflammation that triggers the evolution, growth, proliferation, angiogenesis, invasion, metastasis, and chemo- resistance, via inducing intracellular molecular signaling pathways. These pathways may be interesting for targeting therapy for controlling the progress of the tumor [208, 209]. GROα is like IL-8 pro-inflammatory chemokine, with different functionality in different stages of the cancer process [210]. To our knowledge GROα has not been analyzed in ovarian cyst fluid before. Malignant tumors are able to escape from immune elimination and instead cytokines turn on to create environment that favors the tumor. A study on fourteen cytokines and growth factors in cyst fluid (GROα not included) reported that immunosuppressive state created by ovarian cancer could be reflected in the cyst fluid, [61]. The suppressive immune cells directly enhance the pathogenesis through the release of various cytokines and chemokines. The antibody profile triggered in the course of tumor development may be detected in the cyst fluid and used as an immunologic fingerprint of the malignant tumor providing information on disease associated proteins that can be used both in diagnosis and therapy. Targeting therapy has to focus on preventing the immune suppressive state of the microenvironment and to normalize the inflammatory state by disrupting critical links in the cytokine networks [46, 211]

Ovarian serous cyst fluid proteome

Serous histology is the most common histology in EOC and responsible for most of the deaths in EOC [18]. By investigating only tumors with serous histology and the differences in protein levels between benign and malignant tumors we might find potential tumor markers that could be used in the diagnosis of EOC. In Paper III we used a modern technique, applying isotope-based quantitative proteomic to explore the cyst fluid from serous tumors to concentrate only on the most deadly tumors. To overcome the problems with high abundant proteins, albumin and immunoglobulin were selectively excluded from the samples before the proteomic analysis. To better detect individual differences the samples were not pooled. In addition to identification of proteins and their changes of abundance, a mix of samples can be analyzed under identical conditions and with minimal variations between the runs [212]. We identified 837 proteins in the ovarian cyst fluid, and 32 proteins were differently expressed between the serous benign and EOC cysts after further exclusion of some immunoglobulins. Astacin-like metalloendopeptidase (ASTL) and serum amyloid A4 (SAA4) were selected for validation. Only a few studies using iTRAQ in EOC have been done previously. Boylan et al. were the first to do immunodepletion prior to the iTRAQ analysis, in the search for biomarkers in serum [213], and Gagné et al. studied protein expression in tissue biopsies [214]. In a study from Lund, phosphatidylinositide 3-kinases/ protein kinase B (PI3K/Akt) signaling pathway, known to induce proliferation and prolong cell survival, was described to be the most significant canonical pathway in EOC tumorigenesis. Moreover, several targets and effectors of this pathway were identified with a help of different proteomic workflows, (iTRAQ/ matrix-assisted laser desorption (MALDI)-TOF MS/ electrospray ionization - quadrupole TOF MS/ MS), and multiple reaction monitoring (MRM) [215]. ASTL, an enzyme active in proteolysis found expressed in the ovary [216], was

one of the most interesting proteins identified in our screening profiling, with the highest fold change and it was highly significantly (p<0.001, FC=8.48) decreased in the malignant samples. However, we were not able to verify the primary results in the validation experiments. Eventually the specific high-affinity antibody needed to detect the modification of ASTL in cyst fluid was lacking [172, 215]. SAA4, the other protein validated, an acute phase protein, had lower significance and fold change (p=004, FC= 1.95) than ASTL, but high peptide detection. SAA4 was also one of the significant proteins that showed up in the SELDI profiling doing it little more interesting to study further. Increased levels of SAA4 were related to tumor progression, in both our SELDI and iTRAQ analysis. These results are in line with previous studies on colon cancer and EOC, were SAA4 has been suggested to be involved in carcinogenesis, with successive increase in expression of SAA4 from early tumorigenesis to more advanced stage, but negative in mucinous histology [217-219]. Early detection with imaging diagnostics in ovarian cyst fluid is speculative but interesting. Perhaps it is possible to label an antibody for SAA4 with nucleotide and then screen with PET-like tool, or as decorated SAA4 magnetite nanoparticles [220].

