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ORIGINAL ARTICLE

Automated pattern-guided principal component analysis vs expert-based immunophenotypic classification of B-cell chronic lymphoproliferative disorders: a step forward in the standardization of clinical immunophenotyping

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Immunophenotypic characterization of B-cell chronic lymphoproliferative disorders (B-CLPD) is becoming increasingly complex due to usage of progressively larger panels of reagents and a high number of World Health Organization (WHO) entities. Typically, data analysis is performed separately for each stained aliquot of a sample; subsequently, an expert interprets the overall immunophenotypic profile (IP) of neoplastic B-cells and assigns it to specific diagnostic categories. We constructed a principal component analysis (PCA)-based tool to guide immunophenotypic classification of B-CLPD. Three reference groups of immunophenotypic data files—B-cell chronic lymphocytic leukemias (B-CLL; *n* = 10), mantle cell (MCL; *n* = 10) and follicular lymphomas (FL; *n* = 10)—were built. Subsequently, each of the 175 cases studied was evaluated and assigned to either one of the three reference groups or to none of them (other B-CLPD). Most cases (89%) were correctly assigned to their corresponding WHO diagnostic group with overall positive and negative predictive values of 89 and 96%, respectively. The efficiency of the PCA-based approach was particularly high among typical B-CLL, MCL and FL vs other B-CLPD cases. In summary, PCA-guided immunophenotypic classification of B-CLPD is a promising tool for standardized interpretation of tumor IP, their classification into well-defined entities and comprehensive evaluation of antibody panels.
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Introduction

Currently, flow cytometers used in most clinical diagnostic laboratories are not equipped with enough multicolor capabilities to address many frequent clinical questions directed to immunophenotyping with only one combination of monoclonal antibodies.[1–7] In order to overcome such limitation, several aliquots of the same sample are stained in parallel with a panel of different, but partially overlapping monoclonal antibody (MAb) combinations.[1,2,8–13] Recently, we have proposed a mathematical approach which allows direct calculation of the immunophenotypic features of individual cellular events for an unlimited number of flow cytometric parameters; the end result is a single data file where individual cells are characterized for all markers tested in a sample, for every individual aliquot measured.[14] This new approach has proven to be of great utility for the automated distinction between normal and neoplastic cells coexisting in peripheral blood (PB), even when the latter are present at very low frequencies.[15] However, no study has been reported so far, in which a similar strategy is applied to compare the immunophenotypic patterns of neoplastic cells from individual patients with hematological malignancies, for example, B-cell chronic lymphoproliferative disorders (B-CLPD), with $\geqslant$1 set of reference cases of well established World Health Organization (WHO) entities; such a tool could be of great help for the interpretation of IPs, particularly in complex or atypical cases.

In this study, we describe an automated pattern-guided principal component analysis (PCA)[14] approach for the classification of flow cytometry data. For its evaluation, a group of small B-cell chronic leukemias and lymphomas was selected as a model to compare the performance of the new approach here proposed vs the WHO classification. Small B-cell CLPD are particularly suited for this type of evaluation because complex, highly heterogeneous and partially overlapping immunophenotypic features are observed within these patients, for the different WHO diagnostic groups.[1,2,9,16–19] Evaluation of the overall IPs of neoplastic B-cells typically requires multiple markers ($n \geqslant 20$) in 3- to 10-color stainings[1,2,9,16–19] and interpretation by highly experienced professionals. Finally, despite clear consensus recommendations and guidelines have been proposed,[7,20–22] variable staining profiles are obtained when different MAb clones, fluorochrome conjugates and commercial sources are used. Because of this and other factors, disturbing levels of variability are generated among different centers as well as among different professionals within the same laboratory, as regards final interpretation of the IPs of neoplastic B-cells in small B-cell CLPD; this is particularly true for cases which display immunophenotypic features that only show partial overlap with well defined entities, for example: atypical chronic lymphocytic leukemia (CLL).[23]

Here, we built a new PCA-based procedure for both assignment of individual cases to specific WHO diagnostic subgroups

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and identification of the most informative markers in differentiating among them. The panel used for the construction of this new approach was chosen among those 3- and 4-color panels, currently in-use, before the design of 8-color EuroFlow panels. Overall, our results suggest that this new procedure can be of great help for the standardization of immunophenotypic interpretation of diagnostic B-CLPD samples, and at the same time it provides a valuable tool for the design of new comprehensive multicolor antibody panels support, both for diagnosis and minimal residual disease monitoring of B-CLPD and other hematological malignancies.

