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REVIEW

Proteomic profiling of human plasma for cancer biomarker discovery

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Over the past decades, substantial advances have been made in both the early diagnosis and accurate prognosis of many cancers because of the impressive development of novel proteomic strategies. However, it remains difficult to standardize proteomic approaches. In addition, the heterogeneity of proteins in distinct tissues results in incomplete population of the whole proteome, which inevitably limits its clinical practice. As one of the most complex proteomes in the human body, the plasma proteome contains secreted proteins originating from multiple organs and tissues, making it a favorable matrix for comprehensive biomarker discovery. Here, we will discuss the roles of plasma proteome profiling in cancer biomarker discovery and validation, and highlight both the inherent advantages and disadvantages. Although several hurdles lay ahead, further advances in this technology will greatly increase our understanding of cancer biology, reveal new biomarkers and biomarker panels, and open a new avenue for more efficient early diagnosis and surveillance of cancer, leading toward personalized medicine.

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1 Introduction

In order to achieve a deep understanding of biological complexity in the postgenome era, systematic studies on proteins and their functions are underway. Proteomic technologies enable the large-scale and in-depth investigation of proteins, in particular, the screening of potential biomarkers in complex biological matrices in a high-throughput manner (Fig. 1). To date, proteomics has become a fundamental component for biological study and is recognized as a key method for novel biomarker discovery and personalized therapy [1], especially after the launch of the human plasma proteome project that was proposed at Human Proteome Organisation 2010 in Sydney [2]. As indicators of pathogenic processes and pharmacologic responses to a therapeutic intervention, biomarkers enable us to accurately predict pathophysiology as well as understand how it is altered by treatments. One of the most important issues is how to choose an appropriate material for analysis. With the purpose of clinical application for cancer early detection, a simple and generally accepted

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Abbreviations: AD, Alzheimer's disease; **AFP**, alpha-fetoprotein; **CEA**, carcinoembryonic antigen; **CRC**, colorectal cancer; **DDA**, data-dependent acquisition; **GVHD**, graft-versus-host disease; **HAP**, high-abundant protein; **HCC**, hepatocellular carcinoma; **IAC**, immunoaffinity chromatography; **IDH1**, isocitrate dehydrogenase 1; **LAP**, low-abundant protein; **LMW**, low molecular weight; **MBP**, metal-binding protein; **MSI**, MS imaging; **NGAL**, neutrophil gelatinase-associated lipocalin; **NSCLC**, nonsmall cell lung cancer; **PSA**, prostate-specific antigen; **uPAR**, urokinase plasminogen activator receptor

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Colour Online: See the article online to view Fig. 1 in colour.

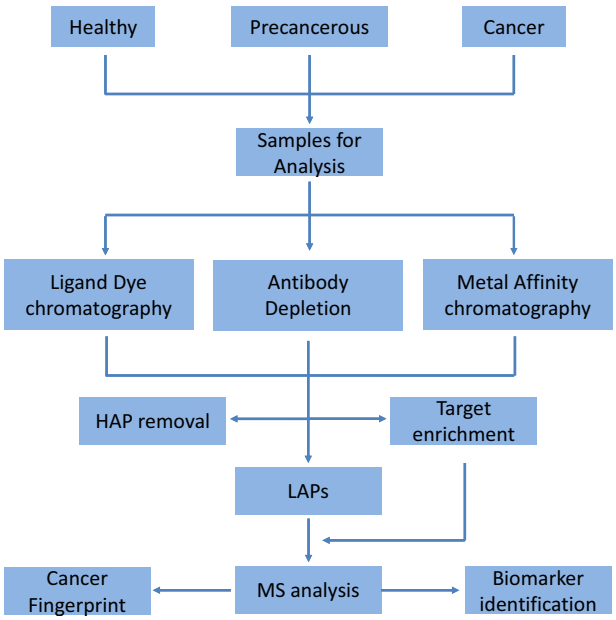

Figure 1. Schematic diagram of a representative plasma proteomics protocol for cancer biomarker discovery. Plasma is obtained from patients and a control cohort, followed by the enrichment of LAPs or removal of unwanted HAPs via various chromatographic techniques. Fractions are then introduced into appropriate MS-based protein analysis platforms, giving either characteristic fingerprints or allowing the identification of specific biomarker candidates.

noninvasive sampling method with wide coverage is desperately needed.

