Our Evolving Approach to the Thyroid Nodule with Indeterminate FNA Cytology

Molecular Testing Platforms

Atil Kargi, M.D.

Associate Professor of Medicine

Division of Endocrinology, Diabetes and Metabolism

University of Miami, Leonard Miller School of Medicine

FACULTY DISCLOSURE

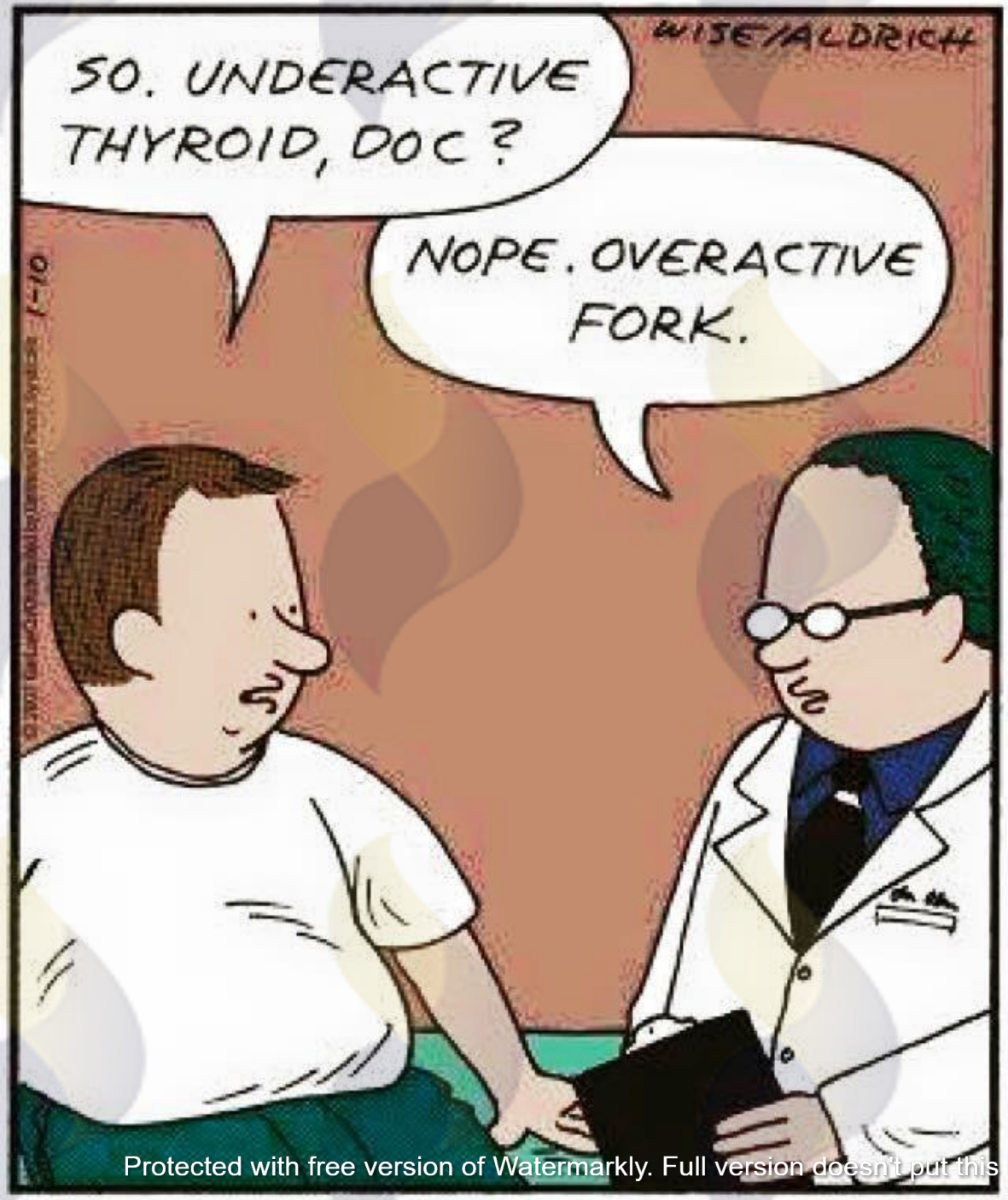
No relevant financial relationship with commercial interests other than the University of Miami

Which molecular test do you use?

1. Afirma GSC
2. Thyroseq Genomic Classifier
3. ThyGeNEXT+ThyraMIR
4. I use more than one test
5. I don’t use molecular testing

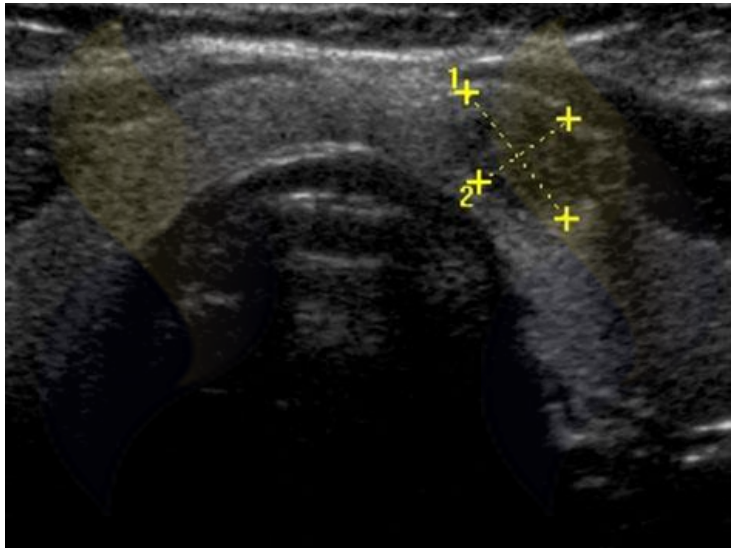
Case Study – Thyroid Nodule

- 55 year old woman referred from “weight loss clinic” where she thyroid ultrasound was done as part of routine evaluation. Her sole complaint is difficulty losing weight.
- She denies recent change in weight, energy levels, heat or cold intolerance, tachycardia, changes in skin hair or bowel movements. She denies *discomfort in the neck, hoarseness or dysphagia.*
- She has no *family history of thyroid cancer* and no prior history *of head and neck irradiation.*
- On physical exam BMI is 27 kg/m2, the thyroid gland is not enlarged and there are no palpable thyroid nodules or cervical lymphadenopathy. Otherwise exam is unremarkable.
- **TSH: 2.1 mIU/L**. CBC, FLP and CMP within normal limits.
- Thyroid ultrasound: **“1.8 cm hypoechoic nodule in left lobe with microcalcifications and increased vascularity”**
- Patient states “I always knew my difficulty losing weight was due to my thyroid”



Case Study – Thyroid Nodule

The patient has USGFNA after discussion of
of risks (negligible) and possibility of non-diagnostic
or indeterminate cytology results.



Cytology:

Atypia of undetermined significance -AUS (Bethesda category III)

What should be the next step?

1. Thyroid lobectomy
2. Ultrasound Follow-up
3. Repeat FNA
4. Afirma GSC
5. Thyroseq v3 GC
6. ThyGeNEXT+ThyraMIR

Bethesda category	Cytology	Frequency	Malignancy rates	Historical management
I	Non-diagnostic/ inadequate	10-15%	1-4% (2-20%)	Repeat FNA
II	Benign	65 -75%	1-4%	US follow-up
III	Atypia/ follicular lesion of undetermined significance	5-15%	10-30% (6-48%)	Repeat FNA or US follow-up or Lobectomy
IV	Follicular neoplasm	5-10%	15-30%	Lobectomy
V	Suspicious for malignancy	3-5%	50-75%	Lobectomy or Thyroidectomy
VI	Malignant	5 %	> 95%	Thyroidectomy

What to do with AUS/FLUS cytology?

