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XPO1 MUTATIONS ARE A NOVEL PREDICTOR OF SHORTER TIME TO FIRST TREATMENT IN EARLY STAGE CLL PATIENTS

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SUMMARY

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western World. In approximately 70% of newly diagnosed cases, CLL presents at an early clinical stage and is managed with a watch & wait strategy. Until now, few clinical and molecular predictors inform on the risk of treatment requirement. On these grounds, we aimed at identifying new molecular markers that may predict early treatment requirement and may help clinicians to better plan the watch & wait strategy in asymptomatic early stage CLL patients. 295 Binet A CLL patients who referred to our institution were subjected to next-generation-sequencing (NGS) in a panel of recurrently mutated genes in CLL. Two validation multicenter cohorts of 402 treatment-naïve Binet A CLL patients (Binet A validation cohort) and 395 untreated Rai 0 CLL patients (Rai 0 validation cohort) were also included and analyzed for *XPO1* mutations. The primary endpoint was time to first treatment (TTFT). In the training cohort, NGS mutational analysis showed that *XPO1* was mutated in 7 (2.4%) patients. In univariate analysis, trisomy 12 (HR 2.42; 95% CI 1.43-4.15; p=0.001), unmutated IGHV genes (HR 4.51; 95% CI 2.83-7.05; p<0.0001) and mutations of *XPO1* (HR 8.88; 95% CI 3.77-20.95; p<0.0001), *NOTCH1* (HR 3.02; 95% CI 1.66-5.51; p<0.001) and *SF3B1* (HR 2.65; 95% CI 1.15-6.10; p=0.022) were associated with a shorter TTFT. By multivariate analysis, *XPO1* mutations (HR 4.24; 95% CI 1.72-10.44; p=0.002) and unmutated IGHV genes (HR 3.43; 95% CI 2.08-5.67; p<0.0001) maintained an independent association with a shorter TTFT. *XPO1* mutational analysis was subsequently investigated in 2 independent multicenter cohorts of early stage CLL patients. In the Binet A validation cohort (N=402 patients), *XPO1* was mutated in 15 (3.7%) patients and was associated with a shorter TTFT (HR 2.59; 95% CI 1.36-4.96; p=0.004). Similarly, also in the Rai 0 validation cohort, (N=395 patients), *XPO1* was mutated in 8 (2.0%) patients and was associated with a shorter TTFT (HR 6.02; 95% CI 15.03-4.96; p<0.001). By combining the training and the validation cohorts (N=1092 patients), a total of 30 somatically

acquired *XPO1* mutations were identified (2.7% of patients). More precisely, 27 (90.0%) mutations affected *XPO1* codon E571 and 3 (10.0%) codon D624. From a clinical perspective, patients carrying either *XPO1* E571 or D624 mutations showed superimposable outcome in terms of TTFT (p=0.345). Based on these results, *XPO1* mutational analysis might be incorporated in other prognostic scores and help clinicians to refine the management of the watch and wait strategy for early stage CLL.

1 INTRODUCTION

1.1 Definition and epidemiology

Chronic lymphocytic leukemia (CLL) is the most common leukemia in the Western World, with an approximate incidence rate of roughly 4.5 new cases per 100,000 individuals annually and a median age at diagnosis of 72 years old (Hallek *et al*., 2008). Males are affected by CLL more than females with a male to female ratio of 1.5-2 (Howlader *et al*., 2016). CLL is characterized by the clonal proliferation and accumulation of mature B cells within the peripheral blood (PB), bone marrow and other lymphatic tissues.

The diagnosis of CLL requires the presence of > $5x10^9$/L clonal B lymphocytes in peripheral blood during at least 3 months. Some morphological traits of CLL can be observed in a blood smear: small, mature lymphocytes, a narrow border of cytoplasm, a dense nucleus without dissemble nucleoli and partially aggregated chromatin and Gumprecht nuclear shadows (Hallek *et al.*, 2018). CLL cells express the B cells antigens: CD19, CD20, CD23 and T-cell surface antigen CD5. The levels of surface immunoglobulin, CD20 and CD79b are low compared with a normal B cell. In those borderline cases, other markers like CD43, CD81, CD200, CD10 and ROR1 can support the diagnosis (Hallek *et al*., 2008; Cheson *et al*., 1996).

CLL is an indolent neoplasia, with a median survival of 12-15 years and requires treatment only in cases of symptomatic disease. Most patients affected by CLL are asymptomatic at diagnosis, while some patients may present systemic B-cell symptoms, lymphadenopathy, hepatosplenomegaly, bone marrow (BM) infiltration, that indicate treatment requirement (Hallek *et al*., 2018). Active disease should be clearly documented to initiate therapy. At least 1 of the following criteria should be met: *i)* evidence of progressive marrow failure as manifested by the development of, or worsening of, anemia and/or thrombocytopenia; *ii)* massive (ie, ≥6 cm below the left costal margin) or progressive or symptomatic splenomegaly; *iii)* bulky lymph nodes (ie, ≥10 cm in longest diameter) or

progressive or symptomatic lymphadenopathy; *iv)* progressive lymphocytosis with an increase of ≥50% over a 2-month period, or lymphocyte doubling time (LDT) <6 months; *v)* autoimmune complications including anemia or thrombocytopenia poorly responsive to corticosteroids; *vi)* symptomatic or functional extranodal involvement; *vii)* disease-related symptoms such a unintentional weight loss, significant fatigue, fever without evidence of infection and night sweats for ≥1 month without evidence of infection.

1.2 Clinical markers in asymptomatic CLL patients

The first prognostic marker used in the clinical management of CLL was the Rai clinical staging system, published in 1975 (Rai *et al*., 1975). The system includes lymphadenopathy, organomegaly, and cytopenia (anemia and thrombocytopenia) to establish five prognostic groups that can be used to predict median survival of newly diagnosed CLL patients. Patients with stage 0 have a median survival of >150 months, with stage I of 101 months, with stage II of 71 months and with stages III or IV of 19 months.

Similarly, a subsequent staging system, namely Binet staging system, was published in 1981. In this score, CLL patients are classified by the number of affected superficial lymphoid tissue regions (cervical lymph nodes, axillary lymph nodes and inguinal lymph nodes), spleen, and liver and by the presence or not of anemia or thrombocytopenia. Binet A patients are characterized by the involvement of less than 3 lymphoid areas and by the absence of anemia or thrombocytopenia. Binet B patients have three or more involved lymphoid areas without anemia or thrombocytopenia. Binet C patients present with anemia and/or thrombocytopenia regardless of the number of involved lymphoid areas (Binet *et al*., 1981). In approximately 70% of newly diagnosed cases, CLL presents at an early clinical stage, namely Binet A, and is managed with a watch & wait strategy.

1.3 Prognostic markers in asymptomatic CLL patients

The clinical course of CLL ranges from a very indolent condition, with a nearly normal life expectancy, to rapidly progressive leading to early death (Raponi *et al*., 2018; Rossi *et al*., 2015). The majority of CLL patients do not need treatment at the time of diagnosis, but they require treatment during their lifetime. This clinical observation is very helpful to identify markers at the time of diagnosis, that may be able to predict the course of the disease. Prognostic biomarkers provide useful information about the biology and the natural history of CLL in the absence and/or independent of the treatment received (Dearden *et al*., 2008).

In the past decade, a large body of genomic investigations have deciphered the genome of CLL. Elucidation of the CLL genomic landscape has substantiated the notion that CLL is not associated with a unique genetic lesion but, conversely, that this leukemia harbors many different genetic abnormalities that may interact and/or surrogate and complement in inducing the development and progression of CLL. Until now, few clinical and molecular predictors inform on the risk of treatment requirement and the impact of CLL gene mutations is not completely understood (Gaidano *et al.*, 2017).

The pattern of tumor growth of untreated CLL has been investigated by analyzing serial longitudinal samples collected between diagnosis and the time of treatment requirement. Two different patterns of growth have been identified. The exponential growth pattern is characterized by a rapid proliferation of the CLL clone, whereas the logistic growth pattern displays a lower rate of progression. These two different patterns of growth associate with peculiar molecular features. CLL cases with an exponential growth are mainly immunoglobulin heavy variable (IGHV) unmutated CLL and have a higher frequency of clonal and subclonal somatic genetic lesions compared to patients with a logistic growth pattern. These different growth patterns, as well as the association between exponential growth, unmutated IGHV genes, and additional genetic lesions, have been validated in an independent cohort of CLL

patients (Gruber *et al.*, 2019).

Other studies have focused on the identification of clinical and molecular features that might identify early stage CLL who are at risk of early progression at the time of diagnosis. Such patients, if identified *a priori*, might benefit from clinical trials comparing early intervention *versus* observation. In this context, the combination of simple clinical features and molecular biomarkers, namely lymphocyte count >15,000/μL, palpable lymph nodes, and unmutated IGHV genes, identifies three different subgroups of Binet A and treatment naïve CLL patients with a high risk of early treatment requirement (Condoluci *et al.,* 2020). This risk model, termed IPS-E (International Prognostic Score-Early), has been validated in several independent series and is a robust tool to inform at the time of diagnosis about the probability that a given CLL patient in early stage disease progresses and needs treatment (Condoluci *et al.,* 2020). Similarly, the second model for Rai 0 CLL identified 17p deletion, 11q deletion, trisomy 12, unmutated and white blood cell count above 32,000/μL as variable associated with shorter TTFT.7 These models do not take into account the clinical impact of gene mutations that may allow a more precise stratification of early stage CLL patients (Cohen *et al.,* 2020).

