The effect of sample storage on the performance and reproducibility of the galactomannan EIA test

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Abstract

Galactomannan enzyme immune assay (GM EIA) is a nonculture test for detecting invasive aspergillosis (IA) forming a key part of diagnosis and management. Recent reports have questioned the reproducibility of indices after sample storage. To investigate this, 198 serum samples (72 from cases and 126 from controls) and 61 plasma samples (24 from cases and 37 from controls), initially tested between 2010 and 2013, were retested to determine any change in index. Data were also collected on circulatory protein levels for false-positive serum samples. Serum indices significantly declined on retesting (median: initial, 0.50, retest, 0.23; \(P<0.0001\)). This was shown to be diagnosis dependent as the decline was apparent on retesting of control samples (median: initial 0.50, retest 0.12; \(P<0.0001\)), but was not evident with case samples (median: initial, 0.80, retest, 0.80; \(P=0.724\)). Plasma samples showed little change on reanalysis after long-term storage at 4°C. Retesting after freezing showed a decrease in index values for controls (median: initial 0.40, retest 0.26; \(P=0.0505\)), but no significant change in cases. Circulatory proteins showed a correlation between serum albumin concentration and difference in index value on retesting. Overall, this study suggests that a lack of reproducibility in GM EIA positivity is only significant when disease is absent. Retesting after freezing helps to differentiate false-positive GM EIA results and, with consecutive positivity, could help to improve accuracy in predicting disease status. The freezing of samples prior to testing could potentially reduce false-positivity rates and the need to retest.

Key words: galactomannan EIA, retesting, aspergillosis, IA diagnosis.

Introduction

Diagnosis and management of invasive aspergillosis (IA) present a clinical challenge in immunocompromised patients, with significant mortality that ranges from 38% to 94% [1]. Effective treatment hinges on early diagnosis [2], but the limitations of classic laboratory methods have resulted in a reliance on clinical suspicion supported by radiological evidence (including nodules, haloes, cavities, and air-crescent signs) [3]. However, these clinical manifestations are not specific to IA, can be transient, and
develop late in disease progression [4]. The most widely accepted nonculture-based mycological test for IA diagnosis is the Platelia *Aspergillus* Ag enzyme immunoassay (EIA; Bio-Rad, Hemel Hempstead, UK), which is included in the mycological criteria used to define fungal disease [5]. The EIA targets galactomannan (GM) circulating in the bloodstream, which is released from actively growing hyphae and detected using rat monoclonal antibody EB-A2. The antibody targets the β-1,3-galactofuranoside side chain of the GM molecule, although it also binds to the galactofuran moiety on other glycoproteins [6]. Performance is variable, with metaanalysis showing pooled sensitivity of 0.71 and pooled specificity of 0.89 in proven cases of IA [7]. As the performance statistics show, both false-positive and false-negative results occur, and several causes for these errors have been documented [8–10], raising concerns about the accuracy of GM testing.

Despite some reports that have confirmed good reproducibility of the EIA [11,12], recent publications [13–15] reported quite different test results. In a large prospective study, not all positive samples, particularly low positives (index: 0.5–0.7), were confirmed on retesting [11]. Johnson et al. found significant (up to 89%) decline in 183 sample index values after either short-term storage at 4°C or long-term storage at −80°C [15]. The researchers reported that the decline was associated with retesting of the initial serum and was not evident when the original ethylenediaminetetraacetic acid (EDTA)–processed supernatant was retested. Only 1.4% of samples in the study were from proven or probable IA cases, limiting the significance of the findings. The authors did not investigate the previous observation that false-positive results showed greater index decline than samples from proven or probable IA cases, although samples from the cases in the latter study were also limited (n = 12).

Here, we examine factors that affect the reproducibility of GM EIA in serum and plasma after storage and determine whether the current strategy of requesting and testing follow-up specimens provides comparable performance to repeat testing of positive samples. The aim was to introduce a simple process that can improve the clinical accuracy of GM EIA.

