Check for updates

Received: 10 November 2021 | Revised: 1 March 2022 | Accepted: 31 March 2022
DOI: 10.1002/cyto.b.22069

ORIGINAL ARTICLE

CLINICAL CYTOMETRY WILEY

Cross-sectional reassessment after 4 years of clinical routine use of AQUIOS CL for absolute T cell quantitation in a university hospital

Léa Lemoine[1] | Christophe Malcus[1] | Françoise Poitevin-Later[1] | Fabienne Venet[1,2] | Guillaume Monneret[1,2] | Morgane Gossez[1,2]

[1]Hospices Civils de Lyon, Edouard Herriot Hospital, Immunology Laboratory, Lyon, France
[2]Université Claude Bernard Lyon 1, Lyon, France

Correspondence
Morgane Gossez, Immunology laboratory - Hôpital Edouard Herriot – Hospices Civils de Lyon, 5 place d'Arsonval, 69437 Lyon CEDEX 03, France.
Email: morgane.gossez@chu-lyon.fr

Abstract

Background: We had previously reported appropriate performances of automated AQUIOS CL cytometer (Beckman Coulter) for regulatory approval of absolute T cell enumeration. However, after 4 years of routine use, we still observed recurrent histogram anomalies that may affect both absolute values and T cell subset percentages results. The objective of the current study was thus to perform a cross-sectional evaluation of these graphical anomalies within a university hospital context, to assess their influence on results and ultimately to propose a standardized decision tree to circumvent graphical disturbances at the time of results validation.

Methods: Eight hundred and sixty-two blood samples were prospectively analyzed on AQUIOS CL. Results were compared to (i) lymphocyte values from complete blood count; (ii) results from manual staining and analysis on Navios cytometer (Beckman Coulter); (iii) results after washing step and reacquisition on AQUIOS CL.

Results: Nearly 75% analyses did not show any graphical anomaly. 20% had single anomaly on "Lymphs (45)" or "Lymphs EV" regions influencing T cells percentages and requiring manual re-gating of "CD3− capture" region. 5% showed concomitant "Lymphs EV" and "Lymphs (45)" anomalies influencing both T cell percentages and absolute counting and requiring additional staining and analysis on Navios. Finally, <1% presented with anomaly on "CD4/CD8" histogram or "CD3+ All" region, influencing both T cell percentages and absolute counting.

Conclusions: Around 25% AQUIOS CL results were flawed due to gating anomalies. In <5% cases, additional back-up procedures should be undertaken to ensure results validity. A simple decision-tree may help in guiding validation process.

KEYWORDS
accreditation, aquios CL, flow cytometry, standardization, T cell

1 | INTRODUCTION

Flow cytometry represents a major tool in clinical laboratory for diagnosis and immune monitoring, especially in the context of hematologic malignancies and immune deficiencies. Moreover, recent rise in immunotherapy use in various contexts (e.g., auto-immunity, infection and cancer) has led to increased interest in immune monitoring to control drug efficacy, within which flow cytometry plays a central role. However, this specialized technique requires specific technical skills, and results may be affected by many parameters (manual staining

 © 2022 International Clinical Cytometry Society.

steps, analyzer setting, fluidic stability, gates definition, operator expertise). All these may hamper inter-laboratory reproducibility. Thus, in the context of EN ISO 15189 accreditation process imposed to medical laboratories in Europe; rigorous flow cytometry standardization is now mandatory (Sack et al., 2013).

AQUIOS CL (Beckman Coulter, Brea, CA, USA) is a single-platform, fully automated volumetric flow cytometry technology, mainly dedicated to T, B and Natural Killer lymphocyte absolute counting. In a pioneer work, we evaluated CD4+ T cell counting on AQUIOS CL and showed that results were in accordance with regulatory approval requirements (i.e., ISO 15189). Results were also in agreement with bead-based CD4+ T cell enumeration protocol used as a reference methodology (Gossez et al., 2017). Similar conclusions were drawn by other groups when evaluating AQUIOS CL performances for total T, B and NK quantitation compared to dual or single-platform (Degandt et al., 2018; Grossi et al., 2018). In resource-limited settings, a 2-color simplified protocol (PanLeucoGate) has been tested on AQUIOS CL for low-cost CD4+ lymphocyte enumeration with suitable results, especially desirable intra- and inter-laboratory variations (Coetzee & Glencross, 2017; Rhodes et al., 2019).

One of the strengths of AQUIOS CL is supposedly its automatic gating strategy, allowing minimal flow cytometry skills and experience from operators. However, in a recent study, Degandt et al., reported substantial percentages (i.e., 30%) of results associated with run notifications, indicating an abnormal cell distribution or population. In such cases, samples had to be rerun on AQUIOS CL or manually gated for better lymphocyte selection (Degandt et al., 2018).

Accordingly, during the last 4 years of daily routine use of AQUIOS CL in our clinical immunology laboratory at a University Hospital (about 100 T cell phenotypings per day), we also observed graphical/histogram abnormalities on a significant number of samples for which we used extra-procedures in order to ensure results sent to clinicians. However, the influence of these anomalies on both cell percentages and absolute counting has never been minutely characterized.

The main objectives of this cross-sectional study was thus to minutely depict graphical anomalies occurring on routine samples analyzed on AQUIOS CL within a university hospital context including mixed patients with various medical aetiologies during a given period of time, to determine their impact on T lymphocyte subset enumeration. A secondary objective was to define a simple decision-tree allowing results release to clinicians.

2 | MATERIALS AND METHODS

2.1 | Cohort

T cells phenotypes were prospectively analyzed from routine work load over a 10 day-period. T cell enumeration had been performed on AQUIOS CL from peripheral blood samples collected in K_3EDTA (BD Vacutainer®, Plymouth, UK), kept at room temperature and analyzed upon arrival within 24 h after collection. In accordance with our Local Ethics Committee's rules, no signed informed consent was needed as leftover samples were used.

2.2 | Collected data

For each sample, the following data were collected: medical department, concomitant complete blood count (CBC) results (including absolute values of leucocytes, total lymphocytes, and proportion of lymphocytes among leucocytes).

2.3 | AQUIOS CL automated method

Details of T cell quantitation method on AQUIOS CL were previously described (Gossez et al., 2017).

