New ELISA for Detecting Primary Biliary Cirrhosis–Specific Antimitochondrial Antibodies

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BACKGROUND: Antimitochondrial antibodies specific for primary biliary cirrhosis (PBC) target the E2 subunits of 2-oxoacid dehydrogenase complexes, in particular the pyruvate dehydrogenase complex (PDC)-E2. Their antigen-specific detection relies on conventional ELISA using purified PDC. More recent assays have employed a hybrid containing the 3 E2-subunits (MIT3). Some PBC sera react with one or the other preparation, suggesting the presence of nonoverlapping epitopes.

METHODS: We have developed an ELISA (anti-M2-3E) using a mixture of purified PDC and MIT3 as antigenic targets. We compared this assay to anti-MIT3 alone, conventional anti-PDC, and indirect immunofluorescence using 173 PBC and 247 disease controls.

RESULTS: The anti-M2-3E ELISA showed a 93.6% diagnostic sensitivity compared with 91.3%, 83.8%, and 87.3% for MIT3, purified PDC, or indirect immunofluorescence, respectively, when all specificities are set to 98.8%. By immunoblotting, anti-M2-3E–positive sera unreactive to purified PDC recognized recombinant E2-subunits of the other 2 complexes, whereas those with no reactivity to MIT3 immunofixed PDC subunits E1α or E1β.

CONCLUSIONS: The diagnostic accuracy of the anti-M2-3E ELISA for detection of antibodies to 2-oxoacid dehydrogenase complexes exceeds that of conventional ELISA and IFL; its novelty derives from the combination of the MIT3 hybrid and purified PDC.

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Received September 26, 2008; accepted January 22, 2009.

Previously published online at DOI: 10.1373/clinchem.2008.118299

Nonstandard abbreviations: PBC, primary biliary cirrhosis; AMA, antimitochondrial antibody; OADC, 2-oxo-acid dehydrogenase complex; M2, mitochondrial 2; PDC, pyruvate dehydrogenase complex; BCOADC, branched-chain oxo-acid dehydrogenase complex (OGDC); and to a lesser extent the E1 and E3 subunits of OADC (1, 4–7). The serological marker of primary biliary cirrhosis (PBC),5 an immune-mediated chronic inflammatory cholestatic liver disease of unknown etiology, is the presence of high-titer antimitochondrial antibodies (AMAs) directed against members of the 2-oxo acid dehydrogenase complex (OADC), also known as mitochondrial 2 (M2) antigen (1–3). Anti-M2 AMAs recognize mainly the E2 subunits of the pyruvate dehydrogenase complex (PDC), branched-chain oxo-acid dehydrogenase complex (BCOADC), and the oxoglutarate dehydrogenase complex (OGDC), and to a lesser extent the E1 and E3 subunits of OADC (1, 4–7). The pathogenic role and the mechanisms responsible for the development of anti-M2 AMA are not clear (8–12); their presence, however, is so closely linked to PBC that the disease is to be questioned in the absence of these antibodies (1, 4, 13). AMAs can also predict future development of PBC for those seropositive cases at early stages with no abnormal cholestatic liver function tests and no symptoms suggestive of cholestatic disease (14, 15).

Accurate detection of AMAs, therefore, is important for establishing diagnosis or predicting disease onset (1, 4). Indirect immunofluorescence (IFL) using rodent kidney/stomach/liver tissue sections or HEP-2 cells as substrates is the gold standard for the detection of liver-related autoantibodies (16), but is time consuming, laborious, and observer dependent and cannot be fully automated (5). IFL patterns are also difficult to interpret in the presence of concurrent antibody reactivity and are unable to provide information regarding the antigen specificity of the observed AMA reactivity (5, 16). To overcome these limitations, ELISAs mainly based on purified PDC from porcine or bovine heart...
mitochondria have been developed but fail to detect AMA-M2 in some 4%–13% of cases with biochemical, histological, and clinical evidence of PBC; most of these cases react with BCOADC-E2 and/or OGDC-E2 (17–19). Moreover, approximately 5% of well-documented PBC patients are negative for AMAs by IFL and do not react with any of the PDC-based preparations (18, 20). These findings have raised questions as to whether AMA-negative PBC cases represent a distinct patient population or simply have AMA titers and specificities not detectable with the conventional IFL and molecular-based assays (9, 13, 21–23). Thus, attempts have been made in the recent past to develop in-house ELISAs based on antigenic mixtures (13, 24) or a hybrid containing the immunodominant PDC-E2, BCOADC-E2, and OGDC-E2 epitopes, designated as MIT3 (because it contains the 3 major mitochondrial epitopes) (23) or BPO (from the abbreviations BCOADC, PDC, and OGDC) (25). Such ELISAs have proven useful in the early detection of AMA reactivity against the most frequently recognized mitochondrial antigens but are unable to detect antibodies against the subdominant PDC-E1α and PDC-E1β epitopes or other mitochondrial antigenic subunits (18, 23, 26–29).

