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SCUOLA DI DOTTORATO DI RICERCA IN ONCOLOGIA CLINICA E SPERIMENTALE E IMMUNOLOGIA

TESI DI DOTTORATO

EXPRESSION AND FUNCTION OF PROTEIN KINASE CK2 IN HODGKIN LYMPHOMA

Direttore: Ch.mo Prof. PAOLA ZANOVELLO

Supervisore: Prof. FRANCESCO PIAZZA

Dottorado: ANDREA VISENTIN

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L'amore che move il sole e l'altre stelle.

DANTE ALIGHIERI (PARADISO XXXIII, v. 145)

INDEX

ABBREVIATIONS

Ab	antibody
ABVD	doxorubicin/bleomycin/vinblastine/dacarbazine
A-AVD	brentuximab vedotin/doxorubicin/vinblastine/dacarbazine
Allo-SCT	allogeneic stem cell transplantation
AML	acute myeloid leukemia
ASCT	autologous stem cell transplantation
ATP	adenosine triphosphate
AV	annexin V
BCP-ALL	B-cell precursor acute lymphoblastic leukemia
BCR	breakpoint cluster region
BEACOPP	bleomycin/etoposide/doxorubicin/cyclophosphamide/vincristine procarbazine/prednisone
BM	bone marrow
BSA	bovine serum albumin
CK2	casein kinase 2
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CR	complete response
CT	computer tomography
DLBCL	diffuse large B-cell lymphoma
DMAT	2dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole
ESR	erythrocytes sedimentation rate
FBS	fetal bovine serum
H&E	haematoxylin & eosin
HL	Hodgkin lymphoma
HRS	Hodgkin and Reed-Sternberg cells
IFRT	involved field radiotherapy
IG	immunoglobulin
IHC	immunohistochemistry
IκB	inhibitor of κB
IKK	IκB kinase

IL-6	interleukin 6
IP3	inositol triphosphate
iPET	interim PET-CT
IPS	international prognostic factors project score
ISRT	involved site radiotherapy
MM	multiple myeloma
MMAE	monomethyl auristatin E
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLPHL	nodular lymphocyte predominat Hodgkin lymphoma
NHL	non-Hodgkin lymphoma
ORR	overall response rate
OS	overall survival
PBS	phosphate buffered saline
PD-1	programmed cell death protein 1
PD-L1	programmed cell death ligand 1
PI	propidium iodine
PET-CT	positron emission tomography with a low-dose CT scan
PFS	progression free survival
PI	propidium iodide
PIP_3	phosphatidylinositol 3,4,5 trisphosphate
PTEN	phosphatase and tensin homolog
R/R	refractory or relapsed patients
SD	standard deviation
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SEER	surveillance, epidemiology and end result database
TAD	transcription activation domain
TAM	tumor-associated macrophages
TBB	4,5,6,7-tetrabromobenzimidazole
TF	transcription factor
TMA	tissue microarray
TNFα	tumor necrosis factor alpha
WB	western blotting
WHO	World Health Organization

AMINO ACIDS ABBREVIATIONS

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
X		generic amino acid

ABSTRACT

Hodgkin lymphoma (HL) is a lymphoid tissue neoplasia accounting for almost 0.6% of all cancers. The neoplastic cells of Hodgkin and Reed-Sternberg cells (HRS) represent less than 1% of all the tumor bulk which is mainly composed by an heterogenous group of reactive cells of the immune system. The abnormalities of transcriptional factors and genes involved in hematopoietic cells differentiation justify the typical immunophenotype of HRS, being positive for CD30, CD15 and the immuno-checkpoint PD-L1 but usually negative for CD20. The survival of neoplastic cells is favored by the activation of NF-κB, JAK/STAT e PI3K pathways, which play a key role in the pathogenesis of this disease.

Protein casein kinase 2 (CK2) is a serine/threonine kinase ubiquitously expressed in eukaryotic cells, constitutively active, consisting of two catalytic (α) and two non-catalytic (β) subunits assembled to form a tetramer. It is involved in a broad variety of cellular processes, among which survival, proliferation, differentiation, DNA damage and other stress responses. This kinase has been found overexpressed in several solid tumors and hematologic malignancies. It has been fully demonstrated that CK2 acts as a potent antiapoptotic factor that promotes a "non-oncogene addiction" phenotype in cancer cells. In particular, it was shown that many B-cell derived tumors, like multiple myeloma, mantle cell lymphoma and chronic lymphocytic leukemia, rely on high CK2 activity and that its genetic and chemical inhibition induces malignant cell death without significantly affecting normal B lymphocytes. However, the involvement of CK2 in the pathogenesis of HL is still unclear.

By means of western blotting, real time PCR, immunofluorescence, flow cytometry and subcellular fractionation we analyzed the expression of CK2 subunit and the effect of Ck2 inhibition in four HL cell lines (L-428, L-540, KM-H2, HDLM-2), Kasumi-1 (a cell lines derived from a patient with acute myeloid leukemia) and age-matched B lymphocytes from a healthy donor, as controls. Immunohistochemistry on tissue microarray was also used. Apoptosis was assessed by flow cytometry using Annexin V/Propidium Iodine test and PARP cleavage by western blotting.

We observed that CK2α was overexpressed while CK β was expressed at lower levels in all HL cell lines as compared to normal B cells. The α catalytic subunit CK2 was localized both in the nucleus and the cytosol, while the β subunit was mainly cytosolic in HL cell lines. These data were confirmed on primary nodes of patients with HL using tissue microarray. This aberrant expression of protein CK2 was not associated with different mRNA levels of

CSNK2A1 and *CSNK2B*, genes coding for the α and β subunit respectively. We observed that CK2 substrates namely NF-κB, STAT3, AKT were constitutively phosphorylated on activatory serine amino acids (NF-κB Ser529, STAT3 Ser727, AKT Ser473, AKT Ser129) in HL cell lines. The pharmacological inhibition of CK2 with the clinical inhibitor CX-4945/silmitasertib mediates time- and dose-dependent apoptosis and dephosphorylation of CK2 targets at activatory residues. Moreover, we demonstrated that silmitasertib was able to decrease the expression of the immuno-checkpoint CD297/PD-L1 but not of CD30, and to enhance the cytotoxicity caused by monomethyl auristatin E (MMAE), the microtubule inhibitor conjugated to an anti-CD30 monoclonal antibody in the brentuximab vedotin drug.

Comprehensively our data point out a pivotal role of CK2a in the survival, as well as the activation of some key signaling pathways, in HL and identify this kinase as a targetable kinase for the development of new effective therapy for this neoplastic disease. Remarkably a skewed expression between CK2 subunits has never been reported in other hematological cancers.

RIASSUNTO

Il linfoma di Hodgkin (HL), neoplasia del tessuto linfoide, impatta per circa lo 0,6% di tutti i nuovi casi di tumori. Le cellule neoplastiche di Hodgkin e Reed-Sternberg (HRS) rappresentano circa l'1% dell'intera massa tumorale che è composta prevalentemente da un infiltrato eterogeneo di cellule reattive non neoplastiche del sistema immunitario. HRS caratteristicamente esprimono il CD30, CD15 ed il *checkpoint* immunologico PD-L1, mentre sono negative per il CD20. L'acquisizione di un'aumentata sopravvivenza è dovuta all'attivazione anomala dei *pathways* NF-κB, JAK/STAT e PI3K.

La proteina casein chinasi 2 (CK2) è una chinasi ubiquitaria nelle cellule eucariotiche, dotata di attività costitutiva, pleiotropica e coinvolta in molti meccanismi della biologia delle cellule normali e tumorali. CK2 è una serin/treonin chinasi costituita da 2 subunità catalitiche (α) e due non catalitiche (β), assemblate a formare un tetramero. Essa è coinvolta in numerosi processi cellulari, tra cui sopravvivenza, proliferazione, differenziamento, risposta al danno del DNA e ad altri tipi di stress cellulare. Livelli anomali di questo enzima sono stati associati sia a tumori solidi che a patologie onco-ematologiche. È stato dimostrato che CK2 agisce come un potente fattore anti-apoptotico promuovendo la "dipendenza non oncogenica" della cellula tumorale. In particolare il mieloma multiplo, il linfoma mantellare e la leucemia linfatica cronica, dipendono dall'elevata attività di CK2 e che la sua inibizione induce l'apoptosi senza danneggiare i linfociti B normali. Tuttavia, il coinvolgimento di CK2 nella patogenesi del HL rimane ancora da chiarire. In questa tesi abbiamo studiato, a livello proteico e genico, la presenza e la funzione di CK2 nel HL.

Mediante *western blotting*, *real time-PCR,* immunofluorescenza, citofluorimetria e frazionamento subcellulare abbiamo analizzato l'espressione delle subunità di CK2 e l'effetto della sua inibizione in quattro linee cellulari di HL (L-428, L-540, KM-H2, HDLM-2), oltre ad una linea cellulare di leucemia mieloide acuta (Kasumi-1) e linfociti B di donatori sani, come controlli. Abbiamo anche utilizzato l'immunoistochimica su un *microarray* di tessuti. L'apoptosi è stata valutata tramite citometria a flusso usando il test Anessina V/Ioduro di Propidio ed il clivaggio di PARP tramite *western blotting*.

Abbiamo evidenziato come CK2α sia sovraespressa mentre CK2β sia espressa a livelli più bassi in tutte le linee cellulari di HL. La subunità catalitica α si localizzata sia nel nucleo che nel citosol, mentre la subunità β è prevalentemente citoplasmatica nelle linee cellulari di HL. Questi dati sono stati confermati su linfonodi di pazienti con linfoma di Hodgkin utilizzato

un *microarray* di tessuti. L'espressione aberrante della proteina CK2 non è associata a differenti livelli di mRNA di *CSNK2A1* e *CSNK2B*, geni codificante per le subunità α e β rispettivamente. Studiandone l'attività della chinasi, abbiamo rilevato come i suoi principali substrati (NF-κB, STAT3, AKT) erano costitutivamente fosforilati in condizioni basali (NF-κB Ser529, STAT3 Ser727, AKT Ser473, AKT Ser129) nelle linee di HL. L'inibizione di CK2, mediante trattamento con CX-4945/silmitasertib, ha determinato l'apoptosi delle cellule di HL in maniera dose e tempo dipendente, accompagnata da una diminuzione della fosforilazione dei substrati di CK2. Inoltre abbiamo osservato che silmitasertib riduceva l'espressione del *checkpoint* immunologico CD274/PD-L1 ma non del CD30, e che era in grado di potenziare l'attività citotossica di monometil auristatina E (MMAE), inibitore dei microtubuli legato ad un anticorpo monoclonale anti-CD30 nel farmaco brentuximab vedotin.

Complessivamente, i nostri dati indicano un ruolo cardine di CK2 nella sopravvivenza come pure nella attivazione di alcune vie del segnale fondamentali nel HL, identificando questa proteina chinasi come potenziale bersaglio per lo sviluppo di nuove strategie terapeutiche per il HL. Inoltre l'espressione sbilanciata delle subunità di CK2 non è mai stata documentata in altre neoplasie ematologiche.

1 INTRODUCTION

1.1 THE PROTEIN CASEIN KINASE 2 (CK2)

1.1.1 OVERWIEV

Protein kinase CK2 is a highly conserved serine/threonine kinase expressed in all eukaryotic cells. It is now abundantly clear that it is very pleiotropic and capable of phosphorylating more than 500 potential substrates in all cellular compartments. CK2 takes part in the regulation of a broad array of cellular processes including proliferation, survival and differentiation (1). Abnormally high CK2 levels have been observed both in solid tumors (breast, prostate, lung, kidney, head and neck) and in hematological malignancies (acute myeloid leukemia, multiple myeloma, chronic lymphocytic leukemia (CLL), mantle cell lymphoma [MCL]). CK2 can provide a specific environment favorable for transformation by combining with oncogenes (e.g. c-Myc) or by the modulation of oncogenic signals (e.g. Wnt pathway) (2). Moreover, CK2 acts as a potent suppressor of apoptosis, therefore, increased CK2 expression in cancer cells sustains survival and blocks the normal apoptotic activity. Based on this involvement in sustaining tumorigenesis, CK2 has recently attracted attention as a potential therapeutic target.

1.1.2 STRUCTURE

CK2 is a hetero-tetrameric holoenzyme consisting of two catalytic α (42kDa in mammals) and two non-catalytic, previously called regulatory, β subunits (28kDa in mammals) (Figure 1). The two catalytic subunits are linked through the β subunits, even if they can perform their activity also in the absence of the regulatory counterpart. CK2 was distinguished among other protein kinases for its ability to phosphorylate serine or threonine residues proximal to acidic amino acids. It was defined a minimal consensus sequence for phosphorylation by CK2 (S/T-X-X-E/D/pS/pY) as well as its unique ability to use either (adenosine triphosphate) ATP or (guanine triphosphate) GTP as a phosphate donor (3).

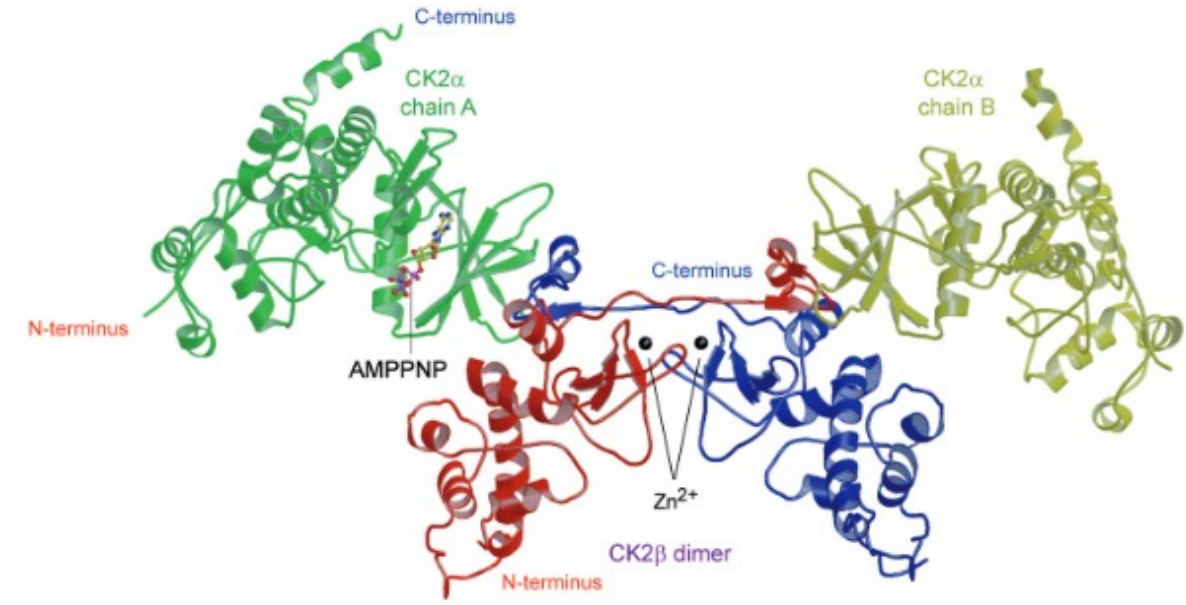


Figure 1. Ribbon diagram illustrating the high-resolution structure of tetrameric CK2 (modified from (4)).

CK2α: The α-subunit is made up of a catalytic core composed of two major folding domains (N- and C-terminal) harboring the active site in between (Figure 1). In humans, two different isoforms of the catalytic subunit (designated CK2α and CK2α'), encoded by distinct genes, were initially characterized. With the exception of their unrelated C-terminal domains, these two isoforms are very similar with approximately 90% identity within their catalytic domain. It is well established that they are closely related and show considerable functional overlap. Indeed, knockout of the gene encoding CK2α' in mice results in viable offspring when heterozygous mice are bred to homozygosity, suggesting that α has the capacity to compensate for α' in the context of viability. However, male are sterile and display defects in spermatogenesis, demonstrating that the functional compensation is not absolute (4-6).

CK2β: Only one known form of the regulatory β subunit has been identified in mammals, but multiple forms have been identified in other organisms, such as *Saccharomyces cerevisiae*. CK2 β is highly conserved among species and x-ray crystallography studies have determined that a dimer of the β subunits forms the core of the tetramer (Figure 1). A large proportion of CK2β has been shown to be phosphorylated on an autophosphorylation site consisting of Ser 2, 3 at its N-terminus and this regulate its proteasome-dependent degradation. CK2β is also phosphorylated at Ser 209 in the C-terminus in a cell-cycle dependent manner by $p34^{cdc2}$ (Figure 2) (5). CK2β monomer has a 'body' consisting of the N-terminal domain and a dimerization

domain, the latter containing the zinc-finger region, characterized by four Cys residues, which mediate the interaction allowing the β dimer to form the core of the holoenzyme. CK2β dimerization precedes catalytic subunit binding and is a prerequisite for the formation of the tetramer. The last 33 amino acids form the 'tail' of the monomer and contain the CK2α-interaction motif. This C- terminal region is responsible for the ability of CK2β to enhance and stabilize CK2 catalytic activity (Figure 2) (5).

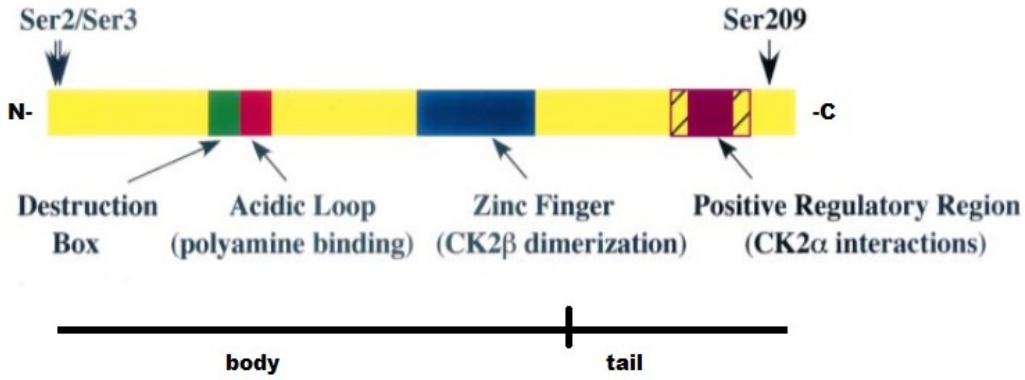


Figure 2. The regulatory CK2β subunit. Linear representation of CK2β, illustrating the main elements within its amino acid sequence (modified from (5)).

1.1.3 FUNCTIONS

CK2 is a signaling enzyme that behaves as an anti-apoptotic agent implying on different cellular functions, kinase pathways and biochemical reactions, which ultimately cooperate to promote cell survival (Figure 4).

Sustains proliferative signaling cascades (Figure 3):

- NF-kB. This transcription factor (TF) is normally sequestered in the cytosol by binding to its inhibitor IkB. CK2 phosphorylates IkB thus promoting its degradation through the proteasome machinery, promotes IKKβ-mediated phosphorylation of NF-kB RELA p65 on Ser356, as well as phosphorylates in Ser529, increasing its transcriptional capability (7);

- PI3K/PTEN/AKT. Here again CK2 operates as a multisite regulator. The tumor suppressor PTEN is the phosphatase that dephosphorylates PIP_3 (phosphatidylinositol 3,4,5-triphosphate), thus maintaining the PI3K/AKT signal down, under resting conditions. It has been demonstrated that phosphorylation of PTEN at Ser 380 by CK2, while regulating PTEN protein stability, has an inhibitory effect on its activity, with the

final result of stimulating AKT-dependent signaling. A second level of CK2 involvement in this pathway is represented by AKT itself: beside a physical interaction between the two kinases, a direct phosphorylation of Ser 129 by CK2 has been identified, which enhances the catalytic activity of AKT. There is also an indirect effect of this CK2-mediated phosphorylation, since it contributes to maintain high levels of phospho Thr 308 by PDK1, by ensuring a stable association with the chaperone protein Hsp90, known to protect Thr 308 from dephosphorylation. Moreover, CK2 down modulation reduces AKT activating phosphorylation at Ser473, mediated by mTOR (8);

- WNT. β-catenin is a transcriptional co-factor in the Wnt signaling pathway. CK2 is a positive regulator of Wnt signaling through phosphorylation of β -catenin at Thr 393, leading to proteasome resistance and increased protein and co-transcriptional activity. β-catenin can therefore enter the nucleus and interact with TFs such as TCF (T-cell factor)/LEF (lymphoid enhancing factor), thus activating Wnt responsive genes, like *MYC* and *CCND1*, that induce proliferation and resistance to apoptosis (9);

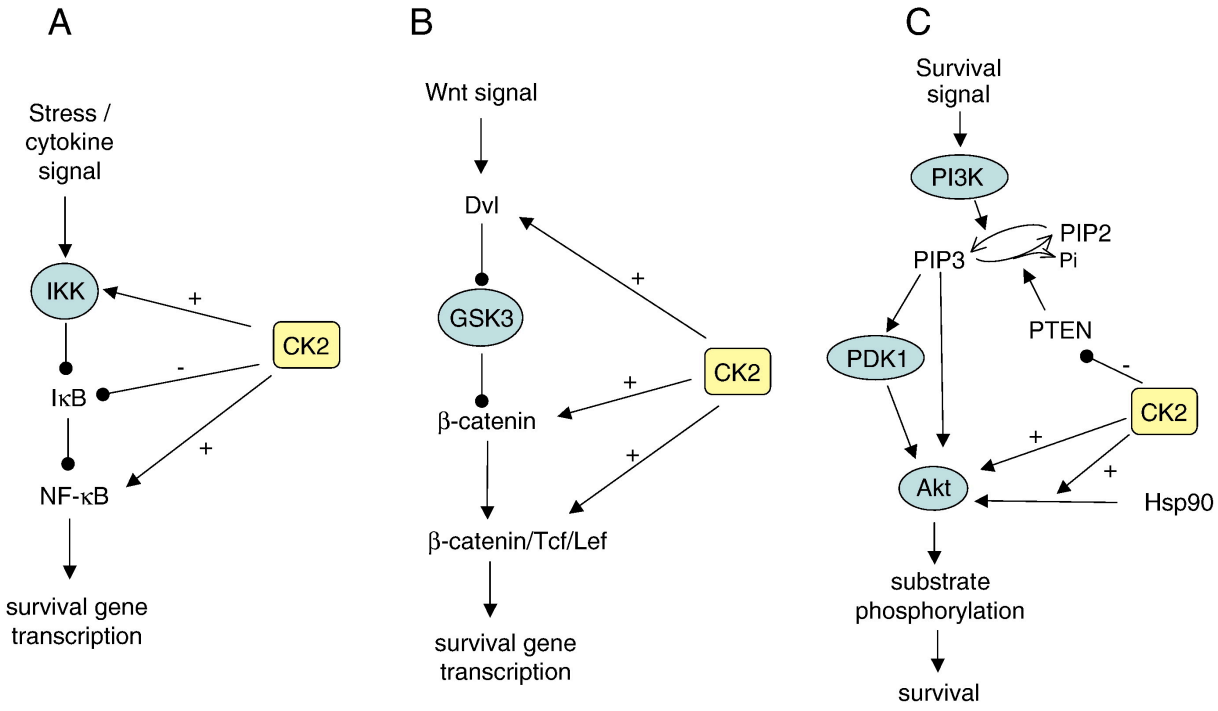


Figure 3. Regulation of pathways di NF-κB (A), Wnt/β-catenina (B) and AKT (C) pathways mediated by CK2. Ruzzene M. *et al* (10).

