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# Multi-centre validation of a flow cytometry method to identify optimal responders to interferon-beta in multiple sclerosis

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*Abbreviations:* CI, Confidence interval; ICC, Intraclass correlation coefficient; IFN, Interferon; MoAb, Monoclonal antibodies; MS, Multiple sclerosis; PC, Participating centres; PMT, Photomultiplier; RRMS, Relapsing-remitting multiple sclerosis; RF, Reference centre; RT, Room temperature

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ABSTRACT

*Background and objectives:* Percentages of blood CD19 + CD5 + B cells and CD8 + perforin + T lymphocytes can predict response to Interferon (IFN)-beta treatment in relapsing-remitting multiple sclerosis (RRMS) patients. We aimed to standardize their detection in a multicenter study, prior to their implementation in clinical practice.
*Methods:* Fourteen hospitals participated in the study. A reference centre was established for comparison studies. Peripheral blood cells of 105 untreated RRMS patients were studied. Every sample was analyzed in duplicate in the participating centre and in the reference one by flow cytometry. When needed, participating centres corrected fluorescence compensations and negative cut-off position following reference centre suggestions. Concordance between results obtained by participating centres and by reference one was evaluated by intraclass correlation coefficients (ICC) and Spearman correlation test. Centre performance was measured by using z-scores values.
*Results:* After results review and corrective actions implementation, overall ICC was 0.86 (CI: 0.81–0.91) for CD19 + CD5 + B cell and 0.89 (CI: 0.85–0.93) for CD8 + perforin + T cell quantification; Spearman r was 0.92 (0.89–0.95; $p < 0.0001$) and 0.92 (0.88–0.95; $p < 0.0001$) respectively. All centres obtained z-scores ≤ 0.5 for both biomarkers.
*Conclusion:* Homogenous percentages of CD19 + CD5 + B cells and CD8 perforin + T lymphocytes can be obtained if suitable compensation values and negative cut-off are pre-established.

## 1. Introduction

IFN-beta is a safe and effective drug for the treatment of RRMS [1,2]. Since response to this treatment is heterogeneous in MS, searching for biomarkers that allow early identification of patients with high probability of showing an optimal response is of great clinical importance.

Blood percentages of CD19 + CD5 + B cells or CD8 + perforin + T cells obtained before initiation of IFN-beta predict response to this treatment in MS [3].

Method standardization is an essential stage preceding the use of biomarkers in clinical practice, especially in flow cytometry where small variations in cut-off position can involve big changes in the results. Standardization of methods in flow cytometry is of great help in the diagnosis of hematopoietic neoplasms [4]. The use of flow cytometry in the diagnosis and management of autoimmune diseases is emerging [5–8], but standardized methods are needed to obtain homogeneous and accurate results.

In this work, we performed a multicentre study to validate blood CD19 + CD5 + B cell and CD8 + perforin + T cell detection by flow cytometry in MS patients as a previous step to their clinical implementation as predictors of response to IFN-beta treatment.

## 2. Patients and methods

### 2.1. Inclusion criteria

105 naïve RRMS patients of 14 Spanish hospitals were included in the study. The study was approved by the Ethics Committees of each hospital. Written informed consent was obtained from all patients before entry. A list of participating centres (PC) is shown in Table 1. Baseline data of patients are shown in Table 2.

### 2.2. Study design and logistics

Samples of 5 ml of heparinized blood were obtained in each PC. One half was stored at room temperature (RT) during 24 h. The other one was shipped at RT to the reference centre (Hospital Ramón y Cajal, Madrid, RC). Percentages of CD19 + CD5 + B cells and CD8 + perforin + T lymphocytes were evaluated in PC and in the RC by a consensual flow cytometry protocol. PC sent their preliminary results to the RC, where they were checked. Suggestions to improve the analysis were made when needed, and data comparison was performed. Experimental design is shown in Fig. 1.

