Mild profile improvement of immune biomarkers in HIV/HCV-coinfected patients who removed hepatitis C after HCV treatment: A prospective study

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Abstract

Objective: There are a lack of consistency among articles in regards to the evolution of peripheral immune biomarkers after HCV therapy. We aimed to detect the most relevant changes in peripheral immune biomarkers among HIV/HCV-coinfected patients who achieved sustained virologic response (SVR) following peg-IFN-α/ribavirin therapy and to evaluate its normalization with respect to an HIV-monoinfected control group.

Methods: We performed a prospective cohort study in 99 HIV/HCV-coinfected patients with samples at baseline (HIV/HCV-b-group) and at week 24 after SVR (HIV/HCV-f-group). We also used a control group of 39 HIV-monoinfected patients (HIV-group) negative for HCV and HBV infections, and who had undetectable HIV viral load and $CD4^+$ >500 cells/mm^3. Peripheral T cell subsets were assessed by flow cytometry and plasma biomarkers by immunoassays.

Results: HIV/HCV-coinfected patients had higher values of in IL-10, IL-4, IP-10, IL-8, IL-1β, IL-18, IL-6, IFN-γ, IL-12p70, TNF-α, sVCAM-1, sICAM-1, and sTNFR-1 than HIV control subjects, both at the beginning and at the end of follow-up. Moreover, three biomarkers ($CD4^+CD38^+$, telomere length, and IL-1RA) were normalized in relation to the control group at the end of follow-up (the HIV/HCV-b group had higher values and the HIV/HCV-f group had similar values as the HIV-group). Additionally, LPS, IL-2, and IL-17A levels were higher in the HIV/HCV-f group than the HIV-group (24 weeks after SVR). During the follow-up, HIV/HCV-coinfected patients had a significant decrease by the end of follow-up in $CD8^+CD45RA^-CD28^+$, $CD4^+CD38^+$, $CD4^+CD25^+CD127^{-/low}$, $CD4^+CD25^+CD127^{-/low}$ $CD45RA^-$, FABP2, LBP, IP-10, sVCAM1. Only $CD4^+CD38^+$ was normalized.

Conclusion: HIV/HCV-patients showed a slight improvement in the overall profile of immune biomarkers after achieving SVR.

Key Words

Chronic hepatitis C; HIV; HCV therapy; biomarkers; inflammation; immune activation

Background

The natural history of human immunodeficiency virus (HIV) involves progressive immunodeficiency, development of acquired immune deficiency syndrome (AIDS), and death in the absence of antiretroviral treatment (1). This immunodeficiency may be reverted by suppressive combination antiretroviral therapy (cART) (2), particularly with early initiation of cART (3). However, a large number of alterations are not completely reversed, such as deficits in $CD4^+$ T helper cell (Th) 1, Th2, Th17, and regulatory $CD4^+$ T cell (Treg) responses (4-9), persistent immune activation (7), systemic inflammation (3), gut mucosal barrier dysfunction and dysbiosis (10-12); which may increase the risk for of both AIDS and non-AIDS-related conditions and death (13, 14).

Hepatitis C virus (HCV) infection is common among HIV-infected subjects, who develop chronic hepatitis C (CHC) over decades. In these patients, the progression of liver fibrosis is faster than for HCV-monoinfected patients (15), with higher rates of cirrhosis, decompensation, hepatocellular carcinoma, and death (16-18). During CHC, HCV infection promotes an immune response to control the viral infection (19), but it also promotes chronic inflammation, non-specific immune activation, immune function dysregulation, and immune senescence, which accelerate liver fibrosis and the development of other comorbidities (19-21). This dysregulation of the immune system can be aggravated by HIV infection (20, 21). In this regard, we recently reported that biomarkers related to $CD4^+$ Tregs, immune activation, bacterial translocation, inflammation, endothelial dysfunction, and coagulopathy were significantly higher in HIV/HCV-coinfected patients than HIV-monoinfected patients (22, 23).

The elimination of HCV infection after HCV therapy (sustained virologic response (SVR)) in HIV/HCV-coinfected patients decreases the risk of clinical events and death (24-26). However, cirrhotic patients who achieve SVR remain at risk of developing hepatocellular carcinoma (27) and extrahepatic cancers (28). Additionally, reversal of cirrhosis after SVR seems to be a slow process and the alterations of the immune system may persist after achieving SVR, particularly in cirrhotic patients (20). Previous studies have described a significant decrease, after SVR with HCV therapy, in peripheral memory T-cells (29) and immune activation (CD4+CD38+ and CD8+CD38+) (29-31), and in plasma levels of biomarkers related to inflammation (interleukin (IL)-6, IFN-γ-inducible protein 10 (IP-10)) (32), bacterial translocation (lipopolysaccharide binding protein, soluble CD14 (sCD14) and fatty acid-binding protein 2 (FABP2)) (30, 32), and endothelial dysfunction (soluble tumor necrosis factor receptor-1 (sTNF-R1), soluble intercellular adhesion molecule 1 (sICAM-1) and soluble vascular cell adhesion molecule 1 (sVCAM-1)) (33-35). However, in the articles there is a lack of consistency in the analyzed biomarkers and the significant biomarkers detected, the timepoints that were used to take the samples after the end of treatment, and the statistical tests used. Additionally, there also are articles that report no changes in these biomarkers (36, 37).

Objective

We proposed this study with a high number of peripheral immune biomarkers (blood and plasma) analysed by multivariate statistical tests based on the hypothesis that elimination of HCV infection could promote an improvement or normalization in peripheral immune biomarkers related to lymphocyte subpopulations (naïve, memory, effector, activated, and Tregs), bacterial translocation, inflammation, endothelial dysfunction, coagulopathy, and immune function in HIV/HCV-coinfected patients who started HCV therapy with peg-IFN-α/ribavirin. We found HIV/HCV-coinfected patients who achieved SVR had decreases in some biomarkers, although most of them were far from normalized.

Patients and methods

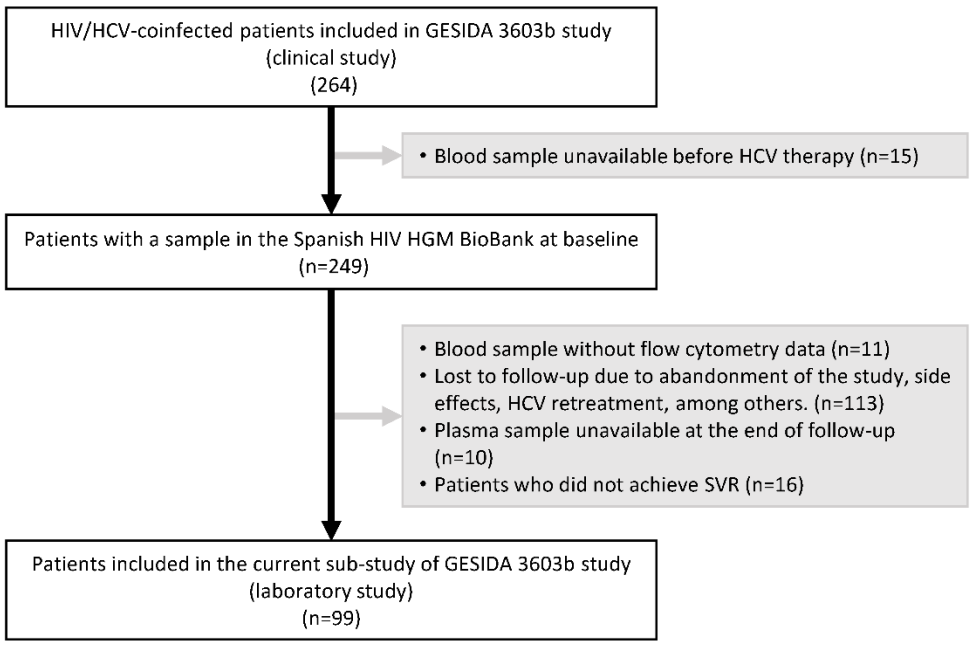
Study subjects

We carried out a prospective cohort study (repeated measures design) in HIV/HCV-coinfected patients from the cohort of "Grupo de Estudio del SIDA" (GESIDA 3603b study; see **Appendix**) enrolled between February 2012 and February 2016 at 14 centres in Spain. This study was performed according to the Declaration of Helsinki and was approved by the Research Ethics Committee of the Instituto de Salud Carlos III (CEI PI 23_2011). Participants gave their written consent before enrolment.

The detailed description of the GESIDA 3603b study has been previously reported (22). All HIV/HCV-coinfected patients received HCV therapy with pegylated interferon (IFN)-alpha plus ribavirin (peg-IFNα+rib) or peg-IFN-α/ribavirin/direct-acting antivirals (DAAs). The selection criteria were: 1) demonstrable HCV plasma viral load and HIV proviral DNA in peripheral blood cells; 2) $CD4^+$ T cell counts ≥200 cells/μL; 3) stable cART ≥6 months or no need for cART according to guidelines; 4) blood sample to performed immunological tests; and 5) a liver stiffness measure (LSM) at baseline. The exclusion criteria were: 1) acute hepatitis C; 2) hepatitis B virus co-infection; and 3) previous diagnosis of hepatic decompensation or hepatocellular carcinoma.

Figure 1 shows a flowchart describing the selection of the HIV/HCV-coinfected patients included in this study. From 264 HIV/HCV-coinfected patients enrolled in the GESIDA 3603b study, 249 had blood samples available before HCV therapy, but 113 patients were lost to follow-up due to dropping out of the study, adverse events, or because non-responder patients received a new HCV treatment. Additionally, 11 patients were discarded by incomplete cytometry data, ten patients did not have their plasma sample available at the end of follow-up, and 16 patients did not achieve SVR. Following these exclusions, the study was conducted longitudinally in 99 HIV/HCV-coinfected patients at baseline (before HCV treatment, HIV/HCV-b-group) and at the end of follow up (week 24 after SVR, HIV/HCV-f-group).

Figure 1. Flow chart describing the inclusion and exclusion criteria of HIV/HCV-coinfected patients included in our study. **Abbreviations**: HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; HGM, Hospital GregorioMarañón; GESIDA, Grupo de Estudio de Sida.



We also used a control group of 39 HIV-monoinfected patients (HIV-group) negative for HCV and HBV infections, and who had undetectable HIV viral load and $CD4^+$ >500 cells/mm^3. This HIV-monoinfected control group has been used in other reports (22, 23), which represents the normality standard for HIV-infected patients without chronic hepatitis viral infection and stable peripheral blood biomarkers.

Clinical data

Clinical and laboratory data were collected prospectively using a standard database via an online form within each center, which were monitored to verify the information collected in the database (22). Alcohol consumption >50 grams/day was considered as high alcohol intake. The time of HIV infection was calculated from the HIV diagnosis date. The time of HCV infection was calculated from the date of the first year they showed some high-risk behaviors for HCV infection (shared needles and other injection paraphernalia, identified sexual contact, use of blood and blood products, and needle piercing) or a diagnosis of acute hepatitis C. The LSM was evaluated by transient elastography (FibroScan®, Echosens, Paris, France) (22).

