Turun yliopisto
University of Turku

OVARIAN CANCER MARKER HE4 IN HORMONE-RELATED GYNECOLOGICAL CONDITIONS AND DIAGNOSIS OF OVARIAN GRANULOSA CELL TUMORS

Marianne Hallamaa

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1278 | Medica - Odontologica | Turku 2017

University of Turku

Faculty of Medicine
Department of Obstetrics and Gynaecology
Department of Physiology
University of Turku Doctoral Programme of Clinical Investigation

Supervised by

Adjunct Professor Antti Perheentupa
Department of Obstetrics and Gynaecology
University of Turku, Finland

Professor Matti Poutanen
Department of Physiology
University of Turku, Finland

Reviewed by

Professor Ulla Puistola
Department of Obstetrics and Gynaecology
University of Oulu, Finland

Adjunct Professor Piia Aarnisalo
Department of Clinical Chemistry
University of Helsinki, Finland

Opponent

Professor Johanna Mäenpää
Department of Obstetrics and Gynaecology
University of Tampere, Finland

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6782-7 (PRINT)
ISBN 978-951-29-6783-4 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy - Turku, Finland 2017

To my family

ABSTRACT

Marianne Hallamaa

Ovarian cancer marker HE4 in hormone-related gynecological conditions and diagnosis of ovarian granulosa cell tumors

University of Turku, Faculty of Medicine, Department of Obstetrics and Gynaecology and Department of Physiology, Doctoral Programme of Clinical Investigation, Turku, Finland

Annales Universitatis Turkuensis 2017

Ovarian cancer is commonly diagnosed at an advanced stage as the early stages are symptom-free. Despite the development in the fields of surgery and chemotherapy, the prognosis remains poor. In order to improve the diagnostic methods, research on biomarkers such as HE4 (Human epididymis protein 4) is actively ongoing. According to previous studies, HE4 is sensitive in detecting even early stages of epithelial ovarian cancer, yet its specificity needs further studies.

Altogether 359 women were included in this study. The aims were to evaluate the performance of serum tumor marker HE4 in benign gynecological conditions and to determine confounding factors in the interpretation of the marker analysis. The usability of epithelial ovarian cancer markers HE4 and CA125 in comparison with inhibin B and AMH was evaluated in the diagnosis and follow-up of ovarian granulosa cell tumors.

HE4 serum concentration was not significantly dependent on hormonal factors, which simplifies the interpretation of the serum HE4 assays particularly in women of fertile age. In tubal pregnancies we detected elevated serum HE4 concentrations, and the tubal epithelium showed more intense and continuous immunohistochemical HE4 staining than normal fallopian tubes.

Combining HE4 with CA125 improves accuracy in ovarian cancer diagnostics. However, normal serum levels of these epithelial ovarian cancer markers do not exclude other ovarian cancer subtypes, which must be kept in mind particularly in premenopausal women. The best serum marker for the diagnosis and follow-up of ovarian granulosa cell tumors is inhibin B, yet its accuracy can be further improved by combining AMH to the analysis.

Keywords: HE4, serum marker, differential diagnosis, CA125, ovarian cancer

TIIVISTELMÄ

Marianne Hallamaa

Munasarjasyövän merkkiaine HE4 hormoniriippuvaisissa gynekologisissa tiloissa sekä munasarjan granuloosasolukasvainten diagnostiikassa

Turun Yliopisto, Lääketieteellinen tiedekunta, Synnytys- ja Naistentautioppi, Fysiologia, Kliininen Tohtoriohjelma, Turku, Suomi

Annales Universitatis Turkuensis 2017

Munasarjasyöpä on diagnosoitaessa useimmiten laajalle levinnyt, koska alkuvaiheen tauti on oireeton. Tämän vuoksi selviytymisennuste on sekä kirurgisten että lääkkeellisten hoitojen kehityksestä huolimatta edelleen huono. Diagnostiikan parantamiseksi tutkimuksissa on kehitetty uusia kasvainmerkkiaineita, kuten HE4 (Human Epididymis Secretory protein 4). HE4 on osoittautunut tehokkaaksi epiteliaalisen munasarjasyövän varhaisdiagnostiikassa, mutta sen tarkkuudesta tarvitaan lisätutkimuksia.

Tutkimukseen osallistui yhteensä 359 naista. Tutkimuksen tarkoituksena oli selvittää kasvainmerkkiaine HE4:n seerumipitoisuuksien vaihtelua erilaisissa hyvänlaatuisissa gynekologisissa tiloissa sekä selvittää mahdollisten virhelähteiden vaikutusta merkkiainepitoisuuksien tulkinnassa. Lisäksi arvioitiin epiteliaalisen munasarjasyövän merkkiaineiden HE4:n ja CA125:n käytettävyyttä munasarjan granuloosasolukasvainten erotusdiagnostiikassa ja seurannassa verrattuna inhibiini B- ja AMH-merkkiaineisiin.

HE4-merkkiaineen seerumipitoisuuksissa ei todettu hormonivaikutuksesta, kuten yhdistelmäehkäisypillereistä, kuukautiskierrosta tai koeputkihoitoon liittyvästä munasarjojen stimulaatiosta, johtuvaa merkittävää vaihtelua. Tämä yksinkertaistaa HE4-määritysten tulkintaa etenkin fertiili-ikäisillä naisilla. Munanjohdinraskaudessa sen sijaan totesimme kohonneita HE4-seerumipitoisuuksia sekä lisääntynyttä voimakasta immunohistokemiallista värjäytymistä munanjohtimen epiteelissä.

Yhdistämällä HE4- ja CA125-määritykset munasarjasyövän diagnostiikassa on päästy parempaan herkkyyteen ja tarkkuuteen. Etenkin hedelmällisessä iässä olevien naisten kohdalla on kuitenkin muistettava, että näiden epiteliaalisen munasarjasyövän merkkiaineiden viiterajoissa olevat pitoisuudet eivät poissulje harvinaisempia munasarjasyöpätyyppejä. Munasarjan granuloosasolukasvainten diagnostiikassa ja seurannassa toimivin kasvainmerkkiaine on inhibiini B, jonka diagnostista osuvuutta voidaan parantaa yhdistämällä määritykseen AMH-pitoisuuden määritys.

Avainsanat: HE4, kasvainmerkkiaine, erotusdiagnostiikka, CA125, munasarjasyöpä

TABLE OF CONTENTS

ABBREVIATIONS

AGCT	Adult-type ovarian granulosa cell tumor
AMH	Anti-Müllerian hormone
AUC	Area under the curve
BMI	Body mass index
BRCA	Breast cancer susceptibility gene
CA125	Cancer antigen 125
CMIA	Chemiluminescent microparticle immunoassay
DF	Disease free
E2	Estradiol
ECLIA	Electrochemiluminescence immunoassay
EIA, ELISA	Enzyme-linked immunosorbent assay
ENDO	Endometriosis group
EOC	Epithelial ovarian cancer
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics
FSH	Follicle stimulating hormone
GEU	Extrauterine pregnancy
GFR	Glomerular filtration rate
GnRH	Gonadotrophin releasing hormone
hCG	Human chorionic gonadotrophin
HE4	Human epididymis protein 4
HNPCC	Hereditary nonpolyposis colorectal cancer
HRT	Hormone replacement therapy
Inh B	Inhibin B
IOTA	International Ovarian Tumor Analysis group
IVF	In vitro fertilization
RMI	Risk of malignancy index
ROC	Receiver operating characteristics
ROMA	Risk of Ovarian Malignancy Algorithm
RRSO	Risk-reducing salpingo-oophorectomy
TVU	Transvaginal ultrasound
WD	With disease

LIST OF ORIGINAL PUBLICATIONS

I Hallamaa M, Suvitie P, Huhtinen K, Matomäki J, Poutanen M, Perheentupa A. Serum HE4 concentration is not dependent on menstrual cycle or hormonal treatment among endometriosis patients and healthy premenopausal women. Gynecol Oncol. 2012 Jun;125(3):667-72.

II Hallamaa M, Huhtinen K, Suvitie P, Perheentupa A. Serum concentrations of HE4 change little during in vitro fertilization. Acta Obstet Gynecol Scand. 2014 Jul; 93(7):640-6.

III Haltia UM, Hallamaa M, Tapper J, Hynninen J, Alfthan H, Kalra B, Ritvos O, Heikinheimo M, Unkila-Kallio L, Perheentupa A, Färkkilä A. Roles of human epididymis protein 4, carbohydrate antigen 125, inhibin B and anti-Müllerian hormone in the differential diagnosis and follow-up of ovarian granulosa cell tumors. Gynecol Oncol. 2017 Jan;144(1):83-89.

IV Hallamaa M, Gabriel M, Huhtinen K, Rantala M, Mäkinen J, Kujari H, Poutanen M, Perheentupa A. Origin of elevated serum HE4 in tubal pregnancy. *Manuscript.*

1. INTRODUCTION

Ovarian cancer is the third most common gynecological cancer and the fourth most common among all cancers in women, causing around 350 deaths yearly in Finland (Finnish Cancer Registry 2014). Despite intensive research and the development of chemotherapeutic options, the prognosis of this disease has improved little over the past decades. The most important reason for this is the often delayed diagnosis. Most ovarian cancers are diagnosed at an advanced stage (III-IV) with a poor prognosis (49.7-17.9%), whereas patients with early stage (I-II) disease have 84.7-92.1% chance of surviving the disease (Heintz et al. 2006). The diagnostic challenge of ovarian cancer is caused by its relatively long symptom-free period. When the often vague symptoms like nausea, stomach pain and ascites appear, the disease is usually already widely spread.

The diagnosis of ovarian cancer is most commonly achieved with the combination of clinical status, ultrasonographic findings and tumor markers. The golden standard of ovarian cancer markers is cancer antigen 125 (CA125), a glycoprotein already introduced four decades ago (Bast et al. 1983). It is used worldwide not only for diagnostic purposes but also to evaluate the effect of ongoing treatment and in follow-up of the patients. CA125, however, has several flaws. It is elevated in many benign gynecological conditions, such as pelvic inflammatory disease, uterine fibroids, pregnancy, ovarian hyperstimulation (Meden and Fattahi-Meibodi) and endometriosis (Fedele et al. 1988, Pittaway and Fayez 1986). In addition, the elevation of serum CA125 levels have been detected in non-gynecological inflammatory diseases such as tuberculosis and thyreoiditis leading to hypothyreosis (Huang et al. 2011, Hashimoto et al. 1989) Some studies have also shown CA125 concentrations to vary within the menstrual cycle (Kafali et al. 2007, Erbağci et al. 1999). The performance of CA125 can be enhanced by combining it with other tumor markers or with ultrasound examination (Jacobs et al. 1988).

One of the most intensively studied novel ovarian cancer markers is Human Epididymis Secretory Protein 4 (HE4). The HE4 gene (WFDC2) expression is amplified in ovarian carcinomas but low in normal tissue (Hellström et al. 2003). HE4 has been found to be more sensitive than CA125 in early-stage ovarian cancer diagnostics (Havrilesky et al. 2008) and in detecting recurrence of the disease (Anastasi et al. 2010). Benign gynecological conditions such as ovarian cysts, uterine fibroids and endometriosis have less impact on serum HE4 than CA125 concentrations (Moore et al. 2012, Huhtinen et al. 2009).

The diagnosis of non-epithelial ovarian cancers, however, requires a different approach. The serum markers HE4 and CA125, as well as the algorithms designed to evaluate the risk of malignancy, do not rule out these less common ovarian cancer subtypes.

The aim of the present study was to determine the effect of hormonal factors on serum HE4 concentrations in comparison with CA125. The HE4 immunoreactivity was evaluated in benign and malignant gynecological tissues, with particular interest in conditions affecting the fallopian tube as a possible origin of serous pelvic cancer. Further, the performance of epithelial cancer markers HE4 and CA125, together with inhibin B and AMH, was evaluated in the diagnosis and follow-up of ovarian granulosa cell tumors.

2. REVIEW OF THE LITERATURE

2.1. HUMAN EPIDIDYMIS SECRETORY PROTEIN 4

2.1.1. Background

HE4 is a stable four-disulphide core protein originally found lining the human epididymis (Kirchhoff et al. 1991). It is a whey acidic type of protein (WAP) comprising approximately 50 amino acids, including eight cysteines in a conserved arrangement (Ranganathan et al. 1999). HE4 is thought to have a role in sperm maturation, yet its function is still somewhat unclear. Protease inhibiting action with significant binding to many seminal fluid proteases has been shown in protein-level studies (Chhikara et al. 2012). The similarities with known leukocyte protease inhibitors in the WFDC protein family also suggest HE4 to have a role in natural immunity (Clauss et al. 2002). The HE4 gene (WFDC2) resides on human chromosome 20q12-13.1. It has been found to be overexpressed in ovarian carcinomas (Schummer et al. 1999), but not in normal ovaries, and being a small, secreted protein, it was introduced as a clinical marker for ovarian cancer (Hellström et al. 2003, Schummer et al. 1999). High HE4 expression has been found in several normal epithelial tissues, such as trachea and salivary gland, and genital tracts in both genders outside of the testes and ovaries. In males, HE4 expression is the highest in the epithelial cells of the epididymal and spermatic ducts, with low and sparse expression in the prostate. The highest expression in females is seen in endometrium, fallopian tubes, endocervical and Bartholin's glands, whereas little or no expression has been detected in myometrium, vulva and ovary. Ovarian cortical inclusion cysts that are lined by metaplastic Müllerian epithelium have shown higher HE4 expression levels than normal ovarian epithelium. (Galgano et al. 2006, Drapkin et al. 2005, Georgakopoulos et al. 2012, Bingle et al. 2002). The WFDC2 gene presents with variable levels of expression also in several distinct parts of the body such as colon, pancreas, kidney tubules, breast tissue, anterior pituitary, thyroid, lacrimal and eccrine glands (Galgano et al. 2006) (**Table 1**).

Altogether five mRNA variants of the HE4 gene with different exonic arrangements have been identified as a result of variable splicing or utilization of alternative promoters (Bingle et al. 2002). In both benign and malignant tissues, HE4-V0, -V1 and -V3 isoforms are most abundant, HE4-V0 depicting the prototype of the HE4 protein. The expression of all HE4 protein isoforms is significantly increased in endometrioid and papillary serous forms of endometrial cancer, whereas increased expression of HE4-V1, -V3 and -V4 variants present with a negative impact on survival of these patients (Jiang et al. 2013). Significant correlation of the protein isoform HE4-V3 and improved prognosis in adenocarcinoma of the lung has also been reported (Tokuishi et al. 2012). The vast majority of studies and methods detecting HE4 expression has been founded on the structure of HE4-V0 with no distinction between the isoforms. However, the

clinical relevance of the structural, and possibly functional, variation of the HE4 molecule appears limited, as HE4-V0 is present in 10-100-fold levels as compared to HE4-V1 and -V3 and in 100-1000-fold levels as compared to HE4-V2 and -V4 in benign tissues, similar finding being present in endometrial carcinoma (Jiang et al. 2013).

Table 1. HE4 protein expression in healthy human tissues. Modified from Drapkin et al. Cancer Res. 2005, Bingle et al. Oncogene. 2002

Tissue examined	Positive/tested (Drapkin et al.2005)	Level of HE4 expression (Bingle et al. 2002)
GI-tract (esophagus, stomach, gallbladder, duodenum, colon, pancreas, liver, spleen)	0/37	n/a
Lymph node	0/7	n/a
Skeletal and cardiac muscle	0/8	n/a
Lung	1/4	weak
Trachea, salivary glands	5/5	strongest
Thyroid	0/6	weak
Kidney	5/6	strong
Brain	0/4	n/a
Breast	4/5	weak
Ovary	0/7	weak
Fallopian tubes	10/10	n/a
Endometrium	4/4	n/a
Cervix	4/4	n/a
Uterus (non-specificated)	n/a	weak
Epididymis	5/5	n/a
Fetal tissues (lung, kidney)	0	weak
Testes	0/4	negative
Prostate	4/7	weak
Pituitary	n/a	weak
Nasal epithelium	n/a	strong

Serum HE4 concentration can be measured using immunometric techniques; enzyme-linked immunosorbent assay (EIA), chemiluminescent microparticle immunoassay (CMIA), electrochemiluminescence immunoassay (ECLIA) and chemiluminescent enzyme immunoassay (CLEIA). There is a commercial kit available for each technique (**Table 2**). The Fujirebio EIA method has been reported to measure 11.3-16.9% higher serum HE4 levels than the equivalent Abbott assay (Bolstad et al. 2011). Comparing the automated CMIA (Abbott Diagnostics) and ECLIA (Roche Diagnostics) assays with EIA as the reference, the bias was an acceptable -3.3% (-6.1 vs. -0.5%) for CMIA and -0.2% (-3.0 vs. 2.5%) for ECLIA, with the bias for ECLIA, however, increasing with elevated values (-28% for HE4>250 pM). Applying chemiluminescence EIA on the automated Lumipulse system (Fujirebio Diagnostics) resulted in a remarkable positive bias of 25.3% (21.8-28.8%) (Ferraro et al. 2016).

Table 2. Laboratory methods for HE4 analysis. Cutoff limits provided by manufacturer.

Technique	Manufacturer	Range (pM)	Detection limit (pM)	Cutoff (pM)
EIA (manual)	Fujirebio	15-900	15	<150
CMIA (automated)	Abbott (Abbott Architecture®)	20-1500	0.18	<70 premenopausal, <140 postmenopausal
ECLIA (automated)	Roche (Elecsys®)	15-1500	15	<140
CLEIA (automated	Fujirebio (Lumipulse®)	20-1500	3.5	<150

Urine measurements with ELISA technique have been studied (Hellstrom et al. 2010, Liao et al. 2015), showing similar diagnostic specificity (94.4%) and sensitivity (86.6% in stage I/II and 89% in stage III/IV ovarian cancer) as previous studies of serum measurements. The discrimination potential of urine HE4 in differentiating ovarian cancer from benign ovarian tumors is considerable, detecting tumors with low malignant potential even better than serum HE4 (Liao et al. 2015).

Considering the significant effect of kidney function on serum HE4 levels, specific ratios between urine creatinine concentration or glomerular filtration rate and urine HE4 have also been calculated, one study showing improvement in diagnostic performance (Hellstrom et al. 2010) and another one not (Macuks et al. 2012). The effect of repetitive freezing-thawing cycles and delayed analysis in room (20°C) or lower (4°) temperature on the stability of HE4 levels has been evaluated by Sandhu et al. (Sandhu et al. 2014) with no significant fluctuation of the levels. The authors also reported no significant difference between HE4 concentrations analyzed from serum or EDTA plasma.

2.1.2. Biological variation

Smoking increases serum HE4 levels, yet the most significant non-malignant cause for elevated HE4 concentrations is renal insufficiency (Bolstad et al. 2011, Park et al. 2011). Ageing increases HE4 levels and age-dependent reference ranges have been suggested (**Figure 1a**). In a meta-analysis by Moore et al. (2012), the serum samples were pooled in age groups by decade and classified to pre- or postmenopausal according to age (<45 or >55) or medical history. Clear increase of serum HE4 concentration by age was demonstrated, menopausal status not being an independent factor (**Figure 1b**). The International Federation of Clinical Chemistry and Laboratory Medicine and the Clinical and Laboratory Standards Institute recommend determining cut points for laboratory values as upper confidence interval for the 95th percentile. The reference limits provided by Fujirebio were estimated in a group of 1,147 pre- and postmenopausal women with 94.4% of HE4 assay value at or below 150 pM. Similar limits for postmenopausal women were suggested by Moore and colleagues (2012), however, they recommend lower thresholds for premenopausal women based on the 95th percentile value of 89.1 pM in their study group of 475 premenopausal women. In Asian population, the 95th percentile cutoff limit for a group of 2,182 women aged 20-65 years was detected to be as low as 30.3 pM (Park et al. 2012).

	Men	Women
Age	Reference limit (90% CI)	Reference limit (90% CI)
18	43.4 (42.2-44.7)	51.5 (50.3-52.6)
22	43.9 (42.6-45.2)	50.2 (49.1-51.3)
26	44.7 (43.3-46.1)	49.4 (48.3-50.6)
30	45.9 (44.5-47.4)	49.1 (48.0-50.3)
34	47.3 (45.8-48.8)	49.1 (48.1-50.3)
38	48.9 (47.4-50.5)	49.6 (38.5-50.8)
42	50.8 (49.2-52.4)	50.3 (49.2-51.5)
46	52.9 (51.3-54.6)	51.3 (50.2-52.5)
50	55.1 (53.5-56.9)	52.6 (51.5-53.7)
54	57.5 (55.9-59.3)	54.1 (52.9-55.2)
58	60.1 (58.4-61.9)	55.8 (54.6-56.9)
62	62.8 (61.1-64.7)	57.7 (56.5-58.8)
66	65.7 (63.9-67.6)	59.7 (58.6-60.9)
70	68.7 (66.8-70.6)	62.0 (60.9-63.2)
74	71.8 (69.9-73.8)	64.4 (63.3-56.5)
78	75.0 (73.1-77.0)	67.0 (65.9-68.2)
82	78.4 (76.4-80.4)	69.7 (68.6-70.9)
86		72.6 (71.5-73.8)

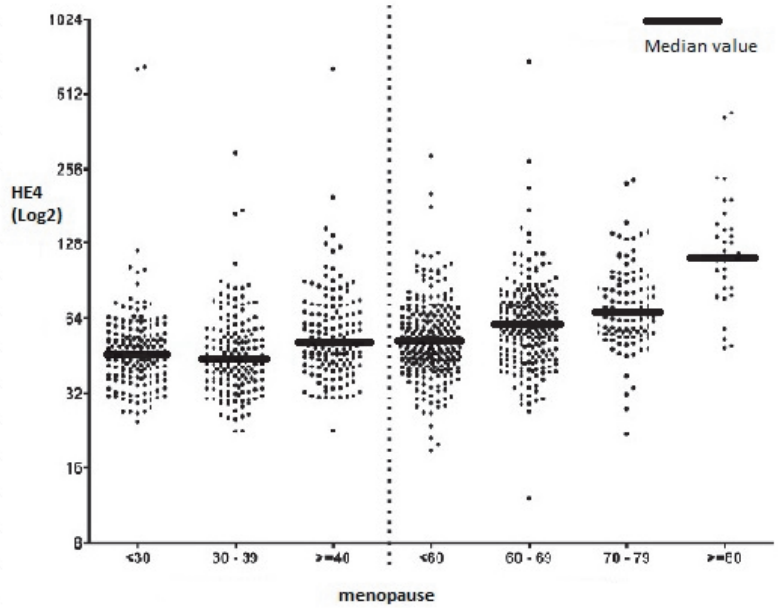


Figure 1a+b. Age-dependent HE4 reference values have been suggested, however menopausal status does not appear to have independent effect on HE4. (modified from Bolstad, 2011 and Moore, 2012)

Pregnancy has in some studies been shown to decrease serum HE4 levels (Moore et al. 2012), in others not (Park et al. 2012, Gucer et al. 2015). Males have 7% lower HE4 levels, however the effect of age is more prominent in men. Overweight decreases serum HE4 concentration by 10% (BMI 30 vs. 20) (Bolstad et al. 2011). Slight fluctuation of serum HE4 concentration throughout the menstrual cycle has been demonstrated (Anastasi, et al. 2010). The history of hormonal contraception use or hormone replacement therapy does not have an effect on the HE4 values (Lowe et al. 2008).

2.1.3. Clinical implications in gynecology

The diagnosis of pelvic tumors comprises a combination of clinical examination, transvaginal ultrasound and serum tumor markers. For three decades, CA125 was the solely used marker for epithelial ovarian cancer. Now, within the past ten years, HE4 analysis has become commercially available, and it has been accepted for worldwide clinical use. In addition to the use in differential diagnosis of EOC (Hellström et al. 2003, Havrilesky et al. 2008), the usability of HE4 in EOC follow-up has been reported, with some evidence that HE4 increase could detect recurrence earlier than CA125 (Anastasi et al. 2010, Innao et al. 2016). However, no studies on the effect of HE4 use in follow-up in patient survival have been published, taken into consideration the clinical effects of the randomized controlled trial by Rustin and colleagues (2010). They presented no survival benefit from early treatment of EOC recurrence based on early CA125 rise as compared to starting treatment only when symptoms occur. In contrast to CA125, elevated serum HE4 concentrations have also been reported to correlate with platinum resistance at the time of the third chemotherapy cycle, suggesting that HE4 might be valuable in treatment planning (Angioli et al. 2014).

In 2009, the HE4 EIA analysis was approved by the American Food and Drug Administration (www.fda.gov) for monitoring patients with ovarian cancer and in 2011 it was approved together with CA125 analysis to estimate the risk of ovarian malignancy.

Besides epithelial ovarian cancer, HE4 may be used in the diagnosis of endometrial cancer (Moore et al. 2008, Huhtinen et al. 2009), serving also as a prognostic factor (Bignotti et al. 2011) and detecting recurrence (Brennan et al. 2015). Different subtypes of ovarian cancer, however, seem to require a different approach. In addition to serous ovarian carcinoma, most clear-cell and endometrioid carcinomas express HE4 in immunohistochemical staining. However, HE4 is expressed only rarely in ovarian mucinous, germ cell and sex cord stromal tumors (Galgano et al. 2006). These less common types of ovarian cancer often present a diagnostic challenge, and the development of targeted specific tumor markers could be very beneficial in clinical work.

