





Performance of Afirma genomic sequencing classifier and histopathological outcome in Bethesda category III thyroid nodules: Initial versus repeat fine-needle aspiration

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Abstract

Background: There is limited data comparing the performance of Afirma Genomic Sequencing Classifier (GSC) in thyroid nodules carrying an initial versus a repeat diagnosis of atypia of undetermined significance (AUS). This study reported an institutional experience in this regard.

Materials and Methods: This retrospective study included consecutive thyroid nodules that had an initial or a repeat AUS diagnosis and had a subsequent GSC diagnostic result (benign or suspicious) from 2017 to 2021. All nodules were followed by surgical intervention or by clinical and/or ultrasound monitoring. GSC's benign call rate (BCR), rate of histology-proven malignancy associated with a suspicious GSC result, and diagnostic parameters of GSC were calculated and compared between the two cohorts (initial versus repeat AUS). Statistical significance was defined with a *p*-value of <.05 for all analysis.

Results: A total of 202 cases fulfilled inclusion criteria, including 67 and 135 thyroid nodules with an initial and a repeat AUS diagnosis, respectively. BCR was 67% and 66% in initial and repeat AUS cohorts, respectively. Rate of histology-proven malignancy associated with a suspicious GSC result were 22% and 24% in initial and repeat AUS cohorts, respectively. Compared with the repeat AUS cohort, the initial AUS cohort showed slightly lower sensitivity (83% vs. 100%), specificity (70% vs. 73%), PPV (23% vs. 24%), NPV (98% vs. 100%), and diagnostic accuracy (72% vs. 75%). Nevertheless, these differences did not reach statistical significance.

Conclusion: GSC demonstrated comparable performance in thyroid nodules with a repeat AUS diagnosis versus nodules with an initial AUS diagnosis.

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KEYWORDS

atypia of undetermined significance (AUS), genomic sequencing classifier (GSC), initial AUS diagnosis, molecular testing, repeat AUS diagnosis, thyroid nodules

1 | INTRODUCTION

As an important modality in evaluation and management of thyroid nodules, fine-needle aspiration (FNA) has been widely utilized in order to distinguish non-neoplastic nodules from neoplastic nodules.¹ Per the 2015 American Thyroid Association (ATA) management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer, FNA cytology of thyroid nodules should be reported using the six diagnostic categories outlined in The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC).² TBSRTC definitively categorizes the majority of aspirated thyroid nodules as either a benign, non-neoplastic nodule (TBSRTC category II, diagnostic rate: 60%–70%) or a malignant nodule (TBSRTC category VI, diagnostic rate: 5%), resulting in a significant reduction of unnecessary surgeries for benign, non-neoplastic nodules while providing useful pre-operative information for malignant nodules. However, approximately 1/3 of the aspirated thyroid nodules fall into indeterminate diagnostic categories (TBSRTC categories III, IV, and V). Compared with category IV (follicular neoplasm/suspicious for follicular neoplasm, FN/SFN) and category V (suspicious for malignancy, SFM), category III (atypia of undetermined significance, AUS) is the most heterogeneous and challenging category. It is not uncommon that thyroid nodules categorized as AUS are proven to be benign upon histologic examination.³ Previous data from our institution showed histology-proven malignancy in 18%–27% of surgically removed AUS nodules while the remaining resected nodules were either non-neoplastic (e.g., benign nodular hyperplasia or lymphocytic thyroiditis) or follicular adenomas upon histologic assessment.^{4,5} Due to its non-negligible risk of malignancy, ATA guidelines have recommended surveillance with ultrasonography, repeat FNA, molecular testing, or diagnostic surgical excision for AUS nodules.²

In more recent years, molecular testing incorporated with FNA evaluation has played an important role in further stratification and management of indeterminate thyroid nodules.^{6,7} Among several commercially available tests, the Afirma Genomic Sequencing Classifier (GSC) was introduced in 2017, and uses next-generation sequencing, incorporating an ensemble model composed of 12 independent classifiers (10,196 genes with 1115 core genes) and 7 other components (parathyroid, medullary thyroid carcinoma, BRAFV600E, RET/PTC1 and RET/PTC3 detection modules, oncocyctic cell index and oncocyctic neoplasm index). Compared with its predecessor, the Gene Expression Classifier (GEC), the newer GSC has demonstrated improved specificity and positive predictive value (PPV) while maintaining a high sensitivity and negative predictive value (NPV).^{8,9} Since 2017, GSC testing has been utilized exclusively in our institution for molecular analysis of thyroid nodules categorized as AUS (TBSRTC category III).

