Review

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Measurement of thyroglobulin, calcitonin, and PTH in FNA washout fluids

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Abstract: Different imaging tools, circulating endocrine markers, and fine-needle aspiration (FNA) cytology are of great importance in the diagnosis and follow-up of different thyroid and parathyroid diseases. Sometimes, however, they are conflicting or inconclusive: interestingly, measuring endocrine markers (i.e. thyroglobulin, calcitonin, parathyroid hormone) in fluids from FNA proved to be a very useful complementary diagnostic tool in such cases. The determination of endocrine markers in fluids other than serum/plasma has been developed in the last years. Although studies have reported overall satisfactory results, a good standardization of procedures has not yet been reached, and further efforts should be made in order to better define preanalytical, analytical, and post-analytical aspects. Here we reviewed critically the literature on the measurement of FNA endocrine markers, focusing on laboratory issues, such as preparation of the sample, choice of solution, and technical features of determination of these markers. Indeed, information for use of FNA-Tg, FNA-CT, and FNA-PTH in clinical practice was also provided.

Keywords: calcitonin; fine-needle aspiration; parathyroid hormone; thyroglobulin; washout.

Introduction

Thyroid diseases are very common, and thyroid carcinomas represent the most frequent endocrine malignancies [1, 2]. Differentiated thyroid carcinomas (DTC), such as papillary (PTC) and follicular (FTC) histologic types, develop from thyrocytes and produce exclusively thyroglobulin (Tg). Then, these patients are followed up over time by monitoring their serum Tg levels and using local and whole-body imaging techniques (i.e. neck ultrasound, whole-body scintiscan, and 18-FDG-PET/TC) [3]. A rate up to 50% of DTC patients have metastatic neck lymph nodes at their initial presentation and/or during postoperative follow-up [3]. Specifically, PTC disseminates via the lymphatic apparatus with metastasis to the regional lymph nodes. While ultrasonography (US) can detect neck lesions suspicious for DTC metastases, fine-needle aspiration (FNA) under US guidance is generally performed to prove the metastatic involvement of these lesions. This approach is essential to allow tailored surgical excision [3]. Because cytologic examination of FNA samples from neck lymph node may be inconclusive or inadequate, the measurement of Tg in fluids from FNA (FNA-Tg) was proposed in the 1990s and has become an essential tool combined to cytology [4, 5]. The results from the literature suggest that FNA-Tg achieve high relevance, particularly in cases involving small, partially cystic, lymph nodes.

More recently, the determination of calcitonin (CT) in washout fluids (FNA-CT) from thyroid nodules suspected for medullary thyroid carcinoma (MTC) has been described [6]. This approach was based on the several limits of cytology in detecting MTC reported in the literature [7]. In this context, a higher accuracy of serum CT with respect to cytology was proven [8], and poor sensitivity of cytology (i.e. 55%–65%) was recorded in single- and multi-center series [9–11]. To adopt the use of FNA-CT plus cytology could reduce false-negative and inconclusive results with sensitivity approaching 100% [6]. This issue has been addressed in the most recently updated version of the ATA guidelines and prompted the board to recommend the use of FNA-CT in patients suspected for MTC with non-conclusive cytologic report [12].

Primary hyperparathyroidism is a not rare disease due to hyperfunctioning parathyroid (HP). These lesions can be single or multiple and have different histologic types, such as adenomas, hyperplasia, or more rarely

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carcinomas. Because the treatment of choice of HP is represented by surgical removal, their identification and localization are pivotal. In this context, several potential limits of different imaging techniques (i.e. US, scintigraphy, magnetic resonance) have been reported [13]. Some years ago, the determination of parathyroid hormone in FNA washout fluids (FNA-PTH) has been proposed as an improving tool in localizing HP [14]. Some controversies have been recorded between the studies on this topic, and the procedure has not been largely diffused.

Tg, CT, and PTH are routinely measured in the blood. The determination of thyroid/parathyroid markers in fluids other than serum/plasma has been developed in the last years. Although studies have reported overall satisfactory results, a good standardization of procedures has not yet been reached, and further efforts should be made in order to better define pre-analytical, analytical, and post-analytical aspects. Here we reviewed critically the literature on the measurement of FNA-Tg, FNA-CT, and FNA-PTH. Published data were screened, main results from these articles were evaluated, and recommendations from international guidelines were summarized. Particular attention was focused on laboratory issues, such as preparation of the sample, choice of the solution, and technical features of the determination of these markers. Finally, information for use of FNA-Tg, FNA-CT, and FNA-PTH in clinical practice was provided.

