



# Ovarian Cancer Biomarkers: Current State and Future Implications from High- Throughput Technologies

Felix Leung<sup>\*,†</sup>, Eleftherios P. Diamandis<sup>\*,†,‡</sup>, Vathany Kulasingam<sup>\*,‡,1</sup>

<sup>\*</sup>Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

<sup>†</sup>Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada

<sup>‡</sup>Department of Clinical Biochemistry, University Health Network, Toronto, Ontario, Canada

<sup>1</sup>Corresponding author: e-mail address: vathany.kulasingam@uhn.ca

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## Abstract

Ovarian cancer remains the most lethal gynecological malignancy worldwide and survival rates have remained unchanged in spite of medical advancements. Much research has been dedicated to the identification of novel biomarkers for this deadly disease, yet it has not been until recently that a few serum-based tests have been added to carbohydrate antigen 125 as Food and Drug Administration-approved tests for ovarian cancer. This lack of success in identifying clinically relevant biomarkers has been largely attributed to poor study design and bias leading to false discoveries or identification of second-tier biomarkers. Fortunately, a better understanding of the guidelines used to assess the clinical utility of a biomarker and the various phases of biomarker development will aid in avoiding such biases. As well, advances in high-throughput technologies have caused a renewed interest in biomarker discovery for ovarian cancer using alternative strategies such as targeted sequencing and proteomics. In this chapter, we will review the current state of ovarian cancer biomarker research with a focus on diagnostic serum markers. Furthermore, we will examine the standard practice guidelines' criteria for acceptance of a biomarker into the clinic as well as emerging high-throughput approaches to the discovery of novel ovarian cancer biomarkers.

## ABBREVIATIONS

**β2M** beta-2 microglobulin

**APOA1** apolipoprotein A1

**AUC** area under the curve

**CA125** carbohydrate antigen 125

**CPG** 27-nor-5β-cholestane-3,7,12,24,25 pentol glucuronide

**ct-DNA** circulating tumor DNA

**EOC** epithelial ovarian carcinoma

**FDA** Food and Drug Administration

**FIGO** International Federation of Gynecology and Obstetrics

**HE4** human epididymis protein 4

**ICRA** Initial Cancer Risk Assessment

**IL6** interleukin-6

**IL8** interleukin-8

**KLK** kallikrein

**LC** liquid chromatography

**LMP** low malignant potential

**LOE** level of evidence

**MALDI** matrix-assisted laser desorption/ionization

**miRNAs** microRNAs

**MLN** mesothelin

**MS** mass spectrometry

**NACB** National Academy of Clinical Biochemistry

**NGS** next-generation sequencing

**NPV** negative predictive value

**OPN** osteopontin

**PLCO** Prostate, Lung, Colorectal, and Ovarian  
**PPV** positive predictive value  
**PRoBE** prospective-specimen-collection, retrospective-blinded-evaluation  
**PSN** prostatic  
**PTM** posttranslational modification  
**RMI** Risk of Malignancy Index  
**ROC** receiver-operating characteristic  
**ROMA** Risk of Ovarian Malignancy Algorithm  
**TAm-Seq** tagged-amplicon deep sequencing  
**TrF** transferrin  
**TT** transthyretin  
**UKCTOCS** United Kingdom Collaborative Trial of Ovarian Cancer Screening  
**UPLC** ultraperformance liquid chromatography  
**VEGF** vascular endothelial growth factor  
**WAP** whey acidic protein



## 1. INTRODUCTION

Ovarian cancer remains the most lethal gynecological malignancy worldwide. While ovarian cancer accounts for only 4% of all malignancies diagnosed in women, over half of the 225,000 new cases eventually succumb to the disease every year worldwide [1]. Despite several known risk factors associated with ovarian cancer, the majority of cases are sporadic and only 5% of the affected population can be attributed to a genetic predisposition. Most hereditary cases of epithelial ovarian carcinoma (EOC) are related to mutations in the *BRCA1/BRCA2* genes [2]. Although at lower frequency, individuals with a family history of mutations in the DNA mismatch repair genes related to Lynch syndrome (hereditary nonpolyposis colorectal cancer) are also at risk of developing ovarian cancer [3]. In addition to hereditary predisposition, other identified risk factors include endocrine, environmental, dietary, and genetic factors. Specifically, advancing age, nulliparity, and hormonal therapy have been associated with increased risk while the use of oral contraceptives, pregnancy, and lactation are associated with decreased risk [4].

Unfortunately, very few ovarian cancer cases are diagnosed at early stages (stage I or II) while the tumor is still localized or confined to the ovary. When early-stage diagnoses are made, it is often characterized by regional, but relatively confined, spread. However, the vast majority are diagnosed at late stages (stage III or IV) with distant spread beyond the abdomen [1]. In tandem with the extremely poor prognosis of late-stage diagnoses, there

is a high death rate of patients with ovarian cancer. To exacerbate the problem, population screening is not suitable with ovarian cancer because of the extremely high specificity (>99.6%) required for a biomarker to achieve an acceptable positive predictive value (PPV) of 10% (due to low disease prevalence). As such, there is an urgent clinical need for novel biomarkers for management of this deadly disease [5].

A biomarker is defined as a quantifiable characteristic that can be objectively measured and evaluated as an indicator of a normal biologic process, a pathogenic process, or a pharmacologic response to a therapeutic intervention [6]. They are typically endogenous molecules that can be measured in bodily fluids or tissues with the ability to discriminate individuals with and without a disease. Biomarkers may appear as various macromolecules, including DNA, mRNA, proteins, metabolites, or processes such as apoptosis, angiogenesis, or proliferation [6]. Within these types of macromolecules, different subtypes may also serve as useful biomarkers. For example, different functional subgroups of proteins, such as enzymes, glycoproteins, and receptors, may serve as useful biomarkers. Additionally, genomic changes such as mutations, amplifications, translocations, and changes in microarray profiles may also be utilized as biomarkers. Biomarkers may be detected in a variety of biofluids, tissues, and cell lines as they are often produced by the diseased tissue itself or by adjacent tissue in response to the presence of disease. By measuring the levels of such markers, biomarkers can be used for a variety of clinical purposes such as population screening, differential diagnosis in symptomatic patients, and for disease staging [7].

The past decade has witnessed an impressive growth in the field of large-scale and high-throughput biology, which is attributed to an era of new technology development. The completion of a number of genome sequencing projects, the discovery of oncogenes and tumor-suppressor genes, and recent advances in genomic and proteomic technologies have had a direct and major impact on our understanding of molecular pathologies. Using high-throughput platforms, hundreds of experiments can be performed simultaneously allowing for the generation of large amounts of data within a relatively short period of time. Coupled with multiplexing and bioinformatics, these technologies have become powerful tools to view numerous genomic and proteomic features (i.e., DNA copy number variation, DNA methylation, and mRNA and protein expression) of various diseases on a global scale. Such technologies have been increasingly exploited in ovarian cancer research in order to elucidate the molecular aspects of the disease. Through genomic, epigenomic, transcriptomic, and proteomic

profiling, there is now evidence that ovarian cancer likely represents a heterogeneous group of diseases that simply share a common anatomical location [8]. It has been postulated that with molecular profiling, ovarian cancer can be classified according to specific “-omic” signatures that may correlate with the tissue of origin, survival, and responsiveness to chemotherapy. If fruitful, this may have enormous ramifications on the clinical management of ovarian cancer patients as these molecular subtypes may indeed represent distinct diseases that should be treated accordingly. Additionally, high-throughput technologies have been applied extensively for the purposes of novel biomarker discovery, specifically, the identification of biomarkers for numerous aspects of ovarian cancer management including early diagnosis, prognosis, prediction, and monitoring disease progression and response to chemotherapy.

This chapter will provide a brief overview of ovarian cancer (covering etiology, pathophysiology, and clinical management). Following this, the focus will be on serum-based biomarkers for the clinical management of this disease—particularly diagnosis. A discussion on how to conduct a biomarker validation study and the phases of biomarker development will be presented. In addition to clinically approved biomarkers, this chapter will cover prominent biomarkers that have emerged from biomarker discovery efforts and have been validated to an extent. Finally, emerging fields of ovarian cancer biomarker discovery will be discussed in the last section with a particular focus on efforts driven by high-throughput technologies.



## **2. OVARIAN CANCER**

### **2.1. Etiology**

There exist several hypotheses that attempt to illustrate the origins of ovarian cancer. A prominent one is the incessant ovulation hypothesis that emerged from observations that women with a greater number of ovulations have an increased risk of ovarian cancer [9]. According to this hypothesis, uninterrupted ovulation leads to a cycle of damage and repair of the ovarian surface epithelium. Due to the constant cellular turnover and overactive repair mechanisms, the surface cells become subjected to an increased risk of developing mutations and consequently malignant evolution. Additionally, increased frequency of ovulation is associated with a greater number of inclusion cysts and other architectural changes in the surface epithelium, such as invaginations. These inclusion cysts and invaginations may create

a suitable environment for ovarian carcinogenesis [10]. Consistent with this hypothesis, women with multiple pregnancies, increased time of lactation, and oral contraceptive use have a lower incidence of ovarian cancer [11–13]. However, a limitation of this hypothesis is the fact that progesterone-based oral contraceptives that do not inhibit ovulation are equally effective as ovulation-inhibiting contraceptives [14]. In addition, women with polycystic ovarian syndrome (a common benign gynecological condition) whose ovulatory cycles are reduced still have a high risk of developing ovarian cancer [15].

The inability of the incessant ovulation hypothesis to explain certain observations thus led to the emergence of the gonadotropin hypothesis. The gonadotropin hypothesis suggests that increased levels of gonadotropins that stimulate ovulation can persist for extended periods of time following menopause and are capable of overstimulating the ovarian surface epithelial cells until carcinogenesis [16–18]. In addition, gonadotropins are able to stimulate the shedding of the ovarian surface epithelial basement membrane, albeit without an ovulatory event [19]. Since inflammation is a well-known precursor of malignancy, the chronic inflammatory processes of the ovarian surface epithelium may be a means by which gonadotropin stimulation and ovulation contribute to ovarian cancer development [17,20]. Furthermore, ovulation is an inflammatory-like process involving inflammatory cytokines and proteolytic enzymes, and their activation ultimately leads to tissue damage.