According to Beckman Coulter recommendations, AQUIOS CL performances were daily assessed by running both Aquios IMMUNO-TROL™ Cells and Aquios IMMUNO-TROL™ Low Cells internal quality controls (Beckman Coulter). The whole gating strategy (Figure 1) is as follows. An initial region "Cells" is drawn on FS/SS plot to eliminate cellular debris (Figure 1a). From "Cells" gate, a SS/CD45 plot with logarithmic scales allows selection and enumeration of leucocytes, excluding remaining red blood cells and cell debris presenting with negative expression of CD45 (Figure 1b). Lymphocyte selection is based on two regions, "Lymphs (45)" and "Lymphs EV", discriminating lymphocytes from monocytes and polymorphonuclear according to CD45 and electronic volume (EV) characteristics respectively (Figure 1c). Then, two Boolean gates are created: "Lymphs All", which favors cell recovery ("Lymphs (45)" OR "Lymphs EV") and "Lymphs", which emphasizes lymphocyte purity ("Lymphs (45)" AND "Lymphs EV"). On the one hand, "Lymphs" is used to select non-T cells on a monoparametric CD3 plot ("CD3−" region) (Figure 1d). On the other hand, "Lymphs All" is used to build a CD3 monoparametric plot allowing selection and enumeration of $CD3^+$ T cells ("CD3+ All" gate) (Figure 1e). From this latter, a CD4/CD8 graph is drawn, enabling quantitation of $CD4^+$ and $CD8^+$ T cells (Figure 1f). Among CD3 negative lymphocytes selected in the "CD3− All" region, those with high SS characteristics which were excluded from the "CD3− region" favoring lymphocyte purity (e.g., NK cells), are identified in a "CD3− Capture" gate (Figure 1g). Finally, absolute value of total lymphocytes is calculated adding cells included in gates: "CD3+ All" (representing T cells), "CD3− "and "CD3− Capture" (containing all non-T cells) (Figure 1h). After this gating strategy, absolute values of lymphocyte subsets are measured by a volumetric technology.

2.4 | Main histogram anomalies observed on AQUIOS CL

Four main types of anomalies were observed on key histograms for cell gating necessary to lymphocyte enumeration on AQUIOS CL (Figure 2).

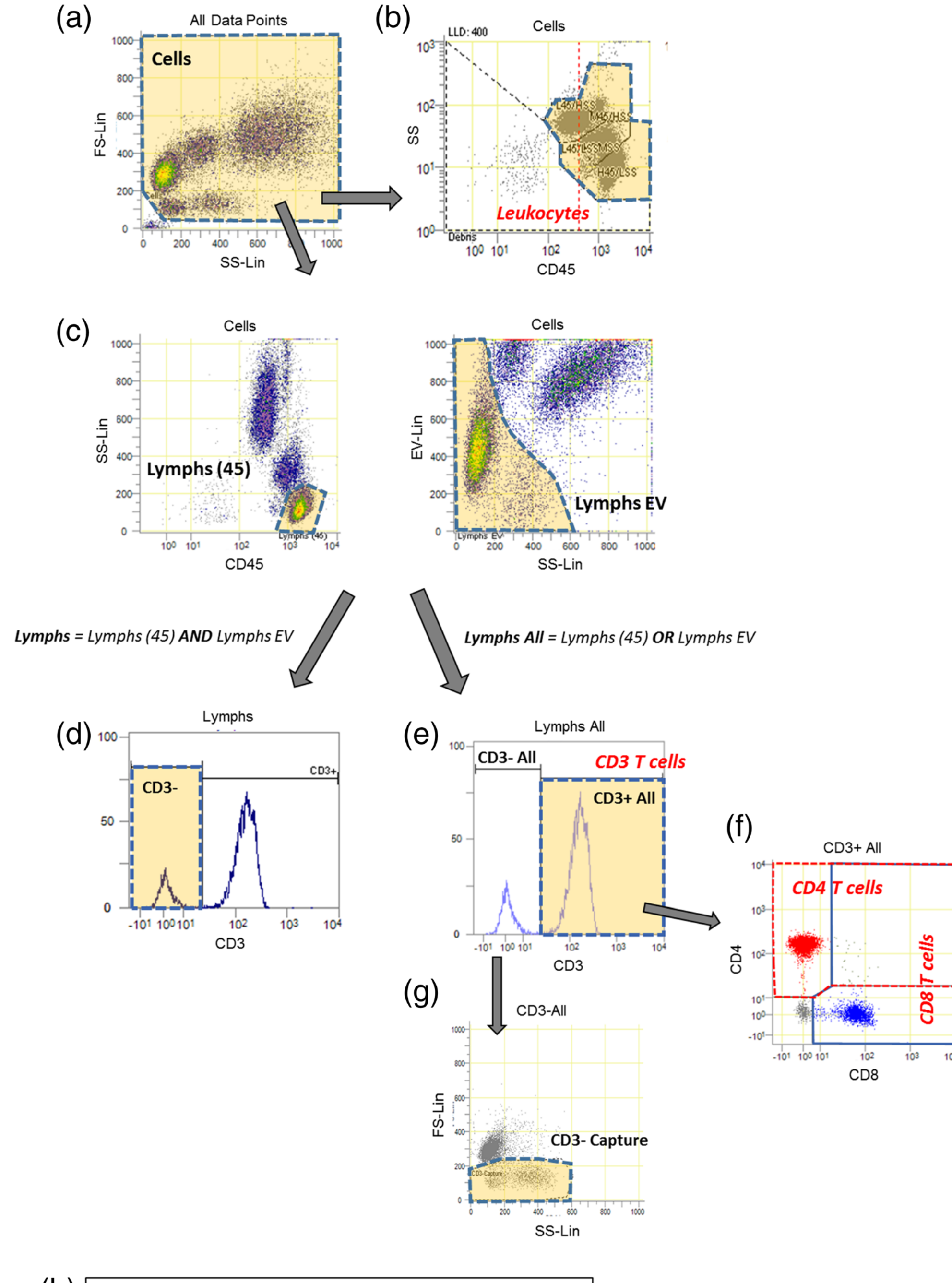


FIGURE 1 AQUIOS CL gating strategy. (a) Region "Cells" allows elimination of low FS and SS cellular debris. (b) Leukocytes are gating and measured on logarithmic SS/CD45 plot from "Cells." (c) Lymphocytes are selected from "Cells" based on two regions "Lymphs (45)" and "Lymphs EV." Then, two Boolean gates are created: "Lymphs All", which favors cell recovery and "Lymphs", which emphasizes lymphocyte purity. Two monoparametric CD3 plots are generated from "Lymphs" (d) and "Lymphs All" (e), in order to select non-T cells ("CD3−" region) and count $CD3^{+}$ T cells ("CD3+ All") respectively. (f) CD4/CD8 histogram is drawn from "CD3+ All" gate, enabling quantitation of $CD4^{+}$ and $CD8^{+}$ T cells. (g) CD3 negative lymphocytes from "CD3− All" region may comprised lymphocytes with high SS characteristics excluded from "CD3− region", which are identified and measured in "CD3− Capture" gate. (H) Total lymphocyte enumeration is calculated adding cells from "CD3+ All" (representing T cells) and ("CD3− "OR "CD3− Capture") (containing all non-T cells) [Color figure can be viewed at wileyonlinelibrary.com]

- Cell dispersion on SS/CD45 histogram (i.e., "Lymphs (45)" gate),
- Cell dispersion on EV/SS histogram (i.e., "Lymphs EV" gate),
- Cell dispersion on CD3 histogram (i.e., "CD3+ All" gate).
- Non-specific staining on CD4/CD8 histogram.

