Heterophilic antibodies may be a cause of falsely low total IGF1 levels

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Abstract

Background: A low serum total IGF1 is considered as a diagnostic indicator of GH deficiency (GHD) in the presence of hypopituitarism. Introduction of IRMA and chemiluminescent immunometric assay (CLIA) IGF1 immunoassays has introduced endogenous antibodies as a new source of interference. In general, this goes unnoticed and might lead to unnecessary diagnostic and therapeutic interventions.

Case: A 56-year-old man was referred with a decline in physical performance, unexplained osteopenia, and weight loss of 3 kg over the past 8 months. Although clinical signs and symptoms were unremarkable, laboratory results pointed to secondary hypothyroidism and secondary hypogonadism. In addition, the serum total IGF1 level (CLIA; Siemens Medical Solutions Diagnostics) was in the low normal range. Two GH stimulation tests were performed, but these tests did not support the diagnosis GHD. Moreover, IGF1 bioactivity measured by the kinase receptor activation assay was normal.

Interference of heterophilic antibodies was considered. After pretreatment with specific heterophilic blocking tubes that contain blocking reagents to eliminate heterophilic antibodies, serum-free thyroxine, testosterone, and IGF1 levels turned out to be normal.

Conclusion: To the best of our knowledge, we here describe the first case in the literature of a patient with low serum total IGF1 levels due to interference from heterophilic antibodies in the used IGF1 immunoassay. When confronted with low-IGF1 levels that do not fit the clinical picture, interference of heterophilic antibodies should be considered in the differential diagnosis.

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Introduction

In 1977, the first RIA for insulin-like growth factor 1 (IGF1) was described by Furlanetto et al. (1). Since then, IGF1 immunoassays have been used to screen patients for the presence of GH deficiency (GHD) or acromegaly. However, when IGF1 immunoassays were applied to larger group of patients, many problems were encountered when these assays were used for these purposes (2). From the beginning, IGF1 immunoassays have been suffering from analytical problems including their reproducibility and accuracy for the detection of IGF1 in human serum.

Almost all IGF1 present in the circulation is bound to IGF binding proteins (IGFBPs) (3). As IGFBPs may interfere with specific antibody binding to IGF1, it is considered essential for a high-quality IGF1 immunoassay that binding protein interference is eliminated (2). Therefore, in virtually all IGF1 immunoassays, techniques such as acid–ethanol extraction or saturation with IGF2 are implemented in order to remove or to neutralize interference by these IGFBPs (4).

Similarly, since variability in IGF1 measurements can lead to erroneous conclusions, it is important that antibodies utilized in IGF1 immunoassays have both high specificity and affinity for IGF1 (5).

Measurement of circulating total IGF1 levels is considered highly sensitive and specific for the diagnosis of acromegaly (6–8). A low circulating IGF1 level in an adult with additional pituitary hormonal deficiencies is considered highly specific for GHD. However, even when the most reliable IGF1 immunoassays are used, only in 57% of severe GHD patients (defined as a peak GH of <2.5 ng/ml in insulin tolerance testing) are circulating IGF1 levels below the 95% confidence interval of the normal range in a healthy population (5).

The introduction of IRMAs and chemiluminescent immunometric assays (CLIA) IGF1 immunoassays has provided enhanced speed and sensitivity for IGF1 measurement and has made it possible to conduct convenient total IGF1 determination without binding protein interference (5). As a consequence, the clinical use of IRMAs and CLIA resulted in a reduction in the number of patients who require provocative GH testing.
However, these new assay technologies may have introduced a new clinical problem when using IGF1 for the diagnosis and evaluation of patients with GHD. We here illustrate this by a case history.

Methods
This study was performed after obtaining the patient’s written informed consent for use of his blood samples for further investigations.

Serum total IGF1 was measured by a solid-phase, enzyme-labeled CLIA Immulite 2000 IGF1 supplied by Siemens Medical Solutions Diagnostics (Los Angeles, CA, USA). In this assay, a murine anti-IGF1 coated to a solid phase (bead; capture antibody) and a polyclonal rabbit anti-IGF1 conjugated to alkaline phosphatase (detection antibody) are used. According to the manufacturer’s instruction, IGFBP interferences are circumvented by an on-board predilution and acidification step (pH < 3.1) to separate IGF1 and IGFBP-3. Once the sample is neutralized again (i.e., restoring pH to 7), IGFBP binding sites are blocked by adding an excess IGF2 in order to prevent reaggregation of IGF1 and IGFBP-3. The within-assay coefficient of variation of the Immulite 2500 IGF1 varies between 3 and 6%. The level of IGF1 was expressed as nanomoles per liter (nM) and was compared with the age-specific normative range values for IGF1. Reference values for IGF1 have been published in a previous report (9).

