Validation of a Single-Platform, Volumetric, CD45-Assisted PanLeucogating Auto40 Flow Cytometer To Determine the Absolute Number and Percentages of CD4 T Cells in Resource-Constrained Settings Using Cameroonian Patients’ Samples

François-Xavier Mbopi-Kéou,a Stefano Mion,b Bertrand Sagnia,c and Laurent Bélec

Laboratoire National de Santé Hygiène Mobile, Ministère de la Santé Publique, Yaoundé, Cameroon, and Faculté de Médecine et des Sciences Biomédicales, Université de Yaoundé I, Yaoundé, Cameroon; Laboratoire de Virologie, Hôpital Européen Georges Pompidou, and Faculté de Médecine Paris Descartes, Université Paris Descartes (Paris V), Sorbonne Paris Cité, Paris, France; and Centre International de Référence Chantal Biya, Yaoundé, Cameroon

The study evaluated the single-platform, volumetric, CD45-assisted PanLeucogating Auto40 flow cytometer (Apogee Flow Systems Ltd., Hemel Hempstead, United Kingdom) for CD4 T cell enumeration, compared to the reference FACSCalibur flow cytometer. Results of absolute counts and percentages of CD4 T cells by Auto40 and FACSCalibur of 234 tripotassium EDTA (K3-EDTA)-blood samples from 146 adults and 88 children (aged from 18 months to 5 years), living in Yaoundé, Cameroon, were highly correlated ($r^2 = 0.97$ and $r^2 = 0.98$, respectively). The mean absolute bias and relative bias between Auto40 and FACSCalibur absolute CD4 T cell counts were +9.6 cells/µl, with limits of agreement from −251 to 270 cells/µl, and +4.1%, with limits of agreement from −16.1 to 24.4%, respectively. The mean absolute bias and relative bias between Auto40 and FACSCalibur absolute CD4 T cell results expressed as percentages were +0.05% CD4 (95% confidence interval [CI], −0.03 to 0.41), with limits of agreement from −6.0 to 5.9% CD4, and +1.0%, with limits of agreement from −32.3 to 34.4%, respectively. The Auto40 counting allowed identification of the majority of adults with CD4 T cell counts below 200 cells/µl (sensitivity, 87%; specificity, 98%) or below 350 cells/µl (sensitivity, 92%; specificity, 98%) and of children with CD4 T cell counts below 750 cells/µl (sensitivity, 82%; specificity, 98%) or below 25% CD4$^+$ (sensitivity, 96%; specificity, 99%). The Auto40 analyzer is a reliable alternative flow cytometer for CD4 T lymphocyte enumeration to be used in routine immunological monitoring according to the WHO recommendations for HIV-infected adults as well as children living in resource-constrained settings.

The 2010 revised guidelines of the World Health Organization (WHO) for scaling up of antiretroviral treatment (ART) in adults and children living in resource-limited settings (35, 36) emphasize the need for laboratory monitoring, based first on immunological assessment by the numeration of CD4 T lymphocytes, mainly to start ART and monitor patients on ART, and second on HIV-1 RNA load in order to monitor treatment efficacy and early therapeutic failure in patients on ART as well as to monitor therapeutic switching (3).

Affordable CD4 T cell counting has gradually become possible by using simple, compact, and robust low-cost new-generation cytometers operating as single-platform volumetric instruments without the use of expensive microbeads (9, 20, 21). The recently developed Auto40 flow cytometer (Apogee Flow Systems Ltd., Hemel Hempstead, United Kingdom) was originally developed for military applications (10). The Auto40 assay is based on a no-lyse procedure (12), which avoids the red blood cell lysis step, thereby reducing assay variability due to changes in assay conditions (time and temperature of incubation) as well as differences in the susceptibility of cells to the lysis reagents (6). The Auto40 analyzer uses a volumetric syringe moved by a stepper motor that draws and delivers a known sample volume. Therefore, its absolute volumetric counting allows the direct determination of the number of cells per unit of sample volume without the need for reference material such as microbeads (9, 19). Due to the stability of its optical bench, the Auto40 may be used on peripheral stationary as well as mobile flow cytometry units (10). The Auto40 analyzer was initially intended for CD4 T cell enumeration based on primary CD4 gating, and it has been validated for measuring absolute CD4 T cell count by reference to the FACSCount system (10). The analyzer has been recently updated for CD4 T cell measurement within 30 min, using PanLeucogating protocol with primary CD45 gating followed by secondary CD4 gating, which allows the user to obtain in a single measurement the CD4 T cell count expressed as both an absolute count and a percentage and, therefore, to address the current WHO recommendations for CD4 T cell measurements in children younger than 5 years (36). The aim of the present study was to evaluate the usefulness of the simplified Auto40 flow cytometry system for CD4 T cell numeration in absolute count and in percentage, compared against a reference flow cytometry method.

