

## ORIGINAL ARTICLE

# ANTIOXIDANTS IN PATIENTS LIVING WITH HIV ON ANTIRETROVIRALS

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### Summary

Oxidative stress is considered predictors of diseases associated with aging (cardiovascular disease, neurodegenerative disease, malignancies, and others) in HIV-negative general population. Antioxidants were investigated in people living with HIV on antiretroviral treatment to determine whether they had an immunosenescent phenotype that might predispose to the development of premature age-related diseases. Clinical studies in this population are controversial.

### Methods

The study was conducted among 213 subjects with HIV, including 172 subjects on antiretro-virals and 41 subjects before the initiation of treatment. The control group consisted of healthy HIV-negative adults. We compared the reduced glutathione and ferric reducing antioxidant power levels in HIV untreated and treated patients and controls. Significant differences were determined by appropriate statistical tests (t-test, Mann–Whitney *U* test, Kolmogorov–Smirnov test, ANOVA, Kruskal–Wallis test). Relationships between continuous variables were quantified using Spearman's rank correlation coefficient.

### Results

Glutathione levels were significantly lower in the treated group compared with the untreated group and controls ( $P < 0.001$ ). Differences in total antioxidant levels between groups were not found.

### Conclusions

Significant decrease of antioxidants was found independent of the virologic status of HIV patients on antiretroviral treatment. Persistence of these abnormal parameters may contribute and predispose to the premature development of diseases associated with aging.

*Key words: Antiretroviral therapy; glutathione; HIV; oxidative stress*

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## Introduction

Evidence suggests that patients infected with RNA viruses, including HIV, are under chronic oxidative stress (OS) (1). Some pathological processes, diseases, and aging, including age-related pathologies, are associated with increased OS (2), which is defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and anti-oxidant defense pathways (3,4). In this context various type of oxygen intermediates are comprised in a general term reactive oxygen species. And different type of oxygen intermediates have a different potential to react with various biological molecules (5). The primary and major sources of endogenous ROS production are mitochondria (5-11). Mitochondria are involved in energy production and regulate innate immunity and the inflammatory response through the intracellular signaling cascade (10).

Antioxidants are involved in the defense mechanism of an organism against pathogens associated with attack by ROS (11). Reduced glutathione (GSH) is part of a well-organized antioxidant defense system. GSH continuously reacts to increased ROS production, thereby reducing the potential oxidative cellular damage (2).

Numerous disorders of cellular redox homeostasis are due to decreased GSH levels. Many different disorders and diseases in human medicine are accompanied by GSH deficiency (2,4,12-14).

Little is known about OS parameters and their association with antiretroviral therapy (ART) in HIV-positive patients. We hypothesized that abnormal levels of the investigated markers (GSH, total antioxidant ability), which represent pathological processes, can be detected in HIV-positive subjects, after ART initiation they return to normal levels.

## Patients and methods

Two hundred and thirteen patients with HIV were enrolled in the study. One hundred and seventy-two subjects with HIV were on ART. The treatment regimen of most patients consisted of two nucleoside reverse transcriptase inhibitors plus another active drug. Another group included forty-one naïve subjects living with HIV before their introduction to ART. There was no patient with another serious acute or chronic disease. Healthy blood donors formed a control group.

## Blood sampling

Blood plasma was collected through a frank venipuncture, on 0.109 mol/l citrate anticoagulant (9+1). Two hours after collection, the plasma supernatant was rapidly decanted to Eppendorf tubes followed by a 15 minutes centrifugation at 1,500 ×g at room temperature. Plasma was rapidly frozen and stored at -80°C. Just before use, plasma was thawed for 15 minutes at 37°C.

