FERROPTOSIS IN CD4+ AND CD8+ T-CELLS IN THE SETTINGS OF HIV INFECTION

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ABSTRACT

Introduction: Elevation of intracellular iron concentration triggers ferroptosis. Understanding the regulation and pathophysiological mechanisms of this process in HIV infection may contribute to antiretroviral therapy (cART) monitoring.

Aim: To perform a correlation analysis of the intracellular labile-bound iron pool (LIP) in CD4+ and CD8+ T cells in association with CD4+, CD8+ T cells absolute count (AC) and CD4/CD8 index in HIV+ individuals on continuous cART with sustained viral suppression.

Material and methods: Peripheral blood samples (Li heparin, n=34) were collected in the course of the routine immune monitoring of HIV+ individuals at four time points during 24 months. Plasma HIV viral load (VL) was determined with the Abbott Real-Time HIV-1 test (sensitivity 40 copies/ml). AC and percentage of CD4+ and CD8+ T cells were determined by direct flow cytometry (Multitest, BD Trucount, FACS Canto II). The intracellular content of LIP in CD4 and CD8 T cells ($LIP_{CD4,}$ $LIP_{CD8)}$ was measured at the beginning of the study, using acetoxymethyl ester and subsequent incubation with a chelator (Deferiprone). LIP was quantified according to the mean fluorescence intensity (MFI) (FACSCanto II, Diva 6.1.2).

Results: In the settings of a higher LIP_{CD4} , high LIP_{CD8} correlated with increased CD8AC (Rho= 0.70, $p<0.05$) up to 11 (min. 6, max. 15) months after LIP measurement., and decreased CD4/CD8 ratio correlated inversely with LIP_{CD8} in all consecutive measurements (Rho= -0.71, $p<0.01$ for all), Importantly, high LIP_{CD8} correlated with a lower CD4AC (Rho= -0.65, $p<0.05$) up to five (min.1, max.8) months after LIP measurement.

Conclusion: The increased concentration of intracellular LIP in CD8 cells in HIV+cART individuals could indicate viral activity in the settings of undetectable HIV VL, directly associated with ongoing cell ferroptosis.

Keywords: HIV, ferroptosis, immune restoration

INTRODUCTION:

Ferroptosis was recently identified as a non-apoptotic, iron- and reactive oxygen species (ROS)-dependent form of lytic cell death characterized by mitochondrial dysfunction, and accumulation of lipid peroxides on biological membranes (1, 2, 3). It has been implicated in a variety of human diseases, including cancer cells' death, neurotoxicity, neurodegenerative diseases, acute renal failure, hepatic inflammation, heart ischemia/reperfusion injury, as well as in HIV, COVID-19, tuberculosis and other infections (3, 4). The role of ferroptosis in viral infections is just beginning to be elucidated.

Ferroptosis results from imbalanced production and degradation of intracellular lipid ROS. Optimal concentrations of ROS are critical for normal cell function and survival. Excessive and uncontrolled production as well as accumulation of ROS due to insufficient cellular antioxidant capacity can be detrimental to the cells (4). When glutathione, the main intracellular enzyme responsible for the reduction of ROS is lacking, cells become susceptible to lipid peroxidation (5, 6). The enzyme lipoxygenase catalyzes this process, utilizing iron as an essential cofactor (7, 8). Thus iron participates in the production of ROS and oxidative stress. Dysregulation of cellular labile iron levels can be triggered via higher accumulation of ferrous iron Fe^{2+} in the cytoplasm (labile iron pool, LIP), increased Heme oxygenase

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1 (HO-1), increased transferrin uptake, reduced expression of ferroportin and depletion of ferritin (4, 8, 9). The transferrine receptor (CD71) can transport exogenous iron ions into the cell by binding to iron storage ferritin, creating an intracellular labile iron pool, which induces the Fenton reaction, leading to the production of ROS (9).