MATERIALS AND METHODS

Clinical specimens and processing. In January 2010, tripotassium EDTA (K3-EDTA)-blood samples obtained by venipuncture in Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) ($n = 234$) were received for routine biological monitoring at the Laboratoire National de Santé Hygiène...
Mobile (LNSHM), Yaoundé, Cameroon, an institutional reference laboratory devoted to HIV screening and monitoring (18). Two aliquots were kept at ambient temperature. No extra specimens were required. All blood samples were unlinked to identifiers. The study was approved by the Cameroon Ministry of Public Health. All participants, including children’s guardians or parents, signed an informed consent.

Each aliquot was first subjected to CD4 T cell count by Auto40 flow cytometer within 1 h at the LNSHM, and a second aliquot was sent within 4 h at ambient temperature to The Chantal Biya International Reference Centre for Research and Prevention of HIV/AIDS (CIRCB), Yaoundé, for immediate measurement by flow cytometry reference analyzer.

**CD4 T cell measurements.** CD4 T cell counting was performed in parallel on two different flow cytometers. The first was the FACSCalibur (Becton Dickinson Immunocytometry System [BDIS], San Jose, CA), a dedicated clinical reference flow cytometer for CD4 T cell counting installed at the CIRCB, using Multitest reagent (BDIS) and MultiSet V2.2 software (BDIS) for calculating the absolute and percentage values of CD4 T cells according to the instructions provided by the manufacturer, as previously described (28, 29). The second was the Auto40 flow cytometer (Apogee Flow Systems Ltd.) equipped with a green laser at 532 nm, a side scatter detector, two fluorescence channels, and means for direct volumetric counting without requiring a red blood cell lysis step.

Direct volumetric CD4 T cell measurements were performed on the Auto40 using phycoerythrin (PE)-conjugated anti-CD4 and PE-Dyomics 649-conjugated anti-CD45 monoclonal antibodies (Apogee Flow Systems Ltd.). The Auto40 analytical procedure avoids the need for a wash step. Briefly, 50 μl of whole EDTA-blood was added into polypropylene test tubes containing predispensed, stabilized monoclonal antibodies. After 25 min of incubation at room temperature in the dark, the blood samples were diluted 1:10 in phosphate-buffered saline. The no-lyse-no-wash stained samples were run on the Auto40 flow cytometer, and the CD4 T cell count was obtained as an absolute number and as a percentage. Analysis on the Auto40 flow cytometer is automatically performed by the built-in software Auto-Lymphocyte (Apogee Flow Systems Ltd.), with the possibility of controlling and assessing the quality of the data analysis. CD45-positive lymphocytes and monocytes were identified by primary gating on bright CD45 fluorescent cells in a CD45 x-axis scatter dot plot scattergram (Fig. 1A and C). The CD4 fluorescent polymorphonuclear cells were excluded from the gating because of their high nuclear density. Within the CD45-positive cells, CD4-positive and CD4-negative lympho-
Precision is the mean of the three precisions calculated for three different counts of CD4 T cells. Measurement, expressed in absolute count and in percentage respectively, 157 and 383 CD4 T cell counts per blood samples of low and medium CD4 T cell counts from the QASI CD4 Immunology, Canada). During the study period, the CIRCB received two Scheme, United Kingdom) and QASI (Quality Assessment Scheme for UKNEQAS (United Kingdom National External Quality Assurance CIRCB was performed on a regular basis using samples provided by were performed by using Calibrite beads (BDIS) and FACSComp software (BDIS). External quality control of the flow cytometry platform at compensation settings were optimized prior to sample acquisition. In addition, weekly calibration and internal quality control of the FACSCalibur instrument, the same lots of reagents were used throughout the study, and manipulations by technicians.