Blood plasma (in this review, the term plasma refers to both plasma and serum) has been considered as a suitable matrix for proteomic analysis for many reasons, with the promise of allowing full screening and identification of cancer biomarkers, with the potential to revolutionize cancer diagnosis and personalized medicine. Firstly, blood has a complex proteome that reflects many tissue proteome subsets [3]. There are over 60 000 miles of vessels including veins, arteries, and capillaries in the human body, where blood flows through every tissue and organ, "sampling" the physical condition of the patient [4]. Secondly, plasma is easy to obtain using standardized formats (standard operating procedures, SOPs) [5]. A noninvasive and acceptable sampling method is vital for widespread use in clinical evaluation, and blood collection is generally accepted and less painful compared with other tissue sampling methods such as bone marrow aspiration. Thirdly, the dynamic nature of plasma reflects diverse physical states of the patient, whereby it is possible to monitor disease progression spatially and temporally, leading toward a more comprehensive understanding of molecular events in tumorigenesis [5]. Lastly, examination of blood has long been introduced into routine biochemical assay, for which a number of key technologies have already been established. Valuable components that have been used for clinical treatment, such as human intravenous immune globulin, can be obtained from the plasma, making it a valuable and accessible biological resource [6]. Thus, taken together, proteomic profiling of human plasma, or suitable animal model systems, affords an attractive landscape for cancer biomarker discovery.

Proteins that are present in plasma originate from a variety of sources. Solid tissues, especially intestine and liver, secrete a large number of proteins that execute their functions in the plasma [7]. The most abundant protein, albumin, comprises approximately half of the total protein content of plasma [4]. While the high-abundant proteins (HAPs) seldom change on cancer initiation, low-abundant proteins (LAPs) secreted by tumor tissues have excellent potential as biomarkers. Intracellular proteins that function within cells may be also released into the blood because of tissue damage and cell death [8]. These tissue leakage products may serve as biomarkers reflecting tissue damage actually induced by tumor formation. Receptor ligands are secreted into the blood, and function either on the cell surface or within cells. As critical upstream signaling molecules, receptor ligands often play important roles in the activation of specific tumorigenesis-related pathways, thus being promising biomarkers for cancer detection [9]. Occasionally, invasive pathogens can bring foreign proteins into the blood stream. Although these exogenous proteins do not seem to associate with tumorigenesis, particular cancer-related infectious organisms may predict the risk of carcinogenesis [10, 11]. Most proteins have both their precursor forms and splice variants as well as PTMs (e.g., glycosylation, phosphorylation, glycosylation, acetylation, methylation, ubiquitination, acetylation, sumoylation, and hydroxylation) and abnormal processing may result in these forms being tumor specific [12]. Finally, there is an enormous repertoire of immunoglobulins (possibly over 10 million different sequences), some of which may be tumor related [13].

The advent of advanced proteomic techniques enables the extensive interrogation of the plasma proteome, which opens a window for novel cancer biomarker discovery. In this review, we briefly summarize the methodology used in plasma proteomic profiling and give some examples of plasma biomarkers or biomarker candidates. Such improvements in proteomic and related technologies will have wide-range application in cancer biomarker discovery and will form the basis for personalized medicine for the discovery and surveillance of this disease.

2 Current proteomic technologies for plasma proteome profiling

2.1 Sample fractionation and separation

The complexity of the human plasma proteome far exceeds the capability of present proteomic technologies, making it virtually impossible to deeply interrogate all plasma proteins in a sample in a single test. One of the widely adopted

strategies is to separate the sample into different fractions according to their unique physicochemical properties. A well-known example is 2D-PAGE, which separates the sample proteome into protein spots on a gel according to their mass and isoelectric point: many thousand proteins may be detected in a single gel image [14]. However, many of the proteins that have potential to be biomarkers are LAPs, whose presence may well be masked by the HAPs. Therefore, extensive fractionation of plasma to remove HAPs and enrich LAPs is typically required prior to proteome analysis [15].

2.1.1 Dye affinity chromatography

Many proteins can bind to the immobilized dyes allowing their purification (ligand dye chromatography), which is a rapid and inexpensive method for crude separation of the proteome [16]. Cibacron blue is a dye that is widely used for HSA removal. Generally, cibacron blue is covalently coupled to a suitable matrix (chromatographic support or magnetic beads), and then HSA can be absorbed and separated from other plasma proteins that flow through the matrix [17]. In addition, the binding of HSA to matrix is reversible. It has been reported that the ionic concentration and pH of the mobile phase are two critical factors affecting the efficiency of HSA binding, and HSA can be eluted via increasing pH from an acidic to a slightly alkaline level [18–20]. Dye affinity chromatography can be also used to enrich a protein of interest, exemplified by the collection of Mullerian inhibiting substance, a secreted glycoprotein in human plasma [21], or the purification of the A33 cell surface antigen that is expressed in more than 95% of human colon cancers [22].