Ferroptosis, oxidative stress, and mitochondrial disruptions are all closely associated and may cooperate to contribute to the residual inflammation and latent viral load in the settings of treated chronic HIV infection (8, 10).

The objective of the present study was to analyze labile bound iron (LIP) in CD4 (LIP_{CD4}) and CD8 T cells (LIP_{CD8}) of HIV+ patients on continuous cART with sustained viral suppression (SVS) in correlation with the routinely monitored immunological parameters CD4 T AC and CD4/CD8 ratio.

MATERIAL AND METHODS

Study design and participants

This study was approved by the Ethical Committee at the National Center of Infectious and Parasitic Diseases, Sofia (Approval number: 2019-026-01). The study was conducted during the follow-up of patients according to European ethical standards.

Peripheral blood samples (Li heparin, n=25) were obtained in the course of routine immune monitoring of HIV+ male individuals, registered at the Specialized Hospital for Active Treatment of Infectious and Parasitic Diseases, Sofia. Four samples per participant were analyzed in the course of 24 months at five-month (range 2-8 mo) intervals,

The inclusion criteria were: continuous cART for more than four years and SVS for at least two years.

Methods: Plasma HIV viral load (VL) was determined with reverse transcription polymerase chain reaction (Abbott Real-Time HIV-1) with the lowest limit of detection at 40 copies/ml.

The absolute count (AC) and percentage of CD4+ and CD8+ T cells were determined by direct flow cytometry (Multitest, BD Trucount, CD3/CD8/CD45/CD4/TRU Count, FACS Canto II) as described before (10).

The intracellular content of LIP was determined at the beginning of the study. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll Paque Plus (Sigma-Aldrich) and incubated with calcein acetoxymethyl ester (CA-AM, Biolegend) followed or not by chelator (Deferiprone, Sigma Aldrich) using the method described by Prus et. al (11) and modified by Emilova et al (10). The difference between the mean fluorescence intensity (ΔMFI) of chelator-treated and untreated cells was used to evaluate the amount of LIP in CD4+ and CD8+T (FACSCanto II, Diva 6.1.2).

Statistical analysis: Between group differences were evaluated with nonparametric Mann–Whitney U test, and data are presented as median, min-max. Relationships between two variables were analyzed by Spearman's rank correlation coefficient. P values less than 0.05, at CI 0.95 were considered significant (SPSS Statistics v.23.0 and Graphpad Prism v.9.0).

RESULTS:

Patients' characteristics are given in Table 1. Despite sustained viral suppression in all participants, immunological responses to cART varied during the follow-up period. Baseline CD4AC and CD4/CD8 ratio ranged from 220 to 1032 cells/μl, and 0.26 to 2.01, respectively. The last measured values ranged from 292 to 1519 cells/μl, and 0.31 to 1.93 respectively. Notably, approximately 45 % of participants had a suboptimal CD4/CD8 ratio (<0.9). A wide range of LIP_{CD4} (median 625, min 83 – max 2935) was observed that was not associated with the age or the immune responses to therapy. Based on the median of LIP_{CD4} two groups were defined: Group A (n=14) with lower than median LIP and B (n=11) with higher than median LIP (320, range 83-594 vs. 1342, range 625-2935, p<0.0001) **(Table 1)**.

Interestingly, in group B a higer LIP_{CD8} was found as compared to group A (LIP_{CD8} 1121, range 389-2968 vs. 444, range 154-1045, p<0.01), indicating concomitant changes of LIP in the two T cell subsets **(Fig.1)**.

While CD4AC and CD4/CD8 did not differ significantly between the groups for the whole follow-up period, a slight decline of CD4 AC was observed at the second determination in group B **(Fig.2)**, as opposed to some increase of CD4 AC in group A **(Table 1, Fig.2).**

At high LIP_{CD4} values (group B), a strong inverse correlation was observed between CD4/CD8 and LIP_{CD8} at all studied points (Rho= -0.65, p<0.05 for all, **fig 3A**), which was associated with an increase of CD8AC (Rho= 0.70, p<0.05, **fig3B**) up to 11 (min. 6,

Table 1. Clinical and laboratory characteristics of study participants.
All data are represented as median, min-max.

