REVIEW

Applications of mass spectrometry for quantitative protein analysis in formalin-fixed paraffin-embedded tissues

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Proteomic analysis of tissues has advanced in recent years as instruments and methodologies have evolved. The ability to retrieve peptides from formalin-fixed paraffin-embedded tissues followed by shotgun or targeted proteomic analysis is offering new opportunities in biomedical research. In particular, access to large collections of clinically annotated samples should enable the detailed analysis of pathologically relevant tissues in a manner previously considered unfeasible. In this paper, we review the current status of proteomic analysis of formalin-fixed paraffin-embedded tissues with a particular focus on targeted approaches and the potential for this technique to be used in clinical research and clinical diagnosis. We also discuss the limitations and perspectives of the technique, particularly with regard to application in clinical diagnosis and drug discovery.

Keywords:
Biomarkers / Biomedicine / Formalin-fixed paraffin-embedded tissue / Oncology / Shotgun proteomics / SRM

1 Introduction

A better understanding of complex diseases goes hand in hand with a constant need for the discovery of novel targets and biomarkers that facilitate disease diagnosis, classification, and treatment. While targets and biomarkers are both relevant in the clinic, the literature mainly focuses on biomarker discovery in clinical research rather than on target discovery. In 2001, the term "biomarker" was defined by the American National Institute of Health as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [1]. One of the preferred biological sources for the quantification of biomarkers is blood as it is easily obtained in a relatively non-invasive manner. However, in certain areas such as oncology, diagnostic, and/or prognostic biomarkers are measured directly in biopsies or surgically resected tumoral tissues to support diagnosis and treatment. Tissue analysis allows direct access to the proteins of interest, at tissue concentrations, without the dilution effect implicit in the analysis of plasma.

In the field of clinical pathology, immunohistochemistry (IHC) represents a useful tool for tumor diagnosis and

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Abbreviations: FF, fresh frozen; **FFPE**, formalin-fixed paraffin-embedded; **IHC**, immunohistochemistry; **LLOD**, lower LOD; **LLOQ**, lower LOQ

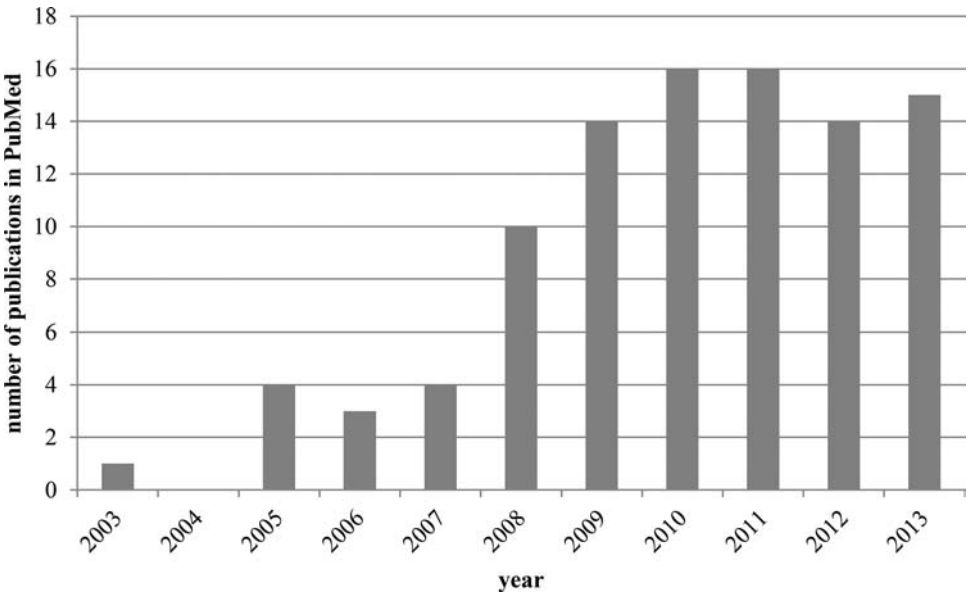

Figure 1. Publications involving proteomics from FFPE tissues. Number of hits in PubMed over the past 10 years in the field of MS-based proteomics in FFPE tissues. Date of search was 19.09.2013 and keywords included "mass spectrometry," "proteomic," and "formalin-fixed." Reviews were not included.