Telomere length

Total DNA was extracted from one million pelleted peripheral blood mononuclear cells with the Wizard® SV Genomic DNA Purification System (Promega, Madison, WI, USA). Relative leukocyte telomere length (rLTL) measurement was performed by monochromatic multiplex real-time quantitative PCR (MMqPCR) based on the method previously described by Cawthon et al (38) and by Hsieh et al (39). The rLTL was expressed as the ratio of the telomere amplification product (T) normalized to a single copy nuclear gene (S) (globin).

Flow cytometry

The expression of surface markers were evaluated in 100μL fresh anticoagulated whole blood, which were stained with the following antibodies: anti-CD38-APC-Cyanine 5.5 (APC-Cy5.5, clone HIT2, Invitrogen, Frederick, MD), anti-CD28-PE (Phycoerythrin, clone CD28.2, Beckman Coulter, Marseille, France), anti-CD57-FITC (Fluorescein, clone NC1, Beckman Coulter, Marseille, France), anti-CD127-PC7 (Phycoerythrin-Cyanin 7, clone R34.34, Beckman Coulter, Marseille, France), anti-CD25-PC5 (Phycoerythrin-Cyanin 5.1, clone B1-49.9, Beckman Coulter, Marseille, France), anti-CD45RA-ECD (Phycoerythrin-Texas Red X, clone 2H4LDH11LDB9, Beckman Coulter, Marseille, France), anti-CD3- PO (Pacific Orange, clone VCHT1, Invitrogen, Frederick, MD), anti-CD8- PB (Pacific Blue, clone SK1, BioLegend, San Diego, CA), and anti-CD4-APC-Cy7 (APC-Cyanine 7, clone OKT4, BioLegend, San Diego, CA).

The antibody mixes and samples were incubated for 20 min at room temperature in the dark and, afterwards, the IMMUNOPREP Reagent System (Beckman Coulter, Galway, Ireland) was added to lyse and fix each sample. We evaluated the fluorescence with a Gallios™ Flow Cytometer (Beckman Coulter, Miami, FL), acquiring a minimum of 200,000 cells in the lymphocyte gate for each sample. We used the Kaluza™ acquisition software (version 1.5; Beckman Coulter, Miami, FL) to analyze the flow cytometry data.

Multiplex immunoassays and ELISA

Plasma samples were collected in the Spanish HIV HGM BioBank and stored until use at –80°C. ProcartaPlex™ multiplex immunoassay (Bender MedSystems GmbH, Vienna, Austria) was used to measure the plasma biomarkers according to the manufacturer's specifications using a Luminex 200™ analyzer (Luminex Corporation, Austin, TX, United States). The plasma biomarkers measured by ELISA multiplex were IL-10, IL-1 receptor antagonist (IL-1RA), IL-4, IP-10, IL-8 (or chemokine (C-X-C motif) ligand 8, CXCL8), monocyte chemoattractant protein-1 (MCP-1)], IL-1β, IL-18, IL-6, tumor necrosis factor alpha (TNF-α), IFN-γ, IL-12p70, IL-2, IL-17A, sICAM1, sVCAM1, sTNFR1, plasminogen activator inhibitor-1 (PAI-1) and D-Dimer.

We also used commercial ELISA for LBP (R&D Systems, Minneapolis, USA), sCD14 and FABP2 (Raybiotech, Georgia, USA), and transforming growth factor beta 1 (TGF-β1; Bender MedSystems GmbH, Vienna, Austria) because the multiplex immunoassay was not available. The lipopolysaccharide (LPS; Hycult Biotech, Uden, The Netherlands) was evaluated by a Limulus amebocyte lysate (LAL) chromogenic endpoint Elisa.

Statistical analysis

The statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) v23.0 software (IBM Corp., Chicago, USA) and the R statistical package version v3.4.1 (R Foundation for Statistical Computing, Vienna, Austria).
The primary outcome variables were the biomarker values. The proportion of missing values was lower than 10% in each biomarker and these missing measures were imputed using the R-package "Hmisc v4.1-1" and the “pmm” algorithm.

We used unadjusted Generalized Linear Models (GLMs) with a gamma distribution (log-link) to analyze the differences in biomarker levels according to independent groups (HIV/HCV-b vs HIV and HIV/HCV-f vs HIV) and related groups (HIV/HCV-f vs HIV/HCV-b). This test provides the arithmetic mean ratio (AMR) of the compared groups and its significance level (*p*-value), which were adjusted by Bonferroni correction for multiple comparisons (*adj-p*-values) to reduce the risk of a spurious result. From the list of significant biomarkers, we performed GLMs adjusted by the main clinical and epidemiological baseline variables to confirm the significant associations found in the unadjusted GLMs. For independent groups (HIV/HCV-b vs HIV and HIV/HCV-f vs HIV), we adjusted the GLM models by gender, age, body mass index, $CD4^+$ T cells nadir (the lowest CD4 count during the follow-up), FIB-4 (a non-invasive liver fibrosis index), and $CD4^+$ T cells. For related groups (HIV/HCV-f vs HIV/HCV-b), we adjusted the GLM models by gender, age, alcohol intake, HCV genotype, HCV viral load, $CD4^+$ T cells nadir, LSM, and $CD4^+$ T cells.

Additionally, we performed a supervised multivariate analysis via partial least squares discriminant analysis (PLS-DA) [R-packages "mixomics v6.0-81"] to model all biomarkers together, which can be correlated (multicollinearity). All biomarker values were normalized by logarithmic transformation (log10) and, subsequently, were auto-scaled (mean centered and then divided by the standard deviation of the variable). The PLS-DA provides the area under the receiver operating characteristics (AUROC) to assess the performance of PLS-DA models. Also, the PLS-DA provides the variable importance in projection (VIP) score of each biomarker for ranking biomarkers, and a VIP score higher than 1 (VIP ≥1) was used for selecting relevant variables.

Results

Patient characteristics

Table 1 shows the baseline characteristics of 99 HIV/HCV-coinfected patients and 39 HIV-monoinfected patients (control group).

Table 1. Clinical and epidemiological characteristics of HIV/HCV-coinfected patients at baseline.

	HIV/HCV	HIV
No.	99	39
Gender (male)	79 (79.8%)	24 (61.5%)
Age (years)	49 (46; 52)	51 (46; 53)
BMI (kg/m^2)	24.7 (21.8; 27.8)	25.3 (23.5; 26.6)
BMI ≥25 kg/m^2	45 (47.4%)	21 (55.3%)
Diabetes	8 (8.1%)	6 (15.8%)
High alcohol intake	44 (44.4%)	1 (3.1%)
HIV acquired by IVDU	74 (74.7%)	-
Prior AIDS	25 (25.3%)	13 (33.3%)
Years since HIV infection	22 (17; 26)	-
Years since HCV diagnosis	21 (14; 25)	-
Previous HCV therapy (IFNα+rib)	45 (45.5%)	-
Antiretroviral therapy		
Non-treated	1 (1%)	-
PI-based	13 (13.1%)	10 (25.6%)
2NRTI+II-based	26 (26.3%)	4 (10.2%)
2NRTI+PI-based	21 (21.2%)	-
2NRTI+NNRTI-based	32 (32.3%)	23 (64.1%)
Others	6 (6.1%)	2 (5.1%)
HIV markers		
Nadir CD4+ T-cells (cells/mm^3)	165 (90; 260)	215 (107; 343)
Nadir CD4+ <200 cells/mm^3	63 (64.9%)	14 (38.9%)
CD4+ T-cells (cells/mm^3)	520.5 (372; 781)	832 (685; 1036)
CD4+ <500 cells/mm^3	45 (45.9%)	0 (0%)
HIV-RNA >50 cp/mL	8 (8.1%)	0 (0%)
HCV markers		
HCV genotype		
1	74 (74.7%)	-
2	2 (2%)	-
3	17 (17.2%)	-
4	6 (6.1%)	-
Log_{10} HCV-RNA (IU/mL)	6.2 (5.7; 6.6)	-
HCV-RNA > 850,000 IU/mL	61 (61.6%)	-
LSM (kPa)	12 (7.8; 18)	-
F0-F1-F2-F3 (<12.5 kPa)	52 (52.5%)	-
F4 (12.5-25 kPa)	31 (31.3%)	-
F4 (25-40 kPa)	10 (10.1%)	-
F4 (>40 kPa)	6 (6.1%)	-

Statistics: Values expressed as absolute number (percentage) and median (interquartile range).

Abbreviations: HCV, hepatitis C virus; HCV-RNA, HCV plasma viral load; HIV, human immunodeficiency virus; LSM, liver stiffness measure; HIV-RNA, HIV plasma viral load; IVDU, intravenous drug user; AIDS, acquired immune deficiency syndrome; IFNα+rib, interferon-alpha plus ribavirin; NNRTI, non-nucleoside analogue HIV reverse transcriptase inhibitor; NRTI, nucleoside analogue HIV reverse.

Profile of immune biomarkers with respect HIV group

At baseline, we compared the HIV/HCV-b group versus the HIV group by an unadjusted GLM analysis (independent groups) (**Figure 2A**; full description in **Supplemental Table 1**). The HIV/HCV-b group had lower values (*adj-p*-values ≤0.05) in replicative senescence (telomere length), while they had higher values (*adj-p*-values ≤0.05) in activated $CD4^+$ T cells ($CD4^+CD38^+$), bacterial translocation (LPS), anti-inflammatory/suppressor function (IL-10, IL-1RA, and IL-4), pro-inflammatory chemokines (IP-10 and IL-8), pro-inflammatory cytokines (IL-1β, IL-18, IL-6, TNF-α, IFN-γ, and IL-12p70), and endothelial dysfunction (sVCAM-1, sICAM-1, and sTNFR-1). From this list of significant biomarkers (*adj-p*-values ≤0.05), adjusted GLM analysis confirmed the significant differences between groups for $CD4^+CD38^+$, telomere length, IL-10, IL-1RA, IL-4, IP-10, IL-8, IL-1β, IL-18, IL-6, IFN-γ, IL-12p70, TNF-α, sVCAM-1, sICAM-1, and sTNFR-1 (*p*-value ≤0.05) (**Figure 3A**).

At the end of the follow-up, the unadjusted GLM analysis (independent groups) between the HIV/HCV-f group and the HIV group (**Figure 2B**; full description in **Supplemental Table 1**) showed that the HIV/HCV-f group had lower values (*adj-p*-values <0.05) in replicative senescence (telomere length) and higher values (*adj-p*-values <0.05) in bacterial translocation (LPS), anti-inflammatory/suppressor function (IL-10, IL-1RA, and IL-4), pro-inflammatory chemokines (IP-10 and IL-8), pro-inflammatory cytokines (IL-1β, IL-18, IL-6, TNF-α, IFN-γ, IL-12p70, IL-2, and IL-17A), endothelial dysfunction (sVCAM-1, sICAM-1, and sTNFR-1), and coagulopathy (D-Dimer). From this list of significant biomarkers (*adj-p*-values ≤0.05), the adjusted GLMs confirmed the significant differences between groups for LPS, IL-10, IL-4, IP-10, IL-8, IL-1β, IL-18, IL-6, IL-2, IFN-γ, IL-12p70, TNF-α, IL-17A, sVCAM-1, sICAM-1, and sTNFR-1 (*p*-value ≤0.05) (**Figure 3B**).

Figure 2. Summary of arithmetic mean ratio (AMR) and level of statistical significance (*adj-p*-values) for the comparison between HIV-monoinfected patients (HIV group) and HIV/HCV-coinfected patients at baseline (HIV/HCV-b) and at the end of follow-up (HIV/HCV-f).