Repeat FNA:

60-70% of the time will reclassify cytology to a different Bethesda category

50% of the time will result in benign cytology - benign cytology has a greater NPV than molecular tests

Second opinion cytology review – may reclassify cytology

Use sonographic characteristics to determine risk of malignancy

Reported cancer risk in AUS/FLUS nodules with suspicious sonographic features is 70-100%

Molecular testing

Haugen B. et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer. THYROID Volume 26, Number 1, 2016

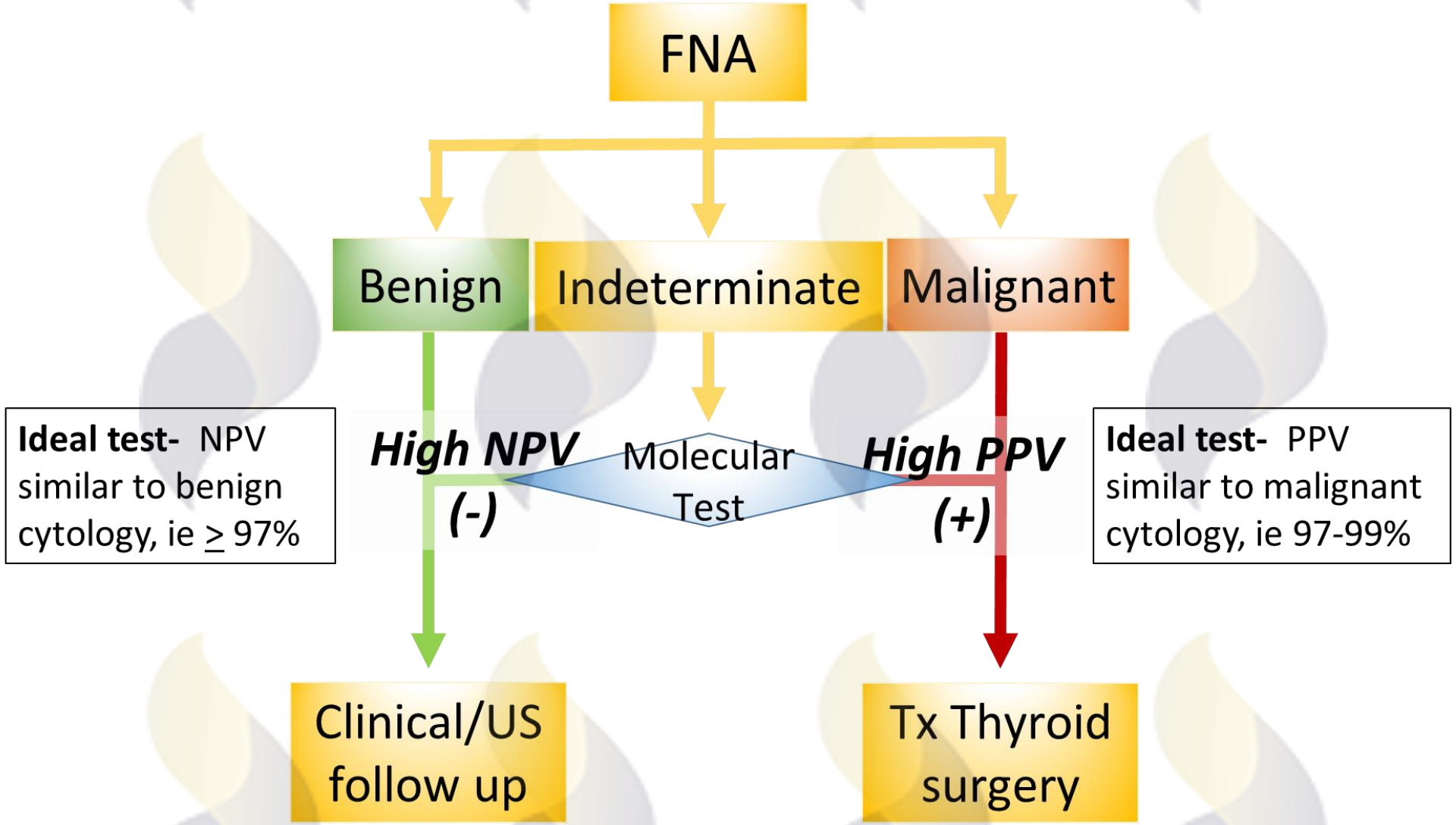
What to do with AUS/FLUS cytology?

RECOMMENDATION 15

For nodules with AUS/FLUS cytology, after consideration of worrisome clinical and **sonographic** features, investigations such ***as repeat FNA or molecular testing*** may be used to supplement malignancy risk assessment in lieu of proceeding directly with a strategy of either surveillance or diagnostic surgery. Informed patient preference and feasibility should be considered in clinical decision-making. (**Weak recommendation, Moderate-quality evidence**)

If repeat FNA cytology and/or molecular testing are not performed or inconclusive, either surveillance or diagnostic surgical excision may be performed for an AUS/FLUS thyroid nodule, depending on clinical risk factors, ***sonographic pattern,*** and patient preference. (**Strong recommendation, Low-quality evidence**)

Haugen B. et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer. THYROID Volume 26, Number 1, 2016



Molecular tests: Factors to consider when interpreting clinical studies

Number of studies

Prevalence of malignancy

Prospective vs retrospective

Single vs multi-center

Blinded vs unblinded

Independent vs industry sponsored

Inclusion B3, B4 and/or B5

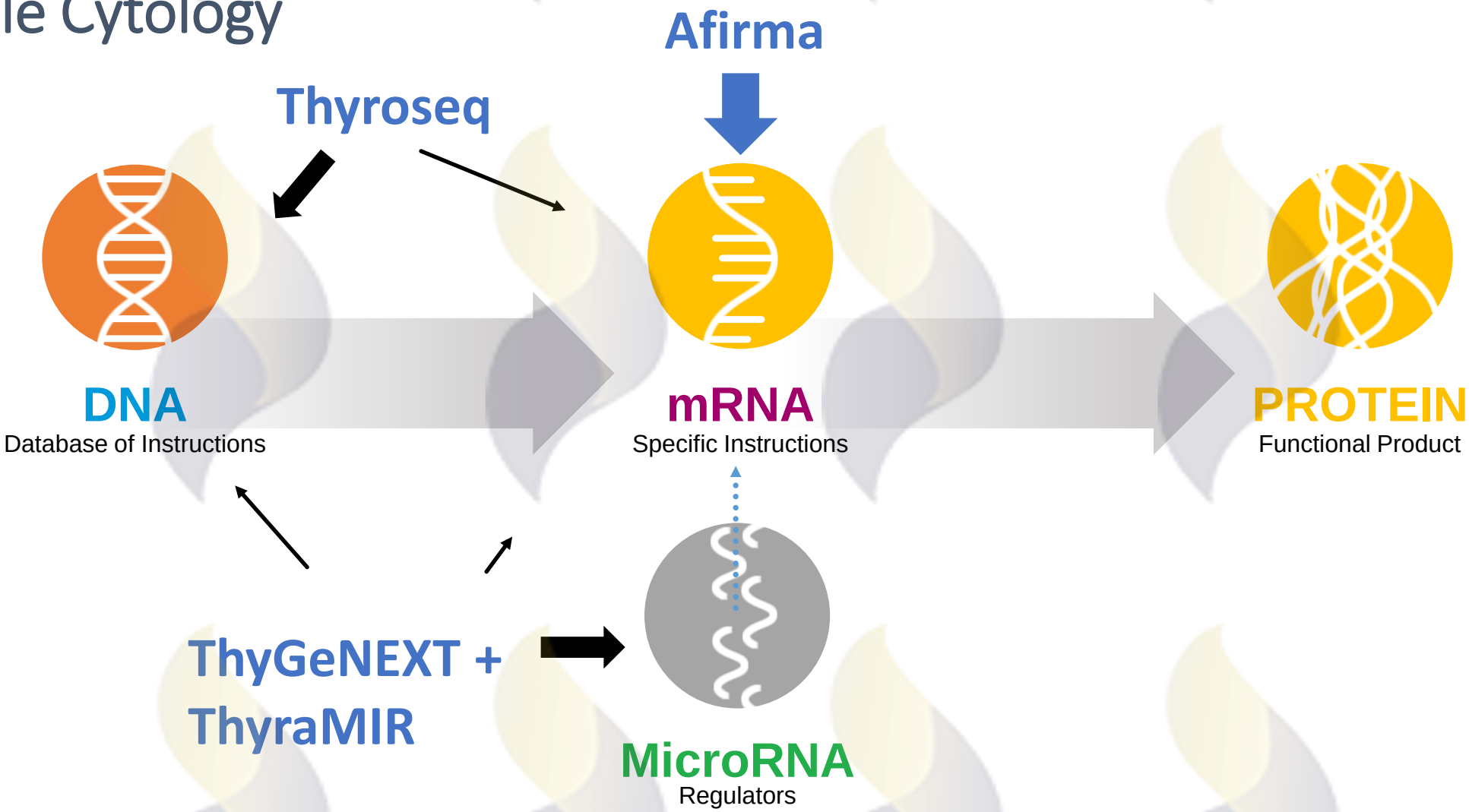
Variety of detailed molecular information precludes "blanket" calculation of PPV