HIV+ participants	Group A low LIP_{CD4}	Group B high LIP_{CD4}	Mann–Whitney U test
Number (n)	14	11	p>0.05
Age (years)	42 27-52	39 26-53	
Time after diagnosis of HIV infection (years)	8.5 6-24	7.0 5-13	
cART duration (years)	7.0 6-20	6.0 5-11	
Baseline* CD4 AC (cells/µl)	557 220-947	633 264-1032	
Second CD4 AC (cells/µl)	638 247-1145	506 232-981	
Third CD4 AC (cells/µl)	737 264-1007	581 312-1123	
Last CD4 AC (cells/µl)	724 292-1125	797 349-1519	
Baseline* CD4/CD8 (ratio)	0.78 0.26-2.01	0.54 0.30-0.99	
Second CD4/CD8 (ratio)	0.75 0.24-1.77	0.49 0.22-1.13	
Third CD4/CD8 (ratio)	0.81 0.30-1.95	0.55 0.39-1.27	
Last CD4/CD8 (ratio)	0.80 0.31-1.93	0.60 0.33-1.24	

*First measurement for the present study

max. 15) months after LIP measurement. In addition, the increase of LIP_{CD8} inversely correlated with CD4AC (Rho= -0.65, p<0.05) up to five (min.2, max.8) months after LIP measurement **(Fig.3C)**.

DISCUSSION:

Immune restoration in the settings of continuous cART has a variable course, and is most often incomplete (13, 14, 15). Our data shows that despite SVS, approximately 45% of the participants had a suboptimal CD4/CD8 ratio (<0.9) as a sign of insufficient immune restoration (14).

The reasons for this are numerous and for the most part are not elucidated. Residual viral reservoirs and low level HIV reactivation are a major concern, but no specific predictive markers or targeted therapy to enhance the recovery of CD4+ AC are available yet. People with an incomplete immune restoration are at higher risk of AIDS and non-AIDS-related morbidity and mortality (16).

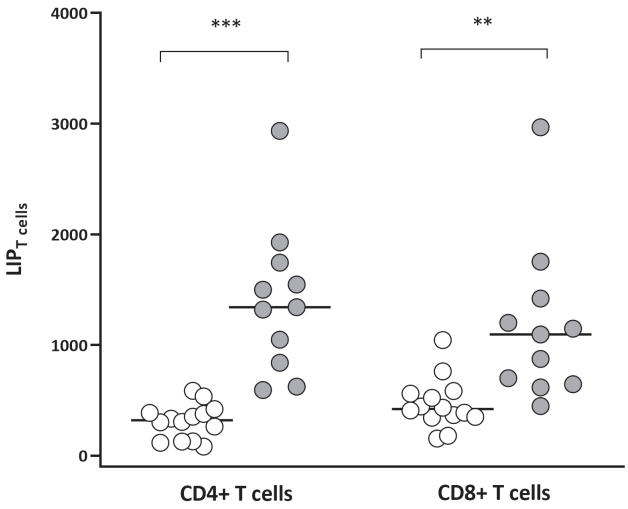

Figure 1. Comparison of MFILIP in CD4+ and CD8+ T cells between group A (open symbols) and B (gray symbols). The difference was statistically significant for CD4+T and CD8+ T cells, ($p<0.0001$ and $p<0.001$).

