An Algorithm for Acetylcholine Receptor Antibody Testing in Patients with Suspected Myasthenia Gravis

To the Editor:

Myasthenia gravis (MG)\(^1\) is a well-characterized autoimmune disease with an estimated prevalence of 1 in 5000 individuals (1). The clinical presentation varies from mild weakness of limited muscle groups (class I or ocular MG) to severe weakness of multiple muscle groups (class V or severe generalized MG). Detection of autoantibodies to the neuromuscular nicotinic acetylcholine receptor (ACHR) has proved useful in assisting in the diagnosis of MG; however, the complexity of this disease, combined with the variety of antibodies associated with MG, has led to multiple attempts to correlate disease severity with antibody detection and concentration. In general, the ACHR antibody concentration is directly proportional to disease severity, but neither the presence nor the absolute concentration of ACHR antibodies correlates with disease severity in any individual patient. ACHR antibodies are specific for MG because they are not detected in healthy individuals or in patients with other autoimmune or neuromuscular disorders; however, their absence does not rule out disease, because only about 85% of confirmed MG patients with generalized disease possess ACHR antibodies (2, 3). ACHR antibodies are less frequently detected in MG patients with mild disease or restricted muscle weakness (2, 3). MG patients without detectable ACHR antibodies often have antibodies to other neuromuscular junction proteins, such as muscle-specific kinase, which is detected in about 70% of seronegative MG patients (3).

The heterogeneous nature of the ACHR antibody response has led to the categorization of ACHR antibodies into 3 types: binding, blocking, and modulating. Assays of binding antibody, measure antibody binding to \(^{125}\text{I-\alpha-bungarotoxin}\)-labeled ACHR. Blocking antibodies interfere with receptor–ligand interaction and are measured by measuring the inhibition by patient serum of \(^{125}\text{I-\alpha-bungarotoxin}\) labeling of ACHR or by serum displacement of \(^{125}\text{I-\alpha-bungarotoxin}\) from bungarotoxin–receptor complexes. The dissociation constants reported for ACHR antibody \([K_D, \text{approximately } 2.35 \times 10^{-11} \text{mol/L}]\) and \(\alpha\)-bungarotoxin \([K_D, \text{approximately } 2.6 \times 10^{-10} \text{mol/L}]\) explain not only the ability of these antagonists to prevent the association of acetylcholine \([K_D, 6.2 \times 10^{-6} \text{mol/L}]\) with its receptor but also the ability of blocking antibodies to displace \(\alpha\)-bungarotoxin from the ACHR. Modulating antibodies accelerate the rate of ACHR internalization by cross-linking adjacent receptors and are detected by measuring the amount of internalized, processed \(^{125}\text{I-\alpha-bungarotoxin}\)-labeled ACHR released from cultured cells (2, 3). In addition to being more technically demanding owing to the requirement of viable cell culture, modulating antibody assays cannot adequately distinguish between blocking antibody–released radioactivity and modulating antibody–released radioactivity and therefore cannot truly distinguish these 2 types of ACHR antibodies.

Different ACHR antibody–testing algorithms have been proposed to elucidate the relative importance of each ACHR antibody subtype with respect to diagnosis and disease severity, but a comparison of the various studies is complicated by the use of alternative methods in different patient populations. In this study, to determine the prevalence and frequency of each type of ACHR antibody, we retrospectively evaluated the presence and concentration of ACHR binding, blocking, and modulating antibodies in 39 380 samples of patient sera submitted from throughout the US to ARUP Laboratories for the assessment of all 3 ACHR antibodies.

Most samples \((n = 34 640, 88\%)\) did not possess detectable ACHR antibodies, whereas one or more ACHR antibody types were detected in 12% \((n = 4740)\) of the clinical samples tested. The clinical status of our patient population was not available to us; however, these data suggest that physicians most often use ACHR antibody testing to rule out rather than to confirm the diagnosis of MG (Fig. 1). In agreement with previous reports \((2, 3)\), our most sensitive assay was the ACHR binding antibody assay, which was positive in 4178 (88%) of the 4740 ACHR antibody–positive serum samples. Modulating antibodies were detected in 70% \((n = 3297)\) of the samples, and blocking antibodies were least prevalent, detected in 65% \((n = 3074)\) of ACHR antibody–positive sera (Fig. 1). Combining binding and blocking ACHR antibody testing identified 97% of the patient population with detectable ACHR antibodies, whereas testing for binding and modulating ACHR antibodies identified only 93% of the ACHR antibody–possessing population. Of the 39 380 samples submitted, only 0.4% \((n = 160)\) tested positive for modulating antibodies in the absence of binding and blocking antibodies.