**Table 1**
Study participants.

| Institution | Number of shipped samples |
|---|---|
| Hospital Universitario Ramón y Cajal, Madrid (Reference centre) | – |
| Hospital Regional Universitario de Málaga, Málaga | 10 |
| Hospital Universitari del Mar, Barcelona | 10 |
| Hospital Universitari Son Espases, Palma de Mallorca | 10 |
| Hospital Universitari Vall d'Hebron, Barcelona | 10 |
| Hospital Universitario Donostia, Donostia-San Sebastián | 10 |
| Hospital Clínico Universitario de Valencia, Valencia | 9 |
| Hospital Clínico Universitario Virgen de la Arrixaca, Murcia | 8 |
| Hospital Universitario Marqués de Valdecilla, Santander | 7 |
| Hospital General Universitario Gregorio Marañón, Madrid | 6 |
| Hospital Clínic Universitari de Barcelona, Barcelona | 5 |
| Hospital San Pedro de Alcántara, Cáceres | 5 |
| Hospital Universitari de la Santa Creu i Sant Pau, Barcelona | 5 |
| Hospital Universitari Germans Trias i Pujol, Badalona | 5 |
| Hospital Universitario de la Princesa, Madrid | 5 |

**Table 2**
Demographic and clinical data of patients included in the study.

| | $n$ = 105 |
|---|---|
| Sex (male/female) | 35/70 |
| Age at disease onset (years) Mdn (25%–75% IQR) | 34.0 (28.0–42.0) |
| Age at study initiation (years) Mdn (25%–75% IQR) | 41.0 (35.0–51.0) |
| Disease duration (years) Mdn (25%–75% IQR) | 2.0 (0.3–11.1) |
| Number of patients who received corticosteroids within the three months prior to sample extraction | 11 |

Footnote: IQR: interquartile range; Mdn: median.

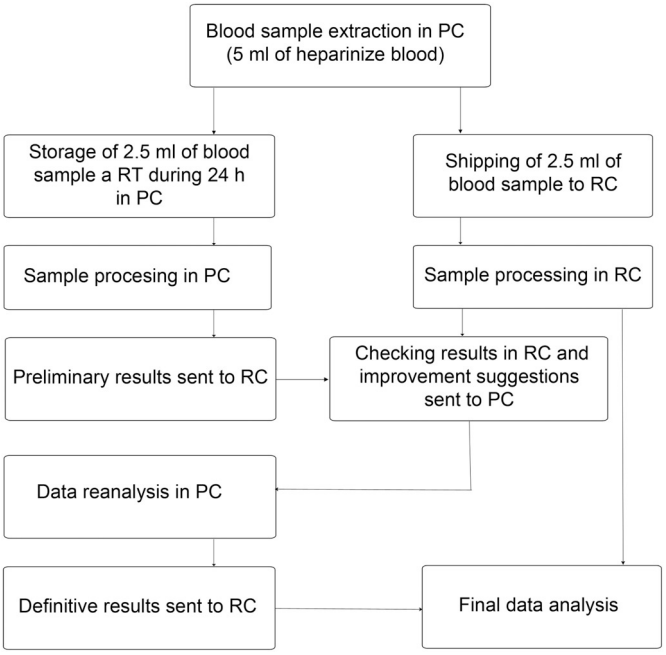


**Fig. 1.** Experimental design. Samples of 5 ml of heparinized blood obtained in participating centre (PC) were divided in two identical aliquots. The first one was stored in the PC at room temperature (RT) during 24 hours (h). The other one was shipped to the reference centre (Hospital Ramón y Cajal, Madrid, RC). Samples were processed at the same time in PC and RC for CD19 + CD5 + B cell and CD8 + perforin + T lymphocyte evaluation. Preliminary data obtained by each PC were sent to RC. There, results were checked and suggestions made to improve the analysis when needed. PC performed reanalysis and sent definitive results to RC where data comparison was made.

### 2.3. Monoclonal antibodies

The following monoclonal antibodies (MoAb) were used (all from BD Biosciences, USA): IgG2b-FITC (clone 27–35), Perforin-FITC (clone δG9), CD8-PE (clone SK1), CD3-PerCP (clone SK7), CD19-PE-Cy7 (clone SJ25C1), CD3-APC (clone SK7), CD5-APC (clone L17F12), CD56-APC (clone NCAM16.2), and CD45-APC-H7 (clone 2D1).

