Immune Activation in Primary Human Immunodeficiency Virus: Influence of Duration of Infection, Treatment, and Substance Use

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Background. Primary human immunodeficiency virus (HIV) is characterized by dynamic changes in viral load and innate and adaptive immune responses; it is unclear the extent to which time from acquisition to antiretroviral therapy (ART) initiation and substance use impact these immunologic changes.

Methods. We studied plasma immune activation biomarkers, viral load, and CD4+ and CD8+ cell counts in participants from the Sabes primary infection study in Peru, who had been randomized to begin ART immediately after diagnosis vs 24 weeks later. We modeled influence of substance use and duration of HIV infection on biomarkers at baseline and over 24 weeks.

Results. Compared to participants enrolled >30 days after HIV acquisition, participants enrolled during acute infection (≤30 days) had higher mean interferon (IFN)–γ and IFN-α2a (1.7-fold and 3.8-fold interquartile range [IQR] higher, respectively). Participants enrolled >30 days after HIV acquisition had higher mean baseline CD8+ cell count (2.7 times the IQR). Alcohol use (positive phosphatidylethanol level) was associated with elevated IFN-γ, tumor necrosis factor alpha (TNF-α), and interleukin 12p70 (IL-12p70), and smoking was associated with higher macrophage inflammatory protein 1α, TNF-α, and IL-12p70. Most biomarkers declined more quickly in participants who initiated ART immediately; however, substance use and duration of HIV infection at enrollment had little influence on rate of decline.

Conclusions. IFN-γ and other biomarkers are elevated during early primary infection, when exposure to HIV antigens is high. Immune activation decreased most quickly in those who started ART during acute/early primary infection. Higher CD8+ cell counts and a trend toward higher soluble CD163 levels during the 30 days after acquisition suggest the onset of compensatory responses and immune exhaustion.

Keywords. antiretroviral therapy initiation; innate immunity; MSM; Peru; primary HIV infection.

Antiretroviral therapy (ART) reduces AIDS-related and non-AIDS-related morbidity and mortality in people with human immunodeficiency virus (PWH); better regimens and earlier initiation have led to significant improvements in life expectancy [1]. Despite suppressive ART, however, both morbidity and mortality remain elevated compared to the general population [2], and differences in health-related behaviors such as alcohol and smoking are unlikely to fully explain the mortality gap [3, 4].

Even when ART is initiated very early after acquisition—within 6 months or less of documented infection—elevation of inflammatory biomarkers over levels seen in human immunodeficiency virus (HIV)–negative populations persist, even when viral load (VL) is suppressed [5, 6]. One study from the RV254 cohort suggested that there may be differences in systemic inflammation and the balance of innate and adaptive immune responses, even between those who differ by mere days in stage of primary HIV infection [6]. It is unclear to what extent these differences are due to incomplete adjustment for VL or substance use. In addition, few studies have followed the trajectory of these responses after ART initiation, and those that have, such as the longitudinal analysis after ART initiation in the Thai acute infection cohort, were limited by small size. Alcohol and other substance use has also been associated with elevated levels of HIV or simian immunodeficiency virus replication, which...
may be mediated by suppression of innate immune responses in the gut and associated dysbiosis [7–11].

Relatively little is known about dynamic changes in inflammatory biomarkers when ART is initiated at different points in primary infection, especially shortly after seroconversion, when HIV infection is more likely to be detected [12]. We examined this question using data from the Sabes study of Peruvian men who have sex with men (MSM) and transgender women (TW). Prior analyses of participants enrolled within 3 months of HIV acquisition demonstrated clinical benefits of initiating ART immediately (during early primary HIV) compared to deferring ART initiation for just 6 months [13, 14]. In this analysis, we assessed longitudinal data from a subset of Sabes study participants to determine whether brief delays in initiation of ART during primary infection result in persistent immunologic changes, ramifications that could lead to higher risk of non-AIDS outcomes [15].