Approximately 15% of MG patients fail to demonstrate any ACHR antibodies; consequently, treatment does not change according to the type of ACHR autoanti-

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1 Nonstandard abbreviations: MG, myasthenia gravis; ACHR, acetylcholine receptor.
body present. These data suggest that the most cost-effective algorithm in the diagnosis of MG is testing for ACHR binding and blocking antibodies with reflex testing for modulating antibodies only in the presence of one or both of these other ACHR antibodies.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest: Employment or Leadership: None declared. Consultant or Advisory Role: None declared. Stock Ownership: None declared. Honoraria: None declared. Research Funding: All financial support was provided by the ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories (an enterprise of the University of Utah) and its Department of Pathology, or the University of Utah Department of Pathology. Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

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Previously published online at DOI: 10.1373/clinchem.2009.140392

Identification of the Hormone Kisspeptin in Amniotic Fluid

To the Editor:

Kisspeptin is the product of the KISS11 (KISS-1 metastasis-suppressor) gene and is the ligand for the G-protein–coupled receptor, now known as the KISS1 receptor (KISS1R)2 (1). Both kisspeptin and KISS1R play a crucial role in the regulation of reproduction and puberty (1). The KISS1 gene encodes a precursor peptide of 143 amino acid residues, which undergoes proteolytic processing to generate kisspetins 10, 13, 14, and 54 (1). These peptides all share the common C-terminal decapeptide necessary for receptor activation (1). Inactivating mutations in the human KISS1 receptor (KISS1R) gene cause hypogonadotropic hypogonadism (1). During pregnancy, circulating plasma kisspeptin concentrations rise by

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1 Human genes: KISS1, KiSS-1 metastasis-suppressor; KISS1R, KISS1 receptor.
2 Nonstandard abbreviations: KISS1R, KISS1 receptor (previous symbol, GPR54); IR, immunoreactivity.
7000-fold in the third trimester, compared with the concentrations in nonpregnant women (2). Plasma markers that are altered during pregnancy, such as β human chorionic gonadotropin, have been used as markers in amniotic fluid and in certain cases may be used as markers to predict fetal outcome. Kisspeptin, however, has not previously been identified in amniotic fluid. The aim of this study was to determine whether kisspeptin is present in amniotic fluid.

After obtaining ethics approval (Hammersmith and Queen Charlotte’s and Chelsea Hospitals Research Ethics Committee no. 06/Q0406/12), we recruited 32 volunteers scheduled to undergo diagnostic amniocentesis at Queen Charlotte’s and Chelsea Hospital, London, UK. Indications for amniocentesis were screening for chromosomal abnormalities or therapeutic amniocentesis for polyhydramnios. All volunteers were in their second or third trimester [mean (SD) gestational age, 17.9 (0.9) weeks] and the mean gestational age at partum was 39.2 (0.28) weeks. Exclusion criteria were marked comorbidity and an age <18 years or >45 years. Medical records were reviewed, and amniotic fluid analysis results, pregnancy complications, and pregnancy and birth outcomes were recorded. In all cases, the amniocentesis was uncomplicated, and the outcome of the pregnancy was a healthy baby with a typical karyotype.

We collected 2 mL of amniotic fluid into sterile containers containing 5000 kallikrein inhibitor units of aprotinin (0.2 mL Trasylol; Bayer). Samples were stored at −20 °C until measurement of kisspeptin immunoreactivity (IR) as previously described (3). To reduce preanalytical factors shown to influence RIA measurement of kisspeptin (4), we collected and stored all samples in an identical fashion.

The peptide was extracted from amniotic fluid with Sep-Pak C18 cartridges (Waters) according to the manufacturer’s instructions, and kisspeptin IR was characterized in amniotic fluid by fast protein liquid chromatography, as previously described (3).

The mean (SE) kisspeptin IR in amniotic fluid was 95.9 (14) pmol/L. There was no correlation between gestational age and kisspeptin concentration in amniotic fluid (P = 0.56; Fig. 1). Amniotic kisspeptin concentrations for the 2 fetus sexes were similar [mean kisspeptin IR, 95.2 (27.8) pmol/L and 105.9 (23.2) pmol/L for male and female fetuses, respectively; P = 0.77]. There was no correlation between kisspeptin concentration in the amniotic fluid and either birth weight (P = 0.67) or gestational age at partum (P = 0.58). Kisspeptin IR eluted as a single peak at a position consistent with the elution profile of kisspeptin 54. The calculated mean chromatographic recovery was 46% (7%) (n = 3).