Materials and methods

Patients and samples

EDTA-anticoagulated PB ($n=99$), bone marrow ($n=105$) samples plus one pleural effusion were collected at diagnosis from 205 patients suffering from small B-cell CLPD—118 males and 87 females; median age of 66 years (range: 26–93 years). According to the WHO criteria,[24] patients were grouped as follows: B-cell chronic lymphocytic leukemias (B-CLL), 120 patients (100 typical and 20 atypical B-CLL cases); lymphoplasmacytic lymphoma, 5 cases; mantle cell lymphoma (MCL), 34 cases; splenic marginal zone lymphoma, 5 cases; mucosa-associated lymphoid tissue lymphoma, 7 cases, and follicular lymphoma (FL), 34 patients.

Median PB lymphocyte counts at diagnosis were of 18.6×10^9 lymphocytes/l (range: 0.8–286 $\times 10^9$ lymphocytes/l); in turn, the mean percentage of neoplastic B-cells in the samples analyzed was of $36.9 \pm 27.9\%$ (range: 0.2–96.8%) —median of 58.2% (range 2.1–96.8%) vs 13.7% (range: 0.2–90.9%) in PB vs bone marrow samples, respectively. All individuals gave their informed consent before entering the study and the study was approved by the local Ethical Committee of the University Hospital of Salamanca (Salamanca, Spain).

Flow cytometry immunophenotyping

For the research purposes of the study, the following multiparameter flow cytometry panel of 3- and 4-color combinations of MAb—fluorescein isothyocyanate/ phycoerythrin/ peridinin chlorophyll protein-cyanin 5.5 (PerCP Cy5.5)/ allophycocyanin (APC)—tested in every individual sample, was used: FMC7/CD24/CD19/CD34, CD22/CD23/CD19/CD20, CD103/CD25/CD19/CD11c, CD43/CD79b/CD19/-, surface immunoglobulin (sIg)λ/(sIg)κ/CD19/CD5, sIgM/CD27/CD19/- and cytoplasmic Cybcl2/CD10/CD19/CD38.

In every case, a stain, lyse and then wash, direct immunofluorescence technique was used following consensus recommendations.[21,25] Briefly, pre-titrated amounts of each MAb in a combination were added to separate aliquots containing $0.12–1 \times 10^6$ white blood cells in 100 μl of sample, depending on the white blood cells count of each sample, for example, appropriate dilution with phosphate-buffered saline (pH = 7.4) was performed for samples with nucleated cell counts $>10 \times 10^9$/l, and gentle mixed. After 15 min of incubation, at room temperature in the darkness, 2 ml of FACS lysing solution (Becton/Dickinson Biosciences—BD, San Jose, CA, USA), diluted 1/10 (v/v) in distilled water, was added; after gentle mixing, another incubation was performed, (10 min at room temperature in the darkness). Samples were then washed in 4 ml phosphate-buffered saline/ aliquot (5 min at 540 g) and measured in a FACSCalibur flow cytometer (BD). For immunophenotypic staining of surface immunoglobulins, the cells were washed twice in 2 ml phosphate-buffered saline with 0.5% albumin/aliquot, before staining with the corresponding antibodies. For immunophenotypic staining of Cybcl2, the Fix and Perm reagent kit (Invitrogen, Carlsbad, CA, USA) was used, strictly following the recommendations of the manufacturer.

Information about 5×10^4 leukocytes/aliquot was acquired and stored, using the CellQUEST software (BD). For samples with low B-cell percentages (<10%), additional information about $\geqslant 5 \times 10^4$ CD19$^+$/SSC^{lo} B-cells was acquired through an electronic *live gate* set on a CD19 vs sideward light scatter (SSC) dot plot, and stored using the CellQUEST software, as previously described.[17]

