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Dynamics of cellular senescence markers after HCV elimination spontaneously or by DAAs in people living with HIV

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ABSTRACT

Background: We identified that acute or chronic Hepatitis C (HCV) infection in people living with HIV (PLWHIV) results in different senescence profiles. However, variations in these profiles after HCV elimination, spontaneously or with direct-acting antivirals (DAAs), remain unclear.
Methods: Longitudinal observational study (48 weeks) in 70 PLWHIV: 23 PLWHIV with active HCV-chronic infection (CHC) before and after HCV eradication with DAAs, 12 PLWHIV who spontaneously clarify the HCV (SC), and 35 controls (HIV). Oxidative stress was quantified at DNA, lipid, protein, and nitrate levels, as well as the antioxidant capacity and glutathione enzyme. The replicative senescence was evaluated by relative telomere length measurement by PCR and twenty-six factors related to Senescence-Associated Secretory Phenotype (SASP) were characterized by Luminex. Differences in senescence markers was evaluated by generalized linear models.
Results: During follow-up, the SC group achieved a significant improvement in glutathione enzyme and lipid peroxidation. The secretion of SASP markers increased but was still lower than that of the HIV group. Overall, the

Abbreviations: 8-OHdG, 8-hydroxydeoxyguanosine; ALT, Alanine transaminase; aAMR, Adjusted Arithmetic median ratio; AMR, Arithmetic median ratio; ART, Antiretroviral treatment; AST, Aspartate aminotransferase; BCA, Bicinchoninic acid; BMI, Body mass index; CHC, Chronically infected with HCV; DAAs, Direct-acting antivirals; DDR, DNA damage response; DNA, Deoxyribonucleic Acid; DNP, Dinitrophenyl; DNPH, 2,4-dinitrophenylhydrazzine; EDTA, Ethylenediaminetetraacetic acid; EGF, Epidermal Growth Factor; FDR, False discovery rate; FGF-2, Fibroblast growth factor 2; GGT, Gamma-Glutamyl Transferase; GLMMs, Generalized linear mixed model; 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; IFN-γ, Interferon-gamma; IL-13, Interleukin-13; IL-15, Interleukin-15; IL-18, Interleukin-18; IL1-RA, Interleukin-1 receptor antagonist; 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; PLWHIV, People living with HIV; ROS, Reactive oxygen species; RTL, Relative telomere length; SASP, Senescence-Associated Secretory Phenotype; SC, Spontaneously clarifiers; SDF-1α, Stromal cell-derived factor 1; SVR, Sustained virological response; TAC, Total non-enzymatic antioxidant capacity; TBA, Thiobarbituric acid; Th1, Type 1 T helper; Th2, Type 2 T helper; TNF-α, Tumor necrosis factor-alpha.

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CHC group reduced the levels of oxidative stress and SASP markers to levels like those of the HIV group. No significant differences in telomere shortening were observed between groups.
Conclusions: As the time since spontaneous resolution of HCV infection increased, patients had an improved senescence profile compared to the HIV group. Elimination of chronic HCV infection by DAAs led to a partial improvement of the senescent profile by restoring oxidative stress levels. However, although some SASP markers reached levels like those of the HIV group, others remained altered.

1. Introduction

Currently, 37.7 million people are living with human immunodeficiency virus (PLWHIV), with 1.5 million new infections annually. Approximately 2.3 million (6.2%) PLWHIV are co-infected with hepatitis C virus (HCV), being most of them chronically infected. Spontaneous clearance occurs in approximately 20% of patients [1], being a rare phenomenon in the context of coinfection [2].

Human immunodeficiency virus (HIV) has become a chronic, non-terminal disease in which patients are aging at an accelerated rate. This aging leads to an increased risk of developing inflammatory diseases inducing premature senescence and development of comorbidities [3]. Thus, PLWHIV experience several stressors besides the virus itself (virus-induced senescence), such as antiretroviral drugs, the higher rate of substance abuse and the presence of other co-infections such as HCV.

HCV causes inflammation and immune activation, leading to clonal depletion and immunosenescence that triggers immunopathological mechanisms that damage the liver [4,5]. This causes DNA damage due to oxidative stress produced directly by the virus itself and indirectly by the host immune response, together with deficits in DNA repair mechanisms [6]. Mitochondrial alterations also occur, where elevated oxidative stress caused by chronic inflammation increases the formation of reactive oxygen species (ROS) and leads to hepatocyte apoptosis [7]. Chronic HCV elimination is possible with the advent of direct-acting antivirals (DAAs), but their access is limited, and a high number of HCV+ individuals are unaware of their status. Some of the immunosenescence mechanisms commented above improve after HCV clearance, but depends on the fibrosis stage of the patient, among other factors. However, it is unknown whether after an acute HCV infection and subsequent spontaneous clearance similar alterations could be observed and whether they persist over time.

HIV/HCV coinfection alter the natural history of both viruses, increases the risk of age-associated comorbidities [8,9], secretion of profibrogenic cytokines and oxidative stress [10], which results in increased immune activation that favours the progression of HIV infection and liver disease [11]. Immune exhaustion leads to progressive loss of immunological memory, a reduction in proliferative capacity and telomere shortening in immune cells in both HCV+ [12] and HIV+ individuals [13]. Telomeres are tandem repeats of a conserved DNA sequence TTAGGG, whose function is to protect the integrity of chromosomes [14,15]. The number of these hexamers is reduced with each cell division and their shortening increases chromosomal instability, leading to a senescent phenotype associated with cellular ageing [15,16]. Thus, telomere length is an index of cellular aging associated with the progression of various diseases [17,18] and even with an increased risk of infection [19].

In this line, HIV/HCV co-infection produces premature virus-induced senescence, which may have implications for the development of long-term sequelae. This phenomenon is indistinguishable from other forms of cellular senescence and is accompanied by a proinflammatory phenotype called the senescence-associated secretory phenotype (SASP) [20], an antiviral mechanism of the senescent cell, which allows limitation of viral replication [21], including production of cytokines, extracellular matrix factors, and coagulation mediators [22].