In addition, by taking advantage of the genetic heterogeneity of CLL, mutations of genes involved in CLL pathogenesis have been tested as biomarkers for identifying early stage CLL patients with a higher risk of progression and treatment requirement. These studies point to mutations of *SF3B1*, *NOTCH1*, *ATM*, and *U1* as molecular predictors of shorter time to first treatment (TTFT) (Shuai *et al*., 2019; Hu *et al.* 2019; Moia *et al*., 2019).

Interestingly, *TP53* disruption is not associated with a shorter TTFT, in line with the notion that *TP53* disruption interacts with treatment with chemotherapeutic agents, but not with a watch and wait strategy that does not expose CLL cells carrying *TP53* disruption to the positive selection pressure exerted by ineffective chemotherapy. The precise role of gene mutations in sorting asymptomatic CLL patients with an imminent risk of treatment

requirement still needs to be clarified and is the current subject of investigations. (Hu et *al*., 2019; Condoluci *et al.,* 2020).

1.4 Management of asymptomatic CLL patients

Approximately 70% of newly diagnosed CLL patients presents in an early stage according to the Binet and Rai staging systems, may never require treatment, and may have a life expectancy similar to the general population (Halek *et al.,* 2018; Binet *et al.,* 1981; Rai *et al.,* 1975). Asymptomatic early stage CLL patients, till now, are managed with a watch and wait strategy, and treatment is started only in case of symptomatic disease including progressive lymphocytosis, enlarged lymph nodes, cytopenia, and systemic symptoms (Hallek *et al.,* 2018). Three clinical trials comparing chlorambucil, fludarabine and fludarabine, cyclophosphamide and rituximab (FCR) *versus* placebo in high risk asymptomatic CLL patients did not prolong survival in early stage asymptomatic CLL patients (Dighiero *et al.,* 1998; Hoechstetter *et al.,* 2017; Herling *et al.,* 2020; Langerbeins *et al.,* 2019). The three above mentioned clinical trials compared chemoimmunotherapy (CIT) *versus* observation, whereas, recently, the phase 3 CLL12 clinical trial compared ibrutinib *versus* observation in asymptomatic CLL patients. Preliminary results have demonstrated a higher progression free survival (PFS) rate in the ibrutinib arm, compared to placebo. However, survival data are not mature enough to demonstrate a clearly advantage of ibrutinib *versus* observation (Langerbeins *et al.,* 2019). Therefore, guidelines still recommend a watch and wait strategy for asymptomatic CLL patients (Hallek *et al.,* 2019).

1.5 The *XPO1* gene

Exportin-1 (XPO1), also called chromosome region maintenance 1 (CRM1), is a pivotal eukaryotic nuclear export protein that carries an extensive array of proteins from the nucleus

to the cytoplasm. XPO1 is a member of the importin-β superfamily of karyopherins that mediates the translocation of numerous RNAs and cellular regulatory proteins, including tumor suppressor proteins (TSPs). Thanks to the energy given by RanGTP, XPO1 can export out of the nucleus some cargo proteins. The cargo proteins of XPO1 include TP53, RB, p21 and other anti-apoptotic proteins. In the cytoplasm, the complex dissociates and XPO1 goes back to the nucleus to repeat its biological activity (Figure 1). XPO1 is overactive in many cancers, and thus has been considered a potential anti-cancer target for decades (Huang et *al*., 2009). XPO1 is a therapeutic target and selective inhibitors of nuclear transport (SINE) such as selinexor are now being investigated in clinical trials in CLL and in other lymphoid malignancies (Lapalombella *et al.*, 2012).

Furthermore, recent studies have focused on the characterization of the molecular alterations in the *XPO1* gene. In primary mediastinal diffuse large B cell lymphoma (PMBL) and classical Hodgkin's lymphoma (cHL) a mutational hotspot affecting the codon E571K (Jardin et *al.*, 2016) has been identified. Missense substitutions targeting *XPO1* have also been previously reported also in other hematological and non-hematological malignancies (Jeromin *et al*., 2014). Pre-clinical models showed that *XPO1* mutations, and in particular the E571K hotspot, seems to enhance the affinity of XPO1 to the cargo proteins that are exported out of the nucleus with a greater extent. In this way, the nucleus is deprived of antiapoptotic proteins that may lead to an uncontrolled cell proliferation. Regarding prognosis, high levels of XPO1 are associated with shorter survival and are a poor prognostic factor in acute myeloid leukemia and in other solid cancers (Kojima *et al.,* 2013 Azmi *et al.,* 2020).

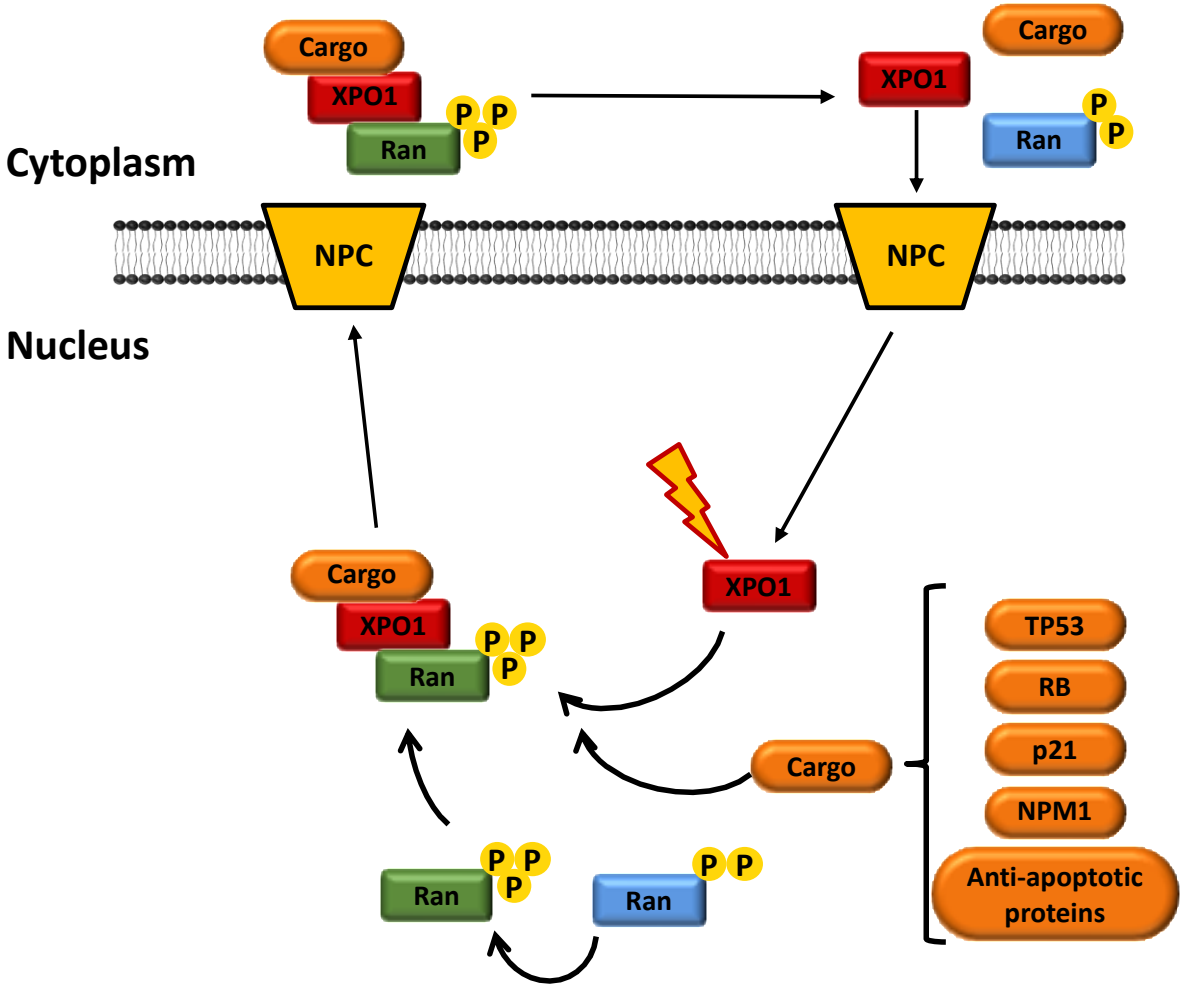

Figure 1: The XPO1 pathway. The XPO1 protein exports nuclear proteins (cargo) from the nucleus to the cytoplasm thanks to the energy provided by the RanGTP. The complex of XPO1, RanGTP and the cargo protein is disassembled in the cytoplasm, cargo is released, and XPO1 is recycled back to the nucleus. *XPO1* mutations (yellow arrow) increased the affinity of XPO1 for cargo proteins thus enhancing their exportation outside the nucleus.

