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Proteomics in cancer research: are we ready for clinical practice?

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Table of contents

Highlights

Abstract

Keywords

1. **Introduction to clinical proteomics**
2. **From clinical proteomics to translational oncology**
 - 2.1. **Potential application of oncoproteomics**
 - 2.1.1. **Pathway analysis and protein interactions**
 - 2.1.2. **Post-translational modifications**
 - 2.1.3. **Biomarkers**
 - 2.2. **Translational oncoproteomics**
 - 2.3. **Proteomic approaches in biomarker discovery and validation**
3. **Challenges in clinical proteomics**
 - 3.1. **Nature and quality of clinical samples**
 - 3.2. **Experimental design**
 - 3.3. **Complexity of the human proteome**
4. **Outlook**
5. **Conclusion**

Acknowledgements

Conflict of interest

References

Short vitae of authors

Highlights

- Oncoproteomics correlates better to the biological phenotype compared genomics and transcriptomics
- With current progress in the field, mass spectrometry-based proteomics has major potential in both biomarker discovery and validation studies
- Limited amount of clinical proteomics results are translated into a clinical setting due to major challenges
- The nature and quality of clinical samples is of tremendous importance for proteomics experiments
- Only proteomics studies with sufficient statistical power and an unbiased study design can deliver potential biomarkers
- Analysis of a highly dynamic and complex proteome requires different fractionation strategies

Abstract

Although genomics has delivered major advances in cancer prognostics, treatment and diagnostics, it still only provides a static image of the situation. To study more dynamic molecular entities, proteomics has been introduced into the cancer research field more than a decade ago. Currently, however, the impact of clinical proteomics on patient management and clinical decision-making is low and the implementations of scientific results in the clinic appear to be scarce. The search for cancer-related biomarkers with proteomics however, has major potential to improve risk assessment, early detection, diagnosis, prognosis, treatment selection and monitoring. In this review, we provide an overview of the transition of oncoproteomics towards translational oncology. We describe which lessons are learned from currently approved protein biomarkers and previous proteomic studies, what the pitfalls and challenges are in clinical proteomics applications, and how proteomic research can be successfully translated into medical practice.

Keywords:

Clinical proteomics * biomarkers * translation to clinic * study design * sample quality * complexity

1. Introduction to clinical proteomics

Since the introduction of the 'omics' technologies, a plethora of new tools to understand complex biological systems became available. While genomics/transcriptomics is well on its way to become common practice in the clinic (e.g. guide therapy decision [1]), other complementary 'omics' technologies need further integration and development to realize their full potential in clinical applications. Studying the proteome, i.e. the protein complement of the genome, is a good way to obtain more information on protein expression and protein modification in cells/tissues/organisms. With proteomics, the systematic and simultaneous analysis of proteins expressed by a certain cell type or tissue has become possible. Proteomics technologies also allow to measure protein abundances, to characterize post-translational modifications (PTMs) and to study protein structure and interactions. Compared to the static genome, the human proteome is more diverse, as it consists of an estimated 500.000 protein isoforms derived from about 20.000 protein-coding genes [2]. This large increase in protein diversity is largely due to alternative splicing and post-translational modification of proteins. Furthermore, it is known that genomic technologies can only provide a static snapshot, as not all gene-encoded proteins are expressed at all the time and in all cells types [3]. In addition, only a poor correlation can be found between abundance levels of transcripts and proteins [4]. These facts all suggest that proteomics applications can be used to bridge the gap between genomic information and functional proteins [5].

The last fifteen years, mass spectrometry (MS)-based proteomic technologies have become the method of choice to analyse complex protein samples. The major driver that propelled proteomics in the clinic are the significant technological advances in the field of mass spectrometry and bioinformatics steadily improving sensitivity, specificity and throughput making the study of several thousands of proteins (and their post-translational modifications) of cells, tissues and body fluids possible in one experiment [6]. To this end, proteomics has been implemented in clinical research to understand the pathophysiology of several diseases [7]. Clinical proteomics can thus be defined as the use of proteomics strategies to answer clinical questions and to improve medical practice at the level of diagnosis, prognosis or therapy [8,9]. More specifically, clinical proteomics focusses on understanding the pathobiology of diseases, characterizing new targets for drug development and therapeutic intervention, and digging for biomarker candidates [6,10].

2. From clinical proteomics to translational oncology

2.1 Potential applications of oncoproteomics

The increased knowledge of the complexity and heterogeneity of cancer of the last ten years made clear that cancer evolves via multiple pathways and is the result of a variety of genetic, molecular and clinical

events. Since malignant transformation involves changes in protein expression, which influences the molecular circuitry in all these cells, protein analysis is a good strategy to characterize regulatory and functional networks, to identify protein modifications and interactions, to study new targets for drug development and to dig for biomarker candidates in the field of oncology. [11,12]. The oncoproteomics field tries to enable biological insights in the carcinogenesis process, to unravel the importance of protein modification (e.g. phosphorylation) in malignancy and to obtain molecular insights into cancer phenotypes. Most efforts in the oncoproteomics field, however, are focused on protein expression profiling across different biological groups with the intention to identify biomarkers that can be used for detection, stratification or prognosis of cancer and cancer therapies. In this review, we will therefore only briefly discuss protein interactions and modification and focus mainly on the search for cancer-related biomarker candidates with proteomic technologies.

2.1.1 Pathway analysis and protein interactions

As a highly complex disease, the dysregulation of multiple signaling pathways and the dynamics of involvement of a variety of components that interact via complex networks in cancer is enormous. Understanding the fundamental basics of the cancer-associated cellular processes such as cell death, cell proliferation and cell differentiation is of crucial importance to improve current therapy and to overcome the high cancer-related mortality rates. Since the dysfunction of biochemical pathways is based at the protein level, the study of the proteome allows the systematic analysis and mapping of cancerous signaling pathways [13]. With the current improvements in mass spectrometry-based technology, analysis of thousands of proteins from clinical samples which could explain the underlying biology becomes possible. This way, proteomics can help to unravel how cellular networks regulate tumor-promoting processes. Proteomics-based pathway analyses are, for example, able to provide evidence for the dysregulation of energy sensing mechanisms in non-small cell lung cancer and its recurrence [14] or to evaluate the apoptosis-induced pathways of new target inhibitors [15].