The aim of this work is to analyse whether HCV clearance in PLWHIV may partially restore cellular damage and immune senescence. We propose a comprehensive approach to assess inflammation, ageing and senescence-related markers in PLWHIV in medium-term follow-up after HCV clearance with DAAs (chronic infection) or spontaneously resolved (after an acute infection). Both HIV and HCV replicate in PBMCs [23], being these cells an excellent non-invasive material to study markers of immune senescence.

2. Material and methods

2.1. Study design

We carried out a multicentre prospective observational study from the COVIHEP cohort (Supplementary Date 1) from three Public Spanish Hospitals in Madrid Autonomous Community: La Paz University Hospital, Infanta Leonor University Hospital and La Princesa University Hospital. 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 enrolment, and the Research Ethics Committee of the Institute of Health Carlos III approved the study (CEI PI 81_2017-v3).

Seventy PLWHIV with different status of HCV infection were enrolled: 1) CHC group (n = 23): PLWHIV with active HCV-chronic infection naïve to any HCV treatment (positive PCR and positive HCV antibodies); 2) SC group (n = 12): PLWHIV who had been acutely infected with HCV and experienced spontaneous viral clearance during the first 6 months after HCV infection (negative PCR and positive HCV antibodies, in the absence of anti-HCV treatment); 3) HIV control group (n = 35): PLWHIV without previous HCV infection (negative PCR and negative HCV antibody). CHC patients were evaluated at baseline and 48 weeks after the achievement of sustained virological response (SVR) with DAAs treatment (Supplementary Data 2). The SC group was sampled with the same follow-up. The HIV group was only evaluated at baseline since it is a well-controlled group (optimized treatment and undetectable viral load), without any intervention regarding the studied outcome (spontaneous HCV elimination or treatment with DAAs). However, we have analysed the clinical, epidemiological, and metabolic characteristics of this group between baseline and at the end of the follow-up, and we have not found significant differences (Supplementary Date 3). All patients received suppressive antiretroviral treatment (ART) during at least one year, were undetectable for HIV during the previous year and had CD4 + T-cells counts $\geq$ 500 cells/mm^3 since at least one year before sample collection. Exclusion criteria were pregnancy, individuals below 18 years old, advance 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 (EDTA) tubes. PBMCs were isolated by density gradient centrifugation method with Lymphoprep ™ and SepMate ™ tubes (Stemcell Technologies) and stored at viability until use. Plasma was clarified and storage at − 80 ^{o}C until use.

2.3. Senescence biomarkers

To characterize cellular senescence, we measured the plasma concentration of ROS, total antioxidant capacity and glutathione reductase enzyme, with different commercial kits following manufacturer instructions unless otherwise specified. We also characterize the replicative senescence and senescence-associated secretory phenotype on each study group.

2.3.1. Oxidative-stress levels in plasma samples

2.3.1.1. ROS damage

2.3.1.1.1. At DNA level. We analysed **DNA damage response** (DDR) by the quantification of the 8-hydroxydeoxyguanosine (8-OHdG) DNA levels, with the DNA Damage Competitive ELISA kit (Thermo Fisher Scientific).

2.3.1.1.2. At protein level. We measured the oxidation of proteins with the **protein carbonyl content** (PCC) assay kit (Sigma-Aldrich) and Pierce bicinchoninic acid (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.

2.3.1.1.3. 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. Slightly differences in manufacturer's instructions were included, as butanol was removed by overnight evaporation at room temperature.

2.3.1.2. Oxidant molecules. We assessed nitric oxide (NO) levels by the Nitric Oxide Assay Kit (Thermo Fisher Scientific).

2.3.1.3. Antioxidant capacity. 2.3.1.3.1. The **total non-enzymatic 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.

2.3.1.3.2. The **reduced glutathione** (GSH) and **oxidised glutathione** (GSSG) were evaluated by the GSH Colorimetric Detection Kit (Thermo Fisher Scientific).

2.4. Secretory phenotype and immune senescence in plasma

We assessed a selection of 26 factors which include cytokines, chemokines, and growth related to SASP [20,24]. All markers were measured in the same sample at the same time by multiplex immunoassays (Luminex xMAP technology). The complete list is available at Supplementary Data 4.

2.5. Replicative senescence in PBMCs

DNA was extracted from PBMCs using the DNA Purification System Kit (Promega Wizard). Quantification of replicative senescence was performed by Monochromatic Multiplex Real-Time Quantitative PCR (MMqPCR) as previously described [25]. Briefly, single-copy gene (β-globin) was used to normalise the number of telomere copies/sample. Relative leukocyte telomere length (RTL) was expressed as the ratio of the telomere amplification product (T) to the single copy gene (S).

2.6. Statistical analysis

For the descriptive study of clinical and epidemiological data of the patients, continuous variables were summarized as median, and categorical as frequency and percentage. Significant differences between categorical data were calculated using the chi-squared test or Fisher's exact test. Kruskal-Wallis and Mann-Whitney U tests were used to compare continuous variables among independent groups. Concentration values were used for oxidative stress markers and raw fluorescence intensity as a relative quantification of the analyte abundances for SASP markers, as previously described [26]. We estimated differences in levels of senescence biomarkers, cytokines, and RTL for paired samples (longitudinal design) by a generalized linear mixed model (GLMMs) (SC-b vs. SC-f), and a generalized linear model (GLMs) for non-paired comparisons (SC-f vs. HIV, CHC-f vs. HIV, and CHC-f vs. SC-f), both with gamma distribution (log-link). GLMs were adjusted by most relevant covariables selected by a stepwise algorithm (p-value<0.15 at each step) from the following list of variables: age, gender, BMI, transmission route, duration of HIV infection, HIV clinical stage, HIV treatment, IFNL4 genotype, HCV genotype and CD4 T nadir. P values were adjusted by false discovery rate (FDR) using Benjamin-Hochberg correction setting a cut-off point of 0.1. 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

Specific clinical and epidemiological characteristics are shown in Supplementary Data 5. Overall, the median age was 50 and 47% were female. The CHC group had the lowest body mass index (BMI) and the maximum time of infection. The SC group showed higher frequency of favourable allele (CC) at interferon lambda 4 (IFNL4) ($p = 0.001$), as expected. Regarding the evolution of biochemical and metabolic characteristics (Supplementary Data 6), similar values were observed among groups, except for alanine transaminase (ALT), as CHC patients at baseline (CHCb) showed significantly higher values. The SC group did not shown differences during the follow-up for any of the biochemical parameters measured, an only reduced ALT levels with respect to HIV controls. The CHC group showed differences during the follow-up only for those markers related to liver function, improving aspartate aminotransferase (AST), ALT and Gamma-Glutamyl Transferase (GGT) markers.