Inclusion of "benign" (premalignant?) neoplasms as positive result

Duration of followup of patients with negative tests and % undergoing surgery

Many studies report findings using older versions of testing platforms

Currently Available Molecular Tests for Indeterminate Thyroid Nodule Cytology

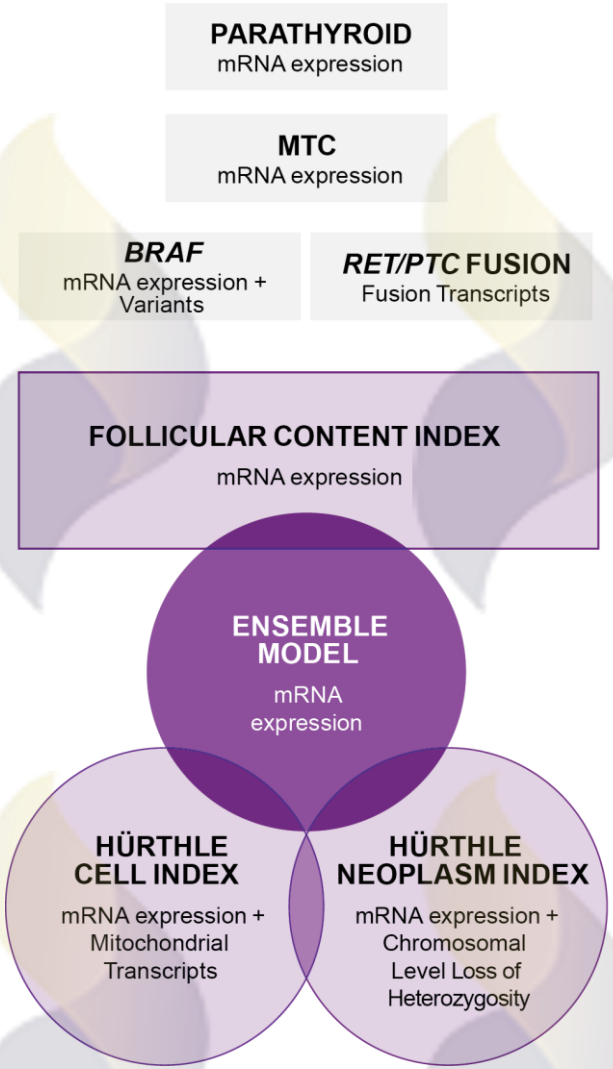


Molecular Test	Genomic sequencing classifier	Multigene genomic classifier	Multiplatform test
Trade Name	Afirma – GSC	ThyroSeq GC (v3)	ThyGeNEXT / ThyraMIR
What is tested?	RNA-seq to assess gene expression with upstream mutation (BRAF v600E), 2 fusions (RET-PTC1/3) Xpression Atlas: 346 genes (761 variants), 130 fusions	NGS DNA and RNA 112 genes (12,135 variants) 120+ fusions Gene expression alterations (19 genes) Copy number alterations (10 chromosomal regions)	NGS DNA and RNA 10 genes 38 fusions 10 miRNAs
	2 dedicated passes Benign/suspicious	1 dedicated pass or cell block/slides Negative/positive	1 dedicated pass or cell block/slides Negative/moderate/positive

Data quality supporting molecular tests

	Afirma GSC	Thyroseq V3	ThyGeNext + ThyraMIR
Analytic Validity (test accuracy and precision)	Yes	Yes	Yes
Clinical Validity that is prospective, multicenter and with blinded central histopathology review	Yes	Yes	No (retrospective study)
Independent post-validation studies	Yes (13)	Yes (10)	No (2 non-independent studies)

Afirma GSC



Initial Classifiers
identify rare neoplasms and lesions with >95% risk of malignancy for more informed treatment decisions

Follicular Content Index
identifies samples with sufficient follicular content

Ensemble Classifier
leverages multiple algorithms to derive the final benign or suspicious result

Hürthle Classifiers
enable correct classification of significantly more Hürthle cell lesions as *benign*

Afirma GSC was validated on 191 of the 210 samples with remaining RNA from Alexander EK, et al. *NEJM* 2012

Four key elements should be considered in clinical validation studies:

BLINDED

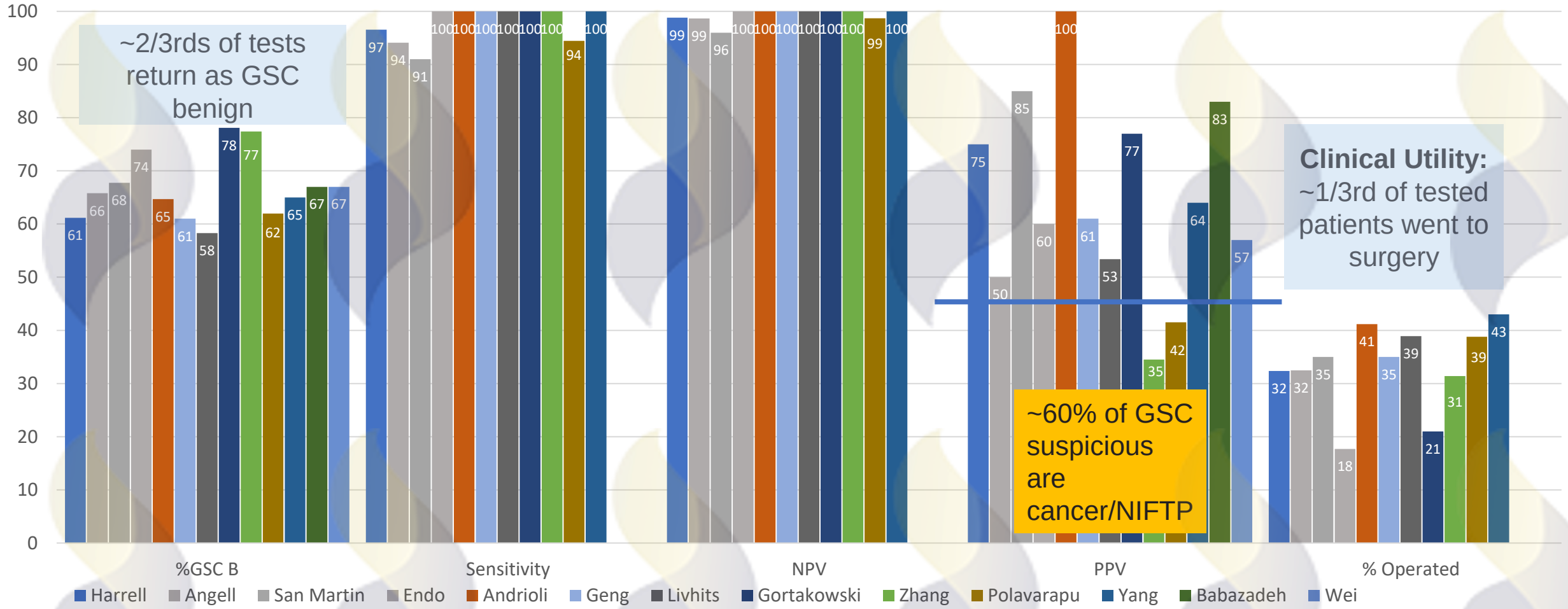
REPRESENTATIVE

MULTICENTER

PROSPECTIVE

1.Patel KN, et al. *JAMA Surg* 2018. PMID: 29799911

Afirma GSC Real-World Independent Clinical Utility: Including All Tested Patients