To overcome this limitation, we have constructed a specially designed hybrid MIT3 clone and mixed it with native PDC antigen. We have taken advantage of this antigenic preparation to develop an ELISA simultaneously detecting antibodies to MIT3 and purified PDC. We compared the diagnostic accuracy of the newly developed anti-MIT3/PDC ELISA, designated as anti-M2-3E antibody ELISA, to that of anti-MIT3 alone, the conventional anti-PDC ELISA, and IFL in PBC patients (n = 173) and a large cohort of disease controls (n = 247).

Materials and Methods

PBC PATIENTS
We obtained serum samples from 173 consecutive PBC patients (160 female; median age 51, range 25–82; mean duration of disease 88 months, range 25–65) attending the liver outpatient clinic of Hospital Clinic, University of Barcelona, Spain, between April 2005 and July 2006. All patients met the internationally accepted histological, biochemical, and clinical criteria diagnostic or compatible with PBC (1, 2, 30). According to Ludwig’s histological classification (31), 90 patients were at stage I, 47 at stage II, 26 at stage III, and 10 were at stage IV. At the time of serum sample collection, all patients were under ursodeoxycholic acid treatment (13–15 mg/kg/day, median duration 53 months, range 1–82).

Presence of clinical signs and symptoms was assessed at presentation and monitored during follow-up visits. All other clinical, laboratory, or diagnostic information was recorded in electronic databases. Disease duration was estimated in accord with current practice as the time from the date of the earliest suspected laboratory and/or clinical evidence of cholestatic liver disease compatible with PBC to the date of the sample collection.

Other causes or features suggestive of coexistent cholestatic liver disease, including drug-induced cholestasis, alcoholic liver disease, hepatitis B and C viruses, human immunodeficiency virus, Wilson disease, hemochromatosis, $\alpha_1$-antitrypsin deficiency, and primary sclerosing cholangitis, were excluded (32, 33).

PATHOLOGICAL AND NORMAL CONTROLS
We tested 647 individuals as controls, including 247 pathological controls (87 with chronic hepatitis B (74 female, median age 45 years, range 19–76; all HBV DNA positive); 113 with chronic hepatitis C (98 female, median age 41 years, range 24–77; all HCV RNA positive), 27 with type 1 autoimmune hepatitis (AIH) (20 female, median age 35 years, range 18–82), and 20 with type 2 AIH (18 female, median age 7 years, range 1–29), all with a score of a definite diagnosis of AIH according to the revised criteria of the International Autoimmune Hepatitis Group (34)] and 400 normal controls (healthy blood donors, 149 female, median age 40 years, range 19–68).

The study was designed in December 2006 when the prototype MIT3 and MIT3/PDC ELISA plates were prepared for autoantibody testing, making this a retrospective cohort study.

In adherence to the Helsinki Principles, informed consent was obtained from all patients whose biological material was used in the present study; the local ethics committees approved the study protocols.

