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Changes in Inflammation but Not in T-Cell Activation Precede Non-AIDS-Defining Events in a Case-Control Study of Patients on Long-term Antiretroviral Therapy

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Background. We examined changes in soluble inflammatory cytokines and T-cell activation after antiretroviral therapy (ART) initiation in an AIDS Clinical Trials Group (ACTG) nested case-control study.

Methods. Cases were 143 human immunodeficiency virus (HIV)–infected adults who developed a non-AIDS event; 315 controls remained event-free. Specimens were tested pre-ART, year 1 post-ART, and at the visit preceding the event. Conditional logistic regression evaluated the associations of biomarker changes with non-AIDS events.

Results. Inflammatory and most activation biomarkers declined from pre-ART to year 1 for cases and controls. Subsequently, inflammatory biomarkers remained mostly stable in controls but not cases. Cellular activation markers generally declined for both cases and controls between year 1 and the pre-event sampling. Controls with greater pre-ART RNA levels or lower $CD4^+$ levels had higher biomarker levels while also experiencing greater biomarker declines in the first year of ART. Changes in biomarkers to year 1 showed no significant associations with non-AIDS events. Cases, however, had significantly greater increases in all plasma biomarkers (but not cellular activation) from year 1 to the visit preceding the event.

Conclusions. Inflammation increases prior to non-AIDS events in treated HIV-infected adults. These biomarker changes may reflect subclinical disease processes or other alterations in the inflammatory environment that causally contribute to disease.

Clinical Trials Registration. NCT00001137.

Keywords. HIV; non-AIDS morbidity; antiretroviral therapy; inflammation; T-cell activation.

Infection with human immunodeficiency virus type 1 (HIV) results in compromised immune function, activation of immune cells, and inflammation [1, 2]. It has been shown that chronic immune activation and inflammation in HIV-infected individuals are consistent predictors of disease progression and AIDS-defining comorbidities [3–7]. Although AIDS mortality has decreased considerably with increased access to antiretroviral therapy (ART), HIV-infected adults experience more non-AIDS-defining morbid events than do uninfected adults, despite ART [1, 8–12].

Recently, several studies have linked elevated levels of inflammatory biomarkers to an increased risk of non-AIDS morbidity and death during ART-mediated viral suppression [13–19]. In one of these studies, Tenorio et al conducted a matched case-control study and showed that higher levels of soluble markers of inflammation and coagulation (interleukin 6 [IL-6], soluble tumor necrosis factor receptors I and II [sTNFR-I and sTNFR-II], kynurenine-to-tryptophan (KT) ratio, and D-dimer) but not T-cell activation predict non-AIDS-related morbid events and death during suppressive ART [20]. Nevertheless, this study and most prior published studies have evaluated the prognostic value of immunologic biomarkers assessed at discrete time points.

There has been ongoing and increasing interest in studying cytokines as biomarkers of HIV disease progression [21–23]. Most biomarker studies of HIV disease progression to date have assessed the impact of a single measurement on future events. A few recent studies have investigated measurement and biological variability by averaging longitudinal levels, thereby increasing the prognostic capacity of the biomarker [17, 24]. Yet, few studies have evaluated whether biomarkers increase within individuals prior to a clinical event, either as a consequence of a preclinical illness or a worsening systemic inflammatory state that eventually manifests as a morbid clinical event. We used the data from Tenorio et al [20] to assess if changes in inflammatory biomarkers are associated with non-AIDS-defining events. We also assessed the associations of biomarker changes after ART with pre-ART factors. A better understanding of inflammation

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and immune activation dynamics during ART might provide insights into the pathogenesis of non-AIDS morbidity, lead to the development of improved prognostic markers, and provide insights into the most appropriate timing of sampling for nested case-control studies.

METHODS

Study Design

We used data from a nested case-control study [20] to examine the association between changes in inflammatory biomarkers and non-AIDS-associated morbidity and nonaccidental death, among ART-naive HIV-infected participants who achieved virologic suppression 1 year after ART initiation in the context of the AIDS Clinical Trials Group (ACTG) 5001 ALLRT (ACTG Longitudinal Linked Randomized Trials) study. ALLRT is a longitudinal cohort study of United States-based HIV-infected participants enrolled after prospective randomization into selected clinical trials conducted by the ACTG [25]. Participants provided written informed consent, and institutional review board approval for ALLRT was obtained by each ACTG site.

All participants were (1) ART-naive when enrolled into an ACTG study, (2) had plasma HIV RNA <400 copies/mL at week 48 of ART, and (3) maintained plasma HIV RNA <400 copies/mL at all subsequent time-points (isolated values >400 copies/mL were allowed if preceding and subsequent values were <400 copies/mL without a change in ART).