### 1. Reduced GSH assay of blood plasma samples

GSH was measured using a colorimetry method based on the reaction between the free thiol group of GSH and 5,5' (2-nitrobenzoic acid). Blood plasma samples were treated with trichloroacetic acid to remove proteins. Then, 200 µl of blood plasma sample (or saline solution as a blank) was added to 600 µl of trichloroacetic acid 2.5% (v/v), and this suspension was shaken and centrifuged at 12,000 ×g for 5 minutes at 4°C. Next, 500 µl of supernatant was neutralized by 125 µl of 0.5 mol/l sodium hydroxide and 400 µl of 0.4 mg/ml 5,5'dithiobis (2-nitrobenzoic acid) was added. Absorbance was measured at 412 nm against the blank and the molar concentration was calculated using the extinction coefficient for conjugate GSH-5-thio-bis-2-nitrobenzoic acid equal to 14,150 M<sup>-1</sup>cm<sup>-1</sup>.

### 2. Ferric reducing antioxidant power (FRAP) assay of blood plasma samples

The FRAP assay is a method to determine the total level of low molecular weight antioxidants in a sample. In the first step, the reagent for the assay was prepared: 2.5 ml of 2,4,6-tris (2-pyridyl)-s-triazine 10 mmol/l in 40 mmol/l HCl was added to another 2.5 ml of 20 mmol/l ferric chloride in water. Then, the mixture was added to 25 ml of 100 mmol/l acetate buffer pH 3.6, shaken, and heated at 37°C for 10 minutes. Blood plasma sample

(or saline solution for blank purposes) in a volume of 30 µl was mixed with 770 µl of deionized water and 200 µl of the aforementioned reagent, shaken, and incubated for 10 minutes. Finally, the sample was centrifuged at 10,000 ×g for 10 minutes. Absorbance was measured against the blank at 593 nm using disposable polystyrene cuvettes. The molar level of antioxidant power expressed by an equivalent concentration of reduced ferric ions was calculated using the extinction coefficient for the chromogenic reagent 26,000 M<sup>-1</sup>cm<sup>-1</sup>.

### Statistical analysis methodology

Descriptive characteristics were expressed by the absolute and relative frequencies for categorical variables and by the median with 5<sup>th</sup> and 95<sup>th</sup> percentile for continuous variables. Parametric (*t*-test, ANOVA) and non-parametric (Mann–Whitney *U* test, Kolmogorov–Smirnov, Kruskal–Wallis) tests were used to compared differences in the distribution of each continuous variable between different groups. The choice of each test depended on several criteria including the number of compared groups and whether the distribution was normal. Multigroup comparison post-hoc tests were utilized to assess between-group differences. Tukey’s test and Benjamini-Hochberg method (FDR) were utilized for cases of ANOVA and Kruskal–Wallis test, respectively. Relationships between categorical variables were performed using Pearson’s  $\chi^2$  test. Relationships between continuous variables were quantified using Spearman’s rank correlation coefficient. Statistical significance of the correlation coefficient was calculated via the asymptotic *t* approximation. Statistical analyses were done with R software, version 3.5.1. P-values less than 0.05 were considered statistically significant.

### Results

#### Markers of OS in HIV naïve subjects and subjects on ART

Table 1 and Fig. 1 show that GSH levels were not significantly different in 30 HIV patients without ART compared with 50 HIV-negative controls (173.9 µmol/l; 181.9 µmol/l; *P* = 0.877). GSH levels were significantly lower in 172 HIV patients on ART compared with 50 HIV-negative controls (138.7 µmol/l; 181.9 µmol/l; *P* < 0.001). GSH levels in HIV patients on ART were significantly lower compared with HIV patients without ART (138.7 µmol/l; 173.9 µmol/l; *P* < 0.001).