CONSTRUCTION OF VECTORS ENCODING FOR BCOADC-E2, PDC-E2, OGDC-E2, AND MIT3
cDNA fragments coding for lipoyl domains of BCOADC-E2 (Swiss-Prot acc. no. P11182), PDC-E2 (P10515), and OGDC-E2 (P36957) were fused, cloned into a prokaryotic expression vector, and expressed in E. coli (23). Briefly, we obtained the fragments by PCR on cDNA IMAGp998A1112130Q (acc. no. BM452811), IMAGp998E1913461Q (acc. no. BU159332), and IMAGp998P0714959Q (acc. no. CK355938) with primers synthesized by MWG Biotech (Table 1) introducing appropriate restriction sites. The PCR reactions were run in a thermal cycler DNA Engine (PTC-200; Bio-Rad Laboratories) for 30 cycles of 30-s denaturation at 94 °C, 45-s annealing at 56 °C,
60-s extension at 72 °C, and finally a 5-min extension for the last cycle. Resulting fragments were digested with the appropriate restriction enzymes (Table 1) and subsequently cloned into linearized pET24d-N that had been digested with NcoI and XhoI as described by Sitaru et al. (35).

**Table 1. Primer sequences for PCR amplification of cDNA fragments of the E2 subunits of PDC, BCOADC, and OGDC.**

<table>
<thead>
<tr>
<th>Subunit and restriction site for individual fragments</th>
<th>Restriction site for MIT3 construct</th>
<th>Primer sequence (5’–&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDC</td>
<td>Esp3I Kpn I</td>
<td>Forward: TATCGTCTCCCATGGTGATCCaaaattatacactggattcct</td>
</tr>
<tr>
<td></td>
<td>XhoI Hind III</td>
<td>Reverse: ATACTCGAGAAGCTTTAAATCTTACCTGTTGGCCT</td>
</tr>
<tr>
<td>BCOADC</td>
<td>NcoI Nco I</td>
<td>Forward: ATACCATGGaggaggtttccagttcagtaagc</td>
</tr>
<tr>
<td></td>
<td>XhoI Kpn I</td>
<td>Reverse: ATACTCGAGGTACCACGGCAGAAGAGTTGC</td>
</tr>
<tr>
<td>OGDC</td>
<td>NcoI Hind III</td>
<td>Forward: ATACCATGGGAAGCTTTAAAATGTGATTAATAGT</td>
</tr>
<tr>
<td></td>
<td>XhoI Xho I</td>
<td>Reverse: ATACTCGAGGcaAGCAGGCCGTTCCCTGAG</td>
</tr>
</tbody>
</table>

**EXPRESSION OF BCOADC-E2, PDC-E2, OGDC-E2, AND MIT3 IN BACTERIA**

Recombinant fusion protein was expressed in *E. coli* RosettaBlue (DE3)/pLacI (Novagen). For protein expression, we used a fresh bacterial colony to inoculate 10 mL LB medium supplemented with 50 mg/L kanamycin and 34 mg/L chloramphenicol. The culture was incubated at 37 °C for 12 h in a bacterial shaker at 130 rpm. We used the primary culture to inoculate 500 mL LB medium with appropriate antibiotics, and the culture was incubated as described until the bacterial suspension reached an A600 nm of 0.5–0.6. Protein expression was induced with 1 mmol/L isopropyl-D-thiogalactopyranoside for 3 h. Bacteria were harvested by centrifugation at 1800 g for 30 min, and pellets were resuspended in 10 mL of 10 mmol/L Tris-HCl, 0.3 mol/L NaCl, 1 mol/L EDTA (pH 8.0). To release the recombinant proteins, the cell suspension was sonicated 3 times for 20 s (Branson Ultraschall) on ice immediately after addition of Triton X-100 (Sigma-Aldrich Chemie) to an end concentration of 1% (wt/vol). Lysed bacteria were centrifuged again at 15000 g for 20 min to sediment insoluble proteins. Pellets were solubilized in 10 mL of 5 mmol/L Tris-HCl, 0.3 mol/L NaCl, 8 mol/L urea, 5 mmol/L imidazole (pH 8.0), and the recombinant protein was purified by immobilized metal chelate affinity chromatography using Ni-NTA Sepharose (Qiagen) with 50 mmol/L sodium acetate, 8 mol/L urea (pH 4.5) as eluent. We determined protein concentrations by measuring at 280 nm on a spectrophotometer (Eppendorf). Expression analysis was performed by SDS-PAGE and immunoblotting using a monoclonal antibody specific to hexahistidine (Merck). For quality control purposes, the preparation was analyzed by MALDI-TOF fingerprinting after SDS-PAGE and tryptic cleavage (IndyMed).