Cases developed a nonaccidental non-AIDS-related death, myocardial infarction (MI), stroke, or a non-AIDS-defining malignancy or serious bacterial infection. For each case, we identified 1–3 virally suppressed ALLRT participants matched for age, sex, pre-ART CD4$^+$ T-cell count (within +50 cells/μL), ART regimen at week 48 (whether protease inhibitor [PI]–containing or not, and whether abacavir-containing or not) and parent (initial randomized) study. Matching for age and sex was necessary as age is a risk factor for the clinical endpoints of interest, and male sex is a known risk factor for cardiovascular disease. Nadir CD4$^+$ T-cell count prior to ART is a risk factor for mortality and cancer. Protease inhibitors and abacavir may be associated with an increased risk for cardiovascular disease. Parent study was chosen as a matching variable to control for possible differences in treatment patterns, demographics, and other factors that could vary by parent study. All controls had endpoint-free follow-up time greater than that of the corresponding case.

Biomarker Measurements

Stored plasma and peripheral blood mononuclear cells (PBMCs) at the time before ART initiation (pre-ART), 1 year (48–64 weeks) after ART initiation, and the time proximal to an event (visit immediately preceding the non-AIDS-defining event [pre-event visit], and a corresponding time point in controls) were tested for the following: soluble markers of inflammation (IL-6, sTNFR- I, and sTNFR-II), monocyte activation (soluble CD14 [sCD14]), interferon-γ inducible protein 10 (CXCL10), and coagulation (D-dimer).

At each visit, whole blood was obtained in tubes containing ethylenediaminetetraacetic acid. Specimens were spun at 400*g* for 10 minutes, and plasma was pipetted and spun again at 800*g* for 10 minutes. Plasma was aliquoted, frozen, and stored at −70°C until assayed. We also performed flow cytometric evaluation of immune activation (HLA-DR$^+$CD38$^+$), exhaustion (PD1$^+$), and senescence (CD57$^+$CD28$^-$) indices on CD4$^+$ and CD8$^+$ T-cells. The T-cell phenotype was characterized in batch by polychromatic flow cytometry on cryopreserved PBMCs that were removed from liquid nitrogen storage, thawed rapidly at 37°C in a water bath, washed, and rested overnight at 37°C in an incubator. The following day, cells were washed and stained for viability with Aqua Live/Dead cell stain kit (Invitrogen) prior to cell surface staining with fluorochrome-conjugated monoclonal antibodies to CD3, CD8, HLA-DR, CD38, CD28, CD57, and PD-1 (BD Biosciences for all) and to CD4 (Invitrogen). After staining, cells were fixed in 2% formaldehyde and analyzed within 24 hours on a LSR2 flow cytometer (BD), using FACSDiva software, version 6.1.1. For the cell-based marker analysis, only results obtained from viable cells and from samples with >100 flow cytometry events were included in the analysis. Detailed procedures for plasma analysis and T-cell phenotyping are described elsewhere [20].

Statistical Analysis

Spearman rank correlations (both unadjusted and partial) were used to assess the associations between biomarker changes at year 1 and at the pre-event time with selected pre-ART factors. Factors examined included HIV RNA load (copies/mL), CD4$^+$ cell count (cells/μL), CD4:CD8 ratio, CD8$^+$ cell count (cells/μL), age, body mass index (BMI; kg/m^2) and waist-to-hip ratio. Levels of soluble markers were $\log_{10}$ transformed in all analyses.

Consistent with the case-control design, conditional logistic regression analysis was used to study the associations of changes of biomarkers at year 1 and at the pre-event time with nonaccidental mortality, MI, stroke, malignancy, and serious bacterial infection and with all events combined. Effects were quantified in terms of the odds ratio (OR) per 1 interquartile range (IQR) on the $\log_{10}$-transformed scale for soluble markers; the IQR was obtained from pooling cases and controls separately at each time point. Supplementary analyses evaluated biomarkers in categories of above vs below the median. Adjusted analyses further controlled for concurrent CD4$^+$ cell count. In a sensitivity analysis, the following additional potential confounders at the time of the biomarker measurement were also evaluated individually in the conditional logistic model: (1) chronic hepatitis B or C infection, (2) smoking status, (3) injection drug use, (4) waist-to-hip ratio, (5) history of clinician-diagnosed diabetes or hypertension, (6) use of antihypertensive or lipid-level lowering

agents, (7) family history of MI, and (8) change in $CD4^+$ cell count after ART initiation. We also checked if there were differential changes in biomarkers with initial ART class regimen.

Analyses were conducted using SAS version 9.4 software (SAS Institute, Cary, North Carolina). Owing to the exploratory nature of the analyses, no corrections were made for multiple comparisons; however, particular attention in interpretation was given to consistencies in observed findings. The significance criterion was a 2-sided P value < .05.