To ensure quality control of the flow cytometric immunophenotyping method with regard to the performance of both the personnel and the instrument, the same lots of reagents were used throughout the study, and all sample preparations and flow cytometric analyses were performed by the same operator for each instrument, as previously reported (25). Adequate training on the use of the reverse pipetting technique and electronic pipette was also provided for each operator. Furthermore, the FACSCalibur photomultiplier tube voltage, sensitivity, and fluorescent compensation settings were optimized prior to sample acquisition. In addition, weekly calibration and internal quality control of the FACSCalibur were performed by using Calibrite beads (BDIS) and FACSComp software (BDIS). External quality control of the flow cytometry platform at CIRCB was performed on a regular basis using samples provided by UKNEQAS (United Kingdom National External Quality Assurance Scheme, United Kingdom) and QASI (Quality Assessment Scheme for Immunology, Canada). During the study period, the CIRCB received two blood samples of low and medium CD4 T cell counts from the QASI CD4 EQA program. The FACSCalibur results for these samples were, respectively, 157 and 383 CD4 T cell counts per μl, within a residual of −5 and −25 and a standard deviation (SD) index of −0.22 and −0.60.

Quality control on the Auto40 flow cytometer was primarily assessed by the use of a calibrated bead sample (Apogee catalog number 1444 for Auto40-green; Apogee Flow Systems Ltd.) at the beginning and end of each session, with the use of stabilized blood reference samples (Cytofix CD4; Caltag-Medsystems Ltd., United Kingdom) as an additional external control.

Assessment of the precision of CD4 T cell counting by Auto40. The intrinsic precision of CD4 T cell counting on the Auto40 instrument was assessed using three different blood samples with CD4 T cell counts of clinical interest. Intrarun (instrument) precision was determined by preparing a large volume (10 ml) of CD45/CD4-stained blood and repeating sample acquisition on the instrument 10 times to determine the instrument’s precision. Intrarun precision, taking into account variations of pipetting made by the technician (tube-to-tube variability), was assessed by repeating the entire CD45/CD4 staining procedure 10 times, including pipetting, sample preparation, staining, and sample acquisition. Interperson variation (between different technicians) was not assessed. Precision was expressed as the coefficient of variance (CV) obtained by dividing the SD of all measurements by their mean (CV% = SD × 100/mean).

Statistical analyses. The Method Validator software, version 1.1.9.0. (Philippe Marquis, France) and the SAS-PC software (version 8.2; SAS Institute, Cary, NC) were used for statistical analyses. First, correlations between the absolute CD4 T cell counts obtained by the reference FACSCalibur and the Auto40 were established by the Passing-Bablok nonparametric method, which has sensitivity to outliers (22). Second, the agreement between FACSCalibur and Auto40 was depicted by difference plots as proposed by Bland and Altman (4, 5) and Pollock et al. (26). The Bland-Altman and Pollock analyses were carried out to calculate the mean absolute and relative bias and limits of agreement, respectively, corresponding to the 95% confidence intervals (95% CI) (± 1.96 × SD) of the mean absolute and relative biases of all paired measurements (5).

To assess the clinical impact of using the Auto40 instead of the FACSCalibur in this setting, the sensitivity and the specificity of the Auto40 were calculated to identify patients whose FACSCalibur CD4 T cell count was below 200 cells/μl, the threshold of immune restoration under ART and the threshold for therapeutic initiation according to the 2006 revised WHO recommendations (34), 350 cells/μl, the new threshold for ART initiation for adults and children aged more than 5 years according to the 2010 revised WHO guidelines (35), or 750 cells/μl with a percentage of CD4+ cells of ≤25%, the new absolute and percent CD4 T cell count WHO thresholds for ART initiation in children aged between 24 and 59 months (36). For clinical significance of the measurement differences for treatment decision, Cohen’s k coefficient was calculated for the study population (8).