2 AIM OF THE THESIS

CLL is a highly heterogeneous disease; some patients may never require treatment, whereas other relapse early after frontline therapy. In approximately 70% of newly diagnosed cases, CLL present at an early clinical stage and is managed with a watch & wait strategy. The IPS-E and the Rai 0 prognostic score identify patients with early treatment requirement but the impact of CLL gene mutations is not completely understood.

Consistently, the aim of this thesis was to identify new molecular markers that may predict early treatment requirement and may help clinicians to better plan the watch & wait strategy in asymptomatic early stage CLL patients.

3 MATERIALS AND METHODS

3.1 Patients

This training cohort included a cohort of 295 Binet A CLL patients. The following biological material was collected: *i*) 295 tumor genomic DNA (gDNA) from PB mononuclear cells at the time of diagnosis; *ii*) paired germline DNA from saliva for 56 cases; and *iii)* normal gDNA from 6 healthy donors was also used to set the experimental and biological background of the deep next generation sequencing (NGS) approach. Patients provided informed consent in accordance with local Institutional Review Board requirements and the Declaration of Helsinki.

The study included also 2 validation cohort. The Binet A validation cohort was of a multicenter cohort of 402 patients from 4 different institutions (Università Vita-Salute San Raffaele and IRCCS Ospedale San Raffaele, Milan, Italy; University of Modena and Reggio Emilia, Modena, Italy; University of Perugia, Perugia, Italy; Sapienza University of Rome, Rome, Italy). The Rai 0 validation cohort was of a multicenter cohort of 395 patients previously described elsewhere (Cohen *et al.*, 2020).

3.2 Fluorescence In Situ Hybridization (FISH)

FISH procedure starts from sample preparation, in which red cells contained in 500µL of PB are lysed in hypotonic solution and, then the white cells in interphase are fixed using a fixative solution composed of acetic acid and methanol. The hybridization step allows the binding of specific probes to the region of interest and the observation of the nucleus using a fluorescence microscope.

For this project, the labelled probes used for the FISH analysis are XCE 12 probe for tris12 detection (MetaSystems, Altlussheim, Germany), XL DLEU/TP53 probe for del17p detection (Cytocell Acquarius, Cambridge, England), XL ATM/11cen locus-specific probe

for del11q detection (Cytocell Acquarius Cambridge, England), and XL DLEU/LAMP probe for del13q detection (MetaSystems Altlussheim, Germany).

3.3 Resequencing gene panel

A targeted resequencing gene panel was designed to include the coding exons plus the splice sites of 10 CLL driver genes known to be implicated in CLL pathogenesis and/or prognosis (size of the target region: 26963 bp).

The gene panel included the most frequently mutated genes in CLL, namely *NOTCH1*, *TP53*, *ATM*, *SF3B1*, *MYD88*, *EGR2*, *XPO1*, *NFKBIE*, *POT1* and *BIRC3* (Landau *et al.*, 2015; Puente *et al.*, 2015).

3.4 Extraction of gDNA for library preparation

Tumor and normal gDNA were extracted by using the "salting out" protocol (Miller *et al.*, 1988). PB was diluted 1:2 with physiological solution (NaCl 0.9%) and then centrifuged in a gradient differentiation Sigma Diagnostic™ Histopaque®-1077 Cell Separation Medium (Sigma-Aldrich, St. Louis, MO, USA) solution to obtain mononuclear white blood cells and granulocytes.

Cells were lysed with Lysis Buffer (Tris-HCl 1M, pH 8.2, NaCl 5M, EDTA 0.5M), SDS 20% and digested with pronase E (20 mg/mL). Samples were incubated at 37°C overnight in a shaking incubator. Proteins were precipitated with 6M NaCl, and subsequently discarded after centrifugation at 3200 rpm for 20 minutes. DNA was isolated by precipitation with pure ethanol and washed three times with 75% ethanol. The excess ethanol was evaporated and the DNA was dissolved with TE buffer (Tris-HCl 1M, pH 8.2 and EDTA 0.5M).

3.5 DNA fragmentation and quantification

Tumor and germline gDNA were quantified using the Quant-iT^{TM} PicoGreen double strand DNA (dsDN)A Assay kit (ThermoFisher Scientific, Eugene, OR, USA). PicoGreen is a molecule that binds selectively to double helix DNA and allows to obtain a precise estimate of the amount of DNA. The fluorimetric reading was performed using the Infinite F200 fluorometer (TECAN, Männedorf, Switzerland) using the Magellan software. The fluorimetric readings were obtained at a wavelength of 485 nanometer (nm) in absorption and 530 nm in emission. For quantification a standard curve was prepared using a DNA of known concentration and performing serial 1:2 scalar dilutions. Quant-iT^{TM} PicoGreen dsDNA Assay kit was used at the 1:200 dilutions.

Five hundred nanograms were sheared through sonication (Covaris M220 focusedultrasonicator, Woburn, MA, USA) before library construction to obtain 250/300-base pair (bp) fragments. The size of the DNA fragments was checked using the Bioanalyzer 2100 (Agilent Technologies, St. Clara, CA, USA).

The Bioanalyzer 2100 was used to verify size of the DNA fragments. This technology is based on a microfluidic plate that allows quantitative and qualitative analysis of DNA almost instantaneously. The High Sensitivity DNA kit was used to evaluate the DNA and library construction to obtain 250/300-bp fragments.

3.6 CAPP-seq library preparation

The NGS libraries for gDNA were constructed using the KAPA Library Preparation Kit (Kapa Biosystems, Wilmington, MA, USA). Hybrid selection was performed with the custom SeqCap EZ Choice Library (Roche NimbleGen, Madison, WI, USA). Multiplexed libraries (n = 10 per run) were sequenced using 300-bp paired-end runs on a MiSeq sequencer (Illumina, San Diego, CA, USA).

3.7 Next Generation Sequencing

The mutational analysis in NGS was performed using the MiSeq (Illumina) platform, which allows for massive high-throughput sequencing of the genomic regions of interest. The sequencing workflow involves the following phases: *(i)* generation of libraries containing the regions of interest; *(ii)* sequencing; and *(iii)* data analysis.

***i*) Libraries generation**

The library preparation using the KAPA Library Preparation Kit (Kapa Biosystems) begins with end repair and A-tailing reaction, which produces end repaired, 5'-phosphorylation, 3'- Atailed dsDNA fragments, followed by the adapter ligation, during which dsDNA adapters with 3'-dTMP overhangs are ligated to 3'-dA-tailed molecules.

***ii)* Next Generation Sequencing**

The MiSeq Illumina sequencer is based on sequencing by synthesis technology, in which DNA libraries are transferred onto a solid support called flowcell, to which they are linked by special adapters. On the flowcell the libraries are amplified by a method called bridge amplification, which generates clusters of identical DNA molecules, each derived from the amplification of a single molecule.

Sequencing is based on the reversible cyclic termination method, with a by-synthesis approach, which includes three steps: the incorporation of the nucleotide, the detection of the fluorescence image and the cut.

In the first phase of the cycle, the DNA polymerase elongates a specific primer by adding a nucleotide covalently bound to a fluorophore. This presents a block on the 3'-OH of ribose which does not allow polymerization with other nucleotides. Each nucleotide base

is bound to a fluorophore of a specific colour. It follows the detection step of the image that recognizes the specific emission wavelength of the fluorophore. Next, the cut removes both the fluorophore and the inhibitory group present at the 3'-OH end, allowing the beginning of a new cycle. Libraries were sequenced by pair-end sequencing using a 300-bp paired-end cycle kit. The library pool was denatured using 0.2N NaOH. An amount of 10 to 12 picomolar (pM) denatured DNA was loaded into the MiSeq reagent cartridge, which also contained all the reagents necessary for the sequencing reaction.

***iii)* NGS data analysis**

During the sequencing run, the integrated software for real-time primary analysis (RTA, Real Time Analysis, Illumina) performs image analysis and identification of the bases and assigns a qualitative score (Phred score) to each base for each cycle.

Once the primary analysis is completed, the MiSeq Reporter (Illumina) software performs a secondary analysis on the data generated by the RTA through a series of procedures that include: *i)* de-multiplexing, in which the data of different samples sequenced are pulled together based on the specific sample index sequences; *ii)* FASTQ generation, which are files containing all the reads obtained from sequencing.

FASTQ sequencing reads were deduped by utilizing the FastUniq v1.1. Then, the deduped FASTQ sequencing reads were locally aligned to the hg19 version of the human genome assembly using the BWA v.0.6.1 software with the default setting, and sorted, indexed and assembled into a mpileup file using SAMtools v.1.

For cases provided with paired normal gDNA, single nucleotide variations and indels were called in tumor gDNA *versus* normal gDNA, with the somatic function of VarScan2. A Z-test was used to compare the variant allele frequency vs the mean allele frequency in unpaired normal gDNA samples to filter out variants below the base-pair resolution

background frequencies across the target region. Only variants that had a significant call in Z-test were retained (Bonferroni adjusted p<4.685157e-7). For cases not provided with paired normal gDNA, single nucleotide variations and indels were called in tumor gDNA with the CNS function of VarScan2.

