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Proteomics, and Metabolomics: Magnetic Resonance Spectroscopy for the Presurgical Screening of Thyroid Nodules

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Abstract: We review the progress and state-of-the-art applications of studies in Magnetic Resonance Spectroscopy (MRS) and Imaging as an aid for diagnosis of thyroid lesions of different nature, especially focusing our attention to those lesions that are cytologically undetermined. It appears that the high-resolution of High-Resolution Magic-Angle-Spinning (HRMAS) MRS improves the overall accuracy of the analysis of thyroid lesions to a point that a significant improvement in the diagnosis of cytologically undetermined lesions can be expected. This analysis, in the meantime, allows a more precise comprehension of the alterations in the metabolic pathways induced by the development of the different tumors. Although these results are promising, at the moment, a clinical application of the method to the common workup of thyroid nodules cannot be used, due to both the limitation in the availability of this technology and the wide range of techniques, that are not uniformly used. The coming future will certainly see a wider application of these methods to the clinical practice in patients affected with thyroid nodules and various other neoplastic diseases.

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INTRODUCTION

Thyroid cancer is usually treated with primary surgery (near-total or total thyroidectomy and lymph nodes dissection if necessary), ablation of the thyroid remnant with radioactive iodine (RAI) (based on the tumor stage) and thyroid-stimulating hormone (TSH) suppressive therapy [1-3].

Follow-up consists of neck ultrasonography (US), basal and after TSH-stimulated thyroglobulin assay [1, 2, 4, 5].

Though thyroid cancer has generally a good prognosis, 10% to 15% of patients with thyroid cancer have recurrent disease, and about 5% will develop metastatic disease not responsive to RAI, and eventually will die from this disease [6].

The knowledge of cancer cell signaling could help us to develop novel anticancer therapies [7-11].

Fine-Needle Aspiration Cytology (FNAC) is currently the most common and efficient method used for the preoperative diagnosis of thyroid nodules. In the majority of cases, the results obtained by FNAC allow a good discrimination between the nodules that can be managed conservatively (thy2 following the American Thyroid Association classification, about 70% of all FNACs) and those requiring surgery (thy4 and thy5, about 10%). The most important limitation of FNAC is its low accuracy in the evaluation of ''follicular-patterned lesions'' (thy3), a situation where the cytology cannot differentiate benign from malignant lesions (follicular adenomas vs follicular thyroid carcinomas/follicular variant of papillary thyroid carcinomas, respectively). This situation leads to a diagnosis of ''undetermined nodule'' in an average 20% of all FNACs, a situation that indicates the necessity of a "diagnostic" surgery, with the only purpose to perform a definitive histology.

With these premises, it is clear that techniques other than cytology, e.g.; molecular biology, flow cytometry, and immunocytochemistry, have been investigated to overcome the limitations of FNAC. Other researchers have investigated the possible presence of biomarkers of thyroid malignancy by using 2D-gel and mass spectrometry-based proteomics.

A few studies have analyzed the possible role of different Magnetic Resonance (MR) techniques, such as Spectroscopy (MRS), in the differentiation between normal and malignant thyroid tissue.

These techniques have been firstly used with the aim of identifying the morphologic features of malignancies, and then, with the improvement in the technology behind the MR, to identify the metabolic differences between thyroid neoplasms (benign or malignant) and normal thyroid tissue. In a second step, the utility of these approaches could be tested with the aim of differentiating between benign and malignant neoplasms. These experiments had generally been performed on operative specimens coming from surgically removed thyroids: the analyses produced the promising results that led to the subsequent phase of the application of the method in the preoperative work-up of thyroid nodules.

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A pre-clinical application of the MRS has been then studied, testing its application on specimens obtained from FNACs performed after the removal of the thyroid gland (ex-vivo), an attempt to determine whether this technology could give a significant improvement in the preoperative diagnosis of the so-called "undetermined" thyroid lesions.

In this paper, we will deal with the historical development and current state of the art of the MRS analysis of thyroid lesions, discussing the results obtained by the different techniques of Nuclear Magnetic Resonance (NMR) and thyroid cancer diagnostics.

Molecular diagnosis of cancer requires analytical tools capable of distinguishing the key biomarkers, that are secondary metabolites, either directly linked to the disease or derived from its induced perturbations.

