Measurement of Calcitonin and Calcitonin Gene–Related Peptide mRNA Refines the Management of Patients with Medullary Thyroid Cancer and May Replace Calcitonin-Stimulation Tests

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Background: Serum calcitonin (sCT) is the main tumor marker for medullary thyroid cancer (MTC), but it has certain limitations. Various sCT assays may have important intra-assay or interassay variation and may yield different and sometimes conflicting results. A pentagastrin- or calcium-stimulation calcitonin (CT) test may be desirable in some situations. Alternatively, or in the absence of the stimulation test, mRNA detection offers the advantages of being more comfortable and less invasive; it only requires blood collection and has no side effects. The objective of this study was to investigate the applicability of measuring calcitonin-related polypeptide alpha (CALCA) gene transcripts (CT-CALCA and calcitonin gene–related peptide [CGRP]-CALCA) in patients with MTC and in relatives diagnosed with a *RET* mutation and to test mRNA as an alternative diagnostic tool for the calcitonin-stimulation test.

Methods: Twenty-three healthy controls and 26 individuals evaluated for MTC were selected, including patients with sporadic or hereditary MTC and *RET* mutation–carrying relatives. For molecular analysis, RNA was extracted from peripheral blood, followed by cDNA synthesis using 3.5 μ g of total RNA. Quantitative real-time polymerase chain reaction (RT-qPCR) was performed with SYBR Green and 200 nM of each primer for the two specific mRNA targets (CT-CALCA or CGRP-CALCA) and normalized with the ribosomal protein S8 as the reference gene.

Results: We detected *CALCA* transcripts in the blood samples and observed a positive correlation between them ($r=0.946$, $p<0.0001$). Both mRNAs also correlated with sCT (CT-CALCA, $r=0.713$, $p<0.0001$; CGRP-CALCA, $r=0.714$, $p<0.0001$). The relative expression of CT-CALCA and CGRP-CALCA presented higher clinical sensitivity (86.67 and 100, respectively), specificity (97.06 and 97.06), positive predictive value (92.86 and 93.75), and negative predictive value (94.29 and 100), than did sCT (73.33, 82.35, 64.71, and 87.50, respectively). In addition, the *CALCA* transcript measurement mirrored the response to the pentagastrin test.

Conclusion: We demonstrate that the measurement of *CALCA* gene transcripts in the bloodstream is feasible and may refine the management of patients with MTC and *RET* mutation–carrying relatives. We propose considering the application of this diagnostic tool as an alternative to the calcitonin-stimulation test.

Introduction

CALCITONIN IS THE MAJOR TUMOR MARKER for medullary thyroid carcinoma (MTC), and it is highly produced, dynamically stored, and rapidly secreted into the blood by the normal calcium-sensing parafollicular C cells and by the C cell–derived MTC (1,2). MTC may occur either sporadically (accounting for almost 75% of the cases) or as part of a familial

syndrome (named multiple endocrine neoplasia [MEN]), a dominantly inherited disorder that is caused by an activating mutation in the *RET* oncogene (3,4).

The technology to measure serum calcitonin (sCT) has evolved during the past four decades (2,5–9). sCT is commonly used for the diagnosis and follow-up of MTC patients (10), and its doubling time is currently considered to be a reliable prognostic indicator and one of the major criteria in

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the management of persistent tumors (11). Moreover, sCT is used as a biochemical screening tool for MTC in relatives with *RET* mutations, frequently assisting with planning the modality of the surgical approach towards prophylactic or therapeutic thyroidectomy (10). Calcitonin measurements can also be of great value in the washout of fine-needle aspiration (FNA) biopsies during the investigation of thyroid nodules because it increases the accuracy of the diagnostic procedure (12).

By contrast, the various sCT assays may have important intra-assay or interassay variation and may yield different and sometimes conflicting results (13,14). Beyond these caveats related to the assay reproducibility, a hook effect in samples with high levels of sCT and a cross-reaction with procalcitonin may happen, although these phenomena are uncommon with two-site monoclonal antibody assays (15–19). On the other hand, heterophilic antibodies (with a prevalence of 1.3% to 3.7%) or anti-calcitonin interference may occur and can sometimes be misleading (20–23).

