

# Metabolomics Methods as a New Diagnostic Tool for Thyroid Nodules

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## Abstract

Carcinoma of the thyroid gland is the most common cancer of the endocrine system. It accounts for approximately 10% of thyroid focal lesions, and the incidence of this cancer is increasing. A valuable technique for differentiating cancerous from benign nodules is fine-needle aspiration biopsy (FNA) with cytological verification. Unfortunately, in 30% of cases, FNA results are not sufficient to determine the proper method of treatment. Therefore, many patients are referred for diagnostic surgery and histopathologic examination. Despite the development of new imaging and molecular diagnostic techniques, no universal marker for pre-surgery identification of malignant changes in the thyroid is available.

Modern measuring techniques, such as nuclear magnetic resonance spectroscopy - NMR - and mass spectrometry - MS, in combination with chemometric analysis led to the development of a new field of biology - metabolomics. Metabolomics allows for the analysis of biochemical processes in biological systems by assessing the metabolome (set of all metabolites - small molecular compounds with a molecular weight <1000 Da - contained in measured biological material). The metabolite profile quantitatively and qualitatively changes in response to disturbances of homeostasis. The promising results of the use of metabolomics methods as diagnostic tools for certain cancers and our experience with using metabolomics methods to differentiate benign from malignant thyroid tumors suggest that this field of science, which has been growing for several years, will improve the diagnosis and differentiation of thyroid cancer.

**Keywords:** Thyroid nodules; Thyroid; Metabolomics; Personalized medicine; Biomarker

## Introduction

Focal thyroid lesions in the form of individual nodules or multinodular goiters are one of the most common problems for endocrinologists and endocrine surgeons. Available sources show that they affect a large population of patients. They are identified by palpation in 2-6% of cases, by ultrasound in 19-35% of cases and by autopsy in 8-65% of cases [1]. It is estimated that approximately 4-12% of thyroid lesions are cancerous, with most being papillary carcinoma lesions [2]. In recent decades, the morbidity of thyroid cancer has increased in both women and men [3-5]. Large tumors with compression symptoms and quickly growing tumors clearly require surgery. Wide access to imaging diagnostics has made it possible to frequently detect small nodules in the thyroid parenchyma in patients without clinical symptoms. A commonly adopted diagnostic and therapeutic strategy sequence is the following: ultrasound - fine-needle biopsy under ultrasound control - decision to perform surgery on the basis of cytology or active surveillance.

Ultrasound tumor characteristics indicating a greater risk of cancer (macro- and micro-calcifications, solid character of the tumor, irregular boundaries, central tumor vasculature, absence of a hypoechoic capsule, greater longitudinal than transverse dimension) do not affect the final diagnosis [6-9]. Other thyroid imaging methods, like computed tomography, standard magnetic resonance imaging and positron emission tomography (PET) are not better at differentiating benign and malignant lesions than ultrasound. There is hope that a new ultrasound technique - elastography - will be better at evaluating the cohesiveness of the tumor and, thus, will better indicate the risk of malignant changes [10,11].

The golden standard for the diagnosis of thyroid nodules is fine-needle biopsy under ultrasound control. The Bethesda system was introduced in 2007 by the National Cancer Institute (NCI) to describe cytopathology of thyroid tumors, and it divides them into 6 categories: nondiagnostic or unsatisfactory (I), benign (II), atypia of

undetermined significance (AUS) or follicular lesion of undetermined significance (FLUS) (III), follicular neoplasm (FN) or suspicious for FN (IV), suspicious for malignancy (V), and malignant (VI) [12]. The risk of malignant changes for each of these groups is as follows: I- 1-4%, II- 0-3%, III- 5-15%, IV- 15-30%, V- 60-75%, and VI- 97-99%. AUS, FLUS, FN and suspicion of malignancy are recognized in 5-42% of cases and act together to form an indeterminate diagnosis; they are all indications for diagnostic surgery [6,13,14].

