Review

Advances in mass spectrometry-based technologies to direct personalized medicine in ovarian cancer

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\textbf{Article Info}

Article history:
Received 1 May 2013
Received in revised form 2 August 2013
Accepted 6 August 2013

Keywords:
Ovarian cancer
Personalized medicine
Mass spectrometry
Proteomics
Chemo-resistance

\textbf{Abstract}

Ovarian cancer is the most lethal gynaecological malignancy in North America and remains one of the most difficult cancers to manage. Although the 5-year survival rates are high when the disease is diagnosed early, this decreases exponentially in late-stage diagnoses and due to the current lack of screening methods, ovarian cancer is often diagnosed in its later stages when the cancer has progressed considerably. To exacerbate this, ovarian cancer patients almost always experience recurrence and resistance to chemotherapy after an initial positive response to treatment. Clearly, new modalities of clinical management are needed for this deadly disease. With the recent advent of high-throughput proteomic technologies, there have been numerous efforts to profile ovarian cancer using mass spectrometry to identify novel biomarkers for various clinical applications including diagnosis, prognosis, therapeutic targets, and monitoring therapeutic response. Identifying such novel biomarkers would allow for better tailoring of disease prevention and treatment on an individual basis in order to improve patient outcome. Unfortunately, traditional bottom-up proteomics

\textit{Abbreviations:} OvCa, ovarian cancer; BRCA 1/2, breast cancer susceptibility gene 1/2; MS, mass spectrometry; PI3K, phosphoinositide 3-kinase; CA125, carbohydrate antigen 125; HE4, human epididymal protein4; FDA, food and drug administration; WFDC2, whey acidic protein four-disulfide core domain protein 2; EOC, epithelial ovarian carcinoma; ROMA, Risk of Ovarian Malignancy Algorithm; LMP, low malignant potential; RMI, Risk of Malignancy Index; m/z, mass-to-charge; PTM, post-translational modification; MALDI, matrix-assisted laser desorption/ionization; ESI, electrospray ionization; BOT, benign ovarian tumour; UPLC, ultra performance liquid chromatography; Q-TOF, quadrupole time-of-flight; LC, liquid chromatography; CPG, 27-nor-5b-cholestane-3,7,12,24,25 pentol glucuronide; AUC, area under the curve; ROC, receiver operator characteristic; PRoBE, prospective-specimen-collection, retrospective-blinded evaluation; VEGF-A, vascular endothelial growth factor A; PFS, progression-free survival; PARP, poly(ADP-ribose) polymerase; 2-DE, 2-dimensional gel electrophoresis; ICAT, isotope-coded affinity tag; TTRAQ, isobaric tag for relative and absolute quantification.

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http://dx.doi.org/10.1016/j.trprot.2013.08.001
1. Introduction

Ovarian cancer (OvCa) is the most lethal of all gynaecological malignancies and is the 5th leading cause of mortality due to cancer in North American women [1]. Despite advances in medicine and technology, the survival rate of women diagnosed with OvCa has remained relatively unchanged over the past three decades [2–4]. Women diagnosed with early-stage OvCa have a 5-year survival rate of approximately 80–90% but this decreases dramatically to 20–30% in late-stage diagnoses [5]. Unfortunately, no reliable mode of screening currently exists for early detection of OvCa and the disease is often asymptomatic during its early stages. As a consequence, most women are diagnosed when the disease has progressed considerably.

In addition to early detection, the treatment and management of OvCa patients faces several challenges. In general, patients diagnosed with advanced disease are managed with surgical cytoreduction and chemotherapy. Although these therapeutic interventions are initially efficacious, patients often experience cancer recurrence, as a result of intrinsic or acquired chemoresistance by cancer stem cells or aberrant expression of oncogenes and tumour suppressor genes in tumour cells. OvCa is not a single disease but rather a heterogeneous group of tumours that can be classified into distinct molecular and histological subtypes, as they can derive from various ovarian and non-ovarian tissues and each display their own germline and somatic mutations. For instance, high-grade serous carcinomas arise from the ovary or fallopian tube and display a high frequency of p53 and BRCA1/2 mutations [6], whereas clear cell and endometrioid tumours have been linked to endometriosis and harbour PI3K mutations [7]. Moreover, mucinous ovarian carcinomas, which comprise the least common subtype, are considered to be secondary metastases to the ovary from other tumours, particularly those found in the gastrointestinal tract [8]. Due to the widespread heterogeneity among ovarian cancers, standard conventional therapies often elicit varying treatment responses within the various subclasses of tumours. For example, clear cell carcinomas often exhibit lower response rates in comparison to high-grade serous tumours following administration of platinum-based drugs [9]. For these reasons, the ability to make definitive subtype diagnoses in order to treat patients accordingly would be extremely useful. The notion of treating patients on such an individual basis, also known as personalized medicine, has thus become a much desired model of care for OvCa patients.