### 2.4. Sample staining

#### 2.4.1. Staining procedure for CD19 + CD5 + B cells

Two aliquots of 100 µl of whole blood were labelled for 20 min at RT in the dark with the following MoAb: T*ube 1*: CD19-PE-Cy7, CD3-APC, CD45-APC-H7; *Tube 2*: CD19-PE-Cy7, CD5-APC, CD45-APC-H7. Erythrocytes were lysed with 3 ml of BD FACS™ Lysing Solution (BD Biosciences), cells washed in saline and analyzed by flow cytometry within a maximum period of 1 h.

#### 2.4.2. Staining procedure for CD8 + perforin + T cells

Two aliquots of 100 µl of whole blood were labelled for 20 min at RT in the dark with CD8-PE, CD3-PerCP, CD56-APC and CD45-APC-H7 MoAb. Cells were washed, fixed and permeabilized with 200 µl of BD Cytofix/Cytoperm™ (BD Biosciences) in the dark at 4 °C for 20 min. Then, they were washed with BD Perm/Wash™ buffer (BD Biosciences) and intracytoplasmic staining was performed for 30 min at 4 °C in the dark with the following MoAb: *Tube 3:* IgG2b-FITC; *Tube 4:* anti-perforin–FITC. Cells were washed twice with BD Perm/Wash™ buffer, resuspended in saline and analyzed by flow cytometry within a maximum period of 1 h.

### 2.5. Flow cytometry

Flow cytometers and analysis software used are listed in Table 3. Calibration beads were always used. Photomultiplier (PMT) voltages and fluorescence compensation parameters were adjusted using a normal donor sample. CD19 + CD5 + B cells and CD8perforin + CD56- T cells were analyzed on viable CD45 + lymphocytes. Mean autofluorescence values were set using appropriate negative controls. A minimum amount of 30.000 viable CD45 + cells per tube were analyzed. Gating strategy is shown in Fig. 2.

### 2.6. Statistical analysis

Statistical analyses were made using StataCorp software V.15.1 (StataCorp LP, USA) and Prism V.6.0 statistical package (GraphPad Software, USA). Concordance between percentages of peripheral blood CD19 + CD5 + B cells and CD8 + perforin + T lymphocytes from PC and the RC was evaluated by using ICC and Spearman test. $p$ values lower than 0.05 were considered as significant. Centre performance was evaluated using z-score values, which were calculated using the following formula: (Absolute value of average of the values obtained by each PC minus average of the values obtained by the reference) divided by (standard deviation of the values obtained by the reference).

## 3. Results

### 3.1. Inter-centre comparison of CD19 + CD5 + B cell and CD8 + perforin + T cell results and Centre performance evaluation before corrective actions

A total of 105 samples were processed. Three samples were eliminated from the study due to a delay in the reception in the RC and another one for a concomitant lymphoma (unexpected finding).

When comparing global results obtained by PC with those obtained by the RC, we found an overall ICC of 0.60 (CI: 0.49–0.71) for CD19 + CD5 + B cell and of 0.80 (CI: 0.73–0.86) for CD8 + perforin + T cell quantification. Spearman test revealed similar results: $r = 0.68$ (CI: 0.56–0.78; $p < 0.0001$) and $r = 0.83$ (CI: 0.75–0.88; $p < 0.0001$) respectively. Correlations are shown in Fig. 3A and C.

We next compared results obtained individually by the 14 PC and those obtained by the RC, using z-score values. Thirteen (92.9%) PC got z-scores values < 1.0 for both biomarkers. In addition, 6 (43.9%) and 11 (78,6%) PC got z-scores ≤ 0.5 for CD19 + CD5 + B cells and CD8 + perforin + T cells respectively (Fig. 4A, C). These results show that concordances were better for CD8 + perforin + T cells than for

