

## Improvement of the Osmotic Fragility (OF) Test in $\alpha$ Thalassemia 1 Screening for HIV-1 Infected Patients by The New Cutoff Values of MCV and MCH

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### Abstract:

**Objective:** To evaluate the efficiencies of the standard  $\alpha$  thalassemia screening regimen in human immunodeficiency virus (HIV) infected patients and improve the efficiencies of this screening regimen using new cutoff values.

**Material and Methods:** A screening process using the osmotic fragility (OF) test, the old cutoff values of mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) at <80 fL and <22 pg, and the new cutoff values at <87 fL and <29 pg was performed in 300 HIV infected patients. After which, a genetic study was performed to detect common  $\alpha$  thalassemia 1 genes deletions composed of Southeast Asian and THAI deletions and also for hemoglobin constant spring and hemoglobin Pakse mutations for  $\alpha$  thalassemia 2 carriers. Screening efficiency was estimated by sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

**Results:** Sensitivity, specificity, PPV, and NPV of the original regimen for thalassemia screening in HIV infected patients were at 50.0%, 95.0%, 39.0% and 96.8%, respectively, demonstrating a high false positivity in HIV-1 infected populations. Therefore, new cutoff values for MCV and MCH were proposed at <87 fL and <29 pg, due to the highest value found in  $\alpha$  thalassemia 1 carriers with HIV-1 infection. These new cutoff values showed higher sensitivity, specificity, PPV, and NPV at 100.0 92.6%, 46.2%, and 100.0%, respectively, and thus enhanced thalassemia screening efficiency in HIV-1 infected patients.

**Conclusion:** Incorporation of the OF test and the new cutoff values for MCV and MCH improve the efficiencies of  $\alpha$  thalassemia 1 screening in HIV-1 infected patients. This helps reduce the cost of confirming positive test results in  $\alpha$  thalassemia screening.

**Keywords:**  $\alpha$  thalassemia, HIV-1, MCH, MCV, OF test

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## Introduction

Thalassemia is a fairly common inherited disorder in Thailand.<sup>1</sup> It is caused by abnormal globin protein synthesis.<sup>2</sup> One of the most severe thalassemia syndromes is hemoglobin (Hb) Bart's hydrops fetalis, which is caused by the deletion of all the  $\alpha$  globin genes.<sup>3</sup> Fetuses with this disease are stillborn while the mothers frequently exhibit preeclampsia, and polyhydramnios.<sup>4</sup> To reduce this condition, screening for the  $\alpha$  thalassemia 1 gene in couples was implemented.<sup>5</sup>

Two methods are used for  $\alpha$  thalassemia 1 screening, the osmotic fragility (OF) test, and the mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). The principle of these two-screening methods is based on the reduction of Hb content in red blood cells, which leads to a disproportion between red blood cell volume and Hb concentration inside the cells, resulting in a positive OF test, and the reduction of MCV and MCH values.<sup>6</sup> Patients who have positive results for any screening test should undergo genetic testing for confirmation. This process is very expensive and less efficient than the screening processes.<sup>7</sup> The efficiency of thalassemia screening is represented by sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV). These were 100.0, 87.1, 84.5 and 100.0% for the OF test and 92.9, 83.9, 37.9 and 99.1% for MCV and MCH.

There are high numbers of human immunodeficiency virus (HIV) infected couples in antenatal care in Thailand, as a result of the reduced rate of HIV antenatal transmission to 1.9% in 2016,<sup>9,10</sup> due to the improved efficiency of the antiviral drugs. All HIV infected couples in this program also underwent normal  $\alpha$  thalassemia screening. However, recent evidence indicates changes in the hematological parameters of HIV-1 infected patients. The major effect caused by antiretroviral drugs is that some of them influence bone marrow suppression via

blocking of nucleic acid synthesis which leads to macrocytosis in HIV infected patients.<sup>11-13</sup> The changes of hematological parameters include Hb levels, MCV and MCH<sup>11</sup> caused by the reduction of Hb in red blood cells, the reduction of red blood volume and the impairment of Hb and blood cell volume. In addition, other evidence also suggesting a high rigidity of red blood cells was observed in patients with HIV infection.<sup>14</sup> These changes may interfere with thalassemia screening by OF test, MCV and MCH, and may result in many patients requiring confirmation testing by deoxyribonucleic acid (DNA) analyses. However, these changes affecting the efficiency of each screening method have not been investigated.

