Adding value in thyroid cancer diagnostic: thyroglobulin and calcitonin measurement in fine needle aspirate washout

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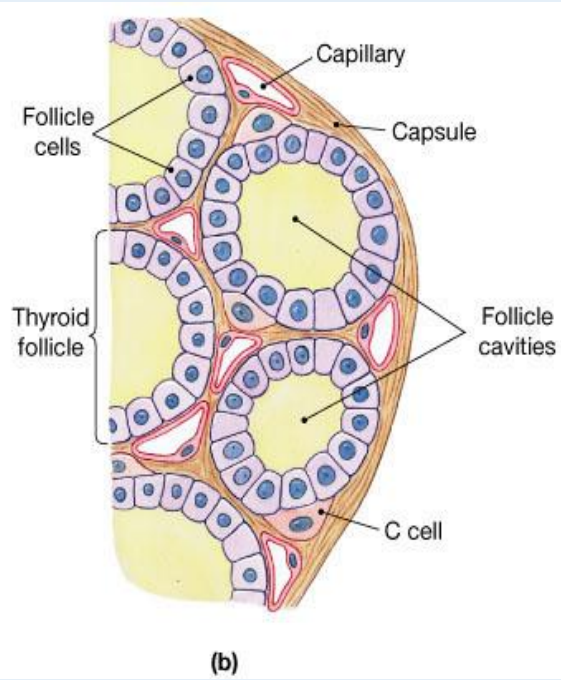
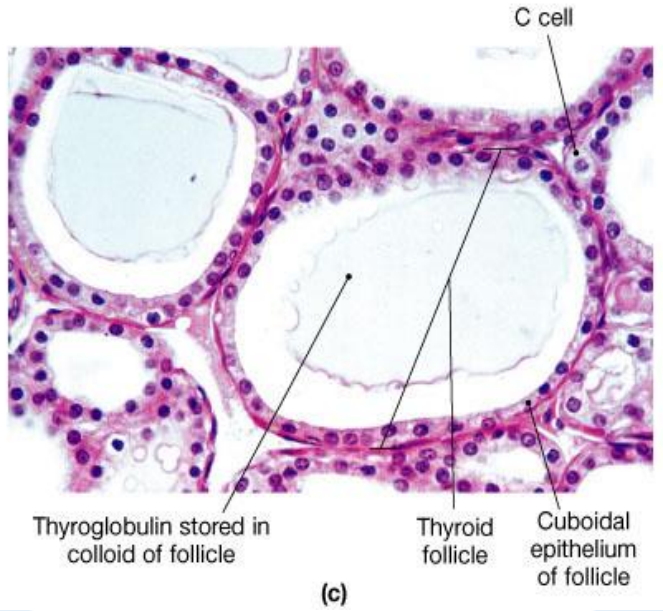
Conflict of interest

Nothing to disclose

Thyroid

- One of the largest endocrine organs
- Function – thyroid hormones synthesis

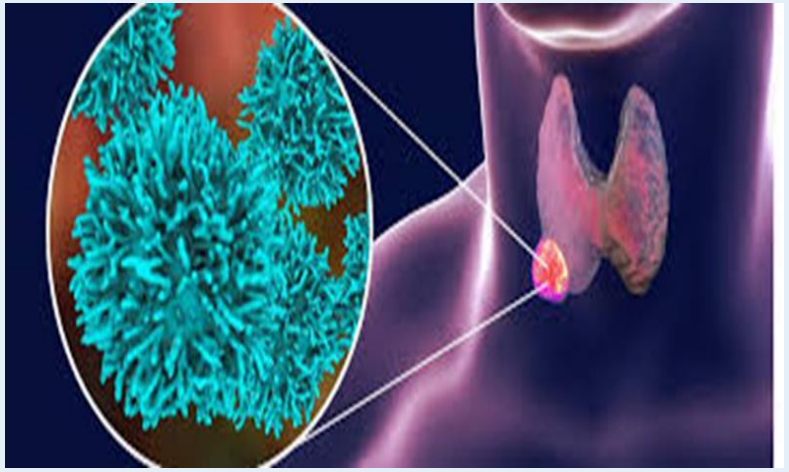




Thyroid tissue

- Follicular cells → **Thyroid hormones (T3, T4)**
- Parafollicular cells (C-Cells) → **Calcitonin**

Thyroid cancers



- Most common endocrine neoplasia - **2.5%** of all cancers and about **90%** of endocrine tumors

*(Siegel, R.L., CA Cancer J. Clin. **2019**, 69, 7–34)*

- Increasing incidence (**4.5%/yr**) – diagnostic techniques improvement, environmental (e.g., radiation, pollution) and lifestyle changes

*(Pellegriti, G. J. Cancer Epidemiol. **2013**, 965212)*

- TC is **the 9th** in the series of cancer in Europe: ≈50,000 newly dg cases and 6000 deaths reported in 2012; 6.3 cases/100,000 persons-year

*(EU Commission Joint Research Center, **2017**)*

Perspective

June 2014

Cancer Research

Projecting Cancer Incidence and Deaths to 2030: The Unexpected Burden of Thyroid, Liver, and Pancreas Cancers in the United States

