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Pitfalls in Thyroid Fine-Needle Aspiration Cytopathology: An Approach to Atypical Findings

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Abstract
Background: Thyroid nodules are prevalent among the general population, thus imposing substantial demands upon healthcare providers to establish effective management paradigms when investigating these lesions. A pivotal component in the diagnostic process involves the cytomorphological evaluation of fine-needle aspiration (FNA) specimens extracted from the nodule under scrutiny. This examination serves the critical purpose of enabling a comprehensive assessment for the risk of either a neoplasm or malignancy, thereby providing the clinical team with the requisite information to render decisions regarding potential surgical intervention and/or a structured clinical follow-up. A subset of FNA specimens obtained from the thyroid gland present a vexing challenge for interpretation and cannot be classified based on cytomorphology as either benign or malignant and are classified as "indeterminate" for neoplasm or malignancy. The indeterminate thyroid FNA diagnosis in the third iteration of the Bethesda classification is termed as "atypia of undetermined significance" (AUS). ***Summary:*** The thyroid FNA specimens classified as "atypical" constitute a perplexing category, necessitating considerations such as repeated cytological evaluations, supplementary molecular analyses, diagnostic lobectomy, or vigilant surveillance. This review article draws upon the most recent Bethesda classification guidelines and delineates various potential pitfalls encountered during the interpretation of atypia observed in thyroid fine-needle aspiration and histopathologic counterparts. Additionally, it proffers strategic algorithms devised to effectively navigate these diagnostic challenges. ***Key Messages:*** It is important to recognize the value of an integrated approach when triaging AUS lesions, considering various clinical, morphological, and sometimes also immunocytochemical or molecular features.

Introduction

The emergence of the Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) marked a groundbreaking leap in the realm of thyroid fine-needle aspiration cytology (FNAC). This innovative reporting system employed a simplified, 6-category format, ushered in a new era of standardized thyroid cytopathology reporting. The first two editions of TBSRTC, introduced in 2010 and further refined in 2017, garnered widespread

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acceptance within the global medical community. In recent years, a series of pivotal developments have unfolded within the field of thyroid pathology, profoundly impacting clinical practice. First, a 5th edition of the World Health Organization (WHO) classification of thyroid tumors has emerged, introducing substantial changes to nomenclature and taxonomy [1, 2]. Second, molecular testing based on molecular profiling of thyroid lesions has ascended as a useful tool in clinical practice, offering invaluable insights into the diagnosis, prognosis, and therapeutic strategies for thyroid cancer. Its growing significance underscores its potential to revolutionize the management of thyroid malignancies. Furthermore, clinical guidelines continue to evolve. Notably, surveillance strategies are gaining prominence as a scientifically grounded approach, particularly for managing subsets of indolent lesions. This shift reflects a paradigmatic transformation in our approach to patient care and underscores the importance of evidence-based management.

Considering the above-mentioned transformative developments, the third edition of the TBSRTC was propelled into existence in 2023 [3]. This updated edition is poised to serve as a vital resource in navigating the evolving landscape of thyroid pathology, providing clinicians and pathologists with the tools necessary to adapt to these progressive changes. The 2023 edition of TBSRTC presents a streamlined set of six reporting category names: I: nondiagnostic, II: benign, III: atypia of undetermined significance (AUS), IV: follicular neoplasm, V: suspicious for malignancy, and VI: malignant [3]. The previous version of this algorithm included alternative names for half of the categories, and these have now been omitted ("unsatisfactory" in category I, "follicular lesion of undetermined significance" in category III, and "suspicious for a follicular neoplasm" in category IV). Moreover, the TBSRTC now recommends that the actual category names are reported along with their numerical designations. This practice serves to prevent any potential confusion, especially when juxtaposed with imaging algorithms such as the Thyroid Imaging and Reporting System (TIRADS) [4].

The inherent robustness of the TBSRTC system resides in its commitment to objective reporting and its capacity to amass prospective series from multiple institutions, enabling the comprehensive analysis of histologic outcomes in patients who underwent surgical procedures. Notably, considering the significant body of extensive research published after the unveiling of the 2017 TBSRTC guidelines, the 2023 TBSRTC reporting scheme incorporates novel data related to the "risk of malignancy" (ROM) specific to each category [3]. While the mean ROM is low for TBSRTC categories I and II (13% and 4%, respectively) and high for categories IV and VI (74% and 97%, respectively), diagnostic categories III and IV remain inadequate in terms of triaging cases for surgery, with ROM at 22% and 30%, respectively. However, it is important to acknowledge the inevitable risk of selection bias, as not all thyroid lesions in the categories II–III undergo surgical excision.

In thyroid cytology, a perplexing conundrum presents itself in the form of the enigmatic "gray zone" inhabited by indeterminate FNAC, concentrated in TBSRTC categories III–IV. While most thyroid lesions can be confidently classified as either benign (TBSRTC II) or malignant (TBSRTC VI), a sizeable cohort of nodules falls within the uncertain realm of indeterminate categorization. The most clinically challenging lesions in terms of indeterminate FNAC associate to the Bethesda category III/AUS, a group that may represent a wide spectrum of atypical cellular features, spanning from architectural to cellular variations. What unites these atypical findings is their shared occurrence in both benign and malignant entities, and even though these features may not alone suffice to warrant a Bethesda VI category diagnosis, they raise legitimate concerns regarding the potential nonbenign nature of the lesion in question [5, 6]. The significant consequence of overcalling thyroid FNAC specimens as "atypical" can lead to unwarranted surgical interventions, which not only pose risks to patients but also burden the healthcare monies. It is therefore an imperative mandate for the practicing diagnostician to maintain a keen awareness of both the common and less recognized pitfalls that permeate the landscape of thyroid cytopathology. This awareness is essential in navigating the intricate web of diagnostic challenges while ensuring accurate patient care.