Maybe the most clinically relevant weakness of CA125 is the fact that elevated CA125 is found only in 50-60% of stage I ovarian cancers (Bast et al.). In some studies, HE4 has been identified as the most sensitive marker in stage I EOC (Moore et al. 2008, Havrilesky et al. 2008). Nevertheless, a recent, large study published opposite results

questioning the superiority of HE4 in early diagnosis (Terry et al. 2016). In conclusion, neither perform exclusively in diagnosing the early stages (Kristjansdottir et al. 2013).

2.1.4. Non-ovarian cancers

HE4 can be elevated also in non-gynecological cancers. A small study of 31 women with a non-gynecological (mostly breast, some gastrointestinal and pancreatic) and nine with non-ovarian gynecological (cervical or endometrial) cancer detected a difference between the non-gynecological cancers and the healthy control group (84 pM vs. 48 pM, p=0.0004), whereas the slight elevation of HE4 levels in non-ovarian gynecological cancers was not significant compared to healthy controls (62 pM vs. 48 pM) (Park et al. 2011). Other neoplasms that show strong HE4 expression are adenocarcinomas of the lung and certain tumors of the salivary gland, thyroid, breast and pancreas and transitional cell tumors of the bladder (Galgano et al. 2006). Some HE4 upregulation has also been found in gastric and pancreatic carcinomas, however, showing no correlation with patient outcome (O'Neal et al. 2013), and only of modest diagnostic value (Huang et al. 2015), thus far with no clinical advantage. Serum HE4 concentrations have been found significantly elevated in primary adenocarcinoma of the lung compared to healthy individuals (Escudero et al. 2011, Hertlein et al. 2012) with potential as a diagnostic and prognostic marker for lung cancer (Iwahori et al. 2012, Lamy et al. 2015, Nagy et al. 2014). Single studies showing serum HE4 increase in pancreatic cancer (Park et al. 2011) and transitional cell cancer of the bladder (Xi et al. 2009) have also been published.

2.2. COMPARISON WITH OTHER MARKERS

2.2.1. CA125

The murine monoclonal antibody OC125 was developed already four decades ago (Bast et al. 1981). It recognizes an antigenic determinant CA125, present in over 80% of epithelial ovarian carcinoma cell lines. CA125 has then, over the years, become the golden standard of ovarian cancer biomarkers in diagnosis, during treatment, follow-up and recurrence detection. CA125 is expressed in epithelial cells of fetal coelomic origin; Müllerian lining, peritoneum, pleura and pericardium and in adults also in fallopian tubes, endometrium, endocervix, pleura and peritoneum, but not in normal ovarian epithelium (Kabawat et al. 1983). The fact that CA125 is produced not only by ovarian cancer cells but also by normal epithelia reactive in many non-malignant conditions causing peritoneal irritation might cause some of the problems in its non-specificity. As a result of its production by the endometrium or resulting from peritoneal irritation, CA125 fluctuates within the menstrual cycle, the highest concentrations in most studies seen during menstruation (Pittaway and Fayez 1987, Bon et al. 1999). Many benign gynecological tumors such as ovarian cystadenomas, uterine fibroids and adenomyosis and endometriomas, as well as other forms of endometriosis, can cause significantly elevated

serum CA125 levels (Giudice et al. 1986, Kitawaki et al. 2005, Barbieri et al. 1986, Meden and Fattahi-Meibodi, Babacan et al. 2014). Pelvic inflammatory disease, pregnancy and ovarian hyperstimulation syndrome can also increase serum CA125 concentration (Jäger et al. 1987, Halila et al. 1986). CA125 has certain limitations also in sensitivity. Only half of the early-stage I-II EOC patients have elevated serum CA125 levels (Bast et al. 2005). Although elevated CA125 is generally observed in over 80% of the ovarian cancer tissues (Rosen et al. 2005), the histology of the tumor plays a significant role in the usability of this marker. In the study by Högdall et al. (2007) 85% of serous, 68% of papillary, 65% of endometrioid, 40% of clear-cell, 36% of undifferentiated adenocarcinomas and only 12% of mucinous cancer types expressed CA125 in tissue arrays.

In order to minimize the effect of non-malignant conditions on serum CA125 concentrations, a method has recently been developed to differentiate the CA125 secreted by cancer cells from the CA125 originated by non-malignant conditions. By applying a particular lectin coating to the plate, the immune assay was able to differentiate CA125 originating from ovarian cancer from the CA125 excreted from benign sources, in particular endometriosis, a known source for false positive CA125 increase (Gidwani et al. 2016).

2.2.2. Novel markers and multiassay analysis

As the value of early diagnosis is indisputable in order to improve the prognosis of ovarian cancer patients, research in the field of biomarkers is very active. However, only a small minority of the new potential molecules detected in the sera of ovarian cancer patients eventually present with any clinical relevance. After the implementation of CA125 decades ago, the only ovarian cancer markers approved by the U.S. Food and Drug Administration (FDA) have been HE4 for ovarian cancer follow-up and OVA1 biomarker panel for optimal referral of patients for surgery in 2009 and the ROMA algorithm for estimating the likelihood of malignancy in case of a pelvic mass in 2011.

Overexpression of the osteopontin gene in EOC was detected in a microarray study by Wang et al. (2001) and further validated as a potential diagnostic marker (Kim et al. 2002). It has, together with kallikrein, been associated with advance in EOC stage and tumor grade, poor primary surgical outcome, the presence of ascites and certain histological types (Bandiera et al. 2013). In a recent review and meta-analysis, the osteopontin studies evaluated were found quite inconsistent in results, nevertheless, concluding a pooled sensitivity of 66% (95% CI: 51%-78%) and specificity of 88% (95% CI: 78-93%). Some diagnostic potential was consequently seen, however, no better than that of CA125 (Hu et al. 2015).

In hope for a diagnostic marker panel Moore et al. studied nine potential biomarkers for their performance in EOC diagnosis. CA125, HE4, SMRP (soluble mesothelin-related peptide), CA72-4, activin, inhibin, osteopontin and EGFR (epidermal growth factor) were all significantly elevated in EOC patients compared with women with benign

gynecological conditions, the only marker with no such finding being ERBB2 (Her2). The most sensitive marker was found to be HE4 (72.9% with set specificity of 95%), followed by SMRP (53.7%) and CA125 with only 43.3% sensitivity. Combining HE4 with CA125 improved sensitivity 76.4% vs. 72.9% (HE4 alone) and 43.3% (CA125 alone) with set specificity of 95%. Adding more biomarkers to this combination did not significantly improve the diagnostic performance (Moore et al. 2008).

Another set of 14 potential EOC detection markers available in commercial kits chosen through literature search and available gene expression data were evaluated for their diagnostic performance in early EOC alone and in combinations (Palmer et al. 2008). Out of the markers MUC16, WFDC2 (HE4), MSLN, IGF2, CHI3L1 (YKL40), MMP7, MIF, PRL, SPP1 (OPN), BMP7, LCN2, IL13RA2, TACSTD1 (EpCam) and AMH, the four with the best performance were MUC16 (CA125), WFDC2 (HE4), MSLN (mesothelin) and MMP7 (matrilysin, matrix metalloproteinase 7). The best combination of markers was CA125 with HE4 as expected, however, in this study they were not found superior to CA125 only in EOC vs. healthy controls, all stages and histologies included.

Ling and colleagues detected downregulation of MicroRNA-451 in EOC (2015), associated with higher stage and poor survival prognosis. A serum proteomic study by Zhang et al. (2004) compared patients with early-stage EOC with healthy controls and identified three potential biomarkers, two of which downregulated in cancer (apolipoprotein A1 and truncated form of transthyretin) and one upregulated (a cleavage fragment of inter-α-trypsin inhibitor heavy chain H4). The combination of these markers combined with CA125 yielded better specificity than CA125 alone in differentiating early (stage I/II) EOC from healthy controls (94% vs. 52% with the sensitivity of 83%, respectively). Marker comparison is presented in **Table 3**.

Table 3. Comparison of novel ovarian cancer markers

	Sensitivity	Specificity	ROC-AUC	Number of cases (ovarian cancer/control)	Author
osteopontin	66 %	88 %	0.85	839/1437	Hu 2015 (meta-analysis of 13 studies)
SMRP	61.2%	90 %	0.82	67/166	Moore 2008
CA72-4	49.2%	90 %	0.78	67/166	Moore 2008
activin	31.3%	90 %	0.69	67/166	Moore 2008
inhibin	8.3%	90 %	0.65	67/166	Moore 2008
MSLN	39 %	95 %	0.73	71/143	Palmer 2008
MMP7	35 %	95 %	0.74	71/143	Palmer 2008
apolipoprotein A1 (↓)+ transthyretin (↓)+ inter-α-trypsin inhibitor heavy chain H4 (↑)	83 %	94 %	0.87	195/308	Zhang 2004

SMRP=soluble mesothelin-related peptide, MSLN= mesothelin, MMP7= matrix metalloproteinase 7, ROC-AUC= receiver operating characteristics, area under the curve

The commercially available OVA1 diagnostic test was developed by Fung and collaborators to aid the clinician in referring pelvic mass patients to the right place of

care. It is comprised of five serum markers; Beta 2 microglobulin, CA125, apoliprotein A1, transferrin and prealbumin, to be combined with clinical examination and anamnesis, not to be used as a screening test. Through a mathematical formula, in which the first two markers are expected to rise and the latter three to decrease in case of ovarian cancer, a particular risk score between 0 and 10 is calculated. The recommended cutoff level is 5 for premenopausal women and 4.4 for postmenopausal, higher scores indicating an increased risk of malignancy (2010).

2.2.3. RMI, ROMA

A Risk of Malignancy Index (RMI) was developed to estimate the probability of malignancy and the need to refer the patient to a tertiary hospital for optimal treatment. RMI is calculated by multiplying the menopausal status by the CA125 value and by certain sonographic features (**Table 4**).

Table 4. Risk of Malignancy Index (RMI) = M x CA125 x U. RMI > 200 = Suspicious for malignancy. Modified from Jacobs et al. 1990.

M (menopausal status) 3 for post- and 1 for premenopausal	
Ultrasound findings (U): = 0 if no ultrasound findings = 1 if one ultrasound finding = 3 if 2-5 ultrasound findings	Bilateral (yes/no) Multilocularity (yes/no) Solid areas (yes/no) Ascites (yes/no) Intra-abdominal metastases (yes/no)

The sensitivity of 85.4% and specificity of 96.9% in differentiating benign from malignant pelvic masses was originally reported with a RMI score of 200. With a score of 50 the index predicted 95% sensitivity with 76% specificity (Jacobs et al. 1990). In clinical practice, a second opinion by an oncologist is, therefore, often recommended when the sum reaches 200.

The usability of RMI is, however, compromised by at least two factors, one of which being the small number of early-stage ovarian cancers expressing CA125. Another disadvantage of this index is the need of specialist training for the ultrasound examination. As a response to the request for a more objective malignancy estimate, the research group of Moore and colleagues calculated an algorithm utilizing both CA125 and HE4 using pooled data from two previously completed unpublished pilot studies (**Table 5**). This Risk of Ovarian Malignancy Algorithm (ROMA) was then validated using a study population of 531 patients with a pelvic mass scheduled for surgery and reported to achieve the sensitivity of 86% (95% CI 80.1-90.8) at set specificity of 75% in differentiating benign neoplasms from ovarian cancer. Using cutoff values of ≥ 13.1% (for premenopausal) and ≥ 27.7% (for postmenopausal) was then recommended for classifying patients into the high-risk group (Moore et al. 2009).

Table 5. Risk of Ovarian Malignancy Algorithm (ROMA). Modified from Moore et al, 2009.

[1]Premenopausal:	Predictive Index (PI)= -12.0+2.38*LN(HE4)+0.0626*LN(CA125)
[2]Postmenopausal:	Predictive Index (PI)= -8.09+1.04*LN(HE4)+0.732*LN(CA125)
Predicted Probability (PP)= exp(PI) / [1 / exp(PI)]	
[1] Premenopausal= Woman who has had a period within one year of the study or who is ≤48 years old if the date of the last menstrual bleeding in unknown.	
[2]Postmenopausal= Woman who has not had a menstrual bleeding within a year prior to the study or who is ≥55 years old if the date of the last menstrual bleeding is unknown.	
Women aged between 48 and 55 years with unknown menstrual history or a hysterectomy in the past were evaluated for menopausal status by measuring serum FSH levels, with ≤ 22mIU/ml considered premenopausal.	

Several studies have later compared these two algorithms with each other, yet with no clear conclusion of superiority (Lennox et al. 2015, Richards et al. 2015, Karlsen et al. 2012, Anton et al. 2012, Van Gorp et al. 2012, Jacob et al. 2011, Moore et al. 2010) (**Table 6**).

Some of the variation of the results is caused by the differences in patient classification. Low malignant potential (LMP, borderline) ovarian tumors, difficult to detect with laboratory markers, are in some studies counted as malignant and in others they are excluded or classified as benign. Results with early (stage I/II) ovarian cancers have also not been reported separately in all studies. In one of the studies (Moore et al. 2010) a diversity of imaging techniques (computed tomography, MRI, ultrasound) was utilized in the RMI calculation, which might have a negative input on its accuracy. Regardless of these results, not all studies have been able to demonstrate a benefit from adding laboratory markers to the subjective assessment by sonography in the hands of an experienced specialist (Van Gorp et al. 2012, Valentin et al. 2009).

Table 6. Studies comparing the diagnostic accuracy of RMI and ROMA algorithms.

Study group	N of patients	Sensitivity ROMA vs. RMI	Specificity ROMA vs. RMI	Patient characteristics
Richards 2015 Aust NZ J Obstet Gynecol	50	n/a PPV 62.5 vs. 47.4%	n/a NPV 73.5 vs.74.2%	Ovarian ca vs. benign pathology
Lennox 2015 Int J Gynecol Cancer	131	54% vs. 68% (stage I) 93% vs. 94% (stage III/IV)	n/a	Different histology groups analyzed
Karlsen 2012 Gynecol Oncol	1,218	94.8% vs. 96.0%	76.5% vs. 81.5% at set sensitivity 94.4% (RMI=200)	Ovarian ca vs. benign pathology
Anton 2012 Clinics (Sao Paolo)	128	74.1% vs. 63% or 83.8% vs. 75.7% (LMP counted benign)	75.8% vs. 92.4%	Ovarian ca vs. benign pathology
Van Gorp 2012 Eur J Cancer	374	84.7% vs. 76.0%	76.8% vs. 92.4%	Ovarian ca vs. benign pathology
Jacob 2011 Gynecol Oncol	127	82.8% vs. 89.6% (LMP excl) 25% vs. 50% (LMP only)	87.3% vs. 98.6%	Ovarian ca vs. benign pathology
Moore 2010 Am J Obstet Gynecol	457	94.3% vs. 84.6% 89% vs. 80.7% (LMP counted malign) 85.3% vs. 64.7% (stage I/II)	n/a	Ovarian ca vs. benign pathology (RMI incl. diverse imaging)
PPV= positive predictive value, NPV= negative predictive value, LMP= low malignant potential				

A recent review by Stukan et al (2015) compared up-to-date studies on the diagnostic performance of the methods differentiating between benign and malignant ovarian masses. The methods evaluated were sonography with particular technical requirements, biomarkers (CA125, CA72-4 and HE4), the biomarker panel OVA1, the IOTA ultrasound models, the Risk of Malignancy Index, Risk of Ovarian Malignancy Algorithm, the Pelvic Masses Score, PMS (combination of a sonographic scan, Doppler values and CA125 value) (Rossi et al. 2011) and different combinations of the separate methods. The best diagnostic accuracy was observed applying the IOTA LR2, RMI and a combination of CA125 and HE4, ROMA, PMS, non-IOTA logistic regression model and a histoscanning score logistic regression model, but none of the above performed superior to the subjective evaluation by ultrasound.

The usability of the algorithms involving menopausal status may be compromised by the fact that the practice for defining the menopausal status varies. Some clinicians refer only to patient age, some to the cessation of regular menstrual bleeding and some to repeatedly elevated serum follicle stimulating hormone (FSH) levels. Age, however, is easily feasible and has a significant effect on HE4 and also independently on EOC risk. Karlsen at al. (2015) replaced menopausal status by age in a mathematical formula comprising HE4 and CA125. This Copenhagen Index (CPH-I) performed equally well as RMI and ROMA in discriminating benign ovarian masses from ovarian cancer.

Considering the significant effect of decreased renal function to HE4 concentrations, Kappelmayer et al. calculated a mathematical model combining glomerular filtration rate (GFR) to serum HE4 and CA125 level measurement, that, however, did not improve the discriminative diagnostic potential (2015).

2.3. DIFFERENTIAL DIAGNOSIS OF PELVIC TUMORS

2.3.1. Benign gynecological conditions

Moore and colleagues analyzed HE4 and CA125 concentrations of 1,042 serum samples from women with benign gynecological disorders such as ovarian and non-ovarian cysts of different etiology, pelvic inflammatory disease, uterine fibroids, ovarian tumors of different histology and endometriosis. Different cutoff values 89.1 pM for pre- and 128 pM for postmenopausal women for HE4 were used for analysis, whereas the standard 35 U/ml was used for CA125. HE4 levels were less frequently (8%) elevated in the benign disease compared to CA125 (29%), $p<0.001$. The most profound difference was observed in the endometriosis group (3% vs. 67%, $p<0.0001$). A heterogeneous group of mucinous and non-specified cystadenomas, adenofibromas and cystadenofibromas was the only group with no difference between HE4 and CA125, with a 20% proposition of increased marker levels for both markers. (2012). Park et al. (2011) analyzed serum HE4 and CA125 concentrations in 176 serum samples, 85 of which were collected from women with non-malignant gynecological diseases such as ovarian cysts, uterine

fibroids, adenomyosis and pelvic inflammatory disease. Median CA125 levels were significantly increased in the benign gynecological disease group compared to healthy individuals (30 vs. 11 kU/l, $p<0.05$), whereas median serum HE4 concentrations between these groups were unchanged (48 pM). Other studies show similar results (Holcomb et al. 2011, Karlsen et al. 2012). A study by Huhtinen and colleagues evaluated HE4 for its diagnostic potential between endometriotic cysts and gynecological malignancies (2009). HE4 was shown effective in differentiating all forms of endometriosis from ovarian and endometrial carcinoma.

2.3.2. Ovarian cancer

2.3.2.1. Prevalence and risk factors

Around 500 new ovarian cancers are diagnosed in Finland every year, comprising approximately 3% of all cancers. Of all ovarian cancers diagnosed between years 2008-2012, 59.6% were diagnosed at an advanced stage (III-IV), having already metastasized or spread within the pelvic cavity. Only 14.1% were limited within the same ovary and 0.7% spread into nearby tissues, whereas 8.4% had metastasized beyond local lymph nodes. More than a quarter of the diagnoses were made in women between 60 and 69 years of age and more than a half between the ages 55 and 74. In 2012, OC caused 336 deaths, being the fifth most deadly cancer among Finnish women (Finnish Cancer Register, National Institute for Health and Welfare 2014). Ovarian cancer is a health challenge predominantly in the Western world with the highest incidences found in Europe and Northern America (Ferlay et al. 2015). The possible socio-economic causes to this difference are, however, unclear. The meta-analysis by Poorolajal et al (2014) showed only a slight connection between overweight and increased ovarian cancer risk, additionally, the somewhat overweight-related polycystic ovary syndrome has been suggested as a risk factor in some studies (Schildkraut et al. 1996), but not in all (Balen 2001). The history of endometriosis has, in several studies, been shown to increase the risk of ovarian cancer, especially those of endometrioid and clear-cell types (Rossing et al. 2008, Brinton et al. 1997, Ogawa et al. 2000). Nulliparity (Cramer et al. 1983, Wittenberg et al. 1999, Adami et al. 1994) has long been known to increase the EOC risk. Large epidemiological studies have linked infertility and the history of infertility treatments with a higher ovarian cancer risk, although in isolated studies the causality has been unclear. In a review by Vlahos et al. (2010) an increased EOC risk was found in women who had received infertility treatments but never conceived, whereas women with a successful pregnancy showed no increase. A Cochrane analysis by Rizzuto and colleagues (2013) reached a similar conclusion, with only a slight increase of risk for borderline ovarian malignancies in subfertile women who had undergone infertility treatments. The effect of hormone replacement therapy (HRT) on the EOC risk remains controversial, most recent studies, however, demonstrate a slight increase of risk (Mørch et al. 2012). The meta-analysis by Zhou et al. (2008) concluded with a summary relative risk of 1.24 from cohort studies and a summary odds ratio of 1.19 from case-cohort

studies for the history of HRT use. In most studies the increase in the risk has been length-of-use dependent, while cessation of hormone use has reduced the risk. Smoking, alcohol consumption and dietary factors have been suspected, yet not convincingly proven, to have a slight risk-magnifying effect. Some hereditary gene mutations linked to ovarian cancer are known. Approximately 5% of the ovarian cancer patients carry the BRCA1 mutation highly predisposing to breast cancer, having an estimated 39-66% risk of getting EOC before the age of 70 (Antoniou et al. 2000). BRCA2 and HNPCC mutations also increase the risk but to a lesser extent (11% and 9%, respectively) (Antoniou et al. 2003, Aarnio et al. 1999). A great majority of the EOC cases remain sporadic, however. The reduction of the ovulatory cycles by nearly any cause, namely pregnancies, lactation, combined oral contraceptive use, late menarche and early menopause decreases EOC risk (Booth et al. 1989, Purdie et al. 1995, Hankinson et al. 1995, Risch et al. 1994), as well as tubal ligation and hysterectomy (Hankinson et al. 1993).

2.3.2.2. Diagnostic methods

Ovarian cancer is most commonly diagnosed at an advanced stage, when the patient is already suffering from nonspecific symptoms as bloating, abdominal discomfort and pain. At that time, the preliminary diagnosis is usually made with imaging techniques, mainly transvaginal sonography, presenting with a suspicious-looking ovarian tumor and often ascites. Serum tumor marker concentrations are then analyzed to further confirm the suspicion, so that the extent of imaging and consecutive surgery can be planned optimally.

Risk prediction models for the differential diagnosis between benign and malignant ovarian tumors have been calculated by The International Ovarian Tumor Analysis (IOTA) study collaboration. The first logistic regression model developed by the IOTA sought for an effective combination of independent variables, aiming at the specificity over 75% with the minimum of 90% sensitivity. As a result, a model (LR1) with twelve most useful variables, out of more than 50 having been evaluated, was provided, including personal history of ovarian cancer, hormonal therapy, age, maximal diameter of the lesion, pain, presence of ascites, blood flow within a solid papillary projection, presence of a solid tumor, maximal diameter of the solid component, irregular internal cyst walls, acoustic shadows and a color score of the blood flow within the tumor.

A simplified model (LR2) was then developed with six variables named: age, ascites, blood flow within a solid papillary projection, diameter of the solid component, irregular internal cyst walls and presence of acoustic shadows (**Table 7**). The multicenter study population of 1,066 patients with benign or malignant ovarian tumors was divided into a developmental data group of 754 patients and a study validation data group (n=312). Within the validation group, with the probability value (risk of malignancy) of 0.10, the AUC was 0.94 with a sensitivity of 93% and a specificity of 76% for LR1 and 0.92 for LR2 with a sensitivity of 89% and a specificity of 73% (Timmerman et al. 2005). In the

IOTA phase II study the aim was to prospectively evaluate the performance of these models with further 1,938 ovarian tumor patients. A temporal validation within the original seven research units (941 patients) resulted in AUC values of 0.95 for LR1 and 0.92 for LR2 and 0.96 for subjective ultrasound assessment in differentiating malignant from benign ovarian tumors. The results for an external validation by 12 new research centers (n=997) were 0.96, 0.95 and 0.95, respectively (Timmerman, et al. 2010). Later studies have come up with similar results (Nunes et al. 2013, Van Holsbeke et al. 2012), the models performing equally or nearly as well as experienced subjective assessment by ultrasound. However, the expertise of the ultrasound examiners naturally affects these results and when the examiner is less experienced, more benefit can be expected from a more structured model with less subjective evaluation (Sayasneh et al. 2013).

Table 7. Logistic regression models to distinguish between the benign and malignant adnexal mass by the IOTA group. Modified from Timmerman et al. 2005.