Statistical relevance and accuracy of proteomic data is supposed to be increased by using multiplexing potential as iTRAQ, through the simultaneous analysis of labeled different biological samples. These powerful tools are able to assess qualitative-quantitative differences in protein profiles between benign and cancer cases [175]. A new study done with iTRAQ showed that molecular signaling cascades working in EOC pathogenesis can be studied with this technique, and tissue-based markers related to specific pathways identified [221]. Multiple studies have been done in the search for more potent biomarker than the 30 year old marker CA125, and significant potential biomarkers have been presented as promising to use in early diagnosis of EOC. Detection performance of multiple markers in a panel has resulted in better detection than a single marker. By assessing protein expression profiles and post-translational

modifications found in health and disease needs to be translated into the clinic, and making it possible to do an appropriate validation [222]. Until recently, the majority of biomarker studies are done on patients with clinical disease, and the tests are taken before operation. Preclinical testing have shown that CA125 levels are increased more than a year before diagnosis of EOC [189]. Validation in samples taken before clinical disease of promising markers may help in the search for early EOC markers.

Appropriate validation of identified potential biomarkers detected in high throughput proteomics is lacking or unsuccessful, and is not only a problem for our group [153, 189]. The specific markers desired could be found in the deep proteome of the EOC cyst fluid, and need to be identified and translated into useable tools in the clinic.

HE4, CA125 and ROMA

Successful surgery is the mainstay of treatment, with no residual tumor left in the end of operation [7, 35]. Appropriate surgery beyond early detection of EOC seems to be one of the most important goals to improve patient's survival and quality of life. Referral of patients with suspicious cancer to expert center for assessment and therapy will increase their chance to a better prognosis, and for the patients with advanced disease it can be crucial to get a better and longer lifetime [7]. It is not only the advanced surgery performed that is of importance but also the multidisciplinary teamwork in the preoperative planning of therapy, during operative activities including qualified surgery and anesthesia, and postoperative intensive care [10, 122]. Very few of the markers presented to be early biomarkers have come into use in the clinic [172]. CA125 is still in top, and now together with the promising biomarker HE4 [138, 178, 179]. The algorithm of the dual marker HE4 and CA125 with menopause status in the

ROMA score could correctly classify patients with pelvic mass into low or high risk of having EOC with 94% sensitivity at 75% specificity [154]. This was the background for our study, evaluation of HE4 and CA125, individually, together, and in the algorithm with menopause, (ROMA), to differ between benign and malignant ovarian cysts [178]. Less than 30% of the women that undergone surgery because of suspicious malignant ovarian cyst had EOC (n=113/373) and 35% if borderline tumors (n=45) were included, and 4% were metastases (n=16). ROMA had best diagnostic power in the postmenopausal cohort (AUC 0.89 vs. 0.83 in premenopausal), whilst lower than in studies, by Van Gorp (AUC 0.90 vs. 0.85), and Kalapothara et al. (AUC 94% vs. 0.73) [223, 224]. CA125 showed generally better performance than HE4, which is in line with Van Gorp et al., but the capacity for HE4 was better than our in differing between benign and stage I EOC (AUC CA125 0.75 respective 0.76, and AUC HE4 0.77 respective 0.72) [223]. Results from Moore et al. were highly in favour of HE4 overall and significantly better than CA125 in the detection of stage I (AUC HE4 0.77 and AUC CA125 0.70) [225]. However, in our study HE4 was complementary to CA125 in the early diagnosis, and more reliable in the younger women with benign disease such as endometriosis, which is in line with other studies [135, 154, 223, 224, 226].

ROMA were reported to be even better than RMI, an algorithm with CA125, menopause status and ultrasound [155], to successfully classify these patients (94% vs. 85% sensitivity for ROMA and RMI respectively, at a set specificity of 75%) [167]. Moore et al. reported higher sensitivity for ROMA in patients with early disease (stage I-II EOC) compared to RMI (85% and 65% respectively) [167]. Several reportes comparing RMI and ROMA have been done, and with conflicting results [167, 223]. In one of the largest ongoing prospective studies, the Danish study, RMI and ROMA were reported as equally in differing between benign and malignant pelvic masses [191]. HE4 and CA125 used together was approved by FDA in September 2011 to aid in referring

