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## Recent developments and applications of quantitative proteomics strategies for high-throughput biomolecular analyses in cancer research

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Innovations in medical technology and dedicated focus from the scientific community have inspired numerous treatment strategies for benign and invasive cancers. While these improvements often lend themselves to more positive prognoses and greater patient longevity, means for early detection and severity stratification have failed to keep pace. Detection and validation of cancer-specific biomarkers hinges on the ability to identify subtype-specific phenotypic and proteomic alterations and the systematic screening of diverse patient groups. For this reason, clinical and scientific research settings rely on high throughput and high sensitivity mass spectrometry methods to discover and quantify unique molecular perturbations in cancer patients. Discussed within is an overview of quantitative proteomics strategies and a summary of recent applications that enable revealing potential biomarkers and treatment targets in prostate, ovarian, breast, and pancreatic cancer in a high throughput manner.

### Introduction

Mass spectrometry (MS) represents a unique and powerful technological platform in investigative biomolecular research. This high sensitivity regime grants access to the discovery and identification of small molecules,<sup>1–3</sup> endogenous peptides,<sup>4–7</sup> proteins,<sup>8–10</sup> and macromolecular complexes.<sup>11–13</sup> The utility of

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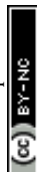
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*University of Wisconsin-Madison, where he continues to pursue novel approaches for glycoconjugate separation, identification, and structural characterization.*



MS is enhanced through the facility of ionizing biomolecule species in solution *via* electrospray ionization<sup>14</sup> (ESI) and matrix-assisted laser desorption/ionization<sup>15–17</sup> (MALDI) that provides a means of producing ions from stationary supports and tissue sections. As well, numerous mass analyzers<sup>18</sup> have been developed to accommodate high-speed and high-resolution measurements. Realizing the full potential and flexibility of modern MS platforms, as well as their ability to decipher complex biological samples, focus has shifted

towards improving instrument efficiency and sample throughput.

Gradual improvements in instrument operational speed, the advent of novel dissociation techniques<sup>19–22</sup> and implementation of multidimensional ion separation regimes<sup>23–25</sup> enable researchers to obtain greater levels of detail from complex mixtures than ever before. However, while shotgun proteomics provides a means for deep proteomic profiling, the typical time course and complexity of a single experiment<sup>26</sup> renders repetitive measurements of numerous samples untenable. For this reason, many have turned to multiplexed quantitative proteomics workflows to provide simultaneous deep proteomic profiling of numerous samples while retaining the ability to assign relative and absolute abundance information.

Quantitative proteomics, now comprised of several distinct strategies, operates under the principle that signal response from any given analyte is related to its abundance within the mixture. As such, should an analyte be identified in numerous samples, the relative intensity of the analyte's signal response in each sample can be used to provide a means of relative or absolute quantitation. However, MS reporting signal is divided into numerous channels depending on the quantity and ionization efficiency of all present biomolecules, which indicates the high variability that can arise from even discrete sample changes. In remedy of this ailment—and to remove run-to-run variation—researchers have employed unique chemical modifiers that often incorporate stable isotopes to label biomolecules within solution. These labels result in a unique mass shift for each sample without altering their retention time in liquid-chromatography. These newly tagged analytes may then be combined, measured simultaneously *via* MS, and then evaluated for the relative abundance of all labeled channels.

These quantitative strategies have provided unique avenues towards the discovery and validation of cancer-specific biomarkers. The ability to analyze numerous samples simultaneously provides researchers not only with a means for high throughput sample profiling, but also a means to uncover what proteomic perturbations are relevant across patients, between control groups, and specific to disease severity and progression. These perturbations and quantitative differences are often discussed in language that is familiar to proteomic researchers but that may create confusion in those coming from adjacent fields of research. Within proteomics, and ubiquitous throughout this review, quantitative differences of proteins, peptides and other biomolecules are described as “up-regulated” or “down-regulated.” These terms are used to describe those species with quantifiable differences against the control, often with statistical significance. Though readers may conjecture that up- or down-regulated protein species are the result of pathway regulation, these hypotheses are often not explored in proteomic literature and may be discussed elsewhere. For this reason, it is important to clarify that differences in regulation are meant only to indicate the quantitative findings presented by the original authors. Regardless of verbiage technicalities, researchers often pursue quantitative proteomics as a facile avenue towards novel biological insight.



Lingjun Li

*Dr Lingjun Li is a Vilas Distinguished Achievement Professor and the Charles Melbourne Johnson Distinguished Chair Professor of Pharmaceutical Sciences and Chemistry at the University of Wisconsin-Madison (UW-Madison). Dr Li received her PhD degree in Analytical Chemistry/Biomolecular Chemistry from the University of Illinois at Urbana-Champaign in 2000. She then did joint postdoctoral research at the*

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Given the significant heterogeneity associated with various cancer subtypes, researchers have sought to employ quantitative proteomics to a litany of biological questions. As seen within, these endeavors have provided seminal insights into the role post-translational modifications play in cancer progression, uncovering up- and down-regulation of biomolecules in disease groups, as well as the efficacy of using protein expression to monitor medical treatment. The true breadth of proteomic cancer research cannot be understated. While quantitative experiments date back several decades, we aim to present a mass spectrometry-centric review. High-throughput quantitative proteomics firmly gained prominence in the early 2000s, providing nearly twenty years of meaningful contributions to cancer detection, identification, and understanding. To provide readers with the most timely and topical review—as well as to provide discussion on future research interests, we have confined our literature review to applications published within the past 5 years. This concise range enables us to provide critical suggestions for researchers seeking to begin or continue their unique cancer research. Here we present a brief introduction to quantitative proteomics methods and recent investigations of prostate, ovarian, breast, and pancreatic cancer.

## Quantitative strategies

Quantitative proteomics has experienced substantial growth over the last two decades, due in large part to the invention and development of high-speed, high-resolution mass spectrometry instrumentation. While there are numerous unique and technically driven means to pursue relative and absolute protein quantitation, most applications fall within one of four major categories: metabolic labeling, isotopic labeling, isobaric labeling, and label-free quantitation. Each method has been thoroughly reviewed and in-depth discussion can be found elsewhere. However, in order to provide rationale behind each strategy for use in investigative cancer research, understanding the principles and key considerations of each is imperative.

### Metabolic labeling

Metabolic labeling is the earliest<sup>27,28</sup> and arguably most traditional method of mass spectrometry-based quantitative proteomics. Taking after the classical Meselson-Stahl<sup>29</sup> experiment that proved the semiconservative nature of DNA replication, more routine use of mass spectrometry for peptide identification revealed that proteins, too, could be metabolically labeled with stable isotopes to provide 'heavy' and 'light' isotopologues. Within these experiments, adjacent cell cultures are provided with either unlabeled, naturally occurring amino acids or amino acids that have been labeled with stable isotopes; this also lends itself to the acronym SILAC, Stable Isotopic Labeling with Amino Acids in Cell Culture.<sup>27,30</sup> Though SILAC has grown to incorporate numerous stable isotopes, the most traditional SILAC strategy is to grow a control group in the presence of <sup>12</sup>C-lysine and <sup>13</sup>N-arginine while providing the experimental group with <sup>13</sup>C-lysine and <sup>15</sup>N-arginine.<sup>31</sup>

During culture growth, these light or heavy amino acids are incorporated into the protein backbones with no effect on protein function, viability or expression. Digesting these cellular proteins with a proteolytic enzyme (*e.g.* trypsin) produces peptides that contain a single labeled or unlabeled residue. Peptides are then combined and analyzed *via* MS, at which point their mass difference can be observed. Evaluating the intensities of the labeled and unlabeled peptide partners allows the relative abundance of peptides and proteins to be determined. Metabolic labeling strategies are of topical interest to groups seeking to reveal how altered growth conditions, drug administration, or environmental perturbations affect protein production and expression. Beyond relative quantitation of proteins and peptides, SILAC-like experiments have been used to probe post-translational modification production and turnover. However, the chief considerations and drawbacks of these methods are (1) the small number of suitable amino acids that may be used for isotope incorporation; (2) poor separation of isotopic envelopes (causing errors in quantitative accuracy); and (3) the inability to incorporate isotopes to biological tissue and biofluid samples. In remedy, researchers may choose to tag proteins and peptides with isotopic labels after extraction and digestion.

### Isotopic tagging

Isotopic tagging, though similar in nature to metabolic incorporation, comes with a higher level of flexibility and customization.<sup>31</sup> Modern research settings have access to a broad array of stable isotopes, the most ubiquitous being <sup>13</sup>C, <sup>15</sup>N, <sup>2</sup>H, and <sup>18</sup>O. These isotopes enable researchers to synthesize their own chemical scaffold while varying the incorporation of these isotopes, creating an array of chemical tags with unique masses that may be functionalized and chemically bound to proteolytic peptides to provide them with a mass difference distinguishable *via* MS.<sup>32,33</sup> In this way, the need for metabolic incorporation is completely removed and experimental peptides can be labeled after extraction and digestion. Similar to metabolic labeling, differences in MS1-level signal intensity between labeled species allow for determination of relative quantitation. Furthermore, isotopic labeling can be used as a means of absolute quantitation, whereby internal calibration curves are created and compared to experimental peptides. Overall, isotopic labeling presents highest utility in instances where the sample collection is relatively small because as sample number increases so does spectral complexity, which can create mass overlap between unique peptide species and produce erroneous quantitation estimates. These limitations in mind, the vast improvements in MS operational speeds, resolving power, and scanning depth begged the question as to whether more efficient chemical labels could leverage these instrumental improvements and eliminate the spectral complexity found in complex isotopic tagging experiments.