The variants called by VarScan 2 were annotated by using the SeattleSeq Annotation 138 tool (http://snp.gs.washington.edu/SeattleSeqAnnotation138) by using the default setting. Variants annotated as SNPs according to dbSNP 138 (with the exception of *TP53* variants that were manually curated and scored as SNPs according to the International Agency for Research on Cancer *TP53* database; http://p53.iarc.fr), intronic variants mapping >2 bp before the start or after the end of coding exons, and synonymous variants were then filtered out.

Among the remaining variants, only protein truncating variants (i.e. indels, stop codons and splice site mutations), as well as missense variants not included in the dbSNP 138 and annotated as somatic in the COSMIC v85 database (https://cancer.sanger.ac.uk/cosmic), were retained.

2.8 Statistical analysis

Medical statistical analysis was performed using SPSS version 24.0. (Chicago, IL, USA). Primary endpoint of survival analysis was time to first treatment (TTFT) defined as the time interval between the date of CLL diagnosis and the date of first CLL treatment. Time to event outcomes were estimated using the Kaplan-Meir method and compared between groups using the Log-Rank test. A false discovery rate approach was used to account for multiple testing, and adjusted p-values were calculated using the Bonferroni correction. The adjusted association between exposure variables and TTFT was estimated by Cox regression. Statistical significance was defined as p value <0.05.

4 RESULTS

4.1 Patient characteristics

Among the 295 CLL cases with early stage Binet A CLL, the median age was 70.8 years old, 136 (46.1%) patients were female and 159 (53.9%) were male, the median lymphocyte count was 8,500/μL, the median hemoglobin level was 14.0 g/dL and the median platelet count was 205,000/μL. The biological features of these patients were consistent with asymptomatic early stage CLL patients. Consistently, 71/285 (24.9%) harbored unmutated IGHV genes, 44/291 (15.1%) had trisomy 12, 12/291 (4.2%) had 17p deletion, 15/291 (5.2%) had 11q deletion and 150/291 (51.5%) had 13q deletion. All clinical and biological characteristics of the studied cohort are reported in Table 1.

Characteristics	Total	Number of patients (%)
Male	N=295	159 (53.8%)
Female		136 (46.1%)
Median age	N=295	70.8 years
Median lymphocyte count	N=295	8,500/μL
Median hemoglobin level	N=294	14.0 g/dL
Median platelet count	N=294	205,000/μL
***IGHV* mutated**	N=285	214 (75.08%)
***IGHV* unmutated**		71 (24.9%)
17p deletion	N=291	12 (4.2%)
No 17p deletion		279 (95.8%)
11q deletion	N=291	15 (5.2%)
No 11q deletion		276 (94.8%)
13q deletion	N=291	150 (51.5%)
No 13q deletion		141 (48.5 %)
Trisomy 12	N=291	44 (15.1%)
No Trisomy 12		247 (84.9%)
Median Follow-up (years)		10.4

Table 1: Clinical data of the 295 CLL patients included in the study.

4.2 Mutational profile of the studied cohort

The mutational analysis, based on targeted resequencing of the genes recurrently mutated in CLL, was completed for all cases, with a coverage >2000x in >80% of the target region (26963 bp) in 90% of patients.

The most frequently mutated gene was *NOTCH1* in 25/295 (8.4%) patients, followed by *TP53* in 17/295 (5.7%), *ATM* in 18/295 (6.1%), *SF3B1* in 10/295 (3.3%), *MYD88* in 12/295 (4.06%), *EGR2* in 6/295 (2.03%), *XPO1* in 7/295 (2.3%), *NFKBIE* in 4/295 (1.3%), *POT1* in 2/295 (0.6%) and *BIRC3* in 1/295 (0.3%) (Figure 2).

In *NOTCH1*, the majority of mutations were frameshift (22/25, 88%) and the c.7541_7542delCT p. P2514fs*4 was the most frequent mutation, present in (17/25, 68%) of patients. In *TP53*, the majority of mutations were missense (16/17, 94.1%) and all mutations clustered in the DNA binding domain of the protein. In *ATM*, the majority of mutations were missense (15/18, 83.3%). In *SF3B1*, all the mutations were missense (10/10, 100%). In *MYD88*, all the mutations were missense (12/12, 100%) and the c.794T>C p.L265P variant was the most frequent mutation, present in (8/12 ,66.6%) patients. In *EGR2*, all mutations were missense (6/6, 100%) and the c.1150C>A p.H384N was the most frequent mutation, presented in (3/6, 50%) patients. In *XPO1*, all mutations were missense (7/7, 100%), 5/7 (71.4%) affected the codon p.E571 and 2/7 (28.6%) the codon p.D624. In *NFKBIE*, all mutations were frameshift (4/4, 100%) and c.759_762delTTAC p.Y254fs*13 was the frequent mutation, present in (4/4, 100%) patients. In *POT1*, the two mutations were missense and the only *BIRC3* mutation was frameshift (c.1281_1285delAAGGG; p.E429fs*7).

4.3 Clinical impact of mutations

After a median follow-up of 10.4 years, 85 (28.8%) patients required treatment. In univariate analysis, molecular characteristics associated with a shorter TTFT were trisomy 12

(HR: 2.42; 95% CI 1.43-4.15; p=0.001), unmutated IGHV genes (HR: 4.51; 95% CI 2.83-7.05; p<0.0001) and mutations of *XPO1* (HR: 8.88; 95% CI 3.77-20.95; p<0.0001), *NOTCH1* (HR: 3.02; 95% CI 1.66-5.51; p<0.001) and *SF3B1* (HR: 2.65; 95% CI 1.15-6.10; p=0.022) (Table 2). The median TTFT for umutated IGHV patients was 4.6 years, and was not reached for mutated IGHV patients (p>0.0001) (Figure 3A). The median TTFT for patients with trisomy 12 was 9.4 years, compared to 21.0 years for patients without (p=0.001) (Figure 3B). The median TTFT for *XPO1* mutated patients was 3.2 years compared to 21.0 years for wild type patients (p<0.0001) (Figure 3C). The median TTFT for *NOTCH1* mutations was 7.1 years, compared to 21.0 years for wild type patients (p<0.001) (Figure 3D). The median TTFT for patients *SF3B1* mutated patients was 4.9 years, compared to 21.0 years for wild type patients (p=0.022) (Figure 3E). Interestingly, neither 17p deletion nor *TP53* mutations associated with a shorter TTFT, in line with the notion that *TP53* disruption interacts with treatment but not with a watch & wait strategy (Figure 4).

By multivariate analysis including variables showing a multiplicity adjusted significant association with TTFT, *XPO1* mutations (HR: 4.24; 95% CI 1.72-10.44; p=0.002) maintained an independent association with a shorter TTFT (Table 2).

	Univariate analysis				Multivariate analysis		
Characteristics	**HR**	**95% CI**	***P***	***P****	**HR**	**95% CI**	***P***
Umutated IGHV	4.51	2.83-7.05	< 0.0001	< 0.0001	3.43	2.08-5.67	< 0.0001
17p deletion	1.96	0.71-5.38	0.191	> 0.999			
Trisomy 12	2.42	1.43-4.15	0.001	0.015	1.73	0.98-3.07	0.059
***NOTCH1* mutation**	3.02	1.66-5.51	< 0.001	0.004	1.40	0.71-2.77	0.327
***SF3B1* mutation**	2.65	1.15-6.10	0.022	0.33			
***TP53* mutation**	1.26	0.46-3.44	0.656	> 0.999			
***XPO1* mutation**	8.88	3.77-20.95	< 0.0001	< 0.0001	4.24	1.72-10.44	0.002

Table 2. Univariate and multivariate analysis in terms of TTFT. P, P-value; P*, Bonferroni correction; CI, confidence interval; HR, hazard ratio.

