UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Department of Surgery, Oncology and Gastroenterology

PhD course in Clinical and Experimental Oncology and Immunology

XXXIV series

Focal Adhesion Kinase (FAK) contribution to the pathogenesis of Chronic Lymphocytic Leukemia

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ABBREVIATIONS

Ab	Antibodies
APC	Allophycocyanin
ATM gene	Ataxia Teleangectasia Mutated
BCL-2	B cell Lymphoma 2
BCR	B cell receptor
BSA	Bovine serum albumin
BTK	Bruton's tyrosine kinase
Ca^{2+}	Calcium ion
C-FAK	C-terminal fragment of FAK
CLL	Chronic Lymphocytic Leukemia
CXCR4	Chemokine (C-X-C motif) receptor 4
DAG	Diacylglycerol
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
F/H	Ficoll/Hypaque
FAK	Focal adhesion kinase
FAT	Focal adhesion-targeting domain
FBS	Fetal Bovine Serum
FC	Flow cytometry
FERM	Band 4.1-ezrin-radixin-moesin
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
FRNK	FAK-related non-kinase
FSC	Forward scatter
HS1	Hematopoietic lineage cell-specific protein-1
HSCs	Hematopoietic stem cells
hTERT	Telomerase reverse transcriptase
Ig	Immunoglobulin
IGHV	Immunoglobulin Heavy Chain Variable Region
IP3	Inositol-1,4,5-triphosphate
ITAM	Immunoreceptor tyrosine-based activation motifs
iwCLL	International Workshop on Chronic Lymphocytic Leukemia
Lyn	Lck/Yes-related Novel Protein Tyrosine Kinase
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
M-CLL	IGHV mutated CLL
MFI	Mean Fluorescence Intensity
MFI	Mean Fluorescence Intensity
MMP	Matrix metalloproteinases
MSCs	Mesenchymal stromal cells
NES	Nuclear export sequence
N-FAK	N-terminal fragment
NK	Natural-Killer
NLCs	Nurse like cells
NLS	Nuclear localization sequence
OS	Overall survival
PARP	Poly-ADP-ribose polymerase
PBS	Phosphate Buffered Saline
PCs	Proliferation centers
PE	Phycoerythrin
PE-Cy7	PE-Cyanine7
PFS	Progression free survival
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphophate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PLCγ	Phospholipase Cγ
PS	Phosphatidylserine
PTK2	Protein Tyrosine Kinase 2
PTPs	Protein-tyrosine phosphatases
RPPA	Reverse Phase Protein Array
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Polyacrylamide gel electrophoresis in SDS
SH2	Src homology 2
SH3	Src-homology 3
SSC	Side scatter
SYK	Spleen tyrosine kinase
TP53	Tumor Protein p53
U-CLL	IGHV unmutated CLL
WB	Western blotting
WBC	White Blood Cells
Y397	Tyrosine 397 FAK phosphorylation site
ZAP-70	Zeta-associated protein of 70kDa

ABSTRACT

Chronic Lymphocytic Leukemia (CLL) is a neoplastic disorder characterized by the clonal expansion and accumulation of B lymphocytes in peripheral blood, bone marrow and lymphoid tissues. CLL pathogenesis involves both intrinsic and extrinsic factors, such as apoptosis dysregulation and altered B-cell receptor (BCR) signaling. The heterogenous disease course can range from an indolent condition to a very aggressive form of leukemia; thus, it is important to identify novel biological features to predict patients' prognosis. Indeed, the research for molecules involved in increased survival and drug resistance of neoplastic B cells is ongoing.

In this context, we focused on the role of Focal Adhesion Kinase (FAK), a 125kDa protein which, upon phosphorylation on Y397, is activated and can recruit numerous signaling molecules. Especially in solid tumors, FAK can regulate several cellular processes, like adhesion, migration, apoptosis, and proliferation. Besides, as a component of focal adhesions, this kinase participates in the dynamic turnover of these structures, a process regulated through proteolytic cleavage by Calpain, a Ca^{2+}-dependent enzyme. The same cellular mechanisms of cytoskeleton remodeling and motility of the tumor cell are regulated by hematopoietic lineage cell-specific protein 1 (HS1) and its homolog Cortactin, over-expressed in CLL and associated with disease aggressiveness.

With this as a background and provided that Cortactin and HS1 can interact with FAK, we hypothesized that FAK might play a key role in CLL pathogenesis and/or maintenance. Therefore, the aim of this three-year PhD project was to characterize FAK functions in CLL cells.

We found that FAK expression was variable among CLL patients, with poor prognosis U-CLL cases showing a down-modulation of the 125kDa full-length form (n=56, 0.39±0.36 *vs* n=67 M-CLL, 0.73±0.72; $p<0.01$, Student's *t* test). Analyzing the presence of other forms of the kinase, we proved that FAK was present also as lower molecular weight bands of 92/94, 84 and 50kDa, compatible with Calpain cleaved fragments. These fragments were found in great amount in the BCR responsive patients, whose receptor signaling is more active, and in which we found a trend toward increased Calpain expression. These cleaved forms were also phosphorylated in Y397, meaning active, and able to relocate inside the cells, since they lacked the domain for the localization near the membrane. We found that cleaved FAK was present inside the nucleus, especially of the responsive patients.

Analyzing the relationship with other molecules involved in CLL aggressiveness, like HS1 and Cortactin, both through flow cytometry and RPPA analysis, we demonstrated that FAK activation was significantly correlated with the over-expression of the two molecules, suggesting that FAK itself may be involved in a pathway for increased aggressiveness of the disease.

To investigate FAK role in tumor survival, we treated CLL cells with 5μM Defactinib, a selective inhibitor for the kinase. After 24 hours of *in vitro* treatment, we demonstrated a reduction in the viability of leukemic lymphocytes (n=46; 33±24% *vs* untreated control, 62±17%, $p<0.0001$, paired Student's *t* test).

In conclusion, we hypothesized that FAK and its cleaved fragments by Calpain are involved in CLL pathogenesis or, in particular, in the more aggressive disease phenotype. Its ability to translocate inside the nucleus and the reported pro-survival functions suggest that FAK is implicated in pathways participating in apoptosis resistance. In addition, the correlation with

other molecules linked to CLL aggressiveness, namely HS1 and Cortactin, indicate an involvement of FAK in promoting a malignant disease phenotype. The apoptotic effects of the FAK inhibitor Defactinib gave further demonstration of the kinase participation in a survival mechanism in CLL.

RIASSUNTO

La Leucemia Linfatica Cronica (LLC) è una malattia linfoproliferativa caratterizzata dall'espansione clonale e dall'accumulo di linfociti B nel sangue periferico, nel midollo osseo e nei tessuti linfoidi. La patogenesi della LLC coinvolge sia fattori intrinseci che estrinseci, come la deregolazione dell'apoptosi e l'alterato *signaling* del recettore delle cellule B (BCR). Il decorso della malattia è molto eterogeneo e può variare da una condizione indolente ad una forma molto aggressiva di leucemia; per questo motivo è fondamentale identificare nuove caratteristiche della malattia che possano dare indicazioni sulla prognosi dei pazienti. Infatti, la ricerca di molecole coinvolte nell'aumentata sopravvivenza e nella resistenza ai farmaci delle cellule B leucemiche è sempre più attuale.

In questo contesto, ci siamo concentrati sul ruolo della chinasi di adesione focale (FAK), una proteina di 125kDa che, in seguito a fosforilazione in Y397, viene attivata e può reclutare numerose proteine di segnale. Specialmente nei tumori solidi, FAK può controllare diversi processi cellulari come l'adesione, la migrazione, l'apoptosi e la proliferazione. Questa chinasi partecipa inoltre al *turnover* dinamico delle adesioni focali di cui fa parte, un processo regolato dal taglio proteolitico di FAK da parte di Calpaina, enzima dipendente dal Ca^{2+} intracellulare. Gli stessi meccanismi di rimodellamento del citoscheletro e di motilità cellulare sono regolati anche da HS1 (*Hematopoietic lineage cell Specific protein-1*) e dalla sua omologa Cortactina, entrambe *over*-espresse e associate a prognosi infausta nella LLC.

Con queste premesse, e considerando che HS1 e Cortactina possono interagire con FAK, abbiamo ipotizzato che la stessa FAK potrebbe svolgere un ruolo chiave nella patogenesi e/o nel mantenimento della LLC. Lo scopo di questo progetto di dottorato è stato, pertanto, quello di caratterizzare le funzioni di FAK nelle cellule di LLC.

Abbiamo dimostrato che l'espressione di FAK nei pazienti con LLC era variabile, con i casi U-LLC a prognosi sfavorevole che mostravano una *down*-modulazione della forma *full-length* da 125kDa (n=56, 0,39±0,36 *vs* n=67 M-LLC, 0,73±0,72; $p<0,01$, test *t* di Student). Analizzando la presenza di altre forme della chinasi, abbiamo dimostrato che FAK era presente anche come bande a peso molecolare di 92/94, 84 e 50kDa, compatibili con un clivaggio mediato dalla proteasi Calpaina. Questi frammenti sono stati trovati in maggiore quantità nei pazienti con BCR responsivo, cioè con un *signaling* più attivo, nei quali abbiamo anche riscontrato una tendenza verso un'aumentata espressione di Calpaina, rispetto ai pazienti con un recettore non-responsivo. Queste forme clivate erano anche fosforilate in Y397, cioè attive, e in grado di "muoversi" all'interno delle cellule, poiché prive del dominio di localizzazione in membrana; FAK clivata, infatti, era presente all'interno del nucleo, specialmente nei pazienti responsivi.

Analizzando la relazione con altre molecole coinvolte nell'aggressività della LLC, come HS1 e Cortactina, sia mediante la citofluorimetria a flusso che l'analisi di RPPA, abbiamo dimostrato che l'attivazione di FAK era significativamente correlata con la sovra-espressione di queste due molecole, suggerendo che FAK stessa potrebbe essere coinvolta in un *pathway* che favorisce una maggiore aggressività della malattia.

Per studiare il ruolo di FAK nella sopravvivenza delle cellule tumorali abbiamo trattato i linfociti di LLC con Defactinib, un inibitore selettivo della chinasi. Dopo 24 ore di trattamento *in*

vitro, abbiamo dimostrato una riduzione della vitalità delle cellule leucemiche (n=46; 33±24% *vs* controllo non trattato, 62±17%, $p<0,0001$, test *t* di Student per dati appaiati).

In conclusione, ipotizziamo che FAK e i suoi frammenti clivati siano coinvolti nella patogenesi della LLC o, in particolare, nel fenotipo più aggressivo della malattia. La sua capacità di traslocare all'interno del nucleo e le riportate funzioni pro-sopravvivenza suggeriscono che FAK sia implicata in un *pathway* di resistenza all'apoptosi. Inoltre, la correlazione con altre molecole legate all'aggressività della LLC, vale a dire HS1 e Cortactina, indicano un coinvolgimento di FAK nel fenotipo maligno della malattia. Gli effetti apoptotici dell'inibizione di FAK via Defactinib hanno dato un'ulteriore dimostrazione della partecipazione di questa chinasi a un meccanismo di sopravvivenza nella LLC.

INTRODUCTION

1. Chronic Lymphocytic Leukemia (CLL)

1.1 Epidemiology and etiology

Chronic Lymphocytic Leukemia (CLL) is a lymphoproliferative disorder featured by the accumulation of long-lived mature clonal B cells in the peripheral blood, bone marrow, and lymphoid tissues. The small CLL lymphocytes show a distinct immune phenotype characterized by expression of B cell markers, such as CD19 and CD20, together with CD5, CD23 and CD200, which are not usually expressed on non-malignant B cells[1].

CLL is the most common adult leukemia in Western countries[2], with an incidence of approximately 4.7 cases per 100,000 people and affects more men than women (a ratio of approximately 1.7:1)[3] (**Fig. 1**). With a median age at diagnosis of 67-72 years[3], CLL can be considered as a disease of the elderly. Nevertheless, it is not unusual to diagnose it in younger individuals from 30 years of age. The incidence increases rapidly with increasing age[4]**.** However, about 10% of CLL patients are younger than 55 years (**Fig. 2**).

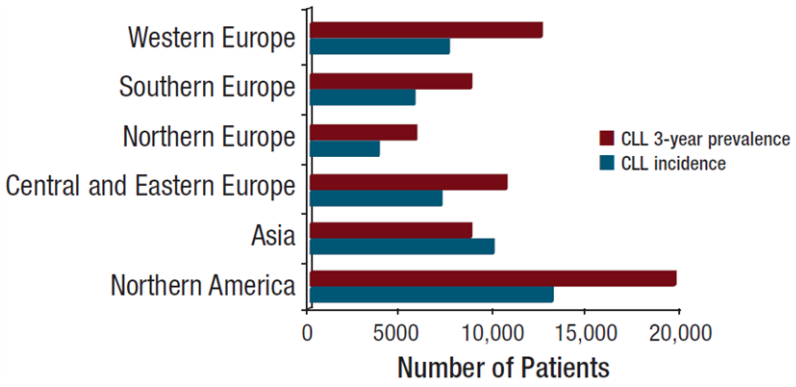

Figure 1. **Worldwide incidence of CLL.** CLL incidence rate per 100,000 inhabitants (from Combest *et al*[5]).

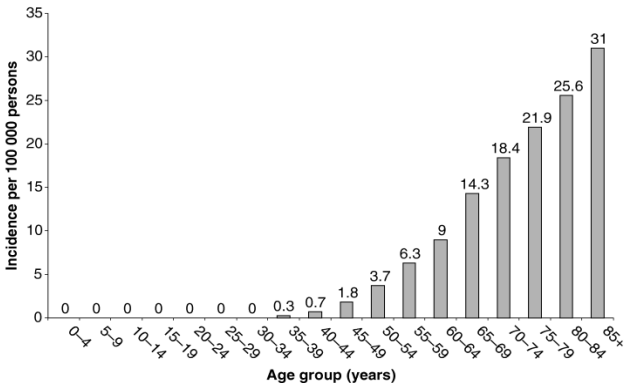

Figure 2. **Incidence of CLL.** Age-specific incidence of CLL (from Redaelli *et al*.[4]).

The etiology of this pathology is still unknown; the exposure to common carcinogens does not seem to be associated with the disease progression. More studies are in progress to assess a potential relation among CLL onset, inflammation, and autoimmune conditions[6]. There is an

inherited genetic susceptibility for CLL. In fact, a familiarity of this pathology is well documented in the 8-10% of cases. Relatives of patients with CLL have 8.5-fold increased risk of developing CLL and other lymphoproliferative disorders compared to the general population, although the basis for this genetic susceptibility remains elusive[7]. Moreover, it has been highlighted the phenomenon of the anticipation in which inherited disease is diagnosed at an earlier age and in a more aggressive form in the later generations of a family[8].

1.2 Clinical features

CLL has a highly variable presentation and clinical course. Most patients are asymptomatic and do not experience disease progression throughout their lifetime, thus living without requiring therapy. The majority of patients, however, will eventually require treatment once presenting an aggressive disease[9].
CLL symptoms can include B symptoms (weight loss, fever, and excessive night sweats), fatigue, increased frequency of infections, which might be associated with hypogammaglobulinemia or autoimmune cytopenia, that could induce immunodeficiency and high mortality for infections. Patients can also show enlarged lymph nodes, hepatomegaly, and splenomegaly[10].

1.3 Diagnosis

The 2018 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[9]:

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 (7-10µm diameter), mature lymphocytes with poor cytoplasm and dense nucleus lacking discernible nucleoli with partially aggregated chromatin. Gumprecht shadows, which are degenerated cells broken during slide preparation, are also present in the peripheral blood smear (**Fig. 3**)[9];

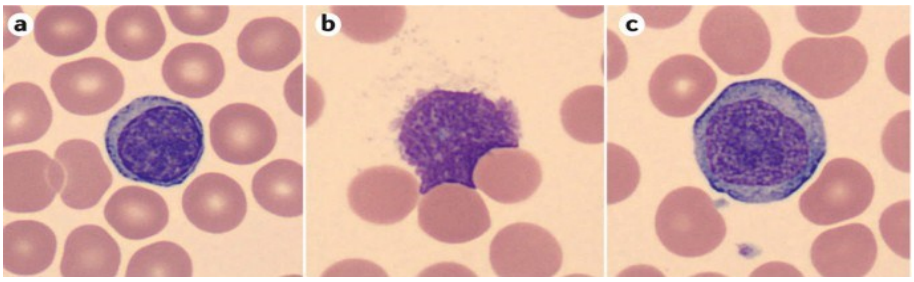

Figure 3. **Peripheral blood smear of CLL.** Wright–Giemsa-stained blood smears showing the typical CLL B lymphocyte (**a**), Gumprecht shadow or smudge cell (**b**) and a prolymphocyte with a prominent nucleolus (**c**). Magnification ×500 (Adapted from Kipps *et al*.[10]).

2. Bone marrow lymphocytic infiltration exceeding 30% of the nucleated cells. 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[9].

3. CLL immunophenotype is characterized by three elements[9]:
   a) the expression of low levels of surface immunoglobulins (Igs) with restricted light chains (κ or λ);
   b) the co-expression of the T-cell antigen CD5 and the B-cell surface antigens CD19, CD20, and CD200; CD200 is of particular importance in the differential diagnosis with mantle cell lymphoma (CD5+ but CD200-);
   c) low levels of CD79b and surface Igs that in CLL appear to be mainly IgM followed by IgD, IgG, and IgA; it is not unusual to find an IgM and IgD co-expression (**Fig. 4**).

In CLL, also T lymphocytes are someway affected, presenting a reduced CD4/CD8 ratio. They show activation markers such as CD25 and HLA-DR. Natural-Killer (NK) cells (CD16+/CD56+) are present in high levels. Several analyses are performed to confirm the diagnosis and to prevent complications: serum protein electrophoresis, Ig dosage, Coombs' test, and analysis of renal and liver function. Before starting an immunotherapy, it is important to assess the absence of viral infections, such as Hepatitis B virus (HBV), Hepatitis C virus (HCV), Cytomegalovirus (CMV), Epstein-Barr virus (EBV), Human Immunodeficiency virus (HIV)[9].

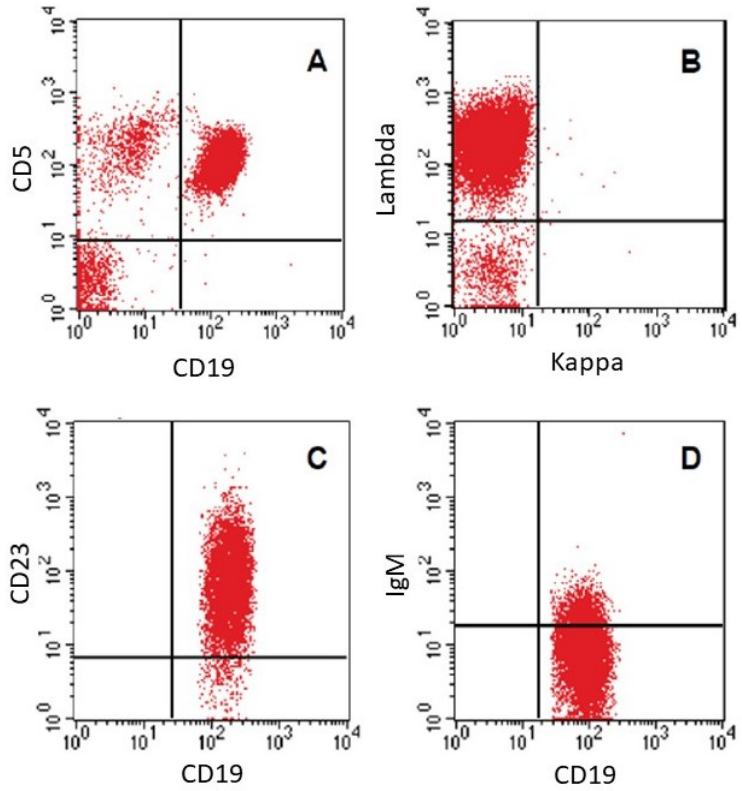

Figure 4. **Cytograms of a representative case of CLL.** B lymphocytes analyzed (CD19+) are positive to CD5 (**A**), express one type of immunoglobulins light chain (λ, **B**), are positive to CD23 (**C**) and to low intensity surface IgM (**D**).

