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POTENTIAL ROLE OF LIN28/LET-7 AXIS IN SEZARY SYNDROME

Settore scientifico disciplinare MED/35

NOME DEL DOTTORANDO

Daniele Fanoni matr. R11910

NOME E COGNOME DEL TUTOR
Prof. Emilio Francesco Berti

NOME E COGNOME DEL COORDINATORE DEL DOTTORATO
Prof. Emilio Francesco Berti

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Sommario

1 INTRODUCTION

1.1 Mycosis fungoides and Sézary syndrome

1.1.1 Brief history

First description of mycosis fungoides (MF) was made by Jean Louis Alibert[1] in 1806. He firstly described cutaneous tumors resembling fungal infection. In 1869, after microscopy studies, Xavier Gillot and Louis Antonine Ranvier hypotesized MF was a lymphoid regeneration in the skin and they called it "lymphadénie cutanée". In 1870 Ernest Bazin described MF as having three stages: nonspecific erythematous (premycotic), plaque (lichenoid) and tumor (fungoides) phases.[2]

Sézary syndrome (SS), originally described by Ernest Besnier and Henri Hallopeau in 1892 as an aggressive cutaneous lymphoma,[3] was then defined by Albert Sézary and Yves Bouvrain in 1938 as a leukaemic variant of MF, characterized by erythroderma, generalized lymphadenopathy and the presence of neoplastic CD4+ lymphocytes (Sézary cells) in the skin, lymph nodes and peripheral blood.[4] In 1968, Marvin A. Lutzner described ultrastucture of "cerebriform" nuclei of Sézary cells.

In 1975 Lutzner and Richard L.Edelson described a group of diseases, including MF and SS, which was named "cutaneous T-cell lymphomas" (CTCL). Early studies to characterize various types of CTCL morphologically, immunologically, clinically and genetically, were carried out during the 80's. First classifications of CTCL were published at the end of the previous century by two distinct international organizations: by European Organization for Research and Treatment of Cancer (EORTC) and by World Health Organization (WHO). These disctint classifications were fused in a WHO-EORTC classification in 2005.[5] The most recent classification is the WHO classification of tumours of haematopoietic and lymphoid tissues printed in 2017.[6]

1.1.2 WHO classification of CTCL

B-cell and T-cell neoplasms are clonal tumours of mature and immature B cells and T cells at various stages of differentiation. Skin can be involved primarily or secondarily However, only those arose in the skin, without involvement in other sites, are considered "primary cutaneous".

Cutaneous T-cell lymphomas (CTCLs) represent 75%-80% primary cutaneous lymphomas. MF e SS are the most frequent indolent and aggressive form among CTCLs, respectively (Table 1).

Table 1. WHO classification of NK/T-cell lymphomas

•Mycosis fungoides (MF) and MF variants
Folliculotropic MF
Pagetoid reticulosis
Granulomatous slack skin
•Sézary syndrome (SS)
•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
•Hydroa vacciniforme-like lymphoproliferative disorder
•Severe mosquito bite hypersensitivity
•Peripheral T-cell lymphomas, NOS and rare subtypes
Primary cutaneous CD8+ aggressive epidermotropic cytotoxic T-cell lymphoma
Primary cutaneous γ/δ T-cell lymphoma
Primary cutaneous CD4+ small/medium T-cell lymphoproliferative disorder
Primary cutaneous acral CD8+ T-cell lymphoma

1.1.3 MF and SS: Definition

Mycosis fungoides is an epidermotropic, primary cutaneous T-cell lymphoma characterized by infiltrates of small to medium-sized T lymphocytes with cerebriform nuclei. The term MF should be used only for classic cases, characterized by the evolution of patches, plaques and tumours, or for variants with a similar clinical course.

SS is defined by the triad of erithroderma, generalized lymphadenopathy, and the presence of clonally related neoplastic T cells with cerebriform nuclei (Sézary cells) in skin, lymph nodes, and peripheral blood. In addition, one or more of the following criteria are required: an absolute Sézary cell count >1000/μl, an expanded CD4+ T-cell population resulting in a CD4:CD8 ratio of >10, and loss of one or more T-cell antigens. SS and MF are closely related neoplasms, but are considered separate entities on the basis of differences in clinical behaviour and cell origin.[7]

1.1.4 Epidemiology

MF is the most common type of cutaneous T-cell lymphoma (4/1,000.000 per year) and accounts for almost 50% of all primary cutaneous lymphomas. Most patients are adult/elderly, but the disease can also be observed in children and adolescents. The male-to-female ratio is 2:1.

SS is a rare disease (0.3/1,000.000 per year), accounting for <5% of all CTCLs. It occurs in adults, characteristically presents in patients aged >60 years, and has a male predominance.

Rare familiar cases of both MF and SS are described,[8] but there are no evidences for a genetic predisposition.

1.1.5 Ethiology

MF is believed to result from chronic antigenic stimulation that leads to uncontrolled clonal expansion and the accumulation of T cell helper memory cells in the skin.[9] In support of this notion, increased numbers of dendritic cells were found in early MF lesions.[10] Specifically, antigen-presenting cell (APC) ligands B7 and CD40 and their respective T cell costimulatory ligands CD28 and CD40L were found to be upregulated in MF lesions.[11, 12] Neoplastic T cells can also express APC ligands, suggesting a possible self-stimulation pathway that leads to T cell expansion. Other studies have shown increased Toll-like receptor expression (Toll-like receptors 2, 4, and 9) by keratinocytes and increased expression of particular human leukocyte antigen class II alleles in MF patients.[13, 14] Toll-like receptor stimulation is seen in inflammatory skin diseases, including psoriasis and chronic allergic contact dermatitis.[15] Although lymphomatoid reactions caused by contact hypersensitivity have been reported, no causal relationship between contact dermatitis or other inflammatory skin conditions and MF/SS have been identified.[16, 17]

Infections, specifically Staphylococcus aureus and associated enterotoxins, may also play an etiologic role in MF. One study found a high rate of S aureus colonization in patients with erythrodermic MF (EMF) and SS, with clinical improvement of both erythroderma and extent of skin disease after antibiotic therapy.[18] Unlike adult T-cell leukemia/lymphoma, which is associated with human T-lymphotropic virus type 1 (HTLV-1), most CTCL patients are serologically HTLV-1−.[19-21] Other investigators have found serologic evidence for Epstein–Barr virus and cytomegalovirus, but there is minimal evidence supporting a viral etiology.[22]

Immunosuppression and/or immunosuppressive therapy may predispose patients to develop CTCL in rare cases after organ transplantation[23-25] and in those with HIV.[26, 27] Occupational factors, such as working in the glass, pottery, and ceramics industry have been studied; however, their role in MF remains controversial.[28, 29] Military exposures, such as herbicide exposure, have been linked to non-Hodgkin lymphoma, although not specifically to CTCL.

1.1.6 Pathogenesis

Mycosis fungoides is characterized by epidermotropism of neoplastic T-cells and by persistent activation and clonal expansion leading to accumulation of T lymphocytes in the skin, lymphnodes and peripheral blood.[9] Tipically, neoplastic T-cells are mature T helper (Th) lymphocytes in both MF and SS. However, recent studies demonstrated that malignant T cells in SS have a T-central memory phenotype, consistent with the clinical presentation of peripheral blood disease, lymphadenopathy, and diffuse erythroderma of the skin. In contrast, neoplastic cells in MF belongs to skin resident T-effector memory cells, a population of T cells that produce inflammatory cytokines and remain stationary within a particular location in the skin. This is remarkably consistent with their clinical ability to produce inflamed skin lesions and to recruit nonmalignant T cells into the skin, giving rise to inflammatory patches and plaques.[7]

Malignant T cells in SS express high levels of L-selectin and CCR7 with lack of expression for both CCR5 and CXCR3. CCR4 is also expressed at high levels, but expression of other skin homing addressins (CLA, CCR6, and CCR10) is variable. Moreover, Sézary cells lack expression of surface molecules CD26 and CD49d with variable expression of CD7.

Increased Th2 lymphocytes in patients with MF and SS lead to a chronic hyperproduction of Th2 cytokines: IL-4, IL-5 and IL-10 and to a deficit of typical Th1 cytokines such as IL-12 and IFN-α. This loop can compromise CD8+ T cell, NK cell, and DC numbers and function, and consequently, the host immune response[30] (Figure 1).

A possible skin homing mechanism of neoplastic cells in SS has been proposed, basing on the lack of expression of CD26. CD26, also known as dipeptidylpeptidase IV, has the ability to cleave and inactivate the chemokine CXCL12 (SDF-1, stromal cell-derived factor 1), which is the CXCR4 ligand. CXCR4 is known to be physiologically involved in regulating the migration mechanisms of certain cell types, such as dendritic and Langerhans cells. This observation, together with the data of the downregulation of CD26, suggested a possible role of CXCR4 in the skin homing of Sézary cells.[31]

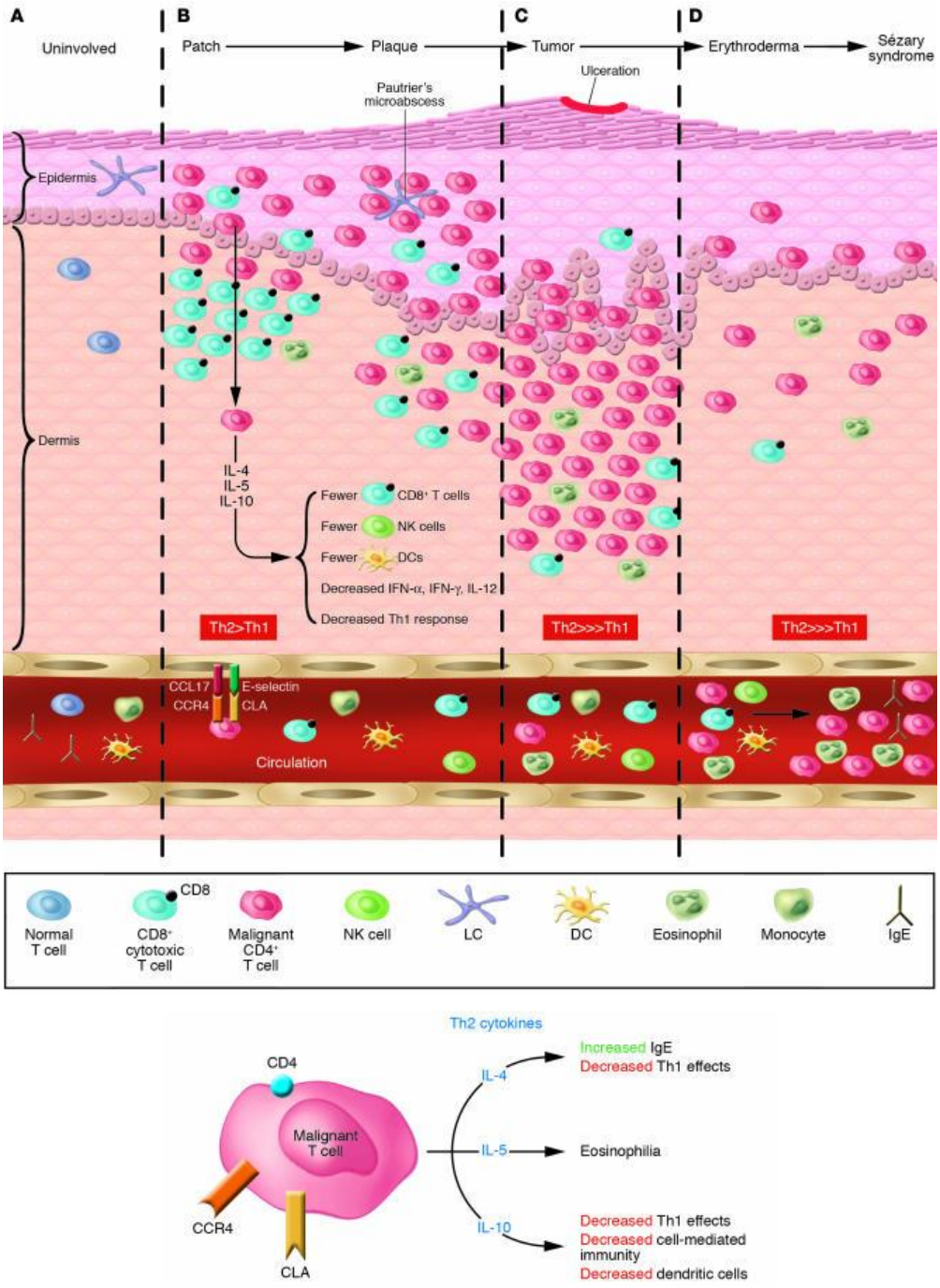

Figure 1. The skin microenvironment in MF progression. (A) Normal skin showing resident Langerhans cells in the epidermis and skin-homing T cells in the dermis and circulation. (B) Patch and plaque MF in which the CD4+ malignant T cells home to the epidermis and collect around Langerhans cells. Of note, in these stages, the epidermal and dermal infiltrate frequently have abundant CD8+ T cells as part of the host immune response. (C) Tumour MF in which the tumour occupies the dermis and subcutaneous tissue and is comprised of primarily malignant T cells and few CD8+ T cells. (D) Erythrodermic MF and SS with detectable circulating malignant T cells that elaborate Th2 cytokines that affect CD8+ T cell, NK cell, and DC numbers and function, and consequently, the host immune response.[30]

Gene expression profiles and genetic analyses lend understanding to the proliferative nature of the malignant T cells and to the immune dysregulation.[32-35] As an example, alterations in expression of certain transcription factors important for T cell differentiation, particularly upregulation of GATA3, and downregulation of expression of STAT4, may account for the Th2 phenotype in most cases.[32, 33]
The loss of nuclear pro-IL16 provides further insight into mechanisms underlying the enhanced proliferation of the malignant T cell population. The presence of nuclear pro-IL16 normally contributes to T cell quiescence and its loss would lead to cell cycle progression.[36] Other valuable insights include the observation that T-plastin is expressed in the malignant T cells of patients with SS; this gene is not normally expressed in hematopoietic cells, and its aberrant expression can facilitate identification of the lymphoma cells for diagnostic and therapeutic purposes.[37]
Other findings include mutations and deletions in chromatin remodeling genes, and gain-of-function mutations in the JAK-STAT pathway.[38]
It is well known that deregulation in STAT3 signaling is one of the molecular mechanism involved in CTCL pathogenesis and cancer progression and many studies demonstrated the efficacy of JAK/STAT inhibitors to induce apoptosis in Sézary cells.[39-41]

1.1.7 Clinical presentation, diagnosis, stadiation and prognosis

Patients in the early stages of MF generally present with patches that may initially appear to be due to non-cancerous skin conditions such as eczema, psoriasis, secondary syphilis, atopic dermatitis, or plaque parapsoriasis. For this reason, diagnosis is often delayed and the interval between the appearance of the first symptoms and the definitive diagnosis can be up to 4-10 years
Clinical observation of the SS patients may evidence "non-specific" signs such as erythroderma, edema, lichenification and itching. From a histological point of view, the skin infiltrate can be similar to that of MF or even have non-specific characteristics. On the other hand, circulating neoplastic cells are characterized by cerebriform nucleus with fine chromatin and scarce cytoplasm, the so-called Sézary cells. In advanced stages SS patients develop alopecia, nail dystrophy, fissures in the palms of the hands and soles of the feet, severe itching and pain in the skin.Often lymph node and in some cases bone marrow can be involved.
Diagnosis of MF and SS, suspected on the basis of the clinical presentation, must always be confirmed by histopathological examination of the skin and molecular analysis.

1.1.7.1 T-cell receptor

The diagnosis of CTCLs classically relies on a combination of clinical, pathological and immunophenotypical criteria. In the last decades, many studies focused on the usefulness of the assessment of TCR clonality in distinguishing benign from malignant cutaneous lymphocytic infiltrates[42-44]

In addition, the detection of a clonal T-cell receptor gene rearrangement in blood and lymph nodes has been introduced, in the new WHO classification, as a staging procedure of MF and SS.

TCR is the antigen recognition molecule used by T lymphocytes. Unlike the B lymphocyte receptor, the TCR does not recognize and bind antigens directly, but recognizes small peptide fragments of pathogenic proteins linked to molecules of major histocompatibility complex (MHC; HLA in humans) on the surface of other cells that present antigens (antigen presenting cells).

TCR is a heterodimer composed by two polypeptide chains: α and β or γ and δ.

T-cells precursors are stimulated by unfully known external signals to differentiate as γ/δ or α/β.

In both cases, TCR structure consists of a variable region (V) which is engaged in antigen binding, a constant region (C), a hinge region (H), a transmembrane region and a cytoplasmic tail. The variable region contains a diversity region (D) and a junction region (J). (Figure 2)[45]

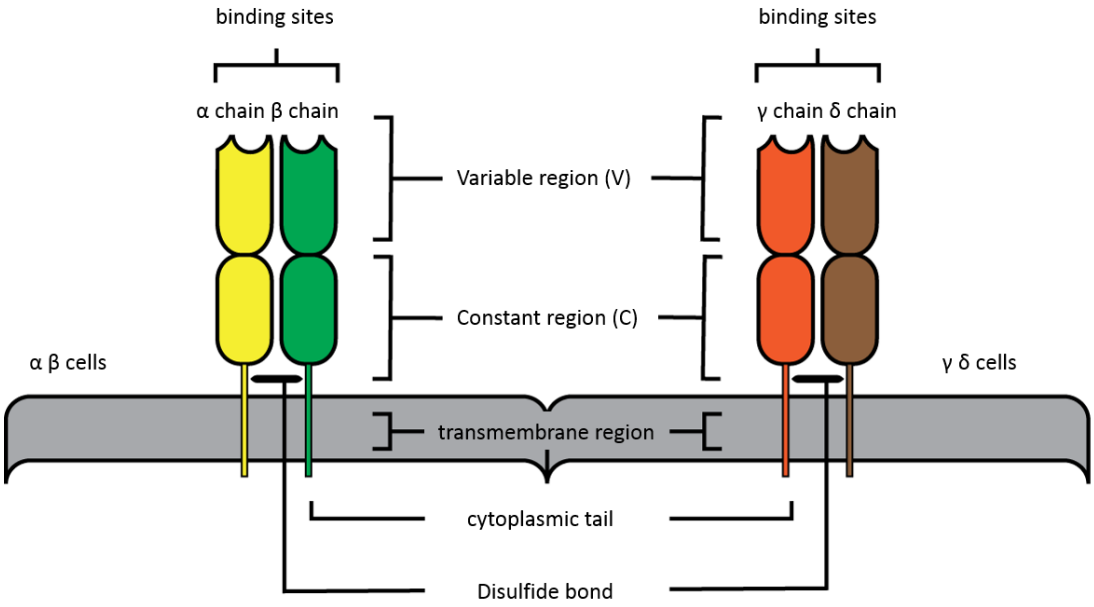

Figure 2. TCR structure

The gene loci coding for the α chain are located on chromosome 14q11 and are made up of 100 V segments, 50 J segments and 1 segment C and are distributed over 80 Kb. The β chain of the TCR is instead found on chromosome 7q35 and consists of germinal form from an aggregate of 30 V segments located away from two other aggregates, each containing a single D segment along with 6 J segments and a single C segment.

The gene loci coding for the γ chain are located on chromosome 7p14-15, while those for the δ chain map to chromosome 14q11 within the gene loci coding for the α chain of the TCR α/β. The genes coding for the γ chain contain V, J and C segments and have a different organization consisting of 15 V segments (of which 8 genes and 7 pseudogenes) and 5 J segments (2 genes and 3 pseudogenes), grouped into 3 functional aggregates of genes. δ chains are encoded by gene segments V, D, J, C; these loci consist of 10 Vδ segments, 3 Dδ segments, 2 Jδ segments and 1 segment for region C. (Figure 3)

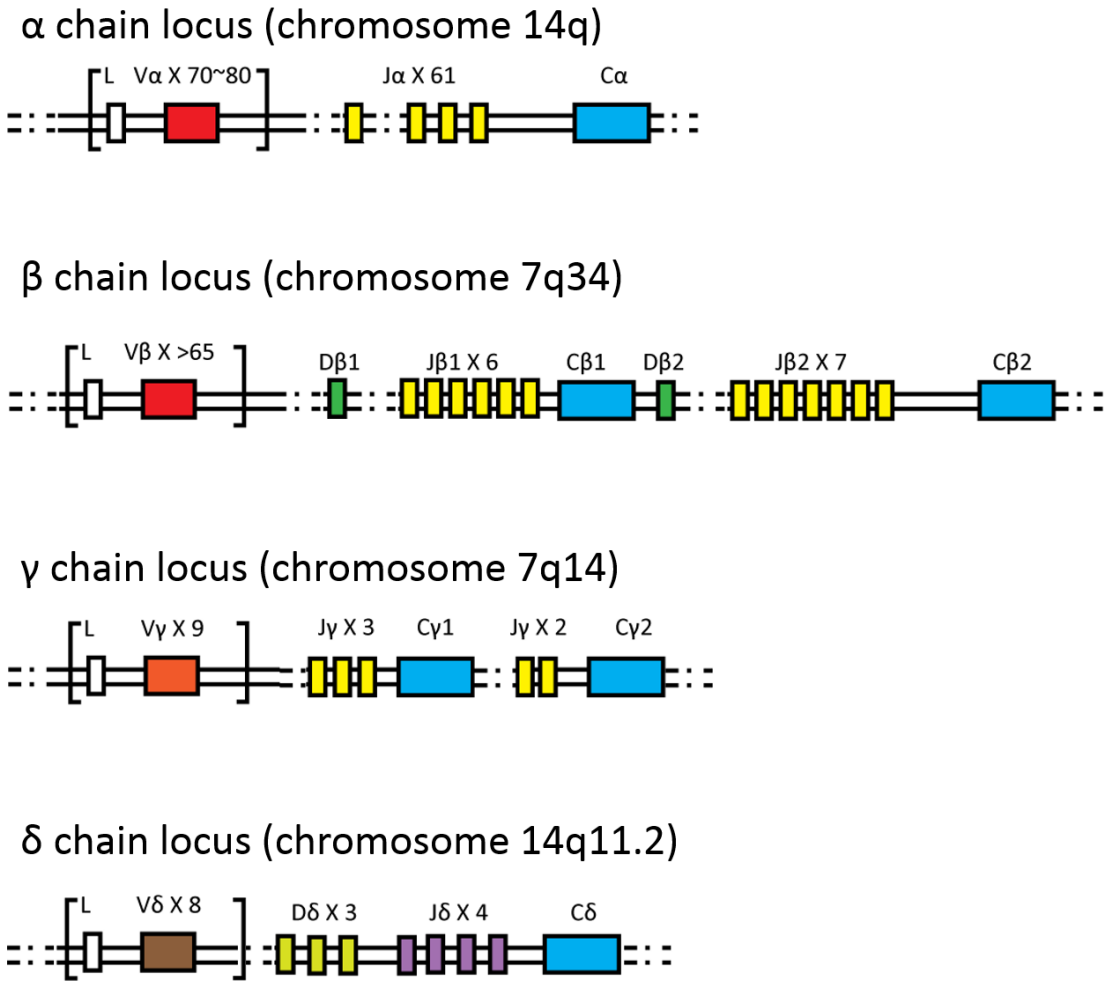

Figure 3. TCR genes

The mRNA production for the synthesis of the α/β and γ/δ chains occurs starting from a series of somatic combinations between the gene segments: for β and δ chains, a D segment is rearranged as a J segment, therefore the dimer DJ rearranges with a V segment to complete the variable domain of the peptide; the transcription of this whole segment gives rise to a primary transcript of RNA containing the domains of the C region; the subsequent splicing of this region leads to the formation of an mRNA which, translated, will give rise to the entire molecule (Figure 4).

