Evaluation of *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARg* Alterations in Different Bethesda Diagnostic Categories: A Multicentric Prospective Study on the Validity of the 7-Gene Panel Test in 1172 Thyroid FNAs Deriving From Different Hospitals in South Italy

Claudio Bellevicine, MD, PhD [1]; Ilaria Migliatico, MD[1]; Roberta Sgariglia, PhD[1]; Mariantonia Nacchio, BS[1]; Elena Vigliar, MD, PhD[1]; Pasquale Pisapia, MD [1]; Antonino Iaccarino, PhD[1]; Dario Bruzzese, PhD[1]; Francesco Fonderico, MD[2]; Domenico Salvatore, MD[1]; Bernadette Biondi, MD, PhD[2]; Stefania Masone, MD, PhD[2]; Vincenzo Novizio, MD[3]; Francesco Scavuzzo, MD[3]; Domenico Serino, MD[3]; Maurizio De Palma, MD[4]; Maria Grazia Chiofalo, MD[5]; Gerardo Botti, MD[6]; Luciano Pezzullo, MD[5]; Vincenzo Nuzzo, MD[7]; Stefano Spiezia, MD[8]; Giovanni De Chiara, MD[9]; Sergio Iorio, MD[10]; Giovanni Conzo, MD[11]; Giovanni Docimo, MD[10]; Antongiulio Faggiano, MD, PhD[12]; Massimo Bongiovanni, MD, PhD [13]; Umberto Malapelle, PhD[1]; Annamaria Colao, MD, PhD[2]; Maria Triassi, MD[1]; and Giancarlo Troncone, MD, PhD [1]; for the Tiroide Network

BACKGROUND: Thyroid fine-needle aspiration (FNA) is a reliable and cost-effective diagnostic tool for establishing the nature of thyroid nodules, although up to 30% of FNAs are still classified as "indeterminate." Molecular testing of FNAs could improve preoperative diagnosis, thereby reducing unnecessary surgery. In this multicenter prospective study the authors investigated, using a 7-gene assay, the distribution and diagnostic impact of *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARg*, the most frequent genomic alterations occurring during thyroid oncogenesis. **METHODS:** In total, of 1172 routine FNAs from 7 centers in southern Italy were classified according to the Bethesda System for Reporting Thyroid Cytopathology. Each specimen was tested, and molecular data were compared with available histology or cytologic follow-up. **RESULTS:** In particular, for atypia of undetermined significance/follicular lesion of undetermined significance cases, the 7-gene test confirmed the high positive predictive value of *BRAF*V600E and *BRAF*-like mutations (80%) and the moderate positive predictive value of *RAS*-like alterations (32.4%), suggesting different surgical management, depending on the type of mutation. The rate of mutation-positive FNAs was strictly related to the risk of malignancy of each diagnostic class, supporting the identification of prognostically relevant diagnostic categories. **CONCLUSIONS:** The 7-gene panel test improves the preoperative risk stratification of indeterminate thyroid FNAs, especially when

Corresponding Author: Giancarlo Troncone, MD, PhD, Department of Public Health, University of Naples Federico II, Via S. Pansini 5, 80131, Naples, Italy (giancarlo.troncone@unina.it).

[1]Department of Public Health, University of Naples Federico II, Naples, Italy; [2]Department of Clinical Medicine and Surgery, University of Naples Federico II, Naples, Italy; [3]Department of Endocrinology, Aziena Ospedaliera di Rilievo Nazionale A. Cardarelli, Naples, Italy; [4]Department of Surgery, Aziena Ospedaliera di Rilievo Nazionale A. Cardarelli, Naples, Italy; [5]Thyroid and Parathyroid Surgery Unit, IRCCS G. Pascale, Naples, Italy; [6]IRCCS G. Pascale, Naples, Italy; [7]Department of Endocrinology, Hospital of the Sea-Azienda Sanitaria Locale Naples 1 Center, Naples, Italy; [8]Department of Endocrine Surgery, Hospital of the Sea-Azienda Sanitaria Locale Naples 1 Center, Naples, Italy; [9]Department of Pathology, Aziena Ospedaliera di Rilievo Nazionale San Giuseppe Moscati, Avellino, Italy; [10]Department of Medical, Surgical, Neurological, Metabolic, and Aging Sciences, University of Campania Luigi Vanvitelli, Caserta, Italy; [11]Department of Cardiothoracic and Respiratory Sciences, University of Campania Luigi Vanvitelli, Caserta, Italy; [12]Department of Experimental Medicine, University of Rome Sapienza, Rome, Italy; [13]Cytopathology, Synlab SA, Lausanne, Switzerland

We thank Paola Merolla for English language editing.

In addition to the authors listed, contributors from the TIRNET Network also include: Livia Barba, Marianna Cacciapuoti, Severo Campione, Pasquale Capasso, Salvatore Carrino, Eduardo Clery, Immacolata Cozzolino, Melania D'Anna, Gianfranco De Dominicis, Filippo De Rosa, Sergio Di Martino, Renato Franco, Diego Gerbasio, Raffele Gianattasio, Eduardo Iervolino, Franca La Vecchia, Carlo Antonio Leone, Gianluca Lombardi, Cristina Luongo, Vincenzo Marotta, Gilda Pontieri, Pasquale Sabatino, Silvia Savastano, Francesca Scognamiglio, and Antonio Sinisi.

Additional supporting information may be found in the online version of this article.

Received: October 4, 2019; **Revised:** November 5, 2019; **Accepted:** November 13, 2019

Published online December 10, 2019 in Wiley Online Library (wileyonlinelibrary.com)

DOI: 10.1002/cncy.22217, wileyonlinelibrary.com

considering the biologic significance of the different types of mutations. Moreover, the rate of mutation-positive FNAs is related to the risk of malignancy of each diagnostic class. ***Cancer Cytopathol* 2020;128:107-118**. © *2019 American Cancer Society*.

KEY WORDS: 7-gene test; cancer; cytopathology; fine-needle aspiration; molecular diagnostics; thyroid.

INTRODUCTION

Thyroid nodules constitute a very common endocrine disease.[1,2] Benign nodules, which are the most frequent, require clinical observation, whereas malignant (MAL) nodules generally undergo surgical resection. Therefore, differentiating between benign and MAL thyroid nodules is crucial in determining clinical management. Currently, thyroid fine-needle aspiration (FNA) represents the most reliable and cost-effective diagnostic tool for establishing the nature of thyroid nodules. Although the introduction of The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) has improved the consistency of diagnostic criteria,[3] FNAs can still give rise to a significant degree of diagnostic uncertainty because of overlapping cytomorphologic features between benign nodules and a subset of MAL nodules (eg, those exhibiting a microfollicular architecture).[4] In particular, TBSRTC identifies 3 indeterminate diagnostic classes, each with a different risk of malignancy (ROM). Atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) is a diagnostic class with a low ROM that usually us managed with FNA repetition, whereas follicular neoplasm/suspicious for follicular neoplasm (FN/SFN) and suspicious for malignancy (SFM) are TBSRTC categories with a higher ROM and are usually managed with surgery. Unfortunately, in the latter categories, postoperative histologic examinations may reveal benign nodules that could have been treated conservatively.[5]

To spare patients from unnecessary surgery, the recently updated TBSRTC has introduced molecular testing as a diagnostic adjunct to FNA cytopathology.[6] Both the US and European Thyroid Associations have promptly endorsed this recommendation, thereby underlining the importance of molecular tests on thyroid FNAs.[7-9] However, no consensus has yet been reached regarding the best molecular strategy. To date, several molecular tests are available, differing for their negative predictive value (NPV) and positive predictive value (PPV). These are also influenced by local factors, including the prevalence of thyroid neoplasm and the malignancy rates in each TBSRTC category of any individual center.[8,10,11] In addition, most molecular assays are proprietary, are centralized in the United States, and are not reimbursed by the European health systems.