1.4 Prognosis

In the past decades, different parameters and biological markers were identified to predict the prognosis of CLL patients. The first markers that have been investigated as potential prognostic factors were age, gender, lymphocyte doubling time, number of prolymphocytes, and bone marrow involvement, but only age and lymphocyte doubling time have been factored into

treatment decisions[11]. Nowadays, clinicians mostly rely on biological and genetic prognostic factors, that have shown to be the most consistent and exploitable from diagnosis.
Given that CLL patient clinical course is extremely variable, it is necessary to stratify patients on the disease gravity in order to propose therapy only to those patients with progressive and/or symptomatic disease[10].

1.4.1 Clinical prognostic factors

1. Clinical staging: There are two widely accepted staging methods in both patient care and clinical trials: the Rai and the Binet system. The original Rai classification was modified to reduce the number of prognostic groups from five to three. Both systems now describe three major subgroups with different clinical outcomes. These two staging systems are simple, inexpensive, and can be applied by physicians worldwide. Both rely exclusively on physical examination and standard laboratory tests[9].
The Rai system is so developed:
   - low-risk disease (stage 0): absolute lymphocytosis >15,000/µl and marrow lymphocytosis >40%;
   - intermediate-risk disease (stage I or II): lymphocytosis, enlarged lymph nodes in any site, and splenomegaly and/or hepatomegaly (lymph nodes being palpable or not);
   - high-risk disease (stage III or IV): disease-related anemia (Hb<110g/l) or thrombocytopenia (as defined by a platelet count $<100x10^9$/l).

The Binet system is based on the number of involved areas, as defined by the presence of lymph nodes with a diameter greater than 1cm or organomegaly, and the presence of anemia or thrombocytopenia. It is subdivided into:
   - stage A. Hb≥100g/l (10g/dl), platelets $\geq100x10^9$/l, and up to 2 lymph node areas involved;
   - stage B. Hb≥100g/l, platelets $\geq100x10^9$/l, and lymphadenopathy greater than that defined for stage A (*i.e.*, 3 or more areas of nodal or organ enlargement);
   - stage C. All patients who have Hb<100g/l and/or a platelet count $<100x10^9$/l, irrespective of lymphadenopathy.

2. Lymphocyte doubling time: it is less than 12 months and it is associated with a worse clinical course.
3. Bone marrow infiltration: a diffuse infiltration pattern, which is typically >30%, correlates with a bad prognosis[9].

1.4.2 Biological prognostic factors

The less recent biological prognostic factors are correlated with the expansion of the leukemic clone. As a consequence, they become indicative only when the disease is worsening. Therefore, their utility is limited since they do not allow to plan therapeutic strategies early on. The biological prognostic factors comprehend:

1. β2 microglobulin: its level is inversely correlated with patient survival. It is related to the lymphocyte doubling time, so that an increase in β2 microglobulin indicates a high neoplastic cell proliferation[12].

2. Thymidine kinase (TK) level: elevated serum thymidine kinase (s-TK) levels predict disease progression in CLL. 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 expect a much longer PFS[13].
3. Soluble CD23: serum CD23 level provides significant additional prognostic information in terms of overall survival (OS) in CLL. Among early-stage patients, sCD23 determination, at diagnosis and during the course of the disease, may help to the early identification of patients who will rapidly progress to upper stages[14].

The progressive discoveries on CLL pathogenesis have identified new prognostic markers that can better determine the clinical course. They describe biological characteristic of the leukemic clone that are crucial to evaluate its proliferation and invasion capability. The study of these markers is performed by flow cytometry, cytogenetic and molecular biology techniques. The main markers are:

1. Mutational status of IGHV - Somatic Hypermutations (SHM) of the Ig heavy chain variable region (IGHV) genes. CLL patients are divided into two subgroups depending on the IGHV genes mutational status: the IGHV unmutated (U-CLL) or mutated (M-CLL) (**Fig. 5**). Conventionally, patients with ≥98% homology to the closest germ-line gene are defined U-CLL and have a poorer prognosis than patients with CLL cells displaying <98% homology, defined M-CLL[15]. In addition, some unrelated CLL patients exhibit almost identical or "stereotyped" B-cell receptor immunoglobulin sequence. Approximately one-third of patients can be grouped into subsets based on shared sequence motifs within the IGHV complementarity-determining region 3 (CDR3)[16]. It seems that some of these subgroups share a similar prognosis. For example, stereotype subset #2 may be associated with an unfavorable prognosis independent of the IGHV mutational status[17].

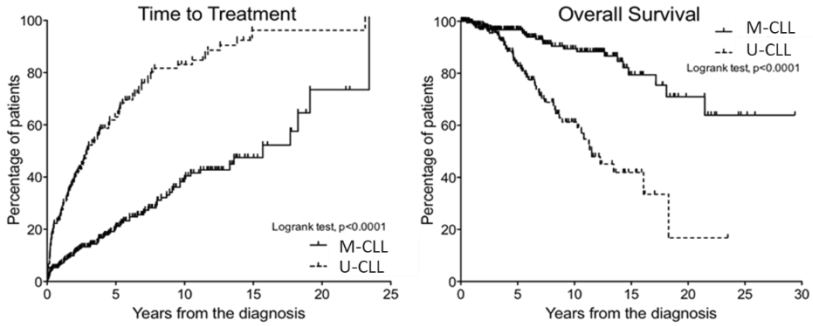

Figure 5. **Time to Treatment and Overall Survival in relationship with IGHV prognostic marker.** The median time to treatment and Overall Survival for mutated (M-CLL) and unmutated (U-CLL) CLL patients (adapted from Visentin *et al*[18]).

2. Chromosomal alterations. CLL is also characterized by a genomic instability that gives rise to several chromosomal aberrations, with 11q, 13q, 17p deletions and 12 trisomy being the most relevant. Clonal genomic aberrations can be identified in approximately 80% of CLL patients by fluorescence in situ hybridization (FISH). While 11q and 17p deletions have been associated with rapid disease progression, the absence of chromosomal abnormalities and the presence of 13q deletion as the sole abnormality are associated with a better prognosis[19] (**Fig. 6**). The presence of high-risk chromosome alterations justifies the use of more aggressive treatment.

Chromosome alterations are independent from IGVH mutational status though higher frequencies of 11q- and 17p- in U-CLL and of 13q- in M-CLL cases are evident[19].

Recently, complex karyotype (CK), defined as the presence of ≥3 chromosomal lesions, has been shown to be a negative prognostic biomarker associated with shorter survival in CLL[20].

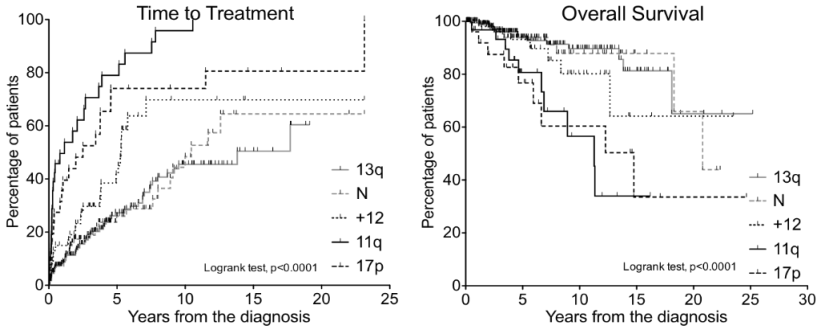

Figure 6. **Time to treatment and probability of survival among patients in the most common chromosomal alterations.** The median survival times and time to treatment for the groups with 17p deletion, 11q deletion, 12q trisomy, normal karyotype, and 13q deletion as single aberration were 0.18, 1.75, 3.81, 11.56, and 8.00 years, respectively (adapted from Visentin *et al*[18]).

3. CD38 expression. CD38 is a transmembrane glycoprotein expressed on the surface of cells in a significant percentage of patients with CLL. A previous study suggested that CD38 expression could be considered as an independent prognostic value in CLL (**Fig. 7**). Cases with CD38+ B cells >30% show a bad prognosis. However, the cut-off to discriminate CD38+ to CD38- is not unanimously defined, oscillating between 20% and 7% of CD38+ B cells[21].

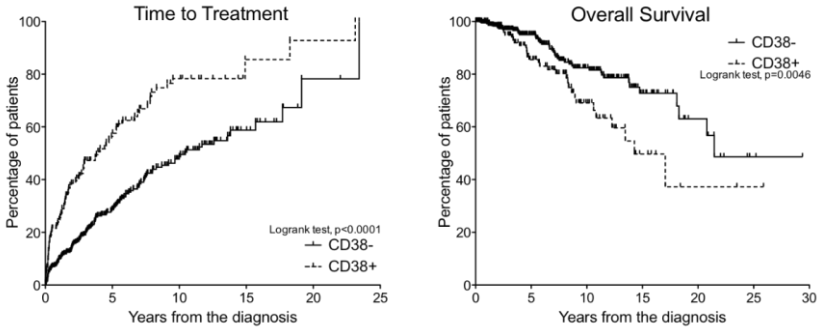

Figure 7. **Time to Treatment and Overall Survival in relationship with CD38 prognostic marker.** The median time to treatment and overall survival for patients with a high (CD38+) or low (CD38-) number of cells expressing this transmembrane glycoprotein (adapted from Visentin *et al*[18]).

4. 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 (Zeta-associated protein of 70kDa) expression. Actually, CD49d is the best flow cytometry-based marker for CLL patients' prognosis stratification[22] (**Fig. 8**).
5. Telomerase expression and telomere length in CLL. Activation of telomerase reverse transcriptase (hTERT) is essential for unlimited cell growth and plays a critical role in tumorigenesis[23]. Recently, the levels of telomerase activity and/or hTERT expression were related to clinical aggressiveness and prognosis in a variety of malignancies, including CLL. CLL cases with high telomerase levels and short telomeres were frequently characterized by an unmutated IGHV status and high-risk chromosomal aberrations. Conversely, CLL cases with

low telomerase levels and long telomeres were associated with a mutated IGHV status and low-risk abnormalities[24] (**Fig. 9**).

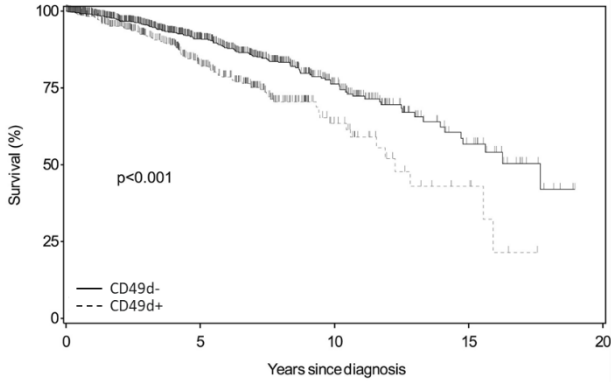

Figure 8. **Overall Survival in relationship with CD49d prognostic marker.** The median survival times for the groups with groups with high (≥30%, CD49d+) or low (CD49d-) expression (adapted from Parikh *et al*[25]).

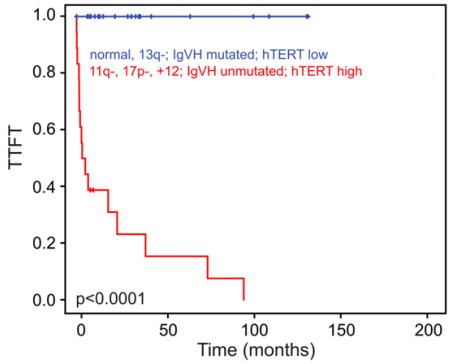

Figure 9. **Curves of treatment-free survival.** Time from diagnosis to first treatment (TTFT) according to IGHV mutational status, chromosomal categories, and hTERT level/telomere length profile (adapted from Rampazzo *et al*[24]).

1.5 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 survival benefit of early therapeutic interventions. When patients have progressive or symptomatic/active disease, treatment should be initiated. The 2018 iwCLL guidelines list some relevant conditions to define symptomatic or active disease[9]:

1. progressive marrow failure (anemia and/or thrombocytopenia; usually Binet stage C or Rai stage III or IV);
2. massive (*i.e.*, ≥6cm below the left costal margin) or progressive or symptomatic splenomegaly;
3. massive nodes (*i.e.*, ≥10cm in longest diameter) or progressive or symptomatic lymphadenopathy;
4. progressive lymphocytosis with an increase of >50% over a 2-month period or 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;

5. autoimmune anemia and/or thrombocytopenia poorly responsive to corticosteroids or other standard therapies;
6. symptomatic or functional extranodal involvement (*e.g.*, skin, kidney, lung, spine);
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.

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 (mAb), rituximab, was the first advancing step; second generation of anti-CD20 mAb, ofatumumab, and an anti-CD52 mAb, alemtuzumab, also came into the clinic, to treat higher risk and refractory patients.
In the last ten years, the improvements in understanding the biology of B cell receptor (BCR) signaling in CLL and its role for the survival of the leukemic cells led to the development of new therapeutic strategies, which involve small molecular inhibitors including ibrutinib, a BTK (Bruton's tyrosine kinase) inhibitor, idelalisib, a PI3Kδ (phosphatidylinositol 3-kinase) inhibitor, and venetoclax, a BCL-2 (B Cell Lymphoma 2) protein antagonist. These improvements provide excellent treatment options for all CLL patients, particularly for older patients with co-morbidities, poor prognostic features, or relapsed/refractory disease. For example, ibrutinib is now considered the standard in first-line therapy for del17p CLL patients[9].
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[9].

1.6 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 and 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[26].
Traditionally, CLL lymphocytes were thought to be derived from *naïve* B cells, but studies starting from 2003 support the derivation of CLL cells from activated antigen-experienced B cells[1]. In 2012, Seifert *et al.*[27] 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 cell of origin of this leukemia[28].

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 expansion of B cells in MBL, which is associated with additional genetic or epigenetic changes, leads to CLL when the lymphocyte count is ≥5,000 cells per µl[28].

CLL cells deriving from post-germinal center B cells, with a T cell-dependent immune stimulation, are characterized by mutated IGHV and are defined as M-CLL. CLL cells deriving from pre-germinal center B cells, with a T cell-independent immune stimulation, express germline IGHV and are defined as U-CLL (**Fig. 10**). The expansion of leukemic clone is due to the accumulation of novel genetic lesions, as well as continued interactions between CLL cells, accessory cells and antigens in the microenvironment of lymphoid tissues[28]. The pathogenesis of CLL involves both intrinsic factors, such as several genetic alterations, and extrinsic ones, mainly represented by the interactions with the microenvironment. Some factors are briefly described below.

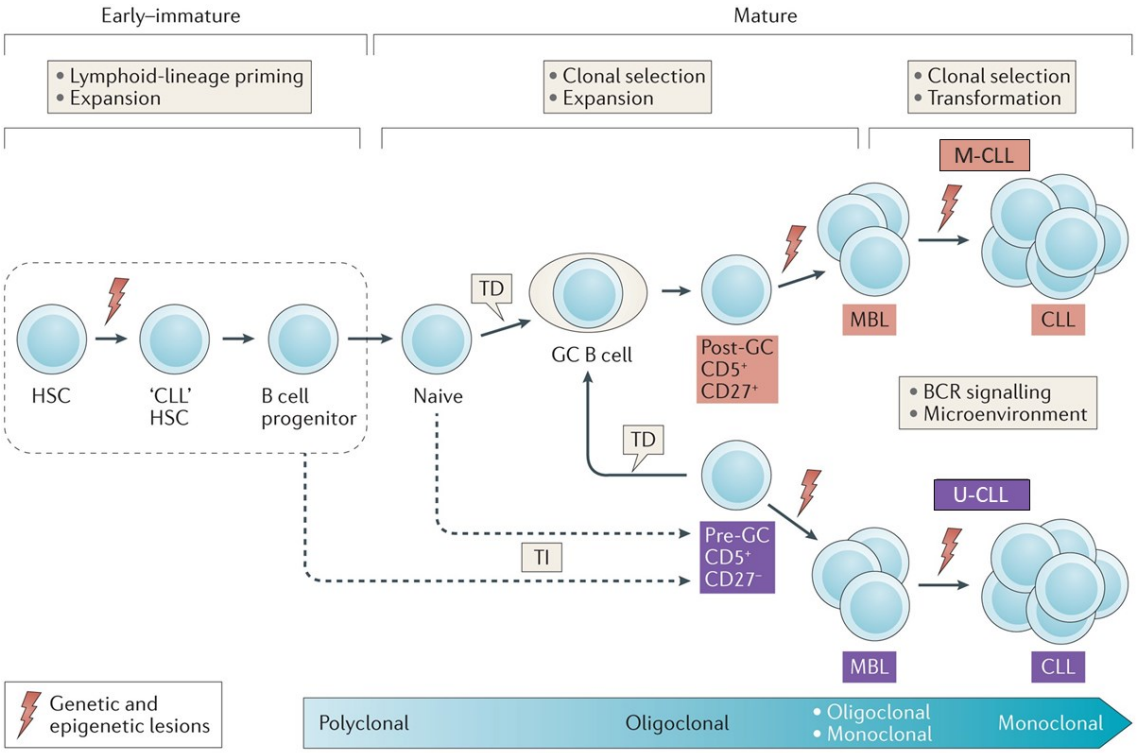

Figure 10. **The cellular origin of CLL subsets.** The IGHV-unmutated CLL cell (U-CLL) derives from a pre-germinal center CD5+ B cell and has an antigen response through a T-independent mechanism. In most cases the lymphocyte undergoes SHM and it is selected in the lymph node based on the affinity of the 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 (M-CLL) 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. TD=T cell-dependent antigen; TI=T cell-independent antigen (image from Fabbri *et al*[1]).

1.6.1 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)[19]. 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[29]. The 7% of patients carry del17p associated with loss of the tumor suppressor gene *TP53* (Tumor Protein p53)[30]. Del11q is found in 18% of patients and is often associated with alterations in ATM gene (Ataxia Telangiectasia 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[19] (**Table 1**).

In 2014, Damm *et al.*[31] identified early mutated genes in the progenitor and/or in mature myeloid fractions of CLL patients. 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 signaling, NOTCH signaling and inflammatory pathways. 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*[32,33].

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 down-regulated 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[34].

Table 1. **CLL genetic alterations.** Most common chromosomal alterations found in CLL patients (adapted from Redaelli *et al.*[4]).

	Type of alteration	Frequency	Clinical correlations
Chromosome 13	Deletions	50–70%	Good prognosis
Chromosome 12	Trisomy	10–30%	Possible high-risk disease
Chromosome 11	Deletions	10–20%	Younger age, more aggressive disease
Chromosome 17	Mutation	1.5–7%	Advanced disease, drug resistance, shorter patient survival

1.6.2 Intrinsic factors: BCR and B cell signaling

BCRs are expressed on B cell membrane and are necessary to bind specific antigen, against which B cells will initiate an antibody response[26]. In normal B cells, several checkpoints of the cell cycle are regulated by BCR signaling[35].

The BCR is a multiprotein complex composed of a ligand-binding trans-membrane immunoglobulin molecule (either IgA, IgD, IgE, IgG or IgM) and the signaling Igα-Igβ (also known as CD79a-CD79b) heterodimer[36] (**Fig. 11**). CLL cells typically co-express IgM and IgD, although at low levels compared to normal B cells.

Binding to a specific antigen induces activation of upstream kinases, including spleen tyrosine kinase (SYK) and the Src kinase Lyn (Lck/Yes-related Novel Protein Tyrosine Kinase), which phosphorylate ITAM domains (immunoreceptor tyrosine-based activation motifs) in the cytoplasmic tails of CD79a and CD79b molecules, thus triggering BCR signaling. Phosphorylation of ITAMs creates docking sites for the recruitment and activation of SYK[35]. This binding, which is performed thanks to two N-terminal Src homology 2 (SH2) domains, leads to a conformational change of SYK which, in its activated form, is able to phosphorylate downstream signaling proteins and neighboring ITAMs, amplifying the signal[28]. BLNK serves as a scaffold protein to bind PLC-γ2 (phospholipase Cγ2) and 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 signaling. The generation of IP3 leads to enzyme activation and calcium influx from the endoplasmic reticulum to the extracellular compartment (**Fig. 12**).