Materials and methods

Search strategy and study selection

A comprehensive computer literature search of the PubMed/MEDLINE, Embase, and Scopus databases was conducted to find published articles on the topic of our review. A beginning date limit was not used, and the search was updated until May 31, 2016, without language restrictions. The authors independently reviewed titles and abstracts of the retrieved articles and reviewed the full text of the papers to assess their definite inclusion. Moreover, to select additional studies and expand the searching of papers to be included, the reference lists of the retrieved articles were screened.

Results

Measurement of thyroglobulin in FNA fluids from lymph nodes suspicious for metastases from differentiated thyroid carcinoma

Clinical data from literature

The measurement of FNA-Tg in cervical lymph nodes/masses suspected to be metastases from DTC has been largely reported (Table 1). This approach was first proposed in 1992 by Pacini et al. [4] and the sensitivity of FNA-Tg (100%) was significantly higher than that of cytology (85%). Remarkably, no FNA-Tg false-positive results were recorded. Later, Frasoldati et al. [25, 26] reported 84% sensitivity and 95% specificity for FNA-Tg, and sensitivity increased up to 95% using the combination of cytology plus FNA-Tg. Also, a sensitivity of 100% of FNA-Tg was described by Cunha and co-workers [17]. Subsequently, these initial data were confirmed in larger series [18]. Due to some discordant results of cutoff levels, other proposals have been reported [16]. More recently, a meta-analysis including 24 studies and 2865 lymph nodes reported overall sensitivity of 95% and specificity of 94%, with significant heterogeneity [27]. However, even if many articles

Table 1: Accuracy of FNA-Tg reported in the main studies.

First author, year [Refs]	Lymph nodes	Analytic method	Adopted cutoff, µg/L	Sensitivity, %	Specificity, %
Pacini, 1992 [4]	23	IRMA	21.7	100	100
Frasoldati, 2004 [15]	73	IRMA	NA	92.3	94.1
Uruno, 2005 [16]	129	NA	Tg-FNA > Tg serum	81.4	NA
Cunha, 2007 [17]	18	CLIA	0.9	100	100
Sigstad, 2007 [18]	24	TR-IFMA	Tg-FNA > Tg serum	100	83.3
Snozek, 2007 [19]	122	CLIA	1.0	100	96.2
Giovanella, 2009 [20]	126	IRMA	1.1	100	100
Kim, 2009 [21]	91	IRMA	50	100	80
Bournaud, 2010 [22]	98	IRMA	0.93	92.3	97.8
Salmashoglu, 2011 [23]	255	IRMA	28.5	100	96.4
Moon, 2013 [24]	528	RIA	1.0	93.2	95.9

CLIA, chemiluminescence immunoassay; ECLIA, electrochemiluminescence immunoassay; IRMA, immunoradiometric assay; NA, not applicable; RIA, radioimmunoassay; TR-IFMA, time-resolved immunofluorimetric assay.

have published, a better standardization of analytical methods and cutoff levels is required.

The issue of the cutoff level of FNA-Tg to discriminate metastatic lymph nodes from non-metastatic ones has been largely debated. In the first studies, the threshold values were reported from 0.9 to 50 μg/L [5, 27]. Also, the metastatic cases diagnosed in presence of thyroid gland and those detected in athyreotic patients were analyzed; substantial overlap of results was found, and FNA-Tg levels up to 88.8 μg/L were recorded in benign lymph nodes from DTC patients still awaiting thyroidectomy [16, 20, 21, 27–30]. Accordingly, different FNA-Tg cutoff was proposed in the presence (36 μg/L) or absence (1.7 μg/L) of thyroid [31]. Meanwhile, two different papers found a unique accurate threshold of 0.93 ng/punction [22] and 28.5 μg/L [23]. However, among 114 and 225 consecutive patients, they enrolled 11% and 35% DTC patients before surgery, respectively. Relevant differences between these two studies are observed in Tg assays and samples management. All in all, both sensitivity and specificity of FNA-Tg was higher in surgically treated DTC than in those waiting for surgery [32, 33] and, consequently, this suggests to combine cytology with FNA-Tg. A cutoff level obtained by "high-sensitivity" assays should provide more accurate data. Snozek and colleagues [19] measured FNA-Tg in 122 samples from 88 athyreotic DTC patients, using immunochemiluminometric assay (CLIA) with functional sensitivity of 0.1 μg/L; 50/52 nonmalignant samples (96.2%) had values ≤ 1 μg/L and 70/70 malignant lesions had levels >1 μg/L. By a different high-sensitivity immunoradiometric assay (IRMA) (functional sensitivity 0.2 μg/L) and adopting a ROC derived cutoff of 1.1 μg/L, the authors of the present review obtained 100% sensitivity, specificity, and accuracy [34]. These results were subsequently confirmed in a large-scale study including 528 cases of FNA-Tg patients [24]. Finally, the presence of serum anti-Tg autoantibodies (TgAb) should influence the measurement of FNA-Tg. Lowering FNA-Tg cutoff levels significantly improved the diagnostic accuracy of FNA-Tg in patients with positive serum TgAb [35]. From a practical point of view, combining FNA-Tg measurement and cytology examination in such cases should be considered [5].