Therefore, the efficiencies of the test were analysed with the aim of enhancing and improving  $\alpha$  thalassemia 1 screening in HIV-1 infected patients in order to reduce the number of the false positive results and also the costs of confirmatory analyses.

## Material and Methods

### HIV infected patients

This was an experimental research study. Subjects were 300 HIV-1 infected patients. Two milliliters of blood were collected from patients during a 1-year follow-up of normal monitoring from June 2015–April 2016. Clinical data including drugs used in the treatment and period of HIV infection were collected. Patients with mild anemia and other signs of iron deficiency were excluded from the study.

The study was approved by the Human Research Ethics Committee of the University of Phayao in January 2015 (No. 3/001/59).

### $\alpha$ thalassemia screening

A blood sample collected from each candidate was used to screen for  $\alpha$  thalassemia 1 by OF test as previously described, and MCV and MCH testing using

complete blood count (CBC) by an automated hematology analyser (Mindry BC-5180). Then, the MCV and MCH of patients were interpreted using standard cutoff values at 80 fL and 27 pg respectively, in order to indicate the likelihood of  $\alpha$  thalassemia 1 carriers. Samples with lower MCV and MCH than the cutoff were defined as screening positive, while scores higher, than the cutoff were defined as negative for  $\alpha$  thalassemia 1. In addition, in the all patients it was confirmed that they were without evidence of the  $\beta$  thalassemia traits by a normal level of Hb A<sub>2</sub>.

#### **DNA analyses of $\alpha$ thalassemia carriers**

Two common  $\alpha$  thalassemia 1 deletions in Thailand: Southeast Asian (SEA) and THAI deletions, which were analysed in all the patients' DNA as described below. DNA was extracted from 500  $\mu$ L of buffy coat using the chloroform extraction method. The DNA was then amplified by 6 specific  $\alpha$  globin gene primers using a multiplex gap polymerase chain reaction, following the method described by Sae-ung et al.<sup>15</sup> Three specific amplification products from these primer pairs were used to indicate the presence of  $\alpha$  thalassemia 1 deletions including 660 bps, 480 bps and 380 bps for SEA deletion, THAI deletion and the control amplicon, respectively. All amplicons were analysed in parallel with positive control for SEA and THAI deletions.

To rule out a change of hematological parameters and to confirm a positive result of OF test from other  $\alpha$  thalassemia carriers, 2 severe  $\alpha$  thalassemia 2 genes that show a correlation with OF test positive were also detected as Hb Pakse (HbPS) and Hb Constant Spring (HbCS). These 2 genes were identified by a multiplex polymerase chain reaction developed by Fucharoen et al.<sup>16</sup> Products of 253 and 183 bps indicated  $\alpha$ PS and  $\alpha$ CS respectively. The result of this amplification was also interpreted in parallel with positive control for HbPS and HbCS.

#### **Defining of appropriate hematological parameter for $\alpha$ thalassemia screening in HIV-1 infected patients**

The data of each subject were collected: age, sex, hematological parameters including Hb, Hct, RBC, MCV, MCH and red cell distribution width (RDW). All data were analyzed for identify differentiation in the sample group that might affect research results, especially the changes of hematological data that cause differences in age or sex. The parameters without of any effect from these factors will be used as tools for  $\alpha$  thalassemia 1 screening. The differences of each hematological parameter were compared between males and females and in 5 different age groups using independent sample t-test and Mann Whitney U test. The parameters given a p-value of more than 0.050 were used in the next defining process.

After that the effect of hematological parameters on the OF test result were also analysed using the independent sample t-test and Kruskal-Wallis one-way Analysis of Variance (ANOVA). In addition, correlations between these parameters and  $\alpha$  thalassemia 1 deletion were analysed using Spearman's rank correlation coefficient with a confidence interval (CI) at 95%.

