FEBS Letters

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REVIEW

The journey towards clinical adoption of MALDI-MS-based imaging proteomics: from current challenges to future expectations

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(Received 3 November 2023, revised 6 December 2023, accepted 9 December 2023)

doi:10.1002/1873-3468.14795

Edited by Metello Enzo Innocenti

Among the spatial omics techniques available, mass spectrometry imaging (MSI) represents one of the most promising owing to its capability to map the distribution of hundreds of peptides and proteins, as well as other classes of biomolecules, within a complex sample background in a multiplexed and relatively high-throughput manner. In particular, matrix-assisted laser desorption/ionisation (MALDI-MSI) has come to the fore and established itself as the most widely used technique in clinical research. However, the march of this technique towards clinical utility has been hindered by issues related to method reproducibility, appropriate biocomputational tools, and data storage. Notwithstanding these challenges, significant progress has been achieved in recent years regarding multiple facets of the technology and has rendered it more suitable for a possible clinical role. As such, there is now more robust and extensive evidence to suggest that the technology has the potential to support clinical decision-making processes under appropriate circumstances. In this review, we will discuss some of the recent developments that have facilitated this progress and outline some of the more promising clinical proteomics applications which have been developed with a clear goal towards implementation in mind.

Keywords: clinical proteomics; machine learning; MALDI-MSI; mass spectrometry; molecular pathology; proteomics; spatial omics

The field of spatial omics, in particular spatial proteomics, is rapidly evolving given that the techniques it encompasses maintain the native spatial relationship between biomolecules and the cellular network in which they are found [1]. This is particularly relevant within a clinical context considering that in certain disease areas, such as oncology and nephrology [2], a high degree of cellular heterogeneity is present and performing bulk proteomics analysis of these tissue samples may lead to the dilution of key molecular information which may help to better characterise its disease status. Moreover, the art of establishing a pathological diagnosis based on the morphological assessment of clinical samples is challenging and may not always provide sufficient information regarding prognosis or predict response to certain therapeutic

Abbreviations

CNN, convolutional neural networks; CSC, cloud storage computing; DESI, desorption electrospray ionisation; dPCR, digital polymerase chain reaction; FCNN, fully connected neural networks; FFPE, formalin-fixed paraffin-embedded; FNA, fine needle aspiration; HSNE, hierarchical stochastic neighbour embedding; IH, immunohistochemical; LDA, linear discriminant analysis; MALDI, matrix-assisted laser desorption/ionisation; MALDI-HiPLEX-IHC, MALDI mass spectrometry-based high-plex immunohistochemistry; MSI, mass spectrometry imaging; NMF, non-negative matrix factorisation; PCA, principal component analysis; PI, post-ionisation; ROI, regions of interest; SOMs, self-organising maps; t-SNE, t-distributed stochastic neighbour embedding; WSI, whole-slide imaging.

treatments, which are crucial for accurately guiding patient management, and for this reason, molecular-based techniques are being more readily turned to as a result. As such, the implementation of spatial proteomics techniques within a clinical setting may serve to support certain diagnostic workflows and pave the way for more personalised pathology.

Among the spatial omics techniques available, mass spectrometry imaging (MSI) represents one of the most promising avenues owing to its capability to map the distribution of hundreds of biomolecules within a complex tissue background in a multiplexed and relatively high-throughput manner. This is in stark contrast to standard histological approaches, and it can thus help to generate a more distinct and complete molecular snapshot of the disease. Moreover, being generally a minimally destructive technique, the tissue specimens may then also be subjected to routine histological staining and evaluation [3] in order to be integrated within the diagnostic workflow.

Desorption electrospray ionisation (DESI) is one such MSI technique that has been regularly employed in clinical research and has heralded a certain degree of promise, without fully taking the leap towards clinical utility. In particular, DESI-MSI has been employed in several oncological contexts, being highlighted to be a feasible approach for the differentiation of prostate cancer, metastatic breast cancers, and thyroid malignancies based upon their metabolomic profiles [4]. Moreover, one of the most promising studies has underlined its potential as a molecular adjunct for performing surgical margin assessment and to determine the completeness of the procedure in pancreatic and breast cancer, where the intraoperative delineation of resection margin is challenging [5,6]. Their findings highlighted that this approach, combined with statistical Lasso model, has the potential to detect tumour involvement at the margin in patients who developed early recurrence. Arguably, this represents one of the most promising avenues for its implementation within a clinical setting and is aided by the more minimal sample preparation, which is required for this MSI technology, being crucial in a context where time is of the essence. However, notwithstanding this particular facet of the technology, its intrinsic ionisation properties render it particularly restricted in the field of clinical proteomics. In fact, only in recent work has DESI-MSI been utilised for the detection of peptides and proteins directly from a tissue section [7] and represents an aspect of the technique which is still in its infancy and requires further development.

