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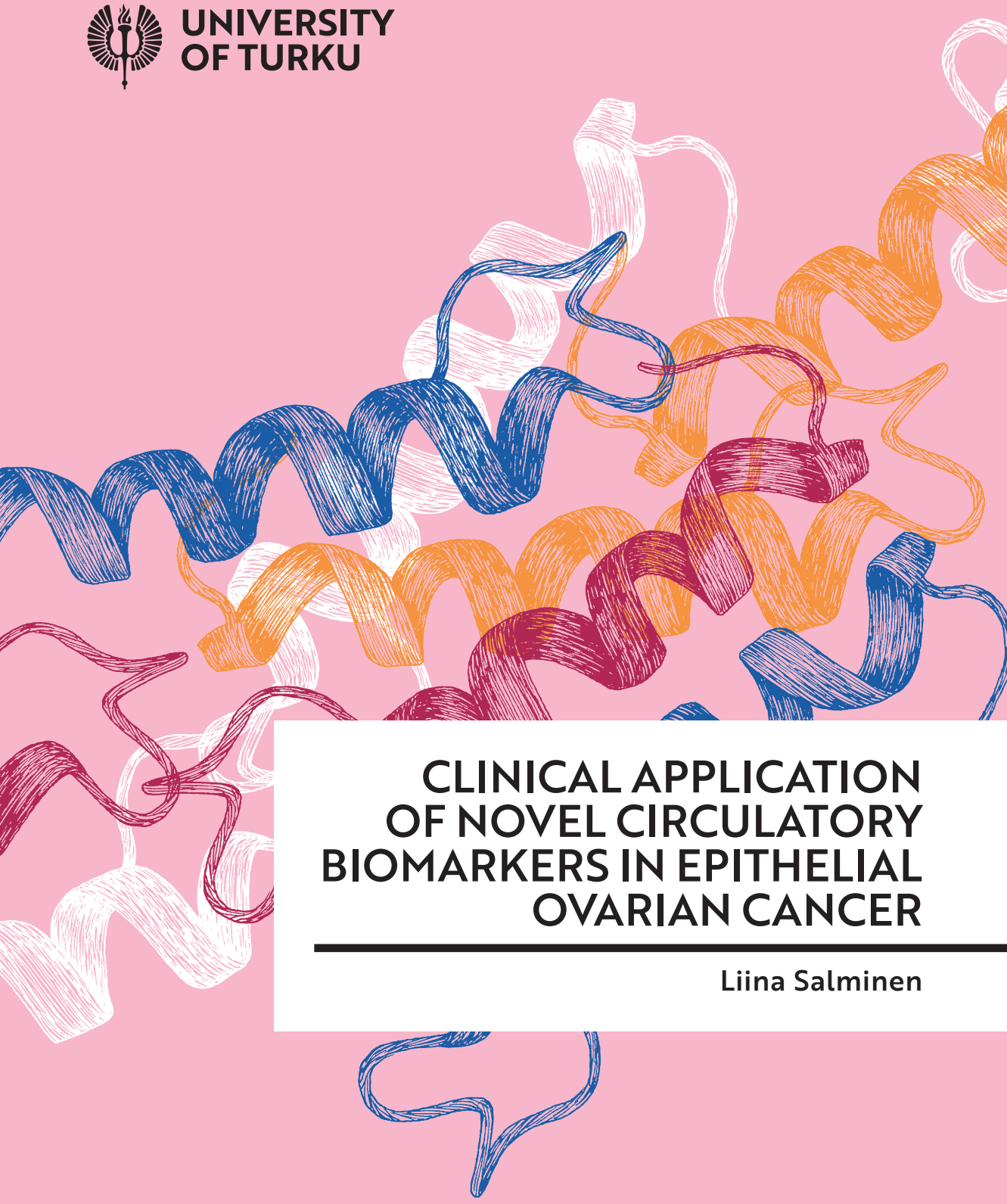

CLINICAL APPLICATION OF NOVEL CIRCULATORY BIOMARKERS IN EPITHELIAL OVARIAN CANCER

Liina Salminen

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To Women

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LIINA SALMINEN: Clinical Application of Novel Circulatory Biomarkers in Epithelial Ovarian Cancer
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ABSTRACT

Epithelial ovarian cancer (EOC) encompasses a heterogenous group of malignancies with a poor overall survival rate. The two root problems behind the poor survival of patients are the lack of precise enough biomarkers to enable screening and early detection of the disease and the development of a chemotherapy resistant, fatal disease.

Cancer antigen 125 (CA125) is currently the only biomarker validated and widely adopted in the diagnosis, treatment monitoring and follow-up of EOC. However, CA125 is not the ideal biomarker, as it is non-specific for EOC and does not reliably express changes in tumor load. In the current study, the feasibility of four novel biomarkers were investigated: CA125-STn and -MGL, human epididymis protein 4 (HE4) and circulating tumor DNA (ctDNA).

This prospective study included 253 women with histologically confirmed EOC, 317 women with benign gynecological diseases and 36 healthy controls. Both CA125-STn and -MGL differentiated EOC from benign diseases with improved specificity compared to conventional CA125. In the longitudinal analyses, HE4, CA125-STn and -MGL, contrarily to CA125, showed good correlation with tumor burden. In addition, HE4 at the time of progression predicted the survival of patients. The longitudinal mutation tracking of plasma ctDNA revealed dynamic, actionable mutations during EOC treatment and follow-up.

CA125-STn and -MGL are EOC-specific biomarkers that showed, similar to HE4, good prognostic potential. The CA125 glycoform assays utilize a robust and affordable measurement technique, which makes them feasible biomarkers also in the clinical setting. Based on the current study, HE4 is an indicator of disease aggressiveness and might be a potential tool in the selection of targeted second line treatments. Similarly, the ctDNA analyses revealed actionable mutations enabling the individual treatment of selected HGSC patients. Overall, these novel biomarkers represent state of the art approaches in the diagnosis, treatment monitoring and follow-up of EOC.

KEYWORDS: Epithelial ovarian cancer, cancer antigen 125, glycoforms, human epididymis protein 4, ctDNA

TURUN YLIOPISTO,
Lääketieteellinen tiedekunta
Synnytys- ja Naistentautioppi
Gynekologinen onkologia
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TIIVISTELMÄ

Epiteliaaliset munasarjasyövät ovat sekalainen ryhmä pahanlaatuisia kasvainsairauksia, joita yhdistää huono selviytymisennuste. Kaksi keskeistä syytä potilaiden huonoon selviytymiseen ovat puute aikaisen diagnostiikan ja seulonnan mahdollistavista merkkiaineista ja hoitoresistentin, loppuvaiheen sairauden kehittyminen.

Cancer antigen 125 (CA125) on tällä hetkellä ainoa validoitu ja laajalti käytetty merkkiaine munasarjasyövän diagnostiikassa, hoitovasteen arvioinnissa ja seurannassa. CA125 ei kuitenkaan ole kyllin hyvä merkkiaine, koska se ei ole spesifinen munasarjasyövälle eikä se luotettavasti osoita muutoksia kasvaintaakassa. Tässä tutkimuksessa selvitettiin neljän uuden kasvainmerkkianeen käytettävyyttä munasarjasyövässä: CA125-STn ja -MGL, human epididymis protein 4 (HE4) ja circulating tumor DNA (ctDNA).

Tutkimuskohortti koostui 253:sta munasarjasyöpää sairastavasta naisesta ja 317:sta naisesta, joilla oli hyvänlaatuinen gynekologinen sairaus. Verrokkiryhmään kuului myös terveitä naisia. Sekä CA125-STn, että -MGL erottivat munasarjasyövän hyvänlaatuisista sairauksista paremmalla tarkkuudella kuin CA125. Pitkittäisanalyyseissä HE4, CA125-STn ja MGL osoittivat hyvää yhteyttä tautitaakkaan, toisin kuin CA125. Lisäksi HE4-taso taudin uusimishetkellä ennusti potilaiden selviytymisaikaa. Pitkittäinen ctDNA-mutaatioprofiilin määrittäminen paljasti hoidon ja seurannan aikaisia, dynaamisia mutaatiota.

CA125-STn ja -MGL ovat munasarjasyöpäspesifejä kasvainmerkkiaineita, jotka osoittivat, samoin kuin HE4, ennusteellista hyötyä munasarjasyövän seurannassa. CA125-STn ja -MGL -paneelit hyödyntävät vakaata ja edullista analysointitekniikkaa, minkä ansiosta ne ovat myös kliiniseltä käytettävyydeltään hyviä. Tämän tutkimuksen perusteella HE4 vaikuttaisi kertovan potilaan munasarjasyövän aggressiivisuudesta ja HE4-määritys saattaa siksi olla mielekäs työkalu yksilöllisten, toisen linjan hoitojen valitsemisessa. Samankaltaisesti, ctDNA-analyysi paljasti kohteita täsmähoidoille valituissa munasarjasyöpäpotilaissa.

AVAINSANAT: Epiteliaalinen munasarjasyöpä, CA125, glykoformit, ctDNA, HE4

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Abbreviations

ARID1A	AT-rich interaction domain 1A
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRCA1/2	Breast cancer genes 1 and 2
CA125	Cancer antigen 125
CADD	Combined Annotation Dependent Depletion
cfDNA	Circulating free DNA
CNA	Copy number alteration
CNV	Copy number variation
COSMIC	The Catalogue of Somatic Mutations in Cancer
CPH I	Copenhagen Index
CT	Computer tomography
ctDNA	Circulating tumor DNA
dbSNP	The Single Nucleotide Polymorphism Database
DNA	Deoxyribonucleic acid
EOC	Epithelial ovarian cancer
FIGO	International Federation of Gynecology and Obstetrics
HE4	Human epididymis protein 4
HGSC	High grade serous ovarian cancer
HR	Homologous recombination
IDS	Interval debulking surgery
KRAS	Kirsten rat sarcoma viral oncogene homolog
LCA	Lens culinaris agglutinin
LGSC	Low grade serous ovarian carcinoma
MAF	Mutant allele frequency
MGL	Macrophage galactose-type lectin
MIS	Minimally invasive surgery
MMR	Mismatch repair
MRD	Minimal residual disease
NACT	Neoadjuvant chemotherapy
OS	Overall survival
PARPi	Poly (ADP-ribose) polymerase inhibitor
PDS	Primary debulking surgery

PFS	Progression free survival
PI3KCA	PIK3 catalytic subunit alpha
PPP2R1A	protein phosphatase 2 scaffold subunit alpha
PTEN	phosphatase and tensin homolog
RECIST	Response Evaluation Criteria in Solid Tumors
RMI	Risk Malignancy Index
RNA	Ribonucleic acid
ROMA	Risk of Ovarian Malignancy Algorithm
SPP	Survival post progression
STn	Sialyl-Thomsen-nouveau
TP53	Tumor protein 53
TVS	Transvaginal sonography
VAF	Variant allele frequency

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Salminen L, Nadeem N, Rolfsen AL, Dørum A, Laajala TD, Grènman S, Hietanen S, Heinosalo T, Perheentupa A, Poutanen M, Bolstad N, Carpén O, Lamminmäki U, Pettersson K, Hynninen J, Huhtinen K. Exploratory analysis of CA125-MGL and -STn glycoforms in the differential diagnostics of pelvic masses. Journal of Applied Laboratory Medicine, 2020 Mar; 5(2): 263–272.

II Salminen L. Nadeem N, Jain S, Grènman S, Carpén O, Hietanen S, Oksa S, Lamminmäki U, Pettersson K, Gidwani K, Huhtinen K, Hynninen J. A longitudinal analysis of CA125 glycoforms in the monitoring and follow-up of high grade serous ovarian cancer. Gynecologic Oncology, 2020 Mar; 156 (3): 689–694.

III Salminen L, Gidwani K, Grènman S, Carpén O, Hietanen S, Pettersson K, Huhtinen K, Hynninen J. HE4 is a prognostic indicator of relapsed high grade serous ovarian carcinoma. Submitted.

IV Oikkonen J, Zhang K, Salminen L, Schulman I, Lavikka K, Andersson N, Ojanperä E, Hietanen S, Grènman S, Lehtonen R, Huhtinen K, Carpén O, Hynninen J, Färkkilä A, Hautaniemi S. Prospective Longitudinal ctDNA Workflow Reveals Clinically Actionable Alterations in Ovarian Cancer. JCO Precision Oncology, 2019 May; 3: 1–12.

1 Introduction

Approximately 295 000 women worldwide, of which 550 women in Finland, received the diagnosis of epithelial ovarian cancer in 2018 (Finnish Cancer Registry, 2018; Bray et al., 2018). EOC is an aggressive malignancy with an unfavorable outcome, as the 5-year survival of patients falters at 44% (Finnish Cancer Registry, 2018). Early diagnosis has proven to be an uphill struggle, and the meager survival rate is mainly due to extensive tumor load at the time of diagnosis. At present, ovarian cancer, alongside cervical cancer, has the highest mortality among patients with gynecological malignancies in developed countries (Bray et al., 2018).

The mainstay of EOC treatment is aggressive cytoreductive surgery with subsequent platinum-based chemotherapy (Benedet et al., 2000). Even though primary, therapy-naïve EOC responds initially well to treatment, most patients eventually develop a treatment-resistant refractory disease, which is another major cause of lethality. This thesis focuses on novel, putative biomarkers in the early diagnostics, treatment monitoring and follow-up of EOC.

Currently, the cancer antigen 125 (CA125) is the gold standard of biomarkers in the diagnosis, treatment monitoring and follow-up of patients with EOC. However, CA125 has several limitations: 1) the serum level of CA125 is elevated in only 50% of early stage EOCs, 2) the correlation of CA125 to tumor volume is questionable, as small amounts of residual disease are present in up to 50% of patients regardless of CA125 normalization and 3) serum CA125 is elevated in various benign diseases and non-EOC malignancies (Bast et al., 2005). Thus, it has become evident that better diagnostic and prognostic EOC biomarkers are needed. Human epididymis protein 4 (HE4) was recently approved as an auxiliary biomarker to CA125 in the diagnosis of EOC. However, HE4 has its own limitations and it is still under debate whether HE4 improves the detection of early stage EOC (Jacob et al., 2011; Kristjansdottir et al., 2013). In addition, the routine measurement of HE4 in EOC treatment monitoring and follow-up is currently not recommended as clinical data on the subject is insufficient (Colombo et al., 2019).

In this study, we prospectively evaluated the diagnostic and prognostic potential of two cancer-specific CA125 glycoform assays (CA125-MGL and -STn) in patients with EOC. These two assays have shown good diagnostic potential in preclinical studies, which made them putative candidates for exploratory clinical trials (Gidwani

et al., 2016, 2019). Longitudinal analyses with the glycoform and HE4 assays were done and the correlation of the biomarkers to tumor burden and their ability to detect recurrent disease was assessed.

The analysis of circulating tumor DNA (ctDNA) represents a non-invasive, state of art approach in EOC diagnostics, prognosis assessment and real-time evaluation of treatment resistance (Barlebo Ahlborn and Østrup, 2019; Zhao, Cardenas and Matei, 2019). CtDNA analysis could be especially valuable in situations when conventional tissue biopsies might not represent the tumor in a comprehensive manner, e.g. heavily pretreated patients or patients with rapidly growing, necrotic tumors. We examined the feasibility of ctDNA mutation tracking in a prospective study and carefully assessed the utility of plasma ctDNA in the real-time detection of actionable mutations during EOC treatment.

2 Review of the Literature

2.1 Epithelial Ovarian Cancer (EOC)

2.1.1 History of EOC Research: From the "Silent Killer" to the "Whispering Disease"

The natural history of ovarian cancer was described for the first time in 1873 by Thomas Spencer Wells, an English physician (Wells, 1873). He presented that ovarian malignancies arise from ovarian surface epithelium, and the theory was further investigated by Wilfred Shaw in 1923; 'The general form of a malignant papillomatous serous cyst implies a development from a papillomatous serous cyst of the ovary... I have been unable to demonstrate histological evidence of any such transition' (Shaw, 1932). Although Dr. Shaw was not able to prove the theory of malignant transformation, it remained the leading theory of ovarian cancer pathogenesis for decades and has since been proven correct in some subtypes of ovarian cancers (Prat, D'Angelo and Espinosa, 2018).

The metaphor of ovarian cancer as the "silent killer" set root promptly due to the apparent lack of symptoms in early stages of disease progression. In the mid twentieth century, ovarian cancer was a recognized disease, but no hope for cure was offered. Researchers mainly focused on the development of palliative methods, such as the alkylating agent hemisulfide mustard, to ease the obstructive symptoms caused by the accumulation of ascites fluid (Seligman et al., 1952). It was decades later that the disease caught the attention of the scientific community at a larger scale, as its incidence appeared to be on the rise: in the 1960s 1 in 100 women was estimated to develop ovarian cancer, while the risk of developing the disease was reported to have risen to 1 in 70 women by the 1980s (Jasen, 2009). Consequently, studies on the long-neglected epidemiology of ovarian cancer were conducted, and emerging evidence of women with increased risk for ovarian cancer was laid out, specifically, low parity and hereditary factors (Jasen, 2009). Simultaneously, the initial study on a platinum-based agent, cisplatin, in the treatment of ovarian cancer was conducted with promising results: 9/34 patients responded to therapy (Wiltshaw and Kroner,

1976), and the results introduced the modern era of ovarian cancer chemotherapy (Steven Piver, 2006). Advancement in the diagnostic field was achieved, as the development of a murine monoclonal antibody in 1979 lead to the development of the cancer antigen 125 (CA125) biomarker assay in 1983 (Bast et al., 1983). The subsequent improvement in diagnostics and increasing opportunities for supportive care enabled a more radical treatment approach, which resulted in improved survival outcomes (Barber, 1986). Recently, the Human Genome Project and further genomic profiling has shifted the scientific thought of ovarian cancer research beyond an "one size fits all" solution (Previs et al., 2017). Generally speaking, the scientific advances of the last century have shifted the image of ovarian cancer from an incurable killer to a manageable disease.

2.1.2 Epidemiology and Etiology

Ovarian cancer is the seventh most common cancer in women worldwide with approximately 295 000 new cases every year (Bray et al., 2018). In Finland, ovarian cancer is the eight most common cancer in women with circa 550 new cases annually (Finnish Cancer Registry, 2018). The incidence of ovarian cancer is declining in developed countries but rising in developing countries, mostly due to increasing life-expectancy (Ferlay et al., 2013). Annually, circa 185 000 women die of ovarian cancer and it is the most lethal gynecological cancer, alongside cervical cancer, in developed countries (age-standardized mortality: 3.8 per 100 000 person-years in high/very-high human development index regions and 4.8 per 100 000 person-years in Finland) (Finnish Cancer Registry, 2018; Bray et al., 2018). Despite the advances in cancer treatment, the 5-year survival rate of ovarian cancer patients falters at 44% as most patients are diagnosed with advanced disease (International Federation of Gynecology and Obstetrics, FIGO, stage III–IV) (Finnish Cancer Registry, 2018). Ovarian cancer is rare in patients < 40 years of age and the majority of ovarian malignancies in young patients are germ cell tumors (Webb et al., 2017). Contrarily, EOC accounts for most of the ovarian cancers in older age groups (Webb et al., 2017).

EOC is a group of heterogenous diseases; consequently, the risk factors vary between the histological subtypes. However, several common risk factors have been determined with various robustness, ranging from established to unlikely risk factors (Table 1). The majority of EOCs are sporadic, but a hereditary predisposition to EOC is well established (Malander et al., 2006; Wentzensen, Poole and Trabert, 2016; Andrews and Mutch, 2017; Webb et al., 2017). Specifically, breast cancer genes 1 and 2 (BRCA1 and BRCA2) mutations causing the breast and ovarian cancer syndrome (14% of EOCs and 17% of HGSC) and in a lesser extent mutations in the mismatch repair genes (MMR-genes) resulting in Lynch syndrome (2% of EOCs) (Malander et al., 2006; Andrews and Mutch, 2017). Women with Lynch syndrome generally

develop EOC at a younger age than women with sporadic EOC, with a predisposition to endometroid and clear cell carcinomas (Helder-Woolderink et al., 2016). Further, women with a history of EOC in the family have an elevated risk of developing EOC regardless of their genetic status (Andrews and Mutch, 2017). Other well-established risk factors are endometriosis, smoking, hormone replacement therapy (HRT) and obesity (Heidemann et al., 2014; Webb et al., 2017). Smoking has been specifically associated with and increased risk of developing mucinous carcinoma (relative risk 1.78; 95% CI 1.52–2.07) (Santucci et al., 2019). In contrast, the continuous use of estrogen only HRT for over 5 years has shown to increase the risk of serous carcinoma, but decreased the risk of mucinous carcinoma (Koskela-Niska et al., 2013). The use of oral contraceptives, pregnancy and tubal ligation have been shown to decrease the risk of EOC (Webb et al., 2017). Women with a history of oral contraceptive use for over 10 years had a 36% to 49% reduction in the risk of developing serous, endometroid or clear cell carcinomas in a study by Wentzensen et al (Wentzensen, Poole and Trabert, 2016). The strongest protective association of pregnancy to EOC was detected in clear cell carcinomas (relative risk 0.35; 95% CI 0.27–0.47), while the risk of developing serous carcinoma was least reduced by pregnancy (relative risk 0.81; 95% CI 0.73–0.90) (Wentzensen, Poole and Trabert, 2016).

