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HSP70/HSF1 axis has a role in the pathogenesis of Chronic Lymphocytic Leukemia and is a druggable target

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
APC	Allophycocyanin
ATM	Ataxia Teleangectasia Mutated
BAD	Bcl-2 Associated Death promoter
BCL-2	B Cell lymphoma-2
BCR	B Cell Receptor
BIRC3	Baculoviral IAP repeat containing 3
BM	Bone Marrow
BMMC	Bone Marrow Mononuclear Cell
BMSC	Bone Marrow Stromal Cell
BOM	Bone Marrow Biopsy
B-PLL	B cell prolymphocytic leukemia
BTK	Bruton's tyrosine kinase
CDR	Complementarity Determining Region
CD40L	CD40 Ligand
CEB	Cytoplasmic Extraction Buffer
CLL	Chronic Lymphocytic Leukemia
CMV	Cytomegalovirus
GC	Germinal Center
DAG	Diacylglycerol
EBV	Epstein-Barr virus
ECL	Enhanced Chemi Luminescence
EDTA	Ethylenediaminetetraacetic Acid
ERK	Extracellular signal Regulated Kinase
Fab	Antigen binding fragment
FBS	Fetal Bovin Serum
Fc	Crystallizable fragment
F/H	Ficoll/Hypaque
FISH	Fluorescence In Situ Hybridization
FITC	Fluorescein isothiocyanate
FSC	Forward Scatter
G-CSF	Granulocyte-Colony Stimulation Factor
GDP	Guanosine diphosphate
GSK3	Glycogen Synthase Kinase 3
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HE	Hematoxylin-Eosin
HIV	Human Immunodeficiecny virus
HLA	Human Leukocyte Antigen
HSF1	Heat shock factor 1
HSP90	Heat shock protein of 90kDa
HSP70	Heat shock protein of 70kDa
HSR	Heat shock response
hTERT	Human Telomerase Reverse Transcriptase
IFN-γ	Interferon-γ
Ig	Immunoglobulin

IGHV	Immunoglobulin heavy chain variable regions
IL	Interleukin
IP-3	Inositol-1,4,5-triphosphate
ITAM	Immunoreceptor Tyrosine-based Activation Motif
mAb	Monoclonal Antiboby
MAP	Microtubule-Associated Protein
MAPK	Mitogen Activated Protein Kinase
MBL	Monoclonal B cell lymphocytosis
MCL-1	Mantle cell lymphoma-1
M-CLL	Mutated-Chronic Lymphocytic Leukemia
MEB	Membrane Extraction Buffer
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
MIIC	MHC-class-II-peptide-loading-compartment
MMP-9	Matrix metalloproteinase - 9
MSC	Mesenchymal Stromal Cell
MTOC	Microtubule Organizing Centre
mTOR	Mammalian Target Of Rapamycin
NEB	Nuclear Extraction Buffer
NF-kB	Nuclear Factor-kappa B
NHL	Non-Hodgkin Lymphoma
NLC	Nurse-like Cell
NK	Natural Killer
OS	Overall Survival
PARP	Poli-ADP-Ribose Polymerase
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFS	Progression Free Survival
PI	Propidium Iodide
PIAS	Protein Inhibitors of Activated STATs
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol 4,5-bisphophate
PL	Prolymphocyte
PLC-γ2	Phospholipase Cγ2
PMN	Granulocyte
PS	Phosphatidylserine
PTK	Protein Tyrosine Kinase
PTP	Protein Tyrosine Phosphatases
R	Rituximab
RBC	Red Blood Cell
RTK	Receptor Tyrosine Kinase
SD	Standard Deviation
SDF-1α	Stromal Derived Factor-1α
SDS-PAGE	Sodium Dodecyl Sulphate/Poly Acrylamide Gel Electrophoresis
Ser	Serine
SFKs	Src Family Kinases
SHM	Somatic Hypermutation
SHIP-1	SH2-domain containing Inositol Phosphatase
sIgM	Surface Immunoglobulin M
SYK	Spleen tyrosine kinase

SOCS	Suppressors of Cytokine Signaling
SRBC	Sheep Red Blood Cell
SC	Side Scatter
STAT	Signal Transducers of Activated Transcription
TC	Tri-Color
Th	T helper lymphocyte
Tyr	Tyrosine
TK	Thymidine Kinase
TP53	Tumor protein 53
U-CLL	Unmutated-Chronic Lymphocytic Leukemia
VH	Heavy chain Variable region
VL	Light chain Variable region
WB	Western blotting
WBC	White Blood Cell
ZAP-70	Zeta-Associated Protein of 70kDa

ABSTRACT

Chronic Lymphocytic Leukemia (CLL) is a neoplastic disorder, characterized by the accumulation of clonal B lymphocytes due to an increased proliferation and the inability to reach programmed cell death. CLL is a heterogeneous disease with some patients asymptomatic, while others experience a more aggressive disorder, treatment failure and a poor overall survival. As a consequence, novel biological and cytogenetic features are crucial to predict patient's prognosis, and the search for key molecules involved in the pathogenesis of the disease is still ongoing.

We previously performed a Reverse Phase Protein Array (RPPA) analysis for the identification of new proteins, which might contribute to the apoptosis resistance and the increased survival of CLL cells. We found the Heat Shock Protein of 70kDa (HSP70) significantly overexpressed in CLL cells with respect to normal controls. HSP70 is a molecular chaperone induced in response to several physiological and environmental insults, thus allowing cells to survive to otherwise lethal conditions. The main responsible of HSP70 transcription is the Heat Shock Factor 1 (HSF1), able to bind to DNA and to activate Heat Shock gene transcription in response to stress.
During this three-year PhD project, we were aimed at characterizing HSP70 and its major regulator, HSF1, in CLL cells.

We found HSP70 and HSF1 both overexpressed (138 CLL patients, 1.24±0.85 *vs* 32 healthy controls, 0.77±0.57; $p<0.01$; 85 CLL patients 0.81±0.77 *vs* 19 healthy controls, 0.30±0.36; $p<0.01$, respectively), correlated to poor prognosis and abnormally localized in the nucleus of CLL cells. We showed that the two protein levels were decreased, and positively correlated to each other (*e.g.* $p<0.05$; $r=0.94$), in patients responding to *in vivo* therapeutic regimens.

To better understand HSP70 and HSF1 role in CLL, we performed *in vitro* inhibition of these proteins using specific drugs (VER-155008, KRIBB11, zafirlukast, fisetin), which led to a dose-dependent apoptosis of leukemic cells from CLL patients (*e.g.* cell viability at 24h: CLL cells alone 63±14%, *vs* CLL cells + 15µM fisetin 35±19%, $p<0.0001$). Moreover, we demonstrated that fisetin was able to bypass the microenvironmental protection when neoplastic B cells were co-cultured with 15µM fisetin and MSCs (*e.g.* 30±17%, 25±19%, 26±15% without MSCs, and 32±14%, 35±23%, 33±22% with MSCs at 24, 48, 72h, respectively).

HSF1 is regulated by its interactions with molecular chaperones, and also by a fine balance of activatory and inhibitory phosphorylations. Since most of HSF1-phosphorylating molecules belong to two RAS-signalling pathways (PI3K/AKT/mTOR and RAF/MEK/ERK), taking advantage from our previous RPPA analysis, we correlated HSP70 to different proteins related to these signals. By a cluster analysis, we divided our patients in $HSP70^{high}$ and $HSP70^{low}$, on the basis of HSP70 protein

levels, demonstrating that the RAS pathway proteins are differently regulated in the two groups. For $HSP70^{high}$ patients, we developed a model based on high AKT-Ser473 levels, in which AKT-Ser473 inhibits GSK3a/b that, in turn, is unable to inhibit HSF1. On the other hand, $HSP70^{low}$ patients present high MEK1/2-Ser217/221 and ERK-Thr202/Tyr204, that negatively regulate HSF1. As a result, the activation of the PI3K/AKT/mTOR pathway leads to a higher expression of HSP70, while an activation of the RAF/MEK/ERK pathway results in HSP70 downregulation.

By RAS pathway inhibition, we validated our model for the aberrant regulation of HSP70/HSF1 axis in CLL. To this purpose, we used resveratrol, a natural polyphenol, able to inhibit AKT and to activate ERK. After 24h treatment with 40μM resveratrol, we observed a reduction in cell viability (61±18% of untreated cells *vs* 54±20%; $p<0.01$) with a decreased protein expression both for HSF1 and HSP70: 1.15±0.69 to 0.50±0.64, and from 1.13±0.69 to 0.54±0.35, respectively; $p<0.01$).

In conclusion, the HSP70 and HSF1 overexpression and correlation with resistance to therapy in CLL, together with the ability of fisetin to induce apoptosis in leukemic B cells bypassing the pro-survival stimuli of tumor microenvironment, represent a starting point for the development of new therapeutic strategies. Moreover, the study of resveratrol effects on leukemic B cells provides new insights on the cross-talk between HSP70/HSF1 axis and RAS signalling in CLL.

RIASSUNTO

La Leucemia Linfatica Cronica (LLC) è una malattia neoplastica, caratterizzata dall'accumulo di linfociti B clonali proliferanti e resistenti alla morte cellulare programmata. Questa leucemia è una malattia eterogenea: alcuni pazienti possono essere asintomatici, mentre altri sperimentano un decorso più aggressivo. Di conseguenza, nuove informazioni biologiche e citogenetiche sono necessarie per prevedere la prognosi del paziente e per curare la malattia attraverso la ricerca di nuove molecole coinvolte nella sua patogenesi.

In precedenza, il nostro laboratorio aveva eseguito un'analisi di RPPA (*Reverse Phase Protein Array*) allo scopo di identificare nuove proteine coinvolte nella resistenza all'apoptosi e nella sopravvivenza delle cellule di LLC. Grazie a questo studio, abbiamo verificato che la *Heat Shock Protein* di 70kDa (HSP70) era significativamente sovraespressa nelle cellule di LLC rispetto ai controlli sani. HSP70 è uno chaperone molecolare indotto in risposta a numerosi insulti fisiologici e ambientali, che consente alle cellule di sopravvivere in condizioni altrimenti letali. Il principale responsabile della trascrizione di HSP70 è *Heat Shock Factor 1* (HSF1), il quale, in risposta allo stress, è in grado di legarsi al DNA ed attivare la trascrizione dei geni dell'*heat shock*.

Durante questo progetto triennale di dottorato, ci siamo focalizzati sulla caratterizzazione di HSP70 e del suo principale regolatore, HSF1, nella LLC.

Abbiamo dimostrato che HSP70 e HSF1 sono sovraespresse (138 pazienti LLC, 1,24 ± 0,85 *vs* 32 controlli sani, 0,77 ± 0,57; p <0,01; 85 pazienti LLC 0,81 ± 0,77 *vs* 19 controlli sani, 0,30 ± 0,36; p <0,01, rispettivamente) e correlate a prognosi sfavorevole nella LLC. Abbiamo inoltre osservato che queste due proteine sono localizzate in modo anomalo nel nucleo delle cellule leucemiche. Nei pazienti che rispondevano a regimi terapeutici *in vivo*, abbiamo evidenziato una diminuzione dei livelli proteici di HSP70 e HSF1 e una correlazione positiva tra gli stessi (p <0,05; r = 0,94).

Per comprendere meglio il ruolo di HSP70 e HSF1 nella LLC, le abbiamo inibite *in vitro* utilizzando molecole specifiche (VER-155008, KRIBB11, zafirlukast, fisetina), che si sono rivelate in grado di indurre apoptosi nelle cellule leucemiche da pazienti con LLC (vitalità a 24 ore: solo cellule LLC 63 ± 14%, *vs* cellule LLC + fisetina 15μM 35 ± 19%, p <0,0001). Inoltre, abbiamo dimostrato che la fisetina era in grado di oltrepassare la protezione fornita dal microambiente quando le cellule B neoplastiche erano poste in co-coltura con MSC e fisetina 15μM (senza MSC: 30 ± 17%, 25 ± 19%, 26 ± 15%; con MSC: 32 ± 14%, 35 ± 23%, 33 ± 22% a 24, 48, 72 ore).

HSF1 non è regolato soltanto dalle sue interazioni con chaperoni molecolari, ma anche da un fine equilibrio tra fosforilazioni attivatorie e inibitorie. Poiché la maggior parte delle molecole che fosforilano HSF1 appartiene a due vie di segnalazione di RAS (PI3K/AKT/mTOR e

RAF/MEK/ERK), sfruttando la nostra precedente analisi RPPA, abbiamo correlato HSP70 a diverse proteine appartenenti a questi *pathway*. Abbiamo diviso i nostri pazienti sulla base dei livelli di HSP70 in due gruppi: $HSP70^{high}$ e $HSP70^{low}$, dimostrando che le proteine dei *pathway* di RAS sono regolate in modo diverso nei due gruppi. Per $HSP70^{high}$ abbiamo sviluppato un modello basato su elevati livelli di AKT-Ser473, che ha la capacità di inibire GSK3a/b che, a sua volta, non è più in grado di inibire HSF1. I pazienti $HSP70^{low}$, invece, presentano elevati livelli di MEK1/2-Ser217/221 e ERK-Thr202/Tyr204, che regolano negativamente HSF1. Di conseguenza, l'attivazione del pathway PI3K/AKT/mTOR porta ad un'espressione più elevata di HSP70, mentre l'attivazione del pathway RAF/MEK/ERK determina una *down*-regolazione di HSP70.

Abbiamo convalidato il nostro modello per la regolazione aberrante dell'asse HSP70/HSF1 nella LLC inibendo alcune molecole dei *pathway* di RAS. A questo scopo, abbiamo usato il resveratrolo, un polifenolo naturale in grado di inibire AKT ed attivare ERK. Dopo 24 ore di trattamento con resveratrolo alla dose di 40μM, abbiamo osservato una riduzione della vitalità cellulare (61 ± 18% delle cellule non trattate contro 54 ± 20% di quelle trattate; $p < 0,01$) ed una ridotta espressione proteica di HSF1 e HSP70 (da 1,15 ± 0,69 a 0,50 ± 0,64 e da 1,13 ± 0,69 a 0,54 ± 0,35, rispettivamente, $p < 0,01$).

In conclusione, la sovraespressione di HSP70 e HSF1 e la loro correlazione con la resistenza alla terapia nella LLC, insieme alla capacità della fisetina di indurre apoptosi nelle cellule B leucemiche bypassando gli stimoli pro-sopravvivenza del microambiente tumorale, rappresentano un punto di partenza per lo sviluppo di nuove strategie terapeutiche. Inoltre, lo studio degli effetti del resveratrolo sulle cellule B leucemiche fornisce nuove informazioni sul *cross-talk* tra l'asse HSP70/HSF1 e la via del segnale di RAS nella LLC.

INTRODUCTION

1. Chronic Lymphocytic Leukemia

1.1 Epidemiology and etiology

Chronic Lymphocytic Leukemia (CLL) is a malignancy of mature clonal CD19/CD5/CD23 positive B lymphocytes (CD19+/CD5+/CD23+), mainly resting in G_0 stage of cell cycle, that accumulate in blood, bone marrow and lymphoid tissues[1]. This accumulation is due both to proliferative activity and to apoptosis resistance of CLL cells.

With an age-adjusted incidence of 4.1/100,000 inhabitants, CLL represents the most common leukemia in the Western countries. A US study, published in 2004, estimated the worldwide incidence of CLL between <1 and 5.5 per 100,000 inhabitants[2] (**Fig. 1**). The incidence increases up to >30/100,000/year at age >80 years. The median age at diagnosis ranges between 67 and 72 years, but about 10% of CLL patients are younger than 55 years. The male/female ratio is 1.7:1[3,4].

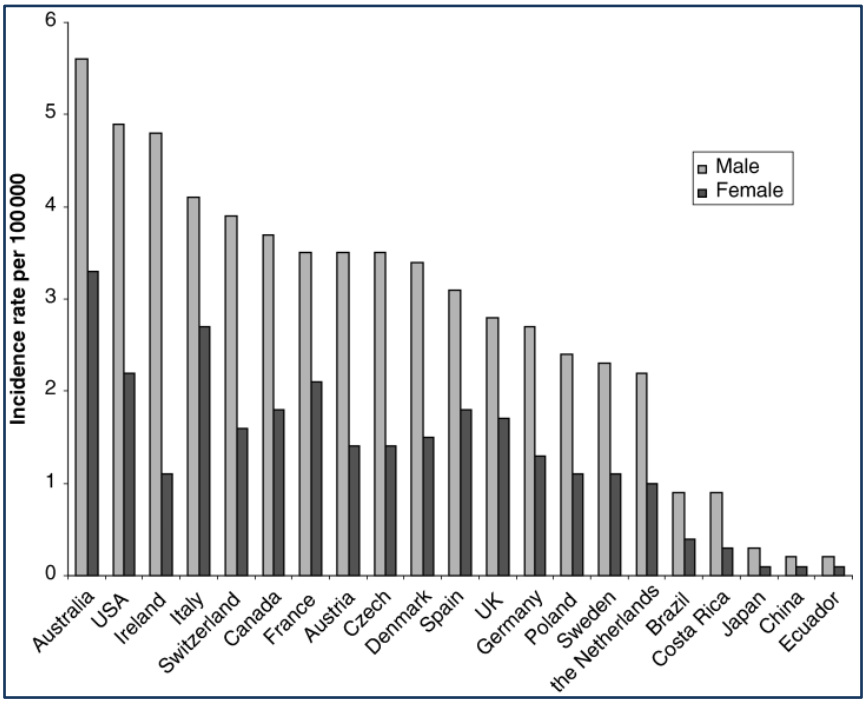

Fig. 1: CLL incidence rate per 100,000 inhabitants (image from Redaelli *et al.*)[2].

The clinical course of CLL is highly heterogenous, with relatively long survival in the majority of patients, and aggressive, treatment-resistant disease in a high-risk subset[5]. In fact, in some cases progressing disease with a poor prognosis occurs, such as the Richter's syndrome, in which CLL changes into a fast-growing diffuse large B cell lymphoma, and B cell prolymphocytic leukemia (B-PLL), characterized by malignant B cells larger than average.

The etiology is still unknown even if studies are in progress to assess a potential relation among CLL onset, inflammation, and autoimmune conditions[6,7]. There is an inherited genetic susceptibility for CLL, with a 8.5-fold increased risk for family members of CLL patients[8]. Regarding environmental factors, the US Department of Veterans Affairs has accepted that the exposure to Agent Orange (a chemical herbicide used by the U.S. military during the Vietnam War) is a risk factor for CLL[9]. Evidence suggests that exposure to insecticides might be a risk factor for CLL, while little data indicate that ionizing radiations can increase the risk of CLL. Furthermore, there is poor evidence that viral infections are risk factors, and epidemiological studies did not demonstrate that blood transfusions can transmit CLL. Dietary or lifestyle factors do not increase the risk of CLL[10].

1.2 Mechanisms of pathogenesis and pathophysiology

B lymphocytes are White Blood Cells (WBC) of the adaptive immune system. In mammals, B cells mature in the bone marrow, while in birds, B cells mature in the lymphoid organ *bursa* of *Fabricius* (the "B" from B cells comes from the name of this organ, where B development was first discovered[11]). Mature B lymphocytes can be induced by T cells to proliferate, switch the isotype, and differentiate into cells secreting antibodies against specific antigens (plasma cells), or into "memory" long-lived stimulated B cells, ready for rapid response to a repeated exposure of the priming antigen[12,13].

Traditionally, CLL lymphocytes were thought to be derived from *naïve* B cells, but recent studies, starting from 2003, support the derivation of CLL cells from activated antigen-experienced B cells[14]. In 2012, Seifert *et al.*[15] carried out a transcriptome analysis of CLL and the main normal B cell subsets from human blood and spleen, pointing out that CD5+ B cells have the most similar gene expression profile of CLL CD5+ cells, and probably represent the cells of origin of this leukemia[16].

As tumorigenesis is a multi-step process, the initiating genetic lesion of CLL probably occurs in the hematopoietic stem cells (HSCs), followed by repetitive antigenic stimulation that leads to additional genetic lesions. HSCs bearing oncogenetic mutations may give rise to B cells with growth/survival advantages, which possibly progress to monoclonal B cell lymphocytosis (MBL).The B cells in MBL become CLL cells by acquiring additional genetic or epigenetic changes[16].

CLL cells deriving from post-germinal center B cells, with a T cell-dependent immune stimulation, are characterized by mutated immunoglobuline heavy-chain variable-region genes (*IGHV*s) and are defined as mutated-CLL (*IGVH*-M). CLL cells deriving from pre-germinal center B cells, with a T cell-independent immune stimulation, express germ-line *IGHV*s and are defined as unmutated-CLL (*IGVH*-UM) (**Fig. 2**). The expansion of leukemic clone is due to accumulation of novel genetic

lesions, as well as continued interactions between CLL cells, accessory cells and antigens in the microenvironment of lymphoid tissues[16].

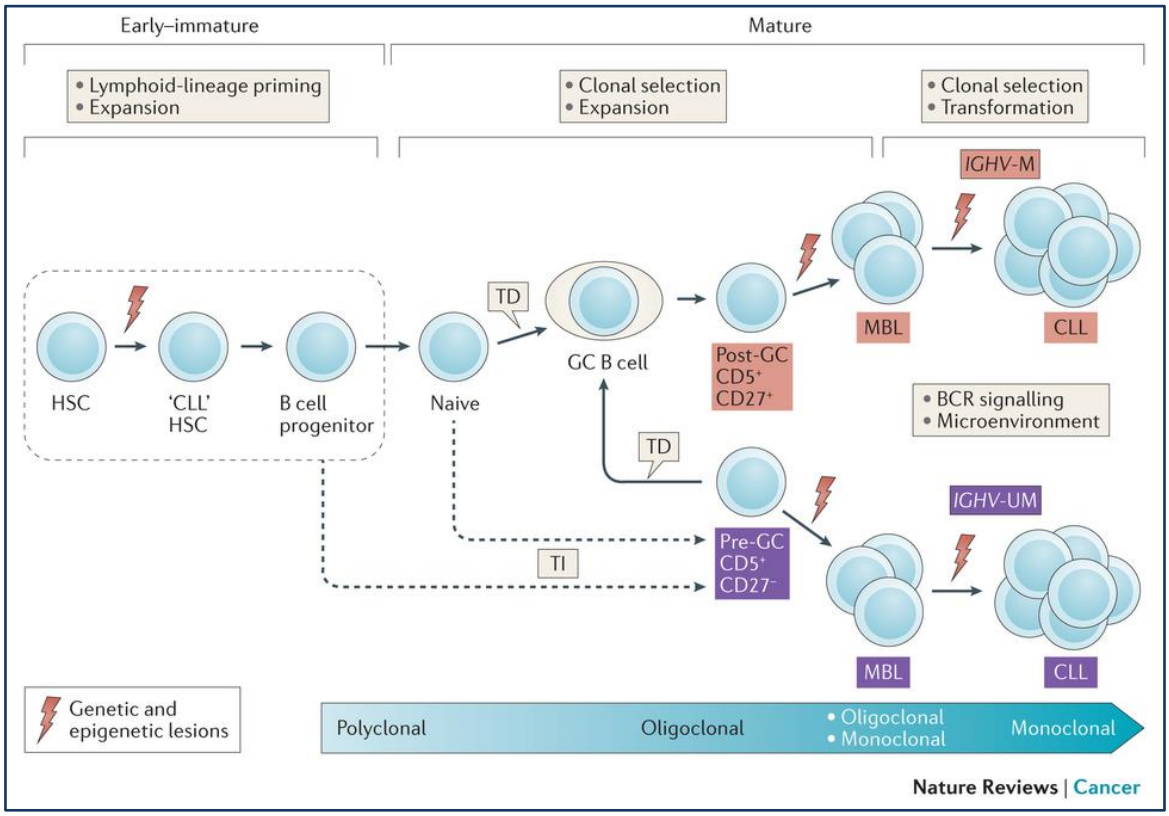

Fig. 2: The cellular origin of CLL subsets. The *IGHV*-unmutated CLL cell (*IGHV*-U) derives from a pre-germinal center CD5+ B cell and has an antigen response through a T-independent mechanism. In most cases the lymphocyte undergoes somatic hypermutation and it is selected in the lymph node based on the affinity of the B cell receptor (BCR) for the antigen: if the affinity is low, the cell dies by apoptosis, while, if the affinity is improved, it will interact with T lymphocytes (Th cells) and with dendritic follicular cells (FDC) in the germinal center (GC). In this case the CLL is defined *IGHV*-mutated CLL (*IGHV*-UM) and probably originates from memory B cells that maintain the expression of IgM and IgD or that have carried out the class switching of the membrane Ig isotype. **GC**=germinal center; **HSC**=hematopoietic stem cell; **TD**=T cell-dependent antigen; **TI**=T cell-independent antigen (image from Fabbri *et al.*)[1].

The pathogenesis of CLL involves both intrinsic factors, such as several genetic alterations, and exstrinsic ones, mainly represented by the interactions with the microenvironment [10]. Some factors are briefly described below.

1.2.1 Intrinsic factors: CLL genetic alterations

CLL genetic alterations include chromosomal alterations, mutations, aberrant expression of miRNAs and epigenetic modifications[10]. Approximately 80% of patients with CLL carry at least one out of four common chromosomal alterations, represented by the trisomy of chromosome 12, the deletion in chromosome 13 (del13q14.3), 11 (del11q), and 17 (del17p)[17]. Del13q14.3 is the most common chromosomal alteration (50% of patients) and involves the DLEU2-mir-15-16 cluster, which regulates the expression of proteins that can inhibit apoptosis or participate in cell cycle progression[18]. The 7% of patients carry del17p associated with loss of the tumour suppressor gene TP53[19]. Del11q is found in 18% of patients and is often associated with alterations in ATM gene

(Ataxia Teleangectasia Mutated), encoding a protein involved in DNA repair. The 16% of patients present the trisomy 12. Each chromosomal alteration is associated with a different clinical outcome[17].

In 2014, Damm *et al.*[20] identified in the progenitor and/or in mature myeloid fractions of CLL patients, early-mutated genes. These mutations are functionally relevant and lead to the accumulation of mutated cells. In CLL the most recurrent somatic mutations have been observed in genes that are involved in DNA damage, mRNA processing, chromatin modification, WNT signalling, NOTCH signalling and inflammatory pathways. Other mutations, such as those found in EGR2 or BRAF genes, can affect B cell-related signalling and transcription[20]. Gene silencing experiments confirm the functional role of several driver mutations. For instance, silencing mutated *WNT* pathway genes leads to a decreased viability of primary CLL cells[10]. In two seminal studies more than 500 CLL samples were characterized by whole-exome or whole-genome sequencing, pointing out that the most frequently mutated genes were *NOTCH1, ATM, BIRC3, SF3B1,* and *TP53*[21,22].

CLL is the first human disease found to be associated with alterations in miRNA. Specifically, mir-15a and mir-16-1 are deleted, altered or downregulated in ~60% of CLL patients and are dysfunctional in a few cases of familial CLL. Reduced expression or loss of these miRNAs can enhance the expression of BCL2 and MCL1 (which encode anti-apoptotic proteins)[23,24]. Moreover, CLL patients with distinctive clinical and biological features are characterized by a differential and dysregulated expression of miRNAs such as miR-29a/b, miR-29c, miR-34b, miR-181b, and miR-3676, which regulate the activity of TCL1A, a protein that promotes the development of CLL in transgenic mice when constitutively expressed in mature B cells[25]. Not only the loss or the reduced expression, but also the increasing of other miRNAs can promote cancer development. The overexpression of mir-155 is associated with enhanced B cell proliferation and lymphomagenesis[26,27]. Genome-wide DNA methylation approaches in CLL show global hypomethylation combined with local hypermethylation, as already observed in other cancers (*e.g.* colon cancer). These studies highlight the differential methylation profiles in *IGHV*-M and *IGHV*-UM CLL, underlining the biological and phenotypic heterogeneity of the disease[28].

This differential methylation in CLL might be a sign of tumor cells adaptive capacity, providing an increased opportunity for somatic mutations within the leukemic clone[10].

1.2.2 Intrinsic factors: BCR and B cell signalling

B cell receptors (BCRs) are expressed on B cell membrane and are necessary to bind specific antigen, against which B cells will initiate an antibody response[12]. In normal B cell several checkpoints of the cell cycle are regulated by BCR signalling[29].