2.4.1 | Complete blood count

Results of lymphocyte count from AQUIOS CL were compared with those from CBC (XN-9000™ automaton, Sysmex, Kobe, Japan) in the hematology routine laboratory of our hospital. If an anomaly was detected on CBC, manual lymphocyte count was then performed by microscopic examination of blood smear (over 100 leukocytes).

2.5 | Lymphocyte phenotyping on Navios (manual staining)

When graphical disturbance on AQUIOS CL did not allow correct lymphocyte selection, samples were reanalyzed with Navios

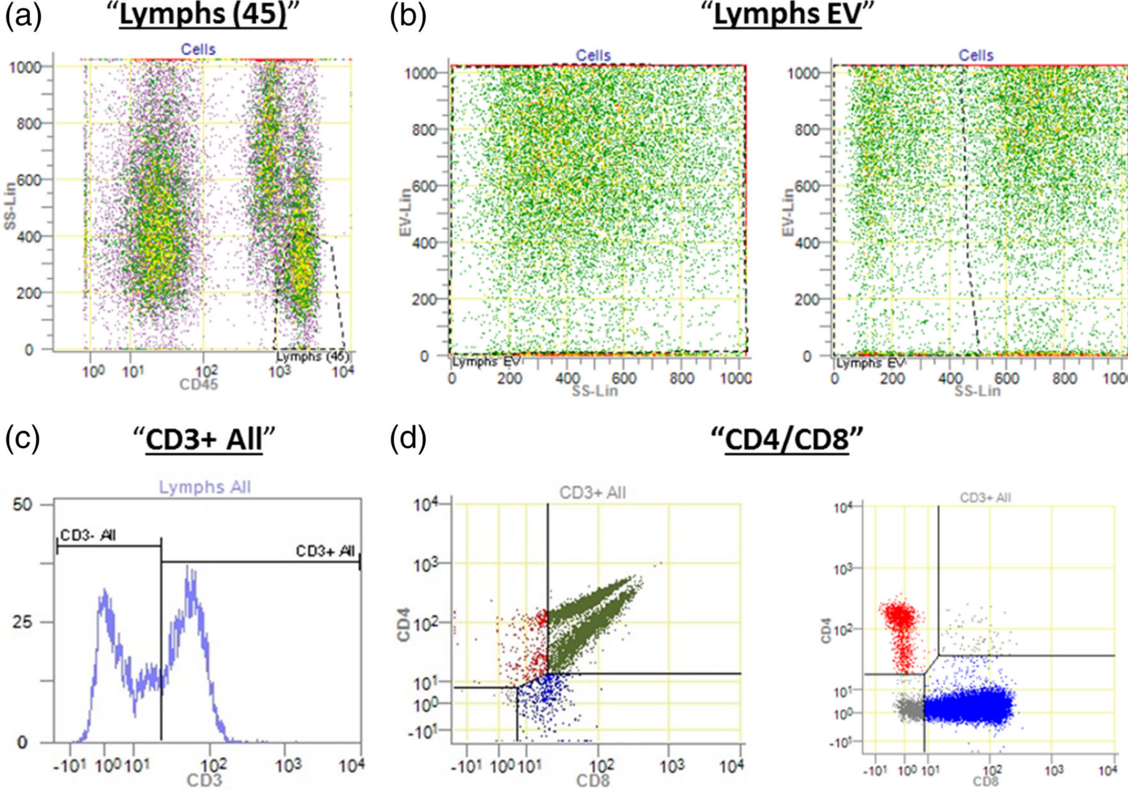


FIGURE 2 Illustrative examples of histogram anomalies observed on AQUIOS CL. Four main types of histogram anomalies were observed: poor erythrocyte lysis and cell dispersion on SS/CD45 (a) or EV/SS plot (b), contamination of CD3+ All region with CD3low cells (monocytes presenting with non-specific staining or abnormal T cells, e.g., hematologic malignancy contexts) (c) and non-specific staining on CD4/CD8 plot (d) [Color figure can be viewed at wileyonlinelibrary.com]

(Beckman Coulter, Brea, CA, USA). Fifty microliter of whole blood was stained with 10 μl of AQUIOS Tetra-1 reagent before lysis of red blood cells by addition of Optilyse C lysing solution (Beckman Coulter). As no counting beads were used in this method, only percentage of lymphocytes among leukocytes and proportions of T cell subpopulations among lymphocytes could be determined on Navios. Thus, we provided absolute values of total lymphocytes, $CD3^+$, $CD4^+$ and $CD8^+$ T cells through a dual-platform method using proportions of lymphocyte subpopulations obtained on Navios and absolute values of leukocytes determined on AQUIOS CL. Navios performances were daily assessed by running both IMMUNO-TROL™ Cells and IMMUNO-TROL™ Low Cells internal quality controls (Beckman Coulter). Calibration beads (Flow-Set and Flow-Check Fluorospheres; Beckman Coulter) were daily run to check for routine alignment, day-to-day and long-term performance validation.

2.6 | Washing step before AQUIOS CL re-analysis

When non-specific staining was observed on AQUIOS CL, 300 μl of whole blood was washed twice in 2 ml PBS and pellet was resuspended in 300 μl of human AB serum before retesting on AQUIOS CL.

