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Analyses of the frequencies of CD235a+CD71+ pre-erythroid cells in peripheral blood and their activity in HIV infected people.

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“Accadono cose che sono come domande.

Passa un minuto, oppure anni, e poi la vita risponde”

-Alessandro Baricco

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I. INTRODUCTION

I.1. Human Immunodeficiency virus infection.

I.1.1. Epidemiological data

Human immunodeficiency virus (HIV) after 32 years from its recognition as a cause of acquired immunodeficiency syndrome (AIDS) is still one of the most challenging problem for the health of people and the health care system in general around the world. Based on World Health Organization reports, by 2015 in whole world were living 36,7 million of people with HIV and in 2015 there were reported 2,1 million of new HIV infections. In the same year the death from AIDS was estimated to be between 940.000-1,3 million [1]. In Europe by the end of 2015 were HIV infection was diagnosed in total in more than 2 million people and in the same year there were reported 153,000 new infections. However, the real number can be much higher as there are many people not recognized as infected [2].

HIV can be transmitted by sexual contact, by injection of infected blood or its products, and during mother to child transmission [3]. New HIV transmissions are taking place mainly during period of recent infections and during the phase of chronic infection in the periods between the interruption of anti-retroviral therapy [4,5]. In Europe, in 2015 the highest proportion of new HIV diagnosis was reported in men who have sex with men (42%), followed by 32% of cases with heterosexual contacts, and 4% after injection drug use [2]. The perinatal transmission accounts for 90% of infections in newborns worldwide, and nearly 330,000 infants are HIV infected each year [6]. Risk factors for HIV transmission include HIV viral load, sexual behaviors, presence of other sexually transmitted infections, lack of circumcision, as well as genetic factors [7,8].

There has been significant progress in last decade in HIV prevention and treatment. The introduction of antiretroviral drugs in 1987 and application in 1996 combination antiretroviral therapy (ART) together with better access and early initiation of ART significantly increased the life expectancy. Nonetheless, it is still on average, 8 years shorter than in HIV negative people [1]. Despite the progress, the burden of HIV infection on the health of affected person remains high, and is mainly caused by late recognition of infection, co-infections, including hepatitis C virus (HCV) and tuberculosis, and co-morbidities such as anemia. Approximately three quarters of the HIV infected in Europe are also chronically infected with HCV. In these people HCV causes accelerated liver disease, cirrhosis and increased mortality rate compared to those with either infection alone [2]. End-stage liver disease in HIV/HCV co-infected people is a leading cause of mortality regardless of the HAART status [3]. People infected with both viruses frequently present anemia. In case of HIV anemia may occur at any stage of the infection [4], whereas in HCV infection is mainly associated with applied therapy, and liver cirrhosis [5,6]. In HIV infected people the prevalence of anemia increases with the severity of HIV disease and its progression. Anemia in these patients is the independent factor of mortality and its reversal decreases mortality rates among HIV infected people [4] [7]. Anemia in HCV infected people is a serious complication during treatment with dual therapy (pegylated interferon-〈 + ribavirin) but also with the therapies that use protease inhibitors [8,9], and causes significant decline of quality of life. Surprisingly, anemia not always was associated with negative outcome, as its fast onset after initiation of dual therapy was found to be associated with better sustained virological response rate [6].

I.1.2. Virology

In 1984, AIDS was confirmed to be mediated by infection with virus to which in 1986 was named HIV. This lentivirus belongs to family of Retroviridae and together with other type of viruses of the same genus is characterized by causing slow, progressive infections. HIV is highly heterogenous, divided to type HIV-1 and HIV-2, several groups, subtypes or clades.

HIV RNA consists of two positive sense strands that code for Gag, Pol, and Env structural proteins. The HIV genome encodes also regulatory proteins Tat and Rev, and accessory proteins Nef, Vif, Vpr and Vpu. Gag protein is further cleaved to matrix, capsid and nucleocapsid proteins, Pol is cleaved to protease, reverse transcriptase and integrase, and Env to gp120 external subunit and gp41 transmembrane subunit.

HIV virus is transmitted mainly through mucosal surfaces where by contact with CD4 T cells expressing $\alpha4\beta7$ integrin can be transported to different tissues. For the entry HIV utilizes its envelope protein (Env) [9]. It binds to CD4 receptor and then to cellular co-receptor. Binding to CD4 can be preceded by contact between Env of the virus and cell surface receptors, such as: heparan sulfate proteoglycans, integrin $\alpha4\beta7$, or pattern recognition receptors. Although, these interactions can bring virus closer virus to CD4 receptor they are not mandatory for its attachment to the cell. The binding between Env protein of the virus and CD4 receptor of the host cells determines the ultimate effect of productive infection. Highly glycosylated gp120 subunit of Env contains five conserved and five variable domains. Variable domains of gp120, undergo frequent mutations and give a virus possibility to escape the immune recognition. The type of the co-receptor on cell surface that HIV binds to is used to classify the virus; HIV R5 are termed those which bind to CCR5 whereas those viruses that bind to CXCR4 are termed X4, and those that bind to both, R5X4. Importance of CCR5 co-receptor was demonstrated by significant resistance to HIV infection of cells of the host with 32 base pair deletion in *ccr5*, termed *CCR5*Δ32. Most spectacular example of such a resistance was demonstrated in HIV infected

patient who underwent stem cell transplants for leukemia. This so called "Berlin patient" HIV had suffered from acute myeloid leukemia for which he received two bone marrow transplants from a donor with a homozygous deletion in CCR5. Shortly after transplantation, the patient ceased antiretroviral therapy and for the last 8 years was always reported HIV negative [10]. After binding of HIV to CD4 and co-receptor, initiates the phase of fusion with cell membrane mediated by gp41 subunit of Env. In this process, opening and stabilization of membrane fusion pore is subsequently followed by release of viral contents to the cytoplasm of the cell after which starts the phase of replication of the virus. Integration of the viral DNA into the genome of the host cells is the first step after entry that is required for productive infection. This step is mediated by integrase protein encoded by virus that is introduced into the cell together with reverse transcriptase, viral RNA and viral core proteins. As the viral DNA is synthesized it associates with integrase and is transported to the nucleus where is integrated with the host DNA to create provirus. Integrated viral DNA is from that moment taking advantage of the cells synthesis mechanisms for transcription and translation of its own material to create new virions. Transcribed into RNA message is transported from the nucleus to the cytoplasm where viral proteins and enzymes are translated and assembled by viral protease to create new virus particles which budd off the host cell. These immature, noninfectious viral particles are transformed into new, mature, infectious viruses by viral proteinase which cleaves Gag polyprotein.

I.1.3. Pathogenesis of HIV infection.

I.1.3.1. HIV entry and infection progression.

Activated T helper cells are main target of HIV as these cells express virus entry receptors; CD4 and chemokine co-receptors, CCR5 or CXCR4, or rarely both. Susceptible to the infection are also other cells that bear entry receptors and among them are resting CD4 T cells, monocytes and macrophages, and dendritic cells . Despite existence of several cell host proteins such as APOBEC3, tetherin, and SAMHD1 that restrict virus replication, accessory proteins of

the virus are capable to counteract these limitations [11]. Transmission of HIV across mucosal membranes is usually established by one founder virus. In this process integrin α4β7 expressed on T cells, mediates the attachment of the virus for its transport to the mucosal tissues [9,12–14]. The founder virus for cell entry is using CCR5 co-receptor rather than CXCR4 and its transmission is followed by a rapid increase in HIV replication that in turn causes significant drop in CD4 T cell counts in peripheral blood as well as in gastrointestinal tract and rise in production of inflammatory mediators. This rapid onset of inflammatory reactions is in contrast to the minimum initial response to other chronic viral infections such as hepatitis C. After acute reaction that may last several weeks, viral load decreases due to development of immune responses and so-called viral set-point is established. During this phase usually very low CD4 T cell counts are found in peripheral blood and their lowest numbers are indicative of so called CD4 nadir [15]. The CD4 T cell nadir is significant predictor of long-term morbidity and immune recovery [7]. Development of immune responses to HIV during first weeks of infection includes differentiation and proliferation of cytotoxic, HIV-specific CD8 T cells that in HLA dependent mode can be effective in killing of productively infected cells but in some cases also cause the emergence of immune escape mutations [16] . In individuals with HLA-B27 allele infected with clade B, CD8 T cells are less prone to become exhausted and their responses are polyfunctional, which means characterized by capability of production of broad range of immunomediators [17]. This phenomenon is one of the reasons of long term control of infection demonstrated by small proportion of HIV infected people termed Long-Term Non-progressors and Elite controllers [18]. In significant majority of HIV infected people CD 8 T cell responses are limited to the narrow range of immune mediators they can produce and are prone to progressive exhaustion which among several factors is characterized by high expression of programmed death 1 (PD-1) on both total and HIV-specific T cells [19]. After acute phase of disease and development of immune response that helps to limit the viral load, infection

progresses and CD4 T cell counts drop below 500 cells/µL and their proportion to CD 8 T cells continues to be less than 1 [20] .

I.1.3.2 Hematopoietic changes

Hematopoietic changes induced by HIV infection are most significantly affecting CD4 T cells and their low counts are caused by direct destruction, reduced production and early senescence [21]. The drop of the number of CD4 T cells is seen in peripheral blood but even more pronounced reduction of their numbers is seen in gastrointestinal tract [22]. In this mucosal tissue, decreased number of CD4 T cells, including those which produce IL-17, together with enhanced inflammatory reactions causes enhanced gastrointestinal tract permeability and in consequence increased plasma concentration of microbial products such as lipopolysaccharides and soluble scavenger receptor (sCD163), that is shading off from the macrophages [23,24]. The number of CD4 T cells declines also due to diminished responsiveness to IL-7, beside IL-2, one of the main growth factors for T cells .

Hematopoietic changes do not concern only CD4 T cells, also development of erythroid cells and other progenitor cells were demonstrated to be affected by HIV. Although it was long debated infection with HIV was demonstrated to take place also in progenitor cells [25]. Direct infection of CD34+ human progenitor cells by HIV was shown to lower the potentials of the development of different cell lineages with erythroid and megakaryocytic cells being most significantly affected [26].

I.1.3.3. Inflammation

Chronic HIV infection is always associated with enhanced processes of immune activation and inflammation, independently of the clinical status of the patient, including elite controllers [27]. Immune activation as well as inflammation are normal physiologic processes that protect host from adverse effects of pathogenic factors and are counteracted efficiently by

immunosuppressive and anti-inflammatory factors. In situation of the continuous presence of foreign material that HIV infection introduces, immune system is persistently activated. Even HIV elite controllers, who are people that had never demonstrated detectable levels of HIV in blood or any clinical symptoms of infection, or those who were on ART, show enhanced status of immune activation in comparison to uninfected people [20,28]. Toll-like receptors (TLR7 and TLR 8) expressed on plasmacytoid dendritic cells constitute the part of innate immune response through which HIV causes immune activation [7]. In this process, production of interferon-α, enhanced leakage of microbial content through the gut mucosal tissue and activation of TLR4 causes enhanced production and release of pro-inflammatory cytokines such as IL-6 and TNF-α. Chronic presence of the HIV antigens is also a cause of continues activation of CD8 T cells that attempt to eliminate infected cells but fail due to the processes of exhaustion and immune senescence [19]. Activated CD8 T cells express enhanced levels of CD38 and HLA-DR receptors and their combined [29] or CD38 expression alone [30,31] on these lymphocytes were found to correlate with disease progression regardless of the time from HIV infection. Although processes of immune activation and related to them inflammation during HIV infection are evident and their association with disease progression established, the role of counteracting mechanisms of immunosuppression are still highly debated. The contribution of immunosuppressive activity of T regulatory cells in disease progression is not clear. These cells in direct cell to cell contact or/and by release of IL-10 may inhibit immune activation and thus diminish inflammatory reactions but may also prevent or inhibit the immune responses that lead to virus elimination [32,33]. Immunosuppressive mechanisms are also mediated by cells through the metabolic activity of enzymes they produce [34,35]. Among these enzymes is an arginase. There are two types of arginase, type I and type II. Type I is expressed in erythroid cells, and type II in extra-hepatic tissues including monocytes, macrophages. Arginase I expression was found to be significantly up-regulated in cells from peripheral blood and lymph nodes of HIV infected people, and blood [36]. Moreover, arginase activity was demonstrated to be significantly

increased in HIV infected people and correlated with severity of disease progression The source of this enzyme in HIV infected people was identified in CD15+ neutrophils. Enhanced arginase activity in HIV infected people was L-arginine depletion and in consequence decreased levels of expression of CD3ζ chain on CD4+ and CD8+ T cells [37]. In animal model, neonatal erythroid cells mediated in arginase type II dependent way downregulation of CD69 expression on T cells during *in vitro* stimulation In this animal model such an activity was associated with enhanced *in vivo* bacterial infectivity [38]. Pre-erythroid cells obtained from human umbilical cord [38] as well as peripheral blood of adults [39] were also demonstrated to have anti-inflammatory activity but whether it was associated with arginase activity was not demonstrated [38].

I.1.3.4. HIV reservoirs and latency

Lack of possibility of elimination of HIV by immune system is not only related to the mechanisms of escape of the virus from different intracellular restriction factors or effector immune responses. It is also related to the possibility of HIV to create the pool of latently infected cells that are "invisible" to the immune system [7,40]. Latency is defined as integration of HIV DNA into the host genome in the absence of virus production. *In vivo* reservoirs of latent HIV viruses were shown in central and transitional memory T cells, and in naive T cells. Latently infected T cells can undergo homeostatic proliferation via stimulation from homeostatic cytokines such as interleukin 7, which further contribute to their long half-life and persistence. Persistence of inflammation, in elite controllers, or patients on ART is suggested to be related to undergoing low-level HIV replication during the reversal of the latency [7]. Although T cells of memory phenotype are possibly the major or the only one reservoir of replication competent virus during ART, the latent virus outside the period of the therapy can be found in other cells, including long debated before, hematopoietic progenitor cells. Among these cells the lineage of megakaryocyte-erythroid progenitors (MEP's) was found to be the most permissive to HIV

infection [26,41]. However, whether the progenitor cells can be the source of replication competent virus remains to be assessed.