The procedure implemented in cases of AUS/FLUS has been widely discussed. Re-biopsy, which is recommended, sometimes changes the diagnosis and helps confirm the decision to perform surgery. For some patients, obtaining multiple biopsies of the same tumor is necessary. The diagnosis of malignancy by re-biopsy delays the implementation of radical surgical treatment of the cancer, and in some cases, exclusion of cancer is not possible without the performance of lobectomy or thyroidectomy [15,16]. Discovering non-cytological markers of malignancy seems particularly important for this category of patients in order to reduce the performance of unnecessary operations, allow for execution of the most tissue-conserving surgery possible and achieve accurate diagnoses allowing for appropriate therapeutic choices to prevent the need for re-peat operations [17].

There are great expectations in genetic research. The development of molecular biology and the possibility of its use in medicine have resulted in the performance of a series of studies on cancer genetics,

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**Received** December 21, 2015; **Accepted** January 11, 2016; **Published** January 13, 2016

**Citation:** Wojtowicz W, Pawelka D, Balcerzak W, Mlynarz P (2016) Metabolomics Methods as a New Diagnostic Tool for Thyroid Nodules. *Metabolomics* 6: 161. doi:10.4172/2153-0769.1000161

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including cancer of the thyroid. Important genetic changes include RAS gene mutations, the PAX8/PPAR $\gamma$  translocation (currently more often present in cancer than in follicular adenoma) and translocation of the proto-oncogene RET/PTC in papillary carcinoma and benign lesions [6]. A new algorithm has been developed for the handling of undefined thyroid tumors, and it involves molecular tests of material obtained by fine needle aspiration as a supplement to cytology analysis (Figure 1) [17].

Xing et al. also highlighted the role of BRAF mutations in assessing the aggressiveness of papillary carcinoma, which may affect surgical treatment decisions [17].

There are also reports in the literature of using microRNA (miRNA) profiles to distinguish papillary carcinoma from normal thyroid tissue and to distinguish follicular carcinoma from follicular adenoma [6]. Reports of miRNA profiles in serum are particularly interesting, as new serum miRNA markers could be measured non-invasively. A study by Yu et al. found that a serum miRNA profile of the miRNAs miR-151-5p, miR-222 and let-7e provided 87.8% and 86.85% sensitivities and 88.8% and 79.5% specificities for the differentiation of papillary carcinoma from benign tumors and healthy controls, respectively. However, there was no significant difference in the level of let-7e between benign nodules and cancerous tissue, which may suggest that the increased serum concentration of let-7e could be due to other factors that are not necessarily related to the presence of a malignant tumor in the thyroid gland [6,18,19].

Detection of increased activity of a number of genes in tumor tissues has resulted in the performance of research to identify markers of thyroid carcinogenesis based on the transcriptional products of those genes in the peripheral blood. The potential markers that have been identified include tissue inhibitor of metalloproteinase-1 (TIMP-1), chitinase 3-like 1 (YKL-40), galectin-3 (Gal-3), cytokeratin 19 (CK-19), and angiopoietin-1 (Ang-1). A recent study by Makki et al. did not confirm differences in the levels of these markers in the serum of patients with benign and malignant thyroid changes [20]. Further studies on the potential markers of carcinogenesis in the thyroid gland are necessary.

A relatively new field of biological science is metabolomics, and it is a method that allows for quantitative measurements of overall metabolomic responses to pathophysiological or genetic changes in living organisms [21]. A schematic of this process is presented in Figure 2 [22].

It seems logical that the next step in the field of cell biology after the assessment of genome transcription products (mRNAs and proteins) is the study of the metabolome. This new manner of examining the processes taking place in living organisms may improve our understanding of the biochemistry of cancer and may help develop powerful diagnostic tools. In recent years, many studies have been published that assessed metabolite profiles using NMR spectroscopy and mass spectrometry in cancers, such as cancers of liver, kidney, ovary, breast, colon, esophagus, prostate and others [22-26].

