

Clinical implementation of novel diagnostic biomarkers for epithelial ovarian cancer

- Can we improve diagnosis?

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To Fredrik, Irma, Siri and Olga

Never get so busy making a living that you forget to make a life.

Abstract

Background: Epithelial ovarian cancer (EOC) is the most lethal gynecologic cancer. The 5-year survival is about 30%, a consequence of failure to establish early diagnosis due to unspecific symptoms. The biomarker serum CA125 and transvaginal ultrasound are used as the “golden standards” for assessing ovarian cysts and pelvic tumors of unknown origin, but early diagnosis is still not achieved and specificity is low. Increased knowledge about EOC specific mutations has revised our understanding of ovarian cancer etiology and heterogeneity. Rare tumor mutations can be detected in liquid biopsies from different compartments.

Aims: To investigate established EOC biomarkers and algorithms in an unselected population of women with ovarian cysts/pelvic tumors. To compare and combine established and new biomarkers and algorithms to improve differential diagnosis of EOC (paper I-III). To explore new ways for early detection of gynecologic cancer, from circulating tumor DNA (ctDNA) and somatic mutations, by mutation specific analysis in liquid biopsies from the genital tract and plasma (paper IV).

Methods: A prospective multicenter trial (paper I-III) was conducted and we also participated in an international multicenter trial (paper IV) with the aim to improve diagnostic accuracy of EOC and to find new screening methods. Serum was collected for analysis of the biomarkers CA125 and HE4. Risk of Malignancy Index (RMI), Risk of Ovarian Malignancy Algorithm (ROMA) and new algorithms were explored. Patient and tumor characteristics were recorded at the time of inclusion and evaluated by multivariate regression analysis (paper I-III). Liquid biopsies were collected from genital tract thin-prep liquids and plasma. Corresponding formalin fixed and paraffin imbedded tissue biopsies were retrieved from the pathology repository. They were analyzed with a multiplex PCR based barcoding of DNA for mutation detection using next generation sequencing (PapSEEK) for rare mutations (paper IV).

Results:

Paper I: At the recommended cut-off level off >35 , CA125 achieved highest sensitivity (SN) for both pre- and postmenopausal women (SN 95.7%; 92.0%), but low specificity (SP) in both pre- and postmenopausal women (Pre-M 59.6%; Post-M 79.5%). HE4 was inferior compared to CA125 in SN but increased in diagnostic

performance with the highest SP (Pre-M 90.9%; Post-M 92.1%). RMI and ROMA were identical in their predictive ability.

Paper II: Three new algorithms were tested and found to perform better than RMI, ROMA or CA125 alone (GOT-1, GOT-2, GOT-3). The addition of HE4 to CA125 or RMI increased SP without hampering SN.

Paper III: Smoking, heart- or kidney failure and endometriosis should be considered when evaluating CA125 and HE4 levels in women assessed for an ovarian cyst/pelvic tumor of unknown origin.

Paper IV: The PapSEEK technique showed impressively high SP (98.6% cervical; 100% endometrial), and when cervical sampling was combined with plasma ctDNA analysis, SN for ovarian cancer was 63%, with retained SP of 100%.

Conclusion: CA125 was superior to HE4 to identify women with ovarian cancer and HE4 was superior to CA125 to identify benign lesions, in this unselected population of women with an ovarian cyst/pelvic mass from the Western region of Sweden. Addition of HE4 to CA125 increased diagnostic accuracy and decreased false positives. Combining established and novel biomarkers and algorithms improved diagnostic accuracy of ovarian tumors. We suggest that HE4 should be incorporated for the differential diagnosis of women with ovarian cysts/pelvic tumors of unknown origin to decrease unnecessary oophorectomies. It is possible to detect somatic mutations and ctDNA from ovarian cancer in plasma, cervical- and endometrial liquid biopsies with high SP. This thesis demonstrate the potential of a protein and gene mutation-based diagnostic test to detect EOC. With improved technique, this might be a potential test for ovarian cancer screening, in the future.

Sammanfattning på svenska

Äggstockscancer eller epitelial äggstockscancer (EOC) är den mest dödliga gynekologiska cancerformen i världen. Den är svår att upptäcka eftersom sjukdomen föregås av ospecifika symtom såsom magsmärter, mättnadskänsla och känsla av uppkördhet. Diagnosen ställs ofta i ett sent skede, då 5-årsöverlevnaden är mindre än 30%. Med förbättrad diagnostik och möjlighet att ställa diagnos i stadium 1 förbättras 5-årsöverlevnaden till nästan 90%. Biomarkören serum CA125 (CA125) och transvaginalt ultraljud (TVU) används som "golden standard" vid bedömning av ovarialcystor/tumörer i lilla bäckenet av oklart ursprung men dessa metoder möjliggör inte att man hittar cancer tidigt. Genom att förbättra vår kunskap om ursprunget för cancer och den genetiska koden s.k. mutationer för olika typer av EOC, kan vi detektera tumörspecifika mutationer, via analyser från vätskebaserade prov från livmoderhals, livmoder slemhinnan eller cirkulerande tumör DNA (ctDNA) i blodet.

Syftet med avhandlingen var dels att utvärdera etablerade EOC biomarkörer inklusive algoritmer eller kombinationer av dessa i en oselektad population av kvinnor med en ovarialcysta/tumör i lilla bäckenet. Därutöver syftade avhandlingen till att fördjupa kunskapen om vilka andra faktorer som påverkar respektive markör och om nya möjligheter för tidig diagnostik existerar såsom cirkulerande tumör DNA (ctDNA) och somatiska mutationer detekterade med mutationsspecifik analys i vätskebaserade biopsier från genitaltrakten och i blodet.

Vi genomförde en prospektiv multicenterstudie (studie I-III) och deltog som ett av flera centrum i en internationell multicenterstudie (studie IV) med syfte att förbättra diagnostik av EOC samt utvärdera potentiella metoder för framtida screening. Blod samlades in för analys av biomarkörerna CA125 och HE4. Risk of Malignancy Index (RMI), Risk of Ovarian Malignancy Algorithm (ROMA) och tre nya algoritmer utvärderades (GOT-1, GOT-2, GOT-3). I samband med inkluderingen noterades för varje patient andra variabler såsom förekomst av rökning, andra sjukdomar, längd, vikt och ärftlighet. Dessa utvärderades med multivariat regressionsanalys. Ultraljudundersökning genomfördes för bedömning av ovarialcystan/-tumören och beräkningar av algoritmerna Risk of Malignancy Index (RMI) och Risk of Ovarian Malignancy Algorithm gjordes (Studie I-III). Dessutom togs vätskebaserade biopsier från blodet, livmoderhals- och livmoderslemhinnan för analys av mutationer kopplade till gynekologisk cancer och cirkulerande tumör-DNA (ctDNA) i blodet. Formalinfixerade vävnadsbiopsier från tumörerna inhämtades från patologen och analyserades. PapSEEK, en metod för att upptäcka små mutationer i DNA eller kromosomavvikelser med gensekvensering, genomfördes på proverna från

livmoder- och livmoderhals-slemhinnan, tumörvävnad och ctDNA i blodprov (studie IV).

Studie I: Vid rekommenderad cut-off >35 visade biomarkören CA125 högst sensitivitet (SN) hos både pre- och postmenopausala patienter (Pre-M 95.7%; Post-M 92.0%). Dessvärre erhöles en lägre specificitet (SP) vilket betydde en hög andel falskt positiva resultat (Pre-M 59.6%; Post-M 79.5%). Biomarkören HE4 hade en sämre sensitivitet än CA125, men specificiteten (SP) var högre både för pre- och postmenopausala kvinnor (Pre-M 90.9%; Post-M 92.1%). Algoritmerna RMI och ROMA var identiska i sin diagnostiska förmåga.

Studie II: Tre nya algoritmer (GOT-1, GOT-2, GOT-3) utvärderades och de hade alla en bättre diagnostisk förmåga än RMI, ROMA eller CA125. Genom att addera HE4 till CA125 eller RMI förbättrades specificiteten utan att sensitiviteten försämrades.

Studie III: Förekomst av rökning, hjärt- och njursjukdom och endometriosis bör övervägas när serumnivåer av CA125 och HE4 evalueras eftersom det finns fler falskt positiva svar bland de patienterna.

Studie IV: PapSEEK-tekniken visade en mycket hög SP (98.6% livmoderhalsen; 100% livmodersslemhinnan). När vi kombinerade vätskebaserad biopsi från livmoderhalsen och ctDNA i blodet förbättrades sensitiviteten för EOC till 63%, med en bibehållen hög specificitet på 100%.

Sammanfattningsvis; vi fann i vårt material, en icke selekterad kohort av kvinnor i den västra delen av Sverige, att CA125 är en bättre markör för att identifiera kvinnor med cancer än HE4. HE4 är bättre än CA125 på att identifiera benigna ovarialcystor/tumörer i lilla bäckenet. Genom att kombinera nya och etablerade biomarkörer och algoritmer förbättrades den diagnostiska noggrannheten och andelen falskt positiva resultat minskade. Vi föreslår att HE4 bör implementeras i utredningen av ovarialcystor/-tumörer i lilla bäckenet av oklart ursprung för att minska antalet onödiga operationer.

Det sista arbetet i avhandlingen visar att det är möjligt att upptäcka mutationer och ctDNA från ovarialcancer i vätskebaserade biopsier från blod, livmoderhals och livmodersslemhinna med hög specificitet. Avhandlingen demonstrerar potentialen i ett framtida protein- och genmutationsbaserat diagnostiskt test för en förbättrad tidig upptäckt av äggstockscancer (EOC) och testet kan eventuellt bli en metod för screening av äggstockscancer.

List of publications

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

- I. Lycke M, Kristjansdottir B, Sundfeldt K.**
A multicenter clinical trial validating the performance of HE4, CA125, risk of ovarian malignancy algorithm and risk of malignancy index.
Gynecol oncol 2018;151:159-165; doi:10.1016/j.ygyno.2018.08.025
- II. Lycke M, Ulfvenborg B, Kristjansdottir B, Sundfeldt K.**
Increased diagnostic accuracy of adnexal tumors with a combination of established algorithms and biomarkers.
J. Clin. Med. 2020, 9, 299; doi:10.3390/jcm9020299
- III. Lycke M, Ulfvenborg B, Lauesgaard J, Kristjansdottir B, Sundfeldt K.**
Consideration should be given smoking, endometriosis, renal function (eGFR) and age when interpreting CA125 and HE4 in ovarian tumor diagnostics.
Manuscript
- IV. Wang Y, Lu L, Douville C, Cohen J.D, Yen T, Kinde I, Sundfeldt K, Kjaer S.K, Hruban R.H, Shih I, Wang T, Kurman R.J, Springer S, Ptak J, Popoli M, Schaefer J, Silliman N, Dobbyn L, Tanner E.J, Angarita A, Lycke M, Jochumsen K, Afsar B, Danilova L, Levine D.A, Jardon K, Zeng X, Arseneau J, Fu L, Diaz Jr L.A, Karchin R, Tomasetti C, Kinzler K.W, Vogelstein B, Fader A.N, Gilbert L, Papadopoulos N.**
Evaluation of liquid from the Papanicolaou test and other liquid biopsies for the detection of endometrial and ovarian cancers.
Sci. Transl. Med. 10, eaap8793 (2018); doi:10.1126/scitranslmed.aap8793

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Paper I-IV

Abbreviations

ARID1A	AT-rich interactive domain-containing protein 1A
AUC	Area under the curve
BMI	Body mass index
BOT	Borderline type tumors
BRAF	V-raf murine sarcoma viral oncogene homolog B1
BRCA1/2	Breast cancer type 1/2 susceptibility protein
CA125	Cancer antigen 125
cfDNA	Cell-free DNA
ctDNA	Circulating tumor DNA
CI	Confidence interval
CPH-1	Copenhagen index 1
CT	Computed tomography
CTC	Circulating tumor cells
CTNNB1	Catenin-interacting protein-1
EOC	Epithelial ovarian cancer
DNA	Deoxyribonucleic acid
eGFR	estimated Glomerular Filtration Rate
ERBB2 (HER2)	Avian erythroblastic leukemia viral homolog 2
FDA	US Food and drug administration
FIGO	International federation of Gynecology and Obstetrics
GOT	Gothenburg index
HE4	Human epididymis protein 4
HGSC	High grade serous carcinoma
IOTA	International Ovarian Tumor Analysis
IL-8	Interleucin-8
KRAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
LGSC	Low grade serous carcinoma
MAF	Mutant Allele Fraction
MCP-1	Macrophage chemoattractant protein-1
MIA2G	Second generation multivariate index
MRI	Magnetic resonance imaging
NGS	Next Generation Sequencing
NPV	Negative predictive value
NÄL	Norra Älvsborgs Länssjukhus

OC	Ovarian cancer
OVA-1	Multivariate index assay
p53	Tumor protein p53
PAD	Pathologic anatomic diagnosis
Pap brush	Papanicolaou brush
pet-CT	Positron emissions tomography CT
PID	Pelvic inflammatory disease
PIKC3CA	Phosphatidylinositol 3-kinase
PLCO	The prostate, lung, colorectal and ovarian trial
PPV	Positive predictive value
Pre-M	Premenopausal
Post-M	Postmenopausal
PTEN	Phosphatase and Tenson Homologue
RCC	Regional cancer center
RMI	Risk of Malignancy Index
ROC	Receiver Operating Characteristics
ROCA	Risk of ovarian cancer algorithm
ROMA	Risk of Malignancy Algorithm
ROMA-P	Modified ROMA algorithm
R-OPS	Rajavithi-Ovarian Cancer predictive Score
SKAS	Skaraborgs Sjukhus Skövde
SN	Sensitivity
SP	Specificity
STIC	Serous tubal intraepithelial carcinoma
SÄS	Södra Älvsborgs Sjukhus
TAO [®] -brush	Intrauterine sampling brush
TP53	Tumor protein p53 gene
TCGA	The Cancer Genome Atlas
TVU	Transvaginal ultrasound
UID	Unique identifier
UKTOCS	The UK collaborative Trial of ovarian cancer screening

Introduction

Epidemiology

Incidence and prevalence

Globally, more than 295,000 women are diagnosed with ovarian cancer (OC) every year, which accounts for 3.4% of all cancers among women (1, 2). The incidence varies geographically and the highest incidence is seen in the eastern (11.4 per 100,000) and central parts (6.0 per 100,000) of Europe and in North America. The incidence is lowest in Southeast Asia and Africa (Figure 1) (2). In Sweden, the annual incidence is around 700 cases (2014), and OC, including tubal cancer, now constitutes roughly 3% of all female cancers (13/100,000 women), and has declined significantly compared to the 1970s (26/100,000) (3, 4). Ovarian cancer is found among women of all age groups, but it is rare under the age of 30. It is considered to be a disease mainly affecting postmenopausal women and the median age at diagnosis is 50-79 years (5).

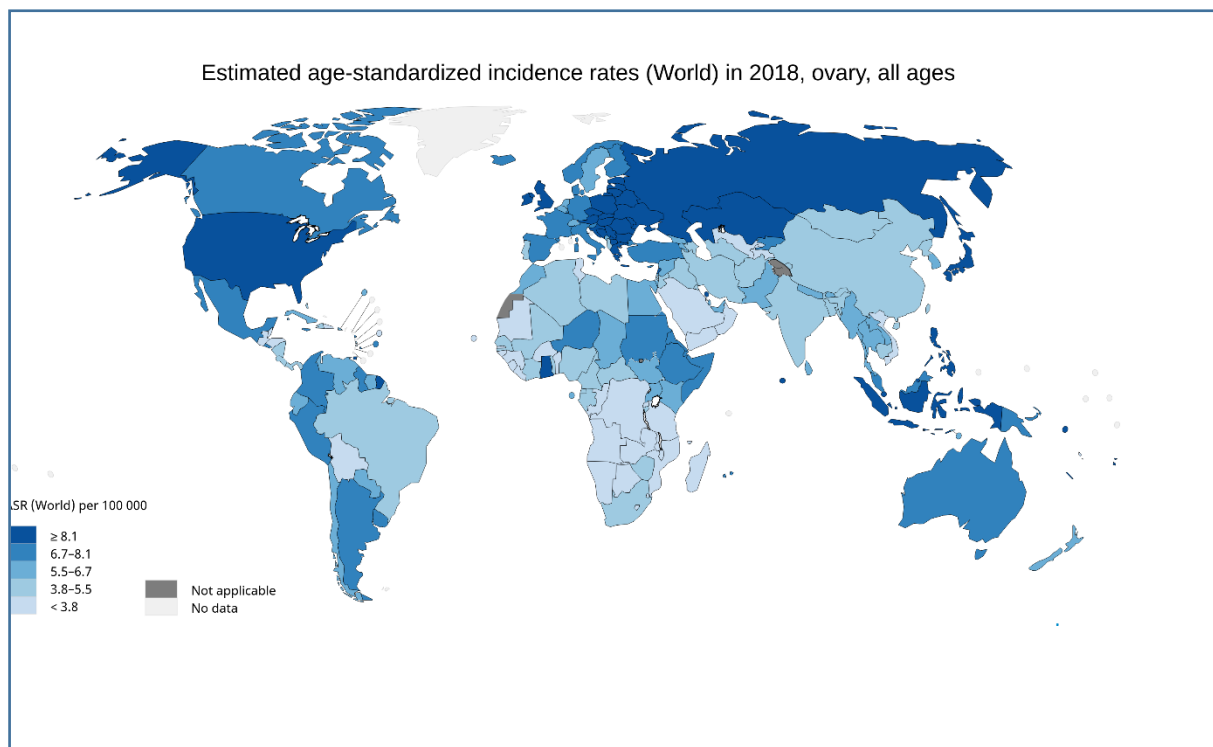


Figure 1. Estimated age-standardized incidence rates of ovarian cancer (world). GLOBOCAN 2018.

Mortality

Ovarian cancer is the most lethal gynecological malignancy worldwide (6) and ranks as number seven most lethal cancer among women (2, 6). This is partly a consequence of the late stage of diagnosis, which today is our major clinical challenge (6). Because the disease typically does not present with symptoms in the early stages most, OC patients have a metastasized cancer already at diagnosis. This phenomenon has led to that we today have almost 185,000 deaths per year in OC worldwide (2). In Sweden disappointing statistical records have been reported with an average of 575 deaths every year (2012-2016 statistics) and the age-standardized mortality rate of 4.9 per 100,000 (Figure 2) (4). The 1-year and 5-year relative survival (RS) rates were of 87 and 49% (2016) (4). In fact, a woman's lifetime risk for acquiring OC is 1 out of 75 and the estimated risk for death in OC is 1 out of 100 (7).

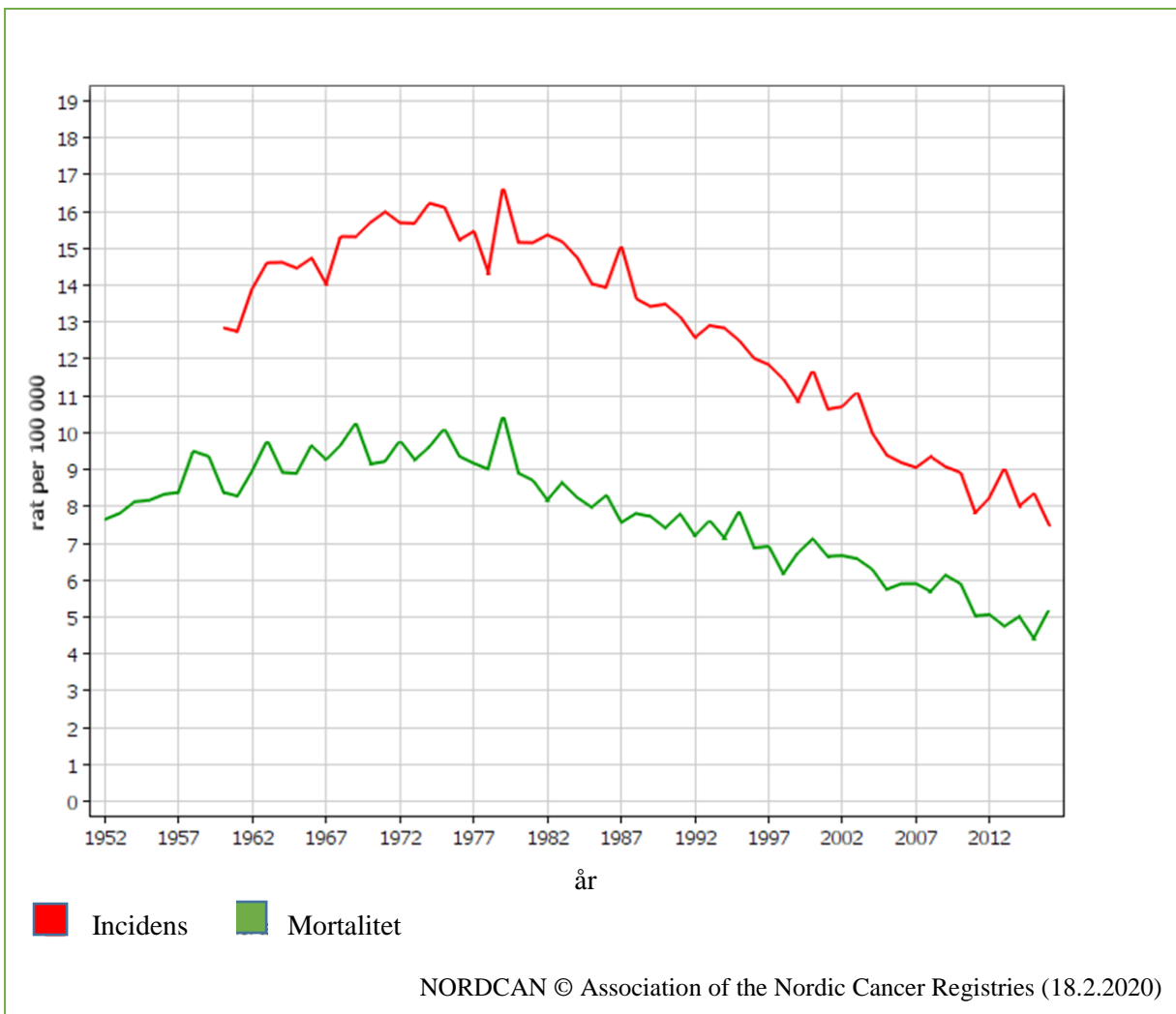


Figure 2. Estimated age-standardized rates of ovarian cancer age 0-85+ in Sweden. NORDCAN 2019.

Etiology

The etiology of OC is complex and still not fully understood. Nearly 95% of OC is derived from the epithelial cells and frequently referred to as epithelial ovarian cancer (EOC). In this context it is worth noting that the coelomic epithelium is the epithelium that lines the surfaces of the abdominal cavity and abdominal organs. Hence, the coelomic epithelium covers the outermost layer of the female gonads and it develops into the peritoneum, pleura and the surface of the ovary. This epithelium is considered to be critically involved in the development of ovarian cancer. Noteworthy, metaplasia of the coelomic epithelium is also linked to endometriosis (8). During embryogenesis the coelomic epithelium will differentiate into granulosa cells upon XX-chromosome directed gonadal sex development. These cells eventually produce anti-Müllerian hormone (AMH) and allows the Müllerian ducts to develop. Therefore, the EOC is a heterogenous disease that differs in histologic subtypes, molecular features, gene expression, pathogenesis and prognosis (9, 10). Whereas, it has traditionally been viewed to evolve from epithelial cells overlaying the ovary, novel studies indicate that most cases of EOC originate from the fallopian tube epithelium, peritoneum or from the endometrium, associated with retrograde menstruation (11, 12). It is important to emphasize that a majority of OC, thus, originate from other gynecological tissues rather than from the ovary itself (1). My thesis will mainly focus on EOC and discuss this disease from many different aspects with special emphasis placed on improved differential diagnosis and early diagnosis in women with an ovarian cyst/pelvic tumor of unknown etiology.

