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Marcadores Prognósticos nas Neoplasias de Tireoide

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Tese de Doutorado

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- Revisão breve sobre o assunto.

- **Artigo 1 (original):** Serum TSH levels as a predictor of malignancy in thyroid nodules: A prospective study; publicado em PLoS One, 2017. Impact Factor: 2.776.

- **Artigo 2 (original):** Role of vascular endothelial growth factor polymorphisms in the pathogenesis of medullary thyroid carcinoma.

Além dos artigos que fazem parte da presente tese, ao longo do período do doutorado, participei de outros projetos que resultaram nos seguintes manuscritos:

- Neoadjuvant multikinase inhibitor in patients with locally advanced unresectable thyroid carcinoma**.** Nava C; Scheffel R, **Cristo AP**; Ferreira CV; Weber S; Zanella AB; Paixão FC; Migliavaca A; Guimaraes JR; Graudenz MS; Maia AL. Front Endocrinol (Lausanne) 2019. Impact Factor: 3.519

- Impact of Serum TSH and Anti-Thyroglobulin Antibody Levels on Lymph Node Fine-Needle Aspiration Thyroglobulin Measurements in Differentiated Thyroid Cancer Patients. Duval MADS, Zanella AB, **Cristo AP**, Faccin CS, Graudenz MS, Maia AL. Eur Thyroid J. 2017. Impact Factor: 3.025

- Increasing diagnostic effectiveness of thyroid nodule evaluation by implementation of cell block preparation in routine US-FNA analysis. **Cristo AP**, Goldstein HF, Faccin CS, Maia AL, Graudenz MS. Arch Endocrinol Metab. 2016. Impact Factor: 1.571

LISTA DE ABREVIATURAS E SIGLAS

ATC - Anaplastic Thyroid Carcinoma

BRAF - Serine/threonine-protein kinase B-Raf

CAT - Carcinoma Anáplasico de Tireoide

CDT - Carcinoma Diferenciado de Tireoide

CEA - antígeno carcinoembrionário

CFT - Carcinoma Folicular de Tireoide

CMT - Carcinoma Medular de Tireoide

CPT - Carcinoma Papilar de Tireoide

DTC - Differentiated Thyroid Carcinoma

FTC - Follicular Thyroid Carcinoma

INCA - Instituto Nacional do Câncer

MAPK - Mitogen-activated protein kinase

miRNA- MicroRNA

MTC - Medullary Thyroid Carcinoma

PAAF - Punção aspirativa por agulha fina

PAX8 - Paired box gene 8

PI3K - Phosphatidylinositol 3-kinase

PPARγ – Peroxisome proliferator-activated receptor γ

PTC - Papillary Thyroid Carcinoma

RET (REarranged during Transfection) - proto-oncogene RET

RET/PTC- RET tyrosine kinase domain rearrangement with different partners

SNP - polimorfismos de nucleotídeo único

TKR - receptores tirosina-cinase

TSH - hormônio tireotrófico

VEGF - Vascular endothelial growth factor

SUMÁRIO

RESUMO

Nódulos tireoidianos são achados clínicos comuns. O câncer de tireoide, em contraste, é raro, embora consista na neoplasia endócrina maligna mais frequente. Um dos principais desafios no manejo de pacientes com neoplasias de tireoide é identificar, além dos parâmetros clínicos, características morfológicas e alterações moleculares capazes de diferenciar tumores que se comportarão de forma mais agressiva. A identificação de marcadores prognósticos confiáveis que determinem já ao diagnóstico, o comportamente biológico do tumor poderá evitar que pacientes de baixo risco sejam expostos a condutas agressivas desnecessárias, e distinguir estes pacientes daqueles que, por apresentarem evolução menos favorável, necessitem de cirurgias mais extensas e acompanhamento mais intenso.

Com os avanços no conhecimento da patogênese das neoplasias tireoidianas, inúmeros estudos vêm investigando marcadores preditivos e prognósticos em relação aos tumores da tireoide, possibilitando o desenvolvimento de novos agentes antineoplásicos. Dessa forma, a tendência na atualidade é de que o tratamento seja feito de forma mais individualizada, considerando o quadro clínico e marcadores, morfológicos, imunohistoquímicos e moleculares do paciente e do tumor.

A primeira parte desta tese compreende uma avaliação prospectiva do papel dos níveis de hormônio tireotrófico (TSH) como preditores de malignidade em nódulos da tireoide em uma amostra de 615 pacientes submetidos à punção aspirativa por agulha fina (PAAF) guiada por ultrassom. O TSH é um fator de crescimento essencial para as células da tireoide e sua via de sinalização é necessária para a expressão de outros fatores de crescimento, receptores e proto-oncogenes. Consequentemente, a supressão do TSH é importante para o manejo clínico do câncer de tireoide. De modo interessante, observamos que níveis séricos mais altos de TSH foram associados a um risco aumentado de malignidade. Pacientes com nódulos malignos apresentaram TSH sérico mais elevado que pacientes com nódulos benignos nos dois ensaios analisados (2.25 vs. 1.50; P = 0.04 e 2.33 vs. 1.27; P = 0.03) e o risco de malignidade foi aproximadamente três vezes maior em pacientes com níveis de TSH $\geq$ 2.26μU/mL do que em pacientes com níveis mais baixos de TSH. Nossos resultados corroboram estudos prévios que sugerem o uso do TSH como ferramenta diagnóstica auxiliar na estratificação do risco de malignidade associado a nódulos tireoidianos bem como na tomada de decisão da conduta terapêutica.

A segunda parte deste trabalho, por sua vez, avaliou em uma coorte de 420 pacientes com carcinoma medular de tireoide (CMT), o papel de polimorfismos de nucleotídeo único (SNPs) do gene VEGF-A na patogênese da doença através da correlação das frequências das variantes com dados clínicos, laboratoriais e prognóstico. Sabe-se que em diversos tumores, incluindo o CMT, há uma superexpressão do VEGF-A e seus receptores, o que possibilita utilizá-los como alvos moleculares para terapias com inibidores multiquinase (MKI) e também como marcadores prognósticos. Os SNPs do VEGF-A rs699947 (C> A), rs833061 (T <C) e rs2010963 (G> C) foram genotipados usando ensaio de TaqMan e os haplótipos foram inferidos usando o programa Phase. 202 (48%) pacientes apresentaram a forma hereditária e 218 (52%) apresentaram a forma esporádica do CMT. A idade média do diagnóstico foi de 40 ± 19.7 anos e 61% eram do sexo feminino. As freqüências alélicas dos SNPs do VEGF-A foram: rs699947 (37.2%), rs833061 (45.6%) e rs2010963 (34.4%). No CTM hereditário, observamos uma associação independente do VEGF-A rs833061 com menor idade ao diagnóstico (genótipo TT) e menor tamanho do tumor (genótipo CC). Além disso, o VEGF-A rs2010963 (genótipo GG) foi associado com menor tamanho tumoral. Para pacientes com CMT esporádico, não foram observadas associações independentes. Nossos resultados evidenciaram relação entre SNPs do gene VEGF-A e fatores prognósticos em CMT sugerindo que essas variantes podem impactar o comportamento tumoral e a apresentação da doença.