Table 1. Risk and protective factors of EOC. Modified from (Webb et al., 2017).

Association	Increased risk	Decreased risk
Established	Family history of EOC	Pregnancy > 6 months
	Endometriosis	Oral contraceptive use
	Smoking	Tubal ligation
	Estrogen only HRT	
	Greater height	
	Obesity	
Probable	Older age at menopause	Breastfeeding
Possible	Younger age at menarche	Older age at last birth
	Combination HRT	Aspirin
	Pelvic inflammatory disease	Vitamin D
	Diabetes mellitus	
	Talc (genital use)	
Unlikely or insufficient evidence	Infertility treatment, hysterectomy without oophorectomy, PCOS, fibroids or ovarian cysts, alcohol intake, diet, physical activity	

HRT: hormone replacement therapy, PCOS: polycystic ovarian syndrome

2.1.3 Pathogenesis

2.1.3.1 Histological Classification

EOC is divided into four distinct cell types based on the normal epithelia in the female reproductive organs: endometroid, clear cell, mucinous and serous (Koshiyama, Matsumura and Konishi, 2014, 2017; Prat, D'Angelo and Espinosa, 2018). It is evident that EOCs are tumors with different clinicopathological and genetic features. Thus, a dualistic model has been established in the histological classification of EOC subtypes into type 1 and type 2 tumors (Kurman and Shih, 2016). Type 1 tumors generally develop from precursory benign ovarian lesions in an adenoma-carcinoma continuum, they are often confined to the ovary and pelvis and are genetically stabile (Kurman and Shih, 2016). Type 1 tumors include endometroid, clear cell, mucinous and low grade serous carcinomas (Kurman and Shih, 2016). It is recognized that endometroid and clear cell carcinomas generally develop from atypical endometriosis, while low grade serous carcinoma evolve from serous borderline tumors in a stepwise fashion (Prat, D'Angelo and Espinosa, 2018). The origin of mucinous carcinoma is incompletely understood, but it is suspected that it develops from benign cystadenomas or mucinous borderline tumors (Prat, D'Angelo and Espinosa, 2018). Type 2 tumors are genetically instable and metastasize aggressively (Kurman and Shih, 2016). There is increasing evidence that type 2 tumors origin from malignant precursory lesions in the distal fallopian tube and/or the ovary i.e. serous tubal intraepithelial carcinoma (STIC) (Piek, Kenemans and Verheijen, 2004). Interestingly, a recent study on the origin of HGSC reported that the ancestral clone of the subsequently observed HGSC was identifiable in the STIC lesion of the patient, further illuminating the origin of HGSC (Labidi-Galy et al., 2017). HGSC is classified as a type 2 tumor (Kurman and Shih, 2016).

2.1.3.2 Molecular Features

In addition to the distinct histological features of the EOC subtypes, the divergent molecular genomics of the diseases have been illuminated in recent years. Molecular tools, such as deep sequencing, ribonucleic acid (RNA) sequencing, epigenomics and proteomics have made it apparent that the molecular heterogeneity of EOC explains, to certain extent, the poor survival outcomes of patients (Kossaï et al., 2018). The main molecular features of the histological subtypes are presented in Table 2.

The large scale genomic analysis of HGSC shows mutations in tumor protein 53(TP53) in 96% and BRCA1/2 mutations in 22% of the tumor samples (The Cancer Genome Atlas Research Network, 2011). In addition, six more significantly mutated genes have been described in 2–6% of HGSC tissue samples (CSMD3, NF1, CDK12,

FAT3, GABRA6, RB1) (The Cancer Genome Atlas Research Network, 2011). Genetic and epigenetic alterations in homologous recombination (HR) pathways are present in up to 50% of HGSCs (Konstantinopoulos et al., 2015). In addition, a high number of copy number alterations (CNAs) has been linked to HGSC, which might, to some extent, be due to the high prevalence of alterations in the HR- pathways (The Cancer Genome Atlas Research Network, 2011; Konstantinopoulos et al., 2015). HGSC immunohistochemistry is positive for CK7, PAX8 and WT, and negative for CK20 (Kossaï et al., 2018)(Table 2).

A low grade serous carcinoma (LGSC) is morphologically similar to HGSC, however, it is characterized by genomic stability and no TP53 mutations (Kossaï et al., 2018). The mutation profile of LGSC differs from that of HGSC, and LGSC tumors generally present mutations in the KRAS or BRAF (Ramalingam, 2016). Endometrioid carcinoma generally show abnormal expression of CTNNB1, PI3KCA, PPP2R1A, PTEN and ARID1A (Kossaï et al., 2018). Interestingly, these mutations have also been found in endometriotic cysts indicating a stepwise malignant transformation (Kossaï et al., 2018). Similar to LGSC, clear cell carcinoma rarely express mutations in TP53 or BRCA 1/2 (Lheureux et al., 2019). Frequent molecular aberrancies presented in clear cell carcinoma include mutated SWI/SNF (SWItch/Sucrose Non-Fermentable) chromatin-remodeling complex genes (specifically ARID1A) 40–60%, PIK3CA 33% and PTEN 37% (Kossaï et al., 2018; Lheureux, Braunstein and Oza, 2019). Mucinous carcinomas are typically heterogeneous tumors and harbor areas of mucinous, benign, borderline and adenocarcinoma origin (Kossaï et al., 2018). The molecular pattern of mucinous carcinoma consists of mutations in KRAS (50–75%) and HER2 (20%), which are generally mutually exclusive (Kossaï et al., 2018; Lheureux, Braunstein and Oza, 2019). Mucinous carcinomas with neither KRAS or HER2 mutations have an increased inclination to disease recurrence and subsequent mortality than tumors with either mutation (Prat, D'Angelo and Espinosa, 2018). The immunophenotypes of the EOC subtypes are presented in Table 2.

Table 2. Histopathological and molecular features of the 5 major subtypes of EOC. Modified from (Kossaï et al., 2018).

	HGSC	LGSC	EC	CCC	MC
Frequency	70%	<5%	10%	5–10%	2–3%
Immunophenotype	CK7+, CK20–, PAX8+, WT1+	CK7+, WT1+, ER+	CK7+, PAX8+, CK20–, WT1–	napsin A+, WT1–, p53–, ER–	CK7+, CK20–, ER–, PR–, WT1–
Precursor lesion	STIC	Low grade malignant potential lesions	Endometriosis	Endometriosis	Borderline mucinous lesions
Molecular abnormalities	TP53, BRCA 1/2	KRAS, BRAF	ARID1A, PTEN	ARID1A, PIC3CA, PTEN	KRAS, HER2

LGSC: low grade serous carcinoma, EC: endometroid carcinoma, CCC: clear cell carcinoma, MC: mucinous carcinoma, STIC: serous tubal intraepithelial carcinoma

2.1.3.3 Spread Patterns

The spread pattern of EOC is unique in comparison to other epithelial cancers, as extra-abdominal spread is rarer and represents late stage disease (Peters et al., 2015). The two main types of EOC spread are tumor cell shedding and metastasis intra-abdominally, and dissemination to the retroperitoneal lymphatic drainage areas. Hematogenous spread has generally been considered rare, but recent studies have suggested that it might be a more common modality of spread than previously thought (Sunila et al., 2015; Coffman et al., 2016).

Preceding metastatic shedding, EOC tumor cells undergo a transition from epithelial-to-mesenchymal and then detach from the primary tumor (Lengyel, 2010). Tumor cells disseminate as single cells or clusters with the physiological flow of the peritoneal fluid. Consequently, the typical intra-abdominal metastatic sites of EOC are the right subdiaphragmatic peritoneum, paracolic gutters and the omentum (van Baal et al., 2017). The dissemination of EOC cells results in the development of peritoneal carcinomatosis; however, the invasion of the peritoneal stroma remains largely superficial (van Baal et al., 2017). In incidental cases, the tumor cells infiltrate the walls of the intestine and bladder.

The lymphatic drainage routes from the ovaries run through the infundibulopelvic and ovarian ligaments, and to some extent through the round ligament of the uterus (Kleppe et al., 2015). Lymphatic metastases are present in up to 85% of patients with advanced EOC (Aletti, 2018) and the typical metastatic sites are the

para-aortic and paracaval regions, the obturator fossa and surrounding the internal iliac arteries (Kleppe et al., 2015). In contrast, supradiaphragmatic lymph node metastases originate mainly from intra-abdominal tumor growth (anterior route of lymphatic drainage) and are considered distant metastases (Mutch and Prat, 2014). Other sites of extra-abdominal metastases include pleural carcinosis and subsequent pleural effusion, inguinal lymph node, and intraparenchymal liver metastases.

2.1.4 Clinical Presentation and Primary Diagnostics

The conventional symptoms associated with EOC are pelvic pain and/or bloating, abdominal distension, a palpable abdominal mass and loss of appetite. Other symptoms include changes in bowel movement, constipation, urinary problems and vaginal bleeding (Ebell, Culp and Radke, 2016). In some cases, a deep venous thromboembolism (DVT) is the first sign of EOC. In fact, EOC is a malignancy with one of the highest rates of concomitant DVTs (Blom et al., 2006). On the whole, the symptoms suggestive of EOC are generally diffuse and become more persistent as the disease advances. Consequently, EOC has been referred to as "the silent killer" as early detection of the disease based on symptoms has remained unlikely.

A physical examination is done at the primary health care center and/or at the gynecologic outpatient clinic when a suspicion of EOC arises. A gynecological examination, including a speculum examination and a bimanual palpation, gives essential information of the mobility of the uterus and the size of the ovaries. Palpable ovaries in a postmenopausal woman is always atypical and further diagnostic investigations are needed. Moreover, a digital rectal examination is considered and all areas with superficial lymph nodes should be carefully assessed. A transvaginal sonography (TVS) is part of the basic diagnostic inspection of these patients. Suspicious TVS findings include ovarian masses >6cm and complex, irregular or bilateral smaller masses (Eskander, Berman and Keder, 2016). The serum levels of cancer antigen 125 (CA125) and occasionally human epididymis protein 4 (HE4) are determined. However, the sensitivity of the CA125 and HE4 assays in the detection of early stage EOC is suboptimal (55% for both assays, Wang et al., 2014) and multimarker panels have been developed to mend the problem: The Risk of Ovarian Malignancy Algorithm (ROMA), Risk Malignancy Index (RMI) and Copenhagen Index (CPH-I) (Jacobs et al., 1990; Van Gorp et al., 2011; Karlsen et al., 2015). These panels build on the biomarkers CA125 and HE4, TVS and/or the patients age/menopausal status. Although, the diagnostic performance of these panels have shown impressive results (AUCs 0.89, 0.86 and 0.88 for ROMA, RMI and CPH-I, respectively) in discriminating early stage EOC from benign diseases, there has been debate on whether these results can be translated into clinical utility (Van Gorp et al., 2011; Karlsen et al., 2015). In all patients, a CT scan of the abdomen and a chest x-ray or a whole-body CT are used to

detect extrapelvic disease preoperatively (Hennessy, Coleman and Markman, 2009). Additionally, the CT scan is a feasible method of evaluating the up-front cytoreductibility of the disease. In patients with ascites and/or pleural effusion, the cytology can be assessed if therapeutic paracentesis is needed. However, waiting for the cytology results is not necessary as the definitive diagnosis is attained from biopsies taken during surgery or a needle biopsy of the carcinosis.

2.1.5 Screening

The evident difficulties in the early detection of ovarian cancer and the notion that the mortality of the disease could be decreased with an early intervention has resulted in studies on ovarian cancer screening. Two randomized controlled trials on the effect of screening on ovarian cancer mortality have been conducted and unfortunately, the screening with CA125, TVS or the Risk of Ovarian Cancer Algorithm did not significantly decrease disease-specific mortality (Buys, 2011; Jacobs et al., 2016). However, the UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) did offer some encouraging results, as the results of the study indicated that a significant reduction in mortality might be seen during a longer follow-up period (Jacobs et al., 2016). The hypothesis is based on the finding that the mortality hazard ratio in the screening group seemed to subside, meanwhile an increasing trend was detected in the no screening group (Jacobs et al., 2016). Currently, the efficacy of ovarian cancer screening is not proven, and the screening methods need to be refined with more specific biomarkers/imaging studies for it to be feasible.

2.1.6 Staging

EOC is staged based on a surgical staging system established by The International Federation of Gynecology and Obstetrics (FIGO) Committee on Gynecologic Oncology (Table 3). The staging procedure includes extrafascial hysterectomy, bilateral salpingo-oophorectomy, radical omentectomy, appendectomy and pelvic and para-aortic lymphadenectomy (Benedet et al., 2000). Biopsies are taken consistently from all suspicious cites (mesentery of the small and large intestines, liver and diaphragm) and cytologic data is collected from peritoneal washings (Heintz et al., 2006). Although the staging system is surgical, the disease stage is confirmed histologically by a pathologist, and changed if necessary. As previously mentioned, a clinical examination is done prior to surgery and it is essential in confirming the disease stage. The FIGO classification corresponds well with the generally used TNM classification of solid tumors.

Stage I and II tumors are confined to the pelvis and are considered early stage (Prat, 2014) (Table 3). Although only 31% of all EOCs are diagnosed at an early stage,

the majority (58%–64%) of endometroid, mucinous and clear cell carcinomas are diagnosed at stage I (Torre et al., 2018). Consequently, these tumors have decent 5 year survival rates of 82%, 71% and 66% for endometroid, mucinous and clear cell carcinoma, respectively (Torre et al., 2018). Stage III tumors have spread intra-abdominally outside the pelvis and/or to the retroperitoneal lymph nodes and stage IV tumors present with distant metastases (pleural effusion, extra-abdominal tumors and growth to the liver parenchyma or other visceral organs) (Prat, 2014) (Table 3). Most of EOCs (60%) are diagnosed at stage III or IV, and patients have meager 5 year survival rates of 41% (stage III) and 20% (stage IV) (Torre et al., 2018). Importantly, 80% of HGSCs are diagnosed at a late stage and this illuminates the aggressive nature of the most common subtype of EOC as the 5 year survival rate of patients diagnosed with HGSC is 43% (Torre et al., 2018).

2.1.7 Prognostic Factors

There are several prognostic factors associated with poor survival outcomes in patients with advanced EOC: old age, poor physical performance status, tumor histology (mucinous and clear cell) and suboptimal cytoreduction (Winter et al., 2007). Similarly, old age, high disease stage and grade, and positive peritoneal washings have been identified to correlate with worse prognosis in early stage EOC (Chan et al., 2008). In addition, emotional disorders worsen the prognosis of elderly patients (Tinquaut et al., 2016). Interestingly, body mass index (BMI) does not seem to play a role in the survival of EOC patients (Kotsopoulos et al., 2012).

Table 3. FIGO 2014 classification system and the corresponding TNM.
Modified from (Prat, 2014).

Stage I. Tumor confined to the ovaries and/or fallopian tube(s)	
IA (T1a, N0-M0)	Tumor limited to one ovary (capsule intact) or fallopian tube; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
IB (T1b, N0-M0)	Tumor limited to both ovaries (capsules intact) or fallopian tubes; no tumor on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings
IC1 (T1c1, N0-M0)	Surgical spill
IC2 (T1c2, N0-M0)	Capsule ruptured before surgery or tumor on ovarian or fallopian tube surface
IC3 (T1c3, N0-M0)	Malignant cells in the ascites or peritoneal washings
Stage II. Tumor confined below the pelvic rim or primary peritoneal cancer	
IIA (T2a, N0-M0)	Extension and/or implants on uterus and/or fallopian tubes and/or ovaries
IIB (T2b, N0-M0)	Extension to other pelvic intraperitoneal tissues
Stage III. Tumor spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes	
IIIA1(T3a2, N0/N1-M0)	Positive retroperitoneal lymph nodes only (cytologically or histologically proven)
IIIA1 (i)	Metastasis up to 10 mm in greatest dimension
IIIA1 (ii)	Metastasis more than 10 mm in greatest dimension
IIIA2	Microscopic extra-pelvic peritoneal involvement with or without positive retroperitoneal lymph nodes
IIIB (T3b, N0/N1-M0)	Macroscopic peritoneal metastasis beyond the pelvis up to 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
IIIC (T3c, N0/N1-M0)	Macroscopic peritoneal metastasis beyond the pelvis more than 2 cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes
Stage IV. Distant metastasis excluding peritoneal metastases	
IVA (any T, any N, M1)	Pleural effusion with positive cytology
IVB (any T, any N, M1)	Parenchymal metastases and metastases to extra-abdominal organs

2.1.8 Treatment of Primary EOC

Cytoreductive surgery followed by intravenous chemotherapy is the foundation of primary EOC treatment. There are two validated strategies for primary treatment: (1) primary debulking surgery (PDS) followed by adjuvant chemotherapy and (2) neoadjuvant chemotherapy (NACT) and interval debulking surgery (IDS) with

subsequent adjuvant chemotherapy (patients with advanced, stage III–IV disease) (Kehoe et al., 2015; Yang et al., 2017) (Figure 1). For optimal treatment results, cytoreductive surgery should be done by a gynecologic oncologist and a comprehensive multidisciplinary approach is recommended (Giede et al., 2005; Fagotti et al., 2016). In addition to the oncological treatments, psycho-oncological support should routinely be provided and the identification of psychosocial distress, sexual dysfunction and psychiatric comorbidity is important (Colombo et al., 2019).

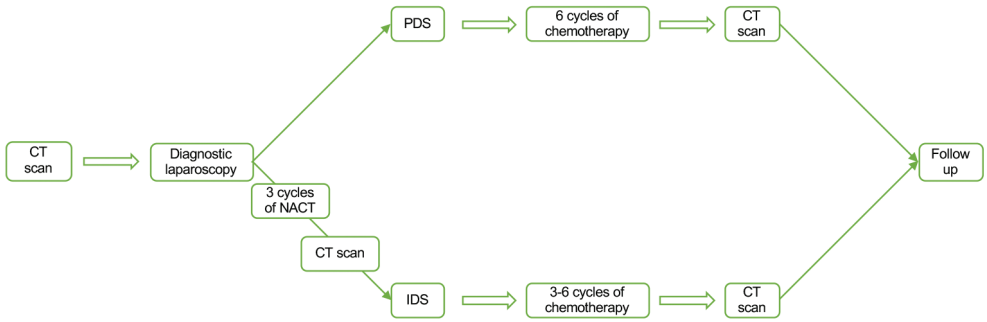


Figure 1. Treatment flowchart of EOC patients in the Turku University Hospital.

2.1.8.1 Laparoscopy

Minimally invasive surgery (MIS) has been implemented in the treatment of primary EOC and it has been proven feasible in the cytoreductive surgery of apparent early stage EOC and in the staging of advanced disease (Gallotta et al., 2014; Fagotti et al., 2016). Still, open surgery is considered the standard approach even in apparent early stage EOC (Colombo et al., 2019).

Concerns regarding MIS in the staging and debulking of early stage EOC include inadequate exposure of the peritoneal surfaces, tumor cell leakage, insufficient lymph node dissection, lack of palpable sensation and the risk of port cite metastasis (Semaan, Abdallah and MacKoul, 2008; Gallotta et al., 2014). These concerns center upon deficient staging, which might lead to the decreased survival of patients. However, the risks are reduced with adequate preoperative workup, careful patient selection and a skillful surgical technique. Consequently, the recurrence rate after MIS for early stage EOC has been reported to be compatible with that of the conventional midline laparotomy (Park et al., 2013).

Explorative laparoscopy is used in the diagnostic work up of patients with advanced stage EOC (Rutten et al., 2012; Zeff, 2018). The aim of the diagnostic procedure is to

improve the prognosis of patients by enabling a comprehensive assessment of operability (probability of complete cytoreduction); intraperitoneal seeding of tumor is easily visualized, the procedure might save the patient from an unnecessary laparotomy and patients with inoperable disease can directly proceed to NACT without the burdensome recovery from laparotomy (Fagotti et al., 2016). Furthermore, cytologic samples and tissue biopsies can be collected during the procedure for the accurate histological classification of the disease.

2.1.8.2 Laparotomy

Debulking surgery (PDS or IDS) through midline laparotomy is the cornerstone of primary EOC treatment (Benedet et al., 2000). Patients with stage IIIC or less extensive disease are generally eligible for up front debulking, while the feasibility of PDS for stage IV disease or bulky tumors should be carefully evaluated to reach the best treatment outcomes (Van Meurs et al., 2013). There is moderate evidence indicating that women treated with NACT and IDS have a similar overall survival as women undergoing PDS (Coleridge et al., 2019). However, PDS should be considered the standard treatment for EOC as there is also contradictory results demonstrating inferior survival in the NACT group regardless of complete cytoreduction in IDS (Gadducci et al., 2017).

As always in surgery, the extent of the procedure should be balanced with the associated increase in morbidity. Modern supportive care enables ultra-radical surgery, in which the range of cytoreduction is extended from the pelvis, omentum and lymph nodes to the upper abdomen and multi-visceral resections (e.g. diaphragmatic, liver, splenic and gastrointestinal resections) (Lepinay et al., 2019). A recent study indicated that the morbidity risk increase with high-complexity surgery, specifically with diaphragmatic resection and gastrointestinal anastomosis (six times increased risk) and multiple bowel resections (eight times increased risk) (Phillips et al., 2019). However, aggressive surgical effort and consequent complete cytoreduction is associated with survival gain and should be strived for whenever feasible (Aletti et al., 2009).

