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Original Article

T-cell Receptor (TCR)-Vβ Repertoire Flow Cytometry for T-cell Lymphoproliferative Disorder: a Retrospective Analysis of a Single-Center Real-Life Laboratory Experience

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Abstract. ***Background*: Discrimination between clonal and reactive cell proliferation is critical for the correct management of T-cell lymphocytosis. Multiparametric flow cytometry (MFC) represents a valuable tool, particularly because it allows the evaluation of the T-cell receptor (TCR) Vβ repertoire to pinpoint eventual clonality in T-cell lymphocytosis. A restricted expansion of a single out of the 24 evaluable families or a "clonogram-off" pattern is highly suggestive of the presence of a clonal T-cell population. However, data available on the concordance between MFC TCR-Vβ repertoire and molecular analysis of TCR gene rearrangements, which is regarded as the gold standard for assessing T-cell clonality, are limited.**

***Objective and methods*: We performed a retrospective monocentric study involving 307 patients referred to our center for lymphocytosis between 2003 and 2024. The aim of our study was to investigate the diagnostic accuracy of MFC TCR-Vβ repertoire analysis and compare its performance with molecular analysis of TCR gene rearrangements for the identification of T-cell clonality.**

***Results*: In clonally restricted cases, MFC TCR-Vβ repertoire analysis demonstrated a restricted expansion of a single Vβ family in 67.5% of cases, while a "clonogram-off" pattern inferred clonality in the remaining 32.5%. For 215 (70%) patients, both MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements were available, showing an absolute concordance (215 out of 215 cases, 100%) between the two methods.**

***Conclusion*: MFC TCR-Vβ repertoire analysis is a rapid, cheap, sensitive, and reliable tool to identify clonal T-cell lymphocytosis. It represents an absolutely valid first-line diagnostic approach, and it should be included in the routine laboratory work-up performed on MFC analysis for peripheral lymphocytosis.**

Keywords: T-cell lymphoproliferative disorders; Clonality; Multiparametric flow-cytometry; Vβ repertoire analysis; TCR rearrangements; TCR-Vβ repertoire MFC analysis.

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Introduction. In peripheral blood lymphocytosis, the discrimination between pathological and reactive lymphocyte proliferation is pivotal for an accurate differential diagnosis and appropriate therapeutic management, especially for T-cell disorders. Approximately 90% of both normal and neoplastic T cells express the αβ T-Cell Receptor (TCR αβ), which is generated through somatic recombination of the V, D, and J gene segments. This recombination process, characterized by nucleotide insertions and deletions, generates a widely diversified TCR repertoire that provides the foundation for adaptive immune specificity (**Figure 1**).[1] Allelic exclusion ensures that each T cell expresses only one TCR, resulting in a repertoire of approximately 2.5 x 10^7 clonotypes, offering broad immune coverage.[2] A smaller proportion of T cells express γδ T-Cell Receptor (TCR γδ), arising through a distinct recombination pathway during early development.[3] In real-world clinical practice, the identification of potential T-cell clonality in cases presenting with peripheral lymphocytosis remains a significant challenge.

Multiparametric flow cytometry (MFC) is an indispensable diagnostic tool for the detection of the pathological nature of peripheral blood (PB) lymphocytosis. While standardized MFC effectively identifies monoclonal B-cell populations through light chain restriction analysis (κ or λ),[4-6] T-cell clonality is more complex to determine and typically necessitates polymerase chain reaction (PCR)-based molecular analysis of TCR gene rearrangements.[7-9] However, T-cell clonality can also be assessed through MFC TCR-