classification. However, this technique relies on specific antibodies for detecting the proteins of interest, the development of which might be time consuming and costly. Moreover, IHC is at best semiquantitative and affords only limited possibilities for multiplexing. In contrast, developments in MS have raised the anticipation for quantitative, reproducible, and highly multiplexed protein assays in tissue. However to date, the implementation of MS for the quantification of proteins in clinical diagnosis has been hampered by time-consuming sample preparation protocols and a lack of sensitivity for low-abundant species [2]. Most critically, however, MS-based tissue proteomics has been traditionally performed on fresh frozen (FF) tissues while clinical samples are generally fixed with formalin and embedded in paraffin (formalin-fixed paraffin-embedded (FFPE)). Indeed, storage of frozen tissues is expensive and difficult from a logistical point of view. Formalin fixation, on the other hand, allows preservation of detailed tissue morphology by forming cross-links between biomolecules and enables storage of samples at room temperature over long periods of time [3]. Therefore, FFPE has become a gold standard in tissue preservation.

While it has been possible for nearly two decades to retrieve information on gene expression levels from FFPE material [4–6], MS analysis of proteins in FFPE tissues is still in its early stages. Nevertheless, this is a growing field (Fig. 1) and some aspects, such as comparability of protein identifications in FF versus FFPE tissues, have already been investigated and reviewed [7, 8]. Despite these advances, there are still many open questions before considering implementation of protein quantification in FFPE tissues in a routine clinical setting. These include defining the nature of protein modifications induced by formalin fixation, evaluating losses occurring during protein extraction, finding solutions for protein content normalization in quantitative assays, and correlating MS-based results with well-established methods such as IHC.

In this paper, we will briefly discuss the current practice in protein retrieval from FFPE tissues prior to MS analysis. We will review recent advances in protein biomarker discovery in clinical research and discuss aspects relevant to targeted protein quantification in FFPE tissues, particularly from a clinical perspective. The analysis of proteins/peptides by MALDI-MS (mostly used in imaging mode, as reviewed by Chatterji et al. [9]) will not be covered in this review as its current application is mainly focused on the analysis of FF tissues, with some few exceptions [10, 11]. Finally, we will discuss the current limitations of MS proteomics on FFPE tissues, but also the possibilities for application in drug discovery or clinical pathology.

2 Comparability of proteomes from FF and FFPE tissues

Formaldehyde preferentially reacts with primary amines (lysine in the case of proteins) or primary amides (asparagine and glutamine) to form cross-links between biomolecules present in the tissue [12]. The intra- and intermolecular cross-links generated this way impair enzymatic activity and often also immunoreactivity [13]. Due to the covalent nature of formaldehyde cross-links, it was long assumed that tissue fixation was incompatible with protein analysis by MS. However, more and more studies have confirmed that heat-induced antigen retrieval used to restore immunoreactivity as well as to reverse cross-links formed among RNA and DNA molecules was also applicable for protein extraction [14]. Layfield et al. were the first to report in 1996 the

amino acid sequencing of a polypeptide corresponding to an immunoglobulin light chain extracted from formalin-fixed tissue [15]. Ikeda et al. then reported extraction of proteins from FFPE tissues followed by Western blot analysis. Their most effective extraction protocol included a 20 min heating step at 100°C followed by a 2 h incubation at 60°C in a RIPA buffer containing 2% w/v SDS, pH 7.6 [16]. Vasilescu et al. reported MS analysis of proteins extracted from cell lines, which had been formalin-fixed in order to cross-link protein complexes. Proteins were extracted at 95°C for 20 min in a buffer containing 2% w/v SDS, pH 6.8, after which they were subjected to SDS-PAGE and digested prior to LC-MS analysis [17]. Later publications have reported similar protocols for protein or peptide extraction from FFPE samples prior to shotgun proteomic analysis on various MS platforms, including RPLC-MS/MS, SELDI-TOF, and MALDI-TOF/TOF [18–21]. It is now well accepted that the key components to extract proteins from FFPE tissues involve heat, a detergent (typically SDS) and an alkaline buffer. The mechanism of antigen retrieval [22], as well as extraction conditions, have been extensively reviewed elsewhere [8, 23–26].

In addition to extraction conditions, other preanalytical parameters are susceptible to influence protein extraction from FFPE tissues. These include ischemic time (time between sample collection and formalin fixation), fixation time (time the sample was left in formalin solution), and storage duration (time from tissue fixation until protein extraction and analysis) [8, 25–28]. It has been demonstrated that a prolonged ischemic time (>60 min) negatively impacts the measurement of HER2 by IHC in FFPE breast tissue [29, 30]. Moreover, duration of formalin fixation affects the extent to which proteins can be recovered. While longer fixation times ensure better preservation of morphological features (in particular for large-sized samples), overall shorter fixation times (consensus being 24 h) almost invariably result in better protein yields. In contrast, tissue dehydration, paraffin-embedding, and storage time of the resulting tissue FFPE blocks appear to play a less significant role. A recent study suggested that storage time of up to 10 years did not significantly impact protein profiling [31].