A pentagastrin- or calcium-stimulation calcitonin test may be desirable in screening for MTC in *RET* mutation-carrying relatives or in the postoperative follow-up of MTC patients. Alternatively, or in the absence of the stimulation test, mRNA detection offers the advantages of being more comfortable and less invasive because it only requires blood collection and lacks other side effects.

The aims of this work were to investigate the applicability of the measurement of calcitonin-related polypeptide alpha (*CALCA*) gene transcripts, particularly calcitonin (*CT-CALCA*) with the co-transcribed calcitonin gene-related peptide (*CGRP-CALCA*) mRNA, and to refine the management of patients with MTC and *RET* mutation-carrying relatives by suggesting an alternative molecular diagnostic tool to the calcitonin-stimulation test.

Patients and Methods

Patients

We selected 26 patients who were consecutively evaluated in our MEN outpatient clinic (Thyroid Unit), at the Division of Endocrinology, Department of Medicine, Escola Paulista de Medicina, Federal University of São Paulo, in São Paulo, Brazil. Fourteen had already been diagnosed with MTC and had undergone total thyroidectomy; six of these patients had an identified *RET* germ-line mutation and eight were typical sporadic cases. The remaining 12 patients were relatives of patients with a *RET* germline mutation and were found to be positive after genetic screening of the proband. In addition, we also studied 23 healthy individuals without any thyroid disorders as controls.

After careful clinical analyses, biochemical (sCT and carcinoembryonic antigen) and imaging studies, these 26 patients were divided into three groups: *RET* mutation-carrying relatives ($n=12$), MTC patients with no evidence of disease after surgery ($n=5$), and MTC patients with biochemical and/or structural disease ($n=9$).

The *RET* mutation-carrying relatives had a blood sample collected before surgery, and disease status (with or without) was defined only after thyroidectomy and confirmatory histopathological analysis. The disease status of MTC patients with no evidence of disease after surgery and MTC patients with biochemical and/or structural disease was determined only after a minimal period of 5 years of follow-up and after

clinical examination and imaging studies (cervical ultrasound and, when indicated according to the American Thyroid Association guidelines, computed tomography and magnetic resonance imaging) (10,24). The 23 healthy controls without a thyroid disorder were considered to be without disease based on clinical and ultrasound examination.

Blood samples were collected for simultaneous sCT measurement and mRNA extraction. Signed letters of informed consent were obtained from all of the patients, and the clinical and molecular diagnostic studies were approved by the university's ethics and research committee (protocol number 1749/06) and conformed to the 1975 Declaration of Helsinki as revised in 1983.

Positive and negative controls for the mRNA measurement assay

TT, an MTC cell line obtained from ATCC (number CRL-2005) through the Rio de Janeiro Cell Bank, was used as a positive control for the *CT-CALCA* and *CGRP-CALCA* mRNAs. The latter also served as the internal control for validation of the *CALCA* major-expressed transcripts. The cells were grown in Dulbecco's modified Eagle's medium (Invitrogen Corp., Carlsbad, CA) that was supplemented with 10% fetal bovine serum (Invitrogen), 100 U/mL of penicillin, and 100 µg/mL streptomycin, and then maintained in a humidified incubator containing 5% carbon dioxide at 37°C, as previously described (21,22). Dissected tissue from a follicular carcinoma was used as a negative control.

sCT assay

We used an immunofluorometric assay developed in our laboratory for the measurement of sCT. This assay is based on two anti-calcitonin monoclonal antibodies, one of which is linked to the solid phase and is specific for the 11–23 amino acid sequence (E1P10), and the other of which is biotinylated and specific for the 17–32 amino acid region (J1P9). The assay's analytic sensitivity is 1 pg/mL, with an upper-normal range limit of 18.4 pg/mL for men and 7.8 pg/mL for women.

Pentagastrin-stimulation calcitonin test

Twelve *RET* mutation-carrying relatives prior to thyroidectomy and four patients who had been already thyroidectomized because of MTC underwent a pentagastrin-stimulation test (Table 1). Samples for sCT measurement were drawn through an indwelling catheter before and 2, 5, 10, and 15 minutes after an intravenous bolus of 0.5 µg/kg of pentagastrin (Pentagastrin™, Cambridge Laboratories Ltd, Newcastle, United Kingdom). All of the samples were centrifuged, and the serum aliquots were immediately stored at –20°C. The test was considered to be positive when there was at least a threefold increase in the sCT level and/or a value above 100 pg/mL after the stimulus (25,26).