In recent years, several studies in this field have been started for two main reasons: the improved availability of analytical techniques such as MRS and Mass Spectrometry (MS), and the consequent development of appropriate statistical tools capable of dealing with the huge mass of spectroscopic data associated to the metabolites presence and variation in concentration. These aspects and the potential of the method have been recently reviewed [12].

In particular, ex-vivo MRS analysis plays a role that is complementary to that of both Inner Magnetic Resonance (IMR) and *in vivo* MRS since these latter tools, although providing excellent morphologic information, are unlikely to capture fine variations in the molecular composition of the lesions, i.e. in their lipid profile.

The first report dealing with the application of proton MRS to cancer diagnosis is the article by Mountford *et al.* [13], while the methodological aspects about the current protocols for the MRS analysis of biopsies can be found in the study by Beckonert *et al.* [14]. An overview of this progressively growing field of application can be found in other studies [15-18].

Thyroid cancer has been the first field where molecular diagnosis was performed using ex-vivo MRS [19-23].

The main issue behind MRS analysis of ex-vivo specimens is the quality of the spectra obtained, that is directly related to the homogeneity of the sample and to its degree of molecular mobility. For this reason, biopsies are not expected to produce high-resolution MRS spectra, if specific techniques are not applied. In the case of thyroid tissue, even if the observed resolution was less than that usually obtained through MRS, the 2D-NMR (COSY) experiment allowed to detect several signals that were assigned to specific molecular features [19].

In this study, the authors reported that two specific signals play a relevant role in the discrimination of lesions of different nature. These two signals are: a methylene ($-CH_2-$) peak at 1.7 ppm, deriving from lipids and lysine, and one at 0.9 ppm, regrouping essentially all the methyl ($-CH_3$) groups from all molecules.

Based on the observation that the MRS-1D spectra of malignant tissue showed a lower content of lipids and higher concentrations of aminoacids when compared to their benign counterpart, the authors assumed the ratio between the two peaks (1.7 and 0.9 ppm) as a tentative indicator for malignancy.

After the analysis of the results obtained from the MRS spectra, and the revision of both cytologic and histologic results obtained from different thyroid lesions, a line was drawn at the value of 1.1, allowing to discriminate the benign tissue (when the ratio is >1.1) from the malignant one (when lower than the threshold).

This method allowed a 100% discrimination between benign and malignant tissues, in specimens obtained from surgically removed thyroid glands.

Another study [20] aimed at verifying the possibility of obtaining the same results from material obtained from FNA, using the same spectral markers to compare the results in cases of citologically undetermined lesions (thy3). This study allowed to elucidate the statistical relevance of analysing small amounts of cells coming from a lesion, a great possibility, in the future, to perform a thorough diagnosis with a relatively non-invasive method. In this study, among the 92 analysed cases, the results obtained from FNAC were discordant from the final histology in only 6.

On the other hand, the analysis produced a low specificity in carcinoma detection (54%), which raised the question about the possible limits and opportunities of establishing the degree of aggressiveness of a lesion before the infiltration has started.

In another study [21], the analysis was performed with the 2D-NMR (COSY) with the aim of achieving a better resolution: the results showed that a second spectral marker, linked to cholesterol, was found at 2.05 ppm. The use of this further signal increased the specificity of the test to 72%. The same group tried to extend this method to material obtained from FNAC, aiming at avoiding surgery performed with the only purpose of obtaining a final diagnosis [22]. However, the quality of the results obtained with the use of these specimens was not significant enough to draw any conclusion.

The authors ascribed their unsatisfying results to the high blood content observed in the material obtained from the aspirations. The blood was virtually absent in samples obtained from surgically removed thyroids, that were devoid of circulating blood. The FNAC samples analysed through the MRS produced spectra in which the discriminatory signals were biased by the ones given by blood cells. The authors concluded that, at that time, it was unclear whether their approach could be ever exported to cells coming from a FNAC performed in the common preoperative workup.

These results were obtained with a very simple spectral indicator and thus the same group, in order to expand their results, set up a sophisticated statistical analysis to evaluate if other and more discriminating markers could have been detected [21]. The information in the MRS spectra was compressed using principal component analysis (PCA), and saving the first 10 principal components (PCs), explaining 97% of the variance. Then, three supervised statistics were created on this reduced dataset: linear and quadratic discriminant analysis, neural networks and genetic programming, which were used in conjunction to assign cases to histological results. This work was a pioneer study in the use of the ad-

vanced statistics that will eventually become the core of metabolomics, and thus provided more indication on potential ways of analysis rather than molecular biomarkers, as part of the spectral information ended up scrambled due to PCA.

