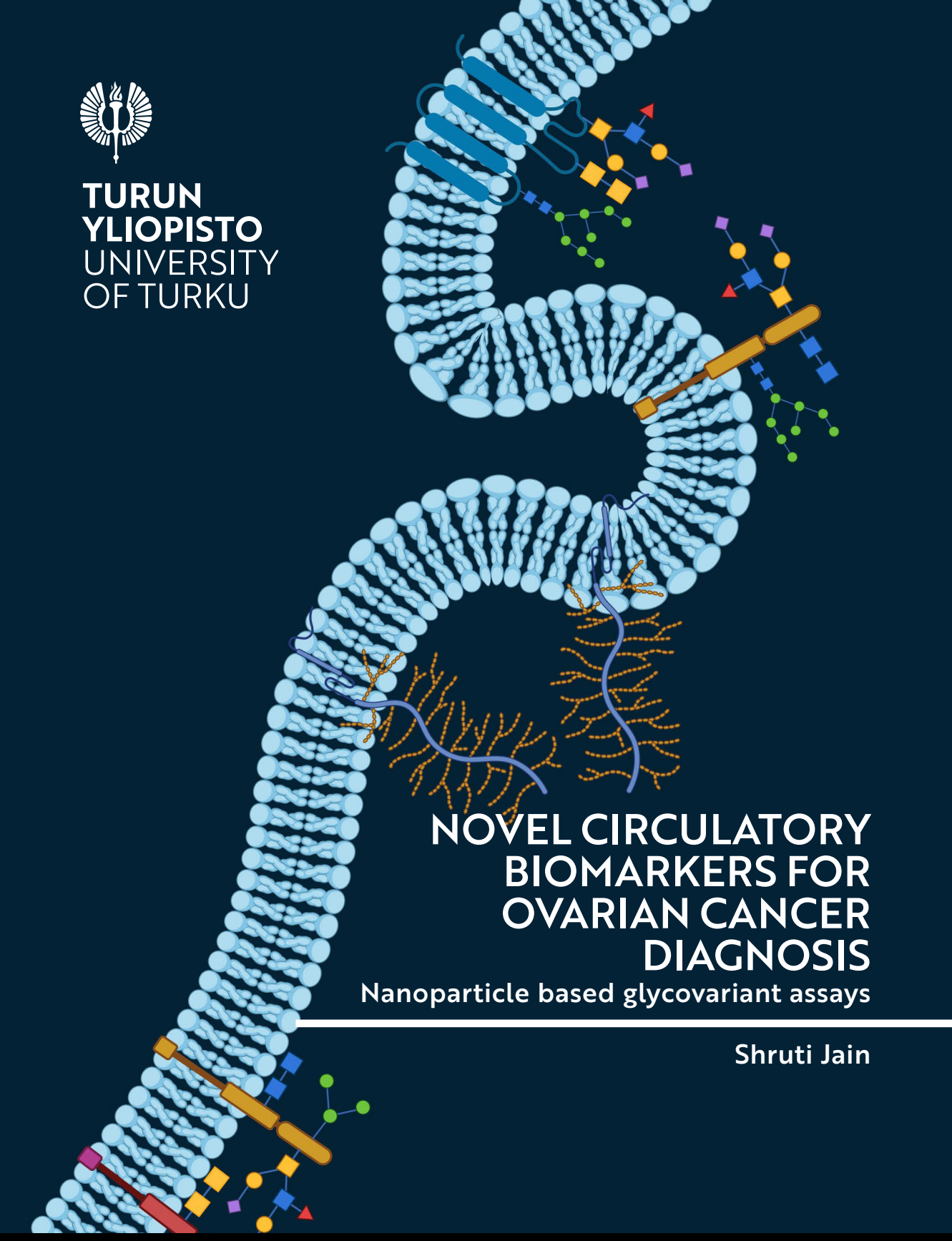

TURUN YLIOPISTO
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NOVEL CIRCULATORY BIOMARKERS FOR OVARIAN CANCER DIAGNOSIS

Nanoparticle based glycovariant assays

Shruti Jain

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माँ- पापा
मम प्रथमो गुरूः।

To Maa-Papa
My first teachers.

UNIVERSITY OF TURKU
Faculty of Technology
Department of Life Technologies
Molecular Biotechnology and Diagnostics
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ABSTRACT

Ovarian cancer is the most lethal gynecological malignancy and the seventh most common cancer worldwide. Current diagnosis options for ovarian cancer suffer from a lack of sensitivity and specificity that lead to either over-diagnosis or missed cancers. Ovarian cancer due to its often-asymptomatic nature in the early stages, leads to late diagnosis and limited treatment options. Therefore, there is an urgent need for the development of sensitive and specific assays involving minimally invasive techniques.

The primary aim of this doctoral thesis was to identify and validate promising novel biomarkers for ovarian cancer diagnosis. The aim was also to explore possibilities to develop a simple assay for the detection of cancer-associated glycoforms directly from human biofluids without any extensive preprocessing. The different studies focused on the development of a europium chelate-labeled nanoparticles (Eu-NPs)- aided immunoassay approach that uses glycan-based markers and their potential combinations for the detection of altered glycans of cancer patients. Several cancer-associated glycoprotein markers in combination with antibodies and lectins were tested to find the best functional biomarkers and their corresponding potential assays. The biomarker assays could significantly discriminate ovarian cancer from benign and healthy controls.

The result of this thesis presents several promising glycovariant biomarkers with a sensitive nanoparticle-based immunoassay approach for the diagnosis of ovarian cancer from unprocessed samples. This assay concept is robust, time-sensitive, requires minimal sample amounts and can be easily adapted to clinical settings and thus presents itself as a promising diagnostic test platform for ovarian cancer.

KEYWORDS: Ovarian cancer, glycovariant, biomarker, europium-nanoparticles, immunoassay

TURUN YLIOPISTO
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TIIVISTELMÄ

Munasarjasyöpä on tappavin gynekologinen syöpä ja maailmanlaajuisesti seitsemänneksi yleisin syöpä. Kaikki tällä hetkellä käytössä olevat munasarjasyövän diagnoosimenetelmät kärsivät herkkyyden ja spesifisyyden puutteesta, mikä johtaa usein joko ylidiagnosointiin tai todellisten syöpätapausten jäämiseen havaitsematta. Munasarjasyöpä on usein oireeton alkuvaiheessa, mikä voi johtaa myöhäiseen diagnoosiin ja rajoitettuihin hoitovaihtoehtoihin. Tästä syystä on tärkeää kehittää uusia, herkempiä ja spesifisempiä diagnostiikkamenetelmiä, jotka eivät vaadi invasiivisia toimenpiteitä.

Tämän väitöskirjan ensisijaisena tavoitteena oli tunnistaa ja validoida lupaavia uusia biomerkkiaineita munasarjasyövän havaitsemiseen. Tavoitteena oli myös kehittää yksinkertainen määritys syöpään liittyvien sokerirakenteiden (glykaanit) havaitsemiseksi suoraan potilasnäytteistä ilman esikäsittelyä. Tutkimukset keskittyivät europiumkelaattileimattujen nanopartikkelien hyödyntämiseen immunomäärityksessä, jossa kohteena olivat glykaanipohjaiset merkkiaineet ja niiden mahdolliset yhdistelmät syöpäpotilaiden muuttuneiden sokerirakenteiden havaitsemiseksi. Useita munasarjasyöpään liittyviä merkkiaineita kokeiltiin yhdessä vasta-aineiden ja lektiinien kanssa parhaiden toiminnallisten biomerkkiaineiden ja niihin sopivien analyysimenetelmien löytämiseksi. Uudet määritykset pystyivät erottelemaan munasarjasyöpäpotilasryhmät hyvänlaatuisista sairauksista ja terveistä kontrolliryhmistä.

Tämän väitöskirjan aikana kehitettiin uusia lupaavia biomerkkiaineita ja immunomäärityksiä munasarjasyövän havaitsemiseen. Kehitetyt määrityskonseptit ovat luotettavia, nopeita ja vaativat ainoastaan pienen määrän potilasnäytettä. Lisäksi immunomääritykset ovat helposti sovellettavissa kliinisiin olosuhteisiin, joka on lupaava lähtökohta uuden munasarjasyöpätestin kehittämiselle.

ASIASANAT: Munasarjasyöpä, glykaani, biomarkkeri, nanopartikkeli, immunomääritys

Table of Contents

Abbreviations

AAL	Aleuria aurantia lectin
AFP	Alpha-fetoprotein
Apo-A1	Apolipoprotein A-1
AUC	Area under the curve
B2M	Beta-2 microglobulin
BSA	Bovine serum albumin
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CA125	Cancer antigen 125
CA15-3	Cancer antigen 15-3
CA19-9	Cancer antigen 19-9
CD63	Cluster of differentiation 63
CEA	Carcinoembryonic antigen
CF	Cyst fluid
CI	Confidence interval
CLEC10A	C-type lectin domain containing 10A
ConA	Concanavalin A
CT	Computed tomography
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EOC	Epithelial ovarian cancer
Eu-NP	Europium chelate-labeled nanoparticles
EV	Extracellular vesicles
F(ab')2	Fragmented antibody
Fc	Fragment crystallizable region
FDA	Food and Drug Administration
FIGO	International Federation of Gynecology and Obstetrics

FSH	Follicle stimulating hormone
GalNAc	N-Acetylgalactosamine
GI	Gastrointestinal
GlcNAc	N-Acetylglucosamine
GLOBOCAN	Global cancer observatory
GnTs	N-acetylglucosaminyltransferases
GV	Glycovariant
HAAAs	Human anti-animal antibodies
HE4	Human epididymis protein 4
HGSC	High-grade serous carcinoma
IL-8	Interleukin-8
ITG	Integrin
KLK3	Kallikrein 3
LCA	Lens culinaris agglutinin
LEL	Large extracellular loop
LGSC	Low-grade serous carcinoma
mAb	Monoclonal antibody
MES	2-(N-morpholino)ethanesulfonic acid
MGL	Macrophage galactose-type lectin
MIF	Macrophage migration inhibitory factor
MUC1	Mucin-1
MUC16	Mucin-16
N or n	Number of samples
NEM	N-Ethylmaleimide
Neu5Ac	N-acetylneuraminic acid
NHS	N-Hydroxysuccinimide
NP	Nanoparticles
NROSS	Normal Risk Ovarian Screening Study
OPN	Osteopontin
PEA	Proximity extension assay
PLA	Proximity ligation assay
PPV	Positive predictive value
PSA	Prostate specific antigen
ROC	Receiver operating characteristic
ROCA	Risk of ovarian cancer algorithm
ROMA	Risk of ovarian malignancy algorithm
RT	Room temperature
SEL	Small extracellular loop
Ser	Serine
sLe^a	Sialyl Lewis^a

SN	Sensitivity
SP	Specificity
STn	Sialyl-Thomsen-nouveau antigen
T	Thomsen–Friedenreich antigen
TAA	Tumor-associated antigens
TEM	Tetraspanin-enriched microdomain
Tf	Transferrin
Thr	Threonine
Tn	Thomsen-nouveau antigen
TRF	Time resolved fluorescence
TTR	Transthyretin
UKCTOCS	UK Collaborative Trial of Ovarian Cancer Screening
VNTR	Variable number tandem repeat
VVL	Vicia villosa lectin
WAP	Whey-acidic protein
WFDC2	WAP four-disulfide core domain protein 2
WGA	Wheat germ agglutinin

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 Jain S, Nadeem N, Ulfenborg B, M Mäkelä M, Ruma SA, Terävä J, Huhtinen K, Leivo J, Kristjansdottir B, Pettersson K, Sundfeldt K, Gidwani K. Diagnostic potential of nanoparticle aided assays for MUC16 and MUC1 glycovariants in ovarian cancer. *International Journal of Cancer*, 2022; 151: 1175-1184.

