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Review

Thyroid and Molecular Testing. Advances in Thyroid Molecular Cytopathology

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Abstract: Thyroid nodules are a common finding in the adult population including the fact that more than 50% of individuals, over the age of 60, have thyroid nodules. The majority have been mostly detected with ultrasonography and 10% by palpation. The majority of these nodules are benign, whereas 5–15% of them are malignant. The pre-operative diagnosis of cancer is a critical challenge in order to ensure that each patient can be treated with the best tailored management with a reduction of unnecessary surgery for benign lesions. Fine needle aspiration cytology (FNAC) represents the first and most important diagnostic tool for the evaluation of thyroid lesions. According to the literature, FNAC is able to render a conclusive diagnosis in up to 70–80% of all cases. For the remaining 20–30% of nodules, cytological diagnoses fall into the category of indeterminate lesions mostly due to the lack of specific morphological features. According to the Bethesda system for reporting thyroid cytopathology (TBSRTC), indeterminate lesions can be sub-stratified into three different subcategories including "atypia of undetermined significance/follicular lesion of undetermined significance-AUS/FLUS"; "follicular or Hürthle cell neoplasm/suspicious for follicular or Hürthle cell neoplasm-FN/SFN"; and "suspicious for malignancy-SFM". Many of these indeterminate lesions undergo repetition or diagnostic lobectomy. Nonetheless, the majority of these cases will have a benign diagnosis due to the fact that the rate of cancer ranges between 6 and 30%. It stands to reason that the application of ancillary technique, mostly molecular testing, emerged as a critical additional tool for those thyroid indeterminate lesions. Since the early 1990s, material collected from cytological samples yields sufficient and adequate cells for the detection of point mutation or gene fusions. Nonetheless, the further availability of new sequencing technologies such as next-generation sequencing (NGS) has led to more comprehensive molecular applications adopted now in clinical use. The current review investigates the multiple advances in the field of molecular testing applied in thyroid cytology.

Keywords: fine needle aspiration cytology; thyroid cancers; indeterminate lesions; molecular testing; personalized medicine

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1. Introduction

Since its widely introduction, in the 1980s, fine-needle aspiration cytology (FNAC) is undoubtedly the first and most important pre-operative diagnostic procedure for the evaluation of thyroid lesions because of its advantages representing by its simplicity, safety, and cost-effectiveness.

Thyroid nodules are commonly found in both pediatric and adult patients characterized by either benign or malignant lesions, with the evidence that the incidence of thyroid carcinoma especially in the USA has increased more than any other cancer.

Despite the differences in the proposed series and the diagnoses in the different classification systems, about 70% of thyroid nodules are benign with only 5–10% reported as "malignant" lesions [1–6]. The remaining 20–25% of them are diagnosed as indeterminate

proliferations" including either benign or malignant lesions, for which a morphological discrimination is not always possible, leading to unnecessary surgical resections (lobectomy and/or total thyroidectomy), psychological implications, and higher health care costs for the patients [7–18]

Although morphology alone is able to provide a correct cytological diagnosis in the majority of lesions, it is not able to make a definitive diagnosis in 100% of cases, demonstrating some flaws that cannot be overcome without the useful support of ancillary techniques.

That said, the use of molecular markers in thyroid nodules has been introduced for diagnostic purposes in the discrimination of the benign and malignant nature of mostly but not exclusively indeterminate lesions, especially to support correct decision making in their management approach. It is univocally stated that many papers have assessed the high diagnostic accuracy of molecular testing when applied on cytological samples [19–34]. In this perspective, the adoption of ancillary techniques, as an integrated tool for a conclusive diagnosis, has become an essential component in the management of several tumors due to the evidence that the knowledge of molecular mechanisms and genetics are linked with tumorigenesis and cancer in the thyroid gland. For that reason, the study of genetic and molecular alterations can be translated and carried out onto clinical practice as an adjuvant and valid tool for diagnosis, management, and prognosis [35–51].

Since the first edition of the Bethesda system for reporting thyroid cytopathology (TBSRTC), the adoption of a standardized cytologic classification system has obtained widespread international acceptance, and has contributed significantly to a more uniform and defined approach and management of thyroid nodules by increasing the quality and reproducibility of thyroid cytology diagnoses [52]. Nonetheless, the first edition of the Bethesda system, launched in 2010, did not address the changes due to the proposal of testing for oncogene mutations which was demonstrated to improve the performance of thyroid FNAC.