#### **Efficiency of $\alpha$ thalassemia screening methods**

To evaluate the efficiency of the OF test in a thalassemia 1 screening, all samples were classified into 4 groups: (1) true positive, a group with positive results for any screening test that carried one  $\alpha$  thalassemia 1 deletion, (2) false positive, a group with positive results for any screening test but did not carry any abnormal  $\alpha$  thalassemia 1 deletion, (3) true negative, a group with negative results for any screening test that did not carry the  $\alpha$  thalassemia 1 deletion and (4) false negative which presented negative results for any screening test but contained one  $\alpha$  thalassemia 1 deletion. The efficiency for screening tests was assessed using sensitivity, specificity, PPV and NPV by the number of patients in each group.

## Results

### The suitable hematological parameters for $\alpha$ thalassemia 1 screening

The hematological parameters of each participant were analysed to identify a suitable parameter for  $\alpha$  Thalassemia 1 screening. Findings demonstrated an almost equal ratio between male and female patients: 121 (40.3%) male and 178 (59.3%) female. Average ages of males and females were 46.6 (45.4–47.8) and 45.3 (44.1–46.4) respectively. Three factors of hematological data differed between the sex groups: Hb, Hct and RBC (Table 1).

Candidates were classified into 5 groups using age: 20–29, 30–39, 40–49, 50–59, and  $\geq 60$ . Most were in the 40–49 age group, 182 subjects (60.6%). Interestingly, 3 hematological factors including Hb, Hct and RBC tended to decrease in higher age groups. In contrast, MCV and MCH showed an increasing trend in higher age groups (Table 2). These analyses suggested that MCV and MCH were not statistically different between age and sex of study groups, and that they could be used as the  $\alpha$  thalassemia screening markers for the patients.

**Table 1** The number and the hematological data of human immunodeficiency virus infected samples classified by sex

Sex	Number (%)	Age	Hb (g/dL)	Hct (g%)	RBC ( $\times 10^6/\mu\text{L}$ )	MCV (fL)	MCH (pg)	RDW (%)
		Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.
Male	121 (40.3)	46.6 $\pm$ 6.4	13.6 $\pm$ 1.59	39.8 $\pm$ 4.41	3.9 $\pm$ 0.73	102.7 $\pm$ 14.8	35.1 $\pm$ 6.3	13.6 $\pm$ 1.7
Female	178 (59.3)	45.3 $\pm$ 7.7	12.3 $\pm$ 1.28	36.3 $\pm$ 3.64	3.6 $\pm$ 0.68	103.0 $\pm$ 15.4	34.8 $\pm$ 6.4	13.3 $\pm$ 1.2
		p-value	<0.0001 <sup>1</sup>	<0.0001 <sup>2</sup>	<0.0001 <sup>2</sup>	0.662 <sup>2</sup>	0.810 <sup>2</sup>	0.088 <sup>2</sup>

<sup>1</sup>p-value from independent sample t-test analyses, <sup>2</sup>p-value from Mann Whitney U test

Hb=hemoglobin, Hct=hematocrit, RBC=red blood cell count, MCV=mean corpuscular volume, MCH=mean corpuscular hemoglobin, RDW=red blood cell distribution width, S.D.=standard deviation