Logistic Regression Model (LR) 1	**Logistic Regression Model (LR) 2**
1. The age of the patient (years)	
2. The presence of ascites (yes = 1, no = 0)	1. The age of the patient (years)
3. The presence of blood flow within a papillary	2. The presence of ascites (yes = 1, no = 0)
projection (yes = 1, no = 0);	3. The presence of blood flow within a papillary projection (yes = 1, no= 0)
4. The largest diameter of the solid component (expressed in mm but with no increase above 50 mm);	4. The maximal diameter of the solid component (expressed in mm but with no increase above 50 mm);
5. Irregular internal cyst walls (yes = 1, no = 0);	5. Irregular internal cyst walls (yes = 1, no = 0);
6. The presence of acoustic shadows (yes = 1, no = 0);	6. The presence of acoustic shadows (yes = 1,no = 0).
7. Personal history of ovarian cancer (yes =1, no = 0);	
8. Current hormonal therapy (yes = 1, no = 0);	
9. The largest diameter of the lesion (mm);	
10. The presence of pain during the scan (yes = 1, no =0);	
11. The presence of a purely solid tumor (yes = 1, no= 0)	
12. The color score (1, 2, 3 or 4).	
The model's estimated probability of malignancy for an adnexal tumor equals $1/(1 + e^{-z})$, where $z = -6.7468 + 0.0326(1) + 1.5513(2) + 1.1737(3) + 0.0496(4) + 1.1421(5) - 2.3550(6) + 1.5985(7) - 0.9983(8) + 0.00841(9) - 0.8577(10) + 0.9281(11) + 0.4916(12)$, and e is the mathematical constant and base value of natural logarithms.	$z = -5.3718 + 0.0354(1) + 1.6159(2) + 1.1768(3) + 0.0697(4) + 0.9586(5) - 2.9486(6$

The IOTA has also implemented simple ultrasound rules to help in differentiating benign ovarian masses from the malignant, using the same study population with 1,066 ovarian tumor patients (Timmerman et al. 2008). A large number of ultrasound variables and their combinations were tested for the best capability to predict the behavior of the tumor. The variables with the highest positive predictive value for malignancy were chosen to form the M-rules, and respectively the findings with the lowest positive predictive value with regard to malignancy were named the B-rules. The performances of different variables were tested alone and in various combinations, resulting in ten simple rules (**Table 8**). None of the ultrasound findings showed sufficient predictive value alone, however, the presence of a solid tumor, ascites or a strong blood flow using Doppler measurement were independent risk factors for malignancy. Acoustic shadowing, the

absence of visible blood flow into the tumor and the presence of a unilocular cyst were indicators of a benign tumor. The rules were later validated in a large study population of 1,938 patients with the diagnostic performance as good as with the subjective assessment (Timmerman, Ameye, et al. 2010). Different risk score systems have been developed for estimating EOC risk in different clinical conditions. Epidemiological factors such as Jewish ethnicity, short or no history of oral contraceptive use, nulliparity, no history of breastfeeding or tubal ligation, dysmenorrhea, endometriosis, polycystic ovaries, obesity and talc use were found to be associated with ovarian cancer in a study population with no known hereditary predisposition for EOC. These factors were used for calculating a risk score system to identify women with an elevated ovarian cancer risk and who would benefit from salpingo-oophorectomy in connection with planned hysterectomy (Vitonis et al. 2011).

Table 8. Ten simple rules for identifying a benign or a malignant tumor by the IOTA group, modified from Timmerman et al. 2008

Rules for predicting a malignant tumor (M-rules)	Rules for predicting a benign tumor (B-rules)
M1 Irregular solid tumor	B1 Unilocular
M2 Presence of ascites	B2 Presence of solid components where the largest solid component has a largest diameter <7 mm
M3 At least four papillary structures	B3 Presence of acoustic shadows
M4 Irregular multilocular solid tumor with largest diameter ≥100 mm	B4 Smooth multilocular tumor with largest diameter <100 mm
M5 Very strong blood flow (color score 4)	B5 No blood flow (color score 1)
If one or more M-rules apply in the absence of a B-rule, the mass is classified as malignant. If one or more B-rules apply in the absence of an M-rule, the mass is classified as benign. If both M-rules and B-rules apply, the mass cannot be classified. If no rule applies, the mass cannot be classified.	

2.3.2.3. Classification and histology

The most widely used classification of OC is shaped by The International Federation of Obstetricians and Gynecologists (FIGO), categorizing OC by the spread of the disease at the time of the diagnosis. The classification system is clinically used to plan the extent of both surgical and chemotherapeutic treatments and to estimate the prognosis of the disease. Stage I tumor involves one or both ovaries and the fallopian tube(s). Stage II tumor grows into an adjacent pelvic organ or is a primarily peritoneal cancer. When the tumor has additionally spread to the peritoneal surfaces outside the pelvic area, but is still within the peritoneal cavity and/or has metastasized to the retroperitoneal lymph nodes, it is classified as stage III. Stage IV involves distant metastases beyond the peritoneal cavity (Prat et al. 2014). Malignant features of ovarian tumors are irregular cellular architecture, nuclear stratification, pleomorphism, mitotic activity and invasive growth. Borderline tumors have similar features except for invasion, whereas tumors are classified as benign when they lack all of these qualities (Underwood J.C.E.1996).

Ovarian malignancies originate from different cells of embryonal origin; coelomic epithelium, mesenchyme, mesonephric or germ cells. Consequently, most tumors can be placed into one of the three major categories: surface epithelial ovarian tumors, also known as carcinomas, (approximately 90% of all ovarian cancers), germ cell tumors (3-7%) and sex cord-stromal tumors (7%) (Prat et al. 2014). Epithelial tumors can be classified as serous, mucinous, clear-cell, endometrioid or transitional cell (Brenner) carcinomas. A further dichotomy (Koshiyama et al. 2014) can be made into type I tumors, including low-grade serous, clear-cell and mucinous histology, and type II tumors, including high-grade serous epithelial carcinomas, high-grade endometrioid and undifferentiated carcinomas, adenocarcinomas and carcinosarcomas, presenting with more aggressive behavior (Vang et al. 2009, Bowtell 2010). The most common germ cell tumors are dysgerminomas, malign teratomas, yolk sac tumors, embryonal and chorionic carcinomas. Granulosa cell and Sertoli-Leydig cell tumors, thecomas, steroid cell tumors and mixed-cell stromal tumors belong to the group of sex cord-stromal tumors (Underwood J.C.E. 1996). The classification of ovarian malignancies according to histology is pictured in **Figure 2**. The less common subtypes of OC are more common in premenopausal, even prepubertal women. They present with a variable clinical picture as well as different treatment options and prognosis.

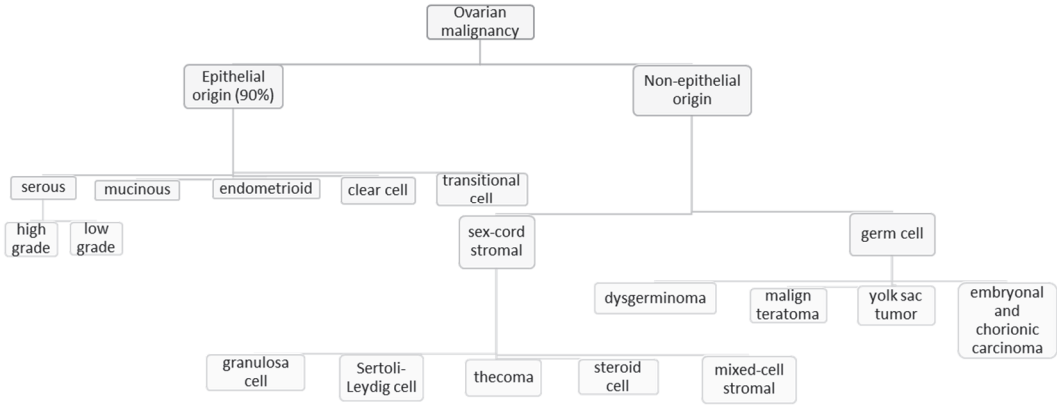


Figure 2. Classification of ovarian malignancies according to the site of origin (modified from Underwood J.C.E. 1996)

2.3.2.4. Granulosa cell tumors of the ovary

Granulosa cell tumors are generally divided into two subtypes, the juvenile and the adult type. The adult type granulosa cell tumor (AGCT) is the second most common ovarian malignancy, making up approximately 5% of all ovarian cancers and 90% of sex cord-derived ovarian cancers. A total of 97% of AGC tumors represent a known somatic mutation in FOXL2 gene (Shah et al. 2009, Jamieson and Fuller 2012). In means of prognosis, AGCT differs remarkably from EOC with a five-year survival of 97-98% vs. 44% (Colombo et al. 2007, Finnish Cancer Registry 2012-2014), as it is more frequently diagnosed at a younger age and at an earlier stage due to its hormone-related symptoms

such as vaginal bleeding (Segal et al. 1995). At the time of diagnosis the ovarian granulosa cell tumor is often relatively large, consisting of solid and cystic components. Representing neoplasms of granulosa cells lining the preovulatory follicle, AGC tumors are known to be hormonally active and to secrete increased concentrations of estradiol, inhibin B and anti-Müllerian hormone (AMH). The growth and the spread tendency of AGCT is relatively slow, and therefore, curative surgery can often be accomplished. However, relapses of the disease may be encountered even decades after primary diagnosis, emphasizing the importance of prolonged follow-up of the patients. Eventually up to 80% of the patients who are diagnosed at an advanced stage, or who experience tumor recurrence, succumb to this disease (Amsterdam and Selvaraj 1997).

The use of the known hormones secreted by ovarian granulosa cells in AGCT diagnostics and follow-up is characterized by their physiological variability, as they are influenced by the age and the menopausal status of the patient. During the reproductive age, most estradiol is produced by the granulosa cells of the ovary by irreversible conversion from androstenedione. AGCTs generally produce significantly increased amounts of estradiol, suggestive of a potential role as a serum marker (Kaye and Davies 1986), however, no reliable correlation between the disease progression and the estradiol levels has been detected (Rey et al. 1996, Lappöhn et al. 1989), possibly due to the loss of androstenedione-producing theca cells in tumor stroma (Schumer and Cannistra 2003).

Inhibin, a granulosa cell growth factor (Hsueh et al. 1987) and a regulator of the FSH secretion (Burger 1993), was first recognized by Lappöhn et al (1989) to correlate with AGCT size and was suggested as a potential tumor marker, being later accepted in AGCT diagnosis and follow-up (Robertson et al. 1999, Healy et al. 1993). Inhibin consists of a biologically less active α-subunit and a β-subunit localized primarily to the ovarian granulosa cells (Woodruff et al. 1988). The subtype βA, which forms inhibin A, has also been detected in the theca cells of a dominant follicle and in the granulosa cells throughout all follicle stages, whereas the expression of the βB-subtype, forming inhibin B, is restricted to small primary follicles (Roberts et al. 1993). With the development of the ELISA method, the subtypes βA and βB could be analyzed separately (Groome et al. 1996), leading to the discovery of the βB subunit being the subtype predominantly secreted by GCT and inhibin B being the specific AGCT marker accepted in clinical use (Petraglia et al. 1998, Robertson et al. 1999).

AMH is expressed by granulosa cells of females at reproductive age, inhibiting excessive recruitment of follicles by FSH. It may be used as a diagnostic or follow-up marker for AGCT either independently or in combination with inhibin B (Long et al. 2000, Färkkilä et al. 2015, Geerts et al. 2009). However, the AMH gene expression has been found to correlate negatively with GCT size (Anttonen et al. 2005), suggesting reduced diagnostic or prognostic value in advanced disease. Regarding markers for epithelial ovarian cancer, CA125 has no significant value in AGCT diagnostics (Stine et al. 2013), whereas studies of HE4 in AGCT diagnostics or follow-up have not been published.

2.4. OVARIAN CANCER SCREENING

A sufficient screening method for a disease requires the possibility to detect the disease at an early, symptom-free stage when the disease is still curable. As any screening method accepted for wide clinical use would involve millions of people, cost-effectiveness must be considered as well as avoidance of unnecessary interventions and morbidity. The relatively low prevalence of EOC and the absence of definitive precursor lesions have increased demands for an EOC screening method for the sensitivity and specificity. Transvaginal ultrasound only has been studied as a screening mode with fairly good detection rate of early-stage cancers (Sato et al. 2000, van Nagell et al. 2000), however, the financial cost is remarkable and special expertise is required. Screening with serum CA125 measurements only (Einhorn et al. 1992, Jacobs et al. 1996) show that CA125 can detect ovarian cancer in asymptomatic postmenopausal women quite effectively, especially during the first years of screening, however, prove no effect on mortality or economic benefit. The most cost-effective mode of screening, involving ultrasound and biomarker assay, has been mathematically calculated to be a multimodal approach with initial CA125 measurement performed once or twice yearly, followed by a sonography examination in case of an elevated CA125 value (≥35U/ml) or doubling of the initial screening value (Urban and Nicole 1997).

The Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial was launched twenty years ago. The first four screening years included a yearly TVU (ovarian or cyst volume >10ml or any cystic tumor with solid or papillary parts resulted in scan positivity) combined with CA125 analysis (threshold value ≥35 U/ml), followed by two years of annual CA125 measurements only. The first part with the combination of screening methods with 30,360 participants resulted in 11.1% of patients having at least one positive result, of which 8.1% had at least one positive TVU and 3.4% had at least one increased CA125 value. Positive TVU scans resulted in more ovarian biopsies than CA125 elevation, however the proportion of invasive cancers per biopsy was considerably higher for the CA125 positives. At the end of the combined screening years 14 surgical operations were performed per one diagnosed cancer. 83% of the cancers had spread (stage III/IV) at the time of diagnosis. TVU resulted in more unnecessary surgery, but the detected cancers were in earlier stage (Partridge et al. 2009). The first results on mortality after a median of 12.4 years follow-up showed no reduction (Buys et al. 2011). Extended mortality results were recently published. In the study population of nearly 70,000 postmenopausal women, no mortality reduction was detected in the median follow-up time of 15, partly up to 19 years, from screening with CA125 and TVU (Pinsky et al. 2016).

A large (n=21,935) pilot screening study by Jacobs et al (1999) randomized women into a control group and a screening group with three annual screens. When CA125 was elevated (≥30 U/ml), a transvaginal ultrasound (TVU) examination was performed and as a third step, the patients were further evaluated by a gynecologic oncologist if the

ovarian volume exceeded 8.2ml. No significant difference in the prevalence of index cancers (ovarian or tubal) was detected between the groups within a seven-year follow-up, however, the scientists suggested a further study about the effect on mortality. A comparative study from the same research group with a computerized CA125-based Risk of Ovarian Cancer Algorithm (ROCA) versus a specific CA125 cutoff level (Menon et al. 2005) proved the screening protocols fairly functional and having decent positive predictive values, however, no statement was made about the effect on mortality and cost-effectiveness.

A further multicenter UK collaborative Trial of Ovarian Cancer Screening (UKCTOCS) study randomized 202,638 postmenopausal women into two different screening arms and a control arm in a 1:1:2 ratio. Women with bilateral oophorectomy, prior ovarian or current non-ovarian cancer or a strong family history of EOC were excluded. The women in the multimodal (MMS) group were screened using ROCA, results triaging the women with no increased risk to annual screening, women with intermediate risk to repeated CA125 testing in three months and high-risk individuals to repeated CA125 testing and TVS within six weeks. The USS screening group with TVS as the primary screening method were triaged to annual screening (no increased risk), repeated TVS in three months (unsatisfactory findings) or a TVS performed by a specialist within six weeks (abnormal findings). After 14 years, a decrease in ovarian cancer mortality of 14% was reported in the MMS group and 11% in the USS group compared to the control group. The most significant reduction of mortality was detected during years 7-14 for both groups (Jacobs et al. 2016). This study challenges a number of earlier studies recommending against screening for ovarian cancer in general population (Buys et al. 2011, Jacobs et al. 1999, Menon et al. 2014).

Women in higher risk of EOC might, however, benefit from a targeted screening program. Karlan et al studied the effectiveness of HE4 as a primary or confirmatory test to select patients for further evaluation with sonography or clinical follow-up. The patients were chosen as high-risk individuals for EOC for either carrying a BRCA mutation, having familial history of EOC or a HNPCC mutation or significant epidemiological risks or certain circulating proteins conferring EOC risk. One study arm included only CA125 measurements as the primary screening method with an HE4 confirmation in case of a positive primary screen and the other included both CA125 and HE4 analysis as primary testing. Person-specific baselines for each marker were calculated in order to detect a possible early rise indicating a malignant process. No advanced cancers were missed by either arm, yet no early-stage cancers were identified by either arm. There was no clear difference between the arms in the capacity to detect cancer, however, including HE4 as a first-line screening method increased the number of interventions compared to CA125 only (2014).

Longitudinal follow-up of CA125 measurements is shown to detect ovarian cancer earlier than using single threshold values (Drescher et al. 2013, Skates et al. 2003, Bast et al. 2002). Significantly elevated serum CA125 levels, although within normal range,

have been associated with adnexal dysplasia among a group of women with hereditary risk for ovarian cancer scheduled for adnexal surgery (Hermsen et al. 2007), possibly indicating the predictive role of CA125 concentrations increasing close to the diagnosis. In a case-control study by Bjørge et al. (2004) serum CA125 levels were found to increase up to 1.5 years prior to ovarian cancer diagnosis. Gislefoss and colleagues (2015) conducted a serum bank study with prediagnostic serial samples from 120 patients with invasive epithelial ovarian cancer. Both HE4 and CA125 were analyzed and compared with healthy controls. A significant increase of HE4 was detected two years before diagnosis, whereas in CA125 a difference was found already four years before the EOC diagnosis was made.

3. AIMS OF THE STUDY

The objective of this study was to evaluate the behavior of the epithelial ovarian cancer marker HE4 in different gynecological conditions and to evaluate its usefulness in the diagnosis and follow-up of ovarian granulosa cell tumors.

The specific aims of the study were:

1. to evaluate the effects of hormonal suppression by oral contraception and hormonal stimulation by gonadotrophins on serum HE4 concentration
2. to explore the effect of ongoing pregnancy as well as spontaneous abortion and tubal pregnancy on serum HE4 concentration and to further assess the immunohistochemical staining of HE4 in different tissues of female reproductive tract with particular interest in conditions affecting the fallopian tube.
3. to evaluate the accuracy of HE4 measurement in the diagnostics and follow-up of ovarian granulosa cell tumors in comparison with CA125, inhibin B and AMH measurements

4. PATIENTS, MATERIALS AND METHODS

4.1. PATIENT CHARACTERISTICS AND STUDY DESIGN

4.1.1. Study I

The first part of this study was started as a part of the Endomet-project (Novel diagnostic tools for endometriosis and their exploitation for prognosis and prevention of complications). The samples were collected between October 2005 and October 2007 in collaboration with Pohjois-Karjala and Päijät-Häme Central Hospitals and Helsinki and Turku University Hospitals.

Altogether 180 women were enrolled in this study, aged between 19 and 48 years. Suspicion of malignancy, pregnancy and pelvic infection were applied as exclusion criteria. The control group consisted of fifty-four asymptomatic women, evaluated during laparoscopic tubal ligation elaborately excluding pelvic pathology, whereas 126 women included in this study were surgically treated for endometriosis. The extent of the disease was evaluated according to the revised American Society for Reproductive Medicine (ASRM) classification of endometriosis and the diagnoses confirmed histopathologically. The women in the control group were significantly older than in the treatment group (39.1 vs. 31.2 years, p>0 001) but the body mass indexes did not differ (p=0.07). Smoking information was not available.

Nearly half (43.3%) of the entire study group used some form of hormonal medication. Combined oral estrogen and progestin contraceptives (CC) were used by 54 subjects (30%), a levonorgestrel-releasing intrauterine device (LNG-IUD) had been inserted into 11 women (6.1%) and 8 (4.4%) of the women had been prescribed pills with progestin only (POP). The remaining five (2.8%) women were categorized into a combination group with either gonadotrophin releasing hormone (GnRH) analogues, aromatase inhibitor or a combination of any of the above. Forty-one (32.5%) of the endometriosis patients used CC, 6 (4.8%) POP, 4 (3.2%) LNG-IUD and 5 patients (3.2%) fell into the combination group. The surgical stages of endometriosis were not different between medication groups. Due to the small sample size in all but the CC group, the other medication groups were not used for statistical comparison in the final analysis.

Serum samples were drawn and endometrial samples (Pipelle de Cornier®, Laboratoire CCD, Paris) taken during the surgery, to be examined by a designated pathologist. The phase of the menstrual cycle was determined and classified into one of five subgroups (0 = non-diagnostic, 1 = proliferative, 2 = secretory, 3+4 = inactive/atrophic and 5 = menstrual). The samples insufficient for diagnosis or otherwise unclassified were excluded from the analysis.

4.1.2. Study II

This prospective trial at the Infertility Unit of Turku University Hospital was conducted between March and June 2010. The cohort consisted of twenty patients with variable etiologies of infertility, scheduled for in vitro fertilization (IVF). The treatment for one of the patients was converted into intrauterine insemination due to poor ovarian response, resulting in discontinuation in this study. The patients were generally healthy, aged between 27 and 39 years with pelvic tumors excluded by sonography. None of the patients had known renal insufficiency and all but two were non-smokers.

A GnRH agonist nafarelin or leuprorelin acetate was administered for long downregulation of the pituitary gland. After withdrawal bleeding, the ovarian suppression was confirmed by transvaginal ultrasound (TVU) and by the presence of hypoestrogenic symptoms. Controlled ovarian hyperstimulation was then induced with recombinant follicle stimulating hormone or menotropin injections and adjusted according to individual ovarian response, estimated by the number and size of the growing follicles, endometrial thickness and estradiol concentrations. Sonographic examinations were performed two to three times during stimulation and when the leading follicle approached the desired size, a human chorionic gonadotrophin (hCG) injection was given 36 hours prior to the transvaginal aspiration of the oocytes. Serum hCG concentrations were measured two weeks after ovum pick-up. Blood samples were drawn for estradiol, HE4 and CA125 measurements at each visit. The day of the first stimulation ultrasound was chosen as the comparative point of time for statistical analysis due to the physiological estradiol values.

4.1.3. Study III

A total of 135 blood samples were collected from 82 adult-type ovarian granulosa cell tumor (AGCT) patients diagnosed between 1962 and 2009 and treated at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital during 2007-2011. The *FOXL2* (c.402C>G; C134W) mutation was detected in all patients. Patient medical records were used for the retrieval of relevant clinical data, including factors such as smoking and serum creatinine levels known to affect some tumor marker analyses. The staging of the tumors at the time of the diagnosis was performed according to the FIGO 2009 criteria. The median age at diagnosis was 60 years and follow-up time 4.7 (2.3-6.4) years. After operative or clinical assessment with sonographic and, when necessary, computed tomography imaging, the samples were dichotomized either into the disease-free (DF) group or the group with macroscopically evident disease (WD). The WD group consisted of 28 preoperative samples drawn within a month before surgery, 5 samples drawn within a month before chemotherapy and 3 samples collected during clinical routine follow-up visits after a minimum of three months from cancer treatment of any kind. All DF samples were drawn during follow-up. Menopausal status was recorded according to patient age and medical history regarding possible surgical interventions, menstrual bleeding, hormone replacement therapy use, and climacteric symptoms.

The malignant control group consisted of 37 patients with epithelial ovarian carcinoma (EOC). The histological classification was high-grade serous carcinoma for 31 patients, endometrioid for 3, mucinous for 2 and clear-cell carcinoma for 1 patient. The median age of the EOC group was 61 years at the time of the diagnosis. One of the EOC patients was a smoker, yet none had renal insufficiency according to the medical records. Forty patients with surgically and histologically confirmed ovarian endometriomas representing benign ovarian tumors were enrolled as benign controls. The median age of the endometriosis patients was 32 years (26-47). None of the patients had known renal insufficiency, while information about cigarette smoking habits was not available. Sample descriptives are pictured in **Table 9**.

Table 9. Description of serum samples in Study III. Modified from Haltia et al. 2017.

	AGCT		EOC	ENDO
	WD	DF		
Number of samples	36	99	37	40
Age of the patient at sample retrieval, years*	60 (36-80)	59 (25-86)	61 (29-79)	32 (26-47)
Primary**	17	n/a	37	40
stage I	17	n/a	5	0
stage II	0	n/a	3	0
stage III	0	n/a	16	15
Stage IV	0	n/a	16	24
Recurrent (serial samples)	19	n/a	0	0
Premenopausal	6	12	7	40
Postmenopausal	30	87	30	0

* Median (range)

** Staging of primary tumors according to FIGO 2009 for AGCTs and EOCs, and according to American Society for Reproductive Medicine in ENDOs.

4.1.4. Study IV

The patients were recruited and serum samples collected for this study in 1994-1996 at the Department of Obstetrics and Gynecology of Turku University Hospital. Relevant clinical data was retrieved from medical records. The patient cohort consisted of 32 tubal pregnancies (GEU), 45 spontaneous abortions and 32 medical abortions. A group of non-pregnant healthy controls (n=77) was used for comparison. The patients were diagnosed by clinical examination combined with transvaginal ultrasound and quantitative hCG measurements. The diagnoses were confirmed by uterine abrasion (in case of spontaneous abortions) or by surgical salpingectomy (in GEU) and consequent histological analysis in all but five GEU and one spontaneous abortion patients, resulting in 27 and 44 patients, respectively. All medical abortions were performed surgically. The duration of the pregnancy was counted from the last menstrual period and when unverified, confirmed by ultrasound. The median age in the medical abortion group was

significantly lower (25 years) and the control group higher (39 years) than in the GEU and spontaneous abortion groups (32 and 30 years, respectively, $p<0.001$). The median duration of pregnancy at the time of analysis was 6.0 (4.3-9.0) weeks for the GEU group, 10.0 (5.3-16.9) weeks for the spontaneous abortion group and 7.8 (4.9-15.3) weeks for the medical abortion group. According to the medical records, none of the patients or controls had known renal insufficiency, while smoking data was not available.