Considering this, matrix-assisted laser desorption/ionisation (MALDI)-MSI has thus established itself as the most widely used technique in pathology MSI [8] due to its capability to map the distribution of a wider range of biomolecules, including peptides as well as proteins. These possibilities have been reflected by a clear boom in the number of studies employing the technique in clinical research, with MALDI-MSI being applied to both tissue and cytological-based samples in a variety of disease contexts, highlighting its potentiality as a tool which can assist in molecular diagnoses. However, along with this hype has come significant expectations for it to be implemented in clinical routine and, unfortunately, the technique struggled to make significant inroads thus far. Important challenges related to method reproducibility have been faced, especially for the management of formalin-fixed paraffin-embedded (FFPE) clinical samples, and have hindered progress somewhat with regard to clinical implementation. Moreover, an increasing demand for single-cell spatial resolutions with sufficient sensitivity and reasonable analysis times has pushed instrumental boundaries and, as a consequence of this, an ever-increasing data load and the need for more sophisticated biostatistical tools to delve through this multi-dimensional data have also driven significant developments.

Despite these challenges, the MSI community has responded in a hugely positive manner and efforts have been made in all of the aforementioned areas that represent barriers to its establishment, rendering the technology more suitable for clinical application. In this review, we will discuss some of the recent developments that have facilitated this progress and outline some of the more promising clinical proteomics applications which have been developed with a clear goal towards implementation in mind.

Recent advancements in spatial proteomics with MALDI-MSI

Methodological advancements

One of the most important challenges overcome in the field of pathology MALDI-MSI has regarded the development of *in situ* trypsin digestion protocols to perform proteomic imaging on FFPE tissue sections [9–11], which represent the gold standard for specimen preservation in pathology units, enabling the long-term storage of samples and the generation of large tissue banks [12]. As a result, FFPE tissue specimens are employed for routine diagnostic assessment and also represent a valuable source of molecular information that can be exploited for tissue typing. Despite the high potential for application in clinical

research and histopathological tissue classification being highlighted in multiple clinical studies [13–15], it is also imperative to demonstrate its robustness in large patient cohorts, across different centres, and throughout a large timescale, especially if the technique is to be implemented within a centralised unit [16]. Accordingly, Buck *et al.* [17] performed a round-robin MSI study on FFPE tissues to investigate the consequences of inter- and intra-centre technical variation on masking biological effects. In doing so, they highlighted that MALDI-MSI data from FFPE samples could be broadly reproduced across multiple centres, with multivariate classifiers reaching, on average, > 90% accuracy. Moreover, this site-to-site reproducibility can also be further improved with the aid of cross-normalisation methods that rely on the computation of spectral intensity profiles for each of the individual mass spectra, along with a reference profile from the individual intensity profiles, before being transformed so that its intensity profile becomes equal to the reference profile [18]. This non-linear and mass-dependent approach not only serves to reduce batch effects in inter-laboratory scenarios but also in cross-protocol scenarios. This desire for reproducible methods has also been supported by a trend towards more accessible and transparent data being shared among various centres, for example through the emergence of the Galaxy framework which has integrated 18 dedicated mass spectrometry imaging tools to facilitate this process [19] and has been shown to be a feasible platform that can be used to classify clinical specimens of FFPE tissue [20].

Whilst not strictly spatial proteomics, but is certainly worth mentioning considering recent trends in this area, the investigation of *N*-glycans linked to the proteins present in pathological tissue specimens has also emerged as a powerful tool in clinical research [21,22]. This is particularly relevant in oncological specimens where abhorrent *N*-glycosylation is commonly observed to be a hallmark of the disease and may also represent a solid indicator of malignant progression [23,24]. In terms of clinical translatability, there may also be similar scope to spatial proteomics considering that protocols were also developed for application on FFPE tissue specimens with similar considerations [23]. However, the relative lack of compatible glycan databases for the annotation of *N*-glycan MALDI-MSI datasets has restricted this aspect somewhat, although the recent addition of the NGlycDB in METASPACE [25], an open-source cloud engine for molecular annotation of MSI data, has aided this aspect and also echoes this trend towards more greater accessibility and transparency. Moreover, trends towards the integration of spatial proteomics data with those obtained from complementary molecular levels, such as the lipidome and *N*-glycome, have also been reported and serve to strengthen the discriminatory power obtained by spatial proteomics. In particular, Denti *et al.* [26] have recently reported the possibility to perform spatial multi-omics of these three molecular classes on a single FFPE tissue section, which can be particularly relevant in instances where clinical tissue may be scarce, such as for low incident diseases or those which require multiple histological and immunohistochemical assessments [27]. This approach represents a further promising avenue considering that it has already been highlighted that using classifiers which span multiple omics levels can improve the correlation of molecular features with clinical endpoints and improve patient stratification [28].

