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MYCOSIS FUNGOIDES AND SEZARY SYNDROME
THE JANUS BIFRONS OF THE CUTANEOUS T-CELL LYMPHOMAS:
EVALUATION OF SPECIFIC PHENOTYPE AND GENETIC ALTERATIONS.

(BIO/13 – APPLIED BIOLOGY)

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ABSTRACT

Mycosis fungoides (MF) and Sézary syndrome (SS) are two variants of cutaneous T-cell lymphomas (CTCL). These entities are considered two distinct diseases, based on phenotypic and genetic analyses. Neoplastic cells in MF seem to derive from T effector memory cells, instead, neoplastic SS cells have a central memory (TCM) profile. Moreover, evidence of a resident memory T cell (TRM) profile in early-stage MF, compared to migratory memory T cells (TMM) profile in advanced-stage MF, seems to explain the clinical behavior of MF during progression. Recent studies have shown more heterogeneity in CTCL phenotypes compared to that theory. Differences between MF and SS also involve the microenvironment, which predominantly expresses Th1 phenotype compared to Th2 phenotype in advanced stages and SS. A series of studies have identified multiple molecular changes in CTCL, showing a heterogeneous landscape of numerous genetic alterations without a strong differential signature between the two diseases. Molecular mutations could be found more frequently in the pathways of epigenetic and/or chromatin regulation, TCR and T-cell/cytokine signaling, JAK/STAT signaling, and phosphoinositide 3-kinases (PI3K)/protein kinase B (Akt) and NF- κ B pathway.

The aim of this project is to delineate the differences between MF and SS about the phenotypic and genetic point of view, taking into account all stages of the diseases.

In the first phase of the project, 15 patients with a new diagnosis of MF and SS were selected, including 5 early-stage MF (IA-IIA), 3 advanced MF (2 III MF, 1 IIB MF), 5 classical SS, 2 non-erythrodermic SS. Neoplastic T-cell immune phenotypes were evaluated on paraffin-embedded formalin-fixed sections of skin biopsies and on CD4+CD7- sorted T cells of the peripheral blood by flow cytometry (except for two CD7+ cases). About the 3 patients with stage IB MF, two of them revealed a TRM phenotype (CD69+CD103+CCR7-CD62L-). In the third of them, T cells aberrantly showed expression of CD69 and also CD62L. One IIA-

MF patient showed expression of CD69 and CD103 (TRM markers) but also a partial expression of CCR7 with the negativity of CD62L, as TMM phenotype. Erythrodermic and tumoral MF patients were characterized by an infiltrate with a TMM phenotype. In SS, 3 patients showed a typical TCM phenotype (CD45RO+CD27+CCR7+CD62L+). Out of 5 CD45RA+ cases, 4 of them evidenced a T naïve phenotype (CD62L+), including an early MF (IIA). One SS showed complete negativity of CD62L and CCR7, arguing a TEMRA phenotype. Immunophenotype of blood samples revealed a better correlation with the skin of SS patients compared to MF patients. Neoplastic T cells in MF mainly had a Th1 phenotype, also in advanced stages, compared to Th2 phenotype of SS patients. This first step of the project confirmed that MF and SS are characterized by heterogeneity of phenotypes with partial correlation to the clinical features. Evidence of the same TCM phenotype in patients with classical and atypical SS suggests that criteria of this disease should be revised, including also non-erythrodermic forms.

In the second phase of the project, we performed a gene expression analysis using a 770-genes panel by NanoString technologies (PanCancer Immune Profiling Panel). To obtain more statically significant data, we included 95 FFPE slides of CTCL. Total RNA from 87 samples was extracted (36 SS and 51 MF at any stage). NanoString data were processed and statistical analysis was performed within the statistical environment R. A principal component analysis was performed on all samples together, but we found a homogeneous group without any evidence of clustering between MF and SS. So, we decided to study separately the two entities. About MF, 12 differentially expressed genes (DEGs) between advanced stages and early stages were found (p-value <0.01): 9 of them resulted upregulated (CCR3, PRAME, FPR2, PMCH, AMBP, TRL7, TNFRSF10C, CFI, HAVCR2) and 3 were down-regulated (KLRB1, CD1B, CD5). Gene ontology (GO) enrichment analysis showed that those genes were significantly enriched in the regulation of the immune system, macrophage activation and Toll-like receptor (TLR) signaling. (FDR <0.05) KEGG pathways analysis revealed a not

significantly representation in any pathways. Then, MF cohort was divided into two groups based on the median expression for each gene and the effects of high or low expression levels on OS were examined using the Kaplan-Meier (KM) survival curve. A list of 39 genes was identified as significant associated with OS (p-value <0.05). The HRs and p-values of those selected 39 genes were calculated through Cox regression model, revealing a 9-genes signature, which did not match with DEGs but showed a high significance from KM survival analysis. The increased expression of the following genes is significantly associated with poor prognosis: CDK1 (HR=2.06), IL6ST (HR=1.49), CCR4 (HR=1.66), ITK (HR=1.78), NOS2A (HR=1.38), IL2RA (HR=2.06), LRRN3 (HR=1.39), DUSP4 (HR=1.74). Instead, the lower expression of CCL26 (HR=0.53, p-value 0.028) is significantly associated to poor prognosis. A prognostic score was developed based on the incidence of each gene of the signature on overall survival and was defined as the linear combination of logarithmically transformed gene expression levels weighted by average Cox regression co-efficient. Patients with a high score of expression of the signature had a significantly poorer prognosis compared to patients with a low score (p-value < 0.05). Similarly, considering advanced versus early stages, the two groups with high score confirmed to have a statistically significant poorer prognosis compared to low score (p-value <0.05). This result is particularly evident comparing the score in early stages. KEGG pathway analysis showed that the 9-genes signature was significantly enriched in cytokine and JAK-STAT signaling. For SS samples, we did not evaluate DEGs because of the absence of different stages like in MF, but we performed the same analyses based on OS. A 14-gene signature was found, characterized by the association of their high expression and poor prognosis. The genes identified were IL12A (HR=2.12), IL5RA (HR=2.80), IFNL2 (HR=2.21), NT5E (HR=1.97), IL18RAP (HR=1.89), ABCB1 (HR=1.74), CCL16 (HR=1.89), CCL1 (HR=1.95), IL22RA2 (HR=1.67), IFNA17 (HR=1.69), C9 (HR=1.79), CCR9 (HR=3.31), SPANXB1 (HR=1.62), TREM1 (HR=1.76). We applied the prognostic score, showing that patients with a high score had a worse prognosis compared to

patients with a low score. GO analysis showed that this 14-gene signature was significantly involved in defense response and Th1 cytokine production (FDR <0.05). KEGG pathway analysis was able to confirm a statistically significant involvement of these genes in JAK-STAT and TLR signaling. In this second phase of the project, we were able to find a gene signature for each disease with a significant prognostic value that could be useful in clinical practice, especially in early stages. Evidence of a strong involvement of JAK-STAT pathway in analysis of both MF and SS is interesting because this pathway is well known to be involved in CTCL pathogenesis and its pharmaceutical inhibition is still studied.

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1. INTRODUCTION

Primary cutaneous lymphomas (PCL) are a heterogeneous group of non-Hodgkin lymphomas characterized by clonal proliferation of mature lymphocytes (B, T or NK) in the epidermis, dermis, subcutaneous tissue and/or adnexa.¹ PCL are rare diseases accounting for around 2% of all lymphomas with an incidence rate of 0.3-1 case per 100.000 person-years. This rate is increasing for unknown reasons.²

Cutaneous T cell lymphomas (CTCL) are the most frequent form of PCL (> 75%) and includes numerous entities which are been classified in different classifications during the years by the World Health Organization (WHO) and the European Organization for Research and Treatment of Cancer (EORTC), based on clinical and morphological evidence. The last one is the 2017 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues.³ (Table 1)

Mycosis fungoides (MF) is the most common CTCL, accounting for around 50-70% of all CTCL, instead Sézary syndrome (SS), the leukemic variant of CTCL, represents 1-3% of cases.²

Cutaneous NK and T cell neoplasms
Mycosis fungoides MF variants • MF-associated follicular mucinosis • Pagetoid Reticulosis • Granulomatous slack skin disease Sézary Syndrome Adult T cell leukemia/lymphoma Primary cutaneous CD30+ T-cell lymphoproliferative disorders • Primary cutaneous anaplastic large cell lymphoma • Lymphomatoid papulosis Subcutaneous panniculitis-like T cell lymphoma Extranodal NK/T cell lymphoma, nasal type Primary cutaneous peripheral T cell lymphoma, rare subtypes • Primary cutaneous gamma delta T-cell lymphoma • Primary cutaneous CD8-positive aggressive epidermotropic cytotoxic T-cell lymphoma • Primary cutaneous acral CD8-positive T-cell lymphoma • Primary cutaneous CD4+ small/medium T-cell lymphoproliferative disorders Peripheral T-cell lymphoma, NOS
Cutaneous B cell neoplasms
Extranodal marginal zone lymphoma of mucosa-associated lymphoid tissue Primary cutaneous follicle centre lymphoma Primary cutaneous diffuse large B-cell lymphoma, leg type Primary cutaneous diffuse large B-cell lymphoma, NOS
Blastic plasmacytoid dendritic cell neoplasm

Table 1. 2017 WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (Modified from: Swerdlow SH et al 2017, 4th edition, Lyon, France³)

1.1 CLINICAL PRESENTATION

Classical MF is characterized by patches and/or plaques which are localized in the first steps of the disease and they tend to be diffuse or evolve to tumors or erythroderma.

The typical initial presentation is characterized by patches or plaques localized in less than 10% of the body surface (30% of the patients) or diffuse in more of the 10% of the body surface (in 35-40% of the patients). These lesions are usually on the flexor surface of the limbs or in the bathing suites areas, even if it can appear anywhere on the body.⁴

MF patches are erythematous and fine scaling and become slightly raised with fixed shape and poikiloderma (atrophy, white and brown areas, telangiectasia) when they evolve to the plaque-stage. Itch is the most common symptom in all stages of the disease. Plaques can be ulcerated.

Around 15-20% of the patients present isolated or diffuse tumors (raised solid lesions greater than 10 mm in diameter) which usually evolved from plaques. “Tumour d’emblée” presentation (not preceded by patches or plaques) is rare. Tumors can be ulcerated, and bacterial superinfection is frequent, leading to sepsis which is the main condition of MF-related mortality in advanced stages.⁵

Erythroderma is present in around 15% of MF patients. By definition, it is a confluent and scaling erythema in more than 80% of the body surface area. In MF, erythrodermic skin is typically atrophic and can be associated with raised plaques and tumors. Palmoplantar keratoderma with onychodystrophy and alopecia are frequently present. Erythrodermic patients usually complain of intense full-body itching and scaling. Dermatopathic lymphadenopathy is frequent and due to severe skin involvement.⁵

The mean time from onset of skin disease and diagnosis is 36 months. A lot of patients present scaling patches in unexposed sun areas. These lesions are relapsing for years and hard to diagnose, configuring the “premycotic phase”.⁶

SS is a distinct subtype of CTCL, and, by definition, it is characterized by a triad of manifestations: 1) erythroderma, 2) diffuse lymphadenopathy, 3) the presence of Sézary cells (SC) in the blood. In particular, the last criterion is necessary to define SS, considering that erythroderma in SS is commonly indistinguishable from erythrodermic MF. Other clinical manifestations include lagophthalmos, alopecia, palmoplantar hyperkeratosis, and onychodystrophy. SS could develop “de novo” or from a long-standing MF.³

SC are large mononuclear T cells with non-granular cytoplasm and cerebriform nucleus. They are also present in the skin with the same morphology and phenotype. To define SS, peripheral blood should contain more than 1000 SC/mm³. Moreover, other diagnostic criteria are the presence, in the peripheral blood, of an increased CD4⁺ clonal population with a CD4/CD8 ratio more than 10, and an increased CD4⁺/CD7⁻ or CD4⁺CD26⁻ population of atypical T cells. Typically, a monoclonal rearrangement of the T-cell receptor is found identical in the skin and in the blood.⁷

Bone marrow involvement is not common.

Frequently, SS patients do not present all criteria in the first steps of the disease, but then they progress to develop the complete clinical picture. SS patients seem to have a worse prognosis compared to erythrodermic MF patients, which have no evidence of blood involvement.⁸

1.2 HISTOPATHOLOGY

1.2.1 Mycosis fungoides

Patch-MF is histologically characterized by a lichenoid or band-like infiltrate in the upper dermis. (Figure 2A) The epidermis is usually not involved, but psoriasiform hyperplasia is possible. Epidermotropism (evidence of atypical lymphocytes residing in the epidermis) is frequent and typical. (Figure 2B) When it is absent, a diagnosis of MF could be a challenge. In a few cases, isolated atypical lymphocytes tend to group within the epidermis forming Pautrier's microabscesses, a hallmark sign of MF. (Figure 2 C,D) Atypical lymphocytes within the epidermis have a bigger nucleus than those in the dermis. Spongiosis could be present. In the upper dermis, beside the band-like infiltrate, fibrosis and oedema are usually present. A perivascular superficial infiltrate, vacuole degeneration of the basal layer of the epidermis with necrotic keratinocytes or incontinentia pigmenti are less frequent histological pictures.⁹

Plaque-MF is characterized by a dense band-like infiltrate in the upper dermis. Pautrier's microabscesses are very frequent. In the lymphoid infiltrate, small pleomorphic cerebriform cells are predominant. In some cases, the infiltrate is diffuse in the dermis, being hard to diagnose.⁹

Tumors are characterized by a dense nodular or diffuse monomorphic infiltrate in all dermis, until the subcutaneous tissue in some cases. Epidermotropism is usually absent. In some case, angiotropism is evident. (Figure 2 E,F) The strong involvement of the eccrine structures is possible, like in syringotropic MF.³

Folliculotropic MF, a variant of MF, is histologically characterized by a dense lymphoid infiltrate around hair follicles with their destruction. Epidermis between hair follicles is usually not involved and epidermotropism is rare. Presence of follicular mucinosis, eosinophils and plasma cells within the infiltrate is frequent.⁹

Large cell transformation (LCT) is defined by the presence of more than 25% of large cells in the total lymphoid infiltrate or forming microscopic nodules. (Figure 2 E) LCT occurs in 20-50% of advanced MF; especially tumor-stage MF, but patch and plaque-stage MF may display LCT less commonly. LCT is a poor prognostic factor, associated with a 5-year survival of <20%.¹⁰

MF is an α/β memory T helper process ($\beta F1+$, CD3+, CD4+, CD5+, CD8-, CD45RO+).¹¹ Less frequently, some cases could have a CD8 ($\beta F1+$, CD3+, CD4-, CD5+, CD8+) or gamma/delta phenotype ($\beta F1-$, CD3+, CD4-, CD5+, CD8-, TCRgamma/delta+). In advanced cases, the loss of pan-T markers is frequent (CD2, CD5, CD7). In plaques and tumors, CD30 expression is possible. Ortonne et al suggested that a low CD4/CD8 ratio in the skin could be a hallmark for MF, but it has not been confirmed.¹² Cytotoxic markers, as TIA-1 and Granzyme-B, are usually negative in MF, but they can rarely express in advanced cases.⁹ T-cell receptor (TCR) clonal rearrangement is common in plaque and tumor-MF, but in only 50% of patch-MF.¹³

1.2.2 Sézary Syndrome

Histological features in SS skin lesions are almost indistinguishable from MF.¹⁴ A spongiotic psoriasiform pattern with a band-like infiltrate is common. Epidermotropism is usually less evident than in MF. Pautrier's microabscesses can be present. Neoplastic lymphocytes are morphologically small and medium pleomorphic cerebriform cells.⁹ Compared to MF, granulomatous inflammatory reaction, follicular mucinosis and LCT are rarer.^{15,16} Neoplastic cells in SS have a T-helper phenotype: CD2+, CD3+, CD4+, CD5+, CD8-. Typically, CD7 and CD26 are negative.¹⁷

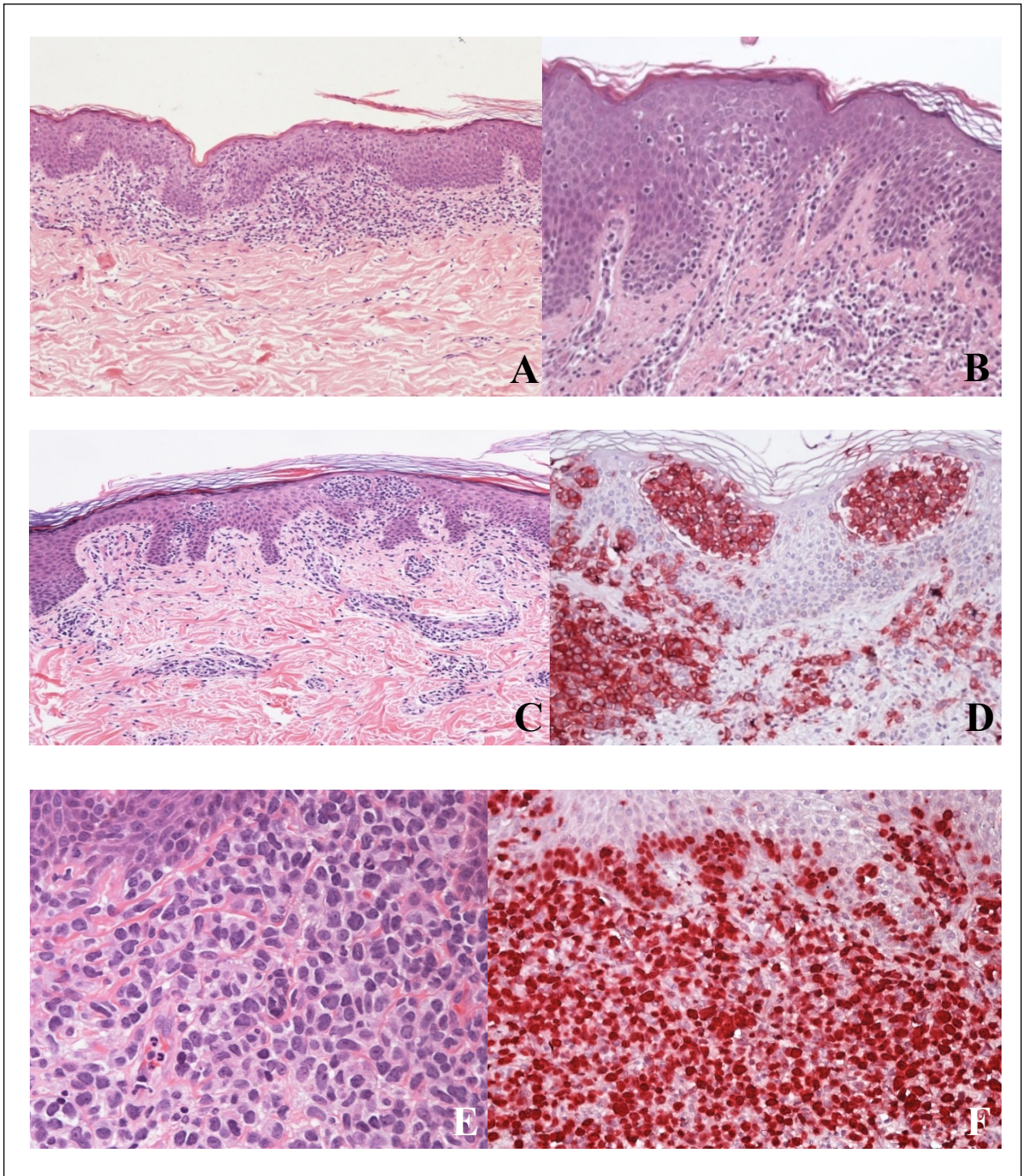


Figure 2. A: epidermotropic band-like infiltrate; B: epidermotropism; C: Pautrier's microabscesses; D: antiCD4 immunostaining shows Pautrier's microabscesses within the epidermis; E: atypical lymphoid infiltrate with LCT; F: proliferation in a tumor (Ki-67++).

1.3 DIAGNOSIS AND STAGING

MF usually starts as a long-standing chronic dermatitis with patches and plaques in less than 10% of the body surface (T1a), especially in bathing suite area.⁴ T1a stage can last for years or progresses with the development of diffuse patches and plaques (T2), tumors (T3) or erythroderma (T4) with or without blood involvement (B2). Lymph nodes or bone marrow involvement is possible but rare. Advanced-stage MF ($\geq$ IIB stage) can show LCT in skin or lymph nodes. Progression from T1 stage to advanced stages is a clinical course of MF, described in around 20% of cases and it takes a lot of years; nevertheless, some cases are characterized by an aggressive course and present tumors or erythroderma at the time of diagnosis.¹⁸

Since 2007, Olsen et al⁷ introduced a new TNMB staging system for MF and SS, which includes the evaluation of the involvement of the skin, lymph nodes, bone marrow, viscera and blood (Table 2, Figure 3)

	Stage	Skin (T)	Lymph Node (N)	Metastasis (M)	Blood (B)
Skin only-patches and plaques	IA	1	0	0	0-1
	IB	2	0	0	0-1
	IIA	1/2	1-2	0	0-1
Tumors	IIB	3	0-2	0	0-1
Erythroderma	IIIA	4	0-2	0	0
	IIIB	4	0-2	0	1
Leukemia	IVA ₁	1-4	0-2	0	2
Nodal disease	IVA ₂	1-4	3	0	0-2
Systemic	IVB	1-4	0-3	1	0-2

Figure 3. ISCL/EORTC revision of MF and SS staging

TNMB stages	
Skin	
T1	limited patches, papules or plaques covering <10% of the skin surface
T1a	patches only
T1b	plaques and patches
T2	patches, papules or plaques covering ≥10% of the skin surface
T2a	patches only
T2b	plaques and patches
T3	one or more tumors (≥1 cm diameter)
T4	confluence of erythema covering ≥80% body surface area
Node	
N0	no clinically abnormal peripheral lymph nodes; biopsy not required
N1	clinically abnormal peripheral lymph nodes; Dutch grade 1 or NCI
LN0-2	
N1a	clone negative
N1b	clone positive
N2	clinically abnormal peripheral lymph nodes; Dutch grade 2 or NCI
LN3	
N2a	clone negative
N2b	clone positive
N3	clinically abnormal peripheral lymph nodes; Dutch grades 2-3 or NCI
	LN 4, clone positive or negative
Nx	clinically abnormal peripheral lymph nodes; no histologic confirmation
Visceral	
M0	no visceral involvement
M1	visceral involvement
Blood	
B0	absence of significant blood involvement: ≤5% of peripheral blood lymphocytes are atypical (Sézary) cells
B0a	clone negative
B0b	clone positive
B1	low blood tumor burden: >5% of peripheral blood lymphocytes are atypical (Sézary) cells but does not meet the criteria of B2
B1a	clone negative
B1b	clone positive
B2	high tumor burden: ≥1000/μL Sézary cells with positive clone

Table 2. ISCL/EORTC revision to the classification of MF and SS

The 2007 update of MF and SS staging introduced the evaluation of the peripheral blood (B stage) at the TNM system because blood involvement is one of the major prognostic factors for MF and SS. Based on the B stage, B0 means the presence of $\leq 5\%$ of atypical cells (Sézary cells) at the blood smear, instead, B2 is defined by the presence of T cell clone in the peripheral blood associated to $\geq 1000/\mu\text{L}$ SC or an increased number of CD4+ or CD3+ cells with a CD4/CD8 ratio ≥ 10 or a high count of CD4+ cells with an abnormal phenotype (loss of CD7 $> 40\%$ or loss of CD26 $> 30\%$). B1 stage is characterized by the presence of SC ($>5\%$) but does not meet the criteria of B2 stage.⁷

Erythrodermic MF, without visceral or bone marrow involvement (T4N0-2M0), was staged in IIIA (T4N0-2**B0**M0) and IIIB (T4N0-2**B1**M0) based on tumor burden in the blood.⁷ High tumor burden in the blood and in the lymph nodes defines the IV stage, which is divided in IVA1 in presence of B3 stage, and IVA2, in case of N3 stage. In case of erythroderma (T4), a low tumor burden in the blood (B0 and B1) characterizes MF in stage III, instead, B2 stage defines SS in stage IVA1 or IVB, based on bone marrow or visceral involvement.⁷ Scarisbrick et al⁸ and Vidulich et al¹⁹ reported that in erythrodermic patients SC count > 10000 cellule/ul is associated to worse prognosis compared to an SC count between 1000 and 10000 cellule/ul. Bone marrow biopsy is recommended in case of blood involvement (B2) or in case of hematological abnormalities.⁷ Bone marrow involvement is included in IVB stage, as well as visceral involvement.