**Table 1.** Patient characteristics in untreated and treated subjects and controls.

		HIV-	HIV+	HIV+ ART	p-value
Number of patients		50 (a)	41 (b)	172 (c)	
sex	female	-	3 (7.3%)	32 (18.6%)	0.129
	male	-	38 (92.7%)	140 (81.4%)	
age		-	37.7 (25.3; 58.6) n = 41	42.1 (27.2; 65.2) n = 172	0.112
	BMI*	-	22.0 (18.5; 28.4) n = 41	24.6 (19.1; 32.8) n = 163	
median (5 <sup>th</sup> ;95 <sup>th</sup> )	FRAP µmol/l	824.0 (603.7; 1135.0) n = 50	812.2 (560.5; 1236.7) n = 30	842.0 (593.6; 1131.2) n = 172	0.775
	GSH µmol/l	181.9 <sup>(c)</sup> (50.9; 231.5) n = 50	173.9 <sup>(c)</sup> (56.8; 239.2) n = 30	138.7 <sup>(a,b)</sup> (85.7; 203.5) n = 172	< 0.001
	viral load/ml	-	44,300 (317; 4,850,000) n = 38	0 (0; 4410) n = 172	< 0.001

\*The body mass index (BMI) was calculated as the weight (kg) divided by the height (m<sup>2</sup>).

The superscripts a-c indicate statistically significant differences between the two categories of a-c.

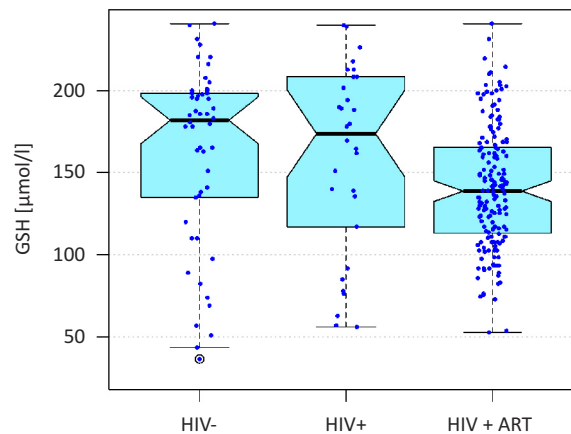
Table 1 and Fig. 2 show that FRAP levels were not significantly lower in 30 HIV patients without ART compared with 50 HIV-negative controls (812.2  $\mu\text{mol/l}$ ; 824.0  $\mu\text{mol/l}$ ;  $P = 0.775$ ). FRAP levels were not significantly different in 172 HIV patients on ART compared with 50 HIV-negative controls (842.0  $\mu\text{mol/l}$ ; 824.0  $\mu\text{mol/l}$ ;  $P = 0.775$ ). FRAP levels in HIV patients on ART were not statistically significant different compared with HIV patients without ART.

#### Markers of OS depend on the viral load (VL) in HIV-positive patients

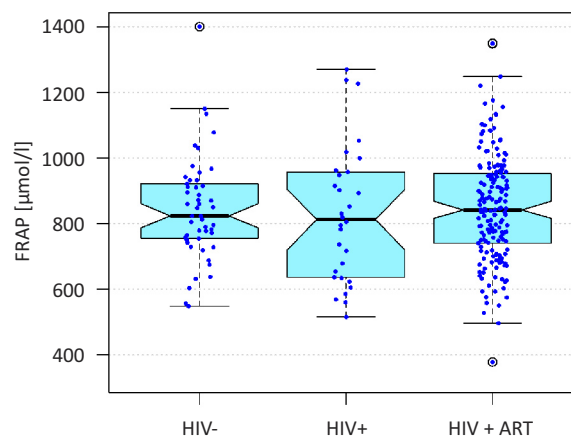
The viral load was 44,300 copies/ml in HIV positive patients without treatment that indicates an intense and ongoing replication of HIV. The viral load was undetectable in HIV-positive patients on treatment, meaning that the virus had significantly suppressed replication (Table 1). There was a statistically significant difference in viral load between the untreated and treated groups ( $P < 0.001$ ).

FRAP was not significantly different between groups without treatment and with treatment regardless of immunologic status and viral suppression. GSH levels were significantly lower in the treated group compared with the naïve group and HIV-negative control group regardless of complete viral suppression and viral load at undetectable level. The median FRAP levels were not different between groups with HIV and the HIV-negative control group.