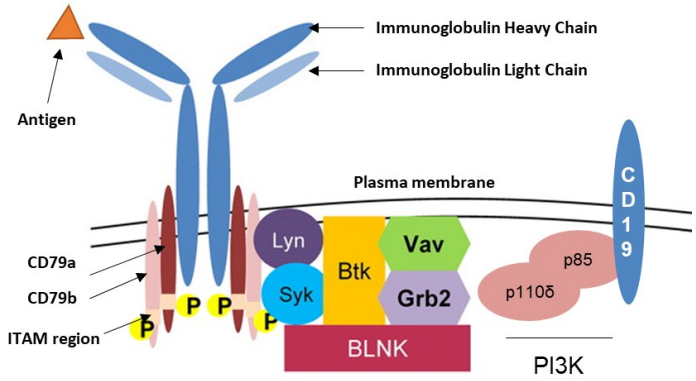

Figure 11. **Schematic representation of the BCR and its signalosome.** BCR is composed of two immunoglobulin heavy and light chains and CD79a and CD79b (Igα/Igβ), which contain ITAMs. After antigen ligation, ITAMs are phosphorylated, creating SH2 binding sites, and recruit kinases and adaptor proteins. The initial signaling complex of the BCR is thus formed (adapted from Woyach *et al*[37]).

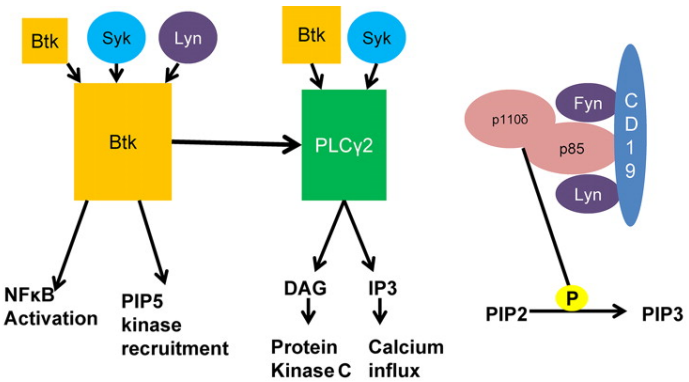

Figure 12. **BCR-induced signal transduction pathways.** Activation of kinases SYK and Lyn inside the BCR signalosome recruits additional kinases leading to activation of three main pathways: (I) BTK, (II) PLC-γ2, and (III) PI3K (p110δ and p85). SYK and Lyn phosphorylate BTK, resulting in the activation of NF-κB 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 (adapted from Woyach *et al*[37]).

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[37].

The activation of signaling proteins, such as BTK or PI3K, leads to the activation of downstream targets, such as AKT/mTOR, NF-κB, mitogen-activated protein kinase (MAPK) and ERK (extracellular signal-regulated kinase), and other molecules, leading to cellular proliferation, survival, or apoptosis[28].

In addition, Lyn recruitment to surface Ig triggers a signaling pathway aimed at activating the cytoskeleton. This involves the hematopoietic lineage cell-specific protein-1 (HS1) protein[38,39] and the related F-actin polymerization, as well as the other kinases involved in BCR signaling and calcium mobilization.

Activation of phosphatases, including SH2 domain containing protein tyrosine phosphatase-1 (SHP1) and SH2 domain containing inositol 5-phosphatases 1/2 (SHIP1/2), and of negative co-receptors (*e.g.* CD22, CD5) contributes to negative regulation of the BCR signaling response[10].

A functional BCR is essential for the survival of mature B cells and in most hematological malignancies, including CLL, there is increasing evidence that BCR signaling plays a relevant role[40]. CLL-BCRs show differential degrees of somatic mutations, which correlate with the clinical prognosis of patients and one third of CLL patients express quasi-identical ("stereotyped") BCRs[41], suggesting that common antigens may be relevant to disease pathogenesis across patients' subsets. As previously explained, the IGHV mutational status identifies two major CLL subsets. The mutated CLL cells (M-CLL) usually show constitutive phosphorylation of signaling proteins, including ERK kinase[42] and reduced levels of responsiveness to BCR stimulation, generally referred to as "anergy"; in contrast, unmutated IGHV (U-CLL) tend to express low-affinity, poly-reactive and self-reactive BCRs[43] and are more responsive to surface immunoglobulin stimulation.

The dysregulation of the BCR signaling pathways in CLL is mainly characterized by constitutive phosphorylation of kinases[37]. 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[35]. In addition, Lyn activates SHIP-1 (SH2-domain-containing inositol phosphatase), which provides negative feedback to BCR signaling by counteracting PI3K activity[10]. At the same time, BCR signaling can be enhanced by ZAP-70, a cytoplasmic tyrosine kinase expressed in T cells, and abnormally found in approximately half of CLL cases[28,44].

We demonstrated that, in B-CLL cells, Lyn is an integral component of an aberrant cytosolic 600kDa complex, where Lyn is associated with Hsp90 (Heat shock protein of 90kDa) through its catalytic domain, HS1 and SHP-1L through its Src-homology 3 (SH3) domain[45]. HS1, one of the most important Lyn substrates, is an F-actin binding protein involved in the apoptosis of several hematopoietic cell lines. HS1 phosphorylation occurs in a sequential model mediated by SYK and Lyn. It seems that tyrosine phosphorylation of cortactin, an HS1 homologous protein involved in

cell motility, occurs by the same mechanism of recruitment of the SRC-family kinases[46]. In addition, both HS1[47] and Cortactin[48,49] have been found over-expressed in CLL and correlated to poor prognosis. Thus, these proteins could have a relevant role in the survival of the neoplastic clone.

Furthermore, the AKT and BTK pathways are also constitutively activated in CLL[37]. These pathways lead to pro-survival signals through their effects on PI3K, PLC-γ2, and NF-κB. 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[37]. 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[50].

This knowledge leads to the development of BCR pathway inhibitors, with promising results in refractory CLL and non-Hodgkin's lymphomas (NHLs). Because of the CLL genetic heterogeneity, the use of multiple drugs or multi-targeted agents is necessary to treat this disease[37].

1.6.3 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[51].

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. 13**).

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 result 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-κB or PI3K/AKT, leading to the over-expression of key antiapoptotic proteins. Together with the microenvironment stimuli, the anti-apoptotic protein BCL-2 is over-expressed, whereas pro-apoptotic proteins such as BAX and BCL-xL are down-regulated[52]. 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[51].

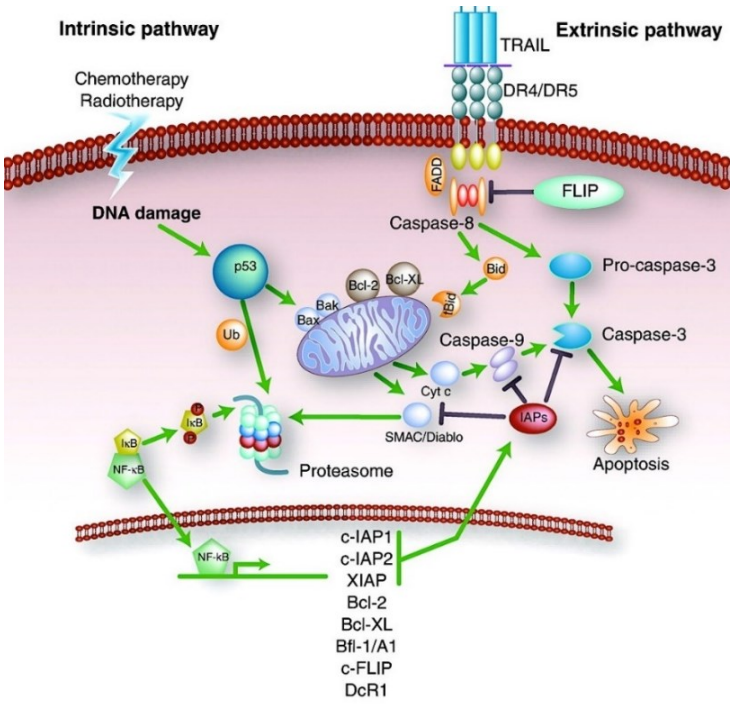

Figure 13. **The molecular mechanisms of apoptosis.** Apoptosis pathways can be initiated via different stimuli, that is, at the plasma membrane by death receptor ligation (extrinsic pathway) or at the mitochondria level (intrinsic pathway). Stimulation of death receptors results in recruitment of the adaptor molecule Fas-associated protein with death domain (FADD) and caspase-8. Caspase-8 initiates apoptosis by direct cleavage of downstream effector caspases. Mitochondria are engaged via the intrinsic pathway, which can be initiated by a variety of stress stimuli, DNA damage, the actions of oncoproteins and tumor suppressor genes (*i.e.*, *TP53*), radiotherapy, and most chemotherapeutic agents. CAD, caspase activated DNase; FAS, fibroblast associated antigen. ICAD, inhibitor of CAD; ROS, reactive oxygen species; TNF, tumor necrosis factor; TRAIL, TNF related apoptosis inducing ligand (image from de Vries *et al*[53]).

Other critical factors are represented by del17p13 and del11q23 that involve, respectively, *TP53* and *ATM*, two tumor-suppressor genes. *TP53* is inactivated in 10-15% of CLL patients, decreasing cell apoptosis and accelerating disease development[52]. P53 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[51].

1.6.4 Extrinsic factors: CLL microenvironment

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[54]. 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 differ 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[55]. Within PCs, CLL cells are exposed to chemokines, integrins, cytokines and survival factors[10].

The NLCs express CXCL12 and CXCL13 chemokines, while MSCs predominantly CXCL12. CLL cells interact with these soluble factors by CXCR4/5 (chemokine C-X-C motif) receptors, which are significantly over-expressed in CLL cells as compared to normal B cells[54]. 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 (**Fig. 14**).

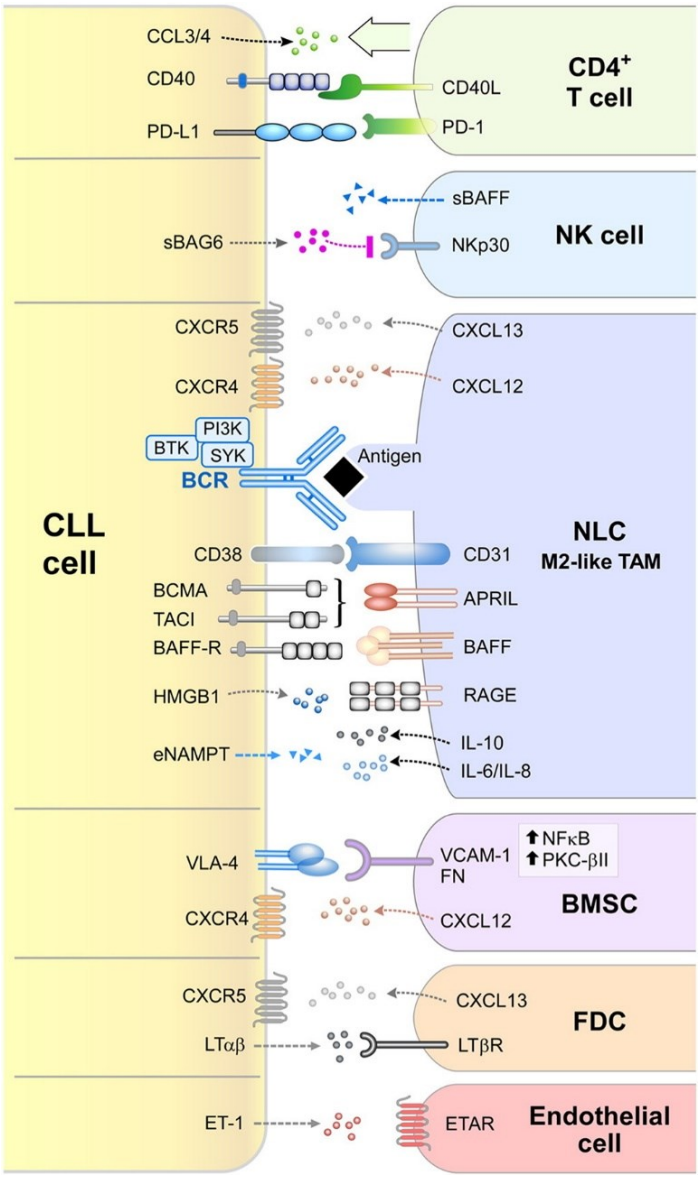

Figure 14. **CLL microenvironment**: CLL cells interact with T-cells, NK cells, NLCs, to bone marrow stromal cells (BMSCs), follicular dendritic cells (FDCs), and endothelial cells through adhesion molecules and chemokine receptors expressed on CLL cells. These interactions, in addition to B-cell receptor engagement, promote CLL survival, proliferation, and homing to tissues (image from Ten Hacken *et al*[56]).

Moreover, CXCR4 receptor involved in maintaining B-cell contact with stromal elements of solid lymphoid tissues, and CD5 normally upregulated after B-cell activation, have been indicated as hypothetical indicators of different trafficking potentials and proliferative histories of CLL cells in the peripheral blood[57,58]. Calissano *et al*. found that CLL clones are composed of subpopulations of cells that proliferate at different rates[58]. Recently divided cells that just migrated out the

lymphoid tissues into the circulation were identified as the proliferative fraction which expresses CD5 at high density and a lower level of surface CXCR4 ($CD5^{bright}CXCR4^{dim}$); whereas $CD5^{dim}CXCR4^{bright}$ cells displaying an increased density of CXCR4 and a low intensity for CD5 were described as the resting compartment of older cells that may be attempting to home back to solid tissues (**Fig. 15**)[57,58]. A deeper study of these two different intraclonal populations could help in finding molecules involved in the increased survival potential of the CLL clone.

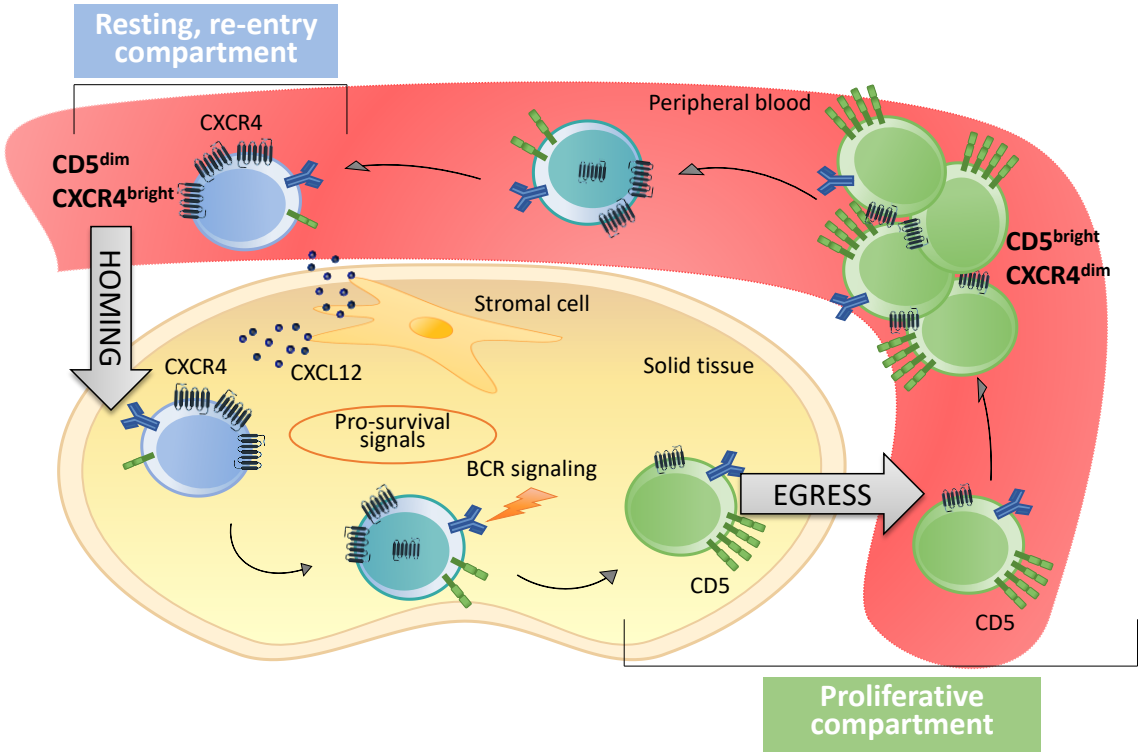

Figure 15. **Hypothetical model of the CLL cell trafficking**. CLL lymphocytes in the stromal microenvironment receive pro-survival stimuli, are activated and divide, upregulating CD5 and internalizing CXCR4. Thus, this recently born cells ($CD5^{bright}CXCR4^{dim}$, proliferative fraction, in green) egress from the solid tissue to get back into the peripheral blood. Remaining in the circulation over time, their proliferation cycle is at rest ($CD5^{dim}CXCR4^{brigh}$, resting fraction, in blue). This leads these cells to re-express CXCR4 to home back to nutrient-rich niches, detecting and following a CXCL12 gradient. Those that do not reenter die by exhaustion.

NLCs express pro-survival signals (*e.g.*, APRIL and BAFF, 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/CD29), expressed by CLL cells, and VCAM-1 and fibronectin, expressed on MSCs, facilitates cell-cell adhesion promoting immune recognition, survival and expansion of the leukemic clone[54] (**Fig. 14**).

Also CLL cells are able to shape the surrounding microenvironment. Indeed, upon BCR activation, CLL cells secrete cytokines, such as CCL3, which attract T cells and monocytes to the niche[59]. 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[54]. 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[59].

2. Focal Adhesion Kinase (FAK)

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase encoded by the *PTK2* (Protein Tyrosine Kinase 2) gene, found on chromosomal region 8q24.3.

2.1 Focal Adhesion Kinase structure

FAK is a ubiquitously expressed 125kDa protein tyrosine kinase which is composed of three major domains: the N-terminal FERM (band 4.1-ezrin-radixin-moesin) domain, the central kinase domain, and the C-terminal focal adhesion-targeting (FAT) domain[60] (**Fig. 16**). Three proline-rich regions connect these domains and act as binding sites for proteins containing a Src-homology 3 (SH3) domain[60].

The FAK FERM domain (aminoacids 1-415) consists of three distinct lobed subdomains (F1, F2 and F3). It functions like a scaffold to mediate protein-protein interaction and connects signals from the extracellular space to the nucleus[61]; for example, it promotes the association of FAK with integrins and activated growth factor receptors[62]. Inside the FERM domain, the major auto-phosphorylation site on tyrosine 397 (Y397) is found. Moreover, it includes a nuclear localization sequence (NLS)[63].

At the other end of the protein, there is the FAT C-terminal domain (aminoacids 677-1052) which also mediates protein-protein interactions. Despite the name, FAK does not bind directly to the cytoplasmic tails of integrins[64] in focal adhesions, whereas the association with integrin receptors is indirect and mediated by the interaction with paxillin and talin[65]. Besides, it presents the phosphorylation site Y925, which mediates the binding of proteins containing the SH2 domain[66].

The central FAK kinase domain (aminoacids 416-676) comprises a folded structure with an ATP-binding cassette, the activation loop, and at least six different tyrosine phosphorylation sites, among which Y397 and Y576/577[67].

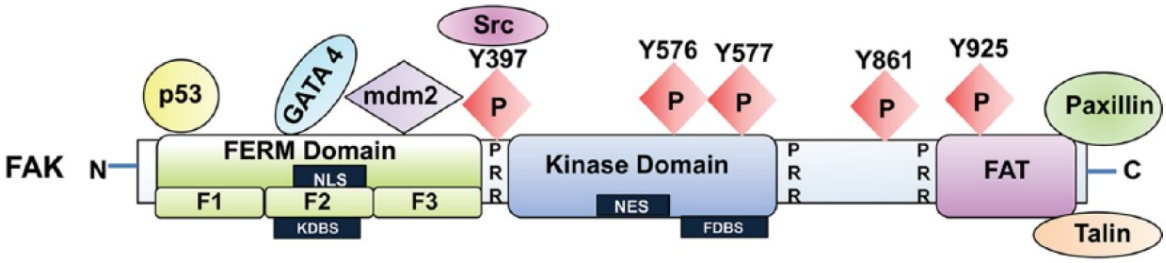

Figure 16. **Schematic structure of FAK.** FAK consists of a N-terminal FERM (band 4.1, ezrin, radixin, moesin) domain, a central kinase domain and a C-terminal FAT (focal adhesion targeting) domain. Many regulatory proteins, such as p53, MDM2 and growth factor receptors, bind to the FAK FERM domain. The FAT domain associates with integrin receptors indirectly via focal adhesion proteins, such as paxillin and talin. Upon Y397 autophosphorylation, Src tyrosine kinase binds to the site, and the FAK-Src complex further phosphorylates the activation loop Y576/577 sites, which are important for the catalytic activity of the kinase. In addition, FAK contains a nuclear export sequence (NES) and a nuclear localization sequence (NLS). Image from Yoon *et al*[68].

