ASSAY INTERFERENCES IN THYROID FUNCTION TESTS

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ASSAY INTERFERENCES IN THYROID FUNCTION TESTS

1. Introduction

2. TSH

3. Tg

4. FT4 and FT3
BIOCHEMICAL DIAGNOSIS OF THYROID FUNCTION DISORDERS

1. TSH NORMAL  (0.4-4.0 mU/l) → euthyroidism

2. TSH DECREASED (<0.4 mU/l)
   a. FT4 increased → primary hyperthyroidism
   b. FT4 normal → subclinical hyperthyroidism
      nonthyroidal illness
   c. FT4 decreased → central hypothyroidism
      severe nonthyroidal illness

3. TSH INCREASED  (>4.0 mU/l)
   a. FT4 increased → TSH producing adenoma
      resistance to thyroid hormone
   b. FT4 normal → subclinical hypothyroidism
      nonthyroidal illness (recovery)
   c. FT4 decreased → primary hypothyroidism
CASE 1
F 46 yr

- not well, tired, often headache
- oligo/amenorrhoea since 8 months
- physical exam unremarkable
- no galactorrhoea

WHAT WOULD YOU DO?
PRL  51 µg/l  slightly increased
FSH  <2.0 U/l  too low for postmenopause
Cortisol  159 nmol/l  decreased
FT4  <2.0 pmol/l  decreased
IGF-1  <5 nmol/l  decreased

DIAGNOSIS: HYPOPITUITARISM
MACROADENOMA (CT-scan)

MANAGEMENT?
• FIRST : HYDROCORTISONE REPLACEMENT

• SECOND : THYROIDININE REPLACEMENT

WHAT IS THE NATURE OF THE PITUITARY MASS?
TSH 178 mU/l markedly elevated
TPO-Ab 2400 kU/l markedly elevated

DIAGNOSIS: PRIMARY AUTOIMMUNE HYPOTHYROIDISM WITH SECONDARY PITUITARY HYPERPLASIA
PITUITARY HYPERPLASIA RESULTING FROM PRIMARY HYPOTHYROIDISM MIMICKING MACROADENOMA

before T4 treatment

after T4 treatment

Young et al., Brit J Neurosurg 1999
CASE 2
F 38 yr

- clinical features of thyrotoxicosis
- TSH persistently normal 0.49 – 2.48 mU/L
- FT4 unequivocally elevated 45 – 82 pmol/L
  → marked discrepancy between clinics and lab

- falsely elevated TSH due to assay interference of heterophilic antibodies
- patient treated with carbimazol
  → heterophilic antibodies disappeared after 10 months but returned when hyperthyroidism relapsed

CASE 3
F 32 yr

- employed in beauty salon; tired
- physical exam unremarkable
- family physician: TSH 12.5 mU/l (elevated) 
  FT4 12.5 pmol/l (normal)

SUBCLINICAL HYPOTHYROIDISM

WHAT DO YOU DO?
F 32 yr

- repeat measurements
  TSH 11.8 mU/l, FT4 13.0 pmol/l

- look for underlying thyroid disease
  TPO antibodies <30 kU/l
  thyroid ultrasound normal

WHAT NOW?
TRIAL OF THYROXINE 100 µg DAILY

- after 3 months still tired
  TSH 10 mU/l, FT4 12 pmol/l

- thyroxine discontinued, 3 months later still tired
  TSH 14 mU/l, FT4 14 pmol/l

PLEASE HELP!
F 32 yr

- observation: TSH did not fall upon L-T4 medication
- suspicion on patient in compliance or assay interference
- consult lab: interference of HAMA in TSH assay
  real TSH 1.5 mU/L = euthyroid
LESSONS

1. In case of abnormal FT4 → measure always TSH
   In case of abnormal TSH → measure always FT4

2. In case of discrepancy between clinics and laboratory:
   In case of discrepancy between thyroid function tests:
   → look for assay interference, consult laboratory
ASSAY INTERFERENCES IN THYROID FUNCTION TESTS

1. Laboratory interference is underrecognized.

2. Routine laboratory techniques can help diagnose this rare entity.

3. Close dialogue between the physician and the laboratory is very important in approaching such cases.
ASSAY INTERFERENCES IN THYROID FUNCTION TESTS

1. Heterophilic antibodies
   - rheumatoid factor

2. Human anti-animal antibodies (HAA)
   - human anti-mouse antibodies (HAMA)

3. Antibodies against TSH, Tg, T4 and T3

4. Drugs (heparin, furosemide)
DEFINITIONS

1. Heterophilic antibodies
   low affinity polyspecific antibodies formed early in the immune response before affinity maturation

2. Rheumatoid factor
   commonly encountered subtype of heterophilic antibody with non-specific affinity for the Fc fragment of human and some animal IgG; it is an IgM

3. Human anti-animal antibodies (HAA)
   high affinity antibodies directed against animal epitopes

MECHANISMS OF ASSAY INTERFERENCE

1. Heterophilic and HAA antibodies
   - capable of cross-linking the capture and detection antibody in a ‘sandwich’ assay → falsely high results
   - prevents cross-linking between capture and detection antibody by binding to either antibody → falsely low results

2. Autoimmune anti-hormone antibodies
   - does not involve cross-linking
   - autoimmune antibody-hormone complex is immunoreactive but biologically inactive
IMMUNOMETRICASSAYS

antigen acts as a bridge between capture and labelled detection antibodies
POSITIVE INTERFERENCE BY HAA AND HETEROPHILIC ANTIBODIES

cross-linking of capture and detection antibodies → falsely high results
NEGATIVE INTERFERENCE BY HAA AND HETEROPHILIC ANTIBODIES

bind capture or detection antibody, prevents cross-linking → falsely low result
ASSAY INTERFERENCES IN THYROID FUNCTION TESTS

