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HAEMATOLOGY

A new approach to the study of Hodgkin lymphoma by flow cytometry

Maria Beatriz Álvarez Flores[1], María Sopeña Corvinos[2], María Medrano Élez[2], Beatriz Soto del Pecho[2], Luz Conejo Sánchez[2], Javier García de la Fe[2], Raquel Guillén Santos[2], Fernado Cava Valenciano[2]

[1]*Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Madrid, Spain;* [2]*Laboratorio Central de la Comunidad de Madrid, San Sebastián de los Reyes, Spain*

Summary

Hodgkin lymphoma (HL) appears to originate from germinal centre B cells but lacks expression of most B cell markers. In contrast to non-Hodgkin B lymphomas, HL is not routinely diagnosed using flow cytometry techniques, and diagnosis is mainly based on immunohistochemical and cytomorphological pathology studies.

Hodgkin and Reed–Sternberg cells are large and fragile, making them difficult to study by flow cytometry. The aim of this study was to characterise the CD71 expression pattern on CD4+ T cells from HL patients and to design a simple flow cytometry algorithm to complement the histopathological diagnosis of HL.

The present study suggests the utility of a conventional staining protocol with a simple panel of seven markers (CD15, CD30, CD4, CD8, CD71, CD3, and CD45) and a well-defined analysis strategy. The proposed algorithm uses the CD71 ratio (calculated as the percentage of CD71+ CD4+ T cells divided by the percentage of CD71+ CD45+ CD3− lymphocytes), with a cut-off of 0.5 to establish diagnosis groups as suggestive ($\geq$0.5) or not suggestive (<0.5) of HL.

In HL, CD71 expression is higher on CD4+ T lymphocytes than on non-T lymphocytes. In addition, the CD4+ T cell population is increased in HL patients, with no change in amounts of CD8+ T cells. Application of the CD71 ratio algorithm yielded a sensitivity of 82% and specificity of 87%, with 84.61% of patients correctly diagnosed.

Although histopathology remains the gold standard for definitive HL diagnosis, the proposed flow cytometry method provides a rapid method to guide the study that would allow a more robust and integrated diagnosis. Moreover, the procedure is easily applicable in most clinical laboratories as it does not require state-of-the-art cytometers and uses standard reagents.

Key words: Hodgkin lymphoma; flow cytometry; CD71; haematology; lymph node; CD4; CD30; CD15.

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INTRODUCTION

The diagnosis of Hodgkin lymphoma (HL) has historically been based on immunohistochemical and cytomorphological pathology studies. Current World Health Organization (WHO 2017) guidelines[1] maintain immunophenotypic profiling by immunohistochemistry techniques as the main diagnostic method for HL. Attempts have been made to diagnose HL using flow cytometry, but the usefulness of this approach is unclear. Although technological advances and increased scientific knowledge have allowed some groups to identify and diagnose HL with high sensitivity and specificity,[2,3] flow cytometry has not been implemented routinely in clinical diagnosis.[4,5]

A likely limitation of flow cytometry for HL diagnosis is the large size and fragility of Hodgkin and Reed–Sternberg (HRS) cells.[4,6,7] Although these malignant cells are generally considered together, Hodgkin cells are large mononuclear cells, whereas Reed–Sternberg cells are multinuclear giant cells.[5,8,9]

HRS cells are usually rare and are surrounded by a reactive inflammatory medium comprising lymphocytes, eosinophils, neutrophils, histiocytes, and plasma cells. HRS cells are also typically surrounded by CD4+ T cells, forming a rosette pattern.[10] Previous studies have shown that the T cell rosette is composed of T helper (Th) cells and T regulatory cells (Tregs).[11,12] The rosetting CD4+ T cells contribute to HRS cell proliferation by secreting factors such as IL-3. Tregs also contribute to HRS cell survival by protecting them against attack by cytotoxic T lymphocytes and natural killer (NK) cells.[13,14] Other authors have reported an increase in Treg numbers in affected lymph nodes, with some studies indicating that these cells have a characteristic phenotype.[15,16]

There is no single, specific marker of HRS cells, and authors use different antibody combinations for flow cytometry, usually targeting the transferrin receptor (CD71) and CD15, CD30, CD40, CD95, CD64, CD45, and CD20 or CD123, in addition to other markers for the detection of Reed–Sternberg cells.[5,17,18] CD71 is frequently expressed on Reed–Sternberg cells[19,20] but is also a general marker of cell proliferation and activation in all cell types, including T

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cells.[21–23] The aim of this study was to characterise the CD71 expression pattern on CD4+ T cells from HL patients and to design a simple flow cytometry algorithm to complement the histopathological diagnosis of HL.

MATERIALS AND METHODS

Patients and samples

A total of 143 suspected lymphoma samples were referred for flow cytometry immunophenotyping. The study included 111 lymph nodes (LN), 25 fine-needle aspirations (FNA), and seven core needle biopsies (CNB).

Preparation of tissue suspensions

Fresh LN tissue was gently mashed with tweezers in 1–3 mL of 0.9% NaCl in water. The homogenate was then dispersed by passing through an insulin needle (Microlance 3; BD, USA).