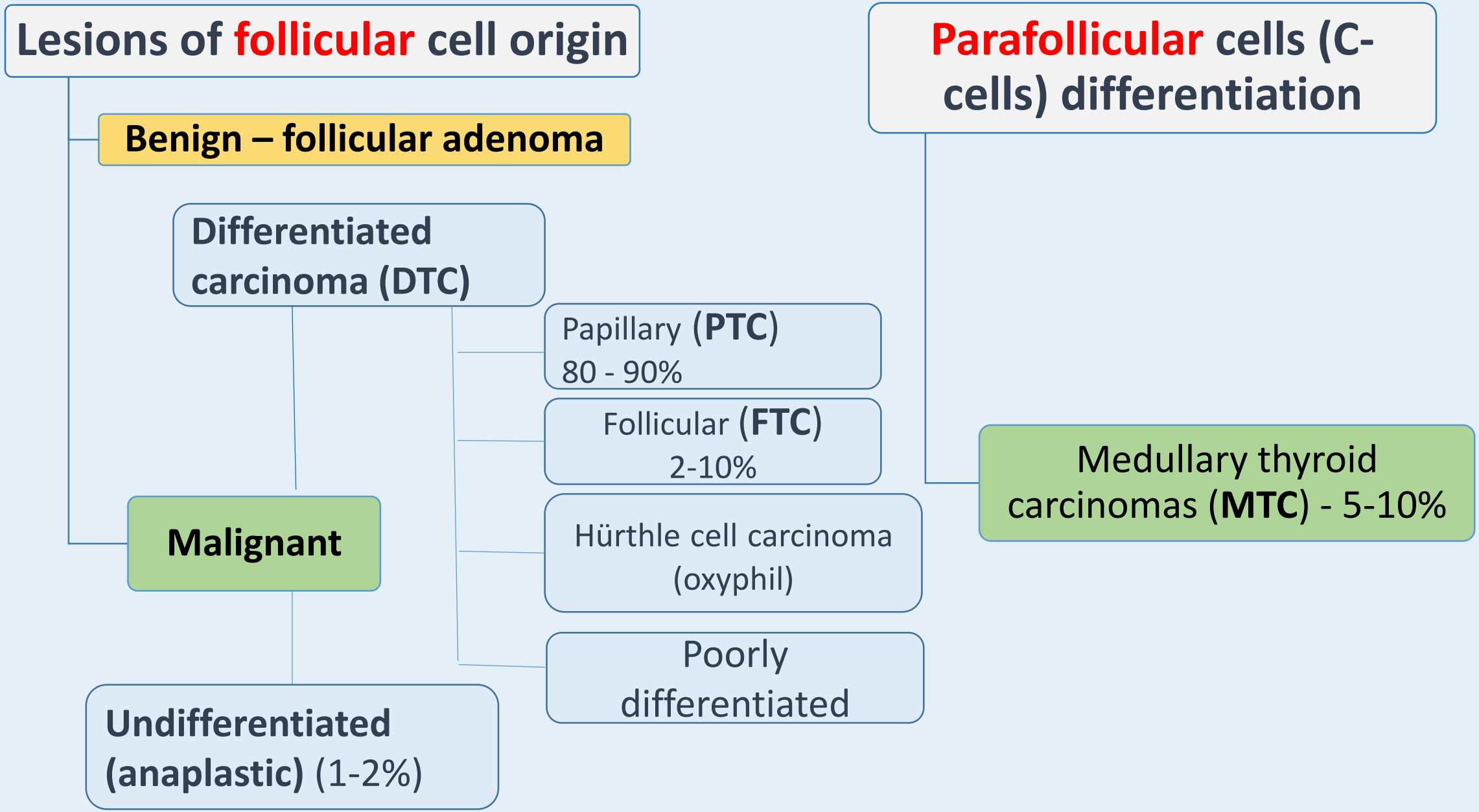
Lola Rahib[1], Benjamin D. Smith[2], Rhonda Aizenberg[1], Allison B. Rosenzweig[1], Julie M. Fleshman[1], and Lynn M. Matrisian[1]

New cases		Total	Women	Men
Thyroid	2010	45,000	34,000	11,000
	2020	92,000	71,000	21,000
	2030	183,000	144,000	39,000
Breast	2010		226,000	
	2020		262,000	
	2030		294,000	

In 2030,
Thyroid will be #2 in women
and #9 in men

Projected death rate stable at 2,000

Histological classification of Epithelial Thyroid Neoplasms



- The diagnostic approach to thyroid cancer - one of the most challenging in endocrine oncology:

- increasing prevalence of thyroid malignancy
- the difficulty to:
    - -distinguish benign from malignant non-functional thyroid nodules,
    - -establish the cervical lymph node involvement during staging,
    - -identifying later recurrences.

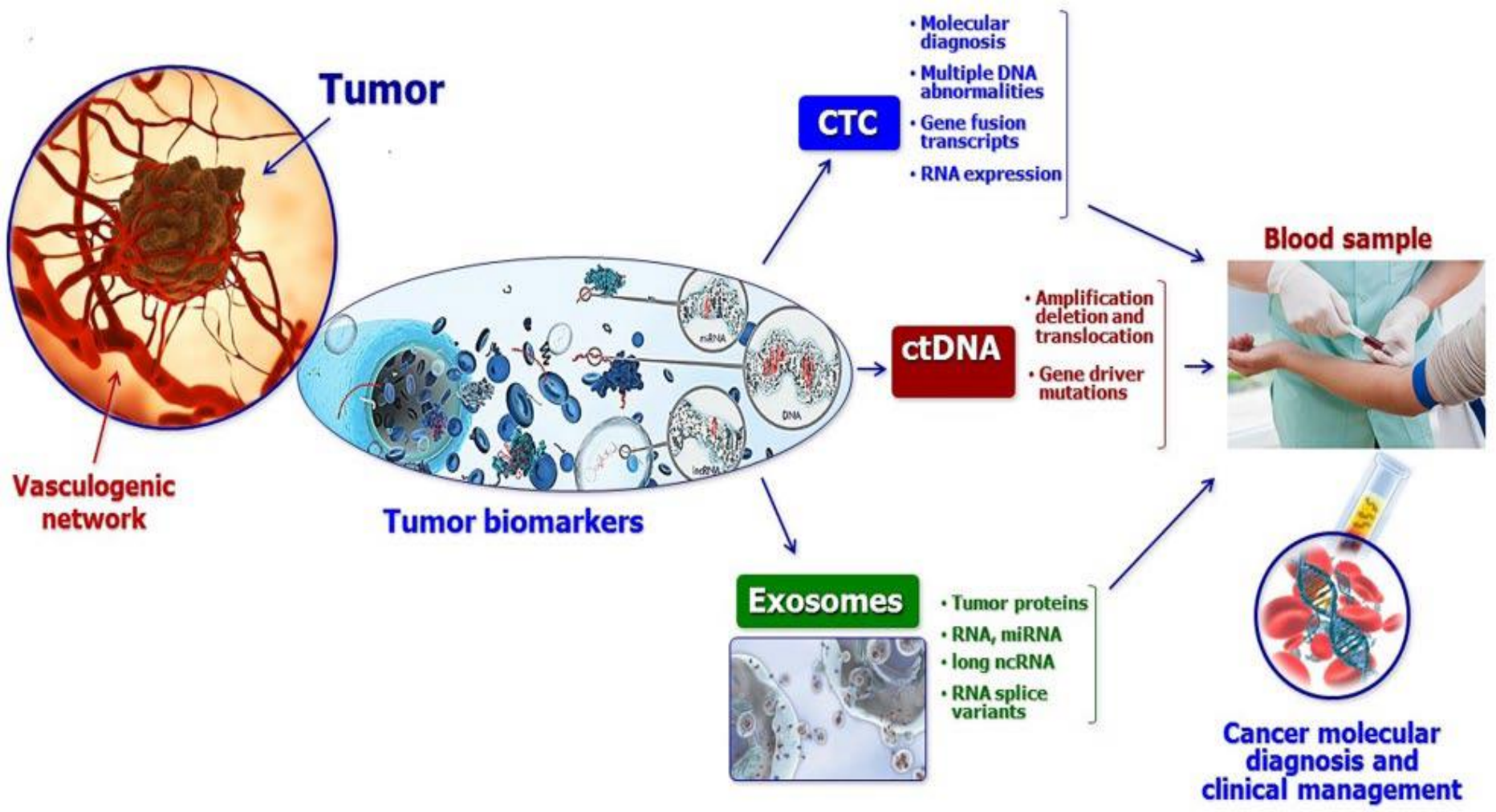
- Main clinical challenges:

- Early diagnostic of high-risk nodules
- Avoiding over-diagnostic and over-treatment in patients with low-risk or benign nodules

- Hormonal thyroid markers: blood tests for thyroid-stimulating hormone (**TSH**), free thyroxine (**FT4**), free triiodothyronine (**FT3**), thyroglobulin (**Tg**), thyroglobulin antibodies (**Tg-Ab**), thyroid peroxidase antibodies (**TPO-Ab**), TSH receptor antibodies (**TRAb)**, and calcitonin (**Ct**).

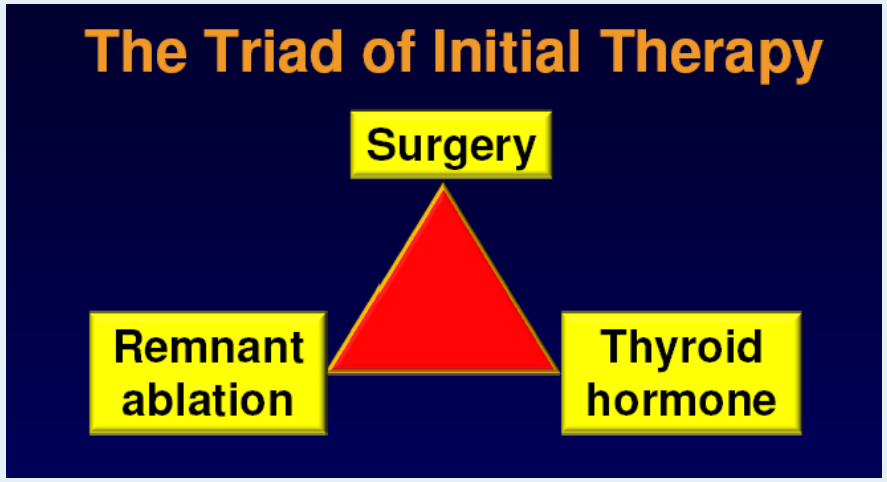
- New clinical and molecular data have allowed the development of new staging systems, predictive and prognostic tools, new approaches to the treatment. *(L. Lamartina et al, F1000 Research 2018, 7)*

Research in thyroid tumors – ***OMICS:*** proteomic signature (specific protein markers e.g. Gal-3), metabolomics (oncometabolites), genomics (tissue/blood/FNAB gene sequencing panels), liquid biopsy - tumor-derived DNA/RNA in plasma extracellular vesicles, circulating cell-free DNA



R. *Palmirotta*, *Therapeutic Advances in Medical Oncology, 2018*

DTC



- Management in **DTC** is effective:
- Early dg (**FNAB of thyroid nodules**)
- Surgical resection
- Medical therapy (ablation I^{131})
- Regular surveillance
- Very good prognosis: ► **But: 10-15% patients - aggressive clinical course**

→ I^{131} resistance (inability to concentrate iodine as the result of impaired expression / function of the sodium/iodine symporter (NIS) or thyroperoxidase (TPO) caused by oncogene-activated signalling that leads to thyrocyte dedifferentiation → death !!!

NCCN Clinical Practice Guidelines in Oncology: Thyroid Carcinoma, vers I. 2013.
Durante C et al., J .Clin Endocrinol Metab, 2006.

DTC - Cervical lymph node (LN) metastases

- in 20% to 50% of patients in the postoperative follow-up period
- It is critical to differentiate metastatic from benign LNs

LNs metastatic – YES – Lymphadenectomy

– NO – Clinical Monitoring

- Fine needle aspiration biopsy of **LN** (**FNAB**) - the **"gold standard"** in the **management of suspicious LNs.**
- Serum TSH, Tg, TgAb
- **Tg** in LN FNAB washout fluid (TG-FNAB) - further increase the diagnostic accuracy, frequently used as a diagnostic adjunct to cytology in patients with DTCs

Thyroglobulin

Tg – heterogenous glycoprotein (600 KDa), produced **exclusively by follicular thyroid cells**, stored as colloid within the thyroid follicles; secretion TSH dependent

Serum Tg level - proportional to the thyroid mass rather than the type of pathology

False positive elevated levels in several medical conditions (goiter, hyperthyroidism, inflammatory or physical thyroid injury, differentiated follicular-cell derived tumors).