Since then, numerous papers and studies have confirmed the useful and relevant diagnostic and prognostic role of genetic alterations in the interpretation of thyroid cytology, leading to the need that a revision of TBSRTC, including molecular analysis, might be appropriate [52]. In fact, the second edition of TBSRTC, released in 2017, focused on some new additional topics including the cytomorphological criteria for FNA classification, reporting terminology, implied ROM–risk of malignancy for each diagnostic category, the role of molecular testing in the different diagnostic categories, and the changes related to the recently described non-invasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) [53–60].

Herein, we summarize the role of molecular application in the different categories of the 2nd edition of TBSRTC.

2. Molecular Testing in Thyroid Lesions and the Bethesda System Categories

Papillary TC (PTC) and follicular TC (FTC) carcinomas arise from follicular cells and they constitute around 90% of all TC, generally with a very good prognosis [1–10].

All the authors and publications have offered a unanimous consensus that FNAC plays an essential role in discriminating benign from malignant thyroid nodules even though the morphological evaluation alone is not able to be diagnostic in all cases and/or to answer all diagnostic questions. For those reasons, many authors have supported the application of ancillary techniques (including immunocytochemistry—ICC and molecular testing) as useful help in improving the performance of FNAC diagnoses and achieving the most appropriate and tailored surgical management [12–20].

The publication from the Thyroid Cancer Genome Atlas clarified the knowledge of the molecular pathology mostly in the field of papillary thyroid carcinoma (PTC) but not only. In fact, the study underlined that two somatic mutations, *BRAF V600E* or a *RAS*-mutations, have a driven role in tumors and that these most common mutations are able to activate the mitogenic-activated protein kinase (MAPK) pathway [40].

After that relevant evidence, in 2015, the American Thyroid Association (ATA) published the revised management guidelines for patients with thyroid nodules and well differentiated TCs (WDTC), recommending the performance of molecular testing in thyroid indeterminate cytology [53]. Specifically, these guidelines suggested that the performance of molecular panels (including also *BRAF*, *RAS*, *RET/PTC*, and *PAX8-PPARγ*) can support a definitive diagnosis in some cases, by improving the accuracy of indeterminate thyroid samples and by stratifying the risk of malignancy (ROM) and thereby reduce the number of unnecessary diagnostic lobectomies and/or thyroidectomies. Furthermore, it is relevant to underline that both the ATA–American thyroid association and the recent 2nd TBSRTC did not endorse any specific molecular test, even though they both reinforced the role of different tests according to the different categories and diagnostic scenarios including, as for Ferris et al., in their ATA, the diagnostic subcategories of the indeterminate lesions [52–54].

In these last decades, different authors highlighted that specific somatic mutations, gene rearrangements, and/or microRNA (miRNA) expression profiles are supported by a high specificity and predictive value for malignant thyroid disease [19–37,40,61–73]. Nonetheless, apart from the validation of single mutation, Nikiforov et al. encouraged the adoption of a broad next-generation sequencing (NGS) panel, leading to a more comprehensive genetic analysis in the diagnosis of indeterminate lesions including nodules with AUS/FLUS and FN/SFN cytology for their best and tailored management [35,36].

Although the use of several "in-house" molecular platforms, mostly defined by own selections by the departments using them, some molecular thyroid tests are commercially available in the USA, including: (a) ThyroSeq (University of Pittsburgh Medical Center [UPMC]/Cytopath Biopsy Lab [CBLPath], Pittsburgh, PA, USA); (b) Afirma gene expression classifier (GEC, Veracyte, South San Francisco, CA, USA); (c) ThyGenX and ThyraMIR (both from Interpace Diagnostics, Parsippany, NJ, USA) [32–36,62–97]. The major issues are represented by the absence of a unique ideal molecular test that is able to simultaneously play a role as "rule-in and/or rule-out malignancy" test (Table 1).

For instance, Thyroseq and ThyGenX tests, having high positive and negative predictive value (PPV and NPV), are likely to be consider as "rule-in malignancy test", whilst the Afirma GEC with its high NPV helps as a "rule-out malignancy" test mostly for indeterminate thyroid lesions [34–37].

Consequently, molecular tests have been introduced in the second edition of TBSRTC for different diagnostic categories such as AUS/FLUS, FN, SM, and malignant entities with the purpose to contribute to a better definition of the risk stratification of thyroid nodules [52]. Herein, a summary of their use in the Bethesda categories with a selective focus on the indeterminate cytologic diagnoses in which their yield are likely to change the management and to better define the prognostic implications [97–112].

Table 1. Characteristics of different thyroid molecular tests.