Besides knowledge on the pathways involved in the carcinogenesis process, also protein-protein interactions have important roles in cellular processes and can deliver crucial insights to cancer biology. Several different strategies exist to isolate these protein complexes, with tandem affinity purification (TAP)-tagged proteins as one of the most frequently used pulldown protocols. MS-based proteomics technologies are able to further analyze these isolated protein complexes. The technical aspects of mapping protein interactions in signaling pathways are out of the scope of this manuscript and reviewed in [16]. One of the examples showing the potential of these strategies for oncological research are the protein-protein interaction studies on the epidermal growth factor receptor (EGFR) activation in stress conditions [17] and how EGFR coordinates diverse biological responses [18].

2.1.2 Post-translational modifications

Besides mapping the cancer-associated pathways and protein-protein interactions, the analysis and quantitative assessment of post-translational modifications (PTMs) is a powerful tool to further understand the signal flow in relevant signaling pathways and networks. As reversible modifications on proteins can alter their structural and functional properties, and thus increase the complexity of the proteome enormously, analysis of the fundamental roles of PTMs in regulating signaling pathways is of great value. PTMs can be of diverse nature, with protein phosphorylation, glycosylation and acetylation as the most studied ones. An overview of PTM analysis using proteomics technologies is provided in Mann et al. [19].

Protein phosphorylation is one of the most studied PTMs in relation to cancers. Unraveling these endogenous phosphorylation events *in vivo* can provide clues for defects in signaling pathways involved in many pathologies, including cancer. Because phosphorylation is a highly dynamic PTM and has a low abundance (compared to non-phosphorylated proteins), phosphoprotein analysis is greatly enhanced by enrichment of phosphopeptides prior to MS-based proteomic analysis [20]. Most 'phosphor'-enriching strategies are based on affinity chromatography where peptides and proteins containing negatively charged phosphate groups are captured onto a positively charged matrix. Another way of enriching phosphopeptides includes the immunoprecipitation by phosphor-specific antibodies. An overview of the technical details regarding phosphoproteomics are provided in several reviews [21-23].

In cancer, phosphorylation and kinases responsible for transferring the phosphate group to proteins are important targets for drug candidates. Indeed, with regard to translational oncology, phosphoproteomic strategies are therefore more and more implemented to, for example, monitor the phosphoproteome after administration of drug components, which are important end points in clinical studies investigating drug pharmacodynamics [24]. As another example, the determination of phosphorylation sites and the protein stoichiometry's are possible indicators of kinase activity and substrate specificity, and thus important information in the search for new drug targets [13].

Besides phosphorylation, the mapping of several other cancer-associated PTMs, are also important in cancer research [25]. Major interest in cancer-associated glycosylation, acetylation, nitrosylation and oxidation are reported [26,27]. An extended overview of these PTMs however, is not in the scope of this review and can be found elsewhere [19].

2.1.3 Biomarkers

For several years now, there is an urge to identify molecules that indicate an alteration of the physiological state of an individual in relation to health and disease, i.e. biomarkers. Because proteomics has the ability to assess both protein production and degradation as well as the characterization of several PTMs, it correlates better with the biological phenotype compared to genomics or transcriptomics data, making the urge for protein biomarkers big [28].

A lot of effort in the field of oncoproteomics is put on the search for biomarkers involved in early cancer detection and diagnosis. It is clear that the more early a malignancy is detected, the more effectively

cancer can be treated, resulting in a dramatic reduction of patient mortality. Early diagnosis of cancer however, is difficult as there is a lack of specific symptoms in early disease. Therefore, if a biomarker is able to detect the disease before clinical symptoms arise, it might have an enormous impact in clinical oncology. Indeed, if the tumor is only small and in a localized stage, resection is feasible and might even be curative, with increasing five year survival rates as a consequence. Currently, however, markers for screening and early detection are limited, and mostly do not possess the acquired sensitivity and specificity. An example is hemoglobin detection in the fecal occult blood test (FOBT) for early diagnosis of colorectal cancer (CRC) [29]. Since proteins are effector molecules influencing signaling pathways, early changes in protein expression might reflect the early transition to malignancy which turns proteins into ideal targets in the search for early detection markers.

Furthermore, determination of changes in protein expression can also be used for the prediction of the therapeutic response. Since responses to treatment of patients at the same clinical stage of cancer might be different, predictive markers are required to optimize treatment decisions [30]. This might be beneficial for example in the treatment of colorectal cancer (CRC) which is currently dependent on the tumor stage. Although stage III CRC patients with lymph node metastasis are usually recommended to undergo adjuvant chemotherapy, only 15 % of these patients actually benefit from the treatment [31]. These different responses might be due to differences in cancer pathophysiology which results in versatile protein expression patterns in patients and finding predictive markers that could predict the therapeutic outcome would allow to optimize clinical decision making. Furthermore, the search for prognostic and surrogate protein markers is also ongoing. Several excellent reviews regarding mass spectrometry-based protein biomarkers can be found in [28,32-34].

2.2 Translational oncoproteomics

The ultimate goal of this interdisciplinary research field is thus to translate basic scientific knowledge into applications in the clinic. Currently, however, the impact of clinical proteomics on patient management and clinical decision-making is insufficient and the implementations of scientific results in the clinic appear to be scarce [9]. Although clinical proteomics did provide substantial advances in understanding the pathobiology and the molecular basis of several diseases that served as potential biomarkers, only a limited amount of these ‘interesting’ biomarker candidates are ultimately translated into a clinical test [35]. This scenario is illustrated by the fact that hundreds of potential cancer biomarker candidates can be found in literature, but only few of them are approved by the Food and Drug Administration (FDA) [32]. A list of FDA-approved tumor protein markers which are presently applied in the clinic is summarized in Table 1. From this list, several of the approved protein markers are already discovered decades ago (e.g. carcinoembryonic antigen (CEA) in mid 1960s), and only a few new markers (or biomarker panels) are approved in recent years.