1. Introduction

2. TSH
   - macroTSH (anti-TSH antibodies)
   - heterophilic antibodies
   - human anti-animal antibodies

3. Tg

4. FT4 and FT3
CASE 4
M 60 yr

- hospitalized for hip fracture after syncope
- polypharmacy including aspirin and atenolol
- sinus bradycardia 55-60 beats per minute
- thyroid function tests to exclude hypothyroidism:
  TSH 232 mU/L, low-normal FT4 10 pmol/l
- clinically euthyroid, no family history of thyroid disease

Loh et al. JCEM 2002; 97: 1823
## PROCEDURES TO IDENTIFY FALSE-POSITIVE TSH

<table>
<thead>
<tr>
<th>Procedure on patient’s serum</th>
<th>TSH results</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated patient’s sample - other assay</td>
<td>122 mU/L 232 mU/L</td>
<td>Different results in various assays suggest interference</td>
</tr>
<tr>
<td>Dilution 10-fold</td>
<td>165 mU/L, 135% recovery</td>
<td>Increasing recovery upon dilution suggests interference</td>
</tr>
<tr>
<td>PEG precipitation and reassay supernatant</td>
<td>3.9 mU/L, 3.2% recovery</td>
<td>Presence of a high-molecular-weight interfering substance</td>
</tr>
<tr>
<td>Heterophile-blocking tube</td>
<td>116 mU/L, 95% recovery</td>
<td>Interference by heterophilic antibodies unlikely</td>
</tr>
<tr>
<td>Incubation with hypothyroid (high TSH) sample</td>
<td>expected 144 mU/L, measured 123 mU/L recovery 85%</td>
<td>Presence of excess TSH binding capacity, likely macro-TSH interference</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td>high-molecular-weight TSH fraction present, increasing after incubation with high TSH sample</td>
<td>Confirms presence of macro-TSH</td>
</tr>
</tbody>
</table>

Loh et al. JCEM 2012; 97: 1823
STRAIGHT FOR INVESTIGATION OF MACRO-TSH

Clinically inconsistent thyrotropin (TSH) result

- Repeat TSH in 3-6 months
- Review clinical history
- Non-discrepant results
- Unlikely assay interference

- Non-linear recovery
  - Perform serial dilution of the sample and measure TSH

- Linear recovery
  - Possible assay interference
    - Likely heterophile antibodies interference
      - Low TSH recovery
        - Elevated
        - Measure rheumatoid factors
          - Incubation of patient serum with a presumably interference-free hypothyroid sample
            - Low TSH recovery
              - Possible macro-TSH interference
                - Gel filtration chromatography
                  - Presence of high molecular weight TSH species
                    - Dissociation of high molecular weight species by acidic elution buffer; increased recovery after incubation with hypothyroid sample
                      - Confirmation of macro-TSH

- Non-discrepant results
  - Likely interference by rheumatoid factors
    - Not elevated
      - High TSH recovery
CASE 5
F 68 yr

- rheumatoid arthritis treated with methotrexate

- feeling well, no complaints

- routine blood test: TSH 171 mU/L, FT4 16.3 pmol/l

- no family history of thyroid disease

- previous treatment with thyroxine stopped because of hyperthyroid symptoms

- rheumatoid factor 735 IU/ml (elevated)

INTERFERENCE OF HETEROPHILIC ANTIBODIES IN TSH ASSAY UNLIKELY

non-linearity of TSH on dilution

preincubation with heterophilic antibody blocking reagent (based on polymeric murine Fab fragments)

INTERFERENCE IN TSH ASSAY CAUSED BY ANTI-RUTHENIUM ANTIBODIES

Polyethyleneglycol precipitation

<table>
<thead>
<tr>
<th></th>
<th>TSH before PEG</th>
<th>TSH after PEG</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient serum</td>
<td>205 mU/L</td>
<td>3.88 mU/L</td>
<td>2%</td>
</tr>
<tr>
<td>Control serum</td>
<td>177 mU/L</td>
<td>138 mU/L</td>
<td>78%</td>
</tr>
</tbody>
</table>

Gel filtration chromatography

- Interfering IgG ■ and IgG aggregates ●
- Free (unbound) TSH * about 28 kDa

### INTERFERENCE BY HETEROPhilIC ANTIBODIES: DIFFERENT RESULTS IN DIFFERENT TSH ASSAYS

<table>
<thead>
<tr>
<th>Assay method</th>
<th>TSH level (mIU/L)</th>
<th>First antibody</th>
<th>Second antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Direct</td>
<td>After 5x dilution</td>
<td></td>
</tr>
<tr>
<td>Abbott Architect</td>
<td>25</td>
<td>48</td>
<td>MM(\alpha)</td>
</tr>
<tr>
<td>Bayer Advia Centaur</td>
<td>8,8</td>
<td>Not tested</td>
<td>SP</td>
</tr>
<tr>
<td>Beckman Coulter DXL</td>
<td>40</td>
<td>Not tested</td>
<td>MM</td>
</tr>
<tr>
<td>Perkin Elmer (Auto)DELFIA*</td>
<td>11</td>
<td>40</td>
<td>MM</td>
</tr>
<tr>
<td>Roche Modular*</td>
<td>108</td>
<td>114</td>
<td>MM</td>
</tr>
<tr>
<td>Siemens DPC Immulite 2000</td>
<td>62</td>
<td>78</td>
<td>MM</td>
</tr>
<tr>
<td>Ortho Diagnostics Vitros Eci</td>
<td>95</td>
<td>98</td>
<td>MM</td>
</tr>
</tbody>
</table>

First antibody is immobilized, second carries label. MM, MM\(\alpha\), MM\(\beta\), mouse monoclonal anti-wholeTSH, anti \(\alpha\)-, anti \(\beta\)-TSH respectively; GP, goat polyclonal anti-whole TSH; SP, sheep polyclonal anti-whole TSH.