RESULTS

Precisions of direct volumetric CD4 T cell measurements by the Auto40. The intra- and interrun precisions were tested using three blood samples with low (<200 cells/μl), intermediate (between 200 to 350 cells/μl), and high (>750 cells/μl) CD4 T cell counts within the ranges of values of medical interest. The results are shown in Table 1. For each level of blood CD4 T cell, CD4 T cell measurements by percentage showed slightly higher intrarun and interrun precisions than those by absolute number.

### Table 1: Intrarun and interrun precision of single-platform, volumetric, CD45-panLeucogating Auto40 flow cytometer for CD4 T cell measurement, expressed in absolute count and in percentage

| Cell group | Range (cells/μl) | Intrarun assay | Interrun assay |
|------------|-----------------|----------------|---------------|
|            | No. (mean ± SD) | Precision (%)  | No. (mean ± SD) | Precision (%)  |
| Measured by absolute count | | | | |
| Low        | <200            | 103 ± 9        | 8.7           | 122 ± 11       | 9.0           |
| Medium     | 200–350         | 415 ± 17       | 4.0           | 431 ± 21       | 4.8           |
| High       | >750            | 921 ± 29       | 3.1           | 937 ± 39       | 4.1           |
| Mean       |                 | 5.2            |               | 5.9           |               |
| Measured by percentage | | | | |
| Low        | <200            | 11.3 ± 0.9     | 7.9           | 11.5 ± 1.0     | 8.6           |
| Medium     | 200–350         | 19.2 ± 0.7     | 3.6           | 19.6 ± 0.8     | 4.0           |
| High       | >750            | 36.0 ± 1.1     | 3.0           | 37.1 ± 1.3     | 3.5           |
| Mean       |                 | 4.8            |               | 5.3           |               |

a Precision is the coefficient of variance (CV) obtained by dividing the SD of all the intrarun or interrun measurements by their mean (CV% = SD × 100/mean); the mean precision is the mean of the three precisions calculated for three different counts of CD4 T cells.

b Mean ± SD of 10 results obtained by repeating 10-fold CD45/CD4-stained blood sample acquisition on Auto40.

c Mean ± SD of 10 results obtained by repeating 10-fold the entire CD45/CD4 procedure and further sample acquisition on Auto40.

d Intrarun precisions obtained with FACSCalibur were 5.2%, 4.1%, and 2.5% for low, medium, and high CD4 T cell counts, respectively.

e Interrun precisions obtained with FACSCalibur were 6.1%, 4.2%, and 2.5% for low, medium, and high CD4 T cell counts, respectively.
Accuracy of direct volumetric CD4 T cell measurements by the Auto40. A total of 234 EDTA-blood samples with correct pre-analytical preparation were obtained from 146 adults (median age, 37 years; 67 males), and 88 children less than 5 years and more than 18 months old (40 males). The absolute and relative biases (and the limits of agreement) for CD4 T cell counts, in absolute numbers and percentages, for 146 adults and 88 children less than 5 years and more than 18 months, including 104 HIV-1-infected individuals (69 adults; 35 children), by the Apogee Auto40 flow cytometer and the FACSCalibur, and in Table 3 at various CD4 T cell count ranges.

The mean ± SD CD4 T cell counts expressed as absolute numbers were 1,010 ± 795 cells/μL (range, 4 to 4,303) by Auto40 and 1,001 ± 801 cells/μL (range, 5 to 4,472) by FACSCalibur (P > 0.5). The non-parametric Passing-Bablok regression analysis of all 234 available T cell results expressed as absolute counts revealed a high correlation between CD4 T cell results by Auto40 and FACSCalibur (r² = 0.97).

