Mini Review

Simona Censi*, Elisabetta Cavedon, Sara Watutantrige Fernando, Susi Barollo, Loris Bertazza, Laura Zambonin, Martina Zaninotto, Diego Faggian, Mario Plebani and Caterina Mian

Calcitonin measurement and immunoassay interference: a case report and literature review

Abstract: Calcitonin (CT) is currently the most sensitive serological marker of C-cell disease [medullary thyroid carcinoma (MTC) and C-cell hyperplasia]. Starting with a report on a case that occurred at our institution, this review focuses on trying to explain the reasons behind the poor specificity and sensitivity of the various CT immunoassays. A 15-year-old patient was referred to our institution in May 2014 for moderately elevated CT levels. Thyroid ultrasonography (US) documented a colloidal goiter. Secondary causes of the hypercalcitoninemia (hyperCT) were ruled out. The mismatch between the clinical picture and the laboratory results prompted us to search for other reasons for the patient's high CT levels, so we applied the heterophilic blocking tube (HBT) procedure to the patient's sera before the CT assay. Using this pretreatment step, his serum CT concentration dropped to <1 ng/L, as measured at the same laboratory. Measuring plasma CT has an important role in screening for C-cell disease, but moderately elevated serum CT levels need to be placed in their clinical context, bearing in mind all the secondary causes of C-cell hyperplasia and the possibility of laboratory interference, before exposing patients to the risks and costs of further tests.

Keywords: calcitonin; medullary thyroid carcinoma; heterophilic antibody.

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*Corresponding author: Dr. Simona Censi, Endocrinology Unit, Department of Medical and Surgical Sciences, University of Padua, Via Ospedale n.105-35128 Padova, Italy,
Tel.: (+39)049.8213003-04-00, Fax: (+39)049.657391,
E-mail: simona.censi@studenti.unipd.it
Elisabetta Cavedon, Sara Watutantrige Fernando, Susi Barollo, Loris Bertazza, Laura Zambonin and Caterina Mian: Endocrinology Unit, Department of Medical and Surgical Sciences, University of Padua, Padua, Italy
Martina Zaninotto, Diego Faggian and Mario Plebani: Laboratory Medicine, Department of Medical and Surgical Sciences, University of Padua, Padua, Italy. http://orcid.org/0000-0002-0270-1711 (M. Plebani)

Introduction

Calcitonin (CT) is a polypeptide hormone composed of 32 amino acids secreted by thyroid C cells. It is currently the most sensitive biochemical marker of C-cell disease (MTC and C-cell hyperplasia).

CT contains an intra-chain disulfide bridge between sequence positions 1 and 7 at the NH2-terminal end of the molecule, and an amide group at the COOH-terminal proline.

CT(1–32) is biosynthesized from the polypeptide precursor procalcitonin (ProCT) [1], a 116-amino-acid prohormone with three constituent peptides: a 57-amino-acid sequence at the amino terminus (NProCT); a centrally-located immature CT containing a terminal glycine; and a 21-amino-acid CT carboxy-terminus peptide 1 (CCP-1). Subsequent enzymatic post-translational processing yields several peptides. In addition to CT(1–32), the serum of normal subjects contains intact ProCT, free NProCT, free CCP-1, and the free conjoined CT-CCP-1 peptide (CT-CCP-1). Because these peptides precede the biosynthesis of CT(1–32), they have collectively been called CT precursors (CTpr). The molar concentration of circulating NProCT is twice as high as that of CT(1–32), both in normal conditions and in MTC. Other neuroendocrine tumors, such as small cell lung cancer (SCLC), carcinoid, pheochromocytoma, and pancreatic islet tumors may exhibit an increase in serum CTpr, so that, unlike the case of MTC, the serum CTpr/CT(1–32) ratio is further increased, probably because these lesions have a shortage of post-translational enzymatic capability. CT secretion by C cells is regulated by serum calcium levels, and CT is metabolized in the liver and kidney.

