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**DEVELOPMENT OF NEW
THERAPEUTIC STRATEGIES
IN MATURE T-CELL NON-
HODGKIN LYMPHOMAS**

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This dissertation is dedicated to all the people who have walked alongside me during these Ph.D. years . Despite the struggles to combine scientific training, clinical duties and family life, I eventually did it, and I have to thank all the persons that made it possible.

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TABLE OF CONTENTS

INTRODUCTION	12
1. T-cell non-Hodgkin Lymphomas	12
<i>Epidemiology and Classification</i>	12
<i>Peripheral T-cell Lymphomas</i>	14
<i>Molecular Basis and Pathogenesis</i>	16
<i>Prognosis and First Line Treatment of Peripheral T-cell Lymphomas</i>	24
<i>Outcome of Relapsed and Refractory Peripheral T-cell Lymphomas</i>	30
2. Tenascin-C	34
<i>The molecule</i>	34
<i>Clinical trials in lymphoid neoplasms</i> ..	39
3. L-Asparaginase	42
<i>The molecule</i>	42
<i>Role of asparaginase activity</i>	46
<i>Role of glutaminase activity</i>	47

	<i>Glutamine and malignant cellular metabolism.....</i>	49
	<i>Mechanism of resistance to L-Asparaginase</i>	50
	<i>Clinical use of L-Asparaginase</i>	53
4.	Scope of the thesis	58
5.	References.....	61

PRIMARY TREATMENT RESPONSE

RATHER THAN FRONT LINE STEM CELL

TRANSPLANTATION IS CRUCIAL FOR

LONG TERM OUTCOME OF PERIPHERAL

T-CELL LYMPHOMAS.....85

1.	Abstract.....	86
2.	Introduction.....	88
3.	Subjects And Methods	91
	<i>Patients</i>	91

	<i>Treatments and outcome evaluation.....</i>	92
	<i>Statistical analysis</i>	93
4.	Results.....	95
	<i>Clinical characteristics</i>	95
	<i>Response and survival in the whole cohort.....</i>	95
	<i>Response and survival according to primary treatment response.....</i>	98
5.	Discussion.....	101
6.	Tables and Figures	107
	<i>Table 1. Patients Characteristics</i>	107
	<i>Table 2. Treatments and Outcome</i>	108
	<i>Table 3. Multivariate analysis for overall survival of the whole cohort</i>	109
	<i>Table 4. Clinical Characteristics of Patients Responsive to Primary Treatment.....</i>	110
	<i>Table 5. Multivariate analysis for overall survival of the responders</i>	111
	<i>Figure 1.</i>	112
	<i>Supplementary Figure 1.</i>	113

7. References..... 114

TENATUMOMAB EVALUATION OF
TENASCIN-C REVEALS PERIPHERAL T-
CELL LYMPHOMA AS ATTRACTIVE
TARGET FOR RADIOIMMUNOTHERAPY ..121

1. Abstract..... 123

2. Introduction..... 125

3. Materials and Methods 128

Patients and Samples..... 128

Immunohistochemistry..... 128

Gene Expression Analysis 131

Statistical Analysis 131

4. Results..... 133

*Immunohistochemical Expression of
Tenascin-C*..... 133

Correlation with Clinical Parameters 136

	<i>Gene Expression of Tenascin-C</i>	137
5.	Discussion.....	139
8.	Tables and Figures	146
	<i>Table 1. Summary of staining characteristics</i>	146
	<i>Table 2. Multivariate analysis for PFS and OS in PTCL patients</i>	147
	<i>Figure 1.</i>	148
	<i>Figure 2</i>	150
	<i>Figure 4</i>	152
	<i>Figure 5</i>	153
	Supplementary Figure 1	155
6.	References.....	156

	EFFECT OF L-ASPARAGINASE IN T-CELL NON-HODGKIN LYMPHOMA: A PRECLINICAL STUDY	163
--	--	-----

1.	Introduction.....	165
----	-------------------	-----

2.	Materials and Methods	170
	<i>Cell lines</i>	170
	<i>Reagents</i>	171
	<i>Alamar blue cytotoxicity assays</i>	172
	<i>Apoptosis measurement</i>	173
	<i>Real-time quantitative PCR for asparagine and glutamine synthetase genes</i>	174
	<i>Detection of ASNS and GS protein</i>	177
	<i>Immunohistochemistry</i>	178
	<i>Statistical analysis</i>	182
3.	Results	183
	<i>ASNase inhibits cell growth and selectively induces cell death of T-cell NHL cell lines</i>	183
	<i>Asparagine synthetases gene expression level and protein quantification</i>	185
	<i>T-cell NHL cell lines are sensitive to glutamine deprivation</i>	186
	<i>Glutaminase activity of ASNase is necessary to inhibit cell growth in T-cell NHL cell lines</i>	188

	<i>Asparagine and glutamine synthetases expression is low in several human T-cell NHL samples</i>	189
4.	Discussion.....	192
9.	Tables and Figures	200
	<i>Table 1.</i>	200
	<i>Figure 1.</i>	202
	<i>Figure 2.</i>	204
	<i>Figure 3</i>	205
	<i>Figure 4.</i>	207
	<i>Figure 5.</i>	208
	<i>Figure 6.</i>	209
	<i>Figure 7.</i>	211
	<i>Figure 8.</i>	213
	<i>Figure 9.</i>	215
5.	References.....	216

CONCLUSION AND FUTURE

PERSPECTIVES	224
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PUBLICATIONS227

INTRODUCTION

1. T-cell non-Hodgkin Lymphomas

Epidemiology and Classification

Non-Hodgkin lymphoma (NHL) is the most common haematological cancer. In 2012, in Italy, the estimated incidence of NHL was 14.5 cases per 100.000 inhabitants with a mortality of 4.4 cases per 100.000 (Ferlay et al., 2013). The World Health Organization (WHO) classify NHL according to the cell of origin in B-cell and T-cell lymphomas (Swerdlow et al., 2008). The latter category includes a heterogeneous and relatively rare group of malignancies accounting for 10-15% of all NHL. The WHO classification identifies nearly 20 different histologies of T-cell NHL, grouped according to the prevalent clinical presentation i.e. leukemic, nodal or extranodal (Swerdlow et al., 2008) (Table 1).

TABLE 1 – 2008 WHO CLASSIFICATION OF PTCL

Peripheral T cell lymphoma, not otherwise specified (PTCL/NOS)
Peripheral T cell lymphoma, specified:
<i>Leukemic:</i>
T cell prolymphocytic leukemia
T cell large granular lymphocytic leukemia
Aggressive NK cell leukemia
Systemic Epstein–Barr virus positive T cell lymphoproliferative disease of childhood (associated with chronic active EBV infection)
Hydroa vaccineforme-like lymphoma
Adult T cell leukemia/lymphoma
<i>Extranodal:</i>
Extranodal NK/T cell lymphoma, nasal type (ENKL)
Enteropathy-associated T cell lymphoma
Hepatosplenic T cell lymphoma
Subcutaneous panniculitis-like T cell lymphoma
Mycosis fungoides
Sézary syndrome
Primary cutaneous anaplastic large-cell lymphoma
Primary cutaneous aggressive epidermotropic CD8+ cytotoxic T cell lymphoma (provisional entity)
Primary cutaneous γ/δ T cell lymphoma
Primary cutaneous small/medium CD4+ T cell lymphoma (provisional entity)
<i>Prevalently nodal:</i>
Angioimmunoblastic T cell lymphoma
Anaplastic large cell lymphoma (ALCL), anaplastic large cell lymphoma kinase (ALK) positive
ALCL, ALK negative (provisional entity)

The systemic nodal/extranodal forms are usually defined as peripheral T-cell lymphomas (PTCL).

Conversely, the primary cutaneous T-cell lymphomas (CTCL), representing the most common forms of cutaneous lymphoma, includes indolent variant with a limited and manageable skin involvement and more aggressive disease characterized by skin tumors or blood involvement and poor prognosis.

Peripheral T-cell Lymphomas

Systemic PTCL includes a wide spectrum of relatively rare disease that have been considered difficult to classify, diagnose and treat (Foss et al., 2011). The morphologic heterogeneity, the lack of molecular markers and the paucity of biological studies on this groups of NHL are the major causes of the difficulties in classification and thus, in diagnosis (O'Connor et al., 2014). A marked variation in the frequency of the various subtypes by geographic region was reported, with PTCL-not otherwise specified (NOS) as the most common

subtype in both North America and Europe, whereas extranodal NK/T-cell lymphoma (ENKL) and adult T cell leukemia/lymphoma (ATLL) common in Asia (Table 2) (Vose et al., 2008).

TABLE 2 – PTCL SUBTYPES BY GEOGRAPHIC REGION

T-cell lymphoma subtype	Europe (%)	North America (%)	Asia (%)
PTCL-NOS	34.3	34.4	22.4
Angioimmunoblastic lymphoma	28.7	16.0	17.9
ALCL, ALK positive	6.4	16.0	3.2
ALCL, ALK negative	9.4	7.8	2.6
ENKL	4.3	5.1	22.4
ATLL	1.0	2.0	25.0
Enteropathy-type	9.1	5.8	1.9
Hepatosplenic	2.3	3.0	0.2
Primary cutaneous ALCL	0.8	5.4	0.7
Subcutaneous panniculitis-like	0.5	1.3	1.3
Unclassifiable T-cell	3.3	2.3	2.4

Adapted from (Vose et al., 2008)

The five most common histologies in Western countries, as identified in an international retrospective survey, are PTCL-NOS (frequency:

34.3%), angioimmunoblastic T-cell lymphoma (AITL) (28.7%), anaplastic large cell lymphoma (ALCL) expressing the anaplastic large cell lymphoma kinase (ALK) (9.4%), enteropathy-associated T-cell lymphoma (EATL) (9.1%) and ALCL ALK negative (6.4%). However, significant differences are present in the literature relatively to the frequency of the subtypes, in particular for ALCL, most probably due to local pattern of referral (Schmitz et al., 2010).

Molecular Basis and Pathogenesis

According to the 2008 WHO classification, lymphoproliferative neoplasms are divided on the basis of morphology, immunophenotype, genetics, and clinical features (Swerdlow et al., 2008). Differently from the B-cell counterpart, classification of mature T-cell lymphoma is hindered by their clinically and pathologically heterogeneity, with inconstant and aberrant

expression of phenotypic markers and lack of recurrent molecular alterations, with the sole exception of ALCL ALK positive (O'Connor et al., 2014). This complexity, summed to the rarity of the disease, poses a great challenge in terms of diagnostic accuracy. Additionally, the paucity of cell lines and animal models hampered until recently the understanding of tumor biology.

The cell of origin of the different forms of mature T-cell lymphomas has been investigated by immunohistochemical analysis. It was reported that AITL and ALCL share a homogeneous effector cell phenotype (CD45RA⁻CD45R0⁺CD27⁻) but differed in the cytotoxic and activation markers, whose expression was observed only in ALCL (Geissinger et al., 2006). Conversely most of the PTCL-NOS evaluated (5 of 8) were CD4⁺ central memory cell (CD45RA⁻/CD45R0⁺/CD27⁺) and four expressed the lymph node homing receptor CCR7. Authors concluded that AILT and ALCL tumor cells correspond to different subsets of

effector cells, while a subset of PTCL-NOS correlates with a non-effector T-cell population. With the availability of antibodies for the T-cell receptor (TCR) isoforms α/β and γ/δ , it has been shown that hepatosplenic T-cell lymphoma and primary cutaneous γ/δ T-cell lymphoma along with a proportion of ENKL, type II EATL and a small minority of PTCL-NOS share a putative common derivation from γ/δ lymphocytes and are characterized by a markedly aggressive clinical course (Tripodo et al., 2009). Additionally, the use of markers of activation (e.g., CD25 and CD30), T regulatory elements (FOXP3), or specialized categories of T lymphocytes such as T- follicular helper (TFH) cells (e.g. CD10, CXCL13, PD-1), supported further immunohistochemical lineage definition. The expression of FTH molecules was revealed especially in AITL and in a subset of PTCL-NOS (Marafioti et al., 2010). However, immunohistochemistry proved as an imprecise tool for the histogenetic definition of PTCL, due to

both technical limitation of the procedure and the aberrant expression or loss of one or more of the T cell-associated markers; as an example, CD10 expression was detected in only 39% of AITL, even when adopting a low cut-off value (Went et al., 2006).

Gene expression profiling (GEP) technology includes several techniques to simultaneously quantify the mRNA expression levels of nearly all the human genes, the resulting transcriptome is subsequently evaluated with the appropriate statistical and bioinformatic tools to identify relevant pathways and interactions between pathways in the biology of the neoplasm. GEP has been applied in several B-cell NHL allowing a better understanding of the cell of origin of the disease and a more accurate diagnosis compared to standard morphologic and immunohistochemical techniques and, more recently, it has been used also to improve the characterization of PTCL (de Leval et al., 2007;

Piccaluga et al., 2007). Additionally, the development of high-throughput sequencing i.e. next-generation sequencing (NGS), allowed to sequence DNA and RNA much more quickly and cheaply than the previously used Sanger sequencing, thus allowing to improve the knowledge of the recurrent the genetic alterations in of PTCL (Odejide et al., 2014; Schatz et al., 2015).

The PTCL-NOS expression profile was found to be similar to activated T lymphocytes, with most cases related to CD4⁺ helper T cells and only a minority with the characteristics of CD8⁺ cytotoxic T-cells (Piccaluga et al., 2007). A unique subgroup of PTCL-NOS with cytotoxic features and adverse prognosis was described (Iqbal et al., 2010a; Iqbal et al., 2010b), however, nearly half of the PTCL-NOS cases resulted uncharacterized by these studies. More recently, in a large study including 372 PTCL patients, two groups of PTCL-NOS have been recognized according to the

expression of GATA3 (33% of the cases) or TBX21 (49%) and their relative target genes, with the first group characterized by adverse prognosis (Iqbal et al., 2014). Additionally, a distinctive activation of therapeutically targetable pathways was recognized, with the NF- κ B and STAT3 pathways overexpressed in the TBX21 group and mTOR and PI3K pathways activated in the GATA3 group.

More compelling evidence permitted to identify the cell of origin for AITL in the follicular helper T-cell, as several studies have shown enrichment of TFH like signatures in this PTCL subgroup along with a marked microenvironmental signature that correspond to the histological findings of mixed inflammatory cells within the areas of neoplastic involvement (de Leval et al., 2007; Iqbal et al., 2010b; Piccaluga et al., 2007). However, TFH like signatures was recorded also in some cases lacking the classic morphologic characteristics of AITL and thus included in the

PTCL-NOS diagnostic category (Iqbal et al., 2010b; Iqbal et al., 2014). These cases shares with AITL the genetic background, including *RHOA* gene mutations, and are included in the upcoming WHO classification in a new provisional entity “*nodal peripheral T-cell lymphoma with TFH phenotype*” (Palomero et al., 2014; Sakata-Yanagimoto et al., 2014; Swerdlow et al., 2016; Yoo et al., 2014).

The GEP signature of ALCL can distinguish cases harboring the ALK fusion protein to those negative for the rearrangement. In fact, ALK positive ALCL is characterized by increased transcript of ALK, CD30, MUC1 and Th17-associated genes (e.g. IL-17A, IL-17F, and ROR- γ) and a concert of regulators of the STAT3 pathways (Piva et al., 2006). In contrast ALK negative ALCL is enriched for expression of MYC and IRF4 target gene signatures as well as for proliferation and mTOR pathway signatures (Weilemann et al., 2015). The distinction between

ALK positive and negative ALCL is further empathized by the different somatic mutations, as JAK1 and/or STAT3 genes (38%), DUSP22 (found in 30% of the cases) and TP63 (8%) that have been identified in ALK negative cases only (Crescenzo et al., 2015; Parrilla Castellar et al., 2014).

Most of ENKL can be identified by signatures typical of the NK cell of origin, along with high expression of cytotoxic molecules as granzyme, and activation of the NF- κ B and JAK/STAT3 pathways (Iqbal et al., 2009; Iqbal et al., 2010a).

It is of interest the observation that PTCL of $\gamma\delta$ derivation shows a similar GEP signature with respect to ENKL, with additional presence of T-cell receptor (TCR)/CD3 complex transcripts (Kucuk et al., 2015). Activating STAT3 mutations and hypermethylation of PRDM1 have been identified in NK-cell malignancies, while STAT5B

mutations have been found in 6% of $\gamma\delta$ PTCL (Kucuk et al., 2011; Kucuk et al., 2015).

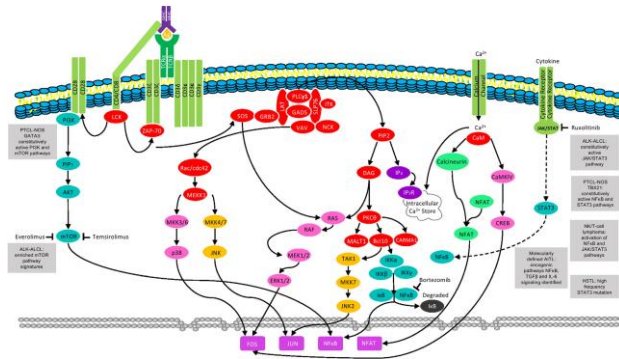


FIGURE 1. CONSTITUTIVELY ACTIVATED PATHWAYS IN PTCL AND THEIR POTENTIAL THERAPEUTIC TARGETS. SCHEME OF PATHWAYS DEREGULATED IN PTCL IDENTIFIED THROUGH INTEGRATION OF GEP AND NGS DATA (IQBAL ET AL., 2016)

Prognosis and First Line Treatment of Peripheral T-cell Lymphomas

Clinical course of PTCL is aggressive and outcome is poor with conventional treatment (Vose et al., 2008). While CHOP-like therapy can cure the disease of most patients with ALCL ALK

positive, there is currently no satisfactory treatment for the other histotypes of PTCL. Despite intensive treatments, prognosis is generally poor with only a minority of patients achieving long term remission.