Molecular Test	ThyroSeq [34–36]	Afirma GSC [32,43,101]	ThyGenX [94]	ThyraMIR [95,97]
Principal method	NGS	mRNA microarray analysis	Multiple PCR + mutations (somatic and rearrangements)	mRNA analysis
NPV	High	High	High (when used with ThyraMIR)	Scant data
PPV	High	Low	High (when used with ThyraMIR)	Scant data
Sensitivity and Specificity	High/High	High/High	High/High	High/High
Material suitable to test	Fresh cytological samples and/or special collection	Fresh cytological samples and/or special collection	Fresh cytological samples and/or special collection	Fresh cytological samples and/or special collection
Clinical relevance	Rule-in test	Rule-out test	Rule-in test	Rule-in and rule-out test
Data analysis	Centralized labs and/or local labs	Centralized labs	Local labs	Local labs

NGS = next-generation sequencing; GEC: gene expression classifier; NPV = negative predictive value; PPV = positive predictive value; RT-PCR = reverse transcription polymerase chain reaction.

3. AUS/FLUS

The 2017 TBSRTC edition maintained the AUS/FLUS category, which was only object of minor changes. Nonetheless, this category still represents a challenge mostly due to the correct interpretation of both architectural and nuclear atypia as well as for the ROM mostly due to the fact that only a minority of AUS/FLUS undergo surgery.

As reported in the 2018 TBSRTC and in agreement with the 2015 ATA guidelines, the best choice for AUS/FLUS is a conservative management including either repeat FNA or the use of molecular testing [52,53]. Although, a repeat FNA would solve the majority of initial AUS/FLUS, about 10–30% of the initial AUS/FLUS would maintain an AUS/FLUS diagnosis at a second repetition. Those latter cases are likely to benefit from the suggested application of mutational testing as added in the TBSRTC 2nd edition.

The ATA guidelines and the second edition of TBSRTC offer the possibility to choose between surgery (typically lobectomy) vs. follow-up observation, depending on a combination of morphological, ancillary yields, and clinical and radiologic findings including the evaluation of clinical risk factors and patient choice [52,53]. The second edition of TBSRTC confirmed the underlined changes in the ROM of an AUS/FLUS nodule mostly based on the architectural and cytologic atypia, ranging from a mean ROM of 47% for those cases with cytologic atypia to only 5% for AUS/FLUS with Hürthle cell atypia [6–11].

In the category of AUS/FLUS, many papers assessed that these subcategory of indeterminate lesions mostly have low risk of malignancy and, when malignant, they frequently result in a histological diagnosis of FVPC, so that the performance of an expanded mutation panel might offer better results in terms of higher sensitivity than $BRAF^{V600E}$ alone, counterbalanced by the diminished specificity due to the increased prevalence of *RAS* mutations [63–91]

In 2007, one of the first and large studies about indeterminate proliferations was conducted by Nikiforov et al. developing a 7-gene molecular test (ThyroSeq v0), composed of a panel of mutations (*BRAF*, *N-/H-/K-RAS*) and translocations of the *RET/PTC* and *PAX8/PPARg* genes [32]. They analyzed a series of 1056 indeterminate lesions and they reported an increased ROM for mutated AUS/FLUS, FN, and SM cases (88%, 87%, and 95%, respectively), compared to 6%, 14%, and 28% in mutation-negative lesions [32]. Specifically, their series included 653 AUS/FLUS [32] with 247 followed by a histological diagnosis. Their yields confirmed the significant increase of cancer risk, from 14% to 87%, in presence of any mutation, and the low cancer risk (6%) in cases characterized by the absence of any mutation (Table 1).

Nevertheless, the application of next-generation sequencing (NGS) technology represented a useful new approach for testing a broad spectrum of point mutations also in the evaluation of indeterminate proliferations. In 2014, after the introduction of the ThyrSeq v1 mutational panel, including 15-genes but without a satisfying NPV, the same authors developed ThyroSeq v2, a new and superior NGS-based assay, applied initially to 143 cases of FN/SFN [34]. ThyroSeq v2 is based on the evaluation of an expanded 56-gene panel composed of several point mutations and gene fusions, resulting in a better NPV [35]. The impact of ThyroSeq v2 for the AUS/FLUS category assessed that its role and yields are mostly linked to the pre-test probability of malignancy for this category. However, the use of NGS for thyroid indeterminate lesions assessed very good results as for: sensitivity of 90.9%, specificity of 92.1%, PPV of 76.9%, and NPV of 97.2% with an overall accuracy of 91.8% [35].

The last version of ThyroSeq v.3 test, released in 2017, included the evaluation of more than 12,000 mutation hotspots and more than 120 gene fusion types [79]. The data from a recent prospective study by Steward et al., including 10 medical centers with 286 cytologic indeterminate lesions, appraised that in the Bethesda III and IV combined nodules, the test reported a 94% sensitivity and 82% specificity, preventing a surgical procedure in up to 61% of the patients [111].

