Analysis of the Physicochemical State of C-Reactive Protein in Different Preparations Including 2 Certified Reference Materials

Malgorzata Rzychon,1 Ingrid Zegers,* and Heinz Schimmel1

BACKGROUND: Standardization of clinical measurements of C-reactive protein (CRP) is based on the availability of reference materials. The serum protein reference material ERM-DA470 was used as a calibrant for various commercially available immunoassays but has now been exhausted. The recently released ERM-DA470k/IFCC was intended to fully replace ERM-DA470. However, the new material was not suited for the certification of CRP because of a bias introduced by the lyophilization process that caused loss of about 20% of CRP measurable by routine immunoassays, compared with the nonlyophilized material that was stored in a liquid frozen state.

METHODS: We investigated the physicochemical state of CRP in a set of 4 lyophilized and 2 nonlyophilized serum-based CRP-containing materials by semi-native gel electrophoresis, Western blotting, and gel filtration.

RESULTS: We detected a monomeric form of CRP (mCRP) in lyophilized materials at a concentration significantly higher than seen in the materials not subjected to lyophilization. Different reconstitution protocols led to variations of the monomeric CRP fraction found in reconstituted, previously lyophilized material.

CONCLUSIONS: Most of the 20% loss in measured CRP after lyophilization of the material can be accounted for by the dissociation of natively pentameric CRP into subunits. The observed dissociation results from lyophilization and subsequent reconstitution of the material at insufficient concentration levels of calcium ions. In the presence of various protein forms, differences in antibody specificity and reactivity between immunoassays and alterations of stoichiometry of antigen–antibody interactions can contribute to the divergence of the measured values.

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C-reactive protein (CRP) is one of the major acute-phase proteins in humans. Its concentrations in serum increase rapidly by several orders of magnitude in the very early stages of infection. Therefore, CRP is widely used as an inflammation marker. Modestly increased CRP concentrations are associated with long-term risk of cardiovascular disease (1–3).

Native CRP (nCRP) is a pentameric protein composed of 5 identical, noncovalently linked subunits of 23 kDa each. Two calcium ions are bound per subunit, and each subunit has a calcium-dependent binding site for phosphorylcholine or macromolecules containing phosphorylcholine. The degree of CRP heterogeneity in vivo remains under dispute. Until now, no structural polymorphism of CRP in humans has been confirmed (1–3).

CRP concentrations are routinely measured by nephelometric and turbidimetric immunoassays, in which quantification depends on comparison of the results with those obtained with a calibrant (4, 5).

The majority of routinely used CRP immunoassays were calibrated with the Certified Reference Material ERM-DA470 (previously distributed under the code BCR-470), released in 1993. The certified value for CRP in ERM-DA470 is traceable to the International System of Units (SI), via the 1st International Standard CRP 85/506, which was used as a calibrant for assigning values to ERM-DA470. The 1st International Standard CRP 85/506 is a residue after freeze-drying of a solution that contained CRP spiked into pooled normal human serum. ERM-DA470 is a lyophilized material produced by a complex processing procedure.
from pooled serum spiked with purified human pentameric CRP. The material was certified for 15 proteins, including CRP as measured by nephelometric, turbidimetric, and single radial immunodiffusion assays. Recently, a new batch of plasma protein reference material was produced to replace the nearly exhausted ERM-DA470 and ensure continuity of the standardization of serum protein measurements. The new material, named ERM-DA470k/IFCC, was processed by a procedure that largely followed the one applied for ERM-DA470 (7). The material was characterized for 12 proteins, but it was not suited for the certification of CRP owing to a bias introduced by the lyophilization process that resulted in a loss of about 20% of CRP measurable by routine immunodiagnosis, compared with the nonlyophilized material that was stored in a liquid frozen state (8). Therefore, another material, ERM-DA472/IFCC, was produced from the same starting material as ERM-DA470k/IFCC but stored in the liquid frozen state instead of in lyophilized form (5). ERM-DA472/IFCC was characterized for CRP (by immunonephelometry and immunoturbidimetry) using the reference material ERM-DA470 as calibrant.