patients likely to have ovarian cancer to specially trained gynecologic oncologists for surgery. Younger women (premenopausal) with endometriosis are often evaluated because of cystic tumor and HE4 seems promising to use in the differential diagnosis of EOC for these patients. In a study by Moore et al., 1042 women with benign gynecologic disease were evaluated, and HE4 was less often elevated than CA125 (8% vs. 29%, $P < 0.001$), and only 3% of the patients with endometriosis had elevated HE4 levels comparing to CA125 in 67% ($P < 0.0001$) [135]. CA125 is often increased in normal pregnancy and is not a reliable marker in that situation, but HE4 is decreased in pregnancy and could be a complement in the diagnostic assessment when dealing with a suspect ovarian cyst in pregnancy [134].

Women presenting with a pelvic mass may be classified more correctly using the combination of HE4 and CA125 [224].Algorithm including HE4 and CA125 together with different imaging technique could further improve the early diagnosis of EOC. Prospective multicenter study is suggested, and further evaluation of the role of the biomarkers, in referral of patients to appropriate level of care, is needed [178].

HE4 and CA125 in type I and type II EOC

It has been suggested that EOC should be divided with regard to molecular genetic changes into slow growing type I, with genetically stable genome (somatic mutations), and aggressive type II tumors, with highly unstable genome (*TP53* and *BRCA1/2)* [18, 31]. High-grade serous tumors are the most threatening and very challenging to diagnose in their early stage, because of unclear preclinical lesion, and consequently more often diagnosed in the late stages than type I EOC, which are not seldom detected incidentally at gynecologic examination by ultrasound [32, 121]. We tested HE4 and CA125

individually and together in the whole study population, and compared benign cysts with type I and type II EOC [179].

Our results showed as expected that the dual marker combination HE4 and CA125 is highly representative for the aggressive type II (AUC 0.93), and surprisingly good tool in the diagnosis of aggressive late type II EOC (AUC 0.99 respective AUC 0.93 type I) [139, 227]. Menopausal status did not affect the diagnostic ability of HE4 and CA125 within type I and type II tumors. However, higher performance of the markers has been achieved in the postmenopausal group in studies comparing benign with all EOC [154, 191, 223, 224]. The diagnostic capacity in the early stage EOC were not sufficient (AUC 0.85 type II vs. AUC 0.73 type I), confirming earlier data, that we lack appropriate markers for early stage disease [8], both for the aggressive and the slow growing type EOC. We missed to identify thirteen cases (11.5%) out of 113 EOC (when both markers were false negative) all in early stage; nine samples were from the type I group and four were type II EOC, and among the six type II EOC included in the study. HE4 could differ benign from borderline cysts, but CA125 did, and CA125 could differ between borderline and type I EOC, but HE4 did not. Borderline is as earlier mentioned a pre-stage to type I EOC [31].

Our findings support the hypothesis that EOC should be looked upon as several different diseases and each histo-type need specific attention in searching and validating biomarkers [28, 179]. The great heterogeneity of EOC, explains a part of the difficulties in finding early markers [87]. Markers found to be expressed in serous and endometrioid EOC are not found in the mucinous and clear cells cancer and vise versa [139]. Mucinous and clear cell cancer is often detected by ultrasound and these histotypes need specific markers to differ from the benign tumors [19, 32]. It is important to diagnose and treat these tumors in an early stage, when complete resection is possible, because of their resistance to

chemotherapy, and bad prognosis if diagnosed in advanced disease [97, 127]. Most of the type II tumors are sensitive to chemotherapy and will get complete response after initial treatment. Unfortunately the majority will relapse after a median time of 10-18 months [7]. The identification of new biomarkers and tools for early diagnosis may also provide new therapeutic targets and strategies to overcome the problem with chemo resistance, and improve the patient's outcome. Ovarian cyst fluid seems to be a valuable source also for these purposes [177].

There is growing body of evidence that some early lesions, implants on the ovaries and peritoneum, are metastases coming from the distal fallopian tube or endometrial fragments from the uterus via retrograde menstruation, and it has recently been suggested that transitional epithelial nests located at the tuboperitoneal junction are the origin of mucinous and transitional cell (Brenner) tumors. Difference in morphology and diversity in biologic behavior and intrinsic gene expression in EOC needs to be respected in future diagnostic studies [28, 75].