The BCR is a macromolecular complex composed of a ligand-binding trans-membrane immunoglobulin molecule (either IgA, IgD, IgE, IgG or IgM) and the signalling Igα-Igβ (also known as CD79a-CD79b) heterodimer (**Fig. 3**)[30].

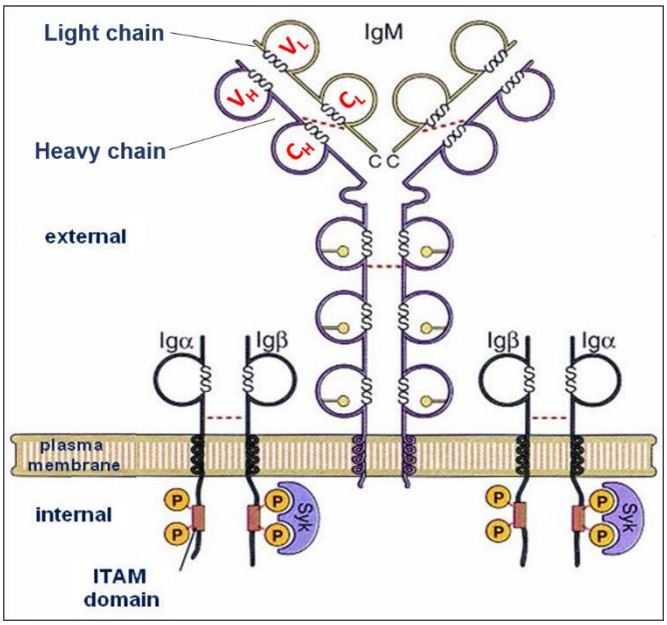

Fig. 3: Schematic representation of the BCR. BCR is composed of two immunoglobulin (Ig) heavy and light chains (variable and constant regions), and CD79a and CD79b (Igα/Igβ), which contain an intracellular activation motif (ITAM) that transmits signals to intracellular tyrosine kinases (for example, SYK and LYN). V_H and V_L=variable regions, C_H and C_L =constant regions (adapted from Abbas *et al.*)[30].

CLL cells typically co-express IgM and IgD, although at low levels compared with normal B cells. The cytoplasmic tails of CD79a and CD79b molecules contain immunoreceptor tyrosine-based activation motifs (ITAM), which can be phosphorylated after the crosslinking of surface immunoglobulin, triggering BCR signalling[10]. The mechanism by which the binding of antigen to the BCR initiates the phosphorylation of ITAM tyrosines is not completely clear, and there are different theories trying to describe this process. The most recent is supported by the so called "dissociation-association model"[31]. On resting B cells, the BCRs form closed autoinhibited oligomers, and the survival signals are maintained by few BCR monomers, in open conformation. The closed BCR oligomers can be dissociated in the presence of a polyvalent antigen, which can bind at the same time more BCR monomers. In this way, the *equilibrium* shifts towards active monomer clusters which drive the signalling[32]. Lipid rafts, which are membrane microdomains, aggregate at the site of BCR and act as platforms for receptor signalling and trafficking. In the absence of antigen, the BCR monomer has a weak affinity for the rafts, but the BCR oligomerization, triggered by polyvalent antigen binding, increases such affinity[33].

The activation of BCRs by the antigen is enhanced by several intracellular components and simultaneously recruits these proteins in a positive feedback loop. Specifically, the Src-related tyrosine kinase (LYN) phosphorylates ITAMs, creating the docking sites for the recruitment and

activation of the spleen tyrosine kinase (SYK)[29]. SYK has two N-terminal SH2 domains that bind to phosphorylated ITAMs. This binding leads to a conformational change of SYK which, in its activated form, is able to phosphorylate downstream signalling proteins and neighbouring ITAMs, amplifying the signal[16,31]. BLNK serves as a scaffold protein to bind PLC-γ2 and Bruton's tyrosine kinase (BTK). Once SYK, BTK, BLNK, VAV, GRB2 and PLC-γ2 are close, PLC-γ2 is dually phosphorylated by BTK and SYK to produce diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) from the phosphatidylinositol 4,5-bisphophate (PIP2). DAG is a classic activator of protein kinase C, which is responsible for many downstream effects of BCR signalling. The generation of IP3 leads to enzyme activation and calcium influx from the endoplasmic reticulum to the extracellular compartment (**Fig. 4**).

Initial phosphorylation events after BCR ligation also activate the PI3K pathway. PI3K is a heterodimer composed of a p110δ catalytic subunit and a p85 regulatory subunit. The regulatory p85 subunit is recruited to the plasma membrane where it associates with LYN. In addition, LYN phosphorylation of CD19 allows p85 to bind to CD19, and this co-association activates the catalytic subunit, p110δ. PI3K then phosphorylates PIP2 to create phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 induces the recruitment of BTK and other kinases, resulting in continued BCR activation[34].

The activation of signalling proteins, such as BTK or PI3K, leads to the activation of downstream targets, such as AKT/mTOR, NF-κB, p38 MAP Kinase (MAPK) and ERK (extracellular signal-regulated kinase), and other molecules, leading to cellular proliferation, survival, or apoptosis[16].

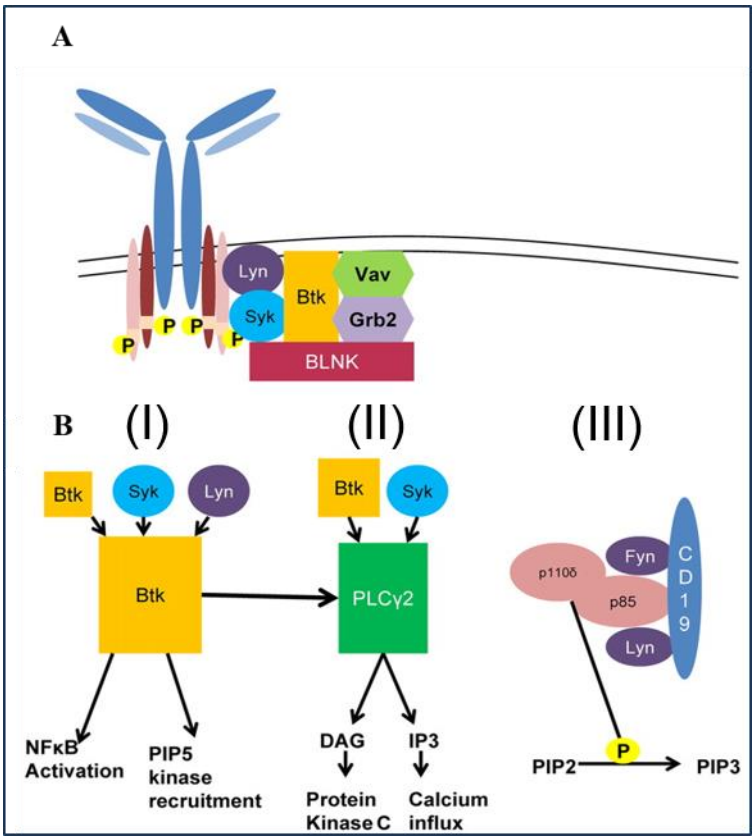

Fig. 4: B-cell receptor signalling in CLL. (A) Phosphorylation of CD79a/CD79b recruits kinases and adaptor proteins, which form the initial signalling complex of the BCR. (B) BCR activation recruits additional kinases leading to activation of three main pathways: (I) BTK, (II) PLC-γ2, and (III) PI3K (p110δ and p85). SYK and LYN phosphorylate BTK, leading to the activation of NFκB and the recruitment of PIP5K. PLC-γ2 is phosphorylated by BTK and SYK and leads to production of DAG and IP3. PI3K activation leads to phosphorylation of PIP2 to PIP3 (image from Woyach *et al.*)[34].

A functional BCR is essential for the survival of mature B cells and in most hematological malignancies, including CLL and non-Hodgkin's lymphoma (NHL)[35]. In CLL, evidence suggests that there is a constitutive BCR signalling *in vivo*, supporting the hypothesis that the surface immunoglobulins are engaged by autoantigens.

The two major CLL subsets, defined by *IGHV* mutational status, reveal *in vivo* contrasting responses toward proliferation or anergy (the inability to respond to BCR-mediated stimuli). The mutated CLL cells are mainly driven towards anergy; in contrast, unmutated leukemic cells are more responsive to surface immunoglobulin stimulation. Anergy is a component of normal B-cell behavior and should increase susceptibility to apoptosis. In leukemic B cells, this susceptibility may be countered by overexpression of the anti-apoptotic BCL-2 protein[36].

Dysregulation of the BCR signalling pathways in CLL is mainly characterized by constitutive phosphorylation of kinases[34]. We demonstrated that LYN kinase is upregulated and constitutively activated in CLL cells with respect to normal B cells, leading to an increased basal total tyrosine phosphorylation and a low responsiveness to BCR ligation[29]. In addition, LYN activates SHIP-1 (SH2-domain-containing inositol phosphatase), which provides negative feedback to BCR signalling by counteracting PI3K activity[10]. At the same time BCR signalling can be enhanced by ZAP70 (the

ζ-associated protein of 70kDa), a cytoplasmic tyrosine kinase expressed in T cells, and abnormally found in approximately half of CLL cases[16,37].

The AKT and BTK pathways are also constitutively activated in CLL[34]. These pathways lead to pro-survival signals through their effects on PI3K, PLC-γ2, and NF-κB. BTK inhibition by the kinase inhibitor PCI-32765 induces apoptosis in a caspase-dependent manner and inhibits both BTK phosphorylation, after IgM ligation, as well as downstream targets of BTK (ERK, NF-κB, and AKT). A subset of CLL patients demonstrates a constitutive phosphorylation of ERK, which is associated with decreased responsiveness to BCR stimulation, like anergic B cells. Moreover, expression of the transcription factor MYC is dependent on ERK1/2 activation after BCR stimulation, suggesting that this pathway is involved in CLL survival and proliferation[34]. Finally, it has been reported that in some CLL cells an alternative transcript of CD79b, ΔCD79b, is upregulated and may explain the reduced BCR expression[38].

This knowledge leads to the development of BCR pathway inhibitors, with promising results in refractory CLL and NHL. Because of the CLL genetic heterogeneity, the use of multiple drugs or multi-targeted agents is necessary to treat this disease[34].

1.2.3 Intrinsic factors: the dysregulation of apoptosis

Apoptosis is a physiological mechanism of programmed cell death, and is essential for development, homeostasis and prevention of tumorigenesis. The apoptotic program escape is one of the hallmarks of cancer and correlates to clinical resistance to therapies. This is particularly true for CLL, characterized by impaired apoptosis[39].

Apoptosis can be mainly triggered through the extrinsic and the intrinsic pathways, and a common execution phase mediated by proteases of the caspase family (**Fig. 5**).

In CLL, a CD5+ subpopulation of B lymphocytes undergoes clonal expansion and progressively accumulate in the bone marrow, lymph nodes and peripheral blood. This situation is due to both CLL defects in apoptotic machinery and the abnormal survival signals delivered by the microenvironment. Leukemic cells in the blood results quiescent and unable to initiate their apoptotic program, although they derived from proliferative pools in the bone marrow and lymph nodes. CLL microenvironment produces chemokines and cytokines that constitutively activate survival pathways, such as NF-kB or PI3K/AKT, leading to the overexpression of key antiapoptotic proteins[39]. Together with the microenvironment stimuli, the anti-apoptotic protein BCL-2 is overexpressed, whereas pro-apoptotic proteins such as BAX and BCL-xL are downregulated[40].

In addition to BCL-2, MCL-1 is a crucial player in impaired apoptosis in CLL cells. This protein is upregulated by PI3K/AKT pathway and MCL-1 silencing is sufficient for inducing CLL cell apoptosis[39].

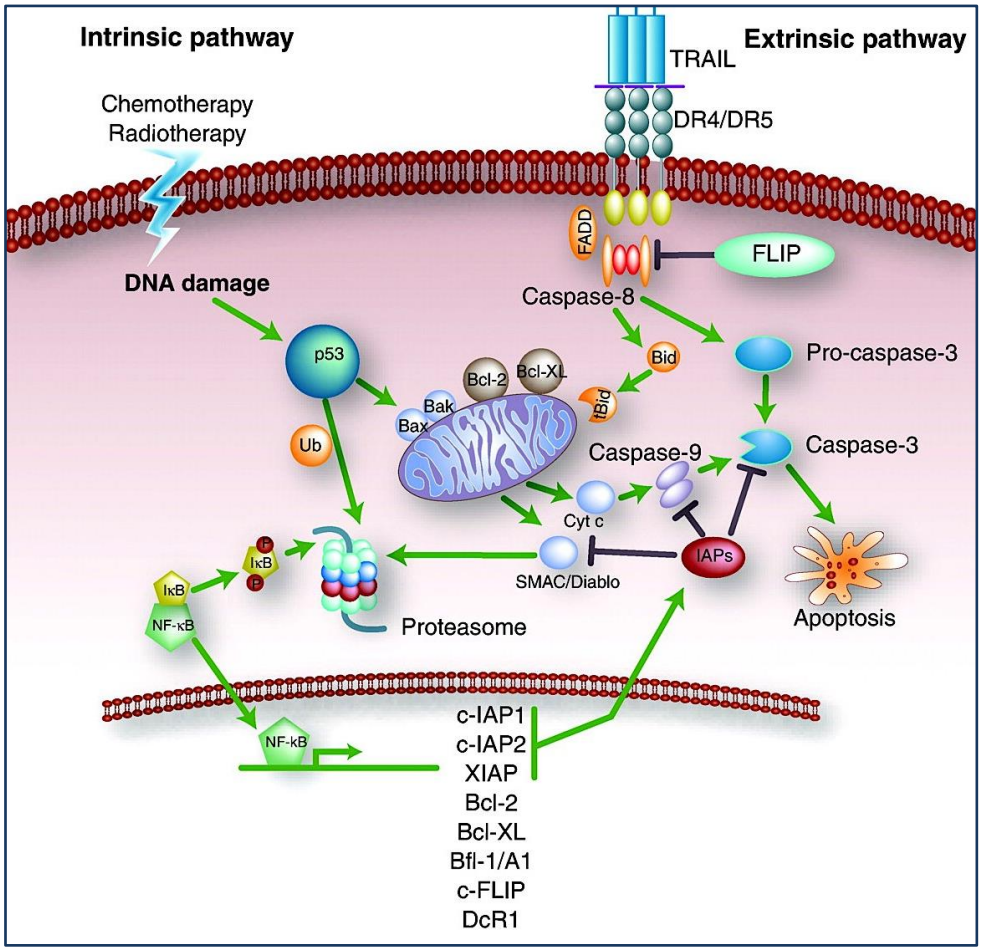

Fig. 5: The molecular mechanisms of apoptosis. Schematic representation of the intrinsic and extrinsic apoptotic pathways. Apoptosis induction via the death receptors can result in activation of both the extrinsic and intrinsic pathways. Chemotherapy and radiotherapy-induced apoptosis is executed via the intrinsic pathways. → activation; ⊣ inhibition (image from de Vries *et al.*)[41].

Other critical factors are represented by del17p13 and del11q23 that involve *TP53* and *ATM*, two tumor-suppressor genes, respectively. TP53 is inactivated in 10–15% of CLL patients, decreasing cell apoptosis and accelerating disease development[40]. TP53 and ATM proteins are central regulators of the DNA-damage-response pathway and their activation leads to cell-cycle arrest, DNA repair, apoptosis, or senescence, depending on the cellular context. Loss of function mutations of TP53 and ATM, even in the absence of a chromosomal deletion, cause adverse effects on patient survival and are associated to CLL chemoresistance[42].

Among novel proteins involved in CLL aberrant cellular proliferation, differentiation, and apoptosis, TOSO, also known as Fas-inhibitory molecule, has been identified. TOSO is a transmembrane protein that inhibits Fas-mediated apoptosis by binding Fas-associated death domain via its C-terminal intracellular domain. In CLL, high levels of TOSO expression correlate with more

aggressive disease, being associated with high leukocyte count, advanced Binet stage, need for chemotherapy and unmutated *IgVH* gene status[43].

1.2.4 Extrinsic factors: CLL microenvironment

Since 1889, when Stephen Paget formulated his "seed and soil" hypothesis, the formation of tumor microenvironment protecting cancer cells emerged as central hallmark of cancer. In CLL the tumor microenvironment, represented by nurse like cells (NLCs), mesenchymal stromal cells (MSCs) and T cells, facilitates leukemic cell survival, proliferation, homing and tissue retention[44]. The observation that CLL cells undergo spontaneous apoptosis when cultured *in vitro*, suggested that the microenvironment may have a crucial role in the survival of CLL cells *in vivo*[1]. CLL cells *in vivo* follow chemokine gradients into lymph nodes, where they form proliferation centers (PCs), which differs from normal germinal centers. PCs are newly formed structures not described in other lymphoproliferative conditions. They contain a pool of proliferating cells that appear as vaguely nodular areas that can be recognized against a monotonous background of small mature-looking lymphocytes[45] (**Fig. 6**). Within PCs, CLL cells are exposed to chemokines, integrins, cytokines and survival factors[10].

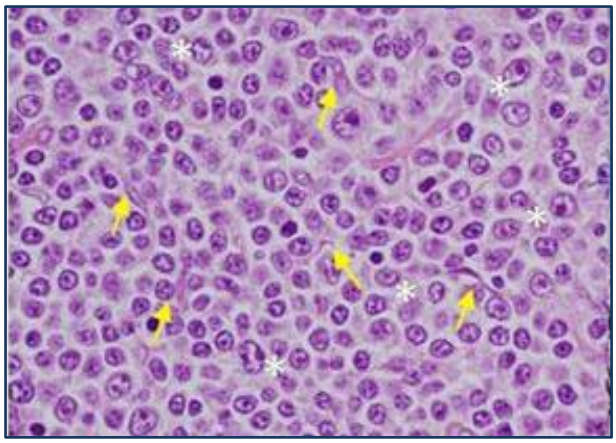

Fig. 6: Hematoxylin-eosin staining (HE) highlighting a proliferation center (PC). Stromal cells not specified are marked with arrows, while neoplastic B prolymphocytes are highlighted with asterisks (image from Herreros *et al.*)[45].

The NLCs express CXCL12 and CXCL13 chemokine, while MSCs predominantly CXCL12. CLL cells interact with these soluble factors by CXCR4/5 receptors, which are significantly overexpressed in CLL as compared to normal B cells[44]. Other chemokine receptors present on leukemic B cells and involved in lymphatic tissue homing are CXCR3 and CCR7. Moreover, the binding of CD31 (on NLCs) and CD38 (on CLL cells) leads to phosphorylation of ZAP-70. The recruitment of ZAP-70 and other kinases to the activated BCR complex increases the capability of leukemic B cells for antigen response.

NLCs express pro-survival signals (*e.g.* BAFF and APRIL, proteins belonging to TNF family) which bind to their respective receptors on leukemic B cells (*e.g.* BCMA, TACI, and BAFF-R). The interaction between VLA-4 (CD49d), expressed by CLL cells and VCAM-1 and fibronectin, expressed in MSCs, facilitates cell-cell adhesion promoting immune recognition, survival and expansion of the leukemic clone[44] (**Fig. 7**).

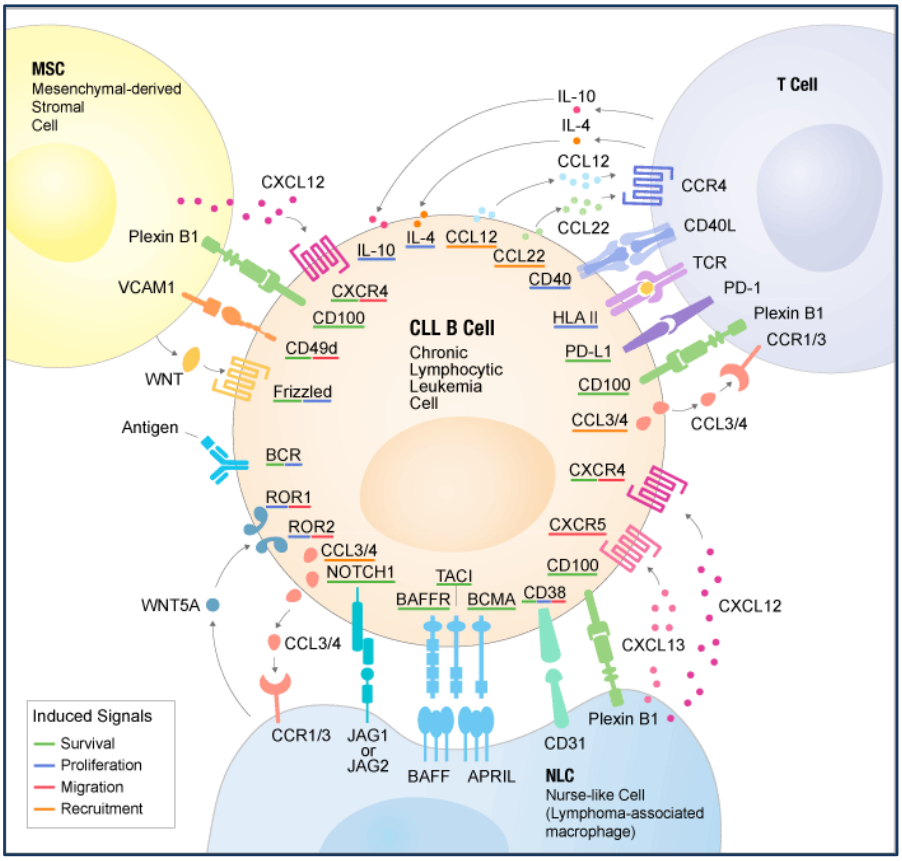

Fig. 7: CLL microenvironment: CLL cells interact with MSC, NLC and T-cells through adhesion molecules and chemokine receptors expressed on CLL cells. These interactions, in addition to B-cell receptor engagement, promote CLL survival, proliferation, and homing to tissues (image from www.abnova.com).

Therefore, CLL cells shape the microenvironment resulting not simply "seeds" that grow in a supportive "soil". Indeed, upon BCR activation, CLL cells secrete cytokines, such as CCL3, which attract T cells and monocytes to the niche[46]. T cells release cytokines, such as IL-4, which can upregulate surface IgM, facilitating the interaction of CLL cells with autoantigens. T cells in PCs express CD40L and interact with CLL cells expressing CD40. This binding causes B cell activation. The expression of the programmed death 1 receptor (PD1) by T cells is also interesting, as the respective ligand, PD-L1, is present on leukemic B cells, which exploit this interaction to resist to antitumor immune reactions[44]. The ability of CLL cells to proliferate and migrate in response to chemokines is promoted by ROR1 (tyrosine-kinase-like transmembrane receptor) pathway, activated by WNT5. High-levels of ROR1 expression in CLL cells are associated with accelerated disease progression.

The most advanced approaches for the molecular targeting of the microenvironment are represented by CXCR4 antagonists with the aim to disrupt the *nexus* between CLL cells and the microenvironment, blocking the pro-survival stimuli[46].

1.3 Diagnosis and clinical features

CLL is a clinical and biological heterogeneous disease. Most of the patients are asymptomatic at the time of diagnosis, becoming aware of the disease after a routine blood count, and do not need treatments. Other patients experience a more aggressive disease, treatment failure and poor overall survival[16]. As a consequence, novel biological and cytogenetic features have become increasingly important in predicting prognosis at the time of diagnosis[47] and the research for molecules involved in apoptosis resistance and increased survival of neoplastic B cells is still ongoing.

CLL symptoms can include fatigue, weight loss, excessive night sweats, abdominal fullness and increased frequency of infections, which might be associated with hypogammaglobulinaemia or autoimmune cytopenia, that could induce immunodeficiency and high mortality for infections. Patients can also show enlarged lymph nodes, hepatomegaly and splenomegaly[10].

The iwCLL (International Workshop on Chronic Lymphocytic Leukemia) guidelines give recommendations on diagnostic criteria for CLL. In most cases the diagnosis of CLL is established by blood counts, blood smear, and immunophenotyping[3]:

1. the initial diagnosis requires detection of ≥5,000 cells per μl of clonal CLL cells for the duration of at least 3 months.

   The lymphocytes in the blood smear are characteristically small, mature lymphocytes with poorly cytoplasm and dense nucleus lacking discernible nucleoli with partially aggregated chromatin. Gumprecht nuclear shadows, which are degenerated cells broken during slide preparation, are also present in the peripheral blood smear (**Fig. 8**)[3];

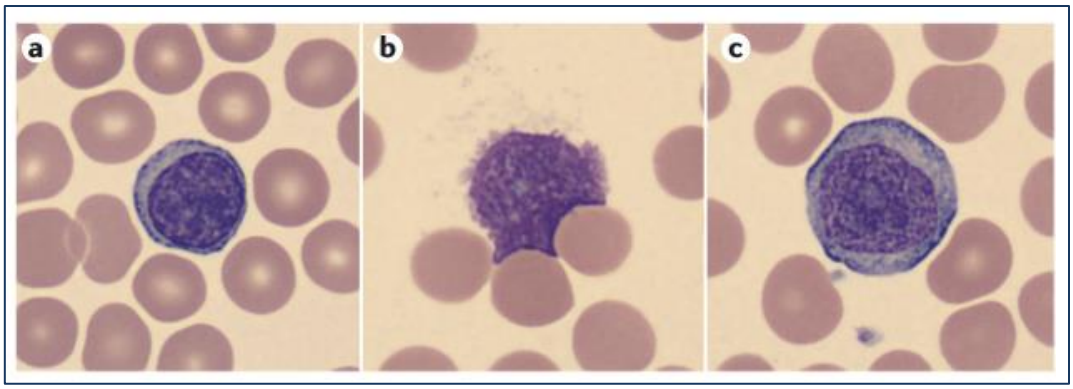

Fig. 8: Peripheral smear of CLL. The peripheral blood smear stained with Giemsa-Wright, shows a typical B lymphocyte of CLL (a), Gumprecht shadow (b) and a prolymphocyte (c) (adapted from Kipps *et al*., 2017)[10].

2. bone marrow lymphocytic infiltration exceeding 30% of the nucleated cells (**Fig. 9**). Although the type of bone marrow infiltration (diffuse *vs* not diffuse) reflects the size of the tumor and provides feedback about the prognosis, recent studies have shown that the prognostic value of Bone Marrow Biopsy (BOM) can be replaced by new prognostic factors[48];

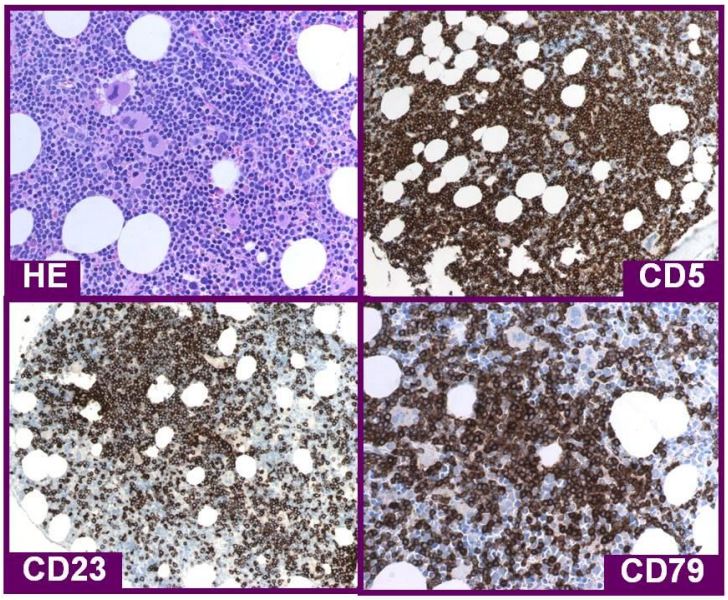

Fig. 9: Bone marrow biopsy of a CLL patient. Preparation stained with hematoxylin-eosin (HE) and three immunohistological pictures showing some diagnostic markers (CD5, CD23, CD79a).

3. CLL immunophenotype is characterized by three elements: i) expression of low levels of surface immunoglobulin with restricted κ-immunoglobulin or λ-immunoglobulin light chains; ii) the co-expression of CD5 (T-cell antigen), CD19, low levels of CD20, CD23 (**Fig. 10**), CD200 (also known as OX-2 membrane glycoprotein), to distinguish CLL from mantle cell lymphoma, and the onco-embryonic surface antigen ROR1 (>95% of patients); iii) low levels of CD79b and mainly IgM and IgD surface immunoglobulin, followed by IgG, and IgA; it is not unusual to find an IgM IgD co-expression[10].