This report is the first to identify kisspeptin in amniotic fluid. Kisspeptin 10 has been shown to inhibit migration and invasion of trophoblast cells in placentation (2); thus, the concentration of kisspeptin in amniotic fluid may be associated with pregnancy outcomes. The concentrations of amniotic fluid kisspeptin (identified as IR in this study) were much lower than those reported for circulating maternal plasma (2). In contrast to maternal plasma concentrations, kisspeptin in amniotic fluid was not observed to increase with gestational age. These findings are perhaps unsurprising, given that a number of amniotic fluid biomarkers display different concentrations during gestation, compared with the concentrations in maternal serum (5). It is possible that the lack of an observed correlation between the kisspeptin concentration in amniotic fluid and gestational age reflects the small number of participants in this study. Amniocentesis is an invasive procedure with an associated risk of miscarriage and is most commonly performed during the second trimester; therefore, the majority of our samples were from women in the second trimester [mean gestational age, 17.9 (0.9) weeks]. Because of this risk, we performed no serial sampling of...
amniotic fluid. The study therefore had a limited scope to investigate correlations between the concentration of kisspeptin in amniotic fluid, the plasma kisspeptin concentration, and gestational age.

In this study, the pregnancy outcome in all cases was a healthy baby with no chromosomal abnormalities identified. It would be interesting to investigate kisspeptin concentrations in amniotic fluid samples from a wider cohort to investigate their utility in predicting pregnancy outcome. Given the high placental expression of KISS1, it is likely that the kisspeptin IR detected in amniotic fluid was derived from the placenta (2). Thus, it would be interesting to examine any correlation between the kisspeptin concentration in amniotic fluid and placental weight.

**Role of Sponsor:** The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

**References**


**Dexamethasone-Suppressed Corticotropin-Releasing Hormone–Stimulation Test Does Not Reliably Diagnose or Predict Recurrence of Cushing Disease**

To the Editor:

First-line treatment for Cushing disease is surgical removal of the adrenocorticotrophin-secreting pituitary tumor. Because the high risk of relapse, it is essential that patients receive long-term postoperative follow-up for disease recurrence through expert clinical evaluation and biochemical assessment of hypercortisolism, including the use of dexamethasone suppression (1). However, no gold-standard test has been shown to accurately predict recurrence (1). The dexamethasone-suppressed corticotropin-releasing hormone–stimulation (LDDST-CRH) test was initially proposed to be more accurate in confirming hypercortisolism than the standard low-dose dexamethasone-suppression test.

**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors’ Disclosures of Potential Conflicts of Interest:** Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

**Employment or Leadership:** None declared.

**Consultant or Advisory Role:** None declared.

**Research Funding:** G.M.K. Nijher, The Wellcome Trust; K.G. Murphy, Biotechnology and Biological Sciences Research Council New Investigator Award; R. Ramachandran, National Institute for Health Research (NIHR); C.N. Jayasena, NIHR; W.S. Dhillo, Higher Education Funding Council for England Clinical Senior Lecturer Award. The Department of Investigative Medicine, Imperial College London is funded by an Integrative Mammalian Biology Capacity Building Award and the NIHR Biomedical Research Centre Funding Scheme.

**Expert Testimony:** None declared.
(LDDST) for the diagnosis of Cushing syndrome (2). The underlying principle of this test is that patients with true hypercortisolism demonstrate suboptimal cortisol suppression by dexamethasone, yet remain responsive to exogenous CRH. More recent studies have challenged the diagnostic accuracy of this test in the diagnosis of Cushing syndrome by demonstrating suboptimal specificity (3–5). The utility of the LDDST-CRH test for postoperative surveillance in patients with previously diagnosed Cushing disease who have been treated with transphenoidal hypophysectomy has not been assessed. Therefore, we investigated the performance of the LDDST-CRH test in this setting.

We identified a subset of 21 patients who had undergone pituitary surgery for Cushing disease and had remained at our center for postoperative surveillance. Each patient had undergone at least 1 postoperative LDDST and LDDST-CRH test as previously described (3). Tests were performed at different time intervals after surgery. Any patients receiving hydrocortisone replacement at the time of this investigation had a 16-h interval between the last dose of hydrocortisone and the first dexamethasone dose. Until March 2006, cortisol was measured by using the Nichols Advantage 1-site chemiluminescence cortisol assay (Nichols Institute Diagnostics). In our laboratory the functional sensitivity estimated from an imprecision profile performed with Nichols reagents was 15 nmol/L (3). Because of the withdrawal of the Nichols Advantage cortisol assay, from March 2006 onward cortisol was measured by using the Immulite® 2000 cortisol assay (Siemens Medical Solutions Diagnostics). The functional

