

# Plasma biomarkers of immune regulation and senescence are associated with incident skeletal-related events in people with HIV: a case-cohort study

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## Title page

**Title:** Plasma biomarkers of immune regulation and senescence are associated with incident skeletal-related events in people with HIV: a case-cohort study

**Running Title:** Immune and senescence biomarkers and SREs in HIV

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## Abstract

**Background:** Persistent immune dysregulation in people with HIV (PWH) on antiretroviral therapy (ART) contributes to an increased risk of skeletal-related events (SREs), serving as a critical paradigm for understanding accelerated aging and age-related diseases. We investigated the association between plasma biomarkers of immune regulation and senescence with incident SRE risk and evaluated potential effect modification by sex.

**Methods:** In a case-cohort study nested within the Spanish CoRIS cohort (65 incident SRE cases; 252 subcohort, including 5 overlapping cases), we quantified 24 baseline biomarkers of immune checkpoints and senescence-associated secretory phenotype factors. We estimated adjusted hazard ratios (aHR) using Borgan II-weighted cause-specific Cox regression models adjusted for clinical confounders (age, region of origin, prior AIDS diagnosis, CD4+ T-cell count, coinfections, and lifestyle factors). Sex-biomarker interactions were assessed as exploratory endpoints.

**Results:** Nineteen biomarkers were independently associated with increased SRE risk ( $p < 0.05$  &  $q < 0.10$ ). Angiogenic factors (VEGF-A; aHR = 1.94; 95% CI, 1.40–2.68), immune checkpoints (PD-L2; aHR = 1.95; 95% CI, 1.09–3.49), and tissue remodeling markers (MMP-1; aHR = 1.91; 95% CI, 1.08–3.39) displayed independent associations, alongside consistent signals for TIM-3, PD-L1, and IL-8. Pairwise correlation analysis demonstrated clustering between checkpoints and inflammatory cytokines. Exploratory interaction analysis indicated that sex modified these associations; signals for seven

markers (e.g., IL-1 $\alpha$ , VEGF-A) were stronger in females, although the limited number of events warrants caution.

**Conclusions:** Circulating biomarkers of immune exhaustion, inflammation, and senescence are independently associated with incident SREs in PWH on long-term ART. Our findings indicate a systemic pro-inflammatory signature and suggest that females exhibit higher sensitivity to inflammatory damage.

**Keywords:** HIV, skeletal-related events, biomarkers, cellular senescence, ageing, immune checkpoints, sex differences

## Introduction

Combination antiretroviral therapy (ART) has transformed human immunodeficiency virus (HIV) infection from a fatal illness into a chronic condition, significantly extending the lifespan of people with HIV (PWH) [1]. Consequently, bone pathologies, encompassing osteoporosis, osteonecrosis, and fragility fractures, termed skeletal-related events (SREs), have emerged as a critical cause of morbidity [2-4]. Notably, SREs occur approximately a decade earlier in PWH compared to the general population [2-4], representing a major challenge in long-term HIV management.

Beyond traditional risk factors [5] and specific ART toxicities [6], persistent immune dysregulation appears to play a central role in HIV-associated bone loss. Even during long-term viral suppression, a state of chronic immune activation and systemic inflammation, known as "inflammaging" [7], persists. This chronic, low-grade inflammatory milieu disrupts bone remodeling,

leading to net bone loss by promoting the differentiation of bone-resorbing osteoclasts while impairing bone-forming osteoblasts [8].

While classic cytokines are known drivers of this process, emerging evidence in the field of osteoimmunology implicates T-cell exhaustion markers (immune checkpoints [ICPs] like PD-1/PD-L1) and SASP factors as critical mediators of tissue ageing and damage. However, while elevated inflammatory markers are linked to mortality in PWH [9], the specific contribution of these immune regulation and senescence biomarkers to incident fracture risk remains largely unexplored.

Furthermore, biological sex may modulate these pathways. Females with HIV face a particularly elevated risk of bone disease, often experiencing more severe decline than males [10]. This vulnerability may be attributed to distinct immunological profiles, as females typically exhibit higher T-cell activation and inflammation than males at similar viral loads [11]. We hypothesize that this sexually dimorphic inflammatory state may render the female skeleton disproportionately sensitive to immune-mediated damage, potentially accelerating bone deterioration independently of traditional ageing mechanisms [5, 10, 12]. Therefore, assessing whether sex modifies the association between these biomarkers and SRE risk is critical for refining risk stratification strategies.

To address these knowledge gaps, we utilized a cohort of PWH on long-term suppressive ART to evaluate the association between a panel of 24 plasma biomarkers (reflecting inflammation, ICPs, and cellular senescence) and the

risk of incident SREs. Additionally, we explored whether these associations are modified by sex, aiming to uncover distinct biological risk profiles contributing to bone disease in PWH.

## **Materials and methods**

### **Study design and participants**

This case-cohort study is nested within the Cohort of the Spanish HIV Research Network (CoRIS), a prospective, open, multicenter cohort of adults with HIV who were ART-naïve at enrollment (details in **Additional File 1**).

Data collection followed the HIV Cohorts Data Exchange Protocol [13] with rigorous annual quality control monitoring [14].

The source population comprised 19,352 participants enrolled in CoRIS as of November 30, 2022. Inclusion criteria for this analysis were: i) age  $\geq 18$  years; ii) receiving stable ART regimen; iii) availability of a cryopreserved plasma sample collected at least 48 weeks after ART initiation (defined as baseline); and iv) suppressed HIV viral load ( $< 50$  copies/mL) at baseline.

The primary outcome was incident SREs, defined as a composite of fragility fractures and osteonecrosis occurring at least three months after baseline.

Following the World Health Organization criteria, fragility fractures were defined as those resulting from low-energy trauma, such as a fall from standing height or less. Fractures resulting from high-impact trauma (e.g., motor vehicle accidents, falls from greater than standing height) and fractures at sites not typically associated with bone fragility (e.g., skull,

facial, or digital fractures) were excluded. Participants with a documented history of SRE prior to baseline were also excluded.

In accordance with the case-cohort design, a comparison subcohort was randomly selected from the eligible source population at baseline, independent of future SRE status. To mitigate protopathic bias, a three-month washout period was applied; therefore, follow-up accrued from three months post-baseline until SRE diagnosis, death, last recorded visit, or administrative censoring (November 30, 2022), whichever occurred first. These criteria yielded a final study population of 312 PWH, comprising 65 incident SRE cases and a random subcohort of 252 individuals (including an overlap of 5 SRE cases).

The study protocol adhered to the ethical principles of the Declaration of Helsinki, was approved by the Research Ethics Committee of the Instituto de Salud Carlos III (CEI PI 87\_2022), and followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidelines (**Additional File 2**).

## **Clinical data and samples**

Demographic, clinical, and virological data were extracted from the CoRIS database. Variables included demographics (age, sex, region of origin, education level), HIV-specific factors (transmission route, prior AIDS-defining events, nadir and baseline CD4+ T-cell counts), and key comorbidities and lifestyle factors (including smoking status and HCV/HBV coinfection). Plasma

samples were processed and stored at -80°C at the Spanish HIV BioBank until analysis.