I.2. HIV/HCV co-infection

HCV co-infections are relatively frequent among HIV infected people since both viruses share the same route of transmission. Recently published results of meta-analyses of the prevalence and burden of HCV co-infection in HIV positive people showed that at the global scale 6,2% of HIV infected people are HCV co-infected [42]. Among HIV/HCV co-infected people, 82,4% are intravenous drug users. One of the major complications of HCV infection is liver disease that causes frequent hospitalizations and death among HIV infected people. Mortality rate among HIV/HCV co-infection is higher in comparison to HIV or HCV alone [43]. Development of liver disease caused by HCV is accelerated in HIV infected people, it develops 12 to 16 years earlier compared with HCV mono-infected. The reason for this accelerated development of liver disease is not known but viral interactions and immunosuppression are among possible reasons [43]. In comparison to HIV and HCV mono-infected, HIV/HCV co-infected people demonstrate more pronounced defects also in immunological parameters. One of the example is higher in this group frequency of activated and exhausted (PD-1 positive) CD4 and CD8 T cells [44]. In HIV infected patients, co-infection with HCV can cause additional changes in the process of hematopoiesis. The liver of HCV infected people is enriched in the number of myeloid dendritic cells but the frequency of stem cell marker (CD34) positive cells is reduced in comparison to the liver disease caused by other factors [45]. High concentrations of soluble scavenger receptor CD163 (sCD163) in plasma of HIV infected [24] as well as in HIV/HCV co-infected patients [46] are seen as an indicator of enhanced permeability of gut mucosal tissue resulting from inflammatory reactions. In HIV/HCV co-infected people increased plasma concentrations of this receptor additionally have a predictive value of liver fibrosis. Released from liver macrophages-Kupffer cells, plasma concentrations of this scavenger receptor

were found to correlate with the advancement of liver fibrosis, being most correlated with the F4 stage, that is liver cirrhosis [46,47]. Although the influence of HIV infection on the development of HCV mediated liver disease is apparent [48] the HCV does not seem to have a major impact on the natural history of HIV infection [43]. HCV co-infection was shown to have no effect on HIV progression [49] or recovery of CD4 T cells after HAART [50].

I.3 Erythroid cells.

Erythroid cells are the earliest differentiated cells of embryo. They develop during so called primitive and definitive form of erythropoiesis [51]. The primitive form takes place during early fetal life and is located in yolk sack of embryos from which is transferred to fetal liver [52]. The cells during this period are large and nucleated. The definitive erythropoiesis takes place during late fetal and post natal life. During this period of the development pre-erythroid cells enucleate and become reticulocytes and later on erythroblasts [53]. Definitive development takes place in fetal liver and continues in bone marrow in adults. Development from proerythroblasts to early reticulocytes takes place in so called erythroblastic islands located in the fetal liver and bone marrow. In these structures erythroid cells surround central macrophage and interact with it through different adhesion molecules among which are integrin $\alpha4\beta1$ [53]. Central macrophages are CD4 positive and serve developing erythroid cells as a supplier of iron and use their scavenger receptor CD163 to eliminate released hemoglobin. Proliferation and differentiation of erythroid cells depends on interaction with central macrophages but it is also dependent from interaction between themselves and from soluble factors of which most important is kidney-derived hormone- erythropoietin (Epo) [54,55]. Epo controls the output of the number of erythroid cells by exerting erythropoietic and anti-apoptotic effects [56]. Although the effect of Epo on the proliferation and differentiation of erythroid cells is predominant, the output of these cells is not only dependent on anti-apoptotic signals. In the presence of physiological concentrations of Epo, erythroid cells which were in direct contact with macrophages proliferate

3 times more efficiently in comparison to the cells without macrophages . This suggests that any changes in macrophage function can result in anemia less responsive to Epo, the phenomenon that can take place during chronic inflammation [57].

Pre-erythroid cells beside being present in high numbers in fetal liver and later on in bone marrow can be found also in human umbilical cord [38]. Human nucleated pre-erythroid cells, in contrast to reticulocytes or erythrocytes, beside CD235a+ (glycophorin A receptor) express also CD71 (transferrin receptor) [58] . Murine pre-erythorid cells, equivalent to those found in humans, are also distinguished by the expression of CD71 but instead of CD235a, the erythroid-lineage-defining molecule TER119 is used [59]. At the molecular level development of erythroid cells is dependent on the on transcription factor - erythroid Kruppel-like factor (EKLF;KLF1). This transcription factor in humans localizes to chromosome 19p13.12–p13.13 and is uniquely expressed in erythroid cells in fetal liver and adult bone marrow [60–62].

I.3.1. Erythroid cells and immune regulation

During erythropoiesis, progenitors cells through the stages of intermediate, nucleated cells of pre-erythroid cells, differentiate into final stage of enucleated erythrocytes. Intermediate, nucleated cells before final maturation can demonstrate activity that is significantly different from finally matured erythrocytes. In 1979, it was showed for the first time that nucleated erythrocytes were able to suppress primary and secondary antibody-mediated responses *in vivo* (17, 19) and later on, these cells were called erythroid immunosuppressor cells (ESC) [63]. It was noted that during erythropoietic disturbances these immature cells can appear in peripheral blood and lymph nodes and cause inhibition of B cell proliferation and humoral immune responses both in mice and humans [64]. In another studies it was observed that pre-erythroid cells could also inhibit proliferative cytotoxic T cell responses (22). Although the underlying mechanism of suppression was not clearly determined, suggestions were made that this effect might be partially mediated through TGF-β and direct cell–cell interactions [65]. Other means by

which immature erythroid cells can modulate immune system is associated with the potential immunomodulatory activity of the cytokines and growth factors they produce. In newborn mice immature erythroid cells were demonstrated to express wide array of mRNA for cytokines such as IL-1α, IL-1β, IL-4, IL-6, and GM-CSF [66]. In newborn mice, production of IL-4 by activated CD4 T cells was changed through the mechanisms dependent on IL-6 released from co-cultured erythroid cells [67]. Anti-inflammatory activity of TER119+CD71+ pre-erythroid cells from the spleens of newborn mice and of CD235a+CD71+ pre-erythroid cells from human umbilical cord was demonstrated *in vitro*. Production of TNF-a by CD11b+ cells from neonatal spleen or from human umbilical cord was significantly augmented when pre-erythroid cells were absent. In both, murine, and human pre-erythroid cells the immunosuppressive activity was mediated by arginase activity and was observed only for neonatal but not cells from adults [38]. Studies performed later however, found that also these cells derived from adult mice have anti-inflammatory properties [39]. The mechanisms of suppressive action of CD235a+CD71+ pre-erythroid cells is arginase dependent but not dependent on changes in the production of reactive oxygen species (ROX), and is not mediated by involvement of other immunosuppressive mediators such as indoleamine 2,3-dioxygenesa (IDO), or transforming growth factor –β (TGF-β) [38].

I.3.2. Erythroid cells and HIV

The perturbation in erythropoiesis and in particular in the development of erythroid progenitor cells was observed in HIV infected people. *In vitro* infection of bone marrow progenitor cells with HIV-2 was shown to inhibit erythropoiesis at the BFU-E and CFU-E stages [68]. Similar effect was later on found also in case of infection with HIV-1. I*n vitro* differentiation of bone marrow derived CD34+ stem cells from HIV-1 infected people naive from anti-retroviral therapy (ART) was found to cause significantly higher accumulation of erythroid burst forming units (BFU-E) in comparison to uninfected people [69]. Such an effect

was not observed anymore after application of ART. Inhibitory effects of HIV infection on development of erythroid cells were found also to be associated with dysregulation of Epo production and signaling. In HIV infected people, naive from ART, serum concentrations of Epo were shown to be lower in comparison to the levels of this hormone, when measured in the same patients after application of ART [70]. Additionally, ex vivo inhibition of the erythropoiesis of progenitor cells, despite the presence of Epo, at the stage of BFU-E, CFU-E in HIV infected ART naive people but not during ART suggests existence of intrinsically refractory mechanisms to the growth effects of this hormone [69]. The accumulation of BFU-E in ART naive patients were associated with chronic inflammation/immune activation but whether the direct cause was related to the bone marrow microenvironment changes and/or HIV infection of progenitor cells was not known. Possibility of the infection of progenitor cells was long time debated and many studies failed to demonstrate proviral material in intermediate hematopoietic cells, till the studies published in 2013 [26,41]. Hematopoietic progenitor cells (HPCs) derived from fetal liver and infected *in vitro* with replication deficient HIV were shown to produce significantly less colonies in comparison to uninfected cells. Most significantly were affected megakaryocyte-erythroid progenitors (MEP) for which no colonies developed. Similar effect was observed in HIV infected humanized mice where bone marrow derived HPCs demonstrated significant defects in colony formations especially for erythroid cells. *In vitro* HIV infection of highly purified, fetal liver, lineage specific cells, demonstrated that all intermediate precursor cells are permissive to infection as all were found positive for viral DNA. Cells belonging to CMP were found to be infected least frequently (1%) whereas those of MEP most frequently (13%). Proviral DNA was also demonstrated in colonies derived from HPCs of bone marrow of humanized mice infected with HIV.

I.4. Anemia in HIV and HCV infection

Deleterious impact of HIV infection on the processes of erythropoiesis caused through different mechanisms can result in development of anemia. Beside mentioned already, direct, deleterious effect of HIV on progenitor cells, reduced blood cell counts and low hemoglobin (Hb) levels can be caused by many other factors associated with HIV infection. Anemia is frequent consequence of opportunistic infections and malignancies to which HIV infected people are susceptible. Lack of supply of micronutrients, including iron, folic acid, and vitamin B12, antiretroviral drugs, such as zidovudine and different myelosuppressive medications can be another cause of anemia [71,72]. The same factors, that indirectly cause anemia in HIV infected people certainly can also affect the development of this disease in in HIV/HCV co-infected people. In case of HCV infection or co-infection however, applied antiviral therapy is the most frequent cause of anemia [73–76]. This side effect of interferon-α (pegIFN-α) and ribavirin, used in antiviral therapy, is caused by bone marrow suppression of erythroid cells differentiation and proliferation and hemolysis [74]. Anemia develops in 30-50% of people on pegIFN-α and ribavirin therapy and its rates are augmented by addition of one of the direct-acting antiviral drugs, enhancing already existing suppressive effect on bone marrow [76,77]. Patients who received anti-HCV therapy can develop anemia at different grades. In triple combination therapy, grade 1 anemia (Hb 9,5-11g/dL) were reported in 36-49% of patients, and the concentrations of Hb below 8,5g/dL (grades 2-3, range 6,5-9,5) in 9% [77].

It is estimated that 63–95% of HIV infected individuals will develop anemia during the course of their disease [78,79]. It may occur at any stage of the disease and its prevalence and severity increases as the disease progresses. Deterioration of quality of life is frequent observations during anemia; resolution of anemia brings significant improvement in quality of life. It has also been shown that anemia is an independent risk factor for mortality and that its reversal improves mortality rates, even after controlling for confounding factors such as CD4

count [80]. In HIV/HCV co-infected people anemia is also a frequent reason of therapy modification, especially in regard of ribavirin dosage [81]. In HIV infected people HAART appears to correct the anemia effectively in a significant number of patients in both adult and pediatric populations [82]. However, ongoing anemia is associated with treatment virologic failure in patients who initiated cART [83]. Despite the widely divergent socioeconomic factors that characterize HIV worldwide, the degree of clinical anemia remains an independent risk factor for predicting mortality from HIV even upon initiation of therapy [84–86].

Anemia in HIV infected people having its bases in the pro-inflammatory state is in many descriptive characteristics similar to anemia of inflammation well described in the aging population [82]. The association between these two type of causes of anemia is important as application of HAART caused significant rise in the number of aging adults with HIV. The relationships between HIV infection, ageing, and inflammation overlap. Among factors that link the anemia of aging and the anemia of HIV are cytokines and their aberrant expression. Pro-inflammatory cytokines including IL-1, IL-6, and TNF-α, are increased in the aging population and linked with multiple comorbidities, including anemia. Similarly, the same pro-inflammatory cytokines have also been linked to HIV infection. However, not only the soluble factors but also cellular changes, including appearance of immature, nucleated pre-erythroid cells, may link both, anemia and inflammation. Their potential to mediate immune regulatory activity demonstrated before may affect the inflammatory status during anemia.

II. HYPOTHESIS

Recent findings on the immunosuppressive role of CD71+erythroid cells in neonates and adults give new light on the development and maturation of immune system. These cells were reported to cause systemic suppression of immune cells' activation at the same time causing increased susceptibility to the progression of the infection. It is suggested that these processes help the immune system to mature without causing harmful, inflammatory reactions. Till now, there were no studies performed to investigate the immunoregulatory activity of pre-erythroid cells during HIV infection. These cells attracted the attention of investigators only as a possible HIV reservoir, and one of the cause of anemia.

For the studies presented here it was hypothesized that pre-erythroid cells although not present in peripheral blood of healthy adults might be found in this tissue during HIV and/or HIV/HCV co-infection and exhibit immunoregulatory effects. The potential findings of pre-erythroid cells in peripheral blood of adults was assumed from several studies that demonstrated before significant influence of HIV on the development of this population of cells. *In vitro* infection of bone marrow progenitor cells with HIV-2, as well as differentiation of bone marrow derived CD34+ stem cells from HIV-1 infected people, naive from anti-retroviral therapy cause significantly higher accumulation of immature erythroid cells. These cells were also shown to harbor viral DNA after *in vitro* HIV infection of hematopoietic progenitor cells. At the bases of potential role of pre-erythroid cells as an important immuneregulatory factor is their capacity to produce arginase and demonstrated in separate studies enhanced activity of this enzyme found during HIV infection. .

Analyses of CD235a+CD71+ pre-erythroid cells in HIV infected people and their function can give a new light in understanding the pathomechanisms of HIV infection. These cells develop during the process of erythropoiesis, the process that is significantly changed

during HIV infection and additionally complicated by HCV mediated co-infection. Anemia developing in many HIV infected and HIV/HCV co-infected people is a co-morbidity during which the presence of pre-erythroid cells and their potential immunoregulatory influence can be most profound. Mechanisms through which these cells were demonstrated to express immunosuppressive activity, that is production of arginase, can be contradictory to expected anti-inflammatory effects during HIV infection since the activity of this enzyme was found to correlate with increased progression of the disease. However, there are no studies that would explain the contribution of CD235a+CD71+ pre-erythroid cells in these processes and whether arginase derived from them is specifically affecting the disease progression.

HIV infection progresses accompanied by immune cell activation and inflammatory reactions, that are present even in subjects whose plasma levels of the virus is undetectable Confirmation of the presence of pre-erythroid cells in peripheral blood of HIV infected people and of their immunoregulatory/inflammatory activity may help to understand the pathomechanisms of HIV disease and lead to improved therapeutical approaches.

III. OBJECTIVES

General objective

The general goal was to examine the participation and the role of human CD235a+ CD71+ pre-erythroid cells in HIV infection and inflammation caused by this virus.

Specific tasks

To meet the goal the following tasks were undertaken:

a) Assessment of the presence of CD71+CD235a+ cells among PBMCs of HIV and HIV/HCV infected people, umbilical cord and healthy controls

b) Analyses of the determinants of the presence of CD235a+CD71+ pre-erythroid cells in HIV and HIV/HCV infected people.

c) In vitro analyses of immunosuppressive activity of CD71+ pre-erythroid cells isolated from PBMCs of umbilical cord and HIV infected people

d) Analyses of arginase activity in serum and PBMCs of tested groups and expression of mRNA for Arg1 to evaluate potential mechanism of immunosuppressive activity of pre-erythroid cells.