### Metabolomics for the diagnosis of thyroid nodules

Studies of the metabolome in thyroid diseases have been performed for approximately 20 years. Studies have used different analytical techniques and have focused on different compound groups. The sample collection methods used are also important. By definition, metabolomic studies allow for the use of samples of various origins, including tissue samples [27,28,37]; breath samples [29]; and biofluids,

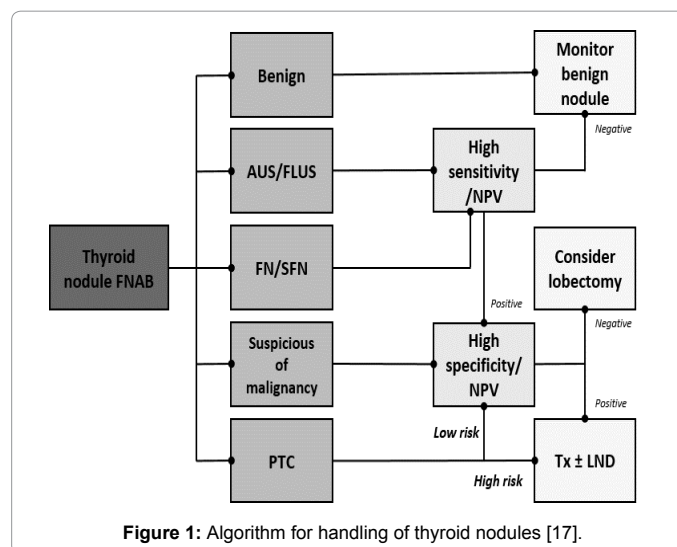


Figure 1: Algorithm for handling of thyroid nodules [17].

such as serum [28,30,31], urine [32], and saliva [33]. This allows metabolomic studies to purely use samples obtained in a less invasive manner (i.e., serum, urine, and saliva). In regards to thyroid cancer, metabolomic studies that have been conducted to date used multiple types of samples and analytical techniques [27,28,34-39,41,43,44,47,53]. Two basic strategies are used in metabolomics. The first is an untargeted approach, where all compounds are tested in a particular sample at a given time. The second is a targeted approach, which focuses on a specific group of compounds in sample. Both approaches are widely used [40].

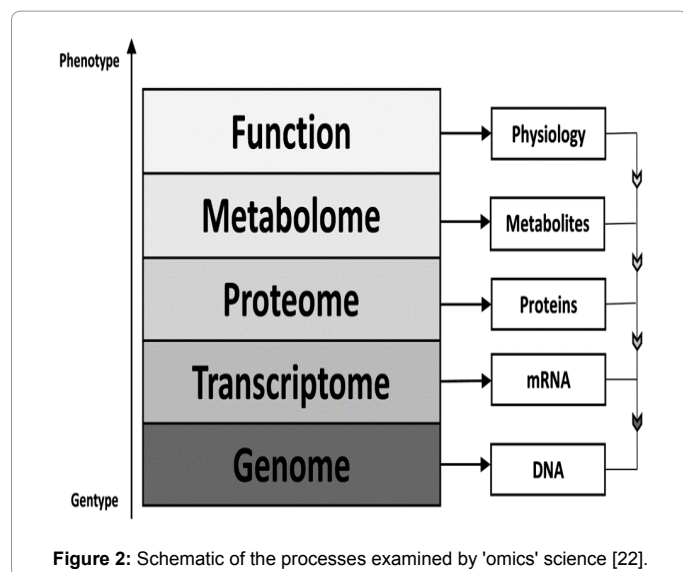
The selected studies described below show that it is possible to use samples from different sources and to use different analytical techniques and different metabolomic strategies for diagnostic support for thyroid cancer. Such studies may facilitate the identification of changes in the thyroid gland.

