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CORSO DI DOTTORATO DI RICERCA IN: ONCOLOGIA E ONCOLOGIA CHIRURGICA
Ciclo di dottorato XXIX

Unlocking new molecular mechanisms and potential therapeutic targets for chronic lymphocytic leukemia

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GENERAL INTRODUCTION

1. Chronic Lymphocytic Leukemia

Chronic lymphocytic leukemia (CLL) represents the most common leukemia in the Western world. It has an age-adjusted incidence rate of 4.5 per 100,000 inhabitants in the United States and a median age at diagnosis of ~70 years (***Fabbri and Dalla-Favera, 2016***). CLL is a malignancy of B-lymphocytes that accumulate in the blood, bone marrow and other lymphoid tissues, and is diagnosed in the presence of ≥5,000 clonal B-lymphocytes per microlitre of peripheral blood persisting for more than 3 months. It is characterized by a neoplastic clonal expansion of CD5+ morphological mature B-lymphocytes (***Zenz et al, 2010***). CLL patients has variable clinical course with overall survival times ranging from months to decades. Some patients have no or minimal signs and symptoms during their entire disease course and have a survival time similar to age-matched controls. Other patients experience rapidly deteriorating blood counts and suffer from symptoms at diagnosis or soon thereafter necessitating therapy. Despite recent major advances in our knowledge of the pathogenesis and in the management of CLL, relapse ultimately occurs in almost all patients who require therapy and the disease remains incurable (***Hallek and Pflug, 2010***).

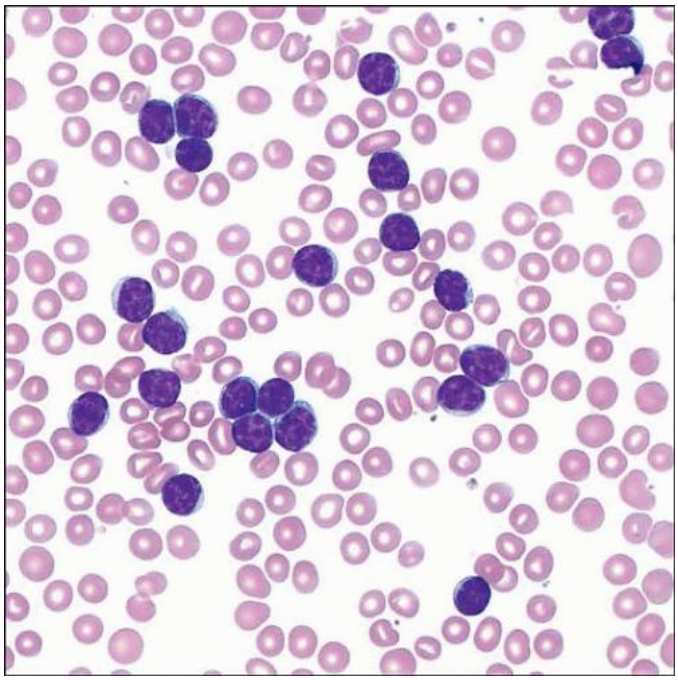


Figure 1. *CLL in the peripheral blood. Marked lymphocytosis is seen; lymphocytes have scant cytoplasm, clumped "soccer ball" chromatin, round nuclei, and indistinct nucleoli.*

2. Prognosis

CLL patients can be very heterogeneous in terms of clinical outcome with some patients showing a life expectancy similar to unaffected individuals with no or delayed need for therapies, while others experience a more aggressive form of the disease with a shorter survival time and early treatment requirements (***Zenz et al, 2010***). A third population of patients has a disease course and a survival expectancy between these two extremes.

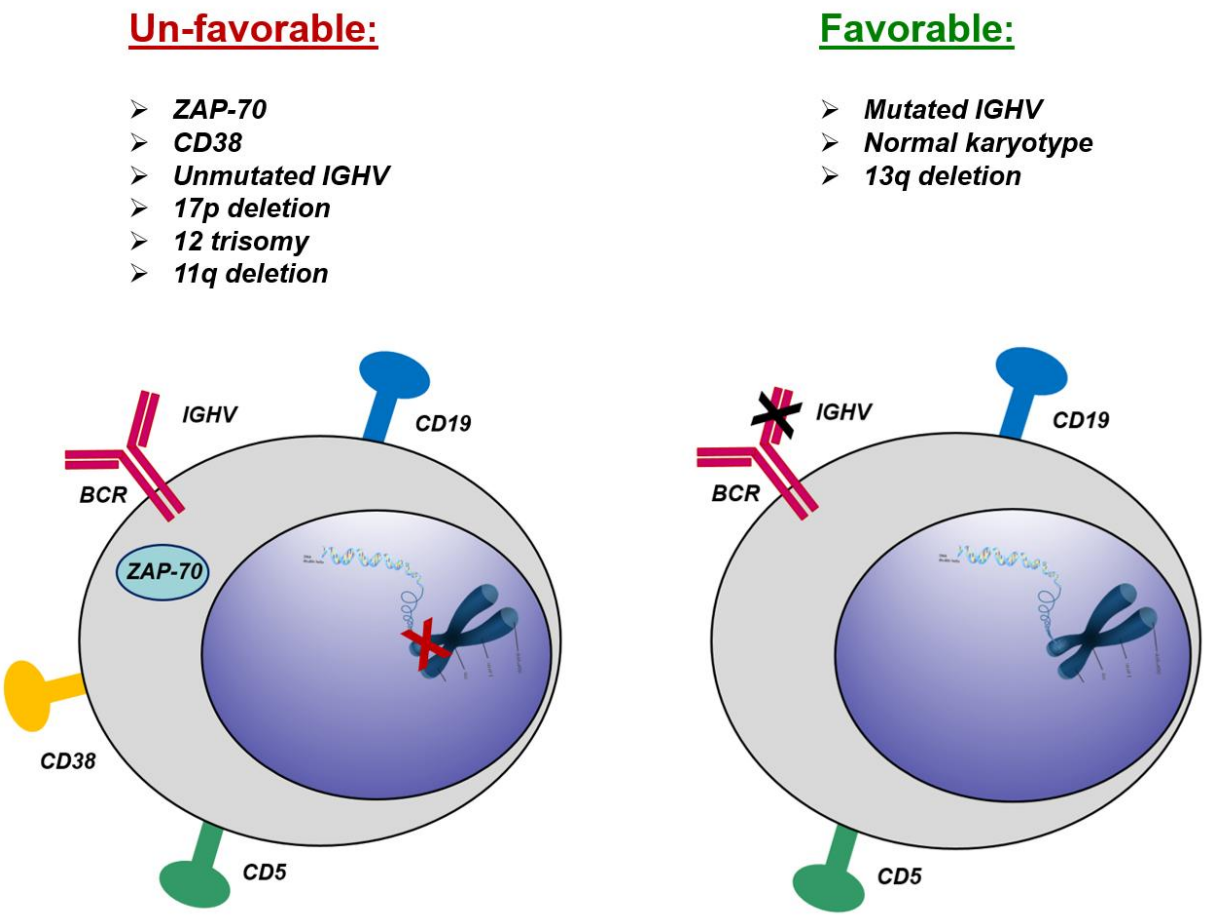


Figure 2. *An illustration of the most important genetic and protein based prognostic markers in relation to the clinical outcome of CLL.*

The standard clinical procedures to estimate prognosis are the clinical staging systems developed by Rai and Binet (***Rai et al, 1975; Binet et al, 1981***). These systems define early (Rai 0, Binet A), intermediate (Rai I/II, Binet B) and advanced (Rai III/IV, Binet C) stage disease with median estimated survival times of > 10, 5–7, and 1–3 years, respectively. However, there is heterogeneity in the course of the disease among individual patients within a single stage group. Several important observations related to the biologic significance of immunoglobulin mutational status, ZAP-70 overexpression, chromosomal

aberrations and CD38 expression have led to the ability to identify patients at high risk for early disease progression (***Kröber et al, 2002; Damle et al, 1999; Hamblin et al, 1999; Döhner et al, 2000***).

The presence or the absence of somatic mutations of the Immunoglobulin Heavy Variable (IGHV) genes in CLL cells correlates with different clinical courses and outcomes (***Hamblin et al, 1999; Damle et al, 1999***). A ≥98% identity of the expressed IGHV gene with the corresponding germline sequence (unmutated CLL) is associated with a more aggressive disease and a shorter median survival time as compared to mutated cases.