IV. MATERIALS and METHODS

Study population

In the cross-sectional study participated 64 HIV infected patients, 34 HIV/HCV-co-infected who were referred to the Clinic of Sacco Hospital, Milan, Italy. HIV infected patients were naive from anti-retroviral therapy whereas HIV/HCV co-infected patients were during ART but were not on anti-HCV therapy. Control group included 11 not infected healthy volunteers. In the study participated 7 mothers at delivery, from who was obtained peripheral blood and the same material from umbilical cord. Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

HIV and HCV infection diagnostic tests

HIV and HCV infection was confirmed by detection of viral RNA by means of PCR. HCV genotype was assessed by reversed hybridization kit Inno-LiPA HCV II (Innogenetics, Belgium). Leukocyte, erythrocytes and platelet counts were performed by routine test. Absolute counts and percentages of CD4 T cells were assessed by means of cytofluorymetry. Biochemical analyses included assessment of plasma levels of alanine transaminase (ALT), aspartat transaminase (AST), total bilirubin, creatinin, prothrombin time, international normalized ratio (INR) were performed by standard methods.

Liver disease analyses

To assess the severity of liver disease in HIV/HCV co-infected patients score of model of end stage liver disease (MELD) and score of the AST to platelet ratio index (APRI) were calculated using following formula: for MELD- $3.78\times\ln[\text{serum bilirubin (mg/dL)}] + 11.2\times\ln[\text{INR}] + 9.57\times\ln[\text{serum creatinine (mg/dL)}] + 6.43$; for APRI: (AST(IU/L)/upper normal

limit)x100/platelets(10^9/L)). Liver fibrosis was also assessed by means of transient elastography (Fibroscan) and reported in the scale of advancement of disease from F1 to F4, with F4 indicating liver cirrhosis.

Cell preparations

Peripheral blood was drawn on the EDTA as an anticoagulant and processed immediately. Peripheral blood mononuclear cells (PBMCs) were isolated by means of centrifugation over the gradient of Biocol of density 1,077 g/L (Euroclone, Pero, Italy) according to the standard procedure. Four parts of peripheral blood diluted twice with phosphate-buffered saline (PBS) were overlaid on 3 parts of Biocol and tubes were centrifuged for 30 min at 400 x g at room temeprature. Aspirated from the interphase PBMCs were washed twice in PBS by pelleting using centrifugation and re-suspending in PBS. After last wash cells were counted in hemocytometer chamber. Freshly isolated PBMCs were used for flowcytometry analyses, separation of CD71+ cells, and cell culture. For gene expression analyses from PBMCs, part of the cells were resuspended in RNA protect (Qiagen, Hilden, Germany) and stored at -80^0C.

CD71 positive cells were isolated directly from mononónuclear cells of umbilical cord and PBMCs of adults by use of biotin labeled anti-CD71 antibodies (eBioscience, San Diego USA). PBMCs were incubated with biotin labeled anti-CD71 antibody (eBioscience, San Diego USA) for 15 min at 4^0C, washed twice with PBS and incubated with pre-washed streptavidine coated beads (Thermo Fisher Scientific, Waltham, USA) for 30 min at 4^0C. Isolated CD71+ cells were stored at -80^0C in RNA protect (Qiagen, Hilden, Germany) for further use. The same procedure was used to deplete CD71+ cells from PBMCs for *in vitro* stimulation experiments.

Flow cytometry

For flow cytometric analyses, $1x10^6$ PBMCs were subjected to 30 minute incubation at 4^0C with antibodies directed against CD235a (PE) and CD71 (FITC) (eBioscience, San Diego USA) or CD3 (PC7), CD8 (FITC), and CD38 (PE) or CD3 (PC7), CD4 (PC5), CD8 (FITC), and

CD69 (ECD) (Beckman Coulter, USA). Controls consisted of cells unstained or incubated at the same conditions with isotype controls. Cells after incubation were washed twice in cold PBS containing 0,04% of sodium azide and 0,1% of bovine serum albumin and resuspended in 2% para – formaldehyde for analyses on flow cytometer (F500, (Beckman Coulter, Brea, USA). Cells were gated on PBMCs based on forward and side scatter and the expression of the receptors was performed using CXP analyses software (Beckman Coulter, Brea, USA).

***In vitro* stimulation studies**

To investigate the effect of pre-erythroid cells on CD69 expression on T cells, PBMCs were depleted of CD71+ cells as described before. The same patients' whole PBMCs and those which were depleted of CD71+ erythroid cells were stimulated or not with PHA (1μg/ml) for 18 hours. All cultures were performed using RPMI1640 supplemented with 10% of FBS, and antibiotics (penicillin at 50IU /ml, Streptomycin at 50 μg/ml) (Euroclone, Pero, Italy) in 24 well plates (Nunc, Roskilde, Danmark). Cells during the stimulation were kept in incubator at humidified atmosphere containing 5% of CO_2 at 37^0C. After the culture, cells were washed and stained with appropriate antibodies, according to the flow cytometry methodology.

Analyses of sCD163, IL-6, and erythropoietin plasma levels.

Soluble CD163 was assessed in plasma by ELISA test (eBioscience, San Diego USA) according to the protocol of manufacturer. The sensitivity limit of the test was 0,3125 ng/ml. Erythropoietin plasma levels were determined by ELISA test (Abcam, Cambridge, UK) according to the protocol of manufacturer. The sensitivity of the test was 0,4 mIU/ml. Interleukin-6 in plasma was measured by use of ELISA high sensitivity kit (Abcam, Cambridge, UK) . The sensitivity of the test was 0,81 pg/ml.

***EKLF, Arg1* gene expression analyses**

Each sample of $5x10^6$ PBMCs were stored in solution of RNA protect (Qiagen, Hilden, Germany) at -80^0C till the moment of RNA extraction. RNA was extracted by use of Absolutely RNA kit (Agilent, Santa Clara, USA) according to the protocol provided by supplier that included step of DNA-se digestion. Final elution of RNA was performed with buffer supplied by manufacturer (tris-EDTA solution). RNA was stored at -80^0C till the moment of cDNA synthesis. Quantity and purity of RNA (A260m,/A280nm) was measured using Tecan Infinite 200Pro (Tecan Austria GmbH, Grodig, Austria) on the same day of cDNA synthesis. For this purpose 2uL of 10nM tris-EDAT as a blank and 2 uL of RNA afterwards was placed in indicated spots of nanoquant plate and measurements were performed using Tecan i-control software. cDNA was synthesized from 20 ng of total RNA using AffinityScript QPCR cDNA synthesis kit (Agilent, Santa Clara, USA) with slight modification of the protocol of manufacturer. In specific, random primers were used to anneal at 25^0C for 10 min followed by 45 min incubation at 42^0C of cDNA synthesis. The reaction was terminated by incubation at 95^0C for 5 min. and cDNA was stored at -20^0C. Expression of *EKLF* and *Arg1* was performed by analyses of absolute copy gene numbers using digital droplet RealTime-PCR (ddRT-PCR, Bio-Rad, Hercules, USA). Primers' sequences for *EFKL* were as follow: Forward primer *5'–CCTGTTTGGTGGTCTCTTCACA-3'*; Reverse *5'-AGGGTCCAT TCGTGGGAAA-3*', for *Arg1:* Forward primer *5'-GTTTCTCAAGCAGACCAGCC*; Reverse *5'-GCTCAAGTGCAGCAAAGAGA*, and for *IL-6:* Forward primer *5'–TCTCCACAAGCGCCTTCG*; Reverse *5'-CTCAGGGCTGAGATGCCG*. Total volume of 20 uL of ddRT-PCR reaction mixture was composed of 10 uL of QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad, Hercules, USA), 500 nM of forward and reverse primers each, and 1uL of cDNA. The ddRT-PCR reaction mixture, together with EvaGreen droplet oil at volume of 70 uL were applied to 8 well cartridge and placed in droplet generator. Created droplets for each sample were transferred to 96-well plate, sealed and placed in T100 thermal cycler (Bio-Rad, Hercules, USA). The following

conditions of PCR were used :95^{0}C;10 min; followed by 35 cycles of 95^{0}C for 30 sek; 60^{0}C for 30 sek with temperature ramp at 2,5^{0}C/s. Absolute copy counts of *EKLF, Arg1, and IL-6* were determined for each sample by droplet reader (Biorad, Hercules, USA) and use of software included with the instrument. Each cDNA sample was run in duplicates and average results show gene copy number per 20uL.

Arginase activity

Arginase activity in plasma was analyzed using commercially available kit (Sigma-Aldrich, Saint Louis, USA). This colorimetric method measures the activity of arginase to catalyze the conversion of arginine to urea and ornithine. The detection limit of the test is 0,3 unit/L in period of 2 hours. Prior to the measurements plasma was depleted from urea using 10kDa Molecular wight cut-off filters (Amicon Ultra centrifugal filters, Ultracel -10K, Merck Millipore Ltd, Tullagreen. Ireland). For this purpose 100 uL of plasma and 400 uL of water were placed inside the cut-off filter and centrifuged at 14,000 x g for 30 min at room temperature. The flow-trough was discarded and to remaining plasma inside the cut-off filter was added 500 ul of water and centrifugation was repeated at the same conditions. volume of plasma remaining in cut-off filter was measured and adjusted with water to amount for total of 80 uL. Plasma depleted of urea was used immediately for arginase activity. To test for it, to each sample of plasma placed in 96-well plate, except blank wells, was added substrate that contained arginine buffer and solution of manganese. Standard consisted of urea solution at 1 mM concentration and water as a blank. The samples were incubated for 2 h at 37^{0}C on shaker. The reaction was stopped by adding urea reagent to all wells. Additionally to blank wells for plasma was added the same volume of substrate that was used in tested wells. The plate was left for 1 h at room temperature on shaker. Due to the turbidity that was developing in plasma after addition of urea all samples and their blanks were centrifuged for 5 min at 13,000 x g at room temperature and

afterwards 200 μl of each was transferred to new wells for measurements. The arginase activity was observed by changes in absorbance read at 430 nm. and calculated using following formula:

$$\text{Arginase activity} = \frac{(A430)\text{sample} - (A430)\text{blank}}{(A430)\text{standard} - (A430)\text{water}} \times \frac{(1\text{mM} \times 50 \times 10^3)}{(V \times T)}$$

1mM - concentration of urea standard
50 - reaction volume
V - sample volume added to well
T - time of the reaction

Arginase activity is expressed in units/L where 1 unit of Arginase is the amount of enzyme that converts 1 umole of L-arginine to ornithine and urea per minute at pH 9.5 and 37^0C.

Statistical analyses

For normally distributed variables and comparison of multiple groups was used one-way analyses of variance and Student *t* test to compare data between two groups. For not normally distributed data were used Kruskal-Wallis and Mann-Whitney tests. Association analyses were performed by Spearman's rank test. All tests were two-sided and P-values less than 0.05 were considered to be statistically significant. Multiple linear regression analyses were performed to predict the presence of pre-erythroid cells with selected in the model predictors specific for tested group. Statistical analysis was performed using Wizard for Mac, by Evan Miller, Version 1.8.20

V. RESULTS

1. **Demographic and clinical characteristics of HIV infected and HIV/HCV co-infected patients.**

All patients in HIV group were chronically infected, naive from ART with the range of time of infection from 2 to 8 years. HIV/HCV co-infected patients and HIV infected patients on ART were treated with combination therapy that did not include the use of zidovudine, medicine which frequently causes anemia. HIV/HCV co-infected patients failed to respond to peg IFN

+ribavirin treatment and their biological material was obtained minimum 1 year after the therapy interruption. General characteristics of patients and immunovirological status of studied groups are shown in **Table 1**. The distribution of demographic parameters, age and sex, between groups was not significantly different, although, majority of patients in 3 groups of infected patients were males. The absolute counts of CD4 T cells, and their frequencies were not statistically different between groups. In group of HIV/HCV co-infected patients were observed on average higher concentrations of plasma bilirubin and lower number of platelets in comparison to other groups causing significant difference in the distribution of these parameters (p=0,002 and p=0,012 respectively). On average, hemoglobin levels were in norm and not significantly different between groups. In HIV infected mean concentration of Hb was13,91 g/dL with fourteen patients in a range from 7,5 to 13 g/dL. Mild and moderate level of anemia in these patients was asymptomatic. In HIV/HCV co-infected group, mean concentration of Hb was 14,6 g/dL with 6 patients in the range between 11-12,6 g/dL. As in case of HIV patients also in those anemia was asymptomatic.

Table1. Clinical and immunovirological characteristics of patients

Parameter	HIV (n=64)	HIV/HCV (n=34)	HIV ART (n=10)	Healthy (n=11)	p- value
Age (years)	40,4±3	41,2±2,0	42±7,2	36,1±7,5	> 0,05
Gender (% female)	28	21,1	33	45,5	> 0,05
HIV VL (copies of RNA/mL)	67973±42658	<37	<37	0	
HCV VL (RNAx10^3 IU/mL)	0	2087151±1396390	0	0	
CD4 [cells/μL],	518±263	658,8±156	768±355	687±95	> 0,05
[%]	36,1±19,6	31,9±4,2	36,6	63±9,3	> 0,05
Hemoglobin [g/dL]	13,91±0,71	14,6±0,65	15,3±1,01	14,9±0,81	> 0,05
Platelets [10^3/μL]	222880±19127	158792±33230	256777,8±63743	210171±93500	p=0,012
Bilirubin	0,57±0,17	1,58±0,42	0,56±0,18	0,69±0,27	p=0,002

Out of the group of HIV/HCV co-infected patients, 27 were examined for the presence of fibrotic changes in the liver. In **Table 2** are demonstrated results of transient elastography examinations, and corresponding to each stage of fibrosis scores of MELD and APRI. In general, scores of MELD and APRI followed the stages of liver fibrosis determined by fibroscan, especially at the level F3-F4.

