Journal for ImmunoTherapy of Cancer

Role of mass spectrometry-based serum proteomics signatures in predicting clinical outcomes and toxicity in patients with cancer treated with immunotherapy

Yeonggyeong Park,[1] Min Jeong Kim,[1] Yoonhee Choi,[2] Na Hyun Kim,[3] Leeseul Kim,[4] Seung Pyo Daniel Hong,[1] Hyung-Gyo Cho,[1] Emma Yu,[5] Young Kwang Chae[6]

To cite: Park Y, Kim MJ, Choi Y, *et al*. Role of mass spectrometry-based serum proteomics signatures in predicting clinical outcomes and toxicity in patients with cancer treated with immunotherapy. *Journal for ImmunoTherapy of Cancer* 2022;**10**:e003566. doi:10.1136/jitc-2021-003566

Accepted 21 January 2022

For numbered affiliations see end of article.

Correspondence to
Dr Young Kwang Chae; young.chae@northwestern.edu

ABSTRACT

Immunotherapy has fundamentally changed the landscape of cancer treatment. However, only a subset of patients respond to immunotherapy, and a significant portion experience immune-related adverse events (irAEs). In addition, the predictive ability of current biomarkers such as programmed death-ligand 1 (PD-L1) remains unreliable and establishing better potential candidate markers is of great importance in selecting patients who would benefit from immunotherapy. Here, we focus on the role of serum-based proteomic tests in predicting the response and toxicity of immunotherapy. Serum proteomic signatures refer to unique patterns of proteins which are associated with immune response in patients with cancer. These protein signatures are derived from patient serum samples based on mass spectrometry and act as biomarkers to predict response to immunotherapy. Using machine learning algorithms, serum proteomic tests were developed through training data sets from advanced non-small cell lung cancer (Host Immune Classifier, Primary Immune Response) and malignant melanoma patients (PerspectIV test). The tests effectively stratified patients into groups with good and poor treatment outcomes independent of PD-L1 expression. Here, we review current evidence in the published literature on three liquid biopsy tests that use biomarkers derived from proteomics and machine learning for use in immuno-oncology. We discuss how these tests may inform patient prognosis as well as guide treatment decisions and predict irAE of immunotherapy. Thus, mass spectrometry-based serum proteomics signatures play an important role in predicting clinical outcomes and toxicity.

INTRODUCTION

Immunotherapy for cancer has advanced significantly in recent years, particularly antibody-based approaches termed immune checkpoint inhibitors (ICIs). ICIs provoke antitumor responses by inhibiting the checkpoint pathways, which include the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) receptors that promote T cell anergy when they bind to cognate ligands (CD80 or CD86 and PD-L1, respectively). Since the approval of ipilimumab by the Food and Drug Administration (FDA) in 2011, multiple other immunotherapy agents have been brought to market, such as pembrolizumab, nivolumab, atezolizumab, and durvalumab.[1 2] Indications for anti-PD-1/PD-L1 in various cancers are expanding and now include high tumor mutational burden (TMB), microsatellite instability (MSI), or mismatch repair deficiency (dMMR).

Although immunotherapy has changed the therapeutic landscape for multiple cancers, only a subset of patients experiences significant survival benefits. For example, clinical studies have shown that 5-year survival for patients with melanoma receiving pembrolizumab is 35%–40%[3]; 4-year survival can be as high as 50% for those receiving nivolumab combined with ipilimumab.[4] In non-small cell lung cancer (NSCLC), 30% of patients with high levels of PD-L1 (≥50% expression) reached 5-year survival.[5 6] Established biomarkers for immunotherapy such as PD-L1 expression and TMB have shown limited reliability in predicting clinical outcomes[7 8]; thus, further exploration into the predictive role of biomarkers that are representative of the host immune response and present in the circulating proteome on a systemic level is warranted.

Mass spectrometry (MS) is a powerful tool for analyzing the proteome. This technique can be combined with machine learning algorithms to identify proteomic signatures that act as potential biomarkers to predict survival outcomes in patients

with cancer treated with immunotherapy. MS allows for a top-down approach, enabling global analysis of intact protein from blood samples.[9] Unlike antibody-based approaches (which have the potential to introduce biases based on the specific proteins being targeted), MS looks at the proteome globally, making it an unbiased assay. Many types of MS exist, each with their own advantages for use. One type, matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) MS uses laser energy to convert analytes into gaseous ions with minimal protein fragmentation. An ionization matrix is used to absorb the laser energy and assist in the ionization of the analytes and the conversion into the gaseous phase. Subsequently, the time required for a given analyte to pass through an electric field and be captured by a detector is measured using the ToF mass spectrometer. The mass-to-charge ratio of each detected ion is recorded and yields a spectrum representative of the specific composition and abundance of analytes.

Additional techniques may be used to identify the proteins represented by specific features in these spectra; however, these can be more complicated. Of greater clinical and scientific value are (1) identifying signatures in these spectra that can distinguish between types of samples (ie, patients who respond to immunotherapy vs patients whose disease is resistant to immunotherapy) and thereby provide a clinically useful classification, and (2) identifying larger biological processes that underlie these observed differences.

Machine learning algorithms have been developed to identify a signature in the mass spectra that distinguishes between patient groups with known clinical phenotypes (eg, response to therapy or nonresponse to therapy), which can then be used to apply classifications to samples from other patients and make clinically actionable predictions (figure 1).[10 11] Bioinformaticians can use techniques such as protein set enrichment analysis (PSEA) to gain insights into the biological systems and mechanisms that give rise to these classifications. Rather than evaluating expression differences protein by protein, the PSEA technique evaluates differences consistent across pre-specified sets of proteins involved in a specific biological function.[12 13] Furthermore, this macro perspective offers protection against identifying isolated random correlations with clinical phenotypes that may not be generalizable.[13]

In addition, mass spectrometry-based glycoproteomics is being investigated as a potential avenue for characterizing the tumor microenvironment. Analyzing post-translational modifications is crucial for understanding the accurate function of proteins. High-throughput mass spectrometry-based glycoproteomics enables the identification of glycoproteins which may provide a more sensitive biomarker in the proteomics field.

In this review, we discuss the performance of MS and machine learning-based serum proteomic tests in real-world settings for predicting immunotherapy responses in patients with either NSCLC or melanoma. In addition, we aim to explore the role of serum proteomic tests in temporally predicting immune-related adverse events (irAEs) and identifying the mechanisms governing irAEs in NSCLC patients treated with immunotherapy.