No significant correlations were detected between GSH and FRAP and viral load.



**Figure 1.** GSH - controls, untreated and treated patients



**Figure 2.** FRAP - controls, untreated and treated patients

## **Discussion**

Aggravation of OS in treated patients was determined by a significant decrease of GSH levels. This conclusion suggests that three key antioxidant enzymes are probably inhibited (superoxide dismutase, glutathione peroxidase, and glutathione reductase). Their inhibition can be caused directly by antiretroviral drugs or their metabolites. Inhibition of antioxidant enzymes consequently leads to the accumulation of ROS molecules (3,4,15,16). This indicated that OS progressed even in the presence of viral suppression and higher CD4<sup>+</sup> lymphocyte counts despite ART, which might be implicated in these processes.

Clinical studies in HIV-infected individuals receiving ART are not conclusive (3-5,12,15,17-19). The results of the current study are in line with the conclusions of Musisi (3). However, Mandas et al. describes the significantly higher levels of ROS in treated than naive (4,17). In contrast to our findings the Awodele et al. study found that HIV-naïve patients had lower systemic GSH levels and higher lipid peroxidation compared with HIV-positive patients with antiretroviral drugs (12,15,19). But the number of patients in these individual studies was low and some parameters were examined using methods different from ours; therefore, a comparison between the current study and previous studies is limited.

Experimental data suggest that ROS production from cells is the primary trigger of a cascade that leads to the expression of an immunosenescent phenotype (7,20,21) and to aberrant innate immune responses, which are considered a common factor that drives sterile inflammatory pathology in many conditions associated with aging (6,22,23). The aggravation of GSH deficiency promoted the functional predominance of OS in treated patients and suggests the important role of GSH homeostasis in HIV pathogenesis. A variety of enzymatic antioxidants may be more sensitive than non-enzymatic ones, which are generally detected by the FRAP assay.

ART has an extremely positive effect on the immunological and clinical condition of patients. Nevertheless, some parameters do not seem to respond to ART and do not return to values comparable to HIV-negative controls. The causes of these responses are likely multifactorial, complex, and mediated via multiple mechanisms. HIV proteins or indirectly pro-inflammatory cytokines may be partially involved in these processes (1,24-27). Mitochondrial dysfunction is caused by HIV itself as well as ART (28,29).

Numerous studies reported that mitochondrial anomalies and OS are associated with disease conditions with a proinflammatory and procoagulant status, including atherosclerosis, neurodegenerative diseases, type 2 diabetes, and renal injury (11,22,30). Furthermore, high ROS levels had a negative effect on genomic integrity resulting in malignancies (10,20).

In this context, our study demonstrated that patients living with HIV on effective ART present with a phenotype that can predispose to this status regardless of virologic suppression and immunologic status.

## **Conclusion**

There is evidence that antioxidant responses are inhibited in patients living with HIV on ART. Inhibition may be due to ART itself, its metabolites or other undefined effects. Currently, there is no detailed pathophysiological explanation for these phenomena. Therefore, anti-oxidant supplementation in parallel with ART might be beneficial for HIV-positive patients and requires further investigation. They remain a key issue for the coming years, and might fundamentally change existing strategies for the treatment of HIV infection.

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## **Conflict of Interest Statement**

The authors have no conflicts of interest regarding the publication of this article.

## Adherence to Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants involved in the study.

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We thank all the study participants.

## Contribution

K.H. and S. S. created this study, collected data and wrote this manuscript  
M.P. performed the lab analyses, and he corrected the final text of the manuscript.  
R. S. participated in data collection and manuscript writing.  
P.H. j. and L.F. they participated in the acquisition of laboratory and clinical data.  
F. Z. dealt with statistical analysis.  
P. H. he managed the entire project and the final data processing.  
The final version of the manuscript was checked and approved by all authors.

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