**Leila Guastapaglia**

# DOSAGEM DE TIROGLOBULINA NO SEGUIMENTO DE PACIENTES COM CARCINOMA DIFERENCIADO DE TIROIDE: INTERFERENTES, NOVOS ENSAIOS E APLICAÇÃO CLÍNICA

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina para obtenção do título de Doutora em Ciências.

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**Orientadora**: Profª. Drª. Rosa Paula Mello Biscolla

**Coorientador:** Prof. Dr. José Gilberto Henriques Vieira

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**ESCOLA PAULISTA DE MEDICINA**

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## Resumo

**Introdução:** O Carcinoma Diferenciado de Tiroide (CDT) é a neoplasia tiroidiana mais comum e tem a dosagem de tiroglobulina (Tg) como principal marcador tumoral. A dosagem de Tg evoluiu muito ao longo dos anos, porém ainda apresenta desafios, apesar de os ensaios imunométricos usuais apresentarem ótimo desempenho. **Objetivo:** O objetivo principal dessa tese é avaliar o papel dos ensaios de Tg e os principais interferentes que resultam em valores falsamente baixos ou altos no seguimento de pacientes com CDT. **Métodos**: No primeiro manuscrito foram selecionados pacientes com doença estrutural e dosagem de Tg subestimadas pelo ensaio imunométrico para avaliação do papel de um novo ensaio competitivo policlonal (Tg-c) desenvolvido no Laboratório de Endocrinologia da UNIFESP com sensibilidade funcional de 20 ng/mL. Os resultados também foram comparados com os obtidos pelo ensaio por cromatografia líquida/espectrometria de massas (LC-MS/MS). No segundo projeto reportamos dois casos de interferência na dosagem de Tg por anticorpos heretófilos e os métodos usados para a investigação. No terceiro projeto foram selecionados 129 pacientes com CDT e anticorpos antitiroglobulina (AcATg) negativos e positivos para a comparação e avaliação do desempenho clínico de três ensaios diferentes de Tg (2 imunométricos e um ensaio por LC-MS/MS). **Resultados:** No primeiro projeto os pacientes que apresentavam doença estrutural e dosagens inapropriadas de Tg pelo ensaio imunométrico apresentaram níveis detectáveis de Tg-c variando entre 22 e 710 ng/mL. No segundo projeto, 2 pacientes que receberam critério de excelente resposta ao tratamento passaram a apresentar dosagens de Tg elevadas durante o seguimento (7,5 e 17 ng/mL) sem a detecção de doença estrutural. Realizamos diluição seriada nas amostras, que mostraram linearidade, porém as dosagens de Tg por outro ensaio imunométrico e por LC-MS/MS resultaram indetectáveis, comprovando a presença de interferentes que levaram a valores falsamente elevados. No terceiro projeto a correlação entre os dois ensaios imunométricos e o ensaio por LC-MS/MS foi substancial (r 0.950-0.985) nos pacientes com AcATg negativos e pobre entre os ensaios imunométricos e por LC-MS/MS (r 0.875-0.878) na presença de AcATg. 58.8% dos pacientes com doença estrutural e AcATg positivos apresentaram dosagem de Tg por LC-MS/MS indetectável. Apesar disso, nesses pacientes, a dosagem de Tg pelo ensaio imunométrico foi em torno de

30% mais baixa do que por LC-MS/MS. A sensibilidade em detectar doença estrutural nos pacientes com AcATg positivos foi maior pelo ensaio imunométrico do que por LC-MS/MS. **Conclusões:** Concluímos que os ensaios imunométricos ainda devem ser usados como método padrão para a dosagem de Tg, porém estão sujeitos a interferentes. Nessas situações, a dosagem de Tg por métodos diferentes como a LC-MS/MS e o ensaio competitivo policlonal ou por outro ensaio imunométrico podem apresentar benefícios.

**Descritores:** Carcinoma diferenciado de tiroide, tiroglobulina, anticorpo antitiroglobulina, anticorpos heterofilos, LC-MS/MS.

## Abstract

**Introduction:** Differentiated Thyroid Carcinoma (DTC) is the most common thyroid neoplasm and has thyroglobulin (Tg) measurement as the main biochemical marker. The measurement of Tg has evolved a lot over the years, but it still poses challenges, despite the usual immunometric assays show great performance. **Objective:** The main aim of this is to evaluate the role of Tg assays and the main interferents that result in falsely low or high values of Tg in the follow-up of DTC patients. **Methods:** In the first manuscript, patients with structural disease and Tg levels underestimated by immunometric assay were selected to evaluate the role of a new polyclonal competitive assay (Tg-c) developed at the Endocrinology Laboratory of UNIFESP with a functional sensitivity of 20 ng/mL. The results were also compared with those obtained by the liquid chromatography/mass spectrometry (LC-MS/MS) assay. In the second study we report two cases of interference in the Tg measurement by heretophilic antibodies and the methods employed for the investigation. In the third manuscript, 129 DTC patients with negative and positive anti-thyroglobulin antibodies (TgAb) were selected for the comparison and evaluation of the clinical performance of three different Tg assays (2 immunometrics assays and LC-MS/MS assay). **Results:** In the first study, patients with structural disease and inappropriate levels of Tg by immunometric assay had detectable Tg-c levels ranging from 22 to 710 ng/mL. In the second manuscript, 2 patients who had criteria of excellent response to treatment began to present elevated Tg levels during follow-up (7.5 and 17 ng/mL) without the detection of structural disease. We performed serial dilution in the samples, which showed linearity, but the Tg measurements by another immunometric assay and by LC-MS/MS were undetectable, proving the presence of interferents that have led to falsely high values. In the third manuscript, the correlation between the two immunometric assays and the LC-MS/MS assay was substantial (r 0.950-0.985) in patients with negative TgAb and poor between the immunometric and LC-MS/MS assays (r 0.875-0.878) in the presence of TgAb. 58.8% of patients with structural disease and positive TgAb had undetectable Tg levels by LC-MS/MS. Despite this, in these patients, the Tg values obtained by the immunometric assay were around 30% lower than by LC-MS/MS. The sensitivity in detecting structural disease in patients with

positive TgAb were higher by the immunometric assays than by LC-MS/MS. **Conclusions:** We conclude that immunometric assays should still be used as a standard method for measuring Tg, but they are prone to interference. In these situations, the measurement of Tg by different methods such as LC-MS/MS and the competitive polyclonal assay or by another immunometric assay may bring benefits.

**Keywords:** Differentiated thyroid carcinoma, thyroglobulin, antithyroglobulin antibody, heterophile antibodies, LC-MS/MS.

## Apresentação

Os três trabalhos apresentados nesta tese originaram-se de ideias que surgiram em 1970 com o desenvolvimento do primeiro radioimunoensaio por Van Herle em 1973. No Brasil o primeiro ensaio para dosagem de tiroglobulina foi publicado na tese de doutorado do Professor Dr. Rui Monteiro de Barros Maciel em 1983: ”*Desenvolvimento de um método radioimunológico para a dosagem de tiroglobulina sérica e sua aplicação no seguimento de pacientes portadores de câncer diferenciado da tiróide¨.*

Com o passar dos anos os radioimunoensaios foram substituídos pelos ensaios imunométricos e contando com a expertise do Professor Dr José Gilberto Henriques Vieira (coorientador desta tese) novos ensaios foram desenvolvidos, incluindo o que é realizado atualmente no Laboratório de Endocrinologia Molecular e Translacional da Universidade Federal de São Paulo - Escola Paulista de Medicina (UNIFESP-EPM) e utilizado no atendimento dos pacientes do ambulatório de Tiroide da Disciplina de Endocrinologia da UNIFESP-EPM): “*Development, characterization and clinical validation of new sensitive immunofluorometric assay for the measurement of serum thyroglobulin. Arq Bras Endocrinol Metab”*, 2012 doi: 10.1590/s0004-27302012000900010.

Dez anos se passaram desde a publicação deste trabalho e apesar da evolução dos ensaios de tiroglobulina, principalmente em relação à sensibilidade funcional que passou de 1 ng/mL para 0,1 ng/mL, os desafios em relação a esta dosagem continuam presentes no dia a dia.

Esta tese de doutorado tem como tema os interferentes na dosagem de tiroglobulina divididos em dois tópicos principais: a presença de anticorpos antitiroglobulina levando a resultados subestimados de tiroglobulina e, a presença de anticorpos heterofilos resultando em valores falsamente altos.

Nesta tese para obtenção do título de Doutor em Ciências, apresentamos, de acordo com as recomendações do Programa de Pós-graduação em Endocrinologia Clínica da Universidade Federal de São Paulo, três artigos científicos que tem por base a discussão da importância da dosagem de tiroglobulina no seguimento de

pacientes com carcinoma diferenciado de tiroide, apresentar os principais desafios nesta dosagem e sugerir algumas alternativas para “resolvê-los”.

**Manuscrito 1**: *“The role of a new polyclonal competitive thyroglobulin assay in the follow-up of patients with differentiated thyroid cancer with structural disease but low levels of serum thyroglobulin by immunometric and LC-MS/MS methods” Endocrine*, 2021 *DOI: 10.1007/s12020-020-02526-8*

**Manuscrito 2:** *"False diagnosis of biochemically recurrent thyroid carcinoma and its unnecessary investigation – the importance of testing for heterophile antibodies*". Submetido na revista *Thyroid (manuscript* ID THY-2022-0201)

**Manuscrito 3:** *“Correlation and clinical performance of three thyroglobulin assays in the management of differentiated thyroid cancer patients”,* em preparação.

## Sumário

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## Lista de abreviaturas, siglas e símbolos

**Ac** Anticorpos

**AcATg** Anticorpos antitiroglobulina

**CDT** Carcinoma Diferenciado de Tiroide

**HAMA** Anticorpos anti-IgG de camundongo (traduzido do inglês: *Human anti-mouse antibodies*)

**INCA** Instituto Nacional de Câncer

**$^{125}$I** $^{125}$Iodo

**LC-MS/MS** Cromatografia líquida/espectrometria de massas (traduzido do inglês: *Liquid Chromatography-Tandem mass Spectrometry*)

**PCI** Pesquisa de corpo inteiro

**SF** Sensibilidade Funcional

**Tg** Tiroglobulina

**TgLT4** Tiroglobulina colhida em uso de levotiroxina

**Tgs** Tiroglobulina estimulada

**TNM** Sistema internacional de classificação de tumores (Do inglês: *Tumor, Lymph Node, Metastasis*)

**TSH** Hormônio Tiroestimulante (traduzido do inglês: *Thyroid Stimulating Hormone*)

# 1 INTRODUÇÃO

O carcinoma diferenciado de tiroide (CDT) representa o câncer endócrino mais comum e o tipo mais prevalente de neoplasia maligna tiroidiana, sendo o subtipo papilífero responsável por 80-85% dos casos, enquanto o folicular responde por cerca de 5-10% (1–3). O câncer de tiroide é considerado o câncer cuja incidência mais cresceu nos últimos anos e atualmente é o quinto tipo de câncer mais comum entre as mulheres. Dados americanos mostram um aumento de incidência acima de 200% desde a década de 1970 (4). Segundo o Instituto Nacional de Câncer (INCA), sua incidência estimada para cada ano do triênio 2020-2022 é de 11.950 casos entre as mulheres e 2.310 entre os homens, o que corresponde ao risco estimado de 2,17 casos a cada 100 mil homens e 11,15 casos a cada 100 mil mulheres (5). Este aumento é atribuído principalmente ao microcarcinoma papilífero que vem sendo cada vez mais detectado pelo aperfeiçoamento de técnicas de imagem e solicitação de exames de forma indiscriminada porém, a taxa de mortalidade praticamente não sofreu modificações ao longo dos anos (6,7).

## 1.1 Tiroglobulina como marcador do carcinoma diferenciado de tiroide

A Tiroglobulina (Tg) é uma proteína heterogênea, com alto peso molecular de 660.000 daltons, que se expressa exclusivamente na célula folicular tiroidiana, é o substrato para a produção dos hormônios tiroidianos e pode ser dosada no sangue periférico de indivíduos que apresentam tecido tiroidiano. A dosagem de Tg é utilizada como o principal marcador bioquímico, com alta sensibilidade e especificidade, para detectar precocemente persistência e recorrência de doença no seguimento de pacientes com CDT (1). Na maioria dos pacientes o tratamento inicial do CDT consiste na tiroidectomia total, embora lobectomia possa ser realizada em casos selecionados. Após tiroidectomia total, a terapia com radioiodo é indicada em todos os carcinomas de alto risco de recorrência e em alguns com riscos intermediário e baixo. Após o tratamento cirúrgico a concentração sérica de Tg atinge o nadir em 3-4 semanas. Entretanto, após a terapia com radioiodo, podem ser necessários alguns meses até que a Tg atinja valores indetectáveis na circulação (8). Valores detectáveis de Tg,

após esse período, sugerem a presença de restos tiroidianos ou persistência de doença (locorregional ou metástases à distância). Já nos pacientes não submetidos ao tratamento com radioiodo, valores baixos de Tg podem ser encontrados devido à presença de restos de tecido tiroidiano normal. Os valores de Tg estão diretamente relacionados à quantidade de tecido tiroidiano remanescente normal ou tumoral (9).

No início do século XXI, quando a sensibilidade do ensaio de Tg era 1,0 ng/mL, foi definido que o paciente seria considerado curado se a dosagem de Tg sob o estímulo do TSH (após o uso de TSH recombinante humano ou pela retirada do hormônio tiroidiano, em hipotiroidismo) fosse inferior a 1-2 ng/mL (10,11). Após o estímulo com TSH ocorre elevação dos valores de Tg em 5-10 vezes quando comparados aos valores basais (em uso de levotiroxina - TgLT4), aumentando, portanto, a sensibilidade da dosagem da Tg. Com o desenvolvimento dos ensaios mais sensíveis demonstrou-se que valores basais inferiores a 0,1-0,2 ng/mL tem boa correlação com a Tg estimulada (Tgs) inferior a 1-2 ng/mL, o que tornou desnecessária a dosagem da Tgs principalmente nos pacientes de baixo e intermediário riscos de recorrência (12–15).

Segundo as recomendações dos principais consensos de manejo do CDT, após 6 meses a 1 ano do tratamento inicial, o paciente é reavaliado e de acordo com as dosagens de Tg, anticorpos antitiroglobulina (AcATg), ultrassonografia cervical e/ou outros exames de imagem é definida a resposta ao tratamento (Estratificação dinâmica de risco). Visto que nem todos os pacientes são submetidos à radioiodoterapia, níveis diferentes de Tg são utilizados para a classificação de resposta nestes pacientes. A tabela 1 discrimina os critérios para classificação de acordo com a resposta após o tratamento (1,9). Após a reavaliação inicial, é recomendado que a Tg seja analisada a cada 6 meses a 1 ano ou com maior frequência nos pacientes sem resposta completa ao tratamento (1).

**Tabela 1: Estratificação dinâmica de risco**

| | Dosagem de Tg* | | AcATg | Imagem |
|---|---|---|---|---|
| | *Com radioiodo* | *Sem radioiodo* | | |
| **Excelente Resposta ao Tratamento** | TgLT4 < 0,2 ng/mL ou Tgs < 1 ng/mL | TgLT4 < 0,2 ng/mL ou Tgs < 2 ng/mL | Ausentes | Negativa |
| **Resposta Estrutural Incompleta** | Qualquer nível | Qualquer nível | Qualquer título | Evidência de doença em exames estruturais ou funcionais |
| **Reposta Bioquímica Incompleta** | TgLT4 > 1 ng/mL ou Tgs > 10 ng/mL | TgLT4 > 5 ng/mL ou Tgs > 10 ng/mL ou Elevação dos valores ao longo do seguimento | Títulos em elevação | Negativa |
| **Resposta Indeterminada** | TgLT4 0,2-1 ng/mL ou Tgs 1-10 ng/mL | TgLT4 0,2-5 ng/mL ou Tgs 2-10 ng/mL | Estáveis ou em declínio | Achados não específicos ou Baixa captação no leito tiroidiano na PCI |

Fonte: Adaptado de Haugen *et at* [1], 2016 e Momesso *et al* [9], 2016.

Tg: Tiroglobulina; AcATg: Anticorpo Antitiroglobulina; TgLT4: Tiroglobulina colhida em uso de levotiroxina; Tgs: Tiroglobulina estimulada (TSH > 30 UI/mL); PCI: Pesquisa de Corpo Inteiro.

*Ensaios de tiroglobulina com sensibilidade funcional < 0,1-0,2 ng/mL.

## 1.2 Ensaios para dosagem de tiroglobulina

A Tg pode ser mensurada por dois diferentes métodos: imunoensaios e cromatografia líquida/espectrometria de massas (LC-MS/MS). Os imunoensaios, por

sua vez, podem ser divididos em ensaio imunométrico ou radioimunoensaio (16). O primeiro método para a dosagem de Tg sérica foi descrito por Van Herle em 1973 e tratava-se de um radioimunoensaio que usava Tg humana marcada com iodo radioativo e um anticorpo gerado em coelhos (17). Após esse primeiro ensaio, houve grande evolução e atualmente dispomos de métodos mais sensíveis, rápidos, automatizados e precisos. O ensaio mais comumente utilizado nos dias de hoje é o ensaio imunométrico por ser totalmente automatizado, rápido e possuir ótima sensibilidade (18,19). Os três métodos disponíveis para a dosagem de Tg possuem diferentes sensibilidades, especificidades e susceptibilidade a interferentes na análise (Tabela 2) (20).

**Tabela 2: Ensaios para a dosagem de Tiroglobulina**

| | | | **Interferência** | | |
|---|---|---|---|---|---|
| **Ensaio** | **Princípio** | **Sensibilidade Funcional (ng/mL)** | **AcATg** | **Anticorpos Heterófilos** | **Tg Heterogênea** |
| Radioimunoensaio | Imunoensaio Competitivo | 0,5-5 | Sim (falso positivo e falso negativo) | Não | Não |
| Ensaio Imunométrico | Imunoensaio Não competitivo | 0,1-0,9 | Sim (falso negativo) | Sim (falso positivo e falso negativo) | Sim (falso negativo) |
| LC-MS/MS | Quantificação direta da Tg | 0,5-1 | Potencialmente não | | |

Fonte: Adaptado de *Algeciras-Schimnich* (20), 2018 e *Spencer e Fatemi* (21), 2013.
AcATg: Anticorpo Antitiroglobulina; Tg: Tiroglobulina; LC-MS/MS: Cromatografia líquida/ espectrometria de massas.

A sensibilidade funcional (SF) ou limite de quantificação para ensaios de Tg é definida como a menor concentração que pode ser medida no soro com um coeficiente de variação menor do que 20% durante um período de 6 a 12 meses usando pelo menos dois lotes diferentes de reagentes e dois instrumentos de calibração (15,22). A sensibilidade funcional deve ser específica de cada ensaio e determinada por um

padrão internacional de calibração (BCR®457) (23,24). Já a sensibilidade analítica ou limite de detecção é definida como o menor valor que um analito pode ser detectado distinguível de zero (22).

É recomendado que as dosagens seriadas de Tg, durante o seguimento dos pacientes com CDT, sejam realizadas pelo mesmo ensaio e preferencialmente pelo mesmo laboratório a fim de evitar variações inter e intraensaios. A variação dos valores de Tg entre os diferentes ensaios ocorre devido à heterogeneidade biológica da estrutura da Tg e ao reconhecimento de diferentes epítopos da molécula de Tg pelos anticorpos (poli ou monoclonais) utilizados no desenho dos ensaios, que variam de cada fabricante (1,20,25,26).

### 1.2.1 Radioimunoensaios

Os radioimunoensaios são ensaios competitivos, pouco utilizados na atualidade devido à sua menor sensibilidade, por serem pouco automatizados e usarem radiofármacos em suas reações, ficando restritos a alguns centros de estudo (27–29). Nesse ensaio, a Tg marcada com $^{125}$Iodo compete com a Tg da amostra pelo anticorpo de captura (anticorpo antitiroglobulina policlonal produzido em coelho). Um segundo anticorpo (IgG anti-coelho) é usado para precipitar o complexo Tg-anticorpo e a radioatividade liberada por esse complexo é lida, sendo inversamente proporcional à quantidade de Tg presente na amostra (Figura 1) (17,30–32).

A dosagem de Tg por radioimunoensaio foi introduzida nos anos 1970 como um método sensível para complementar o seguimento de pacientes com CDT (17,33). A SF inicial variava de 5-15 ng/mL e não era suficiente para detectar valores baixos de Tg em pacientes com doença persistente/recorrente. Atualmente a SF varia entre 0,5 e 5 ng/mL (27–29). Esse ensaio é reconhecido por ser menos susceptível à interferência pelos AcATg por utilizar anticorpos policlonais que podem reconhecer epítopos de Tg mesmo quando ligados aos AcATg endógenos (18,27,34,35).

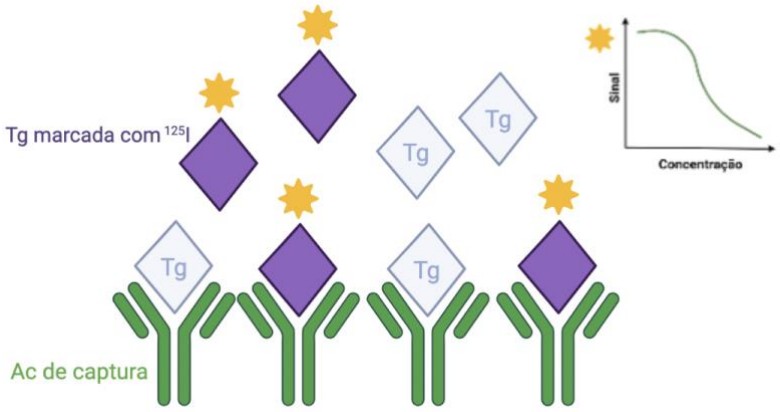


**Figura 1. Representação esquemática do radioimunonensaio.**
Tg: Tiroglobulina; Ac: anticorpo; $^{125}$I: $^{125}$Iodo.

### 1.2.2 Ensaios imunométricos

Os ensaios imunométricos são baseados na interação entre antígenos e anticorpos e inclui a) uma fase inicial (fase sólida) na qual o anticorpo de captura liga-se ao analito da amostra (Tg); e b) uma fase na qual o anticorpo de detecção, acoplado a uma molécula sinalizadora, se liga em uma outra região (epítopo) da Tg capturada, criando um “sanduíche”. O sinal liberado após a formação do “sanduíche” é então mensurado, determinando, de forma direta, a concentração da Tg (Figura 2) (19,30,36–38).