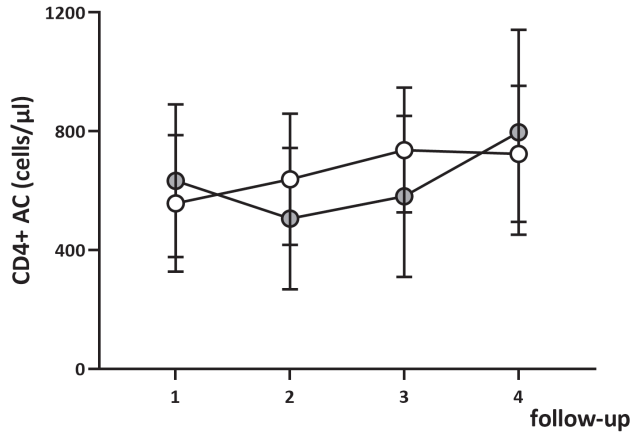

Figure 2. CD4 absolute count in group A (open symbols) and B (gray symbols) during the follow-up period ($p>0.05$).

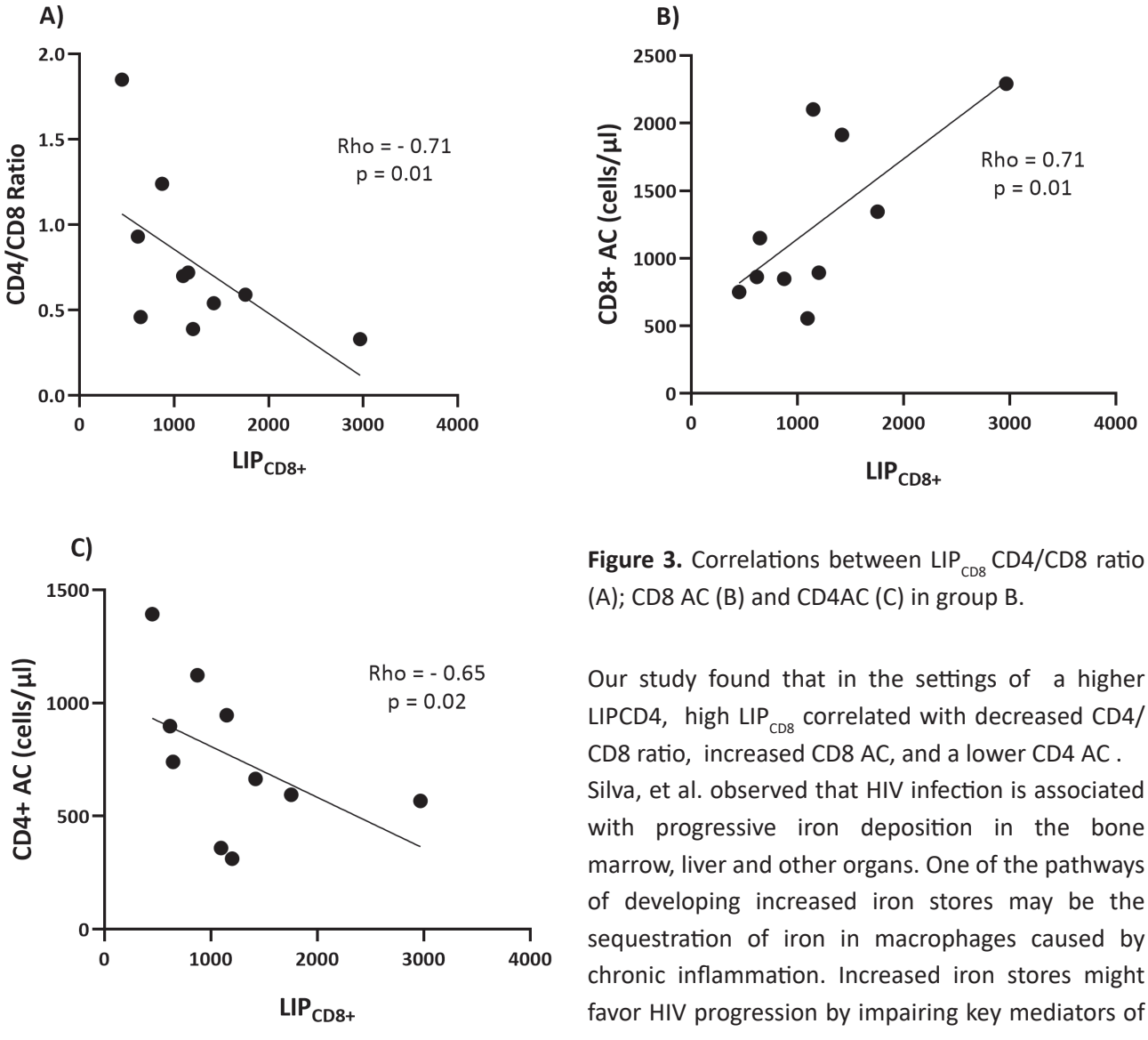

Figure 3. Correlations between LIP_{CD8} CD4/CD8 ratio (A); CD8 AC (B) and CD4AC (C) in group B.

Our study found that in the settings of a higher LIPCD4, high LIP_{CD8} correlated with decreased CD4/CD8 ratio, increased CD8 AC, and a lower CD4 AC . Silva, et al. observed that HIV infection is associated with progressive iron deposition in the bone marrow, liver and other organs. One of the pathways of developing increased iron stores may be the sequestration of iron in macrophages caused by chronic inflammation. Increased iron stores might favor HIV progression by impairing key mediators of

the host response. (12)

Our previous results clearly show that chronic HIV infection affects the regulation of iron turnover leading to increased LIP in the settings of SVS, independently of age, baseline CD4AC, CD4/CD8, HIV VL, and cART duration (12). It is well known that Fenton reaction depends on labile iron concentrations and leads to the formation of free radicals, and intracellular accumulation of ROS (4, 18, 19).

In addition, we have shown that, in HIV+cART+ individuals with SVS, the amount of ROS in CD4+ T cells correlated inversely with the CD4/CD8 ratio, suggesting that the low level of immune activation in patients with suboptimal ratio might reflect reactivation of latent HIV reservoirs (17, 20). The intracellular levels of ROS in monocytes from HIV+ individuals are associated with high viral load. The subsequent iron depletion in other cell compartments induces lysosomal ferritin degradation, iron loading of lysosomes, lysosomal ROS production, lysosomal and mitochondrial lipid membrane permeabilization and cell death with features reminiscent of ferroptosis (4).