Early studies showed that T-cell compared to B-cell phenotype in aggressive NHL was associated with adverse prognosis with 5-year overall survival (OS) rates of 41% vs. 53%, respectively (Gisselbrecht et al., 1998). More recently, in a large multi-national retrospective study the 5-year OS and event-free survival (EFS) for the main PTCL subtypes were 32% and 20% for PTCL-NOS, 32% and 18% for AITL, 70% and 60% for ALCL ALK positive, 49% and 36% for ALCL ALK negative, 32% and 29% for nasal ENKL, 32% and 6% for extra-nasal ENKL, 14% and 12% for ATLL (Vose et al., 2008). Several efforts have been made to improve standard CHOP therapy during the last three decades. A large randomized phase III trial evaluated MACOP-B versus CHOP for the treatment of aggressive NHL

(Jerkeman et al., 1999). Fourteen cases, including intestinal and angioimmunoblastic subtypes, were classified as PTCL and 36 cases were defined as ALCL. Patients with PTCL had a worse 5-year OS (17%) compared to diffuse large B cell lymphoma (61%). The outcome of cases with ALCL was similar to that of diffuse large B cell lymphoma. The German High-Grade Non-Hodgkin Lymphoma Study Group (DSHNHL) recently reported the outcome of PTCL patients enrolled in 7 different phase II and III trials (Schmitz et al., 2010). The three-year OS and EFS for the main PTCL subtypes were 54% and 41% for PTCL-NOS, 67% and 50% for AITL, 90% and 76% for ALCL ALK positive, 62% and 46% for ALCL ALK negative. Treatment in the different studies consisted of CHOP regimens given every 14 or 21 days for 6 or 8 cycles; CHOP with the addition of etoposide; escalating standard doses of cyclophosphamide, doxorubicin, etoposide, and prednisone in younger (≤ 60 years) patients. Only

the addition of etoposide in younger patients with normal LDH was shown to be beneficial, while intensive CHOP-like regimens (e.g. Mega-CHOEP) were detrimental to survival. In a recently published phase III trial, eighty-eight PTCL patients were randomized to receive CHOP-21 or a more intensive ABVD-like regimen (VIP-rABVD) (Simon et al., 2010). Primary end point of the study was the improvement of 2-year EFS. The overall response rate (ORR) was 62% (complete remission, CR: 39%), similar in both arms. Responses in the PTCL subtypes were 64% for ALCL, 53% for AITL and 29% for PTCL-NOS. The 2-year EFS rate was 45% ($\pm 8\%$) with VIP-rABVD and 41% ($\pm 7\%$) with CHOP-21. Thus, despite the unsatisfactory results, currently CHOP-21 with or without the addition of etoposide is the most commonly used regimen for PTCL. Interestingly, current evidence indicate that the use of anthracycline is not associated to superior

outcome in PTCL, with the exception of ALCL ALK positive histology (Vose et al., 2008).

The incorporation of monoclonal antibodies in first-line treatment regimens for PTCL has been explored. Alemtuzumab is an IgG1 kappa monoclonal antibody specific for the cell surface glycoprotein CD52, and have shown substantial efficacy in CD52-expressing B and T-cell lymphomas as a single agent. Several trials evaluated the combination of alemtuzumab with standard CHOP therapy. In a phase II study, 24 newly diagnosed PTCL patients were treated with 30 mg of alemtuzumab given subcutaneously and CHOP every 28 days for up to 8 cycles (Gallamini et al., 2007; Mak et al., 2013). Response was good, with a 71% of patients achieving a CR. The 2 year OS and failure free survival were 53% and 48%, respectively. The good treatment results were however hampered by major infectious toxicity, with life-threatening infections occurring in one-quarter of the patients. The Dutch-Belgian

Hemato-Oncology Group (HOVON) group reported disappointingly similar results with a more intensified C-CHOP program (Kluin-Nelemans et al., 2011). CHOP was given every 14 days while alemtuzumab was administered at 30 mg three times every cycle. The ORR of the 20 patients enrolled was 90% (65% CR) with a 2-year OS and EFS of 55% and 27%, respectively. Major toxicities were represented by 7 cases of neutropenic fever, one CMV retinitis and 3 patients developing secondary Epstein-Barr virus (EBV)-related lymphoma.

Given the high risk of early and late relapse, consolidation with autologous stem-cell transplantation (SCT) has been explored as part of the up-front treatment after induction therapy. In a large phase II trial enrolling 160 advanced PTCL patients (with the exclusion of ALCL ALK positive) autologous SCT was used as consolidation after 6 cycles of standard CHOP with or without etoposide (CHOEP). Authors

reported a 56% of CR at 3 months after the end of the therapy and a 5-year OS and progression-free survival (PFS) of 51% (95% CI, 43% to 59%) and 44% (95% CI, 36% to 52%), respectively (d'Amore et al., 2012). Allogeneic SCT has been proposed as consolidation therapy in responsive PTCL, but its role is still under investigation with only few studies currently available. Despite this procedure is reserved only for a minority of young and fit patients, the reported results are interestingly good and provide evidence of a graft versus T-cell lymphoma effect (Corradini et al., 2014).

Outcome of Relapsed and Refractory Peripheral T-cell Lymphomas

Most of the patients with PTCL will never respond to first-line treatment or will relapse. We have recently underlined this issue in a retrospective study of 209 PTCL patients treated at our Center. While only 63% of the patients achieve

a complete remission (CR) after first-line treatment, 32% of those remitters still experience a relapse. Outcome of relapsed/refractory PTCL is poor. In the absence of hematopoietic stem-cell transplant, treatment is usually palliative, with a median progression-free survival (PFS) and overall survival (OS) of 3.7 months and 6.5 months respectively (Mak et al., 2013). Allogeneic or autologous hematopoietic stem cell transplantation (SCT) are generally offered to patients with chemosensitive disease to offer a chance of long-term survival, with a OS ranging from 36% to 81% (Corradini et al., 2004; Dodero et al., 2011; Kyriakou et al., 2008; Rodriguez et al., 2001). In a retrospective study reporting the Center for International Blood and Marrow Transplant Research (CIBMTR) data of 183 PTCL patients receiving SCT not in first CR, the 3-year OS and PFS reported was of 53% and 42% for those receiving autologous SCT and 41% and 31% for

allogeneic SCT patients, respectively (Smith et al., 2013).

Drugs currently approved for relapsed/refractory setting are few. FDA-approved treatment in relapsed/refractory PTCL i.e. romidepsin, pralatrexate and belinostat do not provide effective solution, as the overall response rate (ORR) is low (29-38%) and the median duration of response insufficient (8.9-13.6 months) (O'Connor et al., 2015; O'Connor et al., 2011; Piekarz et al., 2011). Conversely, brentuximab vedotin (BV), an anti-CD30 antibody conjugated to the potent anti-microtubule agent monomethylauristatin, had shown a significant clinical activity. In a phase II trial on 58 ALCL patients the overall response rate (ORR) was 86% with a 57% of complete remission (Pro et al., 2012). The median durations of overall response and CR were 12.6 and 13.2 months, respectively. Other subtypes of PTCL other than ALCL can express CD30, but the expression is less frequent

and variable. The use of BV in 34 relapsed/refractory CD30 positive PTCL have been recently reported (Horwitz et al., 2014). The ORR and CR rate was 41% and 24%, respectively, with AITL histology showing the best results (CR in 38%). On the basis of the relevant activity, BV has been tested in first-line associated with standard chemotherapy. In a phase II trial reported as an abstract, BV was associated to CHP (vincristine was omitted due to overlapping neurotoxicity) in 26 patients with CD30 positive PTCL (Fanale et al., 2014). The reported exciting ORR and CR rate of 85% and 62% spurred the launch of a randomized double-blind phase III trial (NCT01777152). Despite the clinical activity of BV, ALCL still experience relapse and refractoriness to this approach and the large majority (about 60-80%) of the other PTCL histology do not express CD30 adequately. Thus, new treatments for this patient population are urgently needed.

2. Tenascin-C

The molecule

Tenascins are large glycoproteins found in embryonic and adult extracellular matrices. Only two of the tenascin members, the original tenascin-C and the more recently discovered tenascin-W, have been shown to be overexpressed in tumours compared to healthy tissues (Brellier and Chiquet-Ehrismann, 2012). Tenascin-C, the first described member of the family, can be transiently expressed during adult life after tissue injury and remodelling, while it is not expressed under physiologic conditions. (Chiquet-Ehrismann et al., 1986). It is a multimodular protein comprising four

distinct domains: an assembly domain, a series of epidermal growth factor-like repeats, a series of fibronectin type III-like repeats, and a C-terminal fibrinogen-like globe (Figure 2) (Midwood et al., 2011).

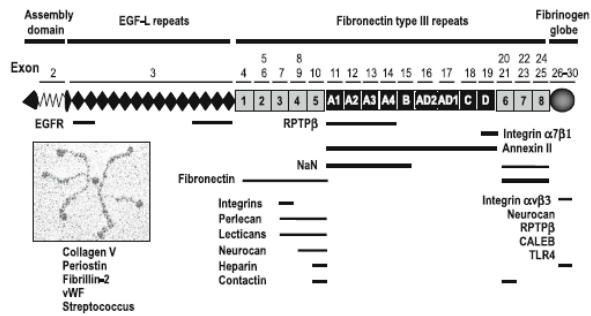


FIGURE 2. THE MODULAR STRUCTURE OF TENASCIN-C AND THE KNOWN BINDING PARTNERS OF EACH TENASCIN DOMAIN (MIDWOOD ET AL., 2011).

Through specific interactions between those domains and cell surface receptors or other extracellular components, tenascin-C mediates a wide range of functions in the embryonic life, during tissue injury and in cancer progression.

Tenascin-C can undergo alternative splicing resulting in large (up to 320 kDa monomer) or small (220 kDa monomer) isoforms. The large variant is preferentially expressed in malignant tissues and is spatially and temporally related to tumor neovascularization and exerts anti-adhesive and immunosuppressive activity (Ghert et al., 2001; Jahkola et al., 1998; Puente Navazo et al., 2001; Zagzag and Capo, 2002). Along with the pivotal role of tenascin-C in neoangiogenesis, the molecule has been shown to promote tumor cell

proliferation in standard cell culture conditions (Chiquet-Ehrismann et al., 1986; Huang et al., 2001). Additionally, the potentiality of tenascin-C in promoting tumor metastasis was reported. Indeed, it has been shown that knocking-down tenascin-C in melanoma cells significantly decreased their capacity to form pulmonary metastases (Fukunaga-Kalabis et al., 2010).

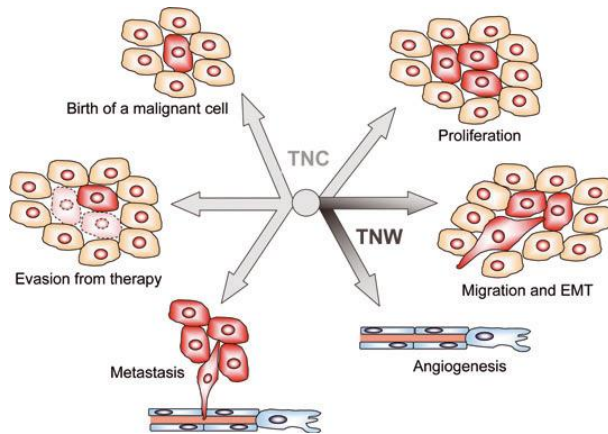


FIGURE 3. SUMMARY OF THE ACTIONS OF TENASCINS IN CANCER (BRELLIER AND CHIQUET-EHRISMANN, 2012)

Most of carcinomas are characterized by the presence of tenascins in the extracellular compartment, and in different setting high expression of the molecules is correlated with poor outcome (Midwood et al., 2011). Several preclinical and clinical studies proved that tenascin-C is a good candidate for antibody mediated therapy in solid cancer (Brellier and Chiquet-Ehrismann, 2012). Similarly, tenascin-C

was reported to be expressed in the hematological setting. In 55 pathological samples of B-cell NHL and Hodgkin disease, the F16 anti-tenascin-C antibody strongly stained nearly all the cases, with tenascin-C being present in the extracellular matrix and the hyperplastic blood vessels (Schliemann et al., 2009).

Clinical trials in lymphoid neoplasms

The clinical feasibility of targeting tenascin-C expressed in the microenvironment of B-cell NHL and Hodgkin lymphoma have been reported. The 81C6 antibody (Neuradiab, Bradmer Pharmaceuticals Inc., Toronto, Canada) was tested in a phase I study enrolling B-cell NHL patients (Rizzieri et al., 2004). Toxicity profile was limited to hematologic events with 4 patients necessitating infusion of autologous bone marrow stem cells. An objective response was observed in 2 of the 9 patients, including one complete remission. More recently, the F16 antibody directed to the extra-

domain A1 of tenascin-C conjugated with ¹³¹I (Tenarad, Philogen SpA, Siena, Italy) was tested in 8 patients with relapsed/refractory Hodgkin lymphoma (Aloj et al., 2014). Toxicity profile was acceptable and mainly limited to hematologic events, objective response was observed in two of the eight patients, including one complete remission. Tenatumomab (ST2146, Sigma Tau S.p.A. R&D, Pomezia, Italy) is a murine IgG2b/k humanized antibody recognizing an epitope within the EGF-like repeats of human tenascin, shared by both small and large tenascin isoforms (De Santis et al., 2006; De Santis et al., 2003; Petronzelli et al., 2005). Tenatumomab have been developed to be applied in the context of the three-step Pretargeted Antibody-Guided Radioimmuno-Therapy (PAGRIT), that consisted of the sequential administration of a biotinylated monoclonal antibody, avidin/streptavidin, and a radiolabeled biotin molecule, leading to specific accumulation of radioactivity at the tumor site

implemented by the multiple valence of avidin/streptavidin toward biotin (Paganelli et al., 2001; Paganelli et al., 1999). Interestingly, in a case report, biotinylated tenatumomab monoclonal antibody and 90Y-biotinDOTA (90Y-ST2210) proved to be extremely effective in a pediatric patient with ALCL ALK positive, relapsing after allogeneic stem cell transplant (Palumbo et al., 2007). However, further development of tenatumomab in PAGRIT was hampered by regulatory hurdles. Tenatumomab has been directly conjugated with ¹³¹I for radio-immunotherapy (RIT) application and is currently in phase I clinical development in tenascin-C expressing cancer (NCT02602067).

3. L-Asparaginase

The molecule

L-asparaginase (ASNase) is an enzyme that hydrolyzes the amino acid L-asparagine to L-aspartic acid and ammonia (Figure 4Figure 1). It is a therapeutic agent currently approved for lymphomas and leukemia; in particular it is a cornerstone of acute lymphoblastic leukemia treatment.

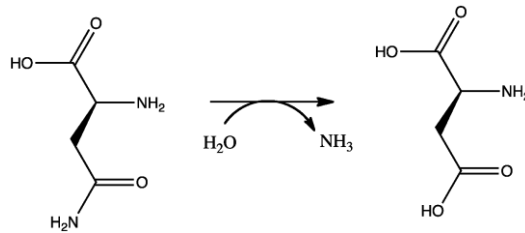


FIGURE 4. ASNASE REACTION

Activity of ASNase was shown early in the fifties by Kidd, that demonstrated the ability of guinea pig serum to control the growth of lymphoid cancer of in vivo mouse systems (Kidd, 1953). Interestingly, it was observed that while guinea pig serum inhibited the growth of lymphoma cell lines *in vivo*, it failed to inhibit the growth of the same cells *in vitro*. However it was not clear at that time which component of guinea pig serum was provided of the cytotoxic effect observed. In 1963, Broome demonstrated that ASNase was responsible for the anti-lymphoma activity (Broome, 1963a; Broome, 1963b). The study spurred the use of ASNase in humans.

However, commercial scale synthesis was necessary for clinical trials and, among the different natural source of the drug, bacteria were identified as the most convenient for this purpose. The formulations currently used in the clinic are derived from two bacteria: *E. coli* and *E. chrysanthemi* (Pieters et al., 2011; Wade et al.,

1968). The most commonly used derives from *E. coli* and it is a tetramer of molecular weight of 138-141 kDa, composed by identical subunits each of which is provided by an active site (Figure 5). L-Asparaginase derived from *E. chrysanthemi* (Erwinase) is a serologically and biochemically distinct form of ASNase, however it shows a similar tetrameric structure and super-imposable pharmacological activity.

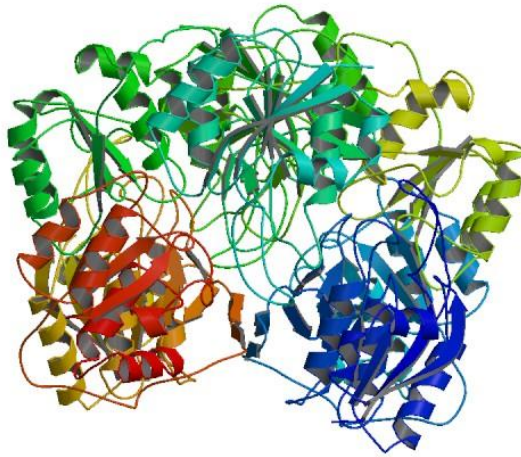


FIGURE 5. CRYSTAL STRUCTURE OF E. COLI ASNASE (SWAIN ET AL., 1993)

As L-asparaginase half life is short and it may induce host response with the production of anti-asparaginase neutralizing antibodies, different formulation have been studied. The use of the pegylated formulation of ASNase can overcome this limitation by altering the immunogenicity of

the protein and allowing a more convenient schedule as it shows a prolonged half-life (Avramis and Tiwari, 2006).