Flow cytometry immunophenotyping

The antibody panel consisted of five tubes (Table 1). Because in most cases the clinical guidance was only to 'rule out lymphoma', the screening analysis included two tubes, one for the detection of B clonality and a second for the percentages of T lymphocyte subpopulations and HL (tubes 1 and 2). When no clonal B cell population was detected in tube 1, a fluorescence minus one control panel (FMO control) was implemented to assess the expression of CD71, CD30, and CD15 (tubes 3, 4, and 5) following the recommendations of Roederer.[24,25] Antibodies were purchased from BD Biosciences (USA). When a clonal B population was detected or any alteration was found indicating suspected T cell pathology, the study was extended to reach a diagnosis and classify the pathological population.

Staining protocol

Each tube contained at least 1×10^6 nucleated cells. For low volume samples, the sample was divided into 3 tubes: two for screening and one reserved for when the study needed to be extended. For the light-chain restriction study, at least 2×10^6 nucleated cells were dispensed and washed twice before staining. Cells were incubated with appropriately titrated fluorescently labeled antibodies for 15 min at room temperature (RT) in the dark. To each sample were added 2 mL of lysis buffer (FACS lysis solution, diluted 1:10 in distilled water; BD Biosciences). Samples were then vortexed gently, incubated for 6 min at RT, and centrifuged at 500×g for 5 min. The supernatant was removed, lysis was stopped by addition of 4 mL phosphate buffered saline (PBS), and samples were centrifuged again and the supernatant removed. Finally, 0.5 mL of PBS was added, and the sample was analysed by flow cytometry as described below.

For Ki67 analysis, the antibody was added after the lysis centrifugation, and the reaction was incubated for 10 min at RT, followed by an additional wash in PBS.

Flow cytometry

All samples were examined by 8-colour flow cytometry in a BD FACSCanto II flow cytometer (BD Biosciences), and data were analysed with Infinicyt software (Cytognos, USA). In all cases, debris was removed by gating in a FSC/SSC dot plot, and doublets were discriminated in a FSC-Area/FSC-High dot plot. Residual normal cells in the patient sample were used as an internal control.

Cytometer standardisation

In order to generate comparable results between patients and over time, we used the BD Cytometer Setup and Tracking System (CS&T), following the recommendations with BD FACSDiva Software Version 6. The photomultiplier voltages were adjusted to unlabelled lysed whole blood cells to obtain optimal PMT voltages for the resolution of dim cell populations.[26] The target values resulting from the PMT optimisation were used for subsequent calibrations to maintain instrument standardisation.[27]

Gating strategy for the HL panel

Once the HL tube was acquired, it was analysed with Infinicyt Software (Cytognos). To select populations, we first removed doublets and debris. Lymphocytes were identified by CD45 expression. The CD4+ T cell population was defined by the CD3+ CD4+ CD8− immunophenotype; non-T lymphocytes (B cells and NK cells) were identified as the CD45+ CD3− population (Fig. 1).

Analysis of CD71, CD30, and CD15 expression

CD71 expression was studied in CD4+ T and non-T lymphocytes. CD15 and CD30 were studied in CD4+ T cells. For the analysis of percentage expression and mean fluorescence intensity (MFI), FMO controls were used (Fig. 2). Some samples showed a slight elevation in CD4 antigen intensity in the CD3+ CD4+ T cell population.

Analysis of CD71 MFI and the CD71 ratio

MFI was measured in the CD71-positive fraction of the CD4+ T and non-T lymphocyte populations. The CD71 ratio was calculated as the percentage of CD71-expressing CD4+ T cells divided by the percentage of CD71-expressing non-T lymphocytes (CD71 ratio = % CD71+ CD4+ T lymphocytes/% CD71+ non-T lymphocytes).

HL diagnosis

HL was diagnosed and classified through a combination of histological examination and immunohistochemical analysis, following the recommendations of the WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.[1] This technique is considered the gold standard for HL diagnosis.

Statistical analysis

Data distribution was assessed with the Kolmogorov–Smirnov test, and the statistical significance of differences was determined with the Mann–Whitney U test or the two-tailed Student t-test, depending on variable distribution. Statistical comparisons were performed with SPSS version 21.0 (IBM, USA). Differences were considered statistically significant at $p<0.05$. Receiver operating characteristic (ROC) curves were plotted and contingency analysed with MedCalc version 18.11.6.

RESULTS

The study of CD4+ and CD8+ T lymphocyte subpopulations detected a higher percentage of Th lymphocytes in HL

Table 1 The antibody panel for B cell clonality screening and Hodgkin lymphoma analysis

Tube	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	V450	V500
1	Kappa	Lambda	CD19	CD5	CD10	CD20	CD45	HLA-DR[a]
2	CD15	CD30	CD4	CD8	CD71	CD3	CD45	Ki67[b]
3	FMO	CD30	CD4	CD8	CD71	CD3	CD45	
4	CD15	FMO	CD4	CD8	CD71	CD3	CD45	
5	CD15	CD30	CD4	CD8	FMO	CD3	CD45	

Tube 1: Analysis of B populations for clone detection. Tubes 2–5: Hodgkin Lymphoma analysis.
APC, allophycocyanin; APC-H7, allophycocyanin H7; FITC, fluorescein isothiocyanate; FMO, fluorescence minus one; PE, phycoerythrin; PECy7, phycoerythrin Cy7; PerCP Cy5–5, peridinin chlorophyll protein; V450, fluorochrome detected in the Pacific Blue channel; V500, fluorochrome detected in the AmCyan channel.
[a] HLA-DR was studied due to previous suspicion of non-Hodgkin's B lymphoma.
[b] Ki67 was studied in three HL cases.