Tumor characteristics

Histopathology

The complex background of EOC has led to that we today divide EOC into subtypes, each with distinct morphological feature and biological behavior. The different histological subtypes differ with regard to their origin, cellular subtypes, molecular features, gene expression and pathogenesis (9). Five main types exist: 1) high-grade serous carcinoma (HGSC) (70%), 2) endometrioid carcinoma (10%), 3) clear cell carcinoma (10%), 4) mucinous carcinoma (3%) and 5) low-grade serous carcinoma (LGSC) (<5%) (Figure 3) (9, 13). Moreover, there are smaller histological types such as seromucinous tumors, Brenner tumors, carcinosarcomas and undifferentiated tumors. Both HGSC and some LGSC are believed to arise from the fallopian tube epithelium and the fimbriated end (11, 14, 15). However, the five main types are

essentially different tumors and, therefore, constitute different diseases. LGSC are associated with borderline tumors and unrelated to hereditary mutations, such as breast cancer type 1/2 susceptibility protein (BRCA1/2) and the Tumor protein p53 (TP53) mutations. HGSC, on the other hand, is not associated with borderline tumors and typically harbor TP53 mutations and BRCA abnormalities. Endometrioid carcinoma, clear-cell carcinoma and seromucinous carcinomas, are possibly associated with endometriosis (16-18). Finally, the origin of mucinous tumors are unknown but some authors suggest they most likely originate from transitional cells at the tubal-mesothelial junction or are metastasis from the gastrointestinal tract (Figure 3) (19, 20).

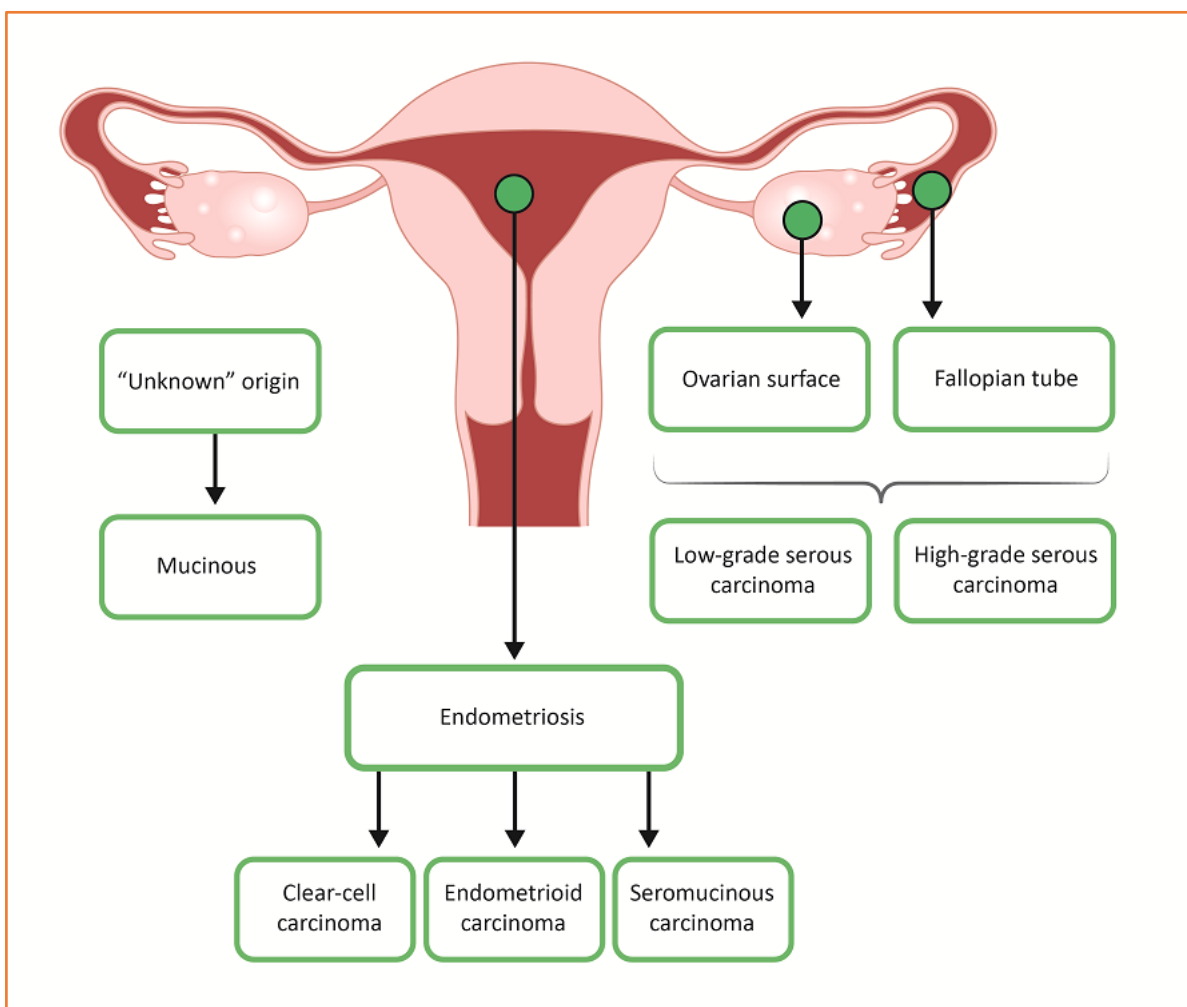


Figure 3. Suggested origin of the EOC “main” subtypes. (Illustration © Jan Funke)

Borderline tumors are a subgroup of epithelial tumors of low malignant potential, as they do not invade the stroma. This is in contrast to the epithelial carcinomas, which readily invade the surrounding stroma. In comparison to benign epithelial tumors (adenomas), borderline tumors can be considered as atypical proliferating epithelium with nuclear atypia. The most common borderline tumors are the serous (55%) and mucinous (40%) types (12). The incidence is 150 cases annually in Sweden, and these tumors account for almost 30% of all epithelial tumors among women under the age of 40 (21). Of note, it is difficult to separate them from malignant tumors when using imaging techniques, such as transvaginal ultrasound (TVU) and computed tomography (CT), and hence complementary diagnostic tools need to be employed. As surgical removal of malignant tumors is an immense challenge there is a need for improved diagnostic tools, especially, since many of the patients with borderline type tumors (BOT) or benign cysts are still fertile and would benefit greatly from less extensive surgery or no surgery at all.

Stage and grade

The recommendations made by the International Federation of Gynecology and Obstetrics (FIGO) are used for staging of EOC. FIGO Stage I-IV, with a higher stage for a more advanced disease, are dependent on their growth path and metastatic status, which has prognostic significance and is also critical for the choice of treatment (Figure 4) (22, 23). Typically a majority (75%) of women diagnosed with EOC have a late stage disease, FIGO III+IV, 5-year survival of <30%. Consequently there is a clear need for earlier diagnosis as the early stage FIGO I+II carries a much better prognosis with a 5-year survival of almost 90%. Thus, early detection is one of the keys to improve the prognosis for EOC patients (24).

The grading of EOC is done according to World Health Organization (WHO) published in 2015 (25). However, during the course of this thesis work, a shift in grading has been implemented due to the improved knowledge about molecular and genetic features of OC. Therefore, we have used both the old and new systems as both tumor stage according to FIGO and type and grade according to WHO critically influence the choice of treatment of EOC. The serous tumors were previously graded according to Silverberg et al and therefore, so were our tumors (26). They were divided into 3 groups (grade 1-3) according to the type of growth, cytological atypia and presence of mitosis. Initially we also used the grading system for endometrioid ovarian cancers as defined for endometrioid tumors in the uterus:

1. Grade 1: well differentiated, with <5% non-squamous or non-morular solid tumor growth.

2. Grade 2: moderately differentiated, with 5-50% non-squamous or non-morular solid tumor growth.
3. Grade 3: poorly differentiated, with >50% non-squamous or non-morular solid tumor growth.

The tumor grade was raised by 1 step if nuclear atypia was present.

The new classification system for EOC involves, as previously, no grading for mucinous and clear-cell type. Seromucinous is a new entity which is associated with endometriosis, and because they harbor AT-rich interactive domain-containing protein 1A (ARID1A) mutations they are more closely related to endometrioid tumors than serous carcinoma (25, 27). Serous- and endometrioid carcinoma are divided into low- and high grade tumors and graded accordingly (25). In fact, previous high-grade endometrioid tumors have been redefined as HGSC (9, 27).

STAGE I: Tumor confined to ovaries	
IA	Tumor limited to 1 ovary or fallopian tube, capsule intact, no tumor on surface, negative peritoneal washings and ascites.
IB	Tumor involves both ovaries or fallopian tubes, otherwise like IA.
	<i>IC Tumor limited to 1 or both ovaries or fallopian tubes including</i>
IC1	Surgical spill.
IC2	Capsule rupture before surgery or tumor on ovarian or tubal surface.
IC3	Malignant cells in the ascites or peritoneal washings.
STAGE II: Tumor involves 1 or both ovaries or the fallopian tubes with pelvic extension (below the pelvic brim) or primary peritoneal cancer	
IIA	Extension and/or implant on uterus and/or Fallopian tubes.
IIB	Extension to other pelvic intraperitoneal tissues.
STAGE III: Tumor involves 1 or both ovaries, or fallopian tube, or a primary peritoneal cancer with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes	
IIIA	(Positive retroperitoneal lymph nodes and /or microscopic metastasis beyond the pelvis.)
IIIA1	Positive retroperitoneal lymph nodes only.
IIIA1(i)	Metastasis ≤10 mm.
IIIA1(ii)	Metastasis >10 mm.
IIIA2	Microscopic, extra pelvic (above the brim) peritoneal involvement ± positive retroperitoneal lymph nodes.
IIIB	Macroscopic, extra pelvic, peritoneal metastasis ≤2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen.
IIIC	Macroscopic, extra pelvic, peritoneal metastasis >2 cm ± positive retroperitoneal lymph nodes. Includes extension to capsule of liver/spleen.
STAGE IV: Distant metastasis excluding peritoneal metastasis	
IVA	Pleural effusion with positive cytology.
IVB	Hepatic and/or splenic parenchymal metastasis, metastasis to extra abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity).
https://www.sgo.org/wp-content/uploads/2012/09/FIGO-Ovarian-Cancer-Staging_1.10.14.pdf	

Figure 4. Staging of ovarian-, tubal- and primary peritoneal cancer according to FIGO.

Dualistic model -Type I and Type II

It is important to note that major advances in our understanding of the pathogenesis of EOC have occurred during the past decades. In particular, the strong link between EOC and the fallopian tube epithelium rather than to the ovary itself has been in focus. HGSC arises from precursor lesions, serous tubal intraepithelial carcinoma (STIC), in the fallopian tube. Localized to the distal fallopian tube or the fimbria in particular, and not from the ovary (28). This was first described in patients with a hereditary risk of OC, i.e. carriers of BRCA1/2 mutations, who were offered to undergo risk-reducing salpingo-oophorectomy (RRSO) (29-31). In the surgical specimens, STIC was found as well as TP53 mutations, in particular. Since the same TP53 mutations were only found in the fallopian tube of these women, it was argued that HGSC, must be derived from the fallopian tube and not from the ovary itself (27, 32, 33). Based on these data Kurman et al proposed a novel progression model including both morphological and molecular features. This simplifying model divides EOC into two different types of tumors; type I and type II (Figure 5) (12, 27, 33-36).

Type I cancers are typically large, cystic and sometimes unilateral. They develop from benign changes implanted in the ovary, which over time become malignant. They are slowly growing and genetically stable (9, 27). Type I tumors comprise of low-grade serous, endometrioid, clear-cell, seromucinous (also called mixed Müllerian tumors), mucinous and Brenner (transitional) tumors. They are proposed to derive from endometriosis sc. endometriosis related tumors (which include endometrioid, clear-cell and seromucinous carcinomas), fallopian tube or primarily the ovary (LGSC), germ cells (mucinous carcinoma) and transitional cells (mucinous carcinoma and Brenner tumors) (27, 37). Except for clear cell carcinomas, type I tumors are considered to be low-grade tumors. Type 1 tumors carry mutations in mismatch repair proteins and signaling proteins involved in cell proliferation such as, V-raf murine sarcoma viral oncogene homolog B1 (BRAF), Kirsten Rat Sarcoma Viral Oncogene Homolog (KRAS), beta-catenin, Phosphatase and Tenson Homologue (PTEN), ARID1, Avian erythroblastic leukemia viral homolog 2 (ERBB (HER2)) but TP53 mutations are not seen (Figure 5) (9, 27, 38). Type I tumors are generally restricted to the ovary when diagnosed (stage 1), and, therefore, they have a more favorable prognosis (9).

Type II tumors account for 75% of all EOC, but are responsible for 90% of all deaths from OC, because they are usually diagnosed at an advanced stage (Table 1) (33, 39). Despite the fact that the response rate to adjuvant chemotherapy is 75%, relapses

occur frequently, and the five-year survival rate is <30% (40). The EOC originates from the fallopian tube epithelium and precursor lesions, STIC, and can develop into aggressive invasive tumors located in the fallopian tube, peritoneum or the ovary. They are characterized by TP53 mutations and are more aggressive, fast growing, and chromosomally instable and harbor frequent amplifications and deletions compared to the slow-growing type I tumors (Figure 5) (9, 27, 38). Type II tumors include HGSC, carcinosarcoma and undifferentiated carcinomas. TP53 mutations are seen in nearly all HGSC patients and in 20% of these tumors also BRCA1/2 mutations are identified (Table 1) (10, 41). Tumors that previously were classified as high grade endometrioid are now classified as HGSC since they also harbor TP53 mutations (27, 42).

Tumors with endometrioid and serous histology are in early stage disease assumed to be associated with a better outcome compared to mucinous and clear-cell histology (40, 43). Clear-cell carcinomas are often diagnosed in an early stage, but are more often associated with a higher frequency of lymph node metastasis (44).

Table 1. Features of the five major subtypes of ovarian carcinoma.

	High-grade serous carcinoma	Low-grade serous carcinoma	Endometrioid	Clear-cell	Mucinous
Frequency	70%	<5%	10%	5-10%	3%
Low- or high-grade	High-grade	Low-grade	Low-grade	High-grade	Low-grade
Dualistic model	Type II	Type I	Type I	Type I	Type I
Precursor lesions	Serous tubal intraepithelial carcinoma (STIC)	Low-grade malignant lesions, serous BOT	Endometriosis, retrograde menstruation	Endometriosis, retrograde menstruation	Low-grade malignant lesions, mucinous BOT
Genetic risc	BRCA1/2		HNPCC/Lynch		
Molecular abnormalities and common mutations	TP 53, BRCA1/2	KRAS, BRAF	PTEN, ARID1A, CTNNB1, PIK3CA, KRAS, CDKN2A, BRAF	ARID1A, PIK3CA, CTNNB1, PTEN, KRAS	KRAS, CDKN2A, PTEN
Usual stage at diagnosis	Advanced	Early or advanced	Early	Early	Early
Prognosis	Poor	Favorable	Favorable	Intermediate	Favorable

Summary overview to highlight differences in origin, prognosis and genetics.

BOT=borderline type tumors, BRCA1/2=breast cancer type 1/2 susceptibility protein, HNPCC=hereditary nonpolyposis colorectal cancer

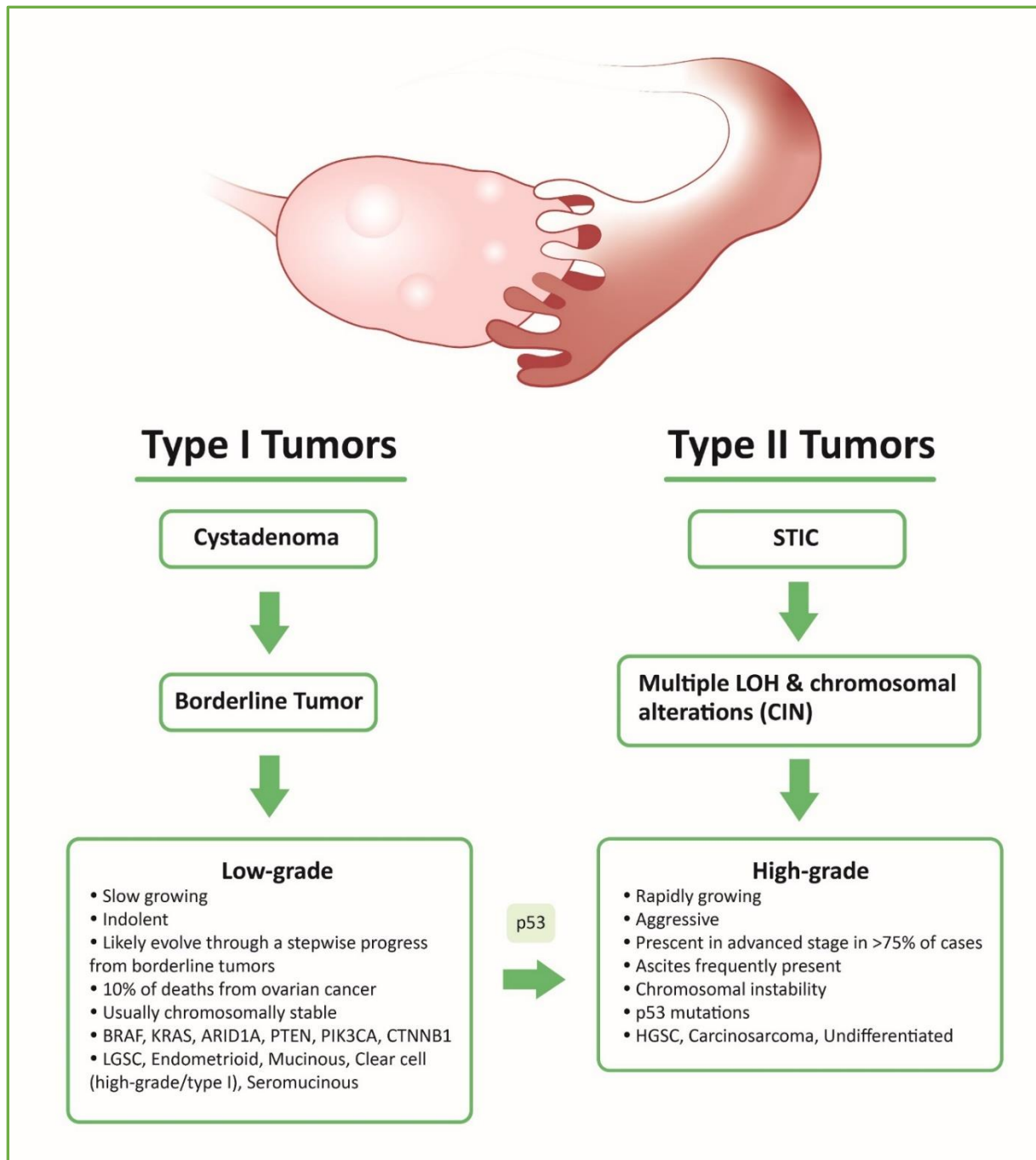


Figure 5. EOC subtypes according to type I and II tumors and the low- and high-grade pathway.

STIC=serous tubal intraepithelial carcinoma, LOH=Loss of heterozygosity, LGSC=low-grade serous carcinoma, HGSC=High-grade serous carcinoma. (Illustration © Jan Funke)

Risk factors for EOC

A number of factors have been associated with an increased risk of EOC including age, obesity, nulliparity, familial history of ovarian cancer and inherited genetic mutations (45). However, genetic inherited mutations are associated with only 5 to 15% of all OC. The most common mutations are BRCA1 and BRCA2, which will be discussed later. Women who have a genetically increased risk of developing OC may benefit from risk-reducing prophylactic surgery after childbirth (46). Noteworthy is that the risk of OC is 3% if you have a first-degree relative with OC and about 15-20% if the patient has a known hereditary risk (47). Also, there might be an increased risk of EOC in women who use hormone replacement therapy (HRT) and in particular estrogen only treated women (48, 49).

The relationship between endometriosis and EOC was first described in 1925 by Sampson *et al.* (50). Between 5- and 15% of the female population suffers from endometriosis and there is an increased risk for clear cell cancer (RR OR 3.05 95% CI 2.43-3.84) and endometrioid cancer (RR OR 2.04 95% CI 1.68-2.48) (51). In a Danish case-control study the relationship between endometriosis and ovarian cancer was shown by the standardized incidence ratio (SIR) to be 1.34 (CI 1.16-1.55) endometrioid (SIR=1.64 (CI 1.09-2.37)) and clear-cell carcinoma (SIR 3.64 (CI 2.36-5.38)) (52). Moreover, endometriosis is detected in 30-55% of all clear cell cancers and in 30-40% of all endometrioid cancers. Endometriosis associated ovarian cancer is more often detected in younger women and more often in earlier stages (53). This being said the overall risk of EOC in women with endometriosis is low, only 3%, and therefore prophylactic bilateral salpingo-oophorectomy (BSO) is not currently recommended as for BRCA1/2 carriers. Genes associated with endometriosis are the tumor suppressor genes TP 53 (5%), ARID1A (50%) and PTEN (20%), as well as the oncogene KRAS (10%) (36, 53).

Endometriosis has been proven correlated to increased risk of ovarian cancer, especially type I endometrioid carcinoma (54). There have been controversies whether other factors of infertility are directly associated with an increased risk of ovarian cancer, although a high number of ovulations and nulliparity have been shown to be associated with an increased risk for EOC (54, 55). The use of fertility medications and the possible increased risk for development of EOC has been controversial and different study results are contradictory but seem to diminish the effect of infertility treatment (56, 57). Nevertheless, during the past decade an explosion of different drugs and treatments have occurred and the impact of

infertility or rather treatment of infertility on risk for EOC must be considered in new trials.

Smoking is yet another known risk factor for EOC with a 50% increased risk for mucinous ovarian carcinoma, while there is no increased risk to develop the other subtypes of OC. On the contrary smoking actually were defined with a tiny decrease in endometrioid and clear-cell carcinomas. This could have to do with the cellular origin of the cancer, endometrioid or clear cell carcinomas (58).

Protective factors

Several factors have been identified to be associated with a lower risk for the development of EOC. A clear risk reduction has been noted for tubal ligation, hysterectomy, parity (especially the first child) as well as a low number of ovulations (54, 55). It should be noted that oral contraceptives that inhibit ovulation have a 20% risk-reducing effect for every 5-year period of use (45, 58). The protective effect is considered to be associated with reduced number of ovulations and continuous even after cessation of treatment (45, 58). Also breastfeeding reduce the life-time risk of EOC (55, 59). Moreover, tubal ligation has been found to reduce the risk for the development of EOC. The largest reduction was seen for endometrioid carcinoma (52%), clear cell carcinoma (42%), but also aggressive mucinous carcinoma (32%) as well as HGSC (20%) (60, 61). In addition, prophylactic oophorectomy in BRCA-positive women has been found to reduce the risk for EOC by 75% (62).

The prophylactic procedure is often performed in premenopausal women with negative effects on the cardiovascular system and unwanted menopausal symptoms. Promising results have shown a risk reduction after salpingectomy with 35-50%. A pooled meta-analyses concluded that the evidence towards the benefit of opportunistic salpingectomy for lifetime risk reduction for EOC were insufficient due to the insufficient evidence and possibly endocrine drawbacks as reported by Muka *et al.* (63, 64). The results from the two ongoing Swedish national RCT trials hysterectomy and opportunistic salpingectomy (HOPPSA) and salpingectomy at time for sterilization (SALSTER) will therefore be exciting.