Instrumental advancements

The scale and scope of single-cell omics technologies has rapidly accelerated in the area of clinical pathology and are promising to provide important insights into human disease [29]. Regarding MALDI-MSI technology, not only recent advancements in tissue preparation protocols, in particular regarding enzyme and matrix deposition aspects, have rendered the technology ever more reproducible [30] but have also made the concept of resolving the spatial localisation of proteins in individual cells a distinct possibility [31]. These advancements represented a particular stumbling block for spatial proteomics considering they are two common sources of bioanalyte delocalisation and underline why MSI of proteins at the single-cell level has trailed behind spatial metabolomics and metabolomics in this aspect [32]. In both instances, however, this possibility has been aided by the routine introduction of MALDI-TOF-MSI instruments that simultaneously move a highly focused laser beam and sample carrier, maintaining a high degree of pixel fidelity [31,33]. In fact, Smith *et al.* [34] exploited these instrumental advancements to highlight that it was possible to generate more distinct protein profiles of single cell types, mesangial cells and podocytes, from within single glomeruli of FFPE renal biopsies. Moreover, increased laser repetition rates of up to 10 kHz also means that this high spatial resolution protein imaging may be performed all within a practical time frame and may be particularly relevant in instances where a timely response is imperative, such as in determining molecular tumour margins [32,35]. However, this technology is also being further advanced to improve the positional accuracy of pixel acquisition and account for topographical variability of the tissue, facilitating spatial proteomics at a 5 μm lateral resolution. For example, this may have significant potential in the typing of complex tumour-

immune environments, where cell localisation and interactions within the tumour microenvironment are increasingly associated with tumour progression and chemoresistance [36]. This may also offer further prospects in the area of cytopathology where it is not uncommon to find only sparse numbers of morphologically abnormal cells and can be challenging to detect using conventional approaches [29,37].

Linked to this desire to achieve a single-cell spatial resolution is also the requirement for increased sensitivity. Accordingly, MALDI-MSI coupled with laser-induced post-ionisation (PI) technology, also called MALDI-2, has been demonstrated to boost the ion yield generated by various biomolecules, especially at higher lateral resolutions [38,39]. This development from Dreisewerd and colleagues has pushed the boundaries for highly sensitive (up to a 100-fold increase with respect to MALDI-1), high lateral resolution, MALDI-MSI of small molecules in pathological tissue, especially when implemented in trapped ion mobility quadrupole time-of-flight mass spectrometers which are capable of resolving highly complex lipid profiles. However, McMillen *et al.* [40] have also demonstrated that MALDI MSI post-ionisation (MALDI-2) can also enhance the ionisation, and thus yield, of tryptic peptides. Employing MALDI-2 they observed significant improvements in terms of signal intensity and molecular coverage, overcoming some of the issues related to the molecular depth provided by MALDI-MSI. In particular, they also noted an approximate 30% increase in the number of proteins that could be identified with two or more tryptic peptides, highlighting its potential to significantly enhance the discovery of possible molecular markers that can then be validated using routine immunohistochemical methods, which should be considered when providing biological context to any proposed proteomic classifiers.

Biocomputational advancements

Naturally, MALDI-MSI acquisitions at higher lateral resolutions and increased speeds have resulted in much larger datasets and an inordinately increased data load. This is also further exacerbated in clinical spatial proteomics studies where large sample cohorts are required to reach sufficient statistical power [41]. This has consequently led to the application of more diligent data analysis techniques, in particular those which are able to reduce the dimensionality of the dataset, extracting only the most relevant features and reducing data load. In particular, linear decomposition techniques, such as principal component analysis (PCA) [41,42] and non-negative matrix factorisation (NMF) [43], have been frequently employed for this purpose. However, t-distributed stochastic neighbour embedding (t-SNE), a probabilistic, non-linear dimension reduction technique has also been applied more readily to spatial proteomics data, including that generated by MALDI-MSI as well as other spatial proteomics techniques such as multiplexed antibody staining [44]. In particular, Abdelmoula *et al.* [45,46] have demonstrated the power of t-SNE for the analyses of highly complex MALDI-MSI datasets, employing it for the detection of distinct tumour subpopulations. However, t-SNE faces limitations in terms of the computational power required to process such large datasets and may not be practical within a routine clinical context. Therefore, hierarchical stochastic neighbour embedding (HSNE) has also been investigated due to its ability to handle large volumes of high-dimensional data with acceptable computational and memory resources [47]. Unfortunately, SNE maps may be different across multiple runs and could limit its clinical applicability where robust biostatistical workflows are required [48].

Clustering algorithms are also able to provide a more simplified representation of the MALDI-MSI dataset, with spectra being clustered together on the basis of their proteomic similarity all whilst maintaining coordinates that are faithful to the original image. These approaches can be particularly advantageous considering that they can be combined with the histological and immunohistochemical images at the disposal of a pathologist in a streamlined manner. Traditionally, bisecting k-means [49] and hierarchical clustering [50] algorithms have been employed to define molecular patterns within MALDI-MSI datasets [51]; however, these approaches may also suffer from lengthy computational times and may be somewhat limited in terms of differentiating morphological structures with only subtle molecular differences given that the calculations can have the tendency to be skewed by outliers which therefore reduce the distance between two similar clusters.