**Table 2** Mean of the hematological data of 300 human immunodeficiency virus infected patients classified by age

Age (years)	Male (%)	Female (%)	Total (%)	Hb (g/dL)	Hct (g%)	RBC ( $\times 10^6/\mu\text{L}$ )	MCV (fL)	MCH (pg)	RDW (%)	
				Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	Mean $\pm$ S.D.	
20–29	3 (2.5)	5 (2.8)	8 (2.6)	12.1 $\pm$ 1.0	37.6 $\pm$ 3.5	4.0 $\pm$ 1.2	100.6 $\pm$ 22.5	34.4 $\pm$ 8.8	13.5 $\pm$ 1.6	
30–39	8 (6.6)	24 (13.5)	32 (10.6)	13.1 $\pm$ 1.5	38.8 $\pm$ 4.8	4.0 $\pm$ 0.7	99.0 $\pm$ 13.9	33.6 $\pm$ 5.5	13.6 $\pm$ 1.3	
40–49	73 (59.8)	109 (61.2)	182 (60.6)	12.7 $\pm$ 1.5	37.3 $\pm$ 4.2	3.7 $\pm$ 0.6	103.1 $\pm$ 14.9	35.2 $\pm$ 6.1	13.5 $\pm$ 1.6	
50–59	34 (27.9)	32 (18.0)	66 (22.0)	13.0 $\pm$ 1.5	38.2 $\pm$ 4.3	3.8 $\pm$ 0.7	103.0 $\pm$ 15.9	34.4 $\pm$ 7.4	13.4 $\pm$ 1.1	
$\geq 60$	4 (3.3)	8 (4.5)	12 (4.0)	13.1 $\pm$ 1.7	38.0 $\pm$ 4.8	3.5 $\pm$ 0.6	109.9 $\pm$ 10.1	38.1 $\pm$ 3.9	13.2 $\pm$ 0.7	
				p-value	0.433 <sup>1</sup>	0.377 <sup>2</sup>	0.197 <sup>2</sup>	0.167 <sup>2</sup>	0.119 <sup>2</sup>	0.584 <sup>2</sup>

<sup>1</sup>p-value from ANOVA analyses <sup>2</sup>p-value from Kruskal–Wallis test

Hb=hemoglobin, Hct=hematocrit, RBC=red blood cell count, MCV=mean corpuscular volume, MCH=mean corpuscular hemoglobin, RDW=red blood cell distribution width, S.D.=standard deviation

### Low efficiency of OF test in $\alpha$ thalassemia 1 screening in HIV-1 infected patients

Results indicated a low efficiency of the OF test in the detection of  $\alpha$  thalassemia carriers in HIV infected patients. The parameters: sensitivity, specificity, PPV and NPV of the OF test were 100.0%, 66.6%, 16.0% and 100.0%, respectively (Table 3). Therefore, it probably suggested that high false positive values occurred while utilizing the OF test as a screening method for  $\alpha$  thalassemia 1 carriers in HIV-1 infected patients, leading to an increased cost of DNA analysis as the confirmation test. This result might be reflected in a change in hematologic features in HIV-1 infected patients. Hematological data of HIV infected patients as OF positive and OF negative were compared to define the differences between these 2 groups.

Nevertheless, only 3 hematological parameters, namely Hb, MCV, and MCH showed mild correlations with  $\alpha$  thalassemia 1, indicated by the spearman  $r$  at 0.268, 0.304, and 0.309 with  $p$ -values of  $5.8 \times 10^{-5}$ ,  $7.9 \times 10^{-12}$  and  $9.9 \times 10^{-11}$  respectively. As previously mentioned, Hb could vary due to differences of age and sex. Only 2 parameters, MCV and MCH, were used in the further study as an  $\alpha$  thalassemia 1 screening tool in HIV-1 infected patients.

### Improvement of $\alpha$ thalassemia 1 screening in HIV-1 infected patients by the new cutoff of MCV and MCH

Another previous method used for  $\alpha$  thalassemia 1 screening in Thailand involves the cutoff values of MCV and MCH at 80 fL and 27 pg respectively. However, HIV-1 infected patients always show increasing MCV and MCH, and this may interfere with the screening result. We demonstrated the efficiency of  $\alpha$  thalassemia 1 screening using MCV <80 fL in HIV-1 infected patients in sensitivity, specificity, PPV and NPV, which were 50.0%, 94.7%, 37.5%, and 96.7%, respectively. Similarly, the efficiency of  $\alpha$  thalassemia 1 screening using MCH <27 pg in sensitivity, specificity, PPV and NPV were 66.7%, 91.5%, 33.3%, and 97.7%, respectively. It was demonstrated that using MCV and MCH for  $\alpha$  thalassemia 1 screening helps improve specificity and PPV, but lowers the sensitivity and NPV of these screening tools. It is suggested that the HIV-1 infected patients may require a new specific cutoff for MCV and MCH in  $\alpha$  thalassemia 1 screening. An investigation was carried out in order to determine the efficiency of the new cutoff compared with the old cutoff.