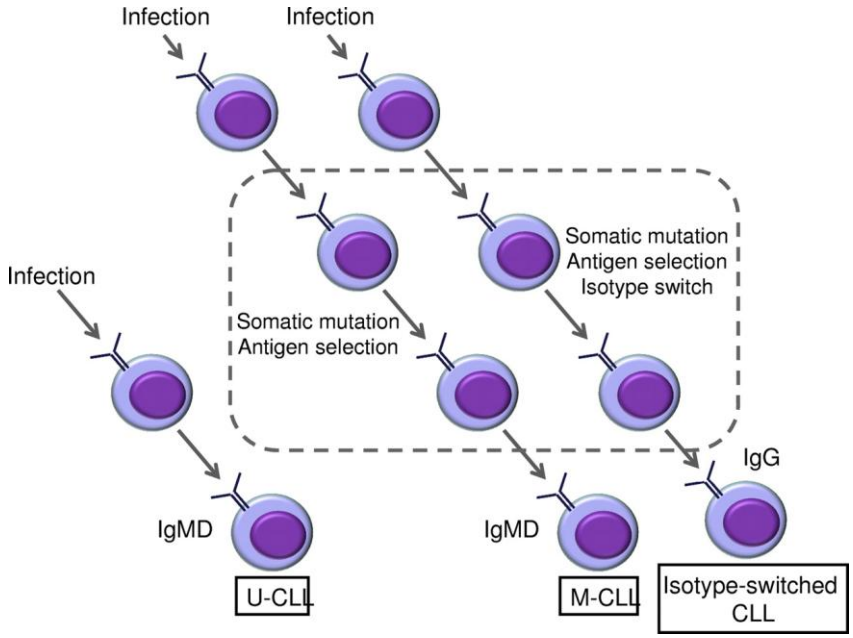


Figure 3. *Pathogenesis of CLL subsets. The two major subsets (unmutated U-CLL and mutated M-CLL) and the isotype-switched variant derive from three different normal B-cell populations with no interconversion (**Stevenson et al, 2011**).*

CD38 expression on the cell surface of CLL cells, is also associated with a more aggressive disease. CD38 positive CLL cases have a shorter time to progression and shorter survival (***Damle et al, 1999***). Gene expression profiling revealed that ZAP70 mRNA is expressed in some CLL cases (***Scielzo et al, 2006***). ZAP70 expression in >20% of the leukemic cells carries an adverse prognostic value (***Crespo et al, 2003***). Initially, ZAP70 was reported as a reliable surrogate for IGHV gene mutation status, as expression of ZAP70 appeared to correlate with U-CLL cases, but later it became apparent that its prognostic value is independent of the mutational status.

3. Chromosomal abnormalities.

CLL cells carry several recurrent genomic alterations, detectable by Fluorescent In Situ Hybridization (FISH). In contrast to other lymphoproliferative disorders, recurrent translocations are not present, while the most typical aberrations lead to loss or gain of genetic material. Some aberrations appear during the course of the disease whereas others can be present very early in the natural history if the disease. Overall, different alterations can be found in up to 80% of CLL cases. More than 50% of the cases show only a single abnormality, 20% carry two and about 10% more than two aberrations (***Dohner et al, 2000***).

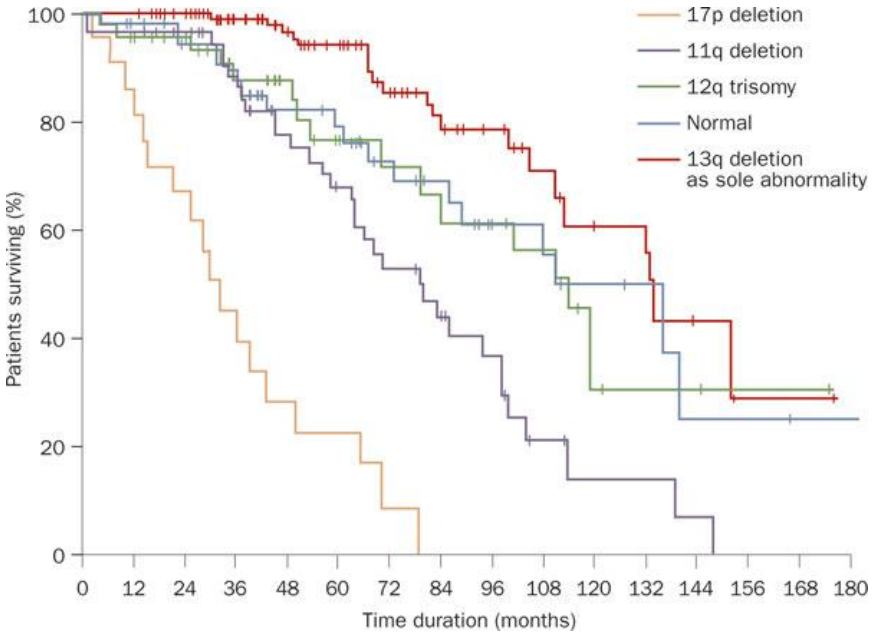


Figure 4. *Patients with certain chromosomal aberrations such as del(17p) or del(11q) have an inferior prognosis compared with patients with trisomy 12, normal karyotype, or del(13q) (**Döhner et al, 2000**).*

Del13q14 is the most frequent alteration (***Cimmino et al, 2005***) and it associates with a favorable clinical course of the disease. On the contrary, the most deleterious aberrations are deletions at 17p (leading to the loss of TP53 gene) and deletions at 11q (associated with reduced ATM expression) correlating with short median survival. Trisomy of chromosome 12 does not significantly affect survival as compared to normal karyotype (***Malek et al, 2013***).

4. 13q14 Deletion

Deletion of 13q14 region, found in more than 50% of CLL patients, is the most common cytogenetic abnormality and has been associated with good prognosis. Several studies have

revealed some insights in the candidate genes located at 13q that could be responsible for CLL pathogenesis, as well as in the prognostic heterogeneity of 13q-deleted patients. With regard to biologic basis underlying 13q deletions, miR-15a and miR16-1, located in the minimal deleted region (MDR), have been described to exhibit a tumoral suppressor function in CLL (***Klein et al, 2010***). Besides these microRNAs, other genes located in 13q, such as DLEU7, could cooperate in the tumoral suppressor activity. In addition, it has been extensively demonstrated that large 13q losses involving RB1 gene are related to shorter overall survival than those small deletions encompassing only miR-15a and miR16-1 (***Calin et al, 2002***).

In contrast to other recurrent abnormalities in CLL, the presence of biallelic losses in 13q has been described in nearly 30% of 13q-deleted CLL patients (***Reddy et al, 2006***). The size of the abnormal clone detected by FISH is variable in CLL patients. It has been described that patients with a higher percentage of altered nuclei have a significantly shorter overall survival. The optimal cut-off point that defines a poorer outcome of 13q deletion differs between 65.5% to 90% (***Hernandez et al, 2009; Dal Bo et al, 2011; Orlandi et al, 2013***). Thus, CLL patients with isolated 13q deletion can be risk-stratified according to the percentage of altered cells by FISH.

Interestingly, deregulation of many relevant cellular pathways has also been shown in those patients with higher percentages of 13q deletion. Among them are remarkably the deregulation of several important miRNAs and overexpression of genes mainly involved in B-cell receptor signaling (e.g., SYK and CD79b), NFκB signaling and prosurvival and antiapoptotic pathways (e.g., Wnt and RAS signaling (***Rodriguez et al, 2012***).

CHAPTER ONE

Role of miR-15a/miR-16-1 and TP53 axis in regulation of telomerase in CLL

1. INTRODUCTION

Telomere and telomerase

Human telomeres are DNA-protein structures consisting of G-rich repeats (TTAGGG), which protects the end of the chromosome from deterioration or from fusion with neighboring chromosomes (***Bryan and Englezou, 1995***). Telomeres are shortened by approximately 50-100 base pairs per cell division in humans (***Allsopp and Vaziri, 1992***). Defects in telomere length have been implicated in the pathology of several age related diseases and premature ageing syndromes, as well as in cancer (***Blasco, 2005***). Human cancer cells have been shown to maintain average telomere length over time (***Englezou et al. 1998***). Human cancer cells appear to have extremely short class of telomeres (***Xu and Blackburn 2007***).

Telomerase is the enzyme responsible for the maintenance of telomeres. Human telomerase is a ribonucleoprotein, consisting of both a protein catalytic subunit, the telomerase reverse transcriptase (hTERT), and an RNA component (hTR) (***Lingner et al, 1997***). Telomerase is able to synthesize new telomeric repeats onto the 3'-end leading stand after DNA replication, allowing for the conventional DNA replication machinery to elongate the 5'-end lagging strand, using the newly extended 3'-end leading strand as a template. Of the two minimal catalytic components of the telomerase hTERT is the limiting component for telomerase activity (***Feng et al, 1995***) and telomerase activity closely correlates with hTERT expression (***Cong et al, 1999***).

Telomerase expression has been found to be up-regulated in approximately 90% of human cancers, which enables the continued and uncontrolled proliferation of the malignant cells that drive tumor growth and progression (***Masutomi et al, 2005***). Reactivation of telomerase in mortal cells stabilizes their telomeres and provides the cell with unlimited proliferative potential (***Vaziri and Benchimol 1998***). The role of telomerase in tumorigenesis is thought to involve the stabilization of critically short telomeres, which

occurs after normal cells have experienced genetic instability and survived an escape from growth arrest similar to crisis. One characteristic of cancer cells is the acquisition of limitless replicative potential (***Hanahan and Weinberg 2000***) and telomerase activity is ultimately required for tumor immortalization (***Huang et al. 2004***).

Telomere and telomerase in CLL

Several studies have focused on the potential prognostic significance of telomere and telomerase interplay in CLL. In hematological malignancies, the shortening of telomere length is accelerated under the increased proliferation pressure and maintained at a short length (***Aalbers et al, 2013***). Significantly shortened telomeres, activation of telomerase and modulation of the expression of telomere-associated proteins are correlated with faster disease progression, reduced response to chemotherapy, and poor prognosis (***Wang et al, 2010***). In CLL patients with high-risk genomic aberrations, (11q or 17p deletions) telomeres are extremely shortened, independently of the stage of disease (***Britt-Compton et al, 2012***). Short telomere length in CLL patients is in line with other classical biological factors of CLL, including unmutated immunoglobulin, CD38 and ZAP-70 positivity (***Roos et al, 2008***). CLL patients with shorter telomere length experienced worse clinical outcomes including shorter progression-free survival and overall survival. Thus telomere length was indicated as a negative prognostic factor in CLL **(*Counter et al, 1995; Sellmann et al 2011***). The expression levels of telomere binding proteins (TRF1, RAP1 and POT1) were reported to be altered in CLL cells (***Poncet et al, 2008***). Increased expression of hTERT and activation of telomerase are universally involved in oncogenesis and the progression of hematopoietic malignancies.