Table 2. Liver fibrosis analyses in HIV/HCV co-infected patients

Fibrosis Stage:*		**MELD	***APRI
No/mild fibrosis	F1 (n=6, 21,4%)	6 (n-3); 7 (n-3)	0,75±0.68
Significant Fibrosis	F2 (n=2, 3,6%)	7 (n-1); 8 (n-1)	1,02±0,85
Advanced Fibrosis	F3 (n=3, 14,3%)	7 (n-3)	1,58±0,24
Cirrhosis	F4 (n=16, 57.2%)	7, (n-2);8 (n-2) 9,(n-3);10 (n-3), 11 (n-3); 12, (n-1);14 (n-1); N-1 ND	2,67±1,82

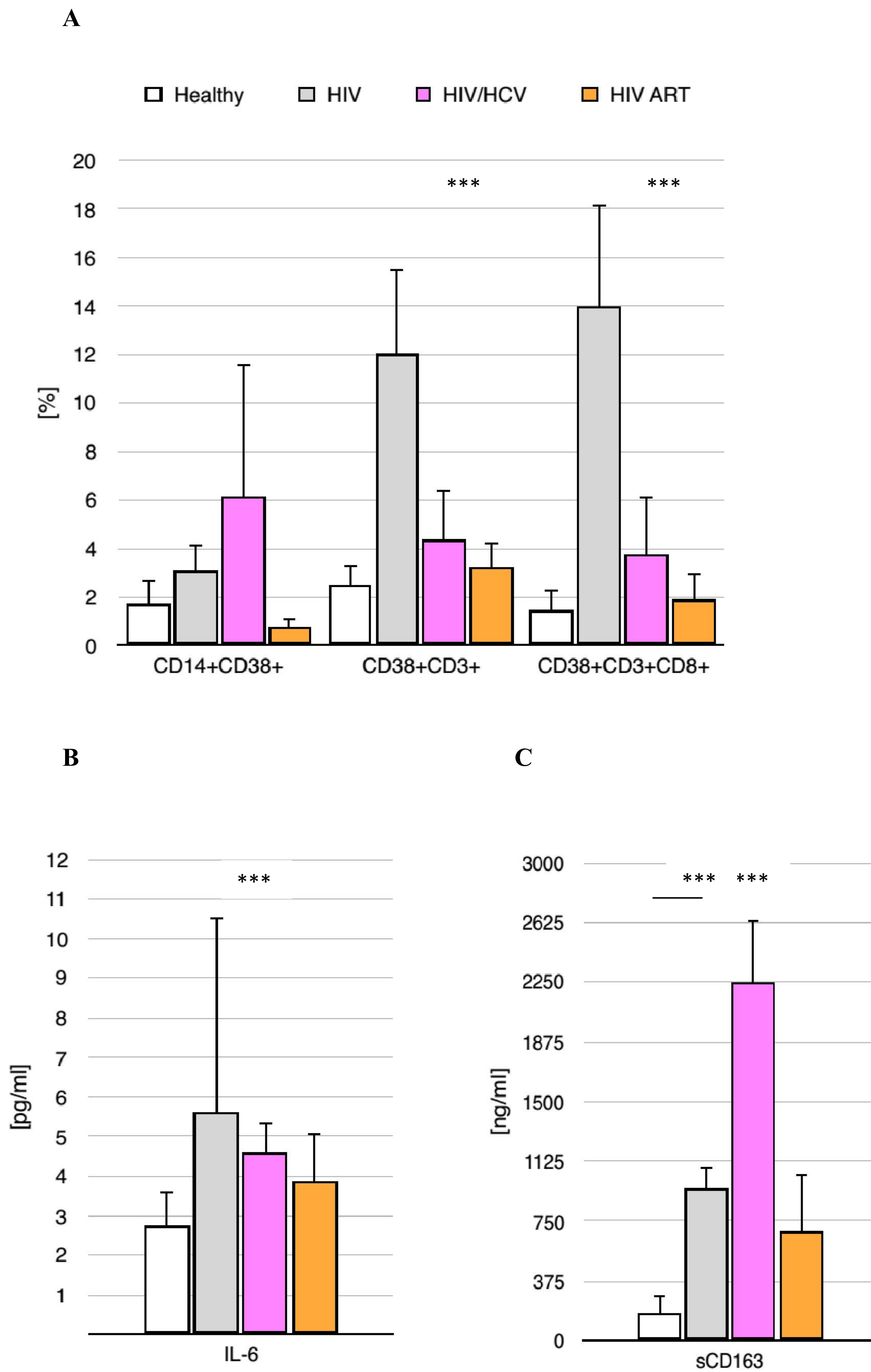
* determined by transient elastography (Fibroscan).

** Model of end stage liver disease score- 3.78×ln[serum bilirubin (mg/dL)] + 11.2×ln[INR] + 9.57×ln[serum creatinine (mg/dL)] + 6.43.

*** The AST to platelet ratio index (APRI) was calculated based on formula: (AST(IU/L)/upper normal limit)x100/platelets(10^9/L)).

To evaluate the immune activation/inflammation status of the studied groups, the expression of CD38 on subpopulations of PBMCs and plasma concentrations of soluble markers, IL-6, sCD163 were measured (**Figure 1**). The expression of CD38 on CD14+ monocytes although higher in HIV infected and HIV/HCV co-infected groups in comparison to healthy controls and HIV ART group was not significantly different. Expression of CD38 on CD3+ T cells was significantly higher in group of HIV infected in comparison to healthy control group and HIV/HCV co-infected as well as HIV ART (p=0,002; p<0,001;p<0,001 respectively). Similar differences between groups were observed also for the expression of CD38 on subpopulation of CD8+ CD3+ T cells (p<0,001;p<0,001,p<0,001 respectively). In case of HIV/HCV co-infected patients the expression of CD38 on CD3+ T cells or CD38+ CD3+ T cells was not significantly different from healthy control group or HIV ART patients. The plasma concentrations of IL-6, although the highest in HIV group, were not significantly different against any of the tested groups (**Figure 1B**). However, in HIV/HCV co-infected, IL-6 concentrations were significantly higher in comparison to healthy controls only (p<0,001). Concentrations of sCD163 in plasma were found to be at significantly different levels between tested groups (**Figure 1 C**; Kruskal-Wallis, p < 0,001). The highest concentrations of this soluble receptor were observed in plasma of HIV/HCV co-infected patients. In this group sCD163 levels were more than 10 times higher than in healthy control group (p<0,001) and more than twice higher than in HIV infected (p<0,001) and HIV on ART (p<0,001). The concentrations of this soluble scavenger receptor in plasma were also significantly higher in HIV infected group in comparison to healthy controls (p<0,001) but not HIV ART group of patients.

Figure 1. Cell activation markers and soluble markers of inflammation in studied groups.



To better characterize the groups tested, associations between immunovirological parameters and the parameters of immune activation and inflammation were estimated **(Table 3)**. In HIV infected patients viral loads were inversely correlated with absolute CD4 T cell counts (p=0.012), positively correlated with the frequency of T cells and monocytes expressing immune activation marker; CD38 (p<0,001; p=0,002, respectively), and with plasma concentrations of inflammation marker - soluble CD163 (p=0,014). In the same group of patients CD4 T cell counts were inversely correlated with the frequency of CD38+ T cells and CD38+ monocytes (p<0,001; p=0.023, respectively) and plasma concentrations of sCD163 (p=0.003). There were also a significant associations between the concentrations of sCD163 and expression of CD38 on CD3+ T cells and CD3+CD8+ T cells (p<0,001;p=0,002 respectively). In group of HIV/HCV co-infected patients (**Table 3**) there were no significant associations found for viral loads of HCV and cell activation and inflammation markers. In this group of patients were observed significant correlations between absolute counts of CD4 T cells, plasma concentrations of sCD163 and expression of CD38 on T cells and on subpopulation of CD8+ T cells, as indicated in the table 3. Additionally, sCD163 concentrations were significantly correlated with the concentrations of IL-6 in this group of patients (p<0,001) but not in HIV monoinfected.

Table 3. Associations between immunovirological parameters with the immunological activation/inflammation covariates in tested groups.

Parameter	Covariate	Correlation	R2	P-value
	HIV infected			
HIV VL [copies of RNA/mL]	CD4 [cells/μL]	-0.386	0.149	0.012
	CD14+CD38+ [%]	0.493	0.243	0.002
	CD3+CD8+CD38+ [%]	0.68	0.463	<0,001
	sCD163 [ng/ml]	0.399	0.159	0.014
CD4 [cells/μL]	CD14+CD38+ [%]	-0.368	0.135	0.023
	CD3+CD8+CD38+ [%]	-0.58	0.337	<0,001
	sCD163 [ng/ml]	-0.468	0.219	0.003
sCD163 [ng/ml]	CD38+CD3+ [%]	0.506	0.256	<0,001
	CD3+CD8+CD38+ [%]	0.421	0.178	0.002
	HIV/HCV co- infected			
CD4 [cells/μL]	CD38+CD3+ [%]	-0.39	0.152	0.033
	CD3+CD8+CD38+ [%]	-0.408	0.166	0.027
sCD163 [ng/ml]	CD38+CD3+ [%]	0.465	0.216	0.006
	CD3+CD8+CD38+ [%]	0.506	0.256	0.003
	IL-6 [pg/ml]	0,636	0,405	<0,001

2. CD235a+CD71+ pre-erythroid cells are present in peripheral blood of HIV and HIV/HCV co-infected patients and their frequency correlates with the expression of *EKLF*.

Cellular analyses

The presence of pre-erythroid cells among PBMCs was examined in HIV and HIV/HCV infected patients, in uninfected healthy controls, and PMCs isolated from umbilical cord blood. In **Figure 2** are shown dot plots representative for each sample in the group demonstrating expression of CD235 and CD71 receptors. The highest frequencies of CD235a+ and CD71+ cells were always found among PBMCs obtained from umbilical cord giving highest mean frequency of CD235a+CD71+ pre-erythroid cells **(Figure 3).** In HIV/HCV co-infected and HIV mono-infected patients frequency of these cells was significantly ($p<0.001$) lower than in cord blood but higher in comparison to healthy controls and HIV ART patients, although not at the statistically significant level.

Figure 2. Flowcytometry quadrant plots demonstrating cells of PBMCs co-expressing pre-erythroid markers : CD235a and CD71

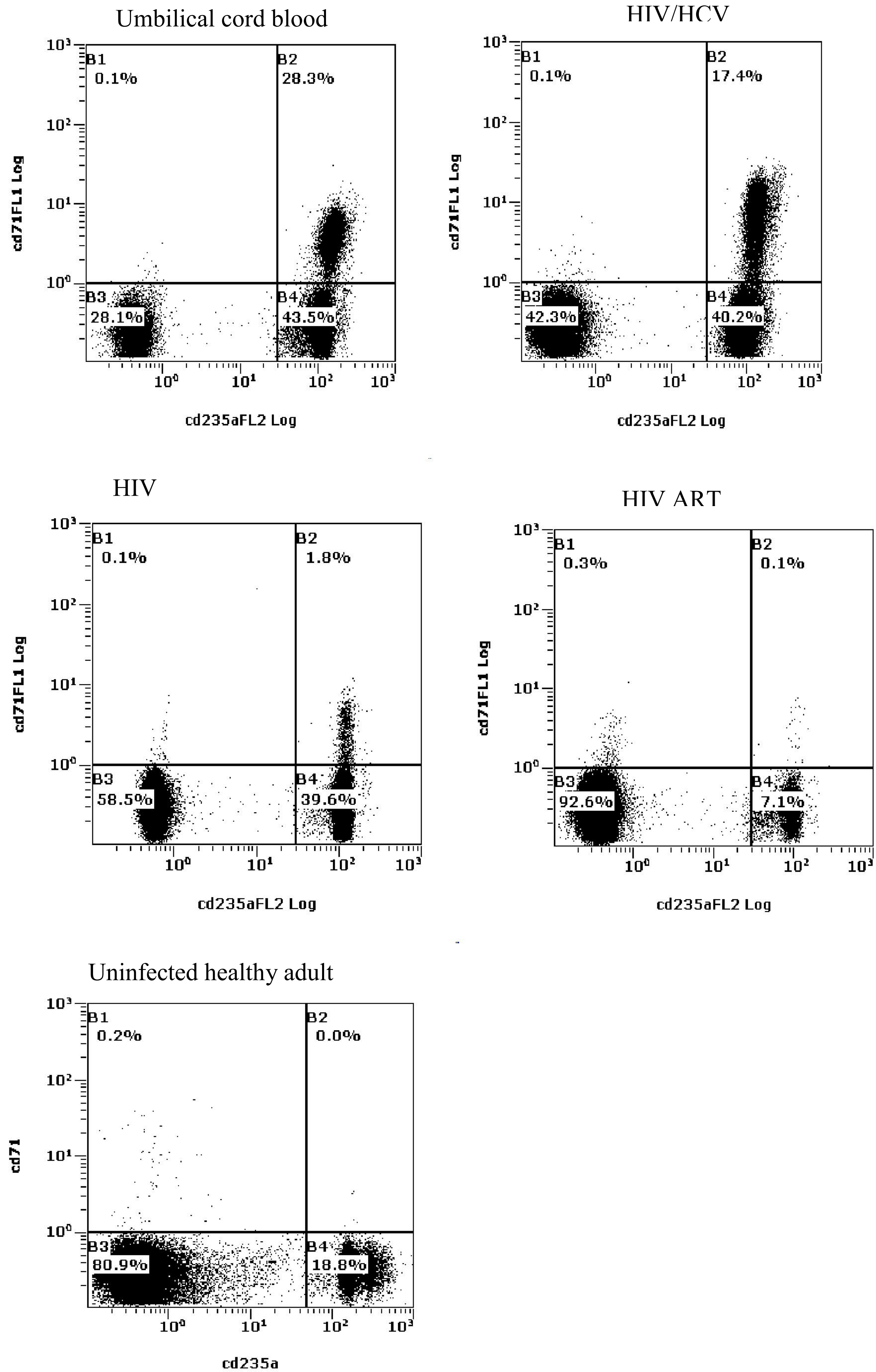
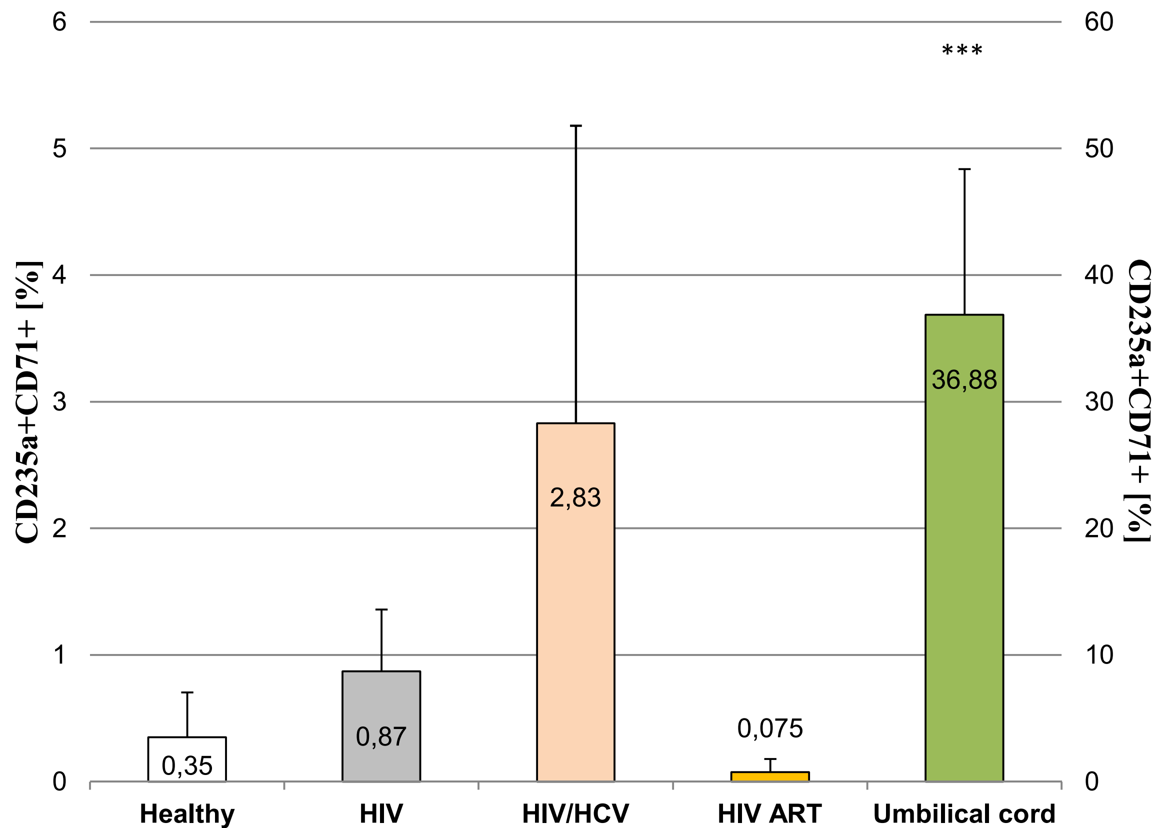


Figure 3. CD235a+CD71+ pre-erythroid cells among PBMCs of adults of tested groups and among mononuclear cells of cord blood.