3.2. Evolution of senescence-associated markers in SC patients

During follow-up, the SC group reduced oxidative stress levels by significantly decreasing lipid peroxidation [AMR= 0.56 (0.42–0.75), $p < 0.001$, q= 0.008] (Fig. 1; Supplementary Data 7). In relation to SASP, SC showed higher levels of soluble factors related to Type 1 T helper (Th1)/Type 2 T helper (Th2), such as Interferon-gamma (IFN-γ) [aAMR= 1.68 (1.17–2.43), $p = 0.005$, q= 0.033], IL-8 [aAMR= 1.31 (1.11–1.55), $p < 0.001$, q= 0.013], interleukin (IL)− 18 [aAMR= 2.39 (1.23–4.64), $p = 0.010$, q= 0.043] and tumor necrosis factor-alpha (TNF-α) [aAMR= 1.45 (1.15–1.82), $p = 0.002$, q= 0.017]; inflammatory cytokines, such as Interleukin-1 receptor antagonist (IL1-RA) [aAMR= 2.26 (1.44–3.54), $p < 0.001$, q= 0.013], and growth factors such as EGF [aAMR= 2.13 (1.20–3.79), $p = 0.010$, q= 0.043], and FGF-2 [aAMR= 1.67 (1.10–2.52), $p = 0.016$, q= 0.059] (Fig. 2; Supplementary Data 8). The chemokine eotaxin lost statistical significance after adjusting for FDR [aAMR= 1.59 (1.01–2.50), $p = 0.047$, q= 0.153].

Compared to the HIV group at the end of the follow-up, the SC group showed an overall improvement of oxidative stress parameters, reducing the GSSG concentration [aAMR= 0.44 (0.22–0.87), $p = 0.019$, q= 0.140] and lipid peroxidation [aAMR= 0.63 (0.42–0.96), $p = 0.031$, q= 0.140] (Fig. 3; Supplementary Data 9).

In relation to the SASP, the SC group showed significantly lower levels of cytokines related to Th1/Th2 response compared to the HIV group, such as Granulocyte Macrophage Colony-Stimulating Factor