Several studies have been performed with the aim of demonstrating equivalence between proteomes retrieved from FFPE and FF tissues using shotgun proteomics. Guzel et al. compared equal areas of microdissected paired FFPE and FF placental parenchyme tissue sections from women with pregnancies complicated by early onset preeclampsia and normotensive control women [32]. An average overlap in protein identities of 60% was observed with no significant difference in the overall number of proteins identified. In another study, Guo et al. reported a protein identification overlap of 83% between FF and FFPE from microdissected glioblastomas [33]. Crockett et al. investigated cell lines derived from a human transformed follicular lymphoma (SUDHL-4) using a complementary Glu-C and trypsin enzymatic digesting step to improve the overall protein identification rate, followed by nanoRPLC-MS/MS [21]. A total of 263 proteins, representing 52% of the total number of proteins identified from FF cells, were found to overlap between both types of samples. More importantly, the GO cellular location and molecular function of the proteins identified from a 3-year-old SUDHL-4 FFPE cell block and a fresh cell lysate were highly similar. In addition, analysis of the FFPE samples provided identification of low-abundance proteins including transcription factors. Tanca et al. used a canine mammary tumor model to compare the proteomic information generated from paired FFPE and FF specimens using gel-based protein fractionation followed by LC-MS/MS and spectral counting quantification [34]. A high level of consistency was seen for all biological and cell localization categories. More significantly, both data sets highlighted comparable protein pathways, suggesting consistent biological information was obtained from both sample types. Interestingly, Tanca et al. observed that high molecular weight proteins were more abundant in FF tissues, possibly because large, intact proteins were more difficult to extract from FFPE material, while basic proteins were overrepresented in FFPE tissues. A similar observation was made in another study where colorectal cancer tumors of three different cellularity levels (low, middle, and high) were analyzed by direct LC-MS/MS (Ducret et al. unpub. data). Mirrored FF and FFPE tissue showed intratissue reproducibility in terms of both protein number and abundance, with an overlap in protein identification of 55% between FFPE and FF tissue. However, the FFPE tissue showed a bias for small structural proteins (actin, calponin, etc.), DNA/RNA-associated proteins (histones, ribosomal proteins), and heat shock proteins, while large, structural multisubunits proteins (myosin, collagen, etc.) and blood proteins (serum albumin, hemoglobin, etc.) were more represented in FF samples.

All of these studies suggest that, while some differences exist with FF samples, the analysis of FFPE tissues provides reliable biological information. It is noteworthy that, while most recently developed extraction protocols appear to achieve nearly equivalent protein yields for FFPE and FF samples, formalin reversal may not be completely achievable. Most notably, publications based on whole protein fractionation, such as 2DE, consistently report lower yields and identification power than peptide-based fractionation methods, possibly because digestion might release analyzable peptides from even partially blocked proteins [35]. Moreover, a lower rate of lysine C-terminal peptides was observed in FFPE compared to FF tissue extracts [28]. There also seems to be differences in extraction recoveries for individual proteins. This fact was elegantly demonstrated by formalin-fixating different solutions of cytoplasmic proteins and HeLa cells as FFPE tissue surrogates [13]. After testing a range of conditions, the optimal extraction pH for lysozyme surrogates was determined to be pH 4, whereas it was pH 6 for carbonic anhydrase. When surrogates containing a mixture of proteins were analyzed, carbonic anhydrase was proportionally underrepresented in the extract. This indicates that multiple extraction conditions might be necessary for comprehensive protein recovery. Consequently, as stated above, some classes of

proteins (e.g. nuclear, cytoskeletal or membrane proteins) will be variably extracted from FFPE tissues. However, although one might expect the cellular localization (e.g. membrane proteins) to majorly impact the extraction efficiency, it seems that the physicochemical properties of the proteins play an even more important role in this regard [13, 21].

The observations discussed above also apply to the characterization and quantification of PTMs such as phosphorylation and *N*-glycosylation. While some studies claimed quantitative recovery from FFPE tissue [36, 37], other investigations [38–40] underlined the need for establishing guidelines and standardized extraction procedures to keep the already naturally occurring biological variability of PTMs in tissue to a minimum. In particular, Gundisch et al. reported that the degree of sensitivity of proteins and phosphoproteins to delayed cold ischemia varied between different patients and tissue types, with some proteins being up- or downregulated in an unspecific and unpredictable fashion while some others, such as glyceraldehyde 3-phosphate dehydrogenase, remained stable across all experiments [39]. However, the quantitative analysis of PTMs from FFPE tissues is still in its infancy and much remains to be done in order for it to become routinely applicable.