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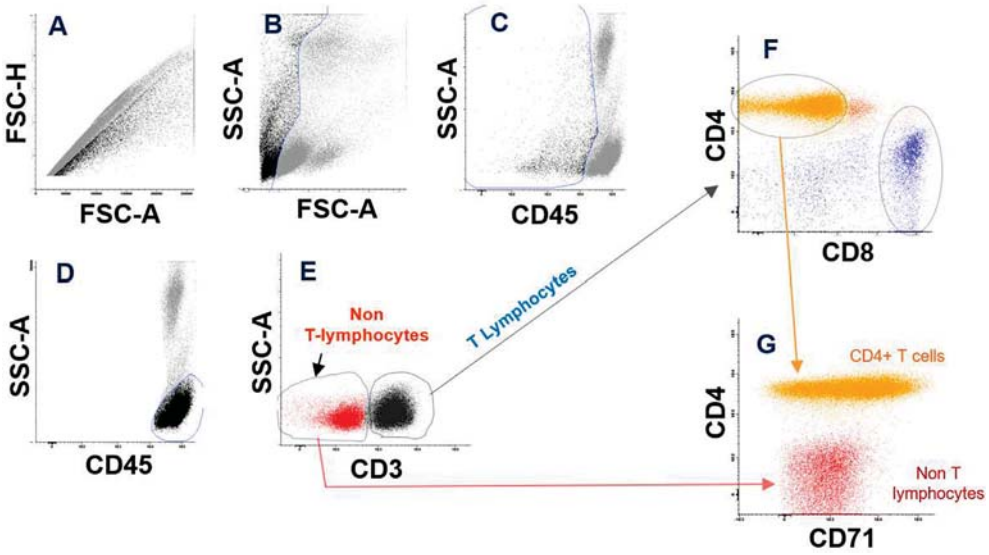

Fig. 1 Representative sequential gating strategy used to identify cell populations in the study of Hodgkin's lymphoma in a lymph node sample. (A–C) Plots used to exclude doublets and debris. (D) SSC-A/CD45 plot for the selection of the lymphocyte population. (E,F) Sequential selection of T lymphocytes and CD4+ T and CD8+ T lymphocyte subpopulations. (G) Depiction of non-T lymphocytes (red) and CD4+ T lymphocytes (orange) for further analysis of CD71 using the fluorescence minus one (FMO) controls (see Fig. 2).

samples than in non-HL samples (43.70% vs 38.85%, $p<0.05$). However, no significant between group differences were found in the percentage of cytotoxic T lymphocytes (23.31% vs 23.67%, $p>0.05$).

HL screening tube analysis revealed a striking elevation of CD71 expression in the CD4+ T cell subpopulation compared with other lymphocytes. Moreover, these CD4+ T cells showed no alterations in CD2, CD5, CD7, CD8, or CD3 antigen expression (data not shown). The percentage CD4+ T cells expressing CD71 was higher in HL samples (49.57%) than in non-HL samples (24.51%) (Mann–Whitney U=4.15, $p<0.001$) (Fig. 3A), as was CD71 MFI (3215.52 vs 2023.42, Mann–Whitney U=3.87, $p<0.001$). A similar pattern was observed for CD30, with expression on 18.59% of CD4+ T cells in HL samples vs 8.82% in non-HL samples (Fig. 3B) and corresponding MFI values of 5081.1 and 3778.95 (Mann–Whitney U=4.15, $p<0.001$). Percentage CD15 expression in T CD4+ cells was also higher in HL samples than in non-HL samples (5.6% and 0.6%, respectively; Mann–Whitney U=2.94, $p<0.001$) (Fig. 3C). CD15 MFI was not measured because of the low percentage expression. Additionally, the CD71 ratio was significantly higher in HL samples (0.71) than in non-HL samples (0.31) (Fig. 3D).

The analysis of CD71 expression in the remaining lymphocyte populations showed higher percentage expression in CD8+ T lymphocytes from HL patients vs non-LH patients (33% and 19.13%, respectively; Mann–Whitney U=3.44, $p<0.001$). In contrast, CD71 expression on B and NK lymphocytes did not differ between the patient groups (28.49% vs 33.2% in HL cases and non-HL samples, respectively).

These findings prompted us to seek a strategy for HL diagnosis according to the percentage CD71 expression. However, the ROC curve established a cut-off of 44% positivity, with a specificity of 89.74% but a sensitivity of just 58.46%. Thus, while the specificity was good, sensitivity was poor, indicating that percentage CD71 expression cannot reliably distinguish between HL and non-HL samples. In contrast, the CD71 ratio proved to be more sensitive and specific. A ROC curve established the optimal CD71 ratio cut-off at 0.50, with an area under the curve (AUC) of 0.897 (two-tailed Student t-test, $p<0.001$) (Fig. 4).