## **Multiplex immunoassays**

Plasma levels of 24 biomarkers related to immune regulation and the senescence-associated secretory phenotype (SASP) were quantified using a Luminex 200TM analyzer (Luminex Corporation, Austin, TX, United States) according to the manufacturer's protocols. The biomarker panel was categorized as follows: 1) ICP molecules: Cluster of differentiation (CD)28, CD80, programmed death 1 (PD-1), PD-ligand 1 (PD-L1), PD-L2, lymphocyte-activation gene 3 (LAG-3), and T-cell immunoglobulin and mucin domain 3 (TIM-3). 2) SASP factors: CD36 Interleukin 1-alpha (IL-1 $\alpha$ ), IL-8 (CXCL8), monocyte chemoattractant protein 1 (MCP-1), MCP-2, tumor necrosis factor beta (TNF- $\beta$ ), tumor necrosis factor receptor I (TNF-RI), TNF-RII, interferon gamma-induced protein 10 (IP-10), stromal cell-derived factor 1-alpha (SDF-1 $\alpha$ ), growth differentiation factor 15 (GDF-15), plasminogen activator inhibitor 1 (PAI-1), epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor A (VEGF-A), and metalloproteinase 1 (MMP-1). As detailed previously [15, 16], analyses were performed using raw fluorescence intensity values expressed in arbitrary units (a.u.). The use of fluorescence intensity values instead of interpolated concentrations was a predefined methodological choice to maximize statistical power and avoid the data loss and bias

associated with values falling outside the standard curve limits, as previously recommended for multiplex assays [15, 16].

To minimize batch effects and ensure comparability across runs, all samples were randomized across plates, ensuring non-differential distribution of SRE cases and the subcohort. Subsequently, we performed inter-plate normalization using Quantile Normalization. This procedure aligns the distribution of fluorescence intensities across all plates, mitigating technical variance, as verified by diagnostic distribution plots and PCA (**Additional File 3**). All assays were performed in a single laboratory using the same lot of reagents.

### **Statistical analysis**

Continuous variables were compared using the Mann-Whitney U test (reported as median and interquartile range [IQR]), while categorical variables were compared using the Chi-square or Fisher's exact test (reported as absolute counts and percentages).

To estimate the risk of incident SREs associated with biomarker levels, we used a Borgan II-weighted cause-specific Cox proportional hazards regression model [17] with robust standard errors [18]. In this model, age was used as the time scale, and death was treated as a competing event (censored at the time of death). Prior to survival analysis, raw biomarker levels were winsorized (at the 10th and 95th percentiles) to mitigate the impact of outliers, log2-transformed, and subjected to inter-plate Quantile Normalization as described above. These transformations were specifically

applied to exclude the non-linear asymptotes of the assay's sigmoidal curve, restricting the analysis to the pseudo-linear dynamic range. This ensures a monotonic relationship and satisfies the log-linear assumption of the Cox proportional hazards models. The proportional hazards assumption was verified for all variables using Schoenfeld residuals. While the assumption was met for the majority of variables, PD-1 and MMP-1 exhibited time-varying effects ( $p < 0.05$ ); for these biomarkers, the reported estimates represent average hazard ratios over the time scale (age). Multivariable models were adjusted for the following potential confounders: sex, region of origin, education, transmission route, prior AIDS diagnosis, CD4+ T cell count, antiretroviral therapy regimen, and coinfection with HCV or HBV. All covariates were treated as fixed baseline variables to evaluate the longitudinal association of the biomarkers at the time of sampling. Additionally, inter-marker relationships were assessed via pairwise Spearman's rank correlation tests. To evaluate the biomarkers collectively and account for potential multicollinearity, we conducted a subcohort-projected Principal Component Cox (PC-Cox) regression analysis. To prevent selection bias inherent in the case-cohort design (where SRE cases are overrepresented), Principal Component Analysis (PCA) was performed strictly on the random subcohort samples ( $n = 252$ ) to establish an unbiased baseline covariance structure. The resulting eigenvector loadings were then projected onto the entire study population ( $n = 312$ ) to calculate population-representative PC scores. The first Principal Component (PC1), representing a

coordinated signature of immune checkpoints and SASP factors, was entered as an independent variable in our Borgan II-weighted cause-specific Cox model, adjusting for the same clinical confounders.

Finally, to assess effect modification by sex, an interaction term between each biomarker and sex was included in the Cox models. P-values for interaction were derived from a Likelihood Ratio Test (LRT).

For descriptive comparisons and correlation analyses, statistical significance was defined as a two-sided p-value  $< 0.050$ . In the longitudinal biomarker analysis, p-values were adjusted using the False Discovery Rate (FDR) method (q-values) [19]. We defined statistical relevance as a  $p < 0.05$  &  $q < 0.10$ . While this threshold is more permissive than confirmatory standards, it was selected to minimize type II errors, maximizing sensitivity to detect signals in this exploratory analysis [20-22]. Regarding effect modification, given the inherent low statistical power of interaction tests in sub-analyses [23], we considered raw p-values (Likelihood ratio test (LRT)  $p < 0.05$ ) as indicative of potential biological signals warranting further investigation, without applying FDR correction to these specific terms.

All statistical analyses were performed using R software (version 4.3.1; R Foundation for Statistical Computing, Vienna, Austria).

## Results

### Participants' characteristics at baseline

**Table 1** summarizes the baseline characteristics of the 312 PWH. The cohort was predominantly male (85.6%; median age 40 years) and mostly of

Spanish origin (60.9%). Sexual transmission was the main route of infection (65.4% MSM). A prior AIDS diagnosis was reported by 13.1% of participants, and the median CD4+ T-cell count was 615 cells/mm<sup>3</sup>. The most common antiretroviral regimen combined nucleoside reverse transcriptase inhibitors (NRTIs) with non-nucleoside reverse transcriptase inhibitors (NNRTIs) (38.8%), followed by regimens containing NRTIs and integrase strand transfer inhibitors (INSTI) (29.2%).

While most demographic and clinical parameters were comparable between SRE cases and the subcohort (**Table 1**), cases were significantly older (median 45 vs. 38 years,  $p<0.001$ ) and exhibited distinct ART exposure profiles. Specifically, NNRTI-based therapies were overrepresented in the SRE group compared to the subcohort (61.5% vs. 32.8%,  $p<0.001$ ). The median follow-up time for the entire study population was 4.62 years (IQR 2.24-7.23).

### **Characterization of incident skeletal-related events**

Of the 65 incident SREs, 60 (92.3%) were fractures and 5 (7.7%) were osteonecrosis cases. The distribution of fracture sites was heterogeneous, predominantly affecting the lower leg ( $n=29$ ; 44.6%) and distal forearm ( $n=15$ ; 23.1%). Less common events included fractures of the upper arm ( $n=4$ ), hip ( $n=3$ ), and ribs ( $n=3$ ), with sporadic cases involving the femoral shaft, shoulder, and vertebral column.

## Baseline biomarker levels

Baseline plasma levels of the 24 biomarkers are detailed in **Table 2**. In unadjusted cross-sectional comparisons, participants who subsequently developed SREs exhibited elevated levels of six biomarkers ( $p < 0.05$ ): VEGF-A, PD-1, IL-1 $\alpha$ , SDF-1 $\alpha$ , TNF-RII, and TIM-3. While only VEGF-A retained statistical significance after FDR correction in this unadjusted analysis ( $q < 0.100$ ), this comparison does not account for time-to-event dynamics or potential confounders. Therefore, to rigorously assess longitudinal association, we prioritized the multivariable survival analysis.