Role of asparaginase activity

The main enzymatic activity of ASNase is to hydrolyze the amino acid L-asparagine to L-aspartic acid and ammonia, thus leading to depletion of the circulating pool of the amino acid (Emadi et al., 2014). Traditionally, the therapeutic activity of ASNase has been attributed to the sensibility to asparagine deprivation of cells unable to synthesize L-asparagine from L-glutamine i.e. devoid of Asparagine Synthetase (ASNS), and thus lack of external source of asparagine leads to selective inhibition of growth of sensitive neoplastic cells. Primary ALL cells and several cell lines exhibit a particularly low level of ASNS expression and are extremely sensitive to asparagine depletion, and reports correlate response to ASNase with ASNS expression both at

mRNA and protein level (Hermanova et al., 2012; Stams et al., 2005; Su et al., 2008).

Role of glutaminase activity

Beside asparagine deamidation, ASNase has a considerable glutaminase activity i.e. L-glutamine is hydrolyzed into glutamic acid and ammonium. As glutamine is the key nitrogen donor for asparagine biosynthesis, lack of glutamine contributes to asparagine deprivation (Wise and Thompson, 2010). Glutaminolytic activity of ASNase was regarded as major culprit for the toxic effect of the drug. However this idea is currently debated, as it becomes clear that glutamine deprivation, along with asparagine hydrolysis, may be responsible for the therapeutic effect. Amino acids exert a critical role in cell biology, as they serve as building blocks for protein synthesis as well as signal transduction messengers transmitting the nutritional status of the entire organism to individual cells. It has been

shown that asparagine and glutamine deficiency can elicit a general transcriptional response termed amino acid response pathway (Gong et al., 1991; Harding et al., 2003; Shan et al., 2010). Upon amino acid depletion, uncharged tRNAs activate general control nonderepressible 2 kinase (GCN2), which phosphorylates subsequently eukaryotic initiation factor 2 alpha (eIF2 α). This latter protein is a fundamental metabolic switch that lowers the rate of global protein synthesis and leads to energy conservation needed for cell survival. Among the effector of eIF2 α is the transcription factor ATF4 leads to expression of several genes involved in cell survival as ASNS, Glutamine Synthetase (GS) and others involved in amino acid synthesis (Aslanian and Kilberg, 2001; Bunpo et al., 2009). However, if the stress cannot be relieved, gene expression then switches to promoting cell death via transcription of the pro-apoptotic transcription factor C/EBP homologous protein (CHOP), also known as growth arrest and DNA damage-

inducible gene 153 (GADD153) (Covini et al., 2012; Harding et al., 2003). Additionally, ASNase mediated depletion of glutamine is responsible for decrease of the mammalian target of rapamycin complex 1 (mTORC1) (Reinert et al., 2006; Willems et al., 2013). This signaling is a master controller of cell growth and division, coupling energy and nutrient abundance to protein synthesis rate by regulating other important kinases, such as S6 kinase (S6K) and Akt (Zoncu et al., 2011). It has been shown that inhibition of mTORC1 with ASNase can induce apoptosis in a model of acute myeloid leukemia (Willems et al., 2013).

Glutamine and malignant cellular metabolism

It has been known for a long time that glutamine is a key element for neoplastic cellular metabolism (Hensley et al., 2013; Schulze and Harris, 2012). While glycolytic pathway is markedly up-regulated, the penultimate step in glycolysis, the conversion of phosphoenolpyruvate

to pyruvate, catalyzed by the enzyme pyruvate kinase, is attenuated and most of the pyruvate is converted to lactic acid and not to acetyl-CoA, necessary for the complete breakdown of glucose in the tricarboxylic acid (TCA) cycle (termed Warburg effect). Thus, most of the fuel for TCA cycle is α -ketoglutarate derived by breakdown of glutamine. TCA cycle in cancer cell is essential both as a source of energy and for production of metabolic intermediates to support cell growth (Reitzer et al., 1979). Lack of glutamine severely impairs neoplastic cell growth and this condition is termed “glutamine addiction”.

Mechanism of resistance to L-Asparaginase

The mechanism of ASNase is still not fully understood as it involves a complex condition derived from amino acid deprivation within a multifaceted interaction among neoplastic and normal tissues, in this context several resistance mechanism have been described (Avramis, 2012).

Primary cell and cell lines up-regulate ASNS mRNA content in response to ASNase treatment *in vitro* and drug-selected ASNase-resistant cell lines exhibit elevated expression of ASNS. As described previously, up regulation of ASNS under the amino acid response pathway is a major mechanism of resistance to ASNase (Figure 6). However, ASNS expression did not proved to be an accurate predictive factor for ASNase susceptibility, as its level measured in patient samples did not consistently showed biological and clinical consequences (Appel et al., 2006; Stams et al., 2003).

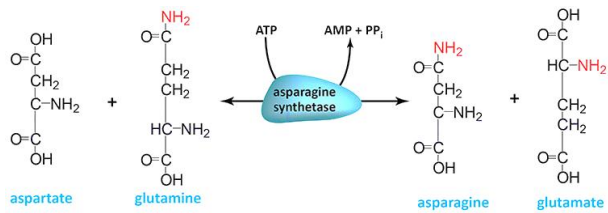


FIGURE 6. ASPARAGINE SYNTHETASIS MECHANISM OF ACTION

Gene-expression patterns of ASNase resistance was tested on leukemia cells from pediatric acute lymphoblastic leukemia patients and compared with treatment outcome (Holleman et al., 2004; Holleman et al., 2006). The resulting 54 gene-probe sets related to ASNase sensitivity had a higher percentage of protein metabolism genes, specifically several ribosomal protein genes (e.g., RPL3, RPL4, RPL5, RPL6, and RPL11), and was independently associated with treatment response (Holleman et al., 2004). Additionally, 15 different genes involved in apoptosis have been linked to apoptotic changes underlying ASNase cytotoxicity (Holleman et al., 2006).

Interaction between normal and neoplastic tissue have been implied, in first instance the input from the de novo biosynthesis of asparagine by the liver. Additionally, it has been shown that also normal adipocytes within bone marrow can replete the glutamine pool necessary to induce ASNase

resistance in acute lymphoblastic leukemia (Behan et al., 2009; Ehsanipour et al., 2013).

A key element of ASNase resistance is represented by the host immunological effects of formation of neutralizing antibodies induced by ASNase protein that can abrogate the enzymatic activity of the drug. Many clinical trials reported the induction of a wide range of human antibodies against the bacterial protein ASNase, and showed a direct link between this immune response and treatment outcome (Avramis, 2012; Panosyan et al., 2004). Replacement of the multiple injections of native *E.coli* ASNase with the long half-life PEG-ASNase have reduced the anti-ASNase antibody formation (Avramis et al., 2002).

Clinical use of L-Asparaginase

The antitumor activity of ASNase has been tested in early study in different hematologic and solid cancers. The particular sensitivity to the drug of acute myeloid and lymphoid leukemias was

reported by several investigators (Hill et al., 1969; Leventhal and Henderson, 1971), but some response was observed in chronic lymphocytic leukemia and disseminated lymphosarcoma (i.e. NHL) while Hodgkin disease and solid tumors were fully resistant (Clarkson et al., 1970). The impressive result of ASNase in acute lymphoblastic leukemia spurred the incorporation of this drug into multi-agent induction regimens. In the last two decades great improvements have been made and intensive ASNase containing regimens showed the best results in this setting (Bassan and Hoelzer, 2011; Rizzari et al., 2014). Clinical development of ASNase-containing regimens in other disease setting have been hampered by the concurrent development of alternative treatments and the toxicity of the drug, especially in the adult setting.

More recently, ASNase emerged as an effective drug in extranodal natural killer/T-cell lymphoma (ENKL), a highly aggressive PTCL

frequent in Asia and rare in Western countries (Jaccard and Hermine, 2011). Up to 90% of the cases are localized to the nasal cavity (nasal type) but a fraction of patients have a disseminated disease (extranasal). Most of the neoplastic cells are positive for NK lineage markers, but in a minority of the cases they show a cytotoxic T-cell phenotype. Additionally, ENKL cells show a constant integration of the Epstein-Barr virus (EBV). Use of ASNase in this setting have been spurred by the preclinical observation that NK-cell tumor cell lines and clinical samples, which are generally resistant to chemotherapy, are sensitive to ASNase treatment (Ando et al., 2005). Additionally, low levels of ASNS expression correlated to in vitro sensitivity to ASNase, with the exception of NK-cell leukemia. In this latter case ASNase were able to induce considerable apoptosis despite elevated ASNS expression at both mRNA and protein levels, due to a glutamine

deprivation-dependent apoptosis, independent from ASNS expression.

The incorporation of ASNase in methotrexate-based multi-agent chemotherapy regimens dramatically improved the outcome of ENKL patients. In a phase II study on 38 patients with advanced stage or relapsed/refractory disease, a chemotherapy including methotrexate, ifosfamide, etoposide, dexamethasone and ASNase (SMILE) followed by autologous or allogeneic SCT resulted in a complete remission rate of 45% and a 1-year overall survival of 55% (Yamaguchi et al., 2011). In a European trial, the combination of L-asparaginase with methotrexate and dexamethasone proved to be effective in 19 relapsed/refractory patients with a complete remission rate of 61% (Jaccard et al., 2011).

Clinical efficacy of L-asparaginase in PTCL (excluding ENKL) is anecdotal. Two case reports described the successful treatment of relapsed/refractory patient with EBV-positive

cytotoxic PTCL. In the first report, subcutaneous PTCL was refractory to standard chemotherapy agents but responded to ASNase (Obama et al., 1999), while the second patients achieved a second complete remission with a combination of ASNase and prednisolone that allowed a subsequent allogeneic SCT (Takahashi et al., 2010). A retrospective study, the incorporation of ASNase in the first line treatment of PTCL patients improved remission but not survival (Yao et al., 2015).

4. Scope of the thesis

Over the past few years several new therapeutic options have become available for relapsed or refractory B-cell non-Hodgkin lymphomas (NHL) and Hodgkin lymphoma disorders (Batlevi et al., 2016; Cheah et al., 2016), but only marginal progress if any have been achieved for mature T-cell NHL (O'Connor et al., 2014). Thus, identification of new treatment strategies and/or new active compounds for T-cell lymphoma is a major unmet need.

In the first part of the thesis we retrospectively evaluated the clinical characteristics, treatments and outcome of PTCL patients treated at our Center over the last two decades. Aim of this study was to reclassify the cases according to the 2008 WHO criteria, define treatment patterns and relative outcome along with the prevalent reason for treatment failure and the role of first-line consolidation therapy with

autologous SCT (Gritti et al., 2015). Our results show that primary response rather than post-remission programs (i.e. autologous SCT) is the crucial determinant of outcome in PTCL, and that a substantial part of the cases is resistant to multi-chemotherapy induction regimens. Basing on this, we preclinically evaluated two different non-chemotherapeutic therapeutic approaches in PTCL.

Taking advantage of the availability of diagnostic specimens of T-cell NHL patients, we showed for the first time in this setting the expression of tenascin-C, a large extracellular glycoprotein overexpressed in cancer that is currently under clinical evaluation as a target for radioimmunotherapy.

In the last part of the thesis, we evaluated the potential therapeutic role of the enzymatic drugs ASNase in PTCL. We first to evaluate in an *in vitro* model of mature T-cell NHL the activity of ASNase and the role of the known mechanism of resistance i.e. asparagine and glutamine

synthetases expression. In a second part we tested the presence of such resistance mechanism in a set of clinical samples covering the biological diversity of the disease, to evaluate if a proportion of PTCL patients might be sensitive to ASNase.

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**PRIMARY TREATMENT RESPONSE
RATHER THAN FRONT LINE STEM
CELL TRANSPLANTATION IS
CRUCIAL FOR LONG TERM
OUTCOME OF PERIPHERAL T-
CELL LYMPHOMAS**

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1. Abstract

Outcome of systemic peripheral T-cell lymphomas (PTCL) is unsatisfactory and no controlled clinical study guides the therapy. Phase II studies suggest to consolidate response achieved after front-line treatment with stem cell transplant (SCT). We retrospectively evaluate the impact of front-line SCT consolidation in a single Center cohort of 209 patients treated during the last two decades. Median age was 49 years (range 15-85) with a prevalence of male sex (61%), advanced stage (68%) while IPI was >2 in 44%. Primary treatment was MACOP-B (39%) CHO(E)P (39%), intensive regimens (18%) or others (4%). Complete response to primary treatment (i.e. before SCT) was 60% (5% partial remission). Forty-four patients further proceeded to SCT while 92 did not receive consolidation. Outcome of primary responders was good, with a 3-year overall survival of 74% (82% in ALCL ALK+ and 69% for the other histologies). By multivariate analysis

a better overall survival was significantly associated with IPI<2 (P=0.001), primary response (P=0.000), and ALCL ALK+ (P=0.012). The multivariate analysis performed on responders, showed that only IPI was predictive of a better survival while ALCL ALK+ and performing SCT were not. Response to primary treatment rather than post-remission programs is the crucial determinant of PTCL outcome.

2. Introduction

Systemic peripheral T-cell lymphomas (PTCL) are a rare and heterogeneous group of non-Hodgkin lymphomas characterized by an aggressive clinical course and poor treatment response.

Several retrospective studies have shown that long-term survival of most PTCL do not exceed 30-40%, with systemic anaplastic large cell lymphoma (ALCL) expressing the anaplastic large cell lymphoma kinase (ALK) protein being the exception (Delabie et al., 2011; Savage et al., 2008; Vose et al., 2008; Weisenburger et al., 2011). The optimal treatment of these lymphomas is still a matter of debate as conventional therapy based on CHOP/CHOP-like regimens provided unsatisfactory outcome (Gisselbrecht et al., 1998; Jerkeman et al., 1999). Attempts to improve response and survival with early treatment intensification led to similarly disappointing results

(Escalon et al., 2005; Gallamini et al., 2007; Kluij-Nelemans et al., 2011; Schmitz et al., 2010; Simon et al., 2010). Several retrospective studies (Chen et al., 2008; Kyriakou et al., 2008; Rodriguez et al., 2003; Smith et al., 2013) and few small-sized phase II trials (Corradini et al., 2006; Mercadal et al., 2008; Nickelsen et al., 2009; Reimer et al., 2009; Rodriguez et al., 2007) suggested that consolidation of the first response with high dose therapy (HDT) followed by autologous stem cell transplant (SCT) is a feasible option and may guarantee better disease control, with long-term overall survival (OS) up to 60-70%. This concept has been tested in a large phase II trial enrolling 160 histologically proved patients that reported an encouraging 5-year OS and progression free survival (PFS) as good as 51% and 44%, respectively (d'Amore et al., 2012). Despite these interesting results, no comparative trial is available and it is not clear whether HDT plus autologous SCT should be considered a standard treatment in

all PTCL subgroups. In addition, the role of allogeneic SCT as front-line treatment for these patients is currently investigated in an ongoing phase II clinical study (Corradini et al., 2012).

In this study, we retrospectively evaluated the outcome of PTCL patients treated in the last two decades at our Center with the aim to evaluate the role of post-remission consolidation with SCT.

3. Subjects And Methods

Patients

Eligible for this retrospective analysis were untreated patients with systemic PTCL diagnosed between January 1990 and December 2012 at “Papa Giovanni XXIII” Hospital (formerly “Ospedali Riuniti”) of Bergamo. Patients treated with a palliative intent, those without pathological material suitable for revision and primary cutaneous T-cell lymphomas were excluded from the study. The diagnostic slides were independently reviewed by two hematopathologists and classified according to the 2008 WHO classification (Swerdlow et al., 2008). When necessary, immunostains were performed i.e. ALK in ALCL. Clinical information was gathered from the electronic charts. When necessary, the paper charts were reviewed. Patient’s informed consent was obtained per institutional regulations in all cases. Approval for the retrospective review of

these records was obtained from the Ethic Committees and the study was conducted in accordance with the Italian laws and the Declaration of Helsinki.

Treatments and outcome evaluation

Treatments included standard CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone), CHOP with etoposide (CHOEP), MACOP-B (Klimo and Connors, 1985) or intensive therapy including both clinical trials (Corradini et al., 2006; Corradini et al., 2012) and intensive acute lymphoblastic leukemia-like schemes (Bassan et al., 2009). Patients proceeding to autologous SCT received an additional stem cell mobilizing cycle (MAD or high-dose cyclophosphamide) followed by HDT according to BEAM (BCNU, etoposide, cytosine arabinoside, and melphalan) plus autologous SCT. Front-line allogeneic SCT was performed within a clinical trial (Corradini et al., 2012). Treatment response

was defined according to the 1999 International Working Group criteria (Cheson et al., 1999) and was recorded after the primary treatment (i.e. CHOP, CHOEP, MACOP-B or intensive therapy) and after SCT consolidation when applicable.