**IMMUNOASSAYS**

We determined the optimum concentrations of reagents at various steps of the immunoassay in preliminary experiments by checkerboard titration. Microtiter plates (Nunc) were coated with recombinant MIT3 (2.5 mg/L) in PBS or a mixture of recombinant MIT3 (2.5 mg/L) and PDC (1 mg/L) from bovine heart mitochondria (Arotec) in PBS overnight at 4 °C, washed with PBS and 0.05% (vol/vol) Tween-20, and blocked for 2 h with PBS and 0.1% (wt/vol) casein. After washing, sera diluted 1:200 in PBS and 0.1% (wt/vol) casein were added and allowed to react for 30 min at room temperature. Bound antibodies were detected using antihuman IgG peroxidase conjugate (Euroimmun) and stained with tetramethylbenzidine (Euroimmun). The reaction was terminated with 100 µL/well 4N H2SO4, and absorbance (A) at 450 nm was read using an automated spectrophotometer (Spectra Mini; Tecan). Conventional ELISA using PDC (anti-M2 ELISA; Euroimmun) and IFL on sections of liver, kidney, and stomach from rat (liver profile 6; Euroimmun) was performed following the manufacturer’s instructions. All tests were performed at the Institute for Experimental Immunology, Euroimmun AG, unless otherwise stated. All antibody measurements were performed under code and blinded to clinical data.
We determined reactivity to individual mitochondrial proteins by immunoblotting using purified PDC (0.25 μg/lane), mitochondrial subfraction isolated from human liver (0.1 μg/lane), and/or individual recombinant (Euroimmun) PDC-E2, BCOADC-E2, or OGDC-E2 antigens (0.1 μg/lane) as antigen source, as described (20, 36, 37). This set of experiments was carried out at the Institute of Liver Studies, King’s College London.

**Statistical analysis**

To determine cutoff values for the ELISA using MIT3, we performed ROC analyses and set values at identical specificity of 98.8% to meet the specificity of IFL testing. We calculated diagnostic accuracy (in percent) as the sum of true positives and true negatives divided by the total number of individuals (patients plus controls) tested. CIs are reported for the 95% range. For multiple linear regression analysis, readings of anti-MIT3 and anti-PDC were chosen as independent variables vs readings of anti-M2-3E as the dependent variable based on the following equation:

\[
\text{coefficient}_{\text{MIT3}} \times \text{OD}_{\text{anti-MIT3}} + \text{coefficient}_{\text{anti-PDC}} \times \text{OD}_{\text{anti-PDC}} = \text{OD}_{\text{anti-M2-3E}}
\]

We then used the coefficients to calculate virtual data for anti-M2-3E based on the data of anti-MIT3 and anti-PDC. All statistical analyses were performed using the EuroStat statistical package (Euroimmun) and MedCalc V9.6.4.0.

**Results**

**Preparation of recombinant proteins**

Recombinant DNAs coding for the E2 subunits of PDC, BCOADC, and OGDC or a fusion protein of the 3 E2 subunits were ligated with prokaryotic expression vectors and expressed in *E. coli*. The proteins, purified by metal chelate affinity chromatography, migrated according to their calculated masses (E2 subunits) at a position marginally exceeding the calculated mass (MIT3) when separated by SDS-PAGE. Identity and full length of all recombinant proteins were verified by MALDI-TOF fingerprinting. An additional minor amount of a 31-kDa protein in the MIT3 preparation was shown to contain the N-terminal BCOADC part of the hybrid protein consistent with His-Tag reactivity (Fig. 1).

**Development of ELISA using MIT3**

To determine the cutoff values of the newly developed immunoassays using MIT3 or mixture of MIT3 with native PDC (designated as M2-3E), we performed an ROC analysis of the ELISA readings with 173 sera from PBC patients and 247 sera from controls, including patients with AIH type 1 (n = 27), AIH type 2 (n = 20), and viral hepatitis (n = 200) (Fig. 2A). From the ROC curve, cutoff values were determined for all ELISAs to give a diagnostic specificity of 98.8%.