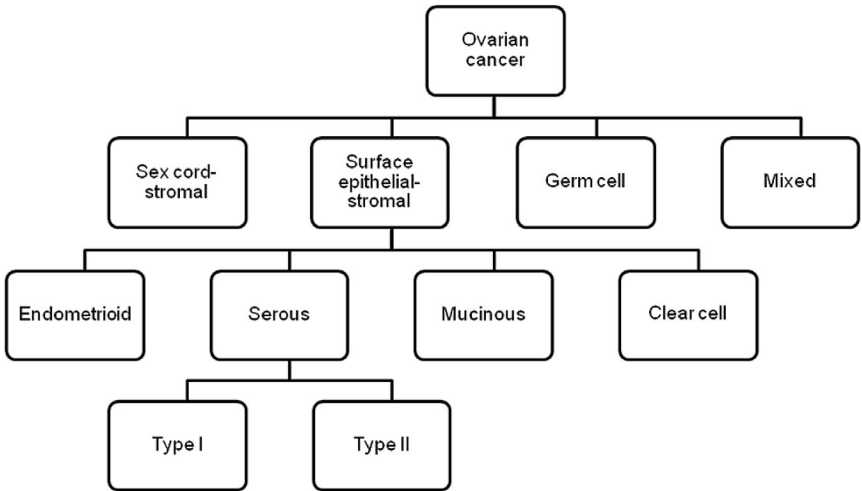
Lastly, the most recent theory hypothesizes that ovarian cancer does not originate in the ovary, but in fact at the distal fallopian tube. This hypothesis is supported by the fact that the majority of early serous malignancies, detected in prophylactic bilateral salpingo-oophorectomies in healthy women, were found in the distal fallopian tube and not the ovary. In addition, analysis of *TP53* mutations in early high-grade serous malignancies of the distal fallopian tube and adjacent bulky carcinomas of the ovary showed shared mutations [21]. Currently, it is almost universally accepted that high-grade serous ovarian carcinomas, for the most part, originate from the distal fallopian tube [22]. However, this theory does not explain endometrioid, mucinous, or clear-cell forms of ovarian cancer.

## 2.2. Pathophysiology

Ovarian cancer is a heterogeneous disease and tumors can be categorized based on histopathological analysis. The majority of tumors of the ovaries

fall into one of three major categories: surface epithelial tumors, sex cord-stromal tumors, and germ cell tumors. Epithelial ovarian cancer, the most lethal among all ovarian malignancies, arises from the cells of epithelial origin and comprises 80% of all ovarian cancer cases [23,24]. Since epithelial ovarian cancer accounts for the majority of ovarian cancers, research is mainly focused on diagnosis and treatment of epithelial ovarian cancer. Epithelial ovarian cancer can be either benign or malignant. The benign tumors seldom spread from the ovaries and are not associated with serious disease [25]. Malignant tumors, however, are known as EOCs. These malignancies have the potential to spread into the peritoneum which in turn acts as a gateway for the malignant cells to spread to the general viscera and beyond. Based on tissue morphology, EOC can be subdivided into four major types: serous, mucinous, endometrioid, and clear-cell carcinomas (Fig. 2.1). In addition, there are other minor types such as malignant Brenner tumors and undifferentiated carcinomas [26]. A brief overview of the four major subtypes of EOC is presented below.

Serous carcinomas of the ovary resemble those of the epithelium of the fallopian tube. It makes up 40–60% of cases and is the most aggressive histological type. Serous carcinomas can be further subdivided into low-grade (Type I) and high-grade (Type II) serous carcinomas [27]. While low-grade carcinomas have a greater probability to be diagnosed at an early stage and are relatively indolent, high-grade carcinomas (which make up the majority of serous carcinomas) are almost exclusively detected when the cancer is



**Figure 2.1** Breakdown of ovarian cancer subtypes.

late-stage and behave aggressively. High-grade serous carcinoma is often characterized by bilateral ovarian involvement and peritoneal membranes with rapid carcinomatosis [26]. Serous carcinomas have a wide spectrum of histological appearances and this may be attributed to the genetic heterogeneity. Most serous carcinomas show papillary and micropapillary architecture with solid areas mixed in with chamber-like open spaces [27]. Other characteristic features of serous carcinomas include the expression of WT1, p53 overexpression and *TP53* mutations, and loss of BRCA1 expression in high-grade tumors [28–30].

Endometrioid tumors are the second most common type of EOC and comprise 10–20% of cases. These tumors were named accordingly due to their morphological resemblance to their endometrial counterparts. Given their similarity to endometrial tissue, most endometrioid tumors are associated with endometriosis, endometrioid borderline tumors, or coexisting tumors of the endometrium [31,32]. Most endometrial carcinomas have either squamous or mucinous differentiation. Molecular features that are characteristic of endometrioid carcinomas include the nuclear expression of the estrogen receptor, the progesterone receptor, and  $\beta$ -catenin [33,34]. In addition, the loci encoding  $\beta$ -catenin, PI3KCA, and PTEN have been reported to have mutations in endometrioid carcinomas [35–37].

Mucinous tumors are much more infrequent, comprising 3% of all epithelial ovarian cancer cases. A defining (and necessary) histological feature of mucinous tumors is the presence of intracytoplasmic mucin [27]. However, obvious mucin expression can be absent in large parts of the tumor [27]. The cells that comprise mucinous carcinomas are very similar to the mucus-secreting goblet cells within the intestinal epithelium and sometimes the endocervical epithelium. Mucinous carcinomas express CK7, lack expression of estrogen receptors, and lack mesothelin (MLN) and fascin expression [38–40]. Finally, *KRAS* and *BRAF* mutations are the predominant genetic aberrations found in mucinous carcinomas [41,42].

Lastly, clear-cell carcinomas account for 10% of malignancies of the ovary. Cells with hobnail configurations (round expansion of clear cytoplasm) are defining features of this histotype [27]. In addition, they often contain polyp-like masses that protrude into the lumen. Clear-cell carcinomas are the most lethal among all EOC subtypes [27]. Interestingly, a large proportion of clear-cell tumors are found with concomitant endometriosis similar to carcinomas of endometrioid origin [27]. Immunohistochemically speaking, clear-cell carcinomas show low estrogen receptor, progesterone

receptor, p53, and mib-1 expression [34]. Mutations in *KRAS* and *PTEN* have also been reported [43,44].

## 2.3. Clinical management

### 2.3.1 Staging and diagnosis of ovarian cancer

Ovarian cancer is classified based on the stage of the disease under the guidelines established by the International Federation of Gynecology and Obstetrics (FIGO). This is based on several criteria including the size of the tumor, the extent of tumor invasion into other tissues, the extent of lymphatic involvement, and the establishment of distal metastases [45]. Typically, ovarian cancer is classified into four stages: I, II, III, and IV, where the first three stages are further subdivided [46]. Briefly, stage I tumors are limited to one or both ovaries with the tumor extending to the surface of the ovary by the third substage. Stage II tumors display pelvic extensions beyond the surface of the ovaries to the uterus, fallopian tubes, and/or other proximal tissues. Stage II tumors often present with ruptured capsules by the third substage. In stage III, the tumor spreads intraperitoneally to distant organs within the abdominal cavity, and finally in stage IV, the tumor cells metastasize via the circulatory and lymphatic system to lymph nodes and other organs in the body, including the pleural space, the hepatic or splenic parenchyma, and the brain [47,48]. Staging at the time of diagnosis is established by thorough surgical examination of the tumor in order to determine the extent of disease progression [2].

Typically, upon suspicion of ovarian cancer (based on symptoms and pelvic examination; more detail in section below about pelvic masses), the levels of serum carbohydrate antigen 125 (CA125) are measured along with transvaginal ultrasonography. In addition, a computed tomography scan of the abdomen and pelvis is performed. Once a tentative diagnosis has been made, exploratory laparotomy is necessary to allow for definitive diagnosis as sampling of the tumor allows for identification of the histology and stage of the tumor [2,49]. An additional purpose of the surgical procedure is to remove as much tumor as possible in a process called optimal debulking or cytoreduction, leaving tumors no larger than 1 cm [50]. Ultimately, surgery is performed to increase the chances of the patient responding to chemotherapy [49,51]. The generally accepted regimen is combination therapy with a platinum compound such as cisplatin or carboplatin and a taxane such as paclitaxel [52]. Currently, FIGO staging is the most reliable prognostic tool as stage I diagnoses are associated with a 5-year survival rate of

80–95%, whereas stage III–IV diagnoses are associated with a markedly decreased 5-year survival rate of 10–30% [53]. In addition to staging, it has been observed that histotype and grade may also be able to predict disease outcome as clear-cell, mucinous, and poorly differentiated carcinomas are associated with poor prognosis [3].

### 2.3.2 Pelvic mass dilemma

Clinically, ovarian cancers often present initially as a pelvic mass of unknown malignant potential. More than 200,000 women undergo exploratory laparotomies for a pelvic mass in the United States each year [54,55]. On average, only 13–21% of pelvic lesions are found to be malignant. In premenopausal women, 10% of masses are malignant, whereas in postmenopausal women 20% are malignant. Accurately discriminating patients with ovarian cancer from benign pelvic lesions is crucial for appropriate treatment planning and patients' outcomes [55]. Recent studies, including meta-analyses, have reported fewer complications, lower risk of reoperation, higher adherence to guidelines, higher fraction of optimal cytoreduction, optimal chemotherapy, and better overall survival for patients with ovarian cancer operated on by gynecologic oncologists, compared to gynecologists or general surgeons [56–62]. Gynecologic oncologists are specially trained to conduct cytoreductive surgery. Despite these advantages, only 30–50% of women with ovarian cancer are referred to gynecologic oncologists [63,64].

Algorithm/biomarkers that enable accurate prediction of the presence of malignancy in women with a pelvic mass are urgently needed and several multiparametric algorithms have been designed for such pelvic mass discrimination. Over 20 years ago, Jacobs *et al.* [65] developed a Risk of Malignancy Index (RMI) that incorporates ultrasound imaging characteristics, menopausal status, and serum CA125 for predicting malignant disease in women presenting with a pelvic mass (Fig. 2.2). Since its introduction, the RMI has reported sensitivities from 71% to 88% and specificities from 74% to 97% [66–69]. Multiple studies have confirmed its clinical utility and it is routinely used in the United Kingdom [70]. However, the reliance of the RMI on serum CA125 means that it has inherited the limitations of CA125 as a biomarker (discussed in the following section) including poor sensitivity for early-stage disease and poor specificity for ovarian cancer overall. Thus, the identification and validation of serum biomarkers in the evaluation of pelvic mass patients remains a particularly focal niche within the field of novel ovarian cancer biomarkers.

In the following sections, both ovarian cancer serum biomarkers approved by the Food and Drug Administration (FDA) and emerging novel

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <p>RMI score= <math>U \times M \times \text{serum CA125}</math></p> <p>U = 0 for ultrasound score of 0</p> <p>= 1 for ultrasound score* of 1</p> <p>= 3 for ultrasound score* of 2–5</p> <p>* Ultrasound scores are determined by assigning the value of 1 for each of the following characteristics seen on ultrasound:</p> <ul style="list-style-type: none"><li>• Multiloculated ovarian cyst</li><li>• Solid component in ovarian mass</li><li>• Bilateral lesion</li><li>• Ascites</li><li>• Evidence of intra-abdominal metastases</li></ul> <p>M = 1 if premenopausal</p> <p>= 3 if postmenopausal</p> <p>An RMI score of 200 or higher warrants preoperative referral to a gynecologic oncologist.</p> |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

**Figure 2.2** Risk of Malignancy Index (RMI). *Reproduced with permission from Ref. [5].*

biomarkers will be discussed. Before a discussion of the current markers, it is important to understand and appreciate the difficulties associated with conducting a “good” biomarker validation study. In this respect, we will first discuss what a tumor marker is and what criteria are used by tumor marker practice guidelines to evaluate the clinical utility of novel biomarkers. Once the criteria are known, it will be easier to appreciate why very few new biomarkers have been introduced into the clinic over the past 30 years. In addition, we will discuss the various phases of biomarker development, which highlights how specific studies should be conducted to bring a marker to the market (from bench to bedside).



