



Asymptomatic Human Immunodeficiency Virus-1 Infection with High CD4⁺ T Cell Count Does Not Alter Iron Metabolism or Hepcidin Levels: The Pilot Study

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ABSTRACT

Introduction: The purpose of the study was to assess hepcidin levels and iron metabolism in otherwise healthy human immunodeficiency virus-1 (HIV-1)-infected males and the influence of antiretroviral therapy on hepcidin production, as data in this group are scarce.

Methods: A total of 89 HIV-1-infected males, 42 on effective antiretroviral therapy (ART)—group A, 47 treatment-naïve—group B, and 27 healthy controls—group C, were enrolled. Erythrocytes parameters, iron metabolism parameters, hepcidin, highly sensitive C-reactive protein (hsCRP), interleukin 6 (IL-6), and soluble transferrin receptor (sTfR) levels were assessed. Conditions related to inflammatory activity, systemic metabolic diseases and iron supplementation were exclusion criteria. Convenience sampling was used.

Results: Median age in HIV-1 group was 33 years, and 27 years in the control group. Median CD4⁺ T-cell count was 724 cells/ μ l in group A, and 488 cells/ μ l in group B ($p = 0.0000$). Nadir CD4⁺ T-cell count was 397 cells/ μ l in group A and 475 cells/ μ l in group B ($p = 0.0001$). Median value of HIV-1 viral load (VL) in group B was 16 900 copies/mL. The hepcidin value was lower in group A than in groups B ($p = 0.0008$) or C ($p = 0.0004$), without differences between groups B and C. The hepcidin value correlated with ferritin in groups A ($r^2 = 0.16$; $p = 0.008$) and B ($r^2 = 0.39$; $p = 0.000$), but not in group C ($r^2 = 0.11$; $p = 0.09$). In group A, the hepcidin value correlated with current CD4⁺ count ($r = 0.48$, $p = 0.0012$), but there was no correlation in group B. There were no correlations of hepcidin values with CD4⁺ T cell nadir in group A ($p = 0.371$) or in group B ($p = 0.477$); ART period ($p = 0.614$); VL in group B ($p = 0.71$). No abnormalities of iron metabolism, hsCRP, IL-6, or sTfR were noted.

Conclusions: Asymptomatic HIV-1 infection does not cause clinically important iron metabolism alterations or increased hepcidin production. Hepcidin values decrease on effective antiretroviral therapy.

Keywords: Antiretroviral therapy; Hepcidin; HIV-1 infection; Inflammation; Iron

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Key Summary Points

Why carry out this study?

Data regarding iron metabolism and hepcidin levels in human immunodeficiency virus-1 (HIV-1)-infected persons without significant immune deficit are lacking.

This study was to assess the impact of stable HIV-1 infection itself and antiretroviral therapy on iron metabolism.

Hepcidin levels and erythrocyte parameters were measured in otherwise healthy young males.

What was learned from the study?

HIV-1 infection with high CD4⁺ T cell count does not alter iron metabolism or erythrocyte parameters.

Hepcidin values decrease on effective antiretroviral therapy and this observation needs further research.

INTRODUCTION

Hepcidin is an 25-aminoacid peptide hormone, produced by hepatocytes, which plays a central role in systemic iron homeostasis—its absorption and distribution throughout the organism. Hepcidin causes degradation of ferroportin, membrane protein transporting the iron. Ferroportin is expressed mainly in duodenal enterocytes and on macrophages. Increased hepcidin levels inhibit the absorption of the alimentary iron from gastrointestinal tract and its utilization from old erythrocytes. It results in decreased serum iron concentration and its availability for hemopoiesis. Thus, hepcidin is a major regulatory iron hormone. Altered hepcidin production and iron metabolism play an important role in the pathogenesis of many acute and chronic infections, as the

microorganisms use the iron acquired from the host for their own life processes [1]. One of the important examples is *Mycobacterium tuberculosis*, which utilizes iron storage in macrophages for its growth, and high hepcidin levels promote iron cellular storage [2]. In chronic hepatitis C virus (HCV) infection, low levels of hepcidin are observed, which result in excessive iron storage in liver tissue [3]. Hepcidin production in the liver is stimulated by the high iron serum levels and inflammatory cytokines, like interleukin-6 (IL-6) [4], so it belongs to the acute phase proteins. Anemia observed in many chronic inflammatory conditions, e.g., systemic autoimmune diseases, is related to persistent stimulation of hepcidin production [5].