METHODS

Literature searches on PubMed and major oncology scientific meetings were conducted up to May 31, 2021. Search terms used for this review included serum proteomics, mass spectrometry, immunotherapy, set enrichment analysis, and biomarkers. Articles that are not related to oncology were excluded. In addition, this review mainly focuses on liquid biopsy tests that are commercially available or in development and four retrospective studies that are summarized in table 1.[14–17]

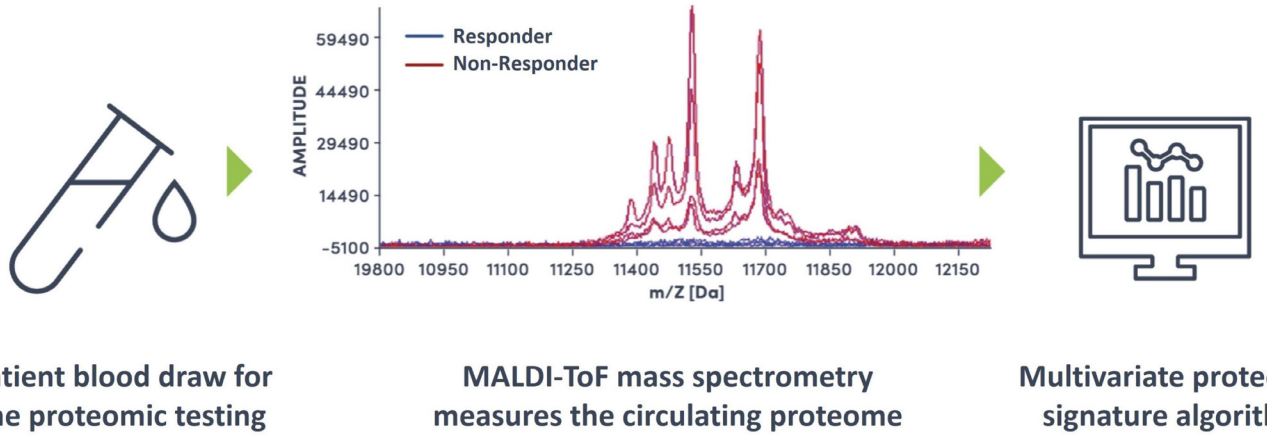

Figure 1 Development of the serum proteomic signatures. To establish a proteomic signature, a patient will first have blood drawn. The patient's serum can then be analyzed with mass spectrometry to identify features specific to that particular patient. The specific peaks can be very complex, making it difficult to manually analyze. Thus, the spectra are analyzed through a machine learning algorithm, which then provides a proteomic signature and an indication of whether the patient will be responsive to immunotherapy.

Table 1 Four retrospective clinical trials based on serum proteomic tests in predicting survival outcomes

Author/study Phase (retrospective)	No of patients	Agents (lines)	Test	Technique	Group (n)	Survival (months)	HR (95% CI)
Chae[14]	47 NSCLC	Nivolumab or pembrolizumab (all lines)	HIC	MALDI-TOF MS	Hot vs Cold (32 vs 15)	mOS NR vs 16.5	HR=0.34 (0.10 to 1.18) p=0.089
Mitchell[15]	83 NSCLC	ICI (nonspecified) monotherapy (first line)	HIC	MALDI-TOF MS	Hot vs Cold (53 vs 30)	mOS NR vs 2.5	HR=0.34 (0.19 to 0.61) p=0.003
	39 NSCLC	ICI (nonspecified) +chemotherapy (first line)	HIC	MALDI-TOF MS	Hot vs Cold (28 vs 11)	mOS NR vs NR	HR=0.66 (0.22 to 1.97) p=0.4533
Muller[16]	116 NSCLC	Nivolumab (second line)	PIR	MALDI-TOF MS	Not resistant vs resistant (75 vs 41)	mOS 11.1 vs 4.3	HR=0.48 (0.30 to 0.77) p=0.002
	116 NSCLC	Nivolumab (second line)	PIR	MALDI-TOF MS	Sensitive vs Not sensitive (32 vs 84)	mOS 17.3 vs 6.0	HR=0.58 (0.38 to 0.87) p=0.009
Xu[17]	36 NSCLC	Nivolumab/ Ipilimumab	PerspectIV	Triple quadrupole MS	Responders vs Non-responders (exact numbers not available)	PFS 70% vs 0% at 18 months	HR=0.11 p<0.001

ICI, immune checkpoint inhibitor; MALDI-TOF, Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF); mOS, median overall survival; MS, mass spectrometry; N, number of patients; NR, non-response; NSCLC, non-small cell lung cancer; PFS, progression-free survival; PFS, progression free survival; PIR, primary immune response.

CURRENT STATE OF SERUM PROTEOMIC TESTS IN CLINIC

The Host Immune Classifier test in lung cancer

The discovery of driver mutations in NSCLC (namely epidermal growth factor receptor, or *EGFR*) and new drugs targeting these mutations (*EGFR*-tyrosine kinase inhibitors, or *EGFR*-TKI) has improved outcomes for many patients with NSCLC. However, not all patients harboring *EGFR* mutations have responded to therapy, and some unselected patients have responded.[18 19] These findings suggest that factors in the host physiology and immune system play a role in determining how patients respond to therapy. This led Taguchi *et al* to apply MALDI-ToF MS and machine learning to the serum proteome to develop what was later termed the Host Immune Classifier (HIC), commercially marketed as the VeriStrat test.[11] To develop the HIC, MALDI-ToF spectra of serum samples from patients with known good versus poor outcomes on *EGFR*-TKI therapy were analyzed with machine learning to determine a proteomic signature that distinguished between outcomes.[11] Other studies have shown that this test is broadly prognostic across multiple different therapies. In addition, it is predictive when considering overall survival of erlotinib relative to chemotherapy in the second line setting, with HIC-Cold patients having worse responses to erlotinib, relative to chemotherapy.[20] Subsequent studies have indicated that these 'HIC-Hot' (aka VeriStrat Good) and 'HIC-Cold' (aka VeriStrat Poor) classifications were not only predictive and prognostic of outcome on *EGFR*-TKI,[20 21] but represented 'Hot' or 'Cold' patient immune status.[22 23]