Compared to the previous staging system, T0 stage (clinically and/or histopathologically suspicious lesions) was removed because clinical staging should be applied only to cases in which the diagnosis has been established.⁷

Based on Olsen et al⁷ recommendation, lymph node biopsy for staging is not necessary in case of absence of palpable lymph adenomegalies. Clinically abnormal lymph nodes are defined as peripheral lymph nodes firm, clustered, fixed or 1.5 cm or larger in diameter on

physical examination.⁷ Histopathological grading for lymph nodes biopsy was also confirmed, based on “Dutch system”. (Table 3)

About visceral involvement, splenomegaly is considered visceral disease without biopsy confirmation when it is evident on physical examination or radiographically. In the case of liver or lung disease or abnormalities of other organs, biopsy confirmation is always requested.⁷

ISCL/EORTC update	Dutch system	NCI-VA classification
N1	Grade 1: dermatopathic lymphadenopathy (DL)	LN0: no atypical lymphocytes LN1: occasional and isolated atypical lymphocytes LN2: many atypical lymphocytes or in 3-6 cell clusters
N2	Grade 2: DL; early involvement by MF (presence of cerebriform nuclei > 7.5 um)	LN3: aggregates of atypical lymphocytes; nodal architecture preserved
N3	Grade 3: partial effacement of LN architecture; many atypical cerebriform mononuclear cells. Grade 4: complete effacement	LN4: partial/complete effacement of nodal architecture by atypical lymphocytes or frankly neoplastic cells

Table 3. Histological staging of lymph nodes in MF and SS.

1.4 PATHOBIOLOGY

Pathogenesis of MF and CTCL, in general (except for Adult T-cell leukemia/lymphoma), is still unknown. It is a sporadic disease without evidence of transmissibility. A presume prolonged antigenic stimulation, by allergens or superantigens in infections, seems to enhance immune responses and subsequently induces mutations in the apoptotic pathway, leading to a clonal proliferation and accumulation of neoplastic T cells.²⁰ Several viruses have been studied to find an infectious origin of MF/SS, like herpes simplex virus, human herpesvirus 6, Epstein-Barr virus, without any evidence to support this hypothesis. On the contrary, the association between *Staphylococcus aureus* colonization and disease activity is well known, supporting the evidence that staphylococcal superantigens are involved in the CTCL pathogenesis by superantigen-stimulated clonal proliferation of T cells.²¹ Exposure to metals, pesticides and organic solvents was associated with increased MF risk, but these observations were not been supported by further well-designed case-control studies.^{22,23}

1.4.1 Neoplastic T-cell phenotype and cytokine profile

Neoplastic cells in MF and SS are characterized by a mature α/β memory T cell phenotype with positivity for CD4, CD45RO and TCR β .²⁴ A subset of MF is characterized by clonal proliferation of CD8⁺ cells with or without a cytotoxic phenotype, but it is never evident in SS. Expression of pan-T cell antigens, like CD3, is usually present, even with aberrations, like loss of CD2 and CD5, which is associated with a worse prognosis.²⁵ In advanced MF, double-positive CD4⁺/CD8⁺ or double-negative (CD4⁻/CD8⁻) phenotypes are possible.²⁶ CD7 is expressed on T cell during early differentiation, but it is usually loss during T-cell homing. Loss of CD7 is considered as a typical feature of CTCL cells but, in the last years, more evidence has shown that some patients (from 10 to 40%) can express CD7 on circulating SC cells. This feature is associated with a longer survival compared to the negativity of CD7.^{3,27} Downregulation of CD26 on CD4⁺ neoplastic T-cells is now considered a hallmark for SC on peripheral blood, easily to detect by flow cytometry. Since its first description, the evidence

of loss of CD26 on SC in the skin and in the peripheral blood has been confirmed by several authors.²⁸⁻³³ CD26 is a costimulatory T cell activation antigen and, in healthy patients, CD4+ T cells with loss of CD26 represent 7% of circulating lymphocytes. The threshold value of 30% of CD4+CD26- cells on total lymphocytes in the peripheral blood suggests the diagnosis of SS and this data has been confirmed in the last staging guidelines.⁷ In a recent European multicenter study, on 59 SS patients and 19 patients with erythrodermic benign dermatosis (EID), loss of CD26 (more than 80% evaluated on total T cells) and/or loss of CD7 (more than 40%) on CD4 T cells was found in 83% of SS patients but never in EID patients.³⁴ CD158k receptor, also called KIR3DL2, was identified as a cell surface marker on SC in 2001³⁵ and its sensibility for the diagnosis of SS was confirmed in several studies.³⁶⁻³⁸ KIR3DL2 is expressed on NK cells but not on normal T lymphocytes. Evidence of KIR3DL2 on SC could precede the classical clinical SS presentation so it could be a useful diagnostic marker for SS.

Alfa-chain of IL-2 receptor (CD25) is heterogeneously expressed on neoplastic T-cells in half of the patients. A mediator of apoptosis, Fas ligand (FasL), is frequently highly expressed on CTCL cells, and its link with the death receptor Fas seems to induce apoptosis on tumor-reactive T cells. Loss of Fas expression, somatic inactivating mutations and deletions of the FAS and ARID1a genes or increased expression of anti-apoptotic proteins such as c-FLIP, induce resistance to FasL-mediated apoptosis in CTCL cells, protecting them for T-cell mediated cytotoxicity.³⁹

Increased expression of programmed death-1 (PD-1) on SS cells, compared to MF and healthy T cells, contributes to the impaired immunity in SS. Moreover, JAK/STAT pathway has been proposed to induce malignant cell expression of PD-L1, an inhibitory B7 family member, which with PD1 has been implicated in benign T cell suppression and tumor immune evasion in CTCL.⁴⁰ Blockade of the signaling pathway with blocking antibodies to PD1 and PD-L1 led to an increase of production of IFN γ in some patients.⁴¹

Sézary cells can also express T-regulatory profile molecules (FOXP-3), which result in suppression of the immune response.²⁶

Recent publications have shown that CTCL neoplastic cells are characterized by various memory T cell phenotypes, which directly correlate with the different clinical behaviors of the CTCL subtypes.⁴² Human skin is the house of numerous memory T cells which contribute to the innate immune and the skin barrier function. T-resident memory cells (TRM) are the subgroup permanently resident in the skin, that works in the localized immunity. Their dysfunction can cause autoimmune or autoinflammatory skin diseases (such as psoriasis, alopecia or contact dermatitis), transplant rejection and malignancies.⁴³ In their skin xenograft model, Watanabe and colleague⁴² transplanted human neonatal foreskin onto immunodeficient mice and also transferred allogenic human peripheral blood cells from unrelated donors. This activation of the T cells induced dermatitis and different subpopulation of memory T cells in the skin graft were shown. A group shortly expressed CD69 and part of these cells also co-expressed CD103, as previously described in skin TRM.^{44,45} Another subgroup expressed CCR7 and L-selectin, two lymph node migration receptors, which describe the T-central memory (TCM) phenotype. They also repeated in the human-engrafted mice the same study previously done by Clark et al⁴⁶ on CTCL patients. After treatment with alemtuzumab (a humanized anti-CD52 antibody), they found a complete depletion of T cells in the blood but some T cells in the skin-graft were spared. These cells expressed CD69 showing that TRM remains in the skin and not able to re-circulate. A subgroup of these TRM also expressed CD103, the α -subunit of the $\alpha E\beta 7$ integrin which binds to epithelial cells by its ligand E-cadherin. These CD69⁺CD103⁺ TRM are more evident in the epidermis than in the dermis, where they are associated with skin appendages (glands and hair follicles).⁴⁷ Authors also found a new intermediate subgroup of T cells, characterized by expression of CCR7 but not L-selectin, so different compared to TCM phenotype. This subset, called migratory memory T-cells (TMM), expresses the skin-homing molecule cutaneous leukocyte antigen

(CLA) and, after treatment of CTCL patients with alemtuzumab, TMM were depleted in both blood and skin, even if much slower compared TCM, showing their ability to recirculate. Given the negativity of L-selectin, they are not able to enter in the lymph nodes from the blood, but authors speculated a specialized recirculation pattern.⁴² Based on these results, the authors suggested the correlation between the sub-phenotypes of the memory T-cells and the clinical behavior of CTCL. Early-stage MF with its fixed and localized skin lesions is characterized by an infiltrate of TRM. Advanced stage MF or SS, with TMM predominant cells in skin lesions, result to have a distinct clinical phenotype characterized by discrete lesions with ill-defined borders; instead, patients with diffuse skin lesions or erythroderma were characterized by malignant T cells with a TCM phenotype in blood and skin.⁴³ (Figure 4)

More recent studies revealed that phenotype of MF/SS neoplastic cells is more plastic and heterogeneous than previously reported, posing a challenge to the model of Watanabe and colleagues⁴² Considering the knowledge about T-cell immunology, also in CTCL, we can find different T cell subsets, corresponding to different stages of antigen experience, functional properties, migration capabilities and their ability to survive and/or self-renew.⁴⁸ Based on surface markers, T naïve cells (TN) which have not yet encountered the antigen, are typically CD45RA+CD45RO-CCR7+CD62L+CD27+CD28+. After antigen stimulation, TN lose CD45RA and express CD45RO, becoming TCM, which maintain the ability to home the secondary lymphoid tissues and self-renew, and T effector memory cells, which loss CCR7 and CD62L and migrate into the site of inflammation. At the last stage of differentiation, terminally effector memory T cells (T EMRA) regain the expression of CD45RA but CD27, CCR7 and CD62L remain negative.⁴⁸⁻⁵²

Other studies, assessing cytokine expression profiles, showed that early MF displays a Th1 profile by producing increased IL2 and interferon- γ (IFN γ), likely as a reflection of antitumor

immune response by IFN γ -secreting CD8⁺/Tia-1⁺ T-cells. In contrast, advanced stage MF and SS are characterized by increased production of Th2-type cytokines (IL4, IL5 and IL10) and cessation of Th1 response contributing to immune failure and secondary malignancies. (Figure 5) This switch from Th1 to Th2 in advanced stages seems to be mediated by a progressive dysregulation of the JAK/STAT pathway and increased expression of the Th2 transcriptional master regulator GATA-3.³⁹ As a demonstration of this theory, a study on multiple skin samples of MF in progression, was conducted to evaluate the differential expression of T-bet, a T-box transcription factor required for Th1 development, and GATA-3. The predominant population found in patch stages were T-bet⁺ cells and in tumor-stage MF were GATA-3⁺ cells, instead, in plaque stage the infiltrate was composed by a mixed of T-bet⁺ and GATA-3⁺ population, suggesting that the Th1 to Th2 transition may occur at this stage.⁵³⁻⁵⁵ Disease progression is also associated to increased expression of Th2 cytokine (e.g. IL4, IL5 and IL13) concomitant to decreasing of Th1 factors such as IFN γ , STAT4 and IL12.³⁹ In addition to Th2 cells, some authors found a subset of SS characterized by malignant T cell Foxp3⁺ but it is still debated whether they represent true T regulatory cells (Treg) or whether FoxP3 expression is secondary to other molecular events that entail immune dysregulation.⁵⁶ Moreover, recent reports claim that malignant T cells in lesional skin of CTCL highly express IL-22 (favoring a Th22 phenotype) but lowly express IL-17 (Th17 phenotype).^{26,39} Despite the discrepant reports regarding IL17 expression, high expression of IL17F seems to be associated with significantly increased risk of disease progression, independently of disease stage.^{57,58} In CTCL, IL22 has been reported to be associated with the expression of CCL20. SiRNA-mediated knockdown of IL22 receptor α in neoplastic CTCL cells seems to inhibit the expression of CCL20, providing a possible link between IL22, the CCR6-CCL20 axis, and malignant dissemination.^{39,57} Phosphorylation of STAT3, the principal mediator of IL-22 signaling was also detected in epidermal keratinocytes and dermal

infiltrating cells in CTCL skin as well as psoriatic skin. It has been reported that IL-22 in psoriatic skin induces epidermal hyperplasia, which is mainly dependent on CCL20 expression and STAT3 phosphorylation. Consistent with detection of pSTAT3 in keratinocytes, acanthosis is often seen in lesional skin of CTCL.⁵⁷

Parallel to the shift in the balance of Th1 and Th2 cytokines, there is a decrease in chemokines that preferentially attract Th1-associated inflammatory cells, such as CXCL9 and CXCL10, highly expressed in early stages, and an increase in chemokines that primarily attract Th2-associated inflammatory cells, such as CCL17, CCL18, CCL22 and CCL26.^{39,59}

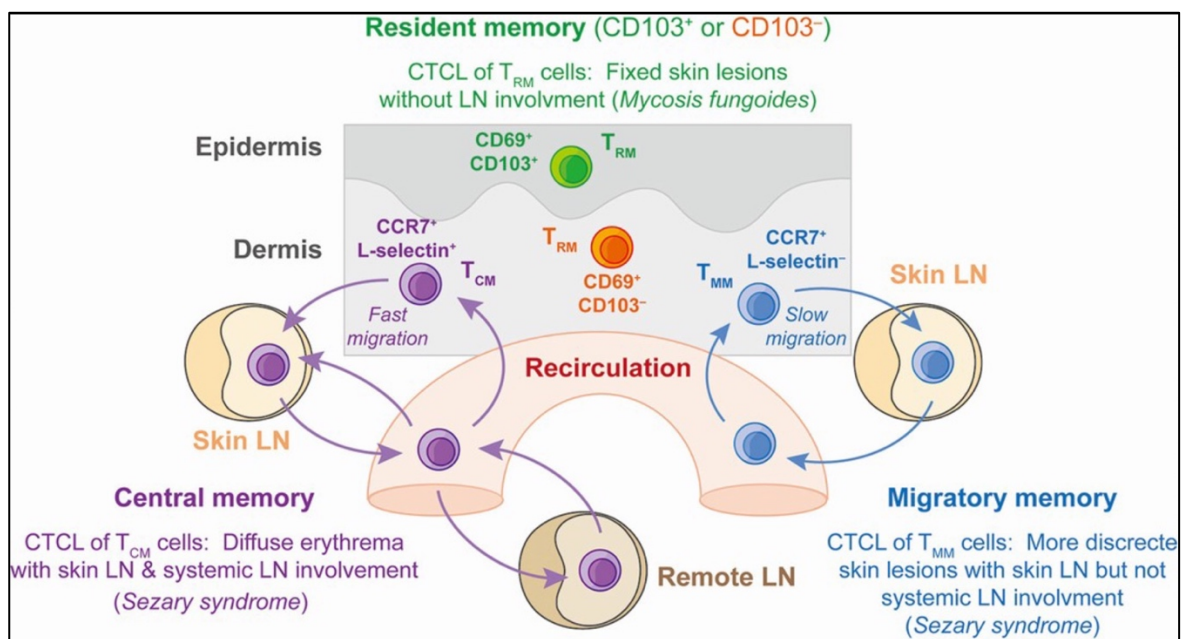


Figure 4. Four subsets of memory T cells with different recirculation pattern and their involvement in CTCL. From: Hochheiser K. Ann Trasl Med 2015;3(20):311

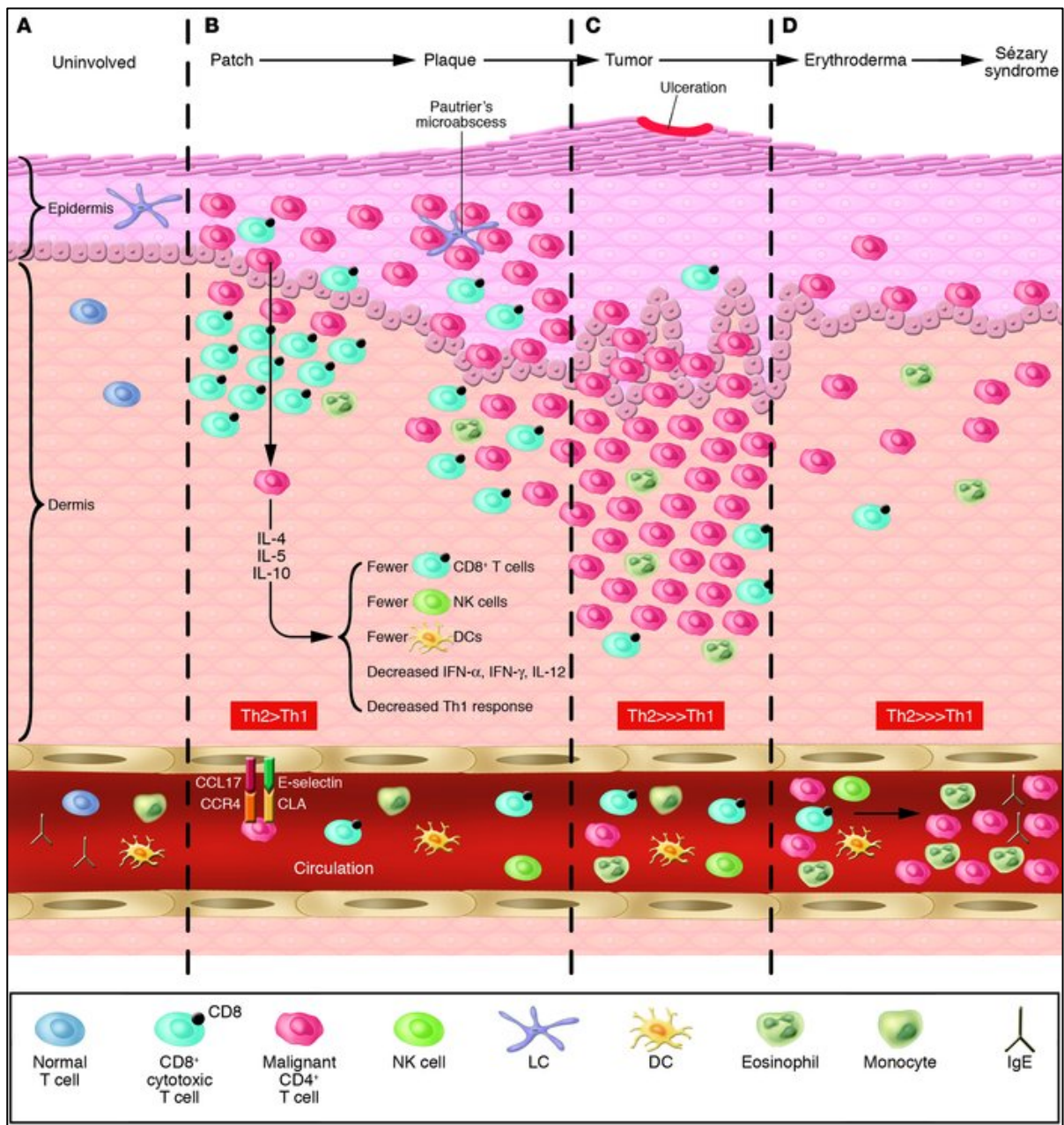


Figure 5. The skin microenvironment in MF and SS. From: Kim EJ.et al. J Clin Invest. 2005;115(4):798-812

1.4.2 Microenvironment

In the last years, numerous studies have shown that genetic mutations in neoplastic cells are not the only players in the pathogenesis and progression of CTCL. An amount of non-malignant immune cells is present in the skin lesions and interacts with neoplastic cells, realizing tumorigenic cytokines which influence the pathogenesis and progression of the

disease. Tumor microenvironment in CTCL includes CD8 T cells, Treg, dendritic cells (DC), macrophages and mast cells.^{39,60} (Figure 5)

In early stages, skin lesions are characterized by an infiltrate of a small population of neoplastic cells mixed to numerous reactive cells, especially CD8+ T infiltrating cells and Treg, which contribute to an antitumor Th1 response. During progression to more advanced stages, evidence of these reactive cells decreases, favoring a tumorigenic response and accumulation of DC. DC are antigen-presenting cells with an opposite possible function: they can induce immune response at a mature state or favoring tolerance at immature state. The close interaction between neoplastic cells and DC was also demonstrated by Berger et al: authors reported the growth of CTCL cells when co-cultured with immature DC but the inhibition of their proliferation with anti-TCR and anti-CD40 antibodies, assuming that the interaction between major histocompatibility complex (MHC) class II on DC and TCR on CTCL cells and CD40 on DC and CD40L on neoplastic cells are able to induce cell proliferation and DC survival.⁶¹ Different DC subsets seem to be related to disease progression: from early patch/plaques stages to advanced stages, the number of mature Langerhans cells tend to decrease compared to immature CD209 positive and CD303 positive DC that tend to be accumulated, favoring the MF immune escape response and tolerance. The release of immunosuppressive cytokines, such as IL4, IL5, IL10, TGF β and VEGF, leads to accumulation of immature DC, prompting the immunosuppression which favors tumor progression.⁶²

Role of macrophages as oncogenic players in many cancers is known. In CTCL, CD163 positive and CD68 positive cells seem to be increased in lesional skin and decreased after the use of skin directed treatment.⁶³ In particular, accumulation of M2-like tumor associated macrophage (TAM) is associated with a worse prognosis and their depletion in xenografted human CTCL cells in mice induces a delay of MF development, angiogenesis and lymphangiogenesis, showing a possible role of M2-like TAM in vascularization, chemotaxis,

tissue remodeling and tumor growth in CTCL.^{39,64} Kara et al demonstrated a decrease of dermal macrophages after MF treatments.⁶⁵

Mast cells are another family of cells of the microenvironment which seem to be involved in development and progression of CTCL, because they were found increased in lesional MF and, particularly, their amount is higher in progressing disease compared to stable disease, correlating with a worse prognosis.⁶⁶⁻⁶⁸

CD4⁺ and CD8⁺ tumor-infiltrating lymphocytes (TIL) were been recently studied also in MF by Murray et al.⁶⁹ Authors showed that TIL are characterized by an “exhausted” phenotype in all stages with the expression of PD1 and TIGIT. This phenotype differs greatly from that of neoplastic lymphocytes, which during tumor progression are more heterogeneous and can show a "cold" (DR⁻), "exhausted" (DR⁺ PD-1⁺), or "evasive" (DR⁺⁺ PD-L1⁺) phenotype. Moreover, TIL show a decreased functional cytotoxicity from early to advanced stages, supporting the evidence of the immune escape of the tumoral cells.⁶⁹

In CTCL skin lesions, eosinophils are activated and release high levels of IL5, IL4 and IL13, inducing neoangiogenesis which helps the tumor growing. Their accumulation in the infiltrate has not been associated with poor prognosis.³⁹

Epidermis and keratinocytes are also involved in CTCL microenvironment. In an organotypic skin model, use of CTCL cells and their supernatants triggered JAK and MAPK induced keratinocyte activation, downregulation of differentiation markers such as keratin 10 and involucrin, epidermal hyperproliferation, disorganized keratinocyte stratification, and decreased barrier formation.⁷⁰

1.4.3 Genomic landscape

SS and MF are both characterized by a similar genetic profile and by great diversity in gene expression, mutations, and chromosomal aberrations.²⁶ The genetic landscape of SS and MF, characterized using genome-wide next-generation sequencing approaches, have revealed that genes, subjected to point mutations and copy number alterations (CNA), take part in cell cycle

regulation, chromatin modification, JAK-STAT signaling, TCR/NK-kB signaling and MAPK signaling. These data suggest that deregulation of these cellular processes underlie lymphomagenesis.⁷¹

The majority of somatic single-nucleotide substitutions observed in SS/MF are C>T transitions (40%-75%),^{72,73} which are much less common in other hematological cancers.⁷² This mutational signature is regarded as caused by UV light exposure when occurring at NpCpG sites,⁷²⁻⁷⁴ or aging when occurring at NpCpC sites. In SS, both aging (43%) and UV radiation (30%) contribute to C>T transitions.^{72,75} At present it is unclear whether exposure to UV light plays a causative role in the onset of the disease.