The major feature of human immunodeficiency virus (HIV) infection is chronic immunological activation, including not only gradual decrease of CD4⁺ T lymphocytes count but also other multiple disturbances: lymphocyte T and monocyte activation, damage of gut-associated lymphoid tissue and excessive bacterial translocation, and alterations in the production of proinflammatory cytokines [6]. The viral Nef protein may play a role in macrophage iron overload as it interacts with human homeostatic iron regulating protein (HFE)—the protein regulating iron storage, as it down-regulates HFE expression on the macrophage surface [7]. The role of iron homeostasis and its associations with chronic inflammatory activation in pathogenesis of HIV infection are poorly known. In vitro studies have shown that high intracellular iron levels induced by high hepcidin levels increase HIV replication, and, moreover, HIV infection may lead to increased cellular iron concentrations [8, 9]. Majority of conducted research have dealt with HIV-infected patients with hematologic disturbances or serious concomitant infections, like tuberculosis or malaria. High iron concentration in macrophages promotes the progression of HIV infection [10, 11], and increases susceptibility to opportunistic infections, such as tuberculosis [2, 12]. On the other hand, untreated HIV infection and HIV-related chronic immune activation increases hepcidin levels. As iron constitutes an essential component for HIV replication in cells, chronic inflammation and

iron storing in cells, e.g., macrophages, may be an important factor increasing viral replication [13], and, moreover, at least partially explain the chronic anemia related to HIV infection.

Data concerning hepcidin levels and iron metabolism in otherwise healthy HIV-positive patients, as well as the influence of antiretroviral therapy on hepcidin production, are scarce and conflicting. This pilot study is an analysis of the hepcidin levels and markers of iron metabolism in young HIV-1-positive male patients without concomitant conditions related to inflammatory processes other than HIV-1 infection, and of the impact of antiretroviral therapy on hepcidin production. The study group has been limited to males only because of preliminary nature of the study and with the purpose of excluding as many additional factors influencing iron metabolism as possible.

METHODS

A total of 89 male HIV-1 infected subjects, Caucasians aged 18–50 years, were enrolled in the study. The HIV-1-infected group consisted of two subgroups: individuals on stable antiretroviral therapy, with undetectable HIV-1 viral load at least 1 year before enrollment (group A, $n = 42$) and treatment-naïve patients without any clinical signs or conditions related to advanced immune deficiency (group B, $n = 47$). A control group consisted of 27 healthy male volunteers, matched for age.

The study group was selected to exclude any additional factors other than HIV-1 infection, influencing the iron homeostasis and inflammatory activation. Only males were included to avoid additional bias in females due to menstrual bleeding and potential loss of iron increasing hepcidin levels. The age range was limited to 50 years to avoid age-related chronic metabolic conditions with potential impact on immune activation. Other exclusion criteria were as follows: anemia with known etiology, current symptomatic or occult not treated syphilis, other current sexually transmitted infections, iron supplementation up to 6 months before enrollment, red blood cells concentrate transfusion up to 6 months, any

acquired immunodeficiency syndrome-defining illness (active or diagnosed in the last 6 months and not cured), any malignancy in the medical history, hepatitis B virus infection defined as positive HBsAg, HCV infection defined as positive anti-HCV, malabsorption, zidovudine use in the current antiretroviral therapy (ART) regimen or within the last 6 months, estimated glomerular filtration rate < 60 ml/min, liver cirrhosis of any etiology, intravenous drug use currently or within last 5 years, alcohol abuse currently or within last 5 years, acute febrile disease up to 1 month before enrollment, any active infection other than HIV-1 infection, and any other conditions related to inflammatory activation (e.g., vein thrombosis). The study group was recruited as the convenience sample.

Collected information included demographic data, history of HIV-1 infection (route of infection, period of antiretroviral treatment and used regimens, maximum and level of HIV-1 viral load, baseline, and nadir and current CD4 + T cell count), past and current concomitant diseases, smoking status.