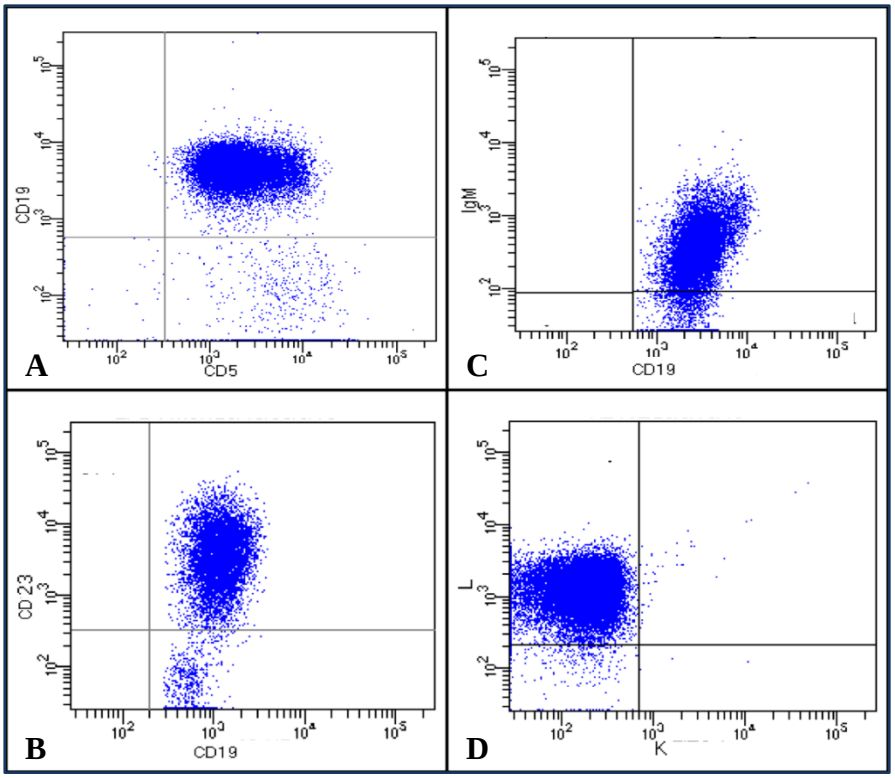

Fig. 10: Cytograms of a representative case of CLL. B lymphocytes analyzed (CD19+) are positive to CD5 **(A)**, to CD23 **(B)**, to low intensity surface IgM (sIgM) **(C)** and express one type of immunoglobuline light chain, λ **(D)**. Gate on live lymphocytes.

Before starting an immunotherapy, it is important to assess the absence of viral infection, such as Hepatitis B virus (HBV), Hepatitis C virus (HCV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Human Immunodeficiency virus (HIV)[48].

1.4 Prognosis

Newly-diagnosed CLL patient clinical course is extremely variable. In fact, some patients remain free of symptoms and are fully active for decades, whereas others rapidly become symptomatic or develop high-risk disease. It is so necessary to stratify patients on the disease gravity in order to define the more appropriate therapy[10].

To stratify CLL patients into prognostic groups, two clinical staging systems are widely used: the Rai staging system (more used in the United States), and the Binet classification (more used in Europe)[10]. These two staging systems are simple, inexpensive, and rely on a physical examination and standard laboratory tests[3].

According to the modified Rai staging system:

1. **low-risk** patients present lymphocytosis with leukemic cells in the blood and/or marrow >30% (former Rai stage 0);
2. **intermediate-risk** patients show lymphocytosis, enlarged nodes in any site, and splenomegaly and/or hepatomegaly (formerly considered Rai stage I or stage II);

3. **high-risk** patients have disease-related anemia (hemoglobin (Hb) levels <11 g/dl) (formerly stage III) or thrombocytopenia (platelet count <100x10^9/L) (formerly stage IV)[3].

The Binet staging system is based on the number of involved areas with the presence of enlarged lymph nodes >1 cm in diameter or organomegaly, and on anemia and thrombocytopenia. The Binet staging system defines:

1. **stage A**: Hb ≥10 g/dL, platelets ≥100x10^9/L;
2. **stage B**: Hb ≥10 g/dL, platelets ≥100x10^9/L and organomegaly greater than that defined for stage A (*i.e.*, 3 or more areas of nodal or organ enlargement);
3. **stage C**: Hb <10 g/dL and/or a platelet count of <100x10^9/L.

The less recent biological prognostic factors correlate with the expansion of the leukemic clone. They become indicative only when the disease is worsening. Their utility is thus limited since they do not allow to plan early therapeutic strategies. The biological prognostic factors comprehend:

1. **β2 microglobulin**: its levels inversely correlate with the survival. It is related with the lymphocyte doubling time. β2 microglobulin increase indicates high neoplastic cell proliferation[49].
2. **Thymidine kinase (s-TK) level**: elevated s-TK levels in CLL patient serum predict disease progression. Patients with s-TK values greater than 7.1U/L have a median progression free survival (PFS) of 8 months, whereas patients with s-TK values ≤7.1U/L show much longer PFS[50].
3. **Soluble CD23 (s-CD23)**: s-CD23 determination in early-stage patients may help to the identification of patients with progression[51].

The new prognostic markers can better determine the clinical course from the diagnosis of the disease, and include:

1. **Somatic Hypermutations** (SHM) of the Ig heavy chain variable region (*IGHV*) genes: CLL patients are divided in two subgroups depending on the *IGHV* genes mutational status defined, unmutated (SHM-), and mutated (SHM+). Conventionally, patients with ≥98% homology to the closest germ-line gene are defined unmutated and have a poorer prognosis than patients with CLL cells displaying <98% homology, defined mutated (**Fig. 11**)[52,53].

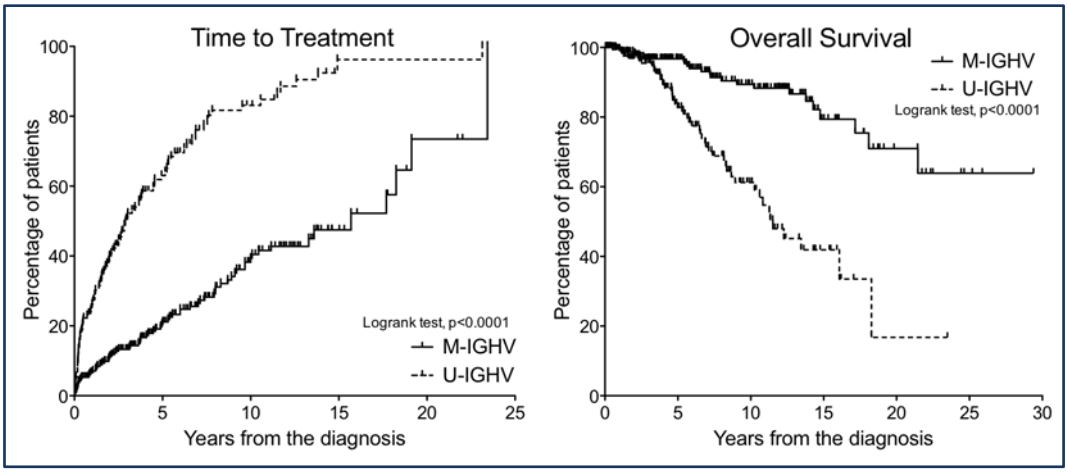

Fig. 11: Time to Treatment and Overall Survival in relationship with *IGHV* prognostic marker. The median time to treatment and Overall Survival for mutated (M-*IGHV*) and unmutated (U-*IGHV*) CLL patients (adapted from Visentin *et al*.) [54].

2. **Chromosomal alterations**. CLL genomic aberrations (del11q, del13q, del17p, and 12 trisomy) can be identified in approximately 80% of CLL patients by fluorescence *in situ* hybridization (FISH). Del11q and del17p have been associated with rapid disease progression, while the absence of other chromosomal abnormalities, and the presence of del13q is associated with a better prognosis[17] (**Fig. 12**). Chromosome alterations are independent from *IGHV* mutational status, although is evident a more frequency of del11q and del17p in unmutated and del13q in mutated cases.

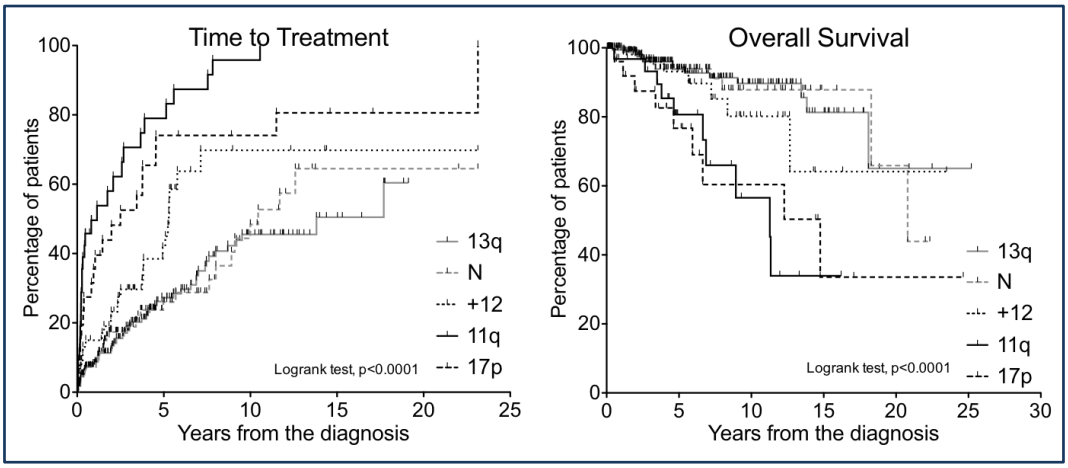

Fig. 12: Time to treatment and probability of survival among patients with common chromosomal alterations. The median survival times and time to treatment for the groups with 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion as single abnormality were 0.18, 1.75, 3.81, 11.56, and 8.00 years, respectively (adapted from Visentin *et al*.) [54].

3. **CD38 expression.** CD38 is a transmembrane glycoprotein that may be overexpressed on the surface of CLL cells. Cases with CD38+ B cells >30% show a bad prognosis (**Fig. 13**). However, the cut-off to discriminate CD38+ to CD38- is not unanimously defined, some authors preferring 30%[54] or 7%[55] of CD38+ B cells[52].

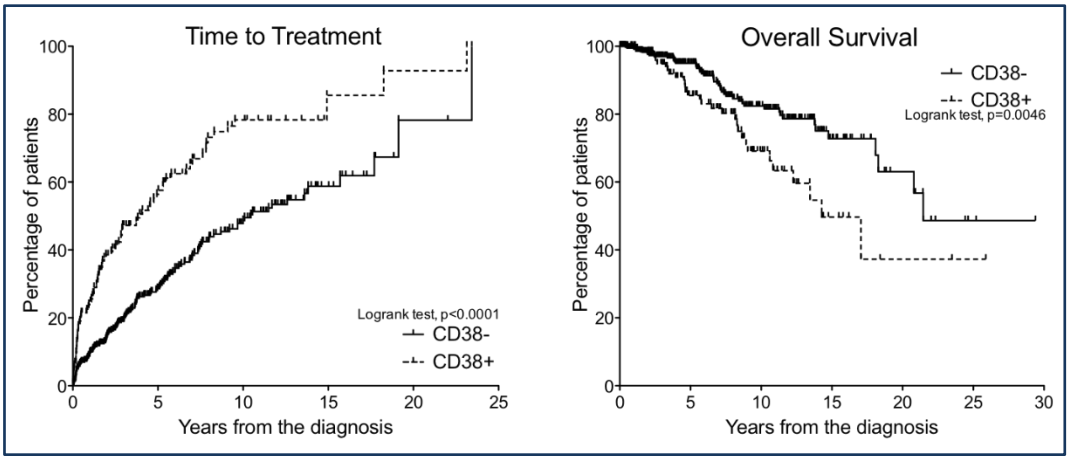

Fig. 13: Time to Treatment and Overall Survival in relationship with CD38 prognostic marker. The median time to treatment and Overall Survival for patients with a high (CD38+) or low (CD38-) number of cells expressing this transmembrane glycoprotein. The threshold for CD38 expression was set at 30%; values ≥30% were defined as CD38+ and < 30% as CD38- (adapted from Visentin *et al.*) [54].

4. **Intracytoplasmatic expression of protein kinase associated to TCR ζ chain of 70kDa (ZAP-70).** Zeta-associated protein of 70kDa (ZAP-70) is a cytoplasmic tyrosine kinase. It is a key signalling molecule for T lymphocytes and NK cells. ZAP-70 expression may reflect an activation state of the malignant clone associated with progressive disease (**Fig. 14**). The expression of ZAP-70 may change over time in CLL, particularly during clinical progression, thus it is important to monitor ZAP-70 expression during the evolution of the disease[56].

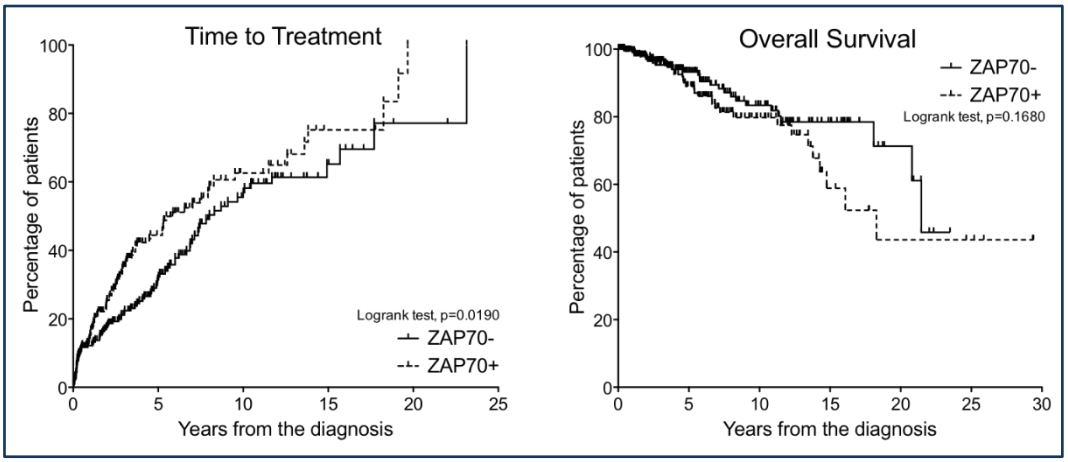

Fig. 14: Time to Treatment and Overall Survival in relationship with ZAP70 prognostic marker. The median time to treatment and overall survival for the groups with high (ZAP70+) or low (ZAP70-) expression of this protein (adapted from Visentin *et al.*) [54].

5. **CD49d expression.** CD49d, the α4 subunit of the integrin heterodimer α4β1, is a surface molecule which promotes microenvironment-mediated proliferation of CLL cells and identifies a subgroup of patients characterized by progressive course and short survival, independent from CD38 or ZAP-70 expression. Actually, CD49d is the best flow cytometry–based marker for CLL patients' prognosis stratification[57].

6. **Telomerase expression and telomere length in CLL**. Activation of telomerase reverse transcriptase (hTERT) plays a critical role in tumorigenesis[58]. Recently, the levels of telomerase activity (TA) and/or hTERT expression were positively correlated to clinical aggressiveness and prognosis in several malignancies, including CLL (**Fig. 15**).

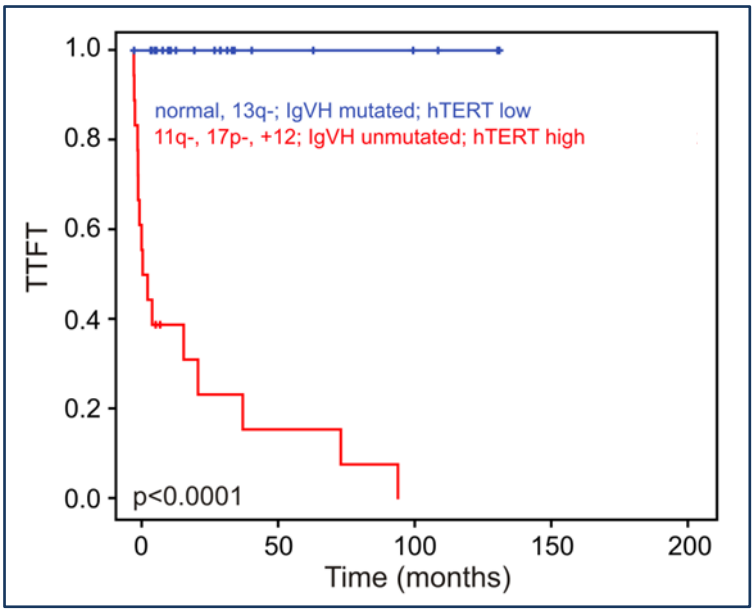

Fig. 15: Curves of treatment-free survival. Time from diagnosis to first treatment (TTFT) according to IgVH mutational status, chromosomal categories, and hTERT level/telomere length profile (adapted from Rampazzo *et al.*)[59].

1.5 From biology into treatment

According to the updated iwCLL guidelines, asymptomatic patients with early-stage disease (Rai 0, Binet A), should be monitored without therapy unless they show disease progression. In fact, studies on early stage disease were unable to show a benefit of early therapeutic interventions. When patients have progressive or symptomatic/active disease, treatment should be initiated. The iwCLL guidelines list some relevant conditions to define symptomatic or active disease[3]:

1. progressive marrow failure (anemia and/or thrombocytopenia; usually Binet stage C or Rai stage III or IV);
2. massive (*i.e.*, ≥6 cm below the left costal margin) or progressive or symptomatic splenomegaly;
3. massive nodes (*i.e.*, ≥10 cm in longest diameter) or progressive or symptomatic lymphadenopathy;
4. progressive lymphocytosis with an increase of >50% over a 2-month period;
5. lymphocyte doubling time (LDT) <6 months; it should be noted that patients with initial blood lymphocyte counts <30,000/mL may require a longer observation period to determine the LDT;

6. autoimmune anemia and/or thrombocytopenia poorly responsive to corticosteroids or other standard therapies;
7. disease-related symptoms, such as unintentional weight loss ≥10% within the previous 6 months, significant fatigue, night sweats, fevers for 2 or more weeks without other evidence of infection.

Moreover, several genes recurrently mutated at diagnosis (*e.g. NOTCH1*, *SF3B1* and *BIRC3*) confer individually a poor outcome in patients with CLL and are associated with adverse clinical and biological features. These observations lead to an integrated mutational and cytogenetic stratification of patients, as follows:

1. **very low-risk:** only 13q14 deleted;
2. **low-risk:** trisomy 12 or patients with normal karyotype;
3. **intermediate-risk**: *NOTCH1* and/or *SF3B1* mutated and/or del11q;
4. **high-risk**: *TP53* and/or *BIRC3* disrupted.

Importantly, ~20% of patients belonging to the low-risk categories were reclassified into higher-risk owing to the presence of *NOTCH1*, *SF3B1* and *TP53* mutations and *BIRC3* disruption, thus significantly improving the accuracy of prediction of clinical evolution (**Fig. 16**).

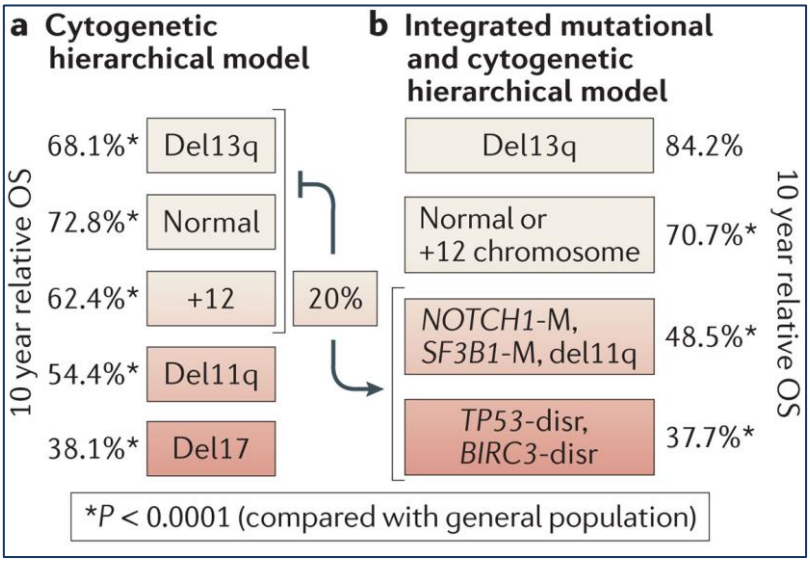

Fig. 16: Integrated mutational and cytogenetic hierarchical model. Ten-year overall survival (OS) relative to a matched general population according to CLL risk class as defined based on the classic cytogenetic (a) or the new mutational and cytogenetic integrated hierarchical schema (b). The accuracy of survival prediction is significantly improved by including NOTCH1, SF3B1 and TP53 mutations and BIRC3 disruption in the model, owing to reclassification of ~20% of low-risk patients into higher-risk classes (image from Fabbri *et al.*)[1].

This integrated mutational and cytogenetic model maintains its independent prognostic value also during disease progression[1].

During the last years, new strategies were developed for CLL treatment in order to create a more personalized approach for patients' therapy. In the 1990s, CLL treatment was generally performed with alkylating agents like chlorambucil and cyclophosphamide and, later, the purine

analogue fludarabine. In the 2000's, the development of the anti-CD20 monoclonal antibody, rituximab, was the first advancing step; second generation of anti-CD20 monoclonal antibody, ofatumumab, and an anti-CD52 monoclonal antibody, alemtuzumab, also came into the clinic, to treat higher risk and refractory patients.

In 2010, combination regimens of fludarabine plus cyclophosphamide (FC) and, successively, FC plus rituximab (FCR) became widely used as a standard frontline therapy for young and fit CLL patients[5,60]. In those years, bendamustine, another alkylating agent, used in combination or not with rituximab (BR), was an important treatment option. CLL trials demonstrated that BR regimen was less active than FCR but, because of the similar survival and better tolerance, BR was accepted as an alternative therapy for olders and patients with co-morbidities.

Despite the initial success of anti-CD20-based chemoimmunotherapy, poor efficacy and short progression free survival was found in patients with del17p or *TP53* mutation.

In the last five years, the improving in understanding the biology of BCR signalling in CLL leads to the development of new therapeutic strategies, which involve small molecular inhibitors including ibrutinib, a BTK inhibitor, idelalisib, a PI3Kδ inhibitor, and venetoclax, a BCL-2 protein antagonist (**Fig. 17**).

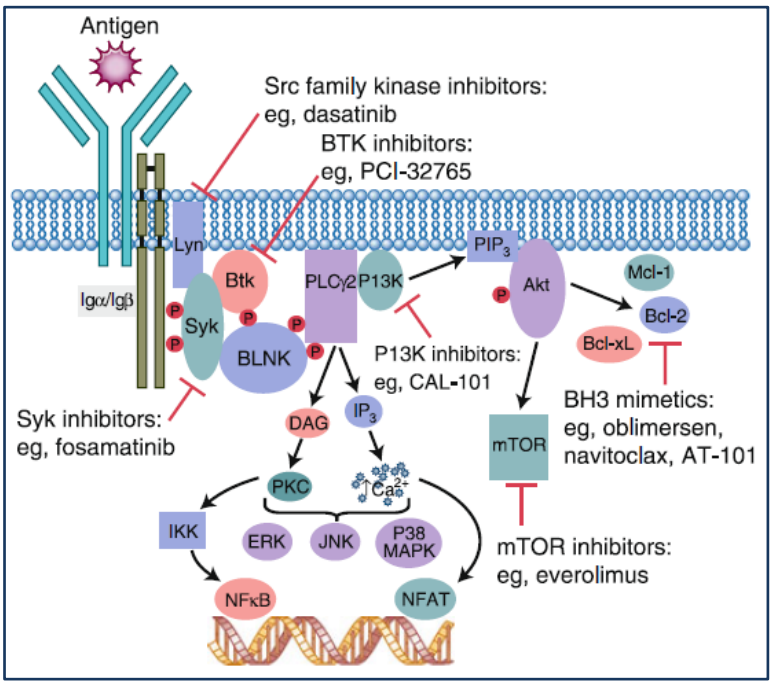

Fig. 17: BCR signalling pathways and targets of novel agents in CLL (image from Riches *et al*., 2011)[61].

These improvements provide excellent treatment options for all CLL patients, particularly for older patients with co-morbidities, poor prognostic features, or relapsed/refractory disease (**Fig. 18**). For example, ibrutinib is now considered the standard in first-line therapy for del17p CLL patients[5,60].

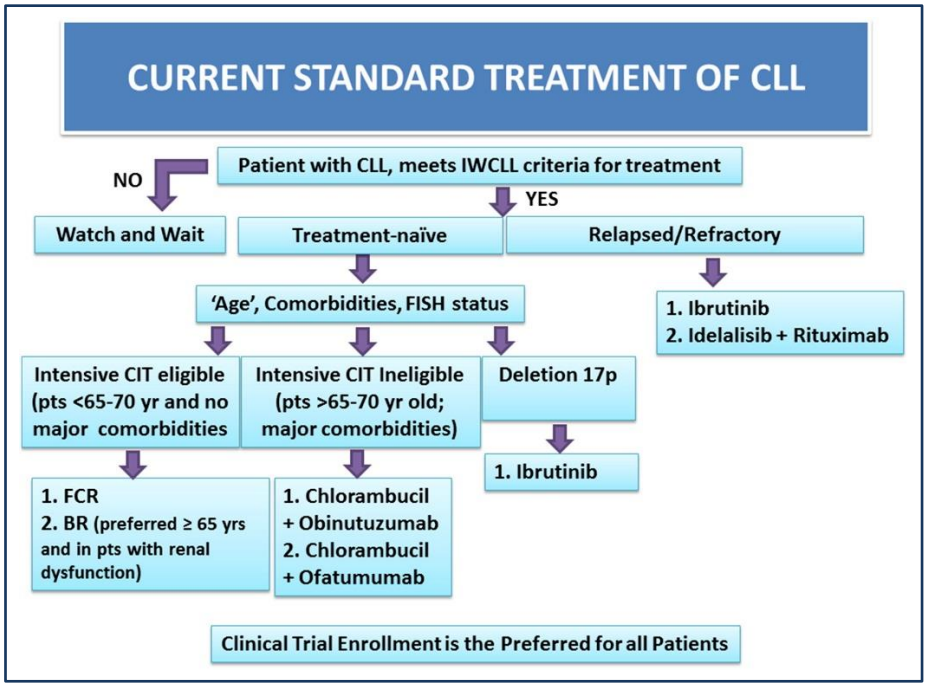

Fig. 18: Treatment algorithm for patients with CLL (image from Jain *et al*., 2016)[62].

It has been hypothesized that these kinase inhibitors may act on several mechanisms, such as BCR activation and microenvironmental interactions. Their activity seems to be independent from genetic lesions, such as *MYD88* and *NF-κB* pathway mutations. However, unidentified genetic lesions may also impinge on the BCR cascade.

Although high-risk patients, with *TP53* disruption and *IGHV*-UM status, initially respond to kinases inhibitors, follow-up studies revealed long-term disease progression. This phenomenon might be explained by treatment resistance caused by acquired mutations in *BTK* (affecting the ibrutinib binding site) or activation of phospholipase Cγ2 (PLCγ2), which acts downstream of BTK.

In the next future, agents specifically targeting other CLL-associated genetic lesions (*e.g. NOTCH1, NF- κB, SF3B1*) may achieve higher specificity and less toxicity[1].

The recently developed treatments options, although encouraging, do not achieve disease eradication. Because of the indolent nature of the disease, the therapies do not aim at complete recovery, but at the control of the expansion of neoplastic cells. In addition to cytoreductive therapy, CLL treatment includes substitutive and support therapies, such as red cell and platelet transfusions, antibiotic therapy, and intravenous immunoglobulin administrations. The optimum duration of treatment with kinase inhibitors, the mechanisms of resistance, the potential role of combinations of targeted agents, and the evaluation of risk of evolution in Richter syndrome have still to be defined trough clinical trials[5].

2. The Heat Shock Protein of 70kDa (HSP70) and the Heat Shock Factor 1 (HSF1)

2.1 The Heat shock proteins

The Heat shock proteins (HSPs) are a family of evolutionarily conserved and ubiquitously expressed molecular chaperones, found in all organisms and in different subcellular compartments[63] (**Fig. 19**).