O ensaio imunométrico foi desenvolvido no início dos anos 1980 e atualmente é o ensaio mais utilizado para a dosagem de Tg por ser rápido, amplamente disponível comercialmente e com boa SF. A primeira geração dos ensaios imunométricos tinha SF de 0,5-1 ng/mL, mas com o avanço do método, atualmente dispomos de ensaios comerciais com SF inferior a 0,1 ng/mL (ensaios de segunda geração). A introdução dos ensaios de segunda geração na prática clínica reduziu a necessidade da mensuração da Tg estimulada pelo TSH durante o acompanhamento de pacientes com CDT (12–15).

A principal limitação dos ensaios imunométricos é a sua susceptibilidade à interferência por AcATg e anticorpos heterófilos (20). Alguns ensaios disponíveis comercialmente utilizam anticorpos direcionados a epítopos não comumente reconhecidos pelos AcATg endógenos e outros utilizam anticorpos policlonais de captura ou detecção visando reduzir essa interferência (39). No entanto, devido à

heterogeneidade dos AcATg endógenos de pacientes com CDT essas estratégias nem sempre são eficazes (40).

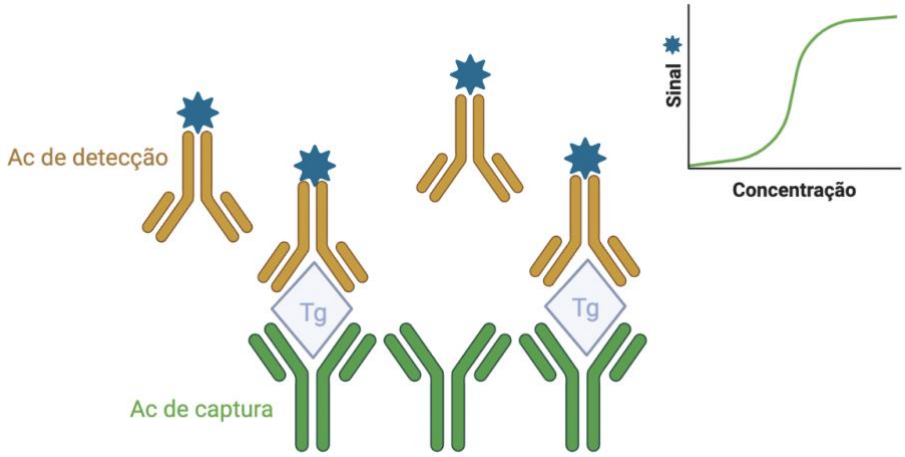


**Figura 2. Representação esquemática do ensaio imunométrico.**
Tg: Tiroglobulina; Ac: anticorpo.

### 1.2.3 Cromatografia líquida/espectrometria de massas (LC-MS/MS)

O método LC-MS/MS é baseado na quantificação direta da Tg após o uso de tripsina para clivar proteínas. Na primeira etapa da análise (fase pré-analítica), há a complexação da Tg livre através da adição de um AcATg monoclonal e após a precipitação das proteínas por "salting out" (adição de sulfato de amônio). Em seguida, a tripsina é adicionada e ocorro a digestão das proteínas grandes, como a Tg, em pequenos peptídeos, o que possibilita a sua mensuração. Os peptídeos específicos da Tg (VIFDANAPVAVR ou FSPDDSAGASALLR) são imunocapturados e separados de outras proteínas por lavagens subsequentes. O anticorpo antipeptídeo é então desconjugado do peptídeo e na próxima etapa há a separação pela cromatografia líquida. Por fim a Tg é detectada usando a espectrometria de massas (Figura 3) (22,31,41). A proteólise com tripsina tem potencial vantagem de quebrar outras proteínas como os AcATg endógenos e anticorpos heterófilos, eliminando-os como interferentes (31,37). O método apresenta boa correlação com o ensaio imunométrico, especialmente nas amostras sem interferentes (42,43).

O primeiro ensaio para dosagem de Tg por LC-MS/MS foi publicado em 2008 por Hoofnagle et al (41) com SF de 2,6 ng/mL. Desde então diversos métodos foram

publicados e a SF tem progressivamente melhorado, atualmente está entre 0,4-1 ng/mL (20,31,42–44).

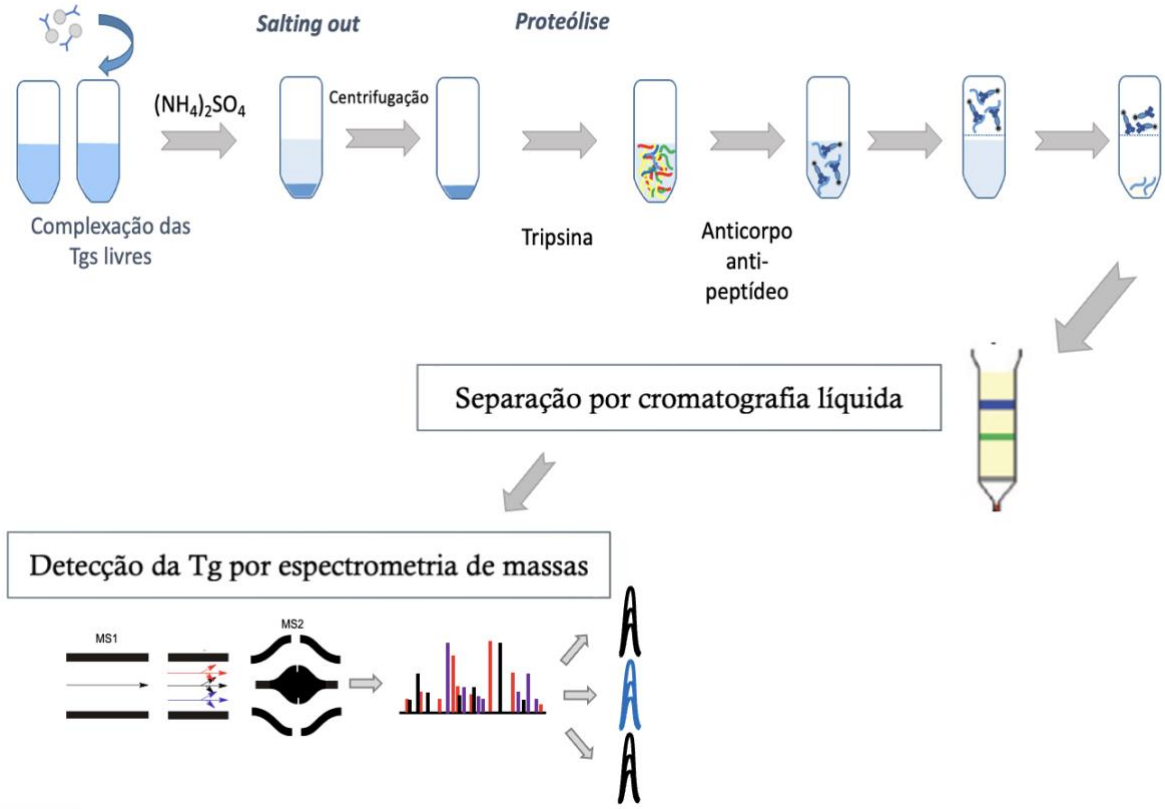


**Figura 3. Representação esquemática da Cromatografia líquida/espectrometria de massas**
Tg: Tiroglobulina

## 1.3 Desafios na dosagem de tiroglobulina

Apesar de todo progresso, ainda enfrentamos desafios nas dosagens de Tg, que podem levar a resultados falsamente elevados ou reduzidos e até mesmo indetectáveis. Nessas situações, a Tg torna-se um marcador tumoral não confiável. A principal interferência se dá pela presença de anticorpos endógenos: autoanticorpos antitiroglobulina e anticorpos heterófilos (45). Uma outra causa que deve ser considerada como desafio na dosagem de Tg é a presença de formas heterogêneas circulantes levando a resultados falsamente baixos (46).

### 1.3.1 Anticorpos heterófilos e Anticorpos antianimais

Anticorpos heterófilos é um termo genérico utilizado para designar qualquer anticorpo anti-IgG que leve a resultados falsos em imunoensaios e podem ser classificados em: a) anticorpos heterófilos “latu senso”: quando são anticorpos produzidos sem a exposição a antígenos específicos, que são considerados de ocorrência natural, chegando a estar presentes em 40% da população, porém com interferência nos ensaios imunométricos muito baixa; e b) anticorpos antianimais: quando produzidos após exposição aguda ou crônica a antígenos específicos e sua interferência pode chegar a 3% (47–49). Os anticorpos anti-IgG de camundongo (HAMA) são os mais comumente encontrados e de maior relevância dentro desse grupo (36,45). A presença de anticorpos heterófilos interfere nos ensaios imunométricos levando, na maior parte dos casos, a resultados falso positivos (caso eles liguem-se aos anticorpos de captura e revelação, formando uma ponte) ou, na sua minoria, a resultados falso negativos (caso liguem-se a apenas um dos anticorpos do ensaio) impedindo a formação do “sanduiche” (Figura 4) (49).

A presença de anticorpos heterófilos deve ser considerada quando os valores de Tg não são compatíveis com o cenário clínico de pacientes com CDT (12,50). A investigação de amostras suspeitas inclui a dosagem de Tg após diluições seriadas para avaliar linearidade, o pré-tratamento das amostras com tubos bloqueadores de anticorpos heterófilos e a dosagem da Tg com outro ensaio imunométrico ou outro método como LC-MS/MS (36,51,52). As amostras são consideradas positivas para interferência por anticorpos heterófilos quando a curva de diluição não é linear, o pré-tratamento com tubos bloqueadores altera significantemente o resultado ou quando os resultados por outros ensaios ou métodos são discrepantes (49). Embora esses passos geralmente sejam suficientes para detectar a presença de anticorpos heterófilos na amostra, eles frequentemente falham em determinar o valor correto da concentração de Tg. Recentemente, a LC-MC/MS foi descrito como método útil nessas situações, uma vez que a tripsina pode digerir os anticorpos heterófilos (42,50).

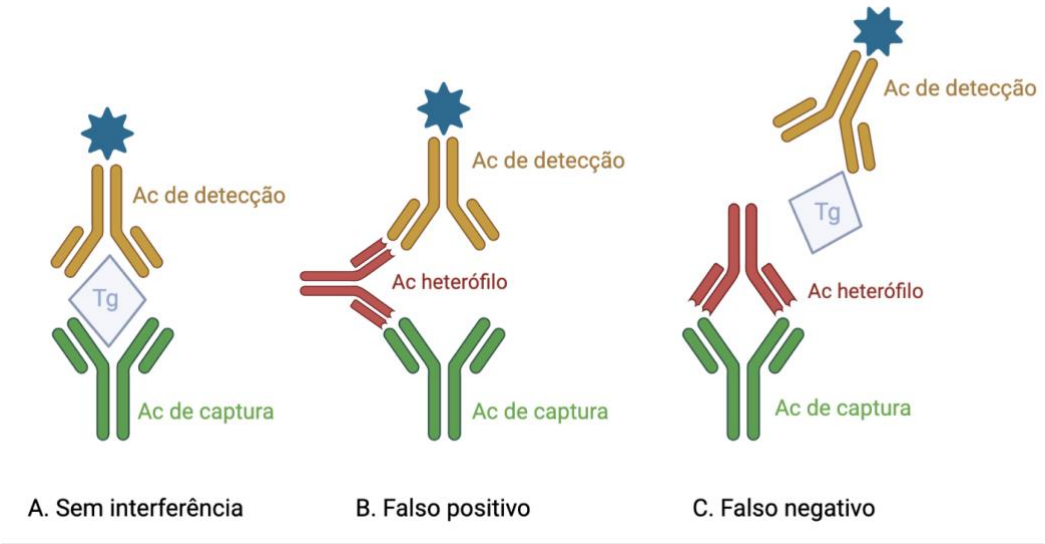


**Figura 4. Representação esquemática do ensaio imunométrico. A, sem interferentes. B, com anticorpos heterófilos resultando em falso positivo. C, com anticorpos heterófilos resultando em falso negativo.**

Tg: Tiroglobulina; Ac: Anticorpo.

### 1.3.2 Anticorpos antitiroglobulina e Heterogeneidade da tiroglobulina circulante

Diferentemente da interferência dos anticorpos heterófilos, a presença de AcATg e a heterogeneidade da Tg circulante podem levar, mais frequentemente, a resultados inapropriadamente baixos ou indetectáveis nos ensaios imunométricos (15,37). A Tg apresenta uma complexa via de metabolização que pode sofrer desregulação nos carcinomas, levando à produção de Tg com isoformas diferentes, as quais podem não ser reconhecidas pelos anticorpos dos ensaios imunométricos usuais (53,54).

Em relação aos AcATg, que estão presentes em 25-30% dos pacientes com CDT, a interferência nos ensaios imunométricos ocorre quando a ligação acontece no mesmo epítopo que seria ocupado pelos anticorpos de captura e/ou de revelação do ensaio não permitindo a formação do “sanduíche” e levando a resultados falsamente baixos (figura 5) (34,37,44,45). Por esse motivo é fundamental que a dosagem de Tg seja sempre realizada junto com a de AcATg, minimizando a possibilidade de que pacientes com doença estrutural não sejam identificados. A interferência dos AcATg na dosagem de Tg é variável entre os pacientes e pouco correlacionada com os seus títulos, o que torna difícil predizer o quanto os valores de Tg serão afetados

considerando apenas a concentração de AcATg (18,31,37,44,55,56). Alguns estudos mostram que sua presença pode subestimar os níveis de Tg em até 80% (34). A interferência de baixos títulos de AcATg (acima da sensibilidade funcional porém abaixo do valor de referência; anticorpos *borderline*) não é clara e alguns autores defendem que mesmo títulos muito baixos ainda podem interferir na dosagem da Tg (27,55,57–59). Isso ocorre pois os valores de referência dos ensaios de AcATg são determinados para o diagnóstico de doença tiroidiana autoimune e não para detectar a interferência nos imunoensaios. Alguns autores postulam que qualquer valor de AcATg acima da SF ou até mesmo do limite de detecção deva ser considerado positivo e potencialmente interferente na dosagem de Tg em pacientes com CDT (27,35,37). Entretanto, estudos recentes mostraram que a interferência de títulos *borderline* de AcATg na dosagem de Tg não é relevante (60,61).

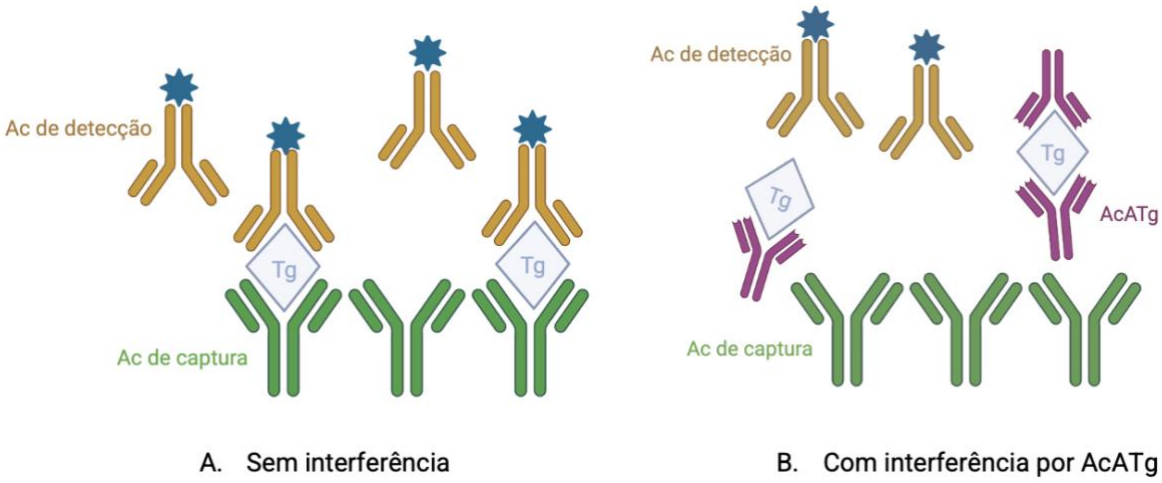


**Figura 5. Representação esquemática do ensaio imunométrico A: sem interferentes e B: com interferência por AcATg endógenos.**

Tg: Tiroglobulina; Ac: anticorpo; AcATg: Anticorpo Antitiroglobulina.

Embora os títulos de AcATg possam ser usados como marcador indireto da presença de Tg (*surrogate marker*) e portanto de persistência ou recorrência do CDT, a determinação da doença em pacientes com AcATg positivos ainda é desafiadora, já que os títulos dos anticorpos podem não se correlacionar exclusivamente com a carga tumoral mas indicar atividade do sistema imune (55,56,61). É fundamental que ao usar o AcATg como marcador tumoral ele seja realizado sempre pelo mesmo ensaio e preferencialmente pelo mesmo laboratório (34,55). Após o tratamento inicial do paciente com CDT é esperado que os títulos de AcATg declinem progressivamente e

negativem em poucos anos (62). O declínio de mais de 50% dos títulos, assim como a persistência de títulos estáveis ao longo do seguimento indicam ausência de doença e boa resposta ao tratamento. Entretanto, a elevação consistente (maior do que 50%) alerta para a presença de doença estrutural (21,56,63). Em virtude de todas as dificuldades no seguimento de pacientes com AcATg positivos, os pacientes são frequentemente submetidos a exames adicionais e tratamentos desnecessários (54).

Os radioimunoensaios são descritos como mais resistentes à interferência por AcATg, devido ao desenho deste ensaio, conforme descrito anteriormente. Entretanto, valores falsamente elevados são frequentemente encontrados (31,37,52,64) e alguns autores demonstraram também a presença de resultados subestimados (25). O determinante da interferência positiva ou negativa depende da interação entre a Tg e AcATg endógenos e a especificidade dos anticorpos utilizados no ensaio (21). A Figura 6 mostra uma das formas de interferência.

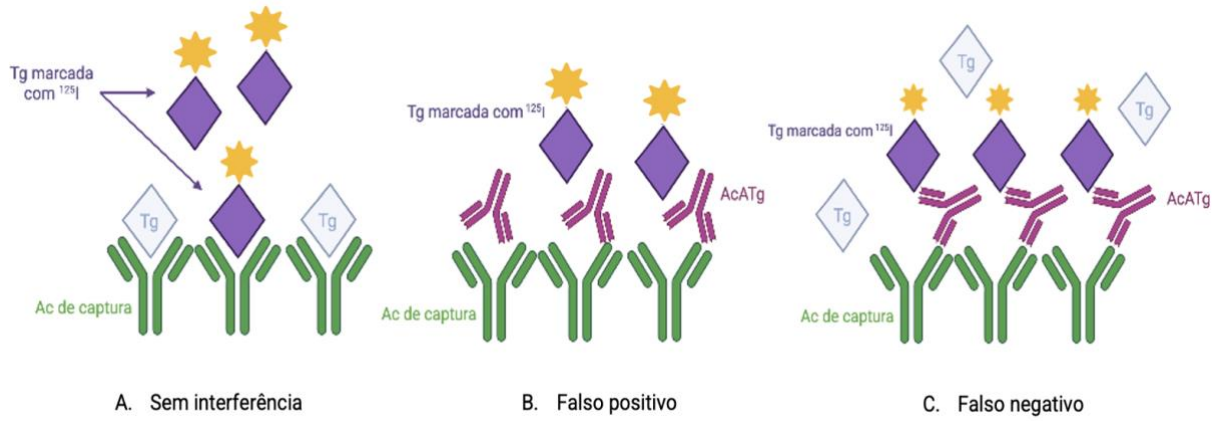


**Figura 6. Representação esquemática do radioimunonensaio A, sem interferentes. B, com interferência de AcATg endógenos resultando em falso positivo. C, com interferência de AcATg endógenos resultando em falso negativo.**

Tg: Tiroglobulina; Ac: anticorpo; AcATg: Anticorpo Antitiroglobulina; $^{125}$I: $^{125}$Iodo.

Em busca de alternativas para quantificar a Tg de pacientes que apresentam interferentes (AcATg e anticorpos heterófilos) ou Tg anômala, a dosagem de Tg por LC-MS/MS vem sendo reportada como uma opção desde 2008 (31,37,41,43), uma vez que nestes casos tende a ser mais precisa que os ensaios imunométricos (54). Alguns estudos mostram benefício adicional em até 20% dos casos de pacientes com CDT e AcATg positivos (34,43). A adequada quantificação da Tg nesses pacientes

reduz os custos com exames desnecessários além do potencial benefício na melhora na qualidade de vida pela correta determinação da presença ou não de doença (37).

Como descrito anteriormente, o ensaio é baseado na quantificação direta de Tg após digestão das proteínas de alto peso molecular com tripsina, separação por cromatografia liquida e detecção por espectrometria de massas. No mesmo processo em que a Tg é clivada, como benefício secundário, há também a digestão dos AcATg e anticorpos heterófilos, eliminando-os como interferentes. Como resultado, a dosagem de Tg por LC-MS/MS é um método mais confiável pois sofre menos interferência desses anticorpos (31,42,43,50,54,65). Embora diversos estudos tenham reportado o seu benefício, dados recentes sugerem que a falha da LC-MS/MS em detectar a Tg nos pacientes com AcATg positivos ocorre em até 40% dos casos (37,43,65).

# 2 OBJETIVOS

## 2.1 Geral

Avaliar o papel dos ensaios de tiroglobulina e seus principais interferentes no seguimento de pacientes com Carcinoma Diferenciado de Tiroide.

## 2.2 Específicos

**Manuscrito 1:** Avaliar o desempenho clínico de um ensaio policlonal competitivo para dosagem de Tg no seguimento de pacientes com Carcinoma Diferenciado de Tiroide que apresentam valores subestimados de Tg e doença estrutural e comparar os resultados aos dos ensaios imunométrico e LC-MS/MS.

**Manuscrito 2:** Discutir a importância da pesquisa de interferentes na dosagem de Tg levando a resultados falso positivos e os métodos usados para sua investigação**.**

**Manuscrito 3:** Comparar e avaliar o desempenho clínico de três ensaios de Tg em pacientes com Carcinoma Diferenciado de Tiroide com anticorpos antirioidianos negativos e positivos.

## 3 MANUSCRITO 1

# *The role of a new polyclonal competitive thyroglobulin assay in the follow-up of patients with differentiated thyroid cancer with structural disease but low levels of serum thyroglobulin by immunometric and LC-MS/MS methods*

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**Abstract**

**PURPOSE:** The aims of this study were to assess the role of an in-house competitive thyroglobulin assay (Tg-c) in the follow-up of metastatic differentiated thyroid carcinoma (DTC) patients who presented underestimated Tg measurements by immunometric assays (Tg-IMA) and to compare the results with IMA and LC-MS/MS Tg methods.

**METHODS:** This prospective study included 40 patients. Twenty-one with metastatic disease: 14 had Tg-IMA levels inappropriately low or undetectable (8 patients with positive and 6 with borderline TgAb) and 7 had high Tg-IMA levels. Nineteen had an excellent response to therapy. The competitive assay employs a polyclonal antibody produced in rabbits immunized with human Tg, Tg labeled with biotin and, for the solid phase separation, a monoclonal anti-rabbit IgG antibody adsorbed to microtiter plates.