Em conjunto, nossos resultados indicam que o conhecimento mais aprofundado de marcadores clinicopatológicos ou moleculares específicos podem melhorar a determinação do prognóstico e comportamento tumoral, auxiliando no diagnóstico e no direcionamento para formas de tratamentos mais adequadas.

ABSTRACT

Thyroid nodules are common clinical findings. Thyroid cancer, in contrast, is rare and consists of the most frequent malignant endocrine neoplasia. One of the main challenges in the management of patients with thyroid neoplasms is to identify in addition to clinical parameters, morphological characteristics and molecular changes that are able to differentiate certain tumors that will behave more aggressively than others. The finding of accurate prognostic markers that determine the severity of the case at diagnosis may avoid low-risk patients from being exposed to unnecessary aggressive medical conduct and distinguish these patients from those who, due to their less favorable evolution, require more extensive surgery and follow-up more intense.

With advances in the knowledge of the pathogenesis of thyroid neoplasms, numerous studies have been investigating predictive and prognostic markers in relation to thyroid tumors, enabling the development of new antineoplastic agents. Thus, the current trend is that the treatment should be more individualized, considering the clinical condition and morphological, immunohistochemical and molecular markers of the patient and the tumor.

The first part of this research work comprises a prospective evaluation in a sample of 615 patients submitted to fine-needle aspiration biopsy (FNAB) guided by ultrasound, the usefulness of thyroid-stimulating hormone (TSH) levels as a predictor of malignancy in thyroid nodules. TSH is a major thyroid cell growth factor, while TSH signaling pathway activation may berequired for the expression of other growth factors, receptors, and proto-oncogenes. Accordingly, TSH suppression is an important therapeutic tool of clinical thyroid cancer management. Interestingly, we observed that higher serum TSH levels were associated with an increased risk of malignancy since patients with malignant nodules presented higher TSH levels than patients with benign nodules in two TSH assays (2.25 vs. 1.50; $P = 0.04$ and 2.33 vs. 1.27; $P = 0.03$) and the risk of malignancy was approximately 3-fold higher in patients with TSH levels ≥ 2.26 μU/mL than in patients with lower TSH levels. Our results corroborate previous studies that suggest the use of TSH as an auxiliary diagnostic tool in the stratification of the risk of malignancy associated with thyroid nodules as well as in the decision of therapeutic conduct.

The second part of this work, in turn, evaluated in a cohort of 420 patients with medullary thyroid carcinoma (MTC), the role of single nucleotide polymorphisms (SNPs) of the *VEGF-A* gene in the pathogenesis of the disease through the correlation of the frequencies with clinical, laboratory and prognostic data. It is known that in several tumors, including MTC, there is an overexpression of VEGF-A and its receptors, which makes it possible to use as molecular targets for therapies with multiquinase inhibitors (MKI) and also as prognostic markers. The VEGF-A SNPs rs699947(C>A), rs833061(T<C) and rs2010963(G>C) were genotyped using TaqMan Genotyping Assays. Haplotypes were inferred using Phase program. Of the 420 MTC patients analyzed, 202 (48%) presented hereditary and 218 (52%) had the sporadic form of disease. The mean age of diagnosis was 40 ± 19.7 years and 61% were female. The minor allele frequencies of VEGF-A SNPs were: rs699947 (37.2%), rs833061 (45.6%), and rs2010963 (34.4%). In hereditary MTC, we observed an independent association of the VEGF-A rs833061 with younger age at diagnosis (TT genotype) and smaller tumor size (CC genotype). Additionally, VEGF-A rs2010963 (GG genotype) was correlated with smaller tumor size.Our results showed a relationship between SNPs of the *VEGF-A* gene and prognostic factors in MTC, suggesting that these variants may impact tumor behavior and disease presentation.

These data suggest that further knowledge of clinicopathological or specific molecular markers that may improve prognostic determination and tumor behavior, may assist in the correct definition of the diagnosis as well as targeting more appropriate forms of treatment.

INTRODUÇÃO

Nódulos tireoidianos são achados clínicos comuns, sendo a prevalência de nódulos palpáveis de tireoide de, aproximadamente, 5% em mulheres e 1% em homens que vivem em áreas iodo-suficientes (1). A grande importância clínica dos nódulos está relacionada à necessidade de exclusão de malignidade (5-10% dos casos). Atualmente, a punção aspirativa por agulha fina (PAAF) guiada por ultrassom é a melhor ferramenta para avaliação e diagnóstico nos nódulos tireoidianos. Nos últimos anos, nódulos incidentais de tireoide vêm sendo diagnosticados com uma frequência crescente através da utilização de técnicas de imagem altamente sensíveis (2, 3). Mais recentemente, o Colégio Americano dos Radiologistas (ACR) também publicou uma classificação ultrassonográfica, conhecida como Thyroid Imaging, Reporting and Data System (TI-RADS). O risco de malignidade é similar em nódulos únicos e múltiplos, portanto, frente a uma tireoide multinodular, devem-se selecionar para punção, nódulos que apresentam características ultrassonográficas de maior risco de malignidade (4 ,5).

O câncer de tireoide é a neoplasia endócrina mais comum, representando aproximadamente 1-1.5% de todos os novos cânceres diagnosticados nos EUA e na Europa, com aumento continuado da incidência nas últimas décadas (6, 7). No Brasil, o Instituto Nacional do Câncer (INCA) estima para cada ano do biênio 2018/2019, 9.610 novos casos de câncer de tireoide (1.570 em homens e 8.040 em mulheres). Esses valores correspondem a um risco estimado de 1.49 casos a cada 100 mil homens e 7.57 casos a cada 100 mil mulheres, sendo o 5º tumor maligno mais frequente em mulheres (INCA; http://www.inca.gov.br).

As neoplasias da tireoide abrangem um grupo heterogêneo de tumores com comportamento clínico, características histológicas, fenotípicas e padrões de disseminação metastáticos distintos (8, 9). O carcinoma diferenciado de tireoide (CDT) é o tipo mais frequente de câncer de tireoide, correspondendo a 80-90% dos casos enquanto as formas mais raras são o carcinoma medular da tireoide (CMT) e o carcinoma anaplásico de tireoide (CAT) (10).

O CDT desenvolve-se a partir das células foliculares da glândula e compreende o carcinoma papilar de tireoide (CPT) e o carcinoma folicular de tireoide (CFT) (11, 12). Em sua maioria, o CDT mantém a capacidade de captar iodo e de responder a

estímulos fisiológicos como o hormônio tireotrófico (TSH). O CDT apresenta excelente prognóstico, mesmo nos casos de doença metastática. A taxa média de sobrevida em 10 anos para pacientes com CPT é de aproximadamente 95% nos casos de doença restrita à tireoide. Em pacientes com doença metastática, a sobrevida média é de 70% e 64% em 10 e 15 anos, respectivamente (13, 14).

O CMT tem origem nas células C tireoidianas e representa em torno de 3-4% dos cânceres da tireoide, podendo ocorrer de forma esporádica (75% dos casos) ou hereditária (25%), como parte da síndrome da neoplasia endócrina múltipla tipo 2 (NEM2). A NEM2 é causada por mutações de linhagem germinativa no proto-oncogene *RET*, sendo o padrão de herança autossômico dominante, sendo subdividida em NEM2A e NEM2B. A NEM2A é caracterizada pela presença de CMT (95%), hiperparatireoidismo (10-20%) e feocromocitoma (30-50%) enquanto indivíduos com NEM2B apresentam CMT (>90%) e feocromocitoma (45%), acompanhados por ganglioneuromas (100%) e habitus marfanoide (65%) (15).