Tg - NOT USEFUL IN THE INITIAL WORK-UP OF NODULAR THYROID DISEASE

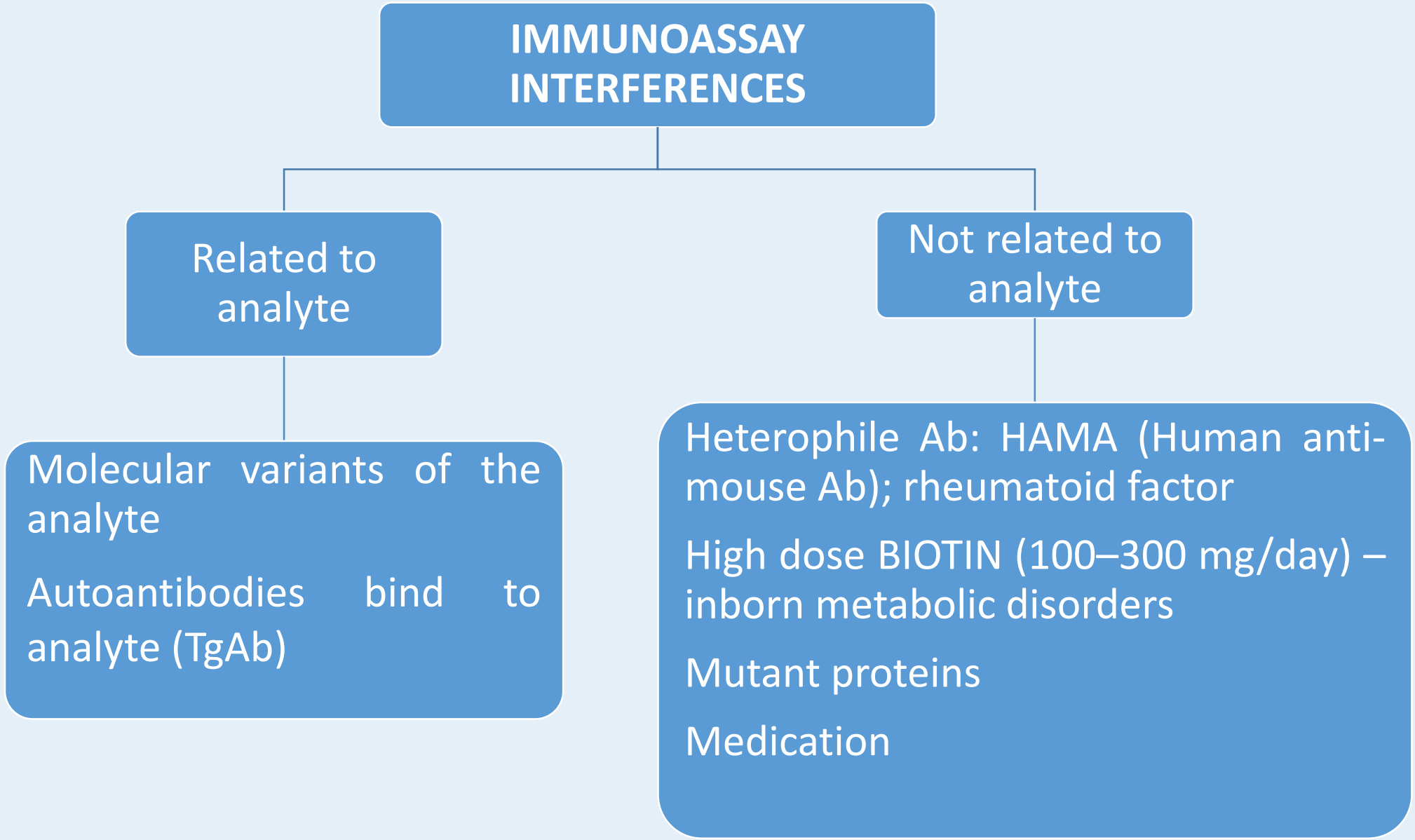
(Routine evaluation of serum Tg for initial evaluation of thyroid nodules is ***not recommended;*** Tg can be elevated in most thyroid diseases and is an ***insensitive*** and ***nonspecific*** test for thyroid cancer*)

**2015 ATA guidelines for adult patients with thyroid nodules and DTC*

BUT

Tg - MARKER OF PERSISTENT OR RECURRENT DISEASE in DTC **patients after total thyroidectomy and radioiodine ablation –** ***treatment effectiveness, recurrences monitoring***

Mind the gap: Tg immunoassay



Adapted from C.Spencer, AACE 2019

Mind the gap: Tg immunoassay

Molecular variants of the analyte - serum Tg heterogeneity – up to 2 fold difference between assays

Autoantibodies bind to analyte (TgAb)

- TgAb have the potential to interfere with any Tg assay
- TgAb prevalence in DTC – **2x** than healthy population
- TgAb assays should not be used interchangeably – differences between IA
- **Recommendation** (*ATA Guidelines, R63*)

- monitoring **TgAb trend, using the same method, as 1st surrogate tumor-marker**

- Tg trend, when detectable, as 2nd surrogate tumor-marker

(Kim et al, JCEM, 2008; Haugen, Thyroid, 2016; Spencer, AACE,2019)

MTC

- MTC - quite rare malignant tumor that originates from C cells

  - sporadic or hereditary variant

- **MTC preoperative diagnostic** – importance for the clinical pathway: *genetic testing for germline RET mutation and surgical approach.*
- Diagnostic accuracy of LNs - FNAB in MTC < in DTC (50-80%)**: various cellular morphologies, low cellularity**