Studies of TBSRTC category III thyroid nodules reported that repeating FNA resulted in a definitive diagnostic entity in 45%–50%

of the cases, in which nearly 45% were recategorized into benign category.^{10,11} A case series from one institution suggested repeat FNA cytology can help optimize molecular testing for a subset of cytologically indeterminate thyroid nodules.¹² Accordingly, performing Afirma GEC only on nodules with repeat Bethesda III/IV cytology would reduce the rate of surgery for benign nodules. Afirma GEC and/or GSC is unlikely to provide benefits in Bethesda III/IV diagnoses that are re-categorized as benign on repeat FNA.^{12,13} At our institution, GSC testing is generally done reflexively if the second FNA has a repeat AUS diagnosis. However, endocrinologists and endocrine surgeons may make individually tailored decisions to collect sample for GSC testing during the initial FNA based on the clinical scenario. Situations that may prompt the provider to collect samples for reflex GSC testing on the initial FNA include: (1) suspicious clinical presentations and/or ultrasound findings, such as history of thyroid cancer in a first-degree relative, exposure to ionizing radiation, or a sonographic pattern that would be classified high risk by ATA guideline, (2) prior non-diagnostic FNA result, and/or (3) patients who are elderly and/or have a long travel time/distance to a FNA clinic for whom a repeat FNA might be especially burdensome.

Recently published data support collection of material for GSC on repeat FNA of nodules with a prior diagnosis of AUS. However, there is limited data regarding the comparison of GSC performance in thyroid nodules with repeat versus initial AUS cytological diagnosis. Different risk of malignancy (ROM), PPV, and NPV between these groups could potentially impact patient management.

The current study was undertaken to compare GSC performance and histological outcomes in thyroid nodules with an initial versus a repeat diagnosis of AUS.

2 | MATERIALS AND METHODS

This retrospective study was approved by the institutional review board (IRB) at the University of Michigan in Ann Arbor, Michigan. The study cohorts included patients (age range: 19–82 years old) with thyroid nodules that underwent FNA and had an initial or a recurrent cytologic diagnosis of AUS (TBSRTC category III) and were tested by GSC (July 2017–June 2021). All the nodules were followed by either surgical intervention or at least 6 months of clinical and/or ultrasound monitoring. Nodules with a GSC testing result of “non-diagnostic” (due to inadequate sampling) and nodules lacking surgical follow-up or appropriate clinical and/or ultrasound monitoring were excluded from the study.

Ultrasound-guided thyroid FNAs were performed by endocrinologists and/or surgeons with cytology-assisted rapid on-site adequacy assessment yielding a combination of smear preparations, ThinPrep slides, and/or cellblocks. Two dedicated passes from each nodule

were also collected into an Afirma-provided fixative vial during the procedure. FNA specimens were then assessed by subspecialty board-certified cytopathologists and diagnoses were reported using TBSRTC. When an initial or a repeat diagnosis of AUS was rendered, the aforementioned pre-collected samples were sent to Veracyte CLIA laboratory (South San Francisco, CA) for Afirma GSC testing.

The following information from individual patients included in this study were collected from electronic medical records: age, sex, and size of thyroid nodule. For those who underwent subsequent surgical interventions, the corresponding histologic diagnoses were noted. For those who did not undergo surgical interventions, the electronic medical record was evaluated to determine the stability of the evaluated nodules via clinical and/or ultrasound monitoring during a period of at least 6 months post-FNA. Nodules that did not develop new high-risk features and showed no marked change in size (less than 20% increase in at least two nodule dimensions with a minimal increase of 2 mm or less than a 50% increase in nodule volume) were considered as clinically stable.² Incidental micro papillary thyroid carcinoma (PTC) within a larger benign nodule was categorized as benign.

Benign call rate (BCR) and diagnostic parameters including sensitivity, specificity, PPV, NPV, and diagnostic accuracy of GSC testing were calculated for each cohort (initial AUS vs. repeat AUS) as follows:

$BCR = \text{number of nodules with benign GSC result} / \text{total number of nodules with GSC testing}$.

$Sensitivity = \text{number of nodules with GSC suspicious result and histology-proven malignancy (true positive)} / \text{number of all histology-proven malignant nodules (true positive + false negative)}$.

$Specificity = \text{number of nodules with GSC benign result and a subsequent surgical and/or clinical benign diagnosis (true negative)} / \text{numbers of all benign nodules (true negative + false positive)}$.