### 3. TUMOR MARKERS

Tumor markers represent a specific subset of biomarkers that are used exclusively for the clinical management of various malignancies. Specifically, tumor markers can be used for screening for cancer, diagnosing the disease, evaluating prognosis, predicting therapeutic response, tumor staging, detecting tumor recurrence or remission, localizing tumor and directing

therapeutic agents, and monitoring therapeutic response [71]. Unfortunately, the usefulness of tumor markers in many of these aspects is relatively limited due to inadequate sensitivity and specificity that the current tumor markers provide. In the context of ovarian cancer, CA125 and human epididymis protein 4 (HE4) are the only individual serum markers approved by the FDA for monitoring therapeutic response and recurrence in ovarian cancer patients (these FDA-approved markers will be discussed in further detail later).

### 3.1. Types of tumor markers

Clinically speaking, biomarkers are classified as being diagnostic, prognostic, or predictive. Diagnostic biomarkers are useful in the detection of disease and in the indication of the type of disease the individual has. To minimize incorrect diagnoses, diagnostic markers are expected to have high sensitivity (the marker is sufficiently elevated in the presence of disease) and specificity (the marker is not elevated in other pathologies). Screening biomarkers are a subset of diagnostic biomarkers where they are used to identify individuals at risk of developing disease within a larger asymptomatic population [72]. Currently, there is no suitable screening marker for ovarian cancer.

Prognostic biomarkers are used once the specific disease has been established and confirmed. These biomarkers are predictors of the course of the disease and its recurrence independent of treatment. However, prognostic biomarkers can influence the type or dosage of therapy provided to the patient—for example, a patient with stage III ovarian cancer (poor prognosis) may require more aggressive treatment than a patient with stage I ovarian cancer (good prognosis). Currently, the FIGO staging is the major prognostic factor for ovarian cancer to determine patient prognosis and course of treatment.

Lastly, predictive biomarkers are predictors of the response to a drug before treatment is initiated. Optimally, predictive biomarkers should be able to stratify individuals as responders or nonresponders to a particular treatment. A hallmark example of a predictive biomarker is HER2/neu expression. In patients diagnosed with breast cancer, the expression of the HER2/neu protein upon immunohistochemical inspection suggests a favorable response to trastuzumab whereas null expression suggests a non-response. Unfortunately, other than definitive diagnosis by biopsy and histopathology, there is currently no single serum-based diagnostic, prognostic,

or predictive biomarker with acceptable sensitivity and specificity for ovarian cancer.

In addition to the aforementioned types of biomarkers, tumor markers can also be used for other purposes that are more specific and relevant to malignancies. These include differential diagnosis in symptomatic patients, estimating tumor volume, evaluating the success of treatment, detecting the recurrence of cancer, and monitoring responses to therapy [71].

3.2. Tumor marker guidelines

A major reason for why so few serum biomarkers have been accepted for routine clinical practice is due to the lack of standardization across tumor marker validation studies. Due to the heterogeneity of methodologies, study design, and patient populations, interpretation of the results of tumor marker studies is vulnerable to biases [73]. These flaws in the design and interpretation of tumor marker studies have thus catalyzed the establishment of practice guidelines for evaluating tumor markers.

The National Academy of Clinical Biochemistry (NACB) Laboratory Medicine Practice Guidelines for Use of Tumor Markers has adopted the level of evidence (LOE) classification scheme to evaluate the clinical utility of various tumor markers. This scheme aims to evaluate the strength of the data presented for a specific marker (Fig. 2.3) [71,74]. The LOE classification system grades tumor marker evidence on a scale from I to V, where an LOE of I represents evidence of the strongest clinical utility. An LOE of I denotes that the evidence is from a single, high-powered, prospective, controlled

| LOE | Description                                                                                                                                          |
|-----|------------------------------------------------------------------------------------------------------------------------------------------------------|
| I   | Evidence is from a high-powered prospective study designed specifically to test the marker for a specific clinical purpose, or from a meta-analysis. |
| II  | Evidence is from a case–control study where testing the marker utility is a secondary to testing the therapeutic hypothesis.                         |
| III | Evidence is from large prospective studies.                                                                                                          |
| IV  | Evidence is from small retrospective studies.                                                                                                        |
| V   | Evidence is from small preliminary studies.                                                                                                          |

**Figure 2.3** Level of evidence (LOE) classification. *Reproduced with permission from Ref. [74].*

study designed specifically to evaluate the tumor marker in question or that the evidence is from a meta-analysis of LOE II/III studies. An LOE of II denotes that the evidence is from data derived from a prospective clinical trial designed to test the therapeutic hypothesis—tumor marker utility is, in fact, a secondary objective of the study. An LOE of III denotes that the evidence is from large prospective studies. An LOE of IV denotes that the evidence is from small retrospective studies. An LOE of V denotes that the evidence is from small pilot studies. Most of the novel biomarker studies being conducted to date and published fall into the category of LOE IV or V. Thus, for a novel biomarker to make its way from the bench to the bedside, a number of well-designed validation studies need to take place to obtain a LOE of I. Below, we discuss some key publications which highlight various phases/studies to conduct to bring a biomarker to the clinic.

### 3.3. Biomarker development

There has been an increased focus on establishing a structure to guide the process of biomarker development in addition to implementing a standardized framework by which to evaluate tumor marker studies. The purpose of such a structure is to provide investigators with specific aims and measures that should be addressed at each step of the process to ensure rigorous study design and minimal bias.

In 2002, Hammond *et al.* put forth a preliminary six-step process in tumor marker development [75,76]. The six steps were as follows: (1) discovery of promising markers, (2) development of assay system, (3) preliminary clinical utility analysis, (4) assay standardization, (5) clinical utility assessment, and (6) validation of assay and clinical utility. While this process was able to outline the stringent analytical characteristics required for a clinical tumor marker test, it was unable to address preanalytical issues (such as sample population and selection criteria) very well. To address such issues, a five-phase process for the development of a screening or early diagnostic biomarker was put forth by Pepe *et al.* [76]. The five phases were as follows: (1) preclinical exploratory studies, (2) clinical assay development for clinical disease, (3) retrospective longitudinal repository studies, (4) prospective screening studies, and (5) cancer-control studies. In these guidelines (please refer to the original publication for further details), each phase is presented with primary and secondary aims, specimen selection criteria, sample size requirements, primary outcome measure, and methods to evaluate results.

Optimally, these items serve as criteria to decide whether a tumor marker can progress to the next phase of biomarker development. While the authors recognize that deviations from the five-phase process may occur, the overall structure should serve as a guiding model to planning and coordinating biomarker—especially screening tumor biomarker—research.

A final study of important note in the field of biomarker development was the first use of the PRoBE (prospective–specimen–collection, retrospective–blinded–evaluation) study design [77]. In this design, specimens are collected prospectively from a cohort representative of the ultimate target population for which the biomarker in question is applicable to. Specimens and relevant clinical data are collected in the absence of knowledge about patient outcome. After the outcome status is known, cases and controls are randomly selected from the cohort and assayed for the biomarker in a fashion blinded to case–control status. Subsequently, the results are collated and unblinded to case–control status, at which point the biomarker can be truly evaluated on its ability to discern cases from controls. This nested case–control study was presented as an example of rigorous and meticulous study design in order to eliminate and avoid common biases often found in biomarker studies. The authors stated that in the ideal situation, the PRoBE study design is used to evaluate putative screening, diagnostic or prognostic markers that display potential in discovery experiments. This way, the quality of discovery research is maintained and the chances that truly clinically useful markers will undergo subsequent evaluation are increased.



## 4. FDA-APPROVED BIOMARKERS

For many decades, CA125 remained the only FDA-approved ovarian cancer marker for monitoring treatment and detecting disease recurrence. In recent years, however, the explosion of high-throughput technology-driven biomarker discovery experiments has led to the approval of three new serum-based tests/algorithms for the management of ovarian cancer. HE4 was approved by the FDA in 2009 for monitoring treatment and detecting disease recurrence. Soon after, the OVA1™ and the Risk of Ovarian Malignancy Algorithm (ROMA) tests were approved by the FDA for the determination of the likelihood of malignancy in premenopausal and postmenopausal women presenting with an adnexal mass. The following sections will discuss these markers (summarized in [Table 2.1](#)).

**Table 2.1** Summary of ovarian cancer serum biomarkers

| Marker                                                                          | Clinical utility                                                                  | Level of evidence | References                        |
|---------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|-------------------|-----------------------------------|
| Carbohydrate antigen 125 <sup>a,b</sup>                                         | Monitoring treatment with chemotherapy                                            | I, II             | <a href="#">[78,79]</a>           |
|                                                                                 | Differential diagnosis of pelvic masses                                           | III               |                                   |
| Human epididymal protein 4 <sup>a,b</sup>                                       | Monitoring treatment with chemotherapy                                            | III, IV           | <a href="#">[80–88]</a>           |
|                                                                                 | Differential diagnosis of pelvic masses                                           |                   |                                   |
| Risk of Ovarian Malignancy Algorithm <sup>a</sup>                               | Supplementary for clinical decision-making for preoperative adnexal mass patients | N/A               | <a href="#">[89–93]</a>           |
| OVA1 <sup>TMa</sup>                                                             | Supplementary for clinical decision-making for preoperative adnexal mass patients | N/A               | <a href="#">[94–104]</a>          |
| Mesothelin <sup>b</sup>                                                         | Differential diagnosis                                                            | N/A               | <a href="#">[105–109]</a>         |
|                                                                                 | Presymptomatic screening                                                          |                   |                                   |
| Interleukins (6 and 8) <sup>b</sup>                                             | Prognosis prediction (IL6)                                                        | IV                | <a href="#">[109–114]</a>         |
|                                                                                 | Presymptomatic screening                                                          | N/A               |                                   |
| B7-H4 <sup>b</sup>                                                              | Differential diagnosis                                                            | N/A               | <a href="#">[109,115,116]</a>     |
|                                                                                 | Presymptomatic screening                                                          |                   |                                   |
| Osteopontin <sup>b</sup>                                                        | Differential diagnosis                                                            | N/A               | <a href="#">[109,114,117–120]</a> |
|                                                                                 | Presymptomatic screening                                                          |                   |                                   |
|                                                                                 | Tumor monitoring                                                                  | III/IV            |                                   |
| Kallikreins (5, 6 <sup>b</sup> , 7, 8 <sup>b</sup> , 9, 10, 11, 13, 14, and 15) | Differential diagnosis                                                            | IV, V             | <a href="#">[109,114,121–136]</a> |
|                                                                                 | Presymptomatic screening                                                          |                   |                                   |
|                                                                                 | Tumor monitoring                                                                  |                   |                                   |
|                                                                                 | Prognosis prediction                                                              |                   |                                   |
| Vascular endothelial growth factor                                              | Prognosis prediction                                                              | N/A               | <a href="#">[137–142]</a>         |
|                                                                                 | Therapeutic target                                                                |                   |                                   |
| Prostasin                                                                       | Differential diagnosis                                                            | IV                | <a href="#">[114,143]</a>         |

<sup>a</sup>FDA approved.