Specific recommendations in guidelines

The ATA guidelines of thyroid nodule and DTC have included FNA-Tg since a long time. The most recent revised version of the text [3] has quoted at Recommendation 32 that "US-guided FNA of sonographically suspicious lymph nodes >8–10 mm in the smallest diameter should be performed to confirm malignancy if this would change management (strong recommendation, moderate-quality evidence); the addition of FNA-Tg washout in the evaluation of suspicious cervical lymph nodes is appropriate in select patients, but interpretation may be difficult in patients with an intact thyroid gland (weak recommendation, low-quality evidence)". Also, the ATA board suggests the measurement of FNA-Tg in particular conditions such as lymph nodes with cystic changes, with inadequate cytology, or with cytologic and US evaluations divergent (i.e. normal cytology from a large lymph node with microcalcifications). On the contrary, ATA experts have underlined that false-positive FNA-Tg may occur (i.e. in case of lymph nodes of central compartment in patient with thyroid). Finally, ATA guidelines report a lack of standardization of FNA-Tg procedures or assays with consequent potential difficult in interpreting data: "standardization including matrix type (phosphate-buffered saline, Tg-free serum, etc.) and volume of diluent matrix would help with interpretation of a Tg washout" [3].

The panel of AACE/AME/ETA thyroid nodules guidelines have stated that "In the presence of suspicious cervical lymphadenopathy, FNA biopsy of both lymph node and suspicious nodule(s) is essential (Grade B)" [28]. These experts suggest to wash the needle in 1 mL of saline solution. Also, even if these guidelines do not assess the cutoff to be used, they underline that in DTC patients previously treated, "the detection of even low thyroglobulin levels by UGFNA should be considered suspicious for malignancy."

Specific guidelines for neck US and US-guided techniques for the management of DTC patient after treatment have been published by ETA [36]. They suggest to report the results of FNA-Tg as "ng/FNA" ("a more suitable result which reflects the quantity of Tg in the needle"), and propose to adopt value <1 ng/FNA as normal, values between 1 and 10 ng/FNA to be compared with the results from cytology, and levels >10 ng/FNA as positive for the presence of tumor tissue. Also, in recommendation 12, they quoted that FNA cytology and FNA-Tg should take into account stage and histology of cancer, size and location of lymph node, and serum Tg level.

The most recent guidelines by AACE/ACE/AME recommend the determination of thyroglobulin, according to clinical indications, on FNA washout of suspicious neck lymph nodes [37].

All in all, according to ATA, AACE/AME/ETA, ETA and AACE/ACE/AME guidelines [3, 28, 36, 37], a relevant role of FNA-Tg in cervical lymph nodes is documented and recognized. Rarely, false-positive and false-negative cases may occur; however, the routine use of this technique in both patients before and after surgery is strongly supported by several high-quality data.

Measurement of calcitonin in FNA fluids from thyroid nodules and lymph nodes of patients with suspicious medullary thyroid carcinoma