However, while giving significantly increased purification factors when used in multidimensional purification protocols, this fractionation method is not totally specific, which limits its use in clinical applications. For example, not only albumin but also other molecules such as NADH and FAD and a number of enzymes are able to interact with cibacron blue [23, 24], which may lead to the loss of potential biomarkers when the albumin is removed from the matrix.

2.1.2 Metal affinity chromatography

Metal-binding proteins (MBPs) bind their substrates and exert their functions through interaction with a particular metal ion so as to maintain structure and perform effect catalysis [25]. Due to their important roles in both normal physiology and pathogenesis, it is important to investigate MBPs and validate their potential roles as cancer biomarkers. Accordingly, IMAC has been applied for MBP enrichment [26]. It has been shown that IMAC processing of human serum allows numerous proteins to be separated according to their different affinity to the corresponding metal ions including cadmium, nickel, zinc, copper and lead [27]. Protein C is a human plasma protein that circulates as an inactive zymogen, whose deficiency may lead to excessive clotting and thrombosis [28, 29]. It has been reported that protein C deficiency may lead to thrombotic complications in breast cancer patients [30]. Making use of its copper-binding property, an IDA-Cu column was used to separate protein C from human plasma, with high activity and recovery without immunoglobulin contamination [29]. In addition, metal affinity chromatography is also suitable for the elimination of some HAPs. Metal-chelated beads (including copper, nickel, zinc, and cobalt) allowed a one-step purification of immunoglobulin G from human plasma (>90% purity) [31], although it was reported that copper-based IMAC may also bind low levels of γ-globulin when enriching the immunoglobulin G [32].

2.1.3 Antibody affinity chromatography

Based on the universal mechanism of highly specific antibody–antigen interaction, antibody affinity chromatography, also known as immunoaffinity chromatography (IAC), has become a popular fractionation method for proteome purification [33]. IAC can serve as an efficient tool for the depletion of many of the HAPs in plasma. In a comparison of four alternative albumin removal techniques, antibody affinity chromatography was shown to be the most efficient, allowing visualization of the highest number of protein spots by 2DE after albumin removal [34]. A common HAP depletion strategy is to mix multiple antibodies in an optimal ratio to remove multiple unwanted proteins simultaneously [35, 36]. Such columns are commercially available (e.g., Agilent MARS, Sigma-Aldrich ProteoPrep 20). In a recent extension of the ultradepletion strategy, plasma was fractionated using dual ion exchange columns (protein repetitive orthogonal offline fractionation) to simplify the proteome, chicken polyclonal IgYs were produced against each fraction, and the purified antibodies were used in an immunodepletion column. Using this support for immunodepletion of human plasma, a total of 165 nonredundant proteins were identified, many being proteins of low abundance [37]. This column has now been used in tandem with an Agilent MARS column to deplete 200 HAP allowing deep mining of the plasma proteome (M. Baker, personal communication). However, it must be remembered that LAPs may bind, either specifically or nonspecifically, to HAPs and thus may be simultaneously removed during HAP depletion, leading to loss of potential biomarkers [36]. In particular, a number of LAPs have been found to be associated with HAPs such as immunoglobulins and albumin [38]. Procedures such as salting-out and molecular sieve filtration may assist the selective remove of HAPs, but need to be further optimized for micropurification [39].

LAPs of interest can also be directly targeted for immunoaffinity enrichment. In a recent publication, the method was applied to the quantitative profiling of cellular Ras and EGFR protein isoforms, as well as serum amyloid A isoforms in plasma [40] and the purification of phosphorylated proteins using antiphosphoprotein antibodies has also been described [41].

An immunoaffinity method for quantitation of peptides in complex digests (called SISCAPA: Stable Isotope Standards and Capture by Anti-Peptide Antibodies) uses antiproteospecific peptide antibodies immobilized onto 100 nL nanoaffinity columns to enrich specific peptides along with spiked stable isotope labeled internal standards of the same sequence followed by detection using MRM [42].

IAC is certainly not without its drawbacks, and the choice of a suitable antibody is fundamental. Polyclonal antibodies recognize different epitopes on the same antigen with distinct affinity. Thus, they have the potential to pull down proteins or protein complexes, even if one or more epitopes are masked. However, they tend to have higher nonspecific binding and it can be difficult to determine pan-elution conditions to release proteins from the numerous epitopes simultaneously [43]. Additionally, polyclonals have a finite supply and vary from animal to animal. It is therefore difficult to obtain a consistent quality of reproducible antibodies from multiple batches or lots. By contrast, monoclonal antibodies possess specific affinity an individual epitope and are a renewable resource [44]. The relatively higher cost of production impacts on their application for clinical use, although the use of highly roboticized high-throughput production platforms will drastically reduce these costs [45].