**Table 3**
Flow cytometers and software specifications.

| PC Code | Flow Cytometer | LEW (B/R) | Fluorescence Channel | | | | | | Analysis Software |
|---|---|---|---|---|---|---|---|---|---|
| | | | FITC | PE | PerCP | PE-Cy7 | APC | APC-Cy7 | |
| Reference | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.7.0 |
| C1 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.6.1.3 |
| C2 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.6.1.3 |
| C3 | Navios (BEC) | 488/638 | 525/40 BP | 585/42 BP | 690/50 BP | 780/60 BP | 660/20 BP | 780/60 BP | Kaluza V.1.3 |
| C4 | LSRII (BB) | 488/633 | 505 LP 530/30 BP | 505 LP 530/30 BP | 685 LP 695/40 BP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FlowJo V.9.9.6 |
| C5 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.8.0.1 |
| C6 | CytoFLEX (BEC) | 488/638 | 525/40 BP | 585/42 BP | 690/50 BP | 780/60 BP | 660/20 BP | 780/60 BP | CytExpert 1.2.11 |
| C7 | Navios (BEC) | 488/638 | 525/40 BP | 575/30 BP | 675/20 BP 695/30 BP | 755 LP | 660/20 BP | 755 LP | Kaluza V.2.0 |
| C8 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.8.0.1 |
| C9 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.6.1.3 |
| C10 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.8.0.1 |
| C11 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.6.1.2 |
| C12 | FACSCanto II (BD) | 488/633 | 502 LP 530/30 BP | 556 LP 585/42 BP | 655 LP 670 LP | 735 LP 780/60 BP | 660/20 BP | 735 LP 780/60 BP | FACSDiva V.6.1.3 |
| C13 | FACSVerse (BD) | 488/640 | 507 LP 586/47 BP | 560 LP 586/42 BP | 565 LP 700/54 BP | 752 LP 783/56 BP | 660/10 BP | 752 LP 783/56 BP | FACSuite V.1.0.6 |
| C14 | Gallios (BEC) | 488/633 | 525/40 BP | 575/30 BP | 675/20 BP | 780/60 BP | 660/20 BP | 755 LP | Kaluza V.1.5 |

Footnote: B: Blue laser; BD: Becton Dickinson and Company; BEC: Beckman Coulter Incorporation; BP: Band Pass; LEW: Laser Excitation Wavelength; LP: Long Pass; PC: Participating centre; R: Red laser.

CD19 + CD5 + B cells quantification which was in agreement with ICC and r values.

### 3.2. Frequent errors and corrective actions

We next investigated the main causes of discordance. The most frequent errors were wrong or inaccurate negative cut-off axis position, and incorrect fluorescence compensation. Other problems appearing occasionally were wrong PMT voltage values, poorly processed samples or miscalculation. Their frequencies are shown in Table 4.

A total of 40 of 101 CD19 + CD5 + B cell percentages (39.6%) and 22 of 101 (21.8%) of CD8 + perforin + T cell quantifications needed corrective actions implementation by RC. A representative example of most common changes is shown in Fig. 5.

### 3.3. Inter-centre comparison of CD19 + CD5 + B cell and CD8 + perforin + T cell results and Centre performance evaluation after corrective actions

Concordance improved considerably after reviewing results. Overall ICC values changed to 0.86 (CI: 0.81–0.91) for CD19 + CD5 + B cells and to 0.89 (CI: 0.85–0.93) for CD8 + perforin + T cells. Spearman r changed to 0.92 in both cases (CI: 0.89–0.95; $p < 0.0001$) and (CI: 0.88–0.95; $p < 0.0001$) respectively (Fig. 3B, D). Performance improved in all centres in which corrective actions were implemented. All participants got z-scores ≤ 0.5 for both biomarkers (Fig. 4B, D).

## 4. Discussion

Standardization is a very important step before implementation of a biomarker in clinical practice. This is especially relevant when flow cytometry is used [9–13]. In this work, we performed a multicentre study to validate blood CD19 CD5 + B cell and CD8 + perforin + T cell detection by flow cytometry prior to their use as predictors of IFN-beta response in MS. We established as RC the centre where the detection method was developed [3].

We used ICC, Spearman r, and z-score values for comparing results. ICC values and Spearman test are broadly used a measure of intercentre agreement [14–19], and *Z*-score is commonly employed in proficiency testing of intercentre comparisons studies as a performance indicator [20,21].

