Original article:

The length of AZT consumption and homocysteine levels are protective factors of macrocytic anemia among adult HIV-infected patients: A case-control study in Central Java Province, Indonesia

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Abstract:

Objective: Macrocytic anemia (MA) is the most common type of anemia in adult HIV-infected patient on azidothymidine (AZT) regimen. The common causes of MA are vitamin B12 and folate deficiency, marked by homocysteinemia. AZT consumption causes homocysteinemia is still controversial. This study aimed to determine the role of AZT consumption and homocysteinemia in developing MA. **Materials and Methods:** This was a case-control study involving adult HIV-infected patient who administered AZT in 12 health care facilities in Central Java Province, Indonesia. Sociodemographic data were obtained through interviews and medical records, while laboratory data included hemoglobin level and mean corpuscular volume (MCV) were measured using the automatic hematology analyzer. Homocysteine level was measured using immunoassay. WHO references was used to diagnose anemia. Macrocytic was determined when MCV >96 fl. The cut-off homocysteinemia was >10 µmol/L. Length of AZT consumption was classified into ≤6 months and >6 months. Data were analyzed using multivariate logistic regression test. **Results:** The population of this study was 503 adult HIV-infected on AZT regimen. In total, there were 116 subjects (age mean±SD: 41,9±9,4) who had MA and 116 controls (age mean±SD: 36,2±8,3) without anemia. Prevalence of anemia was 29.4% and the majority (78.4%) had MA. The odds of having MA among adult HIV infected patient on AZT regimen > 6 months was 0.25 times compared to patients who were on AZT regimen ≤6 months (95% CI 0.08-0.72, p=0.011), homocysteinemia was protective factor of MA (OR 0.43,95% CI 0.24-0.79, p=0.006). **Conclusion:** The length of AZT consumption >6 months and homocysteinemia are protective factors of MA among adult HIV-infected patient.

Keywords: homocysteine, macrocytic anemia, HIV, AZT

Introduction

Human Immunodeficiency Virus (HIV) infection has emerged as one of worldwide health problems. The number of people with HIV / acquired immune deficiency syndrome (AIDS) (PLWHA) has continued to increase since 1980s.[1] The Joint United Nations Programme on HIV/AIDS (UNAIDS) in 2017 reported 36,9 million people worldwide living with HIV/AIDS, with 630.000 among them are Indonesian. In 2017, Indonesia was the third country in Asia-Pacific with the highest rate of new human immunodeficiency virus (HIV) infection. Indonesia was also the second country in Asia-Pacific with the highest mortality rate due to acquired immunodeficiency syndrome (AIDS).[2]

Studies have shown that 11,7-92% of HIV patients also suffer from anemia.[3] The cause of anemia in HIV patients is presumed to be multifactorial, thus making it challenging to identify the cause of anemia as well as the appropriate treatment.[4] The most important contributing factors in anemia include bone marrow infiltration, use of myelosuppressive drugs, the HIV

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infection itself, opportunistic infections, or nutrition factors caused by iron, vitamin A, vitamin B12, and folate deficiency.[5–7]

HIV primarily targets CD4+ cells and without treatment this leads to the collapse of the host immune system and ultimately death.[8] *Azido thymidine* (AZT) is the nucleoside reverse transcriptase inhibitor (NRTIs) drug of choice recommended by WHO for AIDS treatment since 1990, especially for developing countries.[9] AZT is one of the highly active antiretroviral therapy (HAART) that is capable of decreasing viral load and improving the immune system in HIV system.[10] Up until December 2018, most HIV patients (96,9%) in Indonesia have received the original first line antiretroviral therapy (ART) regimen, including AZT.[11]

