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Retrospective cytological evaluation of indeterminate thyroid nodules according to the British Thyroid Association 2014 classification and comparison of clinical evaluation and outcomes

Massimo GIUSTI[†1], Barbara MASSA[2], Margherita BALESTRA[1], Paola CALAMARO[2], Stefano GAY[1], Simone SCHIAFFINO[3], Giovanni TURTULICI[3], Simonetta ZUPO[4], Eleonora MONTI[1], Gianluca ANSALDO[5]

([1]*Endocrinology Unit, San Martino University Hospital, I-16100 Genoa, Italy*)

([2]*Cytopathology and Pathology Unit, San Martino University Hospital, I-16100 Genoa, Italy*)

([3]*Radiology Unit, Evangelico Hospital, I-16100 Genoa, Italy*)

([4]*Molecular Diagnostic Unit, San Martino University Hospital, I-16100 Genoa, Italy*)

([5]*Endocrinology Surgery Unit, San Martino University Hospital, I-16100 Genoa, Italy*)

[†]E-mail: magius@unige.it; omsv1416@gmail.com

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Abstract: The cytology of 130 indeterminate nodules (Thy 3) was retrospectively reviewed according to the British Thyroid Association 2014 classification. Nodules were divided into Thy 3a (atypical features) and Thy 3f (follicular lesion) categories. Histology was available as a reference for 97 nodules. Pre-surgical evaluations comprised biochemical tests, color-Doppler ultrasonography (US), semi-quantitative elastography-US (USE), contrast-enhanced US (CEUS), and mutation analysis from cytological slides. Thyroid malignancy was the final diagnosis for 19% of surgically-treated nodules. No statistically significant difference in the risk of malignancy was found between Thy 3a (26%) and Thy 3f (14%) nodules. Histology of the Thy 3a and Thy 3f nodules showed a higher incidence of Hurtle cell adenomas in Thy 3f (29%) than in Thy 3a (3%) nodules (P=0.01). The only pre-surgical difference concerned the *BRAF* V600E mutation, which was positive in some Thy 3a but not in any Thy 3f nodules (P=0.04). Receiver-operating characteristic (ROC) analysis was used to obtain cut-off values from US (score), USE (ELX 2/1 strain index), and CEUS (time-to-peak index and peak index) data. The cut-off values were similar for Thy 3a and Thy 3f nodules. Data showed that malignancy can be suspected if the US score is >2, ELX 1/2 strain index >1, time-to-peak index >1, and peak index <1. In a sub-group of 24 revised nodules (12 Thy 3a and 12 Thy 3f) with histology as a reference, the diagnostic power of cumulative pre-surgical analysis by means of US, USE, and CEUS showed high positive and negative predictive values (83% and 100%, respectively) for the presence of malignancy in Thy 3a and Thy 3f nodules. In conclusion, in our series of revised Thy 3 nodules, malignancy was low and displayed no significant differences between Thy 3a and Thy 3f categories. The use of cut-offs based on histology as a reference could reduce surgery. Our data support the conviction that, in mutation-negative Thy 3a and Thy 3f nodules, observation should be the first choice when not all instrumental results are suspect.

Key words: Indeterminate thyroid nodules; British Thyroid Association 2014 classification; Clinical evaluation; Outcome

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1 Introduction

Evaluation of thyroid nodules by means of fine-needle aspiration biopsy (FNAB) under ultrasonography (US) guidance is recognized as the best

ORCID: Massimo GIUSTI, http://orcid.org/0000-0002-1269-8376

diagnostic tool for distinguishing malignant from benign lesions (Castro and Gharib, 2003; Cooper *et al*., 2009). The ultimate aim of FNAB is to reassure the patient and to avoid surgery, if not otherwise indicated (Castro and Gharib, 2003). It is essential that cytopathologists and endocrinologists use a standardized reporting system for thyroid FNABs in order to communicate findings more accurately and clearly. The Bethesda System for Reporting Thyroid Cytopathology (BSRTC) (Cibas and Ali, 2009; Jo *et al*., 2010), Japan Thyroid Association (JTA) Reporting System (Kakudo *et al*., 2014), and British Thyroid Association (BTA) classification (BTA, 2014) are the most widely used systems. All of these classifications now split cytological results into several diagnostic categories, each of which is associated with a different cancer risk, ranging from "probably absent" to "highly probable", and with different evidence-based clinical management (Cibas and Ali, 2009; Jo *et al*., 2010; BTA, 2014; Kakudo *et al*., 2014). The main limitation of FNAB is that it may yield non-diagnostic and indeterminate results. Ideally, the rate of non-diagnostic results should be less than 10% (Cibas and Ali, 2009) of all FNAB, but rates as high as 20% are sometimes reported (Alexander *et al*., 2002) and the management of these cases requires integrated clinical, cytological, and US re-evaluation (Moon *et al*., 2015). Different percentages of indeterminate results are reported in the literature. In a meta-analysis of 25445 thyroid FNAB samples from 8 studies using the BSRTC, indeterminate results accounted for 20%; 10% of all samples were diagnosed as category III (atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS)) and 10% as category IV (follicular neoplasm/suspicious for follicular neoplasm (FN/SFN)) (Bongiovanni *et al*., 2012). In a cohort of 3843 FNAB, scored according to the cytological reporting system recommended by the JTA, indeterminate lesions were 10%, but only a few cases were sub-classified from "probably benign" to "probably malignant" in order to evaluate the reproducibility of this classification (Kakudo *et al*., 2015). Finally, in a cohort of 100065 patients with nodules scored according to the BTA classification, an indeterminate Thy 3 category was reported in 5446 (5.4%) (Rago *et al*., 2014). In a sub-group of 1520 fully evaluable Thy 3 nodules, Rago *et al*. (2014) reported that 77% were re-classified as Thy 3A (equivalent to BSRTC category III) and 33% as Thy 3B (equivalent to BSRTC category IV). The sub-classification of indeterminate lesions seems to be crucial in order to define the risk of malignancy. According to the 2009 explanatory notes issued by Cibas and Ali (2009), in the BSRTC classification the risk of malignancy increased more than doubles on passing from diagnostic criteria III (5%–15%) to diagnostic criteria IV (15%–30%), though more recent papers have indicated very different ranges. In the meta-analysis by Bongiovanni *et al*. (2012), the overall value of malignancy was reported to be 16% according to diagnostic criteria III and 26% according to diagnostic criteria IV, but cytological-histological correlation was available for only about 70% of nodules. In studies in which cytological-histological correlation has been available in BSRTC category III lesions, the risk of malignancy has been seen to fluctuate widely, from 19% to 77% (Dincer *et al*., 2013; Choi *et al*., 2014; Cuhaci *et al*., 2014; Ho *et al*., 2014; Hyeon *et al*., 2014; Rosario, 2014; Yoon *et al*., 2014; Deniwar *et al*., 2015; Kapila *et al*., 2015; Yoo *et al*., 2015). Also, there was a significant increase in the malignancy rate from sub-category FLUS to sub-category AUS in some (Choi *et al*., 2014), but not all (Cuhaci *et al*., 2014), investigations. By contrast, in BSRTC category IV, the risk of malignancy (27%–34%) reported in the most recent literature (Ohori *et al*., 2013; Nikiforov *et al*., 2014; Deniwar *et al*., 2015; Kapila *et al*., 2015) seems to be closer to that previously observed (Cibas and Ali, 2009).