As is the trend with computational imaging as a whole, deep learning methods have also come to the fore in the field of pathology MSI and can overcome some of the issues related to the aforementioned techniques in terms of scalability, nonlinearity, and efficiency and render them particularly adept to handling highly complex MALDI-MSI data. Convolutional neural networks (CNN) have been successfully applied to perform tumour classification [52,53], showing their promise even for the typing of challenging, real-world datasets. However, these approaches relied upon the use of processed and labelled data, which may not always be available or demand a significant degree of effort to be created. Accordingly, approaches based

upon fully connected neural networks (FCNN) which bypass the need for underlying spectral preprocessing and feature determination have been proposed [54], culminating in the development of *massNet*, a scalable deep learning architecture to perform probabilistic pixel-based classification directly from mass spectral data with massive dimensionality [55], which was shown to be capable of automatically learning predictive features from large-scale MSI data. Very recently, Self-Organising Maps (SOMs) have also been investigated for the classification of cytological samples. In the work by Nobile *et al.* [56], they were able to detect molecularly distinct cells using only raw mass spectral information and indicate pathological regions of interest (ROI), which represent one feasible means for clinical implementation given that it may also facilitate more in-depth morphological assessment of small cell clusters that may go undetected in case of whole slide assessment.

This relationship between molecular and morphological classification also represents a possible future vision of how MALDI-MSI technology could be implemented within a pathologist's workflow, especially considering that the application of computational analysis of histological images using machine learning applications has also emerged [56,57]. This concept was nicely highlighted by Ščupáková *et al.* [58] where the output of morphometric classification was combined with MALDI-MSI data obtained at a 10 μm lateral resolution. Whilst this work utilised spatial lipidomics data, considering the aforementioned works, it is not unforeseen that this approach could also be adapted to spatial proteomics data in due course. However, the work also underlined certain challenges related to the co-registration of the molecular and morphometric images, which will become an even greater bottleneck when spatial resolutions towards 5 μm are employed. Whilst in this study an approach exploiting laser ablation patterns was used to estimate that the alignment error between the MALDI-MSI dataset and H&E image was below 10 μm, it is evident that there remains the need for further improvements in this area, for example by employing transformation factors to best align the two images [59].

Another important bottleneck which may have a downstream impact on clinical implementation surrounds data storage and accessibility. As the field of pathology readily turns to digital whole slide images (WSI) [59,60], the amount of data generated continues to increase. However, to put this into context, digital histological images obtained with a 40× magnification may have a data size towards 4GB, depending on the compression type used [61]. However, an equivalent raw MALDI-MSI dataset obtained with a 10 μm lateral resolution would dwarf this (tens of GBs) and place further strain on the IT structure of a clinical centre. Whilst the implementation of Cloud Storage Computing (CSC) may alleviate some of this strain [62], it is apparent that the most appropriate means of data compression would need to be determined in order to integrate the most relevant output whilst minimising network load. Similar questions linked to data governance may also arise and whilst there are some possible strategies with regard to omics data generated as part of clinical research [62,63], these questions will also have to be addressed prior to clinical implementation.

Towards implementation: promising clinical applications

Mass spectrometry imaging analysis has the potential to contribute to digital pathology workflows, providing an adjunctive molecular overview of pathological tissue. In recent decades, the number of studies focusing on the discovery of novel diagnostic, prognostic, and predictive biomarkers using mass spectrometry imaging approaches has increased exponentially, but only few of them are wholly dedicated to the implementation of MSI as a novel tool in clinical pathology. Notwithstanding that mass spectrometry techniques commonly require specialised personnel, its role in the routine clinical workflows could still be realised. In fact, an easy, rapid, robust and cost-effective automated system based on MALDI analysis has already found a relevant role as a routine tool for microbial identification (MALDI Biotyper) in clinical microbiology laboratories [64]. In light of this, the MSI community in joint collaboration with pathologists is working hard to reach the ambitious, but also realistic, aim to bring the technology closer to implementation. In the following sections, we will further discuss the application of MALDI-MSI and its use as a potential diagnostic and/or follow-up decision-making tool in the clinical routine of both tissue pathology and cytopathology.