2.1.8.3 Medical Treatment

The standard intravenous (i.v.) chemotherapy regimen for all patients with EOC is six cycles of paclitaxel (175mg/ m2) combined with carboplatin (AUC 5–6) (Benedet et al., 2000). There is strong evidence that also patients with early stage EOC have long term survival benefits from i.v. chemotherapy (Lawrie et al., 2015). However, it is still uncertain whether the survival benefit outweighs the adverse effects of chemotherapy in patients with low grade stage IA disease, and the adjuvant chemotherapy of these patients may be omitted (Lawrie et al., 2015).

Bevacizumab, an anti-angiogenic agent, increases the progression free survival (PFS) of especially high risk EOC patients (stage IV or III + residual tumor) and should be included in the primary treatment of these patients (González Martín et al., 2019). In addition, bevacizumab have been shown to increase the overall survival (OS) of high risk patients in a phase 3 randomized study (Oza et al., 2015). Bevacizumab is also validated in the treatment of recurrent EOC, both in a platinum sensitive and resistant relapse (Pujade-Lauraine et al., 2014; Aghajanian et al., 2015).

In addition to bevacizumab, another group of molecular targeted therapy, namely PARPi, has recently been introduced in the treatment of EOC. PARPis exhibit synthetic lethality specifically on cancer cells with homologous recombination (HR) deficiency, which is present in approximately half of HGSCs (80–90% of all EOCs) (Kossaï et al., 2018). Presently, there are three PARPi approved for this intent: olaparib, niraparib and rucaparib (Colombo et al., 2019). The strongest clinical evidence for the use of PARPis is, at present, in its use as a maintenance therapy after a response to platinum-based chemotherapy in the first line setting as well as in the second line or higher (Colombo et al., 2019). Similar to bevacizumab, PARPis have been shown to increase the PFS of EOC patients and this effect is most notable in, but not limited to, patients with a BRCA mutation (Colombo et al., 2019).

The utility of immunotherapy as a treatment modality in EOC is currently being explored in clinical trials. The mutation of normal cells to cancer cells is a process that takes place daily in every human and consequently, the immune system is highly trained in the elimination of cancer cells (Pietzner et al., 2018). Cancer cells have three distinct ways of breaching the protection mechanisms: down-regulation of the antigen presentation with subsequent impaired tumor cell recognition by T-cells, the creation of an immunosuppressive microenvironment and the utilization of the safety feature of the host's immune system called the "immune checkpoint" (Pietzner et al., 2018). Immune checkpoints are complex constructs that protect healthy cells by blocking T-cells from lethal action despite specific antigen recognition (Pietzner et al., 2018). Checkpoint inhibitors, i.e. nivolumab, pembrolizumab, durvalumab and tremelimumab, target this mechanism hijacked by cancer cells and consequently restore the anti-tumor activity of T-cells (Wang, Liu and Zou, 2019). An initial report on the efficacy of nivolumab in EOC treatment presented promising results as nine patients of twenty responded to the treatment (Hamanishi et al., 2015). In addition to checkpoint inhibitors, the adoptive transfer of ex vivo - expanded tumor-specific T-cells and cancer vaccines are being investigated as immunotherapy options in EOC (Wang, Liu and Zou, 2019).

2.2 Serological and Genomic Biomarkers of EOC

2.2.1 Cancer Antigen 125 (CA125)

The CA125 immunoassay was developed and described for the first time in 1983 (Bast et al., 1983). In the initial report, 1% of 888 healthy individuals had serum CA125 levels exceeding 35.0 U/ml and a normal range for serum CA125 was determined accordingly. The first version of the CA125 assay utilized only OC125 (CA125 I) as the capture antibody and another epitope group, M11, has since been added to the assay (CA125 II) (Duffy et al., 2005). The assay utilizing M11 and OC125 as capture antibodies is the assay generally in use today. The CA125 assay has since its development become a well validated biomarker assay in EOC diagnostics, monitoring and follow-up, and it is currently the only widely recognized biomarker assay used in the clinical setting (Bast, 2010; Pignata et al., 2011; Zhang, Cai and Zhong, 2011).

2.2.1.1 The CA125 Molecule and Glycosylation

The CA125 molecule is a large (200–2000 kDa) mucin-type transmembrane glycoprotein with an extensive amount of N- and O-glycans (249 potential N-glycosylation and over 3700 O-glycosylation sites) (Saldova et al., 2013). CA125 consists of a short cytoplasmic tail, a transmembrane domain and an extracellular domain, which contains the binding site of OC125 and M11 (O'Brien et al., 2001). The epithelial growth factor receptor signal transduction pathway is linked to the release of CA125 to the bloodstream (Scholler, Urban and Gene, 2007). The exact function of CA125 is poorly understood, but its structure indicates a possible immunological role (O'Brien et al., 2001).

Glycosylation is an enzymatic process that fuses saccharides to other saccharides, lipids or proteins (Fuster and Esko, 2005). Deviant glycosylation is a characteristic feature in human cancers and initial reports of aberrant glycosylation were published over five decades ago (Hakomori and Murakami, 1968). The typical cancer-associated changes in glycosylation are sialylation, fucosylation, O-glycan truncation and N- and O-linked glycan branching (Hakomori, 2002). The aberrant glycosylation of serum CA125 in patients with ovarian cancer is particularly notable in N-glycans: ovarian cancer patients have increased rates of core-fucosylated bi-antennary monosialylated glycans and decreased rates in mostly bisecting bi-antennary and non-fucosylated glycans compared to healthy controls (Saldova et al., 2013). As the changes in glycosylation are cancer specific, the quantification of glycans with their protein backbone is a feasible tool for increasing the diagnostic and prognostic potential

of current glycoprotein biomarkers, e.g. CA125 (Pinho and Reis, 2015). Promising results have been attained with several biomarkers in other cancers, including breast, liver and pancreatic cancer (Matsuura, Kawai and Ilirai, 1993; Okuyama et al., 2006; Wi et al., 2016; Terävä et al., 2019).

2.2.1.2 CA125 in Healthy Women

The serum level of CA125 vary in healthy women and several factors influencing the variance of serum CA125 have been identified. Premenopausal women generally have higher levels of CA125 (mean value of 18 U/ml) compared to postmenopausal patients (mean value of 12.0 U/ml) (Bon et al., 1996). Further, fluctuation in the serum CA125 levels have been detected during different phases of the menstrual cycle, highest values arising during the menses (McLemore et al., 2012). Elevated serum CA125 levels have also been associated with pregnancy (Wang et al., 2018). Lower baseline CA125 levels have been detected in women with a history of smoking, regular coffee consumption and hysterectomy (Pauler et al., 2001). In contrast, women with a prior non-ovarian cancer diagnosis have been reported to have increased baseline serum CA125 levels (Pauler et al., 2001).

2.2.1.3 CA125 and EOC

Elevated serum CA125 levels are detected in approximately 80–90% of patients with EOC (Duffy et al., 2005). However, only circa 50% of patients with early stage EOC present with elevated serum CA125 levels (Zhang, Cai and Zhong, 2011). Consequently, it has been established that the CA125 assay lacks the required sensitivity and specificity for screening purposes (Sölétormos et al., 2016). In the differential diagnostic of pelvic masses, the CA125 assay is used as a part of a multimodal approach (clinical examination, TVS and serum CA125) for increased accuracy (Sturgeon et al., 2008) as elevated serum CA125 is largely unspecific for EOC. Several other malignancies, typically adenocarcinomas (e.g. breast, pancreas, endometrial and cervical), express CA125 (Massuger et al., 1997; Liu et al., 2016; Bian et al., 2017; Wang et al., 2017). A further limitation of CA125 is its elevation in various non-cancerous diseases, most importantly diseases of gynecologic origin (e.g. ovarian cysts, endometriosis and inflammations) but also in conditions irritating the peritoneum, pleura or pericardium (e.g. liver cirrhosis with ascites and congestive heart failure with pleural effusion) (Xiao and Liu, 2003; R. G. Moore et al., 2012; K. H. C. Li et al., 2018). In EOC diagnosis, CA125 is most suited for detecting serous, endometroid and clear cell carcinomas and is less frequently elevated in mucinous carcinomas (Duffy et al., 2005).

The measurement of serum CA125 is the standard method in the treatment monitoring and follow-up of EOC (Duffy et al., 2005; Sturgeon et al., 2008).

In treatment monitoring, serum samples are optimally drawn 2 weeks before the start of treatments and subsequently every 2–4 weeks during chemotherapy (Sturgeon et al., 2008). A rising serum value of CA125 correlates with disease progression in 90% of patients and persistently elevated CA125 values equal with persistent disease in 95% of patients (Bast et al., 2005). However, the CA125 assay has its limitations in the monitoring of patients with EOC, as small volumes of persistent disease might be present in up to 50% of patients regardless of CA125 normalization (Bast et al., 2005). Despite its limitations, CA125 is currently the only biomarker used in the treatment response evaluation of patients with EOC (John et al., 2011). The prognostic value of CA125 has been widely investigated, and the nadir value and rapid normalization of serum CA125 have shown prognostic potential (Bast et al., 2005; Van Altena et al., 2010; Zeng et al., 2017). It has also been indicated that the preoperative level of serum CA125 might be a feasible tool for the assessment of operability (Chi et al., 2000; Kang et al., 2010), but there have also been contradictory results (Barlow et al., 2006; Mury et al., 2011).

The feasibility of serum CA125 measurement in the follow-up of EOC was questioned in a large multicenter trial, as the authors did not detect a survival benefit from an early intervention to asymptomatic, CA125 positive recurrence compared to intervention in a later, symptomatic phase (Rustin et al., 2010). There are no published studies investigating the feasibility of treating early, asymptomatic recurrence in the current era of PARPis and other precision drugs and consequently, the retainment from early intervention needs to be re-evaluated. However, follow-up with CA125 is currently recommended especially if patients are considered for studies on second line treatments, patients do not have regular (3 monthly) follow-up visits/imaging studies, secondary surgery is a treatment option in case of recurrence or the patient is included in a clinical study (Verheijen et al., 2012). The serum level of CA125 is generally elevated approximately 3 months before symptomatic recurrence (Bast et al., 2005).

2.2.1.4 CA125 Glycoforms and EOC

A few approaches have been described in the quantification of aberrantly glycosylated circulatory CA125 in patients with EOC. A microarray-based approach measuring two aberrant glycoforms (Neu5Acα2,6GalNAc [STn] and GalNAc [Tn]), showed promise in improving the performance of the conventional CA125 assay in EOC diagnostics (Chen et al., 2013). A similar, lectin-based, immunoassay approach was described in 2016 (Gidwani et al., 2016). The immunoassay utilized macrophage galactose-type lectin (MGL) with a nanoparticle component (Gidwani et al., 2016). Recently, an analogous assay utilizing Sialyl-Thomsen-nouveau antibody (STn) with a nanoparticle component showed increased diagnostic potential in differentiating

CA125 from benign and malignant sources (Gidwani et al., 2019). Lectins are a family of small proteins with the capability of binding to carbohydrates, which makes them putative tools in the exploration of aberrant glycosylation. However, the applicability of lectins has been restricted by their low binding affinity to carbohydrates (Gidwani et al., 2020). Both the CA125-MGL and-STn assays utilized nanoparticles to increase the problematically low affinity of lectins and antibodies: a number of lectins or antibodies was fixed onto the nanoparticles to create a reporter with increased reactivity while maintaining the original specificity of the lectin/antibody (Gidwani et al., 2020). In addition, the polystyrene nanoparticles contain approximately 30000 fluorescent europium (Eu) chelates, which results in a significant signal amplification (Gidwani et al., 2020). Another, non-lectin, approach to glycoprofiling was recently described, in which an automated glyprofiling platform showed promising results in the diagnosis of EOC (Flaherty et al., 2019). The platform analyzed the glycans of six glycoproteins, however, not those of CA125 (Flaherty et al., 2019). In conclusion, glycoprofiling of the CA125 molecule is a promising tool in improving the conventional CA125 assay and enabling a more cancer specific approach to EOC diagnostics. Studies on the prognostic potential of CA125 glycoforms have not been previously conducted.

2.2.2 Human Epididymis Protein 4 (HE4)

Human epididymis protein 4 (HE4) is encoded by the WFDC2 gene (chromosome 20q12–13) and central to its structure is a whey acidic protein (WAP) type four disulphide core domain (Bingle, Singleton and Bingle, 2002). The structure of HE4 is similar to known leukocyte protease inhibitors and it has been suggested that HE4 might play a role in natural immunity (Bingle, Singleton and Bingle, 2002; Clauss, Lilja and Lundwall, 2002). HE4 was initially identified in 1991 by Kirchhoff et al, who hypothesized HE4 to be solely expressed by epididymal tissue (Kirchhoff et al., 1991). However, more recent studies have shown HE4 immunoreactivity in several other tissue microarrays, including the fallopian tubes, salivary glands, pancreaticobiliary system and thyroid gland (Galgano, Hampton and Frierson, 2006). Interestingly, the variance of serum HE4 is relatively small between the sexes (7% lower serum concentration in males) implying that the majority of circulatory HE4 is derived from other sources than the female reproductive organs (Bolstad et al., 2012). The upper 95th percentile of 150.0 pmol/L is validated as the cut off for the HE4 assay as reported by the assay manufacturer (Fujirebio Diagnostics Inc, Malvern PA, USA). However, a two-threshold system has since been suggested (70.0 pmol/L for premenopausal patients and 140.0 pmol/L for postmenopausal patients) as it is acknowledged that the serum HE4 levels increase with age (Dochez et al., 2019).

2.2.2.1 HE4 in Healthy Women

Several physiological and demographical factors affect the concentration of circulatory HE4 in healthy women. The effect of age on serum HE4 levels have shown to be notable and a study by Bolstad et al described a 37% increase in serum HE4 in women aged 60 compared to those of 20 years of age (Bolstad et al., 2012). However, the longitudinal HE4 kinetics associated with aging seem to be complex and non-linear, modelled by a higher degree polynomial (Bolstad et al., 2012). Similar findings have been reported by other researchers, and the most substantial increase in circulatory HE4 has been observed in women over the age of 55 and the positive trend continues until 80 years of age (Moore et al., 2012; Urban et al., 2012). The association of the menopausal status and serum HE4 has been investigated, and currently there is no robust evidence on the menopause affecting serum HE4 levels. Moore et al examined a subgroup of premenopausal women aged 40 years and older and postmenopausal women aged 60 years and less, and detected no difference in the circulatory HE4 levels between the groups (Moore et al., 2012). Escudero et al detected a modest elevation in the HE4 levels of postmenopausal women compared to premenopausal women, however; the difference was not statistically significant (Escudero et al., 2011). Contradictory results have also been reported, as menopausal status was significantly affecting the serum HE4 levels in a Chinese population of 618 healthy women (Tian et al., 2015). Indeed, more comprehensive studies on the subject are warranted.

A few studies have been conducted on the kinetics of HE4 during the normal menstrual cycle of healthy women and currently the matter is still under debate. A recent study detected a significant elevation in circulatory HE4 levels in women during ovulation, although the absolute rise in serum HE4 was modest (7.1 pmol one day after the luteinizing hormone surge) (Moore et al., 2017). Contradictory results were presented by Hallamaa et al in a study, in which no significant changes were detected in the serum HE4 levels during the proliferative, secretory, inactive/atrophic or menstrual phase of the cycle (Hallamaa et al., 2012). Regarding the effect of pregnancy on circulatory HE4, a study on 67 pregnant women showed significantly lower HE4 levels compared to non-pregnant controls (Moore et al., 2012). However, the longitudinal trend of HE4 during pregnancy was consistent, and HE4 might be a feasible biomarker in the triage and monitoring of pelvic masses during pregnancy with certain adjustment (Moore et al., 2012; Qu et al., 2016).

Behavioral factors affecting the circulatory HE4 levels in healthy women include smoking, weight and obesity (Qu et al., 2016). Studies have detected 10% lower serum HE4 levels in obese women (body mass index 30), and the serum HE4 levels in current smokers have been reported to be significantly elevated with 20–30% compared to non-smokers (Bolstad et al., 2012; Urban et al., 2012). Primarily, the deviant HE4 concentrations are linked to metabolic changes associated with obesity and the inflammatory response in the airways related to smoking (Qu et al., 2016).

2.2.2.2 HE4 in non-Ovarian Malignancies

The upregulation of HE4 expression is a typical attribute of carcinogenesis in a variety of non-ovarian tumors. A comprehensive analysis on the tissue expression of HE4 was conducted by Galgano et al, and especially endometrial, pancreaticobiliary, lung and salivary gland malignancies showed increased HE4 expression profiles (Figure 2) (Galgano, Hampton and Frierson, 2006). Supportive results were presented in a study on gastrointestinal carcinomas, of which specifically stomach and pancreatic carcinoma showed increased expression of HE4 (O'Neal et al., 2013). Interestingly, the HE4 upregulation in the tumor tissues of several malignancies were comparable to the level of HE4 upregulation in serous ovarian carcinoma (Galgano, Hampton and Frierson, 2006).

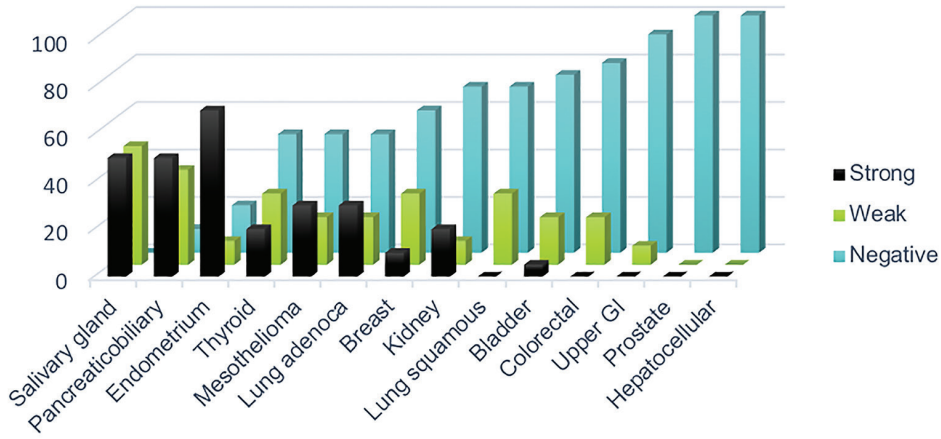


Figure 2. HE4 immunoreactivity in the tumor tissue (%) of non-ovarian malignancies. Modified from (Galgano, Hampton and Frierson, 2006).

Increased tissue expression does not systematically translate to elevated levels of circulatory biomarkers, but there is increasing evidence on the utility of serum HE4 in the diagnostics of several non-ovarian malignancies. Specifically, elevated concentrations of HE4 has been detected in the serum of patients with endometrial, breast, non-small cell lung cancer (NSCLC) and primary liver cancer (Escudero et al., 2011; Gündüz et al., 2016; He et al., 2019). However, the clinical applicability of HE4 in non-gynecological malignancies is still to be validated. HE4 has shown potential in the diagnosis of endometrial carcinoma (EC), however; the results of a recent meta-analysis indicate that the studies on HE4 and EC diagnosis are quite heterogeneous and studies with larger, homogeneous cohorts are needed to further

elucidate the clinical applicability of HE4 (Li et al., 2018). Additionally, EC is generally diagnosed at an early stage due to specific symptoms (Cymbaluk-Płoska et al., 2017), which limits the need for diagnostic markers.

2.2.2.3 HE4 and EOC

A systematic overexpression of HE4 has been associated with EOC; however, the upregulation of HE4 expression varies between the different histotypes of the disease (Figure 3) (Galgano, Hampton and Frierson, 2006). Overall, HE4 has shown to be a feasible EOC biomarker, as the upregulated tissue expression is well translated to increased levels of circulatory HE4 in EOC patients (Hellström et al., 2003). Similar to the HE4 tumor tissue expression profiles, highest serum HE4 concentrations have been reported in serous carcinomas, while the serum HE4 concentration is generally low in mucinous carcinomas (Galgano, Hampton and Frierson, 2006; Kristjansdottir et al., 2013).

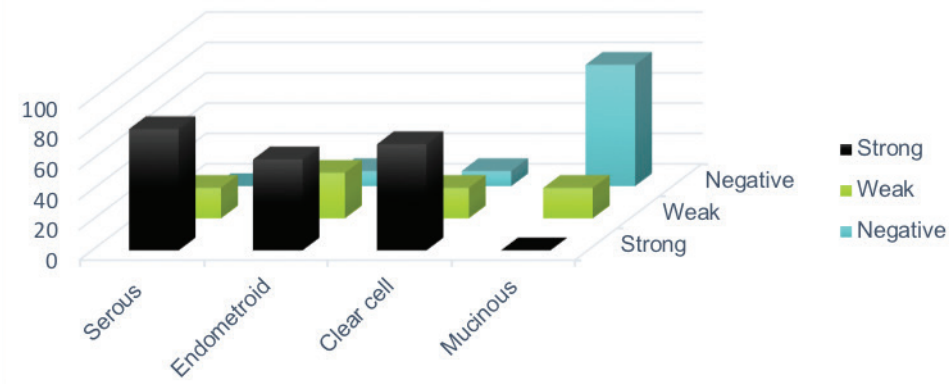


Figure 3. HE4 immunoreactivity in the tumor tissue (%) of the different EOC histotypes. Modified from (Galgano, Hampton and Frierson, 2006).

