



# Molecular markers for the classification of cytologically indeterminate thyroid nodules

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## Abstract

**Background** The diagnosis of indeterminate lesions of the thyroid is a challenge in cytopathology practice. Indeed, up to 30% of cases lack the morphological features needed to provide definitive classification. Molecular tests have been developed to assist in the diagnosis of these indeterminate cases. The first studies dealing with the preoperative molecular evaluation of FNA samples focused on the analysis of *BRAF*<sup>V600E</sup> or on the combined evaluation of two or three genetic alterations. The sensitivity of molecular testing was then improved through the introduction of gene panels, which became available for clinical use in the late 2000s.

Two different categories of molecular tests have been developed, the ‘rule-out’ methods, which aim to reduce the avoidable treatment of benign nodules, and the ‘rule-in’ tests that have the purpose to optimize surgical management. The genetic evaluation of indeterminate thyroid nodules is predicted to improve patient care, particularly if molecular tests are used appropriately and with the awareness of their advantages and weaknesses. The main disadvantage of these tests is the cost, which makes them rarely used in Europe. To overcome this limitation, customized panels have been set up, which are able to detect the most frequent genetic alterations of thyroid cancer.

**Conclusions** In the present review, the most recent available versions of commercial molecular tests and of custom, non-commercial panels are described. Their characteristics and accuracy in the differential diagnosis of indeterminate nodules, namely Bethesda classes III (Atypical follicular lesion of undetermined significance, AUS/FLUS) and IV (Suspicious for follicular neoplasm, FN/SFN) are fully analyzed and discussed.

**Keywords** Indeterminate nodules · AUS/FLUS · FN/SFN · Afirma<sup>®</sup> · Thyroseq<sup>®</sup> · BRAF

## Introduction

Although fine-needle aspiration (FNA) is the gold-standard technique for the preurgical diagnosis of thyroid nodules, around 25% of cases lack the features needed for a

definitive diagnosis and are classified as indeterminate [1]. Most of the indeterminate cases are submitted to surgery, though only the minority of cases (10–40%) will be found to be malignant [2]. In the last decades, with the aim to improve the presurgical diagnosis in indeterminate thyroid nodules, thus reducing the number of unneeded operations, and the consequent expenses and risks, attention has been focused on the preoperative molecular characterization of the nodules. Accordingly, different tests have been developed taking advantage of the major advancements in the knowledge of the genetic bases of thyroid cancer (TC). In this context, the Thyroid Cancer Genome Atlas [3] recently reported the extensive characterization of the most prevalent TC, namely papillary thyroid cancer (PTC), significantly reducing the number of tumors without known genetic driver. Those findings allowed to reclassify PTCs into 2 molecular subtypes, identified as *BRAF*-like and *RAS*-like. Genetic alteration associated to *BRAF*-like

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gene expression profile, such as *BRAF*<sup>V600E</sup> mutation and *RET* fusions are virtually diagnostic of cancer. On the contrary, *RAS*-like mutations, such as *RAS*, *PTEN*, *EIF1AX* mutations and *PPARG* fusions, are associated with either malign or benign follicular neoplasms [4, 5]. Mutations in *TP53* or in *TERT* promoter, in particular when associated with other tumor driver alterations, are frequently found in clinically aggressive thyroid cancer, including poorly differentiated and anaplastic thyroid carcinoma [6]. Differently, copy number alterations (CNA) and mutations in mitochondrial DNA are characteristic of Hürthle cell carcinoma [7].

The first studies dealing with the preoperative molecular evaluation of FNA samples, focused on the analysis of *BRAF*<sup>V600E</sup>, which is the most common PTC mutation [8–46]. However, since many TCs are driven by other mutations, testing for *BRAF*<sup>V600E</sup> alone did not provide sufficiently high negative predictive value (NPV) to avoid surgery for nodules negative for this mutation. In the same years, other Authors proposed the combined evaluation of two or three genetic alterations, such as *BRAF*<sup>V600E</sup> and *RET* fusions [47, 48], or *BRAF*<sup>V600E</sup>, *RET* and *TRK* fusions [49]. The sensitivity of molecular testing was further improved through the introduction of gene panels, which became available for clinical use in the late 2000s. In addition to *BRAF*<sup>V600E</sup>, they tested for several other common genes mutated in TC, and these typically “rule-in” tests panels were able to identify as mutated ~70% of cases. The first panel contributed by Nikiforov et al. in 2011, was a 7-genes molecular test (ThyroSeq<sup>®</sup> v0) composed of a panel of mutations (*BRAF*, *N*-, *H*-, *K-RAS*) and gene fusions (*RET/PTC*, *PAX8/PPARG*). In this seminal study they prospectively analyzed 247 AUS/FLUS and 214 FN/SFN nodules with histological follow-up, reporting a high specificity (97–99%) and a PPV of 88%, but a low sensitivity (57–63%) and a NPV of 86–94%, associated to a cancer prevalence of 14–27% and a residual cancer risk of 6–14% in samples with negative result [50]. The advent of the next-generation sequencing technology promoted the expansion of genotyping panels for thyroid FNA cytology [51] with novel ThyroSeq<sup>®</sup> panels testing for a progressively increasing number of genetic alterations, with a resulting higher sensitivity [52, 53]. In 2012, a “rule-out” test was introduced, namely the Afirma<sup>®</sup> test, which does not rely on detecting gene mutations but is based on the analysis of expression changes in 167 genes. The Afirma<sup>®</sup> test evaluates the gene expression profiles, reports the result as either “benign” or “suspicious”, and has a high NPV [54].

Additional approaches for molecular testing include the analysis of microRNAs (miRNAs) expression. MiRNAs are small noncoding RNAs implicated in gene regulation and several miRNAs have been found dysregulated in thyroid cancer [55–59]. Although different miRNAs have been proposed in different studies, 15 miRNAs could be considered

as the more accurate to discriminate benign from malign lesions with a high sensitivity and specificity [60].

Based on the results obtained by these molecular tests in the preoperative evaluation of thyroid nodules, International and National guidelines [61, 62] recommend the genetic evaluation, whenever possible, for the diagnosis of indeterminate nodules. The main disadvantage of these tests is the high cost [63], which makes them rarely used in Europe. To overcome this limitation, some Authors report data on more limited, customized “rule-in” panels which are able to detect the most frequent genetic alterations of TC, even though with lower sensitivities with respect to the NGS and gene expression profile large panels.

In the present review, the most recent available versions of commercial molecular tests are reported. The accuracy of those test, the pros and cons and their present exploitation in clinical practice are fully analyzed. The reliability of custom panels is described, too. To note, all the data reported refer to indeterminate nodules, namely Bethesda classes III (Atypical follicular lesion of undetermined significance, AUS/FLUS) and IV (Suspicious for follicular neoplasm, FN/SFN) [1], since the most important indication and appropriateness of these tests is for the differential diagnosis of this type of nodules.

## Methods

### Literature search

We performed a PubMed search for studies published between 2009 and 2019 exploring the performance of “rule-in” and “rule-out” panels and including more than four genes and/or miRNAs, exclusively in AUS/FLUS or FN/SFN cytology. Meanwhile, we checked the references of each included paper to identify additional relevant publications.

### Inclusion criteria for studies

1. Indeterminate thyroid results via fine-needle aspiration (FNA) that included Bethesda classes AUS/FLUS or FN/SFN (more than 20 cases).
2. Histopathologic results diagnosis from surgical specimens as gold reference standard for benign or malignant nodules.

### Exclusion criteria for studies

1. Opinions, reviews, commentary, case reports, and insufficient data.
2. Absence of surgical histopathology results.
3. Studies written in languages other than English.
4. Studies on pediatric populations.

5. Studies in which Bethesda III and IV categories cannot be separated from Bethesda classes V.

San Francisco, California), and ThyGenX/ThyraMIR (Interpace Diagnostics, Inc, Parsippany, New Jersey). The RosettaGX Reveal (Rosetta Genomics, Inc, Philadelphia, Pennsylvania) has been recently removed from the market (Table 1).

## Commercial tests

Three tests are commercially available in the United States, based on the analysis of DNA/RNA sequencing data, of mRNA or microRNA expression profiles, or combination of these methods: ThyroSeq<sup>®</sup> v3 (CBLPath, Inc, Rye Brook, New York, and University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania), Afirma<sup>®</sup> (Veracyte, Inc, South

## ThyroSeq v3

The ThyroSeq<sup>®</sup> v3 Genomic Classifier (GC), released for clinical use in 2018, is the enhanced version of the previous ThyroSeq<sup>®</sup> v2 [52]. The main advantages of the new version of this “rule-in” method are the larger number of genes mutation hotspots and gene fusions analyzed, the analysis

**Table 1** Characteristics of the most recent available versions of commercial molecular tests

	ThyroSeq <sup>®</sup> v3	Afirma <sup>®</sup> GSC	ThyGeNEXT/ThyraMIR <sup>®</sup>	RosettaGX Reveal <sup>TM*</sup>
Methodology	NGS	mRNA gene expression	NGS/microRNA expression	microRNA expression
Substrate	1–2 drops from first FNA pass (if adequate cellularity) or 1 dedicated cell pass	2 dedicated FNA passes	1 dedicated FNA pass	Routinely stained direct smears
Mutations/fusions	112 genes (12,135 variants)/> 120 fusions	<i>BRAF</i> mutations/RET-PTC1, RET-PTC3 fusions	10 genes (42 variants)/28 fusions	None
Gene expression	19 genes	1115 genes	None	None
microRNA expression	None	None	10 mRNA	24 mRNA
CNA	10 chromosomal regions	LOH	None	None
Assessment of thyroid follicular cell content	Yes	Yes	Yes	Yes
Marker for parathyroid	Yes	Yes	Yes	No
Marker for MTC	Yes	Yes	Yes	No
Data analysis	Local or centralized	Centralized	Local	Local
Price (\$)	4056 (v2)	6400 (GEC)	1675 (ThyGeNEXT) 4000 (ThyraMIR)	3700
Validation studies	Steward et al. (2019) [65]	Patel et al. (2018) [91]	Labourier et al. (2015) [99]	Lithwick-Yanai et al. (2017) [102]
Bethesda III–IV (n)	154–93	114–76	58–51	150
Prevalence of cancer III–IV (%)	23–35	25–22	32	21
Sensitivity III–IV (%)	91–97	93–88	94–82	74
Specificity III–IV (%)	85–75	71–64	80–91	74
NPV III–IV (%)	97–98	97–95	97–91	92
PPV III–IV (%)	64–68	51–42	68–82	43
Hürthle cell lesions (number)	49	26	na	na
Prevalence of cancer (%)	20	35	na	na
Sensitivity (%)	100	90	na	na
Specificity (%)	67	59		
NPV (%)	100	91		
PPV (%)	43	53		