Personalized medicine is defined as the utilization of an individual’s biological profile to guide decisions in the prevention and clinical management of diseases. Within OvCa, it has become increasingly apparent that each subtype represents a distinct genetic and etiological disease that simply shares a common anatomical location. Thus, it is imperative to delineate the differences between each subtype as well as understand the molecular processes by which tumours acquire resistance in order to construct therapeutic interventions that could be tailored on an individual basis. Such

have not yielded any markers able to pass stringent clinical validation. As a result, many alternative strategies have recently emerged where mass spectrometry is employed in a complementary fashion to traditional shotgun proteomics. In this review, we will examine such complementary mass spectrometry-based biomarker discovery efforts with a focus on early diagnostic markers and markers of chemoresistance.

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approaches to personalized medicine has been the focus of the majority of OvCa studies as comprehensive characterization of the subtypes would greatly aid in the development of subtype-specific management, which in turn would greatly improve patient outcome.

With the recent advent of high-throughput technologies, numerous studies have been undertaken to profile the subtypes of OvCa using genomic, transcriptomic and proteomic approaches in order to identify subpopulations that could potentially benefit from personalized medicine. Specifically, proteomic profiling of OvCa has mainly revolved around the analysis of OvCa cell lines, tissues, and proximal fluids using mass spectrometry (MS). This has led to the identification of numerous altered protein expression patterns of the disease. The study of protein expression in OvCa has been increasingly important as proteins are the mediators of all biological processes and the molecular targets of the majority of drugs. Moreover, the proteome integrates the cellular genetic information and environmental influences. As such, MS has been increasingly implemented as this platform allows for the simultaneous examination of thousands of proteins in biospecimens relevant to OvCa, and has more recently been applied to identification of post-translational modifications and metabolic changes that occur during the disease. 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 deliver the expected goods (novel biomarkers that will have an impact on clinical management). As such, a number of alternative approaches to biomarker discovery have emerged utilizing the power of MS.

In this review, an overview of several different MS-based initiatives to uncover markers and signatures of OvCa will be discussed such as glycomics and metabolomics (Fig. 1). In the latter half of the review, various comparative proteomic studies that uncover mechanisms of chemoresistance – in particular, the efforts to find novel therapeutic targets or markers for the purposes of monitoring or predicting treatment response will be examined (Fig. 1).

2. Current status of OvCa diagnosis

Current modalities for detecting OvCa are primarily based on imaging and serological biomarkers. Women who are suspected to have a mass (of unknown origin) through physical pelvic examination will be subjected to transvaginal ultrasonography and a blood test for carbohydrate antigen-125 (CA125). Once the presence of a mass has been confirmed, its malignant potential must be determined through exploratory laparotomy and subsequent biopsies. Unfortunately, these techniques suffer from low specificity, are invasive and carry their own inherent risks; as such, there has been an increased focus on developing serum-based detection methods due to their efficiency and non-invasiveness.

Since its discovery in 1981 by Bast et al. [10], CA125 – also known as mucin 16 – still remains the best serum biomarker for the management of OvCa. It was identified through the development of a monoclonal antibody (OC125) that displayed reactivity with epithelial ovarian carcinoma (EOC) cell lines and tissues from OvCa 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. 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 [11]. As a result, CA125 displays poor specificity for OvCa as increased CA125 levels can be a result of other pathological states such as heart failure, peritoneal infection, pericarditis, and benign gynecological conditions [12–14]. For these reasons, CA125 is not approved for OvCa screening or for the detection of early disease.

Human epididymal protein 4 (HE4) is another serum OvCa biomarker that is becoming increasingly used in the clinic since its approval by the FDA in 2009. Also known by its gene name WFDC2 (whey acidic protein four-disulfide core domain protein 2), HE4 was initially identified as an mRNA transcript specific to the distal epididymal tissue [15]. 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 [16–19]. Furthermore, Drapkin et al. showed that HE4 is relatively specific to the serous subtype of epithelial ovarian carcinomas (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 [20]. 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. concluded that HE4 may be comparable to CA125 as a monitoring serum tumour marker as both displayed a sensitivity of 80% and a specificity of 95% when used to classify blinded late stage cases and healthy controls [21]. HE4 was approved by the FDA in 2009 as a serum marker for monitoring recurrence of ovarian cancer.

A final approach to OvCa diagnosis that is becoming increasingly prevalent is the use of multimarker panels derived from high-throughput discovery efforts. The rationale is that the use of multiple markers may provide a more accurate representation of whether or not disease is present especially when the disease (such as OvCa) is heterogeneous across different individuals. In a study by Yurkovetsky et al., it was determined that from a list of 56 potential OvCa serum biomarkers, a panel of CA125, HE4, carinoembryonic antigen, and vascular cell adhesion molecule 1 displayed a sensitivity of 86% for early-stage OvCa and 93% for late-stage OvCa at a set specificity of 98% when used to diagnose OvCa patients from healthy controls [22]. The authors were able to further validate this model on an independent blinded validation cohort while additionally showing that the panel was specific to OvCa as it displayed sensitivities of 33% for benign pelvic disease, 6% for breast cancer, 0% for colorectal cancer, and 36% for lung cancer.
Furthermore, two other multimarker-based algorithms have recently gained FDA-approval for the discrimination of benign versus malignant pelvic masses – the Risk of Ovarian Malignancy Algorithm (ROMA) and the OVA1™ test. 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 (beta-2 microglobulin, transferrin, transthyretin, apolipoprotein A1). The ROMA has been investigated in multiple studies that have confirmed its clinical utility. In one such study, Moore et al. combined serum HE4 and CA125 with menopausal status to create the predictive logistic regression model/algorith known as ROMA. A total of 531 patients consisting of 352 benign tumours, 129 EOCs, 22 low malignant potential (LMP) tumours, 6 non-EOCs and 22 non-ovarian cancers were evaluated. It was determined that ROMA could distinguish benign tumours from EOCs and LMP tumours with 89% sensitivity and 75% specificity. 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. In a subsequent study, the authors had confirmed the superiority of ROMA over the existing Risk of Malignancy Index (RMI) in identifying women who will develop EOC when they initially present with a pelvic mass of unknown malignant potential [23]. In this study, the ROMA had achieved a sensitivity of 94% compared to 85% for the RMI at a set specificity of 75% for discriminating benign pelvic masses from EOCs in a cohort of 457 pelvic mass patients. While the OVA1™ test showed promise during the clinical trial leading up to its approval by the FDA as a supplementary for clinical decision-making for preoperative adnexal mass patients, subsequent studies have reported conflicting results. Moore et al. [24] reported that the addition of the seven biomarkers identified by the inventors of the OVA1™ test 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 [25,26].