MALDI-MSI as a complementary molecular tool in tissue-based pathology

The disease contexts that could benefit most greatly from the implementation of MALDI-MSI in the clinic range all the way from the field of oncology to rare diseases (i.e. amyloidosis). Moreover, when tissue biopsy material is limited due to minimally invasive

procedures, as in the case of lung tissue biopsies for cancer diagnosis, the possibility to combine multiple omics information from low sample amounts is of paramount importance. For example, non-small cell lung cancer subtyping is crucial for selecting the most appropriate chemotherapy treatment, however, even if in most cases the diagnosis is based solely on tissue morphology, in some cases immunohistochemistry using multiple antibodies is also required and one single tissue section may not be sufficient for obtaining an accurate diagnosis. Kazdal et al. [65] have thus proposed a novel and easy workflow which combined MALDI-MSI proteomics and digital polymerase chain reaction (dPCR) genetic analysis for the analysis of limited tissue material. Moreover, Janßen et al. [66] developed a classification algorithm using neural network/linear discriminant analysis (LDA) that was trained on TMA lung tissue sections before being validated and tested on specific areas of whole tissue sections (areas with high tumour cell content, low amount of necrosis, and high scan quality), showing a 99.0% and 98.3% test accuracy on a single spectra level and a 100.0% test accuracy on whole section level for the neural network and LDA, respectively. Notwithstanding this promise, the implementation of such a classification algorithm in the clinic may still face some challenges given that the approach requires areas with high tumour cell content to be annotated by a pathologist. Manual annotation is a time-consuming process and, since the technology is rapidly moving towards higher spatial resolution analysis, reaching the single-cell level, such a manual approach may not be practically feasible in clinical routine. In light of this, artificial intelligence approaches which are able to determine spectral patterns within MALDI-MSI datasets and aid in the automatic selection of regions of interest are already under development and will certainly encourage the advancement of the technology into the pathological unit [56].

Establishing an accurate diagnosis, classification, and typing of amyloid deposits remains an open question and is essential in the decision-making process for prognosis evaluation and to select the most adequate treatment for patients. Up until now, Congo red staining in combination with immunohistochemistry, because of specificity and sensitivity issues, is not always able to unravel this clinical conundrum alone. As such, a combined approach using laser capture microdissection and mass spectrometry shotgun proteomics has already been established in the clinical practice for the subtyping of amyloid deposits from different organs [67]. However, given that MALDI-MSI is a minimally destructive technique, capable of resolving even the smallest of amyloid deposits from within a complex tissue background, it could support pathologists in the diagnosis of this challenging group of rare diseases. Despite the relatively low number of samples analysed, the potentiality of this approach has already been shown in cardiac (n = 7 training amyloid samples and n = 66 validation amyloid samples [68,69]), pulmonary (n = 46 amyloid samples [68,69]), and gastrointestinal amyloidosis (n = 65 amyloid samples [68,69]) where MALDI-MSI was able, independently from Congo red staining and immunohistochemistry, to detect and classify amyloidosis based on the presence of the amyloid precursor protein, highlighting an organ and amyloid type-specific signature. Even though large steps forward have been made in the last 10 years with regard to the development of MALDI-MSI-based tools in tissue pathology, considerations related to robust sample preparation, appropriate biocomputational tools with easy-to-interpret outputs, as well as cost-associated issues still need to be refined in order to render its implementation more practical.

MALDI-MSI as a complementary molecular tool in cytopathology

As has been reported in the area of tissue-based pathology, significant strides have also been made in recent years with regard to the use of MALDI-MSI in cytopathology, particularly within the diagnostic context of indeterminate thyroid nodules. As previously touched upon, for the implementation of such high-throughput technology in clinical routine, many aspects have to be evaluated, both in terms of robust, reliable, straightforward, and fast sample preparation workflow, as well as in terms of time and cost of the analysis and consumables. Naturally, this should also be concluded by an easy-to-use software tool with a clear and single read-out. Most of these aspects have already been evaluated in the application of MALDI-MSI to thyroid fine needle aspiration (FNA) biopsy samples. Piga *et al.* developed a robust sample preparation method for cytological thyroid samples using liquid-based cytological preparation with cytocentrifugation that (a) facilitated the removal of haemoglobin, a common ion suppressant encountered in MALDI-MS proteomics; (b) significantly reduced the sample size, from 2 to 4 cm as in standard smear deposition to a cytological spot of 6 mm; (c) enabled eight cytospin spots to be deposited on one single glass slide, instead of only one cytological smear per slide; (d) significantly reduced the time of MALDI-MSI analysis for one single sample from more than 15 h to only