MATERIALS AND METHODS

Study Design and Participants
We performed a subgroup analysis from participants in the Sabes study [13, 14]. Between 2013 and 2017, the Sabes study evaluated an HIV treatment-as-prevention intervention among MSM and TW, the populations most affected by HIV in Lima, Peru. This 3-step screen, rescreen, and treat study design has been published [13]. In brief, of 2685 MSM and TW who reported behaviors associated with high risk for HIV and who were HIV uninfected at the screening visit, 2109 entered a longitudinal cohort. During the 2-year follow-up period, these participants were tested monthly by point-of-care third-generation HIV immunoassay and for HIV type 1 (HIV-1) RNA if negative. Sabes participants with incident HIV infections were eligible for the treatment phase of the study if at the time of diagnosis they were HIV seronegative and plasma HIV-1 RNA positive, or HIV seropositive with a negative HIV third-generation antibody test or HIV RNA within the prior 3 months. Participants were randomized 1:1 within strata defined by serostatus at diagnosis to immediate (at a mean of <1 day [range, 0–6 days]) vs deferred ART initiation (beginning at 24 weeks or when local criteria were met) and followed for clinical events over 1 year. Of the 216 randomized participants, all participants with plasma biomarkers assessed at baseline or longitudinally through 24 weeks were included in this subanalysis. Participants were selected for biomarker analysis if they were co-enrolled in a related observational study that evaluated neurologic or reservoir outcomes [16, 17], had near-complete samples at the selected timepoints, and started ART at the designated visit (either at enrollment in the immediate arm or at week 24 in the deferred arm).

Self-reported demographic, risk behavior, and substance use data were collected using computer-assisted self-interview. Smoking status (current smoker or not) was collected using the 2015 Spanish-language National Health and Nutrition Examination Survey tobacco use questionnaire [18]. Substance use (marijuana, cocaine [paste, powder, injection, crack], hallucinogens, inhalants, heroin, methamphetamine, and tranquilizers) in the past 3 months was collected at baseline and quarterly. Self-reported alcohol use in the past 30 days was collected using the timeline follow-back questionnaire and the Quick Drinking Screen at baseline [19–22].

Laboratory Measurements
We evaluated a panel of markers of immune activation at enrollment (week 0) and 1, 2, 4, 8, 12, and 24 weeks thereafter. CD4, CD8, and VL were evaluated at the same timepoints. A custom Meso Scale Discovery (MSD) U-PLEX chemiluminescent immunoassay panel (Meso Scale Diagnostics, Rockville, Maryland) was used to test for tumor necrosis factor (TNF)–α, TNF-β, interferon (IFN)–α2a, macrophage inflammatory protein (MIP)–1α, MIP-1β, IFN–γ–inducible protein 10 (IP-10), interleukin (IL)–4, IL–8, IL–7, IL–2, IL–1α, IL–1β, IL–12p70, IL–12p70, IL–16, IFN–γ, IL–10, monocyte chemotactic protein (MCP)–1, and stromal cell–derived factor (SDF)–1α, in plasma. We also quantified soluble CD163 (sCD163) and soluble CD14 (sCD14) using an enzyme-linked immunosorbent assay (Quantikine ELISA, R&D Systems, Minneapolis, Minnesota). Vascular cell adhesion molecule 1 (VCAM-1) was quantified with Luminex assay (Human Magnetic Luminex Assay, R&D Systems). For the MSD and Luminex assays, we followed manufacturer instructions [13]. Plasma was additionally analyzed for levels of high-sensitivity C-reactive protein (CRP) (Siemens Healthcare Diagnostics, Newark, Delaware) and D-dimer with Diazyme reagents (Diazyme Laboratories, San Diego, California) in the same specimens, as well as CD4+ and CD8+ T cells (cells per microliter). Phosphatidylethanol (PEth, a direct alcohol biomarker) was measured in dried blood spots (United States Drug Testing Laboratories, Des Plaines, Illinois), using the following cutoffs for PEth 16.0/18.1: negative (nondrinkers or low drinkers) ≤8 ng/mL, positive >8 ng/mL [23]. Biomarker data are available for 97 participants at the enrollment visit, of which 57 participants had a smaller panel of biomarkers available for the multiple timepoints through 24 weeks.