### Magnetic resonance spectroscopy-based metabolomic studies of thyroid nodules

In 1994, Delbridge et al. described differentiating thyroid cancer tissue from normal thyroid gland tissue using proton magnetic resonance spectroscopy with 100% sensitivity. Furthermore, they were able to distinguish two groups of benign adenomas, one with spectrum changes similar to those seen in healthy tissues and one with spectrum changes comparable to those in thyroid cancer tissues, based on the difference in the ratio of two NMR resonances regions, lipids at 1.7 ppm and amino acids at 0.9 ppm. In normal tissue, the ratio was above 1.1, and in cancer tissue, it was below 1.1 [34].

Mackinnon et al. expanded on the studies of thyroid cancer performed by the Mountford group using NMR methods with the application of two-dimensional (COSY) spectra, and this allowed signals that overlapped on one-dimensional spectra to be distinguished from one another. Ninety-three samples from patients after subtotal or total thyroidectomy were used in that study, and the results indicated that the incidence of cholesterol/cholesteryl esters and di-/triglycerides cross peaks could be used to distinguish carcinomas from benign lesions [41].

Promising results were obtained by King et al. [42] and were similar to previously reported results [41]. In that study, 13 samples were examined by proton magnetic resonance spectroscopy. Eight samples



were classified as thyroid cancers, and 5 samples were classified as healthy controls. The characteristic region of the spectrum originating from FA was shown to have diagnostic potential, and the presence of a choline signal also had diagnostic potential, with a high choline/creatinine ratio indicating cancerous tissue.

The findings of that study were confirmed by Gupta et al. who conducted an *in vivo* study involving analysis of the choline resonance signal by MR spectroscopy. A choline resonance signal was identified in all 8 of the cancer tissue samples analyzed in contrast to only 1 of the 17 benign tissue samples analyzed. This method proved to be 100% sensitive and 94.11% specific for the identification of thyroid cancer, which is in accordance with the results of King et al. [42]. The presence of a choline signal in the cells of a malignant tumor may be caused by an increased choline concentration due to rapid cancer cell proliferation [35].

Other studies have also demonstrated the possibility of using MRS as an alternative diagnostic method. However, *in vivo* MRS can result in inaccurate spectra, hindering the achievement of an accurate diagnosis due to the impacts of the surrounding fat, tumor size, tumor location, the act of the patient breathing and swelling near the tumor [44].

The results obtained in previous studies [34,35,41,42] were confirmed seventeen years later by Jordan et al. using <sup>1</sup>H-MAS NMR spectroscopy. They obtained a characteristic spectrum of papillary carcinoma that allowed for the differentiation of cancer, follicular adenoma and normal tissue, and this was confirmed histologically by FNA biopsy. As for the study described above, the distinction between the groups was based on the ratio of two NMR resonances - lipids at 1.7 ppm and amino acid residues at 0.9 ppm. Although that study only examined 13 pairs of samples, it shows the possibility of the use of <sup>1</sup>H-MAS in addition to FNA for the differentiation of follicular tumors [44].

The next studies that clearly demonstrated the usefulness of HRMAS-NMR in the diagnosis of thyroid tumors were performed by Torregrossa et al. [43]. They examined tissue samples from surgical biopsies of 72 patients. The study allowed for distinction of benign, malignant and normal tissue samples. The group of benign lesions

included adenoma (A) and nodular goiter (GN), while the group of malignant lesions included anaplastic carcinoma (AC), papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). Multivariate data analysis (MDA) was performed, which allowed for the generation of predictive models, and the results were confirmed by ROC curve analysis, which revealed an AUC of 0.77. Based on the OPLS-DA loadings, these studies revealed the set of essential metabolites that indicated changes in the thyroid gland. The most important changes in metabolites that indicated malignancy were increase in lactate and taurine and decreases in choline, phosphocholine, myo-inositol and scylloinositol. In this study, decreases in unknown compounds were also observed in the malignant group [43]. The observed changes in metabolite levels are associated with the Warburg effect [45] and other phenomena that affect metabolism during carcinogenesis [30,46,47].

The presented studies [30,34,35,41,42,43,44] showed that among the metabolites that are possible biomarkers, those involved in lipid metabolism play a crucial role in carcinogenesis [48]. Therefore, lipidomics trends associated with carcinogenesis have been identified; however, while MS spectrometry is the method of choice for investigating lipids, NMR studies were also performed.