Workflow algorithm for flow cytometry diagnosis of HL

Because CD30 and CD15 in the HL diagnostic algorithm did not improve discrimination between HL and non-HL samples, the workflow considered only the CD71 ratio. On receipt of a request for a lymph node study (CNB or FNA), the patient's history was checked for records of any previous immunophenotypic study with the presence of an abnormal lymphoid population. If a clonal population had previously appeared or the patient had already been diagnosed, the study was expanded to search for this population. In the absence of previous studies, the HL screening panel was applied. The B population study tube was always applied, as it is possible to find synchronous diseases with clonal B populations.

If a clonal B population was detected, the study was extended in most cases to analyse the expression of CD22, CD23, CD79b, CD200, CD38, FMC7, CD43, and BCL-2 in the target population. But if a phenotypic abnormality was detected in the T antigens from the HL screening panel (in addition to the CD3, CD45, CD4, CD8, CD15, and CD30 antigens included in the tube), then CD2, CD7, and CD5 antigens were also studied in the suspect population, together with CD10, CD26, CD16, CD56, CD57, TCRab, TCRgd, or CD1a, depending on the alterations found previously.

Upon detection of a non-haematological population, an immature B or T population, plasma cells, or other lineages, more specific panels were applied.

If no pathological B population and no T antigen abnormalities were detected, the study was expanded to rule out HL. Once the screening tube had been analysed as described above, samples with a CD71 ratio ≥ 0.5 were considered suggestive of HL. Samples with a CD71 ratio <0.5 are considered not suggestive of HL (Fig. 5).

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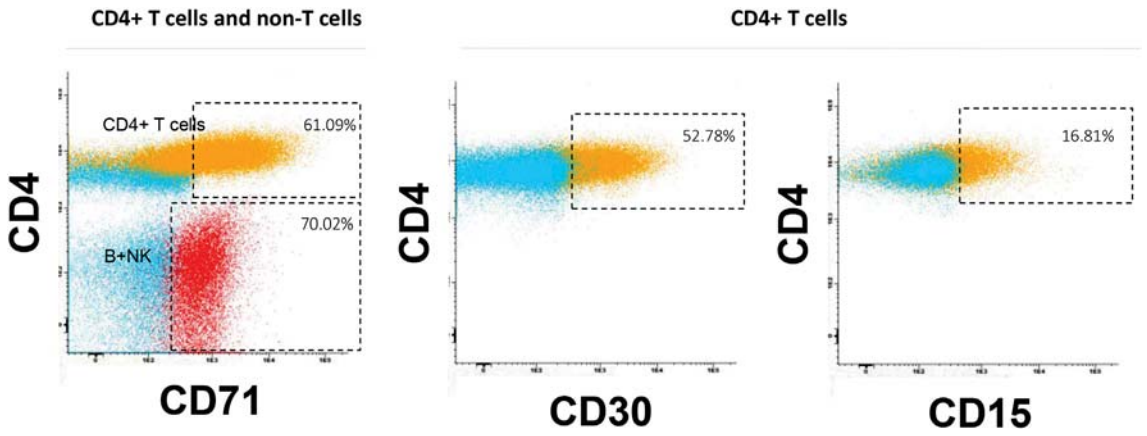

Fig. 2 Representative example of the analysis of CD71, CD30, and CD15 in a lymph node sample. Fluorescence minus one (FMO) controls were used to calculate the percentage expression of CD30 and CD15 in CD4+ T cells and the percentage expression and MFI of CD71 in CD4+ T and non-T lymphocytes. A reference image was created of the FMO control for each antigen (in blue) and overlaid on the corresponding antigen plot of the fully stained tube. CD4+ T cells are depicted in orange and non-T cells in red. FMO, fluorescence minus one; MFI, mean fluorescence intensity.

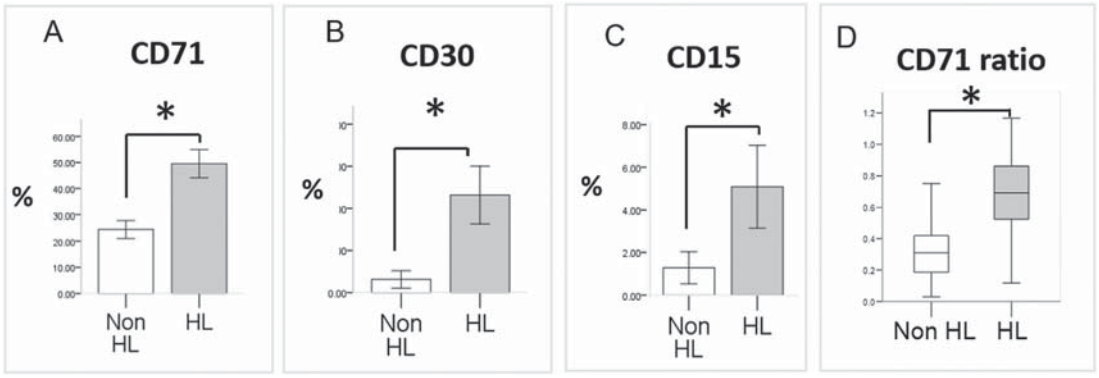

Fig. 3 (A–C) Percentage expression of (A) CD71, (B) CD30, and (C) CD15 in T CD4+ lymphocytes from lymph node samples of HL and non-HL patients. (D) CD71 ratio in HL and non-HL samples. Box and whisker plots indicate median, upper quartile, and lower quartile. Mann–Whitney U test; *p<0.01 for HL vs non-HL.