II Jain S, Santhi PP, Vinod R, Ruma SA, Huhtinen K, Pettersson K, Sundfeldt K, Leivo J, Gidwani K. Aberrant glycosylation of α3 integrins as diagnostic markers in epithelial ovarian cancer. *Clinica Chimica Acta*, 2023; 543: 117323.

III Jain S, Alexander HR, Ulfenborg B, Ruma SA, Lundin E, Hynninen J, Gidwani K, Pettersson K, Huhtinen K, Sundfeldt K, Idahl A. Glycovariant biomarkers of CA125 and CA15-3 with HE4 provides improved EOC detection: A multi-cohort study in Sweden and Finland. (manuscript)

1 Introduction

Ovarian cancer is one of the most formidable challenges in women's health, often emerging quietly and advancing with few warning signs. One of the greatest challenges in combating ovarian cancer is its frequent diagnosis at an advanced stage, largely due to its asymptomatic nature in the early phases. This delayed detection drastically limits treatment options, contributing to a five-year survival rate of only 30-40%. [1] The lack of reliable early detection methods means that fewer than 25% of cases are caught at this more treatable stage.

Current diagnostic methods, such as the CA125 blood test, have significant limitations. While CA125 can indicate the presence of ovarian cancer, it is not specific to this disease and can be elevated in various benign conditions. [2] Another biomarker, HE4, shows promise in distinguishing ovarian cancer from benign pelvic masses, yet it also falls short in early detection, particularly in asymptomatic cases. [3] Despite the FDA's approval of CA125 and HE4 as biomarkers, their primary use is in monitoring treatment response and recurrence rather than in initial screening, underscoring the critical need for more sensitive and specific biomarkers.

The focus of recent research, including the work presented in this doctoral thesis, has been on identifying novel biomarkers for ovarian cancer diagnosis and developing simple, effective assays that can detect cancer-associated glycoforms directly from biofluids with minimal preprocessing. The studies presented here, explored the use of europium chelate-labeled nanoparticles (Eu-NPs) in an immunoassay approach, leveraging glycan-based markers to enhance detection sensitivity. This nanoparticle-based technique, integrated with time-resolved fluorescence (TRF) technology, shows significant promise. It offers a robust, rapid, and minimally invasive method for glycan profiling, capable of discriminating ovarian cancer from benign and healthy controls with high specificity and sensitivity.

The thesis includes different studies, where the diagnostic potential of cancer-specific MUC16/CA125 and MUC1/CA15-3 glycoform assays ($MUC16^{STn}$, $MUC16^{MGL}$, $MUC1^{STn}$, $MUC1^{Tn}$) are evaluated in different clinical cohorts of serum and cyst fluids. Additionally, glycoforms of integrin alpha-3 and tetraspanin CD63 ($ITGA3^{STn}$, $CD63^{STn}$) are also tested in a proof-of-principle study with cyst fluids and ascitic fluid samples.

2 Review of the Literature

2.1 Cancer biomarker history

The discovery of the first-ever cancer biomarker, the light chain of immunoglobulin, in the urine of myeloma patients, marked a significant milestone in the field of cancer diagnostics in 1847. Today, clinicians still use this marker for myeloma diagnosis, albeit with modern quantification techniques. [4] Between 1930 and 1960, scientists identified various hormones, enzymes, and proteins whose concentration was altered in biological fluids from cancer patients. However, the modern era of monitoring malignant disease began in the 1960s with the discovery of alpha-fetoprotein and carcinoembryonic antigen (CEA). Immunological techniques such as radioimmunoassay facilitated the detection of these markers. In the 1980s, hybridoma technology enabled the development of the ovarian epithelial cancer marker carbohydrate antigen CA125. Prostate-specific antigen (PSA [KLK3]), discovered in 1980, is considered one of the most effective cancer markers to date. [5]

Numerous methods exist for the discovery of biomarkers, one of which is the utilization of glycomics approach. Glycosylation, a posttranslational protein modification, is a common occurrence in which covalently linked carbohydrates modify almost all cell surface and secreted proteins. Glycans play crucial roles in various biological processes, including cell-cell communication, immune responses, and disease development, such as cancer. [6]

The study of glycosylation, the process of adding carbohydrate chains to proteins and lipids (glycoproteins and glycolipids), dates back to the mid-20th century. In the 1960s and 1970s, scientists began to notice differences in glycan structures between normal and cancer cells. These differences included altered glycosylation patterns and the presence of tumor-associated antigens (TAA) on the surface of cancer cells. [7] These observations laid the foundation for using glycans as potential cancer biomarkers. It was in the early 21st century that glycomics gained prominence in cancer biomarker discovery. [8] Researchers realized that changes in glycan structures could serve as indicators of cancer and other diseases. For example, the altered glycosylation of certain glycoproteins, such as prostate-specific antigen (PSA) in prostate cancer, became a focus of study. [9] The history of glycomics in the context of cancer biomarker discovery is a relatively recent development, but it

has rapidly expanded with advances in technology and our understanding of glycan biology. Glycomics continues to be an exciting area of research with the potential to revolutionize cancer diagnostics and treatment.

2.2 Ovarian cancer

Ovarian cancer compared to other cancers is a relatively rare but deadly malignancy. According to GLOBOCAN, ovarian cancer mortality in the world in 2022 was approximately 64% of the incident cases for females of all-ages. This mortality increased to ~75% for 50 and above aged females and ~84% for 60-years and above of the incident cases. The age-standardized ovarian cancer incidence and mortality rates for Europe, Oceania and North America were higher than the World per 100 000 females aged 50-years and above. (**Figure 1**)

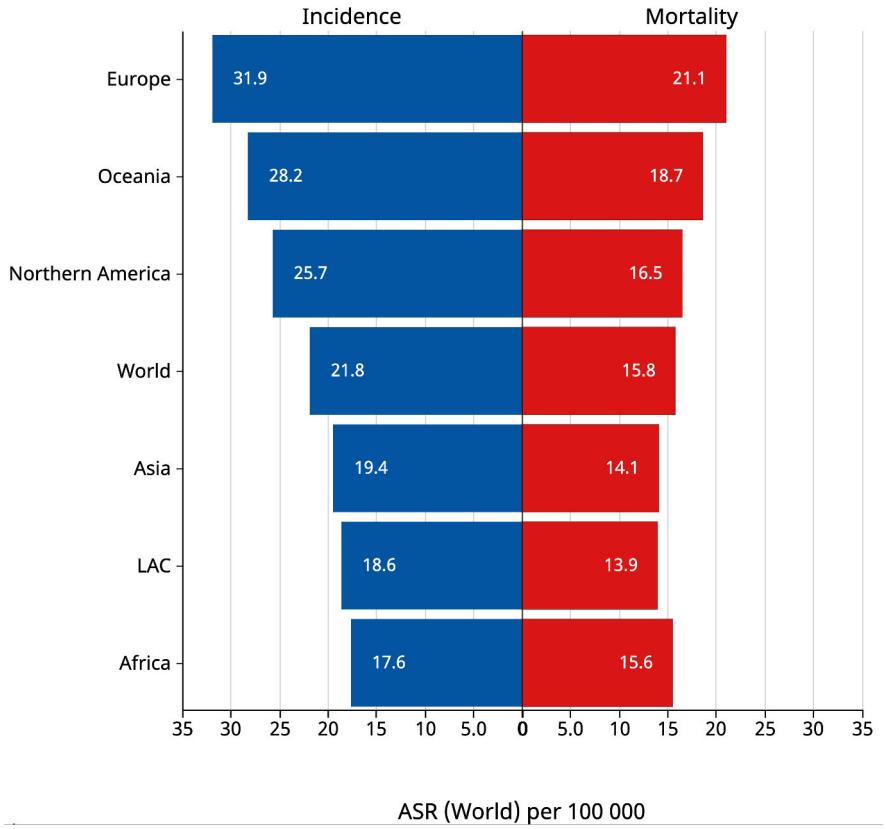

Figure 1. Age-standardized rate (world) per 100 000, incidence and mortality of ovarian cancer in females, aged 50 - 85+, in 2022. Y-axis represents different geographical regions, Europe, Oceania, North America, Asia, Latin America and the Carribean (LAC), Africa and the World. (GLOBOCAN, 2022)

According to the American Cancer Society, the risk factors for ovarian cancer include advanced age, with most cases occurring in women aged 60 years and above; family history of ovarian, breast, or colorectal cancer; genetic mutations such as BRCA1 and BRCA2; and reproductive history, including nulliparity (never having given birth). Other factors include hormone replacement therapy, endometriosis, and obesity. Preventive factors include the use of oral contraceptives, breastfeeding, and tubal ligation.

2.2.1 Types of ovarian cancer

Epithelial ovarian cancer is the most common type (~90%) of ovarian cancer. Other less common types of ovarian cancer include germ cell tumours, stromal tumours and sarcomas. There are many subtypes of epithelial ovarian cancer. The most common include serous, endometrioid, clear cell and mucinous carcinoma.

Serous carcinomas are the most common type and make up more than 50% of all epithelial ovarian cancers. They are classified as low-grade or high-grade serous carcinoma, depending on how the tumor cells look compared to normal tissue. High-grade cancer cells grow and spread faster than those of low-grade. Endometrioid carcinomas grow slowly and can be linked to endometriosis. Clear cell carcinomas are rare and can also be linked to endometriosis. Mucinous carcinomas are also rare and can be difficult to diagnose.

2.2.2 Ovarian cancer detection – screening trials and biomarkers

Ovarian cancer is often referred to as the "silent killer" because it is typically diagnosed at an advanced stage when it has already spread. Early detection is crucial for improving outcomes, but ovarian cancer lacks effective screening methods comparable to those for some other cancers like breast or colon cancer. Ovarian cancer is typically diagnosed at a late stage, resulting in a 5-year survival rate of only 30-40%. [1] Over 75% of cases are identified at advanced stages (III/IV), where the cure rate falls below 30%, while less than 25% are caught early (I/II), when 70-90% of patients can be effectively treated with surgery and chemotherapy. [10,11] Currently, there is no reliable method for early detection of ovarian cancer.