As observed in other high-income countries, including in Italy, the overuse of ultrasound screening in asymptomatic patients has led to an increased detection of thyroid nodules and the overtreatment of low-risk papillary carcinomas.[12,13] Moreover, thyroid nodules and thyroid neoplasm are very common in the Campania region (southern Italy), where the high incidence is attributed in part to volcanic activity.[14] This higher incidence may be caused either by the ingestion of tap water containing volcanic mineral elements, such as vanadium, or by soil radioactivity.[15] Consequently, in our FNA practice over the last decade, we have experienced an increase in patients referred for FNA (65%; data not shown). Because FNA may yield indeterminate results, molecular tests can be useful tools to discriminate between patients with indeterminate thyroid FNA results who require surgery and those who may be treated more conservatively. However, to work at their best, molecular tests need to be not only accurate and reliable but also sustainable. To this end, samples should be tested locally and not outsourced overseas. Furthermore, although next-generation sequencing (NGS) is technically feasible even on thyroid cytology smears yielding low nucleic acid quantity/quality,[16] comprehensive thyroid cancer-specific gene panels (e.g., ThyroSeq) are currently limited to highly specialized reference laboratories.

Conversely, a feasible option is the 7-gene assay.[17] Covering the 7 most frequent genomic alterations occurring during thyroid oncogenesis (ie, *BRAF*V600E, *HRAS*, *NRAS*, *KRAS*, *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARg*), this assay exhibits a sensitivity and a specificity ranging from 18% to 100% and from 82% to 100%, respectively.[10] Such discrepant parameters may result from local differences in cancer prevalence and cytopathology practice patterns. Moreover, whereas *BRAF*V600E and *RET/PTC* mutations (grouped together as BRAF-like mutations) are highly specific for malignant outcomes, *RAS* and *RAS*-like mutations (*PAX8/PPARg* and no *BRAF*V600E alterations) are also detected in benign

thyroid nodules as follicular adenomas or low-risk neoplasms (ie, noninvasive follicular thyroid neoplasm with papillary-like nuclear features [NIFTP]).[18-20]

Therefore, in 2016, we set out to investigate whether the detection of *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARg* alterations in TBSRTC classes could improve the diagnostic accuracy of the indeterminate thyroid FNA categories. With this objective, we established a network called Tiroide Network (TIRNET) to coordinate the execution of molecular tests on thyroid FNAs across the main hospitals of the Campania region (southern Italy) that specialize in diagnosing and treating thyroid nodules. In this multicenter prospective study, we analyzed the results from the first 2 years of routine application of the 7-gene panel on 1172 thyroid FNAs and compared them with those from previous studies.

MATERIALS AND METHODS

Patients and Samples

In March 2016, we set up a regional network called TIRNET to coordinate the execution of 7-gene molecular testing on thyroid FNAs in the major hospitals of the Campania region (namely, Colli Hospital, Aziena Ospedaliera di Rilievo Nazionale A. Cardarelli, National Tumor Institute Pascale, San Giuseppe Moscati Hospital, Azienda Ospedaliera Universitaria Vanvitelli, and Hospital of the Sea-Azienda Sanitaria Locale Naples 1) and in our institution (University of Naples Hospital Federico II). Patients who presented with thyroid nodules and were referred for FNA underwent aspiration either at the above-mentioned hospitals or at our institution. The microscopic diagnosis was made by the local cytopathologists using TBSRTC categories. Because this study was prospective and was meant to assess the contribution of molecular testing in actual clinical practice, no cytologic or histologic revision was undertaken.

FNAs that fell into the nondiagnostic (class I) and benign (class II) TBSRTC categories were not referred for molecular testing. Thus, 1172 thyroid FNAs—featuring either AUS/FLUS (n = 755), FN/SFN (n = 140), SFM (n = 111), or MAL (n = 166) diagnoses—were tested. Mutational analysis of 162 FNA samples from this cohort was reported in our previous study.[17] Although current guidelines do not recommend testing SFM FNAs, because of its high ROM or MAL cases,[6,8] we deemed it necessary to do so to compare the distribution of the mutations across the different TBSRTC classes.

Generally, the efficacy of the 7-gene test in refining the diagnosis of thyroid FNAs classified as AUS/FLUS, FN/SFN, and SFM is verified by histology. However, because repeated FNAs are usually recommended for AUS/FLUS, in this study, we considered FNA repetition as a valid endpoint only when the repeated cytology outcome was benign. A similar approach has already been suggested by Brandler et al[21] and Eszlinger et al[22] for the audit of their FNA series. By contrast, cases with a persistent AUS/FLUS diagnosis or those featuring TBSRTC classes with a higher ROM but without an available histologic diagnosis were excluded.

Finally, the distribution of mutated FNAs in the indeterminate AUS/FLUS and FN/SFN categories was compared with the distribution reported in other studies.[22-25] The current study was approved by the Ethics Committee "Carlo Romano" of the University of Naples Federico II (protocol 155/15/ES1).

Detection of BRAF, RAS, RET/PTC, *and* PAX8/PPARg *Alterations*

Depending on the different practices adopted by the local hospitals in the TIRNET network, FNA biopsies were performed under ultrasound guidance by either endocrinologists or radiologists (possibly with rapid on-site adequacy assessment by a cytopathologist), or directly by interventional cytopathologists.[26] In a preclinical validation study performed with serially diluted samples harboring known mutations, the sensitivity of the 7-gene test (EntroGen, Inc) was detected at 1% (see Supporting Table 1 and Supporting Fig. 1). Moreover, the optimal method for collecting and processing the specimen for the molecular test was established and standardized across the different hospitals. Briefly, after FNA, smears for the microscopic diagnosis were prepared. Then, the performing physician collected an aliquot of the aspirated material in a vial of nuclease-free water (Invitrogen, Ambion; Thermo Fisher Scientific). In every hospital, vials were stored at −20°C until cytologic evaluation by local cytopathologist. In cases classified as AUS/FLUS, FN/SFN, SFM, and MAL, the vials referred from the other hospitals (external FNAs) were placed on dry ice zip-lock bags to avoid DNA and RNA degradation and transported to our molecular laboratory by a courier. In case of nondiagnostic or benign specimens, the vials were disposed of. The vials obtained from FNAs performed in our institution (internal FNAs) were kept unfrozen and further