ART treatment is known to help to decrease morbidity and mortality in HIV patients. However, the use of ART is limited due to its toxic side effect towards hematopoiesis, liver, skin, and the musculoskeletal system.[10,12] Long term ART therapy with AZT was also reported to be associated with bone marrow suppression and mostly can cause anemia.[3,10,13,14] Laboratory findings also proved that AZT causes cytotoxicity in erythroid and myeloid precursor in the bone marrow.[10] The risk of anemia in AZT therapy was reported to be higher compared to other ART such as stavudine and tenofovir.[9] Assefa et al found that there is a high proportion of HIV patients with anemia after 6 months of therapy with AZT.[4] Other study mentioned that anemia most often occurs two to 48 weeks after AZT initial therapy, with the peak incidence at 24 weeks (6 months).[15] This result is consistent with another study which showed that anemia was found 6 months after the first therapy.[14,16] Tadele et al. also found that the prevalence of macrocytic anemia is high (80.6%) after 6 months of therapy with AZT.[17] Most anemia (57,1%) in HIV patients treated with AZT was macrocytic anemia.[7] AZT acts as a thymidine analogue and can interfere with DNA synthesis, as well as inducing macrocytic anemia.[18] The most common cause of macrocytic anemia is vitamin B12 and folate deficiency.[19–21]

Homocysteine (Hcy) is a better laboratory marker for vitamin B12 deficiency compared to vitamin B12 serum levels.[22,23] Hcy is originated from demethylated methionine amino acid which was further catabolized through trans-sulfuration into cysteine or remethylated into methionine. Methionine remethylation needs cofactors, which includes folate and vitamin B12. Hcy concentration is regulated by many factors such as age, genetically determined changes in metabolic enzymes, nutritional factors including vitamin B6, B12, and folate. Folate is important to keep normal Hcy serum levels. Folate also plays an important part in two biochemical cycles, which are DNA biosynthesis and carbon metabolisms such as DNA, lipid, and protein methylation.[24,25] Up until now, several researchers had studied how Hcy levels can affect HIV patients, but the results were controversial. Look et al. found hyperhomocysteinemia (HHcy) in 35% of HIV patients who received HAART.[26] On the contrary, a lower prevalence of 12,3% and 16,4% was found in other studies.[27] Studies regarding the role of AZT treatment in homocysteinemia in HIV patients with macrocytic anemia has never been reported before.

Materials and Methods

This study was a case-control study, conducted at 12 health facilities in Indonesia, including Balai Kesehatan Masyarakat (Balkesmas) Semarang, RSUP Dr. Kariadi, RSUD Tugurejo, RSI Sultan Agung, RS Panti Wilasa, Puskesmas Halmahera, RSUD dr. Soewondo Kendal, RSUD Demak, RSUD dr. Loekmono Hadi Kudus, RSUD RA Kartini Jepara, RSUD RAA Soewondo Pati dan RSUD Dr. R. Soedjati Purwodadi during April-December 2018. Inclusion criteria in this study were adult HIV patients aged 20 to 59[28] who received AZT treatment for ≥4 weeks and was not pregnant during the conduction of this study. Inclusion criteria for case group were patients with macrocytic anaemia (with MCV levels of >96 fL, haemoglobin (Hb) levels of <12g/dL for women and <13g/dL for men). Exclusion criteria were alcoholism, consumption of anticonvulsant drugs and chemotherapy, hyperthyroid, liver disease, haemodialysis, myelodysplastic syndrome, and aplastic anaemia. Exclusion criteria were obtained from interview and patient's medical record. Inclusion criteria of control group were subjects who did not have anaemia (normal Hb levels).

This study had been proved by Ethics Committee for Medical and Health Research (KEPK), Faculty of Medicine Diponegoro University / RSUP Dr. Kariadi (No. 123/EC/FK-RSDK/III/2018) and all subjects signed the informed consent before participating in the study. Interviews and medical record investigation were conducted to obtain sociodemographic characteristics. Venous blood sampling of 5 cc was done for laboratory studies, including Hb, MCV, and Hcy levels.Sociodemographic characteristics include age, sex, education, occupation, marital status, high-risk behaviour, duration of AZT treatment, and body mass index. Education level was divided into low and

high education level. Low education level was further divided into never received formal education /did not graduate elementary school, graduated elementary school, and graduated junior high school. Meanwhile, high education level was divided into graduated senior high school and university. Duration of AZT treatment was categorized into >6 months and ≤ 6 months.[4,15]