**RESULTS:** All 14 patients with structural disease and underestimated levels of Tg-IMA presented detectable Tg-c levels. The median Tg-c level in the group with positive TgAb was 183 µg/L (range: 22 to 710 µg/L), and 58 µg/L (range 23-148 µg/L) in the borderline TgAb group. The levels of Tg-LC-MS/MS were detectable in some patients (range < 0.5-18 µg/L). All 7 patients with high Tg-IMA presented also high levels of Tg-c. Only 2/19 patients with excellent response had Tg-c levels above the functional sensitivity.

**CONCLUSION:** The competitive assay was able to detect Tg in all patients, even in the presence of serum TgAb and may be an option in patients with underestimated Tg-IMA and relevant structural disease.

**Keywords:** thyroglobulin, LC-MS/MS, competitive assay, thyroglobulin antibodies and thyroid cancer

## Introduction

Serum thyroglobulin (Tg) is the main biochemical marker and an independent predictor of disease persistence and recurrence in differentiated thyroid carcinoma (DTC) patients. After treatment with total thyroidectomy and radioiodine ablation, undetectable Tg measured under TSH suppression by immunometric assays (Tg-IMA) with functional sensitivity (FS) of 0.1 μg/L indicates an excellent response to treatment. On the other hand, Tg elevation may indicate recurrence of the disease [1–3].

Since the first Tg method described by Van Herle in 1973 [4], different assays have been used in the follow-up of DTC patients, such as the immunometric assays (IMA) and, more recently, liquid chromatography/tandem mass spectrometry (LC-MS/MS) [5,6]. However, the following pitfalls persist in clinical practice: 1) the presence of heterophilic antibodies and endogenous human antimouse IgG antibodies (HAMA) [5–8]; 2) the presence of thyroglobulin autoantibodies (TgAb) [5,6,9–12]; and 3) the production of anomalous Tg by the tumor, resulting in heterogeneity of the circulating Tg, not identified by routine IMA [12–15]. In the presence of HAMA, falsely high Tg levels can be observed, although less frequently false low levels can also be found [7]. On the other hand, in the presence of TgAb and anomalous Tg, underestimated Tg values are usually observed in patients with metastatic disease [11,12,16]. The presence of TgAb, which occurs in up to 25 to 30% of DTC patients, may interfere with Tg measurement and lead to inappropriately low or even undetectable levels when IMA are used. The interference is variable between patients and loosely correlated with TgAb concentrations [5,6,10–12]. The TgAb, in these patients can be used as a surrogate tumor marker because the serum levels are not correlated with the tumor load but rather indicate the activity of the immune system [13]. On the other hand, the persistence of low levels of TgAb is not always associated with recurrent or persistent disease. The interference of detectable TgAb concentrations below the reference limit (borderline TgAb) in Tg-IMA is not clear and some authors defend that they can really interfere in this assay [10,16].

Since 2008, Tg measurement by liquid chromatography/tandem mass spectrometry (Tg-LC-MS/MS) has been proposed in the follow-up of DTC patients, mainly in those who present positive TgAb. The assay uses trypsin to cleave all proteins, including TgAb, potentially eliminating them as interferers and could be an alternative in these patients [5,17]. However, some studies have shown that the

measurement of Tg by LC-MS/MS still fails in 23-43% of TgAb-positive samples and might not show superiority to Tg measured by IMA [5,11,18].

Therefore, the goals of this study were to assess the role of an in-house, competitive Tg assay in the follow-up of metastatic DTC patients who presented underestimated (undetectable or inappropriately low) Tg measurements by IMA and to compare the results with those of IMA and LC-MS/MS Tg assays.

## Materials and Methods

### *Design*

This was a prospective study conducted at the Thyroid Disease Center in the Division of Endocrinology, Department of Medicine, *Escola Paulista de Medicina, Universidade Federal de São Paulo* (in São Paulo, Brazil) and approved by the Institutional Ethics Committee. Signed informed consent was obtained from all patients.

### *Patients*

From 600 DTC patients followed in our center, we selected 40 patients for this study. Group 1 included 14 patients presenting structural disease and underestimated (inappropriately low or undetectable) Tg-IMA levels. These 14 patients (16 samples) were divided into Group 1a, presenting positive TgAb (8 patients, 9 samples) and Group 1b, presenting borderline TgAb levels [i.e. below manufacturer cut off (MCO) and above FS] confirmed by two different assays (6 patients, 7 samples). Ten patients in Group 1 also had Tg measured by LC-MS/MS, in addition to Tg-IMA.

Group 2 included 7 patients with structural disease and high Tg-IMA measurements and were selected as the disease control group. Group 3 included 19 patients with excellent response to therapy (undetectable Tg-IMA, negative TgAb and cervical ultrasonography) and were considered the non-disease control group.

Structural disease was defined as evidence of disease in neck ultrasound and cytological analyses, whole body scans with uptake out of the thyroid bed, computed tomography (CT) or positron emission tomography-computed tomography (PET/CT). The variables analyzed included patient age at diagnosis, sex, thyroid cancer histological type, therapy and activity of radioiodine received. All samples were collected under levothyroxine therapy and low TSH levels. An excellent response to

therapy was defined based on the ATA consensus of 2015[1].

*In-house Competitive Tg Assay (Tg-c)*

This assay employs a polyclonal anti-Tg antibody produced in our laboratory in the 1980s and used for several years in a traditional Tg radioimmunoassay [19,20]. To obtain this Tg antibody, rabbits were immunized with human Tg by the multiple injection protocol: the Tg used for immunization was obtained from thyroid samples of patients submitted to subtotal thyroidectomy for the treatment of Graves' disease and purified by ammonium sulfate precipitation [19].

This new Tg-c assay was conducted in 12x75 mm polypropylene tubes; 250 µL of patient samples or Tg standards (CRM457) were pipetted in each tube, followed by 250 µL of Tg antibody in a final dilution of 1/5,000 in 50 mM Tris-HCl, pH 7.75, with 0.5% BSA and 0.05% bovine gamma globulin; after brief mixing, the samples were incubated for 72 hours at 4 °C. Tg standards were diluted in Tg-free human sera. To obtain Tg-free human sera, a pool of Tg and TgAb-negative samples were first treated with active charcoal (40 g/L); after 7 hours of mixing, the lot was centrifuged at 13,000 rpm for 30 minutes at 4 °C; this serum was then filtered and submitted to immune affinity with sepharose 4B coupled to an anti-Tg monoclonal antibody (produced in our laboratories against the same Tg preparation used for the polyclonal production). After 5 hours of mixing, the serum was separated by pouring the gel into a column and filtering through a 0.45 µm filter. The standard curve includes samples containing the Tg reference preparation (CRM457) at 400, 80, 16 and 3.2 µg/L diluted in the Tg-free serum.

After a 72-hour incubation, 250 µL of biotinylated Tg, diluted in 50 mM of Tris buffer with 0.5% BSA and 0.05% bovine gamma globulin containing 2% normal mouse serum, in a 1/5,000 dilution were added, followed by a 48-hour incubation at 4 °C. Tg from Sigma was used for biotinylation with EZ-Link Sulfo Biotin (Pierce Biotechnology, Rockford, USA). We used 20 µg of biotin and 2 mg of Tg in this reaction.

Solid phase separation employed microtiter plates (Nunc, Roskield, Denmark) coated with a monoclonal antibody produced at our laboratories against rabbit IgG (2 µg/well). Two hundred microliters were pipetted in triplicate, and the plates were incubated for 5 hours at room temperature. After 12 cycles of washing with 50 mM Tris-HCl buffer, 200 µL of streptavidin labeled with Europium (1/2,000, Perkin Elmer, Turku, Finland) was added to each well; the plates were then incubated for 30 minutes

under agitation and washed again 12 times with Tris-HCl buffer. In the final step, 200 µL of fluorescence solution (Wallac Oy, Turku, Finland) was added to all the wells, and the fluorescence was read in a time-resolved fluorometer (Victor MultilabelCounter, Perkin Elmer, Turku, Finland). The results were analyzed using Multicalc program 2.2 (Perkin Elmer).

For a sample with a mean value of 6.3 µg/L (n=7), the intra-assay coefficient of variation was 17%, and for a mean value of 164.3 µg/L (n=7), it was 2.3%. The interassay coefficient of variation was 22% for a mean of 5.3 µg/L and 7.6% for a mean value of 142.3 µg/L. The analytical sensitivity of the assay was defined as the lowest amount of Tg that can be differentiated from the zero point with 95% confidence and was on the order of 9.4 µg/L, with a functional sensitivity (FS) of 20 µg/L, calculated based on 8 assays performed over a 4-month period.

*Serum assays: Tg, TgAb and TSH*

During the study period, the assays used for Tg and TgAb measurements changed. The Tg-IMA assays used were:

(i) chemiluminescent, Beckman Access immunoassay (Beckman Coulter, Fullerton, CA, USA), with FS 0.1 µg/L. Analytical sensitivity was 0.01 µg/L; intra-assay coefficient of variation (CV) was 1.4% for a pool with a mean value of 4.2 µg/L, 1.4% for a pool with a mean value of 21.6 µg/L, 4.4% for a pool with a mean value of 130.4 µg/L, and 2.0% for a pool with a mean value of 344.7 µg/L. Inter-assay CV was 1.7%, 1.8%, 4.9% and 4.0% for the same pools.

(ii) electrochemiluminescent Roche®, FS 0.1 µg/L. Intra-assay CV was 2.2% for a pool with a mean value of 1.11 µg/L, 1.2% for a pool with a mean value of 1.59 µg/L, 3.0% for a pool with a mean value of 89.3 µg/L, 2.5% for a pool with a mean value of 247 µg/L and 1.9% for a pool with a mean value of 470 µg/L. Inter-assay CV was 3.0%, 2.6%, 4.2%, 3.2% and 3.8% for the same pools.

(iii) in-house immunofluorometric assay, FS 0.5 µg/L. Intra-assay CV of 7.2% for a pool with a mean of 0.67 µg/L, 3.7% for a pool with a mean value of 2.9 µg/L, and 4.9% for a pool with a mean value of 158.0 µg/L. Inter-assay CV was 15.8%, 12.5%, and 10.8% for the same pools. [21].

The Tg-LC-MS/MS was performed at Mayo Medical Laboratories (FS 0.5 µg/L).

TgAb assays were as follows: indirect electrochemiluminescent immunoassay, Roche® (MCO less than 115 kIU/L, limit of detection of 10 kIU/L and limit of quantification of 15 kIU/L) and in-house immunofluorometric assay (MCO less than 40

kIU/L, FS of 15 kIU/L). Between-run precision was <5% along the standard curve. [22] These assays were standardized against the International Reference Preparation 65/93 standard.

TSH levels were measured using an in-house immunofluorometric assay with FS 0.05 mIU/L [23].

*Statistical analyses*

The data are presented as the median, minimum and maximum values. Based on the Tg detection limit and functional sensitivity of the competitive assay, nonparametric tests were considered the best options for the analysis. We used the Wilcoxon test to compare Tg values in two groups and Kruskal-Wallis for analysis with more than two groups. The statistical analysis was performed with IBM SPSS Statistics for Windows, Version 26.0, released in 2019 (IBM Corp., Armonk, N.Y., U.S.A.). A value of $p < 0.05$ was considered significant.

## Results

Table 1 describes the characteristics of the 40 patients included in the study. Thirty-six were women (90%), and the median age at diagnosis was 39 years (range: 10 to 81 years). All patients had been treated with total or near total thyroidectomy, 36/40 patients had papillary thyroid carcinoma (PTC), and all but two had received radioiodine ($^{131}$I) treatment (30 to 950 mCi). Nine patients (22.5%) presented positive TgAb.

In Group 1a (underestimated Tg-IMA levels with positive TgAb), Tg-IMA varied from < 0.1 to 5.1 µg/L (7/8 were undetectable), with median of 0.50 µg/L, confirming the fact that Tg-IMA is not a good disease marker in the presence of TgAb. Seven out of 8 patients had Tg measured by LC-MS/MS, with median of 0.8 µg/L (range: < 0.5-18 µg/L) and in 4 of them the measurements were < 2 µg/L. All patients had positive Tg-c and the median was 183 µg/L (range: 22 to 710 µg/L), showing that this assay can be useful in these patients. Figure 1 describes the results of Tg from group 1 in the 3 different assays. Table 2 depicts patients from Group 1a (patients 1-8).

In Group 1b (underestimated Tg-IMA levels with borderline TgAb; Table 2, patients 9-14), the median Tg-IMA was 0.50 µg/L (range < 0.1- 1.4 µg/L) and just one patient presented detectable Tg-IMA. On the other hand, Tg-c was detectable in all of

the patients and varied from 23 to 148 µg/L, with median of 58 µg/L. Only 3 patients in this group had Tg measured by LC-MS/MS: 2 had results that were detectable but below 2 µg/L (patients 9 and 11; table 2) and 1 patient (patient 10; table 2) presented negative Tg-LC-MS/MS.

In Group 2 (structural disease and high Tg-IMA measurements), Tg-IMA varied from 7.2 to 4440 µg/L and high levels of Tg-c assay were also observed (range of Tg-c: 41 to > 1000 µg/L) (Figure 2). In Group 3 (patients classified as having an excellent response to therapy) all but 2 patients had Tg-c up to FS (20 µg/L) (Figure 2).

## Discussion

The follow-up of DTC patients with structural disease and negative Tg measurements is challenging. Underestimated levels of Tg measured by IMA can be observed in patients with anomalous Tg produced by the tumor and in those with positive TgAb [5,6,9–15]. In these patients, a TgAb trend can be used as surrogate marker, although imprecise, and they still pose a significant challenge due to the uncertainty that arises regarding their disease status [11,13]. The presence of TgAb is not a strong predictor of the presence of residual or recurrent thyroid cancer but rather indicate the activity of the immune system [13,24]. The TgAb may persist for many years in low concentrations without clear evidence of disease and these patients frequently undergo several image studies that may not identify clinically significant disease [16,25,26]. In these cases a Tg measured by a different assay might be useful [13]. The Tg measurement by LC-MS/MS has been described as having no interference from TgAb and could be an option when TgAb are positive. However, recent studies have shown that this measurement can fail in up to 40% of patients, which can lead to a misclassification of patients as having an excellent response to therapy [5,18,26].

In this study, we describe a competitive Tg assay that could be useful in patients with relevant structural disease presenting Tg-IMA levels that are undetectable or inappropriately low. The Tg-c levels were positive in all 14 patients with structural disease and underestimated Tg-IMA measurements (Group 1). The highest Tg-c level (710 µg/L) was found in a patient with extensive lung and neck metastasis (Table 2, patient 4) and the lowest Tg-c was 22 µg/L in a patient with extensive local disease (Table 2, patient 6). Although local cervical disease can be expected in patients with

negative Tg-IMA, we found detectable Tg-c in these patients (Table 2, patients 6,7,10,12). Tg-c measurements were also high in Group 2 (disease control group), validating this assay as a reliable marker.

Therefore, this competitive assay was able to identify/measure Tg in patients with positive TgAb (Group 1a), which may indicate a potential use of this assay in this situation. The difference probably derives from the fact that the Tg-c method uses polyclonal antibodies with wide epitope specificities that have the potential to measure a larger range of abnormal tumor-derived thyroglobulin isoforms, in contrast with IMA methods, which employ monoclonal antibodies with restricted epitope specificities [6,21].

Five out of 6 patients from Group 1b (borderline TgAb) had negative Tg-IMA in the presence of structural disease, showing that, even in the presence of very low levels of TgAb (under MCO), Tg can not be detected by some IMA, as described previously [6,11]. In this group, Tg-c levels were also detectable in all patients.

We must be aware that there are no standardized TgAb methods for use in the follow-up of DTC patients and currently there is no consensus in the literature on how to define a positive test for TgAb in DTC patients [13]. The MCO level used to consider a patient with positive or negative TgAb is optimized for diagnosing autoimmune disease/thyroiditis and not for detecting TgAb interference in Tg measurements [6,10,16]. Some authors support that the FS should be used instead of the MCO to define a positive TgAb test in DTC patients [13,16]. The interference from borderline TgAb in Tg-IMA is not clear, with some studies reporting that they could interfere in the measurement of Tg by IMA, leading to false negative results [10,11,16,27], while recent studies showed that this interference may not be relevant [9,28]. In these last studies, none of the patients with undetectable Tg and borderline TgAb had metastatic disease and when recurrence was detected, all patients had positive levels of Tg-IMA and/or TgAb above MCO.

Borderline TgAb are not a major concern in clinical practice of DTC follow-up. According to 2015 ATA consensus patients are considered as having excellent response to therapy when undetectable Tg-IMA, negative TgAb (considered if under MCO) and negative cervical US are found and they are not candidate for further investigation. If borderline TgAb measurements were considered as a positive result it would lead to greater changes in the management of these patients with additional investigation and TSH suppression needed [1]. However, in our opinion, due to the

possible interference in Tg-IMA measurement, patients with borderline TgAb should be considered for a more attentive follow-up.

Another aspect to be considered is that although TgAb had been measured by two different assays, this does not guarantee that the borderline results were not positive by a third assay, and an interference of TgAb may have led to underestimated Tg-IMA results. The current TgAb assays used are highly variable and not interchangeable. As shown by *Spencer et al* [6], the concordance between 12 different TgAb assays was only 10%, and 33% of the samples had positive TgAb by just one method. It is important to highlight that most patients (5/6) in Group 1b had positive (above MCO) TgAb at the beginning of the follow-up, but borderline TgAb when samples were collected for the Tg-c measurement.

It is also noteworthy that the presence of structural disease can happen in 0.4% of patients with negative Tg-IMA and undetectable TgAb. These patients may have anomalous forms of Tg produced by the tumor or nonsecreting DTC [9,12–14]. We believe that the patients from group 1b (borderline TgAb) are not non-secreting DTC patients because Tg was detected in all of them by the competitive assay.

Regarding the 10 patients from Group 1 who had Tg measured by LC-MS/MS, 5 patients presented low levels (range: 0.5-2.0 µg/L), 3 patients presented levels above 2 µg/L (2.6; 3.1 and 18 µg/L), and 2 patients presented undetectable Tg-LC-MS/MS values (under 0.5 µg/L). It is worth noting that the presence of very low levels of Tg-LC-MS/MS (under 2 µg/L) is not expected to be found in patients with significant structural disease. Among these patients, as observed in Table 2, patients 1, 5, 9 and 11 presented distant metastasis, low levels of Tg-LC-MS/MS and undetectable Tg-IMA but positive Tg-c values (minimum of 27 µg/L in a patient with pulmonary micro nodules and neck disease, and maximum of 605 µg/L in a patient with lung metastasis- patients 11 and 1; table 2, respectively). The patients 6, 7 and 10 (table 2) presented disease confined to the neck: patient 10 had undetectable Tg-LC-MS/MS and Tg-IMA but detectable Tg-c; patient 6 had low levels of Tg-LC-MS/MS, undetectable Tg-IMA and the Tg-c was detectable, and patient 7 presented positive levels of Tg-LC-MS/MS (2.6 µg/L) and Tg-c (71 µg/L) but undetectable Tg-IMA.

Regarding Group 3 (patients classified as excellent response to therapy), 2 patients had detectable Tg-c and were, therefore, reclassified as indeterminate response. These patients have been followed for 8 and 16 years, and have no evidence of disease currently. We believe that these results could be a false positive

of Tg-c. They also had undetectable TgAb, showing that it is unlikely that TgAb could have interfered with Tg-IMA measurements.

A limitation of our study is that the functional sensitivity of the assay was higher than those reported for IMA and LC-MS/MS. This finding could be explained by the use of unpurified antigens (Tg) and polyclonal antibodies. Similar results were found in other competitive assays, such as the one described by *Black et al* and *Crane et al* [29,30].

In conclusion, although Tg-IMA remains the standard method to be used in the follow-up of DTC patients, false negative or inappropriately low results can be observed in patients with structural disease. In these patients, Tg-LC-MS/MS can yield an additional benefit, but its measurement can still fail. The competitive Tg assay described in this study showed benefit for patients with both positive and borderline TgAb who had relevant structural disease (even in diseases confined to the neck) and underestimated Tg-IMA and Tg-LC-MS/MS. The results suggest that Tg-c may be useful in these situations.

**Compliance with Ethical Standards:**

**Funding:** This study was supported by Fleury, Medicina e Saúde.

**Conflict of Interest:** Rosa Paula M. Biscolla, José Gilberto H. Vieira and Rui M.B. Maciel are investigators of the Fleury Group.

**Ethics Approval:** All procedures performed in this study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Consent to participate:** All subjects signed the written informed consent and the institute's committee on human research approved the study protocol.

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**Figures:**

**Fig. 1** Results of Tg from group 1 in the 3 assays

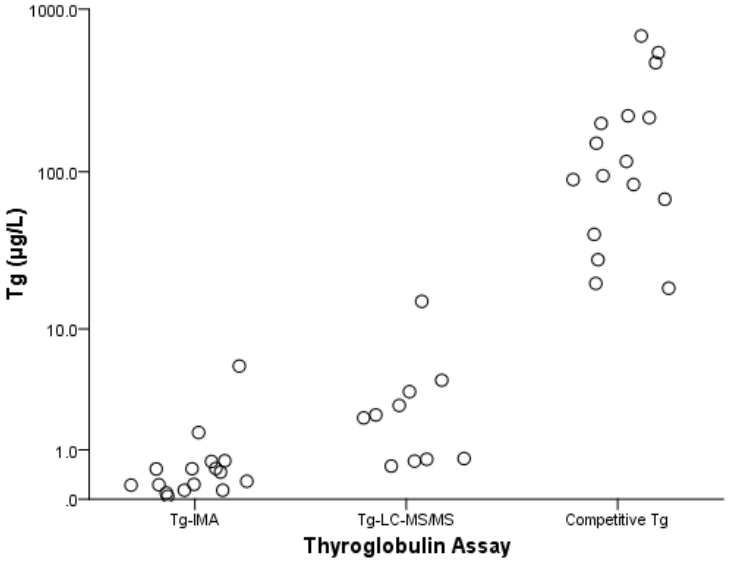
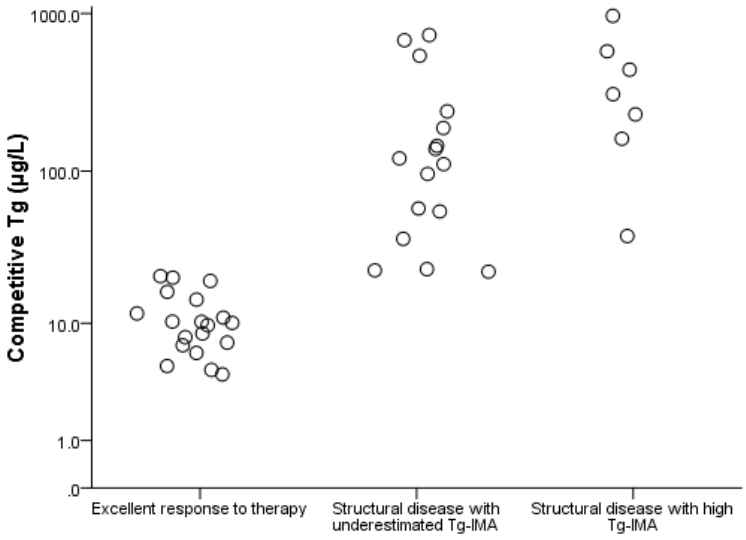



**Fig. 2** Results of Tg-c in the 3 groups



**Tables:**

Table 1. Patient Characteristics

| Group | 1a | 1b | 2 | 3 |
|---|---|---|---|---|
| Number of patients | 8 | 6 | 7 | 19 |
| Age at diagnosis (years) | 28 (13-81) | 44.5 (23-58) | 22 (10-70) | 43 (24-60) |
| Sex, female | 7 (87.5%) | 5 (83.3%) | 6 (85.7%) | 18 (94.7%) |
| Histological type | | | | |
| - PTC | 8 (100%) | 5 (83.3%) | 6 (85.7%) | 17 (89.5%) |
| - FTC | 0 | 0 | 1 (14.3%) | 2 (10.5%) |
| - Mixed | 0 | 1 (16.7%) | 0 | 0 |
| $^{131}$I Activity (mCi) | 250 (150-800) | 350 (150-550)* | 490 (200-950)* | 150 (30-300) |
| Positive TgAb | 8 (100%) | 0** | 1 (14.3%) | 0 |
| Initial risk of recurrence | | | | |
| - low | 0 | 0 | 1 (14.3%) | 12 (63.6%) |
| - intermediate | 5 (62.5%) | 2 (33.3%) | 1 (14.3%) | 7 (36.8%) |
| - high | 3 (37.5%) | 4 (66.7%) | 3 (42.8%) | 0 |
| - unknow | 0 | 0 | 2 (28.6%) | 0 |

PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; TgAb: thyroglobulin autoantibodies. Values are presented as numbers (%) or medians (range).