Clinical data from literature

A number of papers investigated the reliability of FNA-CT in diagnosing MTC (Table 2). The first papers were published in 2007 [38, 39]. In these series, 100% MTC lesions were detected by FNA-CT, while only 62% and 80% had positive cytology. Boi et al. [38] proposed an "arbitrary" FNA-CT cutoff of 36 ng/L corresponding to three times the highest value found in non-medullary lesions. After these preliminary investigations, other studies with different design were published. The multicenter experience of the authors of the present review showed that among 34 patients with a primary MTC (i.e. thyroid nodule), 21 (62%), and 34 (100%) were identified by conventional cytology, and FNA-CT, respectively [41]. Based on these data, a cutoff of 39.6 ng/L was calculated for practice use. Remarkably, a proportion of these subjects had serum CT quite lower. An interesting prospective study [40] compared FNA-CT to basal and pentagastrin-stimulated CT and cytology; the recorded sensitivities were 100% for FNA-CT (using a cutoff of 17 ng/L), 93.7% for basal CT, 87.5% for stimulated CT and 12.5% for cytology. A threshold of 10.4 ng/L for FNA-CT was found by De Crea et al. [42] after retrospective ROC analysis in patients followed up after initial surgery; according to this finding, 16/18 MTC nodules/lymph nodes could be identified. In addition, 15/18 MTC patients had high FNA-CT/CT ratio [42]. Finally, only one paper aimed to calculate a reference range for FNA-CT [43]. In a series of 78 non-medullary thyroid nodules from 54 patients, no significant difference of FNA-CT levels was found whether washing the needle in saline solution or specific buffer. The calculated that the 97.5th upper FNA-CT value was 8.50 ng/L for saline and 7.43 ng/L for the buffer solution. Remarkably, all authors reported detectable levels of FNA-CT (i.e. higher than the functional sensitivity of the method employed) in non-medullary thyroid nodules. It should be speculated that C-cells may be entrapped in FNA sample especially in samples from the middle/upper thyroid lobes [44–46]. This finding supports the need to develop a fixed cutoff. To date, the threshold found [43] in non-medullary thyroid nodules seems more applicable for clinical practice, until new data will be reported.

Specific recommendations in guidelines

The results from the above studies have been promising, and the most updated version of MTC guidelines by ATA [12] has introduced this approach for thyroid nodules with Recommendation 19 (grade B): "FNA findings that are inconclusive or suggestive of MTC should have CT measured in the FNA washout fluid and immunohistochemical staining of the FNA sample to detect the presence of markers". The specific cutoff level for FNA-CT that has to be adopted is not reported by ATA.

The use of FNA-CT in neck lymph nodes is described in AACE/AME/ETA guidelines [28]. There, it has been reported that when metastatic lymph nodes from MTC are suspected, the measurement of FNA-CT increases the diagnostic sensitivity and specificity of cytology. Also, the panel has established that FNA-CT levels >50 ng/L should be regarded as suspicious and values >100 ng/L are nearly diagnostic of metastatic MTC. These guidelines did not report specific items on the use of FNA-CT in thyroid nodules.

The most recent guidelines by AACE/ACE/AME indicate that FNA-CT can be used in enlarged lymph nodes of patients with MTC or in suspicious thyroid nodules of patients at risk for MTC or MEN2 syndrome [37].

According to ATA, AACE/AME/ETA, and AACE/ACE/AME guidelines, FNA-CT has to be used in cervical lymph nodule, suspicious for metastatic MTC [12, 28, 37]. Regarding the use of FNA-CT in thyroid nodule, a main issue still unresolved might be related to the selection of patients who need to be submitted to FNA-CT. Due to limitation of cytology and the lack of universal consensus

Table 2: Accuracy of FNA-CT reported in the main studies.

First author, year [Refs]	Lesions (nodules or lmph nodes)	Analytic method	Adopted cutoff, ng/L	Sensitivity, %	Specificity, %
Boi, 2007 [38]	36	CLIA	36	100	100
Kudo, 2007 [39]	14	NA	67	100	ND
Diazzi, 2013 [40]	60	CLIA	17	100	88.8
Trimboli, 2014 [41]	90	CLIA	39.6	100	100
De Crea, 2014 [42]	62	CLIA	10.4	89	100

CLIA, chemiluminescence immunoassay; NA, not applicable, ND not evaluated.

for evaluating serum CT in thyroid nodule patients, a non-negligible rate of MTC may have a delay in the diagnosis. To measure serum CT in all patients undergoing thyroid FNA and to use FNA-CT in those subjects with elevated serum levels might provide some advantages [6, 47]: to improve the selection of patients at risk for MTC, allow the use of FNA-CT in the same FNA sample and, of high relevance in clinical practice, provide useful information to the cytopathologist. The latter could have a careful morphological assessment and apply ancillary immunocytochemical tests, when indicated [6].