There are fewer reports of risk stratification in the BTA sub-classification. Deandrea *et al*. (2010) stratified 294 Thy 3 nodules, for which the malignancy rate was 17%, into three categories: "follicular lesion of indeterminate significance", "follicular neoplasm", and "Hurtle-cell neoplasm". They found that the percentage of malignant cases in each category was significantly different (5%, 25%, and 23%, respectively). More recently, Rago *et al*. (2014) reported a significant increase in the risk of malignancy between Thy 3A (or Thy 3a) (19%) and Thy 3B (or Thy 3f) (41%).

In indeterminate categories, pre-surgical evaluations for risk management now comprise biochemical tests, color-Doppler US, semi-quantitative elastography-US (USE), contrast-enhanced US (CEUS), and mutation analysis carried out on cytological slides. To our knowledge, no studies have yet focused on risk management of Thy 3 nodules

sub-classified as Thy 3a (atypical features) or Thy 3f (follicular lesion) (BTA, 2014). Only Rago *et al*. (2014) reported a significant correlation between US features and Thy 3B cytology.

Recently, we assessed the diagnostic power of cumulative pre-surgical analysis by means of US, USE, and CEUS in Thy 3 nodules (Giusti *et al*., 2014). In the present study, nodules initially classified as Thy 3 were retrospectively evaluated after their re-classification into Thy 3a and Thy 3f categories. A new, refined, histology-based cut-off is now available for risk stratification.

2 Materials and methods

2.1 Patients

This was a retrospective study. Our database was reviewed in order to pick out patients who had been diagnosed from January 2011 to December 2014 as having a category Thy 3 (indeterminate lesion) according to "The British Thyroid Association Guidelines for the management of thyroid cancer in adults classification" (BTA, 2007). During this period, FNAB was performed on 1932 nodules: 9% of nodules were diagnosed as Thy 3; 10% were non-diagnostic (Thy 1), 76% were benign (Thy 2), 2% were suspicious for malignancy (Thy 4), and 3% were malignant (Thy 5). The slides of 133 Thy 3 nodules (80% of Thy 3 records) were reviewed according to the BTA 2014 classification, which subdivides the Thy 3 category into two sub-classes: Thy 3a and Thy 3f (BTA, 2014). In 20% of cases, slides were no longer available in our cytological bank, while in three of the cases reviewed, the available slides were not adequate for application of the new cytological classification. Thus, 130 Thy 3 nodules were reviewed; the new Thy classification was compared with US features (score) and with the results of molecular biology analysis carried out on FNAB material when available, USE, and CEUS. This final study group consisted of 102 females and 28 males with an age range of 18–86 years (mean, (55.6±14.5) years) at the time of FNAB. All these patients were considered candidates for surgery in accordance with the cytological results. In 55% of these patients, the nodules were found in a multinodular goiter, while 45% had a uninodular goiter. In 23 patients, laboratory data were compatible with Hashimoto's thyroiditis, while 2 patients had a single nodule in a diffuse toxic goiter. Levo-thyroxine was being taken by 18% of patients, either for hypothyroidism (n=9) or as a thyroid stimulating hormone (TSH)-reducing therapy (n=14). Six patients were on methimazole therapy for pre-toxic muitinodular goiter (n=4) or toxic diffuse goiter. Data collection and subsequent analysis were performed in compliance with the Helsinki declaration and approved by the University of Genoa Ethics Committee.