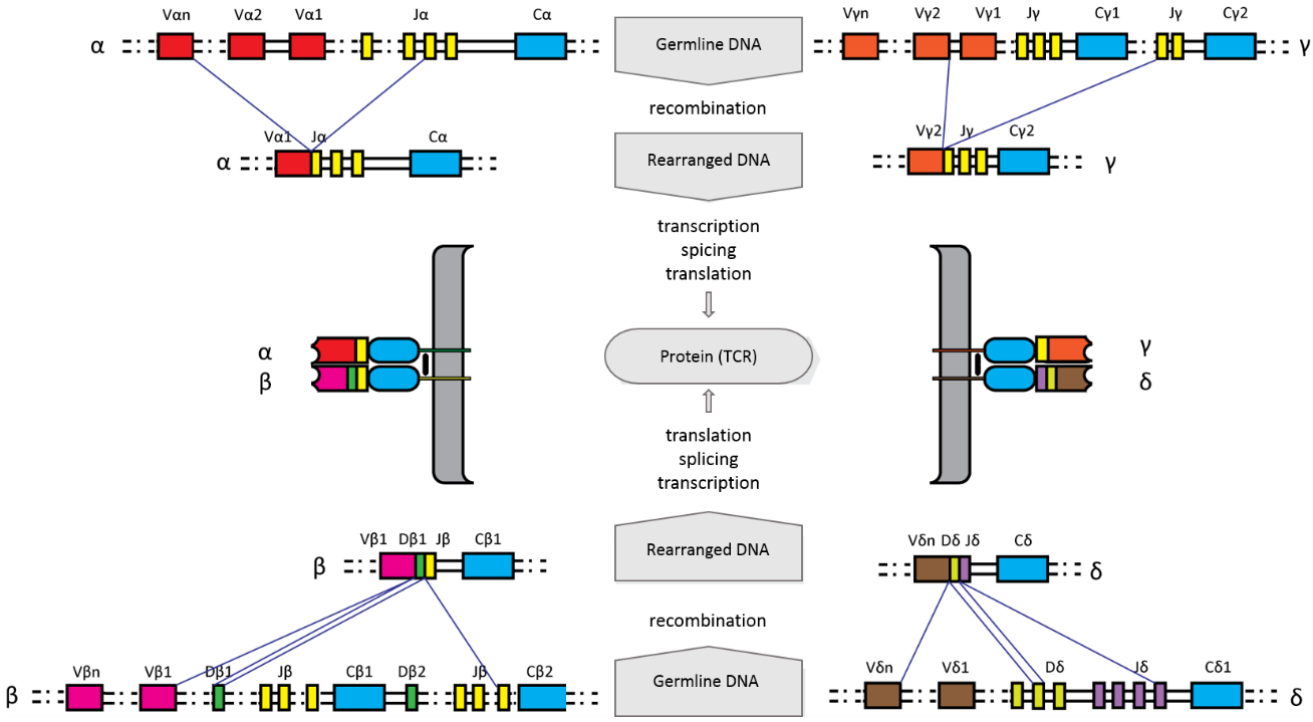

Figure 4. TCR rearrangements.

α and γ chain formation mechanism is similar to the machinery for β and δ maturation. However, the rearrangement directly involves the V and J segments due to the lack of the D segments.

Since the genes for δ chain are located within α loci (in particular between the Vα and Jα segments), any rearrangement of the gene segments fot the α chain leads to a complete deletion of

the δ gene. This means that during the first VJα rearrangement δ locus is deleted and the cell will no longer be able to generate a γ/δ receptor.

In order to further increase the variability, at junctional region there are also nucleotides insertion and deletion mechanisms between the V, D, J segments for the β and δ chains and between V and J segments for the α and γ chains. Added nucleotides are called "P" and "N": the P nucleotide lead to palindromic sequences at the end of the gene segments, while N nucleotides are not encoded starting from a template, but added by the deoxinuleotidyl enzyme terminal transferase (TdT). The total number of added nucleotides is random and the insertion can cause the break of the open reading frame of the coding sequence, leading to non-productive rearrangements.

Since each T-lymphocyte can express just one TCR molecule (a clone) one can detect TCR rearrangement by PCR (clonality).

1.1.7.2 Staging

Staging of MF/SS is based on a tumour-node-metastasis (TNM) classification system originally devised in 1979.[46] A revision and expansion that also includes blood involvement (TNMB) has been published in 2007 and is generally used today.[47] Recent studies have supported the prognostic relevance of these newly refined stages (Table 2).[48-51]

Additionally, histological findings that might be of prognostic importance but which are not accounted for by the TNMB classification are the infiltration of hair follicles (folliculotropism) and a finding of >25% of large cells in the dermal infiltrate (large cell transformation)[47]

Table 2

TNMB staging for mycosis fungoides and Sézary syndrome
Skin
T1 Limited patches, papules, and/or plaques covering <10% of the skin surface. May further stratify into T1a (patch only) versus T1b (plaque ± patch).
T2 Patches, papules, or plaques covering ≥10% of the skin surface. May further stratify into T2a (patch only) versus T2b (plaque ± patch).
T3 One or more tumours (≥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; histopathology

Dutch grade 1 or NCI LN_{0-2}

N1a Clone negative

N1b Clone positive

N2 Clinically abnormal peripheral lymph nodes; histopathology

Dutch grade 2 or NCI LN_3

N2a Clone negative

N2b Clone positive

N3 Clinically abnormal peripheral lymph nodes; histopathology

Dutch grades 3e4 or NCI LN_4;

clone positive or negative

Nx Clinically abnormal peripheral lymph nodes; no histologic confirmation

Visceral

M0 No visceral organ involvement

M1 Visceral involvement (must have pathology confirmation and organ involved should be specified)

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 tumour 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 blood tumour burden: ≥1000/mL Sézary cells with positive clone

SS is staged as T4 N2/3/x M0 B2.

1.1.7.3 Prognosis

Clinical staging in CTCL patients is closely related to the prognosis. (Table 3).[48, 52]

Table 3. Prognosis in CTCL

	Stage						
	IA	IB	IIA	IIB	III	IVA	IVB
5 years global survival (%)	100	96	68	80	40	0	0
10 years global survival (%)	98	83	68	42	20	0	0
Median survival (years)	>32	12.6	10	4.7	3.9	2.5	1.7

Prognosis in MF patients is variable. Body surface area involved, type of lesions (patches, plaques or tumors) and the involvement of lymph nodes or viscera represent independent prognostic factors for survival.[47, 50, 51, 53-55] In fact, while patients with minor skin disease (<10% of the surface area) have an excellent prognosis (5-year survival around 97%), stage IV patients (including SS patients) have a worse prognosis, with a median survival of only 13 months.[52]

Advanced age is associated with a poorer overall survival (OS). Skin stage, folliculotropism, large-cell transformation (LCT), and elevated lactate dehydrogenase (LDH) are independently associated with worse OS. These prognostic factors gave rise to the prognostic index score, developed by the Cutaneous Lymphoma International Consortium study, for patients with advanced MF/SS.[51] Stage IV, age greater than 60 years, LCT, and increased LDH were combined into a 3-tier prognostic index model. These risk groups had significantly different 5-year survival rates regardless of patient stage (IIB–IV): low risk (68%), intermediate risk (44%), and high risk (28%).

1.1.8 Therapies

Since the first description of MF and SS, a great number of therapeutic options have been introduced ranging from topical steroids to multiple chemotherapy passing through immunotherapies (interferons, retinoids and photopheresis) and, more recently, also targeted approaches with humanized monoclonal antibodies. However, because in MF/SS the majority of available treatments are rarely able to induce long-term remissions, therapies are aimed at improving the quality of life. The rare exceptions to this are allogeneic stem cell transplantation (alloSCT) in advanced disease, particularly in SS.

Table 4. Therapeutical approaches in MF and SS

	Treatment
Early stage MF	
Topical treatments	- corticosteroids
	- nitrogen mustard
	- carmustine
	- rexinoid (bexarotene)
	- phototherapy: PUVA, UVB-nb
	- Photodynamic therapy (PDT)
	- radiotherapy
Early-stage refractory MF	
combined therapy	-UVA+psoralene(PUVA) or UVB(311nm) and IFN-α
	- UVA+psoralene(PUVA) or UVB(311nm) and low doses bexarotene
Advanced-stages MF and SS	
biological therapy	- IFN-α
	- retinoids/rexinoids (bexarotene)
	- extracorporeal photopheresis (ECP)
	- Alemtuzumab (anti CD52)
combined therapy	- IFN-α and phototherapy
	- IFN-α and retinoids/rexinoids (bexarotene)
	- retinoids and phototherapy
	- ECP and IFN-α
	- ECP and retinoids/rexinoids
systemic chemotherapy	
single agents	- pegylated doxorubicin
	- purine/pyrimidine analogs
	- pentostatin
	- gemcitabine
multi-agents	- CHOP and CHOP-like
Stem cells transplant	- autologus
	- allogeneic

- non-myeloablative allogeneic

Other experimental therapies

topical
- toll-like receptor (TLR) antagonists
- phophocholine

systemic
- Brentuximab (anti CD30)
- Mogamulizumab (anti CCR4)
- histone deacetylase inhibitors (HDAC)
  - -Vorinostat
  - -Resminostat
  - -Belinostat
  - -Romidepsin
- purine nucleoside phosphorylase inhibitors
- Denileukin diftitox (Ontak®)

1.1.8.1 Topical therapies

Differently from MF, in SS patients no topical therapies are really effective. However, topical agents can be useful as adjuvant therapy in the management of patients.

1.1.8.2 Systemic therapies

Retinoids (incl. bexarotene)

Retinoids are derivatives of vitamin A. All-trans retinoic acid, isotretinoin, etretinate, acitretin and, more recently, bexarotene and alitretinoin have been used for the treatment of cutaneous T-cell lymphomas alone or in combination since the early 1980s.[56, 57] Among these bexarotene stands out through its specific binding to the retinoid-X-receptor (thus termed a 'rexinoid'); it is the only member of the group that was specifically developed and has received approval for the treatment of CTCL.[58-61] According to its label, bexarotene is indicated for the treatment of cutaneous manifestations of advanced stage CTCL in patients who are refractory to at least one prior systemic therapy with a reported overall response rate of 45%. In clinical practice, bexarotene has been used as primary systemic therapy and has shown efficacy also in extracutaneous involvement.[60, 62, 63] The other most commonly used although not approved and less thoroughly studied retinoids are

acitretin (which has replaced its prodrugetretinate in the 1990s) and isotretinoin.[56] Due to heterogeneity of the published evidence and since no direct comparisons exist no conclusion as to superiority in clinical efficacy of one substance over the other can be made. Retinoids are generally well tolerated and share a common adverse effect profile with variable individual symptoms depending on the substance used. Most commonly observed are drying of the skin and mucous-membranes, elevated blood lipids, and in the case of bexarotene central hypothyroidism requiring thyroid hormone substitution in most patients.[64] All retinoids are teratogenic.

With retinoids as monotherapy moderate response rates can be achieved in MF/SS, the substances thus are commonly used in combination (see below) or in maintenance (see below) since they appear safe with long-term use.

Interferon (IFN)-α

Three types of recombinant interferons (IFN-α, IFN-β, IFN-γ) are currently available for therapeutic use with IFN-α existing also in a pegylated form. Therapeutic activity of IFN-α in CTCL was initially reported by Bunn et al., in 1984.[65] The same author some years later summarised the then pertinent evidence and concluded that all of the recombinant IFNs are active for the treatment of MF and SS.[66] However, only recombinant IFN-α has been studied in more detail, has received approval for the treatment of CTCL and remains the most widely used IFN in the treatment of MF/SS.[66] Various treatment and dose escalation schedules have been used with individual doses ranging from 3 million units (MU) to 18 MU applied subcutaneously either three times per week or daily. A commonly used regimen is to start with 3 MU three times weekly with dose escalation upon insufficient response and tapering for maintenance. Side-effects are dose dependent and include flu-like symptoms, elevated transaminases, leukopenia, thrombocytopaenia, and, probably under-recognized mental depression, cardiac arrhythmias, and thyroid dysfunction.[66, 67] Similar to the literature on the older retinoids (with the exception of bexarotene, see above), published evidence on theclinical efficacy of IFN-α suffers from heterogeneity in treatment schedule, patient selection, and methodology. Thus, reported overall response rates range from 0 to 80% without a clear correlation between dose and response.[66]

IFN-α combined with retinoids

Reports on the combined use of IFN-α and retinoids appeared beginning from the late 1980s.[68-71] Etretinate or isotretinoin have been used in these small heterogenous studies, which showed that the combination is tolerable without unexpected toxicity and is able to induce and maintain clinical responses. In the prospective randomised study by Stadler et al. acitretin was used in combination

with IFN-α and compared with the IFN-α /PUVA combination in 82 patients with early stage MF.[72] Although overall response rates did not differ between treatment groups (90.5% versus 90%, respectively) the rate of CR was higher with IFN-α /PUVA (70%) compared with the acitretin combination (38%). The study clearly shows that IFN-α plus PUVA is superior to IFN-α plus acitretin in terms of time to remission and CR rate. However, its results should not be interpreted as an argument to dismiss the latter combination since its efficacy, shown in earlier less stringent trials, was confirmed and it fulfils a need for combination therapy in patients insufficiently responding to monotherapy when access to PUVA is limited. More recently the combination of tretinoin (all-transretinoic acid) with IFN-α has been compared with IFN-α combined with low-dose methotrexate in an open prospective non-randomised trial.[73] Reportedly, both regimens were of similar efficacy and toxicity. In another small trial IFN-α was added to bexarotene upon incomplete remission after 8 weeks with no apparent benefit of the combination.[74] Taken together, the accumulated evidence confirms the clinical applicability of IFN-α retinoid combinations in MF. At the sametime it fails to demonstrate the superiority of any retinoid over the other and of the various combination regimens over monotherapy. Thus a combination of IFN-α and retinoids is recommended when monotherapy with either substance has failed and when the IFN-α /PUVA combination is contraindicated or unavailable.

IFN-α or retinoids combined with Skin directed Therapies (SDT)

The combination of PUVA with systemic retinoids was initially developed to improve efficacy and reduce potential side-effects of photochemotherapy in the treatment of psoriasis.[75] Subsequently, the concept was carried over to CTCL and investigated in a small series of uncontrolled studies and case collections.[76-78] Etretinate and acitretin were used in these studies from which no conclusion as to superiority of the combination over phototherapy alone can be made. With the systematic development and regulatory approval of bexarotene for the treatment of CTCL interest in the combination of this substance with phototherapy led to the publication of a number of reports.[79-83] Outstanding among these studies is a randomised phase III trial conducted by the EORTC Cutaneous Lymphoma Task Force where bexarotene combined with PUVA was compared to PUVA alone in early stage (IB-IIA) MF. The study was closed prematurely due to low accrual and thus did not reach its primary end-point (overall response rate). However, while confirming the safety of the combination its results indicate no significant difference in response rate and response duration between treatments.[81] The first small study about the use of combining IFN-α and PUVA for the treatment of CTCL appeared in 1990 and described complete remission in 12 out of a total of 15

patients.[84] A number of further small studies and case series followed[85-90] using various IFN-α dose schedules and PUVA regimens. Taken together these reports demonstrate that no increase in toxicity occurs with the combination but leave open the question whether it is more effective compared to monotherapy. Safety and efficacy IFN-α plus PUVA were confirmed by the above mentioned prospective trial[72] leaving, however, the issue of superiority compared to either monotherapy unresolved.

Other SDT that can be combined with systemic treatments are topical corticosteroids, nbUVB and localised radiotherapy (see above). Although not systematically studied these options are used based on institutional and personal experience and might prove useful on an individual basis. In summary, current evidence does not support the use of combinations of SDT with systemic therapies as firstline option in early stages of MF. However, when systemic therapy is indicated in more advanced stages adding on of an effective SDT might shorten time to response and alleviate symptoms more quickly and effectively.

Extracorporeal photopheresis

Extracorporeal photopheresis (ECP; which has also been variously called photopheresis, extracorporeal photochemotherapy, or extracorporeal photoimmunotherapy) is a form of phototherapy where blood is exposed extracorporeally to the photoactivated drug 8-methoxypsoralen (8-MOP). The use of ECP was first reported in 1987 by Edelson et al. in CTCL for which it is approved in Europe and the US.[91] Other indications where ECP is used include systemic sclerosis, graft-versus-host disease, solid organ transplant rejection, and Crohn's disease.[92] ECP has an excellent safety profile with almost absent adverse events and details on the recommended prescription, schedule, and other practical issues have been recently published.[93] Since the original publication by Edelson et al. who reported a response rate of 73% (with most of the patients having T4 disease) a number of case series and retrospective studies confirming the efficacy of photopheresis particularly in patients with erythrodermic MF and SS have been published with response rates around 60%.[94-96] Remarkably in most of these reports ECP was used in combination with other agents and modalities, including retinoids, interferons, PUVA, and others, demonstrating on the one hand that ECP can be safely combined with many other agents available for the treatment of MF/SS, and leaving open, on the other hand, the question of superiority of any combination over the other and over monotherapy.

Targeted immunotherapy

Since the introduction of monoclonal antibodies into cancer therapy in the 1990s a number of

recombinant immunoglobulins and other protein constructs have also been developed for and tried in non-Hodgkin lymphomas, with rituximab as a most remarkable example of success in B-cell lymphomas.[97] Some agents have also demonstrated activity in CTCL and it is to be expected that in the near future new antibodies and antibody-constructs will enter the clinics.[98] Denileukin diftitox was developed for the treatment of CTCL and became the first fusion toxin to be approved. It is a recombinant protein consisting of interleukin (IL)-2 linked to the catalytic domain of diphtheria toxin genetically engineered with the intention to target cells expressing the IL-2 receptor.[99] Its activity in the treatment of CTCL has been demonstrated in two phase III trials with overall response rates of 30% and 44% and an acceptable safety profile although grade 3 and 4 capillary leak syndrome was observed in 4% of patients.[100, 101] Denileukin diftitox is currently unavailable and did not obtain marketing authorisation in Europe.

Alemtuzumab is a humanised recombinant IgG1 monoclonal antibody against the CD52 cell surface glycoprotein, which is expressed on normal and malignant B and T lymphocytes but not on haematopoietic progenitors. Alemtuzumab was initially developed and approved for the treatment of lymphoid malignancies. More recently its immunosuppressive effects have been utilised to successfully treat multiple sclerosis.[102, 103] Although alemtuzumab is currently commercialised only for multiple sclerosis it is still available for the treatment of lymphoid neoplasms through a special access programme. Overall response rates of more than 50% have been obtained in MF/SS using the standard dose of 30 mg intravenous (i.v.), three times weekly. At this dosage immunosuppression and opportunistic infections are the most common, sometimes severe adverse events.[104-106] From these studies and a recent long-term observation it appears that alemtuzumab is effective primarily in patients with erythroderma (T4) and blood involvement (B1) and may be able to induce long-term remissions in selected patients.[107] With the intention to reduce toxicity while maintaining efficacy low dose regimens have been introduced.[108-110] Doses up to 15 mg s.c. every other day were used and in small patient series response rates similar to those reported from earlier studies were observed without relevant infectious complications when single doses did not exceed 10 mg.

Brentuximab vedotin is an antibody-drug conjugate consisting of an anti-CD30 IgG1 antibody attached to monomethyl auristatin E, a microtubule-disrupting agent, through a protease-cleavable linker.[111] Upon internalisation into CD30 expressing cells the linker is cleaved and monomethyl auristatin E released into the cell to induce cell cycle arrest. The drug is currently approved in Europe and the USA for the treatment of adult patients with relapsed or refractory

CD30+ Hodgkin lymphoma (HL), patients with CD30+HL at increased risk of relapse or progression following autologous stem cell transplantation, and adult patients with relapsed or refractory systemic anaplastic large cell lymphoma (sALCL). The safety and efficacy of brentuximab vedotin in CTCL has been investigated in two phases II and one very recently reported phase III trials.[112, 113] In one of these studies 32 patients with MF/SS and any level of CD30 expression were included. An overall response rate of 70% observed in patients with a wide range of CD30 expression and a lower likelihood of response if CD30 was expressed in less than 5% of cells as assessed by immunohistochemistry.[113] In the other study 48 patients with CD30+ CTCL (incl. lymphomatoid papulosis, primary cutaneous anaplastic large cell lymphoma, and CD30+ MF/SS) were included with an overall response rate of 73% in the total study population and of 54% in patients with MF/SS. The main toxicities consisted of peripheral neuropathy, that can be dose-limiting, severe, and long-lasting, neutropenia, that can be severe; fatigue, nausea and alopecia.

Mogamulizumab is a humanized monoclonal antibody targeting the CC chemokine receptor 4 (CCR4) expressed on tumour cells of adult T-cell leukaemia-lymphoma (ATLL) and other T-cell lymphomas. The antibody is modified in the composition of its carbohydrates ('glyco-engineered') to enhance its antibody-dependent cell-mediated cytotoxic (ADCC) activity.[114] Currently the drug is approved in Japan for relapsed or refractory CCR4+ peripheral T-cell lymphoma and CTCL. In 3 early phase studies a total population of 48 patients with relapsed CCR4+ CTCL, pretreated MF and SS were treated with mogamulizumab with overall response rates between 38% and 29% mainly in leukaemic CTCL variants. Reported side-effects were mostly low grade and included chills, fever, rash, nausea, headache and infusion-related reactions.[115-117] In 2018 mogamulizumab was approved by the Food and Drug Administration (FDA) for the treatment of refractory MF and SS after at least one prior systemic therapy.[118] Moreover in 2020 also the Agenzia Italiana del farmaco (AIFA) approved mogamulizumab for the treatment of MF and SS after a prior systemic therapy.