Considerations - Limitations

Neither CA125 nor HE4 are specific markers for EOC, both are increased in ovarian cancer and in various other malignancies and in benign diseases as well. Age is a variable that must be taken into account in the interpretation of their results. CA125 levels decrease with increasing age and therefore relative high levels are found in healthy young women, whilst HE4 levels does the opposite, increase significantly with age [134, 228]. Traditionally the cut-off value of < 35 U/mL is used when CA125 is studied, but currently no cut-off value has been recommended for HE4. According to the manufacturer (Fujirebio Diagnostics Inc.), 23 of 347 women (6.6%) have HE4 >150pM. Among healthy women a

cut-off value of 70 pM was suggested [154]. In a later study the same author used 115 pmol/L for healthy woman as a group, and 89 pmol/L respectively 128 pmol/L in pre- and post menopause [134]. Different threshold values for HE4, pre- respective post- menopausal, and these values may vary depending upon the study population [134]. In our thesis the study population is highly selected, patients already diagnosed and scheduled to operation for an ovarian cyst at a tertiary center. We calculated cut-off for HE4 (premenopausal 71.8 pM and postmenopausal 85 pM) and for ROMA (premenopausal 17.3% and postmenopausal 26%) for this group, and specificity of 75% was used in line with Moore et al. [154]. Recently, in a study of asymptomatic women with high risk of EOC, Urban et al. recommend age-specific thresholds for every decade based on HE4 levels to achieve 95% specificity, because of significantly rapid rise in HE4 after the age of 55, (HE4 ranged from < 41.4 pmol/L for 30 years to 82.1 pmol/L for 80 years) [165].

HE4 is highly dependent on alteration in renal function. In patients with chronic kidney disease the levels of HE4 may be elevated without any cancer disease present, but CA125 was affected only in patients with severe renal failure [229]. In a recent study HE4 was reported to be a fibroblast-derived mediator of renal fibrosis [230]. High levels of HE4 should include control of renal status or eventually presence of coherent kidney disease, and HE4 results should be interpreted cautiously in women with renal disorders.

Meta-analyses have been done on the diagnostic performance of HE4, CA125 and ROMA in women with pelvic mass, and some have been in favor of HE4 and ROMA in identification of ovarian cancer [231-233], and another in favor of CA125 but with higher specificity for both HE4 and ROMA [234]. There was major heterogeneity in the studies design, size, and number of cases and characteristics of the patient populations, cut-off values for HE4, analytical methods, and comparisons between groups. Healthy women, alternatively

women with benign tumors were compared to EOC, malignant including borderline or all malignant tumors, in the different studies. Consequently, the interpretation of the diagnostic performance of HE4 and CA125 is varying between the different published studies [231-234].

Patient's characteristics in this thesis differ from the normal distribution one could expect. The patients were already scheduled for operation because of a suspicious cystic ovarian cancer at a center of excellence, and include therefore relative low proportion of benign tumors (58%). The factum that we only included cystic tumors and only analyzed tumors with ovarian origin, may give some explanation for the high proportion of "old women" in the study; 77% of the women with benign tumor were postmenopausal respective 81% in the malignant group. The healthy "young women" with cysts are planned for operation at the "benign" gynecologic team in our hospital, and were therefore not invited into the study, whilst the older women, because of their health status and coherent diseases, and their general higher risk of actually having an ovarian cancer, were planned for operation by surgeons of the gynecologic oncology team. High number of postmenopausal women in the benign group favors CA125, which generates low levels of CA125 but high levels of HE4, which may have implications on the statistical outcome. Solid ovarian tumors were not included in the study, and these are more often highly malignant than the large cystic tumors [181]. The combination of "old women" with cysts can explain the high proportion (50%) of early stage tumors, with 19.5% (22 of 113 EOC) in stage I grade I EOC , and the low percent (63%) of aggressive type II EOC compared to studies by Kurman and Braicu (75% and 84% respectively) [18, 97]. Type I tumors are more often diagnosed in stage I, like in our study 69% compared to 38% in type II EOC. Moreover, women with type I are reported to be younger than women with type II [97, 235]. However, our type I cohort were relative old with 79% in post menopause respective 83% in type II EOC.