2.2 Thyroid function tests

TSH and free-T4 (f-T4) were measured, as already reported (Giusti *et al.*, 2014), by means of ultrasensitive chemiluminescence immunoassay (Roche Diagnostics, Mannheim, Germany). Normal ranges are 0.3–4.2 mIU/L for TSH and 12.0–22.0 pmol/L for f-T4. Thyroperoxidase antibodies (TPOAbs) were evaluated by means of the Dia Sorin assay (Saluggia, Italy); concentrations <100 mIU/L were regarded as negative. Serum calcitonin (CT) was assayed by chemiluminescence immunoassay (Dia Sorin); in our laboratory, the upper limit of the normal CT range is 10 ng/L (Giusti *et al.*, 2014).

2.3 Thyroid US, USE, and CEUS

All patients were examined as previously reported (Giusti *et al*., 2013; 2014). Nodules were examined by means of conventional high-resolution US with a color-Doppler module (MyLab Five, Esaote Biomedica, Genoa, Italy) equipped with a 7.5 MHz linear probe. In accordance with US guidelines (Moon *et al*., 2011), the following parameters were investigated: echogenicity vs. non-nodular tissue, presence or absence of a halo sign, presence or absence of microcalcifications, and presence or absence of an inner nodular flow pattern. All USE examinations were performed by the same radiologist (GT) with more than five years of experience by means of a MyLab 70 XvG US scanner (Esaote Biomedica) equipped with an LA-522 linear probe working in the range of 7–12 MHz and software for the quantification of the USE features of the tissue. The elasticity score (ELX 2/1) index was calculated at the same depth as the ratio between the elasticity feature of the selected region-of-interest (ROI) located on US-normal thyroid tissue and the ROI of the nodule under investigation. As previously reported (Giusti *et al*., 2013), we considered that the ELX 2/1 index directly reported on the screen of the equipment.

CEUS images were acquired by the MyLab 70 US scanner, as previously reported (Giusti *et al*., 2013), by using a non-destructive US mode after bolus injection of SonoVue (4.8 ml; Bracco, Milan, Italy). CEUS video-clips were digitally recorded and analyzed by means of Q-Contrast software V.4.0. (Bracco). Time-intensity curves within selected ROI and color maps were acquired. Nodule and healthy thyroid tissue values of peak contrast enhancement and time-to-peak (TTP) were calculated. Peak and TTP are reported as indexes (P index, TTP index) derived from the ratio between the values from the ROI of the nodule and the ROI of normal thyroid tissue (Giusti *et al.*, 2013).

2.4 FNAB cytology and histology

US-assisted FNAB was performed by an endocrinologist (MG) with more than 20 years of experience by means of a 22-gauge needle attached to a 10-ml syringe. On average, two passes were performed for each nodule. Aspirates were smeared onto glass slides, air-dried, and stained with My Grunwald-Giemsa, and fixed with CytoLyt (Marlborough, MA, USA), and stained with Papanicolau. All cytological samples were evaluated by the same pathologist (BM) with more than 10 years' experience in the pathologic analysis of thyroid cancer. The same pathologist performed the re-evaluation of FNAB samples in comparison with the initial Thy 3 cytological diagnosis. The results of cytological re-evaluation are reported according to the BTA 2014 classification (BTA, 2014); the Thy 3a category was assigned when atypical features were present but not sufficient to assign the specimen to any of the other categories. This category includes specimens with increased cellularity with small uniform follicular organization and scant colloid, follicular cells with small nuclei, and regularly distributed chromatin. The Thy 3f category was assigned when a follicular neoplasm was suspected, but did not present sufficient features for inclusion in the Thy 4 category (suspicious for malignancy). The Thy 3f category includes specimens with hyper cellularity and absence of colloid, well-defined nests, rarely with overlapping nuclei, follicular cells with enlarged nuclei, sparse or irregularly distributed chromatin, rare prominent nucleoli and occasional mitoses. Thy 3a and Thy 3f categories correspond to Bethesda category III (AUS/FLUS) and Bethesda category IV (FN/SFN) neoplasm (Bongiovanni *et al*., 2012). Cytological re-evaluation was performed in a blind condition versus the final histology, which was available after surgery on formalin-fixed, paraffin-embedded tissue in accordance with the World Health Organization guidelines (de Lellis and Willimas, 2004).

2.5 Molecular biology analysis

Somatic point mutations in the *BRAF* and *RAS* genes were determined on cytological material smeared on a slide after the pathologist had verified the adequacy of the sample and selected areas with the highest number of neoplastic cells (>50%). After removal of the coverslip, DNA was extracted from selected areas by means of a "home-made" buffer (pH 8, 1% Tween). The optimal number of cells suitable for molecular studies should be 100 or more. The methods used to study the mutation were the direct Sanger sequencing method (according to the recommendations of the Italian Association of Medical Oncology (AIOM) and the Italian Society of Pathology and Cytology (SIAPEC)) and real-time polymerase chain reaction (PCR) with commercial kits approved for clinical use. The choice of the method was based on the material and the amount of DNA extracted and assayed. Whenever possible, both procedures were performed. The laboratory was accredited by Bureau Veritas International Organization for Standardization (ISO) 9001:2008 and the external quality control for the determination of *BRAF* and *RAS* mutations was promoted by AIOM in 2012 SIAPEC (Monti *et al*., 2015).