The blood samples for *CALCA* transcripts measurement were collected before the pentagastrin infusion through the same catheter.

RNA blood extraction, cDNA synthesis and quantitative real-time polymerase chain reaction

Blood samples (4 mL) from each patient were collected in tubes containing EDTA, and total-RNA isolation and cDNA

TABLE 1. CLINICAL, PATHOLOGICAL, BIOCHEMICAL, AND MOLECULAR FEATURES OF THE 49 SUBJECTS ENROLLED IN THE STUDY

Patient no.	Sex	RET germline mutation	Basal sCT (pg/mL)	Pentagastrin-stimulated sCT (pg/mL)	CT mRNA (RE)	CGRP mRNA (RE)	Clinical staging and pathological analysis
Thyroid-healthy controls							
1–23	10 male, 13 female	n/a	1–21	n/a	Undetectable to 3.50	0.01–4.46	n/a
RET mutation-carrying relatives							
24	Male	p.Val804Met	1	11.1	0.53	1.03	n/a
25	Female	p.Cys609Ser	6.4	6.3	0.48	1.39	n/a
26	Male	p.Val804Met	3.9	10.6	5.14	3.52	No MTC
27	Female	p.Cys634Arg	1090	n/a	104.97	63.38	n/a
28	Female	p.Glu768Asp	26	432	18.23	15.62	T1aNxMx, CCH
29	Male	p.Tyr791Asn	2.7	8.4	1.48	2.55	n/a
30	Male	p.Cys634Arg	50	434	56.6	19.67	T1aNxMx, CCH
31	Female	p.Cys634Tyr	10.4	n/a	0.80	1.12	n/a
		p.Tyr791Phe					
32	Female	p.Glu768Asp	4.6	57	5.11	5.66	CCH
33	Female	p.Cys634Tyr	26.3	52	0.42	2.34	No MTC
		p.Tyr791Phe					
34	Female	p.Cys634Tyr	7.3	68	2.74	28.78	MTC, T1NxMx
		p.Tyr791Phe					
35	Male	p.Cys634Gly	29.2	65	3.23	3.27	No MTC or CCH
MTC after surgery without evidence of disease							
36	Male	None	3.9	2.4	1.05	3.44	T1bN0Mx
37	Female	p.Cys634Gly	1.9	n/a	0.69	0.4	T1amN0Mx
38	Male	p.Cys634Arg	3.5	n/a	33.77	16.25	T2mN1bMx
39	Female	None	1.4	4.1	1.10	2.04	T1bN0Mx
40	Female	None	1	1	2.50	1.17	T1bNxMx
MTC with biochemical and/or structural disease							
41	Male	None	5777	n/a	429,451.53	162,134.05	T3N1bM1
42	Female	None	18	n/a	1912.6	1463.82	T2N1Mx
43	Male	p.Cys609Ser	156	n/a	1.51	3.68	n/a
44	Male	None	67,200	n/a	94,514.64	320,551.38	T3N1Mx
45	Female	p.Met918Thr	20,000	n/a	17.49	11.68	T3mN1M1
46	Female	None	1078	n/a	8.91	5.2	T1bN1bM1
47	Male	None	6410	n/a	533.61	297.21	T4amN1bM1
48	Female	p.Cys634Arg	7.3	77	8.35	6.18	T2mN1M0
49	Female	p.Met918Thr	2834	n/a	83.64	55.78	n/a
Cell line and tissue controls							
PC	n/a	TT cell line	n/a	n/a	271,130.58	163,785.92	
NC	n/a	None	n/a	n/a	0	0	Follicular carcinoma

Clinical status, germ-line *RET* analysis, basal and pentagastrin-stimulated sCT (pg/mL), relative expression (RE) of the CT-CALCA and CGRP-CALCA mRNAs, and pathological findings with the TNM staging (24) of each individual. Positive (PC) and negative (NC) controls and the RE of CT-CALCA and CGRP-CALCA are shown. Basal sCT cutoffs are 18.4 pg/mL for men and 7.8 pg/mL for women. CT-CALCA RE cutoff is 4.3 and CGRP-CALCA is 3.6. A positive cell line (PC) and a negative tissue control (NC) are included at the bottom of the table.