## Association between plasma biomarkers and incident SRE risk

Adjusted associations between baseline plasma biomarkers and SRE risk are detailed in **Figure 1** (full description in **Additional File 4**). Multivariable survival analysis revealed 17 biomarkers independently associated with SRE risk ( $p < 0.05$  &  $q < 0.10$ ).

Thirteen biomarkers demonstrated independent associations with an adjusted hazard ratio (aHR)  $> 1.50$ . These included markers of immune exhaustion, angiogenesis, and tissue remodeling: PD-L2 (aHR = 1.95 [95% CI: 1.09–3.49]), VEGF-A (1.94 [1.40–2.68]), and MMP-1 (1.91 [1.08–3.39]).

Significant associations were also observed for TIM-3, PD-L1, IL-8, IL-1 $\alpha$ , MCP-1, HGF, TNF-RII, CD28, SDF-1 $\alpha$ , and GDF-15 (all aHRs  $> 1.50$ ).

Additionally, FGF-2, PD-1, EGF, IP-10, LAG-3, and MCP-2 exhibited moderate but statistically significant associations (aHR range: 1.29–1.45).

Analysis of Schoenfeld residuals revealed that PD-1 and MMP-1 exhibited time-varying effects ( $p < 0.05$ ), indicating that their association with incident SREs fluctuates across the age time-scale. Consequently, their reported estimates represent average hazard ratios over the follow-up period. To capture the collective contribution of the biomarker panel, we evaluated the coordinated systemic signature derived from the subcohort-projected PCA. The first Principal Component (PC1) captured 62.9% of the total proteomic variance within the subcohort. In the unadjusted Borgan II-weighted cause-specific Cox regression, this projected PC1 systemic signature was significantly associated with SRE risk (HR = 1.08 [95% CI: 1.01–1.15];  $p = 0.032$ ). In the fully adjusted multivariable model, the association remained significant, with the systemic biomarker signature (PC1) independently associated with an increased hazard of SREs (aHR = 1.11 [95% CI: 1.02–1.21];  $p = 0.021$ ). The individual loadings for PC1 are detailed in **Additional File 5**.

### **Baseline biomarker correlations**

Pairwise Spearman's correlation analysis revealed a positive correlation among the 24 plasma biomarkers (Figure 2). Restricting the analysis to strong correlations (Spearman's  $\rho > 0.50$ ; all  $q < 0.100$ ) highlighted significant crosstalk between functional categories. Notably, ICPs (specifically PD-1, PD-L1, and TIM-3) displayed strong positive correlations with SASP biomarkers. This association extended to both proinflammatory cytokines (e.g., IL-1 $\alpha$ , IL-8, TNF-RII) and tissue remodeling factors (e.g., MMP-1, VEGF-A,

HGF), which clustered tightly with ICPs. These findings suggest a synergistic systemic inflammatory milieu where immune exhaustion and senescence-driven secretory pathways are co-activated.

### **Modification of the biomarker-SRE risk association by sex**

Baseline biomarker profiles were comparable between sexes in both study groups (SRE cases and subcohort), with no statistically significant differences remaining after FDR correction (**Additional File 6**; all  $q > 0.100$ ). However, our exploratory analysis suggested sex as a potential effect modifier (**Additional File 7**). By prioritizing raw p-values (LRT  $p < 0.05$ ) to detect signals despite the limited power of interaction tests [23], we identified potential interactions for PD-1, IL-1 $\alpha$ , TNF-RII, SDF-1 $\alpha$ , GDF-15, HGF, and VEGF-A. For all seven analytes, the association with incident SREs was more pronounced in females, although these estimates must be interpreted with caution due to the limited number of events in this subgroup.

Two interaction patterns emerged (**Figure 3**): associations appeared to be either restricted to females (TNF-RII, SDF-1 $\alpha$ , GDF-15, HGF; female aHRs 2.36–4.92 vs. non-significant male aHRs) or present in both but potentially amplified in females (PD-1, IL-1 $\alpha$ , VEGF-A). For instance, the estimated risk associated with IL-1 $\alpha$  was nearly double in women (aHR = 2.97 [95% CI: 1.52–5.82]) compared to men (aHR = 1.51 [95% CI: 1.09–2.08]).

Regarding age, SRE incidence did not differ significantly between women aged <50 and >50 years (27.3% vs. 25.0%;  $p=0.999$ ). However, given the small number of events in the female subgroup, this analysis was

underpowered to detect differences based on age-defined menopausal status.

## **Discussion**

Despite effective ART, SREs remain a significant cause of morbidity in PWH [8]. In this case-cohort study, we identified a distinct plasma proteomic signature, comprising ICPs and SASP factors, that is independently associated with the risk of incident SREs. Notably, our exploratory analysis suggests that sex may act as an effect modifier of these associations, with inflammatory drivers exhibiting stronger associations in females than in males. These findings suggest that the pathophysiological mechanisms underlying HIV-associated bone loss could be sexually dimorphic, challenging uniform risk stratification strategies.

### **Systemic inflammatory signature and bone remodeling**

Even with effective viral suppression, PWH experience 'inflammaging' [7], a chronic inflammatory state that drives non-AIDS comorbidities, including bone disease [8]. Persistent viral reservoirs likely exacerbate this systemic dysregulation; notably, viral proteins such as HIV-1 Tat can directly promote osteoclastogenesis via receptor activator of nuclear factor kappa-B ligand (RANKL) induction despite ART [24]. Our findings align with this model: participants who subsequently developed SREs exhibited a coordinated upregulation of inflammatory signals at baseline. Rather than isolated abnormalities, we identified a biomarker profile involving the co-expression of ICPs (CD28, PD-1, PD-L1, PD-L2, and TIM-3) and SASP factors. This

signature spans established pathways of chemotaxis, angiogenesis, and tissue remodeling, reflecting a systemic pro-inflammatory milieu anteceding the clinical event. This is supported by our PC-Cox analysis, where a signature representing 62.9% of the total biomarker variance was independently associated with a significantly higher hazard of clinical fracture, suggesting that the coordinated co-expression of these pathways is associated with bone fragility.

The simultaneous elevation of immune checkpoints (CD28, PD-1, PD-L2, TIM-3) suggests a persistent state of T-cell differentiation and exhaustion. In the context of bone biology, this immunophenotype is relevant, as differentiated T-cells are known sources of RANKL [25], the primary mediator of osteoclastogenesis. The observed association with SASP components further supports the presence of a pro-resorptive environment; cytokines such as IL-1 $\alpha$ , IL-8, and TNF-RII have been shown in experimental models to potentiate osteoclast activity [26] while inhibiting osteoblast function [27]. Thus, the co-elevation of these markers may reflect a systemic signaling profile that favors the uncoupling of bone remodeling.

Beyond systemic activation, our data suggest a mechanism of enhanced immune cell trafficking to the bone niche. The association with MCP-1 aligns with its function as a potent chemoattractant for monocytes [28], potentially expanding the local pool of osteoclast precursors [29]. This effect is likely synergistic with IP-10, which recruits activated Th1 cells to inflamed tissues [30]. Furthermore, the upregulation of SDF-1 $\alpha$ , typically a homing signal for

osteoblasts, might reflect a compensatory but dysregulated state of high bone turnover [31].