* Averaged results from two labs

PREVALENCE OF INTERFERENCE IN TSH IMMUNOASSAYS

681 subjects with subclinical hypothyroidism

polyethylene glycol extraction (PEG)

117 subjects with PEG-precipitable TSH ratio’s >75%

gel filtration chromatography

11 subjects with TSH eluted at >100 kDa

8/11 anti-TSH autoantibodies (IgG)

1/11 HAMAS

2/11 non-IgG

PREVALENCE 1.62%

Hattori et al. CE 2014
ASSAY INTERFERENCES IN THYROID FUNCTION TESTS

1. Introduction

2. TSH

3. Tg
   - TgAb (anti-thyroglobulin antibodies)
   - heterophilic antibodies
   - human anti-animal antibodies

4. FT4 and FT3
RELEVANCE OF SERUM Tg AND INTERFERENCE OF TgAb in Tg ASSAYS

1. Serum Tg is the primary biochemical marker of differentiated thyroid carcinoma (DTC)

2. TgAb interfere with Tg immunometric assays → falsely low or undetectable Tg values that can mask disease

3. Guidelines: every Tg test have TgAb measured simultaneously and quantitatively by immunoassay

4. TgAb trend reflects changes in thyroid tissue mass → TgAb serve as surrogate postoperative tumor marker
TRENDS IN TgAb VERSUS RISK OF PERSISTENT OR RECURRENT DISEASE

# TgAb Trends in DTC Patients with > 3 Years of Follow-up

<table>
<thead>
<tr>
<th>TgAb Trend</th>
<th>Disease detected during follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 50% fall from initial value</td>
<td>&lt; 3% disease $^{(5,7,47)}$</td>
</tr>
<tr>
<td>stable (&lt; 50% change)</td>
<td>~20% disease $^{(2,5,54)}$</td>
</tr>
<tr>
<td>&gt; 50% rise</td>
<td>~40% disease $^{(9,47)}$</td>
</tr>
</tbody>
</table>

Became TgAb-negative @ median ~ 4 years

Spencer & Fatemi. BPRCEM 2013; 27: 701
1. TgAb are present in 20-30% of patients with differentiated thyroid carcinoma.

2. Tg-IMA (automated 2nd generation immunometric assay) is rapidly becoming standard of care because superior functional sensitivity (0.05 – 0.10 μg/L).

3. Tg-IMA: TgAb cause falsely low or undetectable Tg.

4. Tg-RIA and Tg-LC-MS/MS resist interference by TgAb, but have an order of magnitude inferior functional sensitivity (0.5 – 1.0 μg/L).
COMPARATIVE Tg VALUES BY IMA, LC-MS/MS, RIA

Red - between method CV>30%
Red - Tg <LoQ of LC-MS/MS

Spencer et al. JCEM 2014; 99: 4589
RELATIONSHIP BETWEEN Tg-RIA AND Tg-IMA IN THE ABSENCE OR PRESENCE OF TgAb

Spencer et al. JCEM 2014; 99: 4589
RELATIONSHIP BETWEEN TgAb (horizontal axis) AND TgAb INTERFERENCE in Tg ASSAY (vertical axis)

interference if Tg-IMA/Tg-RIA ratio is <75%

AS = analytic sensitivity of TgAb assay; MC = manufacturer cut-off for detecting TgAb

Spencer et al. JCEM 2011; 96: 1283
RELATION BETWEEN Tg AND TgAb AS A FUNCTION OF TgAb INTERFERENCE

(present if ratio Tg-IMA / Tg -RIA is <75%)

Spencer et al. JCEM 2011; 96: 3615 and Spencer et al. BRPCEM 2013; 27: 701
PERCENT OF SERA WITH UNDETECTABLE Tg-IMA (<0.1 μg/L) ASSOCIATED WITH DETECTABLE Tg-RIA (≥1.0 μg/L)

Spencer et al. JCEM 2011; 96: 3615 and Spencer et al. BRPCEM 2013; 27: 701
DIAGNOSTIC PERFORMANCE OF HIGHLY SENSITIVE Tg DURING L-T4 IN DTC FOLLOW-UP: A META-ANALYSIS

1. Unstimulated basal hsTg (using assays with FS ≤0.1 μg/L) has very high negative predictive value but suboptimal positive predictive value in monitoring DTC patients.