Hepcidin 25 (bioactive) was measured using a commercially available test—an enzyme-linked immunosorbent assay (DRG Instruments, Marburg, Germany). The range of the assay was between 0.153 and 81 ng/mL. Human soluble transferrin receptor (sTfR) was measured using Quantikine® IVD® Soluble Transferrin Receptor ELISA (R&D Systems, Minneapolis, MN, USA). Interleukin-6 (IL-6) and highly sensitive C-reactive protein (hsCRP) were measured using Quantikine® ELISA (R&D Systems). HIV-1 viral load (VL) was determined by real-time PCR assay (COBAS TaqMan HIV-1 Test v.2.0; Roche Diagnostics, Basel, Switzerland). The isolation of HIV-RNA was performed using a System Viral Nucleic Acid Kit (Roche Diagnostics). The CD4 + T cell count was determined by flow cytometry using the FacsCount Becton Dickinson system (BD Biosciences, San Jose, CA, USA). Biochemical tests, complete blood count, iron level, total iron binding capacity (TIBC), ferritin, liver function tests, and creatinine level, were measured with the use of standard commercially available methods as part of routine medical care of HIV-1-infected patients.

Statistical Analysis

The continuous variables were tested for normal distribution with the Shapiro–Wilk test. To compare two groups, the Student's *t* test or the Mann–Whitney *U* test were used depending on data distribution. To compare more than two groups, the Kruskal–Wallis rank sum test was used with post hoc analysis if needed. Pearson's correlation test was used to analyze the correlation of selected variables (the relationship between the variables was linear). The exact Fisher test was used for comparison of nominal data. The *p* values of less than 0.05 were considered statistically significant, but for data with comparison of multiple variables, the Bonferroni correction was used with *p* values of less than 0.006 considered to be statistically significant.

Calculations were performed using Statistica 12.0 software for Windows (StatSoft, Tulsa, OK, USA).

Statement of Ethics Compliance

The study has been conducted in accordance with the Helsinki Declaration of 1964, and its later amendments. The study was approved by the Bioethical Committee of Wrocław Medical University, Wrocław, Poland (consent No. 365/2014). All the participants gave written informed consent before any procedures related to the study. The participants provided consent for publication if any identifying information was included in the manuscript.

RESULTS

The characteristics of the study group are presented in the Table 1.

In group B, eight individuals had HIV-1 VL > 100,000 copies/mL (17%), among them, in two persons, VL was > 1,000,000 copies/mL. A total of five patients with high HIV-1 VL had CD4⁺ count < 350 cells/μl (62.5%), whereas in the entire group B, in ten persons, we found CD4⁺ count < 350 cells/μl (21.3%).

In all treated HIV-1-infected patients, the standard ART three-drug regimens were used. Majority of patients (*n* = 39, 92.9%) were treated with tenofovir disoproxil (TDF)/emtricitabine (FTC) as a backbone two nucleos(t)ide reverse transcriptase inhibitors (N(t)RTI), and in three individuals (7.1%) abacavir (ABC)/lamivudine (3TC) were used. As a third drug protease inhibitor (PI) in 14 individuals (33.3%), non-nucleoside reverse transcriptase inhibitor (NNRTI) in 26 patients (61.9%) or integrase inhibitor (INSTI) in two persons (4.8%) were administered.

No significant abnormalities in platelet level, liver enzymes levels, total bilirubin level, creatinine level, or hsCRP level in any participants were observed. Also, levels of factors potentially causing anemia, as folic acid and vitamin B12 levels were normal in all the participants. There were no significant difference of these parameters between the A, B, and C groups.

Parameters of red blood cells and iron metabolism are presented in Table 2.

For mean corpuscular volume (MCV) values, significant differences in post hoc tests between groups A and B (*p* = 0.0001) and groups A and C (*p* = 0.0001) were observed. Between the B and C groups, the difference was nonsignificant (*p* = 0.67). The iron level in the post hoc tests was significantly lower in group A compared to the healthy controls (group C) (*p* = 0.019), although still within the normal range in all the subjects in group A, whereas there was no difference between group B and the healthy controls (*p* = 0.18). Ferritin levels were different between groups A and C (*p* = 0.01), but no differences were apparent between groups A and B or B and C (*p* = 0.34 and *p* = 0.33, respectively). In the subjects with ferritin levels above the upper normal range, no alterations in other iron metabolism parameters were observed.