O curso clínico do CMT pode variar de um tumor indolente que permanece inalterado por anos para uma forma agressiva associada a uma alta taxa de mortalidade. A doença metastática progressiva constitui o principal desafio no manejo de pacientes com CMT uma vez que as opções terapêuticas disponíveis apresentam resultados pouco expressivos. A terapia inicial para o CMT é a tireoidectomia com ou sem dissecção de linfonodos por compartimentos. Já na doença metastática avançada, os objetivos da cirurgia são paliativos com vistas a minimizar complicações e comorbidades levando-se em consideração a expectativa e a qualidade de vida do paciente. Os tratamentos para o CMT metastático avançado consistem na possível ressecção do tumor, na radioterapia por feixe externo (EBRT) ou na administração de terapia sistêmica (16). De modo geral, o prognóstico do CMT é considerado menos favorável do que o prognóstico de tumores derivados de células foliculares diferenciados. De fato, apesar de representar de 3 a 4% dos tumores tireoidianos, o CMT é responsável por uma taxa de mortalidade de 13% (15-18).

O CAT ou carcinoma indiferenciado é uma forma extremamente rara e agressiva, representando entre 1% e 2% dos tumores tireoidianos. O CAT pode se originar de novo ou ser resultado da progressão e/ou desdiferenciação dos carcinomas diferenciados. Aproximadamente 50% dos pacientes com CAT tiveram carcinoma diferenciado prévio ou coexistente. Em contraste com as células do CDT, as células no

CAT não preservam as características e funções biológicas das células foliculares normais da glândula como captação de iodo, síntese de tireoglobulina e dependência de TSH. O curso clínico do CAT é caracterizado por doença local agressiva, altas taxas de metástases e rápido desfecho fatal (20-50% de todas as mortes por câncer de tireoide). O tempo médio de sobrevida é de aproximadamente 5 a 6 meses e apenas 10 a 15% dos pacientes sobrevivem 2 anos após a apresentação da doença (19-21).

Marcadores Clínico-Patológicos e Moleculares de Prognóstico

A presença de nódulos na tireoide é bastante comum. A etiologia da doença nodular da tireoide é multifatorial, compreendendo um espectro que vai do pequeno nódulo achado de forma incidental a um grande bócio multinodular intra-torácico. Fatores de risco para malignidade vêm sendo estabelecidos para direcionar o diagnóstico, prognóstico e manejo do paciente com nódulo tireoidiano (22).

Marcadores de risco de malignidade e prognósticos mais ou menos favoráveis incluem idade, sexo, história familiar de câncer de tireoide, exposição à radiação ionizante, nível de TSH sérico, entre outros (23). Características ultrassonográficas como nódulos sólidos, hipoecogênicos, com microcalcificações, mal delimitados e com aumento de fluxo nodular central ao Doppler podem ser consideradas suspeitas para malignidade, apesar de não ser possível diferenciar lesões benignas de malignas somente pelo exame ultrassonográfico (3, 24, 25).

A prevalência de nódulos tireoidianos aumenta com o avanço da idade. Em indivíduos mais velhos o achado de um fenótipo histológico de maior risco é mais provável, como variantes mais agressivas de CPT, carcinomas pouco diferenciados ou CAT (26). Outro parâmetro clínico objeto de estudos relacionado com risco de malignidade é o TSH sérico. O TSH é um fator de crescimento essencial das células da tireoide e sua via de sinalização é necessária para a expressão de outros fatores de crescimento, receptores e proto-oncogenes. Estudos prévios sugerem o uso do TSH como ferramenta diagnóstica auxiliar na estratificação do risco de malignidade associado a nódulos tireoidianos e também no direcionamento da conduta terapêutica (27, 28).

Dessa forma, o reconhecimento de marcadores prognósticos é essencial não apenas para a exclusão da malignidade em nódulos mas, principalmente, para

complementar o diagnóstico nos casos de câncer de tireoide. Os tumores tireoidianos apesar de apresentarem histologia e características clínicas bastante diversas, são indolentes na maioria dos casos, com resultados satisfatórios através da terapia convencional disponível. No entanto, alguns tumores podem ocorrer sob formas agressivas associadas a altas taxas de mortalidade e prognósticos desfavoráveis uma vez que as opções de tratamento para estes casos são restritas tornando a eficácia terapêutica limitada e incerta (29).

Apesar das controvérsias e mudanças nos últimos anos acerca da classificação morfológica, é irrefutável que o câncer de tireoide manifesta um espectro de morfologias que se correlacionam com o comportamento clínico (23, 30). Informações sobre o significado dos vários componentes histológicos e suas características incluindo arquitetura, padrão de crescimento, citologia e padrões de invasão têm impactado a determinação do diagnóstico e manejo dos pacientes. Além disso, marcadores expressos pelo tumor e identificados por imunohistoquímica podem colaborar em conjunto com outras características morfológicas na determinação do prognóstico (31).

A abordagem terapêutica atual inclui vigilância ativa, ressecção cirúrgica que pode ser parcial ou total, com ou sem dissecção de linfonodos, radioterapia, radioiodo ou ainda, terapias moleculares alvo-específicas. As novas estratificações de risco apontam para a necessidade da análise do perfil prognóstico do paciente, características do tumor e uso racional dos recursos considerando os riscos de subtratamento ou tratamento exagerado (30, 32).

Atualmente, a identificação de biomarcadores que tenham a capacidade de fornecer características específicas do tipo tumoral e da sua progressão, além de informações sobre o comportamento biológico do tumor, como agressividade e capacidade de disseminação, constitui um desafio importante no tratamento das neoplasias tireoidianas (30, 33).

Neste contexto, a angiogênese tem sido relacionada à taxa de crescimento tumoral e prognóstico através da expressão de marcadores associados ao comportamento do tumor. Esse mecanismo complexo fornece nutrientes essenciais para o desenvolvimento tumoral como oxigênio, enzimas proteolíticas, hormônios e fatores de crescimento e tem como um de seus principais reguladores o fator de crescimento endotelial vascular (VEGF) (34).

Estudo realizado pelo nosso grupo demonstrou que o VEGF e seus receptores estão superexpressos e associados a prognóstico menos favorável em CMT, sugerindo

que essas moléculas podem ser consideradas biomarcadores e também possíveis alvos terapêuticos para tumores não ressecáveis ou metastáticos (35). Em relação ao *VEGF-A*, um estudo multicêntrico observou uma correlação entre alguns polimorfismos de nucleotídeo único (SNPs) comuns ao gene e risco de recorrência estrutural doença ou sobrevida livre de doença em pacientes com câncer de tireoide diferenciado não-avançado (papilar e folicular) (36).

Nas últimas décadas, observou-se um progresso considerável na compreensão dos mecanismos moleculares envolvidos na patogênese do câncer de tireoide (10). As anormalidades genéticas mais comuns no CPT são a ativação aberrante da via de sinalização celular mitogen-activated protein kinase (MAPK), mutações pontuais no gene *BRAF* ou *RAS* e rearranjos RET/PTC. O CFT está frequentemente associado à ativação das vias fosfatidilinositol-3-cinase (PI3K) e MAPK, mutações de *NRAS*, rearranjos como PAX8/PPARγ, e outros eventos (37-39).