The selection of the best fluorochromes for standardization in flow cytometry was previously proved [22]. In addition, our work shows that, apart from minor technical issues or human errors, the main problems in standardization are observed in instrument setting adjustments (i.e. PMT voltages and compensation values) [4] and in manual negative cut-off establishment [23]. Since those variables are subjectively set up, the use of standardized analysis templates for flow cytometry assays are needed to obtain homogeneous results. In this way, our corrective actions clearly improved overall concordance and z-scores obtained by PC. In fact, after revision, overall ICC values and Spearman r were above 0.80 and z-scores below 0.5, which are considered as optimal agreement values, for all centres.

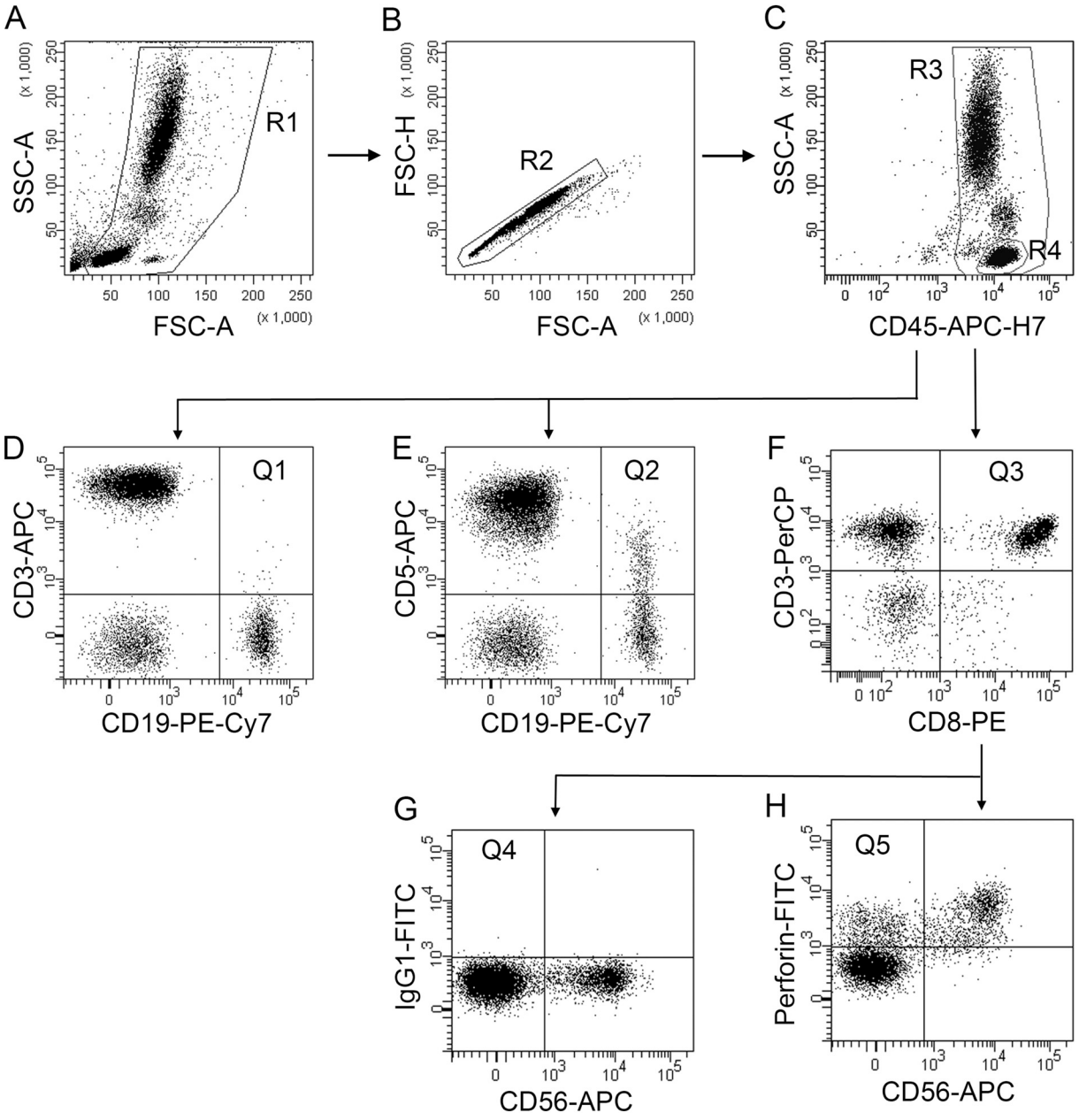


**Fig. 2.** Representative images of gating strategy for flow cytometry analysis. Cells were first gated to exclude debris and apoptotic cells (panel A, gate R1). Single cells (panel B, gate R2) were further analyzed for their CD45 staining to identify leukocytes (panel C, gate R3) and lymphocytes (panel C, gate R4). An anti-CD3-APC antibody was used for CD19+ B lymphocytes autoflorescence evaluation in the APC channel (panel D, quadrant Q1) and for negative cut-off position establishment. Percentages of CD19+CD5+ B cells were obtained from quadrant Q2 in panel E. Expression of CD3 and CD8 identified total CD8+ T cells (panel F, quadrant Q3). An IgG1-FITC antibody was used as isotype control of perforin staining on CD3+CD8+CD56- cells (panel G, quadrant Q4). Percentages of CD3+CD8+CD56- perforin+ T cells (named CD8+perforin+ T cells) were obtained from quadrant Q5 in panel H. Panels D-F are gated on lymphocytes (panel C, gate R4). Panels G and H are gated on CD3+CD8+ T cells (quadrant Q3 in panel F).