To test the contribution of both MIT3 and PDC for the ELISA using the mixture, we compared the quantitative results of all 3 ELISAs. Calculated values based on anti-MIT3 and anti-PDC (see multiple linear regression analysis in “Materials and Methods”) showed a high correlation coefficient of \( R = 0.993 \) compared with the measured values from anti-M2-3E (Fig. 2B). The next best correlation coefficient (between anti-MIT3 and anti-M2-3E) was significantly lower (\( R = 0.977, P < 0.0001 \)).

**ELISA testing**

On the basis of the cutoff defined by ROC analysis, we compared the 2 new immunoassays (anti-MIT3 or anti-M2-3E) with a commercially available anti-PDC ELISA that uses only biochemically purified PDC and with IFL on rat liver, kidney, and stomach sections.
shown in Fig. 2A, antibody reactivity to M2-3E was present in 162 of 173 (93.6%) patients with PBC, 1 of 47 (2.1%) with AIH, and 2 of 200 (1.0%) with viral hepatitis. Thus, the diagnostic sensitivity and specificity of the anti-M2-3E ELISA were 93.6% (162/173) and 98.8% (244/247), respectively. Four of 400 healthy donors (1.0%) showed low antibody reactivity to M2-3E (1.02–1.26× cutoff), including 2 positive by the anti-MIT3 ELISA. None of them (1 woman, 3 men, age 32–42 years) fulfilled the criteria for a probable or definite diagnosis of PBC or had biochemical evidence of cholestasis or chronic liver disease at the time of blood collection.

In comparison, the ELISA systems using MIT3 or biochemically purified PDC individually showed lower diagnostic sensitivities at 98.8% specificity, 91.3% (158/173) and 83.8% (145/173) (Table 2). With the identical cohort of sera, IFL showed diagnostic sensitivity and specificity of 87.3% (151/173) and 98.8% (244/247), respectively, with an overall agreement in the group of PBC patients between the anti-M2-3E ELISA and IFL of 86.7% (150/173) (Fig. 3).

PBC patients positive for anti-M2-3E did not differ significantly from those who were negative in terms of clinical or biochemical parameters such as age [59.8 (1.0) vs 61.6 (2.4) years], sex (5.7% vs 9.1% male), duration of disease [94.1 (5.1) vs 92.8 (16.5) months], Mayo risk score [4.17 (0.07) vs 4.21 (0.16)], and bilirubin concentration [0.88 (0.05) vs 0.89 (0.10) mg/dL]. No significant differences were found for anti-M2-3E readings when sera were subgrouped by histological stages 1–4 [1.72 (0.06), 1.61 (0.11), 1.60 (0.16), and 1.40 (0.41), respectively] or when anti-MIT3, anti-PDC, or AMAs by IFL were used as discriminators.

**IMMUNOBLOTTING**

Twelve anti-M2–negative/anti-MIT3–positive patients from whom an appropriate volume of serum was available were tested for reactivity to individual PDC-E2, BCOADC-E2, or OGDC-E2; 11 of them had no reactivity to PDC-E2, including 10 with significant responses to either BCOADC-E2 and/or OGDC-E2 antigens (7 to BCOADC-E2 alone, 1 to OGDC-E2 alone, and 2 to BCOADC-E2/OGDC-E2).

Two anti-MIT3–negative PBC samples with reactivity by either anti-M2-3E or conventional anti-PDC ELISA were analyzed by immunoblotting for reactivity to individual PDC subunits using purified PDC as antigen source (Fig. 4). Both sera immunofixed bands corresponding to the molecular masses of PDC-E1 and/or PDC-E1β in either purified PDC (Fig. 4A) or human liver mitochondrial subfractions (Fig. 4B).

**Discussion**

Our results demonstrate that the anti-M2-3E ELISA increases the diagnostic accuracy of AMAs compared with an anti-MIT3 ELISA tested alone, conventional anti-PDC ELISA, or IFL. The key to the increased sensitivity is the antigenic substrate, which combines a de-
signed MIT3 antigen with purified PDC. The anti-M2-E3 ELISA gives a positive reading in 93.6% PBC patients, a sensitivity considerably higher than that given by a PDC-based anti-PDC ELISA (83.8%) and the conventional IFL (87.3%) and slightly higher than that of a MIT3-based ELISA (91.3%) (Table 2). The high diagnostic sensitivity was achieved while maintaining a specificity (98.8%) comparable to that of the less sensitive systems. The few weak positives among the healthy blood donors might be considered "false" because of their sex and age distribution and because there is no clinical information known to us that would suggest diagnosis of PBC. However, two of the donors were also positive in the anti-MIT3 ELISA.

