

## CLINICAL STUDY

# Reference range of serum calcitonin levels in humans: influence of calcitonin assays, sex, age, and cigarette smoking

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## Abstract

**Objective:** The objective of this study was to re-evaluate the adult  $C_T$  reference values determined by five different immunoassays and by introducing criteria for selecting control subjects.

**Design:** A prospective multicenter study.

**Patients:** Three hundred and seventy-five clinically euthyroid subjects.

**Methods:** We used five different  $C_T$  immunoassays. Sera were assayed for the concentration of TSH, gastrin, procalcitonin, urea, calcium, and anti-thyroperoxidase antibodies.

**Results:** Screening for the various potential causes of hypercalcitoninemia led to the exclusion of 23% of the sera. Our reference value analysis dealt with 287 subjects (142 men and 145 women). The proportion of samples in which no  $C_T$  was detected varied from 56% (for assay D) to 88% (for assay C). We observed significant correlations (whose magnitude depended on the assay used) between  $C_T$  levels and age or body mass index (BMI) (primarily in men). The distribution of  $C_T$  levels showed that 4.7, 9.8, 2.5, 6.5, and 8.0% of the values were over 10 pg/ml respectively. These values corresponded essentially to samples from 11 male subjects (median age: 55 years), most of whom were smokers. The highest  $C_T$  values were around twice as high in men than women, and were higher in smokers than non-smokers.

**Conclusion:** In clinical practice (and after having excluded the usual causes of raised  $C_T$  levels), the interpretation of  $C_T$  assay results must take into account i) the method used; ii) the patient's gender, age, and weight; and iii) the potential influence of cigarette smoking.

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## Introduction

Medullary thyroid cancer (MTC) is a rare malignant tumor of the parafollicular cells (also called 'C-cells'). It occurs in 5–10% of thyroid tumors (1) and has a prevalence of between 0.3 and 1.37% in nodular thyroid disease (2–9). Calcitonin ( $C_T$ ) is a marker for both initial diagnosis and monitoring of patients with residual or recurrent MTC (10, 11). The sensitivity of serum basal  $C_T$  assays can be improved by performing a pentagastrin stimulation test or through high-calcium perfusion. Serum  $C_T$  assays are indicated in nodular thyroid patients if there is a family history of MTC, multiple endocrine neoplasia type 2, or non-defined thyroid cancer (1). In Europe, several researchers have recommended the systematic use of serum  $C_T$  assays in all nodular thyroid patients, in order to identify sporadic MTC as early as possible (2–5, 7–9), optimize surgical treatment (12), and obtain better survival (13).

However,  $C_T$  is not a specific marker for MTC because raised serum  $C_T$  levels can equally be observed in other circumstances, including C-cell hyperplasia (CCH) (14, 15), other endocrine tumors (pulmonary and pancreatic tumors) (16), kidney failure (17, 18) and, more rarely, in auto-immune thyroid disease (19, 20), hypergastrinemia (21), sepsis (22), type 1A pseudohypoparathyroidism (23), and in the presence of heterophilic antibodies (24). The prevalence of hypercalcitoninemia not due to MTC varies from 0.3 to 4.5% (2–9). The assay's high cost and lack of specificity (25) has dissuaded health authorities in the USA from adopting systematic testing (1, 26).

In contrast to other biological parameters in thyroid function testing (thyroid-stimulating hormone (TSH) and/or anti-thyroid antibodies), there are no published criteria for the selection of control subjects in order to establish  $C_T$  reference values (27). Certain cases of hypercalcitoninemia could be due to inappropriate  $C_T$  reference values because the latter depend on the assay

type and indeed, for a given assay, the country in which it is used (3, 4, 28, 29).

The aim of the present study was to re-evaluate  $C_T$  reference values by introducing criteria for the selection of control subjects, envisaging the use of different reference values for men and women respectively, and looking for a possible correlation between  $C_T$  levels and subject's age.

## Materials and methods

### Patients

In this prospective multicenter study, 375 sera were sampled in subjects with no medical history of thyroid disease. No goiter was apparent and neck palpation did not reveal any abnormalities. Any subject was taking medication that could have interfered with thyroid function testing (with the exception of estrogens). Sera were aliquoted and stored at  $-20^\circ\text{C}$  prior to analysis.