Analysis of Hb and MCV levels were conducted using automatic haematology analyser Sysmex Type XN-1000, and quality of machine and reagent was controlled prior to analysis. Subjects were later categorized into macrocytic anaemia if levels of MCV> 96 fLand Hb <13g/dL in men, and <12 g/dL in women.[29] Subjects diagnosed with macrocytic anaemia were categorized into case group, while subjects without anaemia were categorized into the control group. Hcy serum levels were measured using the immunoassay method with Axis Homocysteine EIA FHCY 1000 reagentand were analysed with the wavelength of 340 nm. The cut-off level for homocysteinemia was >10 µmol/L.[30,31]

Data were descriptively analysed, and normality was tested using Kolmogorov-Smirnov test. Numeric data from both groups were compared using unpaired t test. Categorical data were analysed using Chi-square and Mann Whitney U. Contributing risk factors were identified with multivariate logistic regression analysis using SPSS 25.0 software.

Results

In total 503 subjects were matched with inclusion criteria which were adults (20–59 years of age), HIV positive who have received AZT ≥ 4 weeks, not pregnant, and signed informed consent. All of 503 subjects were interviewed and blood was collected for Hb and MCV analysis. There were 148 subjects found with anemia and the rest were without anemia. From 148 subjects with anemia, there were 116 subjects found with MCV >96fL (macrocytic anemia), later categorized into case group. Control group were 116 subjects with normal Hb selected by consecutive sampling.
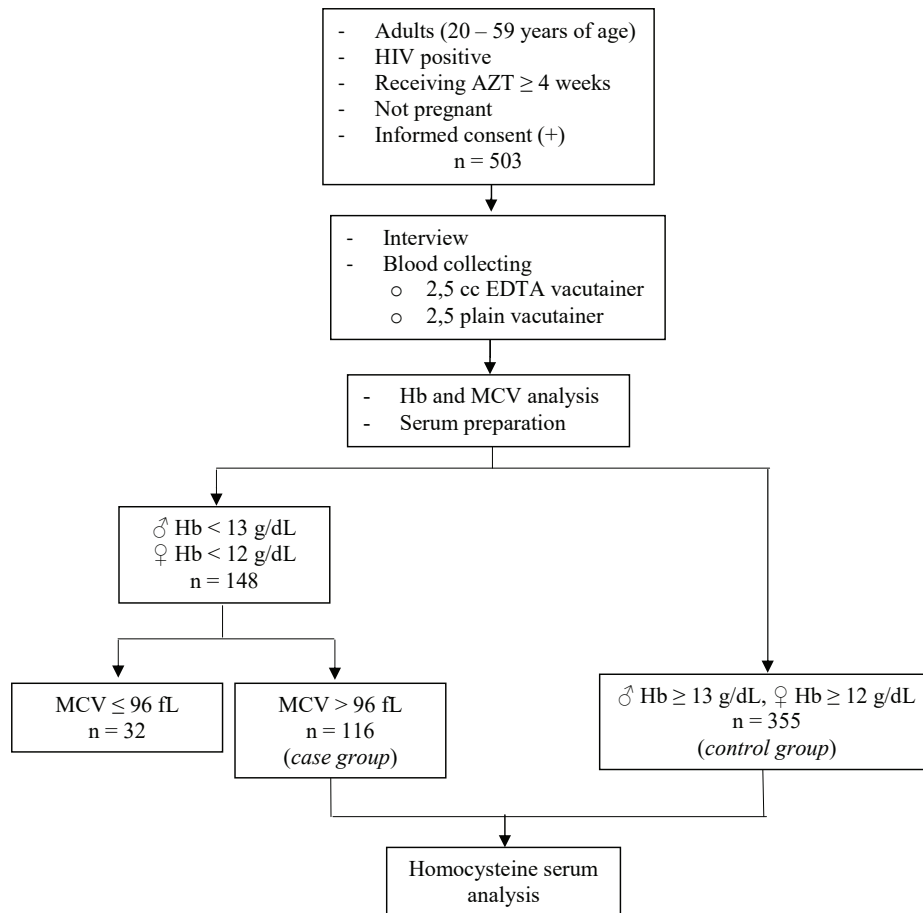