Haematopoietic stem cell transplantation

The first transfer of haematopoietic stem cells from allogeneic bone marrow to terminally ill patients was published in 1957 by E. Donnall Thomas who was awarded the Nobel Prize for his achievements in 1990.[119] In the meantime the technique has been refined through advances in immunological understanding and with the development of efficient protocols for stem cell collection from peripheral and umbilical cord blood, conditioning and support of engraftment after transplantation. Major indications today still include haematological malignancies but have been extended to hereditary bone marrow disease such as thalassaemia and sickle cell anaemia. The first report on autologous stem cell transplantation (ASCT) after total body irradiation in MF appeared in 1991 and described complete remission in five out of six patients with early relapse in three of the responders.[120] Other small case series confirmed that although aggressive treatment with ASCT rescue is feasible and able to induce remissions almost all patients will eventually relapse.[121] Consequently this approach has been abandoned in MF/SS and is not recommended in this consensus. With allogeneic stem cell transplantation (alloSCT) on the other hand durable remissions have been achieved in CTCL and (with the exception of localised radiotherapy for unilesional MF) remains the only treatment option in MF/SS with curative intention. The published evidence from retrospective studies and case series on alloSCT in CTCL comprises nine studies on a total of approximately 250 patients.[122-130] A comprehensive summary and review has been published recently.[121] Both, myeloablative and reduced-intensity conditioning have been used with similar efficacy and lower complication rates including reduced non-relapse mortality (NRM) and lower rates of chronic graft versus host disease (GvHD) in the latter. Graft versus lymphoma (GvL) effect appears to be important for induction and maintenance of remission and donor lymphocyte infusions and tapering of immunosuppression have been demonstrated to induce secondary remission. In the study with the longest reported observation time overall survival was 46% and 44% at 5 and 7 years after transplant, respectively, with 22% NRM [165].[130] In summary, alloSCT, particularly using reduced-intensity conditioning, is able to induce long-term remissions in a substantial percentage of patients with MF/SS although at the price of a high rate of treatment related morbidity and mortality. Consequently, patient selection is difficult, requires careful counseling and should focus mainly on younger, well performing patients suffering from advanced stages of the disease, with a low tumour burden at the time of transplantation and at the same time a high predictable risk of progression and poor prognosis.

Histone deacetylase inhibitors

Histone deacetylases (HDAC) are a class of ubiquitously expressed enzymes, that catalyse the removal of acetyl groups from histones and by this are key regulators of epigenetic regulation of transcription. Specific pharmacological inhibitors of HDAC have been developed and investigated in preclinical and clinical studies for their potential as novel anti tumour agents that work through modification of the epigenetic aberrations associated with cancer.[131] Based on the results of pivotal trials three substances, vorinostat, romidepsin, and belinostat are currently approved by the FDA for treatment of cutaneous manifestations in patients with cutaneous T-cell lymphoma (CTCL) who have progressive, persistent or recurrent disease on or following two systemic therapies (vorinostat); treatment of cutaneous T-cell lymphoma (CTCL) in patients who have received at least one prior systemic therapy and treatment of peripheral T-cell lymphoma (PTCL) in patients who have received at least one prior therapy (romidepsin); and for treatment of patients with relapsed or refractory peripheral T-cell lymphoma (belinostat).[55, 132-136] Efficacy and toxicity of these substances are similar with a reported overall response rate of about 30% and class-as well as substance specific toxicities, most notably gastrointestinal side-effects, thrombocytopaenia, QTc prolongation, and deep vein thrombosis with vorinostat. New substances are in development and the clinical efficacy and toxicity of HDAC inhibitors in CTCL have been recently reviewed.[137]

Chemotherapy

Conventional single agent and combination chemotherapy have been used for the treatment of non-Hodgkin lymphoma since the 1970s with the (C)yclo-phosphamide-(H)ydroxydaunorubicin-(O)ncovin-(P)rednosone or (P)rednisolone [CHOP] regimen evolving as a long-standing standard option for aggressive disease. At the same time this and a number of other combinations and single agents have been tried in CTCL with variable, but generally short-lived success.A comprehensive review on these early experiences is published.[138] Already in 1989, the results of a seminal prospective randomised trial comparing early aggressive with stage-adapted therapy restricted (poly-)chemotherapy to patients with advanced disease, a restriction still applying today [8]. In the meantime novel chemotherapeutic agents with activity in MF and SS have been developed. Among these, promising results with acceptable toxicity have been obtained with pegylated liposomal doxorubicin[139-144] and gemcitabine.[145-149] Treatment regimens in these studies largely followed established dosage recommendations as described for their approved indications. In an EORTC-sponsored prospective multicentre trial Dummer et al. could demonstrate an acceptable safety profile and an overall response rate of 40.8% in 49 patients with pre-treated (2 previous therapies) advanced stage (IIB, IVA, or IVB) MF using pegylated liposomal doxorubicin at 20 mg/m^2

biweekly. Median duration of response was 6 months, similar to what has been reported for other chemotherapy regimens in this high risk population.[143] Gemcitabine was also investigated in combination with bexarotene in a phase II protocol resulting in poor response rates and increased toxicity compared to the single agents leading to the conclusion that this combination should be avoided.[150] A number of other cytotoxic agents have been tried in CTCL including the purine analogues (deoxycoformycin, 2-chlorodeoxyadenosine, fludarabine), bendamustine and others.[151-156] However, limited published evidence precludes inclusion of these substances in the present recommendations.Two other chemotherapeutic agents are included in these recommendations and thus will be mentioned briefly: Chlorambucil is an alkylating agent that was developed in the 1950s for the treatment of chronic lymphocytic leukaemia and non-Hodgkin lymphomas.[157] It can be administered by mouth. In combination with low dose prednisone it was introduced for the treatment of SS in the 1970s by Winkelmann.[158, 159] The original regimen consists of continuous treatment with 2-6 mg/day of chlorambucil and prednisone at an initial dose of 20 mg/day to be tapered to 0-10 mg/day. Although more recently a variant with intermittent dosing was described in a small patient series to be as effective as the original regimen the original prescription is still recommended.[160] However, since in addition to myelosuppression prolonged exposure to chlorambucil carries a leukemogenic risk long-term continuous use should be avoided.[161] Methotrexate was developed as a cytotoxic antifolate in the wake of the 1950s breakthrough of anticancer chemotherapy for the treatment of childhood leukaemias.[162] Soon afterwards its usefulness for treatment of psoriasis and rheumatoid arthritis was demonstrated and low-dose once-weekly methotrexate has become a well-tolerated, standard treatment for non-oncological conditions.[163] There are only few studies on the use methotrexate in various dosing for the treatment of MF/SS that have been reviewed earlier [9]. Since then, additional experience on the safe combination of methotrexate with bexarotene and IFN-α, respectively, have been published.[73, 164]
No conclusion, however, as to the superiority of these combinations over monotherapy is possible and no recommendation as to the optimal use of these regimens can be made.

1.2 Genomic studies

In the last decades, many molecular studies on SS were carried out both before and after Next generation sequencing (NGS) era, such as array-based Comparative Genomic Hybridization (a-CGH), Gene Expression Profiling (GEP), microRNA profiling or sequencing studies, namely Whole Genome sequencing (WGS), Whole exome sequencing (WES) and RNA-sequencing. All these studies highlighted numerous copy number alterations, structural changes such as

translocations or gene-fusions and many mutations. However, no recurrent aberrations nor a specific profile, were found. Different studies evidenced the possible pathogenic role of some recurrent genes such as CyclinD and its repressors p15 and p16, RB1, p53, PTEN, PI3K, AKT, MYC, RAS family, NF-kB and genes encompassed in JAK/STAT signaling pathway.

The most involved pathways underlined by these studies are apoptosis (FAS and TNFRSF10A), the JAK-STAT pathway (JAK1, JAK3, STAT3, STAT5B), the NF-κB pathway (TNFRSF1B and NFKB2), TCR signaling (PRKCQ, PDCD1, CD28, CARD11, and PLCG1), and effectors of T cell differentiation and skin homing (ZEB1 and CCR4). Recurrent mutations are also found in the MAPK pathway (KRAS, BRAF, MAPK1), the PI3K/AKT pathway (PTEN and RHOA), epigenetic regulators (ARID1A, DNMT3A, and KMT2C), cell cycle regulators (CDKN2A and RB), and regulators of genome integrity (TP53 and ATM).

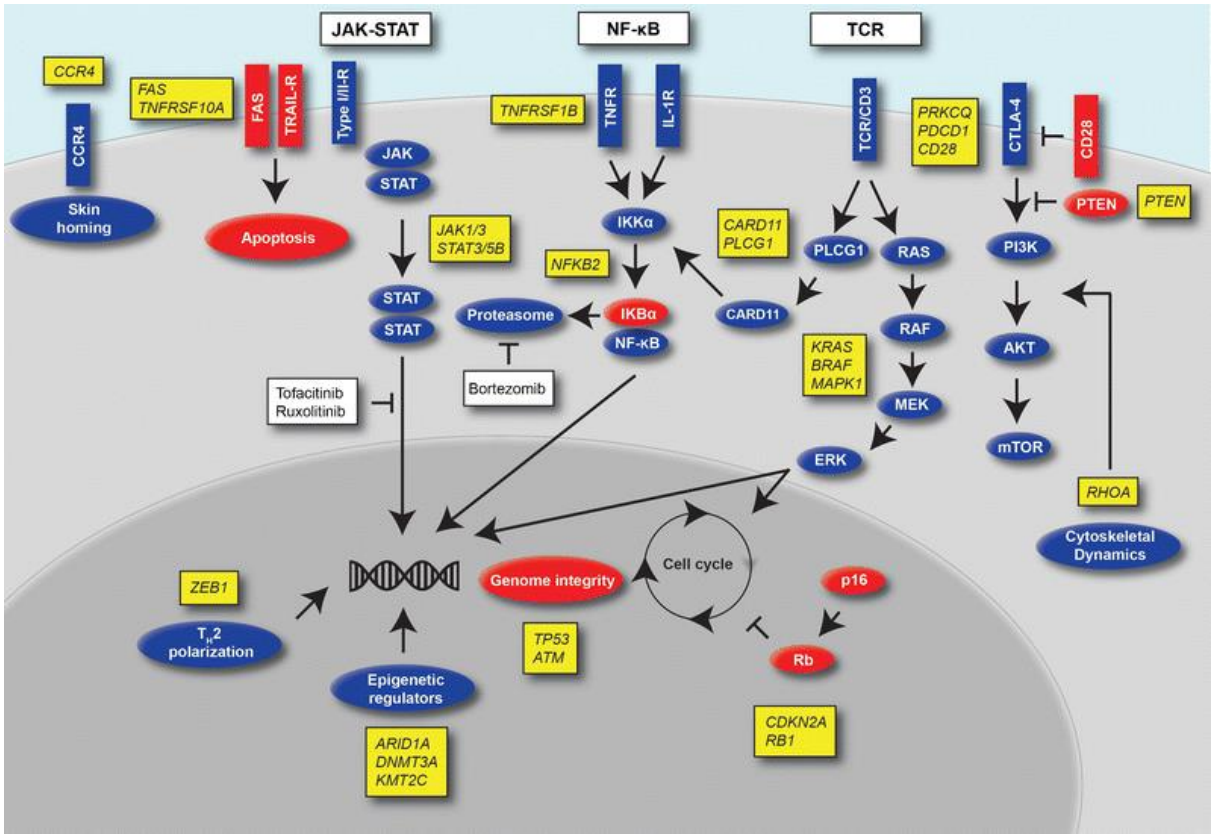

Figure 5. Recurrently mutated signaling pathways in CTCL[165]

1.2.1 NFkB

From a mechanistic point of view, CCR4 overexpression has been described specifically in transformed CTCL compared with nonmalignant cells,[166-168] and its inhibition can lead to CTCL cell death concomitant with suppression of Treg activity.[169] In contrast, different mutations affecting members of the NFkB pathway (such as TRAF6, RELB, or CARD11) have been found by Vaqué et

al[170]. It is well-known that NFkB activity regulates the expression of multiple key T-cell cytokines such as CCL17 and CCL22 (CCR4 ligands) or interleukin 4 (IL-4), IL-6, and IL10, which may confer survival advantages in an autocrine or paracrine manner through the activation of the JAK/STAT pathway.[170]

1.2.2 JAK-STAT signaling

Recurrent mutations in the JAK-STAT pathway are widely described in CTCL.[34, 35, 171-173] Activation of JAK-STAT signaling normally occurs after binding of cognate cytokines to cytokine receptors at the cell surface. This results in STAT protein dimerization leading to nuclear translocation and transcriptional activation of genes regulating T cell proliferation, differentiation, and apoptosis. The JAK-STAT pathway has previously been shown to be hyperactivated in most CTCLs by several groups.[39, 174-177]

Recurrent gain-of-function mutations predicted to hyperactivate JAK1, JAK3, STAT3, and STAT5B[35, 171, 173, 178] were found. Moreover, some authors described mutations in JAK1/JAK3[35, 173, 178] which has been shown to lead to constitutive kinase activation[179, 180] and mutations in STAT3/STAT5B, clustered in the portion of the gene encoding the Src-like homology 2 (SH2) domain, which is responsible for protein dimerization.[35, 171] These activating SH2 domain mutations lead to increased protein phosphorylation, dimerization, and nuclear localization.[181] In addition, copy number gains of STAT3 and STAT5B were also common,[171] a change that has previously been shown in other cancers to be oncogenic in itself.[182]

These mutations are potentially targetable with JAK or STAT inhibitors. Tofacitinib is an oral JAK1/3 inhibitor recently FDA-approved for the treatment of rheumatoid arthritis[183] and psoriatic arthritis.[184] Ruxolitinib is an oral JAK1/2 inhibitor, recently FDA-approved for the treatment of polycythemia vera,[185] myelofibrosis[186] and acute graft versus host disease.[187] McGirt and colleagues showed that tofacitinib inhibited proliferation of JAK3-mutant CTCL cell lines.[173] Follow-up work showed that ruxolitinib was broadly active in CTCL cases with both JAK1 and JAK3-activating mutations.[178] Similarly, da Silva Almeida and colleagues found that both tofacitinib and ruxolitinib exhibited high antitumor activity against a JAK3 mutant CTCL cell line.[35] It remains to be seen whether these agents have any clinical activity in patients and, if so, whether mutational status predicts responses. In other hematologic malignancies, the ability of these agents to induce remission has been somewhat limited because of the development of drug resistance.[188]

1.2.3 Other oncogenes and tumor suppressors

Focal deletions were described in other well-known tumor suppressor genes such as PTEN, RB1,

CDKN2A, ARID1A, DNMT3A and ZEB1.[34, 35, 171, 189-191]

Studies using both comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) array technologies demonstrated that SS is characterized by specific chromosomal gains and deletions. Among these, loss of chromosome 10q, including the PTEN locus at 10q23, appears to be particularly common in these individuals.[192-194]

PTEN is a nonredundant lipid phosphatase whose main role is to antagonize the PI3K signaling,[195] a relevant pathway for cancer development with several members representing potential targets for therapeutics. PTEN is one of the most frequently lost or mutated genes in human cancers,[196] including T-cell malignancies as T-cell acute lymphoblastic leukemia[197] and CTCL, as already demonstrated by loss of heterozygosity (LOH) found within the PTEN locus of mycosis fungoides (MF).[198]

PTEN plays a critical role in T-cell development; in fact, conditional loss of PTEN in thymocytes leads to T-cell lymphomas. Conversely, PTEN deletion in mature T-cell subsets induces autoimmunity, increased cytokine releasing, and, in general, a PI3K/AKT hyperactivation through T-cell receptor also in absence of CD28 co-stimulation, thus indicating that PTEN imposes stringent environmental signals for an appropriate T-cell activation.[199]

In addition to the mutations and genetic alterations, PTEN abundance appears to be finely regulated at the transcriptional/translational level by microRNAs (miRNAs),[195] competitive endogenous pseudogene RNAs,[200] and promoter hypermethylation,[201] and at the posttranslational level by phosphorylation and ubiquitination.[201] Defects of all of theese mechanisms may have critical consequences for PTEN levels and tumorigenesis.

Mao et al, showed SS cases with CCND1 copy number loss also having RB1 loss and suggested that that CCND1 and RB1 losses may result from chromosome instabilities occurring in CTCL progression.[202] In fact, there are some evidence showing an association of RB1 deletion/inactivation and copy number loss of CCND1.[203, 204]

ZEB1 is a zinc-finger-containing transcriptional repressor of IL2. In normal T cells it is essential for the correct development during hematopoiesis.[205-209] ZEB1 is a candidate tumor suppressor gene in adult T-cell leukemia/lymphoma (ATLL), where it contributes to TGF-β1-mediated growth suppression resistance of malignant CD4+ T cells and ZEB1 mutant mice frequently undergo spontaneous CD4+ T-cell lymphomas.[210, 211] Wang et al firstly described a significant focal deletion in 10p11.23 including ZEB1.[34] Moreover, Caprini et al reported the same focal loss with a correspondent transcript downregulation of ZEB1 in SS. In the same paper, they demonstrated a

tumor suppressor role for ZEB1 in SS by survival analysis showing a worse outcome for patients carrying ZEB1 homozygous loss. Using ZEB1 knockout cell lines, they verified that ZEB1 has a role in controlling intracellular reactive oxygen species production affecting viability and apoptosis of SS cells.[191]

Another putative tumor suppressor described as harboring inactivating mutations in SS patients is ARID1A.[34] Functional studies suggested a role of ARID1A in cell cycle checkpoint machinery, possibly through p53-and/or pRb-dependent signaling cascades.[212] Moreover, ARID1A is required for cell cycle exit, since in the absence of functional ARID1A, cell cycle arrest is delayed.[213]

All these results suggest the presence of a high genomic instability in neoplastic cells, probably a secondary event, considering that no tumor-driving events were demonstrated. A possible role of epigenetic factors remains unclear and still needs to be elucidated.

1.2.4 Genes and deregulated pathways involved in apoptosis

One of the suggested pathogenic mechanism in SS is apoptosis dysregulation. Defects in apoptosis are presumed to lead to accumulation of the malignant T cells in vivo. Multiple defects of proapoptotic pathways have been identified, including decreased expression of Fas (CD95), which may be due to hypermethylation of the Fas gene promoter.[214, 215] Other defects include increased expression of the apoptosis inhibitor cFLIP, as well as tumor cell loss of TRAIL-receptor 2 leading to resistance to TRAIL.

Loss of functional p53 activity has been reported in many cancers and deletion of 17p13.1, which includes the TP53 gene locus, has been described in SS.[192, 216] Moreover, Lamprecht et al has recently demonstrated that p53 is nonfunctional in the majority of SS cell lines and patient samples.[216]

Cell death resistance in SS is maintained upstream of the NFκB and MAPK pathways through altered T-cell receptor signaling and activation-in-duced cell death (AICD). These alterations are mainly cau-sed by alterations in phospholipase γ1 (PLCγ1) activity.[217] The exact role of PLCγ1 is controversial in the literature, as both protein deficiency – leading to decreased calcium- and ROS-induced AICD – as well as activating mutations – resul-ting in enhanced NFAT, MAPK, and NFκB signaling – have been described.[170, 217, 218] Thus, further studies are required to ascertain the exact significance as well as the frequency of these mutations. AICD resistance in SC is further maintained by an overexpression of the E3 ubiquitin ligase c-CBL[219] and defective function of T-cell FAS signalling.[215, 220, 221]

1.3 MicroRNAs

microRNA (miRNA) are small non-coding RNAs that regulate gene expression via post transcriptional silencing of target genes. MicroRNAs are a group of small single-stranded non-coding RNAs that have been identified in many organisms.

They have important roles in fundamental biological processes such as cell development, differentiation, proliferation and apoptosis.

Their fundamental role is that of negatively regulate gene expression at the post-transcriptional level (post-transcriptional gene silencing, PTGS).

MiRNAs act by recognizing specific mRNA targets in order to determine their degradation or repression of translation.

The function of many RNA is not known, but some of them have been demonstrated having a role in physiological and pathological processes:

• They can have a role in proliferation, apoptosis and cell differentiation.

• They can be regulated in human diseases

• They can be involved in tumor origin.

1.3.1 MicroRNAs in Sézary syndrome

To the best of my knowledge, there are few papers describing a possible involvement of miRNAs in SS. The most described deregulated miRNAs are mir214, mir199a and mir486.[222-224] These miRNAs resulted upregulated in different studies. In fact, Narducci et al in 2011 found these three miRNAs as the most upregulated by a microRNA expression profiling experiment. In 2012, Qin et al, revealed the same three small RNAs to be the most differentially expressed in SS by a deep-sequencing approach.[223] In 2017 Benoit et al confirm the high expression of mir214 in SS cells.[225] Mir199a was firstly described in 2011 by Ballabio et al,[226] and later, Narducci et al[222] and Qin et al[223] confirmed the upregualtion of this miRNA.. Other miRNAs were described as deregulated in SS. For example, mir21, mir181 and mir122 were found upregulated,[227, 228] while mir31 and let-7 family members were found downregulated in one study.[222]

1.3.2 Let-7 family members

Let-7 family members are widely reported as tumor suppressor miRNAs and they are strongly downregulated in many cancers.[229-234] Particularly let-7a is known to target many oncogenes including c-Myc indeed its upregulation inhibits cell proliferation by reducing the expression of c-Myc.[235]

Another target of let-7a is c-MET and its downstream signaling pathways such as RAS, PI3K, STAT3 and β-catenin pathways[235, 236] Let-7b has several oncogenes as targets such as HMGA1, HMGA2, CCND2 and KRAS, and loss of expression of let-7b has been demonstrated in many cancers comprising acute lymphoblastic leukemia.[237]

Cancer stem cells, tumor microenviroment, inflammation and epithelial to mesenchimal transition (EMT) are all systems that concur to development, progression and aggressiveness of tumors.[238-240] To note that members of let 7 family are strongly invovolved in disregualtion of these systems.[235]

Let-7 was shown to be downregulated in many cancers[241] whereas re-expression of the let-7 suppressed the growth of tumor cells. Moreover, let-7 and some of its targets, such as IL6, STAT3, Akt and NFκB, are directly involved in the switch from inflammation to cancer.[242, 243] Two important let-7 targets are HMGA1 and HMGA2 genes, which are implicated also in the transition from epithelial tio mesenchymal.[244, 245]

It is well demonstrated that let-7 family members negatively regulate IL6,[242, 246] RAS genes,[247] c-Myc,[248] HMGA1,[249-251] HMGA2 ,[250-252] and CCND1.[253, 254] Notably, all these genes are involved in the pathogenesis of a wide range of tumors including SS.(Figure 6).

