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Factors associated with oxidative stress in virologically suppressed people living with HIV on long-term antiretroviral therapy

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Abstract

Background Oxidative stress (OS) is the imbalance between oxidant and antioxidant molecules, in favour of oxidants, that has been associated with an increased risk of morbidity and mortality in ART-treated people living with HIV (PLWH). We aimed to assess factors associated with OS in virologically suppressed PLWH on long-term modern ART.

Method In this cross-sectional study we evaluated OS by measuring both the levels of derivatives-reactive oxygen metabolites (d-ROMs) and the biological antioxidant potential (BAP). We also calculated the BAP/d-ROMs ratio, (OS index, OSi); a cut-off value < 7.3 indicated OS. Factors associated with OS markers were explored by linear regression model.

Results We enrolled 299 experienced PLWH with virological suppression (HIV-RNA < 50cps/mL). The mean of the d-ROMs levels was 409 UCARR (95%CI 394–422), whereas the mean of the BAP levels was 1.809 µmol/L (95%CI 1706–1851). The OSi mean value was 4.84, and 91.6% of the participants were below the cut-off value. By regression analysis, higher production of oxidants was associated with female sex ($p < 0.001$), current exposition to PIs ($p = 0.030$) and HCV co-infection ($p = 0.006$). Higher antioxidant capacity was correlated with higher HDL levels ($p = 0.001$). A lower OSi was associated with female sex ($p = 0.003$) and the current use of triple vs. dual regimen ($p = 0.036$). The OSi correlated negatively with cholesterol levels ($p = 0.002$) and positively with HDL ($p < 0.001$).

Conclusions Virologically suppressed PLWH on long-term ART showed a marked OS. Female sex, the exposure to PIs, and HCV co-infection were associated with higher oxidants, while higher HDL levels were linked to better antioxidant capacity. Interestingly, dual therapy, especially INSTI-based regimens, was associated with lower oxidative stress compared to triple therapy.

Keywords HIV, Oxidative stress, Antioxidant defence, d-ROM test, BAP test

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Introduction

Modern antiretroviral therapy (ART), which results in high rates of viral replication suppression, has transformed HIV infection from a progressive, typically fatal infection to a chronic and manageable disease. As ART is unable to eradicate the virus, people living with HIV (PLWH) must maintain lifelong treatment. PLWH on effective ART have a longer life expectancy and decreased morbidity and mortality than untreated patients [1]. However, ART-treated suppressed PLWH still present persistent chronic immune activation and inflammation; this condition can trigger a vicious cycle that exacerbates immune dysfunctions and inflammation [2, 3] and leads to an increased risk of developing aging-associated and non-AIDS co-morbidities in PLWH as compared to the general population [4, 5]. It has been shown that these conditions are characterized by an altered redox status, i.e., oxidative stress (OS), a condition in which there is an imbalance between oxidant and antioxidant species that has shifted in favour of the oxidants. Several previous studies have shown that OS levels can be associated with morbidity and mortality in ART-treated PLWH independently of the levels of inflammation markers and other routine prognostic markers [6–8]. The most abundant oxidant species, i.e., free radicals, are reactive oxygen species (ROS); they are very highly reactive molecules, due to the unpaired electron in their external shell. At low/moderate concentrations, they are needed for physiological activities such as intracellular cell signaling and homeostasis, immune defence against pathogens, and induction of the mitogenic response. The source of free radicals can be endogenous as a natural product of the normal cellular metabolism of oxygen (i.e., superoxide ion radical, lipoxygenase, NADPH dependent oxidase), or exogenous (i.e., alcohol or smoking). However, the human body is equipped with an antioxidant protection system that counteracts the free radical toxicity and consists of endogenous antioxidants (i.e., enzymes superoxide dismutase, catalase and glutathione peroxidase and non-enzymatic molecules bilirubin, glutathione, vitamin E, beta carotene, albumin and uric acid) and exogenous antioxidants (i.e., Vitamin C or Vitamin E) [9]. Under normal conditions this antioxidant system can defend against oxidant stress both by neutralizing ROS and by maintaining the redox balance of the biological molecules. Free radicals generated in excessive and uncontrollable amounts under OS conditions cause damage to DNA, proteins and lipids, which can severely compromise cellular longevity and contribute to disease development [10].

It has been demonstrated that HIV infection induces oxidative damage by the deregulation of OS pathways. In the absence of ART, the viral Tat protein induces a cascade of reactions with the subsequent escalation of ROS production by mitochondrial generation of superoxide anion [11]. It has also been found that HIV indirectly inhibits the activity of the antioxidant enzymes and causes the depletion of circulatory endogenous antioxidants. During HIV infection, an increase in oxidative damage is correlated with disease progression [8].

However, it has also been demonstrated that ART might contribute to tilting the antioxidant-oxidant balance of the host by increasing the chemically reactive species in circulation. Thus, PLWH show high OS process and antiretroviral therapy could play a role in enhancing this effect. In fact, several studies, mostly conducted in the early ART era, reported an increase in OS during ART in addition to the persistent redox imbalance associated with HIV infection [12–14]. Furthermore, other reports show that protease inhibitors (PIs), as well as other antiretroviral drugs, trigger massive ROS production in various cell types [15–18].

However, it is difficult to well characterize and measure the pattern of OS, especially in a complex setting such as that of ART-treated PLWH.

To date, studies concerning the OS in PLWH on effective modern ART are still scanty.

In the present study, we comprehensively assessed both plasma total oxidant and antioxidant levels in order to explore the factors associated with the OS in long-term ART-experienced PLWH with virological suppression.