### Materials and methods

### Samples and study design

As part of the local routine neutropenic care pathway, hematology patients at high risk of IA are regularly tested by *Aspergillus* polymerase chain reaction (PCR) and EIA [3]. Using this strategy, EIA testing demonstrated good clinical utility, particularly when combined with PCR [16]. EDTA whole blood (4-ml vacutainer, K2 EDTA spray; Becton Dickinson, Oxford, UK) and clotted blood (6-ml vacutainer with no additive; Becton, Dickinson, Oxford, UK) are concurrently sent for fungal diagnostic investigations. Blood samples are processed on the day of receipt, with serum and plasma removed and stored at 4°C until testing, which generally occurs (70%) within 72 h of receipt. Given the twice weekly batch testing and the ability to easily attain new samples, prospective EIA-positive results were confirmed by follow-up studies of new samples. Serum samples are stored at −80°C for internal quality assurance and performance assessment purposes.

An electronic search of EIA results generated between 2010 and 2013 was performed to identify both negative (index, <0.5) and positive (index, ≥0.5) samples. Samples were selected according to original year of testing and EIA result. Available samples were stratified to give balanced numbers of each index value per year. Samples were randomly selected with regard to sample numbers per patient and to whether they were from defined cases or controls. Samples were assigned as case or control after retesting. Serum samples, originally stored at −80°C, were retested within 24 h of thawing. Positive samples were selected to represent weak positives (index, 0.5–1.0), moderate positives (index, 1.0–2.0), and strong positives (index, >2.0). To provide a standardized definition of disease, samples were classified as originating from cases (proven, histological evidence; probable, definitive radiology plus mycological data; possible, IA definitive radiology only) or controls (host factors with no clinical evidence of invasive fungal disease [IFD]) according to the revised European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) criteria [5]. As EIA is used as a disease-defining criterion, PCR was included as mycological evidence in order to support host factors and clinical evidence, with the potential to replace EIA. All patients were independently disease stratified prior to the start of the study. Plasma was initially tested by EIA as part of a previous assessment to compare performance in serum and plasma [17]. All samples were taken in 2012 and stored at 4°C until retesting in 2013. After retesting, plasma samples were frozen at −80°C for 1 week prior to thawing and again tested to investigate the effects of freeze–thaw on reproducibility.

All testing was performed over a 4-week period in July 2013. The study was performed as a retrospective, anonymous assessment of quality for the EIA, with no impact on patient management. All clinical information was gathered as part of routine care.
GM EIA

Repeat testing with Platelia Aspergillus EIA was performed according to the manufacturer’s instructions by the same operator and using a single lot number. Optical densities (OD) were read at 450/620 nm (Multiscan FC; Thermo Scientific, Basingstoke, UK). Indices were calculated by dividing the sample OD by the mean OD of two threshold controls included in the kit. All runs were required to meet the validity criteria designated by the manufacturer. Positive (index, >2.0) and negative (index, <0.4) controls were also included to confirm validity. Samples were considered positive if the sample index was ≥0.5.

Statistical analysis

In investigating the reproducibility of the GM index, agreement between initial and retest results was determined in both a qualitative manner (positive vs. negative result) and quantitative manner (comparison of median initial index vs. retest index). Data were analyzed in order to examine the effects of storage time, strength of initial index value, and sample source (cases vs. controls) on assay reproducibility.

Data were plotted using scatter diagrams, and linear regression was performed in order to determine any significant trends between parameters. In addition, Spearman correlation coefficient was calculated to determine significant correlation between indices. To test for population normality, the Kolmogorov–Smirnov, D’Agostino and Pearson, and Shapiro–Wilks tests were applied. Because both the serum and plasma populations were non-Gaussian, significance between initial and retest values was determined using the Wilcoxon match-pairs signed-rank test with a two-tailed P value of 0.05. A Kappa statistic was calculated to test inter assay qualitative agreement between all samples and for cases and controls individually. Receiver operator characteristic curve analysis was used to assess the effect of storage on overall diagnostic performance. Positive results were also analyzed according to whether or not they were part of a series of consecutive positives (≥2 positives) and linked to a diagnosis of proven or probable IFD.