When in the inactive state, FAK is found in a closed conformation, where the FERM domain blocks the kinase domain, resulting in an auto-inhibited structure[69]. When FAK gets in contact with membranes rich in PIP2, the FERM domain dissociates from the central catalytic domain, which

opens up and exposes the Y397 for phosphorylation, resulting in the active conformation of the protein[69]. In addition, a nuclear export sequence (NES) can be found in this central domain.
When FAK is subjected to alternative splicing, an autonomous C-terminal FAT domain is generated, which is called FAK-related non-kinase (FRNK). FRNK is a truncated 41-kD protein identical to the C-terminal domain of FAK. It localizes to focal adhesions through the FAT sequence but lacks catalytic activity[70]. Previous studies suggest FRNK may regulate FAK by acting as a competitive inhibitor[71].

2.2 Focal Adhesion Kinase activation and functions

The most characterized FAK activation event involves integrin receptor clustering upon the binding of cells to the extracellular matrix (ECM). Once recruited to focal contacts thanks to the indirect interactions between the C-terminal FAT domain and integrins, FAK dimerizes and auto-phosphorylates at Tyr397. Phosphorylated Tyr397 acts as a molecular scaffold for the binding of SH2-domain-containing proteins, such as SRC-family kinases, PLCγ, growth-factor-receptor-bound protein-7 (GRB7), and the p85 subunit of PI3K[64] (**Fig. 17**). In return, SRCs phosphorylate FAK on Y576 and Y577 within the activation loop of the kinase domain. This results in FAK maximal catalytic activation[72].
Additional phosphorylation sites are found in the C-terminal FAT domain at Y861 and Y925, which provides binding sites for SH2 domain-containing proteins[73]. For example, GRB2 binding to FAK leads to the activation of Ras and MAPK cascade[65] and the release of FAK from the FAs.
The activity of FAK can also be modulated positively or negatively by the action of protein-tyrosine phosphatases (PTPs), which are abundant in FAs[74]. In particular, PTPα plays a prominent role in the regulation of FA remodeling and in cell adhesion and signaling[75].
In addition to the kinase-dependent activities mostly associated with integrin-related signaling at focal adhesions, FAK can also have kinase-independent functions. FAK participates in many protein-protein interactions and works as a scaffold, both at focal adhesions and inside the nucleus. This is associated with FERM-mediated interactions and to the presence of the NLS.

2.3 FAK in the nucleus

Because FAK associates with focal adhesion proteins and is an important mediator of integrin signaling, it was believed to localize limitedly to the cytosol and plasma membrane. However, a functional NLS and a NES have been identified in the FAK FERM domain and in the central kinase domain[76]. Thus, FAK has the ability to act as a regulator of nuclear proteins and gene expression, by interacting with certain transcription factors (NANOG, TAF9, MEF2, RUNX1, and RNA polymerase II), and epigenetic modulators (HDAC1, MBD2, and Sin3a)[77]. Although the role of nuclear FAK is not fully understood, several studies have shown that FAK translocation inside the nucleus can promote ubiquitination and proteasomal degradation of nuclear factors (e.g., p53 and GATA4) by forming a complex with E3 ligases (e.g., MDM2 and CHIP) (**Fig. 17**)[78].
FAK and p53 proteins can directly interact in the cells through their N-terminal domains[79]. FAK can suppress transcriptional activity of p53 by sequestering it from apoptotic signaling and in turn

p53 can regulate FAK by inhibiting its promoter for gene transcription. Thus, FAK and p53 can be regulated through a feedback mechanism[80].

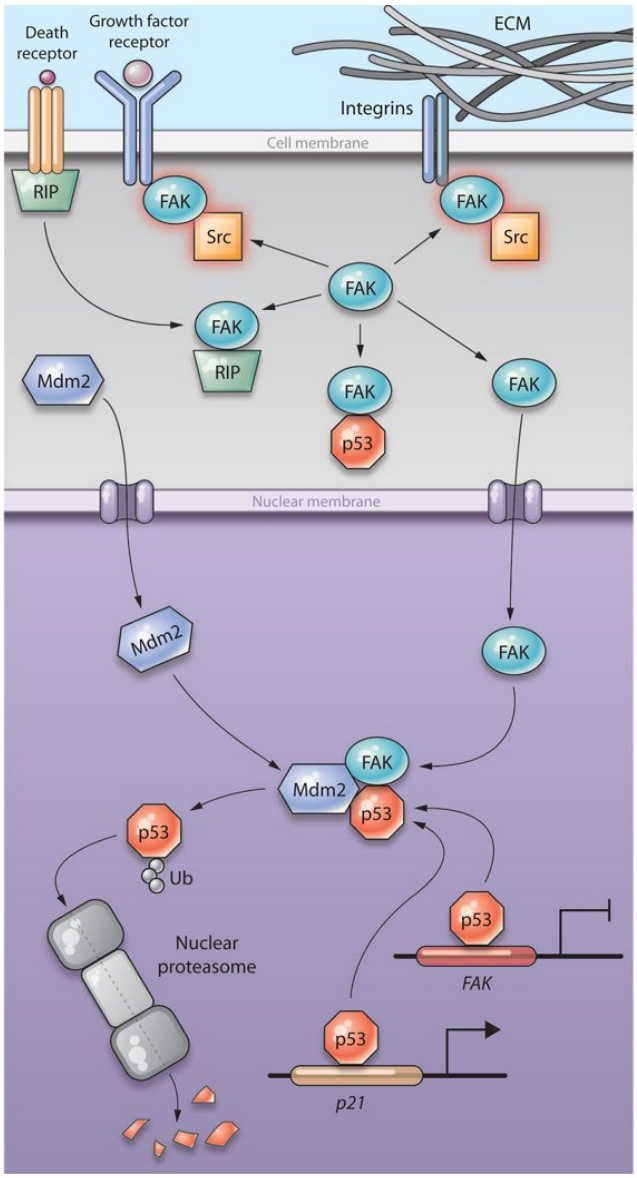

Figure 17. **Focal adhesion kinase interactions in the cytoplasm and in the nucleus.** Schematic representation of FAK interactions inside the cell. FAK is normally recruited to activated integrins at focal adhesions, and growth factor receptors at the cell surface. Through its kinase activity, FAK activates downstream signaling proteins, such as the kinase Src. FAK can also shuttle between the cytoplasm and the nucleus to mediate pro-survival or proapoptotic signaling. In addition, it can also have kinase-independent activities, like acting as a scaffold protein to bind to p53 and the E3 ubiquitin ligase MDM2, facilitating the ubiquitination and degradation of p53. This results in increased cell survival and serves as a feedforward mechanism to increase the abundance of FAK, by decreasing the p53-mediated repression of the FAK gene. Ub, ubiquitin. Image from Cance *et al*[81].

2.4 FAK cleavage

Besides being important in the cell adhesion in association with integrins, FAK has a critical role in regulating focal adhesion dynamics[82]. Evidence supports a role for the Calpain family in controlling adhesion structures disassembly[83].

Calpains are a family of highly conserved cysteine proteases, activated by increased intracellular calcium[84]. Among the various proteins encoded by the 16 known genes of the mammalian Calpain gene superfamily, there are two well-known Calpain isoforms which are ubiquitously

expressed. Calpain 1 requires calcium concentrations in the micromolar range to be activated, therefore it is also called μ-Calpain; on the other hand, Calpain 2 (m-Calpain) is activated by millimolar concentrations of intracellular calcium[85]. Calpains have been reported to orchestrate migration, proliferation and apoptosis in several cell types[86,87]. More than 100 proteins have been identified as Calpain substrates, among which transmembrane receptors, signaling enzymes, transcription factors and cytoskeletal proteins. Both Calpain 1 and Calpain 2 mediate the cleavage of the focal adhesion complex, including FAK[88], paxillin[89], talin[90], α-actinin[91], and β3 integrin[92], contributing to focal adhesion disassembly[93].

In most cases, these intracellular calcium-dependent proteases do not lead to the extensive degradation of their substrates, but instead Calpains cleave proteins in a limited fashion, generating stable protein fragments which may have functions that differ from the one of the original intact form. Proteolysis is directed toward a specific recognition sequence, even though no single consensus sequence has been found yet, and therefore Calpain cleavage is supposed to be controlled also by the detection of secondary structures[94].

Studies have determined that the preferred cleavage site where Calpain cuts FAK is located between the two C-terminal proline-rich regions after S-745, resulting in a C-terminal fragment similar in size to the FRNK[95]. Therefore, the cleavage results in the generation of a N-terminal (N-FAK) fragment and C-terminal (C-FAK) fragment (**Fig. 18**). N-FAK was reported to be of 80kDa and should encompass the FERM domain and the kinase domain, besides containing also the activatory residue Y397, the SH3 binding domain and the NLS signal[96]. On the other hand, C-FAK is a 35kDa fragment which may be considered similar to the endogenous FRNK. Since C-FAK, but not N-FAK, contains the FAT domain for localization on the membrane, Calpain cleavage of FAK in these two fragments may facilitate the dissociation of the kinase from the focal adhesions and their disassembly[96–98], and allow the translocation of N-FAK inside the nucleus.

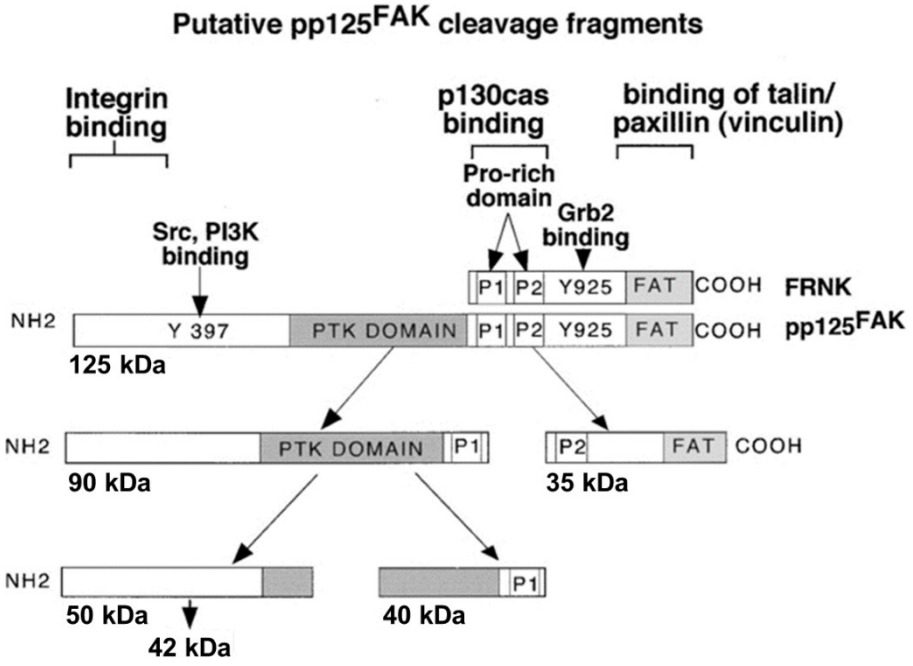

Figure 18. **FAK activity can be regulated by cleavage.** Putative cleavage sites and fragments generated by Calpain proteasic cut. Protein-binding domains of FAK and of FRNK, as well as molecular weights of the fragments, are indicated. pp125FAK, full-length FAK of 125kDa. Adapted from Carragher *et al*[99].

2.5 Roles of FAK in cancer

FAK can interact with many proteins and function as an adaptor at sites of cell attachment to the ECM, besides transmitting signals in the inside of the cell from adhesion and growth factor receptors. Given that both FAK and these receptors are often upregulated in tumor cells, their synergistic action might control the altered growth of tumor cells.

2.5.1 FAK expression in cancer

A number of articles have associated FAK expression with cancer. FAK mRNA was found to be over-expressed in 49 human tissue samples, particularly in 17 of 20 invasive tumors, and in all 15 metastatic tumors of different origins, while no FAK mRNA was detected in 6 normal tissue samples[100]. Larger datasets, such as The Cancer Genome Atlas, reveal that FAK mRNA levels are higher in serous ovarian tumors (~37%)[101] and invasive breast cancers (~26%)[102] and these increased levels are correlated with poor overall patient survival[103,104]. **Fig. 19** shows other human malignancies[105] in which FAK mRNA is over-expressed.

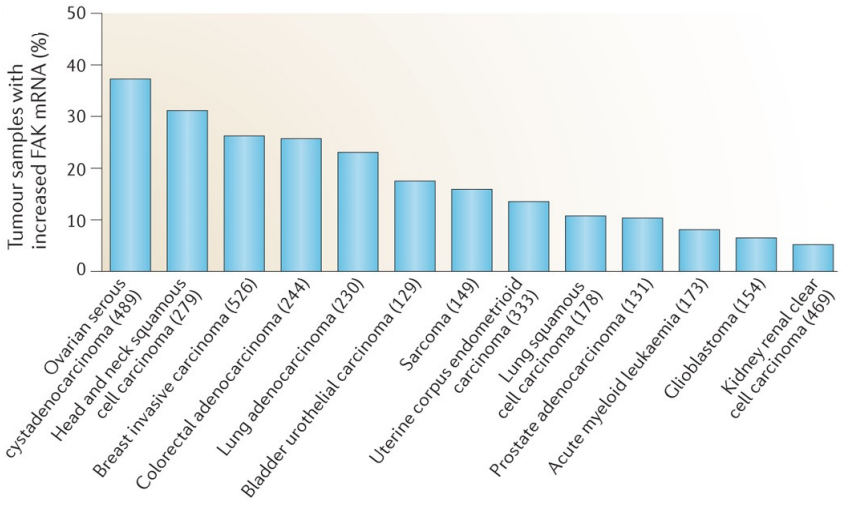

Figure 19. **Increased FAK mRNA levels are found in several human malignancies.** Percentage of tumor samples with increased levels of FAK mRNA. Numbers of tumors analyzed are shown in parentheses on the x-axis. Adapted from Sulzmaier *et al*[106].

Regarding FAK protein expression, immunohistochemical and immunoblotting analyses on malignant human samples showed that this kinase is over-expressed in thyroid, prostatic, cervical, colon, rectum, oral epithelium, and ovarian cancer[107]. Besides, increased FAK expression and activity have been frequently correlated with malignant or metastatic disease and poor patient prognosis[107].

The mechanisms that lie beneath the greater expression of FAK in cancer cells have not yet been established. Moreover, differently from usual oncogenes, just few missense mutations within PTK2, the gene encoding for FAK, are found in tumors[101]. Nonetheless, single nucleotide polymorphisms on chromosomal region 8q24.3, encompassing PTK2 gene, were associated with predisposition to ovarian cancer[108].

FAK role in hematological malignancies has been less investigated compared to solid tumors. A study of FAK expression by immunohistochemistry (IHC) showed that the majority of B-cell

lymphomas exhibited the highest expression of the protein[109]. On the other hand, in leukemias and lymphomas of T cells and in myelomas FAK was absent[109].
FAK down-modulation has been demonstrated also in some solid tumors, like in cervical cancer, where the decreased expression was associated with poor patient outcome[110], and in colorectal carcinoma, where FAK was reduced in the liver metastases[111].
Despite all these studies about altered FAK expression in human cancers, FAK gene promoter has not been studied extensively. A recent study identified the tumor suppressor p53 as one of the transcription factors that can bind to FAK promoter, and this association leads to the suppression of FAK expression[112]. This evidence assumes that p53 transcriptional control of FAK is occurring in normal conditions. Thus, it would be interesting to establish whether *TP53* mutations or deletions, frequently found in cancer settings, can contribute to the altered FAK expression.
Concerning FAK activation in tumors, studies on arrays of malignant tissues using the phosphospecific antibody recognizing FAK Y397 autophosphorylation site revealed that it increases along with tumor progression[105]. In another study on ovarian tissue, phospho-FAK-Y397 was found in invasive tumors, but not in normal epithelium[113].

2.5.2 FAK functions in cancer

FAK has been shown to have many functions in cancer, like motility, metastasis, invasion, angiogenesis, and cell survival (**Fig. 20**).
The anti-apoptotic role of FAK was mainly related to detachment-induced cell death or anoikis, first reported by Frisch *et al*. in kidney epithelial cells[114]. This process was dependent on FAK-Y397 phosphorylation and kinase activity. Likewise, in the HL60 leukemia cell line FAK over-expression, as well as its Y397 phosphorylation, led to resistance to apoptosis[115]. Survival signals coming from integrins and other extracellular stimuli induce the kinase-dependent signaling pathways of FAK, triggering the PI3K-AKT cascade[105].
FAK function in cancer motility and invasion is associated with the promotion of epithelial-to-mesenchymal transition (EMT)[116]. This requires modifications in focal adhesion and cytoskeletal dynamics, expression of matrix metalloproteinases (MMPs) to facilitate ECM invasion, and ultimately leads to an invasive cell phenotype.
Focal adhesion turnover is controlled by targeted focal adhesion protein proteolysis. By live-cell imaging techniques it was demonstrated that FAK regulates the cycle of assembly and disassembly of these adhesions[82]. FAK signaling activates the MAPK pathway, as well as the protease Calpain-2, which mediates cleavage of talin, paxillin or FAK. In addition, dynamic rearrangement of the actin cytoskeleton is necessary for cell movement[74]. The interaction between the proline-rich sites of FAK and the actin-binding protein cortactin results in its phosphorylation and contributes to focal adhesion regulation[117].

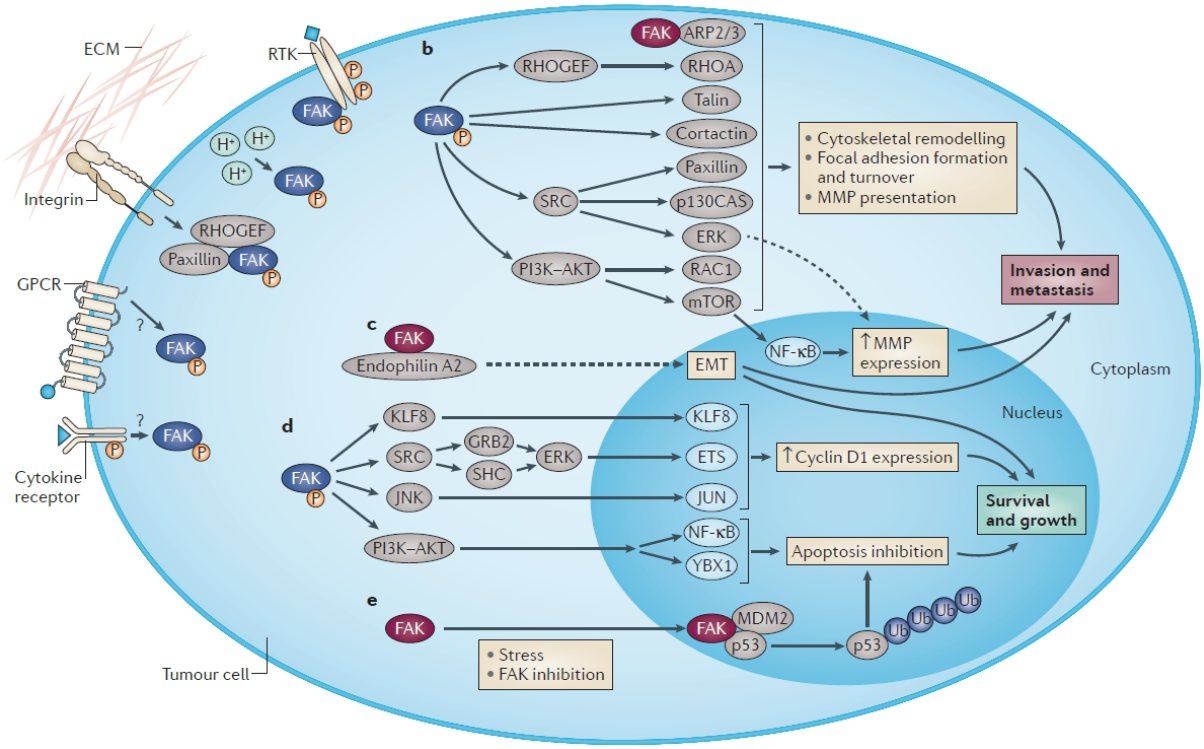

Figure 20. **FAK roles in cancer cells.** FAK takes part in many signaling pathways, both through kinase-dependent (shown in blue) and kinase-independent (red) functions. FAK can be activated near the cell membrane by, for example, integrins, receptor tyrosine kinases (RTKs), and cytokine receptors. Then, active FAK can increase cell motility through effects on ARP2/3, talin or cortactin, and SRC- or PI3K-mediated signaling. This results in cytoskeletal remodeling, focal adhesion turnover, and expression of MMPs, enhancing cell invasion and tumor metastasis. FAK can also induce cell cycle progression through cyclin D1 increased expression and inhibit apoptosis by PI3K-AKT transcriptional effects on nuclear factor-κB (NF-κB) or Y-box-binding protein 1 (YBX1). Inside the nucleus, FAK works as a scaffold for p53 and MDM2 in a kinase-independent manner, increasing p53 polyubiquitylation (Ub) and degradation, thereby promoting cell survival. GRB2, growth factor receptor-bound 2; P, phosphorylation; SHC, SRC homology 2 domain-containing. Image from Sulzmaier *et al*[106].