processed. As previously reported,[17] DNA and RNA were simultaneously extracted using the AllPrep DNA/RNA Kit (Qiagen) and then analyzed by a real-time polymerase chain reaction (RT-PCR)-based procedure on the QuantStudio 5 platform (Applied Biosystems; Thermo Fisher Scientific) with the EntroGen Thyroid Cancer Mutation Analysis Panel Kit (EntroGen, Inc). This mutation panel is able to detect DNA point mutations in *BRAF*V600E, *KRAS* codons 12 and 13, *NRAS* codon 61, and *HRAS* codons 12, 13, and 61. Furthermore, it can detect RNA fusion mutations in *RET/PTC1* (fusion between the *RET* and *CCDC6* genes), *RET/PTC3* (fusion between the *RET* and *NCOA4* genes), and *PAX8/PPARg* fusions through a 1-step procedure that combines complementary DNA synthesis and RT-PCR. The resulting RT-PCR amplification curves were observed on QuantStudio design and analysis software (version 1.2; Thermo Fisher Scientific). Because we tested cytology specimens that can yield a limited amount of nucleic acid, we did not set a minimum requirement for either DNA or RNA. In fact, the samples were considered evaluable when the amplification cycle threshold was <35 cycles and were considered not evaluable when the PCR for 1 or more mutation probes failed, yielding mutation-negative FNAs in all other PCR probes. Samples showing amplification curves with a cycle threshold and/or shape of uncertain interpretation were tested on a more sensitive NGS platform (NGS Ion Torrent Personal Genome Machine; Thermo Fisher Scientific), as described elsewhere.[27] However, as shown in Supporting Table 1, the average DNA and RNA quantities yielded for the FNAs were 6.64 and 7.94 ng/μL, respectively. The results of molecular tests were communicated to all treating clinicians in the TIRNET Network.

Statistical Analysis

Numerical variables were described by using either mean ± SD values or medians with ranges (minimum-maximum) in case of skewed distribution. Categorical variables were summarized using absolute frequencies and percentages. Differences between groups were assessed accordingly by using the Student *t* test (for independent samples), the Mann-Whitney *U* test, and the chi-square test. A chi-square test for trend was also used to verify the association between the mutation-positive FNAs and the TBSRTC classes. The diagnostic performance of the 7-gene test in indeterminate FNAs was assessed by computing sensitivity, specificity, NPV, and PPV. Likelihood ratios with the corresponding 95% CIs were further computed.

To compare the FNA frequency and ROM of the TBSRTC classes in our cohort with those observed in the literature, a random effect meta-analysis of rates was performed. Heterogeneity was measured using the I^2 statistic, with values of 25%, 50%, and 75% indicating low, moderate and high heterogeneity, respectively. All statistical analyses were performed using the statistical platform R (R Core Team. *R. A Language and Environment for Statistical Computing. R Foundation for Statistical Computing*; 2018. Accessed September 2019. https://www.r-project.org/).

RESULTS

Distribution of Genomic Alterations and Inadequate Molecular Tests in Different TBSRTC Classes

Overall, 1172 thyroid FNAs were analyzed in the 2-year study period. The majority of patients referred for FNAs were female (76.4%), the mean age was 49.5 years (range, 10-84 years), and the median nodule size was 15 mm (range, 4-61 mm). FNAs yielded the following diagnostic results: 755 nodules were diagnosed as AUS/FLUS, 140 were diagnosed as FN/SFN, 111 were diagnosed as SFM, and 166 were diagnosed as MAL. Molecular testing was inadequate in 103 of 1172 samples (8.8%). However, only 10 of 103 samples (9.7%) revealed inadequate PCR results for both DNA and RNA; most inadequate FNAs were caused by insufficient RNA (91 of 103 samples; 88.3%). Regarding the rate of inadequate molecular results in each diagnostic class (93 of 755 samples [12.3% of AUS/FLUS FNAs]; 4 of 140 samples [2.8% of FN/SFN FNAs]; 5 of 111 samples [4.5% of SFM FNAs]; and 1 of 166 samples [0.6% of MAL FNAs]), most inadequate samples (12.3%) belonged to the AUS/FLUS category. Although a correlation between the inadequacy of these samples and their cellularity was not carried out, we argue that, because hypocellular FNA is classified more frequently as AUS/FLUS, at least several inadequate cases were because of the low cellular content of the specimens.

Six cases showed amplification curves with a cycle threshold and a shape of uncertain interpretation and were tested using NGS, as described above (see Materials and Methods). In 5 of these 6 cases, the uncertain

RT-PCR results reflected a low mutated allele frequency (2 *BRAF*V600E and 3 *NRAS* mutations), as demonstrated by NGS. In 1 case, the sequence of BRAF exon 15 revealed a *BRAF*K601E mutation.[17]

Of 1172 thyroid FNAs, 1069 (91.2%) yielded adequate molecular results. The distribution of the 7-gene test results in the different TBSRTC classes is summarized in Figure 1A. A statistically significant trend ($P < .001$) of mutation-positive FNAs was observed in TBSRTC classes: the frequency of mutation-positive cases increased from the lower ROM AUS/FLUS diagnostic class (139 of 662 FNAs; 21%) to the upper ROM MAL diagnostic class (135 of 165 FNAs; 81.8%). In addition, regarding the mutated FNAs, the high-risk *BRAF*V600E and *BRAF*-like mutations were noted more frequently in the higher ROM SFM (54 of 68 FNAs; 79.4%) and MAL (133 of 135 FNAs; 98.5%) diagnostic classes. By contrast, *RAS* and *RAS*-like alterations were more frequently observed in the lower risk AUS/FLUS (114 of 139 FNAs; 82%) and FN/SFN (27 of 37 FNAs; 73%) diagnostic classes ($P < .001$) (Fig. 1B).

Whereas mutation-positive FNAs were associated with younger age (44.8 ± 15.1 vs 52.1 ± 14.5 years; $P < .001$), the molecular results were similarly distributed across sexes ($P = .494$). A smaller nodule size was associated with a mutation-positive FNA (median, 14 mm [range, 4-60 mm] vs 16 mm [range, 5-61 mm]; $P \leq .001$). In particular, among mutation-positive FNAs, *BRAF*-like mutations were more frequent in nodules smaller than those harboring *RAS*-like alterations (median, 12 mm [range, 40-52 mm] vs 17 mm [range, 6-60 mm]; $P < .001$). Comparing our results with those obtained from previous studies in which the 7-gene test was adopted,[22-25] we noted a significant degree of heterogeneity in the distribution of mutated FNAs across the AUS/FLUS ($I^2 = 74\%$; $P = .004$) and FN/SFN ($I^2 = 72\%$; $P = .006$) categories (Fig. 2).