Many studies demonstrated that downregulation of let-7 is due to its direct interaction with Lin28A or Lin28B. Lin28 depletion resulted in specific increases in all let-7 family members.[255-258] On contrast Nishi et al demonstrated that let-7b is downregulated in leukemic cells via DNA hypermethylation of its regulatory region.[259] Moreover in pancreatic cancer cell lines let-7 downregulation has been reported to be linked to a constitutively-active STAT3.[249]

MYC is at the same time target and regulator of let-7s. Indeed, the expression of let-7 members is controlled by c-Myc protein which binds to their promoters. It is known that let-7s are downregulated in cell lines with MYC overexpression and that inhibition of c-Myc through the use of chemical agents leads to increased expression of let-7 members demonstrating a direct action of c-Myc on these miRNAs. The presence of binding sites for let-7 was highlighted in the 3' UTR region of MYC. According to the proposed model, let-7 members would be able to directly bind the MYC promoter and to inhibit it. It has been shown that let-7 overexpression in cell lines reduced MYC mRNA levels. Therefore, there is a double negative feedback loop between MYC and let-7.

Additionally, the c-Myc protein regulates the biogenesis of let-7 also by the stimulation of Lin28,[260] which can block the let-7 maturation.[261] Moreover, c-Myc stimulates the expression of HMGA1[262] and IL6.[246] NRAS is suggested to have an impact on HMGA2 biogenesis.[263] HMGA2 on the other hand influences HMGA1, its gene product in turn regulates the expression of c-Myc.[264] (Figure 6).

Hillion and colleagues reported a positive correlation between HMGA1 and STAT3 in a subset of primary human acute lymphoblastic leukemia samples.[265] In line with this, HMGA1 was described

to bind the STAT3 promoter and to upregulate its expression in malignant human hematopoietic cells[265] (Figure 6). The transcription factor STAT3 mediates uncontrolled growth, angiogenesis, and survival of cells and has a great potential as target in cancer therapies.[266] Remarkably, Iliopoulos et al. identified STAT3 binding sites in the promoters of the miRNAs miR-181b and miR-21[243] (Figure 6). These tiny regulators in turn where found to block PTEN (Figure 6), stimulating the activity of NFκ B.[243] The tumor suppressor PTEN functions as an antagonist of PI3K by dephosphorylating its product PIP3[267] (Figure 6).

As interleukin 6 plays a pivotal role during the transition from innate to acquired immunity, IL-6 has been shown to skew T-cell differentiation towards Th2;[268] thus, it makes IL-6 a promising lead in the context of CTCL pathogenesis. IL-6 was initially considered to be a pro-inflammatory cytokine; however, we currently know that it also has anti-inflammatory activity.[269] Moreover, IL-6 is particularly interesting due to its involvement in the differentiation of immune cells toward Th2 phenotype by STAT3 signaling pathway. Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is responsible for cytokine signaling resulting in proliferation, migration and survival of cell. Deregulation of JAK/STAT pathway has been demonstrated in various cancers.[270] In particular, constitutive activation of STAT3 has been shown to play an essential role in CTCLs, particularly in SS.[39, 40, 175-177, 181, 192, 224] JAK/STAT pathway deregulation seems to be crucial also for CTCL progression.

Finally, in two independent studies (Narducci et al.[222] and Corti et al.[271]) a downregulation of some members of let-7 family, particularly let-7a and let-7b, was demonstrated in SS by using microRNA microarrays.

Particularly, in a previous unpublished study from our group we found a clear split in miRNA expression between SS patients and controls. Of particular interest let-7b was the most downregulated in our patients series and comparison of miRNA profile and CGH data showed that all patients with gains in 8q (MYC) have also let-7b downregulation.[271] We found 24 miRNA differentially expressed between SS patients and healthy controls, including miR-214, miR-486 and miR-21 yet described and validated by Narducci et al.[222] and Qin et al.[223] Amplification of 8q (MYC) found on the same patients with let-7b downregulation and LIN28/let-7b link with STAT3/miR21 activation suggest a possible key role of let-7 family members (particularly let-7b) in SS. Finally, let-7 family members expression lead to apoptosis by Bcl-XL repression. In a recent study, Adams et al demonstrates that, in a wide group of haematologic malignancies, Let-7b downregulation lead to apoptosis blockage and that restoring let-7 normal expression downregulates Bcl-XL with a consequent upregulation of caspase-3 and apoptotic activity in neoplastic cells.[272]

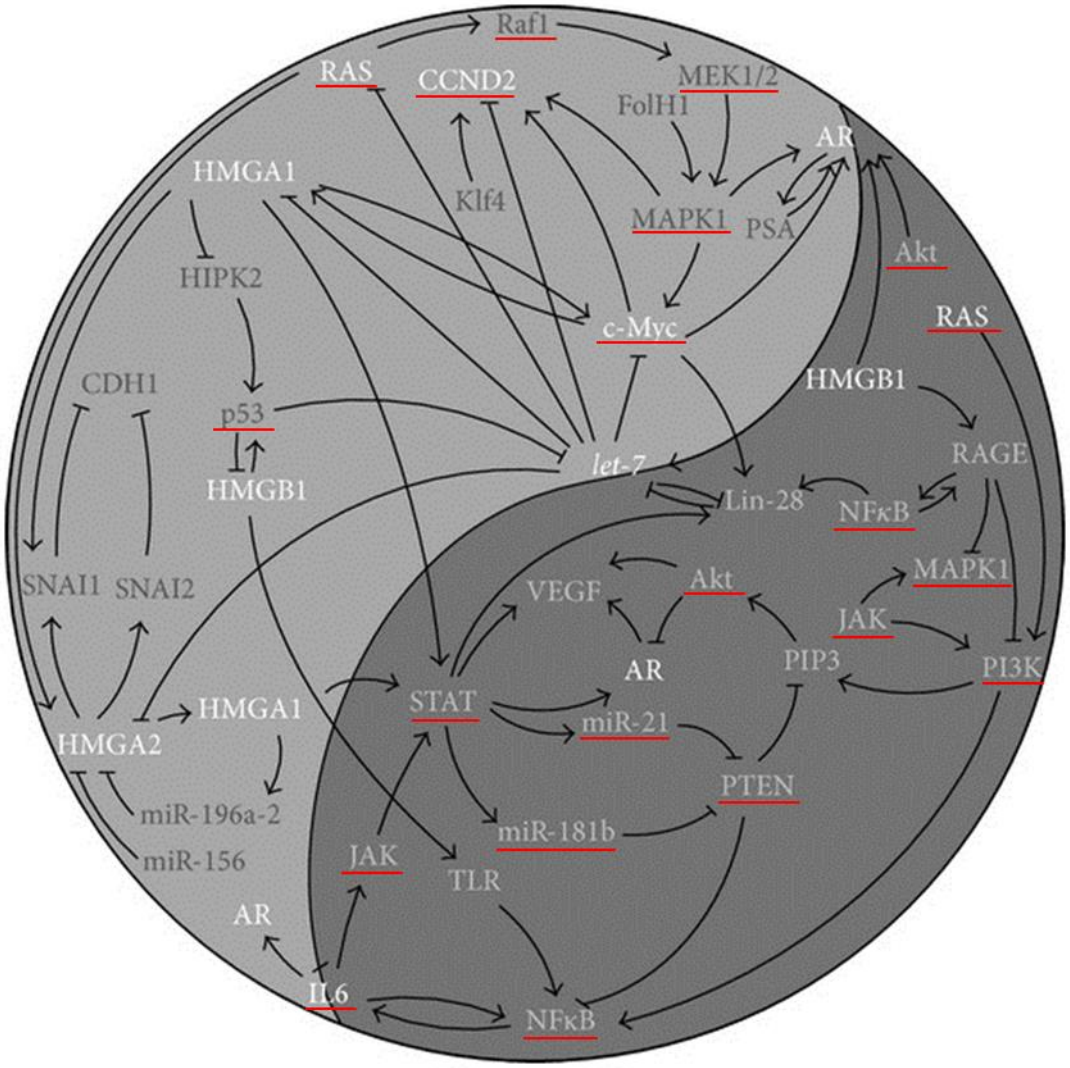

Figure 6.[235] This figure represents the described interactions between let-7 and the reviewed let-7 associated targets (in white letters) with other genes which are as well commonly deregulated in human cancers (in gray letters). The indicated interactions are on transcriptional, posttranscriptional or posttranslational level. Many genes are also been described to have a role in SS (underlined in red).

2 AIM OF THIS STUDY

The goal of this project was to study in deep some data obtained in our previous miRNA profiling study [271] in which 24 miRNAs were evidenced to be de-regulated in SS with respect to healthy donors used as controls. Among these, some miRNAs as miR21, mir199 and mir233 have been already described to have a role in SS pathogenesis. On the other hand, let-7 family members' that we found strongly downregulated in SS are not yet investigated in detail in this neoplasia.

A databank-based search about let-7 showed that they interact with many molecules widely described as main actors in SS such as c-Myc and STAT3, both negatively regulated by let-7b and both hyperactive in SS (REF) or with miR21 (overexpressed in SS) which is activated by STAT3 and possibly indirectly regulated by let-7b (REF): Another study proposed a mechanism feedback loop involved during the switching between inflammation and cancer.[243]

In this process the main molecules involved are again many of the oncogenes and onco-suppressors yet described in SS such as STAT3, miR21, PTEN, Akt e NF-kB. In the proposed feedback let-7b and its repressor Lin28B should play a key role. Moreover, it is well demonstrated that in about 15% of tumors one between Lin28A or B (the direct repressors of let-7 microRNAs) is re-activated leading to a let-7 downregulation and to a hyper- expression of let-7 targets, such as KRAS, MYC and HMGA2. These tumors are refractory to many therapies and give rise to recurrent and more deadly disease. In fact, Lin28A/B via a let-7 dependent mechanism promotes metastasis and resistance to several frontline cancer treatments including ionizing radiation and multiple chemotherapies (including Gemcytabine, commonly used in SS).

Overall these data indicate that Let-7/Lin28 could play and important role in SS e for this reason, I decided to investigate in detail on this topic, taking advantage from a large cohort of SS samples, through the following tasks:

1) to assess expression levels of let-7b, let-7c by means of qRT-PCR analysis
2) to assess the concurrent expression levels of Lin28A and Lin28B in order to verify a possible relationship between Let7 and Lin28 molecules by means of qRT-PCR analysis
3) to assess the relationship between let7 expression levels and response to therapy

As apoptosis is considered one of the pathologic mechanisms involved in SS and let-7 is involved in apoptosis regulation I investigated a possible relationship between let7 expression level and up-

regulated anti-apoptotic protein levels highlighted in our SS patients by protein array and immunohistochemistry

3 MATERIALS AND METHODS

3.1 Patients and controls

For this study patients affected by SS and healthy donors were recruited at Dermatology Unit of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico of Milan.

From each SS patient a tissue sample (skin biopsy) and a peripheral blood sample using Ethylene Diamine Tetraacetic Acid (EDTA) were collected.

As controls EDTA peripheral blood samples was collected from healthy donors. Each group (patients and controls) released their informed consent according to the Declaration of Helsinki Principles.

At the time of surgery, all tissue samples were formalin fixed and then paraffin embedded for histopathological analysis.

Diagnosis of SS was made according to WHO classification, considering clinical aspects, flow cytometry results, histology and T-cell clonality. In this study were included 26 SS patients (15 males and 11 females) with a median age of 69 years (ranging from 17 to 91). Moreover 15 healthy donors (HD) and 3 cell lines derived from CTCL patients, namely Hut78, HH and H9, were included in the study. Cell lines were suitable only for qRT-PCR experiments. Depending on material availability patients and HD were analyzed for qRT-PCR, protein analysis and IHC experiments as summerized in Table 7

For each patient follow-up data and response to therapy were collected.

Table 7. Baseline characteristics and analysis performed on patients and healthy donors

Patient n°	Gender	Age	qRT-PCR	Protein array	Immunohistochemistry
1	male	75	x		
2	male	91		x	x
3	male	68	x	x	
4	female	69	x		x
5	male	72	x		x
6	male	80		x	
7	male	25	x	x	
8	female	59	x	x	x
9	male	69		x	x
10	male	75		x	x
11	male	83		x	
12	female	72	x	x	
13	male	55	x		x
14	female	42	x	x	x
15	male	65	x	x	x
16	male	78		x	
17	female	50	x	x	x
18	male	17	x	x	x
19	female	82	x		
20	male	63	x	x	x
21	female	50	x		
22	female	68	x		
23	female	75		x	x
24	female	72	x		x
25	male	70		x	x
26	female	50	x	x	x
Control n°	**Gender**	**Age**			
1	male	62	x		
2	male	65	x		
3	female	54	x		
4	female	52	x		
5	female	55	x		
6	male	62	x		
7	female	68	x		
8	female	66	x		
9	female	69	x		
10	female	74		x	
11	male	82		x	
12	male	76		x	
13	female	61		x	
14	female	70		x	
15	female	43		x	

3.2 Cells isolation

EDTA blood was drawn from patients and controls, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density centrifugation. Subsequently, CD4+ T-cells were isolated by using magnetic-beads conjugated antibodies (CD14 depletion and CD4 positive selection). In order to obtain only CD4+ lymphocytes, CD4+ cells other than lymphocytes (monocytes) needed to be depleted. For this reason, before the positive selection with CD4+ magnetic-beads conjugated antibodies, CD14+ cells (monocytes) were depleted by magnetic-beads conjugated antibodies anti-CD14.

Immunomagnetic cell separation is based on antibodies coupled to magnetic beads. During incubation with a cell suspension, the antibody/bead complex binds to cells expressing the corresponding epitope. When the cell suspension is placed into a magnetic field, magnetically labeled cells are retained, while unlabeled cells can be removed. To recover the labeled cells, the sample is removed from the magnetic field.

Depletion of an unwanted cell type is usually used to remove a certain cell type from a mixture of cells. The unwanted cells are magnetically labeled. During separation, the unlabeled target cells are collected in the flow-through fraction whereas the unwanted cell type is retained within the column. In our case CD14+ were depleted.

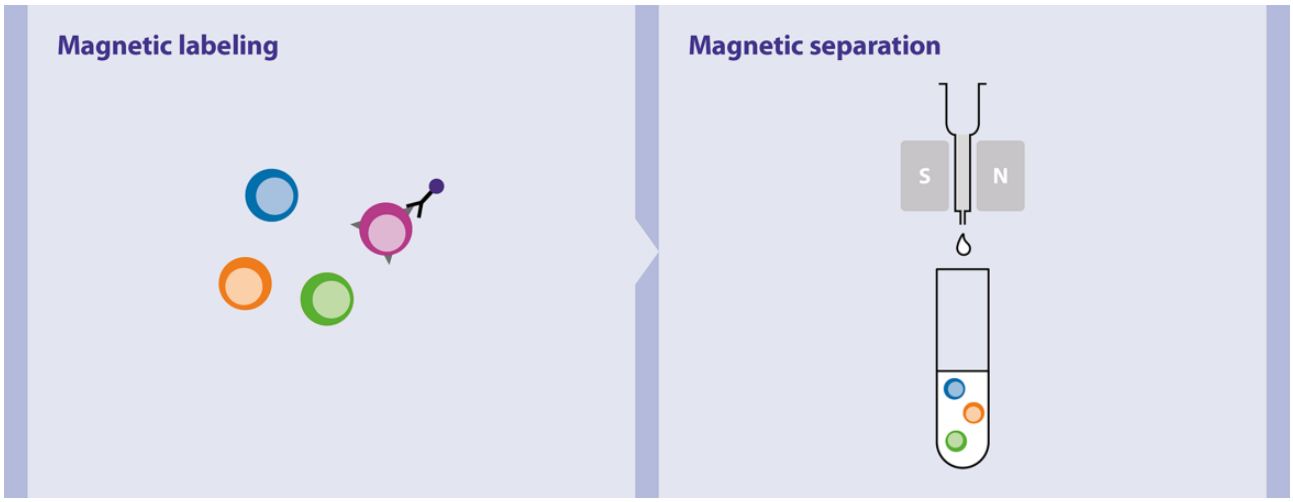

Figure 7. Example of negative selection by magnetic-beads conjugated antibodies

Positive selection means that a particular target cell type is magnetically labeled. During separation, the column is placed in the magnetic field of the MACS Separator. Magnetically labeled cells are retained within the column, whereas unlabeled cells flow through. After a washing step, the column is removed from the magnetic field, and the target cells are eluted from the column. Specific MACS MicroBeads are available for the positive selection of numerous cell types and cell subsets.

Positive selection can be performed by direct or indirect magnetic labeling. In the case of direct magnetic labeling, the cell type–specific MicroBeads directly bind to antigens on the cell surface. For an indirect labeling approach, primary antibodies that bind to cell surface antigens and MicroBeads that bind to the primary antibodies are added to the cells separately in a two-step procedure.

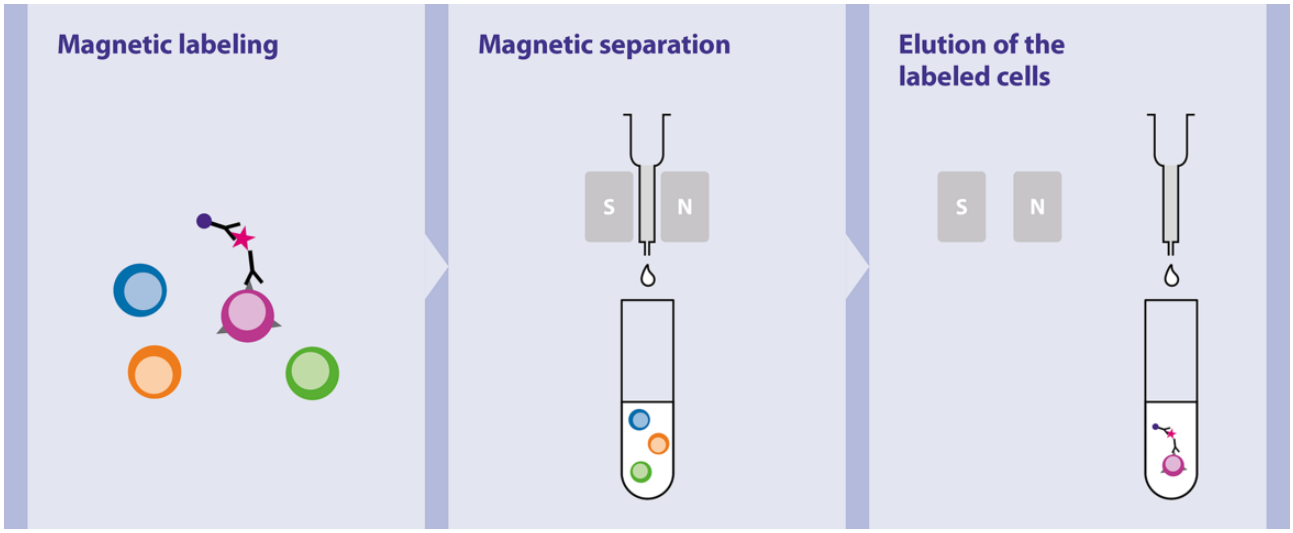

Figure 8. Example of positive selection by magnetic-beads conjugated antibodies

Our strategy was CD14+ depletion followed by CD4+ positive selection.

This approach is useful if an important marker for the target cells is also expressed on a fraction of undesired cells. To enable positive selection of the target cells based on this marker, the fraction of undesired cells needs to be depleted first. To this end, the undesired cells are magnetically labeled via antigens distinct from that common marker. During separation, the labeled cells are retained in the column. The flow-through fraction contains the target cells. These cells can then be labeled with MACS MicroBeads for that marker, and the target cells are isolated by positive selection

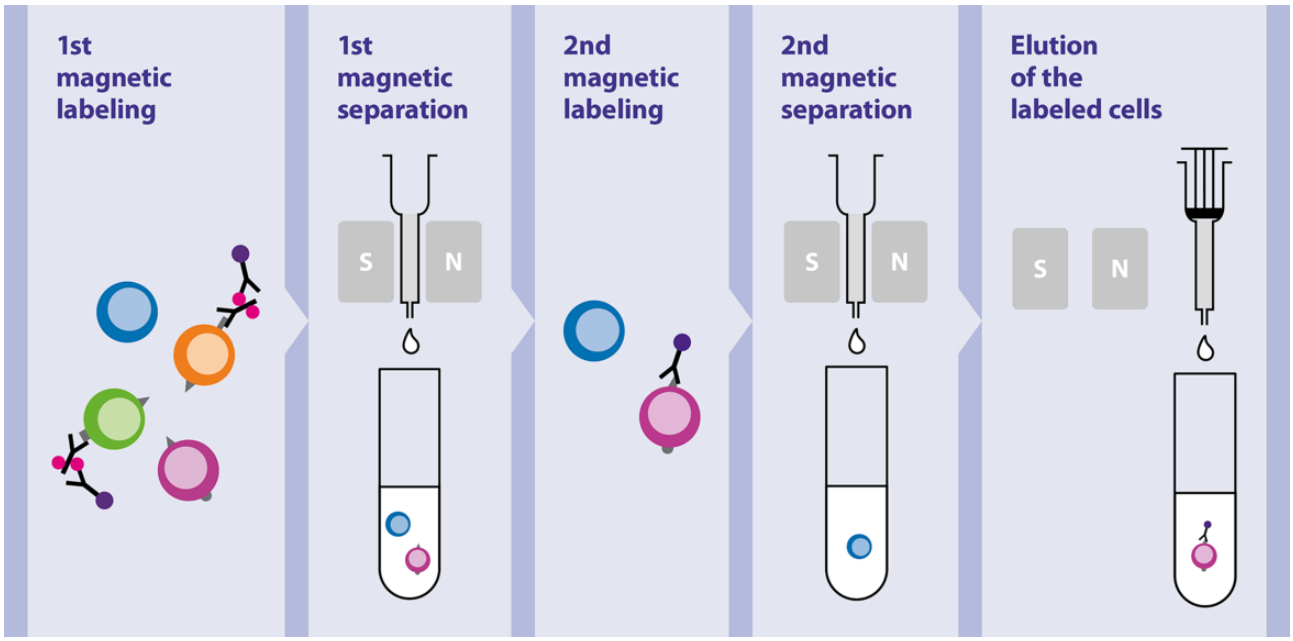

Figure 9. Example of depletion followed by positive selection by magnetic-beads conjugated antibodies

When possible, CD4+ isolated cells were divided into two different aliquots, one for RNA extraction and the other for protein extraction.