*One patient in the group did not receive radioiodine.

** Borderline TgAb (i.e. below manufacturer cut off and above functional sensitivity)

Table 2: Description of patients from Group 1

| Patient | Histology/ *variant* | Structural disease | TgAb* (kIU/L) | Tg-IMA (µg/L) | Tg-LC-MS/MS (µg/L) | Tg-c (µg/L) |
|---|---|---|---|---|---|---|
| 1 | PTC | Lung | > 4000 | < 0.1 | 0.8 | 605 |
| 2 | PTC/*fv* | Neck + lung | 245 | < 0.5 | 3.1 | 183 |
| 3 | PTC | Neck + lung + trachea | 1778 | < 0.5 | < 0.5 | 124 |
| 4 | PTC/*fv* | Neck + lung | 1319 | 5.1 | 18 | 710 |
| 5 | PTC/*fv* | Neck + lung | 2010 | < 0.5 | 1.8 | 520 |
| 6 | PTC | Neck | 130 | < 0.1 | 0.7 | 22 |
| 7a** | PTC/*fv, sclerosing* | Neck | 170 | < 0.5 | 2.6 | 71 |
| 7b** | PTC/*fv, sclerosing* | Neck | 187 | < 0.5 | NA | 89 |
| 8 | PTC | Lung + mediastinum | 1800 | < 0.5 | NA | 225 |
| 9 | PTC/*fv* | Neck + mediastinum | 64 | < 0.1 | 1.5 | 58 |
| 10 | PTC | Neck | 24 | < 0.1 | < 0.5 | 23 |
| 11 | PTC/*oncocytic* | Neck + pulmonary micronodules | 93 | < 0.5 | 1.8 | 27 |
| 12 | PTC | Neck | 15.1 | < 0.5 | NA | 148 |
| 13a** | PTC/*fv* + DTC non-specified | Neck + lung | 102 | 1.4 | NA | 143 |
| 13b** | PTC/*fv* + DTC non-specified | Neck + lung | 97 | < 0.5 | NA | 104 |
| 14 | PTC/*sclerosing* | Neck + pulmonary micronodules | 15 | < 0.5 | NA | 33 |

PTC: papillary thyroid carcinoma; *fv*: follicular variant, DTC: differentiated thyroid carcinoma; TgAb: antithyroglobulin antibodies; NA: not available

* TgAb measured by indirect electrochemiluminescent immunoassay, Roche® (manufacturer cut off less than 115 kIU/L, Limit of quantification 15 kIU/L).

** Patients 7 and 13 had 2 samples analyzed in different situations.

## 4 MANUSCRITO 2

# *False diagnosis of biochemically recurrent thyroid carcinoma and its unnecessary investigation – the importance of testing for heterophile antibodies*

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**Running title:** Thyroglobulin levels and heterophile antibodies.

**Key words:** heterophile antibodies, thyroglobulin, mass spectrometry and thyroid cancer

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## Abstract

**Background:** Thyroglobulin (Tg) levels are important to predict recurrence in differentiated thyroid cancer patients. However, false-positive results can hence the request of unnecessary tests and treatments.

**Methods:** We reported two cases of interference in thyroglobulin measurement and the workup to investigate them.

**Results:** Both patients achieved an excellent response to therapy after total thyroidectomy and one patient had also received radioiodine treatment. During the follow-up, Tg levels increased and there was no evidence of recurrent disease in the imaging studies. The Tg levels by the Access platform were positive but the results by Elecsys platform and LC-MS/MS were undetectable, leading to the hypothesis of heterophile antibodies (HAbs) interference.

**Conclusion:** The possibility of HAbs interference must be considered when the Tg levels do not fit in the clinical picture. The measurement of Tg by another immunoassay or by LC-MS/MS may be useful in these situations.

## Introduction

The measurement of thyroglobulin (Tg) is an important tool to predict disease persistence and recurrence in differentiated thyroid carcinoma (DTC) patients after treatment with total thyroidectomy and radioiodine ablation. Undetectable Tg measured under TSH suppression by immunometric assays (IMAs) with high functional sensitivity (< 0.1 ng/mL) indicates an excellent response to treatment (1, 2). Although the IMAs used in the laboratory routinely are sensitive enough to detect early disease recurrence during follow-up, some pitfalls still exist in clinical practice, including the following: the presence of thyroglobulin autoantibodies (TgAbs) in 15-20% of DTC patients, heterophile antibodies and tumor production of anomalous Tg isoforms. In these situations, Tg becomes an unreliable tumor marker (3–6). Although TgAbs are the most important confounder affecting the accurate determination of Tg by IMAs and, therefore, should always be measured, they can cause falsely low or undetectable readings (5, 7). On the other hand, a positive result is generally assumed to be real and not usually suspected for interference, which leads to the misdiagnosis of an incomplete response to therapy and hence the request for unnecessary tests and treatments (8–10).

Heterophile antibodies (HAbs) can bind to the antibodies employed in IMAs, leading to false-positive (more frequently) or false-negative results (11). In the literature, the reported prevalence of HAbs interference in thyroglobulin assays ranges from 0.4 to 3% (3, 11–13). The presence of HAbs should be considered in DTC patients whose Tg measurements are not consistent with clinical and imaging findings. In those patients, the workup of samples with suspected HAbs includes Tg measurement after serial dilutions, pretreatment with HAbs blocking reagents/tubes and reassaying with different immunoassay platform or methodology (14, 15). The measurement by liquid chromatography–tandem mass spectrometry (LC–MS/MS) is useful in these situations (9, 16). The LC–MS/MS assay is based on the direct quantification of Tg after using trypsin to cleave proteins. In this process, large proteins, such as Tg, are broken into small peptides, which enables their measurement by mass spectrometry. Then, a specific Tg peptide is separated by liquid chromatography and finally detected using mass spectrometry. In the same process in which Tg is cleaved, as a secondary benefit, there is also the digestion of antibodies, eliminating them as interferers (17).

We report two cases of HAbs interference in thyroglobulin measurements leading to unnecessary investigation and the workup used to find out this interference.

**Patients**

Patient #1: A 41-year-old woman with no other comorbidities was assessed for a persistently high serum Tg level after total thyroidectomy. She underwent total thyroidectomy two years prior to the current study. The pathology report showed a 1.6 cm follicular variant of papillary carcinoma with minimum extra thyroid extension, and the patient was classified as having an intermediate risk of recurrence (American Thyroid Association- ATA). She was also treated with 150 mCi of radioactive iodine (RAI) after levothyroxine withdrawn. The posttherapy whole-body scan (WBS) showed uptake in the thyroid bed, and the Tg level was 10.7 ng/mL (TSH 82 mUI/L), with negative TgAbs. After the initial treatment, she presented with undetectable Tg levels under levothyroxine therapy (Tg-LT4) and, after stimulation with recombinant human TSH (rhTSH), was classified as having an excellent response to therapy. One year later, the Tg-LT4 level increased to 7.5 ng/mL, and the high value was confirmed in a second measurement performed in another sample (5.3 ng/mL). She underwent a neck ultrasound (US), WBS and $^{18}$FDG PET/CT scan that showed no uptake or suspicious images, and the Tg level stimulated by rhTSH (Tg-rhTSH) was 7.9 ng/mL. Since the imaging studies were negative, she was reclassified as having a biochemical incomplete response to therapy. Nine months later, her Tg-LT4 level increased to 10.6 ng/mL, and she underwent another WBS and $^{18}$FDG PET/CT scan, both of which were negative; her Tg-rhTSH level was 14.8 ng/mL. The high serum Tg level prompted a further evaluation with abdominal MRI that did not show suspicious images. Two lymph nodes were identified in the neck by US, and guided biopsy showed lymphoid cells with undetectable Tg in the needle washout. As structural disease was not detected, the patient was referred to our laboratory to evaluate for interference in the Tg measurements. The Tg level as measured by the Access platform (Beckman Coulter, Fullerton, CA, USA) was 30 ng/mL, and the serial dilutions were linear (Table 1). We performed Tg retesting using another IMA platform (Elecsys II, Roche) and the LC–MS/MS from Mayo Medical Laboratories (functional sensitivity < 0.5 ng/mL). Both measurements were undetectable, confirming the interference from HAbs.

Patient #2: A 36-year-old woman underwent total thyroidectomy for papillary thyroid microcarcinoma. She was stratified as having a low risk of recurrence by the ATA consensus and was not treated with adjuvant RAI therapy. During the follow-up, she had both undetectable Tg-LT4 and Tg-rhTSH levels and a negative WBS. After nine years, the Tg-LT4 level increased to 17 ng/mL under similar levels of TSH, and TgAbs were negative. She was evaluated with neck ultrasound and WBS, both of which were negative, and the Tg-rhTSH level was 12 ng/mL. The negative results from the WBS and the paradoxical decrease in the Tg-rhTSH level compared with the Tg-LT4 level raised the suspicion of assay interference. The Tg level as measured by the Access platform was 8.9 ng/mL, and the serial dilutions showed linearity. The Tg levels as measured by the Elecsys platform and by LC–MS/MS were undetectable (Table 1).

## Discussion

The follow-up of DTC patients is based on the measurements of Tg and TgAbs and neck ultrasound (1). Tg elevation artifacts can lead to unnecessary tests and treatments, which may include RAI and even invasive procedures (9, 10). Although HAbs do not frequently interfere with the measurement of Tg, the possibility must be considered when the Tg levels do not fit in the clinical picture. Here, we present two cases in which the high and erroneous results of the Tg, due to interference from HAbs, led to the diagnosis of a biochemically incomplete response to therapy and unnecessary extensive investigations.

The term heterophile antibody is used to describe any antibody that may cause false results in immunoassays by binding to the assay antibodies. Although their interference in immunoassays is similar, HAbs can be classified into 3 groups: 1) human anti-animal antibody when there is a known exposure to animal antibodies (among them, the anti-mouse antibodies (HAMAs) are the most important, as most antibodies used in the immunoassays are derived from mice); 2) heterophile antibody when the exposure to the antigen is unknown; 3) and rheumatoid factors with cross reactivity to assay antibodies (15). HAbs generally bind to both the capture and detection antibodies in sandwich assays, forming a bridge that simulates the presence of an analyte leading to false-positive results in its absence or falsely increased measurements when the analyte is present (Figure 1-2). HAbs may also bind to the capture and/or detection antibody, preventing the binding of the analyte and the

formation of the antigen-antibody sandwich complex, causing false-negative or falsely low results (Figure 1-3). This last situation occurs less frequently and may be more challenging to detect (3, 11, 13, 18).

There is not a consensus in the literature on the frequency of HAbs interference in immunoassays. The rates of interference may also depend on the type of assay used (18). Preissner et al. (3) reported a rate of 3% false-positive results in 1106 serum Tg samples analyzed by the Access platform; Giovanella et al. (11) showed that HAbs interference in Tg measurements could be found in 1% of the samples analyzed by the Immulite platform. They also reported some falsely lowered Tg values, although false higher concentrations were the majority. Verburg et al. (12) reported a similar rate of interference in the Tg measurements on the BRAHMS platform (2/201 in DTC samples, 0/52 in controls). In our cases, the interference was only observed on the Access platform and not on the Elecsys II platform.

A false-positive measurement of Tg should be suspected when the Tg level does not fit the clinical picture (low-risk patients, negative neck US, unexpected detectable Tg after years of initial therapy) in the absence of an increment in the Tg measurement after TSH stimulation and/or detectable Tg in the presence of TgAb and no suspicious images (9, 19). An investigation of suspected HAbs samples includes a) serial dilutions to evaluate linearity; b) pretreatment with heterophile-blocking reagents/tubes; c) reassay with a different immunoassay, since samples that show interference in one particular assay may not present the same problem using an assay from another manufacturer; or d) Tg measurement by another method, such as LC-MS/MS (14, 15, 20). Samples are considered contaminated by HAbs when the dilution curve is nonlinear (results deviate more than +/- 20% from the undiluted sample concentration after correction for the dilution factors), pretreatment with HAb-blocking reagents substantially alters the results or when the values from other assays are not similarly increased (3). Although the first steps can generally detect the presence of HAbs, they frequently fail to provide accurate Tg concentration values. Recently, the measurement of Tg by LC–MS/MS was described as useful in these situations, since serum pretreatment with proteolytic enzymes destroys HAbs. Netzel et al. (16) and Barbesino et al. (9) reported cases in which the traditional workup failed to provide accurate Tg results, although it was able to detect HAbs interference in most cases. These authors proposed that samples suspected for HAbs interference should always

be initially evaluated by LC–MS/MS. Although the results obtained by LC–MS/MS measurements are precise, this method is not available in many centers.

In the cases reported here, the low probability of tumor recurrence, the increase in Tg levels after achieving an excellent response to therapy, the absence of disease in imaging studies and the lack of increment in the Tg-rhTSH level compared with the Tg-LT4 level prompted the investigation for interferers. We initially proceeded the investigation with Tg measurements in the Access platform after dilutions of 1:2 and 1:5 from the neat sample. In both cases, the dilution curve was unexpectedly linear, not allowing the confirmation of the presence of HAbs interference. Since we do not have HAb-blocking reagents or tubes available in our country, the next step was to retest the samples using a different immunoassay platform (Roche Elecsys II), which resulted in undetectable Tg levels. The last step was to measure the Tg levels by the LC–MS/MS method. The values were also undetectable, confirming HAbs interference.

The two cases reported in this study show the importance of investigating HAbs in patients whose Tg levels are not compatible with the ongoing follow-up before exposing them to unnecessary tests and treatments. It is worth noting that a positive result is generally assumed to be real and not usually suspected for interferents, which leads to the misdiagnosis of an incomplete response to therapy. In the cases we reported, measurements after serial dilutions did not show the interference of HAbs. On the other hand, the measurement using a different IMA platform or LC–MS/MS was essential for the workup of HAbs interference in those patients.

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Writing – review and editing. Rosa Paula Mello Biscolla: Conceptualization; Writing – review and editing.

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**Statement of Ethics**

The patients provided written informed consent for publishing this data in the report.

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Table 1. Access Tg concentrations neat and diluted, Elecsys Tg II Roche and LC–MS/MS Tg concentrations.

| | Access Tg, ng/mL | | | | |
|---|---|---|---|---|---|
| Patient | Neat | x2* | x5* | Elecsys Tg II Roche, ng/mL | LC–MS/MS Tg, ng/mL |
| 1 | 30 | 29 | 28 | < 0.1 | < 0.5 |
| 2 | 8.9 | 9 | 10 | < 0.1 | < 0.5 |
| * x2 and x5: 1:2 and 1:5 dilutions from neat, respectively | | | | | |

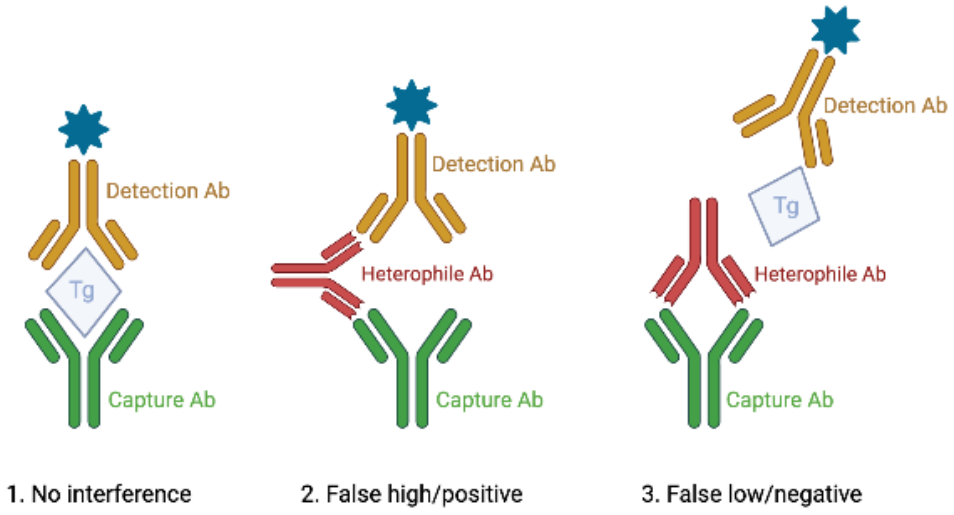


Figure 1: 1, Schematic depiction of immunometric assays for the measurement of Tg without interference; 2, Schematic depiction of false-positive/false high interference for heterophile antibody; 3, Schematic depiction of false-negative/false low interference for heterophile antibody. *Tg* = thyroglobulin; *Ab* = antibody.

## 5 MANUSCRITO 3

# *Correlation and clinical performance of three thyroglobulin assays in the management of differentiated thyroid cancer patients*

**Abstract:**

**Introduction:** Thyroglobulin (Tg) is the main biochemical marker of Differentiated Thyroid Cancer (DTC). Usual immunometric assays (IMA) are prone to interference by autoantibodies which can lead to erroneous results. LC-MS/MS has been described as an assay that may provide accurate results, however the literature reported up to 40% of undetectable rates in patients with positive thyroglobulin antibodies (TgAb) and structural disease. **Objective:** Compare and evaluate the clinical performance of three different Tg assays in DTC patients with positive and negative TgAb. **Methods:** 129 DTC patients with negative (97) and positive TgAb (32) had Tg measured by LC-MS/MS assay (Tg-MS) with functional sensitivity of 0.7 ng/mL and at least one of the 2 IMAs (by Beckman and Roche) with FS of 0.1 ng/mL. TgAb were measure in at least 2 assays in TgAb negative samples. **Results:** Correlation coefficients between the assays in negative samples were r 0.950-0.985 and in positive samples were r 0.875-0.878 between LC-MS/MS and IMAs. The overall concordance rates ranged from 87.5% to 99%. In negative TgAb patients Tg measured by LC-MS/MS was 6.7-29% higher than IMAs. In positive TgAb samples LC-MS/MS detected Tg in one sample more than IMAs and 58.8% of the patients with structural disease from this group had undetectable Tg-MS and 47% by IMAs. Sensitivity in detecting structural disease in positive TgAb samples were 41.2% for LC-MS/MS and 53.3% for IMAs and 35% of the patients with positive TgAb had lower Tg results in the IMAs (10-63% lower). **Conclusion:** The assays correlated well in negative TgAb samples but had poor correlation in the positive samples between IMAs and LC-MS/MS assay. Tg measured by IMAs had better clinical performance in patients with positive TgAb.

**Introduction:**

Differentiated Thyroid Carcinoma (DTC) is the most common endocrine cancer. DTC, developing from normal thyroid follicular cells, accounts for almost 95% of all thyroid cancers. Thyroid tissue is the only source of thyroglobulin (Tg) production, which enables Tg to be used as the main biomarker of DTC with high sensitivity and specificity for early detection of persistent and recurrent disease after thyroidectomy and radioiodine ablation (1,2). National and international guidelines attribute an important role to Tg measurements for the determination of response to therapy, which impacts additional investigations and therapies and frequency of follow-up (1–3). Thyroglobulin can be measured by three different methods: immunometric assays (IMA), radioimmunoassays (RIA) and, more recently, liquid chromatography/tandem mass spectrometry (LC-MS/MS) (4,5). The most used method nowadays is the IMA, which is fully automated, fast and has an excellent sensitivity (5,6).

Tg autoantibodies (TgAb) occur in approximately 25-30% of DTC patients (7,8), and IMA are susceptible to its interference, resulting in false low or undetectable measurements since TgAb can mask the epitopes used by reagent antibodies of the assays to identify the Tg (4,9,10). RIA are less susceptible to TgAb interference due to the competitive nature of the assay and the use of polyclonal antibodies (4,8,11) although false high results can be observed (5,12,13). Recently, LC MS/MS Tg methods has been described as an alternative when TgAb are present (5,13–15). LC-MS/MS is based on specific peptide quantitation following tryptic digestion of all proteins, including TgAb and other antibodies such as heterophile antibodies, and immunocapture of Tg-specific peptides (13–17). As a result, Tg measured by LC-MS/MS (Tg-MS) should allow accurate Tg quantification in TgAb positive samples (10,14). Tg-MS is offered at clinical laboratories as the method to be performed in the presence of TgAb instead of the measurement of Tg by IMA (Tg- IMA) (18,19). However, the usefulness of Tg-MS in thyroid cancer management in clinical practice has no clear advantage, since undetectable Tg values were reported in 20-44% of patients with positive TgAb and recurrent/persistent disease (5,18–20). Despite this data, Tg-MS can show higher results than Tg-IMA suggesting that it may be of benefit in some patients with positive TgAb (5,19,20). Its effectiveness has also been reported in samples without TgAb and others interferers (13,16,18,20).

The aim of this prospective study was to compare and evaluate the clinical performance of three different Tg assays in DTC patients with positive and negative TgAb.