Telomerase activity, hTERT levels and telomere length were all investigated in CLL patients. High telomerase activity is a predictor of a shorter survival in CLL (***Bechter, et al 1998***). It has been also reported that, poor prognosis Ig-unmutated CLL subgroup has higher telomerase activity, as compared with an Ig-mutated subgroup (***Damle et al, 2004***). Moreover, CLL patients expressing high hTERT levels have significantly shorter survival than hTERT negative CLL patients, regardless of disease stage (***Tchirkov et al, 2004***).

We have previously shown that high levels of hTERT and short telomere length were associated with a poor clinical outcome of CLL patients (***Rampazzo et al, 2012***). The

poorest prognosis was found in the group with high hTERT levels and short telomeres, whereas CLL with low hTERT levels and long telomeres had a more indolent clinical behavior. We have also showed that most of the CLL cases with 13q14 deletions have lower levels of hTERT when compared to CLL patients with other chromosomal abnormalities (***Rampazzo et al, 2012***).

The tumor suppressor gene TP53 and telomerase interaction

The tumor suppressor gene TP53 is a sequence-specific transcription factor that can mediate many downstream effects such as growth arrest and apoptosis through activation or repression of its target genes. The TP53 gene is mutated in a number of hematologic malignancies but the frequency of mutations tends to be low in most lymphoid malignancies and in CLL (***Rossi et al, 2009***). TP53 mutations represent strong predictors of poor survival and refractoriness in CLL and they have a well-established clinical relevance and direct implications for the management of CLL (***Dohner et al, 1995***).

Telomerase enzymatic activity can be regulated at multiple levels, including alternative splicing, chaperone-mediated folding and nuclear translocation; however, the major control mechanism of telomerase regulation seems to be at the level of hTERT transcription (***Kyo and Inoue, 2002***). Several studies correlated the TP53 and telomerase activity during carcinogenesis. Telomerase and TP53 are frequently associated with human cancers, and activation of telomerase and inactivation of TP53 is involved in cancer cell immortalization. For example, overexpression of wild-type TP53 was shown to down-regulate telomerase enzymatic activity in a number of cancer cell lines independent of its effects on growth arrest and apoptosis (***Kamusoto et al, 1999***). This observation was attributed to transcriptional repression of hTERT by wild-type TP53 since it was preceded by down-regulation of hTERT mRNA (***Kamusoto et al, 1999***). This conclusion was supported by promoter activity studies demonstrating the ability of TP53 to repress hTERT promoter-reporter constructs. In an additional study, activation of exogenous temperature-sensitive TP53 in BL41 Burkitt's lymphoma cells triggered rapid down-regulation of hTERT mRNA expression independent of the induction of the TP53 target gene p21 (***Xu et al, 2000***).

MicroRNA-15a and microRNA-16-1 mediated regulation of TP53 in CLL

MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression (***Eulalio et al, 2008; Pillai et al, 2007***). MiRNAs represent one of the major regulatory family of genes in eukaryotic cells by inducing translational repression and transcript degradation (***Lim et al, 2005; Pillai et al 2007***). MiRNA profiles can distinguish normal B cells from malignant CLL cells and have been associated with prognosis, progression, and drug resistance in CLL patients (***Ferracin et al, 2010***). It has been shown that, a cluster of two microRNA genes, miR-15a and miR-16-1, was located within the 13q14.3 deleted region (***Calin et al, 2002***). Since 13q14 deletion is the most frequent genomic aberration in CLL, miR-15a/16-1 expression was found downregulated in ~66% of CLL cases (***Calin et al, 2002***). The importance of miR-15a/16-1 was confirmed also in mouse model of CLL. A point mutation causing a decrease in miR-16-1 expression in lymphoid tissues lead to elevated levels of antiapoptotic protein Bcl-2 (***Raveche et al, 2007***). MiR-15a/16-1 and BCL2 expression levels were found inversely correlated in CLL, and downregulation of these microRNAs in leukemic cell lines resulted in an increase of Bcl2 expression with consequent inhibition of apoptosis (***Cimmino et al, 2005***). Moreover, microarray experiments performed on CLL patients with high vs low levels of miR-15a/16-1 identified a gene signature which also contains MCL1, an antiapoptotic BCL-2 family member associated with B-CLL cell survival and chemotherapy resistance (***Calin et al, 2007***). More importantly, it has been also shown that, the tumor suppressor protein TP53 is directly regulated by miR-15a/miR-16-1 in CLL. The loss of miR-15a/miR-16-1 expression, represented by CLLs with 13q deletions, not only shifts the balance toward higher levels of the BCL2 and MCL 1, but also toward higher levels of the TP53 **(*Fabbri et al, 2011*)**. TP53 represented the molecular link between the miR-15a/miR-16-1 clusters. Consequently, in patients with CLLs with 13q deletions, while the number of apoptotic cells may decrease because of the increased levels of antiapoptotic proteins, the TP53 tumor suppressor pathway remains intact, thus keeping the increase in tumor burden relatively low.

2. AIMS

Several studies have focused on the potential prognostic significance of **telomerase** (TERT) in CLL. We previously showed that most of the CLL cases with **13q14 deletion** have lower levels of TERT than CLL with other chromosomal abnormalities. **miR-15a** and **miR-16-1**, targets of 13q14 deletion in CLL; may directly down-regulate tumor suppressor protein **TP53** which is known to be an important negative regulator of telomerase. Based on these evidences, we hypothesized that, in patients with del13q, low TERT levels may be a consequence of the loss of miR-15a and miR-16-1 that in turn regulate TP53 levels. Following this reasoning, during my PhD studies **we aimed** at **determining the miR-15a/mi-R16-1 levels in CLL patients with or without the 13q14 deletion and correlate them to both TP53 and TERT transcripts**.

3. MATHERIALS AND METHODS

Patients

Peripheral blood cells were collected from 155 CLL patients who attended the Haematology Section, Clinical and Experimental Medicine, University of Padova. This study included 99 CLL with the 13q14 deletion as the sole chromosomal abnormality detected by fluorescence in situ hybridization (FISH), and 56 CLL with no chromosomal abnormalities detected by FISH (i.e., 13q14.3, 17p13.1[TP53], and 11q22.3[ATM] deletions, and trisomy 12). CLL cases with TP53 mutations were excluded from this study. The median (interquartile) ages of patients with CLL with normal cytogenetcs profile and with the sole 13q14 deletion were 68 (60-75) and 70 (63-76) years, respectively (p=0.342). All samples were collected at the time of diagnosis, and all patients were untreated at the time of sampling. IGVH gene status was assessed as previously described (***Terrin et al, 2007***). The percent of IGVH unmutated cases did not statistically differ between the two groups (21% vs 23%; p=0.517). The median (interquartile) follow-up time from blood sampling was 55 (27-96) months. Time from diagnosis to first treatment (TTFT) was considered as a marker for time to disease progression. Informed consent was obtained according to the Helsinki declaration and the study was approved by the local Ethics Committees.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed on standard cytogenetic preparations from peripheral blood. The slides were hybridized with the multicolour probe sets LSI p53/LSI ATM and LSI D13S319/LSI 13q34/ CEP12 (Vysis-Abbott, Des Plaines, IL, USA) according to the manufacturer's protocol. Three hundred interphase nuclei were analyzed for each probe. The cut-off for positive values (mean of normal control ±3 standard deviation) determined from ten cytogenetically normal samples was 4% for centromere 12 trisomy (+12), and 10% for del 11q22.3(11q-), del13q14.3 (13q-) and del 17p13.1 (17p-). The B-CLL cases with 11q- or 17p- and 13q- (n=12) were included in the group of 11q-,17p-.

Primers, probes and standard curve construction for the quantification of hTERT transcripts

To quantify the mRNA of hTERT, set of AT primers were designed. The forward primer AT1 (5'-CGGAAGAGTGTCTGGAGCAA-3') and the reverse primer AT2 (5'-GGATGAAGCGGAGTCTGGA-3') bind to nucleotide sequences nt 1784-1803 and nt 1904-1926, respectively, (Genbank accession number: AF015950), located upstream of the RT motif 1 of the hTERT gene. Amplification with AT1 and AT2 primers allowed the detection of all hTERT transcripts. The fluorogenic probes AT (FAM 5'-TTGCAAAGCATTGGAATCAGACAGCAC-3' TAMRA), which recognize sequences located within the products amplified by AT1/AT2 primer pairs, were synthesized by PE Applied Biosystems. The standard reference curve to quantitate hTERT-AT transcripts was performed using serial 5-fold dilutions of the hTERT amplicon we constructed as follows: RNA from BL41 cells, strongly expressing the hTERT gene, was extracted and retrotranscribed as described above, and cDNA was amplified using primers F (5'-ATGTCACGGAGACCACGTTT-3') and R (5'-AGAGCAGCGTGGAGAGGAT-3') which bind to nucleotide sequences located upstream and downnstream, respectively, of regions recognized by AT1/AT2 primer pairs. Amplification was performed in a 50 µl mixture containing 1µl Taq Gold (5 U/µl; Applied Biosystems), 5 µl Buffer 10X (500 mM KCl, 100mM Tris-HCl, pH 8.3), 3µl $MgCl_2$ (25mM), 200µM dNTP, and 35 pmol of each F and R primer, and carried out for 35 cycles, each consisting of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at and 72°C, in a thermal cycler (9600 Perkin Elmer). The amplicon of 840 bp was run on an agarose gel, purified by using NucleoSpin Extract kit (M-Medical) according to the manufacturer's instructions, and quantified by spectrophotometer.