Technological advancements play a vital role in the detection and diagnosis of ovarian cancer. Imaging techniques, such as transvaginal ultrasound and computed tomography (CT) scans, are valuable tools for identifying tumors and assessing their size and spread. However, both methods struggle to reliably differentiate between benign and malignant masses and often fail to detect small or early-stage tumors, resulting in false positives and negatives. Additionally, transvaginal ultrasound's

accuracy is highly operator-dependent, while CT scans involve significant costs and radiation exposure, making them less suitable for routine screening. Blood tests, such as the CA125 test, were developed to measure a specific protein marker that could indicate the presence of ovarian cancer. [12] CA125 is not specific to ovarian cancer and can be elevated in various benign conditions like endometriosis, ovarian cysts and other cancers. [2] Similarly, HE4, another blood biomarker, also struggles with early and asymptomatic detection. While CA125 is slightly better at differentiating ovarian cancer from healthy individuals, HE4 is more effective at distinguishing cancer from benign pelvic masses. [3] However, neither test is sufficiently sensitive for early-stage detection, highlighting the urgent need for new biomarkers.

It's important to note that the FDA has approved CA125 and HE4 as biomarkers for ovarian cancer, but their use is typically in monitoring treatment response and recurrence rather than as standalone screening tools. Additionally, the FDA has also approved a set of algorithms or indexes known as Ova1®, ROMA® (Risk of Ovarian Malignancy Algorithm), and Overa®. [13] These multivariate index assays offer a valuable tool for healthcare professionals in assessing the likelihood of malignancy in ovarian tumors before surgery. They are not true diagnostic tests, but rather triage or referral tests. These tests have two critical requirements, a mass has been confirmed on imaging and the ovarian tumor has already been determined to require surgery. [14] Ova1® combines multiple biomarkers, including CA125, to provide a more comprehensive risk assessment. [15,16] ROMA®, on the other hand, takes into account CA125 and HE4 levels to calculate the risk of malignancy. [17] Overa® is another algorithm that combines CA125 and HE4 with three other markers to improve the accuracy of preoperative assessments, ultimately aiding in more informed treatment decisions for patients with suspected ovarian cancer. [18] (**Table 1**)

Recent studies suggest that 2-stage strategies, where rising biomarkers prompt imaging, can achieve better specificity. In the NROSS and UKCTOCS studies, rising CA125 analyzed with ROCA triggered transvaginal sonography in 2-3% of participants, resulting in 99.6% specificity and only 2-4 operations needed to diagnose each ovarian cancer. [19,20] ROCA assesses the risk by evaluating the pattern of CA125 fluctuations, considering individual baseline levels and rate of increase. This dynamic approach enhances the specificity and sensitivity of ovarian cancer screening, particularly when integrated into a two-stage strategy where abnormal ROCA results prompt further imaging tests. The UKCTOCS multimodal approach also showed a 20% decrease in mortality among cases that developed after 7 years of screening. [21] CA125 alone is limited, as only 80% of ovarian cancers express it, indicating the need for multiple biomarkers to detect early-stage cancers missed by current algorithms. [22] Tumor-derived proteins are present at higher concentrations near to their source, hence analysis of tumor cells, tissues, and

proximal fluids for differentially expressed proteins is being explored for biomarker discovery. [23]

Several studies have explored various methods for early detection and diagnosis of ovarian cancer. Wang et al. (2018) introduced PapSEEK, a test combining assays for mutations in 18 genes and aneuploidy, demonstrating a sensitivity of 33% in 245 ovarian cancer patients, with higher sensitivity (63%) when combined with plasma ctDNA testing. Specificity was around 99%, indicating a low false positive rate. [24] Enroth et al. (2019) used the proximity extension assay (PEA) to compare circulating plasma levels of 593 proteins, developing a multiplex PEA test with 11 biomarkers and age, showing an AUC of 0.94, PPV of 0.92, sensitivity of 0.85, and specificity of 0.93 for detecting ovarian cancer stages I–IV. [25] Guo et al. (2019) identified a four-biomarker panel (CA125, OPN, MIF, and anti-IL-8 autoantibodies) that detected 82% of early-stage ovarian cancers, outperforming CA125 alone (65%). [26] The CancerSEEK study introduced a blood test covering eight cancers, with a sensitivity of 98% for ovarian cancer and a false positive rate below 1%, currently enrolling women for further validation. The CancerSEEK test assessed the levels of circulating proteins and mutations in cell-free DNA. The test though could not determine the cancer type in patients that tested positive and the overall detection for all early-stage cancers was below 50%. [27]

Ovarian cancer screening is recommended primarily for women at high risk due to family history or genetic mutations. Routine screening for the general population is not currently recommended because it can lead to false positives and unnecessary surgeries. Research into more effective screening methods and biomarkers for ovarian cancer is ongoing, with the aim of improving early detection.

Table 1. List of FDA approved protein markers for ovarian cancer. Abbreviations: Tf, Transferrin; TTR, Transthyretin; Apo-A1, Apolipoprotein A-1; B2M, Beta-2 microglobulin; FSH, Follicle stimulating hormone.

BIOMARKER	SPECIMEN	METHODOLOGY	CLINICAL USE
CA125	Serum, plasma	Immunoassay	Monitoring disease progression, response to therapy
HE4	Serum	Immunoassay	Monitoring recurrence or progression of disease
ROMA (HE4+CA125)	Serum	Immunoassay	Prediction of malignancy
OVA1 (CA125 + Tf + TTR + Apo-A1 + B2M)	Serum	Immunoassay	Prediction of malignancy
Overa (CA125 + HE4 + FSH + Apo-A1 + Tf)	Serum	Immunoassay	Prediction of malignancy

2.3 Biofluids in biomarker discovery and interferences in immunoassays

Biofluids such as blood, urine, saliva, etc are indispensable in the diagnostic landscape, offering a non-invasive or minimally invasive means to uncover biomarkers that can lead to early detection, accurate diagnosis, and monitoring of diseases. Each biofluid serves as a window into the body's physiological and pathological processes. Diagnosing cancer using biofluids presents several challenges. The complexity of biofluids and the heterogeneity of cancer itself can complicate biomarker discovery. Variability in biofluid composition, influenced by factors such as patient lifestyle, medication, and underlying health conditions, can obscure the detection of cancer-specific biomarkers. The presence of highly abundant proteins or other molecules in these fluids can mask the presence of critical but low-abundance cancer markers, necessitating advanced techniques to enhance detection sensitivity. The collection, handling, and storage of biofluids must also be meticulously controlled to avoid degradation or contamination, which could lead to false results or misdiagnosis. Serum, cyst fluids and ascitic fluid have been used in the different studies (Study I, II, III) in this thesis.

Serum, the fluid portion of blood after clotting, is most commonly used in cancer diagnostics due to its rich composition of proteins, hormones, and antibodies. Its broad representation of systemic processes makes it an ideal medium for detecting biomarkers associated with various cancers. Serum is readily accessible, and its stability allows repeated testing and large-scale screening. However, in serum, the high levels of abundant proteins, such as albumin, can overshadow the detection of low-abundance cancer biomarkers. [28]

Cyst fluids, collected from pathological cysts in organs such as the ovaries or pancreas, are particularly useful in diagnosing and monitoring cancers associated with these tissues. These fluids are directly linked to the tumor microenvironment, offering a concentrated source of potential biomarkers that are highly specific to the cancerous process within the cyst. The main advantage of cyst fluids is their ability to reveal biomarkers that may not be detectable in more systemic biofluids like serum. However, the invasive nature of collecting cyst fluids limits their routine use. [29]

Ascitic fluid, which accumulates in the abdominal cavity, is more relevant for ovarian cancer, gastrointestinal cancers, and peritoneal carcinomatosis. The fluid reflects the local tumor environment, making it a rich source of cancer-specific biomarkers. It provides direct insight into abdominal malignancies, which can be crucial for detecting and staging cancers like ovarian cancer. However, the invasive procedure required to collect ascitic fluid (paracentesis) limits its use to cases where the benefits outweigh the risks. Also, the potential for contamination with blood or other substances and the variability in ascitic fluid composition depending on the

underlying cancer can pose challenges for consistent and reliable biomarker detection. The high protein content of ascitic fluid can also dilute specific cancer biomarkers, complicating their detection. [30,31]

The accuracy of immunoassays can be compromised by various interferences. Heterophilic antibodies, which can bind non-specifically to animal antibodies used in assays, often cause false-positive results. Rheumatoid factors, particularly in patients with rheumatoid arthritis, can also interfere by interacting with assay immunoglobulins. Autoantibodies, prevalent in autoimmune diseases, may bind to target antigens or assay antibodies, leading to erroneous biomarker quantification. Human anti-animal antibodies (HAAAs) present another challenge by causing cross-reactivity in assays that utilize animal-derived antibodies. The high-dose hook effect, where an excess of analyte leads to a paradoxically low signal, can result in underestimation of the biomarker. Matrix effects, influenced by the composition of the biological sample, and cross-reactivity, where unintended antigens are targeted, can both significantly alter assay results. Additionally, complement proteins, when activated during sample handling, can interfere with antibody binding, and various substances like lipids, bilirubin, and hemoglobin can cause turbidity or direct interactions that skew results. Biotin, a commonly encountered substance, can disrupt biotin-streptavidin-based assays, leading to false results. Assay-specific factors, including antibody quality and reagent stability, can introduce variability in results. To mitigate these interferences, strategies such as blocking agents, pre-treating samples, and improving assay design could be used. Rigorous validation and quality control practices are also crucial to ensure consistent and accurate performance in cancer biomarker detection. [32–34]

2.4 Glycoproteins as cancer biomarkers

Majority of the markers currently used in clinics are serum glycoproteins. Serum glycoproteins have emerged as valuable cancer biomarkers due to their potential to reflect the physiological and pathological changes associated with cancer development and progression. Serum glycoproteins, such as alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), and cancer antigen 125 (CA125), have been extensively studied as cancer biomarkers. These glycoproteins are secreted into the bloodstream by cancer cells and are detectable through blood tests, making them accessible for non-invasive diagnostic purposes. For example, AFP is commonly used as a biomarker for hepatocellular carcinoma, while CEA is associated with colorectal cancer. Elevated levels of these glycoproteins in the serum can indicate the presence of cancer, and monitoring their levels can aid in disease diagnosis, prognosis, and treatment response assessment. [35–37]

However, despite their diagnostic and prognostic value, the use of serum glycoproteins as cancer biomarkers is not without limitations. One major limitation is the lack of specificity, as elevated levels of these glycoproteins can also be observed in non-cancerous conditions, leading to false-positive results. Additionally, the sensitivity of these biomarkers may not be effective in detecting early-stage cancers or small tumor burdens. Factors such as inflammation, liver disease, and pregnancy can influence the levels of serum glycoproteins, leading to potential confounding factors in their interpretation as cancer biomarkers.

2.4.1 Mucin glycoproteins

Mucin glycoproteins are large, complex molecules that play crucial roles in various physiological processes. These glycoproteins are characterized by the presence of numerous O-glycans, which are carbohydrate chains attached to the protein backbone. Mucins can exist in different forms, including large polymeric (gel-forming) structures and small monomeric (soluble) forms, and are found in mucus on the surfaces of tracheobronchial, gastrointestinal, and genitourinary epithelium. [38]

One of the distinguishing features of mucin glycoproteins is the presence of variable number tandem repeat (VNTR) regions, which contain hundreds of O-glycans with remarkable variability. In fact, O-glycans may constitute up to 80% of the molecular weight of mucins. This structural diversity allows mucins to perform a wide range of functions. For instance, they are involved in signal transduction, cell-cell adhesion, and even exhibit exceptional homogenic glycoforms that serve as antifreeze agents in fish. Furthermore, mucins are known to mediate fertilization and are involved in various other biological processes. [38]

Importantly, alterations in mucin glycoproteins have been implicated in many human diseases, particularly in cancers. MUC1, MUC4, MUC13, and MUC16 are among the most common mucins that have been found to be modified in various types of cancer. Targeting the aberrant glycans on these mucins could hold promise for detecting cancer.