Several physiological and pathological conditions can be associated with a rise in CT levels, the most common of which are: male sex, smoking, drugs (glucocorticoids, proton pump inhibitors, β-blockers, glucagon), non-thyroid disorders (hypergastrinemia, hypercalcemia, neuroendocrine tumors, chronic renal failure) and thyroid

diseases (thyroid carcinoma and chronic autoimmune thyroiditis). Another not uncommon, but often underestimated cause of secondary hyperCT is CT immunoassay interference [2].

Starting with a report on a case that occurred at our institution, this review focuses on trying to explain the reasons behind the poor specificity and sensitivity of the various CT immunoassays.

Several other cases of immunoassay interference retrieved from the literature are also discussed.

Materials and methods

Serum calcitonin assay

Serum CT was measured with a commercially-available fully-automated chemiluminescent immunometric assay (CLIA) LIAISON CT II-Gen (DiaSorin Inc., Stillwater, MN, USA), which has an analytical sensitivity of ≤1 ng/L and a functional sensitivity of ≤3 ng/L. A reference upper limit of 10 ng/L is adopted at our institution.

RET analysis

Genomic DNA was extracted from peripheral blood using the QIAamp DNA Mini kit (QIAGEN, Milano, Italy) according to the manufacturer's protocol. Exons 5, 8, 10, 11 and 13–16 of the RET (covering 98% of multiple endocrine neoplasia type 2 (MEN2) a, 95% of MEN2 b and 95% of familiar MTC mutations) were examined by direct sequencing, as described elsewhere [3]. Polymerase chain reactions (PCRs) were performed using primers designed to flank the splice junctions of target exons. Amplified products were sequenced on an ABI 3730 analyzer (Applied Biosystems, Foster City, CA, USA), using BigDye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA). Sequences were analyzed using seqscape software (Applied Biosystems, Foster City, CA, USA). Informed consent for the genetic analyses was obtained from the all patients.

Heterophilic blocking tube

The HBT (Scantibodies, Santee, CA, USA) contains a unique blocking reagent consisting of specific binders that inactivate heterophilic antibodies; it was employed according to the manufacturer's instructions, using 500 μL of sample. The reagent is in the form of a lyophilized pellet at the bottom of the tube. Once the specific binders have bound to the heterophilic antibodies, the antibodies are no longer able to cause immunoassay interference.

Clinical case

A 15-year-old patient was referred to our institution in May 2014 with moderately elevated CT levels. CT assay was requested by his primary care physician as part of his screening for thyroid diseases. The patient had a history of mild subclinical non-autoimmune hypothyroidism and he had suffered from a tibial osteomyelitis a few years earlier. His family history revealed no members with MEN2. His serum CT was 50.5 ng/L and its elevation was confirmed by the same laboratory in a second sample (68.8 ng/L). This moderately high CT level was then further confirmed at our own laboratory (73.9 ng/L). Thyroid US documented a colloidal goiter. Secondary causes of the hyperCT were ruled out. A calcium stimulation test could not be performed due to a severe sinus bradycardia (<40 beats per minute) [4] emerging on cardiological examination. A pentagastrin (Pg) stimulation test could not be performed because Pg has recently become unavailable in Italy, as in most European countries.
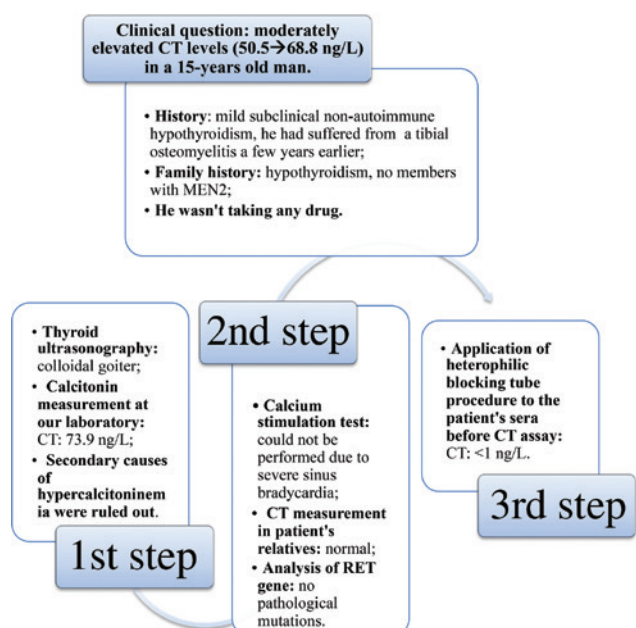
Figure 1: Schematic of clinical and laboratory case management of moderately elevated calcitonin (CT) levels.
MEN2, multiple endocrine neoplasia type 2.