The fact that oncogenic alterations in SS much more frequently result from CNAs rather than single-nucleotide substitutions suggests that the former may play an important role in SS lymphomagenesis.⁷⁴ Choi and colleagues reported complex genomic rearrangements associated with deletion of tumor suppressors in SS, suggesting that phenomena such as chromothripsis or chromoplexy could be key events in the development of CTCL. These authors proposed that deregulation of a group of DNA-cutting enzymes encoded by recombination activated genes may be implicated in the characteristic genomic instability of SS.⁷⁴

About cell cycle, cyclin-dependent kinases inhibitors (CKI) encoded by CDKN1A and CDKN2A are found deleted in 1% and 40% of SS cases, respectively. Deletions of ATM, RB1 and TP53 are frequent as well.^{72,74}

The JAK-STAT pathway is central to cytokine stimulation in T cells. Cytokine-bound receptors activate JAK kinases, which then activate STAT proteins. Activated STAT proteins dimerize and translocate to the nucleus where they induce expression of genes responsible for T-cell proliferation, differentiation and apoptosis.⁷⁶ Point mutations either predicted or confirmed to be gain-of-function are reported in JAK1 (0.9% of cases), JAK3 (2.7%), STAT3 (0.9%) and STAT5B (3.6%).⁷⁷ Hyperactive JAK and STAT proteins often result from

structural changes in their functional domains. For instance, amino acid substitutions in the pseudokinase domain of JAK proteins enhance their tyrosine kinase activity while changes in the SH2 domain of STAT proteins are predicted to enhance dimerization. Copy number gains of JAK2 (13% of cases), STAT3 (60%) and STAT5B (60%) are frequent and have been shown to correlate with increased expression.⁷⁷ Moreover, aberrant activation of STAT5 in the malignant T cells seems to induce expression of MiR-155 which subsequently inhibits the expression of Stat4.⁷⁸

Similarly to the JAK-STAT pathway, the MAPK pathway propagates towards the nucleus extracellular signals received through cytokine receptors. These signals elicit cellular processes such as growth, proliferation and apoptosis.⁷⁹ Subsets of SS/MF patients carry established gain-of-function mutations (<1%) in several members of this pathway such as KRAS, NRAS, BRAF, MAP2K1 and MAPK1.⁷⁷

In CTCL, hyperactive TCR signaling might drive the uncontrolled proliferation of malignant T cells. Genetic alterations reported in SS/MF target a variety of components of the TCR pathway.^{72,74,80,81} CD28 is a co-stimulatory molecule of the TCR complex, which by engaging ligand B7 (CD80 and CD86) on antigen-presenting cells (APCs), co-signals for T-cell activation.⁸² Increased affinity of CD28 for B7 due to amino acid substitutions or gene fusions results in augmented TCR signaling. Point mutations in CD28 occur in 3.6% of cases while in-frame gene fusions (i.e., CD28-ICOS and CD28-CTLA4) have been observed in a small number of cases.^{72,77,80}

Downstream to TCR activation, phospholipase PLC γ 1 catalyzes the production of second messengers (i.e. DAG and IP3), which in turn, activate downstream kinases.⁸² Point mutations in the catalytic core of PLC γ 1 (10% of cases) and copy number gains (5%) increase second messenger production which enhance antigen-dependent T-cell activation.⁷⁷ Furthermore, point mutations in DAG-activated kinases PRKCB and PRKCQ (<1% of cases) and amplification of the latter (30%) may contribute to TCR signaling deregulation.⁷⁷ However,

the functional relevance of these alterations remains uncertain. Gene expression changes triggered by TCR activation are effective through canonical NF- κ B signaling and other downstream pathways.⁸² CARD11 encodes a scaffold protein needed for the activation of transcription factor complex NF- κ B. In SS/MF, point mutations (5.4% of cases) and copy number gains (23%) involving CARD11 lead to increased NF- κ B activation.^{77,80,81} In addition, recurrent deletions (2.5% of cases) of the C-terminal auto-inhibitory I κ B domain of NFKB2 are expected to render it constitutively active.^{77,80}

Besides the TCR complex, other receptors can also elicit canonical NF- κ B signaling in CTCL. Membrane receptor TNFR2 (encoded by TNFRSF1B) initiates NF- κ B signaling upon binding to ligand TNF α . Reported activating point mutations in TNFRSF1B enable constitutive NF- κ B signaling without the need of a ligand.⁸⁰ Overall, point mutations and amplifications involving TNFRSF1B are reported in 2.2% and 2.5% of cases, respectively.^{77,80} In addition, TNFAIP3, which encodes an inhibitor of TNF α -induced NF- κ B signaling, is deleted in 25% of SS cases.⁸⁰

2. RESEARCH PROJECT AIM

The exact pathogenesis of SS and MF is still unknown and diagnosis and therapy in “atypical cases” is often a challenge, so, the overall aim of this project was to better define the immunophenotypic profile of SS and MF and their relationship by the investigation of the expression of T-helper subset markers. Determining intra-tumoral heterogeneity is a challenge but could be useful for understanding pathogenesis and evolution to perform a better diagnosis and treatment. Considering that the study of the balance between appropriate skin defense response and dysregulated responses could be the key to understanding the pathogenesis and treatment of MF and SS, my project advanced in the second part with a wide molecular study not only on neoplastic cells but also on the main players in the skin microenvironment.

3. MATERIALS AND METHODS

3.1 PHENOTYPE STUDY

3.1.1 Patient selection

In the first phase of the study, based on the identification of phenotype of neoplastic T cell, we selected patients seen at the CTCL Clinic of the Fondazione IRCCS Ca' Granda- Ospedale Maggiore Policlinico di Milano, University of Milan. Patients were characterized by a new and untreated diagnosis of:

- classical SS
- Erythrodermic MF without B2 stage
- MF associated with B2 stage

Diagnosis of MF and SS should be met the WHO/EORTC criteria for these diseases.³

Histological sections were reviewed to confirm the diagnosis.

At the time of diagnosis, we collected samples from lesional skin for immunohistochemistry analysis and peripheral blood for cytofluorimetry

3.1.2 Immunohistochemistry on paraffin-embedded tissue

At the time of diagnosis, a new skin punch biopsy was performed for immunohistochemical analysis for each patient. Tissue samples were fixed in buffered formalin, dehydrated, embedded in paraffin wax and sectioned. After deparaffinizing and rehydrating, each section was immersed in EDTA 0.05 M pH8, boiled 3 times for 5 min in a pressure cooker and washed with TBS buffer.⁸³ Each section was placed on the Dako cytometry automated immunostainer and incubated with the specific monoclonal antibody at room temperature for 45 min, washed with tris-buffered saline (TBS), pH 7.6, and incubated in a biotinylated goat antimouse and antirabbit immunoglobulins (DakoREAL™, cod.K5005, Dako cytometry, Glostrup, Denmark) at room temperature for 30 min. After incubation with the secondary antibody and a new wash with TBS, pH 7.6, sections were incubated with streptavidin

conjugated to alkaline phosphatase (Dako REAL™, cod. K5005, Dako cytometry, Glostrup, Denmark) at room temperature for 30 min. A red chromogen solution was prepared as indicated by Dako REAL™ data sheet and used as enzyme substrate according to Cordell et al.⁸⁴ Finally, each section was counter-stained in Mayer's Hematoxylin solution and cover slipped using Vectamount (Vector Laboratories). Monoclonal antibodies used in the study were:

- to determine malignant T cells: CD2 (mouse, AB75, Dako), CD3 (polyclonal rabbit, Dako), CD4 (mouse, 4B12, Dako), CD5 (mouse, 4C7, Novocastra), CD7 (mouse, CBC.37, Dako), CD8 (mouse, C8/144b, Dako), CD26 (mouse, DPPIV, R&D Systems), CD45RA (mouse, 4KB5, Dako), CD45RO (mouse, UCHL1, Dako), PD1 (mouse, NAT105, Abcam),
- for TCM subset: CD27 (rabbit, EPR8569, Abcam), CD62L (rabbit, Abcam), CCR7 (rabbit, Y59, Abcam),
- for TRM subset: CD69 (mouse, 15B5G2, Novus Biologicals), CD103 (rabbit, EPR4166(2), Abcam),
- for Th1 subset: T-BET (mouse, 4B10, Santa Cruz Biotechnology), CXCR3/CD183 (rabbit, Gennova)
- for Th2 subset and skin-homing markers: CCR4/CD194 (rabbit, Abcam), CLA/CD162 (mouse, SELPLG/PSGL-LD/62, LSBIO), GATA3 (mouse, L50-823, Biolegend).

3.1.3 Flow cytometry on peripheral blood

Fresh peripheral blood from patient samples were collected in lithium heparine tubes. For flow cytometry T cells evaluation, a 10-colors panel of anti-human fluorochrome-conjugated monoclonal antibodies were employed. All monoclonal antibodies, fluorochromes and clones used are reported in table 4 (BD Biosciences, San Jose, CA). Each antibody was properly titrated for a given staining of 100 µL volume of whole blood and the optimal concentration was determined by comparison with the isotype matched controls. After the cell staining, erythrocytes were lysed with NH₄Cl lysis buffer (Pharmlyse, BD Biosciences). Following the

washing with PBS 1X buffer, samples were acquired using a BD FACS Aria SORP cell sorter equipped with five lasers: 355 nm UV laser, 405 nm violet laser, 488 nm blue laser, 561 nm yellow-green laser and 647 nm red laser. Acquisition and analysis were ruled out following the exclusion of cell doublets and the stopping gate criterion was set at 50,000 lymphocyte events in the forward scatter (FSC) versus side scatter (SSC) dot plot. Data were analyzed using FACSDiva (ver. 8.0 BD).

moAb	Clone	Color
CD3	SK7	APCH7
CD4	SK3	BUV 661
CD7	M-T701	V450
CD8	RPA-T8	BUV 395
CD26	L272	BB700
CD45	2D1	APCH7
CD62L	DREG-56	PECy7
CD162	HECA-452	FITC
CCR4	1G1	BV786
CCR7	150503	APC
CXCR3	1C6/CXCR3	PE

Table 4. Monoclonal antibodies, fluorochromes and clones used for flow cytometry analysis (BD Biosciences, San Jose, CA)

3.2 GENE EXPRESSION PROFILING

3.2.1 Patient selection

For this second phase of the project, based on gene expression analysis on RNA on paraffin-embedded skin specimens, we decided to increase the number of patients selected in order to obtain a significant number of cases to analyze. So, we selected 95 patients affected by MF in all stages and SS from the database of the UOC Dermatologia, Fondazione IRCCS Ca' Granda – Ospedale Maggiore Policlinico, characterized by the availability of the paraffin-embedded skin biopsies. MF and SS previously untreated for CTCL were collected. Patients studied in the first phase of the project were included. Patients were staged according to the 2007 MF and SS staging revision.⁷ We collected demographic data and clinical course. The date of the last follow-up examination and the cause of death were confirmed from the clinical records. The patients' families and physicians were queried with regard to the cause of death, whenever possible.

About SS patients, we divided them in indolent and aggressive based on clinical course: slowly growing erythroderma, low tumor burden in the peripheral blood with stable lymphocytosis during clinical course without the requirement of high-grade chemotherapy or allogeneic stem-cell transplant.

3.2.2 Total RNA extraction and NanoString nCounter Assay

Total RNA was extracted from 3 sections of 15- μ m of each FFPE sample using RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Invitrogen). Yield of extracted RNA was assessed using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). RNA quality was scored according to DV200 value (% of RNA fragments >200 nucleotides) with the Agilent RNA 6000 Nano Chip Kit (BioAnalyzer, Agilent Technologies); only the samples with a DV200 value >20% passed the QC step.

Gene expression analysis was performed at the Hemato-Lympho-Pathology Lab, European Institute of Oncology (IEO) in Milan (Prof. S. Pileri). Gene expression analysis was measured

by the NanoString nCounter Analysis System (NanoString Technologies) with nCounter PanCancer Immune Profiling Panel that performs multiplex gene expression analysis with 770 genes from 14 different immune cell types, common checkpoint inhibitors, CT antigens, and genes covering both the adaptive and innate immune response. (Table 5)

The Nanostring system computes the relative abundance of each mRNA transcript of interest, through a multiplexed hybridization assay and digital readouts of fluorescent barcoded probes that are hybridized to each transcript. The nCounter CodeSet containing capture and reporter probes was hybridized to 400 ng of total RNA for 20 hours at 65 °C, according to the manufacturer's instructions. Hybridized samples were loaded into the nCounter Prep Station for post-hybridization processing. Target mRNA was assessed with nCounter Digital Analyzer, using maximum scan resolution.

IMMUNE CELL TYPES				
	Cell Type	Description	Panel Genes	
Adaptive Immune Response	B Cells	Perform several roles, including generating and presenting antibodies, cytokine production, and lymphoid tissue organization.	BLK, CD19, CR2 (CD21), HLA-DQB, MS4A1 (CD20), TNFRSF17 (CD269)	
	T Cells	Play a central role in immunity and distinguished from other lymphocytes (e.g., B cells) by the presence of a T cell receptor (TCR) on the cell surface.	CD2, CD3E, CD3G, CD6	
	Helper T Cells		A subset of CD3+CD4+ effector T cells that secrete cytokines with different activities.	ANP32B (APRIL), BATF, NUP107, CD28 (CD28), ICOS (CD278)
		T _H 1	Produce IL-2 and IFN γ and promote cellular immunity by acting on CD8+ cytotoxic T cells, NK cells and macrophages.	CD38, CSF2 (GM-CSF), IFNG, IL12RB2, LTA, CTLA4 (CD152), TXB21, STAT4
		T _H 2	Produce IL-4, IL-5 and IL-13 and promote humoral immunity by acting on B cells.	CXCR6 (CD186), GATA3, IL26, LAIR2 (CD306), PMCH, SMAD2, STAT6
		T _H 17	Produce IL-17A, IL-17F, IL-21 and IL-22 and promote anti-microbial inflammation.	IL17A, IL17RA (CD217), RORC
		T _{FH}	Trigger the formation and maintenance of germinal centers through the expression of CD40L and the secretion of IL-21 and IL-4, thereby playing a critical role in mediating the selection and survival of B cells.	CXCL13, MAF, PDCD1 (CD279), BCL6
		Regulatory T Cells (T _{reg})	CD3+CD4+ T cells that inhibit effector B and T cells and play a central role in suppression of autoimmune responses.	FOXP3
	Memory T Cells	T _{cm} (central memory)	Educated T cells that rapidly respond to antigen. Central memory T cells express L-selectin and CCR7; they secrete IL-2, but not IFN γ or IL-4.	ATM, DOCK9, NEFL, REPS1, USP9Y
		T _{em} (effector memory)	Educated T cells that rapidly respond to antigen. Effector memory T cells do not express L-selectin or CCR7; they secrete effector cytokines like IFN γ and IL-4.	AKT3, CCR2 (CD192), EWSR1 (EWS), LTK, NFATC4
Innate Immune Response	Cytotoxic (CD8) T Cells	Effector T cells with cytotoxic granules that interact with target cells expressing cognate antigen and promote apoptosis of target cells.	CD8A (CD8), CD8B (CD8B), FLT3LG, GZMM (MET1), PRF1	
	Gamma Delta T Cells (T $\gamma\delta$)	Express surface antigen recognition complex type 2 and represent a small percentage of the peripheral T cell population. Functions span innate and adaptive immune responses, including direct cytotoxicity and establishment of memory phenotypes.	CD160, FEZ1, TARP (TCRG)	
	Natural Killer (NK) Cells		Provide a rapid cytotoxic response to virally infected cells and tumors. These cells also play a role in the adaptive immune response by readily adjusting to the immediate environment and formulating antigen-specific immunological memory.	BCL2, FUT5, NCR1 (CD335), ZNF205
		CD56 _{bright}	Constitute the majority of NK cells in secondary lymphoid tissues. Abundant cytokine producers and weakly cytotoxic before activation.	FOXJ1, MPPED1, PLA2G6, RRAD
		CD56 _{dim}	Constitute the majority of NK cells in the periphery and are more cytotoxic than CD56 _{bright} cells.	GTF3C1, GZMB, IL21R (CD360)
	Dendritic Cells	Conventional (Myeloid) Dendritic Cells (DC)	Cells that process antigen material and present it on the cell surface to T cells, thereby acting as messengers between the innate and adaptive immune systems.	CCL13, CCL17, CCL22 (MDC), CD209 (CD209), HSD11B1
		IDC (Immature)	Play a critical role in initiating tumor immunity. Tumor cells can exploit the functional roles of IDCs for tumor progression via release of soluble factors such as VEGF.	CD1A, CD1B, CD1E, F13A1, SYT17
		aDC (activated)	Promote the induction of the adaptive immune response by presenting captured antigen to naive T cells.	CCL1, EBI3, IDO1 (INDO), LAMP3 (CD208), OAS3
		Plasmacytoid Dendritic Cells (pDC)	Similar in appearance to plasma cells and share many characteristics with myeloid dendritic cells. These cells can produce high levels of IFN α .	IL3RA (CD123)
		Macrophages	Scavengers of dead or dying cells and cellular debris. Macrophages have roles in innate immunity by secreting pro-inflammatory and anti-inflammatory cytokines.	APOE, CCL7 (FIC), CD68, CHIT1, CXCL5, MARCO, MSR1 (CD204)
Granulocytes	Mast Cells	Granulocytes that can influence tumor cell proliferation and invasion and promote organization of the tumor microenvironment by modulating the immune response.	CMA1, CTSG, KIT (CD117), MS4A2, PRG2, TPSAB1	
	Neutrophils	Phagocytic granulocytes that act as first-responders and migrate towards a site of inflammation. Typically a hallmark of acute inflammation.	CSF3R (CD114), FPR2, MME (CD10)	
	Eosinophils	White blood cells responsible for combating multicellular parasites and some types of infections.	CCR3 (CD193), IL5RA (CD125), PTGDR2 (CD294; GPR44), SMPD3, THBS1	

Table 5. Immune cell types and populations involved in the response to cancer and corresponding population-specific genes included in the PanCancer Immune Profiling Panel (from: www.nanosttring.com)

3.2.3 NanoString data processing and statistical analysis

All the statistical analyses were conducted within the statistical environment R v4.0.2

(<https://www.r-project.org/>)

3.2.3.1 Clustering and Principal component analysis (PCA)

Pheatmap package was used to perform and display hierarchical cluster analysis. Principal component analysis (PCA) was implemented by prcomp function from the stats package, while ggplot2 package was used to visualize the results.

3.2.3.2 Identification of differentially expressed genes (DEGs)

Nanostring mRNA counts were first log2 transformed and normalized with the NanoStringNorm package. Geometric mean of reference genes was used to remove the technique variation and background expression. Differential analysis was conducted with linear models in the limma package from Bioconductor repository. Comparison among different groups were carried out using linear contrasts within the linear model frame work, moderated t statistics was applied to determine the differences in the gene expression levels between groups with empirical Bayes approach. The Benjamini-Hochberg procedure was then implemented to adjust the p-values to control the false discovery rates at 5%.

3.2.3.3 Kaplan–Meier (KM) survival analysis and univariable Cox regression

Association of patient's survival time and gene expression was assessed using survival package, including Kaplan–Meier (KM) survival analysis and univariable Cox regression. The cutoff criterion was set to KM and cox p-values < 0.05 for screening of the survival-related gene at overall survival.