2.6 Statistical analysis

Non-parametric tests were used to compare averages; the correlation coefficient (r) was calculated by means of Spearman's correlation (Sr) (GraphPad 6.0 Software, San Diego, CA, USA). Data are reported as mean±standard error of mean (SEM) if not otherwise reported. Significance was set at $P \leq 0.05$. A US score (from 0 to 5) was arbitrarily calculated for the nodule under evaluation, with one point being assigned for the presence of each of the following radiological findings: solid, hypo-echoic, microcalcification, internal vascularization, and irregular shape (Giusti *et al*., 2013; 2014). The diagnostic value of the ELX 2/1 strain index from USE or the P index and TTP index from CEUS in distinguishing between benign and malignant nodules was analyzed by means

of cut-off values derived from receiver-operating characteristic (ROC) curves and the calculated area under the curve (AUC). After using this curve to establish a cut-off point, we established sensitivity and specificity values and likelihood ratios. The cumulative results from US, USE, and CEUS were evaluated for sensitivity, specificity, positive predictive value, negative predictive value, and accuracy. Both Thy 3a and Thy 3f nodules that fitted all experimental cut-off points obtained from ROC curves were considered true positive if they proved malignant and false positive if they proved benign on histological examination. In addition, both Thy 3a and Thy 3f nodules that did not fit all experimental cut-off points obtained from ROC curves were considered true negative if they proved benign and false negative if they proved malignant on histology.

3 Results

3.1 Risk of malignancy in revised Thy 3 nodules

The management of the Thy 3 nodules in which cytological re-evaluation was performed is reported in Fig. 1. Three patients (n=1 Thy 3a and n=2 Thy 3f) were lost after the first FNAB, while 5 (n=1 Thy 3a and n=4 Thy 3f) are still under observation in other centers. One patient (Thy 3f) died of a disease unrelated to the thyroid nodule soon after FNAB; another (Thy 3f) was excluded from the study owing to comorbidities. One patient (Thy 3f) refused surgery and any further FNAB. In 24 patients for whom surgery was delayed, and who had a new benign (Thy 2) cytological diagnosis, cytological re-evaluation showed Thy 2, Thy 3a, and Thy 3f classifications of 12, 7, and 3 nodules, respectively (Fig. 1). Histology was available for 97 nodules (75%). Benign histology was observed in all nodules in which the retrospective cytological diagnosis was Thy 2, while malignant histology was found in all nodules in which the retrospective cytological diagnosis was Thy 4. Ten out of 39 (26%) and 7 out of 49 (14%) nodules reassigned to Thy 3a and Thy 3f classifications, respectively, showed malignant histology. The risk of malignancy was not statistically different between Thy 3a and Thy 3f nodules (Fisher's test P=0.28); the risk of malignancy in the nodules still classified as Thy 3 (Thy 3a plus Thy 3f) after revision was 19%. An additional 7 patients had separate microcarcinomas incidentally identified on surgery. If these incidentally discovered thyroid cancers are included, the cumulative rate of malignancy in our resected glands with initial Thy 3 nodules becomes 28%.

The histologic outcomes of the Thy 3a and Thy 3f nodules after surgery are reported in Table 1. The only significant difference observed was the more frequent incidence of Hurtle cell adenomas in benign Thy 3f nodules (29%) than in benign Thy 3a (3%) nodules (Fisher's test P=0.01; Table 1).

3.2 Clinical data and US in revised Thy 3 nodules

Age, sex distribution, and nodular size did not differ significantly between Thy 3 nodules reclassified as Thy 3a and those reclassified as Thy 3f (Table 2). Owing to the long time between the first and last observation, not all clinical data were available for all nodules. The results of the thyroid function test, irrespective of any therapy underway, were similar for Thy 3a and Thy 3f nodules (Table 2). The *BRAF* V600E mutation was positive in only four Thy 3a nodules; this finding was statistically significant (Fisher's test; P=0.04). Two different mutations (*NRAS*, *BRAF* K601E) were noted in two Thy 3f nodules. The US score, ELX 2/1 strain ratio, TTP index, and P index were, on average, similar between Thy 3a and Thy 3f nodules (Table 2).

3.3 US, USE, and CEUS in revised Thy 3 nodules and histological outcomes

A significant correlation was seen between cumulative US findings and histology in both Thy 3a (n=37, Sr=0.49; P=0.002) and Thy 3f (n=47, Sr=0.42; P=0.003) nodules. The ROC curves were obtained with an AUC of 0.95±0.00 (P<0.0001) for Thy 3a and 0.98±0.01 (P<0.0001) for Thy 3f. By establishing a cut-off level that classified nodules with a US score of $\geq$2 as malignant, we were able to achieve a sensitivity of 73% and a specificity of 100% for Thy 3a, with a likelihood ratio of 5.0, and a sensitivity of 74%, and a specificity of 100% for Thy 3f, with a likelihood ratio of 5.2. A significant correlation was seen between the ELX 2/1 index and histology only in Thy 3f (n=28, Sr=0.43; P=0.02) nodules. In ROC analysis, however, significant AUCs were observed for both Thy 3a (n=21, 0.96±0.02; P<0.0001) and Thy 3f (n=28, 0.95±0.02; P<0.0001). By establishing a cut-off level that classified nodules with an ELX 2/1 index of $\geq$1 as malignant, we were able to achieve a