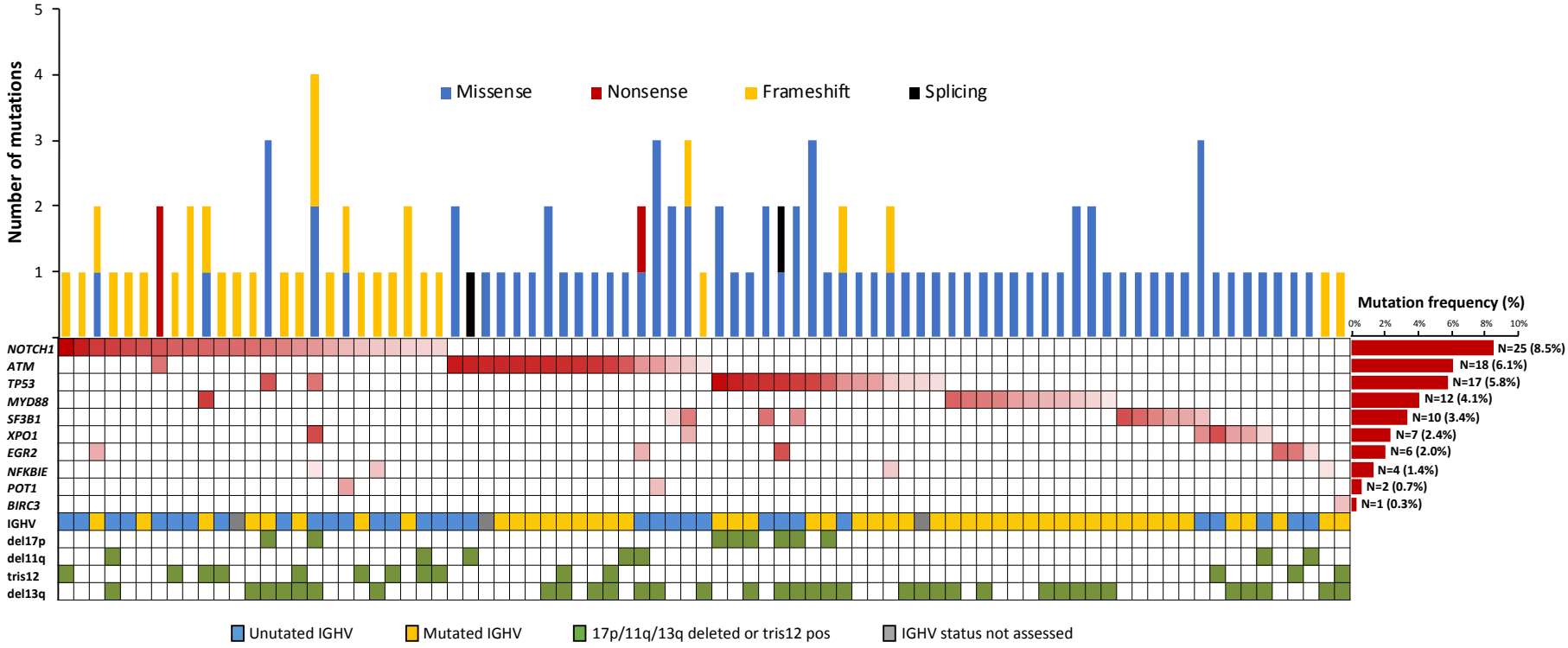

Figure 2. Overview on prevalence and molecular spectrum of non-synonymous somatic mutations discovered in the studied cohort. Heatmap representing the mutational profile of 83 Binet A CLL patients with at least one somatic non-synonymous mutations. Each column represents one patient, each row represents one gene. The fraction of patients with mutations in each gene is plotted on the right. The number and types of aberrations are plotted above the heatmap.

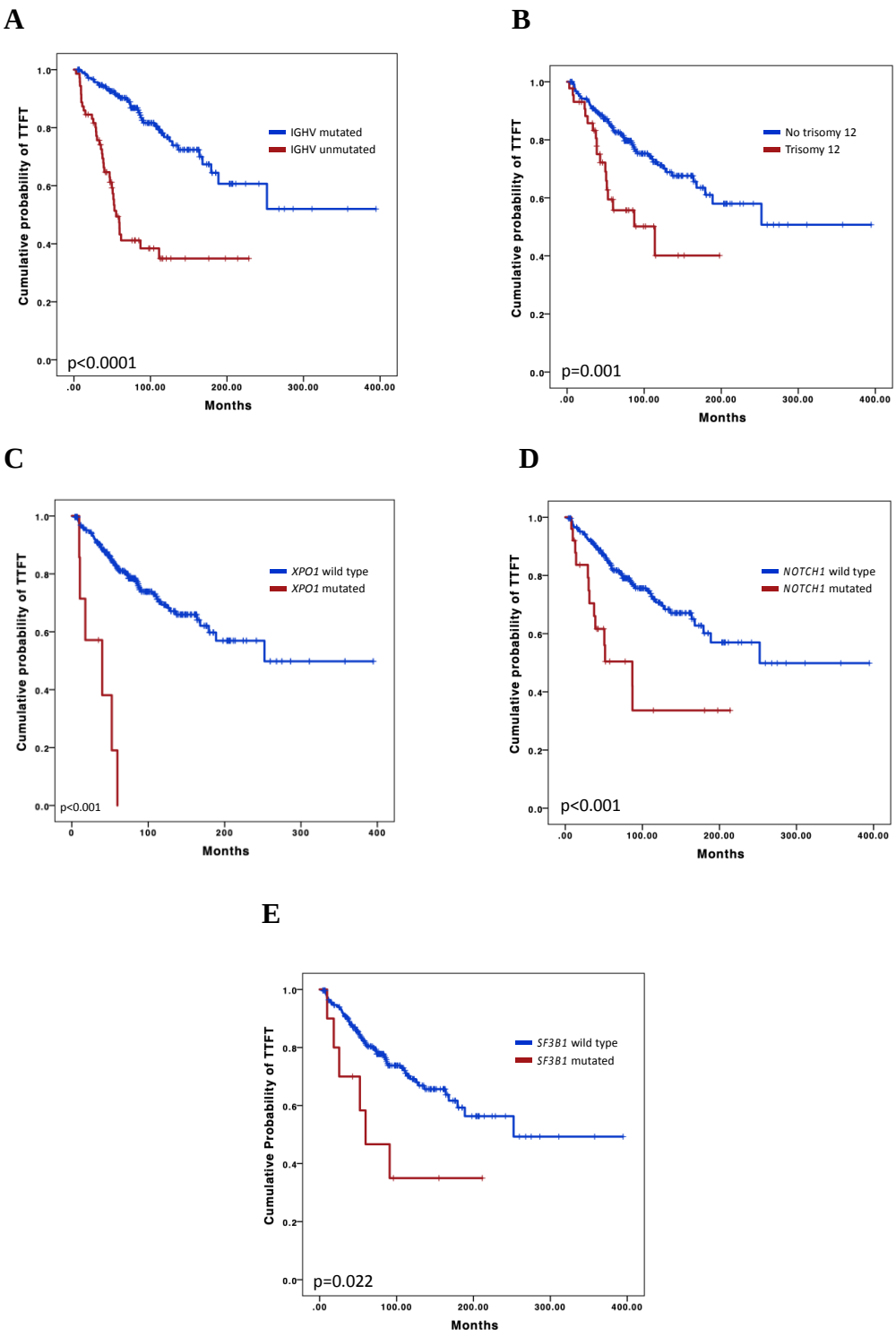

Figure 3. Biological features associated with shorter TTFT. Kaplan-Meier estimates of TTFT of IGHV mutational status (A), Trisomy 12 (B), *XPO1* mutations (C), *NOTCH1* mutations (D) and *SF3B1* mutations (E). Cases harboring unmutated IGHV, Trisomy 12 and *XPO1*, *NOTCH1* and *SF3B1* mutations are represented by the red line. Mutated IGHV patients and wild type patients are represented by the blue line. The Log-rank statistics p values are indicated adjacent curves.

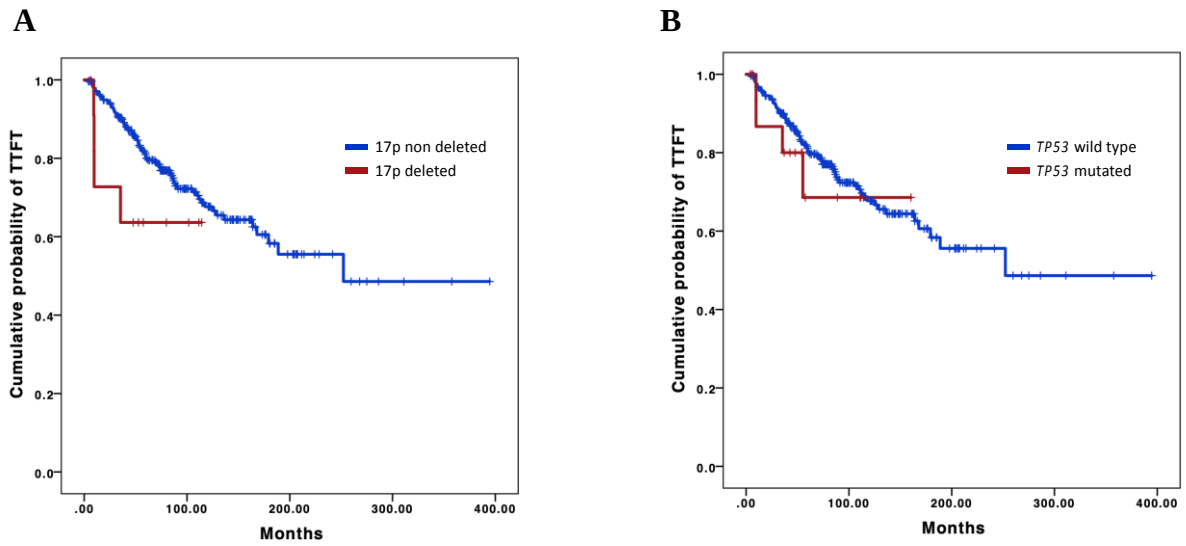

Figure 4. Clinical impact of 17p deletion and *TP53* mutations in terms of TTFT. Kaplan-Meier estimates of 17p deletions (A) and *TP53* mutations (B). Cases harboring 17p deletion and *TP53* mutations are represented by the red line. Wild type patients are represented by the blue line.