\*This test is not yet available

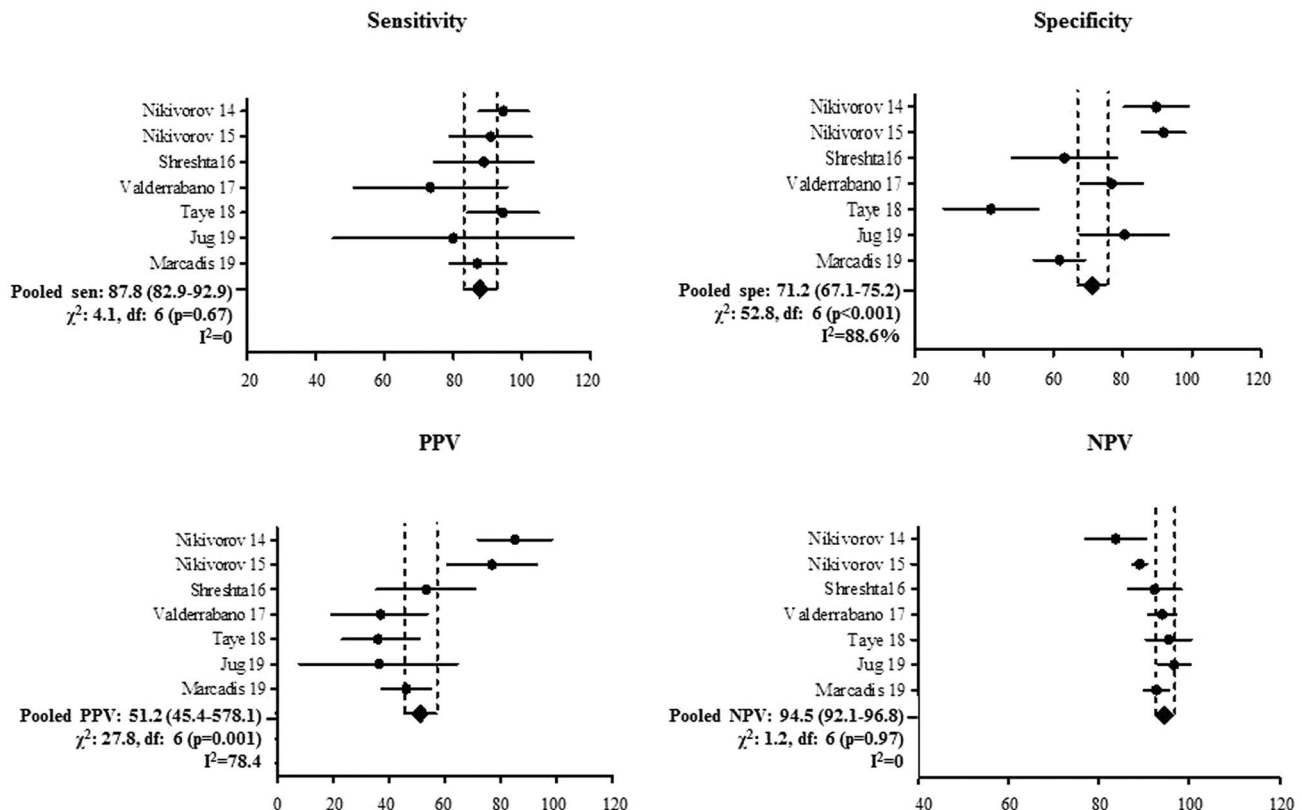
CNA Copy Number Alterations, NGS Next-Generation Sequencing, GSC Genomic Sequencing Classifier, GEC Gene Expression Classifier, LOH Loss Of Heterozygosity; References into brackets; MTC medullary thyroid cancer, NPV negative predictive value, PPV positive predictive value, na not available

of DNA copy number alterations (CNA), and an improved accuracy for the detection of oncocytic (Hürthle cell) tumors [64]. ThyroSeq<sup>®</sup> v3 is based on a targeted next-generation sequencing of DNA and RNA to analyze 112 genes providing information on more than 12,000 hotspot mutations and more than 120 fusions, gene expression alterations in 19 genes, and CNAs in 10 genomic regions. Quality control steps include gene expression analysis for markers to determine adequate thyroid follicular cell content, as well as markers to detect medullary thyroid carcinoma and non-thyroidal tissues (e.g., parathyroid tissue, metastatic carcinoma) (Table 1). The genomic classifier that the test uses is based on a score from 0 to 2 points for each genetic alteration, proportional to its association with cancer. GC scores of 0 or 1 are considered negative for malignancy (with the latter reported as “currently negative” to indicate nodules with low-risk mutations for which active surveillance and repeat FNA could be considered), while GC scores  $\geq 2$  are considered positive results. Among nodules with positive results, ThyroSeq<sup>®</sup> v3 provides further information on preoperative risk stratification based on the type of detected alterations and on their allelic frequency.

The test performance was validated in a multi-institutional, prospective, blinded study [65]. In that study, 257

nodules with indeterminate cytology were analyzed and resected tissue samples were obtained for histopathological diagnosis. ThyroSeq<sup>®</sup> v3 showed 94% sensitivity, 82% specificity, 97% NPV and 66% PPV among 247 Bethesda III/IV cases with a prevalence of malignancy of 28%. The new version of the test demonstrated an improved sensitivity, but lower specificity and PPV compared to the previous version (ThyroSeq<sup>®</sup> v2; 93% and 83%, respectively) [52]. ThyroSeq<sup>®</sup> v3 has been shown to be extremely useful in the identification of Hurthle cell carcinomas (NPV: 100%), while only 43% of adenomas were correctly classified.

Post-validation studies are available only for the ThyroSeq<sup>®</sup> v2 [52, 53, 66–70], and confirmed high NPV (94.5%, 95% CI 92.1–96.8%), but reported lower sensitivity (87.9%, 95% CI 82.9–92.9), specificity (71.2%, 95% CI 67.1–75.2%) and PPV (51.2%, 95% CI 45.4–57.1%) in comparison to the validation studies (Fig. 1 and Supplemental Table 1). Moreover, considering a pre-test probability of 25.6, a positive post-test probability of 54.3%, and a negative post-test probability of 5.5% were reached.



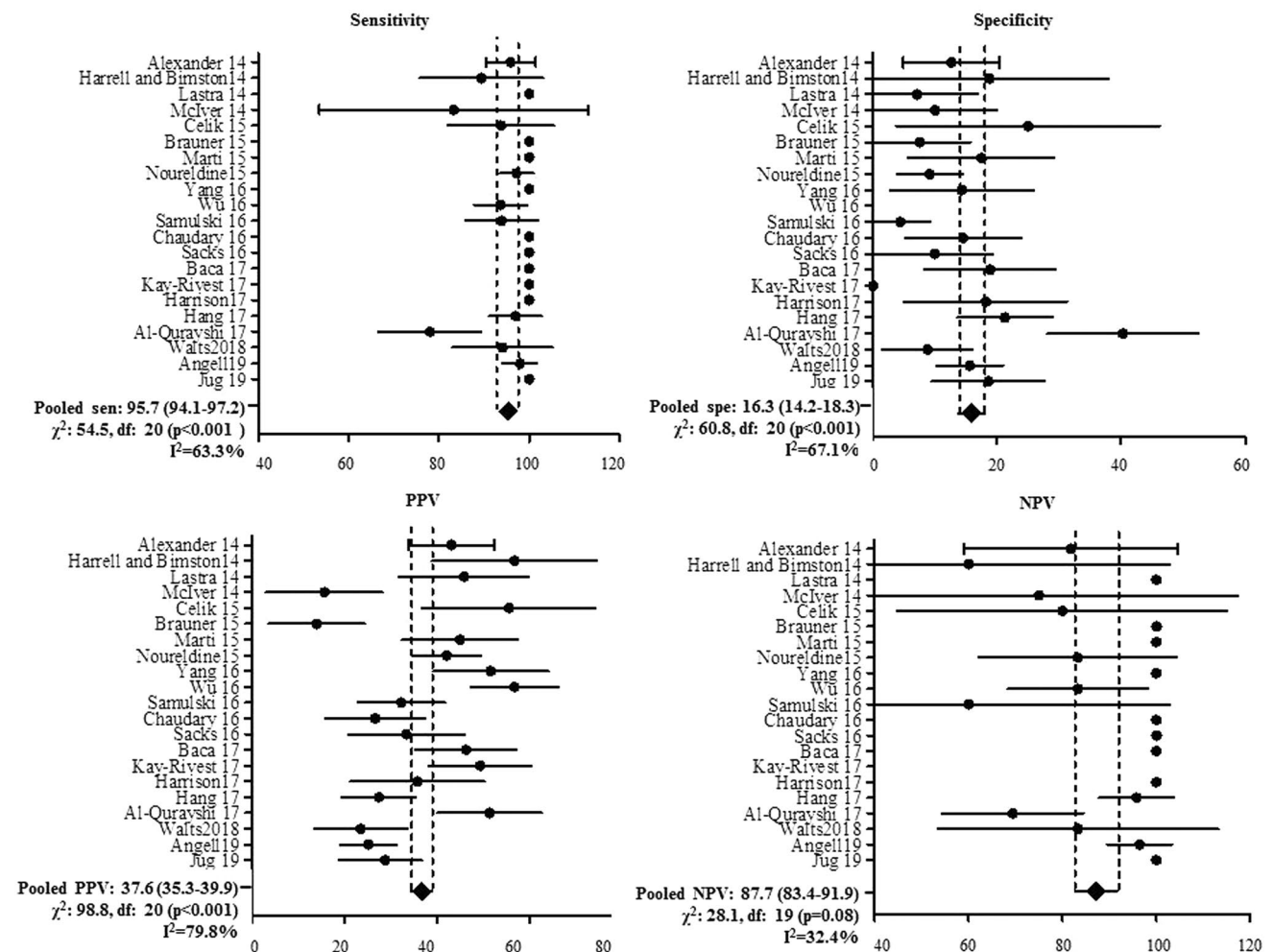
**Fig. 1** Forest plots for sensitivity, specificity, Positive and Negative Predictive Values (PPV, NPV) for ThyroSeq<sup>®</sup> v2. The first Author and the year of publication are indicated