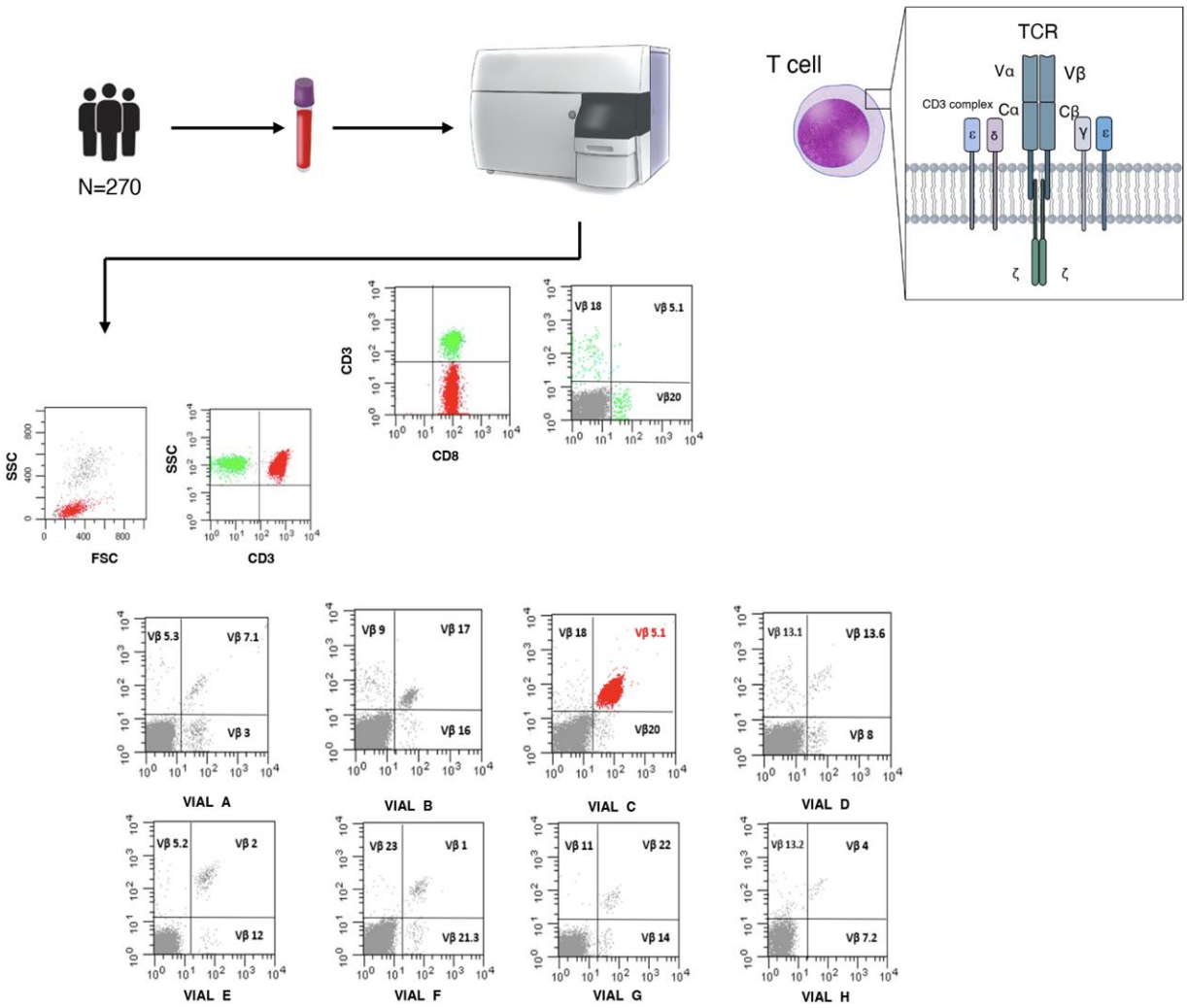

Figure 1. MFC TCR-Vβ repertoire dot plot of a TLD CD4+. Representative dot plot showing a T lymphoproliferative disorder CD4+: Red dots in the SSC/CD3 plot identify the pathological population of T CD4+ cells with TCR -Vβ repertoire analysis showing an aberrant expansion of the VB5.1 family (VIAL C plot); green ones are consistent with the residual T lymphocytes CD8+ with normal family distribution (upper right plots).

Vβ repertoire analysis. The use of anti-Vβ antibodies, targeting 24 distinct Vβ families, enables the identification of a significant part of clonal T cells by detecting expansion of a single Vβ family. Furthermore, in approximately 30% of cases, clonality can be suggested by the absence of detectable Vβ proteins, likely due to a clonal T-cell population expressing a Vβ protein outside the assay panel's coverage.[10-11] Therefore, combining MFC TCR-Vβ repertoire analysis with baseline immunophenotypic profiling is an effective strategy to pinpoint a clonal T-cell lymphocytosis, potentially eliminating the need for molecular analysis of TCR gene rearrangement assay as a first-line diagnostic approach.[11-13] A limited number of studies have explored the concordance between MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements in identifying T-cell clonality.[14-17]

Hence, this retrospective study analyzes samples from 307 patients referred to our center for PB lymphocytosis, comparing results from MFC TCR-Vβ repertoire analysis with molecular analysis of TCR gene rearrangements (detected by PCR and DNA sequencing).

The primary aim of this study is to evaluate, over a two-decade period, the concordance between these two methods in detecting T-cell clonality and assess the usefulness of MFC TCR-Vβ repertoire analysis as a first-level approach in peripheral T cell lymphoproliferative disorders.

Materials and methods.

Study population. We retrospectively collected MFC data from 307 patients with peripheral T-cell lymphocytosis referred to our center at diagnosis from April 2003 to May 2024 (**Figure 1**).

For this analysis, based on MFC findings, the diagnostic entities were categorized into T-cell lymphoproliferative diseases (TLD), T-NK lymphoproliferative diseases (T-NKLDs), and polyclonal T lymphocytosis (PTL). All 307 patients underwent MFC evaluation. In 215 patients (70%), both MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements were performed. For 55 cases (18%), only MFC TCR-Vβ repertoire analysis was available, and in 37 patients (12%), only molecular analysis of TCR gene rearrangements was performed (**Figure 2**). Most of these 37 cases, lacking MFC TCR-Vβ repertoire analysis, were collected around 2003, when Vβ repertoire analysis was not routinely used in clinical practice at our institute. All patients signed the informed consent for the analysis of their samples and the use of their clinical data for scientific purposes.