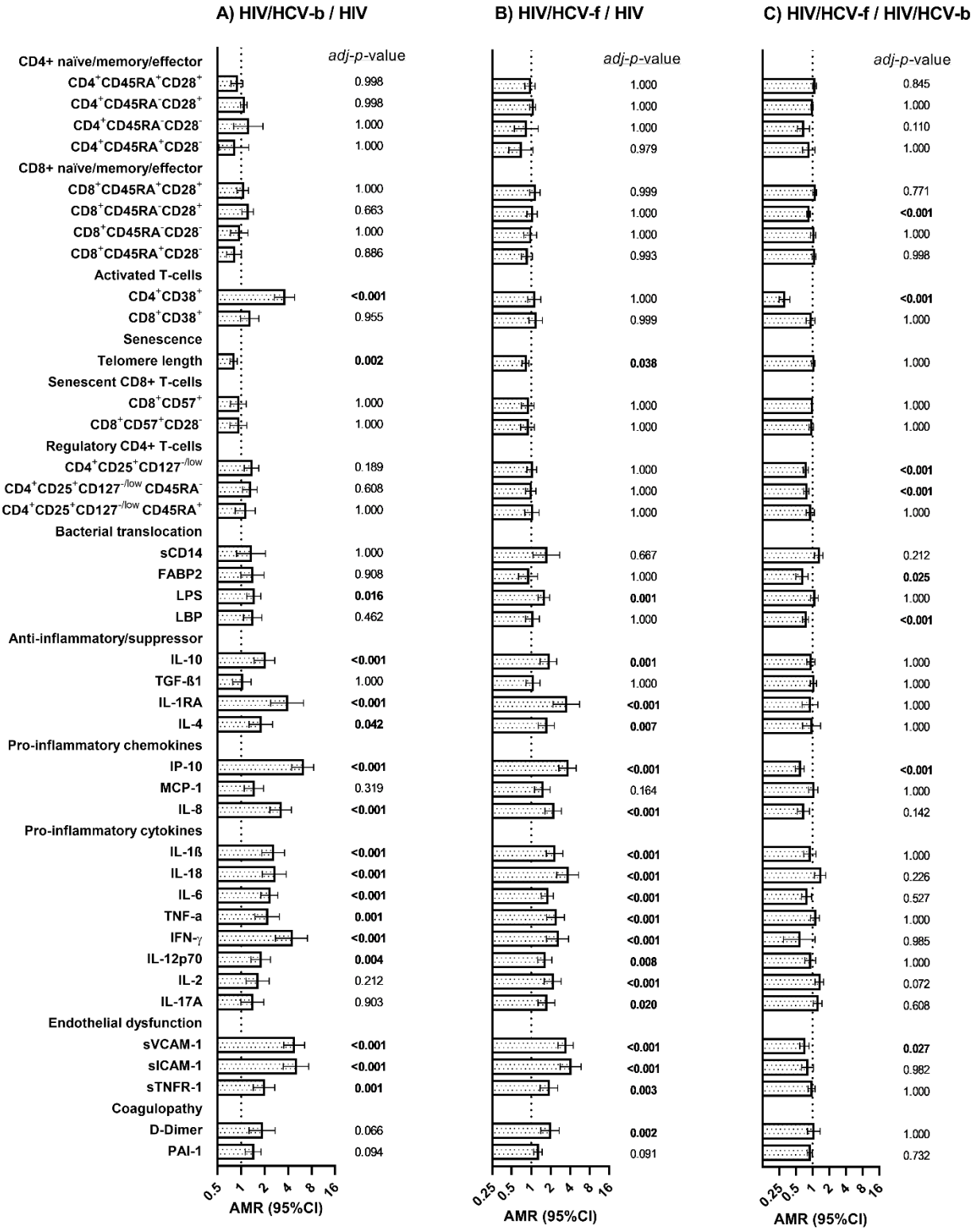
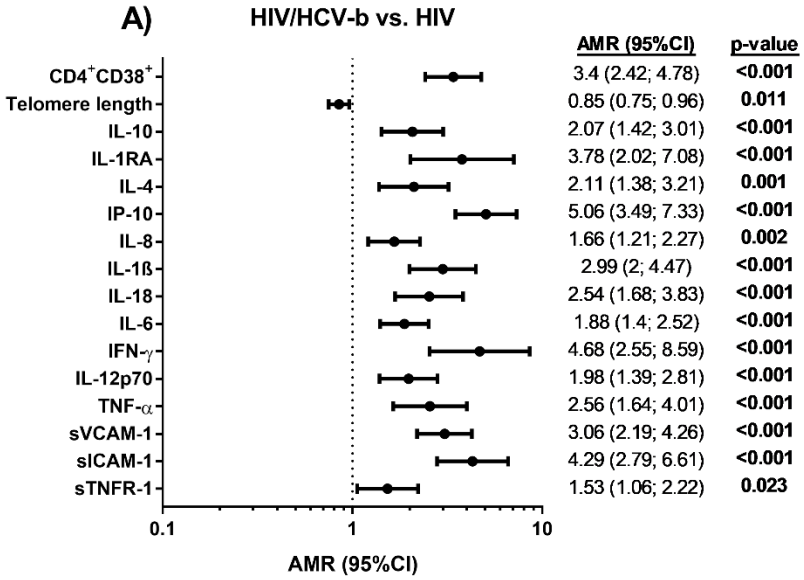
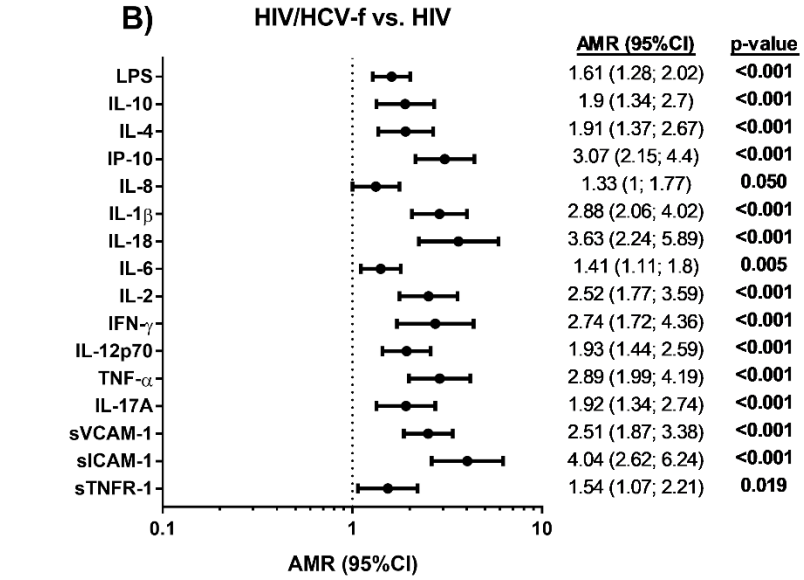
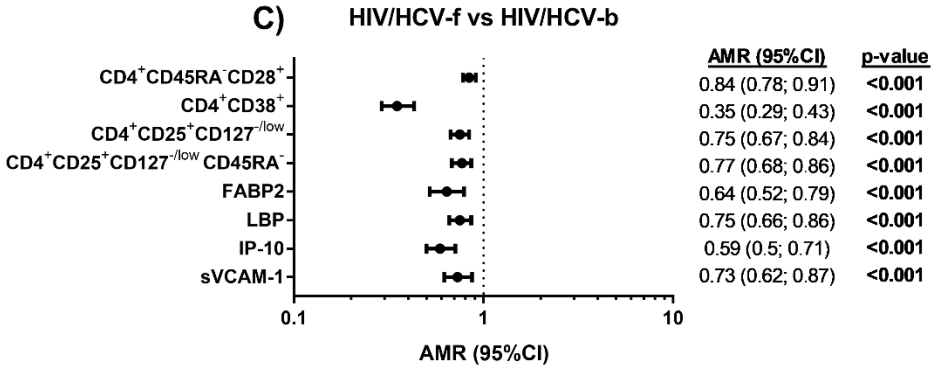


Figure 3. Summary of Generalized Linear Models (GLMs) adjusted by the main clinical and epidemiological characteristics for the comparison of HIV-monoinfected patients (HIV group) and HIV/HCV-coinfected patients at baseline (HIV/HCV-b) and at the end of follow-up (HIV/HCV-f).







We also stratified HIV/HCV-coinfected patients by $CD4^+$ (500 cells/mm^3), cirrhosis (F4), and normalized ALT (40 IU/mL) to analyze whether the severity of liver disease and immune status affect the comparison with the HIV control group, both at baseline (see **Supplemental Table 2**) and at the end of follow-up (see **Supplemental Table 3**). Overall, we found that the pattern of significant differences with respect to the HIV group was similar for a high number of biomarkers, although we also observed different patterns for some biomarkers.

Longitudinal dynamics of the profile of immune biomarkers

The unadjusted GLM analysis (repeated measurements) (**Figure 2C**; full description in **Supplemental Table 1**) showed that the HIV/HCV-f group had lower values than the HIV/HCV-b group (*adj-p*-values <0.05) in early memory $CD8^+$ T cells ($CD8^+CD45RA^-CD28^+$), activated $CD4^+$ T cells ($CD4^+CD38^+$), $CD4^+$ Tregs ($CD4^+CD25^+CD127^{-/low}$), memory $CD4^+$ Tregs ($CD4^+CD25^+CD127^{-/low}CD45RA^-$), bacterial translocation (FABP2 and LBP), pro-inflammatory chemokines (IP-10), and endothelial dysfunction (sVCAM1).

From this list of significant biomarkers (*adj-p*-values ≤0.05), the adjusted GLMs confirmed the significant differences between groups for $CD8^+CD45RA^-CD28^+$, $CD4^+CD38^+$, $CD4^+CD25^+CD127^{-/low}$, $CD4^+CD25^+CD127^{-/low}$ $CD45RA^-$, FABP2, LBP, IP-10, and sVCAM1 (*p*-values <0.05) (**Figure 3C**).