These proteins are commonly known as "heat-shock" proteins because of the method first used in 1974 to upregulate their expression[64]. The upregulation of these genes is called the heat shock response (HSR), although HSPs can be triggered by different stresses that damage proteins. HSPs are upregulated when cells undergo physiological and environmental stresses (hyperthermia, ischemia, anoxia, toxins or ultra violet (UV) light, viral particles, surgical/emotional/mechanical stress)[63].

There are also several HSPs, such as HSC70, GRP78, MTP70, and HSP90, which are constitutively expressed under normal conditions[64]. In fact, HSPs are involved in physiological cellular processes with the functions of folding new synthetized proteins, assembling multiprotein complexes, transporting/sorting of proteins to correct subcellular compartments, cell-cycle controlling and signalling. More recently, HSPs have been involved in antigen presentation to specialized cells of the immune system[65].

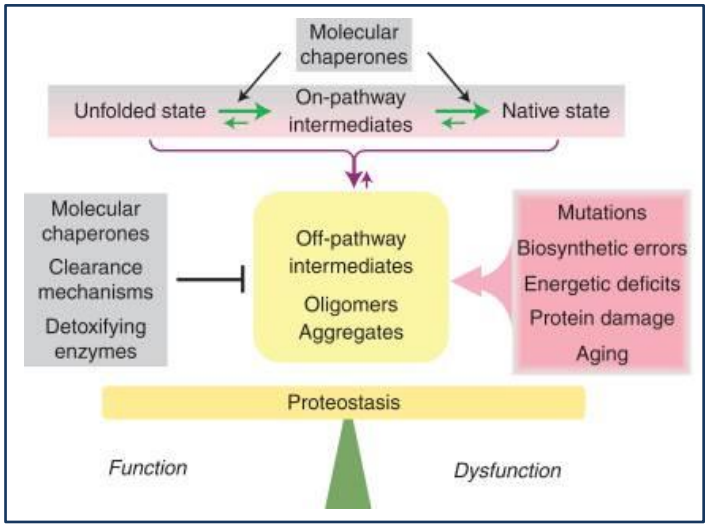

Fig. 19: **Cellular protein homeostasis (proteostasis).** To maintain proteins in a functionally folded state, cells must balance intrinsic and extrinsic forces that perturb protein folding. Molecular chaperones participate at multiple levels in protein biogenesis assisting in the *de novo* folding and preventing deleterious intermolecular interactions (image from Gidalevitz *et al*.)[66].

Mammalian HSPs are divided into six families (**Table 1**), based on their relative molecular weight: the small HSPs (sHSPs) consisting in 11 ubiquitous molecular chaperones encoded by *HSPB* genes; the HSP40 family, containing 49 members that are encoded by *DNAJ* genes; the chaperonin family of HSP60, that consists of 14 members, which are encoded by *HSPD, HSPE, CCT* and other genes; the HSP70 family, containing 13 members encoded by *HSPA* gene family; the HSP90 family,

encoded by *HSPC* gene family and the family of large HSPs, consisting in HSP110 and GRP170, encoded by *HSPH* gene family.[63] HSPs can also be subdivided based on their mechanism of action, into ATP-independent and ATP-dependent proteins[67].

Table 1: HSPs classification according to the protein data bank japan, PDBJ.

Hsp family			
Family (weight)	**Procaryotes**	**Eucaryotes**	**Function**
Hsp10 (10kDa)	GroES	Hsp10	Serve as co-chaperones for Hsp60
Hsp40 (40kDa)	DnaJ	Hsp40	Serve as co-chaperones for Hsp70
Hsp60 (60kDa)	GroEL	Hsp60, TRiC	Assist protein folding
Hsp70 (70kDa)	DnaK	Hsp70, Hsc70, Hsp110	Assist protein folding and involved in transport of proteins to organelles
Hsp90 (90kDa)	HtpG	Hsp83, Hsp89, Hsp90	Stabilize misfolded proteins and regulate the activity of signalling proteins, steroid hormone receptors, and tyrosine kinases
Hsp100 (100kDa)	ClpA, ClpB, ClpX	Hsp104	Regulate protein aggregation, and thermotolerance

2.2 The HSP70 family

The HSP70 family is the most conserved in evolution: it is found in all organisms, from archaebacteria and plants to humans[68]. Interestingly, all eukaryotes have more than one gene encoding HSP70 proteins: the fungus *Blastocladiella emersonii* has 10 putative family members; the yeast contains 8 HSP70 genes; the human HSP70 family comprises at least 13 gene products that differ from each other by amino acid sequence, expression level and sub-cellular localization (**Table 2**). The inducible and the constitutive forms of the HSP70s, called HSP72 (or simply HSP70) and HSP73, respectively, are expressed prevalently in the cytosol, while other HSP70 members are mitochondrial (mtHSP70) or localized in the endoplasmic reticulum (vHSP78). The HSP70 family comprises surface and extracellular proteins and different HSP70s are selectively expressed on the cell surface of viral/bacterial infected cells or of tumor cells. Extracellular HSP70s exist in free soluble forms, complexed to antigenic peptides, or associated with exosomes[68].

HSP70s are assisted by several co-chaperones that constitute up to 3% of the total protein mass of unstressed cells. The co-chaperones are essential for HSP70 activity, since they trigger the binding to the client proteins[69].

Table 2: HSP70 family proteins (adapted from J. Radons, 2016)[70].

Protein	Alternative name	Cellular localization	Chromosome	Stress-inducibile
HspA1A	Hsp70-1, Hsp72, HspA1, Hsp70-1A, Hsp70i	Cytosol, nucleus, cell. membrane, extracell. exosomes	6p21.3	Yes
HspA1B	Hsp70-2, Hsp70-1B	Cytosol, nucleus, cell. membrane, extracell. exosomes	6p21.3	Yes
HspA1L	Hsp70-1L, Hsp70-hom, Hsp70-1t, Hum70t	Cytosol, nucleus	6p21.3	No
HspA2	Heat shock 70 kD protein 2, Hsp70.2	Cytosol, nucleus, cell. membrane, extracell. exosomes	14q24.1	No
HspA5	Hsp70-5, BiP, Grp78, Mif-2 ER	ER, extracell. exosomes	9q33.3	No
HspA6	Hsp70-5, Hsp70B’	Cytosol, extracell. exosomes	1q23	Yes
HspA7	Hsp70-7, Hsp70B	Blood microparticles, extracell. exosomes	1q23.3	Yes
HspA8	Hsp70-8, Hsc70, Hsc71, Hsp71, Hsp73	Cytosol, nucleus, cell. membrane, extracell. exosomes	11q24.1	No
HspA9	Hsp70-9, Grp75, HspA9B, MOT, MOT2	Mitochondria, nucleus	5q31.1	No
HspA12A	Hsp70-12A, FLJ13874, KIAA0417	extracell. and intracell. exosomes	10q26.12	No
HspA12B	Hsp70-12B, RP23-32L15.1	Endothelial cells, intracellular, blood plasma	20p13	No
HspA13	Hsp70-13, Stch	ER, extracellular exosomes, microsomes	21q11	No
HspA14	Hsp70-14, Hsp70L1	Cytosol, cell. membrane	10p13	Yes

The human HSP70 proteins display a domain structure consisting of: (i) a conserved ATPase domain (Nucleotide-binding domain, NBD); (ii) a middle region with protease sensitive sites; (iii) a peptide/substrate binding domain (PBD or SBD) and a G/P-rich C-terminal region containing an EEVD-motif enabling the proteins to bind co-chaperones and other HSPs[68] (**Fig. 20**).

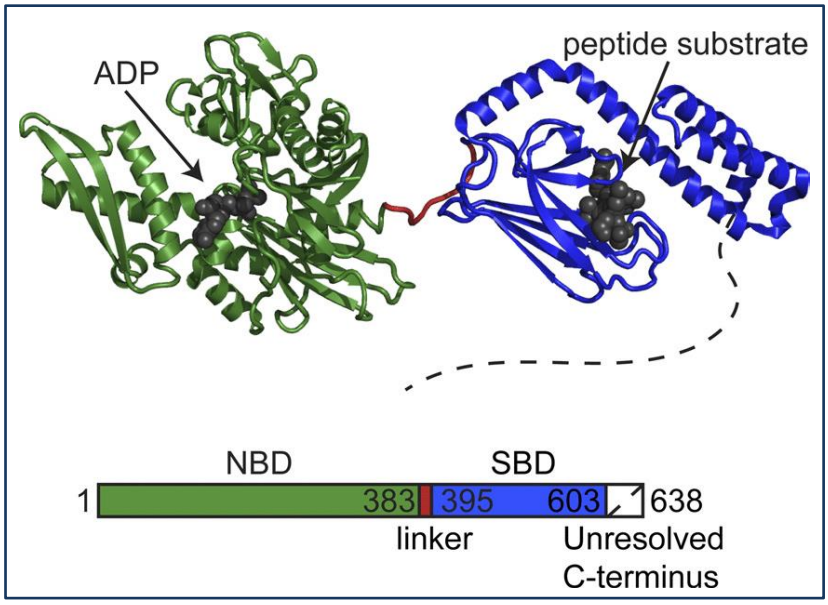

Fig. 20: HSP70 structure in a substrate-bound conformation. The NBD (green) is joined to the SBD (blue) through a short interdomain linker (red). A peptide substrate is sandwiched between the SBD α-sandwich subdomain and β-helical lid, with 35 crystallographically unresolved residues in the C-terminal tail. (image from Smock *et al.*)[71].

2.2.1 Proposed model of HSP70 reaction cycle

HSP70 inactive form binds to an ATP molecule, the NBD and the SBD domains are docked, the substrate binding groove (SBG) domain is open, and HSP70 exhibits low affinity for its substrate. HSP70 co-chaperones [*e.g.* HSP40 family proteins, HOP, also known as stress-inducible protein 1 (STI-1), and BAG-1 (BCL2-binding athanogene-1)], facilitate ATP hydrolysis and nucleotide exchange. Homodimeric HSP40s bind to unfolded polypeptides via their C-terminal SBD region, to prevent protein aggregation, and present them to HSP70 by interacting with its N-terminal ATPase domain. This interaction induces HSP70 conformational change, which stimulates ATP hydrolysis and mediates the closing of SBG domain. HSP70 is now bound to ADP, the docking between the NBD and the SBD domains is interrupted and the chaperone exhibits high affinity for its substrate. HOP stabilizes client proteins and mediates their transfer to HSP90 in a coordinated folding process. ADP is released through BAG1 interaction with HSP70 ATPase domain, followed by a conformational change to release the substrate. This cycle is repeated till the client protein is correctly folded[70] (**Fig. 21**).

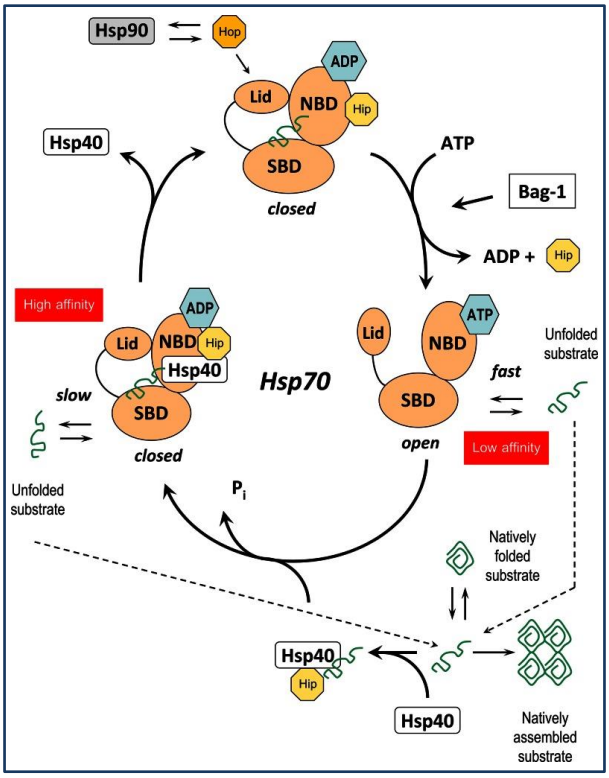

Fig. 21: Proposed model of the HSP70 chaperone reaction cycle. In the ATP-bound state, the NBD and the SBD domains are docked, the SBG is open, and HSP70 exhibits low affinity and fast exchange rates for its substrate. ATP hydrolysis is facilitated by J-domain proteins (JDPs) of the HSP40/DNAJ family and nucleotide exchange by nucleotide exchange factors (NEFs) such as BAG-1. Hsp40 interacts with the N-terminal ATPase domain of HSP70 thereby inducing a conformational change which stimulates ATP hydrolysis and mediates the closing of SBG. In the ADP-bound state, the docking between the two domains of HSP70 is interrupted and the chaperone exhibits high affinity and slow exchange rates for its substrate. Coupling to other chaperones is mediated by HOP (STI-1) which binds to the C-terminal domain of HSP70 and HSP90, thereby passing clients to HSP90. The released substrate can be either folded into the native protein, rebound to HSP70, or natively assembled into oligomers (image from Radons *et al.*)[70].

If the polypeptide is unable to proceed to its native state after multiple cycles of binding and release from the chaperone, HSP70 presents the misfolded polypeptide to the ubiquitin/proteasome system for degradation[72]. This last process is mediated by CHIP (the co-chaperone carboxyl terminus of HSP70-interacting protein) and BAG1. CHIP is both a co-chaperone and an E3 ubiquitin ligase, and it mediates ubiquitination of substrates presented by HSP70. BAG1 has an ubiquitin-like domain (Ubl), which allows its simultaneous binding to HSP70 and the proteasome, facilitating the degradation of substrates[73].

2.3 HSF1 and the Heat shock response

The HSR, consisting in HSP upregulation, is an ancient and sophisticated cytoprotective mechanism, which preserves organismal survival and longevity in proteotoxic stress conditions. The HSR assumes high importance in human pathology, as HSP levels increase in cancer, promoting tumorigenesis, while decline in protein aggregation disorders such as Alzheimer's disease.

The heat shock factors (HSFs) are proteins which regulate the HSR, by promoting *HSP* genes transcription. Known vertebrate HSFs are HSF1, 2, 3, 4 and HSFY[63]. These transcription factors are

characterized by a highly conserved N-terminal helix-turn-helix DNA binding domain and a C-terminal transactivation domain. Among the HSFs, HSF1 is considered the master regulator of the HSR. HSF1 is also involved in processes associated with development, growth, fertility and longevity[74]. Under nonstress conditions, HSF1 exists as inactive monomer in association with HSP70 and HSP90, in the cytoplasm. In response to stress, when the levels of unfolded proteins increase, HSPs release HSF1 and act as molecular chaperones for unfolded molecules. HSF1 is phosphorylated by several kinases, trimerizes, and translocates into the nucleus, where it binds the heat shock elements (HSEs), triggering *HSP* genes transcription[70] (**Fig. 22**).

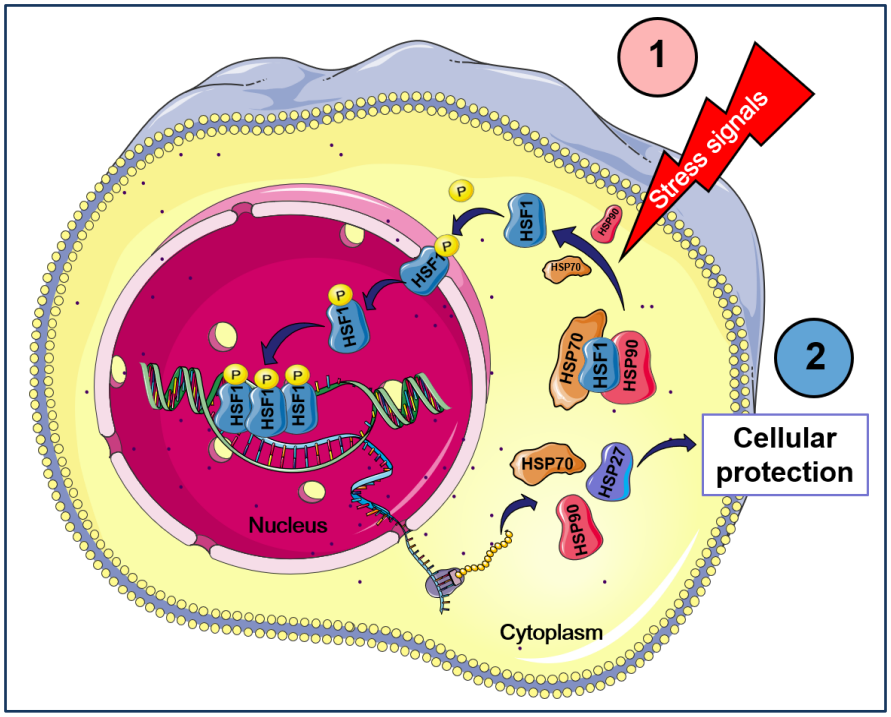

Fig. 22: Role of HSF1 in modulating HSPs expression. Under unstressed conditions, HSF1 is sequestered in the cytoplasm by HSPs (HSP90, HSP70). In response to stress (1) (high temperatures, metal toxicity, reactive oxygen species (ROS), amino acid analogues, hypoxia, ischemia, hypertrophy, aging, apoptosis, inflammation, and viral or bacterial infection), HSPs dissociate from the complex, allowing the activation of HSF1. Following nuclear translocation, HSF1 binds to specific HSE upstream of the heat shock genes promoters to activate transcription of *HSP* genes, which promote cellular protection and survival (2) (image realyzed with Servier Medical Art at https://smart.servier.com).

When stress signals decay, HSP90, HSP70, HSP40, and heat shock factor binding protein 1 (HSBP1) negatively regulate HSF1, binding to its transactivation domain and promoting its monomerization and consequent release from DNA[70].

HSF1 activity is regulated by its interaction with other proteins, and by a fine balance of phosphorylations mediated by different, and some still unknown, kinases (**Fig. 23**). Phosphorylations of Ser230 (by CaMKII), Ser320 (by PKA), Ser326 (by mTORC1) and Thr142 (by CK2) during stress conditions are essential for the transcriptional activity of HSF1. On the other hand, phosphorylation of several serine residues like Ser121 (by MK2 kinase), Ser303 (by GSK3), Ser307 (by ERK) and Ser363 (by JNK/SAPK or PKC) represses HSF1 activity. Phosphorylation of Ser419 by PLK1 is

involved in the accumulation of HSF1 in the nucleus. Other post-translational modifications (i.e. sumoylation and acetylation) induce HSF1 activation. Aberrations in RAS/RAF/MEK/ERK as well as RAS/PI3K/ PTEN/AKT pathways, which change the balance between the phosphorylation and dephosphorylation of HSF1, may activate this transcription factor[74]. At the same time, inhibition of the PI3K/AKT/GSK3 pathway causes a decreased expression of HSF1 and a downregulation of HSPs.

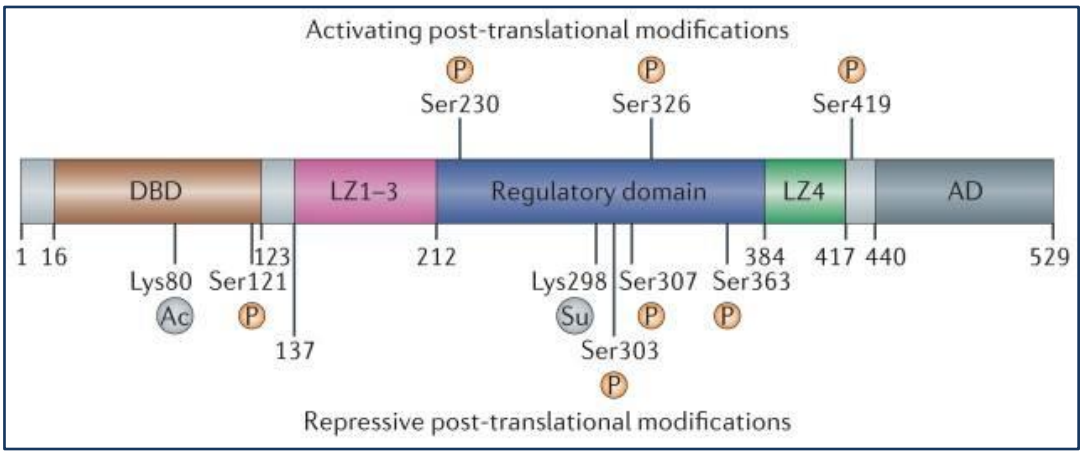

Fig. 23: HSF1 regulation by post-translational modifications in response to cellular stress. HSF1 is hyperphosphorylated on up to 12 serine residues; this occurs in parallel with HSF1- dependent transactivation. HSF1 activity is also repressed by constitutive phosphorylation of Ser303 and Ser307, and by stress-responsive sumoylation of Lys298 (represented on the figure as 'Su'). The binding of HSF1 to DNA is inhibited by the acetylation of Lys80 (represented on the figure as 'Ac'). AD, activation domain; DBD, DNA binding domain; LZ1, leucine zipper domain 1 (image from Neef *et al.*)[75].

2.4 HSP70 and HSF1 in cancer

The expression of HSPs can contribute to the development of several diseases including cancer, where their cytoprotective functions in response to stress are dysregulated. In addition to suppression of apoptosis, dysregulation of HSPs attend tumor proliferation, invasiveness, metastasis, and development of acquired resistance to treatment[63]. Since HSP27, HSP70, and HSP90 expression levels and activities are significantly higher in malignancies, it has been assumed that HSF1 is involved in tumor initiation, being the master regulator of HSPs expression. Therefore, the inhibition of HSPs and HSF1 represents a novel therapeutic strategy for cancer therapy[63].

Cancer cells, characterized by higher metabolic needs and aberrantly activated signalling pathways, show a higher demand for chaperone cytoprotective functions. HSF1 transcriptional activity in highly tumorigenic cells, regulates cancer-specific genes, which are different from genes activated during the normal HSR. The involvement of HSF1 in the regulation of cell cycle, apoptosis, metabolism, adhesion and other processes was observed in breast, colon, and lung tumor cells where HSF1 levels correlate with poor outcomes[74]. Elevated RAS/MAPK signalling levels lead to HSF1 activation (via phosphorylation of Ser326), which precedes malignancy, allowing cells to withstand a wide range of proteotoxic insults, even during the early stages of carcinogenesis[70]. HSF1 is

hyperphosphorylated in stressed cells and this phosphorylation pattern is probably essential for the regulation of genes transcription. When cells are subjected to pro-malignant signalling, HSF1 becomes dephosphorylated on Ser303 and phosphorylated on Ser326[74] (**Fig. 24**). HSF1 activation is also accompanied by sumoylation, a post translational modification and/or deacetylation though the deacetylase sirtuin-1, a factor associated with cancer[76].

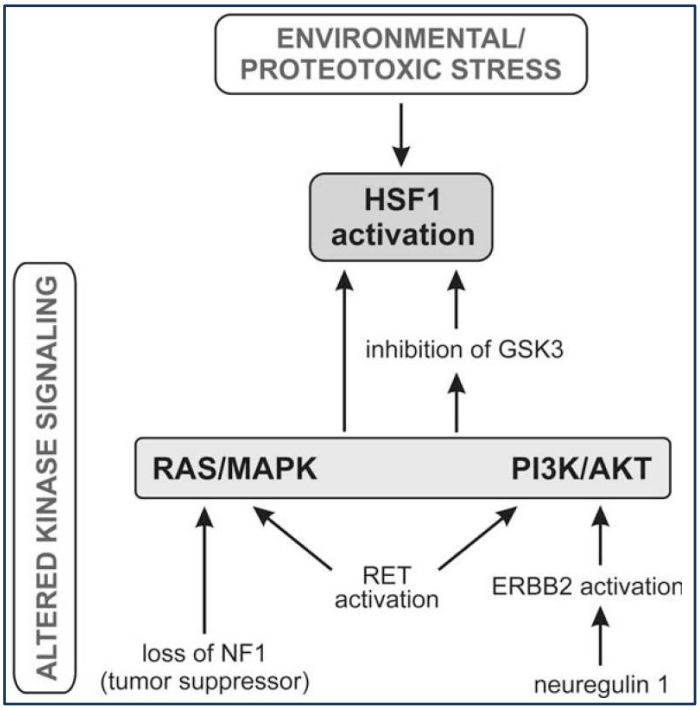

Fig. 24: Different ways of HSF1 activation due to proteotoxic stress or altered kinase signalling. A simple loss of the NF1 tumor suppressor, followed by elevated RAS/MAPK signalling, leads to HSF1 activation via phosphorylation of Ser326. Such activation precedes malignancy. Moreover, it has been found that RET stimulation (which can transduce signalling through PI3K/AKT, PLCG1/G2, RAS/ERK, MAPK, and JNK pathways), leads to increased HSF1 activity. HSF1 becomes transcriptionally active also following exposure of cells to neuregulin 1 (NRG1). NRG1 triggers intracellular signalling cascades through PI3K/AKT, resulting in the inhibition of GSK3, which is HSF1 antagonist. Consistently, inhibition of the PI3K/AKT/GSK3 pathway causes decreased expression of HSF1 and down-regulation of HSPs (image from Vydra *et al.*)[74].

HSF1 and HSPs are implicated in seven of the eight hallmarks of cancer: maintenance of proliferative signals, cell death resistance, inhibition of replicative senescence, induction of tumor angiogenesis, activation of invasion and metastasis, reprogramming of energy metabolism and evasion of immune surveillance[76].

Five important HSP70 family members are implicated in cancer development including stress-inducible HSP70s (cytosolic HSP70 and HSPA6) and constitutively expressed HSP70s (cytosolic HSC70, mitochondrial mortalin, and GRP7, localized in the ER). HSP70 acts as a survival factor during carcinogenesis, suppressing apoptosis through inhibition of both intrinsic and extrinsic pathways. In intrinsic apoptotic pathway, HSP70 binds to BAX, a pro-apoptotic BCL-2 family member, blocking its mitochondrial translocation and leading to the inhibition of APAF-1 recruitment, necessary for the apoptosome. In the extrinsic pathway of apoptosis, HSP70 binds to death receptors DR4/5, inhibiting the formation of the death-inducing signalling complex (DISC) in

BCR-ABL expressing cells. Moreover, HSP70 affected caspase-dependent programmed cell death, by interacting with apoptosis-inducing factor (AIF) and directly blocking AIF-induced chromatin condensation.

HSP70 overexpression is associated with disease progression and with a poor prognosis in melanoma, colon, breast, liver, colorectal, and lung cancers[63]. HSP70 is abundantly expressed during the progression of chronic myeloid leukemia: in HL-60/BCR-ABL and K562 cells, the increased expression of HSP70 enables resistance to imatinib-mediated cell death[63].

In tumors, HSP70 may also be expressed irrespective of HSF1 transcriptional activity. A possible candidate for HSP70 synthesis, in the absence of stress, is represented by the transcription factors signal transducer and activator of transcription 1 and 3 (STAT1/3). STAT3 binds to HSPA1 promoter sequences, different from the HSEs, and competes with HSF1 for HSP70 expression. In contrast, STAT1 recognizes interferon-γ-activated sequences without competing with HSF1 for transcription. The P53 family member ΔNp63α, the deacetylase and longevity factor sirtuin-1, and the mTOR complex 1 (mTORC1) have been identified as putative cancer-related HSF1 regulators in tumors, contributing to HSP70 overexpression by stimulating HSF1 expression and/or activity.

At post-transcriptional level, HSP70 protein is regulated by miRNAs. Although global alterations in miRNAs can be observed in several diseases, including cancer, little is known on the role of miRNAs in the regulation of the HSP expression. In mouse cardiac tissues, miR-1, miR-21, and miR-24 upregulate HSF1 and HSP70 after exposure to ischemia. In contrast, miR-1432-3p is a negative regulator of HSPA1B in pancreatic ductal adenocarcinoma cells.

About post-translational processing of HSP70s, although some amino acid residues are phosphorylated, the effect of these modifications on HSP70 function remains unclear. Phosphorylation of Ser400 in HSP70 is critical for the nuclear location of the chaperone. Further post-translational modifications of HSP70s involve acetylation (HSP70-1/2, HSP70-8, HSP70-9, HSP70-12A), malonylation (HSP70-9), methylation (HSP70-1/2, HSP70.2, HSP70-5, HSP70-6, HSP70-8), and conjugation with the ubiquitin-like interferon-inducible protein ISG15 (HSP70-8). Methylation represents a critical step in the post-translational modification of HSP70s: in the case of HSP70-8, trimethylation alters the affinity of the chaperone for both the monomeric and fibrillar forms of the Parkinson disease-associated protein α-synuclein[70].