3.2.3.4 Gene expression signature-based prognostic risk score

Genes resulting from KM and Cox regression analysis were used to create a prognostic scoring system based on this formula:

$$\sum_{i=1}^{n.genes} (gene\ i\ Cox\ regression\ coefficient) \times (gene\ i\ expression\ level)$$

The patients were ranked by their prognostic scores and divided into two cohorts (high/low). Kaplan-Meier plots were constructed, and a long-rank test was implemented to evaluate differences in OS for MF and SS patients.

4. RESULTS

4.1 PHENOTYPE STUDY

Fifteen patients have been included in this part of the study with the following characteristics:

- 5 patients affected by early-stage MF (IA-IIA)
- 3 patients with advanced MF (2 erythrodermic MF, 1 tumoral MF)
- 5 patients with classical SS
- 2 patients with non-erythrodermic SS (clinic-pathological evidence of MF on the skin and presence of B2 stage in the blood as defined by 2007 revision of the staging.⁷

We decided to analyze peripheral blood of advanced MF and SS, because, based on current knowledge, in early-stage MF the probability to find circulating atypical MF cells is extremely low. Peripheral blood samples have been analyzed by flow cytometry, sorting CD4+CD7- T cells. In 2 cases of SS characterized by CD4+CD7+ Sézary cells, we sorted CD4+CD7+ cells.

Tables 6a e 6b include immunohistochemical features of our patients.

Figure 6 shows immunohistochemical staining in some patients.

Table 7 shows flow cytometry results.

Figure 8-10 shows dot plots of blood CD4+ SC of some patients.

The skin infiltrate was characterized by CD3+CD4+ T cells in all cases but one IA MF which was CD3+CD8+. Skin-homing markers CCR4 and CLA were positive in almost all cases, except for 2 MF cases negative for CCR4. Ten cases of 15 studied (67%) showed a typical memory phenotype with the positivity of CD45RO. About the other 5 patients (1 IIA-MF, 4 IVA1-SS), a co-expression of CD45RA and CD62L in the skin and blood samples, corresponding to a TN phenotype, was seen in all cases except for one SS, characterized by negativity of the CD62L, like in terminally differentiated effector memory T cell (T EMRA). This last data was not correlated with the same phenotype in the blood.

About MF, of the 3 patients with stage IB MF (localized patches and plaques), two of them revealed a TRM phenotype of the neoplastic cells in the skin, because of positivity of CD69 and CD103 and negativity of CCR7 and CD62L. In the third of them, T cells aberrantly showed expression of CD69 and also CD62L. One patient with early IIA MF (pt 3) showed expression of CD69 and CD103 (TRM markers) but also a partial expression of CCR7 with negativity of CD62L, as TMM phenotype. Erythrodermic MF (stage III) and tumoral MF (stage IIB) patients were characterized by an infiltrate with a TMM phenotype (CCR7+CD62L-) but also expressed TRM markers (CD69 and CD103). This data did not correlate with immunophenotype of sorted CD4+ cells in the peripheral blood: the count of CCR7+CD62L- cells were a smaller number compared to CCR7-CD62L+ group, which were the majority.

In contrast, patients affected by SS with or without erythroderma showed in all cases the expression of CCR7+ CD62L+ in the skin and in the blood, except for one case who lacked the CCR7 and the patient previously reported with the positivity of CD45RA but the negativity of CCR7 and CD62L. The last one was the CD7+ variant. The 3 CD45RO+SS patients showed a typical TCM phenotype characterized by co-expression of CD27+CCR7+CD62L+.

In case 9, we were able to perform an immunophenotypic study in two different stages of disease (early MF before and during SS progression with B2 blood involvement). In both stages, skin samples were characterized by a typical TCM phenotype which correlated with the same phenotype of the blood cells. (Figure 7)

Expression of PD1 was strongly positive in 5 out of 7 SS patients, as expected, and partially expressed in 2 IIA MF and IIB MF.

Neoplastic T cells in MF mainly had a Th1 phenotype, with the expression of T-bet in all early stages and also in the two advanced stages; instead, SS patients showed a Th2 phenotype in all cases through the expression of GATA3.

Pts	MF/SS	stage	CD2	CD3	CD4	CD8	CD7	CD26	PD1	CD45RA	CD45RO
1	MF	III	+	+	+	-	+/-	-	-	-	+
2	MF	IIA	+	+	+	-	+	+	+	+	-
3	MF	IIA	+	+	+	-	-	-	+	-	+
4	MF	IB	+	+	+	-	-	+	-	-	+
5	MF	IB	+	+	+	-	-	+	-	-	+
6	MF	IA	+	+	-	+	-	-	-	-	+
7	MF	IIB	+	+	+	-	+/-	-	+	-	+
8	SS	IVA1	+	+	+	-	-	-	+	+	-
9	MF	IIA	+	+	+	-	+/-	-	+	-	+
	aSS	IVA1	+	+	+	-	+/-	-	+	-	+
10	SS	IVA1	+	+	+	-	-	-	-	+	-
11	SS	IVA1	+	+	+	-	+	-	-	+	-
12	aSS	IVA1	+	+	+	-	+/-	-	+	+	-
13	SS	IVA1	+	+	+	-	+	-	+	-	+
14	MF	III	+	+	+	-	+	-	-	-	+
15	SS	IVA1	+	+	+	-	-	-	+	-	+

Table 6a. Immunohistochemical features of MF and SS patients.

Pts	MF/SS	stage	CD27	CD62L	CCR7	CD69	CD103	Tbet	CXCR3	GATA3	CCR4	CLA
1	MF	III	+	-	+	+/-	+	+	-	-	+	+
2	MF	IIA	+	+/-	+	+	+	+	-	-	+	+
3	MF	IIA	+	-	+	+	+	-	+	+	+	+
4	MF	IB	-	-	-	+	+	+	-	+	+	+
5	MF	IB	-	+	-	+	+	+	-	-	+	+
6	MF	IA	+	-	-	+	+	+	+	-	-	+
7	MF	IIB	+	-	+	+	+	+	-	+	-	+
8	SS	IVA1	+	+	-	+	+	-	-	+	+	+
9	MF	IIA	+	+	+	-	-	+	-	+	+	+
	aSS	IVA1	+	+	+	-	-	-	-	+	+	+
10	SS	IVA1	+	+	+	-	-	-	-	+	+	+
11	SS	IVA1	+	-	-	-	-	-	-	+	+	+
12	aSS	IVA1	+	+	+	-	++	-	-	+	+	+
13	SS	IVA1	+	+	+	-	-	-	-	+	+	+
14	MF	III	+	-	+	+/-	+	-	-	+	+	+
15	SS	IVA1	+	+	+	-	-	-	-	+	+	+

Table 6b. Immunohistochemical features of MF and SS patients.

Pt	Dx	cells/ul	% on lymph		% on CD3+CD4+CD7-						
		Lymph	CD4+	CD4+ CD7-	CCR7+ CD62L-	CCR7+ CD62L+	CCR7- CD62L+	CXCR3+	CLA+	CD26+	CCR4+
1	MF	2840	65,2	24,5	0,4	6,3	81,2	72,7	30,2	2,9	100,0
7	MF	690	41,7	10,5	4,3	19,7	54,5	58,9	47,4	56,3	100,0
8	SS	1670	39,6	3,9	9,8	46,3	22,8	64,1	14,9	44,6	100,0
9	SS	2420	92,8	87,3	23,4	73,0	1,9	14,0	0,2	5,2	76,0
12	SS	4570	91,8	73,4	0,9	97,2	1,7	1,3	2,3	2,7	100,0
14	MF	1200	36,7	11,1	3,0	26,8	26,6	82,8	4,7	41,9	100,0

				CD4+ CD7+	% on CD3+CD4+CD7+						
11	SS	1500	80,2	71,4	3,0	31,8	34,2	37,6	6,6	19,6	100,0
13	SS	1820	63,5	86,0	5,9	72,4	8,9	15,0	8,9	19,6	100,0

Table 7. Flow cytometry of MF and SS patients

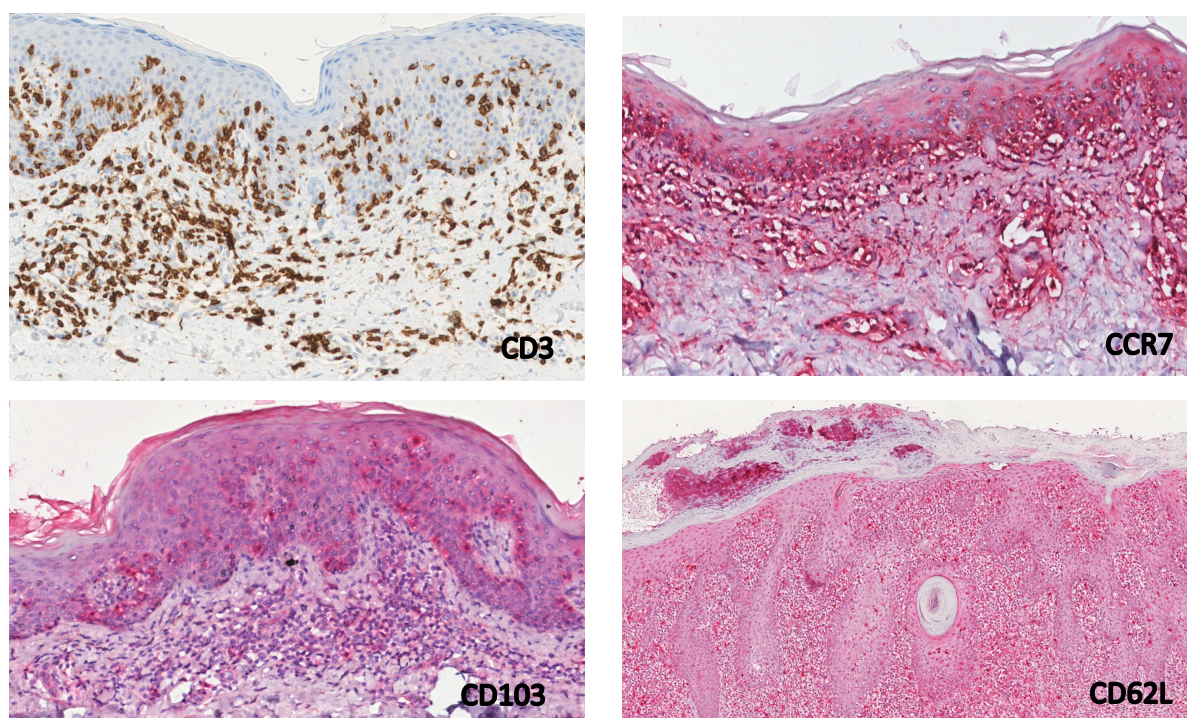


Figure 6. Immunohistochemical staining in some patients of the projects

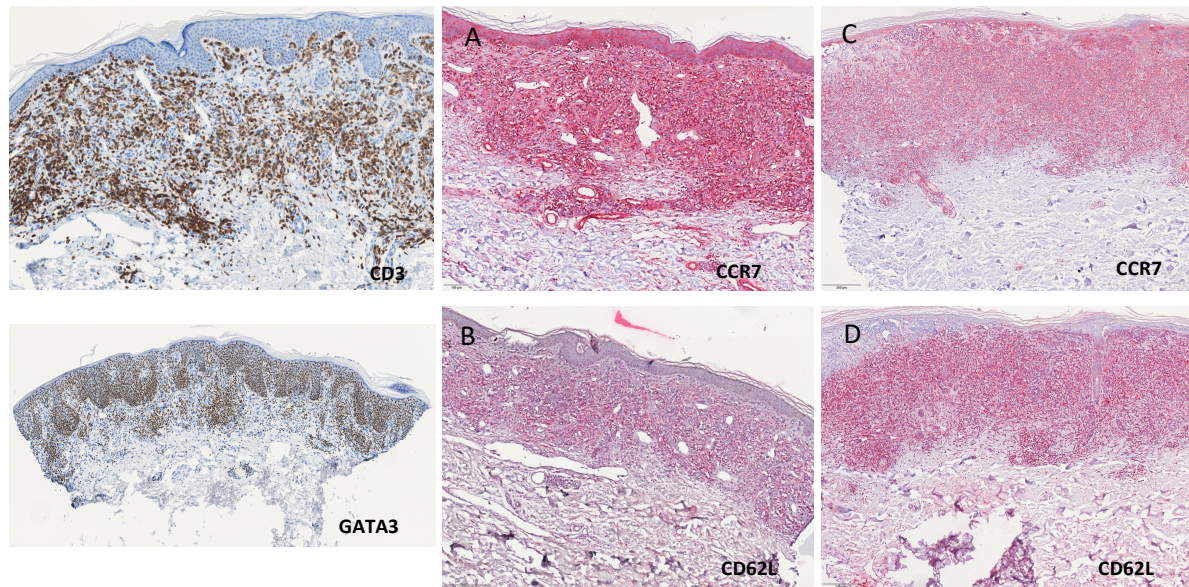


Figure 7. Immunohistochemical staining of patient 9. A,B: CCR7 and CD62L expression in SS skin sample; C,D: CCR7 and CD62L expression in MF sample.

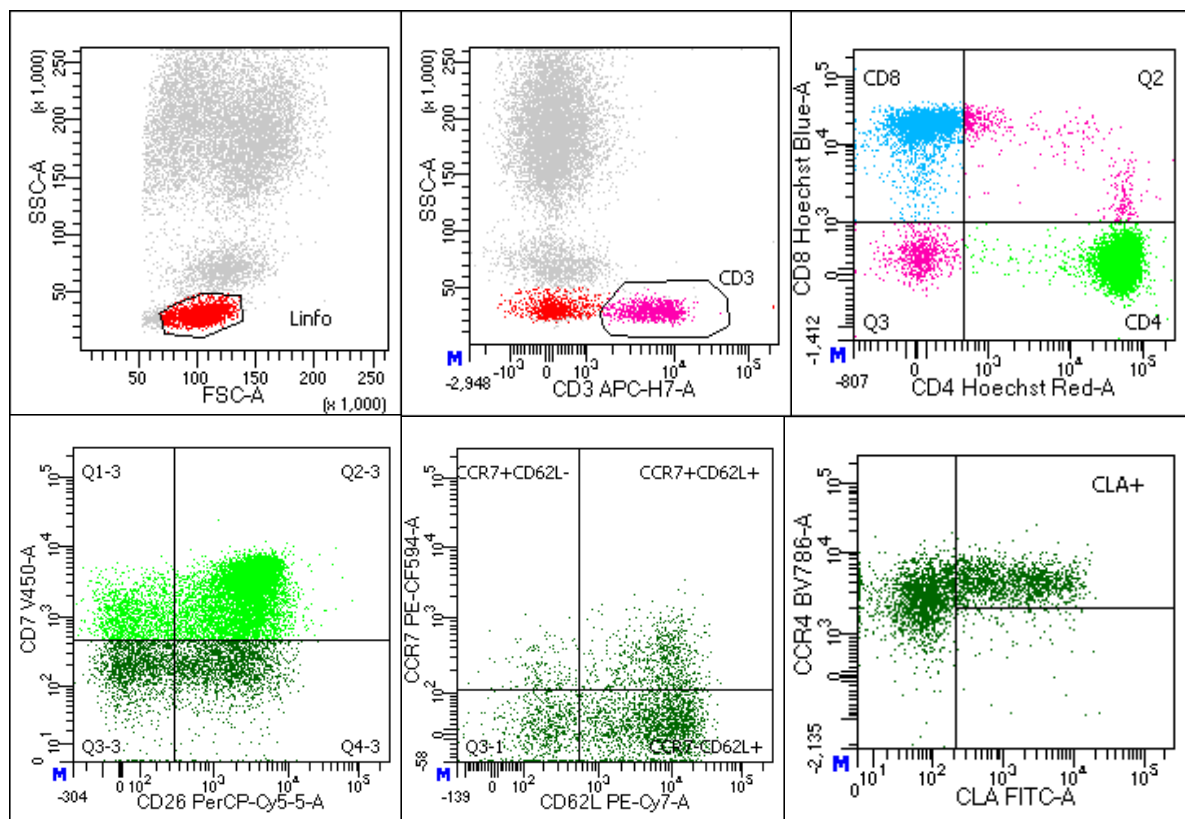


Figure 8. Dot plots of blood CD4+ SC of patient 7 (IIB MF)

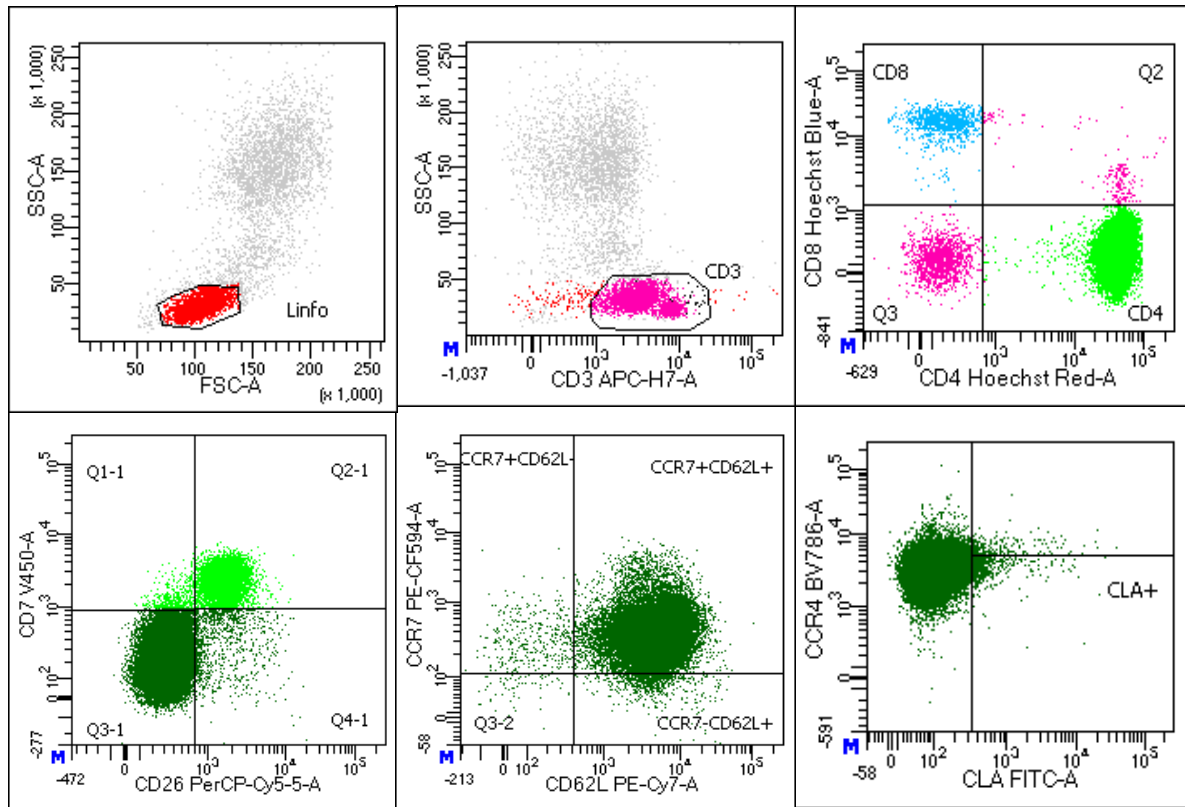


Figure 9. Dot plots of blood CD4+ SC of patient 12 (atypical SS)

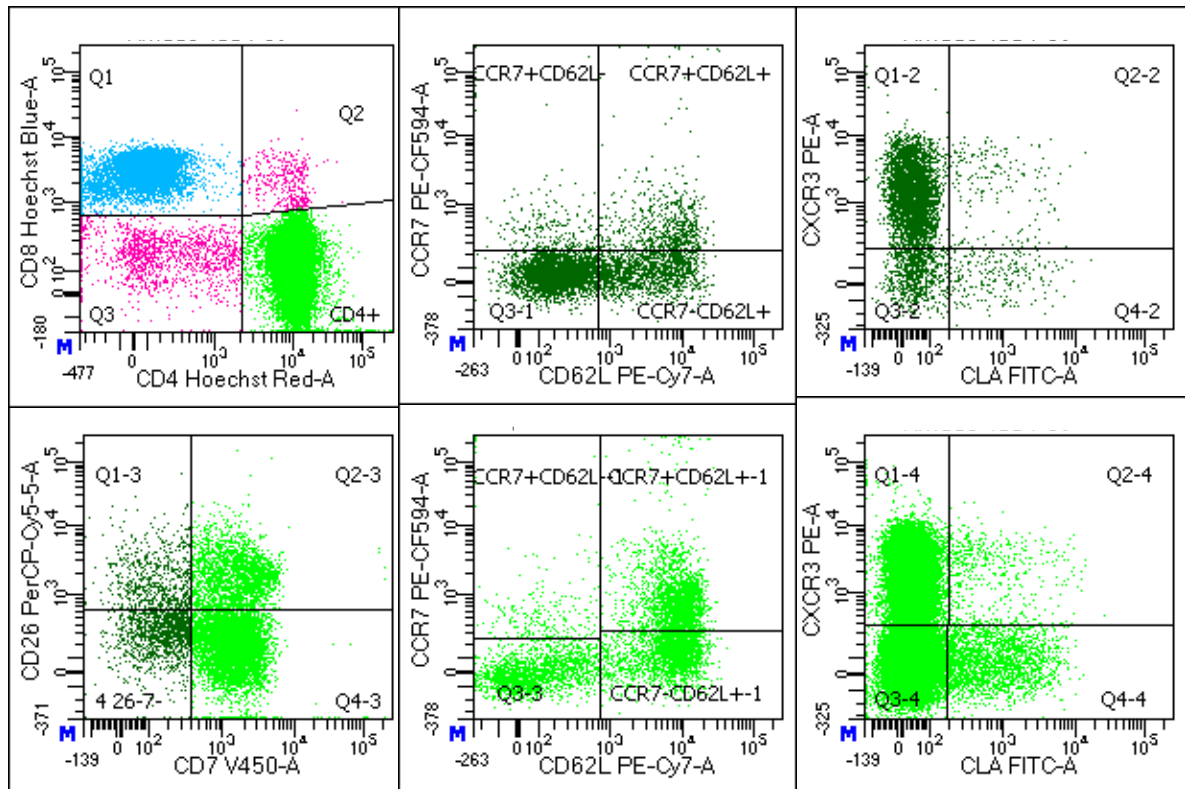


Figure 10. Dot plots of blood CD4+ SC of patient 11 (CD7+ SS)

4.2 GENE EXPRESSION PROFILING

From a total of 95 patients initially identified, 87 of these were analyzed. We only removed 8 samples due to insufficient concentration of RNA. We included 36 SS patients (31 stage IVA1, 4 IVA2, 1 IVB) and 51 MF patients, divided into 26 early-stages (2 IA, 13 IB, 11 IIA) and 25 advanced stages (16 IIB, 2 IVA1, 2 IVA2).