4.4 Validation of *XPO1* mutations as a predictor of shorter TTFT

In order to validate the value of *XPO1* mutations as a molecular predictor of shorter TTFT in early stage CLL patients, we collected and analyze two multicenter cohorts of early stage CLL patients composed of 402 Binet A and of 395 Rai 0 CLL patients, respectively. In the Binet A CLL cohort, *XPO1* was mutated in 15 (3.7%) patients and significantly associated with a shorter TTFT with a median TTFT of 60.4 months compared to 138.6 months in wild type cases (Fig. 5A). Similar results were observed in the Rai 0 cohort in which *XPO1* was mutated in 8 (2.0%) patients and significantly associated with a shorter TTFT with a median TTFT of 40.0 months compared to 169.0 months for wild type patients (Fig. 5B).

By combining the training and the validation cohorts (N=1092 patients), patients carrying either *XPO1* E571 (N=27) or D624 (N=3) mutations showed superimposable outcome in terms of TTFT (p=0.345) (Fig. 6).

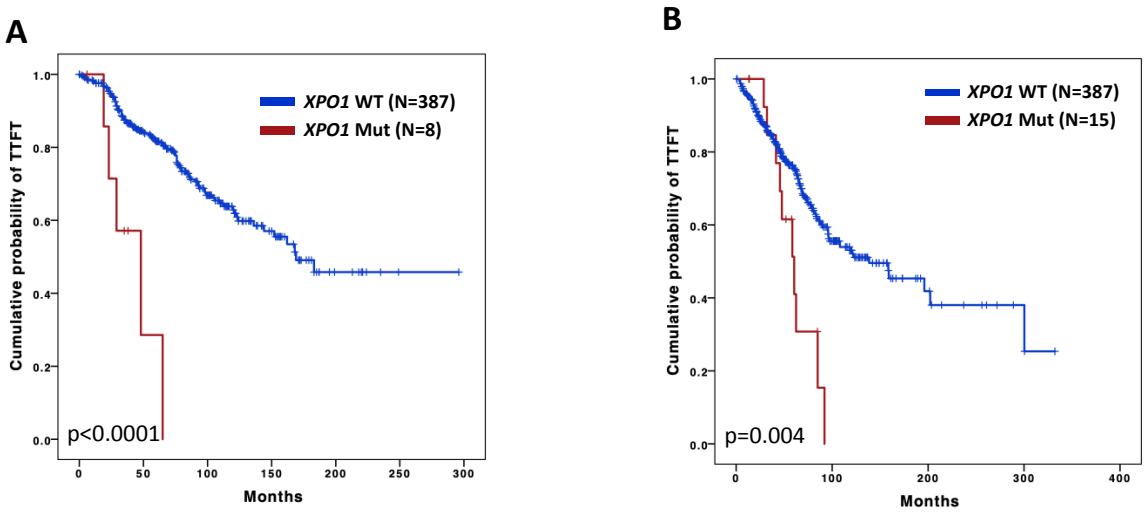

Figure 5. Clinical impact of *XPO1* mutations in terms of TTFT in the validation cohorts. Kaplan-Meier estimates of TTFT in the 1st validation cohort of 402 Binet A CLL patients (A), and in the 2nd validation cohort of 395 Rai 0 CLL patients (B). Cases harboring *XPO1* mutations are represented by the red line and wild type cases are represented by the blue line. The Log-rank statistics p values are indicated adjacent curves.

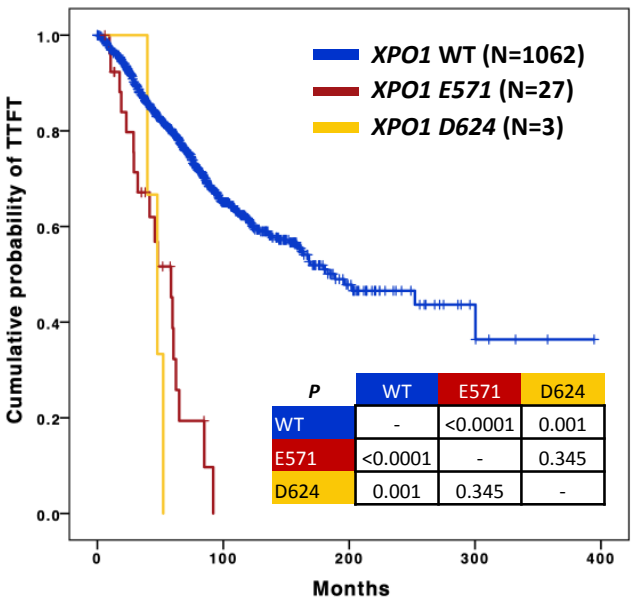

Figure 6. Clinical impact of different *XPO1* mutations in terms of TTFT. Kaplan-Meier estimates of TTFT according *XPO1* mutation type combining the training and the validation cohorts. Patients with *XPO1* mutation in codon E571 are represented by the red line, in codon D624 by the yellow line and wild type cases by the blue line. The Log-rank statistics p values are indicated adjacent curves.

5 DISCUSSION

Through a NGS approach, we performed a targeted resequencing of the 10 most recurrently mutated genes in CLL (Puente *et al.,* 2015; Landau *et al.,* 2015) to identify new molecular markers that may predict early treatment requirement and may help clinicians to better plan the watch & wait strategy in asymptomatic early stage CLL patients.

In a cohort of 295 CLL patients, mutations of the *XPO1* gene, encoding for exportin 1 which mediates the nuclear export of proteins and RNA, sorted out as an independent predictor of shorter TTFT. These initial results were validated in two independent cohorts of early stage CLL patients. Both in the Binet A CLL cohort as well as in the Rai 0 validation cohort, *XPO1* mutations significantly associated with a shorter TTFT. Moreover, since most of mutations, approximately 90%, affect the glutamic acid in position 571, polymerase chain reaction based methods may be used to identify *XPO1* mutation in a simple and time effective manner.

Proper nucleocytoplasmic partitioning of proteins is essential to cellular homeostasis. Exportin-1 (XPO1) is one of the most important human protein devoted to this role (Azmi *et al.,* 2020). Different proteins that are involved in different biological processes are exported by XPO1, including tumor suppressor proteins such as p53 and proteins that inhibit the NF-κB pathway, essential for CLL survival and proliferation (Taylor *et al.,* 2020; Xu *et al.*. 2012). Furthermore, *XPO1* dysregulation is associated with resistance to several standard-of-care therapies, including chemotherapies and targeted therapies, making it an attractive target of novel cancer therapies. Molecular consequences of *XPO1* mutations in CLL are still lacking but *XPO1* mutations alter the NF-κB and the *TP53* pathways that are essential in CLL pathogenesis and treatment resistance and further biological studies may decipher and identify novel biological and therapeutic implications.

Approximately 70% of newly diagnosed CLL patients presents in an early stage according to the Binet and Rai staging systems, may never require treatment, and may have a

life expectancy similar to the general population (Halek *et al.,* 2018; Binet *et al.,* 1981; Rai *et al.,* 1975). Despite the indolent behavior of CLL in the majority of cases, some patients have a CLL clone with a high proliferation rate that may lead to early treatment requirement due to progressive lymphocytosis, enlarged lymph nodes, cytopenia, and systemic symptoms (Halek *et al.,* 2018). Asymptomatic early stage CLL patients, are managed with a watch and wait strategy, and treatment is started only in case of symptomatic disease (Halek *et al.,* 2018). Until now, few clinical and molecular predictors inform on the risk of treatment requirement and the impact of CLL gene mutations is not completely understood (Shuai *et al.,* 2019; Hu *et al.,* 2019).

Recent studies have focused on the identification of clinical and molecular features that might identify early stage CLL who are at risk of early progression at the time of diagnosis. Such patients, if identified a priori, might benefit from clinical trials comparing early intervention *versus* observation. In this context, the combination of simple clinical features and molecular biomarkers, namely lymphocyte count >15,000/μl, palpable lymph nodes, and unmutated IGHV genes, identifies three different subgroups of Binet A and treatment naïve CLL patients with a high risk of early treatment requirement (Condoluci *et al.,* 2020). This risk model, termed IPS-E , has been validated in several independent series and is a robust tool to inform at the time of diagnosis about the probability that a given CLL patient in early stage disease progresses and needs treatment (Condoluci *et al.,* 2020). Similarly, a second model devised for Rai 0 CLL patients identified 17p deletion, 11q deletion, trisomy 12, unmutated and white blood cell count above 32,000/μL as variable associated with shorter TTFT (Cohen *et al.,* 2020). Since *XPO1* mutations significantly associate with a significantly shorter TTFT in both Binet A and Rai 0 CLL patients, the integration of these mutations into the IPS-E and in the Rai 0 prognostic model might improve the prediction of TTFT in early stage CLL patients.

In the present study, we have arbitrarily chosen to keep a threshold of variant allele frequency (VAF) above 5%. This decision, on one hand, helped us to protect against sequencing error or against the detection of minor subclones with little biological and clinical relevance. Conversely, on the other hand, our strategy did not allow us to evaluate the potential clinical impact of mutations with a VAF less than 5%.

In conclusion, *XPO1* mutations are an independent predictor of shorter TTFT and might help clinicians in a better management of the watch & wait strategy for Binet A CLL patients. In addition, functional studies are needed to identify the biological consequences underlying the supposed higher proliferation rate of *XPO1* mutated cells.

REFERENCES

Arnaoutov A, Dasso M. Ran-GTP regulates kinetochore attachment in somatic cells. *Cell Cycle*. 2006; 4:1161-1165.

