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Inflammation burden score in multidrug-resistant HIV-1 infection

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SUMMARY

Objectives: Four-class drug-resistant (4DR) people living with HIV (PLWH) are a fragile population with a high burden of disease. No data on their inflammation and T-cell exhaustion markers are currently available.
Methods: Inflammation, immune activation and microbial translocation biomarkers were measured through ELISA in 30 4DR-PLWH with HIV-1 RNA ≥ 50 copies/mL, 30 non-viremic 4DR-PLWH and 20 non-viremic non-4DR-PLWH. Groups were matched by age, gender and smoking habit. T-cell activation and exhaustion markers were assessed by flow cytometry in 4DR-PLWH. An inflammation burden score (IBS) was calculated from soluble marker levels and associated factors were estimated through multivariate regression.
Results: The highest plasma biomarker concentrations were observed in viremic 4DR-PLWH, the lowest ones in non-4DR-PLWH. Endotoxin core immunoglobulin G showed an opposite trend. Among 4DR-PLWH, CD38/HLA-DR and PD-1 were more expressed on $CD4^+$ ($p = 0.019$ and 0.034, respectively) and $CD8^+$ ($p = 0.002$ and 0.032, respectively) cells of viremic compared to non-viremic subjects. An increased IBS was significantly associated with 4DR condition, higher values of viral load and a previous cancer diagnosis.
Conclusions: Multidrug-resistant HIV infection is associated with a higher IBS, even when viremia is undetectable. Therapeutic approaches aimed to reduce inflammation and T-cell exhaustion in 4DR-PLWH need to be investigated.

Introduction

Nowadays, the vast majority of individuals living with human immunodeficiency virus (HIV) with access to antiretroviral therapy (ART) achieve virologic suppression, although drug resistance is still an unsolved problem that may dramatically reduce therapeutic options for this population.

In a European multicenter cohort study, the cumulative proportion of people living with HIV (PLWH) who had developed triple class virologic failure [i.e. virologic failure of two nucleoside reverse

Abbreviations: AIDS, acquire immunodeficiency syndrome; ART, antiretroviral therapy; BDG, (1,3)-β-D-glucan; CRP, C-reactive protein; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DMEM, Dulbecco's Modified Eagle Medium; EndoCAb, endotoxin core immunoglobulin G; FBS, fetal bovine serum; HAVCR2 or Tim-3, hepatitis A virus cellular receptor 2; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; hs, high sensitivity; IBS, inflammation burden score; IL, interleukin; INSTI, integrase strand transfer inhibitor; IQR, interquartile range; MACE, major adverse cardiovascular event; MDR, multidrug-resistant; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitors; PBMC, peripheral blood mononuclear cell; PDCD1 or PD-1, programmed cell death protein 1; PI, protease inhibitor; PLWH, people living with HIV; PYFU, person-years of follow-up; s, soluble; TNF, tumor necrosis factor; 4DR, four-class drug-resistant; 95%CI, 95% confidence interval

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transcriptase inhibitors (NRTIs), one non-nucleoside reverse transcriptase inhibitor (NNRTI), and one boosted protease inhibitor (PI)] by 9 years from the start of ART was 8.6%.[1] According to more recent Italian epidemiological data, the prevalence of multidrug resistance [defined as at least one major resistant mutation in at least three different drug classes among NRTIs, NNRTIs, PIs and integrase strand transfer inhibitors (INSTIs)] among ART-experienced subjects with HIV was estimated at approximately 9% in the period 2011–2018.[2] Individuals with multidrug-resistant (MDR) strains are usually characterized by a complex clinical history with previous or current uncontrolled viral replication, determining adaptive immune response depletion and occurrence of opportunistic infections. Actually, the incidence of acquired immunodeficiency syndrome (AIDS) defining events and the mortality rate in this population are particularly elevated [1.2/100 person-years of follow-up (PYFU) and 1.4/100 PYFU, respectively].[3] Among MDR-PLWH, it is possible to identify individuals with four-class drug-resistant [(4DR) resistant to NRTIs, NNRTIs, PIs and INSTIs] viruses, that were quantified as about 2% of treatment-experienced PLWH in Italy in 2011–2018.[2] These subjects show a further increased risk of developing AIDS-related events (2.65/100 PYFU) or death for any cause (1.76/100 PYFU), together with a high incidence of non-AIDS-related events (4.71/100 PYFU).[4]

In non-4DR-PLWH on ART, morbidity and mortality remain higher, as compared to HIV-uninfected individuals;[5,6] this may be partially explained by inflammation and immune activation, whose plasma biomarker levels are associated with the rate of non-AIDS-related events[7–14] and decrease but do not revert to normal, once viremia has been suppressed.[14–17] Therefore, the proinflammatory environment recognizes a multifactorial origin, including not only viral replication, but also herpes and hepatitis coinfections[18–20] and microbial translocation, due to increased gut permeability and reduced gut-associated lymphoid tissue response.[21–24] Furthermore, in non-4DR-PLWH the inefficient antiviral T-cell response results in an aberrant expression of immune checkpoint molecules,[25–27] determining an impairment in cytokine production, in order to prevent immune-mediated tissue damage.

In 4DR-PLWH, major drivers of inflammatory burden may include episodes of virologic failure, AIDS events, and long-term exposure to antiretroviral drugs, including currently no longer used compounds with high toxicity. Nevertheless, to our knowledge, there are no available data on the effects of HIV multidrug resistance on inflammation.

The main objective of the current study was to evaluate the impact of four-class drug resistance on inflammation, immune activation, microbial translocation, and T-cell exhaustion markers, in PLWH included in the PRESTIGIO Registry. Thus, we compared plasma levels of soluble biomarkers among 4DR-PLWH with suppressed or unsuppressed viral load and individuals with non-4DR HIV. We also evaluated the expression of exhaustion and activation markers on T cells in the 4DR groups and factors associated with global inflammatory burden in the overall study population.