Immulite 2500 assays were used to measure the following hormonal parameters: free thyroxine (FT4; by a solid-phase, chemiluminescent, competitive analog immunoassay), TSH (by a solid-phase, two-site CLIA), FSH (by a solid-phase, two-site CLIA), LH (by a solid-phase, two-site CLIA), testosterone (by a solid-phase, competitive chemiluminescent enzyme immunoassay), and cortisol (by a solid-phase, two-site competitive chemiluminescent enzyme immunoassay) and compared with the respective normative range values for these hormones. All blood samples were assayed, but cortisol was remeasured after pretreatment with specific heterophilic blocking tubes (HBTs; Scantibodies Laboratory Inc., Santee, CA, USA). The HBT contains a unique blocking reagent composed of specific binders, which inactivate heterophilic antibodies, and allows for the rapid and simple elimination of false-positive heterophilic interference in plasma or serum for sandwich immunoassays. Each tube contains enough reagents to inactivate the heterophilic antibodies in 500 μl of sample. The reagent is in the form of a lyophilized pellet at the bottom of the tube. One tube was used for each sample. After pipetting each sample into the bottom of a tube and mixing the sample with the reagent, each sample was incubated for 1 h in HBT at room temperature (18–28 °C) according to the manufacturer’s instructions (www.scantibodies.com).

After pretreatment with HBT, blood samples were considered free from heterophilic antibody interference. GH was measured by the Immulite 2500 using a solid-phase, two-sites CLIA.

IGF1 kinase receptor activation assay
Circulating IGF1 bioactivity was measured using an in-house IGF1 kinase receptor activation assay (IGF1 KIRA) as was previously described (10, 11). The methodology of this latter assay is based on another principle than that of IGF1 immunoassays. Briefly, IGF1 binding to the IGF1 receptor (IGF1R) results in autophosphorylation of tyrosine residues located within the intracellular kinase domain, being the first step in the intracellular signal cascade. The IGF1 KIRA uses human embryonic renal cells stably transfected with cDNA of the human IGF1R gene (293 EBNA IGF1R) and quantifies phosphorylation of tyrosine residues of the IGF1R to assess IGF1 bioactivity. Since the IGF1 KIRA is based on a totally different method than IGF1 immunoassays, it has no susceptibility to interference by heterophilic antibodies.

Case history
A 56-year-old male patient was referred to the outpatient clinic with a medical history of decline in physical performance, forgetfulness, unexplained osteopenia, and weight loss (about 3 kg over the past 8 months). The patient had no history of previous exposure to animal proteins, but in his childhood he had lived on a farm and was exposed to diverse animal species. Although laboratory results pointed to secondary hypothyroidism and secondary hypogonadism (Table 1), these diagnoses were not clearly supported by clinical signs and symptoms. In addition, the serum total IGF1 level was within the low normal range, and serum cortisol level excluded adrenal insufficiency (Table 1). Two GH stimulation tests were performed, but their results did not support GHD (after GH-releasing hormone (GHRH) stimulation: peak serum GH: 6.4 μg/l (normal > 5.0 μg/l); after arginine plus GHRH: peak serum GH 17.3 μg/l (normal > 16.5 μg/l)). Magnetic resonance imaging of the brain demonstrated a normal pituitary gland with no abnormalities.

Interference with heterophilic antibodies was considered as an explanation for the anomalous laboratory results. After pretreatment with specific HBT, serum-FT4 and testosterone turned out to be normal (Table 1). Circulating IGF1 bioactivity measured by the IGF1 KIRA prior to HBT treatment was within the normal range. Therefore, we also considered interference of heterophilic antibodies as explanation for the low normal total IGF1 levels. After pretreatment with HBT, total IGF1 level also turned out
to be normal (Table 1). When we repeated the IGF1 KIRA measurement in HBT treated serum, there was no evidence for interference of heterophilic antibodies in the IGF1 bioassay (Table 1).

### Discussion

Since there was an obvious mismatch in our patient between clinical findings and laboratory results for FT4 and testosterone, we suspected that the values of both hormones were falsely low due to interference with heterophilic antibodies. Our suspicion was confirmed after pretreatment of the blood samples with HBT, since levels were in the normal range afterwards.

The IGF1 KIRA was not susceptible to interference by heterophilic antibodies. As IGF1 bioactivity measured by the IGF1 KIRA proved to be around the median of the normal range, interference of heterophilic antibodies was also considered as an explanation for the low normal serum total IGF1 levels as measured in the Immulite assay. Indeed, when serum of our patient was pretreated with heterophilic blocking tube (HBT), the total IGF1 level also turned out to be around the median of the age-specific normal range.

Our results demonstrate that the Immulite assays for total IGF1, FT$_4$, and testosterone are susceptible to interference by heterophilic antibodies. Although in the manufacturer’s manual of these assays, it is mentioned that this type of interference may cause anomalous results, to our knowledge this is the first report in literature that describes this phenomenon for an IGF1 immunoassay.