Diagnosis

The early symptoms of OC are nonspecific and include bloating, dyspepsia, gas pains and backache (65). The routine examination of patients with pelvic symptoms include biomarkers, TVU and bimanual palpation with clinical evaluation (66, 67). Because clinical signs and symptoms are vague and the examination and laboratory testing are seldom conclusive there is an urgent need to improve our efforts to develop better tools and protocols to make early and accurate diagnosis possible. Needless to say, today we experience both a doctor's and a patient's delay in diagnosing EOC (65). Moreover, due to the low incidence of EOC, early detection strategies must achieve a high sensitivity >75%, and an even higher specificity >99.6% to achieve a positive predictive value (PPV) of at least 10% (68, 69). There is an urgent need for improved early detection of OC and much better differential diagnosis to improve the five year survival rate. Additionally, due to the high false positive diagnostic results not only unnecessary surgical complications with increased morbidity can be encountered, but additional hormonal substitution is required as there is an increased risk for cardiovascular mortality in these women (63).

There is growing evidence that screening for OC can improve the clinical outcome for the patients. In several countries, including Sweden, there are screening programs for breast-, colon- and cervical cancer, associated with a significant reduction in mortality (70-72). The key to success is the understanding of the natural history of the cancer and the existence of precursor lesions that can be diagnosed and treated (71). A screening test should be validated, reproducible, non-invasive and inexpensive. More importantly, the test should have a high specificity and negative predictive value (NPV) to avoid the false positive cases. For diseases with a low prevalence in the population, such as EOC, we require from the screening test a very high specificity and sensitivity (>75%) (73, 74). However, the high frequency of false positive cases in OC screening is highly problematic and this is why general screening for EOC cannot be recommended yet (75). Diagnostic tests should be used in women who has symptoms with the intention to identify patients with risk of having a malignancy. A diagnostic test requires a high sensitivity and positive predictive value (PPV) to avoid false negative testing. High specificity is also preferable, due to low count of false positive test results. Unfortunately, the current diagnostic tools allow us to bring together clinical assessments, CT scan, TVU and laboratory testing for markers that are not restricted solely to EOC (76). This combination does not adequately discriminate between EOC and benign tumors.

Hence, there is a significant overrepresentation of benign cases that are subject to surgery and, therefore, too many women, including those with benign tumors, are castrated with subsequent requirement for hormonal substitution (77).

Imaging techniques

There are different methods to diagnose ovarian cyst and other pelvic tumors. The different methods aims to facilitate mapping of tumor spread and to be of help in the pre-operative decision of possible treatment strategies. These different methods include CT, magnetic resonance imaging (MRI), positron emissions tomography CT (Pet-CT), and clinical assessment in combination with ultrasound.

CT has a limited value when it comes to evaluation of tumors in the small pelvis. The method is rarely specific and is more often used when it comes to evaluating metastasized disease. MRI is the one of the imaging techniques that has the greatest specificity and sensitivity in separating benign from malignant tumors (78). PET-CT is not frequently used whereas TVU is the first choice for evaluation of a suspect adnexal tumor. It is easy to use, available at most gynecological clinics and inexpensive compared to other imaging methods, and the adnexal findings can be classified according to different TVU algorithms. The international ovarian tumor analysis group (IOTA) have developed criteria and definitions for diagnosing ovarian tumors (79, 80). Simple rules are categorized with five malignant (M) and five benign (B) criteria's, were the definition of benign includes no M criteria present (79, 80). With the "pattern recognition" adnexal tumors can be classified according to ultrasound criteria. An experienced TVU expert can separate malignant from benign tumors with a sensitivity and specificity of 90% and 94%, respectively (81).

In this thesis work classifications according to the Risk of Malignancy Index (RMI) criteria, presented below were used (82, 83).

Biomarkers

A diagnostic biomarker is one that detects and confirms disease and it must be reliable, precise and its detection must be reproducible (84). Moreover, it can also be used to monitor the status of a disease condition following treatment and alert for signs of relapse (84). A tumor biomarker for clinical use takes time to establish and it happens stepwise from its initial discovery in the neoplastic tissue to the translational and clinical phases of application (85, 86). Finally, successful clinical implementation of a biomarker and its diagnostic importance, complementing other tools, is often cumbersome and slow. Nevertheless, novel biomarkers will become

available as we introduce liquid biopsies, which allows for analyze of cell-free deoxyribonucleic acid (cfDNA), circulating tumor DNA (ctDNA), exosomes and microRNA and the use of epigenetic biomarkers for diagnosing EOC in a more personalized way (87). Indeed, we already have several new biomarkers for EOC found either in blood, urine or ascites (88, 89). However, at present only few of these biomarkers have been approved by the U.S Food and drug administration (FDA) for clinical use. Still the most appreciated biomarkers for EOC are the Cancer Antigen 125 (CA125) and Human epididymis protein 4 (HE4) antigens. I will briefly summarize what we know about these biomarkers.

CA125

The discovery of CA125, a transmembrane glycoprotein encoded by the MUC 16 gene located to chromosome 19, became important in the diagnosis of OC (90-92). However, CA125 was only approved for monitoring treatment and for detecting recurrence of disease by the FDA, and not for preoperative diagnosis (76, 92, 93). Serum levels of CA125 are elevated (>35 U/mL) in 80% of EOC-patients in stages III and IV, but in early stages <50% of EOC score positive for CA125 (94). In addition, this biomarker is elevated in only 30-50% of mucinous cancers, therefore, only a significant correlation between histopathological scoring, FIGO stage and preoperative CA125 levels are seen in a minority of EOC cases (42, 94-96). Unfortunately, the serum CA125 levels can also be elevated in other, both benign and malignant diseases, affecting pleura, pericardium or the peritoneum. These non-specific reactions make CA125 less useful as a diagnostic tool for EOC. In fact, cervical-, breast-, colon-, pancreatic-, lung-, gastric- and liver cancer can all exhibit elevated CA125 serum levels (97-99). Also, benign gynecological disorders including endometriosis, pregnancy, menstrual cycle variations, pelvic inflammatory disease (PID), myoma, salpingitis as well as other non-gynecological conditions, such as cirrhosis, peritoneal inflammation, pericarditis, renal failure, liver disease and congestive heart failure, can exhibit elevated serum CA125 levels (100-104). Serum levels of CA125 are, therefore, a poor diagnostic tool for EOC in early stage and it is critical to know that false positive CA125 serum levels may be found in fertile woman. Thus, CA125 is not a specific biomarker for OC.

HE4

Attempts in the early 90's to identify complementing biomarkers for improved and early detection of EOC resulted in the discovery of the human epididymis 4 (HE4) antigen (105). Similar to CA125 it is a glycoprotein that is overexpressed in some EOC patients. It was first thought to be a more promising biomarker for EOC than

CA125 (106, 107). However, it turned out that HE4 could not be detected in some types of EOC and, therefore, HE4 cannot be used as a stand-alone diagnostic marker for EOC. HE4 is a low molecular weight (13 kilodalton) protein, member of the whey acidic proteins (WAP)-gene family of protease inhibitors. These proteins are characterized by nearly 50 amino acid long sequences with eight highly conserved cysteine residues forming four disulfide bridges (108). The mature glycosylated secretory form (25 kilodalton) contains two WAP four-disulfide core (WFDC) domains encompassing the eight cysteine residues, and is encoded from chromosome 20q12-13.1(109). Elevated serum levels of HE4 are found to be highly associated with endometrioid (100%) and serous carcinomas (93%), whereas only 50% of clear cell carcinoma and 0% of cancers with mucinous histology show enhanced levels of HE4, similar to CA125 (86, 110). Unlike assessments of CA125 levels, no standard cut-off level has been agreed on for HE4 and, hence, different cut-off levels are used by different manufacturers. This is problematic, but an even more confounding factor is the fact that HE4 levels are increased by age (111-115). Nevertheless, serum HE4 levels have been shown to have a higher sensitivity than serum levels of CA125 for diagnosing EOC of stage I and stage III-IV in fertile women (116-122). Importantly, in contrast to CA125, HE4 is not elevated during pregnancy or in endometriosis (112, 113, 116, 123, 124). However, women suffering from renal failure or smokers can have elevated levels of serum HE4 (125).

Several studies have compared the diagnostic value of HE4 as opposed to serum CA125 determinations but the results are difficult to compare due to different analytical platforms, used cut offs and compositions of study populations (116-122). Several groups have calculated optimal cut offs for their study population or used statistical fixed thresholds such as a specificity of 75%. Using different analytical platforms have shown to give rise to a difference in marker value of up to 14% (111). In addition to analyzing CA125 and HE4 alone, several groups have also evaluated a combination of these indicating a higher diagnostic accuracy than used alone (121, 122, 126, 127).

Algorithms

RMI

Because serum levels of CA125 or HE4 alone are not conclusive for diagnosing EOC several combinations with other parameters have been developed and implemented. In particular, the combination of these biomarkers, menopausal status and TVU in an algorithm, Risk of Malignancy Index (RMI), was developed in the 1990s to facilitate the referral of patients to the gynecologists (82). RMI predicts whether the

pelvic mass is a cancer or a benign cyst and it is based on a combination of serum CA125 levels, ultrasound findings and menopausal status. RMI relies on gynecologic ultrasound expertise. The RMI assessment has been modified by Tingulstad *et al.* into RMI 2 in 1996 and further modified into RMI 3 in 1999 (83, 128). The differences between RMI 1, RMI 2 and RMI 3 include an increased emphasis on ultrasound findings and menopausal status (Figure 6). All of the algorithms (RMI I-III) recommends a cut-off value of 200.

RMI calculation U x M x CA125

RMI 1 <i>Jacobs et al. 1996 (73)</i>	Ultrasound (U)	0=imaging score 0 1=imaging score 1 2=imaging score 2-5
	Menopause (M)	1=premenopausal 3=postmenopausal
RMI 2 <i>Tingulstad et al. 1996 (112)</i>	Ultrasound (U)	0-1=imaging score 1 2-5=imaging score 4
	Menopause (M)	1=premenopausal 4=postmenopausal
RMI 3 <i>Tingulstad et al. 1999 (74)</i>	Ultrasound (U)	0-1=imaging score 1 2-5=imaging score 3
	Menopause (M)	1=premenopausal 3=postmenopausal

Cut-off for RMI >200 increased risk for EOC

Figure 6. Algorithm for the Risk of Malignancy Index (RMI) calculations.

Overview of the different RMI calculations (RMI I-III). Differences in values of ultrasound calculation and menopausal status as visualized.

EOC=Epithelial ovarian cancer.

ROMA

Another algorithm developed for EOC differential diagnostics is named Risk Of Malignancy Algorithm (ROMA), and was developed as a tool to triage patients into high or low cancer risk and to find appropriate clinical treatment (118). In contrast to RMI, the ROMA algorithm uses serum levels of both HE4 and CA125 as well as the menopausal status, but ultrasound investigations are not included (Figure 7). Because ultrasound is not used it is believed that ROMA is particularly suitable for primary care (118).

Roma predictive index (PI)

Postmenopausal: $PI = -12.0 + 2.38 \times \text{LN (HE4)} + 0.0626 \times \text{LN (CA125)}$

Postmenopausal: $PI = -8.09 + 1.04 \times \text{LN (HE4)} + 0.732 \times \text{LN (CA125)}$

Predicted probability (%) calculated as ROMA %:

$$\frac{\exp (PI)}{1 + \exp (PI)} \times 100$$

Cut off for ROMA: Premenopausal $\geq 11.4\%$

Postmenopausal $\geq 29.9\%$

Figure 7. Algorithm for calculation of the Risk of Malignancy Algorithm (ROMA).

Moore et al. Gynecol Oncol. 2009;112(1):40-6 (ref 117).

The human genome, DNA Helix and mutations

The human body has approximately 200 different cell types. As cells die and get replaced by new cells it is through cell division, a process that is strictly regulated by specific genes. The human genome consists of over 22,000 genes and a majority of these genes encode different proteins that regulate or are responsible for various cellular functions. This is why all changes in cell function can be monitored through different protein activities. It is, thus, important to remember that tumor development is a consequence of gene mutations, deletions, rearrangements and altered gene regulation and is also reflected in the change of protein functions (10, 129). The mechanism behind cancer development is complex and not fully understood, but genetic changes are frequently found in cancer cells. In fact, genomic instability (GIN) is a hallmark of cancer development with single nucleotide changes to large-scale cytogenetic alterations (129). Subsequently, GIN can affect not only DNA replication and cell cycle progression and checkpoint control, but also have an effect on chromosome segregation and DNA repair. Two main groups of GIN exist: 1) nucleotide instability and 2) chromosomal instability (CIN), including chromosomal rearrangements. Hence, GIN is strongly correlated to tumor progression and associated with poor prognosis. Cancer genomes are often highly complex and contains several mutations besides alterations in chromosomes. Tumor protein p53 (p53) is one of the proteins counteracting GIN (130). In general p53 activates cell cycle arrest and loss of p53 allows cancer cells to develop (131). The EOC with most prominent TP53 mutations is the HGSC.

Another abnormality is aneuploidy, which is when the cell has an abnormal number of chromosomes. Detection of aneuploidy has sparked new hope in early diagnosis

of EOC as >90% of the cancers host aneuploidy and in particular ovarian and endometrial cancers have abnormalities located on chromosomes 7q and 8q (132).

Next generation sequencing and early EOC diagnosis

Today a biomarker does not necessarily has to be a protein detected in blood or any other body fluid. The detection of single cells with genetic changes associated with cancer development is a growing field and much hope exist as to that this technology will help early diagnosis of EOC. To facilitate such investigations liquid biopsies have been introduced and whole cells or cell free DNA (cfDNA) can be sampled and analyzed. In this way we get access to circulating tumor DNA (ctDNA) or mRNA. ctDNA is likely derived from apoptotic cells and can therefore represent a specific tumor marker (133). The next generation sequencing (NGS) technology platform has revolutionized detection of genetic cancer abnormalities and uses a massively parallel sequencing (MPS) strategy, to provide high-throughput sequencing that rapidly can screen millions of DNA or RNA strands. NGS can be used to analyze the presence of specific mutations or whole genome abnormalities. Thus, the NGS technology allows us to sequence the whole genome or shorter genomic regions at a very high speed with excellent resolution. This possibility has accelerated the work to identify new mutated genes or other genetic abnormalities in cancer cells. Further expectations are that the NGS technology will help us to diagnose EOC at an early stage as well as suggest appropriate treatment for a particular cancer.

Because EOC grow with cystic compartments, originate in the fallopian tube and can metastasize through the blood or in the peritoneum we can expect to find circulating tumor cells (CTC), ctDNA or tumor DNA in not only blood but also in urine, cyst fluids, cervical and endometrial smears. These cells or their DNA can be sampled and subjected to NGS analysis (134, 135). If we can identify tumor specific mutations in these liquid biopsies it may also be possible to diagnose cancers at an early stage and be used for screening purposes. The fast development of NGS and advanced DNA or RNA sequencing has enabled a promising new arsenal of non-invasive diagnostic possibilities. It is expected that future improvements of these techniques will greatly help in early diagnosis of EOC and, subsequently, the much improved success of treatments of these cancers, personalized treatment, treatment response monitoring and early relapse detection (135).

Somatic and germline mutations-option for diagnosis?

The somatic mutations that are most important for the onset of EOC are tumor suppressor genes such as TP53, BRCA1/BRCA2 and PTEN (18).

Mutations in PTEN have been found in both endometriosis-associated ovarian cancer and in benign endometriosis cysts. ARID1A is present in 50% of clear cell and in 40% of endometrioid cancers (136). Also, signaling pathway genes, such as KRAS and ERBB2 have been found to carry mutations as TP53, which is involved in DNA repair processes (Table 1). BRCA1/2 mutations are detected in almost 20% of HGSC (41). For women under the age of 40 who carries BRCA1/2, the risk for EOC is 3%, and can increase to 10% by the age of 50 (137). The successively increasing risk for EOC up to the age of 80 is higher for carriers of BRCA 1 (49%) than for carriers of BRCA 2 (21%) (138). For carriers of BRCA1/2 gene mutations diagnosed with EOC, the HGSC is the most common type. Another hereditary condition with EOC is associated with Lynch syndrome, also called hereditary nonpolyposis colorectal cancer (HNPCC), and Lynch syndrome is also associated with endometrial carcinoma (27-71%). Approximately 90% of patients with Lynch syndrome have a mutation in either mutL homolog 1 (MLH1) or mutS homolog 2 (MSH2) and synchronous endometrial cancer is detected in 20% of the women diagnosed with EOC (139). Additionally, mutations in BRCA1 interacting protein C-terminal helicase 1 (BRIP1), RAD51 paralog C (RAD51C) and RAD51 paralog D (RAD51D) are associated with an increased risk for EOC among women over 50 years of age. At present no other malignancies are highly associated with pathogen variants of these genes (140, 141). Genes associated with endometriosis are the tumor suppressor genes TP 53 (5%), ARID1A (50%) and PTEN (20%), as well as oncogenic KRAS (10%) (36, 53).

Prognostic factors

Several prognostic factors have been identified for EOC. For example, the Gynecologic Oncology Group (GOG) studies in the early 90's identified presence of age, ascites, residual disease and histological type to be critical for the prognosis (142, 143). Especially age has been consistently identified as an independent prognostic factor for the outcome of OC (43). Indeed, the prognostic effect of a younger age was persistent even after adjustments for stage, grade and surgical treatment of OC (5). Moreover, almost 30% of patients with EOC are diagnosed with a stage 1 cancer with a 5-year overall survival of >80% (144).

Residual tumor after cytoreductive surgery is another important independent factor for the outcome of EOC (43, 145-147). Bristow et al performed a meta-analysis and

included nearly 6900 women with advanced EOC, and found that with every 10% reduction of residual tumor tissue the median survival of the patient group increased by 5.5%. Improved survival was observed with a residual tumor of <10 mm after surgery (43, 147). Of course, no remaining residual disease after cytoreductive surgery will give the highest progression free survival (PFS) and overall survival (OS) (147, 148). Therefore, referral of patients with advanced OC to specialized tertiary surgical centers for cytoreductive surgery is warranted and should be given the highest priority (147, 149, 150).

Aims

The overall aim of this project was to evaluate new and established biomarkers and algorithms in order to optimize accurate diagnosis of EOC and to eliminate any differential diagnosis in women with unclear tumors in the small pelvis. The ultimate aim was to improve early diagnosis of EOC so that more tumor specific treatments can be developed.

The specific objectives of this PhD project were:

- To test the hypothesis that the biomarker HE4 and algorithm ROMA will improve early detection and differential diagnosis in an unselected population of women diagnosed with an unknown ovarian cyst/pelvic tumor compared to CA125 and RMI (Paper 1).
- To test the hypothesis that adding biomarkers or ultrasound measurements to already established algorithms or biomarkers will improve differential diagnosis of EOC (Paper 2).
- To test the hypothesis that HE4 assessments improve differential diagnosis in women with a benign ovarian disease and moreover increase our knowledge about different potential confounders affecting CA125 and HE4 (Paper 3).
- To test the hypothesis that new approaches, using somatic mutation analysis from liquid biopsies in combination with analysis of circulating tumor DNA and aneuploidy, will improve early detection of EOC and endometrial cancer (Paper 4).

Patients and Methods

This section provides an overview of the patients and methods that were used in the thesis work. For a more detailed description, the reader is referred to the individual papers.

This thesis was based on materials that were sampled in a multicenter study with the name Biomarkers of ovarian cancer (BIOMOVCA) (Papers I-III). It was a validation study built on a previous study performed by our group (121). The BIOMOVCA study was undertaken in collaboration with The Regional Cancer Center (RCC) West for ovarian cancers. Sampling of patients was done from September 2013 until February 2016 for Papers I-III, while for Paper IV the patient material was part of an international multicenter study conducted by Prof. B Vogelstein at Johns Hopkins University, Baltimore, USA.

BIOMOVCA (Paper I-III)

Study design

This project was conducted in collaboration with the ovarian cancer group at the RCC West and all hospitals performing gynecological surgery in the Western Healthcare Region of Sweden and the county of Halland. Women were enrolled by both secondary (SKAS, SÄS, NÄL, Varberg, Halmstad) and tertiary (Sahlgrenska University Hospital) centers, prospectively and consecutively. Based on the study design, only patients from surgical units treating gynecological malignancies were included at Sahlgrenska University Hospital. However, both malignant and benign cases were included from the secondary surgical centers. Women with highly suspected advanced ovarian cancer were referred to the tertiary center of Sahlgrenska University Hospital, according to the established practice and they were only entered once in the BIOMOVCA study.

Power and group sizes were based on statistical estimations of results gained in a previous study performed by our group (121). Since the earlier study enrolled patients with suspected ovarian cancer that had been referred to Sahlgrenska University Hospital from specialized surgeons in gynecological oncology, they can be considered to represent a selected sample material. Therefore, the sampling

method used for BIOMOVCA was set out to secure a representative cohort of women, including women of all age groups and patients with benign lesions. For reference, approximately 21% of the patients entered into BIOMOVCA were diagnosed with malignant lesions as compared to roughly 15% (1 out of 7-10), which was suggested in the study by van Nagell *et al.* (77). In the Partheen *et al.* study, 30% of the women included were diagnosed with malignant lesions, while the study population of BIOMOVCA resemble a more “normal” population, in line with 15%. In Western Sweden around 600 patients are examined every year for suspected pelvic tumor/ovarian cysts. Based on previous experience we estimated that approximately 150 of these 600 patients could be included in the BIOMOVCA study every year. This calculation gave us the estimated time for the trial of 2 years. Hence, the proposed group size was 300 patients and according to our calculations of power we planned for an interim analysis of our material after 150 patients had been enrolled. Importantly, all participating doctors at the different hospitals were informed about the details of the study and we obtained signed agreements for how to store and transport all sampled materials, including serum and base data sheets, as described in more detail in Paper I. Contact persons were identified at the different collaborating hospitals and these individuals were in continuous contact with the research group at Sahlgrenska, reporting on patient records for our coded registrations. In this way we could provide monthly updates on included patients made by the individual hospitals and reports were sent to all representatives in the RCC West Ovarian Cancer Group. The study was registered in the National Institute of Health clinical trial registry (ClinicalTrials.gov NCT03193671).

Statistical considerations – establishing adequate sample sizes

The first interim analysis of the data set was conducted in May 2014 with 158 eligible patients enrolled. The statistical evaluation was performed with ROMA and it gave a sensitivity (SN) of 91.7% (95% confidence interval 0.78-0.98). Based on the results a confidence interval (CI) of 0.83-0.97, was determined, i.e. a margin of error of +/- 7.1%, when reaching a final sample size of 300. Hence, a sufficient SN was achieved but the CI was too wide. Therefore, if we enlarged the sample size to 400 observations so the CI would become narrower with a margin of error of +/- 6%. We decided to perform a new interim assessment after an additional 150 patients and a total of 400 enrolled patients. The second interim analysis was conducted in January 2015 with 319 observations. We found that the SN for ROMA was similar to the first analysis at 91.3% and with a CI 0.78-0.98, which still was too wide. The prevalence of EOC was 18% for the study population. We then extended our study to include over 600 patients, and at the end of 2015 we had collected the desired number of

entries and could close further inclusions into the BIOMOVCA study in late February 2016. In the final cohort, a total of 684 patients were enrolled and a prevalence of 21% EOC.

Baseline data protocol

The design of the study protocol was done in co-operation with the RCC West ovarian cancer group and the study section at the Department of Clinical Chemistry at the Sahlgrenska University Hospital. For each enrolled patient a unique code and baseline data sheet, reporting on lifestyle and health status parameters as well as information from the TVU examination and menopausal status, was obtained. The information was entered into an exclusive protocol registry (Figure 8). The same code was also used to tag the serum samples collected. All women were clinically assessed by a physician. TVU investigations were conducted by gynecologists under training as well as experts on gynecological ultrasound.