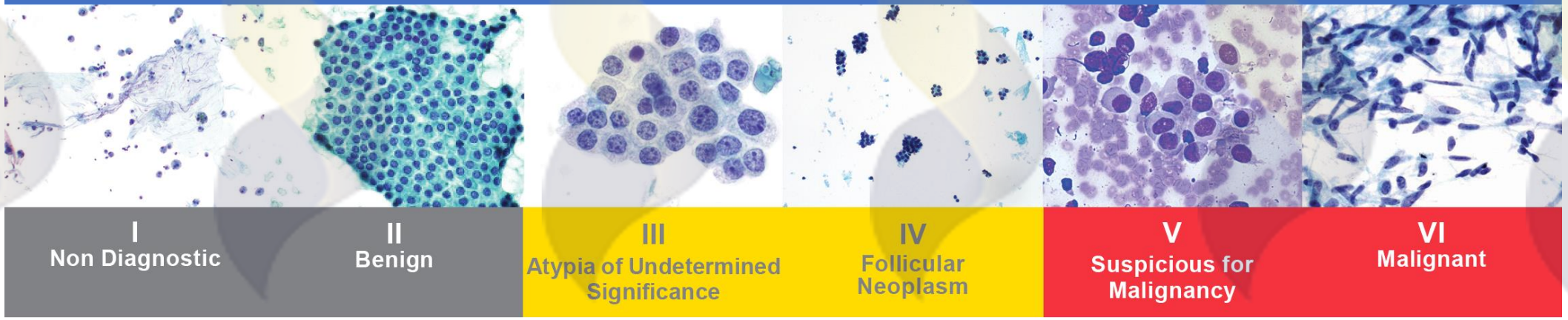


Harrell et al. Endocrine Practice 2018, Endo et al. Thyroid 2019, Angell et al Thyroid 2019, San Martin et al. JCEM 2019, Wei et al. Cancer Cytopathology 2019, Andrioli et al. Endocrine Pathology 2020, Geng et al. Cytopathology 2020, Livhits et al. JAMA Oncology 2020, Gortakowski et al. Thyroid 2021, Zhang et al. Diagnostic Cytopathology 2021, Polavarapu JES 2021, Yang J Am Soc Cytopath 2021, Babazadeh Surgery, 2022

GSC Benign unoperated counted as true negative
GSC Suspicious unoperated excluded

Where Does the Xpression Atlas Fit in the Diagnostic Flow?

The Xpression Atlas Provides Additional Genomic Information among Cases Likely Headed to Surgery



I	II	III	IV	V	VI
Non Diagnostic	Benign	Atypia of Undetermined Significance	Follicular Neoplasm	Suspicious for Malignancy	Malignant

INDETERMINATE

BENIGN
4% ROM

Afirma®
GENOMIC SEQUENCING CLASSIFIER

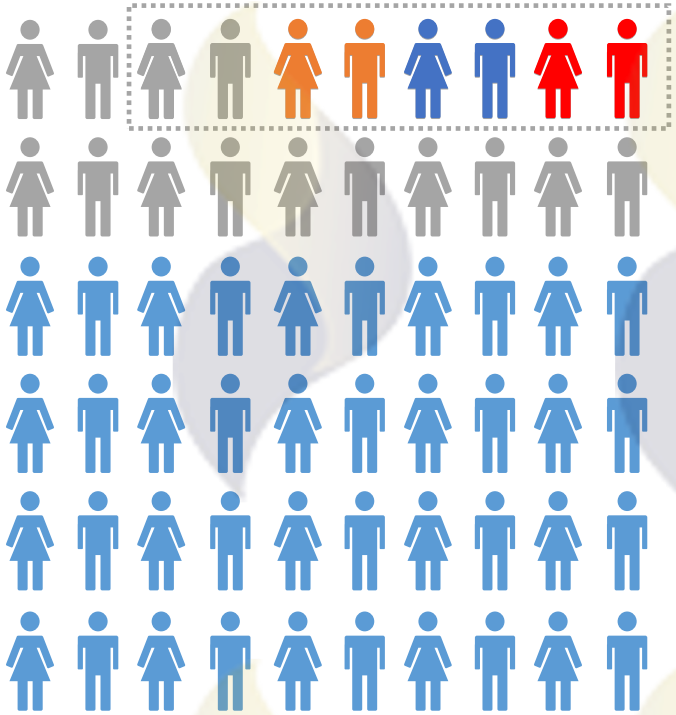
SUSPICIOUS
~50% ROM

Malignancy Classifiers +
Xpression Atlas

veracyte

The Afirma XA Result May Further Refine the Afirma GSC Suspicious Result of ~50% Risk of Malignancy (ROM)

The Afirma GSC classifier identifies ~1/3 of cyto-indeterminate samples as **Afirma GSC suspicious** (~50% ROM)



Of those, 44%[1] have an identified alteration (variant/fusion)

These cases may benefit when a clinically relevant or potentially relevant alteration is found where the associated ROM may increase to

- **~75%** (e.g. NRAS:p.Q61R)
- **>95%** (e.g. ETV6/NTRK3)
- **>99%** (e.g RET:p.M918T)

- If a variant/fusion is not identified with Afirma XA the ROM remains ~50%[2]

1. Hu et al. JCEM 2021 PMID 34009369
2. Angell TE, et al. Front Endocrinol 2019.

Afirma GSC + Xpression Atlas Report

RESULTS

Nodule: A 1.45 cm, Lower Right

CYTOPATHOLOGY

I	II	III	IV	V	VI
Non Diagnostic	Benign	**Atypia of Undetermined Significance**	Suspicious for Follicular Neoplasm	Suspicious for Malignancy	Malignant

AFIRMA GENOMIC SEQUENCING CLASSIFIER

Suspicious (Risk of Malignancy ~50%)

MTC: Negative
Parathyroid: Negative

AFIRMA XPRESSION ATLAS

***BRAF*:p:K601E c.1801A>G**

***BRAF*:p:V600E c. 1799T>A:** Negative
***RET/PTC1, RET/PTC3*:** Not Detected

Clinical Relevance	Risk of Malignancy	Associated Neoplasm Type	FDA Approved Therapy#
Potential clinical significance in thyroid cancer	~50%[11]	Follicular neoplasms (FA, NIFTP, FVPTC, FTC)	No alteration-specific therapy currently approved

RESULTS INTERPRETATION

The result of this 1.45cm Bethesda III nodule A is Afirma GSC Suspicious and *BRAF*:p:K601E c.1801A>G positive which suggests a risk of cancer of ~50%.[11] This genomic alteration is associated with follicular neoplasms (FA, NIFTP, FVPTC, FTC) and a *RAS*-like profile, which includes rates of lymph node metastases and extrathyroidal extension that are lower than *BRAF* V600E-like neoplasms, but higher than Non-*BRAF*-Non-*RAS*-like neoplasms.[9,10] Clinical correlation and surgical resection should be considered.

Afirma suspicious nodules are mostly neoplasms

Table 1. Diagnosis on Surgical Pathology Follow-Up of Cases With Suspicious Afirma Results (n=48)[a]

General Category	Specific Diagnosis
Malignant (22 cases; 46%)	FV-PTC: 16 cases (72.7%) Classic PTC: 3 cases (13.6%) Follicular carcinoma: 3 cases (13.6%)
Benign (26 cases; 54%)	Follicular adenoma: 9 cases (34.6%) Hurthle cell adenoma: 14 cases (53.8%) Adenomatoid nodule: 3 cases (11.5%)

Abbreviations: FV-PTC, follicular variant of papillary thyroid carcinoma, PTC, papillary thyroid carcinoma.
[a] Percentages do not add up to 100 due to rounding.

Lastra RR et al, Cancer Cytopathology 2014

Afirma suspicious nodules that are benign on final histopathology are generally monoclonal neoplasms (FA, NIFTP, HA)

Afirma benign nodules are more often hyperplastic nodules.

