From bench to bedside: discovery of ovarian cancer biomarkers using high-throughput technologies in the past decade

Ovarian cancer is the most lethal gynecological malignancy and survival of this disease has remained relatively unchanged over the past 30 years. A contributing factor to this has been the lack of reliable biomarkers for the clinical management of ovarian cancer. Rapid advances in high-throughput technologies over the past decade has allowed for new and exciting opportunities for biomarker discovery in the field of ovarian cancer, especially with respect to serum biomarkers that can be used for various clinical applications. This review highlights the major genomic and proteomic studies dedicated to ovarian cancer biomarker discovery over the past decade. An emphasis will be placed on the HE4, Risk of Malignancy Algorithm (ROMA) and OVA1™ serum-based tests/algorithms that have recently been approved by the US FDA as ovarian cancer biomarkers.

KEYWORDS: biomarker ▪ HE4 ▪ high-throughput technology ▪ OVA1™ ▪ ovarian cancer ▪ Risk of Malignancy Algorithm

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Ovarian cancer is the most lethal gynecological malignancy and the fifth-leading cause of mortality in North American women [1]. Despite advances in medicine and technology, the survival rate of women diagnosed with ovarian cancer has remained relatively unchanged over the past 30 years [2–4]. For women diagnosed with early-stage ovarian cancer, the 5-year survival rate is approximately 80–90% [5], but this decreases dramatically to 20–30% [6] in late-stage diagnoses. Unfortunately, no reliable mode of screening currently exists for early detection of ovarian cancer, and the disease is often asymptomatic during its early stages. As a consequence, most women are diagnosed when the disease has progressed considerably. Patients diagnosed with advanced disease are managed with surgical cytoreduction and chemotherapy, yet many experience resistance to chemotherapy and relapse. Taken together, ovarian cancer remains a difficult malignancy to manage clinically.

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 probably represents a heterogeneous group of diseases that simply share a common anatomical location [7]. 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 purpose of novel biomarker discovery; specifically, the identification of potential markers for numerous aspects of ovarian cancer management, including diagnosis, prognosis, prediction, recurrence, and monitoring. This review examines some of the landmark high-throughput studies dedicated to the identification of ovarian cancer biomarkers in

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the past decade. Emphasis will be placed on the recent US FDA-approved markers/algorithms – HE4, Risk of Malignancy Algorithm (ROMA) and OVA1™ – that have come about from these studies.

Genomics

Ovarian cancer is defined by a myriad of genetic abnormalities that result in progressive genomic instability [8]. For example, it has been known for a long time that germline mutations in *BRCA1* and *BRCA2* are present in approximately 10% of ovarian cancers and have been postulated to predispose affected individuals to oncogenic transformation and genomic instability [9]. It is also known that loss-of-function mutations in the tumor suppressor *TP53* gene are present in 60–80% of ovarian cancer cases, and may be implicated in the pathogenesis of the disease [10]. With the aid of high-throughput genomic technologies, we have been able to identify many more genetic markers that may be implicated in ovarian cancer. These new technologies include next-generation sequencing, microarrays and array comparative genomic hydridization. Next-generation sequencing, which involves the global deciphering of nucleotide sequences, has allowed for copy number variation, mutational and methylation analysis. Microarrays, which couple oligonucleotide hybridization with the chemical generation of a detectable signal to determine the relative abundance of specific loci in a sample, have allowed for the transcriptional profiling. Array comparative genomic hybridization, a variation of the microarray platform tailored to genomic copy number analyses, has allowed for identification of chromosomal amplification and deletion events. In the past 10 years, over 400 publications have been dedicated to genomic, transcriptomic and epigenomic analyses of ovarian cancer. Below, the authors review a landmark paper that is seen as the most comprehensive genomic study in ovarian cancer to date.