Despite the initial excitement over such multimarker panels, more multi-institutional studies are required before the true clinical applicability of these new tests/algorithms can be determined.

Consequently, there is now a renewed interest for the discovery of novel serum biomarkers, especially for those 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 proteomic experiments have been irreproducible and unable to pass independent, blinded validation experiments. This may be because upregulated proteins in the serum of OvCa patients are often acute phase reactants that are a reflection of the epiphenomena not specific to OvCa. 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 OvCa. Specifically, a screening test for OvCa needs to display a sensitivity of more than 75%, and a specificity of more than 99.6% to attain a PPV of 10% [27,28]. No biomarker has yet to achieve this level of performance.

3. Emerging MS approaches for the discovery of biomarkers to diagnose EOC

As stated previously, proteomic studies in OvCa 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 m/z (mass-to-charge) 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 [29]. Such MS-based discovery experiments – also known as shotgun proteomics – have represented the majority of OvCa biomarker studies. Since 2002,
over 100 studies have been published investigating the proteome of various biological samples relevant to OvCa for novel biomarkers including serum, proximal fluid, cell lines, and tumoral tissues. Unfortunately, very few of these putative markers have passed clinical validation due to inadequate sensitivity and specificity for OvCa. As a result, a number of strategies for OvCa biomarker discovery beyond classical MS-based proteomics have emerged in the past decade. In the following sections, we will examine some of these recent alternative approaches that are being increasingly adopted in the search for novel OvCa biomarkers.

### 3.1. Glycomics

Glycomics is the global study of proteins with carbohydrate post-translational 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 [30]. 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 (these will be discussed later), major advances in both pre-analytical separation methods and MS have allowed for increasingly comprehensive characterization of glycomes and cancer-specific glycoproteins [31,32]. With respect to OvCa, the majority of glycomic-based biomarker studies have employed the use of matrix-assisted laser desorption/ionization (MALDI) MS coupled with extensive pre-analytical enrichment methods for glycans (such as peptide-N-glycosidase digestion, chromatographic separation, and solid phase permethylation) [30].

In a study by Alley Jr. et al., the serum glycomes of 20 healthy control women and 30 OvCa patients were investigated with a specific focus on quantitative profiling of the asparagines-linked oligosaccharides (N-linked glycans) through MALDI MS [33]. Overall, it was observed that the OvCa glycomes had increased tri- and tetra-branched structure with variable sialylation and fucosylation. Further analysis of the immunoglobulin G-associated glycans revealed an increase in α-galactosylated structures in the OvCa glycomes and together, these glycan patterns could be used to distinguish the OvCa 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 another related study, Saldova et al. analyzed total serum N-linked glycans in the serum of healthy controls and patients with OvCa, benign gynaecological conditions and other gynaecological cancers using MALDI MS and electrospray ionization (ESI) MS [34]. From these analyses, it was reported that the OvCa glycome had an increased expression of core fucosylated, α-galactosyl biantennary glycans and sialyl Lewis x. As well, the authors identified altered glycosylation patterns on acute-phase proteins such as haptoglobin, α1-acid glycoprotein, α1-antichymotrypsin and IgG.

Li et al. had also utilized MALDI MS to characterize glycome of serum derived from OvCa patients and healthy controls [35]. In the subsequent analyses, four glycoproteins of 517, 370, 250 and 163 kilodalton corresponding to two forms of apolipoprotein B-199, fibronectin and immunoglobulin A1, respectively, were identified as upregulated in the serum of OvCa patients compared to controls. The glycans subsequently isolated from these parent proteins consisted of O- and N-linked glycans that were distinguishable from the corresponding glycans present in the serum of healthy controls.

Despite the wealth of information that has been accumulated, glycomic-based biomarkers have yet to pass any clinical validation in OvCa. As mentioned previously, 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 OvCa, it is unclear whether such changes are truly OvCa-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 OvCa 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 [31]. It has been suggested that cancer-driven glycosylation aberrations may be traced to the isomeric level where differential expression of certain isomers are indicative of the presence of specific malignancies. Thus, further investigation into resolution of glycomics-profiling by isomers may reveal critical information. 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 [36]. Therefore, development of a robust, quantitative method for glycan-based biomarkers is urgently needed in order to validate candidates that arise from discovery studies.