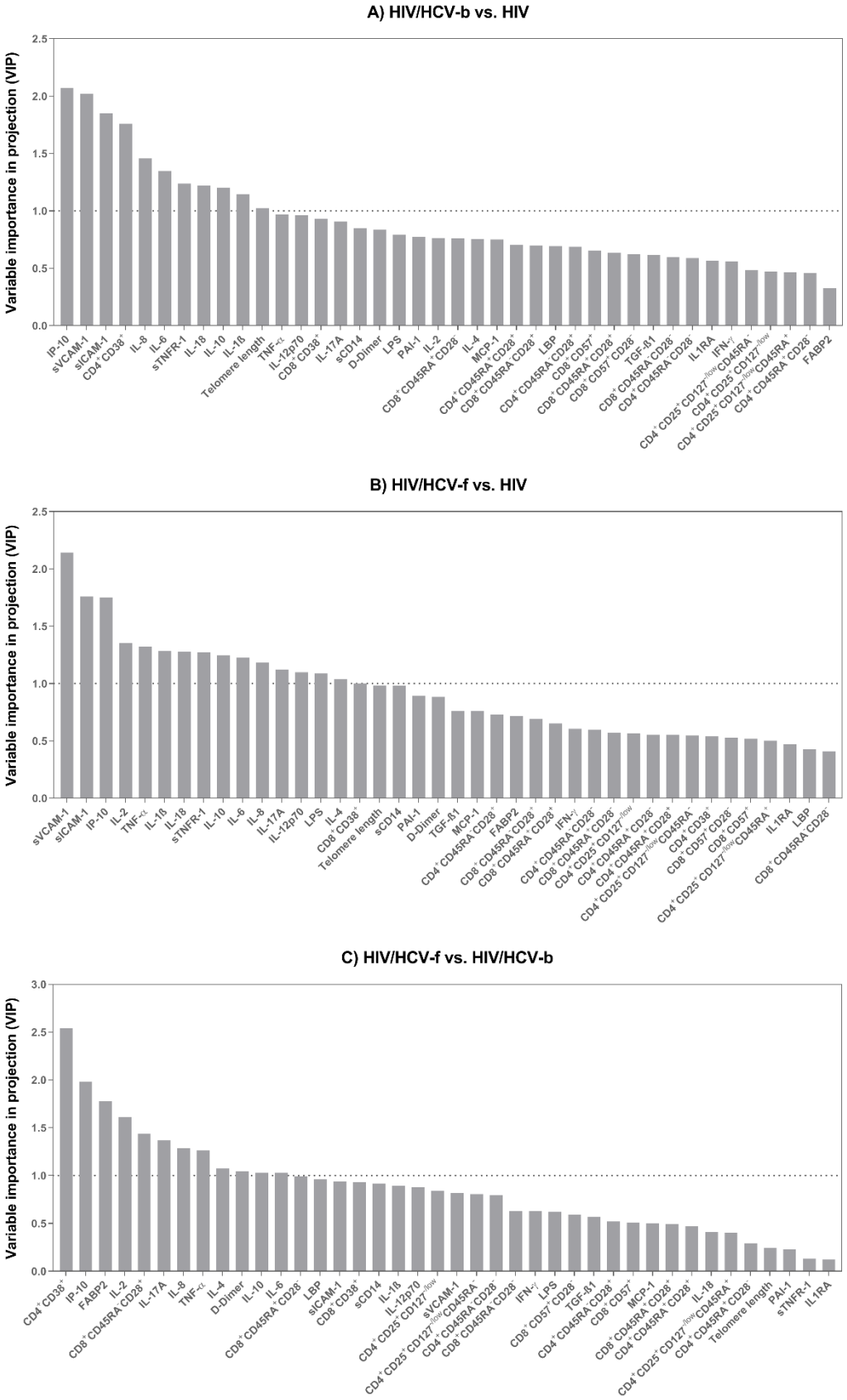
Identification of key biomarkers

The PLS-DA graphics are shown in the **Supplemental Figure 1**. The AUROC was higher than 0.95 at baseline and at the end of follow-up (independent groups), and higher than 0.85 during the follow-up (repeated measurements).

The VIP scores from the PLS-DAs were used to classify and identify the most relevant biomarkers (**Figure 4**). At baseline, the biomarkers with VIP score ≥1 were IP-10, sVCAM-1, sICAM-1, $CD4^+CD38^+$, IL-8, IL-6, sTNFR1, IL-18, IL-10, IL-1ß, and telomere length (**Figure 4A**). At the end of follow-up, the biomarkers with VIP score ≥1 were sVCAM-1, sICAM-1, IP-10, IL-2, TNF-α, IL-1ß, IL-18, sTNFR-1, IL-10, IL-6, IL-8, IL-17A, IL-12p70, LPS, and IL-4 (**Figure 4B**). The biomarkers selected at baseline and at the end of follow-up were already significantly different between groups by GLM analysis, confirming the relevance of these biomarkers. The biomarkers shared in the two analyses were related to a suppressor cytokine (IL-10), inflammation (IL-1β, IL-8, IL-6, IL-18, and IP-10), and endothelial dysfunction (sVCAM-1, sICAM-1, and sTNFR-1). $CD4^+CD38^+$ and telomere length were only found at baseline; while LPS, IL-2, TNF-α, IL-17A, IL-12p70, and IL-4 were only found at the end of follow-up.

During the follow-up (repeated measurements), the biomarkers with VIP score ≥1 were $CD4^+CD38^+$, IP-10, FABP2, IL-2, $CD8^+CD45RA^-CD28^+$, IL-17A, IL-8, TNF-α, IL-4, D-Dimmer, IL-10, and IL-6 (**Figure 4C**). Of these, $CD4^+CD38^+$, IP-10, FABP2, and $CD8^+CD45RA^-CD28^+$ were also contained in the list from the GLM analysis.

Figure 4. Summary of values of variable importance in projection (VIP) for the comparison between HIV-monoinfected patients (HIV group) and HIV/HCV-coinfected patients at baseline (HIV/HCV-b) and at the end of follow-up (HIV/HCV-f).



Discussion

In this study, we evaluated medium-term changes (baseline vs. 24 weeks after SVR) in peripheral immune biomarkers in a cohort of HIV/HCV-coinfected patients, with respect to a control group (HIV-group) and with respect to the baseline samples (repeated measurements). The major findings were as follows:

1) HIV/HCV-coinfected patients had higher values of in IL-10, IL-4, IP-10, IL-8, IL-1β, IL-18, IL-6, IFN-γ, IL-12p70, TNF-α, sVCAM-1, sICAM-1, and sTNFR-1 than HIV control subjects, both at the beginning and at the end of follow-up. Moreover, three biomarkers ($CD4^+CD38^+$, telomere length, and IL-1RA) were normalized in relation to the control group at the end of follow-up (the HIV/HCV-b group had higher values and the HIV/HCV-f group had similar values as the HIV-group). Additionally, LPS, IL-2, and IL-17A levels were higher in the HIV/HCV-f group than the HIV-group (24 weeks after SVR).

2) During the follow-up, HIV/HCV-coinfected patients had a significant decrease by the end of follow-up in $CD8^+CD45RA^-CD28^+$, $CD4^+CD38^+$, $CD4^+CD25^+CD127^{-/low}$, $CD4^+CD25^+CD127^{-/low}$ $CD45RA^-$, FABP2, LBP, IP-10, sVCAM1.

Our results stress the important of inflammation and deregulation of the immune system after achieving SVR, which could shed light on the clinical evolution of HIV-infected patients that eliminate HCV with HCV therapy.

Bacterial translocation biomarkers

Bacterial translocation is the passage of live bacteria or its products from the gastrointestinal tract to extra-intestinal sites, causing a stimulation of host immune cells, the synthesis of pro-inflammatory cytokines, and an overexpression of chronic activation markers (40). Bacterial translocation is very common in HIV/HCV-coinfected patients (40), and it promotes the development of comorbidities and the progression of HIV infection (41) and liver disease (42). In our study, HIV/HCV-coinfected patients had higher LPS levels than the HIV group during follow-up (repeated measures), but these differences were only confirmed by adjusted GLM analysis at 24 weeks after SVR. Moreover, HIV/HCV-coinfected patients had a significant decrease in FABP2 and LBP at the end of follow-up (repeated measures). Thus, these bacterial translocation biomarkers could indicate a risk of developing comorbidities and liver disease progression in HIV/HCV-coinfected patients who achieved SVR.

Anti-inflammatory biomarkers

HIV infection causes a deregulation of the immune system that is not entirely reversed by suppressive cART (5) and is related to gut mucosal barrier dysfunction, inflammation, and immune activation (43). During CHC, the immune system also plays a key role in cirrhosis progression (19-21). In our study, HIV/HCV-coinfected patients had higher plasma IL-10 and IL-4 values than the HIV group during all follow-up. These two biomarkers did not have a significant change during follow-up (repeated measures). IL-10 promotes a suppressive effect, inhibiting the synthesis of several kinds of cytokines (proinflammatory, Th1, Th2, and Th17), and preventing an exacerbated immune response and the subsequent tissue damage (44). However, IL-10 may also induce and sustain immune exhaustion, promote persistence of viral infections, and be related to the progression of HIV and HCV infections (45). IL-4 promotes a Th2 response and blocks the production of Th1 pro-inflammatory cytokines; but IL-4 also promotes the resolution of injuries and favors the healing process via increasing the deposit of collagen (46). During HIV infection, IL-4 levels increase with disease progression, which entails a change of a Th1 cytokine profile to Th2 (47). In liver diseases, IL-4 and IL-10 promote profibrotic effects by activating intrahepatic myofibroblasts that synthesize and secrete collagen, but they also have a protective role since they promote the resolution of liver injury (48). In our study, the elevated levels of IL-10 and IL-4 found at the end of the follow-up

could indicate a remarkable imbalance in the immune system, perhaps to heal liver injury caused by hepatitis C and to compensate for the exacerbated inflammatory response.

Our study also revealed that there were biomarkers that indicated an improvement in inflammation and immune activation after SVR. HIV/HCV-coinfected patients had higher plasma IL-1RA values than the HIV-group at baseline, but not at the end of follow-up (values normalized compared to the HIV control group). IL-1RA has an anti-inflammatory effect by blocking the IL-1 receptor, and its levels are increased with immune activation and inflammation (49). High IL-1RA levels are related to the progression to AIDS (50) and cirrhosis (51). The normalization of IL-1RA at 24 weeks after SVR could indicate a slight decrease in immune activation and inflammation, despite there not being differences in the analysis of repeated measures. Furthermore, both HIV and HCV infections promote increased numbers and function of anti-inflammatory Tregs, which secrete two cytokines with inhibitory functions (IL-10 and TGF-β), which prevent excessive immune activation during these viral infections (52, 53). In our study, we found a significant decrease (repeated measures) in values of Tregs ($CD4^+CD25^+CD127^{-/low}$ and $CD4^+CD25^+CD127^{-/low}$ $CD45RA^-$) in HCV/HIV-coinfected patients.

Inflammation biomarkers

Inflammation and immune activation are typical of both HIV and HCV infections and HIV/HCV coinfection (19, 22, 54), and they play a major role in the pathogenesis of CHC (40, 55). It has been found that plasma levels of biomarkers related to inflammation and immune activation are not completely normalized under cART in HIV-infected patients (43, 54). Thus, this chronic inflammation may impair immune recovery and promote non-AIDS-linked comorbidities (43, 54). Inflammation also promotes endothelial dysfunction, which is related to the development of liver diseases and cardiovascular events (56), and immune activation and immune senescence (20).

Changes in inflammation and endothelial dysfunction biomarkers during HCV therapy have already been previously described in HIV/HCV-coinfected patients, particularly when patients achieved SVR. Guzman-Fulgencio *et al.* did not find any significant decreases between baseline and SVR for a set of Th1/Th2/Th9/Th17/Th22 cytokines (57). However, SVR has been associated with a decrease in sVCAM-1 and sICAM-1 (33-35). In the current study, the HIV/HCV-group had higher levels of inflammation (IP-10, IL-8, IL-1β, IL-18, IL-6, IFN-γ, IL-12p70, and TNF-α) and endothelial dysfunction (sVCAM-1, sICAM-1, and sTNFR-1) than the HIV group at baseline and at the end of follow-up (24 weeks after SVR) after adjusting for the most relevant epidemiological and clinical covariates. Additionally, other pro-inflammatory biomarkers (IL-2, and IL-17A) were higher in HIV/HCV-coinfected patients than the HIV-group only at 24 weeks after SVR, despite not finding differences in the analysis of repeated measures. Therefore, medium-term inflammation is maintained, despite having achieved SVR, which could promote a higher risk of AIDS progression (43, 54), CHC progression and death (40, 55). However, we also found a decrease in IP-10 and sVCAM-1 levels during follow-up (repeated measures), although these biomarkers did not reach values of the HIV control group. Plasma IP-10 and sVCAM-1 levels are linked to liver disease severity in HIV/HCV-coinfected patients (22, 35, 58, 59) and their descent could also indicate an improvement in liver disease.

