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RESEARCH ARTICLE

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HCV spontaneous clearers showed low senescence profile in people living with HIV under long ART

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Abstract

Coinfection with hepatitis C virus (HCV) and human immunodeficiency virus (HIV) increases immune activation, inflammation, and oxidative stress that could lead to premature senescence. Different HCV infections, either acute or chronic infection, could lead to distinct premature cellular senescence in people living with HIV (PLWHIV). Observational study in 116 PLWHIV under antiretroviral treatment with different HCV status: (i) $n = 45$ chronically infected with HCV (CHC); (ii) $n = 36$ individuals who spontaneously clarify HCV (SC); (iii) $n = 35$ HIV controls. Oxidative stress biomarkers were analyzed at lipid, DNA, protein, and nitrates levels, as well as

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; aAMR, adjusted arithmetic mean ratio; AIDS, acquired immunodeficiency syndrome; AMR, arithmetic mean ratio; ART, antiretroviral treatment; BCA, bicinchoninic acid; BMI, body mass index; CHC, chronically infected with HCV; DDR, DNA damage response; DNA, deoxyribonucleic acid; DNP, dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazzine; EDTA, ethylenediaminetetraacetic acid; FDR, false discovery rate; GLMs, generalized linear model; GM-CSF, granulocyte macrophage colony-stimulating factor; Gro-α, growth-regulated oncogene-alpha; GSH, reduced glutathione; GSSG, oxidized glutathione; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFNL4, interferon lambda 4; IFN-γ, interferon-gamma; *IFN-λ3*, interferon lambda 3 gene; IL-13, interleukin-13; IL-15, interleukin-15; IL-1α, interleukin-1 alpha; IL-1β, interleukin-1 beta; IL-2, interleukin-2; IL-6, interleukin-6; IL-7, interleukin-7; IL-8, interleukin-8; IP-10, C-X-C motif chemokine ligand 10; MDA, malondialdehyde; MMqPCR, monochromatic multiplex real-time quantitative PCR; NO, nitric oxide; PBMCs, peripheral blood mononuclear cells; PCC, protein carbonyl content; PCR, polymerase chain reaction; PIGF-1, placental growth factor 1; PLWHIV, people living with HIV; RNA, ribonucleic acid; ROS, reactive oxygen species; RTL, relative telomere length; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SASP, senescence-associated secretory phenotype; SC, spontaneously clarifiers; SCF, stem cell factor; TAC, total nonenzymatic antioxidant capacity; TBA, thiobarbituric acid; Th1, type 1 T helper; Th2, type 2 T helper; TNF-α, tumor necrosis factor-alpha; TNF-β, tumor necrosis factor-beta.

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antioxidant capacity and glutathione reductase enzyme. Replicative senescence was evaluated by relative telomere length (RTL) measurement. Additionally, 26 markers of Senescence-Associated Secretory Phenotype (SASP) were analyzed by multiplex immunoassays (Luminex xMAP technology). Differences were evaluated by generalized linear model (GLMs) adjusted by most significant covariates. The SC group had a senescence signature similar to the HIV control group and slightly lower SASP levels. However, significant differences were observed with respect to the CHC group, where an increase in the nitrate concentration [adjusted arithmetic mean ratio, aAMR = 1.73 (1.27–2.35), $p < 0.001$, $q = 0.009$] and the secretion of 13 SASP-associated factors [granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-β, interleukin (IL)-1β, IL-2, IL-8, IL-13, tumor necrosis factor (TNF)-α, IL-1α, IL-1RA, IL-7, IL-15, C-X-C motif chemokine ligand 10 (IP-10), stem cell factor (SCF); $q < 0.1$)] was detected. The CHC group also showed higher values of IL-1α, IP-10, and placental growth factor 1 (PlGF-1) than HIV controls. The SC group showed a slightly lower senescence profile than the HIV group, which could indicate a more efficient control of viral-induced senescence due to their immune strengths. Chronic HCV infection in PLWHIV led to an increase in nitrate and elevated SASP biomarkers favoring the establishment of viral persistence.

KEYWORDS
HCV/HIV, oxidative stress, SASP, senescence, spontaneous clearance, telomere

1 | INTRODUCTION

Hepatitis C (HCV) and human immunodeficiency virus (HIV) infections remain a serious public health problem. Coinfection affects 1/10 of the HIV-positive population worldwide, exacerbating the natural history of both viruses, accelerating liver fibrosis, and even reducing life expectancy.[1] After an acute infection, spontaneous clearance may occur in approximately 20% of patients within 6 months of infection without any treatment,[2] although it is a rare phenomenon in the context of coinfection with HIV.[3] Besides other coinfections, genetic background, clinical and virological factors also affect HCV spontaneous clearance. Thus, genetic variation at *interferon lambda 3 gene* (*IFN-λ$_3$*), formerly named as *IL28B*, is strongly associated with spontaneous clearance of HCV and also response to treatment.[4] Additionally, female gender also shows better prognosis of HCV infection, with lower HCV RNA viral loads and higher spontaneous clearance.[5]

Although many studies have tried to unravel the factors that predispose to HCV spontaneous clearance, few studies have delved into their clinical consequences. Some studies have pointed out that patients that cleared the infection are at lower risk of mortality and liver-related death than patients who are chronically infected.[6] Conversely, HCV chronic infection showed similar impact on peripheral blood cells (PBMCs) than an acute infection followed by spontaneous resolution.[7] Therefore, more studies are essential to understand the potential immune consequences after HCV resolution.