■ Cancer Genome Atlas Research Network

In 2011, the Cancer Genome Atlas Research (TCGA) Network published a study on the genomic and epigenomic alterations present in high-grade serous ovarian cancer in order to identify genetic markers that may be implicated in pathogenesis, be indicative of clinical outcome and be representative of potential therapeutic targets [11]. Four hundred and eighty-nine high-grade serous ovarian adenocarcinomas were analyzed for mRNA expression, miRNA expression, promoter methylation and DNA copy number; 316 of these tumors were additionally analyzed for exomic sequencing. Mutational analyses were performed through exome capture sequencing to identify loci at which high-grade serous ovarian cancers were most frequently mutated. The authors found that *TP53* was mutated in 96% of the tumor samples, and infrequent but statistically recurrent mutations were also found in *BRCA1*, *CSMD3*, *NF1*, *CDK12*, *FAT3*, *GABRA6*, *GRCA2* and *RB1*. In addition, 113 significant focal DNA copy number aberrations and promoter methylation events involving 168 genes were reported. Finally, 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. Although these subtypes did not correlate with survival data, the authors were able to produce a 193-gene transcriptional signature that correlated with overall survival. The predictive power of this gene signature was successfully validated on an independent set of 255 high-grade serous ovarian cancer samples and on three independent expression data sets.

While the true impact of the TCGA study has yet to be realized due to the massive amount of data accumulated, it has provided a global view of the molecular alterations and abnormalities that occur in high-grade serous ovarian carcinoma. The unique mutational spectrum of high-grade carcinomas delineated by this study can potentially aid in the discrimination of serous carcinoma subtypes and encourages further investigation into molecular subtype-stratified care. The 193-gene transcriptional signature may prove to be an applicable prognostic tool for high-grade serous carcinoma diagnoses if subsequent validation studies are successful. Overall, the catalog of genomic aberrations created from this study can serve as a basis for future studies into genomic-based markers of high-grade serous ovarian cancer. One of the limitations of this study, however, is that it did not examine other histotypes of ovarian cancer, such as mucinous, endometrioid and clear-cell carcinomas.

Proteomics

The study of protein expression in ovarian cancer has been equally important in the

high-throughput era, as proteins are the mediators of all biological processes and the molecular targets of the majority of drugs. Moreover, the proteome integrates cellular genetic information and environmental influences. Proteins expressed by ovarian tumors can be secreted and shed into proximal areas and make their way into the blood circulation. Furthermore, proteins are relatively large and stable molecules and can thus be detected more easily than their nucleic acid counterparts. Proteomic studies in ovarian cancer have been performed mainly through mass spectrometry (MS), as this platform allows for the simultaneous examination of thousands of proteins in a biological sample. In a typical MS-based experiment, proteins are converted to peptides through enzyme digestion. These peptides can be fractionated offline or placed directly into the mass spectrometer for separation and ionization. Following ionization, the peptides are fragmented in a process known as collision-induced dissociation. The mass-to-charge (m/z) ratios of the product ions provide information on the amino acid sequence of the peptide, which can be subsequently identified through the mass spectrum generated and bioinformatics [12]. Here, the authors review some of the major approaches to MS-based ovarian cancer biomarker discovery.

■ Proteomic profiling

In 2002, a study published by Petricoin and colleagues reported a proteomic algorithm that could discriminate serum of women with ovarian cancer and healthy individuals with a sensitivity of 100% (95% CI: 93–100), specificity of 95% (95% CI: 87–99) and a positive predictive value (PPV) of 94% (95% CI: 84–99) [13]. Instead of identifying specific protein candidates, the authors observed a fingerprint of m/z ratios that appeared to be able to distinguish between the two groups. To generate this algorithm, serum samples from 50 healthy women and 50 ovarian cancer patients were subjected to proteomic analysis on SELDI-TOF MS. In a subsequent blinded validation cohort, the proteomic pattern correctly classified 63 out of 66 (92% specificity) noncancer controls and 50 out of 50 (100% sensitivity) ovarian cancer samples.