3 Protein biomarker discovery in FFPE tissues by MS

The discovery of new biomarkers in FFPE tissues using MS has been typically performed using an untargeted shotgun approach wherein samples are digested using a proteolytic enzyme to generate peptides prior to LC-MS analysis. One of the main advantages of an untargeted approach is the ability to analyze samples in an unbiased (i.e. hypothesis-free) approach with respect to the characterization and the relative quantification of the proteins within the dynamic range of the mass spectrometer. However, the stochastic nature of the identification process (as the mass spectrometer is set up to fragment as many peaks as possible) and the finite scanning speed of the instrument limit both the number of proteins that can be confidently identified and the reproducibility of the measurement. One strategy to improve the odd for characterizing a biomarker of interest in FFPE tissues has been to microdissect specific cells of interest. Additionally, peptide-based (or more rarely, protein-based, due to the difficulty to reproducibly extract intact proteins from FFPE tissues) fractionation methods, such as IEF or bidimensional liquid fractionation, have been shown to significantly increase the number of proteins identified in a shotgun approach, at the cost of complex sample processing schemes and significantly longer measurement times. As for untargeted proteomic studies performed on FF tissues, studies on FFPE tissues usually lead to the identification of several hundred to several thousand proteins without and with prior fractionation, respectively.

The discovery of differentially regulated proteins in shotgun proteomic experiments has been typically relying on the co-detection of internal standards for normalization purposes (both technical and biological). However, the difficulty in generating appropriate reference proteome standards in the FFPE paradigm has constrained the use of the otherwise widespread stable isotope-based protein quantification methods, such as SILAC [41]. Recently, a few publications reported the relative quantification of proteins based on differential chemical labeling of peptides, such as iTRAQ [42, 43]. Also, binary comparisons can make use of $H_2{}^{18}O$-based tryptic digestion to increase analytical precision [18]. Nevertheless, a large majority of discovery proteomics studies in FFPE tissue have used label-free quantification methods, such as spectral counting, to derive differential protein abundance by comparing peptide counts [44]. As a consequence of the substantially reduced accuracy compared to intensity-based methods, hardly any experimental design will reach significance except for the very extreme large changes.

In spite of those limitations, a large number of biomarker discovery studies using an untargeted approach have been conducted in the last 5 years, the outcomes of which have been recently reviewed [8, 22, 24, 25]. Not surprisingly, most studies focus on oncology, most likely due to the large number of annotated samples in tissue repositories. Examples of nononcology studies include the analysis of renal tissues from diabetic patients [42], to find markers of nephropathy, and the investigation of open oral human papilloma virus lesions to differentiate patients that may be co-infected with the human immunodeficiency virus [45]. Overall, those studies share two general characteristics: a large variety of analytical methods and a usually very low number of biological replicates [8, 24], resulting in most shotgun proteomic experiments to remain underpowered. The use of modern high-resolution mass spectrometers has considerably improved our ability to confidently identify thousands of proteins in complex mixtures [46]. However, the multiplicity of experimental designs, combined with the numerous experimental variables to be taken into account, renders data interpretation extremely difficult. Finally, it is only very recently that generic biostatistical models have been proposed to take advantage of the rich but complex MS data that are generated in such experiments [47, 48]. It is, therefore, encouraging that in nearly all cases where there was a subsequent verification step either by IHC, SRM, or other targeted methods, candidate protein markers could be positively associated with the investigated disease. This indicates that the results obtained from untargeted proteomic analysis of FFPE tissues are consistent with well-established targeted techniques.

In summary, shotgun proteomics experiments will remain an important tool in clinical research to provide lists of protein candidates for biomarker discovery but the need for extensive sample preparation will naturally prevent the use of such a strategy for routine clinical applications. In the future, we expect the release of novel types of mass spectrometers to further increase the range of proteins amenable to quantification, the data of which should be better taken into account by biostatistical models. With respect to SRM, untargeted

proteomics experiments are useful tools to generate a list of suitable peptides for development of SRM assays.

4 Protein quantification in FFPE tissues using targeted MS approaches

While targeted methods such as SRM require prior knowledge of the analytes (such as *m/z* of the precursor and fragment ions), it has the advantage of being highly specific, reproducible, and provides a larger dynamic range than shotgun proteomics, while retaining a degree capacity to analyze multiple proteins. SRM is most often performed on triple-stage quadrupole instruments, with the first and the third quadrupoles acting as *m/z* filters and resulting in high specificity, whereas the second quadrupole serves as a collision cell [49]. The monitoring of several transitions for a given peptide, as well as monitoring several peptides per protein, further increases the specificity of this method.