| Group 1: LDDST-CRH test failed to predict subsequent recurrence of Cushing disease |
|-----------------|--------|--------|
| Patient | Time after surgery, months | Serum cortisol, nmol/L |
| 1 | 1 | <30 | 68 |
| 5 | 35 | 178 |
| 11 | 95 | 213 |
| 16 | <30 | 69 |
| 19 | 89 | 213 |
| 2 | 3 | 5 | 3 7 0 |
| 3 | 107 | 268 |
| 4 | 46 | 178 |
| 12 | 34 | 89 |
| 18 | 100 | NA |

| Group 2: Recurrence of Cushing disease confirmed by LDDST and LDDST-CRH at first assessment |
|-----------------|--------|--------|
| Patient | Time after surgery, months | Serum cortisol, nmol/L |
| 5 | 10 | 249 | 516 |
| 6 | 13 | 53 | 70 |

| Group 3: Failed LDDST-CRH test, but in disease remission when assessed clinically and by LDDST |
|-----------------|--------|--------|
| Patient | Time after surgery, months | Serum cortisol, nmol/L |
| 7 | 15 | 43 | 34 |
| 28 | 50 | 52 |
| 41 | 36 | 40 |
| 53 | 32 | 30 |
| 83 | <30 | 46 |
| 106 | <30 | <30 |
| 9 | 1 | 32 | 39 |
| 14 | <30 | <30 |
| 34 | <30 | <30 |
| 10 | 99 | <30 | 165 |
| 110 | <30 | <30 |
| 11 | 66 | 32 | 94 |
| 84 | <30 | 187 |
| 98 | <30 | <30 |
| 12 | 48 | 31 | 33 |
| 66 | <30 | 87 |
| 72 | <30 | 201 |
| 78 | <30 | 140 |

Continued on page 1033
sensitivity of the cortisol assay in our laboratory with the use of Siemens reagents was 27 nmol/L. Disease recurrence was confirmed by the development over time of clinical features that aroused suspicion of hypercortisolism, in conjunction with a failed LDDST, as defined by a serum cortisol \(>50 \text{ nmol/L} \) at time \(=48 \text{ h} \). The absence of clinical features of hypercortisolism and suppression of serum cortisol to \(\leq50 \text{ nmol/L} \) after LDDST confirmed disease remission. At the completion of the LDDST-CRH test, a serum cortisol result of \(<38 \text{ nmol/L} \) was considered to exclude disease recurrence (3). For those patients who passed the LDDST (cortisol \(\leq50 \text{ nmol/L} \) but failed the LDDST-CRH test (cortisol \(>38 \text{ nmol/L} \)), adequate suppression of serum cortisol on completion of at least 1 subsequent LDDST was required to confirm remission, along with documented lack of evolving clinical features of Cushing disease.

No patients suffered relapse in the first 6 months postoperatively, and the mean (SEM) duration of follow-up was 6.9 (1.3) years. Data for the patient cohort are shown in Table 1. During long-term follow-up, 6 patients (28%) developed recurrent disease. Among these patients, 4 demonstrated failure to suppress serum cortisol adequately to \(<38 \text{ nmol/L} \) after an LDDST-CRH test, despite results showing suppression to \(\leq50 \text{ nmol/L} \) during an LDDST (group 1). In these 4 patients, recurrence of Cushing disease was detected at a mean time of 10.8 (3.7) months earlier by the LDDST-CRH test than the LDDST test.

There were 15 remaining patients who did not develop clinically observed recurrent hypercortisolism, and the diagnosis of remission was supported by suppression of serum cortisol to \(\leq50 \text{ nmol/L} \) after an LDDST. Eight of these patients showed failure to suppress serum cortisol adequately to \(<38 \text{ nmol/L} \) after an LDDST-CRH test (group 3). All 8 patients were still in clinical remission at the time of this report, and furthermore, these patients underwent a subsequent LDDST that confirmed adequate cortisol suppression to \(\leq50 \text{ nmol/L} \). Therefore, the LDDST-CRH test had a diagnostic specificity of 47% for excluding recurrent Cushing disease. Interestingly, of these 8 patients with a false-positive LDDST-CRH test result, 5 passed a subsequent LDDST-CRH test without any clinical intervention. These patients have remained in remission for a mean period of 107 (24.3) months. This disease-free period is comparable to that seen in the patients in group 4, who have displayed adequate cortisol suppression detected by use of both tests and have remained in clinical and biochemical remission for a mean period of 102 (29.8) months.