Indeed, this study generated much excitement over the potential of proteomic patterns as a reliable screening test for ovarian cancer. Unfortunately, the study was met with much skepticism upon careful examination of the methodology and results. A major criticism involved the misleading PPV of 94% quoted by the authors. Contrary to the results, it was determined that the true PPV was 0.8% based on the sensitivity, specificity and incidence of ovarian cancer reported by Petricoin and colleagues [14]. The incorrect PPV was a result of the authors having designed the cohort used for the screening analyses to have a prevalence of ovarian cancer of approximately 50% despite ovarian cancer being a relatively infrequent disease in the general population. Subsequent limitations of this approach that were identified included: sample collection and storage artifact biases; the failure to identify well-established cancer biomarkers; bias caused by high-abundance serum proteins; and the lack of concordance between peaks generated at different institutions [15–18]. Despite the promising results, the pitfalls of the study highlighted the importance of stringent planning in screening analyses during sample collection and validation and cohort design. Baggerly and colleagues further showed that background noise misconstrued as true signals can also discriminate between patients with cancer and those without [19]. Although research into proteomic patterns as cancer biomarkers continues, few studies have shown clinical applicability as they often do not successfully pass independent validation.

■ Identification of candidate markers

Unlike the generation of mass spectra patterns as a diagnostic tool, the majority of ovarian cancer proteomic studies have been focused on identifying specific proteins as novel biomarkers. Since 2002, over 100 studies have been published mining the proteome of various biospecimens relevant to ovarian cancer for novel biomarkers including serum, proximal fluid, cell lines and tumoral tissues. In serum proteomic studies, the focus has been on the identification of diagnostic proteins that display higher concentrations in the serum of ovarian cancer patients compared with the serum of healthy individuals. Unfortunately, the complexity of the plasma proteome results in a diverse range of concentrations of analytes (over ten orders of magnitude). Without any up-front fractionation, the successful measurement of these serum proteins is beyond the current capabilities of mass spectrometers (decrease in ionization efficiency). As a result, in most serum proteome studies, high-abundance proteins such as albumin and immunoglobulins are removed before MS analysis. One disadvantage of this approach is the disregard for the hypothesis

that these high-abundance proteins may harbor smaller proteins/peptides with diagnostic utility. Nonetheless, MS is limited by its sensitivity for low-abundance proteins. Few biomarkers from serum discovery studies have passed validation and the majority of proteins identified as 'novel biomarkers' are often acute-phase reactants with limited clinical utility [20]. The serum proteomes in animal models of ovarian cancer have also been examined for novel biomarkers [21,22], with a recent study confirming the upregulation of secreted/shed proteins in the plasma of ovarian cancer patients compared with healthy controls that were originally identified through MS analysis on the serum of ovarian cancer mouse models [23].

Proximal fluids such as ascites and interstitial fluid have been recognized as a potential source of novel biomarkers because of their close contact with tumoral tissue. Proximal fluids contain tumoral cells and secreted factors from the tumor microenvironment, and thus, may serve as the most accurate representation of malignancy compared with other biological fluids. To date, there have only been a few in-depth proteomic analyses of proximal fluids, with the most recent one having identified 769 proteins in the soluble portion of tumoral interstitial fluid [24]. Although the authors state that the proteins may represent local secreted factors that are detectable in serum and hold clinical utility, these results have yet to be validated.

Ovarian cancer cell lines have served as a promising platform for biomarker discovery because of the hypothesis that the conditioned media represents proteins secreted/shed by tumoral cells, and that these proteins may end up in the circulation. A drawback of using cell lines to identify novel biomarkers is that an *in vitro* system cannot recapitulate the heterogeneity of ovarian cancers *in vivo*, and that it lacks the tumor–host microenvironment. Two recent studies by Li and colleagues [25] and Cicchillitti and colleagues [26] deciphered the proteomes of chemoresistant human ovarian carcinoma cell lines through MALDI MS and liquid chromatography-tandem MS. Numerous proteins involved in DNA damage repair, metabolic enzymes, cell cycle and apoptosis, and cell stress response were identified as potential markers for chemoresistance, but again, these results have yet to be validated beyond an *in vitro* system.

