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Proteomics for the diagnosis of thyroid lesions: preliminary report

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Objective: Matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry (IMS) is a unique proteomic technology that explores the spatial distribution of biomolecules directly *in situ*, thus integrating molecular and morphological information. The possibility of correlating distribution maps of multiple analyses with cytological features makes it an ideal research tool for discovering new diagnostic markers. A previous study showed that MALDI-IMS could help discrimination between different types of thyroid lesions, especially papillary thyroid carcinoma (PTC); the present feasibility study on *ex vivo* fine needle aspiration (FNA) smears describes its potential in detecting new proteomic targets of other thyroid lesions (follicular lesions, medullary carcinoma).

Methods: MALDI-IMS was conducted on *ex vivo* FNAs obtained from surgical specimens and corresponding *in vivo* samples. Differences between proteomic profiles of different thyroid lesions were compared.

Results: Comparing the protein profiles of hyperplastic nodules obtained from three different patients with each other, and with a new PTC, showed a high degree of concordance, indicating good reproducibility of the IMS technology on cytological samples, suggesting its potential as a tool for biomarker discovery. Furthermore, comparison of the average proteomic profiles of hyperplastic nodules with a Hürthle cell adenoma revealed significant differences, underlying the capability of MALDI-IMS to distinguish between different thyroid lesions. Finally, the proteomic profile of medullary thyroid carcinoma was also characterized.

Conclusions: Our results confirmed the possible role of MALDI-IMS in the search for diagnostic targets of PTC and follicular lesions, which could be applied in larger trials aimed at the identification of proteins, convertible to cost-effective diagnostic tools such as immunohistochemistry. These tests could be used to analyse *in vivo* cytological smears, improving the preoperative diagnosis of indeterminate thyroid nodules.

Keywords: Matrix-assisted laser desorption/ionization, imaging mass spectrometry, MALDI-IMS; thyroid follicular lesions, thyroid carcinoma; proteomics, fine needle aspiration cytology

Introduction

Unfortunately, 15–20% of thyroid fine needle aspiration (FNA) biopsies cannot be diagnosed conclusively using this technique alone and are considered to be 'indeterminate for malignancy', representing a problem in terms of both the pursuance of standardized treatments and costs.[1–3] Recently, different molecular tests pointing to abnormal molecular mechanisms of thyroid cancer, such as genetic testing (BRAF V600E, NRAS codon 61, HRAS codon 61, KRAS codon 12/13 point mutations and RET/PTC1, RET/PTC3, PAX8/PPAR rearrangements)[4] and gene expression classifiers[5] on FNA specimens, have been proposed in order to improve the pre-operative risk assessment of malignancy in indeterminate nodules. Nevertheless, the best techniques are still under

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debate for both economical and practical reasons.[6] Matrix-assisted laser desorption ionization/imaging mass spectrometry (MALDI-IMS) is a unique technology, which explores the spatial distribution of biomolecules directly *in situ*, far exceeding the capabilities of microscopy by providing hundreds of different molecular images from a single scan without the need for target-specific reagents.[7] This technology provides simultaneous correlation between the spatial distribution of proteins and the morphological features of pathological specimens. The first pilot study performed by our group focused on the most frequent and problematic lesion in thyroid cytopathology: papillary thyroid carcinoma (PTC).[8] Preliminary data on PTC underlined the potential of MALDI-IMS as a strategic tool for biomarker discovery in cytological specimens. In that feasibility study, we demonstrated that MALDI-IMS profiles can be potentially integrated with genetic information (all frequent genetic alterations in the mitogen-activated protein kinase pathway or PI3/AKT pathway).

In the present study, we investigate the potential application of MALDI-IMS in thyroid cytopathology for other lesions that are difficult to diagnose. Moreover, we suggest, based on these results, that larger trials should be included in proteomic thyroid pathology research in order to promote investigations that could potentially enable the discovery of more feasible and cost-effective diagnostic tools (immunohistochemistry).[9–11]