## Materials and methods:

### Samples, Study Groups and Study Design:

This is a prospective study conducted at the Thyroid Disease Center in the Division of Endocrinology, Escola Paulista de Medicina, Universidade Federal de São Paulo and Grupo Fleury (in São Paulo, Brazil) and approved by the Institutional Ethics Review Board from both centers (4.281.528 and NP-452). Signed informed consent was obtained from all patients.

Samples were collected from 129 DTC patients followed in the Thyroid Disease Center between September 2020 and October 2021. Samples were stored at -20º C and sent to the Grupo Fleury where they were taken out of storage and all the measurements were performed. The 129 patients were divided into three groups (Table 1): A) Undetectable Tg-IMA and negative TgAb: Patients with excellent response to therapy (45 samples); B) Detectable Tg-IMA and negative TgAb: Patients with structural incomplete response, biochemical incomplete response, and indeterminate response to therapy with negative TgAb (52 samples); C) Positive TgAb (32 samples). Group 3 was subdivided in two groups: C1: structural incomplete response (17 samples) and C2: indeterminate or biochemical incomplete response to therapy (15 samples). The response to therapy was established according to the American Thyroid Association (ATA) consensus of 2015 for patients who have undergone radioiodine treatment (1) and to the criteria proposed by *Momesso et al*. for patients who have not undergone radioiodine treatment (21,22) (Supplemental table 1). Structural disease was defined as evidence of disease in neck ultrasound (US) and cytological analyses, whole body scans with uptake out of the thyroid bed, computed tomography (CT) or positron emission tomography-computed tomography (PET/CT). Groups A and B were selected to evaluate the performance of the Tg-MS in the negative TgAb samples with negative Tg-IMA (Group A) and positive Tg-IMA

(Group B) and Group C in the TgAb positive specimens. The variables analyzed included patient age at thyroid cancer diagnosis, sex, thyroid cancer histological type, initial risk of recurrence and activity of radioiodine received.

**Table 1.** Study Groups

| Group | Criteria | | Response to therapy | n |
|---|---|---|---|---|
| | Tg | TgAb | | |
| **A** | Neg | Neg | Excellent | 45 |
| **B** | Pos | Neg | **Total** | 52 |
| | | | Structural incomplete | 25 |
| | | | Biochemical incomplete | 20 |
| | | | Indeterminate | 7 |
| **C** | Any value | Pos | **Total** | 32 |
| **C1** | | | Structural incomplete | 17 |
| **C2** | | | Indeterminate or Biochemical incomplete | 15 |

Tg: Thyroglobulin; TgAb: Thyroglobulin Antibodies; Neg: negative; Pos: positive.

**Description of the Tg and TgAb assays:**

**Immunometric Tg Assays:**

All patients had Tg measurements by LC-MS/MS and at least by one IMA. All samples were collected under levothyroxine therapy. Positive results were defined as any value above functional sensitivity (FS) and negative results, any value below the FS.

(i) Chemiluminescent, Beckman Access immunoassay (Beckman Coulter, Fullerton, CA, USA; Tg-Beckman in manuscript), with FS 0.1 ng/mL. Analytical sensitivity was 0.01 ng/mL; intra-assay CV was 1.4% for a pool with a mean value of 4.2 ng/mL, 1.4% for a pool with a mean value of 21.6 ng/mL, 4.4% for a pool with a mean value of 130.4 ng/mL and 2.0% for a pool with a mean value of 344.7 ng/mL. Inter-assay CV was 1.7, 1.8, 4.9, and 4.0% for the same pools.

(ii) Electrochemiluminescent Roche (Tg-Roche in manuscript), FS 0.1 ng/mL. Intra-assay CV was 2.2% for a pool with a mean value of 1.11 ng/mL, 1.2% for a pool

with a mean value of 1.59 ng/mL, 3.0% for a pool with a mean value of 89.3 ng/mL, 2.5% for a pool with a mean value of 247 ng/mL, and 1.9% for a pool with a mean value of 470 ng/mL. Inter-assay CV was 3.0, 2.6, 4.2, 3.2, and 3.8% for the same pools.

**LC-MS/MS assay:**

The samples were analyzed by the directed proteomics method developed by Grupo Fleury. The method was based on the work of *Kushinir et al* (15) with the following modifications: Sample preparation included a step of complexing free Tg in serum with monoclonal antibody, followed by a precipitation process by salting out. Then, the samples were reduced and submitted to the enzymatic digestion process with trypsin. After digestion, an immunoenrichment step was carried out at the peptide level using a polyclonal antibody (Covance). The eluate was injected into a liquid microchromatography system (NanoUltimate 3000, Thermo Fisher Scientific) followed by identification in a high-resolution mass spectrometer (Q-Exactive, Thermo Fisher Scientific). The mass transitions were the same as those described by *Kushinir et al* (15) but based on a parallel reaction monitoring (PRM) method in the orbitrap hybrid quadrupole analyzer. The methodology was validated according to the procedures recommended by the Clinical Laboratory Improvement Amendments (CLIA) and the National Committee for Clinical Laboratory Standards (NCCLS). The functional sensitivity was 0.7 ng/mL, and the intra-assay CV was 11.8% for a pool with a mean value of 6.5 ng/mL, 14.1% for a pool with a mean value of 37.9 ng/mL and 9.1% for a pool with a mean value of 136.1 ng/mL.

**TgAb Assays:**

Negative TgAb was considered when TgAb were undetectable at least by two methods, aiming to minimize misclassification. For this analysis, a level above FS or above the limit of quantification on any TgAb assay was reported as a positive result.

(i) Roche Elecsys anti-Thyroglobulin (Roche Diagnostics, TgAb-R in manuscript): indirect electrochemiluminescent competitive immunoassay; manufacturer cutoff (MCO) < 115 kIU/L, limit of detection of 10 kIU/L and limit of quantification of 15 kIU/L.

(ii) ADVIA Centaur anti-Thyroglobulin assay (Siemens Healthcare Diagnostic Inc., TgAb-S1 in manuscript): direct chemiluminescent competitive immunoassay; MCO 60 kIU/L, limit of detection of 10 kIU/L and FS of 30 kIU/L.

(iii) Atellica IM Anti-Thyroglobulin II (Siemens Healthcare Diagnostic Inc., TgAb-S2 in manuscript): chemiluminescent immunometric assay; MCO 4.5 kIU/L, limit of detection and limit of quantification of 0.9 kIU/L.

### Statistical analysis

For statistical analyses, we used the software GraphPad Prism, version 9 (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as mean and range, for normally distributed data, or as median and range for non-normally distributed data. The correlation between assays were assessed using Spearman's correlation coefficient and deming linear regression. Strength of the correlations were determined by the McBride scale. The McBride scale considers an $r < 0.90$ as poor, an $r = 0.90$ to 0.95 as a moderate correlation; meanwhile, an $r = 0.95$ to 0.99 is substantial, and $r > 0.99$ is almost perfect (23). We also estimated the diagnostic accuracy of Tg-MS and Tg-IMA for detecting structural disease in patients who had positive TgAb by calculating sensitivity, specificity, positive predictive value, and negative predictive value. Spearman's correlation was calculated to investigate the relationship between TgAb titers and Tg-MS. The results were considered significant when their p values were $< 0.05$.

## Results:

### Patients Characteristics:

Table 2 describes patients characteristics in each group. Mean age at diagnosis was 42 years (range 6-83 years); 86% (111/129) were female and 90.7% (117/129) had papillary thyroid cancer. All patients underwent total thyroidectomy. Of the 129 patients, 24.8% (32/129) had positive TgAb in at least one assay and 75.2% (97/129) had negative Tg in at least 2 assays.

**Table 2.** Patient characteristics in Groups A, B and C.

| Characteristics | Group A | Group B | Group C |
|---|---|---|---|
| **Number of patients** | 45 | 52 | 32 |
| **Age at diagnosis (years)** | 45 (21-70) | 39.5 (6-83) | 40.5 (9-72) |
| **Female** | 93.3% (42/45) | 76.9% (12/52) | 90.6% (29/32) |
| **Histological type** | | | |
| **PTC** | 88.9% (40/45) | 90.4% (47/52) | 90.6% (29/32) |
| **FTC** | 11.1% (5/45) | 3.8% (2/52) | 3.1% (1/32) |
| **HTC** | 0 | 1.9% (1/52) | 0 |
| **Mixed** | 0 | 1.9% (1/52) | 3.1% (1/32) |
| **Poorly differentiated** | 0 | 1.9% (1/52) | 3.1% (1/32) |
| **Aggressive variant (PTC tumors)** | 5% (2/40) | 14.2% (7/49)<br>2 unknow | 21.4% (6/28)<br>1 unknow |
| **Multifocality** | 23.2% (10/43)<br>2 unknow | 13.7% (7/51)<br>1 unknow | 12.9% (4/31)<br>1 unknow |
| **Initial risk of recurrence** | | | |
| **low** | 55.6% (25/45) | 17.3% (9/52) | 12.5% (4/32) |
| **intermediate** | 37.8% (17/45) | 28.8% (15/52) | 46.9% (15/32) |
| **high** | 2.2% (1/45) | 51.9% (27/52) | 34.4% (11/32) |
| **unknow** | 4.4% (2/45) | 2% (1/52) | 6.2% (2/32) |
| **$^{131}$I Treatment** | 95.5% (43/45) | 90.4% (49/52) | 81.2% (26/32) |
| **$^{131}$I Activity (mCi)** | 150 (30-550) | 400 (100-1000) | 300 (100-850)* |
| **TgAb** | negative | negative | positive |

PTC: papillary thyroid carcinoma; FTC: follicular thyroid carcinoma; HTC: hurtle cell carcinoma; TgAb: Thyroglobulin Antibodies
Values are presented as % (numbers) and mean or medians (range).
* Activity not available in one patient.

All patients in Group A had negative Tg-IMA, TgAb and negative neck US. Group B (positive Tg-IMA and negative TgAB) had 48% (25/52) of patients with structural disease, 38.5% (20/52) with biochemical incomplete response and 13.5% (7/52) with indeterminate response to therapy. Among the patients with structural disease, 48% (12/25) had distant metastasis (lungs, bone, mediastinum and kidney) and 52% (13/25) disease confined to the neck, confirmed by cytological analysis (Supplemental table 2 describes the patients with structural disease).

Table 3 describes characteristics of the patients with positive TgAb (Group C). Seventeen out of 32 patients (53.1%) had structural disease (Group C1; patients 1-17). Among the patients with structural disease, 64.7% of the patients (11/17) had

distant metastasis (lungs, bone, mediastinum, adrenal, brain, liver) and 35.3% (6/17) presented disease confined to the neck, all confirmed by cytological analysis. The other 15 patients from Group C (Group C2; table 3, patients 18-32) had no structural disease identified in the imaging exams and were therefore classified as indeterminate response to therapy (80%; 12/15) and biochemical response to therapy (20%; 3/15).

**Table 3.** Description of the patients from Group C (positive TgAb)

| Patient | Tg-MS (ng/mL) | Tg-Beckman (ng/mL) | Tg-Roche (ng/mL) | Response to therapy | Location of structural disease | Radioiodine therapy |
|---|---|---|---|---|---|---|
| **1** | < 0.7 | N/A | < 0.1 | Structural disease | Lung | Yes |
| **2** | 3.1 | N/A | 2.5 | Structural disease | Lung, mediastinum | Yes |
| **3** | < 0.7 | < 0.1 | < 0.1 | Structural disease | Lung, neck | Yes |
| **4** | 40.4 | 23 | 36.6 | Structural disease | Lung, neck | Yes |
| **5** | 297 | 171 | 263.2 | Structural disease | Lung, brain, bone | Yes |
| **6** | < 0.7 | 0.74 | 1.74 | Structural disease | Lung | Yes |
| **7** | < 0.7 | < 0.1 | < 0.1 | Structural disease | Lung | Yes |
| **8** | < 0.7 | < 0.1 | < 0.1 | Structural disease | Lung | Yes |
| **9** | 1857.4 | 1945 | 2309 | Structural disease | Lung | Yes |
| **10** | 5011 | 5771 | 5445 | Structural disease | Lung, bone, brain, liver, adrenal, neck | Yes |
| **11** | 1371.7 | 670 | 500 | Structural disease | Lung | Yes |
| **12** | < 0.7 | < 0.1 | N/A | Structural disease | Neck | Yes |
| **13** | < 0.7 | < 0.1 | < 0.1 | Structural disease | Neck | Yes |
| **14** | < 0.7 | < 0.1 | < 0.1 | Structural disease | Neck | Yes |
| **15** | < 0.7 | < 0.1 | < 0.1 | Structural disease | Neck | Yes |
| **16** | 3.3 | 2.9 | 2.26 | Structural disease | Neck | Yes |
| **17** | < 0,7 | 0.42 | N/A | Structural disease | Neck | Yes |
| **18** | 2.0 | < 0.1 | < 0.1 | Indeterminate | - | No |
| **19** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | No |
| **20** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | Yes |
| **21** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | Yes |
| **22** | 1.0 | 0.8 | 1.03 | Biochemical | - | Yes |
| **23** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | Yes |
| **24** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | Yes |

| | | | | | | |
|---|---|---|---|---|---|---|
| **25** | < 0.7 | < 0.1 | < 0.1 | Biochemical | - | Yes |
| **26** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | No |
| **27** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | No |
| **28** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | No |
| **29** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | Yes |
| **30** | 2.3 | 1.2 | 1.62 | Biochemical | - | Yes |
| **31** | < 0.7 | < 0.1 | < 0.1 | Indeterminate | - | Yes |
| **32** | < 0.7 | < 0.1 | 0.14 | Indeterminate | - | No |

Tg-MS: Thyroglobulin measured by LC-MS/MS; Tg-Beckman: Thyroglobulin measured by Beckman immunometric assay; Tg-Roche: Thyroglobulin measured by Roche immunometric assay; N/A: not available.

**Comparing the performance of Tg-MS and Tg-IMA in samples with negative TgAb (Groups A and B):**

All patients from Group A had negative Tg results by the three assays. In Group B the range of Tg measurements were: Tg-Beckman < 0.1-697 ng/mL, Tg-Roche 0.14-889 ng/mL and Tg-MS < 0.7-986.2 ng/mL. Figure 1 shows the results of Tg in the three assays.

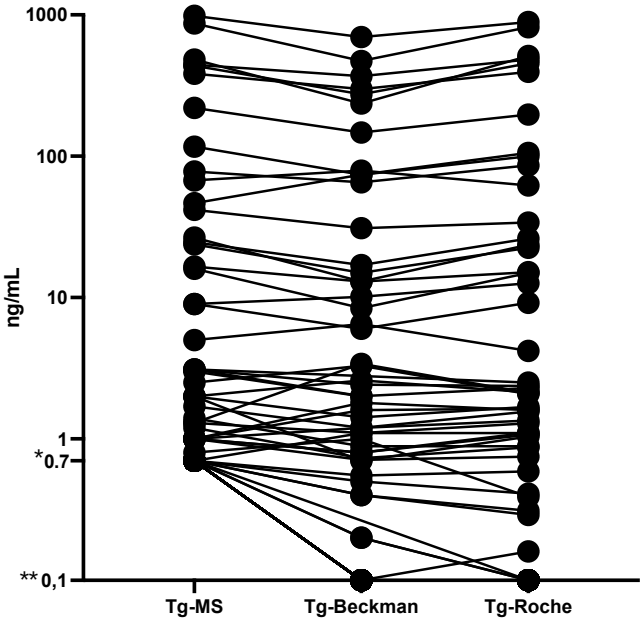


**Figure 1.** Tg results in samples with negative TgAb in the 3 assays.

*FS Tg-MS (0.7 ng/mL); **FS Tg-IMAs: 0.1 ng/mL.

The overall concordance of positive and negative results between Tg-IMAs and Tg-MS was 91.8% (89/97) when the FS of each assay was used (Tg-Beckman showed agreement in one more sample than Tg-Roche). This concordance improved to 96.9% (94/97) if a 0.7 ng/mL cutoff was used for the Tg-IMAs, indicating that the cases classified as negative by Tg-MS and positive by Tg-IMAs had values between 0.1– 0.7 ng/mL, ie, the discrepancies were due to the differences in the assays' FS. The concordance between Tg-Roche and Tg-Beckman was 99% (just one sample discordant). Method comparison between the Tg-IMAs and Tg-MS in TgAb negative specimens correlated well: correlation coefficient between Tg-MS and Tg-Beckman was r 0.950; Tg-MS and Tg-Roche were r 0.956; and the best correlation coefficient was seen between Tg-Beckman and Tg-Roche (r 0.982) (Figures 2A and 2B). Correlations were similar when analyzed only positive Tg-IMA samples (Group B) separately (r 0.954-0.985) (Supplemental Figure 3).

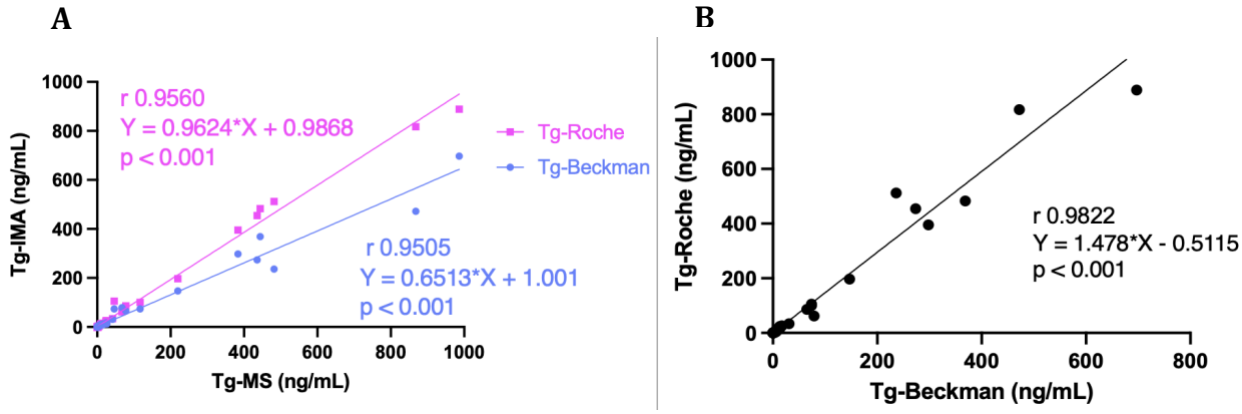


**Figure 2.** Comparison between Tg assays in TgAb negative samples. 2A: Correlations between Tg-MS and Tg-Beckman and Tg-MS and Tg-Roche; 2B: Correlation between Tg-Beckman and Tg-Roche.

In samples with Tg levels above 0.7 ng/mL (n=42), we assessed the average of the differences between the assays. The difference between Tg-MS and Tg-Beckman had average of +29% (range -62 to +186%), between Tg-MS and Tg-Roche was

+6.7% (range -45- +105%) and between Tg-Beckman and Tg-Roche -3% (range -44- + 156).

Among patients from Group B, in the patients with structural disease, Tg-IMA was detectable in all samples but one (only by Tg-Beckman; Tg-Roche was positive) and Tg-MS was undetectable in 5 out of 25 patients (20%). These patients presented only neck disease and Tg-IMAs were below Tg-MS' FS of 0.7 ng/mL (range 0.23- 0.59 ng/mL) (Supplemental table 2). In patients with biochemical incomplete and indeterminate response to therapy, 20/27 (74%) had positive Tg results by the two IMAs and by LC-MS/MS, excluding the presence of interferers (Supplemental table 4). Among the 7 patients with discordant results, when the FS was considered 0.7 ng/mL, just 3 patients (patients 5, 6 and 7 in table 4) maintained the disagreement. False positive results in Tg-IMAs were excluded by serial dilutions in the 3 patients.

**Table 4.** Tg levels in patients with biochemical incomplete or indeterminate response to therapy and negative TgAb with discordant results between IMAs and LC-MS/MS method.

| Patient | Tg-MS (ng/mL) | Tg-Beckman (ng/mL) | | Tg-Roche (ng/mL) | |
|---|---|---|---|---|---|
| | | FS 0.1 | FS 0.7* | FS 0.1 | FS 0.7* |
| **1** | < 0.7 | 0.3 | < 0.7 | 0.14 | < 0.7 |
| **2** | < 0.7 | 0.4 | < 0.7 | 0.16 | < 0.7 |
| **3** | < 0.7 | 0.4 | < 0.7 | 0.45 | < 0.7 |
| **4** | < 0.7 | 0.32 | < 0.7 | 0.47 | < 0.7 |
| **5** | **< 0.7** | **0.7** | | 0.67 | < 0.7 |
| **6** | **< 0.7** | 0.58 | < 0.7 | **0.74** | |
| **7** | **< 0.7** | **1.1** | | **1.29** | |

Tg-MS: Thyroglobulin measured by LC-MS/MS; Tg-Beckman: Thyroglobulin measured by Beckman immunometric assay; Tg-Roche: Thyroglobulin measured by Roche immunometric assay.
*FS considered 0.7 ng/mL in all assays.

**Comparing the performance of Tg-MS and Tg-IMA in samples with positive TgAb (Group C):**

In Group C, Tg-MS ranged from < 0.7-5011 ng/mL, Tg-Beckman < 0.1-5771 ng/mL and Tg-Roche < 0.1-5445 ng/mL (Figure 4). Negative results of Tg were observed in 20/32 patients (62.5%) in IMAs and in 22/32 (68.8%) in the LC-MS/MS assay. The overall concordance in TgAb positive samples between Tg-IMAs and Tg-MS was 87.5% (28/32). Adjusting the Tg-IMAs' FS to 0.7 ng/mL (FS of Tg-MS) the concordance increases to 93.8% (30/32 patients). Tg-IMAs agreement was 96.4% (27/28).

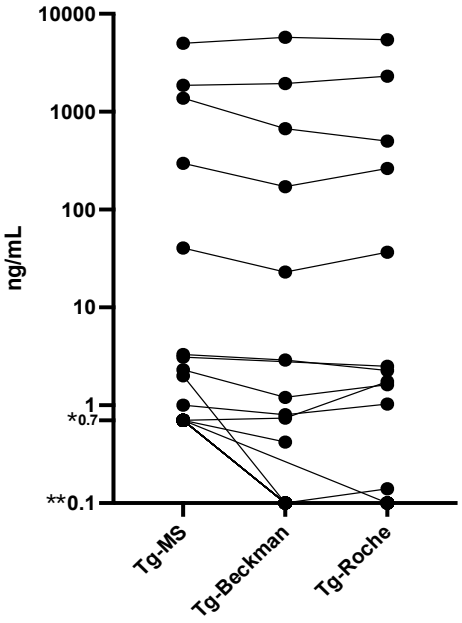


**Figure 4.** Tg results in samples with positive TgAb in the 3 assays.

*FS Tg-MS (0.7 ng/mL); **FS Tg-IMAS: 0.1 ng/mL.