Concurrently, the data reveal a dysregulated tissue remodeling environment, driven primarily by markers of angiogenesis and matrix degradation. The strong association of VEGF-A and MMP-1 with incident SREs aligns with their biological roles: VEGF-A has been linked to osteoclast recruitment and survival [32], while MMP-1 mediates collagen breakdown [33]. Against this backdrop, the concurrent elevation of growth factors (EGF, FGF-2, HGF) likely represents a compensatory but ultimately failed anabolic response to ongoing damage [34, 35]. In a chronic inflammatory milieu, marked by the stress-responsive cytokine GDF-15 [36], these repair signals may be subverted, fueling pathological turnover rather than physiological healing. SASP biomarkers are also associated with metabolic disturbances, such as obesity and insulin resistance, which are prevalent in PWH and can negatively impact bone remodeling. This 'metabolic-bone axis' suggests that systemic senescence may act as a common pathway linking metabolic comorbidities with increased skeletal fragility.

Collectively, these data support the hypothesis that a distinct inflammatory endotype, driven by immune exhaustion and aberrant tissue remodeling, is associated with bone fragility in PWH. While prior studies have linked isolated inflammatory markers to bone disease [37, 38], our multiparametric approach provides a methodological advance. By simultaneously evaluating 24 analytes, we move beyond the study of isolated cytokines to identify a

coordinated biological signature of systemic dysregulation that precedes clinical fracture.

### **Sex-specific effect modification**

Beyond the general associations, a notable exploratory finding was the potential effect modification by sex. This observation aligns with clinical reports that women with HIV experience an earlier and more severe decline in bone health compared to men [10]. While previous research has linked inflammation to SRE risk [39] and documented sex-based immunological differences [40], our data suggest that these factors may interact: the association between key biomarkers (including PD-1, IL-1 $\alpha$ , VEGF-A, and GDF-15) and adverse bone outcomes was significantly stronger in females. This pattern is consistent with evidence from other HIV-associated comorbidities [12, 41]. A plausible biological explanation involves the interplay between the typically higher immune response mounted by females and hormonal status [41, 42]. In the context of chronic HIV stimulation, this heightened immune reactivity might render the female skeleton disproportionately sensitive to inflammatory damage. While this process could be exacerbated by the loss of bone-protective estrogen during the menopausal transition [43], our study was underpowered to assess the interplay between chronic immune dysregulation and menopausal status. The limited sample size of the female subgroup precludes conclusions regarding whether these inflammatory drivers act independently of, or synergistically with, estrogen loss.

These findings suggest that a universal biomarker approach for estimating bone disease risk in PWH may be suboptimal. Instead, clinical risk stratification might benefit from sex-specific strategies, potentially incorporating distinct biomarker panels or calibrated risk thresholds for shared markers.

## **Clinical Implications**

It is worth noting that a significant proportion of fractures occurred in the lower leg. While these are not always included in the traditional fragility fractures category—which typically focuses on the hip, spine, humerus, and wrist—all events in our study met the strict criteria for low-energy trauma. In relatively young populations of PWH (median age 40 years), low-energy fractures at non-traditional sites are increasingly recognized as early clinical indicators of compromised bone quality and are established risk factors for future major skeletal events.

## **Strengths and limitations**

Our study has limitations. First, the limited sample size of female participants constrained statistical power, resulting in wide confidence intervals for sex-stratified estimates. Furthermore, the sparsity of events in the female subgroup may lead to unstable and inflated effect sizes, meaning that the exact magnitude of the hazard ratios reported for females should be viewed as preliminary. While the detection of significant interactions suggests potential biological differences, these findings should be interpreted as hypothesis-generating and require validation in larger cohorts. Second,

certain traditional risk factors for bone disease, such as menopausal status, bone mineral density, renal function, body mass index, insulin resistance, alcohol consumption, and corticosteroid use, were not systematically available for most participants at the study baseline within the CoRIS database, leaving the potential for residual confounding. However, we adjusted for a set of key demographic and HIV-specific variables. Given the number of incident SREs, the inclusion of additional covariates would have risked overfitting the models, compromising the stability of the hazard ratio estimates. Third, biomarkers were assessed at a single time point, precluding the analysis of inflammatory trajectories. Similarly, clinical covariates were adjusted for at baseline and not treated as time-varying variables, which may not capture the impact of subsequent clinical changes during follow-up. Fourth, using arbitrary units (fluorescence intensity) precludes the direct comparison of hazard ratio magnitudes across different biomarkers. Because antibody affinity is analyte-specific, a uniform increase in log<sub>2</sub>-transformed signal does not correspond to an equivalent change in absolute concentration. Consequently, the reported estimates identify independent associations with SRE risk but cannot be used to rank the relative biological potency of the biomarkers. Furthermore, due to the limited number of events and the high multicollinearity between certain biomarkers, we were unable to include multiple biomarkers in a single multivariable model. Consequently, we cannot definitively determine whether the observed associations for certain markers are independent of other highly correlated proteins. Our

findings should therefore be interpreted as a coordinated proteomic pattern rather than a collection of mutually exclusive independent factors.

Despite these limitations, the study possesses several key strengths. First, it is nested within a large, well-characterized cohort with long-term follow-up, where the case-cohort design provided an efficient and statistically valid framework for analyzing rare outcomes. Second, we performed a multiparametric analysis of 24 biomarkers, enabling a systematic evaluation of immune regulation rather than relying on single markers. A strength of our study is the implementation of a subcohort-projected PCA-Cox regression to evaluate the biomarkers collectively. By restricting the PCA to the random subcohort, we obtained unbiased, population-representative principal components, preventing the selection bias that typically arises when performing standard dimensionality reduction on case-cohort datasets with overrepresented cases. Additionally, a sensitivity analysis was performed by comparing the univariable Cox regression results using winsorized versus unwinsorized log<sub>2</sub>-transformed biomarker levels. The results showed consistency in both effect sizes (HR) and significance levels. Furthermore, no significant differences in coefficients were detected (Wald test  $p > 0.05$  for all), demonstrating that the winsorization process stabilized the models against technical outlier-driven attenuation without introducing statistical bias. Third, our statistical approach minimized bias by using age as the time scale in the Cox models, which minimizes bias associated with time-on-study [44]. This multivariable survival analysis unveiled longitudinal associations

that were not apparent in unadjusted cross-sectional comparisons. By incorporating the time-to-event dimension and rigorously adjusting for confounders, this approach captures the longitudinal association of these biomarkers more accurately than simple rank-based tests, demonstrating that their impact lies in the hazard rate over time. Finally, the treatment of death as a competing event prevented the overestimation of risk often observed in standard survival analyses of ageing populations [45].

## Conclusions

In conclusion, a coordinated plasma signature involving immune exhaustion, inflammation, and senescence markers is independently associated with incident SREs in PWH on long-term ART. These findings suggest that immune exhaustion and senescence-driven inflammation may contribute to bone fragility. Furthermore, our exploratory analysis suggests that sex may modify this relationship, with inflammatory drivers exhibiting stronger associations in females. Collectively, these results support moving beyond traditional clinical risk factors to evaluate sex-specific risk stratification strategies that integrate these biomarkers.