Although these results should be confirmed in larger multi-centre cohorts, they indicate the inter-centre reproducibility of CD19+CD5+ B cell and CD8+perforin+ T cell evaluation to be used as biomarkers in MS, when standardized instrument setting values and negative cut-off are established.

## Conflicts of interest

AO received personal fees or grants from Merck, Novartis, Biogen, Roche, Teva and Sanofi- Genzyme. FG-G received personal fees, grants or non-financial support from Almirall, Bayer, Merck, Novartis, Biogen,

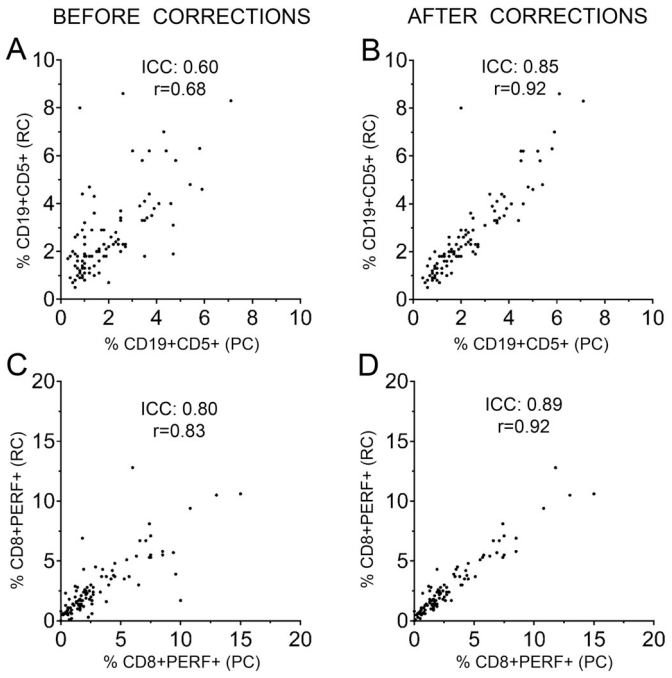


**Fig. 3.** Correlations between the percentages of blood CD19 + CD5 + B cells (A, B) and CD8 + perforin + T lymphocytes (C, D) obtained by participating centres (PC) and by the reference centre (RC) before (A, C) and after (B, D) corrective actions. ICC: Intraclass correlation coefficient; PERF: perforin.

**Table 4**
Frequent errors for CD19 + CD5 + B cell or CD8 + perforin + T cell quantification ($n$ = 101 samples) detected before validation.

| Type of error | Number of samples (percentage of total samples) | |
|---|---|---|
| | CD19 + CD5 + | CD8 + perforin + |
| Wrong or inaccurate negative cut-off axis position | 22 (21.8%) | 15 (14.9%) |
| Incorrect fluorescence compensation | 10 (9.9%) | 4 (4.0%) |
| Wrong PMT voltage values | 4 (4.0%) | 3 (3.0%) |
| Poorly processed samples | 3 (3.0%) | 0 (0.0%) |
| Miscalculation | 1 (0.99%) | 0 (0.0%) |

Footnote: PMT: Photomultiplier.