<sup>b</sup>Under investigation by PLCO study.

## 4.1. CA125

CA125, also known as mucin 16, is a large glycoprotein encoded by the *MUC16* gene. Ranging from 200 to 2000 kDa, CA125 is a transmembrane protein composed of 249 N- and over 3700 O-linked glycosylation sites [144]. Structurally, CA125 is composed of a short cytoplasmic tail, a transmembrane domain, and a large extracellular structure with extensive glycosylation [145]. Due to its strong links to ovarian cancer, many studies have been undertaken to elucidate the function of CA125 and determine if it plays a role in carcinogenesis. Despite this, the biological function of CA125 remains poorly understood. It has been suggested that CA125 may have some immunomodulatory activities via the extracellular interactions through its bound oligosaccharides [146]. As well, CA125 has been shown to interact with MLN, which is produced by the peritoneal epithelium among other cell types [147–149]. As such, the interaction between CA125 and MLN could potentially contribute to metastasis by promoting peritoneal implantation of ovarian cancer cells [150–152].

Since its discovery in 1981 by Bast *et al.* [153], CA125 still remains the best serum biomarker for ovarian cancer. It was identified through the development of a monoclonal antibody (OC125) that displayed reactivity with EOC cell lines and tissues from ovarian cancer patients. Currently, CA125 is approved as a serum marker for both monitoring treatment with chemotherapy and differential diagnosis of patients presenting with a pelvic mass, though the evidence for the latter use stems only from large prospective studies. The standard clinical cut-off for CA125 is 35 kU/L although serum levels have been shown to fluctuate depending on race, menstrual cycle time point, and presence of nonovarian cancer pathologies [71,154–156].

Many studies have investigated the diagnostic value of CA125 in ovarian cancer due to its strong performance as a marker to monitor therapeutic response and detect recurrence. Unfortunately, a major caveat of CA125 is that it is produced by coelomic epithelium which is the progenitor for mesothelial, Müllerian, pleural, pericardial, and peritoneal tissues [157–159]. As a result, CA125 displays poor specificity for ovarian cancer as increased CA125 levels can be a result of other pathological states such as heart failure, peritoneal infection, pericarditis, and benign gynecological conditions [160–162]. Additionally, CA125 is often not elevated in early-stage disease or in nonserous histotypes of ovarian carcinoma [5]. For these reasons, CA125 is not approved for ovarian cancer screening or for the

detection of early disease on its own. The Prostate, Lung, Colorectal, and Ovarian (PLCO) and the United Kingdom Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) screening trials represent two of the largest prospective trials worldwide examining the clinical utility of CA125 in screening for ovarian cancer in asymptomatic women [78,79]. The results of these landmark studies will definitively show whether or not there is an overall survival benefit to screening asymptomatic women with ultrasound, with ultrasound plus CA125, or no screening at all. Although the studies have not been completed yet, interim results have demonstrated that at least among women in the USA, screening with CA125 and transvaginal ultrasound does not reduce mortality rates compared with standard care [163].

## 4.2. HE4

Also known by its gene name *WFDC2* (whey acidic protein four-disulfide core domain protein 2), HE4 is a 25 kDa glycosylated protein that consists of a single peptide and two whey acidic protein (WAP) domains that contain a four-disulfide core composed of eight cysteine residues [164,165]. The gene is located on chromosome 20q12-13.1, in proximity to other gene members of the WAP domain family. Functionally, HE4 is suggested to play a role in host defense because of its ability to bind lipopolysaccharides and other bacterial moieties, as well as demonstrating antiprotease and anti-inflammatory activities [166].

HE4 was initially identified as an mRNA transcript specific to the distal epididymal tissue [164]. Subsequent studies demonstrated that this glycoprotein is expressed in several human tissues such as the respiratory tract and the nasopharynx and in several cancer cell lines [167]. Through microarray gene-expression profiling, it was discovered that HE4 was moderately expressed in lung adenocarcinomas, breast carcinomas, transitional cell endometrial carcinomas, and pancreatic carcinomas, but consistently highly expressed in ovarian carcinomas [168–171]. Furthermore, Drapkin *et al.* [172] showed that HE4 is relatively specific to the serous subtype of EOCs, as expression was observed in approximately 93% of serous carcinomas but it was also present in a smaller proportion of endometrioid, mucinous, and clear-cell carcinomas. Taken together, there was strong evidence that this secreted glycoprotein was a putative serum marker for ovarian cancer.

In a pilot study measuring serum levels of HE4 in ovarian cancer patients, Hellstrom *et al.* [80] concluded that HE4 may be comparable to CA125 as a monitoring serum tumor marker as both displayed a sensitivity of 80% and a

specificity of 95% when used to classify blinded late-stage cases and healthy controls. HE4 was approved by the FDA in 2009 as a serum marker for monitoring recurrence of ovarian cancer using a clinical cut-off of 150 pmol/L [81]. Since FDA approval, however, there have been conflicting results as to the true clinical utility of serum HE4 as a marker for ovarian cancer. Holcomb *et al.* reported that in a cohort of 229 premenopausal women (of which 85% had benign disease, 8% had epithelial ovarian cancer, and 7% had borderline tumors), CA125 and HE4 demonstrated sensitivities of 83.3% and 88.9%, respectively, for epithelial ovarian cancer detection [82]. However, HE4 markedly outperformed CA125 with a specificity of 91.8% versus 59.5%. The greater performance of HE4 may be due to its superior specificity compared to CA125 as it is unaffected by benign pelvic diseases such as endometriosis [83–85]. Recent meta-analyses have reported similar results, demonstrating that HE4 displays greater performance than CA125 in terms of differential diagnosis between benign pelvic disease and ovarian cancer [86,87]. Conversely, some studies have found no benefit in adding HE4 to CA125 for the diagnosis of ovarian cancer. In a study investigating 1218 patients, it was found that CA125 and HE4 demonstrated specificities of 62.2% and 63.4%, respectively, at a fixed sensitivity of 94.4% for ovarian cancer detection [88]. Unfortunately, the only conclusion that can be reached is that the true diagnostic utility of HE4 cannot be evaluated without sufficiently powered prospective trials.

### 4.3. ROMA

ROMA is a serum-based algorithm that combines serum CA125 and HE4 values with menopausal status in order to derive a score indicating the likelihood of malignancy in adnexal mass patients (Fig. 2.4). Following FDA approval of HE4, Moore *et al.* investigated if the dual combination of HE4 and CA125 could be applied to pelvic mass discrimination in a prospective multicenter double-blinded trial [89]. In this study, HE4 and CA125 were combined with menopausal status to create the predictive logistic regression model/algorithm known as ROMA. They found that ROMA could distinguish benign tumors from EOCs and low malignant potential (LMP) tumors with 88.7% sensitivity, 74.7% specificity, 60.1% PPV, and 93.9% negative predictive value (NPV). Though the algorithm performed much better in the postmenopausal population, the authors were able to confirm the clinical utility of ROMA to aid in stratifying patients with a pelvic mass into risk groups.

|                                                                                                                           |                                       |
|---------------------------------------------------------------------------------------------------------------------------|---------------------------------------|
| ROMA score considers:                                                                                                     |                                       |
| <ul style="list-style-type: none"><li>• Serum HE4 level</li><li>• Serum CA125 level</li><li>• Menopausal status</li></ul> |                                       |
| Premenopausal patients:                                                                                                   |                                       |
| ROMA score $\geq 1.31$                                                                                                    | High likelihood of finding malignancy |
| ROMA score $< 1.31$                                                                                                       | Low likelihood of finding malignancy  |
| Postmenopausal patients:                                                                                                  |                                       |
| ROMA score $\geq 2.77$                                                                                                    | High likelihood of finding malignancy |
| ROMA score $< 2.77$                                                                                                       | Low likelihood of finding malignancy  |

**Figure 2.4** Risk of Malignancy Algorithm (ROMA). *Reproduced with permission from Ref. [5].*

ROMA was approved by the FDA for use in the preoperative evaluation of an ovarian tumor in combination with a clinical or radiologic evaluation in the fall of 2011 [90]. The approved algorithm incorporates the serum levels of HE4 and CA125 with menopausal status to generate a score that indicates the likelihood of malignancy. Thus far, ROMA has been approved only on the Abbott ARCHITECT CA125 assay (Abbott Laboratories, Ltd.) platform, in conjunction with a manual HE4 enzyme immunometric assay. Premenopausal patients have a cut-off of 1.31 and postmenopausal patients have a cut-off of 2.77, where scores below the cut-offs suggest a low risk of EOC and scores equal to or above the cut-offs suggest a high risk of EOC. A limitation of the ROMA is that specimens with rheumatoid factor levels over 250 IU/mL will interfere with the ROMA score and should not be tested on this algorithm.

In the validation study leading up to FDA approval, 512 patients were examined in a prospective, blinded clinical trial that compared ROMA to the Initial Cancer Risk Assessment (ICRA), which incorporates serum CA125, presence of ascites, evidence of metastasis, and family history for referral to a gynecologic oncologist [91]. By itself, the ROMA displayed higher sensitivity and NPV compared to the ICRA but poorer specificity and PPV. Following FDA approval, there have been numerous studies seeking to compare the efficacies of the ROMA with other algorithms for the

differential diagnosis of patients with a pelvic mass. Overall, there have been conflicting reports as to how well ROMA performs as a pelvic mass discrimination test. Some studies have confirmed the benefit of ROMA over either HE4 or CA125 alone [92] and others have stated that ROMA does not outperform current modalities for pelvic mass discrimination such as sonography [93]. Clearly, more multicenter studies are needed to truly assess the clinical utility of the ROMA.

