Research Article

Evaluation of Surrogate Markers for Prediction of CD4 Counts in People Living with Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome

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Abstract

India is a developing country where resources are limited. HIV/ AIDS is an ominous public health problem faced by our population and the affordability of patients for 3-6 monthly monitoring of CD4 counts becomes difficult for most patients. The intent of the study was to identify parameters on complete blood counts that can predict a CD4 count of <200/μL. We found that an absolute lymphocyte count obtained by a 5-part cell counter <1250/μL is predictive of a CD4 count <200/μL with a sensitivity and specificity of 87.3% and 70.0% respectively. In addition, a haemoglobin value <11.15g/dL is also a good predictor of the CD4 count <200/μL. The combination of both Hb <11.15g/dl and ALC counter <1250/μL was like a confirmatory test with a specificity of 92.2% and a NPV of 79.5%. Hemoglobin and absolute lymphocyte counts obtained on automated cell counter are robust, cost-effective and easily available methods to follow up PLHA patients and patients on ART. These can effectively predict the CD4 count <200/μL and are especially useful in a developing country where the cost of these tests is one-fifth of flow cytometry.

Keywords: CD4 count <200/μL; Surrogate; Predictor; Monitoring; Absolute lymphocyte count

Abbreviations: HIV: Human Immunodeficiency Virus; AIDS: Acquired Immune Deficiency Syndrome; PLHA: Patients Living with HIV/AIDS; ALC: Absolute Lymphocyte Count; ALC Counter: Absolute lymphocyte Count Obtained on Automated Cell Counter; ALC FCM: Absolute Lymphocyte Count Obtained on Flowcytometer; ART: Antiretroviral Therapy; OIs: Opportunistic Infections; IRIS: Immune Reconstitution Inflammatory Syndrome; DLC: Differential Leucocyte Count; TLC: Total Leucocyte Count; ROC: Receiver-Operator-Characteristic; PPV: Positive Predictive Value; NPV: Negative Predictive Value

Introduction

Care of patients living with human immunodeficiency virus (HIV)/ acquired immune deficiency syndrome (AIDS) [PLHA] is considered as chronic care. It requires both clinical and laboratory monitoring. CD4 cells are the primary target of the HIV and loss of CD4 cells results in weakening of the immune response and renders the host susceptible to infections leading ultimately to AIDS. Measurement of CD4 cell counts in PLHA is done for initiation and monitoring of antiretroviral therapy (ART); initiation of prophylactic therapy for opportunistic infections (OIs);staging by WHO Clinical Classification; diagnosis of Immune Reconstitution Inflammatory Syndrome (IRIS) and to change/ switch ART [1].

In India, till recently, CD4 count <350 cells/μL was used as a cut-off for starting ART in patients classified as WHO clinical stage I and II while all patients with clinical stage III and IV had to be started on ART [2]. However, recently the WHO upgraded their guidelines and stated that irrespective of CD4 cell counts all patients should receive ART as it reduces morbidity and mortality [1]. This recommendation has also been accepted by the Indian authorities [3]. Absolute CD4 count has to be used for monitoring disease activity as per the recommendations at 3-6 month intervals [1-3].

An increase or decrease in CD4 count is indicative of immunological treatment success or failure. In many studies, increase in CD4 cell counts >200/μLis an indicator of success of therapy [4,5]. Also, severe OIs set in with a fall in CD4 count <200/μL [6]. In many resource constrained countries, CD4 count is not readily available. In India, with the introduction of cheap generic drugs for ART, the therapy is available to all patients but they cannot afford the recommended 3 to 6 monthly CD4 counts. Shapiro et al suggested the use of absolute lymphocyte count (ALC) as the predictor of CD4 count in 1998 [7]. A study from India found a total lymphocyte count (equivalent to ALC) of 1200/μL and Haemoglobin(Hb) <12 g/dL to be good predictors of CD4 <200/μL in ART naïve HIV cases [8]. From our centre, data of 2004-05 was published addressing the issue of surrogate markers to predict CD4 count <200/μL and it was found that an ALC of ≤1520/μL has high sensitivity (78%) for a CD4 cell count of ≤200/μL [9]. However, the data from the above studies [7-9] may not be applicable in the present scenario as ART is recommended for all cases now. The earlier studies assessed either only treatment-naïve cases [8] or the CD4 count assessment had been done using a dual-platform approach [9]. Dual platform approach is less accurate than the single platform estimation, which is currently being used at our centre and is a better method for estimation of CD4 and CD8 counts and the CD4:CD8 ratio [10].