### Afirma® gene expression classifier (GEC) and genomic sequencing classifier (GSC)

The Afirma® Gene Expression Classifier (GEC, Veracyte) is a microarray-based test that uses a proprietary algorithm to predict benign lesions (“rule-out” method). The algorithm involves 2 steps. The first step screens for the expression of 25 genes to identify rare neoplasms such as medullary thyroid carcinoma (MTC). Only not excluded samples proceed to the second step, which evaluates the expression profile of further 142 genes to classify indeterminate thyroid nodules into either benign (GEC-B) or suspicious (GEC-S) categories. The test was validated in a multicenter, prospective, blinded study [54] involving 210 nodules of the two indeterminate categories Bethesda III, IV, with a pre-test malignancy rate of 24 and 25%, respectively. Authors showed high sensitivity (87%), but modest specificity (53%); the NPV and PPV were 95 and 94% and 38 and 37% in the two indeterminate categories, respectively. Differently, in

one post-validation study a high frequency of false negative results was recorded [71]. It is worth noting that the interpretation of the above mentioned results requires caution because of the small fraction of GEC-B nodules addressed to surgery in the clinical practice. Moreover, benign Hürthle cell nodules, which represents a large proportion of Bethesda III/IV categories, are frequently falsely classified as GEC-S [72–75]. Meta-analysis of all the available studies using Afirma® and with available histological diagnosis [66, 71–90], showed a pooled sensitivity (95.7%, 95% CI 94.1–97.2%), specificity (16.4%, 95% CI 14.2–18.3%), PPV (37.6%, 95% CI 35.3–39.9%) and NPV (87.7%, 95% CI 83.4–91.9%) of the test (Fig. 2 and Supplemental Table 2). Considering a pre-test probability of 34.5, a positive post-test probability of 37.6%, and a negative post-test probability of 12.3% were reached.

To overcome the modest specificity and PPV of GEC, the Afirma BRAF test was introduced, which assays the expression profile together with *BRAF*<sup>V600E</sup> mutation [34]. However,



**Fig. 2** Forest plots for sensitivity, specificity, Positive and Negative Predictive Values (PPV, NPV) for Afirma® Gene Expression Classifier (GEC). The first Author and the year of publication are indicated

the investigation of *BRAF* mutation did not increase the PPV, mostly due to the low prevalence of classical variants of PTC in Bethesda III and IV nodules. Recently, the next-generation Afirma<sup>®</sup> Genomic Sequencing Classifier (GSC) has been developed to analyze the expression profile of 1115 genes, with RNA-Seq methodology, and including the possibility to detect single nucleotide variants, fusions, and copy number variations in the coding region of the genome [91]. The GSC includes several quality control steps, such as the screening for the expression profile of parathyroid cells and the assessment of follicular cell content. The GSC can detect mitochondrial transcripts, and CNAs for the analysis of Hürthle cell lesions (Hürthle classifier), too. The GSC was validated on the same cohort used for the first generation Afirma<sup>®</sup> GEC, showing increased specificity (from 53 to 68%) and PPV (from 38 to 47%) while maintaining high sensitivity and NPV (Table 1). Furthermore, the GSC showed a highest specificity and PPV in Hürthle cell adenomas compared to GEC. Independent reports comparing the performance of GSC with that of GEC confirmed these results [76, 92–94]. A broader test panel (Xpression Atlas) was developed to detect additional alterations, involved in thyroid neoplasms (761 variants in 346 genes and 130 fusions) [95]. Of note, in both GSC and Xpression Atlas, mutations in the not transcribed portion of the genome, such as in the *TERT* promoter, are not included. Xpression Atlas was intended for Bethesda III/IV nodules with a GSC suspicious (GSC-S) result. However, the impact of the addition of novel variants on improving the risk stratification of thyroid nodules remains to be established.

The Afirma<sup>®</sup> GEC was developed to reduce the morbidity and the cost of repeated FNAC and/or of unnecessary thyroid surgery, but contrasting results have been obtained in different settings regarding its actual impact. Indeed, it has been reported that after the availability of this test the number of indeterminate cytologies has increased without a significant reduction of surgical procedures [66, 75, 77, 78, 96, 97], and the cost-effectiveness of the test in the clinical practice has been questioned [8]. On the other hand, in hypothetical modeling, molecular test resulted considerably more cost-effective than diagnostic lobectomy, being ThyroSeq<sup>®</sup> v3 more cost-effective than GSC [98].

### ThyGeNEXT/ThyraMIR<sup>®</sup>

ThyGeNEXT<sup>®</sup> is a targeted next-generation sequencing test developed by Interpace Diagnostics that evaluates mutations in 10 genes (*BRAF*, *H*-, *K*-, and *N-RAS*, *TERT*, *ALK*, *GNAS*, *RET*, *PTEN*, and *PIK3CA*) and 38 different gene fusions (involving *ALK*, *BRAF*, *NTRK*-1, -2, and -3, *PPARG*, *RET*, and *THADA*).

To increase the sensitivity and NPV of the genotyping panel, Interpace Diagnostic pairs this test with a

complementary miRNA expression classifier called ThyraMIR<sup>®</sup>. Samples for which no mutations or gene fusions are detected by the targeted sequencing test, undergo further risk stratification with ThyraMIR<sup>®</sup> which is based on the expression pattern of 10 miRNAs (miR-29b-1-5p, miR-31-5p, miR-138-1-3p, miR-139-5p, miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375, miR-551b-3p).

The miRNA classifiers were developed using miRNA expression data determined by RT-qPCR on a case–control training set consisted of 240 surgical specimens [99].

The test includes expression analysis for transcripts to confirm the thyroid follicular cell content and detect sampling of parathyroid tissue and markers associated with medullary thyroid carcinoma (miR-375 and *RET* mutations) (Table 1).

The combined test was clinically validated using and earlier version of the NGS-based test called ThyGenX<sup>®</sup>, which analyzes 7 genes (*BRAF*, *H*-, *K*-, and *N-RAS* genes) and 3 gene fusions (*PAX8-PPARG*, *RET-PTC1*, and *RET-PTC3*), together with ThyraMIR<sup>®</sup>. Among 109 Bethesda III/IV cases with a 32% prevalence of cancer, ThyGenX/ThyraMIR<sup>®</sup> together demonstrated 89% sensitivity, 85% specificity, 94% NPV, 74% PPV, and a 61% benign call rate.

Baniz et al. 2019 [100] reported the establishment of an additional level to the two-level miRNA classifier described by Labourier et al. [99]. The Authors showed that this miRNA sub-classification offers the opportunity to support non-surgical management in patients with weak or no driver mutations for low levels microRNA status while supporting the need diagnostic lobectomy for high microRNA status.

Additional post validation studies are certainly needed to better determine the accuracy of ThyGeNEXT/ThyraMIR<sup>®</sup>.

### Rosetta GX reveal<sup>™</sup>

The Rosetta GX Reveal<sup>™</sup> Thyroid Classifier (Rosetta Genomics Philadelphia, PA) was a validated test to measure the expression pattern of 24 miRNAs, found to be up- or down-regulated in PTC, directly on RNA extracted from stained FNA smears prepared for initial cytological evaluation [101]. The advantage of the methodology was that it obviated the need to perform an additional collection of material for molecular testing after the fine needle aspiration, since miRNAs were analyzed from the same sample used for cytological examination. The test is no longer commercially available. The test used algorithms to classify indeterminate thyroid nodules into benign, suspicious for malignancy or positive for medullary carcinoma. Markers associated with thyroid epithelial cells were also included (Table 1).