On the other hand, the Afirma gene expression classifier (GEC) represents another commonly used molecular test for indeterminate thyroid proliferations, which is based

on the opposite concept to predict and "rule in" benign diagnoses [37,83–95]. In 2012, Alexander et al. introduced the Afirma GEC was firstly analyzed in a key study including 265 indeterminate thyroid lesions out of 4812 FNAC cases from a multicenter trial [37]. They found 95% NPV for AUS/FLUS lesions and 94% for FN with an associated malignancy rate of 24% and 25%, respectively. The Afirma GEC test evaluates the expression of 167 genes including 142 genes in the main classifier (benign or suspicious) and 25 smaller gene expression panels to identify rare neoplasms [37]. Nonetheless, whilst the Afirma GEC reduced the ROM mostly for AUS/FLUS and SFN/FN categories, the evidence of its low NPV and PPV for the suspicious for malignancy (SFM) category limited its performance for SFM.

Many other publications from different groups assessed the use of Afirma GEC in AUS/FLUS lesions [46–50]. Unanimously, the data confirmed that those AUS/FLUS with architectural atypia have more frequently (at around 50% of cases) a negative GEC result than in AUS/FLUS with cytologic atypia or cytologic plus architectural atypia, which are characterized by a suspicious yield. In this regard, a negative result is associated with a ROM decreasing from 24% to 5%, confirming that it is likely to suggest that observation over surgery is the best choice for patients with a negative GEC test [48]. Furthermore, an AUS/FLUS lesion with a Hürthle cell pattern endows with a low rate of GEC benign results and a very low risk of malignancy [50].

The new version, a next-generation Afirma genomic sequencing classifier (GSC) included gene expression, but also the presence of DNA variants, fusions, copy number variants, and other information that may be predictive of thyroid cancer [82–84]. The new version has not altered the high original sensitivity but significantly increased its specificity, reducing the number of necessary surgeries in patients with indeterminate cytological reports to 30% of them.

For the category of AUS/FLUS, the results of Afirma GEC testing vary, depending on the different features of atypia. Specifically, in 65% of AUS nodules with architectural atypia (65%), they found a benign GEC, whilst it was 59% in AUS with nuclear atypia (59%) or 38% in AUS with both nuclear and architectural atypia. Patients with GEC suspicious nodules had higher ROM in cases with both architectural and nuclear atypia (57%) than in cases with architectural or nuclear atypia alone (19% and 45%, respectively) [82–88]. San Martin et al. compared a retrospective series of Bethesda III and IV nodules tested with GEC or GSC in an academic center between December 2011 and September 2018 [85]. Their results confirmed an overall surgery rate decrease from 47.8% in the GEC group to 34.7% in the GSC group ($p = 0.25$). Furthermore, GSC turned out to have a statistically significant higher specificity (94% vs. 60%, $p < 0.01$) and positive predictive value (PPV) (85.3% vs. 40%, $p < 0.01$) than GEC. On the other hand, sensitivity and negative predictive value (NPV) dropped with GSC (97.0% vs. 90.6% and 98.6% vs. 96.3%, respectively) [85].

Harrell et al. compared their experience with GSC (11 months) and their prior experience with the GEC (86.5 years) [86]. Specifically, GSC reduced the number of suspicious indeterminate nodules (38.8%), whilst the global number of them was higher when compared to that reported by GEC (58.4%). There was a decrease in the percentage of oncocytic nodules classified as suspicious in the GSC group (82.7% suspicious by GEC and 35.3% classified as suspicious by GSC). Their conclusions led to the evidence that GSC is useful in further reducing the number of indeterminate thyroid nodules that undergo surgery by improving the specificity and maintaining a valuable sensitivity. An important role is attributed to the significant improvement in the specificity of the Afirma GSC test in oncocytic cytologic aspirates [86]

In 2016, the adoption of the new terminology of NIFTP instead of non-invasive and encapsulated follicular variant of PTC has significant reflections into their cytological diagnoses [42,55–61,112–121]. Different series documented that the majority of NIFTP are frequently found in the indeterminate categories with 31% in the AUS/FLUS, 26.6% in the FN/SFN, and 24.3% in the SFM [112–121]. Those NIFTP, diagnosed in the AUS/FLUS category, are likely to lead to a decrease of the overall ROM for AUS/FLUS, even though

there has been a univocal opinion concerning the fact that surgical excision is the reported gold-standard of treatment for NIFTP [16,17]. A morphological diagnosis of NIFTP on cytology can only be suggested so that molecular testing has been evaluated in order to add significant clues [55–60]. Among them, the diagnostic role of the Afirma test to detect NIFTP has been controversial even if NIFTP is often associated with suspicious Afirma GEC results [83–90] The evaluation of genetic alterations (including somatic mutations and/or chromosomal rearrangements) demonstrated that NIFTP has a different molecular profile from PTC characterized by *RAS* mutations (*NRAS, KRAS*) in up to 60% of cases, *PAX8/PPARg* or $BRAF^{K601E}$, in contrast to the frequent $BRAF^{V600E}$ and *RET/PTC* alterations observed in PTC.