The initial study on the diagnostic potential of serum HE4 in EOC was conducted by Hellström et al in 2003, and subsequently, several prospective studies have confirmed the introductory findings (Moore et al., 2008; Abdel-Azeez et al., 2010; Holcomb et al., 2011; Jacob et al., 2011; Van Gorp et al., 2011; Chan et al., 2013). The area under the curve (AUC) of HE4 has been reported to vary from 0,86 to 0,93 between studies in the diagnosis of EOC (Moore, Brown, Miller, Skates, et al., 2008; Holcomb et al., 2011; Jacob et al., 2011; Van Gorp et al., 2011; Chan et al., 2013).

However, there is some heterogeneity in the control groups used, as some studies included healthy controls and others compared EOC patients to those with a benign pelvic mass, and consequently, the AUCs reported are not completely comparable. Based on these previous studies, the diagnostic performance of the CA125 and HE4 assays seems to be similar in advanced EOC (no significant differences in the ROC curve comparisons). Interestingly, Moore et al and Jacob et al reported HE4 to be valuable in the diagnosis of early stage EOC, with a significantly higher sensitivity compared to CA125 ($p < 0.001$) (Moore et al., 2008; Jacob et al., 2011). However, contradictory results have also been reported (Kristjansdottir et al., 2013).

Moreover, there is some evidence on the superiority of HE4 over CA125 in dividing patients with tumors of borderline malignancy (BOT) from EOC, but the results are conflicting (Holcomb et al., 2011; Jacob et al., 2011; Wang, Tao and Ying, 2019). As HE4 is generally not expressed in benign gynecologic diseases, specifically endometriosis (Huhtinen et al., 2009; Zapardiel et al., 2016), it is a feasible auxiliary biomarker in the differential diagnosis of pelvic masses. The most prevalent source of falsely elevated serum HE4 concentrations is chronic kidney disease, and elevated circulatory HE4 concentrations are already measured at mild levels of kidney failure (eGFR 60–89 mL/min/1.73m2) (Nagy et al., 2012).

There is some controversy on whether the longitudinal kinetics of serum HE4 is in concordance with the treatment outcomes of patients and the kinetics of CA125. There are two studies addressing this matter, of which the first one was conducted in a proof-of-concept manner, including 11 patients with consistent dynamics of HE4 throughout the treatment regimen (Hynninen et al., 2011). The other study conducted by Ferraro et al evaluated a heterogeneous cohort of 43 patients, in which HE4 was not as reliable as CA125 in the treatment monitoring and follow-up of OC (Ferraro et al., 2018). However, the cohort consisted of partly non-epithelial ovarian malignancies and included patients with renal impairment, which naturally influenced the results.

Studies on the prognostic utility of serum HE4 have been conducted with inconsistent results. A recent meta-analysis concluded that a high preoperative HE4 level might be a predictor of poor OS and PFS, however, the 14 studies included in the analysis were quite heterogeneous and the matter needs to be studied further (Yuan et al., 2018). A handful of studies have evaluated the association of preoperative HE4 levels to the amount of residual disease with promising results. Angioli et al proposed a preoperative HE4 cut off of 262.0 pmol/L, which discriminated patients with subsequent complete cytoreduction from those with a suboptimal amount of residual disease with a sensitivity and specificity of 86.1% and 89.5%, respectively (Angioli et al., 2013). A similar analysis was conducted by Braicu et al, in which the preoperative HE4 <235.0 pmol/L predicted optimal cytoreduction with a sensitivity of 76.6% and a specificity of 47.3% (Braicu et al., 2013). However, the sensitivity of diagnostic laparoscopy has been reported to be 71% and 95% within studies, with the accuracy of

90% (Fagotti et al., 2005; Van De Vrie et al., 2019). Obviously, diagnostic laparoscopy is an invasive procedure, and the measurement of serum HE4 in combination with a preoperative CT scan might be a feasible auxiliary method in the evaluation of up front cytoreductibility. Although the results on the association of preoperative HE4 and residual disease are indicative of HE4 being a potential indicator of tumor load, there is currently no studies in which the preoperative serum HE4 concentration is directly compared to the tumor load assessed during surgery and/or by CT scan. One study has evaluated the association of the HE4 nadir to different end points, specifically to treatment response and PFS (Vallius et al., 2017). Interestingly, lower HE4 nadir levels were associated with a better response to treatment and a longer PFS (Vallius et al., 2017).

A few studies have addressed the utility of HE4 in the detection of EOC relapse (Capriglione et al., 2017). Anastasi et al conducted a study, in which elevated serum HE4 concentrations were detected with a lead time of 5–8 months to serum CA125 preceding the detection of recurrent disease (Anastasi et al., 2010). The sensitivity and specificity of the combination of HE4 and CA125 in the detection of recurrent OC was evaluated in one of the studies with interesting results: a sensitivity of 76% (vs 35% with CA125 alone) and a specificity of 100% (vs 59% with CA125 alone) was reached (Plotti et al., 2012). In addition, HE4 showed potential in a recent study, in which 7/8 patients with CA125-negative disease had positive serum HE4 levels at disease follow-up and progression (Plotti et al., 2019). Studies on the prognostic value of HE4 at progression have not been published to date.

Currently, there is no clear consensus on the benefit of including HE4 in the treatment monitoring and follow-up of patients with EOC and consequently, the routine measurement of HE4 is not recommended to this end (Colombo et al., 2019). Studies with larger, homogeneous cohorts are needed for the further exploration of the utility of serum HE4 measurement during EOC treatment and recurrence.

2.2.3 Circulating Tumor DNA (ctDNA)

The presence of cell free DNA (cfDNA) in the human bloodstream was initially described in 1948 (Mandel and Metais, 1948). It has since been detected that patients with cancer have elevated levels of circulatory cfDNA, of which the majority is ctDNA (Stroun et al., 1989; Barlebo Ahlborn and Østrup, 2019). There are three different sources to cfDNA in the circulatory compartment of cancer patients (healthy cells, tumor cells and tumor microenvironmental cells), which need to be acknowledged when evaluating the cfDNA burden of a patient (Thierry et al., 2016). Further, the amount of cfDNA is dependent not only on its release from cells but also on its clearance from the circulatory system. The data on cfDNA clearance is limited, but a study on fetal cfDNA suggests a biphasic clearance of cfDNA: the initial rapid phase

had a half time of approximately 1h, and the subsequent slower phase had a half time of circa 13h (Yu et al., 2013). However, data on ctDNA clearance in patients with colorectal cancer showed a monophasic ctDNA clearance half time of 114min after complete cytoreduction (Diehl et al., 2008). The elimination of cfDNA occurs mainly through enzymatic degradation in the bloodstream (DNase I, plasma factor VII–activating protease, and factor H), entrapment and elimination in the liver and spleen, and secretion from the kidneys (Yu et al., 2013; Stephan et al., 2014; Kustanovich et al., 2019). Tumor cells leak their DNA to the extracellular compartment through apoptosis, necrosis, phagocytosis, oncosis, and active secretion (Thierry et al., 2016). The fragmentation of ctDNA has shown to be high compared to the cfDNA of healthy individuals, and the majority of ctDNA fragments are below 145 bp (Mouliere et al., 2011; Thierry et al., 2016). The increasing genomic analyses of cfDNA have shed light on the presence of leukemia-associated genetic mutations in the circulatory system of otherwise healthy individuals (Luis et al., 2019). This phenomenon, clonal hematopoiesis, is strongly associated with aging and is linked to mutations within long-lived hematopoietic stem cells (Luis et al., 2019). Clonal hematopoiesis is a possible confounding factor in the ctDNA analyses and should be acknowledged especially in the elderly.

Although cfDNA can be viewed as a passive component in the circulatory system, there is increasing evidence of its functional role in intercellular communication, transformation of other cells and immunomodulation (Thierry et al., 2016; Kustanovich et al., 2019). Most intriguing from an oncologic perspective is the ability of ctDNA to foster tumor progression by transfecting healthy cells and subsequently forming distant metastases (Trejo-Becerril et al., 2012). These studies suggest that ctDNA might be a feasible therapeutic target in addition to its potential as a diagnostic tool.

2.2.3.1 CtDNA as a Liquid Biopsy Component in Cancer

CtDNA is typically extracted from blood and it carries information of the genetic and epigenetic aberrations of the tumor (Kustanovich et al., 2019). Different detection technologies can be utilized, including next generation sequencing (NGS), digital-PCR platforms, real-time PCR platforms, mass-spectrometry technology and detection of hypermethylation in ctDNA (Elazezy and Joosse, 2018).

Studies measuring the serum or plasma concentration of ctDNA in cancer patients generally link the amount of ctDNA to tumor volume either by imaging studies or disease stage (Reece et al., 2019). Nevertheless, the early detection of malignancies with ctDNA has proven to be difficult, as early stage tumors shed less DNA (50% of patients have detectable plasma ctDNA vs 82% of patients with metastasized disease) and consequently, the background noise of regular cfDNA

is emphasized (Bettegowda et al., 2014). Another recent study reported 71% and 59% rates of ctDNA positive patients with early stage colorectal and breast cancer, respectively (Phallen et al., 2017). Currently, the sensitivity of ctDNA as a screening tool is inferior to conventional screening methods (Barlebo Ahlborn and Østrup, 2019). Although the feasibility of ctDNA plasma concentration measurement alone in early cancer diagnostics is debatable, its diagnostic potential might be improved by including analyses of ctDNA gene methylation to detect epigenetic deregulation of genes (Elazezy and Joosse, 2018). The dualistic approach yielded promising results in a recent proof-of-concept study on renal cell carcinoma, in which a diagnostic accuracy of 100% was reached (Skrypkina et al., 2016).

The quantification of the ctDNA concentration has been studied in the detection of minimal residual disease (MRD) after surgery and in cancer relapse (Reece et al., 2019). However, a more advantageous method for detecting MRD and disease relapse is currently thought to be the mutation tracking of ctDNA (Elazezy and Joosse, 2018). Several studies have been conducted on ctDNA mutation dynamics perioperatively and in relapse (Tie et al., 2016; Ng et al., 2017; Murray et al., 2018; Chen et al., 2019). These studies on colorectal and lung cancer indicate that the detection of ctDNA after apparent complete cytoreduction surgery is associated with MRD and subsequent relapse (Tie et al., 2016; Ng et al., 2017; Murray et al., 2018). Interestingly, there were patients in two of these studies that had persistently elevated plasma ctDNA without clinical or radiological indication of recurrent disease in the frame of the follow-up period (Tie et al., 2016; Ng et al., 2017). However, late recurrence remains an explanation of the phenomenon.

The possibilities of ctDNA in the monitoring of cancer treatment have attracted scientific interest, as more precise tools enable the discontinuation of ineffective therapy and allows the implementation of more suitable ones. With novel precision drugs, a comprehensive knowledge of the tumor mutation profile is needed for the design of optimal treatment plans. However, inaccuracies might occur with traditional tissue biopsy, as tumors often contain subclones and mutations may vary between metastases (Reece et al., 2019). In addition, the mutation profile of the tumor might progress and change during treatment resulting in treatment resistance (Ahlborn et al., 2018). Recent studies have indicated a good correlation (70–90%) between the tumor mutation profile attained from tissue biopsy and that of liquid biopsy (Phallen et al., 2017; Chen et al., 2019). Consequently, longitudinal tracking of the ctDNA mutation profile has been proven feasible in detecting and tracking therapy resistance in colorectal and other solid cancers (Misale et al., 2012; Ahlborn et al., 2018). However, there are limitations in the utilization of liquid biopsy in the mapping of the tumor mutation profile, e.g. the biopsy might show low levels of or excessively fragmented ctDNA, which results in a substandard picture of the tumor mutation profile. An overview of the advantages and disadvantages of conventional tissue biopsy and liquid biopsy are presented in Table 4.

Table 4. Advantages and disadvantages of tissue biopsy and liquid biopsy. Modified from (Barlebo Ahlborn and Østrup, 2019).

Type of biopsy	Advantages	Disadvantages
Solid (tissue)	Standard detection method	Invasive and expensive
	Possibility of cytopathology	Possible complications
	High concentration of tumor cells	Not real-time detection
	Identification of mutation relevant for targeted therapies	Do not capture heterogeneity (unless multiple biopsies)
	Possibility for RNA (transcriptome profiling and gene-expression analysis) and protein analysis	Some tumors are not accessible
Liquid (ctDNA)	Minimally invasive (e.g. blood, saliva urine, CSF)	RNA and protein analyses not possible
	Minimal procedural risk	Short DNA-fragment length
	Repeatable	Low levels of ctDNA
	Real-time measurement	Challenges with pre-analytical factors
	Marker of therapy effect and surgery	Limited clinical applications
	Possibility of capturing tumor heterogeneity	Individual levels between patients and cancer types

Abbreviations: CSF: cerebrospinal fluid

2.2.3.2 CtDNA and EOC

EOCs are known to be histologically and molecularly heterogeneous, and especially HGSC is characterized by frequent chromosomal instability (Cancer Genome Atlas Research Network et al., 2011; Giannopoulou, Kasimir-Bauer and Lianidou, 2018). Recent studies on the ctDNA of EOC patients have been directed at chromosomal abnormalities, somatic mutations and aberrant methylation (Giannopoulou, Kasimir-Bauer and Lianidou, 2018). So far, the studies on early stage EOC diagnosis with ctDNA have presented ambivalent and complicated results: several studies show good discrimination in the cfDNA of controls and cancer patients (Zhang et al., 2013; Cohen et al., 2016; Widschwendter et al., 2017); however, contradictory results have also been reported (No et al., 2012). A recent meta-analysis including the data of 1125 patients and 1244 controls (22 studies) showed a pooled area under the curve (AUC) of 0.896 for ctDNA, which is similar to the AUC of CA125 (0.883) (Li et al., 2019). However, the comprehensive differences in methodology and laboratory environments were reported as significant confounding factors. Although there is

increasing evidence of the utility of ctDNA in EOC diagnostics, standardized and prospective studies are warranted for a more robust level of evidence.

As in other solid tumors, the longitudinal tracking of ctDNA has been demonstrated to be a beneficial method in the detection of MRD during the treatment and asymptomatic relapse of patients with EOC (Forshew et al., 2012; Martignetti et al., 2014; Pereira et al., 2015; Parkinson et al., 2016; Kim et al., 2019). Pereira et al demonstrated that the serial measurement of ctDNA during treatment performed similarly as the conventional CA125 monitoring. In addition, elevated plasma concentrations of ctDNA were detected on average 7 months before detectable relapse with radiologic or conventional serologic methods (Pereira et al., 2015). A case study showed that longitudinal ctDNA tracking is a promising tool in reveling new tumor-specific gene fusions, which might enable the use of precision drugs in selected patients (Martignetti et al., 2014). Further, the authors concluded that ctDNA monitoring results were consistent with the presence of residual disease (Martignetti et al., 2014). Parkinson et al studied the concentration of tumor protein 53 (TP53) mutations in EOC patients and similarly suggested that the ctDNA load correlates well with tumor volume. The longitudinal tracking of ctDNA also gave prognostic information, as the decrease of <60% in the TP53 mutant allele fraction (MAF) after the initial cycle of chemotherapy predicted disease relapse within 6 months (Parkinson et al., 2016). A recent study also evaluated the utility of TP53 mutation tracking as a modality in EOC treatment monitoring, and concluded that the longitudinal tracking of TP53 is clinically efficient and outperforms CA125 (Kim et al., 2019). Currently, the strongest evidence on the clinical utility of ctDNA in patients with EOC lies in the treatment monitoring of EOC and detection of MRD (Asante et al., 2020).

The selective pressure induced by conventional chemotherapy (e.g. platinum agents) or even targeted drugs, such as PARPis, might eventually result in the transformation of EOC cells through additional gain or reversion of genetic aberrations (Asante et al., 2020). A recent study detected several mutations associated with treatment resistance in the plasma ctDNA of three EOC patients (RB1, MTOR, ZEB2, CES4A, BUB1 and PARP8) after they received treatment with either cisplatin, paclitaxel or liposomal doxorubicin (Murtaza et al., 2013). Similar results were reported in an earlier study, in which a de novo mutation of the tyrosine kinase domain (exon 21) of EGFR was detected in the ctDNA of one patient with EOC (Forshew et al., 2012). An essential initiator in both platinum and PARPi resistance in BRCA-mutant EOC is the reverse mutation of the BRCA gene (Lin et al., 2019). A BRCA reverse mutation results in the regained function of the opening reading frame and a subsequent functional homologous recombination repair (Christie and Bowtell, 2017). Interestingly, a recent study detected the BRCA reverse mutations to be generally subclonal, which indicates a multifactorial resistance mechanism or that the resistant cancer cells might interact with and protect more sensitive cells (Christie and Bowtell, 2017). However, these studies on the potential of ctDNA in guiding treatment

decisions utilized panels with a specific set of genes (a few to tens of genes), which decreases the probability of detecting relevant mutations. In conclusion, the genetic factors behind acquired treatment resistance in EOCs are heterogeneous, and further studies on the utility of ctDNA tracking in mutation-driven resistance are needed to elucidate these mechanisms.

3 Aims of the study

The principal aim of this thesis was to evaluate the potential of novel biomarkers in ovarian cancer diagnosis, treatment monitoring and follow-up. In addition, we aimed at paving the way for future, more comprehensive validation studies on these biomarkers.

The distinct aims of the study were:

1. To evaluate the applicability of CA125 glycoform measurement in the differential diagnostics of pelvic masses.
2. To examine the utility of longitudinal CA125 glycoform measurement in the treatment monitoring and follow-up of HGSC.
3. To analyze comprehensively the feasibility of serum HE4 measurement in the treatment monitoring and prognostic stratification of HGSC.
4. To evaluate the benefit of ctDNA tracking in the timely identification of therapy resistant HGSC.

4 Materials and Methods

4.1 Patients, Study Design and Protocol

The study population was comprised of two cohorts prospectively collected in tertiary referral hospitals in Finland (I, II, III, IV) and Norway (I). In Turku University Hospital, Finland, patients have been recruited since 2009 for a clinical study on EOC biomarkers (ClinicalTrials.cov identifier: NCT01276574) and patients recruited between 2009 and 2019 were included in the current study. Women with endometriosis and healthy controls recruited for the ENDOMET study were included in the benign control group in the Study I (ClinicalTrials.cov identifier NCT01301885). The Norwegian cohort was recruited at the Oslo University Hospital during 2012–2015.

The inclusion criteria of the study were histologically confirmed EOC (I, II, III, IV), a benign gynecologic disease (I) and age of majority (I, II, III, IV). In addition, all patients included in Study II and III had at least three longitudinal serum samples taken during treatment. In addition, specific inclusion criteria in Study III was normal serum creatinine and/or glomerular filtration rate. The mean BMI of the patients included in Study III was 26.49 kg/m2. The specific characteristics of the study populations are presented in Table 5.

The diagnosis of EOC was based on a clinical examination, TVS, serum biomarker levels (CA125 and HE4) and a CT scan, and the diagnosis was histologically confirmed with tissue biopsies. Baseline study serum and plasma samples were drawn at the time of diagnosis, prior to any surgical or oncological interventions. Patients underwent a diagnostic laparoscopy and a treatment decision of either PDS or NACT was made by a team of experienced gynecologic oncologists. The amount of residual disease after cytoreductive surgery was evaluated by the operating gynecologic oncologist.

Table 5. Patient characteristics per study.

	Study I	Study II	Study III	Study IV
N (EOC)	232	122	143	12
N (benign)	317	0	0	0
N (healthy)	36	0	0	0
FIGO (2014)				
I	50	2	0	0
II	10	3	1	1
III	122	77	85	9
IV	43	40	57	2
Histology				
HGSC	158	122	143	12
LGSC	24	0	0	0
Clear cell	15	0	0	0
Endometroid	23	0	0	0
Mucinous	12	0	0	0

In the Turku cohort, the tumor spread in the abdominal cavity and retroperitoneum was carefully assessed, and a previously described disease dissemination score was calculated (Table 6) (II, III) (Isoviita et al., 2019). Patients were divided into a low tumor load group (dissemination score 0–12) and a high tumor load group (dissemination score 13–21). Platinum- and taxane-based chemotherapy was routinely administered; however, 16 patients received single agent carboplatin because of frailty. Longitudinal serum/plasma samples were collected in the Turku cohort before each cycle of chemotherapy during primary treatment and at follow-up visits to the outpatient clinic approximately every 3–6 months (II, III, IV).

We determined the concentrations of serum CA125 and its glycoforms (CA125-MGL and -STn) from the baseline samples of patients with confirmed EOC, benign gynecologic diseases (benign tumors, adnexal torsion, pelvic inflammatory disease and endometriosis) and healthy controls (I). In patients with HGSC, longitudinal serum CA125, HE4, CA125-MGL and -STn measurements were done from samples collected during primary chemotherapy, follow-up and until first progression (II, III). Longitudinal plasma samples acquired from 12 HGSC patients were used in the identification of actionable ctDNA mutations (IV).

Table 6. Disease dissemination score, range 0–21. Modified from (Isoviita et al., 2019).