n/a, not available or not applicable; CT, calcitonin; sCT, serum CT; CGRP, calcitonin gene-related peptide; CALCA, calcitonin-related polypeptide alpha; MTC, medullary thyroid cancer; CCH, C-cell hyperplasia.

synthesis were performed as previously reported (27–29), with the exception of using 3.5 μ g of total RNA for the cDNA synthesis. The cDNA was quantified using the NanoVue Plus spectrophotometer (GE Healthcare, Buckinghamshire, United Kingdom) and was considered adequate when the A260/A280 ratio was between 1.8 and 2.0. The total RNA was treated with DNase from the TURBO DNA-free kit (Ambion, Austin, TX) and was reverse-transcribed to cDNA using the Super-Script III Reverse Transcriptase kit (Invitrogen) with oligo (dT) 12–18 primers (Invitrogen) and 1 U of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen) in a reaction

volume of 30 μ L and diluted with UltraPure DNase/RNase-Free Distilled Water (Invitrogen) to final volume of 60 μ L. An aliquot of 2 μ L of the newly synthesized cDNA was used in 20 μ L polymerase chain reaction (PCR) amplifications containing 10 μ L of SYBR Green PCR Master Mix and 200 nM of each primer for the two specific mRNA targets (CT-CALCA or CGRP-CALCA) or for the reference gene (ribosomal protein S8, *RPS8*). All of the primers were designed using the Primer3 software (<http://frodo.wi.mit.edu>), avoiding single nucleotide polymorphisms within the primer binding sites and similar transcripts, as follows: CTF 5'-ATC TAA GCG GTG CGG TAA TC-3'; CTR

5'-CTT GTT GAA GTC CTG CGT GT-3'; CGRPF 5'-CCC AGA AGA GAG CCT GTG ACA-3'; CGRPR 5'-CTT CAC CAC ACC CCC TGA TC-3'; RPS8 5'-AAC AAG AAA TAC CGT GCC C-3'; and RPS8 3'-GTA CGA ACC AGC TCG TTA TTA G-5.

The quantitative real-time PCR (qRT-PCR) amplifications for each subject were performed in triplicate within 40 cycles using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA), according to the manufacturer's protocol. All 49 samples (in triplicate) were run under the same PCR cycling conditions. The threshold cycle (C_q , formerly C_t) was obtained using the Applied Biosystems software and was averaged with $SD \leq 1$. The CT-CALCA and CGRP-CALCA mRNA expression was standardized using blood samples collected from individuals prior to thyroidectomy in whom the histopathological analysis ruled out both MTC and C-cell hyperplasia (CCH), as well as thyroiditis. The relative expression (RE) for CT-CALCA and CGRP-CALCA mRNA was calculated by the formula $2^{(Cs-Rs)/2(Cn-Rn)}$, where C_s is the C_t cycle number for CT-CALCA or CGRP-CALCA in the samples, R_s is the C_t value found for RPS8 of each sample, C_n is the mean value of CT-CALCA or CGRP-CALCA C_t value of individuals without evidence of disease, and R_n is the RPS8 C_t value in the same individuals. This protocol is currently used by our group for studying other thyroid transcripts (27–29).

Statistical analysis

All of the biochemical (sCT) and molecular data (RT-qPCR) values were log-transformed before statistical analysis. The RT-qPCR cutoff values for the CT-CALCA and CGRP-CALCA mRNA were calculated using a receiving operating characteristic (ROC) curve. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using the formulas of Galen and Gambino (30,31). The statistical correlation strength between the CT-CALCA and CGRP-CALCA transcripts and between each transcript and the sCT was calculated using the Pearson test. The two-group analysis was performed using the unpaired Student's t test; for more than two groups, we used analysis of variance, including a *post hoc* analysis with a Bonferroni correction. All of the data were analyzed using the StatView software, and we considered a p value of 0.05 to be statistically significant.