Activation biomarkers

Inflammation promotes immune activation, and vice versa (20, 43). The $CD38^+$ expression on $CD4^+$ T cells is a marker of immune activation that is increased in HIV/HCV-coinfected patients more than in HIV-monoinfected patients (22). In our study, HIV/HCV-coinfected patients at baseline had higher values in activated $CD4^+$ T cells ($CD4^+CD38^+$) than the HIV-group, but these biomarker values had a significant decrease and normalization in comparison to the HIV-

group throughout the follow-up. A reduction in $CD4^+CD38^+$ after HCV elimination has been related to a reduction in proviral HIV-DNA and microbial translocation markers in HIV/HCV-coinfected patients (30, 31). Thus, the decreased CD38 expression in $CD4^+$ T cells could indicate a decreased risk of both AIDS and CHC progression in our HIV/HCV-coinfected patients. Moreover, HIV/HCV-coinfected patients showed a significant decrease in early memory $CD8^+$ T cells ($CD8^+CD45RA^-CD28^+$) at the end of follow-up. During HCV infection, viral antigens promote immune activation and the expansion of memory $CD8^+$ T cells, but HCV eradication promotes a decrease in memory $CD8^+$ T cells (19). Thus, the decrease in early memory $CD8^+$ T cells may indicate a decreased immune activation at 24 weeks after SVR.

Replicative senescence

Immunosenescence is promoted by persistent inflammation and systemic immune activation. When telomerase reverse transcriptase activity is decreased or missing, telomere length gradually decreases, and when it is reduced to a critical size, cells undergo senescence and/or apoptosis (43). HIV infection, HCV coinfection, and some antiretroviral drugs are related to shortened telomere length (replicative senescence) in HIV-infected patients (43). Senescent cells are stably viable, remain metabolically active, and most belong to the so-called secretory phenotype (secretory-associated senescence phenotype, or SASP). These cells secrete molecules that can be dangerous, such as proinflammatory cytokines and chemokines, grow factors, proteases, nitric oxide and reactive oxygen species. This immunosenescence involves immune deregulation that promotes persistent inflammation and systemic immune activation, becoming a vicious cycle (43). In our study, the HIV/HCV-b group had shorter telomeres than the HIV-group, but these differences disappeared by the end of follow-up (HIV/HCV-f group vs. HIV-group), indicating that it does not get worse.

Strengths and limitations of the study

Due to several aspects, our study is more scientifically robust than most of the previous works: i) the sample size and number of biomarkers evaluated were large; ii) the changes in plasma biomarkers were evaluated at 24 weeks after SVR; iii) we used more suitable statistical analyses for a patient study, such as multivariate analyses adjusted for epidemiological and clinical covariates.

Our study also has some limitations: i) Despite including almost 100 patients with repeated measures, the sample size of our study might not be enough to detect differences between groups in some of biomarkers studied. ii) HIV/HCV-coinfected patients met the inclusion criteria to initiate HCV treatment (see the patient section), which may have introduced a selection bias. iii) The matching of the control group to compare with the HIV/HCV-coinfected patients is not perfect. On the one hand, we used a control group of HIV-monoinfected patients (HIV-control group) without chronic hepatitis viral infection and stable peripheral blood biomarkers, but around 45% of the HCV/HIV coinfection group had CD4+ <500 cells/mm^3, compared to 0% in the HIV-control group. The differences in the characteristics of HIV/HCV-coinfected patients made it impossible to form an HIV-control group with similar characteristics. Instead, we decided to form a control group to assess which markers were approaching normal after eliminating HCV infection. On the other hand, we did not have control group of HCV-monoinfected patients with a distribution of liver disease stage similar to the HIV/HCV-coinfected patients, particularly in cirrhosis (F4). However, it is important to take into account that when we stratified our HIV/HCV-coinfected patients by CD4 (500 cells/mm^3), cirrhosis (F4), and ALT (40 IU/mL), we found almost similar trends with respect to the HIV-control group. iv) HCV treatment was based on IFN-containing therapies, which cause deleterious effects on immune system markers despite HCV eradication per se, even though we took the last sample at 24 weeks after SVR. This may be an important factor for the differences with previous studies, particularly in studies based on IFN-free therapy. It is also important to note

that 45% of the subjects had previously passed another cycle of IFN-based therapy, which could have an impact on patients' immune parameters. However, these patients were not discarded because IFN-containing therapies in non-responders do not appear to protect against CHC progression in the long term (60).

Conclusions

In conclusion, HIV/HCV-coinfected patients showed a slight improvement in the overall profile of peripheral immune biomarkers compared to HIV-monoinfected patients after achieving SVR with peg-IFN-α/ribavirin. Nevertheless, HIV/HCV patients who achieved SVR showed an improvement in some biomarkers compared to baseline samples, although most biomarkers were far from normalized. More long-term studies are needed to evaluate whether these biomarkers tend to be like those of HIV-monoinfected patients who never had chronic hepatitis C.

List of abbreviations

Human immunodeficiency virus (HIV)

Acquired immune deficiency syndrome (AIDS)

Combination antiretroviral therapy (cART)

Hepatitis C virus (HCV)

Chronic hepatitis C (CHC)

Sustained virologic response (SVR)

Regulatory CD4+ T cells (Tregs)

T helper (Th)

Interferon (IFN)

Interferon-alpha plus ribavirin (peg-IFNα+rib)

Direct-acting antivirals (DAAs)

Liver stiffness measure (LSM)

HIV/HCV-coinfected patients at baseline (HIV/HCV-b-group)

HIV/HCV-coinfected patients at the end of follow-up (HIV/HCV-f-group)

HIV-monoinfected patients (HIV-group)

Cluster of differentiation (CDXX)

Interleukin (IL-XX)

IFN-γ-inducible protein 10 (IP-10)

Monocyte chemoattractant protein-1 (MCP-1)

Tumor necrosis factor alpha (TNF-α)

Soluble intercellular cell adhesion molecule 1 (sICAM1)

Soluble vascular cell adhesion molecule 1 (sVCAM1)

Soluble tumor necrosis factor receptor 1 (sTNFR1)

Plasminogen activator inhibitor-1 (PAI-1)

Lipopolysaccharide (LPS)

Lipopolysaccharide binding protein (LBP)

Soluble CD14 (sCD14)

Fatty acid-binding protein 2 (FABP2)

Transforming growth factor beta (TGF-β)

Supplemental Table (ST)

Generalized Linear Models (GLM)

Significance level (*p*-value)

Significance level adjusted by Bonferroni correction (*adj-p*-values)

Partial least squares discriminant analysis (PLS-DA)

Area under the receiver operating characteristics (AUROC)

Variable importance in projection (VIP)

Supplemental Figure (SF)

Declarations

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analyzed during the current study may be available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Supervision and visualization: SR.

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Not applicable

Appendix

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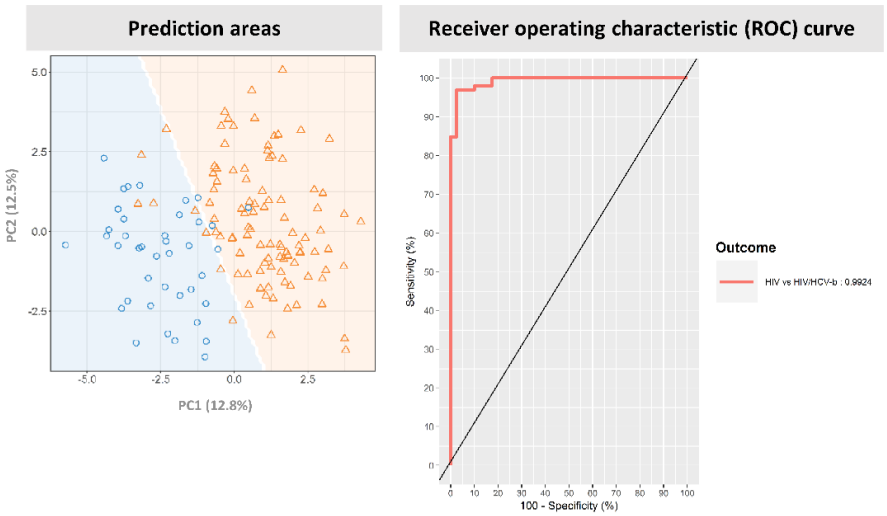
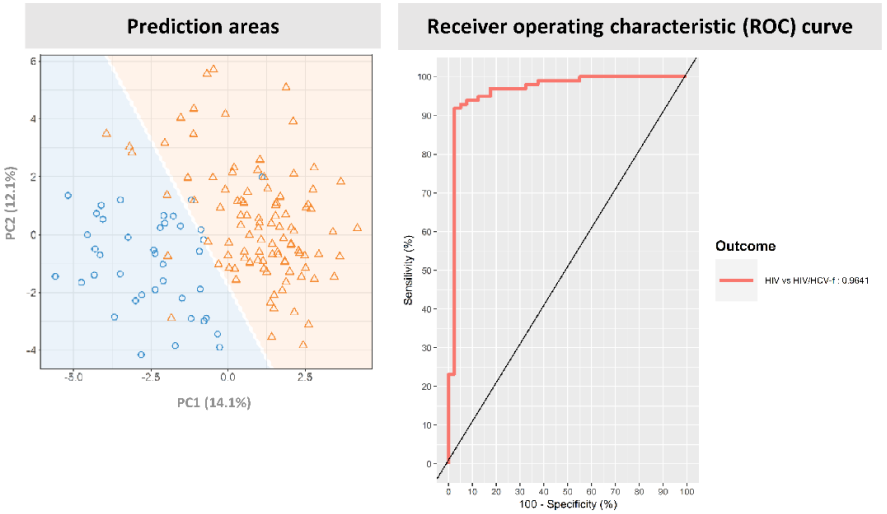
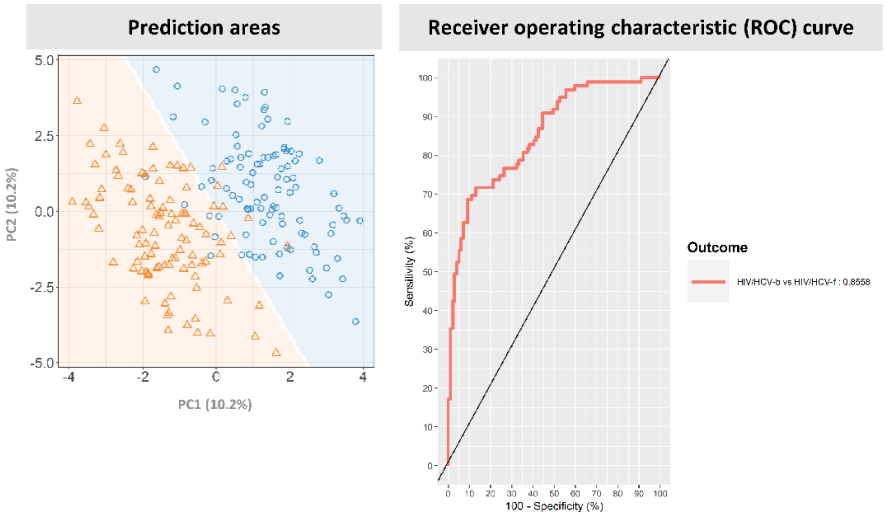
Supplemental Figure 1. Prediction area visualization (left) and ROC curve (right) for the profile of immune biomarkers using a PLSDA. (A), at baseline; (B), at the end of follow-up; (C), during follow-up. **Statistics**: Prediction area for each class were defined by two main components (PC1 and PC2) and calculated by a PLS-DA model ("centroids.dist") in the mixOmincs package. This method defines surfaces around samples that belong to the same predicted class. The ROC curve and AUC averaged were calculated including all components from our final model. **Abbreviations**: PLS-DA, partial least squares discriminant analysis; ROC, receiver operating characteristic; AUC, area under ROC Curve.