Results

Sample characteristics

A total of 198 serum samples from 107 hematology patients were tested, of which 72 were obtained from 20 cases and 126 samples from 87 control patients with host factors but no evidence of fungal disease. Using the 2008 EORTC/MSG criteria, there were 2 proven, 17 probable, and 1 possible case of IA [5]. A total of 61 plasma samples from 14 hematology patients were analyzed, including 24 specimens from 4 probable cases of IA and 37 samples from 10 control patients. Fifty-nine plasma samples (22 from cases and 37 from controls) were available for retesting after being frozen at −80°C for 1 week.

Analysis of serum samples

During initial testing, 119 samples (60.1%) generated a positive (≥0.5) index and 79 (39.9%) were negative by EIA. These results pertain to 53 and 66 positive samples from cases (mean index, 1.7; 95% confidence interval [CI], 1.3–2.1) and control patients (mean index, 0.9; 95% CI, 0.7–1.2), respectively. Forty-nine of the 53 (92.4%) positive samples from cases were part of a consecutive series (≥2) of positive samples compared with 14 of 66 (21.2%) of positive samples from controls (P < 0.0001).

On retesting, the positivity rate decreased significantly to 73/198 (36.9%), and observed qualitative agreement with the initial result was 74.7% (95% CI, 66.1–81.8) and the Kappa statistic was 0.52. The observed agreement and Kappa statistic were higher for samples from actual cases than those of controls. The observed agreement and Kappa statistic for cases were 87.5% and 0.72, respectively, compared with 67.5% and 0.37 for controls (P = 0.002).

Of the 53 initially positive samples derived from cases, 44 (83.0%) were confirmed positive on retest compared with 27/66 (40.9%) for controls (P < 0.0001). A total of 48 samples were positive at initial testing and negative on repeat examination compared with only 2 samples (2.5%) that were negative on initial testing but positive on repeat testing. Of the 48 nonreproducible positive EIA results, 29 (53%) initially generated an index between 0.5 and 0.7, 10 had an index between 0.8 and 1.0, 7 an index between 1.1 and 2.0, and 2 an initial index >2.0, representing 49.2%, 50.0%, 38.9%, and 10%, respectively, of results generated at that particular index.

The median GM indexes when originally tested and retested for the overall population; cases and controls are shown in Table 1. The GM index for the overall population was significantly lower on retesting (difference in median index, 0.27; P < 0.0001). Analysis by disease status shows that although there was no significant difference on retesting samples for cases (difference, 0; P = 0.724), patients with no evidence of IFD showed a significant reduction in index values (difference, 0.38; P < 0.0001). Linear regression analysis showed the correlation between original and retest index was significantly less for controls compared with cases (Fig. 1.) The Spearman coefficient for cases was 0.86 (95% CI, 0.78–0.91) compared with 0.25 (95% CI, 0.07–0.41) for samples originating from control patients.

The overall diagnostic performance of GM EIA testing serum was improved after freezing at −80°C (Fig. 2a).
There was no correlation associated with the time stored between testing and reproducibility of the index (cases, $P = 0.77$; controls, $P = 0.14$) or between the time taken to process the initial sample and difference in indices (cases, $P = 0.944$; controls, $P = 0.814$).

### Analysis of plasma samples

Thirty samples on initial testing (49.2%) generated a positive (≥0.5) index and 31 (50.8%) samples were negative by EIA. On retesting of samples stored at 4°C, 33 (54.1%) were positive and 28 (45.9%) were negative by EIA. On retesting samples stored at −80°C, positivity was 19/59 (32.2%), a 17.0% reduction compared with the initial result, although this did not reach significance ($P = 0.0658$).

For samples stored at 4°C, observed qualitative agreement with the initial result and Kappa statistic was 88.5% (95% CI, 73.6–95.8) and 0.771, respectively, representing excellent agreement between the results and significantly greater reproducibility in plasma compared with serum ($P = 0.025$). Observed agreement and Kappa statistic was similar for cases (83.3%; 95% CI, 55.9–95.7; 0.664) and controls (91.9%; 95% CI, 72.1–98.4; 0.85; $P = 0.8490$). Thirteen samples from cases were initially positive and 11 (84.6) confirmed positive on retest compared with 17/17 (100%) for controls (difference 15.4%; $P = 0.7949$). Only two samples were positive at the initial test and negative on repeat, whereas five samples were negative when initially tested but positive on repeat.