IN VIVO MAGNETIC RESONANCE APPROACHES: NON-INVASIVE PROTON (^{1}H) MRS TECHNIQUE

To try to overcome the insufficient accuracy of pre-surgical FNAC-based diagnosis using a non-invasive method, *in vivo* characterization of malignant thyroid lesions has been developed over the past decade using MRS. King *et al.* [24] succeeded in discriminating thyroid carcinomas from normal thyroid tissue based on the comparison of their respective ^{1}H MRS spectra: 8 patients with histologically confirmed thyroid cancerous tumors larger than 1 cm^3 (3 anaplastic, 2 papillary, 1 follicular carcinomas and 2 metastatic nodes from papillary carcinomas), and 5 lesion-free volunteers participated to this study. Proton MRS spectrum of each patient was recorded using a 1.5T whole-body imaging system (Gyroscan ACS-NT, Philips, Best, The Netherlands), and significant differences in the spectral profiles were observed in 5 malignant samples. In addition to an increase of aminoacids and di- and tri-glycerides in carcinomas, the authors found that choline and creatine signals were only present in material coming from carcinomas, whereas no trace of such markers were observed in patients with normal thyroids. Therefore, choline/creatine ratio was chosen as a marker of malignancy: this ratio ranged from 1.6 (in cases of well-differentiated follicular carcinoma) to 9.4 (in thyroids with anaplastic thyroid cancer). However, in 2 patients with cancer (1 anaplastic cancer and 1 metastatic lesion, 25% of the whole study population), these signals could not be detected either. Creatine signal was also not detected in one patient with anaplastic carcinoma.

According to the authors, this was due to mere technical issues, such as the low sensitivity of the technique, and they also pointed out the possible limitation of their method in the analysis of cystic lesions.

Gupta *et al.* [25] used the same approach to differentiate between malignant (n=8, papillary carcinomas) and benign lesions (n= 18, with 14 colloid lesions, 2 lymphocytic thyroiditis, 1 follicular adenoma, and 1 cyst) in patients with solitary thyroid nodules. As previously reported, the discrimination was based on the presence of choline signal on the ^{1}H-MRS spectra of malignant samples, leading to a method that demonstrated a sensitivity of 100% and a specificity of 88%.

EX-VIVO MAGNETIC RESONANCE APPROACHES: HIGH-RESOLUTION (HR) AND HIGH-RESOLUTION MAGIC-ANGLE-SPINNING (HRMAS) MRS

Yoshioka *et al.* [26] were one of the first research groups to use HR-MRS with the aim of characterizing thyroid cancer. Using an 80MHz WP-80SY-WG-NMR spectrometer (Bruker biospin, Karlsruhe, Germany), they extracted the total lipid content of 15 papillary thyroid carcinomas, 13 follicular adenomas and their corresponding normal tissues (n=27), and their results showed a significantly different spectrum between normal thyroid tissue and papillary carcinoma than that obtained through ^{1}H-MRS.

The average concentrations of the main lipids were then compared between each group and Kruskal-Wallis one-way ANOVA were used to highlight significant differences. The results demonstrated that thyroid cancer was characterized by higher concentrations of cholesterol and lower levels of dolichol, when compared to normal thyroid tissue. Thyroid carcinoma and adenoma could be then differentiated on the basis of their dolichol content, which was at the level of normal tissues in adenoma samples. According to the authors, the low level of dolichol and the high level of cholesterol in cancer might be the consequence of the perturbation of the isoprenoids biosynthesis pathways (in this pathway dolichol and cholesterol are major products) in cancer metabolism; on the opposite side, their concentrations are preserved in thyroid adenomas. Moreover, as a parallel result, it was found that phosphatidylcholine and sphyngomyelin contents were the only significant markers for the discrimination between adenoma and normal tissue, even if they failed to differentiate between follicular adenoma and papillary cancer.

More recently, three research groups investigated the potential of NMR-based metabolomics in the diagnosis of thyroid cancer. The advantage of the metabolomics approach is that it allows an untargeted analysis of the overall metabolites that constitutes a biological organism.

The highest molecular discrimination of the NMR analysis performed on ex-vivo thyroid tissues had to wait for the development of HRMAS and its application to medical studies, firstly demonstrated in 1996 on lymph node tissues [27]. In the following lines we will briefly discuss the specificities of this method, before reviewing its contribution to the diagnosis of thyroid lesions.