No CMT, mutações na linhagem germinativa no proto-oncogene *RET* (REarranged during Transfection) são responsáveis pela forma hereditária da doença e mutações somáticas no *RET* são observadas em casos esporádicos (15, 16). A presença de mutação somática do códon M918T do *RET*, os níveis séricos de calcitonina e antígeno carcinoembrionário (CEA) e os respectivos tempos de duplicação e o número de linfonodos e compartimentos acometidos são os principais indicadores prognósticos nesses pacientes (1, 15, 16).

O CAT compreende tumores mais complexos geneticamente com múltiplas alterações em genes que codificam receptores tirosina-cinase (TKR) e vias como PI3K/AKT/mTor (19).

Dessa forma, mutações, rearranjos genéticos, alterações nas vias de sinalização e outros potenciais fatores moleculares como os microRNAs (miRNA) e os SNPs estão sendo estudados como marcadores prognósticos e podem proporcionar terapias direcionadas visto que estão associados com progressão, metástases e risco de recorrência da doença (37).

A avaliação de possíveis indicadores prognósticos é fundamental para que terapias mais adequadas sejam oferecidas para cada caso visto que esse conjunto de fatores é individual e determinante para a maior ou menor eficácia do tratamento, além de identificar pacientes com maiores riscos de recorrência e morbidades associadas. Em vista do exposto, o objetivo deste estudo foi avaliar o impacto no curso clínico das neoplasias de tireoide de alguns fatores de risco e marcadores prognósticos já bem

estabelecidos como idade, sexo, características ultrassonográficas, entre outros, assim como marcadores ainda controversos na literatura como o TSH e os polimorfismos no gene *VEGF*, envolvidos no processo de angiogênese.

Parte 1

Serum TSH levels as a predictor of malignancy in thyroid nodules: A prospective study.

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PLOS ONE

RESEARCH ARTICLE

Serum TSH levels as a predictor of malignancy in thyroid nodules: A prospective study

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Abstract

Background

The role of serum TSH concentrations as a predictor of malignancy of thyroid nodule remains unclear.

Objective

To prospectively evaluate the usefulness of serum TSH levels as a predictor of malignancy in thyroid nodules.

Methods

Patients with thyroid nodule(s) who underwent fine-needle aspiration biopsy under ultrasonographic guidance in a tertiary, university-based hospital were consecutively evaluated. Patients with known thyroid cancer and/or patients receiving thyroid medication were excluded. Serum TSH levels were measured by two differents methodologies, chemiluminescent (CLIA) and electrochemiluminscent immunoassay (ECLIA). Anatomopathological exam of tissue samples obtained at thyroidectomy was considered the gold standard for the diagnosis of thyroid cancer.

Results

A total of 615 patients participated in the study. The mean age was 55.9±14.7 years, and 544 (88.5%) were female. The median TSH values were 1.48 and 1.55 μU/mL, using CLIA and ECLIA, respectively. One-hundred-sixty patients underwent thyroidectomy and the final diagnoses were malignant in 47(29.4%) patients. TSH levels were higher in patients with malignant than in those with benign nodules in both TSH assays: 2.25 vs. 1.50; P = 0.04 (CLIA) and 2.33 vs. 1.27; P = 0.03 (ECLIA). Further analysis using binary logistic regression identified elevated TSH levels, a family history of thyroid cancer, the presence of microcalcifications, and

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solitary nodule on US as independent risk factors for malignancy in patients with thyroid nodules. Additional analyses using TSH levels as a categorical variable, defined by ROC curve analysis, showed that the risk of malignancy was approximately 3-fold higher in patients with TSH levels ≥2.26 μU/mL than in patients with lower TSH levels (P = 0.00).

Conclusions

Higher serum TSH levels are associated with an increased risk of thyroid cancer in patients with thyroid nodules. Using TSH levels as an adjunctive diagnostic test for stratifying the risk of malignancy associated with a thyroid nodule may help on defining the best therapeutic approaches.

Introduction

Palpable thyroid nodules are a common disorder detected in 4–7% of adults in the general population and in 19–67% of patients who undergo high-resolution ultrasound [1–5]. In contrast, thyroid cancer is rare. However, in most cases, thyroid carcinoma presents clinically as a nodule (solitary or in a multinodular gland) that is indistinguishable from benign neoplasia. The challenge for clinicians, therefore, is to distinguish malignant (5–10%) from benign thyroid nodules[4,5]. Of note, the last National Cancer Institute State Cancer Profile has shown that the incidence of thyroid cancer is rising faster than that of any other malignant neoplasia [6].

Fine-needle aspiration biopsy (FNAB) is the gold standard for evaluating patients with thyroid nodules, and it is currently the most reliable diagnostic technique for evaluating thyroid nodules under ultrasound guidance[5,7]. Diagnostic results are obtained in most cases, as inadequate specimens (nondiagnostic or unsatisfactory) occur in only 5–10% of cases. However, FNAB cannot reliably rule out cancer in 20–30% of nodules, reported as indeterminate for malignancy [8–10] The Bethesda classification [11] recognizes three specific cytological diagnoses characterized by indeterminate cytology. The predicted probability of cancer is 5–10% in Bethesda III patients, 20–30% in Bethesda IV patients, and 50–75% in Bethesda V patients, but variability has been noted at different centers [8,10] and most patients with indeterminate cytology undergo surgery to establish the histopathologic diagnosis. However, only 10–20% of the thyroid nodules with indeterminate cytology (Bethesda III and IV) are malignant[8,12–14]. New diagnostic approaches based on thyroid cancer molecular biomarkers have recently been studied, and some are already introduced in clinical settings. Currently, the most successful panels testing for mutations in thyroid FNAB samples are those testing for BRAF and RAS point mutations and RET/PTC and PAX8/PPARγ rearrangements, as well as TRK rearrangements [15, 16]. Although the use of these molecular tools have been validated in some studies, these tests are expensive (and not cost-effectivity depending of thyroid cancer prevalence), and their impact on patient management remains debatable[17–20].

The role of TSH as a predictor of thyroid nodule malignancy has been evaluated by several studies in the last years. Since Boelaert has reported parallel increases in malignancy risk and serum TSH levels[21], several other authors have investigated the relationship between serum TSH levels and thyroid cancer with conflicting results[22–24]. A recent meta-analysis found a positive association between higher serum TSH levels and increased risk of thyroid cancer[25]. However, the analysis had some limitations, as all of its included studies were cross-sectional, and the vast majority was retrospective (only two prospective studies). In contrast, the EPIC

study, which is based on a huge European cohort, demonstrated a negative association between TSH levels and thyroid cancer risk[26]. Although the EPIC study is a large prospective study, it is a case-control study featuring control subjects who were chosen from a group of cancer-free participants constituting the EPIC cohort (healthy population), thus, which may limit its conclusions.

Here, we aimed to evaluate the role of serum TSH levels as predictor of thyroid nodule malignancy in a cohort of patients with thyroid nodules in a tertiary, university-based hospital.