2.7 | Statistical methods

Agreement between AQUIOS CL and CBC leucocyte and lymphocyte absolute counts were based on Ricos et al., minimal desirable specifications for total error (23.2% and 26.4% respectively) (Ricos et al., 1999). Agreement between total lymphocytes, CD3, CD4 and CD8 T cells percentages and absolute counts from two methods (e.g., AQUIOS CL vs. Navios or washing step) were assessed on Bland–Altman plots, agreement limits drawn using 2.77 × Standard Deviation (SD) rule according to ISO 5725-6:1994 recommendations (Accuracy [trueness and precision] of measurement methods and results — Part 6: Use in practice of accuracy values. https://www.iso.org/obp/ui/#iso:std:iso:5725:-6:ed-1:v1:en).

3 | RESULTS

3.1 | Cohort characteristics

Over a 10 days period, a total of 1009 lymphocyte phenotypings were prospectively considered, among which 147 were excluded from analysis because of unavailable CBC or lymphocytosis above AQUIOS CL linearity limit (i.e., 60×10^9 cells/L). The final cohort comprised 862 analyses related to 834 patients (28 patients had

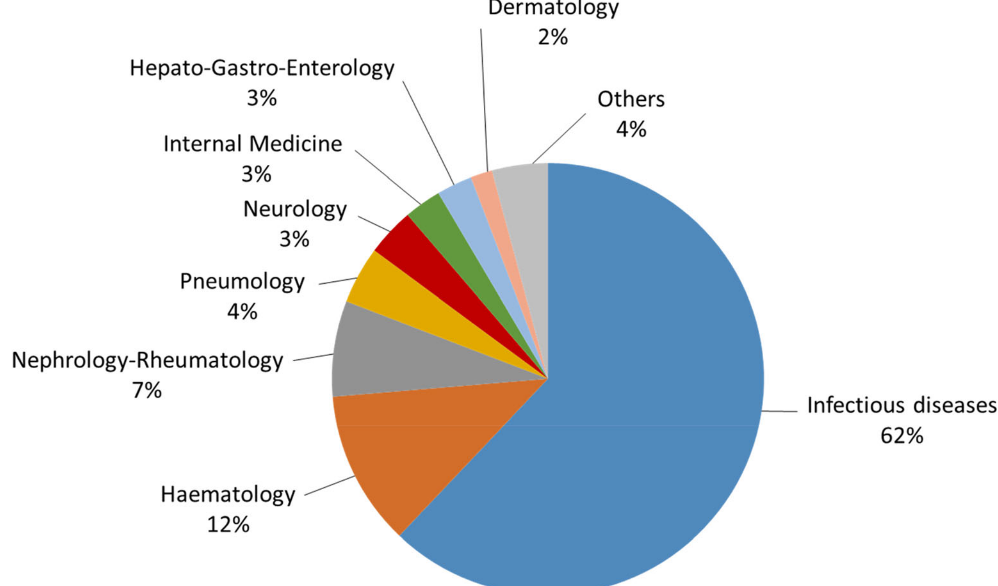


FIGURE 3 Hospital departments prescribing T cell phenotypes. Distribution of hospital departments prescribing T cell phenotypes within the cohort of 862 analyses [Color figure can be viewed at wileyonlinelibrary.com]

two samples over the period). Lymphocyte phenotyping was mainly prescribed for infectious diseases follow-up (62%), but also to patients consulting or hospitalized in other departments such as hematology (12%), nephro-rheumatology (7%). All aetiologies are depicted in Figure 3. Noteworthy, a quarter of T cells phenotypes were associated with complementary phenotypes such as B and NK cells enumeration. Thus, not only absolute count of CD4 and CD8 T cells were requested in these analyses but also percentages of lymphocyte subsets.

3.2 | Histogram anomalies on AQUIOS CL T cell phenotypes

Among 862 analyses, 643 (74.6%) did not show any abnormality in the main plots used for the AQUIOS CL gating strategy to characterize lymphocyte subpopulations. By comparing lymphocyte absolute value obtained with AQUIOS CL and concomitant CBC, 640 on 643 results (99.5%) were concordant between the two methods (data not shown). It is important to note that the 3 "discordant" CBC had been evaluated manually on a microscope (this counting method considers only 100 cells and is known to depend on the operator) making results potentially questionable. Overall, we concluded that AQUIOS CL analysis without any plot disturbance could be trustily validated without any additional operation.

Among 862 analyses, 219 (25.4%) showed one or more plot disturbances. Majority of abnormalities affected EV/SS plot, and to a lesser extent SS/CD45 plot, making it difficult to properly discriminate lymphocytes from monocytes in "Lymphs EV" and "Lymphs (45)" gates. Anomalies on CD3 monoparametric histogram allowing enumeration of T cells ("CD3+ All") and on CD4/CD8 plot used to determine CD4 and CD8 T cell subpopulations were less frequently observed (Figure S1).

3.3 | Influence of anomalies on results

We next investigated whether the 219 anomalies described above may lead to false results. Table 1 summarizes description of each type of anomaly and their impact on T cell enumeration.

3.3.1 | Dual anomaly on both "Lymphs (45)" and "Lymphs EV" gates

We observed 40 analyses (18.2% of total analyses with graphical disturbance) with major anomalies on both "Lymphs (45)" and "Lymphs EV", associated with poor erythrocyte lysis (Figure 2a,b). This phenomenon jeopardized correct discrimination of lymphocytes and, led to disturbance of "CD3+ All" region and CD4/CD8 plot. We compared results with those obtained after manual staining and lecture on Navios cytometer. 70% samples had significantly discrepant results between methods either on proportions or absolute values of $CD3^+$, $CD4^+$ or $CD8^+$ T cells (Figure 4).

3.3.2 | Single anomaly on either "Lymphs (45)" or "Lymphs EV" gates

We observed 164 analyses (74.9% of disturbed analyses) with a single anomaly on either "Lymphs (45)" or "Lymphs EV" gates (most cases). We compared results with those obtained on Navios and noticed a lower proportion of $CD3^+$ cells on AQUIOS CL (Figure 5a). Underestimation of T cell proportion was due to a contamination of "CD3− Capture" gate by monocytes with low FS (Figure 5a). Indeed, modification of the gating excluding FS^{low} cells observed below monocyte main population corrected CD3 proportion (Figure 5b). Noteworthy, contamination of "CD3− Capture" gate had no impact on absolute values of $CD3^+$, $CD4^+$ or $CD8^+$ T lymphocytes.