Activates oncogenes:

- CK2 cooperates with proto-oncogenes such as c-Myc, c-Myb, c-Jun, Ha-Ras and A-Raf, thus promoting cell survival (1, 10);

Confers resistance to cell death:

- Many intracellular proteins, as Bid, Max, HS1, presenilin, are phosphorylated by CK2, and phosphorylation protects these molecules from caspase-mediated cleavage. Caspase-9 itself falls in this category, since its phosphorylation by CK2 protects it from caspase-8 cleavage (5, 10) (Figure 4);

Modulates DNA damage stress response:

- CK2 dependent phosphorylation of p53 at Ser 392 increases in response to UV irradiation. One consequence thereof is a decrease in the pro apoptotic function of p53 observed after UV-induced DNA damage (11);

Promotes cell cycle progression:

- CK2 is required for progression through G1/S and G2/M phases of the cell cycle. CK2 associates with the mitotic spindle and was phosphorylated in mitotic cells, where it interacts with Pin1, an essential regulator of cell division. CK2 participates in the regulation of proteins that have important functions associated with cell cycle progression: topoisomerase II, $p34^{cdc2}$, cdc34, $p27^{kip}$, MDM2, $p21^{WAF}$ and p53 (5).

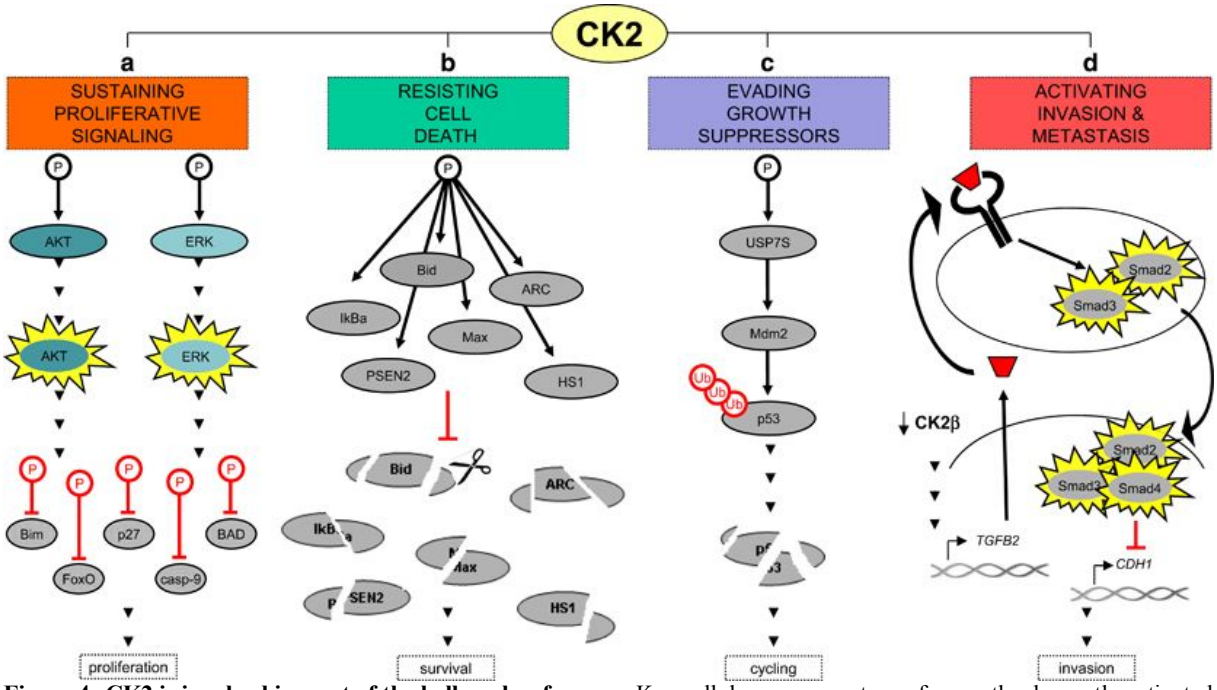


Figure 4. CK2 is involved in most of the hallmarks of cancer. Key cellular components are frequently aberrantly activated in many cancers partly because of an augmented CK2 activity, causing proliferation, survival, cell cycle progression and tissue invasion. (**a**) CK2 direct phosphorylation activates AKT and ERK, which in turn phosphorylate proapoptotic proteins. (**b**) CK2 phosphorylates pro-survival proteins preventing their caspase-mediated cleavage. (**c**) One instance of CK2 regulation of Mdm2/p53 axis. (**d**) One instance of CK2 involvement in metastasis and spreading: CK2β depletion induces the epithelial–mesenchymal transition through a TGFβ autocrine mechanism. Adapted from Mandato et al. (12).

1.1.4 REGULATION

Many eukaryotic protein kinases are strictly regulated by phosphorylation and conformational changes in their activation segment, due to their importance as key components of a wide variety of signaling pathways. In CK2, however, this activation segment is characterized by a striking rigidity and is invariable, allowing the enzyme to adopt a stable active conformation. There are two reasons for this:

1. The activation loop is in extensive contact with the conformationally invariant N-terminal domain, the removal of which causes loss of CK2 catalytic activity.
2. The Mg^{2+} binding loop contains an unusual motif: DWG instead of DFG. The Trp substituting the canonical Phe allows an additional hydrogen bond that disfavors any conformational change.

The stabilization of the active conformation by these constraints provides the rationale for the 'constitutive activity' of CK2 that is mostly independent from stimuli, such as phosphorylation, second messengers and interaction with regulatory proteins. However, it has been reported that CK2β can be phosphorylated in a cell cycle-dependent manner within Ser209 in the C-terminal domain and can undergo auto-phosphorylation in the activation loop at Ser2-3, but these events do not cause a dramatic change in the enzyme activity (Figure 2). It is important to notice that small molecules can, at least in part, regulate CK2 activity: negatively charged compounds can inhibit, while positive charged compounds activate the enzyme. The major mechanism of CK2 regulation in cells seems to be the interaction with the chaperone Hsp90 and its kinase-specific co-chaperone Cdc37. CK2 phosphorylates Hsp90 and Cdc37, thus allowing the recruitment of Hsp90 to the client kinase-Cdc37 complex and inducing the efficient activation of these kinases. Interestingly, CK2 itself is a client of the Hsp90-Cdc37 machinery, suggesting that it mediates a positive auto regulatory feedback loop (4, 5, 13).

1.1.5 INVOLVEMENT OF CK2 IN CANCER

Deregulation of protein kinases frequently underlies many human diseases, with special reference to cancer. More than a half of the oncogenes encode kinases that are usually found mutated in tumors. This paradigm is hardly applicable to CK2, which presents a high constitutive activity and has never been reported to be genetically mutated in neoplasia. For these reasons, CK2 cannot be defined as an oncogene, but rather a potent anti-apoptotic factor,

deeply involved in oncogenesis. In fact, it contributes to create a cellular environment favorable to the establishment and maintenance of a tumor phenotype and cancer cells become dependent on CK2 overexpression for their survival and response to increased cellular stress resulting from oncogenic activation. This phenomenon is called 'non-oncogene addiction' (2, 14). Within a population, a minority of cells can stochastically present high CK2 levels. If an oncogenic mutation occurs in these cells, they can better escape apoptosis and proliferate, leading to a progressive selection of mutated cells and a parallel progressive establishment of a malignant phenotype. Overexpression of CK2 has been well documented in a broad array of cancers, including solid tumors, as breast, colorectal, renal, lung and prostate and hematologic malignancies, like MM, and acute myeloid leukemia, among others. Malignant cells, which depend on CK2 overexpression for their proliferation and viability, are more sensitive to CK2 downregulation than their normal counterparts (10). Recently, a growing number of studies suggested that this kinase could act as a powerful 'oncogenic non-oncogene' in lymphoid tumors. Indeed, CK2 was found overexpressed and essential for growth in MM, MCL, CLL and B-cell precursor acute lymphoblastic leukemia (BCP-ALL) (15).

CK2 IN ACUTE LEUKEMIAS

In human precursor leukemias/lymphomas a role for CK2 was firstly hypothesized by virtue of the interaction of the α subunits with the BCR (breakpoint cluster region) moiety of p190 or p210 BCR-ABL fusion oncoproteins, generated by the chromosomal translocation t(9;22)(q34; q11). This translocation occurs in a fraction of BCP-ALL and in the majority of chronic myeloid leukemia (CML) cases (16). CK2 was shown to sustain survival of BCR-ABL positive cells and its inhibition with small ATP-competitive compounds, such as 4,5,6,7-tetrabromobenzimidazole or 2dimethylamino-4,5,6,7-tetrabromo-1H- benzimidazole, resulted in growth arrest (16, 17). CK2 was demonstrated to be instrumental for a proper activation of the PI3K/PTEN/AKT signaling cascade in BCP-ALL. Gomes *et al.*(18) showed that in adult BCP-ALL cells CK2 is overexpressed/hyperactive. Considering the well-known involvement of CK2 in PTEN protein stability, being CK2-dependent phosphorylation a signal for PTEN stabilization and functional inactivation, the authors challenged BCP-ALL cells with CK2 inhibitor CX-4945, with the remarkable result of causing growth arrest associated with a drop of PI3K/AKT activity (19). In addition, parallel research has identified a potential role for CK2 in the regulation of the half-life of the TF Ikaros, which is mutated in pediatric and adult BCP-ALL (20). A balance between protein phosphatase PP1 and CK2 was found to determine Ikaros

stability and transcriptional activity, with CK2-phosphorylated Ikaros displaying reduced protein stability and functional activation (21, 22). Taken together, these data suggest the relevance of investigating the role of CK2 in Ikaros mutated BCP-ALL, in which this TF displays a loss of function in 5% of cases.

Among acute myeloid leukemia (AML), higher levels of CK2 were associated with an adverse prognosis. In fact, the overexpression of CK2 kinase drive the activation of PI3K/AKT/mTOR cascade and the deregulation of p53 protein. In AML the pharmacological inhibition of CK2 with CX-4945 was able to hit not only the leukemic cells but the subpopulation of leukemic stem cells, triggering the p53-dependent cellular apoptosis (23).

CK2 IN MULTIPLE MYELOMA AND MANTLE CELL LYMPHOMA

CK2 was shown to be overactive and overexpressed in MM and MCL cell lines and primary tumor samples. CK2α knockdown or inhibition with TBB, TBB-derived agents or with CX-4945 caused MM cell apoptosis, not counteracted by the addition of growth factors, such as interleukin 6 (IL-6) and insulin-like growth factor-1. Moreover, CK2 silencing or inhibition was associated to IκBα stabilization and decreased NF-κB transcriptional activity (24). It was also demonstrated that CK2 positively regulates STAT3 and NF-κB-dependent signaling, both in MM and in MCL cells. CK2 down modulation is associated with a reduction in NF-κB phospho-Ser 529 and Ser 536, and STAT3 phospho-Ser 727 levels [23]. Indeed, there is robust evidence that the phosphorylation of these serine residues takes part in the modulation of the transcriptional activation of both proteins. Furthermore, a role for CK2 in the sensitivity of MM as well as MCL cells to novel therapeutic agents was clearly demonstrated. CK2 lies downstream of the endoplasmic reticulum-stress induction by HSP90 inhibitors as well as proteasome inhibition by bortezomib. Double inhibition of HSP90 and CK2 strongly synergizes *in vitro* and in mouse xenotransplant *in vivo* models in inducing MM cell apoptosis (25). Moreover, downregulation of the kinase profoundly influences cellular response to bortezomib. In conditions of CK2 blockade, MM and MCL cells became much more prone to bortezomib-induced cytotoxicity and this was accompanied by an increase in the proteotoxic response, as documented by the raise in ubiquitylated proteins found in these cells (25, 26). In addition to this, signals sustained by CK2 might promote the formation of a pro-survival *milieu* between bone marrow stromal and MM cells. In stromal cells, CK2 stimulates the expression of growth signals, including tumor necrosis factor-α (TNF-α) and IL-6, which activate NF-κB and STAT3 TFs in MM cells (27). In an *in vitro* model of MM cells' culture in the presence of bone marrow

stromal cells, the down modulation of CK2 activity with CX-4945 induced a significant amount of MM cell death minimally affecting the stroma. CX-4945 demonstrated its ability to counteract the stromal support to malignant cells, which may widely contribute to resistance (27).

CK2 IN CHRONIC LYMPHOCYTIC LEUKEMIA

Several lines of evidence suggest a central role for CK2 also in mature B lymphoid tumors, including CLL. Jaeger *et al.* found high levels of CK2β phospho-Ser 209 in primary samples derived from 44 CLL patients (28). The phosphorylation of CK2β on this residue is known to modulate CK2 enzymatic activity and target binding (5). Similarly to BCP-ALL, CK2 inhibition was associated with a decrease in PI3K/AKT functional activation, due to a reduction of PTEN phosphorylation on Ser 380 and AKT on Ser 473, and with an increase in cell death (28). Martins *et al.* confirmed the strong pro-survival function of CK2 in CLL by showing that primary CLL cells are characterized by increased levels of CK2 α and β subunits (29). CK2 inhibition was found to be coupled with inactivation of PKC, increased PTEN activity and apoptosis, especially in CLL cells isolated from patients with advanced disease stage. Importantly, normal B lymphocytes were only slightly affected by the treatment with CK2 inhibitors (29). In another work, Martins *et al.* confirmed the pro apoptotic effect of CX-4945 against primary CLL cells and cell lines (30). Remarkably, the cytotoxic effect of the drug was not reversed by stromal co-culture. *In vivo*, the combination of CX-4945 with fludarabine, which is used for the treatment of CLL, led to a significant reduction of tumor growth, as compared with single treatments. Another work put into light that CX-4945 cooperates with two compounds currently approved for the treatment of relapsed/refractory CLL and NHLs, the BTK inhibitor ibrutinib and the PI3Kδ inhibitor idelalisib (31), providing strong rationale for the introduction of CX-4945 in combination therapies also with inhibitors of the BCR cascade.

1.2 CK2 INHIBITORS

It has been frequently reported that malignant cells, with abnormally high CK2 levels, are more susceptible to CK2 inhibitors-induced apoptosis, than their normal counterparts (10). Interest in developing small molecule inhibitors of CK2 increased with the identification of ATP-binding sites specific chemotypes. However, as with many inhibitors of other kinases, questions regarding their specificity arose immediately. This cautionary note has to be

considered especially for these kinds of compounds that are competitors of ATP, since ATP is the substrate for all protein kinase family members in addition to a vast array of other cellular enzymes. The ATP binding site of CK2 is smaller than most of the other kinases, because of the presence of unique bulky residues, which allow for the design of very selective and specific low molecular weight ATP- competitive inhibitors (32).

Many ATP-competitive inhibitors of CK2 have already been reported in the literature, such as 5,6,-dicloro-1(b-D-ribofuranosil)benzimidazolo, 4,5,6,7-tetrabromo-2-azabenzimidazolo (TBB) and its derivative 2-dimetilamino-4,5,6,7-tetrabromo-1H-benzimidazolo, but they did not reach human clinical trials, except for CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthy ridine-8-carboxylic acid; silmitasertib) (32). (Figure 5).

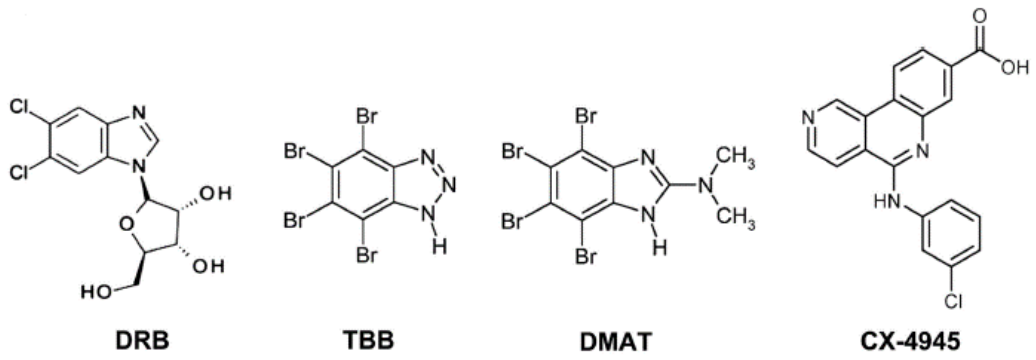


Figure 5. Molecular structure of some CK2 inhibitors. 5,6,-dicloro-1(b-D-ribofuranosyl) benzimidazol (DRB), 4,5,6,7-tetrabromo-2-azabenzimidazol2 (TBB) and its derivative 2-dimethylamino-4,5,6,7-tetrabromo-1H-benzimidazole (DMAT), acid 5-(3-clorofenilamino)benzo(c)(2,6)naptiridin-8-carbosilic (CX-4945, Silmitasertib). Buontempo F. et al (33).

This last compound is a selective, potent, orally bioavailable inhibitor of CK2α subunits, whose anti-tumor activity has been validated in cancer cell lines and murine xenograft models (34). It was designated by Cylene Pharmaceuticals and entered phase I clinical trials for advanced solid tumors and multiple myeloma (MM) (NCT00891280, ClinicalTrials.gov) and, more recently, a randomized study that compares antitumor activity in cholangiocarcinoma patients receiving the standard of care gemcitabine plus cisplatin versus CX-4945 with gemcitabine plus cisplatin (NCT02128282, ClinicalTrials.gov). During phase I trial, CX-4945 has been safe and well tolerated, achieved a serum maximum concentration of 15μM, demonstrated a clear dose-dependent pharmacodynamic response and the capacity to kill tumor cells in patients. The crystal structure of human CK2α in complex with CX-4945 shows two direct protein- inhibitor hydrogen bonds. Two well-ordered water molecules mediate additional contacts between the carboxylate group of CX-4945 and CK2α**.** This extensive combination of

direct and water-mediated hydrogen bonds and van der Waals contacts between CX-4945 and CK2α establishes the structural basis for the high affinity binding of the inhibitor (35). Downregulation of CK2 activity with CX-4945 boosts cytotoxicity in hematological cancer cells; this points out that the kinase may be a valid druggable anti-cancer target to be employed in the treatment of hematological malignancies (36).

1.3 B LYMPHOCYTES

B lymphocytes are a population of cells expressing clonally diverse surface immunoglobulin receptors recognizing specific antigenic epitopes. These cells are key components of the adaptive immunity, responding to pathogens by proliferation, differentiation and antibody (Ab) production (37). Human B-cell development encompasses a *continuum* of stages that begin in primary lymphoid tissues (fetal liver and fetal/adult bone marrow), with functional maturation in secondary lymphoid tissues (lymph nodes and spleen). Early B-cell development is characterized by the rearrangement of immunoglobulin heavy and light chain loci and the assembly of the pre-B-cell receptor BCR, which is fundamental for B-cell development and survival in periphery. Antigen-induced B-cell activation and differentiation in secondary lymphoid tissues are mediated by changes in gene expression that give rise to the germinal center reaction. The germinal center reaction is characterized by clonal expansion, class switch recombination, somatic hypermutation and selection for a high affinity unique antigenic epitope of B-cell receptor. The result of this reaction is the generation of plasma blasts, secreting antibodies while still dividing, and short-lived plasma cells, secreting antigen-specific germ line-encoded antibodies. Persistent Ag-specific antibody titers derive from long-lived PCs that migrate to the BM and can persist without self-replenishment or turnover. On the other hand, B lymphocytes not selected during the germinal center reaction undergo apoptosis. The aforementioned developmental stages have malignant counterparts that reflect the expansion of a dominant subclone leading to development of leukemias and lymphomas (38). The majority of human B-cell tumors arise from mature B-cells recruited into the germinal center reaction (Figure 6). Germinal center B-cell lymphomas are divided into Hodgkin and NHLs. The latter group comprises Burkitt's lymphoma, follicular lymphoma and diffuse large B-cell lymphoma (DLBCL). Development and function of B-cells are principally affected by signaling via NF-kB, PI3K/AKT and JAK/STAT signaling.

