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Research Article

Histopathological Characterization and Development of Objective Diagnostic Criteria in Mycosis Fungoides

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Abstract

Background: Mycosis Fungoides is a subtype of Cutaneous T Cell Lymphomas, often difficult to diagnose in its early stages due to its histopathological resemblance to inflammatory dermatoses. Objectives: To refine and enhance the diagnostic criteria for Mycosis Fungoides by utilizing digital scanning and analysis systems for the quantitative evaluation of immunohistochemical markers. Methods: A retrospective longitudinal observational descriptive study based on the reevaluation and comparison of diagnostic criteria (histological and immunohistochemical) of patients with a clinical suspicion of MF, creating two groups: patients whose diagnosis, despite requiring numerous biopsies, was finally Mycosis Fungoides (MF Group), and patients who, despite clinical suspicion, always had a diagnosis of Inflammatory Dermatoses (Control Group). The study was conducted on 62 patients with clinical suspicion of MF who underwent punch biopsies between 2000 and 2022 at Severo Ochoa University Hospital. Histological and immunohistochemical markers (CD2, CD3, CD4, CD5, CD7, CD8, TOX) were assessed using APERIO AT2 LEICA BIOSYSTEMS scanner and CIVAGENIUS software, ensuring objective and reproducible results. Results: Basal epidermotropism, lymphocytic atypia, and peri-lymphocytic halo showed strong associations with MF diagnosis. Significant differences were observed in the CD3/CD8 and CD5/CD8 ratios between Mycosis Fungoides and Inflammatory Dermatoses groups. High TOX antibody expression correlated with early-stage MF diagnosis. These findings suggest the potential of combining marker ratios and TOX expression with digital analysis to improve diagnostic accuracy. Conclusions: Digital scanning and automated analysis systems significantly enhance the precision and efficiency of Mycosis Fungoides diagnosis. Implementing these methods in routine practice can reduce diagnostic delays and improve patient outcomes by differentiating Mycosis Fungoides from similar dermatological conditions more effectively.

Keywords

Mycosis Fungoides, Tox Antibody, Inflammatory Dermatoses, Epidermotropism, Algorithm, Immunohistochemical Markers

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1. Introduction

Mycosis Fungoides (MF) is classified as a distinct clinical and pathological subtype of Cutaneous T Cell Lymphomas. This disease is marked by an infiltration of small to medium-sized helper T lymphocytes within the epidermis. In cytotoxic variants, however, the malignant cells may be medium or large, particularly in the more advanced stages. In approximately 95% of cases, the infiltrate consists of mature CD4+ (memory) T lymphocytes, while the remaining 2-5% present a CD8+ phenotype. During the early (pre-mycotic) stages of MF, the neoplastic cells typically represent a minority within the infiltrate, with helper T cells being predominant. (1) [1].

From a histological perspective, MF is characterized by a proliferation of atypical lymphocytes known as cerebriform lymphocytes, which show a marked tropism for the epidermis. The classic histological appearance of pre-mycotic forms usually features a patchy or lichenoid (band-like) infiltrate along with an expanded and slightly fibrotic dermis. The epidermis can be acanthotic, atrophic, or normal, with epidermotropism and lymphocytic atypia being sparse in these phases.

Diagnosing MF in its early stages is one of the greatest challenges in clinical dermatology and dermatopathology, and it is considered crucial to enable appropriate and timely management, as well as to avoid potentially harmful treatments that could worsen skin lesions and promote disease progression. Clinical and histological features can be nonspecific or merely suggestive, requiring a certain understanding of lymphocyte ontogeny, and sometimes the use of specific techniques to demonstrate, among other things, cellular phenotypes, clonality, cytogenetic characteristics, and even the presence of viral genetic material [2-7].

Although for some time, molecular genetic analysis of the T-cell receptor (TCR) gene has been considered one of the most useful complementary tests that reveal the neoplastic nature of the infiltrate, it is only positive in 50% of cases, being more frequent in plaque and tumor stages [8, 9].

More modern techniques, such as high-throughput sequencing of the TCR, have shown greater sensitivity and specificity than conventional polymerase chain reaction of TCRγ in distinguishing lymphoproliferative processes from inflammatory dermatoses (ID) [10-13].

Among the most novel findings is the discovery of the Thymocyte selection-associated high mobility group box protein (TOX), considered a critical regulator of early T cell development. According to some authors, positive TOX expression supports the diagnosis of MF and may also be related to disease progression, as it is higher in more advanced stages (plaque-type lesions).

According to current guidelines, conventional manual visual immunohistochemical evaluation of CD2, CD3, CD4, CD5, CD7, CD8, and TOX is very subjective, tedious, time-consuming, and lacks reproducibility, yet is still considered essential for the diagnosis of MF. Additionally, there is no universal system to determine a specific positivity and/or establish cut-off points that help discriminate between histologically similar entities such as some IDs and MF [14-16].

Therefore, the present study aims to reevaluate the diagnostic (immunohistopathological) criteria of each selected patient using digital scanning and analysis systems (Scan Scope and Aperio Leica Image Analysis Workstation), which offer automated analyses and algorithms from which a more precise, rapid, and objective evaluation of the case with high reproducibility indices can be obtained. With these, we will be able to achieve a more accurate diagnosis, reduce the time to diagnosis, and initiate more efficient treatment.

Justification and Hypothesis

In its early stages (patch phase MF is characterized by a patchy inflammatory lymphocytic infiltrate that is often subtle and nonspecific, making diagnosis extremely challenging due to its similarity with other IDs. The scoring systems and algorithms proposed so far have demonstrated low specificity. The immunohistochemical evaluation included in many of these has been performed manually/semi-quantitatively, resulting in significant inter- and intra-observer variability, low reproducibility, and objectivity.

Our hypothesis is that through digital scanning systems and analysis of immunostained samples using the corresponding algorithm, combined with histopathological criteria, a more precise, rapid, and objective evaluation of the case can be achieved with high reproducibility.