Merge of flow cytometry data files and calculation of flow cytometric data

Merge of data files corresponding to different aliquots of each individual sample and calculation of flow cytometry data were performed as previously described in detail,[14] after gating on CD19$^+$ neoplastic B-cells. Briefly, CD19$^+$ neoplastic B-cells were selected for each data file with the INFINICYT software (Cytognos SL, Salamanca, Spain), using conventional gating strategies based on their unique patterns of antigen expression,[19] as illustrated in Supplementary Figure 1. Information restricted to the selected neoplastic B-cells was stored in new separate data files corresponding to each individual sample aliquot. Then, data about neoplastic B-cells contained in each of these new data files for each multicolor staining performed on individual samples was merged into a single data file using the INFINICYT software program. Afterward, information about each individual parameter contained in this new merged file, which was not actually measured for an individual event, was calculated for the overall panel of markers analyzed; such calculation was done for each event measured using the calculation function of the INFINICYT software, based on nearest-neighbour statistical tools.[26,27] For this purpose, those three parameters which were measured in common in every multicolor staining, forward light scatter (FSC) and SSC, as well as CD19 PerCP Cy5.5, were used to search for each event's nearest-neighbour. All other immunophenotypic parameters were only measured for the subset of cellular events corresponding to the specific multicolor staining from the whole multi-tube panel where they were specifically assessed; calculation of the values for each of these latter parameters (for individual cellular events) where they were not directly assessed, was based on the assignment of those values observed for their nearest-neighbour event contained in another aliquot of the same sample, for which staining for those specific parameters had been performed.

After merging the original 4-color (6-parameter) data files and calculating the 'missing' values initially lacking for each individual event, a single data file containing information about all parameters measured in all multicolor stainings, for each of the events recorded, was obtained. Therefore, each of the merged/calculated data file finally contained information about all parameters measured ($n=20$); which were: FSC, SSC, CD19, CD22, CD23, CD20, CD103, CD25, CD11c, FMC7, CD24, CD34, CD43, CD79b, sIgM, CD27, CD5, Cybcl2, CD10 and CD38, for each of the $\geqslant 2.0 \times 10^5$ events analyzed per sample (four aliquots/sample each containing information about $\geqslant 5 \times 10^4$ B-cell events). sIgκ and sIgλ measurements were excluded from the calculated data files as light chain restriction varies among sIgκ+ and sIgλ+ cases, and thus its staining can not be used as a single parameter for disease classification.

Generation of reference data files for specific small B-cell CLPD WHO entities

Three reference groups corresponding to three different small B-cell CLPD entities (typical B-CLL, MCL and FL) were generated with a subgroup of 30 B-CLPD cases. For these three groups, 10 typical B-CLL, 10 MCL and 10 FL cases were randomly selected from the 205 small B-cell CLPD cases analyzed. Supplementary Table 1 shows detailed phenotypic and genetic features of these 30 reference cases (reference data set), as well as of the other 175 patients (testing set) analyzed.

Afterward, PCA was applied and graphically visualized through the automated population separator (APS) view of the INFINICYT software (Figures 1a–c). In this APS view, the first (*x* axis) and second (*y* axis) principal components are used to produce a bidimensional representation of phenotypic profiles. Each principal component is a linear combination of parameters with distinct weights, allowing for a bidimensional representation with most of the information of the original higher dimension space being preserved. We opted for PCA for two reasons: (1) it reduces dimensionality of feature space by restricting attention to those directions along which the scatter is greater; (2) linear combinations are easy to compute. The first and second principal components were used since others (third, fourth and so on) did not provide significantly relevant additional information for the discrimination among cases with different diagnosis.

In the next step, each case was tested (PCA) against the three ‘reference-groups’ in a one vs one comparison: B-CLL vs MCL, B-CLL vs FL and MCL vs FL, (Figures 1d–f, respectively), for a total of 525 comparisons (175 cases tested for three comparisons/case). The set of 30 reference cases were excluded in this testing out of sample phase. For each comparison, individual data files corresponding to neoplastic B-cells from each sample were merged with each of the three previously constructed pairs of reference data files. For classification purposes, the first vs second principal components of the PCA transformation,[15,28] were considered (APS representation shown in Figure 1).