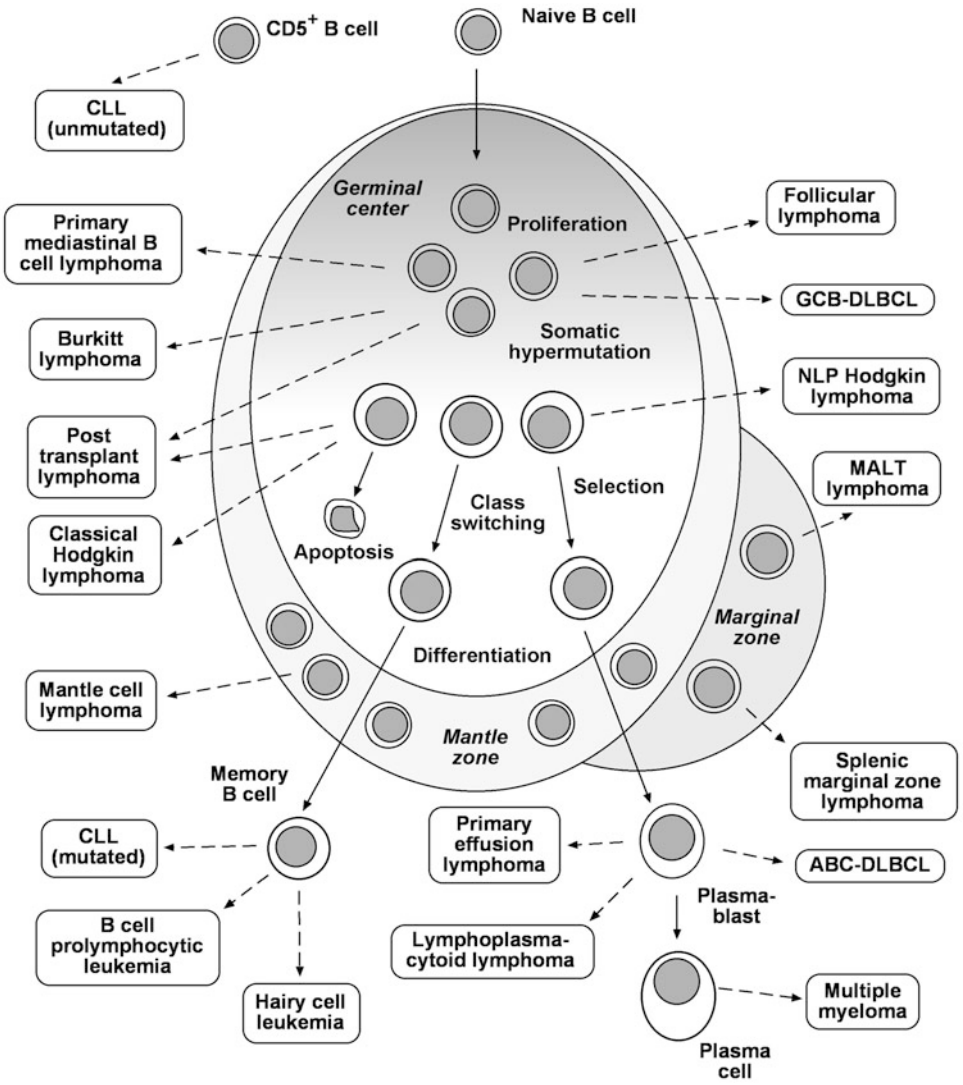


Figure 6. Germinal center reaction and cellular origin of human B-cell lymphomas. Shown are the main steps in mature B cell differentiation in the GC and the presumed cellular origin of human B cell lymphomas.

1.3.1 NF-kB PATHWAY

The NF-κB TF family regulates the expression of a great variety of genes involved in many diverse cellular processes, such as inflammatory and immune responses, growth, survival and development. These factors are normally activated in a tightly regulated manner, as a response to numerous signals, including cytokines, pathogens, and injuries (39). This family consists of five members: p50, p52, p65/RelA, c-Rel and RelB, which share an N-terminal domain responsible for DNA binding and dimerization. Dimers bind to κB sites within promoters/enhancers of target genes and regulate transcription through the recruitment of coactivators or corepressors. Only p65 (RelA), c-Rel and RelB present a transcription activation domain at the C-terminal, which is necessary for positive regulation of gene expression (40). Three distinct NF-kB pathways have been described: the canonical, the alternative and the

atypical. In the canonical pathway, proinflammatory signals like cytokines (e.g. TNFα), pathogen- and danger- associated molecular patterns, and the BCR/TCR activate the dimer p50/p65 (Figure 7), while the alternative pathway is triggered by BAFF or some viruses that direct the activation of p52/RelB. The atypical pathway, on the other hand, is activated in response to DNA damage (41).

In their inactive state, dimers are associated with one of three IkB proteins, IkBα, IkBβ or IkBε that help in keeping them in the cytoplasm. IkB degradation depends on its prior phosphorylation by the IkB kinase complex that consists of the IkB kinases IKKα, IKKβ and the regulatory subunit IKKγ/NEMO. The most extensively studied member of the family is IkBα. During activation of the NF-κB signaling pathway, IkBα is rapidly degraded in a proteasome-dependent manner, leading to release and nuclear migration of p65/p50, which is the primary target of this inhibitor (Figure 7). Transcription is further regulated through posttranslational modifications of NF-kB, like phosphorylation, that modify the ability of the dimers to interact with specific coactivators/corepressors in response to distinct stimuli.

Many protein kinases modify the TF RelA enhancing its transactivation potential:

- PKA phosphorylates RelA at Ser 276, after IkBα degradation, promoting the interaction of p65 with the transcriptional coactivators CBP and p300;
- IKKβ phosphorylates RelA at Ser 536 in the TAD. This phosphor-residue is involved in the regulation of transcriptional activity, nuclear localization and protein stability and mutations thereof disrupt the interaction of RELA with CBP/p300;
- CK2 phosphorylates RelA at Ser 529 in the TAD (Figure 7). The phosphorylation of this residue alters the association with basal components of the transcriptional machinery and may therefore be involved in the regulation of gene expression (40).

It is currently largely unclear how NF-κB dimers control key parameters of the target gene-specific response. Each individual NF-κB activating stimulus leads to the induction of a specific overlapping and distinct subset of genes (42). Constitutive NF-κB activation contributes to the growth and malignancy of cancer cells and affects tumor response to chemotherapy. In particular, it has been demonstrated that it can promote continuous lymphocyte cycling and survival and is a critical pathogenetic factor in lymphomas (43). It is well known that a wide variety of genetic alterations induce an aberrant activation of the

canonical NF-κB signaling pathway in human lymphomas, such as Hodgkin lymphoma (HL), mucosa associated lymphoid tissue lymphoma and activated B-cell like-DLBCL.

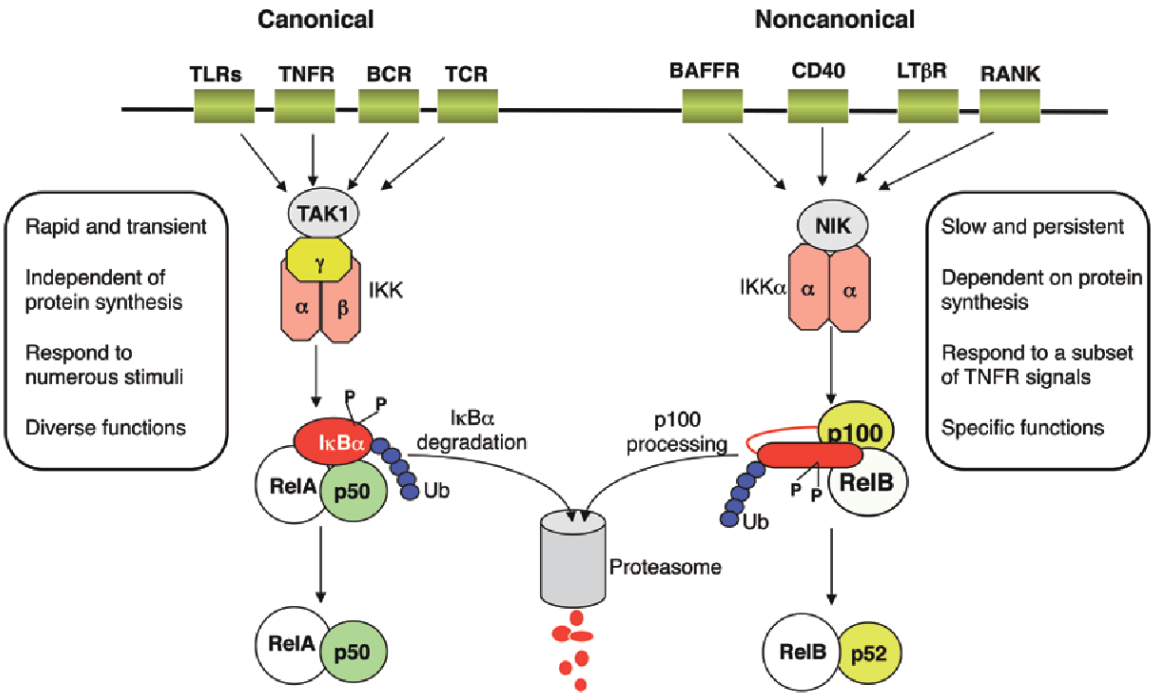


Figure 7. Canonical and non-canonical NF-κB signaling pathways. Canonical pathway is triggered by numerous signals, including those mediated by innate and adaptive immune receptors. It involves activation of IKK complex by Tak1, IKK-mediated IκBα phosphorylation, and subsequent degradation, resulting in rapid and transient nuclear translocation of the prototypical NF-κB heterodimer RelA/p50. Non-canonical NF-κB pathway relies on phosphorylation-induced p100 processing, which is triggered by signaling from a subset of TNFR members. This pathway is dependent on NIK and IKKα, but not on the trimeric IKK complex, and mediates the persistent activation of RelB/p52 complex.

1.3.2 PI3K/AKT PATHWAY

Phosphorylated lipids are produced at cellular membranes during signaling events and are responsible for the recruitment and activation of various cytoplasmic signaling components (44). PI3Ks are a family of lipid kinase enzymes that produce 3'-phosphorylated phosphoinositides that act as second messenger to redirect intracellular proteins to cellular membranes. Among these proteins, which present a PH domain that binds to PIP_3, are BTK, AKT and PDK1. The Ser/Thr kinase AKT is the major mediator of PI3K signaling. Through the regulation of multiple distinct targets, AKT controls the equilibrium between survival and apoptosis, quiescence and proliferation, as well as cell metabolism and differentiation (45). After its membrane recruitment, PDK1 phosphorylates AKT at Thr308 and mTORC2 at Ser 473, thus enhancing AKT activity (46). It has been shown that CK2 can further phosphorylate AKT at Ser129 enhancing Ser473 phosphorylation, thus supporting the view that CK2 action induces conformational changes in AKT that render the kinase more prone to activation (47)

(Figure 8). AKT directly phosphorylates the kinase GSK3 and FOXO TFs, and indirectly stimulates mTOR and NF-kB. Phosphorylation of GSK3 turns off the catalytic activity of this enzyme, by inactivating this protein, which negatively regulates c-Myc and cyclin-D (45). Phosphorylation of FOXOs, instead, induces re-localization of these TFs from the nucleus to the cytoplasm, where they are degraded by the proteasome. Since active FOXOs promote cell death, through the control of the expression of pro apoptotic proteins, like BIM and FasL, reduction in FOXO factors is considered an important event in the pathology of cancer (45). The termination of PI3K/AKT signaling by degradation of PIP_3 is mediated by SHIP and PTEN phosphatases that are able to generate PIP_2 (44). CK2 can phosphorylate and inhibit PTEN, boosting AKT signaling (Figure 8). Activation of the PI3K/AKT cascade is a common feature of most human cancers and B-cell derived tumors represent no exception. Convincing evidence indicate a high AKT and mTOR basal activation in B-cell leukemias, lymphomas and MM. In addition to this, PTEN is often inactivated in human cancers, supporting its role as a fundamental tumor suppressor (44).

1.3.3 JAK/STAT PATHWAY

Different hormones (e.g. erythropoietin, prolactin) and cytokines (e.g. IL-6) use receptors for cytokines not presenting intrinsic kinase activity, differently from Receptor Tyrone Kinases, but acquiring it upon binding with the specific ligand. This interaction enables the recruitment, at the cytosolic portion of the receptor, of tyrosine kinases that in turn phosphorylate various substrates, inducing the transduction of different signals (e.g. proliferation, survival, etc.). JAK/STAT signaling is one of the most investigated and important pathways involved in tumorigenesis and cancer cell sustainment. Four different JAKs (Janus Family Kinase) and seven STATs (Signal Transducer and Activator of Transcription) have been previously identified that, through combinations of homo- and hetero-dimers, are able to transduce signals from more than 40 cytokines (48). Deregulation of JAK/STAT signaling pathways, leading to inappropriate activation of the transcriptional factors STAT, has been identified in several hematological malignancies regulating the survival, proliferation and metabolism of neoplastic cells (48). Among JAKs, JAK2 has a key role in the activation of JAK/STAT pathway due to its tyrosine kinase activity, while STAT3 is a transcriptional factor that once phosphorylated in Tyr705, dimerizes through the SH2 domain, migrates into the nucleus where it regulates the transcription of pro-survival and anti-apoptotic genes such as BCL-2 and MCL-1. While STAT3 phosphorylation on Tyr705 is known to be the critical activator of JAK/STAT pathway, the phosphorylation on Ser727 likely tunes the transcriptional

activity of STAT3 by interacting with other STAT proteins or transcriptional cofactors including AP-1 and p300.

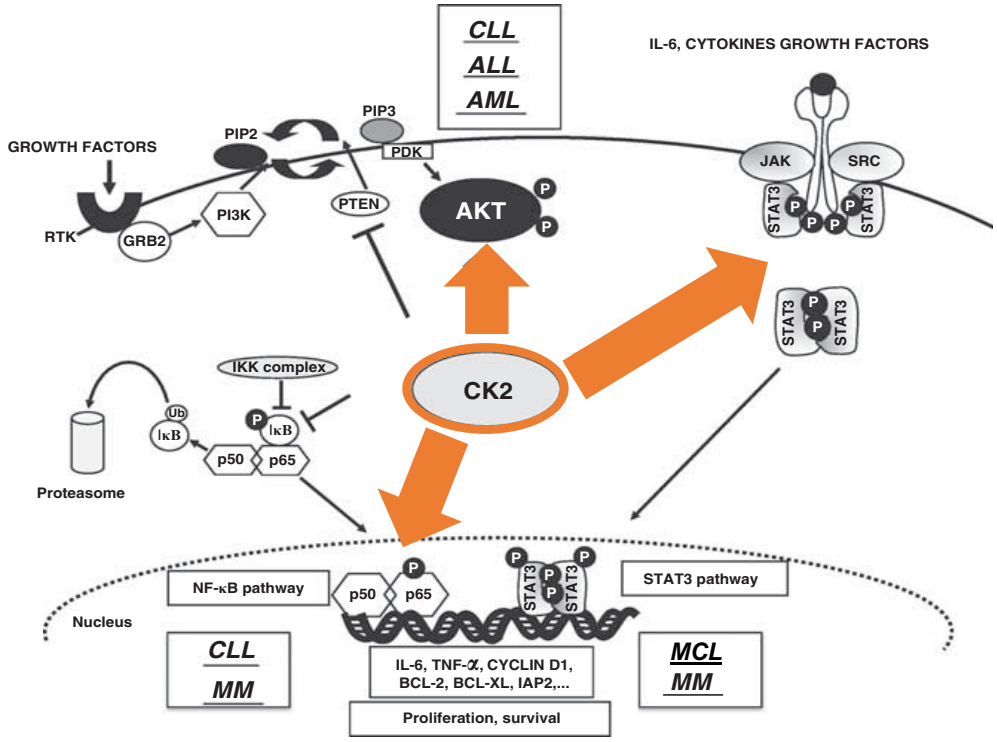


Figure 8. Involvement of protein kinase CK2 in blood tumor-associated signaling pathways. Among the several signaling pathways regulated by CK2, the modulation of the AKT/PKB/PI3K/PTEN cascade may be critical for the survival and proliferation of ALL, CLL and AML tumor cells; in MCL and in MM CK2 regulates the extent of JAK/STAT activation downstream of cytokine/growth factor signaling; CK2-mediated phosphorylation of IkBα in its PEST domain leads to IkBα inhibition through proteasome degradation and CK2 phosphorylation of NF-kB p65 on Ser529 causes the activation of the NF-kB pathway and this could be important for CLL and MM cell survival and resistance to cytotoxic therapies. Adapted from Piazza et al (15).

1.4 THE HODGKIN LYMPHOMA

Lymphomas are cancers arising from lymphoid tissue. Lymphomas are characterized by cellular proliferation that causes lymph nodes enlargement and/or extra-nodal localizations as opposite to leukemia which arose from the bone marrow causing leukocytosis and cytopenia in the peripheral blood. However, bone marrow localization is very rare in HL. HL account for a peculiar group of neoplasia that are different from other lymphoma, the so-called non-Hodgkin lymphoma, due to the involvement of a singular nodal area, most commonly the lymph nodes of the left supraclavicular space and the anterior mediastinum, spread for contiguity and the uncommon extra-nodal localization, such as lung, liver and bone. A pathognomonic sign of this disease is the finding, among reactive leukocytes, of the characteristic, multi-nucleated Hodgkin/Reed-Sternberg (HRS) cells of classic HL or the Lymphocytic-histiocytic variant, also known as "popcorn cells" due to the nuclear shape, of the nodular lymphocyte predominant-HL (NLPHL) subtype. HRS are usually rare in the neoplastic tissue, accounting for almost 0,1-2% of all the cells. HRS cells are able to produce and release several soluble molecules and cytokines to attract reactive B and T lymphocytes, macrophages, neutrophil and eosinophil granulocytes in order to create and immuno-suppressive microenvironment that sustains and support the survival of neoplastic cells (49).

1.4.1 EPIDEMIOLOGY

HL is an uncommon disease, since there are estimated 62,000 new cases every year in the world with an incidence of almost 3 new cases every 100,000 people/year (Figure 9), and is slightly more common in males with a male/female ratio of 1,5:1. HL accounts for 0.5% of all new cancer and impacts on 0.2% of all cancer death. to A characteristic of this lymphoma is the bimodal distribution of incidence of classical HL in western country. There are two peaks of incidence indeed, the first between 15 and 30 years, and the second between 50 and 75 years. As shown in Figure 9 the incidence of classical HL is stable across decades while the mortality is slowly decreasing. Death due HL is more common in elderly patients, in fact the median age at death is 68 years. About NLPHL the distribution of cases had a single peak at 40 years with a male/female ratio of 3:1. World widely HL is more common in rich and western countries.

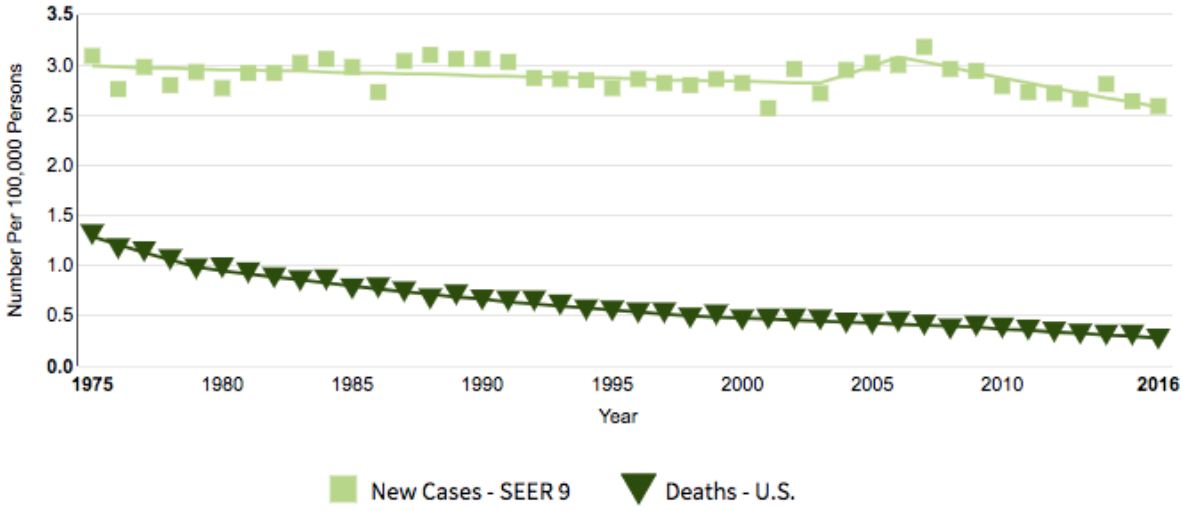


Figure 9. Incidence and mortality of cHL from the Surveillance, Epidemiology and End Result (SEER) database.

1.4.2 CLINICAL CHARACTERISTICS

HL causes enlargement of unpainful lymph nodes, usually localized in the neck, supraclavicular area and in the mediastinum, while abdominal localizations are less common, as well as the extra-nodal involvements such as lung, liver and bone (49). According to different pathological features like morphology, phenotype of tumor cells and composition of inflammatory surrounding cells in the tumor bulk, the World Health Organization (WHO) classification of hematopoietic and lymphoid tissue tumors classifies HL in two different entities, the classical HL and the NLPHL (50). Classical HL can be further classified in different subtypes according the morphology on HRS cells and reactive microenvironment:

- Nodular sclerosis: is the most common histology accounting for 60-70% of all cases, is more common in young females and the diseases is usually localized in the supraclavicular area;
- Mixed cellularity: less common that the former, almost the 25% of cases, but it is the most common histology among HIV patients;
- Lymphocyte rich: rare and accounting for 4-5% of all cases;
- Lymphocyte depleted: very rare in HIV- patients but associated with a worse prognosis.