Materials and methods

Study design

This was a monocentric cross-sectional study that enrolled PLWH from September 2022 to March 2023. We consecutively collected blood samples of PLWH who routinely attended our healthcare facilities at the Department of Infectious Diseases of the University Hospital "Fondazione Policlinico Universitario A. Gemelli IRCCS-Università Cattolica del Sacro Cuore" in Rome, Italy. Concurrently with the blood sampling, we collected demographic, therapeutic, clinical, and viro-immunological parameters for each participant from our electronic database. The inclusion criteria were PLWH of both sexes, over 18 years of age and on ART and being on virological suppression (HIV-RNA < 50cps/mL). The study was conducted in accordance with the Good Clinical Practice and ethical principles of the Declaration of Helsinki. The protocol was reviewed and approved by our local Ethics Committees (ID4477 14/10/2021). Written informed consent was obtained from all participants.

The blood samples of participants were collected in tubes with heparin, in the early morning after an overnight fast. Immediately after the blood draw, plasma samples were separated by centrifugation. To avoid repeated freeze-thaw cycles we divided the sample into 0.2mL aliquots and immediately stored at −80∘C until use.

Assessment of plasma total oxidant levels

We quantified plasma total oxidant levels by using the d-ROMs (Reactive Oxygen Metabolites test (#MC001, Diacron International, Grosseto, Italy) [19, 20]. This is a colorimetric determination of hydroperoxides content (ROOH); these are intermediate oxidative products of lipids, peptides and amino acids, and relatively more stable compounds than their relative parent free radicals. Specifically, this test photometrically quantifies the colored-derivate between the product of hydroperoxides with iron that is released from plasma proteins by an acidic buffer (i.e., reagent R2), according to Fenton's reaction, and a substituted aromatic amine solubilized in a chromogenic mixture, reagent R1.

We followed the manufacturers' instructions with some modifications to adapt the protocol to 96-well microplates. Briefly, we prepared the working solution (R1:R2 = 1:100) by mixing 2 µl of R1 and 190 µl of R2. The remaining 10 µl of R2 l was pipetted into an empty well in which 1 µl of sample was dissolved to reach the total final volume of 200µL. This step allowed the small volume of the sample to dissolve evenly in the reagent. In order to have the reaction start at the same time for all of the samples, we added the working solution with a multichannel to all the wells. Then we incubated the microplate at 37 °C for 75 min. and then immediately read it by measuring the absorbance at 505 nm. The values are expressed in arbitrary units (UCARR), with 1 UCARR corresponding to 0.08mg H_2O_2/dL [19, 20].

Assessment of plasma total antioxidant levels

We also quantified the plasma total antioxidant barrier by using the BAP (biological antioxidant potential) test (#MC436, Diacron International, Grosseto, Italy) [21]. The BAP test is based on the capacity of a coloured solution of ferric ions Fe^{3+}, reagent R2, complexed to a particular chromogen, reagent R1, to decolour when the ferric ions Fe^{3+} are reduced to ferrous ions Fe^{2+} by adding an antioxidant system, i.e., a blood plasma sample. We followed the manufacturers' instructions with some modifications to adapt the protocol to 96-well microplates. Briefly, to adapt the working solution R2:R1 = 1:20 volume we mixed 19 µl of R2 and 380 µl of R1. We added 3.8ul of the sample to the empty wells and then added the working solution with a multichannel to start the reaction simultaneously for each sample. After incubating for 5 min at 37 °C, the absorbance measurement at 505 nm was performed. The results were expressed as µmol of ferric ions reducing antioxidants per litre of the sample (µmol/L) [21].

Standards and settings

For both the d-ROM and the BAP test, we used a calibrator and two control sera ''Low" and "High" (#MC030, #MC031, #MC032, Diacron International, Grosseto, Italy) for each run. We also added a plasma sample from one PLWH as further reference.

The inter-assay and intra-assay coefficients of variation (CV%) ranged from 4.6 to 7.6 for the d-ROM, and 3.9 to7 for the BAP test.

All the standards and plasma samples were assessed in triplicate. Replicates that did not meet the CV% values were discarded and were rerun. The same plasma sample from each participant was used to simultaneously assess the oxidation degree and the antioxidant capacity.

The spectrophotometer measurements and the 37 °C incubation were performed using the Multiskan™ GO Microplate Spectrophotometer (Thermo Fischer Scientific, Waltham, MA, USA).

For each sample, we evaluated the d-ROMs test result, which was adopted as a measure of the oxidation degree, and the BAP test result, which indicated the antioxidant capacity of the tested plasma.

Moreover, for each sample we calculated the BAP/d-ROMs ratio, used as an index of the balance between the antioxidant and oxidant species and expressed as the oxidative stress index (OSi). The cut-off value for the BAP/d-ROMs ratio was set at 7.3; a value lower than 7.3 was defined as an oxidized type and a higher or equal value was considered normal [22–24].

Statistical analysis

Continuous variables were described as medians (IQR) or means (95%CIs). Categorical variables were presented as frequency counts. D-ROM and BAP values were normally distributed, according to the Shapiro–Wilk test, and parametric analyses were performed. We explored the demographic, clinical, therapeutic, viro-immunological and laboratory factors associated with d-ROMs levels, BAP levels and OSi values by using a linear regression analysis. A Pearson correlation analysis was used to correlate d-ROM and BAP levels. We performed a backward stepwise multivariate logistic regression analysis to verify the association of OS by including confounders and all relevant variables related to the outcome of the univariate regression. No outliers had to be removed from the analysis. For all of the statistical analyses, a p value lower than 0.05 was statistically significant. All of the statistical analyses were performed using SPSS (IBM SPSS Statistics for Windows, Version 21.0. Chicago, IL, USA).