We collected *ex vivo* FNAs using a 22-gauge needle from thyroidectomy specimens sent for routine histological examination at the Department of Pathology, San Gerardo Hospital, Monza, Italy. For each nodule of this feasibility study (n = 11), a final histopathological report was also available. Tables 1 and 2 report the clinical and pathological features of the patients. We attained a cytological sample from targeted nodules of thyroidectomy specimens, using fine needles (25G), within 15 minutes after surgery. This procedure allowed us to obtain fresh cytological samples from perfectly corresponding *in vivo* FNA biopsy samples. In addition, this method allowed us to gain a large number of thyroid cells without artefacts and with minimal thyroid tissue waste. Finally, the method gained ethical approval, as we are currently unable to perform the study on *in vivo* FNAs. 4
Briefly, for each target nodule, different passes were performed. A Diff-Quik®-stained slide was prepared 5
for adequacy evaluation; other passes were smeared onto conductive indium tin oxide (ITO) glass slides as targets for mass spectrometric analysis. Each slide was thawed under vacuum for 30 minutes and then washed sequentially in ethanol, as described previously.[8] Matrix deposition for MALDI analysis was performed by spraying sinapinic acid using the ImagePrep automated spraying system (Bruker Daltonics, Bremen, Germany). All the mass spectra were acquired in linear positive mode in the mass range 4000–25 000 Th, with an UltrafleXtreme mass 6

Table 2. Clinico-pathological features of a previously published group of patients with papillary thyroid carcinoma (adapted from reference 8)

	Clinical data			
Patients	Histology	Age (years)	Sex	Size (cm)
7	cvPTC	49	Female	15
8	cvPTC	49	Female	9
9	cvPTC	42	Female	9
10	fvPTC	44	Male	7

cv, conventional variant; fv, follicular variant; PTC, papillary thyroid carcinoma.

Table 1. Clinical and pathological characteristics of the patients enrolled in this study

	Clinical data				
Patients	Histology	Surgical indication	Age (years)	Sex	Size (cm)
1	Hp	MG	70	Female	2
2	Hp	MG; incidental contralateral PTC	52	Female	2
3	Hp	MG	58	Female	2
4	MTC	MG	81	Female	1
5	HCA and Hp	MG	72	Female	2.5 and 2
6	cvPTC	PTC	44	Female	2.1

cvPTC, conventional variant of papillary thyroid carcinoma; HCA, Hürthle cell follicular adenoma; Hp, hyperplastic nodules; MG, multinodular goitre; MTC, medullary thyroid carcinoma.

spectrometer (Bruker Daltonics), equipped with a Smartbeam laser (Nd:YAG/355 nm) operating at a frequency of 2 kHz. Spectra were collected with a laser diameter of approximately 50 μm and at a spatial resolution of 80 μm. After MALDI analysis, the matrix was removed and the slide was stained with Giemsa or haematoxylin and eosin. Finally, the cytological specimens were converted to digital format by scanning the slide through a ScanScope CS digital scanner (Aperio, Park Center Dr., Vista, CA, USA), thus allowing the direct overlap of images and enabling the integration of morphological features with the molecular information obtained through MALDI-IMS.

The pathologist selected the areas of interest, favouring areas with a high density of homogeneous and well-preserved cells and excluding areas with colloid, macrophages, lymphocytes or artefacts. The protein profiles obtained from these areas were subject to statistical analysis in order to define proteomic patterns and to characterize specific thyroid lesions. Spectra were normalized, aligned and denoised before statistical analysis. Statistical analysis was performed using ClinProTools, as described previously.[8] Signals were considered to be statistically different between the groups when the receiver operating characteristic (ROC) analysis showed an area under the curve (AUC) of greater than 0.80 and the Student's *t*-test a *P* value of less than 0.05. mMass (freely available at www.mmass.org) was used to generate average protein profiles from the different types of lesions. In addition, similarity between the previously published PTC series[8] and the new PTC case was evaluated with Radviz using the open source Orange 2.7 software (http://orange.biolab.si/). The Radviz plot shows the distribution of every single spectrum (about 150 spectra/sample) in an area limited by a circumference on which the masses (*m/z*) present in the spectra are displayed in such a way that the distances between the single spectrum and the masses are inversely proportional to the intensity of the masses themselves.

Evaluation of the analytical variability of the method was performed by analysing three smears extracted from hyperplastic nodules (Hp) from control patients (Figure 1). The average protein profiles obtained from the three patients were comparable, showing a mean number of 135 ± 4 proteins (signal-to-noise ratio = 3) with a standard deviation below 20% of their intensities, showing the reproducibility of the technology and a low biological variability.