Ev. frågor till Maria.Lycke@vgregion.se eller Karin.Sundfeldt@gu.se

Biomarkörer för ovarialcancer projektet - FOU VG-regionen

1. Namn och personnummer:

2. Längd: _____ Vikt: _____

3. Rökning? ja () nej ()

4. Ärförlighet ovarial- eller äggstockscancer? ja () nej ()

5. Annan tumörsjukdom? ja () nej () Vilken? _____

6. Hjärt/kärlsjukdom ja () nej () Vad? _____

7. Njursjukdom ja () nej () Vad? _____

8. HRT (ej lokalt)? ja () nej () Antal år _____

9. Antal födda barn? _____

10. Ultraljudsundersökning Datum (ååmmdd): _____

	JA	NEJ
Flerrummig cysta		
Solida områden		
Bilaterala cystor		
Ascites		
Intraperitoneala metastaser		

11. Menopausstatus

Post-menopausal ja () nej () Vilket år? _____
(1 års blödningsfrihet el. hysterektomerad > 50 år)

Figure 8. Base data sheet.

An exclusive protocol registry of lifestyle and biological parameters including menopausal status, entered for every patient included in the BIOMOVCA study and coded with a unique code.

Serum and tumor PAD samples

All women included in this study received surgical treatment according to agreed clinical practice used at the local hospital, and enrollment into BIOMOVCA had no impact on the surgery performed. Serum samples were collected when patients were enrolled for surgery or at the time of admission and preoperative work-up. As indicated in Paper I, the coded samples collected at SKAS, SÄS and NÄL were shipped to the study section at Sahlgrenska University Hospital the following day for storage at -80 C°. Coded samples from Varberg and Halmstad were stored at the separate including hospitals, as described. Serum sample analysis was performed at the Department of Clinical Chemistry at the Sahlgrenska University Hospital and assessments of HE4, CA125, creatinine, and N terminal brain natriuretic peptide (NT-pro-BNP) were determined using the Cobas 6000 or 8000 instruments (151).

Tissue biopsies were examined by an experienced pathologist for pathologic anatomic diagnosis (PAD), which included histological assessment and grading. All EOC PAD specimens were additionally screened by a pathologist specialized in gynecologic oncology at the Sahlgrenska University Hospital. Staging of tumors was done according to the recommendations given by the International Federation of Gynecology and Obstetrics (FIGO) 2014 standards (Figure 4). The correct cancer diagnose, grade and stage were obtained from the National Information Network for cancer treatment (INCA) and the information was entered into the BIOMOVCA registry. Diagnoses of benign tumors were retrieved from the pathological reports entered into Melior or “vårdadministrativt system” (VAS), the hospital journal charts used in Western Healthcare Region and Halland, respectively.

Analysis of biomarkers

In earlier studies assessments of HE4 and CA125 have been done by enzyme linked immunosorbent assays (ELISA). This is a standard technology in clinical laboratories with a high acceptance and excellent reproducibility. However, it is not the most sensitive assay to determine proteins in biological samples. In 2010 FDA approved an automated HE4-assay based on electrochemiluminescence immune-assay (ECLIA), to mitigate the shortcomings of ELISA. Determinations of CA125 II, HE4, NT-pro-BNP and creatinine concentrations were therefore done on the of ECLIA Cobas 6000® or 8000®, e602 and e701 analyzer (Roche Diagnostics Scandinavia, Stockholm, Sweden). The ECLIA assay is a highly sensitive and specific method for assessing these proteins. Briefly, CA125 and HE4 were determined by two types of antibodies in combination with an electrode potential leading to a photon emission as visualized for HE4 in figure 9. The reference value

for a positive sample was set to >35 U/ mL for CA125 (90), and for HE4 we used predetermined thresholds >70 and >140 pmol/L for premenopausal and postmenopausal women, respectively (152-154).

PCR and NGS

Polymerase chain reaction (PCR) and next generation sequencing (NGS) were used for identifications of mutations in gene sequences, as described in some detail in Paper IV. Briefly, whereas PCR is used to amplify or copy a specific DNA sequence, NGS can rapidly sequence the whole genome, or particular genes or target regions of interest. With NGS the targeted sequencing approach can include the genome (the protein-coding portion of the genome), specific genes or targets within genes or mitochondrial DNA. The NGS technology enables the search for rare mutations at large scale and provides unprecedented insights into how and when malignancies may occur in human cells. In fact, assessments of genetic and epigenetic mutations in tumor cells has greatly advanced cancer research, which is also seen in basic research on OC. The NGS technology will significantly contribute to the discovery of additional rare mutations and help in the early diagnosis of EOC. Because of the requirement of lower DNA input compared to previous technologies, NGS has enabled cancer research, where sometimes rare somatic mutations are present. Such as discovering residual tumor cells in the margins after surgery and monitoring effectiveness of cancer therapies by analyzing liquid biopsies for cancer cell gene signatures. Importantly, it will enable improvements in early detection of cancer, which for EOC is of outermost importance.

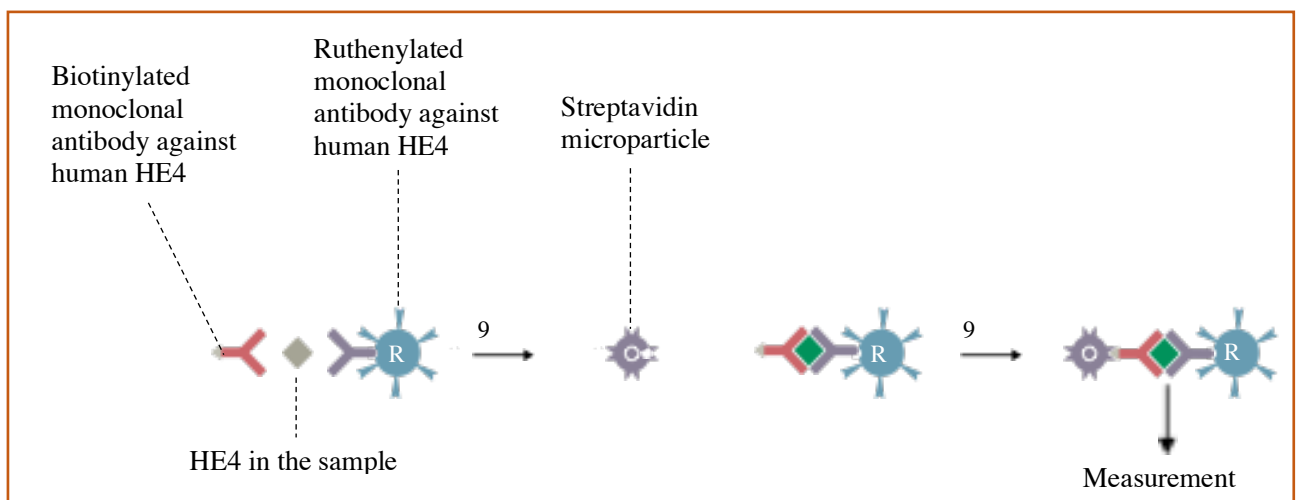


Figure 9. ElectroChemiLuminescence immunoassay (ECLIA) detection.

Two types of antibodies were respectively used to make a “sandwich”: One antibody recognizing the analyte was labeled with ruthenium and the other with biotin. With the addition of streptavidin, then of tripropylamine (TPA) and the application of electrode potential, ruthenium was excited, leading to photon emission. (Illustration from Cobas Roche Diagnostics, with permission from Roche Diagnostics Scandinavia AB).

Pap smear and TAO brush sampling

Pap smear test was initially invented by Papanicolaou in the 1920s and simplified in 1957 by A Hilliard (155). It has revolutionized the prevention of cervical cancer throughout the world, where screening programs for cervical cancer are implemented. Incidence and mortality have been reduced by more than 75% (156). Since the traditional pap-smears have been replaced by a liquid-based method, the possibility for DNA detection as well as cytological analysis have been possible in a non-invasive easy-to-use test. Cells, not only from the cervix but also from the endometrium in the uterus cavity and the tubal lining including the ovaries, shed from the tumors via the tubes, and can be identified from samples taken from the cervix. A previous pilot study, evaluated DNA collected from routinely taken pap smears. The results indicated a promising step towards a screening program for all gynecologic malignancies in the future with this method. With massively parallel sequencing, mutations in 100% of endometrial cancers and 41% of ovarian cancers were detected both in the tumor and the liquid Pap smear (157). As described, identification of tumor cells from other gynecological organs was made possible. To be able to get even closer to the original tumor, and in that way perhaps identify more tumor DNA, the TAO brush (The Indiana University Medical Center Endometrial Sampler) was used in this project (Paper IV). It was introduced as early as 1993 and approved by the FDA for general medical use. Initially it was used for endometrial sampling and cytology analyses of the brushing specimen from the endometrium (158). It has an outer sheath to avoid contamination from the vagina and endocervix, which is pulled back when applied in the uterus cavity. The brush is then rotated 360° counterclockwise for collection of DNA. The outer sheath is then pushed forward again and the TAO brush removed, and immediately placed in thin-prep buffers for disposal of DNA described above (Figure 10).

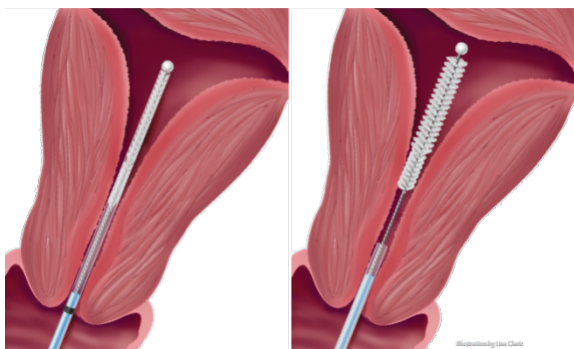


Figure 10. Tao Brush™ IUMC Endometrial Sampler. A 3.5 cm long flexible brush that allows sampling from the endometrial cavity. A plastic sheet protects the brush from endocervical and vaginal contamination during insertion and extraction.

(Illustration and permission from www.cookmedical.com/p/tao)

Here follows a short summary of the methods used in the individual papers that were discussed:

Paper I

In Paper I we included 638 patients selected from the cohort of 684 women using the exclusion criteria described in Paper I (Figure 11). Patients were categorized according to the diagnosis into three main groups; benign tumors (B), borderline type tumors (BOT) and malignant tumors (M). The M group was further subdivided into epithelial ovarian cancers (EOC) and a metastasis group, which consisted of non-epithelial ovarian cancers (Non-EOC) and metastasis of non-ovarian origin to the ovary. The EOC group was subdivided into early (FIGO stage I+II) and late stage (FIGO stage III+IV) tumors, as well as type I and type II tumors according to the dualistic model. All women were classified into premenopausal (Pre-M) or postmenopausal (Post-M) status groups. Assessments of serum levels of CA125 and HE4, and calculations of RMI and ROMA were performed as described in detail in Paper I.

In Paper I we used RMI 1, calculated according to the criteria described by Jacobs *et al.*, whereas in Paper II we used RMI 3 calculations described by Tingulstad *et al.* (82, 83). The difference between the two algorithms is the value yielded by the ultrasound score. The ultrasound was evaluated according to 5 different parameters: multi-locular cyst, solid parts, bilateral cysts, presence of ascites and presence of intraabdominal metastases, and results in one point each. A unilocular cyst gives zero points in RMI 1, while in RMI 3 it will give a score of 1 (Figure 6). The different RMI calculations have been reviewed to compare the accuracy of the predictive models and it was found that the RMI 1 (Jacobs *et al.*) reached the highest sensitivity and specificity at cut-off 200 (159). The Swedish National Guidelines for OC diagnosis recommended RMI 1 at the time of writing Paper I (82).

The statistical analyses were performed using STATA 13.1 (Stata Corp, Texas, USA). Categorical variables were presented as count and percentages, whereas the descriptive statistics were presented as median and range. Diagnostic performance was assessed by sensitivity (SN), specificity (SP), negative predictive value (NPV) and positive predictive value (PPV) for HE4, CA125, RMI and ROMA. Statistics were calculated for the individual groups subdivided for menopausal status accordingly; B vs EOC, B vs EOC+BOT, B vs EOC FIGO I+II (early), and B vs

EOC FIGO III+IV (late). Diagnostic accuracy was evaluated through receiver operating characteristics (ROC) and area under the curve (AUC) calculations. Statistical differences between groups for the separate markers were assessed using the Mann-Whitney U test. All tests were two-tailed and p -value <0.05 were considered statistically significant. We could have used a parametric test for calculating statistical significance in the main group B vs EOC, (without adjustment according to menopausal status), but the sub-groups were small and these data sets were skewed with mean $>$ median values, which is why a non-parametrical test was chosen in all analyses. Whereas SN and SP are descriptors of the diagnostic ability of a test, they cannot directly indicate the probability if a patient is ill or not. Hence, it is critical with a high SN for OC patients as we want to find all cancer patients while a high SP, will help exclude healthy individuals. We therefore calculated the predictive values NPV and PPV, which are dependent on SN and SP as well as the prevalence of the disease. For diseases with a low prevalence, such as OC, a high SP is needed to maintain a high PPV, which is why PPV can be difficult to include as a predictor of OC. On the other hand, ROC AUC visualizes the proportion of true positives and the percentage of false positives in a diagnostic test at different thresholds. The challenge is to find a numerical threshold that gives a reasonable balance between high SN and low false positive results. In fact, this is the most common approach to evaluate the diagnostic ability of these types of tests.

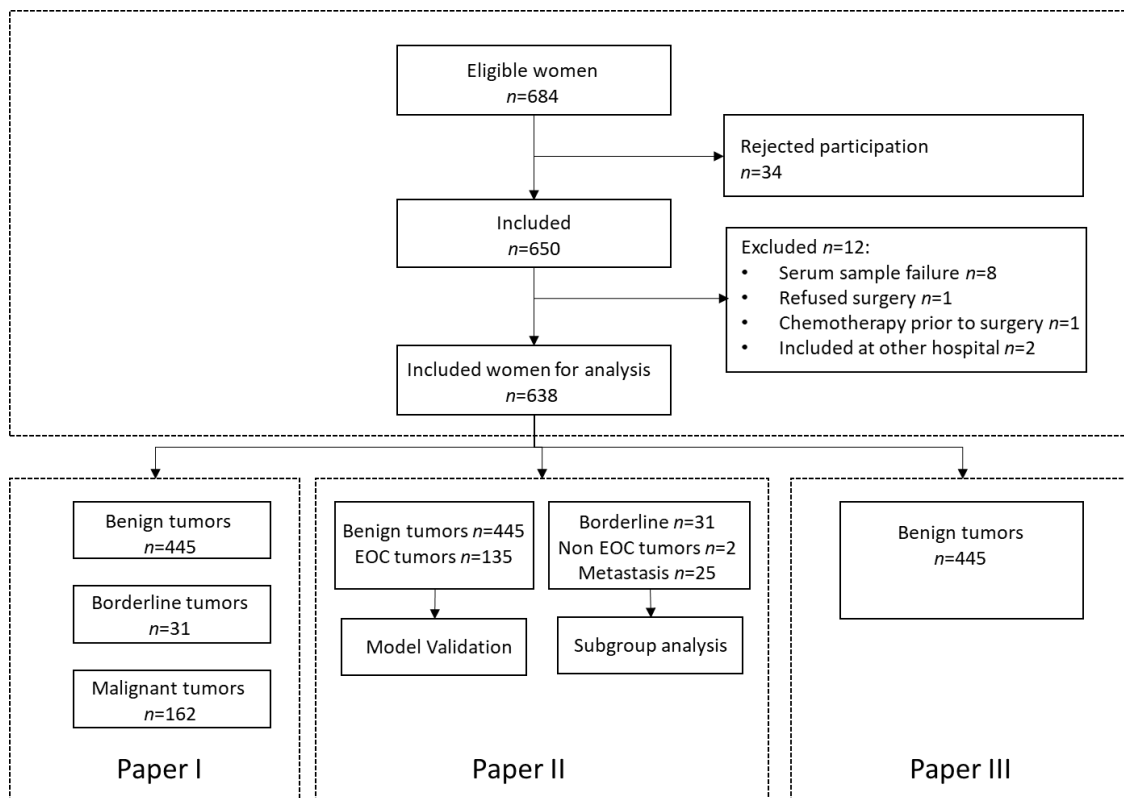


Figure 11. Flowchart of eligible patients for the BIOMOVCA study (paper I-III).

Paper II

The same study population and exclusion criteria were used as described for Paper I (Figure 11). The metastasis to the ovary group (n=25) and non-epithelial ovarian cancer tumors (n=2) were grouped together. Patients with EOC tumors were also subdivided according to molecular genetics and histopathological type I and type II. BOT, metastasis to the ovary and non-EOC tumors were analyzed in subgroups, as described in Paper II. RMI 3 was used according to Tingulstad *et al.*, which was preferable from a statistical modeling perspective, as more patients will be included in the study (including women with a unilocular single-sided cyst), taking into account the expected normal distribution of women with benign ovarian cysts. We used the R version 3.6.1 (R Core Team 2019). The aim of this study was to improve already established markers (RMI, ROMA and CA125) by combining them with HE4 or TVU. Logistic regression analysis was used since our primary response variable were binary (cancer/no cancer) and our data contained several outliers, not normally distributed. Three logistic regression models were fitted: RMI with the addition of HE4, ROMA with the addition of TVU score and the dual combination CA125 and HE4. RMI, CA125 and HE4 were log₂-transformed before fitting the models to reduce the effects of extreme values. The benign and EOC samples were coded with a binary variable, where benign=0 and EOC=1 (cases).

The algorithms were established according to the following template:

$$Y=a+b_1x_1+b_2x_2$$

The three new logistic regression models were named Gothenburg index (GOT)-1, GOT-2 and GOT-3 (Figure 12).

$$\text{GOT-1} = 0.62 \times \log_2(\text{RMI}) + 1.05 \times \log_2(\text{HE4})$$

$$\text{GOT-2} = 0.59 \times \log_2(\text{CA125}) + 1.31 \times \log_2(\text{HE4})$$

$$\text{GOT-3} = 7.02 \times \text{ROMA} + 0.68 \times \text{TVU}$$

Figure 12. Overview of the logistic regression models named Gothenburg index (GOT)-1, GOT-2 and GOT-3 established according to the following template $Y=a+b_1x_1+b_2x_2$. The intercept (a) was dropped for simplicity.

To investigate the performance of the new models for discriminating between benign and EOC tumors, a target sensitivity (target SN) was calculated using the markers (RMI, ROMA, CA125) with established cut-off values (Paper II). From target SN, specificity (SP) and ROC AUC were calculated to determine the diagnostic capacity. Additionally, we used a target specificity (SP 75%) and calculated GOT 1-3 sensitivity in order to compare our results with other research groups in the field. A set SP of 75% is an often used SP for diagnostics, but an acceptance for as many as 25% false positive cases can be discussed (118, 160).

The likelihood ratio test was used for comparison between GOT 1-3 and their baseline models. A p -value of <0.05 was considered statistically significant.

Models were validated using leave-one-out cross fold validation, with the dataset of benign and EOC diagnosed women ($n=580$). Thus, the cohort was split into a training set and a validation set 580 times, with 579 samples in the training set and the remaining sample in the test set. Inside the cross-validation loop, the model was fit with the training set and evaluated on the test set. Performance of the model was calculated based on all 580 iterations, to obtain a realistic estimate of SN, SP and ROC AUC.

Improving and exploring new and old algorithms can preferably be conducted on two separate study cohorts, using one for model preparation and one cohort for testing (external validation). However, since we only had one cohort for both model preparation and validation, we used leave-one-out cross validation to mimic external validation.

Paper III

The aim of this study was to examine how different lifestyles and biological factors impacted on serum levels of the biomarkers CA125 and HE4. For this purpose we used the benign cohort described in Paper I, consisting of 445 women diagnosed with a benign ovarian cyst (Figure 11).

At the time for enrolment a base data sheet was compiled by the handling physician for each patient. The data sheet included information about age, smoking habits, weight, length, endometriosis, parity, hormone replacement therapy (HRT), renal

disease, heart disease, other cancer diseases and heredity for ovarian- and/or breast cancer (Figure 8).

Body mass index (BMI) was calculated according to the standardized procedure, weight and length in the following formula kg/m^2 . As a measure of heart failure NT-pro-BNP was analyzed. It is a natriuretic peptide that is released from the myocardial tissue as a result of myocardial wall extension.

Estimated glomerular filtration rate (eGFR) is a measure of the glomerular filtration and refers to the amount of fluid that is filtrated from the glomerular capillaries to the tubuli system per minute in the nephrons. As a measure for renal failure, serum creatinine was analyzed and eGFR was calculated according to the revised Lund-Malmö equation (161). With impaired renal function, eGFR will decrease. Commonly, normal renal function is associated to $\text{eGFR} > 90 \text{ ml/min/1.73}$.

The descriptive statistics were presented as mean values and standard deviations (SD) as well as median values and range. Categorical variables were presented as mean counts and percentages. As in Paper II, statistical analyses were done with the R version 3.6.1 (R Core Team 2019). A multivariate logistic regression model was created to distinguish between false positives (FP) and true negatives (TN), predicted using CA125 and HE4 with established cut-off values. As described, odds ratios (OR) and the 95% confidence interval (95% CI) of predictors were calculated for the risk of being classified as a false positive by the marker. The following categorical predictors were included and dichotomized to fit the model: pelvic inflammatory disease (PID), endometriosis, HRT, smoking, other cancer diseases and heredity for ovarian- and/or breast cancer. The following continuous predictors were included: age, eGFR, NT-pro-BNP, BMI and parity. Overall, we identified missing data for in women of the 445 enrolled and of these were 61 missing heredity, four smoking and four other cancer diseases. All missing values were imputed as not present and replaced with 0=no. Whereas a linear regression model could not be applied to the data due to non-normal distribution, i.e even after log transformation as for HE4, in particular, we used logistic regression analysis. The likelihood ratio test was used to assess the statistical significance of the separate predictors. Benjamini-Hochberg correction was performed to adjust for multiple testing. Adjusted p -value of < 0.05 was considered statistically significant. Because of the logistic regression model approach, we were limited to examine only the change (increase or decrease) in serum biomarker levels relative to a threshold level, and not the actual concentration of the biomarker. However, the statistically significant predictors showed strong

evidence for association. Noteworthy, imputing missing values as not present was considered reasonable, since the values were not listed in the base data sheet and therefore interpreted as not present. The results should be interpreted with some caution since, not finding an association does not mean that it does not exist.

Paper IV

This was an international multicenter study of women diagnosed with or suspicion of having EOC or endometrial cancer (EC), and was coordinated and executed by prof. Vogelstein. The cohort consisted of 1658 women and samples were collected and analyzed retrospectively at the Ludwig Center for Cancer Genetics and Therapeutics, at John Hopkins University School of Medicine, in Baltimore, USA. The patient material represented women attending gynecological clinics at six different centers, including three centers in the U.S, and one center in Canada, Denmark and Sweden, respectively. The sampling involved Pap-brush sampling from the cervix only at the different centers. One center in the US also sampled TAO brush specimens from the uterine cavity. This way cells or tumor DNA, not only from the cervix, but also from the endometrium in the uterine cavity, or from the tubal lining or the ovaries, could be sampled. Plasma was withdrawn prior to surgery in cell-free DNA blood collection tubes (BCT, Streck tubes 2189629), used for stabilizing nucleated blood cells allowing isolation of cell-free DNA (Figure 13).

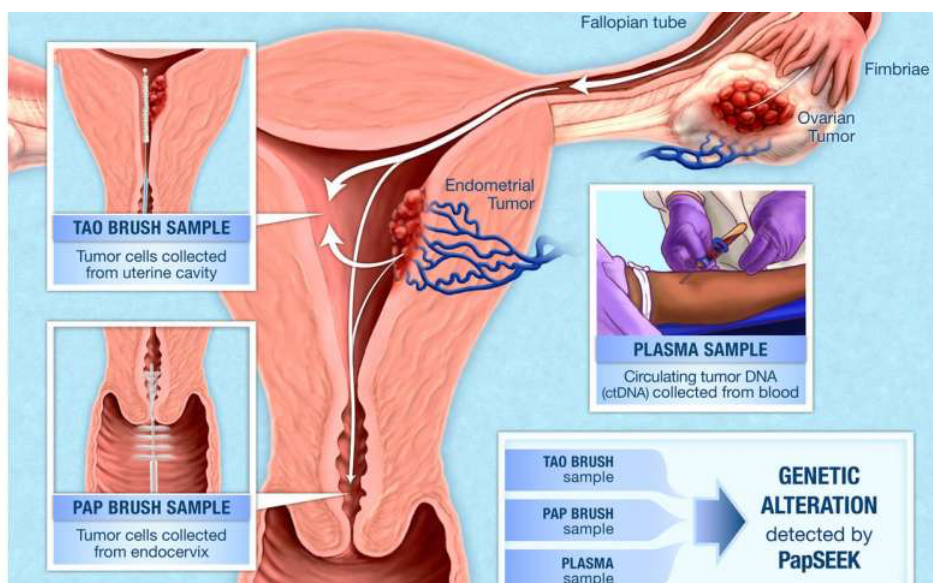


Figure 13. PapSEEK tests for the detection of tumor DNA in the Pap brush, TAO brush and plasma samples.