2.2 Sample analysis

MS-based proteomic approaches lay the foundation for proteome analysis, providing powerful tools to simultaneously study thousands of proteins in a fast, sensitive, and high-throughput manner [46]. The basic principle is that samples are ionized to yield ions with different m/z, which possess different motor patterns in the same electric or magnetic field [47]. The separated ions are detected to allow the production of a mass spectrum and subsequent identification of protein samples from the characteristic mass and fragmentation pattern [47, 48].

2.2.1 MALDI–TOF MS

A number of key technologies have emerged for the routine mass spectrometric analysis of proteins, peptides, and protein fragments. Foremost among these are MALDI–TOF and LC–MS/MS. In the MALDI–TOF MS approach, proteins are cocrystallized with appropriate UV-absorbing compounds (e.g., 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid, or DHB) on a target plate onto which a pulsed-UV laser beam is focused to produce ions, which are introduced into a TOF in a flight tube to measure the time taken to travel a fixed distance. From these data, the m/z can be determined and a spectrogram generated. For example, 2D-DIGE has been coupled with MALDI–TOF MS to analyze the plasma proteome of patients with cervical squamous cell carcinoma, with the results showing a distinctive fingerprint compared with normal controls [49]. In an extension of this technology, affinity reagents (e.g., antibodies and aptamers) have been attached to the chip for selective detection of biomarkers [50].

A novel technique that combines proteomics and histology, termed MALDI MS imaging (MSI), has been developed for molecular profiling and biomarker discovery [51]. MALDI MSI not only quantifies the targeted proteins, but also maps this information to the corresponding tissue areas giving information on their spatial distribution [51, 52]. This has led to the discovery of several cancer biomarkers, facilitating more accurate diagnosis and prognosis [53, 54]. Chan et al. have shown that S100A11 and ferritin light chain are two potential biomarkers of renal cell carcinoma using MALDI MSI based proteomic analysis of four surgical tissue samples [55]. Another MALDI MSI study revealed aberrant phosphatidylinositol expression profiles in prostate cancer clinical samples compared with benign prostate epithelium [56], further illustrating that MALDI MSI can serve as a powerful tool for cancer biomarker discovery.

Attempts are being made to identify biomarkers on the basis of protein pattern analysis. This requires (1) the choice of appropriate healthy specimens as negative controls to rule out the interference of other factors that may cause ambiguous protein expression patterns changes, (2) an MS/MS process to identify biomarker candidates, and (3) the biomarker/biomarker panel validation in large patient cohorts to demonstrate their reliability before clinical application [57].

2.2.2 LC–MS/MS

Advances in LC–MS/MS-based proteomic technologies over the last decade have enabled the identification and quantification of numerous potential biomarkers through either bottom-up or top-down approaches. In the bottom-up strategy, protein samples are digested into peptides (either enzymatically using enzymes such as trypsin, chymotrypsin, LysC, or AspN, or chemically, e.g., CNBr or formic acid) prior to separation of the peptide fragments by HPLC, where peptides are eluted into the mass spectrometer according to their respective physicochemical properties [58, 59]. Reversed phase and/or ion exchange modes are most commonly used. Eluted peptides are ionized in the mass spectrometer and separated according to their unique m/z. In the initial MS scan, the abundance and m/z of all ions eluting at a given time are measured. In the MS2, some or all detected ions are further fragmented allowing the specific peptide sequence to be obtained: the abundances and m/z of the fragments are measured and recorded [60]. By contrast, the top-down approach allows the direct analysis of intact proteins without an enzymatic digestion procedure [61]. In particular, the top-down approach allows degradation products, sequence variants, and combinations of PTMs to be precisely identified. One of the representative technologies is ITMS, where intact proteins

are ionized and trapped in a combination of electric or magnetic fields, followed by sequential release and measurement [62, 63]. In particular, Orbitrap MS has become a prominent ITMS-based technique due to its high resolution, mass accuracy, and sensitivity [64]. Orbitrap MS has revealed several plasma proteins including thrombospondin-1 and 5, alpha-1B-glycoprotein, as promising biomarkers of breast cancer [65]. Recently, Cheon et al. performed a top-down analysis of the low molecular weight (LMW) proteome in human plasma, and identified many LMW proteins, including platelet factor 4 that is a potential biomarker for colorectal cancer [66]. Six of the LMW proteins were further validated by Western blot analysis.