Most of the FDA-approved tumor markers are blood-based markers that enable the discrimination between malignant and benign states, as a complement on the regular imaging modalities, such as CT scans [36]. The majority of these blood-based tumor markers are nevertheless only efficient to detect

late-stage tumors. Cancer antigen (CA) 15-3, for example, is a blood based tumor marker for breast cancer, but cannot be used for early diagnosis purposes due to low sensitivity [37]. As most of the currently available screenings test for cancer mostly lack sensitivity and/or specificity to be useful in population-wide screening programs. The quest to find protein biomarkers able to perform early cancer diagnosis, is still ongoing. [38].

On the other hand, most of these blood-based markers are helpful for disease staging and monitoring, and are thus mostly used for patients with an established disease to monitor disease recurrence or reduction [36]. The major disadvantage is however that most of them are not cancer-type specific [39]. In CRC for example, blood-based CEA levels are compared before and after treatment and checked regularly (mostly every three months) in order to detect disease recurrence. Abnormal levels of CEA in the blood however, are not specific for CRC nor for malignancy as elevated CEA expression is also demonstrated in breast, lung, gastric and pancreatic cancer but also in different inflammatory diseases such as inflammatory bowel disease and pancreatitis [40]. In 2009, a new serum-based tumor marker for ovarian cancer, human epididymis protein 4 (HE4) was approved by the FDA for epithelial ovarian cancer. Compared to CA-125, the 'gold standard' of ovarian cancer detection, HE4 had a higher sensitivity (43.3% vs 72.9%) and a specificity of 95%. Despite its potential, HE4 is currently only approved for ovarian cancer monitoring recurrence or progression, since no established cut-off for a positive HE4 test is available [41]. Interestingly, most recent developed blood-based protein markers are 'panels of multiple biomarkers' rather than single proteins. In ovarian cancer, the combination of CA-125 and HE4 (called the ROMA test = Risk of Ovarian Malignancy Algorithm) was able to discriminate malignant vs. benign cases, with a sensitivity of 74.2% for early stage ovarian cancer and up to 91.7 % sensitivity for later stages [42]. Another biomarker panel for ovarian cancer is the OVA1 test. This test combines CA-125 with four additional tumor-secreted protein markers : beta-2-microglobulin, apolipoprotein A1, prealbumin, and transferrin [43]. Expression levels of this OVA-1 panel are able to evaluate an ovarian/pelvic mass prior to surgery and indicate a woman's likelihood of malignancy. The test improves the accuracy of diagnosis, which is important as it identifies women who will benefit from referral to a gynecologic oncologist for surgery notwithstanding that other clinical/radiologic results indicate the absence of ovarian cancer. Although this might seem a minor result, it is demonstrated that clinical outcome is much better for ovarian cancer patients treated by gynecologic oncologists [44].

Besides blood-based markers, some FDA-approved protein biomarkers for cancer can be detected in other non-invasive matrices, such as urine or feces. Expression of tissue-based approved markers, used in pathology labs to diagnose/predict/prognose the resected tissue with cancer, are on the other hand mostly evaluated in concert with both cell type and location.

Besides the markers approved by the FDA, several other protein-based markers (or panels) are validated in large populations or implemented in clinical trials. In the CALGB80303 trial for example, VEGF-D, SDF-1 and Ang-1 were tested as predictive markers for the (lack of) benefit of bevacizumab treatment in pancreatic cancer patients [45]. In a recent publication of a phase III trial of Gregorc et al 2014, for example, the predictive value of a proteomics signature in blood of non-small cell lung cancer patients was tested and appeared to successfully classify patients benefitting from EGFR-targeted therapy (erlotinib) compared to chemotherapy (trial is registered with ClinicalTrials.gov, number NCT00989690)

[46]. Another recently published result from a phase II trial indicates that differential expression of hepatic proteins is able to predict the tumor response in patients with colorectal liver metastasis [47]. These examples nicely demonstrate that the proteomics technology is more and more integrated in the clinic.

2.3 Proteomic approaches in biomarker discovery and validation

A mass spectrometry-based proteomics biomarker study typically starts with a discovery experiment, where differences in protein expression in small case-control cohorts are assessed in a semi-quantitative approach. In these discovery experiments, different proteomics platforms can be chosen: a gel-based platform, a gel-free setup and an MS-imaging system. An overview of these three platforms can be found in Figure 1.

Within the 20 years of oncoproteomics, two dimensional poly-acrylamide gel electrophoresis (2D-PAGE) is historically the most frequently used analytical protein separation technique [48]. In gel-based approaches, samples are homogenized and cells are lysed before proteins are extracted. The extracted proteins first undergo isoelectric focusing (IEF), which separates them based on their isoelectric point (pI). In a second dimension, the proteins are separated according to molecular weight via sodium dodecyl sulphate (SDS)-PAGE [49]. After staining the gels, thousands of protein spots can be visualized. Although these spot maps provide an overview of the proteome, protein quantitation in 2D-GE experiments is hampered by several factors, including the limited range of detection of staining methods and a considerable gel-to-gel variation making quantitative comparison of spot patterns and further statistical interpretation challenging. These limitations were partially overcome by the introduction of two dimensional differential gel electrophoresis (2D-DIGE) [50], which enables the analysis of more than one sample in a single 2D gel. In 2D-DIGE, proteins are fluorescently labelled with Cy2, Cy3 and Cy5 prior to 2D-gel separation. In the 2D-DIGE set-up, the Cy3 and Cy5 dyes are used to label proteins from two biologically independent samples. The third fluorophore, Cy2, is used to label an internal standard, usually a pool comprising equal amounts of each of the experimental samples being compared. This internal standard ensures that all proteins present in the samples are represented, facilitating normalization and both intra- and inter-gel matching [51]. After the 2D separation, the gel is imaged using different excitation and emission wavelengths for the three dyes and the obtained images can be used to quantify the protein differences between samples using specialized software. Protein spots of interest are typically excised from the gel, digested in-gel using trypsin and analysed via mass spectrometry. Although gel-based approaches were the method of choice 10 years ago and are still widely used, currently gel-free shotgun approaches are the method of choice [52], as they allow a high-throughput analysis of protein samples in a non-targeted way.