4. Follicular Neoplasm (FN/SFN)

The use of molecular testing was also investigated in the FN/SFN category. Data concerning the use of Thyroseq v0 by Nikiforov et al. included 247 FN/SFN cases with 214 having histological follow-up [32]. Thirty-three (87%) out the 38 mutated resected nodules found to be histologically malignant and all resulting in BRAF and PAX8/PPARγ mutated cases. As for the AUS/FLUS category, the yields obtained from FN/SFN resulted in 57% sensitivity, 97% specificity, 86% diagnostic accuracy, 87% PPV, and 86% NPV [32].

Thus, ThyroSeq v2, performed for the analysis of 143 retrospectively and prospectively collected FN/SFN nodules confirmed a 90% sensitivity, 93% specificity, 83% PPV, and 96% NPV [35]. Significantly relevant, also in this paper, the authors confirmed the high specificity of point mutations such as $BRAF^{V600E}$, *TERT*, *TP53*, *PIK3CA*, and any gene fusion in 100% of malignant cases [35]. Additionally, the high PPV and NPV obtained from their studies, assesses that ThyroSeq v2 may perform as both a "rule-out" and "rule-in" test for FN/SFN, representing a valid additional test in selecting those patients eligible for total thyroidectomy [35,65–76,96–110]. A marginal role is played in the evaluation of Hürthle cell nodules. In fact, molecular application is not so relevant in discriminating Hürthle cell carcinoma versus adenoma as also demonstrated by the fact that some of these genetic alterations such as *RET/PTC1-3* rearrangements and *RAS* mutations have been reported in both Hürthle cell adenomas and carcinomas [96–110].

The application of the Afirma testing for the FN/SFN demonstrated a 7.2% reduction of thyroidectomy [83–90]. However, in several studies, indeterminate nodules with oncocytic predominance were defined by a lower specificity or higher false positive rate in GEC tests. [83–92]. Brauner et al. included a cohort of 122 oncocytic predominant nodules identified as GEC suspicious but resulting in benign pathologies. Reporting data from their single academic tertiary center, Endo et al. showed that GSC improved specificity and PPV while maintaining high sensitivity and NPV compared with GEC in thyroid lesions diagnosed as AUS/FLUS and FN/SFN [87]. Furthermore, they had an increase in the benign rate in GSC compared with GEC, as a result of fewer false positive results.

Nonetheless, the updated version of the Afirma (GSC) is able to improve performance for Hürthle cell lesions with increased specificity of 59% compared with just 12% with the original Afirma GEC [82–84].

Angell et al. evaluated 600 nodules in 563 patients tested with either GEC (n = 486) or GSC (n = 114). Specifically, among the SFN/FN category, the benign rate for the GEC and GSC were similar (p = 0.68), but for cytology suspicious for Hürthle cell neoplasm, the benign rate with GSC was 68.2% compared to the benign rate for GEC of 16.4% ($p < 0.0001$) [90]. These data supported the better performance of GSC, able to lead to further reduction in surgical management.

Geng et al. present their experience with GEC in 167 indeterminate lesions and GSC in 133 indeterminate nodules [89]. They found that, based on molecular testing, surgical resection could have been avoided in 61% with GSC, compared to 49% with the GEC test. They concluded that GSC had a better test performance than GEC, suggesting the evidence that GSC is more useful in identifying more cases as benign and limiting the number of unnecessary surgeries [89].

In another study, Hangell et al. used the Afirma® Xpression Atlas (XA) able to detect gene variants and fusions in thyroid indeterminate FNA samples with a panel of 511 genes using whole-transcriptome RNA-sequencing [88]. They focused their evaluation on cytologically indeterminate nodules with a Afirma GSC suspicious, Bethesda V/VI nodules, or known thyroid metastases. They documented high intra and inter-reproducibility ranging from 89% to 94% and inter-lab accuracy (90%). XA was able to identify multiple variants and fusions previously described across the spectrum of thyroid cancers, increasing the opportunities for additional approved or investigational-targeted therapies. Among Bethesda III/IV nodules, the sensitivity of XA as a standalone test was 49%. They concluded that when the Afirma genomic sequencing classifier (GSC) is used first among Bethesda III/IV nodules as a rule-out test, XA supplements genomic insight among those that are GSC suspicious [88]. Their data clinically and analytically validated the use of XA among GSC suspicious, or Bethesda V/VI nodules. The genomic information provided by XA may add important insights for clinical decision making precision medicine in a broad range of FNA sample types.