Diagnostic Performance of the 7-Gene Test in Indeterminate FNAs

The histologic diagnosis was available for 86 of 755 (11.4%) AUS/FLUS cases, 34 of 140 (24.3%) FN/SFN cases, and 57 of 111 (51.4%) SFM cases, whereas a benign cytologic diagnosis was available in 30 of 755 (3.9%) AUS/FLUS cases.

Among the 86 AUS/FLUS FNAs with available histology, the 7-gene test detected 46 of 86 (53.5%) mutated FNAs, including 9 mutations (6 *BRAF*, 1 *HRAS*, 1 *NRAS*, and 1 *PAX8-PPARg*) in 14 FNAs histologically diagnosed as papillary thyroid carcinomas (PTCs), classical variant (cvPTCs); 6 mutations (2 *BRAF*, 1 *HRAS*, 2 *NRAS*, and 1 *PAX8-PPARg*) in 10 FNAs diagnosed as PTCs, follicular variant (fvPTCs); 1 mutation (*NRAS*) in 1 FNA diagnosed as NIFTP; 1 mutation (*NRAS*) in 1 FNA histologically diagnosed as medullary thyroid carcinomas; 1 mutation (*NRAS*) in 1 FNA diagnosed as diffuse sclerosing variant PTC; 2 mutations (2 *HRAS*) in 3 FNAs histologically diagnosed as follicular thyroid carcinomas; only 26 mutations, mostly *RAS* or *RAS*-like (24 of 26 mutations; 92.3%) were retrieved in benign neoplastic and

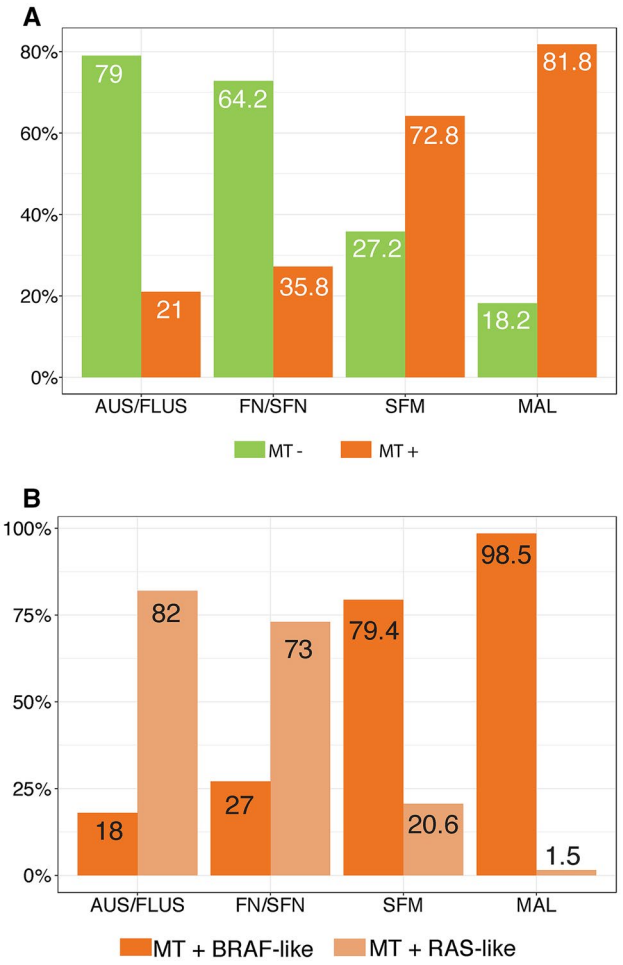

Figure 1. The percent distribution of (A) mutated and nonmutated fine-needle aspirations (FNAs) and (B) *BRAF*-like and *RAS*-like mutations is illustrated in different diagnostic categories according to The Bethesda System for Reporting Thyroid Cytopathology. AUS/FLUS indicates atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; MAL, malignant; MT–, mutation negative; MT+, mutation positive; SFM, suspicious for malignancy.

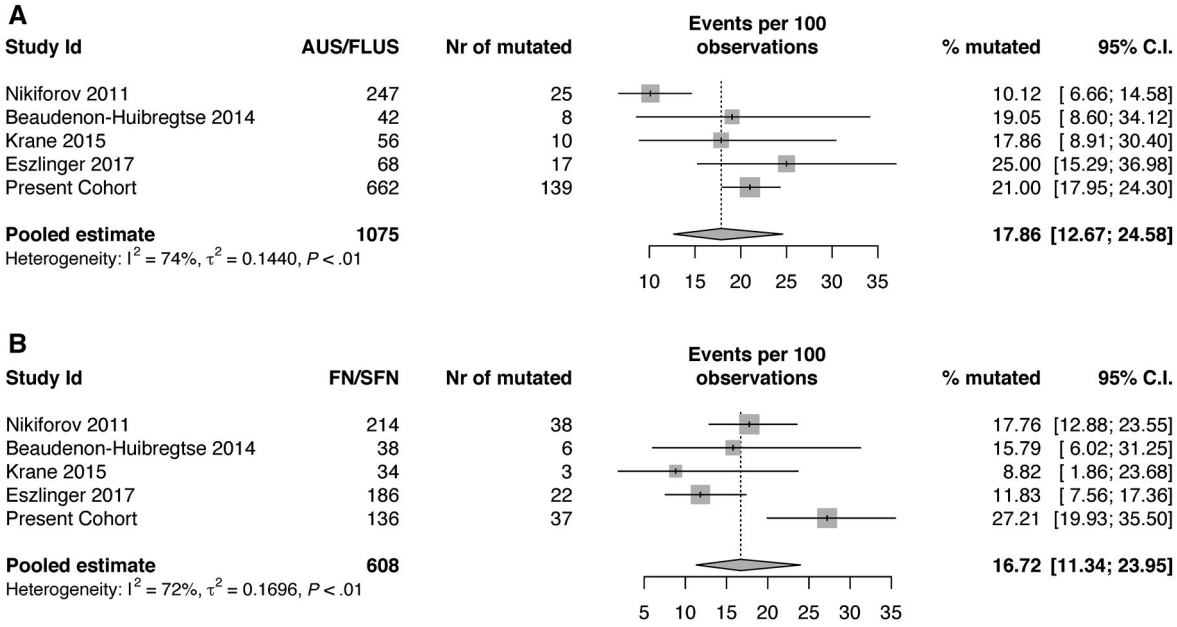

Figure 2. The distribution of mutated fine-needle aspirations (FNAs) classified (A) as atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) and (B) follicular neoplasm/suspicious for follicular neoplasm;(FN/SFN) from different series shows a high degree of heterogeneity.[22-25]

nonneoplastic nodules with histologic follow-up available (56 of 86 mutations; 65.1%) (see Table 1).