We thus carried out the present study with the aim of looking for surrogate markers on cell counters which may predict a critically low CD4 count at diagnosis or a fall in CD4 count <200/μl while on ART therapy. This would be able to restrict the CD4 estimation only in those with the surrogate markers being positive as most PLHA in India cannot afford regular counts, due to expenses.

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Materials and Methods

A retrospective review of all samples submitted for an absolute CD4 and CD8 counts from January 2015 to February 2016 at a tertiary care center was performed. There were a total of 328 samples that were analysed for absolute CD4 and CD8 counts. All samples that fulfilled the following inclusion criteria were taken. These were- 1) Diagnosed cases of HIV/AIDS 2) Age >12 years 3) Availability of both haemogram and flow cytometry CD4 count results. Exclusion criteria were: 1) Age <12 years 2) patients whose samples were submitted for CD4, CD8 counts as a part of primary immunodeficiency workup and were negative for HIV/AIDS. The pediatric patients <12 years of age were excluded as generally the ALC is higher and the classification of severity is made by different cut-offs. A total of 297 samples from 257 patients fulfilled the inclusion and exclusion criteria and were analysed.

All samples were initially run on an automated cell counter (DxH, Beckman Coulter, Fullerton, California, USA) to obtain the complete haemogram with differential leucocyte counts (DLC). The parameters noted from the complete haemogram were: Hb, total leucocyte count (TLC) and absolute lymphocyte count (ALC counter). Subsequently, multiparametric flow cytometric analysis for simultaneous identification and enumeration of total CD3+, total CD4+, total CD8+, dual CD3+CD4+, dualCD3+CD8+ lymphocyte percentages and absolute counts in whole blood were done using a FC500™ flow cytometer (Beckman Coulter, Fullerton, California, USA). This was a single platform approach using CYTO-STAT tetra CHROME™ CD45-FITC/CD56-RD1/CD19-ECD/CD3-PC5 monoclonal antibody reagent and the Flow-Count Fluorosphere™ beads in 1:1 ratio with whole blood specimen. Immunoprep™ (Beckman Coulter, Fullerton, California, USA) reagent was used for red cell lysis and no washing was performed. Using this procedure, values of absolute lymphocyte count (ALC FCM), CD3%, absolute CD3 count, CD4%, absolute CD4 count, CD8%, absolute CD8 count and CD4:CD8 ratio was determined for all samples. The absolute counts were determined using the following formula:

$$\frac{\text{Total no. of cells of interest counted} \times \text{Flowcount fluorospheres assayed concentration}}{\text{Total no. of Flowcount fluorospheres counted}}$$

The instrument standardisation was performed daily with the help of Flow-Check fluoro-spheres™ (Beckman Coulter, Fullerton, California, USA) for the optical alignment and fluidics and Flow-Set™ (Beckman Coulter, Fullerton, California, USA) fluorospheres to optimise it for quantitative analysis of leucocytes. Daily quality-control was performed using the Immuno-Trol cells™ (Beckman Coulter, Fullerton, California, USA).

Statistical Analysis

The data base was created in Microsoft Excel and analysed using SPSS (ver 14.0). Descriptive statistics like proportions and measures of central tendency and dispersion were calculated for each variable. As majority of the variables showed skewed distribution, non-parametric correlation (Spearman's ρ) was performed. Receiver-operator-characteristic (ROC) curves were generated and sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated for the appropriate cut off for ALC counter, ALC FCM (flowcytometry based) and Hb against CD4 count <200/μL. Bivariate linear regression was also carried out using appropriate cut off values. A model of multivariate regression (binary logistic) was created using the cut off values generated from ROC curves to predict CD4 count <200/μL. A p value of <0.05 was taken as significant.

Results

A total of 297 samples were available for analysis from 257 cases. Of these 257 patients, 86 patients (33.5%) were on ART. Of these, 46 patients had only one sample available for CD4 counts in the study duration. The male to female ratio was 1.58:1 with there being 182 (61.3%) males and 115 (38.7%) females. The median age of the patients was 50 years (range, 12 to 79 years). The results of the automated cell counter and the single platform absolute CD3, CD4 and CD8 counts are presented in Table 1.