#### 4.4. OVA1

The OVA1™ markers—CA125, beta-2 microglobulin ( $\beta$ 2M), transferrin (TrF), transthyretin (TT), and apolipoprotein A1 (APOA1)—were identified through proteomic studies with the exception of CA125. Using surface-enhanced laser desorption and ionization time-of-flight mass spectrometry (MS), Zhang *et al.* [94] performed proteomic profiling on the serum of 503 women (153 invasive EOCs, 42 other ovarian cancers, 166 benign pelvic masses, and 142 healthy controls). Three proteins were identified as putative early-stage ovarian cancer biomarkers: APO1A (downregulated in cancer), a truncated form of TT (downregulated in cancer), and a cleavage fragment of inter- $\alpha$ -trypsin inhibitor heavy chain H4 (upregulated in cancer). Following this initial study, a multi-institutional follow-up study determined that the seven candidates that showed the most promise were inter- $\alpha$ -trypsin inhibitor heavy chain H4, TT, APOA1, hepcidin, TrF, connective-tissue activating protein 3, and  $\beta$ 2M [95]. Quantitative immunoassays only existed for  $\beta$ 2M, TrF, TT, and APOA1, and thus, the final algorithm incorporated only these four markers along with CA125 and menopausal status to generate the OVA1™ test.

Using the OvaCalc software (Vermillion, Inc.), the values from each variable are combined and converted into an ovarian malignancy risk index score (Fig. 2.5). For premenopausal patients, an OVA1™ score of less than 5.0 indicates a low probability of malignancy while 5.0 or above indicates a high probability of malignancy. For postmenopausal patients, an OVA1™ score less than 4.4 indicates a low probability of malignancy while 4.4 or above indicates a high probability of malignancy. A limitation of the OVA1™ test is that triglycerides greater than 4.5 g/L or rheumatoid factor greater than 250 IU/mL will interfere with the biomarker assays [96].

The OVA1™ test obtained clearance from the FDA in September 2009 as a supplementary for clinical decision-making for preoperative adnexal mass patients [97]. It should be noted that the FDA cautions against the

|                                                                                                                                                                                                                              |                                       |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|
| OVA1™ score considers:                                                                                                                                                                                                       |                                       |
| <ul style="list-style-type: none"><li>• Serum CA125 level</li><li>• Serum beta-2 microglobulin level</li><li>• Serum transferrin level</li><li>• Serum transthyretin level</li><li>• Serum apolipoprotein A1 level</li></ul> |                                       |
| Premenopausal patients:                                                                                                                                                                                                      |                                       |
| OVA1™ score ≥ 5.0                                                                                                                                                                                                            | High likelihood of finding malignancy |
| OVA1™ score < 5.0                                                                                                                                                                                                            | Low likelihood of finding malignancy  |
| Postmenopausal patients:                                                                                                                                                                                                     |                                       |
| OVA1™ score ≥ 4.4                                                                                                                                                                                                            | High likelihood of finding malignancy |
| OVA1™ score < 4.4                                                                                                                                                                                                            | Low likelihood of finding malignancy  |

**Figure 2.5** The OVA1™ test. *Reproduced with permission from Ref. [5].*

use of the OVA1™ test in the absence of an independent clinical evaluation and the test is not to be used as a screening test or as a deciding factor of whether a pelvic mass patient should continue with surgery. The clinical trial leading to the FDA approval of OVA1™ reported a sensitivity of 92.5%, a specificity of 42.8%, a PPV of 42.3%, and a NPV of 92.7% [98,99]. According to the results of the trial, OVA1™ improved presurgical assessments for both general physicians and gynecologic oncologists as sensitivity increased from 72.2% to 91.7% for general physicians and from 77.5% to 98.9% for gynecologic oncologists.

However, recent studies investigating OVA1™ and variations using different combinations of the markers identified by Zhang *et al.* have reported conflicting results [100]. Moore *et al.* [101] reported that the addition of the seven biomarkers identified by Zhang *et al.* [95,100] to CA125 did not improve the sensitivity for preclinical diagnosis compared to CA125 alone, but other studies have reported the benefits of adding different combinations of the seven biomarkers to CA125 for distinguishing benign from malignant pelvic masses [102,103]. As seen in subsequent studies, there is much dispute over which combination of the seven candidates perform the best and whether they complement CA125. Similar to the ROMA, more multi-institutional studies are needed before the clinical applicability of OVA1™ can be determined. Due to the conflicting evidence, clinicians

have noted the importance of recognizing when the use of the OVA1™ test is appropriate [104]. Currently, it is consensually agreed upon that the OVA1 test should not act as a substitute to clinical decision-making for adnexal mass patients—clinical assessment and/or RMI scoring should still take precedence over any conclusions drawn from the OVA1 test. As well, OVA1 should “never be used as a screening test for women without an adnexal mass” due to the LOE regarding its utility as a screening tool.



## 5. OTHER PROMINENT BIOMARKERS

Numerous putative ovarian cancer markers have been studied over the years across multiple validation cohorts but have yet to gain FDA approval. For the majority of these markers, while they continue to perform relatively well as diagnostic markers, they fail to outperform the existing markers used in clinical practice and they are unable to fulfill the criteria for clinical niches which are in need of serum markers. Despite this, the NACB still recognizes many of these “second-tier” markers as having potential clinical utility despite having a limited LOE (summarized in Table 2.1). In addition to CA125, the PLCO screening trial is currently investigating many of these “second-tier” markers in order to truly evaluate their diagnostic potential (Fig. 2.6).

### 5.1. PLCO markers

#### 5.1.1 Mesothelin

MLN is a glycosylphosphatidylinositol-linked cell surface molecule expressed by mesothelial cells. It is present in normal mesothelium and has been detected in patients with mesothelioma, ovarian cancer, pancreatic cancer, and squamous cell carcinoma [105]. MLN may also be biologically relevant to ovarian cancer due to its potential role in peritoneal implantation and metastasis through its interactions with CA125 [106]. McIntosh *et al.* observed that MLN was elevated in the serum of 76% of ovarian cancer patients and displayed complementarity to CA125 in early detection of ovarian cancer [107]. Specifically, MLN displayed a sensitivity of 60% and a specificity of 98% when used to identify ovarian cancer patients from healthy controls. When combined with CA125, the two biomarkers together produced a higher sensitivity (86.5%) than CA125 (78.8%) or MLN (59.6%) alone at 98% specificity. Furthermore, the sensitivity for CA125 and MLN in combination at a set specificity of 98% was higher (44.1%) than CA125 (37.2%) or MLN (28.8%) alone when used to discriminate ovarian cancer patients from

|               |        |             |        |
|---------------|--------|-------------|--------|
| CA15-3        | MMP3   | MSLN        | KRT19  |
| IGF2          | MMP9   | CEACAM5     | FAS    |
| KLK6          | CA72-4 | IL10        | CA125  |
| MPO           | EGFR   | TNF         | ITIH4  |
| Transthyretin | MIF    | MMP2        | MMP7   |
| FSH           | B7-H4  | HAMP        | PPBP   |
| IGFBP2        | GH1    | TF          | KLK8   |
| TSHB          | SPON2  | SERPINE1    | LHB    |
| TNFRSF1B      | HE4    | IL2RA       | CA19-9 |
| B2M           | IL6R   | CCL11       | LEP    |
| IGFBP1        | SLPI   | Osteopontin | IL8    |
| ERBB2         | PRL    | VCAM1       | APOA1  |

**Figure 2.6** List of PLCO candidates under investigation.

patients with benign ovarian tumors (BOTs). MLN was investigated in another study by Badgwell *et al.* in the serum and urine of ovarian cancer patients and patients with LMP tumors [108]. Compared to patients with benign pelvic masses and healthy controls, 42% of early-stage ovarian cancer cases had elevated MLN in urine but only 12% displayed elevated MLN in corresponding serum at a set specificity of 95%. Similarly, 75% of cases with advanced disease had elevated MLN in the urine compared to 48% in serum. In the PLCO trial specimens, it was found that MLN displayed a sensitivity of 35% at a set specificity of 95% when comparing all cases to healthy controls in “phase II” diagnostic sera [109]. When comparing only early-stage cases to healthy controls, however, the sensitivity fell at 12% at a set specificity of 95%. Furthermore, MLN displayed a sensitivity of 40% at a set specificity of 95% in a subsequent cohort of “phase III” prediagnostic sera.

**5.1.2 Interleukin-6 and interleukin-8**

Interleukin-6 (IL6) and interleukin-8 (IL8) are acute-phase reactants associated with promoting inflammation and recruiting leukocytes. In addition to their immunomodulatory activities, IL6 and IL8 have been implicated in

aspects of tumor growth, disease progression, and/or treatment [110]. In a study by Scambia *et al.*, high levels of IL6 were found in 50% of 114 patients with primary ovarian cancer though it did not outperform nor display complementarity with CA125 [111]. It was also found that high serum IL6 was associated with poor prognosis and this has been observed in other studies [112]. A similar study found that a panel of CA125 with C-reactive protein, serum amyloid A, IL6, and IL8 demonstrated a sensitivity and specificity of 94.1% and 93.1%, respectively, for detection of ovarian cancer [113]. From the available evidence, the NACB has designated IL6 as a potentially useful serum marker for the prediction of prognosis in ovarian cancer patients, albeit still in the “research/discovery” phase with an LOE of IV [114]. As individual markers, IL6 and IL8 are currently under investigation in the PLCO phase II study and IL8 in the PLCO phase III study as well [109].

#### **5.1.3 B7-H4**

B7-H4 is a 282-aa surface protein that is expressed on a variety of immune cells and functions as a negative regulator of T-cell responses. B7-H4 may promote malignant transformation. Tringler *et al.* found that B7-H4 expression was consistently higher in serous, endometrioid and clear-cell ovarian carcinomas compared with mucinous carcinomas or normal ovarian tissues [115]. In a related study, Simon *et al.* investigated the levels of B7-H4 in more than 2500 serum samples, ascites fluids, and tissue lysates [116]. The authors found that B7-H4 was significantly elevated in ovarian cancer tissue lysates compared to normal ovarian tissue lysates; B7-H4 was present at relatively low levels in all serum but showed slight elevations in the serum of ovarian cancer patients compared to healthy controls or patients with benign gynecologic conditions. Finally, the sensitivity at a set specificity of 97% increased from 52% for CA125 alone to 65% when used in combination with B7-H4 in early-stage patients. B7-H4 has also been investigated in the PLCO trial specimens [109]. In the “phase II” diagnostic sera, B7-H4 was able to discriminate all cases from healthy controls with a sensitivity of 35% at a set specificity of 95%, though this decreased to 19% when inspecting only early-stage cases versus healthy controls. Finally, B7-H4 was able to retain a sensitivity of 36% at a set specificity of 95% in “phase III” prediagnostic sera.