Aiming to evaluate the possible role of MALDI-IMS in the controversial characterization of follicular neoplasms, we compared the average protein profile from the three Hps with a Hürthle cell follicular adenoma (HCA, Figure 2). We detected a panel of six differentially expressed proteins (Figure 3). In detail, proteins detected at 4965, 6278 and 6651 Th were up-regulated in hyperplastic lesions, whereas proteins at 7263, 8294 and 8310 Th were up-regulated in HCA (Figures 2 and 3). Moreover, the proteomic profile obtained from an Hp present in the thyroid of the same HCA-affected patient (Hp2) was compared with that of a control patient (Hp1) and no significant differences were observed (Figure 3).

We also compared the proteomic profile of the HCA with that of a medullary thyroid carcinoma (MTC, Figure 2). The results showed that the proteins down-regulated in the HCA were significantly down-regulated even in the MTC when compared with hyperplastic lesions. Nevertheless, the proteins up-regulated in the HCA were not up-regulated in the MTC (Figure 3). Furthermore, by comparing the proteomic profile obtained from the MTC with the profile of Hps, we detected several differentially expressed proteins.

MALDI-IMS results from patients with cvPTC (cv, conventional variant) were also compared with those obtained from the different types of thyroid lesions described above. A new patient with cvPTC was enrolled and the results were compared with those described previously.[8] We evaluated the six differentially expressed proteins (Figure 3) in this new patient with cvPTC (Table 1: patient 6). The results showed that the expression of these six proteins was different from that of the benign cases (Figure 3), but similar to that of MTC. Then, the comparison of PTC with MTC peaks (Figures 2 and 4a) indicated other differentially expressed proteins that could be used to distinguish between the two malignant entities. To evaluate the reproducibility of MALDI-IMS technology also in malignant lesions, we compared our previously published PTC series[8] with the new one. The results showed that the proteomic profile of the new case (patient 6) was comparable with the average proteomic profile of patients 7, 8 and 9 (Figure 4a). Moreover, the Radviz plot showed that the distributions of the two different series overlapped, once again indicating a good reproducibility in terms of biological variability (Figure 4b). Despite the fact that the proteomic profiles of fvPTC (fv, follicular variant) and cvPTC were

LOW RESOLUTION

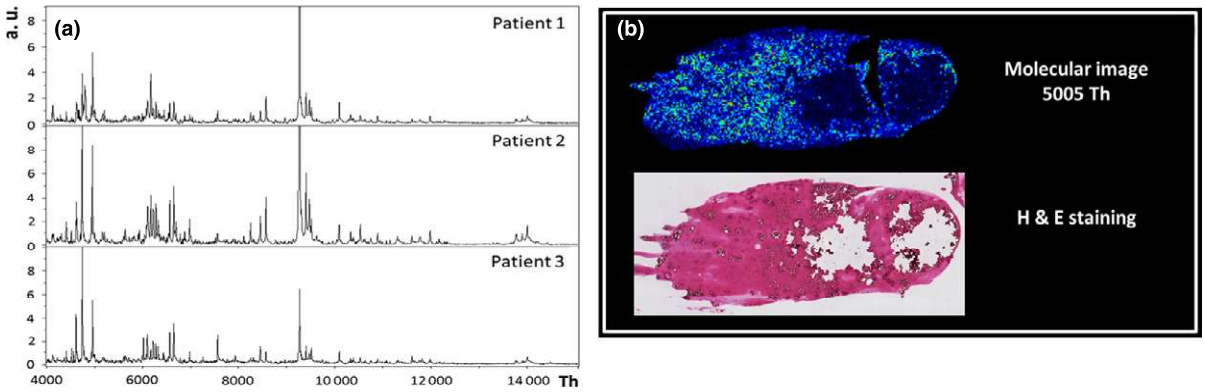

Figure 1. Protein profiles of hyperplastic nodules (n = 3). (a) Zoomed region of the spectra in the mass range 4000–15 000 Th of cytological samples obtained from the three patients. The intensity of the protein signal is expressed as arbitrary units (a.u.). (b) Example of morphological and matrix-assisted laser desorption ionization/imaging mass spectrometry (MALDI-IMS) molecular results obtained on the cytological sample of patient 2 (hyperplastic nodule, ×1 magnification). H & E, haematoxylin and eosin. 10