### TABLE 2 CD4 T cell counts in absolute numbers and percentages in adults and children by the Apogee Auto40 flow cytometer and the FACSCalibur

| Parameter and patient group | Result by age group | Result by HIV-1 status |
|-----------------------------|---------------------|-----------------------|
|                             | Adults              | Children              | Negative | Positive |
| No. of patients             | 146                 | 88                    | 130      | 104      |
| Absolute no. (cells/μL)     |                     |                       |          |          |
| Apogee Auto40 result (mean ± SD) | 661 ± 441         | 1,589 ± 908           | 1,424 ± 750 | 492 ± 488 |
| FACSCalibur result (mean ± SD) | 658 ± 440         | 1,569 ± 933           | 1,427 ± 748 | 467 ± 482 |
| Absolute bias (limits of agreement [mean ± 95% CI]) | 3.6 (−195.7, 202.9) | 20.5 (−318.5, 359.5) | −2.6 (−305.6, 300.4) | 25.5 (−163.5, 214.5) |
| Relative (%) bias (limits of agreement [mean ± 95% CI]) | 4.5 (−41.2, 50.2) | 3.7 (−24.3, 31.5) | 0.2 (−22.3, 22.7) | 9.3 (−44.7, 63.3) |
| % CD4 cell group            |                     |                       |          |          |
| Apogee Auto40 result (mean ± SD) | 31.7 ± 13.0        | 32.5 ± 12.2           | 35.5 ± 12.4 | 21.0 ± 12.0 |
| FACSCalibur result (mean ± SD) | 31.9 ± 12.7        | 32.7 ± 13.0           | 35.5 ± 9.6  | 21.0 ± 9.8  |
| Absolute bias (limits of agreement [mean ± 95% CI]) | 0.1 (−6.0, 6.2) | −0.2 (−5.9, 5.4) | −0.1 (−6.5, 6.3) | 0.0 (−5.2, 5.3) |
| Relative (%) bias (limits of agreement [mean ± 95% CI]) | 1.4 (−38.4, 41.2) | -2.75 (−17.9, 23.4) | -0.0 (−22.1, 21.9) | 2.4 (−41.3, 46.1) |

*CD4 T cell counts, in absolute numbers and percentages, for 146 adults and 88 children less than 5 years and more than 18 months, including 104 HIV-1-infected individuals (69 adults; 35 children), by the Apogee Auto40 flow cytometer at the Laboratoire National de Santé Hygiène Mobile, Yaoundé, Cameroon, and by the FACSCalibur at The Chantal Biya International Reference Centre for Research and Prevention of HIV/AIDS, Yaoundé, Cameroon.

*The Bland-Altman analysis was carried out to calculate the absolute bias and limits of agreement, which are the 95% confidence intervals (±1.96 × SD) of the mean biases of all paired measurements in a given category.

*The Pollock analysis was carried out to calculate the relative bias and limits of agreement, which are the 95% confidence intervals (±1.96 × SD) of the mean biases of all paired measurements in a given category.

### TABLE 3 CD4 T cell counts by absolute count and percentage, expressed by T-cell group

| Parameter and patient group | Result by group size |
|-----------------------------|---------------------|
|                             | Low (<200 cells/μL) | Medium (200-350 cells/μL) | High (>350 cells/μL) |
| No. of patients             | 17                  | 34                    | 183                |
| Absolute no. (cells/μL)     |                     |                       |                   |
| Apogee Auto40 result (mean ± SD) | 103 ± 84           | 297 ± 68              | 1,227 ± 767        |
| FACSCalibur result (mean ± SD) | 85 ± 62            | 283 ± 46              | 1,219 ± 773        |
| Absolute bias (limits of agreement [mean ± 95% CI]) | 18.8 (−66.8, +104.4) | 14.0 (−75.6, +103.6) | 8.1 (−289.3, +305.5) |
| Relative (%) bias (limits of agreement [mean ± 95% CI]) | 42.5 (−57.8, +142.8) | 4.7 (−25.1, +34.6) | 1.5 (−27.7, +30.7) |
| % CD4 cell group            |                     |                       |                   |
| Apogee Auto40 result (mean ± SD) | 7.9 ± 4.7          | 17.8 ± 8.9            | 33.1 ± 11.1        |
| FACSCalibur result (mean ± SD) | 7.5 ± 4.4          | 17.4 ± 9.1            | 33.2 ± 10.8        |
| Absolute bias (limits of agreement [mean ± 95% CI]) | 0.4 (−5.7, +6.4) | 0.4 (−4.2, +4.9) | −0.2 (−6.5, +6.2) |
| Relative (%) bias (limits of agreement [mean ± 95% CI]) | 9.6 (−88.7, +107.9) | 3.1 (−21.4, +87.6) | −0.2 (−22.3, +22.0) |