Direct proteomic analysis of tumoral tissue has also been explored as a source of biomarkers, as all potential protein markers should theoretically originate from the tumor itself, whereupon processes such as leaky capillaries, tumoral production of proteases, and cellular apoptosis cause the release of these proteins into the peritoneal space. However, its limitation lies in the cellular heterogeneity of the tissue; that is, the presence of stromal and vascular cells. Nontumoral cells can contaminate proteomic analyses with highly abundant but nonspecific proteins and thus hinder tissue-directed biomarker identification. To alleviate this, researchers are attempting to develop strategies to isolate as much of the tumoral portion of the tissue sample as possible prior to proteomic analyses. For example, Cadron and colleagues demonstrated that the combination of laser capture microdissection with SELDI-TOF MS on ovarian cancer tissue could generate protein profiles with the ability to discriminate between platinum-sensitive and platinum-resistant patients [27]. Proteins/peptides represented by the discriminatory proteomic peaks may be novel predictive biomarkers, but this will require further investigation as well as identification of the specific proteins/peptides.

Despite the vast number of proteomic studies dedicated to biomarker discovery, very few of these putative markers have passed clinical validation. Candidate biomarkers identified thus far through MS often display weak-to-moderate sensitivity and specificity for ovarian cancer. Thus, a growing strategy to overcome this limitation is a systems biology approach to biomarker discovery that integrates MS with other high-throughput technologies to gain a more comprehensive view of ovarian cancer [28,29]. Comparison of the proteomes of different biospecimen sources may also help to identify proteins that are consistently upregulated in ovarian cancer, and thus, have a higher potential to be novel serological biomarkers. This in turn may help to produce markers with both high sensitivity and high specificity for ovarian cancer.

■ Other prominent strategies

A number of strategies for ovarian cancer biomarker discovery beyond classical MS-based proteomics have emerged in the past decade. One such strategy has been the investigation of the peptidome, or the low-molecular-weight proteome, of biological fluids relevant to ovarian cancer. The low-molecular-weight proteome of both blood and ascites fluid are believed to contain many potential diagnostic peptides. Although peptidomics is in its infancy, there

have already been a few studies that report the utility of peptides for ovarian cancer diagnostics [30,31]. Glycoproteomics, the global study of proteins with post-translational carbohydrate modifications, has also served as a growing avenue for biomarker discovery over the past decade. The rationale for this branch of proteomics is that post-translational modifications may be differentially regulated in cancer, and thus the glycosylated forms of key proteins could have potential diagnostic value. Recent studies highlighted the differential glycosylation of human KLK6 in ovarian cancer compared with normal individuals and even differential glycosylation patterns between chemosensitive and chemoresistant ovarian cancer patients [32,33]. The identification of autoantibody signatures in serum has also been investigated for ovarian biomarker discovery as detection of immunological responses to tumorigenesis may help to detect early-stage disease. Murphy and colleagues identified autoantibodies against p53 and the novel autoantigens α-adducin and endosulfin-α as potential diagnostic markers through protein array screening [34]. A final approach that has been gaining popularity, but has yet to be investigated in ovarian cancer, is MS-based imaging of cancer tissues to identify markers that may be shed into the extracellular space [35]. All of these approaches to biomarker discovery have their advantages and disadvantages, and many of the candidate biomarkers 'discovered' by these strategies have yet to pass validation.

FDA-approved biomarkers

Since its discovery in 1981 by Bast Jr and colleagues [36], CA125 – also known as mucin 16 – still remains the best serum biomarker for ovarian cancer. It was identified through the development of a monoclonal antibody (OC125) that displayed reactivity with epithelial ovarian carcinoma (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. 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 [37–39]. 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 [40–42]. For these reasons, CA125 is not approved for ovarian cancer screening or for the detection of early disease.