Supplemental Table 1. Summary of blood and plasma biomarkers in the control groups (HIV-monoinfected patients) and HIV/HCV-coinfected patients who achieved sustained virologic response (baseline and end of follow-up).

		HIV/HCV-coinfected patients		Cross-sectional analysis		Longitudinal analysis	*p*-values (*)			*adj-p*-values (**)		
	HIV (0)	Basal (1)	Final (2)	HIV/HCV (1) vs. HIV (0)	HIV/HCV (2) vs. HIV (0)	HIV/HCV (2) vs HIV/HCV (1)	0-1	0-2	1-2	0-1	0-2	1-2
CD4+ naïve/memory/effector												
$CD4^+CD45RA^+CD28^+$	38.5 (23.4; 48.1)	33.1 (24; 41.7)	35.5 (26.3; 44.7)	0.88 (0.74; 1.05)	0.96 (0.8; 1.15)	1.08 (1; 1.17)	.147	.631	**.045**	.998	1.000	.845
$CD4^+CD45RA^-CD28^+$	54.3 (45.7; 68.4)	60.3 (51.3; 69.2)	58.9 (51.3; 69.2)	1.08 (0.98; 1.19)	1.06 (0.95; 1.17)	0.98 (0.94; 1.03)	.141	.286	.402	.998	1.000	1.000
$CD4^+CD45RA^-CD28^-$	2.6 (1; 4.8)	2.2 (0.8; 5.4)	1.3 (0.6; 3.3)	1.23 (0.8; 1.9)	0.84 (0.55; 1.29)	0.68 (0.53; 0.88)	.344	.431	**.003**	1.000	1.000	.110
$CD4^+CD45RA^+CD28^-$	1.1 (0.4; 3.8)	1.5 (0.4; 3.2)	0.9 (0.4; 3.2)	0.81 (0.52; 1.25)	0.69 (0.45; 1.06)	0.85 (0.66; 1.1)	.338	.092	.225	1.000	.979	1.000
CD8+ naïve/memory/effector												
$CD8^+CD45RA^+CD28^+$	26.2 (17.1; 38.9)	28.8 (20; 37.2)	31.6 (21.4; 42.7)	1.05 (0.88; 1.24)	1.14 (0.95; 1.38)	1.09 (1.01; 1.18)	.611	.169	**.036**	1.000	.999	.771
$CD8^+CD45RA^-CD28^+$	23.5 (15.8; 33.3)	29.5 (20; 37.2)	25.1 (18.2; 32.4)	1.21 (1.02; 1.44)	1.03 (0.86; 1.24)	0.85 (0.79; 0.91)	**.027**	.766	**.000**	.663	1.000	**.000**
$CD8^+CD45RA^-CD28^-$	13.7 (10.4; 23.8)	13.8 (8.3; 20.4)	13.2 (9.1; 20.9)	0.94 (0.73; 1.22)	0.97 (0.77; 1.22)	1.03 (0.92; 1.14)	.647	.778	.638	1.000	1.000	1.000
$CD8^+CD45RA^+CD28^-$	28.3 (20.7; 37.5)	22.9 (13.5; 34.7)	23.4 (16.6; 32.4)	0.81 (0.65; 1)	0.86 (0.71; 1.04)	1.06 (0.98; 1.15)	.053	.116	.147	.886	.993	.998
Activated T-cells												
$CD4^+CD38^+$	2.8 (2; 5.6)	8.3 (4.5; 22.4)	3.9 (2.6; 6)	3.59 (2.66; 4.85)	1.12 (0.89; 1.41)	0.31 (0.25; 0.39)	**.000**	.323	**.000**	**.000**	1.000	**.000**
$CD8^+CD38^+$	7.1 (4.6; 10.7)	11 (6.9; 18.2)	11.5 (7.4; 18.2)	1.28 (0.98; 1.69)	1.18 (0.93; 1.5)	0.92 (0.77; 1.1)	.074	.162	.380	.955	.999	1.000
Senescence												
Telomere length	0.1 (0.1; 0.1)	0.1 (0.1; 0.1)	0.1 (0.1; 0.1)	0.8 (0.72; 0.89)	0.82 (0.72; 0.92)	1.02 (0.95; 1.1)	**.000**	**.001**	.538	**.002**	**.038**	1.000
Senescent CD8+ T-cells												
$CD8^+CD57^+$	27.7 (16.2; 38.5)	24 (14.1; 36.3)	25.1 (15.1; 34.7)	0.92 (0.73; 1.16)	0.89 (0.71; 1.11)	0.97 (0.89; 1.05)	.478	.298	.400	1.000	1.000	1.000
$CD8^+CD57^+CD28^-$	24.6 (13.4; 34.1)	21.4 (12; 33.1)	21.9 (11; 30.2)	0.92 (0.72; 1.18)	0.88 (0.69; 1.12)	0.95 (0.87; 1.04)	.516	.283	.284	1.000	1.000	1.000
Regulatory CD4+ T-cells												
$CD4^+CD25^+CD127^{-/low}$	6.1 (4.9; 7.5)	8.3 (6.3; 10)	6.2 (4.3; 7.4)	1.36 (1.1; 1.68)	1.02 (0.86; 1.22)	0.75 (0.68; 0.84)	**.005**	.791	**.000**	.189	1.000	**.000**
$CD4^+CD25^+CD127^{-/low}CD45RA^-$	7.9 (6.4; 10.8)	11 (8.1; 13.5)	8.1 (4.9; 11.7)	1.29 (1.04; 1.6)	0.99 (0.82; 1.19)	0.77 (0.69; 0.86)	**.023**	.930	**.000**	.608	1.000	**.000**
$CD4^+CD25^+CD127^{-/low}CD45RA^+$	3.2 (1.8; 5.5)	3.2 (1.9; 4.6)	3 (1.9; 4.4)	1.13 (0.84; 1.52)	1.03 (0.8; 1.32)	0.91 (0.77; 1.08)	.432	.838	.290	1.000	1.000	1.000
Bacterial translocation												

sCD14 (μg/mL)	3.6 (2; 5.3)	4.8 (3.2; 7.8)	5.9 (3.6; 10.7)	1.33 (0.87; 2.05)	1.71 (1.06; 2.76)	1.29 (1.08; 1.54)	.193	**.027**	**.006**	1.000	.667	.212
FABP2	0.6 (0.4; 1.4)	0.7 (0.4; 2)	0.6 (0.2; 1.1)	1.39 (0.99; 1.96)	0.9 (0.64; 1.26)	0.65 (0.5; 0.83)	.058	.549	**.001**	.908	1.000	**.025**
LPS	1 (0.8; 1.4)	1.4 (1.1; 1.9)	1.5 (1.1; 2.1)	1.45 (1.18; 1.78)	1.57 (1.28; 1.92)	1.08 (0.92; 1.26)	**.000**	**.000**	.335	**.016**	**.001**	1.000
LBP	0.8 (0.5; 1.4)	1.1 (0.6; 1.8)	0.8 (0.5; 1.4)	1.4 (1.07; 1.84)	1.05 (0.82; 1.34)	0.75 (0.66; 0.85)	**.015**	.707	**.000**	.462	1.000	**.000**
Anti-inflammatory/suppressor												
IL-10	0.8 (0.4; 1.5)	1.6 (0.7; 3)	1.6 (0.9; 2.8)	1.99 (1.47; 2.7)	1.85 (1.38; 2.48)	0.93 (0.79; 1.1)	**.000**	**.000**	.388	**.000**	**.001**	1.000
TGF-β1	32.8 (18; 48.3)	29.5 (14.8; 57.5)	31.6 (18.6; 58.9)	1.03 (0.78; 1.34)	1.06 (0.83; 1.37)	1.04 (0.91; 1.18)	.854	.632	.582	1.000	1.000	1.000
IL-1RA	145.5 (113.2; 214.3)	158.5 (70.8; 354.8)	208.9 (66.1; 467.7)	3.88 (2.39; 6.29)	3.48 (2.18; 5.56)	0.9 (0.65; 1.24)	**.000**	**.000**	.513	**.000**	**.000**	1.000
IL-4	2.8 (1.6; 4.4)	3.4 (1.7; 7.1)	4.6 (2.6; 6.8)	1.78 (1.26; 2.52)	1.71 (1.29; 2.27)	0.96 (0.66; 1.39)	**.001**	**.000**	.830	**.042**	**.007**	1.000
Proinflammatory chemokines												
IP-10	29.5 (18.8; 47.8)	166 (85.1; 288.4)	102.3 (52.5; 195)	6.15 (4.45; 8.51)	3.62 (2.67; 4.92)	0.59 (0.49; 0.71)	**.000**	**.000**	**.000**	**.000**	**.000**	**.000**
MCP-1	27.6 (14.3; 36.7)	30.9 (16.2; 52.5)	27.5 (17.8; 57.5)	1.45 (1.1; 1.93)	1.49 (1.13; 1.96)	1.02 (0.85; 1.24)	**.010**	**.004**	.807	.319	.164	1.000
IL-8	2.5 (1.2; 3.2)	4.6 (3; 8.1)	3.7 (2.6; 5.9)	3.2 (2.33; 4.39)	2.19 (1.64; 2.92)	0.68 (0.53; 0.88)	**.000**	**.000**	**.004**	**.000**	**.000**	.142
Pro-inflammatory cytokines												
IL-1β	0.6 (0.4; 1)	1.2 (0.6; 2.3)	1.4 (0.6; 2.5)	2.57 (1.84; 3.6)	2.29 (1.71; 3.08)	0.89 (0.69; 1.15)	**.000**	**.000**	.385	**.000**	**.000**	1.000
IL-18	114 (64.4; 184.9)	275.4 (117.5; 549.5)	263 (109.6; 794.3)	2.66 (1.87; 3.79)	3.66 (2.48; 5.41)	1.37 (1.09; 1.73)	**.000**	**.000**	**.006**	**.000**	**.000**	.226
IL-6	3.4 (2.6; 3.9)	5.6 (3.6; 7.9)	5 (3.4; 7.1)	2.29 (1.78; 2.95)	1.77 (1.43; 2.2)	0.77 (0.63; 0.96)	**.000**	**.000**	**.019**	**.000**	**.000**	.527
TNF-α	1.4 (0.5; 2.9)	3 (0.9; 6.2)	3.6 (2.1; 6.6)	2.15 (1.5; 3.08)	2.38 (1.74; 3.26)	1.11 (0.92; 1.33)	**.000**	**.000**	.279	**.001**	**.000**	1.000
IFN-γ	5.9 (3.2; 9)	7.6 (2.2; 26.9)	6.9 (2.3; 21.9)	4.41 (2.75; 7.08)	2.55 (1.72; 3.79)	0.58 (0.3; 1.11)	**.000**	**.000**	.100	**.000**	**.000**	.985
IL-12p70	1.5 (1.1; 2.3)	2.7 (1.4; 4)	3 (1.7; 4.2)	1.78 (1.33; 2.37)	1.62 (1.26; 2.09)	0.91 (0.73; 1.14)	**.000**	**.000**	.412	**.004**	**.008**	1.000
IL-2	2.2 (1; 3.6)	3.5 (1; 8.1)	6 (3.5; 10)	1.62 (1.15; 2.28)	2.15 (1.59; 2.9)	1.33 (1.11; 1.58)	**.006**	**.000**	**.002**	.212	**.000**	.072
IL-17A	1.3 (0.5; 2.1)	1.4 (0.6; 3.5)	2.1 (1.3; 4)	1.39 (0.99; 1.95)	1.71 (1.27; 2.32)	1.23 (1.03; 1.48)	.057	**.001**	**.023**	.903	**.020**	.608
Endothelial dysfunction												
sVCAM-1	334.2 (220.5; 554.4)	1698.2 (812.8; 3162.3)	1349 (776.2; 1995.3)	4.77 (3.51; 6.49)	3.4 (2.59; 4.46)	0.71 (0.59; 0.87)	**.000**	**.000**	**.001**	**.000**	**.000**	**.027**
sICAM-1	562.1 (311.7; 1137.2)	2187.8 (1288.2; 3981.1)	1659.6 (955; 3090.3)	5.03 (3.47; 7.31)	4.06 (2.79; 5.9)	0.81 (0.63; 1.04)	**.000**	**.000**	.095	**.000**	**.000**	.982
sTNFR-1	1.1 (0.2; 2)	1.7 (1.3; 2.7)	1.9 (1.2; 3)	1.96 (1.43; 2.69)	1.88 (1.38; 2.57)	0.96 (0.83; 1.11)	**.000**	**.000**	.586	**.001**	**.003**	1.000
Coagulopathy												
D-Dimer	23.4 (14.7; 45.9)	30.2 (15.1; 74.1)	49 (24; 70.8)	1.85 (1.26; 2.72)	1.95 (1.41; 2.7)	1.05 (0.81; 1.37)	**.002**	**.000**	.693	.066	**.002**	1.000
PAI-1	6.6 (5.1; 8.4)	10 (6.2; 13.8)	10 (6.3; 11.5)	1.43 (1.13; 1.8)	1.27 (1.09; 1.48)	0.89 (0.8; 0.99)	**.002**	**.002**	**.032**	.094	.091	.732