TABLE 1 Anomalies description and impact on T cell enumeration

Type of histogram anomaly	Number of samples	Impact on T cell % quantitation	Impact on T cell absolute enumeration
None	643	No	No
Dual alteration on "Lymphs (45)" AND "Lymphs EV"	40	Yes	Yes
Single alteration on "Lymphs (45)" or "Lymphs EV" with disturbed CD3− Capture gate	164	Yes	No
Non-specific staining on CD4/CD8	10	Yes	Yes
Altered "CD3+ All" gate	5	Yes	Yes

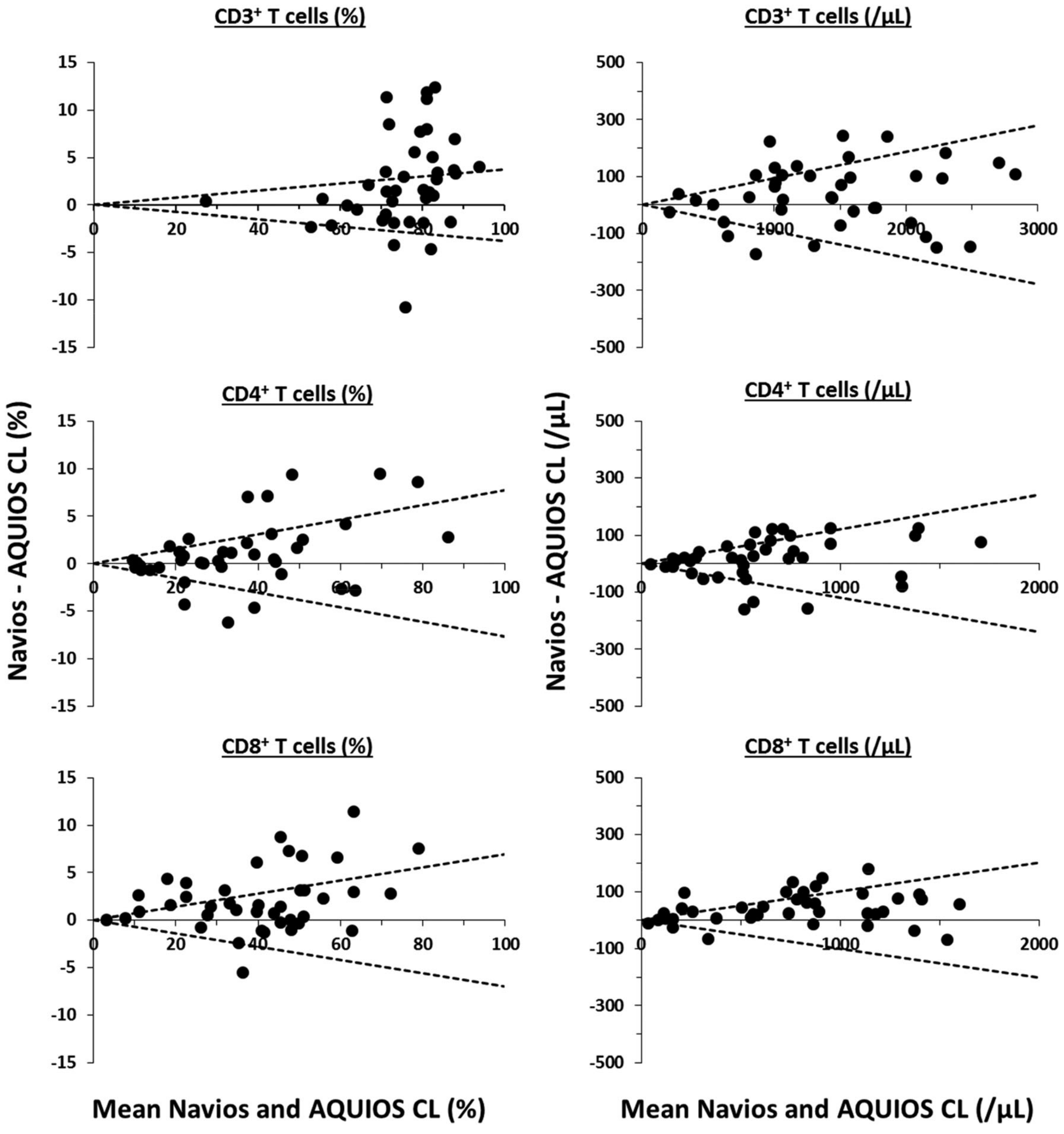


FIGURE 4 Agreement of T cell enumeration on Navios and AQUIOS CL with dual alterations on both "Lymphs EV" and "Lymphs (45)." Bland–Altman plots from paired samples run on AQUIOS CL and Navios. Values on the *x*-axis indicate the average of the results from both flow cytometry methods for each pair of samples, whereas values on the y axis represent the difference in the results. Dashed lines represent limits of agreement between results from both cytometers. Comparisons are presented for both percentages and absolute values of $CD3^+$, $CD4^+$ and $CD8^+$ T cells. Results for one sample with high absolute T cell count (i.e., 3979 $CD3^+$ cells/µl) are not shown on the figure but were within agreement limits