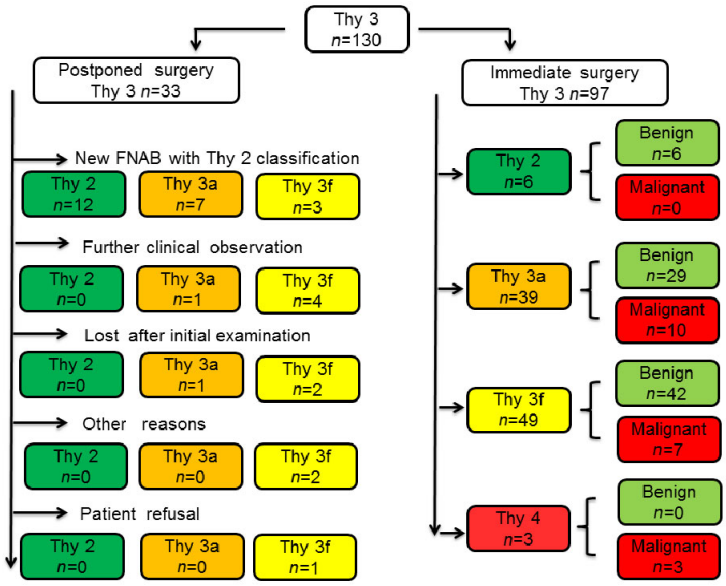

Fig. 1 Flow schematic of Thy 3 nodules managed between 2011 and 2014 and re-evaluated according to the BTA 2014 classification

Malignancy is specific to the targeted nodule; other nodules were not considered. In 6 cases, no further clinical data were available owing to patient loss, death due to causes unrelated to thyroid disease, other severe co-morbidity, or patient refusal

Table 1 Histology of the revised Thy 3 thyroid nodules in which Thy 3a and Thy 3f cytology was found according to BTA 2014 criteria

Histologic outcome	Thy 3a	Thy 3f	*P*-value
Differentiated thyroid carcinoma			
Total	10	7	
PTC	3	1	ns
FvPTC	5	2	ns
FTC	1	2	ns
Hurtle carcinoma	0	1	ns
Medullary thyroid carcinoma	1	0	ns
Indeterminate potential of malignancy	0	1	ns
Malignant with foci of lymphocytic thyroiditis	2	1	ns
Benign histology			
Total	29	42	
Hyperplastic nodule	19	21	ns
Follicular adenoma	9	9	ns
Hurtle cell adenoma	1	12	0.01
Benign with foci of lymphocytic thyroiditis	5	10	ns
Benign with incidental microcarcinomas	3	4	ns

PTC: papillary thyroid carcinoma; FvPTC: follicular variant of PTC; FTC: follicular thyroid carcinoma; ns: not significant

Table 2 Clinical data for Thy 3 nodules re-classified as Thy 3a and Thy 3f according to BTA 2014 criteria

Clinical data	Thy 3a	Thy 3f	*P*-value
Age (year)	55.1±14.2	55.1±14.9	ns
Sex (female/male)	33/15	51/10	0.11
Nodular size (mm)	25.3±1.8	21.8±1.2	0.17
f-T4 (pmol/L)	15.6±0.4 (41)	15.1±0.4 (57)	ns
TSH (mIU/L)	1.64±0.18 (43)	2.03±0.24 (58)	ns
Calcitonin (ng/L)	2.8±0.5 (39)	2.3±0.2 (48)	ns
Positive TPOAbs (%)	41 (27)	35 (29)	ns
BRAF V600E mutation (positive/negative)[1]	4/18	0/26	0.04
US score[2]	2.0±0.1 (47)	2.1±0.1 (57)	ns
ELX 2/1 strain index[3]	1.40±0.09 (27)	1.32±0.07 (32)	ns
TTP index[4]	1.10±0.09 (27)	1.16±0.14 (27)	ns
P index[4]	1.11±0.08 (27)	0.94±0.03 (27)	0.17

The number of evaluable nodules in each category is shown in brackets. [1] The *BRAF* V600E mutation was evaluated on 51 nodules reclassified as Thy 3a or Thy 3f according to the BTA 2014 classification. *BRAF* was not detectable in 3 cases (n=1 Thy 3a and n=2 Thy 3f). [2] Scores ranging from 0 to 5; see Section 2.6 for US score evaluation. [3] The ELX 2/1 strain index was evaluated by semi-quantitative US, as previously reported (Giusti *et al*., 2013; 2014); see Section 2.6 for ELX 2/1 strain index measurement. [4] Time-to-peak (TTP) and peak (P) indices were evaluated by CEUS, as previously reported (Giusti *et al*., 2013; 2014); see Section 2.6 for CEUS data analysis. Data are expressed as mean±SD, number, or mean±SD (number) except positive TPOAbs. TPOAbs: thyroperoxidase antibodies; ns: not significant

sensitivity of 90% and a specificity of 81% for Thy 3a, with a likelihood ratio of 4.1, and a sensitivity of 86% and a specificity of 87% for Thy 3f, with a likelihood ratio of 4.4. CEUS was available for only 42 revised Thy 3 nodules; a significant inverse correlation was found only between the P index obtained for Thy 3f nodules and their histology (n=21, Sr=−0.51; P=0.02). Significant AUCs from ROC curves were obtained for the P index (Thy 3a: 0.88±0.03, P<0.0001; Thy 3f: 0.86±0.04, P<0.0001) and the TTP index (Thy 3a: 0.93±0.02, P<0.0001; Thy 3f: 0.90±0.03, P<0.0001). By establishing a cut-off level that classified Thy 3a and Thy 3f nodules with a P index of ≤0.99 as malignant, we were able to achieve a sensitivity of 43% and a specificity of 81% for Thy 3a nodules, with a likelihood ratio of 2.2, and a sensitivity of 33% and a specificity of 81% for Thy 3f, with a likelihood ratio of 1.7. The TTP index cut-offs were ≥0.96 for Thy 3a (sensitivity 71%, specificity 81%; likelihood ratio 3.7) and ≥0.98 for Thy 3f (sensitivity 71%, specificity 81%; likelihood ratio 3.7).