Biological euthyroidism was confirmed by a TSH assay with a functional sensitivity  $<0.01$  mUI/l (TSH architect from Abbott Diagnostics), for which the reference values range from 0.3 to 3.6 mUI/l. In order to eliminate other potential causes of raised  $C_T$ , we determined calcemia and uremia at all the investigating centers. Tests for gastrin (GASK-PR from CisBio International, Gif-sur-Yvette, France; normal range 30 to 120 pg/ml), TSH, procalcitonin (sensitive ProCT on Kryptor from BRAHMS, Berlin, Germany),  $C_T$ , and anti-thyroperoxidase antibodies (TPOAb from BRAHMS; positive if  $>60$  UI/l) were all performed in the same laboratory.

### $C_T$ assays

Five different  $C_T$  immunoassays (based on radioisotopic, enzymatic, or luminescent labels) were tested: assay A, immunoradiometric assay-human calcitonin (IRMA-hCT) (CisBio International); assay B,  $C_T$  on Advantage (Nichols Institute, San Clemente, CA, USA); assay C,  $C_T$  on Immulite 2000 (DPC, La Garenne-Colombes, France); assay D, Calcitonin-ELISA 7024 (Biomerica, Newport Beach, CA, USA); and assay E, CT-USA-IRMA, KIP0429 (Biosource, Nivelles, Belgium). All recognize the mature

form of  $C_T$ , and the main characteristics of each kit are given in Table 1. The assays were performed as single runs, according to the respective manufacturer's recommendations. Two of the assays (B and C) are automated. The inter-assay coefficients of variation were  $<10\%$  for all measured  $C_T$  levels with the automated assays (B and C), and  $<10\%$  only for the  $C_T$  concentrations higher than 10 pg/ml with the manual assays (A, D, and E).

Correlation coefficients were calculated using Spearman's non-parametric test. The median  $C_T$  values for the smoker and non-smoker females and males were compared by the Mann-Whitney test. The significance threshold was set to  $P < 0.05$ .

## Results

### Elimination of sera

Eighty-eight sera (23%) were eliminated due to abnormal TSH levels ( $n=12$ ), and/or the presence of anti-thyroperoxidase antibodies TPOAb ( $n=34$ ) and/or high gastrinemia, uremia, and calcemia values (in 13, 40, and 2 cases respectively). Several sera were eliminated for at least two reasons. All procalcitonin levels were in the normal range. The data reveal that auto-immune status does not greatly interfere with  $C_T$  levels because only 1 of the 34 patients (i.e. 3%) presented a  $C_T$  level  $>10$  pg/ml according to all the five methods. In contrast, high urea levels were associated with a higher frequency of  $C_T$  levels  $>10$  pg/ml. Depending on the assay used, the proportion ranged from 5% (in assay C) to 23% (assay B) of the patients.

A 61 year old male subject undergoing post-operative and post-radiotherapy follow-up for a non-secreting pituitary macroadenoma (with no known thyroid disease) presented high  $C_T$  levels, ranging from 313 to 473 pg/ml according to the various assays. The neck palpation was negative. The patient underwent thyroid echography (revealing a 1 cm thyroid nodule on the left lobe) and then a total thyroidectomy with dissection of the central and left jugular-carotid lymph nodes. Histological examination confirmed the presence of an 8 mm MTC in the left lobe, whereas the right thyroid

**Table 1** Main characteristics of the five assays evaluated in the study.

Assays	A	B	C	D	E
Antibodies	2 M	2 M	1 M+1 P	2 M	2 M
Tracer	$^{125}\text{I}$	Chemiluminescent	Chemiluminescent	Enzymatic	$^{125}\text{I}$
Pre-treatment	Yes	No	No	No	No
Length	24 h	45 min	45 min	5 h	24 h
Range	8.4–1530	3.5–1500	5–2000	12.3–300	10–1000
LD (pg/ml)	1.5	1	1	1	1
RI (pg/ml)	$<10$	$<5$ for women $<12$ for men	$<10$	$<13$ women $<30$ for men	$<10$
FSe (pg/ml)	5.5	2	2	4	4

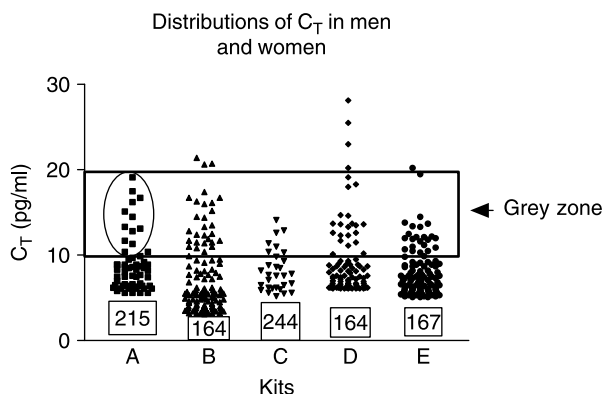
M, monoclonal; P, polyclonal; DL, detection limit; RI, reference interval; FSe, functional sensitivity.

lobe was normal. We did not observe recurrent or left jugular-carotid metastatic adenopathy. A negative test for the rearranged during transfection (RET) proto-oncogene mutation indicated that this patient presented a sporadic MTC. The post-operative baseline serum  $C_T$  level was < 3 pg/ml.