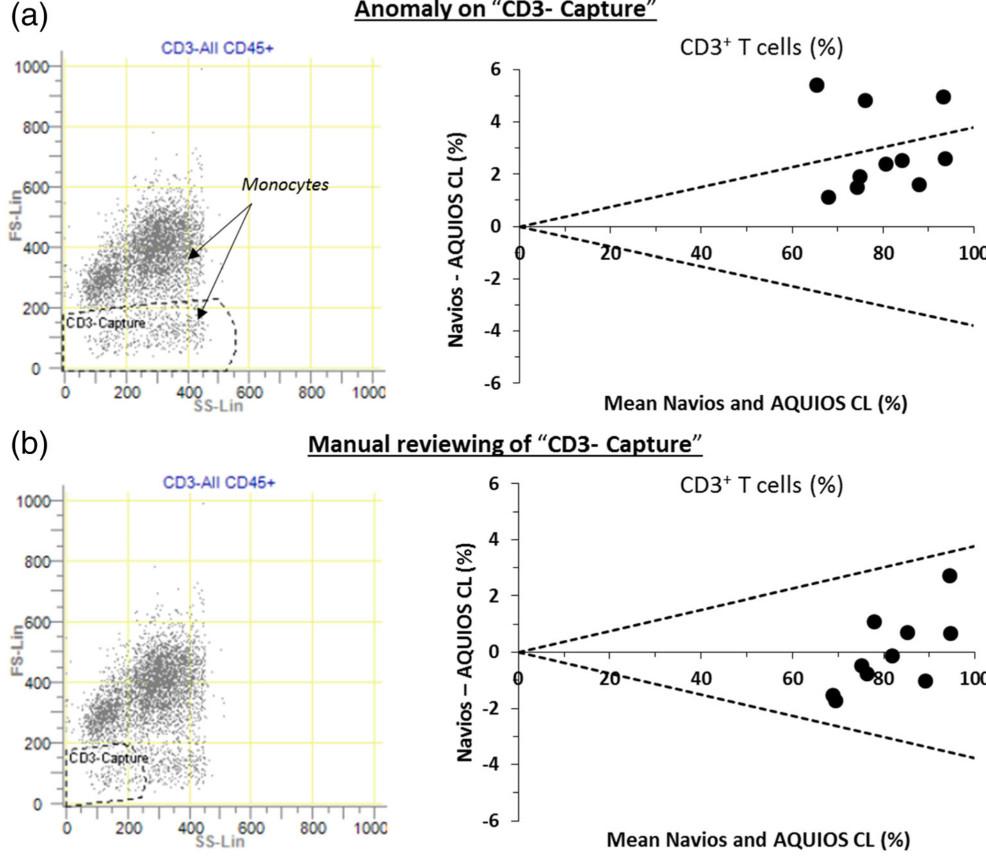


FIGURE 5 Impact and management of "CD3− capture" alteration. (a) Single "Lymphs EV" or "Lymphs (45)" alteration was associated with presence of monocytes on FS/SS plot containing "CD3− Capture" gate. Contamination of "CD3− Capture" by monocytes led to decreased percentages of $CD3^{+}$ T cells on AQUIOS CL compared to Navios. (b) Simple manual reviewing of "CD3− Capture" gate allowed to correct underestimation of $CD3^{+}$ T cell proportions [Color figure can be viewed at wileyonlinelibrary.com]

3.3.3 | Anomaly on CD4/CD8 plot

We observed 10 analyses (4.6% of disturbed analyses) with disturbance on CD4/CD8 plot whose aspects suggested non-specific staining. After a washing step, samples were rerun on AQUIOS CL and graphical anomalies were perfectly corrected in all samples (Figure S2A), allowing correct T cell characterization. Non-specific staining had a significant impact mainly on CD3 and CD8 T cells enumeration, on both absolute values and percentages (Figure S2B).

3.3.4 | Altered "CD3+ all" gate

Finally, we observed five cases (2.3% of disturbed analyses) that presented with disturbed isolated "CD3+ All" gate. Washing procedure was inefficient to correct anomalies on AQUIOS CL (data not shown) and samples had to be analyzed with Navios back-up procedure. All T cell subset percentages and absolute values were discrepant between Navios and AQUIOS CL (Figure S3). In four out of five cases, T cell phenotypes had been prescribed within medical follow up of lymphoma evolution.

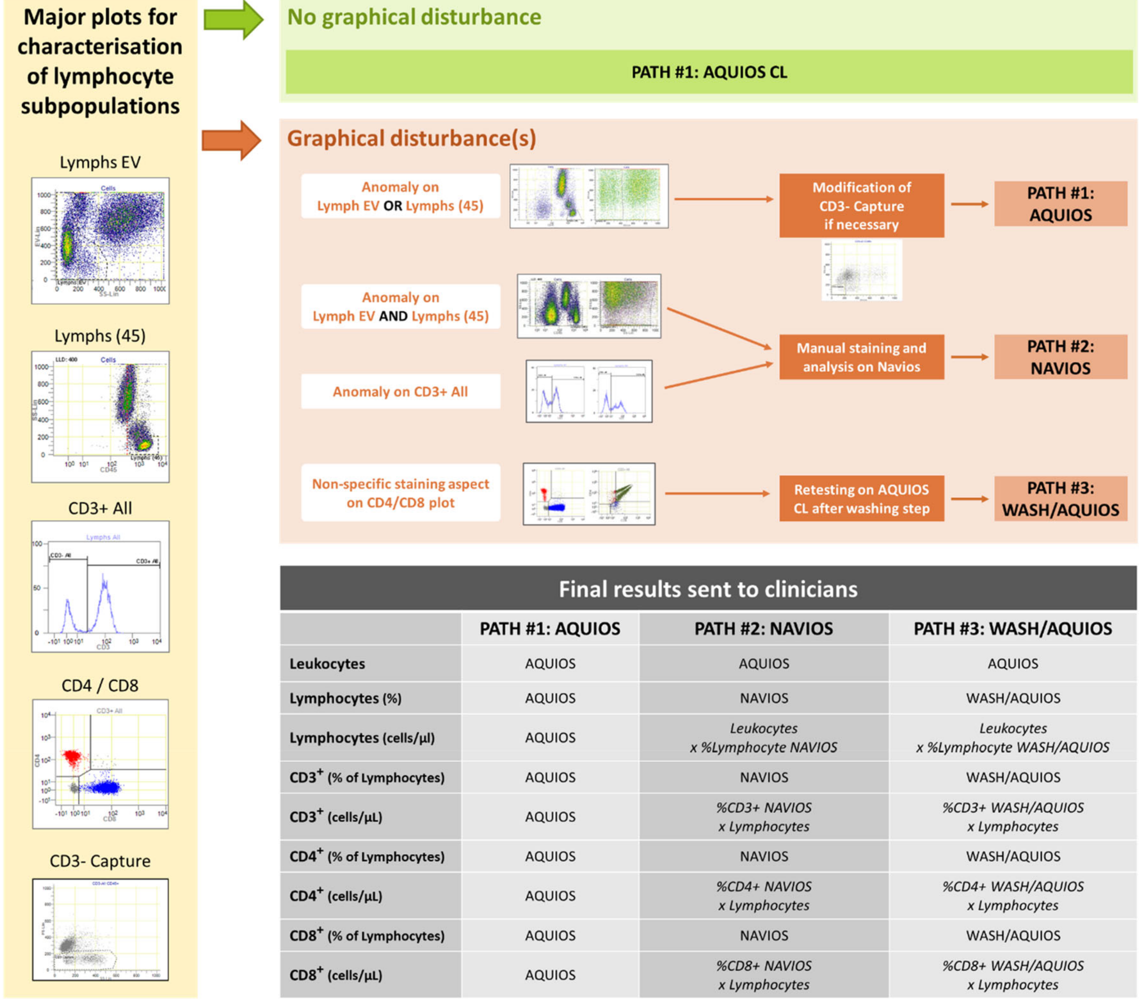
3.4 | Decision tree

Based on these observations (Table 1), we defined a decision tree (Figure 6) in order to guide validation step. It is based on the five main graphs used by AQUIOS Tetra algorithm to characterize lymphocytes and it describes the measures to be undertaken (back-up manual staining or washing step) depending on graphical anomalies on histograms. When retrospectively applied to the present cohort of 862 analyses, this decision tree would have permitted management and results release to clinicians of all analyses.