Wang et al., *Sci. Transl. Med.* 10, eaap8793 (2018).

All specimens were coded and stored at -80° before being shipped to the laboratory in Baltimore. Indeed, using this technique, a previous study has detected mutations in 100% of endometrial cancers and 41% of the ovarian cancers (157). The study also involved re-reviewing the tumor PAD by pathologists specialized in gynecological malignancies at the Baltimore center.

Somatic mutation-specific detection and aneuploidy (PapSEEK)

By using whole genome sequencing, mutations typical for the different histological subgroups of ovarian- or endometrial cancers can be detected (10). Hence, the NGS analysis in Paper IV was developed based on detection of mutations in selected genes (16 or 18 genes) as described in Table 2 (10, 157, 162). Countable mutations were identified on the basis of the following criteria: a) mutations were present in the COSMIC database of somatic mutations of human cancer cells (163) or b) nonsense mutations, out-of-frame insertions, splice site mutations or gene deletions that were located in tumor suppressor genes (163). To score positive, a sample had to be positive either for a somatic mutation (Safe-seq) or aneuploidy (Fast-seq), and was then given a PapSEEK positive score. Even if a test result was negative for a somatic mutation in any of the 18 genes assessed, the PapSEEK test could still be positive if aneuploidy was detected. COSMIC is a catalogue of somatic mutations, a high-resolution resource in the genetics of human cancer (163). It encompasses all forms of human cancer, from large scale tumors to extremely rare variants. The database contains 1,235,846 tumor samples and 4,067,689 observed coding mutations (version 78, September 2016) (163). NGS have disadvantages and can therefore not be used in general to detect rare low-count variants due to the high error rate which occurs during the sequence process. DNA changes in the end product after sequencing may be a) errors that are introduced when the template of interest is produced, b) errors during the amplification step when the library are produced or c) errors during amplification in the instrument used. Compared to NGS, the Safe-seq technology (used on both Pap brush, TAO brush and plasma samples) has enabled more reliable sequencing data for the detection of rare mutations, which are typifying EOC. First a unique identifier (UID) is added to one primer in each pair in every DNA template to be analyzed. The UIDs consists of 14 degenerate bases with an equal chance of being an A, C, T or G. Secondly, amplification of the template of interest is carried out with the unique identifier to create many daughter molecules containing the identical sequence (called UID family). If a mutation is present in the template molecule, which was used in the amplification process, it should also be present in every daughter molecule containing that UID. An UID family where at

least 95% of the family members contain the identical mutation is called a supermutant (Figure 14) (164).

Fast-seq is an amplicon-based approach for detection of aneuploidy. One single primer pair is used to amplify 38.000 long interspersed nuclear elements (LINEs) with PCR, where read depth at a locus is compared with the overall mean. The sequence data is analyzed with Within-Sample AneupLoidy DetectiOn (WALDO), which is an algorithm for amplicon-based aneuploidy detection. It incorporates a support vector machine (SVM), a learning model that categorizes samples as either aneuploid or euploid, using a non-probabilistic binary linear classifier (Figure 14).

Table 2. Driver mutations evaluated for plasma, pap- and TAO brush samples.

Driver mutations			
18 somatic mutations detection in Pap- and TAO brush samples	Tumor specific	16 somatic mutations detection in plasma	Tumor specific
AKT1	EC	AKT1	EC
APC	EC	BRAF	OC
BRAF	OC	CDKN2A	OC
CDKN2A	OC	CTNNB1	OC, EC
CTNNB1	OC, EC	EGFR	EC
EGFR	EC	FBXW7	EC
FBXW7	EC	FGFR2	EC
FGFR2	EC	KRAS	OC, EC
KRAS	OC, EC	NRAS	EC
MAPK1	EC	PIK3CA	OC, EC
NRAS	EC	PPP2R1A	OC, EC
PIK3CA	OC, EC	PTEN	OC, EC
PIK3R1	EC	TP53	OC, EC
POLE	EC, OC	GNAS	OC
PPP2R1A	OC, EC	HRAS	EC, OC
PTEN	OC, EC	NRAS	EC
RNF43	EC		
TP53	OC, EC		

Description of the somatic mutation detection for plasma and Pap- and TAO brush samples. Additionally a description for each mutation is stated for which cancer diagnosis they are most commonly found in.

OC=ovarian cancer; EC=endometrial cancer

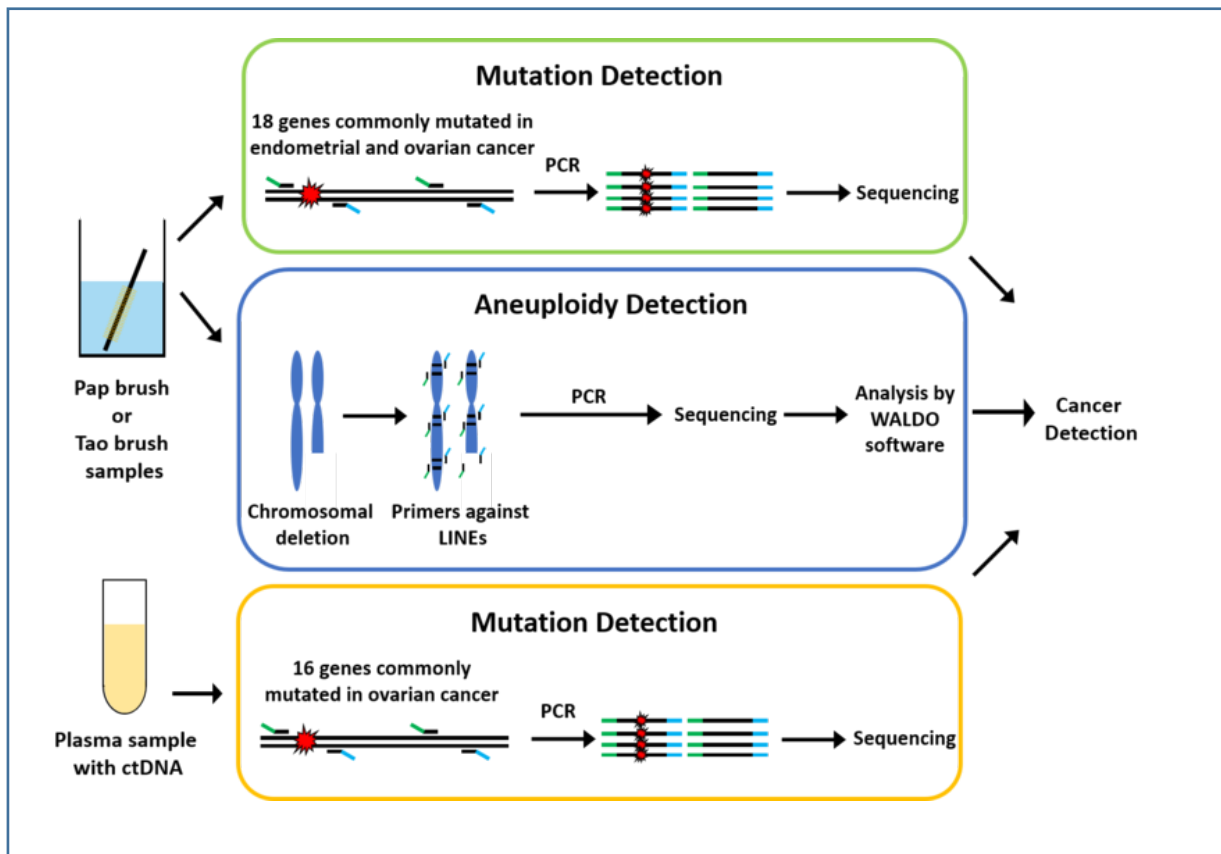


Figure 14. Illustration of the PapSEEK workflow.
(Chloe2019/Wikimedia/CC BY 4.0/<https://creativecommons.org/licenses/by-sa/4.0/>)

The descriptive statistics of this study were presented as the mean with 95% confidence intervals (CI). The analysis was conducted using a median mutant allele fraction (MAF)-based approach. The distribution of mutations found in the control group was compared with MAFs of all mutations to perform a mutation-specific normalization. The MAF was defined as the ratio between the number of supermutants divided by the number of UIDs. By comparing the normalized MAFs of each mutation with a reference distribution of normalized MAFs, a p -value was calculated. Each sample was analyzed in two duplicate wells and assessed using Stouffer's Z score method. The assumption (null hypothesis) on which the p -value (then transformed to a Z -score) was calculated for each well is that the well contains no driver mutations and only technical artifacts, which follow the reference distribution build from the normal controls. To compare the sensitivity of PapSEEK on tumor samples using either Pap-brush or TAO brush, a McNemar test was used. Ten-fold cross validation was used to calculate SN and SP for Pap- and TAO brush,

and plasma samples. Confidence intervals were calculated for SN and SP, assuming binominal distributions. A p -value of <0.05 was considered statistically significant.

Ethical considerations

Ethical approval was obtained for all four Papers in this thesis from the regional ethical committee at Gothenburg University. Paper I-III from ref: 139-13 and Paper IV from ref: 510-13. All participating women received written and oral information of the trials and had an opportunity to decline participation either directly or at any time after inclusion.

Study population in papers I-III

A multicenter investigation of 684 patients was undertaken, out of which 638 were eligible and enrolled in the BIOMOVCA study (Figure 11). A total of 46 patients were excluded according to criteria agreed upon prior to initiation of the study. Thirtyfour women declined to participate, while eight women had serum sample collection failures, one patient refused to undergo surgery, two patients were entered twice at different hospitals, and one woman had neoadjuvant chemotherapy treatment prior to surgery. The distribution of the patients enrolled into BIOMOVCA is visualized in figure 15. The majority of patients were collected at the Sahlgrenska University Hospital and the regional hospital NÄL (70%).

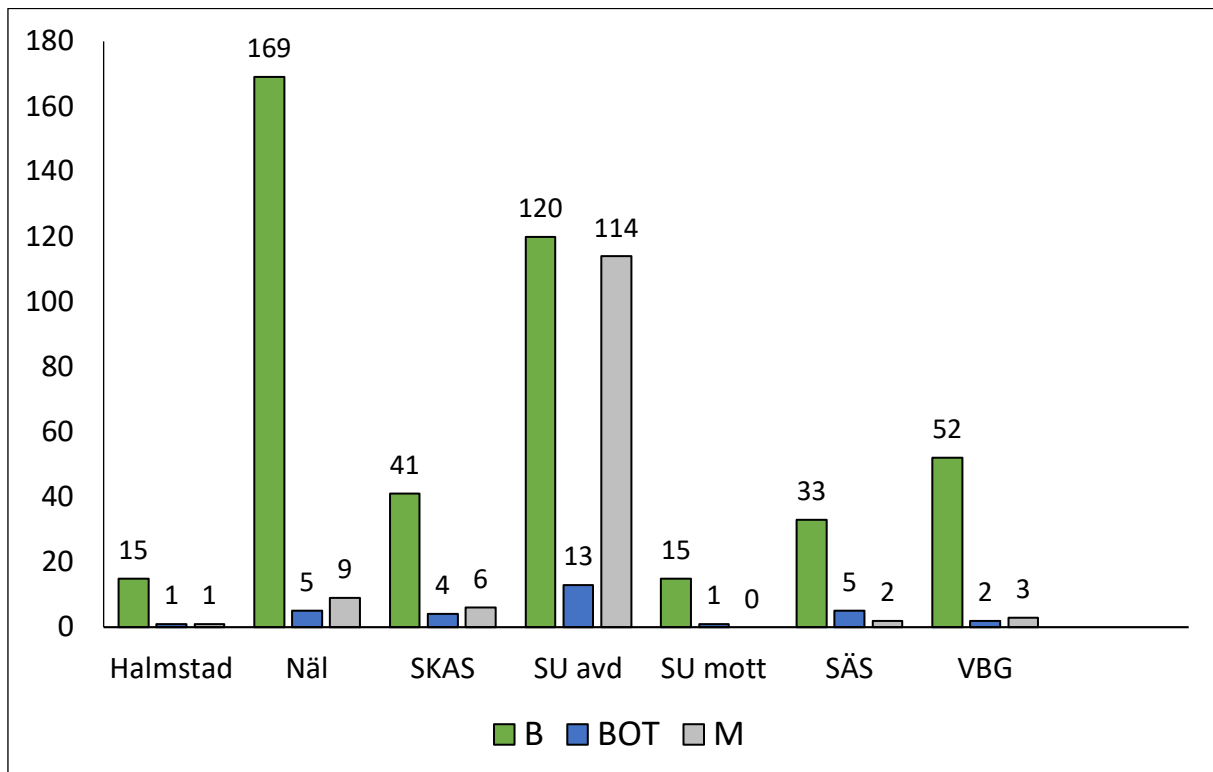


Figure 15. Included patients divided by benign (B), borderline (BOT) and EOC tumors (M) for the separate hospitals and units participating in the BIOMOVCA study (Paper I-III).

Subgrouping of the patients according to histology gave three main categories; benign (B), borderline type (BOT) or malignant EOC tumors (Figure 16). The distribution of EOC histology and stage is shown in figure 17. Noteworthy, women with the malignant EOCs, were 12 years older than the group with benign tumors, which was also expected since EOC is more common among older women. This fact also had an impact on the overall serum levels of HE4 and CA125, since HE4 values have been reported to be higher in post-menopausal women.

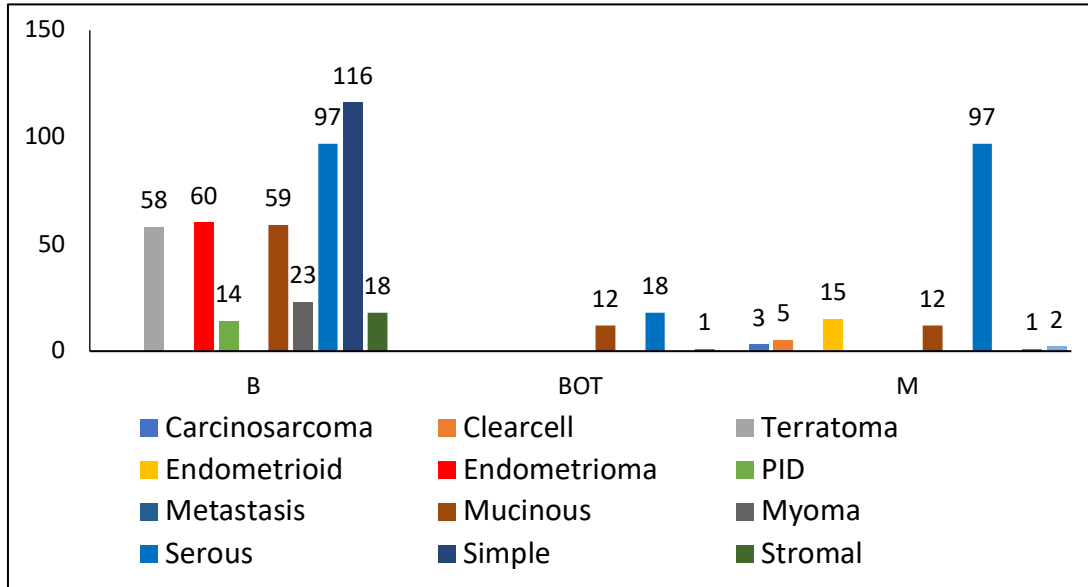


Figure 16. Included patients divided by histology and benign (B), borderline (BOT) and EOC tumors (M) in the BIOMOVCA study (Paper I-III).

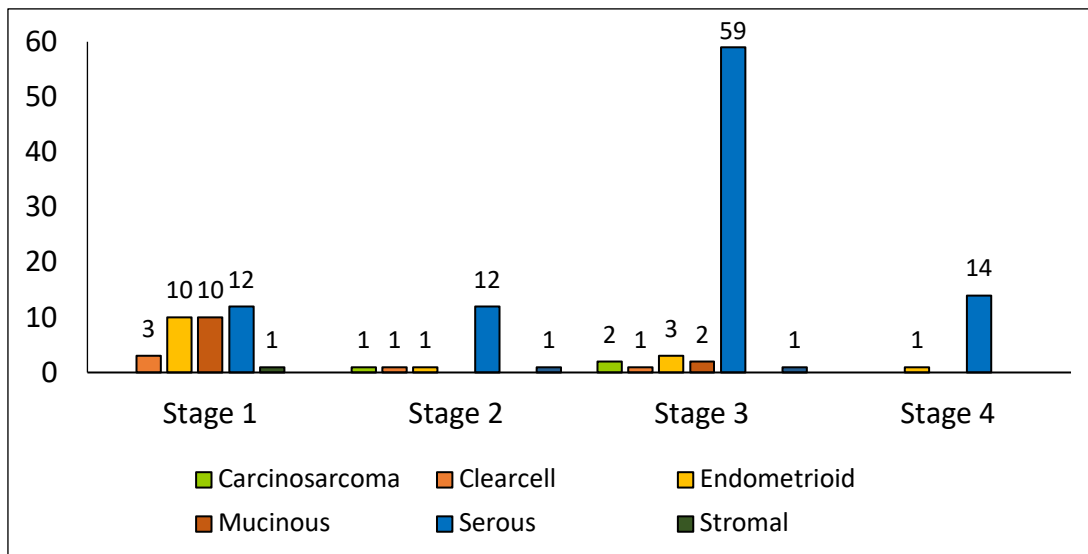


Figure 17. Included patients diagnosed with epithelial ovarian cancer (EOC) divided by histology and stage in the BIOMOVCA study (Paper I-III).

Paper I

The aim of Paper I was to validate the serum biomarkers CA125 and HE4 alone or in the context of the RMI and ROMA algorithms in an unbiased cohort of women diagnosed with ovarian cysts/pelvic tumors. The purpose was to find out whether any of these diagnostic procedures (HE4 and ROMA) could be recommended for implementation in routine pre-operative diagnostic assessments/triage of women with an ovarian cyst/pelvic tumor of unknown origin.

All these markers were found useful for diagnostic purposes to discriminate patients with EOC from benign conditions in our cohort of unselected women diagnosed with an ovarian cyst/pelvic tumor.

Statistically significant results were obtained for all assessments; i.e concentrations of HE4 or CA125 and the algorithms RMI and ROMA, enabling us to discriminate between benign from EOC, benign from EOC+BOT, benign from early stage EOC tumors (FIGO I+II), and benign from late stage EOC tumors (FIGO III+IV). No statistical significance was seen for HE4 assessments when comparing benign and BOT tumors in the postmenopausal group, or CA125 assessments in the premenopausal group.

Comparison between benign tumors vs. EOC, showed that serum CA125 levels achieved the highest sensitivity in both pre- and postmenopausal women (95.7 Pre-M; 92.0 Post-M). Assessments of serum HE4 levels was less predictive (82.6 Pre-M; 72.3 Post-M) (Table 3). However, serum HE4 levels outperformed CA125 levels in specificity. CA125 gave 40.4% false positive assessments in the Pre-M group and 20.5% false positives in the Post-M group, whereas HE4 determinations gave 9.1% false positives in the Pre-M group and 7.9% false positives in the Post-M group (Table 3). Thus, assessments of serum HE4 was superior to CA125 determinations in detecting healthy women. The algorithms assessed for triage of women, RMI and ROMA, were almost identical in their predictive ability for differential diagnostics (ROC AUC RMI Pre-M: 0.88, Post-M: 0.85; ROC AUC ROMA Pre-M+Post-M: 0.84). These results support the notion that ROMA, which does not include TVU, could be recommended for general practice diagnostic work up when EOC is suspected as it only includes menopausal status and serum samples.

Table 3. Diagnostic ability for HE4, CA125, Risk of Malignancy Index (RMI), and Risk of Malignancy Algorithm (ROMA) comparing benign (B) disease with malignant (M) disease.

EOC Pre-M (n=23)				
Diagnostic Test	HE4 (>70 pmol/mL)	CA125 (>35 U/mL)	RMI (>200)	ROMA (≥11.4)
SN	82.6	95.7	87.0	87.0
SP	90.9	59.6	89.6	80.9
ROC AUC (95% CI)	0.867 (0.786-0.950)	0.776 (0.723-0.829)	0.883 (0.810-0.956)	0.839 (0.764-0.914)
PPV	47.5	19.1	45.5	31.3
NPV	98.1	99.3	98.6	98.4
EOC Post-M (n=112)				
Diagnostic Test	HE4 (>140 pmol/L)	CA125 (>35 U/mL)	RMI (>200)	ROMA (≥29.9)
SN	72.3	92.0	89.3	91.1
SP	92.1	79.5	80.5	77.2
ROC AUC (95% CI)	0.822 (0.777-0.867)	0.857 (0.820-0.895)	0.849 (0.810-0.888)	0.841 (0.803-0.880)
PPV	82.7	70.1	70.4	67.5
NPV	86.5	95.0	93.5	94.3

M=epithelial ovarian cancer (EOC), SN=Sensitivity, SP=specificity, ROC AUC=Receiver Operating Characteristics area under the curve, PPV=positive predictive value, NPV=negative predictive value, Pre-M=premenopausal, Post-M=postmenopausal

Paper II

The aim of this study was to improve the diagnostic toolbox to better discriminate benign from malignant conditions in women with an ovarian cyst/pelvic tumor of unknown etiology.

In Paper II three new models, GOT 1-3, were explored and compared with established diagnostic markers (RMI, ROMA, CA125) in an attempt to improve the ability to discriminate benign from malignant conditions in women with an ovarian cyst/pelvic tumor (Figure 12).

The distribution of EOC according to stage and the dualistic model type I and type II are visualized in table 4. Ninety-nine patients (73.3%) were diagnosed with type II tumors and 36 (26.7%) with type I tumors. These results are in line with reported distribution of the dualistic model and indicates that the cohort reflects the common composition of tumors described in the introduction (33).

We found that all new models showed statistically significant improvements in their predictive ability as compared to the baseline models, $p < 0.001$. The addition of serum assessments of HE4 to RMI (GOT-1) or CA125 (GOT-2) increased specificity (SP) to 86% and 79% (84% RMI; 68% CA125), at target sensitivity (target SN) of 92 and 93%, respectively. A larger increase was seen in sensitivity (SN) at a set specificity (SP) of 75% for GOT-2 as compared to that observed with GOT-1. This effect could be explained by the already high sensitivity for the baseline model of RMI. The addition of TVU to the ROMA algorithm (GOT-3) improved specificity at target sensitivity for both pre-M (88%) and post-M (80%) patients compared to the baseline model of ROMA (81% Pre-M; 77% Post-M). At a set specificity of 75% the sensitivity increased for both pre-M and Post-M women. The addition of HE4 assessments and TVU improved the diagnostic performance of both RMI and ROMA. The effect was especially pronounced after adding TVU to ROMA when the false positives were reduced for premenopausal women, as was the effect when HE4 assessments were added to CA125 assessments.