### 3.2. 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 [37–40].
With respect to OvCa, metabolomics-based biomarker discovery efforts have focused primarily on patient serum/plasma and urine samples. In three independent studies, metabolic profiling of urine from OvCa patients using mass spectrometry were able to identify numerous metabolites with the ability to discriminate between healthy controls and OvCa patients. Zhang et al. were able to identify 22 metabolites that were able to discriminate between EOC (n = 40) from benign ovarian tumours (BOT; n = 62) and healthy controls (n = 54) through ultra-performance liquid chromatography (UPLC) quadrupole time-of-flight (Q-TOF) MS analysis of urine samples from the said cohorts [41]. Nine of these metabolites (imidazol-5-yl-pyruvate, N4-acetylcytidine, pseudouridine, succinic acid, (S)-reticuline, N-acetylenuraminic acid, 3-sialyl-N-acetyllactosamine, β-nicotinamide mononucleotide, and 3′-sialyllactose) 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., metabolic analysis of OvCa urine through hydrophilic interaction chromatography and reversed-phase liquid chromatography MS identified five metabolites (two of unknown identities, pseudouridine, fragment of pseudouridine, and phytophosphosine) that were specific to OvCa patients and were significantly upregulated compared to healthy controls and BOT patients [42]. Woo et al. identified three putative urinary metabolite-based biomarkers for OvCa (1-methyladenosine, 3-methyluridine, and 4-androstene-3,17-dione) through liquid chromatography (LC) MS analysis [43]. The authors noted that the putative metabolic markers were also highly involved in oxidative DNA damage and DNA methylation processes and thus, metabolomic approaches are efficient in characterizing metabolic networks present in malignant stages in addition to identifying diagnostic markers.

Similarly, serum/plasma metabolomic studies have revealed potential diagnostic markers for OvCa. In three separate studies, UPLC/MS coupled with partial least-squares discriminant analysis was employed to identify metabolic differences between OvCa patients and controls. Chen et al. identified 27-nor-5β-cholestan-3,7,12,24,25 pentol glucuronide (C5G) as a metabolic biomarker to discriminate EOC from BOT [44]. In a subsequent validation cohort, serum C5G displayed an area under the curve (AUC) of 0.750 in receiver operator characteristic (ROC) curve analysis for stage I cancer with a sensitivity and specificity of 70% and 77%, respectively. Through employing UPLC MS, Fan et al. identified eight candidate biomarkers (demethylphyloquinone, ganglioside, lysophospholipids, ceramides, phytophosphosine, ceramides, N′-formylkynurenine) 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 [45]. Zhang et al. also identified six candidate biomarkers (2 of unknown identity, 2-piperidinone, L-tryptophan, LysoPC(18:3), and LysoPC(14:0)) for distinguishing EOC from BOT [46]. In subsequent independent validation, the combination of the 6 metabolites yielded a comparable AUC (0.840) to that of CA125 (0.875) overall, but a greater AUC among premenopausal patients (0.780 and 0.692 respectively).

Urinary and serum metabolomics remains a promising avenue for OvCa 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 OvCa. Unfortunately, MS-based metabolomics still faces major limitations preventing its introduction into the clinic for OvCa 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) [47]. 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 metabolic 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 inter-study reproducibility.

3.3. 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 OvCa is one such technology. The low-molecular-weight proteome of both blood and ascites fluid are 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 OvCa diagnostics. Fredolini et al. reported approximately 51 serum peptidomic markers that were unique to OvCa patients compared to patients with BOT [48]. On the contrary, Timms et al. recently reported that MALDI MS peptide profiles were unable to accurately diagnosis OvCa from healthy controls, though the endogenous peptides could provide some diagnostic insight [49]. However, it has been noted that a limitation of peptidomic-based approaches is that discriminating peptides bound to carrier proteins (such as albumin) may be lost during offline sample processing. To this end, there exists some studies that have attempted to mitigate this through enriching for and/or isolating serum carrier proteins prior to mass spectrometric analysis to identify novel peptide-based OvCa biomarkers. In one such study by Lowenthal et al., albumin from pooled sera of OvCa patients and non-cancer controls were isolated and subjected to gel electrophoretic separation to extract the bound proteins and peptides [50]. Subsequent reversed-phase MS/MS analysis of the albumin-bound proteins and peptides revealed over 700 peptides and
predicted proteins that have not been previously reported in serum databases. Furthermore, proteolytic fragments of the cancer-related protein BRCA2 were identified and verified through Western blotting and peptide immunocompetition. In a related study, Lopez et al. utilized affinity chromatography coupled with MALDI MS to decipher the carrier-protein bound peptidome [51]. From this, the authors were able to identify a 9 peptide-based biomarker panel that could discriminate stage I OvCa patients from unaffected healthy controls with a sensitivity of 93% and specificity of 97%. Unfortunately, both of these studies were mainly discovery efforts to establish a reliable and reproducible workflow for the analysis of carrier protein-bound peptides and have yet to validate their putative OvCa markers in independent cohorts.