Measurement of PTH in FNA fluids from suspicious hyperplastic parathyroids

Clinical data from literature

A few papers have been published. Initially, Doppman et al. [54] reported seven cases of enlarged parathyroids with measurement of both PTH and Tg in washout (Table 3). Later, several studies demonstrated the relevance to measure FNA-PTH to localize parathyroid adenomas [14, 15, 48–53, 55–67]. In these papers, FNA-PTH achieved a sensitivity ranging from 70% to 100% and a specificity from 75% to 100%. Also, the reliability of FNA-PTH, in detection of parathyroid adenomas was higher than that of cytology [14, 52, 54, 64, 67, 68] and also than that of MIBI [51–53, 58, 61–65]. These authors concluded that FNA-PTH can be used to establish the nature of the mass, discriminate parathyroid gland from thyroid lesions/tissue or cervical lymph nodes, improving the surgical approach in the majority of patients. Regarding the cutoff for FNA-PTH, no significant data have been published; thus, there is no consensus on reference range and their upper reference limit. Several proposals have been reported, ranging from 20 L [48] to 1000 ng/L [50]; some authors, to simplify the matter, have suggested considering positive any FNA-PTH value higher than the respective serum PTH concentration [51, 53, 60, 64, 65, 67]. To avoid misclassification due to circadian and seasonal rhythms and to physical exercise [69], which can influence serum PTH concentrations, and blood contamination which can potentially affect FNA-PTH levels, we suggest as positive cutoff the FNA-PTH/serum PTH ratio ≥ 2 (Table 6). We also advise to measure FNA-PTH in the same laboratory with the same sample preparation and the same IMA method [70, 71]. Some potential minor complications were reported during surgery of patients who underwent FNA of parathyroid; there, 6% of cases had signs of inflammatory responses; consequently, a conversion from mini-invasive to open surgical approaches was required in some cases [72].

Specific recommendations in guidelines

No specific relevant guidelines on this topic have been published. Also, no specific recommendation on FNA-PTH levels and diagnostic cutoff has been quoted.

Considerations on FNA-Tg, FNA-CT, and FNA-PTH testing

The measurement of FNA-Tg, FNA-CT, and FNA-PTH has been recently developed and largely diffused worldwide in the last years. The technique is easy to perform, without a dedicated needle: samples can be collected from FNA for cytology by washing out the needle, after dispensation of the specimen onto the appropriate slides. Despite the achievement of satisfactory results, the determination of thyroid and parathyroid markers in fluids other than blood poses today one of the major challenges to laboratory medicine due to the lack of international standards for the performance and interpretation of the technique.

Table 3: Accuracy of FNA-PTH reported in the main studies.

First author, year [Refs]	Patients	Analytic method	Adopted cutoff, ng/L	Sensitivity, %	Specificity, %
Sacks, 1994 [48]	45	IRMA	20	82	100
Kiblut, 2004 [49]	170[a]	CLIA	1000	87	75
Conrad, 2006 [50]	66	ECLIA	1000	80	100
Kwak, 2009 [51]	18	IRMA	PTH-FNA > PTH-Serum	92.9	100
Boi, 2012 [52]	43	CLIA	103	100	100
Kuzu, 2016 [53]	57	NA	PTH-FNA > PTH-Serum	89	100

[a]One hundred patients with primary hyperparathyroidism; 50 patients with secondary hyperparathyroidism; 20 patients with thyroid diseases. CLIA, chemiluminescence immunoassay; ECLIA, electrochemiluminescence immunoassay; FNA, fine-needle aspiration; IRMA, immunoradiometric assay; NA, not applicable.

Pre-analytical factors: collection, preparation e management of the sample

The first issue to be addressed is the appropriate sampling: samples should be representative of the lesion in the lymph node or in the thyroid bed. However, unlike the FNA cytology, it is possible to make a diagnosis using FNA markers even though no epithelial cells were aspirated, since Tg, CT, and PTH present high levels both inside and in the neighboring area of the lesion [73]. In Kudo's series, the sample of one of the five patients was classified as "inadequate" for cytology, because no epithelial cells were detected [39]. Despite that, the FNA-CT level of the specimen was very high (17,000 ng/L) and was diagnosed as having MTC. Similarly, Uruno et al. [16] demonstrated that six of 16 cases, considered ''inadequate'' by FNA cytology, had high FNAB-Tg values and were diagnosed as having metastasis. In addition, Cignarelli et al. [74] showed the utility of FNA-Tg in the diagnosis of poor cellular material from cystic metastasis.

Different protocols about the pre-analytical step have been published and they vary from one another, often giving minimal detail about the needle gauge to use (21–27 G), the volume to aspirate, the volume of washout, the number of needle washouts, the dilution to use for washout, and the temperature appropriate to maintain the stability of the sample. In various studies, the volume of fluid used to wash the FNA needle ranges from 0.5 to 3.0 mL, and 1.0 mL is the amount most widely utilized [17, 19, 21, 23, 36, 75–77]. Some authors suggest rinsing the needle several times and collecting the full amount of washout fluid in a single tube. The medium used to wash the needle differs among studies, from marker-free serum (provided in the assay kit) to 0.9% saline solution. The latter is the most used option because easily accessible and inexpensive (Tables 4–7) [17, 19, 75].

Table 4: Features of measurement of FNA-Tg in neck lymph nodes.