Materials and methods

Study design and population

This is a matched-cohort study on PLWH, on ART, with ≥18 years of age.

Inflammatory burden was evaluated in 80 individuals, classified into three groups: i) 30 4DR-PLWH with HIV-1 RNA ≥ 50 copies/mL, from the PRESTIGIO Registry; ii) 30 4DR-PLWH with HIV-1 RNA < 50 copies/mL, from the PRESTIGIO Registry; iii) 20 non-4DR-PLWH with HIV-1 RNA < 50 copies/mL. In order to minimize the possible role of confounding factors on evaluated markers, exclusion criteria for this study included the presence of opportunistic diseases (except for Kaposi's sarcoma) or intercurrent infections at the time of sampling, according to clinical evaluation and standard diagnostic criteria, and the assumption of non-steroidal anti-inflammatory drugs, anti-cytomegalovirus antivirals or trimethoprim/sulfamethoxazole (except for the dosage 160/800 mg 3 times/week) at the time of sampling. Groups were matched by age (± 5 years), gender, and smoking habit.

The PRESTIGIO Registry is an Italian, observational, prospective, multicenter, annual collection of demographic, clinical, laboratory, virological, and treatment data of heavily treatment-experienced individuals with 4DR HIV. It includes PLWH with the following characteristics: i) age > 14 years; ii) documented genotypic resistance to NRTIs, NNRTIs and PIs; iii) either genotypic resistance to INSTIs or a history of virologic failure to an INSTI-based regimen, in the absence of an integrase genotype. Genotypic resistance to a drug class was defined as at least intermediate resistance to at least one drug in the class, according to the Stanford HIV database algorithm (version 9.0, hivdb.stanford.edu).

The establishment of the PRESTIGIO Registry was approved (protocol number: 41/int/December 2017) by the San Raffaele Scientific Institute Ethical Committee. Thirty-five Infectious Diseases clinical centers, well distributed across Italy, are actively participating following approval of the study protocol by the local ethical committees. This data collection is registered into the clinicaltrial.gov website (identifier: NCT04098315). The use of anonymized cryopreserved samples and personal data for research studies is regulated by a patient informed consent.

The control non-4DR individuals had been enrolled in the MODAt study,[28,29] a prospective, multicenter, open-label, non-inferiority, randomized, 96-week trial comparing efficacy of atazanavir/ritonavir monotherapy versus atazanavir/ritonavir-based triple therapy. These subjects had signed a written informed consent to collect and analyze cryopreserved plasma samples and to use personal data for research purposes. An inclusion criterion of the MODAt trial was the absence of virologic failure in the clinical history of PLWH included in the analysis. Only samples collected at the baseline (before the ART switch) or from individuals in the triple therapy arm (the control group) were used for our study.

Enzyme-linked immunosorbent assays

Cryopreserved plasma samples were thawed and analyzed for the following soluble biomarkers: i) high sensitivity C-reactive protein [(hs-CRP) R&D Systems, Minneapolis, MN, catalog number DCRP00], an acute phase reactant whose concentrations are significantly higher in individuals with acute and, particularly, chronic HIV infection, as compared to uninfected controls, even after ART start;[16] ii) interleukin 6 [(IL6) R&D Systems, HS600B], a pro-inflammatory cytokine increased in PLWH, that seems to reduce without normalizing with ART;[13,30] iii) tumor necrosis factor α [(TNF-α) R&D Systems, DTA00C], another inflammatory cytokine produced by several cell types, including monocytes/macrophages and T lymphocytes, whose levels are elevated in subjects with HIV;[10,16] iv) D-dimer (Diagnostica Stago, Asnières sur Seine, France, reference Asserachrom D-Di), an indicator of coagulation cascade activation, strictly intertwined with inflammation, with known increased concentrations in chronic HIV infection, even during ART;[10,14,16,17] v) soluble CD163 [(sCD163) R&D Systems, DC1630], a marker of monocyte/macrophage activation, whose levels are elevated in PLWH and do not normalize with therapy;[17] vi) CXCL13 (R&D Systems, DCX130), a chemokine involved in B-cell activation, whose concentrations are increased in individuals with chronic HIV infection and tend to reduce after ART start;[15] vii) sCD14 (R&D Systems, DC140), a soluble form of a receptor involved in response to lipopolysaccharide [(LPS), a microbial by-product that is released in several processes, including bacterial translocation], whose levels are elevated in PLWH and maybe influenced by the ART;[10,16,17,30] viii)

endotoxin core immunoglobulin G [(EndoCAb) Fine Test, Wuhan, China, catalog number EH4142], an antibody elicited by LPS, whose concentrations are interestingly reduced in chronic HIV infection as a probable result of B-cell dysfunction, favoring uncontrolled gut translocation;[22] and ix) (1,3)-β-D-glucan [(BDG) MyBioSource, San Diego, CA, catalog number MBS756415], recently proposed as specific indicator of fungal translocation,[23] a process that requires further studies in PLWH.

All plasma samples were ethylenediamine tetraacetic acid (EDTA)-anticoagulated and stored at − 80 °C without freeze-thaw cycles until the analysis, in order to perform an accurate cytokine measurement[31,32].

An inflammation burden score (IBS) was defined using a methodology similar to previous investigations about inflammation among PLWH.[9,10,13,30,33–35] In the current study, it was a composite score, equal to the number of plasma biomarkers with an abnormal level. An abnormal level was defined as a value at or above the 75th percentile (elevated) for inflammation (hs-CRP, IL6, TNF-α, D-dimer), immune activation (sCD163, CXCL13) and microbial translocation (BDG, sCD14) biomarkers; for EndoCAb, an abnormal level was considered equivalent to a concentration at or below the 25th percentile (reduced). The 25th and the 75th percentiles were calculated by non-transformed biomarker values, obtained from the whole study population. All the considered biomarkers had an equal weight in IBS calculation. The IBS had a range of 0–9, with 0 corresponding to no abnormalities in indicator levels and 9 corresponding to having all the biomarkers with an abnormal value.