This study is the first to assess the use of the LDDST-CRH test as a predictor of recurrence of Cushing disease in patients previously treated by pituitary surgery. The performance of this test is consistent with preoperative studies demonstrating suboptimal specificity for the diagnosis of Cushing syndrome (3–5). Our results suggest that this test is not an accurate predictor of recurrence and in fact may promote unnecessary, more intense surveillance. Therefore, we

<table>
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<th>Table 1. Summary of LDDST-CRH and LDDST results and time spent in remission for 21 patients who underwent follow-up after pituitary surgery for Cushing disease. (Continued from page 1032)</th>
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<td><strong>Patient</strong></td>
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*NA, test result not available.*

For an LDDST, pass is taken as suppression of serum cortisol to \(\leq50 \text{ nmol/L} \) and for a LDDST-CRH test, suppression to \(<38 \text{ nmol/L} \).

Letters to the Editor

Clinical Chemistry 56:6 (2010) 1033
do not advocate the routine use of the LDDST-CRH test for the detection of recurrent hypercortisolism in patients who have undergone pituitary surgery for Cushing disease.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honouraria: None declared.
Research Funding: V. Salem, Medical Research Council Clinical Research Training Fellowship; W. Dhillo and N. Martin, Higher Education Funding Council for England Clinical Senior Lecturer Awards.
Expert Testimony: None declared.
Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Previously published online at DOI: 10.1373/clinchem.2010.143263

Hemolysis Interferes with the Detection of Anti–Tissue Transglutaminase Antibodies in Celiac Disease

To the Editor:

Detection of anti–tissue transglutaminase (tTG) antibodies by the use of human recombinant or erythrocyte tTG-IgA–based ELISA assays is 1 of the preferred tests for diagnosing celiac disease (CD) (1). However, studies comparing different tTG kits have revealed variable sensitivities, raising concern in clinical practice (2). Erythrocytes (RBC) contain tTG (3). In patients, upon hemolysis, the endogenous RBC-tTG released may immunoprecipitate with anti-tTG antibodies and interfere with their detection. Newer anti–deamidated gliadin peptide (DGP)-IgA assays, however, should not be affected (4). To assess the effect of hemolysis on tTG-IgA titers, we used stored sera from 9 patients with biopsy-confirmed, active CD who gave informed consent for study participation. Samples were divided into 3 groups (n = 3, in each) according to the tTG-IgA concentrations after thawing [high titer (>185 U), intermediate titer (100–140 U), and borderline titer (20–50 U)]. A whole-blood sample from 1 tTG/DGP-seronegative patient, which contained 149 g/L of hemoglobin (HGB), was hemolyzed by freezing and thawing until >90% of cells were lysed, then serially diluted (1:2, 1:5, 1:10, 1:50, 1:100, 1:500) in PBS to obtain HGB concentrations of 67.1, 26.8, 13.4, 2.7, 1.3, and 0.27 g/L, respectively, and finally, added to each sample at a 1:1 ratio. For the tTG sequestration experiment, human recombinant tTG from Diarect AG was added for a final concentration of 0.04, 0.02, 0.01, and 0.002 g/L. Undiluted serum was used as the initial titer reference, and serum diluted 1:2 in PBS as the control. Antibody titers were quantified by using 2 ELISA kits: QUANTA Lite h-tTG IgA (human erythrocyte tTG-IgA based) and Gliadin II (DGP-IgA based) from INOVA Diagnostics, Inc. Assays were performed blinded following manufacturer’s instructions. Differences between groups were evaluated by using the Mann–Whitney U-test, with P values <0.05 considered significant.

We found that addition of hemolyzed blood (HB) to sera of
patients with active CD led to reduction of anti-tTG, but not anti-DGP antibodies in all groups, with significant reduction in the intermediate- and borderline-titer groups. Overall mean titer losses of anti-tTG vs anti-DGP antibodies in the high-titer, intermediate-titer, and borderline-titer groups were 36% vs 13%, 45% vs 3% ($P = 0.026$), and 51% vs 2% ($P = 0.0022$), respectively. We also found that addition of increasing concentrations of HGB reduced the titers of anti-tTG, but not of anti-DGP, in a concentration-dependent manner and induced negative anti-tTG results in samples with low tTG antibody concentrations. The anti-tTG titer decreased 2%–65%, 1%–81%, and 16%–74% (for HGB concentrations of 0.3–67.1 g/L) in the high-, intermediate-, and borderline-titer groups, respectively, compared with a decrease of 10%–16%, 4%–8%, and 7%–3% in the anti-DGP titer. In all groups, tTG titer reduction was greater with higher concentrations of HB/HGB and gradually recovered when the red tinge started to disappear at approximately 13 g/L of HGB, until complete visual disappearance (approximately 0.3 g/L HGB). In the intermediate- and borderline-titer groups, titer reduction induced false-negative results (<20 U) with the anti-tTG but not anti-DGP assays for HGB concentrations ≥13 or ≥0.3 g/L, respectively (Fig. 1). A third finding was that addition of increasing concentrations of exogenous tTG (recombinant human tTG) to intermediate-titer sera caused a significant reduction in anti-tTG assay titers similar to that seen with HGB (range, 32%–82%; mean, 69%) compared with that of anti-DGP titers (mean, 18%; range, 1%–38%; $P = 0.0159$).