*CD4 T cell counting in absolute count and percentage in 234 individuals, by the Apogee Auto40 flow cytometer at the Laboratoire National de Santé Hygiène Mobile, Yaoundé, Cameroon, and by the FACSCalibur at The Chantal Biya International Reference Centre for Research and Prevention of HIV/AIDS, Yaoundé, Cameroon, at various CD4 T cell count ranges according to the FACSCalibur results.

*The Bland-Altman analysis was carried out to calculate the absolute bias and limits of agreement which are the 95% confidence intervals (±1.96 × SD) of the mean biases of all paired measurements in a given category.

*The Pollock analysis was carried out to calculate the relative bias and limits of agreement which are the 95% confidence intervals (±1.96 × SD) of the mean biases of all paired measurements in a given category.

*Results in absolute CD4 T cell counts by Auto40 and FACSCalibur were correlated using the nonparametric Passing-Bablok regression analysis (<200 cells/μL: r² = 0.87, slope = 2.6, intercept = 1.17; 200-350 cells/μL: r² = 0.81, slope = 1.17, intercept = 1.17; >350 cells/μL: r² = 0.98, slope = 1.01, intercept = 1.01).

*Results in percentage CD4 T cell counts by Auto40 and FACSCalibur were correlated using the nonparametric Passing-Bablok regression analysis (<200 cells/μL: r² = 0.89, slope = 1.00, intercept = 0.0; 200-350 cells/μL: r² = 0.96, slope = 1.00, intercept = 0.0; >350 cells/μL: r² = 0.97, slope = 1.00, intercept = 0.0).
with a slope of 1.01 (95% CI, 0.98 to 1.04) and an intercept of +7.1 (95% CI, −6.9 to 23.6) (Fig. 2A). The mean absolute bias measured by Bland-Altman analysis and the mean relative bias measured by Pollock analysis between Apogee Auto40 flow cytometer and FACSCalibur CD4 T cell results over the entire range of CD4 T cell results were 9.6 cells/μl (95% CI, 7.5 to 26.8), with limits of agreement from −251 to 270 cells/μl (Fig. 2B), and +4.1%, with limits of agreement from −16.1 to 24.4%, respectively.

The mean ± SD CD4 T cell counts as percentages were 29.1% ± 12.8% (range, 1 to 70) by Auto40 and 29.0% ± 13.1% (range, 1 to 75) by FACSCalibur (P > 0.5). The results of CD4 T cell counts as percentages by Auto40 and FACSCalibur were highly correlated by regression analysis (r² = 0.98), with a slope of 0.99 (95% CI, 0.95 to 1.02) and an intercept of +0.1 (95% CI, −0.9 to 1.3) (Fig. 2C). The mean absolute bias and relative bias between Apogee Auto40 and FACSCalibur CD4 T cell results, as percentages, were +0.05% (95% CI, −0.03 to 0.41), with limits of agreement from −6.0 to 5.9% (Fig. 2C), and +1.0%, with limits of agreement from −32.3 to 34.4%, respectively.

Sensitivity and specificity to identify clinically relevant thresholds by the Auto40. The sensitivity and specificity of CD4 T cell counting by the Auto40 to identify patients having less than (or more than) 200 CD4 T cells/μl, 350 CD4 T cells/μl, 750 CD4 T cells/μl, and 25% CD4⁺ cells were calculated for the 234 available CD4 T cell count measurements and are depicted in Table 4. Whatever threshold was considered, the concordance between the Auto40 and FACSCalibur methods was high, and the decision did not differ between study blood samples according to both methods (κ = 0.95 to 0.97; P < 0.01).