The membrane HSP70 (mHSP70) is involved in maintaining cancer cell stability, protecting cells from lethal damage induced by environmental stressors. Radio(chemo)therapy enhances cell surface expression of HSP70 on tumor cells, thus rendering integral mHSP70 a tumor-specific recognition structure for therapeutic interventions[70].

2.5 HSP70 and HSF1 as druggable targets in CLL

HSPs overexpression is correlated to poor prognosis in several hematological malignancies, such as Acute Myeloid Leukaemia (AML), and Myelodysplastic Syndrome (MDS). HSP70s preserve the functional activity of mutated proteins or products of gene recombination, typical of some cancers. Unlike normal cells, most malignant cells aberrantly express HSPs (*e.g.* HSP70, HSP25, HSP90) to face the multitude of stresses at different stages of tumorigenesis as well as therapeutic treatment[63]. This addiction for HSP70, and for its major regulator HSF1, in malignancy could be exploited to design the most appropriate pharmacological approach targeting HSF/HSP aberrant axis to promote cancer cell apoptosis, minimizing the effects on the normal cells.

HSP70, for example, stabilizes mutated P53, which is not more able to induce cell cycle arrest or apoptosis[77]. Moreover, HSP70 forms complex with BAG3 (one of the largest BAG proteins), which sustains several key oncogenes, suppressing apoptosis. Accordingly, the silencing of BAG3 in tumor lines sensitizes the cells to chemotherapy, suggesting that the BAG3-HSP70 complex may be an attractive drug target[78].

Many HSP90 and HSP70 client proteins hold important functions in the development and promotion of cancer. Unfortunately, the inhibition of HSP90 activates the HSR, upregulating other HSP expression, in a mechanism which could counteract the apoptotic effects of such inhibition. Moreover, the toxicity of combined treatment with HSP70 and HSP90 inhibitors remains to be understood[79].

HSP70 inhibitors can be divided into three main categories: small molecular inhibitors, protein aptamers, and antibodies[63] (**Fig. 25**).

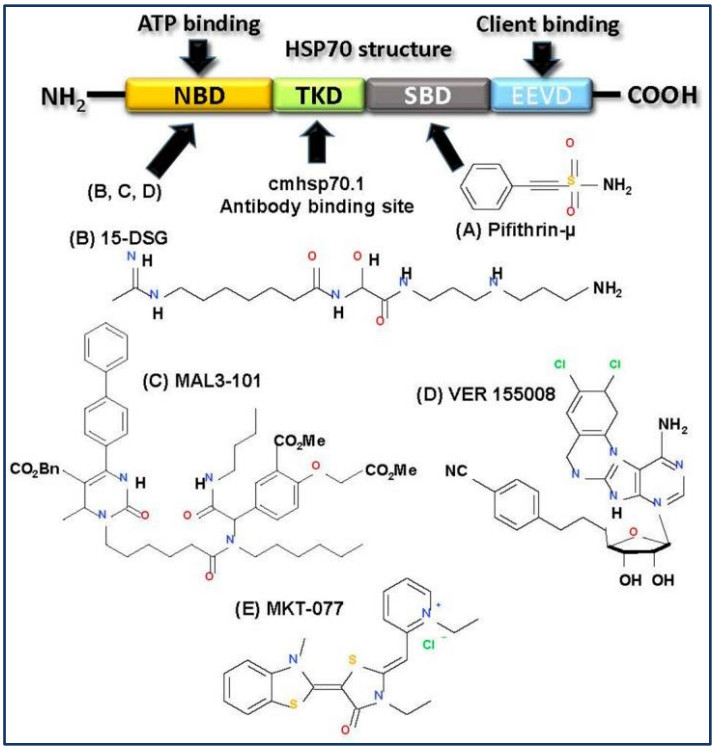

Fig. 25: HSP70 targeting in cancer. HSP70 consists of two major domains: the nucleotide binding domain (NBD) and the C-terminal peptide binding domain (PBD). PBD contains the substrate binding domain (SBD), TKD (epitope sequence) and a carboxyl-terminal chaperone EEVD motif. (A) The aptamer A17 (not depicted) and inhibitor pifithrin-μ bind to SBD. TKD is the extracellular epitope of HSP70 that is recognized and bound by the antibody cmhsp70.1 binds when it is exposed on the surface of tumor cells generating immune responses; (B) 15-DSG, (C) MAL3-101, (D) VER 155008 and (E) MKT-077 are important small molecule inhibitors that specifically binds to the NBD and inhibit HSP70 action (image from Chatterjee *et al.*)[63].

Although the promising results obtained *in vitro*, it must be noticed that it is difficult to bring HSP70 inhibitors to the clinic, because of their off-target effects and potential tossicity[79]. An alternative approach could be the targeting of HSF1, considering that tumor cells are more dependent on this protein than normal cells for proliferation and survival[79].

Another possible therapeutic approach could be the targeting of RAS signalling pathways (PI3K/AKT/mTOR and RAF/MEK/ERK) known to regulate HSF1/HSP70 axis in chronic myeloid leukemia and in multiple myeloma[80,81].

AIM OF THE STUDY

Despite the introduction of innovative therapies, CLL still remains incurable. To definitely eradicate the disease, new molecules able to abrogate apoptosis resistance and increased survival of neoplastic B lymphocytes have to be developed.

In our recent proteomic study[82], we found that HSP70, a protein with anti-apoptotic and cytoprotective function is overexpressed in CLL cells compared to normal controls. The modulation of HSPs mediated by chemical inhibitors is yet not usable in the clinical practice for the reduced specificity and the high toxicity in monotherapy. We explored an alternative approach based on the downmodulation of HSP70 by the inhibition of HSF1, HSP70 transcription factor. HSF1 activity is regulated by several phosphorylations from different kinases, most of which belonging to RAS signalling pathways (PI3K/AKT/mTOR and MEK/ERK), dysregulated in CLL cells.

During the PhD project we analyzed:

- expression, localization and activation status of HSP70 and HSF1 in CLL cells and their possible correlation with the main prognostic factors;
- the correlation between HSP70 and HSF1 expression levels and response to therapy in CLL patients;
- the effect of the inhibition of HSP70 and HSF1 in CLL cells with specific compounds, also by targeting RAS-signalling pathways.

MATERIALS AND METHODS

1. Patients

Leukemic B cells were obtained from 138 CLL patients, aged between 54 and 90 years, enrolled by the Hematology Division (chief Prof. G. Semenzato), Padua University School of Medicine. Normal B lymphocytes were collected from peripheral blood of 32 healthy subjects. Written informed consent was received from patients according to the Declaration of Helsinki, and the ethic approval for the present study was obtained from the local ethic committee.

2. Isolation of B lymphocytes from peripheral blood

2.1 Purification of B lymphocytes

B lymphocytes were isolated from heparinized peripheral venous blood of CLL patients. Blood mononuclear cells (PBMCs) were obtained proceeding with a layering on Ficoll/Hypaque (F/H) (Amersham Biosciences; San Francisco, CA) (**Fig. 26**).

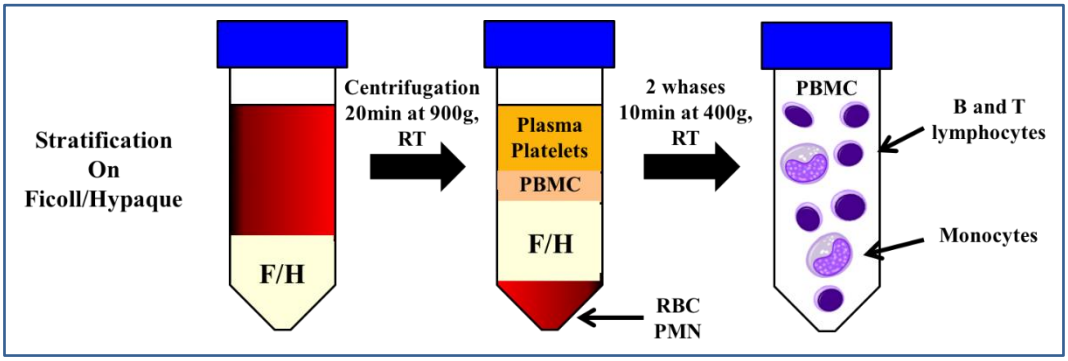

Fig. 26: Isolation of mononuclear cells from peripheral blood by stratification on Ficoll/Hipaque. By centrifugation on F/H, mononuclear cells were isolated from peripheral blood. Mononuclear cells and platelets were concentrated above the layer of F/H because they have lower density; on the contrary, the red blood cells (RBCs) and granulocytes (PMNs) have a higher density than the F/H and collect on the bottom of the tube.

This method exploits the difference of density of mononuclear cells (lymphocytes and monocytes) with respect to the other elements of the blood. Mononuclear cells, which have lower density, focus on the layer of F/H, while the red blood cells and granulocytes are collected on the bottom of the tube. Considered the high WBC count in CLL patients, peripheral blood was first diluted in 1:6 ratio with 0.9% sodium chloride (saline) at room temperature (RT), gently agitated, and later layered slowly over F/H solution. We proceeded with a centrifugation at 900g for 20 min at 20°C, without brake. The ring of mononuclear cells formed at F/H interface was aspirated and subjected to two successive washes with saline by centrifugation at 400g for 10 minutes at 20°C (Figure 19). The pellet was resuspended in an adequate amount of saline, and the cells were counted in a Burker chamber.

2.2 Purification of B lymphocytes with sheep red blood cells (SRBCs)

In most CLL cases, the percentage of leukemic B cells was greater than 90% of total PBMCs isolated.

When the cell population had more than 10% of T lymphocytes, we further performed the purification of B lymphocytes by SRBC method. This purification allows the removal of T cells from the other mononuclear cells, taking advantage from their ability to bind SRBCs and form complexes, called "rosettes". The SRBCs, in fact, express on their surface a specific receptor for the T lymphocyte marker CD2; SRBCs treatment with neuraminidase make more accessible the receptor for CD2 binding.

Aliquots of PBMCs ($25x10^6$) were transferred into a 10ml centrifuge tube and 1ml of SRBC treated with neuraminidase was added. PBMCs and SRBCs were then incubated at 37°C for 25 min, centrifuged at 4°C for 10 min at 200g, without brake, and subjected to a new incubation at 4°C for 30 min. The supernatant was then aspirated and culture medium RPMI 1640 (Invitrogen; Paisley, UK) was added. The mixture was gently resuspended, layered over F/H solution, and centrifuged at 4°C for 20 min at 900g without brake. T cells, surrounded by SRBCs, accumulated to the bottom of the tube, having a density greater than F/H, while the non-T mononuclear cells (monocytes and B cells) were concentrated above the F/H layer. The interface layer, which contains B cells, was transferred into a 10ml tube. Two washes with saline were performed at 400g at 20°C for 10 min and, in the end, cells were resuspended and counted in a Burker chamber (**Fig. 27**).

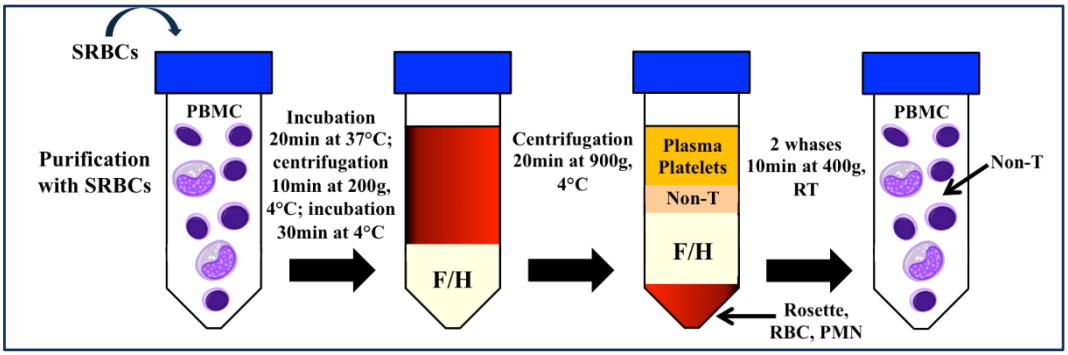

Fig. 27: Purification with SRBCs. When the mononuclear cells obtained by separation over F/H contained a percentage of lymphocytes T≥10%, we used SRBCs to remove them. This method exploits the ability of T cells to bind, through their marker CD2, to SRBCs treated with neuraminidase and forming rosettes that are removed by further stratification on F/H.

2.3 Purification of B lymphocytes using RosetteSep kit

We used the RosetteSep kit (StemCell Technologies; Vancouver, CND) to obtain B cells from whole blood of normal healthy donors. The kit consists of a cocktail of antibodies directed against surface antigens expressed by hematopoietic cells (CD2, CD3, CD16, CD36, CD56, CD66b) and

glycophorin A expressed by red blood cells. This mixture of antibodies binds "not-B" cells and red blood cells creating immunerosettes. In this way, CD19+ B lymphocytes were isolated from whole blood by negative selection.

Each ml of blood was incubated with 50µl of RosetteSep at RT for 20 minutes. The samples were then diluted 1:2 with PBS (Phosphate Buffered Saline)1X + 2% FBS (Fetal Bovine Serum), gently agitated, and then layered over F/H. We proceeded with a centrifugation at 900g for 30 minutes at RT, followed by the aspiration of the ring formed at the F/H interface containing B cells. It was resuspended in PBS1X + 2% FBS and centrifuged at 400g for 10 minutes. Finally, the cells were resuspended in PBS1X and counted in a Burker chamber (**Fig. 28**).

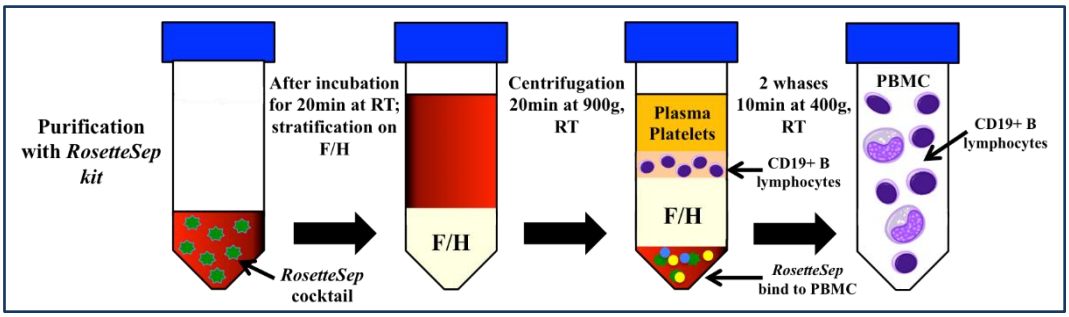

Fig. 28: Purification with RosetteSep kit. CD19+ B lymphocytes were isolated from whole blood of healthy donors by negative selection. 10ml of venous whole blood were incubated for 20 min at RT with 500µl of RosetteSep. Afterwards, through stratification on F/H, we get the CD19+ B cells, which are concentrated just above the layer of F/H, while the rest of the cells related to the rosettes were collected on the bottom of the tube.

3. Isolation of MSCs from bone marrow blood

Mesenchymal stromal cells (MSCs) were isolated from the BM blood of CLL patients. After F/H stratification, BM mononuclear cells (BMMCs) were plated and incubated at 37°C in humidified atmosphere containing 5% CO_2 and allowed to attach for 7 days; the non-adherent fraction was discarded, and adherent cells were maintained until confluence and for further expansion. MSC phenotype was characterized through the expression of CD14, CD31, CD34, CD73, CD90, CD45, and CD105 markers. The capability of MSCs to differentiate into mesenchymal lineages (*e.g.* adipocytes and osteocytes) was assessed (**Fig. 29**).

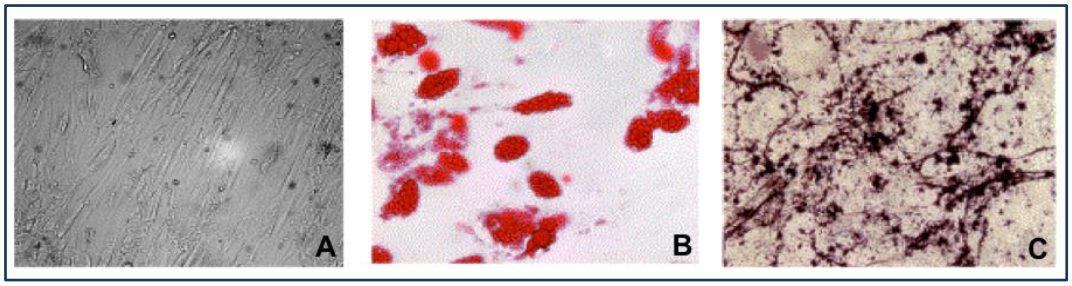

Fig. 29: Different lineages from MSCs. **A.** Undifferentiated MSCs; **B.** MSCs differentiated into adipocytes - Oil red stained; **C.** MSCs differentiated into osteocytes - von Kossa stained.

4. Cell cultures condition

Aliquots of CD19+/CD5+ B and CD5+ T lymphocytes obtained from patients with CLL, and normal B lymphocytes (CD19+) were cultured in RPMI 1640 medium with 2% FBS and antibiotics at a concentration of $2x10^6$/ml in 24- or 48-wells plates. Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 with or without: VER-155088 (0, 5, 20 and 40μM); zafirlukast (0, 35, 50 and 70μM); fisetin (0, 1, 15 and 30μM); KRIBB11 (0, 5 and 10μM); idelalisib (0, 5μM), and resveratrol (0, 40μM) (Selleckchem; Munich, Germany); for experiments, the 24h time-point was chosen.

5. CLL cells/MSCs co-culture

Some experiments were performed on MSC layer. For co-culture experiments, $1x10^5$/well MSCs from CLL patient bone marrow, were seeded into 24 well plates and incubated before the experiment at 37˚C in 5% CO_2, up to confluence. CLL cells were then added to MSCs layer at a ratio of 20:1, alone and with fisetin 15μM for 24, 48 and 72h, to evaluate a possible resistance to this agent due to the co-culture. CLL cells were also plated under the same conditions but not in co-culture with MSCs, in order to compare the results.

6. Subcellular Fractionation

The subcellular fractionation is a technique that allows the study of proteins in different cellular compartments. The fractions that are obtained are defined "enrichment" of the following components: cytoplasmic/soluble, of the membrane, microsomal, mitochondrial and nuclear.

We used the commercial kit “Subcellular Protein Fractionation Kit for Cultured Cells” (Thermo Fisher Scientific; Waltham, MA, USA), containing detergents which separate cytoplasmic, membrane and nuclear proteins.

10^7 lymphocytes were centrifuged and incubated with different buffers following manufacture’s protocol. The buffers are: Cytoplasmic Extraction Buffer (CEB), Membrane Extraction Buffer (MEB) and the Nuclear Extraction Buffer (NEB) with the addition of phosphatase and protease inhibitors (**Fig. 30**).

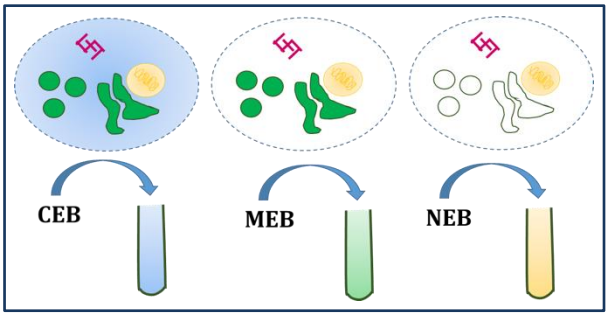

Fig. 30: Representation of cell fractionation protocol. Proteins were collected using several buffers. Cytoplasmic Extraction Buffer (CEB), Membrane Extraction Buffer (MEB) and the Nuclear Extraction Buffer (NEB).

7. Preparation of cell lysates

Cells (5×10^5 for each assay) were prepared by cell lyses with[83]:

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

To use these lysates for SDS-PAGE (polyacrylamide gel electrophoresis) analysis, the follow substances were added:

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

Subsequently, the lysates were vortex, and boiled at of 100°C for 5 minutes.

8. Polyacrylamide gel electrophoresis in SDS (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 an 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 allow concentrates 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.

9. 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 non-specific sites saturation. Follows the incubation for 2 hours and 30 min at RT of the primary Abs, diluted in "dilution buffer" (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% BSA).

For our study we used the following antibodies: anti-HSP70 (Abcam; Cambridge, UK); anti-HSF1 and anti-HSF1Ser326 (Enzo Life Sciences, Inc.; Farmingdale, NY, USA); phospho-Tyr, PARP (Cell Signaling Technology Inc.; Danvers, MA, USA), Lyn (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Lyn-Tyr396 (Epitomics Onc.; Burlingame, CA, USA), anti-α-tubulin and anti-β-actin (Sigma-Aldrich; Saint Louis, MO, USA).

Three washes of 10 min, each at RT, were subsequently performed using "washing buffer" (TRIS-HCl 1M, NaCl 3M, Tween20 0.1% 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 Chemi Luminescence system (ECL) (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 with the Ag-Ab complex, give rise to an oxidation reaction with light emission. The membrane was finally revealed into the

ImageQuant LAS 600 (Amersham Biosciences) and the bands were scanned and quantified by densitometry, using the program ImageQuantTL (Amersham Biosciences), supplied with the instrument.

10. 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), which emits a fluorescence signal at 530nm (green), phycoerythrin (PE) emitting at 585nm, tri-color (TC) that emits at 667nm when hit by a monochromatic laser beam with λ equal to 488nm, and finally, the allophycocyanin (APC) that emits a fluorescence signal at 690nm when excited by a laser beam with λ of 635nm. The cell samples were analyzed by the flow cytometer FACSCanto A (Becton Dickinson; Franklin Lakes, NJ) and data obtained were processed using the FACSDiva 7 software.

10.1 Immunophenotypic analysis

The immunophenotypic analysis on lymphocytes obtained from peripheral blood of CLL patients was performed through flow cytometry. All patients express the typical phenotypic profile according to standard criteria for CLL diagnosis[84].
The immunophenotyping is based on the identification of surface and intracellular Ag using mAb conjugated with fluorochromes. The presence of a certain Ag is revealed and used as an indicator of belonging to a cell line as well as its level of maturation. For apoptosis analysis 20.000 events were acquired.

10.2 Apoptosis analysis

Apoptosis of pathological B lymphocytes was assessed using the Annexin V Apoptosis Detection Kit (Becton Dickinson; Franklin Lakes, NJ, USA).
During the early stages of apoptosis, the plasma membrane undergoes profound changes that indicate the status of apoptotic cells to macrophages, which ensure their elimination. Phosphatidylserine (PS), a negatively charged aminophospholipid normally expressed only on the inner side of the plasma membrane, is exposed on the outer surface. The Annexin V is a protein that, in the presence of high concentrations of Ca^{2+}, recognizes and binds selectively the PS, making it useful for the identification of apoptotic cells that expose the phospholipid on their surface. Aliquots of $250x10^3$ cells were harvested, washed, and incubated for 10 min in the dark and at RT 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 (**Fig. 31**).

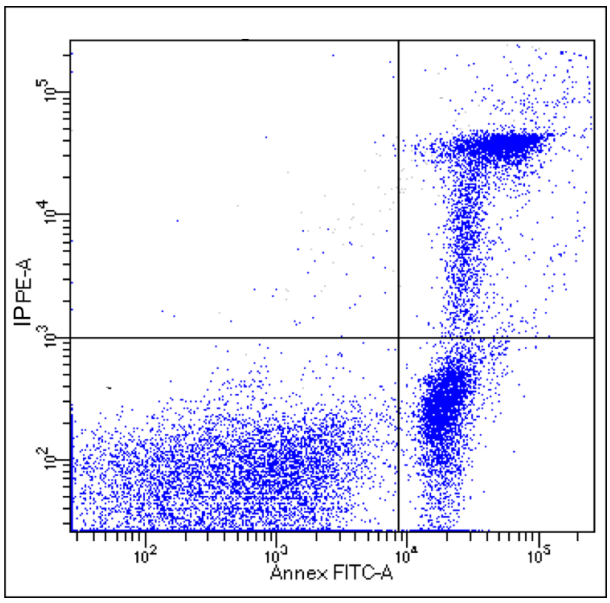

Fig. 31: A representative cytogram of Annex V/IP Test.

For each sample 20.000 events were acquired, and the number of apoptotic cells was expressed as percentage of Annexin V positive cells in the total cells analyzed.

12. Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software Inc.; La Jolla, CA, USA) by Student's *t* test, paired Student's *t* test, and ANOVA. Overall survival and Time to first treatment were calculated from date of diagnosis, and curves were constructed using the method of Kaplan and Meier. Identification of differentially activated or expressed proteins in different subgroups of patients was obtained through Mann-Whitney test. Selected pathways were evaluated through Global test analysis[82] performed with R (www.r-project.org). Data were considered statistically significant when p values were <0.05.

RESULTS

1. HSP70 and HSF1 are overexpressed in CLL cells

We previously found that HSP70, a chaperone allowing cells to survive to lethal conditions, was significantly overexpressed in B cells from CLL patients *versus* normal controls[82]. The main regulatory molecule for the transcription of HSP70 is the Heat Shock Factor 1 (HSF1) that, in response to stress, activates Heat Shock gene transcription[85].

Herein we extended our previous study to a larger cohort of patients and controls, adding HSF1 analysis. Although CLL cells showed a considerable variability of HSP70 levels (range: 0.03-4.46), a significant difference of expression was observed between malignant (n=138, 1.24±0.85) and normal B cells (n=32, 0.77±0.57; p<0.01, Student's *t* test). Again, we found a considerable variation of HSF1 levels in CLL cells (from 0.03 to 4.34); however, we demonstrated a significant difference in HSF1 expression between malignant (n=85, 0.81±0.77) *vs* normal B cells (n=19, 0.30±0.36; p<0.01, Student's *t* test). The two proteins positively correlate in the same patient's sample (n=85; p<0.0001, Pearson's correlation index) (**Fig. 32**).

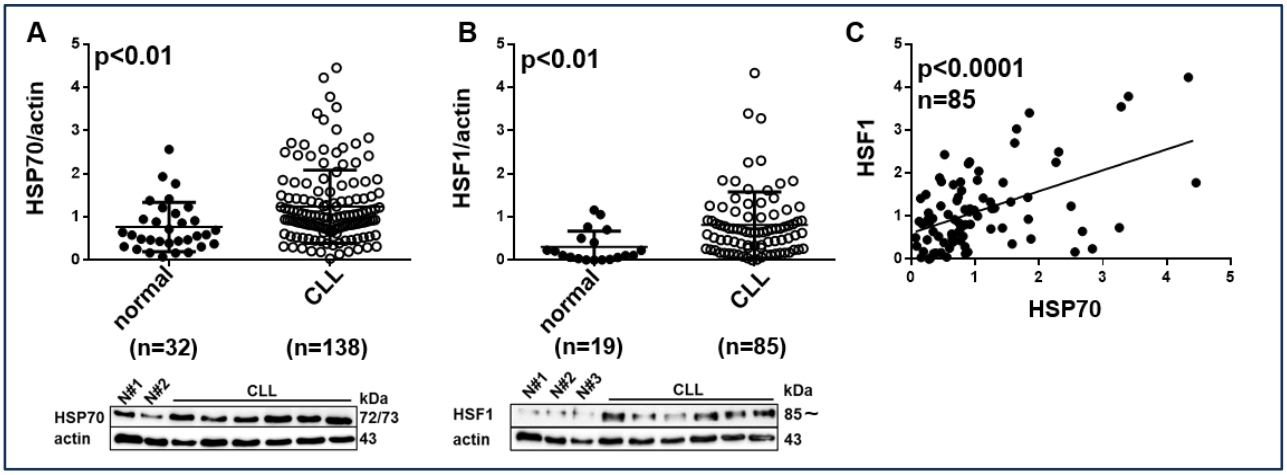

Fig. 32: Expression of HSP70 and HSF1 proteins in CLL. Lysates obtained from normal and CLL cells were loaded on SDS/PAGE and analyzed by immunostaining with anti-HSP70 and anti-HSF1. Actin was the loading control. **A.** Densitometry of HSP70/actin ratio of B cells from 138 CLL patients and 32 healthy subjects (p<0.01, Student's *t* test). WB is representative of all the samples analyzed. **B.** Densitometry of HSF1/actin ratio of B cells from 85 CLL patients and 19 healthy subjects (p<0.01, Student's *t* test). WB is representative of all the samples analyzed. **C.** Pearson's correlation between HSP70/actin and HSF1/actin densitometry of 85 CLL patients' samples immunostained with anti-HSP70 and re-probed with anti-HSF1 (p<0.0001); N=normal.