Statistical analysis

The long-term outcome was assessed in terms of overall survival (OS), progression-free survival (PFS) and disease-free survival (DFS) defined according to Cheson criteria (Cheson et al., 1999). The OS was defined as the time from the start of treatment to death for any cause. The PFS was defined as the time from the start of treatment to disease progression or death for any cause. The DFS was defined as the time from documentation of response to relapse or death for any cause. All the variables were analyzed by the Kaplan-Meier method. Patients were censored at the date of last contact, follow-up was updated in September 2013 and all living patients had been observed at least

once in the previous 3 months. Differences in survival between groups were identified by generalized log-rank analysis. Multivariate analysis for survival was performed by Cox regression model; proportional hazards assumption was tested. A chi-squared test for independence was used to compare patient's characteristics according to SCT consolidation.

4. Results

Clinical characteristics

Two-hundred and nine patients were included in the retrospective analysis. The baseline clinical characteristics are summarized in Table 1. Median age at diagnosis was 49 years with a male predominance (61%). There was a prevalence of ALCL (51%), particularly ALK positive (33%). In the majority of the cases (68%), patients had an advanced stage disease with an intermediate-high or high International Prognostic Index (IPI) (1993) in 44%. Primary treatment was MACOP-B in most of the cases until 2003 and CHOP or CHOEP later on while 18% of the patients received intensive treatments (Table 2).

Response and survival in the whole cohort

Overall response rate to primary treatment (i.e. before SCT consolidation) was 65% with a complete response (CR) achieved in 60% of

patients (Table 2). Of note, 13% of the patients died early during treatment (<6 months), with a similar incidence among the three treatment categories. Consolidation of first response with SCT was performed in 44 patients (21%). In most of these cases (N=41/44, 93%) the stem cell source was autologous while an allogeneic SCT was performed only in 3 cases. Six of the seven patients performing autologous SCT in partial remission (PR) achieved the CR. Of the three patients undergoing allogeneic SCT, one deceased by pulmonary aspergillosis shortly after engraftment, one deceased by grade IV cutaneous and intestinal acute graft versus host disease (GVHD) at day 64 and the last patient is alive and well after nearly 5 years.

Overall survival, PFS and DFS of the whole cohort are presented in Figure 1, while the impact of the different histology on clinical outcome is shown in Supplementary Figure 1.

It was not possible to evaluate the impact of specific histology on outcome due to the relatively small sample size. Interestingly, the 5 patients with Enteropathy-Associated T-cell Lymphoma (EATL) who performed autologous SCT remained all disease free after a median follow-up of 5.61 (0.63 – 7.93) years.

By univariate analysis, an inferior clinical outcome was related to IPI >2 [Hazard Ratio (HR) 2.90, P<0.001], Prognostic Index for PTCL-unspecified (PIT) (Gallamini et al., 2004) >2 (HR 3.29, P<0.001), the male sex (HR 1.60, P=0.016), the lack of a clinical response to primary treatment (HR 6.61, P<0.001), PTCL not otherwise specified (NOS) histology compared with ALCL ALK positive (HR 2.54, P<0.001) and MACOP-B treatment (HR 0.59, P=0.040) while year of treatment >2003 (HR 1.32, P=0.153) did not show any impact. By multivariate analysis IPI, ALCL ALK positive histology and primary treatment

response were independent predictors of outcome (Table 3).

Response and survival according to primary treatment response

Overall survival according to primary treatment response shows that patients responding (CR/PR) to primary treatment fares well, with a global 3-year OS of 74% (Figure 1D) that was 82% in ALCL ALK positive patients and 69% for the other histologies. With the exception of age, the presenting clinical characteristic of patient who received or not a SCT after achieving a clinical response did not significantly differ (Table 4). Of note, autologous SCT converted a PR in CR in 7 out of 8 cases (88%). By multivariate analysis, only IPI >2 was associated with an inferior survival (HR 2.37, P=0.018), while the clinical benefit related to SCT consolidation and ALCL ALK positive histology was not confirmed (Table 5). When excluding ALCL ALK positive patients

from the analysis, the results did not change and performing SCT was not associated to better prognosis (HR 0.84, P=0.609).

The clinical outcome of the 45 patients not responding to primary treatment was remarkably poor, with a 1-year OS of 19% (Figure 1D) that was as low as 4% when excluding ALCL ALK positive patients. For these patients a salvage autologous SCT was performed in 7 cases (16%) and an allogeneic SCT in 6 (13%). Long-term survivors were only 6 patients (all with a diagnosis of ALCL ALK positive) who received either an allogeneic (N=3) or autologous (N=3) SCT. On the contrary, outcome of patients treated with chemotherapy only was very poor, with only one long-term survivor that, however, experienced multiple chemosensitive relapse.

Twenty-eight patients (13%) died early during treatment. The median age of this latter cohort of patients was 62.0 years (range 35-84), 21 patients (75%) showed an IPI >2 and the disease

was symptomatic in 20 cases (71%). In most of the cases (89%) cause of death was infection, in large part associated to evidence of insufficient disease control.

5. Discussion

Over the last two decades, consolidation of first-response with HDT and autologous SCT has been explored in systemic PTCL with the intent to reduce the unacceptable high relapse rate of this group of lymphomas. The heterogeneity and relative rarity of these diseases was a great limit to perform informative trials and only few, small sized non-comparative prospective studies have been available until recently. Several studies have shown a long-term (>3 years) OS ranging from 48 to 73% after HDT and autologous SCT (Corradini et al., 2006; Mercadal et al., 2008; Reimer et al., 2009; Rodriguez et al., 2007; Smith et al., 2013). Recently, a large phase II trial including 160 histologically proven PTCL, with the exclusion of ALCL ALK positive, reported encouraging survival data, with a 5-year OS and PFS of 51% and 44%, using a dose dense CHOEP-14 induction therapy (CHOP-14 if age >60 years) and

autologous SCT in responders (d'Amore et al., 2012). More recently, allogeneic SCT has been as well explored in first-line setting (Corradini et al., 2012).

In this study, we present the outcome of a large cohort of histologically confirmed PTCL patients derived from a single Institution and followed up for a prolonged period of time. To our knowledge, this is the first report that, though retrospectively, describes the outcome of responding patients according to subsequent consolidation strategy. Our results indicate that response to primary treatment is the key variable for the long term prognosis and consolidation with HDT and SCT may not be crucially important for complete responders. Obviously, we could observe the efficacy of SCT consolidation in converting partial to complete response. We included in the current analysis patients with ALK positive ALCL, that are not usually considered eligible for consolidation therapy, despite the severe prognosis

of high risk disease (Savage et al., 2008). Reason for this choice were the homogeneous good prognosis across histologies in responding patients, confirmed by multivariate analysis, and the similar distribution of ALCL ALK positive among the two subgroups (with and without SCT consolidation). Additionally, we could confirm that the results of the study did not change excluding ALCL ALK positive patients. Despite the relatively large sample size, our study resulted too small to address the heterogeneity of PTCL. This was particularly evident for EATL, as we could observe a trend toward a better outcome of patients performing autologous SCT, data in line with a recent report (Jantunen et al., 2013).

The role of allogeneic transplantation as a front line consolidation treatment option could not be addressed by this study and is the matter of investigation of a different ad hoc designed clinical trial. However, in our limited experience, 2 out of 3 patients who underwent a first-line allogeneic

SCT died in CR and this emphasize that this procedure must remain strictly limited to either an experimental setting or a rescue salvage program. Therefore, while waiting for additional data, allogeneic SCT consolidation should be used with caution in patients responding to a first line treatment.

The primary determinant of outcome resulted to be treatment response, and despite several clinical trials have been addressing the unsatisfactory outcome of PTCL in the last two decades, 6 to 8 cycles of chemotherapy with CHOP with or without etoposide (CHOEP) still remains the standard (Dearden et al., 2011; The International Non-Hodgkin Lymphoma Prognostic Factors Project, 1993). Several novel molecules are currently facing the clinical ground and there is high expectation from the combination of standard chemotherapy with newer biological agents (Savage, 2011). Despite the rapid growth of clinical trials in PTCL driven by new drugs, the

role of consolidation with HDT and autologous SCT remains not addressed, yet.

Conversely, since primary response is such a key factor for outcome, there is an urgent need to develop strategies aimed at the early identification of poor responders. In this respect, the role of interim 18F-fluorodeoxyglucose–positron emission tomography (FDG–PET) remains controversial but in general, differently from Hodgkin disease (Gallamini et al., 2012), it does not provide informative data as to chemosensitivity of this group of patients (Cahu et al., 2011; Casulo et al., 2013; Pellegrini et al., 2012).

In conclusion, our study underlines the crucial importance of increasing the quality of response to primary treatment and the need of new drugs or innovative treatment strategies to achieve such a goal. Among these latter treatment strategies, the role of a consolidation of first remissions with autologous stem cell transplantation should always be considered

(Lunning and Horwitz, 2013) but its benefit remains to be proven by well conducted comparative, prospective, clinical trials.

6. Tables and Figures

Table 1. Patients Characteristics

	N	%
All patients	209	100
Age		
<i>Median (range)</i>	49.3 (15 – 85)	
<i>≥60</i>	69	33%
Gender		
<i>Male</i>	128	61%
Histology		
<i>PTCL-NOS</i>	67	32%
<i>ALCL ALK positive</i>	70	34%
<i>ALCL ALK negative</i>	36	17%
<i>AITL</i>	21	10%
<i>EATL</i>	10	5%
<i>Others</i>	5	2%
Ann Arbor Stage		
<i>III – IV</i>	143	68%
ECOG PS		
<i>≥ 2</i>	87	42%
Serum LDH Level		
<i>> ULN</i>	92	44%
Extranodal Sites		
<i>>1</i>	68	33%
Bone marrow involvement		
<i>Yes</i>	22*	12%*
IPI		
<i>>2</i>	91	44%
PIT		
<i>>2</i>	26*	15%*

Legend: * N= 179; *PTCL-NOS: Peripheral T-Cell Lymphoma Not Otherwise*

Specified; ALCL: Anaplastic Large-Cell Lymphoma; ALK: Anaplastic Large Cell Lymphoma Kinase; EATL: Enteropathy-Associated T-cell Lymphoma; AITL: Angioimmunoblastic T-cell Lymphoma; Others: includes hepatosplenic T-cell lymphoma (N=3) and extranodal T/NK-cell lymphoma nasal type (N=2);

Table 2. Treatments and Outcome

	N	%
All patients	209	100
Primary treatment		
<i>CHO(E)P</i>	82	39%
<i>MACOP-B</i>	81	39%
<i>Intensive regimens</i>	37	18%
<i>Others</i>	9	4%
Response to primary treatment		
<i>CR</i>	126	60%
<i>PR</i>	10	5%
<i>SD/PD</i>	45	22%
<i>Early death</i>	28	13%

Legend: *Others: includes MetAspDex regimen, ACVBP or ACOD; CR: Complete Remission; PR: Partial Remission; SD: Stable Disease; PD: Progressive Disease; SCT: Stem Cell Transplant. Early death indicates patients deceasing <6 months from diagnosis.*

Table 3. Multivariate analysis for overall survival of the whole cohort

	Hazard Ratio	CI 95%	P	3-year OS	10-year OS
Age					
< 60	1.00			59%	47%
≥ 60	1.20	0.77 – 1.88	0.425	38%	26%
IPI					
≤ 2	1.00			68%	56%
> 2	1.82	1.21 – 2.74	0.004	32%	21%
Primary treatment response					
NR/EXITUS	1.00			12%	11%
CR/PR	0.14	0.09 – 0.22	0.000	74%	57%
Histology					
ALCL ALK positive	1.00			70%	56%
Others	2.78	1.68 – 4.59	0.000	43%	33%
Chemotherapy scheme					
CHO(E)P	1.00			42%	28%
MACOP-B	1.00	0.57 – 1.76	0.999	62%	50%
Intensified	0.78	0.46 – 1.34	0.376	51%	45%
Year of treatment					
≤ 2003	1.00			57%	44%
> 2003	0.77	0.48 – 1.25	0.291	44%	-

**Table 4. Clinical Characteristics of Patients
Responsive to Primary Treatment**

	With SCT		Without SCT		P
	N	%	N	%	
All patients	44	32%	92	68%	
Age					
<i>Median (range)</i>	43.53 (19– 62)		51.33 (17 – 85)		0.0002
<i>≥60</i>	2	5%	37	40%	0.000
Sex					
<i>Male</i>	28	64%	53	57%	0.503
Histology					
<i>PTCL-NOS</i>	9	20%	28	30%	0.061
<i>ALCL ALK positive</i>	15	34%	36	39%	
<i>ALCL ALK negative</i>	9	20%	15	16%	
<i>AITL</i>	4	9%	11	12%	
<i>EATL</i>	5	11%	1	1%	
<i>Others</i>	2	5%	1	1%	
Ann Arbor Stage					
<i>III – IV</i>	29	66%	55	60%	0.492
IPI					
<i>>2</i>	14	32%	29	32%	0.972
PIT					
<i>>2</i>	5	13%	3	4%	0.077
Relapse/progression					
<i>Yes</i>	10	24%	32	36%	0.164
Primary treatment response					
<i>CR</i>	37	84%	89	97%	0.013
<i>PR</i>	7	16%	3	3%	
Transplant					
<i>Autologous</i>	41	93%	-	-	-
<i>Allogeneic</i>	3	7%	-	-	-
Final response (after SCT)					

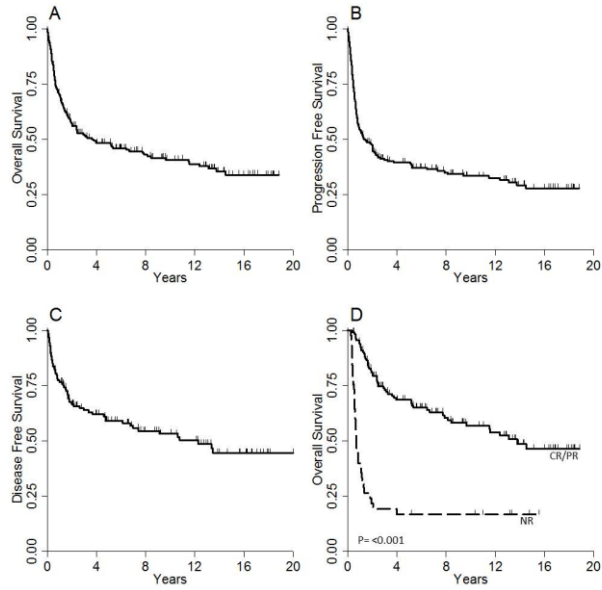
CR	43	98%	-	-	-
PR	1	2%	-	-	-

Legend: PTCL-NOS: Peripheral T-Cell Lymphoma Not Otherwise Specified; ALCL: Anaplastic Large-Cell Lymphoma; ALK: Anaplastic Large Cell Lymphoma Kinase; EATL: Enteropathy-Associated T-cell Lymphoma; AITL: Angioimmunoblastic T-cell Lymphoma; Others: includes hepatosplenic T-cell lymphoma (N=3) and extranodal T/NK-cell lymphoma nasal type (N=2); SCT: Stem Cell Transplant

Table 5. Multivariate analysis for overall survival of the responders

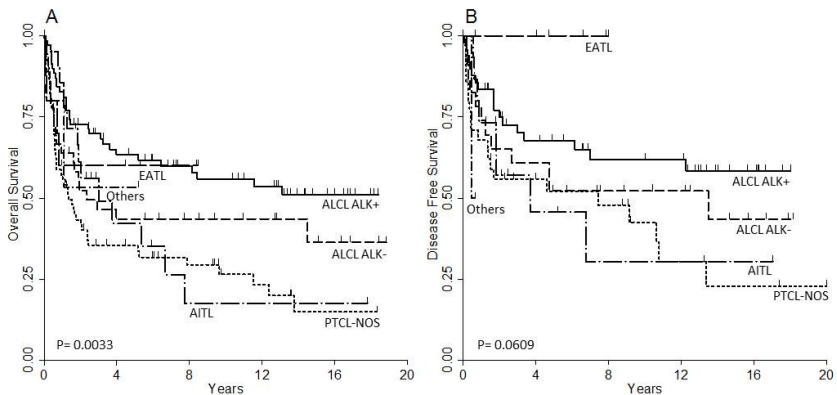
Variables	Hazard Ratio	CI 95%	P	3-year OS	10-year OS
Age					
< 60	1.00			78%	60%
≥ 60	1.08	0.52 – 2.27	0.830	63%	47%
IPI					
≤ 2	1.00			80%	65%
> 2	2.36	1.28 – 4.33	0.006	61%	39%
1st line SCT consolidation					
<i>Performed</i>	0.70	0.36 – 1.38	0.303	79%	63%
Histology					
<i>ALCL ALK positive</i>	1.00			82%	65%
<i>Others</i>	1.74	0.93 – 3.27	0.083	69%	52%
Chemotherapy scheme					
<i>CHO(E)P</i>	1.00			69%	46%
<i>MACOP-B</i>	1.15	0.59 – 2.25	0.679	74%	58%
<i>Intensified</i>	0.90	0.36 – 2.24	0.818	80%	75%

Figure 1.



Legend: Overall survival (A), progression-free survival (B) and disease free survival (C) in the whole cohort. Overall survival according to primary treatment response (E). CR: complete remission; PR: partial remission; NR: no response, including stable and progressive disease.

Supplementary Figure 1.



Legend: Overall survival (A) and progression-free survival (B) according to histology in the whole cohort. PTCL-NOS: Peripheral T-Cell Lymphoma Not Otherwise Specified; ALCL: Anaplastic Large-Cell Lymphoma; ALK: Anaplastic Large Cell Lymphoma Kinase; EATL: Enteropathy-Associated T-cell Lymphoma; AITL: Angioimmunoblastic T-cell Lymphoma; Others: includes hepatosplenic T-cell lymphoma and extranodal T/NK-cell lymphoma nasal type.