In summary (Figure 6), 94% of analyses from the cohort could be validated on AQUIOS CL as is or after simple regating of CD3− Capture region ("PATH #1"); 5% of analyses had to be manually stained and run on Navios cytometer ("PATH #2"); 1% of analyses needed a washing step before reanalysis on AQUIOS CL ("PATH #3").

4 | DISCUSSION

AQUIOS CL has been launched as an automated flow cytometer for rapid CD4+ T absolute counting usable by non-expert technical staff. In a preliminary work (Gossez et al., 2017), just after AQUIOS CL installation in our lab, we noticed several histogram abnormalities that precluded release of clinical results without verification upon usual phenotyping (i.e., on Navios), especially for determination of % of lymphocyte subsets. Accordingly, Degandt et al., described a high number of notifications on AQUIOS CL analyses (35%), which could lead to "revision fatigue" of the users (Degandt et al., 2018). In accordance with Degandt's observations, and despite a better grasp of histograms anomalies, we were still facing those issues



	PATH #1: AQUIOS	PATH #2: NAVIOS	PATH #3: WASH/AQUIOS
Leukocytes	AQUIOS	AQUIOS	AQUIOS
Lymphocytes (%)	AQUIOS	NAVIOS	WASH/AQUIOS
Lymphocytes (cells/µl)	AQUIOS	*Leukocytes x %Lymphocyte NAVIOS*	*Leukocytes x %Lymphocyte WASH/AQUIOS*
$CD3^+$ (% of Lymphocytes)	AQUIOS	NAVIOS	WASH/AQUIOS
$CD3^+$ (cells/µL)	AQUIOS	*%CD3+ NAVIOS x Lymphocytes*	*%CD3+ WASH/AQUIOS x Lymphocytes*
$CD4^+$ (% of Lymphocytes)	AQUIOS	NAVIOS	WASH/AQUIOS
$CD4^+$ (cells/µL)	AQUIOS	*%CD4+ NAVIOS x Lymphocytes*	*%CD4+ WASH/AQUIOS x Lymphocytes*
$CD8^+$ (% of Lymphocytes)	AQUIOS	NAVIOS	WASH/AQUIOS
$CD8^+$ (cells/µL)	AQUIOS	*%CD8+ NAVIOS x Lymphocytes*	*%CD8+ WASH/AQUIOS x Lymphocytes*

FIGURE 6 Decision tree for standardized T cell phenotype analysis on AQUIOS CL. The decision tree is based on five main plots influencing characterization of lymphocyte subpopulations. Their normal aspects are illustrated on the left side of the figure. Depending on the aspect of these plots, three pathways have been determined. If no graphical disturbance is observed, results from AQUIOS CL can be validated without further exploration (PATH #1). If the only anomaly observed involves "Lymph EV" or "Lymps (45)", CD3− Capture might have to be reviewed manually before validation of AQUIOS CL results (PATH #1). In case of concomitant alteration of "Lymphs EV" and "Lymphs (45)" or any anomaly of "CD3+ All", manual staining and analysis on Navios must be performed (PATH #2). In that case, absolute values will be recalculated using percentages of lymphocyte populations measured on Navios and leucocyte enumeration from AQUIOS CL (unaffected by observed anomalies). Finally, when non-specific staining aspect is observed on CD4/CD8 plot, sample must be washed and rerun on AQUIOS CL (PATH #3). Absolute values will be recalculated using percentages of lymphocyte populations measured on washed sample and leucocyte enumeration from unwashed sample (unaffected by observed anomalies) [Color figure can be viewed at wileyonlinelibrary.com]

after 4 years of daily practice. Therefore, we wondered to what extent these graphical anomalies may have influenced both cell percentages and absolute counting in a clinical immunology laboratory belonging to a university hospital. From our routine work load, we designed a cross-sectional study (10 working days) to minutely depict histogram anomalies and their influence on both % and absolute count of lymphocyte subsets and to set-up a decision tree so as to standardize our clinical practice when facing such histogram anomalies.

The first important result was to describe the variety of clinical contexts in which T cell enumeration is requested by clinicians in our university hospital. Although the majority of prescriptions were expectedly associated with HIV-infected patients follow-up, over one third were related to patients with non-infectious diseases. In addition, beyond CD4 T cell count, one quarter of prescriptions were associated with additional phenotypes (e.g., B or NK cells or naïve/memory T cells characterization). Thus, not only absolute count but also quantitation of lymphocyte subset percentages are required. This implies that, within a university hospital context, AQUIOS CL must be suitable for both absolute and percentage counting.

The second important result is to report that 75% of analyses (out of 862) performed on AQUIOS CL did not show any anomaly on the main plots used to characterize lymphocyte subpopulations. In

these cases, total lymphocyte count was perfectly concordant with CBC results. Thus, AQUIOS CL results, in the absence of any plot disturbance, can be validated without any additional process (i.e., manual gating, CBC check, alternate phenotyping).

Then, in accordance to Degandt et al. (2018) we found graphical disturbances on 25% analyses of our cohort, confirming that this phenomenon is not anecdotal. The most frequent (164 of 219) anomaly was a single disturbed "Lymphs (45)" or "Lymphs EV" region, mainly associated with altered "CD3−Capture" region. This anomaly had significant impact on T cells percentages but not on absolute values. As a solution, a simple manual re-gating procedure of "CD3−Capture" allowed to correct results. Overall, this means that 94% results (i.e., 643 without anomaly plus 164 with single disturbed "Lymphs (45)" or "Lymphs EV" region) can be released based on AQUIOS without additional bench procedure.

In 10 cases, we observed histograms evocative of non-specific staining. This was easily resolved by a washing step before re-analysis on AQUIOS CL. Dual anomalies affecting both "Lymphs EV" and "Lymphs (45)" were reported in 40 cases for which additional staining and cell analysis on Navios were mandatory to deliver accurate results. Lastly, in five cases, "CD3+ All" gate was disturbed. Of note, four out of these five cases referred to T cell phenotypes within follow up of lymphoma evolution. Again, in such cases, cell analysis on Navios was mandatory. In summary, results from AQUIOS CL were usable for 95% analyses (including manual gating of CD3− Capture when necessary). Alternate staining/analysis based on back-up procedures was mandatory in the remaining 5% cases. Interestingly, those phenotypes were more often prescribed by hematology and dermatology departments, where patients often present with lymphocyte malignancies such as lymphoma (Figure S4). To circumvent these drawbacks, we propose a decision-tree to help guiding the validation step (Figure 6). Its applicability to other centres/hospitals needs to be further investigated.