COLOR

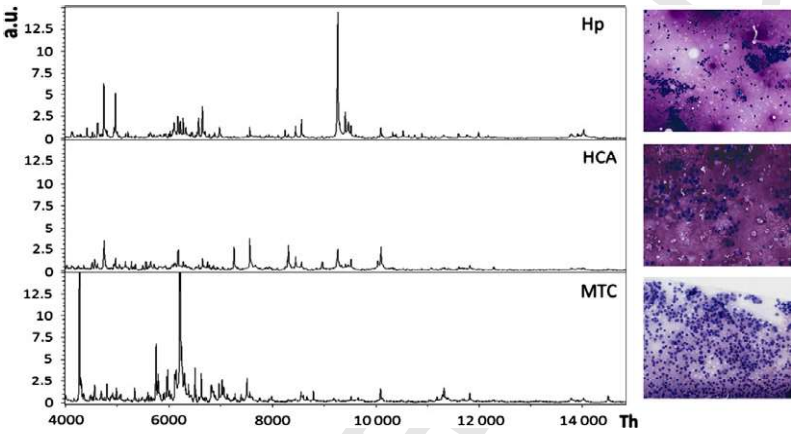

Figure 2. Comparison of the average proteomic profiles of hyperplastic nodules (Hp, n = 3), Hürthle cell follicular adenoma (HCA) and medullary thyroid carcinoma (MTC). Left: zoomed region of the spectra in the mass range 4000–15 000 Th. The intensity of the protein signal is expressed as arbitrary units (a.u.). Right: Giemsa staining underlines the difficulties in differential diagnosis based on morphological features only (×20 magnification).

rather similar, MALDI-IMS still highlighted proteins with altered expression, discriminating between the two histological subtypes. In particular, we detected several potential targets useful in the differential diagnosis between fvPTC and cvPTC (Figure 4a, arrows).

This pilot study has investigated the possible application of the proteomic technique known as MALDI-IMS in different types of thyroid lesion. Considering that the spectra are collected by MALDI-IMS, with a laser diameter of approximately 50 μm and at a spatial resolution of 80 μm, the application of this technique in cytological smears inevitably examines the comparison of clusters of cells (and not an individual cell). However, it allows the acquisition of a large number of spectra/samples, more than 10 000 per sample, thus improving the analytical robustness of the results. Moreover, the IMS approach allows an automated deposition of the matrix, thus providing higher analytical reproducibility. One of the most significant benefits of MALDI-IMS technology is the possibility to integrate proteomic results with classic morphological data, exploring the whole cytological specimen. As a result, the pathologist can direct the evaluation of the proteomic results, focusing the data elaboration solely on the selected areas of interest (e.g. areas with diffuse microfollicular architecture or with monomorphic features in follicular lesions, areas with atypia or non-conventional features in other contexts) and excluding morphologically different areas (e.g. poor cellular zones, clearly hyperplastic or