2.6 FAK inhibitors

As FAK drives several tumor-supporting signaling pathways[105,106], small molecules inhibiting FAK are being developed. In mouse models, FAK inhibition prevents tumor growth, metastasis and angiogenesis[106]. FAK inhibitors have also been used in combination with cytotoxic drugs[118].

The most well-characterized inhibitors of FAK are ATP-competitive kinase inhibitors. These are designed to bind residues surrounding the ATP-binding pocket of FAK, which is found similar in numerous kinases, thus potentially leading to off-target effects. In preclinical models, these FAK inhibitors effectively decrease FAK Y397 autophosphorylation and prevent cell movement[119].

In 2008 and 2009, Phase I clinical trials began with FAK inhibitors by Pfizer PF-562,271 (now termed VS-6062; ClinicalTrials.gov identifier: NCT00666926)[120] and by GSK (GSK2256098; ClinicalTrials.gov identifier: NCT00996671). Low adverse events were found for both compounds. A later generation small molecule, PF-04554878 (now named VS-6063 or Defactinib), showed more favorable pharmacokinetics, and a Phase I trial by Verastem identified some patients with ovarian, colorectal or bile duct tumors who exhibited stable disease (ClinicalTrials.gov identifier: NCT00787033)[118]. New trials were initiated with Defactinib: a Phase II trial is testing responses to FAK inhibition in patients with KRAS-mutant non-small-cell lung cancer (ClinicalTrials.gov

identifier: NCT01951690). Moreover, Defactinib is being tested in combination with paclitaxel, a microtubule-stabilizing agent, in ovarian carcinoma cells in a Phase I/Ib study (ClinicalTrials.gov identifier: NCT01778803)[106].

However, also the kinase-independent functions of FAK have to be taken into account when developing new approaches to inhibit FAK. The scaffolding functions of FAK can be blocked by allosteric inhibitors that bind to distinct domain sites to disrupt different protein–protein interactions. These include compounds of limited complexity (relative molecular mass <300), termed C4, Y11, Y15 and R2[106]. For example, a proposed mechanism for R2 is the blocking of FAK interaction with p53. These scaffold inhibitors have shown anti-tumor activity in xenograft mouse models and in combination with other chemotherapeutics[106].

AIM OF THE STUDY

Focal Adhesion Kinase (FAK) has been studied in many solid tumors and has been shown to be correlated with cancer progression and aggressiveness. Few papers have been published regarding the role of the kinase in oncohematology. Thus, we wanted to explore if FAK may have a role also in Chronic Lymphocytic Leukemia (CLL) pathogenesis.
FAK is involved in many signaling pathways, including those of integrins and chemokine receptors, key in tumor development. Its interaction with molecules implicated in CLL aggressiveness, like the cytoskeletal-connected proteins Cortactin and hematopoietic lineage cell-specific protein 1 (HS1), may suggest an involvement of FAK in promoting a malignant disease phenotype.
Thus, in this PhD project we performed:

- evaluation of FAK expression in a large cohort of CLL patients and healthy controls;
- analysis of FAK phosphorylation and regulation by Calpain proteolytic cleavage;
- evaluation of FAK subcellular localization in CLL B lymphocytes;
- study of FAK relationships with molecules involved in the same pathways of cellular adhesion and migration, *i.e.* HS1 and Cortactin;
- investigation of FAK inhibition effects on the survival of CLL cells.

For this purpose, we employed molecular biology techniques, flow cytometry and cell colture.

MATERIALS AND METHODS

1. Patients

Leukemic B cells were obtained from 142 CLL patients. We analyzed CLL patients (82 males and 60 females), aged between 32 and 85 years, enrolled by the Hematology Division (chief Prof. L. Trentin), University of Padua, School of Medicine. Written inform consent was obtained according to the Declaration of Helsinki.

Clinical characteristics of patients are listed in **Table 2**. In particular, we reported: the number of WBCs, the percentage of lymphocytes and the mutational status of IGHV genes. All neoplastic B cells of the 142 patients examined were positive for CD5, CD19 and CD23 markers, typically co-expressed in CLL.

B lymphocytes obtained from peripheral blood of 10 age-matched healthy subjects were used as controls.

Table 2. **Characteristics of the patients enrolled in the study.**

Patients	142
Median age, years (range)	62 (32-85)
Male/Female	82/60
WBCs count, x10^9/l (range)	46,000 (4,000-330,000)
Lymphocytes, % (range)	97 (15-99)
IGHV Mutated/Unmutated/nd*	67/56/19

*Immunoglobulin heavy chain variable region (IGHV) mutational status: patients with ≥98% homology from the most similar germline gene in the expressed V_H genes were defined unmutated; mutated cases were defined as those in which the CLL cells displayed <98% homology in the expressed V_H gene; nd = not determined.

2. Isolation of B lymphocytes from peripheral blood

2.1 Purification of B lymphocytes

B lymphocytes were isolated from peripheral blood of CLL patients. From a sample of venous blood collected in EDTA, peripheral blood mononuclear cells (PBMCs) were obtained proceeding with a layering on Ficoll/Hypaque (F/H) (Lymphosep, Biowest; Nuaillé, France). This method exploits the difference of density of mononuclear cells (lymphocytes and monocytes) with respect to the other elements of the blood. Peripheral blood was diluted with 0.9% sodium chloride (saline) at room temperature (RT) and layered slowly over F/H solution. We proceeded with a centrifugation at 900g for 20 minutes 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 10min at 20°C (**Fig. 21**). The pellet was resuspended in an adequate amount of saline and the cells were counted in a Burker chamber.

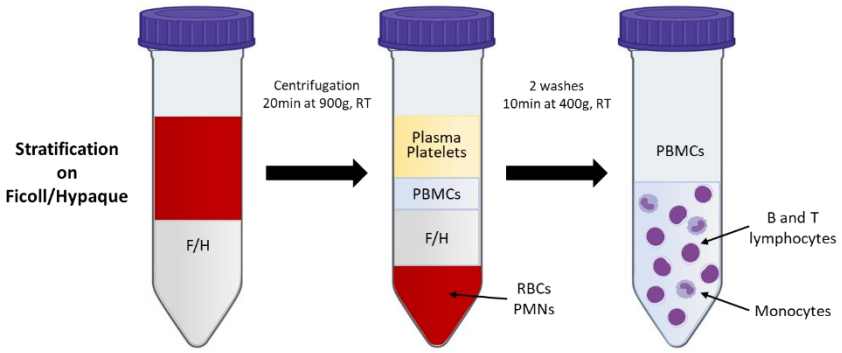

Figure 21. **Isolation of mononuclear cells from peripheral blood by stratification on Ficoll/Hypaque.** By centrifugation on F/H, mononuclear cells were isolated from peripheral blood. Mononuclear cells and platelets were concentrated above the layer of F/H because they have lower density; on the contrary, the red blood cells (RBCs) and granulocytes (PMNs) have a higher density than the F/H and collect on the bottom of the tube.

2.2 Purification of B lymphocytes using RosetteSep kit

We used the RosetteSep kit (StemCell Technologies; Vancouver, CND) to enrich the PBMCs in B cells, especially from whole blood of normal healthy donors. The kit consists of a cocktail of antibodies (Abs) 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 Abs binds "not-B" cells and red blood cells creating immunerosettes. In this way, CD19+ B lymphocytes were isolated by negative selection.

Each ml of blood was incubated with 50µl of RosetteSep at RT for 20min. The samples were then diluted 1:2 with PBS (Phosphate Buffered Saline) 1X + 2% FBS (Fetal Bovine Serum; Euroclone; Milan, Italy), gently agitated, and then layered over F/H. We proceeded with a centrifugation at 900g for 30min 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 10min. Finally, the cells were resuspended in PBS1X and counted in a Burker chamber (**Fig. 22**).

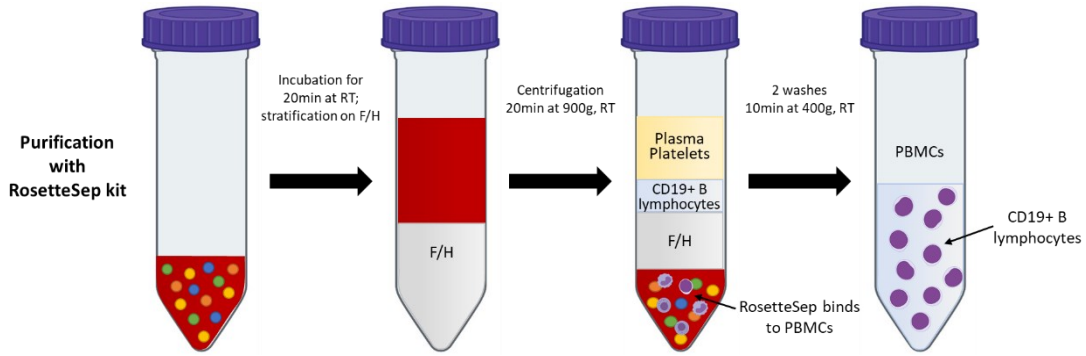

Figure 22. **Purification with RosetteSep kit.** CD19+ B lymphocytes were isolated from whole blood by negative selection. 10ml of venous whole blood were incubated for 20min at RT with 500µl of RosetteSep. Afterwards, through stratification on F/H, we get the CD19+ B cells, which are concentrated just 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. Cell culture conditions

Leukemic B lymphocytes (CD19+/CD5+) obtained from patients with CLL were cultured in RPMI 1640 medium (Thermo Scientific; Rockford, IL, USA) with 2% FBS (Euroclone) and antibiotics (5% Penicillin/Streptomycin; Euroclone) 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 the drug Defactinib (Selleck Chemicals; Munich, Germany) to the final concentration of 5µM for 24hours. After the treatment, cell viability was evaluated by the Annexin V/PI test and by the analysis of PARP (Poly-ADP-ribose polymerase) cleavage using western blot.

4. 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 as "enrichment" of the following components: cytoplasm soluble, membrane, microsomes, mitochondria and nuclei.

We used the commercial Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific) containing detergents which can separate cytoplasmic, membrane and nuclear proteins. 10^7 B lymphocytes were centrifuged and incubated with different buffers following manufacturer's protocol. The buffers used were: Cytoplasmic Extraction Buffer (CEB), Membrane Extraction Buffer (MEB) and the Nuclear Extraction Buffer (NEB) with the addition of phosphatase and protease inhibitors. Therefore, we were able to obtain both cytoplasmic and nuclear protein fractions. These were consequently subjected to western blotting, after appropriate preparation, for the analysis of differential protein expression in cellular compartments.

5. Preparation of cell lysates

Cells (5×10^5 for each assay) were lysed in 30µl of the following buffer:

- Tris(hydroxymethyl)aminomethane-hydrochloride (TRIS-HCl) pH 6.8 20mM;
- sodium chloride (NaCl) 150mM;
- Ethylenediaminetetraacetic acid (EDTA) 2mM;
- ethylene glycol tetra-acetic 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 (sodium dodecyl sulphate)-PAGE (polyacrylamide gel electrophoresis) analysis, the following substances were added:

- 10% glycerol;
- 2% sodium dodecyl sulphate (SDS);
- 1% β-mercaptoethanol;
- Dye (Pyronin) (Sigma-Aldrich; Saint Louis, MO, USA).

Subsequently, the lysates were mixed and heated at 100°C for 5 minutes.

6. 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 pH6.8), which allows the concentration of the protein samples, so that they are all aligned at the start of electrophoresis.
- Running gel (Tris-HCl 1.5M at pH8.8), in which the real separation of proteins occurs.

The plate size of 10x8cm is fixed in the Hoefer Mighty Small-If 250 Scientific Instruments machine (Thermo Scientific). The electrophoresis was run for about 2 hours at 25mA.

7. Western blotting

The western blotting (WB) or immunoblotting is used to detect specific proteins inside a heterogeneous mixture. 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 30min. 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 overnight incubation at +4°C of the primary (Abs), diluted in a dilution buffer (50mM Tris-HCl, pH 7.5, 150mM NaCl, 1% BSA). For our study we used the following Abs: anti-FAK (Merck Millipore; Burlington, MA, USA); anti-phospho-FAK Y397, anti-PARP, anti-μ-Calpain (Cell signaling Technology Inc.; Danvers, MA, USA); anti-HS1 (kindly provided by Prof. Brunati, University of Padua); anti-Cortactin (Abcam; Cambridge, UK); anti-β-actin (Sigma-Aldrich).

Three washes were subsequently performed using a washing buffer (TRIS-HCl 1M, NaCl 3M, Tween20 0.1% at pH 7.5). Membranes were then incubated for 1 hour with a secondary anti-IgG Ab. This is conjugated with horseradish peroxidase (Perkin Elmer; Waltham, MA, USA) and diluted in the dilution buffer. After three additional washes with washing buffer, the membranes were subjected to the detection of the Ab with the enhanced Chemiluminescence system (ECL; Euroclone): each 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 resulting antigen-Ab complex, give rise to an oxidation reaction with light emission. The membrane was finally revealed into the Amersham

Imager 600 (GE Healthcare; Chicago, IL, USA) and the protein bands were scanned and quantified by densitometry, using the Image J program.

8. Reverse phase protein assay (RPPA)

Reverse phase protein array (RPPA) analysis was previously performed on 57 CLL patients and 11 healthy subjects to assess the expression of several proteins, including those related to the cytoskeleton. As previously described in Frezzato *et al*[121], cells were lysed, and proteins were quantified by the Bradford method; lysates were then diluted to ≤1mg/mL in a mixture of Novex Tris-glycine SDS sample buffer (Thermo Scientific) and 2-mercaptoethanol. Lysates were stored at −80°C and boiled for 8min immediately before the array was performed. Afterwards, they were loaded into a 384-well plate and serially diluted with lysis buffer in 4-point dilution curves. Also positive controls were added, using commercial cell-line lysates. Samples were printed in duplicate onto nitrocellulose-coated glass slides (FAST slides, Whatman/Schleicher & Schuell; Florham Park, NJ). Before Ab staining, the arrays were blocked for 1hr at RT in blocking solution (2g I-Block; Tropix; Bedford, MA, and 0.1% Tween-20 in 1L of PBS 1×). Blocked arrays were stained with Abs on an automated slide stainer (Dako Autostainer Plus; Dako; Carpinteria, CA) using the CSA kit (Dako). Slides were scanned on an Epson (Shinjuku; Tokyo, Japan) Perfection V350 scanner at 600 dpi. The images were analyzed with Microvigene Software (VigeneTech Inc.; Carlisle, MA) to extract numeric intensity values from the array images.

9. Flow cytometry (FC)

This technique allows a multiparametric evaluation of antigenic characteristics of the single cells while flowing through a liquid medium, thanks to the use of Abs conjugated with fluorochromes, which, when excited, emit visible and fluorescent light.

The fluorochromes used in this thesis were: fluorescein isothiocyanate (FITC) and AlexaFluor®488, which emit a fluorescence signal at 530nm, phycoerythrin (PE) emitting at 585nm and PE-Cyanine7 (PE-Cy7) emitting at 780nm when hit by a monochromatic laser beam with λ equal to 488nm; the allophycocyanin (APC) and AlexaFluor®647 that emit a fluorescence signal at 660nm when excited by a laser beam with λ of 633nm; and finally, AlexaFluor®405 which emits a signal at 450nm when hit by the 405nm laser.

The cell samples were analyzed by the flow cytometer FACSCanto II (Becton Dickinson; Franklin Lakes, NJ) and data obtained were processed using the FACSDiva 7 software.

9.1 Immunophenotypic analysis

The immunophenotypic analysis on lymphocytes obtained from peripheral blood of CLL patients was performed through FC. All patients express the typical phenotypic profile according to the standard criteria for CLL diagnosis[9]. The immunophenotyping is based on the identification of surface and intracellular Ag using mAbs 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 this study, the forward scatter (FSC) and the side scatter (SSC), proportional to cell size and complexity, respectively, were used to identify lymphocytes among all the different cell types in peripheral blood. For the cells inside the "lymphocyte gate", fluorescent Abs were used to determine the percentage of CD19+/CD5+ cells and the percentage of cells expressing IgM and/or IgD on the cell surface. The following Abs were used: anti-CD5-FITC (BD Biosciences; Franklin Lakes, NJ, USA), anti-CD19-APC (Aczon; Monte San Pietro, BO, IT), anti-IgM-FITC (Agilent Dako; Santa Clara, CA, USA), anti-IgD-PE (BD Biosciences).

9.2 Apoptosis analysis

Apoptosis of pathological B lymphocytes after cell culture was assessed using the Annexin V Apoptosis Detection Kit (Immunostep; Salamanca, Spain). 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. An example of these modifications is phosphatidylserine (PS), a negatively charged aminophospholipid normally expressed only on the inner side of the plasma membrane, which is exposed on the outer surface during apoptosis. The Annexin V is a protein that, in the presence of high concentrations of calcium (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 10min 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 (PE) provided by the kit, which can penetrate only the damaged membranes of late apoptotic and necrotic cells. After the incubation, 100µl of binding buffer were added and cells were analyzed. For each sample 20.000 events were acquired, and the number of viable cells was expressed as percentage of Annexin V/PI double negative cells over the total cells analyzed.

9.3 Analysis of Calcium mobilization

Calcium (Ca^{2+}) mobilization was measured using the fluorogenic probe Fluo3-AM, which emission peak is at 526nm, thus it can be visualized in the FITC channel in FC. Its emitted fluorescence increases with increasing concentrations of Ca^{2+}. $500x10^3$ cells were incubated in 250µl of RPMI 1640 containing 10% FCS and 4µM Fluo3-AM (Invitrogen; Paisley, UK) for 30min at 37°C. Cells were then washed and resuspended at $1×10^6$ cells/mL in PBS 1X at RT. Thus, cells were analyzed for background fluorescence (i.e., of unstimulated cells) for 30sec, followed by addition of 20µg/mL goat $F(ab')_2$ anti–human IgM or IgD (Southern Biotechnology; Cambridge, UK) and data acquisition for further 5min. To confirm that absence of anti-IgM/IgD–induced Ca^{2+} fluxes observed in some samples was not due to technical artefacts, these samples were subsequently treated with 1µM ionomycin (Sigma-Aldrich) as positive control, which elicited robust Ca^{2+} fluxes in all samples. Data were acquired on a BD FACSCanto II and analyzed using FlowJo software (Tree Star; Ashland, OR, USA).

9.4 Analysis of Cortactin and HS1 expression

Peripheral blood of CLL patients (40µl for each tube) was collected and stained with surface monoclonal Abs anti-CD19-PeCy7 (BD Biosciences), CD5-FITC (Abcam) for 10min at RT. Therefore, cells were washed, fixed and permeabilized with FIX & PERM™ Cell Permeabilization Kit (Thermo Scientific) following manufacturer's instructions. During the permeabilization step, in one tube also the anti-HS1 AlexaFluor®647 (Cell signaling Technology Inc.) was added; another tube was added with the anti-Cortactin AlexaFluor®488 Ab (Merck Millipore); a third tube was used as a control and incubated with the isotypic control. After a final wash, 30,000 total events were acquired, and samples were gated on intact cells by FSC *vs* SSC. For analysis, a second gating step on CD19+ cells was used. HS1 and Cortactin expression was assessed as the difference between the Mean Fluorescence Intensity (MFI) of the marked tubes and the MFI of the isotypic control.