In the subgroups analyses of the EOC group we divided the patients into early and late stage tumors and into type I and type II tumors. Whereas GOT-1 improved in specificity in both early (GOT-1 86%; RMI 84%) and late stage tumor groups (GOT-1 90%; RMI 84%), GOT-2 improved specificity for both early and late stage tumor groups, and additionally sensitivity, at a set specificity of 75%, for early stage tumors (SN GOT-2 85%; SN CA125 75%). The improvements for GOT-2 included improved diagnostic accuracy for early stage tumors (ROC AUC GOT-2 0.88; CA125 0.84), as well as late stage tumors (ROC AUC GOT-2 0.98; CA125 0.96). The results were similar when the analysis took into consideration the type I and II tumor subgroupings.

A prior multicenter study modified the ROMA algorithm by adding age instead of menopausal status, Copenhagen Index (CPH-1) (165). This index is a modified regression model from an established algorithm as ours in Paper II. Menopausal status was replaced by age due to the suggested correlation between elevated serum levels of HE4 and increased age, after the age of 50 (111, 114). To further analyze the possible comparison with alternative algorithms and marker tests from other groups CPH-1 was calculated for our cohort (not reported in Paper II). We calculated specificity (83%) as suggested by the authors, using the sensitivity (92%) at RMI >200 cutoff (as suggested by the authors), sensitivity (95%) and 75% specificity and AUC 0.95 (95% CI 0.93-0.97). The results of CPH-1 in our cohort were comparable with GOT 1-3 (Paper II).

Table 4. Epithelial ovarian cancer (EOC) classification according to histology, stage and dualistic model type I and type II.

Histology	Stage	Grade			Total n=135 (%)
		Lowgrade (G1) Type I	Moderate (G2) Type II	Highgrade (G3) Type II	
Serous (All)	I	5		7	12 (8,9)
	II	3		9	12 (8,9)
	III	4	6	49	59 (43,7)
	IV	1		13	14 (10,4)
Total		13 (9,6)	6 (4,4)	78 (57,8)	97 (71,9)
Mucinous	I	10			10 (7,4)
	II				
	III	2			2 (1,5)
	IV				
Total		12 (8,9)			12 (8,9)
Endometrioid All)	I	5	4	1	10 (7,4)
	II			1	1 (0,7)
	III			3	3 (2,2)
	IV		1		1 (0,7)
Total		5 (3,7)	5 (3,7)	5 (3,7)	15 (11,1)
Clearcell	I	3			3 (2,2)
	II	1			1 (0,7)
	III	1			1 (0,7)
	IV				
Total		5			5 (3,7)
Stromal	I	1			1 (0,7)
	II				
	III				
	IV				
Total		1			1 (0,7)
Undifferentiated	I				
	II			1	1 (0,7)
	III			1	1 (0,7)
	IV				
Total			2		2 (1,5)
Carsinosarcoma	I				
	II			1	1 (0,7)
	III			2	2 (1,5)
	IV				
Total			3		3 (2,2)

There were 52 women with early stage (FIGO I+II) disease and 83 women with late stage (FIGO III+IV) disease. 27% of the patients type I and 73% type II according to the dualistic model.

Paper III

The aim of study III was to investigate the possible effect of different predictors on the serum markers CA125 and HE4 in the benign group. This was done to test the hypothesis that HE4 assessments could improve the diagnosis of women with a benign ovarian cyst.

We selected and analyzed the benign cohort of the 445 patient cohort in BIOMOVCA. A total number of 137 patients (44 Pre-M; 93 Post-M) had false positive results with CA125 assessments and 38 patients (21 Pre-M; 17 Post-M) had false positive results with HE4 assessments (Figure 18). To reduce the proportion of women with benign cysts who undergo unnecessary surgery, and become subject for hormonal treatment due to castration, we investigated different possible confounders ability to give rise to a false positive result.

In the multivariate logistic regression analyses, eGFR, as a measure of renal failure, and smoking were significantly associated with increased levels of HE4 in serum above the threshold value (adj p -value <0.001). NT-pro-BNP, as a measure of heart failure, was also significantly associated with an increased level of HE4 (adj p -value <0.05), but with an OR value of 1.0, indicating poor clinical relevance. Serum HE4 levels increased with age, especially in postmenopausal women after the age of 50, which is in line with observations reported by other groups. Interestingly, age was not found to be a significant predictor in our multivariate regression model. Nevertheless, as there are two different cut-off values for HE4 assessments depending on the menopausal status our results were adjusted accordingly (>70 pmol/L Pre-M; >140 pmol/L Post-M). The only predictor that was significantly associated with false positive CA125 levels was endometriosis (adj p -value <0.001), confirming that endometriosis can bring about false positive results of CA125 assessments (100, 103, 104).

Additionally we explored the HE4 and CA125 predictors applied to different EOC histologies that lead to false negative results (tumors classified as benign). However, because of the few patients in this cohort we were unable to develop neither a linear nor a logistic regression model, for CA125 assessments. A total of 10 women (1 Pre-M; 9 Post-M) had false negative results for the predictive value of CA125 and 35 women for HE4 assessments, respectively (Figure 19) (4 Pre-M; 31 Post-M). Importantly, the demographics of the cohort was similar for patients with malignant EOC and benign conditions (Paper III) (Table 5).

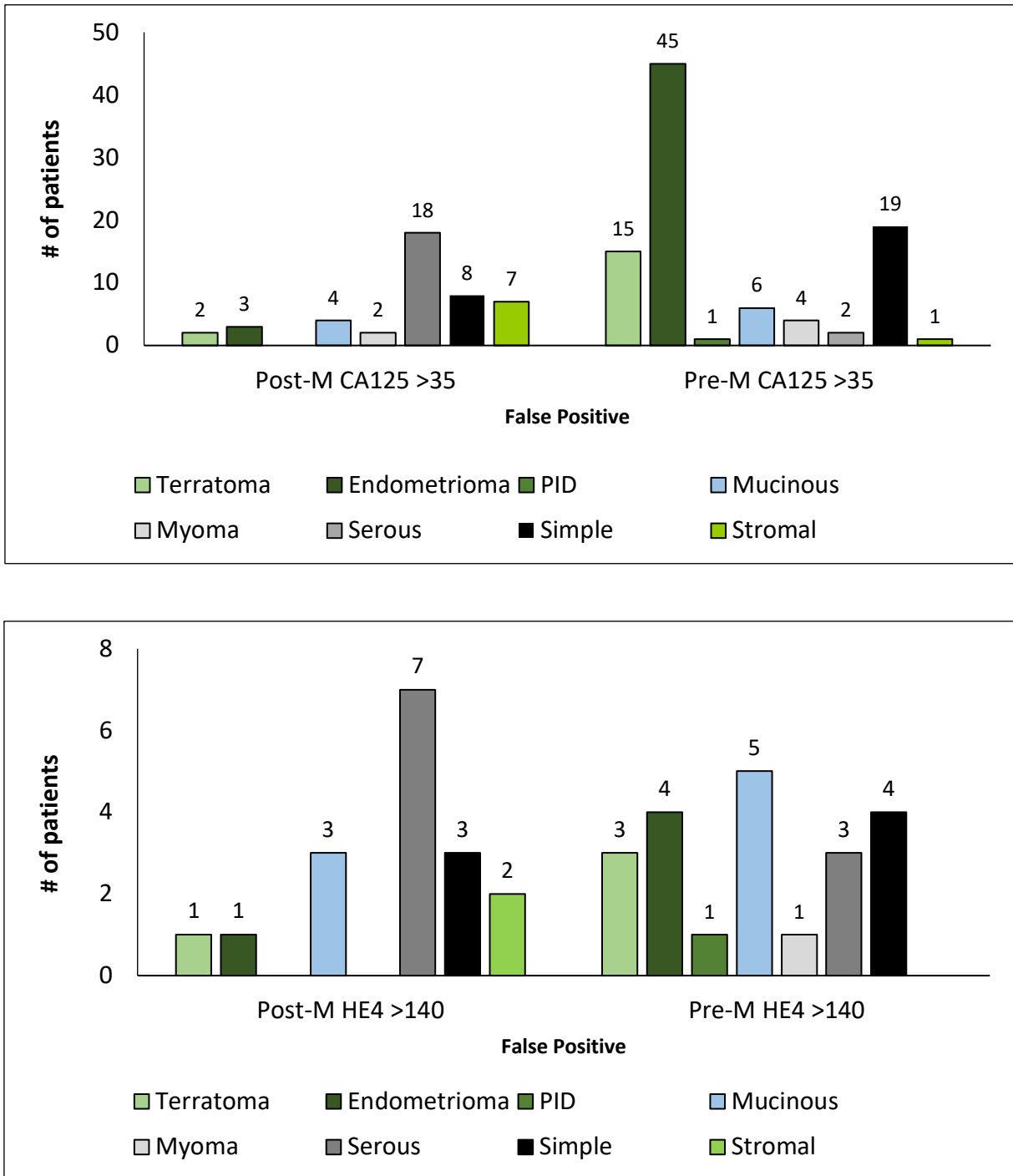


Figure 18. False positive (FP) results for HE4 and CA125 for the BIOMOVCA study (Paper I-III). The results visualized according to menopausal status and histology.

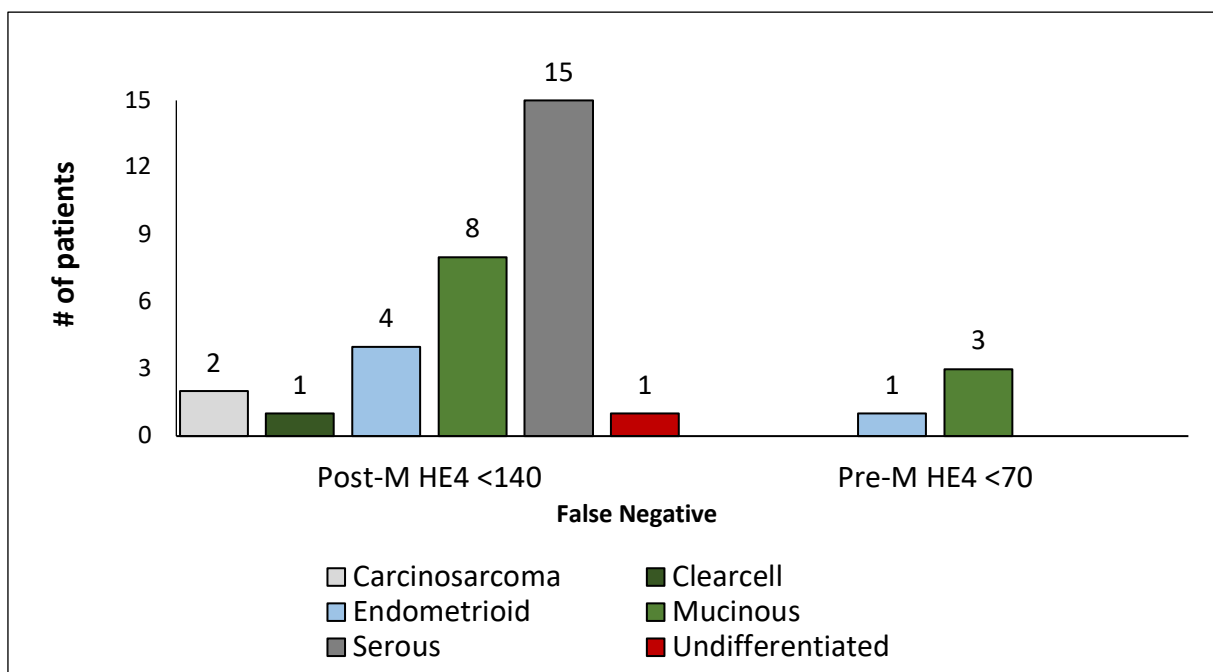
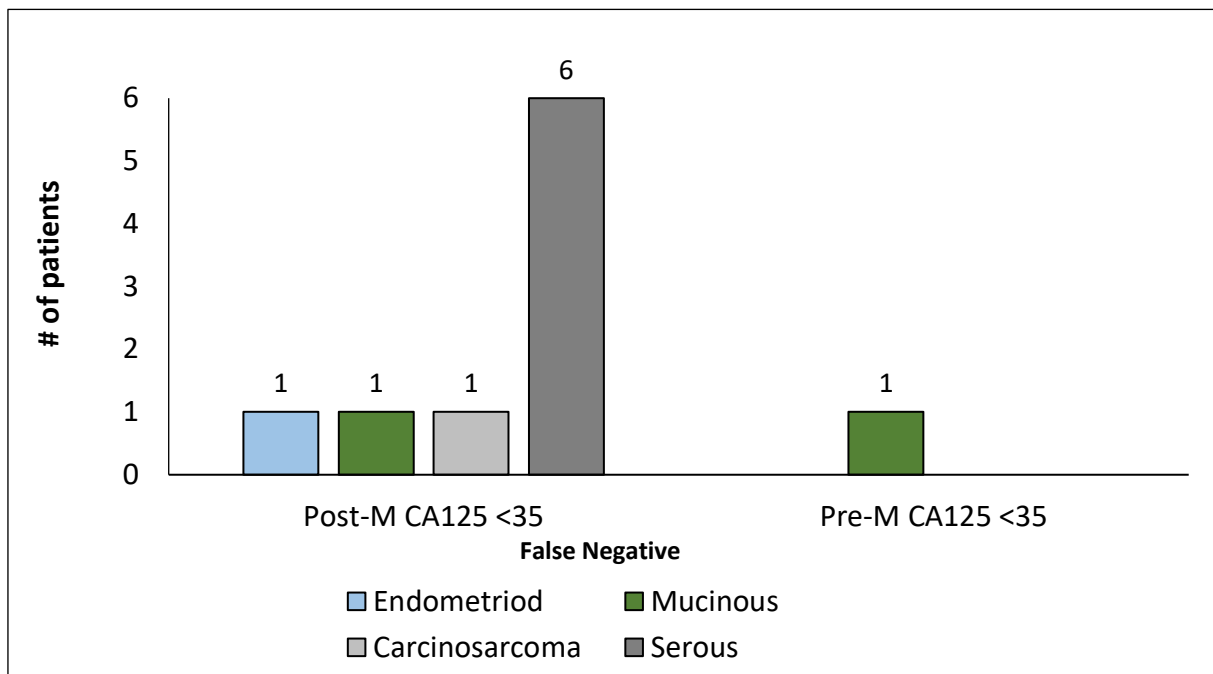


Figure 19. False negative (FN) results for HE4 and CA125 for the BIOMOVCA study (Paper I-III). The results visualized according to menopausal status and histology.

Table 5. Demographics of the study population with malignant disease.

Biological and lifestyle factors	Malignant EOC		
	Pre-M (N)	Post-M (N)	Total N (%)
Number of patients (N)	23	112	135
Age (mean)	44.26	66.46	62.67
BMI (mean)	27.08	26.37	26.49
Smoking	5	11	16 (11.9)
Heredity	3	8	11 (8.1)
Other cancer disease	1	16	17 (12.6)
Heart disease	2	32	34 (25.2)
Kidney disease	1	4	5 (3.7)
Endometriosis	4	7	11 (8.1)
HRT	0	5	5 (3.7)
Children (0)	7	13	20 (14.8)
Children (1-2)	11	61	72 (53.3)
Children (>2)	5	38	43 (31.9)

Biological and lifestyle factors noted for the 135 women with epithelial ovarian cancer (EOC) diagnosis.

N=number of women, children are denoted as 0=no children, 1-2=no more than 2 children, and >2=more than 2 children, BMI=Body Mass Index, HRT=Hormone replacement therapy, Pre-M=Premenopausal, Post-M=Postmenopausal.

The multivariate logistic regression model created for the malignant group had similar predictors as the model for the benign group (Table 6). Early stage disease (p -value <0.001) and other cancer diseases (p -value <0.01) were predictors statistically associated with false negative results of HE4 assessments. Increased knowledge about the diverse etiologies of EOC during the past decades has led to speculations as to how the different EOC histologies may affect the use of biomarkers for diagnosis. Unfortunately, the analysis could not be performed for CA125 due to the small group of only 10 women with false negative results. Mucinous histology was the only predictor that statistically could be linked to false negative results of serum HE4 determinations (p -value <0.001). For both the pre- and postmenopausal women with malignant disease included, median serum levels of HE4, when mucinous histology, was below cut-off (Pre-M 49.9; Post-M 75.2) (Table 7). Moreover, in false positive results, no association between different histologies and HE4 determinations was seen. For CA125 calculations, mucinous, serous, simple and stromal histology were all associated with a low risk of a false positive result. None of these histologies had a median concentration of CA125 above the cut-off for either pre- or postmenopausal patients (table 2, Paper III).

Table 6. Multivariate logistic regression analyses of different predictors for HE4 and CA125.

Predictors	HE4				CA125			
	OR	95% CI	<i>p</i> -value	adj <i>p</i> -value	OR	95% CI	<i>p</i> -value	adj <i>p</i> -value
Stage early	10.44	3.84 - 32.97	<0.001	<0.001	5.97	1.26 - 44.59	<0.05	0.254
Endometriosis	3.44	0.69 - 19.33	0.133	0.292	3.82	0.39 - 31.72	0.229	0.562
Age	1.00	0.94 - 1.06	0.922	0.922	0.98	0.91 - 1.07	0.664	0.664
eGFR	1.45	0.53 - 4.19	0.474	0.579	0.37	0.07 - 1.89	0.227	0.562
'NT-pro-BNP'	1.00	1.00 - 1.00	0.198	0.363	1.00	1.00 - 1.00	0.605	0.664
HRT	8.77	0.76 - 92.88	0.080	0.220	0.00	NA	0.416	0.562
BMI	1.01	0.90 - 1.12	0.857	0.922	0.94	0.77 - 1.11	0.455	0.562
Smoking	0.46	0.06 - 2.27	0.352	0.553	0.00	NA	0.117	0.562
Children	1.54	0.98 - 2.51	0.060	0.218	1.30	0.66 - 2.67	0.444	0.562
'Other cancer'	8.41	2.31 - 35.55	<0.01	<0.01	0.46	0.02 - 3.04	0.460	0.562
Heridity	2.12	0.25 - 13.19	0.455	0.579	0.00	NA	0.284	0.562

Thirtyfive women (31 Pre-M; 4 Post-M) had false positive serum values for HE4 and 10 women (9 Pre-M; 1 Post-M) had false positive serum values for CA125 when using established cut offs from the manufacturer. Multivariate logistic regression analyses were performed with different predictors for HE4 and CA125 separately. *P*-values were calculated and adjusted (adj *p*-value) for multiple testing with Benjamin-Hochberg correction.

PID=pelvic inflammatory disease, eGFR=estimated glomerular renal filtration, NT-pro-BNP=N-terminal prohormone of brain natriuretic peptide, HRT=hormone replacement therapy, BMI=body mass index, OR=odds ratio, 95 % CI=95% confidence interval.

Table 7. Serum levels for HE4 and CA125 according to EOC histologic subtypes.

Histology	N	Age (mean)	HE4				CA125			
			Mean	Std dev	Median	Range	Mean	Std dev	Median	Range
Pre-M										
Clearcell	3	37.7	157.3	29.3	169.0	124.0-179.0	256.0	149.3	180.0	160.0-428.0
Endometrioid	4	47.75	568.3	655.5	357.5	58.2-1500.0	1075.8	1596.5	414.5	51.0-3423.0
Mucinous	4	41.5	63.4	30.6	49.9	44.7-109.0	117.8	71.7	123.0	25.0-200.0
Serous	12	45.7	567.6	510.3	395.0	108.0-1500.0	1701.3	1803.9	1134.0	139.0-5000.0
Total	23	44.3	426.5	487.2	179.0	44.7-1500.0	1128.6	1564.1	261.0	25.0-5000.0
Post-M										
Carcinosarcoma	3	68.7	543.8	828.1	66.8	64.6-1500.0	64.3	32.1	61.0	34.0-98.0
Clearcell	2	57.5	317.2	349.1	317.2	70.3-564.0	61.0	0.0	61.0	61.0-61.0
Endometrioid	11	70.4	464.0	395.2	414.0	53.7-1151.0	298.4	302.0	148.0	16.0-823.0
Mucinous	8	65.4	77.9	23.3	75.2	43.9-124.0	77.5	42.2	72.0	29.0-134.0
Serous	85	66.1	626.3	533.1	407.0	48.3-1500.0	1131.6	1557.1	529.0	9.4-6710.0
Stromal	1	79.0	473.0	NA	473.0	473.0	5000.0	NA	5000.0	5000.0
Undifferentiated	2	62.0	183.5	126.6	183.5	93.9-273.0	426.0	415.8	426.0	132.0-720.0
Total	112	66.5	554.2	517.2	332.5	43.9-1500.0	948.7	1468.0	368.5	9.4-6710.0

Serum levels presented as mean, std dev, median and range.

N=number, Std dev=standard deviation, Pre-M=Premenopausal, Post-M=Postmenopausal

Paper IV

The aim of the study was to increase the sensitivity for detection of ovarian cancers by screening for gene mutations and aneuploidy using genital tract and circulating DNA samples from 1658 patients in a multicenter trial.

In total there were 1915 samples from 1658 women included in this multicenter trial. 1002 healthy controls, 402 women with endometrial cancer and 254 women with EOC.

Pap-brush samples of 245 women diagnosed with ovarian cancer were assessed, of whom 33% (CI 95% 0.27-0.39) were PapSEEK positive, including 34% early stage (FIGO I+II) and 33% late stage (FIGO III+IV). Of the 382 women with pap-brush samples diagnosed with endometrial cancer, 81% (CI 95% 0.77-0.85) were PapSEEK positive. Only 1.4% were PapSEEK positive in the healthy control group of 714 women. The fraction of mutations found in the primary tumors were higher in the endometrial tumors than in the ovarian 97% and 73%, respectively.

TAO brush samples of 51 women diagnosed with ovarian cancer were assessed, of whom 45% (95% CI 0.31-0.60) were PapSEEK positive, 47% early stage (FIGO I+II) and 44% late stage (FIGO III+IV). TAO brush samples from 123 women diagnosed with endometrial cancer were evaluated and 93% (CI 95% 0.87-0.97) were PapSEEK positive. None of the 125 women in the control group had positive results. The fraction of mutations found in the primary tumor as well as in the TAO brush samples were higher in the endometrial cancer samples (97%) than in the ovarian cancer samples (53%).

Since both Pap brush and TAO brush samples were collected further away from the primary tumor in ovarian cancer compared to endometrial cancer and the fact that there are sometimes anatomical disorders that prevent testing for mutations with Pap- and TAO brushes, the hypothesis of detection of mutations in circulating tumor DNA (ctDNA) from plasma were tested in 83 women diagnosed with ovarian cancer. Primers were designed for shorter DNA fragments within 16 genes, due to the fragmented and small size of the ctDNA. The assay were additionally tested on 192 healthy controls, with a specificity of 100%. Sixteen of these women had detectable mutations in the pap brush sample, 19 had a detectable mutation in their ctDNA and 17 had detectable mutations in both pap brush samples and ctDNA. In total 63% of the 83 women assessed were positive for either of the two (Pap-brush or plasma) tests.

The specificity of PapSEEK for both Pap- and TAO brush samples were very high, resulting in few false positive results, indicating the high precision in ruling out the true healthy cases. The most commonly mutated genes found using Safe-Seqs with Pap brush were PTEN (64%), TP53 (41%), PIK3CA (31%), PIK2R1 (29%) and KRAS (18%) for endometrial cancers. TP53 was the most common mutation found for ovarian cancer for both pap brush samples (74%) and TAO brush samples (86%), which is consistent with previous studies of EOC and HGSC (10).