The possible dehydration effect of the samples due to prolonged storage was estimated by measuring sodium concentrations prior to the CA125 and HE4 laboratory assays. The median sodium concentration of all patients was 140.7 mmol/l and therefore within the normal range (137-145 mmol/l) in all study groups. The clearly dehydrated samples with sodium concentrations ≥ 165 mmol/l were excluded from the analysis. Individual corrections for evaporation were made to all marker samples according to the formula introduced by Andersson et al. (Andersson et al. 2007) with the sodium concentration of 140 mml/l considered the reference. The statistical analysis was performed before and after the evaporation correction with no effect on the results.

To further explore the source of the elevated HE4 in tubal pregnancy, different tissue samples from gynecological surgery performed in 2008-2015 were retrieved from the archives of the Department of Pathology, Turku University Hospital and the Pathology Laboratory of Southwestern Finland Ltd., according to the SNOMED-coded (Systematized Nomenclature of Medicine) histopathological diagnosis. Altogether 102 formalin-fixed paraffin-embedded tissue sections were collected according to their histopathological diagnosis. The samples consisted of 9 surgically removed tubal pregnancies, uterine vacuum evacuation samples from spontaneous (n=10) and medical (n=7) abortions, proliferative (n=10) and secretory (n=12) endometrial samples resulting from therapeutic curettage, normal ovarian (n=12) and tubal tissue (n=10) surgically removed for benign indications and endometrial (n=11), ovarian serous (n=11) and tubal carcinoma (n=10). One of the patients was a known BRCA carrier and one had a prophylactic salpingo-oophorectomy due to hormone receptor-positive breast cancer. Other indications for tubo-ovarial surgery included different benign ovarian cysts, sterilization, uterine fibroids, endometrial hyperplasia, adenomyosis and an intestinal tumor later defined as cancer of the small intestine. Prior to the staining procedure, all histological samples were microscopically re-evaluated by a designated pathologist to verify the representativeness of the sections.

4.2. SERUM MARKER ANALYSES

4.2.1. CA125 and HE4

Venous blood samples from each subject were centrifuged, serum separated and stored in -20° or -80° until analysis. HE4 samples were analyzed by enzyme-linked immunosorbent assay (ELISA) analysis (Fujirebio Diagnostics inc., Malvern, PA, USA)

according to the manufacturer's instructions. In our clinic (the Laboratory of Turku University Hospital), the cutoff line of 70 pmol/l is applied for premenopausal and 140 pmol/l for postmenopausal women, whereas the reference limit of 150 pM is provided by the manufacturer.

CA125 analysis was performed with ELISA by Fujirebio (Study I), electrochemiluminescence immunoassay (ECLIA, Roche Diagnostics GmBH, Mannheim, Germany) (Studies II and IV) or chemiluminescent microparticle immunoassay (CMIA) on Abbott Architect i2000 system (Abbott diagnostics, Abbott Park, IL, U.S) (Study III). 35 IU/ml was considered the reference limit for CA125.

4.2.2. Inhibin B, AMH, creatinine (Study III)

Inhibin B (ng/l) levels were analyzed by inhibin B Gen II Elisa (Beckman Coulter) with <200 ng/l as the cutoff limit for premenopausal and <16 ng/l for postmenopausal women, according to the manufacturer's instructions. Creatinine (µmol/l) was analyzed with an enzymatic assay on Roche Modular 8000 clinical chemistry analyzer (Roche Diagnostics, Penzberg, Germany) as routine in the Helsinki University Hospital (HUSLAB). Renal function was evaluated by calculating the glomerular filtration rate (GFR) (Levey et al. 2009) and considered impaired when the GFR was less than 60ml/min /1.73m^2, as defined by the National Kidney Foundation (2002). AMH was analyzed from plasma samples with an ultrasensitive, first-generation AMH ELISA assay from AnshLabs (Webster, TX, USA) with the reference limits of <13 µg/l for premenopausal and <0.2 µg/l for postmenopausal women, provided by the manufacturer.

4.2.3. Estradiol (Study II, Study III), Sodium (Study IV)

Estradiol measurements for Study II were performed with ECLIA (Roche GmBH, Mannheim, Germany) according to the routine procedures at the Turku University Hospital, and with fluoroimmunometric assay (AutoDELFIA™, Wallac, Turku, Finland) for Study III. Sodium concentrations for Study IV were analyzed with automatized indirect ion-selective analyzer (modular ISE 1800, Roche GmBH, Mannheim, Germany) according to the routine procedures at Turku University Hospital.

4.3. IMMUNOHISTOCHEMISTRY (STUDY IV)

Formalin-fixed paraffin–embedded samples were cut at 5 µm, deparaffinized and rehydrated. Antigen retrieval was performed using heat-mediated high pH antigen retrieving solution buffer (ab972) by Abcam (Abcam plc, Cambridge, UK) at pH 9.0 in a pressure cooker for 20 minutes and allowed to cool at room temperature for 30 minutes. After cooling, the samples were transferred into hot rinse and allowed to cool for another 5 minutes before a wash in phosphate-buffered saline solution with Tween (PBS-T, pH 7.4). Blocking against non-specific binding was done with bovine serum albumin (3%

BSA – PBS – 0.05% Tween) for 30 minutes at room temperature. The slides were incubated overnight at 4°C with monoclonal antibody against HE4 (clone 12A2, 2.5µg/ml, Fujirebio Diagnostic Inc., Malvern, PA, USA). Endogenous peroxidase activity was inhibited with 3% hydrogen peroxide solution (H_2O_2) for 20 minutes at room temperature. After rinsing in PBS-Tween, sections were incubated with Dako EnVision+ System-HRP labeled polymer against mouse IgG (K4001) for 30 minutes, washed and stained with Liquid DAB Substrate Chromogen system (Agilent corp., CA, USA) and counterstained with Mayer's hematoxylin. After rinsing with tap water and dH_2O, the sections were dehydrated and mounted. Parallel controls were performed by omitting the primary antibody to verify the absence of non-specific staining in negative controls, using the antibody of the same IgG isotype, clonality, conjugate, and host species as the primary antibody in isotype control and blocking with the recombinant protein. Slides were scanned for further analysis with the panoramic 250 Flash series digital slide scanner from 3DHISTECH Ltd (Budapest, Hungary).

4.4. STATISTICAL ANALYSIS

4.4.1. Study I

Rightly skewed dependent variable CA125 was log-transformed before statistical analysis. The baseline clinical characteristics and concentration of the markers between groups were tested using the independent samples t-test. The associations between the age and continuous outcome variables (HE4 and CA125 concentrations) were studied using Pearson's correlation coefficient. The surgical stages of the disease were compared between the different medical groups using the Kruskal-Wallis test. Two different linear models were fitted in order to study the differences in the means of HE4 and CA125 concentrations. Endometriosis, endometrial histology and the interaction between the two variables were used as predictors in the first analysis, and endometriosis, medication and the interaction between the two variables were used as predictors in the second analysis. In both analyses the effect of the interaction was assessed first, and if the interaction was significant, the associations were studied separately in healthy women and women with endometriosis. Pairwise comparisons between the endometrial histology groups and the medication groups were done using Tukey's test. Statistical analyses were performed using PASW Statistics 18 or SAS for Windows version 9.2. A p-value of < 0.05 was considered statistically significant.

4.4.2. Study II

As the distribution of CA125, HE4 and E2 was right-skewed, the values were log-transformed prior to statistical analysis. Pearson's correlation coefficient was used to estimate the association between HE4, CA125 and E2 concentrations. The samples were divided into groups of non-pregnant and pregnant women, the hCG cutoff level being

50U/l and differences between CA125, HE4 and E2 concentrations were analyzed using Two Way Repeated Measures Analysis of Variance with post hoc Holm-Sidak method for pairwise comparisons. Day 5 after ovarian stimulation was chosen as the comparison point of time, as it was closest to the physiological E2 concentration. Statistical analyses were performed using SigmaStat 3.1 (Systat Software, Inc, USA). A p-value of <0.05 was considered significant.

4.4.3. Study III

The levels of the markers did not follow a normal distribution, and the between-group comparisons were therefore analyzed using the Mann-Whitney U test or Spearman's Rho. Receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) values were calculated, together with their 95% confidence intervals. All the study samples were included in the ROC analyses. Due to the rarity of the disease, multiple circulating tumor marker measurements (both pre-treatment (WD) and follow-up (WD or DF) samples) of the AGCT patients were included to increase the precision of the estimates of sensitivity and specificity. As the repeated measurements ROC curves gave falsely optimistic estimates, the measurements were utilized as independent data. Thus, the ROC curves should be viewed as descriptive only. For the ROC curve calculations, observations below the detection limit were replaced with DL/2 values. Correlated ROC curves were compared nonparametrically. The associations between the continuous variables and the disease status (AGCT WD, EOC, ENDO, AGCT DF) were studied using a mixed-model repeated measures analysis if all the values were above the detection limit and with a mixed effects Tobit model if some variables were below the detection limit. For the ROC analysis using the cutoff data, the samples were dichotomized as either high or normal based on the cutoff levels. Statistical analyses were done using JMP Pro, version 11.0 and SAS, version 9.3 (SAS Inc., Cary, NC, U.S.).

4.4.4. Study IV

The dependent variables CA125 and HE4 were right-skewed and therefore log-transformed before statistical analysis. The baseline clinical characteristics and differences between CA125 and HE4 concentrations in different study groups were analyzed using independent samples t-test. The associations between the duration of the pregnancy and the continuous outcome variables (HE4 and CA125) were tested using Pearson's correlation coefficient. Statistical analysis was performed using SPSS Statistics version 22 (IBM Corp, Armonk, NY, U.S.). A p-value of <0.05 was considered statistically significant.

5. RESULTS

5.1. THE EFFECT OF MENSTRUAL CYCLE AND THE USE OF HORMONAL CONTRACEPTION ON SERUM HE4 (STUDY I)

A total number of 180 women were enrolled in this study, consisting of endometriosis patients (n=126) and healthy controls (n=54). Patient characteristics are summarized in **Table 10**.

Table 10: Patient characteristics. Modified from Hallamaa et al. 2012

	Women with endometriosis (%)	Healthy women (%)	Total (%)
Number of patients	126 (70%)	54 (30%)	180
Patient age (average)	31	39	34
BMI (average)	23,7	24,7	24
Hormonal treatment (%)	56 (44,4%)	22 (40,7%)	78 (43,3%)
Combined contraception users (%)	41 (32,5%)	13 (24,1%)	54 (30%)
Other hormones (%)	15 (11,9%)	9 (16,7%)	24 (13,3%)
No hormones (%)	70 (55,6%)	32 (59,3%)	102 (56,7%)
Endometrial samples (n)	114	51	165
Phase of the menstrual cycle			
Proliferative	24 (21,1%)	8 (15,7%)	32 (19,4%)
Secretory	41 (36,0%)	19 (37,3%)	60 (36,4%)
Inactive/atrophic	41 (36,0%)	20 (39,2%)	61 (37,0%)
Menstrual	5 (4,4%)	2 (3,9%)	7 (4,2%)
Non-diagnostic	3 (2,6%)	2 (3,9%)	5 (3,0%)

Although the endometriosis and healthy groups were different in means of age (31.2 vs. 39.1 years, respectively, $p<0.001$), there was no correlation between HE4 or CA125 and age. There was no significant difference in the median serum concentrations of HE4 between the endometriosis patients and the control group (43.5 vs. 41.2 pM, respectively), the entire study population presenting with a median HE4 concentration of 43.0 pM.

No significant interaction between the menstrual phase and endometriosis was detected ($p=0.35$) suggesting the effect of menstrual cycle on HE4 levels to be similar in the endometriosis patients and the healthy controls and allowing evaluation of the entire study group as one. The median HE4 concentration in the proliferative phase of the menstrual cycle was 43.0 pM, 44.6 pM in the secretory, 40.7 pM in the inactive/atrophic and 42.7 pM in the menstrual phase of the menstrual cycle. Pairwise comparison showed no significant differences between the menstrual phases, the greatest difference detected between the secretory and the inactive/atrophic phases (44.6 vs. 40.7 pM, respectively, $p=0.074$).

The effect of endometriosis on CA125 was significant as expected (25.8 U/l for endometriosis patients vs. 6.8 U/l in healthy women, p<0.001). One third of the endometriosis patients presented with levels above 35 IU/l, the upper limit of reference range in clinical use. The effect of the menstrual cycle was more remarkable in endometriosis patients, and therefore the groups were analyzed separately. In the endometriosis group, serum CA125 concentrations were higher in the secretory (38.8 U/ml, p=0.002) and the proliferative phases (36.2 U/ml, p=0.004) than in the inactive/atrophic phase (15.4 U/ml). No such cycle-dependent variation was detected in the group of healthy women.

However, when the effect of the menstrual cycle on marker levels was analyzed separately in women who did not take hormonal contraception, there was no longer a difference between the groups. Furthermore, no cycle-dependent fluctuation of the serum markers was seen in either healthy women or women with endometriosis.

There was no difference between the study groups regarding the effect of hormonal medication on HE4 (p=0.74) and CA125 (p=0.86), therefore, the analyses for each marker were performed on the entire study population. All medication groups were first analyzed separately with no effect of any medication on the serum HE4 or CA125 levels (**Figure 3**), however, due to the small sample size in all but the CC group, the groups for other medication were not used for statistical comparison in the final analysis. No significant difference was then detected between the women taking combined oral contraceptives and the women with no hormonal medication for either HE4 (p=0.28) or CA125 (p=0.4).

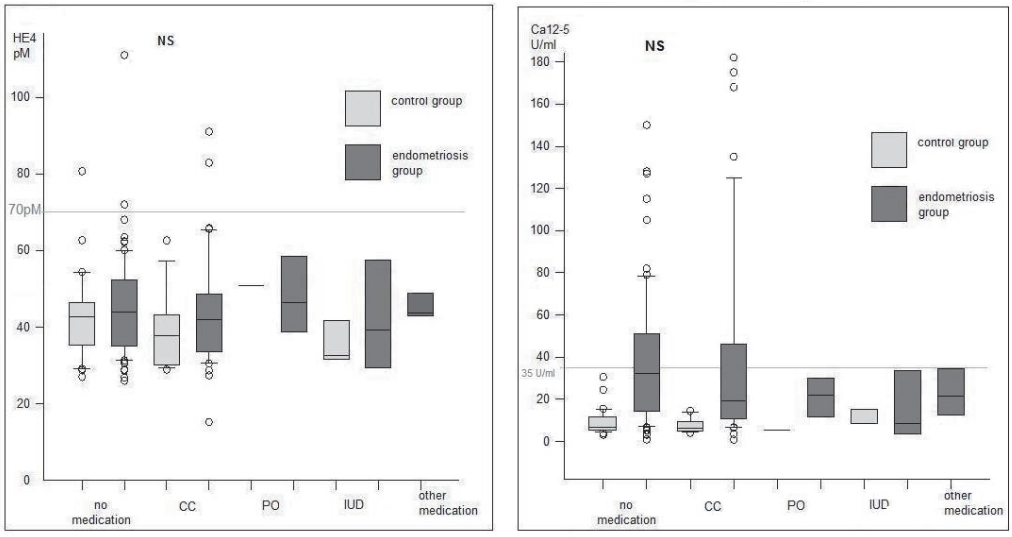


Figure 3. The effect of different forms of medication on serum HE4 and CA125 concentrations.
CC= combined contraceptives, PO= progestin only, IUD= intrauterine device

5.2. SERUM HE4 VARIATION DURING IVF TREATMENT (STUDY II)

This study consisted of twenty women planned for IVF treatment (Patient demographics in **Table 11**). One discontinued the study due to poor response to FSH stimulation and conversion of the treatment to intrauterine insemination.

Table 11. Patient demography. Modified from Hallamaa et al. 2014.

Patient	Age	Body mass ir	Smoking	Cause of infertility	Semen analysis
1	38	26,4	no	nud (oligo-ovulation)	normal
2	34	24,2	no	reduced ovarian reserve, suspected endometriosis, hyperprolactinemia	asthenozoospermia
3	31	27,8	no	male factor	azoospermia propter sytostatica
4	32	18,8	no	male factor, oligo-ovulation	oligozoospermia
5	31	21,1	no	male factor	oligoastenoterato-zoospermia, immunization
6**	38	22,1	no	male factor, oligo-ovulation	asthenoteratozoospermia
7	39	21,1	no	male factor	immunization
8	34	29,8	no	endometriosis*	normal/mild asthenozoospermia
9	38	27,9	no	nud	normal
10	32	31,6	no	male factor, oligo-ovulation	asthenozoospermia
11	31	21,3	no	male factor	obstructive azoospermia
12	33	23,2	yes	endometriosis *	normal
13	29	20	no	endometriosis *	normal
14	27	22,6	yes	male factor	teratozoospermia
15	33	24	no	nud	normal
16	35	27	no	nud	normal
17	31	20	no	male factor	immunization
18	27	19,6	no	endometriosis	normal
19	33	31,6	no	male factor, anovulation, PCOS	asthenozoospermia, immunization
20	29	23,5	no	endometriosis*	normal

*laparoscopically confirmed
** discontinued due to poor ovarian response

The estradiol concentration at the first ultrasound visit, approximately five days from the stimulation start, was the closest (1.3 nM) to the physiological concentration, and therefore, chosen for baseline comparison. Serum estradiol levels reached the median concentration of 5.06 nM during FSH stimulation, however, the interindividual variation was remarkable (1.32-18.0 nM), demonstrating differences in the gonadotrophin response reflecting ovarian reserve.

Serum HE4 concentrations increased remotely during the stimulation, showing gradual ascent from 38.6 pM to 52.2 pM on the day of the ovum pick-up ($p<0.05$, **Figure 4**). By the time of the pregnancy test 14 days later, the HE4 concentrations had decreased back

to the level of GnRH suppression. No significant age-dependency was noted in this small group of patients, except on the day of hCG test, when women over 35 years of age had lower HE4 concentrations than younger women (30.7 pM vs. 40.3 pM, p=0.032).

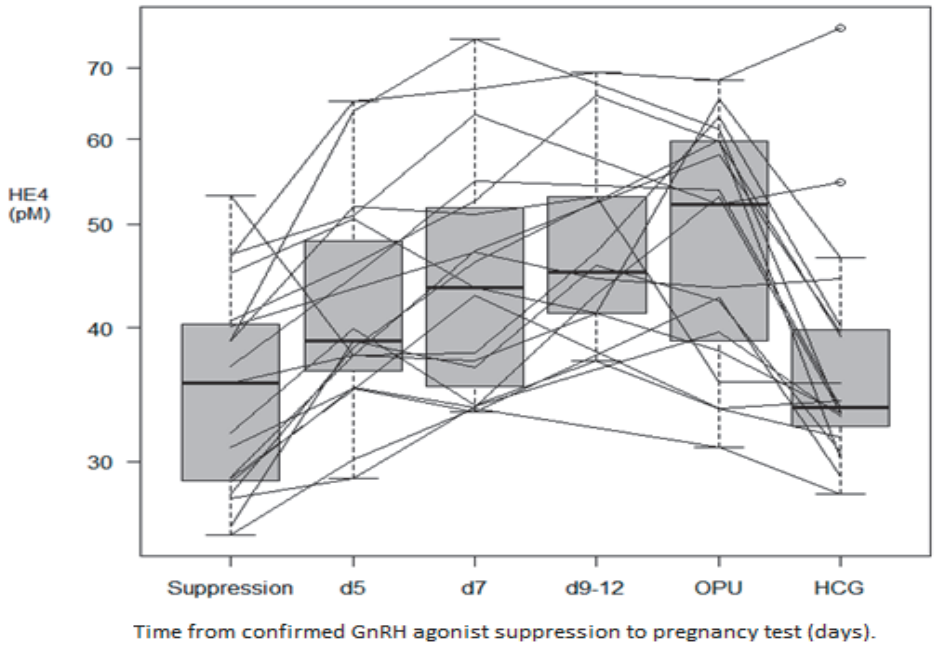


Figure 4. Serum HE4 concentrations during gonadotrophin stimulation. D5 = day 5 from the start of the suppression, d7 = day 7 from the start of the suppression, d9-12 = days 9-12 from the suppression start, OPU = transvaginal ovum pick-up, HCG = day of pregnancy test.

When analyzed separately, the women achieving pregnancy (n=8), had the median HE4 concentration of 39.6 pM, whereas the HE4 concentration in non-pregnant women was 33.4 pM, showing a significant decrease compared to the first stimulation visit ($p<0.05$, **Figure 5a**). Altogether, only two single samples of different patients (74.4 pM at the second stimulation visit and 76.3 pM at the time of the pregnancy test) rose slightly above the threshold limit, 70 pM for premenopausal women, considered at Turku University Hospital, and none over the limit of 150 pM suggested by the manufacturer.

Serum CA125 levels were low from the beginning of the ovarian suppression (median 17.0 U/ml) throughout the FSH stimulation (16.6-18.5 U/ml, **Figure 5b**), and hCG injection had no effect on serum CA125 concentration (18.5U/ml). At the time of the pregnancy test, however, the serum CA125 concentration increased to 37.2 U/ml in pregnant and 39.0 U/ml in non-pregnant women, both significantly higher than the levels at the first stimulation ultrasound ($p<0.001$). CA125 concentrations above the clinically accepted cutoff limit of 35 U/ml were detected in 20 samples, half of them in women with endometriosis, presenting with elevated CA125 levels throughout the study period.

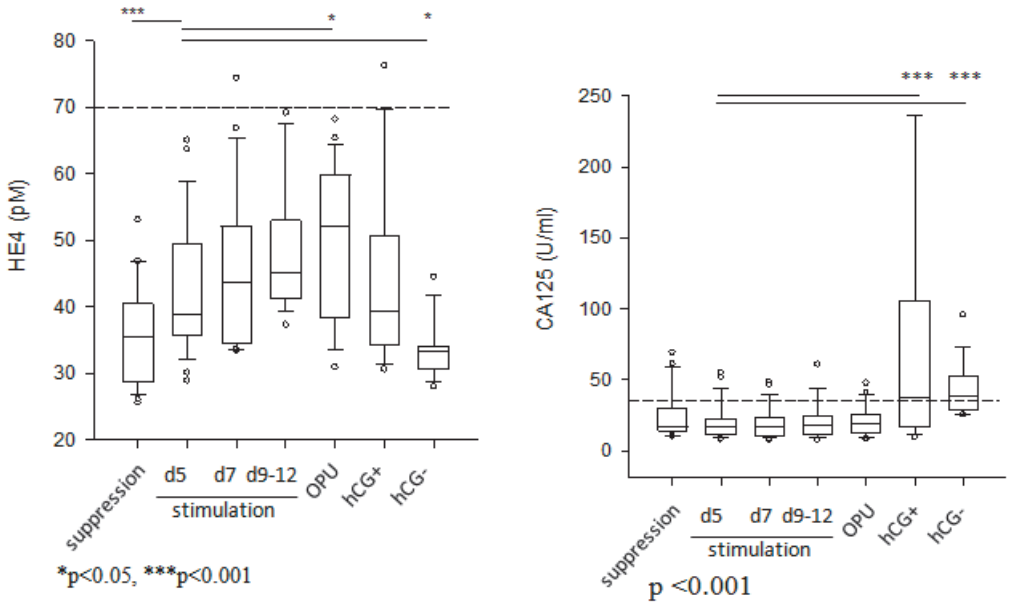


Figure 5a and b. Serum HE4 and CA125 concentrations during IVF stimulation. D5 = day 5 from the start of the suppression, d7 = day 7 from the start of the suppression, d9-12 = days 9-12 from the suppression start, OPU = transvaginal ovum pick-up, HCG+ = day of the pregnancy test (hCG positive), HCG- = day of the pregnancy test (hCG negative). Modified from Hallamaa et al. 2014.

5.3. THE COMPARISON OF TUMOR MARKERS CA125, HE4, INHIBIN B AND AMH IN AGCT DIAGNOSIS (STUDY III)

The epithelial ovarian cancer markers HE4 and CA125 were significantly elevated in the EOC group compared to all other groups ($p<0.0001$ in all pairwise comparisons, **Figure 6**). Both AGCT groups presented with increased HE4 levels compared to ENDO group, however, this difference was mainly interpreted to be due the remarkable age difference between these groups. HE4 ($p=0.034$) and CA125 ($p=0.0001$) levels were also higher in the AGCT WD patients compared to the DF group. However, after excluding patients with renal insufficiency, no further difference in HE4 between the WD and DF groups could be observed. The CA125 elevation in the AGCT WD group was moderate, only 25% of the values exceeding the reference limit of 35 U/ml.

There were four AGCT patients with elevated (>150 pM) HE4 concentrations, one of them in the WD group. All but one had eminent renal failure presenting with decreased glomerular filtration rate. Only one AGCT patient had elevated CA125 levels throughout her three samples, probably associated with her known endometriosis. Furthermore, neither HE4 nor CA125 levels correlated with tumor size or stage.

Inhibin B and AMH were significantly higher in the AGCT WD patients compared with any other group ($p<0.0001$ in all pairwise comparisons except $p<0.05$ for WD vs. ENDO for AMH, **Figure 6**). The ENDO group presented with significantly increased AMH and

inhibin B concentrations compared to the WD and the EOC groups consisting of a significantly older, mainly postmenopausal patient cohort.