Statistics: Values were expressed as median (interquartile range), arithmetic mean ratio (AMR), and 95% of confidence interval (95%CI). (*), *P*-values were calculated by Generalized Linear Models (GLMs) for independent groups and repeated measurements. (**), *P*-values were adjusted by Bonferroni correction for multiple comparisons.

Abbreviations: HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; CDXX, cluster of differentiation; sCD14, soluble CD14; LPS, lipopolysaccharide; FABP2, fatty acid-binding protein 2; LBP, lipopolysaccharide binding protein; IL, interleukin; IP-10, IFN-γ-inducible protein 10; IL-1RA, interleukin-1 receptor antagonist; sVCAM-1, soluble vascular cell adhesion molecule 1; sICAM-1, soluble intercellular cell adhesion molecule 1; sTNFR-1, soluble tumor necrosis factor receptor 1; MCP-1, monocyte chemoattractant protein-1, PAI-1, plasminogen activator inhibitor-1; TGF-β1, transforming growth factor beta 1; IFN-γ, Interferon gamma; IL, interleukin; T-reg, regulatory CD4+ T-cells; TNF-α, tumor necrosis factor alpha.

Supplemental Table 2. Summary of statistical significance between HIV/HCV-coinfected patients (HIV/HCV-group) and HIV-monoinfected patients (HIV group) for blood and plasma biomarkers at baseline.

			CD4+ T-cells strata				Cirrhosis (F4) strata				ALT strata			
	HIV/HCV-group vs HIV-group		>500 cells/mL vs HIV-group		<500 cells/mL vs HIV-group		F4 vs HIV-group		No-F4 vs HIV-group		>40 IU/mL vs HIV-group		<40 IU/mL vs HIV-group	
	p-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)
CD4+ naïve/memory/effector (%)														
$CD4^+CD45RA^+CD28^+$	0.171	0.999	0.754	0.999	**0.026**	0.650	0.076	0.957	0.512	0.999	0.067	0.937	0.616	0.999
$CD4^+CD45RA^-CD28^+$	0.145	0.998	0.731	0.999	**0.018**	0.522	0.064	0.929	0.462	0.999	0.090	0.977	0.851	0.999
$CD4^+CD45RA^-CD28^-$	0.780	0.999	0.242	0.999	0.413	0.999	0.712	0.999	0.898	0.999	0.748	0.999	0.067	0.936
$CD4^+CD45RA^+CD28^-$	0.738	0.999	0.561	0.999	0.972	0.999	0.183	0.999	0.487	0.999	0.609	0.999	0.766	0.999
CD8+ naïve/memory/effector (%)														
$CD8^+CD45RA^+CD28^+$	0.443	0.999	0.213	0.999	0.986	0.999	0.291	0.999	0.755	0.999	0.809	0.999	0.056	0.899
$CD8^+CD45RA^-CD28^+$	**0.022**	0.586	**0.023**	0.608	0.084	0.971	0.113	0.992	**0.015**	0.455	**0.038**	0.787	0.052	0.881
$CD8^+CD45RA^-CD28^-$	0.558	0.999	0.571	0.999	0.647	0.999	0.234	0.999	0.898	0.999	0.778	0.999	0.233	0.999
$CD8^+CD45RA^+CD28^-$	**0.035**	0.760	**0.010**	0.324	0.311	0.999	0.152	0.999	**0.024**	0.624	0.057	0.905	0.071	0.948
Activated T-cells (%)														
$CD4^+CD38^+$	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
$CD8^+CD38^+$	**0.004**	0.164	0.095	0.982	**0.001**	**0.021**	**0.020**	0.551	**0.008**	0.267	**0.006**	0.225	**0.048**	0.861
Senescence														
Telomere length	**0.003**	0.115	**0.039**	0.794	**0.001**	**0.049**	**<0.001**	**0.018**	0.082	0.968	**0.001**	0.055	0.278	0.999
Senescent CD8+ T-cells (%)														
$CD8^+CD57^+$	0.289	0.999	0.146	0.998	0.738	0.999	0.319	0.999	0.383	0.999	0.359	0.999	0.308	0.999
$CD8^+CD57^+CD28^-$	0.364	0.999	0.210	0.999	0.785	0.999	0.421	0.999	0.429	0.999	0.475	0.999	0.286	0.999
Regulatory CD4+ T-cells (%)														
$CD4^+CD25^+CD127^{-/low}$	**<0.001**	**0.004**	**0.008**	0.284	**<0.001**	**0.001**	**0.002**	0.078	**<0.001**	**0.008**	**<0.001**	**0.001**	0.145	0.998
$CD4^+CD25^+CD127^{-/low}CD45RA^-$	**0.001**	**0.042**	**0.007**	0.248	**0.002**	0.077	**0.026**	0.648	**<0.001**	**0.019**	**0.001**	0.050	**0.040**	0.804
$CD4^+CD25^+CD127^{-/low}CD45RA^+$	0.606	0.999	0.124	0.995	0.428	0.999	0.503	0.999	0.804	0.999	0.566	0.999	0.888	0.999
Bacterial translocation														
sCD14 (µg/mL)	**0.018**	0.523	**0.029**	0.695	0.051	0.875	**0.034**	0.749	**0.042**	0.822	0.106	0.989	**0.001**	**0.022**
FABP2 (ng/mL)	0.805	0.999	0.251	0.999	0.393	0.999	0.873	0.999	0.786	0.999	0.731	0.999	0.900	0.999
LPS (UE/mL)	**<0.001**	**0.009**	**0.002**	0.060	**0.001**	**0.033**	**0.003**	0.129	**<0.001**	**0.015**	**<0.001**	**0.010**	**0.028**	0.680
LBP (µg/mL)	**0.037**	0.780	**0.036**	0.770	0.125	0.995	**0.004**	0.153	0.397	0.999	**0.029**	0.698	0.339	0.999
Anti-inflammatory/suppressor														
IL-10 (pg/mL)	**<0.001**	**0.013**	**<0.001**	**0.010**	**0.009**	0.314	**0.005**	0.181	**<0.001**	**0.019**	**<0.001**	**0.016**	**0.025**	0.635
TGF-β1 (ng/mL)	0.457	0.999	0.482	0.999	0.549	0.999	0.339	0.999	0.713	0.999	0.448	0.999	0.695	0.999
IL1RA (pg/mL)	0.784	0.999	0.854	0.999	0.758	0.999	0.967	0.999	0.666	0.999	0.960	0.999	0.433	0.999

IL-4 (pg/mL)	0.156	0.999	0.091	0.978	0.459	0.999	0.987	0.999	**0.015**	0.462	0.253	0.999	0.110	0.990
Proinflammatory chemokines														
IP-10 (pg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**
MCP1 (pg/mL)	0.094	0.981	0.110	0.991	0.184	0.999	0.129	0.996	0.152	0.999	0.136	0.997	0.136	0.997
IL-8 (pg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.003**	**<0.001**	**<0.001**	**0.002**	0.087
Pro-inflammatory cytokines														
IL-1β (pg/mL)	**<0.001**	**0.015**	**<0.001**	**0.004**	**0.027**	0.665	**0.017**	0.493	**<0.001**	**0.005**	**0.002**	0.065	**0.001**	**0.050**
IL-18 (pg/mL)	**<0.001**	**0.001**	**<0.001**	**0.002**	**0.001**	**0.048**	**<0.001**	**0.004**	**<0.001**	**0.019**	**<0.001**	**0.001**	**0.010**	0.334
IL-6 (pg/mL)	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.002**	0.088	**<0.001**	**<0.001**	**0.021**	0.573
TNF-α (pg/mL)	**0.005**	0.184	**0.002**	0.071	0.086	0.972	0.169	0.999	**<0.001**	**0.018**	**0.009**	0.317	**0.027**	0.665
IFN-γ (pg/mL)	0.173	0.999	0.515	0.999	0.067	0.937	0.461	0.999	0.101	0.986	0.123	0.995	0.766	0.999
IL-12p70 (pg/mL)	**0.005**	0.168	**0.007**	0.258	**0.023**	0.598	0.159	0.999	**<0.001**	**0.017**	**0.008**	0.268	**0.033**	0.737
IL-2 (pg/mL)	0.513	0.999	0.186	0.999	0.777	0.999	0.414	0.999	0.057	0.903	0.693	0.999	0.265	0.999
IL-17A (pg/mL)	0.635	0.999	0.277	0.999	0.725	0.999	0.397	0.999	0.104	0.988	0.818	0.999	0.338	0.999
Endothelial dysfunction														
sVCAM1 (µg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
sICAM1 (µg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
sTNFR1 (ng/mL)	**0.001**	**0.028**	**0.021**	0.569	**<0.001**	**0.007**	**0.002**	0.066	**0.005**	0.173	**<0.001**	**0.015**	0.136	0.997
Coagulopathy														
D-Dimer (ng/mL)	0.167	0.999	0.765	0.999	**0.023**	0.607	0.086	0.972	0.462	0.999	0.060	0.917	0.562	0.999
PAI-1 (ng/mL)	**0.001**	**0.041**	**0.012**	0.386	**0.001**	**0.037**	**0.004**	0.139	**0.004**	0.155	**<0.001**	**0.015**	0.259	0.999