Chronic HCV coinfected with HIV increases immune activation, which favors the progression of HIV infection and the development of liver disease leading to premature senescence.[8] Senescent cells often exhibit high levels of various forms of damage accumulation overtime in an old organism, including DNA damage and oxidative modifications.[9] In addition, coinfection can lead to T-cell exhaustion, mainly in chronic infections.[10] Progressive loss of immunological memory correlates with reduced proliferative capacity and telomere shortening in T cells. In fact, telomere shortening has been observed in circulating lymphocytes from both HCV+[11] and HIV+ patients.[12] Telomeres are nucleoprotein structures located at the end of eukaryotic chromosomes formed by repeated DNA sequences (TTAGGG), which maintain the integrity of the chromosome. In each round of replication, telomeres become progressively shorter and when they reach a critical length, cell cycle arrest, cellular senescence, and apoptosis occur.[13] Thus, telomere length is a hallmark of cellular aging associated with the progression of several diseases[14,15] and an increased risk of infection.[16]

HIV and HCV infections produce an indistinguishable form of cellular senescence that shares features of chronic inflammation in aging,[17] accompanied by a pro-inflammatory phenotype called the Senescence-Associated Secretory Phenotype (SASP).[18] This phenotype includes the production of cytokines, extracellular matrix factors, and coagulation mediators,[19] which acts as an antiviral mechanism of the senescent cell and allows limitation of viral replication.[20] However, whatever its primary function, SASP can have

both beneficial effects and detrimental consequences.[19] Some of these parameters improve after HCV elimination, but there is increasing evidence that inflammatory markers are not restored after HCV elimination.[21] This may result in increased comorbidities and mortality in patients who have overcome chronic HCV infection after successful treatment,[22] but there is very little information on patients recovered from spontaneous clearance and even fewer studies focused on the assessment of senescence in these patients.

Therefore, we propose a comprehensive approach to assess inflammation, aging, and senescence-related markers in people living with HIV (PLWHIV) after HCV spontaneous clearance with respect to HCV chronically infected (CHC) and HIV controls. The aim of this work is to analyze the impact of an acute HCV infection followed by a spontaneous clearance and chronic infection on senescence markers which may help to identify patients at risk of developing premature senescence-related diseases and thus anticipate therapeutic interventions in PLWHIV under long ART.

2 | METHODS

2.1 | Study design

We carried out a prospective observational multicenter study from the COVIHEP cohort (Supporting Information: File 1) from five Public Spanish Hospitals in Madrid Autonomous Community: Hospital Universitario La Paz, Hospital Universitario 12 de Octubre, Hospital Universitario Infanta Leonor, Hospital Universitario La Princesa and Hospital Puerta de Hierro. Samples were processed at the National Center for Microbiology, Institute of Health Carlos III, Madrid, Spain. The study was conducted in accordance with the Declaration of Helsinki; all patients gave their written consent before enrollment, and an Institutional Review Board approved this study approved study (CEI PI 81_2017-v3).

One hundred sixteen PLWHIV with different statuses to HCV infection were enrolled: (1) 45 patients with active HCV-chronic infection naïve to any HCV treatment (CHC group) [positive polymerase chain reaction (PCR) and positive HCV antibodies]; (2) 36 HIV patients who spontaneously cleared HCV within 6 months of HCV infection [spontaneously clarifiers (SC) group] (negative PCR and positive HCV antibodies, in the absence of anti-HCV treatment); (3) 35 HIV monoinfected patients without evidence of previous HCV infection (HIV control group) (negative PCR and negative HCV antibody). All patients received suppressive antiretroviral treatment (ART) during at least 1 year and were undetectable for HIV during the previous year, with CD4+ T-cells counts ≥ 500 cells/mm^3 since at least 1 year before sample collection. Patients were followed up every 6 months to monitor HIV infection and HCV screening according to clinical guidelines.[23] Exclusion criteria were pregnancy, individuals below 18 years old, previously HCV treatment, advanced liver fibrosis (>F3), clinical evidence of hepatic decompensation, active drug or alcohol addiction, alcohol-induced liver injury, hepatitis B virus (HBV) active infection, opportunistic infections, and other concomitant diseases such as diabetes, neoplasia, autoimmune disease, among others. Clinical and epidemiology data were obtained from medical records.

2.2 | Biological material

Peripheral venous blood samples were collected in ethylenediaminetetraacetic acid tubes. PBMCs were isolated by density gradient centrifugation method with Lymphoprep™ and SepMate™ tubes (Stemcell Technologies) and stored at viability until use. Plasma was obtained after density gradient centrifugation, clarified, and storage at −80°C until use.

2.3 | Senescence biomarkers in plasma

The cellular senescence was assessed by reactive oxygen species (ROS) markers levels, total antioxidant capacity, glutathione, and SASP levels.

2.4 | Oxidative stress

2.4.1 | ROS markers

ROS damage

At *DNA level*: We measured the 8-hydroxydeoxyguanosine (8-OHdG) DNA levels which reflect the *DNA damage response* (DDR), with the DNA Damage Competitive ELISA kit (Thermo Fisher Scientific).

At *protein level*: We analyzed the oxidation of proteins with the *protein carbonyl content* (PCC) assay kit (Sigma-Aldrich) and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). The carbonyl content was determined by the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazzine (DNPH) leading to the formation of stable dinitrophenyl (DNP) hydrazine adducts.

At *lipid level*: We evaluated *lipid peroxidation*, with the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich), which measured the formation of malondialdehyde (MDA) combined with thiobarbituric acid (TBA) by absorbance. The manufacturer's instructions were followed, with the slight exception that the butanol was removed by overnight evaporation at room temperature.

Oxidant molecules

Nitrate levels. We assessed nitrate by the Nitric Oxide Assay Kit (Thermo Fisher Scientific), following manufacturer instructions.

2.4.2 | Antioxidant capacity

The *total nonenzymatic antioxidant capacity* (TAC) was evaluated with the Total Antioxidant Capacity Assay Kit (Sigma-Aldrich) without Protein Mask, which account for lipid-soluble (tocopherols,

carotenes, vitamin A) and water-soluble (glutathione) antioxidants indicative of their ability to counteract oxidative stress-induced damage in cells.

We determined the *reduced glutathione* (GSH), and *oxidized glutathione* (GSSG) by the GSH Colorimetric Detection Kit (Thermo Fisher Scientific).

2.5 | SASP

We assessed a selection of 26 markers associated to SASP, including cytokines, chemokines, and growth factors[18,24] (complete list is available in Supporting Information: File 2). All markers were analyzed with a multiplex immunoassay (Luminex xMAP technology).