After storage at −80°C, observed agreement and Kappa statistic were higher for cases (100%; 95% CI, 85.1–100; 1.0) than controls (75.7%; 95% CI, 59.9–86.6; 0.49; $P = 0.0199$); 100% of case samples ($n = 11$) were confirmed on retest compared with 8/17 (47.1%) for controls ($P = 0.0039$). No samples that were initially negative tested positive on repeat. The overall diagnostic performance of GM EIA testing plasma was improved after freezing at −80°C (Fig. 2b).

The median GM indexes when originally tested and after retesting are shown in Table 2. There was no significant difference in the median indices when retested samples were stored at 4°C, and linear regression analysis generated similar Spearman coefficients for all populations (Fig. 3). After freezing at −80°C, there was a trend toward a reduction in index on retesting, driven by a reduction associated with the control population. Linear regression generated a Spearman coefficient of 0.96 (95% CI, 0.90–0.98) for cases compared with 0.69 (95% CI, 0.47–0.83) for control patients (Fig. 3).

### Blood protein data

To assess the impact of common circulatory proteins on initial and retest EIA index values, data were analyzed for 65 false-positive serum samples. Forty-four had C-reactive protein (CRP) data available and 64 had albumin, total protein, and calculated globulin concentrations available within ±5 days of sample receipt. Regression analysis that compared blood levels of each constituent against both initial and retest indices and the difference between the two showed that there were no associations with CRP, total protein, or globulins. However, there was a significant correlation between serum albumin concentration and difference in initial and retest values, with a Spearman coefficient of −0.348 ($P = 0.0048$), highlighting a larger reduction in indices with increasing albumin concentration (Fig. 4). Mann–Whitney testing showed a significant difference in albumin levels between positive and negative retests ($P = 0.0005$), with retest positives having a lower mean albumin concentration (24.35 g/L; 95% CI, 21.29–27.41) compared with retest negatives (31.66 g/L; 95% CI, 29.27–34.04).

### Discussion

For a disease where diagnosis remains problematic, it is important that the most widely available laboratory test, that is, GM EIA, be accurate and reproducible. As GM EIA is

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**Table 1.** Comparison of galactomannan enzyme immune assay reproducibility when testing serum samples.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Overall (N = 198)</th>
<th>Case (N = 72)</th>
<th>Control (N = 126)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial index</td>
<td>Retest index</td>
<td>Initial index</td>
</tr>
<tr>
<td>Range</td>
<td>&lt;0.2–7.1</td>
<td>&lt;0.2–5.7</td>
<td>&lt;0.2–6.4</td>
</tr>
<tr>
<td>25th percentile</td>
<td>0.30</td>
<td>0.07</td>
<td>0.80</td>
</tr>
<tr>
<td>Median galactomannan index</td>
<td>0.50</td>
<td>0.23</td>
<td>0.80</td>
</tr>
<tr>
<td>75th percentile</td>
<td>0.80</td>
<td>0.70</td>
<td>1.40</td>
</tr>
<tr>
<td>Wilcoxon $P$ value*</td>
<td>&lt;0.00001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Two-tailed $P$ value for the differences between the initial and retest median galactomannan indices for each population.*
Figure 1. Linear regression analysis comparing original index and retest index values when testing serum.

Figure 2. Receiver operator characteristic (ROC) curves showing performance pre- and post-freezing at −80°C for (a) serum samples and (b) plasma samples. Note that given the design of the study, which contains a disproportionate number of false-positive results that do not represent typical rates encountered in routine testing, ROC curve analysis should not be used to determine performance parameters, only the improvement in overall diagnostic performance on retesting, as represented by the area under the curve (AUC).