A targeted metabolomics approach was used by Y. Yoshioka et al. to examine thyroid carcinogenesis, and they examined 61 tissue samples divided into 4 groups - control (27), papillary cancer (15), adenoma (13), and Basedow disease (6). <sup>1</sup>H-NMR measurements were performed on lipids extracted using the Folch's method [49]. The compounds that had different levels between groups were dolichols, cholesterol, and cholines contained in phosphatidylcholine plus sphingomyelin (PC+SPH). The level of dolichols was lower in the cancer and Basedow groups but was normal in the adenoma samples. Cholesterol was only higher in the cancer samples. The level of acyl chain double bonds and the level of PC+SPH were only significantly higher in the cancer samples compared to the normal tissue samples. The results indicate a disturbance in the isoprenoid synthesis pathway in the papillary cancer samples [37], and this may be related to a defect in a feedback mechanism that is controlled by the intracellular concentration of cholesterol [50].

Another group of compounds used for cancer identification and description in targeted metabolomics approaches is the phospholipids. The results of plasma <sup>31</sup>P NMR spectroscopy studies demonstrated the possibility of distinguishing between hypothyroid patients with cancer, hypothyroid patients in remission and healthy controls. The groups of hypothyroid patients with cancer have lower levels of PE+SM (phosphatidylethanolamine+sphingomyelin) and PC (phosphatidylcholine) than subjects in remission [30]. These changes in the levels of PE + SM and PC in patients with cancer may be caused by tumor metabolism, namely increased metabolism of phospholipids to allow for greater proliferation of tumor cells [38].

In our studies [27], tissue PBS extracts were used to compare groups of patients with different changes in the thyroid gland. The samples were divided into four groups - healthy control (H), non-neoplastic nodules (NN), follicular adenoma (FA) and malignant thyroid cancer (TC). Using chemometric tools, differences between groups in the relative concentrations of specific metabolites were recognized. For all comparisons, OPLS-DA models were used. For two types of benign changes - FA and NN - the key changes observed were



increased concentrations of the branched chain amino acids isoleucine and valine and decreased concentrations of citrate and N-acetylated compounds in the FA group. In comparison FA and TC highest influence on separation had scyllo-inositol and myo-inositol together and also lower levels of methionine and lactate in relation to FA. The TC group was distinguished from the NN group based on differences in the levels of BCAAs, lactate, scyllo-inositol, myo-inositol, citrate and NACs.

Changes in metabolites levels in thyroid lesion can be transition point between healthy and development of thyroid cancer. The main differences observed between healthy tissue extracts samples and samples from thyroids altered in any way were lower levels of lipids, alanine, methionine, acetone, glutamate, glycine, lactate, tyrosine, phenylalanine and hypoxanthine, and the differences that were specific to cancerous thyroid tissues were lower levels of scyllo- and myo-inositol. The differences in those metabolites may be related to increased membrane biosynthesis, tumor development, the Warburg phenomenon and osmoregulation of cancer cells, indicating the possibility of cancer progression in the thyroid gland.

### Mass spectrometry-based metabolomic studies of thyroid nodules

As previously mentioned there are two types of metabolomics approaches, untargeted and targeted. The main goal of metabolomics is to determine the levels of compounds in samples and the relationships to the increases and decreases in those levels with clinical conditions.