Teasing out "Atypia" in Thyroid Gland

The term "atypia" within the context of TBSRTC III-AUS encompasses a spectrum of nuclear and architectural changes that collectively are not sufficient to render to classify thyroid FNA specimens as TBSRTC categories IV–VI. In general, thyroid FNA specimens that are categorized as AUS exhibit one of the following: (a) group of cells exhibiting mild to moderate nuclear atypia either in the form of nuclear pleomorphism or features reminiscent of papillary thyroid carcinoma (PTC) and (b) cells arranged in microfollicles, solid, trabecular, or papillary formations (architectural atypia) (Fig. 1, 2) [3, 7]. These atypical features come with their associated pitfalls, and

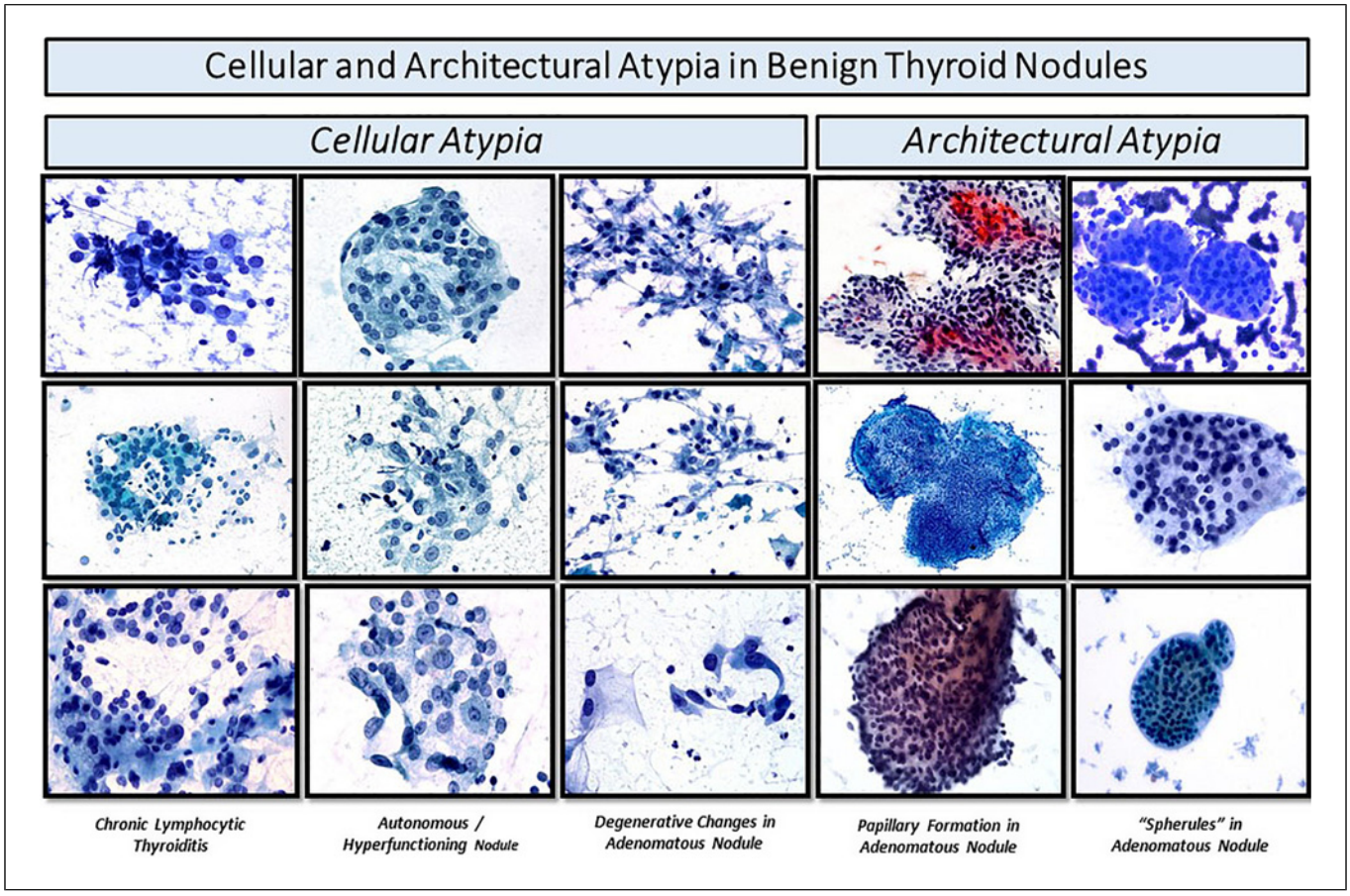

Fig. 1. Examples of cellular and architectural atypia in FNAC of Bethesda III category diagnoses ultimately confirmed benign by histopathology.

care must be taken not to overinterpret subtle findings as malignant. In addition to nuclear and architectural atypia, various unusual findings in other cellular or stromal elements may also serve as the reason for an AUS diagnosis.

While this review primarily emphasizes TBSRTC, it is essential to underscore that various national thyroid FNA cytological classification schemes, such as those employed in the UK and Italy, have undergone extensive validation through meta-analyses. These validations include calculations of the ROM for all diagnostic categories, with a particular focus on indeterminate categories. The Italian system subdivides indeterminate nodules based on atypical features; a similar strategy has also been incorporated in the 3rd edition of TBSRTC. In contrast, the UK system (UK RCPath) consolidates cases with nuclear and architectural atypia into a single category [8]. In the following sections, different types of atypia are listed, along with their clinical implications.

Nuclear/Cellular Atypia

Thyroid FNA specimens classified as AUS based on nuclear atypia are associated with a higher ROM as compared to cases with isolated architectural atypia [9–12]. Several studies have compared the ROM in which FNAC samples were subdivided based on whether they exhibited nuclear and/or architectural atypia, in which combined nuclear and architectural atypia has the highest ROM and is often shown to represent PTC on the histologic follow-up. However, isolated nuclear atypia has a somewhat lower ROM, and the ROM for cases with architectural atypia is generally much lower [9, 13, 14]. A similar trend has also been observed in thyroid FNA specimens from pediatric patients, where it was observed that the nuclear atypia as opposed to architectural atypia exhibited a clear-cut association with malignancy [15].

The idea that nuclear atypia carries the greatest significance regarding the ROM aligns harmoniously with