Figure 1. Consort chart

Hematology examination was conducted in 503 adult HIV patients, consisting of 248 (49,3%) males and 255 (50,7%) females. The average age (±SD) was 37,9 (8,75) years old. There were 421 (83,7%) subjects with macrocytosis (MCV> 96fL). There were148 subjects (29,4%) found with anemia, and 116 subjects (78,4%) among them had macrocytic anemia and then were categorized into the case group (age mean±SD: 41,9±9,4). Control group consisted of 116 subjects(age mean±SD: 36,2±8,3) without anemia. The characteristics of subjects can be seen in table 1.

Table 1. Sociodemographic and laboratory characteristics of all subjects

Subjects' characteristics	Case group n = 116	Control group n = 116	pvalue
Sex, n (%)			<0,001*
• Male	39 (33,62%)	74 (63,79%)	
• Female	77 (66,38%)	42 (36,21%)	
Age (year), mean±SD	41,9±9,4	36,2±8,3	<0,001$^\alpha$
Age			<0,001*
• 20-29 years	10 (8,62%)	26 (22,41%)	
• 30-39 years	46 (39,65%)	55 (47,42%)	
• 40-49 years	31 (26,72%)	25 (21,55%)	
• 50-59 years	29 (25,01%)	10 (8,62%)	
Marital status, n (%)			<0,001*
• Single	7 (6,03%)	50 (43,10%)	
• Married	58 (50,00%)	48 (41,37%)	
• Widower/widow	51 (43,97%)	18 (15,53%)	
Educational status, n (%)			<0,001*
• Low	74 (63,79%)	37 (31,89%)	
• High	42 (36,21%)	79 (68,11%)	
Employment, n (%)			0,021¥
• Unemployed	3 (2,58%)	4 (3,44%)	
• Students	0 (0,00%)	2 (1,72%)	
• Housewife	49 (43,10%)	16 (13,79%)	
• Employee	41 (34,48%)	77(66,37%)	
• Entrepreneur	23 (19,84%)	17 (14,68%)	
Riskbehavior factors, n (%)	7 (6,0%)	39 (33,6%)	0,001¥
• Homosexual	102 (87,9%)	62 (53,4%)	
• Heterosexual	3 (2,6%)	8 (6,9%)	
• Bisexual	3 (2,6%)	6 (5,2%)	
• Injecting Drug User	1 (0,9%)	1 (0,9%)	
• Others			
Body Mass Index (kg/m²)	22,15 ± 3,66	23,14 ± 3,64	0,048$^\alpha$
Length of AZT consumption (months)	15 (12,9%)	6 (5,2%)	0,039*
	101 (87,1%)	110 (94,8%)	
• ≤ 6 months			
• > 6 months			
Homocysteine level±SD (µmol/L)	10,2 ± 5,5	12,9 ± 8,2	<0,001$^\alpha$
Category ofhomocysteine level			<0,001*
• ≤ 10 µmol/L	74 (36,21%)	42 (63,79%)	
• > 10 µmol/L	42 (63,79%)	74 (36,21%)	

*Chi square$^\alpha$t-independent ¥Mann Whitney U

Table 2. The risk factors ofsex, age, length of AZT, homocysteinemia among case and control group