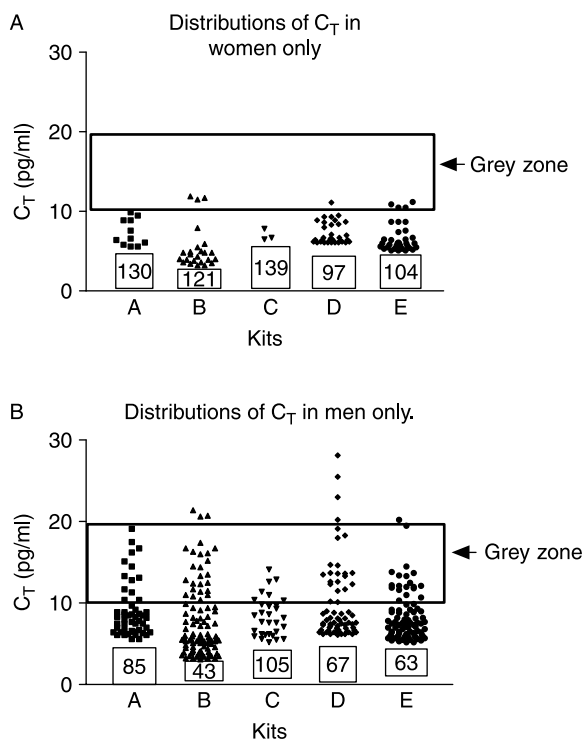
**Reference ranges of  $C_T$  levels**

Hence, our  $C_T$  reference value analysis dealt with 287 sera (142 men and 145 women; median age: 45 years (range 17–83 years)). Among the men, 45 were active smokers and 3 were former smokers (i.e., 33.8%), whereas 27 women were smokers (i.e., 18.6%). The  $C_T$  concentration distributions established with the five assays are shown in Figs 1 and 2A and B. The indicated detection thresholds correspond to the functional sensitivity concentrations stated by the manufacturers. The number of undetectable results varied according to the detection threshold of each method and ranged from 154 (56%, method D) to 244 (88%, method C). For three assays, it was possible to dissociate the reference values for men and for women with sufficient precision. The maximum concentrations observed in men were twice those recorded in women.

Then, we sought to identify correlations between  $C_T$  levels and patient age and BMI in all the subjects, in non-smokers, and in women and men respectively. Significant correlations between  $C_T$  and age were revealed for three assays (A, C, and E) for all the subjects and the non-smokers, although the correlation coefficients were low ( $r$  between 0.128 and 0.180,  $P < 0.05$ ). Drawing a distinction between male and female subjects revealed correlations with age for four assays (i.e., all except assay D) in men ( $r$  between 0.123 and 0.265,  $P < 0.05$ ). However, these correlations were absent in the samples from women, with the exception of a negative correlation for assay B. In terms of the BMI, significant correlations were observed with the majority of the assays (four out of five) for all the subjects and for



**Figure 1**  $C_T$  concentration distributions established with the five assays. The numbers of undetectable  $C_T$  levels, with each assay, were noted in the rectangles near the x-axis.

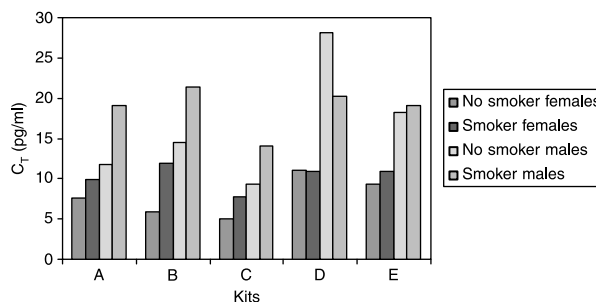


**Figure 2**  $C_T$  concentration distributions established with the five assays in (A) women and (B) men separately. The numbers of the undetectable  $C_T$  levels were noted in the rectangles near the x-axis.

the men alone. These correlations were generally not seen for the women and the non-smokers.

**Interference with cigarette smoking**

After excluding 88 sera, 11 sera from male subjects (median age: 55 years) presented concordant  $C_T$  levels of > 10 pg/ml in at least three different assays (Fig. 1, assay A in the oval line). All the latter subjects were active smokers, except for one former smoker and one who had never smoked. The maximum  $C_T$  concentration observed was 21.4 pg/ml.