Roche, Teva and Sanofi- Genzyme. JEM-R received personal fees from Merck, Novartis, Biogen and Roche. LMV received payment for lecturing or research grants from Merck, Novartis, Biogen, Roche and Sanofi- Genzyme. LQ received research funds or personal fees from the GBS-CIDP Foundation International, GRIFOLS, CSL Behring and Sanofi-Genzyme. MG-G declares personal fees from Merck, Novartis, Biogen, Teva and Sanofi-Genzyme. TC-T reports personal fees from Bayer, Biogen, Merck, Novartis, Roche, Sanofi-Genzyme and Teva. VM-L has received consulting or speaking fees from Almirall, Biogen, Sanofi-Genzyme, Merck-Serono, Novartis, Roche, Terumo and Teva. The remaining authors declare no conflicts of interest.

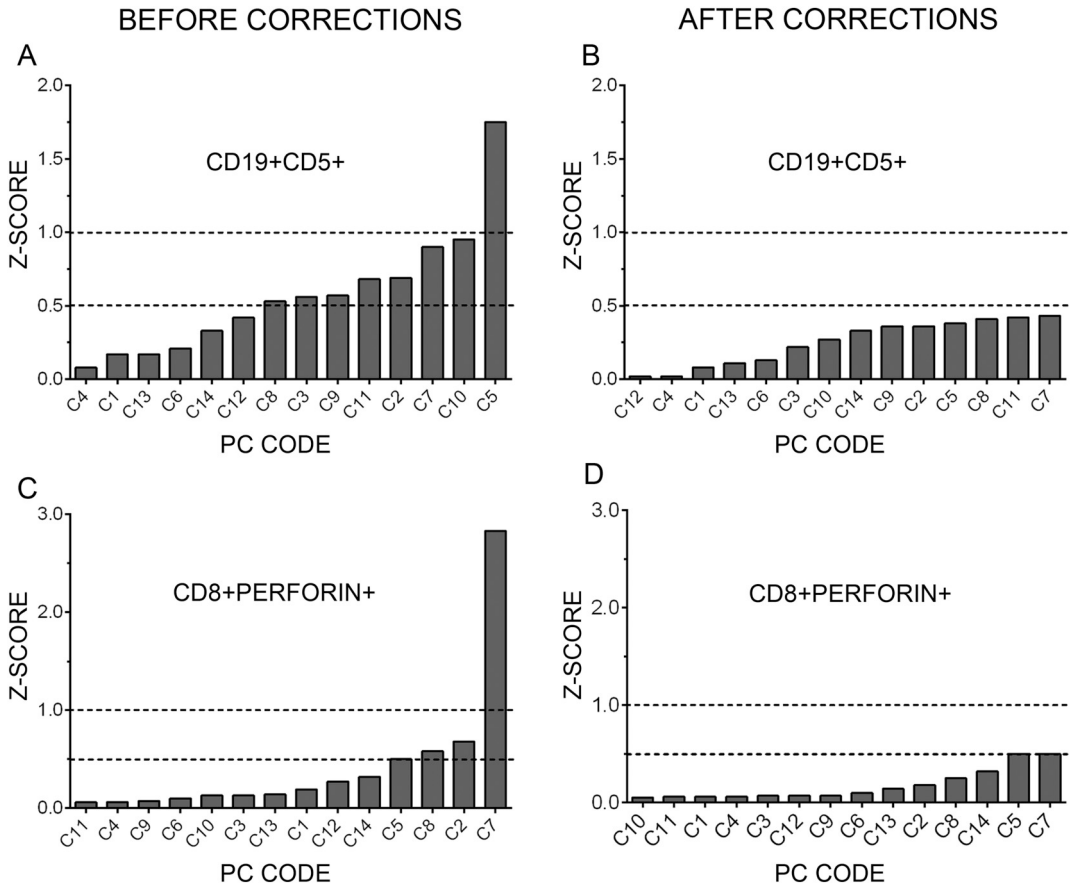


**Fig. 4.** $Z$-score values obtained by every participating centre (PC) for blood percentages of CD19 + CD5 + B cells (A, B) and for CD8 + perforin + T lymphocytes (C, D), before (A, C) and after (B, D) corrective actions.