Materials and methods

Consecutive patients with thyroid nodule(s) who underwent FNAB under ultrasonographic guidance (US-FNAB) between 2012 and March of 2016 in the Thyroid Unit of the Hospital de Clínicas de Porto Alegre, a tertiary, university-based hospital, were prospectively evaluated. The geographical area of the study is iodine sufficient[27]. All patients were referred for evaluation of thyroid nodules and underwent a complete history and physical examination. Patients with known thyroid cancer and/or patients receiving thyroid-related medication were excluded. TSH levels, ultrasound characteristics of the nodules, demographic and clinical data were compared in patients with benign and malignant thyroid nodule. The project was approved by the Research Ethics Committee of the Hospital de Clínicas de Porto Alegre (GPPG 140538). The study was conducted in accordance with the ethical standards of the local institutional, national research committee and with the Helsinki declaration. The local institutional and national research committee did not request informed consent, as the data were analyzed anonymously and the assistance of patients follow routine clinical indications.

Laboratory evaluation

Serum TSH levels were measured by two different methodologies, chemiluminescent immunoassay (CLIA) (ADVIA Centaur XP; Siemens, Tarrytown, NY), with interassay coefficient of variation of 5.3% over the range 0.35–5.50 μU/mL, were used until November 2014. After that, TSH levels were measured by electrochemiluminscent immunoassay (ECLIA) (Roche Diagnóstica, São Paulo, Brasil), with interassay coefficient of variation of 3.11%, reference values: 0.27–4.20 uUI/mL. The thyroid peroxidase antibodies (TPO-Ab) was evaluated by chemiluminescent immunoassay (IMMULITE Systems, Siemens Healthcare Diagnostics, Tarrytown, NY), reference value inferior of 34.0UI/mL.

Ultrasound examination

All patients underwent thyroid US-FNAB, which was performed by the same radiologist using a high-resolution ALOKA ultrasound device with a 7.5 MHz linear transducer (Tokyo, Japan). The number of thyroid nodules, as well as sizes, US characteristics (echogenicity, the presence of microcalcification, halos, contours, and shape), and the presence of cervical lymphadenopathy were documented in the medical records of all patients.

FNAB, cytological and histological diagnosis

FNA and cyto-cell block testing. US-FNA was performed with a disposable needle (21G) connected to a 10 ml disposable syringe[14]. Multidirectional aspiration was performed in dominant nodules in patients with multinodular goiters and/or suspicious nodules on ultrasonography[5,7]. Rapid on-site evaluations of all fine-needle aspiration specimens were performed to determine the adequacy of each specimen. A thyroid FNA specimen was considered satisfactory if at least 6 groups of follicular cells were present, and each group comprised

at least 10 cells. Immediate on-site reaspiration was performed in cases in which specimens considered inadequate for diagnosis were obtained. Six cytology slides were prepared for each patient, four of which were air-dried and immediately stained via the May Grümwald Giemsa technique. The other two slides were immediately fixed in ethanol 96° and subsequently stained via the Papanicolaou technique. The residual hemorrhagic aspirated in the syringe and needle was fixed in 10% formalin. Then, the aspirated material was centrifuged, paraffin-embedded (cell block), sectioned and stained with hematoxylin and eosin to serve as a complementary diagnostic specimen to the FNA specimen. A sample was considered viable if it contained a sufficient number of cells with intact morphology (60 cells from at least 6 groups of 10 follicular cells).

Two independent pathologists reviewed the cytological and histological slides of each case together and assigned the samples to a final diagnostic category. Cytological aspirate adequacy was defined according to the recommendations of the *Papanicolaou Society of Cytopathology Task Force on Standards of Practice* (1996), and the cytological results were classified into the following 6 diagnostic categories according to the criteria of the Bethesda System for Cytological Classification of Thyroid Nodules: 1) non-diagnostic or unsatisfactory, 2) benign, 3) atypia of undetermined significance, 4) a follicular neoplasm or suspicious for a follicular neoplasm, 5) suspicious for malignancy and 6) malignant.

Cell-block slides were reviewed for the presence of cellular elements and classified into the following two categories: 1) diagnostic and 2) non-diagnostic. Cell-block US-FNBA specimens were used as adjunctive diagnostic specimens, and the results were described as cyto-cell blocks[14].

Anatomopathological examinations of tissue samples obtained at thyroidectomy were carried out according to the World Health Organization Guidelines, and the pathology reports pertaining to these samples were considered the gold standard for the diagnosis of thyroid cancer. The pathology reports provided information regarding AJCC/UICC TNM staging and the presence of invasion. The risk of recurrence was evaluated according to the ATA thyroid cancer guidelines[5,7].

Statistical analysis

The clinical, laboratory, ultrasonography and cytological data, which are reported as the mean–standard deviation (SD) values, or as the median with percentiles 25 and 75 (continuous variables), or as absolute numbers and percentages (categorical variables), were compared using an unpaired Student's t-test, Mann–Whitney U-test, or chi-square test, as appropriate. The influence of clinical factors on the risk of thyroid cancer was investigated using binary logistic regression analysis. The differences in the cancer rates between groups with determined TSH levels, considering ROC curve cutoff value, were calculated using odds ratios and 95 percent confidence intervals. Statistical analysis of the results was performed with SPSS software (Statistical Package for Social Sciences) version 18.0.

Results

Patient characteristics

A total of 886 patients with thyroid nodules were consecutively evaluated. Of them, 234 were excluded due to thyroid hormone replacement therapy and/or a previous diagnosis of thyroid cancer, and 37 patients because they did not have available TSH measurement. Thus, 615 patients participated in the study. The mean of age of the participants was 55.9 ±14.7 years, and 544 (88.5%) were female. Solitary nodules were noted in 226 patients (36.7%). As mentioned in the Materials and Methods section, two different assays were used to measure the