The application of MS for the analysis of FFPE tissues is likely to gain importance in the future and it may become a helpful tool in clinical research and diagnosis, providing that a number of conditions are fulfilled. Compared to IHC, targeted MS analysis allows multiplexing of analytes and is more quantitative. However, in order to be implemented in pharmaceutical research or the clinic, it will need to undergo extensive analytical validation and to demonstrate an increased benefit compared to IHC. In opposition to proteins, the quantification by MS of small molecule drugs and their metabolites has been performed routinely for many years in clinical laboratories and stringent criteria apply to these assays. Guidelines for this purpose have been released by the American Food and Drug Administration (http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf). If proteins are to be quantified by MS for pharmaceutical research and clinical diagnosis purposes, the developed assays will need to show robustness and reliability comparable to that of existing assays used for small molecules and metabolites [2].

Table 1 summarizes current applications of targeted MS methods in FFPE tissues. The size of the different studies in terms of number of samples was very variable, ranging from 3 [50] to 55 samples [51].

Even though the procedure for analytical validation of proteomic studies still needs to be established and standardized, several of the studies in the literature contain an analytical validation step, with variations in the approaches and extent of validation. Analytical precision is often assessed and reported CVs are typically below 20–30% [32, 52–57]. Indeed precision is a criterion for selecting individual peptides and transitions. Considering the chemical modifications induced in proteins by formalin fixation, which are not completely understood [25], the reported CVs were surprisingly low. Variability across different slices of the same tumor was represented by CVs below 25%. While this is higher than some clinical standards, it shows promise for clinical use [57]. Linearity has been assessed with $R^2 \geq 0.97$ over dynamic ranges spanning up to —three to four orders of magnitude [32, 56]. The definition of the lower LOD (LLOD) and lower LOQ (LLOQ) has not yet been standardized. LLOD and LLOQ were defined by a threshold of the S/N (typically S/N > 3 for LLOD and S/N > 10 for LLOQ) or as the lowest measured concentration with a CV or a relative error below 20–25% or a combination of both [50, 53, 54]. These criteria are approaching the threshold set by the Food and Drug Administration for small molecules, where the CV at LLOQ should not exceed 20% (http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf). The reported on column levels for LLODs and LLOQs for studies in FFPE tissues were in the picogram [50] or attomol [53, 54] range.

The effect of background matrix on linearity and reproducibility was compared in three different sections of the same tissue [32]. While linearity and reproducibility in the three different sections were comparable, differences in the slope of the three regression curves suggest matrix-dependent signal enhancement/suppression due to the presence in the sample of species competing for ionization. Such effects should always be accounted for when performing an assay in a complex and variable background matrix [58], but they are often missing in the literature.

A few studies did not report any analytical validation of their assay and the conclusions derived from these studies should therefore be taken with caution [37, 51, 59, 60].

The addition of heavy isotope-labeled internal standards is critical to the quantification and precision and is usually used to account for variations in ionization efficiency. While most studies used ^{13}C- and ^{15}N-labeled standards [50, 53, 54, 59], one study used a deuterium-labeled standard [51], while another used synthetic peptides with a glycine insertion in the sequence [32]. This last option might work well provided that the retention time of the analyte and standard are close to each other and that the standard has a unique sequence in order to avoid signal contamination. The authors showed via direct infusion that the ionization efficiency was similar for the analyte and its internal standard. However, it would additionally be useful to check for ionization suppression effects in the elution window of both peaks. Two further studies used a stable isotope-labeled β-actin peptide spiked at a constant concentration of 20 fmol/μL for quantification [52, 57]. This option is not recommended as there is a relatively high risk of fluctuations in signal intensities throughout the chromatographic run, and these fluctuations will not be compensated for with an internal standard monitored at a single retention time throughout the analysis. Three studies have reported no use of internal standards for quantification [37, 56, 60].