$PPV = \text{true positive} / \text{all nodules with GSC suspicious result (true positive + false positive)}$.

$NPV = \text{true negative} / \text{all nodules with GSC benign result (true negative + false negative)}$.

$Diagnostic\ accuracy = (\text{true positive} + \text{true negative}) / \text{total number of nodules}$.

The above parameters were compared between two cohorts (initial AUS vs. repeat AUS) using Social Science Statistics (<https://www.socscistatistics.com/tests/>). Pearson's chi-square or Fisher exact test for categorical variables and student *t* test for continuous variables were performed. Statistical significance was defined as a two-tailed *p*-value of <.05 for all analysis.

3 | RESULTS

3.1 | Study cohort

During the 4-year study period, a total of 3572 thyroid FNAs were performed, and an initial diagnosis of AUS was rendered in 745 (21%) nodules. Of these AUS nodules, 226 (30%) underwent repeat FNA. The study included a total of 202 thyroid nodules which fulfilled inclusion criteria, including 67 nodules with an initial AUS diagnosis and

TABLE 1 Clinical characteristics of the study cohort.

Characteristic	Initial AUS	Repeat AUS	<i>p</i> -value
Number of nodules	67	135	
Sex: F	53	90	.07
M	14	45	
Patient age (years)	52 (30–77)	55 (19–82)	.38
Nodule size (cm)			.56
1–2	28	58	
2–4	35	72	
> 4	4	5	

Abbreviations: AUS, atypia of undetermined significance; F, female; M, male.

135 nodules with a repeat AUS diagnosis. There was no significant difference in the distribution of patient demographics and thyroid nodule size between the two cohorts. The mean age of patients in the initial AUS cohort (52 years) was similar to that of the repeat AUS cohort (55 years). Female predominance was seen in both initial AUS (79%) and repeat AUS (67%) cohorts. Nodules measuring ≤ 4 cm represented the majority in both the initial AUS (94%) and the repeat AUS (96%) cohort (Table 1).

3.2 | BCR and follow-up of GSC- benign nodules

A benign result of GSC testing was obtained in a similar proportion of thyroid nodules with an initial AUS diagnosis (44/67, BCR = 66%) compared with that of thyroid nodules with repeat AUS diagnosis (90/135, BCR = 67%) (Table 2). Most of the thyroid nodules with an initial AUS diagnosis (26/44 = 59%) or a repeat AUS diagnosis (69/90 = 77%) were deemed stable after at least 6 months (range: 6–63 months, average: 21 months) of clinical and/or ultrasound monitoring. Of surgically treated GSC-benign nodules (18/44 = 41%), histopathological examination revealed mainly non-neoplastic changes, predominantly consisting of nodular hyperplasia and rarely, lymphocytic (Hashimoto's) thyroiditis. Adenomas were found in both the initial AUS cohort (three follicular adenomas and one oncocytic adenoma) and the repeat AUS cohort (two follicular adenomas and one oncocytic adenoma). A single case of PTC was documented in a GSC-benign nodule with an initial diagnosis of AUS. Subsequent resection specimen revealed multifocal well-differentiated PTC, with the largest focus measuring 0.5 cm. No malignancy was identified in the repeat AUS cohort (Table 3, Figure 1).

3.3 | Follow-up of GSC-suspicious nodules

A suspicious result of GSC testing was obtained in 23 of 67 (34%) thyroid nodules with an initial AUS diagnosis and 45 out of 135 (33%) thyroid nodules with a repeat AUS diagnosis, respectively. Most GSC-suspicious nodules (65/68 = 96%) underwent surgical interventions. Of these surgically treated suspicious nodules, 17 (77%) with an initial

Column1	GSC benign	GSC suspicious	Proportion of GSC benign	<i>p</i> -value
Initial AUS	44	23	66%	.88
Repeat AUS	90	45	67%	

TABLE 2 GSC results of initial and repeat AUS diagnosis.

Abbreviations: AUS, atypia of undetermined significance; GSC, genomic sequencing classifier.

TABLE 3 Clinical Follow-up and histopathological diagnoses of GSC benign nodules.

Diagnosis	Initial AUS	Repeat AUS
GSC-benign	44 (66%)	90 (67%)
Clinically stable	26 (59%)	69 (77%)
Surgically treated	18 (41%)	21 (23%)
Nodular hyperplasia	12	16
Hashimoto thyroiditis	1	2
Follicular adenoma	3	2
Oncocytic adenoma	1	1
PTC	1	

Abbreviations: AUS, atypia of undetermined significance; GSC, genomic sequencing classifier.