2. The activatory Ser326 of HSF1 is phosphorylated in CLL cells

When HSF1 is investigated by WB analysis, although the main molecular weight (MW) observed is around 85kDa, other additional bands are detected at different MW, due to post-translational modification (*i.e.* phosphorylation, acetylation or sumoylation). HSF1 regulation, in fact, is not only mediated by the interaction with HSP70, but also by specific phosphorylations at activatory/inhibitory sites. We analyzed the phosphorylation of HSF1 at Serine326 (HSF1-Ser326),

the key activatory site[86]. WB analysis showed that HSF1 is phosphorylated in Ser326 in the majority of CLL patients analyzed (51/62 CLL patients) with respect to six healthy controls (**Fig. 33**). These data suggest that HSF1 is activated in CLL cells.

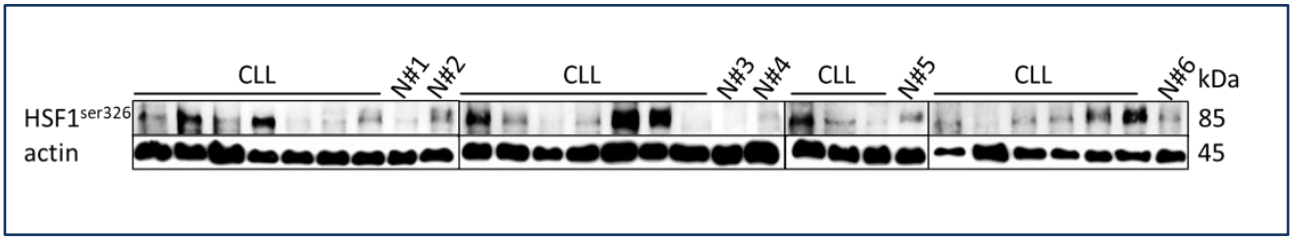

Fig. 33: Expression of HSF1-Ser326 in CLL. Representative WB of all the samples analyzed (62 CLL patients and 6 healthy controls). Lysates obtained from normal and CLL cells were loaded on SDS/PAGE and analyzed by immunostaining with anti-HSF1-Ser326. Actin was the loading control.

3. HSP70 and HSF1 are localized in the nucleus of CLL cells

To better comprehend the role of HSP70 and HSF1 in CLL, we investigated their subcellular localization. We found HSP70 and HSF1 both in the nucleus and in the cytosol of CLL cells, whereas we observed neither HSF1 nor HSP70 in the nuclear fraction, but only in the cytoplasm of normal B cells ($p<0.001$, Student's *t* test) (**Fig. 34**; **Fig. 35**). These results, together with the presence of activated HSF1 (HSF1-Ser326) in the majority of CLL cases, support the hypothesis of an aberrant activation of HSF1/HSP70 axis in CLL.

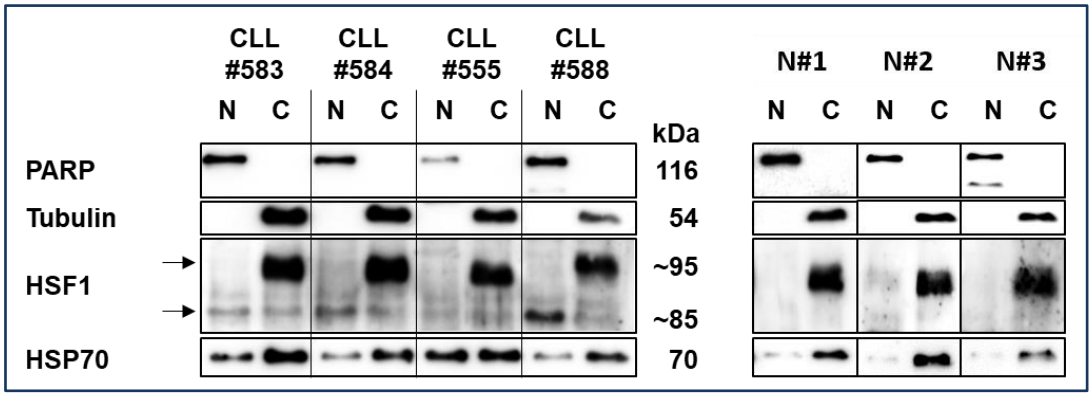

Fig. 34: Comparable aliquots of the different fractions (nuclei= N and cytosols= C) were loaded on SDS/PAGE and immunostained with anti-HSP70, anti-HSF1, anti-tubulin (cytosolic marker) and anti-PARP (nuclear marker). Figure is representative of different experiments performed on 13 CLL patients and 6 normal controls (N#).

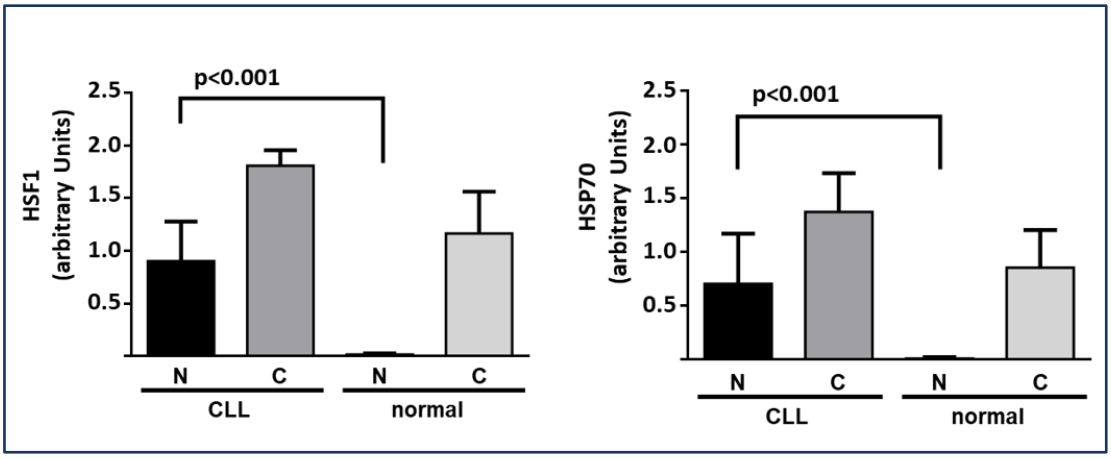

Fig. 35: Histograms show the mean±SD of HSF1 (left) and HSP70 (right) expression detected in the nuclear (N) and cytosolic (C) fraction of all the CLL patients (n= 13) and the normal controls (n= 6) analyzed (CLL nuclear fraction vs normal B cells nuclear fraction, p<0.001 for both proteins; Student's *t* test).

4. HSP70, HSF1 and HSF1-Ser326 expression levels are positively correlated to poor prognosis in CLL

We evaluated the potential correlations among HSP70, HSF1, and HSF1-Ser326 protein expression levels and CLL prognostic factors, including immunoglobulin heavy-chain variable region (*IGHV*) mutational status, leukemic cell expression of CD38 and ZAP70 proteins, and cytogenetic abnormalities (del17p13, del11q23, del13q14 and trisomy 12). We found a positive trend of increased expression of HSP70, HSF1 and HSF1-Ser326 in poor prognosis patients (*IGHV*^{unmutated}, 17p-/11q-/12+ karyotype, ZAP70^{pos} and CD38^{pos}). In particular**,** HSP70 levels were as follows: *IGHV*^{unmutated} (1.21±0.82) *vs IGHV*^{mutated} patients (1.11±0.64); 17p-/11q-/12+ (1.10±0.63) *vs* normal karyotype/13q- patients (1.05±0.65); ZAP70^{pos} (1.25±0.79) *vs* ZAP70^{neg} patients (1.09±0.68); CD38^{pos} (1.28±0.76) *vs* CD38^{neg} patients (1.10±0.73) (**Fig. 36**).

As regard HSF1, the values were: *IGHV*^{unmutated} (1.04±1.03) *vs IGHV*^{mutated} patients (0.75±0.58); in 17p-/11q-/12+ (0.94±0.70) *vs* normal karyotype/13q- patients (0.62±0.44, p<0.05 Student's *t* test); ZAP70^{pos} (0.90±0.77) *vs* ZAP70^{neg} patients (0.60±0.47); CD38^{pos} (0.93±0.66) *vs* CD38^{neg} patients (0.65±0.51) (**Fig. 36**).

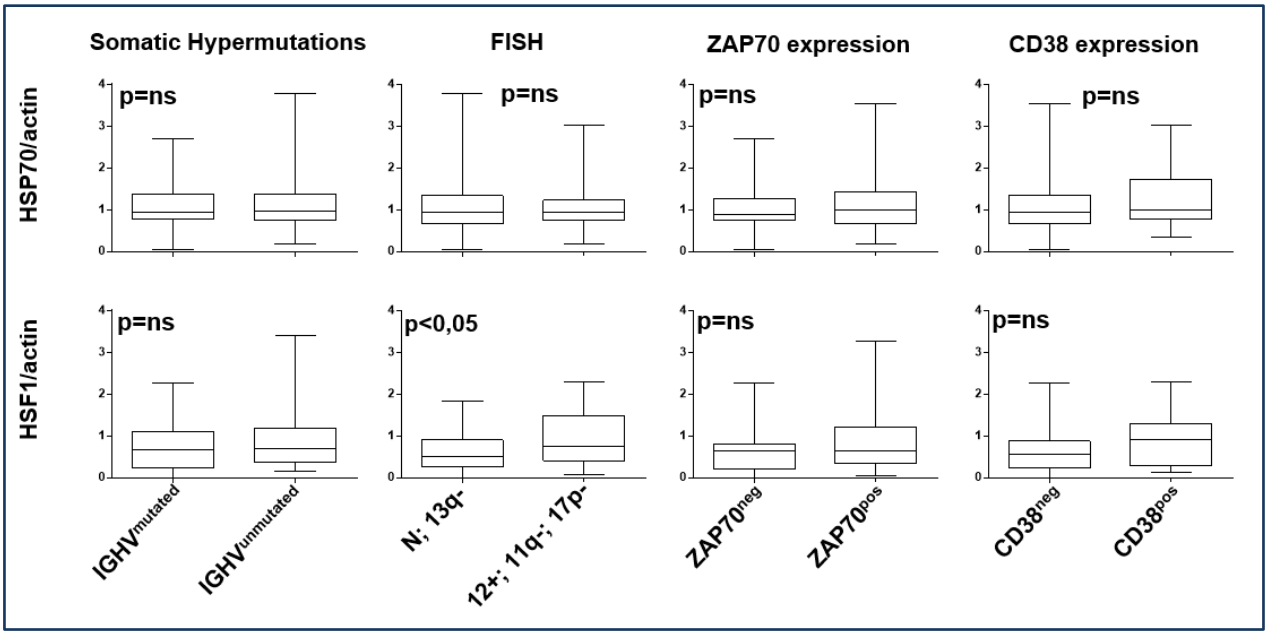

Fig. 36: Correlation of HSP70 and HSF1 expression with CLL prognostic factors. Data obtained in experiments shown in **Fig. 32 A** and **B** of this manuscript were correlated with CLL prognostic factors. Upper box plots report comparisons of HSP70 protein expression between: mutated *vs* unmutated; N/13q- *vs* 12+/11q-/17p-; ZAP70^{neg} *vs* ZAP70^{pos}; CD38^{neg} *vs* CD38^{pos}. Lower box plots report comparisons of HSF1 protein expression between: mutated *vs* unmutated; N/13q- *vs* 12+/11q-/17p- ($p<0.05$, Student's *t* test); ZAP70^{neg} *vs* ZAP70^{pos}; CD38^{neg} *vs* CD38^{pos}. N= normal karyotype.

A positive trend of expression, correlating with poor prognosis, resulted also for HSF1-Ser326. HSF1-Ser326 levels were as follows: *IGHV*^{unmutated} (0.70±0.80) *vs IGHV*^{mutated} patients (0.25±0.32, $p<0.05$ Student's *t* test); 17p-/11q-/12+ (0.52±0.50) *vs* normal karyotype/13q- patients (0.51±0.68); ZAP70^{pos} (0.76±0.82) *vs* ZAP70^{neg} patients (0.25±0.30, $p<0.05$ Student's *t* test); CD38^{pos} (0.52±0.56) *vs* CD38^{neg} patients (0.56±0.68) (**Fig. 37**).

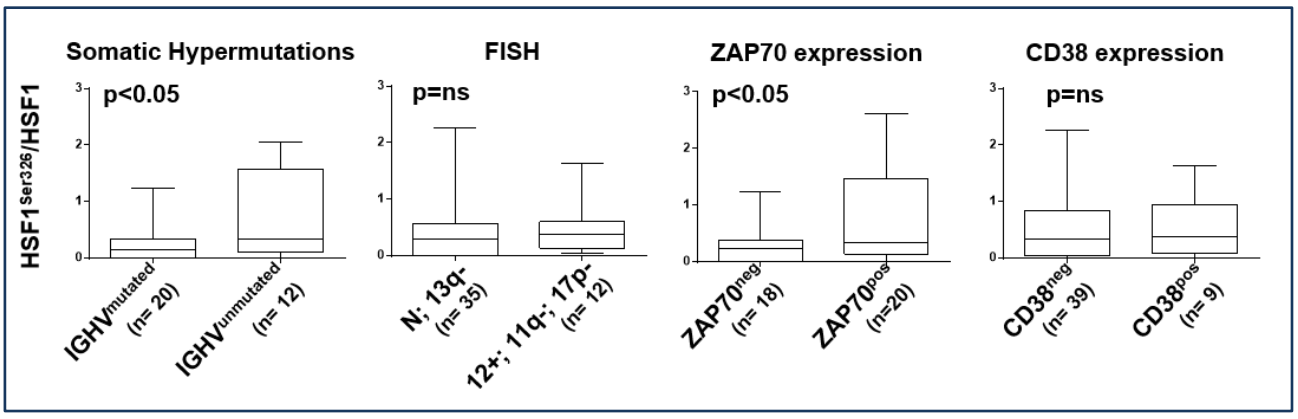

Fig. 37: Correlation of HSF1-Ser326 expression with CLL prognostic factors. Data obtained by densitometric analysis of HSF1-Ser326/HSF1 ratio were correlated with CLL prognostic factors. Box plots report comparisons of HSF1-Ser326 protein expression between: mutated *vs* unmutated ($p<0.05$, Student's *t* test); N/13q- *vs* 12+/11q-/17p-; ZAP70^{neg} *vs* ZAP70^{pos} ($p<0.05$, Student's *t* test); CD38^{neg} vs CD38^{pos} (p= ns, Student's *t* test). N= normal karyotype; ns=not significant.

Because of the high variability in HSF1-Ser326 expression levels among CLL patients, we deepened the analysis separating patients in HSF1-Ser326^{low} and HSF1-Ser326^{high} using the median of HSF1-Ser326 expression levels in CLL patients analyzed as cut-off (=0.33) (**Fig. 38**).

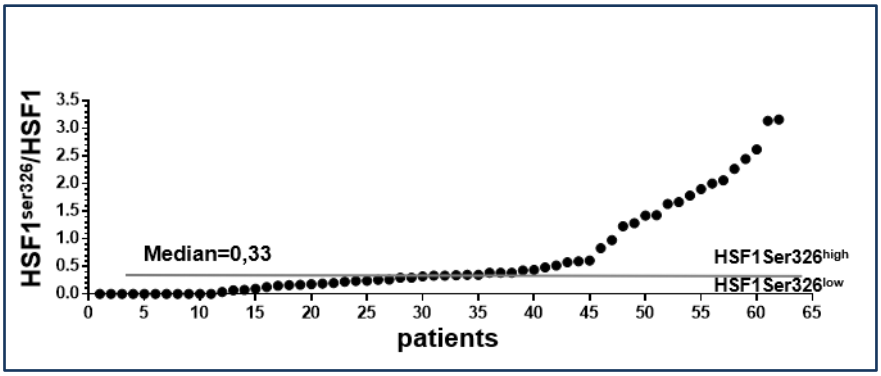

Fig. 38: Cut-off for HSF1-Ser326 high or low. Graph shows all the values obtained by the densitometric analysis of HSF1-Ser326/HSF1 ratio. The cut-off chosen to divide the patients analyzed in HSF1-Ser326^{high} and HSF1-Ser326^{low} was the median.

We found that ZAP70^{pos} patients had a higher phosphorylation in HSF1-Ser326 with respect to ZAP70^{neg} patients ($p<0.0001$, Fisher's exact test) (**Fig. 39**, **A**). Moreover, we grouped patients according to the Integrated CLL Scoring System (ICSS)[54] observing how HSF1 phosphorylation at Ser326 was mainly present in patients with intermediate or high risk (**Fig. 39**, **B**). HSF1-Ser326 levels were as follows: intermediate or high (int/high) ICSS (0.76±0.72) vs low ICSS (0.25±0.33; $p<0.01$, Student's *t* test) (**Fig. 39**, **B**).

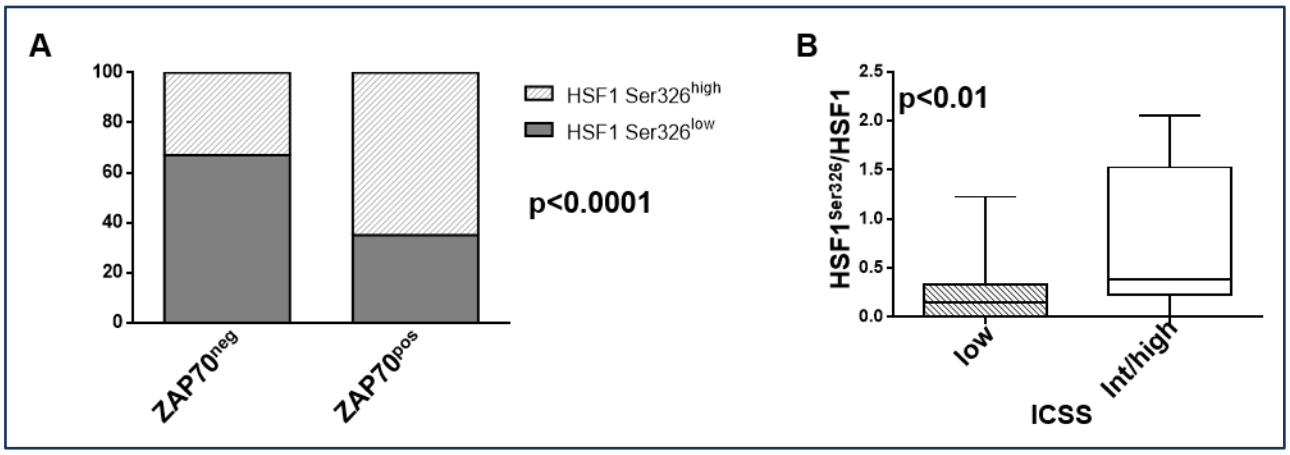

Fig. 39: A. Correlation of HSF1-Ser326 high and low with ZAP70. Histograms represent Fisher's exact test subdivision of ZAP70^{neg} and ZAP70^{pos} patients in groups presenting high or low levels of HSF1-Ser326 ($p<0.0001$).
B. Correlation of HSF1-Ser326 expression with ICSS. Box plot report comparisons of HSF1-Ser326 expression between patients with low ICSS *vs* patients with intermediate or high ICSS ($p<0.01$, Student's *t* test).

We estimated overall survival and time to first treatment in CLL patients expressing high or low levels of HSF1 and HSF1-Ser326. Regarding total HSF1, Kaplan-Meier analysis did not show significant differences between overall survival and time to first treatment for patients with high and low HSF1 expression (**Fig. 40**, **A-B**). The same analysis, performed for the presence or not of HSF1-Ser326 (**Fig. 40**, **C-D**), although not significant, suggested for a better outcome in patients expressing low levels of HSF1-Ser326.

The cut-off for HSF1 high and low is 0.70 as median of HSF1 protein levels in all the CLL patients analyzed, while the cut-off for HSF1-Ser326 is the presence or not of the phosphorylation.

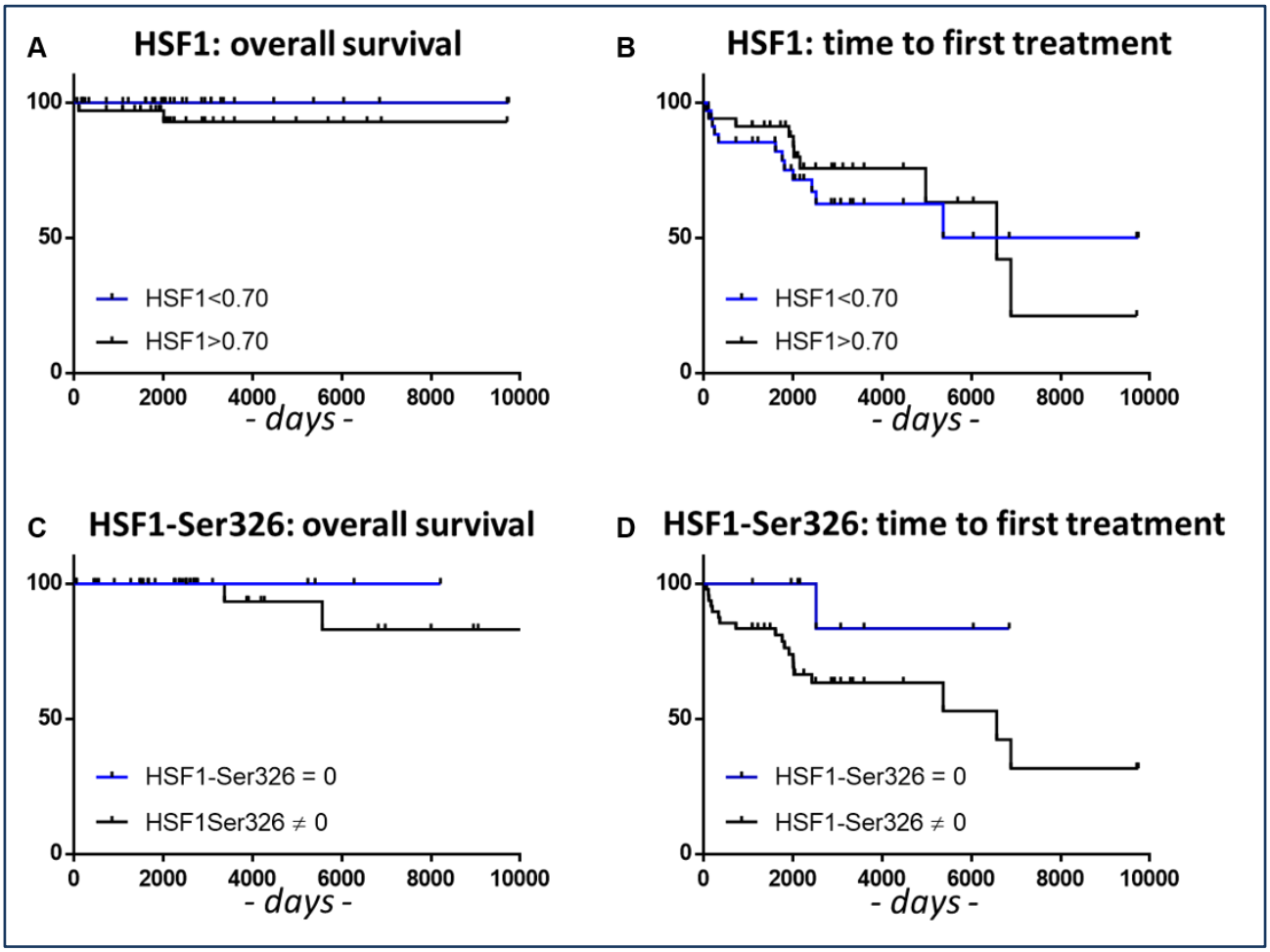

Fig. 40: Overall survival (**A**) and Time to first treatment (**B**) comparison between patients presenting high levels of HSF1 protein (HSF1>0.70, black line) and low levels (HSF1<0.70, blue line); 0.70 is the median of HSF1 protein levels calculated in all patients analyzed. Overall survival (**C**) and Time to first treatment (**D**) comparison between patients presenting HSF1-Ser326 (HSF1≠0, black line) and non-presenting HSF1-Ser326 (HSF1-Ser326=0, blue line).

5. HSP70 and HSF1 decreased after therapy in CLL responsive patients

We found that both HSP70 and HSF1 were reduced after treatment in patients responding to chemo-immunotherapy (Rituximab plus Bendamustine, #297, #445 and #270) (**Fig. 41**, **I-II-III**). In non-responding patients (#74 and #447) (**Fig. 41**, **IV-V**), we observed a correlation between HSP70 and HSF1 expression levels and the course of the disease. Indeed, HSP70 and HSF1 initially reduced, and then increased at the patient's relapse. At the end of our analysis, patients #297, #445 and #270 were without palpable lymphadenopathy or hepatosplenomegaly. They were subjected to subsequent cycles of R-B to complete the ongoing therapy. Patients #74 and #447 relapsed at the end of the analysis and started another therapy.

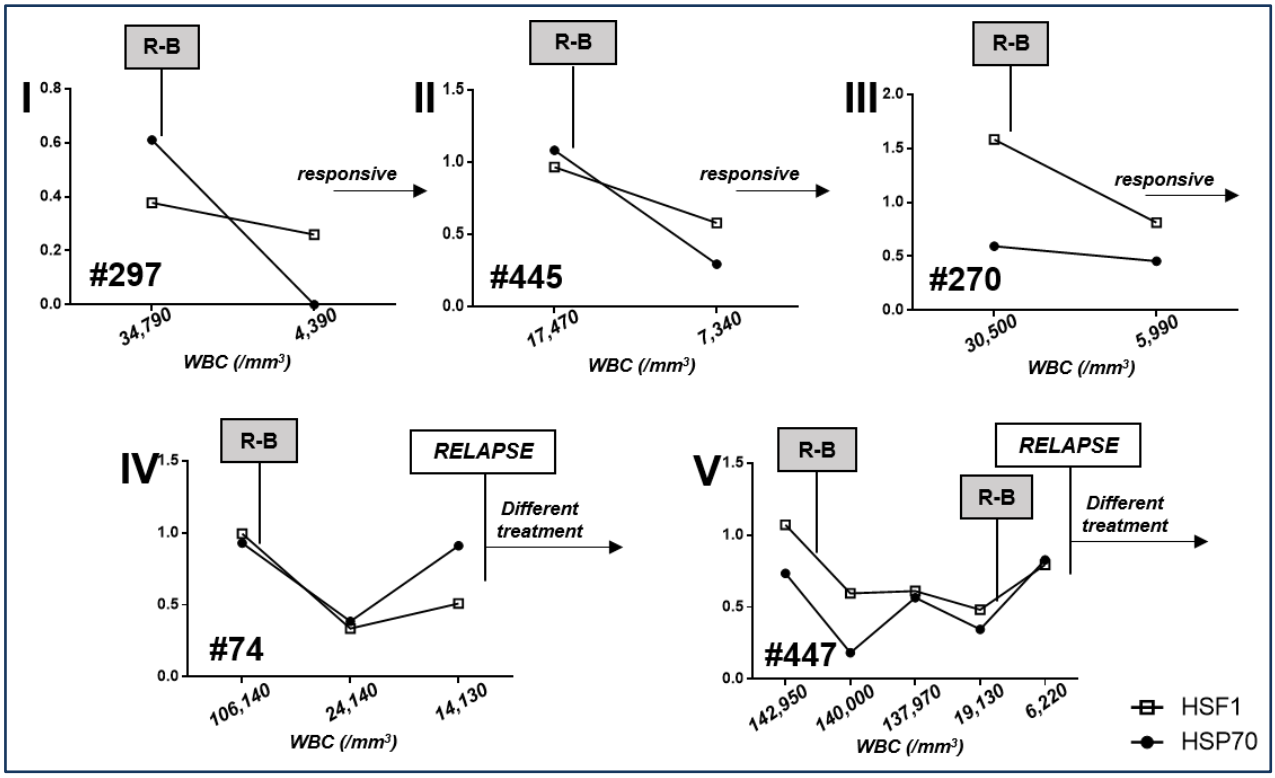

Fig. 41: Effect of *in vivo* therapy on HSP70 and HSF1 protein levels. HSP70 and HSF1 expression were analyzed by WB in 5 patients following R-Benda (R-B) containing regimen. HSP70 and HSF1 protein levels were determined in freshly isolated and purified leukemic B cells before and after 1 or 2 cycles of therapy. X-side reports WBC count (/mm^3) while y-side reports values of HSP70 and HSF1 expression. Protocol R-B: 375mg/m^2 Rituximab at day 1 of first cycle and 500mg/m^2 Rituximab at day 1 of cycles 2, 3, 4, 5 and 6; 90mg/m^2 Bendamustine at first-line and 70mg/m^2 Bendamustine at relapse at days 2 and 3 of cycles 1, 2, 3, 4, 5 and 6. R= Rituximab; Benda= Bendamustine; WBC= White Blood Cells.