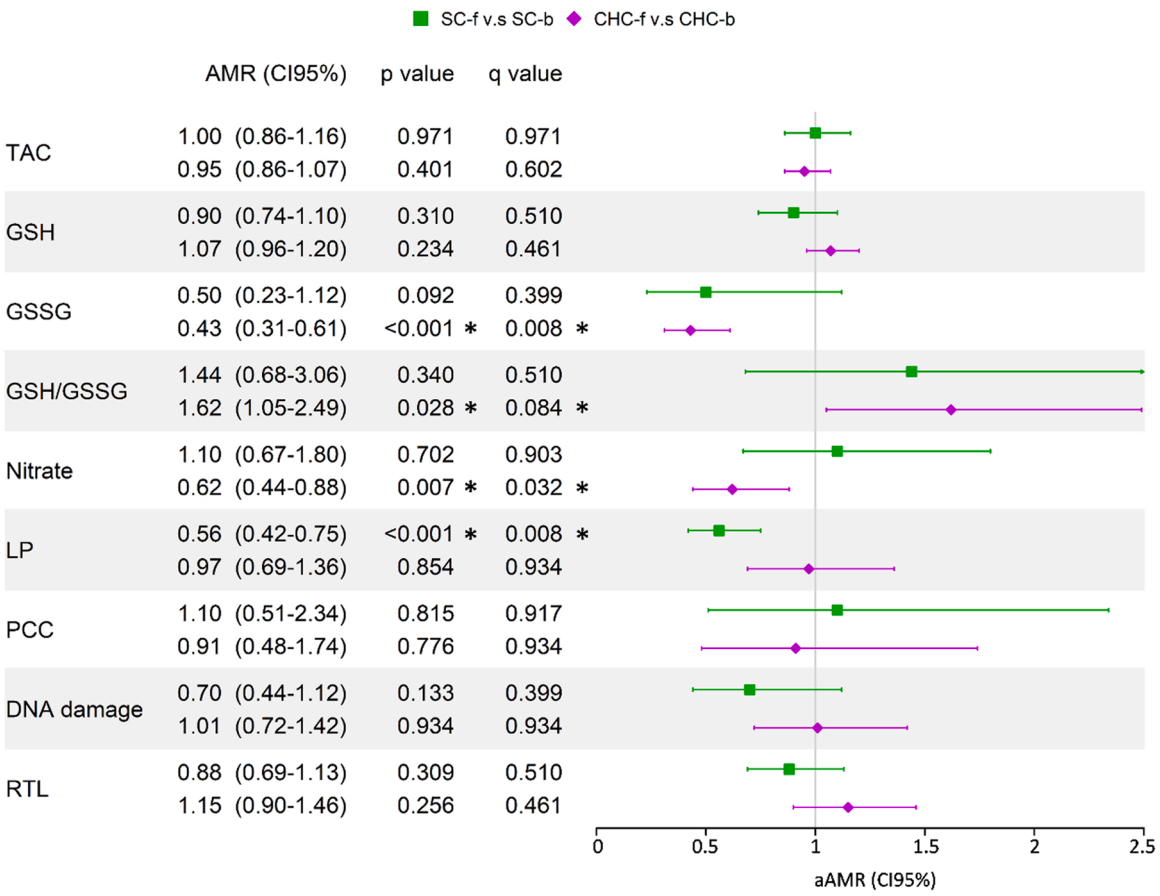

Fig. 1. Evolution of oxidative stress levels during 48 weeks of follow-up in the SC and CHC groups. Values are expressed as arithmetic median ratio adjusted by the most significant variables (aAMR) obtained using a generalized linear mixed model. Statistically significant data are highlighted with * ($p < 0.5$, $q<0.1$). Abbreviations: SC-b, PLWHIV who spontaneously clarify HCV at baseline; SC-f, PLWHIV patients who spontaneously clarify HCV after 48 weeks of follow-up; CHC-b, PLWHIV chronically infected by HCV at baseline; CHC-f, PLWHIV chronically infected by HCV 48 weeks after achievement sustained virological response (SVR) with the new direct-acting antivirals agents (DAAs); TAC, total antioxidant capacity; GSH, reduced glutathione; GSSG, oxidized glutathione; LP, lipid peroxidation; PCC, protein carbonyl content; RTL, relative telomere length; q, corrected level of significance by false discovery rate.

(GM-CSF) [aAMR= 0.78 (0.66–0.93), p = 0.006, q= 0.047], IL-1β [aAMR= 0.76 (0.60–0.95), p = 0.018, q= 0.059], IL-6 [aAMR= 0.72 (0.57–0.90), p = 0.005, q= 0.047] and IL-13 [aAMR= 0.74 (0.59–0.94), p = 0.014, q= 0.059], and the inflammatory cytokine IL-7 [aAMR= 0.82 (0.69–0.96), p = 0.017, q= 0.059], and a tendency to reduce IL-2 [aAMR= 0.79 (0.64–0.99), p = 0.040, q= 0.116]. In addition, they showed a slight increase in other inflammatory cytokines, IL-1α [aAMR= 1.43 (1.10–1.86), p = 0.008, q= 0.047] and IL1-RA [aAMR= 1.83 (1.16–2.89), p = 0.009, q= 0.047], as well as the chemokine SDF-1α [aAMR= 1.39 (1.12–1.72), p = 0.003, q= 0.047] (Fig. 4; Supplementary Data 10).

3.3. Evolution of senescence-associated markers after HCV elimination

The CHC group accomplished an improvement of GSH/GSSH ratio [AMR= 1.62 (1.05–2.49), p = 0.028, q= 0.084] by a significant reduction of GSSG concentration [AMR= 0.43 (0.31–0.61), p < 0.001, q= 0.008], as well as a pronounced decrease in nitrate concentration [AMR= 0.62 (0.44–0.88), p = 0.007, q= 0.032] (Fig. 1; Supplementary Data 7). In addition, they showed a significant reduction in IL-13 [aAMR= 0.76 (0.63–0.93), p = 0.009, q= 0.078] and a tendency to reduce IL-2 [aAMR= 0.81 (0.66–0.99), p = 0.035, q= 0.150], IL-15 [aAMR= 0.80 (0.67–0.96), p = 0.018, q= 0.117] and C-X-C motif chemokine ligand 10 (IP-10) [aAMR= 0.73 (0.54–0.98), p = 0.034, q= 0.150]. By contrast, they also showed a higher levels of Growth-regulated oncogene-alpha (Gro-α) [aAMR= 1.19 (1.05–1.34), p = 0.005, q= 0.065], and Rantes [aAMR= 1.28 (1.09–1.52), p = 0.003, q= 0.065] (Fig. 2; Supplementary Data 8), and a tendency to increase eotaxin [aAMR= 1.42 (1.01–2.01), p = 0.046, q= 0.150] and SDF-1α [aAMR= 1.15 (1.00–1.31), p = 0.045, q= 0.150].

After eliminate HCV by DAAs at the end of the follow-up, the CHC group showed a similar profile to the HIV control group at oxidative stress level (Fig. 3; Supplementary Data 11). They also showed a similar secretory profile to the HIV group, less by an increase in the concentration of Gro-α [aAMR= 1.25 (1.10–1.41), p < 0.001, q= 0.008] and FGF-2 [aAMR= 1.25 (1.12–1.39), p < 0.001, q= 0.008] (Fig. 4; Supplementary Data 12). The CHC group also showed a tendency to reduce the levels of IL-2 [aAMR= 0.82 (0.69–0.97), p = 0.023, q= 0.120], IL-6 [aAMR= 0.82 (0.68–0.99), p = 0.041, q= 0.178], and IL-7 [aAMR= 0.87 (0.76–0.99), p = 0.049, q= 0.179] and a slight increase of IL-1α [aAMR= 1.24 (1.04–1.49), p = 0.017, q= 0.111) compared to HIV individuals.

3.4. Senescence-associated markers in acute vs. chronic HCV long-term infection

After 48 weeks of HCV elimination trough DAAs the CHC group still maintained higher levels of oxidative parameters than the SC group, such as lipid peroxidation [aAMR= 1.68 (1.05–2.68), p = 0.029, q= 0.087] and a lower glutathione ratio [aAMR= 0.45 (0.30–0.70), p < 0.001, q= 0.008] mainly due to a higher GSSG concentration [aAMR= 2.26 (1.27–4.04), p = 0.006, q= 0.027] (Fig. 3;