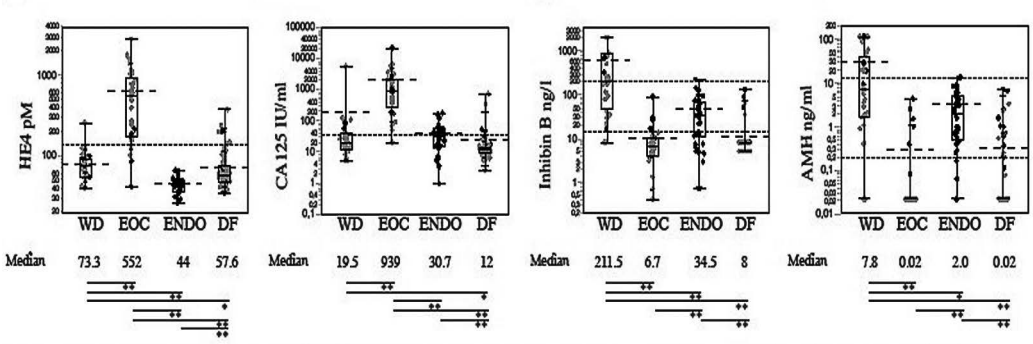


Figure 6. Serum HE4, CA125, inhibin B and AMH concentrations between study groups of granulosa cell tumor patients with disease (WD) and disease free (DF), epithelial ovarian cancer (EOC) and ovarian endometriosis (ENDO) . All markers presented with logarithmic scale. *p<0.05, **p<0.0001. Modified from Haltia et al. 2017.

HE4, CA125, inhibin B and AMH and their combinations were then evaluated for their capacity for differentiating AGCT from endometriosis and EOC. Using continuous data in receiver operating characteristics (ROC) curve analysis, all single markers were able to differentiate AGCT from EOC well with subsequent AUCs of 0.92-0.97, whereas combining any of the markers did not add significant value to the diagnosis (**Figure 7a**). When differentiating between AGCT and ENDO, the accuracies of single markers were lower, CA125 showing poorest specificity with AUC of only 0.60 and HE4 with AUC 0.88. The combination of HE4 and inhibin B yielded the best results (**Figure 7b**). When distinguishing AGCT WD patients from DF patients, inhibin B showed the best potential with AUC 0.97. Adding AMH with the single marker AUC of 0.91 to inhibin, the combination yielded an AUC of 0.98 (**Figure 7c**).

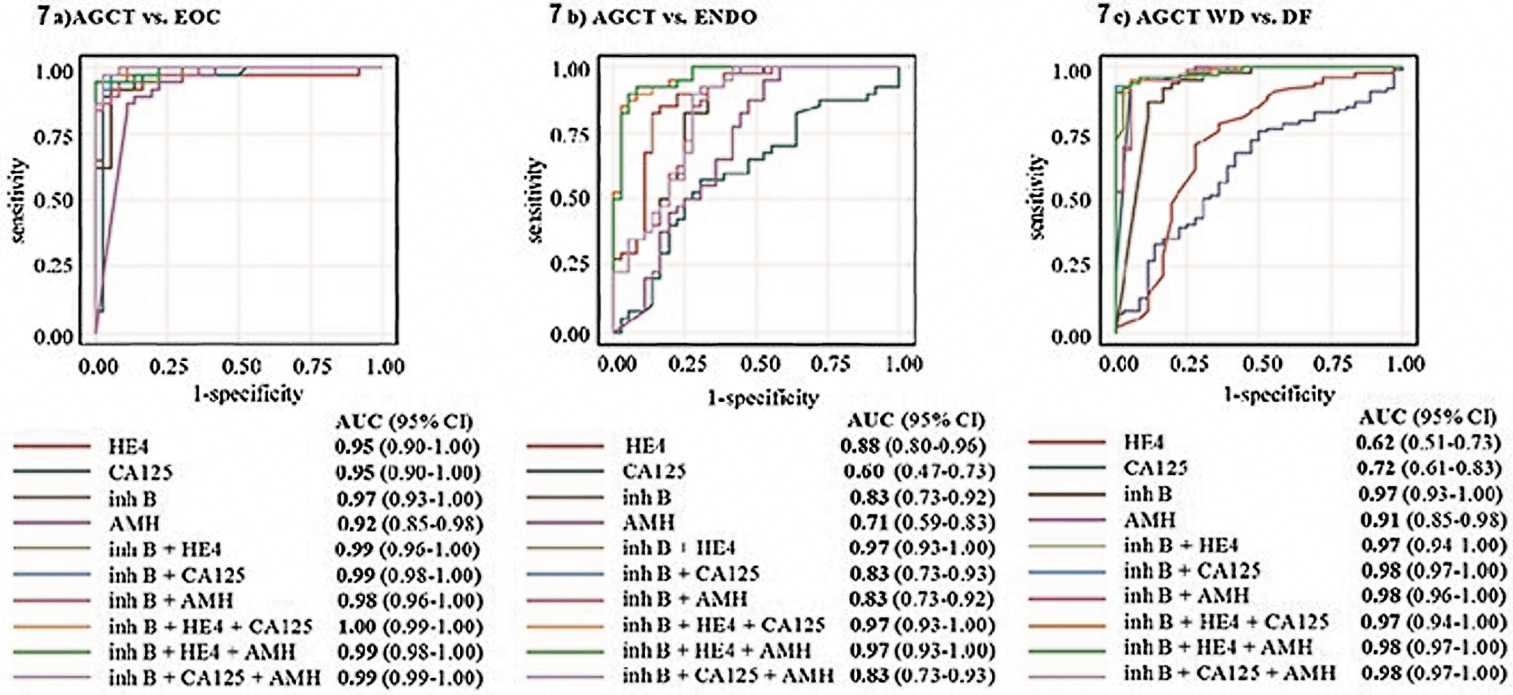


Figure 7. ROC-AUC analyses presenting the accuracy of HE4, CA125, inhibin B and AMH and their combinations in differentiating between AGCT and EOC (7a), AGCT and ENDO (7b) and AGCT WD and DF (7c).
AGCT= adult-type granulosa cell tumor, EOC= epithelial ovarian cancer, ENDO= ovarian endometrioma, WD= with disease, DF= disease free.
Modified from Haltia et al. 2017.

Similar analysis was also performed with the data dichotomized into normal or elevated by the cutoff limits of each marker, taking into consideration the age-related differences between the groups. Particularly, in the AGCT vs. ENDO analysis, utilizing the cutoff levels, the diagnostic accuracy of inhibin B (AUC 0.95) and AMH (AUC 0.89) improved. In ROC comparison analysis using either the cutoff values or the continuous data, the epithelial markers HE4 and CA125 performed equally well in distinguishing AGCT from EOC or ENDO, however, they were inferior to inhibin B and AMH in differentiating AGCT from ENDO and detecting AGCT in WD vs. DF. Combining other markers to inhibin B added no value in AGCT vs. EOC. However, combining either CA125 ($p=0.041$) or AMH ($p=0.036$) to inhibin increased the accuracy compared to inhibin B alone when differentiating between AGCT and ENDO. As a follow-up marker, inhibin B and AMH performed equally and combining other markers added value to inhibin B only.

5.4. THE SOURCE OF ELEVATED HE4 IN TUBAL PREGNANCY (STUDY IV)

The serum samples from 27 women with tubal pregnancy, 44 women with spontaneous abortion and 32 women with an ongoing pregnancy, applying for medical abortion were analyzed for serum HE4 and CA125 concentrations. 77 healthy, non-pregnant women were enrolled for comparison.

The median HE4 levels of GEU patients were significantly higher (51.6 pM) than those in the spontaneous abortion group (42.6 pM, $p=0.001$) and the non-pregnant control group (40.7 pM, $p<0.001$). HE4 concentrations in the medical abortion group were significantly higher (50.4 pM) than those in the spontaneous abortion group ($p=0.03$) and in the non-pregnant control group ($p<0.001$), whereas there was no significant difference between the GEU and medical abortion groups (**Figure 8**).

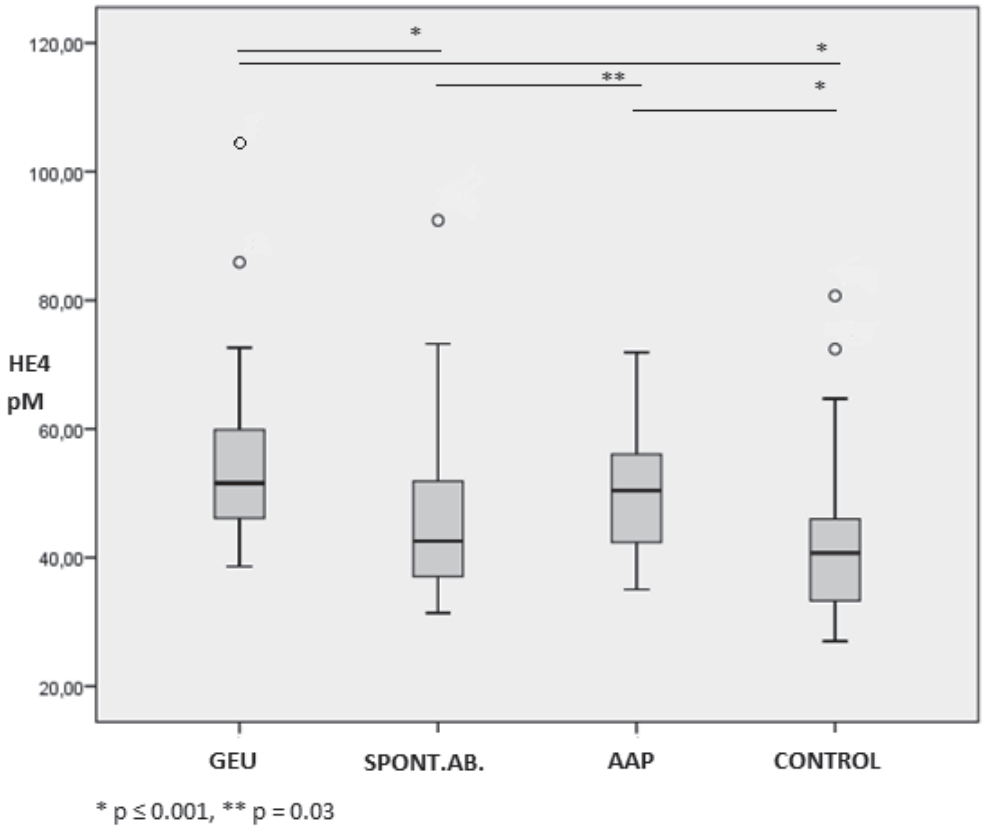


Figure 8. Differences in serum HE4 concentrations between study groups. GEU= tubal pregnancy, SPONT.AB= spontaneous abortion, AAP= medical abortion.

The median CA125 concentration for the GEU group was 21.6U/ml, 31.2 U/ml for the spontaneous abortion group, 39.0 U/ml for the medical abortion group and 8.1 U/ml for the healthy control group. There were no significant differences in the median CA125 concentrations between the different subgroups of pregnant women, however all three pregnancy groups had higher CA125 levels than the healthy controls ($p<0.001$). The duration of the pregnancy did not correlate with serum HE4 or CA125 levels.

All serous ovarian carcinomas showed strong positive staining in HE4 immunohistochemistry. In normal ovaries, the surface epithelium and stroma were immunonegative, whereas the epithelium in the cortical inclusion cysts of Müllerian origin stained strongly (**Figure 9**). Endometrial glands both in proliferative and secretory phase were positive and endometrial stroma negative. The neoplastic glands in endometrial adenocarcinoma stained strongly. The staining in the normal fallopian tubal epithelium was relatively strong, yet discontinuous and tubal stroma was negative (**Figure 10a**). All placental tissue, both epithelium and stroma, was immunonegative irrespective of preceding pregnancy status (tubal, spontaneous or medical abortion). In tubal pregnancies, however, the tubal epithelium staining appeared stronger in intensity

and was continuously distributed over the entire epithelial layer in contrast to the normal fallopian tubes evaluated (**Figure 10b**). Tubal carcinoma stained continuously and strongly (**Figure 11**).

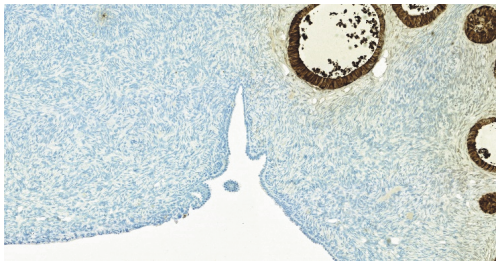
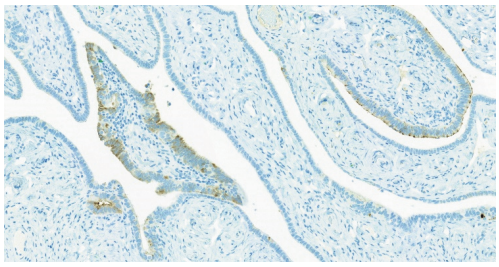
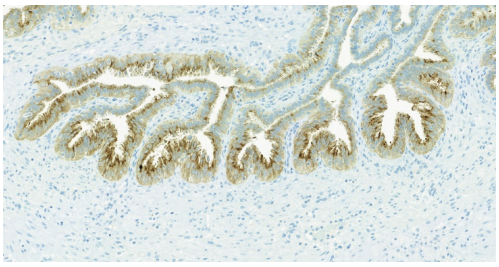


Figure 9. Normal ovary with immunonegative stroma and surface epithelium. Cortical inclusion cyst presents with strong immunohistochemical staining.



10a



10b

Figures 10a and b. Normal fallopian tube epithelium presenting with discontinuous immunoreactivity and variable intensity (**10a**), Consistent immunohistochemical staining over the entire tubal epithelium in tubal pregnancy (**10b**).

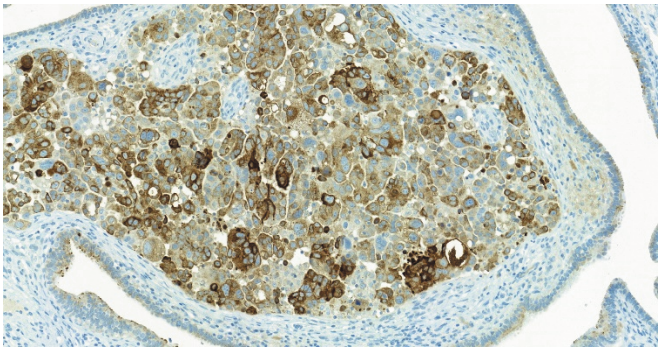


Figure 11. Strong, diffuse staining of the neoplastic epithelium in tubal carcinoma.

6. DISCUSSION

In spite of ambitious research and great advances in ovarian cancer treatment, no significant improvement in the prognosis has been achieved in the last decades. The challenge lies within the long symptom-free period in the early stages of cancer, which often delays the diagnosis until the disease is widely spread and beyond any means of curative treatment. The development of the population-based screening algorithms has been impeded by the rarity of the disease, combined with no clear, detectable pre-malignant condition that could reliably be detected by financially conceivable, non-invasive diagnosing methods. Biomarker research and improvement in knowledge are important for clinicians who are struggling to maintain the delicate balance between the increased morbidity of unnecessary surgery due to false positive diagnostic findings and the risk of missing an early-stage ovarian cancer that could potentially be cured.

6.1. BIOLOGICAL VARIATION AND CONFOUNDING FACTORS IN SERUM MARKER ANALYSIS

Although some of the biological variation of tumor marker concentrations may be subtle, it is of importance that the properties of markers are well known. In the first study, the serum HE4 concentration did not show significant variation during the menstrual cycle. We, however, only divided the menstrual cycle into follicular, secretory and menstrual phases. In a previous study (Anastasi et al. 2010), a slight, yet statistically significant elevation of serum HE4 in the ovulatory phase (45.3 pM) compared to the follicular phase (39.1 pM, $p<0.0002$) was documented. The finding was evident only in women under 35 years, which may further confirm the association. However, the timing of the samples as being ovulatory was done only by counting days of the cycle, ovulation being confirmed retrospectively by the luteal phase progesterone increase.

In our second study, serum HE4 concentrations did not increase significantly during the supraphysiological ovarian stimulation, supported by the findings that normal ovaries show only low if any expression of HE4 (Galgano et al. 2006, Georgakopoulos et al. 2012, Drapkin et al. 2005), therefore expected to show little dependence on the hormonal regulation by gonadotrophins. Also, ovarian suppression by hormonal contraceptive use had no effect on serum HE4 levels in our first study. The HE4 concentration at the time of the ovum pick-up, however, was significantly increased as compared to the first stimulation visit. The oocyte retrieval usually takes place three to five days after the last ultrasound visit, with up to three days of possible gonadotrophin stimulation and a subsequent hCG injection resulting in a luteinizing effect and releasing the ova into the follicular fluid. Elevated serum CA125 levels have been detected in ovarian hyperstimulation syndrome (OHSS) (Jäger, Diedrich, and Wildt 1987) as well as during standard IVF treatment (Unkila-Kallio et al. 2000), up to the extent that it was evaluated

as a prognostic marker for IVF (Miller et al. 1996), nonetheless, concluded to be of no clinical relevance (Urbancsek et al. 2005, Noci et al. 1999). No such elevation of CA125 concentration during IVF treatment was detected in our Study II. Nevertheless, in contrast to HE4, CA125 is expressed in the peritoneal cavity (Kabawat et al. 1983) and elevated due to several unspecific causes of peritoneal irritation (Meden and Fattahi-Meibodi). A small study of nine patients, experiencing severe OHSS with subsequent ascites formation, reported normal HE4 mean values of 42.89 pM, however with no comparison to pre-treatment values (Hatzipetros et al. 2013). Our finding of the slight elevation of HE4 after the ovarian stimulation might be explained by interim changes in the fluid balance and glomerular filtration related to the controlled ovarian hyperstimulation rather than by hormonal causes.

Regarding CA125, the cycle-dependent variation was much stronger in endometriosis patients, a phenomenon previously recognized (Kafali et al. 2004), presenting with higher levels in both the proliferative and the secretory phase compared to the inactive/atrophic phase. Unlike in several previous studies (Pittaway and Fayez 1987, Bon et al. 1999), no increase during the menstrual phase was detected in our study population, possibly due to the small study population or the relatively high prevalence of hormonal contraceptive use. Furthermore, when the analysis was repeated with the exclusion of the endometriosis patients taking hormonal contraceptives, the cycle-dependent variation was no longer seen. This might be explained by the patients with more active endometriosis and more intensive symptoms using more hormonal medication, although there was no difference in the ASRM staging between the different medication groups.

The limitations for clinical use of the tumor marker CA125 have long been recognized and have prompted numerous studies for novel markers. Endometriosis does not affect serum HE4 levels (Huhtinen et al. 2009, Kadija et al. 2012, Moore et al. 2012), whereas endometriosis is a long-known source of elevated CA125 levels (Patton et al. 1986), findings further confirmed by our results in Study I and Study III. The effect of endometriosis on CA125 is evident to the extent of CA125 being evaluated as a diagnostic marker for endometriosis. A recent meta-analysis (Hirsch et al. 2016) concluded that CA125 can be used to further strengthen the suspicion of endometriosis with the pooled specificity of 93%, however, the negative predictive value is poor with the sensitivity of 52%. Furthermore, the diagnostic accuracy improved with more severe disease. However, an increased risk of ovarian cancer, particularly the subtypes of clear-cell, low-grade serous and endometrioid ovarian carcinomas has been associated with endometriosis (Pearce et al. 2012, Melin et al. 2006, Rossing et al. 2008), and even a stronger correlation has been published between histologically confirmed, more severe forms of endometriosis and EOC (Lee et al. 2015). Therefore, the relevance of the significantly increased CA125 levels in patients with endometriosis, although a benign disease, ought to be discussed if ovarian cancer screening protocols for high-risk patients were to be implemented.

In our Study III population, consisting of AGCT (with and without active disease) and endometriosis patients, only four women without EOC had HE4 concentrations higher than 150 pM. All but one of them had clinically relevant renal failure. The creatinine levels of all samples correlated with HE4 levels, supporting previous studies reporting renal failure as the most significant non-malignant cause for elevated HE4, whether resulting from reduced renal secretion or increased secretion by damaged tubular cells (Bolstad et al. 2011, Park et al. 2011). Measuring serum creatinine levels together with HE4 would, therefore, be advisable, particularly in postmenopausal women and women with comorbidities.

6.2. IMPLEMENTATION OF REFERENCE LIMITS

A fairly linear increase of HE4 with age has been depicted with no evident correlation to menopausal status, and age-dependent reference limits for HE4 have been suggested (Bolstad et al. 2011, Moore et al. 2012). It is generally acknowledged that differential diagnosis of pelvic tumors requires clinical judgment considering the clinical status and personal risk factors of the patient, and it may not be solely based on serum marker levels. However, certain cutoffs for serum markers are expected to be clinically applicable. The reference limits for HE4 concentration provided by manufacturers are variable; 150 pM in pre- and postmenopausal women for manual EIA by Fujirebio, 70 pM in pre- and 140 pM in postmenopausal women for automated CMIA by Abbott and 140 pM for automated ECLIA by Roche. Remarkable differences in results between automated assays and manual EIA have been reported (Bolstad et al. 2011, Ferraro et al. 2016), highlighting the importance of automated assay calibration and consideration of differences between reagents and methods used.

Although statistically significant, the fluctuation previously reported for HE4 during the menstrual cycle (Anastasi et al. 2010) or our findings of HE4 increase at the time of ovum pick-up and tubal pregnancy, the changes have all been subtle and far below cutoff limits. Furthermore, although HE4 has been shown to differentiate between early-stage EOC and benign ovarian tumors (Richards et al. 2015), the HE4 levels in both groups have been remarkably below any reference limits, challenging the validation of cutoffs and favoring more research in the implementation of individual longitudinal measurements. Therefore, it can be questioned, whether clinical guidelines for HE4 reference limits can be determined in a similar way as cutoffs for CA125, as level 35 U/ml has been implemented in worldwide clinical use.

6.3. SERUM MARKERS IN THE DIAGNOSIS OF NON-EPITHELIAL OVARIAN CANCER

In our Study III the ENDO group was significantly younger than the EOC and AGCT groups, resulting in an expected increase of the AGCT markers inhibin B and AMH in

comparison with other groups, as both of these markers are secreted by normal, functional ovaries. Particularly in premenopausal women, distinguishing between ovarian malignancy and a benign pelvic mass can be challenging due to hormone-related confounding factors and different distribution of ovarian cancer types in younger age groups. Tumors originating from germ cells, such as teratomas, dysgerminomas and yolk sac tumors, dominate in prepuberty and childhood, whereas the probability of a tumor being of epithelial origin, as well as the probability of malignancy, increase with age, with the peak incidence in the fifth and sixth decades of life (Merino and Jaffe 1993). Out of non-serous epithelial ovarian cancers, endometrioid cancers have shown elevated HE4 concentrations, whereas mucinous and other more uncommon cancers have shown a low and variable HE4 increase (Escudero et al. 2011, Hertlein et al. 2012). The National Comprehensive Cancer Network (NCCN) recommendation (2016) in suspicion of ovarian malignancy of a less common origin is the measurement of inhibin, AFP and beta hCG in addition to CA125.

According to our results (Study III), inhibin B has the best accuracy in AGCT diagnosis, however particularly in the subset of patients with ovarian endometriomas, adding AMH to inhibin B measurement is beneficial for diagnostic accuracy. Therefore, the measurement of both markers, AMH and inhibin B, could be recommended for the differential diagnosis of premenopausal women, whereas inhibin B solely is adequate for diagnosing AGCT in postmenopausal women.

As a follow-up marker, distinguishing AGCT WD patients from DF patients, inhibin B and AMH performed equally well, with no improvement in performance resulting from their combination. In a previous study with a different AMH assay, the combination of inhibin B and AMH improved the diagnostic accuracy slightly (Färkkilä et al. 2015), however, a review by Geerts (2009) concluded no significant difference between the performances of AMH and inhibin B, nevertheless, emphasizing the usability of a marker in GCT follow-up only when the preoperative level had been elevated.

Non-epithelial tumors of the ovary require a different approach in means of diagnosis, and it must be kept in mind that normal concentrations of epithelial cancer markers HE4 and CA125 do not rule out all ovarian cancers as our results from a study population of AGCT patients showed. All algorithms developed for estimating the risk of ovarian malignancy are aimed at detecting the most common, epithelial form of ovarian cancer.

6.4. IMPORTANCE OF CORRECT DIAGNOSIS

The correct primary diagnosis of cancer, following referral to the optimal place for surgical staging and treatment, has a definitive impact on patient survival (Engelen et al. 2006, Earle et al. 2006). Particularly AGCT patients, often diagnosed at an earlier stage with better prognosis than EOC patients, have a remarkable possibility for definitive primary surgery, which improves patient survival rates (Fotopoulou et al. 2010, Miller

et al. 1997). The importance of operative treatment performed by a gynecologic oncologist is further verified by the studies showing that tumor spill into the abdominal cavity worsens prognosis (Auranen et al. 2007, Wilson et al. 2015).