Calcitonin

Ct - 32-amino-acid polypeptide hormone, product of the C-cells

Serum Ct – *sensitive biomarker for **MTC** diagnostic and follow-up*

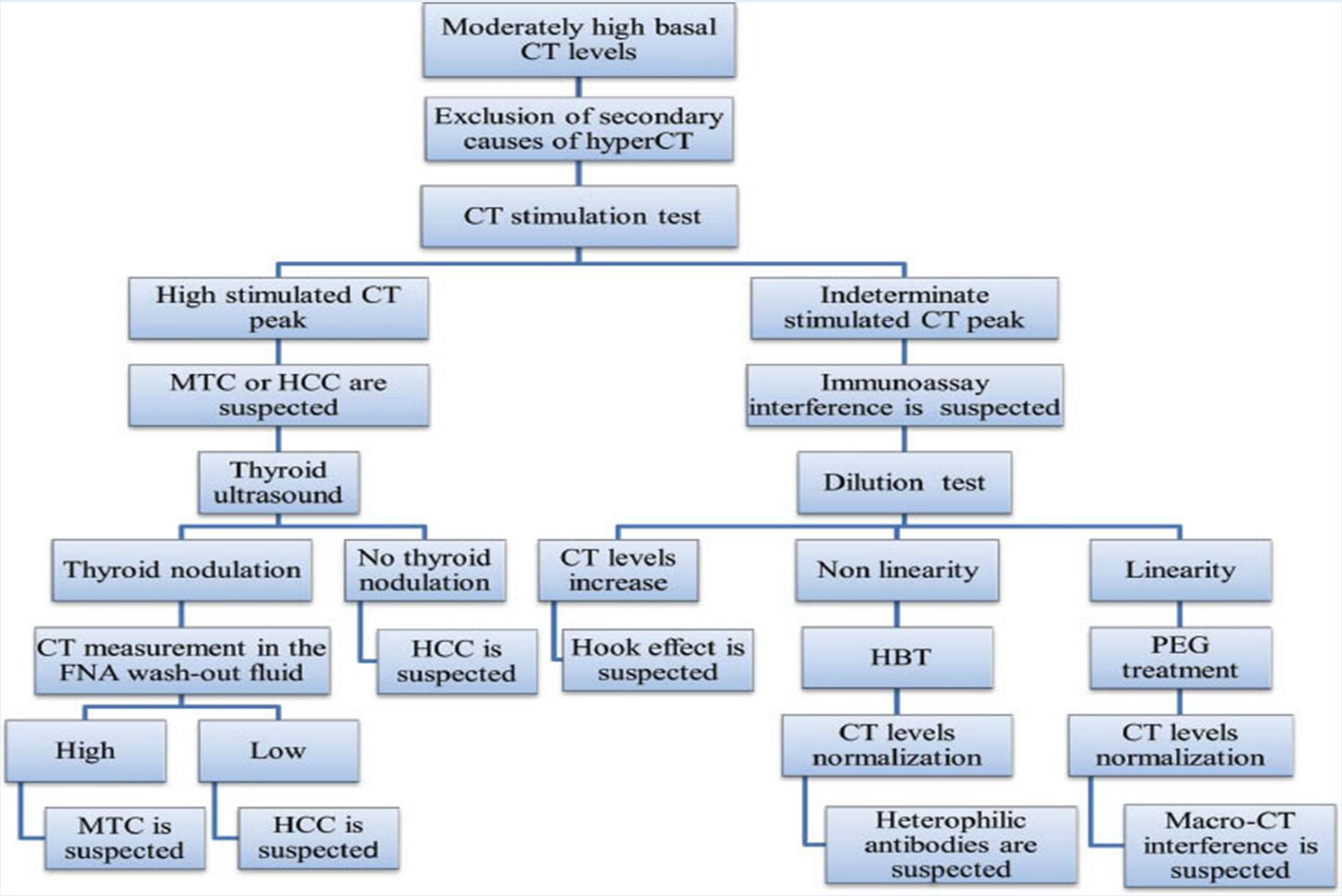
Ct immunoassays pitfalls

- **Technical challenges** in calcitonin assays:

- *sample stability* (2hr at RT) - transported to the laboratory on ice, processed rapidly, and frozen before analysis
- *biphasic half-life* (<30 minutes at physiological levels and up to 30 hours at high levels)
- *Immunoassay interferences* - presence of calcitonin isoforms and fragments

- False increased Ct levels: **non-thyroid disorders**, bacterial infections, pregnancy and lactation; **certain medications** (e.g., proton pump inhibitors, glucagon-like peptide-1 agonists, β-blockers); **smoking**

- Ct can be stimulated by **food and alcohol intake** - *samples drawn in the morning after an overnight fast*



Algorithm integrating clinical, radiology and laboratory testing for moderately elevated Ct levels.

FNA, fine-needle aspiration; HBT, heterophilic blocking tube; HCC, C-cell hyperplasia; hyperCT, hypercalcitoninemia; macroCT, macrocalcitonin; MTC, medullary thyroid carcinoma; PEG, polyethylene glycol.

Censi S, Clin Chem Lab Med 2016

Analytical aspects

Tg

(1) Tg assays with *a functional sensitivity of <0,1ng/mL;*

(2) Calibrated against the *certified reference material BCRVR 457;*

(3) ***The use of the same Tg assay over time;***

(4) Reflex **TgAb** in all samples tested for Tg - identifying samples with potentially falsely ↓or ↑ Tg .

Ct

(1) Ct assays with *a functional sensitivity of <1 pg/mL;*

(2) Calibrated against the *certified reference material IRP WHO ref std 89/620;*

(3) ***The use of the same Ct assay over time;***

Standardization in Tg and Ct assays has reduced the inter-method variability, however, ***significant differences can still be observed between Tg /Ct conc. when the same sample is measured by different methods.***

A. Algeciras-Schimnich, Critical Reviews in Clinical Laboratory Sciences,2018; Netzel et al, J Clin Endocrinol Metab. 2015

TG AND CT IN FNAB WASHOUT

- US-FNAB – gold-standard method in TC:

➢ Dg: grey zone - indeterminate thyroid nodules cytologies Bethesda 3&4 (10-30%) cases with intermediate risk of malignancy - atypia/follicular lesions of undetermined significance → probability of malignancy is too high for watchful waiting but insufficient to warrant a total thyroidectomy

➢ Monitoring: **FNAB from lymph nodes** in indeterminate structural residual disease

→ Tumor markers (Tg, Ct, Cyfra-21) measurement in FNAB wash-out of lymph nodes/thyroid nodules

Tg and Ct in FNAB washout

- **DTC: Tg-FNAB** from **LN** – increases the dg accuracy, particularly in cystic nodes, indeterminate cytology, divergent US and cytological evaluation

- **MTC**: **Ct-FNAB** in the case of inconclusive cytological results (ATA recommendation, 2016)

(Trimboli P, Clin Chem Lab Med. 2017;55:914–925)

- **FNA fluids**:

➢ Unique specimen type for biomarker analysis

➢ Special considerations – handling, stability, validation, interpretation

- Most often the washout is performed by rinsing the FNAB needle with a small volume (0.5 - 5 ml) of **0.9% saline solution** immediately after the cellular component of the biopsy has been expelled for cytological examination.
- Variations of this procedure: **PBS or Tg/Ct-free serum.**

SINCE MATRIX EFFECT COULD BE ASSAY DEPENDENT, THIS SHOULD BE EVALUATED BY THE LABORATORY PERFORMING Tg/Ct-FNAB.