2.4.1.1 MUC16 / CA125

MUC16, also known as CA125, is a significant biomarker that was first identified by Bob Bast and his colleagues in 1981. This discovery, made using a cell line (OVCA 433) from a patient with serous papillary cystadenocarcinoma of the ovary, marked a crucial advancement in the field of cancer biomarker research. [39] CA125 is a mucin-type molecule with a high molecular mass ranging from 200 to 2000 kDa. It is characterized by an abundance of N- and O-glycans, with over 249 potential N-

glycosylation and more than 3700 O-glycosylation sites. Approximately 24%-28% of its composition consists of carbohydrates. [40] CA125/MUC16 is expressed by epithelial ovarian tumors as well as some pathologic and normal tissues.

The protein core of CA125 is composed of a large glycosylated extracellular structure, a transmembrane domain, and a short cytoplasmic tail of 32 amino acids. This membrane-bound protein is released into bodily fluids in high concentrations, making it a valuable target for diagnosis of epithelial ovarian cancer (EOC). CA125 is characterized by a high content of proline, threonine, and serine in the N-terminal region tandem repeats, as well as non-tandem repeats in the C-terminal region. Despite its established role as a biomarker, the biological function of CA125 in both normal and diseased individuals remains incompletely understood. However, evidence suggests its involvement in cell-mediated immune responses, indicating potential roles beyond its traditional use as a cancer biomarker. [41]

2.4.1.2 MUC1 / CA15-3

MUC1, also known as CA15-3 glycoprotein, is a protein encoded by the MUC1 gene. This glycoprotein consists of two peptide fragments: the longer N-terminal subunit (MUC1-N) and the shorter C-terminal subunit (MUC1-C), which remain associated through stable hydrogen bonds. MUC1 is extensively O-glycosylated and moderately N-glycosylated, contributing to 50–90% of its total weight. Its weight can vary between 250 and 500 kDa based on the number of tandem repeats and the degree of glycosylation. [42]

Tumor-associated MUC1 differs from that expressed in normal cells, exhibiting different biochemical features and cellular distribution. For instance, MUC1 in breast cancer cells mostly exhibits Core 1 O-glycans, unlike the extensively branched Core 2 O-glycans found in normally expressed MUC1. Carbohydrate antigen 15-3 (CA15-3, MUC1) is commonly used for the detection of breast cancer, while carbohydrate antigen 19-9 (CA19-9, sLe^a antigen, found on several glycoproteins including MUC1) is used for the detection of pancreatic cancer. [43]

2.4.2 HE4 glycoprotein

Human Epididymis Protein 4 (HE4) is a 25 kDa secreted glycoprotein predominantly expressed in the tissues of the epididymis, lung, and trachea. [44] This protein is classified under the whey-acidic-protein (WAP) four-disulfide core domain (WFDC2) family, indicating that it possesses protease inhibitor activity, particularly interacting with serine proteases such as Prss35 and Prss23, which have been linked to kidney fibrosis in mouse models. [45] The mature HE4 polypeptide features one consensus N-glycosylation site at position 14, suggesting that its glycosylation status

could affect its migration, as ovarian carcinomas predominantly secrete HE4 as an N-glycosylated protein. [44] Located on chromosome 20, the HE4 gene (WFDC2) is often found in amplified segments associated with various cancers, including breast, ovarian, colon, pancreatic, and lung cancers. [46]

HE4 is used as a biomarker in the diagnosis and prognosis of ovarian and endometrial cancers. The combination of serum HE4 with CA125 has demonstrated improved sensitivity in identifying malignant conditions without compromising specificity, which has facilitated the development of the Risk of Ovarian Malignancy Algorithm (ROMA) for distinguishing between malignant and benign pelvic masses. HE4 can be detected in cases where CA125 levels are undetectable, making it a reliable option for detecting recurrences and in identifying early-stage ovarian cancer. [47]

2.4.3 Tetraspanins and integrins

Tetraspanins are a diverse group of surface glycoproteins that play a critical role in organizing and regulating various cellular processes. Characterized by their four transmembrane domains, tetraspanins are involved in the formation of tetraspanin-enriched microdomains (TEMs) within the cell membrane. [48] These microdomains function as scaffolding platforms, bringing together different proteins, including integrins, growth factor receptors, and other tetraspanins, to modulate cell signaling, adhesion, motility, and membrane trafficking. Major tetraspanins such as CD9, CD24, CD63, CD81, and CD151 are known for their association with critical cancer-related processes including invasion, metastasis, motility, tumor initiation, progression, promotion, and angiogenesis. [49]

CD63 was the first tetraspanin to be identified and is a well-studied member of the tetraspanin family, known for its involvement in intracellular trafficking and cell signaling. [50] Structurally, CD63, like other tetraspanins, contains four transmembrane helices that anchor it to the cell membrane. Between these helices are two extracellular loops: a small extracellular loop (SEL) and a large extracellular loop (LEL). The LEL is particularly significant as it contains regions crucial for protein-protein interactions and post-translational modifications, such as glycosylation. CD63 is a known marker for exosomes, and its presence on the exosomal surface is often used to isolate and study these vesicles. The presence of CD63-positive exosomes in bodily fluids such as blood, urine, and saliva can serve as a non-invasive biomarker for cancer. [51]

Integrins (ITGs) are a diverse family of glycoproteins that play a crucial role in cancer progression by facilitating the migration and invasion of cancer cells into the extracellular matrix (ECM). [52] These heterodimeric molecules consist of two integral glycoprotein subunits, alpha (α) and beta (β), which pair to form functional

receptors. The role of ITGs in cancer progression and metastasis is well-documented, with specific subtypes such as α2, α3, α6, αv, β1, and β4 showing significant expression in ovarian cancer cells. [53]

Alterations in ITG glycosylation, particularly N-glycosylations like sialylation and core fucosylation, are frequently observed in tumors and modulate ITG functions during cancer progression. Additionally, truncated O-glycan structures like T, Tn, and sialyl-Tn (STn) are expressed early in tumorigenesis and are linked to poor survival in carcinoma patients. [54]

2.5 Glycosylation changes in cancer

Glycosylation alterations in cancer, particularly protein glycosylation, have gained significant attention due to their potential impact on tumor progression, metastasis, and immune evasion. N-glycosylation and O-glycosylation, two major types of protein glycosylation, undergo significant changes in cancer cells, leading to the generation of aberrant glycoforms that contribute to the malignant phenotype.

In cancer, N-glycosylation alterations are commonly observed, leading to the generation of truncated and branched N-glycans. These changes are associated with the activation of specific glycosyltransferases and glycosidases, resulting in the aberrant processing of N-glycans. For example, increased expression of enzymes such as N-acetylglucosaminyltransferases (GnTs) and fucosyltransferases has been reported in various cancer types, leading to the synthesis of complex and highly branched N-glycans. [55]

O-glycosylation alterations in cancer are characterized by the abnormal expression of O-glycan core structures and the dysregulation of O-glycan elongation enzymes. Mucin-type O-glycans, commonly found in cancer-associated mucins, exhibit truncated and hypoglycosylated structures in cancer cells. These changes are mediated by the dysregulation of core 1 synthase and other glycosyltransferases, leading to the exposure of Tn and sialyl-Tn antigens, which are associated with poor prognosis in cancer patients. [56]

Glycosylation changes in cancer have significant implications for tumor biology and clinical outcomes. Aberrant protein glycosylation contributes to the modulation of cell surface receptors, adhesion molecules, and immune checkpoints, influencing tumor cell interactions with the microenvironment and immune system. Moreover, altered glycosylation patterns can serve as potential biomarkers for cancer diagnosis and prognosis and identifying the specific glycoforms could lead to early detection of cancer. [57]

2.6 Tools to detect glycosylation

2.6.1 Antibodies

Antibodies are powerful tools in the study of glycosylations, particularly in the detection and analysis of specific glycan structures such as the Sialyl-Tn (STn) and Tn antigens. The Sialyl-Tn antigen (STn) is a short O-glycan characterized by the presence of a sialic acid (Neu5Ac in human) residue linked via an α2,6 bond to an N-acetylgalactosamine (GalNAc) that is α-O-linked to a serine or threonine residue (Neu5Acα2-6GalNAcα-O-Ser/Thr). This specific glycan structure is notably simple, comprising only two sugar residues. The presence of the sialic acid at carbon 6 of GalNAc inhibits the formation of more complex glycan cores typically found in mucin-type O-glycans. [58] Despite its simplicity, the STn antigen plays a critical role in cancer biology due to its altered expression patterns in malignant tissues.

STn is weakly expressed in fetal and normal adult tissues, but it is aberrantly overexpressed in more than 80% of human carcinomas, including those of the pancreas, colorectal region, and ovaries. This overexpression is strongly associated with adverse outcomes and reduced overall survival, making STn a key onco-fetal antigen. [58] Its expression in cancerous tissues contrasts sharply with its absence in normal ovarian epithelium, highlighting its specificity as a cancer marker. The presence of STn in serum is typically due to the secretion of O-glycoproteins or the shedding of tumor cells into the bloodstream, which generally occurs in advanced stages of cancer. High levels of STn have been detected in the sera of patients with various types of cancers, with frequencies ranging from 11% to 86% depending on the type, including gastric, pancreatic, ovarian, and colorectal cancers. [59,60] Monoclonal antibodies against STn were among the first to demonstrate that this antigen is overexpressed in cancer cells compared to healthy cells. These antibodies have since become essential tools for studying the role of STn in tumorigenesis and for developing diagnostic assays.

Tn antigen, which is closely related to STn, is an even simpler glycan structure, consisting solely of GalNAc α-O-linked to serine or threonine without the sialic acid modification. While Tn is also associated with cancer, its expression patterns differ slightly, making it another useful target for cancer biomarker research. [61] The detection and analysis of Tn and STn antigens via specific antibodies provide valuable insights into the glycosylation changes that accompany cancer, offering promising avenues for early diagnosis, prognosis, and potentially targeted therapies.

2.6.2 Lectins

Lectins are carbohydrate-binding proteins of non-immune origin that exhibit high specificity for sugar moieties. The ability of lectins to bind specific glycan structures makes them especially useful in detecting glycosylation changes associated with various diseases, including cancer.