Afterward, mean PCA 1 and PCA 2 values were calculated for the neoplastic B-cell events corresponding to each tested case and the reference cases and represented in the PCA space (APS view of the first vs second principal components) as a single square dot (Figures 1g–i). The tested case was then assigned to its nearest reference entity in the APS space, except if it fell outside the three reference groups, to which it was compared; in this latter situation, patients were classified as different from all three reference groups (for example, other B-CLPD). Finally, we compared the results of PCA-analysis of only immunophenotypes against the full WHO clinical diagnosis established on the basis of the patients' clinical features, histopathology and cytogenetics besides conventional immunophenotyping. Subsequently, we calculated the sensitivity, specificity, positive predictive (PPV) and negative predictive (NPV) values of the new (PCA-guided) approach for the diagnosis of B-cell CLPD, using the WHO classification as a gold standard.

Other statistical methods

All numerical and coded data derived from flow cytometric studies were introduced in a database using the SPSS program (SPSS 15.0, Chicago, IL, USA). For each continuous variable analyzed, mean values and their standard deviation (s.d.), as well as the median and the 95% confidence interval, were calculated. In order to assess the statistical significance of differences observed between groups, the Mann–Whitney *U*-test was used. *P*-values <0.05 were considered to be associated with statistical significance.

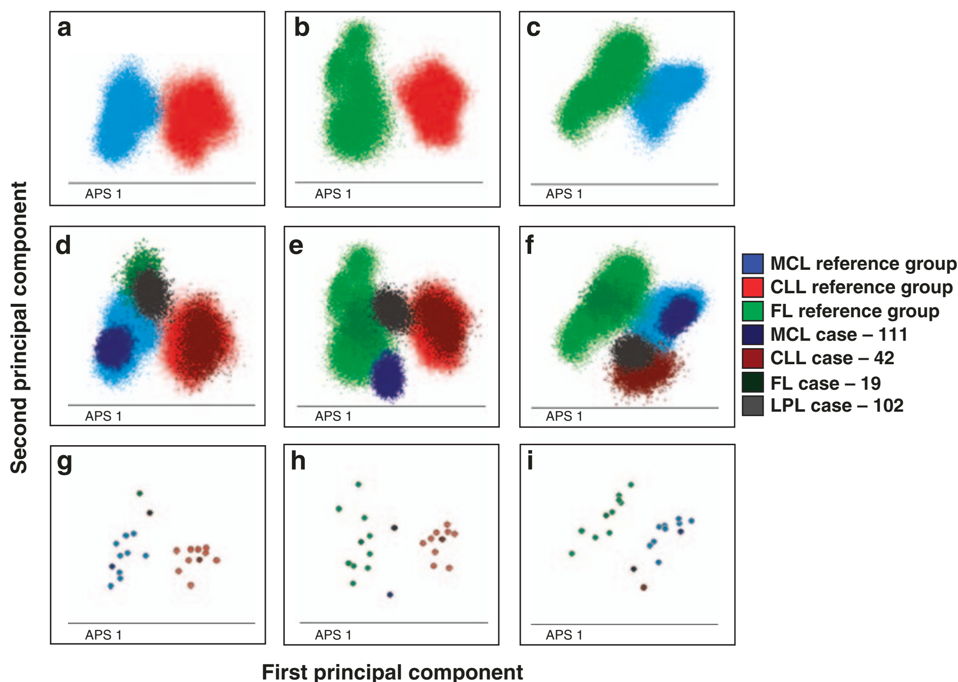

Figure 1 Illustrating example of the CLL vs MCL (**a**, **d** and **g**), CLL vs FL (**b**, **e** and **h**) and FL vs MCL (**c**, **f** and **i**) one vs one comparisons of flow cytometry data files corresponding to the three B-CLPD reference groups as classified by the PCA projections (first vs second principal components). The PCA based classification profile obtained for four cases tested is displayed: a typical CLL (brown dots), one FL (dark green dots), a MCL (dark blue dots) and a lymphoplasmacytic lymphoma (LPL; black dots). In **a**–**f**, each dot corresponds to a single cell event, whereas in panels **g**–**h**, mean principal component 1 vs principal component 2 values for each case (same PCA as in panels **d**–**f**, respectively), are shown.