### Isobaric labeling

As mass spectrometry technology continued to develop, it became obvious that the spectral complexity associated with





high-throughput metabolic labeling and isotopic tagging experiments directly counteracted any instrumental improvements. As such, it became pertinent to find a method for absolute and relative quantitation that alleviate the ailments posed by multiplexed labeling methods while still retaining the facility in quantitative measurements. Remembering that isotopic tags may be constructed to provide a high number of labeling channels, each with a distinct mass difference of  $>1$  Da, isobaric labels correct for this inherent mass difference using a balancing group.<sup>34</sup> When implemented, these isobaric labels display virtually indistinguishable masses at the MS<sup>1</sup> level, reducing the spectral complexity of high-throughput experiments. Upon selection of a labeled analyte, MS dissociation causes the isobaric tags to fragment and produce 'reporter ions' of unique mass. In this way labeled analytes may be selected and fragmented, providing identification and quantitative information in a single step. As a result, the reduced spectral complexity at the MS<sup>1</sup> level promotes greater profiling depth of complex samples and provides equivalent quantitative accuracy. The most popular examples of commercial isobaric labels are iTRAQ, Isotopic Tags for Relative and Absolute Quantitation<sup>35</sup> and TMT, Tandem Mass Tags.<sup>36</sup> However, the broad utility of isobaric labeling has garnered significant attention from the research community, resulting in numerous novel quantitative labeling strategies<sup>37,38</sup> that promote quantitative accuracy at significantly reduced cost.

### Label-free and reaction monitoring

Finally, in instances where sample labeling may not be preferred (*i.e.*, precious samples, low-abundance molecules of interest, or instances where protein targets are known), label-free and reaction monitoring methods provide a suitable alternative.<sup>39</sup> Label-free quantitation serves to provide relative quantitation between samples by comparing area-under-curve for detected analytes. This method, though steadily improving with better instrumentation and software tools, is highly susceptible to changes in sample composition, can result in missing values, and is lower throughput than labeled methods. However, label-free quantitation does still represent a meaningful entry point in discovery-based quantitative proteomics, often providing deep sample profiling and elucidating targets for future analyses. In contrast, reaction monitoring workflows (*e.g.* multiple reaction monitoring, select reaction monitoring, *etc.*) may be considered one of the most accurate quantitative strategies, being most suited to targeted analyses and instances when internal standards are readily available. Though reaction monitoring strategies are often tailored to fit unique experimental conditions, all workflows bear resemblance to a basic strategy. First, serial dilutions of a purified or synthetic peptide standard are analyzed *via* targeted MS/MS. In these targeted analyses, the biomolecule(s) of interest are subjected to MS dissociation, with the various fragments observed and recorded. As each biomolecule will provide a unique transition/fragment, the prevalence of these transitions may be used as a proxy for overall biomolecule abundance. In this way, absolute and relative quantitation information can be determined

without the need for chemical labeling while eliminating concerns over sample and spectral complexity. Often, it is preferential to incorporate an isotope-encoded standard,<sup>40,41</sup> enabling rapid analyses and high quantitative accuracy. Given the variety of quantitative strategies, it is of topical importance to evaluate their efficacy and provide understanding of quantitative accuracy.

### Diagnostic accuracy

As quantitative proteomics continues to mature, discussions over quantitative accuracy will continue to be a vanguard consideration. Recently, Dowle *et al.*<sup>42</sup> provided an in-depth comparison of multiple quantitative strategies and should be evaluated independently by interested parties. Within all quantitative strategies, the primary diagnostic for accuracy and utility are metrics built on specificity and sensitivity. Measures of specificity (*i.e.* proportion of correctly-identified true positives) and sensitivity (*i.e.* proportion of correctly-identified true negatives), may be combined into a single metric. This receiver operating characteristic (ROC) is often viewed as a curve with high sensitivity and specificity representing a value close to 1. As demonstrated by Dowle, several commonly used quantitative strategies display high ROC values, providing detailed considerations of the method most appropriate for a range of experiments. This work may serve as a helpful guide when entering or expanding quantitative proteomics experiments.

Taken together, metabolic labeling, isotopic tagging, isobaric labeling, and label-free strategies provide a wealth of entry points into quantitative proteomics. This access in mind, the growing needs of the medical community combined with the ever-increasing access to mass spectrometry technology necessitate the utilization and expansion of investigational proteomics to aid in discovering and validating cancer-specific biomarkers.

## Prostate cancer

The second leading cancer type in men, prostate cancer is estimated to affect around 12 percent of all men during their lifetime and currently affects over 3 million men within the United States, with the majority of individuals diagnosed being at least 65 and older.<sup>43</sup> Androgen deprivation, the first means of therapeutic intervention, can lead to the progression of castration-resistant prostate cancer (CRPC) in some men, a more aggressive stage of cancer resulting in poor prognosis and survival, with the majority of men developing metastases prior to or following diagnosis.<sup>44</sup> Further analyses of the literature have characterized these CRPC subtypes and demonstrated the growing emergence of CRPC phenotypes that have either low or negative AR expression for which there are few targeted therapeutics.<sup>45</sup> The growing heterogeneity in prostate cancer subtype underscores the urgency to elucidate and discover novel molecular mechanisms underlying pathogenesis for all subtypes. The use of mass spectrometry (MS)-based quantitative proteomics for prostate cancer research in recent years has been a



driving force to exploit the factors underlying tumorigenesis and metastasis.

### Cellular and tissue analyses

Investigations often profile quantitative differences in the proteome *via* patient-derived tissue samples, cellular models, or genetically engineered mouse models such as the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. One such study by Zhang *et al.*<sup>46</sup> utilized a label-free approach to quantify differences in expression between the prostate glands of TRAMP *versus* wild-type littermates. Through generation and an in-depth analysis of the quantitative proteomics data, the authors were able to predict and validate the role of platelet-derived growth factor (PDGF)-B overexpression in increased proliferation, thereby highlighting the therapeutic potential of targeting PDGF signaling within prostate cancer. Other label-free approaches have utilized patient-derived tissue samples to profile global differences, including the work of Müller *et al.*<sup>47</sup> using formalin fixed, paraffin embedded tissue from radical prostatectomy, which focused on characterizing differences between nonmetastasizing tumors, metastasizing primary tumors and their distant nodal metastases. Although the analysis had only five biological replicates per tumor type, significant differences in expression were measured that allowed for clear distinction of each and presented several potential proteins whose increased expression in metastatic tumors could be targeted in future therapeutic studies. However, a smaller sample set warrants further investigation into these proteins as potential targets with a larger sample cohort.

Methods that incorporate stable isotopes into the peptide backbone, such as SILAC, allow for direct comparison of identical peptides across sample types and is more robust to instrumentational variation compared to unlabeled approaches. Recently, SILAC was used to examine extracellular vesicles (EVs) and the impact that upregulated  $\alpha(1,6)$ -fucosyltransferase (FUT8) expression had on biogenesis of these secreted biomolecules.<sup>48</sup> This was one of the first reports to map the systematic impact of an overexpressed glycosyltransferase on the EV proteome, specifically of a glycosyltransferase with known oncogenic activity.<sup>49,50</sup> FUT8 overexpression showed a decrease in EVs produced compared to wild-type cells and further analysis of intact glycopeptides from LAPC4 EVs showed marked differences in glycosite occupancy between EV populations and revealed a shift in glycoform composition. Miao *et al.*<sup>51</sup> combined the SILAC approach with parallel-reaction-monitoring (PRM) methods to discern differential kinase expression in two bone metastasis-derived prostate lines, PC3 and PC3MLN4.<sup>51</sup> Of the kinases that were quantified and found to be differentially expressed, most notably different was mitogen-activated protein kinase kinase kinase 4 (MAP4K4), a kinase previously observed to play a role in ovarian cancer.<sup>52</sup> One final example using the SILAC strategy by Sbrissa *et al.*<sup>53</sup> investigated the mechanisms of bone metastasis by determining CXCR4-interacting proteins through overexpression and knockdown of CXCR4 in PC3 cell lines. Proteomic analysis found one unexpected protein, phosphatidylinositol 4-kinase III  $\alpha$

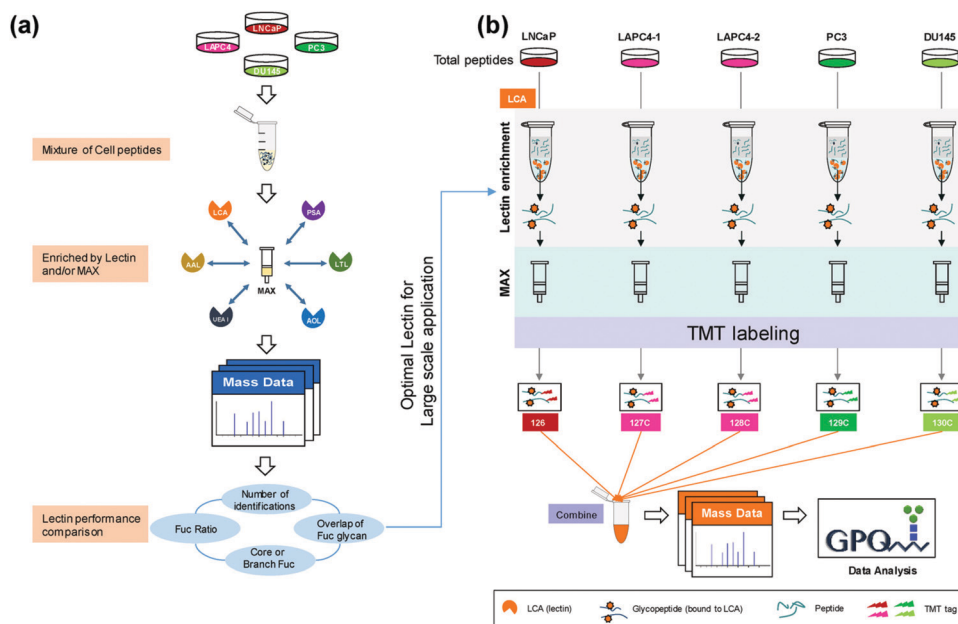
(PI4KIII $\alpha$ ), to be upregulated and it was found to localize with CXCR4 to lipid rafts and thus promote cancer cell invasion through increasing phosphatidylinositol-4-phosphate production. The discovery of this novel interaction between chemokine receptor and PI4KIII $\alpha$  and its regulation on tumor cell invasion requires more detailed experiments characterizing the specific molecular details regarding receptor-kinase communication.