Sources of different results in Tg-FNAB analysis

Immunoassay

- Different functional sensit (0.1-3 ng/ml)

Pre-analytical phase:

- Needle pass
- Matrix (PBS, 0,9% Saline, Dil)
-Wash-out volume(0.5-1mL)

Pre-op vs. post-op :

- Athyreotic pts
- TSH suppress. ↓ Tg & Tg-FNAB

TgAb interferences:

- **NO** (Baskin HJ, Boi F et al)
- **YES** (falsely low Tg-FNAB)

(Jeon S.J.)

G.Grani&A.Fumarola, JCEM, 2014

Tg/Ct-FNAB cut-off values

Debate – lack of consensus:

1. **M+2SD** from the Tg for negative lymph nodes (Cignarelli M, 2003; Boi, 2007)
2. **ROC** curve analysis (Lee YH, 2010; de Crea, 2014; Kihara,2018)
3. **Tg-FNAB/Tg serum, Ct-FNAB/Ct serum** (Chung J,2014; Kihara, 2018)
4. The **upper value of the serum Tg reference interval** (Kim DW, 2012)
5. **Tg-FNAB cutoff = serum Tg** (when detectable) (de Crea, 2014)
6. **Absolute Tg content** (Kahramangil B, 2019)

The diagnostic performance of Tg-FNAB

The diagnostic performance Tg-FNAB is superior in **athyreotic patients**

Tg-FNAB	Cut-off	Sensitivity	Specificity	First author
Athyreotic patients	**1.0 ng/ml**	93%	96%	*Moon JH, JCEM 2013*
	1.0 ng/ml	96.9%	94.1%	*Grani G, JCEM 2014*
	2.5 ng/ml	94%	100%	*Kahramangil B., Surgery, in press, 2019*
Before thyroidectomy	**1 ng/ml**	**86.2%**	**90.2%**	*Grani G, JCEM, 2014*

Thyroglobulin in Lymph Node Fine-Needle Aspiration Washout: A Systematic Review and Meta-analysis of Diagnostic Accuracy

Grani G, JCEM, 2014

Giorgio Grani and Angela Fumarola

Table 3. Pooled Sensitivities, Specificities, Likelihood Ratios, and DORs From Main and Subgroup Meta-analyses

Studies (Refs.)	LNs, n	Sensitivity, % (95% CI)	Specificity, % (95% CI)	Positive Likelihood Ratio (95% CI)	Negative Likelihood Ratio (95% CI)	DOR (95% CI)	I^2, %	Heterogeneity χ^2 (*P* Value)
All, n = 24	2865	95.0 (93.7–96.0)	94.5 (93.2–95.7)	15.807 (10.200–24.497)	0.063 (0.040–0.101)	338.91 (164.82–696.88)	65.7	67.09 (<.001)
Thyroid presence								
Only patients before thyroidectomy, n = 9 (6, 13, 16, 21, 22, 28, 29, 36, 38)	410	86.2 (80.9–90.5)	90.2 (85.1–94.0)	6.184 (4.140–9.238)	0.167 (0.100–0.280)	56.621 (22.535–142.26)	37.3	12.76 (.121)
Only patients after thyroidectomy, n = 13 (6, 16, 21, 24, 26, 28–32, 36, 37, 40)	1007	96.9 (94.9–98.2)	94.1 (91.7–96.0)	17.909 (7.972–40.230)	0.063 (0.040–0.098)	407.65 (198.67–836.46)	0.0	9.35 (.673)
Anti-TgAb								
TgAb-positive patients, n = 3 (16, 24, 26)	69	89.2 (74.6–97.0)	96.9 (83.8–99.9)	13.116 (3.391–50.735)	0.110 (0.015–0.828)	105.54 (12.173–915.09)	22.6	2.59 (.275)
TgAb-negative patients, n = 3 (16, 24, 26)	392	96.8 (93.2–98.8)	97.0 (93.7–98.9)	26.242 (12.683–54.296)	0.039 (0.003–0.469)	982.91 (231.28–4177.3)	7.3	2.16 (.340)
Cutoff								
0.9–1.1 ng/FNA, n = 8 (13, 18, 21, 24, 28, 34, 37, 38)	1252	94.8 (92.8–96.4)	91.2 (88.7–93.2)	13.356 (3.704–48.162)	0.064 (0.028–0.149)	266.81 (45.528–1563.6)	89.4	66.31 (<.001)
10 ng/FNA, n = 5 (26–28, 38, 40)	745	87.7 (83.8–91.0)	94.2 (91.4–96.3)	12.853 (5.920–27.908)	0.133 (0.048–0.370)	104.39 (22.527–483.79)	85.1	26.91 (<.001)
Serum Tg, n = 3 (19, 25, 36)	264	97.3 (93.8–99.1)	94.9 (87.4–98.6)	11.552 (2.845–46.905)	0.038 (0.017–0.084)	423.51 (69.509–2580.4)	31.7	2.93 (.231)
Country								
Korea, n = 9 (19, 25–29, 31, 34, 38)	1579	92.9 (90.8–94.6)	94.3 (92.5–95.8)	12.919 (7.526–22.177)	0.073 (0.037–0.144)	189.56 (65.868–545.55)	80.9	41.92 (<.001)
Italy, n = 6 (4, 6, 13, 16, 20, 22)	325	92.2 (86.7–95.9)	98.3 (95.0–99.6)	20.168 (8.593–47.334)	0.114 (0.062–0.209)	258.72 (83.996–796.90)	0.0	4.78 (.444)
United States, n = 3 (30, 32, 37)	299	97.6 (93.1–99.5)	87.4 (81.5–91.9)	11.611 (2.396–56.271)	0.041 (0.006–0.284)	347.43 (61.067–1976.6)	22.2	2.57 (.276)