Correlations between Tg-IMAs and Tg-MS in positive TgAb samples were worse than the observed in negative TgAb group (Figure 5 A and B). The correlation coefficient between Tg-MS and Tg-Beckman was r 0.875 and between Tg-MS and Tg-Roche was r 0.878. Correlation between Tg-Beckman and Tg Roche was better (r 0.965) than when each one was compared with Tg-MS.

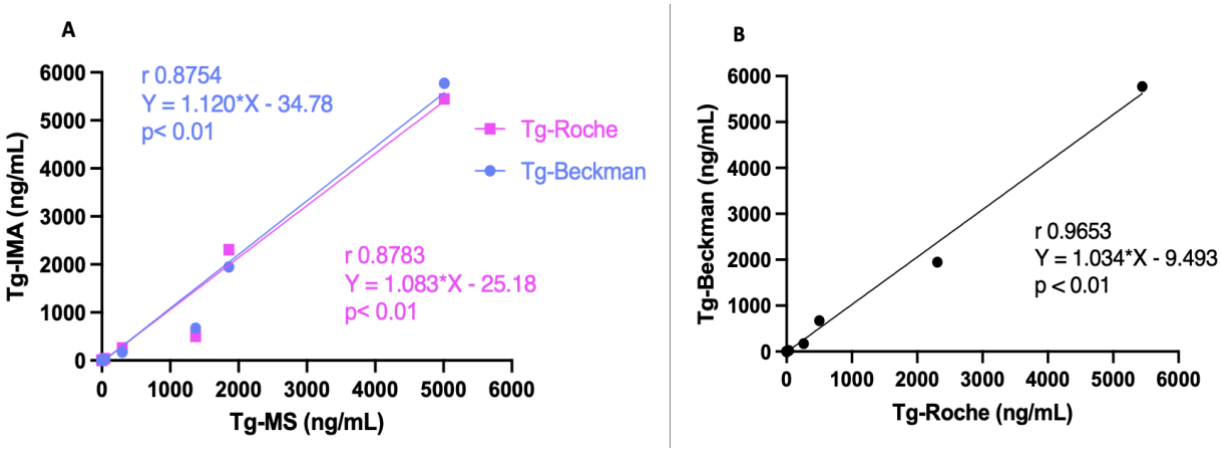


**Figure 5.** Comparison between Tg assays in TgAb positive samples. 5A: Correlations between Tg-MS and Tg-Beckman and Tg-MS and Tg-Roche; 5B: Correlation between Tg-Beckman and Tg-Roche.

In patients with structural disease and positive TgAb (Group C1; table 3, patients 1-17) 8/17 (47%, patients 1,3,7,8,12-15) had negative Tg IMAs results and surprisingly 10/17 patients (58.8%; patients 1,3,6-8,12-15,17) presented negative Tg-MS. Two patients (11.8%) had positive Tg only in IMAs (table 3, patients 6 and 17). Considering the 11 patients with distant metastasis (patients 1-11), 4 (36.4%) had negative Tg in all assays, 6 (54.5%) positive Tg in all assays (patients 2,4,5,9-11), therefore Tg-MS was negative in 5/11 (45.5%) of these patients. One patient with lung metastasis had Tg-MS undetectable but Tg-Beckman was 0.74 ng/mL and Tg-Roche 1.74 ng/mL (table 3, patient 6). The remaining 6 patients with structural disease presented disease confined to the neck (patients 12-17), 4 patients (66.7%) presented negative results in all assays, one (16.7%) had positive Tg in the 3 assays (patient 16) and one positive Tg just in the Beckman assay (patient 17 with cervical lymph nodes, undetectable Tg-MS and Tg-Beckman of 0.42 ng/mL - below the FS of LC-MS/MS). The overall performance in detecting Tg, when considering the FS of 0.7 ng/mL, in Group C1 was better in Tg-IMAs (47% vs 41.2%).

Among the other 15 patients from Group C (biochemical incomplete or indeterminate response to therapy; Group C2; table 3, patients 18-32) Tg was negative in all the three assays in 11/15 (73.3%) of the samples, 12/15 (80%) in Tg-MS and 12/15 (80%) in IMAs. Two patients (13.3%) had positive Tg in all assays and the other 2 patients (13.3%) had discordant results: one (6.7%) had positive Tg-MS and negative

Tg-IMAs (Patient 18; Tg-MS of 2 ng/mL and Tg-IMA undetectable in both assays) and the other one had negative Tg-MS and positive Tg-IMA (Patient 32; Tg-Roche 0.14 ng/mL, below Tg-MS' FS).

Although only one patient with positive TgAb and negative Tg-IMAs (1/20; 5% table 3, patient 18 and in table 5 patient 10) had detectable Tg measured by LC-MS/MS, seven (7/9; 77,8%) had higher results by Tg-MS, besides the positivity in both IMA and LC-MS/MS methods. The average degree of underestimation was 35.8% and varied between 12-51% (p 0.003) for the Beckman assay and average of 27.5%, range 10-63% (p 0.01) for Tg-Roche. These patients are represented in the table 5, patients 1-7. Similarly, one patient (table 3, patient 6 and in table 5, patient 11) had positive Tg just by IMAs (when IMAs' FS was adjusted to 0.7 ng/mL) and other 3 (3/9; 33,3%; table 5, patients 7-9) had Tg-IMAs values higher than Tg-MS (3-24% higher). TgAb titers did not show significant correlation with Tg-IMA underestimation in Spearman's correlation (p 0.46-0.84).

**Table 5.** Relationship between Tg results in patients with positive TgAb and Tg-IMAs > 0.7 ng/mL.

| Patient | Tg-MS (ng/mL) | Tg-Beckman (ng/mL) | Tg-Roche (ng/mL) | Tg-MS vs Tg-Beckman | Tg-MS vs Tg-Roche | TgAb-R (kIU/L) | TgAb-S1 (kIU/L) | TgAb-S2 (kIU/L) |
|---|---|---|---|---|---|---|---|---|
| **1** | 1371.7 | 670 | 500 | -51% | -63% | 21.2 | < 10 | < 0.9 |
| **2** | 2.3 | 1.2 | 1.62 | -47% | -30% | 24.1 | N/A | < 0.9 |
| **3** | 40.4 | 23 | 36.6 | -43% | -10% | 1609 | 882 | >1000 |
| **4** | 3.3 | 2.9 | 2.26 | -12% | -31% | < 10 | < 10 | 7 |
| **5** | 297 | 171 | 263.2 | -42% | -11% | 660 | 839 | 118 |
| **6** | 3.1 | N/A | 2.5 | N/A | -20% | 245 | N/A | N/A |
| **7** | 1.0 | 0.8 | 1.03 | -20% | +3% | 305 | 194 | 6.7 |
| **8** | 1857.4 | 1945 | 2309 | +5% | +24% | 19.7 | < 10 | < 0.9 |
| **9** | 5011 | 5771 | 5445 | +15% | +8% | 24.4 | 29 | < 0.9 |
| **10** | 2.0 | < 0.1 | < 0.1 | - | - | 167.7 | 107 | 76.6 |
| **11** | < 0.7 | 0.74 | 1.74 | - | - | 1367 | >2500 | 131 |

Tg-MS: Thyroglobulin measured by LC-MS/MS; Tg-Beckman: Thyroglobulin measured by Beckman immunometric assay; Tg-Roche: Thyroglobulin measured by Roche immunometric assay, TgAb-R: Thyroglobulin antibodies measured by Roche assay; TgAb-S1: Thyroglobulin antibodies measured by Siemens Centaur assay; TgAb-S2: Thyroglobulin antibodies measured by Siemens Atellica IM assay.

We studied the accuracy of Tg-MS for detecting structural disease in TgAb positive patients, once the most valuable use of Tg-MS is in this specific group. For this analysis we excluded the patients who have not been treated with radioiodine ablation and had positive Tg by any method (table 3, patients 18 and 32) because the detectable Tg in these patients may be due to residual normal thyroid tissue. In this analysis, the sensitivity was 41.2%, specificity 84.6%, positive predictive value (PPV) 77.8% and negative predictive value (NPV) 52.4%. We then analyzed the accuracy of Tg-IMAs in determining disease in these patients. As the concordance between Tg-Roche and Tg-Beckman were 100% in these samples, the results were the same: sensitivity 53.3%, specificity 84.6%, PPV 80% and NPV 61.1%. Using the Tg-MS cutoff of 0.7 ng/mL for Tg-IMAs did not alter the results. Results are shown in tables 6 A and B.

**Table 6.** Diagnostic accuracy of Tg-MS (Figure 6A) and Tg-IMAs (Table 6B) for detecting structural disease in TgAb positive patients.

| Tg-MS | Structural Disease | | Total |
|---|---|---|---|
| | Yes | No | |
| Positive | 7 | 2 | 9 |
| Negative | 10 | 11 | 21 |
| Total | 17 | 13 | 30 |

| Tg-IMA | Structural Disease | | Total |
|---|---|---|---|
| | Yes | No | |
| Positive | 8 | 2 | 10 |
| Negative | 7 | 11 | 18 |
| Total | 15 | 13 | 28 |

Tg-MS: Thyroglobulin measured by LC-MS/MS; Tg-IMA: Thyroglobulin measured by Beckman and Roche immunometric assays.

**Concordance between TgAb assays:**

The overall positivity of TgAb in the three assays were: 15.4% in TgAb-S1, 21.1% in TgAb-S2 and 23.9% in TgAb-R. The overall concordance (positive vs negative) between the 3 assays was 93.8% (91/97 samples). The best concordance was seen between TgAb-S1 and TgAb-S2: 98% (96/98). The concordances between TgAb-S1 and Tg-R and between TgAb-S2 and Tg-R were similar: 94.8% (92/97) and 94.6% (106/112) respectively (Table 7). Among the samples with measurements of TgAb by the 3 assays, 16.5% (16/97) had positive TgAb in the 3 assays, one in 2 assays (1%) and 5 samples (5.1%) had TgAb positive by just one method (4 by TgAb-R and one by TgAb-S2.

**Table 7.** Concordance between TgAb methods

| TgAb assay | TgAb-S1 | TgAb-R |
|---|---|---|
| **TgAb-S2** | 98% (92/97) | 94.6% (106/112) |
| **TgAb-R** | 94.8% (92/97) | - |

**Discussion:**

In clinical practice is not infrequent the change in Tg assay during the follow-up of DTC patients and Tg interassay differences are a complicating factor that may impact in therapeutic decisions. The discrepancy between assays are frequently reviewed in literature and it is highly variable (4,5,5,19,24–26). TgAb pose the biggest challenge in the management of DTC patients as they might interfere in Tg measurement by IMAs. Underestimated or even undetectable Tg values are seen in up to 98% of the samples and there is no correlation with TgAb titers (5,13,19,20,27,28). The trend of TgAb titers can be used as surrogate marker, despite being inaccurate (19,27,29) as the presence of TgAb is not a strong predictor of structural disease but rather indicates the activity of the immune system (27,28). Measurement of Tg by LC-MS/MS has been reported as a method that can provide accurate results when TgAb are present, however it has failed to detect structural disease in about 40% of patients so far (5,18–20). In this study we present the comparison between two IMAs and one LC-MS/MS assay for the measurement of Tg

in negative and positive TgAb samples and the clinical performance in 129 DTC patients.

The overall correlations coefficients between the 3 methods were substantial in the TgAb negative samples (r 0.950 – 0.985; figure 2 and supplemental figure 3), but there was poor correlation between Tg-MS and Tg-IMAs in the presence of TgAb (r 0.875 - 0.878; figure 5). In the samples with negative TgAb, regarding Group A (negative Tg-IMA) the results of the 3 assays were 100% concordant, as expected: all samples had negative results. The overall concordance of positive and negative results in TgAb negative samples (Groups A and B) was also good, almost 92% between IMAs and LC-MS/MS assay and 99% between IMAs. As the FS of the methods are different, we reanalyzed the data considering the cutoff for positivity in IMAs of 0.7 ng/mL (the same as LC-MS/MS). This adjustment improved the agreement to 96.9%. These results are in concordance with the literature (12,13,15). The correlations reported by *Kushnir at al* (15) and *Wheeler et al* (12) between a Tg-MS and Tg-Beckman in TgAb negative samples (r 0.980 and 0.99 respectively) were similar to our result (r 0.9505). *Netzel et al (5)* reported 95-99% concordance rates between Tg-MS and Tg-IMAs using the same negative cut-off for all assays. For assessment of the average percentual differences between the methods we used samples with Tg levels above 0.7 ng/mL. The difference average of +29% was found between Tg-MS and Tg-Beckman, +6.7% between Tg-MS and Tg-Roche and -3% between Tg-Beckman and Tg-Roche (overall range of -62 to +186%). The between-methods biases in Tg assays are well known and indicate that the assays may not be used interchangeably. The standardization against BRC-457 reduces the variability but do not prevent it from existing. It is important that postoperative Tg monitoring in DTC patients be made using the same manufacturers method and preferably in the same laboratory (4,24,25,30). Molecular heterogeneity in Tg derived tumor is the likely cause of the method variability. Different epitopes may be masked or exposed when Tg structure is abnormal (25,31).

In the patients with structural disease, Tg-IMA was detectable in all samples, but Tg-MS was undetectable in 20% of them. All these patients presented disease confined to the neck and Tg-IMAs below Tg-MS' FS. To our knowledge the results of Tg-MS in this particular group of patients (structural disease confined to neck) have

not been addressed yet. This result raises caution regarding the use of Tg-MS to detect DTC persistence/recurrence of low amount and under Levothyroxine therapy. In patients with biochemical and indeterminate response to therapy (positive Tg-IMA and absence of structural disease), Tg-MS had discordant results with Tg-IMAs in just 3 samples. In these cases, interferents were ruled out by serial dilutions and therefore negative Tg-MS levels did not change the clinical status of any patient. It is always a concern whether patients with positive Tg and negative imaging exams have false positive Tg results due to interfering antibodies or true positive values caused by structural diseases not found. In some of these cases, Tg-MS might help because the trypsin employed in the assay to cleave proteins may digest interfering antibodies and provide an accurate Tg level (16,32).

The correlations coefficients found between Tg-MS and Tg-IMAs in Group C (TgAb positive samples) were not as good the as the ones found in the TgAb negative groups, as expected (Figures 5A and 5B). This result was also reported by other studies in the literature (12,13,15,20). However, the concordance rates between the assays were good, especially when considered the FS of 0.7 ng/mL for all of them.

Some aspects should be considered about Tg-MS in positive TgAb patients:

(i) The first important finding in TgAb positive specimens is that Tg-MS had advantage over Tg-IMAs (both assays) in detecting Tg in just 1/20 (5%) patient with negative Tg-IMA. However, this patient classified as indeterminate response to therapy (table 4, patient 18), did not undergo radioiodine treatment and, therefore, this Tg value may be due to normal thyroid tissue. This rate is in concordance to the rates found by *Netzel et al* (16) but discordant to the reports made by *Kushinir et al* (15) and *Spencer et al* (19). *Netzel et al* reported that 8% additional samples were detected by Tg-MS as compared to Tg-Beckman and Tg-Roche but *Kushnir et al* and *Spencer et al* found additional benefit in 23% when compared to Tg-Beckman. The FS of the LC-MS/MS methods used in these studies were 0.5 ng/mL, what may have contributed to the differences between ours and their results.

(ii) Second important finding is that in the subgroup with structural disease (Group C1, n=17) Tg was undetectable in 58.8% of the measurements by LC-MS/MS method and 47% in the IMAs, showing that the assessment of Tg-MS had no

superiority in these patients. Similar results were reported by *Azmat et al.*, *Netzel et al., Spencer et al and Nishihara et al* in LC-MS/MS methods with FS between 0.4-0.5 ng/mL. They found that 43.7%, 44%, 38%, 31.6% respectively, of the patients with structural disease and positive TgAb had undetectable Tg-MS (5,18–20). These differences may also be explained by the lower FS of the LC-MS/MS methods, since many detectable values were below 0.7 ng/mL. Even when considered the higher FS of 0.7 ng/mL, Tg-IMAs had better performance in detecting Tg in these patients (47% vs 41.2%). Two out of the 17 patients (11.8%) with positive TgAb (one with lung metastasis and one with neck disease; table 3 patients 6 and 17) had positive Tg just by IMAs but the detection in one of them (patient 17) was due to the lower FS of Tg-IMA. In the patients with distant metastasis similar results were observed: 36.4% negative Tg in all assays and 45.5% in Tg-MS. Although Tg was detectable in 7/11 patients with distant metastasis, the values were lower than expected in at least 3 of them, even when Tg-MS was detectable (table 3, patient 2 with lung and mediastinum disease and Tg-MS of 3.1 ng/mL; patient 4 with Tg-MS 40.4 and massive lung and cervical disease, and patient 6 with lung metastasis and Tg-Roche of 1.74 ng/mL). Among patients with disease confined to the neck, 66.7% (4/6) had undetectable Tg by all assays (table 3, patients 12-15): two of them had lymph nodes smaller than 1 cm (patients 12 and 13), and the others had diseases greater than 1 cm (patients 14 and 15). Only 2 patients presented detectable Tg (one by all assays and one by Tg-Beckman; table 3, patients 16 and 17). This contrasts with the finding in the patients with negative TgAb and structural disease confined to the neck: all patients had positive Tg in at least one assay even when diseases were smaller than 1 cm. We are aware that patients with small lymph nodes may have negative serum Tg even in the absence of TgAb, as already described in the literature (1,33) but it is not expected in larger diseases. Regarding Group C2 (positive TgAb and absence of structural disease), 80% of the patients presented undetectable Tg-MS and, as described before, just one patient had detectable Tg in LC-MS/MS assay and negative by IMAs. Tg-IMAs were also negative in 80% of the group. These patients have been followed for an average of 6 years and did not present structural disease until now.

(iii) Third finding to be highlighted is that only 7 samples with positive TgAb and detectable Tg-MS had lower Tg-IMAs results. The average degree of underestimation was 35.8% for Tg-Beckman and 27.5% for Tg-Roche (overall range -63 to -11%). We

also observed that 3 samples had higher levels of Tg-IMAs than Tg-MS ranging from 3-24%. *Clarke et al* (13) did not find any lower results in Tg-MS compared to Tg-IMA. *Spencer et al* (25,34) reported that underestimated Tg-IMA values can be found in up to 98% of the TgAb positive samples and the degree of underestimation results can reach 80% when compared to the levels obtained by LC-MS/MS and RAI. *Netzel et al* (5) reported that the type of assay is more decisive in underestimating Tg than the titers of TgAb. They reported that Tg-Beckman and Tg-Roche have a negative bias around 50% relative to TgAb negative samples. In fact, we did not find correlation between TgAb titers and degree of underestimation in any Tg and TgAb assays as shown also by other authors (13,20,27,28). It is important to observe that Tg-MS was also higher (not as higher as in TgAb positive samples) in some TgAb negative specimens: the average of differences between Tg-MS and Tg-IMAs was +29% for Tg-Beckman and +6.7% for Tg-Roche. Therefore, we cannot assume that the underestimated levels of Tg-IMAs are caused exclusively by TgAb interference, but also by the difference of the results between the assays.

(iv) The fourth important result is that, in Group C, the sensitivity of detecting structural disease was higher with Tg-IMAs (53.3%) as compared to Tg-MS (41.2%), indicating no additional benefit in disease detection by measuring Tg-MS in TgAb positive patients. Specificities and PPV had similar results for both methods. In fact, 2 patients with structural disease had detectable Tg-IMA but undetectable Tg-MS but in one of them Tg-IMA was below Tg-MS' FS. *Azmat et al* (18) also reported better sensitivity in Tg-Beckman compared to a LC-MS/MS with FS of 0.5 ng/mL (62.5% vs 56.3%). They found better sensitivity and NPV than we did for Tg-MS, probably related to the better FS of the LC-MS/MS used, similar specificity but our PPV was higher, probably due to the higher frequency of structural disease in our population. Another aspect that can have contributed to this last finding is that they did not mention if patients who have not undergone radioiodine treatment were excluded for this analyze, so their rate of false positive may be bigger. These results demonstrate that at the current FS of the Tg-IMAs of 0.1 ng/mL, there was no benefit in measuring Tg-MS. However, if Tg-MS had an improved FS, it is possible that its performance would also improve.

Some hypotheses can be raised upon the undetectable Tg measurements by LC-MC/MS methods in patients with structural disease and positive TgAb, other than the lower FS. First, the presence of heterogeneous forms of Tg derived tumor (altered by Tg tumor gene mutations or posttranslational modifications) that alters the trypsin-cleavage sites would prevent efficient digestion of the Tg peptide being monitored in the assay, leading to undetectable Tg results in some patients. Monitoring more than one peptide by Tg-MS could help circumvent this potential issue (6,20,35). Second, the presence of TgAb binded to Tg can also difficult the trypsin digestion of Tg. Even with adequate trypsin digestion, the tryptic fragment generated may not present the same charge/mass ratio as the used as reference, thus preventing its recognition by LC-MS/MS analysis (35). Third, the presence of TgAb may increase Tg-TgAb complexes metabolic clearance due to its greater immune elimination from blood stream and distort the relationship between tissue Tg and the circulating Tg concentration (20,36,37). Finally, it is possible that some patients with DTC do not secrete Tg, despite having well-differentiated pathology. These last 2 hypotheses would result in undetectable serum Tg in both Tg-MS and immunoassays. We believe that our patients with structural disease and positive TgAb are not non-secretors because they either presented positive Tg-IMA or detectable Tg in the needle washout fluid from lymph nodes.

The concordances between TgAb assays were high. For the 3 assays we found 93.8% of agreement and the best concordance was between TgAb-S1 and TgAb-S2. This is an important result that was not observed in the literature. As described by *Spencer et al* (4), the agreement between 12 different TgAb assays was only 10%, and 33% of the samples had positive TgAb by just one method. In our study, among the samples with measurements of TgAb by the 3 methods, only 5 samples (5.1%) had TgAb positive by just one method. *Netzel et al* (5) reported detectable TgAb by only 1 of 4 assays in 15% of the samples. We believe that, when suspected interference in Tg measurement occurs, TgAb should be assessed by at least 2 methods to minimize the chance of misclassification.

The strengths of this study are that it is a prospective study in which all measurements were performed in the same sample and TgAb was measured by three assays in almost all specimens. Another aspect to be highlighted is that the study was

conducted in a dedicated DTC center clinical management where the staff is composed of experienced doctors. There are some limitations in this study. First, the number of patients with structural disease and positive TgAb was small. Second, the FS of the LC-MS/MS method is suboptimal what may have led to false-negative results, but it is similar to others Tg-MS' FS reported in the literature (13,15,19,20). As published before (1,33), Tg levels in structural disease confined to the neck are frequently very low or even undetectable. This emphasizes the importance of assay sensitivity for detecting residual thyroid cancer. Lastly, we did not evaluate the Tg-MS measurements after TSH stimulation what may have improved accuracy. However, in patients with positive TgAb, a blunted response of Tg to stimulation has been reported (11).