3.4 Autoantibody signatures

The identification of autoantibody signatures in serum has also been investigated for OvCa biomarker discovery. OvCa is often characterized by the complex network of inflammatory cytokines present in the microenvironment and the involve-ment of immune-related cells such as tumour-associated macrophages. As such, populations of anti-tumour 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 OvCa, Barua et al. identified 11 proteins as immunoreactive ovarian antigens through LC MS [52]. Although this was the first study to identify immunoreactive ovarian antigens by serum anti-tumour antibodies, the authors recognized the fact that the ovarian antigens could not discriminate laying hens with nonmalignant ovarian conditions from those with OvCa. Philip et al. investigated the immunoproteome of OvCa and healthy control sera, as well as that of the conditioned media of the OVCAR3 and SKOV3-A2 cell lines [53]. Overall, 8 autoantibody-reactive autoantigens were identified that were present in all five cancer serum composites and in both cell lines: A-kinase anchor protein 9, eukaryotic translation initiation factor 4, midasin, RAD50, talin 1, vinculin, vimentin, and centrosome-associated protein 350. Furthermore, the authors identified a subset of the MS-generated autoantigens that were implicated in both humoral (B-cell) and cell-mediated (T-cell) immunity. However, the suggested novel autoantibody biomarkers for OvCa 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.

3.5 MALDI-MS imaging

A final approach that has been gaining popularity is MALDI MS imaging of cancer tissues to identify markers that may be shed into the extracellular space. In this technique, tissues are directly subjected to ionization and mass analysis to generate an array of mass spectra for all positions across the tissue specimen. As a result, the protein content of specific regions of interest can be determined, as well as the spatial distribution of specific proteins across the tissue [54]. El Ayed et al. was able to identify the reg-alpha fragment of the 11S proteasome activator complex as a putative biomarker through correlative analyses between MALDI MS imaging and immunohistochemical analysis with an anti-reg-alpha C-terminal antibody [55]. Expression of this protein was validated using Western blot and PCR on the SKOV3 OvCa cell line. However, the authors did not validate overexpression of the marker in clinical samples. Liu et al. were able to extend the approach of MALDI-MS imaging by combining it with lipodemic analysis via MS and transcriptomic analysis via laser capture microdissection to demonstrate that not only are sulfatides upregulated in OvCa, but that their expression is also localized to the carcinoma regions as opposed to surrounding stromal and normal ovarian tissue [56]. Although the authors were able to correlate proteomic data with other high-throughput technologies, the data remains preliminary and discovery-based. Further investigation into specific sulfatides and validation in clinical samples is needed to decipher their true clinical utility for OvCa diagnosis.

Overall, huge advances have been made in the past decade in terms of innovative uses of MS. No longer are biomarker discovery studies focused on only proteomic profiling, but are now investigating downstream molecules on a global scale as markers of OvCa. This paradigm shift represents the changing perspectives on OvCa pathophysiology in that it is no longer a genetic disease, but a complex network of proteins, extracellular interactions and inflammation that leads to malignancy. Despite the advances in technology and throughput, however, many OvCa biomarker discovery studies continue to fail to produce markers that can truly pass clinical validation across multiple independent cohorts and this has been attributed to poor study design and biases. As a result, there have been efforts to implement more stringent and standardized protocols for biomarker evaluations to alleviate these issues. In 2008, Pepe et al. described a variation of a nested case–control study for the purposes of biomarker evaluation (for example between subjects with OvCa and subjects without OvCa) termed prospective-specimen-collection, retrospective-blinded evaluation (PROBE) which has begun to gain prominence in recent biomarker studies [57]. A recent study by Lee et al. in 2011 investigating the ability of a panel of 7 biomarkers in addition to CA125 to diagnose preclinical OvCa also represents the importance of robust study design to truly assess novel OvCa biomarkers. As opposed to previous studies that had reported successful validation of the addition of the 7-biomarker panel to CA125, Lee et al. were able to confirm that the biomarker panel did not in fact improve preclinical OvCa diagnosis compared to CA125 alone. The authors were able to attribute this to the fact that earlier studies were incorrectly using postdiagnostically collected sera as opposed to truly prediagnostic sera. Despite the wealth of advances in MS-based biomarker discovery efforts for OvCa, it is clear that the majority of such approaches still face many biological and technical challenges that must be addressed before this new generation of biomarkers can be introduced into the clinic.

4 Current approaches in the treatment of EOC

As efforts to improve the early detection of OvCa remain ongoing, numerous research initiatives are also focused on
expanding existing treatment strategies, through the incorporation of newly developed targeted therapies into the clinic. In the following section, we will briefly highlight current strategies used in the treatment of OvCa, as well as underline recent advances made towards the use of molecular targeted therapies in OvCa patient care.

Unlike other solid cancers, the treatment of OvCa has progressed very little over the past few decades, as the first-line treatment for advanced-stage patients continues to be a combination of surgical debulking with platinum-based chemotherapy (carboplatin or cisplatin) [58]. Although treatment can prolong survival, many patients are left with residual disease, and ultimately face cancer recurrence. Moreover, another limitation of standard chemotherapy is the development of drug resistance, as most patients become unresponsive to additional rounds of chemotherapy. As such, the urgent need to identify therapeutic targets that can overcome chemoresistance has led to strategies that target the tumour microenvironment, specifically angiogenesis, as well as therapies targeting molecular pathways that are frequently expressed in OvCa tumours [59,60].