In shotgun approaches, also referred to as peptide-centric approaches, samples are homogenized, cells are lysed and the extracted proteins are enzymatically digested into peptides. The resulting peptides are further separated using (mostly nano) liquid chromatography (LC) and analysed with (high resolution) tandem mass spectrometry. To obtain quantitative data, and thus identify proteins and measure their

abundance changes across multiple samples, a subdivision in label-based and label-free methodologies can be made. Although all quantitative labelling strategies have their strengths and weaknesses, the assumption that 'the earlier the label is introduced into the workflow, the lower the error in measurement will be' is appropriate for every strategy [53]. While metabolic (stable isotope labelling by amino acids in cell culture (SILAC)) and enzymatic ($^{16}\text{O}/^{18}\text{O}$ stable isotope) strategies exist, chemical labelling of peptide/protein termini or chemically reactive amino acid side chains are preferred in clinical proteomics. Isotope-coded affinity tags (ICAT) for example, exist in an isotopically light and heavy version of the tag that can label peptides of two different clinical samples [54]. However, because these labelling techniques (SILAC, $^{16}\text{O}/^{18}\text{O}$ and ICAT) make the acquired mass spectrum (MS^1) even more complicated, they are less convenient when complex samples need to be analysed. To that end, isobaric tags are more advantageous because the labels offer multiplexing capability which gives the opportunity to analyse larger sample sets and reduce the measurement time considerably [55]. Two kinds of amine-reactive isobaric tags are commercially available: Tandem Mass Tags (TMT) (2-plex, 6-plex or 10-plex) and isobaric Tags for Relative and Absolute Quantification (iTRAQ) (4-plex or 8-plex). Both TMT and iTRAQ reagents contain a reporter group and an amino-reactive group, spaced by a balancer group which generates an identical mass for all tags. Relative quantification of the differentially labelled peptides is achieved by the generation of a unique reporter ion upon fragmentation of the peptide precursor. The ratio between the signal intensities of these reporter ions in tandem mass spectra reflects the relative expression levels of the peptides in the different multiplexed samples [56]. Because relative quantitative information is gathered here in the low m/z region of the MS^2 spectra instead of the MS^1 spectra, proteome analysis of complex samples is more applicable.

Besides the introduction of labels to allow quantification, label-free approaches can also be used in clinical shotgun proteomics experiments and are often preferred when a large amount of samples (>50) need to be analysed. In these approaches, the preparation (protein extraction, reduction, alkylation, digestion) is performed for each sample separately and is followed by individual LC-MS/MS analyses [57]. Protein quantification can be based on spectral counting, where the number of fragment-ion spectra (MS/MS) acquired for peptides of a given protein are counted and compared between different samples. However, because this quantification method relies 'simply' on counting the acquired spectra, but ignores the different physicochemical properties of peptides, spectral counting method is considered less precise compared to other quantification methods. Label-free quantification can also be performed by the measurement of peptide ion intensities or equivalently ion counting. Here, the chromatographic peak area of a peptide ion of a particular charge and mass has to be determined. This area under the curve is used to compare relative abundances of peptides across different samples based on the m/z and chromatographic retention time of the ion. However, post-processing of raw LC-MS data is required and involves several computational methods that allow feature detection, alignment of the retention times, but also include noise reduction, optimal peak picking and normalization of MS intensities in order to enable comparison of peptides across experiments [58].

A third MS-based approach for biomarker discovery comprises mass spectrometry imaging (MSI). MSI has the major benefit that it allows measurement of intact tissue sections [59] such that the spatial information is maintained, where other approaches require the homogenization of the sample. During

MSI, a virtual equidistant grid is imposed on the tissue section. For each grid point a mass spectrum is generated and the distribution of different m/z values can be analysed. This imaging technique can thus provide spatial and mass distributions and the relative abundances of a large number of biomolecules (e.g. peptides, proteins and metabolites) directly *in situ* from a tissue in a single experiment [60]. The unique advantage of MSI relies in the possibility to produce spatially resolved mass spectrometric data without destroying the tissue morphology, making a correlation with histological results possible. However, major issues regarding protein/peptide identification still exist.

The new candidate markers that are found with aforementioned MS-based discovery techniques, require extensive verification and validation, preferably with orthogonal methodologies. In the verification stage, each biomarker candidate is quantified for a limited number (10s to 100s) of clinical samples, while validation requires a large-scale study (100s-1000s of samples) to fully unlock the biomarker potential in terms of specificity, sensitivity and predictive values (Figure 2)[61].

The verification and validation of protein biomarkers can be conducted via two different analytical platforms: an antibody-based setup or a mass spectrometry-based platform. Mostly, antibody-based technologies are preferred when only a few biomarkers need to be verified in a large amount of samples and the antibody against the targeted protein or a kit (e.g. enzyme linked Immune sorbent assay (ELISA)) is commercially available. However, many interesting biomarker candidates lack commercially available antibodies which is a major pitfall as the development of new monoclonal antibodies and new ELISA assays are extremely expensive and time-consuming [62]. Furthermore, although it is possible to multiplex ELISA technology for the simultaneous detection of multiple proteins (e.g. Luminex platform, microplate-based multiplex kits etc.), most of these commercially available tools only work with a predefined set of proteins (e.g. pathway or function related).

In cases when no immunoassay is available or a higher specificity is required, mass spectrometry-based applications can be implemented. Rather than quantifying thousands of proteins in a non-targeted way in the discovery phase, verification and validation is based on the targeted measurements of peptides which are unambiguous for a certain protein. In multiple reaction monitoring (MRM), a specific peptide of interest and (a) discriminating fragmentation ion(s) (i.e., a transition) are selected, and by relying on the combination of precursor (=peptide) mass selection as well as fragment ion mass selection, all other acquired signals of the complex clinical sample can be ignored and the peptide of interest can be quantified [63,64]. MRM is therefore ideal to analyze and quantify up to hundred protein markers in a large number of samples [65]. Recent developments in targeted strategies also introduced parallel reaction monitoring (PRM) which allows high resolution, full scan MS/MS data from which transitions can be extracted [66,67]. MRM and PRM have the potential to speed up the validation of candidate biomarkers due to their large multiplex capabilities combined with excellent limits of detection [68]. Development of MS-based targeted methods however still take several months for a specific type of biomarker. Furthermore, the production of stable isotope-labeled standards (SIS) peptides that allow absolute quantification is still costly [61]. An overview of all mass spectrometry-based proteomic technologies applied in biomarker discovery and verification is provided in several excellent reviews [52,53,69,70].