Hemolysis, visually detected by a red tinge in the serum/plasma, is commonly seen in clinical practice. Usually caused by improper transportation or venipuncture and occasionally by medical conditions, hemolysis is one of the most common causes of specimen rejection by laboratories, and requires the specimen to be redrawn (5). Visible hemolysis begins at around 0.5 g/L of HGB and is evident above 1.3 g/L (5). Our study demonstrated that hemolysis interferes with the detection of anti-tTG antibodies, leading to false-negative anti-tTG-IgA results when the sample is visibly hemolyzed. This interference is most significant when gross hemolysis is present (approximately 2.5 g/L). The anti-DGP-IgA assay, however, was unaffected by hemolysis. This phenomenon was not limited to the INOVA human–RBC–based tTG assay but was also seen with recombinant tTG–based assays (The Binding Site; Eu–tTG IgA Umana, Eurospital; and EliA Celikey, Phadia) (data not shown). Similar concentration-dependent titer reduction by addition of exogenous recombinant human tTG suggests that sequestration of anti-tTG antibodies by endogenous RBC-tTG is the mechanism responsible. These findings could explain some false-negative results seen in CD diagnosis when tTG-IgA assays are used. Clinicians and laboratories should strive to avoid hemolysis. If it is detected, however, physicians should be notified and blood samples redrawn. If these actions are not possible, samples should be quantified for anti-DGP antibodies.

In conclusion, hemolysis can interfere with diagnosis of CD by sequestration of anti-tTG antibodies, especially in patients with low titers of tTG-IgA. Consequently, anti-tTG should not be determined in samples with visible hemolysis. Anti-DGP serological tests are...
not affected by hemolysis and are suggested when hemolysis is suspected.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: G. L. Norman, INOVA Diagnostics.
Consultant or Advisory Role: P. Green, Alvine Pharmaceuticals and Alba Therapeutics.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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Previously published online at DOI: 10.1373/clinchem.2009.141242

High Glucose Upregulates C-Reactive Protein Synthesis in Macrophages

To the Editor:

C-reactive protein (CRP)1 released from hepatocytes during the acute-phase response is a diagnostically sensitive systemic marker for inflammation; CRP also demonstrates substantial proinflammatory effects (1). Although CRP might have an important role in the pathogenesis and prediction of coronary heart diseases (2), the factors influencing its concentration are not yet well understood. Recent histologic investigations have demonstrated that CRP is present in the human arterial intima of atherosclerotic lesions (3) and is located in macrophages of the arterial plaque. In addition, macrophages have been shown to produce CRP mRNA (4).

Diabetes mellitus is associated with premature and accelerated atherosclerosis (5), and hyperglycemia accelerates atherosclerosis by inducing vascular dysfunction and an increased inflammatory burden. Our goal was to analyze the possible modulation of CRP production in macrophages after their exposure to high glucose concentrations.

J-774A.1 macrophage-like cells (ATCC) were plated in DMEM with 100 000 U/L penicillin, 100 mg/L streptomycin, and 2 mmol/L glutamine (P/S/G), and used within 7 days. THP-1 human monocyte cells were maintained in RPMI-1640 medium with P/S/G, and macrophage induction was achieved by incubation with phorbol myristate acetate. Cells were incubated with either 5–40 mmol/L glucose for 18 h or with 22 mmol/L mannitol (an osmotic control that did not appreciably affect CRP cellular production).

We extracted cellular RNA with MasterPure™ RNA Purification Kit (Epicentre Biotechnologies) and prepared cDNA with the Verso™ cDNA Kit (Thermo Scientific) according the manufacturers’ instructions. CRP production was measured by quantitative real-time PCR by means of Rotor-Gene 6000 (Corbett Life Science/Qiagen) amplification with ABSolute Blue QPCR ROX Mix (Thermo Scientific) and primers and probes for the CRP2 (C-reactive protein,

1 Nonstandard abbreviations: CRP, C-reactive protein; P/S/G, 100 000 U/L penicillin, 100 mg/L streptomycin, and 2 mmol/L glutamine.
2 Human genes: CRP, C-reactive protein, pentraxin-related; ACTB, actin, beta.
pentraxin-related) and ACTB (actin, beta) genes (PrimerDesign) (6).