Results

We enrolled 299 PLWH who met the inclusion criteria. The participants' characteristics at the time of blood collection are reported in Table 1. Most of the participants were males (75.6%), with a median age of 52 (IQR 44–60) years. Most participants (97.7%) had undetectable viremia (i.e., HIV-RNA target not detected, TND). A similar

Table 1 Characteristics of PLWH enrolled in the study ($N = 299$)

	Whole population $n = 299$
Sex, *n* (%)	
Male	226 (75.6)
Female	73 (24.4)
Age,years,median (IQR)	52 (44–60)
Caucasian,n (%)	269 (90.0)
Risk factor,n (%)	
MSM	138 (46.2)
Heterosexual	118 (39.5)
PWID	15 (5.0)
Unknown/others	28 (9.3)
Time since HIV diagnosis,years,median (IQR)	12.0 (7.3–20.1)
Time on ART,years,median (IQR)	10.6 (7.2–15.7)
BMI,median (IQR)	25.0 (21–28)
Smoking habits[a], n (%)	154 (51.5)
Currently smoking	152 (50.8)
Quit smoking	2 (0.67)
Alcohol use[b], n (%)	133 (44.5)
CD4 cell count nadir,cells/mm^3, median (IQR)	214 (72–340)
Zenith HIV-RNA,Log$_{10}$copie/mL,median (IQR)	5.04 (4.46–5.46)
CD4 cell count,cells/mm^3, median (IQR)	690 (491–874)
CD8 cell count,cells/mm^3, median (IQR)	662 (503–878)
CD4/CD8 ratio,median (IQR)	1.01 (0.76–1.38)
HIV-RNA TND (0 copy/mL),n (%)	292 (97.7)
Past AIDS-defining events,(CDC C),n (%)	78 (26.1)
History of cardiovascular diseases[c], n (%)	48 (16.1)
History of cancer[d], n (%)	30 (10.0)
Co-infection,n (%)	
HCV Co-infection[e]	37 (12.4)
HBV Co-infection[f]	5 (1.7)
Comorbidities,n (%)	
Hypertension	44 (14.7)
Diabetes	16 (5.4)
Psychiatric	44 (14.7)
ART,n (%)[g]	
Triple regimen	156 (52.8)
2NRTIs + INSTI	98 (63.3)
FTC/TAF/BIC	76 (77.5)
FTC/TAF + DTG	14 (14.3)
FTC/TAF + RGV	8 (8.2)
2NRTIs + NNRTI	43 (27.2)
FTC/TAF/RPV	25 (58.1)
3TC/TDF/DOR	18 (41.9)
2NRTIs + PI/b	15 (9.5)
FTC/TAF/DRV/COBI	
Dual regimen	141 (47.2)
NRTI + INSTI	133 (95.0)
3TC/DTG	133 (100.0)
NRTI + PI/b	8 (5.0)
3TC + DRV/COBI	8 (100.0)
Concomitant drugs,n (%)	
Statins	31 (10.4)
Vitamins	67 (22.4)
Lipid Nutritional Supplements	50 (16.7)

Table 1 (continued)

	Whole population *n* = 299
NSAIDs	7 (2.3)
Lipid profile,median (IQR)	
Cholesterol,mg/dL	182 (154–205)
Triglycerides,mg/dL	104 (74–151)
HDL,mg/dL	46 (39–54)
Previous blip[h], n (%)	121 (40.5)
Previous failure[i], n (%)	85 (28.4)
Time since last detectable viral load (≥ 50cps/mL),years,median (IQR)	7.9 (5.0-12.1)
Cumulative time with viral load < 50cps/mL,years,median (IQR)	9.04 (5.9–13.2)

Abbreviations BMI: Body mass index; PWID: people who inject drugs; MSM: Men who have sex with men; ART: Antiretroviral therapy; HCV: Hepatitis C; HBV: Hepatitis B; THN: target not detected; NRTIs: Nucleoside reverse transcriptase inhibitors; NNRTIs; Non-nucleoside reverse transcriptase inhibitors; INSTIs: Integrase Inhibitors; PI: Protease Inhibitor. NSAIDs: Non-steroid anti-inflammatory drugs

[a] An adult who has smoked 100 cigarettes in his or her lifetime and who currently smokes cigarettes and who had quit smoking in the previous 6 months. All the current smokers smoke ≥ 10 cigarettes per day; [b] ≥2 alcoholic unit/day; [c] Includes cardiomyopathy, ischaemic stroke and myocardial infarction; [d] Previous non-aids related tumor. [e] Defined as HCV RNA detected. [f] Defined as HBcAb positive. [g] Two PLWH were on non-conventional ART. [h] Defined as a single HIV-RNA values between 50–999 copies/mL. [i] Defines as single HIV-RNA value equal to1,000 or at least two consecutive values between 50 – 1,000 copies/mL