General discussion

Methodological considerations

A major strength of this thesis work is the multicenter approach. To avoid recruitment of patients only from tertiary centers, we turned to the different hospitals in the Western Region of Sweden and asked for their participation. This strategy was used to reduce the number of cases with advanced ovarian cancer disease (tertiary center population), and to include a more unbiased cohort of patients with an ovarian cyst/pelvic tumor. Although centralization of patients with advanced disease to tertiary centers has improved survival and success of surgical interventions, the tertiary center patients poorly reflect a normal distribution of women identified with ovarian cysts or symptoms of pelvic tumors (147, 149, 150). It is particularly important to evaluate new diagnostic tools in the context of what a first line screening can do to predict ovarian cancer. Assays with high specificity are critical in the selection of women in need of advanced surgery (149, 150). Moreover, a diagnostic tool should have a predictive value based on high sensitivity to avoid false negative cases, but equally important high specificity to minimize the number of false positive cases. Our aim was to investigate established diagnostic tools/criteria in an unbiased cohort and to calculate if we could improve the predictive value of the diagnostic tools in such a setting. Advanced operative cases are often few at small hospitals and patients with benign cysts are less frequent at tertiary centers. We planned to undertake a study that included all first line clinics to allow us to calculate predictive values of different diagnostic approaches in an unbiased cohort of women with ovarian cysts or suspected pelvic tumors. Only a few prior studies have used a similar clinical setting (Table 8 and 9) (118, 120, 160, 166-170) .

Random errors

Random errors affect precision of a result. Sample size is an important factor to consider when statistical uncertainty and random errors should be reduced. A larger sample size decreases statistical uncertainty and improves precision. This approach allows us to analyze random samples and obtain confident estimates based on the sample. This means that the target population, must have been given the opportunity to be selected. One way to adjust for or avoid random errors is to conduct sample size calculations, to predict if the sample size is big enough to test a statistical hypothesis.

For example, this is applicable when confidence intervals (CI) are calculated to describe the random error and to calculate the margin of error. With a larger sample, and a narrower CI the statistical uncertainty will decrease. During the present trial two interim analyses were conducted to assess whether a sufficient sample size had been obtained to allow for a reasonable CI. As the margin of error was considered too high and the CI too wide in the interim analyses, the sample size was extended from 300 observations to 684. A wide CI means a lower precision. In Paper I-IV the level of confidence was set to 95%.

Systematic errors

Systematic errors or biases are not affected by sample size but instead takes into account study design, analysis and data collection. It can be divided into three types: selection bias, information bias and confounding elements. These can arise during the planning of the study or during the implementation of the trial. These factors can introduce non-random errors to the study result and might lead to incorrect conclusions.

Selection bias

A selection bias is introduced when the sample is distorted and does not reflect the actual intended target population (171). Paper I-III were conducted on the same patient cohort from a large multicenter trial. Paper IV was based on a large international multicenter trial. In Paper I-III there was a discrepancy between the expected and the actual contribution of patients from the different centers (Figure 15). Therefore, some women were diagnosed with an ovarian cyst but not enrolled for the study, potentially creating healthcare access bias. Another potential bias is when a patient enrolled at one hospital, but actually belongs to another hospital and community. This could create a selection bias, since the sample does not represent the actual population you aim to study. One way to avoid this type of selection bias could be to determine the number of patients that each unit should include prior to start, but in the present trial this was not done. In the earlier study the incidence of EOC was higher (30%) than in the present study (21%) and a majority of the women included in that study were postmenopausal and included at the tertiary center (121). This discrepancy gave a less representative sample and formed the basis for the decision not to determine the number of patients included at the separate units. It further reflects a filter referral bias addressing different levels of complexity in healthcare: from primary, secondary to a tertiary level (172). Inclusion of all patients from all different health care levels, as was done in the present study (Paper I-III), could be a way to mitigate this type of possible selection bias. Data collected for

clinical evaluation in a multicenter setting should be preferred over single center patient inclusions since the sample selection and size become more generalizable.

Information bias

An information bias occurs when improper data are introduced during the inclusion phase. For instance, if the time between inclusion and the time of surgery is too long, the disease can become more advanced, and the assessment with TVU and serum samples may no longer accurately reflect the diagnosis. In the present trial the study setup guaranteed that the time until surgery was as short as possible. A median of 6 days (mean 14 days) was observed between clinical assessment, serum collection and time of surgery. Another bias regarding information is the fact that many individuals, e.g. patients, doctors and nurses, were involved in the inclusion procedure. The potential diverse degree of urgency depending on the individuals' interest to participate in the study, may have affected the inclusion (Paper I-IV). An important question is whether all women eligible to be enrolled in the study received the same and correct information. It can be speculated that this might have been the reason why 34 women declined participation (Paper I).

Another type of information bias is underreporting bias. All base line data of the participating women were recorded at the time of inclusion and not afterwards, to avoid recall bias. In the present trial several examples of when information bias can be introduced exist. For example, body weight and height figures are often not reported accurately due to concern about personal weight or height. At the time of inclusion all patients were asked to step up on the scale to avoid this type of bias.

Smoking is another example when people tend to state "no smoking" since they might be embarrassed to tell the truth. In total, there were 95 women that stated that they were smokers in the cohort out of 638 women. It is thus difficult to know if the remaining women were truly non-smokers, but statistically 11% of the female population in Sweden are smokers (173), which is comparable to figures in Paper III in which 15% stated that they were smokers.

Endometriosis is a PAD verified diagnosis, but several women might assume they have endometriosis due to discussions with their gynecologist on this topic, e.g. due to severe dysmenorrhea. For Paper I-III all reported endometriosis were PAD verified to avoid bias, either noted in the PAD report for the present surgery or found in the medical chart from previous surgery (Paper III). Eight women without PAD verified endometriosis were excluded from the group endometriosis.

There could have been mistakes in the classification of EOC when PAD diagnosis was set if the microscopy was performed by different pathologists, as several different molecular and histopathological features apply to the EOC diagnosis. However, to limit this bias all the EOC patients had their PAD re-reviewed by pathologists specialized in gynecological malignant diseases.

Confounders

A confounder is a variable that causes an inaccurate association between a dependent and independent variable. This situation can occur if the different groups studied lack comparability e.g. in terms of socioeconomic status, group size, age and gender. Such factors that can be assumed to affect the outcome. None of the studies in this thesis had matched controls, why this phenomenon might occur and may be considered a limitation in the study setup. In Papers I-III, the women with benign ovarian cysts were used as a control group, but they were not matched with the groups with cancer diagnosis (EOC). The same comparisons were chosen in Paper IV, where the healthy controls were women with a benign condition and not linked to the cases with malignant disease, but included in the study for the same purpose as the women with cancer.

This study setup was chosen (Paper I-III) since ovarian cancer is a rare disease. By choosing the multi-center study setting, we aimed to avoid a skewed distribution regarding age and diagnosis. In Paper III, we adjusted for confounders and evaluated the associations between different variables and the outcome.

A challenge when creating a multivariate logistic regression model is to decide which predictors to include, since too many predictors will result in less statistical power. For that reason, we chose to include the lifestyle and biological factors noted for each patient in Paper III. These parameters were the main factors we wanted to analyze. Endometriosis and pelvic inflammatory disease (PID) were the only histological predictors included, since there exists a strong relationship between ovarian cancer and these variables as reported by several groups (17, 51-53).

There is always a risk that the predictors or variables excluded for the logistic regression analysis will affect the outcome of the analysis, such as the variables chosen. The aim was to test if the variables included actually had an impact on the outcome.

External and internal validity

With external validity we imply whether results obtained are generalizable. This is dependent on the degree of internal validity of the results (174). The calculation of statistical power was conducted before the onset of the study (Paper I-II) and we tested benign tumors vs. all cases of EOC based on data reported in a previous publication (121). When comparing these groups in Paper I and II, we assumed that the multicenter trial would include a large sample and because of these results would also be generalizable. For subgroup analyses the internal validity was low, due to the small sample sizes, and this represents a limitation in the thesis. Nevertheless, the results obtained in our study are of great importance and lends support to similar results previously reported. Noteworthy, conducting a multicenter trial without hampering or changing the routine treatment protocol, as for example according to national guidelines, could be difficult. Inclusion of a control group of healthy women without any history of an ovarian tumor to our study could have decreased the possible selection biases for Paper III. For Paper I and II, on the contrary, this would not have been of value since these studies evaluated the performance of the biomarkers for possible clinical diagnostic applications. Including a separate group of healthy controls in Paper IV could have been favorable, since screening for cancer was the intended use of the test. Another factor of value could have been to calculate how many patients each participating center could contribute with, to avoid selection bias.

Another important threat to external validity is the complexity of the model created and the risk for overfitting the model and available sample. In Paper III we chose the lifestyle and biological factors, which is one way to avoid overfitting as described under confounders. In Paper II we conducted both model preparation and validation on the same cohort. This could be a threat to external validation, but to prevent that we used leave-one-out cross validation to mimic external validation. Not having an external cohort to conduct validation of the model can be seen as a limitation.

Another important consideration is the interaction among variables. The effect of one variable on the outcome, such as positive cancer diagnosis, may depend on the value of another variable (175). In Paper III for instance, renal failure was found to be associated with elevated serum levels of HE4, thus implying false positive cancer diagnosis. Renal function decreases by age, and age was found to be associated with increased HE4 levels. Thus, these two variables might interact, increased age and decrease renal function, and can affect serum levels of HE4. This will further be important if HE4 measurements are used to determine treatment response in chemotherapy. Chemotherapy is filtered by the kidneys and decreases renal function.

Therefore, elevated HE4 levels for a postmenopausal woman treated with chemotherapy for an EOC tumor may not be a sign for either treatment response failure or relapse rather than the age or chemotherapy dependent effect on the renal function (111, 176).

Discussion and future perspectives

In this thesis we have validated assessments of the biomarkers HE4, CA125, and the algorithms RMI and ROMA, in a cohort of women investigated for an ovarian cyst/pelvic tumor of unknown origin. The study specifically addressed the diagnostic success of these parameters to identify women with an early stage malignant disease. In addition the correct referral of patients with a suspected malignant disease, and the ability to distinguish them from benign conditions, was studied. To secure an unbiased approach we collected patients attending all gynecological clinics in Western Sweden. These women were considered to represent the normal population of women diagnosed with an unknown ovarian cyst/pelvic tumor (Paper I). Furthermore, our aim was to investigate whether combinations of biomarkers and algorithms could significantly improve specificity and sensitivity of diagnostic evaluation and improve differential diagnosis of patients with ovarian cysts/pelvic tumors (Paper II). A scoring system with too many false positive samples would negatively affect the credibility of the diagnostic accuracy as too many benign conditions would be selected for surgery. Our ambition was to increase specificity of the diagnostic toolbox. However, equally important was that we could justify reasonably good sensitivity of the diagnostic arsenal since a low sensitivity would miss patients in need of surgical interventions. In addition, confounding factors may influence the level of biomarkers in serum and, hence, indirectly contribute to poor diagnostic accuracy of the serum concentration assessed. Therefore, we investigated different predictors to increase the clinical validity of the diagnostic assessments in a subgroup of women with benign conditions (Paper III). Importantly, not all EOC subtypes develop large tumors accessible with imaging techniques, such as TVU, for diagnosis. The most common EOC subtype, HGSC, most often originate from the fallopian tube. It evolves from several microscopic small changes that are difficult to detect before they develop into advanced disease. Accordingly the need for alternative diagnostic methods are entitled. In the final paper, we look into future perspectives to improve clinical diagnosis by employing genetic markers strongly linked to cancer.

Together with a group at Johns Hopkins University Hospital in Baltimore, USA, a multicenter analysis investigated whether mutations typifying EOC could be used to arrive at a diagnostic toolbox with sufficiently high predictive value to better serve the need for accurate diagnosis in women with ovarian cancer. Today we consider EOC quite an heterogeneous group of diseases. Knowledge about EOC-specific mutations and access to an easy screening system of DNA in liquid biopsies taken from the genital tract or as a simple blood test from women with pelvic tumors, would greatly improve early diagnosis. This might help to limit the number of patients that unnecessarily undergo surgical interventions, while having a benign condition (Paper IV). At the same time molecular investigations will hopefully also lead to a better understanding of the etiology of EOC. Altogether, progress in this field will not only improve differential diagnostics, but also enable personalized treatments and therapies.

Are serum HE4 levels or the ROMA algorithm a diagnostic instrument ready for clinical implementation?

Early detection of EOC and an improved diagnostic specificity to avoid unnecessary castration of fertile women is of critical importance to increase survival rates and limit morbidity of unnecessary surgery in benign conditions. In recent years, a plethora of biomarkers and algorithms, as well as various combinations of these have been investigated to improve diagnosis of EOC (Table 8 and 9). To evaluate the possible use of serum HE4 determinations and the ROMA algorithm in everyday clinical practice, we evaluated samples from an unselected population of women diagnosed with ovarian cyst/pelvic tumor. Whereas we specifically wanted to evaluate HE4 and ROMA's validity as diagnostic predictors that could improve early diagnosis of EOC in clinical practice, several previous studies have addressed this question, but used either a pre-set specificity and/or sensitivity (75-95%) of HE4 levels (Table 8)(117, 120, 152, 160, 166, 177). Yet other studies have analyzed the biomarkers on different analytical platforms with different cut-off levels (74, 118, 119, 178-180). In this study we defined clear criteria for eligibility, selected methods for recruiting patients and used an automated analytic platform with cut-offs recommended by the manufacturer, in a multicenter setting.

Table 8. Overview of studies evaluating the diagnostic performance of HE4, CA125, RMI and ROMA, comparing benign and malignant EOC cases.

Cases	Study design	Center	Cut-off HE4	SN (CA125/HE4/RMI/ROMA)	SP (CA125/HE4/RMI/ROMA)	SET SP (75%/90%/95%)	ROC AUC (CA125/HE4/RMI/ROMA)	Platform CA125/HE4	HP	Year	Author
374	P	S		81.6/78.1/NA/NA	80 (CA125)	75 (HE4)	0.87/0.84	Architect/ Fujirebio	T	2011	Partheen <i>et al.</i>
387	P	M	150	87.0/65.2/NA/87.0	68.3/98.5/NA/86.1		NA	Abbott/ Fujirebio	T	2016	Romagnolo <i>et al.</i>
128	P	S	70	70.4/79.6/63.0/74.1	74.2/66.7/92.4/75.8		0.80/0.78/0.86/0.82	Roche/ Fujirebio		2012	Anton <i>et al.</i>
50	P	S	70/140	NA	NA		0.61/0.76/0.69/0.74	Abbott	T	2015	Richards <i>et al.</i>
180	P	S	140	86.2/86.2/NA/93.1	78.9/87.4/NA/90.7		0.91/0.92/NA/NA	Roche	T	2014	Ortiz-Munoz <i>et al.</i>
361	P	S	70	Pre: 91.0/84.0/66.0/84.0 Post: 91.0/91.0/68.0/91.0	Pre: 62.0/100.0/75.0/100.0 Post: 65.0/96.0/58.0/77.0		Pre: 0.93/0.95/0.91/0.95 Post: 0.90/0.96/0.89/0.95	Abbott	T	2014	Stiekema <i>et al.</i>
1218	P	S		91.7/91.3/96.0/94.8		75	0.93/0.94/0.96/0.95	Abbott	T	2012	Karlsen <i>et al.</i>
260	P	S	70/140	86.4/69.7/80.3/87.9	52.7/86.0/79.6/68.8		0.82/0.84/0.88/0.89	Roche	T	2015	Yanaranop <i>et al.</i>
374	P	S		NA/NA/76.0/84.7	NA/NA/92.4/76.8		NA/NA/0.93/0.89	Fujirebio	T	2012	Van Gorp <i>et al.</i>
213	P	S	70	79/71/77/75	62/90/82/88		0.81/0.82/0.85/0.84	Abbott		2016	Al Musalhi <i>et al.</i>
160	P	S	70	69.0/86.2/NA/89.6	90.1/85.9/NA/87.3		0.89/0.91/NA/0.93	Fujirebio	T	2011	Jacob <i>et al.</i>
233	P	S		61.2/77.6/NA/NA		90	0.84/0.91/NA/NA	Fujirebio	T	2008	Moore <i>et al.</i>
784	P	M		Pre: 95/75/90/80 Post: 80/82/84/86		75	Pre: 0.80/0.76/0.82/0.77 Post: 0.82/0.79/0.85/0.84	Fujirebio		2018	Liest <i>et al.</i>
531	P	M		NA/NA/NA/88.7	NA/NA/NA/74.7		NA	Abbott/ Fujirebio		2009	Moore <i>et al.</i>
457	P	M		NA/NA/84.6/94.3		75	NA/NA/0.87/0.95	Abbott/ Fujirebio		2010	Moore <i>et al.</i>
151	P	S	37 CA125/ 30 HE4	83.0/98.0/NA/NA	100/100/NA/NA		0.91/0.99/NA/NA	Liaison/ Fujirebio	T	2009	Montagnana
225	P	M		78.6/78.6/NA/NA		95	0.92/0.96/NA/NA	Fujirebio		2009	Huhtinen
389	P	S	70	79.5/74.5/NA/84.9	81.6/83.3/NA/79.7		0.88/0.86/NA/0.90	Fujirebio	T	2011	Van Gorp <i>et al.</i>
419	P	S	70/140	Pre: 92.3/84.6/NA/84.6 Post: 94.3/78.2/NA/93.1	Pre: 59.4/94.2/NA/81.2 Post: 82.3/99.0/NA/84.4		NA	Abbott	T	2011	Bandiera
1590	P	M		Post: 66.7/53.8/64.1/69.2		90	0.85/0.80/0.86/0.85	Roche		2020	Gentry-Maharaj <i>et al.</i>

EOC=epithelial ovarian cancer; ROC AUC=receiver operating characteristics area under the curve; P=prospective; S=single center; M=multi center; SN=sensitivity; SP=specificity; HP=high-risk population: T=tertiary center; NA=not applicable

As described in this thesis, EOC is often diagnosed at a late stage. This is often due to the asymptomatic nature of EOC at the early stages. Thus, when a woman is investigated for an unknown pelvic tumor it is of great importance to correctly identify malignancies, while securing that no false positives are registered. In this way we can improve the decision of which patients should be referred to a tertiary center for further investigations. At present, the Swedish Western Health Care Region (WHCR) consensus is to assess serum levels of CA125 together with imaging techniques including either TVU by a gynecologist with variable experience, CT scan or MRI. Investigations of a suspected pelvic tumor should be undertaken according to the guidelines recommended by the “national health care program”. These include TVU assessments for RMI, or the pattern recognition protocol or the more subjective IOTA simple rules (181). Neither serum levels of CA125 nor the RMI algorithm alone carry a sufficiently high predictive value for the diagnosis of EOC, and therefore we investigated whether serum assessments of HE4 or ROMA would be better from a diagnostic perspective. Several studies prior to ours have validated the diagnostic ability of serum assessments of CA125 and HE4, as well as employed the RMI and ROMA algorithms. Surprisingly, many have evaluated these parameters only in a high-risk population linked to a tertiary center (Table 8 and 9). This implies a highly selected population with dominance of elderly women (above 60 years), as these are overrepresented in EOC (74, 121, 165, 178, 179). Women with less advanced tumors may therefore not be included in these settings. It is important to thoroughly validate the diagnostic value of new biomarkers and algorithms in the regional setting before implementing them into clinical practice.

We found that serum CA125 levels showed the highest sensitivity (Pre-M 95.7; Post-M 92.0) and HE4 levels the highest specificity (Pre-M 90.9; Post-M 92.1) in discriminating EOC tumors from benign tumors (Paper I). These results agree well with findings reported by other groups, albeit they were smaller, and trials that have used recommended cut-off values (180, 182-184)(Table 8). Moreover, we found that the combination of serum levels of both CA125 and HE4 in the new algorithm GOT-2 improved specificity from 68% (CA125) to 79%, which confirms what has previously been proposed (ref partheen, moore). We believe that the addition of serum levels of HE4 is the single most important parameter to improve today’s diagnostic arsenal based on CA125 assessments, and RMI or ROMA algorithms (Paper I-III). HE4 assessments should, therefore, be implemented in clinical practice when investigating women for a possible EOC.

Table 9. Overview of studies evaluating different multi-marker tests and different algorithms, comparing benign and malignant EOC cases.

Test	Cases	Study design	Center	FDA	Panels of biomarkers	Subject characteristics	SN	SP	SN SP 75%	ROC UAC	Platform	HP	Year	Author
MIA (OVA1)	524	P	M	Y	CA125, beta 2 microglobulin, TRF, transthyretin, apolipoprotein A-1	NA	93.0	43.0			Roche/Siemens Healthcare Diagnostics		2011	Ueland
MIA2G (Overa)	493	P	M	Y	CA125, HE4, FSH, TRF, apolipoprotein A-1	NA	91.3	69.1		0.92	Roche		2016	Coleman
ROMA-P	413	P	S		HE4, CA125	Age	85.0	98.0		0.92	Roche/Abbott	T	2016	Chudecka Glaz
CPH-1	1610	P	M		HE4, CA125	Age	82.0	88.4	90.6	0.93	Roche/Abbott/Fujirebio	T	2015	Karlsen
ROMA	531	P	M	Y	HE4, CA125	Menopausal status	88.7	74.7			Abbott/Fujirebio		2009	Moore
R-OPS	266	P	S		HE4, CA125	TVU, Age	93.9	79.9		0.95	Roche	T	2016	Yanaranop
RMI I	143	P	S		CA125	TVU, menopausal status	85.4	96.9			Abbott		1990	Jacobs
RMI II	173	P	S		CA125	TVU, menopausal status	80.0	92.0			Abbott		1996	Tingulstad
RMI III	365	P	M		CA125	TVU, menopausal status	0,71	0,92			Abbott		1999	Tingulstad
Multimarker	184	P	S		CA125, HE4, YKL-40, transthyretin, ApoA1, beta-2-microglobulin, transferrin, LPA	NA	94.0	76.3		0.83	Abbott	T	2019	Moore
ROCA	6532	RCT	M		CA125	TVU		99.8		0.93	Centocor		2005	Menon
Triple screen	218	P	S		HE4, CA125	Symptom index	79.0	91.0			Achitect	T	2017	Goff
Symptom index	607	P	M			Pelvic/abdominal pain, increased abdominal size /bloating, difficulty eating/feeling full	<50 years: 86.7; >50 years 66.7	<50 years: 86.7; >50 years: 90			NA		2006	Goff

EOC=epithelial ovarian cancer; ROC AUC=receiver operating characteristics area under the curve; P=prospective; RCT=Randomized controlled trial; S=single center; M=multi center; FDA=US Food and drug administration; SN=sensitivity; SP=specificity; HP=high-risk population: T=tertiary center; NA=not applicable; Y=yes

Protein-based algorithms and multimarker tests

Over the past decades a number of different protein-based algorithms and multimarker tests have been identified and tested, in an attempt to improve early detection of EOC and facilitate referral of patients with malignant disease (Table 9). Despite extensive evaluations of diagnostic parameters based on single markers, algorithms or combinations in multimarker tests, none of these have received the widespread acceptance for early detection of EOC.

In Paper I, both RMI and ROMA were comparable in their diagnostic accuracy in discriminating EOC from benign tumors. As ROMA does not require advanced TVU it can be argued that ROMA should be preferred over RMI in the first line of clinical primary care. This allows referral of patients to tertiary centers or a gynecologist for TVU assessments (RMI), or assessments with any of the IOTA certified algorithms introduced (185-188). However, in our study ROMA did not outperform RMI as other groups have reported (160, 180). We rather found that both the ultrasound assessments, by any gynecologist at hand, and the addition of HE4 levels, significantly improved the differential diagnostic potential (Paper II). In our models we have, therefore, added either HE4 assessments or the TVU score to RMI, CA125 or ROMA determinations. The results of Paper II pointed to only a slightly increased diagnostic accuracy (ROC AUC) in discriminating benign from malignant tumors; GOT-1 0.95 vs RMI 0.95; GOT-2 0.94 vs CA125 0.92; GOT-3 0.94 vs ROMA 0.93. Nevertheless, these results were calculated using established cut-off levels and clearly showed that the addition of HE4 improves specificity for both RMI (84% RMI; 86% GOT-1) and in particular for the specificity of CA125 assessments (68% CA125; 79% GOT-2). These improvements were higher for CA125 assessments alone with regard to specificity but also sensitivity, which can be explained by the already high performance of RMI in our cohort. The addition of HE4 assessments to RMI will not be as tangible as for CA125.