#### **5.1.4 Osteopontin**

Osteopontin (OPN) is a glycoprotein that functions in bone remodeling as well as in immunoregulatory roles. Additionally, OPN has been shown to

have a major role in tumorigenesis, tumor invasion, and metastasis with reported associations with breast, prostate, and ovarian cancer [173]. With regard to ovarian cancer, OPN was initially detected by a cDNA microarray study of ovarian cell lines and human ovarian surface epithelium where it was found to be higher in ovarian cancer compared its healthy counterparts [174]. A follow-up study validated these findings in terms of mRNA expression in ovarian cancer cell lines and tissues and in terms of serum protein levels [175]. In studies investigating the utility of OPN as a serum monitoring biomarker, it was found that OPN correlated well with disease recurrence, presence of ascites, and bulk of disease [117,118]. While inferior to CA125 in predicting therapy response, OPN rose early in 90% of patients developing recurrent disease. As a diagnostic biomarker, Nakae *et al.* reported a sensitivity of 81.3% for OPN alone. When combined with CA125, sensitivity increased to 93.8% although with a specificity of only 33.7% [119]. The potential complementarity between OPN and CA125 was also demonstrated by Mor *et al.* where a panel of OPN with leptin, prolactin, and insulin-like growth factor demonstrated a sensitivity of 96% and a specificity of 94% [120]. In the PLCO study, OPN has so far only been investigated in the “phase III” prediagnostic sera where it displayed a sensitivity of 11% at a set specificity of 95% [109]. Based on the currently available information, the NACB has designated OPN as a tumor-monitoring marker for ovarian cancer with an LOE of III, IV [114].

### 5.1.5 Kallikreins

The kallikreins (KLKs) are a family of 15 serine proteases encoded by a group of genes located on chromosome 19q13 which participate in a diverse range of cellular processes and pathways through regulating proteolytic cascades. KLKs have been implicated in both the promotion and inhibition of carcinogenesis, angiogenesis, and metastasis. KLKs 4, 5, 6, 7, 8, 9, 10, 11, 13, 14, and 15 have been shown to demonstrate some clinical utility in the detection, diagnosis, prognosis, and monitoring therapeutic response of ovarian cancer, although not all have been extensively investigated as serum markers [83,121–136]. Microarray studies have confirmed the overexpression of KLK6 and KLK10 in 66% and 56% of patients with ovarian cancer, respectively, and KLK10 was elevated in 35% of CA125-negative patients. Furthermore, a combination of CA125 and KLK10 increased sensitivity by 21% compared to CA125 alone in diagnosis of stage I and II ovarian cancer patients [83,129]. Elevated KLK8 levels have been reported to be associated with favorable outcomes [127]. McIntosh *et al.* reported that KLK11 was

able to distinguish ovarian cancer cases from healthy controls and displayed improved specificity than CA125 due to its lower sensitivity for benign gynecological conditions [131]. Additionally, Diamandis *et al.* reported elevated serum KLK11 in 70% of ovarian cancer patients at a set specificity of 95% [124]. Currently, KLK6 and KLK8 are under investigation in the PLCO study as early diagnostic markers. Thus far, only KLK6 has available data—in the “phase II” diagnostic sera, KLK6 displayed a sensitivity of 36% for all cases versus healthy controls and a sensitivity of 12% for early-stage cases versus healthy controls at set specificities of 95% [109]. The sensitivity slightly decreased to 32% in the “phase III” prediagnostic sera. Despite not having all been studied in serum cohorts, the NACB has designated KLKs 5, 6, 7, 8, 9, 10, 11, 13, 14, and 15 as serum markers with clinical utility in the differential diagnosis, tumor monitoring, and prognosis prediction in ovarian cancer with an LOE of IV, V [114].

## 5.2. Other markers

### 5.2.1 Vascular endothelial growth factor

Vascular endothelial growth factor (VEGF) is a glycosylated growth factor that mediates vasculogenesis and angiogenesis. Expression studies have shown that VEGF is present in the theca layer of the ovarian follicle and in the epithelium of the ovary and fallopian tube [176]. VEGF expression has been reported to be associated with poor survival at the tissue level as well as in the serum [137–142]. In one such study, preoperative serum VEGF levels were analyzed in 151 ovarian cancer patients [140]. The authors demonstrated that serum VEGF was significantly higher in patients with ovarian cancer compared to those with benign or LMP tumors. At a cut-off of 246 pg/mL, serum VEGF was able to differentiate malignant from benign ovarian masses with a sensitivity of 74%, specificity of 71%, PPV of 88%, and a NPV of 48%. As a prognostic marker, multivariate analysis showed that higher FIGO stage, presence of residual tumor mass after primary surgery, and higher serum VEGF (>380 pg/mL) were independently associated with a poor prognosis. In early-stage ovarian cancer patients, tumor grading and serum VEGF were the only independent predictors of survival. The authors suggested that serum VEGF had more potential as a prognostic biomarker rather than a diagnostic marker. While not being explored as a diagnostic marker, VEGF has become an attractive marker to investigate in ovarian cancer especially as a therapeutic target—bevacizumab is an angiogenesis inhibitor through its inhibition of VEGF and is currently under investigation as ovarian cancer therapy.

### 5.2.2 Prostasin

Prostasin (PSN) is a serine protease involved in the regulation of epithelial sodium channels. PSN was identified as a potential novel biomarker for ovarian cancer through microarray transcriptional profiling [143]. PSN was found to be overexpressed in ovarian cancer cell lines compared to normal ovarian cell lines and this was subsequently validated with real-time PCR. The authors further investigated PSN at the protein level in the serum of ovarian cancer patients and healthy controls. It was shown that a combination of CA125 and PSN resulted in an improved sensitivity (92%) and specificity (94%) compared with CA125 alone (sensitivity of 64.9% at a set specificity of 94%) and PSN (sensitivity of 51.4% at a specificity of 94%). Although PSN is not being investigated in the PLCO study, the NACB has designated the marker as a differential diagnostic marker for ovarian cancer with an LOE of IV [114].



## 6. EMERGING BIOMARKER RESEARCH

Due to the relative lack of biomarkers that have successfully transitioned from initial identification to clinical validation and implementation, researchers have begun to explore novel approaches to ovarian cancer biomarker discovery. The rapid advancements in high-throughput technologies, especially in next-generation sequencing (NGS) and MS, have further encouraged such alternative approaches to biomarker discovery. In the following section, we will review recent studies investigating the use of microRNA (miRNA) profiling, targeted proteomics, and circulating tumor DNA (ct-DNA) as surrogate biomarkers for ovarian cancer.

### 6.1. MicroRNAs

miRNAs are short (18–25 nucleotides) noncoding gene-regulatory RNA molecules that are becoming increasingly important in the context of carcinogenesis. Due to their ubiquitous roles in biological and cellular processes, deregulation of miRNA expression is now recognized as a hallmark feature of many malignancies [177]. With regard to ovarian cancer, it is strongly suggested that the disruption of oncogenes and tumor-suppressor genes is due in part to this deregulation of miRNAs, consequently encouraging the initiation and progression of carcinogenesis [177,178]. With the delineation of the miRNA signature of ovarian cancer in 2007 and 2008, there has been a surge of interest in the biological significance of miRNAs in ovarian cancer [177–179]. Coupled with the fact that these small molecules are

extremely stable and are present in detectable quantities in the circulation, miRNA has gained attention as a novel family of biomarkers for the management of ovarian cancer.

### 6.1.1 Diagnosis

Differences in serum miRNAs between healthy controls and patients with ovarian cancer were reported by Resnick *et al.* [180]. The authors identified 21 miRNAs that were differentially expressed between serum of ovarian cancer patients and healthy controls. Subsequent analysis revealed that five miRNAs (miR-21, miR-29a, miR-92, miR-93, and miR-126) were found to be overexpressed and three miRNAs (miR-127, miR-155, and miR-99) were decreased in the serum of patients with ovarian cancer, and it was suggested that these differentially expressed miRNA could be potentially used to establish a panel of miRNAs as biomarkers for ovarian cancer. In a similar study, Chen *et al.* used an *in silico* approach to mining all existing miRNA expression profiling studies for ovarian cancer [181]. Through a miRNA ranking system that considered the number of comparisons in agreement and direction of differential expression, five putative miRNA markers were identified—four were upregulated in ovarian cancer (miR-200a, miR-200b, miR-200c, and miR-141) and one was downregulated in ovarian cancer (miR100). The five miRNAs were validated in EOC tissues using quantitative real-time PCR. The Cancer Genome Atlas Network has recently cataloged the most comprehensive set of molecular aberrations in ovarian cancers to date [182]. In this study, 489 high-grade serous ovarian adenocarcinomas were analyzed for mRNA expression, miRNA expression, promoter methylation, and DNA copy number. Integrative analyses of the high-throughput data identified four ovarian cancer transcriptional subtypes (immunoreactive, differentiated, proliferative, and mesenchymal), three miRNA subtypes, and four promoter methylation subtypes. Despite the wealth of information gained from this study, there has yet to be any clinical validation of the miRNA subtypes identified.

### 6.1.2 Prognosis

As mentioned previously, miR-100 was reported to be downregulated in EOC. However, the clinical significance and functional roles of miR-100 expression in EOC were not well defined. Peng *et al.* have reported that underexpression of miR-100 was found to be associated with advanced-stage, higher serum CA125 and lymph node involvement [183]. Unsurprisingly, miR-100 underexpression was correlated with shorter overall survival

of patients with EOC, and multivariate analysis showed that the status of miR-100 expression was an independent predictor of overall survival. Functionally, it was demonstrated that miR-100 could affect the growth of ovarian cancer cells through its regulation of polo-like kinase 1 expression. Together, these results suggest that miR-100 underexpression may be reflective of a poor prognosis and this is related to the fact that miR-100 can function as a tumor suppressor by targeting *PLK1* in EOC. In a related study, patterns of miRNA expression in 487 high-grade serous tumors revealed multiple tumor subtypes and a set of 34 miRNAs was predictive of overall patient survival [184]. Finally, Bagnoli *et al.* had also delineated a miRNA signature associated with early relapse in advanced-stage patients [185]. The signature consisted of 32 differentially expressed miRNAs in early versus late relapsing patients.

### 6.1.3 Therapeutic resistance

miR-93 has been shown to be significantly upregulated in cisplatin-resistant ovarian cancer cells and negatively correlates with *PTEN* expression in ovarian cancer tissues [186]. Fu *et al.* demonstrated that overexpression and knockdown of miR-93 regulates apoptotic activity and as a consequence cisplatin chemosensitivity in ovarian cells. Furthermore, miR-93 could directly target *PTEN* and participated in the regulation of the Akt/PKB signaling pathway. Through targeting *PTEN*, miR-93 has the potential to cause constitutive activation of the mitogenic Akt/PKB pathway, thus contributing to carcinogenesis. The miR-34 family also has a strong role in regulating the p53 pathway in ovarian cancer. Zhang *et al.* have shown that the miR-449a, miR-449b, and miR-192 family of miRNAs may have similar roles [187]. The expressions of miR-449a/b, miR-34b, and miR-34c were found to be 19- to 21-fold elevated after p53 activation by a genotoxic agent. Thus, miR-449a/b, miR-34b, and miR-34c represent potential tumor-suppressor miRNAs that can be used as surrogate biomarkers of cisplatin resistance due to their involvement in the p53 pathway. Their inactivation may contribute to the carcinogenesis and progression of serous ovarian carcinomas.