a defined mycological factor in the EORTC-MSG criteria, assessment of performance may be associated with incorporation bias, particularly when determining clinical validity (e.g., sensitivity/specificity) [5]. This study was designed to determine whether reproducible and/or consecutive positive EIA results were associated with patients who have specific clinical evidence of disease. Radiology was the main indicator of IA, with GM EIA alone not providing sufficient evidence to achieve any level of diagnosis. Consequently, proven, probable, and possible IA cases were included [5]. Notably, the number of positive GM EIA results was similar in both designated cases and controls. All cases, including the possible case (radiological evidence but GM EIA negative), were *Aspergillus* PCR positive, and replacement of GM EIA with PCR as the mycological criteria maintained the same number of cases, albeit the possible case would now be considered probable. Researchers studying reproducibility showed that serum index values declined significantly on retesting [13–15,18], but differentiation between cases and controls was limited. The results of the present investigation support these findings but suggest that the limited reproducibility is a marker of false positivity. On initial testing, 49.4% of the patient population without evidence of IFD had at least one EIA-positive result. However, on retesting, this number fell to 19.5%, whereas overall 87.5% of proven or probable cases remained EIA positive. Ninety-four percent of cases had consecutive EIA-positive samples compared with 16% of control patients (<0.0001). Consequently, retesting of a positive sample or consecutive positive samples could provide further evidence as to a patient’s disease status. Testing follow-up specimens in
Table 2. Comparison of galactomannan enzyme immune assay reproducibility when testing plasma samples.

<table>
<thead>
<tr>
<th>Population</th>
<th>Overall (N = 61)</th>
<th>Case (N = 24)</th>
<th>Control (N = 37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial index</td>
<td>Retest index</td>
<td>Retest index</td>
<td></td>
</tr>
<tr>
<td>(4°C)</td>
<td>(−80°C)</td>
<td>(4°C)</td>
<td>(−80°C)</td>
</tr>
<tr>
<td>Median index</td>
<td>0.10–3.60</td>
<td>0.24–1.69</td>
<td>0.08–4.78</td>
</tr>
<tr>
<td>25th percentile</td>
<td>0.10</td>
<td>0.24</td>
<td>0.08–4.78</td>
</tr>
<tr>
<td>Median index</td>
<td>0.10</td>
<td>0.24</td>
<td>0.08–4.78</td>
</tr>
<tr>
<td>75th percentile</td>
<td>0.40</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Median index</td>
<td>0.10</td>
<td>0.24</td>
<td>0.08–4.78</td>
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<td>Median index</td>
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<tr>
<td>75th percentile</td>
<td>0.40</td>
<td>0.31</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Wilcoxon P value

*Two-tailed P value for the differences between the initial and retest median galactomannan indices for each population.

Figure 3. Linear regression analysis comparing original index and retest index values when testing plasma.

order to attain consecutive positive results has an advantage over repeat testing of the same sample because, to our knowledge, most studies of this nature perform retrospective retesting and do not provide sufficient evidence as to the conditions and duration of storage that would be necessary to remove false positivity through repeat testing. Previously, plasma testing was shown to generate greater indices than serum [17]. In the current study, plasma showed consistently better qualitative agreement than sera on retesting, both before and after freezing. While the plasma consistency provided better statistical accuracy, it diminished the ability to differentiate between true and false positives, particularly when retesting after storage at 4°C. However, numbers are limited, and further research is required for confirmation. As plasma samples are collected with EDTA-anticoagulant and one of the initial EIA steps involves boiling in the presence of EDTA, which also provides good reproducibility when retesting, it is possible that EDTA stabilizes the samples [15].
To test this, plasma samples, tested twice prior to freezing at −80°C, were retested after this process. Freezing decreased the index value for controls and led to a >50% reduction in false positives, although sample numbers were limited (n = 59). This suggests that the freezing of samples prior to testing could reduce false-positive EIA results and supports the results generated when serum was retested after freezing. The effect with plasma was less evident and may be a result of considerably less storage time at −80°C. Further studies are required to determine the duration of freezing that could be incorporated in order to provide optimal performance without limiting clinical utility through increased turnaround times. This would need to be done for both serum and plasma and may not be feasible in routine practice. Given the previous performance benefits of testing plasma, it would be easy to recommend its use over serum [17]. However, until the optimal storage time at −80°C is confirmed and compared with that for serum, it is not feasible to do so.

False-positive specimens were analyzed according to blood protein concentrations. No correlation between CRP and total protein concentrations was found, but there was an association between albumin concentration and change in index on retest. False-positive samples that retested negative had a significantly higher albumin concentration, suggesting a role in the preservation of false positivity, possibly by binding the cross-reacting substance (Fig. 4). There was no explanation for samples that were negative on initial testing but positive on repeat, although human error cannot be excluded; automated processing would minimize this problem.