A drawback of HE4 assessments is their increased levels with age and, since EOC patients often are elderly women, the detected HE4 level could be confounded by age (111, 114, 115). Several groups, have also reported increased serum levels of HE4 with increased age, as we found in Paper I and visualized in Paper III. Because of this, two research groups modified the ROMA algorithm and incorporated age instead of menopausal status, Copenhagen index 1(CPH-1) and ROMA-P (165, 189). The ROC AUC, in the study by Karlsen *et al.*, was comparable for RMI, ROMA and CPH-1 (0.96, 0.95 and 0.96, respectively), and for the modified ROMA-P compared to ROMA (ROC AUC 0.923 and 0.934, respectively). Hence, incorporating age

instead of menopausal status did not contribute to an improvement of the predictive value of the ROMA algorithm for OC (165, 189). This result was perhaps unexpected as the cohorts were taken from a tertiary setting and hosted an elderly cohort compared to unselected patients from a normal outpatient clinic, but further strengthens that the two cut offs adjusted for menopausal status as used in this thesis is sufficient. In fact, when we tested the impact of age in Paper III in our multivariate regression model we did not find that age was a predictor associated with false positive determinations of HE4 levels, in line with Karlsen *et al.* (165). This could be explained by the fact that age already is taken into account when assessing serum levels of HE4, since we use two different cut-off, already adjusting for the menopausal status. In fact, when we calculated CPH-1 in the current cohort the results were comparable (AUC 0.95) to GOT-I, -II and -III (AUC 0.94-0.95). This result indicates that established cut-offs adjusted for menopausal status gives the same result as an age-adjusted algorithm.

A similar model named R-OPS (similar to GOT-1) has been proposed by Yanaranop *et al.* (Table 9) (190). Both models achieved similar ROC AUC (0.95) and a higher specificity (GOT-1 86% and R-OPS 80%) compared to RMI or ROMA (75% and 70%, respectively). A difference between our study and that of Yanaranop *et al.* is that the latter used a tertiary center to recruit their patients and, therefore, their EOC level was 30%, as compared to 21% in our study. Moreover, all their TVUs were performed by an experienced sonographer, which may explain their high sensitivity (94%)(190). These results are, therefore, not applicable to a normal clinical setting, where the TVU examiner may be more or less experienced gynecologists, as in our multicenter cohort of women.

Several multimarker approaches to improve the diagnostic arsenal to discriminate EOC from benign conditions have been reported (Table 9). The Multivariate index assay (OVA1) was the first multimarker test to be approved by the FDA in 2009 for presurgical risk assessments (170, 191). The OVA1 included analyses of Apolipoprotein A-1, transthyretin, beta-2-microglobulin, transferrin (TRF) and CA125. It improved the sensitivity compared to assessments by CA125 alone, but unfortunately with poor specificity (54%) and consequently a high number of false positive cases were reported (170). To improve specificity, Coleman *et al.* developed the second-generation of multivariate index assays, the MIA2G-assay, which included assessments of Apolipoprotein A-1, CA125, HE4, follicle stimulating hormone (FSH) and TRF, which was approved by the FDA in 2016 (169, 192). This assay was better but still doubtful since many false positive cases were reported (SP

69%), albeit the sensitivity was excellent (91%) (169). Therefore, we can conclude that all three algorithms GOT-1, -2 and -3 performed better than these predecessors with a comparable sensitivity, but a much higher specificity (Table 9). Recently Moore *et al.*, one of the inventors of the ROMA algorithm, published a multivariate analysis model with 8-parameters (chitinase-3-like protein (YKL-40), transthyretin, Apolipoprotein A-1, beta-2-microglobulin, transferrin, lysophosphatidic acid (LPA), CA125, HE4 and menopausal status), which reached a comparable diagnostic accuracy of AUC 0.95 similar to GOT-1 (0.94) (Table 9) (168). Interestingly, they found no statistically significant improvement compared to ROMA alone (p -value=0.078).

These results indicate that adding more parameters, such as multiple protein biomarkers, or inventing new expensive assays, is not a cost-effective way forward and does not necessarily improve the diagnostic accuracy of the algorithms (193). In fact, just combining the well-known biomarkers CA125 and HE4 with TVU assessments clearly brings higher sensitivity and specificity to the diagnostic assessments of EOC. Thus, our study clearly supports the conclusion that using protein biomarkers already validated for EOC in different populations of women, perhaps together with TVU, using established cut-off levels for scoring positive samples, is the way forward for improved differential diagnosis of an ovarian cyst/pelvic tumor of unknown origin. We hope that with increasing knowledge of different co-factors impacting on the markers, we have identified the most efficient path forward towards improved predictive and accurate differential diagnosis of ovarian cancer.

Triage – the impact of different ultrasound assessments

In this thesis (Papers I-III) both experienced gynecologists and gynecologists under training performed the TVU for RMI calculations. The IOTA group has presented several different TVU approaches to evaluate pelvic tumors to improve triage and referral of patients with suspected cancer to tertiary centers (79, 179, 187, 188). Pattern recognition to evaluate tumors based on defined criteria, or the subjective use of a risk prediction model, IOTA simple rules, have been proposed as alternatives to RMI (194). These models are used to calculate the malignancy risk. The ADNEX model was developed to further predict malignancy and the precise type of adnexal pathology (195, 196). It should be emphasized that when TVU is performed by an experienced sonographer, it often outperforms ROMA and RMI and other algorithms (195, 197, 198). For example, the IOTA group reported that RMI (SN 75%; SP 92%) was inferior to the logistic regression model 2 (LR2) (SN 93%; SP 84%), and the

simple rules (SN 93%; SP 80%) model. This emanated in a recommendation that a two-step procedure should be used, based on simple rules, and an assessment (pattern recognition) based on observations made by an expert ultrasound examiner (SN 91%; SP 91%). Using this approach it was claimed that more patients with malignancies would be referred to a tertiary center with specialist ultrasound competence (187). If our patients had been assessed according to the IOTA simple rules, our results could perhaps be further improved. Simple rules are often referred to as superior to other TVU-based algorithms, such as RMI, even in the hands of an unexperienced sonographer (199). However, recent data show that the use of simple rules demand training and knowledge in both terminology and assessments before implementation, which is why it can be questioned whether the performers are correctly classified as non-experienced sonographer (200). In a recent publication, 479 patients were assessed with TVU by a general gynecologist and it was observed that the IOTA analysis (SN 83.8%; SP 92%) was superior to the RMI algorithm (SN 77.2%; SP 86,8%), but as many as 18% of the assessments using IOTA simple rules were inconclusive and needed expert evaluations (201).

Access to an expert sonographer is limited in a general clinical setting (202). Moreover, since RMI includes serum levels of CA125 only, while ROMA with the addition of HE4 is more adequate and reliable, especially in patients that are premenopausal. Hence, the ROMA algorithm, based on measurable parameters only, is advantageous to use as a first line diagnostic assessment as it does not rely on specialists for the clinical assessment or TVU.

When using biomarkers, should we suspect that lifestyle factors or comorbidity will impact on their diagnostic value?

The results in Paper I and II clearly show that the addition of HE4 determinations in serum adds to the diagnostic arsenal and allows us to discriminate better between benign and malignant conditions, especially in the premenopausal group. Several factors have been proposed to impact on the interpretation of HE4 levels, such as smoking, age, renal failure, hormonal treatment, pregnancy, menstrual cycle, BMI, heart failure and analysis with different platforms (111-115, 120, 125, 176, 203, 204). The strength of our study (Paper I-III) is the clinical setting, the large cohort of women and that we used recommended cut-offs. In this thesis we additionally aimed at investigate different predictors associated with a false negative outcome when truly malignant tumors were eventually diagnosed. In fact, it is particularly noteworthy that in our study the false negative cases were few; with 10 cases for CA125 and 35 for HE4 assessments. No results were obtained for the sub-analyses

of false negative CA125 values, due to the few cases detected. On the other hand, we looked more carefully into the false negative group of HE4 determinations. In our cohort, assessments of HE4 was unable to detect malignancies of mucinous histology, indicated by both the serum levels of HE4 (Table 7) and the multivariate regression model. These results are in line with prior knowledge of HE4 inability to detect mucinous carcinoma (86, 110).

On the other hand high levels of false positive CA125 assessments can be expected (31%), especially in the group of premenopausal women (40%). This finding confirms prior observations claiming that CA125 assessments have several drawbacks. The results further support that the combination with HE4 assessments has clear advantages as a diagnostic marker in the premenopausal group. As aforementioned, both biomarkers can be expected to be elevated in several benign conditions or found elevated with other malignancies, not just for EOC (205). It is mandatory that the examiner penetrates the patients' medical and gynecologic history, including smoking habits, renal failure, endometriosis, as well as age, when analyzing serum levels of HE4 and CA125 as showed in Paper III (103, 111-115, 120, 176, 203, 204, 206). Thus, the patient history is a critical component and should be acknowledged accordingly in a comprehensive diagnostic effort to discriminate benign from malignant tumors in the pelvis of women.

EOC – is a heterogenous group of ovarian cancers

Today it has become increasingly clear that EOC is not one disease, but rather includes highly heterogenous diseases with different origins as well as mutations that characterize them (129, 207). The histopathologic classification of EOC and how we can integrate molecular genetic features with morphology are going to have a strong impact on the diagnostics and treatments of EOC and cancer in general. The dualistic model takes into account histopathological classifications and integrates genetic findings, which have provided a path to the future where molecular and morphological pathogenesis interact (27). For example, the integration of precise mutations in the different subtypes have become cornerstones in the improvement of the diagnostic arsenal for EOC, with separate origin, aggressivity and prognosis. These observations address the urgent need for pin-pointed diagnostic markers. EOC analysis using high-throughput next generation sequencing (NGS) techniques has positively impacted our ability to determine EOC and subgroup the malignant conditions, as we can better separate them from benign conditions. The TCGA library of selected genomic alterations at the DNA, RNA, epigenetic and protein levels has become the basis for several trials, using NGS techniques in the

assessments of EOC and HGSC (10). A common denominator in these novel approaches is the liquid biopsy, which allows us to search for tumor cells or DNA. In addition, some EOC relevant protein biomarkers, such as serum CA125 and HE4, have been identified and evaluated in this thesis, for an improved diagnostic arsenal for EOC assessments. In the future, several different approaches for detection and possibly screening using molecular techniques will be available. These are now being evaluated in both discovery and analytical validation trials, but it will take time before they can be clinically implemented (208-210). However, already today we can conclude that mutations identified, with ctDNA in plasma increased the sensitivity of the analysis as compared to a PapSEEK/Pap brush (cervical biopsy) analysis alone from 33% to 63%. This is quite a remarkable finding, but it should be noted that a drawback of ctDNA assessments is the natural introduction of mutations due to aging that are not associated with cancers. Hence, ctDNA mutations do not necessarily have to imply cancer. In Paper IV we attempted to separate these two elements to adjust for ageing. We compared the actual mutations found in the cancer specimens and the mutations found in the liquid biopsies. We found that 97% of the endometrial tumors had at least one mutation in both the brush samples (Pap brush and TAO brush) as well as in the primary tumor. For the ovarian tumors, on the other hand, 73% and 53% had the same detectable mutations in primary tumors as for Pap- and TAO brush samples, respectively. The results were promising, but still improvements of this diagnostic tool need to be added before we can rely on them in the clinical practice for an accurate diagnosis of ovarian malignancies. The results for the ovarian tumors could be explained and further support the knowledge of tumor heterogeneity, since only a small proportion of the tumor was sampled and sequenced (211).

Can screening for EOC ever become a preventive step to reduce the number of cases in the future?

It is exciting to consider that a future possibility could be to introduce screening programs for ovarian cancer in conjunction with the existing national cervical screening program. In fact, several attempts to screen for ovarian cancer have already been done, but the outcome of these have been discouraging, and presently it is not recommended (212-214). This is because of the high level of false positive results when using the established diagnostic tools and the lack of data supporting an increase in ovarian cancer survival in screening programs (215). It must be emphasized that ovarian cancer is often diagnosed in an advanced stage of disease, because it can be completely asymptomatic initially. However, in the future more accurate diagnostic tools such as genetic markers for ovarian cancers, and screening

programs as for cervical- breast- and colon cancer, may be introduced. In Paper IV we evaluated such a genetic approach for diagnosing ovarian cancer using two different non-invasive techniques to screen for gynecological cancer in a non-high-risk population. If the PapSEEK analysis can be used as a diagnostic test, it should perform better than e.g. assessments of CA125 in serum (Paper I). Unfortunately, the sensitivity of PapSEEK was low; 33% for Pap brush samples and 45% for TAO-brush samples. CA125 assessments in serum had a much better predictive value in this thesis; sensitivity of 95.7% (CA125 Pre-M), and 92% (CA125 Post-M), in Paper I. At present CA125 assessments carry a much better value as a diagnostic marker for detecting ovarian malignant disease compared to the PapSEEK analysis after Pap brush and TAO brush sample collection. An overriding problem with established diagnostic tools for EOC is that their sensitivity may be high, but the specificity is often rather low. With these genetic markers the opposite was found and the specificity of the PapSEEK analysis was close to 100% for both Pap-and TAO brush samples, compared to 59.6% (Pre-M) and 79.5% (Post-M) for assessments of CA125 (Paper I). HE4 assessments, on the other hand, reached higher sensitivity (Pre-M 82.6%; Post-M 72.3%) and close to the specificity of the PapSEEK analysis (HE4 Pre-M 90.9%; HE4 Post-M 92.1%). In Paper IV a subgroup analysis was conducted to test if the sensitivity could be improved. Detection of specific mutations in ctDNA found in plasma and from Pap brush samples identified an improved sensitivity of 63%, when scoring one of the two tests as a positive sample. These results are interesting for the future, but still the sensitivity is too low to recommend this approach as a screening tool for ovarian cancer.

One general problem can be that advanced cases of ovarian cancer may have anatomical changes that can prevent shedding of cancer cells. Detection of tumor DNA in the uterine cavity or cervix upon local sampling may also be difficult. In addition, another problem may be women with prior tubal ligation or salpingectomy. Several other methods have been evaluated such as vaginal self-swabs, tampons or uterine lavage, unfortunately with little success and insufficient predictive values for general screening (216, 217). Two large randomized controlled trials (RCT) have been conducted with the aim to screen for ovarian cancer: the UK collaborative Trial of Ovarian Cancer Screening (UKTOCS) trial (n=202,546) and the Prostate, Lung, colorectal and Ovarian (PLCO) trial (n=68,447). In addition, the Kentucky cancer screening program has evaluated almost 40,000 American women with annual screening with TVU in a population/control study. They have reported increased specificity and PPV of 20-24.7%, which means that over time using this annual investigation method only 3 or 4 women with a benign diagnosis will go through

surgery to find one cancer (66, 218, 219). The UKTOCS trial had two intervention arms. The first arm was a multimodal screening (MMS) strategy, in which the women went through CA125 assessments annually and the follow-up was determined by the risk of ovarian cancer algorithm (ROCA) to either CA125 assessment after 6 weeks, 3 months, or TVU assessments according to the risk calculation of the algorithm (220). It should be noted that ROCA calculates a statistical risk of OC from serial measurement of serum levels of CA125 rather than from a single sample cut-off level (167, 221). The second intervention arm included annual TVU and a control group with no screening. However, neither of these screening models were successful, as 0.34% of the women tested in the control group died in OC, while 0.29% in the MMS arm and 0.30% in the TVU arm died in OC (p -value = 0.23) (212). The screening also revealed that 44% in the MMS arm had at least one false positive result, and accordingly general screening was not recommended (222). A similar observation was done in the PLCO trial of women in which CA125 testing and TVU was performed annually in the intervention arm. The relative frequency of death in OC was similar to that of the control group that did not undergo any screening (0.31% intervention arm; 0.29% control arm) (212, 213). Of note, the complication rate for the women who underwent surgery was 15% higher in women with benign diagnose as compared to the UKTOCS trial, in which 3.1-3.5% complication rate was reported. Thus, screening for ovarian cancer has not been successful and the consensus today is that screening for ovarian cancer in the general population of women should not be recommended (215). Screening in high risk populations for ovarian cancer is conducted in the US with annual TVU and CA125, but not recommended in the UK, as it apparently does not detect early stage cancers (222, 223). Current guidelines in Sweden do not recommend annual screening for ovarian cancer with TVU and/or CA125 assessments.

Are there other approaches for liquid biopsies sampling?

Due to the anatomy of the female reproductive tract, EOC afflicted tissues, cells or DNA may be difficult to sample. As symptoms often are subclinical, patients come to the medical attention at a late stage of disease. However, circulating tumor cells (CTCs) contribute to the metastatic process and shedding of tumor cfDNA or ctDNA into the blood circulation is most likely to occur at an advanced stage of disease. Detection of CTCs in the bloodstream in early stage cancer has been disappointing with low sensitivity for different types of primary cancers including ovarian. Therefore, hope has been given to ctDNA detection as a means to analyze small amounts of materials from an ovarian cancer. Even a small fraction of ctDNA can undergo extensive analysis, which implies a revolution of high-throughput

techniques and allows a better understanding of cancer biology. Liquid biopsies are non-invasive, inexpensive and tolerable for most women, but not as sensitive when sampled from blood as when taken from the genital tract closer to the primary tumors. Hence, samplings from the peritoneal fluid, uterine lavage, cyst fluid or tampons have been evaluated (217, 224, 225). Sampling with Pap- and TAO brush from the cervix and the endometrial lining of the uterine cavity was used in Paper IV, as an alternative to liquid biopsies in blood for improved early diagnostics and as potential screening source. Yet another source of sampling is the direct sampling from ovarian cyst fluid, and in an earlier study we could demonstrate that such fluid contained high level of proteins, monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) levels that were increased in borderline and early stage malignant cysts compared to benign cysts. Thus, malignant disease could indirectly be spotted by enhanced inflammatory markers in the ovarian cyst fluid and detected early in the disease (226). The same authors observed that ovarian cyst fluid harbored other potential biomarkers for EOC such as Apolipoprotein C-III. This biomarker was significantly increased in malignant ovarian cysts compared to benign ($p=0.001$), and could be used for early detection of ovarian cancer. Together with prof Vogelstein's group in Baltimore, ovarian cyst fluids were analyzed and found to include tumor specific mutations, 100% concordant, with the mutations found in corresponding primary EOC (225). The tumor-specific mutations were detectable in 83% in borderline type tumors, 77% of type I and 100% type II tumors (225). Intraabdominal fine needle aspiration of ovarian cysts for detection of malignancy, is not recommended. A puncture of the cyst for cytology or liquid biopsy gene sequencing before surgery might cause leakage of tumor cells, increased risk of metastasis and upstaging of the disease. As of yet, no groundbreaking solution of how to perform preoperative diagnostic sampling of ovarian cysts in situ has been put forward. Puncture in an already metastasized disease is on the contrary not contraindicated, but recommended for the decision of treatment.

Whereas the present sampling techniques are yet to be refined ongoing work is clearly pointing to that early diagnosis of ovarian cancer can be achieved with liquid biopsies in the future. Screening for specific mutations has already shown improved specificity in diagnosing ovarian cancers, although sensitivity is still an imminent problem. Detection of tumor DNA in a thin prep fluid sample is a brilliant idea, but the approach still needs to be improved. The diagnostic accuracy was higher for endometrial cancer than for ovarian cancers. This is not unexpected, as access to ovarian tissue is limited at this location and a large proportion of patients enrolled in the study were suffering from advanced disease, often with obliterated fallopian

tubes, which easily explains the discrepant results when comparing endometrial and ovarian cancer diagnosis using this technique. Additionally, for the 18 and 16 genes respectively assessed in Paper IV, mutations were more specific for endometrial cancer and several important ovarian cancer mutations such as ARID1A and BRCA1/2 were not assessed (table 2). We believe that a larger and prospective trial needs to be undertaken to better evaluate the benefits of the PapSEEK method for early diagnosis of ovarian cancers. Additionally, including the combination of analysis of protein biomarkers and a healthy control group would be of interest in the future. It may still be early days of the liquid biopsy approach to acquire predictive tools for ovarian cancer diagnosis. However, our work in Paper IV hold promise and clearly identifies a way forward.

One treatment does not fit all

Diagnostic tools with high specificity and sensitivity have been lacking for assessment of ovarian cancer. With the much-improved knowledge about the molecular mechanisms and different oncogenetic features in EOC, the origin of EOC have to be considered. Thus, today we know that EOC is as a group of heterogeneous cancers, which partly explains why generalized treatment is hard to achieve. There are at least two key factors that may improve survival of women diagnosed with EOC: 1) screening for EOC and 2) individualized treatment. Identifying different biomarkers is another example of a future strategic development for improving early diagnosis, which also could take into account histological staging of the different cancer subtypes. This approach can hopefully improve the predictive value of a diagnostic test and suggest better personalized treatments. There are a few good examples. Adjuvant treatment with anti-VEGF antibody (bevacizumab) in combination with a platinum-based chemotherapy increases progression free survival (PFS), but not overall survival (OS), in advanced ovarian cancer. A drawback with this strategy is the lack of molecular markers that could be used to diagnose who would benefit from a particular treatment. Another encouraging example is EOC patients with BRCA mutations. When treated with poly ADP ribose polymerase- (PARP) inhibitors, they have exhibited prolonged PFS. This holds promise for future development of better diagnostic tools (227, 228). The high amount of TP53 mutations in HGSC makes targeted treatment against TP53 mutations attractive (10). There are ongoing phase II trials, and hopefully a TP53-targeted treatment can be implemented in clinical routine in the future (229).

Conclusion

- At the recommended cut-off level of >35 , assessments of CA125 outperformed serum levels of HE4 in diagnosing EOC, but as many as 40% of the women with benign disease in the cohort had false positive CA125 levels. On the other hand, assessments of HE4, using recommended cut-off levels outperformed CA125 in identifying the benign cases and, hence, helps reducing the false positive results (Paper I).
- The diagnostic ability of the algorithms RMI and ROMA was comparable in this cohort using the recommended cut-off levels, supporting that the ROMA algorithm can be used as a first line triage tool, since it does not rely on a specialist for ultrasound assessments (Paper I).
- Combining assessments of HE4 in serum with CA125 determinations or with RMI significantly improved the specificity for diagnosing ovarian cancer. In addition, adding TVU analysis to the ROMA algorithm improved specificity for EOC in both pre- and postmenopausal women as compared to routine diagnostic assessments (the baseline models) (Paper II).
- It is important to penetrate the medical and gynecological history and acknowledge factors such as age, smoking habits, presence of heart disease, renal failure, and microscopy-verified endometriosis before concluding the relevance of increased serum levels of HE4 or CA125 in patients with a suspected ovarian cyst/pelvic tumor (Paper III).
- Serum levels of HE4 should be included in clinical investigations of a suspected ovarian cyst/pelvic tumor in pre-menopausal women.
- The new diagnostic possibilities that come with liquid biopsies and high throughput molecular analysis of gene mutations have improved the specificity of EOC diagnosis and allowed us to effectively discriminate between benign conditions and malignant tumors (Paper IV).

- Sampling of DNA from cervical or endometrial liquid biopsies has made rare mutation detection simple and when combined with detection of ctDNA sensitivity can be increased without hampering specificity (paper IV).
- This thesis demonstrates that if we combine the right parameters we can significantly improve the prognostic value of the EOC-diagnosis and it may help us to identify malignant disease at an early stage and improve differential diagnosis.
- High-throughput molecular analyses has given us an increased understanding of the etiology of EOC and hopefully this will lead to more specific diagnostic tests and individualized therapies.
- It can be foreseen that clinical implementation of the new diagnostic tools may take time, but we should be more optimistic today as we have seen promising new developments.
- Even though new molecular genetics holds promise, we still can explore the use of biomarkers, such as serum levels of CA125 and HE4. If combined with an algorithm, such as RMI, these assessments could have immediate clinical implications for improving the accuracy of EOC diagnosis.

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