## List of abbreviations

**aHR**, adjusted hazard ratio

**AIDS**, acquired immune deficiency syndrome

**ART**, antiretroviral therapy

**a.u.**, arbitrary units

**CD**, cluster of differentiation

**CI**, confidence interval

**CoRIS**, Cohort of the Spanish HIV Research Network

**EGF**, epidermal growth factor

**FDR**, false discovery rate

**FGF-2**, fibroblast growth factor 2

**GDF-15**, growth differentiation factor 15

**HBV**, hepatitis B virus

**HCV**, hepatitis C virus

**HGF**, hepatocyte growth factor

**HIV**, human immunodeficiency virus

**ICPs**, immune checkpoints

**IL**, interleukin

**InSTI**, integrase strand transfer inhibitor

**IP-10**, interferon gamma-induced protein 10

**IQR**, interquartile range

**LAG-3**, lymphocyte-activation gene 3

**LRT**, Likelihood ratio test

**MCP-1**, monocyte chemoattractant protein 1

**MCP-2**, monocyte chemoattractant protein 2

**MMP-1**, matrix metalloproteinase 1

**MSM**, men who have sex with men

**NNRTI**, non-nucleoside reverse transcriptase inhibitor

**NRTI**, nucleoside reverse transcriptase inhibitor

**PAI-1**, plasminogen activator inhibitor 1

**PD-1**, programmed cell death protein 1

**PD-L1**, programmed death-ligand 1

**PD-L2**, programmed death-ligand 2

**PWH**, people with HIV

**RANKL**, receptor activator of nuclear factor kappa-B ligand

**SASP**, senescence-associated secretory phenotype

**SDF-1 $\alpha$** , stromal cell-derived factor 1-alpha

**SREs**, skeletal-related events

**STROBE**, Strengthening the Reporting of Observational Studies in Epidemiology

**TIM-3**, T-cell immunoglobulin and mucin-domain containing-3

**TNF- $\beta$** , tumor necrosis factor beta

**TNF-RI**, tumor necrosis factor receptor I

**TNF-RII**, tumor necrosis factor receptor II

**VEGF-A**, vascular endothelial growth factor A

**WHO**, World Health Organization

## **Declarations**

### **Ethics approval and consent to participate**

The study was approved by the Research Ethics Committee of the Institute of Health Carlos III (CEI PI 87\_2022) and was conducted following the Declaration of Helsinki.

Each CoRIS participant provided his or her written informed consent prior to enrolling in this study. The CoRIS cohort was approved by the Research Ethic Committee of the Gregorio Marañón Hospital.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets are available from the corresponding author on reasonable request.

### **Competing interest**

The authors declare they have no competing interest.

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## **Authors' contributions**

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Investigation: CPM and RME.

Formal analysis: RME.

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Writing – Review & Editing: MR, IM, MAJS and SR.

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## Figure legends

### **Figure 1. Association between baseline plasma biomarker levels and the risk of incident skeletal-related events (SREs).**

**Statistics:** The forest plot displays adjusted Hazard Ratios (aHR) and 95% Confidence Intervals (95% CI) for the association between log2-transformed baseline biomarker levels and SRE risk. Estimates were derived from Borgan II-weighted cause-specific Cox proportional hazards models, adjusted for relevant confounders as detailed in the Methods section. The vertical dotted line indicates an aHR of 1 (no association). P-values and q-values (False Discovery Rate adjusted) are shown on the right; statistically significant associations ( $p < 0.05$  &  $q < 0.10$ ) are shown in bold. Analysis of Schoenfeld residuals indicated time-varying effects for PD-1 and MMP-1 ( $p < 0.05$ ); consequently, their reported estimates represent average hazard ratios over the follow-up period.

**Abbreviations:** aHR, adjusted hazard ratio; CI, confidence interval; FGF, fibroblast growth factor; GDF, growth differentiation factor; HGF, hepatocyte growth factor; ICPs, immune checkpoints; IL, interleukin; IP-10, interferon gamma-induced protein 10; LAG-3, lymphocyte-activation gene 3; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; PAI-1, plasminogen activator inhibitor 1; PD-1, programmed cell death protein 1; PD-L, programmed death-ligand; SASP, senescence-associated secretory phenotype; SDF-1 $\alpha$ , stromal cell-derived factor 1-alpha; TIM-3, T-cell immunoglobulin and mucin-domain containing-3; TNF, tumor necrosis factor;

TNF-R, tumor necrosis factor receptor; VEGF-A, vascular endothelial growth factor A.

**Figure 2. Inter-marker correlation profile at baseline.**

**Statistics:** The heatmap displays pairwise Spearman's rank correlation coefficients ( $\rho$ ) between the 24 plasma biomarkers. The color scale indicates the strength of the association, with darker orange representing stronger positive correlations. All displayed correlations reached statistical significance ( $p < 0.05$ ).

**Abbreviations:**  $\rho$ , Spearman's rank correlation coefficient; CD, cluster of differentiation; EGF, epidermal growth factor; FGF, fibroblast growth factor; GDF, growth differentiation factor; HGF, hepatocyte growth factor; IL, interleukin; IP, interferon gamma-induced protein; LAG, lymphocyte activation gene; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; PAI, Plasminogen Activator Inhibitor; PD-1, programmed cell death protein; PD-L, programmed death-ligand; SDF, stromal cell-derived factor; TIM, T-cell immunoglobulin and mucin domain; TNF, tumour necrosis factor; TNF-R, tumour necrosis factor receptor; VEGF, vascular endothelial growth factor.