	Points			
Anatomic location	**0**	**1**	**2**	**3**
Pelvic carcinomatosis	No	Yes		
Subdiaphragmatic surface carcinomatosis	No	Yes		
Carcinomatosis around the peritoneal cavity	No	Yes		
Small bowel mesentery carcinomatosis	No	Yes		
Small bowel mesentery retraction	No	Yes		
Large bowel mesentery carcinomatosis	No	Yes		
Small bowel serosa carcinomatosis	No	Yes		
Large bowel serosa carcinomatosis	No	Yes		
Invasion to bowel mucosa	No	Yes		
Largest omental nodule, cm	-	<2	2–5	>5
Largest right ovary nodule, cm	-	<10	≥10	
Largest left ovary nodule, cm	-	<10	≥10	
Pelvic lymph node metastasis, suspected	No	Yes		
Para-aortic lymph node metastasis, suspected	No	Yes		
Spleen metastasis	No	Yes		
Invasion to abdominal wall	No	Yes		
Invasion to liver surface	No	Yes		

4.2 Laboratory Methods

4.2.1 CA125 (I, II, III)

Venous whole blood samples (10ml) were collected into serum separation tubes. Samples were incubated for 30–60 minutes at room temperature and centrifuged for 15 minutes. Serum was separated and stored in -70 – -80 °C. The serum CA125 values (U/ml) for the Turku EOC cohort and benign controls were determined using a clinically well-established ECLIA method on the Cobas e 601 instrument or a Modular E170 automatic analyzer (linearity 2.0–5000.0 U/ml, inter-assay coefficient of variability, CV%, 0.7–3.1 and intra-assay CV% 1.8–6.5) (Roche Diagnostic GmbH, Mannheim, Germany). The Oslo EOC cohort and endometriosis controls were measured manually using a CA125 EIA kit (linearity 1.5–500.0 U/ml, inter-assay CV% 3.1–4.0 and intra-assay CV% 2.9–4.4) (Fujirebio Diagnostics Inc., Malvern, PA, USA) in accordance with the guidelines of the manufacturer. The correlation of the assays is good, and they have comparable slopes, r=0.989 (y=0.99 + 5.3) (Fujirebio). Moreover, the assays have the same cut-off of 35.0 U/ml.

4.2.2 CA125-MGL and -STn (I, II)

The measurements of serum CA125-MGL and -STn with in-house time resolved fluorometry (TRF) immunoassays were performed by a blinded investigator in an identical fashion. Firstly, biotinylated capture Ov185 monoclonal antibody or Ov185 F(ab')2 (50 ng/30 µl /well) were immobilized to streptavidin-coated low-fluorescence microtiter wells (Kaivogen Oy, Turku, Finland) in the assay buffer for 60 min at room temperature (RT) without shaking. After washing twice, 25 µl of standard (OVCAR-3 cell line purified CA125) or diluted serum sample was added in triplicates and incubated for 60 min at RT with shaking. Samples were diluted 1:5 and 1:10 in buffer solution for CA125-MGL and CA125-STn, respectively. After washing twice for CA125-MGL and four times for CA125-STn, the captured CA125 antigen was incubated with the Eu+3-chelate-doped Fluoro-MaxTM polystyrene nanoparticles (NPs) (Seradyn Inc., Indianapolis, IN) conjugated with human lectin-MGL (1×107 /25 µl /well) or with STn-specific antibody (5×106/25 µl /well) for 90 minutes at RT with shaking. After incubation, the wells were washed six times with wash buffer. The time-resolved fluorescence for Eu+3 was then measured from dry wells using VictorTM 1420 Multilabel counter. The linearity of the CA125-STn assay was 0.0–500.0 U/m and the intra-assay CV% was <10.0. The linearity of the CA125-MGL assay was 0.0–100.0 U/ml and the intra-assay CV% was <15.0. The inter-assay CV% for both of the assays was <20.0. The principle of the assays is schematically shown in Figure 4.

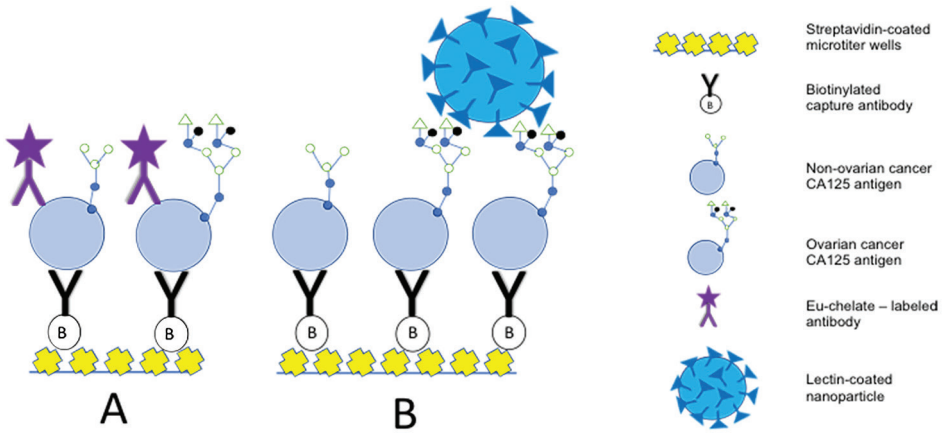


Figure 4. Schematic pictures of A) the conventional CA125 immunoassay, in which the capture and tracer monoclonal antibodies detect protein epitopes of CA125 and B) the CA125-MGL and -STn assays, in which the lectin or antibody coated nanoparticles bind to cancer specific CA125 glycan structures.

4.2.3 HE4 (III)

Venous whole blood samples (10ml) were drawn into serum separation tubes. Samples were incubated for 30–60 minutes at room temperature and centrifuged for 15 minutes. Serum was separated and stored in -70 – -80 °C. Samples were thawed to +4 °C prior to analyses. The serum HE4 concentrations were determined with the EIA method according to the instructions provided by the manufacturer (linearity 20.0–1500.0 pmol/L, inter-assay CV% < 3.2 and intra-assay CV% < 3.5) (Fujirebio Diagnostics Inc., Malvern, PA, USA).

4.2.4 CtDNA (IV)

Venous whole blood (2 x 10ml) samples were drawn into EDTA tubes, shaken 8–10 times and centrifuged for 10 minutes at 2000 x g. The plasma was separated and centrifuged further for 10 minutes. The above-mentioned initial processing of the samples was done within a timeframe of 30 minutes from the phlebotomy. The plasma samples were aliquoted and stored at -80 °C.

Tissue and ascites samples for the evaluation of ctDNA and tumor tissue DNA correlation were collected during diagnostic laparoscopy and debulking surgery, and samples from the ovary and omentum were promoted. Ascites samples were analyzed for patients with a recurrent disease if tumor tissue samples were not evaluable. The extraction of the tissue DNA was done with the AllPrep DNA/RNA kit (Qiagen) at the University of Turku. Whole blood buffy coat samples for the detection of germline mutations were taken at baseline and the DNA was extracted by Auria biobank.

Plasma samples with sufficient concentrations and quality of DNA were sent to BGI Genomics (BGI Europe A/S, Denmark) for library preparation and sequencing. The Oseq™ ctDNA solid tumor panel with 508 actionable genes were utilized for the mutation tracking of ctDNA. The samples were subjected to 1000X coverage. Tissue and blood cell (germline) samples were similarly sequenced at BGI Genomics and the samples were subjected to 200X coverage. BGI is a provider of genomic sequencing and proteomic services with over 20 years of experience in the field.

The analysis pipeline is presented in Figure 5. Variant detection and filtering were performed in Anduril (Ovaska et al., 2010), a bioinformatics workflow optimized for large data sets. Somatic single nucleotide variants (SNVs) were detected from mapped and cleaned sequencing data with MuTect2. Matched tumor-normal pairs and a panel of normals were used for the filtering of systematic artifacts of sequencing. All SNVs and indels were reported as variant allele frequency (VAF), to achieve a more robust measurement of sequencing depth and DNA content than read counts. Variant annotations were made in Annovar (Wang, Li and Hakonarson, 2010) with the databases RefSeq (O'Leary et al., 2016), COSMIC (Forbes et al.,

2017) and dbSNP, and the CADD functional scores (Kircher et al., 2014). The inclusion criteria for variants were 1) at least three existing reads supporting the variant allele and a VAF of >0.01 in at least one sample 2) a minimum coverage of 100 in plasma and 30 in tumor tissue samples and 3) a matched blood control sample with a minimum coverage of 30, VAF <0.01 and a maximum of 2 reads supporting the variant allele. Further mutation filtering included the detection of likely spurious mutations by identification of clusters of variants shared by at least three patients and not previously reported in databases. A CADD score filter (only mutations with a score of >15) was used to exclude non-pathogenic mutations. The detection of copy number alterations (CNAs) were performed with PanelDoc (http://github.com/ammawla/PanelDoC) and CNVPanelizer (http://bioconductor.org/packages/CNVPanelizer/). PanelDoc was chosen for initial CNA detection and calling, as it showed higher sensitivity and correlation than CNVPanelizer in low-purity plasma samples. Consequently, CNVPanelizer was used for the verification of the calls. This procedure ensured that the CNA calls were not based on errors in the PanelDoc software. A read depth ratio of 0.694 was considered as the upper threshold for loss of function and 1.652 was similarly considered the lower threshold for gain. The SNP array analyses and targeted sequencing results of each patients CNA profiles in plasma and tissue samples were compared. This procedure ensured the presence and quantity of ctDNA in the plasma samples. CNAs previously acknowledged as actionable in treatment related toxicity, drug sensitivity and resistance were prioritized. The portion of ctDNA (tumor content) in plasma cfDNA was determined by tracking the TP53 mutation VAF. In patients with a tissue TP53VAF below 40%, other somatic mutations were used for the tumor content estimate. The tracking of longitudinal mutation changes was done by identifying the largest proportion of significantly decreased variants during chemotherapy.

Putative, clinically actionable mutations acknowledged by ESCAT (the European Society for Medical Oncology Scale for Clinical Actionability of Molecular Targets) were validated through immunohistochemistry and in situ hybridization in patients with the existing mutations in tumor tissue (ERBB2 amplification and/or mTOR pathway activation).

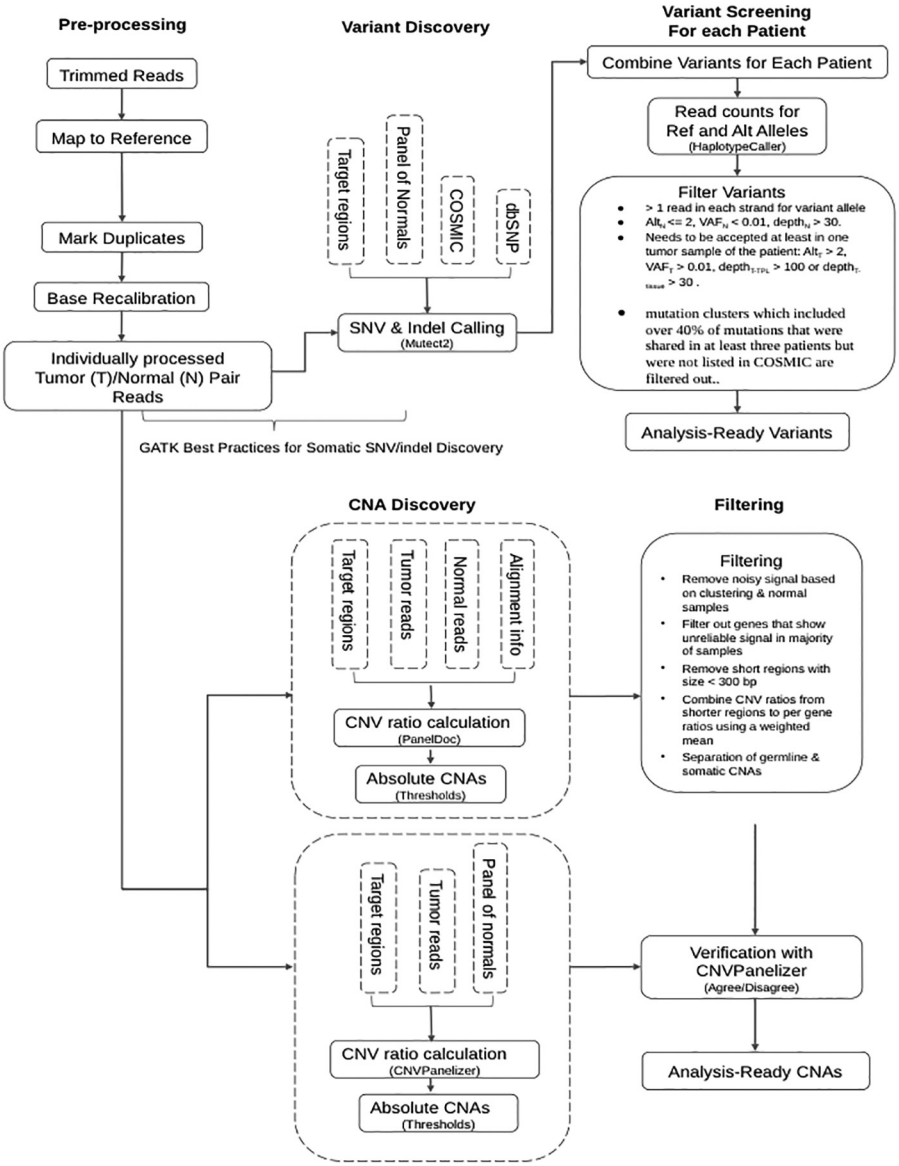


Figure 5. Analysis pipeline for detection of mutations and CNAs. From the Study IV.

4.3 Definitions

The clinically established cut off of 35.0 U/ml was used for the conventional CA125 assay. Cut offs determined in earlier studies were used for the CA125-MGL and -STn assays: 2.0 U/ml and 10.0 U/ml, respectively (I) (Gidwani et al., 2016, 2019).

Complete cytoreduction was defined as 0mm residual disease. The nadir value was defined as the lowest conventional serum CA125 value during primary treatment or within 3 months after treatment. The response to primary treatment was determined according to the Response Evaluation Criteria in Solid Tumors (RESIST) version 1.1 criteria (John et al., 2011). The response was evaluated after the conclusion of primary treatment and it was based on a clinical examination, a CT scan and the serum CA125 level. Patients were classified as complete responders, partial responders or to have stable or progressive disease. Disease progression was determined with serological and/or radiologic criteria also in accordance with the RESIST 1.1 guidelines (John et al., 2011). Progression free survival (PFS) was calculated from diagnosis to relapse detection.

4.4 Statistical Analyses

The statistical analyses were run on R (Version 3.3.3.) and IBM SPSS software (IBM Corp. Released 2017. IBM SPSS Statistics for Macintosh, Version 25.0. Armonk, NY: IBM Corp). $P < 0.05$ was considered statistically significant in all analyses.

4.4.1 Study I

Initially, a sample size calculation was made to ensure a sufficient statistical power for the study. The calculation was done for both the CA125-MGL and -STn assays, and an appropriate statistical power of 80% was reached. The medians, 25th and 75th quartiles of the Turku and Oslo cohorts were compared, and they were combined for further analyses, as the behavior of the biomarkers were similar in the two cohorts. An age bias was detected, and linear regression was utilized for all biomarker assays in order to correct for the age-related trends. The normality of the biomarker values in different diagnostic groups were assessed visually and statistically with the Shapiro-Wilk test. A logarithmic transformation was made to correct for the skewness of the data. The Levene's test was run to evaluate the equality of variances in the biomarker levels of different diagnostic groups. The one-way analysis of variance (ANOVA) test was then used to compare the differences in means between diagnostic groups. The confidence intervals were also assessed visually. Multiple comparisons were performed with the Tukey's honest significant difference (HSD) test or the Games-Howell test, according to the homogeneity of variances.

To evaluate the diagnostic potential of the biomarkers, we generated receiver operating characteristic (ROC) curves in different subgroups. The areas under the curves (AUCs) were compared with the DeLong method (DeLong, DeLong and Clarke-Pearson, 1988). In addition, sensitivities for each biomarker assay were calculated at a fixed specificity of 90% to further examine the diagnostic feasibility of the markers.

4.4.2 Study II

The longitudinal dynamics of the biomarkers during treatment were evaluated separately in patients treated with PDS and NACT. The medians, 25th and 75th quartiles were similar in both of the treatment arms and the groups were combined for the subsequent analyses. We made a logarithmic transformation to correct for the non-gaussian skewness of the data, and baseline and preoperative biomarker values were compared to different end points (tumor load, residual disease, treatment response, PFS and OS) with the one-way ANOVA and t tests. Nadir values were determined for each biomarker assay and the optimal nadir cut offs to PFS were measured from ROC curves with the Youden index method (Youden, 1950). PFS was dichotomized as progression vs. no progression, and the median follow-up in the progression free group was 19.6 months. We evaluated the correlation of the nadir values to PFS with Kaplan-Meier survival curves, and the statistical significance was benchmarked with the log rank test and the Cox's proportional hazards model. Clinically relevant factors were included in the multivariate model in a stepwise fashion. To evaluate disease relapse detection, we determined the serum biomarker concentrations from three separate follow-up samples preceding the detection of relapse. The dynamics of the biomarkers during follow-up were evaluated and the number of patients with positive serum biomarker levels at each time point (exceeding the cut off) were calculated.

4.4.3 Study III

The biomarker kinetics during treatment were evaluated separately for the PDS and NACT treatment arms for the CA125 and HE4 assays, respectively. A logarithmic transformation was run to correct for the skewness of the data. We evaluated the association of the baseline HE4 and CA125 concentrations to different end points (residual disease, tumor load) with t tests. The feasibility of the biomarker nadir values to predict platinum resistant progression were evaluated with Kaplan-Meier curves, and the statistical significance was explored with the log rank test and the survival curves were also assessed visually. The median nadir values, 14.0 U/ml and 57.2 pmol/L, for the CA125 and HE4 assays were used as cut offs. The association of the

biomarker values at progression and survival (PFS, OS) were evaluated with Kaplan-Meier curves and the Cox's proportional hazards model. As OS has been suggested to be a suboptimal end point in cancer patients with a long survival post progression (SPP) (>6 months), we further evaluated the association of the biomarker levels at progression to SPP with Kaplan-Meier curves and the Cox's proportional hazards model in an identical manner as described above.

4.4.4 Study IV

Study IV was designed and carried out as a proof-of-concept study. Fishers exact test was run to detect significant changes in mutation frequency.

4.5 Ethical Aspects

All study participants signed a written consent at the time of enrolment. The data was handled and analyzed according to the information security laws of the European Union. The study was evaluated and accepted by the Ethics Committee in the Hospital District of Southwest Finland (I, II, III, IV) and the Regional Ethics Committee in South East Norway (I).

5 Results

The results presented here are reported in the original Studies I–IV. Additional, unpublished data on the potential of the conventional CA125, CA125-STn and -MGL assays to differentiate EOC from non-EOC malignancies are reported in chapter 5.1.1. The results of the longitudinal studies (II and III) are presented in the same chapters for the reader's convenience, despite the slight differences in the study cohorts. In addition, unpublished, auxiliary results on the performance of the CA125 glycoforms and HE4 are presented in Chapter 5.2.3.

5.1 CA125 Glycoforms and EOC Diagnosis (I)

5.1.1 CA125 Glycoforms are EOC-specific Biomarkers

The median CA125, CA125-STn and -MGL concentrations were calculated for each histological subgroup of EOC, benign gynecological diseases, non-EOC malignancies and healthy controls. All three biomarker assays presented significantly elevated serum concentration in serous, clear cell and endometroid carcinomas compared to benign gynecological diseases (Table 7). However, mucinous carcinoma was not adequately detected by any of the assays ($p=0.103$, $p=1.00$ and $p=0.157$ for the CA125, CA125-STn and -MGL assays, respectively).

The control cohort with healthy women was compared to those with benign gynecological diseases to evaluate the EOC-specificity of the glycoform assays. The conventional CA125 assay presented significantly elevated serum concentrations in benign gynecological tumors ($p=0.001$), pelvic inflammation and adnexal torsion ($p=0.047$), and endometriosis ($p<0.001$) (Table 7). Altogether, the conventional CA125 assay presented falsely elevated serum levels in 36.6% (116/317) of patients with benign diseases (Table 8). Contrarily, significantly elevated CA125-STn concentrations were detected only in endometriosis ($p<0.001$), and not in benign tumors ($p=0.14$), pelvic inflammation or adnexal torsion ($p=0.58$). The CA125-MGL assay showed significantly elevated concentrations in benign tumors $p=0.011$) and endometriosis ($p<0.001$). The CA125-STn and -MGL assays presented concentrations exceeding the cut offs in 17.0% and 27.8% of patients with benign gynecological

Table 7. Biomarker medians, 25th and 75th quartiles for the different diagnostic groups. P-values from the comparisons of means are presented. Reference 1 is healthy controls and the biomarker means of benign gynecological diseases are compared to it. Reference 2 is the mean of benign gynecologic diseases to which the means of the different EOC histologies are compared to. Modified from Study I. * P<0.05.