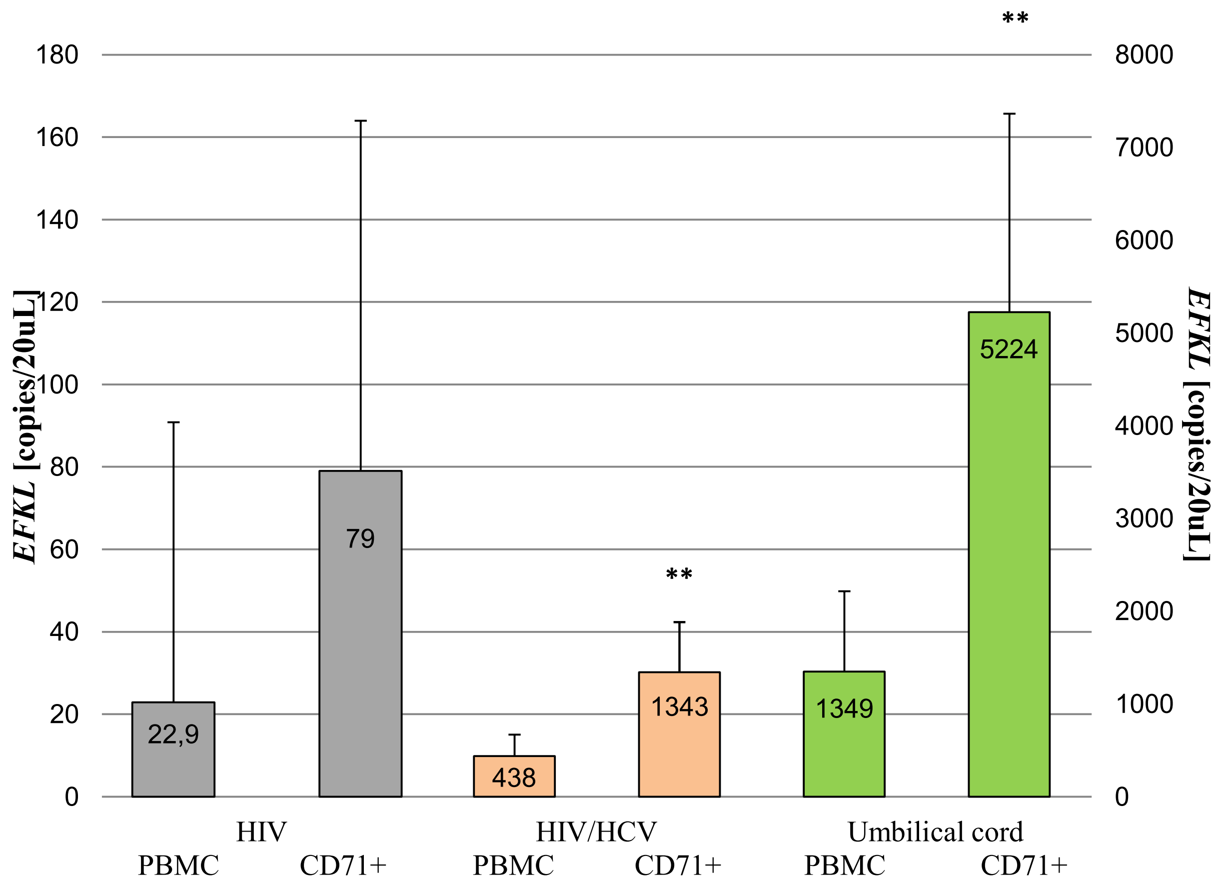
***- (Kruskal-Wallis p<0,001)

Molecular analyses

To ascertain the specificity of observations based on the cell surface markers in determination of pre-erythroid cells, expression of gene for transcription factor – *EKLF,* was measured. This factor is uniquely expressed in erythroid cells and regulates many genes important in their development. It was hypothesized that the gene expression of this erythroid specific transcription factor in PBMCs should follow the findings

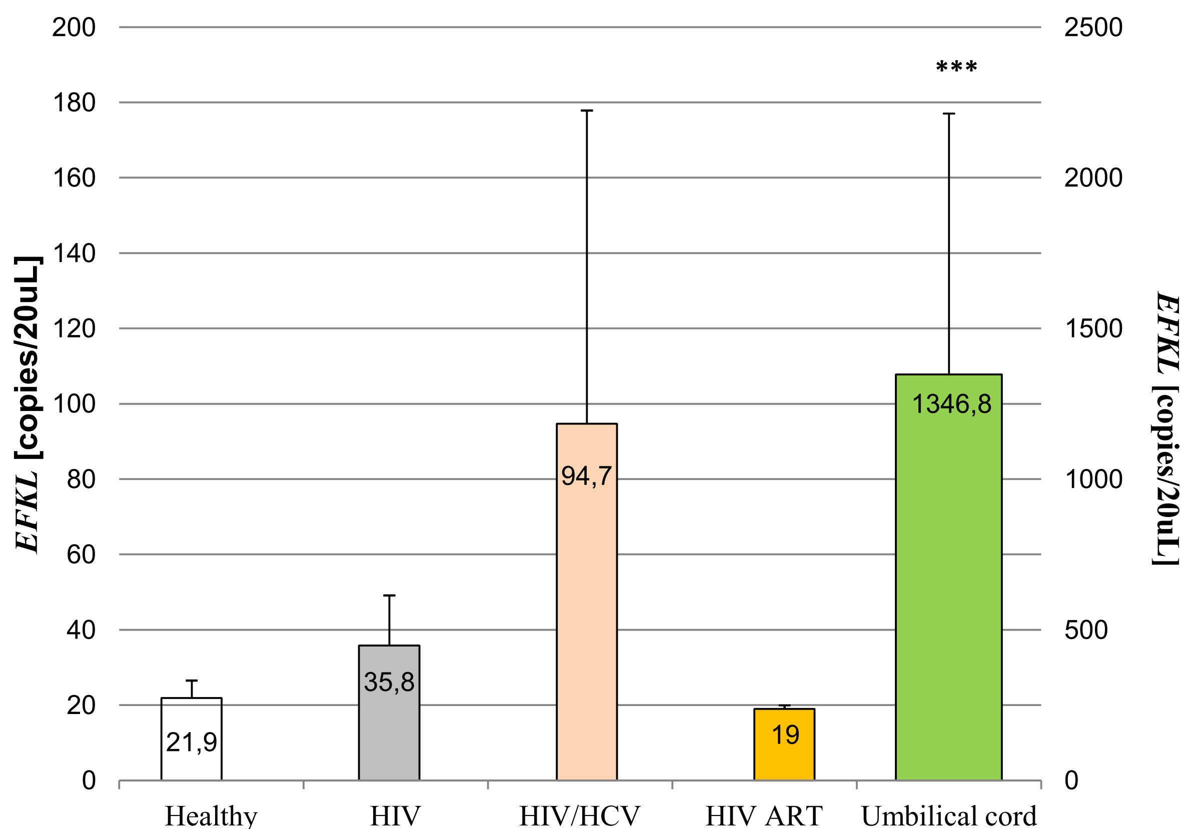
performed at the cellular level. For this reason, from PBMCs were isolated CD71+ cells, and the expression of *EFKL* was compared between separated and all PBMCs of the same sample. In positively selected CD71+ pre-erythroid cells from umbilical cord was observed significantly higher expression of *EKLF* (paired t-test, p=0,008) than in non separated PBMCs (**Figure 4**). Similar effect was observed in case of cells derived from HIV/HCV co-infected patients (paired t-test, p=0,009). Moreover, the expression of EKLF in PBMCs of HIV/HCV co-infected patients correlated significantly (p=0,049) with the frequency of CD235a+CD71+ pre-erythroid cells. In group of HIV infected patients, the expression of *EKLF* was on average 3 times higher in isolated, CD71+ cells in comparison to PBMCs but due to high deviation from the mean this difference was not statistically significant. But, statistically significant correlation (p=0,009) between absolute *EKLF* gene copy numbers and the frequency of

Figure 4. Expression of *EFKL* in PBMCs and in isolated, CD71+ positive cells obtained from 3 different groups of patients.



CD235a+CD71+ pre-erythroid cells in peripheral blood of this group of patients was observed. Analyses of the differences in the *EKLF* gene copy numbers between PBMCs of HIV infected patients, healthy volunteers, and PBMCs of HIV ART, and umbilical cord resemble to those seen at the level of cellular analyses of expression of CD235a and CD71 receptors (**Figure 5**). Significantly higher copy numbers of *EFKL* expression in PBMCs of umbilical cord in comparison to healthy controls and 3 groups of infected patients ($p < 0{,}001$) were observed. The expression of this gene in HIV infected patients and HIV/HCV co-infected in comparison to healthy controls, although higher, was not statistically significant (**Figure 5**).

Figure 5. ***EFKL*** **expression in PBMCs of examined groups**



3. CD235a+CD71+ pre-erythroid cells in HIV/HCV co-infected patients with liver fibrosis, and their association with plasma concentrations of sCD163 in HIV/HCV and HIV infected patients.

In group of HIV/HCV co-infected patients there were 16 people suffering from liver cirrhosis confirmed by fibroscan analyses. In tested group of HIV/HCV co-infected patients MELD and APRI values as a markers of liver fibrosis were significantly higher in liver cirrhotic patients. (**Table 4**; p <0,001 for both). Liver cirrhosis in these patients was marked also with significantly higher plasma levels of EPO (p=0,01) and two pro-inflammatory factors, sCD163, and IL-6 (p <0,001, p= 0,004, respectively). Although in liver cirrhotic patients the frequency of CD235a+CD71+ pre-erythroid cells was higher in comparison to non cirrhotics, the difference was not statistically significant (p=0,006).

Table 4. Comparison between liver cirrhotic and not cirrhotic patients of HIV/HCV co-infected group in selected parameters.

Parameter	Liver cirrhotic (n=16)	No liver cirrhotic (n=18)	Significance* (p-value)
MELD	9,73±1,06	6,8±0,45	<0,001
APRI	2,67±0,97	0.78±0,43	<0,001
sCD163 [ng/ml]	2775+420	1607±512	<0,001
EPO [mIU/ml]	12,52±9,5	4,0±1,38	0,01
IL-6 [pg/ml]	5,38±0,83	3,49±1,06	0,004
CD235a+CD71+ [%]	5,22±4,75	0,25±0,27	0,06

* Mann-Whitney

It has been suggested before that sCD163 is a marker of liver fibrosis in HCV as well as in HIV/HCV co-infected people. This study confirms previous reports. In the group of HIV/HCV co-infected the mean plasma concentration of sCD163 was significantly higher in liver cirrhotic patients in comparison to those without liver cirrhosis (2,775 ng/ml; 1607 ng/ml; respectively; Mann-Whitney, p < 0,001). The concentrations of this soluble receptor in multivariate regression model were found to be significantly associated with liver cirrhosis and the frequency of CD38+ CD8+ T cells but not with plasma concentrations of IL-6 or the frequency of pre-erythroid cells (**Figure 6**). Included in the regression model, HCV viral loads did not show any association. sCD163 is also regarded as an important marker of inflammation related to increased leakage of bacterial antigens from the gut in HIV infected patients. To test if CD235a+CD71+ pre-erythroid cells associate with this pro-inflammatory marker, multivariate regression analyses were performed. In this model were included: CD38 expression on T cells, absolute count of CD4 T cells, and HIV viral load, In HIV patients sCD163correlated significantly with the frequency of CD38 positive CD8+ T cells (**Figure 7**). but the association with other factors, including the frequency of CD235a+CD71+ pre-erythroid cells was not statistically significant.

Figure 6. Detereminants of plasma levels of sCD163 in the group of HIV/HCV co-infected patients.