CRP in sections on slides was stained with antihuman CRP antibody (Sigma–Aldrich) and subsequently counterstained with secondary antibody (antimouse fluorescein isothiocyanate–conjugated IgG; Sigma–Aldrich) (7). All control sections were processed without primary antibody. The slides were photographed with the aid of a fluorescence digital microscope camera [Zeiss Axioskop 2 plus microscope, with image-processing software; Image-Pro Plus 6.0 (Media Cybernetics)]. Light intensity and contrast were calibrated with an appropriate control section. Image-Pro Plus 6.0 software (Media Cybernetics) was used to quantify CRP on the slides, and CRP was measured by ELISA (Mouse CRP 96-well ELISA; Life Diagnostics) as recommended by the manufacturer. Lactate dehydrogenase was measured to assess cell viability, and incubation with 5–40 mmol/L glucose did not affect cell viability. Analysis of variance was used for statistical analyses. Results are expressed as the mean (SD).

THP-1 macrophages incubated with 30 or 40 mmol/L glucose exhibited increased production of CRP mRNA [61% and 109%, respectively; mean ratio of CRP mRNA to \( \beta \)-actin mRNA, 1.144 (0.08) and 1.487 (0.09), respectively], compared with cells incubated with only 5 mmol/L glucose [ratio of CRP mRNA to \( \beta \)-actin mRNA, 0.71 (0.03)]. J-774A.1 mouse macrophages incubated with 40 mmol/L glucose increased CRP mRNA production by 313% [ratio of CRP mRNA to \( \beta \)-actin mRNA, 1.28 (0.05)], compared with cells incubated with only 5 mmol/L glucose [ratio of CRP mRNA to \( \beta \)-actin mRNA, 0.31 (0.01)].

When macrophages were incubated with increasing glucose concentrations (5–40 mmol/L) for 18 h and analyzed by immunohistochemistry for CRP protein content, the intensity of fluorescein isothiocyanate staining of the cells was proportional to the detected CRP concentration. Cells incubated with 20, 30, or 40 mmol/L glucose exhibited increased cellular CRP concentrations (19%, 48%, and 103%, respectively), compared with control cells incubated with 5 mmol/L glucose (Fig. 1). Measured CRP concentrations in media harvested from incubated macrophages treated with 40 mmol/L glucose were significantly higher (by 2.5-fold, 8.8 \( \mu \)g/L) than the concentrations in media harvested from incubated macrophages treated with 5 mmol/L glucose (3.5 \( \mu \)g/L).

We hypothesize that CRP secreted by arterial macrophages could be up-regulated under atherogenic conditions such as diabetes and could therefore actively accelerate inflammatory processes in atherosclerotic lesions. Macrophages exposed to high glucose concentrations modulate the regulation of cellular CRP expression and CRP protein biosynthesis and secretion. Therefore, proinflammatory effects mediated by CRP in the arterial wall could be triggered by CRP secreted locally by macrophages, in addition to the effect of circulating, hepatic-derived CRP. Use of pharmacologic therapy to modulate CRP synthesis at the site of the atherosclerotic lesion possibly could reduce the inflammatory cascade markedly during atherosclerosis development and hence help prevent coronary heart disease.

**Fig. 1.** Effect of glucose on CRP protein in THP-1 macrophages, as measured by quantitative immunohistochemistry with a fluorescence digital microscope camera (n = 4).

*\( P = 0.00016, \) vs 5 mmol/L glucose; #\( P = 0.0035, \) vs 5 mmol/L glucose.

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**Author Contributions:** All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

**Authors’ Disclosures of Potential Conflicts of Interest:** No authors declared any potential conflicts of interest.
Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

References

Interference in a Glucose Dehydrogenase–Based Glucose Meter

To the Editor:

The US Food and Drug Administration (FDA) has issued a second alert to warn of serious errors with certain blood glucose–monitoring strips that use glucose dehydrogenase pyrroloquinoline quinine (GDH-PQQ) methods. Various nonglucose sugars, including maltose, galactose, and xylose, increase the glucose concentrations reported by these methods. Nonglucose sugars are present in a variety of pharmaceutical preparations, including Extraneal (icodextrin) peritoneal dialysis solution and various immunoglobulin preparations. At least 13 deaths associated with erroneous meter measurements of glucose in patients receiving such pharmaceutical preparations have been reported to the FDA.

A 76-year-old man at our institution had 3 blood glucose readings that unexpectedly were >22.2 mmol/L (>400 mg/dL) on a point-of-care (POC) testing meter that used a GDH-PQQ method (Roche Accu-Chek Inform). By contrast, glucose measurements made with a hexokinase method (Abbott Architect) at about the same times were in the range of 7.2–11.2 mmol/L (129–202 mg/dL), suggesting the possibility of a positive interference in the POC method. The patient’s medication list contained none of the pharmaceutical preparations listed in the FDA alert as producing interference in the POC testing method and none that were known to contain nonglucose sugars.