Usually patients with localized disease do not suffer of systemic symptoms, as opposite patients with a more advanced disease are more likely to suffer of B symptoms like fever >38°C, night sweet and weight loss >10% with 6 months. Disease progression is peculiar, involving

first the lymph nodes with spread for contiguity and through the lymphatic system to other near lymph nodes, and subsequently to affect lungs, spleen, liver, bone and very rarely the bone marrow (49). The clinical presentation of a lymphoma can mimic several other diseases, but usually it is characterized by mass or systemic effect. HL is usually characterized by a mass, an enlarged lymph node of the neck or of the chest, which can be asymptomatic or causing different complications according to the region involved such as superior vena cava compression, mediastinal syndrome, deep vein thrombosis, or much rarely spinal compression and obstructive icterus. Other typical symptoms are pruritus and nodal pain after drinking alcoholics. Blood exams may show lymphocytopenia, increased erythrocytes sedimentation rate (ESR) and/or C reactive protein, and less frequently hypo-albuminemia and anemia.

1.2.3 DIAGNOSIS, STAGINING AND PROGNOSIS

The diagnostic process of patients with lymphomas required several analyses with the aim to highlight the spread of the diseases. This process star with patient medical history, physical examination in order to identify palpable masses; complete blood count, biochemistry evaluating liver and kidney functions, serum levels of lactic dehydrogenase, ESR, C reactive protein, protein electrophoresis; computer tomography (CT) scan of neck-thorax-abdomen-pelvis and 18-FDG PET-CT scan (positron emission tomography with 18-fluorodeoxyglucose as metabolic tracker together with a low-dose CT scan).

Staging, i.e. the assessment of tumor extension, was done according with Ann Arbor classification, proposed in 1971 and subsequently updated, which took into consideration the number of involved area, the localization of the lymph nodes above or down to the diaphragm and extra-nodal sites (49). The most updated stating system is the Lugano classification (51) reported in Table I.

Patients at early stage (I-IIA) are classified for the presence of unfavorable markers according to the German Hodgkin study group criteria: ESR>50 if B symptoms are absent or >30 if B symptoms are present, mediastinal mass ratio >0.33, nodal sites >2 and any extra-nodal lesions (52). Patients at advanced stage disease (IIB-III-IV) are furtherly stratified according to the International Prognostic Score (IPS) which includes male gender, age >44years, stage IV disease, hemoglobin<105g/L, albumin<4g/dL, white blood cells >15.000/mm^3, lymphocyte count <8% of all white cells or <600/mm^3 (53). While the evaluation of unfavorable prognostic markers in early-staged patients is useful to plan the number of

chemotherapy cycles, the role of IPS for advanced-staged patients in the PET-CT era is questionable, since the prognostic and predictive roles of PET-CT after 2 cycles of therapy is a stronger prognosticator than IPS staging (54). HL is relatively curable since almost 85% of patients achieved long-term complete remission and is cured. The rate of progression-free survival is about 95% for patients at early stage diseases, while it decreases to 75-80% for those with a more advanced stage.

Table I. Revised staging system of Lugano classification (51).

STAGE	INVOLVEMENT	EXTRANODAL STATUS
LIMITED		
I	One node or a group of adjacent nodes	single extra-nodal lesions without nodal involvement
II	Two or more nodal groups on the same side of the diaphragm	limited contiguous extranodal involvement
ADVANCED		
III	Nodes on both sides of the diaphragm or nodes above the diaphragm with spleen involvement	n.a.
IV		Additional non-contiguous extra-lymphatic involvement

1.4.4 TREATMENT

EARLY STAGE FAVORABLE DISEASE

Combined-modality treatment consisting of a brief chemotherapy followed by radiotherapy (RT) RT was shown to result in superior tumor control compared with RT alone (55). Two or three cycles of doxorubicin/bleomycin/vinblastine/dacarbazine (ABVD) followed by conventionally fractionated RT represent the standard of care for limited-stage HL. A large multicenter trial in which patients were randomly assigned to either two or four cycles of ABVD followed by either 20 or 30 Gy involved-field RT (IFRT) showed similar freedom from treatment failure (FFTF) and overall survival (OS) rates for all treatment groups. Thus, the least toxic approach consisting of two cycles of ABVD followed by 20 Gy IFRT appears to be sufficient for favorable limited stage HL (52). However, the current RT guidelines of the international lymphoma radiation oncology group recommend involved-site RT (ISRT) after chemotherapy in limited stages. Although ISRT has not been randomly compared with IFRT

in a prospective study, there is accumulating evidence of excellent disease control with these smaller RT fields (56). The question of whether RT can be omitted in selected patients with complete metabolic response at interim PET (iPET) is a matter of debate. Several randomized trials addressing this issue have been conducted within the last years. The available data consistently demonstrate a progression-free survival (PFS) advantage for patients treated with combined-modality approaches despite a negative PET. Thus, a patient group that can be safely treated with chemotherapy alone could not yet be defined (57, 58). However, as patients treated with chemotherapy alone still have a good overall prognosis, this approach may be offered to individual patients when the late risk of delivering RT is thought to outweigh the short-term benefit of improved disease control. Early treatment intensification appears to improve the prognosis of patients with a positive iPET. A large randomized study including patients with favorable and unfavorable early-stage HL revealed a significantly reduced relapse rate in those patients with a positive iPET after two cycles of ABVD who completed chemotherapy with two cycles of BEACOPP escalated (bleomycin/etoposide/doxorubicin/cyclophosphamide/ vincristine/procarbazine/prednisone in escalated dose) instead of one or two additional cycles of ABVD before involved-node RT (57). Patients with a positive iPET after two cycles of ABVD should be treated with two cycles of BEACOPPescalated before ISRT.

EARLY STAGE UNFAVORABLE

Also these patients with HL are usually treated with combined modality approaches, i.e. a short course of chemotherapy+RT. Four cycles of ABVD followed by conventionally fractionated RT at 30Gy are widely considered standard of care for early staged unfavorable patients HL (52). In patients ≤60 years who are eligible for a more intensive treatment, this standard is challenged by a protocol consisting of two cycles of BEACOPPescalated followed by two cycles of ABVD and RT at 30Gy. After a median follow-up of 43months, the freedom from treatment failure with this protocol was superior in comparison with four cycles of ABVD followed by RT. However, an advantage in OS could not be shown (59). The question of whether RT is dispensable for early stage unfavorable patients with complete metabolic response at iPET is still unanswered. A large randomized study failed to demonstrate non-inferiority of chemotherapy alone as compared with combined modality treatment in patients with a negative iPET (57). However, as patients treated with chemotherapy alone still have a good overall prognosis, this approach may be offered to individual patients when the late risk of delivering RT is thought to outweigh the short-term benefit of improved disease control.

Early treatment intensification appears to improve the prognosis of patients with a positive iPET. A randomized study including patients with both favorable and unfavorable limited-stage HL revealed a significantly reduced relapse rate in patients with a positive iPET after two cycles of ABVD who completed chemotherapy with two cycles of BEACOPP escalated instead of one or two additional cycles of ABVD before INRT (57). Patients with a positive iPET after two cycles of ABVD should be treated with two cycles of BEACOPPescalated before ISRT. Due to the relevant bleomycin-induced toxicity observed in older individuals receiving more than two cycles of ABVD, bleomycin should be discontinued after the second cycle of chemotherapy in patients >60 years (60).

ADVANCED STAGE DISEASE

These patients with HL are usually treated with chemotherapy alone, while additional RT is confined to patients with residual disease after chemotherapy. Patients ≤60 years are treated with either six cycles ABVD or four/six cycles of BEACOPP escalated, optionally followed by localized RT (61, 62). When ABVD is applied, the omission of bleomycin, i.e. the use of doxorubicin/vinblastine/dacarbazine (AVD) from the 3rd cycle for negative iPET cases should be considered, especially in elderly patients and those at an increased risk for lung toxicity. However, a randomized multicenter study was not able to exclude a PFS difference of >5% at 3 years (63). The question of whether consolidating RT can be safely omitted in patients who have a negative PET after two cycles of ABVD or at the end of chemotherapy has also not yet been definitively answered. However, two randomized trials show no PFS improvement when RT was administrated to patients with both iPET and end-of-therapy PET negative (58, 64). There is no randomized study evaluating the role of early treatment intensification in advanced stage patients who have a positive iPET after two cycles of ABVD. However, several non-randomized studies have suggested that patients with advanced HL who have a positive iPET have a better prognosis after switching from ABVD to intensified protocols, such as BEACOPP escalated, than continuing ABVD (53, 63, 65, 66). A recent randomized study demonstrated an improved modified 2-year PFS after six cycles of brentuximab vedotin in combination with AVD (A-AVD) as compared with standard ABVD. However, A-AVD was associated with an increased rate of neuropathy and hematological toxicity (67). The 2-year follow-up showed a significant advance of A-AVD over ABVD for stage IV patients (67). In patients receiving BEACOPPescalated, treatment can be safely reduced to a total of only 4 cycles in the case of a negative iPET compared with a total of six cycles for PET-positive

patients [19]. In addition, RT can be restricted to the patients with PET-positive residual lymphoma ≥2.5 cm after 4 and 6 cycles of BEACOPP escalated, respectively (63, 68). Several trials randomly comparing ABVD and BEACOPP escalated have shown a superior tumor control and a non-significant trend towards a better OS with BEACOPP escalated (69, 70). A network meta-analysis including 9993 patients also revealed a significantly better OS with BEACOPP escalated when compared with ABVD. The survival benefit was 10% at 5 years (71). However, given the relevant acute toxicity of BEACOPP escalated, appropriate surveillance and supportive care must be available when this protocol is used. In patients>60 years, the BEACOPP regimen should not be given, since an increased rate of acute toxicities and treatment-related mortality has been observed (72). Thus, ABVD-based chemotherapy represents the standard of care for older HL patients who are fit enough for multi-agent therapy. However, due to the relevant bleomycin-induced toxicity observed in older individuals receiving more than two cycles of ABVD, bleomycin should be discontinued after the second chemotherapy cycle in this patient group [12].

RELAPSE DISEASE

For most patients with refractory or relapsed (R/R) HL, the treatment of choice consists of high-dose chemotherapy followed by autologous stem cell transplantation (ASCT) (73). High-risk patients may benefit from tandem ASCT (74). Consolidating treatment with the antibody-drug conjugate brentuximab vedotin, anti-CD30 monoclonal antibody conjugated with the microtubule inhibitor monomethyl auristatin E (MMAE), following high-dose chemotherapy (like BEAM, FEAM or BeEAM poly-chemotherapy) HDCT and ASCT had shown to improve the tumor control in patients presenting with at least one of the following risk factors: primary disease progression, early disease recurrence <12 months after the end of first-line treatment and extranodal disease at the time of relapse (75).

Salvage regimens such as dexamethasone/high-dose cytarabine/cisplatin, ifosfamide/gemcitabine/vinorelbine, ifosfamide/carboplatin/etoposide or bendamustine/ gemcitabine/vinorelbine are given to reduce the tumor burden and mobilize stem cells before ASCT (76, 77). In some patients, single-agent brentuximab vedotin results in a negative PET and may therefore be sufficient as salvage therapy before ASCT (78). Achieving a negative PET should be the goal of salvage therapy, irrespective of the applied protocol, because a complete metabolic response before ASCT was associated with an improved clinical outcome [31]. The role of RT before ASCT is not defined. However, its use may be discussed in patients

with single PET-positive lymph nodes after salvage therapy.

The use of brentuximab vedotin represents an option in patients failing ASCT. The phase II PIVOTAL trial included 102 R/R patients with classical HL who relapsed after ASCT or at least 2 previous lines of therapy. Patients were treated with brentuximab vedotin 1.8 mg/kg by intravenous infusion every 3 weeks. In the absence of disease progression or severe toxicity, patients can receive a maximum of 16 cycles. The primary end point was overall objective response rate (ORR) determined by an independent radiology review. The study demonstrated an ORR of 75% with single-agent brentuximab vedotin and the drug was approved for the treatment of such patients (79). A recent follow-up analysis of the study revealed a 5-year PFS and OS of 22% and 41%, respectively (80) (Figure 10). Patients who achieved a complete response (CR) to brentuximab vedotin (n=34) had estimated OS and PFS rates of 64% and 52%, respectively. The median OS and PFS were not reached in CR patients, with 13 patients (38% of all CR patients) remaining in follow-up and in remission at study closure. Of the 13 patients, 4 received consolidative hematopoietic allogeneic stem cell transplant, and 9 (9% of all enrolled patients) remain in sustained CR without receiving any further anticancer therapy after treatment with brentuximab vedotin (Figure 10).

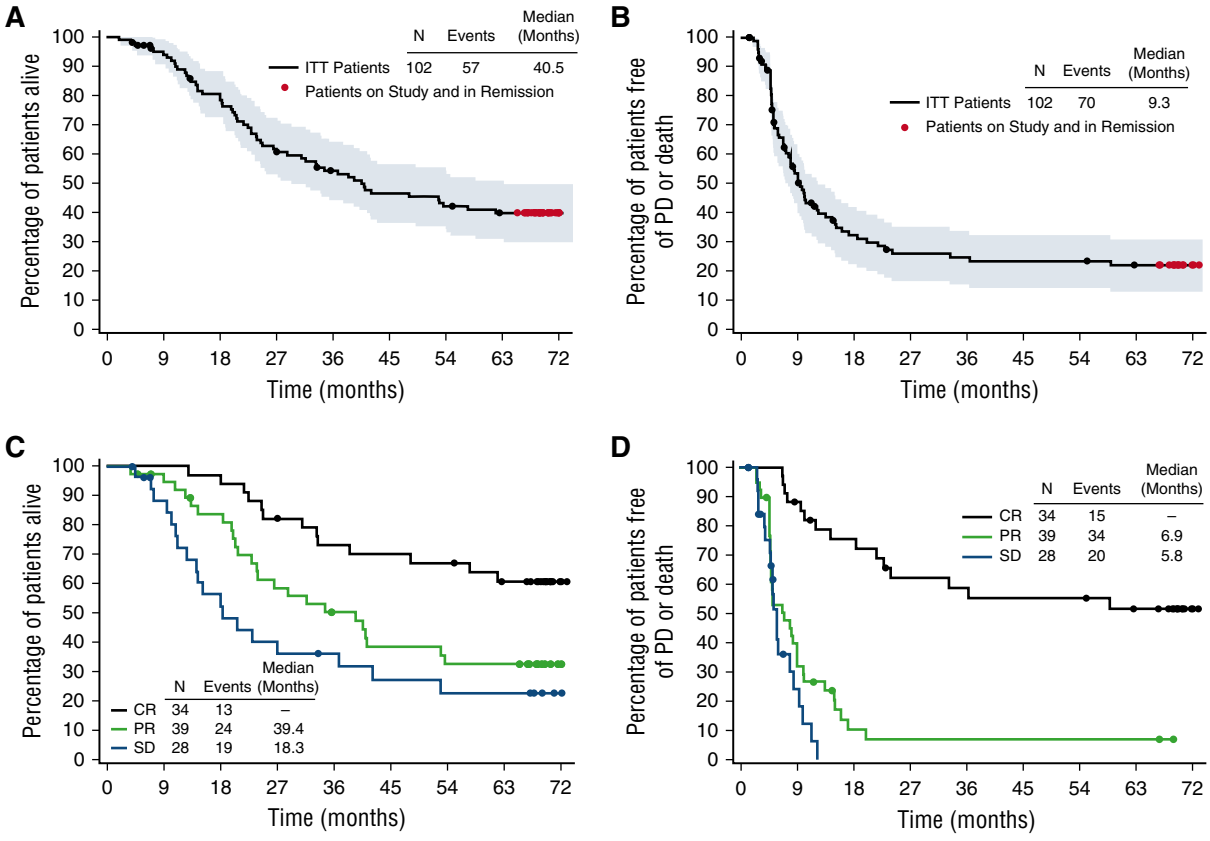


Figure 10. Summary of 5-year follow-up results of Pivotal trial. Overall survival and progression-free survival were analyzed using Kaplan-Meier methodology and are shown (A-B) overall and (C-D) by best treatment response (80).

However, most patients received additional treatment following brentuximab vedotin. The most common treatment-related adverse events were peripheral sensory neuropathy, nausea, fatigue, neutropenia, and diarrhea. Of the patients who experienced treatment-emergent peripheral neuropathy, 88% experienced either resolution (73%) or improvement (14%) in symptoms after treatment discontinuation (79, 80).

Antibodies targeting the programmed cell death protein-1 (PD-1) pathway represent another novel treatment option for patients with multiple relapses. The phase Ib study KEYNOTE-013 tested the safety and efficacy of the anti-PD-1 antibody pembrolizumab in patients with hematologic malignancies (81). The study also enrolled patients with R/R HL whose disease progressed on or after treatment with brentuximab vedotin. Patients received pembrolizumab, 10 mg/kg every 2 weeks, until disease progression. Response to treatment was assessed at week 12 and every 8 weeks thereafter. Principal end points were safety and CR rate. Thirty-one patients were enrolled, 55% received more than four lines of therapy and 71% had relapsed after ASCT. Five patients (16%) experienced grade 3 drug-related adverse events; there were no grade 4 adverse events or deaths related to treatment. The CR rate was 16% and 48% of patients achieved a partial remission, for an overall response rate of 65%. Most of the responses (70%) lasted longer than 6 months, with a median follow-up of 17 months. The PFS rate was 69% and 46% at 6 and 13 months, respectively. These encouraging results led to the development of the phase II KEYNOTE-087 trial (82) to study pembrolizumab in three cohorts of R/R HL patients, defined on the basis of lymphoma progression after 1) (ASCT) and subsequent brentuximab vedotin, 2) salvage chemotherapy and brentuximab vedotin, and thus, ineligible for ASCT because of chemo-resistant disease, and 3) ASCT, but without brentuximab vedotin after transplantation. Patients received pembrolizumab 200 mg once every 3 weeks. Response was assessed every 12 weeks. The primary end points were ORR by central review and safety. A total of 210 patients were enrolled and treated (69 in cohort 1, 81 in cohort 2, and 60 in cohort 3). After a median follow-up of 27.6 months the ORR by independent review was 72% with 28% CR and 44% partial response. By cohort, ORRs and CRs were 77% and 26% for cohort 1, 67% and 26% for cohort 2, and 73% and 32% for cohort 3 (Figure 11). The median duration of response was 16.5 months in all patients, 22 months for cohort 1, 11 months for cohort 2 and 24 months for cohort 3. The median PFS was not reached in all patients with CR, 14 months for patients with partial response, 11 months for patients with stable disease (Figure 11). Median overall survival was not reached in all patients or in any cohort. Treatment-related adverse events of any grade

occurred in 73% patients and grade 3/4 occurred in 12%) patients, most commonly, neutropenia 2% and diarrhea 1%; none resulted in death. Adverse events led to treatment discontinuation in 7% patients.

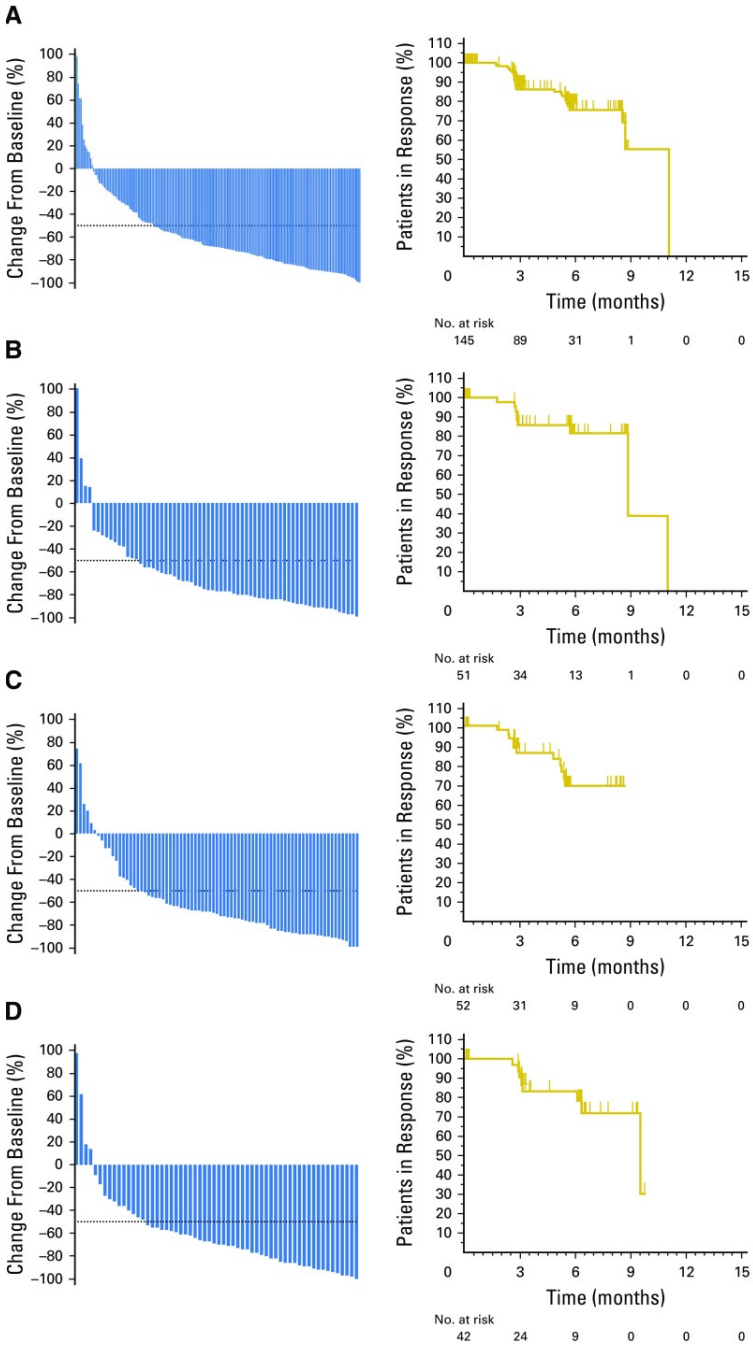


Figure 11. Decrease from baseline in tumor burden (left) and Kaplan-Meier estimates of response duration (right) on the basis of central review in patients with response. (A) All cohorts, (B) cohort 1, (C) cohort 2 and (D) cohort 3.