2.6 | Senescence biomarkers in PBMCs

2.6.1 | Replicative senescence

DNA was extracted from PBMCs using the DNA Purification System Kit (Promega Wizard). We performed the quantification of replicative senescence by monochromatic multiplex real-time quantitative PCR (MMqPCR) as previously described.[25] To normalize and control the number of telomere copies/sample we used the single-copy gene (β-globin). Relative leukocyte telomere length (RTL) was expressed as the ratio of the telomere amplification product (T) to that of a single copy gene (S).

2.6.2 | Statistical analysis of epidemiological data

For the descriptive study of clinical and epidemiological data of the patients, continuous variables were summarized as median, and categorical as frequency and percentage. Concentration values are used for oxidative stress markers and raw fluorescence intensity as a relative quantification of the analyte abundances for SASP markers, as previously described.[26] Significant differences between categorical data were calculated using the χ^2 test or Fisher's exact test. Kruskal–Wallis and Mann–Whitney *U* tests were used to compare continuous variables among independent groups. Univariate and multivariate analysis with generalized linear models (GLM), gamma distribution (log-link), were carry out to estimate differences in senescence biomarkers levels and RTL among groups. The GLM was adjusted for the most significant variables (age, gender, body mass index [BMI], transmission route, duration of HIV infection, HIV clinical stage, HIV treatment, interferon lambda 4 (IFNL4) genotype, HCV genotype, and CD4 T nadir) with a stepwise approach. *q* Value ≤ 0.1 [*p* value corrected for the false discovery rate (FDR) by Benjamini–Hochberg correction] were considered significant. IBM® SPSS Statistics (v.19) and statistical software R (v 3.2.0) (www.r-project.org) were used for all statistical analyses.

3 | RESULTS

3.1 | Epidemiological and clinical characteristics of the patients

Epidemiological characteristics are shown in Table 1. Overall, the median age was 50 years, and 44.8% were female. Both weight and BMI showed significant differences between groups, with the coinfected group showing the lowest values. The median number of years of HIV infection was significantly lower in the control group than CHC. Most of patients included in the SC and CHC groups acquired both HIV and HCV at the same time, except for a small percentage who were already HIV+ when they acquired HCV (7/36 in the SC group; 14/45 in the CHC group), but differences were not significant ($p = 0.234$). Patients who had been in contact with HCV were mainly prior intravenous drug users ($p < 0.001$). SC group showed higher frequency of favorable allele (CC) at *IFNL4* ($p = 0.001$). Regarding lymphocyte count, we observed a higher CD4+ T cells count ($p = 0.04$) and lower nadir CD4+ T cells ($p = 0.02$) in the SC group compared to the HIV+ group.

Regarding metabolic characteristics, biochemical parameters related to liver function were significantly increased in CHC group (Table 2).

3.2 | Senescence profile analysis among different HCV statuses in PLWHIV

The senescence profile between groups was evaluated by a GLM model adjusted by age, sex and *IFN-λ*$_3$ genotype, which were selected by a stepwise procedure.

3.2.1 | Senescence-associated markers after HCV acute infection (SC) versus HIV

Overall, we did not find any differences in oxidative stress levels and replicative senescence between SC and HIV groups (Figure 1, Supporting Information: File 3). However, in relation to the SASP, the SC group showed significantly higher concentrations of interleukin (IL)-1α [adjusted arithmetic mean ratio, aAMR = 1.31 (1.04–1.64), $p = 0.02$, $q = 0.23$] and lower levels of tumor necrosis factor-alpha (TNF-α) [aAMR = 0.83 (0.70–0.98), $p = 0.09$, $q = 0.25$], IL-7 [aAMR= 0.87 (0.77-0.98), $p = 0.03$, $q = 0.25$] and C-X-C motif chemokine ligand 10 (IP-10) [aAMR = 0.77 (0.60–0.99), $p = 0.04$, $q = 0.25$] compared to HIV control group (Figure 2, Supporting Information: File 4), although none of these markers retained statistical significance after adjustment for FDR ($q < 0.1$).

3.2.2 | Senescence-associated markers in HCV long-term infection (CHC) versus HIV

CHC patients showed a tendency to increase higher nitrate levels [aAMR = 1.40 (1.07–1.84), $p = 0.02$, $q = 0.14$] and similar replicative

TABLE 1 Epidemiological and clinical characteristics of the study population.

	Total	HIV	SC	CHC	*p* Value[a]	*p* Value[b]	*p* Value[c]	*p* Value[d]
No.	116	35	36	45	-	-	-	-
Gender (female)	52/116 (44.8%)	18/35 (51.4%)	16/36 (44.4%)	18/45 (40.0%)	0.59	0.56	0.31	0.69
Age (years)	50/116 (45–54)	49/35 (41–56)	52/36 (48-55)	50/45 (45–53)	0.37	0.33	0.90	0.16
BMI (kg/m^2)	23.68 (21.29–26.26)	24.61 (22.34–27.26)	24.72 (22.05–28.04)	22.48 (20.81–25.72)	**0.04***	0.93	**0.04***	**0.04***
HIV infection (years)	19.84 (8.83–26.06)	17.13 (5.67–22.22)	22.98 (11.63–28.01)	21.34 (9.59–27.06)	0.07	**0.03***	0.09	0.47
Transmission route								
IVDU	47/103 (45.6%)	0/29 (0.00%)	21/32 (65.6%)	26/42 (61.9%)	**<0.001***	**<0.001***	**<0.001***	0.83
IVDU + sexual	2/103 (1.9%)	0/29 (0.00%)	1/32 (3.1%)	1/42 (2.4%)				
Sexual	53/103 (51.5%)	29/29 (100%)	10/32 (31.3%)	14/42 (33.3%)				
Vertical	1/103 (1.0%)	0/29 (0.00%)	0/32 (0.00%)	1/42 (2.4%)				
Time of HIV/HCV acquisition								
HIV/HCV cotransmission	-	-	29/36 (80.6%)	31/45 (68.9%)	-	-	-	0.234
HCV acquistion after HIV infection	-	-	7/36 (19.4%)	14/45 (31.1%)				
HIV clinical stage								
A	57/107 (53.3%)	22/32 (68.8%)	13/32 (40.6%)	22/43 (51.2%)	0.20	0.07	0.22	0.56
B	20107 (18.7%)	5/32 (15.6%)	8/32 (25.0%)	7/43 (16.3%)				
C	30107 (28.6%)	5/32 (15.6%)	11/32 (34.4%)	14/43 (32.6%)				
IFNL4 genotype								
CC	59/116 (50.9%)	18/35 (51.4%)	27/36 (75.0%)	14/45 (31.1%)	**0.001***	**0.03***	0.09	**<0.001***
CT	47/116 (40.5%)	16/35 (45.7%)	6/36 (16.7%)	25/45 (55.6%)				
TT	10/116 (8.6%)	1/35 (2.9%)	3/36 (8.3%)	6/45 (13.3%)				
ART regimen								
INSTIs	47/115 (40.9%)	17/35 (48.6%)	12/36 (33.3%)	18/44 (40.9%)	0.45	0.15	0.81	0.53
PIs	10/115 (8.7%)	2/35 (5.7%)	3/36 (8.3%)	5/44 (11.4%)				