Results

As described above, three reference groups of 10 cases each were established and compared in a one vs one basis; these three reference groups contained 'reference' typical B-CLL, FL and MCL cases colored in red, green and blue in Figure 1, respectively. Then, each of the one vs one comparison was represented in a PCA space defined by the first vs the second principal components (Figures 1a–c). Each case from the test group was compared in the same three PCA spaces against the pairs of B-CLL vs MCL, B-CLL vs FL and MCL vs FL reference groups, as illustrated for four different cases in Figures 1g–i. Based on these comparisons, each case was classified as typical CLL, MCL, FL or other B-CLPD. For each comparison, we recorded the sequence of parameters which had the greater weight in the discrimination between each pair of diagnostic B-CLPD entities and their relative contribution to such discrimination value for each comparison (Supplementary Table 2). The most informative markers were: (1) CD23, CD5, CD27, CD10, CD43, CD20 and CD38 for the discrimination between B-CLL vs FL; (2) CD23, CD38, CD20, sIgM, CD79b and FMC7 for B-CLL vs MCL and (3) CD5, sIgM, CD27, CD10, CD25 and CD19 for the distinction between MCL vs FL (Supplementary Table 2).

Table 1 Sensitivity, specificity and both positive and negative predictive values of the PCA-based classification approach for B-CLPD according to the expression of CD22, CD23, CD20, CD103, CD25, CD11c, CD43, CD79b, FMC7, CD24, CD34, CD5, sIgM, CD27, Cybcl2, CD10 and CD38 on neoplastic B-cells

Diagnostic Subgroups	*Sensitivity*	*Specificity*	*Positive predictive value*	*Negative predictive value*
Total B-CLL	91%	100%	100%	88%
Typical B-CLL	99%	100%	100%	99%
Atypical B-CLL	55%	100%	100%	89%
MCL	97%	96%	77%	99%
FL	92%	98%	85%	99%
Other	59%	94%	53%	96%
Total	89%	96%	89%	96%

Abbreviations: B-CLPD, B-cell chronic lymphoproliferative diseases; B-CLL, B-cell chronic lymphocytic leukemia; MCL, mantle cell lymphoma; FL, follicular lymphoma; PCA, principal component analysis.

Overall, the efficiency of the approach here evaluated for correct assignment of the 175 B-CLPD cases analyzed to the three pre-established reference groups was of 89% ($n=155/175$) with a specificity of 89% and a sensitivity of 96%; the positive and negative predictive values were of 89 and 96%, respectively (Table 1).

In detail, 88/89 (99%) typical B-CLL cases and 11/20 (55%) atypical B-CLL patients were correctly assigned to the B-CLL group. From the other 9 atypical B-CLL patients, 8 were classified as not clearly belonging to any of the three reference groups, and the other case was misclassified as MCL (Table 2 and Supplementary Table 3). Interestingly, 5/8 misclassified cases of atypical B-CLL had multiple atypical phenotypic characteristics (for example, $CD20^{hi}$, $FMC7^{hi}$ and $CD79b^{hi}$). Furthermore, these atypical B-CLL cases had trisomy 12, either as the sole genetic abnormality ($n=3$) or in association with del(13q) ($n=1$) or del(17q) ($n=1$), 1/8 showed del(17q) and 1/8 had del(13q) (Supplementary Table 1); in turn, the misclassified case (case ID: 29), showed del(11q) in the absence of t(11;14) (Supplementary Table 1). Based on these results, the sensitivity and specificity reached for typical B-CLL were of 99 and 100%, respectively, and for all B-CLL cases—typical plus atypical—of 91 and 100%, respectively (PPV of 100% and NPV of 88%) (Table 1).

Regarding MCL cases, 23/24 (96.8%) patients were correctly assigned to the MCL (Table 2 and Supplementary Table 3); the other MCL patient was misclassified as FL; of note this case (case ID: 120) showed t(11;14) in association with del(17p), besides an atypical $CD5^{-}$ immunophenotype, in the absence of t(14;18) (Table 2 and Supplementary Table 3). Based on these results, a sensitivity of 97% and a specificity of 96% were obtained for the MCL group, with a NPV of 99% and a PPV of 77% (Table 1).

Similarly, most (22/24 cases; 91.7%) FL cases were also correctly assigned to the FL group (Table 2 and Supplementary Table 3). Of note, the two misclassified cases were assigned to the MCL group; interestingly, these two patients could not be diagnosed on histopathological grounds alone, but they both showed the presence of t(14;18) in association with trisomy 12, and no t(11;14); one of these cases had a $CD5^{+}$ phenotype (Supplementary Table 1). Based on these results, the overall sensitivity and specificity for correct assignment of FL cases were of 92 and 98%, respectively (PPV of 85% and NPV of 99%) (Table 1).