With this in mind, it is clear that measuring hundreds of candidates in hundreds of samples becomes an uncertain path with low return on investment as a large number of candidates need to be validated before a (set of) biomarker(s) can be found. Therefore, it is extremely important to perform a high quality discovery study, so that the number of false positive biomarker candidates due to the lack of proper study design, low quality samples etc., can be minimized. Furthermore, as the costs of the verification/validation procedure are high, many research groups do not even start the verification phase and just publish their discovery results. This *modus operandi* partially explains why most of the published biomarker candidates never reaches clinical practice.

3. Challenges in clinical proteomics

Although clinical proteomics did allow to provide substantial advances in understanding the pathobiology and molecular basis of several diseases, and initial findings in the biomarker-field seemed promising, only a limited amount of these ‘interesting’ biomarker candidates are ultimately translated into a clinical setting [35]. This paucity of approved biomarkers is not due to a lack of effort. The major problem here is that most of the proposed biomarker candidates are unlikely to meet the high bar of clinical validation [71]. This discrepancy between the effort to find new biomarkers and the actual number of biomarkers in medical practice reflects the challenges that are still present in clinical biomarker discovery approaches. An overview of the pitfalls and obstacles that are currently present in clinical proteomics applications with a focus on protein biomarker discovery in cancer research are summarized in Figure 3. Although some of these challenges are important for proteomic researchers (e.g. it is necessary to include quality check points in proteomics workflows to ensure that high-quality end results are obtained [72,73]), some others are of major importance for both clinicians and scientists. The latter are described in more detail below. Furthermore, table 2 represent the current limits of application of proteomics in translational oncology.

3.1 Nature and quality of clinical samples

In clinical proteomics studies, such as biomarker discovery studies, the quality of the study depends on the quality of starting material. If low quality samples are used, no trustworthy results can be obtained, regardless of how accurate the rest of the experiment is [74]. Only if this first pitfall, also known as ‘garbage in = garbage out’, is circumvented, good biomarker candidates can be found. This is, of course, under the premise that the experiment is designed in such a manner that it optimally answers the formalized research hypotheses. The topic of experimental design will be fully discussed in the next section.

To this end, one of the major challenges researchers are confronted with is obtaining high quality samples which are compatible with clinical proteomics technologies. A first step towards obtaining high-quality samples is the set-up of biobanks which collects, stores and processes biological materials, and

their associated genetic, epidemiological, or outcome information. Biobanks can thus be seen as biorepositories that store bio-specimens (e.g. plasma or fresh frozen tissue samples) for research purposes and allow thus to obtain cohorts of samples in a relatively short time period [75]. However, the nature and quality of the sampled material is of tremendous importance in clinical proteomics applications. Still in too many cases, samples are collected in conditions that are not compatible with mass spectrometry-based proteomics. A case in point here is the collection of plasma several hours after blood withdrawal and without the addition of protease inhibitors that limit protein degradation. Furthermore, pathology biorepositories with formalin-fixed, paraffin-embedded (FFPE) tissues are a valuable source of clinical samples but appear suboptimal in regard to proteomics analysis [76].

Besides the nature of the obtained samples, improper handling of samples does also influence the quality of the samples (e.g. sample contamination, protein degradation, etc.) and thus the obtained results. Method of transport and storage of samples, for example, are of tremendous importance because these protein biomarker candidates may degrade when samples are not processed or stored immediately after their collection. Resected tumor material, for example, should be fixed or frozen as soon as possible after resection. However, in current practice, these samples reach pathology labs mostly in a time frame of hours rather than minutes, rendering lower quality samples. If resected tissues are stored several hours later, the protein expression can change (as a result of diminished blood flow etc.) or protein degradation due to the release of proteases can occur. These changes do not reflect the initial protein expression alterations where the researchers are interested in, and should therefore be avoided. Ideally, the storage (e.g. frozen in liquid nitrogen) should be handled in minutes after resection, however, a more realistic time frame will be between 10 minutes and 30 minutes. Therefore, international standard operating procedures for all kinds of samples are necessary which include very short processing times and storage guidelines [77]. In most cases, clinical samples are also already manipulated before the proteomic actual analysis is performed [78]. These manipulations include freeze-thaw cycles, temperature of storage, time before storage, type of anticoagulant used when collecting blood samples, etc.,... Lee and colleagues for example, studied the influence of the temperature, freeze-thaw cycles and different protein extraction procedures on the urinary proteome of one healthy volunteer [79]. Rai and colleagues summarized the effects of pre-analytical variables in human plasma and serum [78]. From both studies, it is clear that deviations in sample handling may significantly alter the proteome which can create differences between comparable samples. Doing so, sample handling can introduce confounding factors in the study. Therefore, it is recommended that samples used in the same clinical proteomics experiment should also be handled by the same protocol [80]. This also includes that patient samples and control samples must be collected in the same time frame, with the same pre-analytical variables, in order to obtain disease-specific results. An overview of the most common pre-analytical variables is provided in table 3.

This problem points out that, unfortunately, there is a conflict between the quality of samples needed by the proteome scientist and the practice of collecting samples in hospitals. Therefore, only if close collaborations with hospitals (oncologists, pathologists etc.) are set up, and the specific demands for sample collection are discussed, high quality samples, compatible with mass spectrometric techniques, can be obtained.