The test was developed using a training set of 375 FNAB smears and was validated using a blinded multicenter retrospective cohort of 189 cytologically

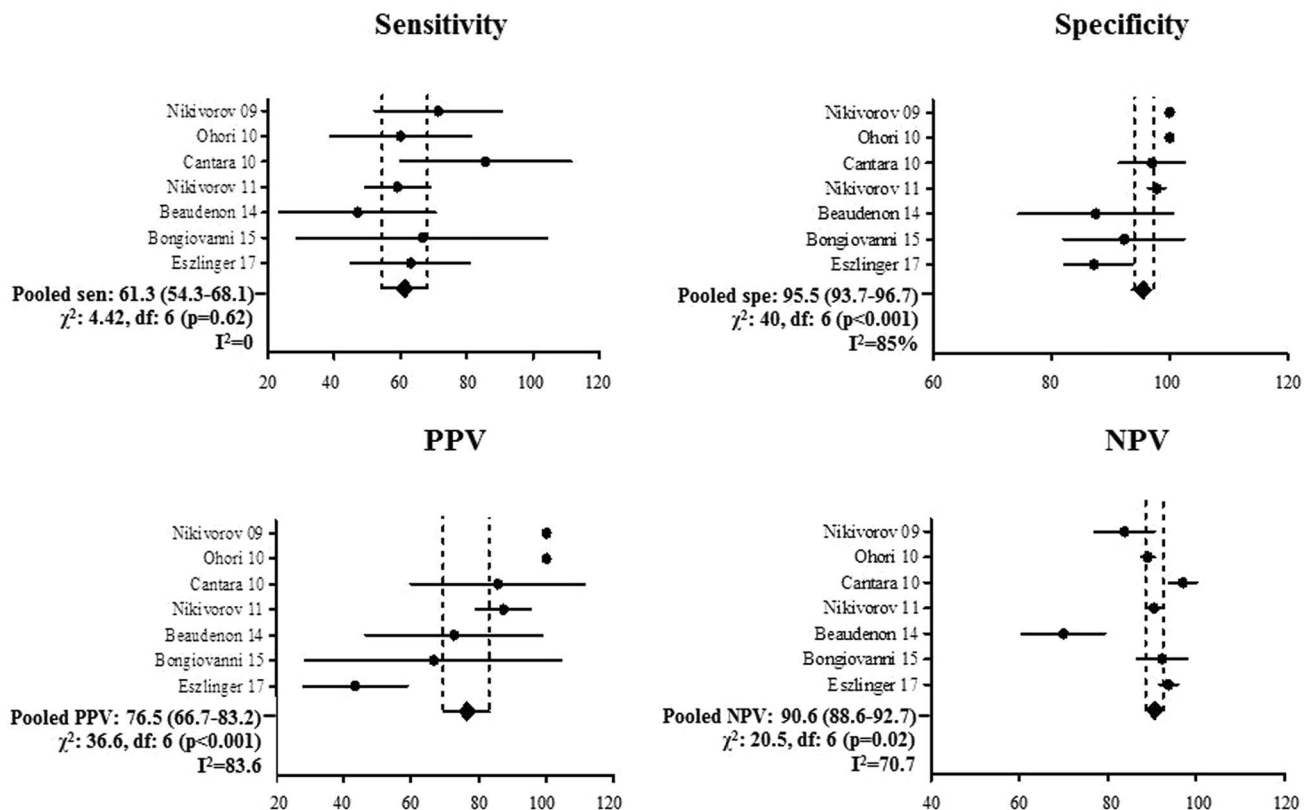
indeterminate cases, including 150 Bethesda III–IV cases, with their corresponding surgical specimens [102]. Considering classes III and IV, this validation study revealed 74% sensitivity and specificity, 43% PPV and 92% NPV, with a malignancy rate of 21%. Of note, since no Hürthle carcinomas were included the validation study, the performance of Rosetta GX Reveal™ in detecting these tumors was not determined.

Walts et al. 2018 retrospectively compared the performance of the Afirma® GEC with that of Rosetta GX Reveal™ in a cohort of 80 Bethesda III–IV thyroid FNAs with surgical follow-up and a rate of malignancy of 20–23% [79]. Rosetta GX Reveal™ demonstrated a higher specificity compared to GEC (60.3% vs 9.5%) but a lower sensitivity (78% vs 94%). Interestingly, Rosetta GX Reveal™ outperformed GEC in the cohort of NIFTP and of Hürthle lesions. A retrospective study was performed in 2018 on a small cohort of 9 Bethesda III–IV thyroid FNAs with a prevalence of cancer of 30%, comparing the Rosetta GX Reveal™ and the ThyGenX/ThyraMIR® combination tests [103]. The 2 tests had similar sensitivities and NPV (85 vs 89%, and 100% for both), while Rosetta GX™ showed a higher specificity (86 vs 71%) and higher PPV (75 vs 60%).

## Non-commercial tests

Although the clinical relevance of the above described commercial tests has been widely recognized, their high cost has prevented their extensive diffusion, particularly in European Countries. As a consequence, “home-made”, customized molecular tests have been developed, many of them never reported in the literature, mainly testing by PCR and direct sequencing *BRAF*<sup>V600E</sup>, *RAS* point mutations and *RET*, *TRK* and *PPARG* fusions (Fig. 3 and Supplemental Table 3).

The first non-commercial panels reported in the literature were based on the analysis of the 7 most frequent genetic alterations in DTC, such as the first Nikiforov’s panel (*BRAF*<sup>V600E</sup> and *BRAF*<sup>K601E</sup>, *RAS* mutations at codons 12, 13, and 61, *PAX8/PPARG*, *RET/PTC* and *TRK* fusions). This panel was tested on 2 series obtaining sensitivities of 60–100%, specificities and PPV of 100%, NPVs of 92–100 in Bethesda III category, with a prevalence of malignancy of 14–17% and sensitivities of 77%, specificities and PPV of 100%, NPVs of 79% in Bethesda IV category, with a prevalence of malignancy of 52% [104, 105]. In the same year, Cantara and co-Authors screened



**Fig. 3** Forest plots for sensitivity, specificity, Positive and Negative Predictive Values (PPV, NPV) for non-commercial 5- and 7-genes panels. The first Author and the year of publication are indicated

the same molecular alterations in 41 indeterminate lesions with a sensitivity and a PPV of 86%, a specificity and NPV of 97% and a risk of malignancy of 17% [106], whereas Beaudenon-Huibregtse et al. found both a lower sensitivity (36/67%) and a NPV (56/86%) in a series of 41 indeterminate cases analyzed by means of the same 7-genes panel, with a risk of malignancy of 50 and 32% in the III and IV categories, respectively [107].

In 2017, there were reported the results obtained in a large German cohort of 254 indeterminate cases analyzed for *BRAF* and *RAS* mutations and *PAX8/PPARG* and *RET/PTC* rearrangements, by pyrosequencing and quantitative PCR, respectively, on air-dried FNA smears [108, 109]. In the AUS/FLUS category they found sensitivity and NPP (58% and 90%, respectively), comparable to those reported by Nikiforov, but a lower specificity (82%) and PPV (41%), with a risk of malignancy of 15%. In the FN/SFN category, the specificity (91%) was similar to that previously reported [104, 107], but the sensitivity was lower (27%), with a risk of malignancy of 17%. The detection of *RAS/PAX8/PPARG* genetic alterations in histologically benign nodules could have affected the specificity in all indeterminate categories, while the low sensitivity in the FN/SFN category was probably due to a very low mutation prevalence in follicular thyroid cancers and in follicular variant PTCs.

Bongiovanni et al. [110], after sampling by laser capture microdissection, applied the 7-gene panel prospectively and retrospectively on 23 FN/SFN, with a malignancy rate of 57%, showing sensitivity and PPV of 67% and specificity and NPV of 92%.

Censi et al. [111] analyzed H-,K-, and N-*RAS*, *TERT* promoter and *BRAF* gene mutations (5-gene panel) in a series of 199 consecutive indeterminate nodules with a sensitivity, specificity, PPV, NPV and risk of malignancy of 50, 78, 37, 84%, and 22% in the AUS/FLUS category, and of 39, 85, 79, 50%, and 58% in the FN/SFN category, respectively. The frequent detection of *RAS* mutation in benign samples, the lack of rearrangement analysis and the introduction of the new NIFTP histopathologic nomenclature may have played a part in the low PPV obtained in this study.

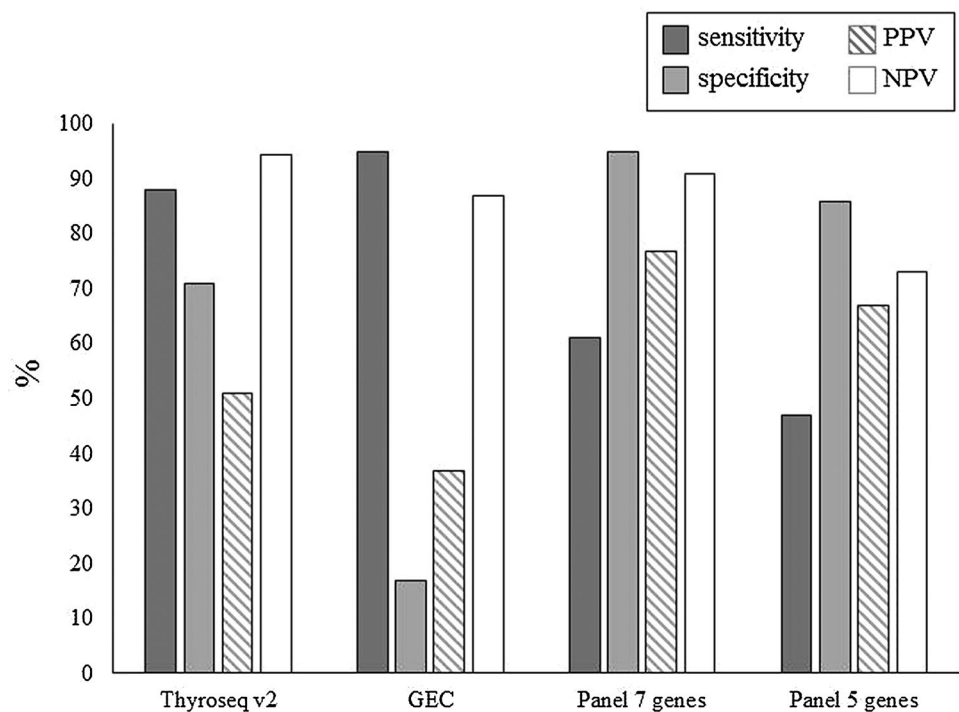
The same 5-gene panel was more recently interrogated on 54 indeterminate nodules showing lower sensitivity (44%) and NPV (67%), but higher specificity and PPV (93 and 85%) [112].