As mentioned previously, the advent of high-throughput technologies has led to a renewed interest in the discovery of novel ovarian cancer biomarkers, especially for serum biomarkers that can complement CA125. A serum-based test is ideal since it would be minimally invasive, requiring a small drawing of blood. Unfortunately, the majority of serum biomarker candidates identified through high-throughput experiments have been irreproducible and unable to pass validation experiments. This may be because upregulated proteins in the serum of ovarian cancer patients are often acute-phase reactants that are a reflection of the epiphenomena rather than specific to ovarian cancer. Furthermore, many serum biomarker discovery studies have focused on identifying diagnostic or disease-screening proteins. Such markers must display an extremely high specificity to reliably rule out those without disease because of the low prevalence of ovarian cancer. Specifically, a screening test for ovarian cancer needs to display a sensitivity of more than 75%, and a specificity of more than 99.6% to attain a PPV of 10% [43,44]. No biomarker has yet been able to achieve this level of performance.

Another unmet clinical need is the ability of serum biomarkers to reliably predict the presence of malignancy in women with a pelvic mass. Patients with ovarian cancer often present initially with a pelvic mass of unknown malignant potential. More than 200,000 women undergo exploratory surgery for a pelvic mass in the USA each year [45,46]. 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 patient outcomes [46]. 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 with gynecologists or general surgeons [47–53]. 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 [54,55]. Algorithms/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 and colleagues 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 [56]. Since its introduction, the RMI has reported sensitivities from 71 to 88% and specificities from 74 to 97% [57–60]. Multiple studies have confirmed its clinical utility and it is routinely used in the UK [61]. Recently, two other algorithms (ROMA and the OVA1 test) gained FDA approval for the discrimination of pelvic masses and were developed using serum biomarkers identified from genomic and proteomic techniques, respectively. The ROMA incorporates serum levels of CA125 and HE4, which was identified through microarray studies, while the OVA1 test incorporates serum levels of CA125 and four other markers identified through MS (β-2 microglobulin [β2M], transferrin [TrF], transthyretin [TT] and apolipoprotein A1 [ApoA1]). Below, the discovery of the ROMA and the OVA1 biomarkers and the studies leading up to and following approval by the FDA is reviewed.

■ HE4

Also known by its gene name *WFDC2*, HE4 is a 25-kDa glycosylated protein that consists of a single peptide and two whey acidic protein domains that contain a four-disulfide core composed of eight cysteine residues [62,63]. The gene is located on chromosome 20q12–13.1, in proximity to other gene members of the whey acidic protein domain family. Functionally, HE4 has been suggested to play a role in host defense because of its ability to bind lipopolysaccharides and other bacterial moieties, as well as demonstrating antiproteinase and anti-inflammatory activities [64].

HE4 was initially identified as an mRNA transcript specific to the distal epididymal tissue [62]. 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 [65]. 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 [66–69]. Furthermore, Drapkin and colleagues 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 [70]. 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 and colleagues 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 [71]. HE4 was approved by the FDA in 2009 as a serum marker for monitoring recurrence of ovarian cancer.