9.5 Analysis of FAK expression in CD5-CXCR4 populations

$1x10^6$ B lymphocytes for each tube were stained with surface monoclonal Abs anti-CD19-PeCy7 (BD Biosciences), CD5-FITC (Abcam) and anti-CXCR4-PE (R&D Systems, bio-techne; Minneapolis, MN, USA) for 10min at RT. Therefore, cells were washed, fixed and permeabilized with FIX & PERM™ Cell Permeabilization Kit (Thermo Scientific) following the manufacturer's instructions. During the permeabilization step, in one tube also the Ab anti-FAK-Alexa Fluor® 405 (Abcam) was added for 10min at RT, while the other tube with no additional Ab was considered as the Fluorescence Minus One (FMO) control. After a final wash, 30,000 total events were acquired, and samples were gated on intact cells by FSC *vs* SSC. For analysis, a second gating step on CD19+ cells was used, and these cells were additionally examined in a density plot of CD5 *vs* CXCR4 to determine the $CD5^{dim}CXCR4^{bright}$ and the $CD5^{bright}CXCR4^{dim}$ populations. In order to evaluate the level of FAK expression in these two populations of the clone, we used the difference between the MFI of fully stained samples and the FMO controls.

10. Statistical analysis

Statistical analyses were performed using Prism (GraphPad Software Inc.; La Jolla, CA, USA) for Student's *t* test, paired Student's *t* test, Pearson's correlation, and Mann-Whitney test. Data are reported as mean ± standard deviation (SD) and were considered statistically significant when *p* values were <0.05.

RESULTS

1. FAK is down-modulated in poor prognosis CLL patients

Focal Adhesion Kinase (FAK) has been found to be over-expressed in many solid tumors. To investigate the function of FAK in CLL, we first examined the expression of FAK in primary samples of CLL.

By WB, we analyzed the expression level of the 125kDa protein FAK in B lymphocytes freshly purified from the peripheral blood of 142 untreated CLL patients and from 10 healthy subjects, used as controls. Results showed a homogeneous level in the healthy samples, whereas in CLL cells FAK expression was really variable, ranging from 0 to 4.67. The analysis did not reveal any significant difference between the control group and the leukemic patients (0.67±0.18 *vs* 0.56±0.55, *p*=ns, Student's *t* test; **Fig. 23A**). CLL is a really heterogeneous disease[9] and this characteristic may therefore explain this result.

For this reason, we divided the patients in groups according to their clinical and biological characteristics. We performed a correlation of the expression data with the most used prognosis factors for the disease[9], observing that, in general, poor prognosis patients presented a trend toward decreased FAK levels. In particular, the difference was statistically significant between the unmutated IGHV patients (U-CLL; n=56, 0.39±0.36) and the mutated counterpart (M-CLL; n=67, 0.73±0.72; *p*<0.01, Student's *t* test; **Fig. 23B**). Thus, these data indicate that 125kDa FAK is down-modulated in the poor prognosis CLL cases.

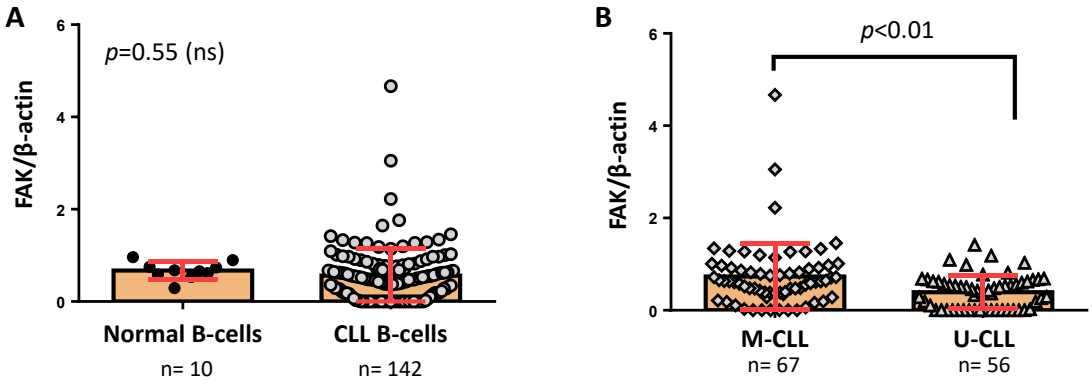

Figure 23. **FAK expression in normal and leukemic B-cells**. By WB we analyzed FAK protein expression in B lymphocytes from 10 healthy controls and from 142 CLL patients. The graphs show the ratio between FAK and β-actin values obtained from the densitometric analysis of their bands on WB membranes. (**A**) When taken as a whole, CLL cells did not show significant differences from the controls and exhibited a very heterogenous expression pattern for FAK. (**B**) When stratifying CLL patients according to the mutational status of IGHV genes, we could observe that FAK was down-modulated in the U-CLL cases, which experience a more aggressive disease, compared to the M-CLL counterpart.

2. FAK is cleaved in CLL patients

FAK promotes focal adhesions formation and maturation and is also involved in their turnover. When focal adhesions are disassembled, cell movement is stimulated[74]. Similarly to the other

components of the adhesion complexes, FAK can be regulated by proteolysis. Protein cleavage is mediated by proteases such as Calpain-2 and Caspase-8[122].

Consequently, we investigated if this occurred also in CLL. Using a different Ab for the kinase (anti-FAK; Merck Millipore) and re-examining our previous WB results, we noticed that in the leukemia patients FAK was not just present as a full-length protein of 125kDa, but also some lower molecular weight bands (92/94, 84 and 50kDa) were detected (**Fig. 24**). Noteworthy, these bands were mostly displayed in the specimens where a down-modulation of FAK whole form (125kDa) was previously found. Normal B cells mainly presented the full-length FAK only. In light of former research from Carragher[99] and others, we hypothesized that the smaller fragments of the protein were the result of the cleavage of FAK by the protease Calpain, since the cleavage products were consistent with its catalytic activity.

For this reason, we further investigated the function of this cleaved forms in our CLL patients.

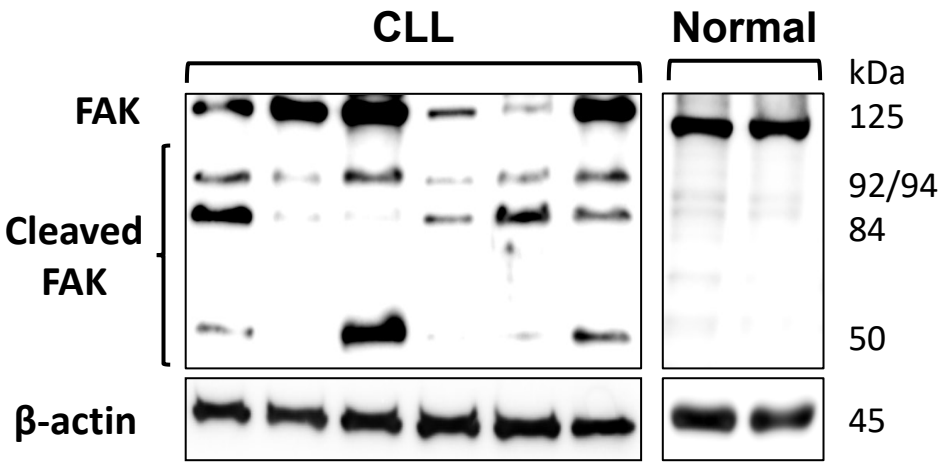

Figure 24. **FAK presents lower molecular weight bands, consistent with a cleavage by Calpain protease**. In CLL samples (n=58), FAK is present both as its full-length form of 125kDa, and also as smaller protein fragments of 92/94, 84 and 50kDa, consistent with proteolytic cleavage of the protein by Calpain. In normal B cells the protein is present mainly as its full-length form of 125kDa.

In a recent work by Łopatniuk *et al*.[123], Calpain levels in CLL were correlated with the clinical stage of the disease, and the protease activity was found to be associated with an increase of anti-apoptotic signaling molecules. To test whether we found differences in the expression of Calpain in our subsets of patients, we analyzed the protein level by WB, which did not prove any significant variation between normal controls (n=6) and CLL patients (n=38), maybe for the low number of analyzed cases (unpaired Mann-Whitney test; **Fig. 25A**). Nonetheless, we could observe a trend according to which U-CLL patients expressed Calpain at higher levels compared to M-CLL, in good agreement with previous research by other groups (**Fig. 25B**).

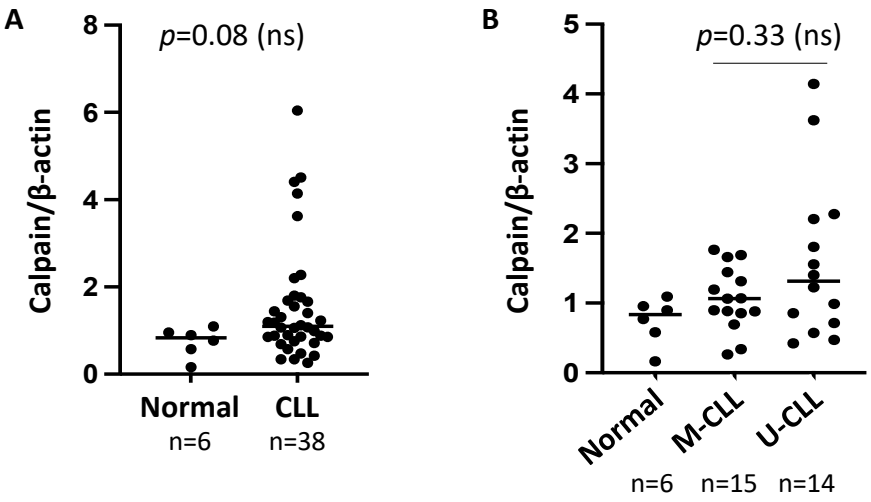

Figure 25. **Expression of Calpain protease in CLL samples**. WB was used to assess the expression level of Calpain protease in B lymphocytes from both normal (n=6) and CLL patients (n=38). The graphs show the ratio between Calpain and β-actin values obtained from the densitometric analysis of their bands on WB membranes. (**A**) Statistical analyses by unpaired Mann-Whitney test found no significant difference of Calpain expression in CLL patients with the control, (**B**) although a trend was present, suggesting an upregulation of the protease in poor prognosis U-CLL (n=14). 9 patients lacked IGHV mutational status evaluation.

3. FAK is active in aggressive CLL

Using an Ab specific for the phosphorylated form of FAK in the Y397 residue, we analyzed the activation status of the protein in the examined CLL samples (n=58). We found out that the poor prognosis patients, U-CLL, who in general displayed a higher amount of the cleaved form of FAK, exhibited also a hyperphosphorylated protein compared to the M-CLL group, both as full-length and as Calpain cleavage products (**Fig. 26**).

Thus, we hypothesized that the cleaved and phosphorylated active version of FAK, found to have functions also inside the nucleus, may have a relevant role in aggressive CLL, initiating a signaling pathway to support the migration potential and to sustain the malignant phenotype of the disease.

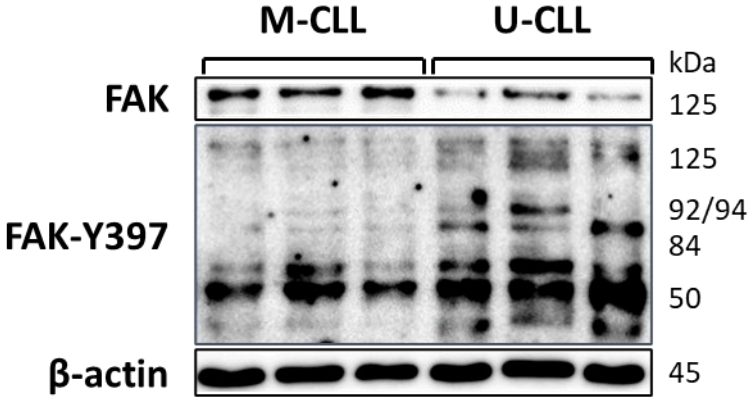

Figure 26. **FAK is more active in poor prognosis CLL.** WB analyses using a specific Ab for the phosphorylated Y397 residue of FAK showed that the protein is predominantly active in the patients with no mutations in the IGHV genes (U-CLL*, on the right*), namely the cases with a more aggressive disease, compared to M-CLL. Besides being activated, in these patients FAK was also cleaved.

4. FAK is cleaved in patients who release Calcium after BCR engagement

Calpain is a calcium-dependent protease. In B lymphocytes cytosolic calcium ion (Ca^{2+}) is one of the key signals for B cell physiological response after the binding of an antigen to the BCR. We explored whether BCR signaling may influence the protease activity of Calpain on FAK, taking into account that not all CLL cases express IgM or IgD on the membrane, and there is evidence that leukemic lymphocytes are heterogeneous also in terms of their ability to respond to stimulation via the antigen receptor[42,43].

By FC, in 40 patients we evaluated Ig expression and the consequent ability of CLL cells to mobilize Ca^{2+}, thus investigating the response of BCR after the addition of anti-IgM and/or IgD $F(ab')_2$. 32 out of the 40 patients taken into consideration presented surface Ig expression; the remaining resulted Ig negative. Among the Ig-expressing patients, 23 cases exhibited a significant increase in the Ca^{2+} fluorescent signal compared to the background fluorescence, denoting a positive response to the stimulation. The other 9 patients, although presenting surface Igs, did not exhibit a considerable increase of the signal after BCR stimulation, similarly to the 8 CLL cases negative for Ig expression; nevertheless, we used ionomycin as control to verify the performance of the Fluo-3AM probe.

After the Ca^{2+} release analysis, we divided the patients in two sets based on the ability to mobilize Ca^{2+}: the first group of patients was considered as responsive to BCR stimulation; the others, both the 9 Ig-expressing and the 8 negative cases, were indicated as non-responsive (**Fig. 27**).

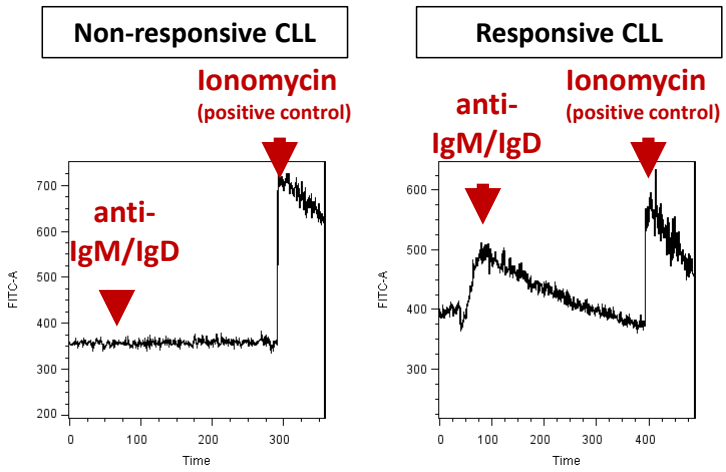

Figure 27. **BCR signaling response in CLL patients.** We analyzed the ability of CLL patients to respond to BCR stimulation in terms of intracellular Ca^{2+} mobilization. We therefore divided the patients in 2 groups: one comprising CLL cases which did not show any significant Ca^{2+} signal after stimulation, labelled as the non-responsive CLL (on the left); and the other, identified as responsive BCR patients, consisting of patients' cells which manifested Ca^{2+} mobilization after BCR engagement (on the right).

In order to investigate whether the activation and function of FAK may have a connection with BCR responsiveness of CLL cells, we analyzed FAK expression and cleavage in these samples, taking into consideration the former classification of samples as responsive or non-responsive. As supposed, the group of responding cases, indicative of the patients who were capable of releasing Ca^{2+} after BCR stimulation, manifested a higher amount of the bands of FAK generated by Calpain cleavage. On the contrary, the non-responsive group, which showed no significant change in Ca^{2+} levels, presented just the full-length of FAK at 125kDa and no or few lighter bands,

consistent with a lower activity of Calpain protease in these patients. In addition, the latter patients showed little phosphorylation of FAK on Y397, while Ca^{2+}-mobilizing cases had a significant higher amount of active FAK (**Fig. 28**).

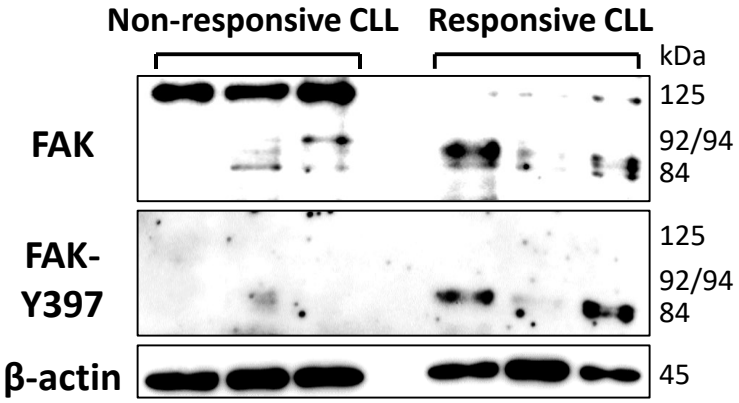

Figure 28. **Responsive BCR patients exhibit cleaved and active FAK**. The group of non-responsive CLL exhibited barely just the non-phosphorylated full-length FAK, whereas in the responsive patients FAK was present as its active and cleaved form.

By WB, we have also analyzed the expression of Calpain in the two sets of patients to check whether there was a difference, not only in relation to the proteasic activity on FAK, but also in the level of expression of the protein itself (**Fig. 29A**). Data revealed that the group of responsive cases showed a trend toward a higher basal level of Calpain, compared to the non-responsive CLL group (*p*=ns, Mann-Whitney test; **Fig. 29B**). Therefore, the augmented cleavage of FAK observed in the responsive patients may be due to a moderate increase in the expression of Calpain protein, which proteasic activity is also easily intensified as a result of the Ca^{2+} mobilization by BCR signaling. This hypothesis will be investigated further in a larger set of patients.

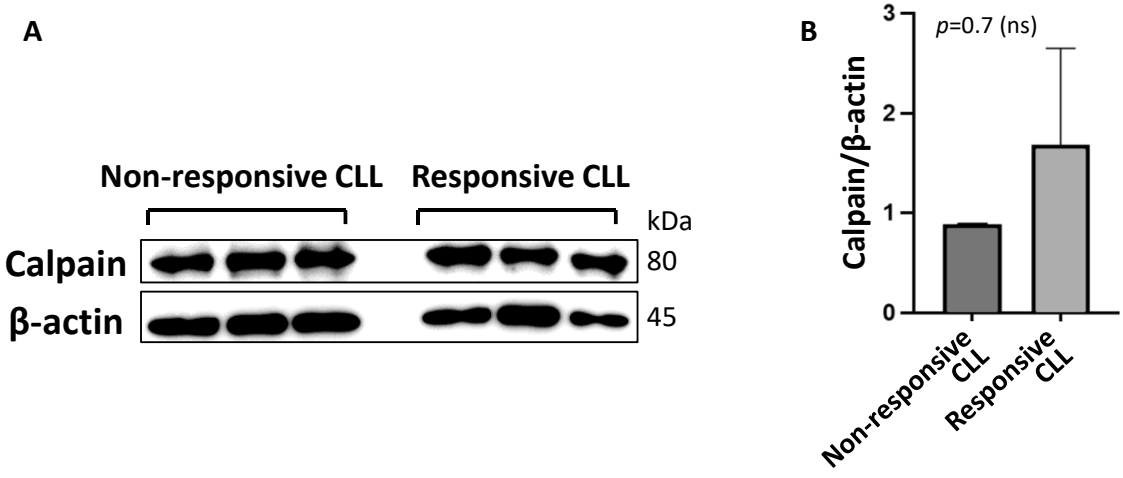

Figure 29. **Calpain basal expression in responsive and non-responsive CLL.** (**A**) The analysis by WB of the expression level of Calpain in the two groups revealed a trend according to which responsive cases showed a higher basal level of Calpain, compared to non-responsive CLL. (**B**) The graph on the right shows the ratio between Calpain and β-actin values obtained from the densitometric analysis of their bands on WB membranes (*p*=ns, Mann-Whitney test).