2. Tg stimulation test:
   - can be avoided with basal hsTg ≤0.1 μg/L (provided TgAb absent)
   - should be considered when hsTg value is above 0.1μg/L

Giovanella et al. JCEM 2014; 99: 440
HETEROPHILIC ANTIBODIES MAY FALSELY INCREASE OR DECREASE Tg-IMA RESULTS

- 406 samples from DTC patients (TgAb positive samples excluded)

- Tg measured before and after incubation in heterophile-blocking tubes (HBT, Scantibodies)

- Mean difference between original and post-HBT Tg values 0.47 ± 0.105 %

- Heterophilic interference = absolute percent difference >3 SD from mean percent difference

- Heterophilc antibodies present in 5/406 samples = 1.2%

HETEROPHILIC ANTIBODIES MAY FALSELY INCREASE OR DECREASE Tg-IMA RESULTS

<table>
<thead>
<tr>
<th>sample</th>
<th>Tg on L-T4</th>
<th>Tg on L-T4</th>
<th>Tg after rhTSH</th>
<th>Tg after rhTSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre-HBT</td>
<td>post-HBT</td>
<td>pre-HBT</td>
<td>Post-HBT</td>
</tr>
<tr>
<td>1</td>
<td>&lt;0.36</td>
<td>4.10</td>
<td>&lt;0.36</td>
<td>10.7</td>
</tr>
<tr>
<td>2</td>
<td>0.98</td>
<td>12.4</td>
<td>1.2</td>
<td>28.2</td>
</tr>
<tr>
<td>3</td>
<td>4.1</td>
<td>&lt;0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>&lt;0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>5.7</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tg as μg/L; HBT = heterophile-blocking tubes

2/5 patients had false-negative or falsely low Tg: both had metastases
3/5 patients had false-positive or falsely high Tg: both without recurrence

<table>
<thead>
<tr>
<th>Peptide Immunoenrichment</th>
<th>Before</th>
<th>After</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteins in plasma</td>
<td>1,796</td>
<td></td>
</tr>
<tr>
<td>Peptides in plasma digest</td>
<td>252,519</td>
<td>253</td>
</tr>
<tr>
<td>Peptides with the correct mass</td>
<td>486</td>
<td>1</td>
</tr>
<tr>
<td>Peptides with the correct mass and fragment</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Peptides with the correct mass, fragment, and retention time</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Hoefnagle & Roth. JCEM 2013; 98: 1343
Tg-LC-MS/MS: CHROMATOGRAM OF PATIENT SAMPLE CONTAINING 5 ng/ml Tg
INTRA-ASSAY, INTER-ASSAY AND TOTAL IMPRECISION OF Tg LC-MS/MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration, ng/mL</th>
<th>Intra-assay, %</th>
<th>Interassay, %</th>
<th>Total, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low 1^a</td>
<td>2.1</td>
<td>6.75</td>
<td>3.67</td>
<td>7.69</td>
</tr>
<tr>
<td>Low QC 1^b</td>
<td>2.3</td>
<td>NA^c</td>
<td>13.9</td>
<td>NA</td>
</tr>
<tr>
<td>Low 2^a</td>
<td>5.7</td>
<td>6.87</td>
<td>5.96</td>
<td>9.10</td>
</tr>
<tr>
<td>Medium QC 2^b</td>
<td>6.5</td>
<td>NA</td>
<td>10.5</td>
<td>NA</td>
</tr>
<tr>
<td>Medium^a</td>
<td>14.8</td>
<td>6.56</td>
<td>5.40</td>
<td>8.50</td>
</tr>
<tr>
<td>High^a</td>
<td>399</td>
<td>3.56</td>
<td>1.71</td>
<td>3.95</td>
</tr>
<tr>
<td>High QC^b</td>
<td>172.8</td>
<td>NA</td>
<td>3.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

^a Samples analyzed in 3 replicates per day over 5 days.
^b Samples analyzed in 1 replicate per day over 20 days.
^c NA, not applicable.
Tg LC-MS/MS COMPARISON WITH Tg IMA

Panel A and B
TgAb negative

Panel C
TgAb positive

23% of samples negative for Tg in IMA had Tg ≥ 0.5 ng/ml by LC-MS/MS

SERUM Tg LC-MS/MS CONCENTRATIONS IN HEALTHY CHILDREN AND ADULTS

# Interference by Heterophilic Antibodies in Tg-IMA (Immulite) and Tg-LC-MS/MS

<table>
<thead>
<tr>
<th>Sample</th>
<th>HAB interference</th>
<th>Neat</th>
<th>x2&lt;sup&gt;a&lt;/sup&gt;</th>
<th>x4&lt;sup&gt;a&lt;/sup&gt;</th>
<th>With HAB blocker</th>
<th>Immulite Tg, μg/L</th>
<th>LC-MS/MS Tg, μg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Negative</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>2</td>
<td>Negative</td>
<td>462</td>
<td>534</td>
<td>479</td>
<td>&gt;462</td>
<td>&gt;300</td>
<td>445</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
<td>194</td>
<td>186</td>
<td>171</td>
<td>196</td>
<td>NA</td>
<td>223</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
<td>99</td>
<td>93</td>
<td>88</td>
<td>100</td>
<td>158</td>
<td>76</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>1.5</td>
<td>1.3</td>
<td>1.1</td>
<td>0.2</td>
<td>NA</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>604</td>
<td>401</td>
<td>340</td>
<td>122</td>
<td>1.2</td>
<td>0.94</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>70.2</td>
<td>51.2</td>
<td>36.7</td>
<td>3.2</td>
<td>&lt;0.20</td>
<td>&lt;0.5</td>
</tr>
</tbody>
</table>

<sup>a</sup> x2 and x4: 1:2 (v/v) and 1:4 (v/v) dilutions from neat, respectively.
<table>
<thead>
<tr>
<th>Method</th>
<th>Principle</th>
<th>Turn-around time</th>
<th>Strengths/pitfalls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radioimmunoassay, RIA</td>
<td>Competitive; limited quantity of polyclonal antibodies</td>
<td>~ 6 days</td>
<td>FS ~ 0.5 μg/L, TgAb interference resistant, HAMA interference no Polyclonal Ab → broad Tg epitope specificity</td>
</tr>
<tr>
<td>Immunometric assay, IMA</td>
<td>Noncompetitive; capture and detection monoclonal antibodies</td>
<td>hours</td>
<td>FS 0.05 – 1.0 μg/L, TgAb interference yes, HAMA interference yes Monoclonal Ab → limited Tg epitope specificity for abnormal tumor Tg isoform</td>
</tr>
<tr>
<td>Liquid chromatography tandem mass spectrometry, LC-MS/MS</td>
<td>Trypsin digestion, immunoaffinity enriched, detection by LC-MS/MS</td>
<td>1-2 days</td>
<td>FS 1.0 – 2.0 μg/L, TgAb interference possible, HAMA interference unlikely Polymorphic tumor Tg may fail to yield target peptides</td>
</tr>
</tbody>
</table>

Spencer & Fatemi. BPRCEM 2013; 27: 701
1. TgAb levels are best assessed using an immunometric assay.