For sera, the comparatively small changes in cases suggest that the β (1–5) GM itself may not undergo loss of antigenicity during freezing and storage. This is supported by the observation that our quality control material showed no significant loss of index after storage (results not shown). In some control patients, false positivity was reproducible and could represent early infection or disease not reaching an EORTC/MSG diagnosis. Alternatively, it may be due to colonization with Aspergillus, where circulating GM is present in the absence of disease [19]. In the patient population that showed a significant decline in GM positivity on retest, it is possible that the EB-A2 antibody was binding another epitope. Multiple reports have shown that in vitro β-lactam antibiotics, total parenteral nutrition, and blood product conditioning fluids resulted in clinical false-positive EIA results [8–10,20,21]. EB-A2 was originally shown to bind to the β (1–5) galactofuranose side chain of GM [22]; however, binding to other galactomannoproteins secreted by A. fumigatus also occurs [6]. Our data suggest that the molecules responsible for false positivity have a lower affinity after freezing and storage, leading to reduced index values for many false positives. Reports on storage of β-lactams suggest that at −70°C, tazobactam and ampicillin remain stable in minimum inhibitory concentration assays for up to 1 year [23,24].

One notable discovery of this study is the stability of plasma samples, even after 12 months of storage. EDTA present in plasma samples has been shown to inhibit the action of some enzymes in blood samples [25]. Although serum contains a smaller proportion of proteins than plasma, it may include molecules that can hamper diagnostic accuracy [26]. Overall, the higher level of enzyme activity in serum could degrade the molecules responsible for false positivity in the Aspergillus EIA over time, while EDTA–plasma may allow both stabilization and adherence to binding proteins of both GM and other epitopes.

The inverse relationship between serum albumin concentrations and repeat EIA index values suggests that higher albumin contributes to lower affinity of false-positive epitopes after storage. Both serum albumin levels and total protein are unstable after freezing; fast freezing, as performed in our laboratory, is known to lead to significant protein denaturation [27,28]. The prerequisite EDTA processing at the beginning of the GM EIA is designed to dissociate complexes and precipitate proteins for removal of potentially cross-reacting substances. The high albumin concentrations may be linked to an epitope that, when dissociated, cross-reacts with the EIA. Alternatively, the albumin could saturate the precipitation process, resulting in entrance of other cross-reacting substances into the EIA. Denaturation of albumin by freezing could help alleviate this problem. The current study is limited in that samples were selected on the basis of time stored and index value and is not representative of the entire hemato–oncology cohort prospectively tested using EIA. Consequently, the incidence of disease and level of false positivity are higher than that seen when a prospective diagnostic service is provided. Performance
statistics would be unreliable and have therefore been omitted. In addition, as positives were confirmed by testing a follow-up sample rather than retesting an aliquot of the initial positive sample, it is not clear whether the latter would be beneficial in differentiating true from false positivity in serum in real time or whether short-term storage at 4°C or −80°C would provide differing results.

In conclusion, our data suggest that decline in GM indices with storage is significantly different between cases and controls, with no significant change in cases. We also show that freezing plays a role in the reduction of the index value for controls. In addition, samples should be assessed to determine if freezing before testing can improve diagnostic accuracy of GM EIA in routine clinical practice. Storage conditions may affect assay performance and could, in part, explain performance discrepancies between different centers evaluating the test. It is important that any new publications describe these conditions and metaanalyses account for these differences when determining pooled performance. Consecutive positive samples and/or confirmed positivity on retest are more likely associated with IA cases.

Declaration of interest

P. L. W. is a founding member of the EAPCRI; received project funding from Myconostica, Lumineux, and Renishaw diagnostics; was sponsored by Myconostica and Gilead Sciences to attend international meetings; and provided consultancy for Renishaw Diagnostics. R. A. B. is a founding member of the EAPCRI; received an educational grant and scientific fellowship award from Gilead Sciences and Pfizer, is a member of the advisory board and speaker bureau for Gilead Sciences, MSD, Astellas, and Pfizer; and was sponsored by Gilead Sciences and Pfizer to attend international meetings. The authors alone are responsible for the content and the writing of the paper. G.K. has no conflicts of interest.

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