Azmi AS, Uddin MH, Mohammad RM. The nuclear export protein XPO1 - from biology to targeted therapy. *Nat Rev Clin Oncol*. 2020. [Epub ahead of print]

Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, Vaugier G, Potron G, Colona P, Oberling F, Thomas M, Tchernia G, Jacquillat C, Boivin P, Lesty C, Duault MT, Monconduit M, Belabbes S, Gremy F. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. *Cancer.* 1981; 48(1):198-206.

Camus V, Miloudi H, Taly A, Sol B, Jardin F. XPO1 in B cell hematological malignancies: from recurrent somatic mutations to targeted therapy. *Journal of Hematology & Oncology* .2017; 10:47.

Camus V, Stamatoullas A, Mareschal S, Viailly PJ, Sarafan-Vasseur N, Bohers E, Dubois S, Picquenot JM, Ruminy P, Maingonnat C, Bertrand P, Cornic M, Tallon-Simon V, Becker S, Veresezan L, Frebourg T, Vera P, Bastard C, Tilly H, Jardin F. Detection and prognostic value of recurrent exportin 1 mutations in tumor and cell-free circulating DNA of patients with classical Hodgkin lymphoma. *Haematologica*. 2016; 101:1094–101.

Catovsky D, Fooks J, Richards S. Prognostic factors in chronic lymphocytic leukaemia: the importance of age, sex and response to treatment in survival. A report from the MRC CLL 1 trial. *British Journal Hematology*. 1989; 72(2):141-149.

Cohen JA, Rossi FM, Zucchetto A, Bomben R, Terzi-di-Bergamo L, Rabe KG, Degan M, Steffan A, Polesel J, Santinelli E, Innocenti I, Cutrona G, D'Arena G, Pozzato G, Zaja F, Chiarenza A, Rossi D, Di Raimondo F, Laurenti L, Gentile M, Morabito F, Neri A, Ferrarini M, Fegan CD, Pepper CJ, Del Poeta G, Parikh SA, Kay NE, Gattei V. A laboratory-based scoring system predicts early treatment in Rai 0 chronic lymphocytic leukemia. *Haematologica*. 2020;105(6):1613-1620.

Condoluci A, Terzi di Bergamo L, Langerbeins P, Hoechstetter MA, Herling CD, De Paoli L, Delgado J, Rabe KG, Gentile M, Doubek M, Mauro FR, Chiodin G, Mattsson M, Bahlo J, Cutrona G, Kotaskova J, Deambrogi C, Ekström Smedby K, Spina V, Bruscaggin A, Wu W, Moia R, Bianchi E, Gerber B, Zucca E, Gillessen S, Ghielmini ME, Cavalli F, Stuessi G, Hess MA, Baumann TS, Neri A, Ferrarini M, Rosenquist R, Forconi F, Foa R, Pospisilova S, Morabito F, Stilgenbauer S, Döhner H, Parikh SA, Wierda WG, Montserrat E, Gaidano G, Hallek M, Rossi D. International Prognostic Score for Asymptomatic Early-stage Chronic Lymphocytic Leukemia. *Blood*. 2020; 135(21):1859-1869.

Dearden C, Wade R, Else M, Richards S, Milligan D, Hamblin T, Catovsky D; UK National Cancer Research Institute (NCRI); Haematological Oncology Clinical Studies Group; NCRI CLL Working Group. The prognostic significance of a positive direct antiglobulin test in chronic lymphocytic leukemia: a beneficial effect of the combination of fludarabine and cyclophosphamide on the incidence of hemolytic anemia. Blood. 2008 Feb 15;111(4):1820-1826.

Dighiero G, Maloum K, Desablens B, Cazin B, Navarro M, Leblay R, Leporrier M, Jaubert J, Lepeu G, Dreyfus B, Binet JL, Travade P. Chlorambucil in indolent chronic lymphocytic leukemia. French Cooperative Group on Chronic Lymphocytic Leukemia. *N Engl J Med*. 1998; 338(21):1506-1514.

Fischer K, Al-Sawaf O, Bahlo J, Fink AM, Tandon M, Dixon M, Robrecht S, Warburton S, Humphrey K, Samoylova O, Liberati AM, Pinilla-Ibarz J, Opat S, Sivcheva L, Le Dû K, Fogliatto LM, Niemann CU, Weinkove R, Robinson S, Kipps TJ, Boettcher S, Tausch E, Humerickhouse R, Eichhorst B, Wendtner CM, Langerak AW, Kreuzer KA, Ritgen M, Goede V, Stilgenbauer S, Mobasher M, Hallek M. Venetoclax and Obinutuzumab in Patients with CLL and Coexisting Conditions. *N Engl J Med*. 2019; 380:2225–2236

Forbes DJ, Travesa A, Nord MS, Bernis C. Nuclear transport factors: global regulation of mitosis. *Curr Opin Cell Biol*. 2015; 35:78–90

Gaidano G, Rossi D. The mutational landscape of chronic lymphocytic leukemia and its impact on prognosis and treatment. Hematology Am Soc Hematol Educ Program. 2017 Dec 8;2017(1):329-337.
Gruber M, Bozic I, Leshchiner I, Livitz D, Stevenson K, Rassenti L, Rosebrock D, Taylor-Weiner A, Olive O, Goyetche R, Fernandes SM, Sun J, Stewart C, Wong A, Cibulskis C, Zhang W, Reiter JG, Gerold JM, Gribben JG, Rai KR, Keating MJ, Brown JR, Neuberg D, Kipps TJ, Nowak MA, Getz G, Wu CJ. Growth Dynamics in Naturally Progressing Chronic Lymphocytic Leukaemia. *Nature*. 2019; 570(7762):474-479.

Gruber M, Bozic I, Leshchiner I, Livitz D, Stevenson K, Rassenti L, Rosebrock D, Taylor-Weiner A, Olive O, Goyetche R, Fernandes SM, Sun J, Stewart C, Wong A, Cibulskis C, Zhang W, Reiter JG, Gerold JM, Gribben JG, Rai KR, Keating MJ, Brown JR, Neuberg D, Kipps TJ, Nowak MA, Getz G, Wu CJ. Growth dynamics in naturally progressing chronic lymphocytic leukaemia. *Nature*. 2019; 570: 474–479.

Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, Hillmen P, Keating M, Montserrat E, Chiorazzi N, Stilgenbauer S, Rai KR, Byrd JC, Eichhorst B, O'Brien S, Robak T, Seymour JF, Kipps TJ. iwCLL guidelines for diagnosis, indications for treatment, response assessment, and supportive management of CLL. *Blood*. 2018; 131(25):2745-2760.

Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, Hillmen P, Keating MJ, Montserrat E, Rai KR, Kipps TJ, Leukemia IWoCL. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. *Blood*. 2008; 111(12):5446-5456.

Hallek M, Cheson BD, Catovsky D, Caligaris-Cappio F, Dighiero G, Döhner H, Hillmen P, Keating MJ, Montserrat E, Rai KR, Kipps TJ; International Workshop on Chronic Lymphocytic Leukemia. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood. 2008 Jun 15;111(12):5446-56.

Hallek M, Shanafelt TD, Eichhorst B. *Chronic lymphocytic leukaemia. Lancet*. 2018; 391(10129):1524-1537.

Hallek M. Chronic lymphocytic leukemia: 2020 update on diagnosis, risk stratification and treatment.

Am J Hematol. 2019 Nov;94(11):1266-1287

Han T, Barcos M, Emrich L, Ozer H, Gajera R, Gomez GA, Reese PA, Minowada J, Bloom ML, Sadamori N. Bone marrow infiltration patterns and their prognostic significance in chronic lymphocytic leukemia: correlations with clinical, immunologic, phenotypic, and cytogenetic data. *J Clin Oncol*. 1984; 2(6):562-570.

Herling CD, Cymbalista F, Groß-Ophoff-Müller C, Bahlo J, Robrecht S, Langerbeins P, Fink AM, Al-Sawaf O, Busch R, Porcher R, Cazin B, Dreyfus B, Ibach S, Leprêtre S, Fischer K, Kaiser F, Eichhorst B, Wentner CM, Hoechstetter MA, Döhner H, Leblond V, Kneba M, Letestu R, Böttcher S, Stilgenbauer S, Hallek M, Levy V. Early treatment with FCR versus watch and wait in patients with stage Binet A high-risk chronic lymphocytic leukemia (CLL): A randomized phase 3 trial. *Leukemia*. 2020.

Hoechstetter MA, Busch R, Eichhorst B, Bühler A, Winkler D, Eckart MJ, Vehling-Kaiser U, Schimke H, Jäger U, Hurtz HJ, Hopfinger G, Hartmann F, Fuss H, Abenhardt W, Blau I, Freier W, Müller L, Goebeler M, Wendtner CM, Bahlo J, Fischer K, Bentz M, Emmerich B, Döhner H, Hallek M, Stilgenbauer S. Early, risk-adapted treatment with fludarabine in Binet stage A chronic lymphocytic leukemia patients: results of the CLL1 trial of the German CLL study group. *Leukemia*. 2017; 31(12):2833-2837.