AUS diagnosis and 32 (74%) with a repeat AUS diagnosis were benign on histologic examination. These were most commonly diagnosed as nodular hyperplasia followed by follicular adenoma and/or oncocytic adenoma. Two non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) cases were documented in the repeat AUS cohort. NIFTP was classified as benign and grouped with follicular/oncocytic adenoma rather than malignancy based on its favorable prognosis and consensus guidelines.¹⁴ Histologically proven malignancy was identified in 5 out of 22 (23%) nodules with an initial AUS diagnosis and 11 out of 43 (26%) nodules with a repeat AUS diagnosis. In the initial AUS cohort, there were 4 cases of PTC and one medullary carcinoma, whereas in the repeat AUS cohort, there were 8 cases of PTC (including 6 conventional and 2 follicular variant), 1 follicular carcinoma, 1 oncocytic carcinoma and 1 medullary carcinoma (Table 4, Figure 1). One patient in the initial AUS cohort and two patients in the repeat AUS cohort declined surgical treatment. During an average of 21 months (24 months for initial AUS, 22 and 18 months for two repeat AUS patients, respectively) of clinical monitoring, their nodules remained stable, and thus were presumed to be benign.

3.4 | Diagnostic performance of GSC in the initial AUS cohort and the repeat AUS cohort

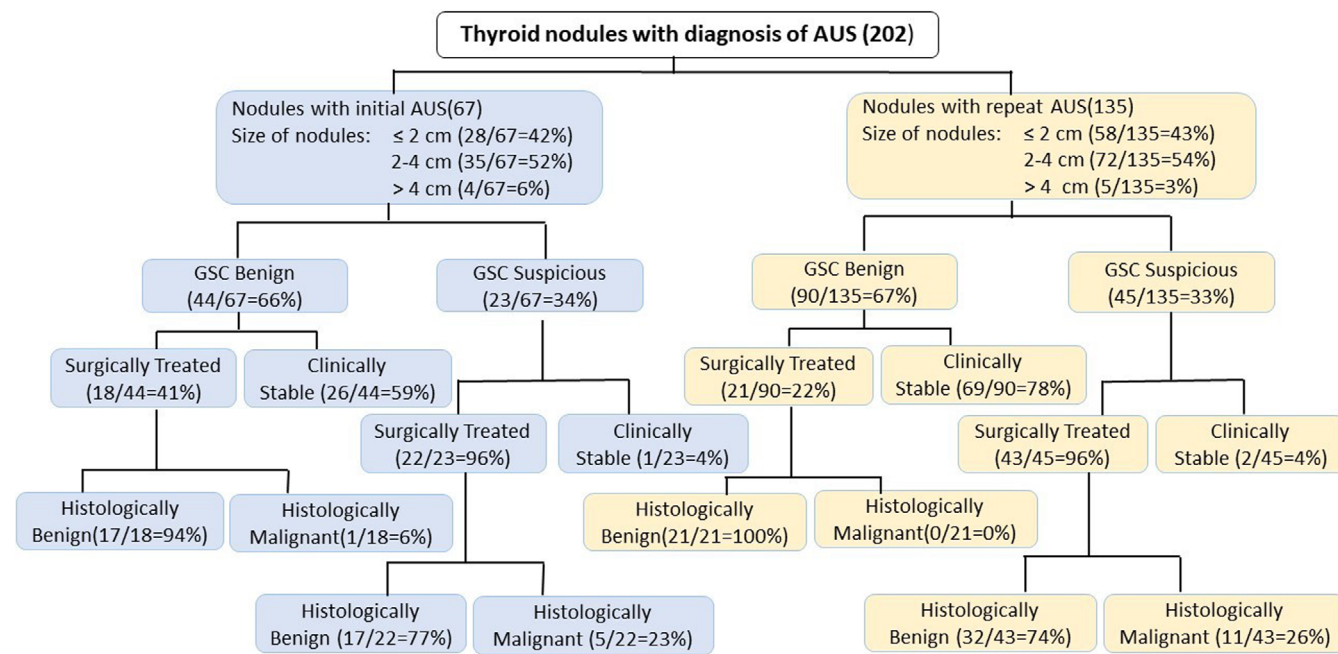
Table 5 compares the diagnostic performance of GSC testing between the initial AUS cohort and repeat AUS cohort. Compared with the repeat AUS cohort, the initial AUS cohort revealed slightly lower sensitivity, specificity, PPV, NPV, and diagnostic accuracy. However, there was no statistically significant difference between the two groups in terms of diagnostic accuracy ($p = .91$).