2.2.3 Multiplex protein quantitation

Depending on the proteomic strategy used, protein quantitation can be either relative or absolute [60]. Data-dependent acquisition (DDA) strategies have been adopted to acquire relative quantification by comparison with spectral libraries, allowing a broad breadth of protein analysis [67, 68]. However, only the most abundant protein subsets can be detected through this approach, and the LAPs that have the potential to be biomarkers are frequently absent [68]. Therefore, in its current format, DDA would not appear to be the preferential tool for plasma proteomic profiling.

MRM (also known as SRM) is capable of absolute quantification and has already contributed to the identification and validation of over a dozen plasma biomarkers [69]. In this technology, using a triple quad mass spectrometer, a proteotypic peptide is selected in Q1. This is then fragmented by collisional excitation with a neutral gas in Q2 and a small number of sequence-specific transition ions analyzed in Q3. The combination of the LC retention time with the proteoprecursor/fragment ion pairs gives a highly specific assay. If a synthetic, stable isotope labeled peptide is used as an internal standard, the concentration can be measured by comparison of the signals from the endogenous forms. It is possible to routinely measure a large number of potential biomarkers in a single run, significantly facilitating their validation. However, MRM only measures preselected proteins and the specific operating conditions have to be carefully optimized [70]. To assist in this, a database (SRMatlas; http://www. srmatlas.org/) that presents a validated compendium of highly specific assays that enable quantification of 99.7% of the 20 277 annotated human proteins has been established. In an extension of this technology (PRM, Q3 of the triple quadrupole is substituted with a high-resolution and accurate mass analyzer allowing the parallel detection of all target product ions in one, concerted high-resolution mass analysis [71].

Recently, data-independent acquisition (including SWATH [72]) has emerged as a novel protein quantification strategy that combines the advantages of DDA and MRM through repeatedly and comprehensively interrogating all of peptides present in a sample [73]. In this technology, consecutive, adjacent precursor ion windows, termed swathes, are rapidly and recursively scanned over the full precursor ion m/z range of the peptide components allowing fragment ion spectra of all precursors within a user defined retention time versus m/z window to be generated. It has been reported that the analysis breadth and depth of data-independent acquisition is close to that of DDA and MRM, respectively [60], highlighting its potential as a generic approach for protein quantification.

Given that the protocols and mechanisms of MS-based proteomic technologies have been recently extensively described [74–76], we have focused on their application in plasma biomarker discovery (Section 3, below).

3 Application of plasma proteomics to cancer biomarker identification

3.1 Prostate cancer

Prostate cancer is the most common cancer among men worldwide [77]. As a gland that synthesizes and secretes biofluid, it is highly likely it will release potential biomarkers during malignant transformation. Prostate-specific antigen (PSA) is a well-characterized biomarker, which has been shown to indicate a risk of prostate cancer when its concentration in plasma is above 4 ng/mL [78]. However, elevated levels of plasma PSA are also found in prostatitis and benign prostatic hyperplasia, which may lead to an over treatment of prostate cancer [79]. Even so, PSA is still widely used as a biomarker for prostate cancer detection due to the lack of better candidates. Already proteomic analysis of plasma has identified a list of potential biomarkers associated with prostate cancer progression. One study used 2DE coupled with DIGE (2D-DIGE) proteomic technology and metabolomic analysis to analyze plasma derived from a prostate tumor-implanted mouse xenograft model. The results suggested that serotransferrin precursor and gelsolin might be two promising biomarkers [80]. Another study reported that the decreased activity of dipeptidyl peptidase 4, but not its abundance, contributed to the metastatic behavior of prostate cancer, suggesting that dipeptidyl peptidase 4 might be an indicator of prostate cancer metastasis [81]. By proteomic profiling of the plasma from a Pten conditional knockout mouse model, 13 new potential biomarkers of metastatic castration-resistance prostate cancer were identified. However, these need to be further validated before application as clinical cancer biomarkers [82]. As discussed above, apart from specific protein biomarkers, characteristic fingerprints of plasma proteomes can also contribute to the biomarker identification and early diagnosis of this cancer [83].

3.2 Liver cancer

Liver cancer or hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide [84].