Of note, the use of an additional procedure is mainly due to the fact that AQUIOS CL does not allow manual gating that could easily permit to get rid of monocyte contamination when "Lymphs EV" and "Lymphs (45)" are disturbed. These limitations might be overcome in the future through the implementation of a new software (AQUIOS CL® Designer Software 2.0) that may allow to modify steps in sample preparation process but also to set up protocols, compensation settings and acquisition templates (Spijkerman et al., 2019). So far, this software has been used in research contexts, allowing to use customized antibody mix, but never in a routine medical laboratory (Koenderman et al., 2021; Spijkerman et al., 2021). This aspect will require further validation.

5 | CONCLUSION

Overall, we extend previous results by confirming the suitability of AQUIOS CL cytometer for most T cell enumerations. However, within a university hospital context including patients with various pathologies, it appears necessary to maintain a back-up procedure available in order to perform "traditional" flow cytometry for 5% of lymphocyte phenotyping. That said, in different clinical settings, depending on patients' medical aetiologies, AQUIOS CL may allow by itself (i.e., without back-up cytometer) to provide accurate results. The necessity to maintain a back-up protocol has to be determined in each centre depending on local patients' specificities. Taking these anomalies into account, we also reported on a decision tree that should simplify the validation process of results from AQUIOS CL. This may help in accreditation process.

ACKNOWLEDGMENTS

The authors would like to thank the whole team from the Immunology Laboratory for their routine work and support during study completion.

CONFLICT OF INTEREST

Authors state no conflict of interest.

AUTHOR CONTRIBUTIONS

Designed the study: Léa Lemoine, Christophe Malcus, Françoise Poitevin-Later, Fabienne Venet, Guillaume Monneret and Morgane Gossez. *Included patients and performed experiments*: Léa Lemoine. *Performed the statistical analyses*: Léa Lemoine and Morgane Gossez. All authors discussed the data, drafted or revised critically the manuscript for important intellectual content, have accepted responsibility for the entire content of this manuscript and approved its submission.

INFORMED CONSENT/ETHICAL APPROVAL

In accordance to our Local Ethics Committee's rules, no signed informed consent was needed as leftover samples were used.

ORCID

Fabienne Venet https://orcid.org/0000-0003-0462-4235
Guillaume Monneret https://orcid.org/0000-0002-9961-5739
Morgane Gossez https://orcid.org/0000-0003-1930-8956

REFERENCES

Coetzee, L. M., & Glencross, D. K. (2017). Performance verification of the new fully automated Aquios flow cytometer PanLeucogate (PLG) platform for CD4-T-lymphocyte enumeration in South Africa. *PLoS One*, *12*(11), e0187456.

Degandt, S., Peeters, B., Jughmans, S., Boeckx, N., & Bossuyt, X. (2018). Analytical performance of an automated volumetric flow cytometer for quantitation of T, B and natural killer lymphocytes. *Clinical Chemistry and Laboratory Medicine*, *56*(8), 1277–1288.

Gossez, M., Malcus, C., Demaret, J., Frater, J., Poitevin-Later, F., & Monneret, G. (2017). Evaluation of a novel automated volumetric flow cytometer for absolute CD4+ T lymphocyte quantitation. *Cytometry Part B: Clinical Cytometry*, *92*(6), 456–464.

Grossi, V., Infantino, M., Meacci, F., Bellio, E., Bellio, V., Ciotta, G., Priami, F., Sarzi-Puttini, P., Atzeni, F., Li Gobbi, F., Damiani, A., Benucci, M., & Manfredi, M. (2018). Comparison of methods and TAT assessment: Volumetric AQUIOS CL and bead-based FACS CANTO II cytometers. *Cytometry Part B: Clinical Cytometry*, *94*(4), 674–678.

Koenderman, L., Siemers, M. J., van Aalst, C., Bongers, S. H., Spijkerman, R., Bindels, B. J. J., Giustarini, G., van Goor, H. M. R., Kaasjager, K. A. H., & Vrisekoop, N. (2021). The systemic immune

response in COVID-19 is associated with a shift to formyl-peptide unresponsive eosinophils. *Cell*, *10*(5), 1109.

Rhodes, D., Carcelain, G., Keeney, M., Parizot, C., Benjamins, D., Genesta, L., Zhang, J., Rohrbach, J., Lawrie, D., & Glencross, D. K. (2019). Assessment of the AQUIOS flow cytometer – An automated sample preparation system for CD4 lymphocyte Pan-Leucogating enumeration. *The African Journal of Laboratory Medicine*, *8*(1), 804.

Ricos, C., Alvarez, V., Cava, F., Garcia-Lario, J. V., Hernandez, A., Jimenez, C. V., Minchinela, J., Perich, C., & Simon, M. (1999). Current databases on biological variation: Pros, cons and progress. *Scandinavian Journal of Clinical and Laboratory Investigation*, *59*(7), 491–500.

Sack, U., Barnett, D., Demirel, G. Y., Fossat, C., Fricke, S., Kafassi, N., Nebe, T., Psarra, K., Steinmann, J., & Lambert, C. (2013). Accreditation of flow cytometry in Europe. *Cytometry Part B: Clinical Cytometry*, *84*(3), 135–142.

Spijkerman, R., Hesselink, L., Bertinetto, C., Bongers, C. C., Hietbrink, F., Vrisekoop, N., Leenen, L. P., Hopman, M. T., Jansen, J. J., & Koenderman, L. (2021). Analysis of human neutrophil phenotypes as biomarker to monitor exercise-induced immune changes. *Journal of Leukocyte Biology*, *109*(4), 833–842.

Spijkerman, R., Hesselink, L., Hellebrekers, P., Vrisekoop, N., Hietbrink, F., Leenen, L. P. H., & Koenderman, L. (2019). Automated flow cytometry enables high performance point-of-care analysis of leukocyte phenotypes. *Journal of Immunological Methods*, *474*, 112–646.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Lemoine, L., Malcus, C., Poitevin-Later, F., Venet, F., Monneret, G., & Gossez, M. (2022). Cross-sectional reassessment after 4 years of clinical routine use of AQUIOS CL for absolute T cell quantitation in a university hospital. *Cytometry Part B: Clinical Cytometry*, 1–10. https://doi.org/10.1002/cyto.b.22069