Quantification of TP53 transcripts

The standard reference curve for quantifying TP53 transcripts was performed using serial 5-fold dilutions of the TP53 amplicon, constructed as follow: one microgram of total RNA extracted from EHEB cell line, expressing wild type TP53, was retro-transcribed into cDNA using the SuperScript TM III RNase Reverse Trancriptase assay (Invitrogen) according to the manufacturer's instructions. The amplicon of 108 bp was run on an agarose gel, purified by using NucleoSpin Extract kit (M-Medical) according to the manufacturer's instructions,

and quantified by spectrophotometer. Then the cDNA (50ng/10uL) of both samples and reference curve, were amplified in real-time PCR with the same kit (TaqMan Gene Expression Assay - Human TP53: Hs01034249_m1 - LifeTechnologies). In particular, amplification of TP53 transcripts was performed in a 20 µl mixture containing 0,25 µl Taq Gold (5 U/µl; Applied Biosystems), 2,5 µl Buffer 10X, 1,5 µl MgCl2 (25mM), 2 uL dNTPs mix (10mM), and 3,5 uL of TaqMan Gene Expression Assay - Human TP53 kit (20X), and carried out for 40 cycles, each consisting of 30 sec at 95°C, 30 sec at 55°C, and 1 min at and 72°C, in a thermal cycler (9600 Perkin Elmer). To normalize TP53 transcripts for the amount of total RNA, 10 µL cDNA from each sample was amplified for the housekeeping gene GAPDH, then values of TP53 were normalized for 10^5 copies of GAPDH.

Evaluation of miRNAs Expression

Reverse transcription (RT)

cDNA for measuring the expression of miR-15a, miR-16-1 and the reference housekeeping gene small nuclear RNA-U6 (RNU6B) was obtained from 15 ng of total RNA available for 101 patients. For each miRNA a specific reverse transcription (RT) was performed according to the manufacturer's instructions using the TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and specific stem-loop RT primers contained in TaqMan® MicroRNA Assays (Thermo Fisher Scientific). Each specific RT reaction for each miRNAs contains 1,5 uL of 10X Buffer, 0,15 uL of dNTPs mix (100mM), 1 uL of Multiscribe RT enzime (50U/uL), 0,2 RNase Inhibitor (20U/uL), 7,65 uL of RNase free water, 0,5 uL of specific RT-primer and 3 uL (15ng) of RNA in a final volume of 20 uL. Each RT reaction was carried out in a thermal cycler (9600 Perkin Elmer) with the following conditions: 30 min at 16°C, 30 min at 42°C and 5 min at 85°C.

qRT-PCR

The qRT-PCR assay for miRNAs expression was performed in a total reaction volume of 20 µL and contained 7.67 µL of nuclease-free water, 10 µL of TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Life Technologies), 1 µL of a TaqMan® MicroRNA Assays probe specific for each microRNA (Life Technologies), and 1.33 µL of cDNA generated by reverse transcription. Amplifications were performed under the following

conditions: 95 °C for 10 min, 50 cycles 95 °C for 15 s and 60 °C for 1 min. The relative expression of miR-15a and miR-16-1 was determined by the 2-ΔCt method. Normalized miR-15a and miR-16-1 expression values for individual patients were calculated as follows: ΔCt miR-15a = Ct miR-15a – Ct RNU6B; ΔCt miR-16-1 = Ct miR-16-1 – Ct RNU6B and normalized expression values = 2-ΔCt. The final normalized miR-15a and miR-16-1 expression values for individual patients were expressed as the mean values calculated from three technical repeats.

Statistical analysis

The distribution of continuous variables, such as TERT and TP53 levels were compared by the Kruskal–Wallis nonparametric test, and the associations between categorical variables were analysed by the x2 test. Spearman's r coefficient (rs) was used for correlations. For each variable, TTFT analysis was performed using the Kaplan-Meier method and compared by the log-rank test. hTERT levels and telomere length were analyzed as dichotomous variables (cut-off: ≤median or <median). Hazard ratios for each category were estimated using univariate Cox proportional hazards models with low risk as the reference class. The independent role of TERT/TP53 level profile in predicting TTFT was tested using a Cox proporzional hazard model. All tests were two-sided, and a $p<0.05$ was considered statistically significant. Statistical analyses were carried out using SAS version 9.1 (SAS institute, Cary, NC, USA).

4. RESULTS

Relative miR-15a and miR-16-1 and TP53 mRNA levels in CLL with or without 13q14 deletion

Since it has been shown that CLL patients with 13q14 deletion lack miR-15a and miR-16-1, our first goal was to examine the relative levels of these miRNA's in our CLL cohort, which was consisting of 13q14 deleted (13q14del) and CLL with no any chromosomal abnormalities (no FISH abnormalities). We observed that levels of both miR-15a ($p=0.009$) and miR-16-1 ($p=0.001$) were significantly lower in 13q14del CLL than in CLL with no FISH abnormalities. The median relative levels of miR-15a and miR-16-1 in 13q14del CLL was 1.97 (ranged between 1.23 – 3.34) and 0.15 (ranged between 0.13-0.31), respectively (Figure 5A and 5B). Instead in CLL patients without chromosomal abnormalities had higher miR-15a and miR-16-1 median values, 2.86 (ranged between 1,90-4.66) and 0.25 (ranged between 0.17-0.37), respectively.

It has been shown that miR-15a and miR-16-1 over-expression in primary CLL cells with 13q14 deletion is associated with a decrease in TP53, both at mRNA and protein levels. To speculate that expression of TP53 is lower due to loss of miR-15a and miR-16-1 because of the deletion in chromosome 13q14, we quantified TP53 mRNA levels in CLL cases with or without 13q14 deletion.

TP53 mRNA levels were measured by RT-qPCR in 13q14del and in CLL without any FISH abnormalities. None of the analyzed samples had TP53 mutation or deletion. We found that TP53 levels were significantly higher in 13q14del CLL than in CLL with no chromosomal abnormalities ($p=0.012$), with median values of 782 [416-1186] vs 582 [304-815] copies/10^6 GAPDH copies, respectively (Figure 5C).

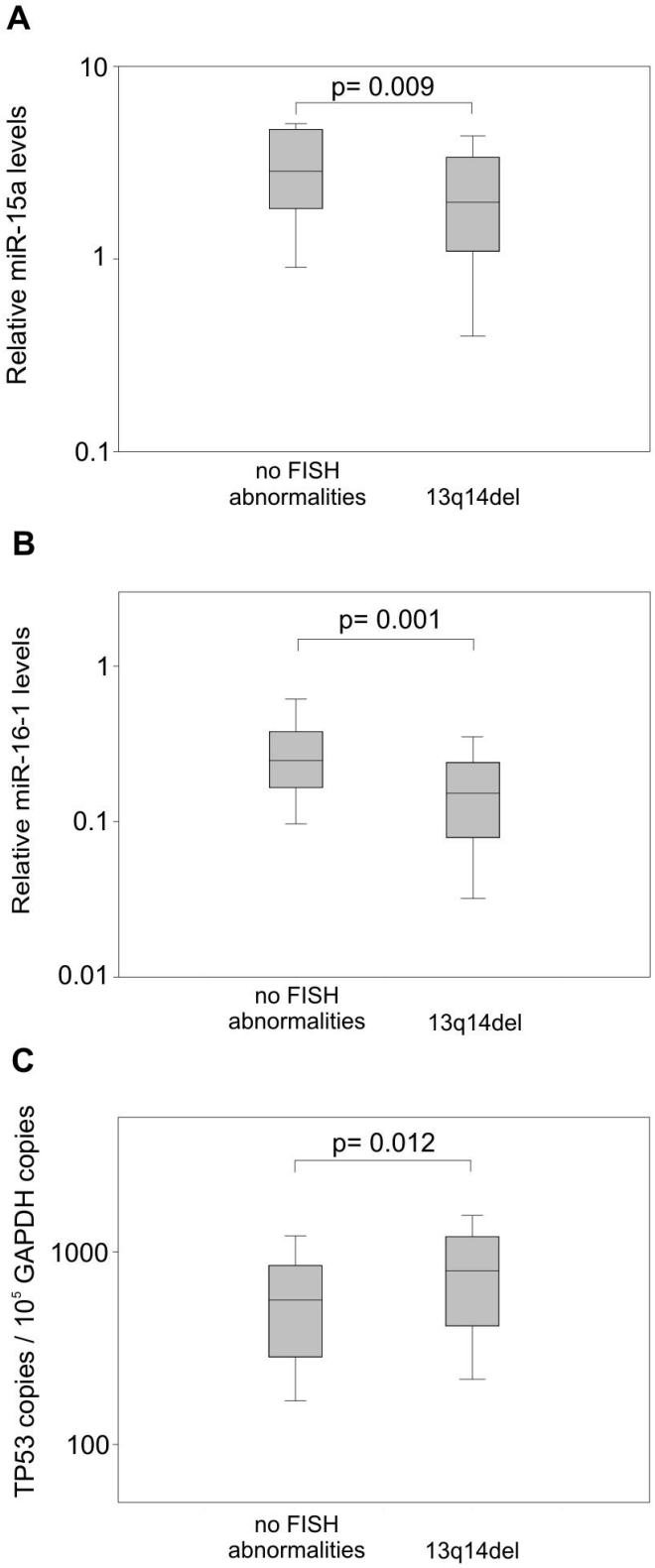


Figure 5. *Relative levels of A) miR-15a, and B) miR-16-1 in CLL with no FISH abnormalities or with 13q14 deletion (13q14del). C) Relative levels of TP53 in CLL with no chromosomal abnormalities or with 13q14 deletion (13q14del). Boxes and whiskers represent 25-75th and 10-90th percentiles, respectively; median is the central line in each box.*

High TP53 levels are associated with reduced levels of miR-15a and miR-16-1

Given that miR-15a and miR-16-1 are encoded at 13q14 and are known to target TP53 mRNA, we related TP53 mRNA levels to miR-15a and miR-16-1 levels in CLL patient with 13q14 deletion. As predicted, the levels of TP53 mRNA significantly and inverse correlated with the levels of miR-15a (rs= -0.775, p<0.0001, Figure 6A) and miR-16-1 (rs= -0.722, p<0.0001, Figure 6B).