Statistics: (*), *P*-values were calculated by Mann-Whitney tests. (**), *P*-values were adjusted by Bonferroni correction for multiple comparisons.
Abbreviations: HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; CDXX, cluster of differentiation; sCD14, soluble CD14; LPS, lipopolysaccharide; FABP2, fatty acid-binding protein 2; LBP, lipopolysaccharide binding protein; IL, interleukin; IP-10, IFN-γ-inducible protein 10; IL-1RA, interleukin-1 receptor antagonist; sVCAM-1, soluble vascular cell adhesion molecule 1; sICAM-1, soluble intercellular cell adhesion molecule 1; sTNFR-1, soluble tumor necrosis factor receptor 1; MCP-1, monocyte chemoattractant protein-1, PAI-1, plasminogen activator inhibitor-1; TGF-β1, transforming growth factor beta 1; IFN-γ, Interferon gamma; IL, interleukin; T-reg, regulatory CD4+ T-cells; TNF-α, tumor necrosis factor alpha.

Supplemental Table 3. Summary of statistical significance between HIV/HCV-coinfected patients (HIV/HCV-group) and HIV-monoinfected patients (HIV group) for blood and plasma biomarkers at the end of follow-up.

			CD4+ T-cells strata				Cirrhosis (F4) strata				ALT strata			
	HIV/HCV-group vs HIV-group		>500 cells/mL vs HIV-group		<500 cells/mL vs HIV-group		F4 vs HIV-group		No-F4 vs HIV-group		>40 IU/mL vs HIV-group		<40 IU/mL vs HIV-group	
	p-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)	*p*-values (*)	*adj-p*-values (**)
CD4+ naïve/memory/effector (%)														
$CD4^+CD45RA^+CD28^+$	0.580	0.999	0.982	0.999	0.283	0.999	0.441	0.999	0.829	0.999	0.220	0.999	0.120	0.994
$CD4^+CD45RA^-CD28^+$	0.286	0.999	0.646	0.999	0.137	0.997	0.127	0.996	0.707	0.999	0.124	0.995	0.490	0.999
$CD4^+CD45RA^-CD28^-$	0.084	0.970	**0.033**	0.744	0.413	0.999	0.080	0.964	0.195	0.999	0.309	0.999	**0.003**	0.109
$CD4^+CD45RA^+CD28^-$	0.359	0.999	0.128	0.996	0.986	0.999	0.331	0.999	0.517	0.999	0.766	0.999	**0.024**	0.620
CD8+ naïve/memory/effector (%)														
$CD8^+CD45RA^+CD28^+$	0.098	0.984	0.117	0.993	0.184	0.999	0.129	0.996	0.162	0.999	0.365	0.999	**0.002**	0.085
$CD8^+CD45RA^-CD28^+$	0.755	0.999	0.404	0.999	0.712	0.999	0.953	0.999	0.631	0.999	0.566	0.999	0.583	0.999
$CD8^+CD45RA^-CD28^-$	0.713	0.999	0.866	0.999	0.616	0.999	0.411	0.999	0.879	0.999	0.996	0.999	0.221	0.999
$CD8^+CD45RA^+CD28^-$	0.088	0.975	**0.014**	0.426	0.698	0.999	0.197	0.999	0.090	0.977	0.172	0.999	0.054	0.890
Activated T-cells (%)														
$CD4^+CD38^+$	0.102	0.987	0.267	0.999	0.070	0.944	0.335	0.999	0.059	0.913	0.087	0.974	0.461	0.999
$CD8^+CD38^+$	**0.001**	**0.030**	**0.009**	0.290	**0.001**	**0.032**	**0.001**	**0.041**	**0.008**	0.267	**0.001**	**0.034**	**0.038**	0.791
Senescence														
Telomere length	**0.005**	0.182	0.072	0.950	**0.001**	**0.046**	**0.001**	**0.025**	0.119	0.994	**0.005**	0.167	0.120	0.994
Senescent CD8+ T-cells (%)														
$CD8^+CD57^+$	0.191	0.999	0.186	0.999	0.342	0.999	0.299	0.999	0.209	0.999	0.220	0.999	0.323	0.999
$CD8^+CD57^+CD28^-$	0.218	0.999	0.218	0.999	0.360	0.999	0.357	0.999	0.215	0.999	0.258	0.999	0.315	0.999
Regulatory CD4+ T-cells (%)														
$CD4^+CD25^+CD127^{-/low}$	0.717	0.999	0.295	0.999	0.603	0.999	0.769	0.999	0.731	0.999	0.431	0.999	0.331	0.999
$CD4^+CD25^+CD127^{-/low}CD45RA^-$	0.759	0.999	0.506	0.999	0.853	0.999	0.615	0.999	0.962	0.999	0.380	0.999	0.158	0.999
$CD4^+CD25^+CD127^{-/low}CD45RA^+$	0.515	0.999	0.122	0.995	0.585	0.999	0.563	0.999	0.570	0.999	0.298	0.999	0.480	0.999
Bacterial translocation														
sCD14 (µg/mL)	**<0.001**	**0.018**	**0.009**	0.312	**<0.001**	**0.010**	**0.004**	0.139	**0.001**	**0.042**	**0.005**	0.170	**<0.001**	**0.003**
FABP2 (ng/mL)	0.085	0.972	0.496	0.999	**0.013**	0.416	0.177	0.999	0.095	0.981	0.182	0.999	**0.037**	0.778
LPS (UE/mL)	**<0.001**	**<0.001**	**<0.001**	**0.008**	**<0.001**	**0.001**	**0.001**	**0.048**	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**0.006**
LBP (µg/mL)	0.699	0.999	0.482	0.999	0.937	0.999	0.633	0.999	0.835	0.999	0.857	0.999	0.424	0.999
Anti-inflammatory/suppressor														
IL-10 (pg/mL)	**<0.001**	**0.007**	**<0.001**	**0.020**	**0.002**	0.084	**0.015**	0.444	**<0.001**	**0.002**	**0.001**	**0.021**	**0.003**	0.120
TGF-β1 (ng/mL)	0.941	0.999	0.458	0.999	0.476	0.999	0.953	0.999	0.854	0.999	0.937	0.999	0.638	0.999
IL1RA (pg/mL)	0.524	0.999	0.106	0.989	0.515	0.999	0.513	0.999	0.637	0.999	0.597	0.999	0.500	0.999

IL-4 (pg/mL)	**0.002**	0.062	**0.002**	0.073	**0.017**	0.488	**0.035**	0.763	**0.001**	**0.025**	**0.007**	0.232	**0.002**	0.085
Proinflammatory chemokines														
IP-10 (pg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.008**
MCP1 (pg/mL)	0.070	0.946	0.178	0.999	0.061	0.918	0.083	0.968	0.146	0.998	0.081	0.966	0.215	0.999
IL-8 (pg/mL)	**<0.001**	**<0.001**	**<0.001**	**0.002**	**<0.001**	**0.001**	**<0.001**	**<0.001**	**0.001**	**0.048**	**<0.001**	**<0.001**	**0.016**	0.467
Pro-inflammatory cytokines														
IL-1β (pg/mL)	**<0.001**	**0.001**	**<0.001**	**0.001**	**0.002**	0.066	**0.002**	0.065	**<0.001**	**0.001**	**<0.001**	**0.016**	**<0.001**	**0.001**
IL-18 (pg/mL)	**<0.001**	**0.002**	**<0.001**	**0.004**	**0.002**	0.075	**0.001**	**0.023**	**<0.001**	**0.012**	**<0.001**	**0.002**	**0.034**	0.751
IL-6 (pg/mL)	**<0.001**	**0.001**	**0.001**	0.058	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.003**	0.113	**<0.001**	**<0.001**	0.106	0.989
TNF-α (pg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.015**	**0.001**	**0.038**	**<0.001**	**<0.001**	**<0.001**	**0.001**	**<0.001**	**0.007**
IFN-γ (pg/mL)	0.280	0.999	0.501	0.999	0.205	0.999	0.639	0.999	0.159	0.999	0.258	0.999	0.627	0.999
IL-12p70 (pg/mL)	**0.001**	**0.028**	**<0.001**	**0.018**	**0.018**	0.513	0.070	0.946	**<0.001**	**0.002**	**0.002**	0.091	**0.003**	0.120
IL-2 (pg/mL)	**<0.001**	**0.007**	**0.001**	**0.034**	**0.001**	**0.039**	**0.032**	0.725	**<0.001**	**<0.001**	**0.001**	**0.049**	**<0.001**	**0.008**
IL-17A (pg/mL)	**0.005**	0.171	**0.002**	0.085	0.067	0.937	0.180	0.999	**<0.001**	**0.013**	**0.011**	0.352	**0.015**	0.453
Endothelial dysfunction														
sVCAM1 (µg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
sICAM1 (µg/mL)	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.001**
sTNFR1 (ng/mL)	**0.001**	**0.023**	**0.031**	0.715	**<0.001**	**0.002**	**0.002**	0.066	**0.003**	0.131	**<0.001**	**0.016**	0.087	0.974
Coagulopathy														
D-Dimer (ng/mL)	**0.002**	0.070	**0.006**	0.215	**0.006**	0.219	**0.003**	0.105	**0.012**	0.390	**<0.001**	**0.014**	0.594	0.999
PAI-1 (ng/mL)	**0.001**	**0.030**	**0.005**	0.189	**0.002**	0.063	**0.015**	0.447	**0.001**	**0.022**	**0.001**	**0.029**	0.054	0.890

Statistics: (*), *P*-values were calculated by Mann-Whitney tests. (**), *P*-values were adjusted by Bonferroni correction for multiple comparisons.
Abbreviations: HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; CDXX, cluster of differentiation; sCD14, soluble CD14; LPS, lipopolysaccharide; FABP2, fatty acid-binding protein 2; LBP, lipopolysaccharide binding protein; IL, interleukin; IP-10, IFN-γ-inducible protein 10; IL-1RA, interleukin-1 receptor antagonist; sVCAM-1, soluble vascular cell adhesion molecule 1; sICAM-1, soluble intercellular cell adhesion molecule 1; sTNFR-1, soluble tumor necrosis factor receptor 1; MCP-1, monocyte chemoattractant protein-1, PAI-1, plasminogen activator inhibitor-1; TGF-β1, transforming growth factor beta 1; IFN-γ, Interferon gamma; IL, interleukin; T-reg, regulatory CD4+ T-cells; TNF-α, tumor necrosis factor alpha.