Pelvic mass is a relevant problem to evaluate in the clinical setting. In the present study all benign cystic tumors were included and analyzed, but only cystic EOC among the malignant cysts, sixteen metastases were excluded. We did not either systematically assess for potential confounders or effect modifiers like other diseases, infection, alcohol or medication. The assessment of benign disease contra ovarian cancer or ovarian metastasis or other types of malignancy localized in the pelvis and abdomen is of more interest than just an ovarian cyst. However, the strength of the study includes prospectively and consecutively selected samples that are well documented. All samples were taken at the time of surgery and the process handling of samples is according to a strict protocol. The pathology was analyzed before test result, and PAD was blinded for the personal in the laboratory. Our results from proteomics were validated in real cases of EOC and not in all malignant or with the borderline tumors, which are more like benign cases, included in the malignant group.

CONCLUSION

We could conclude that ovarian cyst fluid can be used in proteomic profiling and that ovarian cyst fluid is a promising source for detection of early biomarkers for EOC. Inflammation is an early event in tumorigenesis and inflammatory response is found in ovarian cyst fluid in early EOC development, in contrast the proinflammatory cytokines were not significantly increased in the blood circulation until later stage. Abundant proteins, similar to blood were present in high amounts in the cyst fluid. By using enriching methods or selective depletion the deep proteome may be more transparent. A specific early markers found in ovarian cyst fluid could possibly be used as molecular imaging targets not only for diagnosis but also in targeted therapy. HE4 and CA125 seem to be promising to use in the evaluation of a malignant suspect ovarian cyst. HE4 was more specific (negative in patients with endometriosis) than CA125 in the benign tumors in premenopausal women, compared to the postmenopausal. HE4 complemented CA125 in the prediction of malignancy in ovarian cysts. HE4 and CA125 are highly representative for the aggressive type II EOC, but failed to properly identify type I EOC tumors. Early diagnosis of ovarian cyst is the key to improve survival and quality of life for patients presenting with a suspicious ovarian cyst. Correct diagnosis of EOC enables accurate referral of patients to specialized tumor centre for evaluating, planning for and performance of the best available therapy in women with malignancy, and unnecessary extensive operations can be avoided in the benign cohort. HE4 and CA125 poorly identify tumors of mucinous subtype. We need specific biomarkers for the various heterogenic diseases that EOC presents to be able to predict the risk of malignancy in ovarian tumors with more accuracy, and ovarian cyst fluid is an excellent source to mine for these markers.

FUTURE PERSPECTIVES

Validation of HE4 and CA125 in women with a pelvic mass in a prospective multicenter study in Västa Götaland and Halland

One of the most urgent lessons from biomarker failures is the lack of validation in appropriate samples before reaching the clinic. We validated HE4 and CA125 in a selected group of women with suspicious malignant cyst already planned for surgery. Adding HE4 to CA125 into the preoperative workup of women with a pelvic tumor could help in the choice of operative procedures. Minimal invasive surgery, such as laparoscopy could be performed in the low risk group, alternative to laparotomy with debulking surgery in the group with high risk for EOC. Before implementation into the clinic a prospective study in appropriate population must be done to further evaluate performance of the markers as a triage instrument in patients with a pelvic mass. A prospective multicenter study, in the region of Västra-Götaland and Halland has started and will evaluate HE4, CA125, RMI and ROMA in a triage of women with pelvic mass. Additionally ultrasound pattern together with HE4 and CA125 will be assessed, and blood tests will be taken to rule out renal- and heart diseases.