Multiparameter flow cytometry

In 4DR-PLWH, multiparameter flow cytometry was performed to quantify the expression of markers of activation [human leukocyte antigen DR (HLA-DR) and CD38] and exhaustion [programmed cell death protein 1 (PDCD1, also known as PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and hepatitis A virus cellular receptor 2 (HAVCR2, also known as Tim-3)] on $CD4^+$ and $CD8^+$ T lymphocytes, which are known to be elevated in PLWH.[25–27,36–38]

Cryopreserved peripheral blood mononuclear cells (PBMCs) were snap-thawed and washed with Dulbecco's Modified Eagle Medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (Euroclone, Pero, Italy). The cells were counted and incubated for 16 h at 37 °C in a 5% CO_2 incubator. Cells were then stained with fluorescently conjugated monoclonal antibodies (Supplementary Table 1). Surface staining was done at room temperature for 15 min with saturating concentrations of antibodies. Stained cells were washed, fixed and run on CytoFLEX S flow cytometer (Beckman Coulter, Miami, FL). At least 50 000 lymphocytes were collected for each sample. Data were compensated (Supplementary Fig. 1) and analyzed using FCS Express 6 (De Novo Software, Pasadena, CA), to determine the proportion of $CD4^+$ and $CD8^+$ T cells expressing each of T-cell markers (CD38, HLA-DR, PD-1, CTLA4, Tim-3) and to evaluate co-expressions (CD38/HLA-DR, PD-1/CTLA4, PD-1/Tim-3).

Statistical methods

The characteristics of the enrolled population at the time of sample collection were described using median [interquartile range (IQR)] or frequency (percentage), both overall and in each group. Comparisons among groups were calculated with Kruskal-Wallis test or Wilcoxon rank-sum test for continuous variables, Chi-square test or Fisher's exact test for categorical ones, as appropriate.

Multivariate linear regression was fitted to determine factors associated with IBS; slopes and 95% confidence interval (95%CI) were reported for factors with a significant effect on IBS. The model included demographic (age) and clinical [hepatitis C virus (HCV) serostatus, use of statin, previous major adverse cardiovascular events (MACEs), previous cancer diagnosis] characteristics with a potential effect on IBS, in addition to ART duration, $CD4^+$ nadir, 4DR status, viral load and $CD4^+$ T-cell count at the time of sampling. MACEs included stroke, acute myocardial infarction, coronary disease requiring revascularization, and congestive heart failure.

Two-sided p-values < 0.05 were considered significant. Statistical analyses were carried out using the SAS software (SAS Institute, Cary, NC, release 9.4), and the SPSS software (Statistical Package for the Social Sciences, SSPS Inc., Chicago, IL, release 26).

Results

Characteristics of the subjects

Overall, 80 PLWH were evaluated: at the time of sampling, median (IQR) age was 51.7 (45.9–55.2) years, 11 (13.8%) were female and 48 (60%) were smokers. Their first HIV positive test dated back to 21.4 (11.8–27.3) years before sampling and they had been on ART since 17.8 (8–23.7) years; a previous AIDS diagnosis occurred in 21 (26.3%) individuals. Median (IQR) $CD4^+$ and $CD8^+$ T cells were 472 (237–766) and 817 (509–1064) cells/mm^3, respectively, with a median (IQR) $CD4^+$ nadir of 156 (47–260) cells/mm^3. Viremic 4DR-PLWH had an HIV-1 RNA of 3.78 (2.1–4.69) log_{10} copies/mL. In terms of comorbidities, 21 (26.3%) had a positive serology for HCV and 3 (3.8%) had a positive hepatitis B surface antigen (HBsAg), 10 (12.5%) had a previous cancer diagnosis, 5 (6.3%) had previous MACEs, and 17 (21.3%) were using statin. Other characteristics are reported in Table 1. ART regimens at the time of sampling are reported in Table 2 and Supplementary Table 2.

Inflammatory burden

Inflammation (hs-CRP, IL6, TNF-α, and D-dimer), immune activation (sCD163 and CXCL13) and microbial translocation (sCD14 and EndoCAb) biomarker levels showed a significant difference among 4DR-PLWH with HIV-1 RNA ≥ 50 copies/mL, virologically suppressed 4DR-PLWH and the non-4DR group (Fig. 1 and Supplementary Table 3). Generally, the highest marker levels were observed among viremic 4DR-PLWH and the lowest ones in non-4DR subjects, even if not all the biomarkers followed this pattern (Fig. 1 and Supplementary Table 3). Remarkably, EndoCAb showed the highest concentrations in non-4DR-PLWH with HIV-1 RNA < 50 copies/mL and the lowest ones in viremic 4DR individuals (Fig. 1D and Supplementary Table 3). Only BDG concentrations were not significantly different among the three groups (Supplementary Table 3).

The proportions of people with abnormal levels of IL6, TNF-α, D-dimer, CXCL13 and sCD14 significantly differed among 4DR-PLWH with HIV-1 RNA ≥ 50 copies/mL, non-viremic 4DR individuals and the non-4DR group (Supplementary Table 3). Furthermore, individuals with an abnormal EndoCAb concentration were significantly more prevalent in the first group, as compared to virologically suppressed subjects, with or without 4DR strains (Supplementary Table 3).

The IBS was significantly higher in 4DR-PLWH with HIV-1 RNA ≥ 50 copies/mL than in those virologically suppressed (p = 0.006); however, non-viremic 4DR-PLWH showed a more elevated IBS, as compared to the non-4DR group (p = 0.027). Twenty-one (70%) viremic 4DR-PLWH had > 2 plasma soluble biomarkers with an abnormal level, against 9 (30%) 4DR-PLWH with HIV-1 RNA < 50 copies/mL and 1 (5%) individual with a non-4DR virus (Fig. 2).