CD4 counts of all samples were correlated with TLC, Hb, ALC counter, and ALC FCM using the non-parametric Spearman correlation co-efficient. The results of these have been depicted in Table 2. The CD4 counts correlated significantly and maximally with the ALC determined both by the automated cell counter and the single platform approach. The bivariate linear regression results given in Table 2 show that almost 50% (49.4%) variability in CD4 count could be predicted by ALC FCM. The same figure for ALC counter was 43.3% making an almost equally good predictor as ALC FCM. The R^2 values for Hb and TLC were lower with predictive power for variability in CD4 count being only 11.6% and 12.4% respectively.

Linear regression analysis was done between ALC counter and ALC FCM was done and showed a high correlation between the two. The result is depicted in Figure 1.

Since a CD4 count of <200/μL is a significant landmark that determines the susceptibility to infections and is diagnostic of stage IV at the first presentation, we tried to correlate this with the cell counter parameters to see if they can predict the low CD4 count. There were 91 samples with a CD4 count of <200/μL. We found that none of the samples with a CD4 count <200/μL had a TLC of <1200/μL. In our 91 patients with CD4 counts below the cut-off, TLC values ranged from 1700 to 24200/μL (Correlation coefficient (ρ=0.032, p=0.761). For Hb, the value ranged between 4.2 and 17.4 g/dL (ρ=0.334, p=0.002) and Hb was below 12g/dL in 68 of 91 (74.7%) samples. This cut-off of Hb was taken from an earlier study [8].

Parameter	Mean ± SD	Median value	Range
TLC (/μL)	7785.29 ± 3995.42	7200	1500-39500
Hemoglobin (g/dL)	11.98 ± 2.43	12.1	4.2-17.4
ALC counter (/μL)	1916.04 ± 1028.05	1800	100-6200
ALC FCM (/μL)	1829.95 ± 1035.62	1702	24-5950
CD3%	74.15 ± 11.4	75.60	6.41-97.61
Absolute CD3 count (/μL)	1265.95 ± 779.5	1115	5-4748
CD4%	24.72 ± 12.86	24.0	1.09-68.9
Absolute CD4 count (/μL)	422.05 ± 326.9	378	5.5-2910
CD8%	46.5 ± 16.03	47.0	0.17-84.24
Absolute CD8 count (/μL)	793.25 ± 563.1	667	2-3708
CD4:CD8 ratio	0.66 ± 0.58	0.51	0.02-3.49

Table 1: Details of the automated cell counter parameters and single platform flow cytometry results.

Parameter	Correlation co-efficient (Spearman)	p value	Bi-variate linear regression		
			R^2	f	p
TLC	0.368	0.000	0.124	41.89	0.000
Hb	0.415	0.000	0.116	38.27	0.000
ALC counter	0.701	0.000	0.433	223.35	0.000
ALC FCM	0.761	0.000	0.494	288.07	0.000