2. Objectives

Primary Objective:

To quantitatively, objectively, and reproducibly determine the expression of immunohistochemical markers (CD2, CD3, CD4, CD5, CD7, CD8, and TOX) through digital scanning and analysis systems in patients with an initial diagnosis of ID who were later diagnosed with MF and those with a final diagnosis of ID. This will enable us to establish benchmarks and cut-off points that allow for the simple and cost-effective discrimination of both entities.

Secondary Objectives:

(1) To evaluate the diagnostic or discriminative capacity of the increased ratios: CD2/CD8, CD3/CD8, CD4/CD8, CD5/CD8 and CD7/CD8 in the diagnosis of early MF.

(2) To determine the degree of correlation between the loss or gain of expression of a specific immunohistochemical marker and the diagnosis of MF.

(3) To describe, determine, and quantify the differences in the expression of immunohistochemical markers between patients diagnosed with MF and those diagnosed with ID.

(4) To determine the utility of combining different im-

munohistochemical markers (CD2, CD3, CD5, CD7, and TOX) to establish a diagnosis of early MF.

(5) To determine the utility of combining immunohistochemical markers with various histopathological characteristics to establish a diagnosis of early MF.

3. Materials and Methods

3.1. Study Design

This is a retrospective longitudinal observational descriptive study based on the reevaluation and comparison of diagnostic criteria (histological and immunohistochemical) of patients with a clinical suspicion of MF, creating two groups: patients whose diagnosis, despite requiring numerous biopsies, was finally MF (MF Group), and patients who, despite clinical suspicion, always had a diagnosis of ID (Control Group).

The Ethics Committee for Research with Medicines of the Severo Ochoa University Hospital (SOUH) approved the study protocol. The study design adhered to the written protocols of the Declaration of Helsinki for biomedical research.

Sample Population and Selection Criteria:

A total of 62 patients and 135 biopsies were selected, aged between 21 and 90 years, with an initial clinical suspicion of MF and at least one punch biopsy performed on the lesion, in the dermatology service of SOUH from 2000 to 2022. Of the 62 patients described, 30 were diagnosed, some after numerous biopsies, with MF CD4+ (MF Group), and 32 with ID. Selection Criteria: (Figure 1).

Inclusion Criteria for MF Group	Inclusion Criteria for Control Group	Exclusion Criteria
• Clinical suspicion of MF	• Clinical suspicion of MF	• Patient without clinical suspicion of MF
• Follow-up in dermatology at SOUH	• Follow-up in dermatology at SOUH	• Follow-up in another service or hospital
• At least 1 punch biopsy	• At least 1 punch biopsy	• Clinical suspicion without biopsy
• Material fixed, processed, stained, and diagnosed at SOUH	• Material fixed, processed, stained, and diagnosed at SOUH	• Material fixed, processed, stained, or diagnosed at another center
• Early clinical stages (patch/plaque)	• Early clinical stages (patch/plaque)	• Patients with atypical infiltrates, but not conclusive for MF
• Paraffin block in good condition	• Paraffin block in good condition	• Advanced stages of MF
• Optimal dilution/concentration of the antibody	• Optimal dilution/concentration of the antibody	• Paraffin blocks in poor condition (poorly fixed, cut, processed, or stained)
• Patients who, despite initial DI biopsies, are finally diagnosed with MF	• Patients who, despite initial DI biopsies, are finally diagnosed with DI	• Errors in digital scanning

Figure 1. *Summary of Selection Criteria for the Group of Patients Diagnosed with MF and the Control Group.*

A search was conducted in the archive, collecting each slide with its corresponding Hematoxylin-Eosin, as well as its paraffin block. Then, 3-micron sections were made with the microtome (Leica HistoCore AUTOCUT), obtaining a total of 7 slides necessary for each of the corresponding immunohistochemical techniques (CD2, CD3, CD4, CD5, CD7, CD8, and TOX). Antibody, clone, commercial source, and concentration: (Figure 2).

ANTIBODY	CLONE	COMMERCIAL SOURCE	CONCENTRATION
CD2	11F11	Leica Biosystems	Ready to use
CD3	LN10	Leica Biosystems	Ready to use
CD4	4B12	Leica Biosystems	Ready to use
CD5	4C7	Leica Biosystems	Ready to use
CD7	LP15	Leica Biosystems	Ready to use
CD8	4B11	Leica Biosystems	Ready to use
TOX	NAN448	Sigma-Aldrich (Merck Group)	1:100

Figure 2. *Summary table of antibodies, clones, commercial sources, and optimal concentrations.*

The histopathological characteristics of each sample were reviewed; changes in the stratum corneum, epidermis, dermis, as well as the presence of basal/disproportionate epidermotropism, lymphocytic atypia, among others. All immunohistochemical markers were evaluated independently by two pathologists.

Sample Scanning Methodology on APERIO AT2 LEICA BIOSYSTEMS Scanner:

Once the immunohistochemical techniques are completed, the samples are scanned using a bright-field scanner (APERIO AT2 LEICA BIOSYSTEMS) with progressive digitization of the sample. A new image (e-Slide) is downloaded in JPG format and incorporated into specific software (CIVAGENIUS).

Digital Analysis Methodology (APERIO LEICA IMAGE ANALYSIS WORKSTATION):

Each immunohistochemistry slide is automatically read using the Aperio Leica Image Analysis Workstation. The dermo-epidermal junction of the 5 most representative areas is included to then calculate an overall average, with a constant analysis area of 250050 µm2 in each of them.

After loading the corresponding algorithm, four results are obtained based on staining intensity (negative-blue, weak-yellow, medium-orange, and strong-red). To analyze and quantify the amount of staining of the CD antibodies present in an image, the "Positive Pixel Count" algorithm is used. This new method we propose provides the total number of stained pixels in each sample instead of the total number of lymphocytes. From our point of view, pixel counting more accurately reflects the amount of staining present in the sample, making it especially easier to interpret those more challenging CD nuclear membrane-type immunohistochemical markers (with less distinct staining). (Figure 3).