T-cell activation and exhaustion

Among individuals with 4DR HIV, CD38/HLA-DR co-expression on $CD4^+$ and $CD8^+$ T cells was significantly higher in viremic subjects than in those virologically suppressed (Table 3). Furthermore, $CD8^+$ T

Table 1
Characteristics of the study population.

Characteristics	Overall	4DR with HIV-1 RNA ≥ 50 copies/mL (Group 1; n = 30)	4DR with HIV-1 RNA < 50 copies/mL (Group 2; n = 30)	Non-4DR with HIV-1 RNA < 50 copies/mL (Group 3; n = 20)	p-value Group 1 vs. 2 vs. 3§	p-value Group 1 vs. 2§§	p-value Group 2 vs. 3§§	p-value Group 1 vs. 3§§
Age (years)	51.72 (45.89–55.19)	51.72 (45.28–55.11)	53.56 (46.6–56.02)	50.66 (46.06–53.76)	0.429	0.511	0.181	0.559
Male gender	69 (86.3%)	25 (83.3%)	25 (83.3%)	19 (95%)	0.423	1.000	0.381	0.381
Smoking habit	48 (60%)	17 (56.7%)	17 (56.7%)	14 (70%)	0.574	1.000	0.387	0.387
HIV-1 RNA (copies/mL)	36 (0.9–244)	6302.5 (127–48800)	0.9 (0.9–36)	0.9 (0.9–36)	< 0.0001	< 0.0001	0.481	< 0.0001
$\log_{10}$ copies/mL	1.69 (1.69–2.39)	3.78 (2.1–4.69)	1.69 (1.69–1.69)	1.69 (1.69–1.69)	< 0.0001	< 0.0001	1.000	< 0.0001
$CD4^+$ T cell count (cells/mm^3)	472 (237–765.5)	181.5 (75–392)	720 (460–840)	531 (433.5–822.5)	< 0.0001	< 0.0001	0.488	< 0.0001
%	26.2 (15.35–32.5)	14 (8.5–23.6)	27.6 (24–32.7)	31.95 (28.1–38.85)	< 0.0001	< 0.0001	0.045	< 0.0001
$CD8^+$ T cell count (cells/mm^3)	817 (509–1064)	672 (468–911)	982.5 (752–1339)	744 (484–908.5)	0.008	0.008	0.007	0.722
%	41.35 (36.65–53.85)	52.6 (37.6–67)	41 (36.9–46.4)	39.15 (29.25–43.55)	0.005	0.017	0.193	0.004
$CD4^+/CD8^+$ ratio	0.64 (0.3–0.86)	0.25 (0.12–0.48)	0.7 (0.49–0.86)	0.86 (0.65–1.08)	< 0.0001	0.0001	0.061	< 0.0001
Years since first HIV positive test	21.43 (11.78–27.28)	22.78 (17.03–27.69)	25.29 (21.05–28.56)	7.41 (4.58–10.83)	< 0.0001	0.487	< 0.0001	< 0.0001
ART duration (years)	17.78 (8.04–23.69)	19.28 (16.69–25.06)	22.05 (16.85–25.2)	4.28 (2.18–6.38)	< 0.0001	0.555	< 0.0001	< 0.0001
Pre-ART HIV-1 RNA (copies/mL)	120,402 (34,050–228,200)	32,935 (2392–163,000)	228,200 (125,639–318,000)	87,376.5 (40,101.5–187,300)	0.144	0.171	0.116	0.342
$\log_{10}$ copies/mL	5.08 (4.53–5.36)	4.52 (3.38–5.21)	5.36 (5.1–5.5)	4.94 (4.6–5.27)	0.144	0.171	0.116	0.342
Previous AIDS diagnosis	21 (26.3%)	12 (40%)	8 (26.7%)	1 (5%)	0.022	0.412	0.067	0.007
$CD4^+$ nadir (cells/mm^3)	155.5 (47–260)	55 (20–133)	195 (36–311)	261.5 (211.5–355)	< 0.0001	0.016	0.011	< 0.0001
Years of HIV-1 RNA < 50 copies/mL	0.44 (0–2.28)	0(0–0)	1.59 (0.44–2.68)	2.06 (1.36–4.28)	< 0.0001	< 0.0001	0.075	< 0.0001
Positive HCV serostatus	21 (26.3%)	10 (33.3%)	7 (23.3%)	4 (20%)	0.412	0.680	0.308	0.153
Positive HBsAg	3 (3.8%)	1 (3.3%)	2 (6.7%)	0 (0%)	0.298	0.778	0.091	0.215
Previous cancer diagnosis	10 (12.5%)	7 (23.3%)	3 (10%)	0 (0%)	0.043	0.299	0.265	0.032
Previous MACE diagnosis	5 (6.3%)	2 (6.7%)	2 (6.7%)	1 (5%)	0.965	1.000	1.000	1.000
Use of statin	17 (21.3%)	3 (10%)	10 (33.3%)	4 (20%)	0.086	0.057	0.353	0.416
Previous arterial HTN diagnosis	25 (31.3%)	11 (36.7%)	10 (33.3%)	4 (20%)	0.439	1.000	0.353	0.345
Total bilirubin (mg/dL)	0.6 (0.34–1.4)	0.35 (0.25–0.57)	0.52 (0.34–0.7)	2.09 (1.45–3.47)	<0.0001	0.051	<0.0001	<0.0001
eGFR (mL/min/1.73 m^2)	95 (80–103.5)	95.5 (82–104)	$87^{76–99}$	101 (94–106)	0.009	0.160	0.001	0.111

Results described by median (interquartile range) or frequency (percentage). Statistical analyses were performed by Kruskal-Wallis test (continuous variables) or Chi-square test (categorical variables) in the comparison between the three groups (§), Wilcoxon rank-sum test (continuous variables) or Fisher's exact test (categorical variables) in the comparisons between Group 1 and 2 or Group 2 and 3 or Group 1 and 3 (§§). Abbreviations: AIDS: acquired immunodeficiency syndrome; ART: antiretroviral therapy; eGFR: estimated glomerular filtration rate; HBsAg: hepatitis B surface antigen; HCV: hepatitis C virus; HIV: human immunodeficiency virus; HTN: hypertension; MACE: major adverse cardiovascular event; vs.: versus; 4DR: four-class drug-resistant; %: percentage.