2. In DTC patients, the LoQ (limit of quantification) of a given TgAb assay should be regarded as the upper normal limit for the presence of TgAbs.

3. Currently there are no methods for overcoming TgAb interference that result in sufficient accuracy and sensitivity of Tg measurement for clinical use.

4. The presence of TgAb should be assessed quantitatively with every Tg measurement.

Verburg et al. Thyroid 2013; 23: 1211
5. Considering the low prevalence of heterophilic antibodies interference, routine testing for the presence of such antibodies is not recommended.

6. Testing for the presence of heterophilic antibodies, commercially available heterophilic antibody blocking tubes can be used. Alternatives are recovery tests and/or serial serum dilutions.

7. In TgAb positive patients, follow-up should be stratified according to the trend of serum TgAb levels.

8. In TgAb positive patients, there is no indication for isolated TSH-stimulated Tg measurement.

Verburg et al. Thyroid 2013; 23: 1211
ALGORITHM FOR TREATMENT AND FOLLOW-UP IN TgAb POSITIVE DTC PATIENTS

Verburg et al. Thyroid 2013; 23: 1211
ASSAY INTERFERENCES IN THYROID FUNCTION TESTS

1. Introduction

2. TSH

3. Tg

4. FT4 and FT3
   - anti-T4 and anti-T3 antibodies
   - heterophilic antibodies
   - drugs (heparin, furosemide)
FORMATS FOR FT4/FT3 IMMUNOASSAYS

- **Two-step ("back-titration") immunoassay**
- **One-step ("analogue") immunoassay**
- **Labeled antibody immunoassay**

Faix, BPRCEM 2013; 631
FT4/FT3 ASSAYS BY EQUILIBRIUM DIALYSIS "INDIRECT" VS "DIRECT" APPROACH

Faix. BPRCEM 2013; 27: 631
T4 AND T3 AUTOANTIBODIES (AAb) INTERFERENCE IN FT4 AND FT3 ASSAYS

Depressed or increased values depend on methodology

1. **Single antibody technique**
   - AAb will result in low values because tracer (labeled T4/T3 or its analog) is bound by AAb as well as by capture antibodies:
     → high amount of tracer detected, hormone value will be **low**

2. **Double-antibody technique**
   - tracer bound by both capture antibody and AAb, but second antibody used in the separation step binds only capture antibody:
     → low amount of tracer is detected, hormone will be **high**

2. **One-step assays** - prone to interference by AAb
   **Two-step assays** - less or not affected by AAb
   - extraction of T4/T3 by antibody-coated tubes or beads followed by washing step: other serum components eliminated before tracer added

3. **Analog tracers** detect AAb more efficiently than non-analog tracers
CASE 6
F 72 yr

- painless swelling in the neck
- slowing down in performance, slight constipation
- longstanding active rheumatoid arthritis

- some puffiness of the face, grating voice
- large nontender firm diffusely enlarged thyroid gland

- TSH 145 mU/L ↑, T4 55 nmol/l ↓, FT4 11.6 pmol/L ↓
- T3 24.3 nmol/l ↑ or <0 nmol/l ↓ (two different laboratories)
- TgAb and microsomal antibodies positive

→ HYPOTHYROID HASHIMOTO GOITER
→ T3 ANTIBODIES

Treated with 150 µg L-T4 daily:
TSH and FT4 normalized, more energy, goiter regressed in size

CASE 7
F 19 yr

- recently discovered swollen thyroid gland
- tired, less appetite

- clinically euthyroid
- small diffuse goiter, normal consistency

- TSH 6.5 mU/L ↑, T4 95 nmol/l, FT4 16.7 pmol/L (normal)
- T3 9.0 nmol/l ↑ or <0 nmol/l ↓ (two different laboratories)
- TgAb and microsomal antibodies strongly positive

→ SUBCLINICAL HYPOTHYROIDISM (HASHIMOTO)
→ T3 ANTIBODIES

No treatment.
Six months later: complaints disappeared, thyroid status the same.

T3 RADIOIMMUNOASSAY IN CASE 6 - EFFECT OF CORRECTION FOR NONSPECIFIC BINDING

**Principle of RIA**
- binding of T3 to serum proteins dissolved by salicylate
- endogenous T3 and 125I-T3 compete for binding sites on rabbit T3-Ab
- separation from unbound T3 from T3 bound to T3-Ab by PEG

**Correction for nonspecific binding**

<table>
<thead>
<tr>
<th></th>
<th>METHOD 1</th>
<th>METHOD 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>125I-T3 bound in plasma</td>
<td>8394 cpm</td>
<td>8394 cpm</td>
</tr>
<tr>
<td>125I-T3 nonspecifically bound</td>
<td>720 cpm</td>
<td>8306 cpm</td>
</tr>
<tr>
<td>(assay control - method 1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(patient control - method 2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>125I-T3 specifically bound</td>
<td>7674 cpm</td>
<td>88 cpm</td>
</tr>
<tr>
<td>→ % bound in plasma</td>
<td>&gt; % B0 *</td>
<td>0.96 %</td>
</tr>
<tr>
<td>→ [T3] in plasma</td>
<td>&lt; 0 nmol/l</td>
<td>≥ 6 nmol/l</td>
</tr>
</tbody>
</table>