The quantification step is rarely described in a detailed manner, and particularly if quantification was performed using a single reference point or a calibration curve (normal or reversed) [61]. For example, Hwang et al. [51] reported use of a single reference point quantification, although this method was shown to be highly inaccurate in the lower and upper range of the quantification domain [61]. Hembrough et al. performed a normal calibration curve in a nonhuman

Table 1. Summary of applications using a targeted approach to quantify proteins from FFPE tissues

Reference	Tissue	SRM quantification preceded by discovery phase	Micro-dissection	Number of proteins/peptides/ transitions monitored	Normalization	Orthogonal verification
Blackler et al. [59]	Brain (rat)	Yes	Yes	15 Proteins 21 peptides three transitions/peptide	Sum of all peptide ratios	No
DeSouza et al. [55]	Endometrium (human)	No	Yes	17 Proteins $\geq$ 2 peptides/protein three transitions/ peptide	Average of actin and tubulin	No
Fu et al. [50]	Aorta (human)	Yes	No	2 Proteins $\geq$ 2 peptides/ protein three transitions/ peptide	No	No
Gamez-Pozo et al. [37]	Lung (human) kidney (human)	Yes	No	Number of proteins: NA 18 peptides one transition/peptide	No	No
Guzel et al. [32]	Placenta (human)	Yes	Yes	One protein two peptides one transition/peptide	Number of cells	IHC
Hembrough et al. [53]	Xenograft tumors of NSCLC (mouse) lung (human)	No	Yes	One protein one peptide/protein three transitions/peptide	Identical sample surface (12 mm^2) and total protein content	ELISA IHC
Hembrough et al. [54]	Breast (human)	No	Yes	Four proteins one peptide/protein three transitions/peptide	Identical sample surface (12 mm^2) and total protein content	ELISA/ electrochemiluminescence IHC
Hwang et al. [51]	Prostate (human)	Yes	No	One protein one peptide/protein two transitions/peptide	Identical sample size (4 μm thick and 2 mm diameter)	No
Myers et al. [52]	Stomach (human) xenograft DiFi and HCT116 tumors (mouse)	Yes	No	13 Proteins $\geq$ 1 peptide/protein four transitions/peptide	Total protein content	Immunoblot
Nishimura et al. [56]	Lung (human)	Yes (separate publication) [72]	Yes	Six proteins one peptide/protein two transitions/peptide	Actin peptide	No
Sprung et al. [57]	Kidney (human)	Yes	No	Number of proteins: NA 114 peptides four transitions/peptide	No	No
Takadate et al. [60]	Pancreas (human)	Yes	Yes	112 Proteins 183 peptides 697 transitions	Actin peptide	IHC

NSCLC, non-small cell lung carcinoma.

complex matrix (peptide mixture of *Pyrococcus furiosus*), as they rightly state that there is no existing standard tissue matrix [53]. They also compared the linear response in the non-human and an expectedly more complex human matrix and showed that the response was similar in both cases, except for one protein where the slope and intercept were different in both backgrounds [54].

Typically, three peptides per protein and three transitions with the highest intensities per peptide are monitored in an SRM assay as this increases specificity [49, 62]. There is also value in monitoring peptides distributed throughout the sequence (constant and variable domains) to monitor the different variants of a protein [62]. However the number of peptides/transitions monitored varies in the literature with between one to two peptides per protein and one to two transitions per peptide [32, 51, 56] up to two to three peptides per protein and three to four transitions per peptide [50, 52, 55]. It is also noteworthy that all studies cited developed SRM assays for unmodified peptides, thus disregarding any potential modifications induced by formalin fixation.

While monitoring several peptides/transitions represents an advantage for specificity, it raises the question of how to deal with the multiple data points generated in order to obtain a single value representing the expression level of a given protein. In the majority of publications, there is no detailed description of how the SRM data were further processed. It is, for example, unclear how two peptides belonging to the same protein but with inconsistent results should be handled. Guzel et al. mentioned expressing the result as the average of both peptides measured [32], while Sprung et al. used the sum of the four transitions for a given peptide [57]. An option for handling this type of data is to use linear mixed-effects models [63, 64]. However, to our knowledge, none of the SRM methods on FFPE tissues cited here have used this strategy yet.

Normalization is particularly critical when working with tissue since the sampled amount is less quantifiable compared to the volume in the case of biological fluids for example. Several options have been considered for normalization. The simplest is to sample defined tissue volumes (e.g. biopsies of identical size) [51]. Others have used cell number [32], total protein content [52], sum of all peptide ratios for each analysis [59], or specific housekeeping proteins, such as actin or an average of actin and tubulin [55, 56, 60]. Hembrough et al. sampled identical surface areas and additionally normalized the result obtained for total protein content [53, 54]. Three studies did not report any normalization for tissue content [37, 50, 57]. A flaw with most of these approaches is that tissue heterogeneity is not accounted for. The number of cells can be obtained when performing microdissection and this measure can therefore account to some extent for tissue heterogeneity. However, the cell number is approximate and adding another normalization parameter, such as total protein content or a housekeeping protein might lead to more accurate results. The option of using the sum of all peptide ratios is interesting, however, it contains the risk of showing a bias toward the proteins of interest included in the assay, which will naturally be more elevated in certain cases (e.g. in diseased tissues versus healthy tissues), although the amount of sampled tissue might originally be identical. The most appropriate normalization factor still needs to be determined and its performance should be assessed as any imprecision or inaccuracy will be reported on the final measurement.