S Guo et al. [28] focused on the evaluation of lipidomic profiles in serum and tissue samples using MS. They obtained 289 serum samples and 36 tissue fragments. The samples were classified into 3 groups: healthy controls (serum-122; tissue-15), malignant thyroid - MTC (serum-124, tissue-16) and benign thyroid - BTT (serum-43, the tissue-6). The goal of the study was to clearly distinguish the control group from the MTC group, the control group from the BTT group and the MTC group from the BTT group. The identification of compounds that were most valuable for differentiating the groups indicated that similar compounds were different between groups in serum and tissues. These compounds included PC (34:1) – (phosphatidylcholine), PC (36:1), PC (38:6), PA (36:2) – (phosphatidic acid), PA (36:3), PA (38:3), PA (38:4), PA (38:5), PA (40:5) and SM (34:1) – (sphingomyelin) [32]. Overexpression of FNAS (fatty acid synthase) and SCD1 (stearoyl-CoA desaturase-1) was observed in the serum and tissues samples from the MTC group. These enzymes can increase the levels of cancerogenic lipids, which are monounsaturated and saturated fatty acids. These metabolites can infiltrate the blood from tumor tissues via diffusion, which may have resulted in the increased level of these metabolites observed in the serum samples of cancer patients [28,51].

With the use of the relatively new technique of imaging mass spectrometry, IMS, Ishikawa S et al. compared papillary cancer tissues (7 samples) to normal tissues with a focus on phospholipids. A small number of samples were analyzed, but this study proved that it is possible to use IMS as a metabolomic diagnostic tool. The study was able to distinguish between papillary cancer and normal tissue samples. The most important phospholipids that were found to have different

concentrations between the two groups were phosphatidylcholine (16:0/18:1 and 16:0/18:2) and sphingomyelin (d18:0/16:1). These compounds are present in higher concentrations in papillary cancer than in healthy tissue [39]. The results also suggest overexpression of SCD1 in papillary cancer [39,52].

A benefit of metabolomic studies is the ability to use samples collected less invasively than those collected via biopsy or surgery, and finding a marker or characteristic profile of metabolites that can be examined in material collected via less invasive approaches is highly desirable. For example, Yao et al. performed a metabolomic study mainly on serum samples. By examining changes in metabolite levels, they attempted to differentiate more global changes occurring in the thyroid gland. The study examined a total of 140 serum samples (30 - papillary thyroid cancer, 80 - benign thyroid nodules, and 30 - healthy controls). Measurements were made using the LC-MS method. The results showed a general decline in the level of metabolites detected in the papillary thyroid cancer patients in relation to the patients with benign changes in the thyroid. Moreover, the two groups were distinguished from one another based on differences in the amount of FFA used in the biochemical reactions (most likely for beta-oxidation and the synthesis of ketone bodies) and the concentration of 3-hydroxybutyric acid, which is the intermediate product in fatty acid metabolism. In the future, differences in the levels of these metabolites may be used to distinguish patients with PTC from those with nodular goiter. However, the most important observations were the differences in the levels of related carnitines and the increased level of 3-hydroxybutyric acid in patients with papillary thyroid cancer; 3-hydroxybutyric acid can be considered a potential biomarker [36].

To further examine the use of samples collected using less invasive methods, L. Guo et al. examined exhaled breath samples. Volatile compounds present in the samples were measured using the GC-MS analytical technique. Ninety-six samples were used in the study and were divided into 3 subgroups: nodular goiter (n=25), malignant nodule (n=39) and control (n=32). Based on MDA, including VIP values, groups of metabolites that could be used to differentiate the three groups were assigned to particular comparisons. For control vs. benign nodule, the most important metabolites were sulfuric acid, cyclohexylmethyl hexyl ester, isolongifolene-5-ol, 3,5-decadien-7-yne, 6-tbutyl-2,2,9,9-tetramethyl, cyclohexanone, 4-hydroxybutyric acid, phenol, and 2,2-dimethyldecane. For control vs malignant, the most important metabolites were cyclohexanone, 4-hydroxybutyric acid, phenol, and 2,2-dimethyldecane, as for control vs benign nodule, but also ethylhexanol, ethyleneglycol mono vinyl ester, cyclopropane, and 1-bromo-1-(3-methyl-1-pentenylidene)-2,2,3,3-tetramethyl. The validation of both models showed AUC values of 1.00 with 100% sensitivity and specificity. For benign vs malignant, the most important metabolites were (3-Methyl-oxiran-2-yl)-methanol, cyclopentane, 1,1,3-trimethyl-3-(2-methyl-2-propenyl) and trans-2-Dodecen-1-ol. In that scenario, AUC value was 0.901, with a sensitivity of 92% and a specificity of 82%. The detected metabolites were related to changes in biochemical pathways involved in cancer metabolism, lipid metabolism regulation by thyroid hormone, and benzene and 4-hydroxybutanoic acid metabolism [53]. The results of this study confirm the changes in metabolites highlighted by Yao et al. [36] (Table 1).