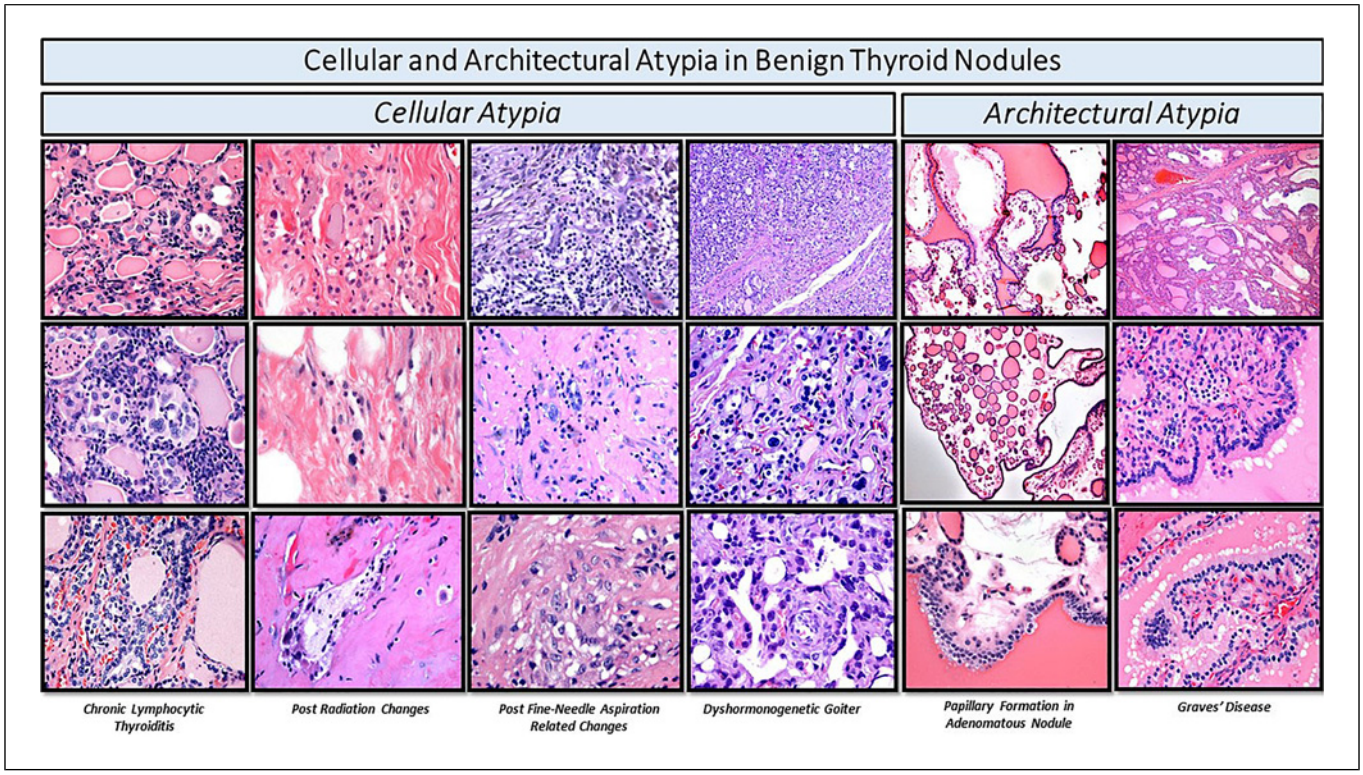

Fig. 2. Histologic features associated with cellular and architectural atypia in FNAC.

the molecular characteristics of AUS lesions. Within this context, instances featuring nuclear atypia demonstrate a heightened occurrence of *BRAF* mutations (Fig. 3) [9, 16]. Conversely, low-risk genetic anomalies typically associated with benign tumors, such as *KRAS* and *PTEN* mutations, as well as *PAX8::PPARG* fusions, were found to be more prevalent in cases characterized solely by follicular patterned tumors, leading to microfollicles in FNA specimens, i.e., architectural atypia [9].

In the AUS category, slight-to-moderate nuclear changes are allowed, such as mild nuclear enlargement, overlapping, nuclear membrane irregularities, and chromatin clearing (Fig. 1). The reason for a good proportion of noninvasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTPs) is preoperatively diagnosed as AUS (Fig. 3) [17]. The finding of multiple, *bona fide* nuclear pseudoinclusions, however, in most instances should raise the suspicion of a *BRAF*-driven tumor, so this nuclear feature is not included among nuclear changes triaging lesions to the AUS category (Fig. 3). To summarize, nuclear atypia in the AUS category should be more prominent that what one would expect from a Bethesda II category lesion, but not sufficient to annotate the tumor as Bethesda V–VI.

In previous 2 editions of the TBSRTC, the interobserver diagnostic reproducibility of the original AUS/FLUS category was, at best, moderate [18]. With the 3rd edition, TBSRTC now separates nuclear atypia from other atypical categories in AUS, which is based on features generally recognizable in the everyday practice of cytology. It is expected that the reproducibility of the AUS category for nuclear atypia will be higher compared to other forms of atypia. This expectation finds support in prior research utilizing the Italian thyroid cytology classification system framework, demonstrating that the subdivision of the indeterminate diagnostic category III conferred increased ROM values for cases with nuclear atypia compared to architectural atypia [19].

Several non-PTC thyroid conditions with occasional nuclear atypia warranting an AUS category diagnosis are highlighted in Table 1 and illustrated in Figures 1 and 2. Notably, various benign thyroid lesions are great mimickers of PTC due to various degrees of nuclear changes, often due to reactive or reparative cellular features. Therefore, many experts advise stringent adherence to the major diagnostic features of PTC to avoid false-positive diagnosis [20]. Interestingly, some authors have also found important genotype-phenotype correlates, with an