Table 2: Non-parametric correlation and bivariate linear regression of CD4 counts with TLC, ALC by both modalities and Hb.
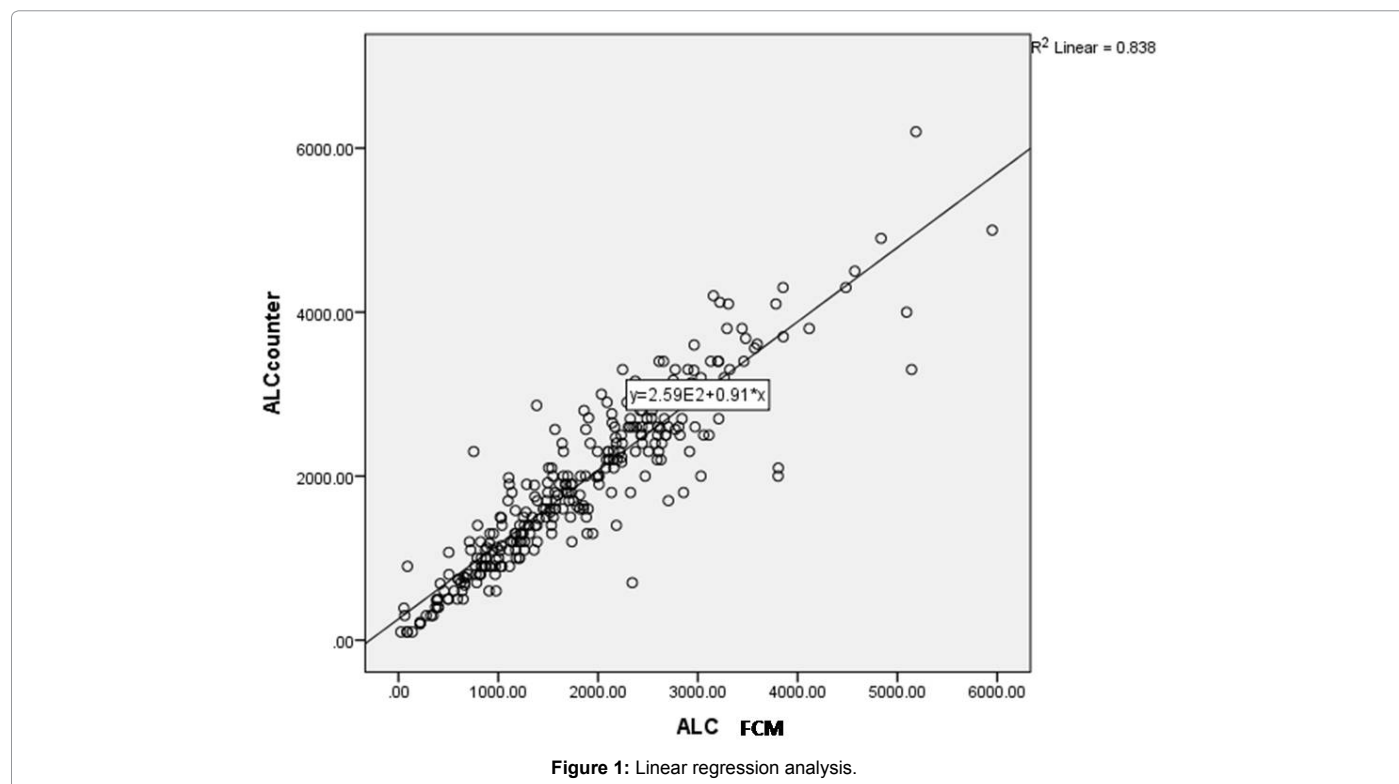
Figure 1: Linear regression analysis.

It was seen that both ALC counter and ALC FCM could predict a CD4 count of <200/μL. The ALC counter ranged between 100-4200/μL (ρ=0.574, p=0.000) and it was seen that 63/91 samples (69.23%) with CD4 count <200/μL had an ALC counter <1200/μL. The ALC FCM ranged between 24-3212/μL (ρ=0.524, p=0.000) and it was seen that 69/91 samples (75.82%) with CD4 count <200/μL had an ALC FCM<1200/μL.

ROC curves were drawn to determine the best cut-off for TLC, Hb, ALC counter and ALC FCM that correlate with a CD4 count of <200/μL. The ROC curves are given in Figure 2 and the statistics are given in Table 3. The cut-off obtained for ALC counter and Hb was <1250/μL and <11.15g/dl, respectively as predictors of CD4 count <200/μL. ALC counter however, had a higher sensitivity, specificity, PPV and NPV than Hb to predict a CD4 count of <200/μL.

A model of multivariate regression (binary logistic) using the cut off values of ALC FCM, ALC counter, Hb and TLC generated from the ROC curves for CD4 <200/μL was made by enter method. The results are displayed in Table 4. TLC lost its value as a predictor of CD4 count <200/μL after applying multivariate regression analysis. However, both ALC counter and Hb had an odds ratio >2 with p<0.05.

Based on the above, we looked at the effectiveness of a combination of Hb<11.15g/dL and ALC <1250/μL as a predictor of a CD4 <200/μl. The combination had a sensitivity of 46.5%, but a very high specificity of 92.23%. The PPV was 72.42% and NPV 79.5%. The sensitivity was low making it a poor screening test but the high specificity made it a very goodconfirmatory test for a CD4<200/μl.