Chemical or enzymatic isotopic labeling strategies allow for labeling of more than cell culture models to study prostate cancer. One approach by Lee *et al.*<sup>54</sup> used biotin—both as an isotopic label and for affinity purification—to systematically label cell-surface proteins that could serve to distinguish adenocarcinoma from neuroendocrine prostate cancer. From this proteogenomic investigation, they systematically validated two candidate antigens: FXYD domain containing ion transport regulator 3 (FXYD3) in prostate adenocarcinoma and CEA cell adhesion molecule 5 (CEACAM5) in neuroendocrine prostate cancer. While additional investigation into targeting these antigens is warranted, such a study demonstrates the utility of quantitative proteomics in discovering and validating new therapeutic targets for advanced prostate cancer.

Much of the quantitative research has shifted to the use of isobaric labeling strategies, which allow for increased multiplexing capabilities and decreased instrument variation through reduced overall runs. Zhou *et al.*<sup>55</sup> used 5-plex TMT labeling to perform a large-scale proteomic quantitation of core fucosylated glycopeptides after selective lectin affinity enrichment to differentiate non-aggressive and aggressive prostate cancer cell models (Fig. 1). Over 20 fucosylated proteins were upregulated in the aggressive cell lines and were involved in processes such as cellular signaling, adhesion and extracellular communication. Identification of these fucosylated proteins and their upregulation in aggressive prostate cancer models establishes these proteins as potential targets for further examination into how their upregulation impacts the aggressive phenotype of the associated model. Another advantage to using TMT labeling is that these tags can undergo synchronous precursor selection (SPS)-MS3 quantitation, which allow for more accurate quantitation. Zhou *et al.*<sup>56</sup> utilized a TMT-SPS-MS3 approach on patient-derived tissue samples with varying prostatic phenotypes to determine differential expression of protein complexes. Low-grade prostate cancer samples were found to have upregulation of complexes involved in RNA splicing and downregulation of those associated with cell adhesion, while high-grade prostate tissue samples had increased assembly of antiapoptotic complexes and a similar lower abundance of complexes involving cell adhesion. Such a comprehensive study of individual protein complexes may give way to determining what protein complexes are critical in distinguishing and diagnosing low- and high-grade cancers.

Comparable to TMT labeling, iTRAQ allows for both relative and absolute quantitation of labeled samples. Höti *et al.*<sup>57</sup> set out to examine the mechanisms underlying androgen resistance through a global proteomic approach using iTRAQ, labeling tryptic peptides from two prostate cancer cell models grown in triplicate.





**Fig. 1** Complete workflow utilized by Zhou *et al.*<sup>55</sup> detailing the quantitative approach to investigate site-specific fucosylation and glycoproteins associated with aggressive prostate cancer phenotypes. The optimized enrichment strategy used to identify glycopeptides contributing to prostate cancer aggressiveness shows promise for application in a variety of cancer glycosylation studies but should also be applied to other prostate cancer models to determine its utility across sample types. Reprinted with permission.

One main realization of the data was that androgen resistance cannot be treated with a single therapeutic, as the mechanisms driving resistance involve multiple independent pathways. While unfortunate, these findings did uncover some of the mechanisms driving resistance, including the PI3K/AKT signaling pathway, mitochondrial dysfunction of oxidative phosphorylation complexes and the multicatalytic 26S proteasome. Zhang *et al.*<sup>58</sup> used two sublines of PC-3M to distinguish unique characteristics of highly- and poorly-metastatic potential in prostate cancer. After validation, two proteins were found to potentially contribute to the higher metastatic potential, matrix metalloproteinase 1 (MMP1) and four and a half LIM domains 1 (FHL1). While FHL1 has been extensively studied in a variety of cancer types, the information collected here suggests a unique role of MMP1 for increasing metastatic potential in prostate cancer, presenting the opportunity for future inspection of both MMP1 and other MMPs. Webber *et al.*<sup>59</sup> performed a stromal cell proteomics analysis to differentiate normal from tumor-reactive stromal phenotypes that drive disease progression. One compelling finding was the loss of aldehyde dehydrogenase (ALDH1A1) expression in altered *versus* normal stromal types, suggesting its potential role as a novel marker of disease-induced changes of the stromal environment. Additional investigations have turned to animal models, as prostate cancers grown *in vivo* reflect interactions that may otherwise be missed in cell culture models. The *Pten*-knockout mouse model<sup>60</sup> was recently examined by Zhang *et al.*<sup>61</sup> through the combined analysis of iTRAQ proteomics and microarray transcriptomics to identify associated molecular changes in mouse prostate carcinogenesis. Both transcriptomic and proteomic data found that immune and inflammation responses were greatly perturbed, in addition

to mediations in central nodal activity through the Akt, NF- $\kappa$ B and P53 signaling pathways.

While tissue-based sampling allows for determination of mechanistic properties of the pathways contributing to tumorigenesis and metastasis, its highly invasive nature is discouraged unless necessary. Even if biopsies are obtained, these analyses are often limited by size constraints, as patient-derived tissues covering all stages of prostate cancer progression can be difficult to obtain in large numbers. Mouse models afford the opportunity to mimic tumor progression and metastasis *in vivo*, but there are still controversies surrounding prostate-based mouse models due to distinct anatomical differences.<sup>62</sup> Cell culture models avoid the translational constraints that other model organisms are bound to, but often omit stromal-epithelial interactions during cell growth, a process that has a great impact on tumor invasiveness and metastatic potential. Additionally, current cell-based models for prostate cancer often either only reflect advanced prostate cancer or require the use of multiple cell lines to cover multiple progression stages, introducing variability that complicates genetic-based analyses. Recent advances in cell-based prostate models have been made that address some of the pitfalls outlined here,<sup>63</sup> so future quantitative studies should be selective in the models they choose when profiling.

### Biofluid analyses

There is a push to develop biomarker strategies involving the collection of biofluids, a less invasive and more cost-effective method of sample collection. Biofluids—such as blood, tissue-based fluid, saliva, or urine—allow for easier monitoring of patient outcomes, as disease progression and treatment responsiveness can be evaluated with frequent patient



sampling. Such biofluid-based monitoring strategies are critical in prostate cancer patients, as a portion of men diagnosed with prostate cancer have tumors that are indolent. One study by Davaliev *et al.*<sup>64</sup> comparatively profiled urine samples using a label-free strategy from patients with prostate cancer, benign prostatic hyperplasia, bladder cancer and renal cancer to determine selective biomarkers for earlier diagnosis of prostate cancer. Of the most promising urinary biomarkers identified by the authors, nine had not yet been associated with prostate cancer, indicating their potential as novel biomarkers and necessitating further research into their associated pathways. Soekmadji *et al.*<sup>65</sup> profiled secretome differences of unlabeled, CD9-positive EVs from cell culture models treated with the hormone dihydrotestosterone (DHT). Their combined analyses determined that DHT treatment increases CD9-positive EV secretion and alters the content of secreted EVs, and in agreement with previous literature highlighting the potential of CD9 EVs as a biomarker for prostate cancer.

Reaction monitoring-based strategies are one label-free approach that are typically used after initial discovery for validation and accurate quantitation of biomarkers. Targeted analysis of urinary EVs was completed by Sequeiros *et al.*<sup>66</sup> using SRM to quantify 64 protein candidate biomarkers for prostate cancer. A two-protein combination (ADSV and TGM4) distinguished patients with benign tissue from those with cancer, and a five-protein panel differentiated high- from low-grade prostate cancer (CD63, GLPK5, SPHM, PSA and PAPP), highlighting the advantages of targeted proteomics as a diagnostic tool in the clinic. Kim *et al.*<sup>67</sup> investigated expressed prostatic secretion samples using SRM-based quantitation to determine molecular signatures for extracapsular prostate cancer. From a pool of over 200 potential candidates, these researchers narrowed the candidate list to include 34 peptides representative of 27 unique proteins with promising results as biomarkers. Karasota *et al.*<sup>68</sup> evaluated the analytical performance of multiple SRM- and PRM-based strategies to

quantitate kallikrein related peptidase 4 (KLK4) in a variety of biofluid samples. Secreted KLK4 was demonstrated to be present in seminal plasma, and for the first time was investigated as a potential biomarker in both seminal plasma and blood. Taken together, the label-free, targeted proteomics methods used for analysis of biofluids offer a reliable tool for biomarker validation and should thus be considered as useful tools for clinical development.

Fujita *et al.*<sup>69</sup> combined two strategies, initially using iTRAQ for urine samples to profile EVs from patients with a high Gleason score.<sup>70</sup> After quantifying 3528 proteins, candidate biomarkers were selected for further quantitation and validation using SRM/MRM. Fatty acid binding protein 5 (FABP5) was highlighted as the most promising biomarker from urinary EVs for the detection and diagnosis of high Gleason score prostate cancer, but further studies would be necessary for confirmation. Yan *et al.*<sup>71</sup> performed an iTRAQ-based analysis on the serum of prostate cancer patients with or without bone metastasis to find potential biomarkers indicative of these metastases. Of the 32 differentially expressed proteins identified, three—CD59, haptoglobin and tetranectin—were selected and validated to be related to prostate cancer bone metastasis, confirming their utility as serum biomarkers. Larkin *et al.*<sup>72</sup> implemented iTRAQ to enhance their proteomic profiling of high-quality serum samples for biomarker discovery. After identification and validation using ELISA, two biomarkers, SAA and TSR1, showed promising results when used in combination with KLK3. However, these results were obtained in a small sample cohort, so further studies with a larger, more diverse sample set are necessary before serious consideration of these proteins as biomarkers. Table 1 summarizes selected prostate cancer biomarkers.