Overall, the pooled sensitivity, specificity, PPV and NPV of the 7-genes molecular test on Bethesda III/IV nodules was 61.3% (95% CI 54.3–68.2%), 95.2% (95% CI 93.7–96.7%), 76.5% (95% CI 69.7–83.2%) and 90.6% (95% CI 88.6–92.7), respectively. Considering a pre-test probability of 20.3, a positive post-test probability of 76.5%, and a negative post-test probability of 9.4% were reached.

The pooled sensitivity of the 5-gene panel was 46.8%, (95% CI 36.7–56.9%), specificity 86.3% (95% CI 81–91.6%), PPV 66.7% (95% CI 55.3–78%) and NPV 73.5% (95% CI 67.3–79.8). Considering a pre-test probability of 36.9, a positive post-test probability of 66.7%, and a negative post-test probability of 26.4% were reached.

As expected, the 5 and 7 gene non-commercial panels are less sensitive, but more specific of the commercial Afirma® and Thyroseq® tests (Fig. 4).

**Fig. 4** The pooled sensitivities, specificities, Positive and Negative Predictive Values (PPV, NPV) for commercial and non-commercial tests





Several non-commercial panels for indeterminate cytologies have been also developed based on the analysis of different miRNAs, being miR-146 the only one tested in all series (Supplemental Table 3) [50, 80, 104–116].

Shen et al. [113] identified and validated a set of four miRNAs (miR-146b, -221, -187 and -30d) in 30 AUS samples, obtaining a sensitivity of 63.6%, specificity of 78.9%, PPV of 64%, and NPV of 79%, with a prevalence of malignancy of 37%.

Santos et al. [114] developed a new molecular classifier test (mir-THYpe) that analyzes the expression profiles of 11 miRNAs (let-7a, miR-103, miR-125a-5p, let-7b, miR-145, RNU48, miR-146b, miR-152, miR-155, miR-200b, and miR-181b) obtained from the same FNA cytology smear slides used to classify the thyroid nodule as indeterminate. In the validation set, the mir-THYpe test reached 100–83% sensitivity, 82–79% specificity, 25–38% PPV, 100–97% NPP, 5–13% cancer prevalence in Bethesda III and IV nodules, respectively. Mazeh et al. analyzed the expression of 6 miRNAs (miR-21, -31, -146b, -187, -221 and -222) in 11 indeterminate FNA samples, and found a sensitivity of 89%, specificity of 100%, PPV of 100% NPV of 66 [115], and a prevalence of malignancy of 63%.

Aside from these panels which analyzed the expression of miRNAs in FNA cytologies, some Authors investigated the use of circulating miRNA, which would represent a simpler and less invasive procedure [117–120]. In particular, Pilli et al. [120] analyzed the expression of two miRNA (mi-95, -190) in the serum of 72 Bethesda III and IV FNAC with an available histological diagnosis, reaching a sensitivity of 71.9%, a specificity of 85%, PPV 79.3% and NNP 79.1%, with a prevalence of malignancy of 44%. Despite these promising results, the analysis of miRNAs in the serum poses some concerns, such as the low level of miRNAs and technical problems associated with the analysis of such samples.

## Molecular testing of NIFTP

Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) is an encapsulated or clearly delimited, noninvasive neoplasm with a follicular growth pattern and nuclear features of PTC. This entity has been established in 2016 after the revision of the outcome of 108 patients with noninvasive follicular variant PTC not treated by radioactive iodine by a working group of thyroid experts [121]. After a follow-up of at least 10 years there was no recurrence recorded, and this peculiar entity was then re-classified as non-malignant. This reclassification aims to avoid overtreatment of patients with an indolent lesion. NIFTPs are associated with “RAS-like” mutations (*RAS*, *BRAF K601E* mutations, *PAX8/PPARG*, *THADA* fusions) [122], and share

gene expression profile with encapsulated follicular-variant PTC, minimally invasive follicular carcinoma and follicular adenoma [80]. Since all the commercial tests described here were developed prior to the nomenclature change, NIFTPs were classified as malignant in the validation sets. Accordingly, in both the validations studies and in the “real-world” clinical settings 95% and 80% of NIFTP were classified as suspicious/malignant by GEC or ThyroSeq® v2, respectively (Supplemental Tables 1 and 2). The reclassification of NIFTP as a benign neoplasm would likely affect the predictive value of these tests.

## Conclusions

The diagnosis of indeterminate lesions of the thyroid is a challenge in cytopathology practice. Indeed, up to 30% of cases lack the morphological features needed to provide definitive classification. The molecular characterization of thyroid nodules has become more easy and exhaustive since the advent, in the last 10 years, of NGS and Gene Expression technologies which have provided better stratification of patients. Two different categories of molecular tests have been developed, the ‘rule-out’ methods, which aim reduce the avoidable treatment of benign nodules, and the ‘rule-in’ tests that have the purpose to optimize surgical management (total thyroidectomy or lobectomy). Although each test has different advantages and limitations in the evaluation of indeterminate FNA samples, they are progressively increasing their performance levels and are predicted to become an integral part of the thyroid nodule evaluation, especially if their cost will be reduced. Finally, it should be highlighted that the genetic characterization of a thyroid nodule has a positive impact not only in the initial treatment but potentially in the follow-up of patients, too. Indeed, some molecular markers, including the most studied *BRAF* and *TERT* promoter mutations, have been shown to harbor a prognostic value and their evaluation is predicted to be of help in the stratification of patients into distinct risk groups and in a better assessment of their outcome.

Moreover, in the era of targeted therapies, knowing the molecular signature of the tumor is crucial for the selection of the most appropriate antineoplastic compound.

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## Compliance with ethical standards

**Conflict of interest** on behalf of all authors, the corresponding author states that there is no conflict of interest.