PCA based on expression profiles of all genes was performed, but only a homogeneous group was found, without any evidence of clustering between MF and SS. So, we decided to study separately the two entities.

4.2.1 Mycosis fungoides

4.2.1.1 Identification of differentially expressed genes (DEGs)

PCA implemented only on MF dataset revealed a quite homogenous group of samples and this data was confirmed by an unsupervised hierarchical clustering analysis. (Figure 11)

We next searched for the differentially expressed genes (DEGs) profiles, comparing advanced stages versus early stages. Twelve genes were highlighted as being statistically significant (p-value <0.01): 9 of them were upregulated in advanced stages (CCR3, PRAME, FPR2, PMCH, AMBP, TRL7, TNFRSF10C, CFI, HAVCR2) and 3 were downregulated (KLRB1, CD1B, CD5). (Table 8)

Performing an unsupervised hierarchical clustering on all those DEGs, there were 3 major clusters. Cluster 1 was well-defined and very homogenous, including 5 advanced patients (4 IIB and 1 III). Cluster 2 was more heterogeneous containing primarily advanced stages (IIB-IVA2) and only 6 early stages (1 IA, 2 IB, 3 IIA). Cluster 3 contained mainly early stages with only 4 advanced stages out of 24 samples. (Figure 12)

Gene Ontology (GO) enrichment analysis, by GSEA tools, of the 12 DEGs previously reported differentially expressed between advanced stage MF and early-stage MF, showed that those genes are significantly enriched in the regulation of the immune system, macrophage activation and Toll-like receptor (TLR) signaling (FDR <0.05). (Figure 13)

We also performed the KEGG pathways analysis of the 12 genes showing DEGs in MF, but, as a result, the 12-DEGs were not significantly represent in any KEGG pathways.

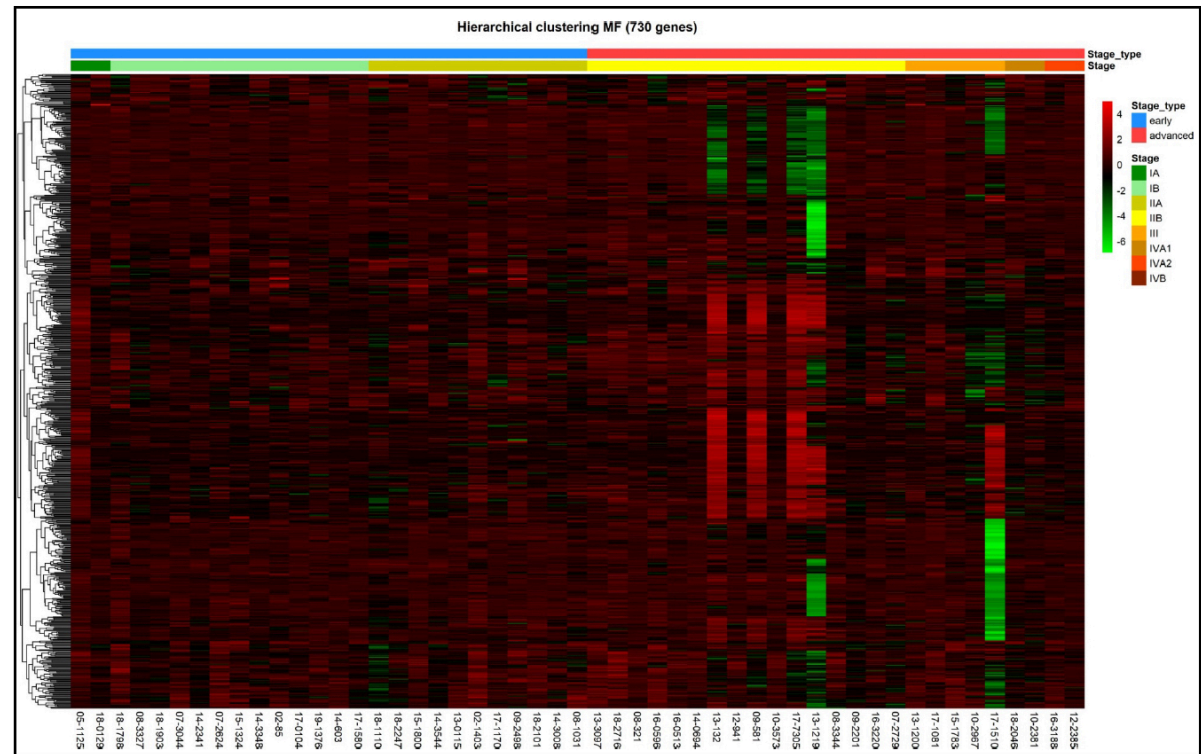


Figure 11. Mycosis fungoides. Hierarchical clustering on all 770 genes of PanCancer Immune Profiling Panel.

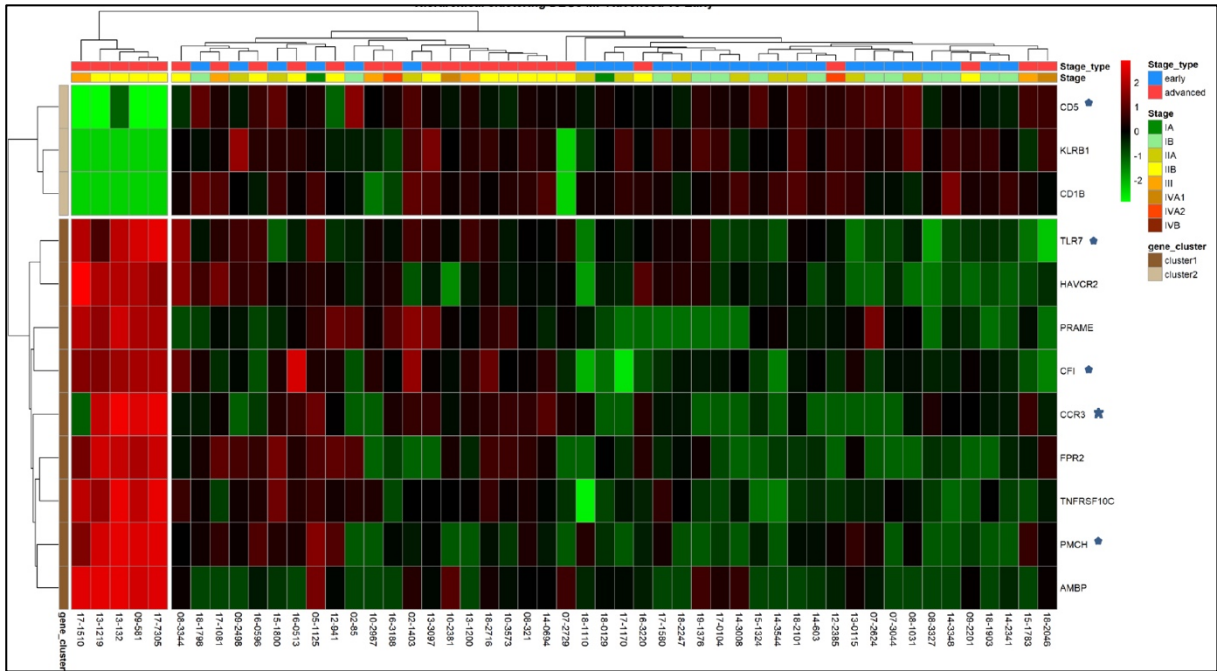


Figure 12. Mycosis fungoides. Hierarchical clustering on differential expressed genes between advanced versus early stages.

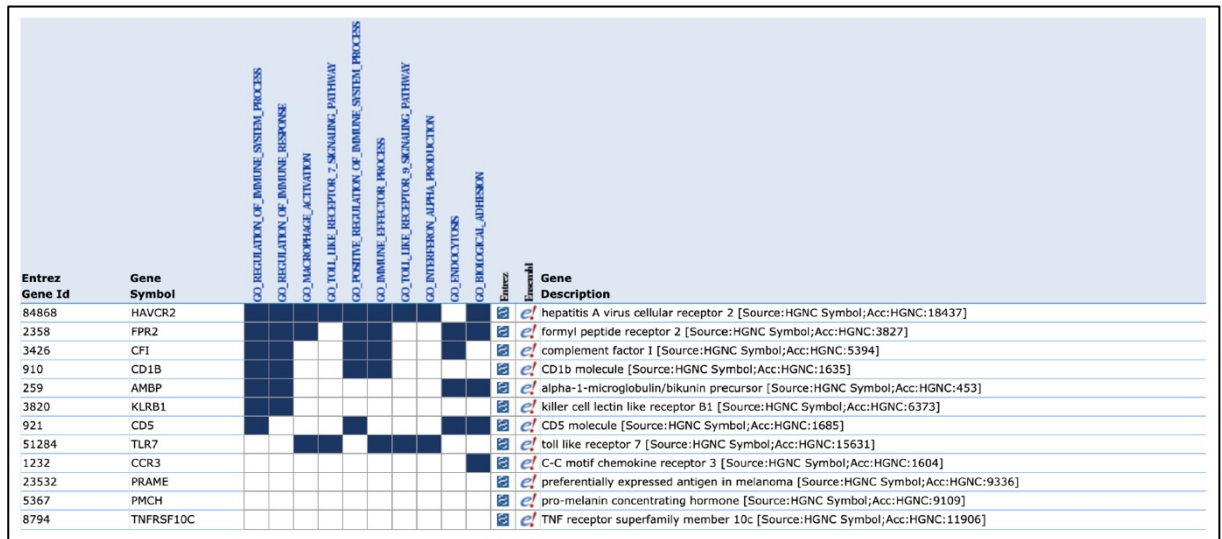


Figure 13. Mycosis fungoides. Gene Ontology analysis of 12 DEGs between advanced MF versus early MF.

Gene_name	logFC	AveExpr	P.Value	adj.P.Val
CCR3	2.352219	2.67251	0.000376	0.274495
PRAME *	2.126617	3.637494	0.005657	0.3072
FPR2	2.101933	3.193308	0.005406	0.3072
PMCH	2.046579	2.814594	0.00697	0.3072
AMBP	1.914559	2.105278	0.008215	0.3072
TLR7	1.278607	4.822729	0.006056	0.3072
TNFRSF10C	1.234925	4.679471	0.007559	0.3072
CFI	0.876832	6.577627	0.006326	0.3072
HAVCR2 *	0.683228	7.531608	0.00251	0.3072
KLRB1	-1.76968	5.521627	0.004289	0.3072
CD1B *	-2.12776	6.24202	0.002232	0.3072
CD5	-2.1692	7.564706	0.001817	0.3072

Table 8. Mycosis fungoides. Differential expression analysis between advanced stages and early stages showed 12 DEGs (9 upregulated and 3 downregulated) with a p-value <0.01

4.2.1.2 Gene expression prognostic signature

To further assess the importance of the 770 genes studied, MF patient cohort was divided into two groups based on the median expression for each gene. Subsequently, the effects of high or low expression levels on OS were examined using the Kaplan-Meier survival curve. A list of 39 genes was identified as significantly associated with OS (p-value <0.05). The hazard ratios (HRs) and p-values of those selected 39 genes were calculated through Cox regression model to discover statistically significant independent genes for predicting OS. This analysis revealed a 9-genes signature, which did not match with DEGs but showed a high significance from Kaplan-Meier survival analysis. The increased expression of the following genes is significantly associated to poor prognosis: CDK1 (HR=2.06), IL6ST (HR=1.49), CCR4 (HR=1.66), ITK (HR=1.78), NOS2A (HR=1.38), IL2RA (HR=2.06), LRRN3 (HR=1.39), DUSP4 (HR=1.74). Instead, the lower expression of CCL26 (HR=0.53, p-value 0.028) is significantly associated with poor prognosis.

A prognostic score was developed based on the incidence of each gene of the signature on overall survival and was defined as the linear combination of logarithmically transformed gene expression levels weighted by average Cox regression co-efficient. The prognostic scores were assigned for all patients and used to predict survival probability. According to this system, Kaplan-Meier analysis based on score of the 9-gene signature showed that patients with a high score of expression have a significantly poorer prognosis compared to patients with a low score (p-value < 0.05). Similarly, considering advanced versus early stages, the two groups with high score confirmed to have a statistically significant poorer prognosis compared to low score (p-value <0.05). This result is particularly evident in early stages, where the group with high score had the worst prognosis compared to the best prognosis of the low score group. (Figure 14)

KEGG pathway analysis, by GSEA tools, showed that the 9 genes with prognostic value are significantly enriched in cytokine and JAK-STAT signaling.

Kaplan Meier analysis was performed for each genes of the prognostic signature, evaluating high vs low expression but also considering advanced and early-stages classification. High expression of CDK1, IL6ST, CCR4, ITK, NOS2A, IL2RA, LRRN3 and DUSP4 was significantly associated to poor prognosis, even when samples were divided in advanced stage and early-stage, except for DUSP4 for whom the statistical significance was not present by stages. On the contrary, low expression of CCL26 correlated with a worse prognosis and this data was confirmed by survival analysis performed on advanced stages versus early stages. (Figures 15-23)

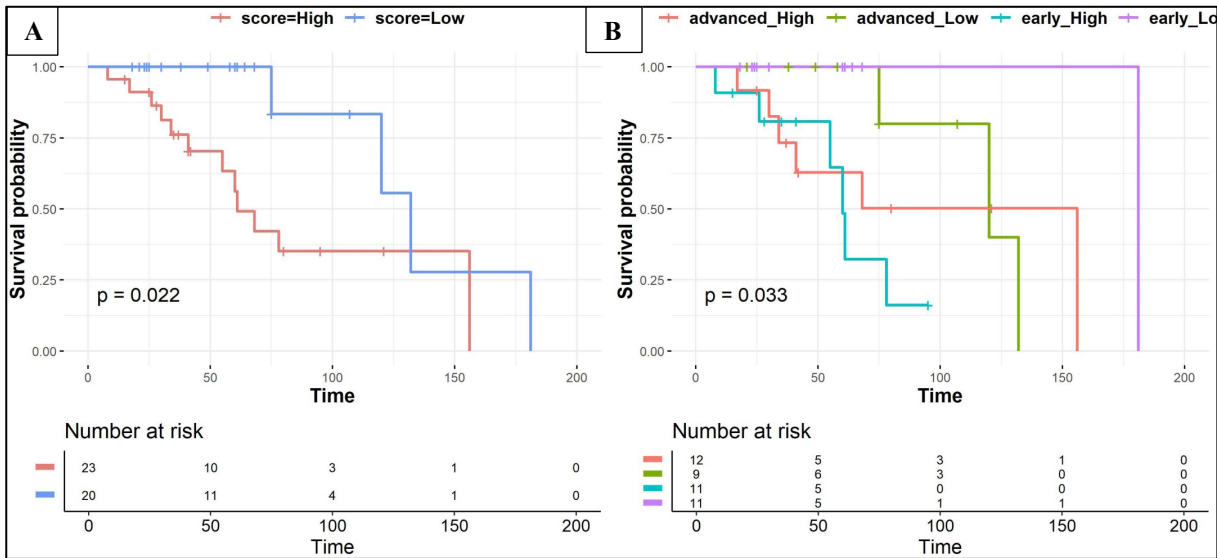


Figure 14. Mycosis fungoides. Prognostic score. A: Kaplan-Meier overall survival curves separated samples in high score with poor prognosis and low score with good prognosis. B: KM curves of advanced and early stages with high and low score.