Statistical Analysis
For cross-sectional analysis, the primary predictor of interest was the time from estimated date of detectable HIV infection (EDDI) to enrollment (ENR). EDDI was estimated from the published Consortium for Evaluation and Performance of HIV Incidence Assays calculator that includes clinical and HIV testing history for participants, as previously described [14, 24]. Multivariate linear regression analysis was used to assess differences in plasma biomarkers between participants with EDDI-to-ENR intervals of ≤30 vs >30 days (eg, acute infection vs later primary infection). Biomarkers (except D-dimer,
high-sensitivity CRP, CD4, and CD8) were log10-transformed to achieve constant variance assumptions and were normalized to the cohort’s interquartile range (IQR) given variable dynamic ranges of biomarkers. Viral load was considered a potential confounder in all models. Additional covariates considered were age, current smoking, and alcohol use. We evaluated several measures of alcohol use given evidence that exposure measurement is imperfect and different methods have variable correlation with biologic outcomes [25]. These included blood PEth, calculated drinks per day, reported alcohol use in the past 30 days, and Alcohol Use Disorders Identification Test (AUDIT) score. Additional covariates were included in models if they changed estimates by ≥10%.

Biomarkers measured longitudinally (TNF-α, MIP-1α, IP-10, IL-7, IL-6, IL-2, IL-1α, IL-12, IFN-γ, IL-10, CD4, CD8, CD4/CD8 ratio, and VL) were evaluated with generalized estimating equations (GEEs) to capture differential time trend of each analyte by immediate or deferred ART arm, including interaction between treatment arm and time. Models were additionally adjusted for age, baseline VL, current smoking, and PEth. We used the Benjamini-Hochberg procedure to correct P values for false discovery [26]. Analyses were performed with SAS software, version 9.4 (SAS Institute, Cary, North Carolina) and Stata software, version 16 (StataCorp, College Station, Texas).

This study received Institutional Review Board approval at the Fred Hutchinson Cancer Research Center and the Bioethics Committee at Asociación Civil Impacta Salud y Educación. All participants provided written informed consent for the Sabes study, including consent for storage and future use of specimens.

RESULTS

Of 97 participants, 32 and 65 had EDDI-to-ENR intervals of ≤30 or >30 days, respectively. Median age was 26 years (IQR, 21–40 years). Forty participants (44%) were current sometime or daily smokers, 54 (48%) had detectable PEth levels, and 22 (23%) had AUDIT scores ≥15. Use of hallucinogens, opiates, amphetamines, and tranquilizers was negligible in the cohort (≤1 person reporting each) and therefore were not further elaborated. The median HIV VL was higher in the ≤30-day EDDI-to-ENR group than in the >30-day group (6.7 [IQR, 6.2–7.0] log10 copies/mL vs 5.4 [IQR, 4.7–6.0] log10 copies/mL, P < .001; Table 1). No baseline covariates differed significantly between ART initiation arms.

Despite Overall Similarity, Some Biomarkers Differ by EDDI-to-ENR Group

While several biomarkers were higher in the EDDI-to-ENR ≤30-day group in unadjusted analysis, adjusting for VL attenuated many of these associations (Figure 1). CD4/CD8 ratio, VCAM-1, IL-10, and IP-10 were most attenuated by VL adjustment. After adjustment for both VL and smoking, most biomarkers were similar between the 2 EDDI-to-ENR groups (Supplementary Table 1). Mean CD8+ cell count in the >30-day group was higher by 2.7 times (95% confidence interval [CI], 1.7–4.4) the IQR (P = .015). Similarly, sCD163 in the >30-day group was higher by 1.4 (95% CI, .96–2.11) times the IQR, but the difference was no longer significant after adjustment (P = .25). Those in the ≤30-day group had mean 1.7-fold (95% CI, 1.2–2.4) and 3.8-fold (95% CI, 1.6–9.1) IQR higher IFN-γ and IFN-α2a levels than the >30-day group (both P = .028).