Though CT measured in the patient's relatives was normal, we first ruled out the possibility of a familial cancer syndrome by analyzing the RET gene in our young patient, which revealed no pathological mutations. The mismatch between the clinical picture and the laboratory results prompted us to search for other reasons for the patient's high CT levels. Though often underestimated, the presence of heterophilic antibodies in the specimen is a potential cause of falsely elevated CT, so the HBT procedure was applied to the patient's sera before the CT assay. Using this pretreatment step, his serum CT concentration dropped to <1 ng/L, measured at the same laboratory (Figure 1).

Discussion

The development of detection methods characterized by adequate analytical sensitivity for the purpose of using CT as a tumor marker has proved clinically useful [5]. In the past, radioimmunoassays (RIAs) were used to measure CT, but they also measured CT precursors. The more specific assays employed later on – immunoradiometric assays (IRMAs) or CLIAs – mainly recognize the monomeric form of serum CT, but false positives and false negatives are still possible.

All immunoassays are based on antigen-antibody reactions. The tracer is chemically bound to an antigen (RIA) or antibody (IRMA/CLIA) and capable of producing a quantitatively measurable signal. Immunoassays can be classified by type of tracer (radioisotopic, enzymatic, fluorescent or chemiluminescent). A target analyte can be assessed by means of competitive or non-competitive assays.

Competitive and non-competitive immunoassays

In a competitive immunoassay (e.g. RIA, FIA, EIA, etc.) the unlabeled target analyte in a sample and a labeled analyte compete to bind an antibody, which is in a limited concentration. The main advantage of a competitive immunoassay lies in that it is suitable for measuring any antigens (protein or small molecule, such as a steroid or thyroid hormone) regardless of its molecular dimensions [6]. RIAs were the first to be used for CT assay in the past, but these assays could measure calcitonin-like proteins as well as CT, and its precursors too. There is a report in the literature (Table 1) of a 17-year-old girl referred to an endocrinologist for thyroid nodules and high plasma CT levels, as measured by RIA (Endocrine Sciences, Calabasas Hills,

Table 1: Clinical cases of immunoassay interference reported in the literature.

No. of patients	Assay principle	Type of interference	Medical history	References
1	RIA, Endocrine Sciences, one-site assay	Probable interference of CT-like proteins	Single thyroid nodule	Uwaifo et al. [7]
2	IRMA, Scantibodies, polyclonal antibodies, one step assay IRMA, CIS Bio International, monoclonal antibodies, one step assay	Hook effect	MTC patients	Tommasi et al. [8]
1	IRMA, Diagnostic Systems, polyclonal antibodies, one-step assay	Hook effect	Widely metastatic MTC	Leboeuf et al. [9]
4	ICMA, Nichols Institute Diagnostics, monoclonal antibodies, two-step assay	Heterophilic antibodies	Multinodular goiter	Papapetrou et al. [10]
1	IRMA, Biosource, monoclonal antibodies, two-step assay	Heterophilic antibodies	Multinodular goiter	Tommasi et al. [11]
1	IRMA, Biosource, monoclonal antibodies, two-step assay	Heterophilic antibodies	Multinodular goiter	Kim et al. [12]
5	CLIA, Siemens Healthcare Diagnostics, monoclonal antibodies, two-step assay	Heterophilic antibodies	Multinodular goiter	Giovanella et al. [13]
3	IFMA, in-house, monoclonal antibodies ECLIA, Roche Diagnostics, monoclonal antibodies, two-step assay	Macro-CT	History of MTC in follow-up and screening for MTC in V804M RET mutation carrier	Alves et al. [14]

CLIA, chemiluminescent immunometric assay; CT, calcitonin; ECLIA, electrochemiluminescence immunoassay; ICMA, immunochemiluminometric assay; IFMA, immunofluorometric assay; IRMA, immunoradiometric assay; Macro-CT, macrocalcitonin; MTC, medullary thyroid carcinoma; RIA, radio immuno assay.