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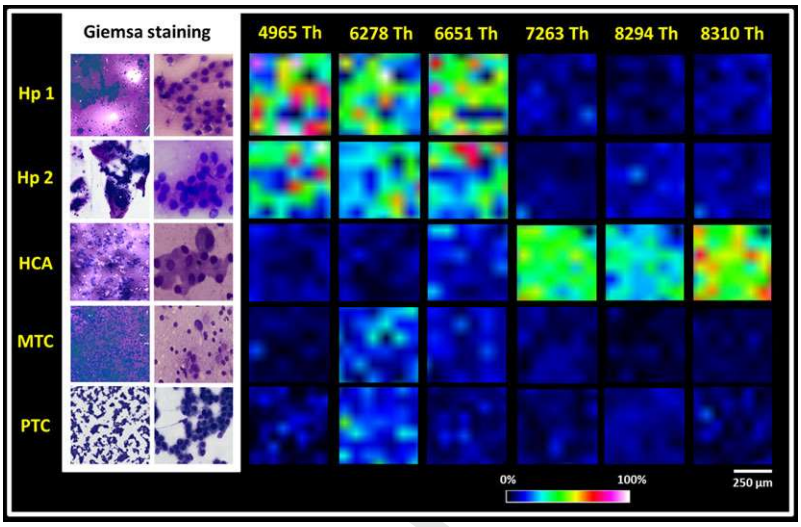
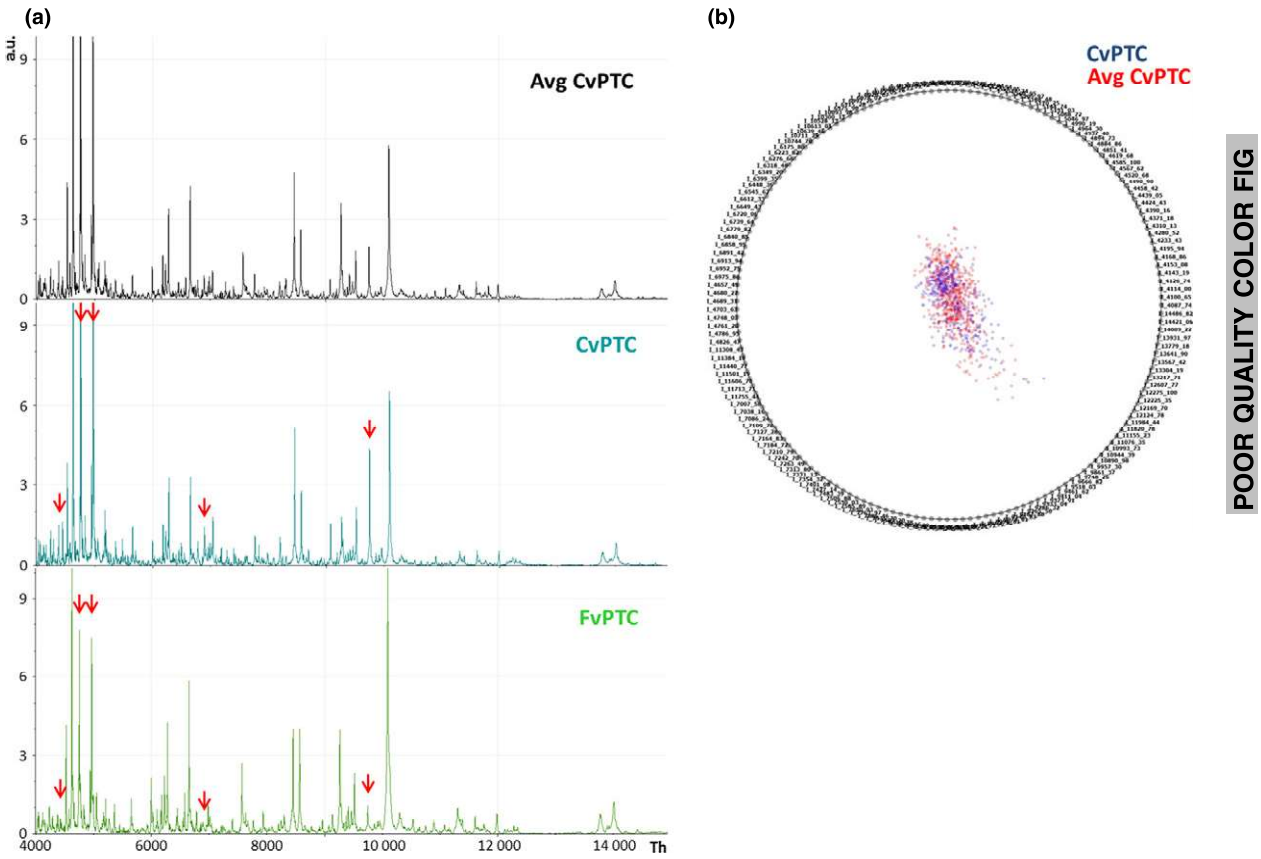


Figure 3. Comparison of matrix-assisted laser desorption ionization/imaging mass spectrometry (MALDI-IMS) results between a hyperplastic lesion of patient 1 (Hp1), a hyperplastic lesion sampled from the patient affected by Hürthle cell follicular adenoma (HCA) (Hp2), HCA, medullary thyroid carcinoma (MTC) and papillary thyroid carcinoma (PTC). The figure shows a panel of six proteins in Hp, HCA, MTC and PTC samples. Proteins at 4965, 6278 and 6651 Th are up-regulated in Hp, whereas proteins at 7263, 8294 and 8310 Th are up-regulated in HCA. MTC and PTC show a similar 'signature of malignancy'. The intensity of the protein signal is expressed as arbitrary units (a.u.) from 0% to 100%.

normal thyroid cells, inflammatory elements, macrophages, foci of ischaemic necrosis in Hürthle cell-type lesions), which may confound the conclusions. The average protein profiles obtained from the three hyperplastic patients were highly comparable and supported the technical reproducibility of the technology, as well as being suggestive of low biological variability.