Overall reliability	High
Solution to be used	Saline, 1 mL
Cutoff to be adopted	< 1 μg/L negative; > 10 μg/L positive
Potential false positives	Ectopic normal thyroid tissue
Potential false negatives	Presence of TgAb, hook effect
Reliability in presence of inadequate FNA cytology	Unchanged
Pre-analytic factors to be considered	Collection, preparation and management of the sample
Post-analytic factors to be considered	Units to express FNA-Tg concentration (ng/FNA units or ng/mL), cutoff
Time of work	1 day
Costs	Up to 15 €

Table 5: Features of measurement of FNA-CT in thyroid nodules and neck lymph nodes.

Overall reliability	High
Solution to be used	Saline, 1 mL
Cutoff to be adopted	< 10 ng/L negative; > 36 ng/L positive
Potential false positives	Unknown assay interferences
Potential false negatives	Hook effect
Reliability in presence of inadequate FNA cytology	Unchanged
Pre-analytic factors to be considered	The physician performing biopsy and the laboratorist must be informed of the suspicious MTC. Collection, preparation e management of the sample
Post-analytic factors to be considered	Units to express FNA-CT concentration (ng/FNA units or ng/L), cut-off
Time of work	1 day
Costs	Up to 20 €

As regards FNA-Tg, the important but still poorly assessed issues of dilution volumes and washing solutions were evaluated at Oncology Institute of Southern Switzerland [20]. In that study, the needles were washed by 1 mL of normal saline solution, and wash aliquots were randomly collected into three different tubes: plain tube, serum separator tube, and lithium-heparin tube. Even if FNA-Tg of the three groups were strongly correlated, the values were significantly higher in plain tubes as compared to the other two; furthermore, plain tubes and serum separator tubes provided 100% sensitivity, whereas false-negatives occurred in lithium–heparin tubes (98% sensitivity). Thus, the pre-analytical phase might introduce significant variability in FNA-Tg, which should be

Table 6: Features of measurement of FNA-PTH.

Overall reliability	High
Solution to be used	Saline, PTH-free serum
Cutoff to be adopted	FNA-PTH/Serum PTH ratio ≥ 2
Potential false positives	PTH truncated fragments
Potential false negatives	Hook effect
Reliability in presence of inadequate FNA cytology	Unchanged
Pre-analytic factors to be considered	Physician performing biopsy and laboratorist must be informed of the hyperparathyroidism. Collection, preparation, and management of the sample
Post-analytic factors to be considered	Units to express FNA-PTH concentration (ng/FNA units or ng/L); cutoff
Time of work	1 day
Costs	Up to 15 €

Table 7: Key points for standardization of FNA-marker measurement.

Sampling	Representative of the lesion in the lymph node or in the thyroid/parathyroid bed
Solution to be used	0.9% saline solution, 1 mL
Collection	Rinse the needle, if possible, two or more times; collect the full amount of washout fluid in a single plain tube and keep on ice CT and PTH samples throught the entire process as they are poorly stable peptides
Pre-treatment	Mix and centrifuge the sample to discard cellular debris coming from blood and tissue contamination
Measurement	Consider potential assay interferences; i.e. if undetectable FNA-marker is found, use dilution or batching to detect a possible "hook effect"
Adopted cutoff	Consider the method used by each individual center to determine the FNA-marker

reduced by using a fixed dilution fluids volume and a plain tube (Table 7) [20].

Also for CT and for PTH, the published papers described different protocols of sample dilution (i.e. 0.5, 1.0, 2.0 mL) and solution (i.e. saline, marker-free serum, dilution buffer) in the tube, but there are no strong data on the ideal fashion to prepare, handle, and store these samples (Tables 5–7) [43]. Undoubtedly, both CT and PTH are poorly stable peptides in serum, and consequently also in FNA, requiring precautions for preservation (i.e. need to be kept on ice throught the entire process) [70, 77].

In addition, it is important to bear in mind that before the measurement, the sample could require a pre-treatment such as mixing and centrifugation to discard cellular debris coming from blood and tissue contamination [78].

Analytical factors: "hook effect", immunoassay interference, analytical variability

Measuring thyroid/parathyroid markers in fluids other than blood poses difficulties to laboratories due to numerous factors (interferences, lack of methodological standards, inadequate functional sensitivity, and analytical variability of the commercially available antibody kits) affecting the accuracy of the results and so making hard the comparison between studies [79]. In addition, the measurement of Tg, CT, and PTH in non-serum/plasma samples is problematic from an analytical perspective due to the lack of experimental data to support the validity of results and absence of formal support for this application by commercial manufacturers [6, 69, 78].