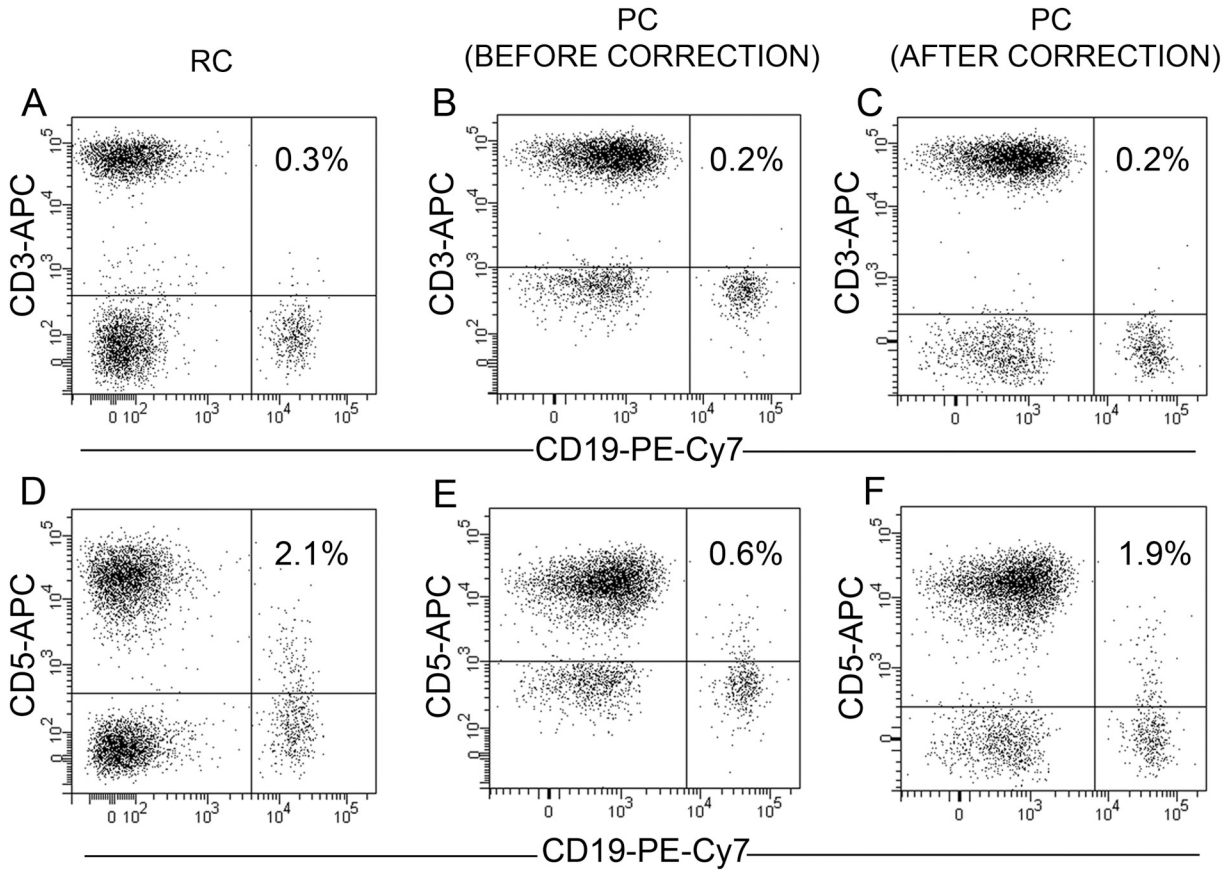


**Fig. 5.** Representative images of a common error for blood CD19 + CD5 + B cell quantification. Dot plots of CD19 vs CD3 (control, panels A, B and C) and of CD19 vs CD5 (CD19 + CD5 + B cell evaluation, panels D, E and F) from a multiple sclerosis patient, obtained by the reference centre (RC; A, D) and by a participating centre (PC), before (B, E) and after (C, F) APC - APC-H7 fluorescence compensation correction. All plots are gated on total lymphocytes.

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