The same behaviour was observed after treatment with new drugs such as kinase inhibitors (*i.e.* Ibrutinib, a BTK inhibitor). Patients #65, #366, #425, #365 are four examples of the strong correlation between HSP70 and HSF1 during treatment, being their protein levels decreased when the patient respond to therapy (**Fig. 42**). We pointed out a significant positive correlation between HSP70 and HSF1 levels during *in vivo* treatment (boxes in **Fig. 42**). At the end of our analysis, patients were in good general conditions and with a satisfactory hematological pattern.

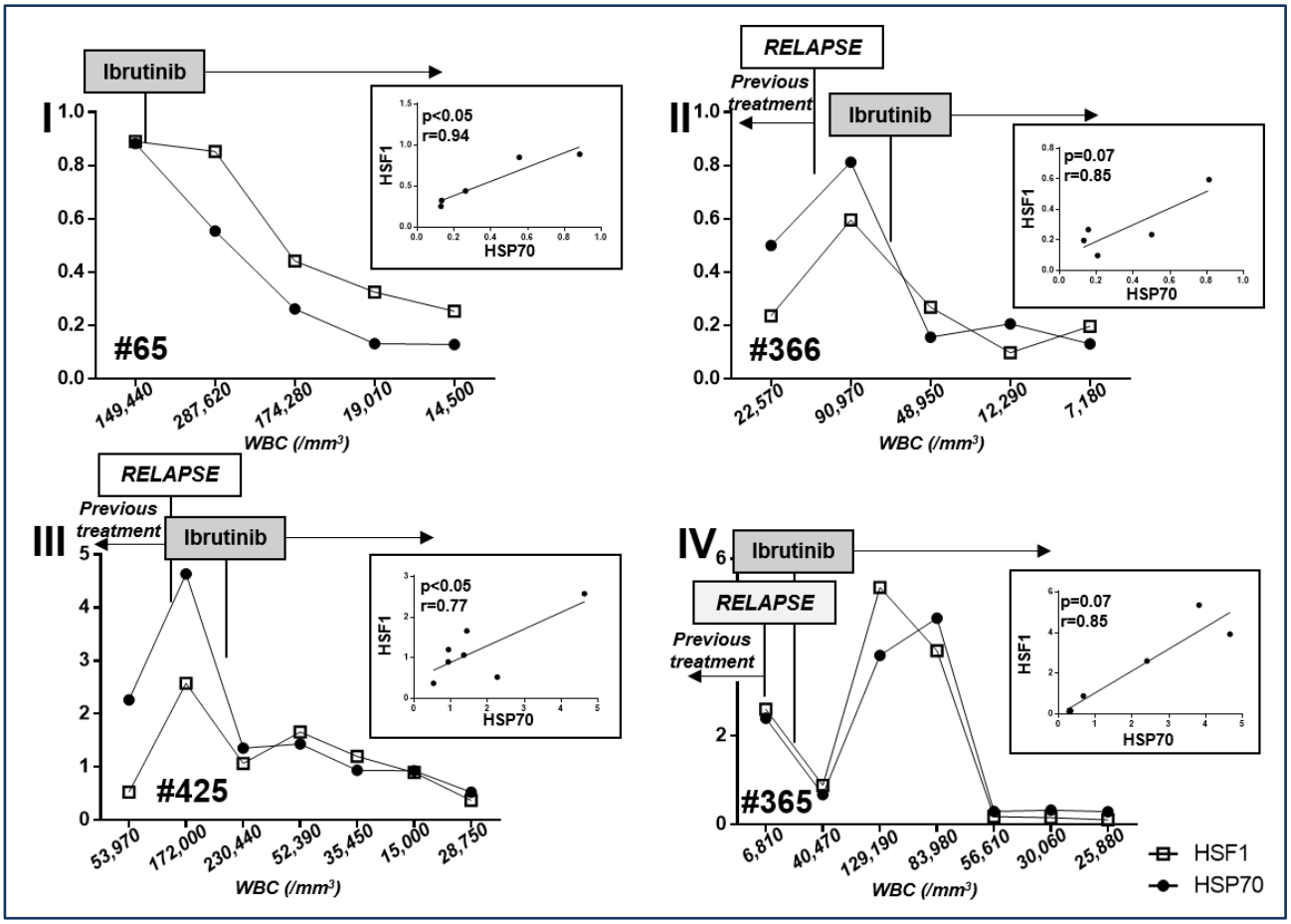

Fig. 42: HSP70 and HSF1 expression were analyzed by WB in 4 patients following Ibrutinib containing regimen. HSP70 and HSF1 protein levels were determined in freshly isolated and purified leukemic B cells before therapy and at different time-points (bi- or tri-monthly). X-side reports WBC count (/mm3) while y-side reports values of HSP70 and HSF1 expression. WBC= White Blood Cells. Protocol Ibrutinib: 420mg Ibrutinib once a day till progression.

6. HSP70/HSF1 axis is a druggable target in CLL cells

6.1 Inhibition of HSP70 by VER-155008 and zafirlukast

To inhibit HSP70 and investigate its role on tumor survival, we treated CLL cells with VER-155008 (an inhibitor of ATPase activity of all HSP70 family members), and, separately, with zafirlukast (a clinically anti-asthmatic agent inhibiting HSP70/HSP40 complex formation).

- *VER-155008*

After 24h treatment with VER-155008 at 5, 20 and 40μM, we observed a reduction in cell viability starting from 5μM (56±10% of living cells), 20μM (26±7%) up to 40μM (13±7%) with respect to untreated cells (65±10%; *p<0.05, **p<0.01 and ***p<0.001, respectively, paired Student's *t* test) (**Fig. 43**).

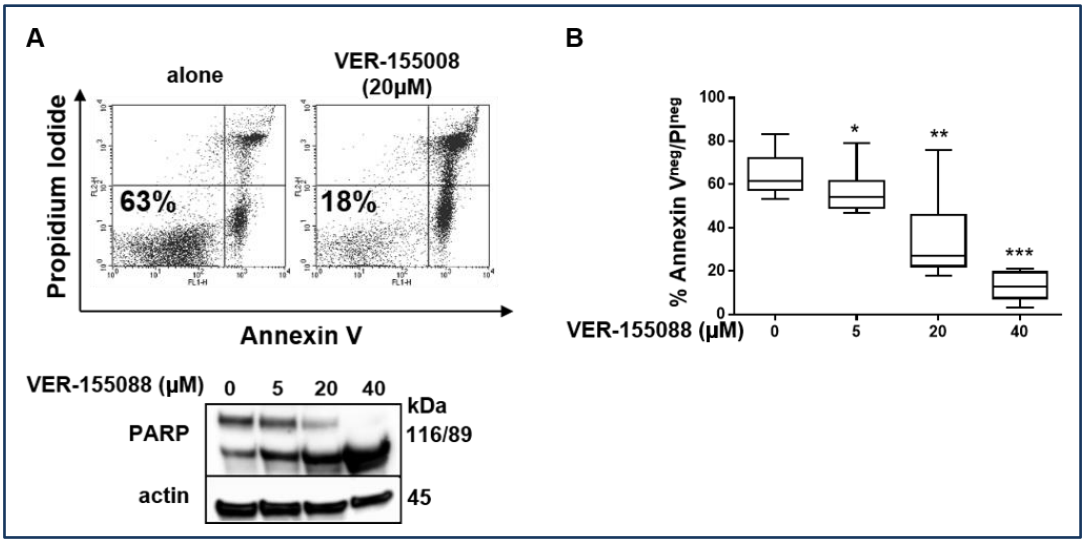

Fig. 43: Effect of VER-155008 on cell viability of leukemic B cells. Cells were cultured alone or in the presence of 5, 20 and 40µM VER-155008 and cell apoptosis was analyzed by annexin V-PI flow cytometric test. **A.** Cytograms and blot report a representative case of all CLL patients analyzed. The percentage reported in the cytograms represents viable Annexin V^{neg}/PI^{neg} cells. In all conditions, the total cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and detected sequentially with anti-PARP (116kDa= full length, 89kDa= cleaved), to highlight apoptosis, and anti-actin. **B.** Box plots report the percentage of Annexin V^{neg}/PI^{neg} cells after 24h treatment. Data are reported as median, lower and upper quartiles, minimum and maximum (*p<0.05, **p<0.01 and ***p<0.001 between alone *vs* 5, 20 and 40µM respectively, paired Student's *t* test).

- *ZAFIRLUKAST*

After 24h treatment with zafirlukast at 35, 50, 70µM we observed a dose dependent B cell apoptosis starting from 35µM (43±25% of living cells), 50µM (27±25%) up to 70µM (19±18%) with respect to untreated cells (64±12%; **p<0.01, **p<0.01 and ****p<0.0001, respectively, paired Student's *t* test) (**Fig. 44**).

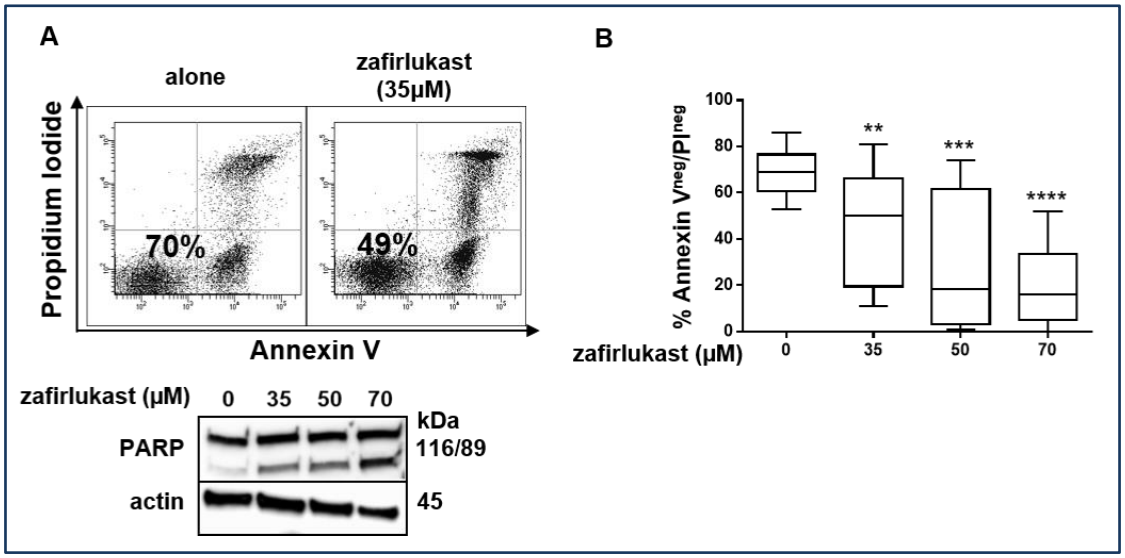

Fig. 44: Effect of zafirlukast on cell viability of leukemic B cells. A. Cytograms and blot report a representative case of CLL patients analyzed, and percentage reported in the cytograms represents viable Annexin V^{neg}/PI^{neg} cells. In all conditions, the total cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and detected sequentially with anti-PARP (116kDa= full length, 89kDa= cleaved), to highlight apoptosis, and anti-actin. **B.** Box plots report the percentage of Annexin V^{neg}/PI^{neg} cells after 24h treatment. Data are reported as median, lower and upper quartiles, minimum and maximum. (**p<0.01, **p<0.01 and ***p<0.0001 between alone *vs* 35, 50 and 70µM respectively, paired Student's *t* test).

6.2 Inhibition of HSF1 by KRIBB11 and fisetin

Because of the known difficulties to utilize HSP70 inhibitors in patients, for their unpredictable off-target effects, we tried an alternative approach by inhibiting HSF1, HSP70 transcription factor.

We used KRIBB11, a molecule that inhibits HSF1 by blocking p-TEFb (positive transcription elongation factor b) recruitment to the *HSP70* gene promoter. Furthermore, we deepened our studies using fisetin, a natural flavonoid which inhibits HSF1 activity by directly preventing its binding to the *HSP70* promoter.

- *KRIBB11*

After 24h treatment with KRIBB11 at 5 and 10µM, we observed a dose dependent B cell apoptosis starting from 5µM (40±19% of living cells) up to 10µM (33±21%) with respect to untreated cells (64±12%; ***p<0.001, paired Student's *t* test) (**Fig. 45**).

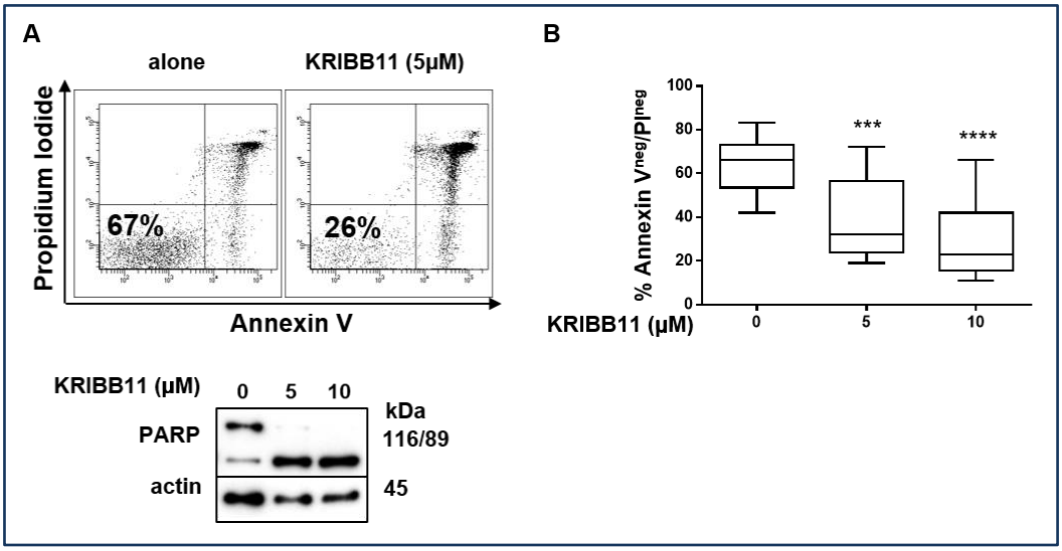

Fig. 45: Effect of KRIBB11 on cell viability of leukemic B cells. A. Cytograms and blot report a representative case of all CLL patients. The percentage reported in the cytograms represents viable Annexin V^{neg}/PI^{neg} cells. In all conditions, the total cell lysates were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and detected sequentially with anti-PARP (116kDa= full length, 89kDa= cleaved), to highlight apoptosis, and anti-actin. **B.** Box plots report the percentage of Annexin V^{neg}/PI^{neg} cells after 24h treatment. Data are reported as median, lower and upper quartiles, minimum and maximum (***p<0.001 and ****p<0.0001 between alone *vs* 5 and 10µM respectively, paired Student's *t* test).

- *FISETIN*

After 24h treatment with fisetin, we observed a reduction in cell viability starting from 15µM (35±19% of living cells) with respect to untreated cells (63±14%, p<0.0001, paired Student's *t* test); further decreased at 30µM (21±10%, p<0.0001, paired Student's *t* test) (**Fig. 46**).

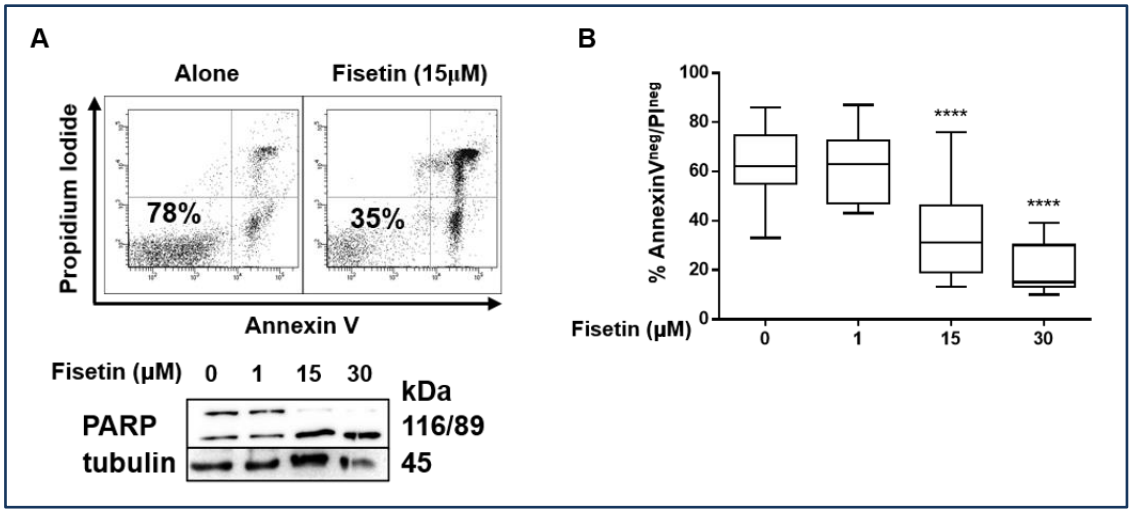

Fig. 46: Effect of fisetin on cell viability of leukemic B cells. A. Cytograms and blot report a representative case of all CLL patients. The percentage represents viable Annexin V^{neg}/PI^{neg} cells. In all conditions, the total cell lysates were subjected to WB and immunostained with anti-PARP (116kDa= full length, 89kDa= cleaved), to highlight apoptosis, and anti-tubulin. **B.** Percentage of Annexin V^{neg}/PI^{neg} cells after 24h fisetin treatment is reported as median, lower and upper quartiles, minimum and maximum (****p<0.0001 between alone *vs* 15 and 30µM, paired Student's *t* test).

When HCT116, a human colon cancer cell line, are heat shocked and cultured with fisetin, proteins as HSP70 and BAG3 are inhibited, and the cells lost the apoptosis protection provided by HSP70/BAG3 complex[87]. BAG3 was found overexpressed and correlated with poor prognosis and resistance to therapy in CLL patients[88]. We demonstrated that fisetin can reduce BAG3 expression in treated cells, and simultaneously increase the expression of 33kDa BAG1, known to interact with the proteasome and increase the degradation of HSP70 client proteins[87] (**Fig. 47**).

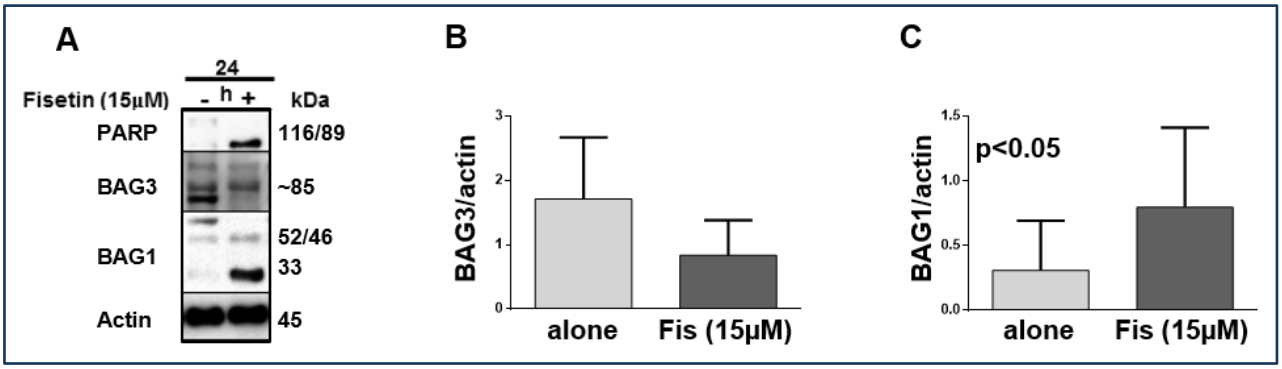

Fig. 47: Effect of fisetin on proteic pattern of leukemic B cells. A. CLL cells were cultured in medium alone (-) or in the presence of 15µM fisetin (+) for 24h, the total cell lysates were subjected to WB and immunostained with anti-PARP, anti-BAG3, anti-BAG1, and anti-actin. WB shows a representative case. **B.** and **C.** Histograms report mean±SD of BAG3/actin and BAG1/actin (p=ns and p<0.05 respectively, paired Student's *t* test).

We performed the same inhibition experiment in the presence of MSCs, representing the tumor niche. MSCs protect CLL cells from apoptosis induced by conventional drugs (Fludarabine, Dexamethasone and Cyclophosphamide)[89]. We demonstrated that MSCs are unable to protect neoplastic B cells from fisetin-mediated apoptosis. We did not observe any significant differences (p= ns) in viability in CLL cell cultured with 15µM fisetin compared to leukemic B cells co-cultured with MSCs and 15µM

fisetin (30±17%, 25±19%, 26±15% without MSCs, and 32±14%, 35±23%, 33±22% with MSCs at 24, 48, 72h, respectively).

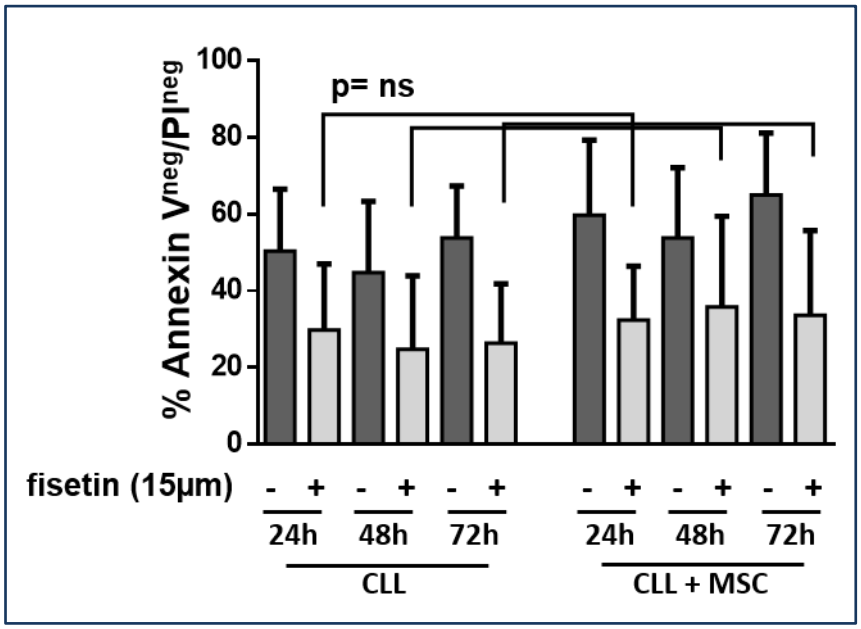

Fig. 48: Percentage viability of CLL cells co-cultured with or without MSCs and fisetin. CLL cells were co-cultured for 24, 48, and 72h with/without MSCs obtained from CLL patients with the addition of 15µM fisetin. The graph shows the mean±SD of Annexin V^{neg}/PI^{neg} cells percentage obtained in 10 cases analysed. MSC: Mesenchymal Stromal Cells.

Flavonoids are known to affect the phosphorylation status of some molecules, particularly PI3K, AKT, MAPKs but also SRC and SYK kinases[90]. We demonstrated that in CLL cells, fisetin affects the activation status of the SRC-family tyrosine kinase LYN, crucial for CLL cell survival[91]. By WB analysis, we reported the decrease of total tyrosine phosphorylation (P-Tyr), which was correlated to neoplastic clone apoptosis (as documented by PARP protein detection), and to the reduced LYN-Tyr396 (LYN activatory site) (**Fig. 49**).

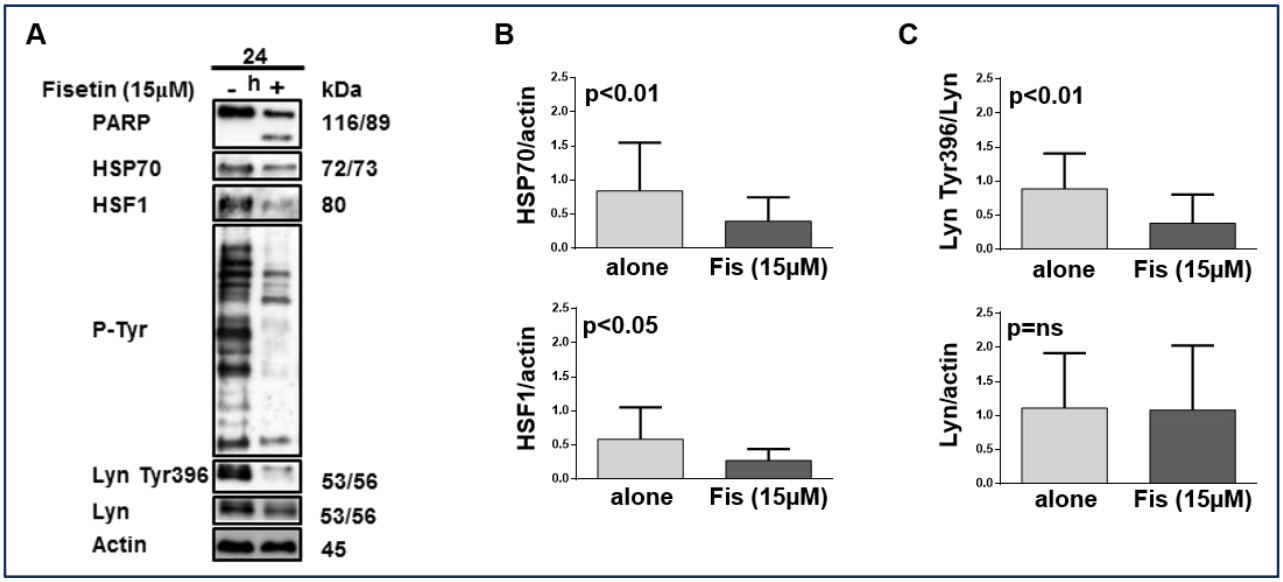

Fig. 49: Effect of fisetin on proteic pattern of leukemic B cells. A. CLL cells were cultured in medium alone (-) or in the presence of 15µM fisetin (+) for 24h, the total cell lysates were subjected to WB and immunostained with anti-P-Tyr, anti-Lyn-Tyr396, anti-Lyn, anti-PARP, and HSP70, anti-HSF1 and anti-actin. WB shows a representative case. **B.** and **C.** Histograms report mean±SD of HSP70/actin and HSF1/actin ($p<0.01$ and $p<0.05$ respectively, paired Student's *t* test), mean±SD of Lyn-Tyr396/Lyn and of Lyn/actin ($p<0.01$ and p=ns respectively, paired Student's *t* test).

7. Idelalisib and resveratrol induce apoptosis in CLL cells by reducing HSF1 and HSP70 protein expression levels

Since most of HSF1-phosphorylating molecules belong to two RAS-signalling pathways (PI3K/AKT/mTOR and RAF/MEK/ERK), taking advantage from our RPPA analysis[82], we correlated HSP70 to different proteins related to these pathways. By a cluster analysis, we divided our patients in $HSP70^{high}$ and $HSP70^{low}$ considering as cut-off the value of the median of HSP70 expression levels calculated by RPPA.