Another NGS technology, commercially known as miRInform (Asuragen, Austin, TX, USA) is ThyGenX (Interpace Diagnostics, Parsippany, NJ), which is a thyroid 8-gene panel, representing a "modified version" of the original gene panel test by Nikiforov et al., able to detect genetic alterations [117,121]. The new version, supported by its specific methodology, documented that the detection of $BRAF^{V600E}$ or *RET/PTC* is associated with 100% ROM, but it is lower and wider for *RAS* (range, 12–87.5%) and *PAX8/PPARg* (range, 50–100%) alterations [117,121]. Despite this evidence, the ROM for wild type indeterminate lesions is not significantly affected: in fact, for AUS/FLUS, it is only slightly higher than that of a benign lesion, whilst for FN/SFN, it is identical to the non-tested cases.

That found, Interpace Diagnostics suggested that ThyraMIR (from Interpace Diagnostics, Parsippany, NJ) might be a valid additional reflex test, for those cases with wild type/negative ThyGenX result that are not *BRAFV600E* or *RET/PTC1-3* mutated 94). In fact, different papers studied the performance of miRNAs on indeterminate thyroid lesions, as some specific miRNAs (e.g., miR-146, 221, 222) are a clue to thyroid well differentiated carcinomas [65–76]. In fact, ThyraMIR is defined as a thyroid microRNA (miRNA) classifier that is able to divide results into "positive" or "negative" categories.

Furthermore, a high sensitivity and specificity is obtained by combining ThyGenX and ThyraMIR as underlined in two different studies including indeterminate thyroid nodules [94,95]. The authors found high sensitivity (94% for AUS/FLUS and 82% for SFN/FN) and specificity (80% for AUS/FLUS and 91% for SFN/FN), with a PPV of 74% and NPV of 94% [94,95]. The application of multi-panel testing offers both important diagnostic information through the definition of specific mutations, and the prognostic role of some of them leading to a more personalized and tailored management also for AUS/FLUS category [94,95].

In a recent paper published by Vielh et al., the authors studied follicular adenomas (FA) and carcinomas (FTC) and they suggested the hypothesis that an analysis of a large series of FA and FTC with their genetic landscape could potentially help to identify a combination of genetic alterations, such as somatic copy number variations (sCNVs) characteristic of FTC. Furthermore, these genetic biomarkers would be useful in developing simple and direct tests that are applicable to cytological specimens to complement cytomorphology [91]. In their study, they firstly included two independent (training and validation) sets of histologically confirmed samples from two comprehensive cancer centers (Gustave Roussy (GR), Villejuif, France, and the University of Texas MD Anderson Cancer Center (UT MDACC), Houston, TX, USA) represented by frozen tissues from 59 FA and 67 FTC [91]. Hence, they included 27 stained FN/SFN with histological follow-up, confirming their previous findings and showing the feasibility of the DNA FISH (DNA fluorescent in situ hybridization) assay. These data assessed that their triple DNA FISH diagnostic assay may be used to identify 50% of FTCs with a high specificity (>98%) and with a low-cost adjunct

to cytomorphology to help further classify follicular neoplasms on already routinely stained cytological specimens [91].

5. NIFTP

NIFTP terminology, defined as "noninvasive follicular thyroid neoplasm with papillary-like nuclear features", was introduced in 2015 in order to replace the encapsulated-noninvasive follicular variant of PTC and to more accurately reflect the biological behavior of the tumor [55–60,112–122]. Its introduction wanted to modify the way the lesion is likely to be clinically approached and perceived by both practitioners and patients. Additionally, the use of NIFTP, allows for more uniformity in reporting for general pathologists less comfortable to exclude overt malignancy with certain nuclear features [55]. Specifically, NIFTP is an exclusively histological diagnosis defined by strict major and minor histological criteria, mostly because NIFTPs are biologically similar to follicular adenomas lacking lymph node metastases and/or recurrence. Nevertheless, the definition of NIFTP underwent some important revisions in 2018 including the lack of any true papillae formation and the exclusion of lesions harboring the *BRAF V600E* mutation and other high-risk genetic abnormalities [123]. It stands to reason that the changes reflected the imperfection of the defined criteria in outcome prediction and the global efforts for improvement.