In our hands, elevated labile iron levels were in strong inverse correlation with CD4 AC and CD4/CD8 ratio up to five months after the measurement. This observation is in line with recent studies on SARS-CoV-2, HCV, and HIV, suggesting a strong link between viral infection and ferroptosis (3, 10, 21, 22, 23, 24, 26). Viruses can trigger ferroptosis by disrupting different stages of cell metabolism, and affect host immune system through various pathways (10). Ferroptosis provides a favourable environment for viral survival, replication, and evasion of host immune response (10,22). Thus, targeting ferroptosis could be a promising approach for antiviral treatment (21, 27).

Our study is limited by the small number of the participants and the lack of established reference values for LIP. Therefore, we defined A and B subgroups somewhat artificially, based on the median LIP_{CD4}.

CONCLUSION:

In conclusion, chronic HIV infection affects iron metabolism and leads to increased LIP in the settings of SVS, and independently of age, baseline CD4AC, CD4/CD8, HIV VL and cART duration. Since elevated LIP promotes HIV replication, and is associated with T-cell dysfunction, exhaustion and ferroptosis, it may serve as a sensitive predictive marker for clinical follow-up. A larger prospective study is justified to assess the independent prognostic significance of LIP, and the undesirable effects of certain cART components on iron homeostasis.

Acknowledgements: The study is supported by the European Fund for regional development through Operational Program Science and Education for Smart Growth, Grant BG05M2OP001-1.002-0001-C04 "Fundamental, Translational and Clinical Investigations on Infections and Immunity" and the National Program for Prevention and Control of HIV and STI in Bulgaria 2021 – 2025

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