The proteomic signature that underlies the HIC Hot or Cold result is determined by eight features (ie, peaks depicting the mass to charge (m/z) ratio) within the mass spectra, identified by a machine learning algorithm. While mass spectrometry does not identify which polypeptides correspond to specific spectral features, subsequent deconvolution analyses have determined that certain spectral features in the HIC signature correspond to isoforms of serum amyloid A1.[23] Pathway analysis showed that the HIC-Cold signature is correlated with the acute phase response pathway, which includes many proteins known to be associated with chronic inflammation. 23 biomarkers have been shown to be associated with HIC classification, with the most significant being C-reactive protein, IL-6, serum amyloid A, CYFRA 21.1, IGF-II, osteopontin, ferritin, TRAIL, and sNeuropilin-1.[23]

Patients classified as HIC-Cold have an immunosuppressive tumor microenvironment with a systemic chronic inflammatory disease state, leading to an ineffective immune response to cancer. Patients classified as HIC-Hot have a responsive immune system without chronic inflammation, promoting an immune response to the tumor and better outcomes with therapies. Notably, this classifier is predictive of outcomes regardless of the type of therapy administered.[24] It has been validated to stratify patients without treatment,[21] as well as those given targeted therapy,[25] chemotherapy,[26] or immunotherapy.[15 27]

In an ongoing real-world clinical study validating the HIC's effectiveness in patients with NSCLC taking immunotherapy and other standard of care regimens, multivariate analysis identified HIC classification as an independent predictor of overall survival when adjusted for ECOG PS (Eastern Cooperative Oncology Group

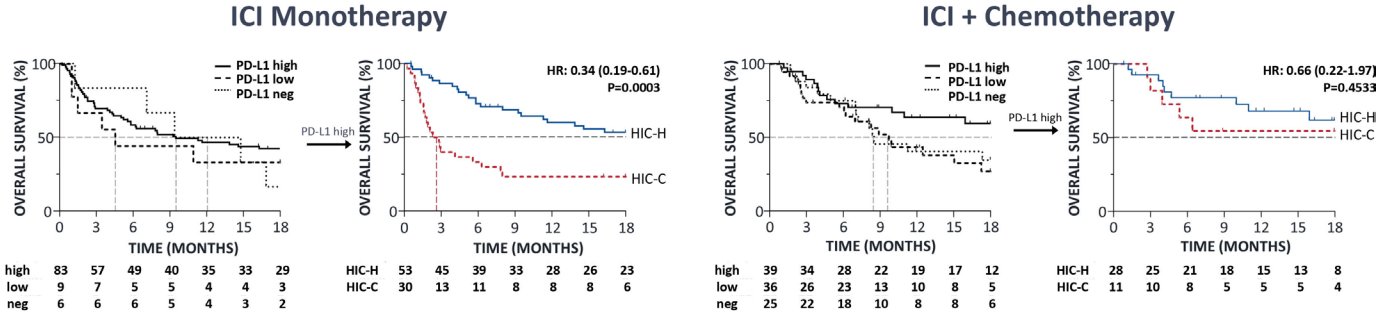

Figure 2 Overall survival (OS) of advanced non-small cell lung cancer (NSCLC) patients, stratified by Host Immune Classifier (HIC) test. Left: When given immune checkpoint inhibitor (ICI) monotherapy, PD-L1 does not clearly stratify patients when considering OS. However, when only the PD-L1 high population is analyzed, HIC testing effectively stratifies patients. Right: When given ICI and chemotherapy, PD-L1 again fails to clearly stratify patients, although OS does seem to improve with time for PD-L1 high patients. When the PD-L1 high population is then subjected to HIC testing, the test can still stratify patients based on performance. Notably, HIC-Cold patients have a much better OS when given ICI and chemotherapy, relative to ICI alone. Conversely, HIC-Hot patients perform similarly with either ICI and chemotherapy or ICI alone.

performance status), histology, age, gender, disease stage, and PD-L1 expression. In fact, when examining first-line chemotherapy, immunotherapy, or combination chemotherapy and immunotherapy, HIC stratified all patients effectively when examining overall survival.[15]

In another analysis of PD-L1 high (≥50%) patients receiving ICI monotherapy, median overall survival was not reached in the HIC-Hot group and these patients had much better survival than HIC-Cold patients (HR=0.34 p=0.0003) (figure 2). Importantly, in the HIC-Cold group, patients treated with ICI/chemotherapy combination had better overall survival than those treated with ICI monotherapy (ICI/chemotherapy vs ICI monotherapy; Not reached vs 2.5 mo).[15] HIC-Cold status is associated with poor prognosis independent of treatment type and PD-L1 expression. Current treatment guidelines recommend single-agent pembrolizumab for patients with ≥50% PD-L1 expression and pembrolizumab/chemotherapy for patients with PD-L1 expression <50%[28 29]; however, the data above argue that patients with HIC-Cold status should be given pembrolizumab/chemotherapy even with high PD-L1 expression. Therefore, HIC may provide information to guide treatment decisions when selecting immunotherapy treatment types in patients with advanced NSCLC.

We conducted a retrospective study with 47 NSCLC patients with no *EGFR*-activating mutations who were subjected to HIC classification between 2016 and 2018. Patients classified as HIC-Hot demonstrated higher progression-free survival (PFS) and overall survival (OS) compared with the HIC-Cold group, regardless of the treatment administered (median PFS of 7.1 vs 4.2 mo; p=0.013 and median OS not reached vs 17.2 mo; p=0.012).[14] When considering NSCLC patients given immunotherapy, HIC-Hot classification was associated with significantly increased PFS (median PFS of 6.2 vs 3.0 mo; p=0.012), while the differences in OS trended towards significance (median OS not reached vs 16.5 mo; p=0.076). Finally, multivariate analysis showed that HIC classification was significantly correlated with PFS and OS in NSCLC patients treated with immunotherapy (HR=0.26, p=0.017 and HR=0.16, p=0.034, respectively). Therefore, this MS-based serum proteomic signature has the potential to serve as a biomarker for survival outcomes in NSCLC patients receiving immunotherapy.