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**TENATUMOMAB EVALUATION
OF TENASCIN-C REVEALS
PERIPHERAL T-CELL
LYMPHOMA AS ATTRACTIVE
TARGET FOR
RADIOIMMUNOTHERAPY**

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1. Abstract

With currently available treatments, the prognosis of T-cell non-Hodgkin lymphoma (NHL) is poor and the identification of new targets is crucial to develop innovative therapeutic strategies. Tenascin-C, a large extracellular glycoprotein overexpressed during the fetal life as well as in solid cancer and B-cell NHL, can be recognized by tenatumomab, a monoclonal antibody binding to an epitope within the EGF-like repeats of the molecule. Since the presence of tenascin-C in T-cell NHL has not been evaluated so far, we used a tenatumomab based immunohistochemistry approach to investigate the expression of tenascin-C in 75 peripheral T-cell lymphoma (PTCL) and 25 cutaneous T-cell lymphoma (CTCL) patients. Data were analyzed in terms of staining intensity, proportion of involved areas and histological pattern, and results were correlated with the baseline clinical characteristics

and outcome. Nearly all of the cases (93%) were tenascin-C positive and 59% of PTCL were characterized by a diffuse (>50% of involved areas) expression. A stromal expression was present in all cases, while a vascular and vascular plus cytoplasmic positivity was revealed in 49% and 23%. Proportion of involved areas correlated with clinical parameter of aggressiveness. In multivariate analysis, presence of vascular pattern of expression was associated to shorter overall survival. A constant overexpression of tenascin-C gene was observed in two independent publicly available datasets. In conclusion, tenascin-C represents an attractive target that sets the bases for exploring the activity of ¹³¹I-tenatumomab in T-cell NHL which has been developed for radio-immunotherapy (RIT) applications and is currently in phase I clinical trial.

2. Introduction

Over the past few years several new therapeutic options have become available for relapsed or refractory B-cell non-Hodgkin lymphomas (NHL) and Hodgkin lymphoma (Batlevi et al., 2016; Cheah et al., 2016), but only marginal progress if any have been achieved for mature T-cell NHL (Coiffier et al., 2012; O'Connor et al., 2015; O'Connor et al., 2011; Pro et al., 2012). T-cell NHL is a heterogeneous and relatively rare group of malignancies representing 7-15% of all NHL (Swerdlow et al., 2008). It includes systemic NHL, defined as peripheral T-cell lymphoma (PTCL), and primary cutaneous T-cell lymphoma (CTCL). While the latter category shows a variable outcome with only patients with advanced stage or transformed disease suffering of adverse outcome, prognosis of systemic PTCL is generally poor with conventional treatment, with the notable exception of low-risk, ALK positive,

anaplastic large cell lymphoma (ALCL) patients (Gritti et al., 2015; Vose et al., 2008). Thus, identification of new therapeutic strategies in T-cell lymphoma is a major unmet need.

Tenascin-C is a large hexameric glycoproteins found in embryonic and adult extracellular matrices (Brellier and Chiquet-Ehrismann, 2012). It can undergo alternative splicing resulting in large (up to 320 kDa monomer) or small (220 kDa monomer) isoforms. The large tenascin-C variant is preferentially expressed in malignant tissues, is spatially and temporally related to tumor neovascularization and may exert anti-adhesive (Ghert et al., 2001; Jahkola et al., 1998; Zagzag and Capo, 2002). and immunosuppressive activity (Puente Navazo et al., 2001). The presence of tenascin-C within tumor infiltrated tissues made it as an attractive candidate for antibody mediated therapy. Based on the remarkable expression of tenascin-C within lymph nodes of patients with B-cell NHL and Hodgkin

lymphoma, radio-immunotherapy (RIT) approaches have been developed (Jaspars et al., 1995; Paganelli et al., 1999; Rizzieri et al., 2005; Schliemann et al., 2009; Vacca et al., 1996) and phase I clinical trials in these settings have shown the feasibility and the potential activity of such a therapeutic approach (Aloj et al., 2014; Rizzieri et al., 2004). Tenatumomab (ST2146, Sigma Tau S.p.A. R&D, Pomezia, Italy) is a murine IgG2b/k humanized antibody recognizing an epitope within the EGF-like repeats of human tenascin, shared by both small and large tenascin isoforms (De Santis et al., 2003). Tenatumomab conjugated with ¹³¹I have been developed for RIT applications, and it is currently in phase I clinical development in tenascin-C expressing cancer (NCT02602067). In the present study, we evaluate the presence of tenascin-C using tenatumomab antibody in a series of diagnostic biopsies of T-cell NHL patients.

3. Materials and Methods

Patients and Samples

Patients with a confirmed diagnosis of T-cell NHL regularly followed at the Hematology Unit of “Papa Giovanni XXIII” Hospital, Bergamo were considered for this study. The diagnosis was done according to the 2008 WHO classification (Swerdlow et al., 2008). Clinical data were gathered from the electronic charts and our data bases. The project was approved by the Hospital Ethic Committee (authorization number 980/2014 of the 19-Jun-2014).

Immunohistochemistry

Standard immunohistochemistry was performed on formalin fixed-paraffin embedded (FFPE) diagnostic samples obtained from our Department of Pathology. Deparaffination, target retrieval and rehydration were performed using a single run with Dako Envision Target Retrieval

Solution low pH (Dako code K800521-2) in Dako Thermostated Bath (Dako PT link) at 97 °C for 15 minutes. Sections were subsequently stained by Dako Autostainer Link 48 using Dako Flex Kit (DAKO code K 800221-1). In particular, the first step was to block each section with Envision Flex Peroxidase blocking reagent for 5 minutes at room temperature and then slides were rinsed in wash buffer (Envision wash Buffer 20x; Dako code K8000). Slides were incubated with tenatumomab (Sigma Tau S.p.A. R&D, Pomezia, Italy), a murine IgG2b/k anti tenascin-C antibody, for 30 minutes at room temperature (1:1000 dilution) and then the solution was removed by washing in buffer solution. Envision Flex HRP was added to each section and they were incubated for 20 minutes at room temperature; then the reaction was stopped by wash buffer. Afterwards Envision Flex DAB substrate was added and incubated for 10 minutes at room temperature and then slides were rinsed by wash buffer. Sections were counterstained with

Envision Flex Hematoxylin (Dako code k8008) for 5 minutes and then they were washed in deionized water. Lastly, slides were dehydrated and coverslips were mounted. Negative controls included slides incubated with a non-relevant isotype-matched antibody. Slides were assessed by three independent experienced investigators (A.G., L.G.S. and L.C.) using light microscopy and staining was scored using an arbitrary scale based on four different levels “no staining”, “weak”, “moderate” and “strong” (0 to 3). A grading system was used to express the proportion of involved areas in each case, as follows: 0% to 25% of involved areas per lesion, 26% to 50%, 51% to 75% and 76% to 100%. In Figure 1 (Panel A and B) is shown a representative panel of the expression level graded in a four-point scale according to staining intensity and proportion of involved areas. Association of tenascin-C with vasculature, stroma or cytoplasm was annotated (Figure 2).

Gene Expression Analysis

We retrieved raw data from two publicly available gene expression dataset i.e. Gene Expression Omnibus data sets GSE19069 (Iqbal et al., 2010), and GSE6338 (Piccaluga et al., 2013). In the two series, 183 unique cases of T-NHL were present, specifically 72 PTCL-not otherwise specifies (NOS), 42 angioimmunoblastic lymphoma (AITL), 35 ALCLs, 20 CTCL, 12 adult T-cell leukemia/lymphoma. Expression value of tenascin-C gene (TNC) was extracted and expressed in relative fluorescence unit, as from original microarray dataset.

Statistical Analysis

All the associations between expression of tenascin-C and clinical characteristics were evaluated with Chi-squared test or Fisher's exact test, as appropriate. Overall survival and progression-free survival were estimated by the Kaplan-Meier method and survival curves were

compared applying the log-rank test. Cox proportional hazard models were used to estimate hazard ratios and 95% confidence intervals in multivariable setting. Proportional hazard assumption was verified for all estimated models. Overall survival (OS) was calculated from diagnosis to death as a result of any cause and progression-free survival (PFS) from diagnosis to disease progression or death as a result of any cause. All reported p-values were two sided and the conventional 5% significance level was fixed. Statistical analysis was performed using SAS software (version 9.4).

4. Results

Immunohistochemical Expression of Tenascin-C

A total of 100 diagnostic biopsies from patients with T-cell NHL were evaluated for immunohistochemical (IHC) tenascin-C expression with tenatumomab. Of the 100 patients evaluated, 75 cases had a diagnosis of PTCL while 25 were CTCL. Among PTCL, 40 patients had a diagnosis of ALCL (ALK negative, n=21 or ALK positive, n=19), PTCL-NOS (n=20), AITL (n=9), T-cell lymphoblastic lymphoma (N=3), enteropathy-associated T-cell lymphoma (N=2), hepatosplenic T-cell lymphoma (N=1). Regarding CTCL, the most common histology was mycosis fungoides (n=13) followed by CD30+ primary cutaneous T-cell NHL (n=6), CD4+ small/medium cell primary cutaneous lymphoma (N=5) and Sézary syndrome (N=1). Data summary of the expression of tenascin-C in T-cell NHL is presented in Table 1.

Tenatumomab revealed the presence of tenascin-C in nearly all cases evaluated (93%). A high proportion of tenascin-C positive areas in pathologic samples (>50%) were shown in half of the patients (Table 1). There was a significantly different distribution among the specific histology ($P=0.0043$). Most of the PTCL (59%) were characterized by a diffuse expression (>50%) of tenascin-C, while in CTCL this was the case only in a minority of patients (24%, $P=0.0027$). In PTCL, diffuse expression (>50%) was detected in 81% of the ALCL ALK negative patients, 78% of AITL and 58% ALCL ALK positive. A lower (<50%) tenascin-C involvement was detected in PTCL NOS (30%) and CTCL (24%). The staining intensity was not significantly different among histologies ($P=0.5791$), but a higher staining intensity (grade 2-3) was more common in AITL (67%), ALCL ALK positive (68%) and negative (57%) and CTCL (64%). A direct correlation was found between tenatumomab staining intensity and

proportion of involved areas by tenascin-C ($P < 0.001$). The histological pattern of tenascin-C expression was most commonly extracellular (stromal in all cases and vascular in 50%) and in some cases intracellular (cytoplasmic, 23%) (Figure 2 and Table 1). Vascular pattern was revealed in 56% of PTCL and 20% of CTCL ($P = 0.0026$). High prevalence of vascular expression was shown in AITL (100%), ALCL ALK negative (74%), PTCL NOS (44%) and ALCL ALK+ (42%). Cytoplasmic positivity for tenascin-C was present only in cases showing a vascular expression and was recorded nearly exclusively in PTCL (20 of 21 positive cases), in particular in AITL (44%), ALCL ALK negative (37%) and positive (32%). A representative panels of immunostainings showing the different pattern of expression of tenascin-C for the main T-cell NHL subtype is shown in Figure 3.

Correlation with Clinical Parameters

Full baseline clinical characteristics and advanced (Ann Arbor III-IV) in the majority of the cases (68%). The international prognostic score (IPI) was low, intermediate-low, intermediate-high and high in 40%, 15%, 22% and 23% respectively. The 5-year OS and PFS were 75% and 59% respectively for ALCL ALK positive, while for the remaining cases of PTCL were 30% and 26%; in CTCL the 5-year OS was 70% (Supplementary Figure 1). No correlation was found between both staining intensity and pattern of expression with the baseline clinical characteristics, including age (P=0.8479 and P=0.3498), stage (P=0.6146 and P=0.3227), LDH (P=0.1179 and P=0.1560), IPI (P=0.2637 and P=0.0871). Conversely, a positive association was found between proportion of involved areas and LDH (P=0.0088) and IPI (P=0.0328). Patients with high (>50%) proportion of involved areas were characterized by a trend toward a reduced OS and PFS (Figure 4).

Multivariate models adjusted for age, gender and IPI were calculated to analyze the impact of the histologic characteristic of tenascin-C expression with PFS and OS in the 75 PTCL patients (Table 2). While staining intensity per se was not prognostic (data not shown), a trend for shorter PFS was found in those cases with high (>50%) proportion of involved areas (P=0.0627). Conversely, the presence of vascular pattern of expression was associated to a trend toward decreased PFS (P=0.0785) and a significantly shorter OS (P=0.0228) by multivariate analysis, mostly related to the adverse outcome of patients with this characteristic and high IPI score.

Gene Expression of Tenascin-C

To further assess the expression of tenascin-C by T-cell NHL, we evaluated the presence at mRNA level of TNC using publicly available reports. Two large dataset of PTCL were used for this analysis (GSE19069 and GSE6338) (Iqbal et

al., 2010; Piccaluga et al., 2013). Gene expression datasets were retrieved and expression value of TNC was extracted for normal and T-cell NHL samples. Significant overexpression of TNC was present in all PTCL subgroups compared to normal resting and activated T-cells in both of the two considered datasets (Figure 5).

5. Discussion

Currently, the majority of the patients diagnosed with T-cell NHL are still orphan of effective treatments (O'Connor et al., 2014). While Brentuximab Vedotin showed a significant activity in relapsed/refractory ALCL, with response rate up to 86% and median PFS of 13.3 months, no satisfactory therapy has been currently reported for the other histologies (Pro et al., 2012). Among the FDA-approved drugs i.e. Romidepsin, Pralatrexate and Belinostat, response occurs in less than 30% of the cases and median PFS do not exceed 4 months (Coiffier et al., 2012; O'Connor et al., 2015; O'Connor et al., 2011). The use of Brentuximab Vedotin in non-ALCL CD30⁺ PTCL was associated with good responses in AITL while a limited benefit was reported for the other histologies (Horwitz et al., 2014). Thus, the research of innovative therapeutic strategies is

currently a major unmet need in this patient population.

In the present study, we evaluated for the first time the IHC expression of tenascin-C using tenatumomab, a monoclonal antibody in clinical development for RIT application. To better characterize protein expression pattern we evaluated several aspects i.e. staining intensity, proportion of involved areas and pattern of expression. Tenatumomab revealed the presence of tenascin-C in nearly all the cases at variable intensity. Additionally, the presence of tenascin-C in T-cell NHL samples with IHC resulted in concordance with its expression at mRNA level reported by GEP analysis from independent, publicly available datasets. Most of the systemic PTCL showed both strong and diffuse tenascin-C expression, especially ALCL and AITL histology. Conversely, in CTCL, staining was less intense and sparse, and tenascin-C was mainly present only in the stromal architecture. Those differences

in expression pattern may be related to the major tissue alteration and neoangiogenesis characterizing aggressive PTCL. Indeed, we found a particularly strong and diffuse expression of tenascin-C in AITL, which is well recognized to show the most prominent vascular component among lymphomas. We report for the first time the correlation of tenascin-C immunohistochemical expression with clinical parameters in a cohort of NHL patients. While tenatumomab staining intensity was not per se prognostic, we indeed found a positive correlation with the proportion of involved areas and clinical aggressive presentation in PTCL. Additionally, we found that the presence of vascular pattern of expression and, maybe, proportion of lymph node involvement are associated with survival outcomes. These findings are probably due to the most prominent lymph node effacement and vascularization in aggressive lymphomas (Cardesa-Salzman et al., 2011), and the high expression of tenascin-C in those poor

prognosis cases is of major interest when considering tenascin-C as a therapeutic target.

Our findings are in line with previous reports in pathological samples of B-cell NHL and Hodgkin disease with different anti tenascin-C antibodies. The F16 anti-tenascin-C antibody (Philogen SpA, Siena, Italy) strongly stained nearly all the cases, with tenascin-C being present in the extracellular matrix and the hyperplastic blood vessels (Schliemann et al., 2009). Interestingly, in addition to NHL, tenascin-C expression has been previously reported also in the cytoplasm of neoplastic cells of several solid tumors such as breast, laryngeal or cholangiocarcinomas (Aishima et al., 2003; Ishihara et al., 1995; Yoshida et al., 1999). Cytoplasmic expression is often reported to be present at the invasive front of the tumor which is generally considered a marker of aggressive behavior and is associated with adverse prognosis (Aishima et al., 2003; Yoshida et al., 1999).

Additionally, it has been reported the direct production of tenascin-C by human carcinoma cell lines (Kawakatsu et al., 1992). Differently from Hodgkin and B-cell NHL, we observed that tenascin-C was expressed in the cytoplasm of PTCL, in particular in a significant proportion of the cases of ALCL (32%) and AITL (44%), and was associated in all of those cases with a diffuse stromal and perivascular tissue expression.

The clinical feasibility of targeting tenascin-C expressed in the microenvironment of B-cell NHL and Hodgkin lymphoma have been previously reported. The 81C6 antibody (Neuradiab, Bradmer Pharmaceuticals Inc., Toronto, Canada) was tested in a phase I study enrolling B-cell NHL patients (Rizzieri et al., 2004). Toxicity profile was limited to hematological events with 4 patients necessitating infusion of autologous bone marrow stem cells. An objective response was observed in 2 of the 9 patients, including one complete remission. More

recently, the F16 antibody directed to the extra-domain A1 of tenascin-C conjugated with ^{131}I (Tenarad, Philogen SpA, Siena, Italy) was tested in 8 patients with relapsed/refractory Hodgkin lymphoma (Aloj et al., 2014). Toxicity profile was acceptable and mainly limited to hematological events, objective response was observed in two of the eight patients, including one complete remission. The anti-tenascin antibody ST2146 (tenatumomab) have been developed to be applied in the context of the three-step Pretargeted Antibody-Guided Radioimmuno-Therapy (PAGRIT), that consisted of the sequential administration of a biotinylated monoclonal antibody, avidin/streptavidin, and a radiolabeled biotin molecule, leading to specific accumulation of radioactivity at the tumor site implemented by the multiple valence of avidin/streptavidin toward biotin. Interestingly, in a case report, biotinylated tenatumomab monoclonal antibody and ^{90}Y -biotinDOTA (90Y-ST2210) proved to be

extremely effective in a pediatric patient with ALCL ALK positive, relapsing after allogeneic stem cell transplant. However, further development of tenatumomab in PAGRIT was hampered by regulatory hurdles. Tenatumomab has been directly conjugated with ^{131}I for radio-immunotherapy (RIT) application and is currently in phase I clinical development in tenascin-C expressing cancer (NCT02602067).