Table 1. Demographics of the 92 Participants Who Contributed Cross-Sectional and Longitudinal (n = 57) Data, a Subset of the Total 216 Participants Randomized Within the Sabes Clinical Study

| Variable                    | Enrolled Within 30 Days of EDDI (n = 32) | Enrolled 31–100 Days From EDDI (n = 65) | P Value |
|-----------------------------|------------------------------------------|------------------------------------------|---------|
| Male sex at birth           | 32 (100)                                 | 65 (100)                                 | 1.00    |
| Gender identity             |                                          |                                          | .25     |
| Transgender female          | 2 (6)                                    | 8 (12)                                   |         |
| Male                         | 29 (91)                                  | 57 (88)                                  |         |
| Other/not reported          | 1 (3)                                    | 0 (0)                                    |         |
| Age, y, mean (IQR)          | 26.4 (22.8–29.7)                         | 27.0 (21.3–30.2)                         | .63     |
| PEth results at ENR<sup>a</sup>, ng/mL |                                   |                                          | .43     |
| <8 (Negative)               | 14 (44)                                  | 28 (43)                                  |         |
| 8–111                       | 14 (44)                                  | 24 (37)                                  |         |
| ≥112                        | 0 (0)                                    | 3 (5)                                    |         |
| Not available               | 4 (12)                                   | ...                                      |         |
| Current smoking             | 16 (50)                                  | 25 (38)                                  | .28     |
| Cocaine use (any form)      | 1 (3)                                    | 3 (5)                                    | .73     |
| Marijuana                   | 3 (9)                                    | 6 (9)                                    | .98     |
| CD4 at ENR<sup>a</sup>, median (IQR) | 406 (276–543)                         | 428 (284–571)                            | .96     |
| HIV VL at ENR<sup>a</sup>, log<sub>10</sub>, median (IQR) | 6.7 (6.2–7.0)                         | 5.4 (4.7–6.0)                            | <.0001  |

Data are presented as No. (%) unless otherwise indicated. Few, if any, participants (1 or none each) reported any use of inhalants, amphetamines, opioids, or hallucinogens and are therefore omitted from these tables.

Abbreviations: EDDI, estimated date of detectable infection; ENR, enrollment; HIV, human immunodeficiency virus; IQR, interquartile range; PEth, phosphatidylethanol, a long-lived alcohol metabolite; VL, viral load.

<sup>a</sup>ENR was generally within 6 days of diagnosis of acute or primary HIV.
Changes in Biomarkers Over Time

Time of ART initiation was significantly associated with decline in levels of TNF-α, MIP-1α, IL-7, IL-12, IFN-γ, and IL-10. All declined more quickly in those who initiated ART immediately (and were thus on ART during the observation period), but in adjusted analyses only the decline in MIP-1α, IL-12, and IL-10 remained significantly more rapid in those treated during the substudy period (Figure 2, Supplementary Table 2). Time between HIV acquisition and enrollment had less impact. Those enrolled within 30 days of EDDI had lower overall levels of IL-6 over time in adjusted analysis; time since EDDI (≤30 vs >30 days) also affected rate of decline of IL-1α (Supplementary Table 2). In GEEs restricted to the immediate ART arm where the impact of time between EDDI and
Figure 2. Change in soluble biomarkers over the first 24 weeks among people with human immunodeficiency virus (HIV) who initiated antiretroviral therapy (ART) immediately vs deferred ART initiation until 24 weeks after HIV diagnosis. Longitudinal analysis with generalized estimating equations was performed with biomarkers obtained at baseline (week 0) and at weeks 1, 2, 4, 8, 12, and 24 after enrollment, using an interaction term for study week and treatment arm, unadjusted for substance use or estimated date of detectable infection. The shaded area displays 95% confidence intervals around the regression line. Interleukins 1α, 2, and 6 are not displayed.

Abbreviations: IFN, interferon; IL, interleukin; IP, interferon-γ-inducible protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor; VL, viral load.
enrollment should be most clear, only decline in IL-1α was impacted by the EDDI-to-ENR interval (faster decline in the ≤30-day group) (Figure 3). A similar but nonsignificant trend was seen in IFN-γ (Figure 3). Linear models best described time-dependent changes compared with quadratic models. Neither age nor baseline VL influenced the rate of change of biomarkers over 24 weeks.

### Influence of Alcohol and Smoking on Biomarkers During Early HIV Infection

At the enrollment visit, average drinks per day, reported alcohol use during the past 30 days, and AUDIT score were not associated with differences in markers of immune activation after adjustment. However, positive PEth level was associated with a 1.3-1.5-, and 1.7-fold IQR higher for IFN-γ, TNF-α, and IL-12p70 (P = .04, .03, and .02, respectively). Compared to current smokers, nonsmokers had a 1.4-, 1.4-, and 1.8-fold IQR higher TNF-α, MIP-1α, and IL-12p70 (P = .04, .03, and .01, respectively). In longitudinal analysis, IL-1α declined more quickly in smokers and in those with positive PEth, and there were no other associations between use of substances and change in biomarkers over time (Supplementary Table 2). Small proportion of participants using substances other than alcohol or smoking (<10% using marijuana and <1% other substances) precluded formal analysis of associations with biomarkers.