The use of quantitative proteomic strategies on patient-derived biofluid samples show promise in the discovery and validation of new biomarkers. Specifically, the KLK family of proteins has been shown in the mentioned literature to have potential in many biofluids and may improve diagnostic

Table 1 Summarized selection of prostate cancer biomarkers

Proposed biomarker	Source	Findings
Platelet-derived growth factor (PDGF)-B <sup>46</sup>	Prostatic tissue	Overexpressed with increased cancer proliferation
$\alpha(1,6)$ -Fucosyltransferase (FUT8) <sup>48–50</sup>	LAPC4 and LNCaP cells	Increased FUT8 expression corresponded with decreased extracellular vesicle production
Mitogen-activated protein kinase kinase 4 (MAP4K4) <sup>51,52</sup>	PC3 and PC3MLN4 cells	Differential expression in metastasis-derived cell lines
Phosphatidylinositol 4-kinase III $\alpha$ (PI4KIII $\alpha$ ) <sup>53</sup>	PC3 cells	Upregulated in PC3 cell lines; promotes cancer cell invasion
FXD domain containing ion transport regulator 3 (FXD3) <sup>54</sup>	PrAd, NEPC cell lines	Biomarker specific to prostate adenocarcinoma
CEA cell adhesion molecule 5 (CEACAM5) <sup>54</sup>	PrAd, NEPC cell lines	Biomarker specific to neuroendocrine cancer
Four and a half LIM domains 1 (FHL1), matrix metalloproteinase 1 (MMP1) <sup>58</sup>	PC-3M sublines	Promote higher metastatic potential
Aldehyde dehydrogenase (ALDH1A1) <sup>59</sup>	Stromal tissue	Loss of expression in altered stromal cell types
Actin-depolymerizing factor (ADSV), transglutaminase 4 (TGM4) <sup>66</sup>	Urine	Differentiates benign and cancerous tissue
CD63 molecule (CD63), glycerol kinase 5 (GLPK5), SPHM sulfohydrolase (SPHM), prostate-specific antigen (PSA) and pappalysin 1 (PAPP) <sup>66</sup>	Urine	Distinguishes high- and low-grade cancer
Kallikrein related peptidase 4 (KLK4) <sup>68</sup>	Seminal fluid	Biomarker available in seminal fluid
Fatty acid binding protein 5 (FABP5)	Urine	Utility in detecting, diagnosing high gleason score prostate cancer
CD59 molecule (CD59), haptoglobin and tetranectin <sup>71</sup>	Serum	Expression correlated to bone metastasis





accuracy further when combined with others. On the other hand, serum biomarkers in prostate cancer deserve a level of scrutiny as demonstrated by prostate-specific antigen (PSA), a currently approved biomarker whose elevation in serum is also associated with benign prostatic hyperplasia (BPH), resulting in high false positive rates.<sup>73</sup> Noting this, prostate cancer biomarkers should be rigorously tested against patients with BPH and other prostatic diseases to ensure accuracy. Such rigorous tests involving larger sample sets can be achieved using the quantitative strategies described above, indicating their potential to advance the knowledge within the field at a rapid pace.

## Pancreatic cancer

The seventh leading cause of cancer-related deaths in the world,<sup>74</sup> pancreatic cancer has rightfully garnered significant attention from clinical research communities. In-depth proteomic analyses have illuminated the highly dynamic nature of post-translational modifications,<sup>75–77</sup> while providing novel insights toward treatment monitoring and severity stratification. The promising results of these profiling experiments in hand, great success has come in the effort to employ quantitative strategies to illuminate dysregulated protein expression, identify treatment pathways, and validate potential biomarkers.

### Tissue analyses

The prevalence of pancreatic cancer across the world's population has necessitated in-depth proteomic analyses of cancerous tissue and model systems. Model cell lines have enabled researchers to identify pertinent biomolecules specific to pancreatic cancer without the need for invasive, repetitive tissue resection. Naturally, the study of cell lines lends itself to the use of SILAC to perform quantitative investigations. Recently, Liu *et al.*<sup>78</sup> performed secretomic analyses of pancreatic cancer cells (PC-1), revealing 161 proteins with altered expression, including 55 proteins not previously reported. As well, they note a combination panel for cadherin 3 (CDH3), plasminogen activator, urokinase (PLAU), and lunatic fringe (LFNG) proteins that may be useful for improving cancer patient prognoses. Beyond this, Marchand *et al.*<sup>79</sup> employed a three-channel SILAC approach to reveal association of transcription factor EB (TFEB) with nuclear proteins upon inhibition of glycogen synthase kinase-3 (GSK3) and mammalian target of rapamycin (mTOR). Moving beyond SILAC experiments, Shi *et al.*<sup>80</sup> used isotopic dimethyl labeling to examine paracrine communication between pancreatic cancer cells and pancreatic stellate cells (PSCs). This experiment provided the knowledge that leukemia inhibitory factor (LIF) is a key paracrine factor from activated PSCs acting on cancer cells. Employing a novel approach, Roberts *et al.*<sup>81</sup> developed a cysteine-reactive fragment-based ligand library to coordinate novel small molecules that impair pancreatic cancer pathogenicity with druggable hotspots for potential cancer therapy. While numerous SILAC and isotopic tagging workflows exist outside the time frame of this review, the relatively small number of recent applications indicates an area of potential focus for researchers examining pancreatic cancer.

Isobaric labeling, however, has seen significant use in the study of pancreatic cancer. Zhang and colleagues<sup>82</sup> have provided a meaningful guide for those seeking to perform isobaric labeling experiments using the commercial TMT<sup>36</sup> offering from ThermoScientific. Beyond this, Perera *et al.*<sup>83</sup> employed TMT labeling to study pancreatic cancer cell metabolism, revealing the MiT/TFE proteins – MITF, TFE3 and TFEB – are decoupled from regulatory mechanisms, increasing expression levels of lysosomal catabolic function essential for pancreatic ductal adenocarcinoma (PDA) growth. As an alternative to TMT, An *et al.*<sup>84</sup> employed iTRAQ in the analysis of serum exosomes from chemotherapy patients (Fig. 2). Of note, this study indicates patient-derived exosomes play a significant role in cancer metastasis. Furthermore, Li *et al.*<sup>85</sup> demonstrated monumental success in broad protein quantitation while analyzing Peripheral Blood Mononuclear Cells (PBMCs).

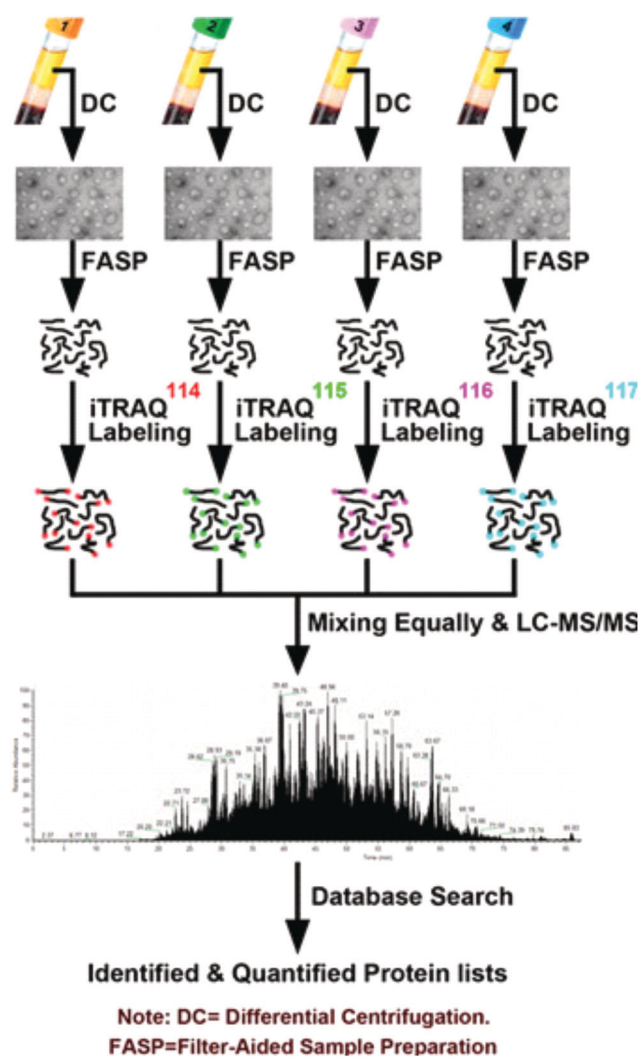


Fig. 2 Workflow described by An *et al.*<sup>84</sup> for the quantitative analysis of chemotherapy patient exosomes through iTRAQ labeling and quantitative mass spectrometry. This example of a facile isobaric labeling proteomics experiment provides deep proteomic profiling of multiple complex samples with lower spectral complexity than isotopic labeling methods. Reprinted with permission.





This study, which employed iTRAQ labeling and 2D-LC-MS quantified 3357 proteins, with 114 being distinguished as dysregulated in the cancer group. These examples of isobaric labeling indicate the broad utility for high throughput analyses of complex pancreatic cancer samples. However, a chief limitation of TMT and iTRAQ is cost, placing their use out-of-reach for many budding research groups. In remedy, Li and colleagues<sup>34</sup> developed Dimethyl Leucine (DiLeu) that provides greater multiplexity<sup>86–88</sup> than commercial options at a fraction of the cost. DiLeu is available as an isotopic,<sup>89</sup> isobaric,<sup>34,86,87</sup> and mass-defect<sup>90</sup> chemical tag and has even been modified to provide absolute quantitation.<sup>89,91</sup> The mass-defect offering, mdDiLeu, has been successfully applied for simultaneous multiomic analysis of pancreatic cancer cells,<sup>92</sup> providing uncompromised access to high throughput small molecule and protein quantitation.