3.2 Experimental design

Currently, one of the major reasons why most biomarker candidates found by proteomics techniques do not survive validation is due to the lack of well-powered experimental designs in discovery studies. In the experimental setup, two things are crucial: 1) the statistical power and 2) the design of the experiment [81]. The statistical power reflects the ability to detect an effect in a population when the effect is present. This power thus indicates the probability of avoiding a false-negative conclusion and must be high enough to obtain reliable statistical results. The statistical power of an experiment depends on three parameters: the effect size (minimal fold change), sample size and total variance [82], with the latter two terms to be determined from the experiment. To obtain an appropriate power (mostly 90% or higher in discovery experiments), it is thus necessary to quantify the sources of variation in that workflow and to limit them as much as possible [72]. The total variation is the result of both technical variation introduced by sample preparation or analytical processes and biological variation which includes factors like gender, age, health status etc.... Although it is not always possible to experimentally determine these variation parameters in a pilot experiment (for example due to the lack of clinical test samples), these preliminary experiments (in general) benefit the accuracy of the power calculations [83]. Furthermore, the sample size numbers necessary to obtain the required statistical power can be computed when good estimations of all previous parameters are obtained [84]. These sample size calculations are necessary as a suboptimal amount of samples gives unreliable statistical results, while oversampling is a waste of materials, money and time. All these parameters together determine the relevance of the results and are interconnected with each other. For example, for a certain power, significance level and effect size, the amount of variation will influence the sample size proportionally. In other words, samples with high variability require more biological replicates than those with low total variation values to assess whether an observed effect is a true effect (Figure 4). Suboptimal estimation of one of the parameters can thus have a negative influence on the reliability of the observed results.

A second threat to the validity of the experiment is related to the bias in the design. Bias can be defined as the systematic erroneous association of group characteristics in such a way that it distorts the comparison with another group [85]. Bias can be present at several levels and is mostly correlated to the 'problem of baseline equality'. If, for example, a comparison between male patients and female healthy volunteers is conducted, a bias is created as the outcome may depend on gender differences rather than on disease state. Other biases can be less evident but unintentionally created for instance when controls collected in one clinical center are compared to patients from another center, when controls are measured at day 1 and patients at day 2 or when different storage duration times are present between the different groups [86], etc.. In literature, some examples of such bias can be found. Villanueva and colleagues, for example, found tumor-specific peptide profiles in prostate, breast and bladder cancer. Although they reported that the described peptide profile in prostate cancer had a specificity and sensitivity of nearly 100%, the samples were not age and gender matched. While all patients were males with a mean age of 67, the control group was significantly younger (mean age 35) and consisted out of more than 50% female healthy volunteers [87]. This, of course, jeopardizes the reliability of the results [86,88]. In a second example from literature, a serum-based protein test was reported able to discriminate women with ovarian cancer from women without ovarian cancer [89]. However, bias was

created between the cancer patients and their age-matched controls as these two groups were derived from judgmentally different sources [90].

To guard against unknown biases, samples should be randomized and changes in the protocol should be avoided during the conduct of a clinical study. This randomization can be achieved by creating an arbitrary but balanced order of sample processing. To achieve this balanced setup, a detailed description of several clinical parameters of all patients (and healthy volunteers) must be available. With this information, it might for example be possible to find control persons that match the patients in the best possible way [91]. For example; patient accrual in the study should follow the guidelines from clinical trials. Furthermore, as the molecular heterogeneity of several tissues is huge, using well defined experimental groups will also benefit the experiment, for example by using only a particular tumor type or tumor stage.

Besides the bias that can be introduced in the setup, the design of the whole experiment is also crucial to obtain high quality results. The lack of a good study design, can jeopardize the whole experiment. An example of such a problem is demonstrated by Hu and colleagues [92]. In their publication,, they illustrate a study where a mass spectrometric analysis of five types of cancer is performed. However, due to lack of design of the study, the obtained results could not be clustered according to cancer type, but rather cluster based on their run dates. This problem could have been overcome when a randomization was achieved by creating an arbitrary but balanced order of sample processing. In a second example, the same group also shows data from a study where the proteomic analysis of 50 cancer tissues did not deliver the results they expected as a change in the protocol halfway the sample processing forced clustering to distinguish protocols rather than obtaining biologically relevant subtypes [92].

So, regardless which proteomic analysis set-up is used, a good experimental design is essential. Therefore, it is also recommended to involve a statistician before the onset of the experiment, as stated by Ronald A. Fisher: “to consult a statistician after an experiment is finished is often merely to ask him to conduct a post mortem examination. He can perhaps say what the experiment died of”. These issues also point out that an intense cooperation between researchers, statisticians and clinicians is crucial to successfully perform a clinical proteomics experiment. In these ‘successful’ experiments differentially regulated proteins are not related to differences in parameters such as age, gender or treatment, but are disease-specific changes.

3.3 Complexity of the human proteome

One of the major challenges in proteomics is the fact that the proteome is highly dynamic, i.e. it is changing in every tissue and at every point in time. Characterizing the wide variety of proteins in diverse tissues combined with a protein concentration range that might exceed ten orders of magnitude, complicates the deep profiling of the human proteome extensively [93]. This large dynamic range unfortunately also outperforms the modern mass spectrometers, as only up to four orders of magnitude can be analyzed [94]. This means that, although current MS techniques are able to detect very low

amounts of proteins (in attomole range), still major difficulties in detecting these low abundant proteins exist when high abundant proteins are present. In clinical samples, it is possible to detect the 5000 most abundant proteins expressed at a certain time point, but challenges remain to detect the lower abundant proteins, and especially the 'dark corner' of the proteome (Figure 5) [94].

Although several extensive pre-fractionations are used to reduce the overall complexity, even in combination with the newest high-resolution instruments, proteomic analysis fails to detect the lowest concentrated part of the proteome. In plasma for example, one of the most accessible sample types in clinical proteomics, the ten most abundant proteins account for 90% of the total plasma protein content, making characterization of the full plasma proteome a major challenge [95]. Furthermore, as low-abundance proteins represent the most promising source of biomarkers, analyzing all these proteins and their isoforms will stay a major and almost infeasible task in the near future. The most recent attempts to map the entire human proteome have shown that, currently, proteomics is capable of mapping 84% of the protein coding genes [96,97]. Besides the fact that not all proteins can be detected, it should be noted that a second complexity-related challenge lies in the uncertainty of protein identification by database searching [97].

The complexity of the human proteome can be circumvented in part by directing the analysis on other sample types. However, although primary cell lines (2D and 3D) or animal models are excellent model systems to reveal insights in pathways and signaling mechanisms, the translation of biomarker candidates towards patient samples is more difficult. Human samples, such as blood or frozen tissues, are therefore in many cases still preferred. However, instead of using a 'whole tissue' proteome, such as blood, sample fractionation strategies are implemented to lower the proteome complexity. This fractionations lead to less complex 'sub-proteomes' and can be performed both at a cellular (e.g. platelets [98], leukocytes [99]) or subcellular (e.g. mitochondria [100], exosomes [101]) level.