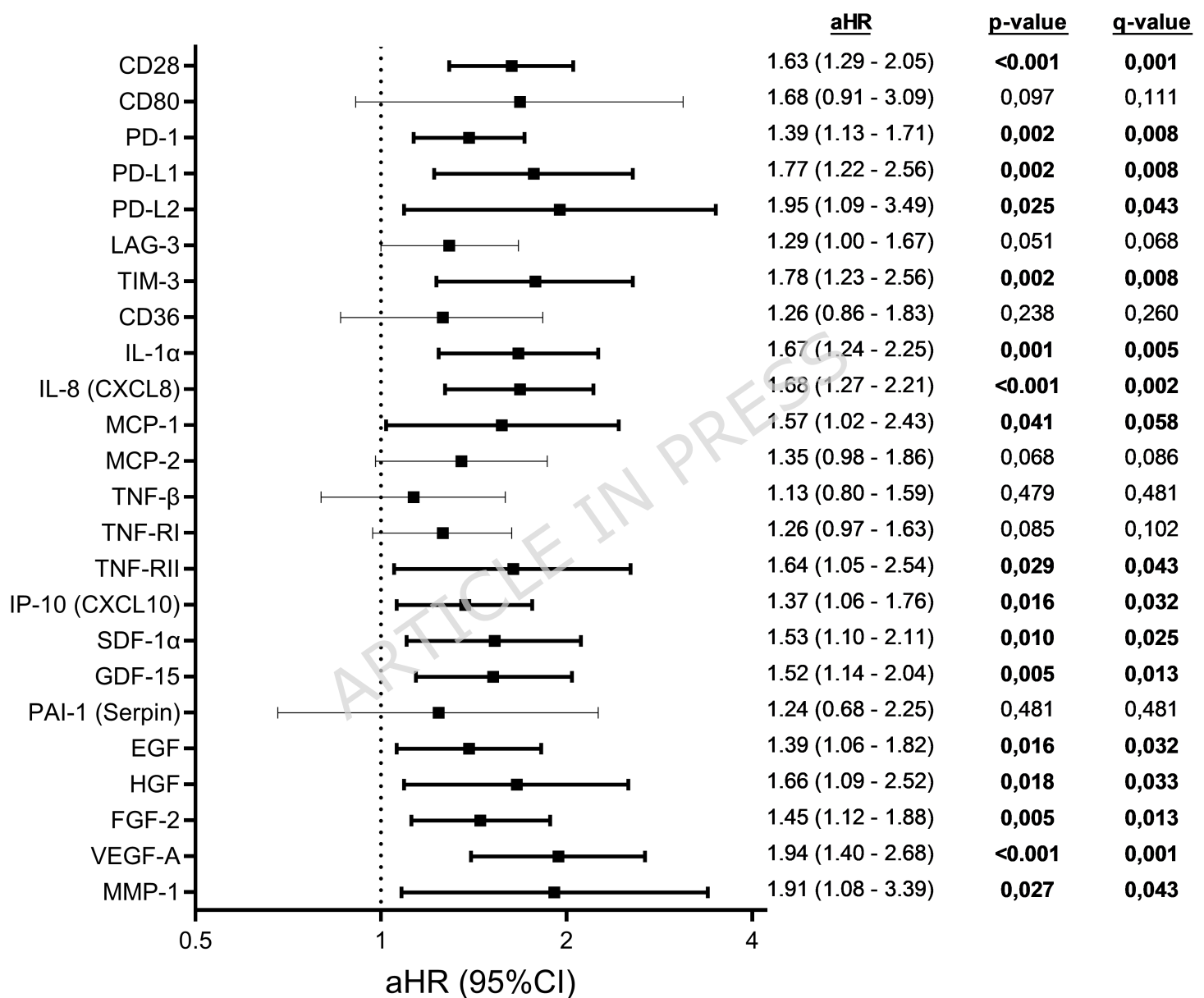
**Figure 3. Exploratory analysis of effect modification by sex on the association between plasma biomarkers and SRE risk.**

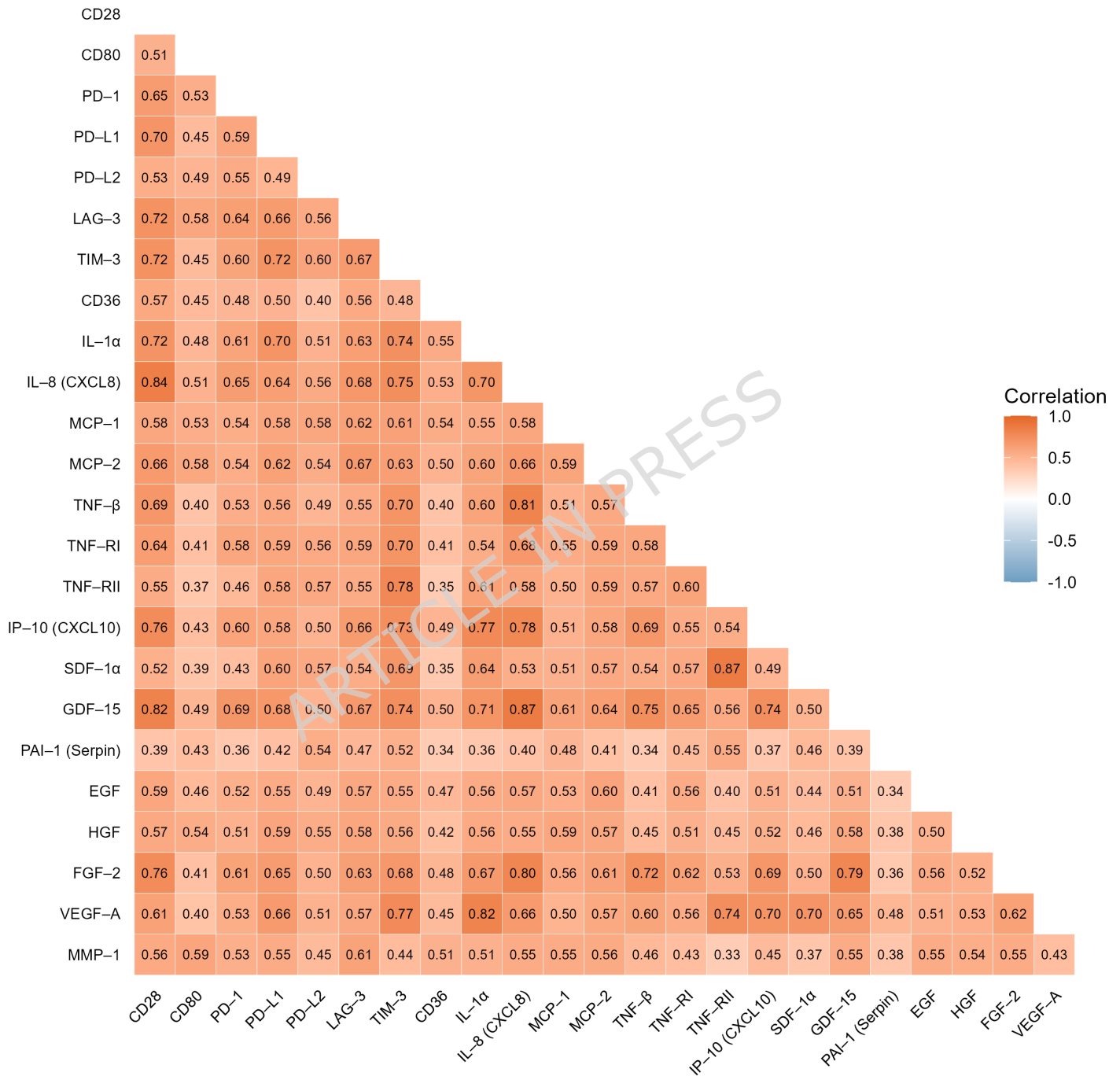
**Statistics:** This forest plot displays sex-stratified adjusted Hazard Ratios (aHR) and 95% Confidence Intervals (95% CI) for the seven biomarkers with statistically significant sex interaction terms. Red symbols/lines represent