Results

CT-CALCA mRNA can be detected in the blood stream

As a proof of principle, we were able to detect and utilize the measurement of the CT-CALCA mRNA and its partner transcript CGRP-CALCA mRNA in the blood of patients with MTC and in those relatives who tested positive for *RET* mutations and were thus at risk for early C-cell disease or MTC. In addition, we detected CT-CALCA in 8 out of 23 thyroid-healthy controls and CGRP-CALCA in all 23. CALCA transcripts were detected in all 26 patients and in the TT cultured cells (the positive control). We were able to measure a very low RE for the CT-CALCA and CGRP-CALCA mRNAs and the values were 0.002 (median 2.62 and maximum 429,451) and 0.001 (median 2.3800 and maximum 32,0551), respectively (Table 1).

In the follicular thyroid carcinoma tissue used as negative control, we detected traces of CALCA transcripts. Thus, after

RE calculation using the RPS8 internal control, the RE was considered to be zero (CT-CALCA, $RE=0.000005$; CGRP-CALCA, $RE=0.000011$; Table 1).

CT-CALCA and CGRP-CALCA mRNAs are correlated

We observed a highly positive correlation between the CT-CALCA mRNA and CGRP-CALCA mRNA ($r=0.946$, $p<0.0001$; Fig. 1A). The RE distribution analysis through an ROC curve could predict the RE cutoffs for the CT-CALCA and CGRP-CALCA mRNAs, which were 4.3 and 3.6, respectively. The area under the curve was 0.923 for CT-CALCA and 0.964 for CALC-CGRP. Moreover, the majority of the subjects yielded CT-CALCA mRNAs with higher RE values than those of the CGRP-CALCA mRNAs.

Based on the defined transcriptional RE cutoffs that were obtained from the ROC curve and the sCT concentration, we noted that the CT-CALCA mRNA diverged from the sCT in 10 out of 49 cases (20.4%) and that the CGRP-CALCA mRNA diverged in 10 out of 49 cases (20.4%). Furthermore, the CT-CALCA and CGRP-CALCA mRNAs diverged in three individuals (11%). The RE of CT-CALCA and CGRP-CALCA in each group of stratified patients is presented in Figure 2, along with the disease status reclassification after thyroidectomy of the *RET* mutation-carrying relatives and the further follow-up of all of the MTC patients.

Upon predictive analysis testing, the CT-CALCA mRNA RE yielded a higher PPV and higher NPV than did the sCT. Moreover, CGRP-CALCA yielded a higher PPV and NPV than CT-CALCA (Table 2). After further analyses of the individuals who presented no evidence of MTC, we could not find any significant differences based on the presence or absence of the thyroid gland when measuring the CT mRNA ($p=0.3144$) or the CGRP mRNA ($p=0.2367$).

CALCA mRNA correlates with sCT and may replace the calcitonin-stimulation test

The CT-CALCA and CGRP-CALCA mRNAs also correlated with the biochemical sCT (Figs. 1B, 1C). There was a positive correlation between the CT-CALCA mRNA and the sCT ($r=0.713$, $p<0.0001$) and between the CGRP-CALCA mRNA and the sCT ($r=0.714$, $p<0.0001$). With regard to only the disease-free individuals, the RE of the CGRP-CALCA mRNA was 1.3-fold higher than that of the CT-CALCA mRNA, but when we considered patients with biochemical and/or structural disease, the RE of the CT-CALCA mRNA was 1.5-fold higher than that of the CGRP-CALCA mRNA. Four individuals exhibited falsely elevated sCT levels; two were healthy controls without evidence of disease and two presented negative CALCA mRNA with no response in the pentagastrin-stimulation test and no MTC or CCH in the surgically removed thyroid tissue. sCT from three healthy controls presented a nonlinear response to the dilution with mouse serum, which may suggest circulating antibody assay interference.