The present study addresses the issue of decreased CRP concentrations measured by immunoassays in ERM-DA470k/IFCC after lyophilization of the material. We evaluate the physicochemical state of CRP in different reference materials.

Materials and Methods

CRP AND CRP-CONTAINING MATERIALS

We obtained CRP of human origin from the Centre for Amyloidosis and Acute Phase Proteins (University College London), with a concentration of 4.07 g/L in 10 mmol/L Tris, 140 mmol/L NaCl, 2 mmol/L CaCl₂, 0.1% NaN₃, pH 8.0 (liquid frozen). ERM-DA470k/IFCC (lyophilized), ERM-DA470 (lyophilized, no longer available), a lyophilized pilot batch produced during feasibility studies for ERM-DA470k/IFCC (precursor batch, called Piloc in (8)), a batch stored at 4 °C (liquid batch), and the liquid frozen material ERM-DA470k/IFCC were from the Institute for Reference Materials and Control. The 1st International Standard, 1986, human CRP (lyophilized) 85/506 (WHO) was purchased from the National Institute for Biological Standards and Control.

Each ampoule of the 1st International Standard CRP 85/506 contains 49 mg per mL. After reconstitution, the solutions were stored at 4 °C and used for up to 1 week.

GEL ELECTROPHORESIS

We performed SDS-PAGE according to the method by Laemmli (9). For native electrophoresis, SDS was omitted from all the buffers, dithiothreitol (DTT) was not added, and samples were not boiled. Semi-native gel electrophoresis was performed as described (10) with SDS concentrations reduced to 0.05 g/L (17 μmol/L, one-twentieth of normal concentrations), without a sample heating step and reducing agents.

WESTERN BLOTTING

After electrophoresis, proteins were transferred onto a polyvinylidene fluoride membrane, blocked with 50 g/L BSA in TBS-T (10 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mL/L Tween20), incubated with anti-CRP antibody produced in rabbit, the IgG fraction of this antiserum (Sigma, 1:1000 in 10 g/L BSA in TBS-T), followed by incubation with antirabbit IgG (whole molecule) peroxidase antibody produced in goat, affinity isolated, adsorbed with human IgG (Sigma, 1:20000 in 10 g/L BSA in TBS-T), and developed
with tetramethylbenzidine substrate for membranes (Sigma). We chose polyclonal antibodies as primary antibodies to ensure detection of multiple forms of CRP in native, semi-native, and denaturing conditions. We estimated the amount of CRP present in analyzed samples by comparing a given signal with a signal produced by known amounts of pure CRP preparation at several concentrations. The protocol was optimized to detect as little as 0.1 ng pure CRP.

SIZE EXCLUSION CHROMATOGRAPHY (GEL FILTRATION)
Gel filtration was performed on a Superdex 200 10/300 GL (GE Healthcare) column in 10 mmol/L HEPES, 140 mmol/L NaCl, 0.5 mmol/L CaCl2, pH 7.2, at room temperature unless stated otherwise. Before gel filtration, samples were passed through a 0.22-μm filter, 100 μL of sample were injected, and fractions of 1 mL (or 0.5 mL) were collected. After gel filtration, fractions were mixed with SDS-PAGE loading buffer containing DTT and stored at −20 °C until analyzed. To estimate the molecular mass of the protein, the column was calibrated with gel filtration standards (Sigma cat. no. MW-GF-200).

PRODUCTION OF MONOMERIC CRP
We produced monomeric CRP by urea/EDTA chelation according to a modified procedure (11): CRP was incubated in 7.2 mol/L urea and 10 mmol/L EDTA at a concentration of 0.4 g/L for 1 h at room temperature, followed by overnight dialysis against 10 mmol/L Tris-HCl, 15 mmol/L NaCl at pH 7.6.

Results
CHARACTERIZATION OF MONOMERIC CRP
We characterized monomeric CRP by gel electrophoresis under native, denaturing, and semi-native conditions. Semi-native gel electrophoresis in the presence of SDS concentrations reduced to one-twentieth of normal concentrations has been described (10) as a method suitable for distinguishing between native and monomeric CRP.