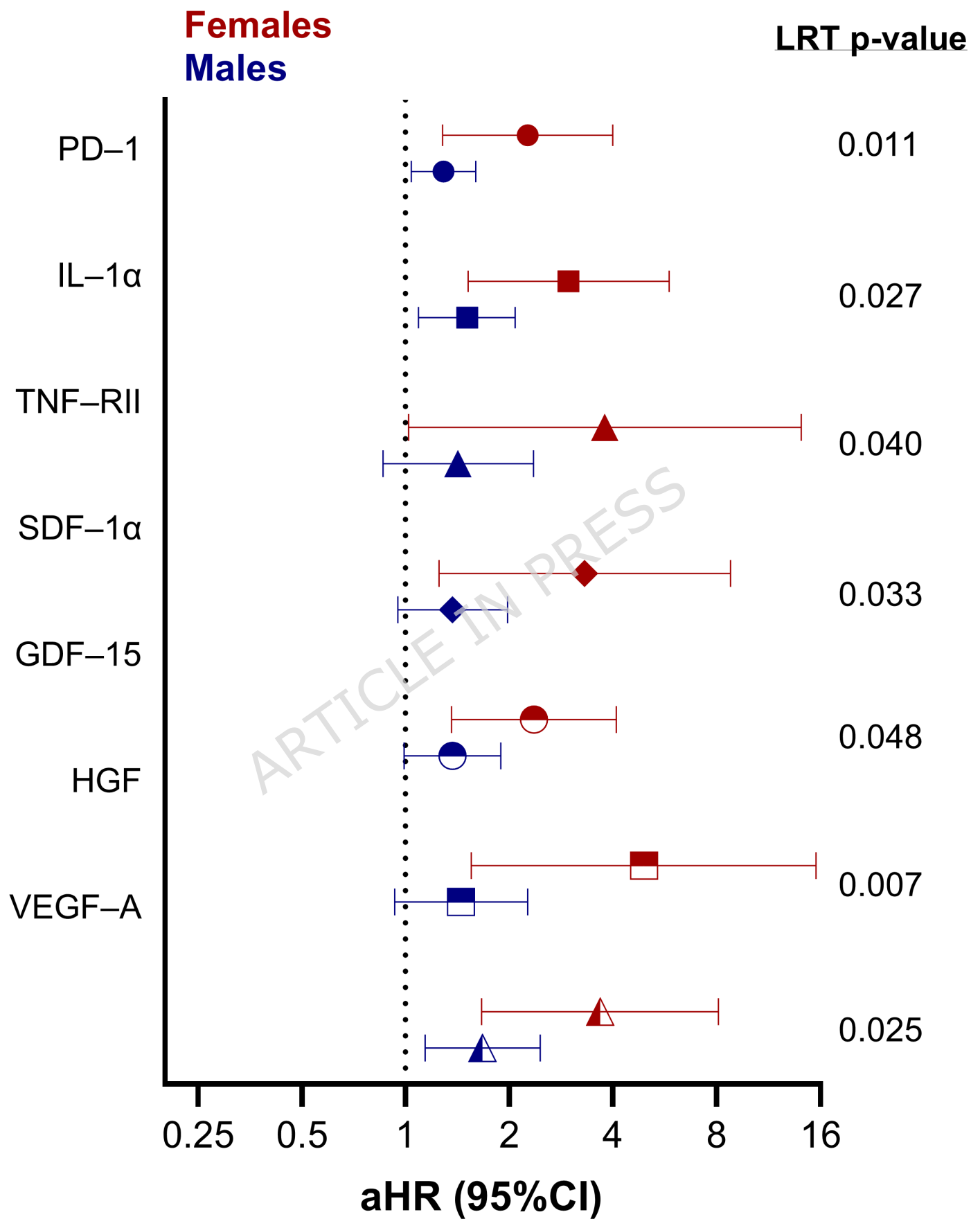
female participants (n=12 events) and blue symbols/lines represent male participants (n=53 events). Estimates are derived from multivariable Cox regression models. The p-value on the right is for the interaction term (Likelihood Ratio Test).

**Abbreviations:** aHR, adjusted hazard ratio; CI, confidence interval; GDF-15, growth differentiation factor 15; HGF, hepatocyte growth factor; IL-1 $\alpha$ , interleukin 1-alpha; PD-1, programmed cell death protein 1; SDF-1 $\alpha$ , stromal cell-derived factor 1-alpha; TNF-RII, tumor necrosis factor receptor II; VEGF-A, vascular endothelial growth factor A.

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**Table 1.** Baseline demographic, clinical, and virological characteristics of people with HIV (PWH) on antiretroviral therapy, stratified by incident skeletal-related events (SREs).

|                                      | All patients  | SREs cases    | Subcohort non-cases | <i>p-value</i>   |
|--------------------------------------|---------------|---------------|---------------------|------------------|
| <b>No.</b>                           | 312           | 65 (20.8%)    | 247 (79.2%)         |                  |
| <b>Age (years)</b>                   | 40 (33–47)    | 45 (39–51)    | 38 (32–45)          | <b>&lt;0.001</b> |
| Age (>50 years), n (%)               | 59 (18.9%)    | 18 (27.7%)    | 41 (16.6%)          | 0.063            |
| <b>Sex (male), n (%)</b>             | 267 (85.6%)   | 53 (81.5%)    | 214 (86.6%)         | 0.399            |
| <b>Region of origin, n (%)</b>       |               |               |                     | 0.127            |
| Spain                                | 190 (60.9%)   | 38 (58.5%)    | 152 (61.5%)         |                  |
| Europe (non-Spain)                   | 38 (12.2%)    | 13 (20.0%)    | 25 (10.1%)          |                  |
| Latin America                        | 67 (21.5%)    | 10 (15.4%)    | 57 (23.1%)          |                  |
| Other/Unknown                        | 17 (5.4%)     | 4 (6.2%)      | 13 (5.3%)           |                  |
| <b>Education level, n (%)</b>        |               |               |                     | 0.186            |
| None or primary                      | 45 (14.4%)    | 11 (16.9%)    | 34 (13.8%)          |                  |
| Secondary                            | 154 (49.4%)   | 38 (58.5%)    | 116 (47.0%)         |                  |
| University                           | 90 (28.8%)    | 13 (20.0%)    | 77 (31.2%)          |                  |
| Other/Unknown                        | 23 (7.4%)     | 3 (4.6%)      | 20 (8.1%)           |                  |
| <b>Smoking status, n (%)</b>         |               |               |                     | 0.126            |
| Never smoker                         | 111 (35.6%)   | 25 (38.5%)    | 86 (34.8%)          |                  |
| Ex-smoker                            | 23 (7.4%)     | 6 (9.2%)      | 17 (6.9%)           |                  |
| Smoker                               | 78 (25.0%)    | 9 (13.8%)     | 69 (27.9%)          |                  |
| Unknown                              | 100 (32.1%)   | 25 (38.5%)    | 75 (30.4%)          |                  |
| <b>HIV transmission route, n (%)</b> |               |               |                     | 0.693            |
| Heterosexual                         | 83 (26.6%)    | 20 (30.8%)    | 63 (25.5%)          |                  |
| MSM                                  | 204 (65.4%)   | 39 (60.0%)    | 165 (66.8%)         |                  |
| IDU                                  | 11 (3.5%)     | 2 (3.1%)      | 9 (3.6%)            |                  |
| Other/Unknown                        | 14 (4.5%)     | 4 (6.2%)      | 10 (4.0%)           |                  |
| <b>Coinfections, n (%)</b>           |               |               |                     |                  |
| HCV coinfection                      | 22 (7.1%)     | 8 (12.3%)     | 14 (5.7%)           | 0.112            |
| HBV coinfection                      | 85 (27.2%)    | 20 (30.8%)    | 65 (26.3%)          | 0.459            |
| <b>HIV-related markers</b>           |               |               |                     |                  |
| Previous AIDS, n (%)                 | 41 (13.1%)    | 8 (12.3%)     | 33 (13.4%)          | 0.986            |
| Nadir CD4+/mm <sup>3</sup>           | 300 (165–419) | 242 (141–399) | 314 (165–433)       | 0.074            |
| Baseline CD4+ /mm <sup>3</sup>       | 615 (427–848) | 597 (401–761) | 625 (436–863)       | 0.369            |
| <b>Baseline CD4+ strata, n (%)</b>   |               |               |                     | 0.551            |
| ≥500                                 | 207 (66.3%)   | 40 (61.5%)    | 167 (67.6%)         |                  |
| 350–499                              | 52 (16.7%)    | 14 (21.5%)    | 38 (15.4%)          |                  |
| <350                                 | 47 (15.1%)    | 9 (13.8%)     | 38 (15.4%)          |                  |
| Unknown                              | 6 (1.9%)      | 2 (3.1%)      | 4 (1.6%)            |                  |
| <b>ART Regimen, n (%)</b>            |               |               |                     | <b>&lt;0.001</b> |