Hepcidin levels in post hoc tests significantly differed between groups A versus B (*p* = 0.0008) and groups A versus C (*p* = 0.0004), while for groups B versus C, the difference was not significant.

Hepcidin levels in the HIV-infected group [median 22.24 (interquartile range, IQR, 8.86–37.56; range 0.85–80.6)] and healthy controls [median 37.2 (IQR 18.3–49.4; range

Table 1 Characteristics of the study group

	HIV-1-infected subjects			<i>p</i>	C (healthy controls), <i>n</i> = 27	<i>p</i> ^a
	All (A+B), <i>n</i> = 89	A (treated), <i>n</i> = 42	B (treatment-naïve), <i>n</i> = 47			
Age [median (IQR, range)]	33 (28–38; 19–50)	34 (30–37; 21–50)	33 (26–40; 19–48)	0.2740	27 (24–42; 22–48)	0.246
BMI [median (IQR, range)]	22.43 (20.9–24.93; 16.95–33.03)	22.46 (20.34–26.88; 18.51–33.03)	22.04 (20.94–24.38; 16.95–29.35)	0.6098	24.49 (22.60–27.17; 19.32–30.56)	0.037
Route of HIV transmission ^b [<i>n</i> (%)]						
MSM	80 (91.95)	35 (85.37)	45 (95.7)			
HET	4 (4.60)	4 (9.76)	0			
BI	1 (1.15)	0	1 (0.98)			
IDU	2 (2.30)	2 (4.88)	0			
CD4 ⁺ T cell count, current; cells/μL [median (IQR, range)]	575 (441–757; 177–1073)	724.5 (577–932; 373–1049)	488 (376–588; 177–1073)	0.0000		
CD4 ⁺ T cell count nadir; cells/μL [median (IQR; range)]	414.5 (297–533; 40–1073)	397 (241–470; 40–707)	475 (353–583; 177–1073)	0.0001		
CD8 ⁺ T cell count; cells/μL [median (IQR; range)]		750.5 (575–1049; 291–2000)	929 (733–1415; 382–1925)	0.014		
CD4/CD8 [median (IQR; range)]		0.85 (0.68–1.27; 0.33–2.52)	0.49 (0.36–0.78; 0.1–1.49)	0.000		
HIV-1 VL at the study; copies/mL [median (IQR, range)]	As in group B	Below the threshold of detection	16,900 (3300–61,300; 462–1,140,000)			
HIV-1 VL—the highest detected; copies/mL [median (IQR, range)]	41,800 (9993.5–92,650; 101–1,140,000)	51,400 (16,620–101,000; 101–901,000)	23,000 (6230–75,500; 462–1,140,000)	0.049		

Table 1 continued

	HIV-1-infected subjects			<i>p</i>	C (healthy controls), <i>n</i> = 27	<i>p</i> ^a
	All (A+B), <i>n</i> = 89	A (treated), <i>n</i> = 42	B (treatment-naïve), <i>n</i> = 47			
Years of treatment [median (IQR, range)]		3 (2–5; 1.5–9)	N/A			
Smoking [<i>n</i> (%)] ^b				0.4263		0.0504
No	35 (57.38)	15 (65.22)	20 (52.63)		21 (80.77)	
Yes	26 (42.62)	8 (34.78)	18 (47.37)		5 (19.23)	

BMI body mass index; *HET* heterosexual; *IDU* intravenous drug use; *IQR* interquartile range; *MSM* men having sex with men; *VL* viral load

^aDifferences between HIV+ group and healthy controls

^bNot all data available

1.2–61.6)] differed significantly ($p = 0.0134$), with very low levels of hepcidin in group A, as shown on Fig. 1.

All the subjects had IL-6 levels below the threshold of detection for the test used (3.13 pg/mL), except one individual in group A with the value 3.3 pg/mL.

Hepcidin level was correlated with ferritin level in groups A ($r^2 = 0.16$; $p = 0.008$) and B ($r^2 = 0.39$; $p = 0.000$), but not correlated in group C ($r^2 = 0.11$; $p = 0.09$).