■ ROMA

In a subsequent study, Moore and colleagues demonstrated that, among a panel of known serum markers for ovarian cancer, HE4 displayed the highest sensitivity for detecting ovarian cancer, particularly in stage I disease [72]. Furthermore, the sensitivity of CA125 increased from 43.3 to 76.4% at a set specificity of 95% when combined with HE4, suggesting that combined HE4 and CA125 may be an accurate predictor of malignancy. The authors went on to investigate whether the dual combination of HE4 and CA125 could be applied to pelvic mass discrimination in a prospective multicenter double-blinded trial [73]. In this study, HE4 and CA125 were combined with menopausal status to create the predictive logistic regression model/algorithm known as ROMA. A total of 531 patients with conditions consisting of 352 benign tumors, 129 EOCs, 22 low malignant potential (LMP) tumors, six non-EOCs and 22 non-ovarian cancers were evaluated. It was found that ROMA could distinguish benign tumors from EOCs and LMP tumors with 88.7% sensitivity, 74.7% specificity, 60.1% PPV, and 93.9% negative predictive value (NPV). Although 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 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 [101]. The approved algorithm incorporates the serum levels of HE4 and CA125 with menopausal status to generate a score that indicates the likelihood of malignancy (**Box 1**). Thus far, ROMA has been approved only on the Abbott ARCHITECT CA125 assay (Abbott Diagnostics, Ltd, Australia) 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-off 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 with 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 [74]. Alone, the ROMA displayed higher sensitivity (81.3 vs 43.8%) and NPV (98.2 vs 95.7%) compared with the ICRA; but poorer specificity (74.5 vs 90.0%) and PPV (18.8 vs 24.1%). When combined, the ICRA and ROMA did not improve in any statistic compared with both algorithms alone. Following FDA approval, there have been numerous studies seeking to compare the efficacies of ROMA with other algorithms for the differential diagnosis of patients with a pelvic mass. The results

Box 1. Risk of Malignancy Algorithm.

▪ ROMA score considers:
  – Serum HE4 level
  – Serum CA125 level
  – Menopausal status
Premenopausal patients
▪ ROMA score ≥1.31: high likelihood of finding malignancy
▪ ROMA score <1.31: low likelihood of finding malignancy
Postmenopausal patients
▪ ROMA score ≥2.77: high likelihood of finding malignancy
▪ ROMA score <2.77: low likelihood of finding malignancy

ROMA: Risk of Malignancy Algorithm.

of several of these studies are summarized in TABLE 1. The PPV ranges from 27 to 89% and the NPV ranges from 81 to 99.6% in these studies [75–79]. 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 [79] and others have stated that ROMA does not outperform current modalities for pelvic mass discrimination such as sonography [77]. A recent study investigating the screening performance of known and putative ovarian cancer biomarkers found that CA125 displayed higher sensitivity than HE4 at a set specificity of 95% in Phase III specimens [80], while another study suggested that the combination of HE4 and CA125 is redundant in the clinic because HE4 is superior to CA125 overall due to its higher specificity and greater ability to detect borderline tumors and early-stage ovarian and tubal tumors [81]. Clearly, more multicenter studies are needed to truly assess the clinical utility of ROMA.

Table 1. Select studies of the Risk of Malignancy Algorithm for discrimination of pelvic masses.						
Study (year)	**Compared cohorts**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Ref.**
Partheen *et al.* (2011)	Benign vs premenopausal malignancy	81.0	75.0	60.7	90.7	[75]
	Benign vs postmenopausal malignancy	87.1	75.0	62.8	90.7	
Molina *et al.* (2011)	Benign vs premenopausal malignancy	74.1	88.9	44.4	87.4	[79]
	Benign vs postmenopausal malignancy	95.2	83.1	88.9	90.2	
Bandiera *et al.* (2011)	Benign vs premenopausal EOC	84.6	81.2	84.6	81.2	[78]
	Benign vs postmenopausal EOC	93.1	84.4	84.4	93.1	
Van Gorp *et al.* (2012)	Benign vs premenopausal malignancy	66.7	87.8	60.5	90.4	[77]
	Benign vs postmenopausal malignancy	91.0	58.8	74.3	83.3	
Moore *et al.* (2010)	Benign vs EOC and LMP	89.0	75	62.3	93.6	[76]
	Benign vs EOC	94.3	75	59.8	97.1	
	Benign vs stage I–II EOC	85.3	75	27.1	97.9	
	Benign vs stage III–IV EOC	98.8	75	52.1	99.6	
EOC: Epithelial ovarian carcinoma; LMP: Low malignant potential; NPV: Negative predictive value; PPV: Positive predictive value.						