Histology	N	Age	CA125 (U/ml)	p-value	CA125-STn (U/ml)	p-value	CA125-MGL(U/ml)	p-value
Healthy	36	37	8.3 (6.3–12.9)	Ref. 1	1.3 (0.6–2.7)	Ref. 1	0.6 (0.1–0.9)	Ref. 1
Benign	317		22.0 (12.9–52.0)	Ref. 2	2.9 (1.5–2.3)	Ref. 2	1.0 (0.5–2.3)	Ref. 2
Neoplasms	126	56	26.0 (15.0–57.5)	0.001*	2.6 (0.8–8.4)	0.14	0.9 (0.5–2.4)	0.011*
Non-neoplastic	8	43	28.0 (11.5–50.5)	0.047*	2.5 (0.8–8.4)	0.58	1.5 (0.2–3.9)	0.96
Endometriosis	183	36	19.0 (11.0–48.6)	<0.001*	3.2 (1.8–8.9)	<0.001*	1.0 (0.6–2.2)	<0.001*
Ovarian cancer	232							
High grade serous	158	65	644.5 (243.3–1438.8)	<0.001*	382.4 (89.9–1071.0)	<0.001*	74.4 (19.8–165.8)	<0.001*
Low grade serous	24	59	308.5 (90.8–844.0)	<0.001*	23.6 (7.5–187.5)	<0.001*	8.0 (3.1–51.3)	<0.001*
Clear cell	15	59	78.7 (45.0–382.0)	0.014*	31.9 (7.6–70.1)	0.035*	6.8 (1.5–24.4)	<0.001*
Endometroid	23	63	175.0 (67.0–593.0)	<0.001*	15.9 (3.6–135.7)	0.001*	11.2 (7.1–27.7)	<0.001*
Mucinous	12	60	77.0 (29.5–179.8)	0.103	4.0 (1.4–15.0)	1.00	3.4 (1.1–13.3)	0.157
Stage (FIGO2014)								
I	50	63	73.0 (38.5–202.8)	<0.001*	9.0 (3.9–46.8)	0.002	6.9 (1.7–12.8)	<0.001*
II	10	61	531.0 (153.5–882.3)	<0.001*	210.0 (45.5–474.0)	<0.001*	58.2 (22.9–108.2)	<0.001*
III	122	65	574.0 (239.0–1265.8)	<0.001*	306.6 (35.8–1050.0)	<0.001*	69.2 (13.6–165.6)	<0.001*
IV	43	62	773.0 (365.0–1716.0)	<0.001*	263.6 (52.3–1121.8)	<0.001*	81.5 (27.7–184.2)	<0.001*
Unknown	7							

diseases, which is significantly less than with the conventional CA125 assay ($p<0.0001$ and $p=0.018$, for the CA125-STn and -MGL assays, respectively) (Table 8).

Table 8. Women (N and %) with benign gynecological diseases or non-EOC malignancies and elevated CA125. Modified from Study I.

Histology	**CA125 (>35.0 U/ml)**		**CA125-MGL (>2.0 U/ml)**		**CA125-STn (>10.0 U/ml)**	
	N	%	N	%	N	%
Benign (all)	**116**	**36.6**	**88**	**27.8**	**54**	**17.0**
Neoplasms	50	39.7	35	27.8	12	9.5
Non-neoplastic	3	37.3	3	37.5	1	12.5
Endometriosis	63	34.4	50	27.3	41	22.4
Non-EOC malignancy*	**16**	**84.2**	**16**	**84.2**	**5**	**26.3**

*7 GI-carcinomas, 1 GIST, 1 biliary carcinoma, 1 SCC in MCT, 1 STUMP, 3 soft tissue sarcomas, 2 uterine endometroid carcinomas, 1 lymphoma, 2 unspecified non-EOC carcinomas

Interestingly, elevated serum CA125-STn levels were detected in particularly few non-EOC malignancies (5/19 patients, 26.3%) (Table 8). In contrast, conventionally measured CA125 and CA125-MGL were elevated in nearly all patients with non-EOC malignancies (16/19 patients, 84.2% for both of the assays) (Table 8). We examined the discriminative potential (EOC vs. non-EOC malignancy) of the assays further with ROC curves, in which the CA125-STn performed with the highest AUC (0.852, CI: 0.785–0.919) (Figure 6).

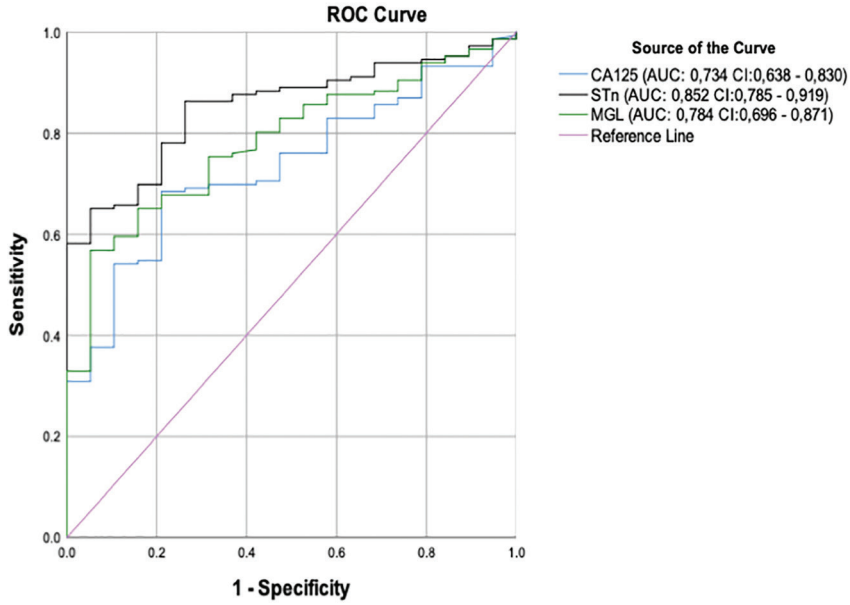


Figure 6. ROC curves of the conventional CA125, CA125-STn and -MGL assays in discriminating EOC (N=232) from non-EOC malignancies (N=19).

5.1.2 The CA125 Glycoforms Improve the Detection of EOC

Patients with marginally elevated concentrations of conventionally measured CA125 generally present a diagnostic challenge for the attending gynecologist. We defined an arbitrary range of 35.0–300.0 U/ml for marginally elevated total CA125 levels to evaluate the applicability of the biomarkers in this subgroup comprehensively. Specific interest was directed at the postmenopausal population, as the number of premenopausal EOC patients in the current cohort was low.

Initially, the whole cohort (EOC N=232, benign N=317) was included in the ROC curve analysis, in which the combination of the CA125-STn and -MGL assays performed with the highest sensitivity at a fixed specificity of 90.0% (61.6%, p=0.014 vs. 54.3% for the conventional CA125 assay). However, the AUC of the combined glycoform assays were similar to that of the conventional CA125 assay (p=0.107) (Table 9). Next, the postmenopausal subpopulation (EOC N=204, benign N=84) was analyzed and interestingly, the combination of the CA125-STn and -MGL exceeded the diagnostic ability of the conventional CA125 assay (p=0.021, AUC comparison, Table 9). This result was further reinforced in the marginally elevated subgroup (EOC N=63, benign N=35), in which the CA125-STn assay was superior in discriminating EOC from benign disease compared to the conventional CA125 assay (Figure 7).

Although both of the glycoform assays exceeded the diagnostic ability of the conventional CA125 assay in this subgroup (p=0.042 and p=0.0023 for CA125-MGL and -STn, respectively), there was no additional diagnostic improvement to the AUC of the CA125-STn assay when the glycoform assays were combined (Table 9).

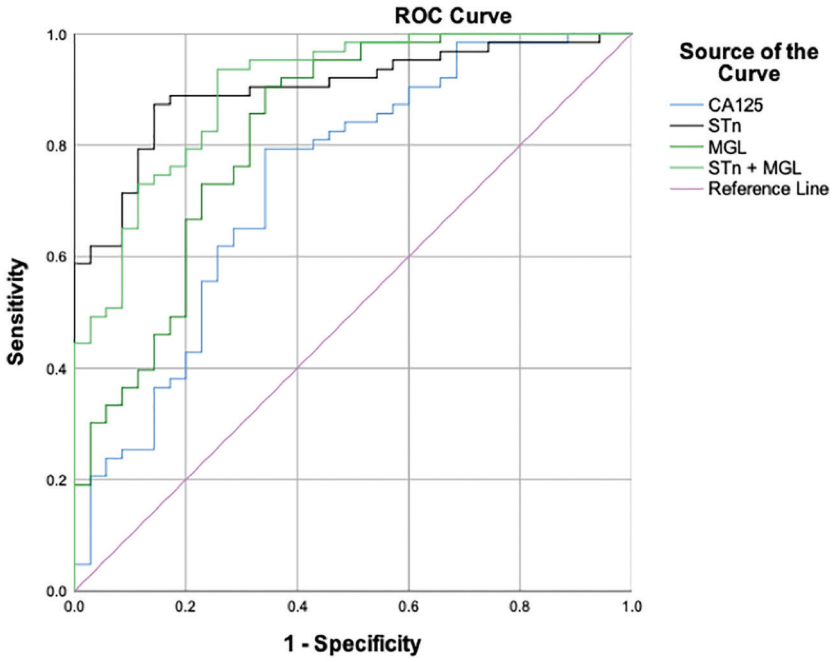


Figure 7. ROC curves of the biomarkers in postmenopausal patients with marginally elevated CA125 (35.0–300.0 U/ml). N EOC: 63, N benign: 35. Modified from Study I.

Table 9. The AUCs, the comparisons of AUCs (p-value) and sensitivities at a fixed specificity for the different biomarkers. Modified from Study I. *P<0.05.

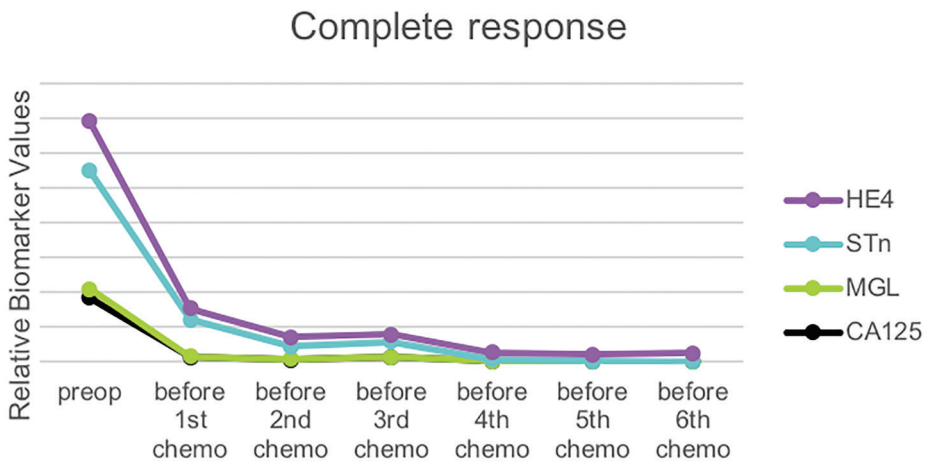
Subgroup	N EOC	N benign	Biomarker	AUC (95% CI)	AUC comparison	Specificity	Sensitivity	p-value
All patients	232	317	CA125	0.78 (0.74–0.83)	Reference	90.0	54.3	**Ref.**
			STn	0.80 (0.76–0.83)	0.75	90.0	59.5	**0.08**
			MGL	0.80 (0.76–0.84)	0.32	90.0	58.6	**0.15**
			STn + MGL	0.81 (0.77–0.85)	0.11	90.0	61.6	**0.01***
Postmenopausal	204	84	CA125	0.90 (0.86–0.93)	Reference	90.0	73.5	**Ref.**
			STn	0.92 (0.89–0.95)	0.18	90.0	84.8	**0.0009***
			MGL	0.91 (0.87–0.94)	0.32	90.0	73.5	**1.0**
			STn + MGL	0.93 (0.90–0.96)	0.02*	90.0	81.9	**0.02***
Postmenopausal	63	35	CA125	0.73 (0.63–0.84)	Reference	90.0	25.4	**Ref.**
CA125 35–300 U/ml			STn	0.90 (0.84–0.96)	0.002*	90.0	79.4	**< 0.001***
			MGL	0.82 (0.73–0.91)	0.04*	90.0	39.7	**0.03***
			STn + MGL	0.90 (0.83–0.96)	0.0004*	90.0	65.1	**< 0.001***

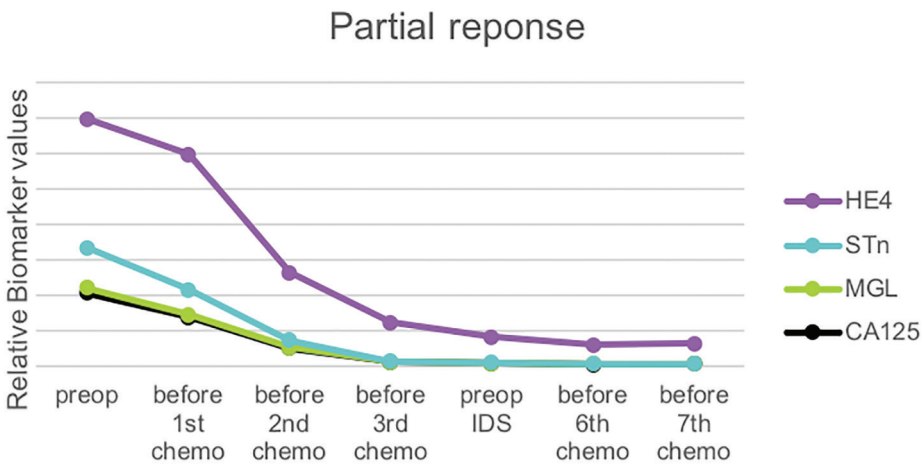
5.2 Longitudinal CA125-STn, -MGL and HE4 Analyses (II, III)

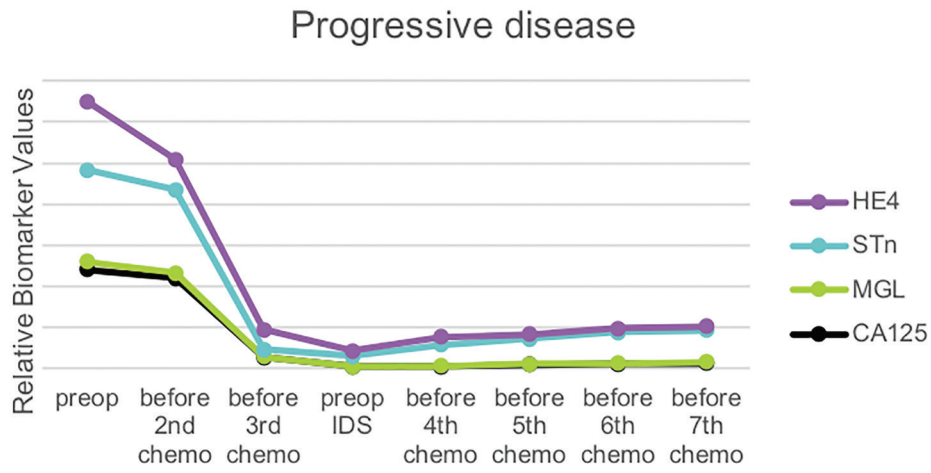
5.2.1 CA125 Glycoforms and HE4 During Primary HGSC Treatment

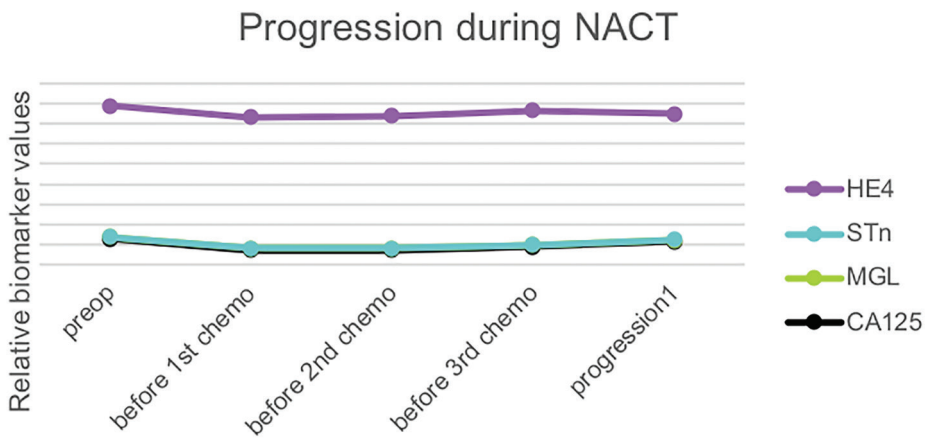
The number of HGSC patients treated with PDS and NACT were proportionate in both of the study cohorts: 45.5% (PDS) and 54.5% (NACT), in Study II and 40.6% (PDS) and 59.4% (NACT) in Study III. The median biomarker concentrations in the PDS groups at baseline were 923.5 U/ml, 431.2 U/ml, 82.5 U/ml for the conventional CA125, CA125-STn and -MGL assays (II), and 918.0 U/ml and 750.5 pmol/L for the conventional CA125 and HE4 assays (III). The median baseline biomarker values in the NACT groups were 709.0 U/ml, 517.2 U/ml, 80.7 U/ml for the conventional CA125, CA125-STn and -MGL assays (II), and 838.7 U/ml and 773.4 pmol/L for the conventional CA125 and HE4 assays (III). Individual biomarker profiles of patients with different responses to treatment were visually evaluated and the kinetics of the biomarkers were found to be consistent (Figure 8). The biomarker concentrations declined most rapidly during the three initial cycles of chemotherapy regardless of the treatment regimen. At the conclusion of primary therapy, the median biomarker concentrations were lower in the PDS group; however, the dissimilarity was not found to be significant and consequently the treatment arms were combined for the prognostic analyses.

Figure 8. Examples of individual biomarker profiles of patients during the primary treatment of HGSC. X-axis: time point during treatment, Y-axis: relative biomarker values (continues on the next page).




Partial reponse
Relative Biomarker values
preop
before 1st chemo
before 2nd chemo
before 3rd chemo
preop IDS
before 6th chemo
before 7th chemo
HE4
STn
MGL
CA125


Progressive disease
Relative Biomarker Values
preop
before 2nd chemo
before 3rd chemo
preop IDS
before 4th chemo
before 5th chemo
before 6th chemo
before 7th chemo
HE4
STn
MGL
CA125


Progression during NACT
Relative biomarker values
preop
before 1st chemo
before 2nd chemo
before 3rd chemo
progression1
HE4
STn
MGL
CA125

5.2.2 The CA125 glycoforms and HE4 are Indicators of Tumor Load

The baseline serum biomarker values were compared to the tumor load assessed during surgery and the CA125-STn, -MGL and HE4 assays, contrarily to CA125, differentiated patients with a high tumor load (dissemination score 13–21) from patients with low tumor load (dissemination score 0–12) (p=0.030, p=0.026 and p<0.0001 respectively) (Table 10). A similar comparison was done on the association between baseline biomarker concentration and residual disease after cytoreductive surgery. Interestingly, a significant difference was detected in the baseline CA125-STn and -MGL levels between patients with subsequent complete (R0) and suboptimal (R>0) cytoreduction (p=0.025 and p=0.022, respectively) (Table 10). Neither the conventional CA125 assay nor the HE4 assay distinguished between completely and deficiently debulked patients (p=0.154 and p=0.054), although HE4 was trending toward statistical significance (Table 10). No significant associations between the baseline biomarker levels and survival were observed.

Table 10. Comparison of baseline biomarker values (median, 25th and 75th quartiles) between subgroups A) Study II B) Study III. Modified from Study II and III. *P<0.05.

A	**CA125 (U/ml)**	**CA125-MGL (U/ml)**	**CA125-STn (U/ml)**
Low tumor load	840.0 (440.0–2070.0)	69.4 (26.7–141.3)	243.9 (62.1–1048.6)
High tumor load	1024.5 (521.3–1846.8)	137.1 (51.6–211.3)	748.4 (258.6–1493.0)
P - value	**0.363**	**0.026***	**0.030***
R0	839.5 (362.0–1592.0)	54.6 (16.5–139.4)	219.7 (21.7–938.0)
R >0	918.0 (462.0–2323.8)	102.4 (33.8–206.9)	515.9 (170.8–1479.5)
P- value	**0.154**	**0.022***	**0.025***

B	**CA125 (U/ml)**	**HE4 (pmol/L)**
Low tumor load	652.0 (272.0–2105.0)	637.8 (346.1–891.0)
High tumor load	1038.0 (543.0–2342.5)	1043.6 (613.5–1189.0)
P - value	**0.067**	**< 0.0001***
R 0	848.2 (274.3–2048.8)	716.8 (314.7–964.1)
R>0	916.0 (489.0–2272.0)	821.4 (476.5–1131.2)
P - value	**0.641**	**0.054**

5.2.3 The CA125-STn and HE4 Nadirs Predict PFS

In Study II, 54.9% of the patients developed disease recurrence during the median follow-up time of 21.5 months (range 2.3–118.6). The median time from the end of primary treatment to the detection of recurrence was 12.8 months (range 1.1–46.0).

The median nadir values were 12.0 U/ml, 2.4 U/ml, 0.6 U/ml and 53.9 pmol/L for the conventional CA125, CA125-STn and -MGL, and HE4 assays, respectively. Optimal cut offs for the biomarker nadir values during primary treatment were independently determined for each assay with the Youden index method and they were as follows: 33.0 U/ml CA125, 0.8 U/ml CA125-STn, 0.6 U/ml CA125-MGL and 54.3 pmol/L HE4. The analyses were run with the optimal cut off values determined for each assay. In the univariate analysis, the nadir value of CA125-STn >0.8 U/ml and the nadir value of HE4 >54.3 pmol/L were significantly associated with shorter PFS (p=0.02 and p<0.001, log rank test) while the nadir values of the conventional CA125 assay (p=0.154, log rank test) and the CA125-MGL assay (p=0.129, log rank test) were not associated with the PFS of patients. The CA125-STn nadir remained an independent prognostic factor in the multivariate analysis, contrarily to other clinical factors (residual disease, disease stage and age). Interestingly, when the nadir value of HE4 >54.3 pmol/L was added to the multivariate model, it emerged as the only statistically significant covariate (p=0.004; HR 2.36 and 95% CI 1.31–4.26) (Table 11). In this model, the CA125-STn nadir >0.8 U/ml had a HR of 2.28 (95% CI 0.87–5.98, p=0.095) (Table 11). The biomarker nadir values were not associated with the OS of patients.