The most important issues are represented by the implication of NIFTP on thyroid cytology and its allocation into the different diagnostic categories. Specifically, different papers reported that NIFTP are frequently diagnosed in the indeterminate categories with 31% in the AUS/FLUS, 26.6% in the FN/SFN, and 24.3% in the SFM [56–60].

It is important to underline, as also stated in the 2nd TBSRTC, that a definitive diagnosis of NIFTP is not possibly delivered on FNAC samples [52]. Nonetheless, the detection of nuclear pseudoinclusions combined with papillary structures are typically seen in cytological samples from PTC, whilst the evidence of a predominantly follicular pattern with less frequent nuclear elongations and grooves cannot exclude a histological diagnosis of NIFTP [56,109].

From a molecular side, NIFTP has a similar mutational profile as other follicular thyroid neoplasms, with frequent *RAS* family mutations and *PAX8-PPAR*γ fusions [55–60, 112–122]. Nonetheless, the analysis of the transcriptomic landscape is highly heterogeneous, justifying the difficulty to gene expression-based cytopathologic classification.

Since now, no specific genetic alterations have been found to link with a definitive diagnosis of NIFTP, although molecular testing has been performed to differentiate NIFTP from other neoplasms [111–115]. Only few recent papers discussed the role of GEC in the diagnosis of NIFTP, confirming that NIFTP is frequently associated with suspicious Afirma GEC results [111,112].

The most relevant evidence, as in different studies, confirmed that NIFTP shows a different molecular profile from PTC [113–115]. On the other hand, none of the papers supported the evidence that there are differences in clinic-pathological or molecular profiles between non-invasive and invasive encapsulated FVPTC cases, except with respect to vascular and capsular invasion [55–60]. This point is against a possible diagnosis of NIFTP on cytological samples [56–60]. Kim et al., including 177 consecutive FVPTCs (74 non-invasive encapsulated, 51 invasive encapsulated, 52 infiltrative), demonstrated that all the molecular yields are in favor of a diagnosis of NIFTP as a neoplasm [120]. Specifically, they documented that any type of *RAS* mutation (*NRAS*, *HRAS*, and *KRAS* mutations) were more likely seen in encapsulated FVPTC (48.6% in non-invasive and 66.7% in invasive) than in infiltrative FVPTC (15.4%). $BRAF^{V600E}$ mutation confirmed to be more commonly described in the classic PTC or invasive FVPTC, as in fact they estimated a similar rate in non-invasive (12.2%) and invasive (11.8%) subtypes of encapsulated FVPTC, and higher in infiltrative FVPTC (34.6%) [120]. For other genetic alteration, i.e., *RET-PTC* rearrangements, they were exclusively found (11.5%) in infiltrative FVPTC.

According to some other authors, many NIFTP series express alterations in *RAS*, *PAX8/PPA*, or $BRAF^{K601E}$, in contrast to the frequent $BRAF^{V600E}$ and *RET/PTC* alterations

observed in PTC [112–123]. For this reason, molecular testing such as ThyroSeq v2 or ThyGenX could be a valid aid to guide surgical management (total vs. hemithyroidectomy) and an accurate cytological diagnosis.

6. Suspicious for Malignancy-SFM

The morphological diagnosis of a nodule as SFM nodule is associated with a high PPV (of around 70% for malignancy), even though the description of NIFTP has lowered the malignancy risk to approximately 50% (range 45–60%) [1–18,52]. As a result, several authors confirmed the limited role of molecular need for SFM nodules [18–37,43,97–100]. Additionally, the ATA guideline recommendations highlighted that molecular testing should be carried out in SFM nodules, only if their results may induce changes in the surgical decision making and extent of surgery [53]. The detection of specific genetic alterations with ThyroSeq and/or ThyGenX/ThyraMIR may have both management and prognostic implications, including the extent of surgery, patient follow-up, and risk of recurrence. Nonetheless, the SFM category also includes malignancies other than PTC, and for that reason, immunocytochemistry can be diagnostically very helpful [100].

Nikiforov et al. diagnsoed 67 cases of SFM out of 1056 cases, finding that 51 had histological follow-up, including 54% with a malignant outcome [32]. Among them, 20 mutations were identified, including 19 malignant histological diagnoses (95%) harboring 10 BRAF ^{V600E}, seven RAS, one RET/PTC, and one PAX8/PPARγ mutations. In their series, RAS-*positive* nodule confirmed to be a benign FA. A cyto-histological correlation, combined with the results of mutational analysis on FNAC, demonstrated 68% sensitivity, 96% specificity, 81% diagnostic accuracy, 95% PPV, and 72% NPV [32]. It is important to underline that also in this category BRAF ^{V600E}, RET/PTC, and *PAX8/PPARγ* mutations were associated with malignancy in close to 100% of nodules.