■ OVA1

Unlike HE4 (discovered via genomic strategies), the OVA1 markers (CA125, β2M, TrF, TT and ApoA1) were identified through proteomic studies, with the exception of CA125. Using SELDI-TOF MS, Zhang and colleagues performed proteomic profiling on the serum of 503 women (153 invasive EOC, 42 other ovarian cancers, 166 benign pelvic masses and 142 healthy controls) [82]. After validation across multiple institutions, three proteins were identified as putative early-stage ovarian cancer biomarkers: ApoA1 (downregulated in cancer), a truncated form of TT (downregulated in cancer), and a cleavage fragment of inter-α-trypsin inhibitor heavy chain H4, ITIH4 (upregulated in cancer). A multivariate index combining these three biomarkers with CA125 displayed higher sensitivity than CA125 alone (74 versus 65%) at a set specificity of 97% and displayed higher specificity than CA125 alone (94 versus 52%) at a set sensitivity of 83%. Following this initial study, a multi-institutional follow-up study was conducted to analyze additional samples for a list of candidates with the highest potential to be early ovarian cancer detection biomarkers. The final list of seven candidates that showed the most promise were: ITIH4, TT, ApoA1, hepcidin (HEPC), TrF, connective-tissue activating protein 3 (CTAP3) and β2M [83]. Quantitative immunoassays only existed for β2M, 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., TX, USA), the values from each variable are combined and converted into an ovarian malignancy risk index score (**Box 2**). 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 [84].

The OVA1 test obtained clearance from the FDA in September 2009 as a supplementary test for clinical decision-making for preoperative adnexal mass patients [102]. It should be noted that the FDA cautions against the 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% [103,104]. According to the results of the trial, OVA1 improved presurgical assessments for both general physicians and gynecologic oncologists, as the sensitivity increased from 72.2 to 91.7% for general physicians, and from 77.5 to 98.9% for gynecologic oncologists. Following FDA approval, a trial with 516 women was performed to assess the benefits of OVA1 for clinical decision-making for general physicians and gynecologic oncologists [85,86]. It was reported that the overall sensitivity of OVA1 for EOCs was 99%, 82% for non-EOCs, 75% for borderline tumors and 94% for secondary metastases to the ovary. Additionally, OVA1 positively identified 76% of the malignancies that had been missed by CA125 and, for gynecologic oncologists, improved sensitivity from 78 to 98%, but decreased specificity from 75 to 26%.

However, recent studies investigating OVA1 and variations using different combinations of the markers identified by Zhang and colleagues have reported conflicting results [87]. The results of these select studies are summarized in **Table 2**. Moore and colleagues [88] reported that the addition of the seven biomarkers identified by Zhang and colleagues [83,87] to CA125 did not improve the sensitivity for preclinical diagnosis compared with 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 [89,90]. A limitation of OVA1 is that all the markers with the exception of CA125 are acute-phase reactants that may be nonspecific for ovarian cancer [82]. As seen in subsequent studies, there is much

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Box 2. The OVA1™ test.

- OVA1™ score considers:
  - Serum CA125 level
  - Serum β-2 microglobulin level
  - Serum transferrin level
  - Serum transthyretin level
  - Serum apolipoprotein A1 level

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

Table 2. Select studies of OVA1™ for discrimination of pelvic masses.