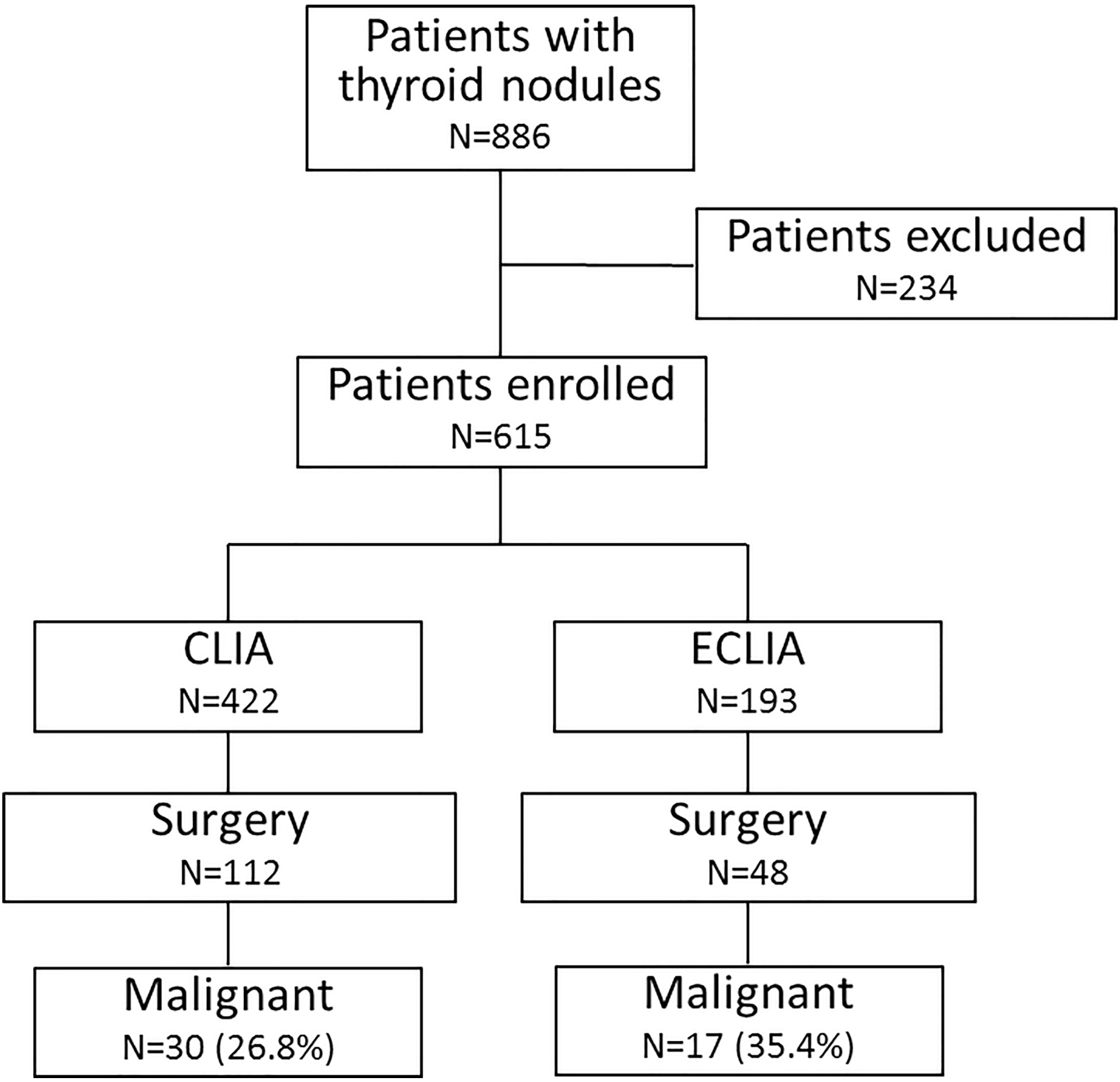

Fig 1. Flow chart of patients who met inclusion/exclusion criteria for the study population. CLIA, Chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay.

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TSH levels during the study period. Since the reference ranges were slightly different between these assays, we have analyzed the patients separately (Fig 1). The median TSH in 422 patients evaluated by CLIA was 1.48 μU/mL (P25: 0.93 and P75: 2.31) while the median TSH was 1.55 μU/mL in 193 patients analyzed by ECLIA, (P25: 0.95 and P75: 2.80). There was no

Table 1. Clinical and oncology features of patients included in the study.

	All Samples	CLIA	ECLIA	P value
	(N = 615)	(N = 422)	(N = 193)	
Age (yr.)	55.9 ± 14.7	56.4 ± 14.0	54.9 ± 16.0	0.25
Female gender (%)	544 (88.5)	378 (89.6)	166 (86)	0.20
Family history of thyroid cancer (%)	37/602 (6.1)	29/409 (7.1)	8/193 (4.1)	0.16
Personal history of other neoplasia (%)	68/604 (11.3)	48/411 (11.7)	20/193 (10.4)	0.63
Exposure to radiation (%)	28/601 (4.7)	23/408 (5.6)	5/188 (2.7)	0.98
Serum TSH (μU/mL)		1.48 (0.93–2.31)	1.55 (0.95–2.80)	0.32
Solitary nodule (%)	226 (36.7)	155 (36.7)	71 (36.8)	0.99
Microcalcifications (%)	106/483 (21.9)	80/292 (19)	26(13.5)	0.00
FNA cyto-cell block (%)				0.00
I—Nondiagnostic	35 (5.7)	20 (4.7)	15 (7.8)	
II—Benign	434 (70.6)	315 (74.6)	119 (61.7)	
III—Indeterminate	56 (9.1)	28 (6.6)	28 (14.5)	
IV—Follicular lesion	50 (8.1)	30 (7.1)	20 (10.4)	
V/VI—Malignant	40 (6.5)	29 (6.9)	11 (5.7)	

Values are the mean ± SD or median (P25-P75). CLIA, chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay. The TSH reference range is 0.35–5.50μU/mL by CLIA and 0.27–4.20μUI/mL by ECLIA. Solitary nodules and microcalcifications were evaluated by ultrasonography.

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significant differences in median TSH values (P = 0.32). Indeed, the correlation between the two different TSH assays showed a strong correlation (r = 0.99). Forty-three out of 261 (16.5%) patients analyzed presented positivity to TPO-Ab.

All patients underwent US-FNBA and had their sample analyzed by cytologic and cell block diagnostic testing. The combined cytological and cell block (cyto-cell) interpretations were as follows: 5.7% (n = 35) unsatisfactory, 70.6% (n = 434) benign, 9.1% (n = 56) indeterminate, 8.1% (n = 50) follicular lesion and 6.5% (n = 40) malignant (Table 1). Surgery was indicated for 170 patients with the following cyto-cell results: 7 nondiagnostic cyto-cell blocks, 30 indeterminate lesions, 43 follicular lesions, 36 malignant lesions and 54 benign lesions, based on clinical (compressive symptoms, recent growing) and ultrasonographic characteristics (large nodules, microcalcification, irregular margin). Final histological data were available for 160 patients, 113 of whom had benign lesions (70.6%), and 47 of whom had malignant lesions (29.3.% of surgical cases and 7.6% of the sample). Of the subgroup evaluated by CLIA, 112 patients underwent surgery and 30 (26.8%) had malignant nodules while 48 out of 193 patients analyzed by ECLIA were thyroidectomized and 17 (35.4%) had thyroid cancer. The characteristics of all enrolled patients are shown in Table 1. The distribution of age, gender, and other clinical features were similar between both TSH assay groups. However, the presence of microcalcification were higher in CLIA group (19 vs. 13.5%; P = 0.00) and the distribution of cytological evaluation showed a higher prevalence of indeterminate category in ECLIA group (6.6 vs. 14.5%; $P<0.001$).

TSH and thyroid cancer

In the group evaluated by CLIA, 91.7% of patients showed serum TSH levels within the normal range; 23 patients (5.5%) presented TSH levels less than 0.35 μU/mL, and 12 (2.8%) had TSH levels greater than 5.5 μU/mL. In patients analyzed by ECLIA, TSH was within the normal range in 87%; 4 (2.1%) patients showed TSH levels less than 0.27 μU/mL and 21 patients (10.9%) have TSH levels greater than 4.20 μU/mL.

Noteworthy, patients with thyroid cancer exhibited higher median TSH levels than patients with benign nodules irrespectively of the assay methodology: 2.25 vs. 1.50 (CLIA;P = 0.04) and 2.33 vs. 1.27 (ECLIA; P = 0.03) (**Fig 2**;Table 2). Moreover, a family history of thyroid cancer, solitary nodules and the presence of microcalcifications were associated with malignancy (Table 2). Further analysis using binary logistic regression identified elevated TSH levels, a family history of thyroid cancer, the presence of microcalcifications and solitary nodule on ultrasonography as independent risk factors for malignancy in patients with thyroid nodules. There were no differences in age, gender, non-thyroid neoplasia history, presence of TPO-Ab positivity or previous radiation exposure history between patients with benign nodules and those with thyroid cancer.