3.4 Predictive value of mutation analysis, US score, USE, and CEUS in revised Thy 3 nodules

Histological outcomes, mutation analysis, and fully evaluable US scores, USE and CEUS were available for only 24 reclassified nodules (n=12 Thy 3a and n=12 Thy 3f) (Table 3). In 9 nodules (n=8 Thy 3a and n=1 Thy 3f) histology was malignant; mutation analysis was positive in 6 nodules (Table 3). The US score (≥2), ELX 1/2 score (≥1), TTP index (≥1), and P index (≤1) were all positive for malignancy for 6 out of 8 (75%) histologically malignant Thy 3a nodules and for 3 out of 12 Thy 3f nodules (25%; 1 with malignancy, 8%). The diagnostic power of the cumulative pre-surgical analyses of Thy 3a and Thy 3f nodules by means of US, USE, and CEUS, considering the experimental cut-off points obtained from ROC curves, was: sensitivity 63% and 14%, specificity 75% and 82%, accuracy 67% and 83%, respectively. The predictive positive value and the negative predictive value for Thy 3a and Thy 3f nodules were 83% and 100%, respectively.

4 Discussion

Current recommendations are in favor of repeating FNAB for a nodule with an indeterminate Bethesda category III diagnosis (Cibas and Ali, 2009; Dincer *et al*., 2013; Rosario, 2014), while surgical resection is recommended for nodules with a confirmed diagnosis of Bethesda category III or a cytological diagnosis of Bethesda category IV (Dincer *et al*., 2013; Rosario, 2014). Moreover, in these latter nodules, a variable rate of malignancy is generally reported, from quite low to fairly elevated (Dincer *et al*., 2013; Ohori *et al*., 2013; Cuhaci *et al*., 2014; Nikiforov *et al*., 2014; Rosario, 2014; Yoon *et al*., 2014; Kapila *et al*., 2015). However, when histology confirms a thyroid cancer, an overall good prognosis is reported (Rago *et al*., 2014; Trimboli *et al*., 2015).

Our patients with Thy 3 cytology were, on average, middle-aged females with a nodule size slightly larger than 20 mm, without any clinical difference after cytological sub-classification as Thy 3a and Thy 3f; this is in agreement with other reports (Dincer *et al*., 2013; Rosario, 2014; Yoo *et al*., 2015). Surgery was undertaken in the majority (75%) of our cases after the first FNAB. The same approach has most frequently been adopted in almost all similar studies (Ohori *et al*., 2013; Ho *et al*., 2014; Hyeon *et al*., 2014; Nikiforov *et al*., 2014; Rago *et al*., 2014; Deniwar *et al*., 2015; Yoo *et al*., 2015), even when a second FNAB was performed (Ho *et al*., 2014; Rosario, 2014; Yoo *et al*., 2015). For our Thy 3 nodules, surgery allowed us to ascertain a low rate of thyroid cancers (19%), which is a percentage close to that reported in studies employing the BTA classification (Mihai *et al*., 2009; Deandrea *et al*., 2010; Giusti *et al*., 2014; Rago *et al*., 2014). In our study, the reclassification of Thy 3 nodules according to BTA 2014 criteria identified a significantly higher incidence of Hurtle adenomas in Thy 3f than in Thy 3a, without a very significant difference between the two sub-categories in the risk of malignancy on histology. Our observed low incidence of malignancy (14%) in nodules reassigned to the Thy 3f category suggests caution owing to the low number of nodules, but does not justify the more aggressive management of nodules reassigned to the Thy 3a category (or BSRTC category III), as reported by some for BSRTC category IV (Cibas and Ali, 2009; Ohori *et al*., 2013; Nikiforov *et al*., 2014; Deniwar *et al*., 2015; Kapila *et al*., 2015).

The reason for the variable rates of malignancy in studies using the BSRTC is unknown. Some authors hypothesize a random variation or institutional

Table 3 Clinical data and outcomes for Thy 3a and Thy 3f nodules with mutation analysis available and full instrumental evaluation

Case No.	Age (year)	Sex	Nodular size (mm)	Mutation analysis[a]	US (score)	USE ELX 2/1 index	CEUS TTP index	CEUS P index	Surgical outcome[b]
Thy 3a									
1	34	M	9	Negative	3	1.9	1.10	0.86	Malignant
2	44	F	24	Negative	2	1.3	1.10	1.40	Benign
3	45	F	30	Positive	4	3.0	1.25	0.69	Malignant
4	45	F	44	Positive	4	1.0	1.62	0.82	Malignant
5	46	F	46	Positive	3	1.7	0.92	2.07	Malignant
6	50	M	32	Negative	2	0.8	0.90	1.10	Malignant
7	54	M	20	Negative	2	1.7	2.40	0.90	Malignant
8	56	F	7	Positive	3	0.8	1.00	1.00	Malignant
9	64	F	16	Negative	3	1.0	1.00	0.98	Malignant
10	65	F	15	Negative	3	1.0	1.00	0.70	Benign
11	68	F	40	Negative	3	1.3	0.70	1.10	Benign
12	77	M	18	Negative	2	0.9	0.90	1.30	Benign
Thy 3f									
1	38	F	20	Negative	1	1.4	0.70	0.75	Benign
2	40	F	18	Negative	5	2.0	1.10	0.98	Malignant
3	40	M	45	Negative	1	0.9	0.94	0.75	Benign
4	42	F	17	Negative	2	1.1	1.10	0.84	Benign
5	46	M	11	Negative	2	1.1	0.98	1.10	Benign
6	58	F	16	Negative	1	1.5	0.90	1.00	Benign
7	64	M	30	Negative	2	0.9	0.98	1.10	Benign
8	65	F	8	Positive	2	1.0	1.20	0.99	Benign
9	65	F	18	Negative	2	1.0	0.98	1.15	Benign
10	66	F	24	Negative	1	2.4	1.00	1.10	Benign
11	75	F	16	Negative	1	1.8	1.00	1.40	Benign
12	78	F	30	Positive	1	0.9	1.00	1.20	Benign