The Primary Immune Response test in NSCLC

The HIC test is useful for stratifying patients broadly, without a focus on any particular therapy; thus, HIC is a prognostic test. While prognostic tests are useful for predicting patient responses, predictive tests have more utility when selecting specific treatment regimens. Another serum-based proteomic test that has been validated in advanced NSCLC patients and effectively stratifies patients based on their response to immunotherapy is the Primary Immune Response (PIR). This test was developed from 116 advanced NSCLC patients treated with second-line nivolumab. Similar to HIC, the PIR test uses multivariate machine learning techniques based on MALDI-ToF MS features from 116 serum samples. The resulting spectra are then used to evaluate proteomic signatures in these patients. When applied to three independent cohorts for survival analysis, this yielded a 274-protein signature that was also evident in the validation samples.[16] Six biological pathways were significantly correlated with PIR (with false discovery rate (FDR) correction). These include acute phase response, acute inflammatory response, wound healing, complement activation, innate immune response, and chronic inflammatory response.[16] Notably, the PIR test was not shown to be associated with PD-L1 expression.[16]

The primary difference between the HIC and PIR tests is evident when considering the specific techniques used. The HIC test is broadly prognostic; it uses MALDI-ToF MS and examines eight features that are then analyzed by a machine learning algorithm to determine whether a sample has systemic chronic inflammation. The PIR test uses a modified version of MALDI-ToF MS known as DeepMALDI. This allows for a much higher resolution (using 274 features) in the final output and is particularly

aimed at identifying a population of patients that would benefit from immunotherapy, thereby functioning as a predictive biomarker.

The classifiers used in the development of PIR test yield three subgroups: Sensitive, Intermediate, and Resistant, with good, intermediate, and poor outcomes, respectively. This test has been validated in advanced NSCLC patients and effectively stratifies patients based on their response to immunotherapy. For differentiating patients with the worst outcome from the remainder of the cohort, the 'Resistant' group was compared with the 'Not resistant' group, which is the combination of sensitive and intermediate subgroups. The median overall survival was 11.1 mo vs 4.3 mo in Not resistant vs Resistant, respectively (HR=0.48, p<0.002).[16] Moreover, pathway analysis of the protein signature showed that acute phase, wound healing, and complement activation pathways were all significantly related to the test classification of Not resistant or Resistant.[13]

Current NCCN guidelines for immunotherapy administration are mainly based on PD-L1 levels.[29–31] However, studies have shown that PD-L1 status does not necessarily correlate with a patient's immune status or response to therapy.[8 32] This test stratified patients into 'Resistant' and 'Not resistant' classifications, independent of PD-L1 expression status and has shown promise for identifying patients most likely to respond to immunotherapy.

The PerspectIV (InterVenn) test

Protein glycosylation is the most abundant and most complex form of post-translational protein modification. Glycosylation profoundly affects protein structure, conformation, and function. For example, one mechanism by which cancer cells evade the host immune system is by expressing sialic acids on their cell surface. These glycosylated cancer cells can be targeted to improve response to cancer immunotherapy.[33] However, the potential role of differential protein glycosylation as biomarkers has so far been limited by the technical complexity of generating and interpreting this information.

InterVenn's AI-enabled, mass spectrometry glycoproteomics platform, PerspectIV, is the first serum glycoproteomic test that assesses post-translational modifications in a site-specific manner across thousands of peptides and glycopeptides.[34] PerspectIV targets proteins involved in the host immune response, such as immunoglobulins, acute phase reactants, and innate immune molecules such as components of the complement system. Mechanistically, PerspectIV employs dynamic multiple reaction monitoring (dMRM) via triple quadrupole mass spectrometry in combination with liquid chromatography to quantify glycopeptides in a site-specific manner,[34] and a sequential deep learning neural network algorithm to automate peak integration from the dMRM and enable high-throughput interrogation of the glycoproteome.[35] As with other diagnostic tests, a major technical challenge for this test is to develop a high-performing signature, with both high sensitivity and specificity.

In a recent study, the PerspectIV platform was leveraged as a liquid biopsy approach to predict response to ICI treatment in metastatic melanoma.[17] Using pretreatment blood samples from 36 patients with metastatic melanoma subsequently treated with nivolumab/ipilimumab or pembrolizumab, 413 glycopeptide signatures were interrogated from 69 serum proteins. Of these, eight glycopeptides with an FDR≤0.1 and p≤0.001 were used to create a multivariable model for PFS. The model yielded a HR of 0.11 at a p value of 10^{-5} for separating treatment responders and non-responders (70% vs 0% PFS, respectively, at 18 months based on score above/below cut-off), as compared with a HR of 1.5, p=0.5 for PD-L1 expression. Although this cohort is small and independent validation would be required, these results suggest that glycoproteomics has great potential to predict response to ICI treatment and to outperform current standard clinical biomarkers such as PD-L1.

Predicting toxicity using serum proteomic tests

Role of the HIC test in predicting toxicity

While stratifying patients who will likely respond to immunotherapy agents is certainly important for physicians, even patients who are expected to respond may experience irAEs. Early recognition of patients who may experience irAEs is important so that appropriate measures can be taken to minimize toxicity and closely monitor patients who are prone to irAEs.

We explore the association between the HIC test and developing irAEs in NSCLC patients treated with immunotherapy. This was conducted by using serum samples from 70 NSCLC patients treated with all treatment regimens and lines of therapy including ICI.[36] Patients identified as HIC-Hot did not show significant difference from HIC-Cold patients in terms of 'Time to first irAE' (p=0.72, HR=0.83, 95% CI=0.29 to 2.32). Further research is required to establish appropriate serum proteomic tests to predict the development of irAE.

Role of the PIR test in predicting toxicity

We also used a PIR testing to investigate the immune reaction with an application on irAEs in patients with NSCLC.[36 37] This is accomplished by classifying patients as either PIR-Not Sensitive or PIR-Sensitive, based on serum biomarkers derived from Deep-MALDI and machine learning.[16] PIR-Not Sensitive patients were those who were likely to have poorer survival outcomes with immunotherapy and also experience irAEs.