In the last few decades, the more specific and sensitive immunometric assays (IMAs) (i.e. IRMA, immunoenzymatic-ELISA and CLIA) have widely replaced older competition-based measurement methods, using radioactive iodine tracers (radioimmunoassay [RIA]) for the measurement of peptides and proteins such as TG, CT, and PTH. Although the use of the most advanced IMAs present several advantages (higher functional sensitivity, shorter incubation time, wider working range, more stable labeled antibody reagent), many technical problems remain to be solved and need to be considered when measuring markers in washout fluids [29].

First, IMAs are subject to the "hook effect": very high concentrations of the marker cause falsely lower results; it is due to the saturation by the antigen of the binding capacity of the capture antibodies on the solid phase support, preventing the sandwich from forming [30]. The "hook effect" is more frequent in one-step, two-site immunoassay, although it can also occur in two-step methods [77]. Samples dilution or batching must be used to detect this effect, especially whether undetectable FNA-marker is found [19, 75].

Second, some types of interference can alter IMA results. As regards Tg, it is widely known that its measurement in serum can be affected by the presence of TgAb. Their presence could mask epitopes used by reagent antibodies, causing false-negative results or at least an underestimation of serum Tg concentration. The possible influence of the antibodies in the determination of FNA-Tg is an issue addressed by several studies with inconclusive results. In particular, Baskin [80] recorded no interference, and then similarly, Boi et al. [31] demonstrated that, although TgAb positivity could determine a slightly underestimation of FNA-Tg concentrations, no false positive cases were found in their data. Meanwhile, some studies described lower FNA-Tg concentrations in washout fluids containing TgAb [18, 76, 81, 82], suggesting that this finding might be due to blood contamination or active intra-nodal TgAb synthesis [83]. Interestingly, in his study, Spencer [84] showed that any detectable level of TgAb, although lower than the cutoff of positivity, has the potential to interfere with Tg measurement relative to its level. Recently, Netzel et al. [85] reported that Tg quantification by liquid chromatography-tandem mass spectrometry (LC-MS/MS) overcame TgAb interference: 20% of TgAb-positive serum samples that tested negative for the presence of Tg by IMA (< 0.1 µg/L) had Tg concentrations ≥ 0.5 µg/L when determined by LC-MS/MS. However, because the current Tg-MS assays have a manual and complex

workflow and above all present a suboptimal functional sensitivity (FS) resulting in misclassification of patients with Tg between 0.1 and 0.5 μg/L, the authors concluded that Tg-IMA with optimal FS should remain the first-line test in TgAb-positive patients. The use of Tg-MS should only be reserved for selected cases with both TgAb positive and Tg undetectable by IMA, where the precise quantification of Tg is considered clinically essential.

In addition, some years ago, a paper [86] described FNA performed in two PTC patients with positive TgAb before and after rhTSH; detectable FNA-Tg levels were found only after rhTSH stimulation. The authors speculated that the Tg excess induced by rhTSH stimulation may 'saturate' all TgAb binding sites, explaining why Tg was only detectable after rhTSH stimulation in their patients. However, the several limitations of that case series (i.e. the accuracy of lymph nodes sampling not definitely confirmed, lack of cytologic examination, first generation Tg assay) do not support this 'rhTSH-stimulated FNA-Tg measurement' approach [35]. Despite these studies, all in all, the influence of TgAb on the clinical performance of FNA-Tg was poor and FNA-Tg levels remained detectable [31].

Of note, the measurement of TgAb could itself be problematic in the washing liquid [27]. As part of the thyroid autoimmunity, the analytical limitations of serum TgAb assays have been reported [87]. In particular, there is a large inter-method variability in results with IMAs for serum TgAb despite the introduction of the International Reference Preparation 65/093 (IRP 65/093) [88]. Further confusion may result from the use of different concentrations or cutoff values for defining 'positive' and 'negative' TgAb results [85].

Another analytical aspect to consider is the large inter-assay variation of FNA-Tg, FNA-CT and FNA-PTH results, obtained by different IMAs, causing important standardization problems and, consequently, confusion in interpretation of the results.

With regards Tg, the significant discordances in serum results (i.e. up to 40%–60%) [29] were reduced (to about 30%) by the introduction of the Certified Reference Material 45 (CRM-45). Such existing variability is due to the differences in the Tg epitope specificity of the antibodies used as reagents in various IMAs and to the heterogeneity of circulating human Tg [88, 89].