[a] Mutation analysis: Thy 3a cases Nos. 3–5 and 8, *BRAF* V600E mutated; Thy 3f case No. 8, *BRAF* K600E mutated and case No. 12, *NRAS* mutated. [b] Malignant histology: Thy 3a: case No. 1, medullary thyroid carcinoma, cases Nos. 3, 4, 5, and 8, PTC, cases Nos. 6, 7, and 9, FvPTC; Thy 3f: case No. 2, Hurtle carcinoma

differences in the interpretation of slides (Ho *et al*., 2014). Another explanation could be that retrospective analyses have been carried out on large series of FNAB records gathered over a period of time when the BTA/BSRTC classifications were not yet used, with cases being reclassified according to medical files rather than a real review of slides (Rago *et al*., 2014). Moreover, the differences in malignancy rates could be due to ethnic differences among the populations evaluated and the adoption of different size-limits of nodules for which FNAB is performed (Yoo *et al*., 2015). Some studies have considered the absence of suspicious US findings on follow-up to be an indicator of benignity, which may also explain the differences in percentages (Ho *et al*., 2014; Rosario, 2014; Yoo *et al*., 2015). Finally, some authors have reported a very poor inter-observer reproducibility for the Thy 3 category, in contrast to the good agreement seen for the other BTA categories (Kocjan *et al*., 2011).

In indeterminate lesions, the need to obtain cytological-histological correlation and to reassure patients is generally cited as the justification for surgery. Other reported reasons for immediate surgery are: US suspicious for malignancy (Rosario, 2014), nodule size (Mehta *et al*., 2013), the rate of nodular increase over time (Nakamura *et al*., 2015), high circulating thyroglobulin levels (Nakamura *et al*., 2015), and compressive symptoms or cosmetic problems (Nakamura *et al*., 2015). Current efforts, however, are directed toward refining risk assessment, identifying clinical features helpful for management and reducing unnecessary surgery.

In a previous study (Giusti *et al*., 2014), we observed that, for cytological Thy 3 nodules, conventional US can be associated with the combined application of USE and CEUS. In a series of 63 nodules classified as Thy 3 according to the BTA, in which cytological and histological correlation was available, we reported a malignancy rate of 21%; by means of ROC analysis, cut-off values were set for the US score (>2 suspicious features), strain index on USE (ELX 2/1 >0.95), and CEUS indexes (P index <0.99; TTP index >0.99). In the present study, the same evaluation was performed after Thy 3 nodules had been split into the two categories Thy 3a and Thy 3f. In this larger study population, and after subclassification of Thy 3 nodules, the sensitivity, specificity, and likelihood ratio obtained from the individual ROC curves for US, USE, and CEUS did not seem to differ between the two Thy 3 groups. Moreover, the present study further indicated that the use of cut-offs based on histology can prompt a policy of observation also of Thy 3a and Thy 3f nodules.

Molecular testing for oncogene mutations (Nikiforov *et al*., 2014) or gene expression (Labourier *et al*., 2015) in materials from FNAB is now available. Nikiforov *et al*. (2014) reported high sensitivity (90%) and specificity (93%) for the ThyroSeq v2 NGS panel in Bethesda category IV nodules. Thus, this extended mutation panel could further increase the sensitivity of molecular analysis by detecting additional malignant nodules. Recently, Labourier *et al*. (2015) reported that the use of multiplatform testing in the clinical setting could potentially result in a 6–7-fold reduction in the number of unnecessary surgeries, thanking to its high negative predictive value in cytological indeterminate thyroid nodules (BSRTC categories III–V). This finding seems to be in contrast to the previous observation that the *BRAF* mutation seems to be less important in follicular neoplasms (Eszlinger *et al*., 2014). In our study, the *BRAF* V600E mutation, the most frequent mutation in thyroid cancer, was found only in the Thy 3a category. The presence of nodules with the *BRAF* V600E mutation only among nodules reclassified as Thy 3a, but not in those reclassified as Thy 3f, is not surprising, given the different distribution of definitive histological diagnoses (Table 1).

However, at present, refined imaging studies and molecular biology are essential for indeterminate lesions before a decision is taken regarding surgery. In our study, 24 nodules with histological diagnoses were fully evaluated according to the revised institutional cut-offs for US, USE, and CEUS. According to these cut-offs, 67% of nodules reclassified as Thy 3a were rightly classified as malignant or benign, while only 25% of them were wrongly considered benign. By contrast, among the nodules reclassified as Thy 3f, the only one that was malignant was rightly classified by ancillary imaging techniques, and 75% were correctly considered benign. In our opinion, at present, ancillary imaging techniques (USE and CEUS) and molecular analysis are evaluations complementary to cytology, and can reduce the number of surgeries and prompt a more observational strategy in Thy 3 nodules, regardless of their sub-stratification. In this respect, gray-scale US remains of major help in stratifying thyroid nodules according to malignancy risk (Horvath *et al*., 2009; Moon *et al*., 2011; 2015) on the basis of the number of US features, such as solidity, hypoechogenicity or marked hypoechogenicity, irregular margins, microcalcifications, and taller-than-wide shape. Our data indicated that indeterminate nodules without suspicious US features, and without other risk factors revealed by molecular biology and ancillary US techniques, can be followed up, as recently reported, by combining the thyroid imaging reporting system and the BSRTC for thyroid nodules with non-diagnostic results on FNAB examination (Moon *et al*., 2015).