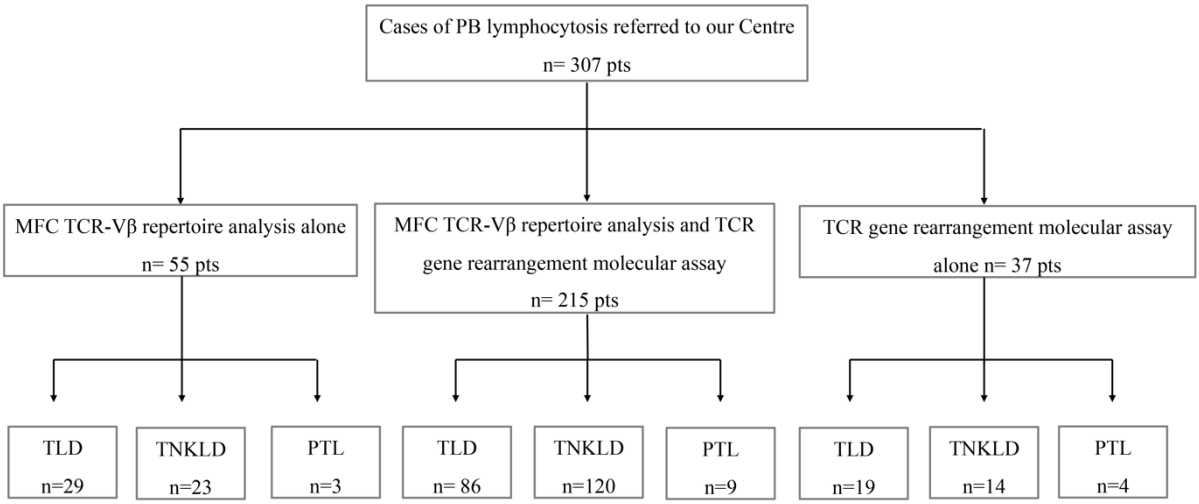

Figure 2. **A cohort distribution flow chart and MFC clustered diagnosis were detected.** Abbreviations: MFC, multiparametric flow cytometry; PTL, polyclonal T lymphocytosis; TCR, T cell receptor; TLD, T lymphoproliferative disease; TNKLD, T-NK lymphoproliferative disease.

Multiparametric Flow Cytometry Analysis. The clustered immunophenotypic diagnosis was assessed by MFC using a combination of monoclonal antibodies (mAbs) recommended by the EuroFlow Consortium.[18-19] Peripheral blood (PB) samples were stained within 24 hours of collection before any treatment, including steroids. Total leukocytes cells were incubated with an appropriate volume of mAbs directed against T, NK, and B lymphoid lineage antigens (CD45, CD3, CD2, CD5, CD7, CD38, CD4, CD8, CD16, CD56, CD57, CD158a, CD158b, TCR-αβ, and TCR-γδ for the lineage T/NK and CD19, CD20, CD22, CD5, CD200, CD23, Ig λ and Ig κ light chains for the lineage B; sourced from Becton Dickinson, San Jose, CA; Società Italiana Chimici, SIC, Life Sciences, Rome, Italy; and Beckman Coulter, Brea, CA). Data on standardized 4-6-8-12 color staining combinations were acquired on FACSCalibur, FACSCanto I, FACSCanto II, or BD FACS Lyric flow cytometers (Becton Dickinson) by collecting at least 50,000 ungated events and analyzed using the Paint-A-Gate and FACSDiva software (Becton Dickinson). Cytometer setup and tracking beads (BD) were used for

daily cytometer optimization. Pathological cells were gated within the total CD45+ leukocyte population, considering that all cases were positive for the pan-leukocyte antigen. Antigen expressions were measured using specific mAbs and compared with values obtained from internal negative controls, which were represented by a population of cells that do not express the antigen of interest and thus remain unlabeled in an antibody-labeled cell-suspension. These controls were exposed to identical conditions (including exposure to the antibody directed to the antigen of interest) as the cell population under investigation. Cell surface antigen expression was estimated by assessing the proportion of positive leukemic cells for each given antigen with a positivity cut-off of equal or more than 20%.

MFC TCR-Vβ repertoire analysis. Evaluation of Vβ expression was performed using the Beckman Coulter Beta Mark TCR Vbeta Repertoire Kit© according to the manufacturer's instructions. The kit is made up of 8 vials, each containing a premixed combination of three monoclonal antibodies specific for a different TCR-Vβ family (one stained with FITC, one with PE, and one with both fluorochromes), thus covering 24 TCR-Vβ antigens and about 70% of the normal human TCR-Vβ repertoire. Information on a minimum of 30.000 events was acquired for each reagent combination. Analysis was performed by gating all CD3+ positive events after CD45 lymphocyte selection on an SSC/CD45 plot. An additional gating strategy, including immunophenotypically aberrant lymphocyte subsets, was used to enhance the accuracy of TCR-Vβ repertoire analysis in cases of small lymphocyte populations. T lymphocytes were further divided into CD4+ and CD8+.

TCR-Vβ repertoire usage was assessed through 8 FITC/PE dot plots, each derived from one different test tube and showing the combination of the three moAb in the FITC, PE, and double positive quadrants. Then, the percentage value of each of the 24 families was calculated and reported on worksheet data. Data analysis was conducted using the Paint-A-Gate and FACSDiva software (Becton Dickinson).[11] Representative plots are reported in **Figure 1**.