Alpha-fetoprotein (AFP) is a plasma glycoprotein whose sugar chains are modified when hepatocytes undergo malignant transformation [85]. It has been shown that two forms of AFP, namely AFP L3 and AFP P4 + P5, are potential biomarkers to distinguish HCC from cirrhosis [85]. In addition, proteomic analysis of plasma extracted from HCC and cirrhosis patients revealed that osteopontin, an extracellular matrix protein, was aberrantly upregulated in plasma from HCC patients, indicating that osteopontin has the potential to be a biomarker of HCC [86]. In another study, in order to investigate glycosylated proteins as potential biomarkers in HCC progression, fucosylated proteins were enriched from the plasma of HCC, hepatitis B virus (HBV) infected, and cirrhosis cases as well as a healthy patient cohort using the fucose-specific aleuria aurantia lectin. The fucosylated fractions were then analyzed by MRM, showing that ceruloplasmin (CERU), alpha-1-antitrypsin (A1AT), alpha-1-antichymotrypsin (AACT), and alpha-1-acid glycoprotein 1 (A1AG1) were four potential HCC biomarkers [87]. Hepatitis C virus contributes to cirrhosis and HCC progression, and a proteomic profiling of plasma extracted from hepatitis C virus induced HCC or cirrhosis cases displayed a number of HCC biomarker candidates, among which galectin-3 binding protein and vitronectin were further validated by ELISA test in a large population [88].

3.3 Lung cancer

Lung cancer is one of the deadliest human cancers worldwide [89]. Patients with advanced nonsmall cell lung cancer (NSCLC) have an extremely low survival rate [90], and biomarkers, or panels of biomarkers, are required for the early diagnosis of this malignancy before metastasis occurs. Comparison of the plasma from several mouse lung cancer models using in-depth quantitative proteomics identified a potential biomarker titf1, also known as Nkx2-1 [91]. In an antibody-based proteomics approach (antibodies are one of the three pillars of the Human Proteome Organisation approach [92]), large monoclonal antibody libraries were used to screen for plasma biomarkers of NSCLC patients. The results showed that complement factor nine , haptoglobin, complement factor H, antichymotrypsin, and leucine-rich α-2 glycoprotein 1 could serve as lung cancer biomarkers [93]. Although some of the biomarkers are not specific (e.g., complement factor H and antichymotrypsin have also been found to be involved in the progression of bladder and pancreatic cancer, respectively [94, 95]), those five potential biomarkers may predict risk of lung cancer as part of a panel, but not individually. It is now generally accepted that, due to the heterogeneity of many diseases, biomarker panels may be required to give adequate sensitivity and specificity. 2D-DIGE coupled with MALDI–TOF/TOF MS proteomic analysis revealed 28 differentially expressed proteins in lung cancer tissue. One of the biomarker candidates, isocitrate dehydrogenase 1 (IDH1), was further confirmed to accumulate in the plasma of NSCLC patients, and RNA interference against IDH1 was shown to decrease the growth of xenograft tumors in a mouse model, indicating that IDH1 may be a promising biomarker of lung cancer [96].

3.4 Pancreatic cancer

Pancreatic cancer remains one of the most deadly cancer types worldwide [97]. Given that those patients diagnosed with pancreatic cancer have an extremely low survival rate (usually <1 year), a reliable biomarker for early detection of this malignancy is desperately needed [98]. LRG was identified as a potential biomarker of pancreatic cancer, using antibody affinity depletion of high-abundance proteins followed by anion-exchange chromatography and 2D-DIGE followed by MS analysis [99]. Several plasma proteins, including transthyretin, apolipoprotein A-I, and plasma retinol-binding protein, have been found to be differentially expressed in plasma from pancreatic cancer patients and healthy controls, but this has not yet been further validated [99]. Faca et al. performed proteomic profiling of the plasma extracted from a pancreatic cancer mouse model, and 165 proteins were shown to present at elevated levels in the pancreatic cancer group compared with the corresponding controls [100]. Five selected proteins including neutrophil gelatinase-associated lipocalin (LCN2), lithostathine 1 (REG1A), regenerating islet-derived protein 3 (REG3), tissue inhibitor of metalloproteinase 1 (TIMP1), and insulin-like growth factor binding protein 4 (IGFBP4) were further evaluated in clinical blood samples from patients with pancreatic cancer, with promising results [100]. In addition, a recent study using both CE coupled to MS (CE-MS) and Chip-MS/MS reported that following plasma sampling from pancreatic cancer mouse model and cells, distinct peptide signatures identifying different stages of pancreatic cancer could be established [101].