3.3 Cell lines

Hut78 (TIB161), H9 (HTB 176), and HH (CRL2105) cell lines established from peripheral blood of CTCL patients were obtained from American Type Culture Collection (ATCC) and grown in complete RPMI 1640 with 10% FBS (Sigma-Aldrich).

3.4 Isolation of total RNA

Total cellular RNA was isolated from CD4+ lymphocytes with the "Purification of Small RNA (Containing miRNA)" protocol using the RNeasy Plus Mini Kit (Qiagen) which allows the purification of total RNA containing small RNAs, such as miRNA from animal and human cells. Briefly, 350 µl of a lysis buffer is added to the sample and cells are disrupted and homogenized immediately by pipetting.

The homogenate is added to a gDNA eliminator spin column and centrifuged for 15 seconds at ≥8000 x g. Discard the column, and save the flow-through. A volume (usually 350 µl) of 70% ethanol is added to the flow-through, mixed thoroughly by vortexing, transferred to an RNeasy Mini

spin column and centrifuged for 15 seconds at ≥8000 x g. A 0.65 volumes (usually 455 µl) of 100% ethanol is added to the flow-through, and centrifuged in an RNeasy MinElute spin column for 15 seconds at ≥8000 x g. The flow-through is now discarded.

After two washing procedures using buffers and ethanol, RNA can be eluted with RNase-free water directly placed to the spin column membrane and centrifuged for 1 min at ≥8000 x g.

This RNA eluate is enriched in various RNAs of <200 nucleotides, including miRNA, 5S rRNA and tRNA. RNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

3.5 Real time RT-PCR

3.5.1 Reverse transcription (RT)

TaqMan® Advanced miRNA Assays protocol allows to prepare cDNA templates from miRNA followed by PCR amplification of the cDNA template and subsequent data analysis.

In the first stage of the workflow mature miRNAs from total RNA are modified by extending the 3' end of the mature transcript through poly(A) addition, then by lengthening the 5’ end by adaptor ligation. The modified miRNAs then underwent universal reverse transcription followed by amplification to increase uniformly the amount of cDNA for all miRNAs (miR-Amp reaction).

The reverse transcription procedure included the following 3 main steps:

Poly(A) tailing reaction.

Starting with a total RNA sample, poly(A) polymerase is used to add a 3’-adenosine tail to the miRNA.

5 µg total RNA were used to perform poly(A) tailing reaction in a total volume of 5 µL containing poly(A) buffer, ATP, poly(A) enzyme and RNase-free water (according to the manufacturer’s instructions).

Samples were placed into a thermal cycler (MyCycler™, Biorad) at 37°C for 45 minutes (polyadenylation), followed by 65°C for 10 minutes (Stop reaction) and a final holding step at 4°C.

Adaptor ligation reaction

The miRNA with poly(A) tail undergoes adaptor ligation at the 5’ end. The adaptor acts as the forward-primer binding site for the miR-Amp reaction. To complete this reaction RNA ligase, an adaptor and PEG 8000 are needed. They were added to the previous 5 µL within a ligase buffer and RNase-free water in a final volume of 15 µL.

Ligation reaction took 60 minutes at 16°C followed by a holding step at 4°C in a thermal cycler (MyCycler™, Biorad).

Reverse transcription reaction

A universal RT primer binds to the 3' poly(A) tail and the miRNA is reverse transcribed. The resulting cDNA is suitable for all TaqMan® Advanced miRNA Assays.

In a final volume of 30 µL RT enzyme, RT universal primers and dNTPs were added within RT buffer and RNase-free water.

Reaction was performed in thermal cycler and consisted of a RT step at 42°C for 15 minutes followed by a stop reaction step at 85°C for 5 minutes.

3.5.2 Initial PCR step to amplify the cDNAs (pre-PCR).

The miR-Amp reaction utilizes universal forward and reverse primers to increase the number of cDNA molecules. 5 µL of RT-PCR product were used as the template for a 50 µL PCR reaction. Briefly, miR-Amp primers (10 µmol/L), miR-Amp Master Mix and RNase free-water were mixed to obtain 45 µL or reaction mix. The cycling protocol consisted of an initial 5-min enzyme activation step at 95 °C followed by 14 cycles of denaturation at 95 °C for 3 s, and annealing at 60 °C for 30 s. Finally a stop reaction step at 99°C for 10 minutes was followed by a hold step at 4°C.

3.5.3 Quantification of let-7b and let-7c expression by real-time RT-PCR.

Real-time RT-PCR was made to quantify the expression of let-7b and let-7c (Assay name has-let-7b-sp and has-let-7c-5p, respectively. Applied Biosystems). As miR-26a is always expressed at the same level in CD4+ T-cells and it was never described having an altered expression in SS, it was selected as housekeeping for miRNAs expression experiments (Assay name has-miR-26a-5p). All PCR experiments were run in duplicate.

Real-time PCR was performed in a total volume of 20 µL per reaction. We placed 5 µL of 1:10 diluted pre-PCR product into a 15 µL reaction mixture that contained RNase-free water, TaqMan® Advanced Master Mix and the specific TaqMan® Advanced miRNA Assays (let-7b, let-7c or miR-26a). The cycling protocol consisted of an initial 20-seconds enzyme activation step at 95°C, followed by 40 cycles of denaturation at 95°C for 1 s, and annealing/extension at 60 °C for 20 seconds. All reactions were performed on a StepOnePlus™ instrument (Applied Biosystems).

3.5.4 Normalization of data

Careful normalization is essential for accurate quantification of miRNAs. All data were evaluated with respect to let-7b and let-7c downregulation by normalizing to the expression of miR-26a and using the 2−ΔΔCt method.

3.5.5 Real-time PCR for let-7 targets and inhibitors

Real-time PCR for some let-7 targets and for their direct inhibitors (Lin28A and Lin28B) was performed with StepOnePlus™ instrument (Applied Biosystems). Among possible let-7 targets were selected 4 genes possibly related to already described pathogenesis mechanisms in SS, namely CCND, MYC, HMGA1 and HMGA2. RNA 18s was choosen as housekeeping gene. Genes expression were evaluated by relative quantification with respect to RNA 18s gene expression and the 2−ΔΔCt method as described above.

3.5.6 Statistical analyses

All quoted p values are two-sided, with 0.05>p>0.01 (*) and P less than 0.01 (**) being considered statistically significant and highly significant, respectively. The Student's t test was applied for all analyses; all statistical tests were two-sided.

3.6 Protein analyses

3.6.1 Total protein extraction

Each sample was placed in 3 ml of cold radioimmunoprecipitation assay (RIPA) buffer (sc-24948) containing protease- and phosphatase-inhibitors. Samples were incubated on ice for 30 min, transferred to microcentrifuge tubes and centrifuged at 10000 x g for 10 min at 4°C. The supernatant was collected and the sample was centrifuged again. The new supernatant fluid was added to the previous one, this mixture representing the total cell lysate. In order to standardize the cell lysate of each tissue sample, the total proteins in each sample were measured by a microBCA kit (ThermoScientific, Waltham, MA, USA).

3.6.2 Protein array

For each sample, a volume containing 100 µg of proteins was loaded in a glass-slide format of apoptosis antibody array (RayBio®, Norcross, GA, USA). The volume to be loaded was calculated by the following formula: volume (expressed in µl) = 100 µg/protein concentration (expressed in

μg/μl). Each glass-slide array contained 8 subarrays and was suitable for 8 samples. Each subarray allowed the evaluation of expression levels of 43 apoptosis molecules in a sample. Normalization of data at the end of the experiment provided semi-quantitative results. The subarray was composed of specific antibodies against target molecules coated on the glass-slide. After hybridization of the tissue lysate, each antibody bound its target molecule and unbound proteins were washed out. The slide was then incubated with biotin-conjugated antibodies against the same target molecule, washed and then incubated with cyanin 3 (Cy3)-conjugated streptavidin, creating a biotin–streptavidin–Cy3 complex detectable using a microarray laser scanner. Using a data extraction software it was possible to convert fluorescent signals into numerical data and, after normalization, to obtain an expression value of signal intensity for each molecule in each sample. The molecules tested were the following: bad, bax, bcl-2, bcl-w, BID, BIM, caspase3, caspase8, CD40, CD40L, cIAP-2, cytoC, DR6, Fas, FasL, HSP27, HSP60, HSP70, HTRA, IGF-I, IGF-II, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IGFBP-5, IGFBP-6, IGF-1sR, livin, p21, p27, p53, SMAC, Survivin, sTNF-R1, sTNF-R2, TNF-alpha, TNF-beta, TRAILR-1, TRAILR-2, TRAILR-3, TRAILR-4, XIAP.

3.7 Immunohistochemistry

As already described above, for each SS patient a lesional skin biopsy was collected at time of surgery to perform routinely histology examinations. Tissue samples were fixed in buffered formalin, dehydrated, embedded in paraffin wax and sectioned; no antigen unmasking pretreatment was needed. After deparaffining and rehydrating, each tissue section was placed on a Dako automated immunostainer (Dako Cytomation, Glostrup, Denmark), and incubated with the specific primary monoclonal antibody at room temperature for 45 min, and then washed with Tris-buffered saline (TBS), pH 7.6, and incubated in peroxidase-conjugated (HRP) goat anti-mouse and anti-rabbit immunoglobulins (Dako FLEX™, code K8024) at room temperature for 30 min. After incubation with the secondary antibodies the sections were washed with TBS, pH 7.6, once again. A Diaminobenzidine (DAB) chromogen solution was prepared as indicated by the Dako FLEX™ datasheet and used as an enzyme substrate, followed by counterstaining with Mayer's haematoxylin, After air drying, each section was coverslipped using the “VectaMount" mounting medium (Vector Laboratories, Burlingame, CA, USA). We used monoclonal antibodies to CyclinD1, c-Myc, HSP-70, p27 and p53.

4 RESULTS

4.1 Let-7 family members are downregulated in SS

Initially, to better clarify whether let-7 family members have a differential expression in SS, expression levels of let-7b and let-7c were compared between the two groups (patients vs controls). In order to establish expression levels a ΔCt was calculated by subtracting the Ct from each miRNA of the test or reference group by the Ct mean of the normalizing control (miR-26a). ΔCT = Ct miRNA (let-7) - mean housekeeping (miR-26a). The fold-change was calculated using the 2−ΔCt (test)/2−ΔCt (control) model, represented by 2−ΔΔCt. Both let-7 miRNAs (let-7b and let-7c) showed differential expression with greater downregulation in SS than in healthy donors.

Let-7b resulted downregulated in 8/18 (44%) patients and in 0/9 (0%) controls while let-7c was shown downregulated in 7/18 (39%) patients and 0/9 (0%) HD. 2 patients showed a downregulation of both let-7b and let-7c miRNAs (11%). Globally, 13/18 (72%) patients had a downregulation in at least one let-7

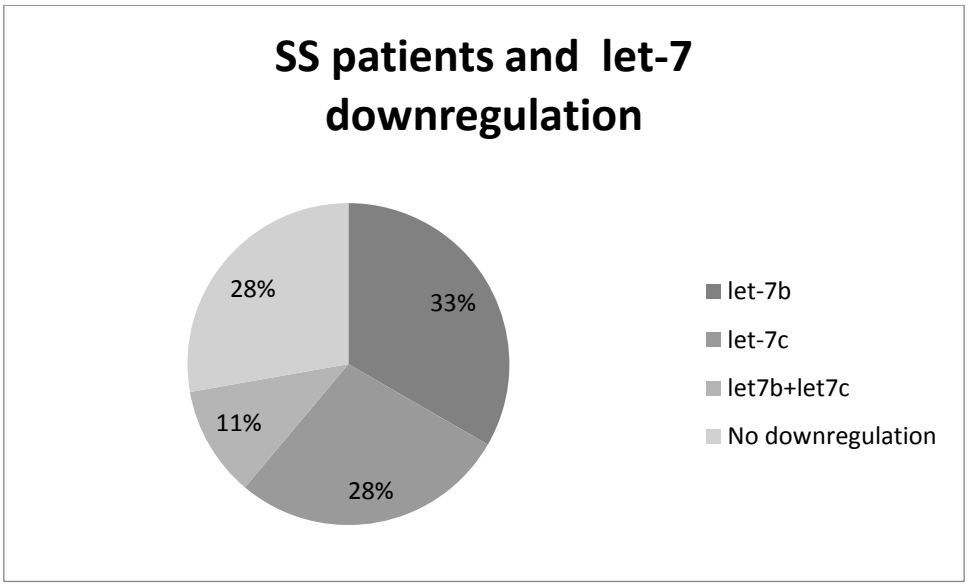

Figure 10. Percentage of SS patients with or without let-7 downregulation. Globally, 72% of SS patients had at least one let-7 member downregulated.

Considering that just half of patients had each let-7 downregulated and considering the small number of cases studied, differences in let-7b and/or let-7c expressions between groups didn't reach a statistically significance. However, it was suddenly clear that expression of both let-7b and let-7c in SS patients was not uniform and some patients showed a downregulation.

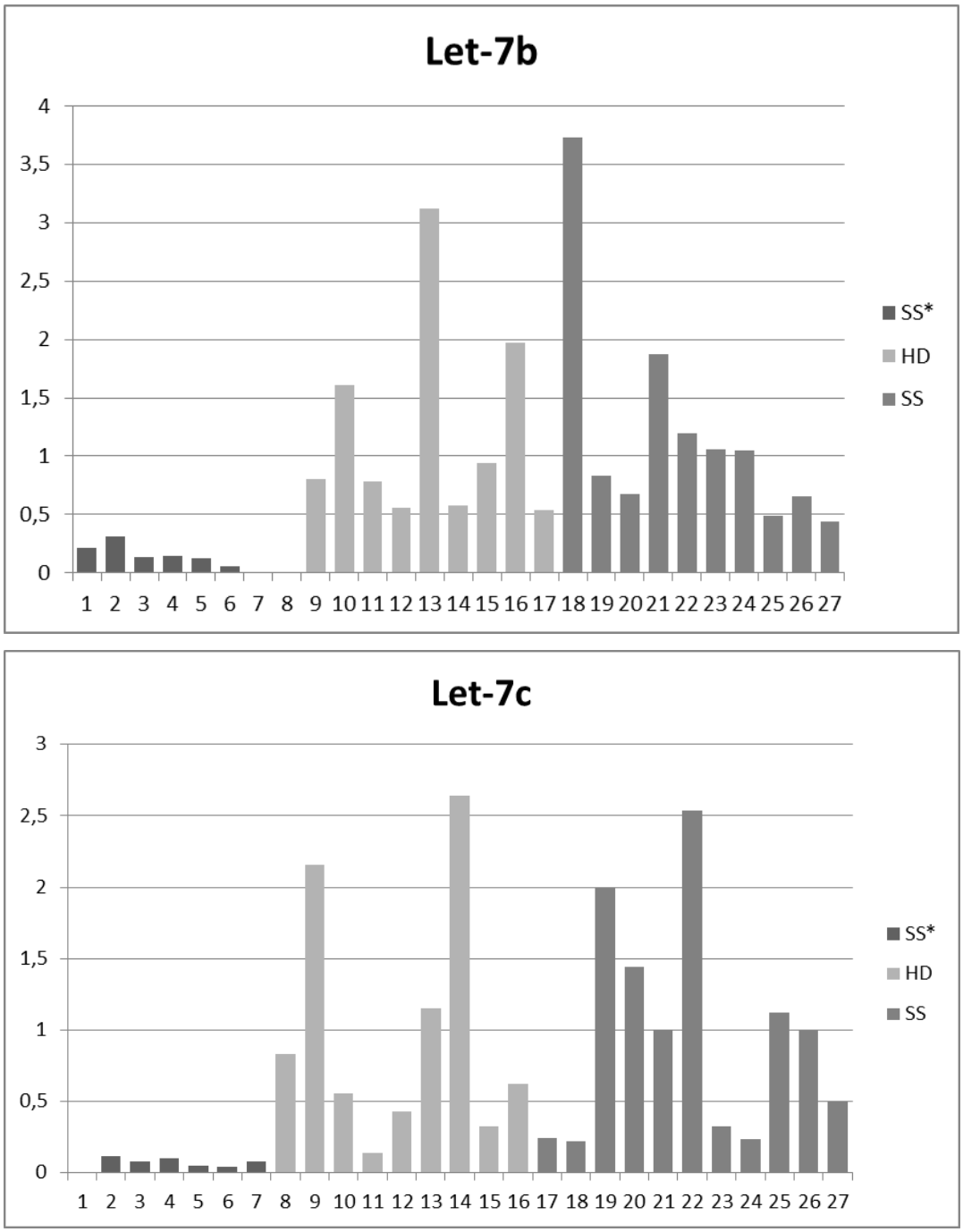

Figure 11. Bar chart showing expression values (2−ΔΔCt) of let-7b and let-7c in SS patients versus healthy donors (HD). Differences were nor statistically significative. Patients' data were not homogeneous. In both graphs about half of patients had normal expression (as HD) while tha other half showed a downregulation.

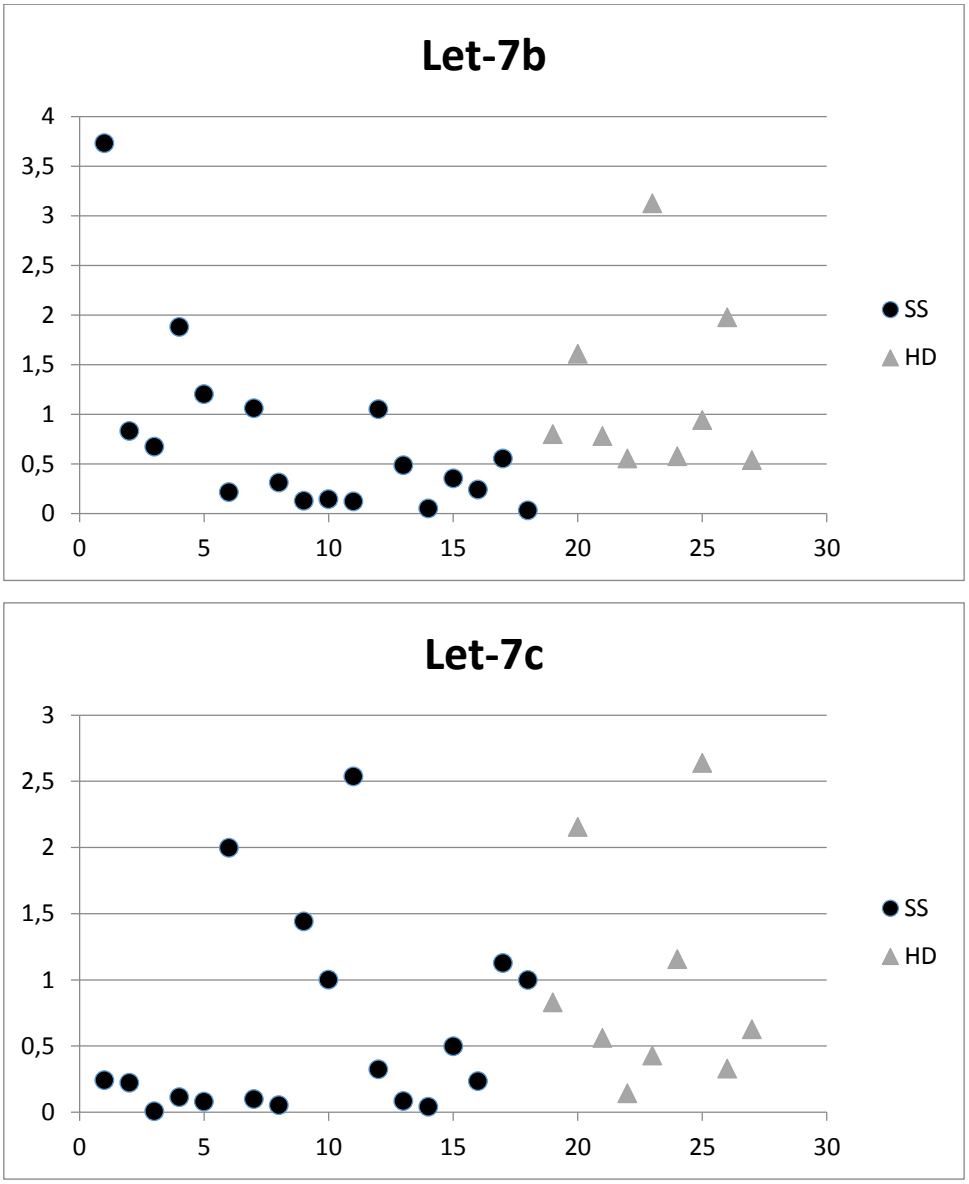

Figure 12. Expression values (2−ΔΔCt) of let-7b and let-7c in SS patients versus healthy donors (HD). Differences were nor statistically significative. Patients' data were not homogeneous. In both graphs about half of patients had normal expression (as HD) while tha other half showed a downregulation.

As patients showed a heterogeneous distribution they were divided in two distinct groups: those with let-7 downregulation (SS*) and those with a normal let-7 expression (SS). Median values of $2^{-\Delta\Delta Ct}$ in SS with altered expression (SS*) resulted lower than in HD with a statistical significance ($p=0.0063$ and $p=0.0020$ for let-7b and let-7c, respectively).