The new cutoff was analysed by comparing hematological data between patients with and without the  $\alpha$  thalassemia 1 gene. MCV and MCH between patients with and without  $\alpha$  thalassemia 1 were analyzed. We found

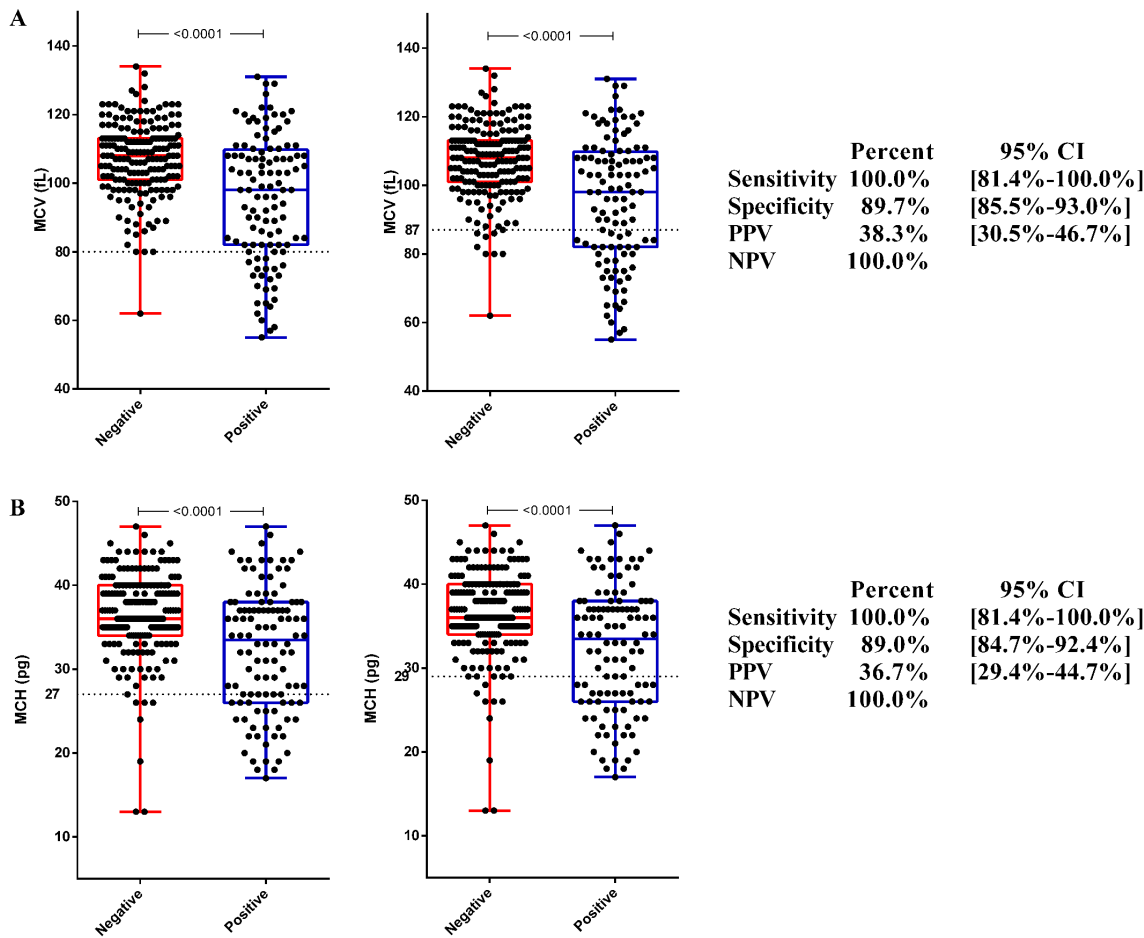
**Table 3** Frequencies of  $\alpha$  thalassemia gene and OF screening efficiency in HIV-1 infected patients