Label-free analyses, too, have seen routine utilization in pancreatic cancer investigations. Wang *et al.*<sup>93</sup> introduced the novel IonStar pipeline for accurate MS1-level protein quantitation. This preliminary example quantified >4000 proteins from 40 biological samples and identified 541 proteins dysregulated groups treated with birinapant and paclitaxel. Later Zhu *et al.*<sup>94</sup> applied the IonStar pipeline to elucidate the relations among relevant signaling pathways during gemcitabine and birinapant treatment. These applications highlight the utility of quantitative proteomics to evaluate treatment efficacy. In a similar vein, Singh *et al.*<sup>95</sup> presented a large-scale, label-free proteomics study to uncover the mechanism by which sanguinarine suppresses cancer proliferation. While quantifying >3100 proteins, 37 biomolecules were identified as differentially expressed, highlighting the pleiotropic effects of sanguinarine. Finally, Zhou *et al.*<sup>96</sup> employed parallel reaction monitoring (PRM) to identify 165 potential biomarkers in pancreatic cancer. During validation, brain acid soluble protein 1 (BASP1) was identified as a novel target for pancreatic cancer therapy and is shown to interact with Wilms tumor protein (WT1).

### Biofluid analyses

Considering the real-world application of investigational proteomics analyses, a topical concern is the need for invasive patient sampling. This in mind, researchers have long sought to identify cancer-specific analytes from biofluids, which may be sampled repeatedly at lower physical and monetary cost to patients. Though metabolic and isotopic labeling are not well represented in pancreatic cancer research in recent years, Jhaveri *et al.*<sup>97</sup> used a novel serum antibody-based SILAC immunoprecipitation approach, denoted as SASI, to identify specific targets expressed in cancer patients post-vaccine therapy. More popular, however, are applications utilizing isobaric labeling. Sogawa *et al.*<sup>98</sup> employed TMT labeling to ascertain that complement component 4 binding protein alpha (C4BPA) and polymeric immunoglobulin receptor (PIGR) expression was significantly higher in preoperative patients than postoperative. Naba *et al.*<sup>99</sup> identified unique expression levels in 35 proteins as pancreatic cancer islets progressed from hyperplastic to angiogenic to insulomas. Yu *et al.*<sup>100</sup> employed iTRAQ to

quantify 4517 proteins in the exosomes of Panc02 and Panc02-H7 cells, notably revealing cancer-derived exosomes promote tumor metastasis. Lin *et al.*<sup>101</sup> and Liu *et al.*<sup>102</sup> further implemented iTRAQ for quantitative evaluations of pancreatic cancer patient serum. An important overlap of these two studies was the identification that apolipoprotein A-1 (APOA1) shows distinct expression in pancreatic cancer patients. Considering this trend was shared between patients expressing carbohydrate antigen (CA) 19-9 and those who are CA19-9-negative, APOA1 presents an area of significant interest moving forward.

Similar to the studies presented in pancreatic cancer tissue analyses, label-free quantitation has been routinely employed in quantification of biofluid proteins. Through this quantitative strategy, Ohmine *et al.*<sup>103</sup> successfully validated deoxycytidine kinase (dCK) as a good predictor of progression-free survival and an effective biomarker of gemcitabine sensitivity. Yoneyama *et al.*<sup>104</sup> identified insulin-like growth factor-binding proteins insulin-like growth factor binding protein 2 (IGFBP2) and IGFBP3 as compensatory biomarkers of pancreatic cancer in instances when CA19-9 screening is inconclusive. Park *et al.*<sup>105</sup> performed a large-scale validation of biomarkers, finding that APOA-IV, APOCIII, IGFBP2, and tissue inhibitor of metalloproteinase 1 (TIMP) were significantly altered in pancreatic cancer. Of note, a panel including CA19-9, APOA-IV, and TIMP1 showed improved performance in distinguishing early pancreatic cancer from pancreatitis. Do *et al.*<sup>106</sup> identified 18 biomarker candidates associated with malignancy in intraductal papillary mucinous neoplasms (IPMNs). Finally, Nigjeh *et al.*<sup>107</sup> developed an optimized data-independent acquisition (DIA) workflow to identify and quantify >14 000 peptides from ~2300 plasma proteins (Fig. 3).

As seen by the numerous examples of pancreatic cancer tissue and biofluid investigation, quantitative proteomics provides a facile entry point into the field of biomarker identification and validation (Table 2). Considering the agreement across several studies that proteins such as APOA1, APOA4, IGFBP and CA19-9 serve as rigorous biomarkers in pancreatic cancer, future studies should investigate the utility of high throughput label-free, PRM, or MRM screening of these biomolecules. Meaningful evaluation of MS-based protein assays in blind studies may demonstrate potential to accurately identify and diagnose pancreatic cancer at scale. Development of these workflows and associated technology will be vital to understanding the risk factors associated with disease onset and progression, as well as the success of current and novel treatment strategies.

## Breast cancer

The high rate of incidence associated with breast cancer, as well as targeted focus drawn from successful advocacy and research fundraising, have shed significant light on the mechanisms of breast cancer. Though this dedicated focus has reduced patient mortality and cancer rates in high income countries,



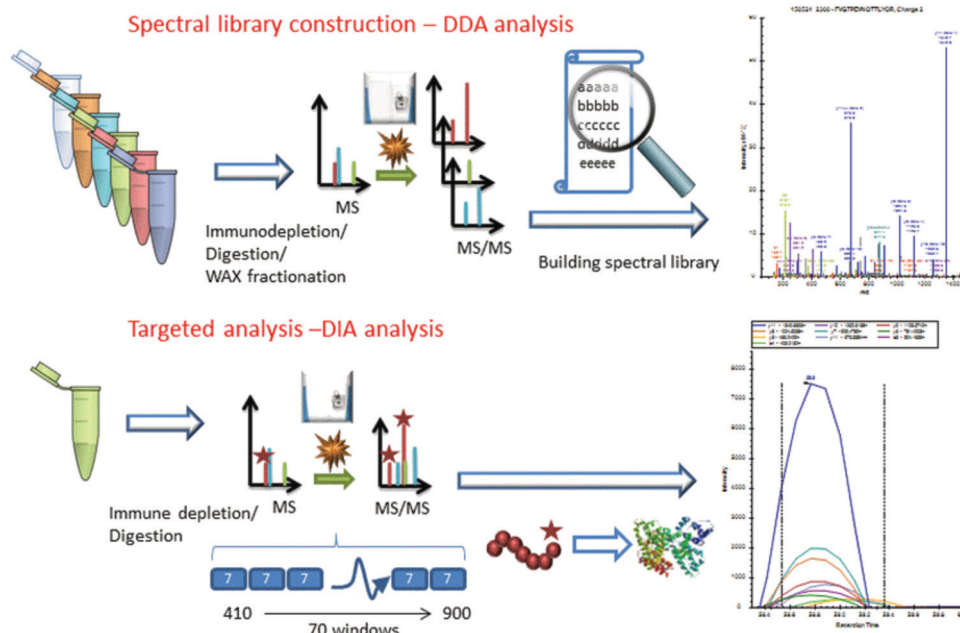


Fig. 3 Workflow implemented by Nigjeh *et al.*<sup>107</sup> Quantitative workflows utilizing isobaric labels present the greatest propensity for deep proteome profiling. However, these workflows are limited by their instrument acquisition speed and cycle time required to select and fragment top precursors. For this reason, implementation of DIA strategies presents the ability to sequence a greater number of peptides in the same amount of time. Though the data processing methods are significantly more involved, DIA workflows are sure to be of critical importance to proteome profiling in the coming years. Reprinted with permission.

Table 2 Summarized selection of pancreatic cancer biomarkers

Proposed biomarker	Source	Findings
Cadherin 3 (CDH3), plasminogen activator, urokinase (PLAU), lunatic fringe (LFNG) <sup>78</sup>	PC-1 cell secretome	Potential for improving cancer patient prognoses
Transcription factor EB (TFEB) <sup>79</sup>	HEK293, PANC1, MIA PaCa-2 cells	Association with nuclear protein upon inhibition of GSK3
Leukemia inhibitory factor (LIF) <sup>80</sup>	Pancreatic stellate cells	Denoted as major paracrine factor
Melanocyte inducing transcription factor (MITF), transcription factor binding to IGHM enhancer 3 (TFE3) and transcription factor EB (TFEB) <sup>83</sup>	Tissue, PDA cells	Decoupled from regulatory mechanisms, promote catabolic function
Brain acid soluble protein 1 (BASP1) <sup>96</sup>	Tissue	Novel cancer therapy target
Complement component 4 binding protein alpha (C4BPA), polymeric immunoglobulin receptor (PIGR) <sup>98</sup>	Serum	Higher expression in preoperative patients than postoperative
Apolipoprotein A-1 (APOA1) <sup>101,102</sup>	Serum	Distinct expression in both CA19-9 positive and CA19-9-deficient patients
Deoxycytidine kinase (dCK) <sup>103</sup>	PK9, CFPac-1, PK1, SUIT-2, and AsPC-1 cells	Predictor of progression-free survival, biomarker of gemcitabine sensitivity
Insulin like growth factor binding protein 2 (IGFBP2) and IGFBP3 <sup>104</sup>	Plasma	Compensatory biomarkers when CA19-9 screening is inconclusive
Insulin-like growth factor binding protein 2 (IGFBP2) tissue inhibitor of metalloproteinase 1 (TIMP1), apolipoprotein A IV (APOA-IV), apolipoprotein CIII APOCIII <sup>105</sup>	Blood	Protein panel highly effective in early detection of pancreatic cancer

developing nations display the opposite trend.<sup>108</sup> Beyond this, breast cancer is of continual interest to the medical community due to the high rate of recurrence and metastasis.<sup>109,110</sup> For these reasons, many have turned to quantitative proteomics to aid in stratifying cancer subtypes and identifying potential biomarkers.

### Tissue and biofluid analyses

Within the timeframe of this review, the majority of quantitative proteomic investigations have been centered on tissue analyses,

often employing model cell lines or resected tumor tissue to determine protein expression. Though few applications have employed metabolic labeling for quantitative analyses, Tyanova *et al.*<sup>111</sup> presented a robust investigation that merged quantitative mass spectrometry with traditional RNA- and DNA-based sequencing strategies. Analyzing 40 tumors that were either estrogen receptor positive, Her2 positive, or triple negative, the authors identified an average of >7000 proteins on average, spanning 8 orders of magnitude in protein intensity. Within this study, they combined their quantitative results with



microarray analyses and machine learning classification to identify potential subtype-specific therapies.