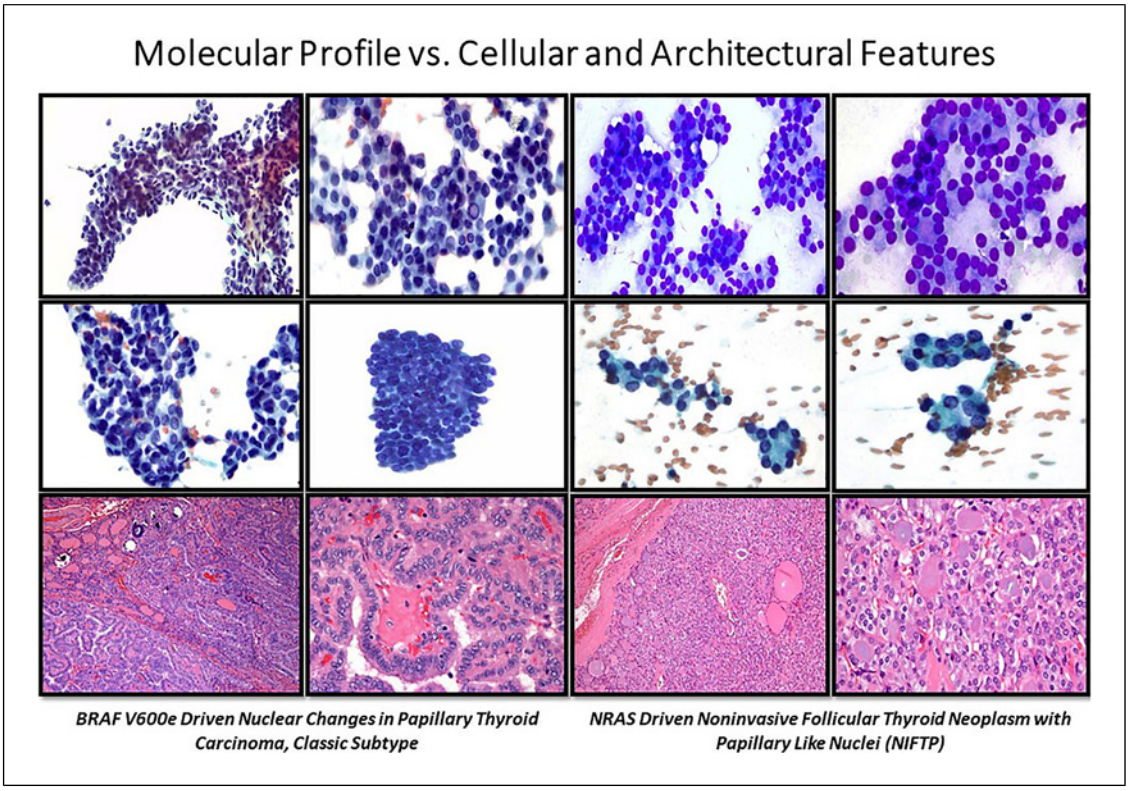

Fig. 3. Cellular/nuclear and architectural features in a *BRAF* V600E-driven, classic PTC compared to *NRAS*-mutated NIFTPs. Note the prominent nuclear atypia and clear-cut papillary architecture in the fine-needle aspirate of a classic PTC compared to the subtle nuclear and architectural atypia with microfollicles of an NIFTP.

Table 1. Common non-PTC thyroid lesions with various types of atypia on FNAC

Histopathologic diagnosis	Nuclear atypia on FNAC	Architectural atypia on FNAC	Other forms of atypia on FNAC
Graves' disease Hashimoto's thyroiditis	Intranuclear grooves –	Microfollicles –	ATD-related atypia Oncocytic atypia, lymphocytic atypia, psammoma bodies
Hyperplastic nodules/TFND	Intranuclear grooves Nuclear pleomorphism	Papillary structures, microfollicles	Amyloid deposits, psammoma bodies
Follicular thyroid tumors	Crowding	Papillary structures, microfollicles	Mucin production, signet ring cells
Oncocytic thyroid tumors	Intranuclear grooves Nuclear pleomorphism Intranuclear inclusions (rare)	Microfollicles	Vanishing tumors on repeat FNA, reactive spindle cells (mesenchymal origin), psammoma bodies
NIFTP	Enlarged, crowding, intranuclear grooves, nuclear chromatin clearing	Microfollicles	–
Hyalinizing trabecular tumor	Intranuclear grooves, pseudo-inclusions	–	–
MTC	Intranuclear grooves pseudoinclusions	–	Amyloid deposits, rarely intracellular pigment (melanin)

PTC, papillary thyroid carcinoma; FNAC, fine-needle aspiration cytology; ATD, antithyroid drug; TFND, thyroid follicular nodular disease; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features.

overrepresentation of *THADA* gene fusions in benign thyroid lesions with occasional nuclear grooves [21]. It is also equally important to note that other malignant tumors of the thyroid gland can display few but not all cytological features of PTC, such as intranuclear grooves or occasional nuclear inclusions, features that may be encountered in medullary thyroid carcinoma (MTC) and oncocytic thyroid carcinoma [22, 23].

Architectural Atypia

Thyroid FNA specimens classified as TBSRTC III-AUS based on architectural atypia show either microfollicles or papillary formation [24]. In cytology literature, a microfollicle is a flatter and poorly circumscribed follicular cell group, which shows nuclear crowding and overlapping. It is well documented in the literature that microfollicles are commonly encountered in both benign and malignant follicular-derived lesions of the thyroid gland. However, some have placed more emphasis even on the presence of few microfollicles as one of the atypical features. It is also prudent to know that sharply defined circumscribed collections of thyroid follicular cells termed as "spherules" can be mistaken for microfollicles, and are usually associated with benign conditions (Fig. 1) [25]. Papillary structures without readily identifiable nuclear features of PTC can be seen as focal or a prominent change in follicular nodular disease, chronic lymphocytic thyroiditis (CLT; also known as Hashimoto's thyroiditis), Graves' disease, oncocytic thyroid tumors, follicular adenoma with papillary architecture, and *DICER1*-mutated thyroid lesions, ranging from thyroid follicular nodular disease to follicular thyroid tumors (Fig. 1, 2) [1, 26–28]. The ROM for cases with architectural atypia in most instances is quite low, but not negligible [9, 13, 14].

Other Forms of Cellular Atypia

In addition to nuclear or architectural atypia, various other cellular characteristics have been documented, which are typically uncommon in thyroid lesions and have the potential to induce diagnostic perplexity. In the following account, we list some of the cellular features, which may be responsible for classifying a thyroid FNA specimen as AUS.

Oncocytic Atypia

When assessing thyroid FNACs, caution must be exercised when diagnosing a potential oncocytic thyroid neoplasm. Oncocytic change, focal to diffuse, can be encountered in various thyroid lesions. Several nonrelated confounders exist, for example, CLT, follicular nodular disease, primary granular cell tumor, autonomous nodule, and intrathyroidal parathyroid adenoma (Fig. 2). Moreover, certain PTC subtypes and subsets of medullar thyroid carcinoma (MTC) may also exhibit oncocytic features. In CLT, the oncocytes represent metaplastic transformation of follicular epithelial cells [29], and care must be taken not to overinterpret oncocytic cell predominance of FNAC specimen as a diagnostic of either TBSRTC III or IV. This is particularly important if the specimen shows an appreciable number of lymphocytes in the background and percolating among the follicular cell groups, and thyroid function tests especially serum TSH and serologic studies showing overt hypothyroidism. It is important to note that oncocytic metaplasia is frequent in adult CLT cases but may not be so prominent in pediatric patients [29].

Clear-Cell Change

Clear-cell change can occur in primary thyroid lesions, with a reported frequency of 1–3%. This phenomenon is most encountered in FTCs but is a more infrequent occurrence in PTCs and oncocytic thyroid carcinoma. The underlying cause of the clear-cell phenotype is believed to stem from intracytoplasmic deposits of glycogen or lipids. While the histologic manifestation of clear-cell change appears to have limited prognostic significance, it poses a significant challenge in terms of differential diagnoses, as follicular thyroid tumors, PTC, MTC, metastatic renal cell carcinoma, and parathyroid tumors all may present with these rare features [30–34].