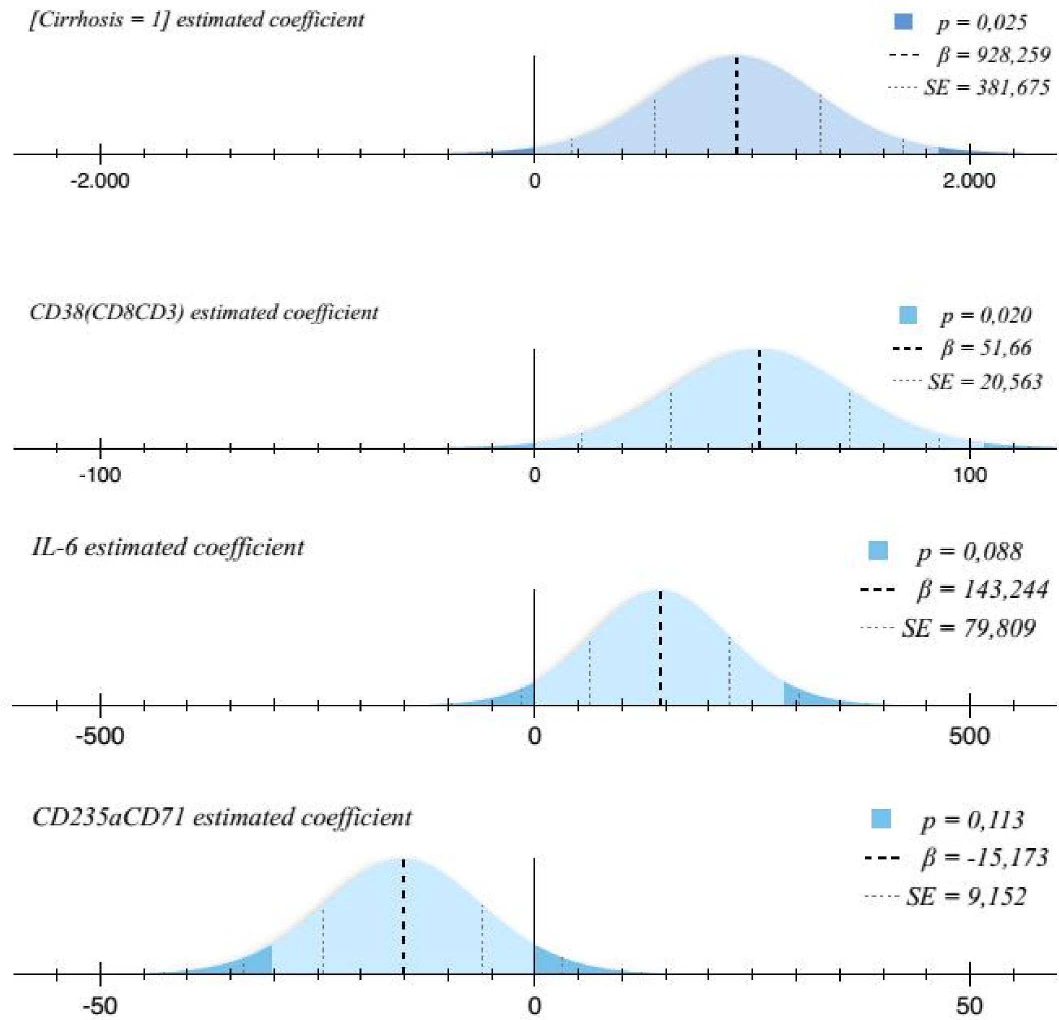
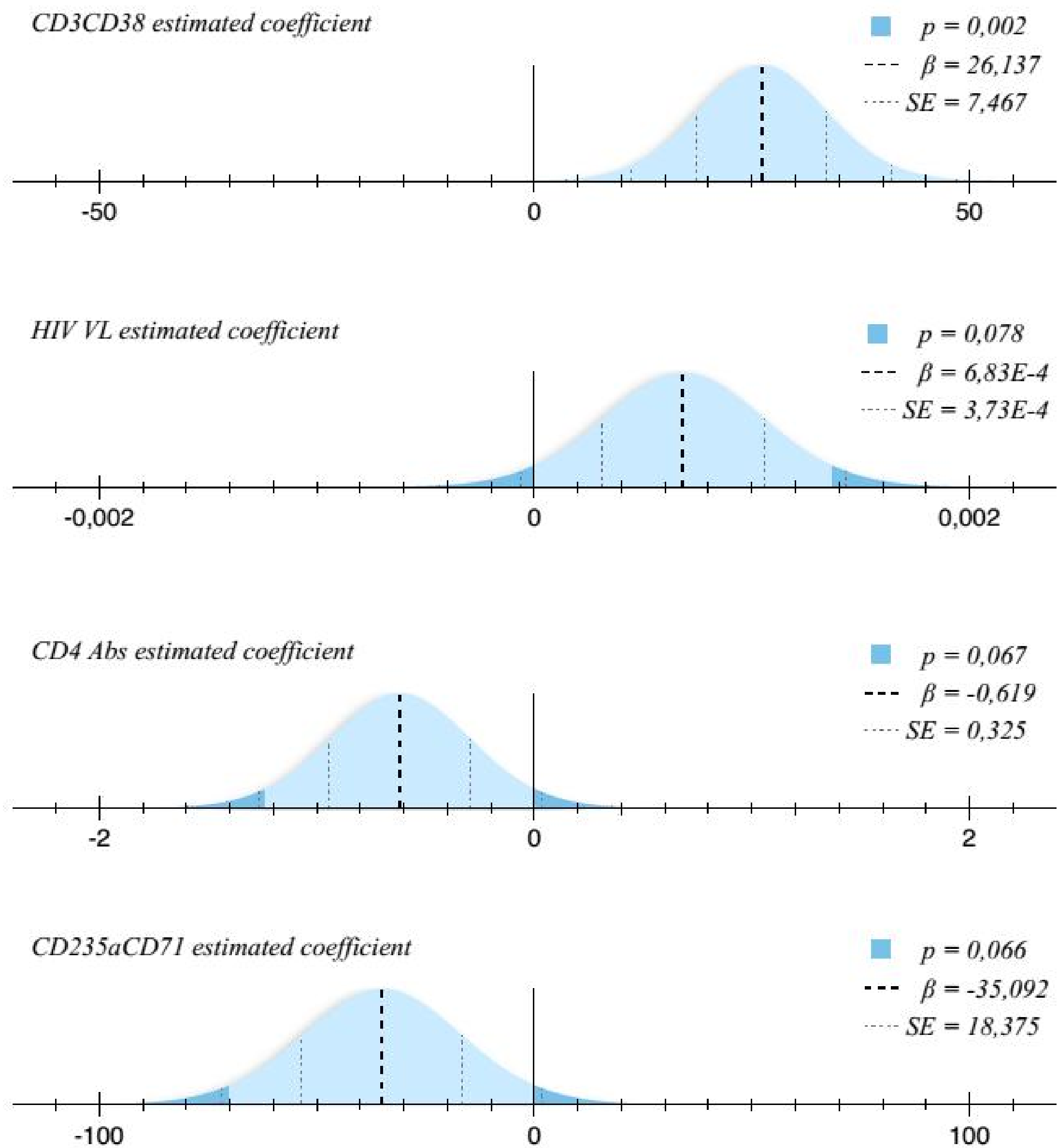


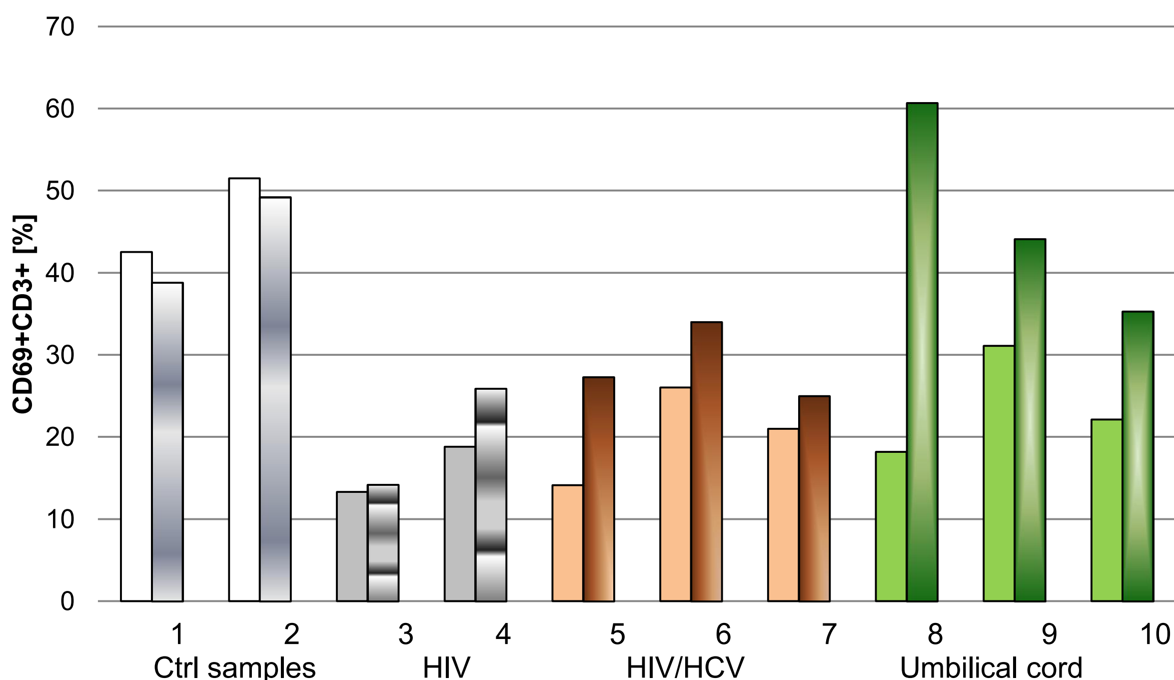
Figure 7. Detereminants of plasma levels of sCD163 in the group of HIV infected patients.



4. Activity of pre-erythroid cells.

To analyze the effect of pre-erythroid cells on immune activation samples with confirmed by flowcytometry presence of >5% of these cells in PBMCs were selected. Part of PBMCs of each sample were used to deplete CD71+ cells by magnetic beads. For each PBMCs used, the efficiency of CD71+ depletion was tested by means of flow cytometry and in none of the samples remained more than 0,02% of pre-erythroid cells. Obtained population of cells, PBMCs and PBMCs without pre-erythroid cells of the same patient, were stimulated with PHA and CD69 expression on CD3+ T cells was examined 18 hours later. In these experiments were used mononuclear cells of umbilical cord, PBMCs of HIV infected, and HIV/HCV co-infected adults. The depletion of CD71+ cells from PBMCs and analyses of CD69 expression on T cells after PHA stimulation were performed also with samples that contained minimal number of CD235a+CD71+ pre-erythroid cells (<0,1%) in PBMC's (Ctrl samples). This control served to ascertain that possible changes in the expression of CD69 are the result of the depletion of CD71+ erythroid cells rather than any other cell subpopulations expressing this receptor. Most apparent differences in frequency of T cells expressing CD69 between CD71+ depleted and not depleted cells after PHA stimulation were observed for the mononuclear cells of umbilical cord (**Figure 8**, samples No 8, 9, and 10). Similar effect, but much less pronounced, were observed in case of samples obtained from HIV infected and HIV/HCV co-infected patients. In case of HIV infected only one of the samples showed relatively apparent increase in CD69 expression when CD71+ pre-erythroid cells were depleted. The effect of increased expression of CD69 on T cells in samples depleted of CD71+ cells were more apparent in HIV/HCV co-infected samples (samples 5,6, and 7). Control samples (No 1 and 2) with low content of pre-erythroid cells (<0,05%) showed no effect of the depletion of pre-erythroid cells but the highest frequency of CD69+ T cells among PBMCs of adults.

Figure 8. The effect of the depletion of CD71+ cells on the expression of CD69 on T cells after *in vitro* stimulation.

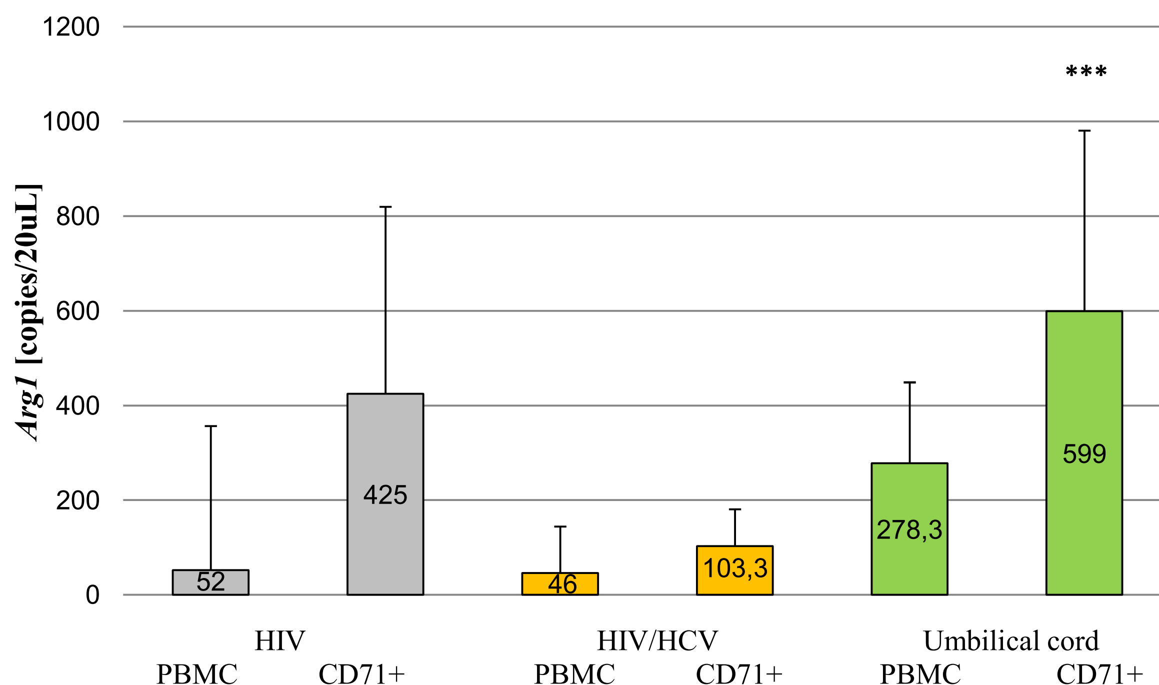


5. Arginase 1 gene expression and arginase activity in plasma of tested groups.

***Arg1* expression**

Anti-inflammatory activity of pre-erythroid cells was suggested before to be mediated by arginase. Similarly to the analyses of *EFKL* gene expression, also *Arg1* was evaluated in CD71+ pre-erythroid cells and compared to unseparated PBMCs of three groups. CD71 positive cells of umbilical cord and HIV infected and HIV/HCV co-infected patients demonstrated on average higher expression of Arg1 in comparison to PBMCs but the differences were statistically not significant (**Figure 9**; Paired t-test; p=NS). Comparison of *Arg1* expression in PBMCs of tested groups showed significantly higher expression of this gene in PBMCs from umbilical cord in comparison to other groups (**Figure 10**; p<0,001). *Arg1* expression in HIV infected patients was not significantly different from HIV/HCV co-infected patients nor both groups were different in this respect from healthy. Expression of Arg1 did not correlate with the percentage of CD235a+CD71+ pre-erythroid cells present in peripheral

Figure 9. Expression of *Arg1* in PBMCs and in isolated, CD71+ positive cells obtained from 3 different groups of patients.

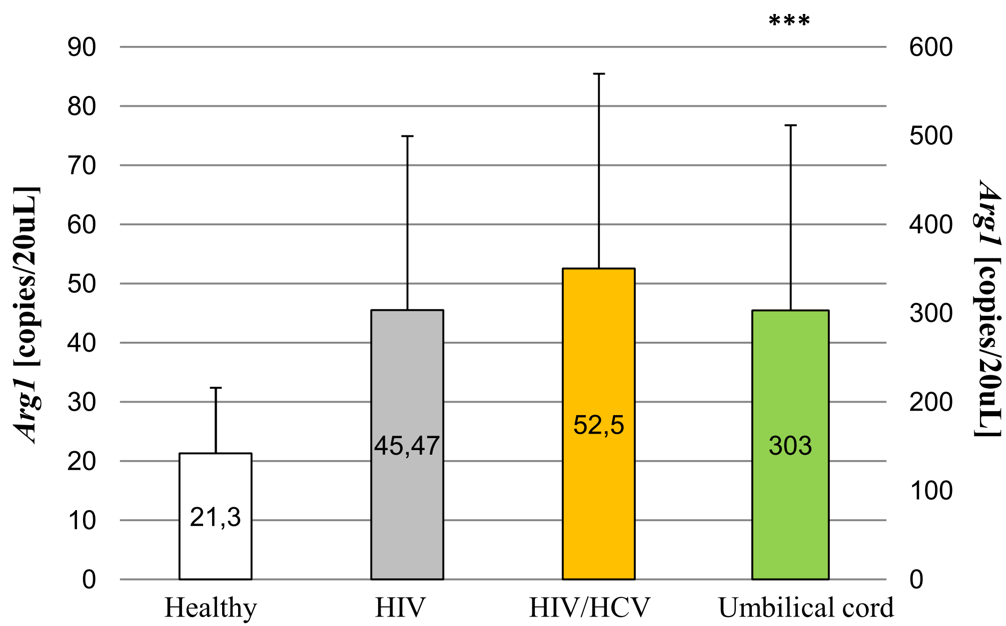


blood of HIV infected patients. However, in group of HIV/HCV co-infected patients *Arg1* correlated with the frequency of CD235a+CD71+ pre-erythroid cells (0,468;0,216;p=0,007), and was even more associated with the expression of *EKLF* (0,949;0,901;p<0,001). In the same group expression of *Arg1* was inversely correlated with Hb (-0,514;0,264;p=0,003) and frequency of CD4 T cells (-0,453;0,205;p=0,033).