Selection and validation of early serous EOC markers

Several proteins from the iTRAQ-study are of interest for further studies. One of them are secreted protein, acidic and rich in cysteine-like 1 (SPARCL1), a glycoprotein down-regulated in EOC, and reported to suppress tumor invasion in variety of tumor types [236]. S100A8 and A9 (Calgranulin A and B) and peroxiredoxin 2 (PRDX2), showed higher expression in the early stage compared to late stage tumors. S100A8 and S100A9 are calcium binding proteins that inhibit casein kinases I and II essential for phosphorylation of

various molecules, which is necessary for normal transcription and translation, and have been found increased in EOC and several other tumors [237, 238]. PRDX2 is H_2O_2 antioxidant protein, is located in the cell nucleus and protects cancer cells from DNA damage-induced cell death, and is suggested to have effect on patient's survival [239]. PRDX1, a family member to PRDX2 increased in EOC as well, and has been correlated to poor survival in serous tumors [240]. Glucose regulated protein 78 (GRP78), increased in EOC and associated to cancer growth and drug resistance. GRP78 is located on the surface of cancerous cells, which make it interesting as a target for both diagnosis and treatment [241, 242]. Isocitrate dehydrogenase 1 (IDHC1) is critical for certain life processes involved in cellular redox homeostasis and normal metabolisms has been associated to platinum resistance [243]. Interestingly, GRP78 and IDHC1, showed increased expression in both of the stage I low-grade serous (type I) tumors included. Low- grade serous EOC is generally known to be chemo-resistant, and these markers could be a potential target to prevent drug resistance. Triosephosphate isomerase1(TPI1) involved in glycolysis and gluconeogenesis, glyceraldehyde-3phosphate dehydrogenase (GAPDH) a key enzyme in glycolysis, and triosine 3-monooxygenase /tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) suggesting a role in regulating insulin sensitivity were as well upregulated in the stage I low grade type I tumors.

Studies on biomarkers, identified by iTRAQ in cyst fluid from serous ovarian cysts that could differ benign from early stage EOC are ongoing in our group. Bioinformatics software, ingenuity pathway analysis (IPA) will be used to select markers to go on with. Selected proteins detected in ovarian cyst fluid will be validated with multiple reaction monitoring (MRM), a targeted, multiplexed assays to screen and quantify proteins in patient plasma samples with high sensitivity, absolute specificity and sufficient throughput.

Noninvasive reliable tests would be the best of choice

The widespread use of Papanicolaou test (Pap-smear) for early detection of cervix cancer leads to reduction in morbidity and mortality from that cancer. Cervical brushing often contains neoplastic cells from the ovary or uterus as well as cells from cervix, but not enough to be used in diagnosis. A technology to detect one mutant template DNA molecular among tens of thousands of normal template molecules has been developed by a research group, at John Hopkins, Baltimore, USA, (PI Professor. Vogelstein). Genes such as *TP53, RAS, PIK3CA and ARID1A* are mutated in EOC and endometrial cancer and these genes can be detected in different biofluids, even when present in small amount. The group mentioned above has studied DNA from liquid Pap- smear specimens to detect DNA from cells shed from endometrial and ovarian cancer that have accumulated in the cervix. EOC was detected in 41% of cases and endometrial cancer was 100% detected [244]. A multicenter prospective study is planned to be performed, to evaluate the ability of liquid based DNA Pap -smear to detect ovarian and endometrial cancer, and our research group will participate in this study. Dr. Vogelstein's group is currently investigating the ovarian cyst fluid with the technology described above, with the purpose to develop a clinically applicable molecular genetic test for use in the diagnosis of EOC and to guide in therapy, in cooperation with our group too.

New surgical technique using specific tissue glue to prevent spill of cyst fluid during abdominal surgery and to minimize the operation has recently been published [248]. Maybe in near future, it will be generally accepted to use minimal invasive needle biopsy or cyst fluid sampling in the preoperative diagnosis assessment, if the diagnostic safety and the accuracy of the diagnosis are improved.

Improvement of biomarkers already detected

Instead of searching for novel biomarkers, improvement of the diagnostic ability of the biomarkers already detected is of interest. The biomarkers are often present in various posttranslational isoforms. Glycosylation promoting or inhibiting tumor cell invasion and metastasis is of crucial importance in current cancer research. Aberrant glycosylation has been observed in EOC and identification of potential glycoprotein biomarkers in proximal fluid (tissue fluid, ovarian cyst fluid and ascites) of EOC [245]. CA125 has multiple different potential glycosylation sites, and differences have been found in N-glycans in serum comparing EOC patients and healthy women. Glycosylated state of CA125 may provide a more specific detection of EOC, and this may also be true for HE4 [246]. We have recently started collaboration, with chemists at the University of Gothenburg, and using ovarian cyst fluid with these questions in focus.