CA, USA) in two consecutive samples [7]. The patient's hyperCT was in contrast with her clinical picture, so the Authors checked for causes of spurious hyperCT. Serial dilutions showed a marked non-linearity in antigen concentrations, thus pointing to the presence of an interfering substance in the patient's plasma. The patient's samples were therefore tested again after plasma extraction, using the same RIA method and CT levels were undetectable. Subsequent analysis with a different immunochemiluminometric assay (ICMA) confirmed that the CT level was below the assay's limit of detection.

Non-competitive immunoassays (IRMA/CLIA) involve two antibodies, one for capture and one for signaling, that recognize two different antigen epitopes, antibodies being in excess [15]. These methods are also called two-site assays, the principle behind them being a "sandwich" formed by the capture antibody, the antigen being measured, and the signal antibody. The signal antibody can be added at the same time as the capture antibody (one-step methods) or at other times during the assay incubation period (two-step methods).

Due to their high specificity, two-site and two-step immunoassays are currently regarded as the most accurate way to measure mature CT levels in serum [16, 17].

Hook effect

If antigens are in extremely high concentrations, then the capture and signal antibodies become saturated with the antigen, preventing the sandwich from forming. When the liquid phase is discarded, most of the antigen is lost together with the signal antibodies, so the resulting antigen concentration is falsely low. This is the so-called "hook effect" and it can only occur in one-step, two-site immunoassays, giving a low signal when the concentration of the target analyte is very high. This phenomenon affects solid-phase assays, where the concentrations of capture antibody may be limiting. It is less likely to occur when a two-step method is used, but it is still possible. If a one-step method uses polyclonal capture and signal antibodies, moreover, the antigen may become sandwiched between two signal antibodies, and this gives rise to a lower concentration when the liquid phase is discarded (Table 1) [8].

The hook effect in CT assays was described in a case report (Table 1) involving a patient with a large tumor burden and high carcinoembryonic antigen (CEA), but low CT levels, which aroused the suspicion of a false negative result [9]. CT was measured with a one-step, two-site IRMA kit using polyclonal antibodies (DSL-7700 ACTIVE

IRMA kit, Diagnostic Systems Laboratories, Inc., Webster, TX, USA). After sera dilutions, the CEA levels remained the same, but the CT levels gradually increased. When the same specimen was tested with another assay that involved a two-step procedure (IRMA-human calcitonin hCT, CIS Bio International, Gif-sur-Yvette, France) and two monoclonal antibodies, CT levels were found high.

## Heterophilic antibodies, definition and etiology

Kaplan and Levinson defined the interference from heterophilic antibodies as "an interference mechanism from human antibodies of any subclass against any part of the animal antibody, where the human antibodies are of sufficient titer and affinity to have analytically significant effect and the immunogen has not been identified" [18].

Heterophilic antibodies are human anti-animal antibodies of the IgG, IgA, IgM or IgE classes. Natural heterophilic antibodies are found in all individuals and their presence is largely independent of the person's age. Heterophilic antibodies have been found in up to 40% of healthy people [19], and can persist in the blood for several months after exposure to animal immunoglobulin (Ig). An anti-mouse monoclonal IgG was still detectable after 10 months in one study [20], and in another report [21] it persisted for up to 30 months after immunoscintigraphy. Both anti-idiotype antibodies (directed against the hypervariable region of the Ig) and anti-isotype antibodies (directed against the constant regions) may develop, though the latter are generally more common than the former [22].

Circulating anti-animal antibodies can arise from iatrogenic and non-iatrogenic causes. The former includes a normal response of the human immune system to the administration of a "foreign" protein antigen. Diagnostic and pharmaceutical agents derived from animal sources are widely used. Some recombinant proteins are purified in monoclonal mouse antibody columns and some of the mouse antibody may become detached and co-purify with the recombinant protein [23]. Blood transfusions are also associated with an increased incidence of anti-animal antibodies, possibly as a result of the infusion of pre-existing human anti-animal antibody or of a foreign antigen in the unit of blood [24]. Some vaccines against viral or bacterial diseases contain animal serum capable of inducing heterophilic antibodies in man [25]. Heterophilic antibodies may often develop in animal handlers too [26].