In conclusion, we found substantial correlations between the assays in negative TgAb samples and poor correlations between Tg-MS and Tg-IMAs in positive TgAb patients. The concordance (positive *vs* negative) between the assays was good regardless the presence of TgAb. In TgAb positive group, the LC-MS/MS assay had advantage in detecting Tg over Tg-IMAs in just 1 patient and almost 60% of the patients with structural disease and positive TgAb had undetectable Tg-MS. Tg-IMAs had better sensitivity in detecting structural disease in the presence of TgAb but were around 30% lower than Tg-MS. Based on these findings, Tg-IMAs with optimal FS should remain the frontline assay in TgAb negative and in also positive patients.

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## Supplements:

**Supplemental Table 1:** Response to therapy

| | **Tg levels*** | | **TgAb** | **Structural image** |
|---|---|---|---|---|
| | *With radioiodine* | *Without radioiodine* | | |
| **Excellent response to therapry** | TgLT4 < 0.2 ng/mL or Tgs < 1 ng/mL | TgLT4 < 0.2 ng/mL or Tgs < 2 ng/mL | Absent | Negative |
| **Structural incomplete response** | Any level | Any level | Any titer | Evidence of structural disease in image or functional studies |
| **Incomplete biochemical response** | TgLT4 > 1 ng/mL or Tgs > 10 ng/mL | TgLT4 > 5 ng/mL or Tgs > 10 ng/mL or Elevation of the levels during follow-up | Rising titers | Negative |
| **Indeterminate** | TgLT4 0.2-1 ng/mL or Tgs 1-10 ng/mL | TgLT4 0.2-5 ng/mL or Tgs 2-10 ng/mL | Stable or declining titers | Non-specific findings or Low uptake in thyroid bed in WBS |

Source: Adapted from *Haugen et at* (1) and *Momesso et al* (21,22).

Tg: Thyroglobulin; TgAb: Thyroglobulin Antibodies; TgLT4: Thyroglobulin measured under levothyroxine therapy; Tgs: Stimulated thyroglobulin (TSH > 30 UI/mL); WBS: Whole body scan.

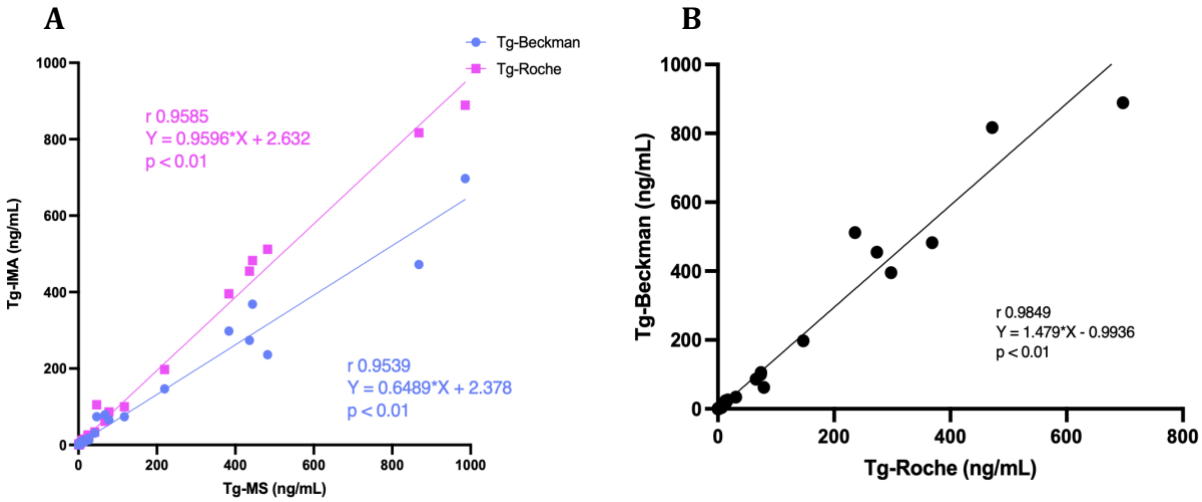
*Thyroglobulin assays with functional sensitivity < 0.1-0.2 ng/mL.

**Supplemental Table 2:** Description of the patients from Group B with structural disease

| Patient | Tg-MS (ng/mL) | Tg-Beckman (ng/mL) | Tg-Roche (ng/mL) | Location of structural disease | Radioiodine activity (mCi) |
|---|---|---|---|---|---|
| **1** | 444 | 368.51 | 482.7 | Lung, bone, kidney | 950 |
| **2** | 41.7 | 30.97 | 33.96 | Lung | 550 |
| **3** | 482.2 | 236 | 512 | Lung | 400 |
| **4** | 24.6 | 17 | 26.15 | Lung, neck | 800 |
| **5** | 26.6 | 13 | 23.64 | Lung | 500 |
| **6** | 2.0 | 1.42 | 1.69 | Lung | 400 |
| **7** | 986.2 | 697 | 889 | Bone | 600 |
| **8** | 383.5 | 298 | 395.7 | Bone, neck | 550 |
| **9** | 219.7 | 147 | 197.3 | Lung, neck | 800 |
| **10** | 2.0 | 0.7 | 1.04 | Lung | 480 |
| **11** | 46.5 | 74 | 105.4 | Lung, neck | 550 |
| **12** | 9.0 | 10.13 | 12.64 | Neck, mediastinum | 250 |
| **13** | < 0.7 | < 0.1 | 0.23 | Neck | 350 |
| **14** | < 0.7 | 0.4 | 0.31 | Neck | 250 |
| **15** | 5.0 | 6.5 | 4.2 | Neck | 250 |
| **16** | < 0.7 | 0.5 | 0.41 | Neck | 600 |
| **17** | 1.0 | 0.8 | 1.07 | Neck | 200 |
| **18** | < 0.7 | 0.4 | 0.29 | Neck | 550 |
| **19** | 16.6 | 13 | 15.14 | Neck | 400 |
| **20** | 868 | 472 | 817 | Neck | 300 |
| **21** | 9.0 | 6.0 | 9.19 | Neck | 250 |
| **22** | 117 | 73.9 | 100 | Neck | 200 |
| **23** | < 0.7 | 0.55 | 0.59 | Neck | 350 |
| **24** | 1.2 | 0.73 | 0.87 | Neck | 630 |
| **25** | 436 | 273.6 | 454.9 | Neck | 0 |

Tg-MS: Thyroglobulin measured by LC-/MS/MS; Tg-Beckman: Thyroglobulin measured by Beckman immunometric assay; Tg-Roche: Thyroglobulin measured by Roche immunometric assay.

**Supplemental Figure 3**. Comparison between Tg assays in samples with positive Tg-IMA and negative TgAb 3A: Correlations between Tg-MS and Tg-Beckman and Tg-MS and Tg-Roche; 3B: Correlation between Tg-Beckman and Tg-Roche.




**Supplemental Table 4.** Tg levels in patients with biochemical incomplete or indeterminate response to therapy and negative TgAb with concordant results.

| Patient | Tg-MS (ng/mL) | Tg-Beckman (ng/mL) | Tg-Roche (ng/mL) |
|---|---|---|---|
| **1** | 2.5 | 3.3 | 2.08 |
| **2** | 1.7 | 1.2 | 1.35 |
| **3** | 1.0 | 0.8 | 1.11 |
| **4** | 16 | 8.4 | 14.92 |
| **5** | 1.0 | 1.8 | 1.6 |
| **6** | 2.0 | 2.6 | 2.14 |
| **7** | 1.3 | 1.1 | 1.1 |
| **8** | 78 | 65.3 | 86.1 |
| **9** | 3.1 | 2.01 | 2.29 |
| **10** | 3.1 | 2.42 | 2.37 |
| **11** | 1.0 | 1.6 | 1.64 |
| **12** | 0.8 | 1.0 | 0.39 |
| **13** | 23.8 | 14.98 | 22.5 |
| **14** | 1.0 | 1.2 | 1.56 |
| **15** | 1.3 | 3.4 | 2.12 |
| **16** | 1.4 | 0.89 | 0.89 |

| | | | |
|---|---|---|---|
| **17** | 1.0 | 0.71 | 0.75 |
| **18** | 67.4 | 79 | 62.02 |
| **19** | 3.0 | 2.0 | N/A |
| **20** | 1.0 | 1.4 | 1.42 |

Tg-MS: Thyroglobulin measured by LC-MS/MS; Tg-Beckman: Thyroglobulin measured by Beckman immunometric assay; Tg-Roche: Thyroglobulin measured by Roche immunometric assay; N/A: not available.

# 6 CONCLUSÕES E CONSIDERAÇÕES FINAIS

A dosagem de Tg continua sendo o melhor marcador tumoral no seguimento dos pacientes com Carcinoma Diferenciado de Tiroide. Os ensaios imunométricos da rotina tem ótimo desempenho, entretanto a presença de interferentes trazem desafios.

Contemplando o objetivo do primeiro manuscrito 1 *"The role of a new polyclonal competitive thyroglobulin assay in the follow-up of patients with differentiated thyroid cancer with structural disease but low levels of serum thyroglobulin by immunometric and LC-MS/MS methods"*, o ensaio competitivo policlonal para a dosagem de Tg, desenvolvido no Laboratório de Endocrinologia da UNIFESP, apresentou ótimo desempenho em pacientes com doença estrutural e valores subestimados de tiroglobulina pelo ensaio imunométrico, em pacientes com AcATg positivos e *borderline.* Quando comparado com a dosagem por LC-MS/MS o ensaio competitivo também mostrou melhor resultado.

Em relação ao objetivo 2, o manuscrito *"False diagnosis of biochemically recurrent thyroid carcinoma and its unnecessary investigation – the importance of testing for heterophile antibodies*" mostrou que investigar pacientes com dosagens elevadas de Tg que não se encaixam no cenário clínico é extremamente importante para evitar exames e tratamentos desnecessários. A avaliação tradicional com diluições seriadas das amostras pode falhar em identificar a interferência e uma nova análise utilizando ensaios diferentes de Tg podem apresentar benefícios nessas situações.

Considerando o objetivo 3, o manuscrito intitulado *"Correlation and clinical performance of three thyroglobulin assays in the management of differentiated thyroid cancer patients"* mostrou boa correlação entre os ensaios imunométricos e por LC-MS/MS nos pacientes com AcATg negativos, porém esse resultado não foi observado em pacientes com AcATg positivos. Não houve benefício na utilização do ensaio por LC-MS/MS para detectar a Tg em pacientes com AcATg positivos e nesse grupo a sensibilidade dos ensaios imunométricos foi melhor para detecção de doença estrutural. Foram encontrados valores mais baixos de Tg nos ensaios imunométricos

do que no ensaio por LC-MS/MS tanto em pacientes com AcATg negativos quanto naqueles com AcATg positivos.

O avanço nos métodos de dosagem de Tg ao longo dos anos permitiu o surgimento de ensaios com ótima acurácia, porém ainda persistem desafios a serem ultrapassados. A interferência pelos AcATg na maioria dos métodos de dosagem de Tg merece destaque visto que 25-30% dos pacientes com CDT tem AcATg positivos e sua presença pode levar a resultados errôneos nos ensaios usuais. O desenvolvimento de métodos que não estejam sujeitos à interferentes é, portanto, de grande interesse e importância.

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## Nota à população

Essa tese de doutorado teve como objetivo estudar os principais interferentes na dosagem de tiroglobulina no seguimento de pacientes acompanhados por câncer de tiroide (principalmente os do tipo papilífero e folicular). O principal marcador do tumor de tiroide é a dosagem de tiroglobulina no sangue e após realizada a cirurgia para retirada completa da glândula tiroide e o tratamento com iodo radioativo, a dosagem baixa ou até indetectável da tiroglobulina indica que o paciente está curado enquanto dosagens elevadas podem indicar presença do tumor.

Porém alguns interferentes podem “atrapalhar” a dosagem de tiroglobulina resultando em valores falsamente baixos ou falsamente altos.

Este trabalho resultou em dados que foram publicados em revista internacional e apresentados em congressos nacionais e internacionais.

Como conclusão dessa tese verificamos que a presença de anticorpos e sua interferência devem ser sempre lembradas frente a um resultado de tiroglobulina que não esteja compatível o quadro clínico do paciente.

# Anexos

## Anexo 1- Termo de Consentimento Livre e Esclarecido (Manuscrito 1)

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**
**Escola Paulista de Medicina, Universidade Federal de São Paulo**

**A. <u>DADOS DE IDENTIFICAÇÃO DO PACIENTE</u>:**

**A.1. Nome do paciente:_____. Iniciais: _____**

**B. <u>DADOS SOBRE A PESQUISA CIENTÍFICA:</u>**

Por favor, leia atentamente estas informações e não tenha nenhum receio de perguntar para esclarecer qualquer dúvida a respeito. A formulação desse documento foi realizada para informá-lo(a) sobre os procedimentos necessários para alcançar o objetivo deste estudo.

**B.1. <u>Título do estudo</u>:**

***Desenvolvimento e validação de um novo ensaio de tiroglobulina em pacientes com doença metastática e dosagem de tiroglobulina indetectável/baixa pelos ensaios de rotina***

**B.2. <u>Pesquisador responsável:</u>**

*Dra. Leila Guastapaglia*
*Dra Rosa Paula Mello Biscolla*

**C. <u>EXPLICAÇÕES AO PACIENTE SOBRE A PESQUISA</u>:**

**C.1. <u>Justificativa e objetivos da pesquisa:</u>**

A pesquisa foi preparada com o objetivo de desenvolver um novo método para dosar a tiroglobulina, que é um marcador do câncer de tiroide no sangue. A tiroglobulina, após o tratamento cirúrgico do câncer de tiroide deve vir baixa ou negativa. Nos casos de valores elevados, devemos pensar em recidiva do câncer no pescoço ou presença de metástases. Porém, alguns pacientes que tem metástases do câncer de tiroide tem resultados de tiroglobulina negativos nos métodos de dosagem que estão disponíveis no mercado atualmente. Esses resultados de tiroglobulina são falsos-negativos (o paciente tem doença, porém o marcador vêm com resultado negativo).

O nosso objetivo é desenvolver um método que consiga detectar a tiroglobulina desses pacientes e comparar com a dosagem da tiroglobulina pelos métodos atuais.

**C.2. <u>Procedimentos que serão utilizados</u>:**

Usaremos amostras do seu sangue que foram colhidas para a dosagem de tiroglobulina durante o seu tratamento no ambulatório de Tiroide, e que estejam guardadas no laboratório da Endocrinologia.

**C.3. <u>Desconfortos e riscos esperados:</u>**

Os pesquisadores verificaram que, em condições similares de protocolo de estudo, raramente os pacientes que participaram das pesquisas referiram algum problema. Esta pesquisa tem risco muito baixo para o paciente. A probabilidade de que você sofra algum dano com conseqüência imediata ou tardia do estudo é mínima. O potencial risco é o vazamento das informações da sua doença.

**C.4. <u>Benefícios:</u>**

Se conseguirmos desenvolver esse novo método, poderemos diagnosticar pacientes com metástases do câncer de tiroide, que poderiam não ter sido diagnosticadas com os métodos de tiroglobulina existentes atualmente.

**D. <u>ESCLARECIMENTOS SOBRE GARANTIAS DO SUJEITO DA PESQUISA:</u>**

**D.1. <u>Confidencialidade:</u>**

A menos que sejam requeridos judicialmente, apenas os examinadores pesquisadores terão acesso aos dados do estudo que identifiquem seu nome. Nenhuma publicação o identificará. Você tem direito de ser mantido atualizado sobre os resultados parciais desta pesquisa. Os dados coletados serão utilizados única e exclusivamente para pesquisa.

**D.2. <u>Despesas</u>:**

Não há despesas pessoais para o participante em qualquer fase do estudo. Também não há compensação financeira relacionada a sua participação. Se existir qualquer despesa adicional, ela será absorvida pelo orçamento da pesquisa.

**D.3. <u>Participação voluntária</u>:**

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Sua participação neste estudo é voluntária. Você pode se recusar a participar bem como desistir do mesmo a qualquer momento, sem qualquer prejuízo de seu tratamento. Por outro lado, sua participação no estudo poderá ser interrompida, sem seu prévio consentimento, pelo médico, caso você não siga as orientações fornecidas ou por problemas administrativos.

**E. INFORMAÇÕES PARA CONTATO EM CASO DE QUAISQUER INTERCORRÊNCIAS:**

Em qualquer etapa deste estudo, o pesquisador irá esclarecer todas as suas dúvidas, sua participação, seus direitos e este termo de consentimento. Você deverá contatar a Dra. Leila Guastapaglia no seguinte endereço: Rua Borges Lagoa, 800; telefone: (11)5549-7255, (11) 5084-5231 ou no celular (11) 99228-1531. Se você tiver alguma dúvida ou consideração sobre a ética desta pesquisa, entre em contato com o Comitê de Ética e Pesquisa (CEP) – R. Botucatu, 572 – 1º. Andar – cj. 14; Fone: 5571-1062, FAX: 5539-7162, E-mail: *cepunifesp@epm.br*

**F. CONSENTIMENTO PÓS-ESCLARECIDO:**

Eu discuti com a Dra. Leila Guastapaglia sobre a minha decisão em participar desse estudo. Ficou claro para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos, as garantias de confidencialidade e de esclarecimentos permanentes. Ficou claro também que minha participação é isenta de despesas e que tenho garantia de acesso a tratamento hospitalar quando necessário. Declaro que concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízo ou perda de qualquer benefício que eu possa ter adquirido ou no meu atendimento neste serviço.

**G. VIAS:**

Uma via deverá ficar com o pesquisador e outra com o participante. Todas as vias serão rubricadas pelo pesquisador principal e pelo participante no momento da aplicação do termo.

_____ Data _____/_____/_____
Assinatura do paciente / representante legal

_____ Data _____/_____/_____
Assinatura da testemunha

Declaro que obtive de forma apropriada e voluntária o Consentimento deste paciente ou representante legal para participação neste estudo.

_____ Data _____/_____/_____
Assinatura do pesquisador principal e carimbo

## Anexo 2- Termo de Consentimento Livre e Esclarecido (Manuscrito 2)

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

Título do Estudo: **Resultado de tiroglobulina falso positivo em pacientes com carcinoma diferenciado de tiroide – importância da pesquisa de anticorpos heterófilos**

Pesquisadores Responsáveis: **Dra Leila Guastapaglia e Dra Rosa Paula Mello Biscolla**

O (A) Senhor (a) está sendo convidado (a) a participar de um RELATO DE CASO. Esse tipo de pesquisa é importante porque destaca alguma situação incomum e/ou fato inusitado do comportamento de uma doença e/ou outra condição clínica. Por favor, leia este documento com bastante atenção antes de assiná-lo. Caso haja alguma palavra ou frase que o (a) senhor (a) não consiga entender, converse com o pesquisador responsável pelo estudo ou com um membro da equipe desta pesquisa para esclarecê-los.

A proposta deste termo de consentimento livre e esclarecido (TCLE) é explicar tudo sobre o relato de caso e solicitar a sua permissão para que o mesmo seja publicado em meios científicos como revistas, congressos e/ou reuniões científicas de profissionais da saúde ou afins.

O objetivo desta pesquisa é relatar um caso e/ou situação clínica específica que ocorreu: Durante o seguimento do seu câncer de tiroide foi evidenciado um alto nível do marcador tiroglobulina no sangue, que levou a investigação de metástases e a tratamentos. Como a investigação de metástases resultou negativa, a presença de interferentes na dosagem da tiroglobulina foi avaliada e mostrou-se positiva. Esse resultado levou a conclusão que o interferente causou um resultado da tiroglobulina falso-positivo. A presença de interferentes na dosagem de tiroglobulina é incomum e pouco suspeitada.

Se o(a) Sr.(a) aceitar esse relato de caso, os procedimentos envolvidos em sua participação são: Coleta dos dados da sua doença e dos resultados de exames pelo prontuário. Não haverá necessidade de colher novos exames ou fazer novos tratamentos. Não serão publicadas fotos ou imagens do seu caso.

A descrição do relato de caso envolve o risco de quebra de confidencialidade (algum dado que possa identificar o(a) sr(a) ser exposto publicamente). Para minimizar esse risco, NENHUM DADO QUE POSSA IDENTIFICAR O(A) SR(A) COMO NOME, CODINOME, INICIAIS, REGISTROS INDIVIDUAIS, INFORMAÇÕES POSTAIS, NÚMEROS DE TELEFONES, ENDEREÇOS ELETRÔNICOS, FOTOGRAFIAS, FIGURAS, CARACTERISTICAS MORFOLÓGICAS (partes do corpo), entre outros serão utilizadas sem sua autorização.

Contudo, este relato de caso também pode trazer benefícios. Os possíveis benefícios resultantes da participação na pesquisa são: alertar os médicos que cuidam de pacientes com carcinomas de tiroide sobre a possível presença de interferentes na dosagem do marcador tiroglobulina e acrescentar informações na literatura sobre essa situação rara. Não haverá benefícios direto aos participantes, porém, contribuirá para o aumento de conhecimento sobre o assunto e poderá beneficiar futuros pacientes.

Sua participação neste relato de caso é totalmente voluntária, ou seja, não é obrigatória. Caso o(a) Sr.(a) decida não participar, ou ainda, desistir de participar e retirar seu consentimento durante a realização do relato de caso, não haverá nenhum prejuízo ao atendimento que você recebe ou possa vir a receber na instituição.

Não está previsto nenhum tipo de pagamento pela sua participação neste relato de caso e o(a) Sr.(a) não terá nenhum custo com respeito aos procedimentos envolvidos.

Caso ocorra algum problema ou dano com o(a) Sr.(a), resultante deste relato de caso, o(a) Sr.(a) receberá todo o atendimento necessário, sem nenhum custo pessoal e pelo tempo que for necessário. Garantimos

indenização diante de eventuais fatos comprovados, com nexo causal com o relato de caso, conforme especifica a Carta Circular nº 166/2018 da CONEP.

É garantido ao Sr.(a), o livre acesso a todas as informações e esclarecimentos adicionais sobre o relato de caso e suas consequências, enfim, tudo o que o(a) Sr.(a) queira saber antes, durante e depois da sua participação. Caso o(a) Sr.(a) tenha dúvidas, poderá entrar em contato com a pesquisadora responsável Dra Rosa Paula Mello Biscolla telefone (11) 5014-7626 ou no celular (11) 97697-9721, endereço Rua General Valdomiro de Lima 508 – Térreo. CEP: 04344-070, São Paulo e email rosapaula.biscolla@grupofleury.com.br ou Dra Leila Guastapaglia no telefone (11) 992281531 e e-mail leilag@hotmail.com ou com o Comitê de Ética em Pesquisa Comitê de Ética em Pesquisa do Grupo Fleury – Rua General Valdomiro de Lima 508 – Térreo. CEP: 04344-070. E-mail: instituto.fleury@grupofleury.com.br

Esse Termo é assinado em duas vias, sendo uma do(a) Sr.(a) e a outra para os pesquisadores.