Multiple linear regression analysis showed that the performance of the anti-M2-3E ELISA can be modeled from the data of the ELISA using the individual antigens with a correlation coefficient of $R^2 = 0.993$ (Fig. 2B), indicating a simple additive effect of both antigens and, at the same time, underlining both their contributions to the ELISA using the mixture.

Compared to conventional anti-PDC ELISA, the anti-MIT3 ELISA has better diagnostic sensitivity (91.3% vs 83.8%) at the same level of specificity (98.8%). Eighteen (10%) PBC cases seropositive for AMAs by anti-MIT3 ELISA were negative by anti-PDC
ELISA (Fig. 3). The increased sensitivity was in part accounted for by the inclusion of BCOADC-E2 and OGDC-E2 in the MIT3 hybrid, because 10 of 12 cases testing negative in the conventional anti-M2 assay, but positive in the anti-MIT3 ELISA, reacted with BCOADC-E2 or OGDC-E2, antigens absent in the conventional assay. Our results are in agreement with previous reports on MIT3 or BPO hybrids showing a 9%–20% increase in the diagnostic sensitivity of AMA detection over assays using purified PDC or recombinant PDC-E2 alone and clearly indicate the need to incorporate BCOADC-E2 and OGDC-E2 into AMA detection systems (13, 23, 25, 38). Of clinical relevance is the fact that of 18 anti-MIT3–positive cases, 5 (27.8%) would be diagnosed as “AMA-negative PBC patients” since AMA, undetectable by conventional anti-PDC ELISA, was also undetectable by IFL (Fig. 3). This finding agrees with studies showing that, in histologically proven PBC, those negative for AMAs by IFL (4%–13%) can be positive for antibodies directed to BCOADC-E2 and OGDC-E2 (17–19).

Comparison of the conventional anti-PDC ELISA with the anti-MIT3 ELISA revealed that among the 145 anti-M2–positive cases, 4 (2.8%) were negative by the anti-MIT3 ELISA and IFL. Two cases immunofixed PDC-E1α and -β, which are contained within purified PDC (Fig. 4). To maximize the performance of the anti-MIT3 ELISA, we have therefore added purified PDC to MIT3 and developed the anti-M2-3E assay, which has slightly better diagnostic sensitivity (93.6% vs 91.3%) at the given diagnostic specificity of 98.8% than the anti-MIT3 ELISA (Table 2). Thus, the anti-M2-3E ELISA enabled us to detect 4 additional cases with AMA reactivity that we would have missed using an anti-MIT3 ELISA alone. All of them were also seronegative by IFL.

In conclusion, an ELISA with high diagnostic sensitivity and specificity for AMA detection is described. Its novelty derives from the combination of a designer MIT3 antigen, containing the major antigenic E2 domains of all 3 2-oxo acid dehydrogenase complexes, with PDC purified from bovine heart. The assay performs at least as well as IFL, the mainstay technique for AMA detection (5, 16). At variance with IFL, the assay is not observer dependent. In view of the fact that the anti-M2-3E ELISA is a new assay, its excellent performance, comparable to that reported by in-house hybrid-based assays (23–25, 38), needs to be externally validated (5).

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: W. Schlumberger, Director Immunochemistry Division, EUROIMMUN AG; W. Stocker, Head, EUROIMMUN AG.

Consultant or Advisory Role: None declared.

Stock Ownership: W. Schlumberger, EUROIMMUN AG; W. Stocker, EUROIMMUN AG.

Honoraria: None declared.

Research Funding: The project was supported in part by a grant from the Instituto de Salud Carlos III, Centro de Investigaciones Biomédicas en Red de Enfermedades Hepáticas y Digestivas, Spain.

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.

Acknowledgment: We thank Professor Harold Baum for helpful comments.
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