If we consider that neoplasms such as FAs and HAs could be considered premalignant and surgical removal is appropriate treatment, the "false positive" rate of the Afirma test would be much lower

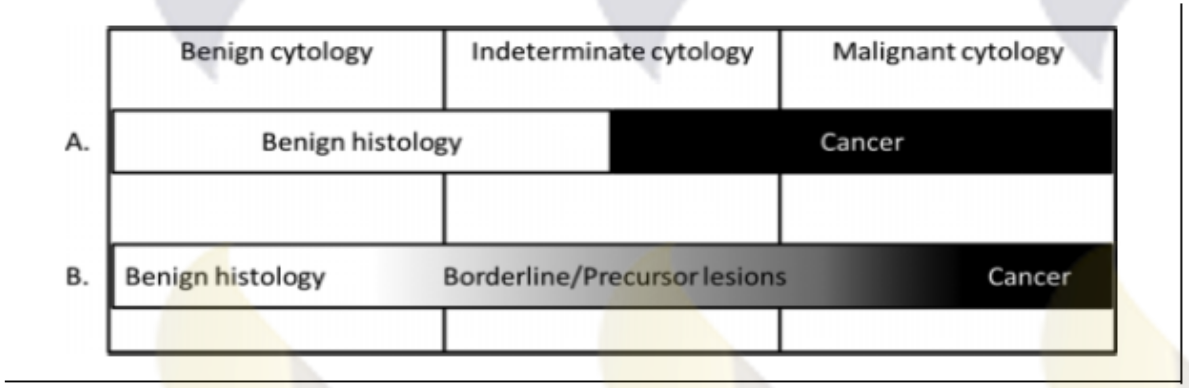
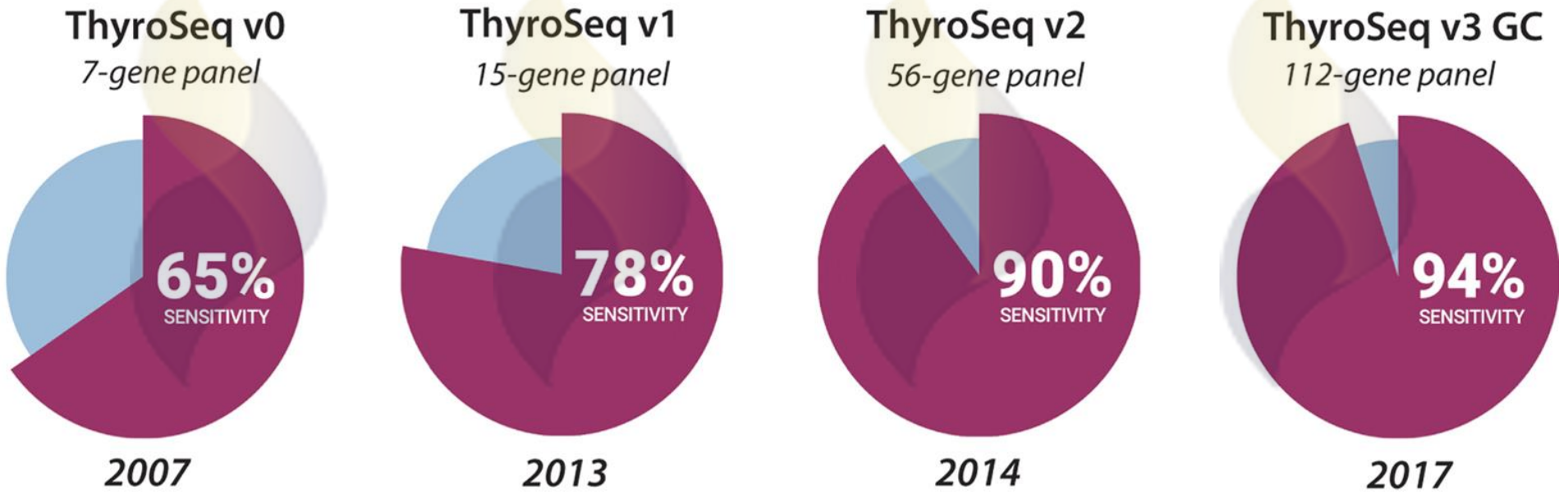


Figure 4. Thyroid cytology–histology correlation. (A) Traditional view of thyroid cytology–histology correlation. (B) Current view of thyroid cytology–histology correlation. Borderline/precursor lesions include neoplastic lesions without clear evidence of invasion or minimally invasive, with or without papillary-like nuclear features.

ThyroSeq Test for ThyroidNodules

Evolution of ThyroSeq Test

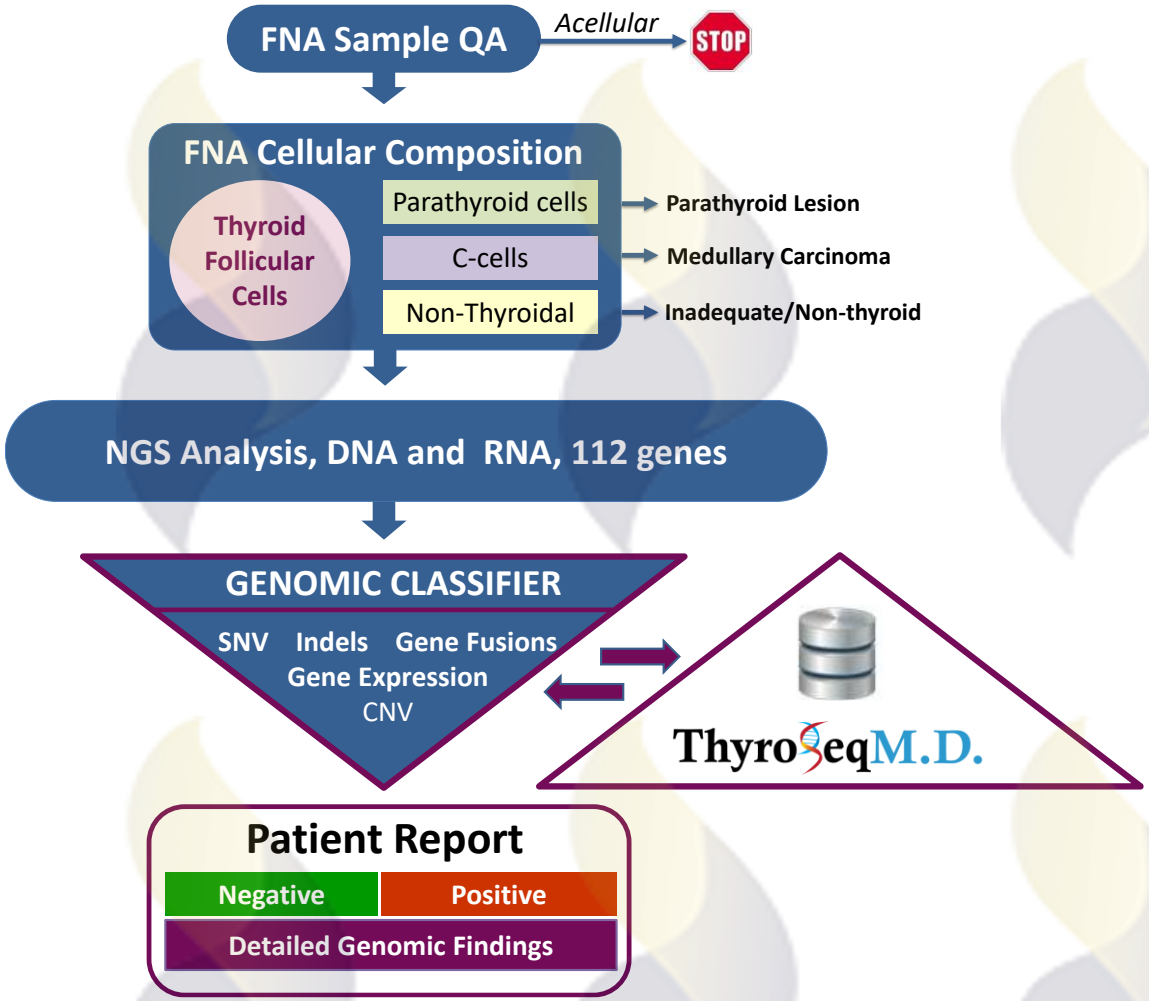


Pre-NGS

Next Generation Sequencing Approach

ThyroSeq v3 Genomic Classifier (GC)