Of 34 FN/SFN FNAs with available histology, the 7-gene test detected 10 of 34 (29.4%) mutated FNAs, including 3 mutations (1 *BRAF*, 1 *HRAS*, and 1 *NRAS*) in 3 FNAs histologically diagnosed as cvPTCs, 5 mutations (2 *BRAF*, 1 *HRAS*, 2 *NRAS*) in 8 FNAs diagnosed as fvPTCs, 1 mutation (*NRAS*) in 9 FNAs diagnosed as follicular adenomas, Hurthle cell type, and 1 mutation (*NRAS*) in 1 FNA histologically diagnosed as adenomatous goiter.

Among the 57 SFM FNAs with available histology, the 7-gene test detected 31 of 57 (54.4%) mutated FNAs, including 25 mutations (24 *BRAF* and 1 *NRAS*) in 34 cvPTCs, 4 mutations (2 *BRAF* and 2 *NRAS*) in 9 fvPTCs, and 2 mutations (2 *BRAF*) in 2 PTCs (1 sclerosing and 1 cystic variant). Thus, the ROM associated with AUS/FLUS, FN/SFN, and SFM cytology was 25.9% (30 of 116 cases), 35.3% (12 of 34 cases), and 93% (53 of 57 cases), respectively.

The sensitivity, specificity, PPV, and NPV of the 7-gene test performed in the AUS/FLUS, FN/SFN, and SFM cases are shown in Table 2 and Figure 3. The PPV of the 7-gene test in AUS/FLUS and FN/SFN FNAs was 42.6% and 80% with an likelihood ratio of 1.85 (95% CI, 1.25-2.75) and 6.67 (95% CI, 1.69-26.35) and a sensitivity of 66.7%. The NPV was 82.8% in AUS/FLUS FNAs and 81.8% in FN/SFN FNAs, with a specificity of 64% and 90%, respectively (Fig. 2A,B). Given the high pretest ROM (93%) of SFM diagnoses, the PPV (100%) and the NPV (9.5%) were not informative over cytology alone (Table 2). It worth noting that, for the AUS/FLUS diagnoses, a significant statistical difference ($P = .01$) was observed between the PPVs of FNAs harboring *BRAF*V600E and *BRAF*-like alterations (80%) and the PPVs of FNAs harboring *RAS* and *RAS*-like mutations (32.4%). Irrespective of TBSRTC classes, *RAS* and *RAS*-like mutations were associated with malignant outcomes in 20 of 47 (42.6%) cases, whereas *BRAF*V600E and *BRAF*-like alterations resulted in malignant outcomes in 39 of 41 cases (95.1%). The histologic and cytologic available follow-up data with the respective molecular results are summarized in Table 1.

DISCUSSION

FNA is widely used to investigate the benign or malignant nature of thyroid nodules and thus can spare patients unnecessary surgery. Yet up to 30% of FNA samples fall into the indeterminate category—an aspect that critically undermines its role in establishing the need for

TABLE 1. Available Histologic and Cytologic Follow-Up With Respective Molecular Results

Histologic and Cytologic Follow-Up in AUS/FLUS FNAs	Results of the 7-Gene Test										Group of Mutations	
	BRAF	*HRAS*	*NRAS*	*KRAS*	*RET/PTC1*	*RET/PTC3*	*PAX8/PPARg*	MT–	NE (PCR Failed)	Total	*BRAF*-Like	*RAS*-Like
AUS/FLUS	10	14	18	1	1		3	58	11	116	10	37
cvPTC	6	1	1				1	5		14	6	3
fvPTC	2	1	2				1	4		10	2	4
PTC, sclerosing variant			1							1		1
FTC		2						1		3		2
MTC			1							1		1
NIFTP			1							1		1
FA	1 (K601E)	4	4					5		14		9
HFA	1	2	3					2	1	9	1	5
AN		2	5	1	1			16	4	29	1	8
HT		1					1	2		4		2
Benign follow-up		1						23	6	30		1

Histologic and Cytologic Follow-Up in FN/SFN FNAs	Results of the 7-Gene Test										Group of Mutations	
	BRAF	*HRAS*	*NRAS*	*KRAS*	*RET/PTC1*	*RET/PTC3*	*PAX8/PPARg*	MT–	NE (PCR Failed)	Total	*BRAF*-Like	*RAS*-Like
FN/SFN	3	2	5					22	2	32	3	7
cvPTC	1	1	1							3	1	2
fvPTC	2	1	2					3		8	2	3
FTC								1		1		
FA								7	1	8		
HFA			1					7	1	9		1
AN			1					3		4		1
HT								1		1		

Histologic and Cytologic Follow-Up in SFM FNAs	Results of the 7-Gene Test										Group of Mutations	
	BRAF	*HRAS*	*NRAS*	*KRAS*	*RET/PTC1*	*RET/PTC3*	*PAX8/PPARg*	MT–	NE (PCR Failed)	Total	*BRAF*-Like	*RAS*-Like
SFM	28		3					21	5	57	28	3
cvPTC	24		1					7	2	34	24	1
fvPTC	2		2					5		9	2	2
PTC, sclerosing variant	1									1	1	
PTC, cystic variant	1									1	1	
PTC, Warthin-Like oncocytic variant								1		1		
MTC								2		2		
NIFTP								1		1		
HTT								1	1	2		

TABLE 1. Continued

Histologic and Cytologic Follow-Up in SFM FNAs	Results of the 7-Gene Test								Group of Mutations	
	BRAF	*HRAS*	*NRAS*	*KRAS*	*RET/PTC1*	*RET/PTC3*	*PAX8/PPARg*	MT–	NE (PCR Failed)	Total	*BRAF*-Like	*RAS*-Like
Parathyroid carcinoma								1		1		
Hurthle cell carcinoma								1		1		
FA								1		1		
HFA								1		1		
AN									1	1		
HT									1	1		

Abbreviations: AN, adenomatous nodule; AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; cvPTC, classical papillary thyroid carcinoma; FA, follicular adenoma; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; FTC, follicular thyroid carcinoma; fvPTC, follicular variant papillary thyroid carcinoma; HFA, follicular adenoma, Hurthle cell type; HT, Hashimoto thyroiditis; HTT, hyalinizing trabecular tumor; MT–, mutation negative; MTC, medullary thyroid carcinoma; NE, not evaluable because of failed PCR; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; PCR, polymerase chain reaction; SFM, suspicious for malignancy.