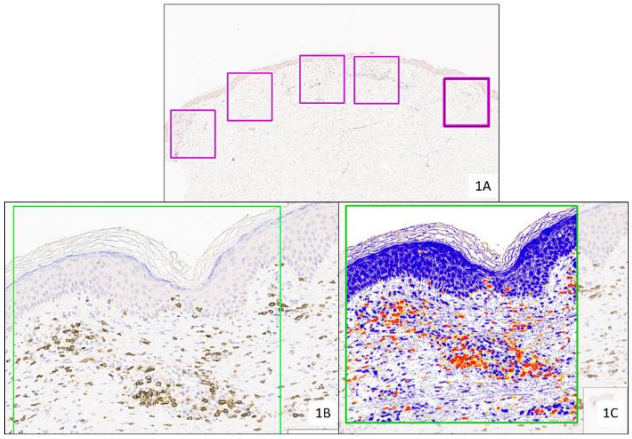


Figure 3. *Composite image. Example of CD5 antibody immunohistochemical staining. 1A. Manual selection of the 5 most representative areas including the dermoepidermal junction. 1B: Nuclear membrane staining with the CD5 antibody. 1C: Automated reading after loading the "positive pixel count" algorithm. Blue: negative, Yellow: weak, Orange: moderate, Red: strong.*

To accurately reflect the number of stained lymphocytes in each sample with the different CD antibodies; the Medium (M) orange and Strong (S) red, positives were summed. The yellow (weak) marking was not considered, as it did not correlate well with weakly stained lymphocytes and often confused this weak staining with artifactual background staining of the sample itself.

To analyze and quantify the amount of TOX antibody staining present in an image, the "Nuclear" algorithm is used, obtaining the total number of stained lymphocytes based on staining intensity (Figure 4).

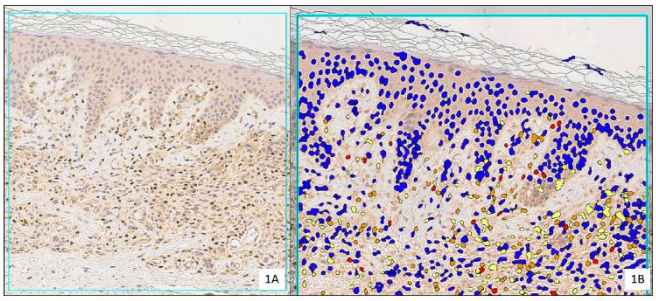


Figure 4. *Composite image. Example of TOX antibody immunohistochemical staining. 1A: Nuclear staining with the TOX antibody. 1B: Automated reading after loading the "nuclear algorithm." Blue: negative, Yellow: weak, Orange: moderate, Red: strong.*

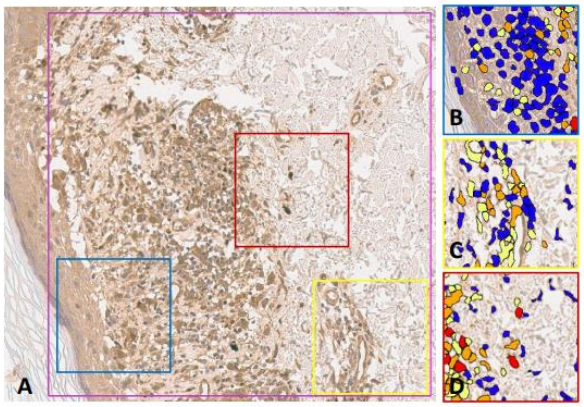


Figure 5. *Immunohistochemical artefactual findings. (A) Example of tissue sample with commonly encountered artifacts; (B) Keratinocyte nuclei misinterpreted as negative lymphocytes (0-blue); (C) Non-representative weak intensity of yellow nuclei (high background staining and erroneous labeling of vascular structures); (D) Non-representative strong intensity of red nuclei (overstained areas), tissue remnants, debris, and/or inaccurately interpreted blurry nuclei.*

Among the 4 categories provided by the algorithmic analysis, the one most accurately and representatively reflecting nuclear staining with this antibody was the orange category (moderate staining, 2+). Similar to other immunohistochemical markers, negative staining (blue marking) should not be

interpreted as total negative lymphocytes, as it includes squamous epithelial cellularity. In our view, categories +1 and +3 do not reflect the true staining pattern and therefore should not be used in interpreting TOX antibody expression. This is likely due to artefactual phenomena during sample digitization. (Figure 5).

This figure represents such artefacts that support the exclusive use of the +2 count (moderate/orange).

None of the loaded algorithms could discriminate between squamous epithelial cells and unstained lymphocytic cells. Both were interpreted as negative (unstained) and thus marked in blue (negative). For this reason, we will use absolute values instead of percentages.

3.2. Statistical Analysis

The study was conducted on a total of 135 samples using Stata® 16.1 Software (Stata Corp, College Station, TX, USA).

CLINICAL VARIABLES	HISTOPATHOLOGICAL VARIABLES
Age	**Corneal layer:** normal / hyperkeratotic (orthokeratotic or parakeratotic).
Sex	**Epidermal layer:** normal / atrophic / acanthotic (regular or irregular) / vacuolar alteration of the basal layer / spongiosis.
Location	**Dermal layer:** dermal expansion / type of inflammatory infiltrate (superficial perivascular, superficial and deep, lichenoid, interstitial or hypodermic).
Clinical stage (patch or plaque)	**Others:** Disproportionate epidermotropism (abundant lymphocytes in spongiotic areas), basal epidermotropism (lymphocytes aligned at the dermoepidermal junction), intraepidermal collections (Darier's nests), blood extravasation, pigment incontinence, lymphocytic atypia, perinuclear lymphocytic halo, folliculotropism, multinucleated giant cells, eosinophils.