Howlader N, Morton LM, Feuer EJ, Besson C, Engels EA. Contributions of Subtypes of Non-Hodgkin Lymphoma to Mortality Trends. *Cancer Epidemiol Biomarkers Prev*. 2016;25(1):174-179.

Hu B, Patel KP, Chen HC, Wang X, Luthra R, Routbort MJ, Kanagal-Shamanna R, Medeiros LJ, Yin CC, Zuo Z, Ok CY, Loghavi S, Tang G, Tambaro FP, Thompson P, Burger J, Jain N, Ferrajoli A, Bose P, Estrov Z, Keating M, Wierda WJ. Association of gene mutations with time-to-first treatment in 384 treatment-naive chronic lymphocytic leukaemia patients. *British Journal Hematology*. 2019; 187:307–318.

Huang WY, Yue L, Qiu WS, Wang LW, Zhou XH, Sun YJ. Prognostic value of CRM1 in pancreas cancer. *Clin Invest Med*. 2009; 32: E315–21

Jain P, Kanagal-Shamanna R, Wierda W, Keating M, Sarwari N, Rozovski U, Thompson P, Burger J, Kantarjian H, Patel KP, Medeiros LJ, Luthra R, Estrov Z. Clinical and molecular characteristics of XPO1 mutations in patients with chronic lymphocytic leukemia. *Am J Hematol*. 2016; 91: E478–9

Jardin F, Pujals A, Pelletier L, Bohers E, Camus V, Mareschal S, Dubois S, Sola B, Ochmann M, Lemonnier F, Viailly PJ, Bertrand P, Maingonnat C, Traverse-Glehen A, Gaulard P, Damotte D, Delarue R, Haioun C, Argueta C, Landesman Y, Salles G, Jais JP, Figeac M, Copie-Bergman C, Molina TJ, Picquenot JM, Cornic M, Fest T, Milpied N, Lemasle E, Stamatoullas A, Moeller P, Dyer MJ, Sundstrom C, Bastard C, Tilly H, Leroy K. Recurrent mutations of the exportin 1 gene (XPO1) and their impact on selective inhibitor of nuclear export compounds sensitivity in primary mediastinal B-cell lymphoma. *Am J Hematol*. 2016; 91:923–30

Jeromin S, Weissmann S, Haferlach C, Dicker F, Bayer K, Grossmann V, Alpermann T, Roller A, Kohlmann A, Haferlach T, Kern W, Schnittger S. SF3B1 mutations correlated to cytogenetics and

mutations in NOTCH1, FBXW7, MYD88, XPO1 and TP53 in 1160 untreated CLL patients. *Leukemia*. 2014; 28:108–17

Johansson P, Eisele L, Klein-Hitpass L, Sellmann L, Dührsen U, Dürig J, Nückel H. Percentage of smudge cells determined on routine blood smears is a novel prognostic factor in chronic lymphocytic leukemia. *Leuk Res*. 2010; 34(7):892-898.

Kojima K, Kornblau SM, Ruvolo V, Dilip A, Duvvuri S, Davis RE, Zhang M, Wang Z, Coombes KR, Zhang N, Qiu YH, Burks JK, Kantarjian H, Shacham S, Kauffman M, Andreeff M. Prognostic impact and targeting of CRM1 in acute myeloid leukemia. *Blood*. 2013; 121:4166–74.

Landau DA, Tausch E, Taylor-Weiner AN, Stewart C, Reiter JG, Bahlo J, Kluth S, Bozic I, Lawrence M, Bottcher S, Carter SL, Cibulskis K, Mertens D, Sougnez CL, Rosenberg M, Hess JM, Edelmann J, Kless S, Kneba M, Ritgen M, Fink A, Fischer K, Gabriel S, Lander ES, Nowak MA, Dohner H, Hallek M, Neuberg D, Getz G, Stilgenbaur S, Wu CJ. Mutations driving CLL and their evolution in progression and relapse. *Nature*. 2015; 526: 525–530.

Langerbeins P, Bahlo J, Rhein C. Ibrutinib versus placebo in patients with asymptomatic, treatment-naïve ealry stage Chronic Lymphocytic Leukemia (CLL): primary endpoint results of the phase 3 double-blind randomized CLL12 trial. *EHA* .2019; LB2602.

Lapalombella R, Sun Q, Williams K, Tangeman L, Jha S, Zhong Y, Goettl V, Mahoney E, Berglund C, Gupta S, Farmer A, Mani R, Johnson AJ, Lucas D, Mo X, Daelemans D, Sandanayaka V, Shechter S, McCauley D, Shacham S, Kauffman M, Chook YM , Byrd JC. Selective inhibitors of nuclear export show that CRM1/XPO1 is a target in chronic lymphocytic leukemia. *Blood.* 2012; 120:4621–4634.

Melo JV, Catovsky D, Gregory WM, Galton DA. The relationship between chronic lymphocytic leukaemia and prolymphocytic leukaemia. IV. Analysis of survival and prognostic features. *Br J Haematol*. 1987; 65(1):23-29.

Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*. 1988;16(3):1215.

Moia R, Favini C, Sagiraju S, Spina V, Bruscaggin A, Rasi S, De Paoli L, Deambrogi C, Scipiani M, Kodipad A A, Rossi D, Gaidano G. XPO1 Mutations May Identify Binet a Chronic Lymphocytic Leukemia Patients with Shorter Time to First Treatment. *Blood* .2019; 134:1743.

Molica S, Alberti A. Prognostic value of the lymphocyte doubling time in chronic lymphocytic leukemia. *Cancer*. 1987; 60(11):2712-2716.

Okamura M, Inose H, Masuda S. RNA export through the NPC in eukaryotes. *Genes.* 2015; 6:124–49 progression and relapse. Nature. 2015 Oct 22;526(7574):525-530

Puente XS, Bea S, Valdes-Mas R, Villamor N, Gutierrez-Abril J, Martin-Subero JI, Munar M, Rubio-Perez C, Jares P, Aymerich M, Baumann T, Beekman R, Belver L, Carrio A, Castellano G, Clot G, Colado E, Colomer D, Costa D, Delgado J, Enjuanes A, Estivill X, Ferrando AA, Gelpi JL, Gonzalez B, Gonzalez S, Gonzalez M, Gut M, Hernandez-Rivas JM, Lopez-Guerra M, Martin-Garcia D, Navarro A, Nicolas P, Orozco M, Payer AR, Pinyol M, Pisano DG, Puente DA, Queiros AC, Quesada V, Romeo-Casabona CM, Royo C, Royo R, Rozman M, Russinol N, Salaverria I, Stamatopoulos K, Stunnenberg HG, Tamborero D, Terol MJ, Valencia A, Lopez-Bigas N, Torrents D, Gut I, Lopez-Guillermo A,

Lopez-Otin C, Campo E. Non-coding recurrent mutations in chronic lymphocytic leukaemia. *Nature*. 2015; 526:519–524.

Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood*. 1975; 46(2):219-34.

Raponi S, Del Giudice I, Marinelli M, Wang J, Cafforio L, Ilari C, Piciocchi A, Messina M, Bonina S, Tavolaro S, Bordyuh M, Mariglia P, Peragine N, Mauro FR, Chiaretti S, Molica S, Gentile M, Visentin A, Trentin L, Rigolin GM, Cuneo A, Diop F, Rossi D, Gaidano G, Guarini A, Rabadan R, Foà R. Genetic landscape of ultra-stable chronic lymphocytic leukemia patients. Ann Oncol. 2018 Apr 1;29(4):966-972.

Rossi D, Terzi-di-Bergamo L, De Paoli L, Cerri M, Ghilardi G, Chiarenza A, Bulian P, Visco C, Mauro FR, Morabito F, Cortelezzi A, Zaja F, Forconi F, Laurenti L, Del Giudice I, Gentile M, Vincelli I, Motta M, Coscia M, Rigolin GM, Tedeschi A, Neri A, Marasca R, Perbellini O, Moreno C, Del Poeta G, Massaia M, Zinzani PL, Montillo M, Cuneo A, Gattei V, Foà R, Gaidano G. Molecular prediction of durable remission after first-line fludarabine-cyclophosphamide-rituximab in chronic lymphocytic leukemia. Blood. 2015 Oct 15;126(16):1921-1924.

Shuai S, Suzuki H, Diaz-Navarro A, Nadeu F, Kumar SA, Gutierrez-Fernandez A, Delgado J, Pinyol M, López-Otín C, Puente XS, Taylor MD, Campo E, Stein LD. The U1 spliceosomal RNA is recurrently mutated in multiple cancers. *Nature.* 2019; 574: 712–716.

Taylor J, Sendino M, Gorelick AN, et al. Altered Nuclear Export Signal Recognition as a Driver of Oncogenesis. *Cancer Discov*. 2019;9(10):1452-1467.

Xu D, Grishin NV, Chook YM. NESdb: a database of NES-containing CRM1 cargoes. *Mol Biol Cell*. 2012;23(18):3673-3676.

Zhang K, Wang M, Tamayo AT, Shacham S, Kauffman M, Lee J, Zhang L, Ou Z, Li C, Sun L, Ford RJ, Pham LV. Novel selective inhibitors of nuclear export CRM1 antagonists for therapy in mantle cell lymphoma. *Exp Hematol*. 2013; 41:67–78.