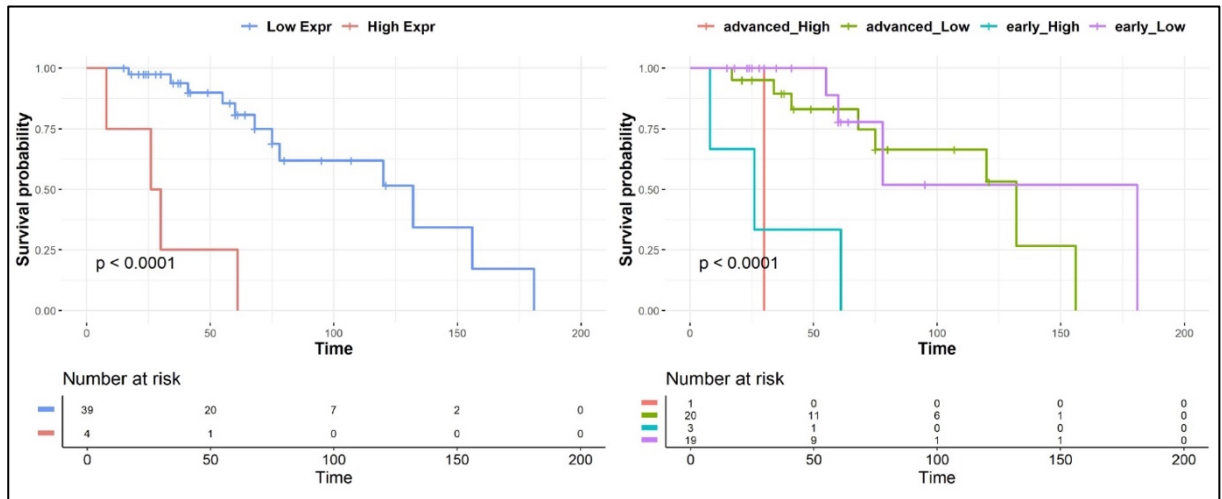


Figure 15. CDK1

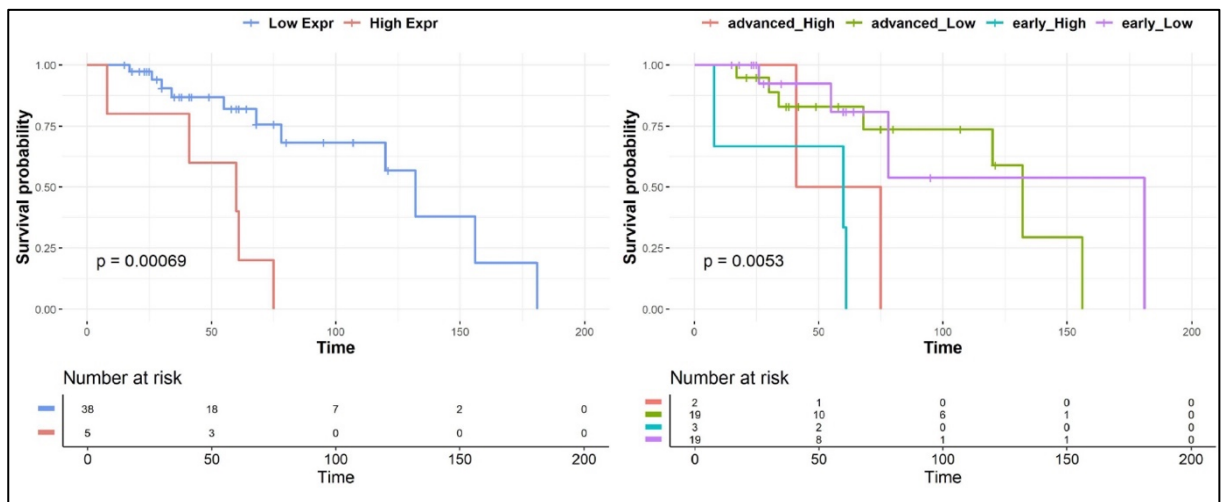


Figure 16. IL6ST

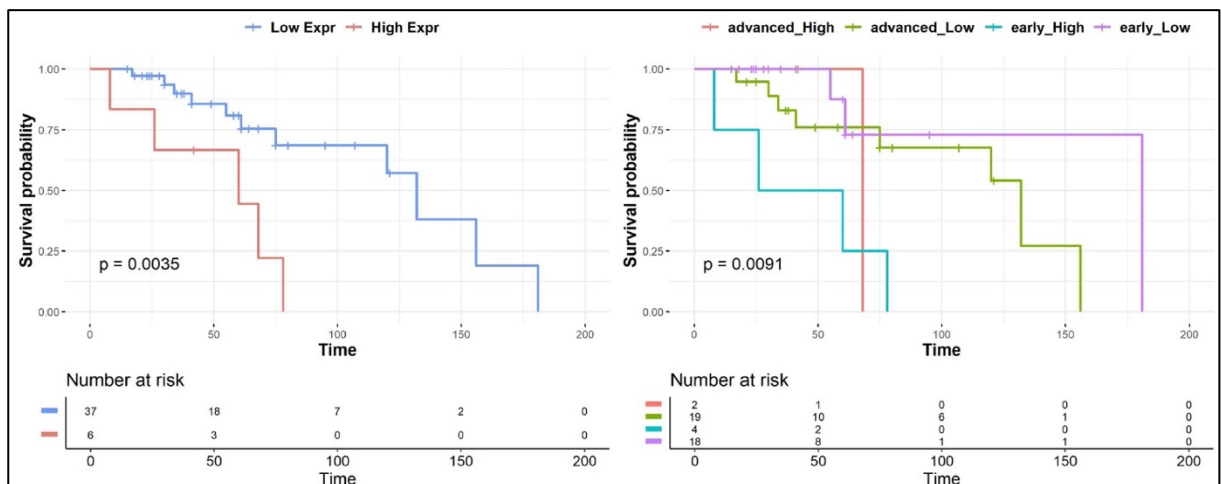


Figure 17. CCR4

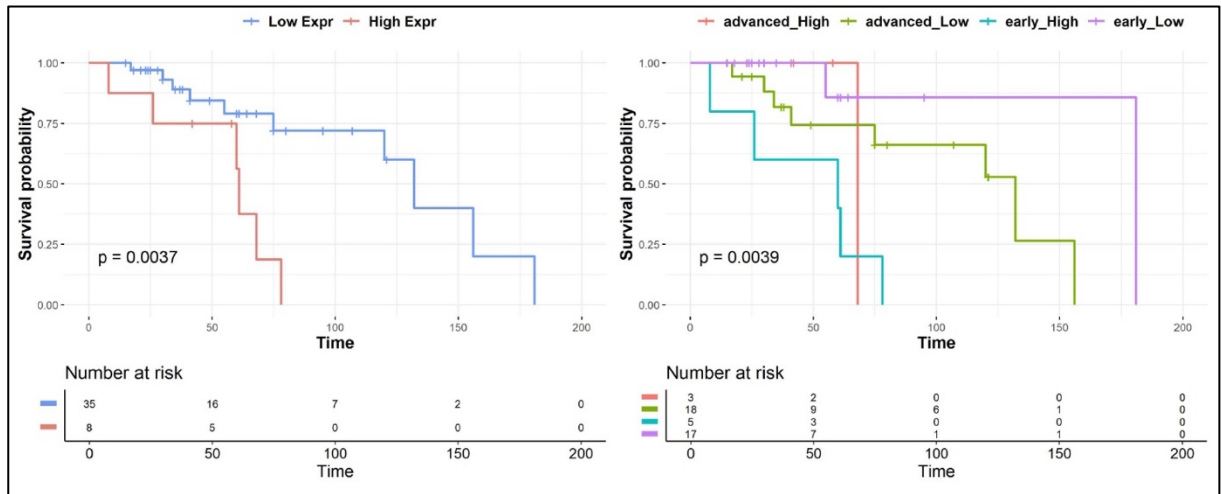


Figure 18. ITK

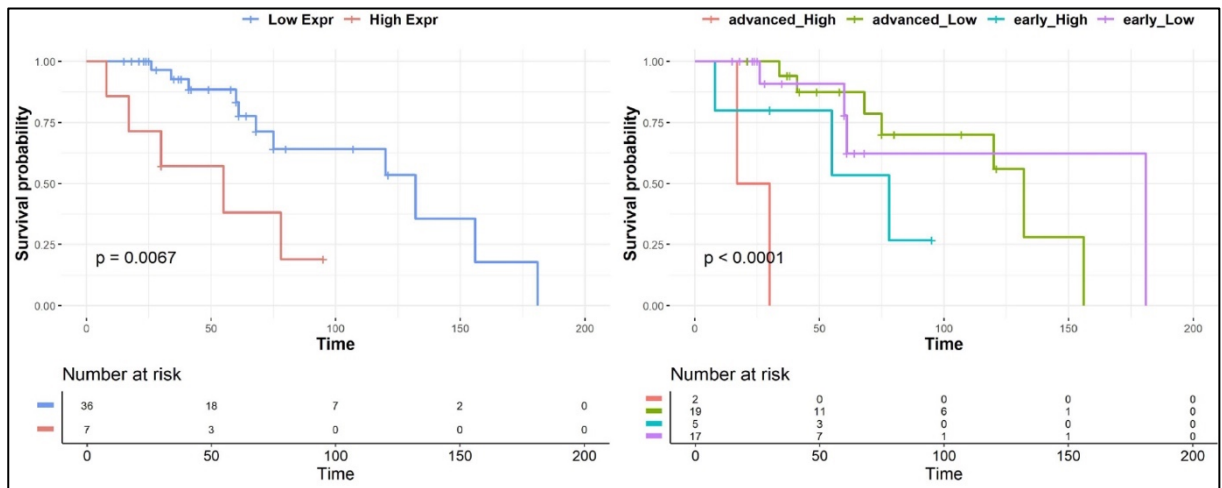


Figure 19. NOS2A

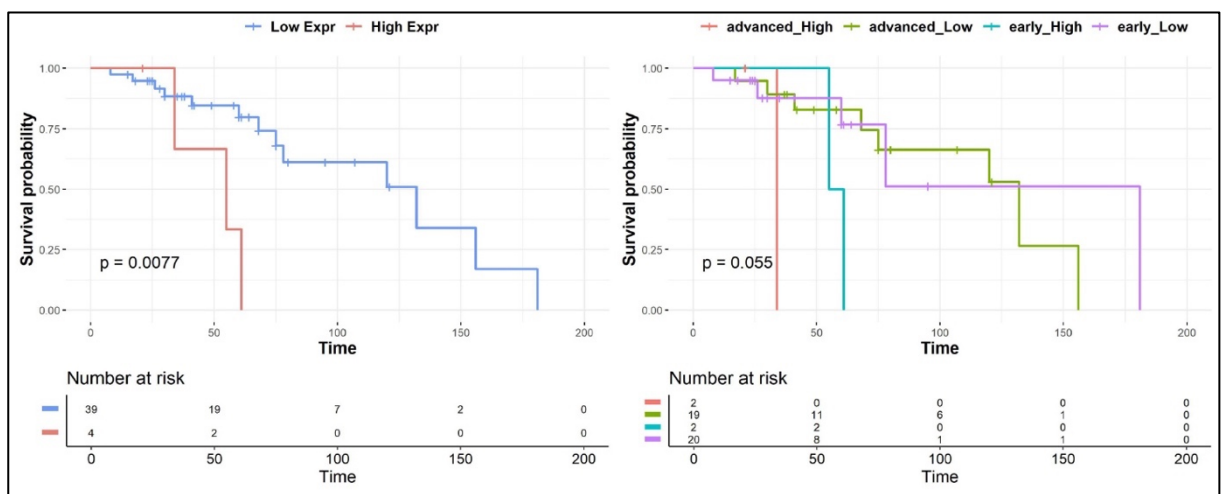


Figure 20. IL2RA

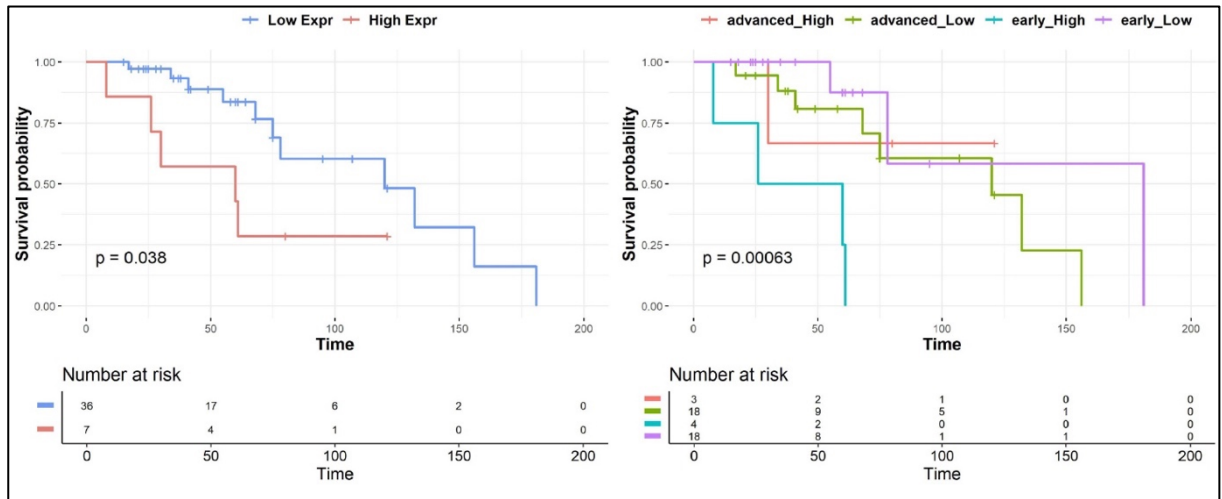


Figure 21. LRRN3

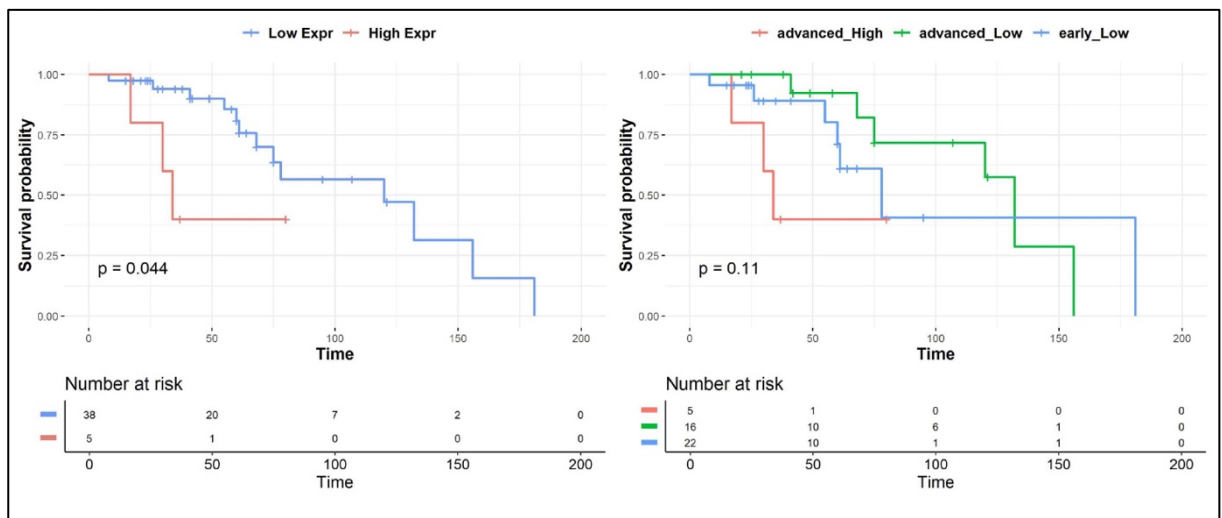


Figure 22. DUSP4

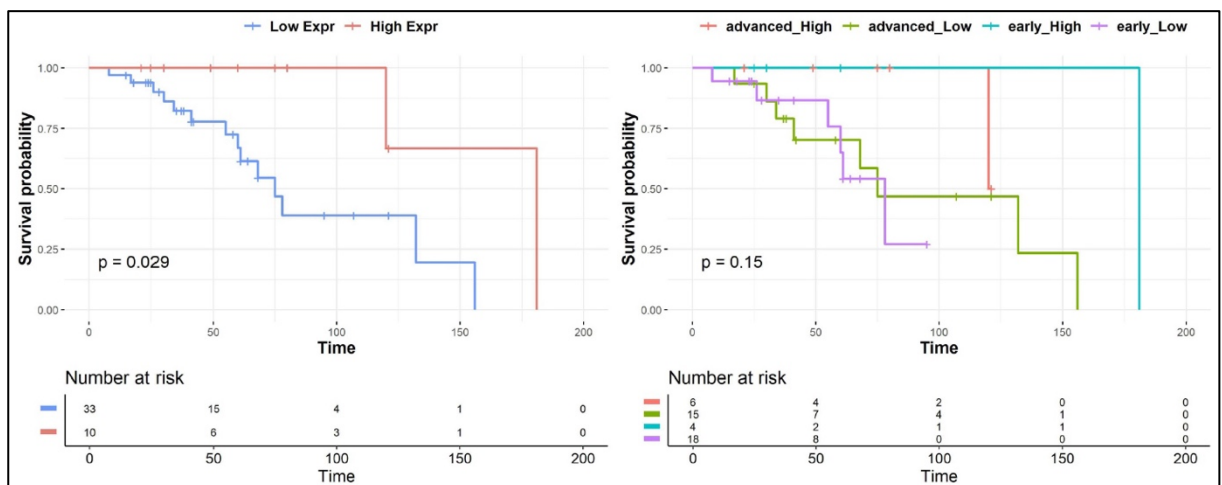


Figure 23. CCL26

4.2.2 Sézary syndrome

PCA showed a quite homogenous group of patients, except for 3 of them (1 IVA1 atypical SS, 1 IVA2 SS also affected by Sarcoma di Kaposi, and 1 IVA2 SS). This data was confirmed by an unsupervised hierarchical clustering analysis. (Figure 24)

As previously done for MF, samples were divided into two groups based on median expression for each gene (low/high). Survival analysis identified 58 genes significantly associated with OS (p-value <0.05). About them, Cox regression model resulted in evidence of 14-gene signature characterized by association of their high expression and poor prognosis. In particular, the genes identified were IL12A (HR=2.12), IL5RA (HR=2.80), IFNL2 (HR=2.21), NT5E (HR=1.97), IL18RAP (HR=1.89), ABCB1 (HR=1.74), CCL16 (HR=1.89), CCL1 (HR=1.95), IL22RA2 (HR=1.67), IFNA17 (HR=1.69), C9 (HR=1.79), CCR9 (HR=3.31), SPANXB1 (HR=1.62), TREM1 (HR=1.76).

This 14-gene signature was next used to calculate the prognostic score for OS, assigning the score to each patient as previously described. Kaplan-Meier analysis was used to determine significant differences in OS among different groups, showing that patients with a high score had a worse prognosis compared to patients with a low score. This prognostic correlation was confirmed evaluating the subgroups of SS with aggressive versus indolent course. In both subgroups, high score was significantly associated with poor prognosis compared to low score. (Figure 25) Looking at Kaplan-Meier analyses on single gene of the signature, group of high expression in indolent SS is not present for all genes, except for CCL1 and TREM1. About TREM1, it is the only one showing a not statistically significant difference between high and low expression in aggressive and early-stages. (Figures 26-39)

GO analysis, performed by GSEA tools on 14 genes associated with prognostic score, showed that these genes are significantly involved in defense response and Th1 cytokine production (FDR <0.05). KEGG pathway analysis of 14-gene signature was able to confirm a statistically significant involvement of these genes in JAK-STAT and TLR signaling. (Figures 40,41)

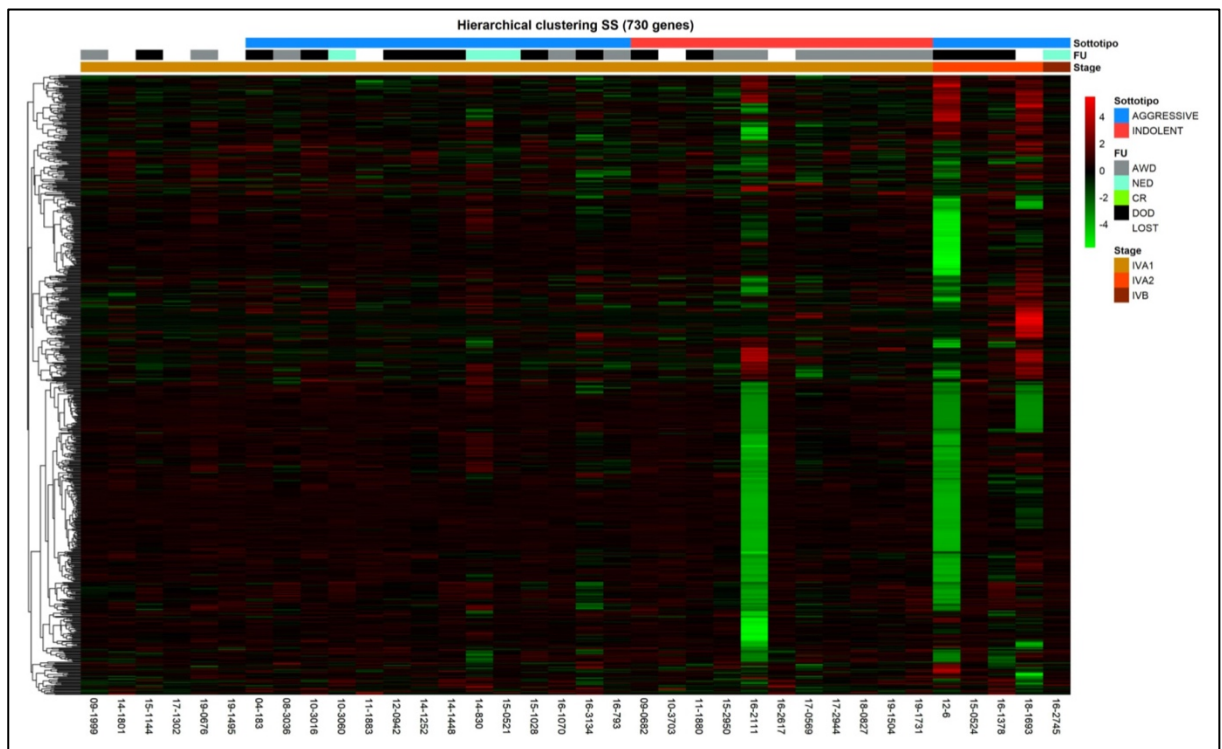


Figure 24. Sézary syndrome. Hierarchical clustering on all 770 genes of PanCancer Immune Profiling Panel.

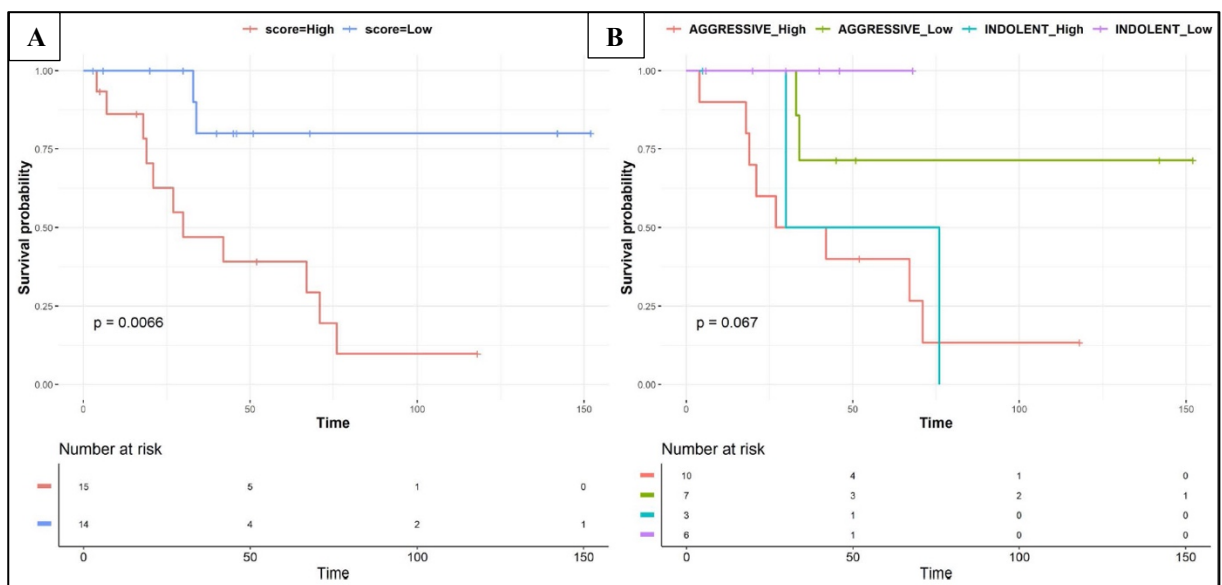


Figure 25. Sézary syndrome. Prognostic score. A: Kaplan-Meier overall survival curves separated samples in high score with poor prognosis and low score with good prognosis. B: KM curves of aggressive and indolent SS with high and low score.