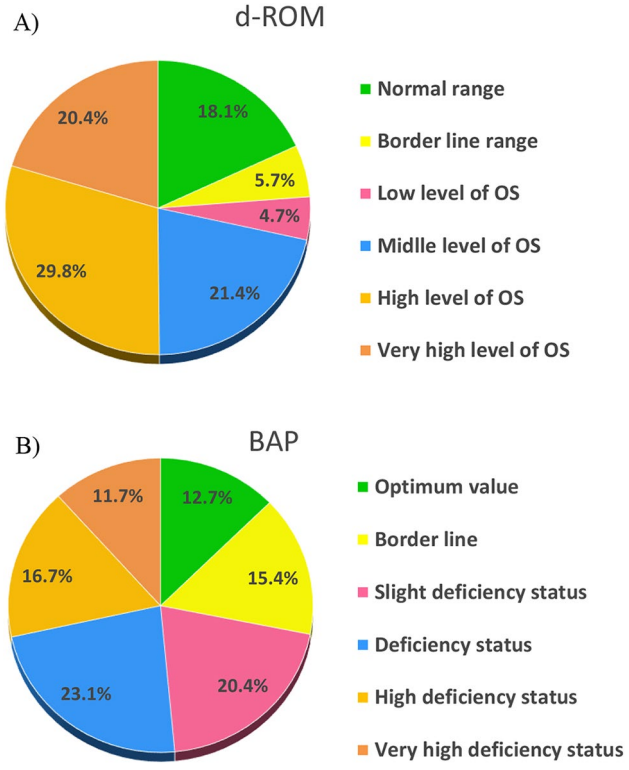

Fig. 1 Pie charts representing the percentage of PLWH with oxidative stress severity. For d-ROM, ranges are represented from normal to very high levels of reactive oxygen metabolites. The manufacturer's reference values of d-ROMs levels were as follows: 250–300 UCARR were considered normal, 301–320 UCARR were borderline range, 321–340 UCARR were low levels, 341–400 UCARR were middle levels, 401–500 UCARR were high levels, and > 500 UCARR very high levels (**A**). For BAP, ranges are represented from optimum to very high deficiency status of the antioxidant potential. The manufacturer's reference values in the µmol/L of the antioxidants were as follows: >2200 optimum value, 2200–2000 borderline status, 2000–1800 slight deficiency status, 1800–1600 deficiency status, 1600–1400 high deficiency status, < 1400 very high deficiency status (**B**)

proportion of PLWH were on a triple standard therapy (52.8%) and a dual regimen (47.2%); an integrase inhibitor (INSTI)-based regimens were the most frequently used by 77.3% of the participants, followed by non-nucleoside reverse transcriptase inhibitors (NNRTIs), i.e., 14.4%, and boosted protease inhibitors (PIs) 7.7%, specifically in all cases was a fixed dose combination consisting of FTC/TAF/DRV/COBI in triple regimen and 3TC+DRV/COBI in dual regimen. All of the participants on dual regimen with NRTI and INSTI was taking the combination 3TC+DTG.

The mean d-ROM levels were 409 (95%CI 394–422) UCARR, which indicates that the population was characterized by a high level of oxidants. Accordingly, considering the breakdown variable based on the manufacturer's reference values, half of the population fell into the high level of oxidants category, i.e., 29.8% were at the high level and 20.4% were at the very high level, while the remaining population, i.e., 21.4%, showed a middle level of oxidants and 28.5% spanned the normal to low level value (Fig. 1A).

Regarding the BAP levels, the mean was 1,809 (95%CI 1,766-1,851) µmol/L, thus indicating an overall deficiency status in the plasma antioxidant barrier capacity. Therefore, half of population fell into the category considered to have a deficiency status; specifically, 23.1% fell into the deficiency category, 16.7% the high deficiency category, and 11.7% the very high deficiency category. A minority of PLWH showed normal values (12.7%) (Fig. 1B).

Overall, we found that the PLWH were characterized by a high level of OS. In fact, the OSi median value was 4.56 (95%CI 3.56–5.62), and when categorized (≥7.3 or <7.3) 91.6% of the participants had values below the cut-off, which indicated a marked imbalance between antioxidant power with respect to hydroperoxide circulation.

Table 2 Univariable and multivariable to predict factors associated with d-ROM, BAP and OSi