In a phase 1b study, nivolumab, another PD-1-blocking antibody, produced a high response in patients with R/R HL, with an acceptable safety profile. Researcher assessed the

clinical benefit and safety of nivolumab monotherapy in patients with classical HL after failure of both autologous stem-cell transplantation and brentuximab vedotin, in the phase 2 CHECKMATE-205 study (83). Patients were enrolled into 3 cohorts by treatment history: brentuximab vedotin-naïve (cohort A), brentuximab vedotin received after ASCT (cohort B), and brentuximab vedotin received before and/or after ASCT (cohort C). Patients received nivolumab intravenously over 60 min at 3 mg/kg every 2 weeks until progression, death, unacceptable toxicity, or withdrawal from study. The primary endpoint was objective response following a prespecified minimum follow-up period of 6 months, assessed by an independent radiological review committee. All patients who received at least one dose of nivolumab were included in the primary and safety analyses. In the extended follow-up (83) 243 patients were treated: 63 in cohort A, 80 in cohort B, and 100 in cohort C. After a median follow-up of 18 months, 40% continued to receive treatment. The ORR was 69% overall and ranged from 65% to 73% in each cohort. Overall, the median duration of response was 17 months, and median progression-free survival was 15 months (Figure 12). Of the 70 patients treated past conventional disease progression, 61% of those evaluable had stable or further reduced target tumor burdens. The most common grade 3 to 4 drug-related adverse events were lipase increases (5%), neutropenia (3%), and ALT increases (3%). Twenty-nine deaths occurred; none were considered treatment related. Pembrolizumab and nivolumab were associated with high response rates, acceptable safety profile and long-term benefits across a broad spectrum of patients with relapsed HL, offering a new treatment paradigm for this disease.

In patients with multiple relapses who have no other treatment options, acceptable remission rates, satisfying quality of life and prolonged survival can be achieved with gemcitabine-based palliative chemotherapy and/or regional RT. In general, patients with multiple relapses should be enrolled in clinical trials evaluating other novel agents whenever possible.

Allogeneic stem cell transplantation represents a potentially curative treatment option for patients failing ASCT and/or achieving a CR with targeted agents. This approach should be considered and discussed in young, chemo-sensitive patients in good general condition after careful evaluation of the risk–benefit ratio (84, 85).

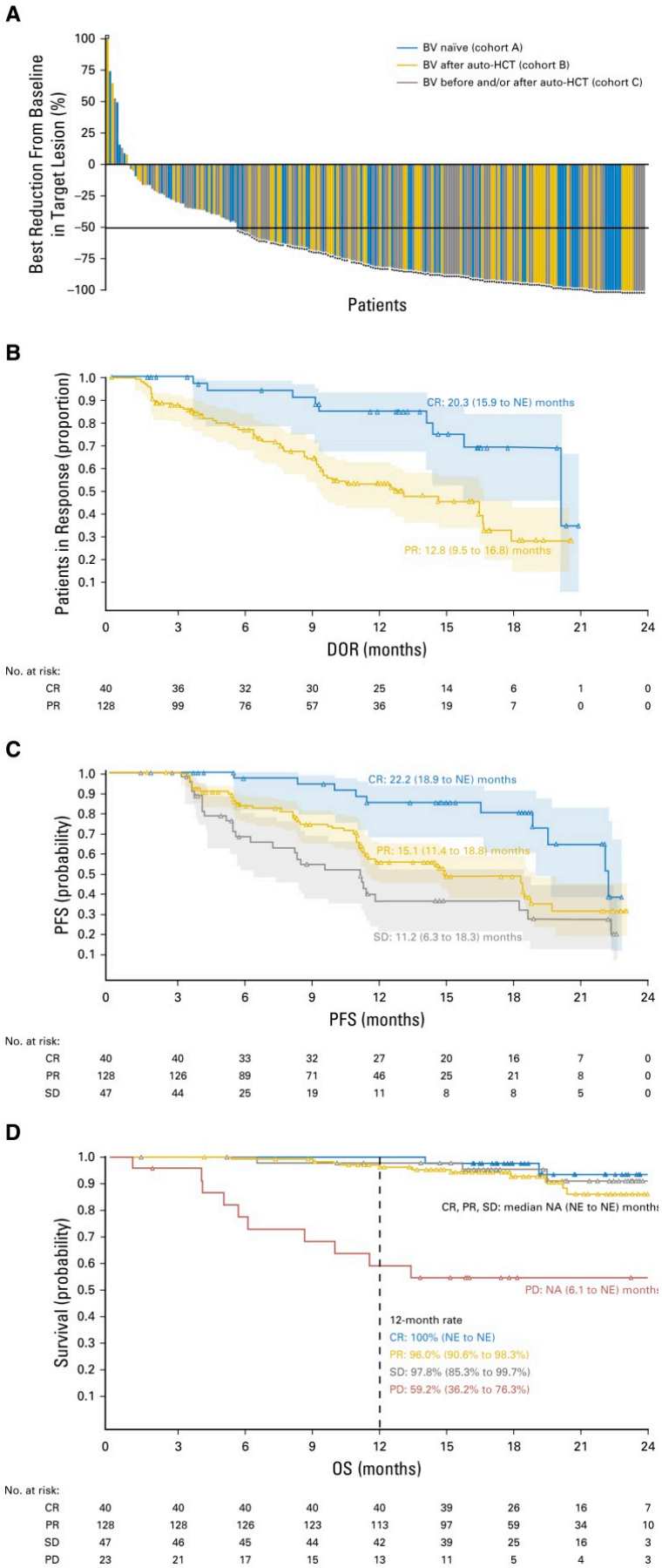


Figure 11. Best change in (A) target lesions, (B) duration of response (DOR), (C) progression-free survival (PFS), and (D) overall survival (OS), according to best overall response (83).

1.4.5 HODGKIN AND REED-STERNBERG CELLS

The HRS cells, shown on Figure 13, are the pathognomonic sign of HL. Morphologically both Hodgkin and Reed-Sternberg cells have a great size (diameter >45μm) and abundant cytoplasm, but the former usually have a polylobate single nucleus while the latter show multiple nuclei (49). From an immuno-phenotypic point of view, HRS cells carry peculiar features since they expressed markers of different hematopoietic cell lines. HRS are likely to undergo reorganization of genetic expression that lead to partial loss of the typical surface markers of B lymphocyte. In fact, HRS express the typical markers of T lymphocytes (CD3, NOTCH1, GATA3), cytotoxic cells (granzyme B and perforin), B lymphocytes (PAX5 and CD20), dendritic cells (CCL17), NK cells (ID2), myeloid cells (CSFR1) and of granulocytes (CD15, about 75-80% of cases). Moreover, all HL express CD30, a TNF receptor, CD40 and the checkpoint inhibitor ligands (PD-L1 and PD-L2).

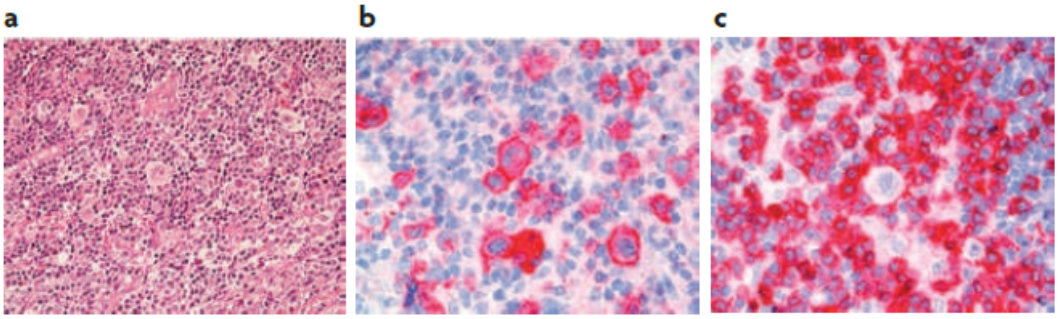


Figure 12. Hodgkin and Reed-Sternberg cells. a) hematoxylin and eosin staining of a case of nodular sclerosis classical Hodgkin lymphoma. **b)** immuno-histochemistry for CD30. **c)** immuno-histochemistry for CD3, showing that normal T lymphocytes surrounding HRS cells. Adapted from Küppers R (86).

The mechanisms that lead to the loss of B-lymphocyte phenotypic markers and to the development of HRS cells are caused by the deregulation of several TFs and genes among crippled germinal center B-cells rescued from apoptosis by EBV infection. For example OCT2, BOB1 and PU-1 induce B-cell genes, EBF1 is a key transcriptional factor of B-cell differentiation, TCF3, PAX5 and NOTCH1 are TFs of T-cell and negative regulators of B-cell differentiation, STAT5A/B and GATA2 are key TFs of hematopoietic stem cells. The deregulation of these proteins leads to a modification of the gene expression profile resulting in only partial expression of the normal immune-phenotype of germinal center B lymphocyte. HRS cells harbor different chromosomal abnormalities and are aneuploid. Some recurrent translocations involve immunoglobulin (IG) loci and other oncogene such as BCL1, BCL2, BCL3, RELA and MYC. The amplification of the chromosomal region 9p24 is a pivotal feature of HL. These abnormalities lead to the overexpression of PD-L1, the ligand of the receptor PD-

1 who is usually expressed on the cell surface of exhausted T lymphocyte, both CD4+ and CD8+ cells. The engagement of PD-L1 with PD-1 triggers the inhibition of cytotoxic reaction by T lymphocytes against neoplastic cells. Other relevant abnormalities have been identified and linked to apoptosis activators and inhibitors as TP53, FAS, caspasis-8, caspasis-10, FADD, BAD, ATM and BCL2. The most common mutations involve JAK/STAT and NF-kB signaling pathways, which are usually activated in cHL. Among the former pathway, gain of function mutations of JAK2 and loss of function mutations SOCS1, a negative regulator of JAK/STAT pathway are commonly found. Regarding the latter pathways mutations of RELA (a member of NF-kB transcriptional factors), BCL3, NFKBIA, NFKBIE, TNFAIP3 (negative regulators of NF-kB signaling) have been recognized. Activation of these two signaling pathways is triggered also by autocrine and/or paracrine signals which lead to the activation of STAT3, STAT5A, STAT5B, STAT6 (86, 87). Other abnormalities have been recognized in the PI3K/AKT and MAPK/ERK pathways, whose inhibition triggers apoptosis and impinges cells proliferation. HRS cells also harbor clonal and somatic mutation within immunoglobulin heavy chain variable region of the B-cell receptor. Usually somatic hypermutations of immunoglobulin genes occur during the process of maturation and activation of B lymphocytes in the germinal centers of lymph nodes. These data further support the hypothesis that HRS derived from mature B lymphocytes (86, 87).

In the NLPHL variant the lympho-histiocytic cells express germinal center B lymphocytes markers like CD20, BCL6 and the enzyme cytidine deaminase which is necessary for the somatic hypermutation and immunoglobulin class switch processes; while CD30 is usually negative. All these features support the notion that Hodgkin lympho-histiocytic cells derive from germinal center B cells (86, 87).

1.4.6 THE MICROENVIRONMENT OF HL

A typical feature of HL is that the neoplastic cells account for almost 1% of all the cells of the tumor bulk, while the remaining cells are normal reactive leukocytes whose proportion depends on the specific HL subtype. Within the microenvironment of the tumor bulk T and B lymphocytes, plasma cells, macrophages, neutrophils, eosinophils and mast cells are found. This complex picture plays a pivotal role in either the initial and the progressive phase of the disease. The presence of reactive leukocytes is fundamental for HRS cells since from one hand HRS cells have the ability to recruit immune cells through the release of chemotactic factors, and on the other hand leukocytes support the survival and the proliferation of neoplastic cells.

Several data reinforce this hypothesis among which, the difficulty of HRS to grow in culture, the short survival of HL cell lines in murine immune-deficiency models, the extreme rarity of HRS in the peripheral blood and a microenvironment enriched of leukocytes even in extra-nodal localization. HRS cells also have to suppress cytotoxic immune cells, such as T lymphocytes, NK cells and tumor-associated macrophages (TAM), that would attack neoplastic cells. These interactions between cytotoxic cells and neoplastic cells involve the so called immune-check points such as PD-1, TIM3, LAG3, OX40. The most studied pathway is the PD-1/PD-L1. In physiological conditions, after binding of PD-1, expressed on T cells, with its cognate ligands PD-L1 or PD-L2, expressed on target cells, T lymphocytes are inhibited. T cells expressed PD-1 after several and continuous activation, the so-called exhausted state. The activation of PD-1/PD-L1 axis and the concomitant inhibition of T cells, allow the end of normal immune responses (Figure 14). In several solid cancers PD-1/PD-L1 axis is aberrantly activated, thus creating an immune-suppressive microenvironment that boosts cancer cells grow. In HL one of the key mechanisms of immune escape is the mediated by PD-1/PD-L1 pathway, expressed by T lymphocytes and HRS, respectively. The activation of this pathway is further supported by the amplification of the 9p24, the locus of PD-L1 and PD-L2. This pathway has become targetable by monoclonal antibodies such as nivolumab and pembrolizumab (anti-PD1 antibodies), or by atezolizumab or durvalumab (anti-PD-L1 antibodies). The clinical application of nivolumab or pembrolizumab proved to be active ad effective in heavily treated HL (88). Several clinical trials evaluating these agents at different clinical time points, either as single agents or in combination of chemotherapy or brentuximab vedotin are ongoing (86-88).

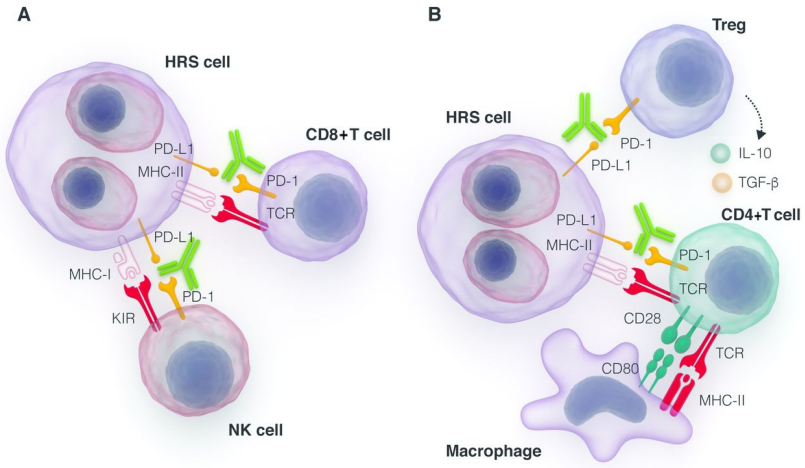


Figure 14. Suppression of anti-tumor T cell responses by the cHL microenvironment. (A) HRS cells and stromal cells secrete cytokines, chemokines, and other soluble immunomodulatory factors which recruit Th2 and regulatory CD4+ T cells and favor the differentiation of tumor-infiltrating T cells into regulatory and Th2 cells. (B) HRS cells evade recognition by CD8+ and CD4+ T cells by downregulating MHC-I and MHC-II and the expression of PD-L1. Vardhana et al (88).

AIM OF THE STUDY

The protein kinase CK2 is a pleiotropic serine/threonine kinase, highly conserved among species and expressed by all kind of eukaryotic cells. During the last decades CK2 has come to attention due to its structure, regulation and peculiar features. In fact, this protein regulates a high number of cell processes, is constitutively active and play a pivotal role in cellular biology, being involved in the regulation of metabolism, DNA-damage response, survival and proliferation. Moreover, it is overexpressed and involved in the pathogenesis of several cancers and hematological malignancies. CK2 is not a classical oncogene, but it is a driver for the development of tumor through "non-oncogene addiction". More recently, CK2 has been studied in hematological malignancies including multiple myeloma, chronic lymphocytic leukemia, acute leukemias and mantle cell lymphoma. However, its role and involvement in classical Hodgkin lymphoma pathogenesis has never been studied.

The aims of this PhD program were to dissect the role of CK2 in HL through the assessment of:

- expression levels of CK2 protein subunits, i.e. the catalytic (CK2α) and the non-catalytic/regulatory (CK2β), and their subcellular localization in models of HL;
- expression levels of CK2 genes, i.e. *CSNK2A1* and *CSNK2B* in models of HL;
- expression levels of CK2α and CK2β in primary lymph nodes from patients with HL;
- The effects of the CK2 inhibitor CX-4945/Silmitasertib on HL cell survival and STAT3, NF-kB and AKT signaling;
- The modulation of surface markers such as CD20, CD30, PD-L1 (CD274) and PD-L2 (CD273) after CK2 inhibition;
- The combined *in vitro* treatment of HL cells lines with CX-4945/Silmitasertib and monomethyl auristatin E (MMAE).

3 MATERIALS AND METHODS

3.1 CELL CULTURE

In this thesis, experiments were performed in 4 HL cell lines, namely L-428, L-540, HDLM-2 and KM-H2 kindly provided from Prof. Carlo-Stella of Humanitas university, Rozzano-Milan, Italy. L-428, L-540 and HDML-2 derived from patients with nodular sclerosis HL, while KM-H2 from a patient with mixed cellularity subtype. The expression of immune-phenotypic markers and clinical characteristics of these HL cell lines are summarized in Table 2 and 3, respectively. All cell lines were EBV negative and displayed the typical surface markers of HL such as CD30 and CD15, but lacked of CD19 and CD20. As positive controls, we used Kasumi-1 cells, an M2 acute myeloid leukemia cell line (Liebniz Institute Germane Collection of Microorganism and Cell Cultures DSMZ, Germania), and leukemic B cells from a CLL patients; as negative control we used age-matched normal B lymphocytes derived from buffy coats. Cells were maintained in flasks, according to manufactures instruction, in controlled atmosphere at 5% CO2, with appropriate medium. L-540, HDML-2 and Kasumi-1 were cultivated with 80% RMPI 1640 (Euroclone, Milan; Italy) and 20% (FBS (*Fetal Bovine Serum*, Euroclone); L-428 and KM-H2 with 90% RPMI 1640 and 10% FBS. five percent antibiotics, penicillin and streptomycin (Euroclone) were added to medium. Medium was changed twice for week and cells were spited when necessary.

Table 2. Immune-phenotypic characterization of HL cell lines.

	CD3	***CD4***	***CD14***	***CD15***	***CD19***	***CD25***	***CD30***	***HLA-DR***
L-428	-	-	-	+	-	-	+	-
L-540	-	-	-	+	-	+	+	-
HDLM-2	-	+	-	+	-	+	+	+
KM-H2	-	-	+	+	-	-	+	+

Table 3. Clinical characteristics of HL cell lines.

	L-428	L-540	HDLM-2	KM-H2
HL subtype	nodular sclerosis	nodular sclerosis	nodular sclerosis	mixed cellularity
AGE	37 years	20 years	74 years	37 years
ORIGIN	pleural effusion	bone marrow	pleural effusion	pleural effusion

3.2 TREATMENT WITH INHIBITORS

$2x10^6$ cells of each HL cell line were resuspended in 1ml of the appropriate medium and plated in 24-well plates. Cells were incubated for 24h, 48h and 72h medium only (alone), or with the CK2 specific inhibitor, CX-4945 (Silmitasertib; Selleck Chemicals; Munich; Germany) at 5, 10 and 15μM concentration or with MMAE (monomethyl auristatin E; Selleck Chemical) at 5nM.

3.3 EVALUATION OF PROTEIN EXPRESSION

The evaluation of protein expression levels among HL cell lines was performed using electrophoretic run on acrylamide gel electrophoresis in 10% SDS-PAGE (Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis) and subsequent western blotting.

3.3.1 Preparation of cell lysates

Cells (5×10^5 for each assay) were prepared by cell lysis with :

- Tris(hydroxymethyl)aminomethane-hydrochloride (TRIS-HCl) pH 6.8 20mM;
- soidum cloride (NaCl) 150mM;
- Ethylenediaminetetraacetic acid (EDTA) 2mM;
- ethylene glycol tetraacetic acid (EGTA) 2mM;
- 0.5% Triton X-100;
- complete protease inhibitor cocktail (Roche; Mannheim, Germany);
- sodium orthovanadate 1mM (Calbiochem; Gibbstown, NJ).

To use these lysates for SDS-PAGE analysis, the following substances were added:

- 10% glycerol;
- 2% sodium dodecyl sulphate (SDS);
- 1% β-mercaptoethanol;
- Dye (Pyronin, Sigma-Aldrich; Milan, IT).

Subsequently, the lysates were vortexed, boiled at 100°C for 5 minutes.