(Continues)

TABLE 1 (Continued)

	Total	HIV	SC	CHC	*p* Value[a]	*p* Value[b]	*p* Value[c]	*p* Value[d]
NRTIs	3/115 (2.6%)	2/35 (5.7%)	0/36 (0%)	1/44 (2.3%)				
NNRTIs	36/115 (31.3%)	7/35 (20%)	17/36 (47.2%)	12/44 (27.3%)				
Bitherapy	15/115 (13%)	6/35 (17.1%)	3/36 (8.3%)	6/44 (13.6%)				
Monotherapy	4/115 (3.5%)	1/35 (2.9%)	1/36 (2.8%)	2/44 (4.5%)				
Lymphocyte count								
CD4+ T (cell/mm^3)	760.40 (595–1013)	818 (674.7–1046.4)	732.7 (559.8–926.1)	712 (530–1047.6)	0.08	**0.04***	0.06	0.92
CD8+ T (cell/mm^3)	863 (650–1150)	942 (792–1368)	871 (598–1141)	790 (634–1048)	0.48	0.41	0.23	0.79
CD4/CD8 ratio	0.87 (0.64–1.17)	1.02 (0.64–1.24)	0.99 (0.74–1.18)	0.81 (0.56–1.00)	0.41	1.00	0.31	0.22
CD4 T nadir	216 (142–302)	257 (180–324)	205 (89–240)	215 (100–324)	0.10	**0.02***	0.34	0.24
HCV genotype								
1	3/16 (2.6%)	-	-	3/15 (6.7%)				
2	-	-	-	-	**<0.001***	-	-	**<0.001***
3	1/16 (0.9%)	-	-	1/15 (2.2%)				
4	12/16 (10.3%)	-	1/1 (2.8%)	11/15 (24.4%)				

Note: Statistics: values are expressed as the absolute numbers (%) and median (interquartile range). *p* Values were estimated by Kruskal–Wallis and Mann–Whitney *U* test for continuous variables and χ^2 or Fisher's exact test for categorical variables. Statistically significant values are represented with * in bold ($p < 0.05$).

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; CHC, chronically infected with HCV; HCV, hepatitis C virus; HIV, human immunodeficiency virus; INSTI, integrase inhibitor; IVDU, intravenous drug user; NNRTI, nonnucleoside reverse transcriptase inhibitors; NRTI, nucleoside analog reverse transcriptase inhibitor; PI, protease inhibitor; SC, spontaneously clarify HCV.

[a]Comparison between all the three groups enrolled in this study.

[b]Comparison between HIV and SC groups.

[c]Comparison between HIV and CHC groups.

[d]Comparison between SC and CHC groups.

TABLE 2 Metabolic characteristics of all groups of patients enrolled in this study.

	Total	HIV	SC	CHC	*p* Value[a]	*p* Value[b]	*p* Value[c]	*p* Value[d]
No.	116	35	36	45	-	-	-	-
Lipid profile								
Glu (mg/dL)	92.00 (86.00–99.00)	92.50 (86.00–97.00)	96.50 (89.00–102.50)	88.00 (86.00–95.00)	0.09	0.13	0.45	**0.04***
Glu ≥ 110 (mg/dL)	11/91 (12.10%)	2/26 (7.70%)	5/28 (17.90%)	4/37 (10.80%)	0.50	0.27	0.68	0.42
TC (mg/dL)	189.50 (170.50–208.50)	185.00 (172.00–205.00)	192.00 (178.00–223.00)	186.00 (166.00–203.00)	0.33	0.52	0.44	0.14
TC ≥ 200 (mg/dL)	33/88 (37.50%)	10/26 (38.50%)	11/27 (40.70%)	12/35 (34.30%)	0.87	0.87	0.74	0.60
LDL (mg/dL)	112.00 (96.50–132.50)	115.50 (100.00–145.50)	115.00 (100.00–133.00)	108.00 (82.00–130.00)	0.33	0.92	0.23	0.20
LDL ≥ 130 (mg/dL)	25/80 (31.30%)	9/24 (37.50%)	6/21 (28.60%)	10/35 (28.60%)	0.73	0.53	0.47	1.00
HDL (mg/dL)	49.00 (40.00–56.50)	46.00 (37.00–55.00)	51.00 (45.00–57.00)	50.00 (40.00–61.00)	0.59	0.36	0.38	0.93
TG (mg/dL)	126.00 (92.50–181.00)	116.00 (95.50–183.50)	131.00 (118.00–182.00)	126.00 (79.00–180.00)	0.22	0.29	0.44	0.10
TG ≥ 200 (mg/dL)	15/84 (17.90%)	5/24 (20.80%)	6/25 (24.00%)	4/35 (11.40%)	0.41	0.79	0.32	0.20
LDL/HDL	2.43 (1.88–2.92)	2.43 (2.10–2.99)	2.42 (1.91–2.95)	2.22 (1.64–2.86)	0.46	0.65	0.27	0.38
AI (TC/HDL)	3.86 (3.29–4.63)	3.95 (3.63–5.14)	4.00 (3.36–4.60)	3.83 (3.02–4.43)	0.38	0.66	0.20	0.34
Low risk	63/80 (78.80%)	17/24 (70.80%)	17/21 (81.00%)	29/35 (82.90%)	0.52	0.43	0.27	0.86
Moderate risk	17/80 (21.30%)	7/24 (29.20%)	4/21 (19.00%)	6/35 (17.10%)	0.52	0.43	0.27	0.86
AIP log (TG/HDL)	0.40 (0.27–0.57)	0.39 (0.30–0.63)	0.40 (0.32–0.62)	0.42 (0.13–0.54)	0.65	0.84	0.48	0.41
High risk	63/80 (78.80%)	22/24 (91.70%)	17/21 (81.00%)	24/35 (68.60%)	0.10	0.29	**0.04***	0.31
LCI	54.93 (33.18–83.74)	60.0 (35.6–123.3)	70.2 (44.1–81.3)	43.3 (23.70–75.70)	0.23	0.84	0.21	0.11
Liver function								
AST (mg/dL)	26.00 (22.00–34.00)	23.50 (20.00–27.00)	23.00 (19.00–26.00)	37.00 (29.00–46.00)	**<0.001***	0.10	**<0.001***	**<0.001***
AST ≥ 40 (mg/dL)	14/90 (15.60%)	2/26 (7.70%)	0/27 (0.00%)	12/37 (32.40%)	**<0.001***	0.14	**0.02***	**0.001***
ALT (mg/dL)	28.00 (22.00–45.00)	27.50 (22.00–33.00)	21.50 (17.00–25.00)	45.00 (32.00–55.00)	**<0.001***	**0.006***	**<0.001***	**<0.001***
ALT ≥ 40 (mg/dL)	27/91 (29.70%)	5/26 (19.20%)	0/28 (0.00%)	22/37 (59.50%)	**<0.001***	**0.02***	**0.001***	**<0.001***
GGT (mg/dL)	33.00 (24.00–52.00)	26.50 (21.00–43.00)	32.00 (26.00–40.00)	48.00 (32.00–89.00)	**0.002***	0.26	**0.002***	**0.009***
GGT ≥ 50 (mg/dL)	24/84 (28.60%)	5/24 (20.80%)	2/26 (7.70%)	17/34 (50.00%)	**<0.001***	0.18	**0.02***	**<0.001***
Albumin	4.40 (4.00–4.50)	4.15 (4.00–4.70)	4.50 (4.40–4.70)	4.40 (4.00–4.40)	0.15	0.30	0.79	**0.04***