3.5 Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer worldwide [102]. It has been reported that a combination of metalloproteinase-1 (TIMP-1) and carcinoembryonic antigen (CEA) may have predictive potential, based on a prospective, population-based study including 4509 individuals [103]. Interestingly, neither biomarker in its own right is particularly useful, with poor sensitivity and selectivity, although CEA is effectively used to monitor recurrence. Neutrophil gelatinase-associated lipocalin (NGAL), also known as LCN2, was shown to be a potential biomarker of colorectal cancer by proteomic profiling of 526 solid tissue and plasma samples [104]. However, NGAL may not be a specific biomarker of colorectal cancer since the plasma level of NGAL is also associated with pancreatic cancer [105]. Another proteomic screening of plasma proteins comparing colorectal adenoma and colorectal carcinoma cases revealed a number of

differentially expressed proteins, including hemoglobin subunit β (HBB), α-2-HS-glycoprotein (AHSG), transthyretin (TTR), zinc-α-2-glycoprotein (ZAG), and retinol-binding protein 4 (RBP4), suggesting that they hold the potential to be biomarkers for colorectal cancer [106]. Interestingly, a number of these proteins were also identified in a quantitative human fecal proteomics study using multiplexed MRM analysis [107]. Through protein microarray analysis, 43 of 8000 proteins were found to be differentially expressed in plasma samples from colorectal cancer patients, among which three proteins, PIM1, MAPKAPK3, and ACVR2B, were further validated by immunoblotting and immunohistochemistry [108]. In a recent study, the Olink Proseek® Multiplex Oncology I proximity extension assay, which enables high-throughput, multiplex immunoassays that measure 92 proteins across 96 samples simultaneously using only 1 μL of serum or plasma was used to analyze the plasma of CRC patients and control samples, showing CEA, IL-8, and prolactin as three potential biomarkers capable of predicting different stages of CRC [109].

A recent series of proteomic-based studies have revealed the importance of the urokinase plasminogen activator receptor (uPAR) in colorectal cancer biology. By coupling coimmunoprecipitation using highly specific monoclonal antibodies with MS analysis, the interactome was revealed comprising a number of known interactants of the urokinase receptor, as well as many novel uPAR protein binding partners, including the epithelial restricted αvβ6 integrin [110]. This interaction was further confirmed using orthogonal *in cellulo* and *in vitro* functional proteomic approaches [111]. The clinical importance of uPAR was established, when, using a prospective registry of consecutive rectal cancers between 1988 and 2001 from the Concord Hospital, Sydney, Australia, for which all nondeceased patients were followed for a minimum of 5 years; it was shown, using highly specific monoclonal antibodies, that epithelial and stromal cell uPAR expression differentially correlates with survival in stage B and C rectal cancer patients [112]. Significantly this differentiates a subset of patients who need no further treatment from a group that has a poorer prognosis.

3.6 Gastric cancer

Gastric cancer is another leading cause of cancer mortality worldwide with median survival of around 1 year for metastatic disease [113]. This low survival rate is partly due to the lack of effective biomarkers, for which plasma proteomics could be revealing [114]. Utilizing IAC to deplete HAPs prior to 2DE and MS-based proteomic profiling, three protein precursors including haptoglobin precursor, complement C4-B precursor, and complement factor I precursor showed distinct expression patterns between plasma samples from patients with gastric cancer and those of healthy controls [115]. Further analysis by Western blot confirmed that the expression of complement factor I precursor in plasma is downregulated in a gastric cancer cohort, suggesting it may be a potential biomarker for the diagnosis of gastric cancer [115]. Chong et al. reported that plasma proteomics profiling of a mouse xenograft model revealed that interalpha-trypsin inhibitor heavy chain H3 was highly expressed in the gastric tumor bearing mice compared with control mice. This was further validated through proteomic analysis of 167 clinical plasma samples [116]. Similarly, apolipoprotein A-I (APOA1) was found to be downregulated in the plasma of gastric mouse xenograft models, suggesting that APOA1 may also be a biomarker of gastric cancer [117]. However, APOA1 has also been found to be involved in the progression of pancreatic cancer as mentioned above [99], indicating that it might be a nonspecific tumor indicator, although it may well be useful as part of a biomarker panel.

Further details of these studies are given in Table 1.