RESULTS

Description of the Study Population

The analysis included 143 cases and 315 controls. Cases and controls had similar pre-ART demographic characteristics (Supplementary Table 1). Overall, 85% of the population was male, the median (Q1–Q3) age was 45 (39–51) years, $CD4^+$ cell count was 215 (76–334) cells/μL, plasma HIV RNA was 4.8 (4.4–5.4) $\log_{10}$ copies/mL. Forty-four percent of the participants initiated an nonnucleoside reverse transcriptase inhibitor (NNRTI) + nucleoside reverse transcriptase inhibitor (NRTI) ART regimen, 28% on a PI + NRTI regimen, 10% a PI + NRTI + NNRTI regimen, 10% an NRTI-only regimen, and 8% on a PI + NNRTI regimen.

Non-AIDS Events

Among 143 cases, non-AIDS-defining events occurred at a median (Q1–Q3) of 2.9 (1.7–4.6) years after ART initiation. There were 21 nonaccidental deaths (2 fatal MIs, 1 fatal stroke, 2 cases of fatal malignancies, 3 fatal serious bacterial infections, and 13 other nonaccidental deaths); 23 non-fatal MIs; 15 nonfatal strokes; 50 nonfatal malignancies (Supplementary Table 2); and 34 nonfatal bacterial infections. The median endpoint-free follow-up time for the controls was 3.3 years after ART initiation.

Soluble Markers of Inflammation and Coagulation

Significant declines (P < .0001) were observed for all biomarkers 1 year after ART initiation for both cases and controls, but no significant differences were detected between cases and controls. Supplementary Table 3 provides a summary of the biomarker levels across time for cases and controls. Cases had marginally greater declines than controls in sCD14 levels (Supplementary Figure 1*A*). Year 1 biomarkers during ART-mediated viral suppression were correlated with pre-ART levels (for controls the Spearman correlation coefficients ranged from 0.48 to 0.67; P < .001), consistent with the hypothesis that the inflammation "set point" during ART is largely determined by the extent of pre-ART inflammation.

Biomarkers remained mostly stable after year 1 for the controls. The only significant change after year 1 was for CXCL10 and sTNFR-II, both of which continued to decline (P < .0001) during the time between year 1 and the pre-event time. On the other hand, among cases, most biomarkers increased between year 1 and the pre-event time-point, despite maintenance of ART-mediated viral suppression. Although cases and controls had similar biomarker levels at year 1, there were consistent and significant differences in biomarker dynamics after this time point. Differences between cases and controls were significant for all biomarkers ($P \le .01$) (greater decreases or lesser increases in controls) during this time period except for CXCL10, for which the difference in decline between cases and controls was only marginally significant (Figure 1A).

We also compared the changes in biomarkers between pre-ART and year 1 by initial ART regimen class (PI + NRTI vs NNRTI + NRTI) separately among cases and controls. No differences in biomarker changes were detected by initial ART regimen (data not shown).

Correlations of Soluble Marker Changes With Pre-ART Factors

We next assessed the associations between pre-ART factors with changes of inflammatory biomarkers from pre-ART to year 1 in the controls (Figure 2A). Higher pre-ART HIV RNA levels were associated with greater declines for all biomarkers ($P \le .01$). These results remained significant after adjustment for pre-ART $CD4^+$ for all markers except for IL-6 and D-dimer. A lower pre-ART $CD4^+$ cell count and a lower pre-ART CD4:CD8 ratio were also associated with greater declines for all biomarkers (P < .001 for all associations). Both HIV RNA levels and $CD4^+$ cell count independently predicted the magnitude of the soluble marker decline in the first year of therapy. The pre-ART $CD8^+$ cell count was not correlated with changes for any of the soluble biomarkers at year 1. Older age pre-ART was correlated with greater sTNFR-II, sCD14, and D-dimer declines ($P \le .03$). Lower pre-ART BMI values were correlated with greater biomarker declines at year 1 ($P \le .01$) and higher pre-ART waist-to-hip ratio was also correlated with greater declines only for sCD14 and D-dimer ($P \le .03$).

Among cases, higher pre-ART HIV RNA correlated with greater biomarker declines at year 1 for all soluble biomarkers ($P \le .03$) except for IL-6. Lower values of pre-ART $CD4^+$, CD4:CD8, and BMI correlated with greater decreases for certain biomarkers at year 1 ($P \le .04$) for the cases, whereas older age correlated with sTNFR-I and sTNFR-II declines ($P \le .04$). $CD8^+$ cell count and waist-to-hip ratio were not correlated with changes in biomarkers to year 1 (Figure 2B).

While lower pre-ART $CD4^+$ cell count and higher pre-ART HIV RNA were associated with greater declines from baseline to year 1, lower pre-ART $CD4^+$ cell count and higher pre-ART HIV RNA correlated with higher levels of biomarkers at year 1 for both cases and controls (for controls: Spearman correlation coefficients at year 1 with pre-ART $CD4^+$ count ranged from −0.17 to −0.13 [P < .02]; correlations with pre-ART HIV RNA ranged from 0.15 to 0.29 [P < .01] for all biomarkers except for D-dimer [P > .05] and sCD14 for correlation with pre-ART HIV RNA at year 1 [P > .05]).