Signet Ring Morphology

Rarely, benign and malignant thyroid tumors may show signet ring cell features [35–38]. The clinical attributes of these lesions are not believed to be different from conventional thyroid tumors of the same type, but the bulk of information is derived from small case series, given the rarity of the diagnosis. The preoperative FNAC is often indeterminate, and most cases undergo diagnostic lobectomy [35].

Mucin-Producing Tumors

Mucinous variants of papillary and follicular thyroid carcinoma have been previously reported, and a similar phenomenon is also encountered in MTC [39–43]. Due to their overall rarity, data are limited regarding incidence and prevalence. On the preoperative level, the lesions should not be confused with primary salivary-type tumors such as secretory carcinoma and mucoepidermoid carcinoma, as well as metastatic tumors with mucin production, such as adenocarcinoma of gastrointestinal, pulmonary, or mammary origin.

Pigmented Lesions

Hemosiderin-laden macrophages are usual findings in thyroid aspirates from adenomatous nodules arising in follicular nodular disease with cystic degeneration and usually do not warrant additional attention. Histiocytic cells can show enlarged elongated nuclei with chromatin clearing and intranuclear grooves, mimicking PTC features [44]. Rarely, atypical histiocytic cells with grooved or contorted nuclei may be noted – which should alert the cytologist of the possibility of isolated Langerhans cell histiocytosis of the thyroid [45, 46]. On postoperative analysis, CD1a and S100 immunoreactivity are noted in the Langerhans' cells, and the lesion is often driven by a *BRAF* V600E mutation [45]. The presence of melanotic pigment in the thyroid ("black thyroid gland") is closely associated with prolonged minocycline therapy but rarely may also represent melanotic MTC [47, 48]. It is crucial to exclude metastatic melanoma when faced with an aspirate showing lesional cells with intracytoplasmic pigment.

Endocrine-Type Degenerative Atypia

Pronounced endocrine-type cellular atypia is characterized by marked nuclear pleomorphism and bizarre cellular features and is observed in the subset of cases representing clinically benign conditions such as Graves' disease treated with antithyroid drug regimen, dyshormonogenetic goiter, and normal thyroid tissue exposed to irradiation (Fig. 2) [49, 50]. In some instances, the preoperative FNAC may be overinterpreted as malignant based on these cellular features, leading to unnecessary surgical intervention. In a recent series, 4.1% of cases with endocrine-type atypia were false positive for malignancy on preoperative FNAC, thus highlighting the need for careful assessment and review of clinical features of seemingly benign lesions with pleomorphic features [50].

Other

Other forms of thyroid atypia can be encountered in thyroid gland, which are unrelated to the tumor morphology and structure, and rather reflective of stromal changes. Some of the most encountered features are listed briefly below.

Lymphocytic Atypia

Lymphocytic aggregates are common in the thyroid and may be indicative of an underlying CLT, subacute thyroiditis (de Quervain's thyroiditis), or silent (focal) thyroiditis (Fig. 1, 2). However, lymphocytic infiltration is also commonly encountered in certain subtypes of PTC, such as the diffuse sclerosing PTC and Warthin-like PTC [51, 52]. As a rule of thumb, lesions with lymphocytic background should only be diagnosed as PTC when all major diagnostic features are present. If the cytologist cannot safely rule out lymphoma, flow cytometry has been shown to be helpful [53].

Vanishing Thyroid Tumors

A rare but acknowledged group of lesions in thyroid cytopathology are the so-called "vanishing thyroid tumors," which are degenerated lesions induced by secondary changes associated to a previous FNAC procedure. This is more commonly encountered in cases where a small gauge needle (22–23 gauge), instead of larger gauge (25–27 gauge), has been used. In a series of 14 cases, TBSRTC V-VI (suspicious for PTC/PTC) and TBSRTC III were the two most common preoperative diagnoses; and 7 of the vanishing thyroid tumors were nondiagnostic on postoperative pathology [54]. Oncocytic thyroid tumors seem particularly overrepresented among cases that degenerate/vanish following FNAB [55, 56]. The mechanistic reason for the abolition of tumor tissue is not known. For the practicing cytopathologist, the entity may be of interest for in cases lacking tumor in the surgical pathology specimen undergoing multiple FNA procedures.

Amyloid Deposits

Amyloid deposits are usually associated with MTC and can be visualized by Congo red staining with the characteristic apple-green birefringence under polarized light. Focal amyloid depositions may also be detected in amyloid goiter – a rare but potential differential diagnosis, which should not be overlooked, especially in patients with an established amyloidosis [57–59].

Desmoid-Like Fibromatosis

A subset of PTCs may present with an associated desmoid-like fibromatosis, a synchronous soft tissue neoplasm driven by *CTNNB1* mutations [60–62]. In some instances, the finding of atypical spindle cells on FNA may lead to diagnostic conundrums. A nuclear beta-catenin staining (preferably performed on the cell block specimen) may be indicative or diagnostic of fibromatosis in such cases.

Psammoma Bodies

As defined, psammoma bodies are small, round to flower-contoured lamellated calcium deposits that can occur in various neoplastic processes throughout the body such as PTC, serous ovarian carcinoma, meningioma, duodenal neuroendocrine tumors, and papillary renal cell carcinoma, to name a few. In thyroid