Publication	Technique	Important changes	Sample type	Amount (n=)
Delbridge et al.	MRS	Lipids 1.7 ppm and 0.9 ppm amino acids regions ratio	Tissue	98
King et al.	MRS	Choline/Creatine ratio and FA	Tissue	13
Mackinnon et al.	2D <sup>1</sup> H MRS	cholesterol/cholesteryl esters and di-/triglycerides	Tissue	93
Gupta et al.	MRS	Choline	Tissue	8
Jordan et al.	<sup>1</sup> H RMAS	Lipids 1.7 ppm and 0.9 ppm amino acids regions ratio	Tissue	13
Toregrossa et al.	<sup>1</sup> H RMAS	Lactate, taurine, choline, phosphocholine, myo-inositol, scylloinositol and unknown compounds	Tissue	72
Yoshioka et al.	<sup>1</sup> H NMR	Dolichols, cholesterol, acyl chain double bounds, PC+SPH	Tissue	61
Raffelt et al	<sup>31</sup> P NMR	PR+SM and PC	Plasma	70
Deja et al.	<sup>1</sup> H NMR	Alanine, methionine, acetone, glutamate, glycine, lactate, tyrosine, phenylalanine, hypoxanthine, scyllo- and myo-inositol	Tissue	64
S Guo et al	FT-ICR-MS	PC(34:1), PC(36:1), PC(38:6), PA(36:2), PA(36:3), PA(38:3), PA(38:4), PA(38:5), PA(40:5) and SM(34:1)	Tissue/serum	36/289
Ishikawa et al.	IMS, MS/MS	PC (16:0/18:1 and 16:0/18:2) and SPH (d18:0/16:1 )	Tissue	7
Yao et al	LC-MS	FFA and 3-hydroxybutyric acid	Serum	140
L Guo et al.	GC-MS	Ethyl hexanol, phenol, 4-hydroxybutanoic acid	Exhaled breath	96

**Table 1:** Summary of results of metabolomic studies of changes in the thyroid gland [27,28,34-39,41,43,44,47,53].

## Summary

Metabolomics methods have great potential in supporting the diagnosis of thyroid lesions and differentiating between different changes to the thyroid gland. The results summarized here confirmed the possible benefits that this field of science brings to medicine.

Assessment of metabolite profiles could be a cheaper alternative to molecular tests of biopsy tissues. The issue with the latter seems to be the small amount of material collected during FNA and the need to fix the material in an alcoholic solution for cytological evaluation. The material obtained for metabolomic assessment should also be used for genetic testing, requiring an additional biopsy and freezing of the specimen in a short period of time. That limitation may not be a technical problem but is an inconvenience for patients, as they have to undergo two biopsies of the same tumor, which increases the risk of complications.

Efforts to use samples collected non-invasively or in ways that are less problematic and dangerous for the patient have led to metabolomics research as an alternative for basic diagnostics. It seems that the most important goal of this method is to be able to clearly separate between subjects with benign tumors, subjects with malignant tumors and healthy subjects. In addition, the use of metabolomics for analytical methods and the processing and analysis of spectra and chromatograms are also increasing. These factors may make it possible to obtain even better results and generate more accurate models of differentiating between subjects for use in certain diagnostic models.

In addition, the protocols used in metabolomics for sample preparation and measurements should be standardized to facilitate the implementation of the results obtained in studies conducted for diagnostic purposes.

## Acknowledgement

This project was supported by Wroclaw University of Technology S40531/Z0303.

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