## References

- Cibas ES, Ali SZ (2017) The 2017 Bethesda system for reporting thyroid cytopathology. *Thyroid* 27:1341–1346
- Ho AS, Sarti EE, Jain KS, Wang H, Nixon JJ, Shaha AR et al (2014) Malignancy rate in thyroid nodules classified as Bethesda category III (AUS/FLUS). *Thyroid* 24:832–839
- Cancer Genome Atlas Research Network (2014) Integrated genomic characterization of papillary thyroid carcinoma. *Cell* 159:676–690
- McFadden DG, Dias-Santagata D, Sadow PM, Lynch KD, Lubitz C, Donovan SE et al (2014) Identification of oncogenic mutations and gene fusions in the follicular variant of papillary thyroid carcinoma. *J Clin Endocrinol Metab* 99:E2457–E2462
- Kim TH, Lee M, Kwon AY, Choe JH, Kim JH, Kim JS et al (2018) Molecular genotyping of the non-invasive encapsulated follicular variant of papillary thyroid carcinoma. *Histopathology* 72:648–661
- Xu B, Ghossein R (2016) Genomic landscape of poorly differentiated and anaplastic thyroid carcinoma. *Endocr Pathol* 27:205–212
- Ganly I, Makarov V, Deraje S, Dong Y, Reznik E, Seshan V et al (2018) Integrated genomic analysis of Hürthle cell cancer reveals oncogenic drivers, recurrent mitochondrial mutations, and unique chromosomal landscapes. *Cancer Cell* 34:256–270.e5
- Seo JY, Choi JR, Moon HJ, Kim EK, Han KH, Kim H et al (2015) Clinical Implication of highly sensitive detection of the BRAFV600E mutation in fine-needle aspirations according to the thyroid Bethesda system in patients with conventional papillary thyroid carcinoma. *Ann Otol Rhinol Laryngol* 124:392–399
- Marchetti I, Lessi F, Mazzanti CM, Bertacca G, Elisei R, Coscio GD et al (2009) A morphomolecular diagnosis of papillary thyroid carcinoma: BRAF V600E detection as an important tool in preoperative evaluation of fine-needle aspirates. *Thyroid* 19:837–842
- Patel A, Klubo-Gwiezdzinska J, Hoperia V, Larin A, Jensen K, Bauer A et al (2011) BRAF(V600E) mutation analysis from May-Grunwald Giemsa-stained cytological samples as an adjunct in identification of high-risk papillary thyroid carcinoma. *Endocr Pathol* 22:195–199
- Kang G, Cho EY, Shin JH, Chung JH, Kim JW, Oh YL (2012) Role of BRAFV600E mutation analysis and second cytologic review of fine-needle aspiration for evaluating thyroid nodule. *Cancer Cytopathol* 120:44–51
- Brahma B, Yulian ED, Ramli M, Setianingsih I, Gautama W, Brahma P et al (2013) Surgical perspective of T1799A BRAF mutation diagnostic value in papillary thyroid carcinoma. *Asian Pac J Cancer Prev* 14:31–37
- Panebianco F, Mazzanti C, Tomei S, Aretini P, Franceschi S, Lessi F et al (2015) The combination of four molecular markers improves thyroid cancer cytologic diagnosis and patient management. *BMC Cancer* 15:918
- Shi Q, Ibrahim A, Herbert K, Carvin M, Randolph M, Post KM et al (2015) Detection of BRAF mutations on direct smears of thyroid fine-needle aspirates through cell transfer technique. *Am J Clin Pathol* 143:500–504
- Rho M, Kim EK, Moon HJ, Yoon JH, Park VY, Han K et al (2017) Clinical parameter for deciding the BRAFV600E mutation test in atypia of undetermined significance/follicular lesion of undetermined significance thyroid nodules: US features according to TIRADS. *Ultrasound Q* 33:284–288
- Rossi M, Lupo S, Rossi R, Franceschetti P, Trasforini G, Bruni S et al (2017) Proposal for a novel management of indeterminate thyroid nodules on the basis of cytopathological subclasses. *Endocrine* 57:98–107
- Seo JW, Jang AL, Suh SH, Park HS, Kang MK, Hong JC (2017) Atypia of undetermined significance on thyroid fine needle aspiration—risk factors for malignancy. *Clin Otolaryngol* 42:234–238
- Kim DS, Kim DW, Heo YJ, Baek JW, Lee YJ, Choo HJ et al (2018) Utility of including BRAF mutation analysis with ultrasonographic and cytological diagnoses in ultrasonography-guided fine-needle aspiration of thyroid nodules. *PLoS One* 13:e0202687
- Rossi ED, Martini M, Capodimonti S, Cenci T, Bilotta M, Pierconti F et al (2018) Morphology combined with ancillary techniques: an algorithm approach for thyroid nodules. *Cytopathology* 29:418–427
- Girlando S, Cuorvo LV, Bonzanini M, Morelli L, Amadori P, Dalla Palma P et al (2010) High prevalence of B-RAF mutation in papillary carcinoma of the thyroid in north-east Italy. *Int J Surg Pathol* 18:173–176
- Nam SY, Han BK, Ko EY, Kang SS, Hahn SY, Hwang JY et al (2010) BRAF V600E mutation analysis of thyroid nodules needle aspirates in relation to their ultrasonographic classification: a potential guide for selection of samples for molecular analysis. *Thyroid* 20:273–279
- Paskas S, Jankovic J, Zivaljevic V, Tatic S, Bozic V, Nikolic A et al (2015) Malignant risk stratification of thyroid FNA specimens with indeterminate cytology based on molecular testing. *Cancer Cytopathol* 123:471–479
- Gay S, Schiaffino S, Santamarena G, Massa B, Ansaldo G, Turtulici G et al (2018) Role of strain elastography and shear-wave elastography in a multiparametric clinical approach to indeterminate cytology thyroid nodules. *Med Sci Monit* 24:6273–6279
- Di Benedetto G, Fabozzi A, Rinaldi C (2013) Clinical management of thyroid nodules with indeterminate cytology: our institutional experience using SIAPEC cytological criteria and V600-BRAF test. *Pathologica* 105:1–4
- Monti E, Bovero M, Mortara L, Pera G, Zupo S, Gugiatti E et al (2015) BRAF Mutations in an Italian regional population: implications for the therapy of thyroid cancer. *Int J Endocrinol* 2015:138734
- Jo YS, Huang S, Kim YJ, Lee IS, Kim SS, Kim JR et al (2009) Diagnostic value of pyrosequencing for the BRAF V600E mutation in ultrasound-guided fine-needle aspiration biopsy samples of thyroid incidentalomas. *Clin Endocrinol (Oxf)* 70:139–144
- Adeniran AJ, Hui P, Chhieng DC, Prasad ML, Schofield K, Theoharis C (2011) BRAF mutation testing of thyroid fine-needle aspiration specimens enhances the predictability of malignancy in thyroid follicular lesions of undetermined significance. *Acta Cytol* 55:570–575
- Kim SK, Hwang TS, Yoo YB, Han HS, Kim DL, Song KH et al (2011) Surgical results of thyroid nodules according to a management guideline based on the BRAF(V600E) mutation status. *J Clin Endocrinol Metab* 96:658–664
- Pelizzo MR, Boschin IM, Barollo S, Pennelli G, Toniato A, Zambonin L et al (2011) BRAF analysis by fine needle aspiration biopsy of thyroid nodules improves preoperative identification of papillary thyroid carcinoma and represents a prognostic factor. A mono-institutional experience. *Clin Chem Lab Med* 49:325–329
- Yeo MK, Liang ZL, Oh T, Moon Y, An S, Kim MK et al (2011) Pyrosequencing cut-off value identifying BRAFV600E mutation in fine needle aspiration samples of thyroid nodules. *Clin Endocrinol (Oxf)* 75:555–560
- Canadas-Garre M, Becerra-Massare P, Lopez de la Torre-Casares M, Villar-del Moral J, Cespedes-Mas S, Vilchez-Joya R et al (2012) Reduction of false negative papillary thyroid carcinomas by the routine analysis of BRAF(T1799A) mutation on

- fine-needle aspiration biopsy specimens: a prospective study of 814 thyroid FNAB patients. *Ann Surg* 255:986–992
32. Lee ST, Kim SW, Ki CS, Jang JH, Shin JH, Oh YL et al (2012) Clinical implication of highly sensitive detection of the BRAF V600E mutation in fine needle aspirations of thyroid nodules: a comparative analysis of three molecular assays in 4585 consecutive cases in a BRAF V600E mutation-prevalent area. *J Clin Endocrinol Metab* 97:2299–2306
  33. Jeong SH, Hong HS, Lee EH, Cha JG, Park JS, Kwak JJ (2013) Outcome of thyroid nodules characterized as atypia of undetermined significance or follicular lesion of undetermined significance and correlation with Ultrasound features and BRAF(V600E) mutation analysis. *AJR Am J Roentgenol* 201:W854–W860
  34. Kloos RT, Reynolds JD, Walsh PS, Wilde JI, Tom EY, Pagan M et al (2013) Does addition of BRAF V600E mutation testing modify sensitivity or specificity of the Afirma gene expression classifier in cytologically indeterminate thyroid nodules? *J Clin Endocrinol Metab* 98:E761–E768
  35. Koh J, Choi JR, Han KH, Kim EK, Yoon JH, Moon HJ et al (2013) Proper indication of BRAF(V600E) mutation testing in fine-needle aspirates of thyroid nodules. *PLoS One* 8:e64505
  36. Agretti P, Nicolai F, Rago T, De Marco G, Molinaro A, Scutari M et al (2014) BRAF mutation analysis in thyroid nodules with indeterminate cytology: our experience on surgical management of patients with thyroid nodules from an area of borderline iodine deficiency. *J Endocrinol Invest* 37:1009–1014
  37. Danilovic DL, Lima EU, Domingues RB, Brandao LG, Hoff AO, Marui S (2014) Pre-operative role of BRAF in the guidance of the surgical approach and prognosis of differentiated thyroid carcinoma. *Eur J Endocrinol* 170:619–625
  38. Johnson SJ, Hardy SA, Roberts C, Bourn D, Mallick U, Perros P (2014) Pilot of BRAF mutation analysis in indeterminate, suspicious and malignant thyroid FNA cytology. *Cytopathology* 25:146–154
  39. Park HJ, Moon JH, Yom CK, Kim KH, Choi JY, Choi SI et al (2014) Thyroid “atypia of undetermined significance” with nuclear atypia has high rates of malignancy and BRAF mutation. *Cancer Cytopathol* 122:512–520
  40. Seo JY, Kim EK, Baek JH, Shin JH, Han KH, Kwak JY (2014) Can ultrasound be as a surrogate marker for diagnosing a papillary thyroid cancer? Comparison with BRAF mutation analysis. *Yonsei Med J* 55:871–878
  41. Seo JY, Kim EK, Kwak JY (2014) Additional BRAF mutation analysis may have additional diagnostic value in thyroid nodules with “suspicious for malignant” cytology alone even when the nodules do not show suspicious US features. *Endocrine* 47:283–289
  42. Marino M, Monzani ML, Brigante G, Cioni K, Madeo B, Santi D et al (2015) High resolution melting is a sensitive, cost-effective, timesaving technique for BRAF V600E detection in thyroid FNAB washing liquid: a prospective cohort study. *Eur Thyroid J* 4:73–81
  43. Park KS, Oh YL, Ki CS, Kim JW (2015) Evaluation of the real-Q BRAF V600E detection assay in fine-needle aspiration samples of thyroid nodules. *J Mol Diagn* 17:431–437
  44. Kim TH, Jeong DJ, Hahn SY, Shin JH, Oh YL, Ki CS et al (2016) Triage of patients with AUS/FLUS on thyroid cytopathology: effectiveness of the multimodal diagnostic techniques. *Cancer Med* 5:769–777
  45. Kowalska A, Kowalik A, Palyga I, Walczyk A, Gasiór-Perczak D, Kopczyński J et al (2016) The usefulness of determining the presence of BRAF V600E mutation in fine-needle aspiration cytology in indeterminate cytological results. *Endokrynol Pol* 67:41–47
  46. Beisa A, Kvietkauskas M, Beisa V, Stoskus M, Ostaneviciute E, Jasiunas E et al (2017) The utility of the Bethesda category and its association with BRAF mutation in the prediction of papillary thyroid cancer stage. *Langenbecks Arch Surg* 402:227–234
  47. Salvatore G, Giannini R, Faviana P, Caleo A, Migliaccio I, Fagin JA et al (2004) Analysis of BRAF point mutation and RET/PTC rearrangement refines the fine-needle aspiration diagnosis of papillary thyroid carcinoma. *J Clin Endocrinol Metab* 89:5175–5180
  48. Domingues R, Mendonça E, Sobrinho L, Bugalho MJ (2005) Searching for RET/PTC rearrangements and BRAF V599E mutation in thyroid aspirates might contribute to establish a preoperative diagnosis of papillary thyroid carcinoma. *Cytopathology* 16:27–31
  49. Sapio MR, Posca D, Raggioli A, Guerra A, Marotta V, Deandrea M et al (2007) Detection of RET/PTC, TRK and BRAF mutations in preoperative diagnosis of thyroid nodules with indeterminate cytological findings. *Clin Endocrinol (Oxf)* 66:678–683
  50. Nikiforov YE, Ohori NP, Hodak SP, Carty SE, LeBeau SO, Ferris RL et al (2011) Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. *J Clin Endocrinol Metab* 96:3390–3397
  51. Nikiforova MN, Wald AI, Roy S, Durso MB, Nikiforov YE (2013) Targeted next-generation sequencing panel (ThyroSeq) for detection of mutations in thyroid cancer. *J Clin Endocrinol Metab* 98:E1852–E1860
  52. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL et al (2014) Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by Thyroseq v2 next generation sequencing assay. *Cancer* 120:3627–3634
  53. Nikiforov YE, Carty SE, Chiosea SI, Coyne C, Duvvuri U, Ferris RL, Gooding WE, LeBeau S, Ohori NP, Seethala RR, Tublin ME, Yip L, Nikiforova MN (2015) Impact of the multi-gene ThyroSeq next-generation sequencing assay on cancer diagnosis in thyroid nodules with atypia of undetermined significance/follicular lesion of undetermined significance cytology. *Thyroid* 25:1217–1223
  54. Alexander EK, Kennedy GC, Baloch ZW, Cibas ES, Chudova D, Diggans J et al (2012) Preoperative diagnosis of benign thyroid nodules with indeterminate cytology. *N Engl J Med* 367:705–715
  55. Chen YT, Kitabayashi N, Zhou XK, Fahey TJ 3rd, Scognamiglio T (2008) MicroRNA analysis as a potential diagnostic tool for papillary thyroid carcinoma. *Mod Pathol* 21:1139–1146
  56. Nikiforova MN, Tseng GC, Steward D, Diorio D, Nikiforov Y (2008) MicroRNA expression profiling of thyroid tumors: biological significance and diagnostic utility. *J Clin Endocrinol Metab* 93:1600–1608
  57. Swierniak M, Wojcicka A, Czetwertynska M, Stachlewska E, Maciag M, Wiechno W et al (2013) In-depth characterization of the microRNA transcriptome in normal thyroid and papillary thyroid carcinoma. *J Clin Endocrinol Metab* 98:E1401–E1409
  58. Mancikova V, Castelblanco E, Pineiro-Yanez E, Perales-Paton J, de Cubas AA, Inglada-Perez L et al (2015) MicroRNA deep-sequencing reveals master regulators of follicular and papillary thyroid tumors. *Mod Pathol* 28:748–757
  59. Yip L, Kelly L, Shuai Y, Armstrong MJ, Nikiforov YE, Carty SE, Nikiforova MN (2011) MicroRNA signature distinguishes the degree of aggressiveness of papillary thyroid carcinoma. *Ann Surg Oncol* 18:2035–2041
  60. Wei WJ, Shen CT, Song HJ, Qiu ZL, Luo QY (2016) MicroRNAs as a potential tool in the differential diagnosis of thyroid cancer: a systematic review and meta-analysis. *Clin Endocrinol (Oxf)* 84:127–133