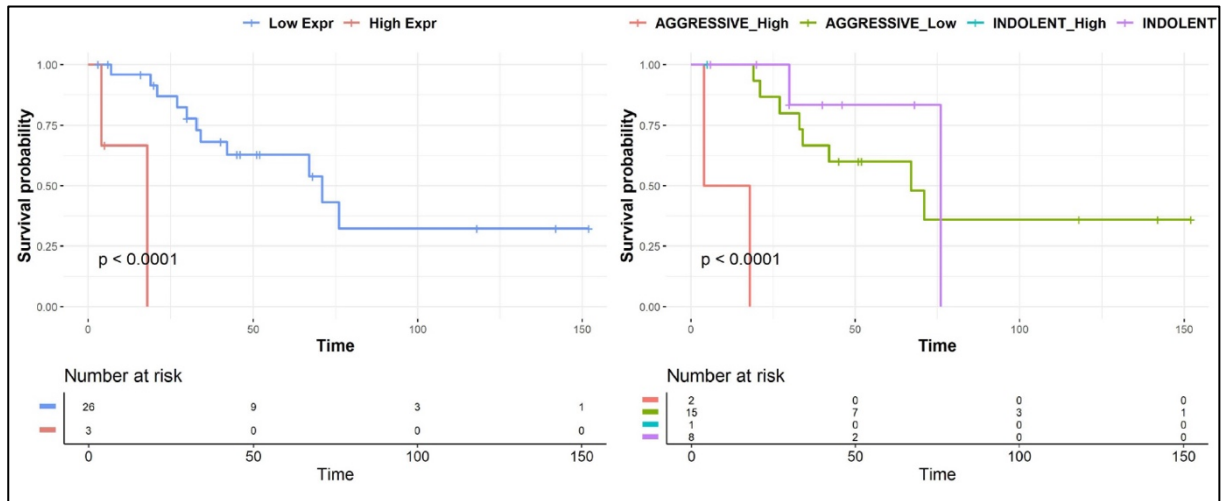


Figure 26. IL12A

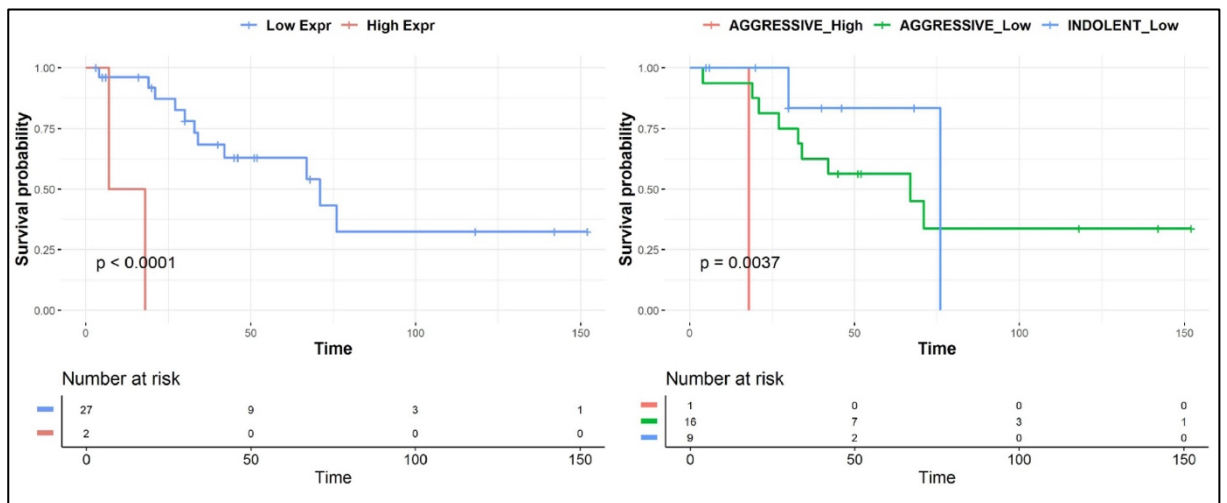


Figure 27. IL5RA

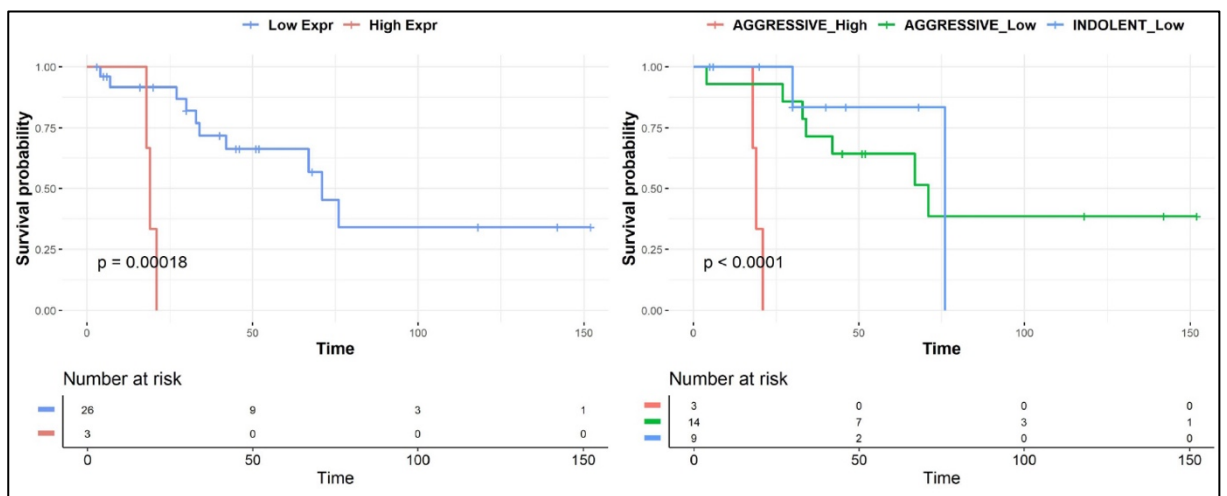


Figure 28. IFNL2

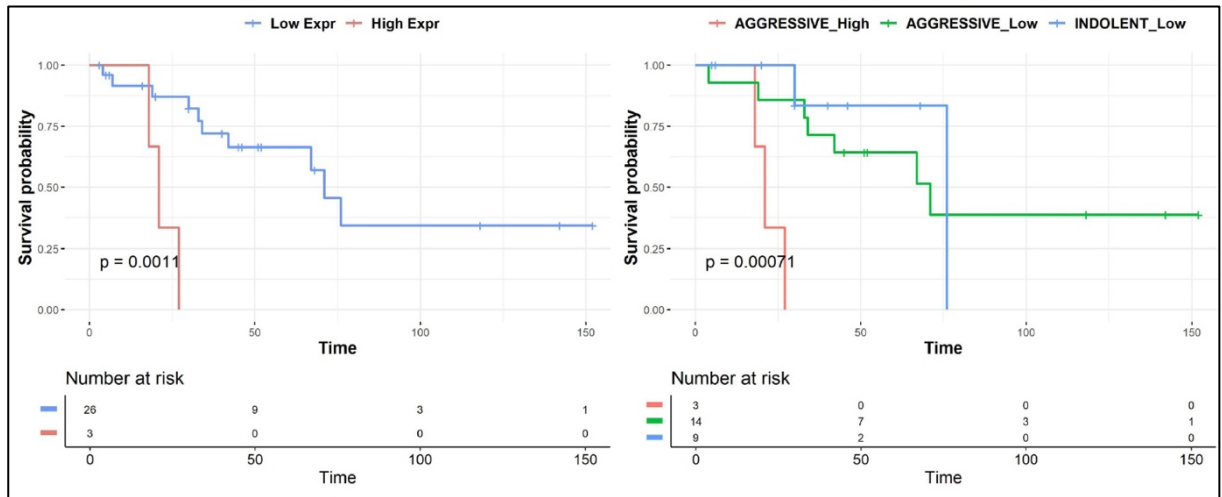


Figure 29. NT5E

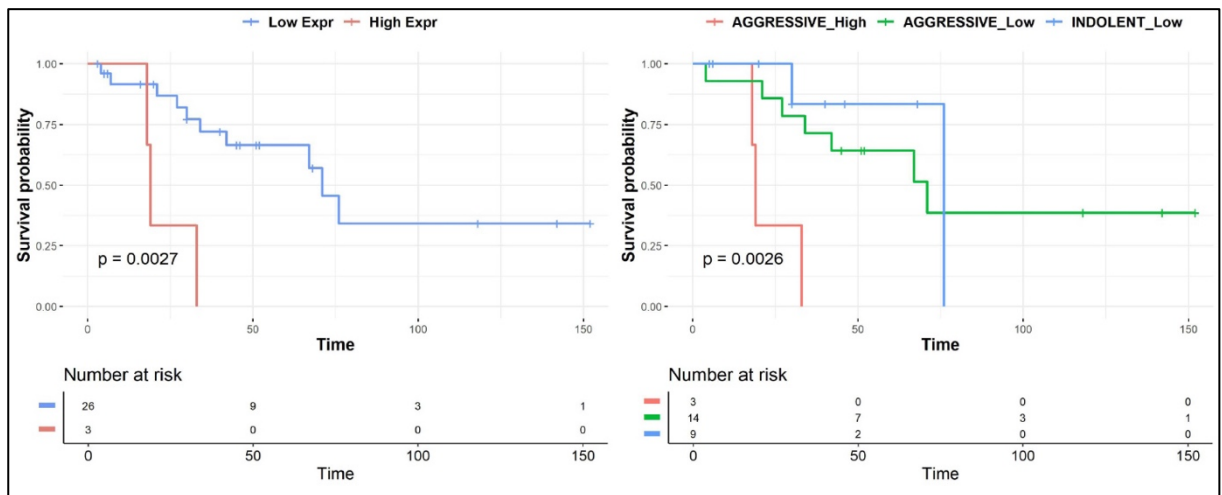


Figure 30. IL18RAP

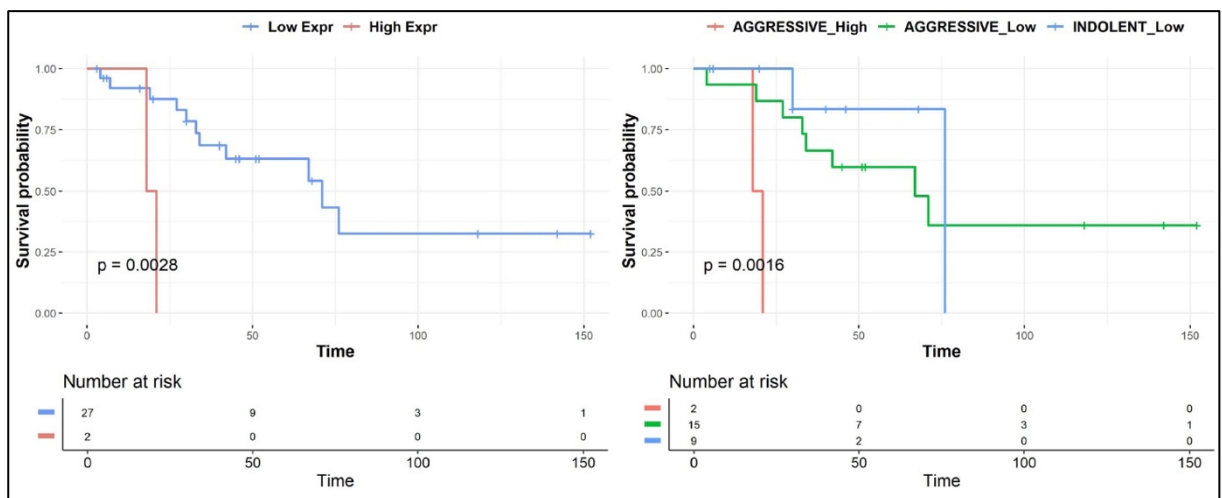


Figure 31. ABCB1

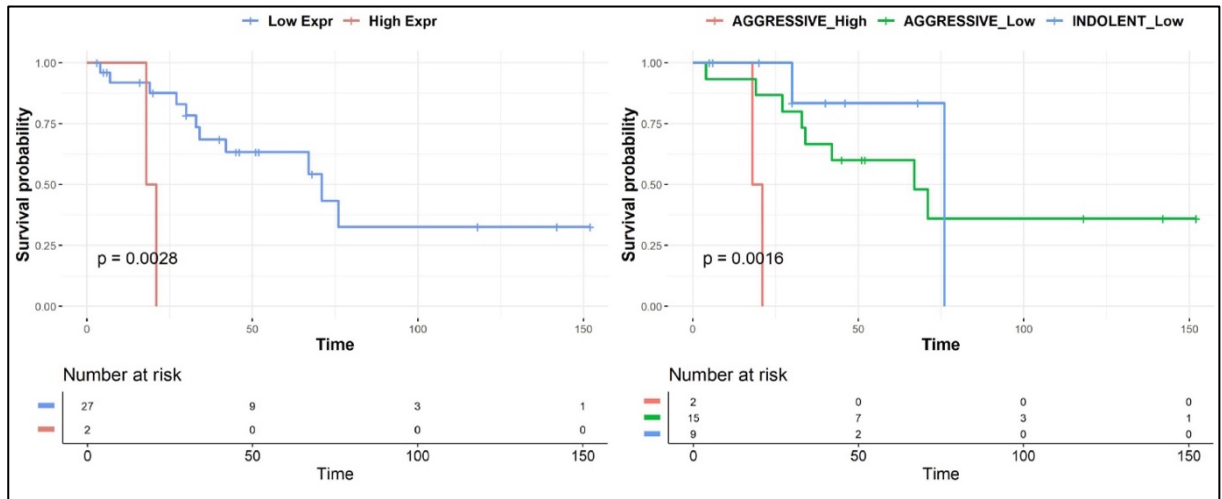


Figure 32. CCL16

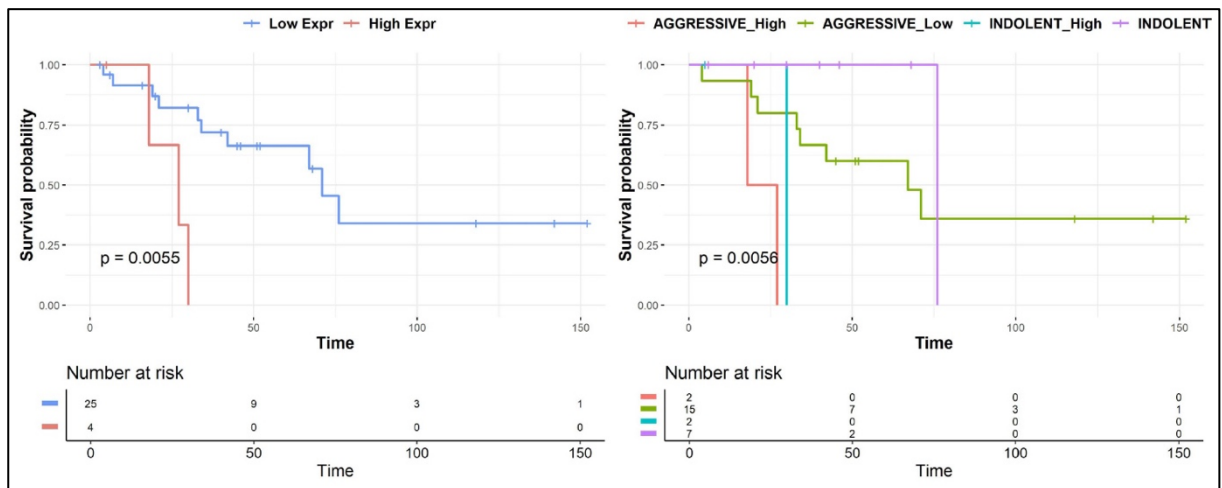


Figure 33. CCL1

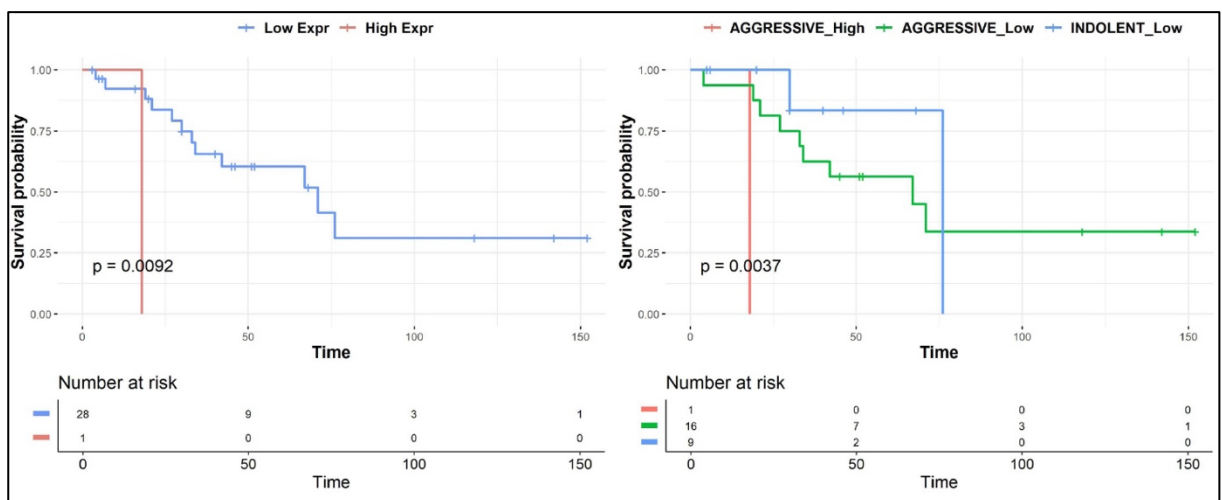


Figure 34. IL22RA2

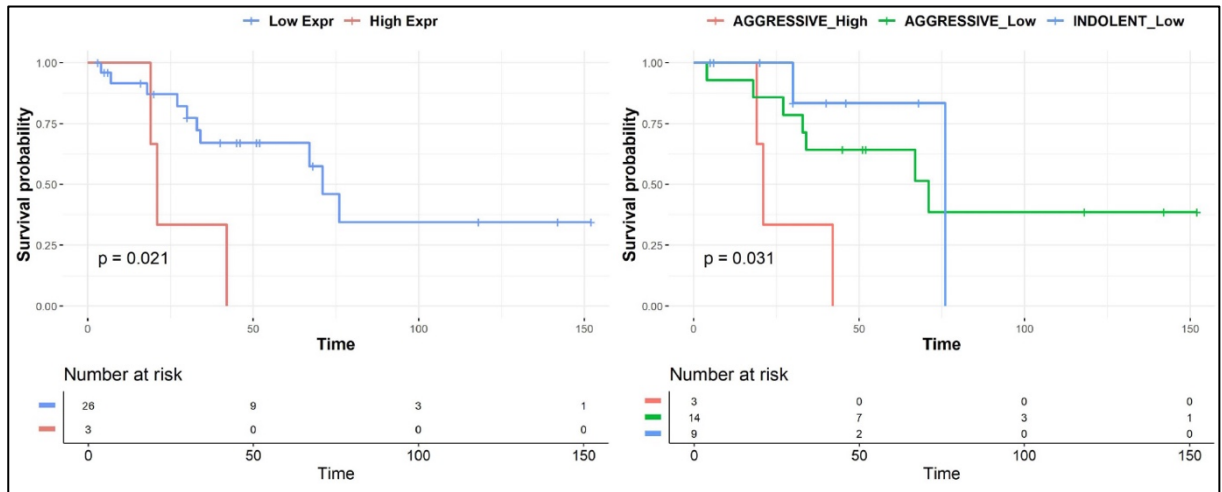


Figure 35. IFNA17

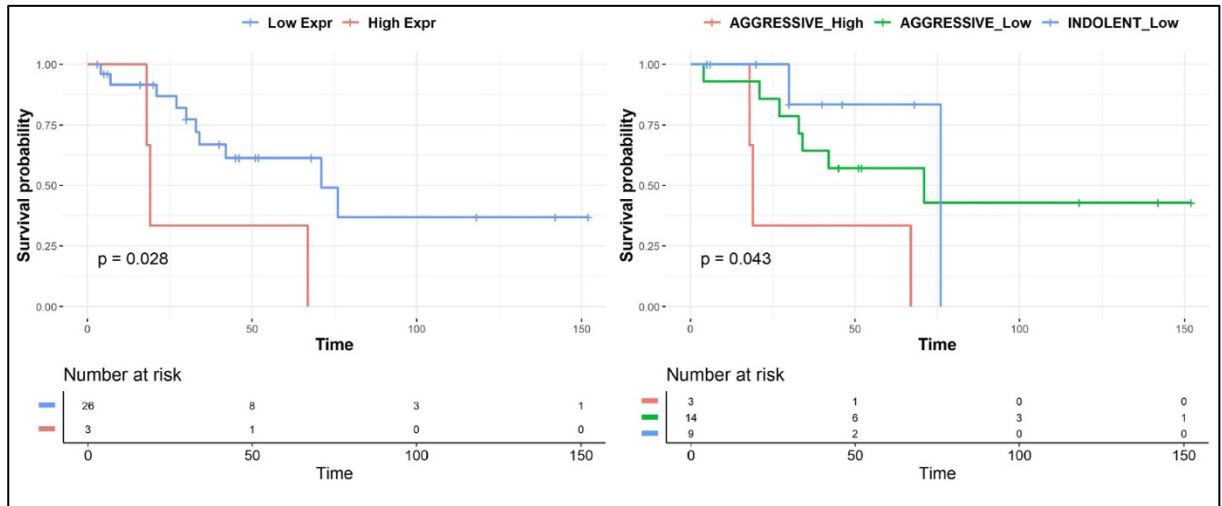


Figure 36. C9

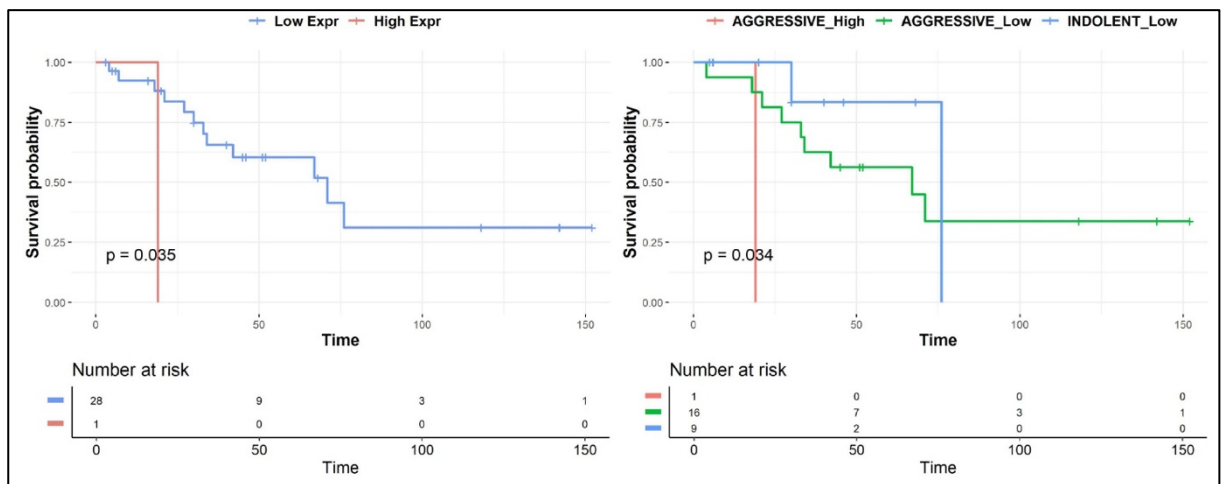


Figure 37. CCR9

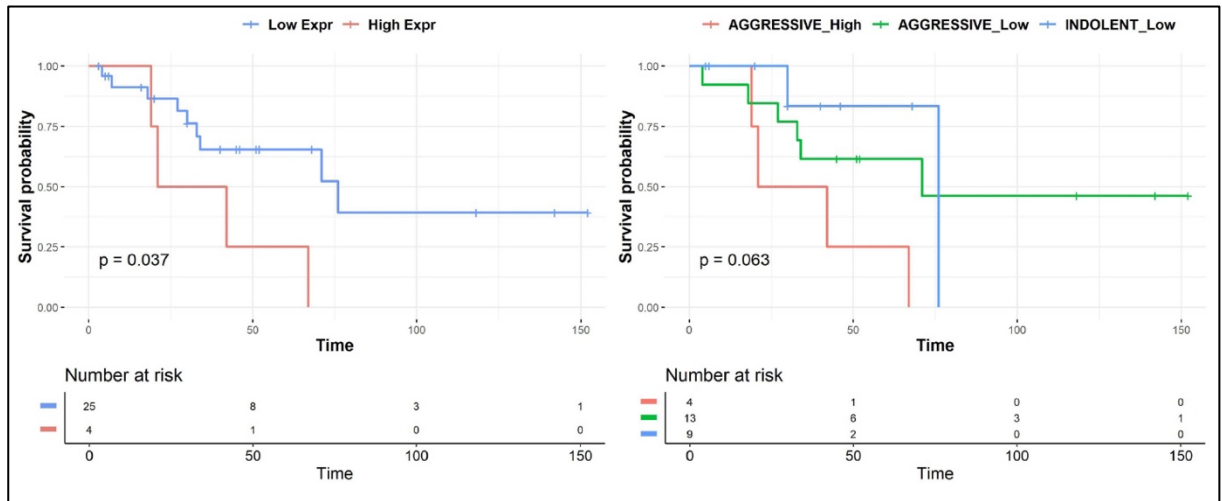


Figure 38. SPANXB1

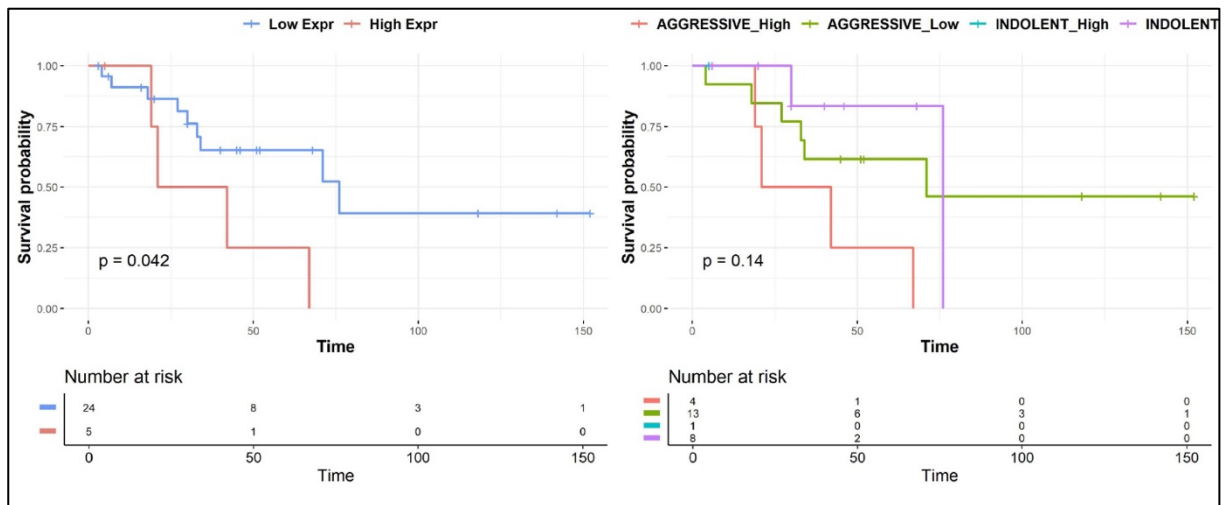


Figure 39. TREM1

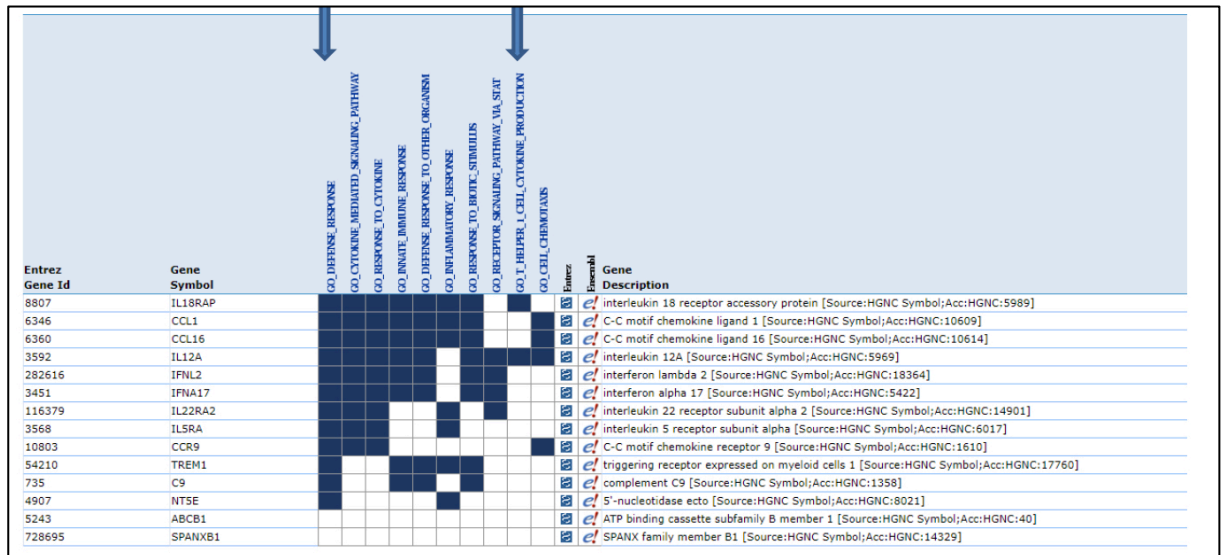


Figure 40. GO analysis, by GSEA browser, showed that 14 genes, associated with high and low risk in SS, are significantly involved in defense response and Th1 cytokine production (FDR<0.05)