Lectins can be broadly classified into plant and animal lectins based on their origin. Plant lectins, such as those found in seeds, grains, and raw legumes, have been widely studied and used in research. Concanavalin A (ConA), for example, was one of the first lectins to be purified on a large scale and has been extensively used in the characterization and purification of sugar-containing molecules. Animal lectins, on the other hand, include families like galectins, selectins, and siglecs, each playing crucial roles in biological processes such as immune response, cell adhesion, and signal transduction. [62]

Glycosylation changes, such as increased fucosylation, sialylation, and overexpression of truncated mucin-type O-glycans, are hallmarks of cancer progression and invasion. Lectins, due to their specificity, are uniquely suited to detect these changes. For instance, the macrophage galactose-type lectin (MGL) has been shown to bind to rare terminal GalNAc structures. Wheat germ agglutinin (WGA), a plant lectin, specifically binds to N-acetylglucosamine (GlcNAc) and sialic acid, both of which are commonly found in the glycan structures that are altered in cancer cells. The use of lectin-based approaches can be applied to tissue lysates, serum samples, or even cell surfaces to screen for novel cancer biomarkers. [62]

2.6.3 Antibodies / lectins as tools to detect glycosylations

The clinical applications of antibodies and lectins in detecting altered glycosylations could be used in cancer diagnostics, providing specificity and sensitivity in various methodologies. One of the most widely used methodologies is the sandwich enzyme-linked immunosorbent assay (ELISA). Akita et al. utilized a sandwich ELISA where CA125 carrying the Sialyl-Tn (STn) antigen was captured by an STn monoclonal antibody. This assay was able to discriminate significantly higher levels of STn-CA125 in the peritoneal fluid of ovarian cancer patients compared to those with endometriosis, highlighting its diagnostic potential. [63] Similarly, Shang et al. demonstrated the effectiveness of an AAL lectin-based magnetic bead ELISA for detecting fucosylated haptoglobin in serum samples from hepatocellular carcinoma patients. This method provided rapid and accurate detection, underscoring the utility of lectin-based assays in clinical diagnostics. [64] Another example is the use of Lens culinaris agglutinin (LCA) lectin in an ELISA-based assay to measure α-fetoprotein (AFP) levels. The assay effectively distinguished between benign and malignant

liver diseases, with malignant patients showing higher levels of lectin-reactive AFPs, thus supporting its role in liver cancer diagnosis. [65]

Lectins have also been integrated into microarray glycoprofiling platforms, which offer high-throughput analysis of glycosylation patterns across multiple samples. A study by Chen et al. in 2013 demonstrated that microarray glycoprofiling of CA125, a well-known ovarian cancer biomarker, could improve differential diagnosis of ovarian cancer. In this study, Vicia villosa lectin (VVL) was used to detect Tn glycoforms on serum-secreted CA125, showing excellent glycan specificity. [66] Despite the promising results, microarray platforms face challenges such as low reproducibility, which limits their widespread clinical adoption. [67]

Proximity Ligation Assays (PLA) represent another innovative application of antibodies and lectins in cancer glycosylation detection. Ricardo et al. in 2015 used PLA to distinguish aberrant glycoforms, such as Tn and STn, of MUC16 and MUC1 in serum from ovarian cancer patients compared to those with benign lesions. This approach allowed for highly specific detection of glycosylation changes, contributing to more accurate cancer diagnosis. [68] Additionally, Zhang et al. in 2018 utilized PLA with ConA lectin to recognize mannose residues on cancer cells, further showcasing the versatility and precision of PLA in detecting specific glycan alterations associated with malignancy. [69] The clinical applications in detecting altered glycosylations in cancer are advancing rapidly, with various methodologies like ELISA, microarray glycoprofiling, PLA and others, offering new avenues for early diagnosis and better patient outcomes.

2.7 Nanoparticle based glycovariant assays

The reliable detection of cancer-specific glycoforms requires a diagnostic platform that is not only specific and robust but also cost-efficient for widespread clinical use. Improving the binding affinity of antibodies and lectins is critical for enhancing the sensitivity of such assays. This can be achieved through the use of europium chelate-doped nanoparticles (Eu-NPs), which provide significant signal amplification and increased functional affinity, or avidity. The integration of Eu-NPs with time-resolved fluorescence (TRF) technology can facilitate the development of a streamlined, rapid two-step protocol for precise glycan profiling.

To address the issue of insufficient affinity in lectins and low-affinity glycan-specific antibodies, large fluorescent nanoparticles are employed. These nanoparticles provide substantial signal amplification by the approximately 30,000 Eu-chelates packed within each 95 nm nanoparticle. These nanoparticles also enable the immobilization of multiple antibodies/lectins, thereby enhancing reactivity through a bioavidity effect while maintaining specificity. The high signal amplification achieved with Eu-NP technology significantly surpasses that of direct

fluorescent reporter coupling. [70] Unlike traditional fluorescence methods, which measure the intensity of emitted light at a single point in time, TRF monitors the decay of fluorescence signals after excitation over a specific time window. This approach allows for the differentiation between fast-decaying background signals and longer-lived fluorescence emissions from the sample of interest. TRF improves signal-to-noise ratios by minimizing interference from short-lived background fluorescence and enhances sensitivity and specificity in detecting fluorescent molecules.

The Eu-NP-assisted TRF technology has demonstrated significant improvements in various cancer diagnostic assays. [71] For example, the use of macrophage galactose-type lectin (MGL) or STn-antibody coated Eu-NPs has been applied in epithelial ovarian cancer diagnosis, showing a 10- to 100-fold enhancement in analytical performance compared to traditional Eu-chelate-labeled lectins. [72,73] This platform has also proven effective in prostate cancer detection using AAL lectin for PSA assays, in breast cancer detection with WGA and MGL lectins for CA15-3 assays, and in profiling surface glycosylation in prostate cancer through urine-derived extracellular vesicles. [74–76]

Further studies have highlighted the versatility of Eu-NP based assays in identifying cancer-associated glycoforms. For instance, aberrant fucosylation of ITGα3 was previously identified in bladder cancer urine samples. [77] Additionally, detecting cancer-associated glycosylation of MUC1 and MUC16 with WGA, alongside the measurement of total CD63 concentration has been shown to aid in the differential diagnosis of primary breast cancer. [78] The use of the Eu-NP-based assay not only enhances sensitivity and specificity but also holds promise for reducing false-positive rates in conventional immunoassays.

3 Aim of the study

The principal aim of this thesis was to identify and evaluate the potential of novel biomarkers in ovarian cancer diagnosis. In addition, we also aimed at developing a simple and sensitive nanoparticle based immunoassay for ovarian cancer detection.

The distinct aims of the study were:

I. To identify new MUC16 and MUC1 based glycovariant biomarkers in cyst fluids and serum in a discovery cohort (n=75), and test the performance of previously reported and new markers in a validation cohort (n=272) for ovarian cancer detection.

II. To establish a proof-of-principle assay for the detection of integrin and tetraspanin glycoisoforms from cyst fluids and ascitic fluid for the diagnosis of ovarian cancer.

III. To validate the best performing markers from study I in a large multi-center study (n=1602) using samples from three different hospital centers in Umeå, Gothenburg and Turku. Aim was also to test the performance of combination biomarkers along with several subtype analysis. Additional unpublished data from tumor histotype analysis and metastatic analysis is also included.

4 Materials and Method

4.1 Study Design

The study population included four cohorts prospectively and consecutively collected at tertiary referral hospitals in Sweden (I, II, III) and Finland (III). The first cohort is from patients recruited for diagnostic and debulking surgery between 2001 and 2010, at the unit for gynecologic cancer surgery at Sahlgrenska University Hospital, Gothenburg, Sweden (I, II). These patients were divided into a discovery and a validation cohort. The discovery cyst fluid cohort (I, II) was based on a previously published study where 8 different mutation markers were analysed in DNA extracted from 77 cyst fluid samples. [79] Blood samples were taken after anesthesia but prior to surgery while CF was aspirated directly after removal of the cyst from the abdomen. In Study I, two samples (one healthy and borderline) were excluded from the discovery cohort, as the paired serum of these patients was not available. The validation cohort in Study I, comprised of 272 paired CF and serum samples. In Study II, six ascitic fluid samples from Turku University Hospital were also included, where 2 were liver cirrhosis as benign condition and 4 epithelial ovarian cancer (EOC).

In Study III, patient cohorts from three different hospital centers were included. In Sweden, at the University hospital of Umeå, the blood samples were collected 1990-2016 from 622 women and at the Sahlgrenska University Hospital in Gothenburg 2016-2019 from 498 women. In Finland, at the Turku University hospital, the blood samples were collected 2009-2019 from 482 women.

In the study population, patients with neo-adjuvant chemotherapy and patients not accepting or understanding informed and written consent were excluded. Handling and processing of samples were standardized for all patients. All tumors were diagnosed, staged and graded according to existing FIGO classification, and reviewed according to FIGO 2014 by specialist in gynecologic pathology. The specific characteristics of the study populations are presented in **Table 2**.

The study design and protocol were approved by the local ethics committee in accordance with the Helsinki Declaration. The studies were approved by the regional ethical review authority in Umeå (Dnr. 2017-376-31), the ethical review authority in

Gothenburg (Dnr. 201-15) and the ethics committee in the Hospital District of Southwest Finland (ETMK 53/180/2009).

Table 2. Patient characteristics per study.

	STUDY I		STUDY II		STUDY III
SAMPLE MATRIX	Discovery – CF & Serum	Validation – CF & Serum	Cyst Fluid	Ascitic Fluid	Serum
ALL PATIENTS	75	272	77	6	1602
N (EOC)	31	67	31	4	596
50 & BELOW AGE GROUP	8	13	8	-	69
ABOVE 50 AGE GROUP	23	54	23	-	527
N (BENIGN)	12	129	12	-	716
50 & BELOW AGE GROUP	2	25	2	-	294
ABOVE 50 AGE GROUP	10	104	10	-	419
N (HEALTHY)	9	58	10	2	-
50 & BELOW AGE GROUP	4	18	4	-	-
ABOVE 50 AGE GROUP	5	40	6	-	-
N (BORDERLINE)	23	18	24	-	115
N (METASTATIC)	-	-	-	-	175
FIGO (2014) - EOC					
I	8	36	8	-	80
II	3	2	3	-	53
III	17	27	17	-	278
IV	3	2	3	-	144
HISTOLOGY - EOC					
HGSC	16	28	16	-	408
LGSC	6	5	6	-	52
CLEAR CELL	1	7	1	-	29
ENDOMETRIOID	6	17	6	-	51
MUCINOUS	1	10	1	-	38

4.2 Reagents

A panel of monoclonal antibodies (mAb) used in the different studies are listed in **Table 3**. Antibodies are either biotinylated with biotin or conjugated on nanoparticles. Some antibodies are also used as $F(ab')_2$, after removing the Fc region using bromelain digestion. The lectins were used as tracers after coating on europium-nanoparticles and are listed in **Table 4**.

The ovarian cancer cell line OVCAR-3 purified CA125 (Fujirebio Diagnostics AB, Göteborg) was used to make standards for MUC16 GVs. An EOC patient's ascitic fluid having 300 U/mL of CA15-3 was used for standards of MUC1 GVs. Yellow streptavidin coated low fluorescence microtitration plates, wash buffer and the assay buffer was obtained from Uniogen Oy (Turku, Finland). Europium (III)-Chelate-doped Fluoro-Max^{TM} polystyrene nanoparticles (95 nm diameter) were acquired from Seradyn Inc. (Indianapolis, IN, USA).

Table 3. Antibodies used in this doctoral study.