In group A, the hepcidin level was correlated with the current CD4⁺ T cell count ($r = 0.48$, $p = 0.0012$), whereas in group B no correlation was observed.

There were no correlations of hepcidin level with CD4⁺ T cell nadir, neither in group A ($p = 0.371$) nor in group B ($p = 0.477$), or with the period of ART in the treated group ($p = 0.614$), HIV-1 VL in group B ($p = 0.71$), the highest HIV VL ($p = 0.21$ for group A, $p = 0.83$ for group B), smoking ($p = 0.632$), body mass index (BMI) ($p = 0.41$, $p = 0.98$, and $p = 0.15$, for groups A, B, and C, respectively), any red blood cell parameters, or with levels of iron, folic acid, vitamin B12, or sTfR. Because of the small number of participants in group A, the impacts of certain classes of antiretroviral medicines (namely, PI, NNRTI, or INSTI), nor

specific N(t)RTIs, such as tenofovir versus abacavir, on analyzed parameters were not assessed.

The small study sample did not allow for conducting multivariate analysis.

Due to the small number of patients, the power was low for non-parametric tests (on average at the level of about 55%), and medium for parametric tests (about 65%). In order to prove the existence of a difference between the selected groups of hepcidin and iron concentration, the study groups would have to be very numerous (in the case of iron concentration assessment, each group should have at least 663 patients, and, in the case of hepcidin, at least 754 subjects).

DISCUSSION

Chronic immune activation is an important clinical feature of HIV infection, determining multiple abnormalities of the host metabolic homeostasis. Antiretroviral therapy by stable suppression of viral replication allows the reversal of such abnormalities in the majority of people living with HIV (PLWH) on effective ART. As hepcidin level increases in the states of chronic inflammation and immune activation, affecting iron homeostasis, and leading to

Table 2 Hematological and iron metabolism parameters

	HIV-1-infected group		Group C (healthy controls)	<i>p</i>
	Group B (treated)	Group A (treatment-naïve)		
RBC; mln/ μ L [mean; SD]	4.89 \pm 0.42	5.08 \pm 0.31	5.18 \pm 0.29	0.004
HGB; g/dL [mean; SD]	15.64 \pm 1.21	15.19 \pm 1.08	15.42 \pm 0.8	0.144
HCT; % [median (IQR; range)]	44.7 (42–47; 40–53)	44.0 (42.2–46; 29–49.8)	43.95 (43–46.3; 40.5–48.8)	0.604
MCH; pg [median (IQR; range)]	32 (31–33; 27–35)	30 (29–31; 24.1–33.1)	29.9 (29–30.6; 25.5–32.6)	0.000 (differences: A vs. B; B vs. C groups)
MCHC; g/dL [mean, SD]	34.87 \pm 1.06	34.42 \pm 1.05	34.74 \pm 1.07	0.126
MCV; fL [mean, SD]	91.91 \pm 4.56	86.91 \pm 4.06	85.91 \pm 3.83	0.000
Iron; μ g/dL [mean, SD]	108.83 \pm 37.96	95.34 \pm 28.26	115.07 \pm 34.58	0.035
TIBC; μ g/dL [median, IQR; range]	346.5 (294–371; 256–414)	328 (299–344; 250–500)	320 (296–358; 245–396)	0.257
UIBC; μ g/dL [median, IQR; range]	235 (193–260; 118–316)	220 (197–261; 122–468)	207 (177–250; 95–292)	0.236
Transferrin saturation; % [mean, SD]	32.48 \pm 10.59	29.96 \pm 9.78	35.93 \pm 11.05	0.062
Ferritin; μ g/L [median, IQR; range]	91 (43–120; 6–742)	100 (54–154; 11–952)	131 (82–227; 13–525)	0.013
sTfR; nmol/L [median, IQR; range]	15.4 (12.3–18.5; 8.1–27.7)	15.9 (11.6–23.4; 3.6–58.7)	17.6 (14.7–22; 9.2–46.8)	0.103
Hepcidin; ng/mL [median (IQR; range)]	12.27 (3.35–27.33; 1.08–63.82)	28.85 (18.56–47.8; 0.85–80.6)	37.2 (18.3–49.4; 1.2–61.6)	0.0001