conservative or surgical treatment. In the current multicenter study, we evidenced that the 7-gene panel test is a useful and reliable ancillary technique for improving the interpretations of indeterminate FNAs. In fact, in our FNA series, the performance of the 7-gene molecular test was in line with the results reported in the literature. More specifically, the sensitivity of the 7-gene test in both AUS/FLUS and FN/SFN FNAs (66.7%) was comparable to that observed by Nikiforov et al,[23] whereas the specificity of the test in FN/SFN FNAs (90%) was comparable to that reported by Eszlinger et al.[22] However, the specificity of mutation-positive FNAs in the AUS/FLUS category (64%) was below the value usually reported in other series, generally ranging from 82% to 100%.[22-25] As expected, such low specificity resulted in a relatively low PPV (42.6%) for the 7-gene test. This has been explained by the low specificity and variability of PPVs in *RAS* and *RAS*-like alterations,[28] which, in our series, were detected more frequently in diagnostic categories with a lower ROM. By contrast, the higher risk molecular *BRAF*V600E and *BRAF*-like alterations were significantly associated with TBSRTC classes that had a higher ROM (ie, SFM and MAL) (Fig. 1B). In fact, when *BRAF*-like and *RAS*-like mutation were considered separately in the AUS/FLUS category, PPV variability between the *BRAF*-like and *RAS*-like alterations (80% vs 32.4%, respectively; $P = .010$) was much more accentuated, justifying the need to manage patients according to the types of mutations. However, in our series, 2 cases that were cytologically classified as AUS/FLUS and harbored *BRAF* mutations were histologically benign (Table 2). One case had a *BRAF*K601E mutation that is biologically related to *RAS*-like mutations and also can be observed in benign neoplasms such as follicular adenoma.[29] Conversely, the second case harbored a *BRAF*V600E mutation that is practically pathognomonic of PTC. To ensure that this mutation was not a false-positive result, we also extracted DNA from the cells scraped from the original smear and confirmed the presence of a *BRAF*V600E mutation in the cytologic specimen. Nonetheless, the histology revealed only a 25-mm Hurthle cell follicular adenoma and post-FNA regressive changes. Although we have demonstrated that the *BRAF*V600E mutation was actually present in the DNA extracted from cells both suspended in the vial and smeared onto a microscopic slide, the absence of a finding of PTC on histology should be considered as a false-positive result from a clinical point of view. We

TABLE 2. Diagnostic Performance of the 7-Gene Test in Fine-Needle Aspirations Diagnosed as Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance, Follicular Neoplasm/Suspicious for Follicular Neoplasm, and Suspicious for Malignancy

FNA Diagnoses	No. of FNAs/Total No. (%)					Likelihood Ratio (95% CI)[a]
	Cytology-Only ROM	Sensitivity	Specificity	PPV	NPV	
AUS/FLUS, n = 116	30/116 (25.9)	20/30 (66.7)	48/75 (64.0)	20/47 (42.6)	48/58 (82.8)	1.85 (1.25-2.75)
FN/SFN, n = 34	12/34 (35.3)	8/12 (66.7)	18/20 (90.0)	8/10 (80.0)	18/22 (81.8)	6.67 (1.69-26.35)
SFM, n = 57	53/57 (93.0)	31/50 (62.0)	2/2 (100)	31/31 (100)	2/21 (9.5)	ND

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; FNA, fine-needle aspiration; ND, not defined; NPV negative predictive value; PPV, positive predictive value; ROM, risk of malignancy; SFM, suspicious for malignancy.

[a]A positive likelihood ratio approaches infinity as specificity is equal to 1, indicating that the molecular test was not informative over cytology alone. The statistical measures are based on a subset of cases, ie, surgically resected nodules and nodules with a benign diagnosis on a repeated FNA.

argue that this could be a case of "vanishing" thyroid tumor, in which FNA induced secondary changes that completely replaced the thyroid carcinoma.[30] In fact, Eze et al, in their classical series of vanishing thyroid tumors after FNA procedure, reported a *BRAF*V600E mutated FNA classified as AUS/FLUS (case 13 from their series) with a negative histology.[30] To also verify the vanishing tumor hypothesis from a molecular standpoint, we performed the test on the formalin-fixed, paraffin-embedded section of the thyroid lobe, including both the Hurthle cell adenoma and the area featuring regressive changes, and no mutations were found.

Finally, 1 AUS/FLUS case harboring a *RET/PTC* fusion resulted in a benign histology result (Table 2). In this case, it should be mentioned that, even if an *RET/PTC* fusion and a *BRAF*V600E mutation are biologically related, the former genomic alteration was also retrieved in benign thyroid lesions.[31] Regarding the SFM category, application of the 7-gene test did not provide any additional clinically useful information for the patient. According to these results, we also observed that the increasing frequency of mutation-positive FNAs was associated with an increased ROM of TBSRTC classes from AUS/FLUS to MAL (Fig. 1A), supporting the establishment of prognostically relevant cytologic diagnostic categories from a molecular standpoint. The association between mutation-positive FNA frequency and ROM has also been observed in various institutions applying the 7-gene test to TBSRTC framework.[22-25] The rate of mutation-positive FNAs is directly proportional to the prevalence of cancer. The significant degree of heterogeneity in the distribution of mutated FNAs in the AUS/FLUS ($I^2 = 74\%$; $P = .004$) and FN/SFN ($I^2 = 72\%$; $P = .006$) categories (Fig. 2) depends on the prevalence of cancer among TBSRTC classes from different studies. In fact, as shown in Table 3,[22-25] the tight association between the rate of mutation-positive FNAs and the ROM for TBSRTC diagnostic classes is evident in the current and other series, also revealing the local variability in the use of Bethesda microscopic criteria, which, in turn, may explain the variable sensitivity, specificity, PPVs, and NPVs in different FNA series that adopted the 7-gene test.[10] That being said, one may argue that thyroid molecular testing combined with the usual quality metrics used in thyroid cytopathology (ie, the AUS:MAL ratio and an AUS/FLUS rate < 10%)[32] could definitely be used as an additional performance measure.

Interestingly, we observed that FNAs with *RAS* and *RAS*-like mutations were obtained from nodules that were larger than those harboring *BRAF*V600E and *BRAF*-like alterations—a finding in line with other experiences suggesting that *RAS*-mutated nodules grow at a faster rate.[33,34]

We acknowledge that the current study suffers from 2 limitations. The first is in regard to the relatively low number of cases with available follow-up. For AUS/FLUS cases, this was because only a minority of these patients underwent excision, and those who were referred for repeat FNAs may have chosen institutions that were not included in our hospital network. Moreover, because the treating physicians were aware of the molecular test results, at least some of these patients may have deferred surgery on the basis of a mutation-negative outcome, limiting our ability to determine true-negative versus false-negative outcomes of the 7-gene test.