Test	Results	$\alpha$ thalassemia genes					Total	Screening efficacies	
		Negative	SEA	Thai	CS	PS		Sensitivity	
OF	Negative	169	0	0	19	0	188	<b>Sensitivity</b>	100.0% (81.4–100.0%)
	Positive	84	17	1	10	0	112	<b>Specificity</b>	66.7% (60.8–72.1%)
Total		253	17	1	29	0	300	<b>PPV</b>	16.1% (13.9–18.4%)
								<b>NPV</b>	100.0%

OF=osmotic fragility test, SEA=southeast asian, CS=constant spring, PS=Pakse, PPV=positive predictive value, NPV=negative predictive value

the range of MCV and MCH in patients without  $\alpha$  thalassemia was 62.0–134.0 fL and 13.0–47.0 pg respectively. The lower level was detected in  $\alpha$  thalassemia 1 patients at 60.0–86.0 fL and 19.0–28.0 pg for MCV and MCH respectively. The new cutoff value was estimated to be the highest MCV and MCH values of the patients with

$\alpha$  thalassemia 1 gene plus one as 87 fL and 29 pg, respectively. Efficiency of the new cutoff was calculated and results could increase specificity and PPV from 66.6% to 89.7% and 16.0% to 36.7% respectively (Figure 1).



**Figure 1** Comparison between the old and the new cutoff values of mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) between  $\alpha$  thalassemia 1 and non-thalassemia 1 carrier

Old (left) and new (right) cutoff value of MCV (A) and MCH (B) were used to define patients with and without  $\alpha$  thalassemia 1 gene. (A) the old cutoff value showed some false negative sample due to a higher MCV level after HIV infection. Increasing of MCV cutoff value from 80 fL to 87 fL helped enhance the sensitivity of the screening test, all patients with  $\alpha$  thalassemia 1 gene were given a positive result. Also, a different of MCH between  $\alpha$  thalassemia carrier and non-thalassemia carrier that could be used as screening criteria. Increasing of MCH cutoff from 27 pg to 29 pg help increased screening efficiencies (B).

The new cutoff values improved the efficiency of  $\alpha$  thalassemia screening in HIV-1 infected patients, significantly. Next, we considered the efficiency of  $\alpha$  thalassemia 1 screening if all methods were used together. The efficiency of screening power from 5 different methods comprising the OF test, MCV, MCH, MCV incorporated with MCH, and MCV incorporated with MCH and the OF test were compared. Results showed the highest efficiencies when all tests were used together, cooperatively having sensitivity, specificity, PPV and NPV screening for HIV-1 infected patients of 100.0%, 92.5%, 46.1% and 100.0% respectively (Table 4).

A diagram for  $\alpha$  thalassemia 1 screening in HIV-1 infected patients is shown in Figure 2. This new cutoff can increase the accuracy of  $\alpha$  thalassemia 1 screening in HIV-1 infected patients and also reduce false positive samples, resulting in the reduction of confirmation costs by DNA analyses.