In a search for other sources of interfering compounds that might contribute to this patient’s high POC glucose readings, we investigated his dietary intake. The patient had been receiving Nepro® with Carb Steady® (NPS), an Abbott Nutrition product that contains, among other ingredients, maltitol and Fibersol®, a modified maltodextrin. Neither maltitol nor Fibersol was listed in the FDA alert or in the product insert as an interference in the GDH-PQQ method, but consideration of their chemical structures and the method of Fibersol manufacture led us to investigate their possible interference in the GDH-PQQ method.

We obtained a bottle of NPS solution from hospital stores, maltitol from Sigma-Aldrich, and Fibersol from Matsutani America. The latter 2 compounds were dissolved in whole blood from a healthy donor to make stock solutions of 5 g/L, similar to the stated concentration of Fibersol in NPS (approximately 6.25 g/L). Increasing volumes of the resulting maltitol or Fibersol solutions, or of NPS, were added to separate portions of whole blood from the same donor. In all steps that involved dissolving solids or mixing liquids, samples were rocked on a Nutator mixer (BD) for 5 min to ensure homogeneity. Glucose was measured in samples of whole blood by use of the Roche meter and in the separated plasma by the hexokinase method.

Both NPS and Fibersol produced results like those seen in the patient (Fig. 1). As expected, NPS and Fibersol had little effect on the results of the hexokinase method, but each increased the glucose concentration reported by the POC method.


doi:10.1373/clinchem.2009.136838

Previously published online at
DOI: 10.1373/clinchem.2009.136838

1 Nonstandard abbreviations: FDA, US Food and Drug Administration; GDH-PQQ, glucose dehydrogenase pyrroloquinoline quinine; POC, point of care; NPS, Nepro® with Carb Steady®.
method \([P < 0.001, \text{linear model test for homogeneity of slopes}]\), performed with SAS program GLM (SAS Institute). The small increase in the hexokinase results probably reflects an effect of glucose from the corn syrup solids in NPS. Fibersol at the highest concentration tested, 830 mg/L (various molecular weights), increased the apparent glucose concentration as measured by the POC method by 3.2 mmol/L (57 mg/dL), from a baseline value of 4.5 mmol/L (82 mg/dL) to 7.7 mmol/L (139 mg/dL). The results shown are from a single experiment and are representative of 2. The POC glucose method and the hexokinase method showed significantly different interferences by NPS and Fibersol \((P < 0.001, \text{homogeneity of slopes})\).

Fig. 1. Effects of carbohydrates on glucose concentrations reported by a glucose meter for whole blood (open symbols) and by a hexokinase method for corresponding plasma (closed symbols). Separate stock solutions of Fibersol and maltitol were made in whole blood. Each had a concentration of 5 g/L. Stock solutions or NPS, which contains approximately 6.5 g/L Fibersol, were added to whole blood (from the same volunteer) at the volume ratios shown on the horizontal axis. Indicated are samples with added NPS (■, ●), added Fibersol solution (○, ●), and added maltitol solution (△, ●). The data points at the far right of the figure are for the Fibersol and maltitol stock solutions. Aspiration errors on the Architect analyzer due to the viscosity of NPS precluded analysis of the undiluted preparation. The \(r^2\) of regression lines (not displayed) for the GDH-PQQ data were 0.994 and 0.993 for the NPS and Fibersol samples, respectively. The data points at the far right of the figure are for the Fibersol and maltitol stock solutions. Aspiration errors on the Architect analyzer due to the viscosity of NPS precluded analysis of the undiluted preparation. The \(r^2\) of regression lines (not displayed) for the GDH-PQQ data were 0.994 and 0.993 for the NPS and Fibersol samples, respectively. The results shown are from a single experiment and are representative of 2. The POC glucose method and the hexokinase method showed significantly different interferences by NPS and Fibersol \((P < 0.001, \text{homogeneity of slopes})\).

We suggest that modified maltodextrins such as Fibersol be added to the list of potential interferences in GDH-PQQ methods for measuring glucose and that a role of modified maltodextrins be considered in cases of unexpectedly high results from POC glucose meters that use GDH-PQQ methods.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: D.E. Bruns, Abbott Diagnostics and Siemens.
Expert Testimony: None declared.
Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

Acknowledgments: We thank Dr. James C. Boyd for assistance with statistical analysis.

References


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Previously published online at DOI: 10.1373/clinchem.2010.143453