Variables	d-ROM						BAP						BAP/d-ROM					
	Univariable			Multivariable			Univariable			Multivariable			Univariable			Multivariable		
	B	95% CI	p	B	95% CI	p	B	95% CI	p	B	95% CI	p	B	95% CI	p	B	95% CI	p
Sex																		
Male(ref)	0			0			0			0			0,0			0		
Female	80,7	49.3 to 112.1	<0.001	88.7	56.5 to 120.9	<0.001	99,6	0.8 to 198.5	0,048	47,7	-54.8 to 150.1	0,361	-0,59	-1.07 to -0.10	0,018	-0,75	-1.24 to -0.25	0,003
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Caucasian	-5,2	-52.0 to 41.5	0,826				-27,5	-169.7 to114.8	0,704				-0,01	-0.71 to 0.69	0,986			
Time since HIV diagnosis, per 10 years increase	2,9	-7.2 to 13.1	0,571				12,9	-17.9 to 43.8	0,409				0,02	-0.14 to 0.17	0,834			
Time on ART, per 10 years increase	18,1	-2.4 to 38.8	0,084				39,2	-23.7 to 102.1	0,221				0,04	-0.35 to 0.27	0,795			
BMI, median (IQR)	0,4	-3.2 to 4.0	0,827				-0,9	-11.9 to 9.9	0,861				0,003	-0.06 to 0.05	0,914			
Smoking habits (n=154)*	17,7	-10.4 to 45.7	0,216				-44,3	-129.7 to 41.0	0,308				-0,32	-0.74 to 0.09	0,129			
Alcohol use	8,5	-19.8 to 36.8	0,554				3,1	-82.9 to 89.1	0,944				-0,02	-0.44 to 0.40	0,924			
CD4 cell count nadir, cells/mm³, per 100 cells increase	0,9	-6.1 to 7.9	0,794				7,2	-14.3 to 28.6	0,514				-0,04	-0.15 to 0.06	0,437			
Zenith HIV-RNA, Log_{10} copie/mL	-9,9	-25.3 to 5.57	0,209				5,6	-41.6 to 52.8	0,815				0,22	-0.02 to 0.44	0,068			
CD4 cell count, cells/mm³, per 100 cell increase	0,15	-0.3 to 0.61	0,54				1,3	-0.2 to2.7	0,080				0,00	-0.008 to 0.007	0,891			
CD8 cell count, cells/mm³, per 100 cell increase	-0,15	-0.6 to 0.30	0,523				1,2	-0.3 to 2.5	0,107				0,004	-0.002 to 0.01	0,202			
CD4/CD8 ratio	18	-7.1 to 43.4	0,158				16,7	-60.2 to 93.6	0,669				-0,30	-0.68 to 0.07	0,114			
HIV-RNA (TND)	-14,1	-92.3 to 64.1	0,723				87,5	-150.1 to 324.9	0,469				0,42	-0.74 to 1.59	0,475			
Past AIDS-defining events, (CDC C)	20,7	-11.2 to 52.7	0,201				-33,1	-130.3 to 64.2	0,504				-0,30	-0.77 to 0.18	0,220			
HCV Co-infection	63,2	23.3 to 103.1	0,002	54,8	16.0 to 93.6	0,006	34,9	-88.4 to 158.1	0,578				-0,48	-1.08 to 0.12	0,119			
HBV Co-infection	-10,3	-120.0 to 99.5	0,854				-100,1	-433.9 to 233.5	0,555				-0,30	-1.94 to 1.34	0,719			
Cardiac diseases	-12,2	-50.5 to 26.1	0,530				-37,9	-154.3 to78.4	0,522				-0,03	-0.61 to 0.54	0,910			
Hypertension	-7,3	-47.0 to 32.3	0,717				-1,6	-122.2 to 119.1	0,980				-0,04	-0.63 to 0.55	0,896			
Diabetes	41,9	-20.3 to 104.3	0,186				-92,8	-282.4 to 96.8	0,336				-0,79	-1.71 to 0.15	0,098			
Neoplasia	36,6	-10.0 to 83.2	0,123				8,6	-133.6 to150.9	0,905				-0,52	-1.22 to 0.18	0,144			
Psychiatric	32,7	-6.7 to 72.3	0,104				27,5	-93.1 to148.1	0,654				-0,28	-0.87 to 0.31	0,359			
ART																		
Triple regimen (ref)	0						0						0.0					
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
ART-based class																		
INSTIs	-0.22 to 0.79	0,271					-19,9	-53.8 to 14.0	0,249				41,6	-61.3 to 144.6	0,427			0,2
NNRTIs	-0.65 to 0.54	0,859					-10,3	-50.4 to 29.8	0,614				-98,1	-219.3 to 232	0,112			-0,0
PIs	-1.38 to 0.19	0,139					66,0	13.7 to 118.3	0,014	55.0	5.48 to 104.6	0,030	69,3	-90.8 to 229.4	0,395			-0,5
Statins	-0.67 to 0.71	0,956					-9,5	-55.6 to 36.7	0,687				34,6	-105.5 to 174.8	0,627			0,0
Vitamin D	-0.22 to 0.78	0,281					-9,1	-42.8 to 24.6	0,595				67,7	-34.5 to 169.8	0,194			0,2
Lipid Nutritional Supplements	-0.76 to 0.37	0,492					-10,2	-47.9 to 27.4	0,593				-93,3	-207.3 to 20.7	0,108			-0,2
NSAIDs	-2.49 to 0.28	0,119					85,3	-7.2 to 177.7	0,070				-148,6	-430.5 to 133.3	0,300			-1,1
Lipid profile																		
Cholesterol, mg/dL	-0.01 to -0.001	0,050	-0,01	-0.01 to -0.003	0,002		0,3	-0.06 to 0.7	0,098				0,1	0.9 to 1.2	0,825			-0,0
Tryglicerides, mg/dL	-0.007 to 0.001	0,054					0,15	-0.1 to 0.4	0,231				-0,2	-0.9 to 0.5	0,523			-0,0
HDL, mg/dL	0.001 to 0.034	0,049	0,03	0.01 to 0.05	<0,001		0,02	-1.1 to 1.2	0,972				6,3	2.9 to 9.7	<0.001	6.1	2.6 to 9.6	0,001	0,0
Previous blip	-0.42 to 0.44	0,966					14,7	-13.8 to 43.3	0,312				9,6	-77.4 to 96.7	0,827			0,0
Previous failure	-0.62 to 0.31	0,502					22,8	-8.3 to 53.8	0,150				22,5	-72.2 to 117.2	0,640			-0,1
Time since last detectable viral load (years)	-0.03 to 0.06	0,640					-0.8	-3.9 to 2.4	0,632				1.2	-8.3 to 10.8	0.800			0.0
	0,01	-0.04 to 0.05	0,836				**Cumulative time with viral load <50cps/mL, (years)**			0,6	-2.2 to 3.4	0,679				3,2	-5.3 to 11.6	0,458

NRTIs; Non-nucleoside reverse transcriptase inhibitors; Abbreviations: BMI: Body mass index; ART: Antiretroviral therapy; HCV: Hepatitis C; HBV: Hepatitis B; NRTIs: Nucleoside reverse transcriptase inhibitors; NN

[illegible] Integrase inhibitors, PIs: Protease inhibitors, NSAIDs: Non-steroidal anti-inflammatory drugs.

*Variable refers to current smokers (n=152) and quit smokers (n=2).

Abbreviations: BMI: Body mass index; ART: Antiretroviral therapy; HCV: Hepatitis C; HBV: Hepatitis B; NRTIs: Nucleoside reverse transcriptase inhibitors; NNRTIs; Non-nucleoside reverse transcriptase inhibitors; INSTIs: Integrase Inhibitors; PIs: Protease Inhibitors. NSAIDs: Non-steroid anti-inflammatory drugs

*Variable refers to current smokers ($n=152$) and quit smokers ($n=2$)

Plasma d-ROM and BAP levels were positively correlated, although this correlation was not statistically significant ($n=299$, $r=0{,}111$, $p=0{,}054$).