Figure 6. *Clinical and histopathological variables for statistical analysis.*

A descriptive analysis of the immunohistopathological characteristics is presented. Regarding the immunohistochemical evaluation, being software-based, inter- and intra-observer variability is null. The variables under study are represented in (Figure 6).

An assessment of missing data and the Shapiro-Wilk test for normality were conducted. To evaluate the association between histological variables and final diagnosis, the chi-square test was used, and Bonferroni correction was applied to identify statistically significant cases.

Effect size was analyzed using Cramer's V coefficient strength of association.

4. Results and Discussion

Of 62 patients, 32 (51.6%) had a final diagnosis of negative ID and 30 (48.4%) had MF. Among the samples with a definitive diagnosis of MF, up to 32.35% were initially diagnosed as ID on the first biopsy, with only 67.65% initially diagnosed as MF. This suggests a diagnostic delay in up to 32.35% of patients, and consequently, a delay in the initiation of treatment.

4.1. Average Diagnostic Time

Literature reports an average time from disease onset to a definitive diagnosis of MF of approximately 2.27 years [17]. In our study, the average time between the first and second biopsy was 2.3 months (SD=9.6 months) regardless of the final diagnosis. However, when data is broken down by final diagnosis, the average time between the 1st and 2nd biopsy for a final negative diagnosis is 4.2 months (SD=13.4 months), while for a final diagnosis of MF it is 0.5 months (SD=1.5 months). The same trend was observed between the times of the 2nd and 3rd biopsies (Figure 7).

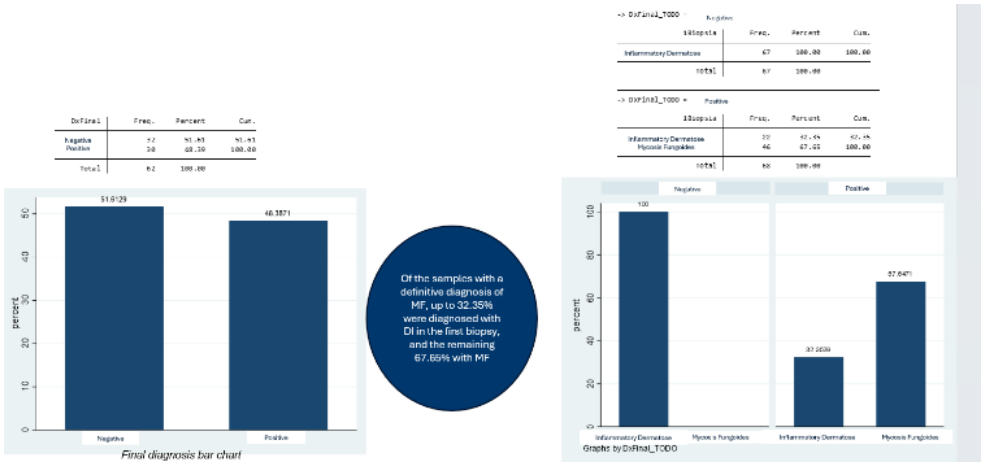


Figure 7. *Avarage diagnostic time. Out of 62 patients, in 30 (48.4%) of them, the final diagnosis was MF, and in 32 (51.6%), it was negative (ID). Of the samples with a definitive diagnosis of MF, 32.35% were diagnosed with ID in the first biopsy, and the remaining 67.65% with MF. In other words, with the current diagnostic methods, we are not able to diagnose up to 32.35% of cases in the first instance, which implies a delay in diagnosis, treatment, and the need to re-biopsy the patient.*

These data suggest that patients with biopsies where the inflammatory infiltrate is at least suspicious are re-biopsied more quickly than those where, despite clinical suspicion of MF, the infiltrate is histologically more inconspicuous.

Histopathological Criteria:

Regarding histopathological criteria, our study corroborates the data published in the literature on their frequency of occurrence. Dermal expansion, lichenoid infiltrate, interstitial infiltrate, basal epidermotropism, lymphocytic atypia, and the perilymphocytic halo are more frequent in patients with a final diagnosis of MF compared to the control group. These differences are statistically significant in all of them with $p<0.05$, and these last three histopathological criteria also show moderate to strong association strengths with Cramer's $V > 0.4$ (Figure 8).