### DISCUSSION

Clinical benefits of ART initiation during acute/early primary infection vs later primary infection have been previously reported in our randomized Sabes study. We extended these findings by assessing innate and adaptive immune responses with soluble biomarkers in a subgroup of Sabes participants. Observations from these analyses include the following. First, most markers of immune activation were higher in those ≤30 days vs >30 days from EDDI (ie, those acute HIV infection vs those later in primary infection), a finding modestly attenuated by adjustment for VL. IFN-γ and other inflammatory markers are higher during this period marked by high exposure to viral antigens, prior to the onset of compensatory responses and immune exhaustion [27]. Levels of sCD163, expressed on M1 macrophages, which work to suppress tissue inflammation, were higher in participants enrolled >30 days from EDDI, perhaps in response to inflammation in the acute period, although this finding was attenuated by adjustment for baseline VL. The >30-day group also had higher levels of CD8; the lower levels of IFN-γ we observed in the >30-day group and proliferation defects observed in other studies suggest reduced CD8 functional capacity during the immediate post–acute infection period [28]. Among participants in the Short Pulse Anti-Retroviral Therapy at Seroconversion study and in our full analysis of the Sabes study, normalization of the CD4/CD8 ratio was seen in those treated during acute infection, but not in those treated after 6 months from infection in the former or after 90 days from infection in the latter [14, 29].

As expected, most biomarkers decreased significantly over time and did so more quickly in those starting ART immediately. Neither age nor baseline VL modified the rate of change in markers over time, even though those with higher VLs also showed higher levels of immune activation. Only longitudinal changes in IL-1α were influenced by alcohol consumption or smoking. Except for IL-1α, rate of biomarker decay appeared unaffected by time since EDDI at enrollment, and those who started treatment immediately had faster declines in only 3 other markers. Although we did not directly evaluate inflammasomes in this study, our findings hint at a complex regulation of acute inflammation that depended on time since HIV infection and exogenous exposures [30].

Our results complement the results of Sereti et al [6], who used a smaller biomarker panel in 78 Thai participants enrolled during early primary infection; baseline differences by stage (based on pooled nucleic acid tests, fourth-generation enzyme immunoassays, and Western blots) were reported for CRP, IL-6, intestinal fatty acid–binding protein (I-FABP), sCD14, D-dimer, and hyaluronic acid. At baseline, our EDDI-to-ENR categories differed by CD4, CD8, sCD163, INF-γ, IL-12p70, and IL-α2a. Sereti et al and others assessed posttreatment biomarker levels and found I-FABP or lipopolysaccharide and sCD14 to be elevated compared to levels in uninfected controls [5, 6, 31, 32]. We report substantial impact of ART initiation in primary infection on multiple inflammatory biomarkers (comparing immediate vs deferred ART initiation). Moreover, among participants who initiated ART immediately after diagnosis, small differences in time since HIV acquisition (≤30 vs >30 days) had little impact on subsequent overall level or rate of change in biomarkers.