Under denaturing and reducing conditions, monomeric CRP runs at an apparent molecular mass of about 25 kDa, which corresponds to the molecular mass of the CRP monomer. No additional sample degradation was observed. In keeping with observations by Taylor et al. (10), monomeric CRP could not be detected in electrophoresis run under native conditions, where only a band of apparent molecular mass of about 150 kDa was observed that corresponded to nCRP. Using semi-native conditions, however, a band of apparent molecular mass of about 50 kDa appeared in tested monomeric CRP samples. This fast migrating band was assumed to be monomeric CRP (10). Based on results of gel electrophoresis, it was observed that using 0.05 g/L SDS did not affect the pentameric state of nCRP, as the untreated protein migrated as only 1 band under these conditions. We further combined the semi-native electrophoresis of the monomeric CRP and nCRP preparations with Western blotting and confirmed that the polyclonal antibodies used are suitable for detection of both pentameric and monomeric CRP (Fig. 1A).

Next, we subjected nCRP and monomeric CRP to gel filtration (Fig. 1B). Pentameric nCRP eluted at 13.6 mL and monomeric CRP at 16.7 mL, which corresponds to a molecular mass of 107 kDa for nCRP and 26 kDa for monomeric CRP. This observation is in agreement with theoretical values.

CHARACTERIZATION OF CRP-CONTAINING MATERIALS BY SEMI-NATIVE GEL ELECTROPHORESIS
We analyzed a set of 4 lyophilized and 2 nonlyophilized serum-based materials containing CRP. After reconsti-
tution of the lyophilized materials, the concentration of CRP in each of the materials was approximately 40 mg/L.

Semi-native electrophoresis combined with Western blotting revealed a band for the molecular mass of about 150 kDa, corresponding to nCRP, in all 6 materials analyzed (Fig. 2). A second band migrating at an apparent molecular mass of about 50 kDa was assumed to correspond to monomeric CRP. It was detected only in the electrophoretic patterns of CRP materials that were lyophilized. It was (nearly) absent in both liquid and liquid-frozen material.

The total content of CRP is roughly the same in the compared samples because of the preparation procedure. It could be concluded that a considerable fraction of CRP is present as monomeric CRP in lyophilized ERM-DA470k/IFCC and in the precursory batch of that material. A lower amount of monomeric CRP was detected in lyophilized ERM-DA470, and even less in the sample of the lyophilized 1st International Standard CRP 85/506. The monomeric CRP was almost undetectable in a sample of the liquid batch stored at 4 °C and completely absent in the liquid-frozen material ERM-DA472/IFCC.

CHARACTERIZATION OF CRP-CONTAINING MATERIALS BY GEL FILTRATION

We analyzed the same 6 serum-based materials containing CRP by gel filtration followed by analysis of the collected fractions by electrophoresis under denaturing and reducing conditions combined with Western blotting detection. Fig. 3A shows the UV absorption chromatogram, dominated by the most abundant proteins present in serum, and Fig. 3B the Western blot, which is specific for CRP only.

From Fig. 3B, it is clear that the most prominent CRP signals for the ERM-DA470k/IFCC material were found in fractions eluted at 13.5–14.5 mL (11.5–15 mL including the peak head and tail) and 16.5–17.5 mL (the peak head and tail ranging from 15.5 to 19 mL). These elution volumes correspond to molecular mass of about 100 kDa and about 23 kDa, and are the same as the elution volumes of pentameric and monomeric CRP, respectively. It was estimated that monomeric CRP accounts for approximately 10% to 20% of the total CRP. Much weaker signals were also detected in fractions eluted at the void volume of the column (7–8 mL) and at 9–9.5 mL (molecules of about 800 kDa).