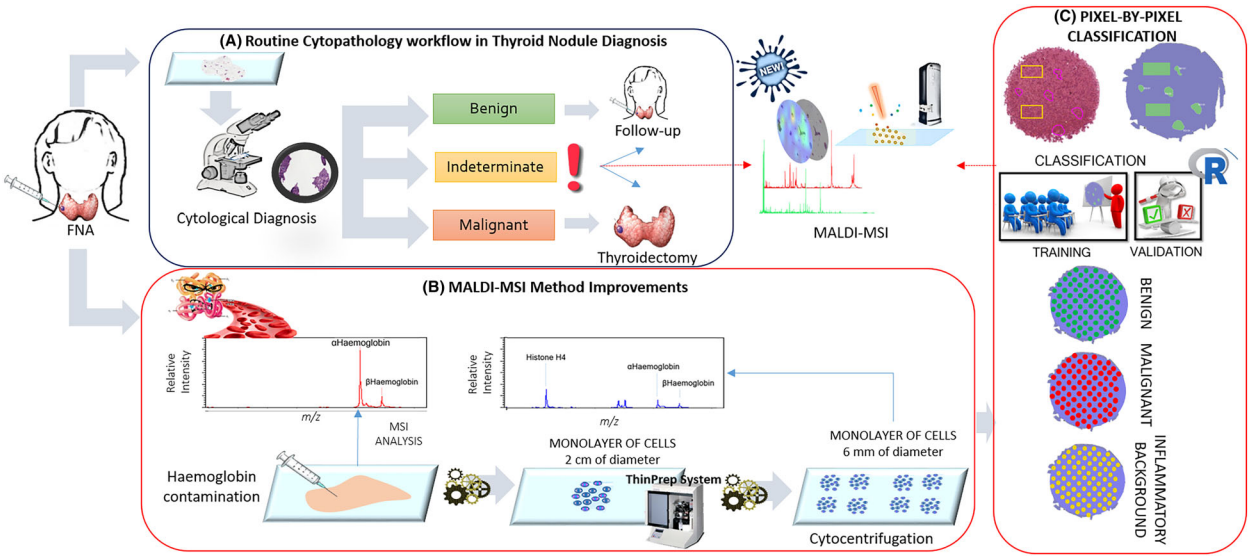

Fig. 1. A schematic overview of the recent methodological and biocomputational advancements made with regard to the utility of MALDI-MSI as a tool in thyroid cytopathology. Fine needle aspiration biopsy samples are collected with the aid of ultrasound guidance directly from the nodule of a patient. Panel (A) describes the routine workflow for the cytological diagnosis of thyroid nodules. Generally, after cytological diagnosis, benign nodules undergo follow-up with repeated FNA biopsy, malignant nodules require thyroidectomy, whilst nodules without a clear-cut benign or malignant diagnosis will be classified as indeterminate lesion and if considered by the pathologist as low-risk will undergo follow-up, if high-risk will undergo thyroidectomy. Recently, MALDI-MSI has been proposed as a novel tool in the diagnosis of these indeterminate thyroid lesions in order to avoid unnecessary thyroidectomy. Panel (B) briefly describes the MALDI-MSI sample preparation improvements made for the analysis of cytological thyroid specimens, in order to have a robust, sensitive, simple, reproducible and fast protocol for proteomics MALDI-MSI analysis. In order to bring MALDI-MSI into the clinical routine workflow there is the need to build a classification model able to correctly classify indeterminate cytological samples. Panel (C) describes a pixel-by-pixel classification approach able to correctly classify single pixel in cytological samples, as benign (green coloured), malignant (red coloured) or inflammatory background (yellow coloured).

approximately 4 h per sample [70,71]. Moreover, with the main scope of bringing the technology to the clinic, since thyroid FNA samples could not always be processed immediately after collection, the authors have also evaluated both morphological and proteomic FNA stability in cytological preservative solution as well as intra-day and inter-day reproducibility, highlighting that samples can be stored at 4 °C for up to 2 weeks and then transferred into glass slide for MALDI-MSI proteomic analysis [71]. This 14-day timeframe could facilitate easy shipment from the collection clinical site to the MALDI-MSI unit, enabling multicentric sample collection. As a whole, the developed method can meet the required analytical criteria but can also be easily collected and shipped by non-specialised personnel. An overview of this progress is presented in Fig. 1.

The authors have then supported these analytical aspects by constructing a MALDI-MSI classifier using a Lasso statistical method previously trained on annotated regions of interest of benign (hyperplastic) and malignant thyrocytes (papillary thyroid carcinoma), as well as regions with an inflammatory background (Hashimoto's Thyroiditis) to classify the whole cytological sample using a pixel-by-pixel classification approach which will allow the direct analysis of MALDI-MSI generated data without requiring the pathologist to manually annotate regions of interest [72–74]. Despite the FNA thyroid samples used in the validation set (n = 170) included samples with inadequate cellularity, the approach showed a specificity of 82.9% and a sensitivity of 43.1%, whilst when the analysis was focused only on a subset of FNAs with adequate cellularity, sensitivity increased to 76.5%. More recently, Capitoli *et al.* [75] suggested a novel workflow, which included MALDI-MSI in the clinical routine, to be used in cases of indeterminate diagnosis. In particular, the authors suggested a three-level diagnostic classification for indeterminate nodules in the pixel-by-pixel approach based on the percentage of malignant and benign pixels present in the whole sample: a number of malignant pixels lower than 7 suggest a benign sample and a ultrasound follow-up at 12 months; a number of malignant pixels higher than

16.7% suggest a malignant sample, and thus a thyroidectomy, whilst in instances where the number of malignant pixels was between 7.0% and 16.7% (defined as a grey zone), this identified nodules which will require a strict ultrasound follow-up, and, eventually associated with a repeat biopsy [75]. Up to now, the classification model is built considering a small class of thyroid nodule lesions such as hyperplastic, papillary thyroid cancer, and Hashimoto's thyroiditis lesions, but the next step will be to enlarge the training cohort to other benign and malignant thyroid lesions as well as to test the model using samples collected in different pathological units. It is also important to stress that whilst this work has focused on thyroid cytopathology, it also has the potential to be utilised in other cytopathological contexts such as cervical squamous cell carcinoma [76] and lung cancer [77].