- Assessment of DNA and RNA adequacy for testing
- FNA cellular composition determination (MTC; parathyroid)
- NGS analysis for four classes of genetic alterations in 112 genes
  - (i) Mutations (>12,000 variants)
  - (ii) Gene fusions (>150 types)
  - (iii) Copy number alterations
  - (iv) Gene expression alterations
- Test result interpretation based on knowledge database of >3,000 cases with known surgical outcome allowing to provide assessment of cancer probability and risk of cancer recurrence



Nikiforova et al. *Cancer* (2018)

Performance of ThyroSeq v3 Test in Thyroid Nodules

JAMA Oncology | Original Investigation

Performance of a Multigene Genomic Classifier in Thyroid Nodules With Indeterminate Cytology

A Prospective Blinded Multicenter Study

David L. Steward, MD; Sally E. Carty, MD; Rebecca S. Sippel, MD; Samantha Peiling Yang, MBBS, MRCP, MMed; Julie A. Sosa, MD, MA; Jennifer A. Sipos, MD; James J. Figge, MD, MBA; Susan Mandel, MD, MPH; Bryan R. Haugen, MD; Kenneth D. Burman, MD; Zubair W. Baloch, MD, PhD; Ricardo V. Lloyd, MD, PhD; Raja R. Seethala, MD; William E. Gooding, MS; Simion I. Chiosea, MD; Cristiane Gomes-Lima, MD; Robert L. Ferris, MD, PhD; Jessica M. Folek, MD; Raheela A. Khawaja, MD; Priya Kundra, MD; Kwok Seng Loh, MBBS; Carrie B. Marshall, MD; Sarah Mayson, MD; Kelly L. McCoy, MD; Min En Nga, MBBS; Kee Yuan Ngiam, MBBS, MRCS, MMed; Marina N. Nikiforova, MD; Jennifer L. Poehls, MD; Matthew D. Ringel, MD; Huaitao Yang, Md, PhD; Linwah Yip, MD; Yuri E. Nikiforov, MD, PhD

- Prospective double-blind multicenter study
- Bethesda III-V cytology with surgical outcome
- 10 study centers; patient recruitment 01/2015-12/2016
- Central pathology review by a panel of 3 pathologist
- Primary outcome: accuracy of detection of cancer+NIFTP

Performance in Bethesda III and IV nodules (n = 247; disease prevalence 28%)

Result	Cancer+NIFTP (n = 68)	Benign (n = 179)	Result
Positive	64	33	Sensitivity, 94 (86-98)
Negative	4	146	Specificity, 82 (75-87)
			NPV, 97 (93-99)
			PPV, 66 (56-75)

Steward DL et al. *JAMA Oncology* (2019)

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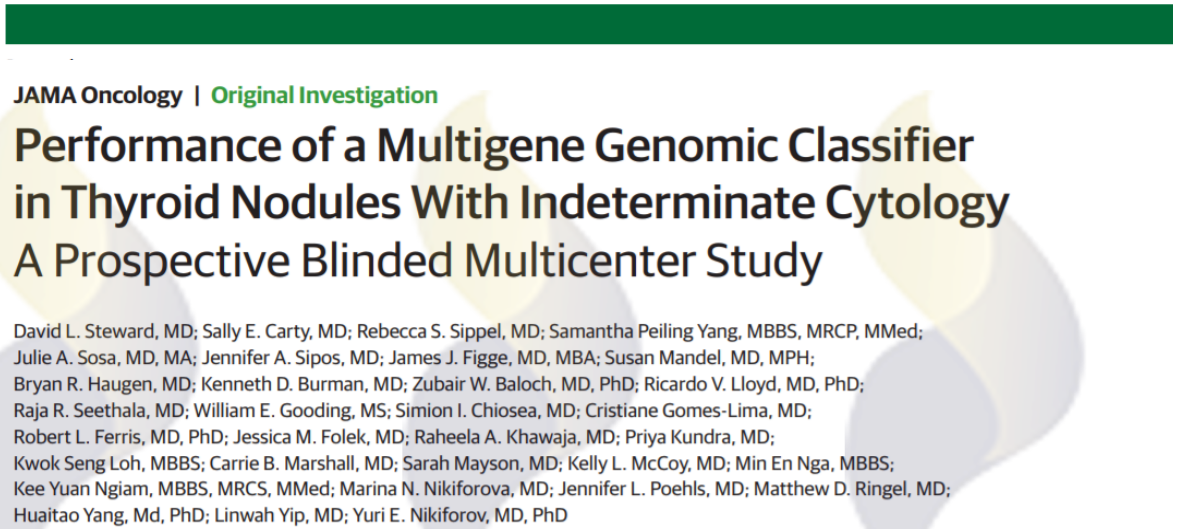

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61% avoidable surgeries

Steward DL et al. JAMA Oncology (2019)

ThyroSeq v3 Performance Across Various Cancers and NIFTP

11/11
NIFTP

24/27
PTC

21/22
PTC, FV

10/10
Hurthle Cell Carcinomas

3/4
Follicular Carcinomas

1/1
Medullary Thyroid Carcinoma

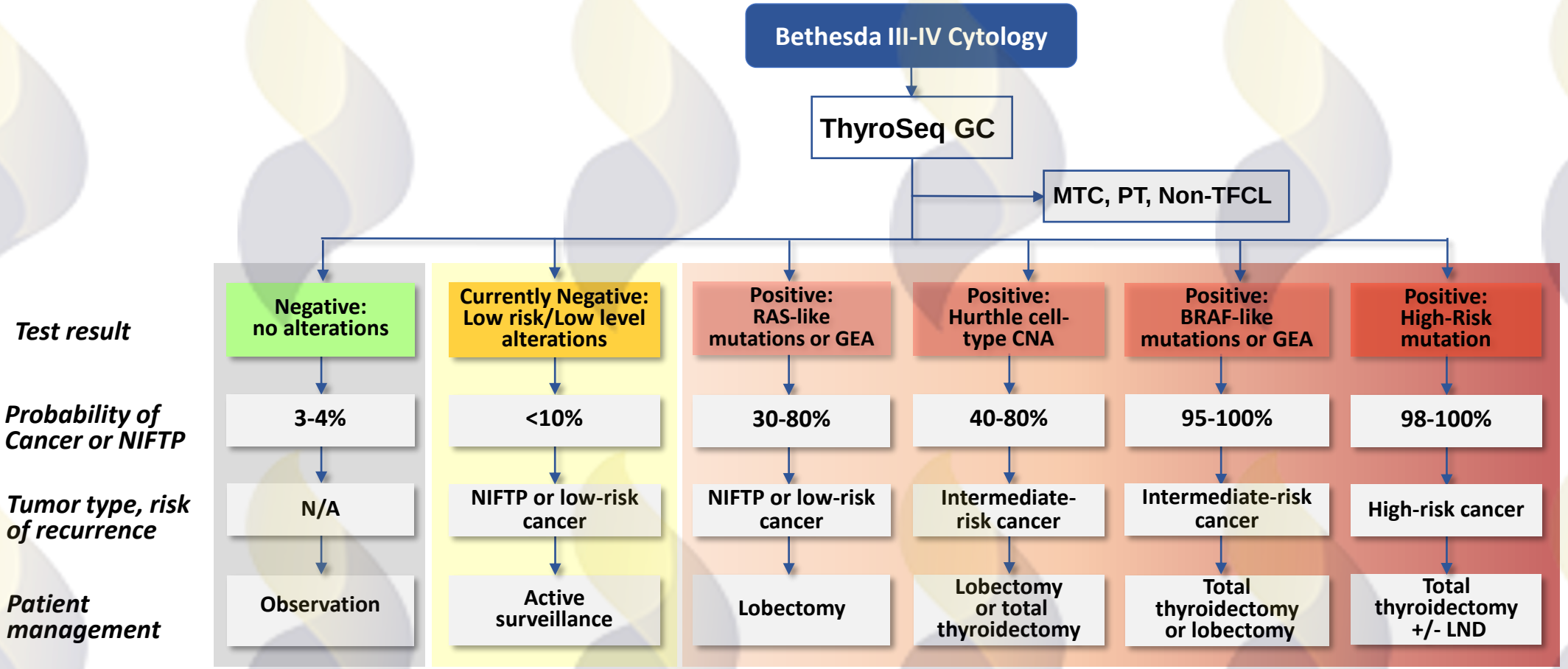
1/1
Metastatic Carcinoma

Steward DL et al. *JAMA Oncology* (2019)