Study (year)	Compared cohorts	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Serum markers used	Ref.
Ware Miller *et al.* (2011)	Benign vs premenopausal malignancy	91.0	43.0	28.0	95.0	OVA1™ markers	[85]
	Benign vs postmenopausal malignancy	95.0	26.0	47.0	88.0		
Moore *et al.* (2012)	Prediagnostic asymptomatic women (diagnosed with cancer later) vs healthy women	35.6% (training set) 26.3% (test set)	95.0% (training set) 93.4% (test set)	–	–	All seven candidate markers with CA125	[88]
Hogdall *et al.* (2011)	Benign and borderline vs ovarian cancer	74.0	95.0	–	–	β2M, TT, TrF	[89]
Clarke *et al.* (2011)	Benign vs stage I/II EOC	88.2	95.4	–	–	ApoA1, TT, CTAP3 with CA125	[90]
	Benign vs stage I/II EOC	86.8	93.7	–	–	All seven candidate markers with CA125	

ApoA1: Apolipoprotein A1; β2M: β-2 microglobulin; CTAP3: Connective-tissue activating protein 3; EOC: Epithelial ovarian carcinoma; NPV: Negative predictive value; PPV: Positive predictive value; TrF: Transferrin; TT: Transthyretin.

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. It should also be mentioned that panels of biomarkers, which may only marginally improve the sensitivity and specificity of single markers, may not be cost-effective. As such, a cost–benefit analysis is necessary and should be performed before bringing a panel of biomarkers to the clinic.

Conclusion
In the past decade, there has been a wealth of information generated from high-throughput studies on ovarian cancer. The advances in genomic and proteomic technologies have presented us with new and exciting opportunities for the discovery of novel biomarkers, an aspect of ovarian cancer that the clinic is lacking. 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. However, despite these successes, very few biomarkers 'discovered' by these strategies have made it past validation studies and clinical trials. The reasons for this obstacle are not a lack of pathophysiological knowledge, powerful techniques or investment funds. The fact is that the difficulties associated with biomarker discovery have been underestimated. Unfortunately, the majority of discovery studies are often marked by deficiencies in both study design and statistical analyses, leading to misinterpretation of the results and exaggeration of positive findings [91]. In addition, patient selection bias and other preanalytical shortcomings along with poor analytical methodology contribute to the lack of novel biomarkers making their way into the clinic.

Future perspective
Although high-throughput technologies still represent a powerful and useful tool, it is imperative that studies are designed meticulously with careful consideration of the biases that may arise from the analytical and the clinical aspects of ovarian cancer biomarker discovery. 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, 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.

Executive summary

Ovarian cancer is the most lethal gynecological malignancy

- Ovarian cancer is often diagnosed when the disease has progressed considerably and the chances of survival are poor.
- The advent of high-throughput technologies has provided a new avenue for biomarker discovery to improve clinical management of ovarian cancer.

Genomics

- Ovarian cancer is a heterogeneous disease with numerous genetic abnormalities implicated in its pathogenesis.
- The Cancer Genome Atlas Research (TCGA) study has provided a comprehensive view of the scope of mutations present in high-grade serous ovarian carcinoma and will serve as a basis for future genetic biomarker studies.

Proteomics

- Proteomics-based biomarker discovery has been explored mainly through mass spectrometry.
- Initial studies of proteomic profiling as a diagnostic marker were promising, but subsequent examination of the results proved otherwise.
- Many proteins have been identified as potential novel biomarkers, but they have yet to pass validation.

US FDA-approved biomarkers

- HE4, identified through microarray studies, was recently approved for monitoring post-treatment ovarian cancer patients.
- The Risk of Malignancy Algorithm (ROMA), which uses the combination of serum CA125 and HE4, and menopausal status, was recently approved for pelvic mass discrimination, but more studies are required to resolve the conflicting findings.
- The OVA1™ test uses a combination of serum CA125 and the serum levels of proteins identified through mass spectrometry, and was recently approved for pelvic mass discrimination, but more validation studies are needed.

Conclusion

- High-throughput technologies are powerful tools for novel biomarker discovery in ovarian cancer, as demonstrated by the recent approval of HE4, ROMA and the OVA1™ tests.
- Careful planning in the study design and subsequent validation studies are critical to biomarker discovery and development and this remains the bottleneck for the majority of biomarker discovery studies.

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