Fig. 2. Comparison of 6 CRP preparations by semi-native PAGE followed by Western blotting detection. Lyophilized materials were reconstituted in water; 1 µL of each material was analyzed. The panel is representative of 2 experiments. Int. St., International Standard

Fig. 3. Gel filtration of 100 µL ERM-DA470k/IFCC reconstituted in water. (A), Gel filtration chromatogram. Fractions of 0.5 mL were collected. (B), SDS-PAGE analysis followed by Western blotting detection of gel filtration fractions; 9 µL of each fraction was analyzed. Both panels are representative of at least 5 experiments.
Together they are estimated to account for <5% of the total CRP and might represent forms of the protein that are complexed or further aggregated.

Gel filtration chromatograms of the remaining 5 materials were almost identical to the chromatogram shown in Fig. 3A. The estimation of the relative amounts of CRP in the collected fractions showed that fractions eluted at about 17 mL contain more monomeric CRP in the case of the lyophilized materials than in the case of liquid-frozen and liquid material (Table 1). The highest content of monomeric CRP was found in ERM-DA470/IFCC and in the precursory batch of that material, and amounted to approximately 10% to 20% of the total CRP. The monomeric CRP accounted for about 5% to 10% of the total CRP in ERM-DA470 and the 1st International Standard CRP 85/506. Less than 1% of total CRP was present in the monomeric form in the liquid-frozen material ERM-DA472/IFCC and the liquid batch material (Table 1).

USE OF HIGH CALCIUM CONCENTRATIONS FOR RECONSTITUTION

To investigate favorable reconstitution conditions, ERM-DA470k/IFCC was reconstituted on ice in 1 mL of 20 mmol/L HEPES, 10 mmol/L CaCl₂, pH 8.0, and analyzed by semi-native gel electrophoresis and gel filtration followed by SDS-PAGE and Western blotting of the collected fractions (Fig. 4). Reconstitution of lyophilized material in 10 mmol/L CaCl₂ led to a substantial reduction of the monomeric CRP content compared with the monomeric CRP content in the same material reconstituted in water. Based on results

### Table 1. Comparison of the content of monomeric CRP (mCRP) in reference materials obtained with and without lyophilization.

<table>
<thead>
<tr>
<th>Analytical method and form of CRP</th>
<th>ERM-DA470</th>
<th>ERM-DA470k/IFCC Precursor batch</th>
<th>1st International Standard CRP 85/506</th>
<th>ERM-DA472/IFCC Liquid batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slowly migrating (nCRP + complexes/aggregates)</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>Fast migrating (mCRP)</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Aggregates/complexes, %</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>nCRP</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>mCRP, %</td>
<td>5–10</td>
<td>10–20</td>
<td>10–20</td>
<td>1–5</td>
</tr>
</tbody>
</table>

*a+++*, Very strong band; ++++, strong band; ++, intermediate band; +, weak band; –, no band visible.
of Western blotting applied to gel filtration fractions, the content of monomeric CRP was reduced to half the original level when gel filtration was run in 0.5 mmol/L CaCl₂, and even more if 10 mmol/L CaCl₂ was used in the gel filtration buffer. When analyzed by semi-native gel electrophoresis combined with Western blotting, monomeric CRP was undetectable in a material reconstituted in high-calcium buffer (Table 2).

**Discussion**

The present study addresses the loss in CRP concentrations observed when reconstituted ERM-DA470k/IFCC is measured by routine immunoassays.

The process of lyophilization is known to generate a variety of stresses that may alter the physicochemical state of proteins (12). In the case of CRP, these alterations are likely to consist of changes, such as aggregation or monomerization, in the multimeric state of a natively pentameric protein.

We investigated the multimeric state of CRP in lyophilized serum-based materials by gel filtration and electrophoresis in semi-native conditions, both followed by Western blotting detection. We compared the results to those obtained for serum-based materials that were not lyophilized. To provide a basis for comparisons, the monomeric form of CRP was produced and its basic properties, such as electrophoretic mobility under various conditions and the elution profile in gel filtration, were characterized in the experimental setup.