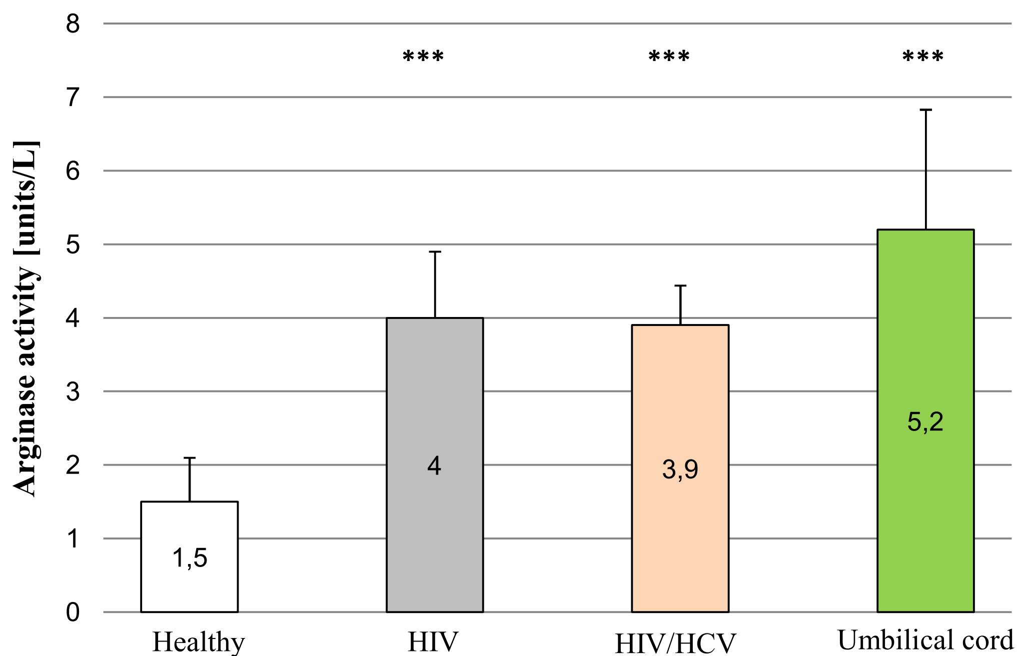
<u>Arginase activity</u>

Arginase activity in plasma was not different between the groups of HIV infected, HIV/HCV co-infected and plasma of umbilical cord blood but significantly different (p<0,001) in comparison to healthy controls **(Figure 11)**. The arginase activity in HIV infected group nor in HIV/HCV co-infected group was found to be associated with the frequency of

Figure 10. Expression of *Arg1* in PBMCs obtained from studied groups of patients.



.**Figure 11. Arginase activity measured in plasma of studied groups.**



CD235a+CD71+ pre-erythroid cells or *EKLF* expression in PBMCs. Activity of this enzyme was not found to be significantly correlated with any of the immunovirological factors examined in both groups.

6. Multivariate regression analyses for prediction of CD235a+CD71+ in HIV infected and HIV/HCV co-infected patients.

In multivariate analyses of prediction of the frequency of CD235a+CD71+ pre-erythroid cells were included patients of HIV and HIV/HCV co-infected patients, and selected covariates: age, sex, absolute count of CD4 T cells, expression of CD38 on CD8 T cells, plasma concentrations of IL-6 and Hb levels, expression of *Arg1* in PBMCs and plasma arginase activity.

These analyses demonstrated that frequency of CD235a+CD71+ pre-erythroid cells is significantly associated with belonging to the group of HIV/HCV co-infected patients (**Figure 12**), male sex, low counts of CD4 T cells, and low expression of CD38 on CD8+T cells. Most significant inverse correlation was observed with the levels of Hb . There was no significant effect of IL-6 protein levels , nor *Arg1* expression or arginase activity

In multivariate analyses on HIV/HCV co-infected group of patients were included additionally sCD163 and HCV VL as a covariates. In those analyses (**Figure 13**) only low Hb concentrations were significantly associated with the presence of pre-erythroid cells. Similarly to co-infected patients, in group of HIV infected, regression model in which was included HIV viral load, demonstrated most significant correlation between pre-erythroid cells and Hb blood levels. However, in contrast to HIV/HCV co-infected the frequencies of pre-erythroid cells were inversely correlated also with sCD163 plasma concentrations and male sex. No significant associations were observed in this group with IL-6, CD4 absolute counts, or expression of CD38 on CD3CD8, nor with *Arg1* gene expression or arginase activity. (**Figure 14**)

Figure 12. Multivariate regression model on the determinants of the frequencies of CD235a+CD71+ pre-erythroid cells in HIV infected and HIV/HCV co-infected patients.

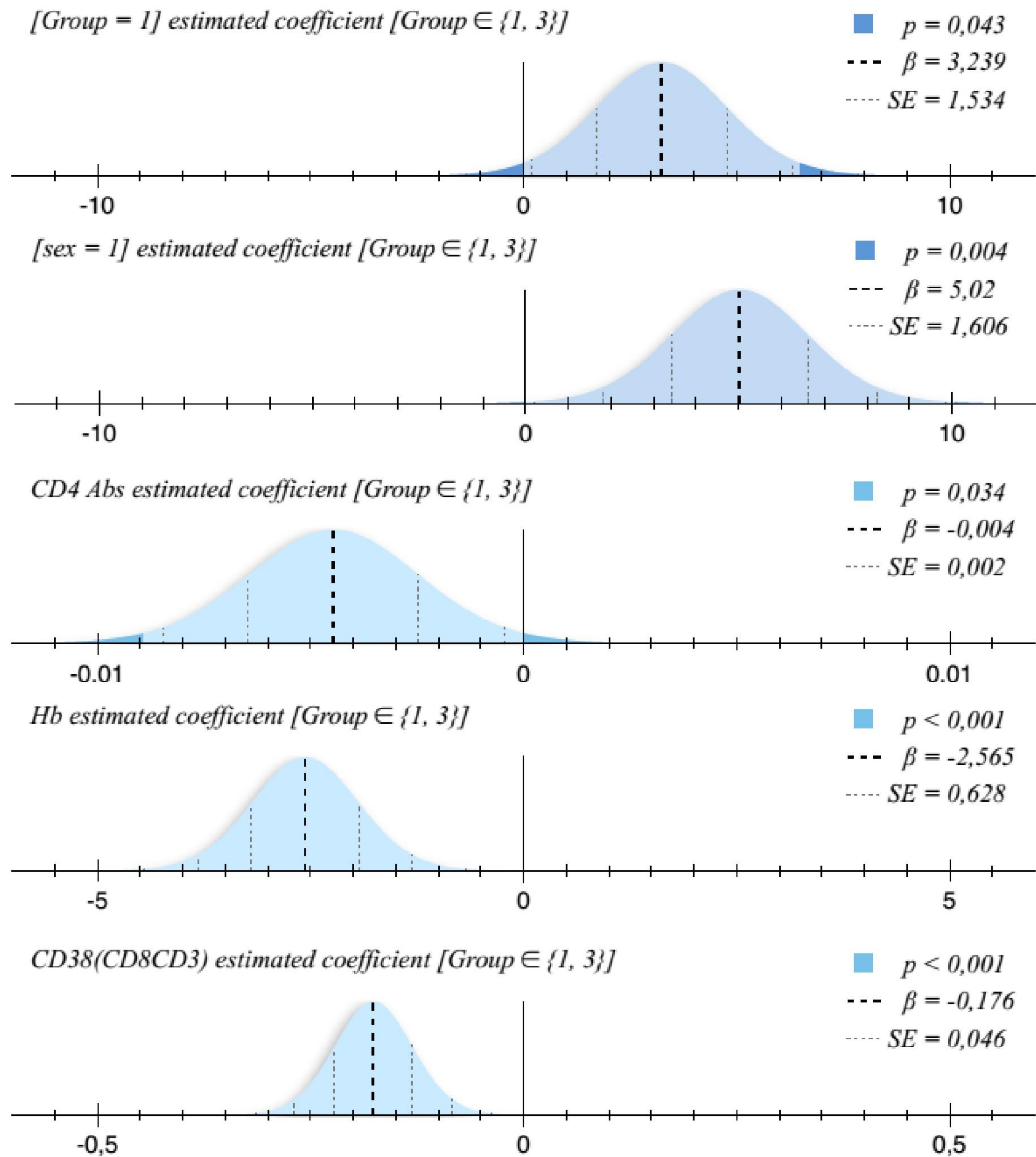


Figure 13. Multivariate regression model on the determinants of the frequencies of CD235a+CD71+ pre-erythroid cells in HIV/HCV co-infected patients.

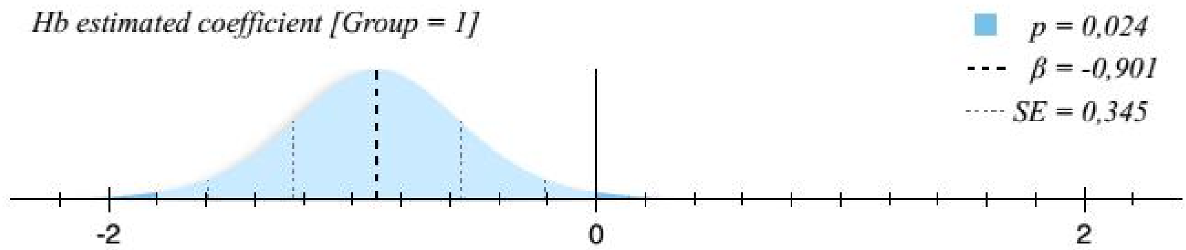
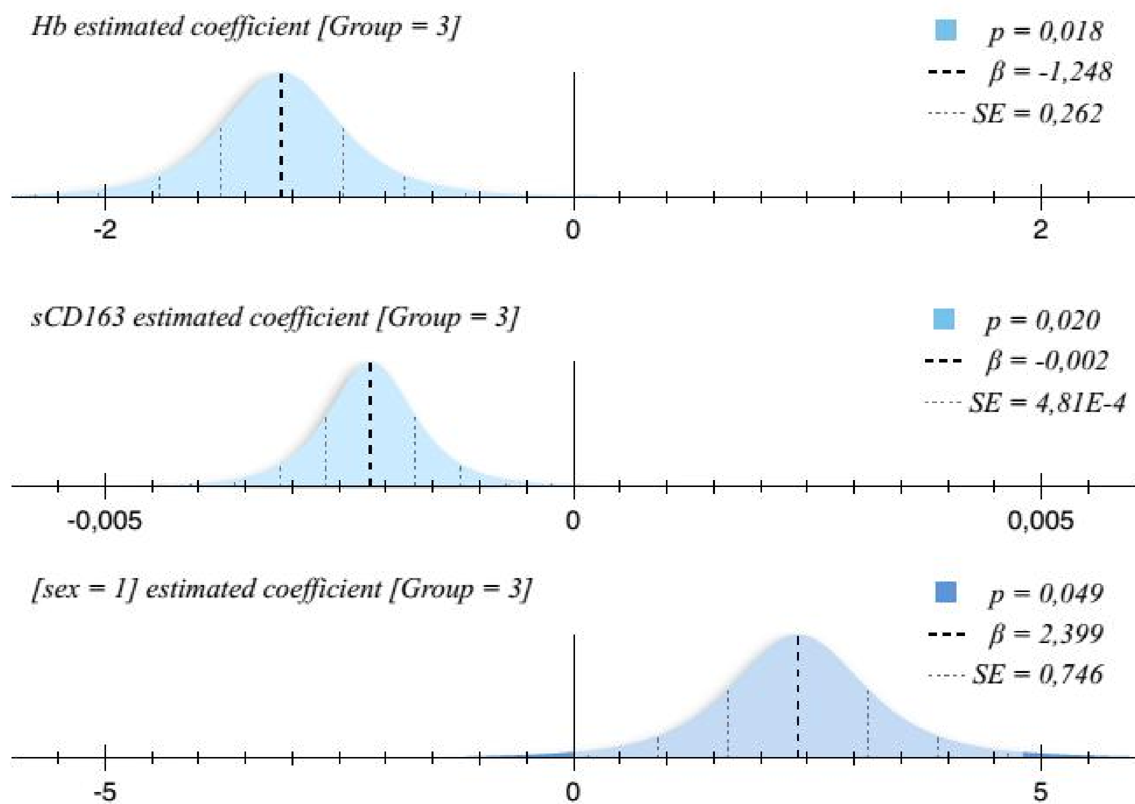


Figure 14. Multivariate regression model on the determinants of the frequencies of CD235a+CD71+ pre-erythroid cells in HIV infected patients.



7. Immune activation/inflammation parameters in anemic and not anemic, HIV infected and HIV/HCV co-infected patients.

From univariate regression analyses it is apparent that the frequency of pre-erythroid cells in peripheral blood of HIV infected and HIV/HCV co-infected patients are determined by erythropoietic factors and most strongly associate with Hb concentrations. Presence of pre-erythroid cells among PBMCs of HIV infected and HIV/HCV co-infected patients were evident in cases where Hb levels were below normal range (<13 g/dL). Based on this observation groups of HIV and HIV/HCV co-infeceted patients were divided into anemic and not anemic and comparison of hematologic, immune activation/inflammation parameters between them were performed (**Table 5A and Table 5B**). In the range of Hb <13g/dL were found 14 patients in HIV infected group and 6 patients in HIV/HCV co-infected. Average concentration of Hb in anemic, HIV infected patients was 11,29±0,95 and 12,075±0,96 in HIV/HCV co-infected patients. These values were significantly lower from the average values of Hb in corresponding subgroups of not anemic patients (15,22±0,45; p<0,001, 15,43±0,43;p<0,001; respectively). The concentrations of EPO were found significantly different between these subgroups only in HIV infected (**Table 5A**). In HIV infected group anemic patients in comparison to not anemic were found significantly higher frequencies of CD235a+CD71+ pre-erythroid cells, and frequencies of cells positive for CD38 expression. Additionally, these patients also demonstrated significantly lower number of CD4 T cells and significantly higher plasma concentrations of sCD163, and IL-6. In HIV/HCV co-infected group, anemic patients demonstrated significantly higher frequencies of CD235a+CD71+ pre-erythroid cells and higher frequencies of CD38+ cells but only on the subpopulation of CD3+CD8+ T cells (**Table 5B**).

Table 5A. Differences in erythropoietic and immunological factors between anemic and not anemic HIV infected patients.