ThyroSeq v3 Test Performance Confirmed in Multiple Independent Studies

Study Institution	Sensitivity	Specificity	NPV	PPV	Benign Call Rate	Study Reference
University of Pennsylvania	93%	90%	98%	68%	71%	Desai D, et al. Cancer Cytopathol. 2020.
McGill University, Montreal, Canada	95%	100%	97%	100%	58%	Chen T, et al. Thyroid. 2020.
University of California, Los Angeles	97%	85%	99%	63%	60%	Livhits M, et al. JAMA Oncol. 2020.
Multicenter clinical validation study	**94%**	**82%**	**97%**	**66%**	**61%**	**Steward DL, et al. JAMA Oncol. 2018.**

Individualized Patient Management Informed by ThyroSeq



Abbreviations: MTC, medullary thyroid cancer; PT, parathyroid; Non-TFCL, non-thyroid follicular cell lesion; GEA, gene expression alterations; CNA, copy number alterations; LND, lymph node dissection

Comparison of Test Performance

Independent, Head-to-Head Test Comparison by UCLA

JAMA Oncology | Original Investigation

Effectiveness of Molecular Testing Techniques for Diagnosis of Indeterminate Thyroid Nodules
A Randomized Clinical Trial

Masha J. Livhits, MD; Catherine Y. Zhu, MD; Eric J. Kuo, MD; Dalena T. Nguyen, MPH; Jiyoon Kim, MS; Chi-Hong Tseng, PhD; Angela M. Leung, MD; Jianyu Rao, MD; Mary Levin, SCT; Michael L. Douek, MD; Katrina R. Beckett, MD; Dianne S. Cheung, MD; Yaroslav A. Gofnung, MD; Stephanie Smooke-Praw, MD; Michael W. Yeh, MD

- Prospective parallel randomized trial
- 372 Bethesda III-IV nodules monthly block randomized to Afirma GSC (n=201) or ThyroSeq v3 (n=171)

Livhits MJ, et al. *JAMA Oncol.* 2021 Jan 1;7(1):70-77.

UCLA Head-to-Head Comparison Results

- ✓ Diagnostic performance of both tests was high and not statistically different
- ✓ Lower % samples inadequate for ThyroSeq v3 testing
- ✓ Higher BCR for ThyroSeq v3
- ✓ Reported ThyroSeq v3 has enhanced use to diagnose and prognosticate thyroid cancer based on specific molecular alterations detected

Study conclusion:

benign molecular test result. In light of these findings, the choice of molecular test may hinge on factors other than diagnostic performance, such as cost, processing time, sample inadequacy rate, and information regarding specific mutations that may guide future treatment.

Samples Inadequate for Testing

4%	9%
ThyroSeq v3	Afirma GSC

p=0.059

Benign Call Rate

60%	53%
ThyroSeq v3	Afirma GSC

p=0.047

Livhits MJ, et al. *JAMA Oncol.* 2021 Jan 1;7(1):70-77.

Afirma GSC and Thyroseq GC clinical performance data

	Afirma – GSC	ThyroSeq v3
NPV	96%	97%
PPV	47%	66%
BCR (clinical validation study)	54%	61%
BCR (clinical utility studies)	66-76%	58-74%

Patel JAMA Surgery 2018;153:817; Steward DL. JAMA Oncol. 2019;5:204-212

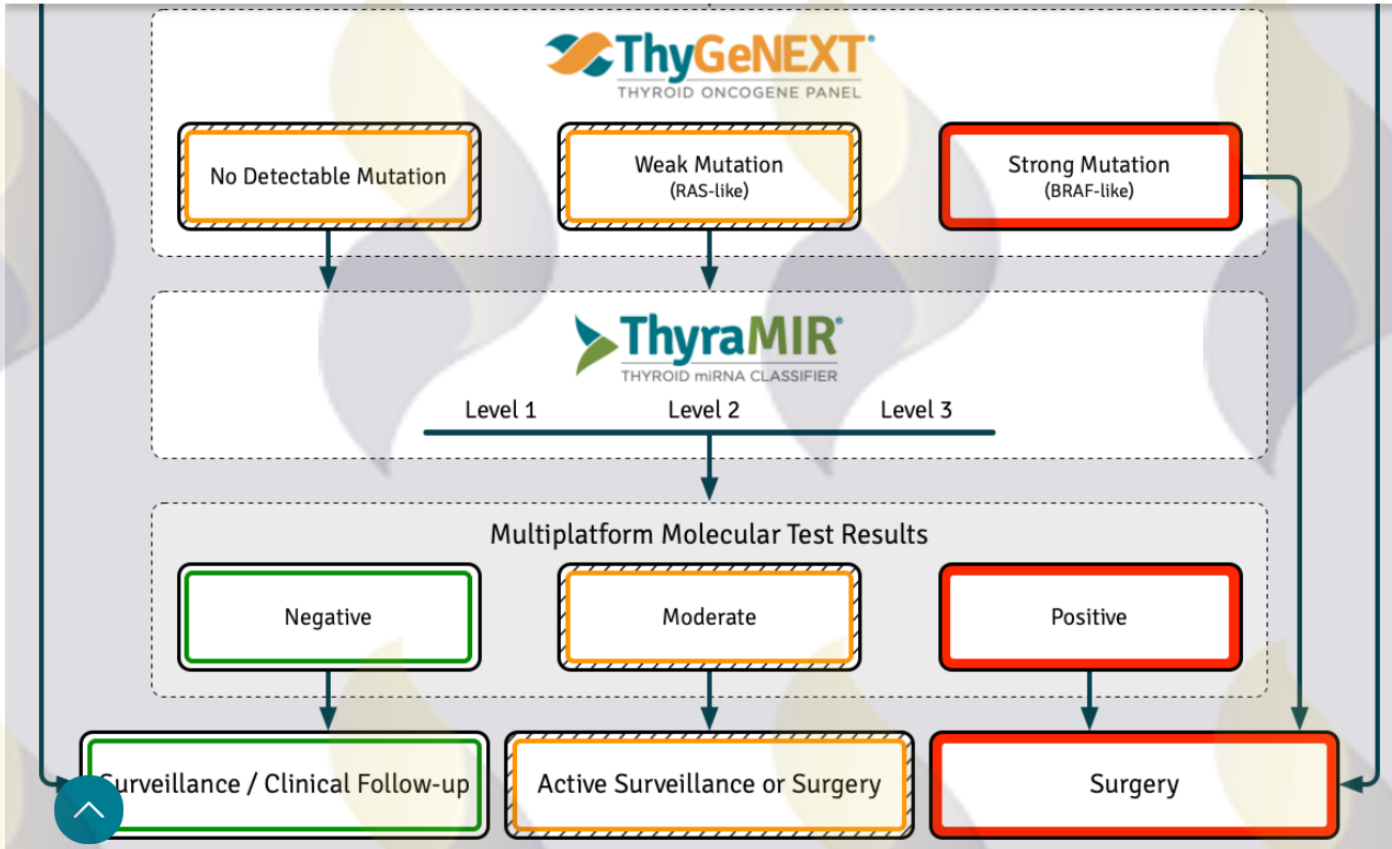
ThyGeNEXT/ThyraMIR

- Step 1: ThyGeNEXT
  - Mutation and gene fusion panel
- Step 2: ThyraMIR
  - Expression of 10 miRNA genes
  - The miRNA analysis may be valuable in predicting behavior of nodules with weak driver mutations (eg, RAS)