ANTIBODY NAME	SPECIFICITY	CLONE	MANUFACTURER	STUDY
Ov185 mAb	CA125 antigen	-	Fujirebio Diagnostics AB	I, III
Ma552 mAb	CA15-3 antigen	-	Fujirebio Diagnostics AB	I, III
STn1242 mAb	Sialylated-Tn antigen	-	Fujirebio Diagnostics AB	I, II, III
Tn	Anti-Tn antigen	5F4	SBH Sciences	I
CD63	CD63 antigen	H5C6	BD Biosciences	II
ITGα3	ITGα3 antigen	IA3	R&D Systems	II

Table 4. Lectins used in this doctoral study.

LECTIN NAME	ABBREVIATION	SPECIFICITY	MANUFACTURER	STUDY
Macrophage galactose lectin (CLEC 10A)	MGL	GalNAc	R&D Systems	I, III
Wheat germ agglutinin	WGA	GlcNAc	Vector Laboratories	I, II

4.3 Preparation of assay reagents

4.3.1 Nanoparticle conjugation with lectins / antibodies

The amino groups of CD63, integrin binding mAbs, STn, Tn and lectins were covalently coupled to the activated carboxyl group of the europium-nanoparticles. Briefly, NP ($5x10^{11}$ - $1x10^{12}$ particles) were applied to nanosep 300 kDa omega centrifugal device (VWR, USA) and washed with conjugation buffer (50 mmol/L MES, pH 6.1). The particles were resuspended using tip sonication and then transferred to a microcentrifuge tube. The surface of these NP was activated for 15 mins with 8 mmol/L NHS (Sigma-Aldrich, USA) and 2.6 mmol/L EDC (Sigma-Aldrich, USA). Antibodies and lectins were coupled to the NP under vigorous shaking for 1.5 h at RT in a solution containing 100 mM NaCl and 500 mM MES pH 6.1. pH was raised with 1 mol/L carbonate buffer and 10 g/L BSA was added to block the remaining active sites on the particle. The solution was then stored overnight at 4 ºC.

Next day, the NP conjugated mixture was again washed, resuspended, and then stored at 4 ºC for a few days before the removal of aggregates. After that, conjugated-NP mixture was centrifuged (2000 rpm, 5 min) to remove noncolloidal aggregates. Then aggregates-free supernatant was transferred to a new tube. The particle concentration was determined by diluting the particles with 0.1% v/v triton-x-100 solution and by comparing with a known standard particle stock concentration. The measurements were performed with 1420 Victor™ Multilabel Counter (PerkinElmer, Finland) and nanoparticle concentrations were determined. The antibody/lectin coated Eu-NP were stored using the buffer 10 mmol/L Tris-HCl, pH 7.8, supplemented with 0.1% BSA and 0.01% sodium azide at 4 ºC. The particles were thoroughly vortexed before every use to disperse the aggregates.

4.3.2 Bromelain digestion of mAb for removal of Fc region

To reduce immunoassay interference, capture mAb Ov185 and Ma552 (I, III) was digested to $F(ab')_2$ fragments with bromelain enzymatic (ID-Diluent 1, Diamed, Cressier, Switzerland) treatment which resulted in the Fc removal of the mAb, following the protocol by [80] with some modifications. Briefly, 40 µL of bromelain was added for 1 mg of the mAb and a $1/10^{th}$ reaction volume of 10X digestion buffer (pH 7.0, 0.5 mol/L Tris-HCl, 1 mol/L NaCl, 30 mmol/L EDTA). After incubation at 37 °C for 2.5 h, $1/10^{th}$ reaction volume of freshly made 0.2 mol/L NEM was added to stop any further enzymatic reaction. Protein G Hitrap-chromatography column (GE Healthcare, Life Sciences, UK) was used to purify the enzymatically digested $F(ab')_2$ fragments. NAP™-5 and NAP™-10 gel-filtration columns were used to change the buffer of the purified fragments with 0.9 g/L NaCl before further modifications.

4.3.3 Biotinylation of antibodies

Anti-CD63 and anti-integrin antibodies along with F(ab')$_2$ fractions of Ov185 and Ma552 were biotinylated with 40-fold molar excess of biotin-isothiocyanate in 50 mmol/L sodium carbonate buffer (pH 9.8) for 4 h at room temperature. Biotinylated antibodies were purified with NAP^{TM}-5 and NAP^{TM}-10 gel-filtration columns with the use of 50 mmol/L Tris-HCl (pH 7.75), containing 150 mmol/L NaCl and 0.5 g/L NaN_3. The biotin labelled antibody was stored in 1 g/L BSA at 4 °C.

4.4 Methods

Immunoassays were performed in this study using a sandwich format as shown in **Figure 2**. Briefly, biotinylated capture antibodies were immobilized on a streptavidin coated low fluorescence microtiter wells in the assay buffer for 60 mins at RT. After two washes, samples diluted in assay buffer were added in triplicates and incubated for 60 mins at RT with shaking. Again, after two washes, lectins/antibodies conjugated with Eu-NPs in assay buffer were added as tracers and incubated for 60 mins at RT with shaking. After six washes, time resolved fluorescence measurement was performed (λex: 340 nm; λem: 615 nm) from dry wells using Hidex Sense (Hidex Oy, Turku, Finland).

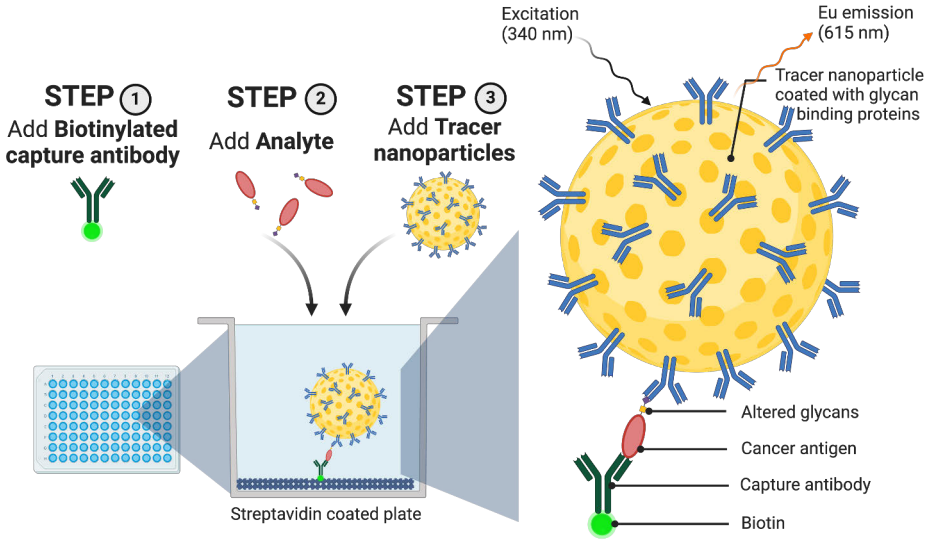

Figure 2. Diagrammatic representation of the methodology of the europium-nanoparticle based immunoassay. Created with BioRender.com

4.4.1 Study I

A paired CF & serum discovery cohort (N=75) was used to test five different MUC16 and MUC1 based GV markers. These were $MUC16^{MGL}$, $MUC16^{STn}$, $MUC1^{STn}$, $MUC1^{Tn}$ and $MUC1^{WGA}$. Best performing markers, $MUC16^{MGL}$, $MUC16^{STn}$ and $MUC1^{STn}$ were then tested in a paired CF & serum validation cohort (N=272). $MUC1^{Tn}$ was not detected in serum and was hence included only in the CF validation cohort. Fujirebio CA125 EIA, CA15-3 EIA and HE4 EIA kits were used according to manufacturer's instructions as reference for comparisons. Stage and age-based subgroup analysis was performed.

4.4.2 Study II

EV and integrin glyco-isoforms are reported in a panel of 77 cyst fluid samples from the Gothenburg discovery cohort. Four different assays are tested. Two as reference immunoassays, $CD63^{IA}$ and $ITG\alpha3^{IA}$, where same antibody was used as biotinylated capture and as Eu-NP coated tracer. The other two are their STn glycovariant assays, $CD63^{STn}$ and $ITG\alpha3^{STn}$, where STn antibodies coated on Eu-NP were used as tracer molecules. These markers were also tested in 6 ascitic fluid samples including 4 EOC and 2 benign liver cirrhosis.

4.4.3 Study III

A large multi-center study (N=1602) was performed using samples from three different hospital centers in Umeå, Gothenburg and Turku. Three GV markers were included for testing, $MUC16^{MGL}$, $MUC16^{STn}$ and $MUC1^{STn}$. These were compared against the reference CA125 EIA, CA15-3 EIA and HE4 EIA Fujirebio kits. Combination markers were tested for benefits. Along with stage and age-based analysis, tumor histotypes were also analyzed for assay performances. A separate analysis of the metastatic samples was performed.

4.5 Statistical analyses

Statistical analysis was performed using IBM SPSS Statistics (version 28), Origin (version 2016) and R-software. The receiver operating characteristic (ROC) curve analysis was carried out by plotting specificity (SP) against sensitivity (SN) of the assay and measuring area under the curve (AUC) at 95% confidence interval (CI). The ROC curves for marker combinations were derived using logistic regression in SPSS. Origin was used to make the boxplots and calculate the p-values using the two-sample t-test, where p-value below 0.05 was considered statistically significant.

5 Results & Discussion

The results presented here are reported in the original Studies I–III. Additional, unpublished data on tumor histotypes and metastatic analyses are reported in section 5.7 and 5.8 respectively.

5.1 Mucin glycoforms are EOC specific biomarkers (I)

Several mucin glycovariant biomarkers were tested in paired cyst fluid and serum samples in a discovery (N=75) and a validation cohort (N=272). For the analysis, malignant samples were measured against the benign and healthy group. Based on the discovery cohort, $MUC16^{STn}$ was the best performing marker in both CF and serum samples. In the CF validation cohort (borderline excluded), detection was increased by 43% with $MUC1^{STn}$ over CA15-3 EIA and 46% with $MUC16^{STn}$ over CA125 EIA at 90% specificity (SP). On combining the MUC1 GVs (STn & Tn), detection increased by 42% over CA125 + CA15-3 EIA. It can be seen in CF, that the immunoassay defined mucin assays CA125 and CA15-3 perform poorly compared to their glycovariants to discriminate EOC from benign and healthy control. In the serum validation cohort, $MUC16^{STn}$ stood out as the best performing marker, as seen also in the discovery cohort. In serum, $MUC16^{STn}$ detected 75% cases at 90% SP showing a 9% improvement over CA125 EIA.

5.2 Integrin glycovariants potential for EOC detection (II)

STn glycoforms were also tested for tetraspanin (CD63) and integrin (ITGA3) based assays for EOC detection. To measure total tetraspanin and integrin amounts CD63 and ITGA3 immunoassays were also included. The STn glycovariant of CD63 shows significant discrimination ($p < 0.005$) of non-malignant with both borderline as well as EOC cases (non-malignant refers to healthy+benign). The median S/B ratio of EOC in $CD63^{STn}$ assay is also 5-fold higher than the $CD63^{IA}$. $ITG\alpha3^{IA}$ also significantly discriminates the EOC samples ($p < 0.01$). The GV of ITGα3 ($ITG\alpha3^{STn}$), significantly discriminates non-malignant with both borderline and EOC

cases ($p < 0.01$). In case of CD63 based assays, the AUC of CD63 was 0.637, which increased to 0.945 with $CD63^{STn}$. The AUC of $ITG\alpha3^{IA}$ and $ITG\alpha3^{STn}$ was 0.912 and 0.940 respectively that increased to 0.976 on combination of the two integrin markers. These markers were also tested in ascitic fluid, where $ITG\alpha3^{STn}$ GV performed best and detected 3 out of 4 EOC compared to 2 benign liver cirrhosis samples.