Discussion

CD4 count <200/μL is an important landmark in the management of PLHA patients as it suggests the patient is in a higher WHO stage at diagnosis and also shows progression in disease while on ART. Even though the generic forms of ART have made it accessible to the entire PLHA population in India, the recommended monitoring of CD4 count

Parameter	Best cut-off	Area under curve (AUC)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Hb	11.15 g/dL	0.732	73.2	67.2	51.3	82.1
TLC	6700/μL	0.660	67.0	57.1	43.2	78.5
ALC counter	1250/μL	0.862	87.3	70.0	70.8	86.5
ALC FCM	1178/μL	0.890	89.0	74.7	74.7	88.8

Table 3: The statistics for all the best cut-off parameters evaluated as predictors of a CD4 count <200/μL

Parameter	Cut- off value	Odds ratio	95% confidence Interval (CI)	p value
ALC FCM	1178/μL	8.56	3.43, 21.39	0.000
TLC	6700/μL	1.54	0.079, 2.99	0.203
Hb	11.15 g/dL	2.13	1.09, 4.17	0.027
ALC counter	1250/μL	2.51	1.01, 6.23	0.048

Table 4: Multivariate (binary logistic) regression for the best cut-off to predict CD 4 count<200/ μL
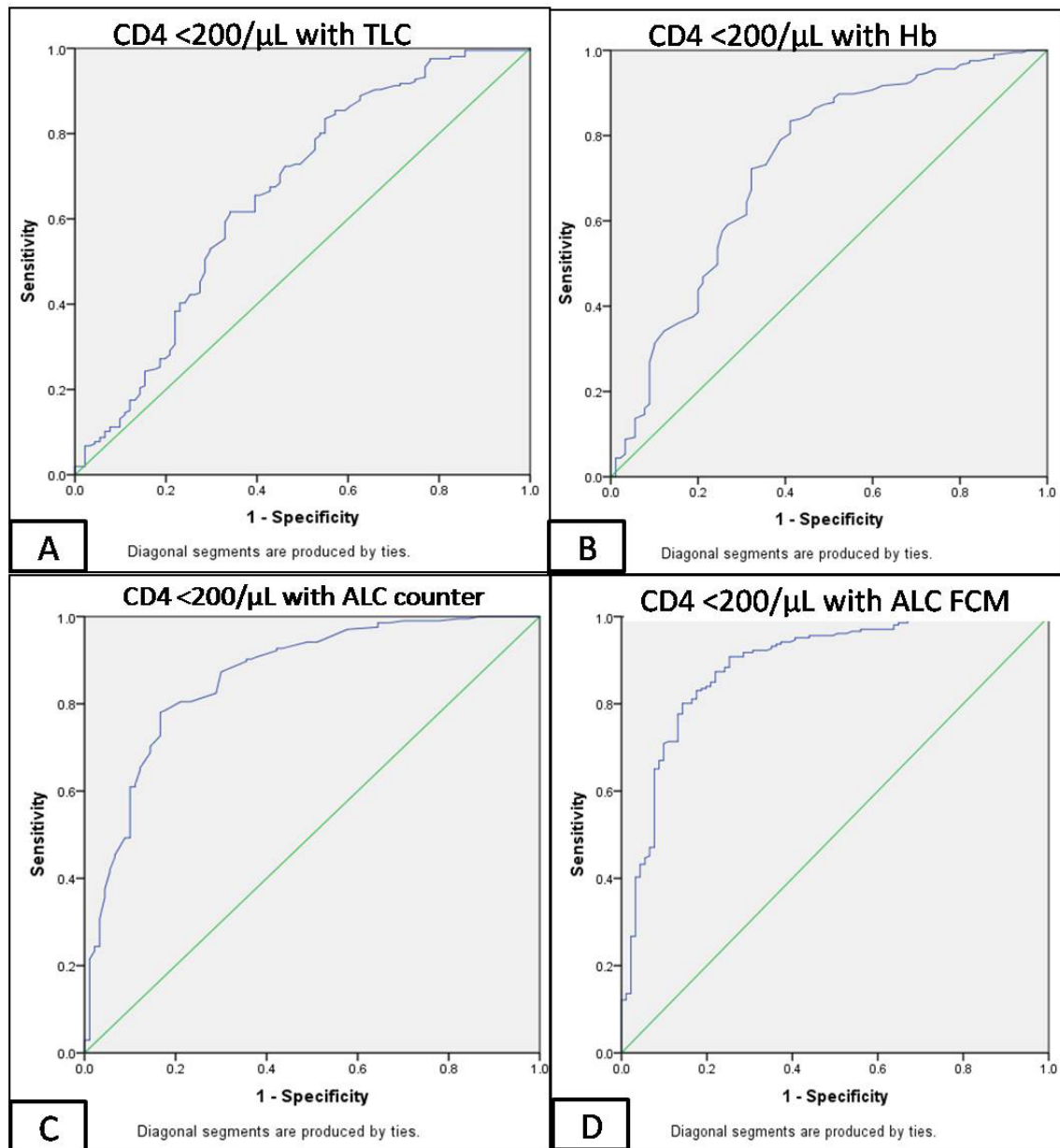
Figure 2: ROC curves.

every 3-6 months is not economically viable. Indices from automated cell counters can indicate the likely stage at diagnosis as well as response to therapy in these patients.