Also for FNA-CT measurement, some analytical problems should be considered, similar to what happens in serum/plasma. In particular, the differences in the antigen structure and in the immunoreactivity, due to different antibodies of the assay scheme, lead to large discordance of the results published in literature [77]. Immunocytochemistry for CT has so far been the only tool to increase the diagnostic accuracy of FNA cytology in detecting MTC.

Finally, cases of MTC with negative serum CT have been described. These cases might have false-negative FNA-CT results. Nevertheless, the number of cases with negative serum CT is negligible, and most of these were undifferentiated tumors or microcarcinomas [6, 90, 91]. On the other side, this measurement seems reliable also in MTC with ultrasound presentation as pure cyst [92].

Like the other two markers (Tg and CT), even the determination of FNA-PTH is characterized by a large inter-method variability, ranging from 1.8- to 4.2-fold in the most common third-generation (3G) commercially available IMAs [68, 69]. Moreover, the measurement of PTH could be affected by the presence of truncated fragments in biological fluids able to cross-react with the antibodies used in IMAs.

Another analytical aspect to consider is the FS of IMA, which greatly influences the performance of the kits. It is defined as the lowest measured concentration of the marker with a coefficient of inter-assay variation of 20% [89] and it represents the lowest clinically relevant value detected by the test. The FS of a method directly influences the cutoff [22]: the greater FS of the currently available assays accounts for the progressive reduction of the suggested diagnostic thresholds. Thus, some authors suggest that the FNA marker cutoff should be adapted to the method of measurement used by each individual center (27). In the first study about the cutoff of FNA-Tg [4], the threshold, defined by mean ± standard deviation, was quite high (21.7 ng/punctuation) and it was due to the low FS of the method used to measure Tg (3 μg/L). In the last decades, IMA has been greatly improved with a very good FS to give reliable results also in the very low concentration range (between 0.1 and 1 μg/L) [84].

Furthermore, when determining the concentration of a marker in fluids other than serum/plasma, we have to consider the so-called matrix effects that are changes of the medium in which the marker is measured. According to this theory, the solution used to wash the needle does not contain the marker but it is characterized by physiochemical properties (pH, polarity, particles of solid lesions), able to induce non-specific interactions with the reagents of the kit, particularly by influencing protein conformation (i.e. antibodies). Thus, matrix effects could represent confounding factors, about which still little information is present in literature [73, 93]. For example, Baskin [80] and Snozek et al. [19] reported slightly higher FNA-Tg levels in physiological saline than in TG-free buffer solution, thus confirming a change in washout solution. Such a matrix

effect might account for the high Tg levels found by some authors in benign LNs [38]. Despite the demonstration of the matrix effect in some studies, the most advanced IMA for serum markers (i.e. Tg, CT and PTH) do not seem to be affected by this type of interference, obtaining comparable results with the use of saline, marker free-serum and kit buffer [73, 75].

All in all, the measurement of a marker in non-serum/non-plasma samples is problematic from an analytical point of view due to the lack of experimental data to support the validity of results and the absence of formal support for this application by commercial manufacturers of assays. The responsibility of the laboratory is thus to determine whether analysis of non-serum/non-plasma samples is listed as an 'intended use' in the information for users provided by manufacturers. In its absence, full analytical validation to regulatory standards by laboratories is required.

Post-analytical factors

The marker measured in the FNA fluid is not the true concentration but it reflects the dilution of the marker left in the needle in the arbitrary selected volume of the washout fluid. Thus, some authors and also the ETA guidelines suggest expressing Tg, CT, and PTH in ng/FNA units [36]. Nevertheless, several studies reported FNA-marker in µg/L or ng/L, allowing for the comparison of the FNA-marker and serum-marker levels.

Moreover, in the interpretation of FNA marker levels, it is important to consider the clinical context of the patient, such as pre/post-thyroidectomy, histopathological diagnosis, and serum TSH concentration [78].

Conclusion for clinical use of FNA-Tg, FNA-CT, and FNA-PTH

Measuring endocrine biochemical markers in FNA washout fluids rapidly emerged as a powerful and relatively cheap tool to refine challenging clinical diagnosis in patients with thyroid/parathyroid tumors. In particular, both FNA-Tg and FNA-CT measurements are now included in current clinical guidelines. Nevertheless, we underline that results should be used in conjunction with information from the clinical evaluation of the patient and other diagnostic tools. A close cooperation between laboratory specialists and clinicians involved in thyroid/parathyroid diseases' care is mandatory to define the most appropriate pre-analytical procedures, to select accurate interpretation criteria, and to properly address cases with conflicting results. In our experience, the presence of laboratory specialist during FNA procedures was relevant, especially during the introduction of these techniques in daily clinical practice, to define an accurate workflow.

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