More popular than SILAC-like experiments, isobaric labeling has been extensively employed in breast cancer investigations. Suman *et al.*<sup>112</sup> employed iTRAQ to identify proteins associated with breast cancer subtypes. Notably, this study indicated fibronectin (FN1), alpha-2-macroglobulin (A2M), complement component-4-binding protein-alpha (C4BPA) and complement factor-B (CFB) were critical to subtype differentiation in both plasma and tissue samples. Calderon-Gonzalez *et al.*<sup>113</sup> further employed this technology to identify 306 differentially expressed proteins in breast cancer cell lines. As well, their study indicates large proline-rich protein (BAG6), ATP-dependent RNA helicase (DDX39), annexin A8 (ANXA8) and cytochrome *c* oxidase subunit 4 (COX4) may serve as useful biomarkers. Gajbhiye *et al.*<sup>114</sup> provided a novel DIA-iTRAQ strategy to uncover proteomic divergence in HER2-enriched cancer cell lines, which allowed for the creation and testing of a 21 protein panel to discriminate cancer and healthy controls. Turning to TMT labeling, Going *et al.*<sup>115</sup> and Clark *et al.*<sup>116</sup> utilized this strategy, illuminating the pathways of action of methoxychalcone in triple negative breast cancer (Fig. 4) and classifying exosomal cargo proteins, respectively. As a cost-effective alternative to these iTRAQ and TMT labeling strategies, DiLeu tagging approach has also successfully been employed in identifying strategies for inhibiting cancer cell proliferation. Within this work, Liu *et al.*<sup>117</sup> revealed that

dynamic methylation of pyruvate kinase M2 (PKM2) directly affect the metabolic activity of cancer cells and promotes cell propagation, migration and metastasis. This study, along with those detailed above, serve to indicate the importance of high-throughput quantitative cancer proteomics, outlining potential targets for future treatment strategies.

A significant entry into quantitative breast cancer proteomics was provided by Johansson *et al.*<sup>118</sup> This study provided in-depth quantitation of 45 breast cancer tumors, spanning each of the 5 PAM50-based molecular classifications. Upon quantitation of 9995 proteins, the authors used these proteome profiles to interpret multiple layers of systems measurements. While each of these studies offered unique insight into uncovering and validating potential biomarkers and investigative strategies, a chief concern among many is the long-term reproducibility of quantitative measurements. Using iTRAQ to quantify proteins from human-in-mouse xenograft tissue, Zhou *et al.*<sup>119</sup> demonstrated that the large majority of quantitative measurements hold consistent over time, but also raised some topical concerns. First, they observed higher variability in quantitation of hydrophilic peptides compared to those of average peptide character, likely due to poor retention of these peptides on column. Second, as researchers have their choice of dissociation methods, this study reveals stepped collision energy offers higher reproducibility between unique measurements. Finally, whereas most commercial software implements a form of scoring to determine the quality of a peptide spectral match (PSM), this study goes further and reveals that a stricter scoring mechanism improves reliability of time-course measurements. This study provides an excellent framework and series of considerations for individuals seeking to begin or improve quantitative mass spectrometry investigations.

Label-free analyses have also been routinely implemented for high throughput biomarker discovery and screening. Among these, Ntai *et al.*<sup>120</sup> compared the quantitation efficiency in bottom-up and top-down analyses of tumor xenografts. Tveitras *et al.*<sup>121</sup> performed comparative analyses of pre-metastatic and metastatic triple negative breast cancer xenograft tissue, uncovering significant changes in expression of haptoglobin, fibrinogen, and thrombospondin-4 and transferrin receptor protein 1 between groups. Wang *et al.*<sup>122</sup> employed a DIA-select reaction monitoring (SRM) approach to reveal distinct proteomic and *N*-glycoproteomic divergence between normal, precancerous, and cancerous tissues. Gamez-Pozo *et al.*<sup>123</sup> integrated label-free MS quantitation with RT-qPCR to definitively distinguish estrogen receptor positive and triple negative cancer subtypes. Nie *et al.*<sup>124</sup> identified 98 differentially expressed proteins when comparing pure breast cancer stem cells and mature luminal cells. Finally, Warmoes *et al.*<sup>125</sup> elucidated 215 proteins that are significantly enriched in BRCA1-deficient secretome. This study highlights the potential of mass spectrometry to provide sensitive identification of biomarkers in instances when traditional ELISA screening may fall short.

These examples of successful quantitative proteomic analyses in breast cancer applications highlight the flexibility and facility

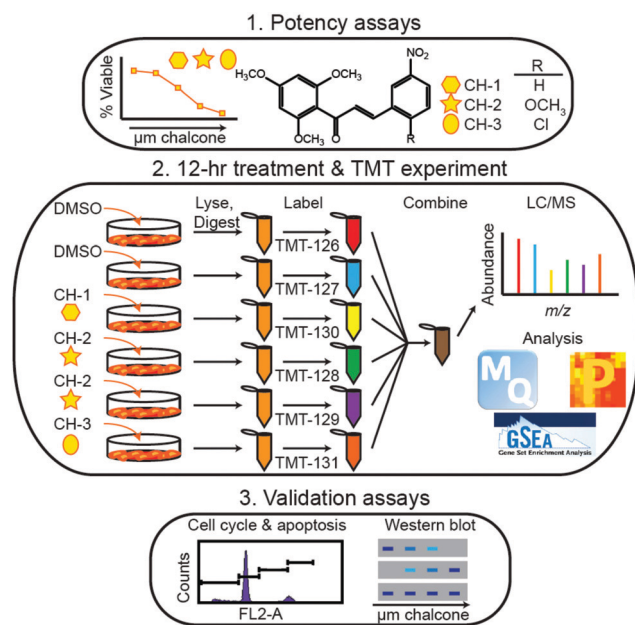


Fig. 4 Representative workflow established by Going *et al.*<sup>115</sup> As quantitative proteomics is critical for discovering and validated biomolecules of interest during periods of disease and treatment, this workflow represents an example of how treatment strategies may be controlled and systematically evaluated. While SILAC methods would be useful in situations where cell growth is monitored, isotopic labeling methods may be considered inherently lower throughput due to the increases in spectral complexity they may provide. Reprinted with permission.



Table 3 Summarized selection of breast cancer biomarkers

Proposed biomarker	Source	Findings
Fibronectin (FN1), alpha-2-macroglobulin (A2M), complement component-4-binding protein-alpha (C4BPA) and complement factor-B (CFB) <sup>112</sup>	Tumor tissue	Critical for subtype differentiation
Large proline-rich protein (BAG6), ATP-dependent RNA helicase (DDX39), annexin A8 (ANXA8) and cytochrome <i>c</i> oxidase subunit 4 (COX4) <sup>113</sup>	MCF7 and T47D, MDA-MB-231, and SK-BR-3 cells	Putative biomarkers for breast cancer
Methylated pyruvate kinase M2 (PKM2) <sup>117</sup>	MCF7, MDA-MB-231, HEK293T cells	Promotes cell propagation, migration and metastasis
Haptoglobin, fibrinogen, and thrombospondin-4 and transferrin receptor protein 1 <sup>121</sup>	Pre-/metastatic xenograft tissue	Reveal <i>N</i> -glycoproteomic divergence between normal, precancerous, and cancerous tissues

of creating novel workflows to answer an array of biological problems. Knowing there have been a significant number of proteomic measurements made prior to the period in review, these examples of biomarker discovery and validation highlight how rigorous protein MS-based screening assays for the confident identification and stratification of breast cancer may be within reach (Table 3). Assays of this kind, devoid of the need for invasive and repetitive tissue sampling, provide a meaningful conduit towards aiding communities that have limited access to dedicated cancer screening centers and provide direct targets for potential novel therapies.

## Ovarian cancer

Although it has an estimated incidence rate of approximately 2% for 2020, ovarian cancer is the deadliest reproductive cancer in women, with an estimated mortality rate of 5% in women diagnosed with any cancer type and 64% for women diagnosed with ovarian cancer.<sup>126</sup> Much emphasis has been placed on the continued research into mechanisms driving ovarian cancer, as late-stage diagnosis of advanced cancer contributes to the high mortality of ovarian cancer. Continued efforts have focused on the identification of critical mechanisms driving disease progression across ovarian cancer subtypes. Quantitative proteomic strategies have continued to increase the depth of knowledge surrounding ovarian cancer and its various subtypes to improve earlier identification strategies and highlight new therapeutic targets.

### Cellular and tissue analyses

Because the majority of diagnosed ovarian cancer cases have already progressed to a more advanced stage, much quantitative research delves into tissue and cellular proteomic profiling to isolate and exploit dysregulated proteins. While only applicable to cellular-based models, SILAC has been implemented in ovarian cell lines and led to the discovery of critical modulators in ovarian disease progression. Musrap *et al.*<sup>127</sup> cultured the ovarian line OV-90 in adherent and non-adherent conditions using SILAC to compare the impacts of cancer aggregate formation on cellular proteomics. After quantifying 1533 proteins in total, they compared expression with other aggregate-forming lines and saw upregulation of CLCA1, which appeared to affect cancer cell aggregation after further siRNA experimentation.

Grassi *et al.*<sup>128</sup> utilized SILAC to quantify epidermal growth factor (EGF)-induced epithelial–mesenchymal transition (EMT) to identify specific mechanisms of this process that may be dysregulated for metastatic purposes. 206 proteins were found to be differentially expressed, some of which included proteins associated with the G1 and G2 checkpoints of the cell cycle, indicating the role of EGF-induced EMT in cell cycle control mechanisms. Another investigation by Ji *et al.*<sup>129</sup> utilized the metabolic strategy to perform an integrated proteomic and *N*-glycoproteomic analysis of ovarian cancer lines that were either doxorubicin-sensitive or -resistant. They quantified 5509 protein groups and identified 1525 high-confidence *N*-glycosites corresponding to 740 glycoproteins. Quantifying the protein abundance allowed these researchers to examine glycoprotein abundances and alterations, which provides unique information into the role of *N*-glycosylation in drug resistance.