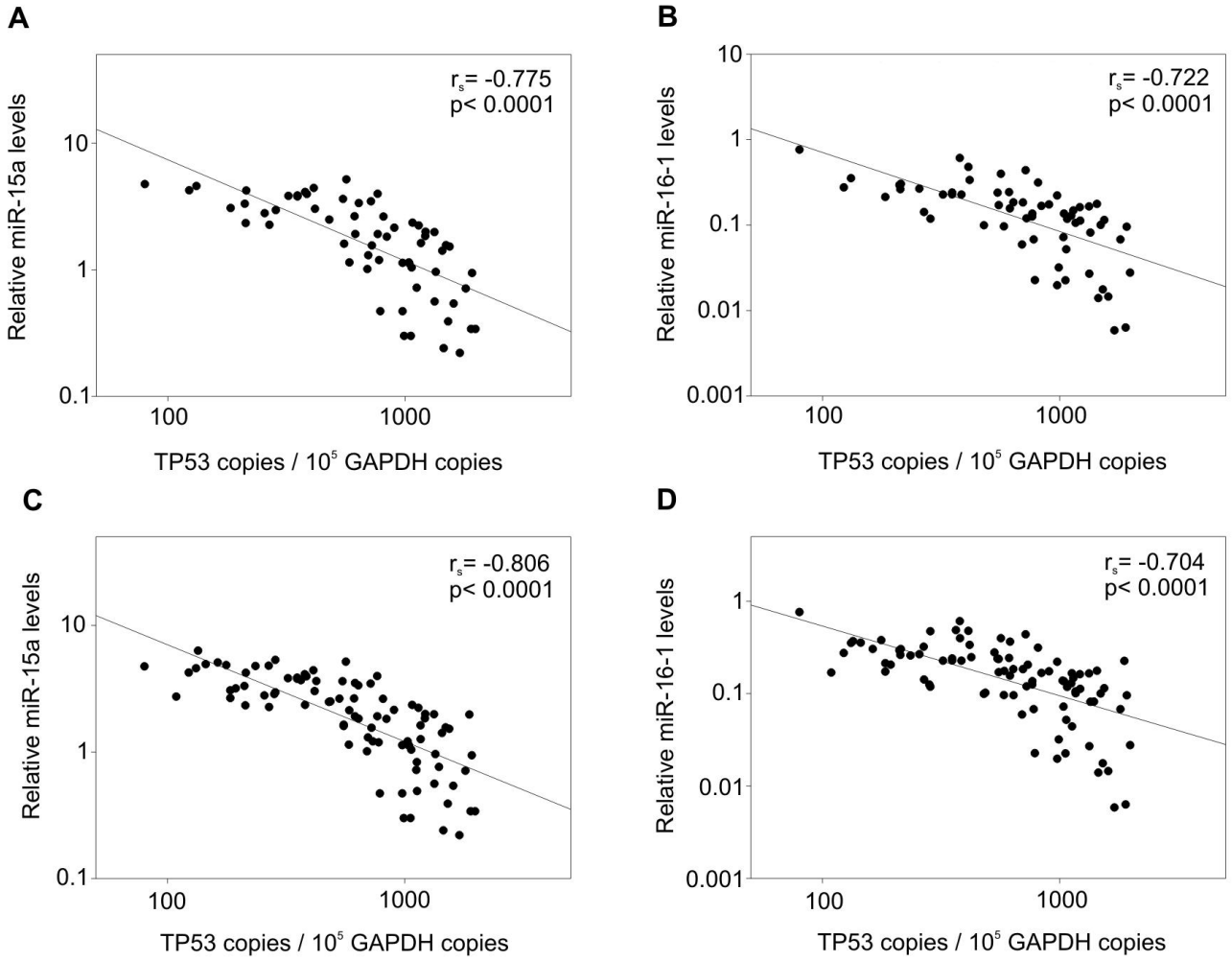


Figure 6*. Relationship between TP53 levels and A) miR-15a, B) miR-16-1 in 13q14del CLL and C) miR-15a, D) miR-16-1 in all CLL. r=Spearman's rho correlation coefficient.*

Moreover, same inverse correlations were observed between the levels of TP53 and miR-15a and miR-16-1 also in CLL cases with no chromosomal abnormalities (rs= -0.806, p<0.0001, Figure 6C, and rs= -0.704, p<0.0001, Figure 6D; respectively). These observations supported a model in which TP53 mRNA levels in CLL are determined by levels of miR-15a and miR-16-1.

Relationship between TP53 and TERT levels

Having established that deletion of miR-15a and miR-16-1 at 13q14 is likely to account for the high TP53 mRNA levels observed in CLL samples without TP53 deletion/mutation, we next sought to explain the reduced expression of telomerase in 13q14 patients. We have previously shown that 13q14del CLL cases expressed lower levels of TERT than CLL cases with other chromosomal abnormalities (***Rampazzo et al, 2012***). TP53 regulates a large array of genes, which mediate tumor-suppressive effects, such as inhibition of cell-cycle progression and induction of apoptosis. TP53 is also a very well-known down-regulator of TERT (***Kanaya et al, 2000; Xu et al, 2000***).

To test this idea, both 13q14del and no FISH deletion CLL samples were analysed for the expression of TERT mRNA levels. We found that 13q14del CLL expressed significantly lower levels of TERT than CLL with normal cytogenetic profile (55 [20-115] vs 73[46-198] copies/10^6 copies GAPDH, p= 0.027; Figure 7A).

Finally, we associated the TERT levels with TP53 mRNA levels and found that TERT mRNA levels negatively correlated with TP53 mRNA levels. Of interest, we found a negative correlation between TP53 and TERT levels only in the 13q14del CLL (rs= -0.359, p= 0.002, Figure 7B), but not in CLL without chromosomal abnormalities (rs= 0.111, p= 0.484, Figure 7C).

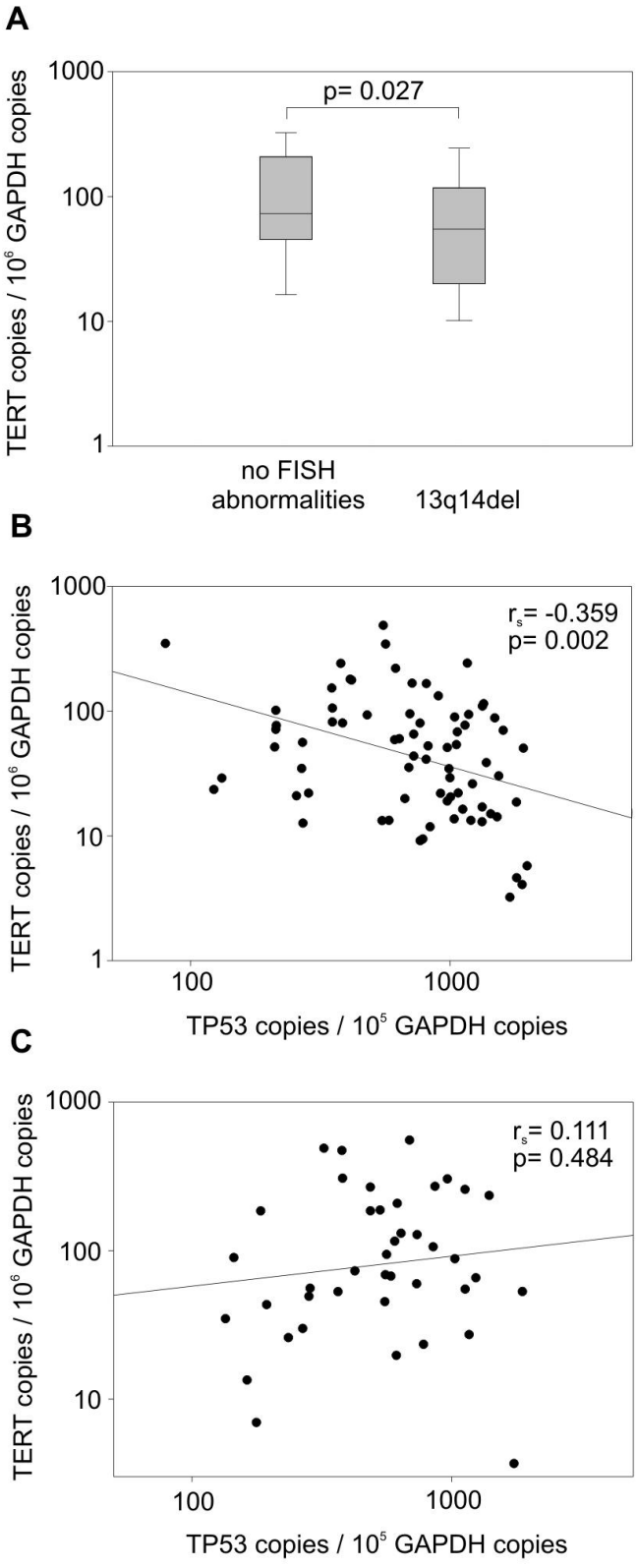


Figure 7*. A) Relative levels of TERT in CLL with no FISH abnormalities or with 13q14 deletion (13q14del). Boxes and whiskers represent 25-75th and 10-90th percentiles, respectively; median is the central line in each box. B) Relationship between TERT and TP53 levels in 13q14del CLL. C) Relationship between TERT and TP53 levels in CLL with no chromosomal abnormalities. r=Spearman's rho correlation coefficient.*