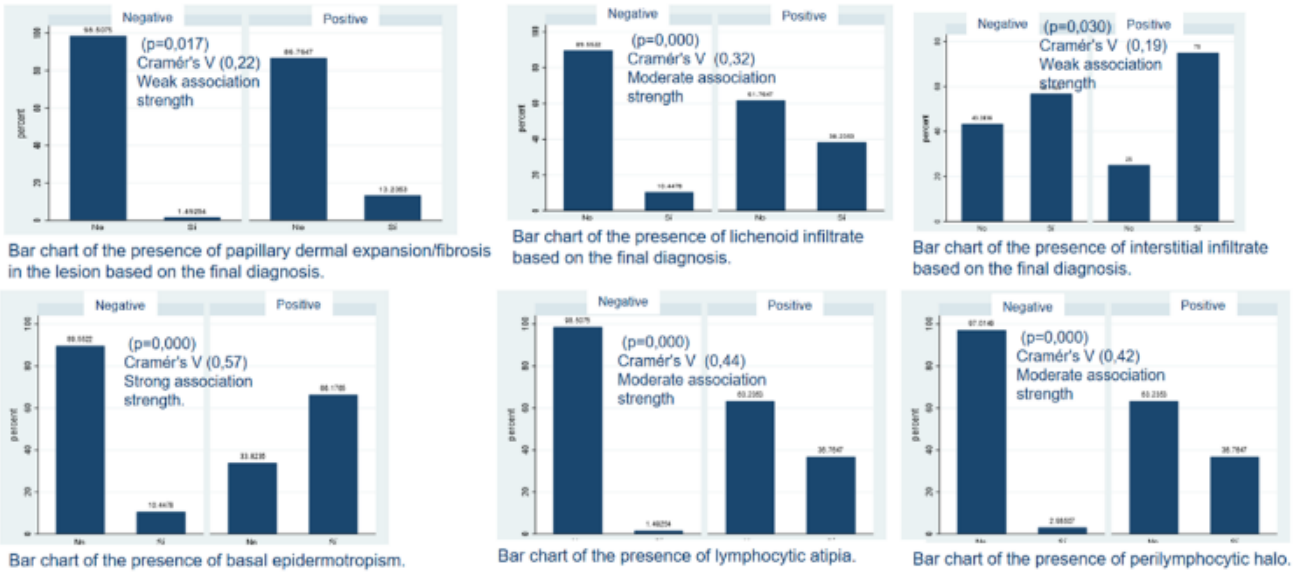
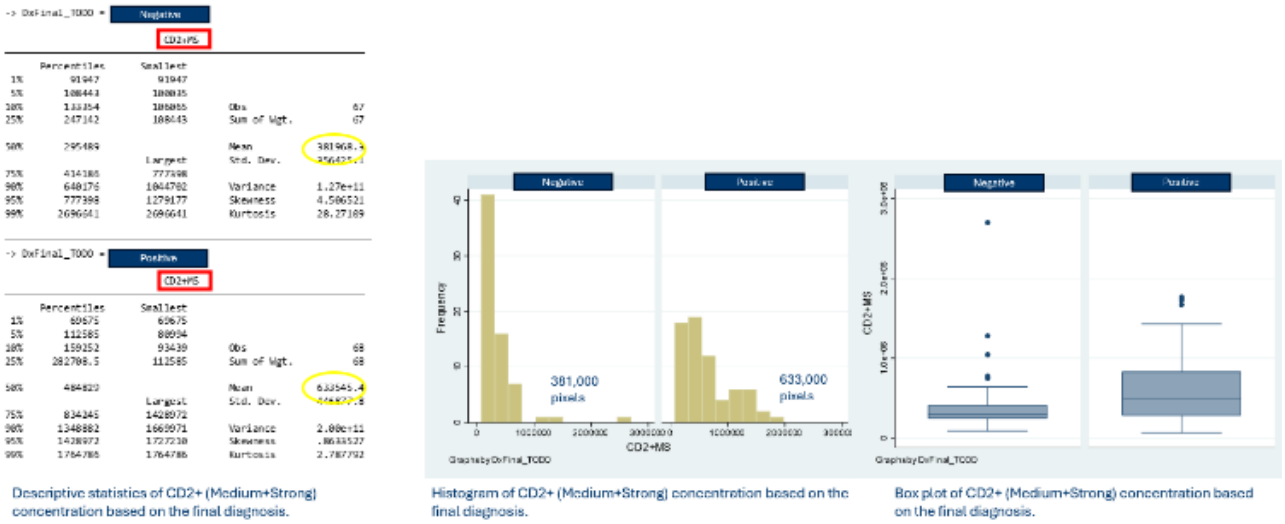


Figure 8. *Frequency of occurrence of histopathological criteria showing a weak / moderate / strong association (Cramer's V: > 0.2) with the diagnosis of MF, also presenting statistically significant differences compared to DI ($p<0.05$).*

Concentration of common markers and their ratios:

For each of the immunohistochemical variables (CD2, CD3, CD4, CD5, CD7, CD8), the differences in mean concentrations based on the final diagnosis were studied (Figure 9).



Descriptive statistics of CD2+ (Medium+Strong) concentration based on the final diagnosis.

Histogram of CD2+ (Medium+Strong) concentration based on the final diagnosis.

Box plot of CD2+ (Medium+Strong) concentration based on the final diagnosis.

Figure 9. *Example of the mean concentrations based on the final diagnosis for CD2 antibody. Patients with a final negative diagnosis (ID group): the average concentration was 381,000 pixels. Whereas in the group of patients with a final positive diagnosis (MF group), it was higher: 633,000 pixels.*

In all cases, the staining concentration was higher in patients with MF compared to the control group (ID) (Figure 10).

IHQ	Pixels (medium + strong) in negative patients. Control group (DI)	SD	Pixels (medium + strong) in positive patients. MF	SD
CD2	381968,3	SD=356425,1	633545,4	SD=446877,8
CD3	446199,9	SD=271546,6	1571980	SD=1089940
CD4	312728,6	SD=240609,3	612898	SD=595380,4
CD5	489711,2	SD=247784,6	1517207	SD=1140925
CD7	238235,7	SD=151753,4	322450,6	SD=299965
CD8	259938,3	SD=177583,5	448309	SD=503527,4

Figure 10. *Summary Table of Differences in Mean Concentrations for Each Immunohistochemical Marker Based on Final Diagnosis (Negative Control Group - DI / Positive for MF).*

To assess the differences in the increse of the mean concentrations of the immunohistochemical markers and the final diagnosis for MF, the non-parametric Mann-Whitney U test was used. Statistically significant differences in mean concentrations between the two groups were observed for the immunohistochemical markers CD2, CD3, CD4, and CD5 with p-values of 0.001, 0.000, 0.000, and 0.000, respectively. The remaining markers did not show significant differences ($p > 0.05$).

As early as 2003, Nicolas Ortonne et al. [18] demonstrated the importance of evaluating the CD3/CD8 ratio for the diagnosis of MF. They retrospectively compared the immunophenotypic characteristics of 30 cases of MF and 28 ID. CD3 was chosen as the exclusive marker for lymphocytes instead of CD4 (previously used) to avoid staining histiocytoid cells. The CD3/CD8 ratio was determined separately in the epidermis and dermis using two methods, one quantitative and the other semi-quantitative. The concordance rates between the two methods were higher in epidermal infiltrates than in dermal ones. The mean CD3/CD8 ratio was significantly higher in patients diagnosed with MF compared to the control cases, concluding that a high CD3/CD8 ratio (>75%) supports the diagnosis of MF, although not absolutely specific.