IQR interquartile range; *HCT* hematocrite; *HGB* hemoglobin; *MCH* mean corpuscular hemoglobin; *MCHC* mean corpuscular hemoglobin concentration; *MCV* mean corpuscular volume; *RBC* red blood cell; *SD* standard deviation; *sTfR* soluble receptor for transferrin; *TIBC* total iron binding capacity; *UIBC* unsaturated iron binding capacity

persistent anemia, and such a phenomenon potentially may be observed in persons with HIV infection [14]. However, in contrast to earlier research, assessing primarily the patients with advanced HIV infection, the analysis of our study group did not demonstrate any important abnormalities in erythrocyte parameters nor clinically significant alterations of iron metabolism in treatment-naïve patients and subjects on ART. Significant rates of anemia in PLWH shown in multiple studies seems to be instead related to advanced HIV infection

complications, such as low CD4⁺ T cell count or concomitant opportunistic infections (e.g., tuberculosis), in addition to malnutrition [15]. Persistent anemia is also observed in individuals on effective ART. Roldan et al. showed that such a phenomenon has no relationship to the hepcidin levels or alterations of iron homeostasis but is rather due to residual inflammation or persistent immune activation despite the successive ART [16]. In our group, even statistically significant differences, e.g., in iron levels between groups A and C, were still negligible

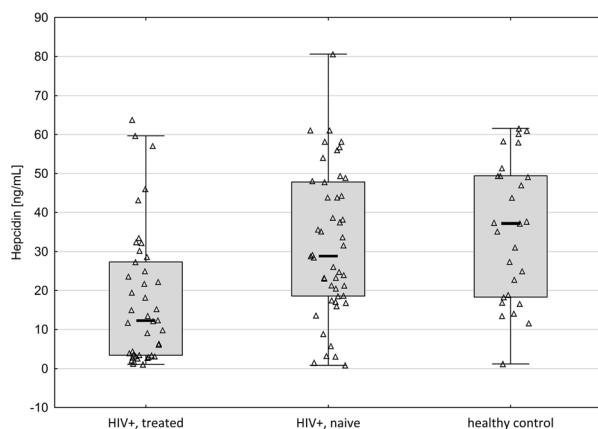


Fig. 1 Hepcidin levels in HIV-1-infected subjects treated with antiretroviral drugs, naïve HIV-1-infected subjects, and healthy controls

clinically, as, in all the participants, the values of iron metabolism and erythrocyte parameters remained within the normal ranges.

The finding of the highest MCV values in group A is consistent with many observations among PLWH treated with nucleos(t)ide reverse transcriptase inhibitors. This phenomenon has been particularly commonly observed with the use of zidovudine and dideoxynucleoside analogs (didanosine, stavudine, zalcitabine), and reflects their substantial mitochondrial toxicity [17, 18]. Macrocytic anemia has been a frequent adverse effect of zidovudine [19], not observed on therapy with other N(t)RTI; however, other drugs from this group, among them tenofovir disoproxil, also demonstrate mild mitochondrial toxicity, which may explain the significant difference in MCV values between naïve and treated individuals. In all study participants, other potential causes of increased MCV, such as vitamin B12 or folic acid deficiency, were absent.

Looking at the impact of ART on iron metabolism, Chang et al. demonstrated the increase of serum iron levels in ART-naïve, HIV-infected individuals [8]. Opposite results were obtained by Malvoisin et al. in a group of 182 HIV-1-infected women on ART; patients with detectable HIV-1 viral load had lower iron levels than those with effective therapy. In our study, there was no difference between groups A and B, and, moreover, no differences in HIV-infected

patients compared to healthy controls were observed. These conflicting data need further investigation. Malvoisin et al. also demonstrated higher levels of hepcidin in individuals with high viremia, which is consistent with our results [20].

Our group B consisted of young males newly diagnosed with HIV-1 infection, in which the cause of testing was previous risky sexual behaviors not the signs of immune deficiency. We may assume that the period of HIV-1 infection in our ART-naïve group was short, and therefore we did not demonstrate the negative consequences of chronic inflammation, even despite the relatively high median HIV-1 viral load.