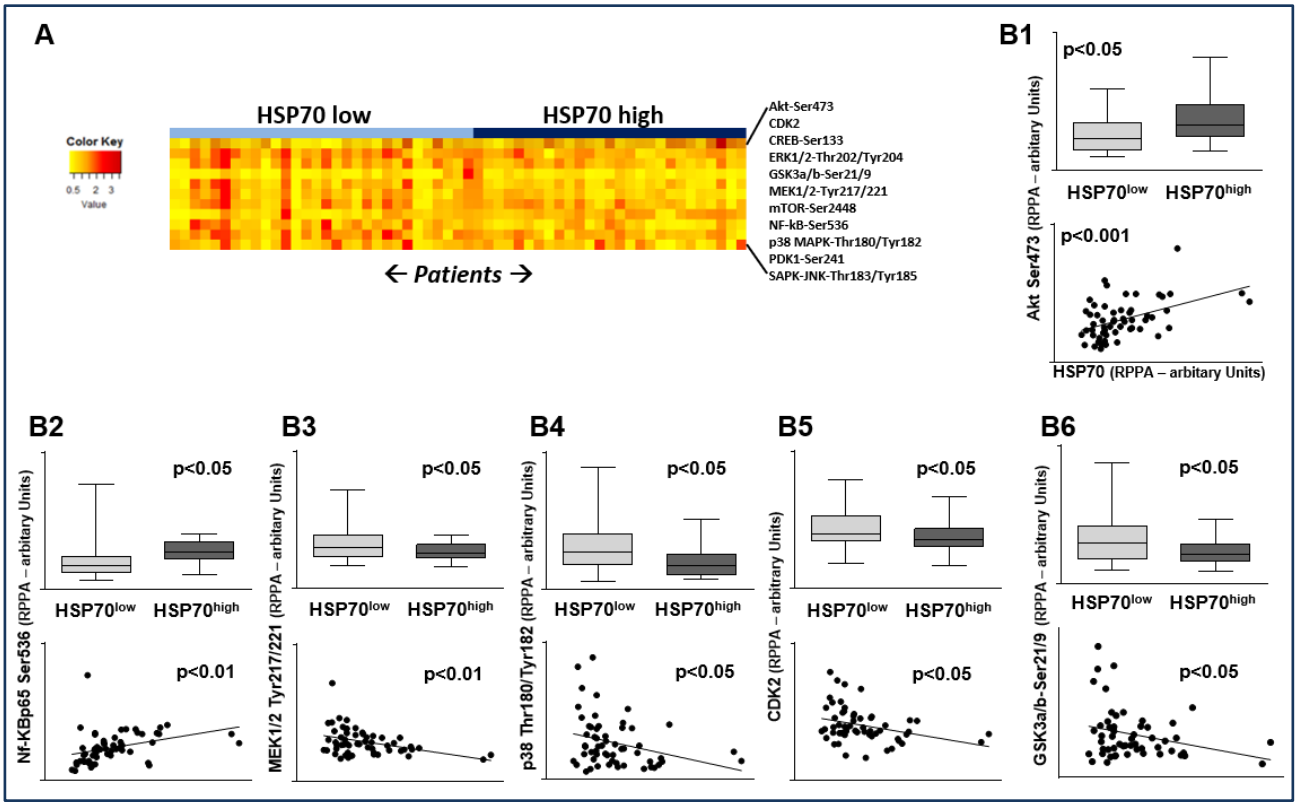

Fig. 50: Proteomic analysis by RPPA of RAF/MEK/ERK and PI3K/AKT/mTOR pathways in CLL cells. A. Heat map shows the expression of the indicated molecules in CLL patients by Global Test analysis. Values refer to our previous RPPA[82] and patients were divided in HSP70^{low} and HSP70^{high} according to the median value of HSP70 obtained in RPPA. **B1-6.** Box-plots report the values, expressed as median, lower and upper quartiles, minimum and maximum, of the protein reported in the Heat map whose difference of expression was significant in HSP70^{low} *vs* HSP70^{high} patients (Akt-Ser473, p<0.05; NF-kB p65-Ser536, p<0.05; MEK1/2-Tyr217/221, p<0.05; p38 MAPK-Thr180/Tyr182, p<0.05; CDK2, p<0.05; GSK3a/b-Ser21/9, p<0.05; Student's *t* test). Data on ERK1/2-Thr202/204 are not reported in this context because not significant, however is observed a trend of over-activation in HSP70^{low} patients, accordingly to what observed for MEK1/2 activation. Pearson's correlations were obtained for proteins indicated in the box-plots with RPPA values obtained for HSP70 (Akt-Ser473 vs HSP70, p<0.001 and r= 0.46; NF-kB p65-Ser536, vs HSP70, p<0.01 and r= 0.35; MEK1/2-Tyr217/221 vs HSP70, p<0.01 and r= -0.38; p38 MAPK-Thr180/Tyr182 vs HSP70, p<0.05 and r= -0.30; CDK2 vs HSP70, p<0.05 and r= -0.32; GSK3a/b-Ser21/9 vs HSP70, p<0.05 and r= -0.27).

We demonstrated that the proteins examined are regulated in a different way between HSP70^{high} and HSP70^{low} patients, and we developed a model to explain this difference (**Fig. 51**). In particular, patients with high expression of HSP70 have high AKT-Ser473, which inhibits GSK3a/b. In its inhibited form, GSK3a/b is no more able to inhibit HSF1. On the other hand, HSP70^{low} patients present high MEK1/2-Ser217/221 and ERK-Thr202/Tyr204, known to negatively regulate HSF1.

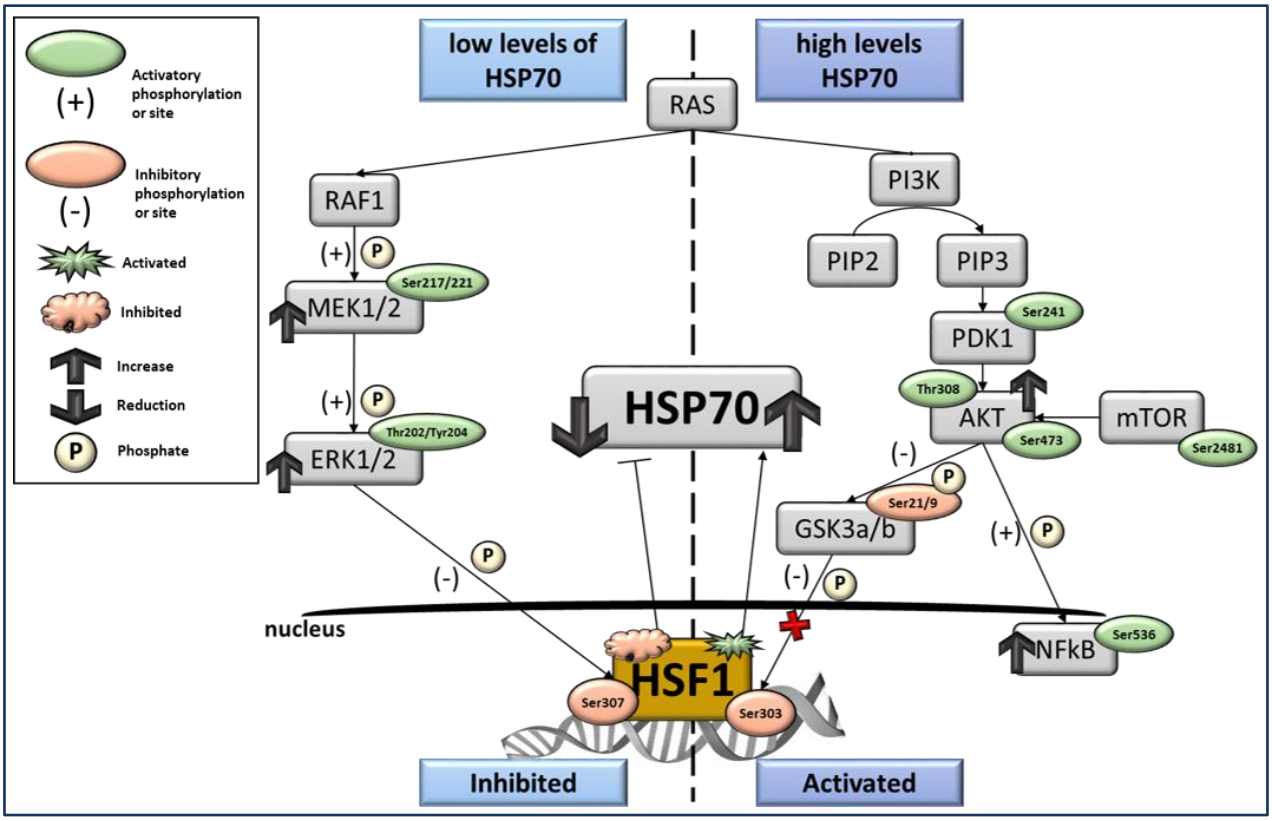

Fig. 51: Model explaining the HSP70 regulation by HSF1 via RAS-dependent pathways, in CLL. In HSP70^{high} patients, we found an increased phosphorylation of AKT-Ser473 that is reflected in a high amount of GSK3a/b phosphorylated at inhibitory Ser21/9. In this way, GSK3a/b is no more able to inhibit HSF1 that, in turn, up-regulates HSP70. In HSP70^{low} patients we found un up-regulation of the RAS-RAF-MEK1/2-ERK1/2 pathway; activated ERK at Thr202/Tyr204 can phosphorylate HSF1 at inhibitory Ser307 thus preventing HSP70 transcription.

To confirm our model for the regulation of HSF1/HSP70 axis in CLL, we use Idelalisib, a selective inhibitor of the phosphatidylinositol 3-kinase p110δ (PI3Kδ), already used in clinical practice. After PI3K inhibition, both HSF1 and HSP70 expression levels decreased, demonstrating an involvement of the RAS/PI3K/AKT pathway in the up-regulation of HSF1/HSP70 axis. This compound is also able to induce apoptosis (PARP cleavage) (**Fig. 52**).

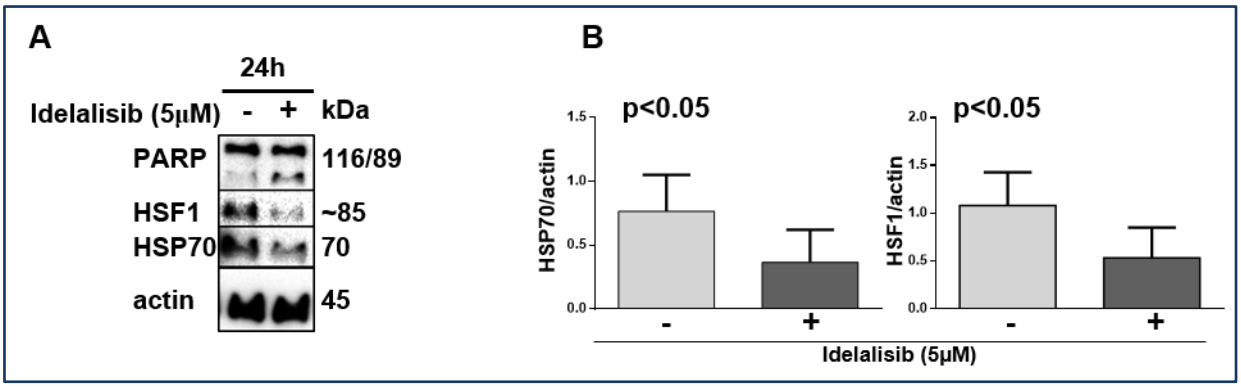

Fig. 52: Effect of idelalisib on apoptosis/HSP70/HSF1 in CLL B cells. CLL cells were cultured in medium alone (-) or in the presence of 5μM idelalisib (+) for 24h. **A.** The total cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose membrane and detected sequentially with: anti-HSF1, anti-HSP70, anti-PARP (116kDa= full length, 89kDa= cleaved), to highlight apoptosis and anti-actin. The figure shows a representative case. **B.** Histograms show the mean±SD of HSP70/act (left) and HSF1/act (right) of all the patients analysed (n= 8; p<0.05 for both proteins, paired Student's *t* test).

To simultaneously affect the two RAS-mediated pathways, we used resveratrol, a natural polyphenol with anti-tumorigenic and anti-oxidant properties[92], able to inhibit AKT and activate ERK. After 24h treatment with 40µM resveratrol, we observed a reduction in cell viability (54±20% of living cells) with respect to untreated cells (61±18%; $p<0.01$, paired Student's *t* test) (data not shown).

The analysis of HSF1 and HSP70 expression levels showed that HSF1 decreased from 1.15±0.69 to 0.50±0.64, and HSP70 from 1.13±0.69 to 0.54±0.35, in CLL cells cultured alone and with resveratrol 40µM, respectively ($p<0.01$, Student's *t* test) (**Fig. 53**).

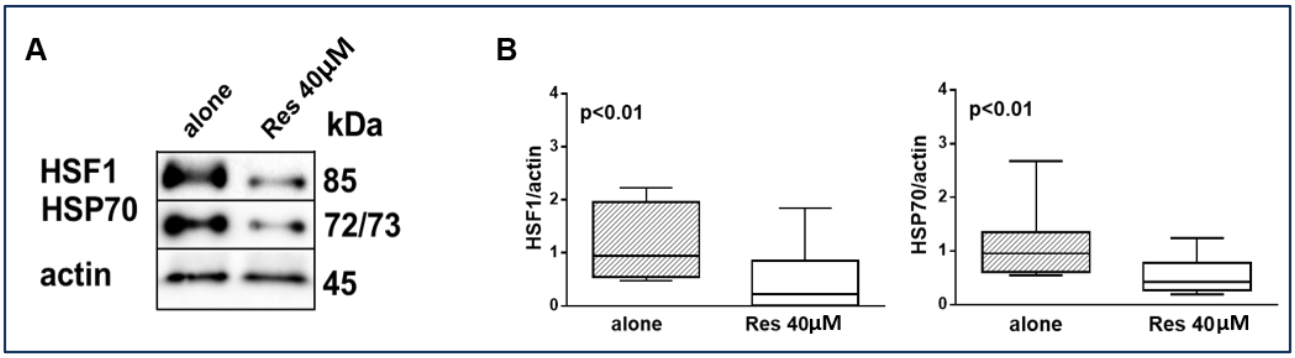

Fig. 53: Effects of Resveratrol on HSF1 and HSP70 expression levels. A. CLL cells were cultured in medium alone or in the presence of 40µM resveratrol (Res) for 24h, the total cell lysates were subjected to WB and immunostained with anti-HSF1, anti-HSP70, and anti-actin. WB shows a representative case. **B.** Histograms report mean±SD of HSF1/actin and HSP70/actin ratio and are comprehensive of 8 cases analyzed ($p<0.01$, Student's *t* test between alone and Res 40µM).

DISCUSSION

We demonstrated that both HSP70 and HSF1 are overexpressed in CLL cells as compared to normal B lymphocytes, and positively correlated to each other in neoplastic cells from patients responding to *in vivo* treatments. We highlighted that HSP70 expression in CLL cells is regulated by the transcription factor HSF1 through RAS-dependent pathways.

We recently identified a series of molecules differently expressed or activated in CLL cells with respect to normal, among which HSP70 emerged as overexpressed in CLL patients[82]. HSP70 assists the folding/re-folding of proteins, prevents aggregate formation, is involved in the transport of proteins, and can block both caspases-dependent and independent apoptosis[93]. Herein, we analyzed the expression levels of HSP70 in a larger cohort of CLL patients and controls, demonstrating that this protein is overexpressed in our patients.

HSP70 inhibits cell death by blocking the recruitment of procaspase-9 to the APAF-1 apoptosome, and is overexpressed in several tumors (*e.g.* breast cancer, lung cancer and ovarian cancer) and hematologic malignancies (*e.g.* acute myeloid leukemia), where it is associated with adverse prognostic factors[82]. It was not surprising to find HSP70 overexpressed in CLL, whose main pathogenetic, intrinsic factor is the lack of an adequate apoptotic response. Other members of the HSPs (*i.e.*, HSP90 and HSP27) are overexpressed in CLL[94], pointing out a possible role for this protein family in the pathogenesis of this disease and offering the rationale for their use as therapeutic targets.

The main regulatory molecule for the transcription of HSP70 is HSF1 that, in response to stress, binds to DNA and activates heat shock gene transcription[95]. We showed that, like HSP70, HSF1 was overexpressed in CLL. Moreover, we found HSF1 phosphorylated at its activatory site, Ser326, in the majority of CLL analysed patients. These findings, together with HSP70/HSF1 positive correlation in CLL cells at basal conditions, highlighted a positive activatory feedback between these two proteins.

To better understand the role of HSP70 and HSF1, we studied their subcellular localization in leukemic cells. HSP70 was localized both in the cytosol and in the nucleus of CLL cells, differently from what observed in normal B cells where HSP70 was found exclusively in the cytosol. Kotoglou and colleagues considered the possibility that nuclear HSP70 could have a DNA-repair activity, together with PARP, in case of single strand breaks during apoptosis[96]. In this way, HSP70 would be able to induce drug-resistance by acting in a terminal phase of the apoptotic processes, downstream with respect to the action of pro-apoptotic agents, such as chemotherapeutics[96].

Under normal conditions, HSF1 resides in the cytosol in a complex with HSP70 and HSP90. When the cell is stressed, the HSPs detached from HSF1, that migrates into the nucleus to trigger the heat shock gene transcription, including *HSP70*[70]. In line with this mechanism, we found HSF1 in the nucleus of leukemic cells where it can act as transcription factor. Seo *et al.* suggested that the overexpression of HSPs does not always show a feedback inhibition of HSF1. In fact, in some cancer cells (*e.g.* human lung carcinoma cells), HSP overexpression promotes the activation of HSF1 resulting in more HSP products, which provide radio- or chemo-resistance to cancer cells[97]. Accordingly, HSP70 overexpression is implicated in a lower response to pharmacological treatment because it favours cancer adaptation to changes induced by chemotherapy in the microenvironment[98]. We demonstrated that HSP70 decreased after therapy in those CLL patients responding to fludarabine- or bendamustine-based regimens[82]. Herein we showed that both HSP70 and HSF1 decreased, and positively correlated each other during treatment, in patients responding to traditional (rituximab-bendamustine) and kinase inhibitor (ibrutinib) regimens.

Although CLL shows very homogeneous phenotypic characteristics, the clinical course of the disease is extremely variable: some patients do not require any pharmacological intervention, while others have an aggressive leukemia since the onset, with poor prognosis. We observed a higher expression of HSP70, HSF1 and HSF1-Ser326 in patients without somatic hypermutation (unmutated) of I*GHV* or severe genetic abnormalities, associated with poor prognosis. On the other side, a lower expression of these proteins was associated with favourable prognosis. Correlations with other prognostic factors, such as CD38 and ZAP70, were similar. Within these data, we highlighted a significant correlation between HSF1 expression levels and chromosomal abnormalities, and HSF1-Ser326 and somatic hypermutations and ZAP70 expression. These data are in line with other studies showing an overexpression of HSP70 in different tumors, including hematological malignancies, and its correlation with an unfavorable prognosis[85,99]. In addition, Steiner *et al*. demonstrated that in acute myeloid leukemia (AML), patients belonging to the 'poor' risk group had more HSP70+ cells, even if they did not detect a significant correlation between HSP70 expression and cytogenetic risk groups ($p=0.53$)[102].

The overexpression of HSP70 and HSF1 and their correlation to poor prognosis in CLL suggest a key role for HSP70/HSF1 axis in neoplastic cell viability. For this reason, we inhibited HSP70 in CLL cells to evaluate the potential apoptotic effects. We previously proved the efficacy of pifithrin-μ, a selective inhibitor of inducible HSP70[82], and a statistically significant cell death rate was also found with VER-155008, an inhibitor of ATPase activity of all HSP70 family members. Moreover, we successfully used the leukotriene antagonist zafirlukast, a clinical anti-asthmatic agent, which also inhibits HSP70/HSP40 complex formation[100]. Despite the promising results obtained with

our experiments and the demonstrated relationship between HSP70 and cancer, HSP70 inhibition could have the side effect of inducing other molecular chaperone expression, as described for HSP90 inhibition, thus resulting toxic for the organism[101]. This is the reason why no progress was made in bringing HSP70 inhibitors to the clinic.

An alternative and attractive approach may be represented by the targeting of the major regulator of HSP70, HSF1. In 2015, Kim *et al*. demonstrated that fisetin, a dietary flavonoid, is able to induce apoptosis in cancer cells by inhibiting HSF1 activity through the blocking of its binding to the HSP70 promoter[87]. In 2016, the cytotoxic profiles of flavonoids, analyzed in two human CLL cell lines, highlighted quercetin and fisetin as potentiating luteolin in killing CLL cells[102]. Moreover, when heat shocked-cells are cultured with fisetin, induction of proteins such as HSP70 and BAG3 are inhibited, thus leading to the loss of the apoptosis protection provided by HSP70/BAG3 complex, and to the contemporaneous anti-apoptotic protein reduction (such as Bcl-2, Bcl-xL, and Mcl-1)[87]. We translated this knowledge in *ex vivo* neoplastic B cells from CLL patients, and we demonstrated that fisetin treatment resulted in a dose-dependent cell apoptosis, accompanied by a reduction in HSP70 and HSF1 expression. Moreover, in our patients fisetin diminished BAG3 expression and simultaneously increased 33kDa-BAG1, known to interact with the proteasome and to lead to the degradation of HSP70 client proteins[87]. Of note, BAG3 was previously found overexpressed and correlated with poor prognosis and resistance to therapy in CLL[88].

In recent years, it was demonstrated that the induction of cellular death by flavonoids is often accompanied by the modulation of activity of different protein kinases. They greatly affect the phosphorylation status of molecules, such as PI3K, AKT, MAPKs and also SRC and SYK kinases[90]. In this context, we demonstrated that in CLL cells, fisetin affects HSF1, with consequent reduction of HSP70 expression and apoptosis induction, and also the activation status of the SRC-family kinase LYN, which is crucial in maintaining CLL cell survival[29,91].

Since most of HSF1-phosphorylating molecules belong to two signalling pathways taking part from RAS (PI3K/AKT/mTOR and RAF/MEK/ERK), we took advantage from our previous proteomic study[82] and correlated HSP70 to different proteins related to these signalling. We demonstrated that the examined proteins behave in a different manner when HSP70 is highly expressed with respect to lower levels. In the first condition, we found an overexpression of AKT-Ser473, an inhibitor of GSK3a/b that, in the inhibited form, prevents HSF1 inhibition with consequent overexpression of HSP70. On the contrary, when HSP70 is less expressed, we found an up-regulation of MEK1/2-Ser217/221 and ERK-Thr202/Tyr204, known to negatively regulate HSF1. These data suggest that in CLL, HSP70 expression is regulated by the modulation of HSF1 activity through the activation of one or the other way triggered by RAS. An activation of the PI3K/AKT/mTOR pathway

leads to a higher expression of HSP70, while an activation of the RAF/MEK/ERK signalling results in HSP70 downregulation. The dissection of signalling pathways connected to HSP70/HSF1 axis in CLL will contribute to define the biology and understand the pathogenesis of this disease. In this work we used resveratrol, a natural phenol, proved to simultaneously affect the two RAS-mediated pathways, inhibiting AKT and activating ERK[80] (**Fig. 54**). We found that this compound can induce apoptosis as consequence to the reduction of both HSF1 and HSP70, thus supporting our model for the aberrant RAS pathways in CLL. Mustafi *et al.* reported a similar mechanism by which resveratrol was able to induce apoptosis by downmodulating HSP70 via AKT pathway in Chronic Myelogenous Leukemia (CML) cell lines[103].

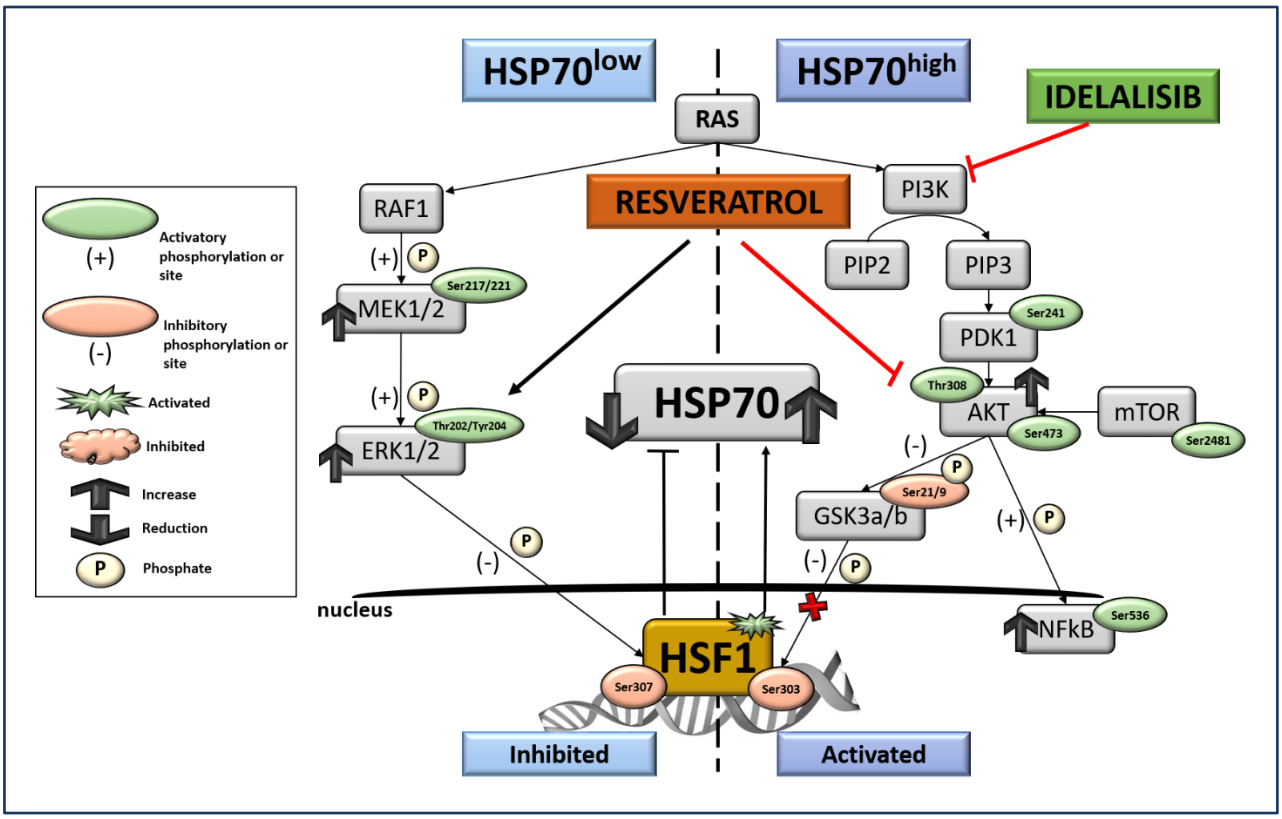

Fig. 54: Targeting of our model for the aberrant RAS signalling pathways in CLL. The two RAS pathways are simultaneously targeted by resveratrol, which inhibits AKT and activates ERK, downmodulating HSP70. Idelalisib inhibits PI3K pathway, preventing the activation of AKT and resulting in the downmodulation of HSP70.

Despite these data, the connections between AKT and MAPK signalling pathways and HSP70 have not been thoroughly explored, and the role of upstream kinases in controlling HSF1 activity was not properly understood[103]. Cotto *et al.* demonstrated that AKT pathway controls the translocation of HSF1 through the phosphorylation of GSK3b at Ser303, which prevents the HSF1 accumulation in the nucleus of normal cells[104]. This data are particularly interesting since we found HSF1 abnormally localized in the nucleus of CLL cells, confirming the dysregulation of the AKT pathway explained by our model (**Fig. 54**). Thus, the activation of GSK3b in presence of resveratrol definitely contributes to the blocking of HSF1 translocation to the nucleus and of HSP70 expression.

An ulterior confirmation of dysregulated RAS signalling pathways in CLL, comes from our experiments using idelalisib, an inhibitor of PI3K. Idelalisib can diminish the expression of both HSF1 and HSP70 (**Fig. 54**), demonstrating an involvement of the PI3K/AKT pathway in the up-regulation of HSF1/HSP70 axis.

In conclusion, HSP70 and HSF1 overexpression and correlation with resistance to therapy in CLL, together with the ability of fisetin to induce apoptosis in leukemic B cells bypassing the pro-survival stimuli of tumor microenvironment, represent a starting point for the development of new therapeutic strategies. In addition, the study of resveratrol effects on leukemic B cells provides new insights on the cross-talk between HSP70/HSF1 axis and RAS signalling in CLL.

We are aimed at further dissecting RAS aberrant pathways in CLL, taking advantage from the double-action mechanism of Resveratrol on these pathways. We are collecting preliminary data on B cells from CLL patients and healthy controls treated with other compounds acting as Resveratrol with the advantage of being more bioavailable and effective: Pterostilbene, Honokiol, and Triacetyl Resveratrol. We plan to analyze the effects of these inhibitors on HSF1/HSP70 axis, further investigating the RAS-pathway model, with the ultimate goal of unravel the pathobiology of CLL.

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