The diagnostic performance of Ct-FNAB

Ct-FNAB/ assay	Cut-off	Sensitivity	Specificity	First author
Immulite, Siemens	**36 pg/ml**	100%	100%	Boi, 2007
Liaison XL, DiaSorin	**10.4 pg/ml**	89%	100%	De Crea, 2014
ECLIA Systems, Roche	**21 pg/ml**	100%	100%	Kihara, 2018

ACTA OTORHINOLARYNGOLOGICA ITALICA 2014;34:399-405

THYROID

Calcitonin measurement in fine-needle aspirate washouts *vs.* cytologic examination for diagnosis of primary or metastatic medullary thyroid carcinoma

Dosaggio della calcitonina nel liquido di lavaggio dell'agoaspirato vs. esame citologico nella diagnosi del carcinoma midollare della tiroide primitivo o metastatico

C. DE CREA[1], M. RAFFAELLI[1], D. MACCORA[1], C. CARROZZA[2], G. CANU[2], G. FADDA[3], R. BELLANTONE[1], C.P. LOMBARDI[1]

[1] UO Chirurgia Endocrina e Metabolica, [2] UO Analisi Ormonali and [3] UO Anatomia Patologica e Istologia, Policlinico "A. Gemelli", Università Cattolica del Sacro Cuore, Rome, Italy

Table II. Accuracy, positive predictive value and negative predictive value of FNAB-C*, FNAB-CT†, FNAB-CT†/sCT¶ ratio, and integration of FNAB-C* with FNAB-CT† and FNAB-CT†/sCT¶.

	Sensitivity	Specificity	Accuracy	Positive predictive value	Negative predictive value
FNAB-C*	50%	100%	85%	100%	83%
FNAB-CT†	89%	100%	97%	100%	96%
FNAB-CT†/sCT¶ ratio	83%	93%	90%	83%	93%
FNAB-C* + FNAB-CT†	94%	100%	98%	100%	98%
FNAB-C* + FNAB-CT†/sCT¶	89%	91%	90%	80%	95%

** FNAB-C: ultrasound-guided fine-needle aspiration biopsy cytology; † FNAB-CT: calcitonin in the needle wash-out; ¶ sCT: serum calcitonin.*

Tg/Ct FNAB - Our Experience

Materials and methods

- The washout - **1 ml saline solution 0.9%**, immediately after the biopsy's cellular component was expelled on the slide for the cytological examination.
- Immunoelectrochemiluminiscent method (ECLIA-Roche)
- Producer validated matrix: ***only serum/plasma***

Immunoassays analytical characteristics in **serum/plasma**

	Tg	Ct
Reference material	BCRVR 457	IRP WHO ref st 89/620
Measuring range	0.04 – 500 ng/ml	0.5 – 2000 pg/ml
LOB	0.02 ng/ml	0.3 pg/ml
LOD (analytical sens)	0.04 ng/ml	0.5 pg/ml
LOQ (functional sens)	**0.1 ng/ml**	**1 pg/ml**
Repeatability (CV%)	2 – 2.2%	1.4 – 1.8%
Reproducibility (CV%)	4.1 – 4.6%	1.6 – 2%

Validation steps for FNAB matrix

- **LOB** (concentration below which analyte-free samples are found with a probability of 95 %) – 10 replicates saline 0.9%;
- **LOD** (based on the Limit of Blank and the standard deviation of low conc. samples) – 10 replicates saline 0.9%;
- **Precision:** repeatability and reproducibility – 10 replicates/run, 2 replicates/10 days, respectively
- **Trueness** of the assay:

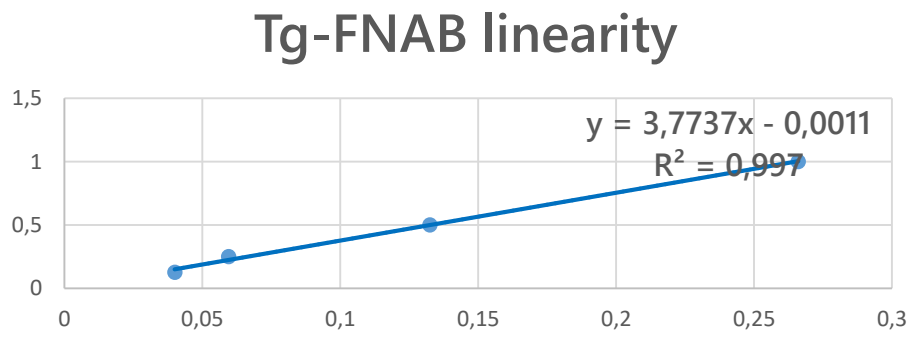
	Tg	Ct	Acceptance criteria
Linear dilution (basal conc.)	0.266ng/ml; 163.4ng/ml; 199.3ng/ml	572.5 pg/ml; 15.14 pg/ml	r2≥0,99 y-intercept ≤2

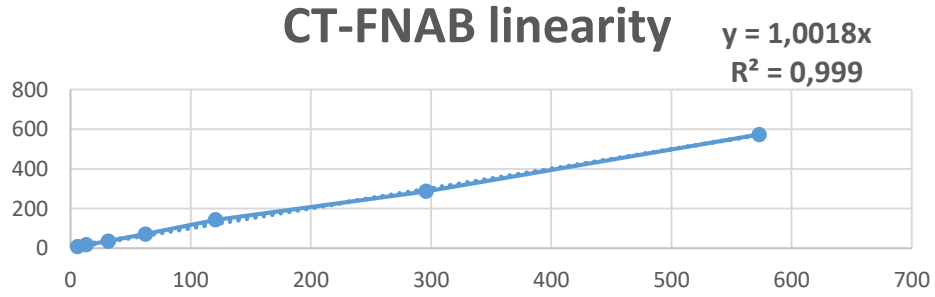
- **Recovery** test (conc. samples spiked with high cal) – accept. crit. 80-120%