Subsequently, we correlated BCR responsiveness with the clinical and prognostic characteristics of the patients, and we found out that the group of responsive patients consisted mainly of the poor prognosis U-CLL cases, while M-CLLs were largely represented in the non-responsive set.

Consequently, we speculate that the ability of CLL cells to positively respond to BCR triggering is linked to the cleavage and activation of FAK, mainly in the poor prognosis patients, as a result of the increase of the level of intracellular Ca^{2+} after stimulation of the receptor. This suggests a possible link between BCR signaling cascade and FAK activation in CLL aggressive disease.

5. FAK is able to translocate inside the nucleus of CLL cells

Our results so far suggest a significant role of FAK in the aggressive phenotype of CLL. Referring to previous research demonstrating a role for the kinase in other compartments other than the cytoplasm, we explored the localization of FAK inside the leukemic cells of our patients.

Using a kit for subcellular fractionation, we purified the proteins from the cytoplasm compartment and from the nucleus. To verify the efficiency of the fractionation method, we used markers for each subcellular fraction, namely tubulin for the cytoplasm content, while PARP was used as a control for the nuclear fraction. After WB of the proteins from the different fractions we purified, we could observe that in the non-responsive patients, which did not show positive BCR response in terms of Ca^{2+} mobilization, FAK was mostly present only in the cytoplasm. Instead, in the responsive CLL samples FAK was able to localize also inside the nuclear compartment, mostly in its cleaved and active form, phosphorylated in Y397 (**Fig. 30**).

Another interesting result was that in both groups, FAK full-length was present only in the cytoplasm. Thus, we hypothesized that the cleavage of FAK by Calpain, eliminating the FAT domain for its localization near the membrane, can trigger and facilitate the translocation into the nucleus. Here, FAK may be directly involved in mechanisms promoting survival and cellular proliferation of the aggressive leukemic cells.

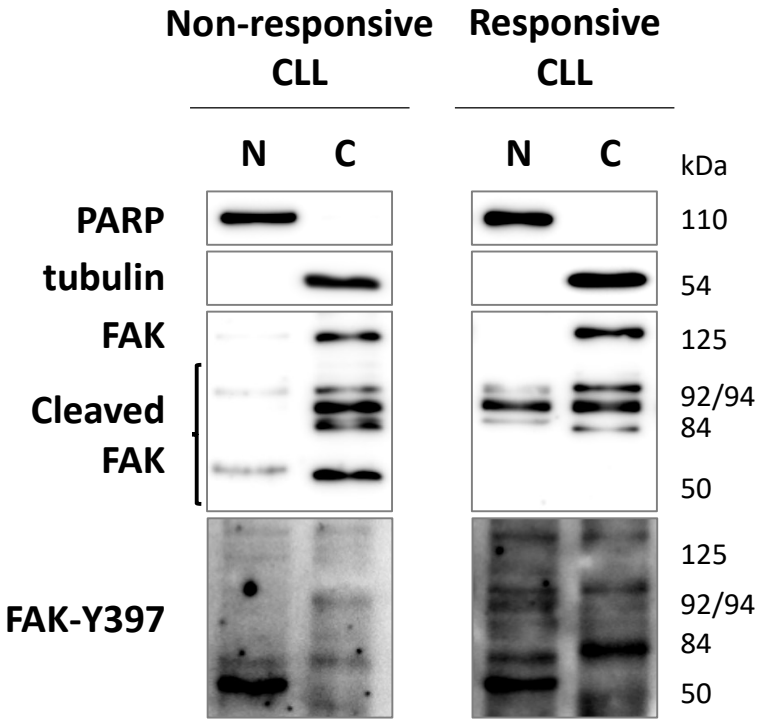

Figure 30. **Calpain fragments of active FAK are present inside the nucleus of responsive BCR patients**. Aliquots of 10^7 cells were lysed with the different buffers provided by the kit for subcellular fractionation, obtaining proteins from the cytoplasmic (C) and nuclear (N) compartments. We found that FAK full-length of 125kDa was present only in the cytoplasm, while cleaved FAK fragments were present also inside the nucleus of cells. In particular, these cleaved forms were found to be phosphorylated in the activatory Y397 in the patients responsive to BCR stimulation.

6. FAK activation correlates with cortactin and HS1 expression

FAK is involved in the engagement of integrins and assembly of focal contacts, playing a major role in cellular adhesion and in metastatic invasion of various cancers. In the same cellular processes, Lyn substrates, like HS1 and its homolog Cortactin, are involved and are relevant for cytoskeleton and ECM remodeling, shape and motility of the tumor cell. In addition, these molecules were found to be associated with CLL pathogenesis and aggressiveness[47–49] and do also interact with FAK.

We therefore investigated the relationship between FAK and these cytoskeletal-associated molecules in CLL. We evaluated HS1 and Cortactin expression by FC, using specific fluorochrome-conjugated Abs against the two cytoplasmic proteins, and we then correlated their levels to FAK expression in the same samples. We found that, parallel to the presence of active/cleaved FAK in the group of patients with positive BCR signal and aggressive disease, there was also an over-expression of the other two pro-survival proteins, Cortactin and HS1, compared to the non-responsive BCR cases (697±399 *vs* 265±137; $p<0.05$ for Cortactin; 1618±1575 *vs* 232±139; $p<0.05$ for HS1, Mann-Whitney test) (**Fig. 31**).

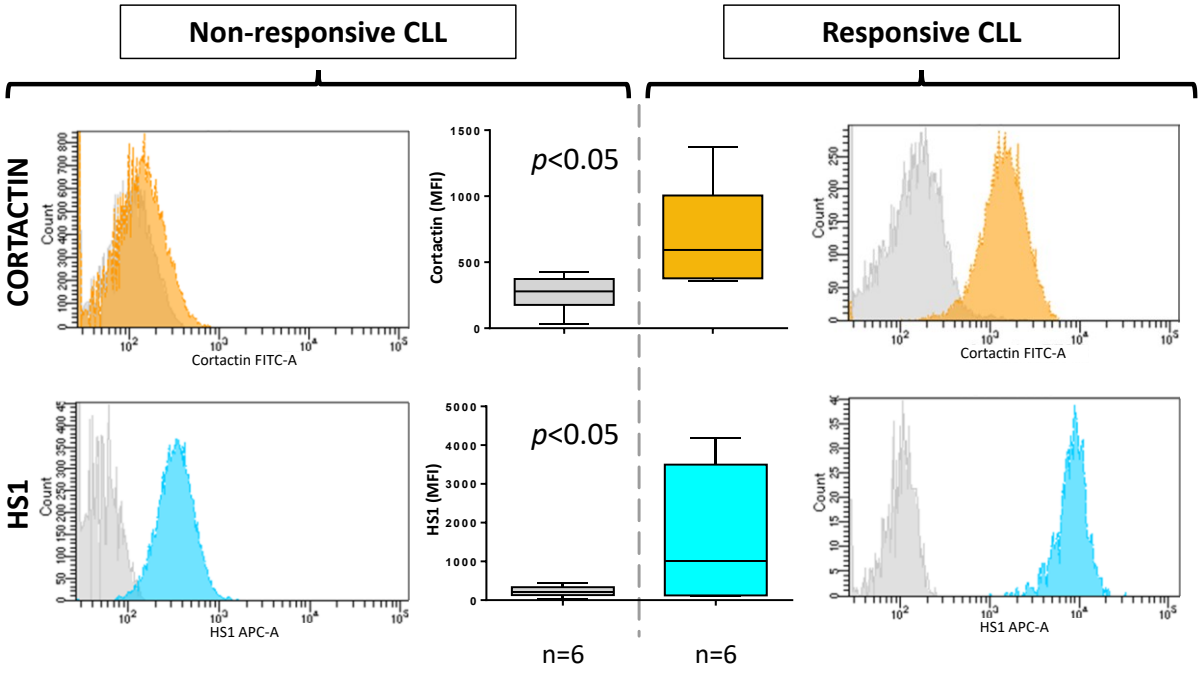

Figure 31. **Responsive BCR patients over-express the cytoskeletal-regulators Cortactin and HS1 together with active/cleaved FAK.** We evaluated HS1 and Cortactin expression by FC, using specific fluorochrome-conjugated Abs against the two cytoplasmic proteins (AlexaFluor®647 and AlexaFluor®488, read in the APC and FITC channels, respectively). We found that, in those patients with responsive BCR and a more aggressive disease (on the right), which previously we demonstrated to have a higher amount of cleaved/active FAK both in the cytoplasm and in the nucleus, Cortactin and HS1 were over-expressed (697±399 for Cortactin; 1618±1575 for HS1) compared to the non-responsive cases (on the left; 265±137, $p<0.05$ for Cortactin; 232±139, $p<0.05$ for HS1, Mann-Whitney test).

This correlation between HS1 and Cortactin over-expression and the presence of active/cleaved FAK was further validated by a previous RPPA analysis, in which the expression of 53 proteins was compared and correlated[121]. Regarding FAK activation, we divided the patients in 2 groups according to the obtained median value of its phosphorylation on Y397, namely FAK-Y397^{low} *vs* FAK-Y397^{high} (**Fig. 32A**). We found that HS1 was significantly over-expressed in the pFAK-high group (29,802±7,354) compared to the samples with decreased phospho-FAK (25,027±6,481;

p<0.05), and so was for Cortactin (365,786±117,921 *vs* 302,955±122,501; *p*=0.05, Student's *t* test, **Fig. 32B**). Besides, a significant positive correlation was proven between HS1 expression levels and FAK activation (r=0.32; *p*<0.05 for HS1). Even though the correlation was not significant for Cortactin (r=0.18; *p*=ns), a trend was visible (**Fig. 32C**).

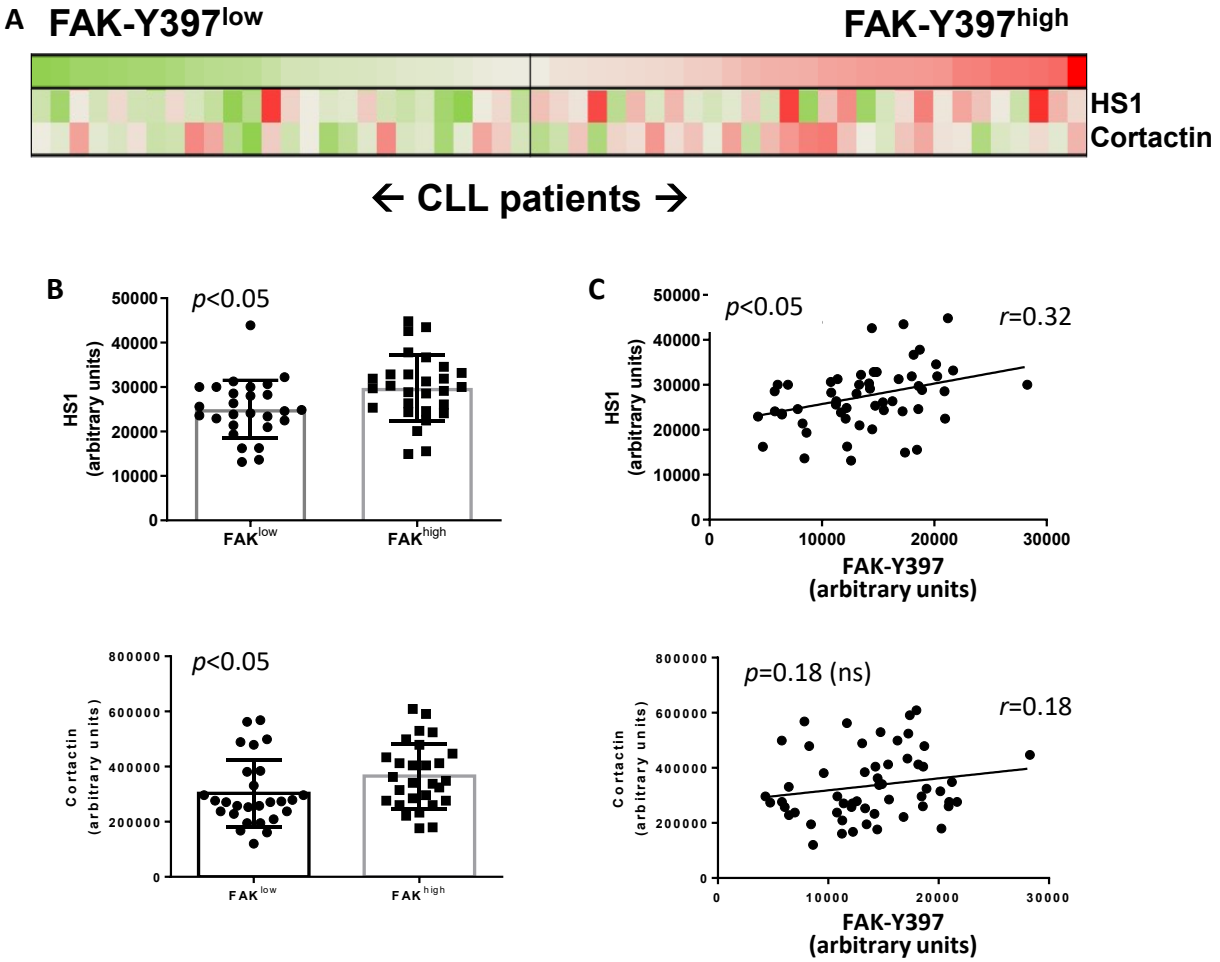

Figure 32. **FAK hyperphosphorylation is correlated with the over-expression of Cortactin and HS1**. HS1 and Cortactin expression and FAK phosphorylation on Y397 were evaluated by RPPA. The analysis was performed on 57 CLL patients and 11 healthy subjects as reported in Frezzato *et al*[121]. (**A**) Patients were divided in 2 groups according to the median value of FAK phosphorylation on Y397, namely pFAK-low *vs* pFAK-high. (**B**) HS1 was found to be significantly over-expressed in the pFAK-high group (29,802±7,354 *vs* 25,027±6,481; *p*<0.05), and so was for Cortactin (365,786±11,7921 *vs* 302,955±122,501; *p*=0.05, Student's *t* test). (**C**) The correlation between HS1 expression levels and FAK activation was significant (*r*=0.32, *p*<0.05 for HS1, Pearson's correlation). A trend was visible for the correlation between Cortactin levels and FAK-Y397 (*r*=0.18, *p*=ns, Pearson's correlation).

7. FAK is more expressed in the proliferative fraction of the leukemic clone

To investigate whether FAK may be implicated in pathways promoting the increased survival of CLL cells, we analyzed its expression in different fractions inside the leukemic clone of each patient. Referring to Calissano *et al*.[58], the surface expression of CXCR4 and cluster designation 5 (CD5) was studied by FC, thus identifying the so-called resting ($CD5^{dim}CXCR4^{bright}$) and proliferative ($CD5^{bright}CXCR4^{dim}$) populations of the clone. For all the samples, the two different fractions of cells were determined using differential surface membrane densities of CXCR4 and CD5. In parallel, also the intracellular level of FAK expression was analyzed by FC. The results, in

terms of difference of the MFI, proved that FAK is significantly more expressed in the proliferative population of the leukemic clone (in green; 452.5±217.4), enriched in recently divided cells that are lymphoid tissue emigrants, compared to the older resting fraction of cells which may have been circulating in the periphery longer (in blue; 351.4±172.1; *p*=0.0001, paired Student's *t* test) (**Fig. 33**).

This result further supports our hypothesis of FAK being involved in the survival potential of CLL.

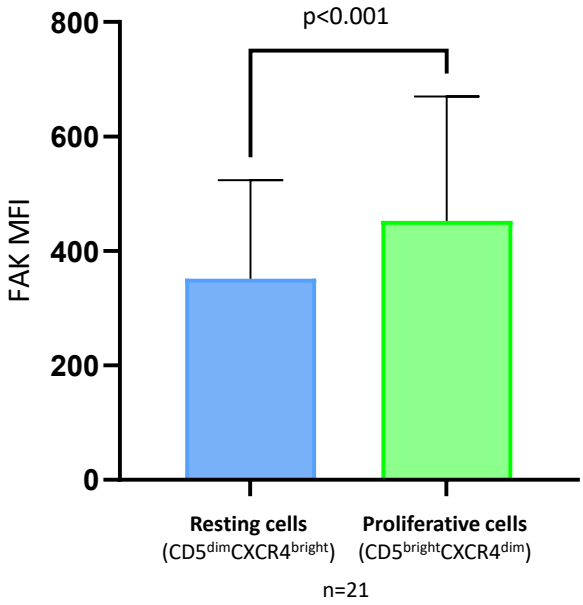

Figure 33. **FAK is over-expressed in the proliferative fraction of the CLL clone**. We analyzed FAK expression by FC in the sub-populations of CLL clones, namely the older resting cells and the proliferative compartment from the peripheral blood of 21 CLL patients. We demonstrated that FAK protein is more expressed in the proliferative fraction of the CLL clone, which recently emigrated from the tissues and divided (452.5±217.4), compared to the older resting cells (351.4±172.1; *p*=0.0001, paired Student's *t* test).

8. FAK inhibition leads to CLL apoptosis

Defactinib is a small molecule inhibitor of FAK, currently being evaluated for potential combination therapies for various solid tumors, that inhibits FAK phosphorylation at the activatory tyrosine Y397.

We examined the effect of FAK inhibition on the survival of B lymphocytes from 46 CLL patients after 24 hours of treatment *in vitro* with 5µM Defactinib. WB analyses revealed that FAK expression was down-modulated after Defactinib treatment. More in detail, we noticed a lower amount of 125kDa FAK in the treated condition (0.47±0.39) compared to untreated cells (0.91±0.90; *p*<0.001, paired Student's *t* test). In addition, FAK activation, determined by the level of autophosphorylation at the Y397 site, was also reduced in the cells treated with Defactinib (1.16±0.80 *vs* 1.81±0.94; *p*<0.0001, paired Student's *t* test) (**Fig. 34**).

FAK inhibition induced significant apoptosis of CLL cells. We assessed the effects on cell viability by WB, determining the level of cleaved PARP, synonym of caspase-dependent death. Indeed, Defactinib caused CLL cells apoptosis, as assessed by the increase of the cleaved PARP in the treated samples compared to the untreated control (**Fig. 34**, bands relative to PARP).

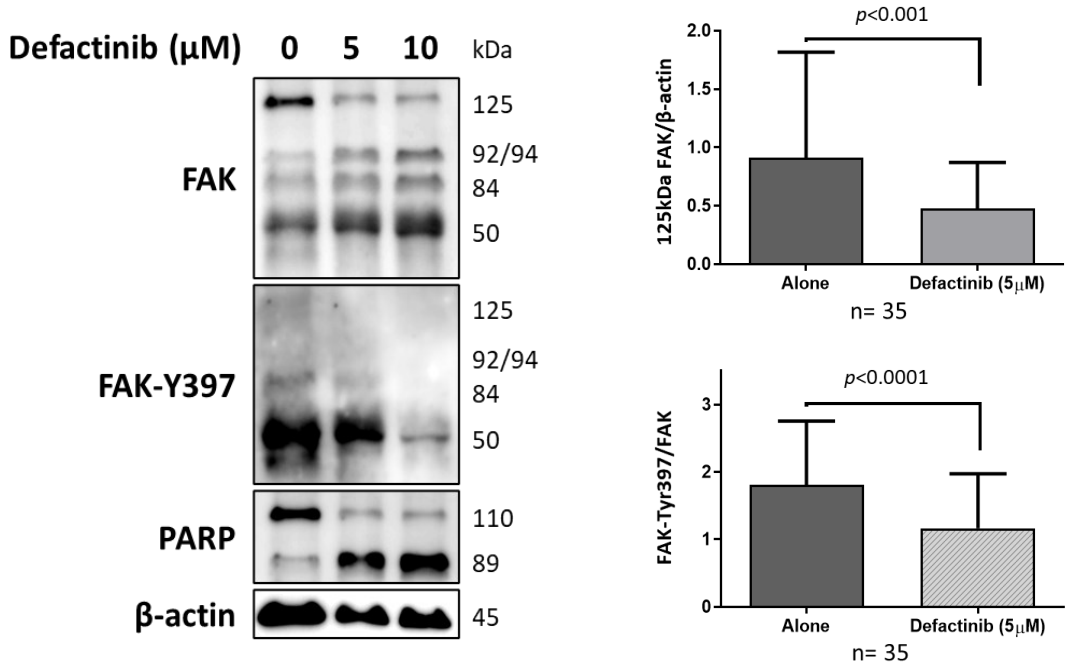

Figure 34. **Defactinib, FAK inhibitor, leads to the down-modulation of FAK expression and phosphorylation, causing CLL apoptosis.** WB analyses revealed that a lower amount of the full-length 125kDa FAK was present after treatment with Defactinib 5μM (0.47±0.39) compared to untreated cells (0.91±0.90; $p<0.001$, paired Student's *t* test). Besides, FAK activation, determined using a phospho-specific Ab for the level of phosphorylation at the Y397 site, was reduced (1.16±0.80 *vs* 1.81±0.94; $p<0.0001$, paired Student's *t* test). FAK inhibition led to the increase of cleaved PARP (89kDa) in the treated samples compared to the untreated control, demonstrating a pro-apoptotic effect of Defactinib in CLL cells.