(Continues)

TABLE 2 (Continued)

	Total	HIV	SC	CHC	*p* Value[a]	*p* Value[b]	*p* Value[c]	*p* Value[d]
ALP	83.00 (66.00–101.00)	77.00 (66.00–94.00)	94.50 (70.00–116.00)	85.00 (65.00–96.00)	0.12	0.06	0.49	0.10
TBIL	0.47 (0.38–0.60)	0.42 (0.40–0.57)	0.40 (0.29–0.55)	0.50 (0.44–0.62)	0.05	0.34	0.10	**0.03***

Note: Statistics: values are expressed as the absolute numbers (%) and median (interquartile range). *p* Values were estimated by Kruskal–Wallis and Mann–Whitney *U* test for continuous variables and χ^2 or Fisher's exact test for categorical variables. Statistically significant values are represented with * in bold ($p < 0.05$).

Abbreviations: AI, atherogenic index (low risk 0.21); ALP, alkaline phosphatase; ALT, alanina aminotransferase; APRI, AST/platelets index; AST, aspartato aminotransferasa; CHC, chronically infected with HCV; FIB-4, fibrosis-4 index; GGT, gamma glutamil transferasa; Glu, glucose; HDL, high-density lipoprotein; HCV, hepatitis C virus; HIV, human immunodeficiency virus; LCI, lipoprotein index [(TC × TG × LDL)/HDL]; LDL, low-density lipoprotein; SC, spontaneously clarify HCV; TBIL, total bilirubin; TC, total cholesterol; TG, triglycerides.

[a]Comparison between all the three groups enrolled in this study.

[b]Comparison between HIV and SC groups.

[c]Comparison between HIV and CHC groups.

[d]Comparison between SC and CHC groups.

senescence compared to the HIV group (Figure 1, Supporting Information: File 5). However, regarding the SASP, CHC showed significantly higher levels of IL-1α [aAMR = 1.90 (1.42–2.55), $p < 0.001$, $q = 0.02$], IP-10 [aAMR = 1.46 (1.14–1.87), $p = 0.003$, $q = 0.03$] and placental growth factor 1 (PIGF-1) [aAMR = 1.36 (1.11–1.67), $p = 0.003$, $q = 0.03$] in comparison to HIV control group (Figure 2, Supporting Information: File 6).

3.2.3 | Senescence-associated markers in chronic HCV long-term infection versus HCV acute infection

(Significant increased levels of nitrate [aAMR = 1.73 (1.27–2.35), $p < 0.001$, $q = 0.008$] were observed in the CHC group compared to the SC group (Figure 1, Supporting Information: File 7), as well as significant higher levels of 13 soluble biomarkers related to immune senescence (Figure 2, Supporting Information: File 8). In particular, cytokines related to the Th1/Th2 response, as granulocyte macrophage colony-stimulating factor (GM-CSF) [aAMR = 1.30 (1.14–1.52), $p = 0.002$, $q = 0.01$], IFN-γ [aAMR = 1.33 (1.05–1.71), $p = 0.02$, $q = 0.05$], IL-1β [aAMR = 1.30 (1.07–1.58), $p = 0.009$, $q = 0.03$], IL-2 [aAMR = 1.20 (1.02–1.42), $p = 0.03$, $q = 0.06$], IL-8 [aAMR = 1.11 (1.01–1.22), $p = 0.03$, $q = 0.06$], IL-13 [aAMR = 1.21 (1.01–1.44), $p = 0.04$, $q = 0.08$], and TNF-α [aAMR = 1.42 (1.17–1.72), $p < 0.001$, $q = 0.006$]; and inflammatory cytokines, such as IL-1α [aAMR = 1.64 (1.16–2.32), $p = 0.005$, $q = 0.02$], IL-1RA [aAMR = 1.57 (1.16–2.13), $p = 0.004$, $q = 0.02$], IL-7 [aAMR = 1.26 (1.10–1.43), $p < 0.001$, $q = 0.006$] and IL-15 [aAMR = 1.40 (1.18–1.65), $p < 0.001$, $q = 0.006$]. The chemokine IP-10 [aAMR = 1.71 (1.34–2.20), $p < 0.001$, $q = 0.006$] and the stem cell factor (SCF) [aAMR = 1.17 (1.03–1.34), $p = 0.02$, $q = 0.05$].