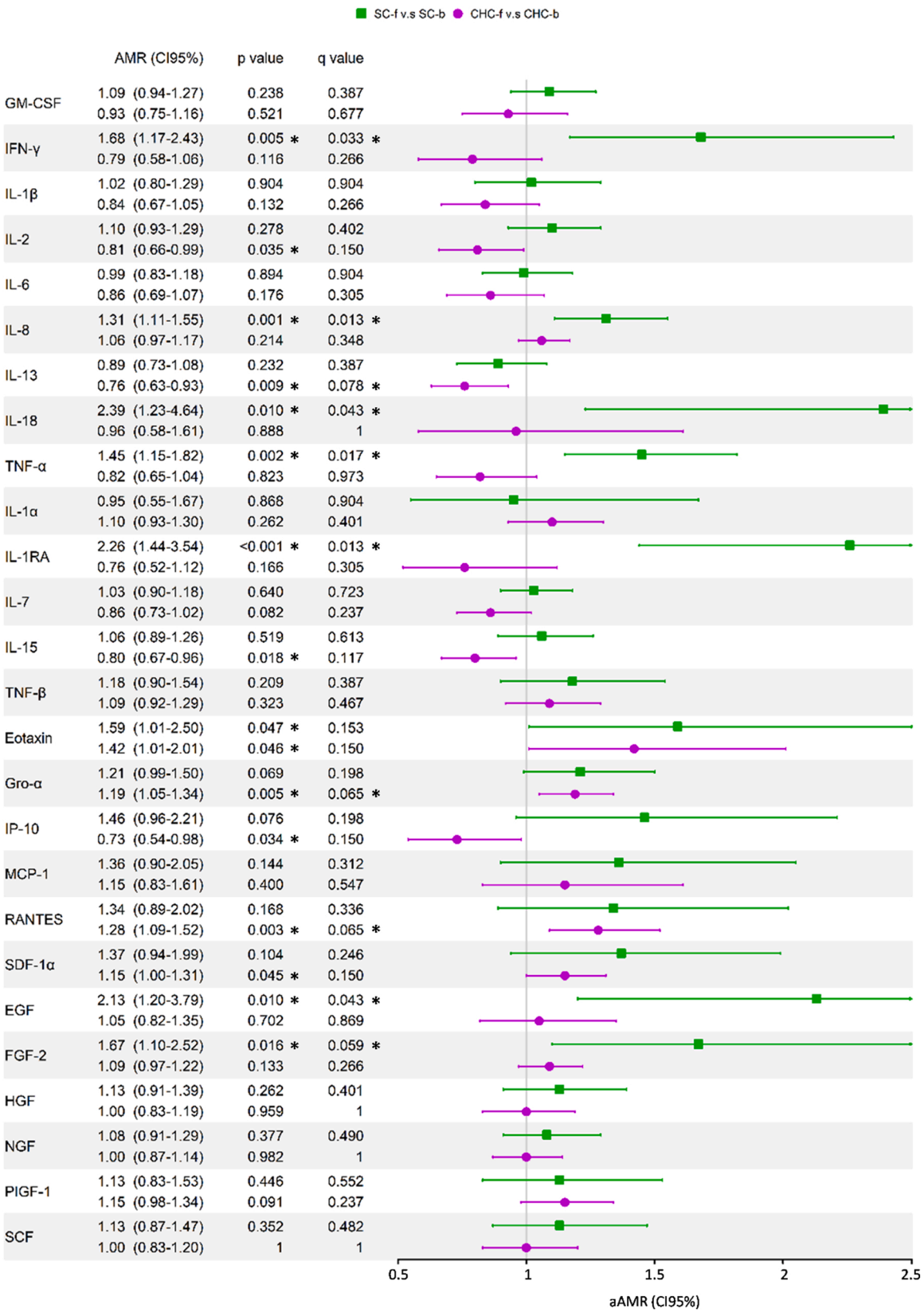

Fig. 2. Evolution of SASP during 48 weeks of follow-up in the SC and CHC groups. Values are expressed as arithmetic median ratio adjusted by the most significant variables (aAMR) obtained using a generalized linear mixed model. Statistically significant data are highlighted with * ($p < 0.5$, $q<0.1$). Abbreviations: SC-b, PLWHIV who spontaneously clarify HCV at baseline; SC-f, PLWHIV patients who spontaneously clarify HCV after 48 weeks of follow-up; CHC-b, PLWHIV chronically infected by HCV at baseline; CHC-f, PLWHIV chronically infected by HCV 48 weeks after achievement sustained virological response (SVR) with the new direct-acting antivirals agents (DAAs); q, corrected level of significance by false discovery rate.