The sensitivity and specificity resulting from this analysis strategy were 82% and 87%, with a positive predictive value (PPV) and negative predictive value (NPV) of 0.80% and 0.85%, respectively. The percentage of correctly diagnosed cases was 84.62%, with a +LR of 6.36 and a −LR of 0.21.

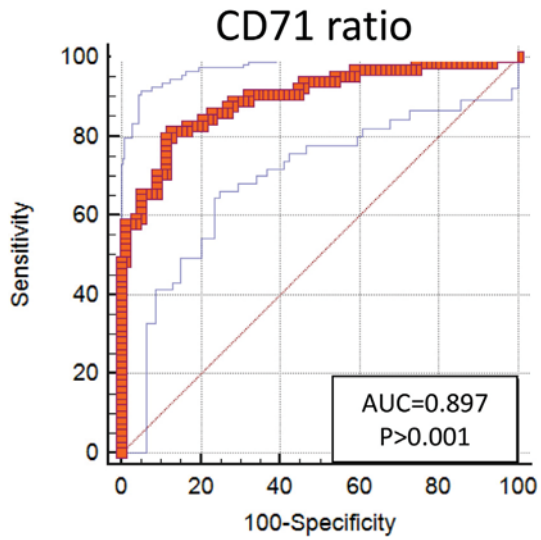

Fig. 4 Receiver operating characteristic (ROC) curve for the determination of the optimal cut-off for the CD71 ratio. The cut-off was set at 0.5, with an area under the ROC curve (AUC) of 0.897.

Among the 143 patient samples studied, the algorithm achieved 121 correct, 12 false negative, and 10 false positive diagnoses (Table 2).

Details of the gold-standard histopathological diagnosis are presented in Table 3.

Algorithm and T cell lymphoma

In our case series, application of the algorithm did not suggest exclusion of HL in any patient who had a final histopathological diagnosis of non-Hodgkin T lymphoma. This may be because in some cases peripheral blood or bone marrow samples sent to the laboratory to rule out chronic lymphoproliferative syndrome or lymphoma had already been studied before acquisition of the lymph node biopsy. When an abnormal T population was detected, the subsequent immunophenotypic study was specifically targeted to detect that population. In any case, immunoglobulin light chains were always studied to rule out synchronous diseases involving B cell populations. In these cases, the HL tube would prove a useful assessment of the expression of CD15 and CD30 but would not rule out HL as this was not the clinical suspicion.

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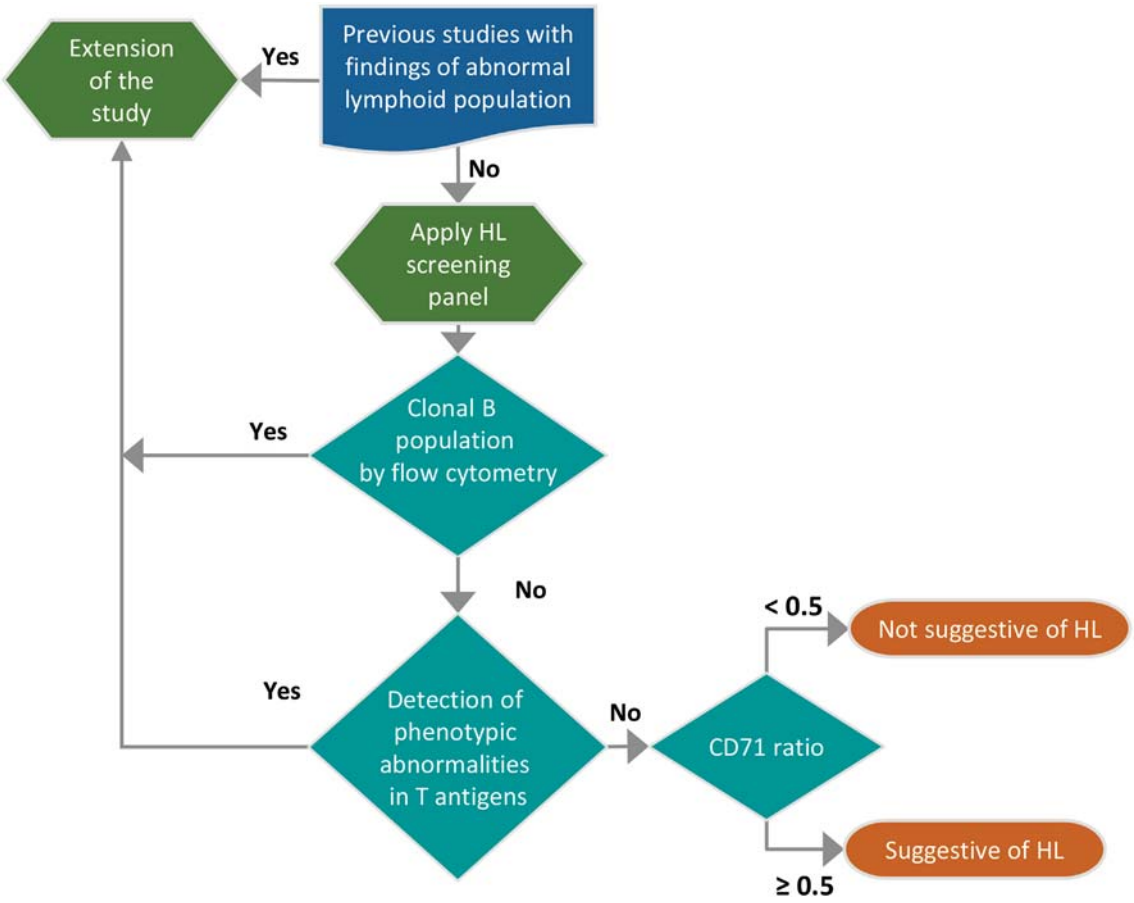