|              |             |            |            |
|--------------|-------------|------------|------------|
| NRTI + InSTI | 91 (29.2%)  | 9 (13.8%)  | 82 (33.2%) |
| NRTI + PI    | 52 (16.7%)  | 10 (15.4%) | 42 (17.0%) |
| NRTI + NNRTI | 121 (38.8%) | 40 (61.5%) | 81 (32.8%) |
| Other        | 40 (12.8%)  | 6 (9.2%)   | 34 (13.8%) |
| Unknown      | 8 (2.6%)    | 0 (0.0%)   | 8 (3.2%)   |

**Statistics:** Data are presented as No. (%) for categorical variables and median (interquartile range [IQR]) for continuous variables. P-values were calculated using the Mann-Whitney U test for continuous variables and the Chi-square test or Fisher's exact test for categorical variables. Statistically significant differences ( $p < 0.05$ ) are highlighted in bold.

**Abbreviations:** AIDS, acquired immune deficiency syndrome; ART, antiretroviral therapy; HBV, hepatitis B virus; HCV, hepatitis C virus; IDU, injection drug use; InSTI, integrase strand transfer inhibitor; MSM, men who have sex with men; NNRTI, non-nucleoside reverse transcriptase inhibitor; NRTI, nucleoside reverse transcriptase inhibitor; PI, protease inhibitor; SREs, skeletal-related events.

*Note: The Non-SRE Subcohort refers to participants from the randomly selected subcohort who did not develop an event during follow-up.*

**Table 2.** Baseline plasma levels of immune regulation and senescence-associated secretory phenotype (SASP) biomarkers, stratified by incident skeletal-related event (SRE) status in people with HIV (PWH) on antiretroviral treatment.

| Category          | Marker         | Yes- SREs               | No- SREs                | <i>p</i> -value | <i>q</i> -value |
|-------------------|----------------|-------------------------|-------------------------|-----------------|-----------------|
| Immune regulation | CD28           | 20.38 (11.10 - 86.97)   | 15.32 (10.23 - 40.60)   | 0.079           | 0.237           |
|                   | CD80           | 11.95 (9.69 - 16.01)    | 11.47 (9.67 - 15.02)    | 0.517           | 0.591           |
|                   | PD-1           | 60.61 (28.45 - 196.60)  | 40.70 (23.56 - 91.92)   | <b>0.016</b>    | 0.132           |
|                   | PD-L1          | 16.04 (12.25 - 29.46)   | 14.84 (10.87 - 21.47)   | 0.110           | 0.264           |
|                   | PD-L2          | 22.17 (16.56 - 33.43)   | 22.61 (17.42 - 30.95)   | 0.704           | 0.735           |
|                   | LAG-3          | 14.27 (9.43 - 22.98)    | 12.35 (9.25 - 19.34)    | 0.232           | 0.309           |
|                   | TIM-3          | 115.55 (76.93 - 215.86) | 98.14 (68.69 - 166.40)  | <b>0.046</b>    | 0.184           |
| SASP              | CD36           | 11.68 (8.95 - 27.59)    | 12.54 (9.58 - 19.08)    | 0.653           | 0.712           |
|                   | IL-1 $\alpha$  | 42.52 (23.56 - 87.44)   | 32.26 (20.52 - 57.56)   | <b>0.020</b>    | 0.132           |
|                   | IL-8           | 59.91 (34.44 - 215.51)  | 49.12 (28.63 - 112.15)  | 0.093           | 0.248           |
|                   | MCP-1          | 18.39 (14.11 - 26.50)   | 17.00 (12.87 - 25.93)   | 0.158           | 0.283           |
|                   | MCP-2          | 20.01 (12.52 - 40.36)   | 18.69 (12.56 - 25.00)   | 0.362           | 0.457           |
|                   | TNF- $\beta$   | 95.85 (38.10 - 213.96)  | 84.69 (44.19 - 178.40)  | 0.905           | 0.905           |
|                   | TNF-RI         | 96.07 (50.09 - 219.47)  | 80.16 (43.61 - 186.66)  | 0.227           | 0.309           |
|                   | TNF-RII        | 120.88 (79.51 - 212.27) | 107.32 (72.08 - 164.30) | <b>0.038</b>    | 0.182           |
|                   | IP-10          | 61.35 (26.76 - 202.09)  | 43.60 (23.49 - 99.12)   | 0.177           | 0.283           |
|                   | SDF-1 $\alpha$ | 44.84 (32.79 - 98.57)   | 39.69 (28.16 - 62.16)   | <b>0.022</b>    | 0.132           |
|                   | GDF-15         | 85.71 (53.81 - 173.26)  | 68.99 (40.17 - 137.84)  | 0.056           | 0.192           |
|                   | PAI-1          | 19.57 (13.36 - 27.19)   | 17.22 (13.27 - 24.42)   | 0.442           | 0.530           |
|                   | EGF            | 16.75 (11.98 - 30.70)   | 14.55 (9.70 - 28.15)    | 0.134           | 0.283           |
|                   | HGF            | 11.20 (8.02 - 19.43)    | 10.11 (7.92 - 14.68)    | 0.173           | 0.283           |
|                   | FGF-2          | 35.21 (20.86 - 131.12)  | 31.99 (18.83 - 63.20)   | 0.195           | 0.293           |
|                   | VEGF-A         | 94.29 (57.66 - 203.27)  | 73.15 (46.15 - 117.29)  | <b>0.003</b>    | <b>0.072</b>    |
|                   | MMP-1          | 9.59 (8.15 - 14.32)     | 9.48 (7.22 - 12.85)     | 0.166           | 0.283           |

**Statistics:** Data are presented as median fluorescence intensity (arbitrary units [a.u.]) and interquartile range (IQR). P-values were calculated using the Mann-Whitney U test. The q-values represent p-values adjusted for multiple testing using the False Discovery Rate (FDR) method. Statistically significant differences ( $p < 0.05$  or  $q < 0.100$ ) are highlighted in bold.

**Abbreviations:** CD, cluster of differentiation; EGF, epidermal growth factor; FGF, fibroblast growth factor; GDF, growth differentiation factor; HGF, hepatocyte growth factor; IL, interleukin; IP, interferon gamma-induced protein; LAG, lymphocyte activation gene; MCP, monocyte chemoattractant protein; MMP, matrix metalloproteinase; PAI, Plasminogen Activator Inhibitor; PD-1, programmed cell death protein; PD-L, programmed death-ligand; SDF, stromal cell-derived factor; TIM, T-cell immunoglobulin and mucin domain; TNF, tumor necrosis factor; TNF-R, tumor necrosis factor receptor; VEGF, vascular endothelial growth factor.

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