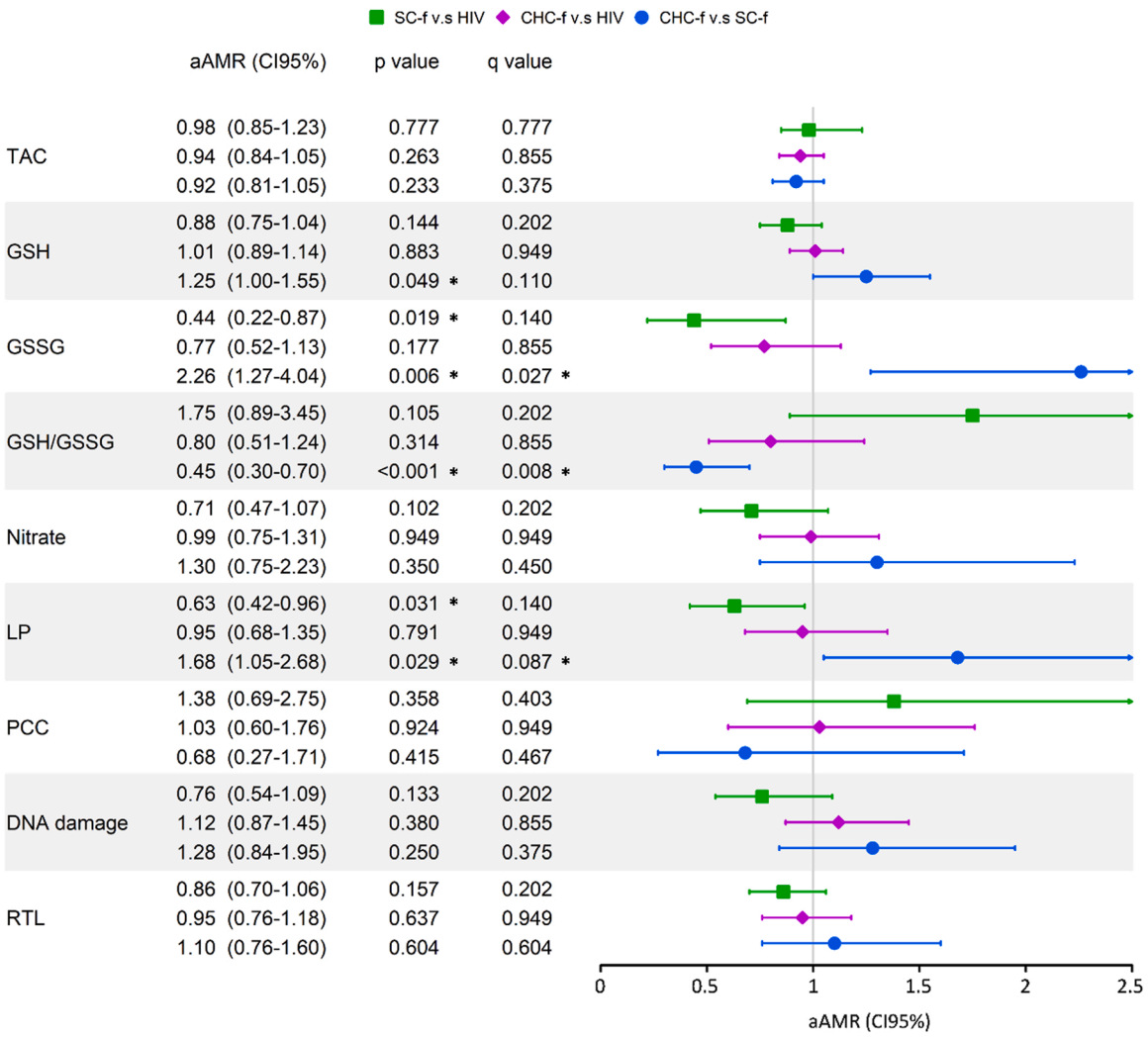

Fig. 3. Comparison of oxidative stress levels between SC, CHC and HIV groups after 48 weeks of follow-up. Values are expressed as arithmetic median ratio adjusted by the most significant variables (aAMR) obtained using a generalised linear model. Statistically significant data are highlighted with * ($p < 0.5$, $q<0.1$). Abbreviations: SC-f, PLWHIV patients who spontaneously clarify HCV after 48 weeks of follow-up; HIV, PLWHIV control group; CHC-f, PLWHIV chronically infected by HCV 48 weeks after achievement sustained virological response (SVR) with the new direct-acting antivirals agents (DAAs); TAC, total antioxidant capacity; GSH, reduced glutathione; GSSG, oxidized glutathione; LP, lipid peroxidation; PCC, protein carbonyl content; RTL, relative telomere length; q, corrected level of significance by false discovery rate.

Supplementary Data 13).

No major differences were obtained at SASP level between groups exposed to HCV, with a slight tendency to reduce IL-1RA [aAMR= 0.59 (0.38–0.91), p = 0.016, q= 0.416] levels in the CHC group (Fig. 4; Supplementary Data 14).

4. Discussion

We describe for the first time the evolution of senescence markers in HIV+ patients who experienced HCV spontaneous resolution and chronic HCV elimination with DAAs. Our data indicate that as the HCV spontaneous clearance time increases, patients experienced a significant reduction of oxidative stress levels. The secretion of factors related to SASP become slightly higher during the follow-up for the SC group, which could be related to normal aging. SASP in this group was still lower than that in the HIV group, despite the lower median age of the HIV group, suggesting that SC patients may dampen ageing-related changes. By contrast, the HCV elimination with DAAs after chronic HCV infection allowed a normalization of oxidative stress markers to HIV control group and a reduction of some SASP markers while others remained altered.

The SC group achieved a pronounced restoration of the reduced microenvironment by an improvement in the glutathione enzyme and lipid peroxidation since the time of clearance increases. GSH is an essential intracellular and extracellular protective antioxidant, whose ratio with respect to GSSG is a marker of oxidative stress. Of note, the concentration of GSH in plasma is in micromolar range, accounting for roughly 0.4% of total blood GSH [27]. We have therefore detected significant and big differences in this biological compartment, but bigger differences could be present at intracellular or total blood level. Previous studies were focused on acute infection, describing an increase in ROS production [28], but few assessed the impact of spontaneous clearance over the long term, and even fewer in HIV/HCV co-infected individuals. Our results show that as the time of spontaneous clearance increases, the restoration of redox balance considerably improves.

To our knowledge, this is the first report analysing cellular senescence profile of SC patients. It has been postulated that people who spontaneously clear HCV have certain immune strengths. Although the mechanisms involved have not yet been well characterized, it has been described a high HCV- and HIV-specific T-cell polyfunctionality [29,30], together with a highly differentiated NK phenotype [31,32].