* B0 or initial binding = % of tracer bound to antibody in the first zero-standard point of the standard curve
SCATCHARD PLOTS OF T3 BINDING TO PLASMA SAMPLES IN 2 PATIENTS WITH T3 ANTIBODIES

PREVALENCE AND SIGNIFICANCE OF T4 AND T3 ANTIBODIES

1. Prevalence
   - dependent on detection method
   - 0 – 1.8% of population
   - 1 – 7% of patients with autoimmune thyroid disease,
     mostly with Hashimoto’s hypothyroidism
   - T3Ab more frequent than T4Ab
   - mostly occurring together with TPOAb and TgAb

2. Investigation
   - elimination of T4/T3 antibodies by polyethylene glycol
     or Protein G (binds to Fc region of all 4 IgG subclasses)

3. Biologic significance
   - almost none
   - relationship with macroangiopathy in type 1 diabetes?
T4 AND T3 ANTIBODIES IN VITILIGO

A

Specificity of THAb

% of patients

T3-Ab 14.20%
T4-Ab 10.00%
T3-Ab+T4-Ab 71.40%
none 4.20%

B

Classes of THAb

IgG-T4 31
IgM-T4 51
IgG-T3 56
IgM-T3 37
IgM+IgG-T4 57
IgM+IgG-T3 60

Colucci et al. Arch Environ Contam Toxicol 2015
# 15 SERA WITH DISCREPANT FT3 VALUES IN ELECSYS AND RIA-GNOST ASSAYS

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>Age, years</th>
<th>TSH, mIU/L</th>
<th>ftT₃, pmol/L</th>
<th>Anti-T₃ Ab, %&lt;3%</th>
<th>ftT₃, pmol/L</th>
<th>Conclusion of the interference analysis report (Roche Diagnostics)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Elecsys</td>
<td>Ria-gnost</td>
<td>Elecsys</td>
<td>Elecsys</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RI a 3.8–7.1</td>
<td>RI B 3.1–6.5</td>
<td>(HBT)</td>
<td>Lot 174865</td>
</tr>
<tr>
<td>1</td>
<td>M</td>
<td>64</td>
<td>0.77</td>
<td>9.7</td>
<td>4.9</td>
<td>9.7</td>
<td>Neg</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>22</td>
<td>1.3</td>
<td>8.3</td>
<td>5.1</td>
<td>8.1</td>
<td>Neg</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>66</td>
<td>2.3</td>
<td>9.4</td>
<td>4.1</td>
<td>20.4</td>
<td>Neg</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>69</td>
<td>1.2</td>
<td>9.4</td>
<td>3.7</td>
<td>8.9</td>
<td>Neg</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>36</td>
<td>4.7</td>
<td>7.8</td>
<td>5.2</td>
<td>7.8</td>
<td>Pos (3.4%)</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>65</td>
<td>1.8</td>
<td>7.1</td>
<td>4.8</td>
<td>ND</td>
<td>Neg</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>68</td>
<td>2.0</td>
<td>7.7</td>
<td>4.3</td>
<td>7.2</td>
<td>Neg</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>77</td>
<td>0.21</td>
<td>7.5</td>
<td>4.1</td>
<td>7.5</td>
<td>Neg</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>61</td>
<td>1.1</td>
<td>7.1</td>
<td>4.8</td>
<td>ND</td>
<td>Neg</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>70</td>
<td>1.8</td>
<td>7.1</td>
<td>3.7</td>
<td>6.9</td>
<td>Neg</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>41</td>
<td>1.3</td>
<td>8.1</td>
<td>4.8</td>
<td>ND</td>
<td>Pos (3.4%)</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>76</td>
<td>0.56</td>
<td>8.5</td>
<td>4.8</td>
<td>8.5</td>
<td>Neg</td>
</tr>
<tr>
<td>13</td>
<td>F</td>
<td>48</td>
<td>1.56</td>
<td>7.7</td>
<td>6.0</td>
<td>8.5</td>
<td>Neg</td>
</tr>
<tr>
<td>14</td>
<td>F</td>
<td>27</td>
<td>1.1</td>
<td>7.7</td>
<td>5.2</td>
<td>7.8</td>
<td>Neg</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>47</td>
<td>2.2</td>
<td>7.8</td>
<td>6.0</td>
<td>7.8</td>
<td>Neg</td>
</tr>
</tbody>
</table>

RI, reference interval. NS, not sent to the manufacturer. ND, not determined. ftT₃ results above the corresponding upper reference limit are indicated in bold. *Reference interval for subjects aged 20–60 years; †reference interval for subjects aged >60 years. *Result at the upper limit of the reference interval.
ANTI-RUTHENIUM ANTIBODY INTERFERENCE

1. Sera containing anti- ruthenium antibodies may interfere in the Elecsys FT3 assay
   - they prevent T3 binding to assay antibodies, yielding falsely high FT3 results.
   - to avoid interference from ruthenium antibodies, a new blocking protein was developed and included in the Elecsys FT3 assay, resulting frequently in normal FT3 results

2. Anti-ruthenium interference may result in falsely high results in competitive Elecsys (i.e. vitamin D and FT4) and low results in the sandwich assay (i.e PTH and TSH)