Molecular analysis of TCR gene rearrangements. Genomic DNA PB samples at diagnosis were screened by Polymerase Chain Reaction (PCR) amplification using the BIOMED-2 primer sets for TCR rearrangements.[20] The products obtained from TCR rearrangements were further analyzed using heteroduplex analysis to discriminate between amplifications derived from monoclonal or polyclonal lymphoid cell populations.[21] Positive PCR products were sequenced using the Big Dye Terminator Cycle Sequencing Reaction Kit and analyzed using an automatic ABI PRISM 3130 DNA genetic analyzer (Applied Biosystems, Foster City, CA). The TCR nucleotide sequences obtained were analyzed as reported.[22,23]

Statistical analysis. The statistical methods employed in the analysis included descriptive statistics, such as the calculation of medians and ranges (minimum and maximum) for continuous variables, including age and various biological markers. Frequencies and percentages were computed for categorical variables, such as diagnostic groups and molecular rearrangements. A chi-squared test was employed to assess the association between categorical variables, with Fisher's Exact Test applied for associations with low cell counts to ensure robustness in small sample sizes. Comparative analyses were conducted to evaluate differences between groups, such as diagnostic subcategories or the presence or absence of molecular rearrangements. A significance level of $p < 0.05$ was adopted for all statistical tests, and all computations were performed using the R statistical software.

Results.

Study population. The median age of the 307 patients included in the analysis was 64 years (range 35-90). At the time of immunophenotypic assessment, the median absolute lymphocyte count was 7.9 x 10^9/L (range 4.0 x 10^9/L - 224.6 x10^9/L), and the median count of detected pathological lymphocyte count was 2.25 x 10^9/L (1.0 x 10^9/L - 215.6 x 10^9/L) (**Supplementary table 1A**).

Overall, 286 patients (93%) expressed an αβ T-Cell Receptor phenotype (sCD3+/TCR αβ+), while 21 patients (7%) exhibited γδ T-cell receptor expression (sCD3+TCR γδ+).

Among the 286 cases expressing αβ T cell Receptor (sCD3+TCR αβ):

- In 126 (44%) patients, the conclusive diagnosis was TLDs, which were further classified as CD4+ (n=60 patients, 47.6%), CD8+ (n=55 patients, 43.7%), CD4+/CD8+ (n=8 patients, 6.3%), and CD4-/CD8- (n=3 patients, 2.4%); none of these cases expressed NK surface antigens.
- In 144 cases (50%), there was variable co-expression of NK cell antigens (CD16, CD56, CD57, CD158a, CD158b) with T-cell antigens, leading to the conclusive diagnosis of T-NKLDs. These were further classified as follows: CD8+ (*n*=102 patients, 70.8%); CD4+ (*n*=16 patients, 11.2%); CD4+/CD8+ (*n*=20 patients, 13.8%); CD4-/CD8- (*n*=6 patients, 4.2%).
- The remaining 16 cases (6%) were diagnosed as reactive T CD8+ lymphocytosis (PTLs) due to normal MFC TCR-Vβ repertoire family expression and/or polyclonal TCR gene rearrangements detected by molecular assay; of these 16 cases, the majority [nine (56%)] showed HLA-DR expression by MFC.

Among the 21 cases (7%) expressing γδ T cell Receptor (sCD3+TCR+ γδ+), eight cases (38%) were classified as TLDs, while the remaining thirteen cases (62%) were classified as T-NKLDs.

In all cases, the residual mature B cell population (CD19+/CD22+/CD20+/CD38+/CD200+) was minimal, with a median count of 0.19 x 10^9/L (0.1 x 10^9/L - 13.44 x 10^9/L) and did not exhibit any κ- or λ light chain restriction or any aberrant antigen expression (**Supplementary table 1A e 1B**).

MFC TCR-Vβ repertoire analysis. All 307 patients presenting with PB lymphocytosis were evaluated with an MFC analysis exploiting a wide moAbs panel that included antibodies directed against T/NK lineage antigens to highlight eventual aberrant antigen expression.

A total of 270 patients (87.9%) included in the analysis had available MFC TCR-Vβ repertoire data. In 170 cases (63%), aberrant expansion of a single TCR-Vβ repertoire family included in the kit was detected. Among all the 24 TCR-Vβ families, Vβ13.1 was the most frequently expanded in 26 patients (15.3%), followed by Vβ8 and Vβ2 in 17 patients each (10%), Vβ17 in 11 patients (6.5%), and Vβ1 in 10 patients (5.9%). Conversely, in 88 cases (32.6%), the TCR-Vβ repertoire analysis revealed the complete absence of expression for any of the TCR-Vβ repertoire families tested ("clonogram-off") (**Figure 3**). The clonogram displayed a "normal" plot (absence of aberrant expansion of any of the TCR-Vβ repertoire families, thus presenting a normal distribution) in all 12 patients (4.4%) with PTLs. All these cases of reactive lymphocytosis were CD8+, and more than half (9 patients, 75%) expressed HLA-DR reactivity antigen. Furthermore, when stratified by clustered diagnosis (**Figure 4A** and **4B**), a similar frequency of expression of Vβ1, Vβ2, Vβ7.2 families and clonogram-off was noted between the TLD and T-NKLD cohorts. Although the frequency of certain MFC TCR-Vβ repertoire families was higher in one diagnostic category compared to another, no statistically significant difference was observed.