Variables associated with reactive oxygen metabolites, antioxidant capacity and oxidative stress were investigated by multivariable linear regression analyses (Table 2). Female sex (versus male sex, adjusted mean change+88.7, 95% CI 56.5 to 120.9, $p<0.001$), current exposition to PI (versus non-PI, adjusted mean change+55.0, 95% CI 5.48 to 104.6, $p=0.030$), active co-infection with hepatitis C (adjusted mean change+54.8, 95% CI 16.0 to 93.6, $p=0.006$) were independently associated to a higher production of reactive oxygen metabolites (d-ROM). Higher HDL levels (per unit increase mg/dL, adjusted mean change+6.1, 95% CI 2.6 to 9.6, $p=0.001$) were independently associated to higher antioxidant capacity (BAP) in our population.

Interestingly, we found that the current use of dual therapies correlated with a low grade of OS (i.e., a higher OSi) (versus triple therapy, adjusted mean change+0.44, 95% CI 0.03 to 0.86, $p=0.036$) Furthermore, our analysis showed that female patients had a significantly higher oxidative status, which was reflected by their lower OSi (versus male sex, adjusted mean change −0.75, 95%CI -1.24 to -0.25, $p=0.003$). The OSi correlated negatively with total cholesterol (per 1 mg/dL increase, adjusted mean change −0.009, 95% CI -0.01/-0.003, $p=0.002$), but was positively associated with HDL (per 1 mg/dL increase, adjusted mean change+0.03, 95% CI 0.01 to 0.05, $p<0.001$).

In a sensitivity analysis, selecting only the largest population, i.e., PLWH on INSTI-based regimen ($n=231$) we found that dual therapies correlated both with a lower d-ROM levels (versus triple therapy, adjusted mean change −40.9, 95% CI -70.8 to -11.1, $p=0.007$). and higher OSi (adjusted mean change+0.69, 95% CI 0.22 to 1.16, $p=0.004$).

In a sub-analysis restricted to females the d-ROM levels were significantly correlated with increasing age (rho=+0.243, $p=0.038$). Moreover, when we further stratified the females by age (“>45 years” and “≤45 years”, considering 45 years as a menopausal transition cut-off) a trend was observed with the older women (>45 years) showing higher d-ROM levels (versus women≤45 years, mean change+66.5, 95% CI -2.60 to +135.6, $p=0.056$).

Furthermore, in an analysis adjusted for sex, age and smoking habits, we observed a negative trend of association between use of Vitamin D and d-ROM (adjusted

mean change −29.7, 95% CI -62.8/+3.4, $p=0.079$) and a positive trend with OSi (adjusted mean change+0.45, 95% CI -0.06/+0.97, $p=0.084$).

Any relation was found when analyzing the three outcomes with TAF, TDF or 3TC based regimen (all p values were not significant).

Discussion

Here we aimed to characterize the redox status of long-term modern ART-treated PLWH with virological suppression and to explore the factors associated with OS in this population.

Besides the implication in the pathogenesis of HIV disease and the progression from the asymptomatic stage to the development of AIDS, in virally-suppressed PLWH it has been show that chronic oxidative stress might contribute to the development of comorbid diseases, aging-associated diseases and overall morbidity [6]. Furthermore, OS was associated with all-cause mortality in treated PLWH, with a persistent predictive performance in virologically-suppressed individuals [7].

In the latter study the authors also found that the sequential addition of biomarkers of inflammation, monocyte activation and coagulation (D- dimer, sCD14 and, hsCRP level) in the model adjusted to predict the occurrence of severe non-AIDS events and death, significantly and gradually improved the predictive performance of the model, with high sensitivity and good accuracy. This finding highlights the relationship between OS and residual inflammation which, however, remains to be fully characterized.

Overall, we observed that our setting of PLWH showed a high level of OS that was determined by high levels of oxidant species and a deficient antioxidant capacity.

In our study we also evaluated the OSi, which is a combined measurement of pro-oxidants and antioxidants in clinical samples. In our population, the mean of this index was very low (4.56) and for the majority of participants (91.6%) was under the cut-off value.

The slight positive correlation between the d-ROMs and BAP parameters, which demonstrate a possible relationship between the pro-oxidant/antioxidant systems, might suggest the existence of an adaptive response mechanism to detoxify oxidative stress-related harmful metabolites [9]. Thus, it seems that a higher degree of stress is correlated with a higher extent of antioxidant capacity, although globally the endogenous antioxidant scavengers are likely to be inadequate to counterbalance the oxidant insult in this setting of PLWH.

Although it has been demonstrated that OS is involved in HIV infection [7, 25–27], the interplay between HIV, ART and OS must still be fully established.

A series of previous studies reported an increase in OS during ART in PLWH, in addition to the persistent redox imbalance associated with HIV infection. Although it must be considered that these studies were mostly conducted in the initial highly active antiretroviral therapy era or earlier, they suggest that previous antiretroviral drugs might cause toxicity and OS in PLWH [12–14]. In this study, PLWH had started treatment an average of 10 years before, thus they mostly underwent a modern regimen. However, they showed a high level of OS that was manifested by a high level of d-ROMs and a low BAP and consequently by a low OSI index.

These results seem to support previous findings and indicate that antiretroviral therapy could play an important role in the exacerbation of OS in PLWH with suppressed viral load.

It has been observed that ART can induce an increase in oxidant generation, a decrease in antioxidant protection or a failure to repair oxidative damage. Antiviral therapy could also have a role in OS resulting from the destruction of tissues and liver cells and the activation of neutrophils and macrophages. The long-term side effects of HIV treatment, which include several metabolic disorders, also have an impact on the oxidant profile [14].

To the best of our knowledge this is the first study that has focused on OS levels in viro-suppressed PLWH who were receiving modern dual or triple ART-regimens. It should be noted that PLWH on less-drug regimens, i.e., dual therapies, showed a significantly low degree of redox imbalance (i.e., higher OSi) than PLWH on triple ART. Furthermore, the sub-analysis restricted to PLWH on INSTI-based regimen, not only confirmed that dual therapies correlated with higher OSi but also highlighted the association with lower d-ROM. These observations are quite relevant since it could be speculated that reducing drug pressure could be associated with a more favourable OS profile. This might also translate in a clinical benefit in the long term (i.e. reduced incidence of aging-associated comorbidities).