To further explore the potential role of TSH levels as a predictor of thyroid cancer, we subdivided the sample into quartiles in accordance with the TSH level distribution of the study (≤0.93, 0.94–1.48, 1.49–2.31 and ≥2.32 μU/mL for CLIA and ≤0.95, 0.96–1.55, 1.56–2.80 and ≥2.81 μU/mL ECLIA). Interestingly, we observed that the frequencies of malignancy in each TSH quartile varies in accordance with TSH levels, for both assays (P = 0.02 and P = 0.04; respectively, Fig 3). For CLIA group, the prevalence of malignancy in patients with TSH levels in the first quartile (≤0.93 μU/mL) was 14.3%, while those in the upper quartile (≥2.32 μU/mL) exhibited a cancer prevalence of 48.3% (OR 5.6; CI 1.35–23.2)(P = 0,01). The prevalence of malignancy in patients evaluated by ECLIA with TSH levels in the first quartile (≤0.95 μU/mL) was 23%, while those in the upper quartile (≥2.81 μU/mL) exhibited a cancer prevalence of 64% (OR 5.83; CI 1.00–34.6)(P = 0,04). Of note, even within normal range of TSH, there was a significantly increased risk of thyroid cancer as TSH increased.

We subsequently performed ROC curve analysis to identify the best TSH cutoff for predicting malignancy. Remarkable, the TSH value of 2.26μU/mL was identified to both assays. Then, we perform additional analyses using TSH levels as a categorical variable, the prevalence of malignancy was higher in those patients with TSH levels >2.26 μU/mL than in patients with lower TSH levels (<2.26 μU/mL) (OR 3.87; CI 1.87–8.01)(P = 0.00). Similar results were

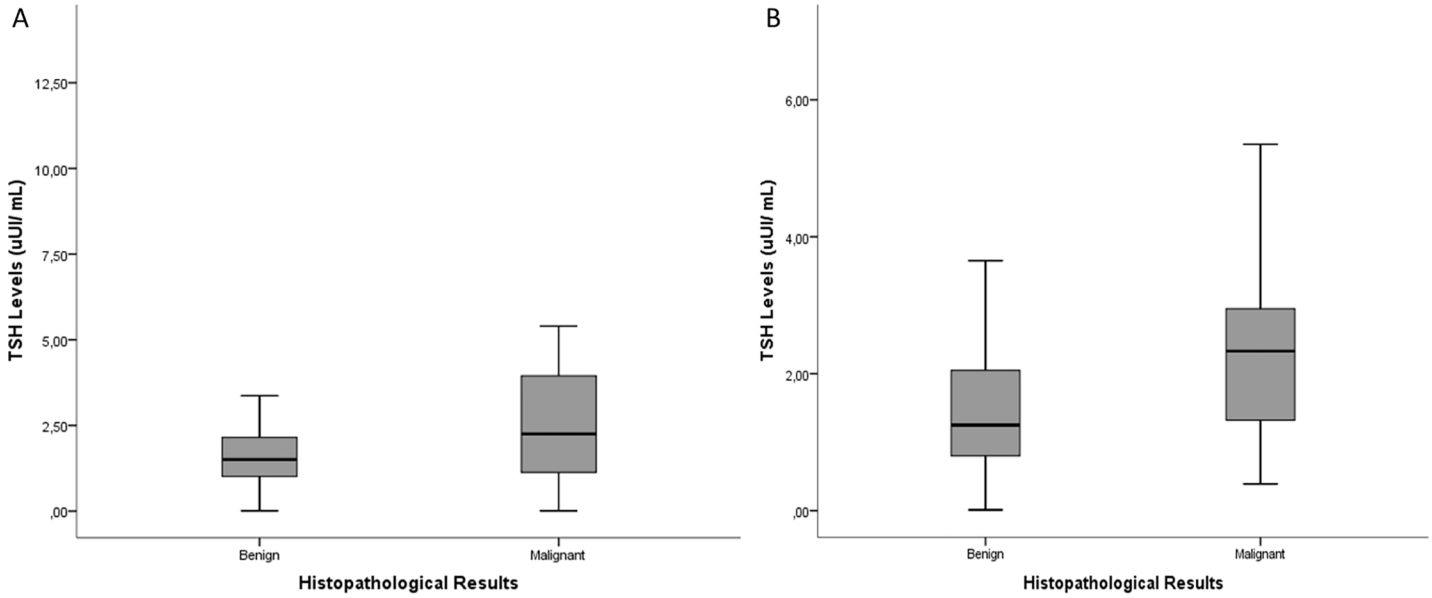

Fig 2. TSH level in malignant and benign thyroid nodules. CLIA, Chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay. Box plot illustrating the median TSH level and the TSH level quartiles and ranges in malignant and benign thyroid nodules for CLIA(A) and ECLIA (B). Patients with thyroid cancer exhibited higher median TSH levels than patients with benign nodules for both methodology. P = 0.04 (A) and P = 0.03 (B).

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Table 2. Characteristics of patients with benign and malignant nodules.

	Malignant nodule	Benign nodule	P value
	(N = 47)	(N = 113)	
Age (yr.)	47.9 ± 13.6	50.9 ± 14.8	0.24
Male gender (%)	10 (21.3)	12 (10.6)	0.07
Family history of thyroid cancer (%)	7 (14.9)	5 (4.4)	0.03*
Personal history of other neoplasia (%)	4 (8.5)	8 (7.1)	0.79
Exposure to radiation (%)	2 (4.2)	4 (3.5)	0.84
Serum TSH (μU/mL)			
CLIA	2.25 (1.12–4.07)	1.50 (0.99–2.17)	0.04
ECLIA	2.33 (1.30–3.06)	1.25 (0.79–2.10)	0.03
Microcalcifications (%)	21 (44.7)	16 (14.2)	0.00*
Solitary nodule (%)	26 (55.3)	42 (37.2)	0.03*
TPO-Ab positively (%)	4/22 (18.2)	6/55 (10.9)	0.39

Values are the mean ± sd or median (P25-P75). CLIA, chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay.The TSH reference range is 0.35–5.50μU/mL by chemiluminescent and 0.27–4.20μU/mL by electrochemiluminscent. Solitary nodules and microcalcifications were evaluated by ultrasonography (during US-FNA).

*Independent variable by binary logistic regression.

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obtained when only patients with normal TSH levels were analyzed (OR 3.42 CI 1.62–7.24) (P = 0.00).

Among the 47 patients with thyroid cancer, three cases harbor medullary thyroid carcinoma and 44 differentiated thyroid carcinoma (DTC). Among patients with DTC, 11 cases were microcarcinoma (25.0%). There were no differences in prevalence of TSH >2.26 μU/mL or distribution of TSH quartile between patients with or without microcarcinoma.

Discussion

In the present prospective study, we demonstrated that patients with malignant thyroid nodules presented higher serum TSH levels than patients with benign nodules.