### DISCUSSION

In the present study, we demonstrated that the Auto40 flow cytometer, operated by a laboratory technician, performs acceptably compared with the FACSCalibur for CD4 T cell measurement expressed as an absolute number as well as a percentage. The Auto40 flow cytometer results as an absolute number and as a percentage gave high levels of correlations with those obtained by the reference flow cytometer method. The correlation was maintained at different CD4 T cell count ranges over all the dynamic range of values in absolute number (up to 4,472 CD4 T cells/μl) as well as in percentage (up to 75% CD4⁺ cells). CD4 T cell counting by CD45-assisted Auto40 allowed us to identify the majority of...
threshold at 25% CD4 for an age of more than 5 years (35). Determinations were done on the 88 available count measurements from children.

Our study confirms the interest in the Auto40 analyzer for CD4 T cell enumeration in developing countries, as recently reported for Senegal by Dieye et al. (10). The Senegalese validation used the Auto40 analyzer, which measured only absolute CD4 T cell numbers, was completed. The technique was found to be easy to carry out and highly reproducible, with low intra- and interrun precisions, less than 10%, which is considered acceptable for clinical use (10). Taken together, these findings demonstrate that the simplified, single-platform, volumetric, CD45-assisted PanLeucogating Auto40 flow cytometer is a reliable alternative flow cytometer for CD4 T lymphocyte enumeration to be used in routine immunological monitoring according to the WHO recommendations for HIV-infected adults as well as children living in resource-constrained settings.

Our study confirms the interest in the Auto40 analyzer for CD4 T cell enumeration in developing countries, as recently reported for Senegal by Dieye et al. (10). The Senegalese validation used the initial Auto40 version based on primary CD4 gating and focused exclusively on CD4 T cell counting in absolute number (10). However, because the CD4 T lymphocyte percentage is essential for monitoring HIV disease progression in children less than 5 years old (21, 35), the primary CD4 gating format of the Auto40 analyzer, which measured only absolute CD4 T cell numbers, was modified by the manufacturer in 2006 to measure the percentage of CD4 T cells. Thus, our observations extend those of the previous study by demonstrating that the updated version of the Auto40 flow cytometer, now based on a PanLeucogating protocol using anti-CD45 and anti-CD4 monoclonal antibodies, is valid for CD4 T cell enumeration not only by absolute number but also by percentage. Finally, the present independent validation of the Auto40 analyzer in the HIV-dedicated reference LNSHM laboratory in Cameroon well fulfilled the WHO recommendations for validation of CD4 T cell assays in resource-poor settings (33) and independently reinforces and extends the previous Senegalese conclusions concerning the clinical interest of the Auto40 flow cytometer for immunological monitoring of HIV-infected patients.

Immunophenotyping using flow cytometry is the reference method for the enumeration of the CD4 marker on T lymphocytes (2). Initially, the use of a large panel of reagents and stringent gating techniques for full lymphocyte subset analysis was shown to be superfluous and expensive (17). The simplified flow cytometric PanLeucogating protocol is based on a sequential strategy by initially gating on the total white blood cell population (CD45-positive population), which then serves as the common denominator to enumerate CD4-positive T lymphocytes instead of primarily gating on the error-prone lymphocyte population (30). The specific use of the side scatter parameter allows for the discrimination of monocytes due to the high side scatter and low CD4 expression of monocytes, thereby enabling accurate CD4-positive T cell enumeration. The CD4-positive lymphocytes are low side scatter and high CD4-expressing cells (11). The PanLeucogating method has been demonstrated to allow accurate CD4 T cell counting when used in dual platforms (11, 23, 24) as well as single platforms, either volumetric (14) or bead based (31, 32).