5.3 STn glycoforms improve EOC detection (III)

In concordance with our previous study I, CA125 EIA is particularly poor at high specificities. $MUC16^{MGL}$ and CA15-3 EIA were the least performing single markers and thus have not been reported. Borderline samples were not included in the analysis and are analysed separately in section 4.6. Metastatic samples are analysed separately in section 4.8 focusing on gynecologic and gastrointestinal (GI) cases. In the EOC (N=596) vs benign (N=716) analysis, $MUC16^{STn}$ performed similar to HE4 EIA detecting 73% cases at high 98% SP, showing an improvement of 22% over CA125 EIA. HE4 on combination with the 2 STn GVs detected 85% cases, which was significantly better compared to CA125+HE4 reference ($p < 0.0001$).

5.4 Stage based analysis (I & III)

Both early and late-stage ovarian cancer in Study I and III were analysed. Only early-stage results are discussed here as it is the clinically more relevant group. In Study I there were 38 early stage EOC. At 90% SP, in CF validation cohort, $MUC1^{STn}$ recognized 31 EOC cases (82% SN) whereas CA125 + CA15-3 EIA recognized only 19 (50% SN). In serum validation, $MUC1^{STn}$ recognized 23 cases (61% SN) where CA125 + HE4 EIA only 17 (45% SN).

In Study III there were 133 early stage EOC cases. At 98% SP, CA125 EIA recognized only 30 cases (23% SN) whereas other single markers recognized 58 ($MUC16^{STn}$), 61 ($MUC1^{STn}$) and 69 (HE4 EIA) cases respectively. On combining HE4 EIA with the 2 STn markers, detection increased to 90 early-stage EOC cases. Serum sample results from Study I and III for various subgroups including tumor histotypes are compiled in **Table 5**.

Table 5. Data compilation of serum samples from Study I (validation cohort) and Study III for various subgroups. Data information: Area under the curve (AUC) at 95% confidence interval (CI) and sensitivity at 90 & 98% specificity is reported for Study I and III respectively.

SUBGROUP	BIOMARKER	N (EOC)	N (BENIGN + HEALTHY)	AUC (95% CI)	SPECIFICITY	SENSITIVITY	STUDY
OVERALL EOC	CA125 EIA	67	187	0.85	90	66	I
VS BENIGN		596	716	0.92	98	51	III
	HE4 EIA	67	187	0.82	90	58	I
		596	716	0.95	98	73	III
	$MUC16^{STn}$	67	187	0.88	90	75	I
		596	716	0.92	98	73	III
	$MUC1^{STn}$	67	187	0.87	90	70	I
		596	716	0.91	98	69	III
	CA125 + HE4 EIA	67	187	0.83	90	61	I
		596	716	0.96	98	74	III
	$MUC16^{STn}$ + $MUC1^{STn}$	67	187	0.90	90	73	I
		596	716	0.93	98	75	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	67	187	0.84	90	64	I
		596	716	0.97	98	85	III

SUBGROUP	BIOMARKER	N (EOC)	N (BENIGN + HEALTHY)	AUC (95% CI)	SPECIFICITY	SENSITIVITY	STUDY
EARLY STAGE	CA125 EIA	38	187	0.76	90	47	I
		133	716	0.83	98	23	III
	HE4 EIA	38	187	0.73	90	42	I
		133	716	0.92	98	52	III
	$MUC16^{STn}$	38	187	0.83	90	61	I
		133	716	0.79	98	44	III
	$MUC1^{STn}$	38	187	0.82	90	61	I
		133	716	0.83	98	46	III
	CA125 + HE4 EIA	38	187	0.74	90	45	I
		133	716	0.92	98	53	III
	$MUC16^{STn}$ + $MUC1^{STn}$	38	187	0.84	90	61	I
		133	716	0.85	98	48	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	38	187	0.76	90	50	I
		133	716	0.94	98	68	III

SUBGROUP	BIOMARKER	N (EOC)	N (BENIGN + HEALTHY)	AUC (95% CI)	SPECIFICITY	SENSITIVITY	STUDY
ABOVE 50	CA125 EIA	54	144	0.85	90	69	I
AGE GROUP		527	419	0.93	98	47	III
	HE4 EIA	54	144	0.83	90	56	I
		527	419	0.94	98	69	III
	$MUC16^{STn}$	54	144	0.89	90	76	I
		527	419	0.93	98	80	III
	$MUC1^{STn}$	54	144	0.90	90	80	I
		527	419	0.92	98	77	III
	CA125 + HE4 EIA	54	144	0.84	90	61	I
		527	419	0.96	98	74	III
	$MUC16^{STn}$ + $MUC1^{STn}$	54	144	0.92	90	76	I
		527	419	0.95	98	84	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	54	144	0.86	90	63	I
		527	419	0.97	98	85	III

SUBGROUP	BIOMARKER	N (EOC)	N (BENIGN + HEALTHY)	AUC (95% CI)	SPECIFICITY	SENSITIVITY	STUDY
HGSC	CA125 EIA	408	716	0.95	98	62	III
	HE4 EIA	408	716	0.98	98	84	III
	$MUC16^{STn}$	408	716	0.95	98	85	III
	$MUC1^{STn}$	408	716	0.94	98	79	III
	CA125 + HE4 EIA	408	716	0.98	98	86	III
	$MUC16^{STn}$ + $MUC1^{STn}$	408	716	0.95	98	86	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	408	716	0.99	98	93	III
LGSC	CA125 EIA	52	716	0.87	98	31	III
	HE4 EIA	52	716	0.88	98	48	III
	$MUC16^{STn}$	52	716	0.89	98	52	III
	$MUC1^{STn}$	52	716	0.76	98	40	III
	CA125 + HE4 EIA	52	716	0.91	98	46	III
	$MUC16^{STn}$ + $MUC1^{STn}$	52	716	0.89	98	52	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	52	716	0.90	98	63	III

SUBGROUP	BIOMARKER	N (EOC)	N (BENIGN + HEALTHY)	AUC (95% CI)	SPECIFICITY	SENSITIVITY	STUDY
CLEAR CELL	CA125 EIA	29	716	0.81	98	21	III
	HE4 EIA	29	716	0.83	98	34	III
	$MUC16^{STn}$	29	716	0.76	98	48	III
	$MUC1^{STn}$	29	716	0.86	98	52	III
	CA125 + HE4 EIA	29	716	0.87	98	28	III
	$MUC16^{STn}$ + $MUC1^{STn}$	29	716	0.87	98	52	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	29	716	0.92	98	62	III
ENDOMETRIOID	CA125 EIA	51	716	0.87	98	31	III
	HE4 EIA	51	716	0.94	98	73	III
	$MUC16^{STn}$	51	716	0.88	98	55	III
	$MUC1^{STn}$	51	716	0.83	98	45	III
	CA125 + HE4 EIA	51	716	0.94	98	73	III
	$MUC16^{STn}$ + $MUC1^{STn}$	51	716	0.89	98	55	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	51	716	0.97	98	80	III

SUBGROUP	BIOMARKER	N (EOC)	N (BENIGN + HEALTHY)	AUC (95% CI)	SPECIFICITY	SENSITIVITY	STUDY
MUCINOUS	CA125 EIA	38	716	0.79	98	16	III
	HE4 EIA	38	716	0.87	98	29	III
	$MUC16^{STn}$	38	716	0.71	98	16	III
	$MUC1^{STn}$	38	716	0.82	98	50	III
	CA125 + HE4 EIA	38	716	0.87	98	29	III
	$MUC16^{STn}$ + $MUC1^{STn}$	38	716	0.82	98	47	III
	HE4 + $MUC16^{STn}$ + $MUC1^{STn}$	38	716	0.89	98	50	III

5.5 Age based analysis (I & III)

Age based analysis was done for the (i) above 50 age group and (ii) 50-years and below age group. In Study I, there were 198 above 50-years aged women, of which 54 presented EOC cases. At 90% SP, in CF validation, $MUC1^{Tn}$ recognized 48 EOC cases whereas CA125 + CA15-3 EIA recognized only 21 cases. In serum validation, $MUC1^{STn}$ recognized 43 cases whereas CA125 + HE4 EIA recognized 32 EOC cases. In Study III, there were 946 above 50-years aged women, of which 527 presented EOC cases. At 98% SP, $MUC16^{STn}$ + $MUC1^{STn}$ GV combination recognized 442 EOC cases whereas CA125 + HE4 EIA recognized 389 cases.

For 50-years and below age group, results from only Study III are discussed. This is because Study I had a low number of only 13 EOC cases of 50-years and below aged women and hence would not be a suitable cohort for this analysis. In Study III, there were 363 women aged 50 and below, of which 69 presented EOC cases. HE4 EIA was the best single marker that recognized 46 out of 69 cases, whereas other markers recognized only 35 ($MUC16^{STn}$), 29 ($MUC1^{STn}$) and 31 (CA125 EIA) cases.

5.6 Borderline Tumors

Borderline tumors are analysed separately from the other groups because they are not considered true cancers as they seldom spread beyond stage I. In Study II, $CD63^{IA}$ discriminates borderline (N=24) significantly from non-malignant cases (N=22) ($p < 0.0001$), whereas there is no significant discrimination of EOC (N=31) from non-malignant samples ($p = 0.23$). The most significant discrimination of borderline with non-malignant cases is seen with $ITGA3^{IA}$ ($p < 0.00005$).

In Study III, 115 borderline cases were present. Mucin glycovariants are more EOC specific and hence showed low borderline tumor detection. At 98% SP, detection was almost negligible (only 5-10%). At 75% SP, STn GVs showed 37-43% detection whereas HE4 EIA showed 45% and CA125 EIA showed 50% borderline tumor detection. (**Figure 3**)

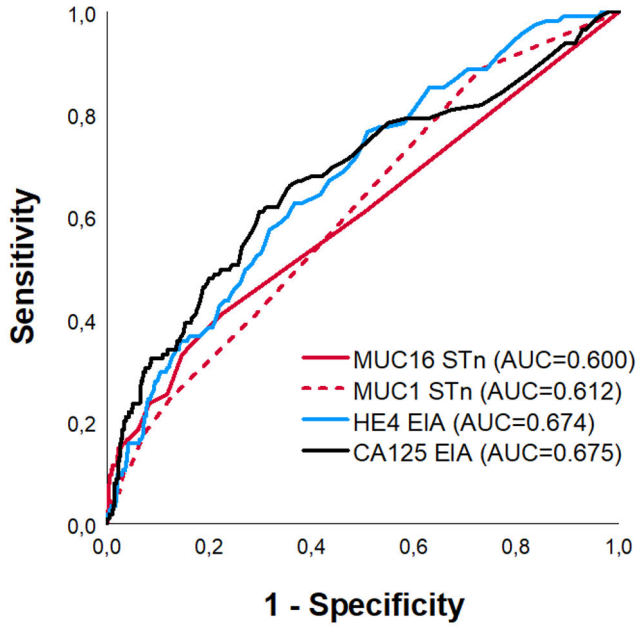

Figure 3. ROC plots for borderline tumors from Study III. STn glycovariants along with HE4 and CA125 EIA are shown for 115 borderline vs 716 benign samples. Data information: AUC at 95% CI is reported.