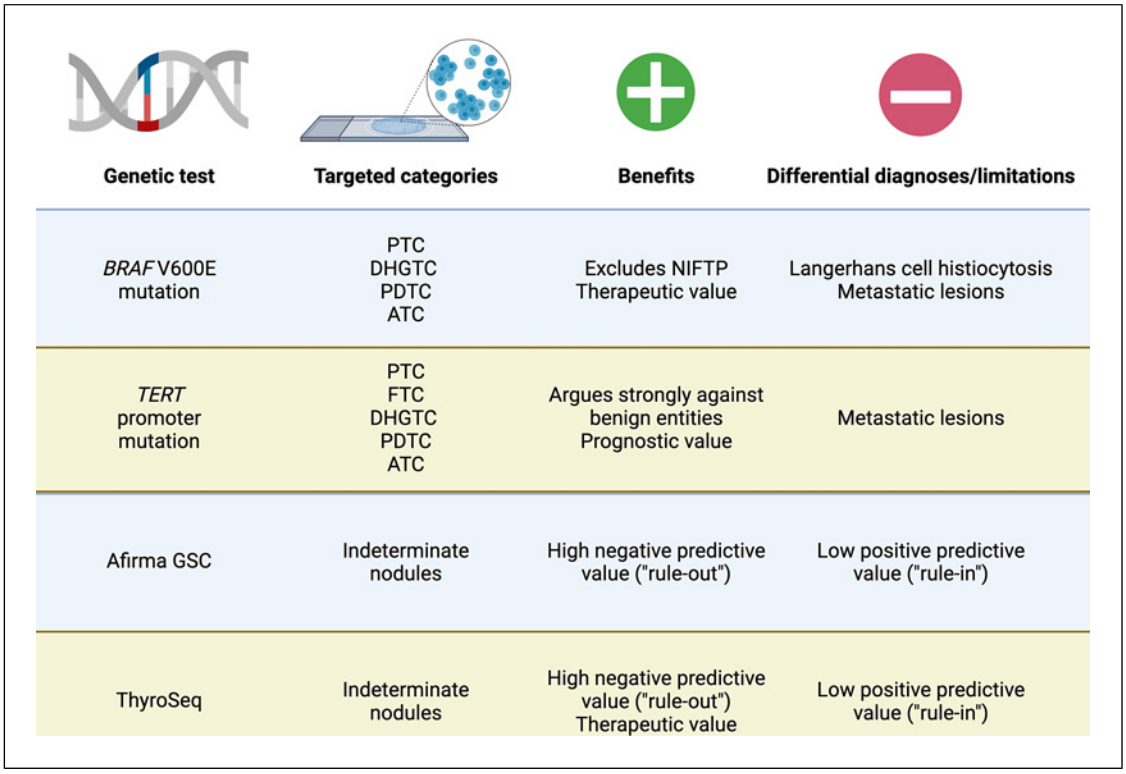

Fig. 4. Overview of useful genetic tests in the preoperative workup of indeterminate thyroid nodules. *BRAF* V600E and *TERT* promoter mutations can be assayed as single-gene tests with high positive predictive values for malignancy, while Afirma® GSC® and ThyroSeq V3® are two well-known multigene panels with a broader range. PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; DHGTC, differentiated high-grade thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; ATC, anaplastic thyroid carcinoma; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features. Created using BioRender.com.

cytopathology, the finding of psammoma bodies may indicate a PTC, but it is important to know that other, non-PTC lesions may occasionally can harbor psammoma bodies, such as oncocytic thyroid tumors, thyroid follicular nodular disease, and CLT [63–65].

Ancillary Technique as a Triage for "Atypical" Thyroid FNA

The selection of ancillary studies to aid triaging of thyroid lesions in the clinical setting is mostly dependent upon specific circumstances: the cytomorphological features of the specimen, and the level of clinical and radiological suspicion regarding malignancy. These adjunct assessments serve to complement the conventional cytological evaluation, playing a pivotal role in shaping thyroid nodule management. Below is a short summary of the most used auxiliary analyses in the preoperative workup of thyroid tumors.

Immunocytochemistry

Although cytomorphological assessment is the cornerstone of the TBSRTC, immunocytochemistry (ICC) may be particularly useful in cases with various forms of atypia. ICC may help to distinguish whether a lesion is a primary tumor or a metastasis to the thyroid gland, as most well-differentiated and high-grade thyroid carcinomas express the nuclear transcription factors TTF1 and PAX8. Moreover, nuclear expression of GATA3 supports a parathyroid origin. Some studies suggest that the Ki-67 proliferation labeling index could be of value cytological preparations from thyroid lesions; however, there are no established criteria for how the proliferation index will be enumerated in FNA specimens. Although malignant thyroid tumors exhibit an increase in Ki-67 compared to benign lesions, there exists a significant overlap between tumor entities [66, 67]. ICC employing antibodies targeting PTC-related markers such as CK19, HBME1, and Galectin-3 has shown some promise in cases reported as AUS, especially for cell block preparation [68, 69].

However, the specificity of these markers will require further investigations in extended series. Similarly, the mutation-specific *BRAF* VE1 antibody has shown encouraging results when applied to cell block material, albeit with a decrease in both sensitivity and specificity when used on conventional smears [70].

Loss of CD56 expression and overexpression of cyclin D1 on ICC are reported to show high predictability for malignant thyroid tumors on subsequent histopathology; however, the literature is limited on this topic [71–73]. Recently, P53 was shown to serve as a valuable diagnostic marker in cytology specimens to identify indeterminate lesions that represent well-differentiated thyroid cancers on subsequent histologic preparations [74].

Single-Gene Analyses

Molecular workup of thyroid nodules is gaining ground as a reliable method in clinical routine practice, especially for cases classified as TBSRTC III and IV. In terms of preoperative assessment, the analyses can be subdivided into single-gene assays and targeted, multigene panels – each with their benefits and downsides. Some of the main hallmarks of these tests are illustrated in Figure 4.

The most straightforward molecular analysis with a clear-cut implication for diagnosis is sequencing of the *BRAF* gene, more specifically codon 600. A somatic *BRAF* V660E mutation is the most frequent recurring event in thyroid cancer and is practically never reported in benign thyroid lesions. Specifically, *BRAF* V600E is reported in approximately 50–60% of PTC and is also found in the majority of high-grade thyroid carcinoma and anaplastic thyroid carcinoma (ATC) derived from a PTC lineage [1]. Therefore, the finding of a *BRAF* V600E mutation is inevitably indicative of malignant thyroid tumor even if the overall cytomorphology is suspicious but not confirmatory of thyroid malignancy. Moreover, the finding of such a mutation would also help the cytopathologist to exclude potential differential diagnoses such as follicular neoplasm and NIFTP, as these two entities should exhibit a *RAS*-like genomic profile and not a *BRAF* mutation [1]. However, it should be noted that metastatic melanoma or adenocarcinoma of the lower gastrointestinal tract also frequently harbors activating *BRAF* mutations, so a combination of clinical history, imaging, and morphological assessment is key to avoid diagnostic pitfalls when interpreting molecular analyses. From a clinical perspective, the 2022 WHO classification of thyroid neoplasia and current ATA guidelines suggest that ATC should undergo *BRAF* mutational testing to allow for potential tailor-made treatment with BRAF inhibitors [1, 75]. Thus, *BRAF* sequencing is recommended for all tertiary thyroid cancer centers. *BRAF* hot spot mutational testing is included in almost all modern next-generation sequencing platforms that interrogate clinically impactful cancer-associated genes, but cheaper, rapid assays that uniquely target this gene are also available. As *BRAF* mutations are clonal in thyroid cancer, multiregional sampling to ensure detection is not required [76, 77]. Many pathology laboratories also incorporate a mutation-specific BRAF antibody in the workup of thyroid tumors, although the validation of this analysis is heavily focused on the postoperative, formalin-fixated paraffin-embedded material rather than on the FNAC specimen [78].