Interference by circulating endogenous antibodies in immunoassays is as old as the technique itself and is a potential problem for all assays employing immunometric method (12). Assays using either polyclonal or monoclonal antibodies may be affected and assays previously reported to be affected by heterophilic antibody interference include those for TSH, tri-iodothyronine, T$_4$, prostate specific antigen (PSA), testosterone, and LH (13–16). Interference from circulating antibodies is specific for an individual patient, and these proteins have the potential to interfere in an unpredictable way with some (but not necessarily all) immunoassay tests used (17). In accordance with this latter possibility, we did not find any indication for heterophilic antibody interference in the serum TSH, LH, and FSH measurements of our patient.

Most papers about heterophilic antibody interference in immunometric assays report an overestimation of the analyte leading to lower concentrations of the analyte after blocking treatments (18). In these false-positive assay outcomes, heterophilic antibodies bind both the solid-phase capture antibody as well as the labeled antibody reagent, linking them together. So it appears as if the analyte in this sample is present and causing the complex. False-negative assay results like those in our patient have also been reported in literature. Two mechanisms have been described: binding and blocking the solid-phase capture antibody or binding and blocking the labeled antibody reagent. However, it is unknown which mechanism was responsible for the false-negative results in our patient.

Circulating anti-animal antibodies can arise from iatrogenic and noniatrogenic causes (19). Administration of mouse antibodies for therapeutic and imaging purposes may lead in many patients to the formation of human anti-mouse antibodies (HAMAs) (20, 21). Such antibodies have a strong affinity for mouse IgG and are a major cause of immunoassay interference (20).

Kaplan & Levinson distinguish specific HAMAs from heterophilic antibodies. (22, 23). Heterophilic antibodies are human antibodies of low avidity that show broad activity against IgGs from several species. These antibodies can arise as a result of occupational exposure of farm workers, from activities such as keeping pets, ingesting animal antigens in food (like cow’s milk), vaccination, infection, or even blood transfusion (19, 24, 25) and may persist for several years (26). The published estimates of the presence of heterophilic antibodies in the normal population vary between 1 and 80% (13, 19).

Nonlinear sample dilution has been recognized as a method to identify samples with interference (12, 27). However, this is not universally successful. If sample dilution happens to result in an apparent elevation in the same range as the undiluted sample, interference may not be detected (28).

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**Table 1** Results of hormonal parameters comparing the original non-pretreatment assay results with the results obtained after pretreatment with heterophilic blocking tube (HBT)*.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal range</th>
<th>Primary result</th>
<th>After HBT$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free T$_4$ (pmol/l)</td>
<td>9–23</td>
<td>&lt;3.9</td>
<td>20.0</td>
</tr>
<tr>
<td>TSH (U/l)</td>
<td>0.55–3.50</td>
<td>0.50</td>
<td>22.0</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>12–33</td>
<td>&lt;0.70</td>
<td>1.3</td>
</tr>
<tr>
<td>LH (U/l)</td>
<td>0.8–7.6</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>FSH (U/l)</td>
<td>0.7–11.1</td>
<td>3.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Total IGF1 (nmol/l)</td>
<td>8–24</td>
<td>9.5</td>
<td>16.1</td>
</tr>
<tr>
<td>IGF1 KIRA (pmol/l)</td>
<td>191–566</td>
<td>374</td>
<td>372</td>
</tr>
<tr>
<td>Cortisol (nmol/l)</td>
<td>&gt;500</td>
<td>650</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not determined.

*After using heterophilic blocking tubes (HBT, Scantibodies Laboratory Inc., USA).
There are several methods to reduce the effects of heterophilic antibodies. Addition of serum of nonimmune animal sera will reduce the effect of heterophilic antibodies in many cases (13). This method works best when the species of the human anti-animal antibody is matched with nonimmune animal serum (13). Therefore, it is routine practice for assay manufacturers to incorporate the serum of nonimmune animal species in the reagents of most methods. Bovine serum has been shown to be more efficient than murine serum (29). It has been suggested that heterophilic antibodies in most patients are bovine IgGs arising from the ingestion of meat and milk (13).

Polymerized IgG, chemical aggregation, and heat aggregation are other methods that have been shown to improve the ability of nonspecific antibodies to reduce interference (13, 28).

Several blocking reagents are commercially available (18). In our study we have used HBT, which contains lyophilized specific binders to inactivate animal antibodies (19). A difference between values for the treated and untreated specimens is interpreted as evidence for heterophilic antibody interference. It is known that this method is not appropriate for every immunoassay. However, after pretreatment with HBT, serum total IGF1 concentration in our patient became like IGF1 bioactivity around the median of the normal range, suggesting that HBT is a suitable technique to eliminate heterophilic antibody interference in the Immulite 2000 IGF1 assay.

In conclusion, the Immulite total IGF1 assay method, in its present form, is susceptible to interference by heterophilic antibodies. Physicians should be aware of this phenomenon because results of circulating total IGF1 levels are often used as a basis for further diagnostic and therapeutic decisions.

Declaration of interest
There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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