Table 11. Associations of the biomarker nadir values and selected clinical variables to PFS with univariate (log rank test) and multivariate analyses (Cox regression model). The hazard ratio (HR) demonstrates the probability of a shorter PFS in patients with a specific clinical attribute. Modified from Study II. *P<0.05.

Clinical variable	Univariate p-value	Multivariate p-value	Multivariate HR	95% CI
Elderly (age>65)	0.20	0.55	1.17	0.70–1.95
Disease stage (FIGO 2014): III vs IV	0.38	0.45	1.23	0.72–2.11
R >0mm	0.80	0.46	1.27	0.67–2.41
CA125 nadir > 33.0 U/ml	0.15	0.60	1.21	0.60–2.41
MGL nadir >0.6 U/ml	0.13	0.88	1.05	0.56–1.95
STn nadir > 0.8 U/ml	0.02*	0.095	2.28	0.87–5.98
HE4 nadir > 54.3 pmol/L	< 0.001*	0.004*	2.36	1.31–4.26

5.2.4 CA125 and HE4 Nadir Values Predict Platinum Resistant Relapse

The applicability of the CA125 and HE4 assays to detect platinum resistant progression was evaluated in the Study III. The median nadir values were 14.0 U/ml (interquartile range 20.0) and 56.7 pmol/L (IQR 54.2) for the CA125 and HE4 assays. Compared to the CA125 assay, an earlier HE4 nadir was detected in 19 (26.8%) patients and 23 (32.4%) patients had a later HE4 nadir. Platinum resistant relapse (PFI <6 months) was diagnosed in 26 (36.6%) patients during the follow-up period. Interestingly, the median CA125 and HE4 nadir values were different ($p<0.0001$, both assays) in the platinum resistant and platinum sensitive subgroups: 28.0 U/ml (IQR 41.0) and 87.7 pmol/L (IQR 103.3) in the platinum resistant group, and 10.5 U/ml (IQR 11.3) and 49.7 pmol/L (IQR 27.07) in the platinum sensitive group. The association of the biomarkers to platinum resistant progression was further evaluated in a time-to-event fashion with the Kaplan-Meier estimator curves. Nonetheless, patients with biomarker nadir values exceeding the median cut offs were at significantly higher risk of developing a platinum resistant progression (log rank test: CA125 $p<0.0001$ and HE4 $p=0.008$).

5.2.5 CA125 Glycoforms and HE4 in Disease Relapse

The potential of the glycoforms to detect disease relapse was evaluated from serial serum samples leading to relapse. Interestingly, the CA125-STn levels increased earlier than the conventionally measured CA125 levels in 37.0% (13/35) of patients preceding the detection of relapse. At the detection of relapse, 80.0% (28/35) of the patients had higher fold increase in the serum CA125-STn concentrations compared to the conventional CA125 concentration. The CA125-MGL assay did not detect disease relapse earlier than the conventional CA125 assay; however, 11.0% (4/35) of patients had a higher fold increase measured with the CA125-MGL assay compared to the conventional CA125 assay. The CA125 glycoform assays detected relapse with similar performance as the conventional CA125 assay in 20.0% (7/35) of patients. Importantly, the glycoform assays detected disease relapse better or with similar performance as the conventional CA125 assay in all patients. Examples of individual progression profiles are presented in Figure 9.

Longitudinal follow-up samples of HE4 were evaluable for 32 patients. A supplementary analysis was done to assess where the HE4 alight on the detection of relapsed disease in comparison to the conventional CA125 and the glycoform assays. The HE4 assay showed earlier increase in 34.0% (11/32) of patients, while the CA125-STn levels increased earlier that the conventional CA125 assay in 31.0% (10/32) of patients. The HE4 and CA125-STn assays showed earlier increase in an equal number of patients ($p=0.80$). Preceding relapse, the CA125-MGL levels did not increase earlier than the conventionally measured CA125. A higher fold increase

was detected in 94.0% (30/32) of patients with the HE4 assay and in 81.0% (26/32) of patients with the CA125-STn assay (p=0.12). Three patients (9.0%) had a higher fold increase measured with the CA125-MGL assay compared to the conventional CA125 assay. A similar performance to that of the conventional CA125 assay was detected in 3.0%, 15.0% and 91.0% of patients with the HE4, CA125-STn and -MGL assays, respectively.

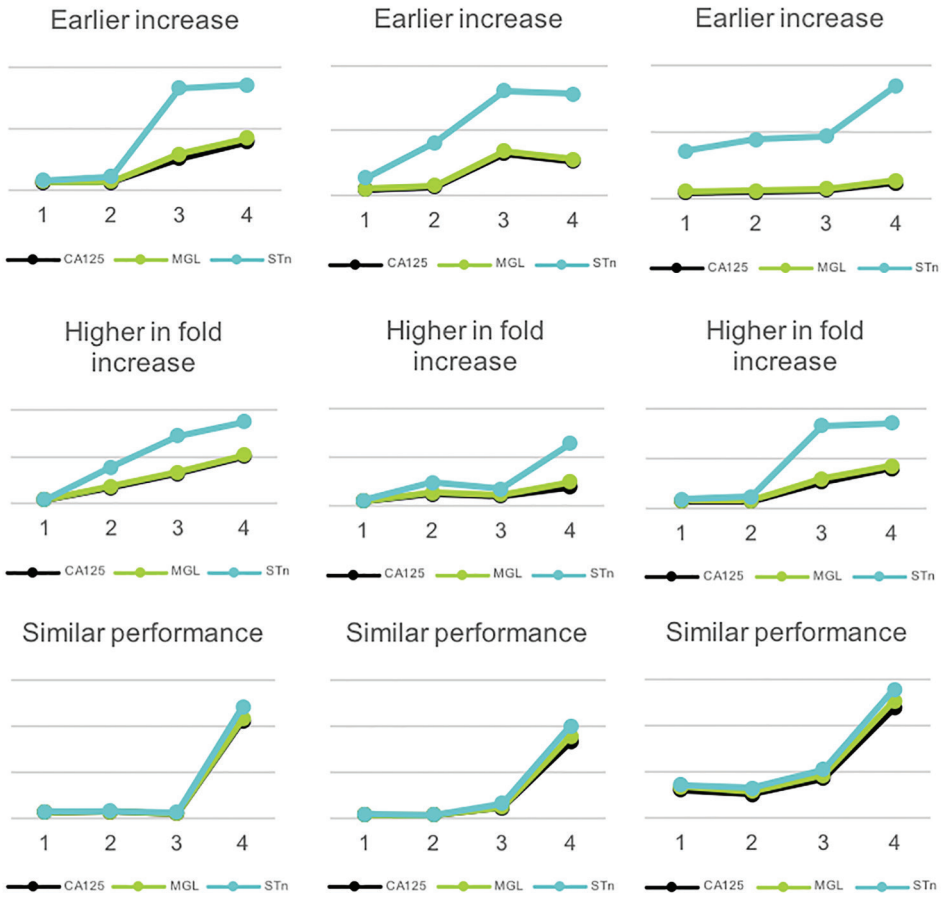


Figure 9. Examples of CA125, CA125-STn and -MGL profiles leading to the detection of relapse (time point 4). Y-axis: the relative biomarker values. Modified from Study II.

5.2.6 HE4 and the Prognosis of Relapsed Disease

The average follow-up time of the patients was 2.5 years (range: 1.5 months to 10.2 years) in Study III. The median survival post progression (SPP) was 16.0 months. At the time of progression, the median CA125 and HE4 concentrations were 162.0 U/ml and 199.2 pmol/L, respectively. Two endpoints (overall survival and survival post progression) were used to comprehensively analyze the applicability of the biomarkers in the prediction of the survival of the patients, as SPP has been suggested as a more appropriate endpoint in patients with a long (>6 months) survival after the detection of relapse (Broglio and Berry, 2009). In the univariate analysis, the serum HE4 concentration of >199.2 pmol/L was associated to both endpoints (OS: p<0.0001 and SPP: p=0.001) (Figure 10). In contrast, the CA125 concentration at relapse was not significantly associated to either of the endpoints (OS: p=0.13 and SPP: p=0.25) (Figure 10). Interestingly, less than 50.0% of the patients with a HE4 concentration >199.2 pmol/L at progression were alive a year after the detection of progression, while half of the patients with serum HE4 <199.2 pmol/l at progression were alive two years after the detection of relapse (Figure 10). All patients with a SPP exceeding three years had a serum HE4 concentration <199.2 pmol/L at progression (Figure 10).

A subset of clinically relevant variables (disease stage, treatment strategy and residual disease) were selected for the multivariate model and each of them was at first evaluated univariately. Patients treated with NACT were found to have a worse OS (p=0.002, log rank test) in the above-mentioned analyses. However, the HE4 concentration >199.2 pmol/L was the only variable independently associated with worse OS and SPP in the multivariate Cox regression model (OS: p=0.002 and SPP: p=0.001) (Table 12).

Table 12. The HE4 serum concentration of >199.2 pmol/L at progression was identified as an independent predictor of a worse overall survival (Cox regression model). Modified from Study III. *P<0.05.

Clinical variable	Univariate p	Multivariate p	Multivariate HR	(95% CI)
Disease stage:III vs IV	0.12	0.19	1.80	0.75–4.33
R > 0mm	0.51	0.43	0.71	0.30–1.67
PDS vs NACT	0.002*	0.07	2.20	0.94–5.15
CA125 >162.0 U/ml	0.13	0.60	1.25	0.53–1.67
HE4 >199.2 pmol/L	< 0.0001*	0.002*	4.83	1.74–13.38

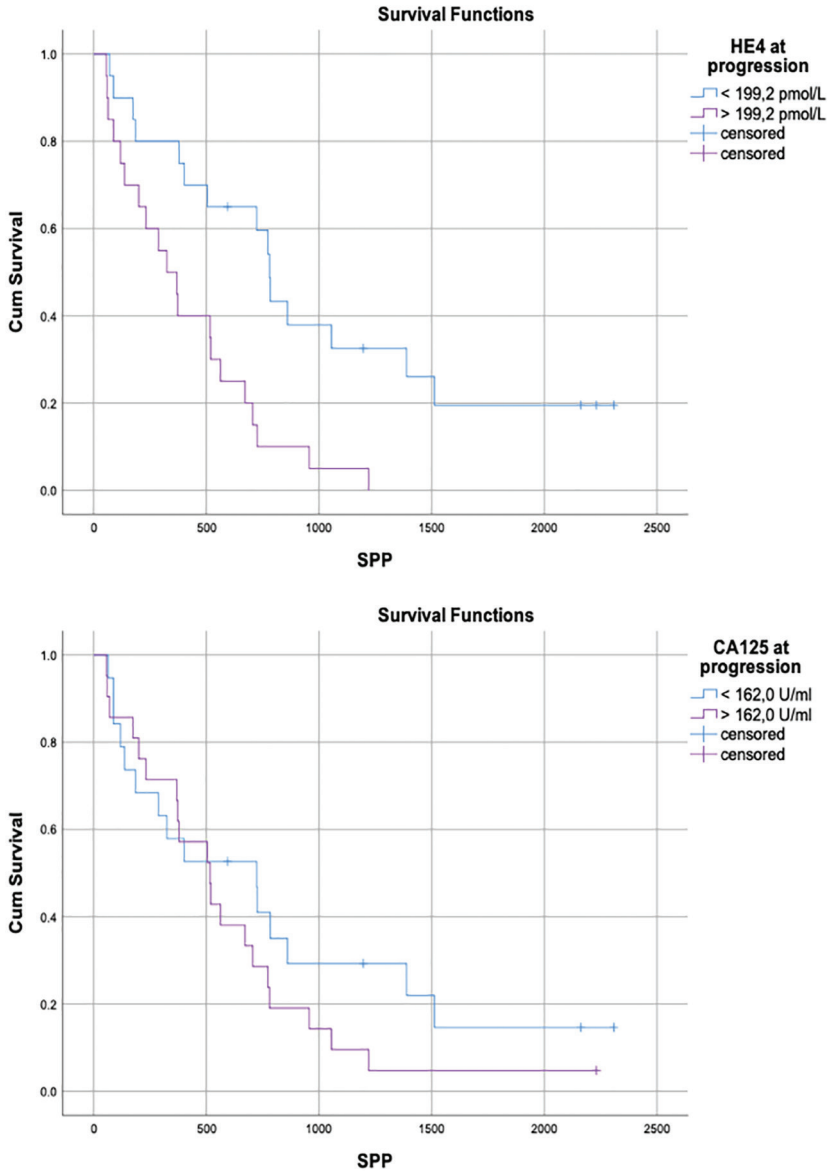


Figure 10. The Kaplan-Meier curves of SPP of patients (N=40) with serum biomarker values below and exceeding the cut offs (medians) at progression. A serum HE4 concentration wof >199.2 pmol/L was significantly associated with the SPP (log rank test, p=0.001). A higher serum CA125 concentration at progression was not significantly associated with the SPP (p=0.252). Modified from Study III.

5.3 Longitudinal Evaluation of ctDNA (IV)

5.3.1 CtDNA and the Tumor Mutation Profile

The Oseq^{TM} ctDNA solid tumor panel with subsequent variant and CNA calling were applied in the analyses. The Oseq^{TM} ctDNA panel is designed for solid tumors including lung cancer, colorectal cancer, ovarian cancer, breast cancer, melanoma and lymphoma. After calling and filtering, 265 mutations in 185 genes and CNA aberrations in 113 genes were approved for further evaluation. The applicability of plasma ctDNA as a snapshot of the tumor tissue mutation environment was evaluated by comparing plasma mutations to tumor biopsies obtained during surgery. Overall, the plasma ctDNA mutation profiles were similar to the mutations detected in tumor tissue (79% median concordance in mutations and 74% in CNAs) (Table 13). The decisive filtering progress and the exclusion of alterations derived from blood cells (germline sample analysis) maximized the likelihood of the detected mutations to be derived from the actual tumor and not confounding factors, i.e. clonal hematopoiesis.

Table 13. The individual mutation concordances between plasma and tissue. The median concordance was high (79%). Modified from Study III.

	Tissue(s)	PFI group	Plasma only (N)	Both (N)	Both (%)	Tissue only (N)	Total
EOC587	Baseline adnexa; IDS liver	poor	16	19	54%	0	35
EOC677	Baseline peritoneum; IDS ovary; prog ascites	poor	3	33	92%	0	36
EOC736	Baseline adnexa; IDS ovary	poor	0	22	100%	0	22
EOC183	Baseline and IDS omentum	poor	4	25	86%	0	29
EOC568	Baseline adnexa; IDS ovary	poor	2	11	85%	0	13
EOC1067	PDS omentum; prog ascites	poor	11	19	61%	1	31
EOC1030	PDS ovary	good	7	14	54%	5	26
EOC49	Baseline and IDS omentum	good	9	36	80%	0	45
EOC415	PDS omentum	good	1	8	80%	1	10
EOC1099	PDS ovary	good	0	5	56%	4	9
EOC429	PDS omentum	good	0	10	77%	3	13
EOC998	PDS ovary	good	6	4	40%	0	10

The frequency of the TP53 mutation was assessed and, not surprisingly, it was detected in all 12 patients. A moderate positive correlation between serum CA125 and the plasma TP53VAF was discovered (median 0.67, range 0.16–0.97). In addition, the CNAs detected with PanelDoc were strongly correlated (0.88) with the ctDNA portion of the cfDNA. In consideration of these results, ctDNA seem a feasible indicator of the tumor mutation environment in HGSC.

5.3.2 CtDNA and Treatment Monitoring

In 75.0% (9/12) of the patients, the ctDNA profile matched the clinical treatment outcome. One patient was excluded from the current analysis, as the baseline plasma sample had a too low tumor content for the accurate estimation of significant changes. The longitudinal changes in the ctDNA mutation profiles were different between patients with a good and a poor response to treatment: good responders were found to have significantly lower amounts of mutations and a higher proportion of mutations with decreasing VAF during primary therapy (p=0.008). Importantly, these differences were already detected early on in the treatment schedule (before the 2nd and 3rd cycles of chemotherapy) (Figure 11).

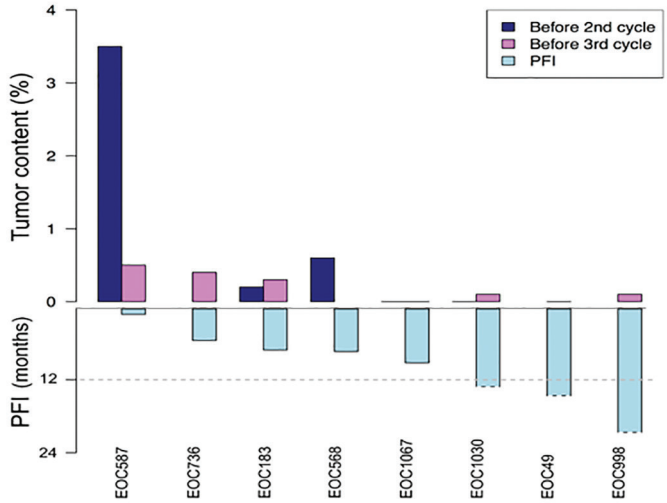


Figure 11. Patients with ctDNA profiles matching the clinical outcome. The reduction of the tumor content (%) in plasma happened during the first cycles of chemotherapy in patients with a good response to treatment, while poor responders showed persistently elevated levels of tumor content during therapy (p=0.008). Modified from Study III.

Next, specific attention was directed at poor responders and the genes with stable or increasing mutation VAFs during treatment. Enriched pathways detected in the poor responders included transcription, p53, chromatin regulatory and DNA double-strand break repair pathways. The chromatin regulatory pathways were the most selectively enriched and included 10 genes, of which some have been associated with DNA replication fork degradation and the development of therapy resistance (KMT2A, KMT2B, KMT2D, KDM5A, and SUZ12).

5.3.3 CtDNA Reveals Actionable Mutations

The motivation for the ctDNA analyses were to obtain a non-invasive snapshot of the actionable mutations in the patients' malignancies. Potential, clinically actionable mutations were detected in the plasma ctDNA of seven patients (Table 13). The mutations were positioned in four engaging complexes: mammalian target of rapamycin (mTOR; patients EOC429, EOC49), DNA repair (patients EOC429, EOC677, and EOC1067), epidermal growth factor receptor (EGFR; patients EOC587, EOC736, and EOC568), and cyclin dependent kinases (CDKs; patient EOC1067).

Mutation mediated over activation of the mTOR-pathway was detected in the plasma ctDNA of patients EOC429 (mutation in the NF1 gene) and EOC49 (mutation in the PTEN gene). Recent studies indicate that ovarian cancer patients with mTOR-pathway amplification might be responsive to phosphatidylinositol 3-kinase/mTOR inhibitors (Mabuchi et al., 2015). Furthermore, carboplatin has been described to effectively influence the apoptosis of cancer cells through the mTOR pathway (Zhou et al., 2018). In the current study, both patients with mTOR overactivation responded well to platinum-based chemotherapy (Table 14). Additionally, EOC429 had a deleterious mutation in the RAD51C gene resulting in HR deficiency, which makes the disease susceptible for platinum-based chemotherapy (Table 14).

Three patients (EOC429, EOC677 and EOC1067) were found to have mutations and CNAs in genes associated with DNA repair. We detected mutations both in BRCA2 and STAG2 in the ctDNA of patient EOC677; however, the frequency of the mutations was low being suggestive of subclonal events. One patient (EOC1067) had a somatic deletion of FANCA with concurrent deletions in CDKN1B and CDKN2B, and although these mutations convey HR-deficiency and PARPi-sensitivity, disease progression was already detected at 8.9 months after the conclusion of the platinum-based chemotherapy (Table 14).

Three patients had mutations or CNAs in the EGFR pathway, which generally make tumors susceptible for EGFR inhibitors (i.e. trastuzumab, a humanized monoclonal anti-ERBB2 antibody). Patient EOC736 had been treated with NACT, subsequent IDS and adjuvant chemotherapy consisting of paclitaxel and carboplatin. Although a complete response to the primary therapy was achieved, the TP53VAF

remained elevated throughout the treatment in the ctDNA analyses. Based on the current study, persistently elevated TP53VAF indicates a suboptimal response to treatments and in patient EOC736 it had already progressed 5 months after the conclusion of primary therapy. Pegylated liposomal doxorubicin (PLD) was selected as the second line treatment. Despite the PLD the patient's condition worsened and the serum CA125 concentration rose (Figure 12). At progression, the patient's ctDNA analysis revealed a high copy-count amplification of the ERBB2 gene. A decision to include trastuzumab in her treatment was done based on the current ctDNA analysis result. Intriguingly, the patient achieved a complete serological response (serum CA125 reduced from 840.0 U/ml to 19.0 U/ml) after the change in chemotherapy from PLD to a combination of trastuzumab, carboplatin and paclitaxel (Figure 12). A CT scan showed a significantly reduced tumor load and a partial response to treatment was concluded in accordance with the RECIST criteria (Eisenhauer et al., 2009).