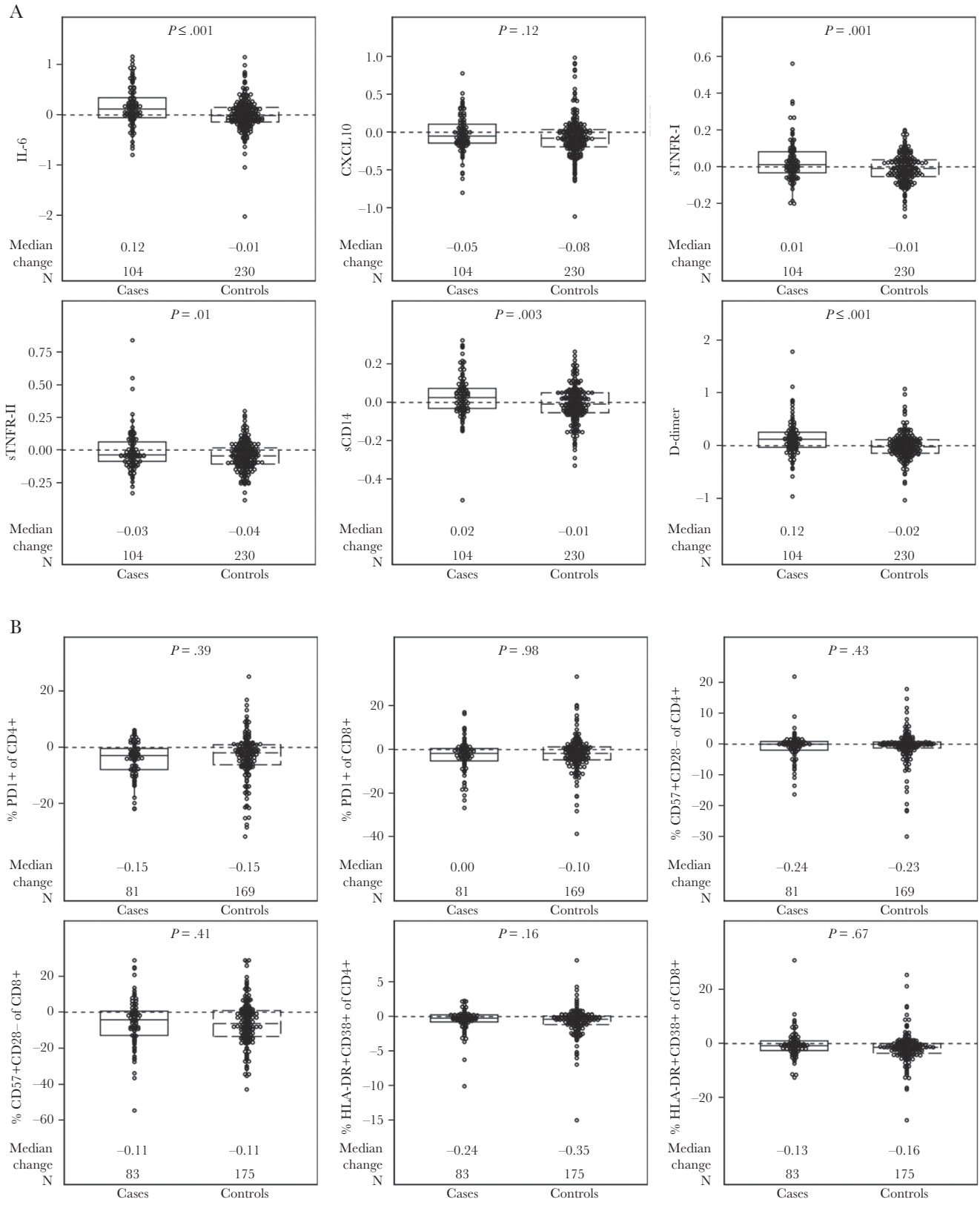

Figure 1. Changes of biomarkers. *A*, Changes of $\log_{10}$-transformed soluble biomarkers from year 1 to the pre-event time. *B*, Changes of T-cell biomarkers from year 1 to the pre-event time. The box plots display the data distribution. Boxes represent the first quartile, median, and the third quartile. Median relative changes are given separately for cases and controls; statistical comparison is based on conditional logistic regression. *P* value from conditional logistic regression model comparing distributions between cases and controls. Abbreviations: CXCL10, interferon-γ inducible protein 10; IL-6, interleukin 6; sTNFR, soluble tumor necrosis factor receptors; CD14, soluble CD14.