4 | DISCUSSION

Our data show similar senescence profile between PLWHIV who spontaneously clarify HCV and HIV monoinfected individuals, but slightly lower levels of SASP. On the other hand, chronic HCV infection in PLWHIV showed higher nitrate concentration and increased plasma levels of SASP biomarkers.

Prior knowledge has shown that HCV infection, together with HIV,[27] promote oxidative stress and senescence,[28,29] especially during chronic infection and increasing with fibrosis progression,[30] probably to facilitate HCV persistence.[31] However, it was unknown how the acute infection and subsequent spontaneous clearance could affect the senescence profile or whether the immune strengths of spontaneous clearance patients allow them to better manage the senescence parameters.

Virus-induced senescence has been previously described for a variety of viruses,[32] as part of an antiviral response mechanism to limit viral replication.[20,33] However, the importance of senescence in acute inflammatory conditions such as respiratory viral infections has been less approached until the COVID-19 outbreak, as it has been mainly addressed in chronic infections. There is evidence that

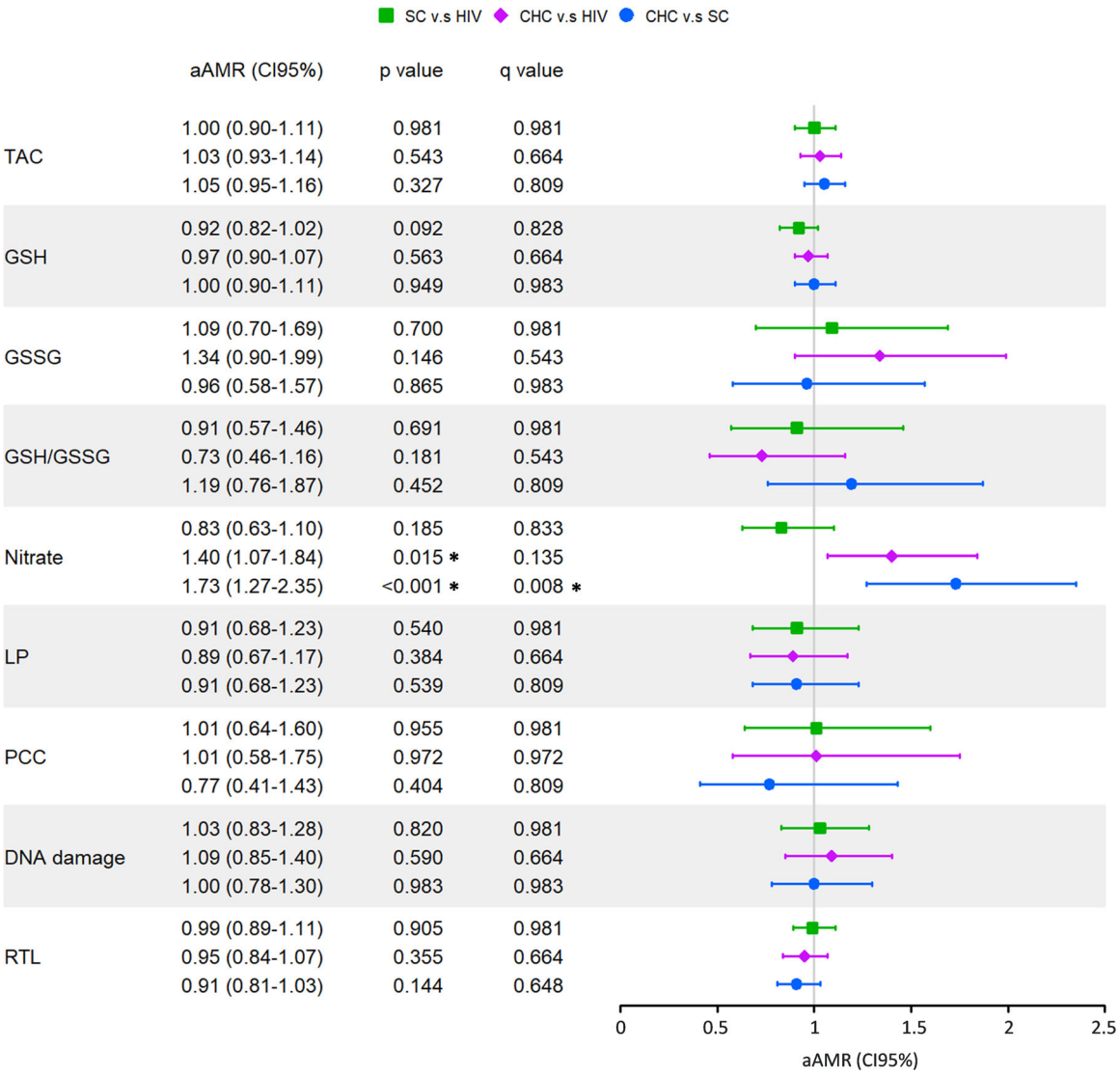

FIGURE 1 Oxidative stress and replicative senescence in people living with HIV with different HCV infection. Values are expressed as adjusted aAMR, obtained using a generalized linear model; *, $p < 0.05$ and $q < 0.1$. aAMR, adjusted arithmetic median rate; CHC, PLWHIV chronically infected with HCV; GSH, reduced glutathione; GSSG, oxidized glutathione; HCV, hepatitis C virus; HIV, human immunodeficiency virus mono-infected patients; LP, lipid peroxidation; PCC, protein carbonyl content; *q*, corrected level of significance by false discovery rate; RTL, relative telomere length; SC, PLWHIV who spontaneously clarify HCV; TAC, total antioxidant capacity.

senescence contributes to the pathophysiology of respiratory viral infections,[34] and a recent report has observed that viruses may convergently contribute to the evolution of human aging.[35] Thus, we assumed that all patients suffered from viral infections in a similar proportion and all of them were recruited previous COVID-19 pandemic.