Patient survival times and recurrence-free years can be simply analyzed and compared as first outcome measures. However, other related issues and humane aspects may be very important to the patient. A certain number of unnecessary surgery is unavoidable in tumor diagnostics, yet the diagnosis ought to be as precise as possible to keep the harms and risks as low as possible regarding laparoscopy vs. laparotomy, need for lymphadenectomy etc. The inevitable psycho-social stress experienced by the patients can be considerable and unnecessarily prolonged if the diagnosis is delayed and unsure instead of direct referral to an oncologic center when needed. Particularly in the studies of cancer-related genetic counseling and cancer screening, the psycho-social aspects have been taken into consideration as the news of a significantly increased cancer risk can have remarkable effects on the quality of life of a person having previously considered herself healthy (Ardern-Jones, et al. 2005, Barrett et al. 2014).

6.5. TUBAL ORIGIN OF OVARIAN CANCER (STUDY IV)

Epithelial ovarian cancer was long considered to originate from the ovarian surface epithelium and EOC hypothesized to result from consequent ovulations resulting in damage to the ovarian surface epithelium, supported by epidemiological studies showing a risk reduction with ovulation reductive measures such as pregnancies and birth control pills and vice versa (Purdie et al. 1995, Risch et al. 1994). With later research and improved knowledge it has, however, become apparent that there can be several etiological factors and ways of pathogenesis within this spectrum of disease.

In our fourth study we measured serum HE4 and CA125 concentrations in different conditions related to early pregnancy. In continuum with our previous studies related to hormone-related effects on HE4, we separately evaluated normal ongoing pregnancies, spontaneous abortions and tubal pregnancies. There is benefit in utilizing these samples drawn a long time ago, in an era of more invasive methods of gynecological treatment. All of the patients in the study were treated surgically and diagnoses confirmed by histology, whereas nowadays nearly all medical and spontaneous abortions are treated medically with no histological samples available. In our study population, the serum HE4 concentration was higher in women with ongoing pregnancy and tubal pregnancy than in women with spontaneous abortions and non-pregnant women. However, the explanation for this is unclear and may be partially explained by socio-demographic differences between the groups, such as smoking habits. In previous studies the effect of pregnancy on serum HE4 has been controversial (Moore et al. 2012, Park et al. 2012). Some tubal pregnancies may present with a functioning placenta and even a living fetus, whereas in most cases the development of the pregnancy is disrupted already at an early stage. Regardless, it is previously known that placental tissue does not express HE4

(Galgano et al. 2006). Our finding of the elevated HE4 concentration in tubal pregnancies as compared to discontinued intrauterine pregnancies prompted us to explore the role of the affected tubal epithelia in this serum marker elevation with immunohistochemistry. The interest was further aroused by the theory of EOC precursor lesions originating from the fallopian tube, and the relatively early increase of HE4 in the early stages of EOC (Moore et al. 2008). Whereas the HE4 immunohistochemical staining in the healthy tubal epithelium was relatively weak and discontinuous, the staining in tubal pregnancies appeared more intensive and consistent throughout the tubal epithelium. All placental cell types were HE4 immunonegative.

The question, whether ovarian carcinoma, peritoneal carcinosis and tubal carcinoma are actually separate diseases or multifocal occurrences of one disease, was asked already in 1981 (Bannatyne and Russell 1981). More recently, the same theory resurfaced when a histopathological examination of fallopian tubes prophylactically removed from women with a high risk for ovarian cancer revealed early dysplastic lesions (Piek et al. 2001). When further cohorts of prophylactically removed fallopian tubes from BRCA-positive women were examined, 4,5-5,7% of the women were diagnosed with occult tubal or ovarian malignancy present, and with the majority of the cancerous lesions observed at the distal end of the tubae (Callahan et al. 2007, Laki et al. 2007), the results suggested that the increased risk of cancer in these women might actually originate from the changes appearing in the fimbriae.

Prophylactic and risk-reducing bilateral salpingo-oophorectomy (RRSO) has been recommended to BRCA carriers as it reduces ovarian cancer risk significantly, up to 80% (Domchek et al. 2010, Finch et al. 2014), resulting also in a 50% risk-reduction in breast cancer and a 75% decrease of overall mortality (Domchek et al. 2010). The timing of the procedure is generally recommended at the age of 35 to 40 years or when there is no more wish for children (Finch et al. 2006, Meindl et al. 2011). The theory of the increased risk of these women arising purely from the tubes and the recognition of the significant ovarian and peritoneal carcinoma reduction in low-risk women due to salpingectomy (Lessard-Anderson et al. 2014) would support the idea of prophylactic salpingectomy already at a younger age followed by a delayed oophorectomy years later. However, thus far the only intervention shown to decrease mortality in BRCA carriers is bilateral salpingo-oophorectomy. In our study population there was one known BRCA carrier with RRSO and one woman with prophylactic salpingo-oophorectomy due to recently diagnosed hormone receptor-positive breast cancer.

6.6. METHODOLOGICAL CONSIDERATIONS, STUDY LIMITATIONS AND STRENGTHS

6.6.1. Retrospective studies

The limitations of retrospective Studies I, III and IV lie in the patient selection and allocation. In Study I, the patients were carefully diagnosed and staged surgically and the phase of the menstrual cycle was determined both histologically and by patient interview. However, the answers and the histological stages were relatively inconsistent when compared with each other, and therefore the analysis was based on histology only. Granulosa cell tumors of the ovary are so rare that although all available serum samples over several decades were included in the analysis, the number of the most valuable preoperative samples, in particular, was limited. Therefore, several samples from different clinical stages of the patients were included to add more power to the analysis, making the statistical analysis and the interpretation of the results more challenging.

6.6.2. Prolonged storage of the samples

The samples for Study IV were collected and frozen already in 1994-96. HE4 has been shown to be stable during repetitive freeze-thaw cycles (Sandhu et al. 2014), however, no data on the effect of such a long storage time has been reported. The probable mechanisms of bias in this view would be vaporization and possible chemical degradation of the biological compound. The effect of vaporization was estimated by sodium measurements and the significantly increased (>165 mmol/l) were excluded from the analysis. A mathematical correction for the evaporation effect was also applied by C/140 * c, where C stands for current Sodium concentration, 140 models average Sodium concentrations, and c is the current HE4 concentration. All statistical analyses were repeated with the corrected values and the differences between the groups remained the same. The degradation of the HE4 molecule is difficult to evaluate, and as the samples from all the groups were handled similarly and stored in similar circumstances, the effect of the prolonged storage is estimated not to be different between groups.

Regarding the tissue samples for immunohistochemistry, the handling of surgical samples, such as the length of the fixation time, is not standardized and may therefore be variable, which could possibly affect the immunohistochemical staining. Nevertheless, all samples in general are handled in the same way, so systematic bias is unlikely to modify the results.

6.6.3. Study population

In Study I, the different medication groups were eventually restricted so significantly that some of the different medication groups needed to be excluded from the publication due to the small sample size. Regarding Study II, as it is previously known that HE4 is

not expressed by a normal ovary (Drapkin et al. 2005), a negative finding was expected and this relatively small sample size was estimated to show a possible trend in serum HE4 concentrations.

Although the data was retrospective to a large extent, the necessary background information was available from well-designed patient questionnaires, and medical records being traceable in all studies. Particularly the accuracy of diagnostics with surgical and histological confirmation of the disease in Study I population is exceptional. The patient cohort in Study III is unique due to the rarity of the disease, with the time span of sample collection dating back for decades.

6.7. FUTURE ASPECTS

It is unlikely that a population-based ovarian cancer screening system will be implemented in the near future. However, targeted screening with multimodal approach, including relatively wide implementation of genetic testing in high-risk families as well as personal and etiological risk factors, could be manageable in the industrialized world, where ovarian cancer is the leading cause of death among gynecological malignancies. Regarding serum marker analyses, calculated models detecting marker elevation with regard to a personal baseline appear more reliable than fixed cutoffs.

Less invasive and cost-effective methods for tumor marker analysis are being developed in combination with modern information technology, such as a possibility of urine sampling for HE4 measurement and further assay interpretation with a mobile application (Wang et al. 2015). In any case, the key to improving the ovarian cancer survival rates is in diagnosis, timely and accurate.

7. CONCLUSIONS

1. HE4 is a relatively stable serum biomarker for ovarian cancer. HE4 is not expressed in normal ovarian tissue, and its serum concentration is not dependent on hormonal contraceptives, menstrual cycle or gonadotrophin stimulation.

2. Inhibin B is the most accurate marker for diagnosis and follow-up of adult-type ovarian granulosa cell tumors. However, when differentiating granulosa cell tumors from ovarian endometriomas, additional serum AMH measurement is beneficial, and the combination of inhibin B and AMH may be recommended for differential diagnosis of pelvic tumors in premenopausal women.

3. The more intensive and continuous HE4 immunohistochemical staining in the fallopian tubes affected by ectopic pregnancy, as compared to normal fallopian tubes, may contribute to the elevated serum HE4 concentration in patients with tubal pregnancy.

8. ACKNOWLEDGEMENTS

This work was carried out in the Department of Obstetrics and Gynaecology and the Department of Physiology, University of Turku during the years 2009-2017. I wish to express my warmest gratitude to the following persons:

Professor, Head of Department, Seija Grénman and Professor Juha Mäkinen at the Department of Obstetrics and Gynaecology for your positive and supporting attitude toward the scientific work carried out in the clinic. You are easy to approach and you always find time in your busy schedule to ponder upon any issues related to either clinical work or research.

My supervisor, Antti Perheentupa, Adjunct Professor, for support and admirable patience. You have taught me the very principles of doing research. You have always been there to answer questions, later guiding me to find the answers. Unlike myself, you never showed any doubt about finishing this project.

My supervisor, Professor Matti Poutanen, for emphasizing the importance of knowing physiology. You brought a deeper insight into my thinking and provided excellent practical facilities for carrying out the laboratory work.

All the co-authors of the original articles for your significant contribution. My warmest thanks go to Kaisa Huhtinen, PhD, for kindly helping with everything in the beginning. You were my interpreter between physiology and gynecology. Pia Suvitie, MD, for both scientific contribution and friendly support while we were working on our respective projects side by side. Anniina Färkkilä, MD, PhD and Ulla-Maija Haltia, MD, for valuable collaboration. Harry Kujari, MD for your expertise and delightful, cynical humor and Michael Gabriel, MD for introducing me to immunohistochemistry. Mika Rantala, MD, is thanked for the contribution in sample collection.

All the scientists and other personnel at the Department of Physiology and the Department of Pathology for having assisted me in the laboratory work and sample collection. The nurses in the gynecological outpatient clinic and gynecological ward for assisting with patient recruitment and sample collection. Jaakko Matomäki for the assistance on statistical analyses.

The official reviewers of this thesis, Professor Ulla Puistola and Adjunct Professor Piia Aarnisalo, for valuable comments and constructive criticism. Virpi Rantanen, Adjunct Professor, and Eila Suvanto, MD, PhD, for your contribution in the steering group of this dissertation project.

My chief Riikka Aaltonen for making it possible to combine clinical and scientific work.

All the patients for participating in this study. You are greatly appreciated for making this research possible.

My colleagues at the Department of Obstetrics and Gynaecology in Turku University Hospital for friendship and collegiality. Particularly the scientifically oriented colleagues for support and inspiration on several occasions on the 12th floor and postgraduate courses and congresses; Johanna H, Pia H, Kaisa K, Carita, Kirsi and others. Sanna O, Sanna S and Rauni for introducing me to endocrinology and for being everyday peer supporters in struggles of combining motherhood and work.

Jonna and Emmi with your families for being such dear friends over the years. Family Söderström for providing excellent travel companionship to brighten up the winters. Suvi, Minna and Mirka for friendship and support when needed.

My parents-in-law, Saini and Eero, for taking such good care of our children.

My parents for endless support and care. My dad, Eero, for encouraging me to study and pursue my dreams since the very first days of school. My mom, Hanne-Maarit, for tirelessly helping out in everyday life and for always being there. I can never thank you enough. My brother, Jere and his family, for friendship and company.

Mikko, my husband and partner, for loving care and endless patience and support over the years. You are the best dad ever. Our precious little girls Lotta, Liina and Mette, you mean everything to me.

This work was financially supported by the Finnish Funding Agency for Technology and Innovation, University of Turku Doctoral Programme of Clinical Investigations, Clinical Research (EVO) funding of Turku University Hospital, Turku University Foundation, The Finnish Medical Society Duodecim and Ida Montin Foundation.

9. REFERENCES

Aarnio, M., R. Sankila, E. Pukkala, R. Salovaara, L. A. Aaltonen, A. de la Chapelle, P. Peltomäki, J. P. Mecklin, and H. J. Järvinen. 1999. "Cancer risk in mutation carriers of DNA-mismatch-repair genes." Int J Cancer 81 (2):214-8.

Adami, H. O., C. C. Hsieh, M. Lambe, D. Trichopoulos, D. Leon, I. Persson, A. Ekbom, and P. O. Janson. 1994. "Parity, age at first childbirth, and risk of ovarian cancer." Lancet 344 (8932):1250-4.

Amsterdam, A., and N. Selvaraj. 1997. "Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes." Endocr Rev 18 (4):435-61.

Anastasi, E, T Granato, GG Marchei, V Viggiani, B Colaprisca, S Comploj, MG Reale, L Frati, and C Midulla. 2010. "Ovarian tumor marker HE4 is differently expressed during the phases of the menstrual cycle in healthy young women." Tumour Biol. 31 (5):411-5.

Anastasi, E, GG Marchei, V Viggiani, G Gennarini, L Frati, and MG Reale. 2010. "HE4: a new potential early biomarker for the recurrence of ovarian cancer." Tumour Biol 31 (2):113-9.

Andersson, A. M., T. K. Jensen, A. Juul, J. H. Petersen, T. Jørgensen, and N. E. Skakkebaek. 2007. "Secular decline in male testosterone and sex hormone binding globulin serum levels in Danish population surveys." J Clin Endocrinol Metab 92 (12):4696-705.

Angioli, R., S. Capriglione, A. Aloisi, F. Guzzo, D. Luvero, A. Miranda, P. Damiani, R. Montera, C. Terranova, and F. Plotti. 2014. "Can HE4 predict platinum response during first-line chemotherapy in ovarian cancer?" Tumour Biol 35 (7):7009-15.

Anton, C., F. M. Carvalho, E. I. Oliveira, G. A. Maciel, E. C. Baracat, and J. P. Carvalho. 2012. "A comparison of CA125, HE4, risk ovarian malignancy algorithm (ROMA), and risk malignancy index (RMI) for the classification of ovarian masses." Clinics (Sao Paulo) 67 (5):437-41.

Antoniou, A. C., S. A. Gayther, J. F. Stratton, B. A. Ponder, and D. F. Easton. 2000. "Risk models for familial ovarian and breast cancer." Genet Epidemiol 18 (2):173-90.

Antoniou, A., P. D. Pharoah, S. Narod, H. A. Risch, J. E. Eyfjord, J. L. Hopper, N. Loman, H. Olsson, O. Johannsson, A. Borg, B. Pasini, P. Radice, S. Manoukian, D. M. Eccles, N. Tang, E. Olah, H. Anton-Culver, E. Warner, J. Lubinski, J. Gronwald, B. Gorski, H. Tulinius, S. Thorlacius, H. Eerola, H. Nevanlinna, K. Syrjäkoski, O. P. Kallioniemi, D. Thompson, C. Evans, J. Peto, F. Lalloo, D. G. Evans, and D. F. Easton. 2003. "Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case Series unselected for family history: a combined analysis of 22 studies." Am J Hum Genet 72 (5):1117-30.

Anttonen, M., L. Unkila-Kallio, A. Leminen, R. Butzow, and M. Heikinheimo. 2005. "High GATA-4 expression associates with aggressive behavior, whereas low anti-Müllerian hormone expression associates with growth potential of ovarian granulosa cell tumors." J Clin Endocrinol Metab 90 (12):6529-35.

Ardern-Jones, A., R. Kenen, and R. Eeles. 2005. "Too much, too soon? Patients and health professionals' views concerning the impact of genetic testing at the time of breast cancer diagnosis in women under the age of 40." Eur J Cancer Care (Engl) 14 (3):272-81.

Auranen, A., J. Sundström, J. Ijäs, and S. Grénman. 2007. "Prognostic factors of ovarian granulosa cell tumor: a study of 35 patients and review of the literature." Int J Gynecol Cancer 17 (5):1011-8.

Babacan, A., C. Kizilaslan, I. Gun, M. Muhcu, E. Mungen, and V. Atay. 2014. "CA 125 and other tumor markers in uterine leiomyomas and their association with lesion characteristics." Int J Clin Exp Med 7 (4):1078-83.

Balen, A. 2001. "Polycystic ovary syndrome and cancer." Hum Reprod Update 7 (6):522-5.

Bandiera, E., L. Zanotti, A. S. Fabricio, E. Bucca, E. Squarcina, C. Romani, R. Tassi, E. Bignotti, P. Todeschini, G. Tognon, C. Romagnolo, M. Gion, E. Sartori, T. Maggino, S. Pecorelli, and A. Ravaggi. 2013. "Cancer antigen 125, human epididymis 4, kallikrein 6, osteopontin and soluble mesothelin-related peptide immunocomplexed with immunoglobulin M in epithelial ovarian cancer diagnosis." Clin Chem Lab Med 51 (9):1815-24.

Bannatyne, P., and P. Russell. 1981. "Early adenocarcinoma of the fallopian tubes. A case for multifocal tumorigenesis." Diagn Gynecol Obstet 3 (1):49-60.

Barbieri, R. L., J. M. Niloff, R. C. Bast, E. Scaetzl, R. W. Kistner, and R. C. Knapp. 1986. "Elevated serum concentrations of CA-125 in patients with advanced endometriosis." Fertil Steril 45 (5):630-4.

Barrett, J., V. Jenkins, V. Farewell, U. Menon, I. Jacobs, J. Kilkerr, A. Ryan, C. Langridge, L. Fallowfield, and UKCTOCS trialists. 2014. "Psychological morbidity associated with ovarian cancer screening: results from more than 23,000 women in the randomised trial of ovarian cancer screening (UKCTOCS)." BJOG 121 (9):1071-9.

Bast, R. C., N. Urban, V. Shridhar, D. Smith, Z. Zhang, S. Skates, K. Lu, J. Liu, D. Fishman, and G. Mills. 2002. "Early detection of ovarian cancer: promise and reality." Cancer Treat Res 107:61-97.

Bast, RC Jr, D Badgwell, Z Lu, R Marquez, D Rosen, J Liu, KA Baggerly, EN Atkinson, S Skates, Z Zhang, A Lokshin, U Menon, I Jacobs, and K Lu. 2005. "New tumor markers: CA125 and beyond." Int J Gynecol Cancer 15 Suppl 3:274-81.

Bast, RC Jr, TL Klug, E St John, E Jenison, JM Niloff, H Lazarus, RS Berkowitz, T Leavitt, CT Griffiths, L

Parker, VR Jr Zurawski, and RC Knapp. 1983. "A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer." N Engl J Med 309 (15):883-7.

Bast, RC Jr., M. Feeney, H. Lazarus, LM. Nadler, RB. Colvin, RC. Knapp. 1981. "Reactivity of a monoclonal antibody with human ovarian carcinoma." 68 (5):1331-1337.

Bignotti, E., M. Ragnoli, L. Zanotti, S. Calza, M. Falchetti, S. Lonardi, S. Bergamelli, E. Bandiera, R. A. Tassi, C. Romani, P. Todeschini, F. E. Odicino, F. Facchetti, S. Pecorelli, and A. Ravaggi. 2011. "Diagnostic and prognostic impact of serum HE4 detection in endometrial carcinoma patients." Br J Cancer 104 (9):1418-25.

Bingle, L., V. Singleton, and C. D. Bingle. 2002. "The putative ovarian tumour marker gene HE4 (WFDC2), is expressed in normal tissues and undergoes complex alternative splicing to yield multiple protein isoforms." Oncogene 21 (17):2768-73.

Bjørge, T., A. K. Lie, E. Hovig, R. E. Gislefoss, S. Hansen, E. Jellum, H. Langseth, K. Nustad, C. G. Tropé, and A. Dørum. 2004. "BRCA1 mutations in ovarian cancer and borderline tumours in Norway: a nested case-control study." Br J Cancer 91 (10):1829-34.

Bolstad, N., M. Oijordsbakken, K. Nustad, and J. Bjerner. 2012. "Human epididymis protein 4 reference limits and natural variation in a Nordic reference population." Tumour Biol. 33(1):141-8.

Bon, GG, P Kenemans, JJ Dekker, PG Hompes, RA Verstraeten, GJ van Kamp, and J Schoemaker. 1999. "Fluctuations in CA 125 and CA 15-3 serum concentrations during spontaneous ovulatory cycles." Hum Reprod 14 (2):566-70.

Booth, M., V. Beral, and P. Smith. 1989. "Risk factors for ovarian cancer: a case-control study." Br J Cancer 60 (4):592-8.

Bowtell, D. D. 2010. "The genesis and evolution of high-grade serous ovarian cancer." Nat Rev Cancer 10 (11):803-8.

Brennan, D. J., A. Hackethal, K. P. Mann, I. Mutz-Dehbalaie, H. Fiegl, C. Marth, and A. Obermair. 2015. "Serum HE4 detects recurrent endometrial cancer in patients undergoing routine clinical surveillance." BMC Cancer 15:33.

Brinton, L. A., G. Gridley, I. Persson, J. Baron, and A. Bergqvist. 1997. "Cancer risk after a hospital discharge diagnosis of endometriosis." Am J Obstet Gynecol 176 (3):572-9.

Burger, H. G. 1993. "Evidence for a negative feedback role of inhibin in follicle stimulating hormone regulation in women." Hum Reprod 8 Suppl 2:129-32.

Buys, S. S., E. Partridge, A. Black, C. C. Johnson, L. Lamerato, C. Isaacs, D. J. Reding, R. T. Greenlee, L. A. Yokochi, B. Kessel, E. D. Crawford, T. R. Church, G. L. Andriole, J. L. Weissfeld, M. N. Fouad, D. Chia, B. O'Brien, L. R. Ragard, J. D. Clapp, J. M. Rathmell, T. L. Riley, P. Hartge, P. F. Pinsky, C. S. Zhu, G. Izmirlian, B. S. Kramer, A. B. Miller, J. L. Xu, P. C. Prorok, J. K. Gohagan, C. D. Berg, and PLCO Project Team. 2011. "Effect of screening on ovarian cancer mortality: the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Randomized Controlled Trial." JAMA 305 (22):2295-303.

Callahan, M. J., C. P. Crum, F. Medeiros, D. W. Kindelberger, J. A. Elvin, J. E. Garber, C. M. Feltmate, R. S. Berkowitz, and M. G. Muto. 2007. "Primary fallopian tube malignancies in BRCA-positive women undergoing surgery for ovarian cancer risk reduction." J Clin Oncol 25 (25):3985-90.

Chhikara, N., M. Saraswat, A. K. Tomar, S. Dey, S. Singh, and S. Yadav. 2012. "Human epididymis protein-4 (HE-4): a novel cross-class protease inhibitor." PLoS One 7 (11):e47672.

Clauss, A., H. Lilja, and A. Lundwall. 2002. "A locus on human chromosome 20 contains several genes expressing protease inhibitor domains with homology to whey acidic protein." Biochem J 368 (Pt 1):233-42.

Colombo, N., G. Parma, V. Zanagnolo, and A. Insinga. 2007. "Management of ovarian stromal cell tumors." J Clin Oncol 25 (20):2944-51.

Cramer, D. W., G. B. Hutchison, W. R. Welch, R. E. Scully, and K. J. Ryan. 1983. "Determinants of ovarian cancer risk. I. Reproductive experiences and family history." J Natl Cancer Inst 71 (4):711-6.

Domchek, S. M., T. M. Friebel, C. F. Singer, D. G. Evans, H. T. Lynch, C. Isaacs, J. E. Garber, S. L. Neuhausen, E. Matloff, R. Eeles, G. Pichert, L. Van t'veer, N. Tung, J. N. Weitzel, F. J. Couch, W. S. Rubinstein, P. A. Ganz, M. B. Daly, O. I. Olopade, G. Tomlinson, J. Schildkraut, J. L. Blum, and T. R. Rebbeck. 2010. "Association of risk-reducing surgery in BRCA1 or BRCA2 mutation carriers with cancer risk and mortality." JAMA 304 (9):967-75.

Drapkin, R., H. H. von Horsten, Y. Lin, S. C. Mok, C. P. Crum, W. R. Welch, and J. L. Hecht. 2005. "Human epididymis protein 4 (HE4) is a secreted glycoprotein that is overexpressed by serous and endometrioid ovarian carcinomas." Cancer Res 65 (6):2162-9.

Drescher, C. W., C. Shah, J. Thorpe, K. O'Briant, G. L. Anderson, C. D. Berg, N. Urban, and M. W. McIntosh. 2013. "Longitudinal screening algorithm that incorporates change over time in CA125 levels identifies ovarian cancer earlier than a single-threshold rule." J Clin Oncol 31 (3):387-92.

Earle, C. C., D. Schrag, B. A. Neville, K. R. Yabroff, M. Topor, A. Fahey, E. L. Trimble, D. C. Bodurka, R. E. Bristow, M. Carney, and J. L. Warren. 2006. "Effect of surgeon specialty on processes of care and outcomes for ovarian cancer patients." J Natl Cancer Inst 98 (3):172-80.