PIR-Sensitive patients were more likely to tolerate immunotherapy without developing irAEs. Patient samples were obtained 3 weeks following the initiation of systemic therapy. Applying PSEA analysis to these samples revealed notable differences between irAE positive and negative groups in the following protein pathways: extracellular matrix remodeling, complement activation, IFN-γ, and immune tolerance (p<0.05).[38]

These results are particularly interesting given that the literature has historically shown that patients with irAEs

tend to have better survival.[39–41] In general, patients that experience irAEs do so because they have an active immune response. Thus, they are more likely to respond to therapy, which allows their immune system to combat the tumor. When examining irAEs in patients stratified with PIR, the results indicate that patients that experience irAEs actually have worse performance relative to those that do not experience irAEs. It is possible that the time required for the development of the first irAE is different between the PIR-Sensitive and PIR-Not sensitive populations. Thus, the PIR test may be detecting a temporal relationship between irAEs and survival, thereby identifying a patient population who are less likely to develop irAEs, without compromising on efficacy.[42]

Given that blood samples are easily accessible, investigating proteomics using liquid biopsy can be very useful for characterizing biological processes, which have the potential to provide insights into understanding the early mechanisms related to irAE development following immunotherapy. Thus, predicting irAE development and selecting those patients who need close monitoring using blood-based proteomics such as PIR should be further investigated.

Summary and validation state of serum proteomic tests for immunotherapy

Of the three serum proteomic tests presented, it is important to note their various histories and status of clinical validation when considering what technical limitations may apply to employing them. For instance, the HIC test has been locked since 2008 and has been extensively validated in numerous independent cohorts (table 2[11 16 17 20 21 38 43]). However, it is also based on fewer spectral features than newer tests (eg, PIR) and was not explicitly designed for immunotherapy, although it has demonstrated applicability there. Nonetheless, in the other therapeutic contexts in which it has been employed, the HIC test has demonstrated significant clinical utility in being broadly prognostic with some predictive abilities,[20 24] as well as impacting physician decisions and improving quality care measures.[44 45] More recent analyses have shown that HIC is also predictive, when considering immunotherapy alone or in combination with chemotherapy for patients with high PD-L1 status.[46]

Relatively early in development, the PIR test underwent independent cohort validation, but prospective clinical validation is pending. Capturing 274 mass spectral features and specifically designed for use in immunotherapy, PIR has demonstrated clinical utility as a predictive of outcomes for immunotherapy[16] and potentially as a predictor of irAEs.[36 37]

Focusing specifically on glycosylated proteins, the PerspectIV test platform is well developed, but requires analytical and independent clinical validation. Part of the power of the PerspectIV approach is the ability to examine hundreds of potential signatures to yield one with high predictive power.[17] Whether this signature can be locked into a test with robust performance remains to be seen. Thus, this newer test faces the technical challenge of avoiding issues of overfitting[47] and maintaining specificity and sensitivity under validation in large, independent cohorts.

Technical and practical limitations of MS-based proteomic tests

Every diagnostic assay has certain limitations that should be considered prior to implementation. Evidently, genomics, transcriptomics, and proteomics each fill a specific set of needs and cannot easily be substituted for one another; thus, each assay will have its own strengths and weaknesses. Many of these limitations are not necessarily specific to MS-based techniques, but rather issues inherent to the biological system being studied. Here, we will discuss the technical and practical limitations associated with proteomic testing, with a focus on MS-based techniques.

Technical limitations

Many proteomic tests are based on a protein signature. For such tests, the quality of the signature itself is limited by the data used to generate the signature. As is true in any instance that requires data extrapolation, the applicability of the signature for the intended use population may vary from the dataset on which it was initially developed. Another technical limitation is that the cut-off value of a given proteomic test may be arbitrary. Many proteomic tests use binary results (eg, good vs poor). This is often done in order to make the interpretation of the

Table 2 Comparison of serum proteomic tests and validation status

	HIC	PIR	PerspectIV
Components in signature (n, type)	8 spectral features[11]	274 spectral features[16]	8 glycoproteins[17]
Date first published* or presented**	2007*[11]	2018**[43]	2019**[17]
Designed for immunotherapy?	No[11]	Yes[16]	Yes[17]
Test locked?	Completed	Completed	Pending
Independent cohort validation?	Completed[21]	Completed[38]	Pending
Prospective clinical validation?	Completed[20]	Pending	Pending

HIC, host immune classifier; PIR, primary immune response.

test results easy for the user; however, there is a need to validate whether the binomial approach is better than a spectrum when predicting response.

In addition, MS-based diagnostics using circulating biomarkers are naturally limited to addressing needs where the circulating proteomic provides sufficient information to address a given problem. While the circulating proteome can provide abundant information about processes such as the immune system and inflammation, it is less suited to capture other processes, such as somatic mutations in tumors. Moreover, oncologic blood-based proteomic assays often measure proteins that are shed into the blood from the tumor. Therefore, the utility of such a test will depend on the amount of shedding, which can vary from patient to patient.

Ideal measurement of the circulating proteome should be conducted in homeostatically-controlled fluid, with little to no temporal fluctuations. While the levels of some proteins vary by time of day or season, many other proteins do not show these variations. Thus, best practices for test development should be considered to avoid possible confounding effects from sample collection artifacts. For example, sample collection protocols for test development should always be uniform across test classification groups without temporal limitations, unless specified for the final test.

Moreover, serum and plasma should be handled appropriately to preserve sample quality over time. It is possible to maintain sample quality at low temperatures, but this can lead to issues with sample transport (ie, reduced convenience and/or increased costs). One alternative solution to this is to store serum and plasma samples dried on to collection devices at ambient temperature for easy and cost-effective shipping to testing laboratories. Furthermore, tests using certain hypothesis-free MS techniques, such as MALDI, cannot be carried out accurately on samples that are heavily hemolyzed, oxidized, or contaminated. MALDI-ToF MS allows for a comprehensive assessment of many proteins in the serum proteome, which can be used for Quality Control purposes (eg, checking for hemolysis, sample oxidation or degradation, or sample contamination).

Traditionally hypothesis-free MS measurements of the circulating proteome were limited by low sensitivity. However, recent developments have expanded the dynamic range of the proteome to span ~4 orders of magnitude. Targeted MS measurements, such as MRM can have very high sensitivity for a panel of known proteins. Finally, to allow MS measurements to be used in test development, it is essential to process the spectral data appropriately to ensure reproducibility of the measurements. Proper processing is crucial in order to compare measurements between samples or patients.

Practical limitations

In addition to technical limitations, practical limitations concerning implementation of the test in a clinical setting should also be considered. Relative to genomics, proteomics is a much newer field and thus less well-established. Therefore, physicians will naturally be weary of using proteomic-based approaches without significant test validation. In addition, physicians will need to undergo proper education of these techniques and their utility before considering using them for patient care. In the clinical setting, most clinicians are not aware of these proteomic tests, given their absence from informed treatment guidelines such as the NCCN guidelines. To this effect, more prospective and randomized clinical trials are required to instill confidence in proteomic assays. Once they are established in prospective and randomized trials, they can then be added to guideline recommendations, making them a part of standard of care.