In conclusion our data shows that tenatumomab is able to reveal the expression of tenascin-C in T-cell NHL, with the strongest expression value in the more aggressive and poor prognosis histological groups. Thus, tenascin-C represents an attractive target that sets the bases for exploring the activity of ^{131}I -tenatumomab in this orphan disease.

8. Tables and Figures

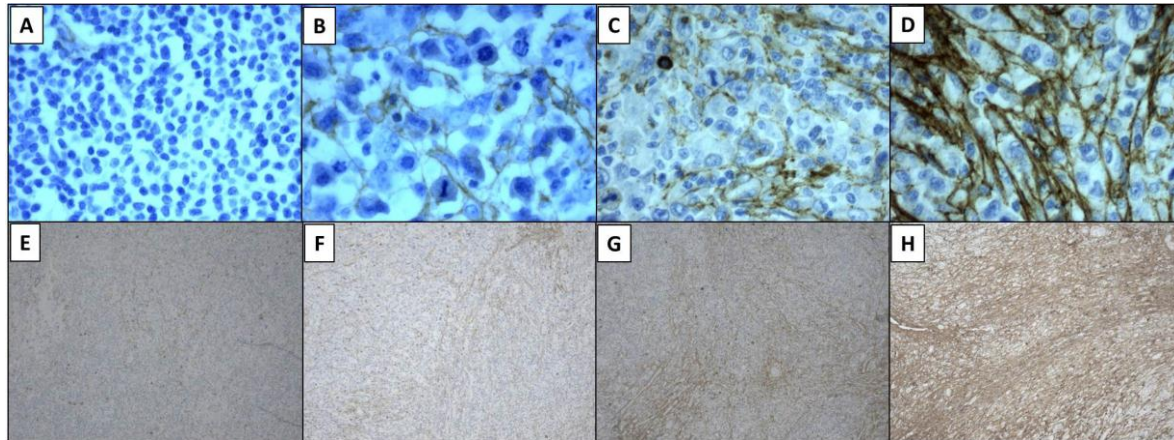
Table 1. Summary of staining characteristics

Histology	N	Staining intensity				Proportion of involved areas (%)				Pattern of expression			
		0	1	2	3	0-25	26-50	51-75	76-100	Negative	Stromal only	Vascular +Stromal only	Vascular + Stromal+ Cytoplasm
<i>Peripheral T-cell lymphoma, not otherwise specified</i>	20	2	10	5	3	5	9	5	1	2	10	5	3
<i>Anaplastic large cell lymphoma, ALK negative</i>	21	2	7	7	5	2	2	14	3	2	5	7	7
<i>Anaplastic large cell lymphoma, ALK positive</i>	19	0	6	7	6	0	8	9	2	0	11	2	6
<i>Angioimmunoblastic T-cell lymphoma</i>	9	0	3	5	1	0	2	6	1	0	0	5	4
<i>Lymphoblastic lymphoma</i>	3	0	1	1	1	1	0	2	0	0	2	1	0
<i>Enteropathy-associated T-cell lymphoma</i>	2	1	0	0	1	1	0	0	1	1	0	1	0
<i>Hepatosplenic T-cell lymphoma</i>	1	0	0	1	0	0	1	0	0	0	1	0	0
PTCL (subtotal)	75	5	27	26	17	9	22	36	8	5	29	21	20
<i>Mycosis fungoides</i>	13	1	4	3	5	1	9	3	0	1	11	0	1
<i>Primary cutaneous anaplastic large cell lymphoma</i>	6	0	2	4	0	1	2	3	0	0	2	4	0
<i>CD4+ small/medium cell primary cutaneous lymphoma</i>	5	1	0	3	1	3	2	0	0	1	4	0	0
<i>Sézary syndrome</i>	1	0	1	0	0	0	1	0	0	0	1	0	0
CTCL (subtotal)	25	2	7	10	6	5	14	6	0	2	18	4	1
Total	100	7	34	36	23	14	36	42	8	7	47	25	21

Table 2. Multivariate analysis for PFS and OS in PTCL patients

Factors	Multivariate for PFS				Multivariate for OS			
	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value	HR (95%CI)	P value
Proportion of involved areas (%)								
0-50	1.00				1.00			
51-100	0.53 (0.27-1.03)	0.0627			0.55 (0.27-1.12)	0.1011		
Pattern of expression								
Negative or Stromal only			1.00				1.00	
Vascular plus Stromal			0.52 (0.25-1.08)	0.0785			0.41 (0.19-0.88)	0.0228
Age								
≤ 60	1.00		1.00		1.00		1.00	
> 60	1.56 (0.70-3.51)	0.2800	1.31 (0.58-2.94)	0.5158	1.92 (0.83-4.42)	0.1256	1.59 (0.71-3.54)	0.2591
Gender								
Female	1.00		1.00		1.00		1.00	
Male	1.17 (0.59-2.31)	0.6506	1.33 (0.66-2.66)	0.4218	1.54 (0.75-3.14)	0.2366	1.81 (0.87-3.80)	0.1144
IPI								
Low	1.00		1.00		1.00		1.00	
Interm-Low	1.71 (0.61-4.81)	0.3121	1.88 (0.68-5.22)	0.226	1.96 (0.66-5.79)	0.2239	2.23 (0.77-6.47)	0.1387
Interm-High	1.81 (0.67-4.92)	0.2446	2.39 (0.91-6.29)	0.0774	1.82 (0.61-5.42)	0.2855	2.36 (0.83-6.75)	0.1089
High	4.10 (1.57-10.68)	0.0039	5.99 (2.12-16.92)	0.0007	5.10 (1.92-13.54)	0.0011	8.41 (2.93-24.14)	<.0001

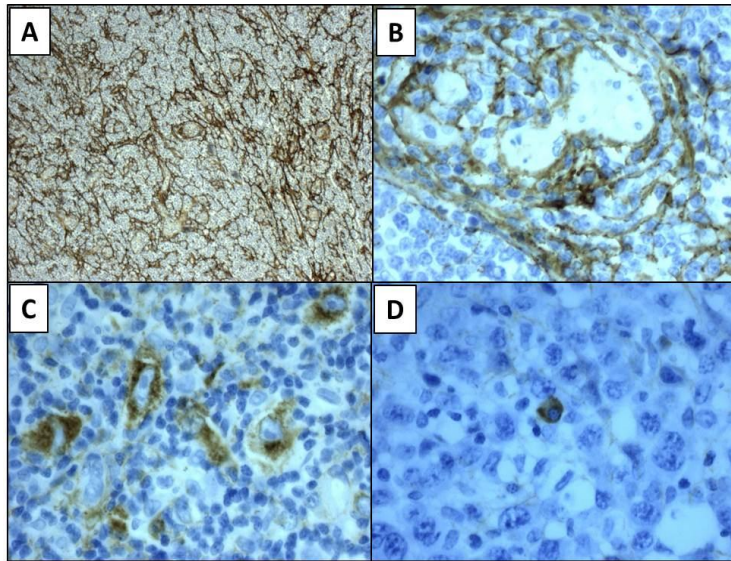
Figure 1.



Legend. Tenascin-C expression was evaluated with tenatumomab antibody and graded according to scoring intensity as no staining (panel A), weak (B), moderate (C) or strong

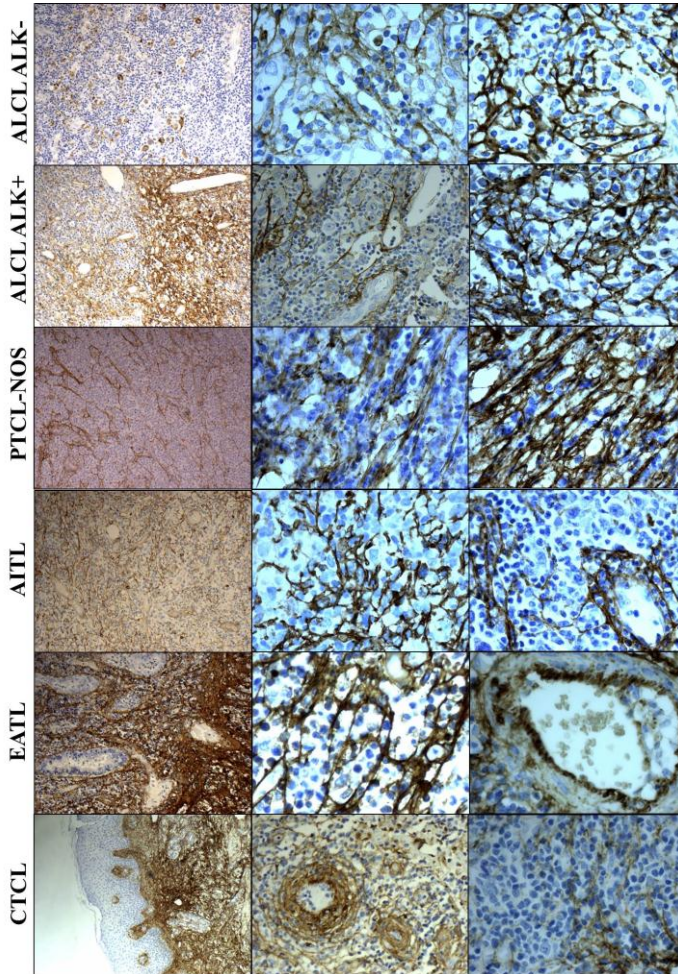
(D). Proportion of involved areas was assessed as follows: 0 = 0% to 25% of involved areas per lesion (E), 1 = 26% to 50% (F), 2 = 51% to 75% (G), 3 = 76% to 100% (H). Magnification 40x (A-D), 10x (E-H).

Figure 2



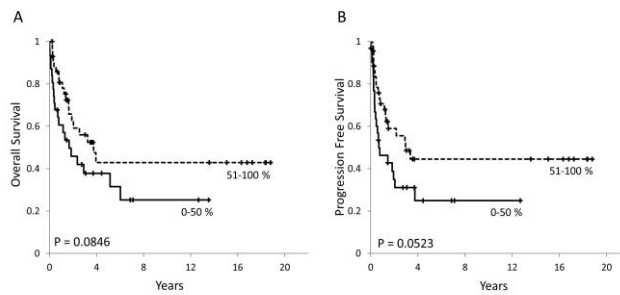
Legend. *Representative panels of pattern tenascin-C expression: stromal (panel A), vascular (B), cytoplasmic in neoplastic lymphocytes (C) and in normal plasma cell (D. Magnification 10x (A), 40x (B-D).*

Figure 3



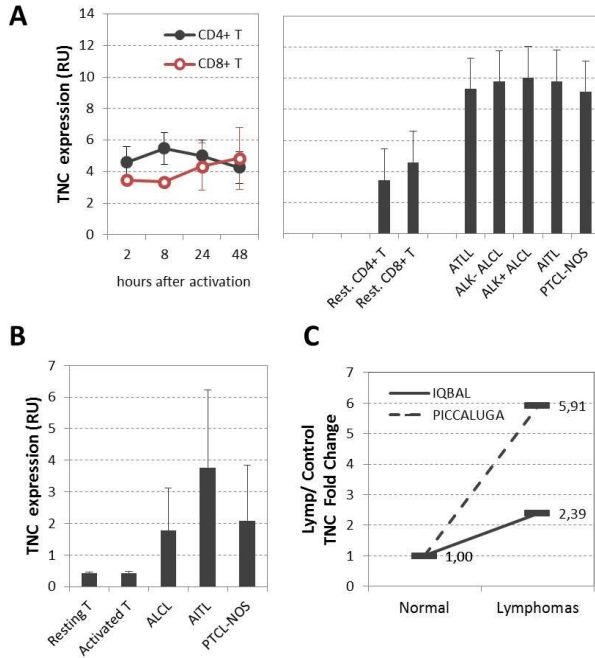
Legend. *Tenascin-C* expression in the different T-NHL subtypes. Magnification: 10x and 40x.

Figure 4



Legend. Overall survival (A) and progression free survival (B) according to the proportion of involved areas by *tenascin-C* in peripheral T-cell lymphoma (N=75).

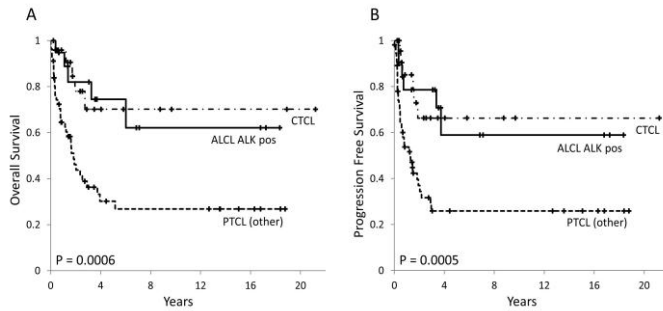
Figure 5



Legend. Overall (A) Gene expression values from Iqbal et al: left panel, TNC time course expression after CD3/CD28-IL12 for CD4+ T-cell (black) and

CD8+ T-cell (red) cells; right panel, TNC expression for resting cells and five type of lymphomas. (B) Gene expression values from Piccaluga et al: TNC expression for resting and activated normal T cells and three types of lymphomas. All expression values are expressed in relative fluorescence unit, as from original microarray dataset. (C) Overall averaged fold change of TNC gene expression between normal tissues (resting or activated non lymphoma cells) and lymphomas for the two considered datasets.

Supplementary Figure 1



Legend. *Overall survival (A) and progression free survival (B) according to diagnosis in the whole cohort. ALCL ALK+: anaplastic large cell lymphoma ALK positive (N=19); PTCL others: peripheral T-cell lymphoma with diagnosis different than ALCL ALK positive (N=56); CTCL: cutaneous T-cell lymphoma (N=25).*

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**EFFECT OF L-ASPARAGINASE IN
T-CELL NON-HODGKIN
LYMPHOMA: A PRECLINICAL
STUDY**

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1. Introduction

Mature T-cell non-Hodgkin lymphoma (NHL) is a heterogeneous and relatively rare group of malignancies representing 7-15% of all NHL (Swerdlow et al., 2008). It includes systemic NHL, defined as peripheral T-cell lymphoma (PTCL), and primary cutaneous T-cell lymphoma (CTCL). Over the last decades only marginal progress have been achieved for the treatment of mature T-cell NHL, and most of the patients suffer of adverse outcome (Coiffier et al., 2012; O'Connor et al., 2015; O'Connor et al., 2011; Pro et al., 2012; Vose et al., 2008). Similarly to B-cell NHL, patients obtaining a complete remission (CR) show a

prolonged survival irrespectively from the chemotherapeutic regimens and/or consolidation strategy adopted (Gritti et al., 2015; Tarella et al., 2014). However, the proportion of patients fully refractory to chemotherapy or suffering of early (<6 months) progression after treatment is as high as 40-50% of the cases (Gritti et al., 2015; Tarella et al., 2014; Vose et al., 2008). Thus, the intrinsic resistance to chemotherapeutic agents is a major issue that has not been yet successfully addressed.

L-asparaginase (ASNase) is an enzyme that hydrolyzes the amino acid L-asparagine to L-aspartic acid and ammonia, leading to depletion of the pool of the amino acid (Avramis, 2012). In cells unable to synthesize L-asparagine from L-glutamine, the unavailability of the amino acids

leads to protein synthesis inhibition, cycle cell arrest in the G1 phase and apoptosis (Emadi et al., 2014). Additionally, ASNase has a considerable glutaminase activity. Besides the role of L-glutamine as nitrogen donor for asparagine biosynthesis, its shortage is currently regarded as of key importance in hindering the neoplastic cellular metabolism (Hensley et al., 2013). ASNase is currently a cornerstone of the treatment of both adult and pediatric acute lymphoblastic leukemia (Bassan and Hoelzer, 2011; Rizzari et al., 2014). More recently, ASNase have been evaluated in extranodal natural killer/T-cell lymphoma (ENKL), a highly aggressive PTCL frequent in Asia and rare in Western countries (Jaccard and Hermine, 2011). Preclinical evidence showed that

NK-cell tumor cell lines and clinical samples, which are generally resistant to chemotherapy, are sensitive to ASNase treatment (Ando et al., 2005). Additionally, low levels of asparagine synthetase (ASNS) expression correlated to in vitro sensitivity to ASNase, with the exception of NK-cell leukemia. In this latter case, ASNase was able to induce considerable apoptosis despite elevated ASNS expression at both mRNA and protein levels, due to a glutamine deprivation-dependent apoptosis, thus underlying the importance of glutamine deprivation in the mechanism of action of ASNase. The incorporation of ASNase in methotrexate-based multi-agent chemotherapy regimens dramatically improved the outcome of

ENKL patients and has become the standard of care (Jaccard et al., 2011; Yamaguchi et al., 2011).