In light of the recent surge of studies looking at miRNAs as surrogate biomarkers, it must be recognized that this field of ovarian cancer biomarkers is still in its infancy. Although they remain stable in the circulation, there currently exists no robust assay that can (1) measure a specific miRNA molecule and translate the measurement to a quantifiable signal and (2) translate a quantifiable signal to a clinically meaningful conclusion.

Unfortunately, the relative abundance measurements that can be accomplished through methods such as real-time PCR are virtually meaningless when comparing between patients. Furthermore, many of the miRNAs mentioned have yet to be validated in independent cohorts. Thus, before miRNAs can be introduced into the clinic as serum markers for ovarian cancer, much effort needs to be placed into assay development and independent validation studies.

## 6.2. Targeted proteomics

With the recent advent of high-throughput technologies, numerous studies have been undertaken to profile ovarian cancer using MS. This has led to the identification of numerous altered protein expression patterns of the disease. The study of protein expression in ovarian cancer has been increasingly important as proteins are the mediators of all biological processes and the molecular targets of the majority of drugs. As such, MS has been increasingly implemented as this platform allows for the simultaneous examination of thousands of proteins in biospecimens relevant to ovarian cancer. Such technologies yield information that may be useful for the diagnosis and treatment of patients through the discovery of markers for prognosis, prediction, disease monitoring, and response to chemotherapy. Despite these advantages and promises, the era of proteomics has yet to identify novel biomarkers with a significant impact on clinical management. As such, a number of alternative approaches to biomarker discovery have emerged utilizing the power of MS.

### 6.2.1 Glycomics

Glycomics is the global study of proteins with carbohydrate posttranslational modifications (PTMs) and has also served as a growing avenue for biomarker discovery over the past decade. The addition of carbohydrates to nascent proteins, also known as glycosylation, is one of the most common PTMs and is biologically implicated in protein folding, stability, localization, and cell communication [188]. Due to its extensive involvement in cellular processes, it is speculated that glycosylation is accordingly affected or differentially regulated in malignant states. As a result, proteins are aberrantly glycosylated and these abnormal glycoforms can be used to detect the presence of disease. While glycomic analysis of biological specimens still faces challenges, major advances in both preanalytical separation methods and MS have allowed for increasingly comprehensive characterization of glycomes and cancer-specific glycoproteins [189,190]. With respect to

ovarian cancer, the majority of glycomic-based biomarker studies have employed the use of matrix-assisted laser desorption/ionization (MALDI) MS coupled with extensive preanalytical enrichment methods for glycans (such as peptide-N-glycosidase digestion, chromatographic separation, and solid-phase permethylation) [188].

In a study by Alley *et al.*, the serum glycomes of 20 healthy control women and 30 ovarian cancer patients were investigated with a specific focus on quantitative profiling of the asparagine-linked oligosaccharides (N-linked glycans) through MALDI MS [191]. Overall, it was observed that the ovarian cancer glycomes had increased tri- and tetra-branched structure with variable sialylation and fucosylation. Further analysis revealed that glycan patterns could be used to distinguish the ovarian cancer patients from the healthy controls. It was, however, noted that cancer patients were all diagnosed with late-stage cancer and further studies with serum from women with stage I/II cancer are needed to truly assess whether these glycomic patterns can be used as early detection markers. In a related study, Saldiva *et al.* analyzed total serum N-linked glycans in the serum of healthy controls and patients with ovarian cancer, benign gynecological conditions, and other gynecological cancers using MALDI MS and electrospray ionization MS [192]. From these analyses, it was reported that the ovarian cancer glycome had an increased expression of three glycan structures. As well, the authors identified altered glycosylation patterns on acute-phase proteins.

Despite the wealth of information that has been accumulated, glycomic-based biomarkers have yet to pass any clinical validation in ovarian cancer. Global investigation of glycosylation and subsequent identification of putative biomarkers remains hampered by biological and technical limitations. While numerous authors have identified unique glycomic profiles for ovarian cancer, it is unclear whether such changes are truly ovarian cancer-driven or simply a result of the metabolic phenomena that ensue after malignancy and inflammation. Thus, additional studies that clearly demonstrate such glycomic changes as being specific to ovarian cancer are required. Due to the heterogeneity and complexity of glycosylation, a prominent technical limitation of glycomics that has been recognized is the limited ability of current MS platforms to distinguish glycome isomers [189]. Finally, a major limitation of glycomic approaches to biomarker discovery is the availability of validation methods. The gold-standard quantitative method for validating putative serum biomarkers is an enzyme-linked immunosorbent assay, which is based on antibody-antigen interactions to generate a detectable (and quantifiable) signal. Unfortunately, analogous

assays for glycan-based epitopes suffer from poor reproducibility. There have been attempts to develop lectin- or antibody-based assays, but these capture methods often display poor specificity for the glycan epitope of interest and low sensitivity [193]. Therefore, development of a robust, quantitative method for glycan-based biomarkers is urgently needed in order to validate candidates that arise from discovery studies.

### 6.2.2 Metabolomics

In addition to glycomics, an equally prominent MS-based strategy for biomarker discovery has been the investigation of the metabolome or the global population of metabolites. Metabolites are the end products of metabolic pathways which in turn are a phenotypic reflection of the biological sample under investigation. Thus, it is reasonable to presume that under a diseased state, metabolic pathways will be altered and the resultant metabolites will indicate such pathological changes. Such metabolic profiling has been increasingly applied to biomarker discovery and has seen some clinical utility in various malignancies such as breast, colon, oral, and prostate cancer [194–196].

With respect to ovarian cancer, metabolomics-based biomarker discovery efforts have focused primarily on patient serum/plasma and urine samples. In two independent studies, metabolomic profiling of urine from ovarian cancer patients using MS was able to identify numerous metabolites with the ability to discriminate between healthy controls and ovarian cancer patients. Zhang *et al.* were able to identify 22 metabolites that were able to discriminate between EOC from BOTs and healthy controls through ultra-performance liquid chromatography (UPLC) quadrupole time-of-flight MS analysis of urine samples from the said cohorts [197]. Nine of these metabolites were also found to be significantly different between different-staged cancers and could reliably distinguish stage I/II from stage III/IV cancers. In a similar study by Chen *et al.*, metabolomic analysis of ovarian cancer urine through hydrophilic interaction chromatography and reversed-phase liquid chromatography (LC) MS identified five metabolites that were specific to ovarian cancer patients and were significantly upregulated compared to healthy controls and BOT patients [198].

Similarly, serum/plasma metabolomic studies have revealed potential diagnostic markers for ovarian cancer. In two separate studies, UPLC MS coupled with partial least-squares discriminant analysis was employed to identify metabolic differences between ovarian cancer patients and controls. Chen *et al.* identified 27-nor-5 $\beta$ -cholestane-3,7,12,24,25 pentol

glucuronide (CPG) as a metabolic biomarker to discriminate EOC from BOT [199]. In a subsequent validation cohort, serum CPG displayed an area under the curve (AUC) of 0.750 in receiver-operating characteristic (ROC) curve analysis for stage I cancer with a sensitivity and specificity of 70% and 77%, respectively. Fan *et al.* identified eight candidate biomarkers for the diagnosis of EOC. The authors were able to further validate these markers in an independent cohort and demonstrated that combining all 8 markers yielded an AUC of 0.941 with a sensitivity of 92% and a specificity of 89% for detecting EOC [200].

Urinary and serum metabolomics remains a promising avenue for ovarian cancer biomarker discovery. The use of metabolites as disease biomarkers is well established (such as elevated glucose for diabetes mellitus), thus lending credence for the use of such metabolites for ovarian cancer. Unfortunately, MS-based metabolomics still faces major limitations preventing its introduction into the clinic for ovarian cancer diagnosis. Biologically, metabolic responses due to malignancy can vary greatly and metabolites may undergo extensive biotransformation from the site of malignancy to biofluid of interest (urine or serum) [201]. Metabolites may even undergo such processing *ex vivo*, and thus, metabolomic studies are susceptible to biases originating from sample collection and storage. Furthermore, metabolites can be influenced by environmental factors such as smoking, sleep patterns, diet, and age. Therefore, such confounding variables can potentially disguise the true effects of malignancy in metabolomic profiling. Future studies will need to focus on the standardization of metabolomic protocols to decrease the chances of introducing such biases and also on intra- and interstudy reproducibility.

### 6.2.3 Peptidomics

Numerous alternative strategies to standard shotgun proteomics have evolved in the past decade in addition to glycomics and metabolomics. The investigation of the peptidome, or the low-molecular weight proteome, of biological fluids relevant to ovarian cancer is one such technology. The low-molecular-weight proteome of both blood and ascites fluid is believed to contain many potential diagnostic peptides. It is hypothesized that metabolic activity increases in tandem with the progression of malignancy and consequently, protease activity increases as well. Thus, endogenous peptides are generated, some of which may be secreted into the surrounding environment where they can theoretically be detected and used to monitor disease. Furthermore, progression of malignancy is also

associated with the degradation of adhesion and cell-to-cell junction proteins, and this may also be another source of endogenous peptides with diagnostic potential. Although peptidomics is in its infancy, there have already been a few studies that report the utility of peptides for ovarian cancer diagnostics. Fredolini *et al.* reported approximately 51 serum peptidomic markers that were unique to ovarian cancer patients compared to patients with BOT [202]. On the contrary, Timms *et al.* recently reported that MALDI MS peptide profiles were unable to accurately diagnose ovarian cancer from healthy controls, though the endogenous peptides could provide some diagnostic insight [203]. Needless to say, greater characterization of the endogenous peptidome of various biospecimens related to ovarian cancer is needed to truly assess whether or not peptide-based biomarkers are clinically useful.

#### **6.2.4 Autoantibody signatures**

The identification of autoantibody signatures in serum has also been investigated for ovarian cancer biomarker discovery. Ovarian cancer is often characterized by the complex network of inflammatory cytokines present in the microenvironment and the involvement of immune-related cells such as tumor-associated macrophages. As such, populations of antitumor antibodies may be present and detection of said immunological responses to tumorigenesis may help to detect early-stage disease. In a laying hen model of human ovarian cancer, Barua *et al.* identified 11 proteins as immunoreactive ovarian antigens through LC MS [204]. Although this was the first study to identify immunoreactive ovarian antigens by serum antitumor antibodies, the authors recognized the fact that the ovarian antigens could not discriminate laying hens with nonmalignant ovarian conditions from those with ovarian cancer. Philip *et al.* investigated the immunoproteome of ovarian cancer and healthy control sera, as well as that of the conditioned media of the ovarian cancer cell lines [205]. Overall, eight autoantibody-reactive autoantigens were identified that were present in all five cancer serum composites and in both cell lines. However, the suggested novel autoantibody biomarkers for ovarian cancer diagnosis were not validated in an independent cohort. Future studies will thus need to address how well such putative autoantibody-based markers perform in independent, blinded validation. Recently, Karabudak *et al.* described a high-throughput, proteomic approach to identifying novel autoantibody biomarkers for ovarian cancer [206]. In this study, the authors employed protein microarray screening in combination with quantitative proteomics to identify autoantibody—as

well as the corresponding autoantigens—serum markers that could distinguish ovarian cancer from nonovarian cancer patients. The three most prominent markers identified were autoantibodies against ezrin, cofilin-1, and PDZ domain-containing protein. It was reported that the three autoantibody markers displayed higher specificity and sensitivity compared to CA125 in preliminary ROC curve analysis—unfortunately, these results were only “validated” in pooled serum samples. Therefore, assaying for these autoantibodies in individual samples in a true validation cohort is required before any conclusions can be made for these novel biomarkers.