It was found that in materials subjected to lyophilization, CRP existed not only in the native pentameric state but also in a monomeric form. In nonlyophilized materials, the monomeric form of CRP either could not be detected at all or was present at concentrations much lower than in lyophilized materials. Although aggregation is considered to be one of the major sources of instability for lyophilized proteins during storage (12), gel filtration analysis of lyophilized material revealed much less CRP in fractions containing molecules of high molecular mass than in the fractions containing monomeric CRP. Comparable amounts of high molecular mass forms of CRP were also found in fractions obtained after gel filtration of liquid frozen material that was not lyophilized. Therefore, it was concluded that the main factor that distinguishes the lyophilized from the nonlyophilized reference materials is the increased amount of CRP present in the dissociated, monomeric form and not the presence of aggregates.

The pentameric structure of native CRP is maintained by van der Waals forces, hydrogen bonding, and salt bridges between adjacent subunits. In addition, the preservation of the overall structural integrity of the molecule requires its interactions with calcium ions (1, 13). When calcium-binding sites are not occupied, the protomers of CRP undergo structural changes and have a looser conformation (1, 13, 14), allowing for weaker interactions between the subunits. Therefore, the different degrees of dissociation of CRP pentamers in the analyzed materials may result from differences in the concentration of calcium. Indeed, reexamination of the processing protocols of the lyophilized materials in question showed that the calcium concentration in ERM-DA470, ERM-DA470k/IFCC, and the precursor batch of the latter was 0.5 mmol/L (8), whereas the 1st International Standard for CRP was produced by spiking CRP into unprocessed human serum, so its calcium concentration is presumably close to the physiological level of 2 mmol/L. Accordingly, the amount of monomeric CRP found in the 1st International Standard for CRP was lower than in the other materials. The variations in content of monomeric CRP among reference materials containing calcium at 0.5 mmol/L may be attributed to slight differences between the applied

<table>
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<tr>
<th>Analytical method</th>
<th>Estimation of the mCRP content in reference material</th>
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<tbody>
<tr>
<td>Semi-native PAGE and Western blotting</td>
<td>++ +</td>
</tr>
<tr>
<td>Gel filtration with 0.5 mmol/L CaCl₂, SDS-PAGE, and Western blotting of gel filtration fractions, %</td>
<td>10–20</td>
</tr>
<tr>
<td>Gel filtration with 10 mmol/L CaCl₂, SDS-PAGE, and Western blotting of gel filtration fractions, %</td>
<td>10–15</td>
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* ++ +, Strong band; –, no band visible.
processing protocols that affected the degree of dissociation of nCRP.

The results show that the destabilization of CRP induced by the lyophilization process is reduced by calcium ions stabilizing CRP when present at sufficiently high concentration. This conclusion is in agreement with literature stating the importance of calcium for the protection of CRP from denaturation \( (2, 10, 11, 15) \). Although Potempa et al. \( (15) \) reported that the formation of monomeric CRP could not be readily achieved just by removal of calcium without concurrent denaturing, other researchers have observed spontaneous dissociation of CRP after prolonged storage in buffer lacking calcium or containing chelator \( (2, 13, 16) \). In the current study, no major dissociation of CRP was observed in materials with calcium concentrations reduced to 0.5 mmol/L that were not subjected to lyophilization.

Apart from lyophilization, the reconstitution step can affect the protein stability \( (12) \). The dried protein may not be able to refold to its native form during reconstitution. The materials in which a decreased signal of CRP was observed were lyophilized and reconstituted in distilled water before analysis. To test whether the reconstitution conditions might have an impact, ERM-DA470k/IFCC was reconstituted in a buffer containing 10 mmol/L CaCl\(_2\) and compared to the material reconstituted in water. The results of this experiment revealed that addition of calcium to the reconstitution buffer resulted in substantial reduction of the monomeric CRP content in the reconstituted material.

One of the possible explanations for this phenomenon is that an addition of calcium promotes the reassociation of CRP protomers that dissociated in the course of lyophilization. This seems rather unlikely, however, as CRP’s failure to renature once dissociated has been convincingly described in literature \( (17) \). Therefore the most plausible explanation is that stress caused by exposure to lyophilization under reduced concentration of calcium does not immediately lead to dissociation of CRP but only destabilizes its pentameric structure. On reconstitution, the destabilized structure either dissociates into protomers or is partially restored, providing that a stabilizing factor such as calcium ions is present in the reconstitution buffer, especially at lower temperature.