Correlations with the percentage of 13q14 deletions detected by FISH

Distribution of data we obtained was clearly variable among 13q14 deleted CLL patients. Some CLL cases showed remarkably high and some low levels of mRNA expressions. We sought to clarify the quantitative variation in mRNA expression between individual cases due to different percentage of 13q14 deletions. It has been shown that the percentage of deleted nuclei refines the prognosis of 13q14del CLL cases. The percentage of the 13414 deletion within each case, was detected by fluorescence in situ hybridization (FISH) analysis.

The different percentage of nuclei carrying the 13q14 deletion in fact explained the variable levels of miRNAs, TP53, and TERT observed within the 13q14del CLL group; indeed, the percentage of nuclei carrying the 13q14 deletion negatively correlated with both miR-15a rs= -0.324, p= 0.006, Figure 8A) and miR-16-1 (rs= -0.288, p= 0.016, Figure 8B) levels.

Moreover, the percentage of nuclei carrying the 13q14 deletion positively correlated with TP53 levels (rs= 0.317, p= 0.004, Figure 8C) and negatively correlated with TERT levels (rs= -0.277, p= 0.007, Figure 8D).

Taken all together, these data support the concept that there is a network among miR-15a/miR-16-1, TP53 and TERT within the 13q14del CLL (Figure 9A).

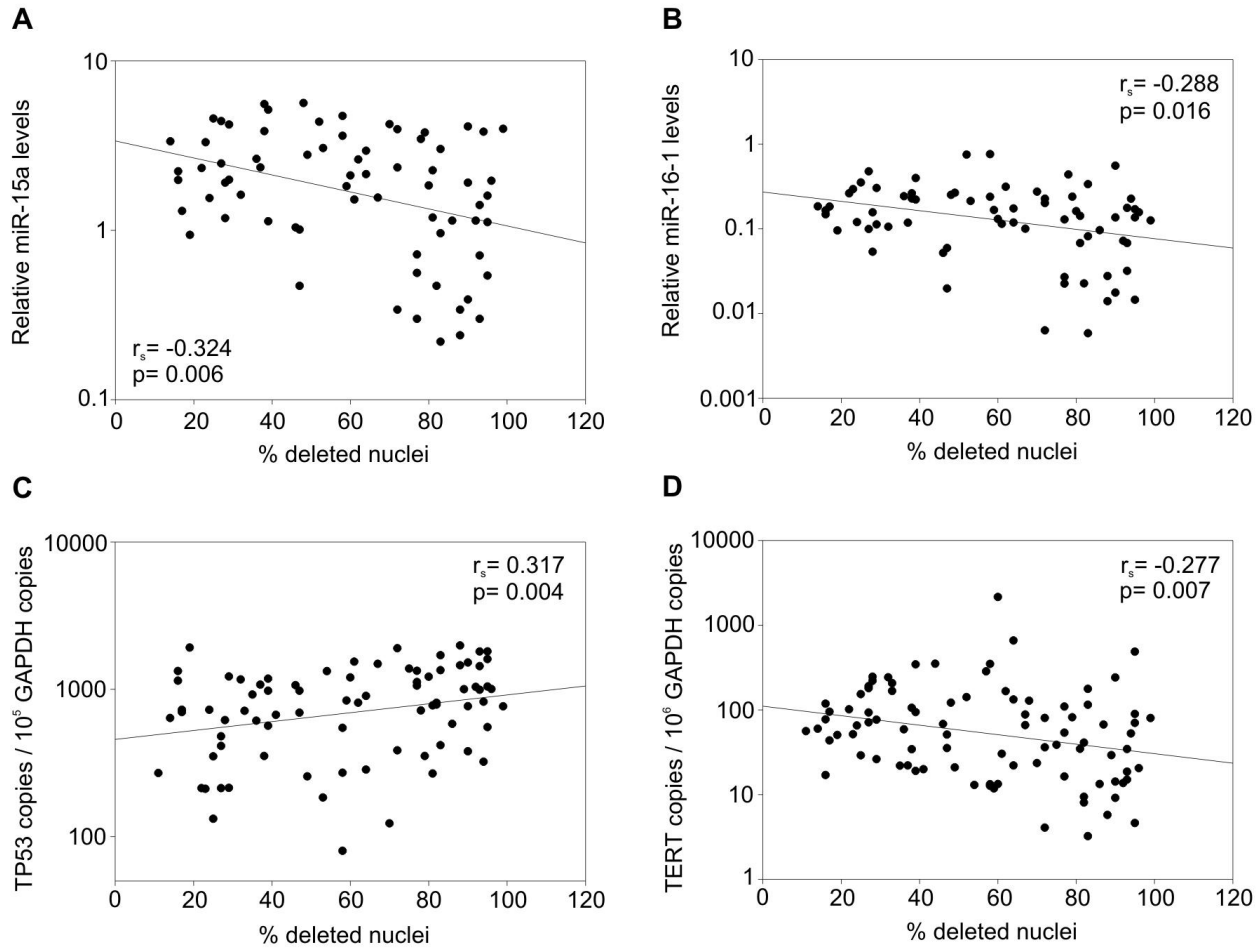


Figure 8*. Relationship between percentage of 13q14 deleted nuclei and A) miR-15a, B) miR-16-1, C) TP53 and D) TERT levels. r=Spearman's rho correlation coefficient.*

Prognostic value of TERT and TP53 levels in CLL

Kaplan-Mayer analysis showed that low TERT levels (below the median) are prognostic of better disease outcome, estimated as TTFT, in the entire cohort of CLL here studied (Table 1), and even in the subgroup of patients with 13q14del CLL (Figure 9B).

Of interest, cases with high TP53 expression have a better prognosis in the subgroup of 13q14del CLL (Figure 9C), but not in the subgroup of CLL with normal cytogenetics profile (Table 2). Within the 13q14del CLL, high TERT/low TP53 levels define the subgroup of cases with the worse prognosis, and multivariate analysis confirmed the independent value of TERT/TP53 profile in relation to IGVH mutational status (Table 3).

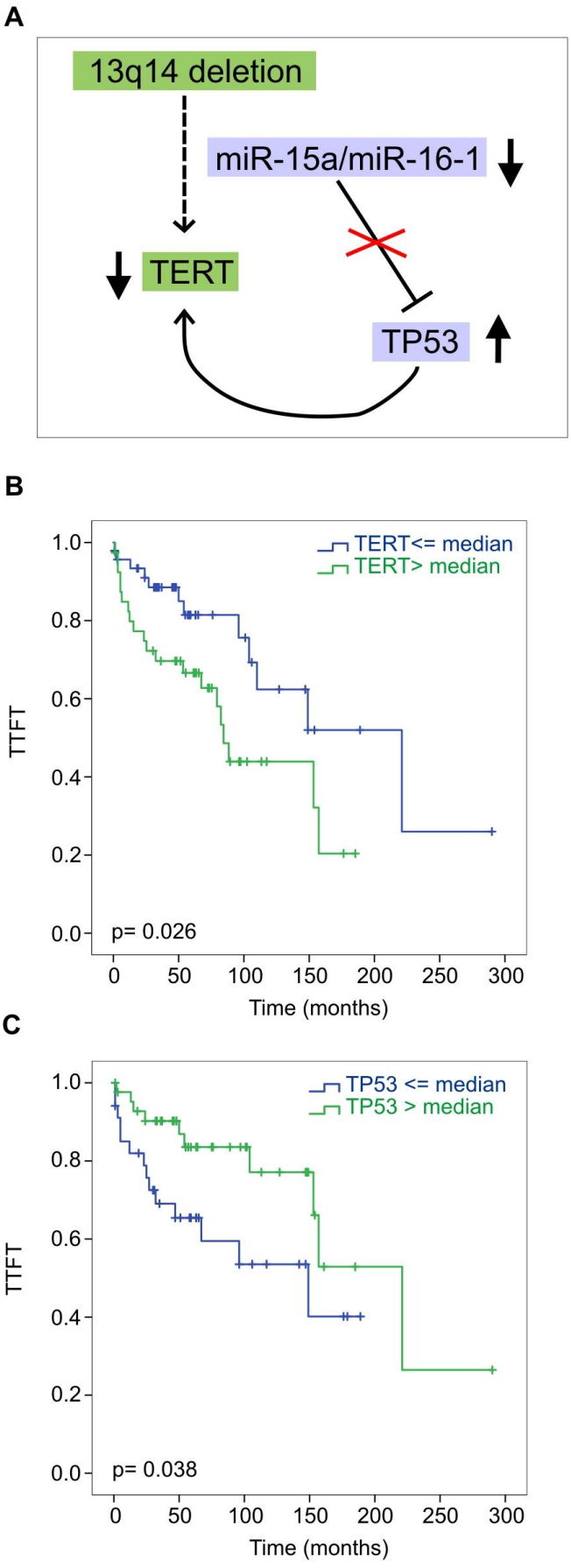


Figure 9. *A) Proposed network between miR-15a/miR-16-1, TP53, and TERT expression. Down-regulation of miR-15a/miR-16-1, due to 13q14 deletion, leads to increased of TP53 levels which, in*

turn, down-regulate levels of TERT, the catalytic component of the telomerase complex. Treatment-free survival [time from diagnosis to first treatment (TTFT)] curves in patients with 13q14del CLL according to B) TERT levels; C) TP53 levels; and D) in patients with different TERT/TP53 profiles.