CONCLUSIONS AND FUTURE DIRECTIONS

With the advent of validated proteomics platforms, serum proteomics are now emerging as effective biomarkers to help guide treatment of certain cancers. The assays currently being developed for clinical use reflect the advantages of employing proteomic analysis. First, the use of top-down proteomics allows for analysis of intact proteins and post-translational modifications. Unlike DNA or RNA-based platforms, this allows for an improved understanding of the host immune response and the tumor microenvironment, as evidenced by the elevated immune markers in both the HIC-Cold group and the PIR-Resistant group among NSCLC patients. Given their informative role in assessing the immune environment, proteomic assays are also demonstrating their use in the prediction of irAEs. irAEs can become a clinically significant factor that can lead to discontinuation of treatment or other adverse outcomes. Proteomic tools such as PIR can be helpful in identifying high-risk groups and allowing for early recognition of irAEs.

Second, these biomarkers are being validated to predict response to various lines of therapy. The HIC test has been validated on samples with traditional platinum-based therapy as well as ICI therapy.[15] As demonstrated in this review, response to immunotherapy in patients can be widely variable; thus, predictive biomarkers that can be used to determine appropriate therapies are crucial. Unfortunately, currently established biomarkers do not always successfully identify patients who have survival benefits from immunotherapy. However, advances in biomarker research have illustrated many other factors that may have predictive abilities for cancer treatment. Recently, genomic alterations such as dMMR, MSI, and TMB have been approved by the FDA.[48] Genomic and proteomic markers under investigation include chromatin remodeling genes, PD-L1 expression, and some types of HLA that confer sensitivity to immunotherapy. In addition, other markers such as alterations in *EGFR*, *KEAP1*, *JAK1/2*, *MDM2*, *PTEN*, *STK11* as well as β2-microglobulin and Wnt/β-catenin signaling alterations are related to resistance to immunotherapy.[48] Finally, the clinical relevance of the tumor microenvironment is

another current area of investigation. Studies have shown that higher concentrations of tumor infiltrating lymphocytes are associated with favorable clinical outcomes.[49] While all of these biomarkers individually provide some benefit to therapeutic decision-making, they are much more powerful when considered collectively, especially in combination with host factors such as age, sex, ethnicity, body mass index, etc.

Although proteomics research carries strong potential for clinical application, the current landscape has limited utility in predicting immunotherapeutic outcomes, due to various factors.

It is important to note that, while proteomics has the power to significantly influence treatment decision making, technical challenges with respect to implementing proteomics into clinical practice should be thoroughly examined. Practically, the HIC and PIR tests require <10 µl serum and proper protocols are placed to ensure that the process of sample preparation will not cause protein degradation. Another consideration for determining the suitability of a given proteomic method is whether it can distinguish between isoforms and/or activation states of a protein. Because MALDI-ToF measures mass/charge ratio, it is able to capture all isoforms of a given protein or polypeptide present in the proteome, including nascent or truncated versions, and various post-translational modifications (eg, phosphorylation, glycosylation, ubiquitination). The HIC test has a quick turnaround time of 36 hours, which is very useful as it can expedite time to treatment in patients with aggressive disease. The PIR test is not yet commercially available and thus has no data on turnaround times.

Diagnostic assays using proteomics, both exclusively and in combination with other technologies, have emerged as clinically valuable tools in the oncology market. Some examples of validated, commercially available assays include the HIC proteomic test provided by Biodesix and TissueCypher provided by Cernostics. Medicare payment rates for proteomic diagnostic assays range from US$2500–US$4000 while genomic assays range from US$1500 to US$6000.[50] However, tests such as HIC are currently fully covered by Medicare and many private insurance companies, thereby ensuring that there are no direct charges to the patient.

Lastly, most of the studies focused on proteomics-based biomarkers are retrospective, limiting their use in broad settings. We anticipate that future clinical trials focused on collecting prospective data will provide more robust information about its real-world predictability. Thus, combining and integrating different biomarkers could potentially improve patient selection for immunotherapy and provide the best overall survival outcomes. Moreover, advances in proteomic technologies are providing new opportunities for more implementation. For instance, next-generation MS techniques such as trapped ion mobility spectrometry time-of-flight (timsToF) can be combined with nanoparticle-based approaches[51] to improve the biological resolution. Using these techniques in combination yields a technology that is very sensitive and reproducible, while still examining relevant peptides, regardless of their relative serum abundance.

Current data suggest a role for incorporating proteomics where other biomarkers are limited in predicting immunotherapy response. For instance, the HIC-Cold group was associated with worse survival outcome despite high PD-L1 expression in NSCLC treated with pembrolizumab monotherapy (NR vs 2.5 mo, HIC-Hot vs HIC-Cold, HR=0.34, n=83). Although further studies are warranted to validate this finding, the serum proteomic signature may be crucial in selecting those who may benefit from chemotherapy in combination with pembrolizumab. Our analysis indicates that current guidelines should integrate proteomics into existing recommendations to aid decision-making in cancer treatment.

The long validation history of the HIC supports a role for machine-learning derived serum proteomic tests in cancer care decision-making in general and in treatment courses involving immunotherapy specifically. Moreover, second generation tests encompassing additional features, such as PIR, may yield additional predictive insights, including prediction of irAEs. A role for tests measuring specific post-translational modifications, such as PerspectIV, is also emerging. Prospective validation of newer proteomic signatures and determination of their role in aspects of care such as physician behavior impact and patient quality measures are therefore promising directions of research to continue to deliver on the potential of immunotherapy and improve on biomarker-informed cancer care.

Author affiliations
[1]Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA
[2]Department of Internal Medicine, NewYork-Presbyterian Queens, Flushing, New York, USA
[3]Department of Internal Medicine, AMITA Health Saint Joseph Hospital Chicago, Chicago, Illinois, USA
[4]Department of Internal Medicine, AMITA Health Saint Francis Hospital Evanston, Evanston, Illinois, USA
[5]Department of Medicine, Northwestern University, Chicago, Illinois, USA
[6]Department of Hematology and Oncology, Northwestern University Feinberg School of Medicine, Chicago, Illinois, USA

Acknowledgements We acknowledge Namratha Sastry, PhD (Biodesix), Rachel Hartfield, PhD (medical writer, contracted by Biodesix), Apurva Dixit (Senior Director of Business Development, InterVenn Bioscience), and Klaus Lindpaintner, MD, MPH (InterVenn) for helpful discussions and support with manuscript preparation.