Clinical efficacy of ASNase in the non-T/NK-cell NHL is anecdotal, and only two case reports described the successful treatment of relapsed/refractory patient with EBV-positive cytotoxic PTCL (Obama et al., 1999; Takahashi et al., 2010).

Given the high proportion of chemo-refractory PTCL, we aimed to evaluate in a preclinical setting the enzymatic drug ASNase and the role of the known mechanism of resistance i.e. ASNS and glutamine synthetases (GS) in T-cell NHL.

2. Materials and Methods

Cell lines

The transformed CTCL cell line HH (ATCC® CRL2105™), the Sézary syndrome cell line HuT-78 (ATCC® TIB-161™), the CD30⁺ anaplastic large cell lymphoma (ALCL) cell line Karpas 299 (Sigma Aldrich, Catalogue No. 06072604), the NK-cell NHL cell lines NK-92 (ATCC® CRL-2407™) were used in the study. Additionally, the acute lymphoblastic leukemia cell line MOLT-4 (ATCC® CRL-1582™) and the blast crisis of chronic myelogenous leukemia cell line K562 (ATCC® CCL-243™) were used as controls. The HuT-78 cell line was grown in

Iscove's Modified Dulbecco's Medium-IMDM (EuroClone, Milano, Italy), supplemented with 20% fetal calf serum (FCS), L-glutamine 2 mM and gentamycin 50 µg/mL. The other cell lines were grown in RPMI 1640 medium (EuroClone, Milano, Italy) supplemented with 10% FCS, L-glutamine 2 mM (Lonza, Verviers, Belgium) and gentamycin 50 µg/mL. The NK-92 cell line was grown in the same conditions but with addition of sodium bicarbonate 1.5 g/L (Monico, Venezia, Italy) and interleukin-2 (IL-2) 500 U/mL.

Reagents

We used three different ASNase, derived from 2 different sources: *E. coli* (Medac GmbH, Wedel, Germany) and from *E. chrysantemi*

(Erwinase®, EUSAPharma, Hemel Hempstead, UK). In the former case both wild type and *E. coli* mutant Q59L, lacking of glutaminase activity were tested (kindly provided by Philip L. Lorenzi, MD, University of Texas MD Anderson Cancer Center, Houston, TX, USA) (Chan et al. 2014).

Alamar blue cytotoxicity assays

Cytotoxicity assays were performed using the Alamar Blue (Serotec Ltd, Oxford, United Kingdom) vital dye essentially as described previously (Amaru Calzada et al., 2013). Briefly, cell lines were plated at 10^5 cells/well in the absence or presence of increasing concentrations (0.1 to 100 IU/mL) of ASNase. After 24 hours of culture, 1/10 volume Alamar blue solution (Bio-

Rad, Hercules, CA, USA) was added, and after an additional 8 hours of incubation, the plates were read in a FLUOstar Omega (BMG LABTECH GmbH, Ortenberg, Germany) with emission at 590 nm. Each experiments was performed at least in triplicate.

Apoptosis measurement

Cells were resuspended at the concentration of 5×10^5 /mL and incubated with 1 or 10 IU/mL ASNase for 48 hours. Cells were subsequently stained with Annexin V (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions, and the percentage of apoptotic and necrotic cells was determined by standard flow cytometry on a

FACSCanto II instrument (BD Biosciences, San Jose, CA, USA). Annexin V⁻ cells represent live cells, while Annexin V⁺ apoptotic cells.

Real-time quantitative PCR for asparagine and glutamine synthetase genes

Total RNA was obtained from $2-5 \times 10^6$ cells using the RNeasy Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. 1 μg of total RNA was retrotranscribed to cDNA in a total volume of 20 μL using SuperScript®Double-Stranded cDNA Synthesis kit (Invitrogen™, Waltham, MA, USA). To quantify the differential expression of ASNS in the different cell lines, we used the quantitative PCR (qPCR) approach, applying the $\Delta\Delta\text{Cp}$

method. This method directly uses the Cp (Crossing point) values generated from a qPCR system to calculate relative gene expression, using a reference gene as normalizer. In a qPCR system, the Cp value is inversely proportional to the transcript amount. The ΔCp is the difference in crossing point between the target and reference genes and the $\Delta\Delta\text{Cp}$ represents the difference between ΔCp in the test sample and ΔCp in the sample used as calibrator (MOLT-4). The results were expressed as fold change calculated with the following formula: $\text{Fold change} = E^{-\Delta\Delta\text{Cp}}$, where E represents the PCR efficiency experimentally determined. As reference gene, the housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used. For ASNS transcript

amplification, we used primers and probe previously described (Leslie et al., 2006): forward primer: 5'-TCAGCCCGCCACATCAC-3'; reverse primer: 5'-CAATGAAGCTATAAGCTTTCTTCAAGTG-3'; Probe: 5'Fam-CTGACCTGCTTACGCCAGATTTTCTTCAA-3'Tamra. For GAPDH transcript amplification, we designed the assay for qPCR employing the Primer3 software available online: forward primer 1: 5'-ATGGTTTACATGTTCCAATATGATTC-3'; reverse primer: 5'-CTCCTGGAAGATGGTGATGG-3'; probe: 5'Fam-CAAGCTTCCCGTTCTCAGCC-3'Tamra. The amplification was performed in a final volume

of 25 μ L containing 5 μ L of cDNA (100 ng RNA equivalent), 300 nM of each primer, 200 nM probe. The thermal cycling conditions were as follow: 2 min enzyme activation at 50 °C and 10 min incubation at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min.

Detection of ASNS and GS protein

Cells were lysed in M-PER extraction reagent (Pierce, Rockford, IL, USA). Equivalent amounts of protein were analyzed by standard Western blotting using anti-ASNS antibody (AbCam, product n° ab40850). Detection was performed using horseradish peroxidase-labeled secondary antibodies (Santa Cruz Biotechnology, Dallas, TX, USA) and Super Signal West Pico

Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA). The protein level of GS was quantified using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's recommendations (LifeSpan BioSciences, product n° LS-F7539).

Immunohistochemistry

Formalin fixed-paraffin embedded (FFPE) diagnostic samples were obtained from T-cell NHL patients in care in the “*Papa Giovanni XXIII*” Hospital of Bergamo. The project was approved by the Hospital Ethic Committee (authorization number 980/2014 of the 19-Jun-2014). The histologic type of each PTCL was determined according to the WHO 2008 classification

(Swerdlow et al., 2008). Clinical information was gathered from the electronic charts. Standard immunohistochemistry for ASNS and GS was performed. Deparaffination, target retrieval and rehydration were performed using a single run with Dako Envision Target Retrieval Solution High pH (Dako code K8000) for ASNS and Low pH (Dako code K800521-2) for GS in Dako Thermostated Bath (Dako PT link) at 97 °C for 30 minutes for ASNS and for 15 minutes for GS. After, sections were stained by Dako Autostainer Link 48 using Dako Flex Kit (DAKO code K 800221-1). In particular, the first step was to block each section with Envision Flex Peroxidase blocking reagent for 5 minutes at room temperature and then slides were rinsed in wash buffer (Envision wash Buffer

20x; Dako code K8000). They were incubated with two antibodies for 30 minutes at room temperature: rabbit monoclonal Anti-Asparagine Synthetase antibody (EP 282y) (Abcam code ab40850) (1:50 dilution) and rabbit polyclonal Anti-Glutamine Synthetase antibody (Abcam ab16802) (1:500 dilution); then the solution was removed by washing in buffer solution. In particular for ASNS a Rabbit Linker (Dako code K8009) was added to improve the signal and after 15 minutes it was removed by a wash in buffer solution. Envision Flex HRP was added to each section and they were incubated for 20 minutes at room temperature; then the reaction was stopped by wash buffer. Afterwards Envision Flex DAB substrate was added and incubated for 10 minutes at room

temperature and then slides were rinsed by wash buffer. Sections were counterstained with Envision Flex Hematoxylin (Dako code k8008) for 5 minutes and then they were washed in deionized water. Lastly, slides were dehydrated and coverslips were mounted. Negative controls included slides incubated with a non-relevant isotype-matched antibody. Slides were assessed by two independent investigators using light microscopy and staining was scored using an arbitrary scale based on four different levels “no staining”, “weak”, “moderate” and “strong” (score 0 to 3). A grading system was used to express the proportion of positive cells in each case, as follows: 0% to 25% of positive cells per lesion (score 1), 26% to 50% (score 2), 51% to 75%

(score 3), and 76% to 100% (score 4). A final score was then determined by summing up the intensity score and the percentage of the positive cells in the tissue, assigning a weighted final score between 0 and 6 for each tissue sample, as previously reported (Dufour et al., 2012).

Statistical analysis

Differences between groups were analyzed using the Student t-test and Mann-Whitney U test. Correlation between parameters was calculated using the Spearman rank correlation test.

3. Results

ASNase inhibits cell growth and selectively induces cell death of T-cell NHL cell lines

The effect of ASNase deriving from *E. coli* on cell viability was assessed in 4 T-cell NHL and in K562 cell lines using the Alamar Blue assay (Figure 1). IC₅₀ values for ASNase cytotoxicity relative to vehicle-treated cells were calculated. The cell lines HUT-78, Karpas299 and NK-92 were sensitive to ASNase (IC₅₀ about 0.5-6 IU/mL, Figure 1B, C, D and F), while the HH and K562 cell lines were resistant up to 100 IU/mL (Figure 1A, E and F).

In order to verify that the difference in sensitivity in the Alamar Blue assay was due to a different apoptotic response, we also investigated in the same cell lines the effect of ASNase on apoptosis by Annexin V binding. Annexin V⁺ apoptotic cells increased significantly after treatment with 1 and 10 IU/mL ASNase of the three sensitive cell lines HUT-78, Karpas299 and NK-92, but not of the resistant HH cell line (Figure 2). These data confirm that ASNase induces cell death of the 3 former cell lines but not of control HH cell line.

Asparagine synthetases gene expression level and protein quantification

In order to investigate the mechanism of sensitivity and resistance to ASNase in the studied cell lines, ASNS expression was investigated. Baseline ASNS mRNA expression was measured by real time quantitative PCR in the 4 T-cell NHL and K562 cell lines using GAPDH as reference gene. The MOLT-4 was used as calibrator to normalize the relative expression of each cell line. HH and Hut-78 showed the highest expression of ASNS, while Karpas299 and NK-92 had the lowest results (Figure 3A). Thus the data suggest that ASNS at least in part correlates with resistance with K562 having the highest ASNS expression level. The four T-cell NHL cell lines were also

evaluated for ASNS protein expression by Western blot. Figure 3B shows the detection of the ASNS protein of 60 kD in most cell lines. ASNS protein level was highest in the HH and K562 resistant cell lines (normalized for beta-actin expression), low for Hut-78 and Karpas299 and undetectable in NK-92 and MOLT4. We conclude that ASNS expression may at least in part explain resistance to ASNase.

T-cell NHL cell lines are sensitive to glutamine deprivation

In several cancers, the neoplastic growth is dependent by external source of glutamine. To evaluate the impact of glutamine addiction of T-cell NHL cell lines we assess the sensibility to

glutamine deprivation. We found that Hut-78, Karpas299 and NK-92 cell lines, whose growth was inhibited by ASNase treatment, were susceptible to glutamine deprivation (Figure 4). Cells expressing the GS enzyme can synthesize the amino acid and overcome the dependence from external source. Thus, we assessed the GS protein level with ELISA. The GS protein level was high in HH cell line, intermediate for the Hut-78 and Karpas299, and very low in NK-92 (Figure 5). We conclude that presence of external source of glutamine is necessary for Hut-78, Karpas299 and NK-92 cell lines and the high GS protein expression may explain the glutamine independence from glutamine of HH cell line.

Glutaminase activity of ASNase is necessary to inhibit cell growth in T-cell NHL cell lines

Along with the asparaginase enzymatic activity, ASNase can deaminate glutamine leading to the depletion of the plasmatic concentration of both amino acids. To investigate the role of glutamine depletion in ASNase action, we evaluated the effect of the three different forms of ASNase, differing in their glutaminase activity: the *E. chrysantemi* enzyme with ten-fold higher glutaminase with respect to the *E. coli* protein, and the mutant form Q59L lacking any glutaminolytic activity. The effect of these three ASNase on cell viability in the 4 T-cell NHL and K562 cell lines and the relative IC_{50} are shown in Figure 6. *E. chrysantemi* ASNase had a significant higher

activity at low concentration (0.1 IU/mL) in Hut-78 ($P < 0.001$) (Fig.6B) and NK-92 ($P = 0.003$) (Fig. 6D), conversely the Q59L ASNase did not show cytotoxic effect at any dose level. These data confirmed the importance of ASNase glutaminase activity in exerting the observed *in vitro* effect.

Asparagine and glutamine synthetases expression is low in several human T-cell NHL samples

In order to assess whether primary T-cell NHL, like the cell lines studied above, express variable amounts of ASNS and GS and determine therefore whether primary T-cell NHL may be sensitive to ASNase treatment, we determined ASNS and GS expression in a panel of 22 diagnostic samples of mature T-cell lymphoma

patients of different subtypes (3 AITL, 7 ALCL ALK positive and negative, 2 AETL, 4 ENKL, 5 PTCL NOS, 1 HSTCL). We first set up the immunohistochemistry and scoring method. An example is shown in Figure 7 and 8. We then analyzed all 22 samples and the results are shown in Table 1. All the samples showed a degree of positivity for ASNS that in 10 of the 22 cases (45%) was low and present in a minority of the cells (score 1). One case was GS negative and 12 (55%) were scored 1 (Table 1). In Figures 9 is schematically represented the values of ASNS (Panel A) and GS (Panel B) score according to PTCL specific histology. Most of the PTCL cases, the HSTCL and a part of the other histologies showed a low expression of ASNS at a similar

level with respect to ENKL cases which should be highly sensitive to ASNase. These data suggest that a proportion of primary T-cell NHL may be sensitive to ASNase treatment similar to ENKL.

4. Discussion

The antitumor activity of ASNase has been tested in early study in different hematologic cancers. The significant sensitivity to the drug of acute lymphoblastic leukemia was reported by several investigators (Hill et al., 1969; Leventhal and Henderson, 1971), and spurred further studies leading to the incorporation of the drug into the currently used multi-agent induction regimens (Bassan and Hoelzer, 2011; Rizzari et al., 2014). However, a response was also observed in acute myeloid leukemia, in some chronic lymphocytic leukemia and in NHL (Clarkson et al., 1970). However, clinical utility of ASNase in these latter

settings has been only occasionally reported (Emadi et al., 2014). More recently, the successful incorporation of AS Nase in multi-drug regimens in ENKL was reported by several clinical trials (Jaccard et al., 2011; Yamaguchi et al., 2011).

Differently from B-cell NHL, classification of mature T-cell NHL is hindered by their clinically and pathologically heterogeneity, with inconstant and aberrant expression of phenotypic markers and lack of recurrent molecular alterations, with the sole exception of ALCL ALK positive (O'Connor et al., 2014). With the advent of gene expression profiling (GEP) technology, this heterogeneity of PTCL was further highlighted and several studies showed a strict similarity between T-cell and NK-cell PTCL

(Iqbal et al., 2010a; Iqbal et al., 2010b; Piccaluga et al., 2007; Piccaluga et al., 2013). Of interest the observation that PTCL of $\gamma\delta$ derivation shows a similar GEP signature with respect to ENKL, with additional presence of T-cell receptor (TCR)/CD3 complex transcripts (Kucuk et al., 2015). Given the great molecular and clinical heterogeneity of T-cell NHL and the strict similarity of some PTCL with the ENKL form, we speculated that a subgroup of T-cell derived NHL may be susceptible to ASNase treatment in a similar fashion as the NK-cell ones.

To investigate this, we evaluate for the first time the preclinical use of ASNase in the non-T/NK-cell NHL. We report that T-cell NHL cell lines show a different degree of sensibility to ASNase that can be similar to the NK-cell NHL

cell line NK-92. All the T-cell NHL cell lines showed a higher expression of ASNS with respect to NK-92. However, sensitive cell lines were characterized by lower ASNS expression compared to the resistant ones. Additionally, sensitive cell lines showed a marked glutamine-addiction, and the key importance of glutamine depletion was confirmed by the different activity observed with the three ASNase forms. In fact, while no effect was seen for the Q59L mutant ASNase lacking of glutaminase activity, the *E. chrysantemi* derived ASNase, with enhanced glutamine activity in comparison to the *E. coli* form, was characterized by significantly lower IC50 values. For several years the mechanism of action of ASNase was thought to be related to its

ability to deplete extracellular levels of asparagine, an amino acid not synthesized by cells lacking of ASNS, such as lymphoblasts (Avramis and Panosyan, 2005). Conversely, glutaminase activity of ASNase was regarded as major culprit for the toxic effect of the drug. However this idea is currently debated, as it becomes clear that glutamine deprivation, along with asparagine hydrolysis, is responsible for the therapeutic effect the drug. It has been reported that in cells with low or intermediate-low expression of ASNS, ASNSase glutaminase activity is determinant in exerting a cytotoxic effect in acute lymphoblastic leukemia (Chan et al., 2014). Additionally, glutamine removal inhibits mTORC1 and induces apoptosis in acute myeloid leukemia cells

(Willems et al., 2013). Thus, our data suggest that the cytotoxic activity of ASNase in T-cell NHL cell lines may be related to the combined effect of depleting asparagine and glutamine.