Furthermore, certain genetic markers have been linked to these

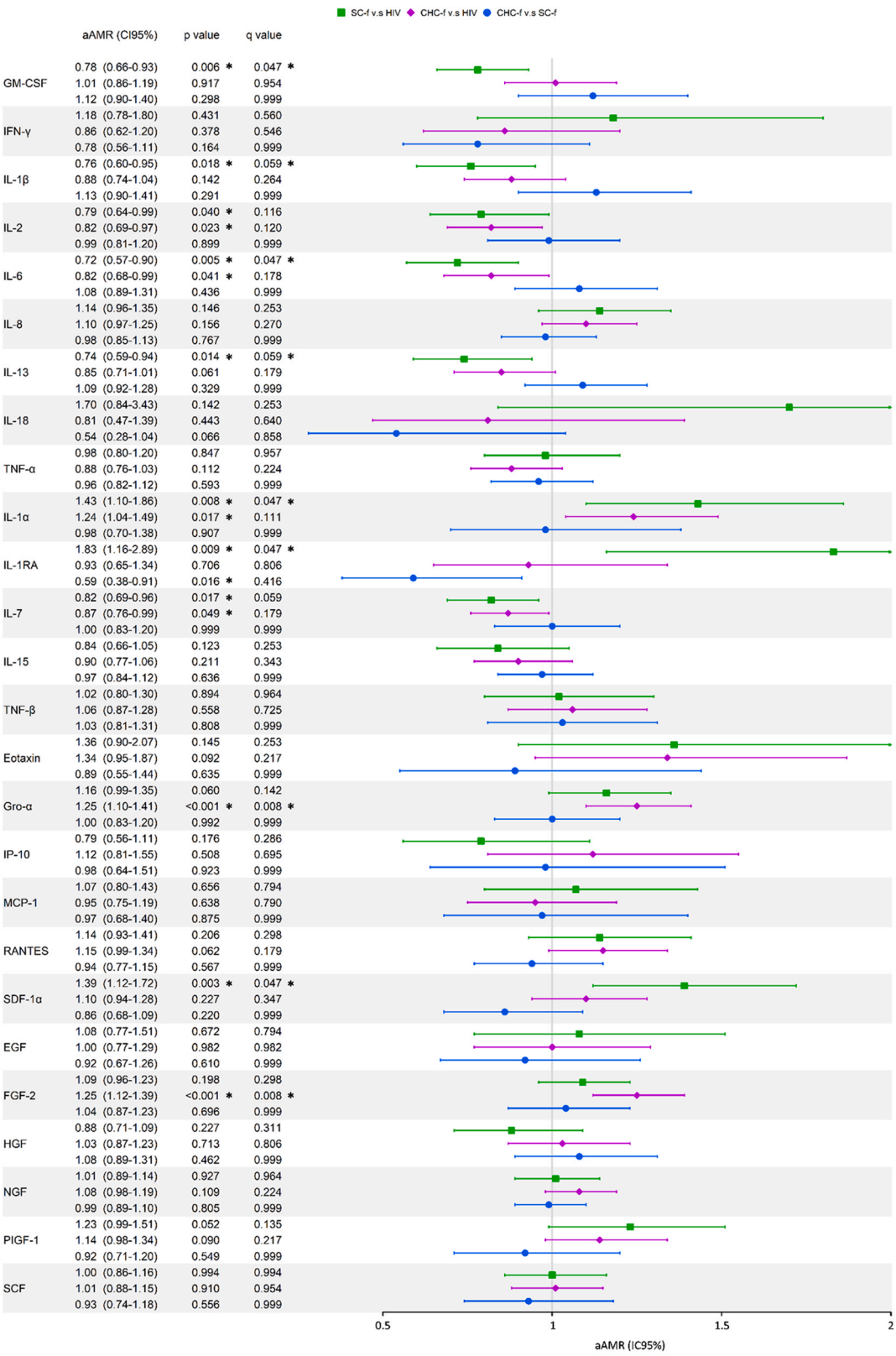

Fig. 4. Comparison of the SASP between SC, CHC and HIV groups after 48 weeks of follow-up. Values are expressed as arithmetic median ratio adjusted by the most significant variables (aAMR) obtained using a generalised linear model. Due to the extreme values of some covariates, the data for cytokine IL-18 are shown unadjusted by AMR. Statistically significant data are highlighted with * ($p < 0.5$, $q<0.1$). Abbreviations: SC-f, PLWHIV patients who spontaneously clarify HCV after 48 weeks of follow-up; HIV, PLWHIV control group; CHC-f, PLWHIV chronically infected by HCV 48 weeks after achievement sustained virological response (SVR) with the new direct-acting antivirals agents (DAAs); q, corrected level of significance by false discovery rate.

individuals, such as interleukin genotypes, HLA alleles, and factors affecting the T-cell response [33–36]. The expression of some of these molecules are also related to the control of HIV viral replication in elite controllers and long-term non-progressors [37,38]. The existence of HIV-elite controllers that spontaneously clear HCV (named super-controllers) [39] suggest that there are some common or additive mechanisms involved in the control of both viruses [39,40]. In fact, Dominguez-Molina et al. (2018) demonstrated that a better T-cell response to HCV corresponded with a better T-cell response to HIV [39]. In particular, they showed an increase of these specific responses in the SC group versus the CHC group, which is in agreement with the lower lipid peroxidation and higher glutathione enzyme observed in the SC group in our study.

On the other hand, it has been suggested that serum oxidants (MDA and GSSG) are elevated by increased lymphocyte and erythrocyte destruction in chronically HIV-infected patients, leading to the release of MDA, GSSG and reduced GSH [41]. In fact, the use of the stress biomarker "MDA" has been proposed as a possible predictor of progression to AIDS [42,43]. Therefore, considering that SC individuals may be developing better control of HIV infection by a better T-cell response, this may partly explain the lower levels of lipid peroxidation derived of HIV infection in the SC group.

Regarding CHC patients, they improved the oxidative stress levels during the follow-up by an elevation of the GSH/GSSG ratio, mainly due to the depletion of GSSG concentration, and reduction in nitrate concentration. Although they still maintained higher concentration of GSSG and lipid peroxidation than the SC group, CHC individuals reached similar oxidative stress levels to the HIV group after HCV elimination by DAAs. It is well known that HCV can now be rapidly and sustainably cleared from chronically infected patients with this antiviral therapy [44], but the consequences of viral elimination on immune system remain elusive, especially in the HIV/HCV co-infection context. Our results agree with previous studies showing an increase of the enzymatic activity of several antioxidant enzymes in chronic HCV mono-infected patients after reaching SVR at the end of treatment with DAAs, such as GSH and GSH peroxidase (GPx) activity [45,46], as well as a decreasing effect for GSSG, among others [46]. In some works, the beneficial effect was independent of HCV genotype and antiviral regimen used [45], which contrasts with another work which associated higher oxidative stress levels with genotype 1b [47].

In relation to the nitrate concentration, previous researches have shown increased plasma nitrate and nitrite levels during chronic HCV infection [48,49]. The greater elevation of oxidative stress markers and lower serum antioxidants in patients coinfected with HIV/HCV versus HIV-monoinfected subjects [41,50,51] hinted that higher oxidative stress status in coinfected subjects was at least partially contributed to HCV infection.