In our study, the mean concentrations for each of the following ratios were quantitatively, objectively, and reproducibly determined based on the combination of pixels (M + S), which accurately reflects the real/original immunohistochemical staining: CD2/CD8, CD3/CD8, CD4/CD8, CD5/CD8, and CD7/CD8 (Figure 11).

Ratios	Average concentration (pixels). Negative/DI patients	SD	Average concentration (pixels). Positive/MF patients	SD
CD2-CD8 (medium+strong)	3.9	4.0	7.6	17.8
CD3-CD8 (medium+strong)	4.8	3.2	26.1	65.5
CD4-CD8 (medium+strong	2.9	1.9	4.5	7.1
CD5-CD8 (medium+strong)	5.6	4.3	17.6	29.0
CD7-CD8 (medium+strong)	2.3	1.6	1.8	1.7

Figure 11. *Summary table of differences in mean concentrations for each ratio based on final diagnosis (Negative Control Group - DI / Positive for MF).*

Consistent with the literature, our study observed an increase in all ratios in patients with MF compared to the control/ID group, except for the CD7-CD8 ratio, which was inverted (higher in ID group). This finding could be explained by the fluctuating nature of CD7, considered by some authors [19] as the first antibody to decrease in concentration (even in early stages of MF) and thus regarded as one of the most specific for the diagnosis.

To assess the differences in the increase means of the ratios CD2/CD8, CD3/CD8, CD4/CD8, CD5/CD8, and decrease CD7/CD8, within the final diagnosis for MF, the non-parametric Mann-Whitney U test was used. Significant differences between the two groups were observed exclusively for the ratios CD3/CD8, CD5/CD8, and CD7/CD8, with p-values of 0.000, 0.000, and 0.004, respectively. The remaining ratios did not show significant differences (p >

0.05). (Figure 12).

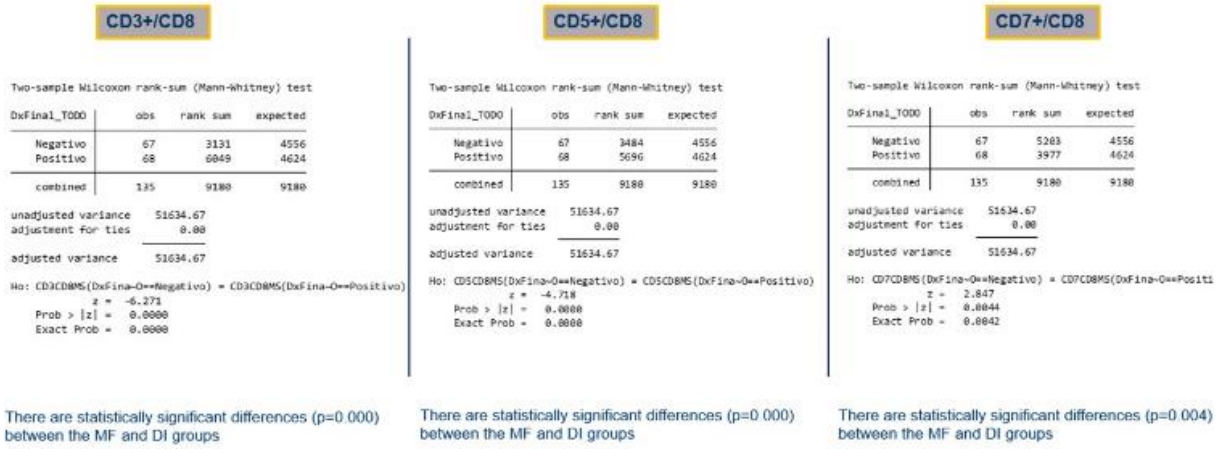


Figure 12. *Mann-Whitney U Test to* assess the differences in the increase means of the ratios *CD2-CD8, CD3-CD8, CD4-CD8, CD5-CD8, and decrease CD7-CD8, within the final diagnosis for MF.*

For those markers (CDs) or ratios that demonstrated significant differences between the control/ID and positive/MF groups, a ROC curve analysis was performed to obtain cut-off points that could help discriminate patients suspected of MF from the control group (ID) in the future. This was done by considering the final diagnosis (negative/ID and positive/MF) as the reference variable and the corresponding immunohistochemical marker or ratio as the diagnostic variable.

Cut-off point tables and graphics. Analysis of the ROC curve of the diagnostic test (CD2, CD3, CD4, CD5), (CD3/CD8, CD5/CD8, CD7/CD8) and TOX (nuclei 2+): annexed document

The choice of cut-off points for CDs and ratios should be personalized, potentially varying based on the desired sensitivity and specificity. From our perspective, since cutaneous T-cell lymphoma has a high potential for progression and a detrimental prognosis if not detected in early stages, selecting a cut-off point with very high sensitivity (even at the expense of specificity) helps minimize the number of false negatives, thereby ensuring that no patients are left undiagnosed.

The high number of false positives resulting from this cut-off selection can be refined by using a relatively novel marker, such as the TOX antibody, choosing a highly specific cut-off point.

1.1 4.2. TOX antibody concentration:

The TOX factor is considered a protein that plays a critical role in T-cell development, specifically in the selection and maturation of thymocytes in the thymus. This protein regulates molecular events during thymocyte selection, determining which T cells will develop and which will be eliminated during maturation in the thymus.