3.3.2 Polyacrylamide gel electrophoresis in SDS-PAGE

The polyacrylamide gel electrophoresis in SDS is one of the methods used to separate a mixture of proteins on the basis of their molecular weight. SDS is a ionic detergent that binds tightly to proteins causing their denaturation. In the presence of an excess of SDS,

approximately 1.4g of detergent will bind to each gram of protein, providing a constant amount of negative charge per unit mass. Therefore, during electrophoresis, all protein-SDS complexes move toward the anode, and thanks to the molecular sieve properties of the gel, their mobility is inversely proportional to their molecular weight. By the migration of standard proteins of known molecular weight simultaneously to samples, it is possible to determine the protein sample weights. SDS polyacrylamide gel is prepared following Laemmli method. The electrophoretic plate consists of two types of gel:

- Stacking gel (Tris-HCl 0.5M at pH 6.8), which allows concentrating the protein samples so that they are all aligned at the start of electrophoresis.
- Running gel (Tris-HCl 1.5M at pH 8.8), in which the real separation of proteins occurs.

The plate size of 10×8cm is fixed in the Hoefer Mighty Small-If 250 Scientific Instruments machine (Amersham Biosciences). The electrophoresis was run for about 2 hours at 25mA.

3.3.3 Western blotting

The western blotting (WB) or immunoblotting is an immunoassay able to detect traces of a specific protein in a heterogeneous mixture, combining the high resolving power of gel electrophoresis with the specificity of the antibodies. The WB is a technique with high sensitivity, able to detect quantities of protein in the order of nanograms. After SDS-PAGE, proteins are transferred onto a nitrocellulose membrane by the action of an electric field, obtained by applying the appropriate current of 350mA for 2 hours and 30 minutes. The buffer used for the transfer consists of: 25mM Tris, 192mM glycine, 20% methanol and 0.1% SDS with a final pH of 8.0. After the transfer, the membrane is left overnight in the saturation buffer consisting of 50mM Tris-HCl, pH 7.5, 150mM NaCl and 5% bovine serum albumin (BSA), for nonspecific sites saturation. Follows the incubation for 2 hours and 30 min at room temperature of the primary Ab, diluted in "dilution buffer" (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% BSA).

For our study, we used the following antibodies: anti-AKT (Cell Signaling), anti-AKT-Ser129 (Cell Signaling), anti-AKT-Ser473 (Cell Signaling), anti-CK2β (Santa Cruz), anti-CK2α (kindly provided by Prof. Maria Ruzzene, University of Padova), anti-NF-κB (Abcam; Cambridge; United Kingdom), anti-NF-κB-Ser529 (Cell Signaling), anti-PARP (Cell Signaling; Danvers; Massachusetts; United States), anti-PD-L1 (Cell Signaling), anti-STAT3 (Cell Signaling), anti-STAT3-Ser727 (Cell Signaling), anti-β-actin (Santa Cruz Biotechnologies; Dallas; Texas; United States), anti-α-tubulin (Sigma Aldrich; Saint Louis;

Missouri; United States). Three washes of 10 min, each at room temperature, were subsequently performed using "washing buffer" (1M Tris-HCl, 3M NaCl, 0.1% Tween at pH 7.5). Membranes were then incubated for 30 minutes with a secondary anti-IgG Ab, obtained against the animal species immunized for the primary Ab. The secondary Ab is conjugated with horseradish peroxidase (Amersham International Biotechnology; Buckingamshire, UK) and diluted in "dilution buffer". After three additional washes with "washing buffer", the membrane was subjected to the detection antibody with the enhanced ChemiLuminescence system (Pierce; Rockford, Illinois): the membrane is incubated for 1 min with 1ml of luminol and 1ml of H_2O_2, which in contact with the peroxidase and, as a result, with the Ag-Ab complex, giving rise to an oxidation reaction with light emission. The membrane was finally revealed into the *Imager 600UV* (GE Healthcare Bio-Sciences) and the bands were quantified by densitometry, using the program *software ImageQuant TL* (GE Healthcare Bio-Sciences), supplied with the instrument.

3.3.4 Subcellular Fractionation

The subcellular fractionation is a technique that allows the study of proteins in different cellular compartments. The obtained fractions are defined "enrichment" of the following components: cytoplasmic/soluble; membrane and microsomal; nuclear. In this thesis, we used a commercial kit (Thermo Scientific, Rockford, IL, USA) supplied with detergents to separate cytoplasmic, membrane and nuclear proteins. Cells were centrifuged and incubated with different buffers following manufacture's protocol. The buffers were: Cytoplasmic Extraction Buffer, Membrane Extraction Buffer and the Nuclear Extraction Buffer with the addition of phosphatase and protease inhibitors 100x (Figure 14). Cellular compartments subsequently underwent SDS-PAGE and western blotting.

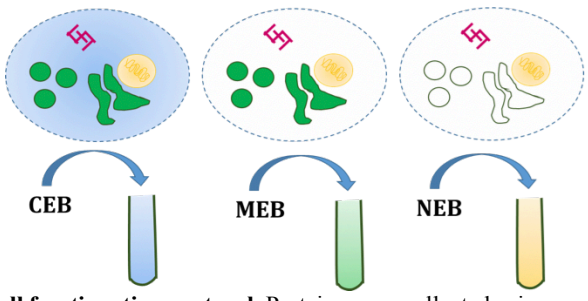


Figure 14. Representation of cell fractionation protocol. Proteins were collected using several buffers. Cytoplasmic Extraction Buffer (CEB), Membrane Extraction Buffer (MEB) and the Nuclear Extraction Buffer (NEB).

3.4 FLOW CYTOMETRY

This technique allows a multiparametric evaluation of antigenic characteristic of the single cells by the analysis of visible and fluorescent light they emit when flow through a liquid medium. The fluorochromes used in this thesis were fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC-H7) and PE-Cy7 which emits a fluorescence signal at 530nm, 585nm, 765nm and 785nm when excited by a laser, respectively. Aliquots of 500,000 HL cell lines were harvested, washed, and incubated for 30 min in the dark and at 4°C with the following antibodies: CD20-APC-H7, CD30-PE (Becton Dickinson; Franklin Lakes, NJ, USA), CD273/PD-L2-FITC (Miltenyi Biotech; Bergisch Gladbach, Germany), CD274/PD-L1-PE-Cy7, CD279/PD-1-PE-Cy7 (Fisher Scientific; Hampton, NH, USA). For each sample, 20,000 events were acquired and analyzed by flow cytometry using the FACSCanto II™ A cytometer and data were processed using the DIVA Software (Becton Dickinson).

3.4.1 Evaluation of apoptosis with Annexin V/Propidium Iodide test

Apoptosis of different cell samples (pathological cells from different HL cell lines, normal B lymphocytes) was assessed using the Annexin V Apoptosis Detection Kit (Valtet Occhiella, Turin, Italy). During the early stages of apoptosis, the plasma membrane undergoes profound changes that indicate the status of apoptotic cells to macrophages, which ensure cell elimination. Phosphatidylserine (PS), a negatively charged aminophospholipid normally expressed only on the inner side of the plasma membrane, which become exposed on the outer surface during apoptosis. The Annexin V is a protein that, in the presence of high concentrations of Ca2+, recognizes and binds selectively the PS, making it useful for the identification of apoptotic cells. Aliquots of $250x10^3$ cells were harvested, washed, and incubated for 10min in the dark and at room temperature with: 100µl of binding buffer, a Ca^{2+}-rich solution that optimizes the binding of Annexin V to the PS, 5µl of Annexin V (FITC), and 5µl of Propidium Iodide (PI) (PE) provided by the kit (1µl/ml final concentration). After the incubation, 100µl of binding buffer were added and cells were analyzed. For each sample 20.000 events were acquired and the number of alive cells was expressed as percentage of Annexin V negative/PI negative cells in the total cells analyzed.

3.5 CONFOCAL MICROSCOPY ANALYSIS

Aliquots of the different cell samples (pathological cells from different HL cell lines and normal B lymphocytes from healthy subjects) were collected, washed and plated on poly-

L-lysine coated slides for 15min at room temperature. Cells were then fixed in 4% paraformaldehyde for 10min, washed twice with PBS1x and permeabilized with 0,1% Triton X-100 (Sigma-Aldrich) for 4min. Before staining, non-specific protein binding were blocked by incubating the slides for at least 30 min in 2% BSA. Cells were then stained with antibodies against CK2α and CK2β (the same used for WB) and DAPI for nuclear staining, washed three times with PBS1x and incubated with anti-mouse-Alexa488 secondary antibody for 30 minutes in the dark. Slides were mounted with cover slips and fluorescence was detected using the UltraView LCI confocal system (Perkin Elmer; Waltham, MA, USA) equipped with a fluorescence filter set for excitation at 488 and 594nm.

3.6 EVALUATION OF mRNA LEVELS

3.6.1 RNA PURIFICATION

RNA was purified using RNeasy mini kit (Qiagen). This procedure represents a well-established technology that combines the selective binding properties of a silica-based membrane with the speed of microspin technology. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and sample is then added to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. RNA is then eluted in water. The procedure provides enrichment for mRNA since most RNAs <200 nucleotides are excluded.

Briefly, cells were collected and washed, removing the medium; then the appropriate volume of RLT lysis buffer, that contains guanidine-thiocyanate, was added (350 µl for $<5x10^6$ cells, 600µl for $5\text{-}9x10^6$ cells). RLT was supplemented with β-mercaptoethanol 1:100 v/v, which inhibits RNases further. Samples were homogenized by vortexing and then 70% ethanol was added. After pipetting, lysed samples were transferred to RNeasy spin columns and centrifuged at 11,000rpm for 15sec, discarding the flow-through. RNA bound to the silica membrane was washed with buffer RW1 and centrifuged at 10,000rpm for 15sec; a mix of DNase and buffer RDD (10µl and 70µl respectively) were added directly on the membrane and kept in incubation for 15-30sec, in order to remove contaminant DNA. Afterwards a series of washes were performed, first of all with 350µl of buffer RW1 at 11,000rpm for 15sec and then with 500µl of buffer RPE (containing ethanol) at 10,000rpm for 15sec. Samples were centrifuged at 12,800rpm for 2min, ensuring that the membrane was dry. At the end RNA was

eluted using 30µl of RNase free water at 11,000rpm for 30sec. Finally, RNA was quantified by means of Nanodrop 1000 (Thermo Scientific).

3.6.2 REVERSE TRANSCRIPTION

Reverse transcription is a reaction exploited by a RNA-dependent polymerase capable of synthesizing a complementary strand of DNA, called cDNA, using a RNA strand as template. RNA was retro-transcribed to cDNA by means of Reverse Transcription System (Promega, USA). AMV, namely avian myeloblastoma virus, is the reverse transcriptase enzyme used, which synthesizes single stranded cDNA from isolated mRNA; it shows polymerase activity from 5' to 3', and RnaseH activity from 3' to 5', degrading the RNA strand when the hybrid cDNA/RNA is formed. The reaction was done in a final volume of 20µl:

- $MgCl_2$ (25mM) 4µL
- reverse transcription 10X buffer 2µL
- dNTPs mix (10mM) 2µL
- Oligo dT primer (0.5mg/ml) 1µL
- RNasin RNase inhibitor 0.5µL
- AMV Reverse Transcriptase 0.6µL
- RNase free H_20 to final volume

Then samples underwent the following thermal protocol:

- 42°C for 15'
- 95°C for 5'
- 4°C maintenance

3.6.3 REAL-TIME PCR

The real-time PCR is a method for gene quantification characterized by high sensibility and specificity. It is called "real-time PCR" because it allows the scientist to observe in "real time" the increase in the amount of DNA as it is amplified. This is possible because the real-time PCR system combines a thermal cycler and an optical reaction module that detects and quantifies fluorophores. Molecules added to the PCR mix, as SYBR Green, bind the amplified DNA and emit a signal that increases in proportion to the rise of the amplified DNA products. An amplification curve is obtained where cycle numbers are found in abscissa and the fluorescence normalized on internal fluorophore in ordinate. At the beginning of the reaction there are only little changes in fluorescence and this is the baseline region; the increasing in

fluorescence above this threshold underlines amplified product formation. From this point on, the reaction maintains an exponential course that degenerates in plateau at the end of the reaction. In the intermediate cycles the curve has a linear course: this is the most important phase since the amount of amplified DNA is correlated with the amount of cDNA expressed in the initial sample. In this linear region a threshold of fluorescence is chosen and from this value it is possible to obtain the Ct (threshold cycle), namely the cycle that are necessary, for the sample, to reach that threshold of emission. If the amount of cDNA present at the beginning in the sample is high, the curve will rise earlier and Ct values will be smaller. As detector dye we used SYBR Green that emits low fluorescence if present in solution; on the contrary the signal becomes stronger if the dye binds to double strand DNA. However SYBR Green is not a selective dye and binds to all DNA, even to primer dimers. For this reason, it is recommended the introduction of a further step after amplification, called dissociation protocol. During this step, temperature rises gradually until all the double strands are de-naturated. This method allows the identification of contaminants or unspecific amplification products since they show different melting points. There is also a dye called ROX that works as an internal reference used by the instrument to normalize the SYBR Green fluorescence.

For the evaluation of gene expression we chose a relative quantification method, using the ΔΔCt formula:

1. ΔCt = Ct (target gene) - Ct (reference gene)
2. ΔΔCt = ΔCt (of treated sample) - ΔCt (of untreated sample, the internal calibrator)
3. 2^-ΔΔC. The “2” value represents the higher efficiency for reaction that means a doubling of the product at every cycle of amplification.

The thermal cycler used was the Sequence Detection System 7000 (Applied Biosystem) and the software was ABI PRISM 7000.

The reagents of the reaction mix were:

- Roche FastStart Universal SYBR Green Master (ROX) 7.5µl
- Forward primer (4pmol/µl) 1µl
- Reverse primer (4pmol/µl) 1µl
- H2O 4.5µl
- cDNA 1µl

FastStart Universal SYBR Green Master (ROX) contains all reagents (except primers and template) needed for running the Real-time PCR. FastStart Taq DNA Polymerase is a hot start polymerase with the following amplification protocol:

- UDG activation 50°C 2'
- Polymerase activation 95°C 10'
- Denaturation 95°C 15''
- Annealing and amplification 60°C 1'

The last three processes were repeated for 40 cycles. Dissociation protocol: increasing temperature from 60°C to 95°C.

In the table below are reported the sequences of the primers used for the Real-Time PCR.

Table 4. Primers for quantitative real time-PCR. β-actin was used to normalize the reaction. The sequences were found using Primer Express program (Applied Biosystem).

GENES	PRIMERS	SEQUENCES (5'-3')
CSNK2A1	forward	TCATGAGCACAGAAAGCTACGA
CSNK2A1	reverse	AATGGCTCCTTCCGAAAGATC
CSNK2B	forward	CCCATTGGCCTTTCAGACAT
CSNK2B	reverse	CCGTGTGATGGTGTCTTGATC
β-ACTIN	forward	CCAGCTCACCATGGATGATG
β-ACTIN	reverse	ATGCCGGAGCCGTTGTC

3.7 TISSUE MICROARRAY

Tissue microarrays (TMAs) were prepared from cases with adequate diagnostic material, as previously described (89). In details, tumor areas enriched in HRS cells were selected and 3 tissue cores (diameter=1 mm) were obtained from each donor block. Appropriate positive and negative controls were also included. The TMA was prepared by using the Galileo TMA CK3500 arrayer (Integrated System Engineering; Milan – Italy). Cases not assessable by TMA (i.e. small tissue samples; TMA cores lacking consistent numbers of HRS cells) were analyzed on whole tissue sections. Immunohistochemical staining for CK2α (EP1963Y, Epitomics, CA, USA) and CK2β (PA5-27416, ThermoFisher, Massachusetts, USA) were performed in duplicate. Antigen retrieval was performed with heat/ethylenediamine tetraacetic acid (EDTA) in an automated immunostainer (University Hospital of Padova). All cases with discordant immunohistochemical results were assessed in joint sessions at the microscope by two hemato-pathologists. The positivity for CK2α and CK2β were graded as: 0 = negative; 1 = positive <30% of HRS; 2 = positive 30-60% HRS or week-moderate intensity; 3 = positive >60% HRS or strong diffuse intensity. Specimens derived for 25 unselected HL patients, 15

(60%) were nodular sclerosis and 9 (35%) mixed cellularity and 1 (5%) lymphocyte depletion histotype. The median age at diagnosis was 35±16 years. Nine patients had an early stage (I-IIA) and 16 had an advanced stage disease (IIB-IV). Twenty-four out of 25 patients were treated with ABVD-based treatment with or without involved field radiotherapy, the remaining with VEPEMB. Interim PET, defined as DS>3, was positive in 4 cases. After a median follow-up of 48 months 9 (36%) patients relapsed and 4 (16%) died.

Table 5. Patients' characteristics.

Patients	Gender	date of born	date diagnosis	Age	Subtype	stage	therapy	iPET	Relapse	data relapse	ASCT	AlloSCT	Death	Follow-up
UPN-1	1	04/01/90	17/05/16	26,37	SN	2B	ABVDx6+RT	neg	yes	23/03/17	yes	no	no	29/08/19
UPN-2	0	05/12/95	13/10/14	18,86	SN	2A	ABVDx4+RT	neg	no		no	no	no	17/06/19
UPN-3	1	16/03/86	19/11/15	29,68	CM	4B	ABVDx6	neg	yes	18/01/17	no	no	no	27/08/19
UPN-4	1	06/11/50	28/03/11	60,39	CM	4B	ABVDx6+RT	neg	yes	04/07/14	yes	no	yes	27/07/18
UPN-5	0	03/10/81	14/04/16	34,53	CM	4B	ABVDx6	neg	yes	12/12/16	yes	no	no	30/05/19
UPN-6	1	09/02/48	10/08/16	68,50	CM	3B	AVDx6	pos	yes	15/02/17	no	no	yes	26/03/19
UPN-7	0	26/07/68	28/07/14	46,01	CM	2B	ABVDx6	neg	no		no	no	no	26/03/19
UPN-8	0	25/02/96	14/07/14	18,39	SN	2B	ABVDx6	pos	yes	20/03/15	yes	no	no	29/04/19
UPN-9	0	18/03/85	21/02/13	27,93	CM	2A	ABVDx4+RT	neg	no		no	no	no	01/07/19
UPN-10	1	24/02/67	19/06/14	47,32	CM	1A	2ABVD+RT	neg	yes	18/03/19	no	no	no	30/05/19
UPN-11	1	26/09/75	15/10/13	38,05	SN	4B	ABVDx6	neg	yes	05/01/15	yes	yes	yes	18/03/19
UPN-12	0	18/06/86	27/05/14	27,94	SN	2A	ABVDx4+RT	neg	yes	04/11/14	yes	yes	no	30/08/19
UPN-13	1	02/01/99	10/06/17	18,44	SN	2B	ABVDx6+RT	neg	no		no	no	no	19/12/18
UPN-14	0	10/01/83	15/05/13	30,35	SN	2A	ABVDx4+RT	neg	no		no	no	no	01/10/18
UPN-15	1	21/06/85	15/01/15	29,57	SN	2B	ABVDx6+RT	neg	no		no	no	no	16/07/19
UPN-16	1	21/06/94	26/03/12	17,76	SN	2A	ABVDx4+RT	neg	no		no	no	no	25/02/19
UPN-17	1	26/09/75	15/05/17	41,64	SN	2A	ABVDx4+RT	neg	no		no	no	no	17/07/19
UPN-18	1	18/09/41	30/06/17	75,78	SN	3A	VEPEMB	neg	no		no	no	no	30/07/19
UPN-19	1	19/01/92	21/11/17	25,84	SN	4B	ABVDx6	neg	yes	06/08/19	no	no	no	02/09/19
UPN-20	1	29/12/96	16/11/17	20,88	SN	2A	ABVDx4+RT	neg	no		no	no	no	21/05/19
UPN-21	0	23/02/91	28/06/17	26,35	SN	2A	ABVDx4+RT	neg	no		no	no	no	15/07/19
UPN-22	1	01/07/89	16/08/09	20,13	SN	4A	ABVDx2+BEACOPPesc+bl	pos	yes	15/12/15	yes	no	no	28/06/19
UPN-23	1	27/04/89	25/01/19	29,74	CM	4A	ABVDx6	neg	no		no	no	no	30/05/19
UPN-24	1	08/09/46	20/01/15	68,37	DL	4B	ABVDx6	neg	no		no	no	no	29/08/19
UPN-25	1	20/10/92	20/04/17	24,50	CM	4B	ABVDx2+BEACOPPesc+bl	pos	yes	17/12/18	no	no	no	30/07/19

3.8 STATISTICAL ANALYSIS

Statistical analysis was performed using Mann-Whitney test, Fisher's exact test, Wilcoxon matched pairs test, Kruskal-Wallis test. Data are reported as mean ± standard deviation (SD) and were considered statistically significant when p values were <0.05 or less.

4 RESULTS

4.1 CK2 SUBUNITS ARE UNBALANCED IN HL

We assessed CK2 expression in HL cell lines. By WB, we found that all the four HL-derived cell lines (L-428, L-540, KM-H2 and HDLM-2) expressed the catalytic subunit of CK2, i.e. CK2α, at much higher levels than normal B lymphocytes (p=0.0044). Conversely, the non-catalytic subunit CK2β was expressed at significantly lower levels as compared to normal B lymphocytes (p=0.0040) (Figure 15A). The median densitometry of CK2α/β-actin from 3 independent experiments for each cell lines was 0.46±0.05, 0.51±0.10, 0.86±0.05, 0.89±0.05 and 0.26±0.09 for L-428, HDLM-2, L-540, KM-H2 and B-cell from heathy donors (Figure 15B). The median CK2α/β-actin ratio in HL cell lines was 2.7-fold higher than B lymphocytes (p<0.0001). The median densitometry of CK2β/β-actin from 3 independent experiments for each cell lines was 0.07±0.03, 0.20±0.02, 0.31±0.10, 0.47±0.10 and 0.98±0.09 for L-428, HDLM-2, L-540, KM-H2 and B-cell from heathy donors (Figure 15C). The median CK2β/β-actin ratio in HL cell lines was 3.8-fold fewer than B lymphocytes (p<0.0001) (Figure 15C). These observations were also confirmed by immunofluorescence (Figure 15D) showing a diffuse strong intensity of CK2α, while CK2β protein was present at lover intensity in HL cell lines. Moreover, CK2α seems to be localized both in the nucleus and the cytosol of HRS cells. To confirmed these data, we studied subcellular protein fractions founding CK2α both the nucleus and cytosolic compartments of HL cell lines, while CK2β was mainly a cytosolic protein (Figure 15E).