Einhorn, N., K. Sjövall, R. C. Knapp, P. Hall, R. E. Scully, R. C. Bast, and V. R. Zurawski. 1992. "Prospective evaluation of serum CA 125 levels for early detection of ovarian cancer." Obstet Gynecol 80 (1):14-8.

Engelen, M. J., H. E. Kos, P. H. Willemse, J. G. Aalders, E. G. de Vries, M. Schaapveld, R. Otter, and A. G. van der Zee. 2006. "Surgery by consultant gynecologic oncologists improves survival in patients with ovarian carcinoma." Cancer 106 (3):589-98.

Erbağci, AB, N Yilmaz, and I Kutlar. 1999. "Menstrual cycle dependent variability for serum tumor markers CEA, AFP, CA 19-9, CA 125 and CA 15-3 in healthy women." Dis Markers 15 (4):259-67.

Escudero, J. M., J. M. Auge, X. Filella, A. Torne, J. Pahisa, and R. Molina. 2011. "Comparison of serum human epididymis protein 4 with cancer antigen 125 as a tumor marker in patients with malignant and nonmalignant diseases." Clin Chem 57 (11):1534-44.

Fedele, L, P Vercellini, L Arcaini, MG da Dalt, and GB Candiani. 1988. "CA 125 in serum, peritoneal fluid, active lesions, and endometrium of patients with endometriosis." Am J Obstet Gynecol 158 (1):166-70.

Ferlay, J., I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. M. Parkin, D. Forman, and F. Bray. 2015. "Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012." Int J Cancer 136 (5):E359-86.

Ferraro, S., S. Borille, A. Carnevale, E. Frusciante, N. Bassani, and M. Panteghini. 2016. "Verification of the harmonization of human epididymis protein 4 assays." Clin Chem Lab Med 54 (10):1635-43.

Finch, A., M. Beiner, J. Lubinski, H. T. Lynch, P. Moller, B. Rosen, J. Murphy, P. Ghadirian, E. Friedman, W. D. Foulkes, C. Kim-Sing, T. Wagner, N. Tung, F. Couch, D. Stoppa-Lyonnet, P. Ainsworth, M. Daly, B. Pasini, R. Gershoni-Baruch, C. Eng, O. I. Olopade, J. McLennan, B. Karlan, J. Weitzel, P. Sun, S. A. Narod, and Hereditary Ovarian Cancer Clinical Study Group. 2006. "Salpingo-oophorectomy and the risk of ovarian, fallopian tube, and peritoneal cancers in women with a BRCA1 or BRCA2 Mutation." JAMA 296 (2):185-92.

Finch, A. P., J. Lubinski, P. Møller, C. F. Singer, B. Karlan, L. Senter, B. Rosen, L. Maehle, P. Ghadirian, C. Cybulski, T. Huzarski, A. Eisen, W. D. Foulkes, C. Kim-Sing, P. Ainsworth, N. Tung, H. T. Lynch, S. Neuhausen, K. A. Metcalfe, I. Thompson, J. Murphy, P. Sun, and S. A. Narod. 2014. "Impact of oophorectomy on cancer incidence and mortality in women with a BRCA1 or BRCA2 mutation." J Clin Oncol 32 (15):1547-53.

Fotopoulou, C., K. Savvatis, E. I. Braicu, V. Brink-Spalink, S. Darb-Esfahani, W. Lichtenegger, and J. Sehouli. 2010. "Adult granulosa cell tumors of the ovary: tumor dissemination pattern at primary and recurrent situation, surgical outcome." Gynecol Oncol 119 (2):285-90.

Fung, E. T. 2010. "A recipe for proteomics diagnostic test development: the OVA1 test, from biomarker discovery to FDA clearance." Clin Chem 56 (2):327-9.

Färkkilä, A., S. Koskela, S. Bryk, H. Alfthan, R. Bützow, A. Leminen, U. Puistola, J. S. Tapanainen, M. Heikinheimo, M. Anttonen, and L. Unkila-Kallio. 2015. "The clinical utility of serum anti-Müllerian hormone in the follow-up of ovarian adult-type granulosa cell tumors--A comparative study with inhibin B." Int J Cancer 137 (7):1661-71.

Galgano, MT, GM Hampton, and HF Jr Frierson. 2006. "Comprehensive analysis of HE4 expression in normal and malignant human tissues." Mod Pathol 19 (6):847-53.

Geerts, I., I. Vergote, P. Neven, and J. Billen. 2009. "The role of inhibins B and antimüllerian hormone for diagnosis and follow-up of granulosa cell tumors." Int J Gynecol Cancer 19 (5):847-55.

Georgakopoulos, P., S. Mehmood, A. Akalin, and K. R. Shroyer. 2012. "Immunohistochemical localization of HE4 in benign, borderline, and malignant lesions of the ovary." Int J Gynecol Pathol 31 (6):517-23.

Gidwani, K., K. Huhtinen, H. Kekki, S. van Vliet, J. Hynninen, N. Koivuviita, A. Perheentupa, M. Poutanen, A. Auranen, S. Grenman, U. Lamminmäki, O. Carpen, Y. van Kooyk, and K. Pettersson. 2016. "A Nanoparticle-Lectin Immunoassay Improves Discrimination of Serum CA125 from Malignant and Benign Sources." Clin Chem 62 (10):1390-400.

Gislefoss, R. E., H. Langseth, N. Bolstad, K. Nustad, and L. Mørkrid. 2015. "HE4 as an Early Detection Biomarker of Epithelial Ovarian Cancer: Investigations in Prediagnostic Specimens From the Janus Serumbank." Int J Gynecol Cancer 25 (9):1608-15.

Giudice, LC, A Jacobs, J Pineda, CE Bell, and L Lippmann. 1986. "Serum levels of CA-125 in patients with endometriosis: a preliminary report." Fertil Steril 45 (6):876-8.

Groome, N. P., P. J. Illingworth, M. O'Brien, R. Pai, F. E. Rodger, J. P. Mather, and A. S. McNeilly. 1996. "Measurement of dimeric inhibin B throughout the human menstrual cycle." J Clin Endocrinol Metab 81 (4):1401-5.

Gucer, F., G. Kiran, E. Canaz, M. Kilinc, H. C. Ekerbicer, F. Avci, H. Kiran, A. Coskun, and D. C. Arikan. 2015. "Serum human epididymis protein 4 can be a useful tumor marker in the differential diagnosis of adnexal masses during pregnancy: a pilot study." Eur J Gynaecol Oncol 36 (4):406-9.

Halila, H., U. H. Stenman, and M. Seppälä. 1986. "Ovarian cancer antigen CA 125 levels in pelvic inflammatory disease and pregnancy." Cancer 57 (7):1327-9.

Hankinson, S. E., G. A. Colditz, D. J. Hunter, W. C. Willett, M. J. Stampfer, B. Rosner, C. H. Hennekens, and F. E. Speizer. 1995. "A prospective study of reproductive factors and risk of epithelial ovarian cancer." Cancer 76 (2):284-90.

Hankinson, S. E., D. J. Hunter, G. A. Colditz, W. C. Willett, M. J. Stampfer, B. Rosner, C. H. Hennekens, and F. E. Speizer. 1993. "Tubal ligation, hysterectomy, and risk of ovarian cancer. A prospective study." JAMA 270 (23):2813-8.

Hashimoto T., F. Matsubara. 1989. "Changes in tumor marker concentration in female patients with hyper-, eu- and hypothyroidism." 36 (6):873-879.

Hatzipetros, I., P. M. Gocze, K. Cziraky, K. Kovacs, E. Kalman, and B. Farkas. 2013. "Assessment of cells in the ascitic fluid of women with ovarian hyperstimulation syndrome: the clinical implications for subsequent ovarian malignancy." Reprod Biol Endocrinol 11 (1):91.

Havrilesky, LJ, CM Whitehead, JM Rubatt, RL Cheek, J Groelke, Q He, DP Malinowski, TJ Fischer, and A Berchuck. 2008. "Evaluation of biomarker panels for

early stage ovarian cancer detection and monitoring for disease recurrence." Gynecol Oncol 110 (3):374-82.

Healy, D. L., H. G. Burger, P. Mamers, T. Jobling, M. Bangah, M. Quinn, P. Grant, A. J. Day, R. Rome, and J. J. Campbell. 1993. "Elevated serum inhibin concentrations in postmenopausal women with ovarian tumors." N Engl J Med 329 (21):1539-42.

Heintz, A. P., F. Odicino, P. Maisonneuve, M. A. Quinn, J. L. Benedet, W. T. Creasman, H. Y. Ngan, S. Pecorelli, and U. Beller. 2006. "Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer." Int J Gynaecol Obstet 95 Suppl 1:S161-92.

Hellstrom, I., P. J. Heagerty, E. M. Swisher, P. Liu, J. Jaffar, K. Agnew, and K. E. Hellstrom. 2010. "Detection of the HE4 protein in urine as a biomarker for ovarian neoplasms." Cancer Lett 296 (1):43-8.

Hellström, I, J Raycraft, M Hayden-Ledbetter, JA Ledbetter, M Schummer, M McIntosh, C Drescher, N Urban, and KE Hellström. 2003. "The HE4 (WFDC2) protein is a biomarker for ovarian carcinoma." Cancer Res 63 (13):3695-700.

Hermsen, B. B., S. von Mensdorff-Pouilly, J. Berkhof, P. J. van Diest, J. J. Gille, F. H. Menko, M. A. Blankenstein, P. Kenemans, and R. H. Verheijen. 2007. "Serum CA-125 in relation to adnexal dysplasia and cancer in women at hereditary high risk of ovarian cancer." J Clin Oncol 25 (11):1383-9.

Hertlein, L., P. Stieber, A. Kirschenhofer, K. Krocker, D. Nagel, M. Lenhard, and A. Burges. 2012. "Human epididymis protein 4 (HE4) in benign and malignant diseases." Clin Chem Lab Med 50 (12):2181-8.

Hirsch, M., J. Duffy, C. J. Davis, M. Nieves Plana, K. S. Khan, and International Collaboration to Harmonise Outcomes and Measures for Endometriosis. 2016. "Diagnostic accuracy of cancer antigen 125 for endometriosis: a systematic review and meta-analysis." BJOG 123 (11):1761-8.

Holcomb, K., Z. Vucetic, M. C. Miller, and R. C. Knapp. 2011. "Human epididymis protein 4 offers superior specificity in the differentiation of benign and malignant adnexal masses in premenopausal women." Am J Obstet Gynecol 205 (4):358.e1-6.

Hsueh, A. J., K. D. Dahl, J. Vaughan, E. Tucker, J. Rivier, C. W. Bardin, and W. Vale. 1987. "Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis." Proc Natl Acad Sci U S A 84 (14):5082-6.

Hu, Z. D., T. T. Wei, M. Yang, N. Ma, Q. Q. Tang, B. D. Qin, H. T. Fu, and R. Q. Zhong. 2015. "Diagnostic value of osteopontin in ovarian cancer: a meta-analysis and systematic review." PLoS One 10 (5):e0126444.

Huang, T., S. W. Jiang, L. Qin, C. Senkowski, C. Lyle, K. Terry, S. Brower, H. Chen, W. Glasgow, Y. Wei, and J. Li. 2015. "Expression and diagnostic value of HE4 in pancreatic adenocarcinoma." Int J Mol Sci 16 (2):2956-70.

Huhtinen, K, P Suvitie, J Hiissa, J Junnila, J Huvila, H Kujari, M Setälä, P Härkki, J Jalkanen, J Fraser, J Mäkinen, A Auranen, M Poutanen, and A Perheentupa. 2009. "Serum HE4 concentration differentiates malignant ovarian tumours from ovarian endometriotic cysts." Br J Cancer 100 (8):1315-9.

Huang WC., CW. Tseng, KM Chang, JY Hsu, JH. Chen, GH. Shen. 2011. "Usefulness of tumor marker CA-125 serum levels for the follow-up of therapeutic responses in tuberculosis patients with and without serositis." Jpn J Inf Dis. 64 (5):367-72

Høgdall, E. V., L. Christensen, S. K. Kjaer, J. Blaakaer, A. Kjaerbye-Thygesen, S. Gayther, I. J. Jacobs, and C. K. Høgdall. 2007. "CA125 expression pattern, prognosis and correlation with serum CA125 in ovarian tumor patients. From The Danish "MALOVA" Ovarian Cancer Study." Gynecol Oncol 104 (3):508-15.

Innao, P., M. Pothisuwan, and P. Pengsa. 2016. "Does Human Epididymis Protein 4 (HE4) Have a Role in Prediction of Recurrent Epithelial Ovarian Cancer." Asian Pac J Cancer Prev 17 (9):4483-4486.

Iwahori, K., H. Suzuki, Y. Kishi, Y. Fujii, R. Uehara, N. Okamoto, M. Kobayashi, T. Hirashima, I. Kawase, and T. Naka. 2012. "Serum HE4 as a diagnostic and prognostic marker for lung cancer." Tumour Biol 33 (4):1141-9.

Jacob, F., M. Meier, R. Caduff, D. Goldstein, T. Pochechueva, N. Hacker, D. Fink, and V. Heinzelmann-Schwarz. 2011. "No benefit from combining HE4 and CA125 as ovarian tumor markers in a clinical setting." Gynecol Oncol 121 (3):487-91.

Jacobs, I, D Oram, J Fairbanks, J Turner, C Frost, and JG Grudzinskas. 1990. "A risk of malignancy index incorporating CA 125, ultrasound and menopausal status for the accurate preoperative diagnosis of ovarian cancer." Br J Obstet Gynaecol 97 (10):922-9.

Jacobs, I. J., U. Menon, A. Ryan, A. Gentry-Maharaj, M. Burnell, J. K. Kalsi, N. N. Amso, S. Apostolidou, E. Benjamin, D. Cruickshank, D. N. Crump, S. K. Davies, A. Dawnay, S. Dobbs, G. Fletcher, J. Ford, K. Godfrey, R. Gunu, M. Habib, R. Hallett, J. Herod, H. Jenkins, C. Karpinskyj, S. Leeson, S. J. Lewis, W. R. Liston, A. Lopes, T. Mould, J. Murdoch, D. Oram, D. J. Rabideau, K. Reynolds, I. Scott, M. W. Seif, A. Sharma, N. Singh, J. Taylor, F. Warburton, M. Widschwendter, K. Williamson, R. Woolas, L. Fallowfield, A. J. McGuire, S. Campbell, M. Parmar, and S. J. Skates. 2016. "Ovarian cancer screening and mortality in the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS): a randomised controlled trial." Lancet 387 (10022):945-56.

Jacobs, I. J., S. Skates, A. P. Davies, R. P. Woolas, A. Jeyerajah, P. Weidemann, K. Sibley, and D. H. Oram. 1996. "Risk of diagnosis of ovarian cancer after raised serum CA 125 concentration: a prospective cohort study." BMJ 313 (7069):1355-8.

Jacobs, I. J., S. J. Skates, N. MacDonald, U. Menon, A. N. Rosenthal, A. P. Davies, R. Woolas, A. R. Jeyarajah, K. Sibley, D. G. Lowe, and D. H. Oram. 1999. "Screening for ovarian cancer: a pilot randomised controlled trial." Lancet 353 (9160):1207-10.

Jacobs, I., I. Stabile, J. Bridges, P. Kemsley, C. Reynolds, J. Grudzinskas, and D. Oram. 1988. "Multimodal

approach to screening for ovarian cancer." Lancet 1 (8580):268-71.

Jamieson, S., and P. J. Fuller. 2012. "Molecular pathogenesis of granulosa cell tumors of the ovary." Endocr Rev 33 (1):109-44.

Jiang, SW., H. Chen, S. Dowdy, A. Fu, J. Attewell, E. Kalogera, R. Drapkin, K. Podratz, R. Broaddus, J. Li. 2013. "HE4 transcription- and splice variants-specific expression in endometrial cancer and correlation with patient survival." Int J Mol Sci 14 (11):22655-77.

Jäger, W., K. Diedrich, and L. Wildt. 1987. "Elevated levels of CA-125 in serum of patients suffering from ovarian hyperstimulation syndrome." Fertil Steril 48 (4):675-8.

Kabawat, SE, RC Jr Bast, AK Bhan, WR Welch, RC Knapp, and RB Colvin. 1983. "Tissue distribution of a coelomic-epithelium-related antigen recognized by the monoclonal antibody OC125." Int J Gynecol Pathol 2 (3):275-85.

Kadija, S., A. Stefanovic, K. Jeremic, M. M. Radojevic, L. Nikolic, I. Markovic, and J. Atanackovic. 2012. "The utility of human epididymal protein 4, cancer antigen 125, and risk for malignancy algorithm in ovarian cancer and endometriosis." Int J Gynecol Cancer 22 (2):238-44.

Kafali, H, H Artunc, and M Erdem. 2007. "Evaluation of factors that may be responsible for cyclic change of CA125 levels during menstrual cycle." Arch Gynecol Obstet 275 (3):175-7.

Kafali, H., H. Artuc, and N. Demir. 2004. "Use of CA125 fluctuation during the menstrual cycle as a tool in the clinical diagnosis of endometriosis; a preliminary report." Eur J Obstet Gynecol Reprod Biol 116 (1):85-8.

Kappelmayer, J., P. Antal-Szalmás, and B. Nagy. 2015. "Human epididymis protein 4 (HE4) in laboratory medicine and an algorithm in renal disorders." Clin Chim Acta 438:35-42.

Karlan, B. Y., J. D. Thorpe, K. Watabayashi, C. W. Drescher, M. R. Palomares, M. Daly, P. J. Paley, P. J. Hillard, M. R. Andersen, G. L. Anderson, R. Drapkin, and N. Urban. 2014. "Use of CA125 and HE4 serum markers to predict ovarian cancer in elevated-risk women." Cancer Epidemiol Biomarkers Prev.

Karlsen, M. A., E. V. Høgdall, I. J. Christensen, C. Borgfeldt, G. Kalapotharakos, L. Zdrazilova-Dubska, J. Chovanec, C. A. Lok, A. Stiekema, I. Mutz-Dehbalaie, A. N. Rosenthal, E. K. Moore, B. A. Schodin, W. W. Sumpaico, K. Sundfeldt, B. Kristjansdottir, I. Zapardiel, and C. K. Høgdall. 2015. "A novel diagnostic index combining HE4, CA125 and age may improve triage of women with suspected ovarian cancer - An international multicenter study in women with an ovarian mass." Gynecol Oncol 138 (3):640-6.

Karlsen, M. A., N. Sandhu, C. Høgdall, I. J. Christensen, L. Nedergaard, L. Lundvall, S. A. Engelholm, A. T. Pedersen, D. Hartwell, M. Lydolph, I. A. Laursen, and E. V. Høgdall. 2012. "Evaluation of HE4, CA125, risk of ovarian malignancy algorithm (ROMA) and risk of malignancy index (RMI) as diagnostic tools of epithelial ovarian cancer in patients with a pelvic mass." Gynecol Oncol 127 (2):379-83.

Kaye, S. B., and E. Davies. 1986. "Cyclophosphamide, adriamycin, and cis-platinum for the treatment of advanced granulosa cell tumor, using serum estradiol as a tumor marker." Gynecol Oncol 24 (2):261-4.

Kim, J. H., S. J. Skates, T. Uede, K. K. Wong, J. O. Schorge, C. M. Feltmate, R. S. Berkowitz, D. W. Cramer, and S. C. Mok. 2002. "Osteopontin as a potential diagnostic biomarker for ovarian cancer." JAMA 287 (13):1671-9.

Kirchhoff, C., I. Habben, R. Ivell, and N. Krull. 1991. "A major human epididymis-specific cDNA encodes a protein with sequence homology to extracellular proteinase inhibitors." Biol Reprod 45 (2):350-7.

Kitawaki, J., H. Ishihara, H. Koshiba, M. Kiyomizu, M. Teramoto, Y. Kitaoka, and H. Honjo. 2005. "Usefulness and limits of CA-125 in diagnosis of endometriosis without associated ovarian endometriomas." Hum Reprod 20 (7):1999-2003.

Koshiyama, M., N. Matsumura, and I. Konishi. 2014. "Recent concepts of ovarian carcinogenesis: type I and type II." Biomed Res Int 2014:934261.

Kristjansdottir, B., K. Levan, K. Partheen, and K. Sundfeldt. 2013. "Diagnostic performance of the biomarkers HE4 and CA125 in type I and type II epithelial ovarian cancer." Gynecol Oncol 131 (1):52-8.

Laki, F., Y. M. Kirova, P. This, C. Plancher, B. Asselain, X. Sastre, D. Stoppa-Lyonnet, R. Salmon, and IC-BOCRSG IC-BOCRSG: Institut Curie - Breast Ovary Cancer Risk Study Group. 2007. "Prophylactic salpingo-oophorectomy in a series of 89 women carrying a BRCA1 or a BRCA2 mutation." Cancer 109 (9):1784-90.

Lamy, P. J., C. Plassot, and J. L. Pujol. 2015. "Serum HE4: An Independent Prognostic Factor in Non-Small Cell Lung Cancer." PLoS One 10 (6):e0128836.

Lappöhn, R. E., H. G. Burger, J. Bouma, M. Bangah, M. Krans, and H. W. de Bruijn. 1989. "Inhibin as a marker for granulosa-cell tumors." N Engl J Med 321 (12):790-3.

Lee, W. L., W. H. Chang, K. C. Wang, C. Y. Guo, Y. J. Chou, N. Huang, H. Y. Huang, M. S. Yen, and P. H. Wang. 2015. "The Risk of Epithelial Ovarian Cancer of Women With Endometriosis May be Varied Greatly if Diagnostic Criteria Are Different: A Nationwide Population-Based Cohort Study." Medicine (Baltimore) 94 (39):e1633.

Lennox, G. K., L. R. Eiriksson, C. J. Reade, F. Leung, G. Mojtahedi, E. G. Atenafu, S. E. Ferguson, J. Murphy, E. P. Diamandis, V. Kulasingam, and M. Q. Bernardini. 2015. "Effectiveness of the risk of malignancy index and the risk of ovarian malignancy algorithm in a cohort of women with ovarian cancer: does histotype and stage matter?" Int J Gynecol Cancer 25 (5):809-14.

Lessard-Anderson, C. R., K. S. Handlogten, R. J. Molitor, S. C. Dowdy, W. A. Cliby, A. L. Weaver, J. S. Sauver, and J. N. Bakkum-Gamez. 2014. "Effect of tubal sterilization technique on risk of serous epithelial

ovarian and primary peritoneal carcinoma." Gynecol Oncol 135 (3):423-7.

Levey, A. S., L. A. Stevens, C. H. Schmid, Y. L. Zhang, A. F. Castro, H. I. Feldman, J. W. Kusek, P. Eggers, F. Van Lente, T. Greene, J. Coresh, and CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration). 2009. "A new equation to estimate glomerular filtration rate." Ann Intern Med 150 (9):604-12.

Liao, J. B., Y. Y. Yip, E. M. Swisher, K. Agnew, K. E. Hellstrom, and I. Hellstrom. 2015. "Detection of the HE4 protein in urine as a biomarker for ovarian neoplasms: Clinical correlates." Gynecol Oncol 137 (3):430-5.

Ling, S., M. Ruiqin, Z. Guohong, and W. Ying. 2015. "Expression and prognostic significance of microRNA-451 in human epithelial ovarian cancer." Eur J Gynaecol Oncol 36 (4):463-8.

Long, W. Q., V. Ranchin, P. Pautier, C. Belville, P. Denizot, H. Cailla, C. Lhommé, J. Y. Picard, J. M. Bidart, and R. Rey. 2000. "Detection of minimal levels of serum anti-Müllerian hormone during follow-up of patients with ovarian granulosa cell tumor by means of a highly sensitive enzyme-linked immunosorbent assay." J Clin Endocrinol Metab 85 (2):540-4.

Lowe, KA, C Shah, E Wallace, G Anderson, P Paley, M McIntosh, MR Andersen, N Scholler, L Bergan, J Thorpe, N Urban, and CW Drescher. 2008. "Effects of personal characteristics on serum CA125, mesothelin, and HE4 levels in healthy postmenopausal women at high-risk for ovarian cancer." Cancer Epidemiol Biomarkers Prev 17 (9):2480-7.

Macuks, R., I. Baidekalna, and S. Donina. 2012. "Urinary concentrations of human epididymis secretory protein 4 (He4) in the diagnosis of ovarian cancer: a case--control study." Asian Pac J Cancer Prev 13 (9):4695-8.

Meden, H, and A Fattahi-Meibodi. 1998. "CA 125 in benign gynecological conditions." Int J Biol Markers 13 (4):231-7.

Meindl, A., N. Ditsch, K. Kast, K. Rhiem, and R. K. Schmutzler. 2011. "Hereditary breast and ovarian cancer: new genes, new treatments, new concepts." Dtsch Arztebl Int 108 (19):323-30.

Melin, A., P. Sparén, I. Persson, and A. Bergqvist. 2006. "Endometriosis and the risk of cancer with special emphasis on ovarian cancer." Hum Reprod 21 (5):1237-42.

Menon, U., M. Griffin, and A. Gentry-Maharaj. 2014. "Ovarian cancer screening--current status, future directions." Gynecol Oncol 132 (2):490-5.