Subjects' characteristics	Case group n = 116	Control group n = 116	Multivariate (OR; CI 95%) p value
Sex, n (%)			
Female	77 (66,38%)	42 (36,21%)	(3,41; 1,84-
Male	39 (33,62%)	74 (63,79%)	6,30)<0,001
Age			<0,001
20-29 years	10 (8,62%)	26 (22,41%)	
30-39 years	46 (39,65%)	55 (47,42%)	(1,95; 0,78-4,90) 0,156
40-49 years	31 (26,72%)	25 (21,55%)	(3,02; 1,11-8,23) 0,031
50-59 years	29 (25,01%)	10 (8,62%)	(9,06; 2,95-27,82) 0,000
Length of AZT consumption,n(%)			
>6 months	101 (87,1%)	110 (94,8%)	(0,25; 0,08-0,72)
≤ 6 months	15 (12,9%)	6 (5,2%)	0,011
Category ofhomocysteine level, n(%)			
> 10 µmol/L	74 (63,79%)	42 (36,21%)	(0,43; 0,24-0,79)
≤ 10 µmol/L	42 (36,21%)	74 (63,79%)	0,006

*Regression logistic test

Based on multivariate analysis results (see table 2), female sex, and age group (age 40-49 and 50-59 years old) were found to be risk factors of macrocytic anemia. Meanwhile, AZT treatment for >6 months and Hcy levels of >10µmol/L were protective factors towards macrocytic anemia incidence in adult HIV patients who received AZT treatment. HIV patients who were treated with AZT aged 40-49 has a higher risk of 3,02 times (95% CI 1,11-8,23; p=0,031), while for patients aged 50-59 the risk is 9,06 times higher (95% CI 2,95-27,82; p=<0,001) for macrocytic anemia in comparison with patients aged 20-29. Odds of female HIV patients who received AZT treatment for >6 months is 3,41 times (95% CI 1,84-6,30; p=<0,001), meanwhile for men the odds is 0,25 times (95% CI 0,05-0,47; p=0,001) compared to odds of HIV patients who received AZT treatment for ≤6 months. Odds of HIV patients with Hcy levels of >10 µmol/L to suffer from anemia is 0,43 times (95% CI 0,24-0,79; p=0,006) compared to odds of HIV patients with Hcy levels of ≤10 µmol/L

Discussion

Anemia is the most common side effect in HIV patients who received AZT treatment.[3] Anemia in HIV patients can cause physical and psychological dysfunction, decrease in quality of life, the progressivity of the disease, and decrease in life

expectancy. Anemia can happen each year due to HIV infection, and the degree of anemia is related to the progressivity of the disease.[32] The pathophysiology of anemia in HIV infection is an important factor in disease management, as well as prevention in morbidity and mortality.[7]

Prevalence of anemia is around 11,7-92,0%, depending on HIV stadium, sex, age, pregnancy status, and whether or not an opportunistic infection is present. Prevalence and severity of anemia increase as the severity of the disease increases.[3] Globally, anemia prevalence in HIV patients who received ART treatment is 23-50%.[32] This study found anemia prevalence of 29,4% in HIV patients who were treated with AZT. This prevalence is consistent with a research result in South West Ethiopia, which was 23,1%.[33] This prevalence is smaller compared to a previous study of HIV patients in Indonesia, which was 49,6%.[34] The mechanism of ACT induced anemia was a disturbance in blood cells progenitor proliferation, which was related to duration and dosage of therapy. Hematology toxicity in most patients happen after 3-6 months, and was reversible.[14,35]

Macrocytic anemia was found in most (116; 78,4%) anemia patient in this study. This number was higher compared to the study from Wahyuwibowo et al, which showed macrocytic anemia of 57,1%. The sample used in the previous study was smaller (54 people) compared to this study.[7]Generally, the most common cause of macrocytic anemia is vitamin B12 and folate deficiency.[19–21]Gastrointestinal complications were often found in HIV patients, such as an increase in inflammation and slow mucosa repair due to virus replication, which results in lamina propia thinning and cause vitamin B12 and folate malabsorption.[7]In New York, 13% of macrocytic anemia case was related to AZT treatment.[36]AZT is a thymidine analogue with the biggest effect towards MCVand is also related to macrocytic anemia.[37]