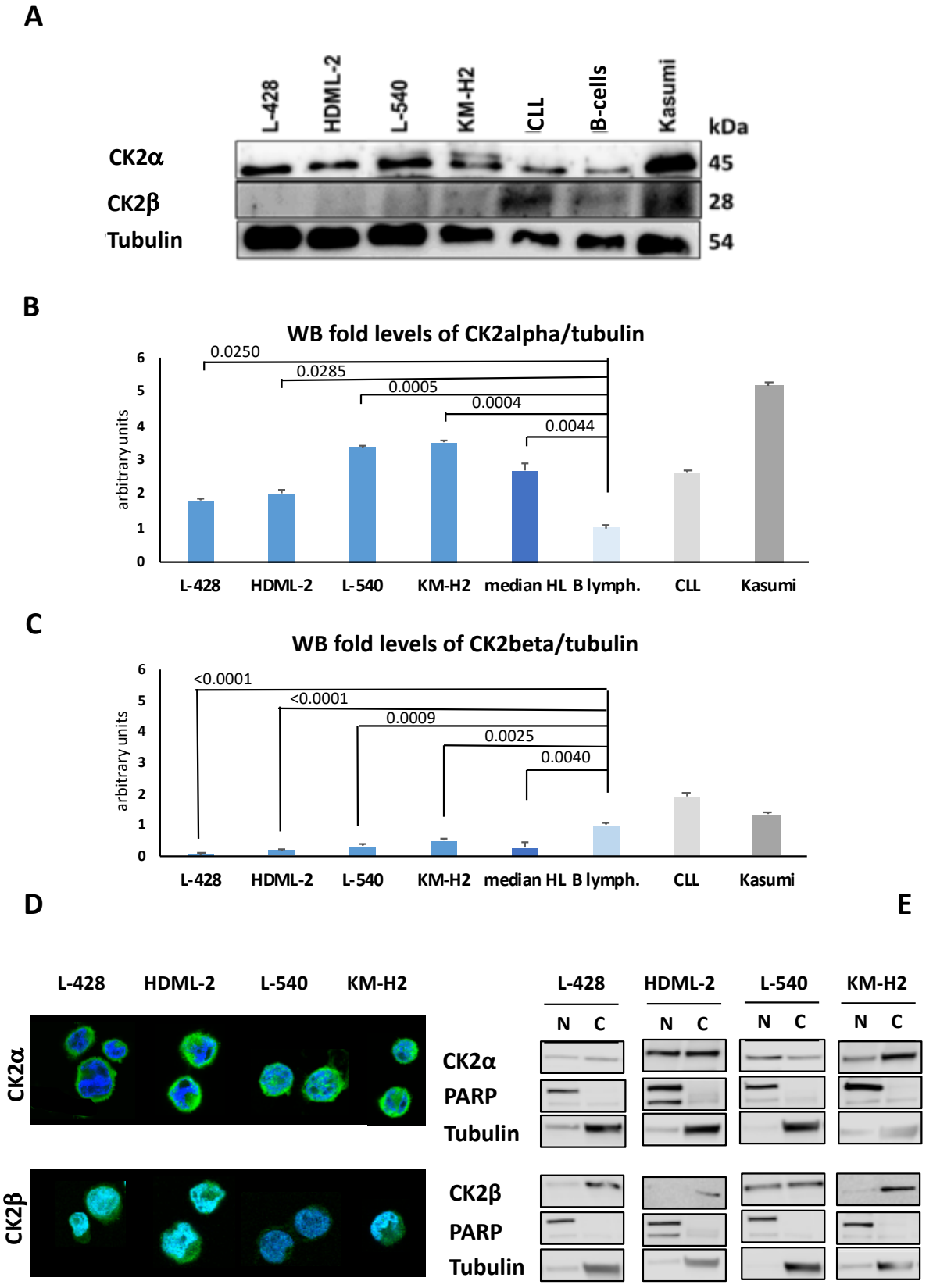
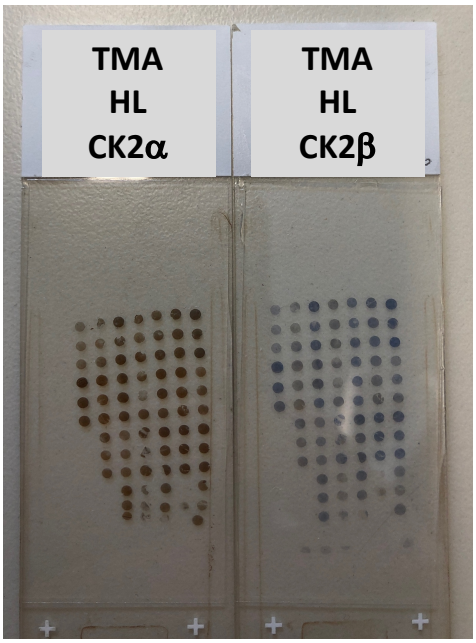


Figure 15. Representative cases of expression of CK2 alpha (α) and beta (β) subunits by western blotting analysis (A), proteins densitometry (B-C), confocal microscopy (D) and nuclear and cytosolic fractions (E).

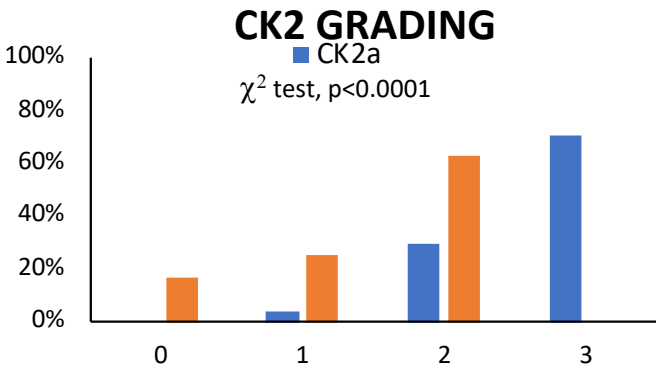
4.2 CK2 SUBUNITS ARE SKEWED IN PATIENTS WITH HL

We performed a tissue microarray of neoplastic lymph nodes derived 30 patients with HL and reactive adenopathy, to evaluated the expression and localization of CK2 subunits in HL patients (Table 5 and Figure 16A). We observed the 71% of patients strongly expressed CK2α in HRS cells (i.e. grade 3), while 29% were at grade 2 and 4% at grade 1 but no one was grade 0 (Figure 16B). Conversely, no patient expressed CK2β at grade 3, 67% of HL patients was grade 2, 21% grade 1 and 17% grade zero (Figure 16B). This skewed expressed was statistically different ($p<0.0001$). Moreover CK2α was expressed both in the nucleus and the cytosol of all HRS, while CKβ was found in the cytosol in 96% of cases but in the nucleus only in 1 patient ($p<0.0001$) (Figure 16C). We also compared the clinical characteristics of our HL patients (age, gender, iPET, relapse, PFS and OS) with the grading of CK2 subunits expression, but we did not find any correlation.

A



B



C

	CK2a	Ck2β
Nuclear	0%	4%
Cytosolic	0%	96%
Nuclear & Cytosolic	100%	0%

Figure 16. Picture of tissue microarray (A), expression of CK2 subunits according to our grading and description of their cellular localization (C).

4.3 COMPARISON OF CK2 mRNA LEVELS

To study whether the unbalance between CK2 subunits were associated with skewed levels of CK2 mRNA, by RT-PCR we analyzed the mRNA of CSNK2A and CSNK2B genes. The median mRNA levels of CSNK2A1/β-actin from 3 independent experiments were 2.76±0.15, 1.73±0.10, 2.45±0.10, 1.52±0.05 and 2.64±1.12 for L-428, HDLM-2, L-540, KM-H2 and B-cell from heathy donor (p value not significant, Figure 17A). The median mRNA levels of CSNK2B/β-actin from 3 independent experiments were 1.06±0.10, 2.19±0.10, 1.32±0.05, 2.06±0.15 and 1.94±1.17 for L-428, HDLM-2, L-540, KM-H2 and B-cell from heathy donor (p value not significant, Figure 17B). Median mRNAs between HL cells lines and normal B lymphocytes were not statistically different.

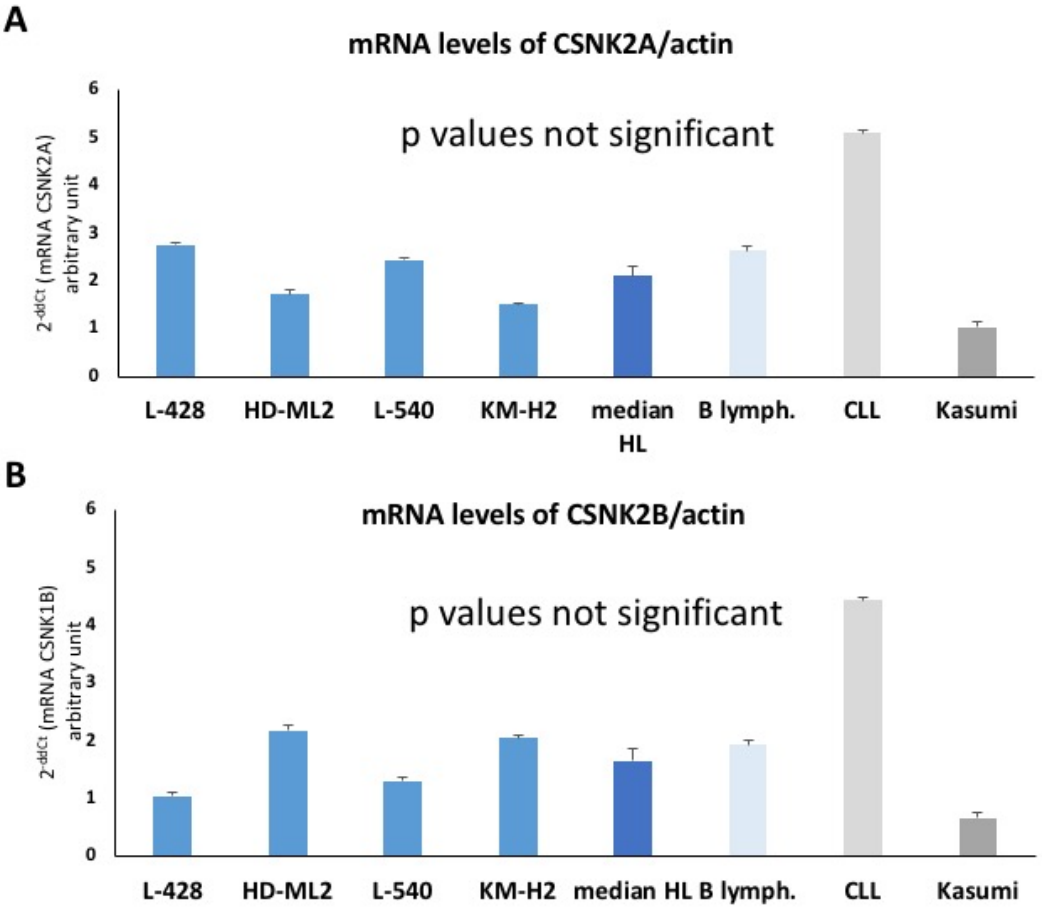


Figure 17. Histogram of mRNA levels of CSNK2A (A) and CSNK2B (B) among HL cell lines, normal B lymphocytes as negative control, chronic lymphocytic leukemia (CLL) and kasumi cells as positive controls.

4.4 CK2 TARGETS ARE PHOSPHORYLATED

In order to evaluate CK2 activity, we performed WB analysis of the expression of CK2 substrates, namely phosphorylated NF-κB on Serine 529 (P-NF-kB S529, Figure 18A) AKT on Serine 473 (P-AKT S473, Figure 18B) and on Serine 129 (P-AKT S129, Figure 18C), and STAT3 on Serine727 (P-STAT3 S727, Figure 18D). These molecules were found to be phosphorylated on CK2 related residues at basal condition, as compared to normal B lymphocytes (12). Proteins phosphorylation occurred at different levels among each cell lines, reflecting the clinical heterogeneity of patients with HL. As summarized in Figure 18E, CK2 can phosphorylated directly STAT3 on S727, NF-kB on S529 and AKT on S129; AKT can also be phosphorylated indirectly on S473 by CK2, through the recruitment of mTOR2 complex. Since AKT, NF-kB and STAT3 play a pivotal role in HL pathogenesis, their basal phosphorylation suggests an important role of CK2 in driving signaling pathways with a pro-growth function in HL.

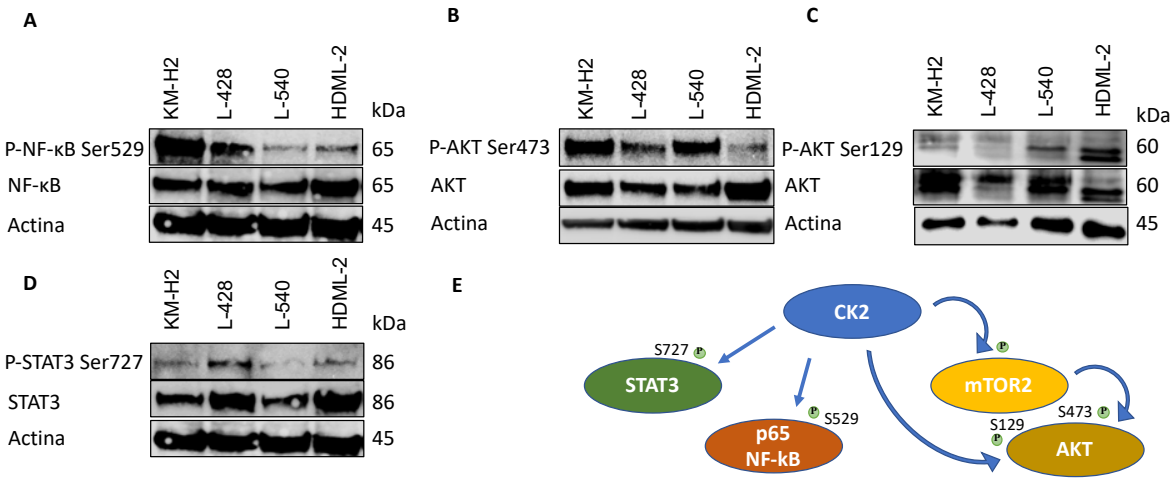


Figure 18. Representative western blotting analysis from 3 cases for each cell lines of CK2 pathways. NF-kB (A), AKT (B and C) and STAT3 (D). Panel E show a schematic representation of CK2 direct and indirect substrates.

4.4 CK2 INHIBITION TRIGGERS HL APOPTOSIS

Since CK2 promotes pro-survival signals, we analyzed the effect of its inhibitions in the survival of HL cell lines. Treatment with the CK2α specific inhibitor CX-4945/silmitasertib, caused a time and dose-dependent apoptosis as assessed by Annexin V/Propidium Iodide flow cytometry test and PARP cleavage by western blotting analysis (Figure 19, Kruskal-Wallis test, $p<0.05$). For all the four HL cell lines, the percentage of viable cells proportionally decreased starting from the dose of 5μM as compared to the mock-treated cells ($p<0.05$). *In-vitro* treatment with silmitasertib at 10μM for 48h was able to reduce the number of alive HL cell lines by half (Figure 19). This point is remarkable since the serum max concentration of CX-4945 achieved is 15μM (90).

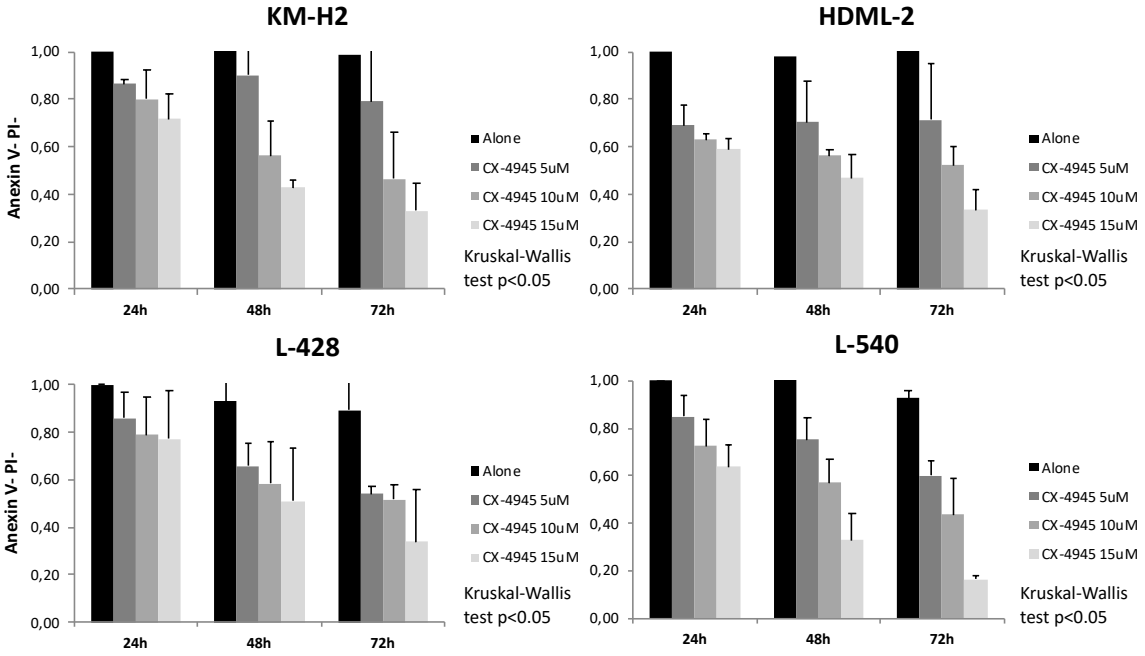


Figure 19. Apoptotic effect of CX-4945 on Hodgkin lymphoma cell lines. Apoptosis was detected through Annexin V propidium iodide (PI) assay by flow cytometry. Alive non-apoptotic cells were Annexin V and propidium iodine double negative.

To shed light on the mechanisms of silmitasertib-induced apoptosis we treated HL cell lines with increasing doses of silmitasertib for 24h, and we observed showed that *in vitro* treatment caused the cleavage of PARP, a marker of apoptosis, but also the dephosphorylation of AKT on Ser473, STAT on Ser727 and NF-kB on Ser529 (Figure 20). Phosphorylations decreased variable among each cell lines, but were almost null in all cell lines when CX-4945 was used at 15μM, excepted for P-STAT3 Ser727 for HDML-2 cells.

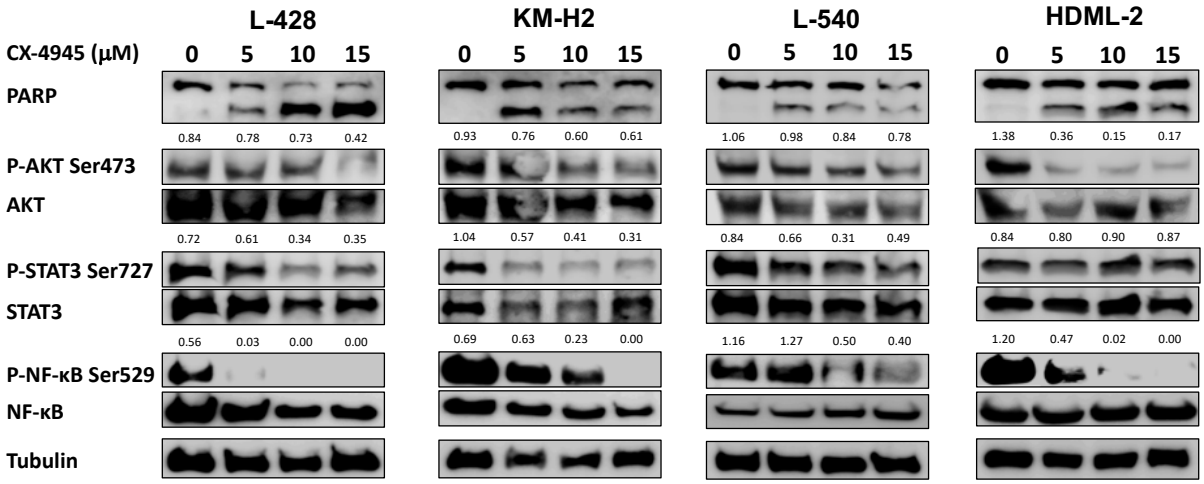


Figure 20. Western blotting analysis of the effects of CK2 inhibition by CX-4945 on CK2 substrates in four HL cell lines. Above the wester blotting are reported the densitometry ratio between the phosphorylated and the total protein.