However, an alternative interpretation could be hypothesized, i.e., the recent less-drug regimens, being highly potent, generally well-tolerated, and convenient with once-daily dosing might facilitate adherence to therapy and consequently limit low-level viral replication. This latter, usually below the limits of detection in the routine clinical assay, may derive from various sources including periodic blips of viral reactivation from latency and/or ongoing viral replication in sanctuary sites. Although this residual viremia appears not to be clinically relevant, it is important to consider that viral replication itself can both be induced by and induce oxidative stress [8].

In the multivariable analysis, we found an association between current exposure to PIs and a higher production of reactive oxygen metabolites.

It has been previously observed that some protease inhibitors can increase chemically reactive species in

circulation, possibly by producing more oxidized metabolites deriving from the interaction between ROS and infected cell biomolecules. Thus, the link between HIV-PI usage and increased ROS production is well established by studies that include numerous cell and tissue types, i.e. macrophages, cardiomyocytes and endothelial cells. However, the exact mechanisms and the sequence of events whereby PI treatment triggers intracellular ROS production are not well understood. Even though the precise sources of PI-induced ROS are still unclear, most studies suggest they have a mitochondrial origin. Therefore, they support the view that the mechanism driving the relationship between PIs and increased OS is a biochemical one, such as mitochondrial interference and activation of the P450 hepatic system by ART when comprising PIs [14–18].

Thus, the mitochondrial overproduction of ROS induced by PIs-usage can lead to a vicious cycle that induces mitochondrial DNA mutations, damages the mitochondrial respiratory chain, alters membrane permeability and influences Ca2+homeostasis and mitochondrial defense systems. All of these changes in mitochondrial function lead to serious downstream consequences and could be associated with severe complications and diseases [28, 29].

Indeed, it has been demonstrated that protease inhibitors inhibits in vitro mitochondrial peptidase processing [30].

Furthermore, ritonavir is a potent substrate and a mechanism-based inhibitor of cytochrome p450 (CYP3A and CYP2D6). To a lesser extent, it also inhibits CYP2C9, CYP2C19 and CYP1A2 enzymes in the liver and the gut [31].

Interestingly, in a more recent study, Ganta et al. showed that ritonavir initially triggers endoplasmic stress which leads to activation of the BAX protein and its translocation to the mitochondria and causes outer membrane permeabilization there by favoring the entry of ritonavir inside the mitochondria and leading to the release of cytochrome c and mitochondria-mediated apoptosis [32].

It has been proposed that PIs by triggering ROS production also leads to the dysregulation of the ubiquitin–proteasome system (UPS), which is a regulatory system that constitutes an important part of the cell's antioxidant defenses [33].

Furthermore, it has been demonstrated that virus protease inhibitors such as ritonavir may be associated with cardiovascular disease, ritonavir directly impair vascular functions and endothelial monolayer permeability through the mechanism of oxidative stress [16–18, 34]. Overall, our results appear to extend this relationship between oxidants and Darunavir boosted with cobicistat.

Our study also shows that females have a significantly higher oxidative status than males; this is reflected by a higher reactive species and a lower OSi index. This finding is not surprising and agrees with the findings of previous studies carried out both in PLWH and in healthy individuals and subjects affected by various diseases. A possible explanation is that in our population, as in previous ones, there were several women of perimenopausal or menopausal age and in this condition there is an increase of ROS production [35, 36]. In fact, the median age of the females was 54 (IQR 43–61) years, which is compatible with the menopause age. Also, when stratifying women by categorical age, choosing "45 years" as the cut-off (considered the age at which menopausal transition begins), we observed a slight correlation between older women and higher levels of d-ROM. By contrast, the females showed significantly higher antioxidant levels, although this correlation lost significance in the multivariable model.

Furthermore, the multivariable linear regression analysis revealed that the OSi index was negatively correlated with total cholesterol but was positively associated with HDL. This finding supports the interrelationship between oxidative status and lipid profile. It is noteworthy that in previous studies it was reported that the oxidative modification of lipoproteins, protein glycation and glucose auto-oxidation, leading to the excessive production of lipid peroxidation products, was associated with progressive levels of cholesterol and overall lipidemia. Thus, it has been observed that an increase in OS levels could be an early event in the evolution of hyperlipidaemia, hypercholesterolemia and finally the risk of cardiovascular diseases [37, 38].

Finally, as expected our analysis showed that co-infection with hepatitis C was associated with a higher production of reactive oxygen metabolites. In fact, it has been demonstrated that HCV has evolved to manipulate the redox status, thus generating the OS that not only causes hepatic damage but also decreases the ability of the cell to work against such pro-oxidant species. It has been shown that HCV proteins localised to mitochondria contribute to increased reactive oxygen species production and cellular apoptosis. Thus, PLWH with chronic HCV-coinfection showed increased oxidative stress with inflammation and disruption of mitochondrial functioning [39–41].

Interestingly, we found a trend between better oxidative status and the administration of vitamin D. The latter is a steroid molecule that primarily influences whole-body calcium homeostasis. The role of this fat-soluble vitamin in the modulation of oxidative stress status in various tissues has also been demonstrated [42]. It could be speculated that supplementation of Vitamin D, as an antioxidant, might be beneficial in certain risk groups,

such as PLWH, due to its action in reducing oxidative stress and endothelial damage.

Our study has limitations that warrant consideration. First, it was designed as a pilot, cross-sectional study. Thus, as several unmeasured or uncontrolled biases can influence the results of the cross-sectional analysis, this should be considered when interpreting our results. We did not analyze a HIV negative group since the main purpose of our exploratory study was to investigate the associated factors in our setting of suppressed PLWH on modern effective ART; in fact, the increase of OS in treated PLWH vs. healthy has already been largely determined [8, 43].