Contributors YP and NHK drafted the manuscript. YP, YKC, MJK and LK performed the literature search. YP developed tables and figures. MJK, SPDH, H-GC and EY helped revising the manuscript. All authors read and approved the final version of the manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests YKC is on the advisory boards for Roche/Genentech, Astrazeneca, Foundation Medicine, Counsyl, Neogenomics, Guardant Health, Boehringher Ingelheim, Biodesix, Immuneoncia, Lilly Oncology, Merck, Takeda, Pfizer, and Tempus. YKC has received research grants from Abbvie, BMS, Biodesix, Lexent Bio, and Freenome.

Patient consent for publication Not applicable.

Ethics approval Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

1 Sharma P, Siddiqui BA, Anandhan S, *et al*. The next decade of immune checkpoint therapy. *Cancer Discov* 2021;11:838–57.
2 Twomey JD, Zhang B. Cancer immunotherapy update: FDA-approved checkpoint inhibitors and companion diagnostics. *AAPS J* 2021;23:39.
3 Hamid O, Robert C, Ribas A, *et al*. Antitumour activity of pembrolizumab in advanced mucosal melanoma: a post-hoc analysis of KEYNOTE-001, 002, 006. *Br J Cancer* 2018;119:670–4.
4 Hodi FS, Chiarion-Sileni V, Gonzalez R, *et al*. Nivolumab plus ipilimumab or nivolumab alone versus ipilimumab alone in advanced melanoma (CheckMate 067): 4-year outcomes of a multicentre, randomised, phase 3 trial. *Lancet Oncol* 2018;19:1480–92.
5 Brahmer JR, Rodriguez-Abreu D, Robinson AG, *et al*. LBA51 KEYNOTE-024 5-year OS update: First-line (1L) pembrolizumab (pembro) vs platinum-based chemotherapy (chemo) in patients (pts) with metastatic NSCLC and PD-L1 tumour proportion score (TPS) ≥50%. *Ann Oncol* 2020;31:S1181–2.
6 Garon EB, Hellmann MD, Rizvi NA, *et al*. Five-year overall survival for patients with advanced Non–small-cell lung cancer treated with pembrolizumab: results from the phase I KEYNOTE-001 study. *J Clin Oncol* 2019;37:JCO1900934.
7 Bai R, Lv Z, Xu D, *et al*. Predictive biomarkers for cancer immunotherapy with immune checkpoint inhibitors. *Biomark Res* 2020;8:34.
8 Doroshow DB, Bhalla S, Beasley MB, *et al*. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. *Nat Rev Clin Oncol* 2021;18:345–62.
9 Hortin GL. The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. *Clin Chem* 2006;52:1223–37.
10 Grigorieva J, Asmellash S, Net L, *et al*. Mass spectrometry-based multivariate proteomic tests for prediction of outcomes on immune checkpoint blockade therapy: the modern analytical approach. *Int J Mol Sci* 2020;21:ijms21030838. doi:10.3390/ijms21030838
11 Taguchi F, Solomon B, Gregorc V, *et al*. Mass spectrometry to classify non-small-cell lung cancer patients for clinical outcome after treatment with epidermal growth factor receptor tyrosine kinase inhibitors: a multicohort cross-institutional study. *J Natl Cancer Inst* 2007;99:838–46.
12 Bessarabova M, Ishkin A, JeBailey L, *et al*. Knowledge-based analysis of proteomics data. *BMC Bioinformatics* 2012;13:S13.
13 Grigorieva J, Asmellash S, Oliveira C, *et al*. Application of protein set enrichment analysis to correlation of protein functional sets with mass spectral features and multivariate proteomic tests. *Clinical Mass Spectrometry* 2020;15:44–53.
14 Chae YK, Kim WB, Davis AA, *et al*. Mass spectrometry-based serum proteomic signature as a potential biomarker for survival in patients with non-small cell lung cancer receiving immunotherapy. *Transl Lung Cancer Res* 2020;9:1015–28.
15 Mitchell RB, Rich P, Walker PR, *et al*. Real-world performance of blood-based host immune profiling in first-line immunotherapy treatment in advanced-stage non-small cell lung cancer. *J Clin Oncol* 2020;38:9545–45.
16 Muller M, Hummelink K, Hurkmans DP, *et al*. A serum protein classifier identifying patients with advanced non-small cell lung cancer who derive clinical benefit from treatment with immune checkpoint inhibitors. *Clin Cancer Res* 2020;26:5188–97.
17 Xu G, Rice R, Huang H. Abstract 387: Glycoproteomics as a powerful liquid biopsy-based predictor of checkpoint-inhibitor treatment response. 112th Annual Meeting of the American Association for Cancer Research 2021.
18 Shepherd FA, Rodrigues Pereira J, Ciuleanu T, *et al*. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005;353:123–32.
19 Thatcher N, Chang A, Parikh P, *et al*. Gefitinib plus best supportive care in previously treated patients with refractory advanced non-small-cell lung cancer: results from a randomised, placebo-controlled, multicentre study (Iressa survival evaluation in lung cancer). *Lancet* 2005;366:1527–37.
20 Gregorc V, Novello S, Lazzari C, *et al*. Predictive value of a proteomic signature in patients with non-small-cell lung cancer treated with second-line erlotinib or chemotherapy (PROSE): a biomarker-stratified, randomised phase 3 trial. *Lancet Oncol* 2014;15:713–21.
21 Carbone DP, Ding K, Roder H, *et al*. Prognostic and predictive role of the VeriStrat plasma test in patients with advanced non-small-cell lung cancer treated with erlotinib or placebo in the NCIC clinical Trials Group BR.21 trial. *J Thorac Oncol* 2012;7:1653–60.
22 Milan E, Lazzari C, Anand S, *et al*. SAA1 is over-expressed in plasma of non small cell lung cancer patients with poor outcome after treatment with epidermal growth factor receptor tyrosine-kinase inhibitors. *J Proteomics* 2012;76 Spec No.:91–101.
23 Fidler MJ, Fhied CL, Roder J, *et al*. The serum-based VeriStrat® test is associated with proinflammatory reactants and clinical outcome in non-small cell lung cancer patients. *BMC Cancer* 2018;18:310.
24 Leal TA, Argento AC, Bhadra K, *et al*. Prognostic performance of proteomic testing in advanced non-small cell lung cancer: a systematic literature review and meta-analysis. *Curr Med Res Opin* 2020;36:1497–505.
25 Buttigliero C, Shepherd FA, Barlesi F, *et al*. Retrospective assessment of a serum proteomic test in a phase III study comparing erlotinib plus placebo with erlotinib plus tivantinib (MARQUEE) in previously treated patients with advanced non-small cell lung cancer. *Oncologist* 2019;24:e251–9.
26 Grossi F, Rijavec E, Genova C, *et al*. Serum proteomic test in advanced non-squamous non-small cell lung cancer treated in first line with standard chemotherapy. *Br J Cancer* 2017;116:36–43.
27 Grossi F, Rijavec E, Biello F, *et al*. P3.02c-074 evaluation of a pretreatment serum tests for nivolumab benefit in patients with non-small cell lung cancer. *J Thor Oncol* 2017;12:S1322.
28 Hanna NH, Schneider BJ, Temin S, *et al*. Therapy for stage IV non-small-cell lung cancer without driver alterations: ASCO and OH (CCO) joint guideline update. *J Clin Oncol* 2020;38:1608–32.
29 Network NCC. Non-Small cell lung cancer (version 2.2020). Available: http://www.nccn.org/default.aspx [Accessed 23 Dec 2019].
30 Ettinger DS, Wood DE, Aggarwal C, *et al*. NCCN guidelines insights: non-small cell lung cancer, version 1.2020. *J Natl Compr Canc Netw* 2019;17:1464–72.
31 Swetter SM, Thompson JA, Albertini MR, *et al*. NCCN Guidelines® insights: melanoma: cutaneous, version 2.2021. *J Natl Compr Canc Netw* 2021;19:364–76.
32 Davis AA, Patel VG. The role of PD-L1 expression as a predictive biomarker: an analysis of all US food and drug administration (FDA) approvals of immune checkpoint inhibitors. *J Immunother Cancer* 2019;7:278.
33 Xiao H, Woods EC, Vukojicic P, *et al*. Precision glycocalyx editing as a strategy for cancer immunotherapy. *Proc Natl Acad Sci U S A* 2016;113:10304–9.
34 Li Q, Kailemia MJ, Merleev AA, *et al*. Site-specific glycosylation quantitation of 50 serum glycoproteins enhanced by predictive glycopeptidomics for improved disease biomarker discovery. *Anal Chem* 2019;91:5433–45.
35 Wu Z, Serie D, Xu G, *et al*. PB-Net: automatic peak integration by sequential deep learning for multiple reaction monitoring. *J Proteomics* 2020;223:103820.
36 Kim L, Chae YK, Jung CM. Potential role of serum proteome in predicting immune-related adverse events from immune checkpoint inhibitors in non-small cell lung cancer. *J Clin Oncol* 2021;39:e21218.
37 Davis AA, Park J, Kim L. Abstract 5527: serum proteomic scores for understanding the mechanisms of immune-related adverse events (irAEs) in non-small cell lung cancer. *Cancer Res* 2020;80:5527.
38 Chae YK, Park J, Iams W, *et al*. P33.06 utilizing serum proteome to understand response and resistance to immune checkpoint inhibitors in advanced non-small cell lung cancer. *J Thor Oncol* 2021;16:S407–8.
39 Shankar B, Zhang J, Naqash AR, *et al*. Multisystem immune-related adverse events associated with immune checkpoint inhibitors for treatment of non-small cell lung cancer. *JAMA Oncol* 2020;6:1952–6.
40 Ricciuti B, Genova C, De Giglio A, *et al*. Impact of immune-related adverse events on survival in patients with advanced non-small cell lung cancer treated with nivolumab: long-term outcomes from a multi-institutional analysis. *J Cancer Res Clin Oncol* 2019;145:479–85.
41 Socinski MA, Jotte RM, Cappuzzo F, *et al*. Pooled analyses of immune-related adverse events (irAEs) and efficacy from the phase 3 trials IMpower130, IMpower132, and IMpower150. *J Clin Oncol* 2021;39:9002.
42 Nam M, Kim L, Cheng W. Abstract 520: potential role of serum proteome in predicting immune-related adverse events from