HEPARIN TREATMENT IN VIVO LEADS TO IN VITRO ARTEFACT: SPURIOUSLY HIGH FT4/FT3

Stockigt & Lim. BPRCEM 2009; 23: 753
**EFFECT OF HEPARIN, TIME AND TEMPERATURE ON IN VITRO GENERATION OF NEFA**

<table>
<thead>
<tr>
<th>Sample storage</th>
<th>Normal subjects</th>
<th>Serum NEFA mmol/l</th>
<th>Diabetic (Serum Tg &gt; 5 mmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Heparin 10 IU</td>
<td>Heparin 25,000 U/day</td>
</tr>
<tr>
<td>Thawed from −80 °C</td>
<td>0.24 ± 0.03</td>
<td>0.30 ± 0.14</td>
<td>0.77 ± 0.11</td>
</tr>
<tr>
<td>4 °C, 7 days</td>
<td>0.41 ± 0.04*</td>
<td>0.36 ± 0.15</td>
<td>2.90 ± 1.10*</td>
</tr>
<tr>
<td>22 °C, 4 h</td>
<td>0.17 ± 0.04</td>
<td>1.30 ± 0.67*</td>
<td>3.29 ± 1.33*</td>
</tr>
<tr>
<td>22 °C, 20 h</td>
<td>0.23 ± 0.05</td>
<td>2.88 ± 1.17*</td>
<td>1.03 ± 0.16*</td>
</tr>
<tr>
<td>37 °C, 4 h</td>
<td>0.19 ± 0.05</td>
<td>3.49 ± 1.18*</td>
<td></td>
</tr>
<tr>
<td>37 °C, 22 h</td>
<td>0.35 ± 0.05*</td>
<td>0.48 ± 0.21*</td>
<td></td>
</tr>
</tbody>
</table>

Serum was frozen at −80 °C after sampling; mean ± SD; n > 4; *p < 0.05.

NEFA, non-esterified fatty acids
FT4 BY ALL THREE METHODS INCREASED AFTER HEPARINIZATION FOR HEMODIALYSIS

Stockigt & Lim. BPRCEM 2009; 23: 753
EFFECT OF FUROSEMIDE ON FT4 IN THREE ASSAYS THAT VARY IN SAMPLE DILUTION

Stockigt & Lim. BPRCEM 2009; 23: 753
ASSAY INTERFERENCES - CONCLUSIONS

1. *When to suspect interference?*
   - discrepancy between clinics and lab
   - discrepancy between various thyroid function tests

2. *Causes?*
   - anti-hormone antibodies
   - heterophilic antibodies, anti-animal antibodies (HAMA)
   - some drugs

3. *Prevalence?*
   - rather rare, except interference of TgAb in Tg assays

4. *Significance?*
   - minor, except loss of Tg as tumour marker if TgAb present

5. *Future?*
   - less interference in isotope dilution LC/MS-MS methods?
## TESTS TO DETECT ASSAY INTERFERENCE

<table>
<thead>
<tr>
<th></th>
<th>Heterophilic antibodies</th>
<th>Autoimmune antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution experiment</td>
<td>Reduction in hormone concentration</td>
<td>Increase in hormone concentration</td>
</tr>
<tr>
<td>Gel filtration chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery experiment by adding exogenous hormone</td>
<td></td>
<td>Reduced recovery (vast capacity of antibody/hormone complex to bind hormone)</td>
</tr>
</tbody>
</table>
CHARACTERISTICS OF Tg ASSAYS

1. **ANALYTICAL SENSITIVITY**
   = lowest concentration that can be reliably distinguished from zero

2. **FUNCTIONAL SENSITIVITY (FS)**
   = measure of imprecision of an assay at a low analyte concentration:
   lowest concentration resulting in a coefficient of variation of 20%

3. **LIMIT OF DETECTION (LoD)**
   = the lowest analyte concentration likely to be reliably detected and
distinguished from the limit of the blank

4. **LIMIT OF QUANTIFICATION (LoQ)**
   = the lowest analyte concentration that can be reliably measured,
   with defined requirements for bias and imprecision, such as the
   total allowable error, often defined as \( \leq 30\% \)
CASE 3 – LESSON

• in case of spuriously high or low results
  • in TSH, FT4 or T3 assays:

→ CONSIDER HAMA’ s, T4-Ab, T3-Ab

→ CONSULT LABORATORY
DETECTION OF HAMA’s

• dilution of sample → parallelism with standard line?

• in case of high TSH: OUR PATIENT:
  1:5 dilution of sample with mouse serum  TSH 1.5 mU/l

• in case of low TSH:
  addition of standard TSH to sample
T4 DISPLACEMENT IN VITRO BY DRUG COMPETITORS AT RELEVANT THERAPEUTIC CONCENTRATIONS