Alternatively, because sampling whole tissue sections does not account for heterogeneity of cellular protein expression, several studies included a microdissection step prior to protein extraction [32, 53–56, 59, 60]. Microdissection of specific cells, however, is a time-consuming process that must be performed by a trained pathologist and the small amount of tissue sampled may lead to sensitivity issues. Nevertheless, several SRM studies on FFPE tissues reported the use of microdissection, which simultaneously decreases sample complexity and increases the dynamic range of the analysis.

Several of the SRM assays developed on FFPE tissues published to date represent confirmation of findings observed during a preliminary discovery phase and orthogonal verification using other approaches is therefore limited in these cases [37, 50, 56, 59]. Myers et al., however, obtained immunoblot data consistent with SRM data [52] and Guzel et al. showed a good correlation between IHC and SRM data in a preeclampsia model, albeit with a limited number of samples [32]. However, in both cases, the degree of correlation was not defined. The largest cohort for orthogonal verification was presented by Takadate et al. in which IHC was used to confirm the association of four candidate prognostic markers and outcomes in 87 cases of pancreatic ductal adenocarcinoma [60]. The association of SRM data with IHC could be verified for four proteins, but further orthogonal verification using Kaplan–Meier curves led to ambiguous results for at least one of these proteins. Hembrough et al. correlated IHC with SRM data for EGFR in ten human non-small cell lung carcinoma xenografts and indicated a correlative trend [53]. In a more recent study, they determined HER2 levels by IHC in two cohorts of 10 and 19 breast cancer samples, but a correlation between IHC and SRM data was observed only in the first cohort, suggesting to the authors that many patients with a 3+ IHC staining for HER2 probably do not express elevated levels of this protein and are therefore unlikely to benefit from trastuzumab [54]. This example illustrates the potential of this type of method for the generation of new hypotheses.

In summary, targeted proteomics on FFPE tissues is slowly moving towards more routine applications, either in pharmaceutical research or in the clinical field. However, much work still needs to be achieved in order to obtain a consensus on several critical issues: sample preparation protocols, quantification, parameters for analytical validation, data processing, normalization, etc. More emphasis is still needed on the comparison with reference methods for protein or gene expression measurement in FFPE tissues (IHC, fluorescence in situ hybridization, etc.), as current reports of MS assays are still ambiguous and there is a lack of quantitative measures assessing how well these methods perform.

5 Perspectives for clinical applications

The analysis of proteins and peptides from FFPE tissues is constantly improving in terms of extraction, detection, and quantification. In particular, the characterization of proteomes from FFPE tissues and their comparison with proteomes from FF tissues are evolving rapidly and have the potential to significantly complement data obtained using expression arrays. Even though a large amount of data is already available through gene expression assays, it remains of importance to obtain protein expression data, due to the limited correlation between protein levels and gene expression [65–67]. Compared to IHC, which is traditionally used in clinical practice to classify tumors according to biomarker expression levels, MS-based protein analysis has the potential to provide a more quantitative assessment of tissue protein expression levels with a dynamic range of quantification of up to five orders of magnitude using SRM [49]. Furthermore, MS enables multiplexing assay formats with the measurement of several analytes in parallel. A key feature of an MS-based method is the analytical specificity enabling the analysis of isoforms, such as those resulting from somatic mutations [68]. Such discrimination is more difficult with IHC as antibodies selectively recognizing specific forms of an epitope rarely are available.

From a clinical and pharmaceutical perspective, analysis of the proteome from retrospective FFPE tissues presents several advantages. Such tissues exist in repositories with a high degree of clinical annotation. Notwithstanding the ethical and consent issues, such samples have great utility in clinical and research settings. The ability to profile large and well-annotated cohorts would facilitate understanding of pathophysiological processes and may enable the discovery of novel biomarkers or therapeutic targets. In particular, accurate protein quantification will be important to unravel deregulated cellular signaling events by providing a representative insight of cellular effectors. This includes PTMs such as phosphorylation, a process whose deregulation is often involved in cancerous cells [37]. There is an increasing recognition that personalized healthcare is of importance in cancer therapy. Functional classification of tumors, disease staging, biomarker definition, patient stratification, and choice of treatment may all be enhanced by the wealth of data generated from FFPE tissues. From a drug discovery perspective, the ability to analyze a target in its tissue environment and to understand its underlying regulation is absolutely essential to test the efficacy and selectivity of novel pharmaceutical agents. However, due to the invasive nature of tissue sampling, human samples are usually restricted to plasma or urine, more rarely cerebrospinal fluid is used. Oncology is a notable exception as tumor resection is often a part of the medical treatment. In this particular domain, the ability to analyze proteins from FFPE tissues combined with tissue collections available have the potential to further allow a deeper understanding of the mechanisms underlying cancer.