Among the non-iatrogenic causes of heterophilic antibodies, one known condition involves the transfer
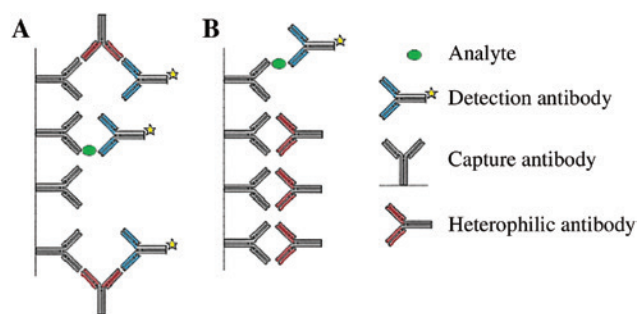
Figure 2: Schematic of heterophilic antibodies and their mechanisms of interference.
(A) False-positive interference. (B) False-negative interference.

of antibodies across the placenta. The transfer of dietary antigens across the gut wall has also been reported, especially in celiac disease. In fact, the absorption of allergens may be facilitated by mucosal damage (as in celiac disease), with the stimulation of antibody production [22].

Mechanisms of interference in immunoassays

In immunometric assays, heterophilic antibodies can form a bridge between capture and signal antibodies, leading to false-positive results in the absence of the target analyte or, if the sample also contains an analyte, to a false elevation in its measured levels (Figure 2). Heterophilic antibody's presence can generate false-negative results if the interfering antibody blocks the antigen-binding site on the capture antibody, preventing any binding of the antigen (Figure 2).

When an interference from heterophilic antibodies is suspected, a dilution test can be performed. The interference of endogenous antibodies in immunoassays can be confirmed by serial dilutions, which demonstrate a marked non-linearity in antigen concentrations [27]. This test is inaccurate in detecting heterophilic antibody interference, however, because other factors may influence the lack of linearity, and some cases with interfering antibodies show a perfect linearity on serial dilution [28].

Another method involves sample pretreatment to neutralize the heterophilic interference. Polyethylene glycol (PEG) Ig precipitation has been proposed, but it is time-consuming, difficult to automate and not very accurate because it is accompanied by an analyte co-precipitation. The interference of heterophilic antibodies can also be tested by using another kit with different detection antibodies, since the interference usually concerns a specific antibody or pair of antibodies.

To prevent heterophilic antibody interference, manufacturers now add blocking reagents in the form of small quantities of animal serum (mouse or goat), polyclonal animal IgGs, or IgG fragments from the same species as the one used to raise the reagent antibody. This technique reduces the interference, but does not always eliminate it completely [22].

Finally, samples can be pretreated with heterophilic blocking reagent (HBR) or HBT to inactivate heterophilic antibodies. These reagents have a higher binding affinity for human anti-animal antibodies (heterophilic antibodies) than non-specific blocking agents (like those used in conventional blocking procedures). They contain a unique blocking reagent composed of specific binders, a proprietary mix of lyophilized mouse anti-human IgM with a high affinity for human anti-animal antibodies that inactivate heterophilic antibodies [22]. Although commonly used when heterophilic antibodies are suspected, these blocking reagents are not always fully effective in preventing heterophilic antibody interference, as previously demonstrated [29].

Interference of heterophilic antibodies in calcitonin assay

Tumor marker assays for human chorionic gonadotropin (hCG), prostate-specific antigen (PSA), cancer antigen 125 (CA 125), CEA, and CT too, have all been reported to suffer from the problem of heterophilic antibody interference, frequently with unwarranted clinical outcomes [30].

Many cases of heterophilic antibodies interfering with CT assays have been described in the literature (Table 1) [10–13], and the prevalence of this interference has been estimated at up to 1.3% in patients with thyroid nodules, and up to 3.7% in the general population [10, 31]. Papapetrou et al. [10] reported on three patients with nodular goiter who underwent surgical neck exploration after an erroneous diagnosis of MTC due to high levels of CT being detected with a two-site immunochemiluminometric assay (Nichols Calcitonin ICMA) employing two monoclonal antibodies (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA): when the HBT procedure was applied, their CT concentrations were normal, suggesting the presence of heterophilic antibodies (Table 1).