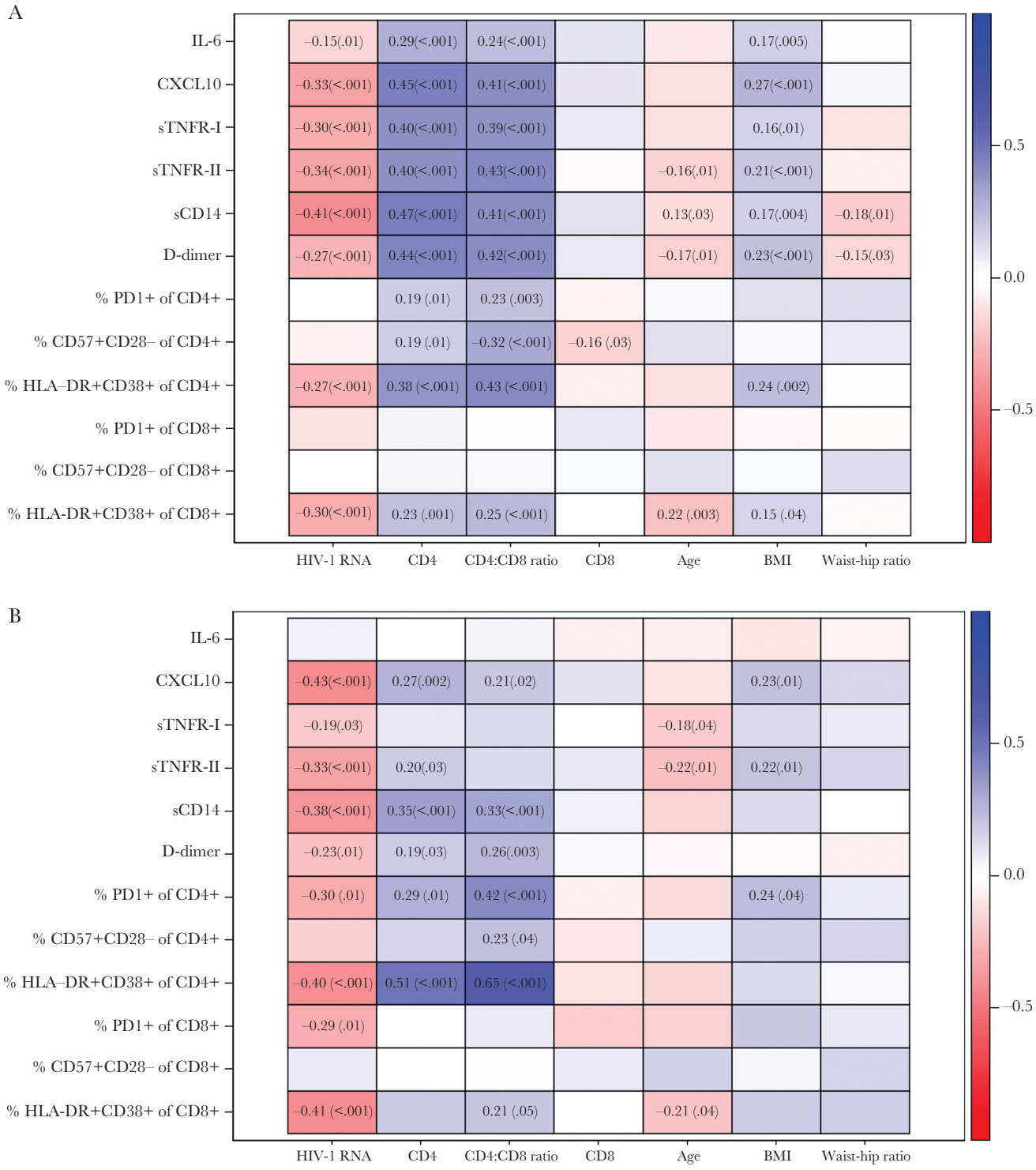

Figure 2. Correlations (*P* values) of pre–antiretroviral therapy (ART) factors with changes of biomarkers. Soluble and T-cell biomarkers from pre-ART to year 1 for the controls (*A*) and from pre-ART to year 1 for the cases (*B*). Negative correlations in red and positive in blue. Correlations (*P* value) if $P < .05$. Correlations comparisons used a Spearman rank test. Abbreviations: BMI, body mass index; CXCL10, interferon-γ inducible protein 10; HIV-1, human immunodeficiency virus type 1; IL-6, interleukin 6; sTNFR, soluble tumor necrosis factor receptors; CD14, soluble CD14.