Targeting the tumour microenvironment through the abrogation of angiogenesis mechanisms has proven to be an effective strategy for advanced OvCa. The importance of new blood vessel formation via increased production of vascular endothelial growth factor A (VEGF-A) in the growth and metastasis of OvCa tumours has led to a series of clinical trials evaluating the efficacy of the VEGF-A inhibitor, bevacizumab, along with conventional chemotherapeutic agents [61,62]. Two phase III clinical trials have shown that administration of bevacizumab during and after carboplatin and paclitaxel treatment can prolong progression-free survival (PFS) in patients with advanced OvCa and for those with high risk of disease progression [61,62]. However, slight decreases in the quality of life of patients were reported with continual bevacizumab treatment [63]. Based on the results of these trials, the use of bevacizumab in combination with standard chemotherapy has been approved in Europe. Moreover, similar increases in PFS were also observed when bevacizumab was administered during and after chemotherapeutic treatment in platinum-sensitive recurrent OvCa [64]. These findings suggest that it may also be useful in the treatment of platinum-sensitive relapsed patients, however, further evaluation is needed to elucidate the appropriate use of bevacizumab in the management of OvCa.

In addition to anti-angiogenic therapies, other promising targeted therapies include those that disrupt aberrant signalling pathways that become activated in OvCa tumours. These include inhibitors against PI3K/AKT/mTOR and Ras/Raf/MEK/ERK pathways, which have higher mutation rates in clear cell/endometrioid and low-grade serous ovarian tumours/mucinous, respectively [60,65]. As a result, these agents provide an attractive option for the treatment of rarer OvCa subtypes, as they typically show no response to standard chemotherapies. Randomized controlled trials are needed to assess the clinical utility of these drugs, as well as their potential to treat patients that have developed resistance to platinum- and taxane-induced cytotoxicity.

Lastly, disrupting DNA repair machinery using Poly(ADP-ribose) polymerase (PARP) inhibitors is a promising strategy for treating OvCa patients harbouring BRCA1 or BRCA2 mutations [60]. As BRCA1/2 proteins are essential to the homologous recombination repair pathway, preventing single-stranded DNA break repair with PARP inhibitors will lead to an accumulation of double-stranded breaks, which will induce apoptosis in BRCA-deficient tumour cells [65]. Whether these inhibitors will have more effectiveness as a single agent or in combination with therapies still requires further investigation, as this may depend on the histological and molecular tumour subtype of the patient.

Overall, it is evident that the future of OvCa treatment and management will involve a combinatorial approach, as conventional therapies will be used in combination with newly developed agents. Further investigation on the appropriate administration of the above therapies will be a focus of upcoming efforts, as ongoing clinical trials will assess the clinical utility of these drugs as well as determine which patients will benefit the most from each therapeutic agent.

5. Comparative proteomics to identify proteins associated with chemoresistance

Despite the major emphasis placed on the search for early detection biomarkers through proteomic profiling and other alternative biomarker discovery efforts, these studies do not allow for the identification of markers that could guide treatment nor predict its response in patients. As such, attempts have been made towards uncovering proteomic changes that occur as a result of chemoresistance. These include profiling chemosensitive and resistant cancer cell lines and tissues, as a starting point in understanding the molecular basis of resistance to chemotherapeutic agents, which will ultimately lead to the identification of markers for treatment response as well as the discovery of novel therapeutic targets. In the following sections, we will describe a few of the emerging cell line-based proteomic strategies, including quantitative proteomics, glycoproteomics, and organellar proteomics to study chemoresistance. In addition, the use of tissue proteomics to complement the above strategies will be discussed.

5.1. Cell line-based proteomics

EOC cell lines provide a valuable biological source for conducting high-throughput proteomics because of their easy manipulation and the ability to mine the proteome in depth. Using the human OvCa cell line, A2780, which was derived from an untreated patient, numerous studies have generated its platinum- and taxane-resistant derivatives in order to compare proteomic changes between the two conditions, or to an inherently resistant cancer cell line, OVCA3 [66–70]. Findings from these studies revealed that protein expression changes occurred in pathways regulating stress response, apoptosis, metabolism, cell cycle, and protein biosynthesis [67,68]. For instance, Ciccihlittli et al. identified disulphide isomerase Erp57 as a novel paclitaxel-resistant marker that forms a complex with TUBB3, and directs microtubule attachment to chromosomes, which is interesting given that paclitaxel targets tubulin [68]. Further studies should examine the effects of Erp57 knockdown on decreasing resistance to paclitaxel.
in other OvCa cell lines, as well as evaluate the potential of ERp57 to be used a marker to monitor therapy and patient outcome. Similar studies incorporated 2-dimensional gel electrophoresis (2-DE) coupled to ESI Q-TOF tandem MS/MS or MALDI-TOF MS in the analysis of A2780 and SKOV3 platinum and taxane-sensitive and -resistant cell lines, and identified numerous potential markers of resistant OvCAs for personalized cancer therapy [69–71]. However, additional evaluation of these proteins in large clinical validation studies is required to elucidate their potential as predictive markers of chemoresistance. Further examination on the role of these proteins in the development of platinum resistance using knockout mouse models will determine their value as potential therapeutic targets. Other cell line model systems of chemoresistance, such as IGROV1 (sensitive) and IGROV1-R10 (resistant) cells have also been employed in the quest to find altered proteomic signatures of resistance, which have been followed up with a kinetic analysis [72,73]. Through this analysis, Le Moguen et al. identified time and concentration-dependent changes in protein levels associated with pathways linked to stress, oxidative stress response, glycolysis, and cell communication [73]. Overall, these initial studies have unravelled potential molecular pathways that become disrupted during chemoresistance. Using this knowledge, specific experiments may be conducted to elucidate the mechanisms underlying resistance, as the above approaches only provided a global snapshot of platinum-resistance associated proteins.