Imaging and targeting approach is of interest

Non invasive techniques such as positron emission tomography (PET) or single photon emission computerized tomography (SPECT) imaging are useful for cancer detection. PET and SPECT imaging shows the chemical function of organs and could be used in targeting selected biomarkers found in the ovarian cyst fluid. Folate receptor α (FR-α), essential for DNA synthesis is over-expressed in 90-95% of EOC. FR-α has been used as a target by tumor specific fluorescence imaging in preoperative guidance of tumor distribution improving both staging and the ability to complete cytoreductive surgery [247].

Targeting approach in therapy is growing, and multiple trials are ongoing. Specific biomarkers indicating chemotherapy resistance enables synthesis of drugs that can be included into the therapy to overcome the challenge of drug

resistance. Magnetic nanoparticles coupled to FR-α to deliver the drugs of choice to the specific tumor site, including multiple drug molecules in one particle, has been performed in vitro [220]. A hundred of distinct micro-RNAs, regulators of genes involved in fundamental cell processes acting in health and disease, have been discovered and are promising to be used in targeted therapy. Developing therapeutic strategies to restore homeostasis by modifying micro-RNA expression may prove to be more comprehensive and successful than targeting individual genes or proteins, as there are only some miRNAs deregulated in cancer [249].

Concluding remarks

Next generation sequencing, next generation biomarkers, and high throughput technologies are continuously developing. Translation of biomarkers detected by these technologies and implementation into clinical practice will be in the near future. Disease specific markers in a panel with combination of proteins and genes, that take into account the great heterogeneity of EOC and the growing evidence that atypical lesions from the fallopian tube and uterus might be the origin of EOC, will hopefully improve the early diagnosis of the different diseases that EOC harbor [28, 179, 250]. Ovarian cyst fluid offers a valuable source for future biomarker discovery and possible targets for therapeutic intervention [177].

SUMMARY IN SWEDISH - SVENSK SAMMANFATTNING

Bakgrund: Äggstockscancer eller epitelial ovarial cancer (EOC) är en av de ledande orsakerna till cancerdöd hos kvinnor i västvärden, men om sjukdomen upptäcks i sin linda finns det goda möjligheter till bot. Tidig diagnos är en riktig utmaning, endast en fjärdedel av tumörerna upptäcks tidigt. Då är cancern begränsad till äggstockarna och den relativa 5-års överlevnad är >90%, detta i motsats till 20-30% då cancern har spridit sig till bukhålan. Under 2011 fick 676 kvinnor diagnosen äggstockscancer och 563 kvinnor dog i sjukdomen det året. Biomarkörer för tidig diagnos saknas och för att ställa diagnosen är invasiv kirurgi inkluderande vävnadsundersökning nödvändig. Avsaknad av prekliniska lesioner, ospecifika symptom, samt äggstockarnas svåråtkomliga läge djupt i bäckenet bidrar till den sena upptäckten av sjukdomen. Tidig diagnos är avgörande för förbättrad överlevnad, då tidig och rätt diagnos möjliggör bästa val av behandling. Operation som utförs på de centra som är specialiserade i tumörkirurgi har visad sig minska morbiditet och förbättra överlevnad vid EOC. CA125 har sedan 30 år används vid diagnos och uppföljning av EOC, men den är tyvärr inte tillförlitlig nog. Trots många års försök att finna någon ersättare har ingen presenterats som riktigt uppnår målet. Nyligen godkände FDA CA125 tillsammans med HE4 en lovande markör, för att användas vid differential diagnostik av bäcken tumör hos kvinnor. Specifika biomarkörer kan vara till hjälp vid tidig diagnos och vätska i tumörens närområde, som cystvätska från ovariet kan innehålla dessa specifika proteiner.

Syfte: Fokus för studierna var att studera möjligheterna för att identifiera nya samt att utvärdera redan etablerade biomarkörer för potentiellt användande vid tidig diagnos. Studierna utfördes på cystvätska och även på blodprover, från kvinnor som opereras för misstänkt äggstockscancer. Ytterligare mål med

studierna var att studera etablerade biomarkörers förmåga att skilja benigna from maligna tumörer och separera långsamt växande typ I tumörer från de mer aggressiva och snabbväxande typ II tumörer.