Expanded mutation panel (ThyGeNEXT)		microRNA risk classifier(ThyraMIR)
DNA variant	Fusions (n) and mRNA	microRNA
BRAF[a]	*BRAF* (3)[b]	miR-31-5p
ALK	*ALK* (2)	miR-29b-1-5p
GNAS	*NTRK* (8)	miR-138-1-3p
HRAS	*PPARg* (5)	miR-139-5p
KRAS	*RET* (14)[b]	miR-146b-5p
NRAS	*THADA* (5)	miR-155
PIK3CA	*NKX2.1*	miR-204-5p
PTEN	*PAX8*	miR-222-3p
RET[b]	*TBP*	miR-375
TERT promoter[b]	*USP33*	miR-551b-3p

Jackson, S, et al. *Diagnostic Cytopathology*. 2020.
Lupo et al. *Diagnostic Cytopathology*. 2020; 48: 1254-1264

ThyGeNEXT+ThyraMIR test algorithm



Patient Management

- Strong driver mutations, such as BRAF V600E, TERT promoter, and RET/PTC help inform consideration of surgical intervention[2-9]
- microRNA classification assists with variable mutational profiles
  - → Lack of detectable mutations[1]
  - → Weak driver mutations, such as RAS[1,12]

Request Info

ThyGeNEXT + ThyraMIR

A. Performance in Bethesda III and IV nodules (n = 178, disease prevalence 30%)

MPTX result	Benign n	Malignant + NIFTP n	Parameter	Observed test performance, % (95% CI)	Prevalence adjusted test performance, % (95% CI)
Negative	77	4	Sensitivity	93 (82-98) Negative threshold	95 (86-99) Negative threshold
Moderate	35	15	Specificity	90 (84-95) Positive threshold	90 (84-95) Positive threshold
Positive	12	35	NPV	95 (88-99)	97 (91-99)
			PPV	74 (60-86)	75 (60-86)
			Moderate ROD	30 (17-44)	39 (32-46)

- The moderate group has a risk of malignancy that is similar to the pre-test risk of malignancy.
- In the validation cohort, a moderate result occurred in 28% of patients.
- benign call rate 46% and positive results in 26%.
- Since this is a non-binary test the interpretation of the moderate risk category in calculating PPV and NPV has a major effect on these predictive parameters.

- Can be sent on 1 dedicated pass, slides or cell blocks. Does not require refrigeration.

Lupo et al. Diagnostic Cytopathology 2020

Current strategy

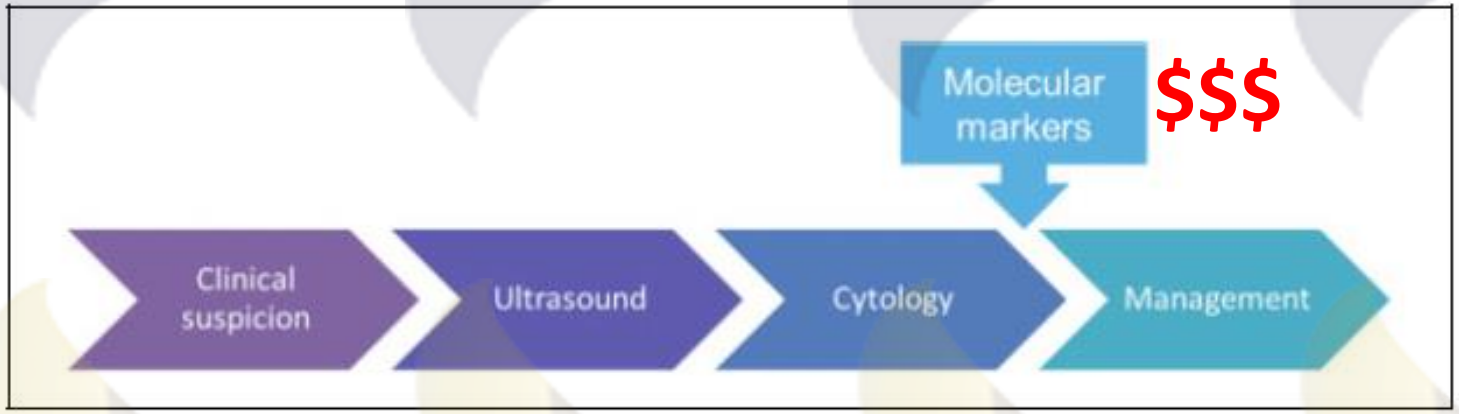


Figure 1. Current diagnostic approach for a newly diagnosed thyroid nodule.

Proposed strategy

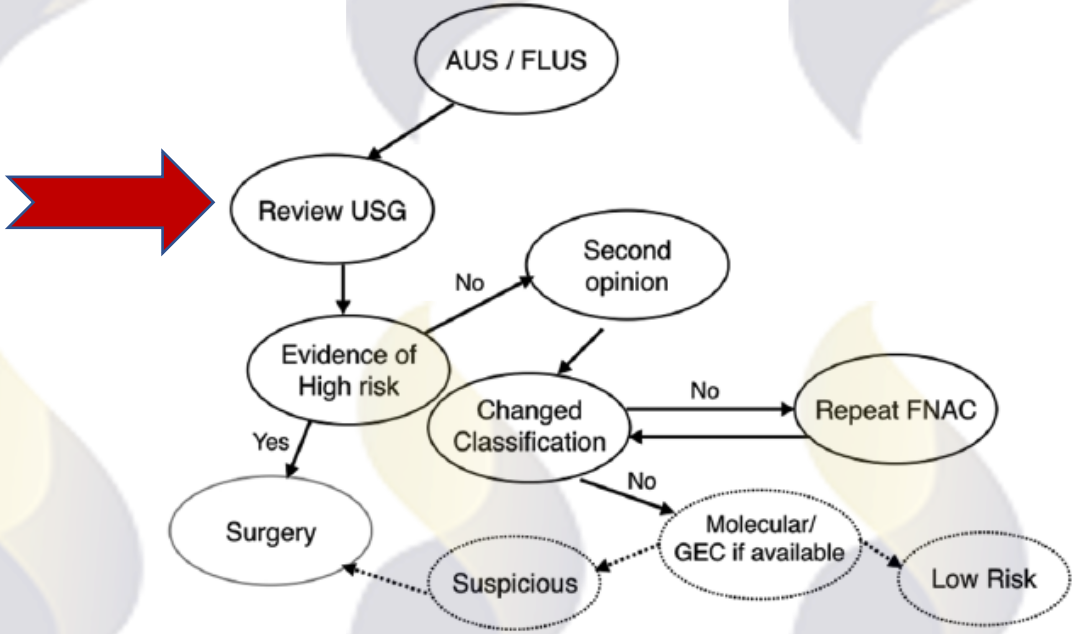


Figure 2: Clinical decision-making in Bethesda III lessons

Molecular tests: Does sonographic risk matter?

University of Miami Studies (Afirma GEC and Thyroseq v2, ATA-US and TIRADS):

- Sonographic risk alone was not an adequate predictor of malignancy
- There was a modest correlation of sonographic risk category with molecular test results
- NPV of both molecular tests was not altered by sonographic risk category
- While 75% of high sonographic risk nodules were Thyroseq positive, NPV remained high in this category
- PPV of Afirma GEC was higher in higher sonographic risk categories, while PPV of Thyroseq was similar in nodules regardless of sonographic risk category

Multicenter Thyroseq and US study:

Neither the ATA nor TI-RADS US scoring systems further informed the risk of cancer/NIFTP beyond that predicted by TSv3.

Arosemena, Kargi et al Thyroid 2020
Figge JJ, Nikiforov YE et al Thyroid 2021

Summary

Molecular tests are valuable tools in the management of thyroid nodules with indeterminate cytology

Testing algorithms have evolved and improved rapidly in the last few years to improve specificity and PPV while maintaining high sensitivity and NPV

Currently there are more robust studies to support the clinical validity and utility of Afirma GSC and Thyroseq v3 GC when compared to ThyGeNEXT+ThyraMIR

There is considerable heterogeneity in methodology and results of various studies of molecular tests

Molecular test performance is not meaningfully altered by sonographic risk

Long-term followup studies of outcomes in patients undergoing molecular testing are needed, especially for non-operated cases with negative test results