From the data for the value assignment of ERM-DA470k/IFCC and ERM-DA472/IFCC \( (5, 6) \), the effect of the lyophilization of serum containing CRP was comparable for all routine immunoassays in the studies. For these and other materials studied, the reduction in amount of CRP measured correlated with the amount of monomeric CRP detected. When approximately 20% of the CRP was monomeric, the measured CRP concentration was about 20% lower. This indicates that the decrease of measured CRP concentration is due to monomeric CRP producing a much lower response in homogeneous immunoassays compared to pentameric CRP.

When Potempa et al. \( (15) \) examined preparations of monomeric CRP for reactivity with multiple antisera raised to purified native nCRP, they concluded that changes occurring during formation of monomeric CRP led to the loss of some of the epitopes of native CRP, which critically affected the immunological reactivity of CRP as well as its physicochemical characteristics. These conclusions were later confirmed in other studies \( (11, 18, 19) \). As all the immunochemical techniques, including those used for routine analysis of CRP, depend on the specificity of the antibodies used, it is very likely that differences in recognition of various forms of CRP by antibodies will affect the results of the CRP quantification. In particular, the attenuated recognition of monomeric CRP by antibodies raised against nCRP would explain a reduced concentration of CRP measured in the reference material ERM-DA470k/IFCC, in which a fraction of CRP is dissociated.

Impaired recognition of monomeric CRP is not the only factor by which partial dissociation of CRP will affect results of its measurements. CRP in human plasma is routinely measured by light-scattering immunoassays that are based on agglutination reactions involving formation of bridges between antibodies and antigens. In these assays, the stoichiometry of the antigen–antibody reaction plays a critical role, since it is associated with the effectiveness of the observed precipitation. The cross-linked structure required to achieve aggregate formation is most efficiently produced by polyvalent antibodies and antigens with multiple antigenic determinants. In the case of CRP, the dissociation of a pentameric molecule reduces the number of antigenic determinants per antigen molecule, and thus the number of viable antibody-binding sites per molecule. Consequently, the efficiency of the formation of a cross-linking structure and the intensity of light scattering will decrease, resulting in attenuated detection of the monomeric protein, especially in assays using monoclonal antibodies.

In clinical chemistry, one important aspect of the suitability of a reference material is its similarity to native human samples. Because the multimeric form of CRP in the analyzed samples affects results of CRP quantification based on immunoassays, the question has been raised whether this concerns the reference material in question only, or if it is a general issue for CRP measurements in native patient samples as well. The body of scientific literature comprises contradictory evidence about the persistence of altered forms of CRP in vivo. Nevertheless, even if present, monomeric CRP is predominantly membrane-bound, and its detection in the circulating blood is rather implausible \( (20) \).
The use of lyophilized reference materials containing monomeric CRP (as is the case for ERM-DA470 and to a lesser extent the 1st International Standard CRP 85/506) for the calibration of immunoassays does not necessarily lead to a substantial bias in measurement results, as long as only methods specific for the pentameric form of CRP are used, and the assays claim to measure native pentameric CRP. In the case where all CRP polypeptides are claimed to be measured, the presence of monomeric CRP, as a reference material artifact, will lead to a break in traceability. For example, LC-MS–based methods are being developed for CRP (21). These methods measure all the polypeptide chains, including those in the monomeric form. The presence of monomeric CRP would lead to a discrepancy between measurement results of routine immunoassays and LC-MS–based methods. Therefore ERM-DA472/IFCC, the certified reference material for CRP, was produced as a liquid-frozen material containing only pentameric CRP.

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.
Honorary: None declared.
Research Funding: The work in this was carried out in part within the EURAMET joint research project Clinbiotrace, which has received funding from the European Community’s Seventh Framework Program, ERA-NET plus, under the JRP contract no. T2 J11.
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