Furthermore, different papers found that $BRAF^{V600E}$ mutation is strictly correlated to specific and distinctive morphological features including architectural and cellular features [105,106]. In detail, Rossi et al. linked mutated $BRAF^{V600E}$ cells with a morphological appearance of "plump features" defined by large polygonal tumor cells with cell height less than twice the width, and having squamoid-like metaplasia with homogeneous, eosinophilic, moderate to abundant cytoplasm, as well as sharing nuclear features of PTC [105,106]. Furthermore, the same authors discovered a peculiar nuclear shape (sickle-shaped feature), which was associated with 100% of $BRAF^{V600E}$ cases and lack in the *BRAF* wild type counterpart. These results were also confirmed by Kwon et al. in a series of 142 SM cases [41]. According to Jara et al., the detection of *BRAF* mutation in SFM nodules has an important implication for determining the extent of surgery [109]

The SFM category is likely to benefit from the introduction, in 2014, by Veracyte of two malignant classifiers to their testing: Afirma MTC and Afirma BRAF, which are both mRNA classifiers, similar to the Afirma GEC. [82–84]. In detail, the former is capable to identify the gene expression signature of MTC, the latter identifies the $BRAF^{V600E}$ mutation. These tests, as suggested by the Veracyte, might be useful for the SFM category or also cases in the positive for malignancy category. To note, the usefulness and role of the *BRAF* mutation is still controversial even though some authors correlated *BRAF* mutation with a more aggressive behavior (e.g., lymph node metastases and extra thyroid infiltration) [22,26,38,39,100].

7. Practical Approach to Indeterminate Lesions Using Also Molecular Testing

The adoption of an algorithm approach is likely to be a valid aid for the adequate management of indeterminate lesions and the identification of malignancy in thyroid nodules [50]. It is important to underline that the algorithm approach is based on the fact that morphology is and remains the central focus of cytological evaluation. In fact, the recognition of the morphological cytological features of thyroid nodules is a crucial first step. Furthermore, the morphological analysis is also able to identify some morphological aspects associated with $BRAF^{V600E}$ mutation, able to increase the ROM for those nodules

to 100%. The recognition of these morphological features straightens the number of cases for the molecular testing with a reduction of time and costs. Then, the use of NGS or small molecular panels is able to support the morphological features as reported in the single subchapters with the subclassification of indeterminate thyroid nodules.

8. Conclusions

In conclusion, it is clear that the correct classification and diagnosis of indeterminate lesions of the thyroid is still a challenge in cytopathology practice. Despite the fact that the evaluation of morphological features is able to solve the majority of diagnostic issues, in the field of indeterminate proliferations, morphology alone is not able to definitively classify all of these indeterminate lesions.

Ancillary molecular testing for indeterminate thyroid FNA has provided better risk stratification and reduced the need for diagnostic thyroid surgery. Different papers showed that several mutation analysis panels are both diagnostic tests and prognostic markers. As previously described, the different molecular testing, including the Afirma GSC, ThyroSeq, and ThyGenX/ThyroMIR are characterized by different advantages and limitations so that they might contribute to a more precise and tailored management [121–124]. Nevertheless, it is relevant to assess that molecular testing still represents only an adjunct to be discussed together with valuable clinical information (e.g., nodule ultrasound size and high-risk ultra-sonographic characteristics) and cytomorphology.

As reported by Livhits et al., in a series of 397 indeterminate lesions, the performance of both the RNA test and DNA-RNA test increased specificity and reduced by 49% the number of nodules to avoid diagnostic surgery [124]. Furthermore, neither the RNA test nor DNA-RNA test has statistically significant difference in performance, in sensitivity (100% vs. 97%, respectively), and specificity (80% vs. 85%, respectively) [124].

However, despite the unique support of molecular testing as an additional and precious tool, one of the limits can be represented by the costs of some of these molecular analyses. Labourier reported the cost-effectiveness of molecular testing in nodules with AUS/FLUS or FN/SFN cytology by using different management strategies: standard of care without molecular testing (StC), gene expression classifier (GEC), and mutation and miRNA testing (MMT). They concluded that molecular testing with high benign diagnostic yield can generate both positive health outcomes (less surgeries, 32%) and positive economic outputs (cost savings, 67%). These results are consistent with previously reported cost-utility data and provide valuable insights for informed decision making by patients, physicians, and payers [61].

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