Applicable to more than just cell culture-based models, isobaric labeling is commonly employed for quantitative experiments applied to ovarian cancer sample sets. Zhang *et al.*<sup>130</sup> used iTRAQ labeling to integrate quantitative proteomics with the transcriptomic profile of ovarian high-grade serous cancer (HGSC) patient biospecimens. Over 3500 proteins were quantified and used in tandem with genomic results to reveal a strong association between specific histone acetylation events and the homologous recombination deficient phenotype seen in patient samples. Hiramatsu *et al.*<sup>131</sup> comparatively profiled HGSC and endometrial carcinoma samples using iTRAQ-based quantitation. Comprehensive analysis revealed 356 quantifiable proteins and identified mitochondrial inner membrane protease subunit 2 (IMP2) and minichromosome maintenance complex component 2 (MCM2) to be modulators of rapid HGSC growth, illustrating the need to examine these two proteins in further ovarian cancer studies.

Alternatively, many other analyses have used the TMT-based isobaric strategy rather than iTRAQ labeling. Recently, Hu *et al.*<sup>132</sup> used an integrated proteomic and glycoproteomic approach with TMT-labeled peptides in their analysis of ovarian HGSC *versus* non-tumor tissues. These authors combined global proteomics, solid-phase extraction of glycosite-containing peptides (SPEG) and glycan identification *via* intact glycopeptide analysis to provide a comprehensive view into *N*-glycoproteomics within ovarian cancer. Their integrated approach yielded promising results, identifying tumor-specific glycosylation and revealing glycosylation enzymes that were





correlated with altered glycosylation status. Yoshimura *et al.*<sup>133</sup> treated neighboring peritoneal mesothelial cells with a micro-RNA shown to be elevated in the serum of ovarian cancer patients to determine its role in cancer invasion and metastasis. The TMT-based proteomics analysis exhibited increased expression of fibronectin and vitronectin, enhancing the ability of the cancer cells to invade the surrounding environment. A straightforward, quantitative comparison of TMT-labeled normal *versus* cancerous ovarian tissue was performed by Qu *et al.*<sup>134</sup> to find differentially expressed proteins that hold promise in elucidating disease progression. Initial analyses found 498 differentially expressed proteins and highlighted chloride intracellular channel protein 1 (CLIC1), which was examined further and ultimately determined to promote tumorigenesis, making it an attractive therapeutic target. Proteogenomic and phosphoproteomic analysis was performed by McDermott *et al.*<sup>135</sup> to characterize mechanisms driving ovarian HGSC functions down to the post-translational level. Global proteomic analysis led to the identification of 10 706 proteins and combined results described a role of histone acetylation as a marker for homologous recombination deficiency, confirming an association earlier proposed by Zhang *et al.*<sup>130</sup> Phosphoproteomics data provided understanding into proliferation-induced replication stress and the impact it has on chromosomal instability in HGSC, implying that mitotic and cyclin-dependent kinases could serve as therapeutic targets after future experimental validation.

Label-free quantitation is frequently employed for ovarian cancer analyses, as the global overview it provides of the proteome allows researchers to identify multiple pathways for further targeted analyses. Chuffa *et al.*<sup>136</sup> used this approach to determine the influence of melatonin treatment on an *in vivo* model of ovarian cancer. Comparative proteomics analyses showed that downregulation of processes involved in cancer signaling was promoted, underlining molecular targets for therapeutic intervention while indicating the feasibility of melatonin supplementation for ovarian cancer patients. Another comparative analysis by Júnior *et al.*<sup>137</sup> explored the effects of P-MAPA, IL-12 or a combination immunotherapy of the two on the SKOV-3 ovarian cancer cell line. After confirming 532 proteins were identified across all groups, it was noted that combination therapy of P-MAPA and IL-12 was most efficient at regulating proteins involved in metabolic processes that may render cancer cells more vulnerable, suggesting that the use of the two therapies concomitantly is a plausible treatment strategy. Coscia *et al.*<sup>138</sup> used a quantitative, label-free approach in tandem with other quantitative strategies to probe the proteomes of platinum-resistant and -sensitive ovarian HGSC patient-derived tissues (Fig. 5). Multi-level quantitative analyses revealed cancer/testis antigen family 45 (CT45) as a prognostic factor through mediation of chemosensitivity, thereby exposing it as an immunotherapy target.

The quantitative tissue analyses outlined here provide multiple protein targets for the development of new targeted therapies. The role of a defective DNA damage response in ovarian cancer is well established, so the multiple studies highlighting histone

acetylation and its role in homologous recombination deficiency is supported by current literature and should be examined in therapeutic development.<sup>139</sup> Additional analyses that examine post-translational modifications simultaneously with proteomics should also be explored, as these studies may highlight other processes outside the DNA damage response that promote cancer progression. The experiments above outline the utility that quantitative proteomic approaches hold in advancing the knowledge of the ovarian cancer field.

### Biofluid analyses

Quantitative analyses that inspect biofluids of ovarian cancer samples provide valuable information about potential biomarkers that allow for earlier detection and diagnosis, a current area of the ovarian cancer field that is in dire need of new research breakthroughs. Isobaric labeling of ovarian biofluids allow scientists to relatively quantify biomarkers that may otherwise go undetected or are lost during depletion of abundant serum proteins such as albumin. Zhang *et al.*<sup>140</sup> profiled exosomes derived from patient plasma using the TMT tagging strategy. When the 225 proteins identified across all samples were quantitatively compared, proteins associated with the coagulation cascade were found to be differentially expressed and may therefore be promising diagnostic factors for ovarian cancer. Zhang *et al.*<sup>141</sup> went on to further profile circulating exosomes of late-stage cancer patients using iTRAQ. After validation, they determined that apolipoprotein E (ApoE) multiplexed with epithelial cell adhesion molecule (EpCAM), plasminogen (PLG), serpin family C member 1 (serpinC1) and complement component 1q (C1q) were able to accurately diagnose ovarian cancer. It was also noted that activation of coagulation cascades was increased in the ovarian cancer cohort due to increased Factor X levels, demonstrating the impact that tumor-derived extracellular vesicles may have on other biological processes. Swiatly *et al.*<sup>142</sup> examined iTRAQ-labeled serum proteins from healthy control, benign ovarian tumor and ovarian cancer patients. Five proteins were found to be differentially expressed within the ovarian cancer group, and three of these coupled to current biomarkers CA125 and HE4 improved diagnostic discrimination between benign and malignant ovarian tumors. Russell *et al.*<sup>143</sup> used iTRAQ to screen preclinical serum samples for detection of early stage biomarkers and initially identified 90 differentially expressed proteins in ovarian cancer cases. A second targeted analysis of 20 selected candidates revealed vitamin K-dependent protein Z (VKDP), an anticoagulant not previously associated with ovarian cancer, as either a novel independent early detection biomarker or concomitantly with CA125 to increase differential diagnostic capabilities.

Although label-free analyses suffer from longer instrument times and potential run-to-run variability, they provide the greatest profiling depth of the multiple quantitative strategies and are vital to finding new ovarian biomarkers. Barnabas *et al.*<sup>144</sup> performed deep proteome profiling of 187 uterine liquid biopsy-derived microvesicles to identify early detection biomarkers. Machine learning algorithms identified a 9-protein