Focusing on the 21 cases expressing TCR γδ+: in 3 patients (14.3%) MFC TCR-Vβ repertoire analysis showed pathological expansion of one family, two of which were confirmed as monoclonal by molecular analysis of TCR gene rearrangements (one sample was not available for molecular analysis); in 12 cases (57.1%) MFC TCR-Vβ repertoire analysis showed a "clonogram off" pattern and for 11 of the 12 patients (91.7%), a confirmed monoclonal restriction was evidenced by molecular analysis of TCR gene rearrangements (one sample was not available for molecular analysis); for the remaining 6 cases (28.6%) MFC TCR-Vβ repertoire analysis was not available (all cases confirmed as monoclonal by molecular analysis of TCR gene rearrangements).

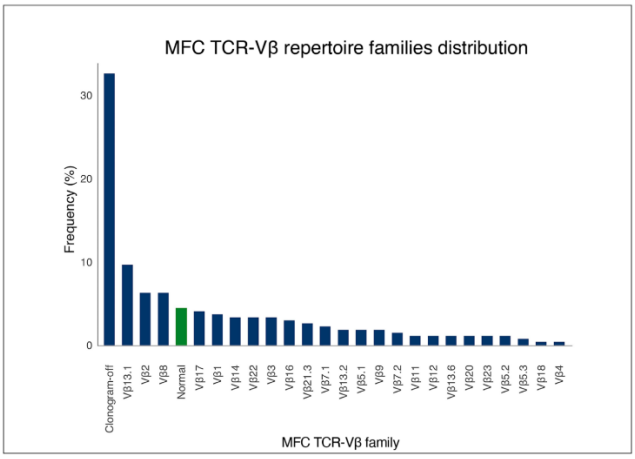

Figure 3. Bar charts illustrating the distribution of MFC TCR-Vβ repertoire families. Abbreviations: PTL, polyclonal T lymphocytosis; TCR, T Cell Receptor; TLD, T lymphoproliferative disease; TNKLD, T-NK lymphoproliferative disease.

Molecular analysis of TCR gene rearrangements. Overall, molecular analysis of TCR gene rearrangement assay (by PCR and DNA sequencing) was performed in 252 patients (82%). The distribution of different families' rearrangements through the entire cohort is shown in **Figure 5**. The most frequent rearrangement detected by PCR was Vγ2-8Jγ1.3 in 110 patients (43.6%), followed by Vγ9Jγ1.3 in 61 patients (24.2%), Vγ 2-8Jγ 1.1 in 52 patients (20.6%), Vγ10Jγ1.3 in 42 patients (16.7%), and Vγ10-11Jγ1.3 in 26 patients (10.3%). Similarly to MFC TCR-Vβ repertoire analysis, no difference was found in the distribution of different families' molecular rearrangement when stratified by diagnosis (**Figure 6A** and **6B**).

In the 37 cases (12 %) for which MFC TCR-Vβ repertoire analysis was not available, only molecular analysis of TCR gene rearrangements was performed.

Of these, four cases (10.8%) exhibited a polyclonal TCR pattern, as suggested by clinical history and baseline immunophenotyping (all cases were sCD3+/CD8+/HLA-DR+), while 33 cases (89.2%) showed monoclonal TCR rearrangement.

Interestingly, in most clonal cases, baseline MFC analysis was already highly suggestive of pathological lymphocytosis, even in the absence of the MFC TCR-Vβ repertoire analysis. In 4 cases (two sCD3+/CD8+/CD20+, one sCD3+/CD4+/CD20+, and one TCRγδ+CD20) there was the rare aberrant CD20 expression; in other 4 cases there was the lack of surface sCD3 antigen expression (sCD3-/cCD3+); in 13 cases there was the aberrant co-expression of NK antigens (CD8+/CD16+/CD56+/CD158+); in 7 cases there was the lack of CD7 antigen expression, while in other 2 cases there was the aberrant co-expression of CD4/CD8 antigens.