4 | DISCUSSION

We reported our institutional experience of Afirma GSC performance in thyroid nodules with a repeat AUS diagnosis versus thyroid nodules with an initial AUS diagnosis. Compared to nodules with a repeat AUS diagnosis, nodules with an initial AUS diagnosis (some with suspicious clinical and/or ultrasound findings) demonstrated similar BCR of GSC testing and rate of histology-proven malignancy in nodules with a suspicious GSC result. Diagnostic parameters of GSC testing, including sensitivity, specificity, PPV, NPV, and diagnostic accuracy did not differ between these two cohorts. These findings indicate that the pre-test probability of malignancy (thus the performance characteristics of Afirma GSC) is similar whether (1) an endocrinologist/surgeon's assessment of concerning clinical/sonographic features is used to refine the selection of a nodule for potential molecular testing based on the initial AUS diagnosis or (2) repeat diagnosis of AUS is used to refine the selection of a nodule for potential molecular testing.

Several studies have demonstrated the importance of repeat FNA in thyroid nodules with an initial AUS diagnosis. Repeat FNA establishes a definitive diagnosis (TBSRTC category II or VI) in up to 50% of nodules with an initial diagnosis of AUS, reducing unnecessary surgical intervention in benign nodules and providing useful pre-operative information for malignant nodules.^{10,11,15,16} However, a notable portion of nodules remained as AUS after repeat aspiration. As such, molecular testing as an adjunct to FNA plays an important role in further stratification of these nodules. Thus, endocrinologists/endocrine surgeons tend to use GSC mainly in nodules with a repeat AUS diagnosis.

Afirma GSC testing is considered a rule-out test. When tested on thyroid nodules categorized as AUS and FN/SFN, studies have shown that GSC has a BCR of 60.0%–78.0%, sensitivity of 94.0%–100%, specificity of 17.0%–94.0%, PPV of 41.0%–85.3%, and NPV of 96.0%–100%. For AUS (BSRTC category III) alone, the following ranges are noted: 59.0%–80.8% for BCR, 85.7%–100% for sensitivity, 24.0%–94.9% for specificity, 52.0%–80.0% for PPV, and 96.3%–100% for NPV.^{17–24} The wide range in test performance may be related to many factors and/or study variations (e.g., prevalence of malignancy associated with the study cohorts, case selection criteria, cases countered as numerator and/or denominator for calculation of diagnostic parameters, etc.). With regard to the latter, our previous study of GSC performance in AUS nodules showed a difference in specificity when only surgically treated nodules (41.2%) were included versus all nodules (82.8%) were included.⁸ In comparison with previous reported studies, the current study showed a relatively lower PPV and ROM in both initial AUS and repeat AUS cohorts. This could be partially explained by the fact that only 2 out of 43 (4%) surgically removed nodules with a repeat AUS diagnosis were subsequently



Abbreviation: AUS: Atypia of Undetermined Significance; GSC: Genomic Sequencing Classifier.

FIGURE 1 Study flow diagram. There was no significant difference between single and repeat AUS thyroid nodules when comparing the GSC benign call rate and rate of malignancy.

TABLE 4 Clinical follow-up and histopathological diagnoses of GSC suspicious nodules.

Diagnosis	Initial AUS	Repeat AUS
GSC-suspicious	23 (34%)	45 (33%)
Clinically stable	1 (4%)	2 (4%)
Surgically treated	22 (96%)	43 (96%)
Benign histopathology		
Nodular hyperplasia	12	19
Follicular adenoma	5	10
Oncocytic adenoma		1
NIFTP ^a		2
Malignant histopathology		
Papillary thyroid carcinoma	4	6
PTC (follicular variant)		2
Follicular carcinoma		1
Oncocytic carcinoma		1
Medullary carcinoma	1	1

Abbreviations: AC, architectural and cytological atypia; AUS, atypia of undetermined significance.

^aNIFTP was grouped with follicular/oncocytic adenoma rather than malignancy based on the currently available consensus that NIFTP has a good prognosis and can be managed as follicular/oncocytic adenoma.

diagnosed NIFTP, which were not classified as malignant (positive) cases in our study. In contrast, most of the previous studies categorized NIFTP into a malignant (positive) category. In these studies, NIFTP represented notable proportion of “malignant” nodules, ranging from 22% to 60%.^{17-19,21,23}

TABLE 5 Diagnostic accuracy of Afirma GSC in thyroid nodules with initial and repeat AUS.