Tg/Ct-FNAB

Assay performance characteristics	LOB (ng/ml)	LOD (ng/ml)	CV intra-assay (%)	CV inter-assay (%)
Tg – 0.9% Saline	<0.04	0.046	5.8	8.7
Ct – 0.9% Saline	<0.5	0.55	3.55	2.6-3.7

	Recovery (%)	y-intercept	r^2
Tg	89.5 - 120	0.0003	0.997
Ct	100 - 114	1.6569	0.999





Case 1 – Dg. Thyroid nodule

US	Solid nodule, isoechogeneity, 34/23mm
Tg-FNAB	**< 0,04 ng/ml**
FNAB cytology	Benign cytology (epidermal or sebaceous cyst **not of follicular origin**)

Case 2 – Monitoring TC

2013 – dg PTC, total thyroidectomy, radioiodine ablation 131I

	2014	2015	2017	Cytology	HP	OBS
TSH	>100 µIU/ml	>100 µIU/ml	0,086* µIU/ml			* TSH suppressed
Tg	**5.21ng/ml**	**8.06ng/ml**	1.23ng/ml			Persistent disease, progressive
TgAb	<20 U/L	<20 U/L	<20 U/L			
Tg-FNAB (left para-tracheal adenopathy)			**4224 ng/ml**	PTC metastasis	Recurrence, PTC metastasis	Prompt to surgery ↓ MONITORING

CONCLUSIONS

I.

Ct and **Tg** are **heterogenous tumor-marker proteins** and are monitored in patients as **serial measurements** in serum – **method continuity is more important than the reference range**

- Changing the method impose re-baselining
- Consider the clinical context when interpreting results

II. *Tg/Ct - FNAB* - complementary tests to cytology in TC

Tg-FNAB **- evaluations of suspicious LN**

- <u>High sens/spec</u> in early detection **nodal metastases in DTC**
- Compare favorably with cytology
- Accurate diagnostic of cases in which cytology is non-diagnostic.
- Maximum dg performance in athyreotic, TgAb negative patients

Ct-FNAB **-** should be considered the standard in **pre-surgical diagnostic work-up of MTC following moderately/high serum Ct** and of **suspicious neck MTC metastases**

III. Tg/Ct-FNAB

Need of standardization: patient selection, collection techniques, analytical methods, cut-off ⇒ **impact on clinical decision**

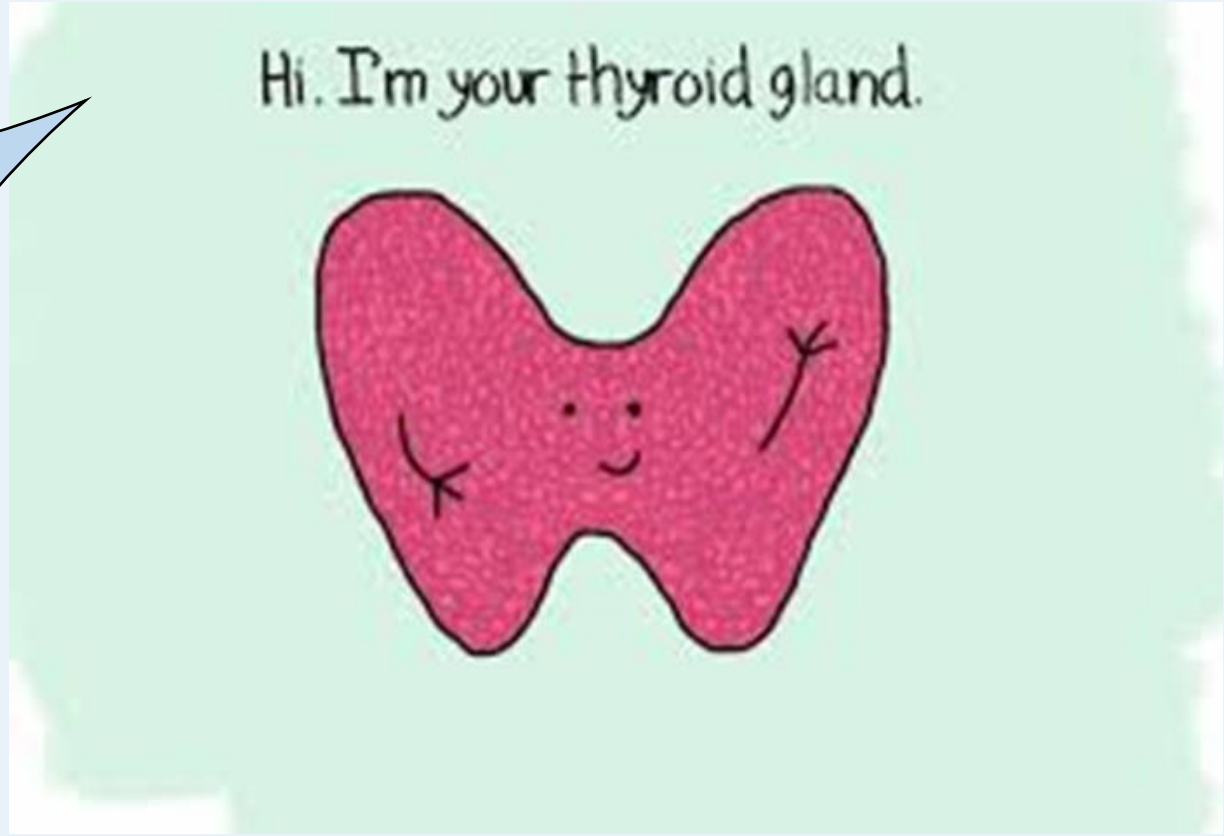
Tg/Ct-FNAB should be integrated, ***without replacing FNAB-cytology*** in evaluation of DTC recurrences and detection of primary or metastatic MTC, respectively

Close communication between all members of the care team ensures the best outcome of the patient!

Ruxandra Dobrescu *Dumitru Ioachim* *Catalina Poalelungi*

Dan Niculescu *Corin Badiu*

Thank you !



Adriana Padure *Liliana Parvu* *Dana Manda* *Suzana Vladoiu*