When CT levels are found moderately high (as often happens when heterophilic antibodies are involved), a calcium/Pg stimulation test is the best way to distinguish secondary hyperCT from a C-cell disease. The clinician should suspect a hyperCT of being false when there is no significant increase in serum CT after calcium or Pg

stimulation. Tommasi et al. [11] described the case of a 73-year-old man with a multinodular goiter and high basal plasma CT levels on monoclonal antibody-based IRMA (CT-US-IRMA; Biosource, Nivelles, Belgium): findings on fine-needle aspiration (FNA) cytology to seek evidence of MTC and a mild increase in plasma CT during the Pg stimulation test gave the impression that the patient's high CT levels might depend on a cross-reaction with heterophilic antibodies. Using two different animal immunoreactive antibody fragments generally included among the assay reagents was evidently not enough to neutralize the heterophilic antibodies' interference in this patient. In fact, the CT levels dropped by more than 80% after the HBT was added (Table 1).

Later on, Kim et al. [12] studied a 31-year-old female with nodular thyroid disease, hyperCT (144.7 ng/L, CT-US-IRMA; Biosource, Nivelles, Belgium), and CEA within normal limits. The patient was otherwise healthy and her medical history was unremarkable. Her family history revealed no members with MTC. There was no evidence of MTC on FNA cytology. When a calcium stimulation test was performed, the lack of response aroused a suspicion of spurious hyperCT, so serum CT was measured using another kit (IRMA-human CT, hCT; CIS Bio International, Gif-sur-Yvette, France) and CT proved undetectable. A dilution test was performed and showed non-linearity. To confirm the suspected interference of heterophilic antibodies, the authors measured serum CT again after applying HBT and its level decreased to 1.56 ng/L with the originally-used kit. It is worth noting that both the first and the second kits used in this case were two-step IRMAs with monoclonal antibodies, which goes to show that the interference is often specific towards a particular antibody, or pair of antibodies (Table 1).

All these cases reported in the literature underscore the importance of HBT/HBR procedures in the clinical management of spurious hyperCT due to heterophilic antibodies.

Macrocalcitonin

A recent paper demonstrated a new source of interference in CT assays, i.e. the presence of macrocalcitonin (macro-CT), responsible for three cases (Table 1) of hyperCT (measured with an in-house kit involving two-site monoclonal antibodies) in the absence of any thyroid C-cell disease [14]. This phenomenon had already been described for some proteins and peptides, and it is due to the formation of macro-aggregates between CT and Igs (mainly IgG) directed against the CT protein. Heterophilic antibody interference was ruled out in these patients because their

CT concentrations remained linear after serial dilutions, and adding an excess of mouse serum to the assay did not modify the CT level. Macro-CT was suspected in the light of a low recovery after PEG treatment, an immunoreactivity for CT as a macroaggregate in gel filtration chromatography and the finding of CT-Ig complexes on protein A-Sepharose analysis.

Problems with comparing CT measurements from different laboratories

As a consequence of the differences in assay formats, the antibodies used and the different CT precursors, a common problem with CT assay concerns the difficulty of comparing different assays. Various forms of CT precursors may be detected in blood samples, as explained earlier. The concentrations of these peptides vary with clinical status, renal function and tissue origin of the CT (normal or ectopic production). When CT is measured with antibodies that do or do not recognize different CT precursors, its level varies depending on the antisera used in the assay. Since various and variable CT isoforms and fragments can be found in some patients, there may be a significant disagreement between different CT assay results, even if the assays are calibrated with the World Health Organization's second international reference preparation. Martinetti et al. [32] compared four IRMAs and RIAs for the purpose of measuring CT: the values they returned were very scattered and a patient's classification according to CT cutoff varied depending on which specific assay was used. The American Thyroid Association suggests that an individual should be followed up using the same CT assay over time. It also advocates that the CT assay used should be noted on every laboratory report, and that laboratories also notify clinicians of any changes to their methods [17].