In this context, Yaohua Zhang et al. [20] conducted research to characterize positive identification markers for MF by comparing MF and DI lesions using high-throughput genomic detection tools (complementary DNA microarrays). Only 19 genes studied showed overexpression in patients diagnosed with MF and not in DI patients. Among them was TOX, a critical regulator of T cell development, especially expressed in CD4+ cells. High sensitivity and specificity were demonstrated in identifying patients with MF-positive biopsies.

The use of high-throughput genomic detection techniques and manual evaluation of TOX nuclear expression incur high costs with very low reproducibility rates. Staining was semi-quantitatively analyzed, establishing 3 categories: negative <10%, weakly positive 10-30%, and strongly positive >30% of total infiltrating lymphocytes. This evaluation method sets wide interpretation ranges, again showing low reproducibility.

In our study, to improve these indices and reduce costs, after performing TOX antibody immunohistochemistry, the sample was scanned using the APERIO AT2 scanner from LEICA BIOSYSTEMS to automatically digitize the slides.

The average concentration of +2 nuclei in the overall sample was 245.3 (SD=361.5 cells). The average concentration of +2 nuclei in the sample with a final negative/ID diagnosis was 132.1 (SD=107.7 nuclei). In contrast, the average concentration of +2 nuclei in the sample with a final positive/MF diagnosis was 356.8 (SD=473.9 nuclei) (Figure 13).

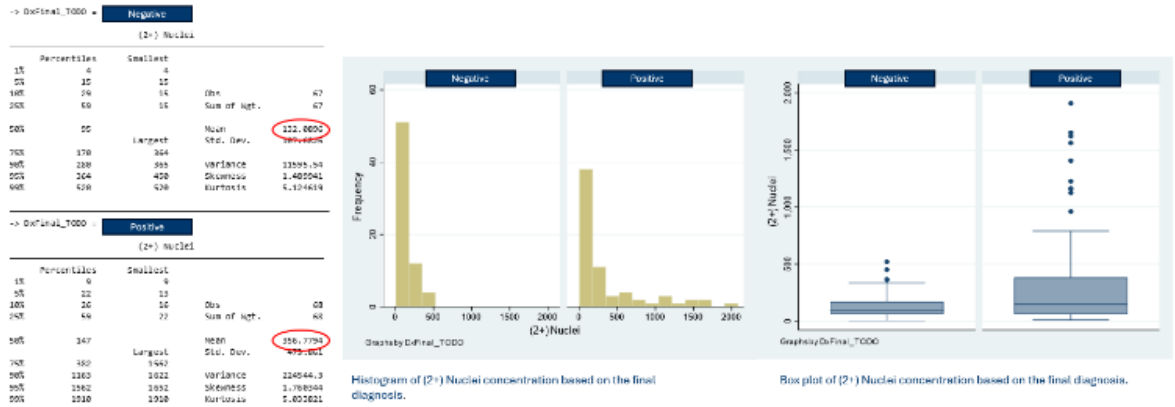


Figure 13. *Average concentration of +2 nuclei with TOX antibody in the overall sample. Average concentration of +2 nuclei in the sample with a final negative/ID diagnosis. Average concentration of +2 nuclei in the sample with a final positive/MF diagnosis.*

After conducting the non-parametric Mann Whitney U test, statistically significant differences (p=0.040) were observed between the average number of +2 nuclei in the group with a positive final diagnosis (MF group) and the group with a negative final diagnosis (ID group) (Figure 14).

Two-sample Wilcoxon rank-sum (Mann-Whitney) test

DxFinal_TODO	obs	rank sum	expected
Negative	67	4088.5	4556
Positive	68	5091.5	4624
combined	135	9180	9180

unadjusted variance 51634.67
adjustment for ties -6.04
adjusted variance 51628.62

Ho: BD(DxFina~O== Negative) = BD(DxFina~O== Positive)
z = -2.057
Prob > |z| = 0.0396
Exact Prob = 0.0395

U de Mann-Whitney test

Figure 14. *Mann Whitney U test to assess the differences in the increase means of TOX antibody, within the final diagnosis for MF.*

No significant differences were found for the other categories (+1, +3).

Additionally, a ROC curve analysis was conducted considering the final diagnosis as the reference variable (negative/ID and positive/MF) and +2 nuclei as the diagnostic variable with an AUC of 0.6 (95% CI: 0.5-0.7). This allows us to establish a specific threshold that could help distinguish cases where the previously proposed methods were uncertain. To achieve this, we detail a series of cutoff points based on specific sensitivity and specificity.

From our perspective, it would be beneficial to consider using more specific cutoff points (>95%) for those samples whose clinical, histopathological, and additional immunohistochemical characteristics (previously described CDs) are compatible or suggestive of an MF diagnosis. With this high specificity, we can strengthen the suspicion diagnosis effectively.

4.2. Binary Logistic Regression

To assess the influence of statistically significant diagnostic tests on the final diagnosis, binary logistic regression tests were performed. Initially, immunohistochemical variables CD2+ (M+S), CD3+ (M+S), CD4+ (M+S), CD5+ (M+S) were evaluated.

Overall, the model was statistically significant with moderate fit (R2 of 46.36%). For both CD2 and CD3 markers, an odds ratio of 1 was obtained, which is statistically significant in both cases with a $p<0.05$, indicating that their presence does not increase or decrease the probability for MF. For the remaining markers, no statistically significant differences were observed (Figure 15).