Behavioral influences in PWH likely contribute to changes in inflammatory markers, independent of the impact of plasma viremia. Studies of the effect of alcohol consumption have reported contrasting effects on cytokines, complicated by different ways of measuring alcohol use. A potential influence is the suppression of gut-associated innate immune function by chronic alcohol consumption; this could contribute to gastrointestinal dysbiosis and higher levels of systemic immune activation and HIV replication, although higher levels of alcohol consumption have been associated with overall lower blood levels of IL-6 and TNF-α [33]. Smoking is also well-known to produce both pro- and anti-inflammatory effects. Smoking can increase systemic IL-6 specifically as well as gastrointestinal inflammation more generally [34, 35]. Smoking also suppresses T-cell function, which can inhibit responses to viral infections [36, 37]. This could possibly explain why nonsmokers exhibited higher levels of some biomarkers associated with antiviral responses at baseline. Cocaine has also been associated with markers of T-cell activation in PWH during chronic infection.
Figure 3. Change in soluble biomarkers over the first 24 weeks among people with human immunodeficiency virus (HIV) comparing those enrolled within 30 vs >30 days from estimated date of detectable HIV infection (EDDI) among those who initiated antiretroviral therapy immediately. Longitudinal analysis with generalized estimating equations was performed with biomarkers obtained at baseline (week 0) and at weeks 1, 2, 4, 8, 12, and 24 after enrollment, using an interaction term for study week and EDDI group. The shaded area displays 95% confidence intervals around the regression line. CD4, CD8, and viral load are not displayed. Abbreviations: EDDI, estimated date of detectable infection; ENR, enrollment; IFN, interferon; IL, interleukin; IP, interferon-γ-inducible protein; MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.
Although prior analyses of chronic immune activation in PWH focused on a disproportionate exposure of this population to smoking, alcohol, and other substances, it has become clearer that the burden of inflammation in PWH is driven by HIV itself, whether through pathogenesis established prior to ART initiation or ongoing immune stimulation during treatment [4, 39, 40].

Our study has several limitations. The relatively small sample of participants with acute HIV limited our ability to identify clinically meaningfully differences compared with later primary infection. We also do not compare our findings to persons without HIV. Small sample size also impacted our ability to exclude the influence of measured variables on longitudinal change in markers and the ability to retain sufficient power to evaluate multiple covariates. The relatively homogeneous participant population and randomized ART allocation, however, reduced potential bias associated with treatment initiation. This study did not include any participants assigned female at birth; thus, results may not be generalizable given known sex differences in immune responses and viral setpoint [41]. Smoking and moderate/high levels of alcohol consumption were common in this cohort, although other substances were not reported with sufficient frequency to be included in this analysis.

This study is unique in its ability to compare inflammatory markers at baseline and over time in persons starting ART during primary HIV. Randomization to time of ART initiation allowed for comparison groups that were similar except for ART initiation. Unique findings of this study include identification of CD8 and sCD163 elevations during postacute infection, interaction of smoking and alcohol with inflammation, and the influence of ART on immune activation during early treatment. Although we found weak associations between substance use and changes in biomarkers of immune activation in the first 6 months after HIV acquisition, alcohol use and smoking were associated with several proinflammatory cytokines at the time of HIV diagnosis. Thus, the frequent care interactions that occur around ART initiation could provide an opportunity for education and counseling to PWH around cessation of smoking and moderation of alcohol to improve overall health outcomes. Concordant with the primary findings of the Sabes study, which showed deleterious effects on clinical outcomes associated with delaying ART by only 6 months during primary infection, this study showed deleterious effects on clinical outcomes associated with delaying ART by only 6 months during primary infection, this study found beneficial immunologic consequences in those who started ART within 30 days of HIV acquisition. Currently, the World Health Organization and all guidelines panels recommend starting ART at the time of HIV diagnosis, but these recommendations are based on data from prevalent infection [1, 42–45]; the beneficial immunologic consequences of starting ART within 30 days of acquisition seen in this study therefore add strength to the rationale for expediting ART initiation in acute or primary infection.

Supplementary Data
Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes
Author contributions. The Sabes clinical study was conceived and implemented by J. R. L., P. G., J. S., and A. D., in addition to a large team at the nongovernmental organization IMPACTA Peru. Subanalysis was designed by T. G. and R. B. I. Laboratory and data processing: J. M., D. P.-S., S. P., R. V., E. W. Statistical analyses: S. R. S., Y. W., S. D., R. B. I. Manuscript preparation: S. R. S., S. D., R. B. I., A. D. All authors have reviewed and agree to the final submitted manuscript.

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Patient consent. The Sabes study was approved by the Fred Hutchinson Cancer Research Center Institutional Review Board (IRB) as well as the Peruvian National Institute of Health and other local IRBs and ethics committees. All participants provided written informed consent.

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Potential conflicts of interest. R. B. I. has received consulting fees from SeaGen and AbbVie, outside the submitted work. A. D. has received consulting fees from SeaGen and research funds from Gilead, outside the submitted work. All other authors report no potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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