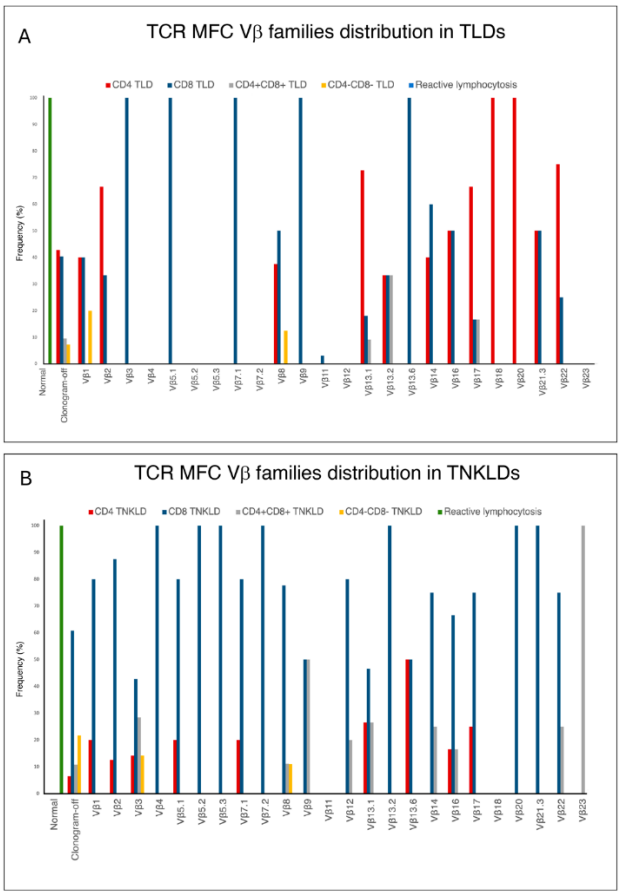

Figure 4. Bar charts illustrating the distribution of MFC TCR-Vβ repertoire families across TLDs cohort (A) and TNKLDs (B) cohort. Abbreviations: PTL, polyclonal T lymphocytosis; TCR, T cell receptor; TLD, T lymphoproliferative disease; TNKLD, T-NK lymphoproliferative disease.

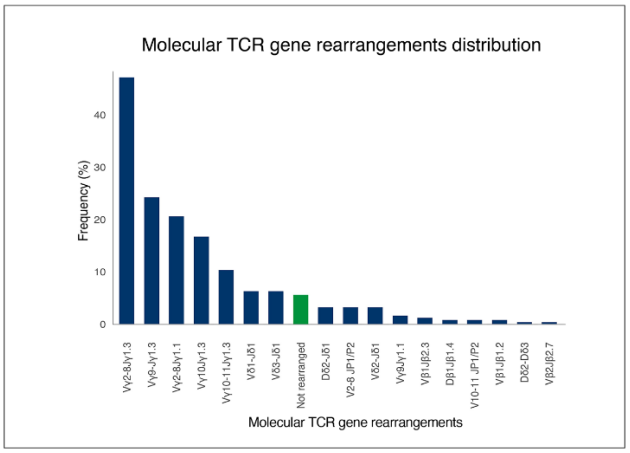

Figure 5. Bar charts illustrating the distribution of molecular TCR gene rearrangements across the whole cohort. Abbreviations: TCR, T cell receptor; TLD, T lymphoproliferative disease; TNKLD, T-NK lymphoproliferative disease.

Correlation between MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangement. We analyzed data from 215 patients (70%) who underwent both MFC and TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements.

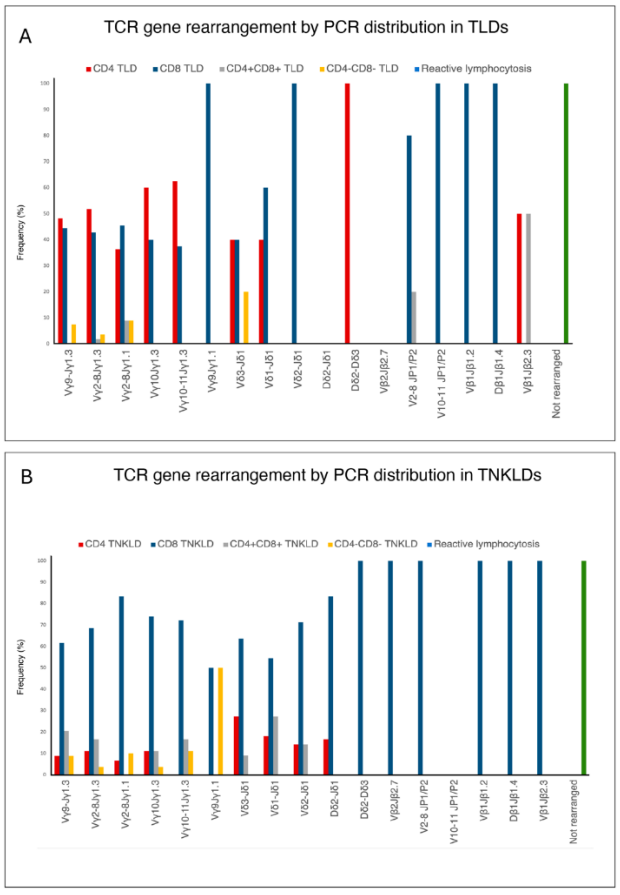

Figure 6. Bar charts illustrating the distribution of molecular TCR gene rearrangements across TLDs cohort (A) and TNKLDs (B) cohort. Abbreviations: TCR, T cell receptor; TLD, T lymphoproliferative disease; TNKLD, T-NK lymphoproliferative disease.

In 136 cases (63.3%), a pathological expansion of a single Vβ family was observed in the MFC TCR-Vβ repertoire analysis. Subsequent molecular analysis of TCR gene rearrangements revealed monoclonal restriction, thus confirming the flow cytometry findings.