5.1.1 Quantitative proteomics

The studies highlighted above employed a qualitative approach to identifying markers of chemoresistance. In order to achieve more accurate protein quantification between different conditions, a few studies have applied labelling techniques as a means to quantify protein expression changes. For instance, isotope labelling via isotope-coded affinity tag (ICAT) and isotopic tag for relative and absolute quantification (iTRAQ) has also been incorporated into comparative proteomic studies as it allows for easy quantification of proteins between different conditions, which is often completed in fewer MS runs compared to non-labelling approaches. In particular, Shetty et al. utilized iTRAQ labelling to identify drug resistant tumour antigens in SKOV3-A2 (cisplatin-resistant), SKOV3-A2-CF (cisplatin-treated), and OVCAR3 (cisplatin-sensitive) OvCa cell lines, which could be used for immunotherapeutic targeting with antigen-specific vaccines [74]. Overall, the authors found that cisplatin treatment of platinum-resistant OvCa cells increased MHC Class I presentation of peptides derived from various proteins implicated in cancer [74]. In another study, iTRAQ was used to quantify protein expression between the cisplatin-sensitive cell line, COC1, and its resistant subline, COC1/DDP, which revealed decreased and increased levels of two proteins, PKM2 and HSPD1, respectively, in resistant cancer cells [75]. Subsequent functional knockdown of PKM2 and HSPD1 revealed that these proteins play a role in cell viability, and therefore, may serve as potential therapeutic targets [75]. Moreover, Stewart et al. used another form of isotope labelling, ICAT, to compare the proteome of sensitive and resistant IGROV-1 cancer cells, in which differentially expressed proteins were then correlated with mRNA expression; however, due to suggested post-transcriptional mechanisms, the majority of candidates did not display the same changes in expression at both the protein and mRNA levels [76].

5.1.2 Glycoproteomics

Besides looking at total protein expression as a whole, another approach to studying chemoresistance involves the study of glycoproteomics. During cancer progression, protein PTMs, particularly glycosylation, display altered expression patterns, which may contribute to the malignancy of the disease as discussed previously. Glycan structures may also contribute to various biological processes that promote tumorigenesis and encourage metastatic behaviour. Therefore, analyzing alterations of glycan structures has been a viable method for the discovery of markers related to chemoresistance. Enrichment and characterization of the glycoproteome from A2780-sensitive and -resistant cell lines has also led to the identification of a few glycoproteins, including CD70, tumour rejection antigen (gp96) 1, triose phosphate isomerase, palmitoyl-protein, thiouesterase 1 precursor and ER-associated DNAJ, which represent putative markers of chemotherapy resistance [66,77]. Interestingly, the majority of proteins identified through glycoprotein enrichment were not uncovered in proteomic analyses of the entire proteome, which underlines its advantage in discovering low-abundant markers of drug resistance [77]. Subsequent validation of these findings in clinically annotated patient tumour samples may lead to the incorporation of these markers into the clinic, which will be important before analyzing these markers as therapeutic targets.

5.1.3 Organellar proteomics

Proteomic technologies have also been applied to characterize the proteomes of subcellular organelles, which is useful for gaining insight into their biological function during various diseased states. It has been recognized that the ability of malignant cells to evade apoptosis may play a major role in the resistance of tumour cells to chemotherapeutic agents. Since mitochondria control the intrinsic apoptotic pathway, a few studies have examined the differential protein expression in the mitochondrial of platinum-resistant OvCa cell lines [78,79]. Using an organellar proteomic approach, Chappell et al. used label-free proteomics to quantify differences in protein expression between cisplatin-sensitive (A2780) and resistant (A2780-CP20) OvCa cell lines, which resulted in elevated expression of ALCAM and AKAP12, and decreased expression of Nestin [78]. In a comparable study, a 2-DE proteomic analysis revealed a decreased expression of prohibitin in platinum-resistant cell lines, which was confirmed in tissues from patients who were resistant to chemotherapy [79]. Taken together, these findings highlight the use of proteomic applications towards the understanding of mitochondrial dysfunction in platinum-resistant OvCa.