3.7 Other disease biomarkers in plasma

Proteomic approaches have also been successfully applied in biomarker discovery for other disease. One example is for Alzheimer's disease (AD), a progressively neurodegenerative disorder that lowers the life quality of many elderly patients [118, 119]. An iTRAQ-MS study of AD and the control plasma proteome showed that Complement 4a, an inflammatory-related protein, is upregulated in the plasma of AD patients. This was further validated by immunoblot and ELISA [120]. Additionally, YKL-40 was shown to be potential biomarker of AD through LC–MS/MS-based proteomic profiling method, and it was validated in another cohort [121]. Graft-versus-host disease (GVHD) is an immunological rejection response that severely limits the use of tissue grafts, such as allogeneic haematopoietic stem cell transplantation [122]. Proteomic analysis of GVHD patient blood and control samples have shown that REG3α was upregulated in the plasma of GVHD patients, and high REG3α concentrations predicted high risk of nonrelapse mortality [123]. Recently, using LC–MS/MS proteomic analysis of plasma samples, a four-biomarker panel composed of ST2, CXCL9, MMP3, and osteopontin was established and shown to efficiently predict and diagnose GVHD [124]. In summary, proteomic technologies are proving to be powerful tools of plasma biomarker discovery both in oncology and other diseases.

4 Conclusion

Cancer development is an extremely complex and heterogeneous event, making it a challenging task to elucidate key effectors among numerous putative factors in a reasonable time frame. However, recent rapid advances in proteomic technologies [125] are facilitating the analysis of protein signaling pathways in a high-throughput manner [8], which is greatly accelerating biomarker discovery and revolutionizing the landscape for disease treatment and diagnosis [126].

Table 1. Details of cancer biomarker studies presented in Section 3

Cancer	(Potential) plasma biomarkers	Peptide fractionation methods	Protein quantitation methods	Number of patient samples	Reference
Prostate cancer	PSA	IAC	RIA	366	[78][a)]
	Serotransferrin precursor Gelsolin	2DE	LC–MS/MS	–	[80][a)]
Liver cancer	AFP L3 AFP P4 + P5	LAC	RIA	361	[85][a)]
	Osteopontin	2DE	LC–MS/MS	312	[86]
	CERU A1AT AACT A1AG1	LAC	MRM MS	40	[87][a)]
	G3BP VTN	2D DIGE	MALDI–TOF MS	67	[88][a)]
Lung cancer	Nkx2-1	AEC and RPC	LC–MS/MS	77	[91]
	C9 Hpt CFH ACT LRG1	LAC	Ion trap MS	301	[93]
	IDH1	2D DIGE	MALDI–TOF MS	161	[96][a)]
Pancreatic cancer	LRG transthyretin APOA1	AEC and 2D DIGE	LC–MS/MS	10	[99]
	LCN2 REG1A REG3 IGFBP4	AEC and RPC	LC–MS/MS	60	[100]
Colorectal cancer	TIMP-1 CEA	IAC	Microarray	4509	[103][a)]
	TTR ZAG RBP4	2DE	MALDI–TOF MS	60	[106][a)]
	PIM1 MAPKAPK3 ACVR2B	–	Microarray	20	[108][a)]
Gastric cancer	C4-B precursor CFI	2DE	MALDI–TOF MS	20	[115]
	ITIH3	HPLC	LC–MS/MS	167	[116]
	APOA1	HPLC	LC–MS/MS	–	[117][a)]

a) Immunodepletion techniques were not used in these protocols.
LAE: lectin-affinity chromatography; AEC: anion exchange chromatography; C9: complement factor nine; CFI: complement factor I; CFH: complement factor H; G3BP: galectin-3 binding protein; Hpt: haptoglobin; ITIH3: interalpha-trypsin inhibitor heavy chain H3; LRG1: leucine-rich α-2 glycoprotein 1; RPC: reversed-phase chromatography; IAC: immunoaffinity chromatography; VTN: vitronectin.

Initiatives such as those recently emanating from multidisciplinary teams (e.g., [127–129]) have enabled very deep profiling of clinical samples with high identification and quantification accuracy, allowing the identification of several disease proteomes (e.g., breast, ovary, and colon). Additionally, large-scale antibody-based initiatives (e.g., [130, 131]) have also revealed a tissue-based map of the human proteome. Such studies give exciting clues to further potential disease-related biomarkers.

Human plasma has been recognized as an excellent matrix for proteomic profiling due to many unique properties, including its general acceptance and the minimally invasive way in which it can be obtained, the wide range of organs that are "sampled", and its routine use in clinical pathology. In spite of significant progress to date, however, some limitations still need to be addressed. The current Plasma Proteome Database (http://www.plasmaproteomedatabase.org/) lists 10 546 proteins: however, plasma concentration ranges are only given for 1278 and validated MRM protocols only exist for 279. Future prospects lie in the further optimization of plasma extraction and storage, the development of novel methods to mine deeper into the proteome (e.g., improved HAP depletion), further improvements in MS-based and associated technologies, and, importantly robust high-throughput methods for the rigorous validation of potential biomarkers in a large cohort. Such studies will clearly play a

key role in advancing personalized medicine with its associated benefits to the global community [132].

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