Other single-gene aberrations with potential clinical implications are *TERT* promoter (*TERTp*) mutations, which are noncoding alterations associated with malignant thyroid tumors exhibiting a particularly poor patient outcome [79–81]. The *TERT* gene encodes the catalytic subunit of the telomerase complex, responsible for maintaining telomere length and allowing immortalization [82]. Two recurrent mutations (denoted "C228T" and "C250T") have been shown to enhance the binding of various transcription factors to the *TERT* promoter, in turn leading to increased *TERT* gene transcription. *TERTp* mutations are observed in small subsets of PTC and FTC with dismal prognosis and are frequently reported in high-grade thyroid carcinomas (differentiated high-grade thyroid carcinoma and poorly differentiated thyroid carcinoma) as well as ATC [1]. On the other hand, *TERTp* mutations are almost never found in benign thyroid lesions, thus providing the pathologist with a marker exhibiting excellent specificity [83]. In some institutions, *TERTp* mutations are routinely assayed as a postoperative analysis in rare, low-risk entities such as follicular thyroid tumor of uncertain malignant potential, given that mutation-positive samples have a real risk of future recurrences [84]. Therefore, it is not surprising to find that studies with *TERTp* mutational testing using digital droplet PCR performed on thyroid FNAC material have shown to exhibit excellent specificity, but a poor sensitivity [85, 86]. As the positive predictive value of a mutation is very high, the finding of such an alteration in a thyroid aspirate would strongly indicate a malignant tumor. For small subsets of cases diagnosed as AUS, the molecular analysis could help to triage certain cases to surgical excision in favor of re-biopsy or surveillance. Other single-gene markers of potential value when triaging cases with indeterminate cytology include *TSH receptor* gene mutations, which are almost always associated with benign disease [87].

Multigene Classifiers

Several multigene classifiers are available to increase the sensitivity of thyroid FNAC analyses, including Veracyte Afirma® Gene Expression Classifier (GEC), Afirma® Genetic Sequence Classifier (GSC), ThyroSeqV3® (Sonic Healthcare USA), RosettaGX® Reveal, and ThyraMIR®. While ThyroSeqV3® combines gene mutation and fusion signatures with the expression of various messenger RNAs (mRNAs), the other panels are focused on mutations, mRNA expression, or microRNA expressional patterns. Most of these genomic classifiers are primarily targeting lesions with an indeterminate cytology, meaning Bethesda categories III–V.

The Afirma®® GEC is developed by Veracyte Inc. and assesses 167 genes in terms of mRNA expression levels and categorizes nodules as benign, suspicious, or nondiagnostic. Recent research highlights the potential of GEC analysis in managing indeterminate cytology results with high negative predictive values, potentially reducing unnecessary surgeries by nearly 50%, particularly in the Bethesda III–IV categories [88, 89]. Building on these initial findings, the subsequently developed Afirma® GSC includes the expression of roughly 10,000 genes, with a focus on 1,115 core genes and several auxiliary components to detect parathyroid origin, MTC, *BRAF* V600E mutations, and *RET::PTC1* and *RET::PTC3* fusions, as well as the presence of oncocytic cells and oncocytic neoplasms. The GSC has proven more specific and sensitive in terms of diagnostic performance than the GEC predecessor when assessing cytologically indeterminate thyroid lesions [90, 91].

ThyroSeqV3® examines various genetic mutations rearrangements and gene expression with high sensitivity and specificity, achieving a high diagnostic accuracy in indeterminate nodules [92]. The algorithm exhibits an exceptionally high negative predictive value but, just like its Afirma®® counterpart, shows a limited positive predictive value for indeterminate cytology [93]. In recent years, ThyroSeq® has shown efficient in triaging NIFTP from infiltrative and encapsulated follicular variants of PTC, making the test of potential clinical use in high-volume centers [94].

Interestingly, studies have explored a more targeted molecular approach, exemplified by the 7-gene classifier developed by an Italian consortium [95]. The 7-gene panel test, which concentrates on key genetic events in thyroid cancer, demonstrated improved preoperative risk stratification of indeterminate nodules. This implies that employing a more focused approach with fewer genetic markers could be a valid strategy in triaging thyroid nodules for subsequent clinical management, while other groups have reported limited success in triaging indeterminate cases using a restricted set of genes [96].

Finally, in the context of genetic testing, it is crucial to emphasize that access to ancillary molecular techniques varies among different institutions globally. The reasons for this discrepancy may be rooted in the cost of the analyses, as implementing and maintaining the methodology can be expensive. Additionally, multigene classifier tests often require national regulatory approval and validation before clinical use, with regulatory processes and requirements varying across countries. Finally, disparities in national guidelines may also influence the adoption of molecular tests into practices; pathology centers may be less motivated to incorporate these tests in regions where they are not widely endorsed. Hence, numerous centers currently depend solely on cytological evaluation, and most national guidelines lack recommendations regarding the molecular workup of thyroid nodules.

Helpful Algorithms in Atypical Cases

It is well established that each thyroid FNA case demands a personalized rather than a generalized approach. Thus, offering precise guidelines for cases exhibiting any form of atypia, nuclear, architectural, or otherwise, can be challenging. Nevertheless, there exists a roadmap that can greatly benefit cytologists when evaluating a thyroid case in the clinical setting.

AUS cases represent a diverse spectrum of benign and malignant thyroid lesions displaying varying forms and extent of atypia. The clinical management may include clinical follow-up, repeat FNA (with or without ancillary studies), or perform a diagnostic lobectomy. In these instances, the value of repeat FNA should not be overlooked in case the initial assessment was indeterminate. It has been shown that cases classified as TBSRTC III or IV that are classified as benign on repeat FNA exhibit an exceedingly minimal risk for NIFTP and/or malignancy, thus casting doubt upon the utility of molecular testing in this specific cohort [97]. Moreover, multigene molecular testing in cases diagnosed as TBSRTC III on repeat FNA increases the positive predictive value of the test by reducing the number of benign cases referred to surgery [98]. Interestingly, oncocytic thyroid neoplasms may be hard to predict even by molecular testing due to their profile being different other follicular derived neoplasms [1, 98].