To date, there are hardly any data on the long-term evaluation of the impact of DAAs. Our results agree with Villani et al. (2020), who observed that after DAAs treatment the circulating redox status of patients affected by chronic hepatitis C improved [45]. Therefore, the elimination of HCV viremia could explain the improvement in oxidative stress observed in the CHC group at the end of the follow-up, characterized by a reduction in nitrate levels and improvement of glutathione enzyme.

We did not observed changes in MDA levels during and at the end of follow-up in the CHC group, but it has been described that the reduction of some markers such as lipid peroxidation begins later than the recovery of glutathione activity after the end of DAAs therapy in HCV mono-infected patients [45]. Recent research has reported a reduction in MDA in HCV+ patients after 8 weeks of DAAs treatment [46]. However, they did not indicate the fibrosis stage of their population, which it is an important factor due to that mono-infected HCV patients with fibrosis stage F0/F1 patients have been previously demonstrated reduced efficacy of DAAs therapy on circulating redox balance compared to F3/F4 patients [45]. This could partially explain why we only observed a slight recovery of oxidative stress biomarkers, given that high fibrosis stages were considered exclusion criteria in our cohort. In addition, it has been reported that PCC levels correlated with ALT serum levels [52], and considering that the CHC group normalized biochemical parameters related to liver function could be necessary long-term studies to determine the clinical impact of DAAs in all these senescence biomarkers and if they will be completely restored.

Oxidative stress may accelerate the cellular aging, which has been associated with a SASP. Surprisingly, SC individuals showed slightly higher levels of SASP after one year of follow-up (IFN-γ, IL-8, IL-18, TNF-α, IL1-RA, EGF, and FGF-2), but we should keep in mind that these changes could be related to normal age-related mechanisms. Thus, although the SC group seem to show an increased cellular aging, the levels of these senescence biomarkers remained similar to the HIV group.

The biological mechanism underlying the lower SASP levels of the SC group is unknown. These patients show a common genetic background, as they significantly carry the favourable genotype for the IFNL4, which is associated with higher rates of HIV and HCV disease resolution [53]. Patients harbouring the favourable genotype are more likely to spontaneously clarify the HCV during the acute infection, showing a vigorous CD8 + and CD4 + T cells responses [33]. Surprisingly, although interferons are generally antiviral proteins, the favourable variant is associated with an impaired production of IFNL4 protein, leading to a lower interferon-stimulated genes (ISGs) expression levels [54]. This scenario would be related to a reduced oxidative stress and proinflammatory cytokine responses, as well as a lower metabolic dysfunction [55]. Similarly, as CHC patients predominantly carry the unfavourable genotype, the higher ISGs production could be an additional factor for a higher senescence profile in this group.

The CHC group partially improved SASP during the follow-up by reducing molecules such as IL-2 and IP-10, both of which are also associated with ongoing tissue damage and progressive liver disease during chronic hepatitis C infection [56]. This is consistent with the reduction of AST, ALT and GGT parameters in the CHC group during the follow-up. However, other important cytokines within the SASP remained stable, such as IL-8 which showed no significant changes compared to baseline, consistent with Villani and colleagues, who followed an HCV mono-infected cohort for 12 weeks after antiviral treatment with DAAs [45]. On the other hand, the CHC group showed similar levels to the HIV group at the end of follow-up after completion of DAAs therapy, with a slight reduction in IL-6, the most prominent interleukin within the SASP [24] and which is known to induce ROS damage [57].

To date, DAAs are the first-choice therapy for effectively eliminating HCV. However, if they are sufficient to block the uncontrolled inflammation and severe liver injury in HCV-infected individuals are still under discussion. While our work showed an overall restoration of SASP after sustained virologic response after DAA therapy, others have reported a long-term risk for progression to persistent hepatic inflammation, liver cirrhosis and hepatocellular carcinoma [58,59].

Therefore, this premature senescence may be an important component of the pathogenesis of HIV/HCV co-infection, as there is an increased immune activation that promotes both progression of HIV infection and liver disease [11].

To interpret our data correctly, we should bear in mind that this is a preliminary study with a limited sample size, which could have limited the possibility of finding statistical significance in some subgroups. However, in spite of this, it should be noted that its longitudinal design allows us greater statistical power than cross-sectional studies. Thus, we have used a GLMM, which adequately accounts for the random effect in our model, limiting the false positive rate and increasing statistical power. In addition, we have applied the FDR correction to limit false-positive results. Further studies would also be of interest to study the evolution of the senesce-markers in PLWHIV after HCV eradication under therapy with longer follow-up.

5. Conclusions

In conclusion, our data provide evidence that as the time of clearance increases, patients who experienced a spontaneous resolution of HCV showed a similar senescence profile to HIV patients without previous HCV infection, with an enhancement of oxidative stress markers. On the other hand, the elimination of chronic HCV infection by DAAs led to an improvement of cellular senescence profile, by a restoration of oxidative stress levels and a partial reduction of SASP-related molecules to levels like those of the HIV group.

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CRediT authorship contribution statement

Verónica Briz, Amanda Fernández-Rodríguez: Funding body, study concept and design**; Luz Martín-Carbonero, Lourdes Domínguez, Pablo Ryan, Ignacio de los Santos**: patient́s selection and clinical data acquisition; **Violeta Lara-Aguilar, Celia Crespo-Bermejo, Sergio Grande-García, María Engracia Cortijo-Alfonso, Sofía Bartolomé -Sánchez, Erick Joan Vidal-Alcántara**: sample preparation and analysis; **Violeta Lara-Aguilar, Daniel Valle-Millares, María Ángeles Jiménez-Sousa, Amanda Fernández-Rodríguez**: Statistical analysis and interpretation of data; **Violeta Lara-Aguilar, Amanda Fernández-Rodríguez**: Writing of the manuscript; **Amanda Fernández-Rodríguez**, **Verónica Briz**: critical revision of the manuscript for relevant intellectual content; **Amanda Fernández-Rodríguez**, **Verónica Briz**: supervision and visualization; **all authors**: read and approved the final manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114664.

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