Association of Soluble Marker Changes With Non-AIDS-Defining Events

Greater declines in biomarkers to year 1 showed no significant associations with non-AIDS-defining events except for sCD14 in the adjusted analyses (Figure 3). Evaluating models including both year 1 levels and changes from pre-ART, levels at year 1 remained highly predictive of subsequent events ($P < .01$) for all

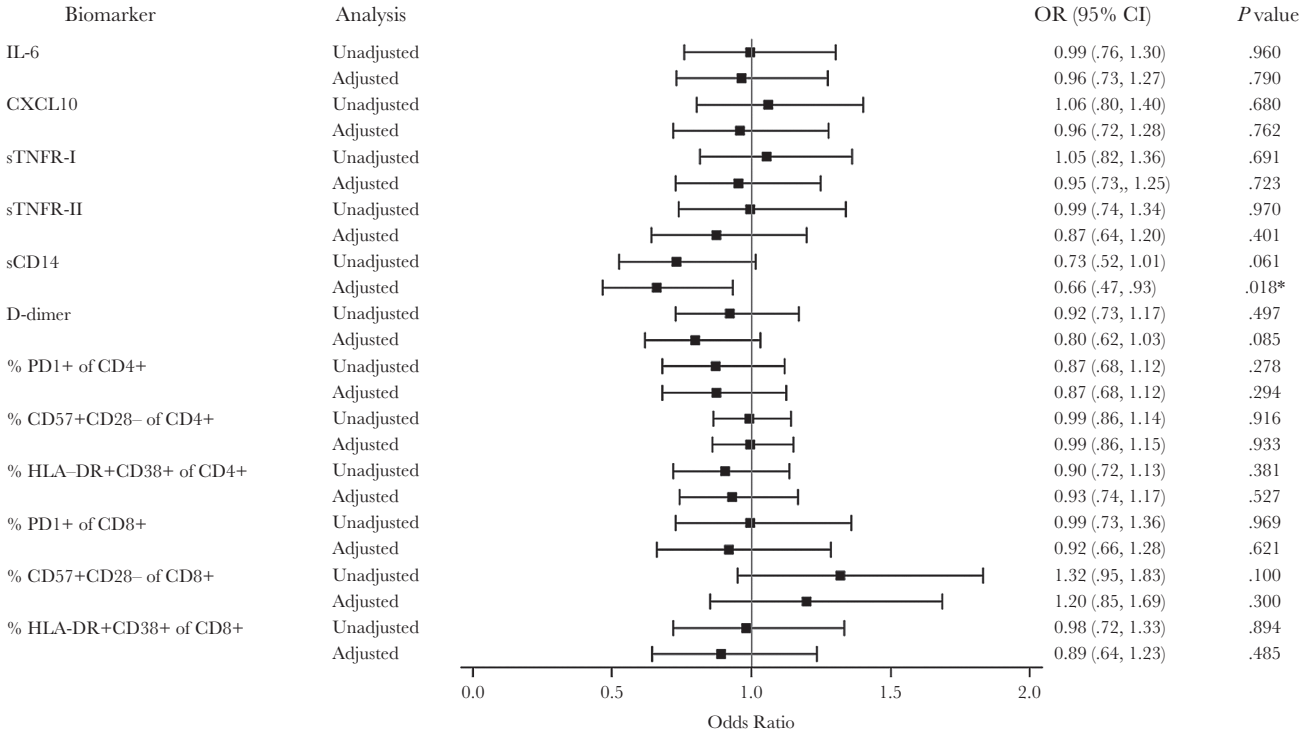

Figure 3. Odds ratios per interquartile range of having a non-AIDS-defining event for biomarker changes from pre–antiretroviral therapy to year 1. Adjusted analyses controlled for concurrent $CD4^+$ T-cell count. *P = .01 to <.05; **P < .01. Abbreviations: CI, confidence interval; CXCL10, interferon inducible protein 10; IL-6, interleukin 6; IQR, interquartile range; OR, odds ratio; sTNFR, soluble tumor necrosis factor receptor.

biomarkers except for CXCL10 as in Tenorio et al [20]. Cases, however, had significantly greater increases in all biomarkers except for CXCL10 from year 1 to the pre-event time (Figures 1A and 4). Results were similar when we analyzed soluble biomarkers in terms of above vs below the median. In sensitivity analyses adjusting for traditional risk factors including pre-ART intravenous drug use, hepatitis B/C, waist-hip ratio, diabetes, hypertension, antihypertensive or lipid-lowering agent usage, smoking and family history of MI, results remained similar to the unadjusted analyses. Results also remained similar after adjusting for initial ART regimen class.

Increases of IL-6, sTNFR-I, sTNFR-II, and D-dimer from year 1 to pre-event time were associated with development of non-AIDS-defining malignancy. Increases of IL-6 and D-dimer were associated with increased mortality. Increases of sCD14 were associated with having serious bacterial infection (Supplementary Table 4). These findings suggest that biomarkers increase during a ramp-up period prior to an event (events occurred at a median of 2.9 years after ART initiation in this study), and that this pre-event increase in biomarkers is for conditions thought to have longer preclinical periods such as malignancies.

T-Cell Activation and Senescence

Declines in the selected activation/checkpoint and senescence markers among T cells from pre-ART to year 1 were significant for both cases and controls ($P < .0001$) with the exception of $CD57^+CD28^-$ percentage among $CD8^+$ cells changing for the cases. No significant differences were detected between cases and controls in T-cell activation changes from pre-ART to year 1, or from year 1 to the pre-event time (Supplementary Figure 1*B* and Figure 1*B*).