Fig. 5 Proposed workflow algorithm for Hodgkin lymphoma (HL) screening by flow cytometry. If the clinical history shows no previous studies and application of the HL screening tubes shows no other signs suggesting a pathological B or T population, then the CD71 ratio is considered. A ratio < 0.5 is considered not suggestive of HL, whereas a ratio ≥0.5 is considered suggestive of HL.

One patient with suspected chronic lymphoproliferative syndrome (with no previous studies) had a final diagnosis of angioimmunoblastic T cell lymphoma (AITL). Screening tubes were run, and the suspected T malignancy population was detected in the immunoglobulin light chain tube from the presence of a CD10+ CD5+ lymphoid population with no CD19 or CD20 expression (Supplementary Fig. 1A, Appendix A). The CD71 ratio was below 0.5 (0.33), but this was not relevant because the findings did not indicate consideration of HL, and the study was extended to consider T cell non-Hodgkin lymphoma.

In an inguinal biopsy referred for polyadenopathy screening, the HL tube screen detected a CD4+dim CD3− CD45+ population, and the subsequent expanded T population study revealed the following phenotype: CD10−, CD15−, CD30−, CD71−, CD2+, CD7+, CD5+, TCRab−, TCRgd−, CD56−, and CD1a+ 51.80%. Histopathology confirmed a diagnosis of T lymphoblastic lymphoma (Supplementary Fig. 1B, Appendix A). The CD71 ratio was 0.3.

One lymph node biopsy was referred for screening to distinguish between suspected HL and anaplastic large cell lymphoma (ALCL). In this case, a more complete screening was performed using an initial tube with CD2+Kappa FITC, CD7+Lambda PE, CD19 PerCP-Cy5.5, CD5 PE-CY7, CD8 APC, CD3 APC-Cy7, and CD45 V450, in addition to the HL tube. The sample had a high CD71 ratio (2.18) and was negative for CD30, and there was no evidence of large cells or phenotypic alterations in the pan-T antigens studied. This suggested that HL should be ruled out, as later confirmed by histopathology.

We also received two other patient samples for the study of ALCL. One patient had already been diagnosed with ALK-negative ALCL and had received treatment; the lymph node biopsy was referred to our unit due to suspicion of disease relapse. A complete panel for ALCL and HL was run to determine the CD71 ratio. Two CD30+ cell populations were observed (Fig. 6A): one with larger cells, greater cell complexity, high CD45 expression, and no detected CD4 expression; and another corresponding to the CD4+CD30+ T lymphocyte population, which showed no phenotypic alterations in CD2, CD7, or CD5 expression (data not shown). The CD71 ratio was high (0.82). The second patient sample was sent to our unit due to a high suspicion of ALCL. The HL tube screen detected the presence of a large CD30+ cell population, with partial expression of CD3 and negativity for CD4. Together with the other immunophenotypic features (CD45++, CD8−, CD57−, CD25++, CD1a−, CD56+, CD2+, TCRab−, TCRgd−, CD14−, and CD5−), these findings suggested ALCL, which was confirmed in the histopathological study (Fig. 6B). The CD71 ratio was 0.25.

A lymph node biopsy referred for suspected lymphoma showed CD30+ cells in the HL tube, corresponding to CD4+ T lymphocytes, with no alterations in CD3 or CD45 expression and no large CD30+ cells. The CD71 ratio was elevated (0.94), and eosinophils were present (Fig. 6C),

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Table 2 Comparison of gold standard diagnosis of Hodgkin lymphoma (HL) with the proposed new flow cytometric approach

Histopathology	Flow cytometry		Total
	HL	Non HL	
HL	53	10	63 (44.05%)
Non HL	12	68	80 (55.94%)
Total	65 (45.45%)	78 (45.54%)	143

Table 3 Histopathological diagnosis (N=143)

Diagnosis	*n* (%)
Hodgkin lymphoma	65 (45.45)
Classic HL	63 (44.05)
Nodular sclerosis classic HL	39 (27.27)
Mixed cellularity classic HL	12 (8.39)
Lymphocyte rich classic HL	4 (8.39)
Nodular lymphocyte predominant HL	2 (2.79)
Non-Hodgkin lymphoma	78 (54.54)
Reactive	55 (39.46)
Absence of malignancy	13 (9.09)
Other lymphoma (non-HL)	3 (2.09)
Metastasis	7 (4.89)

Final histopathological diagnosis in all samples analysed.

suggesting that HL could be ruled out. This patient was finally diagnosed with mixed cellularity classical Hodgkin lymphoma (MCcHL).