Our preliminary report demonstrating the application of MALDI-IMS in PTC showed interesting results;[8] therefore, the second stage of this feasibility test examined the possible role of MALDI-IMS in the controversial characterization of non-papillary neoplasms.[4] None of the genetic-based diagnostic novelties appears to solve the issue definitively.[6] Proteomics, as a new tool of research, could approach the problem from a different point of view and could lead to significant discoveries. In this sense, the potential of MALDI-IMS was evaluated on the basis of its ability to differentiate between hyperplastic proliferations and truly clonal follicular lesions with clinical and biological malignant progression. For this purpose, the average protein profile of Hps from three different patients was used as a reference spectrum for the comparative analysis with an HCA, detecting a panel of six differentially expressed proteins. We confirmed that these differences were a result of intrinsic proteomic characteristics of the lesion and not caused by biological variability of the patient. Indeed, the expression of these proteins was compared with the profile of an Hp present in the thyroid of the same patient with HCA. The proteomic profile obtained from this Hp was statistically comparable with the results of the three control patients, but significantly different from the HCA. These data only provide a preliminary insight, but clearly reveal the potential role of MALDI-IMS as a supporting tool for the development of a panel of proteins useful for the prediction of the malignant risk of follicular lesions. These results open up future perspectives for the investigation of the ability of MALDI-IMS to characterize follicular adenoma and follicular carcinoma in larger series. The possibility of distinguishing malignant and benign thyroid nodules by the identification of specific protein patterns for different thyroid tumours and on different types of thyroid specimens, both cytological and histological, by MALDI-IMS proteomic analysis should be confirmed with a wider cohort of patients. As a result, this would ensure a high statistical power for the discovery of promising markers based on proteomic profiling experiments.

In this pilot study, we also compared the proteomic profile of MTC with HCA. This investigation tackled a classic cytological diagnostic challenge and further highlighted the methodological innovation of MALDI-IMS. The results showed that the three proteins down-regulated in the HCA were significantly down-regulated even in the MTC when compared with hyperplastic lesions. Nevertheless, the proteins up-regulated in HCA were not up-regulated in the MTC. Furthermore, by comparing the proteomic profile obtained from MTC with that from hyperplastic lesions, we detected several differentially expressed proteins.



POOR QUALITY COLOR FIG

Figure 4. (a) Comparison of the average proteomic profiles of our previously published series[8] [three conventional variant papillary thyroid carcinomas (cvPTC), patients 7, 8 and 9], a new cvPTC (patient 6) and a previously published follicular variant (fvPTC) (patient 10). The zoomed region of the spectrum in the mass range 4000–15 000 Th shows the most significant differences in the proteomic profile of fvPTC versus cvPTC (arrows). The intensity of the protein signal is expressed as arbitrary units (a.u.). (b) The Radviz plot shows the distribution of every single spectrum of the previously published series (Avg cvPTC and fvPTC) and the new cvPTC sample (cvPTC). The two distributions are overlapped, indicating that there are no significant differences between the two groups.

Finally, the capabilities of MALDI-IMS in the frequent diagnostic challenge related to PTC were evaluated. The results showed a panel of six proteins differentially expressed in a new cvPTC versus benign cases. In particular, this result showed that MALDI-IMS could assist in the differential diagnosis between Hürthle cell-type lesions and PTC, a well-described diagnostic problem.[12] For both PTC and MTC, this panel, which we considered as an example of a 'signature of malignancy', was very similar; however, we found other different peaks that could be used to differentiate MTC from PTC. Furthermore, no differences were observed between the average profile of our previously published data[8] and the new cvPTC. In addition, the distribution, reproduced from the previously published series,[8] reveals the possible existence of a 'PTC-specific' proteomic signature and confirms the good reproducibility of the method also in terms of biological variability. This technique could also identify minimal differences between various histological variants (fvPTC versus cvPTC).

In the era of molecular biology, proteomics could prove to be a novel approach for defining diagnostic targets in thyroid pathology. However, as the MALDI-IMS approach requires high expertise and advanced instrumentation, this could potentially limit its application to the research field. These preliminary results encourage the startup of a large trial which could reinforce the association between proteomic profiles and already well-known genetic alterations in thyroid diseases, and identify the most powerful

proteomic targets through tandem mass spectrometry (MS/MS) acquired directly on tissue by MALDI-LIFT-TOF/TOF and/or by a specific shot-gun proteomic technique based on nanoLC-ESI-MS/MS. These technologies are able to identify the genetic counterpart of the proteomic findings through the query of protein databases with raw data. In addition, any possible post-translational modifications present on these proteins will be characterized. Knowledge of the identity of these proteins could be transferred into cost-effective and routine diagnostic tools, such as immunohistochemistry, that could be used on cytological *in vivo* smears, to improve the pre-operative diagnosis of indeterminate thyroid nodules.

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