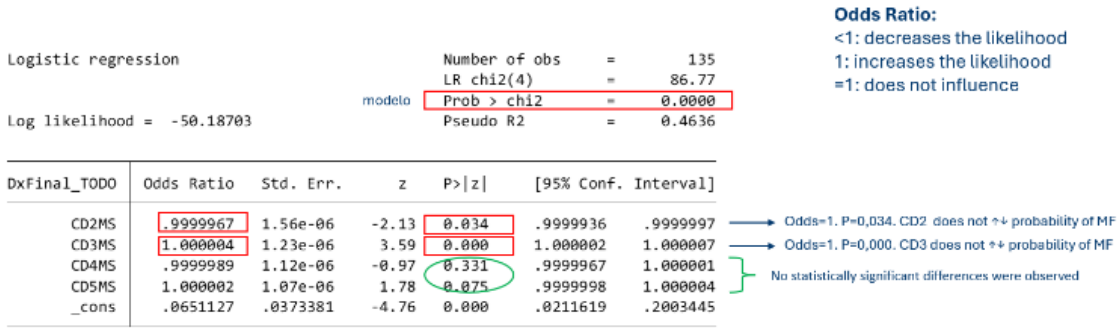


Figure 15. *Binary Logistic Regression was performed to assess the influence of statistically significant diagnostic tests on the final diagnosis. Immunohistochemical variables CD2+ (M+S), CD3+ (M+S), CD4+ (M+S), CD5+ (M+S) were evaluated.*

The same test was conducted to study the influence of immunohistochemical variables expressed in ratios: CD3+/CD8+ (M+S), CD5+/CD8+ (M+S), and CD7+/CD8+ (M+S), on the final diagnosis.

In this case, a statistically significant model was observed (p=0.000) with an R2 adjustment of 27.81%. For the CD3:CD8 ratio, the odds ratio was >1 (1.4) with a p<0.05. This suggests that a high CD3/CD8 ratio increases the likelihood of a diagnosis of MF. However, for the CD7/CD8 ratio, the odds ratio is <1 (0.68) with a p<0.05, suggesting that its presence (or high concentration) decreases the likelihood of a diagnosis of MF (a protective factor). (Figure 16).

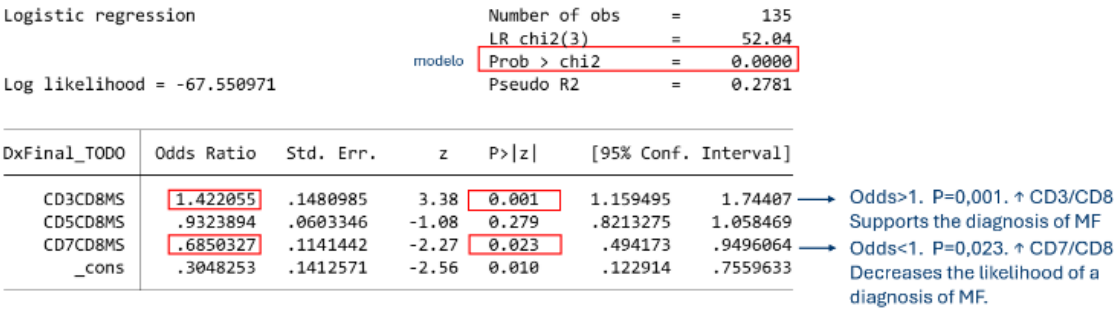


Figure 16. *Binary Logistic Regression was performed to assess the influence of statistically significant diagnostic tests on the final diagnosis. Ratios: CD3+/CD8+ (M+S), CD5+/CD8+ (M+S), and CD7+/CD8+ (M+S) were evaluated.*

4.3. Study Limitations

(1) Most biopsies were punch type, resulting in limited material. Some paraffin blocks had no viable tissue left after initial microtome cuts, preventing complete immunohistochemical analysis, leading to the exclusion of these blocks and patients from the study.

(2) Although the sample size seemed small, it was sufficient to demonstrate significant differences between MF-diagnosed patients and the control/ID group. A larger sample size might yield more significant conclusions.

(3) Five specific areas of the biopsy are manually selected for analyzing immunohistochemical marker expression. In cases of scarce infiltrate, this selection can be subjective. To increase reproducibility and reduce intra-observer subjectivity, a single pathologist made the selections.

(4) The specific dilution for the TOX antibody depended on the antigenic capacity of the tissue. To avoid variations, blocks older than the year 2000 were excluded.

(5) The algorithm provides different results for immunohistochemical expression intensities. Statistical results may vary depending on the intensity parameter used. In this study, "medium" and "strong" intensities were summed to better reflect marker expression. For TOX, the +2 nuclei intensity was used for similar reasons.

5. Conclusions

(1) Median diagnostic times vary depending on histological findings from the initial biopsy. Re-biopsy occurs more promptly in cases where the initial biopsy is at least suspicious.

(2) Histopathological features showing a moderate to strong association (V de Cramer: >0.4) with MF diagnosis and also demonstrating statistically significant differences compared to ID include basal epidermotro-

pism, lymphocytic atypia, and peri-lymphocytic halo.

(3) Immunohistochemical markers CD2, CD3, CD4, and CD5 are useful in the diagnosis of early-stage MF, showing statistically significant differences compared to the control group (patients with a final diagnosis of ID).

(4) Increases in CD3/CD8 and CD5/CD8 ratios are useful in the diagnosis of early-stage MF, presenting statistically significant differences compared to the control group (patients with a final diagnosis of ID).

(5) The CD7/CD8 ratio shows an inverse relationship, being higher in patients with a final diagnosis of ID and showing statistically significant differences compared to the group of patients with a final diagnosis of MF.

(6) Elevated levels of TOX antibody expression are useful in the diagnosis of early-stage MF, showing statistically significant differences compared to the control group.

(7) Obtaining a high CD3/CD8 ratio increases the odds of obtaining a positive final diagnosis for MF.

(8) Obtaining a high CD7/CD8 ratio decreases the odds of obtaining a positive diagnosis for MF.

Abbreviations

MF	Mycosis Fungoides
TCR	T-cell Receptor
ID	Inflammatory Dermatoses
TOX	Thymocyte Selection-Associated High Mobility Group Box Protein
SD	Standard Deviation
CI	Confidence Interval
SOUH	Severo Ochoa University Hospital
M+S	Medium + Strong

Author Contributions

The manuscript has been read and approved by all authors.

Conflicts of Interest

The authors declare no conflicts of interest.

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