61. Haugen BR, Alexander EK, Bible KC, Doherty GM, Mandel SJ, Nikiforov YE et al (2016) 2015 American thyroid association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American thyroid association guidelines task force on thyroid nodules and differentiated thyroid cancer. *Thyroid* 26:1–133
62. Pacini F, Basolo F, Bellantone R, Boni G, Cannizzaro MA, De Palma M et al (2018) Italian consensus on diagnosis and treatment of differentiated thyroid cancer: joint statements of six Italian societies. *J Endocrinol Invest* 41:849–876
63. Sciacchitano S, Lavra L, Ulivieri A, Magi F, De Francesco GP, Bellotti C et al (2017) Comparative analysis of diagnostic performance, feasibility and cost of different test-methods for thyroid nodules with indeterminate cytology. *Oncotarget* 8:49421–49442
64. Nikiforova MN, Mercurio S, Wald AI, Barbi de Moura M, Callenberg K, Santana-Santos L et al (2018) Analytical performance of the ThyroSeq v3 genomic classifier for cancer diagnosis in thyroid nodules. *Cancer* 124:1682–1690
65. Steward DL, Carty SE, Sippel RS, Yang SP, Sosa JA, Sips JA et al (2019) Performance of a multigene genomic classifier in thyroid nodules with indeterminate cytology: a prospective blinded multicenter study. *JAMA Oncol* 5:204–212
66. Jug R, Parajuli S, Ahmadi S, Jiang XS (2019) Negative results on thyroid molecular testing decrease rates of surgery for indeterminate thyroid nodules. *Endocr Pathol* 30:134–137
67. Shrestha RT, Evasovich MR, Amin K, Radulescu A, Sanghvi TS, Nelson AC et al (2016) Correlation between histological diagnosis and mutational panel testing of thyroid nodules: a two-year institutional experience. *Thyroid* 26:1068–1076
68. Valderrabano P, Khazai L, Leon ME, Thompson ZJ, Ma Z, Chung CH et al (2017) Evaluation of ThyroSeq v2 performance in thyroid nodules with indeterminate cytology. *Endocr Relat Cancer* 24:127–136
69. Taye A, Gurciullo D, Miles BA, Gupta A, Owen RP, Inabnet WB 3rd et al (2018) Clinical performance of a next-generation sequencing assay (ThyroSeq v2) in the evaluation of indeterminate thyroid nodules. *Surgery* 163:97–103
70. Marcadis AR, Valderrabano P, Ho AS, Tepe J, Swartzwelder CE, Byrd S, Sacks WL et al (2019) Interinstitutional variation in predictive value of the ThyroSeq v2 genomic classifier for cytologically indeterminate thyroid nodules. *Surgery* 165:17–24
71. Al-Qurayshi Z, Deniwar A, Thethi T, Mallik T, Srivastav S, Murad F et al (2017) Association of malignancy prevalence with test properties and performance of the gene expression classifier in indeterminate thyroid nodules. *JAMA Otolaryngol Head Neck Surg* 143:403–408
72. Harrell RM, Bimston DN (2014) Surgical utility of Afirma: effects of high cancer prevalence and oncocyctic cell types in patients with indeterminate thyroid cytology. *Endocr Pract* 20:364–369
73. Lastra RR, Pramick MR, Crammer CJ, LiVolsi VA, Baloch ZW (2014) Implications of a suspicious Afirma test result in thyroid fine-needle aspiration cytology: an institutional experience. *Cancer Cytopathol* 122:737–744
74. Brauner E, Holmes BJ, Krane JF, Nishino M, Zurakowski D, Hennessey JV et al (2015) Performance of the Afirma gene expression classifier in Hürthle cell thyroid nodules differs from other indeterminate thyroid nodules. *Thyroid* 25:789–796
75. Yang SE, Sullivan PS, Zhang J, Govind R, Levin MR, Rao JY et al (2016) Has Afirma gene expression classifier testing refined the indeterminate thyroid category in cytology? *Cancer Cytopathol* 124:100–109
76. Angell TE, Heller HT, Cibas ES, Barletta JA, Kim MI, Krane JF et al (2019) Independent comparison of the afirma genomic sequencing classifier and gene expression classifier for cytologically indeterminate thyroid nodules. *Thyroid* 29:650–656
77. Sacks WL, Bose S, Zumsteg ZS, Wong R, Shiao SL, Braunstein GD et al (2016) Impact of Afirma gene expression classifier on cytopathology diagnosis and rate of thyroidectomy. *Cancer* 124:722–728
78. Wu JX, Young S, Hung ML, Li N, Yang SE, Cheung DS et al (2016) Clinical factors influencing the performance of gene expression classifier testing in indeterminate thyroid nodules. *Thyroid* 26:916–922
79. Walts AE, Sacks WL, Wu HH, Randolph ML, Bose S (2018) A retrospective analysis of the performance of the RosettaGX® Reveal™ thyroid miRNA and the Afirma gene expression classifiers in a cohort of cytologically indeterminate thyroid nodules. *Diagn Cytopathol* 46:901–907
80. Wong KS, Angell TE, Strickland KC, Alexander EK, Cibas ES, Krane JF et al (2016) Noninvasive follicular variant of papillary thyroid carcinoma and the Afirma gene expression classifier. *Thyroid* 26:911–915
81. Alexander EK, Schorr M, Klopper J, Kim C, Sips J, Nabhan F et al (2014) Multicenter clinical experience with the Afirma gene expression classifier. *J Clin Endocrinol Metab* 99:119–125
82. McIver B, Castro MR, Morris JC, Bernet V, Smallridge R, Henry M et al (2014) An independent study of a gene expression classifier (Afirma) in the evaluation of cytologically indeterminate thyroid nodules. *J Clin Endocrinol Metab* 99:4069–4077
83. Celik B, Whetsell CR, Nassar A (2015) Afirma GEC and thyroid lesions: an institutional experience. *Diagn Cytopathol* 43:966–970
84. Marti JL, Avadhani V, Donatelli LA, Niyogi S, Wang B, Wong RJ et al (2015) Wide inter-institutional variation in performance of a molecular classifier for indeterminate thyroid nodules. *Ann Surg Oncol* 22:3996–4001
85. Noureldine SI, Olson MT, Agrawal N, Prescott JD, Zeiger MA, Tufano RP (2015) Effect of gene expression classifier molecular testing on the surgical decision-making process for patients with thyroid nodules. *JAMA Otolaryngol Head Neck Surg* 141:1082–1088
86. Samulski TD, LiVolsi VA, Wong LQ, Baloch Z (2016) Usage trends and performance characteristics of a “gene expression classifier” in the management of thyroid nodules: an institutional experience. *Diagn Cytopathol* 44:867–873
87. Baca SC, Wong KS, Strickland KC, Heller HT, Kim MI, Barletta JA et al (2017) Qualifiers of atypia in the cytologic diagnosis of thyroid nodules are associated with different Afirma gene expression classifier results and clinical outcomes. *Cancer Cytopathol* 125:313–322
88. Kay-Rivest E, Tibbo J, Bouhabel S, Tamilia M, Leboeuf R, Forest VI et al (2017) The first Canadian experience with the Afirma® gene expression classifier test. *J Otolaryngol Head Neck Surg* 46:25
89. Harrison G, Sosa JA, Jiang X (2017) Evaluation of the Afirma gene expression classifier in repeat indeterminate thyroid nodules. *Arch Pathol Lab Med* 141:985–989
90. Hang JF, Westra WH, Cooper DS, Ali SZ (2017) The impact of noninvasive follicular thyroid neoplasm with papillary-like nuclear features on the performance of the Afirma gene expression classifier. *Cancer Cytopathol* 125:683–691
91. Patel KN, Angell TE, Babiary J, Barth NM, Blevins T, Duh QY et al (2018) Performance of a genomic sequencing classifier for the preoperative diagnosis of cytologically indeterminate thyroid nodules. *JAMA Surg* 153:817–824
92. Harrell RM, Eyerly-Webb SA, Golding AC, Edwards CM, Bimston DN (2019) Statistical comparison of Afirma GSC and Afirma GEC outcomes in a community endocrine surgical practice: early findings. *Endocr Pract* 25:161–164
93. Endo M, Nabhan F, Porter K, Roll K, Shirley L, Azaryan I et al (2019) Afirma gene sequencing classifier compared to gene