immunotherapy in non-small cell lung cancer. *Cancer Res* 2021;81520.
43 Smit EF, Aerts JG, Muller M, *et al*. Prediction of primary resistance to anti-PD1 therapy (APD1) in second-line NSCLC. *Ann Oncol* 2018;29:mdy269.068
44 Page RD, Argento AC, Nash DB, *et al*. The role of proteomic testing in improving prognosis and care planning quality measures for lung cancer. *Manag Care* 2017;26:37–47.
45 Page RD, Arnaud AM. Precision medicine: estimated clinical and economic outcomes of using a predictive and prognostic biomarker to avoid ineffective therapies in advanced non-small cell lung cancer. the American College of medical quality (ACMQ) annual meeting. Washington DC 2017.
46 Rich P, Mitchell RB, Schaefer E, *et al*. Real-world performance of blood-based proteomic profiling in first-line immunotherapy treatment in advanced stage non-small cell lung cancer. *J Immunother Cancer* 2021;9:e002989.
47 Hernández B, Parnell A, Pennington SR. Why have so few proteomic biomarkers "survived" validation? (Sample size and independent validation considerations). *Proteomics* 2014;14:1587–92.
48 Fountzilas E, Kurzrock R, Hiep Vo H, *et al*. Wedding of molecular alterations and immune checkpoint blockade: genomics as a matchmaker. *J Natl Cancer Inst* 2021:1634–47. doi:10.1093/jnci/djab067
49 Tumeh PC, Harview CL, Yearley JH, *et al*. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* 2014;515:568–71.
50 Medicare clinical laboratory fee schedule (CLFS), 2019. Available: https://www.cms.gov/license/ama?file=/files/zip/21clabq3.zip [Accessed 20 Sep 2021].
51 Blume JE, Manning WC, Troiano G, *et al*. Rapid, deep and precise profiling of the plasma proteome with multi-nanoparticle protein corona. *Nat Commun* 2020;11:3662.