<table>
<thead>
<tr>
<th>Medication</th>
<th>aAffinity for TBG (relative to T4 × 10³)</th>
<th>bFree Fraction percent</th>
<th>cTherapeutic Concentration umol/l</th>
<th>cT4% displacement (undiluted serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenclofenac</td>
<td>1.5</td>
<td>0.5</td>
<td>270</td>
<td>90</td>
</tr>
<tr>
<td>Aspirin</td>
<td>0.03</td>
<td>11</td>
<td>1800</td>
<td>62</td>
</tr>
<tr>
<td>Meclofenamic acid</td>
<td>8</td>
<td>0.2</td>
<td>60</td>
<td>39</td>
</tr>
<tr>
<td>Diflunisal</td>
<td>0.07</td>
<td>0.7</td>
<td>320</td>
<td>37</td>
</tr>
<tr>
<td>Mefenamic acid</td>
<td>2.7</td>
<td>0.6</td>
<td>80</td>
<td>31</td>
</tr>
<tr>
<td>Phenylbutazone</td>
<td>0.04</td>
<td>0.9</td>
<td>320</td>
<td>31</td>
</tr>
<tr>
<td>Flufenamic acid</td>
<td>0.6</td>
<td>0.2</td>
<td>70</td>
<td>10</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>3.9</td>
<td>0.5</td>
<td>10</td>
<td>7</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>–</td>
<td>–</td>
<td>40</td>
<td>44d</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>0.3</td>
<td>5.8</td>
<td>80</td>
<td>13–45d</td>
</tr>
<tr>
<td>Furosemide*</td>
<td>11</td>
<td>1.8</td>
<td>3–30</td>
<td>5–30°</td>
</tr>
<tr>
<td>Iopanoic acid†</td>
<td>0.1</td>
<td>1.2</td>
<td>200</td>
<td>8</td>
</tr>
<tr>
<td>Heparin</td>
<td>&lt;0.01</td>
<td>–</td>
<td>2</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

a Affinity for TBG in isolation relative to thyroxine at 4°C (×10³) (data from ref 24).
b Undiluted serum, spectrophotometric method, data from ref 34.
c Data from refs 22, 27, 31.
d Data from ref 27.
e Effect at high dosage >500 mg/day; effect at lower doses if renal function impaired.
f Concentrations during oral cholecystography.

Stockigt & Lim. BPRCEM 2009; 23: 753
EFFECT OF OLEIC ACID ON FT4 FRACTION WITH PROGRESSIVE DILUTION

*equilibrium dialysis*

Stockigt & Lim. BPRCEM 2009; 23: 753
FUROSEMIDE-INDUCED INCREASE IN FT4 FRACTION IS PROGRESSIVELY LOST WITH SAMPLE DILUTION by equilibrium dialysis.

Stockigt & Lim. BPRCEM 2009; 23: 753
CASE 9
F 36 yr

- Graves’ hyperthyroidism
- treatment with 30 mg methimazol and 125 μg thyroxine daily since nine months
- no complaints, feels perfectly well
- clinically euthyroid, goiter has decreased in size
• TSH 0.07 mU/l (suppressed)
  FT4 20 pmol/l (normal)
  T3 2.05 nmol/l (normal)

• three months ago the same lab results

INTERPRETATION? WHAT DO YOU DO?
too much thyroxine? too little methimazole?
TSH RECEPTOR IN PITUITARY FOLLICULO-STELLATE CELLS

TSH-R on FS cells in the pituitary

Prummel et al. JCEM 2000; 85 (Nov):4347-53
DO TSH-R ANTIBODIES INFLUENCE PITUITARY TSH SECRETION?

Does TSH also correlate with TBII values?

Prospective study in 45 consecutive patients with Graves’ hyperthyroidism, treated with “block and replacement” therapy: MMI or PTU + L-Thyroxine.

Aim: Are TSH levels lower in TBII +ve patients than in TBII -ve patients three months after reaching euthyroidism?

Euthyroidism: normal FT4 and TT3 in the absence of elevated TSH

Brokken et al. JCEM (Sept) 2003; 88:4135-8
Characteristics at 3 months euthyroidism: TBII +ve (22) versus TBII -ve (23)

Free T4 pM

FT3 Index

TBII

NS

negative positive

NS

negative positive
Characteristics at 3 months euthyroidism: TBII +ve (22) versus TBII -ve (23)

P = 0.015

Brokken et al. JCEM (Sept) 2003; 88:4135-8
CASE 9 - LESSON

- TSH may remain suppressed for a long time in Graves’ hyperthyroidism

- explanation: suppression of TSH release via binding of TBII to pituitary TSH receptors

- don’t use TSH to guide medication (unless TSH becomes elevated)
CASE 10
F 46 yr

- not well, tired, often headache
- oligo/amenorrhoea since 8 months
- physical exam unremarkable
- no galactorrhoea

WHAT WOULD YOU DO?
- **PRL**: 35 µg/l, slightly increased (N:<25 µg/l)
- **LH**: 3.0 U/l, no premature menopause
- **FSH**: 2.0 U/l
- **TSH**: 2.0 mU/l, normal (N: 0.4-4.0 mU/l)
- **cortisol**: 250 nmol/l, normal (N: 200-550 nmol/l)

- **MRI pituitary**: adenoma 1.2 x 1.4 cm
  - no pressure on optic chiasm

**WHAT NEXT?**
• α-subunits: 1.0 U/l increased (N: <0.80 U/l)

• diagnosis: clinically nonfunctioning pituitary adenoma

• proposal: transsphenoidal hypophysectomy

OKAY?
NOT OKAY – RULE OUT PREOPERATIVELY

- central adrenal insufficiency → metyrapone test
  insulin tolerance test

- central hypothyroidism → FT4 7.0 pmol/l
  (N: 10-23 pmol/l)
TSH IN CENTRAL HYPOTHYROIDISM CAN BE DECREASED, NORMAL, OR SLIGHTLY INCREASED
DISCREPANCY BETWEEN BIOACTIVITY AND IMMUNO-REACTIVITY OF TSH IN CENTRAL HYPOTHYROIDISM

* $P<0.001$ vs control groups

Persani et al., JCEM 2000
CASE 10 - LESSON

NORMAL TSH = EUTHYROIDISM

EXCEPT:

TSH CAN BE NORMAL (OR SLIGHTLY ELEVATED) IN CENTRAL HYPOTHYROIDISM