- expression classifier in indeterminate thyroid nodules. *Thyroid* 29:1115–1124
94. San Martin VT, Lawrence L, Bena J, Madhun NZ, Berber E, Elsheikh TM et al (2019) Real world comparison of Afirma GEC and GSC for the assessment of cytologically indeterminate thyroid nodules. *J Clin Endocrinol Metab* Oct 26 pii: dgz099
  95. Angell TE, Wirth LJ, Cabanillas ME, Shindo ML, Cibas ES, Babiarz JE et al (2019) Analytical and clinical validation of expressed variants and fusions from the whole transcriptome of thyroid FNA samples. *Front Endocrinol (Lausanne)* 10:612
  96. Chaudhary S, Hou Y, Shen R, Hooda S, Li Z (2016) Impact of the Afirma gene expression classifier result on the surgical management of thyroid nodules with category III/IV cytology and its correlation with surgical outcome. *Acta Cytol* 60:205–210
  97. Abeykoon JP, Mueller L, Dong F, Chintakuntlawar AV, Paludo J, Mortada R (2016) The effect of implementing gene expression classifier on outcomes of thyroid nodules with indeterminate cytology. *Horm Cancer* 7:272–278
  98. Nicholson KJ, Roberts MS, McCoy KL, Carty SE, Yip L (2019) Molecular testing versus diagnostic lobectomy in Bethesda III/IV thyroid nodules: a cost-effectiveness analysis. *Thyroid* 29:1237–1243
  99. Labourier E, Shifrin A, Busseniers AE, Lupo MA, Manganelli ML, Andrus B et al (2015) Molecular testing for miRNA, mRNA, and DNA on fine-needle aspiration improves the pre-operative diagnosis of thyroid nodules with indeterminate cytology. *J Clin Endocrinol Metab* 100:2743–2750
  100. Banizs AB, Silverman JF (2019) The utility of combined mutation analysis and microRNA classification in reclassifying cancer risk of cytologically indeterminate thyroid nodules. *Diagn Cytopathol* 47:268–274
  101. Benjamin H, Schnitzer-Perlman T, Shtabsky A, Vandenburg CJ, Ali SZ, Kolar Z et al (2016) Analytical validity of a microRNA-based assay for diagnosing indeterminate thyroid FNA smears from routinely prepared cytology slides. *Cancer Cytopathol* 124:711–721
  102. Lithwick-Yanai G, Dromi N, Shtabsky A, Morgenstern S, Strenov Y, Feinmesser M et al (2017) Multicentre validation of a microRNA-based assay for diagnosing indeterminate thyroid nodules utilising fine needle aspirate smears. *J Clin Pathol* 70:500–507
  103. Partyka KL, Randolph ML, Lawrence KA, Cramer H, Wu HH (2018) Utilization of direct smears of thyroid fine-needle aspirates for ancillary molecular testing: a comparison of two proprietary testing platforms. *Diagn Cytopathol* 46:320–325
  104. Nikiforov YE, Steward DL, Robinson-Smith TM, Haugen BR, Klopper JP, Zhu Z et al (2009) Molecular testing for mutations in improving the fine-needle aspiration diagnosis of thyroid nodules. *J Clin Endocrinol Metab* 94:2092–2098
  105. Otori NP, Nikiforova MN, Schoedel KE, LeBeau SO, Hodak SP, Seethala RR et al (2010) Contribution of molecular testing to thyroid fine-needle aspiration cytology of “follicular lesion of undetermined significance/atypia of undetermined significance”. *Cancer Cytopathol* 118:17–23
  106. Cantara S, Capezzone M, Marchisotta S, Capuano S, Busonero G, Toti P et al (2010) Impact of proto-oncogene mutation detection in cytological specimens from thyroid nodules improves the diagnostic accuracy of cytology. *J Clin Endocrinol Metab* 95:1365–1369
  107. Beaudenon-Huibregtse S, Alexander EK, Guttler RB, Hershan JM, Babu V, Blevins TC et al (2014) Centralized molecular testing for oncogenic gene mutations complements the local cytopathologic diagnosis of thyroid nodules. *Thyroid* 24:1479–1487
  108. Eszlinger M, Kroghdahl A, Münz S, Rehfeld C, Precht Jensen EM, Ferraz C et al (2014) Impact of molecular screening for point mutations and rearrangements in routine air-dried fine-needle aspiration samples of thyroid nodules. *Thyroid* 24:305–313
  109. Eszlinger M, Böhme K, Ullmann M, Görke F, Siebolts U, Neumann A et al (2017) Evaluation of a 2-year routine application of molecular testing of thyroid fine-needle aspirations using a seven-gene panel in a primary referral setting in Germany. *Thyroid* 27:402–411
  110. Bongiovanni M, Molinari F, Eszlinger M, Paschke R, Barizzi J, Merlo E et al (2015) Laser capture microdissection is a valuable tool in the preoperative molecular screening of follicular lesions of the thyroid: an institutional experience. *Cytopathology* 26:288–296
  111. Censi S, Cavedon E, Bertazza L, Galuppini F, Watutantrige-Fernando S, De Lazzari P et al (2017) Frequency and significance of Ras, Tert promoter, and Braf mutations in cytologically indeterminate thyroid nodules: a monocentric case series at a tertiary-level endocrinology unit. *Front Endocrinol (Lausanne)* 8:273
  112. Macerola E, Rago T, Proietti A, Basolo F, Vitti P (2019) The mutational analysis in the diagnostic work-up of thyroid nodules: the real impact in a center with large experience in thyroid cytopathology. *J Endocrinol Invest* 42:157–166
  113. Shen R, Liyanarachchi S, Li W, Wakely PE Jr, Saji M, Huang J et al (2012) MicroRNA signature in thyroid fine needle aspiration cytology applied to “atypia of undetermined significance” cases. *Thyroid* 22:9–16
  114. Santos MTD, Buzolin AL, Gama RR, Silva ECAD, Duflath RM, Figueiredo DLA, Carvalho AL (2018) Molecular classification of thyroid nodules with indeterminate cytology: development and validation of a highly sensitive and specific new miRNA-based classifier test using fine-needle aspiration smear slides. *Thyroid*
  115. Maze H, Deutch T, Karas A, Bogardus KA, Mizrahi I, Gura-Wahnon D, et al (2018) Next-Generation Sequencing Identifies a Highly Accurate miRNA Panel That Distinguishes Well-Differentiated Thyroid Cancer from Benign Thyroid Nodules. *Cancer Epidemiol Biomarker Cancer Epidemiol Biomarkers Prev* 27:858–63
  116. Pagan M, Kloos RT, Lin CF, Travers KJ, Matsuzaki H, Tom EY et al (2016) The diagnostic application of RNA sequencing in patients with thyroid cancer: an analysis of 851 variants and 133 fusions in 524 genes. *BMC Bioinformatics* 17(Suppl. 1):6
  117. Lee YS, Lim YS, Lee JC, Wang SG, Park HY, Kim SY et al (2015) Differential expression levels of plasma-derived miR-146b and miR-155 in papillary thyroid cancer. *Oral Oncol* 51:77–83
  118. Yu S, Liu Y, Wang J, Guo Z, Zhang Q, Yu F et al (2012) Circulating microRNA profiles as potential biomarkers for diagnosis of papillary thyroid carcinoma. *J Clin Endocrinol Metab* 97:2084–2092
  119. Cantara S, Pilli T, Sebastiani G, Cevenini G, Busonero G, Cardinale S et al (2014) Circulating miRNA95 and miRNA190 are sensitive markers for the differential diagnosis of thyroid nodules in a caucasian population. *J Clin Endocrinol Metab* 99:4190–4198
  120. Pilli T, Cantara S, Marzocchi C, Cardinale S, Santini C, Cevenini G et al (2017) Diagnostic value of circulating microRNA-95 and -190 in the differential diagnosis of thyroid nodules: a validation study in 1000 consecutive patients. *Thyroid* 27:1053–1057
  121. Nikiforov YE, Sethala RR, Tallini G, Baloch ZW, Basolo F, Thompson LD et al (2016) Nomenclature revision for encapsulated follicular variant of papillary thyroid carcinoma: a

paradigm shift to reduce overtreatment of indolent tumors.  
JAMA Oncol 2:1023–1029

122. Brandler TC, Liu CZ, Cho M, Zhou F, Cangiarella J, Yee-Chang M et al (2018) Does noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) have a unique molecular profile? Am J Clin Pathol 150:451–460

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