Table 2
Antiretroviral therapy regimens taken by the study population at the time of sampling.

ART regimen	Overall	4DR with HIV-1 RNA ≥ 50 copies/mL (Group 1; n = 30)	4DR with HIV-1 RNA < 50 copies/mL (Group 2; n = 30)	Non-4DR with HIV-1 RNA < 50 copies/mL (Group 3; n = 20)	p-value
2-drug regimen	13 (16.25%)	2 (6.67%)	11 (36.67%)	0 (0%)	< 0.0001
1 boosted PI + 2 NRTIs	22 (27.5%)	2 (6.67%)	0 (0%)	20 (100%)	
1 INSTI + 2 NRTIs	2 (2.5%)	1 (3.33%)	1 (3.33%)	0 (0%)	
Complex regimen	43 (53.75%)	25 (83.33%)	18 (60%)	0 (0%)	

Results described by frequency (percentage). Statistical analyses were performed by Chi-square test. A complex regimen was defined as ≥ 3-drug combination different from 1 boosted PI + 2 NRTIs or 1 INSTI + 2 NRTIs or 1 NNRTI + 2 NRTIs. Abbreviations: ART: antiretroviral therapy; HIV: human immunodeficiency virus; INSTI: integrase strand transfer inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; NRTI: nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; 4DR: four-class drug-resistant.