Column 1	Initial AUS	Repeat AUS	p-value
Sensitivity	83%	100%	1
Specificity	70%	73%	1
PPV	23%	24%	1
NPV	98%	100%	1
Diagnostic accuracy	72%	75%	.91

Abbreviations: AUS, atypia of undetermined significance; GSC, genomic sequencing classifier; NPV, negative predictive value; PPV, positive predictive value.

A majority of previously published studies on GSC testing included nodules with a repeat AUS and/or FN/SFN diagnosis.^{12,17,20,25,26} Few studies included nodules with an initial AUS and/or FN/SFN diagnosis only or a mixture of nodules with an initial and a repeat AUS and/or FN/SFN diagnosis.^{19,22} The decision to obtain molecular testing for cytologically indeterminate thyroid nodules was based on joint decision-making between providers and patients. However, criteria regarding case selection (initial AUS diagnosis vs. repeat AUS diagnosis) was not clarified. The current study consisted of two separate cohorts (repeat AUS vs. initial AUS), although the study cohort was enriched with thyroid nodules with repeat AUS diagnosis. Clinical factors leading to sample collection for GSC testing on initial FNA included concern for a high-risk nodule, advanced patient age, and long travel distances for healthcare, among others. Presumably, those nodules with suspicious clinical presentations and/or ultrasound findings have a higher pre-test probability of malignancy, potentially impacting GSC testing performance in different

NPV and PPV between these two cohorts. Similar GSC testing BCR and ROM between thyroid nodules with initial and repeat AUS diagnoses in the current study may be partially explained by the selection bias. It is important to note that our initial AUS cohort could be enriched for nodules with high-risk clinical and/or radiologic features. As such, these findings may not necessarily generalize to all nodules with an initial AUS diagnosis. It is noteworthy to mention that a study of the Afirma gene expression classifier (GEC) by Baca et al demonstrated an identical malignant risk for an initial versus repeated AUS diagnosis in GEC-suspicious thyroid nodules and statistically similar results for an initial versus repeated indeterminate cytology. Therefore, they claimed that repeat FNA did not improve the PPV of Afirma GEC testing of AUS nodules.²⁷ On the contrary, some studies demonstrated an increased rate of histology-proven malignancy in thyroid nodules with pre-operative repeat FNA while reducing unnecessary surgical treatment of benign thyroid nodules.^{11,16} Further, other authors note that repeat FNA cytology can guide the selection of cytologically indeterminate thyroid nodules that warrant molecular test (GEC).^{12,13}

In our study, GSC testing parameters are comparable. However, it is noted that the repeat FNA cohort had a higher sensitivity than the initial FNA cohort (100% vs. 83%). However, the relatively high sensitivity in both cohorts is reassuring that majority of patients with histologically proven thyroid cancer will have suspicious GSC results. The result in our study suggests that there is the potential for decreasing the number of FNAs for patients who undergo GSC testing. However, the cost-effectiveness associated with expanding GSC testing to more initial AUS diagnoses is beyond the scope of this study. Limitations of the current study were small case cohort and relatively short follow-up periods for some non-surgically treated AUS nodules. As molecular testing has gained more acceptance and popularity, there may be a tendency to collect specimens for molecular testing at the time of the first FNA due to various reasons. This approach may provide more information as this cohort expands in subsequent studies.

AUTHOR CONTRIBUTIONS

Xiaobing Jin: Acquired, analyzed, interpreted data and resource materials, drafted the manuscript and contributed significant revisions on subsequent drafts, contributed to final approval of version to be published, and agrees to be accountable for all aspects of the work in ensuring questions related to accuracy or integrity of any part of the work. **Madelyn Lew:** Contributed revisions for intellectual content, contributed to final approval of version to be published, and agrees to be accountable for all aspects of the work in ensuring questions related to accuracy or integrity of any part of the work. **Liron Pantanowitz:** Contributed revisions for intellectual content, contributed to final approval of version to be published, and agrees to be accountable for all aspects of the work in ensuring questions related to accuracy or integrity of any part of the work. **Jennifer J. Iyengar:** Contributed revisions for intellectual content, contributed to final approval of version to be published, and agrees to be accountable for all aspects of the work in ensuring questions related to accuracy or integrity of any part of the work. **Megan R. Haymart:** Contributed revisions for intellectual content, contributed to final approval of

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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