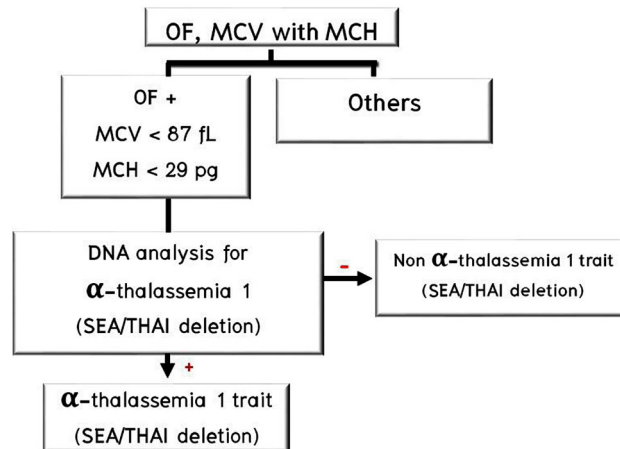
## Discussion

The data obtained revealed the differences in Hb, Hct, and RBC may be partly due to the diverse RBC production of different sexes and ages. In addition, the reduction of these 3 factors may be either the result of an infection of hematopoietic stem cells by the HIV virus or an inhibitory effect through cytokine release by activated immune cells. There is a previous study that demonstrated an increase of Interleukin 18 and Stem Cell Factor in HIV-1 infected patient serum.<sup>17</sup> These cytokines can induce lymphocyte production and drive CD34 positive stem cells to lymphoid progenitor cells instead of erythroid progenitor cells affecting RBC reduction. In addition, the reduced erythropoietin secretion in the elderly also causes changes in hematological data.<sup>18-20</sup> However, not only the effect from the previous factors, there is evidence which demonstrated a physiological change to the elderly also contributes to a low Hb and Hct level.<sup>21</sup> Taken together, these 3 parameters may alter the previous criteria for the diagnosis of  $\alpha$  thalassemia 1 carrier.

**Table 4** Efficiencies of  $\alpha$  thalassemia 1 screening in human immunodeficiency virus infected patients using variety parameter combination

Efficiencies index	OF (%)	MCV 87 fL (%)	MCH 29 pg (%)	MCV+MCH (%)	MCV+MCH+OF (%)
Sensitivity	100.0	100.0	100.0	100.0	100.0
Specificity	66.6	89.7	89.0	89.0	92.5
PPV	16.0	38.3	36.7	36.7	46.1
NPV	100.0	100.0	100.0	100.0	100.0

OF=osmotic fragility test, MCV=mean corpuscular volume, MCH=mean corpuscular hemoglobin, PPV=positive predictive value, NPV=negative predictive value



**Figure 2** Improvement of  $\alpha$  thalassemia screening scheme in human immunodeficiency virus infected patients.

OF test, MCV, and MCH have been used cooperatively in  $\alpha$  thalassemia screening in HIV-1 infected patients. The sample with OF positive, MCV less than 87 fL and MCH less than 29 pg were defined as screening positive for  $\alpha$  thalassemia 1. In contrast, any patients does not confine to the criteria was defined as screening negative for  $\alpha$  thalassemia 1.

Our results show that the changes of these parameters affect  $\alpha$  thalassemia screening using the OF-test, MCV, and MCH. The low screening efficiencies of the OF test may be a reflection of the side effects of the antiviral drugs and HIV-1 virus itself.<sup>19,22,23</sup> And these could interfere with mineral absorption and result in changes of cell protein synthesis and reduced fatty acid on the cell membrane. The consequences may increase the rigidity in RBC's infected patients, resulting in false positives.<sup>24</sup> Recently, Quiros-Roldan and colleagues demonstrated that inflammation cytokines, such as IL-6 and IL-8, are correlated with low Hb levels in HIV-1 infected patients. Therefore, these may lead to false positives in OF screening, due to a lower Hb content in red blood cells.<sup>22</sup>

Interestingly, the new cutoff values for MCV and MCH in our findings were higher than the conventional ones. This is a concern due to the increase of MCV and MCH in HIV infected patients. Some evidence suggests

that the antiviral drug is another factor involved in the change of lipid levels in the patients. Increasing the high-density lipid cholesterol significantly correlates with the MCV in the patients with azidothymidine therapy.<sup>21</sup> Other evidence indicated that HIV infected patients showed defects in vitamin B12 and folate metabolism, causing the impairment of DNA synthesis, thus resulting in megaloblastic changes in red blood cells.<sup>24-26</sup> These new cutoff values help increase the specificity and PPV of the screening test. The higher specificity and PPV lead to more trustworthiness of the screening process, and it is suggested that HIV-1 infected patients who are given the positive results by this screening method have more chance to be  $\alpha$  thalassemia 1 carriers than with the previous screening method.

### Conclusion

$\alpha$  thalassemia 1 screening using the OF test and MCV and MCH with the old cutoff values at 80 fL and 27 pg indicated low screening efficiencies for HIV-1



infected patients. The new cutoff values for MCV and MCH were, therefore, determined to improve  $\alpha$  thalassemia 1 screening efficiencies in HIV-1 infected patients. The incorporation of the OF test with the new cutoff of MCV and MCH improved screening efficiencies and reduced false positive results in  $\alpha$  thalassemia 1 screening in HIV-1 infected patients.

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