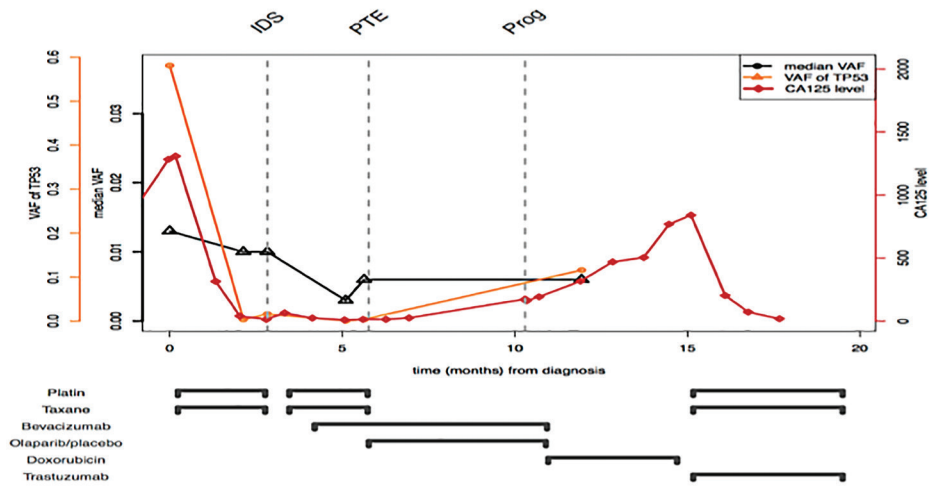


Figure 12. At progression, a high copy-count amplification of the ERBB2 gene was detected in the ctDNA of patient EOC736. Despite treatment with PLD the patient's condition worsened and serum CA125 concentrations began to rise. Based on the ctDNA analysis, trastuzumab was added to the treatment regimen and a partial response was achieved (RECIST criteria). Modified from Study IV.

Table 14. Clinically interesting actionable variants. Modified from Study IV.

Patient	PFI	Alteration	VAF or CNA ratio in plasma (% of ctDNA)	VAF or CNA ratio in tumor tissue	Biologic effect	Drugs associated	ESCAT tier
EOC429	>18	NF1 p. Q1754X	0.340 (98)	0.556	PI3K/AKT/mTOR pathway activation	PI3K/mTORi sensitivity	III
		RAD51C del germline	0.620	0.270	HR deficiency	PARPi sensitivity	III
EOC49	>14	PTEN p. A333V	0.005 (100)	0.080	PI3K/AKT/mTOR pathway activation	PI3K/mTORi sensitivity	III
EOC677	3.5	BRCA2 p. G602E	0.018 (75)	0.002	HR deficiency	PARPi sensitivity	I
		STAG2 p. C347F	0.016 (67)	0.031	HR deficiency	PARPi sensitivity	IV
EOC1067	8.9	FANCA del	0.620	0.410	HR deficiency	PARPi sensitivity	IV
		CDKN1B del	0.670	0.700	Loss of cell cycle control	CDK2/4i sensitivity	IV
		CDKN2B del	0.630	0.420	Loss of cell cycle control	CDK4/6i sensitivity	IV
EOC736	5.2	ERBB2 amp	7.130	10.150	EGFR pathway activation	HER2i and trastuzumab sensitivity	II
EOC568	7.0	ERBB4 p. H374Y	0.009 (39)	0.092	EGFR pathway activation	ERBBi and lapatinib sensitivity	IV
EOC587	0.9	MAP2K1 p. A19T	0.111 (20)	0.134	EGFR pathway inactivation	EGFRi resistance	IV
		MAPK1 amp	2.540	2.740	EGFR pathway inactivation	EGFRi resistance	IV

6 Discussion

Epithelial ovarian cancer is characterized by an unfavorable outcome and it is, alongside cervical cancer, the most lethal gynecological malignancy in the developed countries (Bray et al., 2018). Early diagnosis has proven to be very difficult, mostly due to the lack of specific symptoms and accurate biomarkers. Although aggressive surgical effort and the implementation of platinum-based chemotherapy have improved the survival of patients, eventually, the vast majority of patients develop a treatment resistant, progressive disease.

CA125 is currently the only biomarker validated and widely used in the diagnosis, treatment monitoring and follow-up of EOC. However, CA125 is not the ideal biomarker as 1) it does not detect early stage EOC with the accuracy needed, 2) it does not precisely portray tumor load and 3) it is unspecific for EOC. Consequently, biomarker research has been a hot topic and it has generated potential, new tumor marker candidates. One of them is HE4, which was recently approved as an auxiliary biomarker for CA125 in the diagnosis of EOC. Unfortunately, HE4 has since been found to be elevated in several other malignancies and non-cancerous diseases, which limits its utility as a diagnostic marker in EOC. All things considered, there is still a dire need for better EOC biomarkers.

6.1 CA125 Glycoforms and EOC Diagnosis

Based on this study, the CA125-STn and -MGL assays detected the most common EOC subtype, HGSC, adequately ($p<0.001$, both assays) and showed good diagnostic applicability particularly in postmenopausal patients. A further diagnostic advantage was achieved when the conventionally measured CA125 was marginally elevated (35.0–300.0 U/ml). In the current cohort, the diagnostic performance of the conventional CA125 assay was slightly inferior to that reported in previous studies (AUC 0.78 vs 0.87) (Wang et al., 2014). Not surprisingly, neither the conventional CA125 assay or the glycoform assays detected the mucinous histotype of EOC, and additional biomarkers might need to be combined for the diagnostic coverage of mucinous carcinoma. Endometroid, low grade serous and clear cell carcinomas were sufficiently detected by all of the biomarker assays. Importantly, the number of patients with a benign disease and elevated CA125 concentrations was markedly

reduced with the application of the glycoform assays. The leading concern regarding the low specificity of the conventional CA125 assay is the subsequent additional testing and emotional anguish after a falsely elevated test. Based on the current results, the glycoform assays might be feasible tools in decreasing these unnecessary, often invasive, diagnostic procedures.

The measurement of glycoforms to increase the diagnostic specificity of conventional biomarkers have also been explored in other cancers (Korekane et al., 2012; Kirwan et al., 2015). Indeed, the LCA-reactive fraction of alpha-fetoprotein (AFP-L3) has been validated as a more specific biomarker than total-AFP and is currently used in the clinical setting in the diagnosis of hepatocellular carcinoma. Little is known about the function of altered glycosylation seen in cancers regardless of the tumor type. It has been hypothesized that altered glycosylation might be part of a larger "metastatic code" of cancers contributing to disease progression and metastasis (Fuster and Esko, 2005). Consequently, it is likely that glycoforms show better prognostic performance than their conventional counterparts.

There are several multimarker panels designed for the differential diagnosis of pelvic masses. Currently, the Risk of Ovarian Malignancy Algorithm (ROMA), Risk of Malignancy Index (RMI) and the Copenhagen Index (CPH-I) are the most widely used in clinical settings (Van Gorp et al., 2011; Anton et al., 2012; Moore et al., 2013). These panels build on the tumor markers CA125 and HE4, TVS and the patients menopausal status/age. However, TVS is an operator-dependent modality and the accuracy of the panels vary. The sensitivity and specificity of the multimarker panels have been reported to be 67% to 94% and 75% to 92% between studies. Based on the current study, the CA125 glycoform assays could bring additive value to the contemporary diagnostic metrics both as individual markers and as putative parts of multimarker panels. The CA125 glycoform assays, similar to CA125 and HE4, present an objective result utilizing a robust technique. A commercialized version of the CA125-STn and -MGL assays would in no way differ from conventional, fully automized sandwich assays.

6.2 CA125 Glycoforms and HE4 in the Preoperative Evaluation and Prognostic Stratification of HGSC

The cohorts used in the longitudinal CA125-STn, -MGL and HE4 analyses differed slightly in the number of patients; however, all patients were diagnosed with HGSC, the biomarker measurement and follow-up schedules were identical and, due to this, the results of these two studies (II and IV) are discussed simultaneously below and in the Chapter 6.3.

In the current study, the longitudinal kinetics of the CA125-STn and -MGL assays were found to be consistent during primary HGSC treatment and comparable to that

of the conventional CA125 assay. Moreover, the dynamics of HE4 during HGSC treatment was consistent, and we did not detect discrepancies between the HE4 and CA125 dynamics in the current cohort contrary to the study by Ferraro et al (Ferraro et al., 2018). However, the cohort analyzed by Ferraro et al. was heterogeneous and it also included non-epithelial ovarian malignancies. Consequently, these two studies are not absolutely comparable.

The main result in the longitudinal biomarker analyses were the association of the baseline CA125-STn, -MGL and HE4 with the tumor load assessed during surgery. Unsurprisingly, the conventional CA125 assay showed no such association, which is in line with the evidence of discrepant CA125 concentrations with actual disease extent (Bast et al., 2005). No optimal cut off values in separating high tumor load patients from those with a low tumor load for the CA125-STn, -MGL or HE4 assays were determined, and the exploration of this subject remains for further studies. Currently, the modalities for the preoperative evaluation of tumor load are contrast enhanced (ce)CT and in selected patients and hospitals positron emission tomography (PET)/CT. In addition, diagnostic laparoscopy is done in many tertiary referral hospitals to achieve the most accurate assessment of operability. The accuracies of these modalities for the assessment of tumor load have been reported to be 57% and 85% for ceCT between studies, 64% and 91% for PET/CT between studies and 90% for diagnostic laparoscopy (Fagotti et al., 2005; Kitajima et al., 2008; Hynninen et al., 2013). Instinctively, the measurement of serum biomarkers would act as a complementary, non-invasive method in the evaluation of operability.

The results of this study indicate that both the CA125-STn and -MGL assays might be better predictors of residual disease than the conventional CA125 and HE4 assays. Our results are in line with previous studies on the utility of the conventional CA125 assay in the prediction of the amount of residual disease (Barlow et al., 2006; Kang et al., 2010; Mury et al., 2011). In the current cohort, baseline HE4 was not significantly associated with the amount of residual disease, although there was a tendency of higher serum HE4 concentrations in patients with macroscopic residual disease. In consequence, our study does not offer further support to the previous studies on HE4 and residual disease (Angioli et al., 2013; Braicu et al., 2013; Vallius et al., 2017). Parallels can be drawn between the amount of residual disease after cytoreductive surgery and the initial tumor load of the patient; however, these two variables do not entirely characterize the same feature as already small amounts of tumor on unresectable anatomic locations might lead to significant quantities of residual disease. Again, no specific cut offs for the biomarkers in the prediction of residual disease were determined in the current study. The HE4 cut offs suggested by Angioli et al and Braicu et al were 267.0 pmol/L and 235.0 pmol/L, respectively, and considering the median HE4 concentration at baseline in the current cohort (806.4 pmol/L) the cut offs appear utterly strict in the two above-mentioned studies (Angioli et al., 2013; Braicu et al., 2013).

In clinical trials, an assessment tool for the timely evaluation of tumor load and the prognostic stratification of patients during primary treatment is needed. Here, the nadir CA125-STn level of >0.8 U/ml was identified as an independent predictor of PFS, while the CA125-MGL and the conventional CA125 assays showed no such association. In the complementary analyses, the HE4 nadir >54.3 pmol/L was also identified as a predictor of PFS and showed stronger correlation to PFS than the CA125-STn assay in the multivariate analysis. In Study II, optimal cut offs for each assay were determined independently and PFS was dichotomized into progression vs no progression, which differs from the analysis in Study III, in which the nadir values were evaluated in the prediction of platinum resistant relapse. In the light of these results, the CA125-STn and HE4 assays seem to identify patients who develop progression more rapidly, even after the 6-month pillar of platinum resistant relapse. In the current cohort, both the conventional CA125 and the HE4 assays were adequate tools in the detection of patients with particularly poor outcomes (PFI <6 months). Our results on the CA125 and HE4 nadir values are in line with previous studies on the subject (Kang, Seo and Park, 2009; Van Altena et al., 2010; Xu et al., 2013; Vallius et al., 2017).

6.3 CA125 Glycoforms and HE4 in Recurrent HGSC

Despite a satisfactory response to primary treatment, most of the patients with HGSC eventually develop recurrent disease. In the literature, the serum concentration of conventionally measured CA125 has been reported to rise approximately 3 months before a subsequent symptomatic recurrence (Bast et al., 2005). However, the results of two large, multicenter trials are contradictory on the concordance of conventionally measured CA125 to radiologic response criteria (Alexandre et al., 2012; Lindemann et al., 2016). In the current study, the CA125-STn showed elevated serum CA125 concentrations prior to that of the conventional assay in 37.0% (13/35) of patients and higher in fold increase in 80.0% (28/35) of patients. The CA125-MGL assay did not detect recurrence earlier than the conventional CA125 assay, but a higher in fold increase was detected in 11,0% (4/35) of the patients. The results indicate that specifically the CA125-STn assay might be able to detect very small amounts of tumor growth and could consequently show better concordance to radiologic response evaluation criteria than the conventional CA125 assay. In the complementary analyses, the HE4 assay performed equally well as the CA125-STn assay in the detection of relapse. This finding is in line with previous results, which have suggested a lead time of 5–8 months to the conventional CA125 assay with the HE4 assay (Anastasi et al., 2010).

Without doubt, the conclusion of the study by Rustin et al, questioning the benefit of routine measurement of conventional CA125 in asymptomatic women after the conclusion of primary therapy (Rustin et al., 2010), raises the question of whether our

result on the earlier detection of asymptomatic recurrence is of clinical value. Firstly, with the development and implementation of new targeted therapies, i.e. PARPi and immunological drugs, the advantage of early treatment of asymptomatic recurrence needs to be re-evaluated, and secondly, well-designed trials with biomarkers already detecting recurrence in small tumor volumes are needed when investigating the clinical applicability of these new therapies.

Based on the current study, the serum HE4 concentration at the time of relapse is associated with the survival of patients and in consideration of this, HE4 might well be an indicator of disease aggressiveness. In contrast, conventionally measured CA125 did not show such an association. Overall, HE4 is a promising auxiliary tool to the conventional CA125 assay in the detection of recurrent disease and it could aid clinicians in choosing the most appropriate second line treatment in this era of targeted therapies.

6.4 Identification of Actionable Mutations in ctDNA

The mechanisms behind acquired treatment resistance are heterogeneous and still unapprehended to a large extent. The results of the current study showed that the tracking of relevant ctDNA mutations during the treatment and follow-up of HGSC is a feasible method in the timely detection of treatment resistance. The ctDNA analysis predicted the treatment response of the patients: poor responders were found to have a higher mutation burden and less mutations with a decreasing VAF during treatment. These findings are in good concordance with the premise that a stable or increasing abundance of mutations result from underlying selection processes associated with specific treatments and the development of treatment resistance (Murtaza et al., 2013). Interestingly, in the current study, these differences were already seen in the early phases of the treatment regimen, i.e. after cytoreductive surgery and after the 2nd cycle of chemotherapy.

Importantly, the ctDNA mutations profiles were found to be comparable to the aberrations in the tumor tissue biopsies taken during surgery, which is a prerequisite for the utilization of ctDNA altogether. The germline and ctDNA comparison increased the likelihood of the mutations originating from the tumor further. Indeed, as indicated by previous studies (Asante et al., 2020), ctDNA appeared to be a feasible method in the real-time assessment of intratumor diversity and clonal evolution. CtDNA analysis might be especially advantageous in situations where conventional biopsies are unattainable or if there is reason to believe that conventional biopsies would not represent the tumor in an up-to-date manner, i.e. heavily pretreated patients, fast-growing and/or necrotic tumors.

Actionable mutations with possible clinical applicability were detected in the ctDNA of seven HGSC patients (58.0%). One patient with a platinum resistant

progression and a poor response to conventional therapy (pegylated liposomal doxorubicin) was detected to have a high-confidence ERBB2 amplification in the ctDNA analysis. ERBB2, a transmembrane receptor tyrosine kinase, is a member of the epidermal growth factor receptor (EGFR) family (Chung et al., 2019). ERBB2 transduces proliferative and survival signals to the cell as a homodimer (without ligand stimulation) or a heterodimer with other EGFR family members upon ligand stimulation (Ménard et al., 2000). Based on the ctDNA results, the treatment of the patient was changed to a combination of trastuzumab, reduced dose carboplatin and paclitaxel, and compellingly, a complete normalization of CA125 was seen after two months of the change in therapy. A previous study reported similar results, in which three patients out of seven (42.8%) with ERBB2 amplifications reached a complete response to treatment after the implementation of trastuzumab (Ray-Coquard et al., 2008). These results indicate that trastuzumab might reverse the therapy resistance of patients with ERBB2 amplification. However, little is known about the exact role of the ERBB2 amplification in the development of therapy resistance, and in vitro analyses have suggested that, instead, the molecules involved in its signaling pathway might be the true protagonists in the development of ERBB2 induced therapy resistance (Ménard et al., 2000). Indeed, the precise mechanism of the anti-tumor activity of trastuzumab is also not completely understood and proposed mechanisms include the inhibition of the ERBB2-mediated pathway and antibody-dependent cell-mediated cytotoxity (Minami et al., 2013). Although ERBB2 amplification is rare in ovarian cancer (5.7%, in situ hybridization) (Chung et al., 2019), it might be an indicator of poor prognosis and an object for targeted therapies and consequently, ERBB2 testing of selected, relapsed HGSC patients deserves consideration.

6.5 Strengths and Limitations

All serological sample analyses were done by a blinded investigator in an identical manner, which is a strength of the study. However, two different kits for the measurement of the conventional CA125 serum concentrations were used and this can be seen as a limitation of the study, despite the good correlation between the two kits. Another limitation of the study is that some of the serum samples were stored for a long time, the oldest samples for 10 years, and the quality of the samples were not controlled at the initiation of the study. Both CA125 and HE4 have been reported to be stable molecules and no degradation was detected after 5 years of storage at -80 °C in a study on the effect of long term storage on CA125 and HE4 concentrations (Sandhu et al., 2014).

The goal when assembling the cohort for Study I was to cover a clinically relevant, heterogeneous subset of patients. Consequently, the diagnostic performance of the assays should be well translatable to real clinical circumstances. However,

the number of patients with rarer histological subtypes of EOC were few, and it is difficult to draw conclusions on the diagnostic potential of the CA125-STn and -MGL assays in these groups as the statistical power is low.

The strengths of the longitudinal studies are the prospective study setting, standardized treatment regimens and response evaluation criteria. A limitation of the studies is the lack of serum samples at one or several individual timepoints in the sample series of some patients. However, the longitudinal kinetics of the biomarkers were consistent throughout the primary treatment and follow-up of the patients. The tumor load was exclusively assessed by the operating gynecologic oncologist and it was not confirmed from imaging studies. A further limitation is that the interobserver variability was not evaluated and, in this context, an observer bias is possible.

We included over 500 cancer-related genes in the ctDNA analysis, which enabled a comprehensive analysis of somatic mutations and CNAs. The obvious limitation of the ctDNA study is the low number of patients. However, the study was designed as a proof-of-concept study and this limitation was evident already at the planning phase of the study.

6.6 Future Perspectives

The diagnostic performance of the CA125-STn and -MGL assays need further assessment and the preferred next step in the validation process is a multicenter trial with an increased heterogeneity in the study population. The current exploratory studies have provided a good foundation for further validation. Moreover, the technical development of the assays is still ongoing, with the goal to reduce the number of false positive cases further. A very high specificity is needed for a biomarker to be potentially applicable as a screening tool, and future studies will show whether the glycoform assays are suitable for the task.

The routine measurement of HE4 during treatment and follow-up is currently not included in the treatment recommendations of HGSC (Colombo et al., 2019). However, in the current study the prognostic potential of HE4 at disease progression was observed. Further studies on the subject are warranted in addition to studies evaluating the feasibility of treating asymptomatic, early progression with new, targeted therapies

The longitudinal tracking of ctDNA mutation profiles show promise in finding therapeutic windows during the primary therapy and follow-up of HGSC. Little is still known about the genetic alterations behind the development of treatment resistance and future studies on ctDNA might shed additional light on the matter.

7 Conclusions

The following conclusions can be drawn based on this work:

1. The CA125 glycoforms, CA125-STn and -MGL, are potential new biomarkers of EOC. Both of the assays showed better EOC-specificity than the conventional CA125 assay.
2. The longitudinal kinetics of the CA125-STn, -MGL and HE4 assays showed good concordance with the conventional CA125 assay and the treatment response of the patients.
3. The baseline concentrations of the CA125-STn, -MGL and HE4 assays were significantly associated with the tumor burden assessed during surgery and these biomarkers might serve as auxiliary tools in the evaluation of up front cytoreductibility.
4. The landscape of EOC treatment is rapidly changing due to the development and implementation of new targeted therapies. The new therapies are specifically changing the notion of recurrent disease and patients live longer with stable diseases. The CA125-STn and HE4 assays are sensitive indicators of recurrence and might be potential tools in the selection of second line treatments. Studies on the feasibility of treating asymptomatic, serological recurrence are warranted.
5. A strongly elevated serum HE4 concentration at progression might be associated with a more aggressive relapse, which could ease the prognostic stratification of patients.
6. A longitudinal ctDNA analysis with a large panel reveals actionable mutations and opens potential therapeutical windows both during the primary therapy and follow-up of patients with HGSC.

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