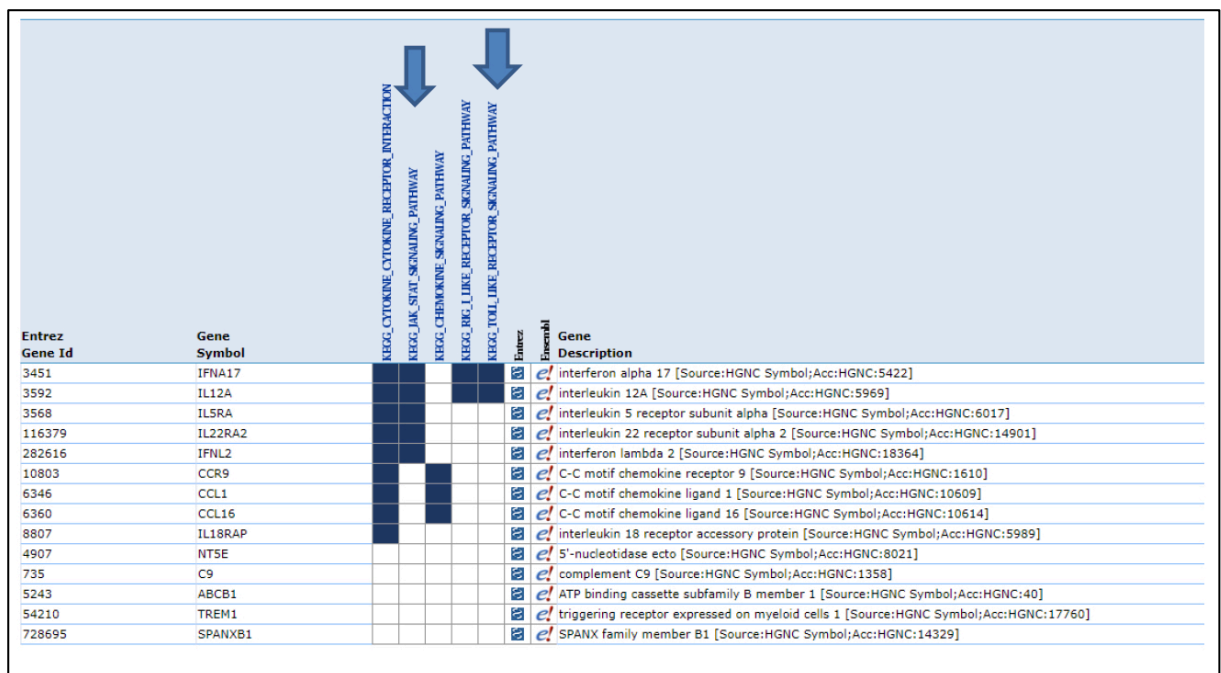


Figure 41. KEGG pathway analysis, by GSEA browser, showed that 14 genes are significantly involved in JAK/STAT and TLR signaling. (FDR<0.05)

5. DISCUSSION

Mycosis fungoides and Sézary syndrome are the two principal forms of CTCL, characterized by distinct clinical features, but their differences or correlation about pathogenesis and development is still a challenge. Studies in literature have frequently taken together these entities without any distinction.

In this PhD project, the first part is based on the study of the phenotype of the neoplastic T cells in MF and SS, in order to evaluate specific differences between the two entities and their correlation with the clinical presentations. Patients studied were a limited group but included cases with classical features and also cases with an atypical presentation, like SS without erythroderma.

In this case series, all patients showed the expression of skin-homing markers CLA and CCR4 on the skin, except two cases of MF (CCR4 negative). In the blood of the patients studied the expression of CCR4 was very high in quite 100% of CD4 cells sorted. CCR4 is the chemokine CCL17/CCL22 receptor, involved in the migration of activated T-cell in the site of inflammation.⁴⁸ CCR4 is expressed in 20-25% of CD4 cells of healthy individuals, but its level in SS and MF is extremely higher, also compared to benign dermatoses.⁸⁵⁻⁸⁷ In particular, SS has a higher level of CCR4 expression compared to MF, confirming the better response to antiCCR4 treatment, called Mogamulizumab, of SS patients compared to MF.^{48,88} Interestingly, 5 of our cases were characterized by CD45RA and not CD45RO as usual, meaning that they have not a T memory phenotype as typically described for MF and SS cells.³ Campbell and colleagues in their papers largely demonstrated that SS cells have a strong expression of CD27, CCR7 and CD62L, which in the normal CD4⁺ T cells corresponding to TCM phenotype; instead, MF cells are usually negative for those surface markers, showing a different T cell origin.^{85,86,89} Further studies revealed that neoplastic cells in advanced MF skin lesions highly express CCR7 showing an intermediate phenotype called

TEM. Campbell et al, about the expression of CD45RA in their samples, reported a variable and unexpected expression because it is usually not present on T memory cells.⁸⁵ Following Horna and colleagues study, evaluation of naïve and memory T phenotype should be based on the expression of CD62L, in addition to CD45RA, because the correlation between these and other markers of T subsets, like CD27, CD44, CD45RO, CD127, CCR7, could be not perfect.⁴⁸ CD62L is a cell adhesion molecule expressed on high endothelial venules, which permits rolling and tethering of leukocytes in the lymphoid tissue and circulation of the TN and TCM in the lymph nodes.⁴⁸ In this study, use of CD62L alone was favored because the lack of CCR7 could be compensated by CXCR4 (a chemokine normally expressed on CTCL) in lymph node-homing.⁴⁸ Based on this explanation, out of 5 CD45RA+ cases, 4 of them evidenced a TN phenotype, including an early MF. One SS showed complete negativity of CD62L and CCR7, arguing a TEMRA phenotype. Taking an overview on all SS cases, we can see that the phenotype is variable ranging from the typical TCM one (2 classical SS and one atypical SS), TN phenotype (2 classical SS and one atypical SS) and TEMRA phenotype (one classical SS). MF cases were more homogeneous showing a large expression of skin resident markers (CD69 and CD103) in almost all cases. In particular, localized MF (IA-IB) were characterized by the typical TRM phenotype; instead, the evidence of CCR7 on neoplastic cells of 2 erythrodermic MF and one IIB MF identified the TMM phenotype in these cases. Findings of MF seems to be more in line with the description of the group of Campbell and colleagues.^{85,86,89} Interestingly, the case 9, the only one with the immunophenotypical analysis in two different stages of disease (before, as early-stage MF, and then, during progression to SS), showed the same TCM phenotype in all stages of the disease and in the blood sample. In this case, evidence of a TCM phenotype in the first diagnosis of MF could be considered a marker of the further progression of the disease, because of the tendency of the neoplastic cells to re-circulate in the blood, lymph nodes and skin since the first evidence of the disease with localized MF skin lesions. This could be

important data for the clinician, who can program therapy schedule and follow up in a different way.

Immunophenotype of blood samples of cases studied, revealed a better correlation with the skin of SS patients compared to MF patients. This disparity between blood and skin-derived neoplastic cells has been recently described in SS patients.^{90,91} Based on the result of this case series, MF seems to be more differentiated by the expression of the skin resident markers, but the number of cases studied is still too small. SS cases are more heterogeneous, in line with the recent findings in the literature,^{26,48,92} which have questioned the “cell of origin” theory of Campbell and colleagues.^{85,86,89} Horna et al, in a large case series, found a highly variable spectrum of naïve/memory T-cell phenotype in CTCL, with no statistically significant correlation with a clinical diagnosis of SS or leukemic MF.⁴⁸ They also showed a shift of phenotype in the same patients during the clinical course (37.5% of cases studied), based on the expression of CD62L. These results induced the authors to conclude that in MF the change in naïve/memory phenotype is an antigen-driven event and these different phenotypes in CTCL reflect the functional status of the SC; so, their evidence should not be used for diagnostic purpose.⁴⁸ In contrast with the dichotomy theory that SS and MF are two distinct diseases based on cells of origin, our findings confirm the heterogeneity of the phenotype in CTCL, suggesting that expression of the markers studied should be used to evaluate clinical presentations and schedule the treatments.

In the second part of the project, we would like to evaluate differences between MF and SS by a different point of view. We decide to evaluate the phenotype of neoplastic cells involved and cells of the microenvironment, by gene expression profiling. We used a wide panel of targeted genes by Nanostring technologies, called PanCancer Immune Profiling Panel, which includes 770 genes identifying different phenotypes of T cells, B cells but also dendritic cells, macrophages, mast cells, neutrophils and natural killer cells.

As the first result of this analysis, MF and SS samples did not cluster in two or more groups, probably due to heterogeneous populations of cells (malignant and non-malignant cells) that we were analyzing; so, we decided to study the two groups of diseases separated.

In MF, we found 12 genes differentially expressed in advanced stages compared to early stages and these genes were found significantly enriched in the regulation of the immune system, macrophage activation and TLR signaling. In particular, CCR3 is the receptor for eotaxins and it is expressed on eosinophil and Th2 cells. CCR3 and eotaxin 3 (CCL26) has been found more expressed in advanced-stages CTCL compared to early stages, as expected in a Th2 cell-rich environment. CCL26 is primarily secreted by non-malignant cells within the lesional CTCL skin, so dermal fibroblasts, keratinocytes and endothelial cells, by IL4 and IL13, contribute to the elevation of CCL26, increasing chemotaxis of CCR3.^{39,93} High expression of CCR3 and CCR4 has been reported to be associated to a poor prognosis in MF and SS.⁹⁴

Involvement of apoptosis was highlighted by evidence of upregulation of TNFRSF10C, a gene member of the Tumor Necrosis Factor (TNF) family. This receptor is not able to induce apoptosis but seems to function as an antagonist receptor that protects cells from apoptosis induced by TNF-related apoptosis-inducing ligand (TRAIL). TNFRSF10C is a p53-regulated DNA damage-inducible gene. Its expression was detected in many normal cells and less in cancer lines. In CTCL, expression of TNFRSF10C has been poorly described; however, sensitivity to TRAIL has been found significantly reduced, so in CTCL, like in other primary hematologic tumors, the resistance to TRAIL-mediated apoptosis could be due to expression of TRAIL decoy receptors, such as DcR1/TNFRSF10C, or also activation of antiapoptotic signaling pathways, such as NF- κ B, or overexpression of anti-apoptotic proteins, such as FLIP.⁹⁶⁻⁹⁸

HAVCR2 gene encodes for T-cell immunoglobulin-3 (TIM-3), a cell surface molecule expressed on IFN γ -producing CD4⁺T helper 1, CD8⁺ T cytotoxic 1 T cell, Treg and innate

immune cells (DC, NK, cells, monocyte).⁹⁹ Tim-3 is a negative regulator of type 1 immunity, involved in antigen-specific tolerance, and a key regulator of effector T cell function.¹⁰⁰

Expression of Tim3 and PD1 on dysfunctional or exhausted T cells suggested that Tim3 and PD-1 have a non-redundant and synergistic function in inhibiting effector T cell responses.¹⁰⁰

Preclinical and clinical studies on solid and hematological tumors showed that co-blockade of Tim-3 and PD-1 pathways is superior to PD-1 pathway blockade alone at improving anti-tumor effector function and suppressing tumor growth.¹⁰⁰⁻¹⁰³ Tim-3 may also have a role in regulating the function of FoxP3⁺ Treg cells. By IL27 induction, expression of Tim-3 and Lag-3 has an important regulatory role in T regulatory 1 cells, suggesting that Tim-3 functions in both effector and regulatory lymphocytes subsets to regulate immune responses at the site of inflammation.¹⁰⁰

PMCH is a precursor of melanin-concentrating hormone (MCH) and it is highly expressed *in-vitro* differentiated human Th2 cells. Expression of PMCH on T reactive cells in nodal follicular lymphoma could be associated with survival and transformation.¹⁰⁴

PRAME encodes a protein that acts as a repressor of retinoic acid receptor and likely confers a growth advantage to cancer cells via this function. Its expression was described firstly in solid cancer, but now the expression of PRAME in hematological malignancies has been widely evaluated and its overexpression has been reported to be a role in the progression of acute leukemia.^{105,106}

We then analyzed the correlation of expression of the genes in the PanCancer Immune Panel and overall survival in both MF and SS group of patients, in order to perform a prognostic score.

In MF, we found a 9-gene prognostic signature for OS, showing its ability to predict survival in our cohort of patients. Moreover, high expression of almost all these genes (CDK1, IL6ST, CCR4, ITK, NOS2A, IL2RA, LRRN3) significantly correlated with a poorer prognosis in

early stages compared to low expression in both advanced and early stages. This data suggests the possible good predictive role of these genes in evaluating prognosis in the early stages.

The 9-gene signature in MF was found to be enriched in the cytokine and JAK-STAT signaling pathway by KEGG pathway analysis. JAK-STAT pathway is well known to be involved in CTCL pathogenesis, like in other non-malignant cutaneous diseases, such as psoriasis and atopic dermatitis.^{71,73,74,81} Pharmaceutical JAK/STAT inhibition is still studied in CTCL patients, like ruxolitinib (JAK1/2 inhibitor) which inhibits neoplastic T cell proliferation and activates apoptosis, and JAK inhibitor tofacitinib, which strongly inhibits miR-155 expression and STAT5 activation.¹⁰⁷ IL2RA, also called CD25, is typically overexpressed in malignant T cells of MF and SS, resulting in a cascade of phosphorylation of several proteins including JAK3, STAT3 and STA5.^{86,107} High expression of CD25 in immunohistochemical studied was significantly associated to advanced-stage disease and correlated with clinical response to denileukin diftitox, a recombinant fusion protein of diptheria toxin and ligand IL2.¹⁰⁸ IL6ST encodes for glycoprotein 130 or CD130, a transmembrane protein receptor of IL6, IL27 and IL31, and leads to activation of JAK/STAT pathway promoting cell survival.¹⁰⁹ DUSP4 encodes for an enzyme member of the dual-specificity protein phosphatase family, which negatively regulates members of the MAPK superfamily.¹¹⁰ DUSP4 is able to induce STAT5 hyper-activation, enhanced IL2 signaling and increased T cell proliferation.¹¹¹

Importance of CCR4 in CTCL is widely known and studied. By a meta-analysis of Van Kester and colleagues, CCR4 was included in the gene signature of tumor-stage MF.¹¹² It is a novel therapeutic target and Mogamulizumab, an anti-CCR4 monoclonal antibody, is indicated in relapsed or refractory MF or SS, who have been treated with at least 1 prior line of therapy.¹¹³

IL-2-inducible T cell kinase (ITK) is a member of the Tec family of non-receptor protein tyrosine kinase. ITK is directly downstream of LCK in the T cell receptor (TCR)-mediated

signal transduction cascade and plays an important role in T cell activation, proliferation, differentiation, and effector cytokine production.¹¹⁴ ITK expression on SC was found associated with poor prognosis.¹¹⁵ An *in vitro* study about the use of ITK-inhibitor (CTA191) on CTCL cell lines showed its potential ability to target and kill malignant T cells, inducing apoptosis and activation of caspase-3/7.¹¹⁶

Our survival analysis on SS patients showed a 14-gene signature for OS and gene expression-based risk score based on expression levels of these 14 genes was able to split patients into two risk categories, even when the score was applied to indolent and aggressive courses. High expression of all 14 genes of the signature was significantly associated with poor prognosis, and this data was strongly confirmed by the significantly poor prognosis of high expression of all genes in aggressive SS. Interestingly, high expression in indolent forms was not found for most of the genes of the signature.

Pathway analysis revealed a significant involvement of 14 genes in JAK/STAT signaling, which is widely described in CTCL pathogenesis as reported above. Surprisingly, some of these genes are significantly involved in Th1 cytokine production, based GO analysis. In particular, IL12A encodes for a subunit of IL12, a cytokine which is involved in JAK/STAT signal transduction pathway through activation of STAT4. Although both Th1 and Th2 cells can bind IL12, only Th1 cells are capable of signaling in response to IL12 and IFN γ . DC and monocytes/macrophages are major producers of IL12 in response to infectious pathogens and are stimulated by activated T cells through CD40/CD40L interaction.^{117,118} DC has been defined in two categories: myeloid dendritic cells (DC1) and plasmacytoid dendritic cells (DC2). DC1 are the major producer of IL12 and drive T-cell differentiation into Th1 response; instead, DC2 stimulate T naïve cells to produce predominantly Th2 cytokines and are recognized to be the major producers of IFN α in response to viral infections.¹¹⁹⁻¹²¹

SS is typically a Th2 disease and production of IL12 is deficient due to a marked decreasing of DC1 population in SS patients.¹²⁰ Our result of high expression of IL12A in SS patients with poorer prognosis should be validated in independent cohorts.

IFNL2 gene encodes IL28, a cytokine distantly related to IL10 family. IL28 is a ligand for the heterodimeric class II cytokine receptor composed of IL10RB and IFNLR1, leading to activation of JAK/STAT signaling pathway through STAT3 and STAT5 activation.¹²²

NT5E encodes CD73, an ectonucleotidase playing a critical role in tumor escape from immune surveillance in numerous solid tumors. CD73 acts by inhibition of clonal expansion, activation and homing to tumor-specific T cells, increasing substantial component of the suppressive capabilities of Tregs and Th17 cells, acceleration of the conversion of anti-tumor type 1 macrophages into pro-tumor type 2 macrophages.¹²³ High expression of CD73 was markedly associated with poor prognosis and distal/local lymph nodes metastasis in different solid cancers.¹²⁴ CD73 has been also evaluated as a therapeutic target: preclinical data on mice and melanoma models reported that blockade of CD73 could enhance the efficacy of anti-CTLA-4 and anti PD1 inhibitors, showing that CD73 is a promising target for cancer immunotherapy.¹²⁴⁻¹²⁶

SPANXB1 is a gene member of the SPANX family of cancer/testis associated genes (CTA), a subgroup of tumors antigens reported in numerous cancers, encompassing promising candidate targets for T-cell based immunotherapy. CTA have been also evaluated in CTCL but never the SPANX family.^{127,128}

IL22RA gene encodes receptor for IL22, which is play important roles in the pathogenesis of various skin diseases. High levels of IL22 were found in skin and serum of CTCL patients, especially in advanced stages, suggesting that IL22 could be a good marker of disease severity in this kind of disease.⁵⁷

CCL1 is a chemokine ligand secreted by activated T cells, endothelial cells, mast cells, monocytes and epithelial cells. Its sole receptor is CCR8, which is preferentially expressed on

Th2 cells and regulatory T cells. CCL1 is a potent attractant for Th2 cells. Its serum levels are found elevated in patients with CTCL, suggesting that this chemokine also may be associated with the Th2-dominant microenvironment in CTCL.^{59,129}

CCR8 is also one of the receptors of CCL16, a chemokine ligand with a chemoattractive role for monocytes and lymphocytes. CCL16 is also implicated in tumor neovascularization.^{130,131}

CCR9 is a chemokine which plays roles in the migration of thymocytes and maturation in normal and inflammatory environments. CCR9 is highly expressed in numerous varieties of hematological and solid cancers. The interaction with its ligand CCL25 can activate many signaling pathways, inducing tumor chemoresistance and metastasis. Blocking of the CCR9/CCL25 signal has been demonstrated to be a potential novel cancer therapy.^{132,133}

5. CONCLUSION

The study of phenotypes in MF and SS of this PhD project permitted to confirm the heterogeneity of the phenotypes of these two entities, especially SS, reflecting the functional state of the neoplastic T cells and their correlation with clinical features, and not only their cell of origin. The deeper analysis by gene expression profiling on a wide targeted panel of genes revealed two distinct prognostic signatures for MF and SS, which significantly correlate with JAK/STAT pathways and contain possible therapeutic targets.

6. FUTURE STUDIES

The next steps of this project have already been planned and include the validation of the results of the gene expression analysis. In particular, we will validate the prognostic score in two independent cohorts of MF and SS patients from two Italian CTCL clinics. Moreover, we will also analyze gene expression on CD4⁺CD26⁻ cells from blood samples of SS patients to compare with the skin samples studied.

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