The CALCA transcript measurement, particularly CGRP-CALCA, mirrors the response to the pentagastrin-stimulation test in all 11 patients tested and agrees in quantification (Table 2). We also compared the patients who responded to the stimulation test with those who did not. After analyzing each transcript, a significant difference between the two groups was observed (Fig. 3). We identified a small overlap between

FIG. 1. *CALCA* transcript intercorrelation and their correlation with sCT. CT-CALCA and CGRP-CALCA correlate with each other (A), CT-CALCA mRNA reflects its peptide counterpart measured in the serum (sCT) (B), and CGRP-CALCA mRNA also correlates with sCT (C). CT, calcitonin; CGRP, calcitonin gene-related peptide; CALCA, calcitonin-related polypeptide alpha.

the 90th percentile of the nonresponsive group and the 10th percentile of the group that was responsive to the pentagastrin-stimulation test for the CT-CALCA RE, but there was no overlap between these groups for the CGRP-CALCA RE (Fig. 3).

FIG. 2. Relative expression of CT-CALCA (A) and CGRP-CALCA (B) in healthy controls, *RET* mutation-carrying relatives, medullary thyroid cancer (MTC) patients with no evidence of disease after surgery, and MTC patients with biochemical and/or structural disease. The healthy controls are represented by white dots, and the gray dots represent individuals with no evidence of the disease. The black dots represent patients with confirmed MTC or C-cell hyperplasia (CCH) after thyroidectomy in the second group; a patient later diagnosed with tumor recurrence in the third group; and patients with evidence of disease in the fourth group. The dashed line represents the receiving operating characteristic (ROC) curve cutoff of the RE of CT-CALCA (4.3) and CGRP-CALCA (3.6). The subjects are in the same order as in Table 1. <LOD, below level of detection.

TABLE 2. SUMMARY OF THE COMPARATIVE STATISTICAL ANALYSIS OF CT-CALCA AND/OR CGRP-CALCA mRNA AND THEIR PROSPECTIVE USE

	Sensitivity	Specificity	PPV	NPV
Serum calcitonin (sCT)	73.33	82.35	64.71	87.50
CT-CALCA mRNA	86.67	97.06	92.86	94.29
CGRP-CALCA mRNA	100	97.06	93.75	100
Post-pentagastrin test	100	100	100	100

Transcripts measured while detectable in the blood stream, basal and pentagastrin-stimulated sCT.

PPV, positive predictive value; NPV, negative predictive value.

FIG. 3. Comparative analysis of the *CALCA* transcripts and their group response to the pentagastrin-stimulation test. A comparative plot of the relative expression of CT-CALCA (**A**) and CGRP-CALCA (**B**) mRNAs between the groups of individuals with and without response to the pentagastrin test—demonstrating the transcripts' ability to distinguish the two groups.

Discussion

The revised American Thyroid Association (ATA) guidelines for patients with thyroid nodules and differentiated thyroid cancer do not recommend sCT measurement in screening patients with thyroid nodules (32). The major problem is the lack of specificity, with a high incidence of false-positive results (23,33). The ATA guidelines for MTC management recommend sCT measurement (without the pentagastrin-stimulation test) during the follow-up of *RET*-mutation carriers and during the follow-up of MTC patients after surgery (10). In patients with thyroid nodular disease and elevated basal sCT, in *RET*-mutation carriers prior to prophylactic surgery, and during the follow-up of MTC patients, the calcitonin-stimulation test is performed in some centers as a strategy to increase sensitivity and specificity (10,34). Although sCT is a good biomarker to diagnose and follow the majority of MTC patients, it may not be sensitive enough to anticipate the diagnosis of small MTCs or CCH while performing family screening (35).

It has been demonstrated that transcriptional splicing of the *CALCA* gene forms three isoforms (36). The first isoform is translated and cleaved to form calcitonin and CCP-I (formerly known as PDN-21 or kalcitonin), the second isoform forms calcitonin and CCP-II, and the third gives rise to CGRP (36). The *CALCA* gene has six exons, each with well-established

contributions to splicing isoforms: exon 1 is not translated; exon 2 encodes the signal peptide; exon 3 forms the amino-terminal peptide; exon 4 encodes calcitonin; and exon 5, CGRP, and exon 6 comprise part of the CGRP but are not translated. Based on the *CALCA* splicing characteristics, we were able to design accurate and specific primers for these transcripts, which were subsequently confirmed by sequencing.