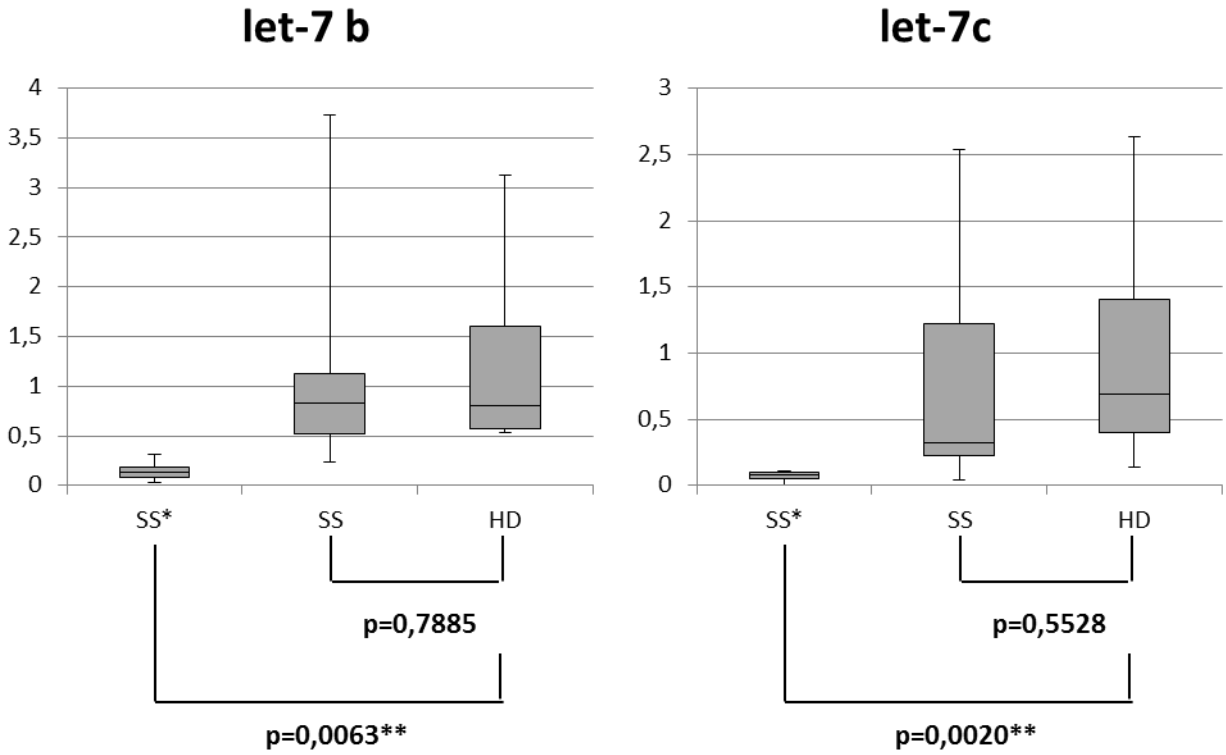

Figure 13. Expression values ($2^{-\Delta\Delta Ct}$) of let-7b and let-7c in SS patients versus healthy donors (HD). Patients were divided in two groups: SS with altered let-7 expression (SS*) (n=8 and n=7, respectively) and SS with let-7 normal expression (SS) (n=10 and n=11, respectively). Differences between SS* and HD are statistically significative. Boxplots representing the minimum, the maximum, the sample median, and the first and third quartile.

Let-7 analysis in cell lines showed a downregulation for let-7b in HUT78, a downregulation of both let-7b and let-7c in H9, while HH presented normal levels for both miRNAs.

4.2 Lin28A is expressed in SS

Lin28A and Lin28B, the direct repressors of let-7 family members, are expressed during the embryonic development. However, no adult cells express these molecules normally.

Analyzing expression levels of Lin28A and Lin28B both resulted unexpressed in HD. In SS patients Lin28B was found unexpressed while Lin28A was found expressed in all the cell lines and only in a 7 out 18 SS patients. Differences between SS and HD didn't reach statistical significance (p=0.1422).

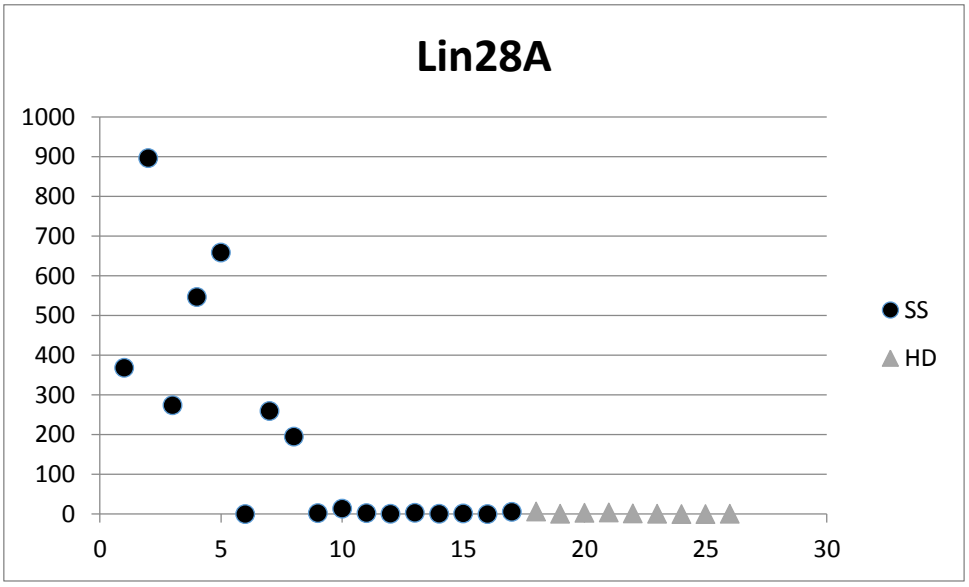

Figure 14. Expression levels of LIN28A in SS (n=18) and HD (n=9). It is clearly visible that 11 patients didn't expressed Lin28A, while 7 of them had a protein overexpression.

Interestingly, looking at patients with Lin28A overexpression 5 out of 7 patients had a let-7c downregulation. Stratifying the patients according to let-7c expression, patients showing a let-7c downregulation were associated to a significant higher level of Lin28A respect to to HD (p=0.048)

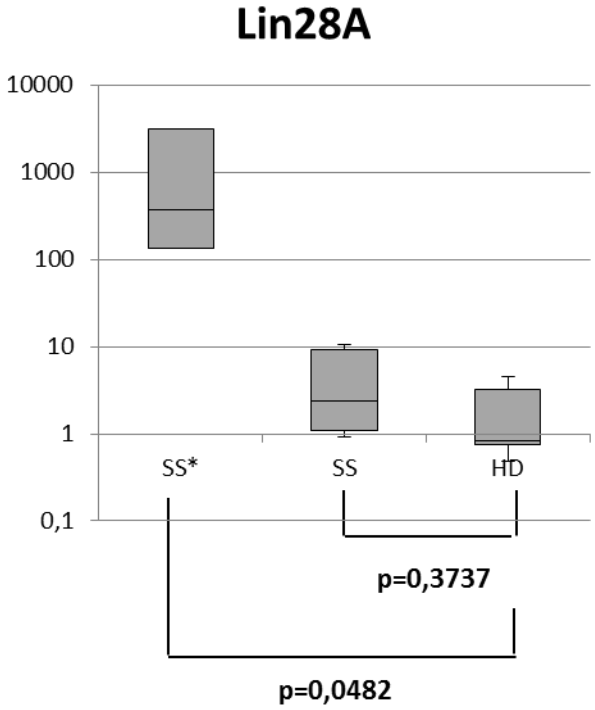

Figure 15. Expression levels of LIN28A in SS*, SS patients with let-7c downregulated (n=7), SS (SS patients with let-7c normal expressed, n=11) and HD (N=9) normalized as expression in HD was 1.

4.3 Let-7 expression and response to therapy

In order to investigate whether let-7 expression influenced response to therapy all SS patients were divided in two groups. A group of patients who received a first line therapy (usually ECP and/or monochemotherapy) standing for a long period of time (>24 months) in stable disease (SD) were considered as "responders" to therapy. Those patients whose after a first line therapy progressed, and often progressed also after a second line therapy, were considered as "non responders".

Table 8. SS patients, let-7 expression and response to therapy

pt. N°	Therapy	Let-7 expression	Responder
1	chlorambucil	normal	Responder
3	ECP+methotrexate; then chlorambucil	Let-7b down	Non-responder
4	ECP; then chlorambucil	Let-7b down	Non-responder
5	ECP; then acitretin; then chlorambucil	normal	Non-responder
7	IFNα; then chlorambucil	Let-7c down	Non-responder
8	ECP; then gemcitabine; then campath	Let-7b down	Non-responder
12	steroids; then gemcitabine	both let-7 down	Non-responder
13	clorambucil+methotrexate; then gemcitabine; then doxorubicin	Let-7b down	Non-responder
14	ECP+IFNα; then gemcitabine; then campath	normal	Non-responder
15	ECP+chlorambucil	Let-7c down	Responder
17	gemcitabine	normal	Responder
18	ECP; then TSEB+gemcitabine	Let-7b down	Non-responder
19	prednisone	normal	Responder
20	ECP; then TSEB+gemcitabine	Let-7c down	Non-responder
21	CHOEP	Let-7c down	Non-responder
22	ECP; then clorambucil; then bexarotene	Let-7b down	Non-responder
24	ECP	both let-7 down	Responder
26	ECP	Let-7c down	Responder

Contingency test (Fisher's test) was applied to understand a possible link between let-7 expression and response to chemotherapy.

Of note, although Fisher's test didn't reach a statistical significance, number of "non responder" patients in let-7 downregulated group was much higher than those in let-7 normal expressed group.

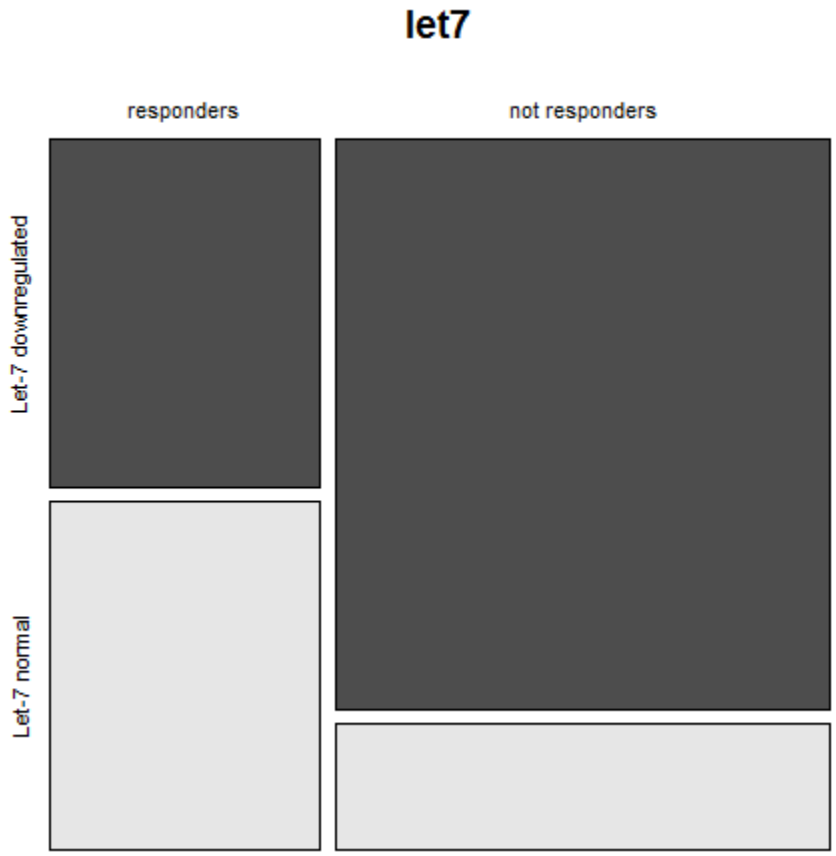

Figure 16. Contingency table data underlined let-7 downregulation in 50% of patients considered "responders" and in 80% of patients considered "non-responders"

4.4 Protein array of apoptosis-related molecules

Since SS cells show a high apoptotic resistance the study next focused on the expression of anti apoptotic protein in purfied CD4+ malignant cells derived from 18 SS patient and 6 HD using a glass slide antibody array in which expression levels of 43 apoptosis-related proteins were measured and normalized on internal controls.

Among the 43 protein studied 11 showed a statistically significative difference between SS and HD. About cyclin-dependent kinase inhibitors p21, p27 and p53 resulted statistically less expressed in SS than in normal controls (78.87 ± 2.18, 0.94 ± 1.77, 10.29 ± 3.70, versus 97.73 ± 8.75, 3.50 ± 6.71 and 15.68 ± 5.84, respectively; with p-values p=0.016, p=0.044 and p=0.043, respectively).

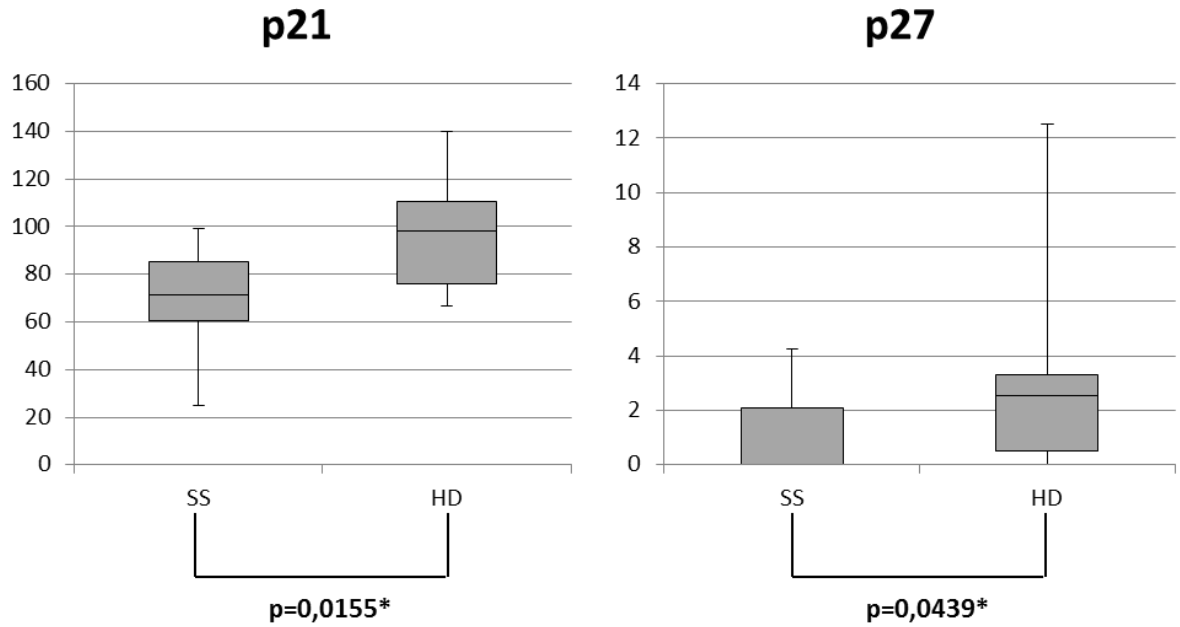

Figure 17. Expression of cell-cycle regulators p21 and p27 detected by apoptosis array. In SS were measured lower levels than in HD. Differences were significative with p=0,0155 and p=0,0439, respectively.

Particularly, p53 already described in SS having a pivotal role in the disease, showed a significant diminished expression in SS patients than in HD. This result was expected and represented just a confirmation.

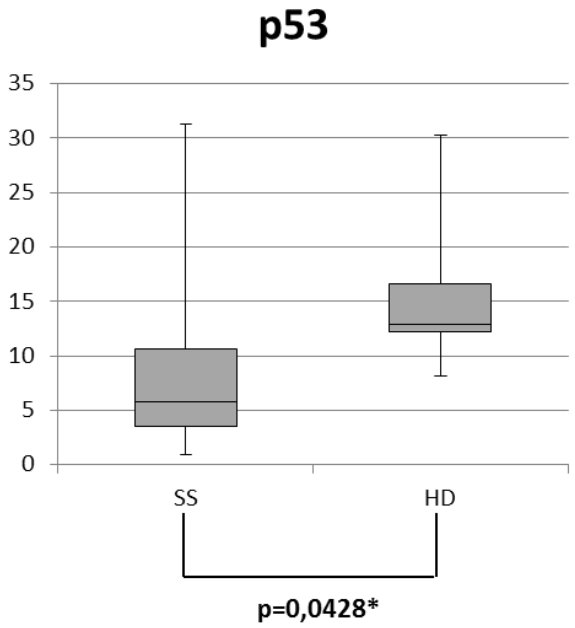

Figure 18. Expression of p53 were significantly lower in SS than in HD (p=0.0428).

In a second step, the study was focalized on finding correlations between apoptosis-related proteins expression and let-7s expression. The goal was to find some molecules with altered expression in SS patients with let-7s downregulation. Among SS patients no proteins with different expression between let-7 normal and let-7 downregulated groups were found. Nevertheless, a higher statistical significance in p53 levels by comparing the group of patients carrying let-7 downregulation (SS*) with HD.

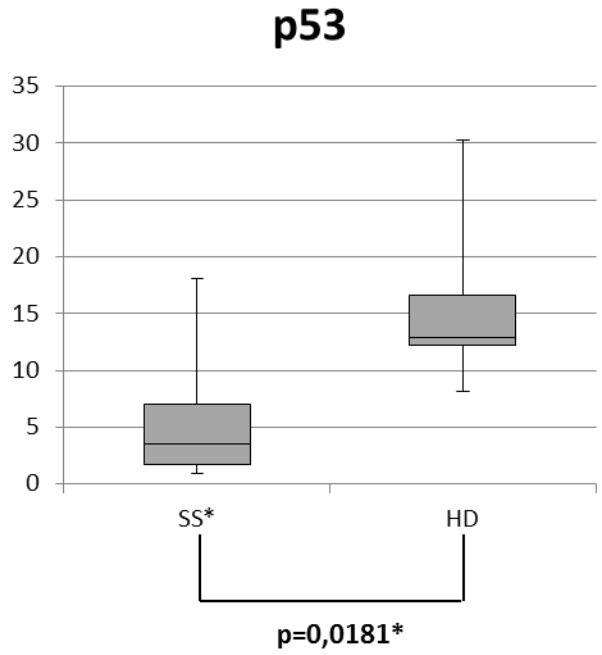

Figure 19. Expression levels of p53 in patients with let-7 downregulation (SS*, n=8) compared to HD

Many pro-apoptotic factors resulted significantly downregulated in SS than in HD such as bad (10.32 ± 6.11 versus 16.33 ± 6.08; p =0.024), bax (13.97 ± 4.23 versus 34.03 ± 5.76; p= 0.01), bcl-w (0.78 ± 2·02 versus 2·68 ± 1.72; p= 0.021) and BID (1.21 ± 2.73 versus 5.30 ± 3.76; p= 0.034), Figure 20.

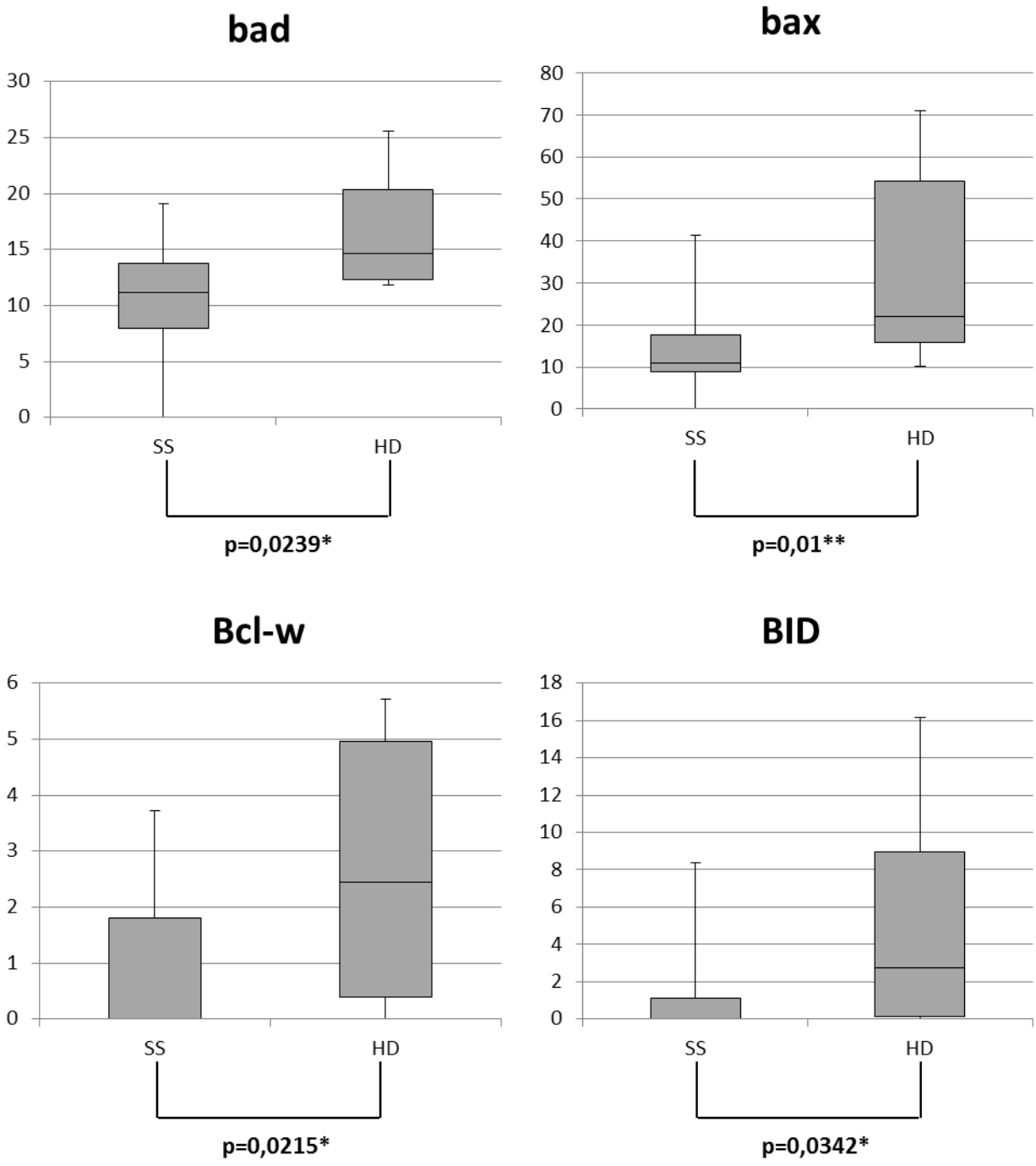

Figure 20. Expression of some pro-apoptotic molecules, belonging to Bcl-2 family, by means of apoptosis array. Bad, bax, Bcl-w and BID resulted significantly downregulated in SS versus HD with p=0,0239, p=0,010, p=0,0215 and p=0,0342, respectively.

Among inhibitors of apoptosis (IAP) family members cIAP-2 and XIAP showed a higher expression in SS than in HD raising statistical significance (6.08 ± 0.66 and 40.12 ± 25.77 versus 1.61 ± 1.55 and 13.97 ± 8.32, respectively; with p-values p=0.024 and p=0.045, respectively).