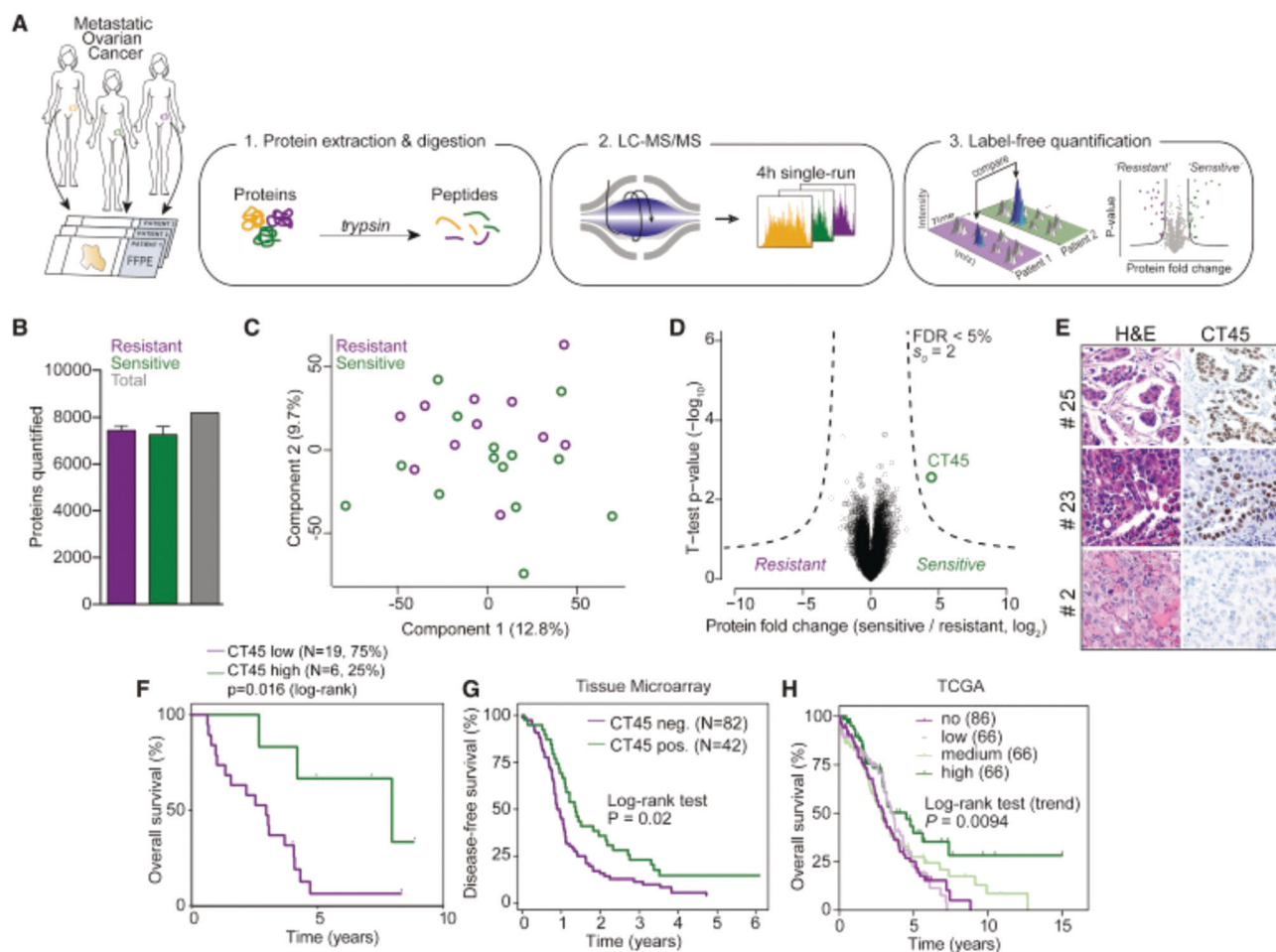


Fig. 5 Analysis by Coscia *et al.*<sup>138</sup> to determine proteomic differences in ovarian cancer tissue samples either resistant or sensitive to platinum-based chemotherapeutics. This strategy identified CT45 as a chemosensitivity modulator and demonstrates the ability of quantitative methods to identify factors that play a role in therapeutic resistance. Reprinted with permission.

signature that correctly identified all Stage I lesions, demonstrating the strength of the panel for future use in early diagnosis. Zhang *et al.*<sup>145</sup> studied the plasma proteins to isolate biomarkers related to chemoresistance of postoperative recurrence. These experiments found a total of six dysregulated proteins that could serve as predictive biomarkers for chemoresistance in ovarian cancer patients. The combination of plasma proteomics and metabolomics was utilized by Ahn *et al.*<sup>146</sup> to discover new molecular signatures of ovarian HGSC. Differential expression of 34 metabolites and 197 proteins was found, with three proteins (phosphopantothenoylcysteine synthetase (PPCS), peripheral myelin protein 2 (PMP2) and tubulin beta class I (TUBB)) and two metabolites (L-carnitine and PC-O) related to the carnitine system established as potential markers of cancer plasticity. Hüttenhain *et al.*<sup>147</sup> created a biomarker development strategy for large-scale SRM studies in ovarian cancer plasma samples. After developing a 5-protein signature for ovarian cancer and testing it against the current ELISA-based standard for biomarker tests, it was found that the SRM-based method had sensitivity measurements that exceeded the current ELISA standard, validating its potential for clinical development and use.

Rauniyar *et al.*<sup>148</sup> also used a more targeted approach, combining data-independent acquisition methods with PRM to improve identification of ovarian cancer serum biomarkers. They demonstrated that ApoA-IV is a more reliable biomarker than previously determined by immunological assays in addition to the identification of C-reactive protein, transferrin and transthyretin as other available ovarian serum markers. Overall, this study validated the use of quantitative mass spectrometry as a more sensitive and reliable method of quantitation compared to immunological-based procedures.

While the quantitative research mentioned here has progressed ovarian cancer research, continuing studies are still necessary to delve deeper into specific mechanisms of novel markers identified. During the review process, many of the identified studies had a tissue-based proteomics approach and minimal studies focused on biofluid samples (Table 4). More studies focusing on the use of biofluids in ovarian cancer research are critical in the development of novel biomarkers for earlier detection and treatment, and the lack of literature compared to tissue-based studies highlights a current area for further quantitative experimentation in ovarian cancer.



Table 4 Summarized selection of ovarian cancer biomarkers

Proposed biomarker	Source	Findings
Calcium-activated chloride channel 1 (CLCA1) <sup>127</sup>	OV-90 cells	Affects cancer cell regulation
Insulin-like growth factor 2 (IMP2) and minichromosome maintenance complex component 2 (MCM2) <sup>131</sup>	HGSC and endometrial tissue	Modulators of rapid high-grade serous cancer growth
Fibronectin and vitronectin <sup>133</sup>	Peritoneal mesothelial cells	Increased expression promotes cancer cell invasion
Chloride intracellular channel protein 1 (CLIC1) <sup>134</sup>	Tissue	Determined to promote tumorigenesis
Histone acetylation <sup>130,135</sup>	Tumor tissue	Marker for homologous recombination deficiency
Phospholipidoleate-palmitoleate anhydride (P-MAPA), interleukin 12 (IL-12) <sup>137</sup>	SKOV-3	Combination immunotherapy is a plausible treatment strategy
Cancer/testis antigen family 45 (CT45) <sup>138</sup>	Tissue	Found to be a prognostic factor
Apolipoprotein E (ApoE), epithelial cell adhesion molecule (EpCAM), plasminogen (PLG), serpin family C member 1 (serpinC1) and complement component 1q (C1q) <sup>141</sup>	Circulating exosomes	Diagnostic markers of ovarian cancer
Vitamin K-dependent protein Z (VKDP)	Preclinical serum	Novel, early detection biomarker
Phosphopantothencycysteine synthetase (PPCS), peripheral myelin protein 2 (PMP2) and tubulin beta class I (TUBB) <sup>146</sup>	Blood, plasma	Potential markers of cancer plasticity
Apolipoprotein IV (ApoA-IV) <sup>148</sup>	Serum	More reliable biomarker compared to benchmark proteins

In particular, studying the microvesicular proteome for the discovery of novel biomarkers has shown great potential both here and in other quantitative applications. Profiling of extracellular vesicles may prove to be a vital key in the prevention of late-stage diagnosis and increasing the overall survival rate of patients diagnosed with ovarian cancer.

## Conclusions and future directions

The various quantitative strategies outlined here have demonstrated the growing utility of MS-based quantitation methods in cancer diagnosis and research. Quantitative analyses of prostate cancer have been frequently performed within the field due to the growing emergence of resistance to first-line treatments and false diagnoses associated with elevated PSA levels. Multiple members of the KLK family were identified as potential biomarkers and further strengthened when detected in combination with other proteins, suggesting their potential for clinical diagnosis. Targeted validation experiments in a cohort spanning all grades of prostate cancer as well as BPH should be performed before serious consideration is given to using these proteins as biomarkers. Pancreatic studies have been relatively successful in determining sets of robust biomarkers for diagnosis and patient stratification. APOA1, APOA4, IGF1, and CA19-9 have been indicated in numerous peer-reviewed studies as critical components for pancreatic cancer screening. Future analyses should focus on high throughput reaction monitoring to rapidly screen for these biomarkers. Breast cancer research has seen limited quantitative proteomics studies in recent years, so future efforts of those investigating new biomarkers and determining mechanisms of carcinogenesis should consider quantitative proteomics strategies in their analyses. The small number of studies highlighted here contribute potential protein panels useful for breast cancer screening, but more large-scale studies that confirm the utility of these proteins as biomarkers are necessary. Ovarian research has seen large numbers of tissue- and cellular-based

quantitation, but there is a lack of biofluid-based experiments. While tissue-based studies provide large amounts of information that guide knowledge of disease mechanisms, biofluid studies offer important insights that could facilitate the identification and development of protein biomarkers for clinical diagnosis. Due to the lack of biomarkers that detect ovarian cancer at an earlier stage, studies covering biofluids are critical and present an understudied area within the ovarian field.

A common drawback of the quantitative studies addressed is that these investigations only determine up- or downregulation of differentially expressed proteins at a single point in time. Time-course evaluations monitoring the differential expression and dynamic changes of these proteins over time could prove to be more useful, as these studies would explain how expression levels change within a single patient over time. In combination with the expression levels across varying disease severity, there is a potential to determine a critical expression level for each stage of cancer progression that determines not only if the patient has cancer, but also the severity of that cancer relative to biomarker concentration levels. Rapid analyses of cancer samples *via* targeted monitoring strategies offer benefits over current immuno-based assays such as ELISA, demonstrating the advantage of MS-based quantitation for detection and prolonged patient monitoring. Another strategy for improving cancer diagnosis is the integration of additional analyses, such as transcriptomics, metabolomics, or analysis of post-translational modifications and associated crosstalk. Many of the studies outlined here utilized a combined approach to their investigations, leading to the successful identification of a specific protein or process with altered expression in both datasets. These integrated approaches help scientists identify mechanisms driving cancer metastasis and treatment resistance, thus demonstrating their growing utility in future studies. Additional efforts should be made towards understanding communication within the tumor microenvironment, as much remains to be known about the interactions that help a tumor transition from localized to metastatic ability.



Finally, studies focusing on single-cell analyses should also be considered for future experiments, as the cellular diversity and heterogeneity provided from such examinations may prove to be critical in understanding specific mechanisms that allow pathogenesis to advance.

Taken together, this review highlights the utility of various quantitative strategies, their associated limitations, and some directions for novel applications in cancer diagnosis and cancer research. As instrumental capabilities continue to grow, it will become necessary for researchers to develop and validate higher throughput labeling strategies that accommodate deeper proteomic profiling. Regardless of the application, quantitative proteomics represents a premier avenue towards cancer biomarker detection, identification, and validation. Continued efforts in the coming years will certainly be centered on the utility of mass spectrometry-based biomarker detection in clinical settings and the development of point-of-care biomolecule screening.

## Author contributions

H. N. M., D. G. D., and L. L. wrote, edited and reviewed the manuscript.

## Conflicts of interest

There are no conflicts of interest to declare.

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