The final decision on whether to undertake observation or surgery of indeterminate nodules may be based on nodular size and growth. However, the meaning of both parameters is controversial. Indeed, while some authors suggested that large nodules are at higher risk of malignancy (McCoy *et al.*, 2007; Wharry *et al*., 2014), others (Kim *et al*., 2014; Nakamura *et al*., 2015) did not support this hypothesis. Size cannot therefore be unequivocally considered a useful means of distinguishing benign from malignant nodules. Moreover, no studies have confirmed that progressive nodular growth is pathognomonic for malignancy. However, Nakamura *et al*. (2015), who used the JTA Reporting System, stated that, for nodules cytologically diagnosed as adenomatous, growth is not, in itself, a risk factor for malignancy, while follicular neoplasms that grow are at higher risk.

Finally, we must consider whether the time spent in ancillary evaluations, such as molecular biology analyses, new echographic techniques, or further

FNAB, will impair the quality of life of patients by increasing their anxiety, or reassure them by helping to avoid unnecessary surgery. To our knowledge, no studies have focused on this aspect in patients with Thy 3 nodules. However, Nou *et al*. (2014) and Trimboli *et al*. (2015) reported no increase in tumor staging in patients awaiting surgery for up to 3–4 years, while Yeh *et al*. (2004) observed a higher rate of vascular and capsular invasion and a higher incidence of persistent disease when the diagnosis of thyroid carcinoma was delayed by an average of more than 2 years. These data, together with the low risk of malignancy seen in both Thy 3a and Thy 3f nodules in our study, justify extending diagnostic studies after the first cytological diagnosis. Surprisingly, the new 2015 American Thyroid Association guidelines for thyroid nodules and differentiated thyroid cancer (Haugen *et al*., 2016) reported only the utility of US and molecular testing in the risk stratification of indeterminate nodules, without mentioning USE and CEUS as further potentially useful techniques. Both our investigation and the recent study conducted by Azizi *et al*. (2015) using shear wave elastography indicated the potential utility of these techniques in improving our ability to assess risk in thyroid nodules at low risk of malignancy, including BSTRC III and IV.

Our study had several limitations: (a) its retrospective, single-center design; (b) the relatively low number of nodules included; (c) the lack of extended follow-up in all non-operated patients, resulting in a possible verification bias; (d) the consideration that the indolent nature of the majority of differentiated thyroid cancers may lead to underestimation of the malignancy rate or, on the other hand, overestimation of the potential of US, USE, and CEUS to diagnose malignancy, which could prompt surgery in nodules without an intrinsic oncologic risk; (e) technical difficulties, such as the fact that thyroid tissue adjacent to the Thy 3 nodule may not be normal, thereby rendering both USE and CEUS impracticable; (f) finally, injection of the contrast agent before CEUS is sometimes refused by patients.

5 Conclusions

In the present study, the rate of malignancy in Thy 3 nodules proved to be fairly low, and no significant differences emerged between the Thy 3a and Thy 3f classes. The only histological difference between the groups was the significantly higher percentage of Hurtle adenomas among Thy 3f nodules. US, USE, and CEUS are all useful technical methods for refining diagnoses of malignancy, thereby reducing false-positive diagnoses and unnecessary surgical interventions. Molecular testing could be widely used in the near future. In our experience, the *BRAF* V600E mutation is a peculiar characteristic of Thy 3a nodules only. A non-surgical strategy can be undertaken more often in Thy 3 nodules, irrespective of their sub-classification as Thy 3a or Thy 3f, when no suspicious data emerge from cumulative instrumental and molecular biology analyses. Our data supported the conviction that, in mutation-negative Thy 3a and Thy 3f nodules, observation should be the first choice when not all instrumental results are suspect. Although larger populations are needed, it is currently impracticable to pool data from different centers where qualitative, semi-quantitative, and quantitative (shear wave) USEs are used.

Compliance with ethics guidelines

Massimo GIUSTI, Barbara MASSA, Margherita BALESTRA, Paola CALAMARO, Stefano GAY, Simone SCHIAFFINO, Giovanni TURTULICI, Simonetta ZUPO, Eleonora MONTI, and Gianluca ANSALDO declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study.

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中文概要

题　目：甲状腺不确定结节的回顾性细胞学评估研究

目　的：回顾性分析了 130 例甲状腺不确定结节的临床评估和预后，以及组织细胞学检测结果，为甲状腺结节的治疗提供合理的治疗方法。

方　法：根据英国甲状腺协会 2014 年分类，回顾性分析了 2011 年 1 月至 2014 年 12 月 130 例甲状腺不确定结节（Thy 3）的细胞学检查。将结节分为 Thy 3a（非典型特征）和 Thy 3f（滤泡病变）两类。比较评估术前生物化学检查、彩色多普勒超声检查（US）、半定量弹性超声成像（USE）、超声造影（CEUS）、细胞学、组织学以及分子生物学突变分析等结果。

结　论：Thy 3 结节的恶性肿瘤发生率较低，且在 Thy 3a 和 Thy 3f 之间没有显著差异。使用基于组织学的截取值作为参考可以降低采用手术的治疗方案。在阴性突变的 Thy 3a 和 Thy 3f 结节中，当不是所有的仪器检测结果都疑是阳性时，组织学观察应该作为首选。

关键词：不确定性甲状腺结节；英国甲状腺协会 2014 年分类；临床评价；预后