Another limitation is the relatively small sample size. Furthermore, we could not explore the influence of socio-economic factors, dietary habits, exercises, and mental health status on OS as potential confounders. The evidence suggests that moderate exercise stimulates beneficial adaptive responses by improving antioxidant defences and redox balance. However, high-intensity exercises can lead to harmful levels of oxidative stress. These effects are significantly influenced by age, gender, ethnicity and socio-economic status, which can affect the generation of reactive species during exercise and the body's ability to manage them. Furthermore, it has been seen that natural antioxidants in the diet are beneficial but that high-dosage supplements might hinder positive adaptations to exercise. Clinically, these insights advocate for personalized exercise programs and nutrition plans that consider individual demographic factors and baseline oxidative stress levels to maximize the health benefits and minimize potential harm [44].

However, we are confident that one strength of the study is technical. Indeed, it should be noted that a major problem in applying OS in clinical medicine has historically been its measurement, considering the complexity of the process and the number of components, oxidants and antioxidants involved. OS can be measured in biological samples using different markers, i.e., both oxidants and antioxidants. No single parameter has yet been recommended as a gold standard and the variety of methods with different sensitivity and reliability makes interpretation difficult. Above all, it has been demonstrated that these individual markers only partially reflect OS. Thus, to overcome such biases, it is advisable to determine the total redox status by evaluating all oxidants and antioxidants simultaneously without excluding their interactions with each other [45, 46].

In this study, we used an integrative approach by using the two validated d-ROMs and BAP tests that have been shown to have high sensitivity and specificity for measuring the total pro-oxidant component and antioxidant capacity, respectively.

Another problem in assessing the OS components is due to the instability of the species involved, particularly ROS, which have a short half-life and high reactivity. In this context, the d-ROMs test provides a useful tool because it determines the contents of hydroperoxides, which are intermediate oxidative products and more stable compounds compared to their relative parent ROS. Alberti et al. investigated the validity of measuring the levels of d-ROMs and demonstrated the existence of a correlation between the d-ROMs test data and those obtained from electron paramagnetic resonance (ESR), which is the gold standard for detecting paramagnetic species such as organic free radicals [47]. Moreover, the d-ROMs test has been validated against ESR method, with reference values determined in a study involving over 4000 healthy people. The expected values in a healthy individual are between 250 and 300 UCARR, while higher values denote a surplus of peroxides indicative of a systemic increase in ROS levels [48].

Overall, we can state that the combined use of d-ROMs and BAP tests has been shown to meet al.most all the criteria of an ideal biomarker of oxidative stress for clinical practice and it is now being applied in the clinical setting for a wide variety of disorders [49–51].

Furthermore, to minimise the experimental issues it should be noted that in our study we adopted rigorous experimental settings, as specified earlier.

Moreover, to investigate the redox balance more thoroughly, we calculated the OSI index for each patient. In fact, the OSI index has been shown to provide a better and more powerful index for evaluating the overall oxidative stress in clinical samples and the establishment of a definitive relationship between oxidative stress and disease status [43–45].

In conclusion, we identified several factors associated to the balance between oxidant/antioxidant species in ART-experienced PLWH with virological suppression, thus contributing to better characterize OS in the recent ART era.

This cross-sectional study, even in the absence of an HIV-negative control group, was assessed in a clinical practice setting, and was useful, for establishing preliminary evidence in planning a future advanced study. It was a "snapshot" of a group of selected homogeneous PLWH populations with long-term suppressed PLWH the majority of whom were primarily on an INSTI-based regimen. Additional studies with a longitudinal controlled design are needed to overcome the limitations due to the exploratory nature of the present study. For instance, based on the results obtained from this pilot study, it might be interesting to conduct a prospective longitudinal study in a setting of long-term suppressed PLWH and to compare OS markers in a group switching to less drug regimen vs. continuing a triple regimen.

Future studies could also evaluate the intricate relationship between residual inflammation and OS in long-term ART-experienced PLWH by incorporating the measurement of inflammation, monocyte activation and coagulation markers, such as CRP, IL-6, D-dimer and sCD14, in order to further enhance the investigation.

In fact, in addition to OS markers, including the above-mentioned levels of inflammation markers could better characterized OS and the overall inflammatory response in treated PLWH.

Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
BAP	Biological Antioxidant Potential
CI	Confident Interval
CV	Coefficients of Variation
d-ROMs	derivatives-Reactive Oxygen Metabolites
DRV	Darunavir
ESR	Electron Paramagnetic Resonance
FTC	Emtricitabine
HCV	Hepatitis C Virus
HDL	High Density Lipoprotein
INSTI	Integrase Strand Transfer Inhibitor
IQR	Interquartile
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors
OS	Oxidative Stress
OSi	Oxidative Stress Index
PIs	Protease Inhibitors
PLWH	People Living with HIV
ROS	Reactive Oxygen Species
TAF	Tenofovir Alafenamide Fumarate

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Author contributions
Manuscript conceptualization: F.L. and S.B. Data collection: A.B., V.I., P.F.S., E.V. Data analysis: F.L. Methodology: F.L. and A.S. Drafting the manuscript: F.L. and S.B. Manuscript editing: A.B., V.I., P.F.S., M.F. and S.D.G. All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets analysed during the current study are available from the corresponding author on reasonable request.

Ethics declarations

Ethics approval and consent to participate
The study was conducted in accordance with the Good Clinical Practice and ethical principles of the Declaration of Helsinki. The protocol was reviewed and approved by our local Ethics Committees (ID4477 14/10/2021). Written informed consent was obtained from all participants.

Competing interests
The authors declare no competing interests.

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