5.7 Histological subtypes (Study III)

Epithelial ovarian cancer is divided into different subtypes based on histology, which is the appearance of the tumor cells. The different histological subtypes from Study III are compiled in **Table 6**. The most common type is serous carcinoma. In high grade serous carcinoma (HGSC) (N=408), all single markers including STn GVs and HE4 performed significantly better ($p < 0.0001$) than CA125 EIA with detection increasing from 62 to 79-85% at 98% SP. In low grade serous carcinoma (LGSC) (N=52), $MUC16^{STn}$ was the best performing single marker (52% SN). Increase in detection was also seen on combining HE4 with the two STn GVs (63% SN). In the other less common subtypes, clear cell carcinoma (N=29), HE4 EIA showed an increase in detection on combining with the two STn GVs from 34% alone to 62% in a 3-marker panel. In endometrioid cancer (N=51), HE4 alone was the best performing marker (73% SN) and the only one that showed a significant increase in detection ($p < 0.001$) compared to CA125 EIA reference. In mucinous carcinoma, $MUC1^{STn}$ GV was the only marker with a significant increase in detection ($p = 0.038$) compared to the reference marker.

Table 6. Data compilation of serum histological subtypes of ovarian cancer from Study III. Data information: Area under the curve (AUC) at 95% confidence interval (CI), and sensitivity (SN) and p-value at 98% specificity (SP) is reported.

SUBGROUP	N (EOC)	N (BENIGN)	MARKERS	AUC	SN (%) AT 98% SP	P-VALUE (98% SP)
HGSC	408	716	CA125 EIA	0.95	62	Reference
			HE4 EIA	0.98	84	< 0.0001
			$MUC16^{STn}$	0.95	85	< 0.0001
			$MUC1^{STn}$	0.94	79	< 0.0001
			CA125+HE4 EIA	0.98	86	Reference
			HE4+both STn	0.99	93	< 0.0001
LGSC	52	716	CA125 EIA	0.87	31	Reference
			HE4 EIA	0.88	48	0.179
			$MUC16^{STn}$	0.89	52	0.065
			$MUC1^{STn}$	0.76	40	0.566
			CA125+HE4 EIA	0.91	46	Reference
			HE4+both STn	0.90	63	0.065
CLEAR CELL	29	716	CA125 EIA	0.81	21	Reference
			HE4 EIA	0.83	34	0.791
			$MUC16^{STn}$	0.76	48	0.325
			$MUC1^{STn}$	0.86	52	0.230
			CA125+HE4 EIA	0.87	28	Reference
			HE4+both STn	0.92	62	0.141
ENDOMETRIOID	51	716	CA125 EIA	0.87	31	Reference
			HE4 EIA	0.94	73	< 0.001
			$MUC16^{STn}$	0.88	55	0.051
			$MUC1^{STn}$	0.83	45	0.355
			CA125+HE4 EIA	0.94	73	Reference
			HE4+both STn	0.97	80	0.303
MUCINOUS	38	716	CA125 EIA	0.79	16	Reference
			HE4 EIA	0.87	29	0.152
			$MUC16^{STn}$	0.71	16	1.000
			$MUC1^{STn}$	0.82	50	0.038
			CA125+HE4 EIA	0.87	29	Reference
			HE4+both STn	0.89	50	0.073

5.8 Metastatic cancers (Study III)

Study III included 175 metastatic cancers, of which the majority were gynecologic (N=99) and GI-cancers (N=39). These cancers form an overlap with the benign group but show discrimination from the EOC cases, thus proving that the markers are more EOC specific. (**Figure 4**) From **Figure 5A**, it can be clearly seen that the MUC16^{STn} GV (72.5% SN, 100% SP) is more EOC specific relative to GI cancers where CA125 and HE4 are both elevated. Whereas very little differences are seen in gynecological cases where all markers are almost equally elevated. (**Figure 5B**)

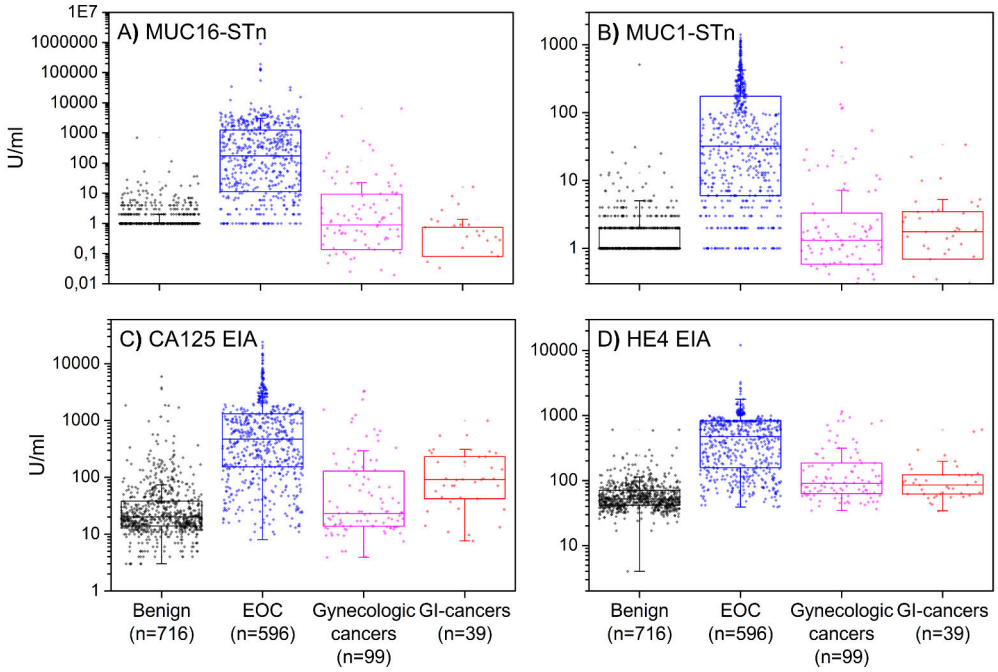

Figure 4. Gynecologic (n=99) and Gastrointestinal (n=39) metastatic cancers are compared against the benign (n=716) and EOC (n=596) samples from Study III. Box plots are shown for two glycovariant (A) MUC16^{STn} (B) MUC1^{STn} and two reference (C) CA125 EIA (D) HE4 EIA assays.

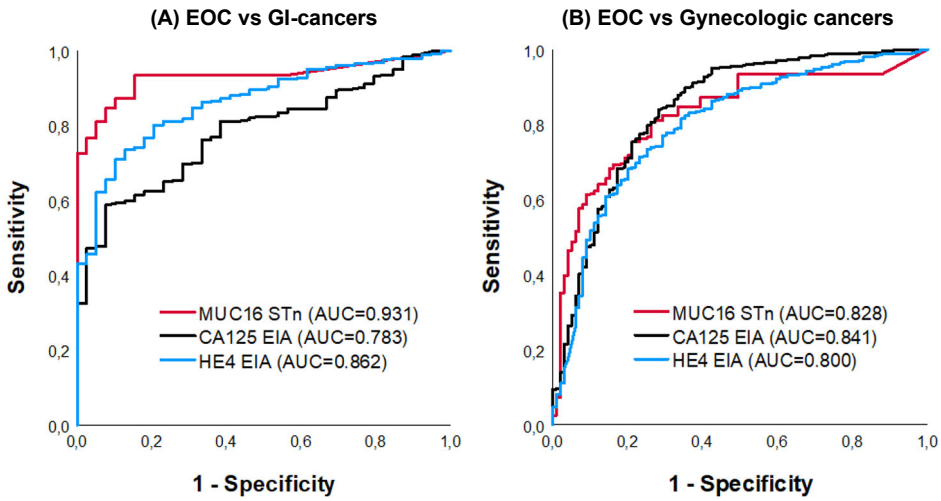

Figure 5. ROC plots showing MUC16^{STn} GV performance against the reference CA125 and HE4 EIA for (A) EOC (n=596) vs metastatic GI-cancers (n=39) and (B) EOC (n=596) vs metastatic gynecologic cancers (n=99).

6 Conclusions

This doctoral thesis was carried out to develop a simple and sensitive assay platform and to explore the potential of novel biomarkers for ovarian cancer diagnosis. Several conclusions can be drawn based on the three studies included in this work.

In Study-I in cyst fluids, STn glycovariant of MUC16 ($MUC16^{STn}$) and STn and Tn glycovariant of MUC1 ($MUC1^{STn}$, $MUC1^{Tn}$) show striking improvement over the conventional CA125 and CA15-3 immunoassays. In serum, $MUC16^{STn}$ showed the best overall diagnostic performance compared to the reference CA125 and HE4 EIA. It was observed that the glycovariants offer the most advantage at high specificity areas.

In Study-II, STn glycoforms of CD63 and ITGA3 ($CD63^{STn}$, $ITGA3^{STn}$) show better discrimination of healthy and benign from EOC cases compared to their immunoassay counterparts ($CD63^{IA}$, $ITGA3^{IA}$). The combination of $ITGA3^{STn}$ and $ITGA3^{IA}$ show improved detection for EOC over their individual performance. Study-II was only performed in cyst fluids and ascitic fluid samples and calls for further studies on blood samples (serum / plasma).

In Study-III, STn glycovariants and HE4 show superior performance relative to the reference CA125 EIA. STn glycovariants provided a clear additive effect on combining with HE4, and significantly improved results were seen in early stage EOC as well as above 50-years aged women, which are the two most clinically relevant subgroups. In 50-years and below age group, HE4 was seen to be the best performing marker. In tumor histotype analysis, STn based biomarkers showed higher detection but because of low number of samples they could not reach significance. It was especially seen in the clear cell group.

It was seen that glycovariants are more EOC-specific, as the borderline tumor detection was found to be very low. On the other hand, the immunoassays for ITGA3 and CD63 ($ITGA3^{IA}$, $CD63^{IA}$) were found to discriminate borderline significantly from non-malignant cases. Higher EOC specificity of glycovariants compared to CA125 and HE4 was also seen from metastatic cancer analysis, though better discrimination of EOC was seen with gastrointestinal cases than gynecological. CA125 is found in many cancers and it should be checked if it is elevated in lung, pancreas, etc.

The biomarkers presented in this study do not cover the whole expanse of ovarian cancer. Still additional markers are needed in combination to identify the missing cases. We are now continuing research on autoantibodies and other glycoprotein biomarkers like CA19-9 to close the gap. Further research on tetraspanins and integrins could also serve this purpose. This study has shown that STn based biomarkers perform better than CA125 in a diagnostic setting and STn markers in combination with HE4 are even better. Their potential as screening markers is a question yet to be answered.

The novel integrin and tetraspanin glycovariant assays look promising and call for future extensive studies. The MUC16 and MUC1 based nanoparticle assisted glycovariant biomarker assays show great potential for ovarian cancer detection. Studies on the feasibility of these markers in a screening context is warranted.

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