Menon, U., S. J. Skates, S. Lewis, A. N. Rosenthal, B. Rufford, K. Sibley, N. Macdonald, A. Dawnay, A. Jeyarajah, R. C. Bast, D. Oram, and I. J. Jacobs. 2005. "Prospective study using the risk of ovarian cancer algorithm to screen for ovarian cancer." J Clin Oncol 23 (31):7919-26.

Merino, M. J., and G. Jaffe. 1993. "Age contrast in ovarian pathology." Cancer 71 (2 Suppl):537-44.

Miller, B. E., B. A. Barron, J. Y. Wan, J. E. Delmore, E. G. Silva, and D. M. Gershenson. 1997. "Prognostic factors in adult granulosa cell tumor of the ovary." Cancer 79 (10):1951-5.

Miller, K. A., J. L. Deaton, and D. E. Pittaway. 1996. "Evaluation of serum CA 125 concentrations as predictors of pregnancy with human in vitro fertilization." Fertil Steril 65 (6):1184-9.

Moore, R. G., A. K. Brown, M. C. Miller, D. Badgwell, Z. Lu, W. J. Allard, C. O. Granai, R. C. Bast, and K. Lu. 2008. "Utility of a novel serum tumor biomarker HE4 in patients with endometrioid adenocarcinoma of the uterus." Gynecol Oncol 110 (2):196-201.

Moore, R. G., M. C. Miller, E. E. Eklund, K. H. Lu, R. C. Bast, and G. Lambert-Messerlian. 2012. "Serum levels of the ovarian cancer biomarker HE4 are decreased in pregnancy and increase with age." Am J Obstet Gynecol 206 (4):349.e1-7.

Moore, R. G., M. C. Miller, M. M. Steinhoff, S. J. Skates, K. H. Lu, G. Lambert-Messerlian, and R. C. Bast. 2012. "Serum HE4 levels are less frequently elevated than CA125 in women with benign gynecologic disorders." Am J Obstet Gynecol 206 (4):351.e1-8.

Moore, RG, AK Brown, MC Miller, S Skates, WJ Allard, T Verch, M Steinhoff, G Messerlian, P DiSilvestro, CO Granai, and RC Jr Bast. 2008. "The use of multiple novel tumor biomarkers for the detection of ovarian carcinoma in patients with a pelvic mass." Gynecol Oncol 108 (2):402-8.

Moore, RG, M Jabre-Raughley, AK Brown, KM Robison, MC Miller, WJ Allard, RJ Kurman, RC Bast, and SJ Skates. 2010. "Comparison of a novel multiple marker assay vs the Risk of Malignancy Index for the prediction of epithelial ovarian cancer in patients with a pelvic mass." Am J Obstet Gynecol.

Moore, RG, DS McMeekin, AK Brown, P DiSilvestro, MC Miller, WJ Allard, W Gajewski, R Kurman, RC Jr Bast, and SJ Skates. 2009. "A novel multiple marker bioassay utilizing HE4 and CA125 for the prediction of ovarian cancer in patients with a pelvic mass." Gynecol Oncol 112 (1):40-6.

Mørch, L. S., E. Løkkegaard, A. H. Andreasen, S. K. Kjaer, and O. Lidegaard. 2012. "Hormone therapy and different ovarian cancers: a national cohort study." Am J Epidemiol 175 (12):1234-42.

Nagy, B., H. P. Bhattoa, Z. Steiber, M. Csobán, M. Szilasi, G. Méhes, M. Müller, J. Lázár, J. Kappelmayer, and P. Antal-Szalmás. 2014. "Serum human epididymis protein 4 (HE4) as a tumor marker in men with lung cancer." Clin Chem Lab Med 52 (11):1639-48.

National Kidney Foundation. 2012. K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification.Am. J. Kidney Dis., 39 (2 Suppl 1) (2002), pp. S1–266

NCCN. Ovarian Cancer Including Fallopian Tube Cancer and Primary Peritoneal Cancer 2016. Available from: http://www.nccn.org/ professionals/physician_gls/pdf/ovarian.pdf.

Noci, I., M. Maggi, R. Biagiotti, A. D'Agata, L. Criscuoli, and M. Marchionni. 1999. "Serum CA-125 values on the day of oocyte retrieval are not predictive of

subsequent pregnancy with in-vitro fertilization." Hum Reprod 14 (7):1773-6.

Nunes, N., G. Ambler, W. L. Hoo, J. Naftalin, X. Foo, M. Widschwendter, and D. Jurkovic. 2013. "A prospective validation of the IOTA logistic regression models (LR1 and LR2) in comparison to subjective pattern recognition for the diagnosis of ovarian cancer." Int J Gynecol Cancer 23 (9):1583-9.

O'Neal, R. L., K. T. Nam, B. J. LaFleur, B. Barlow, K. Nozaki, H. J. Lee, W. H. Kim, H. K. Yang, C. Shi, A. Maitra, E. Montgomery, M. K. Washington, W. El Rifai, R. I. Drapkin, and J. R. Goldenring. 2013. "Human epididymis protein 4 is up-regulated in gastric and pancreatic adenocarcinomas." Hum Pathol 44 (5):734-42.

Ogawa, S., T. Kaku, S. Amada, H. Kobayashi, T. Hirakawa, K. Ariyoshi, T. Kamura, and H. Nakano. 2000. "Ovarian endometriosis associated with ovarian carcinoma: a clinicopathological and immunohistochemical study." Gynecol Oncol 77 (2):298-304.

Palmer, C., X. Duan, S. Hawley, N. Scholler, J. D. Thorpe, R. A. Sahota, M. Q. Wong, A. Wray, L. A. Bergan, C. W. Drescher, M. W. McIntosh, P. O. Brown, B. H. Nelson, and N. Urban. 2008. "Systematic evaluation of candidate blood markers for detecting ovarian cancer." PLoS One 3 (7):e2633.

Park, Y., Y. Kim, E. Y. Lee, J. H. Lee, and H. S. Kim. 2012. "Reference ranges for HE4 and CA125 in a large Asian population by automated assays and diagnostic performances for ovarian cancer." Int J Cancer 130 (5):1136-44.

Park, Y., J. H. Lee, D. J. Hong, E. Y. Lee, and H. S. Kim. 2011. "Diagnostic performances of HE4 and CA125 for the detection of ovarian cancer from patients with various gynecologic and non-gynecologic diseases." Clin Biochem 44 (10-11):884-8.

Partridge, E., A. R. Kreimer, R. T. Greenlee, C. Williams, J. L. Xu, T. R. Church, B. Kessel, C. C. Johnson, J. L. Weissfeld, C. Isaacs, G. L. Andriole, S. Ogden, L. R. Ragard, S. S. Buys, and PLCO Project Team. 2009. "Results from four rounds of ovarian cancer screening in a randomized trial." Obstet Gynecol 113 (4):775-82.

Patton, P. E., C. S. Field, R. W. Harms, and C. B. Coulam. 1986. "CA-125 levels in endometriosis." Fertil Steril 45 (6):770-3.

Pearce, C. L., C. Templeman, M. A. Rossing, A. Lee, A. M. Near, P. M. Webb, C. M. Nagle, J. A. Doherty, K. L. Cushing-Haugen, K. G. Wicklund, J. Chang-Claude, R. Hein, G. Lurie, L. R. Wilkens, M. E. Carney, M. T. Goodman, K. Moysich, S. K. Kjaer, E. Hogdall, A. Jensen, E. L. Goode, B. L. Fridley, M. C. Larson, J. M. Schildkraut, R. T. Palmieri, D. W. Cramer, K. L. Terry, A. F. Vitonis, L. J. Titus, A. Ziogas, W. Brewster, H. Anton-Culver, A. Gentry-Maharaj, S. J. Ramus, A. R. Anderson, D. Brueggmann, P. A. Fasching, S. A. Gayther, D. G. Huntsman, U. Menon, R. B. Ness, M. C. Pike, H. Risch, A. H. Wu, A. Berchuck, and Ovarian Cancer Association Consortium. 2012. "Association between endometriosis and risk of histological subtypes of ovarian cancer: a pooled analysis of case-control studies." Lancet Oncol 13 (4):385-94.

Petraglia, F., S. Luisi, P. Pautier, J. C. Sabourin, R. Rey, C. Lhomme, and J. M. Bidart. 1998. "Inhibin B is the major form of inhibin/activin family secreted by granulosa cell tumors." J Clin Endocrinol Metab 83 (3):1029-32.

Piek, J. M., P. J. van Diest, R. P. Zweemer, J. W. Jansen, R. J. Poort-Keesom, F. H. Menko, J. J. Gille, A. P. Jongsma, G. Pals, P. Kenemans, and R. H. Verheijen. 2001. "Dysplastic changes in prophylactically removed Fallopian tubes of women predisposed to developing ovarian cancer." J Pathol 195 (4):451-6.

Pinsky, P. F., K. Yu, B. S. Kramer, A. Black, S. S. Buys, E. Partridge, J. Gohagan, C. D. Berg, and P. C. Prorok. 2016. "Extended mortality results for ovarian cancer screening in the PLCO trial with median 15years follow-up." Gynecol Oncol 143 (2):270-275.

Pittaway, D. E., and J. A. Fayez. 1987. "Serum CA-125 antigen levels increase during menses." Am J Obstet Gynecol 156 (1):75-6.

Pittaway, DE, and JA Fayez. 1986. "The use of CA-125 in the diagnosis and management of endometriosis." Fertil Steril 46 (5):790-5.

Poorolajal, J., E. Jenabi, and S. Z. Masoumi. 2014. "Body mass index effects on risk of ovarian cancer: a meta-analysis." Asian Pac J Cancer Prev 15 (18):7665-71.

Prat, J., and FIGO Committee on Gynecologic Oncology. 2014. "Staging classification for cancer of the ovary, fallopian tube, and peritoneum." Int J Gynaecol Obstet 124 (1):1-5.

Purdie, D., A. Green, C. Bain, V. Siskind, B. Ward, N. Hacker, M. Quinn, G. Wright, P. Russell, and B. Susil. 1995. "Reproductive and other factors and risk of epithelial ovarian cancer: an Australian case-control study. Survey of Women's Health Study Group." Int J Cancer 62 (6):678-84.

Ranganathan, S., K. J. Simpson, D. C. Shaw, and K. R. Nicholas. 1999. "The whey acidic protein family: a new signature motif and three-dimensional structure by comparative modeling." J Mol Graph Model 17 (2):106-13, 134-6.

Rey, R. A., C. Lhommé, I. Marcillac, N. Lahlou, P. Duvillard, N. Josso, and J. M. Bidart. 1996. "Antimüllerian hormone as a serum marker of granulosa cell tumorsof the ovary: comparative study with serum alpha-inhibin and estradiol." Am J Obstet Gynecol 174 (3):958-65.

Richards, A., U. Herbst, J. Manalang, S. Pather, S. Saidi, T. Tejada-Berges, K. Tan, P. Williams, and J. Carter. 2015. "HE4, CA125, the Risk of Malignancy Algorithm and the Risk of Malignancy Index and complex pelvic masses - a prospective comparison in the pre-operative evaluation of pelvic masses in an Australian population." Aust N Z J Obstet Gynaecol.

Risch, H. A., L. D. Marrett, and G. R. Howe. 1994. "Parity, contraception, infertility, and the risk of epithelial ovarian cancer." Am J Epidemiol 140 (7):585-97.

Rizzuto, I., R. F. Behrens, and L. A. Smith. 2013. "Risk of ovarian cancer in women treated with ovarian stimulating drugs for infertility." Cochrane Database Syst Rev 8:CD008215.

Roberts, V. J., S. Barth, A. el-Roeiy, and S. S. Yen. 1993. "Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle." J Clin Endocrinol Metab 77 (5):1402-10.

Robertson, D. M., N. Cahir, H. G. Burger, P. Mamers, and N. Groome. 1999. "Inhibin forms in serum from postmenopausal women with ovarian cancers." Clin Endocrinol (Oxf) 50 (3):381-6.

Rosen, DG, L Wang, JN Atkinson, Y Yu, KH Lu, EP Diamandis, I Hellstrom, SC Mok, J Liu, and RC Jr Bast. 2005. "Potential markers that complement expression of CA125 in epithelial ovarian cancer." Gynecol Oncol 99 (2):267-77.

Rossi, A., C. Braghin, F. Soldano, M. Isola, V. Capodicasa, A. P. Londero, L. Forzano, and D. Marchesoni. 2011. "A proposal for a new scoring system to evaluate pelvic masses: Pelvic Masses Score (PMS)." Eur J Obstet Gynecol Reprod Biol 157 (1):84-8.

Rossing, M. A., K. L. Cushing-Haugen, K. G. Wicklund, J. A. Doherty, and N. S. Weiss. 2008. "Risk of epithelial ovarian cancer in relation to benign ovarian conditions and ovarian surgery." Cancer Causes Control 19 (10):1357-64.

Rustin, G. J., M. E. van der Burg, C. L. Griffin, D. Guthrie, A. Lamont, G. C. Jayson, G. Kristensen, C. Mediola, C. Coens, W. Qian, M. K. Parmar, A. M. Swart, MRC OV05, and EORTC 55955 investigators. 2010. "Early versus delayed treatment of relapsed ovarian cancer (MRC OV05/EORTC 55955): a randomised trial." Lancet 376 (9747):1155-63.

Sandhu, N., M. A. Karlsen, C. Høgdall, I. A. Laursen, I. J. Christensen, and E. V. Høgdall. 2014. "Stability of HE4 and CA125 in blood samples from patients diagnosed with ovarian cancer." Scand J Clin Lab Invest 74 (6):477-84.

Sato, S., Y. Yokoyama, T. Sakamoto, M. Futagami, and Y. Saito. 2000. "Usefulness of mass screening for ovarian carcinoma using transvaginal ultrasonography." Cancer 89 (3):582-8.

Sayasneh, A., L. Wynants, J. Preisler, J. Kaijser, S. Johnson, C. Stalder, R. Husicka, Y. Abdallah, F. Raslan, A. Drought, A. A. Smith, S. Ghaem-Maghami, E. Epstein, B. Van Calster, D. Timmerman, and T. Bourne. 2013. "Multicentre external validation of IOTA prediction models and RMI by operators with varied training." Br J Cancer 108 (12):2448-54.

Schildkraut, J. M., P. J. Schwingl, E. Bastos, A. Evanoff, and C. Hughes. 1996. "Epithelial ovarian cancer risk among women with polycystic ovary syndrome." Obstet Gynecol 88 (4 Pt 1):554-9.

Schumer, S. T., and S. A. Cannistra. 2003. "Granulosa cell tumor of the ovary." J Clin Oncol 21 (6):1180-9.

Schummer, M, WV Ng, RE Bumgarner, PS Nelson, B Schummer, DW Bednarski, L Hassell, RL Baldwin, BY Karlan, and L Hood. 1999. "Comparative hybridization of an array of 21,500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas." Gene 238 (2):375-85.

Segal, R., A. D. DePetrillo, and G. Thomas. 1995. "Clinical review of adult granulosa cell tumors of the ovary." Gynecol Oncol 56 (3):338-44.

Shah, S. P., M. Köbel, J. Senz, R. D. Morin, B. A. Clarke, K. C. Wiegand, G. Leung, A. Zayed, E. Mehl, S. E. Kalloger, M. Sun, R. Giuliany, E. Yorida, S. Jones, R. Varhol, K. D. Swenerton, D. Miller, P. B. Clement, C. Crane, J. Madore, D. Provencher, P. Leung, A. DeFazio, J. Khattra, G. Turashvili, Y. Zhao, T. Zeng, J. N. Glover, B. Vanderhyden, C. Zhao, C. A. Parkinson, M. Jimenez-Linan, D. D. Bowtell, A. M. Mes-Masson, J. D. Brenton, S. A. Aparicio, N. Boyd, M. Hirst, C. B. Gilks, M. Marra, and D. G. Huntsman. 2009. "Mutation of FOXL2 in granulosa-cell tumors of the ovary." N Engl J Med 360 (26):2719-29.

Skates, S. J., U. Menon, N. MacDonald, A. N. Rosenthal, D. H. Oram, R. C. Knapp, and I. J. Jacobs. 2003. "Calculation of the risk of ovarian cancer from serial CA-125 values for preclinical detection in postmenopausal women." J Clin Oncol 21 (10 Suppl):206s-210s.

Stine, J. E., A. Suri, P. A. Gehrig, M. Chiu, B. K. Erickson, W. K. Huh, J. T. Soper, L. Van Le, M. G. Conner, and K. H. Kim. 2013. "Pre-operative imaging with CA125 is a poor predictor for granulosa cell tumors." Gynecol Oncol 131 (1):59-62.

Stukan, M., M. Dudziak, K. Ratajczak, and J. P. Grabowski. 2015. "Usefulness of diagnostic indices comprising clinical, sonographic, and biomarker data for discriminating benign from malignant ovarian masses." J Ultrasound Med 34 (2):207-17.

Terry, K. L., H. Schock, R. T. Fortner, A. Hüsing, R. N. Fichorova, H. S. Yamamoto, A. F. Vitonis, T. Johnson, K. Overvad, A. Tjønneland, M. C. Boutron-Ruault, S. Mesrine, G. Severi, L. Dossus, S. Rinaldi, H. Boeing, V. Benetou, P. Lagiou, A. Trichopoulou, V. Krogh, E. Kuhn, S. Panico, H. B. Bueno-de-Mesquita, N. C. Onland-Moret, P. H. Peeters, I. T. Gram, E. Weiderpass, E. J. Duell, M. J. Sanchez, E. Ardanaz, N. Etxezarreta, C. Navarro, A. Idahl, E. Lundin, K. Jirström, J. Manjer, N. J. Wareham, K. T. Khaw, K. Smith Byrne, R. C. Travis, M. J. Gunter, M. A. Merritt, E. Riboli, D. Cramer, and R. Kaaks. 2016. "A prospective evaluation of early detection biomarkers for ovarian cancer in the European EPIC cohort." Clin Cancer Res. 22(18):4664-75.

Timmerman, D., L. Ameye, D. Fischerova, E. Epstein, G. B. Melis, S. Guerriero, C. Van Holsbeke, L. Savelli, R. Fruscio, A. A. Lissoni, A. C. Testa, J. Veldman, I. Vergote, S. Van Huffel, T. Bourne, and L. Valentin. 2010. "Simple ultrasound rules to distinguish between benign and malignant adnexal masses before surgery: prospective validation by IOTA group." BMJ 341:c6839.

Timmerman, D., A. C. Testa, T. Bourne, L. Ameye, D. Jurkovic, C. Van Holsbeke, D. Paladini, B. Van Calster, I. Vergote, S. Van Huffel, and L. Valentin. 2008. "Simple ultrasound-based rules for the diagnosis of ovarian cancer." Ultrasound Obstet Gynecol 31 (6):681-90.

Timmerman, D., A. C. Testa, T. Bourne, E. Ferrazzi, L. Ameye, M. L. Konstantinovic, B. Van Calster, W. P. Collins, I. Vergote, S. Van Huffel, L. Valentin, and International Ovarian Tumor Analysis Group. 2005. "Logistic regression model to distinguish between the benign and malignant adnexal mass before surgery: a multicenter study by the International Ovarian Tumor Analysis Group." J Clin Oncol 23 (34):8794-801.

Timmerman, D., B. Van Calster, A. C. Testa, S. Guerriero, D. Fischerova, A. A. Lissoni, C. Van Holsbeke, R. Fruscio, A. Czekierdowski, D. Jurkovic, L. Savelli, I. Vergote, T. Bourne, S. Van Huffel, and L. Valentin. 2010. "Ovarian cancer prediction in adnexal masses using ultrasound-based logistic regression models: a temporal and external validation study by the IOTA group." Ultrasound Obstet Gynecol 36 (2):226-34.

Tokuishi, K., S. Yamashita, K. Ohbo, and K. Kawahara. 2012. Tumour Biol 33 (1):103-9. Splice variant HE4-V3 expression is associated with favorable prognosis in pulmonary adenocarcinoma.

Underwood J.C.E. General and Systemathic Pathology. 1996. Second edition. Churchill Livingstone

Unkila-Kallio, L, A Tiitinen, H Alfthan, P Vuorela, U Stenman, and O Ylikorkala. 2000. "Effect of an in vitro fertilization program on serum CA 125, tumor-associated trypsin inhibitor, free beta-subunit of human chorionic gonadotropin, and common alpha-subunit of glycoprotein hormones." Fertil Steril 74 (6):1125-32.

Urban, and Nicole. 1997. "Use of a stochastic simulation model to identify an efficient protocol for ovarian cancer screening." Controlled Clinical Trials 18:251-270.

Urbancsek, J, EE Hauzman, A Murber, AR Lagarde, T Rabe, Z Papp, and T Strowitzki. 2005. "Serum CA-125 and inhibin B levels in the prediction of ovarian response to gonadotropin stimulation in in vitro fertilization cycles." Gynecol Endocrinol 21 (1):38-44.

Valentin, L., D. Jurkovic, B. Van Calster, A. Testa, C. Van Holsbeke, T. Bourne, I. Vergote, S. Van Huffel, and D. Timmerman. 2009. "Adding a single CA 125 measurement to ultrasound imaging performed by an experienced examiner does not improve preoperative discrimination between benign and malignant adnexal masses." Ultrasound Obstet Gynecol 34 (3):345-54.

Van Gorp, T., J. Veldman, B. Van Calster, I. Cadron, K. Leunen, F. Amant, D. Timmerman, and I. Vergote. 2012. "Subjective assessment by ultrasound is superior to the risk of malignancy index (RMI) or the risk of ovarian malignancy algorithm (ROMA) in discriminating benign from malignant adnexal masses." Eur J Cancer 48 (11):1649-56.

Van Holsbeke, C., B. Van Calster, T. Bourne, S. Ajossa, A. C. Testa, S. Guerriero, R. Fruscio, A. A. Lissoni, A. Czekierdowski, L. Savelli, S. Van Huffel, L. Valentin, and D. Timmerman. 2012. "External validation of diagnostic models to estimate the risk of malignancy in adnexal masses." Clin Cancer Res 18 (3):815-25.

van Nagell, JR Jr, PD DePriest, MB Reedy, HH Gallion, FR Ueland, EJ Pavlik, and RJ Kryscio. 2000. "The efficacy of transvaginal sonographic screening in asymptomatic women at risk for ovarian cancer." Gynecol Oncol 77 (3):350-6.

Vang, R., I.M Shih, and R. J. Kurman. 2009. "Ovarian low-grade and high-grade serous carcinoma: pathogenesis, clinicopathologic and molecular biologic features, and diagnostic problems." Adv Anat Pathol 16 (5):267-82.

Vitonis, A. F., L. Titus-Ernstoff, and D. W. Cramer. 2011. "Assessing ovarian cancer risk when considering elective oophorectomy at the time of hysterectomy." Obstet Gynecol 117 (5):1042-50.

Vlahos, N. F., K. P. Economopoulos, and G. Creatsas. 2010. "Fertility drugs and ovarian cancer risk: a critical review of the literature." Ann N Y Acad Sci 1205:214-9.

Wang, S., R. Akbas, and U. Demirci. 2015. "Microchip ELISA coupled with cell phone to detect ovarian cancer HE4 biomarker in urine." Methods Mol Biol 1256:111-21.

Wilson, M. K., P. Fong, S. Mesnage, K. Chrystal, A. Shelling, K. Payne, H. Mackay, L. Wang, S. Laframboise, M. Rouzbahman, W. Levin, and A. M. Oza. 2015. "Stage I granulosa cell tumours: A management conundrum? Results of long-term follow up." Gynecol Oncol 138 (2):285-91.

Wittenberg, J., L. S. Cook, M. A. Rossing, N. S. Weiss, and L. Wittenberg. 1999. "Reproductive risk factors for mucinous and non-mucinous epithelial ovarian cancer." Epidemiology 10 (6):761-3.

Wong, K. K., R. S. Cheng, and S. C. Mok. 2001. "Identification of differentially expressed genes from ovarian cancer cells by MICROMAX cDNA microarray system." Biotechniques 30 (3):670-5.

Woodruff, T. K., J. D'Agostino, N. B. Schwartz, and K. E. Mayo. 1988. "Dynamic changes in inhibin messenger RNAs in rat ovarian follicles during the reproductive cycle." Science 239 (4845):1296-9.

Xi, Z., M. LinLin, and T. Ye. 2009. "Human epididymis protein 4 is a biomarker for transitional cell carcinoma in the urinary system." J Clin Lab Anal 23 (6):357-61.

Zhang, Z., R. C. Bast, Y. Yu, J. Li, L. J. Sokoll, A. J. Rai, J. M. Rosenzweig, B. Cameron, Y. Y. Wang, X. Y. Meng, A. Berchuck, C. Van Haaften-Day, N. F. Hacker, H. W. de Bruijn, A. G. van der Zee, I. J. Jacobs, E. T. Fung, and D. W. Chan. 2004. "Three biomarkers identified from serum proteomic analysis for the detection of early stage ovarian cancer." Cancer Res 64 (16):5882-90.

Zhou, B., Q. Sun, R. Cong, H. Gu, N. Tang, L. Yang, and B. Wang. 2008. "Hormone replacement therapy and ovarian cancer risk: a meta-analysis." Gynecol Oncol 108 (3):641-51.