MALDI mass spectrometry-based high-plex immunohistochemistry (MALDI-HiPLEX-IHC)

Conventional immunohistochemical (IHC) staining of tissue specimens (fresh frozen or FFPE) is a simple and robust technique that is employed both in research as well as in routine clinical diagnostic laboratories, enabling tissue sections to be stained with individual protein expression markers and provide additional, objective, molecular information to support routine morphologic examination [78]. However, in recent years, the advent of personalised medicine has underlined the necessity to obtain more extensive molecular information in order to achieve a more accurate, personalised, diagnosis. In light of this, conventional IHC has become largely insufficient given that the analysis of multiple biomarkers using conventional IHC assays is not only time-consuming but also requires a larger number of serial tissue sections, which in some cases may not be available due to scarce tissue biopsy material and, when available, the cellular composition of the section may vary throughout the tissue block. Therefore, multiplexed immunohistochemistry combined with fluorescence microscopy could overcome some of the problems associated with conventional IHC, enabling the evaluation of multiple protein biomarkers (more than 30 different antibody stains) from one single tissue section [79–81]. Despite the progress made with multiplexed IHC approaches, the protocol still involves multiple step cycles of antibody staining and stripping, rendering it time-consuming both analytically as well as from a biocomputational standpoint. Given the frequency in which IHC is required in routine pathological workflows, the scope for possible implementation of techniques able to achieve multiplex IHC in a high-throughput manner is quite evident and this field has progressed rapidly in recent years [82,83]. In this multiplex framework, however, MALDI mass spectrometry-based high-plex immunohistochemistry (MALDI-HiPLEX-IHC) represents a particular novelty with promising potential to take large progress in the precision medicine and digital pathology era [84–86]. This new approach, utilising MALDI-MSI technology, is capable of simultaneously mapping the tissue distribution, in one single tissue slide, of up to 12 known protein expression markers using photocleavable mass-tag probes [84]. A significant advantage of this MALDI-HiPLEX-IHC approach with respect to multiplex IHC is that these antibodies can be mapped in one single, relatively high-throughput, analysis (Fig. 2). Moreover, data from these protein expression markers can also be combined with untargeted spatial multi-omics data obtained from the same tissue section, which may help to reveal the complex biological jigsaw behind pathological states [12,26]. In challenging scenarios, such as in the diagnosis of breast cancer, the use of this multiomic MALDI-HiPLEX-IHC approach could offer crucial molecular information that could complement the digital pathology workflow, especially considering that these tumours still present open challenges in terms of pathological diagnosis, prognostic classification, and selection of appropriate therapeutic treatments [87]. Whilst much progress has been made regarding the molecular subtyping of breast cancer by mass spectrometry imaging approaches [88–90], at multiple omics levels, the most significant step forward in a clinical context could regard the integration of multi-level molecular information in one single workflow, such as the one required for MALDI-HiPLEX-IHC.

Concluding remarks

As we move towards an era of personalised medicine, there is a growing need for molecular tools to provide support for pathologists and aid in disease characterisation. In the context of tissue and cytological samples, MSI represents an ideal tool to achieve this goal considering its spatially resolved nature along with relatively high throughput. Among these MSI techniques, MALDI-MSI has been associated with the greatest promise in terms of MSI-based spatial proteomics and it was hoped that this would result in rapid advancements towards clinical implementation. However, despite this promise, obstacles related to data reproducibility, large-scale validation, and the utility of practical biocomputational tools have represented