**Declaração de Consentimento**

Acredito ter sido suficientemente esclarecido a respeito das informações que li ou que foram lidas para mim, descrevendo o estudo "Resultado de tiroglobulina falso positivo em pacientes com carcinoma diferenciado de tiroide – importância da pesquisa de anticorpos heterófilos". Ficaram claros para mim quais são os propósitos do estudo, os procedimentos a serem realizados, seus desconfortos e riscos. Concordo voluntariamente em participar deste estudo e poderei retirar o meu consentimento a qualquer momento, sem penalidades ou prejuízo ao meu atendimento no Fleury Medicina e Saúde.

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Assinatura do paciente/representante legal Data / /

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Assinatura da testemunha Data / /

(Somente para o responsável do projeto)
Declaro que obtive de forma apropriada e voluntária o Consentimento Livre e Esclarecido deste paciente ou representante legal para a participação neste estudo.

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Assinatura do responsável pelo estudo Data / /

## Anexo 3- Termo de Consentimento Livre e Esclarecido (Manuscrito 3)

**Universidade Federal de São Paulo**
Campus São Paulo
Escola Paulista de Medicina
Departamento de Medicina

UNIFESP 25 ANOS
Universidade pública, conhecimento público

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**

**Título do Projeto de Pesquisa:** VALIDAÇÃO CLÍNICA DE UM NOVO ENSAIO PARA A DOSAGEM DE TIROGLOBULINA SÉRICA POR CROMATOGRAFIA LÍQUIDA/ ESPECTROMETRIA DE MASSAS (LC-MS/MS)

**Pesquisadores Responsáveis:** Dra. Rosa Paula Mello Biscolla e Dra. Leila Guastapaglia.

**Local onde será realizada a pesquisa:** Centro de Diabetes. Rua Estado de Israel, 639, Vila Clementino, São Paulo-SP.

Você está sendo convidado(a) a participar, como voluntário(a), da pesquisa acima especificada. O convite está sendo feito a você porque o seus exames de sangue não são compatíveis com o seus exames de imagem ou você fará parte do grupo controle, caso seus exames sejam compatíveis. Sua contribuição é importante, porém, você não deve participar contra a sua vontade.

Antes de decidir se você quer participar, é importante que você entenda porque esta pesquisa está sendo realizada, todos os procedimentos envolvidos, os possíveis benefícios, riscos e desconfortos que serão descritos e explicados abaixo.

A qualquer momento, antes, durante e depois da pesquisa, você poderá solicitar maiores esclarecimentos, recusar-se a participar ou desistir de participar. Em todos esses casos você não será prejudicado, penalizado ou responsabilizado de nenhuma forma.

Em caso de dúvidas sobre a pesquisa, você poderá entrar em contato com o pesquisador responsável Dra Rosa Paula Mello Biscolla, nos telefones (11) 50899214, celular (11) 999126446 e e-mail rosapaula29@gmail.com ou Dra Leila Guastapaglia nos telefones (11) 50899214, celular (11) 992281531 e e-mail leilag@hotmail.com. Este estudo foi analisado por um Comitê de Ética em Pesquisa (CEP) que é um órgão que protege o bem-estar dos participantes de pesquisas. O CEP é responsável pela avaliação e acompanhamento dos aspectos éticos de todas as pesquisas envolvendo seres humanos, visando garantir a dignidade, os direitos, a segurança e o bem-estar dos participantes de pesquisas. Caso você tenha dúvidas e/ou perguntas sobre seus direitos como participante deste estudo ou se estiver insatisfeito com a maneira como o estudo está sendo realizado, entre em contato com o Comitê de Ética em Pesquisa (CEP) da Universidade Federal de São Paulo, situado na Rua Botucatu, 740, CEP 04023-900 – Vila Clementino, São Paulo/SP, telefones (11) 5571-1062 ou (11) 5539-7162, às segundas, terças, quintas e sextas, das 09:00 às 12:00hs ou pelo e-mail cep@unifesp.br.

Todas as informações coletadas neste estudo serão confidenciais (seu nome jamais será divulgado). Somente o pesquisador e/ou equipe de pesquisa terão conhecimento de sua identidade e



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Rua Pedro de Toledo, 720, 2º andar - Vila Clementino - São Paulo - SP
(11) 5576-4848 ramal 2609 ou 3027, departamento.medicina@unifesp.br

| Rubrica do Pesquisador Principal | Rubrica do(a) Participante da Pesquisa |
|---|---|

nos comprometemos a mantê-la em sigilo. Os dados coletados serão utilizados apenas para esta pesquisa.

Após ser apresentado(a) e esclarecido(a) sobre as informações da pesquisa, no caso de aceitar fazer parte como voluntário(a), você deverá rubricar todas as páginas e assinar ao final deste documento elaborado em duas vias. Cada via também será rubricada em todas as páginas e assinada pelo pesquisador responsável, devendo uma via ficar com você, para que possa consultá-la sempre que necessário.

**INFORMAÇÕES IMPORTANTES QUE VOCÊ PRECISA SABER SOBRE A PESQUISA**

**Justificativa para realização da pesquisa:** A pesquisa foi preparada com o objetivo de avaliar um novo método para dosar a tiroglobulina (que é um marcador do câncer de tiroide no sangue), que foi desenvolvido pelo laboratório Fleury. A tiroglobulina, após o tratamento cirúrgico do câncer de tiroide deve vir baixa ou negativa. Nos casos de valores elevados, devemos pensar em retorno do câncer no pescoço ou presença de metástases a distância. Porém, alguns pacientes que tem metástases do câncer de tiroide tem resultados de tiroglobulina negativos nos métodos de dosagem que estão disponíveis no mercado atualmente. Esses resultados de tiroglobulina são falsos-negativos (o paciente tem doença, porém o marcador vêm com resultado negativo). Isso acontece principalmente quando o paciente possui também anticorpos contra a tiroglobulina (anticorpos anti-tiroglobulina).

**Objetivos da pesquisa:** O nosso objetivo é validar um método que consiga detectar a tiroglobulina desses pacientes e também nos pacientes que não tenham anticorpos e comparar com a dosagem da tiroglobulina pelos métodos atuais.

**População da pesquisa:** A população alvo são pacientes portadores de câncer de tiroide que apresentem exames de sangue e de imagem não concordantes, especialmente aqueles que possuem anticorpos anti-tiroglobulina positivos. Será necessário ter um grupo controle para validar o novo método de dosagem de tiroglobulina. Nesse grupo controle serão incluídos pacientes com exames concordantes: aqueles que apresentem metástases e tiroglobulina positiva ou aqueles que não tenham mais doença com exames de imagem negativos e tiroglobulina também negativa.

**Procedimentos aos quais será submetido(a):**

✓ Os pacientes com critérios para participar do estudo serão selecionados dentre aqueles que são seguidos no ambulatório de câncer de tiroide.

✓ O paciente que tenha interesse em participar receberá todos os esclarecimentos necessários sobre a pesquisa e assinará este termo de consentimento livre e esclarecido.



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|---|---|

✓ Usaremos amostras do seu sangue que foram colhidas para a dosagem de tiroglobulina durante o seu seguimento no ambulatório de Câncer de Tiroide. Em alguns casos, caso a amostra não esteja adequada, poderá ser necessário a coleta de uma segunda amostra de sangue (que será realizada da mesma forma que ocorre habitualmente), com data a ser combinada pelo pesquisador e paciente. O volume de sangue que será coletado é de 10 mL.

✓ Nesse amostra de sangue serão dosados os exames que rotineiramente acontecem para o seu seguimento e uma parte será enviada para o laboratório Fleury para a dosagem da tiroglobulina pelo novo método. Após as dosagens, o restante da amostra será descartado.

✓ Não será necessário consultas extras e você receberá os resultados dos exames nas suas próximas consultas.

**Riscos em participar da pesquisa:** Os pesquisadores verificaram que, em condições similares de protocolo de estudo, raramente os pacientes que participaram das pesquisas referiram algum problema. Esta pesquisa tem risco muito baixo para o paciente. A probabilidade de que você sofra algum dano com consequência imediata ou tardia do estudo é mínima. O potencial risco é o vazamento das informações da sua doença e caso seja necessário a coleta de uma nova amostra de sangue poderão haver os desconfortos habituais relacionados a coleta do sangue como hematomas.

**Benefícios em participar da pesquisa:** Se conseguirmos validar esse novo método, haverá uma novo tipo de exame disponível para os pacientes com câncer de tiroide. Além disso, poderemos diagnosticar pacientes com metástases do câncer de tiroide, que poderiam não ter sido diagnosticados com os métodos de tiroglobulina existentes atualmente.

**Forma de acompanhamento do tratamento:** Você continuará o seu seguimento rotineiro no ambulatório de câncer de tiroide.

**Privacidade e confidencialidade:** Os pesquisadores se comprometem a tratar seus dados de forma anonimizada, com privacidade e confidencialidade. Solicitamos autorização para consulta do seu prontuário.

**Acesso a resultados parciais ou finais da pesquisa:** você receberá os resultados dos exames nas suas próximas consultas.

**Custos envolvidos pela participação da pesquisa:** a participação na pesquisa não envolve custos, tampouco compensações financeiras. Se houver gastos, como de transporte e alimentação, eles serão ressarcidos.

**Danos e indenizações:** Se ocorrer qualquer problema ou dano pessoal durante ou após os procedimentos aos quais o Sr. (Sra.) será submetido(a), lhe será garantido o direito a tratamento imediato e gratuito na Instituição, não excluindo a possibilidade de indenização determinada por lei, se o dano for decorrente da pesquisa.



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|---|---|

**Consentimento do participante**

Eu, abaixo assinado, declaro que concordo em participar desse estudo como voluntário(a) de pesquisa. Fui devidamente informado(a) e esclarecido(a) sobre o objetivo desta pesquisa, que li ou foram lidos para mim, os procedimentos nela envolvidos, assim como os possíveis riscos e benefícios decorrentes de minha participação e esclareci todas as minhas dúvidas. Foi-me garantido que eu posso me recusar a participar e retirar meu consentimento a qualquer momento, sem que isto me cause qualquer prejuízo, penalidade ou responsabilidade. Autorizo a divulgação dos dados obtidos neste estudo mantendo em sigilo minha identidade. Informo que recebi uma via deste documento com todas as páginas rubricadas e assinadas por mim e pelo Pesquisador Responsável.

Nome do(a) participante:_____

Endereço:_____

RG:_____; CPF: _____

Assinatura: _____ local e data:_____

**Declaração do pesquisador**

Declaro que obtive de forma apropriada e voluntária, o Consentimentos Livre e Esclarecido deste participante (ou representante legal) para a participação neste estudo. Declaro ainda que me comprometo a cumpri todos os termos aqui descritos.

Nome do Pesquisador: _____

Assinatura: _____ Local/data:_____

Nome do auxiliar de pesquisa/testemunha: _____

Assinatura: _____ Local/data:_____



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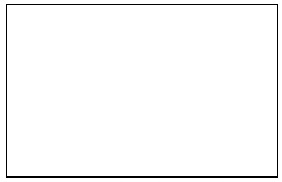
| Rubrica do Pesquisador Principal | Rubrica do(a) Participante da Pesquisa |
|---|---|



Assinatura Datiloscópica *(se não alfabetizado)*

Presenciei a solicitação de consentimento, esclarecimentos sobre a pesquisa e aceite do participante.

Testemunhas (não ligadas à equipe de pesquisadores)

Nome: _____; Assinatura: _____)



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| Rubrica do Pesquisador Principal | Rubrica do(a) Participante da Pesquisa |
|---|---|

## Anexo 4- Parecer do CEP e Plataforma Brasil (Manuscrito 1)

UNIVERSIDADE FEDERAL DE SÃO PAULO - HOSPITAL SÃO PAULO UNIFESP-HSP

**PARECER CONSUBSTANCIADO DO CEP**

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** Desenvolvimento e validação de um novo ensaio de tiroglobulina em pacientes com doença metastática e dosagem de tiroglobulina indetectável/baixa pelos ensaios de rotina

**Pesquisador:** Leila Guastapaglia

**Área Temática:**

**Versão:** 2

**CAAE:** 57296816.9.0000.5505

**Instituição Proponente:** Universidade Federal de São Paulo - UNIFESP/EPM

**Patrocinador Principal:** Fleury S.A
Universidade Federal de São Paulo - UNIFESP/EPM

**DADOS DO PARECER**

**Número do Parecer:** 1.669.236

**Apresentação do Projeto:**

Trata-se da apresentação de respostas de pendencias apontadas no parecer inicial.

Projeto CEP/UNIFESP n: 0853/2016 A tiroglobulina (Tg) sérica é o principal marcador bioquímico utilizado para detectar persistência e recorrência de doença em pacientes em tratamento para Câncer Diferenciado de Tiróide (CDT). Atualmente dispomos de ensaios sensíveis, automatizados e precisos, porém esses ensaios ainda podem falhar em
detectar pacientes com doença metástatica por resultados falsamente indetectáveis ou desproporcionalmente baixos.

**Objetivo da Pesquisa:**

Hipótese: A dosagem de tiroglobulina por ensaio competitivo pode ser utilizada como marcador de metástases em pacientes com dosagens de tiroglobulina indetectável ou baixa pelos ensaios de rotina.- Objetivo Primário: Desenvolver e validar um novo ensaio de Tg competitivo para ser usado em pacientes com doença estrutural (metastática) e Tg indetectável/ baixa nos ensaios imunométricos de rotina.
- Objetivo Secundário: Comparar o novo ensaio de Tg competitivo ao método padrão-ouro para

**Endereço:** Rua Botucatu, 572 1º Andar Conj. 14
**Bairro:** VILA CLEMENTINO **CEP:** 04.023-061
**UF:** SP **Município:** SAO PAULO
**Telefone:** (11)5571-1062 **Fax:** (11)5539-7162 **E-mail:** secretaria.cepunifesp@gmail.com

## Anexo 5- Parecer do CEP e Plataforma Brasil (Manuscrito 2)

INSTITUTO FLEURY/SP

**PARECER CONSUBSTANCIADO DO CEP**

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** RESULTADO DE TIROGLOBULINA FALSO POSITIVO EM PACIENTES COM CARCINOMA DIFERENCIADO DE TIROIDE - IMPORTÂNCIA DA PESQUISA DE ANTICORPOS HETERÓFILOS

**Pesquisador:** Rosa Paula Mello Biscolla

**Área Temática:**

**Versão:** 2

**CAAE:** 40461420.8.0000.5474

**Instituição Proponente:** Fleury S.A

**Patrocinador Principal:** Financiamento Próprio

**DADOS DO PARECER**

**Número do Parecer:** 4.609.486

**Apresentação do Projeto:**

Trata-se de descrição de casos pontuais sobre resultado de tiroglobulina.

**Objetivo da Pesquisa:**

Descrição de casos

**Avaliação dos Riscos e Benefícios:**

Beneficios potenciais maiores que os riscos.

**Comentários e Considerações sobre a Pesquisa:**

Casos de interesse clínico e laboratorial.

**Considerações sobre os Termos de apresentação obrigatória:**

Adequados

**Conclusões ou Pendências e Lista de Inadequações:**

Não há inadequações.

**Considerações Finais a critério do CEP:**

**Este parecer foi elaborado baseado nos documentos abaixo relacionados:**

**Endereço:** Avenida General Valdomiro de Lima, 508, 1º andar
**Bairro:** Jabaquara **CEP:** 04.344-903
**UF:** SP **Município:** SAO PAULO
**Telefone:** (11)5014-7771 **Fax:** (11)5014-7425 **E-mail:** instituto.fleury@grupofleury.com.br

## Anexo 6- Parecer do CEP e Plataforma Brasil (Manuscrito 3)

INSTITUTO FLEURY/SP

**PARECER CONSUBSTANCIADO DO CEP**

**Elaborado pela Instituição Coparticipante**

**DADOS DO PROJETO DE PESQUISA**

**Título da Pesquisa:** VALIDAÇÃO CLÍNICA DE UM NOVO ENSAIO PARA A DOSAGEM DE TIROGLOBULINA SÉRICA POR CROMATOGRAFIA LÍQUIDA/ ESPECTROMETRIA DE MASSAS (LC-MS/MS)

**Pesquisador:** Rosa Paula Mello Biscolla

**Área Temática:**

**Versão:** 1

**CAAE:** 35353020.2.3001.5474

**Instituição Proponente:** Fleury S.A

**Patrocinador Principal:** Fleury S.A

**DADOS DO PARECER**

**Número do Parecer:** 4.281.528

**Apresentação do Projeto:**

Projeto realizado em conjunto com a UNIFESP que visa validar novo ensaio para dosagem de tiroglobulina sérica por cromatografialíquida/ espectrometriade massas (lcms/ms)

**Objetivo da Pesquisa:**

validar novo ensaio para dosagem de tiroglobulina sérica por cromatografia líquida/ espectrometriade massas (lcms/ms)e comparar com o método padrão.

**Avaliação dos Riscos e Benefícios:**

Riscos reduzidos pois se trata de validação de método.

**Comentários e Considerações sobre a Pesquisa:**

Projeto de importancia metodologica e que

**Considerações sobre os Termos de apresentação obrigatória:**

adequados. Responde adequadamente os questionamentos levantados.

**Conclusões ou Pendências e Lista de Inadequações:**

Não há inadequações

**Considerações Finais a critério do CEP:**

**Endereço:** Avenida General Valdomiro de Lima, 508, 1º andar
**Bairro:** Jabaquara **CEP:** 04.344-903
**UF:** SP **Município:** SAO PAULO
**Telefone:** (11)5014-7771 **Fax:** (11)5014-7425 **E-mail:** instituto.fleury@grupofleury.com.br

## Anexo 7- Parecer do Grupo Fleury (Manuscrito 1)

GrupoFleury

**Carta de Anuência**

A quem interessar possa,

Declaramos, para os devidos fins, que após análise do projeto de pesquisa, intitulado **"Desenvolvimento e validação de um novo ensaio de tiroglobulina em pacientes com doença estrutural e dosagem de tiroglobulina indetectável/ baixa pelos ensaios de rotina"** dos pesquisadores Leila Guastapaglia, José Gilberto Henriques Vieira e Rosa Paula Mello Biscolla, o mesmo foi aprovado para desenvolvimento em parceria com o Grupo Fleury. As etapas experimentais serão desenvolvidas em nossa área de pesquisa e desenvolvimento, que possui os equipamentos e reagentes necessários para tal.

São Paulo, 17 de junho de 2016.

Flávia Helena da Silva
Coordenadora de Pesquisa e Desenvolvimento
Grupo Fleury

Flávia Helena da Silva
Coordenadora de Pesquisa
e Desenvolvimento

Av. General Valdomiro de Lima, 508 – 04344-903 – São Paulo - SP

## Anexo 8- Parecer do Grupo Fleury (Manuscrito 2)

GrupoFleury

### Parecer sobre projeto de Pesquisa

Prezada pesquisadora,

Agradecemos pela submissão do seu projeto **RESULTADO DE TIROGLOBULINA FALSO POSITIVO EM PACIENTES COM CARCINOMA DIFERENCIADO DE TIROIDE – IMPORTÂNCIA DA PESQUISA DE ANTICORPOS HETERÓFILOS**.

Temos a satisfação de informar que a sua proposta foi aprovada nos termos dos critérios estabelecidos pelo Grupo Fleury, com orçamento total de **R$900,00**.

Para que possamos dar início à condução do projeto é necessário atendimento aos seguintes passos:

- Aprovação do projeto pelo(s) Comitê(s) de Ética em Pesquisa da(s) instituição(ões) envolvida(s);
- Assinatura do Termo de Cooperação Técnica (pelos representantes legais das instituições envolvidas), nos casos de projeto em parceria.

Segue o link do site do Grupo Fleury, com orientações sobre como submeter o projeto ao CEP (Comitê de Ética em Pesquisa): http://www.grupofleury.com.br/SitePages/inovacao/submissao-projetos-cep.aspx#conteudo

A submissão do projeto ao CEP, deverá ocorrer através da Plataforma Brasil, no seguinte endereço: http://plataformabrasil.saude.gov.br/login.jsf

Para a identificação do CEP do Grupo Fleury, quando da submissão do projeto, basta inserir no campo de busca do CEP o seguinte CNPJ: 60.840.055/0001-31.

## Anexo 9- Parecer do Grupo Fleury (Manuscrito 3)

GrupoFleury

### Parecer sobre projeto de Pesquisa

Prezado pesquisador,

Agradecemos pela submissão do seu projeto **VALIDAÇÃO CLÍNICA DE UM NOVO ENSAIO PARA A DOSAGEM DE TIROGLOBULINA SÉRICA POR CROMATOGRAFIA LÍQUIDA/ ESPECTROMETRIA DE MASSAS (LC-MS/MS)**.

Temos a satisfação de informar que a sua proposta foi aprovada nos termos dos critérios estabelecidos pelo Grupo Fleury, com orçamento total de **R$26.000,00** a ser utilizado com a realização dos testes diagnósticos e da prestação de serviço de estatística.

Para que possamos dar início à condução do projeto é necessário atendimento aos seguintes passos:

- Aprovação do projeto pelo(s) Comitê(s) de Ética em Pesquisa da(s) instituição(ões) envolvida(s);
- Assinatura do Termo de Cooperação Técnica (pelos representantes legais das instituições envolvidas), nos casos de projeto em parceria.

Segue o link do site do Grupo Fleury, com orientações sobre como submeter o projeto ao CEP (Comitê de Ética em Pesquisa): http://www.grupofleury.com.br/SitePages/inovacao/submissao-projetos-cep.aspx#conteudo

A submissão do projeto ao CEP, deverá ocorrer através da Plataforma Brasil, no seguinte endereço: http://plataformabrasil.saude.gov.br/login.jsf

## Trabalhos apresentados em Congressos durante a Pós-graduação

1. **Apresentação Oral em Congresso:**

   - “Desenvolvimento e validação de um novo ensaio de tiroglobulina em pacientes com doença estrutural e dosagem de tiroglobulina indetectável/ baixa pelos ensaios de rotina” – Encontro Brasileiro de Endocrinologia, 05/2016

2. **Apresentações em Congresso na Forma de Pôster:**

   - “Comparison Between Three Thyroglobulin Assays: Immunometric, Polyclonal Competitive and Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS), in Papillary Thyroid Cancer Patients with Metastatic Disease” - Congresso da Endocine Society 04/2017, disponível em https://endo.confex.com/endo/2017endo/meetingapp.cgi/Paper/31325

   - “Validation of a new competitive thyroglobulin assay in Differentiated Thyroid Cancer patients with structural disease and comparison with immunometric assay and LC-MS/MS results” – Congresso Mundial de Câncer de Tiroide, 07/2017

   - “Diagnostic pitfall of biochemically recurrent thyroid carcinoma: the importance of testing for heterophile antibodies” – Encontro Brasileiro de Tiroide 04/2022