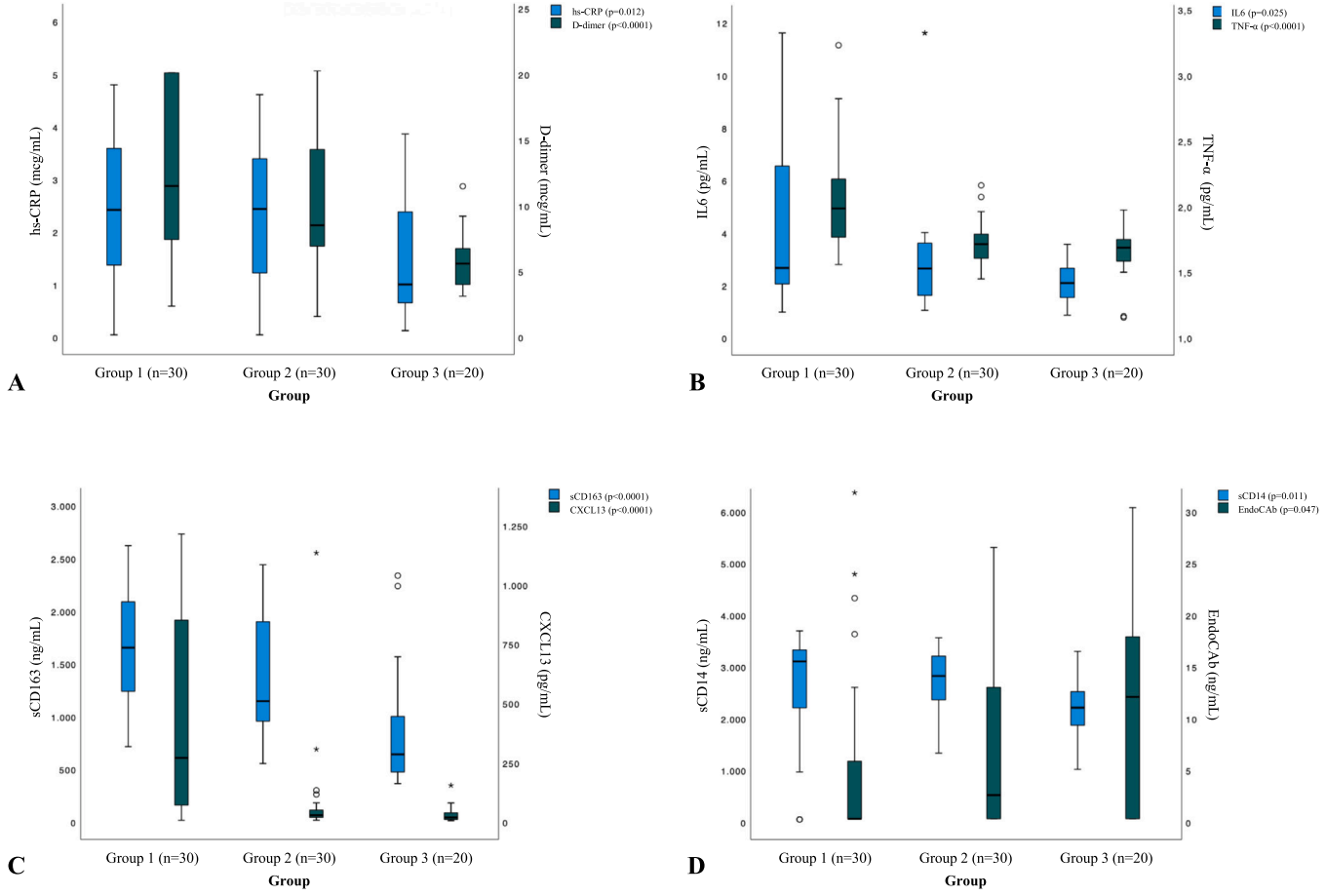

Fig. 1. Plasma levels of inflammation, immune activation and microbial translocation soluble biomarkers in viremic 4DR-, non-viremic 4DR-, and non-4DR-PLWH. Groups were matched by age (± 5 years), gender and smoking habit. The boxes represent the middle 50% of the data values for each group. The horizontal line across the box marks the median value. The error bars identify the minimum value and the maximum, excluding outliers. Mild outliers (circles) are defined as (> 3rd quartile + 1.5 *IQR) or (< 1st quartile - 1.5 *IQR). Extreme outliers (stars) are defined as (> 3rd quartile + 3 *IQR) or (< 1st quartile - 3 *IQR). Statistical analyses were performed by Kruskal-Wallis test and Wilcoxon rank-sum test, with statistical significance at $p < 0.05$. Group 1: people living with four-class drug-resistant HIV with HIV-1 RNA ≥ 50 copies/mL. Group 2: people living with four-class drug-resistant HIV with HIV-1 RNA < 50 copies/mL. Group 3: people living with non-four-class drug-resistant HIV with HIV-1 RNA < 50 copies/mL. Abbreviations: CRP: C-reactive protein; EndoCAb: endotoxin core immunoglobulin G; hs: high sensitivity; HIV: human immunodeficiency virus; IL: interleukin; IQR: interquartile range; PLWH: people living with human immunodeficiency virus; s: soluble; TNF: tumor necrosis factor; 4DR: four-class drug-resistant.

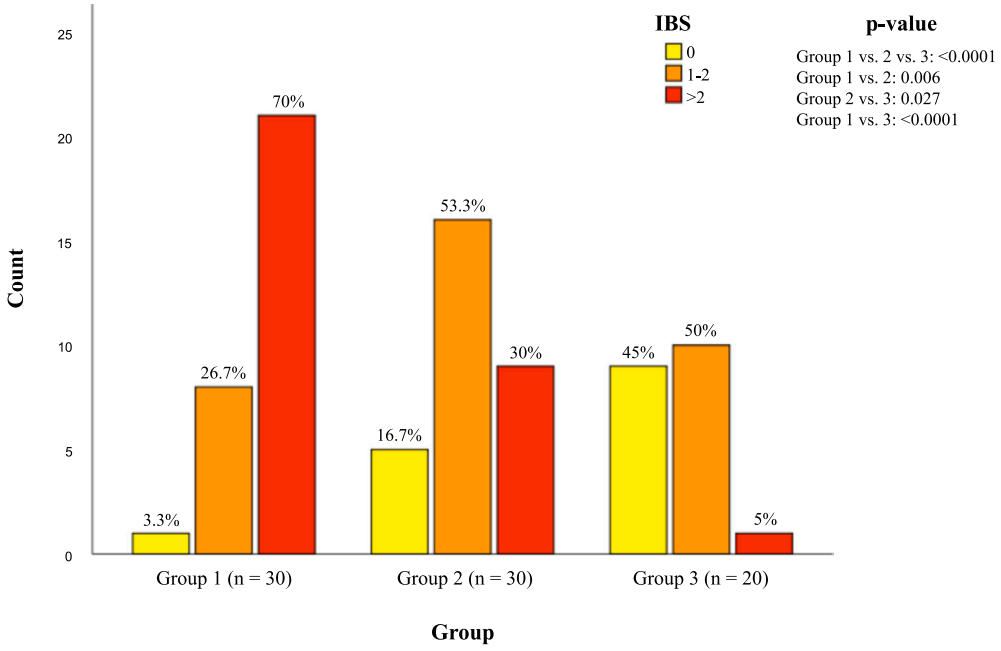

Fig. 2. Inflammation burden score distribution in viremic 4DR-, non-viremic 4DR-, and non-4DR-PLWH. Groups were matched by age (± 5 years), gender and smoking habit. Data are shown as frequency (percentage). Statistics were performed by Kruskal-Wallis test and Wilcoxon rank-sum test, with statistical significance at $p < 0.05$. Group 1: people living with four-class drug-resistant HIV with HIV-1 RNA ≥ 50 copies/mL. Group 2: people living with four-class drug-resistant HIV with HIV-1 RNA < 50 copies/mL. Group 3: people living with non-four-class drug-resistant HIV with HIV-1 RNA < 50 copies/mL. Abbreviations: HIV: human immunodeficiency virus; IBS: inflammation burden score; PLWH: people living with human immunodeficiency virus; vs.: versus; 4DR: four-class drug-resistant; %: percentage.

Table 3
T-cell activation and exhaustion markers in 4DR-PLWH.

Biomarkers	4DR with HIV-1 RNA ≥ 50 copies/mL (Group 1; n = 30)	4DR with HIV-1 RNA < 50 copies/mL (Group 2; n = 30)	p-value
$CD38^+$, % of $CD4^+$ T cells	35.29 (12.38–39.03)	20.28 (11.72–41.08)	0.514
$HLA\text{-}DR^+$, % of $CD4^+$ T cells	12.32 (5.41–14.75)	4.99 (3.53–7.74)	0.037
$CD38^+HLA\text{-}DR^+$, % of $CD4^+$ T cells	4.41 (2.32–5.32)	1.88 (0.82–2.76)	0.019
$PD\text{-}1^+$, % of $CD4^+$ T cells	47.68 (40.83–62.51)	35.78 (28.22–46.89)	0.034
$CTLA4^+$, % of $CD4^+$ T cells	1.47 (0.49–2.32)	0.85 (0.42–1.52)	0.382
$Tim\text{-}3^+$, % of $CD4^+$ T cells	0.98 (0.4–1.54)	1.23 (0.85–1.77)	0.279
$PD\text{-}1^+CTLA4^+$, % of $CD4^+$ T cells	0.49 (0.17–1.23)	0.43 (0.32–0.75)	0.849
$PD\text{-}1^+Tim\text{-}3^+$, % of $CD4^+$ T cells	0.49 (0.17–1.23)	0.43 (0.32–0.75)	0.849
$CD38^+$, % of $CD8^+$ T cells	33.48 (8.68–40.49)	10.24 (6.57–18.06)	0.034
$HLA\text{-}DR^+$, % of $CD8^+$ T cells	13.84 (10.93–18.2)	8.14 (4.92–14.75)	0.018
$CD38^+HLA\text{-}DR^+$, % of $CD8^+$ T cells	6.1 (3.9–7.52)	1.86 (0.98–3.46)	0.002
$PD\text{-}1^+$, % of $CD8^+$ T cells	48.94 (44.71–59.56)	39.63 (29.75–51.6)	0.032
$CTLA4^+$, % of $CD8^+$ T cells	1.09 (0.6–1.72)	0.37 (0.25–0.97)	0.077
$Tim\text{-}3^+$, % of $CD8^+$ T cells	0.95 (0.69–1.78)	1.31 (0.88–1.63)	0.474
$PD\text{-}1^+CTLA4^+$, % of $CD8^+$ T cells	0.3 (0.22–0.64)	0.08 (0.01–0.28)	0.057
$PD\text{-}1^+Tim\text{-}3^+$, % of $CD8^+$ T cells	0.51 (0.32–0.7)	0.47 (0.34–0.79)	0.738