Table 1. Time and Hazard ratio of *IGVH* status, *TERT* levels and *TP53* levels for their effects on disease progression in all CLL cohort

	TTFT[x] months (95% CI)▪	**p-value log-rank**	**Hazard ratio (95% CI)▪**	**HR p-value**
***IGVH* status**		< 0.0001		
IGVH mutated	211 (107;221)		1	
IGVH unmutated	44 (18;157)		4.0 (2.1;7.4)	< 0.0001
***TERT* levels**		0.008		
TERT low*	149 (107;-)		1	
TERT high*	82 (67;157)		2.2 (1.2;4.0)	0.009
***TP53* levels**		0.123		
TP53 low*	109 (72;-)		1.7 (0.9;3.3)	0.127
TP53 high*	157 (149;-)		1	

**TERT or TP53 low: TERT or TP53 <= median; TERT or TP53 high: TERT or TP53 > median; xTTFT: time to first treatment; ▪CI: Confidence Interval*

Table 2. Time and Hazard ratio of *IGVH* status, *TERT* levels and *TP53* levels for their effects on disease progression in CLL with no chromosomal aberrations				
	TTFT[x] months (95% CI)[▪]	**p-value log-rank**	**Hazard ratio (95% CI)[▪]**	**HR p-value**
***IGVH* status**		0.007		
IGVH mutated	109 (86;-)		1	
IGVH unmutated	56 (14;68)		6.0 (1.4;25.4)	0.015
***TERT* levels**		0.047		
TERT low*	109 (86;-)		1	
TERT high*	72 (45;-)		2.9 (0.97;8.7)	0.057
***TP53* levels**		0.969		
TP53 low*	109 (72;-)		1.0 (0.3;3.4)	0.973
TP53 high*	- (20;-)		1	
***TERT&TP53* levels**		0.590		
TERT high* & *TP53* high*	- (1;-)		1.0 (0.3;3.4)	0.966
TERT low* & *TP53* low*	109 (86;-)		0.5 (0.1;1.5)	0.197
TERT high* & *TP53* low*	72 (44;-)		1	
TERT low* & *TP53* high*	- (18;-)		0.4 (0.1;2.2)	0.321

**TERT or TP53 low: TERT or TP53 <= median; TERT or TP53 high: TERT or TP53 > median; xTTFT: time to first treatment; ▪CI: Confidence Interval*

Table 3. Time and Hazard ratio of *IGVH* status, *TERT* levels and *TP53* levels for their effects on disease progression in CLL with 13q14 deletion				
	TTFT[x] months (95% CI)[▪]	**p-value log-rank**	**Hazard ratio (95% CI)[▪]**	**HR p-value**
***IGVH* status**		< 0.0001		
IGVH mutated	211 (110;-)		1	
IGVH unmutated	32 (6;157)		4.1 (1.9;8.6)	0.0003
***TERT* levels**		0.026		
TERT low*	211 (104;-)		1	
TERT high*	84 (53;157)		2.2 (1.1;4.7)	0.030
***TP53* levels**		0.038		
TP53 low*	- (32;-)		2.3 (1.02;5.5)	0.041
TP53 high*	157 (149;-)		1	
***TERT*&*TP53* levels**		0.012		
TERT high* & *TP53* high*	157 (153;-)		0.2 (0.06;0.8)	0.025
TERT low* & *TP53* low*	- (96;-)		0.3 (0.07;1.0)	0.047
TERT high* & *TP53* low*	32 (5;-)		1	
TERT low* & *TP53* high*	221 (104;-)		0.3 (0.1;0.8)	0.013
***TERT*, *TP53* levels & *IGVH* status**		< 0.0001		
Other; *IGVH* unmutated	157 (1;157)		0.3 (0.1;0.7)	0.006
Other; *IGVH* mutated	221 (104;-)		0.1 (0.03;0.4)	0.0004
TERT high* & *TP53* low*; *IGVH* unmutated	12 (1;32)		1	
TERT low* & *TP53* high*; *IGVH* mutated	- (25;-)		0.4 (0.1;1.0)	0.040

***TERT or TP53 low: TERT or TP53 <= median; TERT or TP53 high: TERT or TP53 > median; xTTFT: time to first treatment; ▪CI: Confidence Interval*

5. DISCUSSION

In CLL, molecular mechanisms resulting in disease development are still not clearly understood; therefore, the identification of factors that could predict the clinical course of CLL represents a crucial objective to manage this malignancy. The discovery that miR-15a/16-1 is a main target of 13q14 deletion in CLL was the initial step in uncovering of one critical mechanism in CLL pathogenesis. As the miR-15a/16-1 cluster targets not only BCL2, but also TP53, (***Fabbri et al, 2011***) the intact TP53 tumor suppressor pathway, due to the loss of miR-15a/16-1 expression, may explain the more indolent form of disease described in the 13q14del CLL.5 Nonetheless, also these patients present variability in their disease outcome. Since TP53 is an important negative regulator of TERT, (***Kanaya et al, 2000; Xu et al 2000***) a prognostic marker of CLL outcome, we investigate the role of miR-15a/miR16-1 and TP53 in relation to TERT levels and CLL outcome.

As expected, we found that levels of both miR-15a and miR-16-1 were lower in 13q14del CLL than in CLL with no chromosomal abnormalities, while TP53 levels were higher in 13qdel CLL. Moreover, levels of miRNAs and TP53 in 13q14del CLL were correlated with percentages of deleted nuclei. It should be noted that miRNAs expression varied also in CLL with no chromosomal aberrations; therefore, other mechanisms than deletion of miRNA cluster in the 13q14 region, should contribute to regulate their expression. It has been recently demonstrated that miR-15a/miR16-1 and TP53 are engaged in a feedback circuitry loop in CLL: increased levels of miR-15a/miR16-1 target and down-regulate TP53 expression, while TP53 binds to its specific binding sites on chromosome 13 and up-regulates the expression of miR-15a/miR-16-1 in CLL with normal cytogenetic profile (***Fabbri et al, 2011***)**.** As the expression on of TP53 is influenced by many factors in CLL pathology (***Wang et al, 2013***) this variability may influence miRNA levels which in turn down-regulate TP53 expression.

An interesting and intriguing result of our study is that levels of TP53 inversely correlated with TERT levels only in 13q14del CLL. At transcriptional level, more than 20 transcription factor-binding sites acting as activators or repressors have been identified within the TERT promoter (***Dolcetti et al, 2012***). Cooperation of MYC and SP1 is required for the full activation of the TERT promoter, while TP53 protein, through its interaction

with SP1, down-regulates TERT (***Kanaya et al, 2000; Xu et al 2000***). It is possible that TP53 acts as a main driver in TERT regulation in 13q14del CLL, while in CLL with no or other chromosomal aberrations TERT is regulated by many other factors.

Notably, we found that TP53 had prognostic value only on 13q14del CLL. This result, together with the finding that a negative correlation between TP53 and TERT levels can be observed only in 13q14sel CLL and not in CLL with normal cytogenetic profile, emphasizes the existence of a network between miR-15a/miR-16-1, TP53 and TERT within the 13q14del CLL. It has recently been advanced by some studies (***Dal Bo et al, 2011; Hernandez et al, 2009; Rodriguez et al, 2012***) that CLL with a high percentage of 13q deletion tend to have a worse prognosis. The deletion of miR-15a and miR-16-1 and the consequent lack of inhibition of the antiapoptotic BCL2 gene may partially support this trend. Nonetheless, miR-15a and miR-16-1 also target the tumor suppressor gene TP53. Hence, the loss of the miR-15a/miR-16-1 cluster due to 13q14 deletion not only shifts the balance toward higher levels of antiapoptotic proteins, but also toward higher levels of tumor suppressor entities, such as TP53. With a cut-off of 80% of 13q14 deleted nuclei (***Hernandez et al, 2009; Rodriguez et al, 2012***) we did not find any significant differences in disease outcome (chi-quadrate 2.307, Log Rank=0.129). This supports the concept that, rather than the percentage of deletion per se, the effects of the deletion influence the disease outcome.

In conclusion, all together these findings indicate that in 13q14del CLL the miR15/16-16-1 cluster and TP53 axis may be an important pathway, which regulates TERT expression and its activity, thus influencing the disease outcome. The regulation of TERT driven by miR-15a/16-1 and TP53 may explain why TERT levels are low in the CLL patients carrying 13q14 deletions, and miR-15a and miR-16-1 may become useful biomarkers or therapeutic targets for CLL cases with 13q14 deletion. Finally, the analysis of TERT/TP53 profile may be particularly useful in refining the prognosis of patients with 13q14del CLL.