The other limitation is that we were unable to study the effect of the reclassification of encapsulated fvPTCs as NIFTPs on the variation in both the ROM and the 7-gene test diagnostic performance because

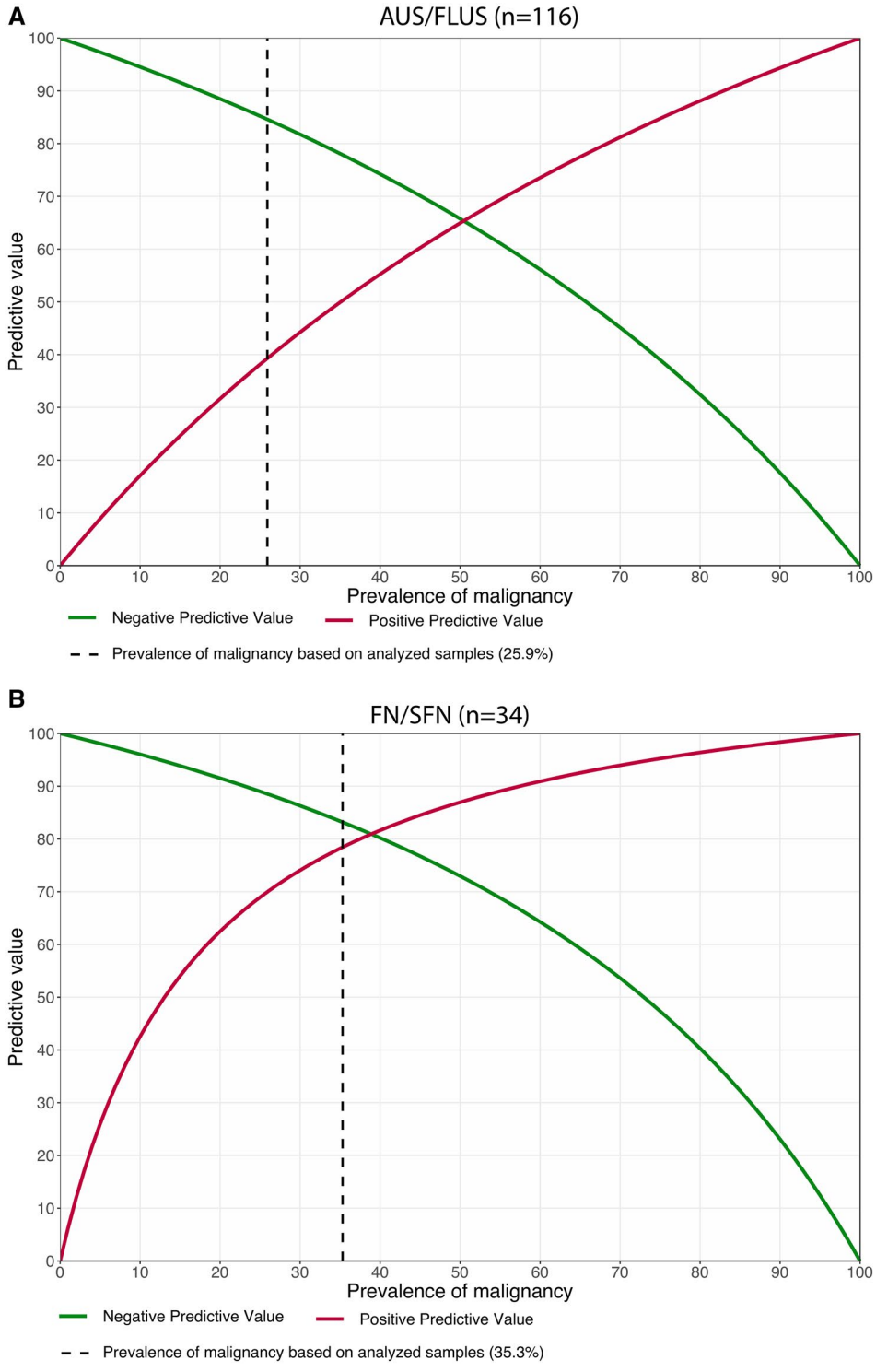

Figure 3. Positive and negative predictive values of the 7-gene test applied to fine-needle aspirations (FNAs) diagnosed as (A) atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) and (B) follicular neoplasm/suspicious for follicular neoplasm (FN/SFN) are illustrated.

only 2 NIFTPs were retrieved from surgical follow-up. Despite these limitations, our study has demonstrated that the 7-gene test is a useful ancillary diagnostic approach to guide the clinical management of patients who have thyroid FNA diagnoses that fall into the indeterminate cytology categories.

TABLE 3. Mutation Frequency and the Risk of Malignancy in Atypia of Undetermined Significance/Follicular Lesion of Undetermined Significance and Follicular Neoplasm/Suspicious for Follicular Neoplasm Fine-Needle Aspirations in Different Series

Series	% Mutated	% ROM[a]
Current cohort		
AUS/FLUS	21.00	25.90
FN/SFN	27.20	35.30
Nikiforov 2011[23]		
AUS/FLUS	10.12	14.00
FN/SFN	17.76	27.00
Beaudenon-Huibregtse 2014[24]		
AUS/FLUS	19.05	50.00
FN/SFN	15.79	32.00
Krane 2015[25]		
AUS/FLUS	17.86	33.00
FN/SFN	8.82	26.00
Eszlinger 2017[22]		
AUS/FLUS	25.00	15.00
FN/SFN	11.83	17.00

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; ROM, risk of malignancy.
[a]The ROM refers to the prevalence of cancer among resected nodules for each Bethesda category.

Conclusions

In the current study, we reported the results from a 2-year prospective application of the 7-gene test in the routine diagnostic setting of different centers in southern Italy. The mutation rates detected in thyroid FNAs were associated with the ROM in each diagnostic class, which, in turn, may be related to the local variability in the use of Bethesda microscopic criteria. Nonetheless, the 7-gene test may represent a valid adjunct technique to risk-stratification analyses based solely on microscopic criteria. This would be especially recommended when physicians are confronted with the challenging interpretation of AUS/FLUS and FN/SFN results and when different types of mutations that have different associations with the occurrence of MAL neoplasms—ie, *BRAF*-like versus *RAS*-like mutations—are considered.

FUNDING SUPPORT

This study was supported by the Campania Region for the Investigation of the Molecular Biology of Thyroid Cancer (grant LR n.24 29/12/2005).

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

AUTHOR CONTRIBUTIONS

Claudio Bellevicine: Conceptualization, data curation, investigation, methodology, project administration, writing–original draft, and writing–review and editing. **Ilaria Migliatico:** Data curation, methodology, formal analysis, and writing–original draft. **Roberta Sgariglia** and **Mariantonia Nacchio:** Data curation, investigation, and validation. **Elena Vigliar:** Investigation and visualization. **Pasquale Pisapia:** Data curation. **Antonino Iaccarino:** Investigation. **Dario Bruzzese:** Data curation, methodology, and formal analysis. **Francesco Fonderico**, **Domenico Salvatore**, **Bernadette Biondi**, **Stefania Masone**, **Vincenzo Novizio**, **Francesco Scavuzzo**, **Domenico Serino**, **Maurizio De Palma**, **Maria Grazia Chiofalo**, **Gerardo Botti**, **Luciano Pezzullo**, **Vincenzo Nuzzo**, **Stefano Spiezia**, **Giovanni De Chiara**, **Sergio Iorio**, **Giovanni Conzo**, **Giovanni Docimo**, and **Antongiulio Faggiano:** Data curation and investigation. **Massimo Bongiovanni:** Validation and writing–review and editing. **Umberto Malapelle:** Investigation, methodology, resources, software, and validation. **Annamaria Colao:** Supervision. **Maria Triassi:** Supervision and funding acquisition. **Giancarlo Troncone:** Conceptualization, data curation, investigation, methodology, project administration, supervision, writing–original draft, and writing–review and editing.