The SC group showed similar levels of oxidative stress markers to the HIV group, which indicates a normalization of oxidative stress after HCV spontaneous resolution. Oxidative stress is an important key in HCV pathogenesis during both acute and chronic stages of inflammation, but little is known about long-term effects after spontaneous resolution in either HCV monoinfected patients or PLWHIV. HCV-induced oxidative stress significantly contributes to hepatic disease, playing a critical role in hepatic fibrogenesis and carcinogenesis.[36] However, patients in our cohort did not show advanced liver fibrosis, and probably the HCV-related oxidative stress has not been fully activated. Previous studies showed that HCV acute infection is characterized by decreased GSH in erythrocytes and increased ROS, viral replication, and apoptotic death.[37] Our data show that these makers are restored after HCV resolution, achieving similar redox environment that those PLWHIV who have never been infected by HCV.

Oxidative stress is a hallmark of chronic HCV infection[38] and is characterized by extensive production of reactive species, resulting in protein, lipid, and DNA damage that ultimately leads to liver injury.[39] The CHC group showed similar oxidative stress to SC individuals, except for an increase in nitrate concentration. IFN-1 stimulate the nitric oxide (NO) synthase enzyme that is expressed in hepatocytes

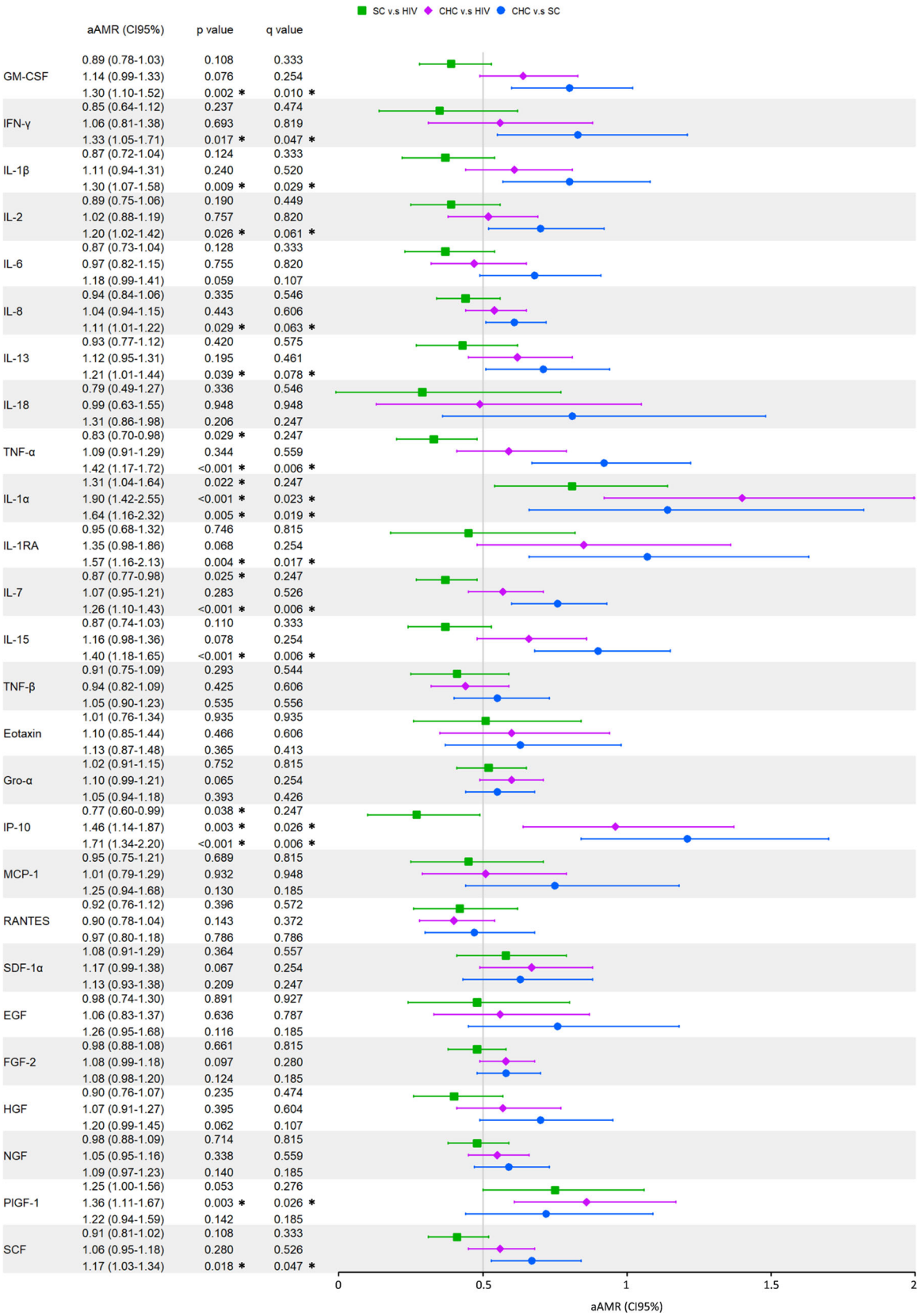

FIGURE 2 (See caption on next page).

and macrophages as the isoform inducible NO synthase.[40] The role of NO in HCV, is still unclear, but previous studies have reported higher concentrations in patients with chronic HCV infection than general population and HCV-seropositive patients,[41] being associated with the progression of liver cirrhosis.[42] However, it has been demonstrated that NO does not directly impact on HCV replication. NO impairs antivirals responses by suppressing type 1 helper T cell responses, favoring the viral persistence in liver cells.[43,44] In general, our results agree with previous findings where a re-establishment of the reduced environment, reduced ROS and increased GSH in chronic phase to promote viral persistence.[37]

Cell damage can also promote replicative senescence through telomere attrition. Overall, our results showed a tendency towards a shorter telomere length in PBMCs from HCV-infected individuals (either SC or CHC) compared to the HIV group, suggesting that HCV infection increases the replicative senescence in PBMCs, a phenomenon that may be due, in part, to the replication of both viruses in these cells.[45,46] This increased replicative senescence seems to be more pronounced in the CHC group, which is in accordance with previous studies that reported a decrease in telomerase activity in chronic infection, even though telomerase activity increases with the activation of the PBMCs in normal conditions.[47] Furthermore, a positive correlation between telomere shortening and fibrosis progression during chronic HCV infection have been found,[48] which could explain the absence of pronounced telomeric alteration in our study since fibrosis was an exclusion criterion in our cohort.