The performance of single-platform, volumetric flow cytometric systems operating with CD45-based gating and generic monoclonal antibodies has been previously reported for a limited number of commercially available analyzers, including mainly the microcapillary-based Guava AutoCD4/CD4% (Merck Millipore, Münster, Germany) (16). Both latter analyzers have proven their usefulness for validating absolute number- and percentage-based CD4 T cell counting in the field (16, 25). These single platforms using only a flow cytometer and volumetric systems allow counting of CD4 T cells in a fixed volume, thus leading to a considerable reduction in the costs of CD4-positive T cell enumeration in comparison to microbead-based systems (2). In addition, the compact low-range single platforms Auto40 and CyFlow SL_3 are portable desktop flow cytometers that do not require optical alignment, can run on a 12-volt car battery, and can be connected to a laptop computer, suggesting their possible use in health care mobile units and thereby making CD4 T cell enumeration available in remote or hard-to-reach locations. One of the additional main features of the Auto40 analyzer is the use of stabilized monoclonal antibodies. The thermostable monoclonal antibodies we used can be kept as long as 1 year at room temperature (75°C). Overall, the use of thermostable reagents increases the accessibility to flow cytometry testing, since the increase in manufacturing costs related to the antibody stabilization procedure does not exceed 15% of the original cost (1).

The accurate determination of CD4 T lymphocytes is crucial for clinical importance for caring for adults or children infected with HIV or suffering from AIDS. Although classical flow cytometry represents the reference method for CD4 T cell enumeration, the feasibility of flow cytometric methods in the field remains controversial in resource-poor settings (13). In the present study, a well-trained technician was able to use the Auto40 flow cytometer with low intra- and interrun variability, less than 10%, which is comparable to results of other published reports using single-platform flow cytometers (7, 15, 16, 25) and is acceptable in routine clinical practice (25). The low observed variability of Auto40 confirmed that single-platform flow cytometric methods are more reproduc-

### Table 4: Sensitivity and specificity of CD4 T cell counting by the Auto40

| Threshold | Sensitivity (%) | Specificity (%) | Cohen’s k coefficient |
|-----------|----------------|----------------|----------------------|
| 200 CD4 T cells/µl | 87 | 98 | 0.97 |
| 350 CD4 T cells/µl | 92 | 98 | 0.96 |
| 750 CD4 T cells/µl | 82 | 98 | 0.95 |
| 25% CD4 | 96 | 99 | 0.96 |

* a The sensitivity and specificity of Auto40 CD4 T cell counting to identify patients having less than (or more than) 200, 350, or 750 CD4 T cells/µl and 25% CD4 cells were calculated on the 234 available CD4 T cell count measurements.

* b Threshold of immune restoration under antiretroviral treatment and the threshold for therapeutic initiation according to the 2006 revised WHO recommendations (34).

* c Determinations were done on the 146 available count measurements from adults.

* d WHO thresholds for antiretroviral treatment initiation in adults and children aged more than 5 years (35).

* e Determination was done on the 146 available count measurements from adults.

* f WHO thresholds for antiretroviral treatment initiation in children aged between 24 and 59 months (36). Determinations were done on the 88 available count measurements from children.

* g A 10% bilateral range (i.e., counts between 190 and 210 CD4 T cells/µl) for the threshold at 200 CD4 T cells/µl and 20% (250 CD4 T cells/µl) for the threshold at 350 CD4 T cells/µl for the threshold at 350 CD4 T cells/µl, counts between 712 and 787 CD4 T cells/µl for the threshold at 750 CD4 T cells/µl, and counts of 23.7 and 26.2% CD4 for the threshold at 25% CD4 was considered similar.
ible than dual-platform methods (27). Finally, higher precisions for high than for low CD4 T cell count have been previously re-
ported for single-platform volumetric flow cytometric methods (16).

In conclusion, the Auto40 flow cytometer constitutes a prom-
ising system for performing single-platform absolute number and
percentage-based CD4 T lymphocyte counts with excellent repro-
ducibility, and it should facilitate wider access to CD4 T cell
enumeration for adults and children with HIV infection who live in
resource-constrained countries.

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We declare that we have no competing interests.

F.-X.M.-K. and L.B. conceived and designed the research; F.-X.M.-K.
and B.S.P. performed the experiments; L.B. and S.M. performed statistical analyses; F.-X.M.-K., L.B., and S.M. analyzed the results and drafted the manuscript.

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