When using the HRMAS, the sample is span around an axis oriented at 54.7 degrees (the "magic angle"), with respect to the magnetic field of the MRS instrument. This method was introduced for the analysis of solids, where it provides a significant enhancement of the resolution due to the removal of the broadening effects that arise from multiple sources. Indeed, a MRS spectrum achieves its optimal resolution only in those specific cases when the molecules of the sample are under very high symmetry, e.g. in liquids in molecular motion, but not in solids.

Thus, the MRS signal without the HRMAS can easily loose a significant part of its resolution.

The sample holder is a ceramic hollow cylinder, deriving from the equipment that was originally devised for the study of hard solids. The volume of the samples that can be analyzed in this tool is about 80 μl, thus making it available for material obtained from a common biopsy.

Alternatives to this sample holder are represented by the cheaper but more fragile pyrex sample holder, that have been developed for specific manufactures, and by a plastic disposable container that simplifies the sample preparation, also avoiding the necessity of cleaning the expensive ceramic objects.

The small vials containing the material are water-tight and fit the ceramic cylinder ("inserts"), so that they can contain the biopsy and a few microliter of heavy water, which helps setting up the experiment for optimal results. The sam-

ple is typically span at rates of 2 to 4 kHz, to avoid spectral artefacts. Fast spinning has been a point of concern due to sample changes that may be induced by the associated centrifugal forces. Recently, a procedure was demonstrated that allows much slower spinning rates (about 400Hz), so that in the future this aspect will be no more a concern [28].

The first study on thyroid lesions using the HRMAS was led by an American research group from the Harvard Medical school, who assessed the potential of this technique (600MHz spectrometer) for the diagnosis of papillary thyroid carcinoma, based on the analysis of cytological (Fine-needle aspiration biopsies – FNAB) and histological samples obtained from 13 patients (4 papillary carcinoma, 4 follicular adenoma and 5 normal tissues) [29]. As already discussed above, HRMAS-MRS has the significant advantage that it needs only a limited amount of tissue to complete a sound analysis, thus allowing the collection of the samples without losing a significant amount of tissue that might interfere with a thorough histologic diagnosis.

Nevertheless, in this study, each sample was also microscopically analyzed at the moment of its collection, to verify the viability of the specimens in terms of cells composition: this was done to avoid any bias that can be given by fibrous tissue, inflammation, colloid, normal thyroid cells, and blood cells.

Following a principal component analysis of the ^{1}H-HRMAS-NMR spectra of the biopsies, 4 PCs were selected according to their correlation with their quantified tissue concentrations (PC1 = % fibrovascular; PC3 = % tumor + % colloid; PC5= % benign epithelia; PC6=% inflammation; PC being the linear combination of the NMR signal areas). A conventional supervised multivariate statistical analysis was then performed on these 4 PCs, and the percent composition of the five represented pathological features was obtained. The resulting canonical scores 1 (linear combination of the PCs and % of pathological features) was able to discriminate the three tissue types from each other. The same coefficients (obtained from PCA and canonical analysis on the biopsies) was then applied to the NMR spectra of specimens obtained from FNAC, leading to a significant discrimination between normal tissue and follicular adenoma only along the second canonical score, whereas it was able to discriminate between normal tissue, follicular adenoma, and papillary cancer in materials obtained from biopsies.

In two separate studies, another group [30, 31] developed a metabolomics approach based on HRMAS-NMR spectroscopy of thyroid biopsies obtained during surgery, in order to characterize thyroid lesions from healthy tissues, and discriminate benign from malignant tumors, in patients undergoing surgery for citologically undetermined lesions (follicular adenoma, follicular carcinoma, or follicular variants of papillary carcinoma at final histology).

Since no extraction is needed to analyze tissues using HRMAS-NMR spectroscopy, a higher concentration of mobile hydrophobic and hydrophilic metabolites can be observed on the same ^{1}H NMR spectra. Therefore, the study showed that benign and malignant thyroid lesions not only demonstrated a higher level of several aminoacids (alanine, cysteine, glutamine, glutamate, isoleucine, leucine, lysine, phenylalanine, serine, tyrosine, valine), lactate and taurine, but also a significantly lower content of fatty acids, with respect to normal tissues.

Furthermore, when this approach was used to analyze the samples that cytology failed to diagnose preoperatively, follicular adenomas and carcinomas as well as papillary carcinomas were analyzed using an orthogonal projections on latent structures discriminant analysis (OPLSDA) [32].