In all, 10 out of the other 17 (58.8%) small B-cell CLPD studied (5 lymphoplasmacytic lymphoma, 7 mucosa-associated

Table 2 Frequency of B-CLPD cases classified according to the WHO, assigned to the different reference subgroups using the proposed phenotype-based PCA classification approach

WHO diagnostic subgroups	*Immunophenotypic subgroups defined by the automated PCA classification approach*			
	B-CLL	*MCL*	*FL*	*Other*
Typical B-CLL	89/90 (99%)	0 (0%)	0 (0%)	1/90 (1%)[a]
Atypical B-CLL	11/20 (55%)	1/20 (5%)[b]	0 (0%)	8/20 (40%)
MCL	0 (0%)	23/24 (96.8%)	1/24 (4.2%)[c]	0 (0%)
FL	0 (0%)	2/24 (9.5%)[d]	22/24 (91.7%)	0 (0%)
Other	0 (0%)	4/17 (23.5%)	3/17 (17.7%)	10/17 (58.8%)

Abbreviations: B-CLPD, B-cell chronic lymphoproliferative disorders; B-CLL, chronic lymphocytic leukemia; MCL; mantle cell lymphoma; FL, follicular lymphoma; PCA, principal component analysis; WHO, World Health Organization.
[a]One B-CLL case with a $CD23^{-}$ phenotype and both del(11q) and del(13q).
[b]Atypical B-CLL case with a $CD20^{+++}$ and $FMC7^{++}$ phenotype.
[c]Case corresponding to a $t(11;14)^{+}$ MCL with a $CD5^{-}$ phenotype and additional del(17p).
[d]Both cases corresponded to a FL with $t(14;18)^{+}$, which were unclassifiable as such on histopathological grounds alone; one of them had a $CD5^{+}$ phenotype in association with trisomy 12.

lymphoid tissue lymphomas and 5 splenic marginal zone lymphoma) were correctly identified as different from B-CLL, FL and MCL. Four of the other seven cases were assigned to the MCL group and three to the FL group (Table 2 and Supplementary Table 3); none of them was misclassified as belonging to the B-CLL group. Thus, the overall sensitivity and specificity for this latter group was of 59 and 94%, respectively (NPV of 53% and PPV 96%).

Discussion

Currently, the utility of immunophenotyping is highly variable and heterogeneous depending on the specific IP of the individual cases investigated.[8,9,11–13,29] As an example, among B-CLPD it is recognized that immunophenotyping is extremely powerful in the differential diagnosis between typical B-CLL, hairy cell leukemia and other disease entities; its reliability progressively decreases from MCL and FL to MZL.[30–32] Several factors contribute to such variability, which include: (i) the lack of robust individual markers to efficiently define each disease entity, leading to the need for interpretation of complex IPs; (ii) the biological variability of individual disease entities, with overlapping features between different WHO diagnostic subgroups and (iii) the lack of standardized criteria and procedures for interpretation of complex flow cytometry profiles.[9,16–19,22]

Initially, efforts have mainly concentrated on the identification of new, highly informative markers;[7,18,30–33] this has led to the use of progressively large panels of reagents. Latter on, standardization efforts have focused on providing recommendations and guidelines as regards: (i) the specific techniques applied for sample preparation and staining, (ii) the most informative, mandatory markers and (iii) scoring systems for standardized clear-cut definition of individual disease entities.[7,20,21] Altogether, these efforts have improved the reproducibility of immunophenotyping, but at the expense of increasing the complexity of interpretation of the flow cytometric data, which typically requires highly-qualified and experienced professionals.[7]

In a certain way, in the last decades an objective technique such as multiparameter flow cytometry immunophenotyping, has progressively evolved into a process based on a relative subjective expert-based interpretation of histograms and bivariate dot plots (for example, 'FCM images') similar to that of conventional pathology ('morphological and histopathological microscopic images').[34] More recently, attempts have been made to apply expert supervised algorithms and approaches (for example, Bayesian algorithms) to a more accurate classification of B-CLPD and other hematological neoplasias.[10,35] However, in these studies, values for individual markers are either expressed as mean/median values (for example, mean fluorescence intensity) for a cell population or they are translated into an arbitrary categorical classification of negative vs positive and dim vs bright patterns of marker expression, before the use of the derived algorithm; this *a priori* manipulation of data, may introduce a bias with a negative impact on the performance of the algorithm used.