REFERENCES

1. Mazzaferri EL, Mazzaferri EL. Management of a solitary thyroid nodule. *N Engl J Med*. 1993;328:553-559.
2. Guth S, Theune U, Aberle J, Galach A, Bamberger CM. Very high prevalence of thyroid nodules detected by high frequency (13 MHz) ultrasound examination. *Eur J Clin Invest*. 2009;39:699-706.
3. Cibas ES, Ali SZ. The Bethesda System for Reporting Thyroid Cytopathology. *Am J Clin Pathol*. 2009;132:658-665.
4. Kocjan G, Chandra A, Cross PA, et al. The interobserver reproducibility of thyroid fine-needle aspiration using the UK Royal College of Pathologists' classification system. *Am J Clin Pathol*. 2011;135:852-859.
5. Nishino M, Wang HH. Should the thyroid AUS/FLUS category be further stratified by malignancy risk? *Cancer Cytopathol*. 2014;122:481-483.
6. Cibas ES, Ali SZ. The 2017 Bethesda System for Reporting Thyroid Cytopathology. *Thyroid*. 2017;27:1341-1346.
7. Haugen BR, Alexander EK, Bible KC, et al. 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*. 2016;26:1-133.
8. Ferris RL, Baloch Z, Bernet V, et al. American Thyroid Association statement on surgical application of molecular profiling for thyroid nodules: current impact on perioperative decision making. *Thyroid*. 2015;25:760-768.
9. Paschke R, Cantara S, Crescenzi A, Jarzab B, Musholt TJ, Sobrinho Simoes M. European Thyroid Association guidelines regarding thyroid nodule molecular fine-needle aspiration cytology diagnostics. *Eur Thyroid J*. 2017;6:115-129.
10. Nishino M. Molecular cytopathology for thyroid nodules: a review of methodology and test performance. *Cancer Cytopathol*. 2016;124:14-27.
11. Valderrabano P, Leon ME, Centeno BA, et al. Institutional prevalence of malignancy of indeterminate thyroid cytology is necessary but insufficient to accurately interpret molecular marker tests. *Eur J Endocrinol*. 2016;174:621-629.
12. Dal Maso L, Panato C, Franceschi S, et al. The impact of overdiagnosis on thyroid cancer epidemic in Italy, 1998-2012. *Eur J Cancer*. 2018;94:6-15.
13. Welch HG, Doherty GM. Saving thyroids—overtreatment of small papillary cancers. *N Engl J Med*. 2018;379:310-312.

14. Biondi B, Arpaia D, Montuori P, et al. Under the shadow of Vesuvius: a risk for thyroid cancer? *Thyroid*. 2012;22:1296-1297.
15. Pellegriti G, De Vathaire F, Scollo C, et al. Papillary thyroid cancer incidence in the volcanic area of Sicily. *J Natl Cancer Inst*. 2009;101:1575-1583.
16. Bellevicine C, Sgariglia R, Malapelle U, et al. Young Investigator Challenge: can the Ion AmpliSeq Cancer Hotspot Panel v2 be used for next-generation sequencing of thyroid FNA samples? *Cancer Cytopathol*. 2016;124:776-784.
17. Bellevicine C, Sgariglia R, Migliatico I, et al. Different qualifiers of AUS/FLUS thyroid FNA have distinct BRAF, RAS, RET/PTC, and PAX8/PPARg alterations. *Cancer Cytopathol*. 2018;126:317-325.
18. Agrawal N, Akbani R, Aksoy BA, et al. Integrated genomic characterization of papillary thyroid carcinoma. *Cell*. 2014;159:676-690.
19. Medici M, Kwong N, Angell TE, et al. The variable phenotype and low-risk nature of RAS-positive thyroid nodules. *BMC Med*. 2015;13:184.
20. Paulson VA, Shivdasani P, Angell TE, et al. NIFTP accounts for over half of "carcinomas" harboring RAS mutations. *Thyroid*. 2017; 27:506-511.
21. Brandler TC, Aziz MS, Coutsouvelis C, et al. Young Investigator Challenge: atypia of undetermined significance in thyroid FNA: standardized terminology without standardized management—a closer look at repeat FNA and quality measures. *Cancer Cytopathol*. 2016;124:37-44.
22. Eszlinger M, Bohme K, Ullmann M, et al. Evaluation of a two-year routine application of molecular testing of thyroid fine needle aspirations (FNA) using a 7-gene-panel in a primary referral setting in Germany. *Thyroid*. 2017;27:402-411.
23. Nikiforov YE, Ohori NP, Hodak SP, et al. 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*. 2011;96:3390-3397.
24. Beaudenon-Huibregtse S, Alexander EK, Guttler RB, et al. Centralized molecular testing for oncogenic gene mutations complements the local cytopathologic diagnosis of thyroid nodules. *Thyroid*. 2014;24:1479-1487.
25. Krane JF, Cibas ES, Alexander EK, Paschke R, Eszlinger M. Molecular analysis of residual ThinPrep material from thyroid FNAs increases diagnostic sensitivity. *Cancer Cytopathol*. 2015;123:356-361.
26. Bellevicine C, Vigliar E, Malapelle U, et al. Cytopathologists can reliably perform ultrasound-guided thyroid fine needle aspiration: a 1-year audit on 3715 consecutive cases. *Cytopathology*. 2016;27:115-121.
27. Malapelle U, Mayo de-Las-Casas C, Rocco D, et al. Development of a gene panel for next-generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients. *Br J Cancer*. 2017;116:802-810.
28. Kim BW. The positive predictive value of *RAS* mutations in cytologically indeterminate thyroid nodules has considerable unexplained variability across studies. *Clin Thyroidol*. 2018;30:269-272.
29. Afkhami M, Karunamurthy A, Chiosea S, et al. Histopathologic and clinical characterization of thyroid tumors carrying the $BRAF^{K601E}$ M mutation. *Thyroid*. 2016;26:242-247.
30. Eze OP, Cai G, Baloch ZW, et al. Vanishing thyroid tumors: a diagnostic dilemma after ultrasonography-guided fine-needle aspiration. *Thyroid*. 2013;23:194-200.
31. Najafian A, Noureldine SI, Azar F, et al. RAS mutations, RET/PTC and PAX8/PPAR-gamma chromosomal rearrangements are also prevalent in benign thyroid lesions? Implications thereof and a systematic review. *Thyroid*. 2017;27:39-48.
32. Krane JF, VanderLaan PA, Faquin WC, Renshaw AA. The atypia of undetermined significance/follicular lesion of undetermined significance:malignant ratio. *Cancer Cytopathol*. 2012;120:111-116.
33. Kakarmath S, Heller HT, Alexander CA, et al. Clinical, sonographic, and pathological characteristics of RAS-positive versus BRAF-positive thyroid carcinoma. *J Clin Endocrinol Metab*. 2016; 101:4938-4944.
34. Puzziello A, Guerra A, Murino A, et al. Benign thyroid nodules with RAS mutation grow faster. *Clin Endocrinol (Oxf)*. 2016;84:736-740.