In the other 70 cases (32.5%), the MFC TCR-Vβ repertoire analysis showed the complete absence of expression in any of the Vβ families tested ("clonogram off"), suggesting a pathological clonogram and, consequentially, pathological lymphocyte expansion, as confirmed by monoclonal restriction through molecular analysis of TCR gene rearrangements.

Although HLA-DR expression in CD8+ lymphocytes is typically suggestive of reactive processes, in eight of the 13 cases (61.5%) presenting HLA-DR antigen expression, the TCR-Vβ repertoire MFC analysis showed pathological family expansion or aberrant "clonogram-off" pattern, finding that was later confirmed by monoclonal rearrangement of the TCR gene. Conversely, the other five HLA DR+ cases (38.5%) showed a normal clonogram pattern, a finding that was later confirmed by the negativity of the TCR gene rearrangement molecular assessment.

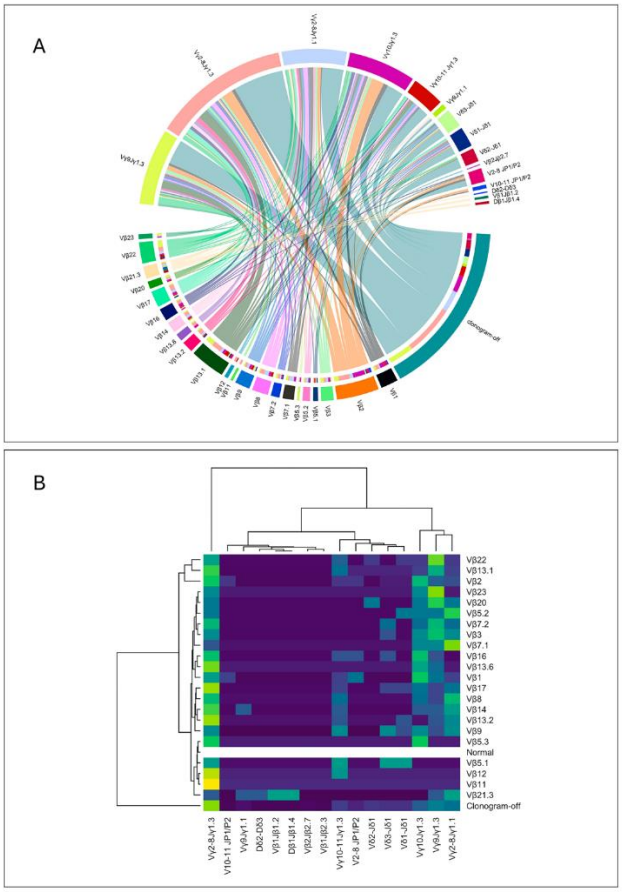

Figure 7. **(A) Chord diagrams and (B) heatmap illustrating the associations between MFC TCR-Vβ repertoire families and TCR gene rearrangements by PCR.** [A] Colored trajectories in the chord diagram join associated MFC TCR -Vβ repertoire families and TCR gene rearrangements, while [B] in the heat map the lighter squares show the degree of association between TCR-Vβ repertoire families and TCR gene rearrangements.

Globally, in 9 out of 215 cases (4.2%) showing a regular distribution of Vβ families ("normal clonogram") by MFC TCR-Vβ repertoire analysis, the molecular analysis of TCR gene rearrangements confirmed the polyclonal nature of the T-cell population in all instances.

Overall, the concordance between MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements was as high as 100% (215 out of 215 cases). In fact, both in cases where molecular analysis of TCR gene rearrangements detected clonality in T-cells and in those where such method showed the absence of clonality, these results were consistent with the results obtained from MFC TCR-Vβ repertoire analysis.

Finally, we evaluated the correlation between MFC TCR-Vβ repertoire families and molecular TCR gene rearrangement families (**Figure 7**). Among TCR-Vβ repertoire findings, "clonogram-off" predominantly correlated with Vγ2-8Jγ1.3 (32.1%), followed by Vγ9Jγ1.3 (15.7%), Vγ2-8Jγ1.3 (13.5%), and Vγ10Jγ1.3 (10.7%) ($p<0.01$). Similarly, Vβ13.1 was frequently associated with Vγ2-8Jγ1.3 (31.0%) or Vγ9Jγ1.3 (24.1%), while Vβ2 correlated primarily with Vγ2-8Jγ1.3 (29.4%) and Vγ10Jγ1.3 (26.4%) ($p<0.01$).

Discussion. The pivotal aim in the diagnostic assessment of peripheral lymphocytosis is the identification of clonal populations, which allows the differentiation between reactive and pathological conditions and thereby guides clinical management. While the MFC detection of clonal B cells is currently standardized through light chain restriction, the assessment of T-cell clonality remains more complex and resource-intensive, presenting significant diagnostic challenges.

The comparison of MFC TCR-Vβ repertoire analysis with alternative clonal detection techniques, such as molecular analysis of TCR gene rearrangements, represents a significant area for discussion. Although the sensitivity of MFC TCR-Vβ repertoire analysis has been explored in the literature, direct comparisons with other methodologies remain limited to small case series.[14-17] Notably, Morice et al.[15] compared MFC TCR-Vβ repertoire analysis with molecular analysis of TCR gene rearrangements (PCR and Southern blot) in 65 peripheral blood samples suspected of TLD. They identified 29 TLD cases by molecular TCR gene rearrangements assay, of which 26 showed clonality also via MFC TCR-Vβ repertoire analysis; conversely, three cases did not exhibit clonality by MFC TCR-Vβ repertoire analysis but resulted rearranged by molecular assay.