5.1.4 Limitations of cell line-based approaches

In general, the aforementioned studies have resulted in an indispensable amount of information regarding molecular mechanisms implicated in chemoresistance, and have provided numerous potential markers that may serve as indicators of drug response. However, several limitations of these
studies prevent the incorporation of these markers into the clinic. For instance, the majority of these studies were conducted on one or two OvCa cell lines, which surely do not capture the heterogeneity of this disease [80]. Since in vitro findings do not always translate to what is observed in vivo, all of these markers need to be confirmed using human samples, such as tissues, serum, and proximal fluids. Another limitation of using in vitro cell lines is that it is not representative of the tumour-host interactions that occur in the cancer microenvironment [80]. Future studies should focus on more targeted approaches that measure specific protein levels in clinically well-defined samples. For example, Kim et al. used selective reaction monitoring-based quantification to measure the levels of a SOD1, which has been shown to prevent chemotherapeutic-induced apoptosis in OvCa cells [81]. As such, this method will be useful for subsequent studies that aim to validate or verify these proteins in various biological samples. Lastly, the results from these studies suggest that numerous proteomic alterations occur during drug resistance. Future studies may benefit by combining these findings to delineate common pathways dysregulated in chemoresistant cells. Targeting molecular pathways may be a more practical approach to treating resistant tumours, and thereby, providing a more effective way for tailoring personalized patient care.

5.2. Tissue proteomics

Biases present in cell line-based models have emphasized the importance of using biological samples that recapitulate the disease, and thus, have led to tissue proteomics as another alternative to understanding chemoresistance. Thus far, a few approaches have been carried out to characterize differential protein expression between primary and recurrent OvCa tissues [82–85]. For example, using quantitative proteomics via ICAT, Pan et al. compared the expression between a chemosensitive and a chemoresistant tissue harvested at primary debulking surgery prior to chemotherapy to identify which proteins are responsive to it [82]. These differential expressions were then correlated with gene expression profiles of similar tissues, which revealed that proteins related to cell junctions and the extracellular matrix, become altered during chemotherapy [82]. Another study used paired primary and recurrent post-chemotherapy samples from high-grade serous OvCa patients to identify numerous proteins elevated in recurrent tissues, which were also confirmed by gene expression analysis [83]. Subsequent knockdown of these proteins in carboplatin-resistant cell lines using short hairpin RNA, identified RELA, the p65 subunit of NF-κB, and STAT5, as modulators of drug resistance [83]. As a result, inhibition of both proteins reduced the chemoresistance potential of cancer cell lines, and therefore, may represent a novel treatment for recurrent OvCa platinum-resistant patients [83]. Interestingly, both studies used an integrated approach to find chemoresistant markers, as they employed gene expression profiling to validate their proteomic discovery data. Perhaps, future efforts may benefit from integrating data obtained from genomic, transcriptomic, and proteomic approaches as means to understanding the molecular basis of chemoresistance. Moreover, Kim et al. used the differential protein expression profiles of chemosensitive and chemoresistant tissues obtained from 2-DE to construct a two marker panel, SGEF and keratin 1, to serve as predictive markers for chemoresistant disease with a sensitivity and specificity of 80% and 92% respectively [84]; however, although promising, these markers require further validation in larger sample cohorts.

Lastly, rather than focusing on individual proteins, biological signalling pathways could also be used as targets for overcoming chemoresistance. A recent study investigated the expression of proteins from molecular pathways associated with OvCa progression [85]. Using reverse phase protein arrays and normalized CA125 values, numerous proteins from the TGF-β pathway were implicated in playing a role in chemoresistance in high-grade serous OvCa [85].

Overall, the importance of using biological tissues for discovery is evident through the various studies that implicate different biological pathways in drug resistance. Given that none or very few protein expression changes are common between the different studies, we have to question whether tissue proteomics is a viable route for investigating chemoresistance. Alternatively, the lack of consistent results may be due to the heterogeneity of the disease as well as patient-to-patient variability. In addition, biases from the methodologies used, including pre-analytical and post-analytical variables, may also have an effect on the variability and reproducibility between studies. Since OvCa is a complex disease, future studies need to be conducted to limit biases by having a standard protocol for tissue collection, using paired primary and recurrent tissue from the same patient, as well as taking into account the subtypes of the disease.

6. Future perspectives

Overall, it is evident that proteomic and MS-based technologies have yielded an indispensable amount of information, which has been useful for the understanding of proteomic alterations that occur during OvCa pathogenesis. In terms of diagnostics, the use of shotgun proteomics has been relatively disappointing due to the wealth of novel markers “identified”, yet few have passed clinical validation. The lack of markers has thus necessitated this surge of innovative MS-based biomarker discovery techniques such as glycomics and metabolomics. Whether or not these techniques will identify the elusive novel biomarker(s) for OvCa remains to be seen as the majority of the approaches, however promising, are still in their infancy and there still exists many technical limitations that have yet to be overcome. On the other hand, proteomic studies aimed at identifying markers of therapeutic response are only beginning to emerge. Although several mechanisms of chemoresistance and potential markers of drug response have been unravelled, these studies are also subjected to their own biases and limitations. Future efforts should focus on using biologically relevant samples that capture the heterogeneity of the disease, as well validating findings in independent sample cohorts.
REFERENCES

[35] Li B, An HJ, Kirmiz C, Lebrilla CB, Lam KS, Miyamoto S. Glycoproteomic analyses of ovarian cancer cell lines and sera from ovarian cancer patients show distinct