These data were confirmed also by FC using the test for apoptosis detection with Annexin V (FITC)/Propidium Iodide (PE): the percentage of the viable cells, identified as the Annexin V/Propidium Iodide double negative cells, was lower after culture with the inhibitor, confirming that Defactinib can cause apoptosis of CLL lymphocytes (62±17% *vs* 33±24%; $p<0.0001$, paired Student's *t* test) (**Fig. 35A**).

Of note, the response to Defactinib in terms of decrease in viability, as measured by the Annexin test, identified two groups of patients, one more sensitive to the drug (circled in **Fig. 35B**). Since we previously demonstrated that FAK is more active in poor prognosis CLL, we plan to investigate the clinical and biologic characteristics of the most responsive group.

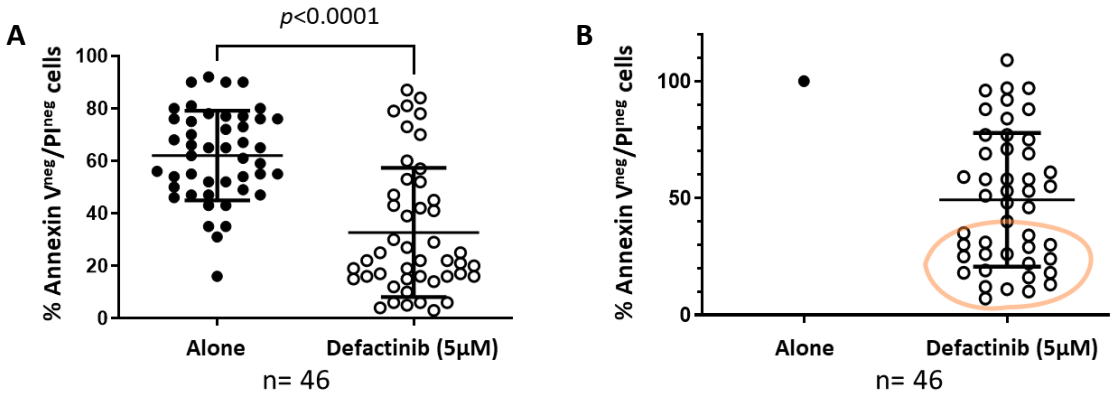

Figure 35. **CLL cells experience apoptosis after FAK inhibition by Defactinib.** (**A**) Treatment with 5μM Defactinib led to apoptosis of CLL, determined as a decrease in the percentage of alive cells (62±17% *vs* 33±24%, $p<0.0001$; paired Student's *t* test). (**B**) When the percentage of viable cells in the control sample was set to 100%, we could observe that after treatment CLL patients could be divided in two groups: the first, under the median value for viability response (circled), which can be considered as sensitive to Defactinib; the other, in which the percentage of viable cells did not change significantly from the untreated condition, could be regarded as not affected by FAK inhibition.

DISCUSSION

During this three-year PhD program, we demonstrated that in CLL lymphocytes FAK is present as cleaved forms and is hyper-phosphorylated, especially in poor prognosis patients. The cleavage of FAK is regulated by Calpain, as strongly suggested by the results in BCR responsive patients. FAK expression correlated to the overexpression of Cortactin and HS1, molecules linked to disease aggressiveness[47,48]. Furthermore, we showed that FAK has a role in the survival potential of CLL, as supported by the induction of apoptosis by the use of its selective inhibitor, Defactinib.

FAK is a non-receptor tyrosine kinase and is a crucial molecule at the crossroad between extracellular environment and intracellular signaling. In fact, it can be activated by interactions with integrins, growth-factor receptors and chemokine receptors. Once phosphorylated, FAK is able to interact with Src and control many signaling pathways, leading to migration, proliferation and survival[106]. For these reasons, FAK has been studied in tumors, especially of solid tissues. Its overexpression has been linked with cancer aggressiveness, even if in some cases also its downmodulation was reported as correlated with poor prognosis[110]. For what concerns the role of the kinase in hematological tumors, few papers have been published. In particular, nothing is known about FAK functions in CLL. Thus, we planned to explore FAK role in CLL considering its involvement in many key signaling pathways connected to cancer development, and especially its relationships with molecules (*i.e.* Lyn, HS1, Cortactin) participating in altered BCR signaling and apoptosis resistance, two main hallmarks of CLL pathogenesis.

Herein, we analyzed the expression and functions of FAK in CLL patients from the Hematology Division of Padua hospital. Firstly, we observed a variable and heterogenous FAK expression in patients, which did not result significant compared to healthy controls. We were able to notice that, among CLL cases, some patients exhibited an evident 125kDa FAK down-modulation.

CLL lymphocytes are known to present a homogenous phenotype, characterized by the expression of CD19/CD5/CD200 surface markers, but disease course is really heterogenous, ranging from an indolent disease, sometimes completely asymptomatic, to a very aggressive form of leukemia which progresses fast and presents short survival. The guidelines from the International Workshop on Chronic Lymphocytic Leukemia (iwCLL) are consensus recommendations aimed at guiding clinicians in diagnosis and treatment of the disease, besides providing a valuable source of information on new biological markers for prognosis. In the last ten years, research in this field has provided increasing evidence on the importance of BCR signaling in this disease, and the mutational status of IGHV genes has become one of the most relevant prognostic factors of CLL. Using the subdivision of patients in poor prognosis U-CLL and in M-CLL with favorable prognosis, we found that the patients characterized by a lower expression of 125kDa FAK were part of the former group. Most of the studies regarding FAK expression and function in oncohematological disorders are about acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). Recher *et al.*[124] demonstrated that FAK was expressed in approximately 40% of AML patient samples, while Tavernier-Tardy and colleagues[125] found that the 65.5% of AML cells was positive for FAK expression. Both research works correlated the kinase expression with poor prognosis. In particular, FAK was associated with enhanced blast

migration and increased cellularity[124] and the patients with over-expression of FAK, as well as of CXCR4 and VLA-4 had a significantly shorter overall survival[125]. Also in ALL, FAK protein expression was proved to be higher in leukemia cells from patients than in lymphocytes from normal controls[126] and it was shown to be activated in Ph+ ALL[127]. Even if the just mentioned research papers on FAK expression in other leukemias, describing FAK over-expression as correlated with dismal disease outcome, are in contrast with our results in CLL, indicating a down-modulation of FAK in patients with unfavorable prognosis, these findings indicate a role for FAK in oncohematological diseases. Of note, above stated authors do not mention any evidence on the investigation beyond FAK full-length form of 125kDa.

As part of focal adhesion complexes, FAK has a crucial role in regulating the dynamics of focal adhesions assembly and disassembly. In particular, it can be subject to proteolytic cleavage by calpain proteases. Calpains are a family of cysteine proteases activated by increased intracellular Ca^{2+}. A recent work by Łopatniuk *et al.*[123] demonstrated that Calpain levels in CLL were correlated with the clinical stage of the disease. By WB analyses we explored Calpain expression in our cohort of patients, suggesting that U-CLL followed a trend towards overexpression of the protease. Thus, we deepened our analysis and found that FAK was present both as its full-length form of 125kDa and also as fragments of lower molecular weight. Literature review suggested that these fragments of 92/94, 84 and 50kDa were compatible with cleavage products by Calpain[99]. In particular, we found a great amount of the Calpain-mediated products of FAK in CLL patients with poor prognosis, *i.e.* U-CLL, where they were also hyperphosphorylated, meaning catalytically active. Our results were supported by a recent work from Sundaramoorthy *et al.*[128] who also deepened the investigation on FAK beyond its 125kDa full-length form. Their study on colon cancer demonstrated that cleavage of FAK by Calpain, after Ca^{2+} administration, increased the kinase activity, enhancing the motility of cancer cells, synonym of adverse outcome[128].

Although this classification is not clear-cut, the absence of mutations in IGHV genes in U-CLL often reflects the presence of a responsive BCR, compared to more anergic receptors on M-CLL cells, which commonly express low levels of surface Igs. We validated that, in contrast to non-responsive cases, the BCRs on responsive cells were able to mobilize Ca^{2+}, manifesting a significant increase of the ion concentration after appropriate stimulation of the surface receptor. Since Calpain catalytic function is regulated by Ca^{2+}, we hypothesized that the protease might be more active in the responsive patients, mainly U-CLL with more aggressive disease. This may explain our results regarding increased expression of FAK cleavage products particularly in poor prognosis cases. Experiments are ongoing in order to better understand the link between BCR activation and Calpain increased activity, since this protease can be activated also by Src kinases, which in CLL are predominantly represented by Lyn, that we previously found overexpressed and hyperactivated in comparison to normal B cells[35].

The most characterized FAK functions are related to integrins signaling, hence mainly localized near to the plasma membrane and in the cytosol. However, FAK has been demonstrated to also have kinase-independent functions, consisting in protein-protein interactions and scaffold activities. In particular, this is associated with the localization of FAK inside the nucleus, possible thanks to the nuclear localization and export sequences (NLS and NES) identified in the FAK FERM domain and in the central kinase domain[76]. Here, FAK and p53 proteins can directly interact in

the cells through their N-terminal domains[79]. FAK can suppress transcriptional activity of p53 by acting as a scaffold protein to bind to p53 and its E3 ubiquitin ligase Mdm2, facilitating the degradation of p53, thus sequestering it from anti-apoptotic signaling[80]. This function designates FAK as a participant involved in a pathway for increased survival. In light of this information, we investigated on the localization of FAK and its cleaved forms inside the B cells of CLL. Using subcellular fractionation, we were able to demonstrate that the full-length form of FAK of 125kDa was present only in the cytosol, while the cleaved/active forms of FAK were localized also inside the nucleus, especially in the patients with responsive BCR, suggesting a role for the kinase in this cellular compartment also in leukemic cells. Comparably, Sundaramoorthy *et al.*[128] found that in colon cancer cells Ca^{2+} administration increased FAK and cleaved FAK (N-FAK) levels in nuclear fractions, where it was able to modulate p53 protein stability, that could be recovered only by inhibition of proteasomal degradation. We therefore suppose that, when Calpain cleaves FAK in the two N-FAK and C-FAK fragments, the FAT domain required for its localization near the membrane is eliminated from N-FAK, which can dissociate from focal adhesions and is facilitated in translocating into the nucleus[96–98]. Here, FAK may be directly involved in mechanisms promoting survival and cellular proliferation of the aggressive leukemic cells.

Moreover, p53 is one of the transcription factors which can bind to FAK gene promoter, counteracting its expression in a negative feedback mechanism. In view of the fact that p53 is inactivated in about the 15% of CLL patients which manifest del17p, the chromosomal region encompassing *TP53* coding sequence, or loss of function mutations in *TP53* gene, and that these cytogenetic lesions correlate to a remarkable unfavorable prognosis, the investigation on FAK-p53 interaction in CLL is certainly to be further explored.

Considering the involvement of FAK in pathways of cellular adhesion, migration, metastatic invasion, and proliferation, we wanted to analyze the relation with other proteins involved in the same mechanisms. We investigated on HS1 and Cortactin, two Lyn substrates that we previously demonstrated to be associated with CLL pathogenesis and aggressiveness[47,48], which do also interact with FAK. Similarly to the kinase under study, HS1 and Cortactin are relevant for cytoskeleton and extracellular matrix remodeling, shape, and motility of the tumor cell. Analyzing protein expression by both flow cytometry and Reverse Phase Protein Array (RPPA) in our CLL patients, we found that FAK expression and phosphorylation at the activatory Y397 residue correlated with overexpression of both HS1 and Cortactin. This prompted us to speculate the existence of a pathway involving FAK-Cortactin-HS1 interactions which ultimately promotes the aggressive phenotype of CLL.

Similarly to other tumors, CLL lymphocytes are dependent on microenvironment signals for enhanced survival capabilities. Leukemic cells circulate in the peripheral blood and accumulate in secondary lymphoid organs to interact with other cells and to receive survival stimulation. An important pathway involved in the regulation of trafficking and retention of B cells is the CXCR4/CXCL12 axis, which allows the homing of lymphocytes in the solid tissues. Recently, it has also been shown that, on CLL cells, CXCR4 undergoes an enhanced recycling, thus contributing to an increased surface expression of the receptor on the cells in the periphery[129]. Calissano *et al.* demonstrated that CLL cells resting in the periphery are characterized by a $CD5^{dim}CXCR4^{bright}$ phenotype and have slow proliferation kinetics[58]. Instead, the egressing lymphocytes, which have been in the secondary lymphoid organs and have thus been stimulated

by the cells in that microenvironment, show a higher proliferative potential and exhibit a $CD5^{bright}CXCR4^{dim}$ phenotype. Analyzing these two subclonal populations in our patients by flow cytometry, we demonstrated that FAK was significantly overexpressed in those CLL cells which were recently born and had egressed from the microenvironment. Thereby this result gave additional support to our hypothesis of FAK being implicated in pathways promoting the increased proliferation of CLL. Likewise, studies on AML cells by Carter *et al.*[130] demonstrated that the activation of FAK by the bone marrow microenvironment promoted leukemia/stroma interactions which support the survival of leukemic cells[130].

As FAK drives several tumor-supporting signaling pathways, small molecules inhibiting the kinase are being developed. One of this is the selective ATP-competitive inhibitor Defactinib, which completed the Phase II clinical trials in patients with KRAS-mutant non-small-cell lung cancer[106]. We thus examined the effect of Defactinib on CLL lymphocytes to analyze whether FAK inhibition could have an effect on the survival of these cells. We demonstrated that Defactinib was effective in inducing apoptosis of CLL cells after 24 hours of *in vitro* treatment at the concentration of 5µM, besides downmodulating FAK expression and phosphorylation. In particular, data from Annexin V/Propidium Iodide apoptosis test revealed the presence of a heterogeneous response to Defactinib treatment among CLL patients, with a group of cases exhibiting higher apoptotic response after the inhibitor administration. We therefore plan to investigate the characteristics of these CLL patients which were more responsive to Defactinib, focusing in particular on the mutations of *TP53* gene and the related chromosomal aberrations.
All the reported results helped us in generating a hypothesis, sustained by literature, by which FAK can be activated by multiple pathways, such as integrins, chemokine receptors and Src kinase, besides being cleaved by Calpain. In BCR responsive patients, which manifest an active receptor signal that leads to a higher intracellular concentration of Ca^{2+}, Calpain protease is increasingly activated. This results in an improved cleavage of FAK and a consequent greater generation of N-terminal fragments of the kinase. These N-FAK fragments lack the FAT domain required for membrane localization, thus they dissociate from focal adhesions and can easily translocate inside the nucleus. Here, N-FAK could counteract the anti-tumoral functions of p53, acting as a scaffold to facilitate its degradation. Back in the cytoplasm, FAK expression and activation is correlated with the over-expression of Cortactin and HS1, cytoskeletal related proteins associated with poor survival in CLL. Therefore, we suppose that these three proteins, FAK, Cortactin and HS1, are involved in a pathway leading to increased survival of CLL cells, ultimately resulting in the malignant phenotype of poor prognosis patients. **Fig. 36** outlines our hypothetical pathway involving BCR/Calpain/FAK activation/HS1 and Cortactin, eventually leading to an aggressive phenotype.

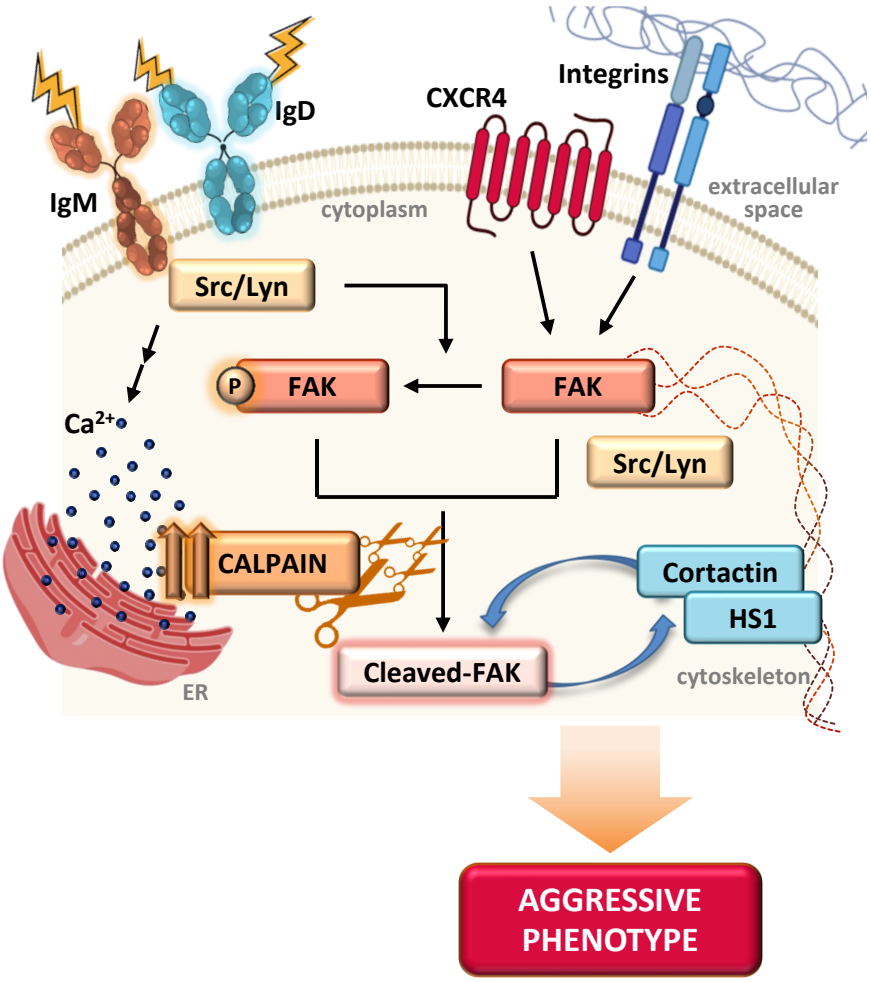

Figure 36. **Hypothetical model of FAK interactions in CLL.** After BCR stimulation, Ca^{2+} is released in the cytoplasm and can activate Calpain protease, which cleaves FAK, thus activating it. Together with HS1 and Cortactin, FAK could be involved in a pathway promoting the malignant phenotype of CLL.

The pro-survival roles of FAK can be counteracted by treatment of cells with Defactinib, a selective kinase inhibitor, which we demonstrated to be effective in leading CLL lymphocytes to significant apoptosis. Other studies in literature revealed that the administration of VS-4718, another inhibitor of FAK, prevented cell growth and induced apoptosis in AML cell lines, especially when combined with ABT-199, inhibitor of BCL-2[131]. Similarly, the silencing of FAK inhibited leukemogenesis in BCR/ABL-transformed ALL cells and increased apoptosis of cancer cells in combination with imatinib[132]. Also, the combinatorial therapy of VS-4718 and dasatinib prolonged survival in a model of B-ALL[133]. We therefore propose that FAK has a decisive role in the survival of poor prognosis CLL cells and could be exploited as a therapeutic target. Future experiments will help in decoding factors that increase the cleavage of the kinase and the sensitivity to FAK inhibition. Despite the recent developments in the search for effective treatments to eradicate the disease, CLL still remains incurable. The identification of the features of the patients more responsive to Defactinib administration may be helpful in identifying a subset of CLL cases which therapy could be implemented with this inhibitor, alone or in combination.

In conclusion, our results suggest that FAK, besides being a possible target for treatment, could be an interesting molecule to study to get additional knowledge on CLL pathobiology or, at least, on the establishment of its malignant phenotype.

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