CGRP is a peptide synthesized in the peripheral and central nervous system of mammals and in others species (37). CGRP is also produced in perivascular nerve terminals and acts as a potent vasodilator (38–40). In sepsis, an increase in the production of CGRP and CT mRNA occurs in adipose tissue and transiently by adherent monocytes (41). In rats, CT and CGRP mRNA are found in normal thyroid tissue (42). The CGRP transcript and its peptide have been detected in MTC tumor tissue (43–45). Moreover, the CGRP peptide has been detected in the plasma of MTC patients, also with response after pentagastrin stimulation (46).

Herein, we have proposed an alternative tool that is similar to the one already in use in the management of differentiated thyroid cancer, using thyroglobulin and thyrotropin receptor mRNAs in the follow-up of patients who have tested positive for anti-thyroglobulin antibodies (47–49). In two previous studies, blood CT mRNA was detected by RT-PCR (50,51). In addition, CT mRNA has also proven useful in FNA biopsies of thyroid nodules for the diagnosis of MTC (52). Following these findings, our work is the first attempt to use RT-qPCR to measure *CALCA* gene transcripts in the blood, and also the first attempt at using CGRP mRNA quantification. Moreover, the present study refined the laboratory tools for use in the management of patients with MTC and of their relatives who are diagnosed with *RET* mutations.

Because the levels of CGRP-CALCA and CT-CALCA mRNA are closely related, and both are positively correlated with the sCT measurements, one can argue that very few spurious non-CALCA transcripts might have occurred. We believe that this positive correlation does not necessarily reflect interdependence because other posttranscriptional events might play a role in this mechanism.

Interestingly, patient 34 (Table 1), who was a *RET* mutation-carrying relative with a basal sCT within the normal range (7.8 pg/mL) and a calcitonin of 68 pg/mL in the stimulation test, presented a high RE of CGRP-CALCA, but a normal RE of CT-CALCA. He underwent thyroidectomy, and MTC was histopathologically confirmed, which may suggest a higher sensitivity of CGRP-CALCA mRNA. After thyroidectomy, patient 38 (Table 1) had low levels of sCT and a high RE of CT-CALCA and CGRP-CALCA mRNAs. However, his most recent tests revealed higher levels of sCT (15 and 8.9 pg/mL); therefore, an FNA biopsy of a 0.7 cm cervical lymph node that we had been closely monitoring was performed, and the cytopathological analysis of this node revealed metastatic MTC. This result suggests that the CT-CALCA and CGRP-CALCA mRNAs could have enabled early detection of tumor recurrence in this case. Conversely, patient 43 (Table 1) exhibited an elevated sCT with a normal RE of the CT-CALCA mRNA and a slightly elevated RE of the CGRP-CALCA mRNA. Thus far, this patient has not exhibited any sign of macroscopic disease after thyroidectomy for MTC, which might suggest other veiled sCT assay interferences, such as heterophilic antibody or a rare CT peptide conformation (52).

We believe that sCT lacks sensitivity and specificity in some clinical situations. In cases with discrepancies between the clinical presentation and the sCT during follow-up, the CALCA mRNAs may help to refine the diagnosis of patients who are apparently disease-free. CALCA transcript quantification showed the capability to distinguish between the group that responded to the pentagastrin-stimulation test and the nonresponsive group.

The stability and the reproducibility of the CT-CALCA mRNA appear to be greater compared with those of its peptide, as suggested in our study. Therefore, we believe that the detection of both transcripts are feasible options for the follow-up of atypical MTC patients and for the staging of asymptomatic *RET* mutation carriers. Ultimately, CALCA transcript measurements could replace the laborious stimulation test as a more comfortable method; however, studies with larger series of patients are clearly necessary to better define the use of the measurements in clinical practice. In light of our findings, we suggest this molecular diagnostic tool as an alternative to the stimulation test for those individuals who have sCT levels that are borderline or mildly above the normal range as well as for asymptomatic *RET* mutation-carrying relatives. Although previous studies suggest that other tissues could produce CT mRNA, based on our results and the correlation with sCT, we believe that mRNA production by the C cell and by the MTC is much higher, and that this type of interference is insufficient to affect the use of CT or CGRP mRNA as potential diagnostic tools (53). We demonstrate that, in the MTC-free patients, the presence of the thyroid gland does not affect the diagnostic power of measuring the CT or CGRP transcripts.

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Author Disclosure Statement

The authors have no conflict of interest to declare.

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