A primary challenge in the field emerges when clonality is inferred indirectly, particularly in cases where dominant MFC TCR-Vβ repertoire family expansions are not observed. In our cohort, 63.3% of cases were identified through MCF TCR-Vβ repertoire family expansion, while 32.5% were deduced based on clonogram-off profiles, consistent with previously published data.[14-16] Langerak et al.[14] reported MFC TCR-Vβ family expansion in 66% of 47 suspected TLD cases, with the remaining 16 cases (44%) showing a clonogram-off pattern and molecular analysis of TCR gene rearrangements revealing monoclonal rearrangements in all these cases.

Accordingly, all MFC TCR-Vβ pathological clonogram cases (both expansion of a single TCR-Vβ repertoire family and clonogram-off) in our study ultimately revealed an underlying clonal process when tested by molecular analysis for TCR gene rearrangements.

Our study, including 307 patients, of whom 215 underwent both MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements, to the best of our knowledge, represents the largest of its kind to date. Various gating strategies and criteria for defining cell clonality have been proposed and employed in the literature.[10-14,20-23] Our approach predominantly focused on CD3+ populations, with a particular emphasis on immunophenotypically aberrant subsets to enhance the

accuracy of MFC TCR-Vβ repertoire analysis. By carefully isolating these aberrant populations, we were able to clearly identify the restriction of individual MFC TCR-Vβ families or the presence of a clonal profile.

Most cases in our cohort were diagnosed as T-lymphoproliferative disease (TLDs and T-NKLDs), while only a few cases were polyclonal/reactive lymphocytosis (PTLs). This is probably due to the careful selection by clinicians, which led only highly suspicious cases to undergo second-level analysis. The rate of concordance between MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements in assessing TCR clonality was high (215 out of 215 cases, 100%), confirming other experiences reported in the literature.[14-17]

Another important finding of our study that needs to be highlighted was the ability of MFC TCR-Vβ repertoire analysis to detect clonality of a T-cell population expressing antigens that are usually associated with reactive conditions, such as HLA-DR, which is often expressed on activated cytotoxic T-cells, a phenomenon previously described in conditions such as viral infections and autoimmune diseases.[24-26] Although not inherently indicative of malignancy, this expression can occasionally obscure an underlying lymphoproliferative disorder.[27] In our series, eight cases (3%) displayed a pattern of reactive lymphocytosis (sCD3+/CD8+/HLA-DR+), yet MFC analysis revealed either a dominant TCR-Vβ expansion or a clonogram-off profile, both suggestive of an underlying clonal process, which was later confirmed by molecular analysis of TCR gene rearrangements. All this highlights the importance of a thorough investigation of persistent lymphocytosis over time, as it may mask an underlying lymphoproliferative disorder (LPD) and suggests the need to add MFC TCR-Vβ repertoire mAbs in routinary MFC panels for T-cell analysis, as this could provide a rapid, cost-effective, and efficient method in detecting LPDs.

Conclusions. MFC TCR-Vβ repertoire analysis is a rapid, cheap, sensitive, and quantitative method for detecting T-cell clonality in patients with suspected T lymphoproliferative disorder.

Our findings show a complete concordance between MFC TCR-Vβ repertoire analysis and molecular analysis of TCR gene rearrangements over a prolonged period of 20 years, supporting its role as an effective first-level approach test, particularly in settings where molecular assay may not be readily available.

Furthermore, the immunophenotypic study of T lymphoid populations together with MFC TCR-Vβ repertoire analysis can be complementary or used alternatively to molecular analysis of TCR gene rearrangements, serving as a reliable tool for guiding the differential diagnostic work-up toward T-cell clonal pathologies. Therefore, we reckon that the use of MFC TCR-Vβ repertoire analysis constitutes an effective first-line diagnostic approach for the detection of peripheral T-cell lymphoproliferative disorders and that it should be incorporated into standard MFC panels when a T-cell disorder is suspected.

Authors contributions. MA and JM: Write an original draft with equal contribution. MLM and SI: flow cytometry assessment. DSI: molecular data analysis, interpretation conceptualization, investigation, formal analysis, and contribution to the writing of the original draft. VB: molecular data analysis and interpretation. MB, MGN, and SI: contribution to the laboratory work. AL: data collection, acquisition, analysis, interpretation, and review of the manuscript. AC: data collection, acquisition, analysis, and interpretation. MLB: review of the manuscript. MSDP: flow cytometry assessment; data collection, acquisition, analysis, and interpretation; conceptualization, investigation, formal analysis, and writing the original draft. MM: Supervision and manuscript editing. All authors have read and agreed to the published version of the manuscript.

Data availability statement. the data that support the findings of this study are available from the corresponding author upon reasonable request.

Informed consent. Written informed consent was collected according to local practice.

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