The levels of IL-6 did not differ in the HIV-1-infected patients and the control group and were low. This shows that asymptomatic HIV-1 infection with relatively high CD4⁺ T cell count is not related to a chronic inflammatory process intense enough to induce excessive hepcidin production, which might lead to iron metabolism disturbances and the developing of chronic anemia.

The very low levels of hepcidin, still with normal serum iron level and erythrocyte parameters, observed in our study in individuals with well-controlled HIV-1 infection, on effective ART, needs further research and verification in larger groups. One of the possible causes might be the influence of antiretroviral drugs (in general or some certain drugs, e.g., tenofovir) on hepcidin production or expression, which has not yet been investigated. Another explanation of this finding may be just a small sample size and a random result. A phenomenon observed in this group, which also needs further investigation, is the positive correlation of hepcidin level with current CD4⁺ T cell count, which has been not described in other studies. Nevertheless, the differences related to the CD4⁺ T cell count in the treated group seem to be clinically insignificant, as we did not find any alterations of iron homeostasis in this group of patients. We also cannot explain the highest levels of hepcidin observed in the control group. The healthy volunteers did not show any disturbances of iron metabolism, potentially related to higher hepcidin

levels. Analysis of the detailed medical history in this group showed no factors potentially related to inflammatory state in these individuals. Moreover, levels of IL-6, which is a well-known activator of hepcidin production [21, 22], were similar in all three groups, and, in almost all subjects were below the threshold of detection of the test used. Too small a study group may be the one of the possible explanations. On the other hand, in comparison with treated and treatment-naïve HIV-1-infected subjects, the obtained results of hepcidin levels were as expected, and significantly higher in persons not treated with ART.

The different times since HIV infection diagnosis between treated and naïve groups potentially may have had an impact on the results, but it was obvious and impossible to avoid, and was among the substantial features differing between the groups, similarly to the potential toxicity of the medicines used in the past.

Of note, all the above described differences in hepcidin levels between the three analyzed groups did not lead to any clinically important iron metabolism alterations or erythrocyte abnormalities, thus they may be so subtle that their impact on iron levels is negligible. Low-power calculations may involve the risk of erroneously excluding the existing differences, but the values of hepcidin and iron concentrations in the analyzed groups were within the normal range, and, in the authors' opinion, demonstrating that the difference is not clinically significant.

CONCLUSIONS

In PLWH with a stable course of HIV-1 infection, without opportunistic infections or serious concomitant diseases, we did not observe iron metabolism alterations related to the induction of hepcidin production in our cross-sectional analysis. Even the observed differences in hepcidin levels in the analyzed groups are so mild that they did not have any impact on significant or clinically important abnormalities in iron levels or erythrocyte parameters. Therefore, iron metabolism disturbances reported in the

PLWH may be limited to the advanced HIV infection stages with intense inflammatory activation, or more related to concomitant conditions being the consequences of deep immune deficiency, such as active tuberculosis [23].

Limitations of the study include the small study group, which among others did not allow for conducting reasonable multivariate analysis. Moreover, longitudinal analysis of the study groups would give a more reliable basis for assessing the trends of hepcidin levels, particularly before and after ART introduction in the naïve group. Another limitation is the enrolling to the study of only young males. On the other hand, such selection of the study group allowed us to exclude an additional variable, which is the potential inhibition of hepcidin production due to anemia related to menstruation bleeding in young women.

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Author Contributions. All authors had access to study data and participated in writing and review of the manuscript. Aleksandra Szymczak contributed to concept and design, drafting the manuscript; Malgorzata Zalewska contributed to study design, acquisition of data

and data analysis; Weronika Rymer contributed to data review, statistical analysis, drafting and review of the manuscript; Ewa Anita Jankowska contributed to the concept and design, analysis, interpretation and critical revision of data.

Disclosures. Aleksandra Szymczak, Malgorzata Zalewska, Weronika Rymer and Ewa Anita Jankowska all confirm that they have no conflicts of interest to declare.

Compliance with Ethics Guidelines. The study has been conducted in accordance with the Helsinki Declaration of 1964, and its later amendments. The study was approved by the Bioethical Committee of Wroclaw Medical University, Wroclaw, Poland (consent No. 365/2014). All the participants gave written informed consent before any procedures related to the study. The participants provided consent for publication if any identifying information is included in the manuscript.

Data Availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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