4. Outlook

Clinical proteomics applications have yield several novel insights into cancer biology and boosted the search for new cancer drug targets the last decades tremendously. In biomarker discovery however, clinical proteomics did lose some of its credibility due to the many, often highly optimistic, reports of new cancer biomarker candidates which could not be reproduced in independent settings [9,34,102]. Currently however, the new guidelines and recommendations for proteomic biomarker discovery studies (including quality control) are more and more implemented, with a multitude of cancer biomarker candidates surviving verification stages and waiting for large-scale validation as a result. Initiatives, such as the Proteome Standardization Initiative (PSI) of the Human Proteome Organization (HUPO) and the Clinical Proteomic Tumor Analysis Consortium (CPTAC) are well-known and of high importance to the field [103,104]. With advances in MS-based targeted technologies for high-throughput validation, large cohorts of biomarker candidates are expected to be measured in a short amount of time which might speed up translation of scientific results to clinical settings. Although the search for the ideal biomarker

for many malignancies remains a challenging initiative, mounting evidence is present that 'omics' technologies provide valuable information on new candidate biomarkers for cancer diagnosis and screening [105]. Indeed, integration of proteomics findings with genomics and transcriptomics data might even be the next step in the 'bench to bedside' process.

5. Conclusion

The progress that has been made in the field of mass spectrometry and protein technology over the past decades, could not deliver the expectations, since the translation of proteomics results into the clinic is lagging behind. Still in too many cases, issues regarding the quality of the samples, the experimental design, the complexity of the human proteome, improper quality control and costly analytical methods in both discovery as well as validation steps devalue these findings. Current efforts of many researchers however, do show that the future might be bright for clinical proteomics applications. In many research projects, multidisciplinary teams (biologists, biostatisticians, oncologists, pathologists,...) are included nowadays which not only provides a link with the clinic, but also take into account that the experimental design is scientifically sound before the experiments are performed. Also, an additional focus in several proteomics research groups towards quality control of the mass spectrometry analysis will be beneficial for future projects. Can this increasing awareness of these clinical proteomics-related challenges and the attempts to tackle them really mean a step forward towards the implementation of protein biomarker candidates in clinical practice? Will the execution of highly controlled workflows in the analysis of high-quality subproteomes (e.g. exosomes, lymphocytes,...) lead towards the protein biomarkers surviving the biomarker pipeline? Chances are huge, but only time can tell.

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Figures:

A. Gel-based proteomics

B. Gel-free proteomics

C. Mass spectrometry imaging

Figure 1: Overview of three complementary proteomics workflows used in cancer biomarker discovery.

Figure 2: Schematic representation of the different stages in the biomarker pipeline.

Figure 3: Schematic representation of the challenges in clinical proteomics applications.

Figure 4: Overview of the influence of the variation of the sample size. If protein expression differences need to be quantified, samples with a large coefficient of variation will need more biological replicates compared to samples with a low coefficient of variation when the same power, same effect size and level of significance are applied.

Figure 5: The distribution of protein abundances can be represented via a bell-shaped curve on a logarithmic protein copy number scale. The figure indicates that detecting the most abundant half of the proteome (i.e. 5000 proteins with more than 10000 copies per cell) is currently possible with the dynamic range of modern mass spectrometers. Deeper analysis in the proteome however, requires very extended fractionation and separation times and has a lower rate in discovery of new proteins. The detection of the 1000 least abundant proteins remains the dark corner of the proteome. Reprinted with permission [94] .

Tables:

Table 1: FDA-approved protein markers used in clinical practice.

<i>Biomarker</i>	<i>Clinical use</i>	<i>Disease</i>	<i>Specimen</i>
<u>Blood-based</u>			
Alpha-fetoprotein	staging	testicular cancer	serum
Human chorionic gonadotropin-beta	staging	testicular cancer	serum
CA 19-9	Monitoring	pancreatic cancer	serum, plasma
CA125	Monitoring	ovarian cancer	serum, plasma
CEA	Monitoring	colon cancer	serum
Thyroglobulin	Monitoring	thyroid cancer	serum, plasma
PSA	screening and monitoring	prostate cancer	serum
CA 15-3	Monitoring	breast cancer	serum, plasma
CA27-29	Monitoring	breast cancer	serum
Fibrin/FDP	Monitoring	colorectal cancer	serum
HER2/NEU	monitoring	breast cancer	serum
Pro2PSA	discriminating malignant vs benign	prostate cancer	serum
HE4	Monitoring	ovarian cancer	serum
ROMA (HE4+CA125)	prediction of malignancy	ovarian cancer	serum
OVA1	prediction of malignancy	ovarian cancer	serum
<u>Urine-based</u>			
NMP22	screening and monitoring	bladder cancer	urine
Fibrin/FDP	Monitoring	bladder/colorectal cancer	urine
BTA	Monitoring	bladder cancer	urine
<u>Tissue-based</u>			
Epidermal growth factor receptor	Therapy selection	colon cancer	tissue
KIT	Diagnosis and therapy selection	gastrointestinal tumor	tissue
Cytokeratins	prognosis	breast cancer	tissue
HER2/NEU	prognosis	breast cancer	tissue
p63 protein	diagnosis	prostate cancer	tissue
Estrogen receptor	prognosis, predictive	breast cancer	tissue
Progesteron receptor	prognosis, predictive	breast cancer	tissue

Table 2: Limits of applications of proteomics.

<ul style="list-style-type: none">* Limited dynamic range of MS-based proteomic technologies Dynamic range of proteins exceeds 10^7, while MS range is limited to 10^4* Not all samples are suited for MS-based analysis Collection and preservation of samples in biobanks is crucial* very complex proteomes need fractionation Separation of proteins in multiple dimensions Working with subproteomes

Table 3: Overview of pre-analytical variables important in proteomic studies.