It has been shown that ASNS expression imparts resistance to ASNase therapeutic activity and a previous report showed that it is a predictive biomarker for ASNase sensitivity in the setting of ovarian cancer (Lorenzi et al., 2006). Thus, we investigated the expression of ASNS in 22 diagnostic samples of PTCL patients. We found that several cases show a very low but quantifiable expression of ASNS, in particular all the ENKL and a part of PTCL NOS. Since ENKL are sensitive to ASNase treatment, our findings suggest that other forms of PTCL may be

susceptible to ASNase. However, it should be noted that other factors e.g. hepatic and adipocyte amino acid synthesis contribute to ASNase sensitivity in vivo (Avramis, 2012; Ehsanipour et al., 2013).

Given the sensibility to glutamine depletion of several T-cell NHL cell lines in our in vitro model, and the key importance of GS expression in overcoming the dependence from external source of glutamine, we assessed the presence of GS in diagnostic PTCL samples. Despite no study correlates GS expression and response to ASNase, it has been previously reported that GS is a mechanism of resistance of ASNase (Avramis, 2012; Tardito et al., 2011). Our results shows that, similarly to ASNS, several

PTCL cases showed a very low GS expression, thus confirming that selected cases of PTCL may be susceptible to ASNase treatment.

In conclusion, our finding shows that in a preclinical setting, several T-cell NHL cell lines are susceptible to ASNase treatment and a proportion of diagnostic T-cell NHL specimen express at low levels two well-known mechanism of resistance to ASNase i.e. ASNS and GS. Our data indicated that ASNase treatment may be useful in selected cases of PTCL. Further studies are necessary to confirm these findings.

9. Tables and Figures

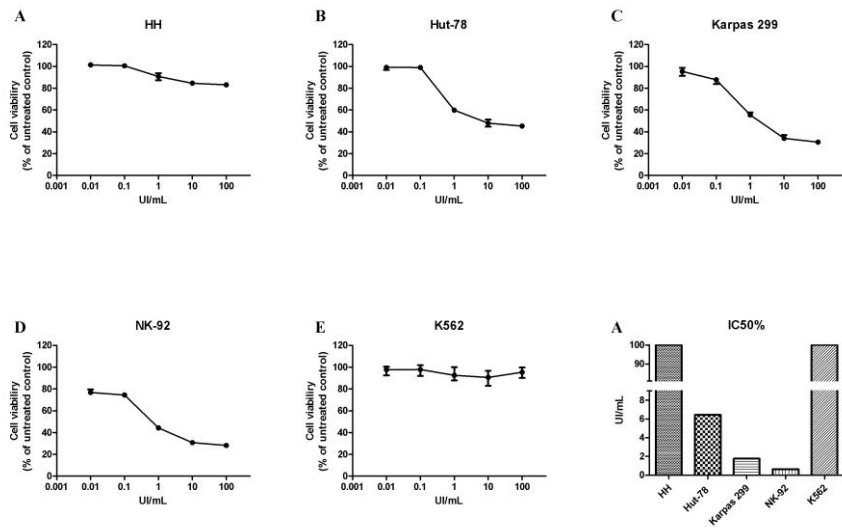
Table 1.

DIAGNOSIS	Asparagine synthetase			Glutamine synthetase		
	Score	Staining intensity	Proportion of positive cells	Score	Staining intensity	Proportion of positive cells
AITL	3	Low positive	51-75%	2	Low positive	26-50%
AITL	1	Low positive	0-25%	1	Low positive	0-25%
AITL	6	Highly positive	76-100%	5	Positive	76-100%
ALCL ALK negative	3	Low positive	51-75%	1	Low positive	0-25%
ALCL ALK negative	1	Low positive	0-25%	1	Low positive	0-25%
ALCL ALK negative	2	Positive	0-25%	1	Low positive	0-25%
ALCL ALK positive	3	Low positive	51-75%	1	Low positive	0-25%
ALCL ALK positive	1	Low positive	0-25%	4	Low positive	76-100%
ALCL ALK positive	3	Highly positive	0-25%	3	Positive	26-50%
ALCL ALK positive	3	Positive	26-50%	4	Positive	50-75%
EATL	3	Highly positive	0-25%	1	Low positive	0-25%
EATL	3	Highly positive	0-25%	2	Low positive	26-50%

HSTCL	1	Low positive	0-25%	1	Low positive	0-25%
ENKL	1	Low positive	0-25%	2	Low positive	26-50
ENKL	1	Low positive	0-25%	1	Low positive	0-25%
ENKL	1	Low positive	0-25%	1	Low positive	0-25%
ENKL	2	Pos	0-25%	6	Highly positive	76-100%
PTCL NOS	2	Low positive	0-25%	2	Low positive	26-50%
PTCL NOS	1	Low positive	0-25%	0	Negative	NEG
PTCL NOS	1	Low positive	0-25%	1	Low positive	0-25%
PTCL NOS	1	Low positive	0-25%	1	Low positive	0-25%
PTCL NOS	2	Low positive	26-50%	1	Low positive	0-25%

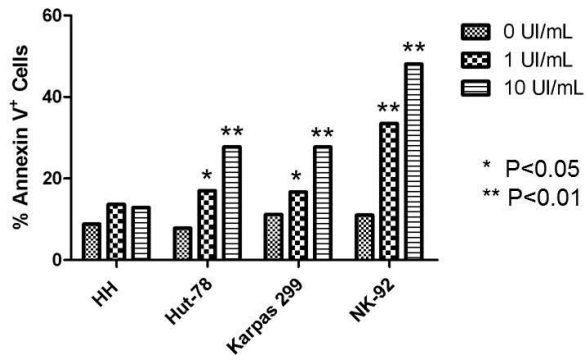
Legend. *AITL: Angioimmunoblastic T-cell Lymphoma; ALCL: Anaplastic Large-Cell Lymphoma; ALK: Anaplastic Large Cell Lymphoma Kinase; EATL: Enteropathy-Associated T-cell Lymphoma; HSTCL: Hepatosplenic T-cell Lymphoma; ENKL: Extranodal T/NK-cell Lymphoma; PTCL-NOS: Peripheral T-Cell Lymphoma Not Otherwise Specified.*

Figure 1.



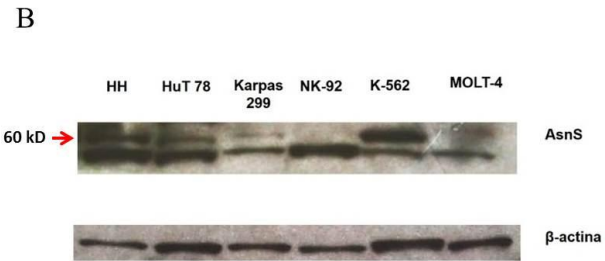
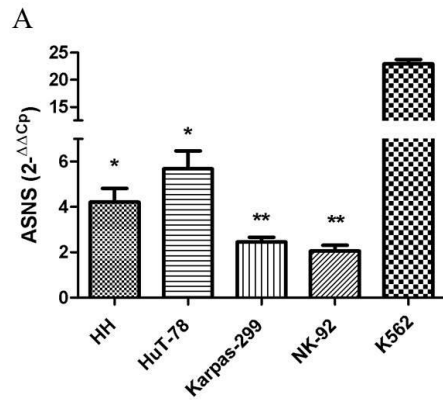
Legend. (A-E) Effect on cell viability at increasing concentration of ASNase. (F) Schematic representation of the IC_{50} values

Figure 2.



Legend. Apoptotic response at increasing concentration of ASNase

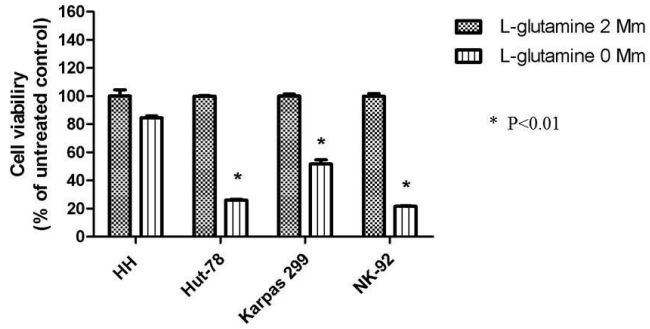
Figure 3



Legend: * $P < 0.01$ vs K562, ** $P < 0.01$ vs HH/Hut-78

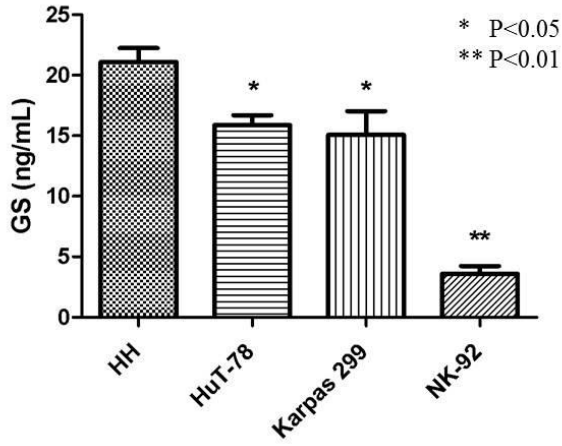
Legend. (A) Baseline asparagine synthetase (ASNS) mRNA expression was studied by real time quantitative PCR with the $\Delta\Delta C_p$ method, using GAPDH as reference gene and ASNS expression by MOLT-4 cell line as calibrator. (B) Cells were lysed in M-PER extraction reagent and Western blotting was performed to analyze baseline ASNS protein expressions

Figure 4.



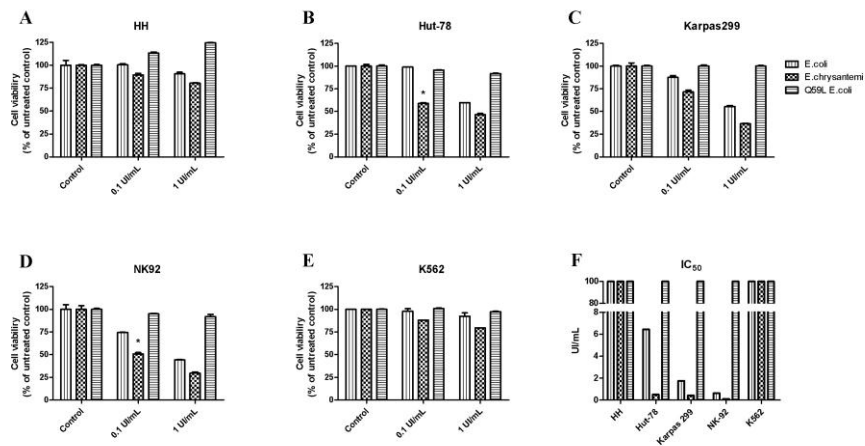
Legend. Effects of glutamine deprivation on cell viability. Cells were cultured for 24 hours with or without 2 mM L-glutamine.

Figure 5.



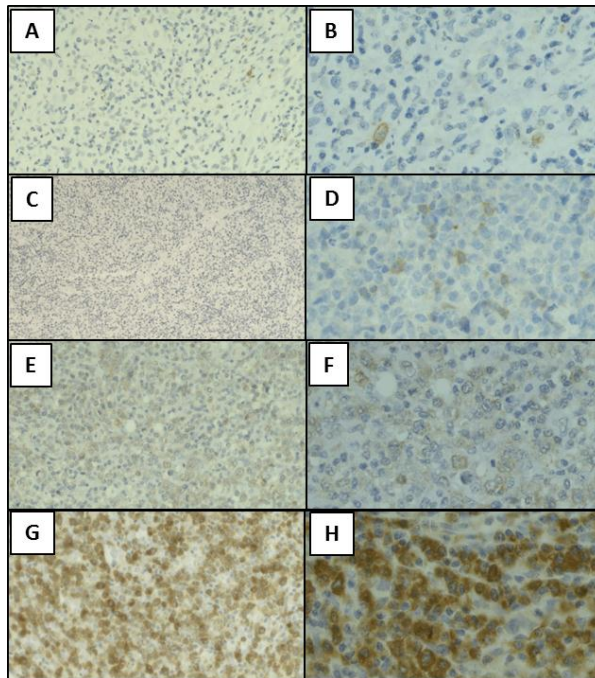
Legend. Baseline glutamine synthetase protein was quantified by ELISA.

Figure 6.



Legend. (A-E) Comparative effect on cell viability at increasing concentration of three different forms of ASNase. (F) Schematic representation of the IC_{50} values of the three different forms of ASNase.

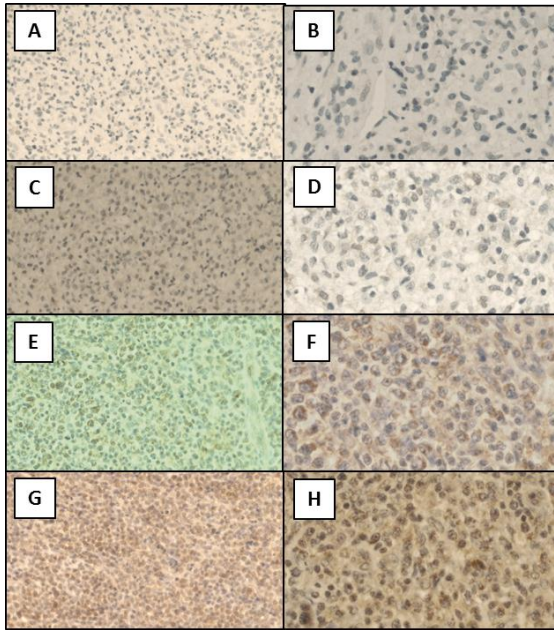
Figure 7.



Legend. Representative panels of ASNS expression in PTCL. (A) and (B) represent a case with low staining intensity and a grading of 0-25%. (C) and (D) represent a case with low staining intensity and a grading of 26-50%. (E) and (F) represent a

case with moderate staining intensity and a grading of 51-75%. (G) and (H) represent a case with high staining intensity and a grading of 76-100% Magnification 40x (B, D, F and H), 20x (C and E) and 10x (A and G).

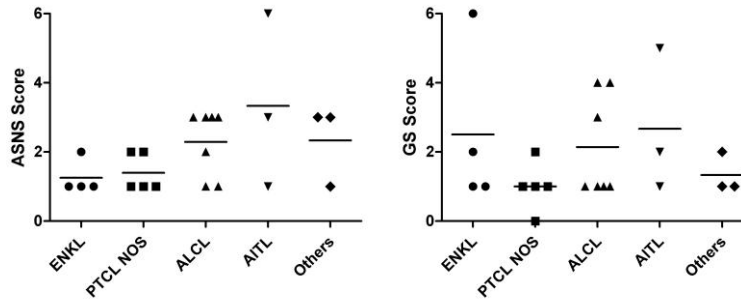
Figure 8.



Legend. Representative panels of GS expression in PTCL. (A) and (B) represent a case with low staining intensity and a grading of 0-25%. (C) and (D) represent a case with low staining intensity and a grading of 26-50%. (E) and (F) represent a

case with moderate staining intensity and a grading of 76-100%. (G) and (H) represent a case with high staining intensity and a grading of 75-100% Magnification 40x (B, D, F and H), 20x (A, C, E and G).

Figure 9.



Legend. Disposition of asparagine synthetase (A) and glutamine synthetase (B) immunohistochemical score according to specific histology.

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CONCLUSION AND FUTURE PERSPECTIVES

Aim of the present thesis was to address the current clinical unmet need in the care of T-cell lymphoma. We started from a clinical perspective, evaluating the diagnostic accuracy and the outcome of a retrospective large cohort of PTCL patients, focusing on the major determinant of outcome i.e. primary response.

Subsequently we moved back and investigated two different therapeutic approaches in a preclinical setting. We showed for the first time that tenascin-C is an attractive marker for targeting the tumor microenvironment of T-cell NHL. While several attempts have been made to

develop RIT approaches for the cure of solid cancer, B-cell NHL and Hodgkin disease, this therapy has not been applied to date in T-cell NHL. Currently, the immunoconjugated anti-tenascin-C antibody ¹³¹I-Tenaumomab is in phase I study in refractory tumors and, if the safety profile permits further study, it may be tested in T-cell NHL.

We also investigated the antitumor activity of the enzyme ASNase in a preclinical model of T-cell NHL, showing that asparagine and glutamine depletion may be useful in selected cases. Further laboratory and clinical studies are necessary to evaluate the role of this molecule. As *E. coli* ASNase is currently approved for the treatment of NHL, we are planning a prospective observational study to assess the clinical utility and biological

correlates of ASNase treatment in relapsed/refractory T-cell NHL.

In conclusion our study has opened novel therapeutic perspectives in the field of T-cell NHL.

PUBLICATIONS

Gritti G, Boschini C, Rossi A, Delaini F, Grassi A, Algarotti A, Micò C, Trezzi R, Gianatti A, Barbui AM, Rambaldi A: “*Primary treatment response rather than front line stem cell transplantation is crucial for long term outcome of peripheral T-cell lymphomas*”. PLoS One. 2015 Mar 27;10(3):e0121822. doi: 10.1371/journal.pone.0121822

Gritti G, Gianatti A, Petronzelli F, Boschini C, Rossi R, Trezzi R, Rossi A, Barbui AM, Rambaldi A: “*Tenascin-C is Highly Expressed in T-Cell non-Hodgkin Lymphomas and Represents an Attractive Target for*

Radioimmunotherapy". Blood 2016 128:4141

(ASH meeting Abstract Book)

Gritti G, Gianatti A, Petronzelli F, De Santis R,
Pavoni C, Rossi RL, Cattaneo L, Spagnoli LG,
Ferrari S, Rossi A, Barbui AM, Rambaldi A:
*"Evaluation of Tenascin-C by Tenatumomab in
Peripheral T-Cell Lymphoma Identifies a New
Target for Radioimmunotherapy"*. Submitted