**Figure 3** Maximum  $C_T$  values observed for each assay in euthyroid controls according to sex and cigarette smoking.

Our data analysis enabled us to separate the values obtained in men from those found in women and to take into account the subject's smoking status. We report the maximum values observed for each assay in Fig. 3. For three assays (A, B, and C), positive smoking status significantly increased the interpretation threshold. This was not true with assays D and E. In addition, we calculated the median  $C_T$  values for the non-smoker and smoker females and males with each assay. We found higher median  $C_T$  concentrations for the smoker versus non-smoker males. The differences were significant with the assays A ( $P=0.018$ ), B ( $P=0.001$ ), C ( $P=0.012$ ), D ( $P=0.036$ ), and E ( $P=0.035$ ). No significant differences were noted with females.

## Discussion

The generalized use of  $C_T$  assays in nodular thyroid disease, the development of new luminescent assay kits, and variations in published reference values prompted us to re-evaluate serum  $C_T$  concentrations by introducing clinical and biological criteria for the selection of control subjects. Our multicenter study collected 375 serum samples and determined the  $C_T$  level in each with five different immunoassays (all with detection limits of around 1 or 1.5 pg/ml). The functional sensitivities stated by the manufacturers ranged from 2 to 5.5 pg/ml. The two automated luminescent assays had the lowest functional sensitivities. It may appear paradoxical for kits D and E to combine such low functional sensitivities with such a high 'factory' calibration value. Therefore, we decided to check the value for kits A and B ourselves because we used these methods routinely. We found that a value of 5.5 pg/ml could only be obtained by introducing an intermediate reference concentration half that of the manufacturer's calibration value. Our results were coherent with those of Bieglmayer *et al.* (30, 31).

Twenty-three percent of the sera were excluded from our analysis. The main reasons for elimination were marked uremia (accounting for 10% of the excluded sera) and the presence of TPOAbs (9.1%). Raised  $C_T$

levels have been described (16–18) in cases of chronic kidney failure and in hemodialyzed patients (in up to 25% of the latter subjects). The mechanism underlying this elevation has not been well established. There are two possible hypotheses – either a modification of clearance or hyper-production – because a correlation between  $C_T$  levels and the intensity of glomerular filtration has not been observed.

The prevalence of MTC is low, and among 1000 patients presenting nodular thyroid disease, only three to five patients will have MTC (2, 3, 9). On the basis of our 375 sera tested, we detected one case of sporadic MTC in a male subject. Systematic use of a  $C_T$  assay enabled optimal care provision for this MTC patient, apparently with complete recovery.

Our reference value study dealt with 287 subjects (142 men and 145 women), i.e., in compliance with a minimum sample size of 120 recommended by Baloch *et al.* (27). The proportion of smokers was higher for the male subjects (33.8%) than the female subjects (18.6%).

In Figs 1 and 2A and B, we show the distribution of  $C_T$  concentrations determined using the five assay kits. It is clearly apparent that a non-negligible proportion of the values is situated above 10 pg/ml (4.7, 9.8, 2.5, 6.5, and 8.0% for assays A, B, C, D, and E respectively). The maximum observed concentration was 28.1 pg/ml (for assay D, which often yielded isolated high values that were not confirmed by the other assays). The cut-off of 10 pg/ml recommended as the normal threshold by the assays A, C, and E (and even 12 pg/ml for B in men) appears to be too low. Our data are more in agreement with the threshold of 20 pg/ml chosen by Pacini's and Elisei's groups in Italy (3, 13). The recent study by Costante *et al.* (32) on 5817 assays indicated a prevalence of hypercalcitoninemia (> 10 pg/ml) of 4.87%, including 0.26% for medullary cancers and 0.12% for CCH. It is noteworthy that the great majority of these cases of hypercalcitoninemia (77%) are due to  $C_T$  levels between 10 and 20 pg/ml. Should we have different reference values for men and for women? Two of the five kits tested suggest doing so in their reference standards. A postmortem study (33) has shown that

**Table 2** Moderately elevated  $C_T$  levels measured, with five assays, in 11 sera from male subjects recorded for cigarette smoking habits.