Macrocytosis related to vitamin B12 or folate deficiency is a direct cause of ineffective erythropoiesis/ dyserythropoiesis. Erythroblast maturity in one marrow requires several important vitamins and cofactors, including vitamin B12 and folic acid. Deficiency in one of those c omponents will result in unsynchronized maturation of erythroblast nuclei and cytoplasm due to a defect in DNA synthesis. Erythrocyte cell size will become bigger/macrocytosis.[38]

To identify vitamin B12 and folate deficiency in subjects, we conducted Hcy levels measurement. Hcy is a good marker in detecting vitamin B12 and folate deficiency.[22,23]Hcy is originated from demethylated methionine amino acid which was further catabolized through trans-sulfuration into cysteine or remethylated into methionine. Methionine remethylation needs cofactors, which includes folate and vitamin B12. Hcy concentration is regulated by many factors such as age, genetically determined changes in metabolic enzymes, nutritional factors including vitamin B6, B12, and folate. Folate is important to preserve normal Hcy serum levels. Folate also plays an important role in two biochemical cycles, which are biosynthesis DNA and carbon metabolism such as DNA, lipid, and protein methylation.[24,25]

The average Hcy levels in subjects with macrocytic anemia (case group) were10,2±5,5 µmol/L. Meanwhile, the average Hcy levels in subjects without anemia (control group) was 12,9±8,2 µmol/L. There was a significant difference in Hcy levels in case group compared to control group with $p=<0,001$ ($p<0,05$). Based on multivariate logistic regression analysis the odds of Hcy levels of >10 µmol/L was 0,43 (95% CI 0,24-0,79), which showed that higher than cut-off Hcy levels can act as a protective factor towards macrocytic anemia compared to Hcy levels of ≤10 µmol/L.

The most common cause of macrocytic anemia is vitamin B12 and folate deficiency, which is usually marked with increase blood Hcy concentration levels.[23]All populations in this study continued AZT treatment for at least 4 weeks. We found 83,7% macrocytosis prevalence of total population. Similar results from previous studies also stated that AZT can cause macroytosis. AZT therapy was reported to be related to mitochondrial toxicity through cellular DNA synthesis disturbance.[39]AZT therapy is the main cause of macrocytosis in HIV patients, however, the mechanism remains unclear. AZT competes with natural trioxide phosphate deoxynucleoside to bind with HIV reverse transcriptase as well as DNA polymerase and mitochondria pol-γ. Obstacles in cellular DNA synthesis can cause a disturbance in erythrocyte cell synthesis, and delay in nucleus development in bone marrow, which will result in macrocytosis.[37]AZT is cytotoxic towards erythroid precursor and myeloid in bone marrow, which

causes anemia as one of its side effects.[10] Aside from that, AZT also acts as thymidine analogue and can interfere with DNA synthesis as well induce macrocytic anemia.[18]

De Larranaga et al found a strong relation between HHcy and folate deficiency, but found no relation between Hcy levels and the side effects of AZT treatment. Based on this fact, we assume that vitamin B12 and folate deficiency did not cause macrocytic anemia in the subjects. It is because that homocystenemia is a protective factor towards macrocytic anemia in this study.[27]

We also found a significant difference between the occurrence of macrocytic anemia in patients treated with AZT for >6 months and ≤6 months (*p*=0,011). Based on multivariate logistic regression analysis, the odds of AZT treatment of >6 months compared to ≤6 months was found to be 0,25 (95% CI 0,08-0,72), which showed that AZT treatment of >6 months is a protective factor towards the occurrence of macrocytic anemia. According to theory, anemia in HIV is affected by the side effects of HIV treatment.[40,41] Use of AZT can cause hematology toxicity which is started with decreasing ATP concentration and depletion of glutathione before the damage of mtDNA. The decrease in glutatione levels can cause an increase in reactive oxygen species (ROS) production, which will cause a disturbance in DNA, protein, and lipid functions.[42–45] Sharma et al found that hematology toxicity in most patients happened in 3-6 months and was reversible.[14,35]