Parameter	Not anemic HIV (n=50)	Anemic HIV (n=14)	p-value
EPO [mIU/ml]	5,14±1,8	16,5±10,5	<0,001
Erythrocytes [cells x 103/uL]	5.176,071±235,646	4.247,857±605,078	<0,001
CD235a+CD71+ [%]	0,417±0,276	2,473±2,033	<0,001
CD4 [cells x 103/uL]	599,6±87	304,2±131	<0,001
CD14+CD38+ [%]	4,2±1,5	8,9±6	0.026
CD3+CD38+ [%]	4,8±1,1	8,9±5,4	0.018
CD3+CD8+CD38+ [%]	10±3	26,8±14,4	<0,001
sCD163 [ng/ml]	866±145	1.244,9±380	0.031
IL-6 [pg/ml]	1,9±2,2	12,56±4,5	0.025
Arg1 [copies/20μL]	50,17±38	30,2±30	0,57

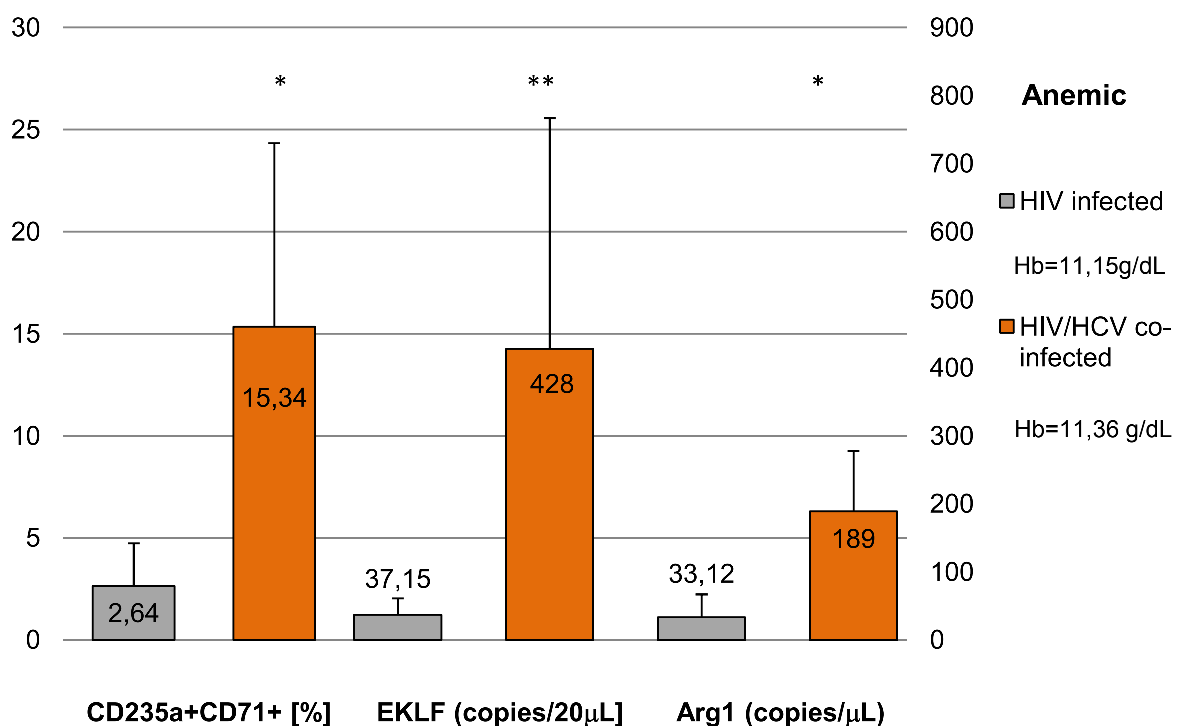
Table 5B. Differences in erythropoietic and immunological factors between anemic and not anemic HIV/HCV co-infected patients.

Parameter	Not anemic HIV/HCV (n=28)	Anemic HIV/HCV (n=6)	p-value
EPO [mIU/ml]	5,832±1,088	18,313±20,703	0,32
Erythrocytes [cells x 103/uL]	4.873,75±195,339	4.046,25±525,544	0,002
CD235a+CD71+ [%]	0,656±0,564	9,629±9,321	0,036
CD4 [cells x 103/uL]	666±143	638±533	0,256
CD14+CD38+ [%]	4,73±5,2	9,94±18,28	0,447
CD3+CD38+ [%]	1,72±0,49	3,98±3,55	0,138
CD3+CD8+CD38+ [%]	3,15±1,32	8,2±7,9	0,025
sCD163 [ng/ml]	2094±474	2697±870	0,162
IL-6 [pg/ml]	4,6±1,9	2,1±2,1	0,064
Arg1 [copies/20µL]	29,8±12,6	114,7±163	0,027

8. Comparison of CD235a+CD71+ pre-erythroid cells in anemic HIV infected and HIV/HCV co-infected patients.

Anemia was found to be the main determinant of the frequencies of CD235a+CD71+ pre-erythroid cells in peripheral blood of virus infected groups. Both, HIV infected, and HIV/HCV co-infected with similar level of anemia (HIV-11,15 g/dL; HIV/HCV-11,36 g/dL) were compared. It was observed that in HIV/HCV co-infected patients frequencies of CD235a+CD71+ pre-erythroid cells and the expression of *EKLF,* and *Arg1* were significantly higher in comparison to HIV infected (Mann-Whitney-p=0,019; p=0,004; p=0,049, respectively).

Figure 15. Comparison of anemic HIV infected and HIV/HCV co-infected patients



* p=0,019
** p=0,004
* p=0,048

VI. DISCUSSION

Processes of hematopoiesis during HIV infection are significantly disturbed, and beside declining number of CD4 T cells, other cell populations, including erythroid cells, undergo aberrant development which can be clinically manifested, for example by anemia. [25,41,87]. Defective erythropoiesis during HIV infection may be caused by different mechanisms, including inadequate responses of developing cells in bone marrow to EPO, direct infection of pro-genitors cells by virus, disruption of the correct architecture of bone marrow [69,70,88]. In result of these abnormalities accumulation of immature erythroid cells can take place [69]. Whether pre-erythroid cells contribute to the pathogenesis of HIV infection is not know. Beside possibility that these cells can carry viral material [41], their other role was not investigated. Pre-erythroid cells, derived from the spleens of murine neonates were recognized as a nucleated cells which actively participate in the regulation of immune responses and inflammation, causing their suppression and its down regulation, respectively [38]. In this model, pre-erythroid cells were demonstrated to cause immune suppression leading to significant enhancement of bacterial infectivity but at the same time they were shown to inhibit inflammatory reactions [38]. Anti-inflammatory activity of TER119+CD71+ pre-erythroid cells from the spleens of newborn mice was demonstrated *in vitro* where the production of TNF-α by adult CD11b+ cells, and expression of CD69 on adult CD8+ cells was significantly decreased after the stimulation. The mechanisms by which these pre-erythroid cells mediate anti-inflammatory reaction was suggested to be dependent from arginase but not indoleamine 2,3-dioxygenesa, transforming growth factor–β. Human CD235a+CD71+ pre-erythroid cells, equivalent to TER119+CD71+ pre-erythroid cells from mice, are found frequently in cord blood but not in peripheral blood of healthy adults [38]. The depletion of CD235a+CD71+ pre-erythroid cells from PBMCs of human cord blood caused significant increase in production of TNF-α after stimulation in comparison to not separated cells, demonstrating that similarly to

mice, human pre-erythroid cells posses anti-inflammatory activity. In this study anti-inflammatory activity was suggested to be a property only of pre-erythroid cells isolated from newborns but not adults [38]. Studies performed later however, confirmed that such an activity can be also mediated by cells isolated from adult mice [39].

Till now, there were no studies performed to investigate the immunoregulatory activity of pre-erythroid cells during HIV infection. These cells attracted the attention of investigators only as a possible HIV reservoir, and one of the cause of anemia [25,41,69]. Although the hypothesis of the study presented in this thesis is based mostly on the findings in animal model and during bacterial infection, potential presence and anti-inflammatory activity of pre-erythroid cells in human adults infected with HIV had significant bases. The presence of pre-erythroid cells in peripheral blood of adults was assumed from the studies which demonstrated significant influence of HIV on the development of this population of cells [41,69]. Infection of bone marrow progenitor cells with HIV-2 *in vitro*, differentiation of bone marrow derived CD34+ stem cells from HIV-1 infected people was shown to cause significant accumulation of immature erythroid cells [69]. Such an effect was not observed anymore after application of the therapy. Inhibitory effects of HIV infection on development of erythroid cells were found also to be associated with dysregulation of Epo production and signaling [70]. It was demonstrated that the development of erythroid cells from *in vitro* HIV infected hematopoietic progenitor cells was significantly reduced, and intermediate precursor cells were permissive to infection and harbored viral DNA [41]. Consideration of the role of pre-erythroid cells in HIV infection was also based on the capacity of these cells to produce arginase, activity of which was before associated with enhanced severity of the progression of HIV disease [37,38].

The results obtained during presented here study demonstrated that CD235a+CD71+ pre-erythroid cells in peripheral blood of HIV infected and HIV/HCV co-infected people are found on average in relatively high frequencies. In contrast, in healthy, uninfected or HIV

infected adults on ART presence of these cells is minimal. To diminish the doubts raised by some authors about the specificity of CD235a+CD71+ receptors for pre-erythroid cells [39], the presence of these cells was confirmed at the molecular level by analyzes of the expression of the gene for erythroid specific transcription factor, *EKLF* [62]. The analyses of the expression of *EKLF* were found to replicate observations at cellular level in all tested groups. Increased frequency of CD235a+CD71+ pre-erythroid cells in peripheral blood of HIV infected patients confirms performed before by other authors observation on the accumulation of immature erythroid cells, and lack of this phenomenon in patients who received ART [69]. Interestingly, ART treatment had no such an effect in some patients of HIV/HCV co-infected group. Despite applied therapy in this group, on average, high frequency of CD235a+CD71+ pre-erythroid cells were seen. The possible effect of this increased number of cells in HIV/HCV co-infected group despite ART was suspected to be related to the liver cirrhosis. In fact, the frequencies of CD235a+CD71+ pre-erythroid cells in some patients with liver cirrhosis were the highest observed. Moreover, liver cirrhotic patients demonstrated higher concentrations of EPO, and pro-inflammatory factors, IL-6, and sCD163 in comparison to patients without liver cirrhosis.

In accordance with previous findings, performed *in vitro* analyses of anti-inflammatory activity of pre-erythroid cells obtained from human umbilical cord confirmed substantial increase in expression of immune activation receptor, CD69, on T cells after stimulation when CD71+ cells were depleted. However, such an effect was not as potent when the cells of HIV infected and HIV/HCV co-infected were used. To understand the mode pre-erythroid cells can mediate their anti-inflammatory activity and how it is presented in different groups, analyzes of the expression of gene coding for *Arg1* and the plasma activity of this enzyme were performed. Selection of this enzyme was based on previous findings showing the expression of arginase in pre-erythroid cells [38] and the association of its activity with HIV disease severity [37]. As in case of *EKLF*, positively selected CD71+ cells from PBMCs of umbilical cord, and peripheral

blood of tested groups were used to test the *Arg1* expression. The results demonstrated that in CD71+ isolated cells *Arg1* expression is augmented in comparison to all PBMCs. Moreover, expression of this gene in PBMCs of HIV/HCV co-infected patients correlated with the frequency of CD235a+CD71+ pre-erythroid cells. To test if possible differences in *Arg1* expression are reflected also at the functional level, analyses of arginase activity in plasma were performed. Obtained results demonstrated that arginase activity on average was significantly increased in HIV and HIV/HCV co-infected patients in comparison to healthy controls. However, the direct link between pre-erythroid cells frequencies and arginase activity was not found. It is possible that the measures of the activity of arginase in plasma are biased by other cellular sources of this enzyme, for example CD15+ neutrophils [37]. In HIV infected patients, arginase activity was found to positively correlate with the frequency of CD15+ neutrophils [37] and severity of disease. In current study plasma arginase activity was not found to associate with any of the markers of disease severity in HIV infected or HIV/HCV co-infected patients.

In multivariate analyses the frequencies of CD235a+CD71+ pre-erythroid cells were found to be predicted by belonging to the group of HIV/HCV co-infected patients, male sex, and inversely associated with the expression of CD38 on CD8 T cells, CD4 T cells absolute counts and Hb plasma concentrations. Adjustment for HCV viral load, and sCD163 plasma concentrations revealed Hb as an independent predictor of the frequency of CD235a+CD71+ pre-erythroid cells in peripheral blood of HIV/HCV co-infected patients. In HIV group adjustment for HIV viral load and sCD163 again demonstrated significant inverse association with Hb concentrations, but also with sCD163 and more frequent presence of these cells in males. Anemic patients demonstrated not only higher number of the pre-erythroid cells but also significantly higher levels of the immune activation/inflammation markers in comparison to not anemic patients of both groups of virus infected people. The differences in the distribution of pre-erythroid cells between the supgroups, were accompanied by similar observations in *Arg1*

expression in HIV/HCV co-infected patients but without contrary effects on immune activation and inflammation parameters. Together, these results show that differently from *in vitro* experiments and contrary to the hypothesis, *in vivo* presence of pre-erythroid cells in peripheral blood does not diminishes immune activation and inflammation during HIV and HIV/HCV co-infection. This hypothesized, immunosuppressive effect of pre-erythroid cells was not observed despite significantly higher frequencies of these cells and *Arg1* expression in HIV/HCV co-infected patients in comparison to HIV infected with similar level of anemia.

In conclusion, this study demonstrates that in HIV and HIV/HCV co-infected people anemia contributes to the appearance of immature erythroid cells in peripheral blood and as a mononucleated cells separate with frequently used in the studies PBMCs. The mechanisms and extent of accumulation of these cells in peripheral blood however are different in patients with or without HCV co-infection. First, because pre-erythroid cells are much more frequent in HIV/HCV co-infected people than in HIV infected, despite similar levels of Hb, and secondly, they are present in HCV co-infected patients despite applied ART. The most suspected cause of this effect is the presence of liver cirrhosis and associated with this disease enhanced levels of EPO. However, in multivariate analyses the connection between pre-erythroid cells and this co-morbidity was not confirmed. The potential anti-inflammatory and/or immunoregulatory effects of pre-erythroid cells observed by others and in *in vtro* of this study were not confirmed by analyses of immune activation and inflammatory parameters *ex vivo*. However, it can't be excluded that this observation can be false negative as the selected parameters where limited to few markers of HIV progression, and immune activation/inflammation such as CD38, IL-6, and sCD163. Additionally, the number of cases studied is also a limiting condition for conclusions to be certain, what is evident especially in regard of liver cirrhotic patients.

What is not leaving any doubt is that anemia has the leading cause behind the appearance of pre-erythroid cells in peripheral blood but if the presence of these cells can have

important immunoregulatory effects and thus influence the disease progression requires more in depth analyses. From this study is also apparent that mostly HIV/HCV co-infected, anemic patients with liver cirrhosis are prone to have relatively high frequencies of these cells in peripheral blood. Taking into the consideration, that it is the group of patients who most frequently fail to respond to direct acting anti-viral drugs against HCV [76], potential impact of pre-erythroid cells can't be excluded, especially that these cells are already present at very moderate decline of Hb concentrations below normal range.

Present study suggests that during HIV infection and HIV/HCV co-infection occurrence of anemia can lead to abnormal accumulation/development of pre-Er's with potentials of modulation of immune responses. However, what are the mechanisms of this activity and how it translates to the disease progression is not clear and requires farther studies. This study contributes in understanding the mechanisms of anemia in HIV infected people by inclusion of cellular component into the observations that before where mostly based on changes of Hb and Epo analyses. This study may help to better understand the association between anemia and increased mortality, increased disease progression, and reduced quality of life of HIV infected people as reported by "Anemia in HIV Working Group" [78]. It may also help to find the explanation for the similarity between HIV-induced and normal ageing inflammatory pathways [82].

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