Sex- and age-related normal CT reference ranges

There are also some issues concerning the normal reference ranges for CT. Most laboratories set their normal CT range at <10 ng/L for both males and females, irrespective of age and anthropometric factors, but normal CT levels depend on both gender and age, and possibly on body weight and thyroid volume too, as recently suggested [33, 34]. In adults at least, primary CT values should be interpreted in the light of gender-specific reference ranges: men have higher CT levels than women, possibly because they have nearly twice as many C cells, as demonstrated

by a post-mortem study [35]. Much the same gender-related differences as in normal basal CT levels are seen after calcium or Pg injection in healthy subjects and MTC patients [36, 37]. Gender-specific differences in both basal and stimulated CT levels have recently been reported in the case of C-cell disease too. Using a two-site automated CLIA, the basal CT cutoff capable of separating non-MTC conditions (including normality and C-cell hyperplasia) from MTC patients was 26 ng/L in females and 68 ng/L in males, while the corresponding calcium-stimulated CT cutoffs were 79 ng/L and 544 ng/L, respectively [4].

The few data available on age-specific CT levels in young children suggest that CT levels are particularly high during the early weeks of life. The reference range for serum CT is wider in children (especially the newborn) than in adults [38]. A recent survey conducted on 2740 subjects aged from 1 day to 16 years, who underwent blood tests for conditions not affecting serum CT, confirmed that the normal range of CT levels is higher in children than in adults, especially during the first 2 years of life (with normal limits up to 48.9 and 14.7 ng/L during the first and second years, respectively) and that CT levels decrease from the third year of life onwards, gradually coming to parallel those of adults [39].

Beyond serum CT

Since cytological evaluation has a poor sensitivity in identifying MTC, being its detection rate of around 56.4% in patients with MTC, as recently estimated [40], measuring CT in the washout fluid after FNA is a valuable tool for the purpose of recognizing MTC [41, 42]. It was recently introduced by the American Thyroid Association in its latest MTC guidelines and recommended for use in the case of inconclusive or MTC-suggestive cytological findings [16].

Given that CT assay suffers from several pre-analytical, analytical and post-analytical problems, a systematic review recently provided a comprehensive analysis on the use of the CT precursor ProCT as a diagnostic marker of MTC [43]. From a pre-analytical point of view, ProCT is a very stable protein, so ProCT samples are easier to manage than CT samples, which need to be kept on ice throughout the entire processing chain. All commercial ProCT assays use the same antibodies too, so they produce comparable results. Post-analytically, in the preoperative diagnosis of MTC, ProCT values correlate with the extent of the disease and with biochemical cure rates. After total thyroidectomy, ProCT serum levels show a specificity of 57%–100% in cured patients, and a sensitivity of 84%–100% in patients

with active/recurrent MTC. ProCT measurement has consequently been suggested as a valuable complementary test in MTC diagnostics for patients with thyroid nodules and increased basal CT levels [44]. One major bias in the use of ProCT as a tumor marker of MTC lies in the possibility of a concurrent bacterial infection. More data are needed, moreover, to establish the optimal ProCT cutoff levels for the management of C-cell disease.

Conclusions

In conclusion, two main points arise from our clinical case.

1. CT measurement
   In addition to the various recommendations on routine CT measurements in patients with thyroid nodular disease, it is important to emphasize that assessing CT without appropriate diagnostic justification can expose patients to unnecessary and even serious risks and psychological stress. This was the case in our patient, whose general practitioner had measured serum CT even before obtaining a neck US. As a result of this unnecessary CT measurement, the patient then underwent further expensive tests. In other medical settings where less clinical expertise is available, this patient might even have undergone unwarranted surgical treatment with lifelong repercussions.