Correlations of T-Cell Activation and Senescence Marker Changes with Pre-ART Factors

For controls, greater decreases in T-cell activation at year 1 correlated with higher pre-ART HIV RNA and older age, and lower $CD4^+$ cell count, lower CD4:CD8 ratio, and lower BMI pre-ART (Figure 2A). Similar trends were observed for the cases (Figure 2B).

Association of T-Cell Activation and Senescence Marker Changes with Non-AIDS-Defining Events

None of the T-cell biomarker changes at year 1 or at the pre-event time was predictive of a non-AIDS-defining event (Figures 3 and 4). Results were similar after adjustment for potential confounders.

DISCUSSION

HIV-infected adults on ART generally have higher levels of inflammation and T-cell activation than do age-matched controls. Single measurements of many biomarkers—particularly

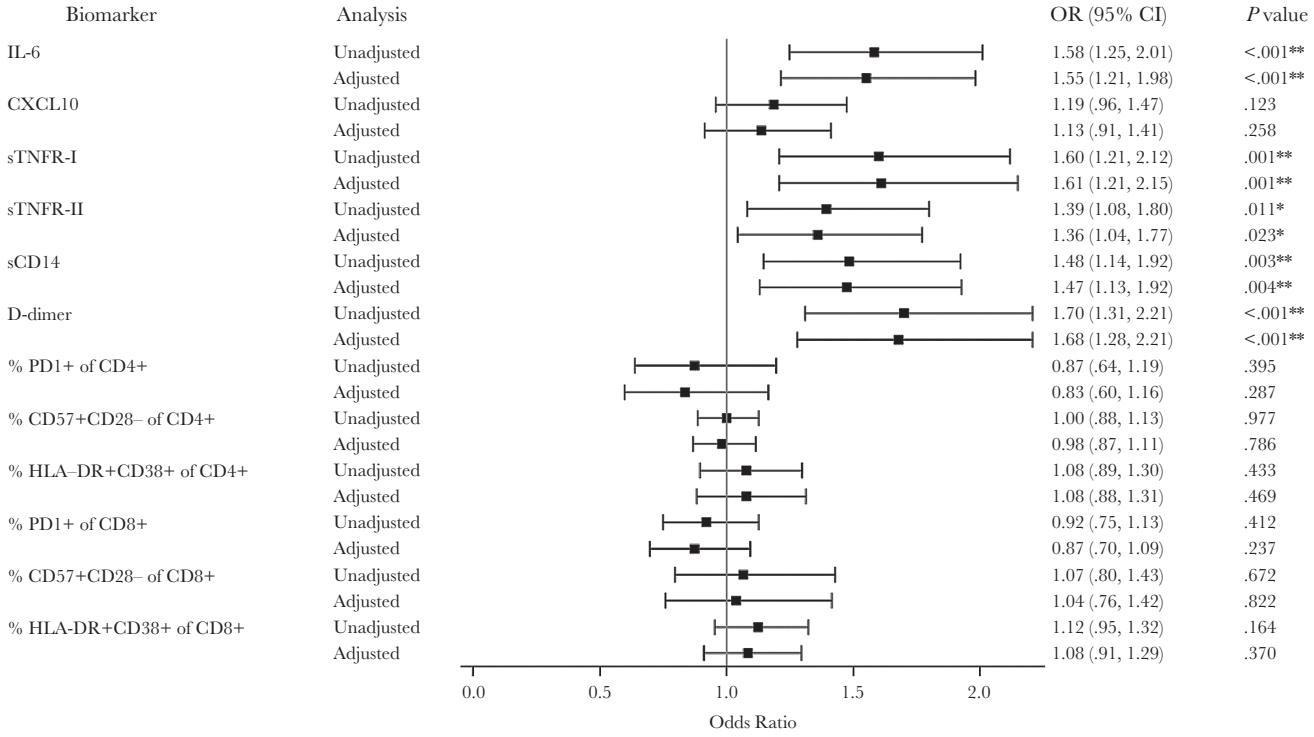

Figure 4. Odds ratios per interquartile range of having a non-AIDS-defining event for biomarker changes from year 1 to the pre-event time. Adjusted analyses controlled for concurrent CD4+ T-cell count. *P = .01 to <.05; **P < .01. Abbreviations: CXCL10, interferon inducible protein 10; IL-6, interleukin 6; IQR, interquartile range; sTNFR, soluble tumor necrosis factor receptor.