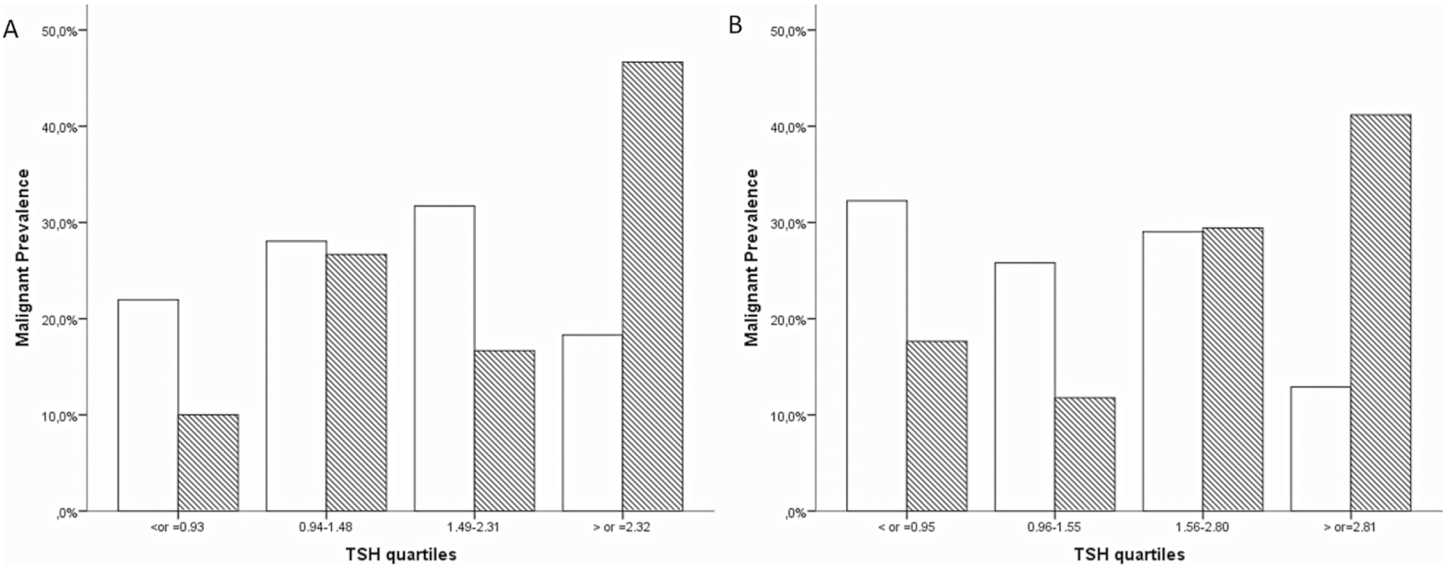

Fig 3. Frequencies of malignant and benign nodules according to TSH quartile. CLIA, Chemiluminescent immunoassay; ECLIA, electrochemiluminscent immunoassay. Frequencies of malignant (dashed bars) and benign (white bars) nodules according to TSH quartile for CLIA(A) and ECLIA (B). A significant increase in the prevalence of malignancy was noted in higher TSH quartiles. P<0.01.

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Accordingly, the prevalence of malignancy was higher in subjects with TSH levels >2.26 μU/mL, as defined via ROC curve analyses. Remarkably, additional analyses using TSH levels as a categorical variable show that the risk of malignancy was higher in patients with TSH levels in upper quartile as compared with patients with lower TSH levels in two different methodologies.

TSH is a major thyroid cell growth factor, while TSH signaling pathway activation may be required for the expression of other growth factors, receptors, and proto-oncogenes[28–30]. Accordingly, TSH suppression is an important therapeutic tool of clinical thyroid cancer management[5,7]. In the last years, several studies have addressed the role of TSH as a predictor of thyroid nodule malignancy but the results are still open to discussion. Here, we have demonstrated that patients with higher TSH levels have increased risk for malignancy. Remarkable, as TSH quartiles increased, the likelihood of malignancy rose, and the odds ratio of thyroid cancer in patients with TSH levels in the upper quartile was 5-fold higher than in those patients with TSH levels in the first quartile (Fig 3). Similar results were observed using the ROC analyses to define the TSH levels. Our observations are in agreement with previous studies [21–25] although contrast with the data from the EPIC study[26]. Noteworthy, however, the EPIC was a case-control study that includes only cancer-free subjects which might be a limiting when compared with our study.

Despite the consistent association between higher TSH levels and malignant nodules shown in most series, including this one, an optimal TSH cutoff value for predicting the risk of cancer has not been yet identified. Indeed, the lack of previous studies validating nomograms or equations intended to determine an optimal TSH cutoff value has limited the use of serum TSH levels as a malignancy predictor. Here, we found the TSH cutoff value (≥2.26 μU/mL) using the best point of a ROC curve for the two different TSH assays used during the study period. Some authors have suggested that while no consensus exists regarding a TSH cutoff value, it may be practical to preferentially perform US-FNAB in nodules less than 1.5 or 2.0 cm exhibiting patterns arousing low or very low suspicion for malignancy on ultrasonography[2,25]. Haymart et al. suggested TSH may play a key role in optimizing surgical interventions when aspirates are suspicious for malignancy[23]. These recommendations might support an adjunctive role for TSH levels when evaluating thyroid nodules.

This study has several strengths, as it included a large number of patients with thyroid nodules who were evaluated at a single institution and did not exclude patients with abnormal TSH levels, which enhances the external validity of its findings and increases the clinical applicability of its data. Also, two TSH methodologies were analyzed with consistent results. Thus, the results presented here may have important clinical implications, since its indicate that TSH levels may help on the diagnosis strategy, in conjunction with clinical, ultrasonographic and cytological features. However, as noted above, TSH should not be used for diagnostic decision-making in isolation. Also, although we demonstrated a prospective study data, the design of our study was not delineated for evaluate the TSH as a causative role in thyroid cancer pathogenesis. In this view, we do not recommend screening for thyroid cancer in patients with chronic TSH elevations nor suppressive treatment for subclinical hypothyroidism and for benign nodular disease.

Conclusions

Higher serum TSH levels are associated with an increased risk of thyroid cancer in patients with thyroid nodules. The use of TSH as an adjunctive diagnostic test for stratifying the risk of malignancy associated with thyroid nodules may have value to decision-making on diagnostic approaches.

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Funding acquisition: Ana Luiza Maia.

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Validation: Lenara Golbert, Ana Patrícia de Cristo.

Visualization: Lenara Golbert.

Writing – original draft: Lenara Golbert.

Writing – review & editing: Lenara Golbert, Marcia Silveira Graudenz, Ana Luiza Maia.

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Conclusão

No presente estudo demonstramos que níveis séricos mais altos de TSH estão associados a um risco aumentado de câncer de tireoide em pacientes com nódulos tireoidianos. Além disso, nossos resultados evidenciaram associações entre polimorfismos do gene *VEGF-A* e fatores prognósticos em CMT, como idade ao diagnóstico, tamanho do tumor, entre outros, sugerindo que essas variantes podem desempenhar um papel importante na patogênese da doença.

Esses dados sugerem que a identificação de marcadores clínicos, morfológicos ou moleculares que tenham a capacidade de predizer malignidade em nódulos ou informar sobre o tipo de tumor que terá um comportamento mais agressivo é de muita relevância para a prática clínica. Dessa forma, esses resultados colaboram com a ampliação do conhecimento acerca do prognóstico e comportamento da doença tireoidiana.

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