Type	Controlable?	Blood	Tissue	Urine
Time of collection	yes	✓	✓	✓
Method of sample collection	yes	✓	✓	✓
Fasting status	yes	✓	✓	✓
Diet	no	✓	✓	✓
Alcohol use	no	✓	✓	✓
Smoking status	no	✓	✓	✓
Medications	no	✓	✓	✓
Age	no	✓	✓	✓
Gender	no	✓	✓	✓
Ethnicity	no	✓	✓	✓
Type of sampling tube	yes	✓		✓
Order of collection	yes	✓	✓	✓
Time from collection to freeze	yes	✓		✓
Temperature of freeze	yes	✓	✓	✓
freeze-thaw cycles	yes	✓	✓	✓
Aliquot size	yes	✓		✓
warm ischemia time	no		✓	
cold ischemia time	no		✓	
Time from resection to freeze	yes		✓	
Time from resection to fixation	yes		✓	
Fixation time	Yes		✓	
Type of fixative	yes		✓	
Time of storage	yes	✓	✓	✓
Transport	yes	✓	✓	✓

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Dr. Evelyne Maes received her Master's degree in Biochemistry and Biotechnology from the University of Leuven (KU Leuven) in 2009. She joined the Functional Genomics and Proteomics research lab (KU

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Inge Mertens

Inge Mertens is coordinator of the Center for Proteomics (CFP), a joined initiative of VITO and University of Antwerp, Belgium. She is also appointed as professor in the department of Molecular Imaging, Pathology, Radiotherapy & Oncology (MIPRO) within University of Antwerp. She is author/coauthor of >30 publications in peer-reviewed journals. In her current position, her main fields of interest are clinical applications of proteomics technology.

Dirk Valkenburg

Dirk Valkenburg received his M.Sc. degree in Electrotechnical and Mathematical engineering from the Catholic University of Leuven (Belgium) in 2004. He received his Ph.D. degree in Mathematics and Statistical Bioinformatics from the University of Hasselt (Belgium) in 2008 and obtained a M.Sc. degree in Biostatistics in 2009 from the same institution. In 2008, he joined the laboratory of Prof. Dr. Geert Verbeke as a Postdoctoral researcher in the Department of Public Health at the Catholic University of Leuven to initiate new activities in the field of statistical bioinformatics and to support omics-related research at the University Hospitals of Leuven. In 2009, Dirk Valkenburg assumed a position as corporate scientist at the Flemish Institute for Applied Research (VITO, Mol, Belgium). Within the Center for Proteomics research facility, Dirk Valkenburg is team leader of 'statistical data analysis and x-omics'. His research is centred on data processing, statistical analysis of various 'omics' data and integrating data workflows. Currently, his research group is focusing on computational tools for the low-level interpretation of 'omics' data. The auxiliary and meta information extracted by the low-level data analysis can be used to augment molecular and clinical data repositories. A key objective in this research line is the use of this information for quality control and quality assurance to assess data integrity. Furthermore, Dirk Valkenburg is a visiting professor at Hasselt University and is affiliated to the Center for Statistics (CenStat). CenStat is a research institute of UHasselt and is internationally renowned, both for its theoretical as well as for its applied research in Biostatistics and Statistical Bioinformatics.

Patrick Pauwels

Patrick Pauwels is professor in molecular oncopathology at the Antwerp University. He is member of the board of directors of the CORE (Center for Oncologic Research) Antwerp. After his studies in medicine at the Catholic University of Leuven (Belgium), he started a residency program in internal medicine. After two years, he switched to a pathology residency program (KUL). He became a pathologist in 1992 and joined the Stichting PAMM (Catharina Hospital), Eindhoven, the Netherlands. His career became academic when he moved to the Maastricht University Hospital (the Netherlands), where he was

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Christian Rolfo

Christian Rolfo is associate Professor in the oncology department at the University Hospital Antwerp, Belgium. He studied at the National University of Córdoba, graduating from medical school in 1996. In 1998, he got a scholarship at the University of Milan for a project in Breast Cancer with the prestigious Dr. Luca Gianni, in one of the European reference centers for cancer. With him, he worked for more than six years, dealing with Breast Cancer, Head and Neck Tumors, Sarcomas and Rare Tumors. During this period he dealt with caring and Phase I Research Protocols, under the direction of Prof. Gianni. In 2003, he obtained the European Oncology Board certification. Dr. Rolfo is also a Doctor of Medicine and Surgery at the University of Catania, Italy. In 2004 he moved to Palma de Mallorca, Spain, where he worked at the Oncology Unit of the Clinica Rotger until September 2012. In 2009, Dr. Rolfo obtained the PhD degree and Doctor Europaeus in Clinical and Experimental Oncology Research with cum laude from the University of Palermo, Italy. From October 2012, he is Senior Staff Member in the Department of Oncology as Associate Professor at the University Hospital Antwerp, Belgium. Currently he is Head of Phase I - Early Clinical Trials Unit and Director of Clinical Trials Management Program. He has a major focus on Clinical Research, Drug Development and Resistance. He is actively working in a research program of Liquid Biopsies in Lung Cancer, specifically in exosomes isolation and circulating tumor DNA. Dr. Rolfo is the author of numerous papers published in various journals and is part of the working group of the ETOP mesothelioma (European Platform Thoracic Oncology), led by Prof. Rolf Stahel. He is also member of the membership Board of IALSC (International Association for the Study of Lung Cancer) and member of prestigious Societies including AACR, BACR, EACR, and ASCO. From 2015 onwards, he is a board member of Drug Development Group for ESMO.

Geert Baggerman

Dr. Geert Baggerman is a biologist specialized in mass spectrometry and proteomics/peptidomics. He procured his masters in Biology at the Catholic university of Leuven in 1997 after which he did a PhD on mass spectrometric analysis of natural peptides in insects in 2002. During his postdoc he, amongst other things, worked on the development of maldi imaging in order to allow the analysis of (neuro-)peptides directly from tissue. In 2008, Geert Baggerman became the director of the center for proteomics and metabolomics (ProMeta) at the Catholic University Leuven. In 2011, Geert transferred to The Center for Proteomics at the university of Antwerp (Belgium). The Center for Proteomics (CFP) is a shared initiative between VITO and University Antwerp to join forces in the field of mass spectrometry. The joint topic is on health research with a strong focus on cancer. Geert Baggerman is employed by both VITO and University Antwerp where he became a professor in 2012.