With a relatively high number of follicular lesions analyzed (30 follicular adenoma and 10 follicular thyroid carcinoma out of the 72 samples), the authors managed to build a robust statistical model, which enabled to discriminate cancerous tissues from benign lesions.

The results obtained showed that thyroid cancer (both papillary and follicular) differed from follicular adenomas by higher concentrations of taurine, lactate, phenylalanine and tyrosine and lower concentrations of myo- and scyllo-inositol, choline, phosphocholine and/or glycerophosphocholine and unknown compounds.

Finally, Deja *et al.* analyzed extracts of homogenized tissue samples using liquid-state NMR-based metabolomics in order to understand the molecular mechanisms involved in the development of thyroid tumors and in particular, thyroid cancer [33].

Healthy tissues, benign lesions (including non-neoplastic nodules), follicular adenomas, and thyroid carcinomas were analyzed, and their respective profiles compared using pairwise OPLSDA. Comparison between healthy tissues and each of the three other tissue types all led to statistically significant discrimination: a higher concentration of several aminoacids (methionine, alanine, glutamate, glycine, tyrosine, phenylalanine) and lactate in tumor samples was also demonstrated. In addition, increased level of hypoxanthine and decreased level of acetone in thyroid lesions were also described, that were not detected in Torregrossa's samples. Also, new markers such as 3-hydroxybutyrate and β-glucose (deregulated only in benign lesions), and phosphocholine and formate (only present in non-neoplastic nodules), were reported. Finally, four metabolites (creatine, scyllo-inositol, myo-inositol and uracil) were found to be selective biomarker candidates for thyroid cancer. Interestingly, the authors commented about the metabolic specificity of follicular adenomas, which possess some of the metabolic features of normal thyroid tissue, while simultaneously displaying part of the metabolic profile associated with thyroid cancer. This, in addition with other evidences (such as epidemiological and demographic data, cases of malignancies found within benign nodules, identical cytological appearance) [34, 35], may indicate an intermediate nature of these benign tumors that could support the theory that follicular thyroid carcinoma might arise from preexisting follicular adenoma, or that, in some cases, a follicular adenoma can be a preneoplastic lesion.

These metabolomics studies came at the final conclusion that specific markers of different tissues (benign and malignant) were correlated with the cell proliferation observed during the development of the tumor (e.g. the increase in amino acid level and the decrease in lipids). Moreover, both the Warburg effect (due to the lactate) and the imbalanced

osmolytic function (due to the differences in myo-inositol and scyllo-inositol), in addition with other markers of tumor aggressiveness (taurine), were characteristic features highlighted in thyroid cancer.

Furthermore, Xia *et al.* [36] used metabolite set enrichment analysis on the discriminant metabolites in order to highlight the more commonly involved metabolic pathways in the development of thyroid cancer. The outcomes obtained by this analysis pointed out that protein biosynthesis were the most affected pathways, as a consequence of the increased proliferation rate of cancer cells, which requires fast supplementation of protein machinery.

CONCLUSION

It appears that the high-resolution of HRMAS-MRS improved the overall accuracy of the analysis of thyroid lesions to a point that a significant improvement in the diagnosis of cytologically undetermined lesions can be expected. This analysis, in the meantime, allows a more precise comprehension of the alterations in the metabolic pathways induced by the development of the different tumors.

Although these results are promising, at the moment, a clinical application of the method to the common workup of thyroid nodules cannot be used, due to both the limitation in the availability of this technology and the wide range of techniques, that are not uniformly used.

The next future will certainly see a wider application of these methods to the clinical practice in patients affected with thyroid nodules and various other neoplastic diseases.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

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Declared none.

ABBREVIATIONS

RAI	=	Radioactive iodine
TSH	=	Thyroid-stimulating hormone
FNAC	=	Fine-Needle Aspiration Cytology
MR	=	Magnetic Resonance
MRS	=	Magnetic Resonance Spectroscopy
NMR	=	Nuclear Magnetic Resonance
MS	=	Mass Spectrometry
IMR	=	Inner Magnetic Resonance
PCs	=	Principal components
PCA	=	Principal component analysis
HR	=	High-Resolution
HRMAS	=	High-Resolution Magic-Angle-Spinning
FNAB	=	Fine-needle aspiration biopsies
OPLSDA	=	Orthogonal projections on latent structures discriminant analysis

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