While MS analysis of FFPE tissue has advanced in recent years and is already showing some impact in medical research, it is important to also acknowledge current limitations, which need to be addressed before this technique can be considered as an option in clinical diagnosis. For example, it is now clear that retrieving peptides or even whole proteins from FFPE tissues is possible and to a significant extent comparable with proteomes from FF tissues. But questions remain to be answered regarding the optimal way to process these types of samples in a standardized manner. A key limitation of both shotgun and targeted proteomics in tissues is the lack of spatial resolution due to the sample preparation process. This aspect can be circumvented by dissecting out an area of interest by laser capture microdissection. However, despite recent advances in automation, laser capture microdissection requires significant pathology expertise, resources, and time, which is not easily compatible even with middle-scale studies (e.g. 50 samples) or a clinical diagnostic application.

Due to the many issues related to MS-based quantification of proteins from FFPE tissues, this technique is still a long way from being adopted in clinical settings. This is in part due to the complexity of the sample preparation procedure, which is relatively long and requires a number of manual steps including many variables, such as antigen retrieval or trypsin digestion. This is in contrast with IHC, where staining is performed by robotic systems with the concomitant speed and reproducibility. Another important aspect is tissue heterogeneity and the requirement for normalization to allow comparison between samples. Tumor samples typically contain a high degree of cellular heterogeneity, represented by various amounts of tumoral cells and stroma, with possibly hypoxic or necrotic areas. Accordingly, normalization can be based, among others, on the surface of dissection area, on an averaged protein or peptide amount (measured using a colorimetric assay), or on the inclusion of housekeeping proteins in the quantification assay. However, there is at this time no consensus within the research community on normalization or an optimal method for expressing protein expression levels in tissue.

Another factor specific to shotgun and SRM proteomics is the use of peptide quantification as a surrogate for protein expression level, with the former not necessarily agreeing in absolute terms with the latter. This factor is true where bottom-up proteomics is performed, but of particular significance in the FFPE setting. Studies with tissue surrogates have shown that >90% of proteins are recovered when using the appropriate extraction protocol, with the extraction rate varying between proteins, probably due to different physicochemical properties [13]. Therefore, the exact proportion of proteins effectively extracted during the antigen retrieval process remains unknown and is probably different for individual proteins. In plasma (and other biofluids), the spiking of known amounts of stable isotope-labeled proteins represents an elegant strategy to account for losses during the digestion and extraction procedure. This approach is not suitable

for tissue as it would require a heavy isotope-labeled protein to be present in the sample in a physiological manner before the sample is fixed with formalin, which is not feasible. However, a relative quantification procedure using a stable isotope-labeled cell culture of the appropriate lineage for normalization might be applicable for tissue if the proteins of interest are physiologically present at appropriate amounts in the standard before formalin fixation [69]. The addition of heavy isotope-labeled peptides in the sample prior to MS analysis therefore remains the best option, although a compromised solution, as the peptides may decay at variable rates depending on the time point of their introduction in the extraction procedure, which in turn may affect the accuracy of measurements [70]. Moreover, preanalytical factors including steps from the surgical removal of the tissue to the MS analysis need to be controlled for, since any preanalytical variation will be reflected in the final result.

Finally, the acceptance of protein quantification by SRM from FFPE tissue in the clinical community will depend on the availability of standardized procedures and quality controls that need to be defined and on the interlaboratory standardization, which must be established. The chemical modifications induced by formalin fixation [7,25,71] and the effects on protein retrieval of parameters such as fixation time and storage time have already been reviewed [8, 25–27] but must be well understood and better managed before quantification of proteins from FFPE tissues can be performed routinely. Correlations of MS with IHC will indicate whether this new technique is suitable for wider clinical use. In addition, mass spectrometers remain a large capital investment for an individual laboratory, although the reagents costs associated with IHC (antibodies) may ultimately compensate for this. While they are increasingly being used in a clinical setting, combining the expert knowledge of the pathologist to examine the tissue morphology and of the analytical chemist to set up analytical methods requires co-ordination and commitment.

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