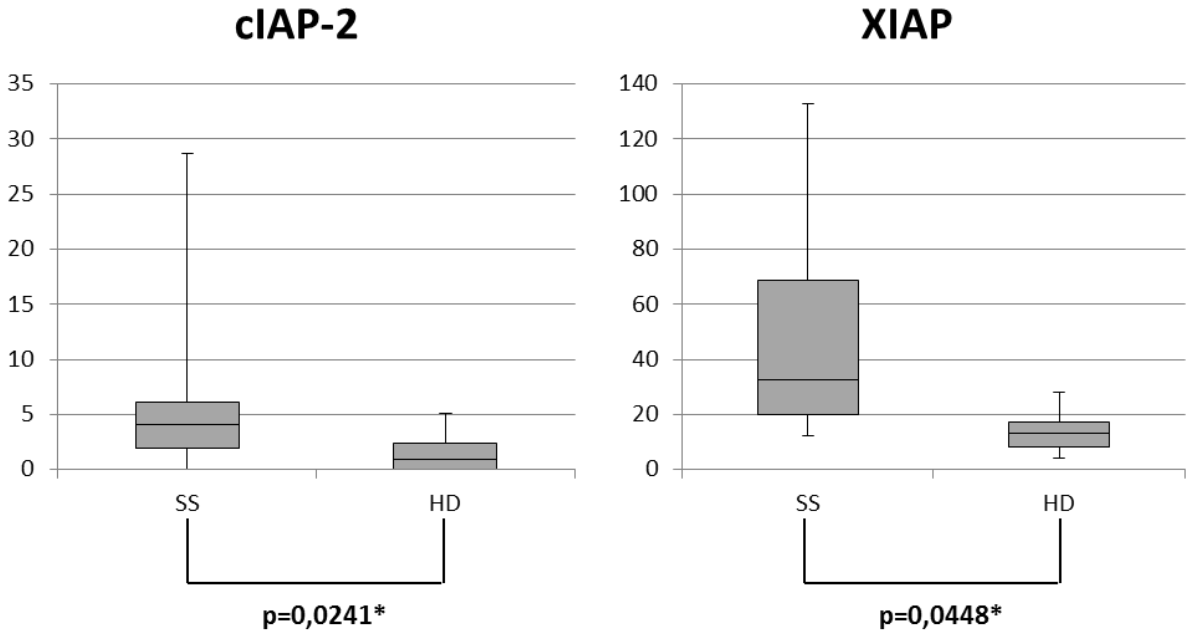

Figure 21. Expression levels of inhibitors of apoptosis (IAP) cIAP-2 and XIAP. Higher levels in SS than in HD reached statistically significance with p=0,0241 and p=0,0448, respectively.

Among heat shock proteins, HSP-60 was found higer expressed in SS than HD (36.86 ± 30.66 versus 16.39 ± 21.9) reaching statistical significance p=0.0431

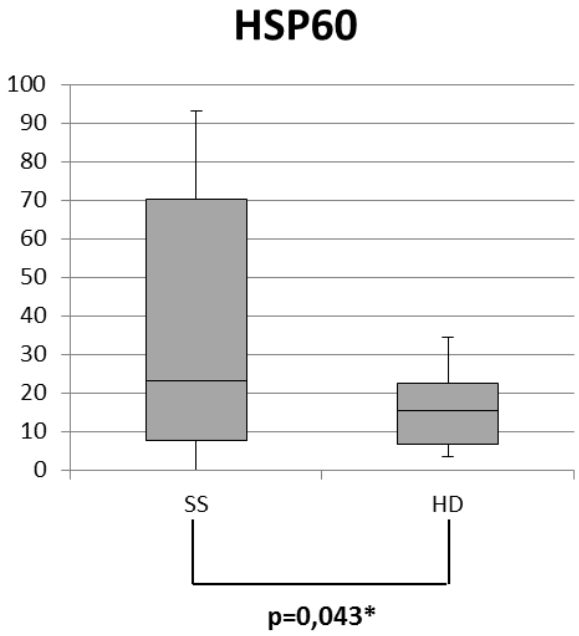

Figure 22. Expression levels of HSP-60

Finally, livin was significantly less expressed in SS (17.94 ± 6.17) than in normal CD4+ lymphocytes (32.53 ± 5.71; p = 0.0044) (Fig.23).

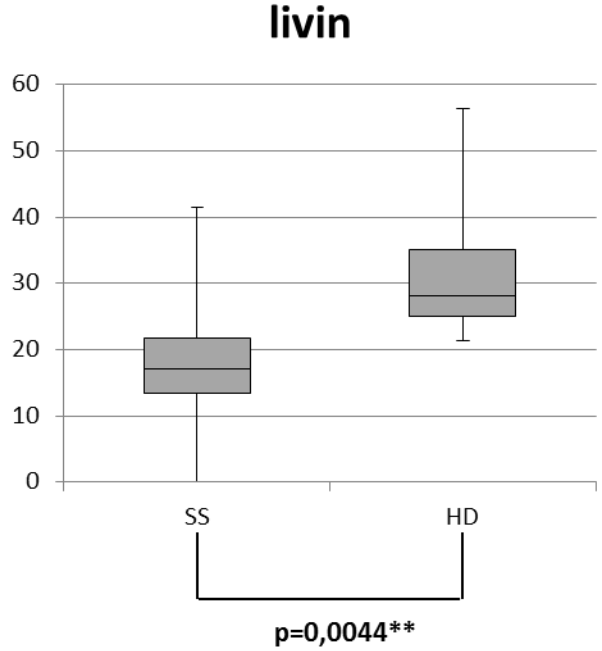

Figure 23. Expression of livin by means of apoptosis array. Downregulation in SS was statistically significant with p=0.0044

4.5 Immunohistochemistry

Immunohistochemical stainings were performed in order to confirm some results obtained by qRT-PCR or by apoptosis array. Among genes/proteins to validate only five targets were choosen because of antibodies availability in the lab. Proteins evaluated by means of immunohistochemistry were: Cyclin D1, c-Myc, HSP-70, p27 and p53.
Results are summarized in Table 9.

Table 9. Immunohistochemical results

Patient n°	CCND1	c-Myc	HSP-70	p27	p53
2	+/-	++	+/-	++	+/-
4	neg	+	+	neg	neg
5	neg	neg	neg	neg	+/-
8	+/-	+/-	++	+	neg
9	+/-	+/-	++	++	neg
10	neg	neg	++	+/-	neg
13	+/-	++	+	++?	+
14	+/-	neg	+	neg	neg
15	+/-	+	+/-	+	neg
17	+/-	++	+/-	neg	neg
18	neg	+	++	neg	neg
20	+/-	++	+	++	neg
23	+/-	+	+/-	-	+/-
24	+	++	++	neg	+/-
25	neg	++	+/-	+/-	+/-
26	neg	+/-	neg	neg	neg

As expected, cyclin D1 was almost all negative in all the patients tested (Figure 24). This result confirmed those already registered by means of qRT-PCR. The lonely patient who showed a discrete positivityof cyclin D1 in tumor cells was patient n°24, who had both let-7 (let-7b and let-7c) downregulated.

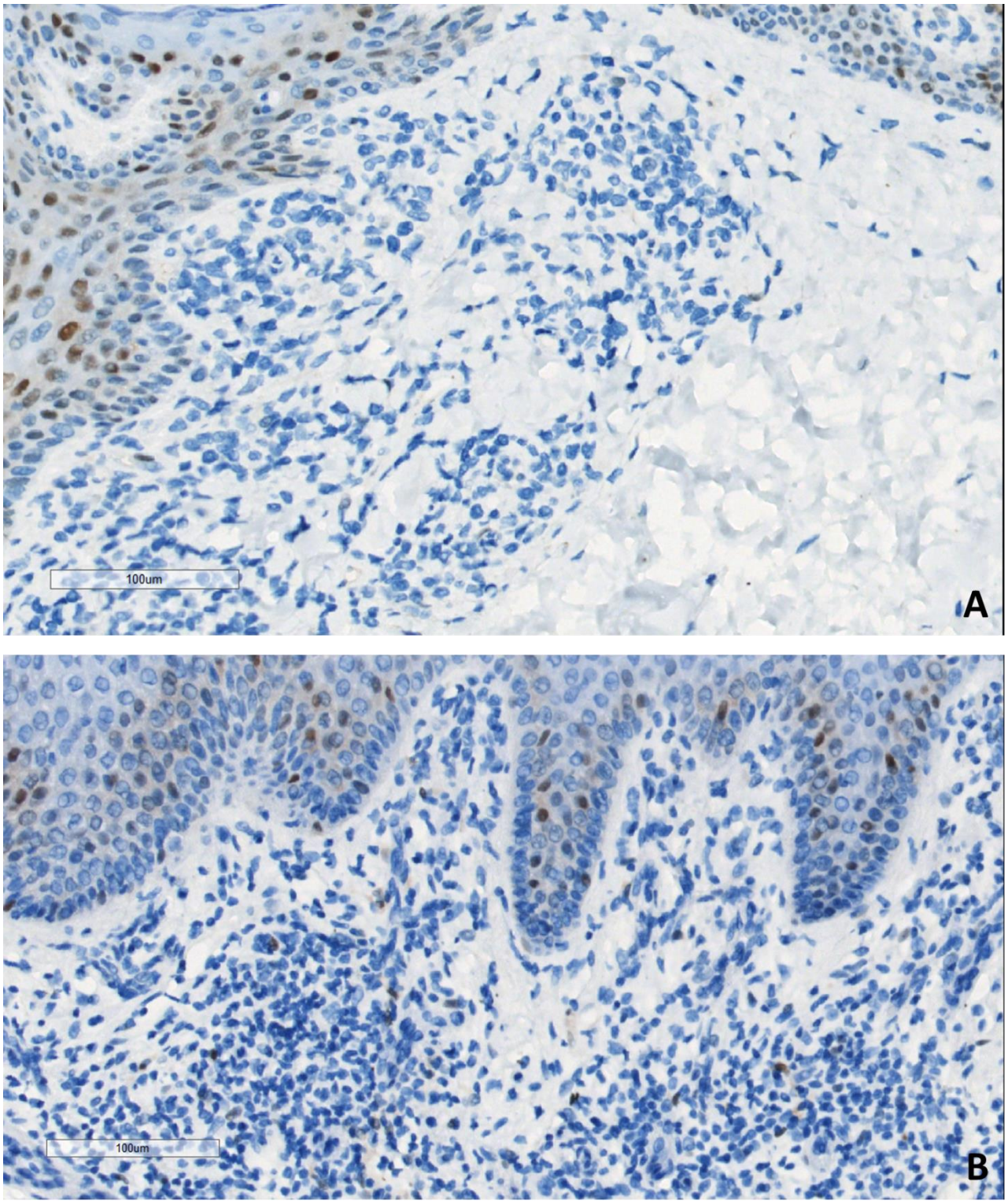

Figure 24. An example of Cyclin D1 negativity in SS tumor cells. Of note the positive control in keratinocyte nuclei. No differences were found in Cyclin D1 expression between SS with let-7 normal expressed (A) and let-7 downregulated (B).

Myc protein was successfully detected by means of immunohistochemistry and it was always positive on Sézary cells (Figure 25). This result was in line with was expected. In fact, c-myc expression is widely known to be abundant in tumor cells of SS. In contrast qRT-PCR wasn't able to detect MYC expression.

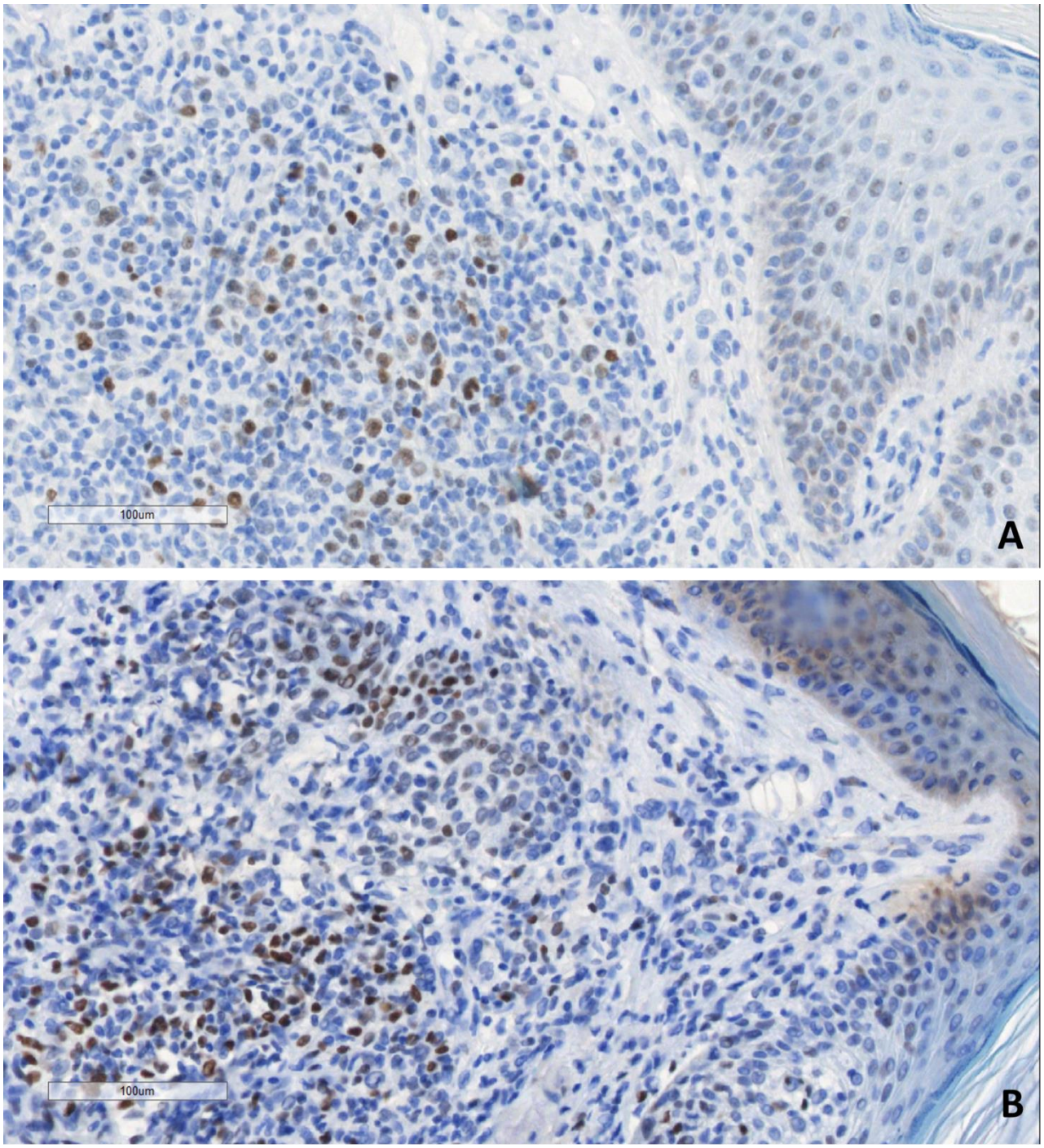

Figure 25. Examples of c-Myc nuclear positivity in SS samples. A relative high number of infiltrating lymphocytes showed nuclear staining, particularly largest and cerebriform cells (Sézary cells). A and B represent patients with let-7 downregulation and let-7 normal expression, respectively.

HSP-70 was found positive in almost all the patients. However, its expression varies from very low expression (+/-) to a diffused expression in both tumor and reactive cells, including macrophages (Figure 26).

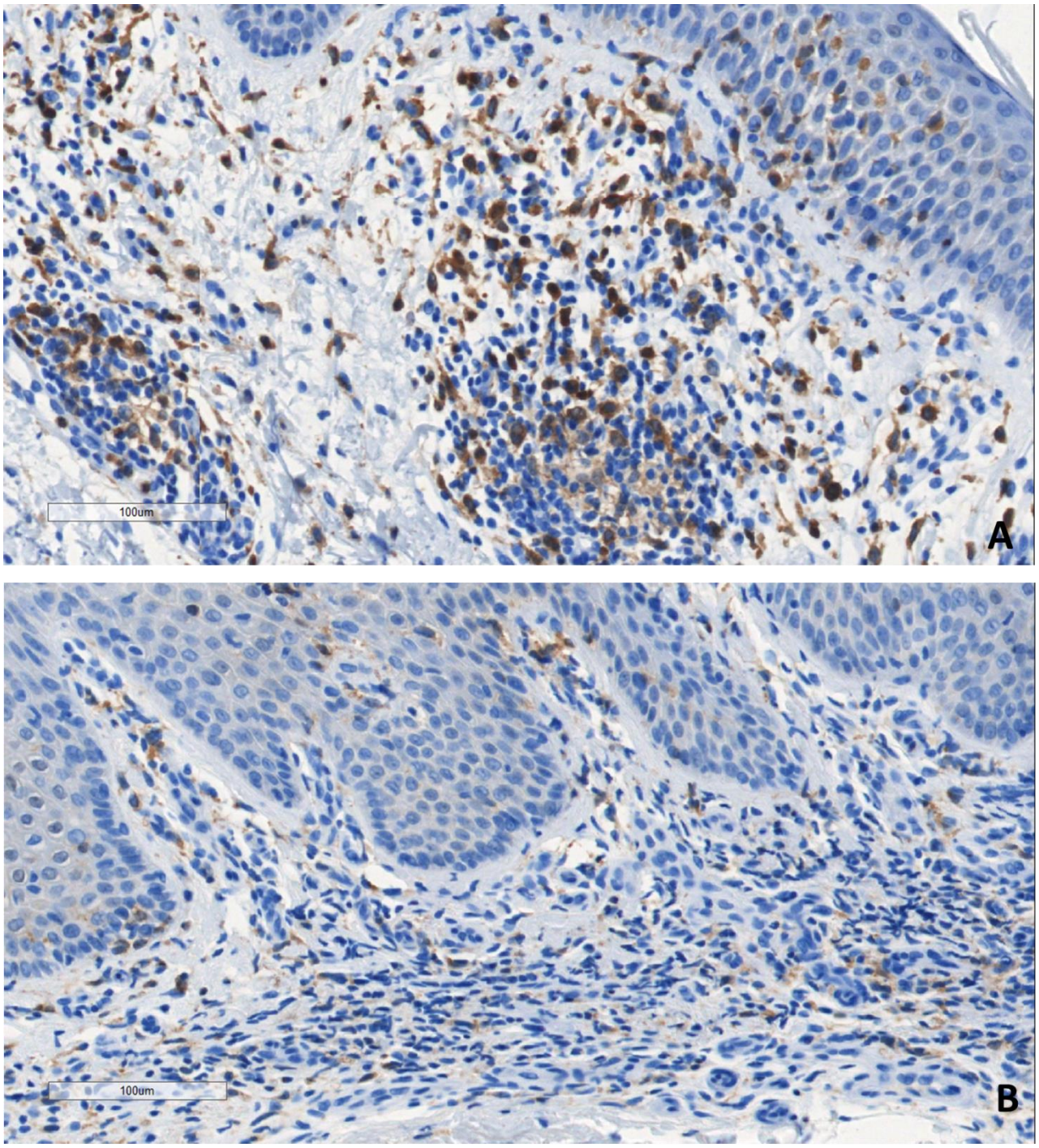

Figure 26. HSP-70 expression varies from diffuse positivity in infiltrating cells, including macrophages (A) to a less intense and spared positivity particularly in SS patients with normal let-7 expression (B).

Cell-cycle regulators p27 and p53 were expected negative. However p27 showed a very variable expression among different samples. On contrast p53 was almost all negative in all the patients (Figure 27). A diminished expression in SS cells was already evidenced by apopotosis array analysis.

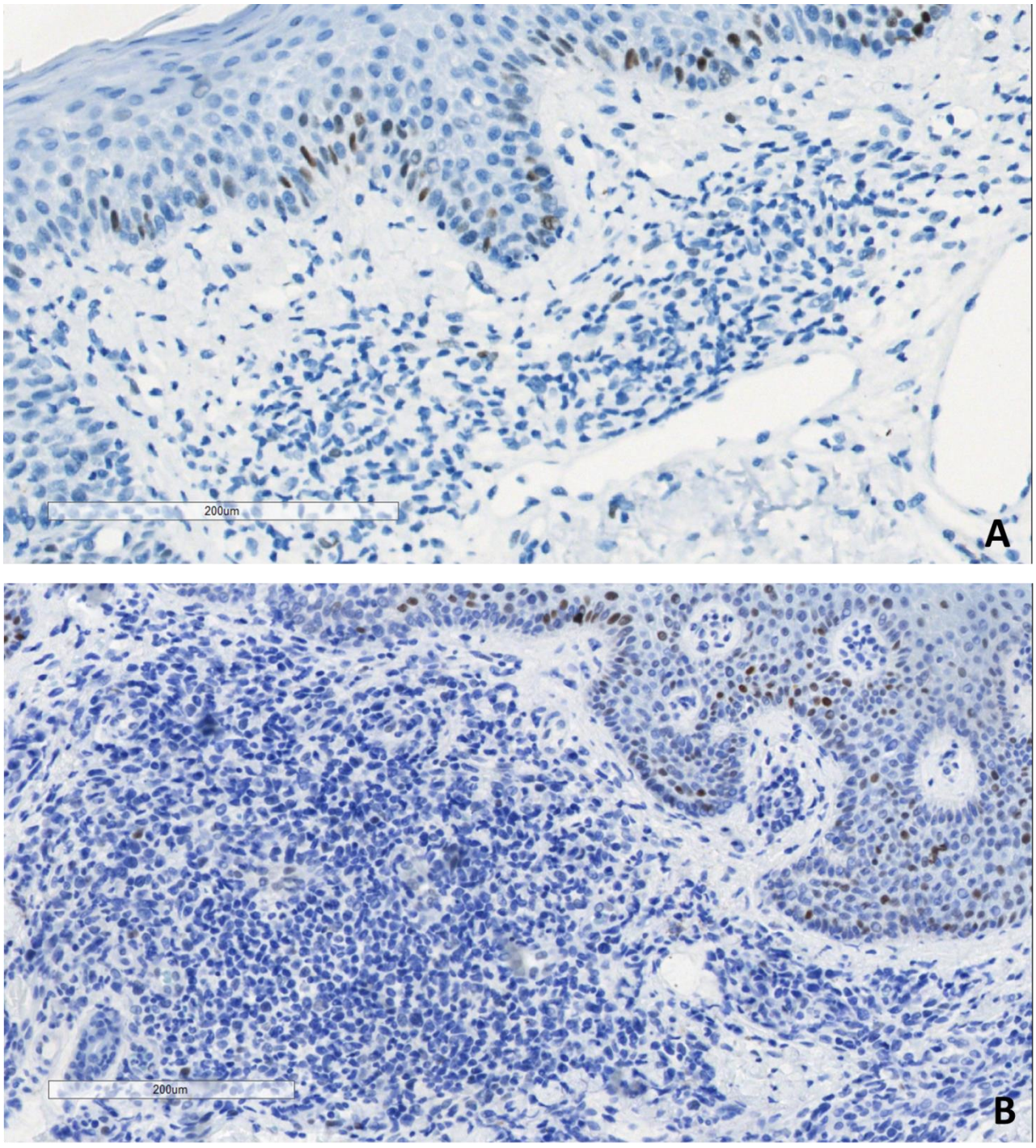

Figure 27. Examples of p53 negativity in SS tumor cells regardless let-7 expression (A and B). Basal keratinocytes served as internal positive control for the reaction.