Telomere length on coinfected patients has been scarcely approached but in different populations and biological material. Grady et al. observed that telomere length in CD4 and CD8 T cells were reduced in CHC patients in comparison to healthy controls,[49] which seem to occur early during infection. After HCV elimination, telomere size recovery of PBMCs was observed, but only in patients with advanced fibrosis,[25] particularly in compensated patients. However, when patients with different liver fibrosis status were accounted, no significant increase of telomere length was observed after HCV elimination,[50] but telomere size was still lower than HIV controls.

In addition, oxidative stress and telomere shorting could lead to SASP associated with cellular aging.[51] Our data showed similar profile of SC and HIV groups, with a general tendency toward a reduction of plasma markers of immune senescence in the SC group. The more robust immune system of these subjects could explain the observed reduction of the inflammatory environment and suggest a more efficient resolution of HCV infection.

By contrast, when SASP plasma levels of SC and CHC patients were compared, we observed a highly different profile. The CHC group showed higher levels of several molecules associated with immune senescence, such as IL-6, IL-8, and TNF-α the most prominent interleukins within the SASP[24,52] and which are also known to induce ROS.[53] In particular, elevated concentrations of the pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-8, and TNF-α were found, cytokines that during normal healthy aging have been shown to be significantly increased compared with younger individuals.[54,55] This also agrees with previous studies, where increased cytokines of Th1 response[56,57] and IP-10[58] were also observed in CHC patients. Likewise, the increase in factors associated with SASP has also been observed in other viral infections such as hepatitis B[59] and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[60] The increase of these markers has been shown mainly in chronic viral hepatitis, increasing with fibrosis progression[30] and in hepatocellular carcinoma,[61] but there is no previous knowledge of these markers on patients without advanced fibrosis.

CHC patients also showed slightly higher levels of IL-1α, IP-10, and PIGF-1 than HIV group. It has been proposed that the level of some factors associated to SASP reflects the progression of liver fibrosis in patients with chronic HCV.[30] But the absence of large differences in the SASP between CHC and HIV groups could be due to the lack of advanced fibrosis present in our cohort, on contrary of previous studies.[50]

To sum up, in SC individuals, short-term exposure to SASP factors drives the recruitment of immune cells to eliminate premalignant and senescent cells, thereby preventing tumorigenesis. However, the long-term exposure to SASP experienced by CHC generates chronic inflammation and could contribute to tumorigenesis and age-associated pathologies.[62,63] Hence, the accumulation of senescence cells in these CHC individuals may significantly affect nonacquired immunodeficiency syndrome (AIDS) events via the SASP. Therefore, although nonadvanced fibrosis was present in CHC group, they could be prone to cancer development in the future.

To interpret our data correctly, we should bear in mind that some clinical and epidemiological variables were statistically different between groups, therefore, they were included in the full statistical model as adjustment variables for accounting their effect on senescence markers. The main limitation that should be considered is the limited sample size which could have restricted the possibility of finding statistical significance in some subgroups. Additional factors not considered, such as common respiratory viral infections may also play a role, but we assume that these events could impact in the same way in all study groups. Future studies on long-term

FIGURE 2 Quantification of senescence-associated secretory phenotype in people living with HIV under different HCV infectious status. Values are expressed as aAMR, obtained using a generalized linear model; *, $p < 0.05$ and $q < 0.1$. aAMR, adjusted arithmetic median rate; CHC, PLWHIV chronically infected with HCV; GM-CSF, granulocyte macrophage colony-stimulating factor; Gro-α, growth-regulated oncogene-alpha; HCV, hepatitis C virus; HIV, human immunodeficiency virus monoinfected patients; IFN, Interferon; IL, interleukin; IP-10, C-X-C motif chemokine ligand 10; PIGF-1, placental growth factor 1; *q*, corrected level of significance by false discovery rate; SC, PLWHIV who spontaneously clarify HCV; SCF, stem cell factor; TNF, tumor necrosis factor.

follow-up would be essential to unravel the long-time effects of HCV-related SASP in liver-related comorbidities on PLWHIV. It also would be interesting to evaluate whether similar results are observed in non-HIV patients, to have a better understanding of the negative consequences of HCV infection and to address possible palliative strategies.

5 | CONCLUSION

In conclusion, PLWHIV who spontaneously resolved an HCV acute infection showed slightly lower senescence profile to monoinfected HIV individuals. On the other hand, PLWHIV with chronic HCV infection led to higher oxidative stress levels for plasma nitrate and biomarkers related to the SASP, favoring the establishment of viral persistence.

AUTHOR CONTRIBUTIONS

Funding body: Amanda Fernández-Rodríguez and Verónica Briz. *Study concept and design*: Amanda Fernández-Rodríguez and Verónica Briz. *Patients' selection and clinical data acquisition*: Luz Martín-Carbonero, Lourdes Domínguez, Pablo Ryan, and Ignacio de los Santos. *Sample preparation and analysis*: Violeta Lara-Aguilar, Celia Crespo-Bermejo, Manuel Llamas-Adán, Sergio Grande-García, María Engracia Cortijo-Alfonso, and Sofía Bartolomé-Sanchez. *Statistical analysis and interpretation of data*: Violeta Lara-Aguilar, Daniel Valle-Millares, Amanda Fernández-Rodríguez, and María Ángeles Jiménez-Sousa. *Writing of the manuscript*: Violeta Lara-Aguilar and Amanda Fernández-Rodríguez. *Critical revision of the manuscript for relevant intellectual content*: Amanda Fernández-Rodríguez and Verónica Briz. *Supervision and visualization*: Amanda Fernández-Rodríguez and Verónica Briz. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The study was reviewed and approved by the Committee of the Institute of Health Carlos III (CEI PI 81_2017-Enmienda 2022). The patients/participants provided their written informed consent to participate in this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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