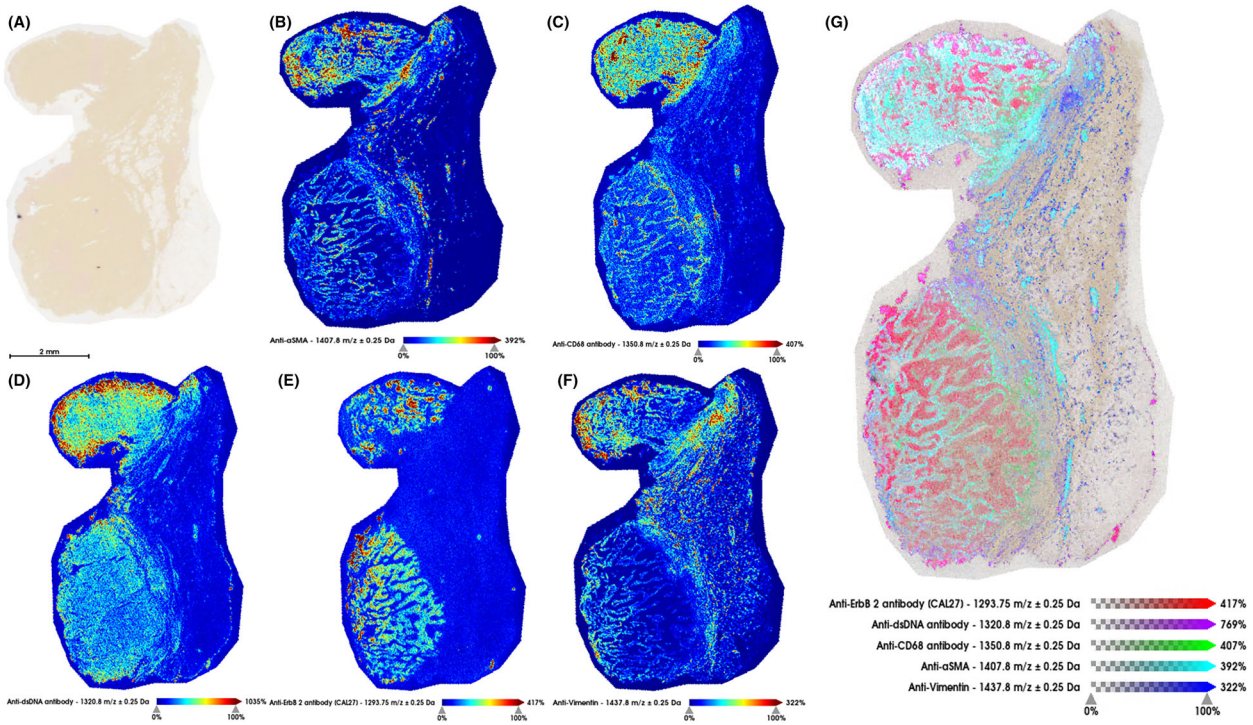

Fig. 2. MALDI-HiPLEX-IHC of human breast cancer tissue showing (A) optical image of the breast cancer tissue, antibody MS-images from (B) Anti-αSMA, (C) Anti-CD68, (D) Anti-dsDNA, (E) Anti-ErbB 2 (HER2), (F) Anti-Vimentin, and (G) composite image of the five antibodies overlaid with the optical image of the human breast cancer tissue. Dimension and intensity scale bars are provided. The dataset used to generate this image was kindly provided by Ambergen and Bruker Daltonics and was produced with their permission.

significant barriers in this regard. However, to tackle these barriers, significant steps have been made in the last few years and we are now in a position where clinical studies employing MALDI-MSI are much better equipped and orientated to achieve this goal of implementation, as highlighted by several promising applications, especially in the area of cytopathology.

Notwithstanding this progress, challenges still remain with regard to the most appropriate biocomputational tools and data storage platforms. Whilst these issues remain without a definitive response, much can be achieved with clear dialogue and interaction between the fields of clinical pathology and MSI. The landscape of pathology is continually adapting to the influx of molecular-based techniques, in combination with machine learning algorithms, and bridging this gap between the two expertise can also help bring MALDI-MSI closer towards clinical utility [91], with the hope that the technology can be combined with histological and clinical data in order to contribute to clinical decision making [91,92]. Naturally, this may not necessarily lead to swift clinical implementation but there is now, more than ever, solid evidence to suggest that the technological foundations have been laid in order to gradually facilitate this transition.

Acknowledgements

The authors would like to thank Ambergen and Bruker Daltonics for their permission to use one of their datasets to generate Fig. 2 presented in this manuscript. The work presented in this manuscript was facilitated by Regione Lombardia: programma degli interventi per la ripresa economica: sviluppo di nuovi accordi di collaborazione con le università per la ricerca, l'innovazione e il trasferimento tecnologico: Regional law n °9/2020, resolution n° 3776/2020, Italian Ministry of Health under the grant 'Ricerca Finalizzata' GR-2021-12374235: Regional law n° 9/2020, resolution n° 3776/2020 under the grant 'Ricerca Finalizzata GR-2019-12368592'. Moreover, this work was funded by the National Plan for NRRP Complementary Investments (PNC, established with the decree-law 6 May 2021, n. 59, converted by law n. 101 of 2021) in the call for the funding of research initiatives for technologies and innovative trajectories in the

health and care sectors (Directorial Decree n. 931 of 06-06-2022) – project n. PNC0000003 – AdvaNced Technologies for Human-centrEd Medicine (project acronym: ANTHEM). This work reflects only the authors' views and opinions, neither the Ministry for University and Research nor the European Commission can be considered responsible for them.

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