In our patient series, no cases of ALCL were detected without prior clinical suspicion, meaning that the algorithm could not be tested sufficiently. However, it seems likely that with a testing strategy that screens for the presence of large CD30+ cells in the HL tube, a possible ALCL would be detected. It is also important to note that, as with HL, the utility of cytometry for the study of anaplastic NHL-T is limited by the fragility of the affected cells, their nodal location, and their frequent low abundance.[28]

ALCL is generally a more aggressive disease than HL, and this consideration can be useful in deciding which type of lymphoma is suspected. Therefore, it is always important to consider the immunophenotypic findings together with the patient's clinical context. If the laboratory does not have access to previous studies and clinical records, the screening tubes could usefully include some T lineage markers, such as CD2, CD7, and CD5.

The proposed flow cytometry approach is applicable to all sample types so long as there is sufficient cellularity (Supplementary Fig. 2, Appendix A).

Analysis of Ki67 expression in three HL lymph node samples showed that Ki67-expressing cells were also CD71-positive, in agreement with previous studies,[21,29,30] and indicating activation and proliferation of CD4+ T lymphocytes (Supplementary Fig. 3, Appendix A).

DISCUSSION

Our results suggest that in HL CD71 expression is higher on CD4+ T lymphocytes than on non-T lymphocytes. In addition, the percentage of Th lymphocytes was higher in HL samples than in non-HL samples, while the percentage of

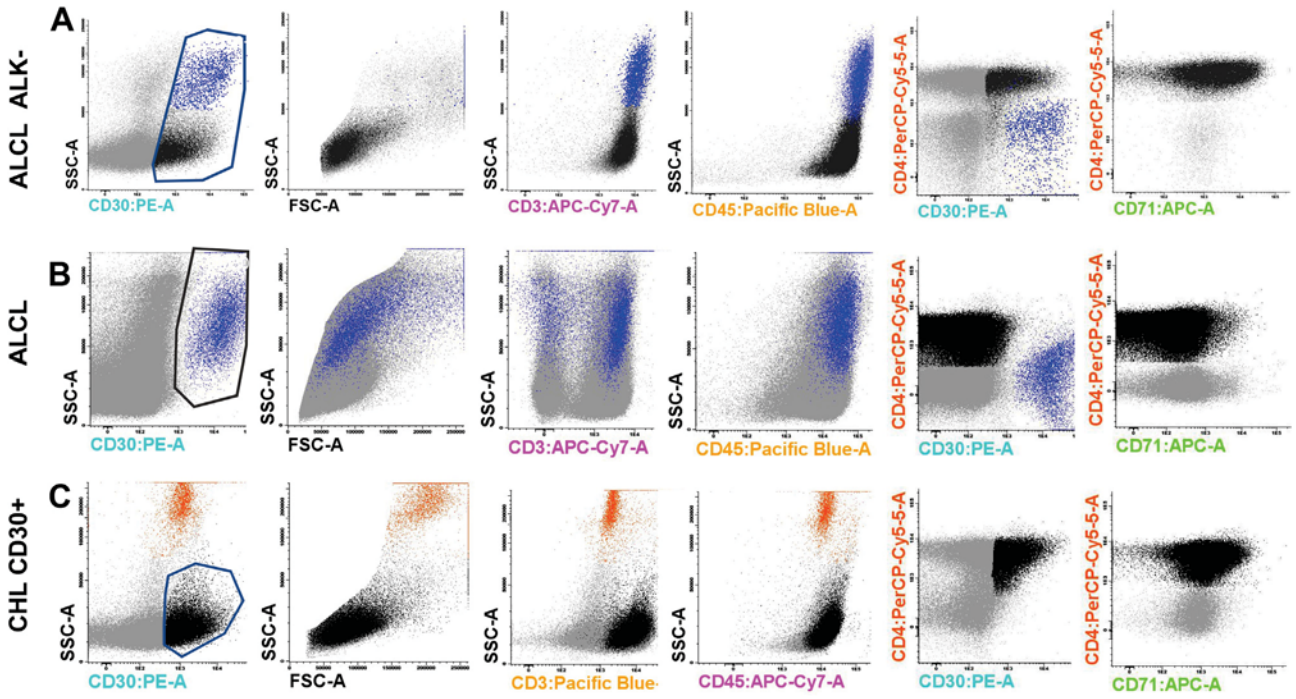

Figure 6 Representation of cell size/complexity and the expression of CD3, CD45, CD4, and CD71 in two cases of CD30+ ALCL (A,B) and a case of classical Hodgkin lymphoma with mixed cellularity (C). (A) ALK− CD30+ ALCL. In the SSC/CD30 plot, two CD30+ populations are observed, one of large cells (blue) and one of smaller cells (black). The large cell population is off the scale in FSC, is positive for CD3 and CD45, shows high cellular complexity, and is negative for CD4. In the CD4+ T population, CD71 expression is high, with a CD71 ratio of 0.82. (B) CD30+ ALCL. Blue depicts a large and complex CD30+ population with partial expression of CD3, high expression of CD45, and negativity for CD4. The CD4+ T cells (black) are CD30− with low CD71 expression and a CD71 ratio of 0.25. (C) Classic CD30+ HL. Eosinophils are present (orange), and a CD30+ population (black) corresponds to CD4+ T lymphocytes, with normal expression of CD3 and CD45, a high percentage of CD71, and a CD71 ratio of 0.94. Unlike the situation in ALCL, large CD30+ cells are absent.