Metod – Resultat: Studiepopulationen inkluderade kvinnor med cystisk tumör i bäckenet med planerad operation på avdelning för tumörkirurgi SU Sahlgrenska. Proverna, både cystvätska och blod, samlades in prospektivt under operation och analyserades retrospektivt. Ett urval av högeffektiva proteomik metoder användes för att leta nya markörer, medan valideringen av de selekterade proteinerna gjordes med ELISA eller Immunoblot. Benigna, borderline tumörer och EOC jämfördes, för vilka signifikanta skillnader, ROC AUC, cut-off värden, sensitivitet och specificitet beräknades.

Del I. SELDI-TOF MS (n=192) användes för att utforska hela cystväskans proteom, vi kunde då identifiera ApoC-III i kombination med PCI som visade god diagnostik förmåga (ROC AUC 0.91%), när CA125 inkluderades stärktes den diagnostiska förmågan ytterligare (0.94%).

Del II. Med Immunoprecipitation MS (n=38) genomsöktes cystvätskans immunoproteom. Proteiner kända för att vara involverade i cancer inflammation användes för att berika materialet och samtidig komma undan problem med proteiner som förekommer i hög koncentration. Två högt signifikanta kemokiner, MCP-1 och Il-8, validerades tillsammans med GROα i serum, (n=256). MCP-1, Il-8, och GROα visade signifikant högre förekomst i cystvätskan än i blod och dessutom upptäcktes skillnader mellan benigna och maligna cystor tidigare i cystvätskan än i perifera cirkulationen.

Del III. Med iTRAQ fokuserade vi på att hitta markörer för serösa EOC, studerades skillnader i proteinuttryck mellan benigna (n=5) och maligna serösa tumörer (n=10). Två av de 87 signifikanta proteiner, SAA4 och ASTL validerades med immunoblot (n=68) i cystvätska och serum. Valideringen

stärkte de tidigare fynden då även prover med blandade histologier visade signifikant (p=0.001) högre förekoms av SAA4 i cystvätska från EOC jämfört med benigna prover.

Del IV. HE4 och CA125 nivåer i plasma mättes med ELISA och deras förmåga att separera benigna cystor och EOC validerades i vårt material (n=374). CA125 visade generellt bättre diagnostisk förmåga än HE4. Dock kunde man påvisa att HE4 kompletterade CA125 i vissa grupper då framförallt bland fertila kvinnor. ROMA riskstratifiering visade högst ROC AUC (0.89) och sensitivitet (87 %) i post menopaus gruppen. Pre menopaus gruppen hade klart sämre resultat (AUC 0.83 och sensitivitet 81 %).

Del V. HE4 och CA125 validerades (n=373), med EOC uppdelad i typ I och typ II. HE4 och CA125 i kombination, visade bäst förmåga när det gällde att upptäcka den aggressiva sorten typ II (AUC 0.93), men var dock inte tillförlitligt vid tidig diagnos (AUC 0.85). Våra resultat visar ett betydligt sämre resultat för HE4 och CA125 i typ I tumörer (AUC 0.79 och 0.73 för tidiga).

Konklusion: Cystvätska från äggstockscysta innehåller betydligt högre koncentration av proteiner jämfört med blod. Inflammatorisk reaktionen är en tidig förändring i cancer utveckling, och förhöjda kemokin nivåer kunde identifieras tidigare i cancerprogressen i cystvätskan än i blod, vilket kan vara en indikation på att cystvätskan är en utmärkt källa för att identifiera tidiga EOC markörer. HE4 uppvisade ett bättre resultat vad det gäller att identifiera premenopausal kvinnor med malign cysta, samt kunde bättre särskilja benigna tumörer än vad enbart CA125 gjorde. Typ II tumörer upptäcks i hög utsträckning med kombinationen HE4 och CA125, men är undermåliga i typ I och överlag i tidiga EOC. Våra resultat stödjer att EOC är flera olika sjukdomar och vid fortsatta biomarkerstudier ska betraktas som sådan. Vi förespråkar därför att en panel med histologispecifika markörer för varje cancertyp kan öka möjligheten att göra en tidig diagnos.

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