In a second step SS patients were grouped basing on let-7 expression status in order to verify whether expression of some molecules was influenced by let-7 expression. Table 10 shows immunohistochemical results in different groups:

Table 10. Immunohistochemistry and let-7 expression

Let-7 status	Patient n°	CCND1	c-Myc	HSP-70	p27	p53
let-7 norm	5	neg	neg	neg	neg	+/-
	14	+/-	neg	+	neg	neg
	17	+/-	++	+/-	neg	neg
let-7b down	4	neg	+	+	neg	neg
	8	+/-	+/-	++	+	neg
	13	+/-	++	+	++	+
	18	neg	+	++	neg	neg
let-7c down	15	+/-	+	+/-	+	neg
	20	+/-	++	+	++	neg
	26	neg	+/-	neg	neg	neg
both let-7 down	24	+	++	++	neg	+/-

As already described above, cyclin D1 showed a strong positivity only in patient n° 24 in which both let-7b and let-7c were found downregulated (Figure 28).

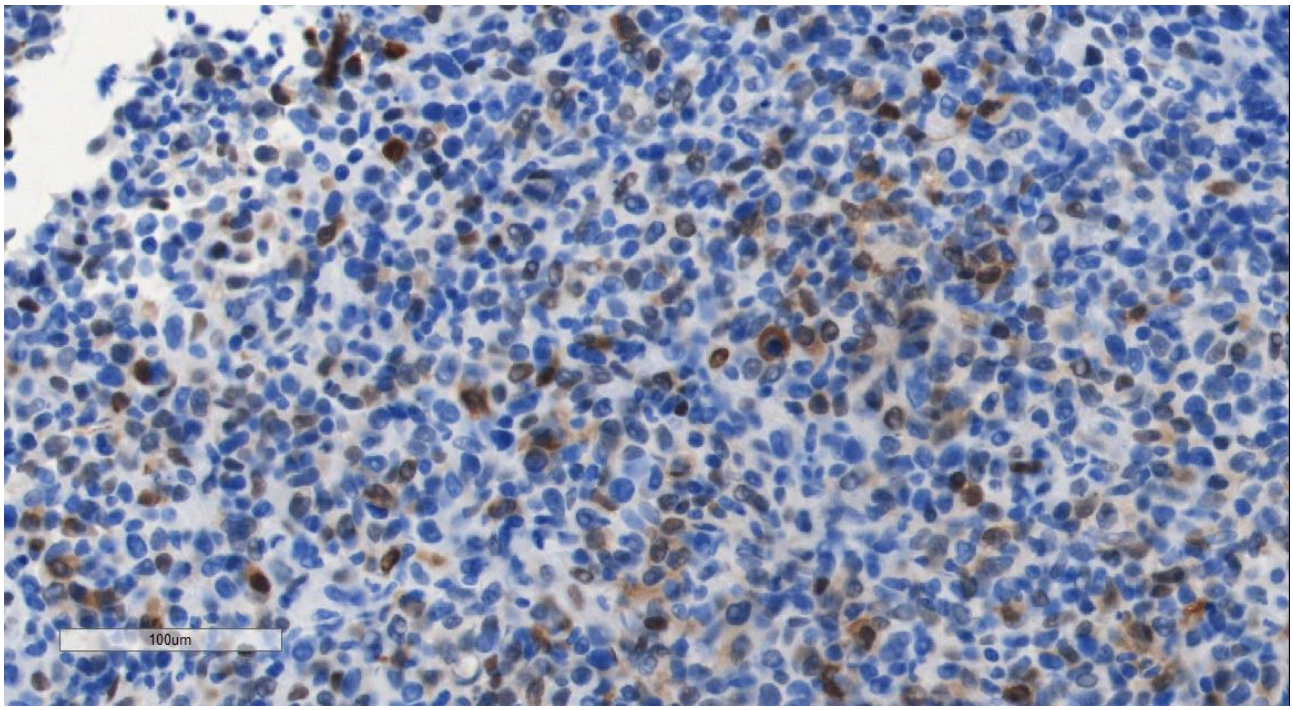

Figure 28. Cyclin D1 expression in patient n° 24. Some infiltrating cells showed an intense positivity. Among these also typical Sézary cells.

No differences were evidenced in p27 and p53 expression. c-Myc was almost all strong positive on tumor cells. However, 2 out of 3 patients with a normal let-7 expression showed a c-Myc negativity by immunohistochemistry (Figure 29).

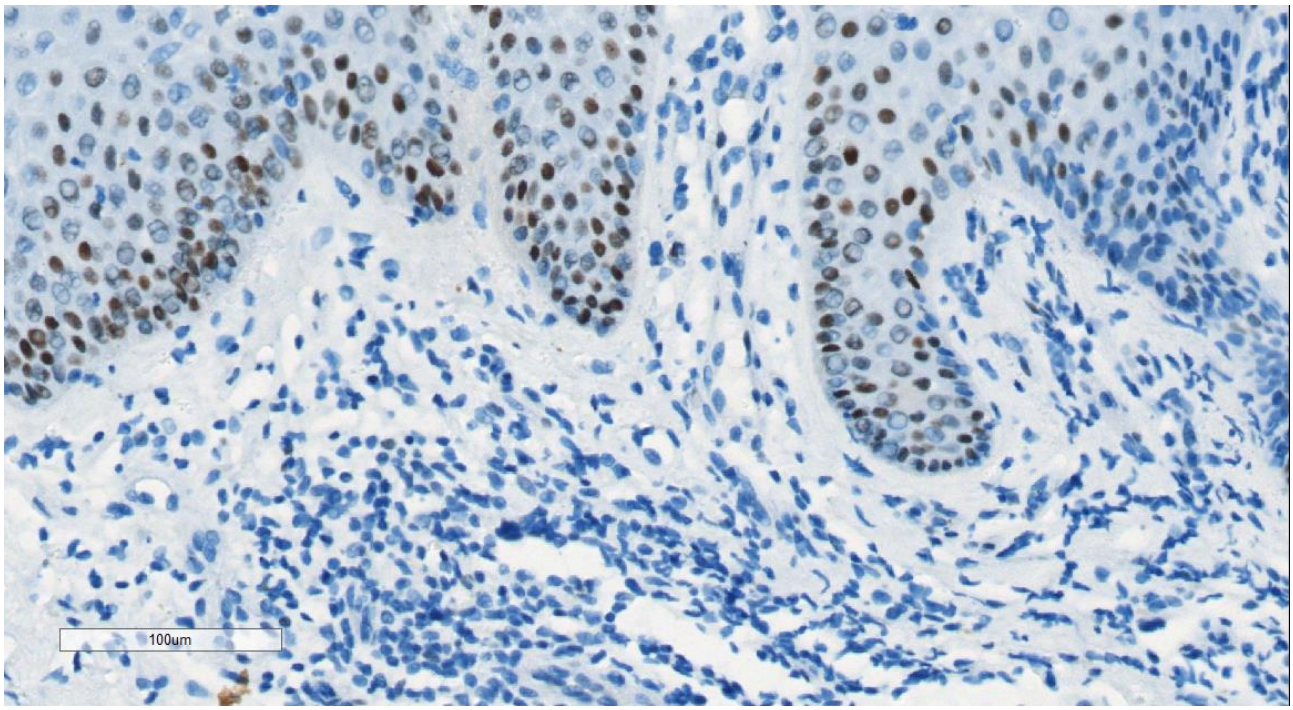

Figure 29. An example of c-Myc negativity in a patient with let-7 normal expression. No cells within dermal infiltrates were stained. Reaction control were keratinocytes showing nuclear staining.

5 DISCUSSION

The present study was focalized on a possible role of Lin28/Let-7 axis in Sézary Syndrome. Let-t family members' downregulation is widely known to play a role in cancer mechanisms and many of their targets were already described to be involved in the pathogenesis or maintenance of SS. Moreover, in some previous studies, let-7 family members resulted downregulated in SS when compared with normal controls.

For these reasons a direct link between let-7 expression in SS and a possible role in the onset or progression of the disease seemed to be plausible.

The first part of the study was dedicated to confirm by qRT-PCR let-7 downregulation in SS. Among let-7 family members just let-7b and let-7c were choosen to be validated. This becacuse they were the members yet seen as the most downregulated in SS patients in a previous miRNA profiling study. qRT-PCR experiments confirmed let-7b and let-7c downregulation in more than 70% of patients versus 0% of healthy controls.

As a first consideration is to note that downregulation was not present in all the patients. This could mean that other members acting on the same targets could be downregulated when both let-7b and let-7c are normally expressed. Otherwise, let-7 members could be involved in some SS mechanism other than onset.

In fact, if let-7 downregulation were the cause of the disease all SS patients would be found downregulated. Moreover, other members of let-7 family should be investigated in order to have a more complete overview.

In a second step of the study the aim was to know whether let-7 downregulation was due to a direct repression by Lin28 molecules.

Expression analyses of Lin28A and Lin28B revealed a complete absence of Lin28B mRNA, while Lin28A was expressed only in a group of SS patients and in none controls.

It is well known that Lin28 expression is mainly confined in embryonic stem cells and no adult tissues expresses normally this protein. Nevertheless, in about 15% of tumors, a reactivation of one between Lin28A or Lin28B is described.[255, 273] In those cancers in which Lin28 is reactivated, one of the let-7 members is always downregulated.

In the present study a significant linear correlation was found between Lin28A expression and let-7c downregulation. In fact, 6 out of 7 patients with let-7c downregulation had a detectable Lin28 expression, while among SS patients without let-7c downregulation only 2 out of 11 patients had a detectable Lin28A expression. This suggest that let-7c downregulation in SS is due to a Lin28A

reactivation. Further studies could demonstrate whether Lin28A repression leads to restored levels of let-7c confirming a direct inhibition via Lin28A. Moreover, studies focused on chemoresistance in cells expressing Lin28A or with Lin28A silencing could address whether Lin28A should be considered a new terapeutical target in SS. On the other hand, Lin28A expression and let-7b downregulation didn't show linear correlation. It is possible that let-7b downregulation was due to other mechanisms and not to a direct repression via Lin28. In fact, Nishi et al demonstrated that let-7b is downregulated in leukemic cells via DNA hypermethylation of its regulatory region.[259] Further studies focused on methylation status of regulatory region of let-7b could unravel this point.

Among let-7 targets only CCND1 expression was detectable. The mRNA of this gene resulted expressed at the same levels in both SS patients and HD. By means of immunohistochemistry, Cyclin D1 sowed similar stainings in all SS patients with rare and scattered positive nuclei among neoplastic cells. No evidences suggested an upregulation of this gene/protein in let-7 downregulated samples, nor in let-7 normal expressed ones.

Unfortunately, nor HMGA1 and HMGA2 nor MYC expression was measurable in both SS and HD samples. This was certainly due to a technical error during the RNA extraction.

At the time of RNA extraction, the protocol used (RNeasy Plus Mini Kit-Qiagen) suggested this procedure in order to obtain small RNAs (containing miRNAs). No other suggestions were present.

At the time of writing (more or less two years later), the same protocol added an appendix (Appendix E) in which additional steps allowed to purify larger RNAs starting from the first spin column used. Here the sentences as they appear in the new version of the protocol: *["If purifying both small RNA (containing miRNA) and larger RNA (>200 nt), save the RNeasy Mini spin column for use in step E13 (the spin column can be stored at 4°C or at room temperature [15–25°C], but not for long periods). Follow steps E6 to E12 to purify small RNA, and then steps E13 to E17 to purify larger RNA.]*

For this reason RNA obtained from CD4+ isolated cells in both SS and HD samples were not suitable for qRT-PCR experiments measuring expression levels of let-7 targets.

Unfortunately, HMGA1 and HMGA2 were not testable by means of immunohistochemistry within this study because of no suitable antibodies in a short period of time.

Considering data obtained in the present study, is not possible to determine whether let-7 downregulation acts or not on HMGA1/2 expression. Further investigations to highlight this point are needed.

Regarding c-Myc expression, 17 SS patients were tested by immunohistochemical stainings. As expected, neoplastic cells were positive in a great proportion of samples. This is the reason why no significative differences of c-myc expression were detected between SS patients with let-7

downregulation and SS patients with let-7 normal levels. Howeveer, 2 out of 3 samples in let-7 normal expressed group showed lower c-myc expression by means of immunohistochemistry. Although the very small number of samples, this result could be interpreted as a less c-myc activation in SS patients without let-7 downregulation. In fact, myc activates Lin28 that repress let-7. The last one can activate itself myc creating a feedback loop.[241, 274, 275] MYC is widely described as amplified in a high percentage of SS[35, 192, 226, 271, 276-279] Gain of 8q was reported being one of the most frequent copy number event in this disease. A possible mechanism could start with 8q gain and the consequent amplification of MYC. This could bring to reactivation of LIN28A that represses let-7 family members. Downregulation of let-7 leads, on his own, to a stimulation of MYC transcription.

As already described above, let-7 downregulation should not to be considered a pivotal event in SS onset. However, the loop mechanism involving Myc/Lin28A/let-7 could lead to a chemoresistance.

Of note, a very high number of patients with let-7 downregulation were non-responders to different chemotherapy approaches. That does not mean that all non-responder patients had let-7 downregulation, so this event is not necessary in chemoresistance. Other studies, with different approaches, should be carried on in order to verify whether chemoresistance is due to or worsened by let-7 downregulation.

Although some authors suggested FAS downregulation in SS as responsible of diminished apoptosis in tumor cells,[215, 220, 221, 280] in the present study no aberrant expression of FAS or of FASL was observed. However, downregulation of proapoptotic Bcl-2 family members emerged from apoptosis array analysis. Among them a statistical significance was reached by diminished expression of Bad, Bax, Bcl-w and BID. To the best of my knowledge no previous studies highlighted a downregulation of these proapoptotic molecules in SS. Nevertheless, there are no studies focused on expression of pro-apoptotic molecules in CTCLs. Further analyses are needed to understand whether these molecules are definitely less expressed in SS than in normal CD4+ lymphocytes. A larger cohort of SS patients could validate the significance of the downregulation of proapoptotic Bcl-2 family members. Moreover, other approaches, such as immunohistochemistry, western blot and qRT-PCR, should be used to confirm these results.

On the other hand, this study highlights a higher expression in SS than in HD for two members of inhibithors of apoptosis (IAPs) family, namely cIAP-2 and XIAP. These molecules are never described to be overexpressed in CTCL. However XIAP is described to play an important role in chemoresistance in diffuse large B-cell lymphomas (DLBCL)[281-284] and in Hodgkin's lymphoma.[284] Moreover a couple of papers describing the effects of new drugs in CTCL showed among

mechanisms of action the power to reduce XIAP and cIAP-2 in CTCLs, including SS.[285, 286] Once again overexpression of XIAP and cIAP-2 seems to influence chemoresistance in CTCLs.

Heat shock proteins (HSPs) are chaperone proteins which are synthesized in response to various stresses and they are highly expressed in different types of cancers, including lymphomas.[286-288] HSPs are not only involved in tumor progression but also in determining their response to treatment.[286, 287, 289, 290] HSP-70, for instance, contributes to the resistance of cells to anti-cancer therapies and its neutralization leads to an apoptosis induction in drug-resistant cells.[286, 287, 290] In the present study two important HSPs have been found highly expressed in SS by using different methods. By means of apoptosis array HSP-60 resulted overexpressed in pathologic CD4+ lymphocytes of SS patients. This result should be validated by using other techniques and in a larger cohort of patients. HSP-60 was identified to regulate apoptosis via interacting with Bax and Bad.[289, 291-295] Interestingly, high levels of HSP-60 are related to low levels of Bax and Bad, highlighting a inverse correlation between HSP-60 levels and those of Bcl-2 proapoptotic members.

HSP-70 was found expressed in SS tissue samples not only by tumor lymphocytes, but also by reactive cells. This is in line with data reported in literature that reported the key role played by HSPs in regulating the immune response within the tumor microenvironment.[287, 296-298] HSPs, in fact, are known to act as immunomodulants.

HSP-70 expression by means of immunohistochemistry varies from small number and scattered cells within infiltrate to diffuse and high-intensity positivity. Of note, only samples from SS patients in which let-7b downregulation was found showed the intense and diffuse pattern, while samples from SS patients having normal levels of let-7b resulted scarcely stained. As HSP-70 was described to act in chemoresitance also in lymphomas,[286-288, 296, 297, 299, 300] it could be interesting verify whether HSP-70 expression in SS is related to drug resistance. In fact, in all world wide guidelines of Sézary treatment both doxorubicin and gemcitabine are suggested as first- or second-line therapy, and high levels of HSP-70 were linked to doxorubicin[290] and gemcitabine[300] refractoriness.

Cyclin-dependent kinase inhibitors (CDKNs) are a group of proteins which can inhibit cyclin-dependent kinases (CDKs) and then stop the cell cycle and/or induce apoptosis. For this reason CDKNs are widely considered tumor suppressors and pro-apoptotic molecules. Many CDKNs are described deregulated in a wide type of cancers including lymphomas. Within this family deletions and/or downregulation of CDKN1A (p21), CDKN1B (p27), CDKN2A (p16) and CDKN2B (p15) have been reported in CTCLs.[35, 277, 301-304]

The present study did not take in account p15 and p16, already widely described and discussed in SS. However, by means of apoptosis array, protein expression levels of both p21 and p27 were measured. Both CDKNs were found lower expressed in SS than in HD. Moreover, both p21 and

p27 were identified to be let-7 targets.[305-307] At the time of writing there are no evidences on a direct link between let-7 downregulation and p21/p27 lower expression. However, future studies could focus on whether let-7 status could regulate CDKNs levels.

About p53 expression in SS, present study confirms previous published data. In fact, TP53 (gene encoding the transcription factor p53) is located in chromosome 17p, widely described as one of the most deleted region in SS.[35, 165, 171, 192, 216] Moreover, some authors have recently demonstrated that p53 is nonfunctional in the majority of SS cell lines and patient samples.[173, 216, 308] In this study p53 was found low expressed in SS by either arrays and immunohistochemistry, as expected. Moreover, considering the let-7 downregulated SS patients, p53 levels were found lower than in the whole SS group. There are some papers that reported a direct link between let-7 and p53. In one of these authors demonstrated a linear correlation between downregulation of p53 and Lin28A expression.[309] They suggested that inhibition of p53 upregulated Lin28A and c-Myc levels and downregulated let-7 expression levels. This mechanism could be the same involved in SS, in which a downregulation of p53 is followed by upregulation of Lin28A and c-Myc with a consequent downregulation of let-7c that could lead to an augmented chemoresistance.

In conclusion, the present study confirmed let-7b or let-7c downregulation in more than 70% of SS patients. Probably, this downregulation didn't affect the pathogenesis of SS. However, patients presenting let-7 downregulation seemed to be more prone to drug-resistance. Possible mechanisms pass through reactivation of Lin28A by p53 or by c-Myc. The first one could be due to a deletion of TP53 or inactivation of protein p53 which leads to a reactivation of Lin28A and subsequently to a downregulation of let-7c and upregulation of c-Myc. As c-Myc overexpression is able to reactivate Lin28A, this starts a loop mechanism. The second mechanism could be due to reactivation of Lin28A by c-Myc. In fact, it's speculable that gain of 8q24 (region containing MYC) leads to a c-Myc overexpression with consequent Lin28A reactivation. Lin28A represses let-7 increasing chemoresistance in tumor cells. Moreover, other dysregulated molecules, namely cIAP-2, XIAP, p21 and p27 asould contribute to drug-resistance in SS. Finally, as the rate of proliferation of SS cells is less than that of other tumor cells, it seems to be likely that the very large number of tumor cells and accumulation of mutations are due to a defect in apoptosis. Many pro-apoptotic factors such as Bax, Bad, Bcl-w and BID, have been found less expressed in SS than in HD. Nevertheless, anti-apoptotic factors cIAP-2 and XIAP resulted highly expressed. Taken together these data suggest the existence of complex mechanisms regulating apoptosis and drug resistance in SS. Further studies, including functional studies, are needed to better understand the role of each molecule in this complex network.

6 LIMITATIONS AND FUTURE PERSPECTIVES

Although with some limitations due to the scarce material available this study highlighted the importance of let-7/Lin28 axis in SS.

Firstly, the study confirmed a downregulation for let-7 family members in SS. The limited number of SS patients included in the present study represented a limitation particularly to obtain a strong statistical analyses. Validation of data with a larger cohort of patients is needed.

Moreover, let-7 family account 10 members, while the study focused only on two of them. Further studies should broaden perspectives by studying other family members as well.

About the role of Lin28A in direct repression of let-7c this study strongly suggests that mechanism as the main involved. However, further insights about the direct contribution of Lin28A in blocking let-7c maturation are required. Particularly, studies focusing on *in vitro* models in which Lin28A expression will be induced could lead to understand whether let-7c downregulation is due to a direct repression by Lin28A.

Results obtained lead to deepen this topic even if remains to clarify whether Lin28/let-7 axis has a role in the pathogenesis of the disease or in chemoresistance.

About let-7 targets it will be possible to investigate which possible let-7 interactants have potential role in oncogenesis and/or chemoresistance.

Future directions will be aimed at analyizing let-7 targets by qRT-PCR, western-blot and immunohistochemistry. Moreover, as let-7b and let-7c were normal expressed in HH cell line, functional studies could be carried out by silencing let-7b and let-7c in HH cells in order to verify which targets change their expression after let-7 downregulation.

Finally, *in vitro* chemosensitivity assays could be carried on to investigate whether let-7 downregulation lead to a major chemoresistance against agents usually used in SS therapy such as gemcitabine, chlorambucil and doxorubicin.

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