Results described by median (interquartile range). Statistical analyses were performed by Wilcoxon rank-sum test. Abbreviations: CTLA4: cytotoxic T-lymphocyte-associated protein 4; HIV: human immunodeficiency virus; HLA: human leukocyte antigen; PD-1: programmed cell death protein 1; PLWH: people living with HIV; Tim-3: hepatitis A virus cellular receptor 2; 4DR: four-class drug-resistant.

cells expressing CD38 or HLA-DR and $CD4^+$ T cells expressing HLA-DR were significantly higher in the first group compared to the second one (Table 3).

Similar results were obtained for T-cell exhaustion: PD-1 was significantly more expressed on $CD4^+$ and $CD8^+$ T lymphocytes of 4DR-PLWH with HIV-1 RNA ≥ 50 copies/mL (Table 3). Other markers of immune exhaustion did not show the same results, even if CTLA4 expression and PD-1/CTLA4 co-expression on $CD8^+$ T cells were marginally higher in the same group (Table 3).

Factors associated to inflammatory burden

After adjusting for age, ART duration, $CD4^+$ T-cell count, $CD4^+$ nadir, HCV serostatus, use of statin and previous MACEs, higher values of the IBS were significantly associated with the 4DR condition (slope = +1.65, 95%CI = 0.02–3.06, p = 0.023), increasing values of viral load (per $1\ log_{10}$ copies/mL higher: slope = +0.66, 95%CI = 0.23–1.09, p = 0.003) and the presence of a previous cancer diagnosis (slope = +2.04, 95%CI = 0.76–3.33, p = 0.002).

Discussion

Our findings showed that individuals with four-class drug-resistant HIV-1, including those virologically suppressed, were characterized by a higher inflammatory burden, as compared to subjects with a non-4DR virus.

Plasma concentrations of all the examined inflammation and immune activation indicators were significantly different among

4DR-PLWH with HIV-1 RNA ≥ 50 copies/mL, virologically suppressed 4DR-PLWH and the non-4DR group. In particular, TNF-α, sCD163, CXCL13 and marginally IL6 and D-dimer levels were higher in viremic 4DR-PLWH than in those with HIV-1 RNA < 50 copies/mL. These results are in line with other clinical studies highlighting that levels of these markers show an improvement in individuals with HIV on effective ART,[14–17] even if not attaining normal levels. However, we found higher concentrations of hs-CRP, D-dimer, sCD163 and marginally IL6 and CXCL13 among individuals with HIV-1 RNA < 50 copies/mL harboring a 4DR virus, as compared to non-4DR-PLWH. This is consistent with the complex clinical history of 4DR-PLWH: by definition, antiretroviral drug resistance selection is strictly linked to prolonged and uncontrolled viral replication in the presence of ART. HIV reservoir is known to be associated with the previous pattern of viral load, including periods of viral suppression or active replication,[39] so it is expected to be elevated in individuals with MDR HIV; an association between inflammation and latent and active viral reservoir had been previously described.[40] Furthermore, non-viremic 4DR-PLWH had a lower $CD4^+$ nadir, as compared to the non-4DR group; in previous studies, this immunological parameter had been shown as related to a faster premature inflamm-ageing.[41]

We obtained similar findings for sCD14, an indirect indicator of gut translocation, whose concentrations were significantly lower in non-viremic non-4DR-PLWH. By contrast, among groups with four-class drug resistance, there was not a significant difference due to the viral load; actually, prior studies did not unanimously prove the reduction of sCD14 levels after the initiation of ART.[16,17]

In our analysis, endotoxin core immunoglobulin G, another indirect biomarker of microbial translocation, was differently expressed among the enrolled groups: in this case, the highest levels were detected in non-4DR-PLWH and the lowest ones in viremic subjects with a 4DR virus. In line with our results, a reduction in plasma EndoCAb concentrations had been observed in PLWH compared to HIV-negative subjects[22]: in case of acute infection, these decreased levels could be due to binding and neutralization of circulating LPS, similarly to what happens during acute microbial translocation [such as sepsis[42]]; in chronic HIV infection, this reduction is opposite to the increase observed in other conditions of chronic microbial translocation [for instance, inflammatory bowel diseases[43]] and might be justified, partially at least, by a dysfunctional and exhausted B-cell response.[44,45] Multidrug resistance, associated with complex clinical history, may be involved in enhancing B-cell exhaustion. Moreover, the aforementioned increase of CXCL13, that we observed in 4DR-PLWH, suggests that a T-follicular-helper-cell dysfunction may contribute to this process, especially in the viremic group. These events could result in a further demodulation of response to bacterial translocation, exacerbating its role of contributor to inflammation and immune activation.

The only analyzed soluble marker whose levels did not vary significantly among the three groups was (1,3)-β-D-glucan, an indicator of fungal translocation. Previous studies were also inconclusive, by reporting both increased[46] and decreased[23] concentrations of this marker in PLWH, as compared to HIV-uninfected controls.