The majority of ART used in HIV treatment is AZT. Several studies showed that after treatment with AZT, a significant improvement in hematology profile was found, including a decrease in anemia prevalence or a significant increase in Hb levels.[3] Enawgaw et al found that HIV patients who received ART experienced an increase in erythrocyte levels six months after their initial treatment started, and it kept gradually increasing.[46] However, Ejeliogu et al stated that Hb levels in patients taking AZT decreased progressively after use within 6 months and subsequently Hb levels will increase gradually.[35] The prevalence, severity of anemia, and degree of macrocytosis lowered after AZT therapy in the 6-12 month.[17] Assefa et al in their study found that anemia prevalence dropped 67% after ART therapy for 12 months. It was further explained that ART treatment can lower the occurrence of opportunistic infection, which will later lower the levels of proinflammation cytokines such as TNF which can suppress erythropoiesis. This indirectly showed the effectivity of ART in reducing HIV associated anemia by reducing the likelihood of opportunistic infection, anemia caused by chronic diseases, and by improving the patient's nutritional status.[4,33] Similar results were concluded by Johannessen et al, who found a significant increase of Hb levels in HIV patients after receiving ART treatment for the first 12 months.[47] Other explanation that would make sense is the fact that ART can suppress HIV virus that directly suppressesthe bone marrow, therefore preventing anemia.[33] AZT is long known to be the cause of anemia in HIV patients, especially in the beginning of ART treatment in HIV patients with initially low Hb levels.[32]

We found that female subjects in this study had a higher risk of 3,41 times compared to male subjects towards macrocytic anemia. Most population (87,3%) in this study had macrocytosis (MCV >96fL). A study by Agarwal et al also showed similar results, in which female HIV patients who received AZT were more prone to anemia.[16] A study by Assefa showed that moderate and severe anemia is more common in female patients in comparison to male patients, before and after 6 months of ART therapy. It was further explained that female patients had a higher risk of routine blood loss through menstruation and labor.[4]

Subjects aged 40-49 had a higher risk of 3,02 times towards macrocytic anemia, while subjects aged 50-59 had a higher risk of 9,06 times in comparison with subjects aged 20-29. Other studies also found a significant correlation between age and occurrence of macrocytic anemia in HIV patients who received AZT treatment, with most subjects aged 25-44.[17] Other studies showed a higher risk of 2,4 times towards anemia as patients get older, particularly in patients over 55 years old. Hematopoietic stem cell showed resistance in erythropoietin increase as patients get older. Furthermore, aging is also correlated with an increase in proinflammatory cytokines which contributes towards EPO resistance.[4]

Limitation includes unmatched case-control design and no analysis on the patients' initial Hb before AZT treatment. We also did not calculate the existence of opportunistic infections and did not consider giving additional therapy such as cotrimoxazole.

Conclusion

In this study, we conclude that AZT therapy of >6 months and homocysteinemia (>10 µmol/L) are protective factors towards macrocytic anemia in HIV patients who received AZT treatment. However, patients aged 40-59, also female patients, are risk factors for macrocytic anemia.

Ethical Approval

Ethical approval for this study was obtained from Faculty of Medicine Diponegoro University-Dr. Kariadi Hospital Ethics committee no. 123/EC/FK-RSDK/III/2018.

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Conflict of interest

There are no conflict of interests declared

Authors Contribution

Data gathering and idea owner of this study: Danis Pertiwi

Study Design: Danis Pertiwi

Data Gathering: Danis Pertiwi

Data Analysis and consultation:Danis Pertiwi, Suradi, Tri Indah Winarni, Ari Natalia Probandari

Writing and submitting of the manuscript:

Danis Pertiwi, Suradi, Tri Indah Winarni, Ari Natalia Probandari

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