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cytotoxic T lymphocytes was the same in both groups. These results are consistent with proposals that HRS cells rely on T helper cells to create a favourable tumour environment and control the cytotoxic response of T and NK lymphocytes.[31,32] Despite the presence of multiple tumour-infiltrating T cells that 'rosette' malignant HRS cells, the failure of these T cells to kill HRS cells strongly suggests that the T cell response in HL is ineffective.

The CD71 expression pattern on CD4+ T cells in HL patients prompted us to analyse whether CD71 expression on tumour-associated lymphocytes could provide the basis for the diagnosis of HL by flow cytometry, which, contrasting its utility in most lymphomas, is of poor diagnostic value in this entity.

The diagnostic algorithm based on the CD71 ratio accurately discriminated between HL and non-HL in a high proportion of samples (84.61%). In two patients, histological analysis of FNAs identified cells suggestive of a chronic lymphoproliferative disorder but without concluding a diagnosis of HL, whereas the new flow cytometry-based immunophenotyping approach indicated HL. In both cases, subsequent histopathological analysis of lymph node biopsies confirmed the final diagnosis of nodular sclerosis classical HL.

Instances of false negative and false positive diagnosis with the cytometry-based approach are summarised in Supplementary Table 1 (Appendix A). Of the 12 false negative results, three were close to the CD71 ratio diagnostic cut-off (0.48, 0.45, and 0.42) and the CD4+ T population included cells expressing CD15 and CD30 (15.81%, 12.0%, and 27.95%, respectively). Cases near the CD71 ratio cut-off and positive for CD15 or CD30 expression could therefore be interpreted as 'HL suspects', with an indication that HL be confirmed or ruled out by pathological anatomy. Another refinement strategy could be to relate the CD71 ratio to the size of the tumour component, since the CD4+, CD71+ T cell percentage would be low in samples with few HRS cells. In patients with NHL-B cells together with clinical signs, another synchronous disease cannot be ruled out, and clinical assessment would be indicated to avoid misdiagnosis of HL.

Of the 10 false positive diagnoses, one patient had a histological diagnosis of NHL-B, although the immunophenotype analysis did not detect a B cell clone. One possible explanation is that the sample sent for flow cytometry might not have been representative, since we usually find very close agreement between pathological anatomy and flow cytometry for the detection of B cell clonal populations, as also reported by other authors.[33,34] Another explanation might be the large size of the cells.[7] It is also important to note that lymph node metastases of lymphoepithelial tumours had very similar characteristics to those of HL in terms of CD71 expression and ratio, so the proposed algorithm would likely have limited ability to discriminate between these diseases. Therefore, it may be necessary to include more markers in order to reduce the number of misdiagnosed cases.

Regarding T-cell lymphomas, some alterations were found in tubes 1 and 2 that alerted us to the possible presence of an abnormal T population and required further study. However, these patients were generally well studied before referral and there was no need for an HL study.

CONCLUSION

The flow cytometry strategy we used achieved a correct positive or negative diagnosis in 84.61% of the patients studied, with a sensitivity and specificity of 82% and 87%. Flow cytometry study of HL is not currently routine in clinical laboratory practice, and this strategy could provide a new tool to support the diagnosis of HL. However, despite the good correlation between the flow cytometry and histopathological analysis, confirmation of the utility of the proposed immunophenotyping approach will require further studies with larger numbers of patients.

The new algorithm describes a single tube, well-defined analysis and interpretation strategy that can be applied in a simple and standardised way in any clinical laboratory without requiring high end instruments. The antibodies are widely used in diagnostic panels for malignant haemopathies, so in-laboratory tuning is highly viable without requiring significant investment in antibodies or additional reagents. Its applicability in samples such as FNA is of special interest, since it provides rapid orientation for the pathological study of HL, which is more limited in these types of samples than in lymph node samples. Flow cytometry of FNAs successfully diagnosed HL, including non-classical types; however, larger sample sizes are needed because nodular lymphocyte predominant HL was detected in only two cases.

Caution should be exercised when the CD71 ratio is close to but below cut-off and when signs and symptoms are suggestive of HL; in these cases, the CD71 ratio may be influenced by the tumour burden if it is very low. Although histopathology remains the definitive diagnostic tool for HL, flow cytometry can provide rapid study guidance and provide a more robust and integrated HL diagnosis. The results presented here provide the basis for further studies of HL by flow cytometry, which should examine larger patient samples and possibly include other antibodies to increase the sensitivity of the technique.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2022.07.005.

Address for correspondence: Dr Maria Beatriz Álvarez Flores, Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Flow Cytometry Unit, Melchor Fernández Almagro, 3, Madrid, 28029, Spain. E-mail: mbalvarez@cnic.es

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