4.5 CK2 MODULATES THE EXPRESSION OF PD-L1 BUT NOT OF CD30

CD30 and PD-L1/CD274 are known surface markers of HL, and targets of the clinically available drugs brentuximab vedotin and the checkpoint inhibitors (i.e. nivolumab and pembrolizumab), respectively. In HL, CD30 the target of brentuximab vedotin is overexpressed by all HRS cells and PD-L1 is expressed on the cell surface due to amplification of the locus of the gene, which is mapped on chromosome 9, and/or by constitutive active TFs STAT3 and NF-kB, required for PD-L1 expression. Since we demonstrated that CK2 regulates STAT3 and NF-kB in HL, we hypothesized that CK2 could be involved in the regulation of PD-L1 expression. To addressed this point, we inhibited CK2α with CX-4945/silmitasertib at 10μM for 48h and we observed the decrease of PD-L1/CD274 mean fluorescence intensity but not of CD30 (Figure 21A). While CD30 and CD20 were unmodified by CK2 inhibition. CD273/PD-L2 was significantly decreased in only HDML-2 cells. Moreover, we confirmed the downregulation of PD-L1 by WB as shown in Figure 21B.

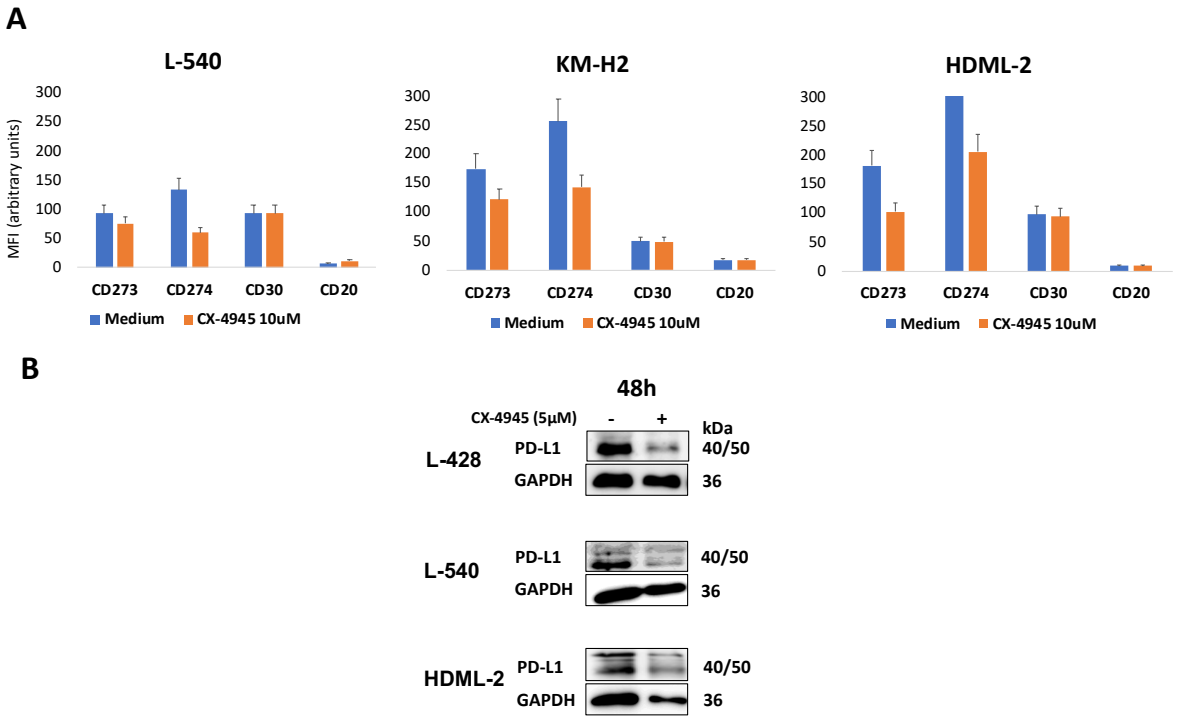


Figure 21. Histograms of MFI and WB of PD1/PD-L1 proteins. Panel A shows histograms of the mediam MFI expression of CD273 (PD-L2), CD274 (PD-L1), CD30 (positive control) and CD20 (negative control) with or without treatment with CX-4945 for 48h in 3 HL cell lines by the independent experiments. Panel B MFI = mean fluorescence intensity.

4.6 CK2 INHIBITION BOOSTS THE ACTIVITY OF MMAE

Monomethyl auristatin E (MMAE) is the microtubules inhibitor conjugated to CD30 monoclonal antibody in the clinically available drug Brentuximab vedotin. Since Brentuximab vedotin/MMAE is clinically active in one third of patients and the duration of response is almost 9 months, the identification of the targeted therapy to combined with it is eagerly needed. In order to assess whether CX-4945/silmitasertib is able to enhance the activity of MMAE we treated three HL cell lines with CX-4945 at 5µM, MMAE at 5nM [dose derived from the literature (91)] or both drugs for 24 or 48 hours. As shown in Figure 23, the combination of CX-4945+MMAE was able to decrease significantly the rate of alive cells as compared to each drug used alone. After 24 hours of treatment the rate of alive cells decreased from 89%, 86% and 87% with MMAE alone to 83% (p=0.0782), 69% (p=0.0093) and 64% (p=0.0051) with CX-4945+MMAE in L428, L540 and HDML-2, respectively (Figure 23). While after 48 hours of *in-vitro* treatment the rate of alive cells decreased from 73%, 70% and 68% with MMAE alone to 55% (p=0.0038), 59% (p=0.0237) and 49% (p=0.0205) with CX-4945+MMAE in L428, L540 and HDML-2, respectively (Figure 23). In particular L-428 cells were the less sensitive while HDML-2 were the most sensitive to the combination therapy.

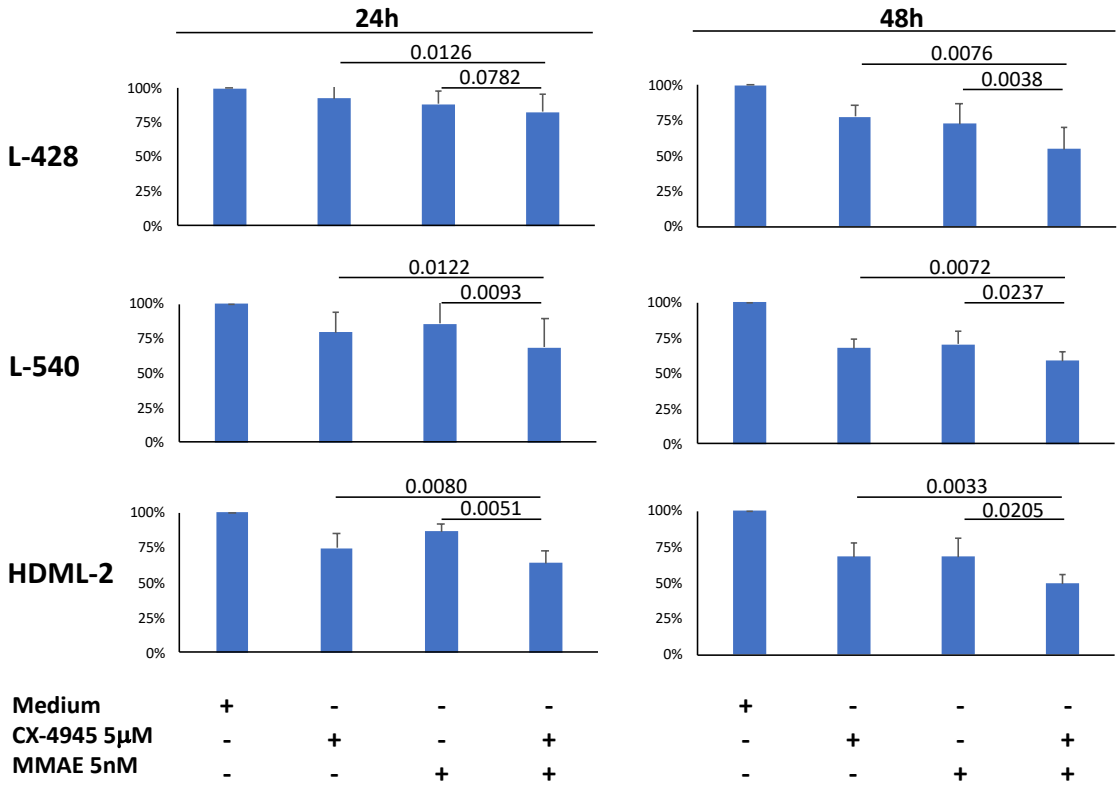


Figure 23. Apoptotic effect of MMAE alone or in combination with CX-4945 in three HL cell lines. HL cells lines were treated till 48hs with CX-4945 and/or MMAE or the combination of both drugs. The rate of alive cells was normalized on cells exposed to medium only. Wilcoxon pairs signed rank test was used to compared paired data and p values are reported.

The enhanced apoptotic effect by the combination of CX-4945 and MMAE was also confirmed by WB (Figure 24). After 24h and 48h of *in vitro* treatment CX-4945+MMAE were able to significantly decrease band of full length PARP and, consequently, to increase the band of the cleaved protein as compared to MMAE-only treated cells (Figure 24).

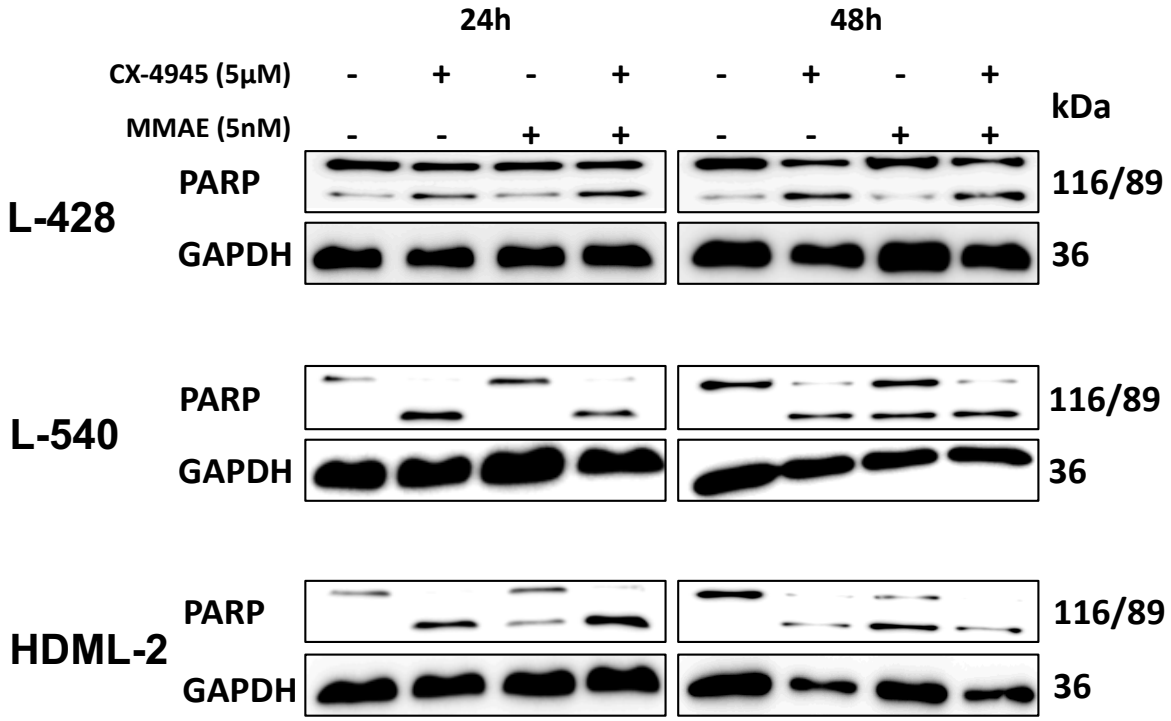


Figure 24. Representative western blotting of PARP protein expression. HL cells lines were treated with CX-4945 and/or MMAE or both drugs for 24 and 48 hours.

5 DISCUSSION

The understanding of the pathogenesis of hematological malignancies has greatly improved during the last decades. In particular, a lot of data has been provided in Hodgkin lymphoma, specifically on the identification of its cellular origin from germinal center B-cells, the microenvironment interaction and various pathways involved in its development and progression (4). These understandings have shed light to remarkable progresses in developing new treatment strategies targeting not only malignant tumor cells, following the use of the anti-CD30 monoclonal antibody brentuximab vedotin, but also the microenvironment, through the use of the checkpoint inhibitors nivolumab and pembrolizumab (5). Despite these innovative and relevant therapeutically results, only 20-30% of relapsed and refractory patients achieve complete remission and long-term disease control. CK2 is a pleiotropic and tetrameric enzyme, composed by 2 catalytic/α and 2 non-catalytic/β, previously called regulatory, subunits, involved in the proliferation, angiogenesis, secretion of growth factors, invasiveness and resistance of solid cancer (2). Indeed, CK2 was found overexpressed, essential for neoplastic cell growth, and its inhibition triggered apoptosis of multiple myeloma, acute leukemia and non-Hodgkin lymphoma cells without a significant impact on normal lymphocytes (3). However, the role of CK2 in Hodgkin lymphoma is unknown and has not been investigated.

With this as a background, in this thesis we characterized CK2 protein and investigated its putative role in the pathogenesis of classical HL.

During this three-year project, we demonstrated that CK2α was overexpressed in HL cell lines as compared to normal B-lymphocytes as assessed by western blotting and confocal microscopy. These data were also confirmed by immunohistochemistry on a tissue microarray derived from 28 patients confirming that the catalytic subunit CK2α was highly expressed both in the cytosol and the nucleous of almost all HRS, while the CK2β was expressed at lower intensity and mainly in the cytosol. This aspect is remarkable, since, to our knowledge, it is the first hematological malignancies with an imbalance between α and β subunits. The expression of CK2α or β did not correlated with histological variants, age, stage and outcome suggesting that this unbalance in occurred in almost all cases and is likely to be necessary for classical HL development.

Since HRS are likely to derived from CD30+ germinal center (i.e. centrocytes re-differentiating into centroblasts) and/or extrafollicular B lymphocytes (i.e. active, proliferating memory B cells), as suggested by phenotypic, IgV gene analyses, exome sequencing and strong

MYC signature (92), we can speculate that a skewed expression of CK2 subunits that we observed in HRS cells could be present also in CD30+ B cells or might be necessary for HL development. For this reason, we planned to isolate the very rare CD30+ B lymphocytes from reactive lymph nodes or tonsils and to study the expression of CK2 subunit in this subset. Remarkably, it has been discovered that the β-subunits of CK2 are not responsible for either activation or inactivation of the catalytic subunits, while β likely shapes the substrate targets of CK2. Hence, to gain additional information about the roles of the individual CK2 subunits, Borgo *et al* (93) generated C2C12 myoblasts entirely devoid either of both catalytic subunits, or of the β-subunit. Authors showed that while CK2α/α'(-/-) cells grow similarly to wild-type cells, the growth of CK2β(-/-) cells is severely impaired, consistent with the hypothesis that not all cellular functions of the β-subunit are mediated by CK2 holoenzyme. To address into the functional implications of the β-subunit authors performed a quantitative proteomics study of CK2β(-/-) cells, leading to the identification and quantification of more than 1200 proteins. Of these, 187 showed a significantly altered expression (fold change ≥ 1.5 or ≤ -1.5) as compared to wild-type cells (93). A functional analysis of these proteins discloses the implication of CK2β in many processes, for example, cell cycle, proliferation, transport, metabolic processes, etc., and in some of which the catalytic subunits of CK2 do not seem to play a relevant role (93). The mechanisms that maintained low protein levels of CK2β, as well as the cellular events that might derived from re-expression of CK2β in HRS, are unknown and will investigated by future researches.

Pivotal signaling molecules in classical HL, such as NF-κB, PI3K/AKT and STAT3, were also found to be phosphorylated in CK2-regulated residues, like AKT-Ser129, AKT-Ser473, NF-κB-Ser529 and STAT3-Ser727, and therefore constitutively activated. We proceeded analyzing the effects of CK2 inhibition with silmitasertib, previously called CX-4945. We demonstrated that all the 4 HL cell lines underwent *in vitro* dose and time-dependent apoptosis after treatment with silmitasertib, together with a reduction of active AKT-Ser473, NF-κB-Ser529 and STAT3-Ser727. However the phosphorylation of STAT3 on Ser727 was unmodified in HDML-2 cells, even at higher doses. Interestingly these cells harbored both STAT3 D661Y mutation in the SH2 domain, and SOCS1, a negative regulator of JAK/STAT pathway, inactivation (94).

The importance of targeting more pathways at the same time was highlighted by a phase I and a II clinical trials evaluating the clinical activity and safety of JAK2 and/or PI3K inhibitors in relapsed refractory patients with HL. Ruxolitinib, an oral available JAK2 inhibitor, approved

for the treatment of chronic myeloproliferative disease, showed disappointing results in HL since the ORR was 9.4%, no patient achieved the complete remission and the median PFS was only 3.5 months. Similarly, idelalisib a selective PI3Kδ inhibitor, approved for the treatment of chronic lymphocytic leukemia and follicular lymphoma, generates an ORR of 20% with 4% CR, but the median PFS was only 2.3 months. However, since both PI3Kδ and JAK/STAT pathways contribute to tumor cell proliferation and survival in B-cell malignancies, their simultaneous inhibition may provide synergistic treatment efficacy. A phase 1 dose-escalation/expansion study assessed the safety, efficacy, pharmacokinetic, and pharmacodynamic of dezapelisib, a selective PI3Kδ inhibitor, as monotherapy or combined with itacitinib, a selective JAK1 inhibitor, in adult patients with relapsed B-cell lymphomas. Final results have been reported. Overall, 114 patients were treated (monotherapy, n=49; combination therapy, n=72). Dezapelisib 100mg twice daily (monotherapy) alone or in combination with itacitinib 300 mg once daily were the recommended phase 2 doses. One dose-limiting toxicity (gastrointestinal bleed secondary to gastric DLBCL regression) occurred with monotherapy. The most common serious adverse events with monotherapy were pneumonia (n=5) and pyrexia (n=4), and with combination P. jiroveci pneumonia (n=5), pneumonia (unrelated to P. jiroveci; n=5), and pyrexia (n=4). Grade 3 or higher transaminase elevations were less common with treatment combination. Dezapelisib was active across the B-cell lymphomas; 63% of patients (5/8) with follicular lymphoma responded to monotherapy. Adding itacitinib provided promising activity in select subtypes, with an ORR of 67% (n=14/21) in classic HL (vs 29% [n=5/17] with monotherapy) and 31% (4/13) in nongerminal center B-cell like DLBCL. These clinical data support the notion that the combined inhibition of more pathways together might be a relevant treatment strategy for highly pretreated patients with HL.

Moreover, we also observed that silmitasertib could improve the clinical activity of brentuximab vedotin and checkpoint inhibitors, since we demonstrated that *in vitro* treatment with silmitasertib was able to trigger the downregulation of PD-L1/CD274, but not of CD30, and to enhance the activity of MMAE, the microtubules inhibitor of brentuximab vedotin. These data will be validated through the survival/metabolic assay, such as the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma, Germany) assay and the calculation of the combination index between drugs using the Chou-Talalay method.

6 CONCLUSIONS

We herein demonstrated classical HL is likely to be the first hematological malignancies with a skewed expression of CK2 subunits, being that CK2a overexpressed in the cytosol and the nucleolus of HRS both in HL cell lines and primary nodes. HRS are highly dependent by CK2α activity since, we observed that *in vitro* treatment is CX-4945/silmitasertib triggered de-phosphorylation of AKT on Ser129 and Ser473, NT-kB in Ser529 and STAT3 on Ser727 and was synthetic lethal in HL. Moreover, CX-4945 also caused downregulation on CD274/PD-L1 but of CD30, and it enhanced the rate of apoptotic effect of MMAE. This point might have a relevant clinical implication, since only a few patients achieved complete remission and the identification of novel drugs able to enhance brentuximab vedotin and/or checkpoint inhibitors is highly needed (Figure 25). In addition, further study on CK2 protein will improve our understanding on HL pathogenesis.

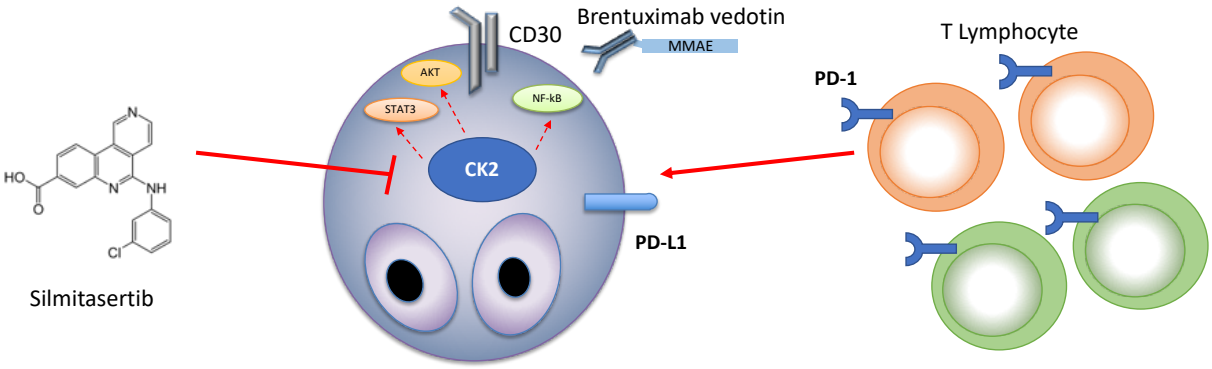


Figure 25. Schematic representation of possible drug combinations of CK2 inhibitor silmitasertib, brentixumab vedotin and anti-PD-L1/PD1 axis.

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8 PUBBLICATIONS

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Summited paper:

1. "JAK2/STAT3 pathway is constitutively activated in chronic lymphocytic leukaemia and its inhibition enhances ibrutinib activity, counteracting bone marrow microenvironment". F Severin*, F Frezzato*, **A Visentin***, V Martini, V Trimarco, S. Carraro, E Tibaldi, AM Brunati, F Piazza, G Semenzato, M Facco, L Trentin. *These authors contributed equally to this work.

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