In our study, we found that ALC counter had a high correlation with ALC FCM and can be relied upon as equivalent to the accurate ALC FCM. On analyzing the correlation between the cell counter values and absolute CD4 count, we found the best Spearman co-efficient with ALC FCM (0.761) which was followed closely by ALC counter (0.701). It was also seen that on linear regression analysis (Figure 1), the R^2 linear was 0.838 indicating that ALC counter is almost equivalent to ALC FCM. Almost 43.3% variability in CD4 count could be predicted by ALC counter. In any scientific study, there are many independent variables associated with the outcome variable. Any single variable predicting >20% variability in outcome is considered as a significant predictor.

The predictive value of TLC and Hb was found to be <20%. Hence only ALC FCM and ALC counter were significant predictors of CD4 count.

Gautam et al. [8] identified Hb<12 g/dL as an indicator for picking up a CD4 count <200/μL. We found that Hb<12g/dL had corrcoeff (ρ)=0.334 to predict CD4 count <200/μL (p=0.002).In our cohort of patients, samples with CD4 count <200/μL, the best cut-off for TLC and Hb to predict CD4 count <200/μL was 6700/μL and 11.15g/dL, respectively. However, TLC had the lowest sensitivity, specificity and PPV and hence, was not a good parameter to determine CD4 count <200/μL.

Best cut-offs were also determined for both ALC counter and ALC FCM to predict CD4 count <200/μL and were 1250/μL and 1178/μL respectively. Both had a sensitivity of >85% with a specificity of ≥70%. The PPV and NPV were >70% and >85% respectively. Our cut-off

was very similar to the 1200/µL reported from Ghana [11] and in an earlier paper from India [8]. The cut-off that we obtained is lower than the 1520/µL recommended by Kakar et al. [9] and higher than 1000/µL recommended by Shapiro et al. [7]. A study from Providence, USA carried out in the emergency department found that ALC <950/µL predicted a CD4 count<200/µL. In addition, they found that ALC >1700/µL was likely to rule out a CD4 count <200/µL [12].

As per multivariate analyses, TLC was statistically insignificant predictor for CD4 count <200/µL. Both ALC counter <1250/µL and Hb <11.15 g/dL had a significant association and can be used to predict a CD4 count <200/µL, though bivariate linear regression did not find Hb to be a robust indicator of CD4 values in all ranges (Table 2). The combination of both Hb<11.15g/dl and ALC counter <1250/µL was like a confirmatory test with a specificity of 92.2% and a NPV of 79.5%. However, ALC<1250/µL is the best surrogate marker with a high sensitivity which can pick up CD4 count<200/µL. Both ART naïve PLHA and PLHA on ART can be monitored by 3monthly haemograms and ALC counter and Hb values below the cut-off either separately or in combination are indicators to do the expensive CD4 count in resource restricted countries where a routine 3-6 monthly CD4 count is not economically feasible. Also, in India, flow cytometry is not available at tier 3 and 4 cities as well as in villages while the automated cell counters are available more readily and the cost of a haemogram is usually <INR 300 while that of flow cytometry is >INR 1500. Hence both the availability and cost of the automated cell counter is better than flow cytometry and is a feasible alternative to an expansive test.

The earlier cut-offs of surrogate markers for picking up low CD4 counts were based on studies done with the less sensitive dual platform method of estimation [9] or were restricted to ART-naïve patients only [7,8], whereas the present study is based on data of patients at diagnosis and follow up and CD4 count has been calculated on the more accurate single platform flow cytometry [10].

Thus, we recommend that in PLHAs, routine monitoring may be done only by ALC and Hb determined on automated cell counters and values <1250/µL and <11.15g/dl, respectively be taken as warning signs of progression of disease/ failure of therapy. We recommend that a CD4 count may be restricted to patients fulfilling the above criteria. This may also mandate a workup for OIs and alternative ART.

Conclusions

ALC and Hb obtained on automated cell counter are robust, cost-effective and easily available methods to follow up PLHA patients and patients on ART. These can effectively predict the CD4 count <200/µL.

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