In this study, we applied and evaluated a mathematical procedure, which has been recently proposed, for the classification of individual patients into pre-established and well-defined WHO diagnostic entities. A detailed description of the mathematical algorithm has been previously reported by our group[15] and they have become widely available as friendly tools incorporated into commercial flow cytometry data analysis software. For this propose, we selected a model of heterogeneous overlapping diseases—small B-CLPD—to perform a retrospective study of a series of 205 PB and bone marrow patient samples (30 samples were used to build the model and the other 175 to test it). The new mathematical tools used allow calculation of the complete immunophenotypic information derived from distinct aliquots of the same sample, for every individual cell measured in all sample aliquots;[14] In addition, they permit generation of reference data files containing information about neoplastic B-cells from several patients which are selected as representative of individual, well-defined WHO entities (for example, B-CLL, MCL and FL); afterward, unsupervised pattern recognition multivariate analysis—for example PCA—[15,28] can be easily applied to compare each case interrogated against (for example, two) reference disease groups. In fact, in our study, to reduce the dimensionality of the data, we just considered the first vs second principal components. Noteworthy, this procedure showed an overall efficiency of >85%, for the classification of a relatively large group of small B-cell CLPD into specific WHO disease groups. To the best of our knowledge, this is the first time that such a procedure, based on information derived from phenotypic profiling of individual tumor cells, is proposed to guide/help the expert on the interpretation of their overall immunophenotypic pattern, in the diagnostic work-up of hematological malignancies. The level of efficiency reached was particularly high among typical CLL, MCL and FL with only a few ($n=4$) discrepant cases: one corresponded to an atypical $CD5^-$ MCL, another to a $CD5^+$ FL and a third case to a FL which could also not be classified as such, based on strict histopathological or immunophenotypic criteria alone.

Despite this, 11% of false or undetermined diagnoses were found which is higher than desirable. Noteworthy, most of the cases were found among those disease entities for which reference IP groups were not used in our study (for example: splenic marginal zone lymphoma, lymphoplasmacytic lymphoma and mucosa-associated lymphoid tissue lymphomas, as well as in atypical B-CLL). Although this could be viewed as a failure of the newly proposed procedure, it more likely reflects the need for additional markers to be included in this MAb panel. In fact, it is generally known that with the restricted panel of reagents used, entities such as splenic marginal zone lymphoma and lymphoplasmacytic lymphoma can not be clearly defined on immunophenotypic grounds, requiring additional markers and information.[25,36,37] Alternatively, some WHO diagnostic groups might actually correspond to a heterogeneous group of different disorders. In line with this latter hypothesis, it should be noted that a major fraction of all atypical B-CLL cases was actually properly classified as such, whereas another subgroup of patients was considered to be clearly different, not only from B-CLL, but also from MCL and FL. Further investigations on larger series of patients in which reference groups for atypical B-CLL and other small B-cell CLPD are also included, are required to define the precise value of this new tool in these and other subtypes of B-CLPD, as well as in acute leukemias and other hematological malignancies.

Overall, these results show that when combined with the mathematical approach used, currently available 3- and 4-color panels work relatively well, but they are suboptimal for the classification of some B-CLPD disease entities. Accordingly, with this new strategy the performance of a panel of reagents can be objectively monitored and evaluated in terms of its rate of failure, pointing to the need for improved panels. However, it should be noted that for each new panel designed, a new set of reference data files, which had been stained with it and measured under comparable conditions, is required.

In summary, here we describe a new powerful tool that can be used in the future to help expert-based interpretation of multiparameter flow cytometry immunophenotypic data in the subclassification of hematological malignances. The proposed strategy may also contribute to a better definition of specific subgroups of diseases and to improve standardization of interpretation of flow cytometry data, and it can be applied for the evaluation of the performance of currently used antibody panels for immunophenotypic classification of B-CLPD and other malignancies.

Conflict of interest

Cytognos SL is part of the UE-supported EuroFlow Research Consortium, and has implemented some of the algorithms described in the present study, in its proprietary software INFINICYT; Cytognos SL has a contract license of several patents owned by the University of Salamanca, of which A Orfao, CE Pedreira and ES Costa are inventors. Other authors declare no competing financial interests.

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