Our multivariate linear regression analysis showed a significant association between HIV four-class drug resistance and IBS. Apparently, this could be justified by the lower $CD4^+$ T-cell count and nadir reported for viremic 4DR-PLWH. By definition, viremic 4DR-PLWH have an active viral replication and a concurrent reduction in $CD4^+$ T-cell count. Moreover, the long history of virologic failures in 4DR population may explain a lower $CD4^+$ nadir, as aforementioned. However, these factors were included in our model and the 4DR status remained significantly related to an increase of IBS, reinforcing the hypothesis about a pro-inflammatory role of previously uncontrolled viral replication. Intriguingly, a higher inflammatory burden in individuals with a MDR virus might partially account for the premature ageing process and the high risk of AIDS- and non-AIDS-related events or deaths, observed in this population.[4] Inflammatory burden was also found to be associated with higher HIV-1 RNA levels and a previous cancer diagnosis. Many studies showed a correlation between HIV-1 viremia and inflammatory marker levels, that are particularly reduced after virologic suppression.[14–17] For what regards the association between cancer and inflammatory burden, inflammation is recognized as a hallmark feature of cancer development and progression[47,48] and many trials have suggested the efficacy of anti-inflammatory therapy in neoplastic patients.[48]

In line with soluble marker findings, among 4DR-PLWH, also cellular biomarkers of immune activation (HLA-DR and CD38/HLA-DR co-expression on $CD4^+$ and $CD8^+$ T cells, and CD38 on $CD8^+$ T cells) were significantly more expressed in individuals with HIV-1 RNA ≥ 50 copies/mL. Similarly, previous studies showed that the expression of these cellular molecules is elevated in PLWH, compared to HIV-uninfected individuals, and tends to diminish with virologic suppression and to increase with virologic failure.[36–38]

We also found that, among 4DR-PLWH, the expression of immune checkpoint molecules on $CD8^+$ (PD-1 and, marginally, CTLA4 and PD-1/CTLA4 co-expression) and $CD4^+$ (PD-1) T cells was increased in viremic subjects, as a probable consequence of excessive T-cell activation. PD-1, probably the best-known immune exhaustion receptor, had been previously reported to be up-regulated on T lymphocytes in individuals with chronic HIV infection and associated with viral load.[25,49] A similar trend had been shown for CTLA4, especially on $CD4^+$ T cells, although its decrease after ART initiation seemed to be modest and slow.[50] As for new inhibitory pathways, we studied the expression of Tim-3, known as a marker of activated but dysfunctional T-cell population in PLWH,[27] but we did not find any significant difference in the two groups of 4DR-PLWH.

Considering the particularly elevated IBS and the higher immune exhaustion that we observed in viremic 4DR-PLWH, an integrated therapeutic approach, including drugs that reduce inflammation (for instance, anti-TNF-α molecules) and microbial translocation (e.g., probiotics or trimethoprim/sulfamethoxazole) or revert T-cell exhaustion (immune checkpoint inhibitors), might be hypothesized for this population with very limited treatment options. However, further studies are required to validate this possible therapeutic strategy.

The current investigation has some limitations that need to be mentioned. Firstly, given the small sample of 4DR-PLWH enrolled, findings from this study are difficult to be generalized to other MDR-PLWH. This constraint is mainly due to the low prevalence of four-class drug resistance among individuals with HIV and the rigorous matching criteria. However, matching by age, gender and smoking habit allowed us to avoid these factors from affecting the analysis of immune dysregulation biomarkers. Furthermore, the absence of data on T-cell activation and exhaustion markers in virologically suppressed non-4DR-PLWH limits the strength of our observations on cellular marker trend, although it has no substantial implications in possible future treatment strategies for viremic 4DR-PLWH. The unavailability of direct measures of gut-epithelial disfunction in the current study can be added to the constraints; nonetheless, we considered indirect indicators (sCD14 and EndoCAb) as appropriate surrogate markers of bacterial translocation, underlying inflammation and immune activation. The lack of a control viremic non-4DR group can be considered another limitation, but in our country this population includes mainly ART-naïve PLWH with younger age, so the baseline characteristics of this group would be extremely different from the three others. Finally, the absence of information on comorbidities (except for HCV, HBV, MACEs, arterial hypertension, use of statin, and cancer) was another constraint; in particular, cytomegalovirus coinfection is recognized as an important contributor to inflammation in PLWH. The role of herpes coinfection in inflamm-ageing is still debated in case of low $CD4^+$ T-cell count,[18,51,52] so it

was not correctly evaluable in viremic 4DR-PLWH, due to their compromised immune response (median $CD4^+$ T-cell count < 200 cells/mm^3). However, in non-viremic groups that had median $CD4^+$ T-cell count > 500 cells/mm^3, the unknown serostatus and viremia did not allow us to exclude a role of cytomegalovirus in the increase of inflammatory burden.

In conclusion, the current study contributes to our knowledge on the effects of HIV multidrug resistance on inflammatory burden. Actually, we provide evidence that people living with MDR HIV-1, especially when viral replication is uncontrolled, are characterized by a higher inflammation burden score compared with non-4DR-PLWH. This potentially offers the opportunity for new therapeutic options aimed at reducing excessive inflammation or reverting T-cell exhaustion, in order to improve the survival and the quality of life of this fragile population.

Author contributions

AC, VS, LG, AP, and TC had full access to all the data in the study and took final responsibility for the integrity of the data, the accuracy of the data analysis, and the decision to submit for publication. AC and VS conceived the study. LG and TC helped in the study design. RC and TC performed the experiments. RC performed flow cytometric data analyses, LG and AP performed statistical analyses. AG, AP, PC, GCM, AB, MZ, and MMS contributed in data collection, administrative or material support. TC, VS and LG wrote the first draft of the manuscript. All the authors contributed in data interpretation and critical revision of the manuscript.

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Availability of data and materials

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

Declaration of interest

None.

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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.jinf.2023.03.011.

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