2. Excluding secondary causes of hypercalcitoninemia
   When moderately elevated CT levels are found, secondary causes or laboratory interferences should always be considered, especially when there are discrepancies between a patient's clinical and biochemical data. It is mandatory to rule out such secondary causes by obtaining a detailed medical history and biochemical tests. A calcium/Pg stimulation test is normally the best way to distinguish secondary from primary hyperCT, but it could not be performed in our patient due to a severe sinus bradycardia: had we performed this test on the strength of a falsely elevated CT measurement, we would have exposed our patient to far from negligible potential cardiac consequences, as recently demonstrated [45].
   When an interference is suspected, CT should be measured with another assay kit. In our case, CT was first measured at two different laboratories, but it subsequently emerged that the two laboratories used the same kit, LIAISON CT II-Gen. To avoid any confusion, all laboratories should state in their report which type of assay they have used. This is
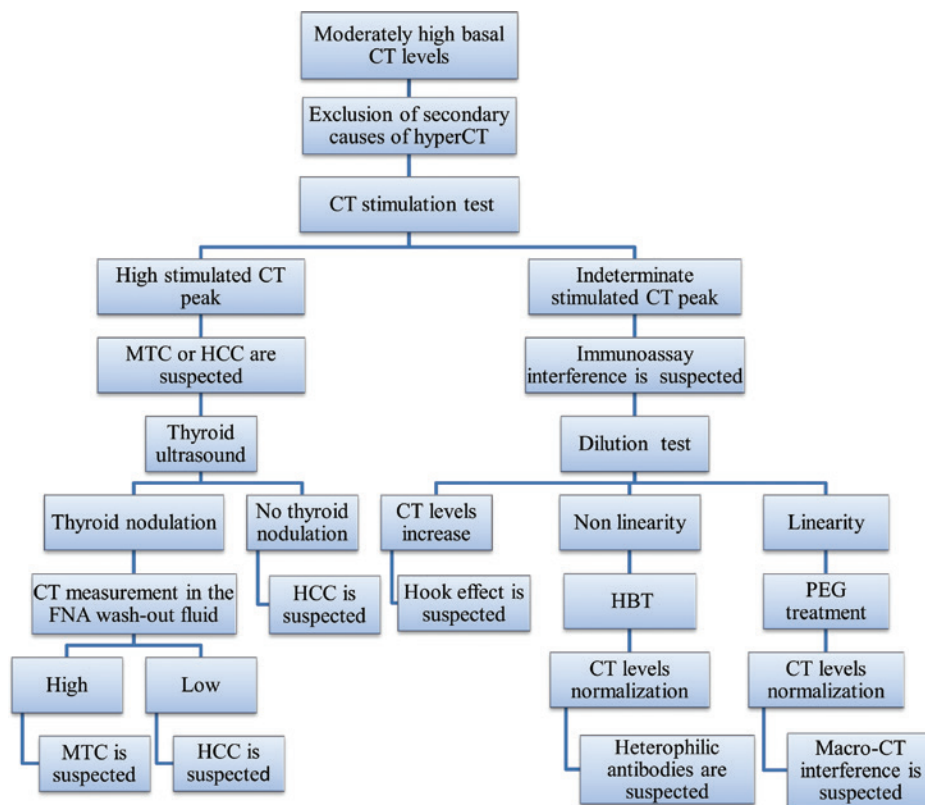
Figure 3: Algorithm integrating clinical, radiology and laboratory testing in the interpretation and follow-up of moderately elevated calcitonin (CT) levels.
FNA, fine-needle aspiration; HBT, heterophilic blocking tube; HCC, C-cell hyperplasia; hyperCT, hypercalcitoninemia; macroCT, macrocalcitonin; MTC, medullary thyroid carcinoma; PEG, polyethylene glycol.

particularly important in the case of CT measurement because of the variety of commercial kits available, the lack of a standardized CT measurement procedure, and the various types of possible interference. Clinicians should know which assay has been used and be aware of its limits.

In conclusion, measuring plasma CT has an important role in screening for C-cell disease, but moderately elevated serum CT levels need to be placed in their clinical context, bearing in mind all the potential secondary causes of C-cell hyperplasia and the possibility of laboratory interference, before exposing patients to the risks and costs of further tests (Figure 3).

Like other similar cases reported in the literature, the case of our patient clearly shows that, even when two-site, two-step IRMAs or CLIAs (the assays with the highest specificity for CT detection) are considered, false-positive results can still occur, and they are more common than might be expected. Heterophilic antibody interference, as well as other not infrequent sources of interference, should always be taken into account as a potential cause

of spurious hyperCT, especially when clinical and biochemical data are inconsistent.

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