While most indeterminate thyroid lesions may be subjected to repeat fine needle aspiration or molecular testing, one must also acknowledge the presence of other

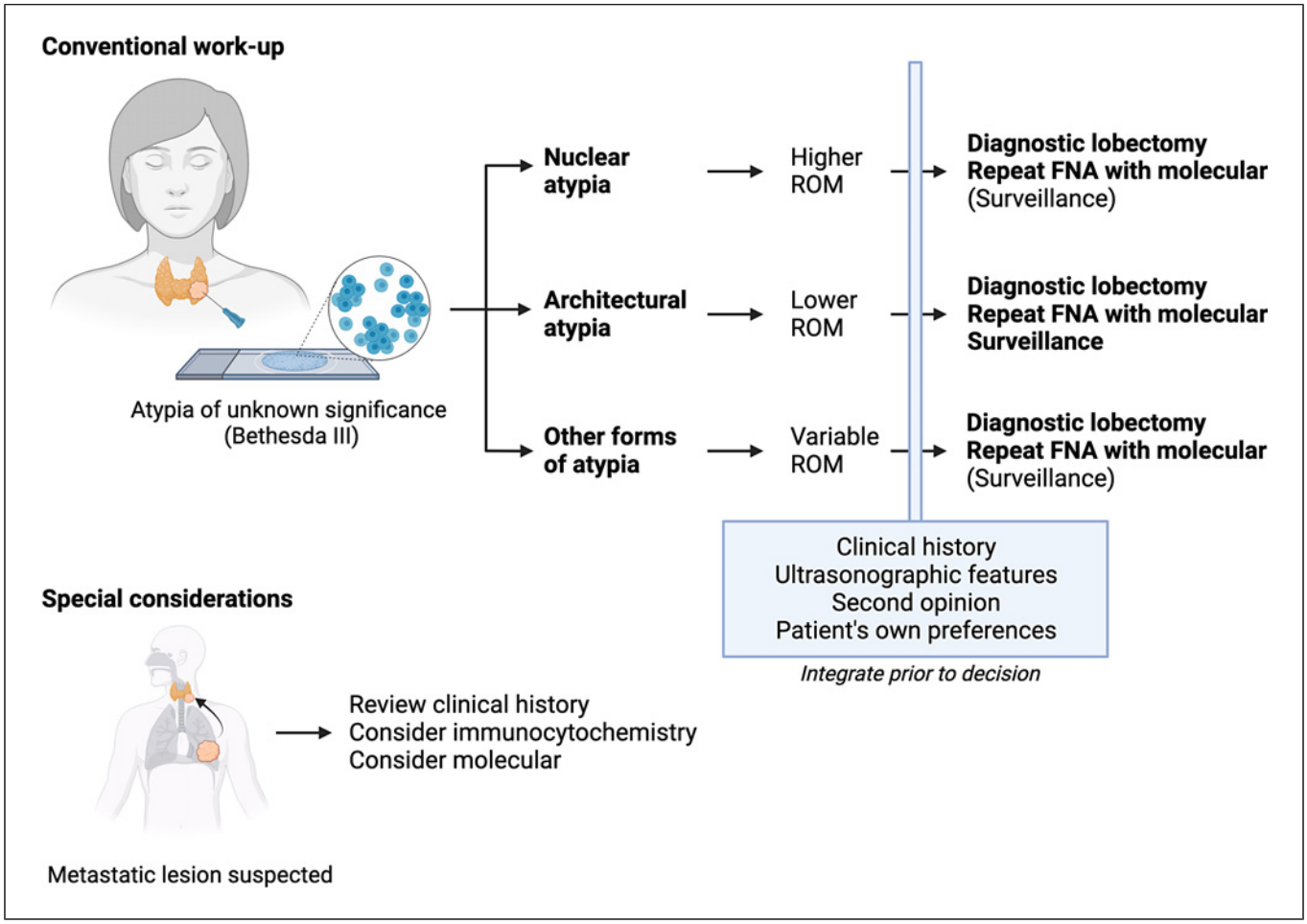

Fig. 5. Schematic representation of clinical triaging of patients with a preoperative Bethesda III category lesion. Thyroid FNA analyses with atypical features stratified into nuclear, architectural, or "other" are associated with various ROM. Preferred clinical workup is highlighted in bold text. Integration of other clinical parameters is imperative for all TBSRTC III lesions. Created using BioRender.com.

clinical methods to valuable in the risk stratification of thyroid nodules, such as integration of histologic, clinical, and ultrasonographic features. It is also important to contemplate the utilization of a core-needle biopsy in specific cases characterized by the insufficient material in cytology or instances that are notably fibrotic and challenging to aspirate. This is particularly pertinent if the clinical presentation raises concerns about malignancy. For instance, the diagnosis of ATC may benefit from a core-needle biopsy, as the increased tissue volume allows for assessments that may not always be feasible on the FNAC material. These assessments may include immunohistochemistry to rule out rare differential diagnoses and contribute to potentially therapeutic evaluations (e.g., BRAF VE1, PD-L1). Furthermore, "rapid on-site evaluation" (ROSE) in thyroid cytology offers immediate feedback to the cytopathologist during the FNAB procedure. This enables the assessment of specimen adequacy and helps identify the potential need for additional passes to obtain a representative sample. Consequently, ROSE contributes to a reduction in the likelihood of inconclusive or nondiagnostic results. Therefore, ROSE enhances the overall diagnostic accuracy of thyroid cytology and minimizes the necessity for repeat FNA procedures [99, 100]. Moreover, from an imaging perspective, the TIRADS algorithm may prove to be particularly helpful, as pairing a structured ultrasonographic examination with the Bethesda system substantially improves preoperative risk assessment of thyroid nodules [101, 102].

Overall, in comprehensive patient management, it is essential to evaluate the FNAC results, associated imaging,

related clinical features, and ancillary tests, including molecular workup, to thoroughly assess the significance of atypia. Therefore, the importance of discussing challenging patient cases in a multidisciplinary tumor board meeting cannot be underestimated. An approach to atypia in thyroid FNAC is illustrated in Figure 5.

Conclusion

Employing the lexicon of TBSRTC, cytopathologists find themselves equipped with a powerful means of conveying thyroid FNA interpretations to referring physicians. This terminology has facilitated communication in a concise, unequivocal, and clinically relevant manner, ultimately enhancing the efficacy of thyroid nodule management. However, there are still several gaps to be filled, most notably in the realm of cytologically indeterminate lesions. It is important to recognize the value of an integrated approach when triaging AUS lesions, considering various clinical, morphological, and sometimes also immunocytochemical or molecular features. Future intensified molecular research regarding the molecular underpinnings of thyroid tumors holds the potential to unveil even more refined and high-resolution risk profiles, possibly enabling translation into trimmed-down, clinically useful single-gene or ICC panels. This has the potential to offer precise preoperative stratification of AUS lesions as part of routine clinical practice, even in cytology centers without access to expensive multigene panels.

Conflict of Interest Statement

Drs. Christofer Juhlin and Zubair Baloch have no relevant interests to disclose that can potential influence the content of this submission.

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