### **6.3. Circulating tumor DNA**

The investigation of cell-free DNA or ct-DNA as surrogate biomarkers for disease is not a novel approach to biomarker discovery. The notion of detecting free DNA in biological fluids as indicators of malignancy has been investigated for over a decade due to numerous advantages. Ct-DNA has the potential to be abundantly present in serum owing to its small molecular size and the fact that tumors often metastasize through the circulatory system. Additionally, numerous malignancies are often defined by hallmark mutations at specific loci, and thus, a diagnostic test probing for precise mutations within ct-DNA could have high specificity. For these reasons, the use of serum ct-DNA as biomarkers of malignancy represents an ideal, noninvasive screening and monitoring tool. While “emerging” is a misnomer with regard to ct-DNA, there has indeed been a recent resurgence of research into ct-DNA as cancer biomarkers and this can be directly attributed to rapidly evolving sequencing technologies. The past decade has witnessed massive improvements in sequencing methods, read length, accuracy, amount of data output, and time required per run [207,208]. Whereas earlier DNA sequencing required slower, laborious methods such as polymerase chain reaction, current NGS platforms operate at a much higher efficiency thus allowing for the generation of greater amounts of data in a short amount of time.

#### **6.3.1 Pre-NGS Era**

Prior to the emergence of ct-DNA, circulating tumor cells were already being examined for their prognostic and predictive significance. In one such study, the authors successfully isolated tumor cells from the sera of ovarian cancer patients but found no correlation between circulating tumor cell numbers and patient outcomes [209]. Similar to circulating tumor cells, much of the earlier ct-DNA research focused on identifying ct-DNA-based

markers for prognosis and for disease surveillance. In terms of diagnosis and/or prognosis, the focus was to identify specific molecular alterations and mutations within ct-DNA that were specific to ovarian cancer. For example, Swisher *et al.* examined p53 mutated sequences in free tumor DNA derived from the blood and ascites fluid of women with EOC [210]. It was found that 50% of the 137 tumors had somatic p53 mutations and that plasma ct-DNA was an independent predictor of decreased survival. However, plasma ct-DNA was detectable in only 30% of the p53-positive cases, and of those, only one was diagnosed as early-stage EOC. Additionally, the authors failed to acknowledge the lack of utility of p53-mutant ct-DNA for nonserous EOCs as p53 mutations are almost exclusively found in high-grade serous EOC. In a similar study, Dobrzycka *et al.* evaluated the prognostic significance of ct-DNA and specific *KRAS* mutations in women diagnosed with EOC [211]. It was found that ct-DNA was detectable in 55 of the 126 patients of which the majority were of the serous histotype. Furthermore, ct-DNA was significantly associated with decreased survival in the serous EOC patients (90.8% for presence of ct-DNA vs. 93.4% for absence of ct-DNA). In terms of *KRAS* mutations, it was shown that mutations in codon 12 were present in 27 of the 126 EOC cases and particularly high in cases of the mucinous histotype. Downstream survival analyses revealed that the presence or absence of *KRAS* mutations significantly affected survival rates in patients diagnosed with mucinous ovarian carcinoma. Similar to the previous study, a limitation of focusing on only *KRAS* mutations is that nonmucinous carcinomas may be missed since *KRAS* mutations are quite specific to the mucinous histotype.

Whereas diagnostic/prognostic studies attempted to identify surrogate biomarkers in the form of specific ct-DNA mutations, disease surveillance studies aimed to establish correlations between total ct-DNA concentration and tumor burden. The rationale was that as an ovarian tumor progressed, greater amounts of tumor-derived DNA would be released into circulation due to increased necrosis and apoptosis. Thus, quantification of total plasma ct-DNA could act as a marker for monitoring disease progression and response to therapy. In a preliminary study by Kamat *et al.*, total plasma cell-free DNA was investigated as a biomarker for monitoring disease through comparing the level of cell-free DNA in late-stage ovarian cancer patients with that of healthy controls [212]. Through probing across three different loci using real-time PCR, the authors determined that total plasma cell-free DNA was elevated in patients with ovarian cancer compared to healthy controls. It was therefore suggested that cell-free DNA could be

used as a marker for disease progression as its plasma levels appeared to correlate with the presence of malignancy. However, the authors did note that the findings were preliminary at best and that because cell-free DNA was measured (as opposed to ct-DNA), it was very likely that both normal and tumor-derived DNA were being detected. In a similar study, cell-free DNA was investigated as a surrogate marker for tumor burden and response to therapy in an orthotopic model of ovarian cancer [213]. Through measuring plasma cell-free DNA via real-time PCR, the authors found that cell-free DNA correlated significantly with tumor burden, apoptotic activity, and response to therapy. As tumor formation progressed after injection of ovarian cancer cells, cell-free DNA increased accordingly. A limitation, however, is that because the authors focused on cell-free DNA and not tumor-specific DNA, contamination from normal genomic DNA could cause an underestimation of the ability of plasma DNA to monitor disease progression.

While studies during the pre-NGS era established the basis for much of the ct-DNA research that would follow, it was clear that the studies were often hampered by the slow, laborious PCR-based methods required to sequence and analyze the ct-DNA. The lack of parallelization and multiplexing meant that often only one specific molecular event was inspected—this is apparent in the previously mentioned studies that focused on only p53 mutations or on only *KRAS* mutations. Ultimately, many preliminary studies misrepresented ovarian cancer due to inspecting single molecular events that do not occur at equal frequencies across the different histotypes.

### **6.3.2 NGS platforms and beyond**

The recent surge of interest in ct-DNA can be attributed to rapidly developing sequencing technologies in which many platforms have evolved beyond the PCR-based Sanger methods. The increasing use of plasma sequencing in prenatal diagnostics has demonstrated the clinical feasibility of cell-free DNA as surrogate biomarkers [214–217]. Furthermore, advances in targeted deep sequencing has allowed for improved detection of mutations across the genome, even if they occur at very low frequencies and/or do not occur at frequently mutated loci [218–220].

In a recent study by Forsheew *et al.*, such a method was developed where amplification and deep sequencing of large genomic regions allowed for the detection of both frequent and infrequent mutations in ct-DNA from the plasma of ovarian cancer [207]. This method, referred to as tagged-amplicon

deep sequencing (TAm-Seq), was able to identify cancer mutations at allele frequencies as low as 2% with a sensitivity and specificity of >97%. Across plasma ct-DNA from 38 patients, the authors were able to identify *TP53* mutations at allelic frequencies of 4–44%. Subsequent validation of the TAm-Seq method using patient-specific digital PCR assays demonstrated strong concordance between the two methods with a correlation coefficient of 0.90. Overall, the TAm-Seq method was able to identify mutations at allelic frequencies of >2% in plasma with a sensitivity of 97.5% and a PPV of 100%. Finally, the authors were able to apply TAm-Seq to monitoring disease progression and response to treatment. Through TAm-Seq sequencing of patient serum during the treatment regimen, it was demonstrated that mutant allelic frequencies correlated strongly with the clinical course of the disease compared to CA125. These results were all validated using digital PCR with excellent concordance. A facet of TAm-Seq that still needs to be improved on is to increase its threshold of detection to <2% allelic frequency. In a similar study by Murtaza *et al.*, exomic sequencing of three ovarian cancer patients throughout their chemotherapy was used to track the genomic evolution of the tumor and identify mutations indicative of acquired drug resistance [208]. Using paired-end sequencing on the Illumina HiSeq2500, it was observed that mutant allelic frequencies in plasma ct-DNA in loci associated with drug resistance significantly increased following treatment. The mutant allelic frequencies identified through exome sequencing were validated using digital PCR.

The notion of “liquid biopsy” through deep sequencing of plasma is becoming increasingly amenable in the management of ovarian cancer as sequencing technologies continue to evolve. In addition to being advantageous to tissue biopsies due to its noninvasiveness, ct-DNA sequencing allows for truly personalized medicine as the mutational profiles generated by each patient are unique, and thus, each patient will be treated on an individual basis. However, NGS platforms still suffer from false positives due to background signals similar to other high-throughput technologies. Ultimately, the presence of mutated tumor DNA can never be assured until validation by single-nucleotide assays such as Sanger sequencing. As well, the use of ct-DNA as surrogate markers suffers from similar limitations as miRNAs in that there exists no standardized assay that can translate ct-DNA into a quantifiable signal that can be compared across patients. Therefore, before ct-DNA-based modalities can be introduced into the clinic, issues regarding analytical sensitivity, background noise, and the lack of an appropriate assay must be addressed first.



## 7. CONCLUSION

Ovarian cancer remains a very difficult malignancy to manage because of the heterogeneity in histology, prognosis, and progression it demonstrates. The major unmet clinical need still remains biomarkers that can accurately diagnose the disease during its early stages because of the significantly higher prognosis early-stage disease is associated with. Unfortunately, the past few decades of biomarker studies for this purpose have not produced fruitful results as the majority of “novel biomarkers” often fail to pass successful clinical validation. This has been attributed to deficiencies in both study design and statistical analyses leading to misinterpretation of the results and exaggeration of positive findings [221]. Fortunately, there is an increasing body of research dedicated to standardizing study design and interpretation in order to mitigate such flaws that are often seen in biomarker studies.

Despite CA125 having been the only FDA-approved serum marker for ovarian cancer for a long time, the advances in genomic and proteomic technologies have presented us with new and exciting opportunities for the discovery of novel biomarkers. As seen by the recent approval of the HE4, ROMA, and OVA1™ tests/algorithms, high-throughput technologies represent a very feasible method of biomarker discovery for various clinical applications in ovarian cancer. As high-throughput platforms continue to evolve, we are able to examine an increasing number of aspects of ovarian cancer in order to identify surrogate markers as seen by the examples of miRNA, glycoproteins, and ct-DNA. However, it is imperative that these high-throughput studies are designed meticulously with careful consideration of biases that have plagued studies in the past. Vast amounts of “-omics” data have already been accumulated for ovarian cancer and if handled appropriately, there are enormous opportunities for the identification of novel biomarkers for disease screening, early diagnosis, prognosis, prediction of therapy response, and therapeutic targeting. These issues should not be seen as a deterrent for high-throughput biomarker discovery, but we should learn from the mistakes of the past so that we may bridge the gap between bench and bedside in the near future.

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