IL-6, D-dimer, sCD14, and sCD163—have been associated with a greater risk of subsequently developing a non-AIDS event or death [1, 13, 18, 20]. The degree to which changes in these biomarkers during ART or prior to an event predict these outcomes has not been well characterized. In our current study, we measured a series of well-defined markers over time in a group of HIV-infected adults who started ART and either developed a non-AIDS event or who remained event free. As expected, we found that most biomarkers decrease during the first year of ART. Biomarker trends during early ART were not predictive of subsequent disease progression; however, levels were highly predictive both pre-ART and at year 1 [20]. Many biomarkers however, increased prior to an event, indicating that changes in inflammation may suggest evolving subclinical disease or that increased inflammation may in fact contribute to disease risk. A role for the latter mechanism is suggested by the results of the main study in which both pre-ART and year 1 (on ART) inflammatory marker levels predicted the occurrence of non-AIDS-defining morbid events [20].

Other groups have assessed biomarker dynamics during ART. Wada et al [21] found that biomarkers were stable after 1 year of viral suppression; they did not, however, assess the association of biomarkers with clinical events. In a subsequent report, Wada et al [19] did assess the association of biomarker levels with mortality and reached the same conclusions as Tenorio et al [20]. Low pre-ART CD4+ cell count and high HIV RNA levels independently predicted the magnitude of biomarker decreases on ART, suggesting that these 2 markers of disease have distinct relationships to inflammation. It is thought that viral replication directly causes an inflammatory response and that ART-mediated declines in replication result in subsequent declines in inflammation. Our data are consistent with this model [26]. These relationships, however, are complex and viral suppression with ART does not normalize the inflammatory biomarkers [27]. Other factors that have been linked to the inflammatory state of HIV infection include dyslipidemia [28] (that we did not assess) and a failure of regulatory immune cell function (also not measured). Of note, lower pre-ART CD4+ cell numbers and higher pre-ART HIV RNA also correlated with higher pre-ART inflammatory marker levels. Lower CD4:CD8 ratio was also linked to the magnitude of decrease in inflammatory biomarkers, but this was entirely related to CD4+ cytopenia.

Tenorio et al showed that higher inflammatory biomarker levels both pre-ART and at year 1 were significantly associated with incident non-AIDS events [20]. This was supported by the strong positive correlations between pre-ART and year 1 biomarker levels as also observed by Gandhi and colleagues [29]. In a study performed in resource-limited countries, Balagopal et al [23] found that the highest quartile of pre-ART CD4+ T-cell activation (≥33.15) and the highest quartile of sCD14 ($\geq 2.80 \times 10^6$) predict subsequent progression to World Health Organization stage 3 or 4 disease or death within 96 weeks on

combination ART after adjusting for baseline gender, age, BMI, baseline CD4+ T-cell count, and hemoglobin. The study did not, however, confirm an association between disease progression on ART and IL-6, tumor necrosis factor–α, or CXCL10. Greater declines in biomarkers to year 1 showed no significant associations with events in our study. Cases, however, had significantly greater increases in all biomarkers from year 1 to the visit preceding the event, at a median of 2.9 years after ART initiation. Greater increases in cases after year 1 may reflect preclinical disease processes [5]. In our study, we observed increases when considering clinical events with longer preclinical periods such as non-AIDS-defining malignancies [20, 30].

T-cell activation/senescence/checkpoint biomarker changes were not predictive of a clinical event in our analyses. This result is consistent with other studies that have shown no association between T-cell activation and clinical outcomes in patients on suppressive ART [15, 20, 31, 32], although some studies have shown associations between CD8+ T-cell activation markers and clinical outcomes [31, 33] and association of baseline CD4+ T-cell activation with outcomes among patients on combination ART [23, 34, 35].

Strengths and limitations of our study include the case-control design that makes it impossible to generate direct estimates of incident morbidity risk related to each of the risk factors evaluated. Furthermore, the matching strategy used in this study is a means of providing a more efficient stratified analysis rather than a direct means of preventing confounding, allowing for the possibility of residual confounding. The population of our study started ART at a median CD4 count of 215 cells/μL. This is a limitation given that CD4 count at initiation of ART has been increasing [36]. Prospective studies will be needed to address the limitations of our study and to corroborate our results that, to our knowledge, are novel, linking temporal biomarker changes on ART to the risk of clinical events.

In conclusion, our results underscore the importance of earlier initiation of ART in HIV-infected adults as low pre-ART CD4+ cell count is linked to higher inflammatory marker levels even after a year of ART. Our additional analysis and the original analysis reported by Tenorio et al [20] support the utility of measuring biomarker levels as well as changes of biomarker levels as predictors of non-AIDS events. Our results combined with those from Wada et al [21] and Balagopal et al [23] support measuring at year 1 or later of ART vs repeated earlier measuring. Individuals who experience clinical events during ART tend to have increases in inflammation biomarkers after ART-mediated viral suppression is achieved. This has implications for the interpretation of studies when biomarkers are measured closer to the occurrence of clinical events. It appears that the pre-ART set point and the levels of biomarkers after ART administration reflect an ongoing pathophysiologic process that is linked to risk for non-AIDS-defining events in the ART era. Interventions targeting inflammation may alter the risk for morbid events in HIV-infected persons well controlled on ART.

Supplementary Data

Supplementary materials are available at *The Journal of 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

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