Patient	Age (years)	A	B	C	D	E	Smoking habit
1	75	14.5	12.5	9.7	12.3	11.9	Deprived
2	54	15.1	13.5	9.6	7.2	12.5	Yes
3	57	13.1	14.4	10.4	8.7	10.3	Yes
4	70	11.4	16.3	8.5	20.2	13.7	No
5	63	16.2	20.7	12.6	13.7	13.4	Yes
6	51	13.3	15.2	9.8	10.2	9.2	Yes
7	56	12.8	16.1	10.3	12.6	12.1	Yes
8	50	17.5	21.4	11.4	12.3	20.2	Yes
9	53	11.3	16.7	8.8	10.1	12.2	Yes
10	36	16.7	16.7	12.9	18.3	14.5	Yes
11	74	19.1	17.4	14.1	11.5	13.3	Yes

physiologically men have twice as many C-cells as women. Precise measurement of  $C_T$  levels in women requires the use of techniques with low functional sensitivity and a measuring range that is well suited to low concentrations (30, 31, 34). The dissociation of  $C_T$  levels in men and women also yields a ratio of 2 (i.e., as for the C-cell numbers). A single normal threshold of 20 pg/ml appears to be too high, in view of the values observed in female non-smokers. Normal more specific  $C_T$  ranges (males versus females and smokers versus non-smokers) will permit the reduction of the gray zone of 'suspiciously elevated  $C_T$  levels' and possibly ameliorate the positive predictive value of basal  $C_T$  for the diagnosis of medullary thyroid carcinoma.

We observed significant correlations between  $C_T$  levels and patient age, the magnitude of which depended on the assay technique used and the population tested. The correlation coefficients were low (around 0.25 in men and half of this value in the overall population) and were more significant in men than women. Verga *et al.* (28) did not detect a correlation between  $C_T$  levels (measured using assay B) and age in a population of 125 children and 98 male and female adults. This discrepancy can perhaps be explained by the fact that higher (and thus more easily detectable) values are found in men. The high proportion of undetectable values in women may bias this approach, and the same is true for correlations between the  $C_T$  concentration and the BMI. Again, the significant correlations essentially concerned our male subjects.

Our in-depth analysis (presented in Table 2) identified 12 sera that corresponded to moderate hypercalcitoninemia. Of the 12 sera samples all were from male subjects (median age: 55), ten of whom were active smokers, one former smoker, and one subject who had never smoked. Hence, cigarette smoking may be a newly identified cause of elevated  $C_T$  levels. This factor has not been taken into account by the previous studies in this field. We found significant differences between the median  $C_T$  concentrations for only non-smoker and smoker males, because the number of smokers was higher in males than females, and because the observed  $C_T$  concentrations were so elevated and measured with a better accuracy. The assays A, B, and C gave the better significance values. The assay D gave non-specific effects (30, 31). The action of tobacco on thyroid follicles is quite well known (35–38). Tobacco use constitutes a risk factor for goiter and its effect is accentuated in the presence of iodine deficiency. Tobacco consumption has contrasting effects on thyroid, i.e., both stimulatory and inhibitory influences (33). The involvement of a variety of mechanisms of action has been evoked: the main toxins cited are thiocyanate (which inhibits iodide transport and organification) and 2,3-OH pyridine (which inhibits thyroxine deiodination). Cigarette smoking can aggravate tissue hypoxia and exert significant immunomodulatory effects (38), and the reversibility of

cessation of tobacco consumption is not yet well understood (36). C-cells are distributed uniformly throughout the organism; they can be found not only in the thyroid and the thymus but also in the liver, lungs, duodenum, and jejunum. The effect of tobacco on pulmonary neuroendocrine cells has received more attention (39), and it has been established that smoking can increase the number of neuroendocrine cells and the secretion of peptides such as  $C_T$ . With respect to thyroid C-cells, a single study on hamsters by Tabassian *et al.* (40) has revealed a  $C_T$  elevation that may be related to the nicotine content of cigarettes. In order to determine the precise mechanisms of tobacco action on thyroid C-cells further studies would be required. Vantyghem *et al.* (41) observed an increase in  $C_T$  concentrations in patients with high chronic alcohol and tobacco consumption and reported that  $C_T$  levels did not normalize after the withdrawal of alcohol, suggesting that tobacco may play a predominant role.

Lastly, we integrated smoking status into our analysis: for assays A, B, and C, cigarette smoking clearly increases the threshold in both women and men, and this phenomenon is not seen with assays D and E, for which the measurement range is shifted toward higher values.

In conclusion, this clinical study shows that all  $C_T$  immunoassays are not equivalent. The most accurate assays allow dissociation of the  $C_T$  normal ranges for males and females. The clinical and biological characterization of our control subjects revealed that men and women differ in terms of the maximum observed  $C_T$  concentrations. Calcitonin levels are correlated with age and BMI (especially in men). Cigarette smoking can increase the concentration of  $C_T$ , and so taking this factor into account will help interpret moderately elevated  $C_T$  levels, especially in men.

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