CHAPTER TWO

Targeting MEK/ERK pathway as a novel therapeutic prospective in CLL

1. INTRODUCTION

B cell receptor complex

Both immature and mature B cells recognize and generate signals for B cell responses to antigens through multi-protein complexes termed B cell receptor (BCR). The BCR is composed of the ligand-recognizing Ig heavy and light chains in noncovalent association with Igα (CD79a) and Igβ (CD79b) transmembrane proteins. Igα and Igβ are associated as disulfide-linked heterodimers and mediate the signal transduction function of the BCR. Antigen-mediated aggregation of BCR complexes is required to trigger and sustain the signaling processes necessary for generating a signal of sufficient strength and duration to elicit responses by immature and mature B cells. The BCR triggers an intracellular signaling cascade that requires immunoregulatory tyrosine activation motifs (ITAMs) present on the cytoplasmic domains of Igα and Igβ (***Coggeshall et al, 2000; Wienands et al, 2000***). Tyrosine residues on these motifs become phosphorylated, and serve as hubs for the recruitment of additional kinases and adaptor proteins, and subsequent activation of multiple signaling cascades (***Hsueh et al, 2000***).

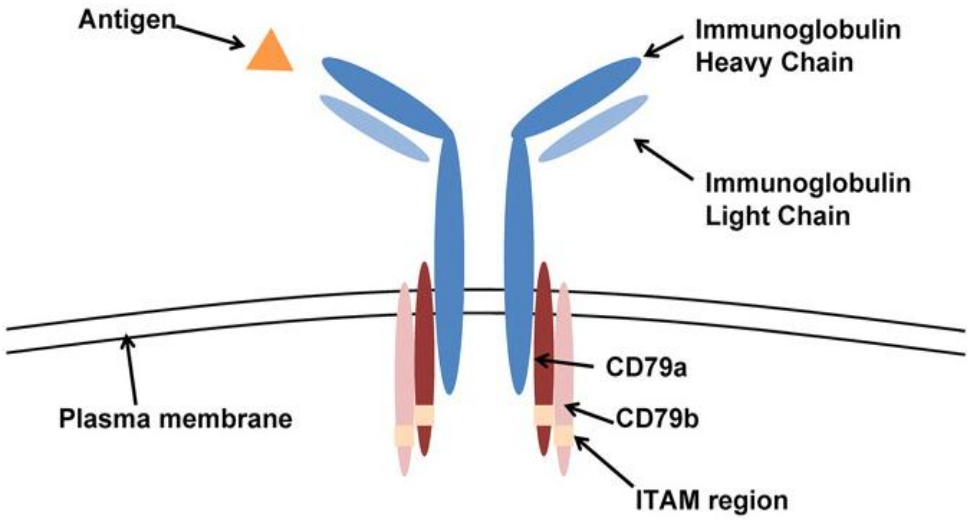


Figure 10. *B cell receptor (BCR) components signaling (**Woyach et al, 2012**). The BCR consists of the antigen-binding heavy chains IgH and light chains IgL that are coupled to the Ig-α (CD79A) and Ig-β (CD79B) signaling subunits.*

BCR signaling in CLL

Analysis of CLL cells isolated from the blood and lymph nodes provided direct evidence for antigen-dependent signaling through the BCR in (**Herishanu et al, 2011**). BCR signaling is likely a key driver of CLL proliferation since *in vitro* BCR activation supports CLL cell survival and proliferation (**Longo et al, 2008; Paterson et al, 2012**). Strong cellular response to BCR activation correlates with a more aggressive disease course of CLL (**Le Roy et al, 2012; Gobessi et al, 2007**). Consistent with these *in vitro* data, the degree of BCR activation in CLL cells *in vivo* correlates with increased tumor proliferation and shorter time to progression (**Herishanu et al, 2011**).

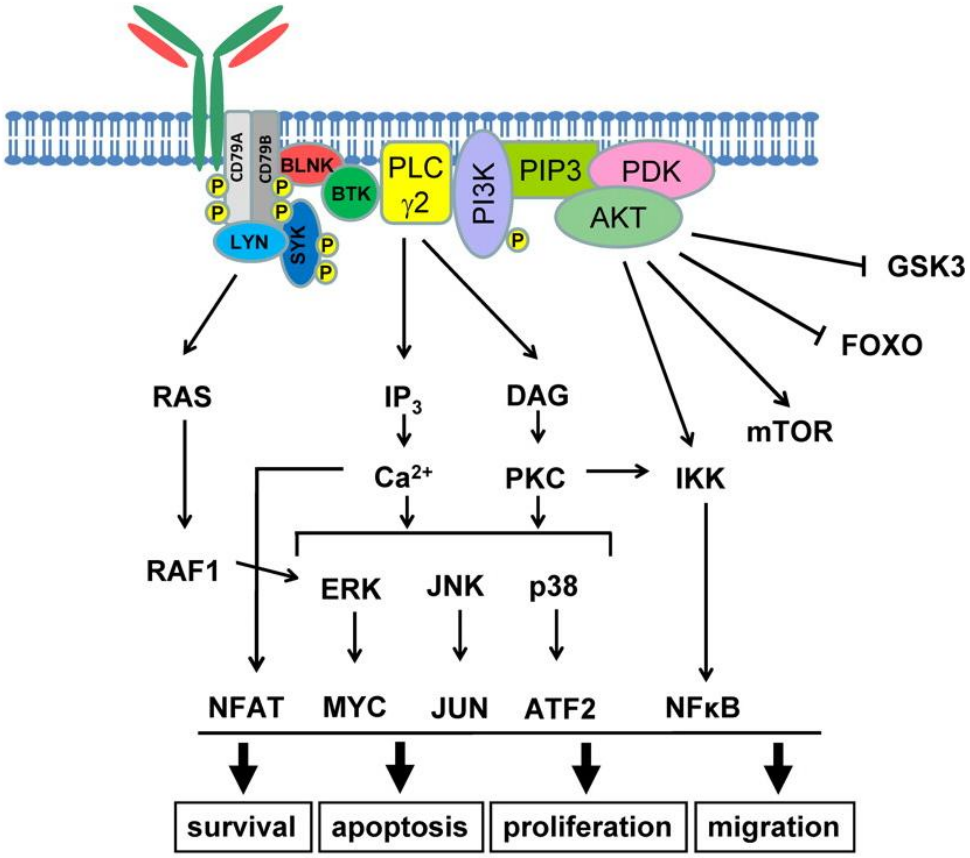


Figure 11. *Antigen encounter and BCR clustering promotes tyrosine phosphorylation of the ITAMs SYK, triggering the activation of a BTK, PI3Kδ, and MAP kinase, leading to cell survival and proliferation (**Bannish et al. 2001**).*

Antigen encounter and BCR clustering promotes tyrosine phosphorylation of the ITAMs by Src-family kinases such as LYN, FYN, and BLK. Phosphorylated ITAMs then recruit spleen tyrosine kinase (SYK) through interactions with its SH2 domains. SYK activation triggers activation of a signaling cascade that engages Bruton's tyrosine kinase (BTK), phosphoinositide 3-kinases (PI3Ks; including PI3Kδ), nuclear factor (NF)-κB, PI3K,

nuclear factor of activated T cells (NF-AT), mitogen-activated protein (MAP) kinase, and RAS signaling pathways, leading to cell survival and proliferation (**Bannish et al. 2001**).

MAPKs pathway

The Mitogen Activated Protein Kinase (MAPK) pathway is one of the most conserved signaling pathways amongst eukaryotes, and controls basic cellular events like proliferation, differentiation, survival and apoptosis. In this pathway, different protein kinases trigger a cascade of signals by sequential phosphorylation events in a hierarchical way. One of the best characterized elements of the MAPK pathway are MEK1/2 and ERK1/2.

MEK1/2

MEK1 and MEK2 are related protein kinases that participate in the RAF-MEK-ERK signal transduction cascade. This cascade participates in the regulation of a large variety of processes including apoptosis, cell cycle progression, cell migration, differentiation, metabolism, and proliferation. Activated RAF directly phosphorylates and activates MEK1/2 at two serine residues (Ser217 and Ser221) that are localized in their activation loop. Two MEK homologues, MEK1 and MEK2 are ubiquitously expressed in mammals. Both of these MEKs sequentially phosphorylate ERK1 and ERK2 at two sites - Tyr185 followed by Thr183 (***Sebolt-Leopold et al, 2004***).

In vitro expression of constitutively active forms of MEK has been shown to result in transformation, giving rise to highly tumorigenic cell lines (**Mansour et al, 1994**). Similarly, MEK has been implicated in the development of a broad range of human tumours (**Hoshino et al, 1999**). Studies carried out in a large number of tumor types have indicated the importance of MEK, which activates ERK, in driving their proliferation and, often, progression.

ERK1/2

MEK1/2 activation induces the downstream phosphorylation of ERK1/2 at level of Threonine 202 and Tyrosine 204. ERK is the most downstream protein of MAPK pathway that acts directly on cellular substrates, and its phosphorylation is associated with different functions, including cell cycle regulation, but also is necessary for regulating lymphocyte activation status and responsiveness to antigens (***Packham et al, 2014***). Several reports

suggest that ERK activity can be modulated in terms of duration and magnitude (***Ebisuya et al, 2005***). In physiological conditions, ERK signalling is tightly controlled by feedback loops at multiple levels, which are essential for maintaining regulated cell growth and homeostasis. Activated ERK phosphorylates a number of substrates, including other kinases and transcription factors, which execute programmes related to cell cycle progression, differentiation, protein translation and evasion from cell death (***Samatar et al, 2014***).

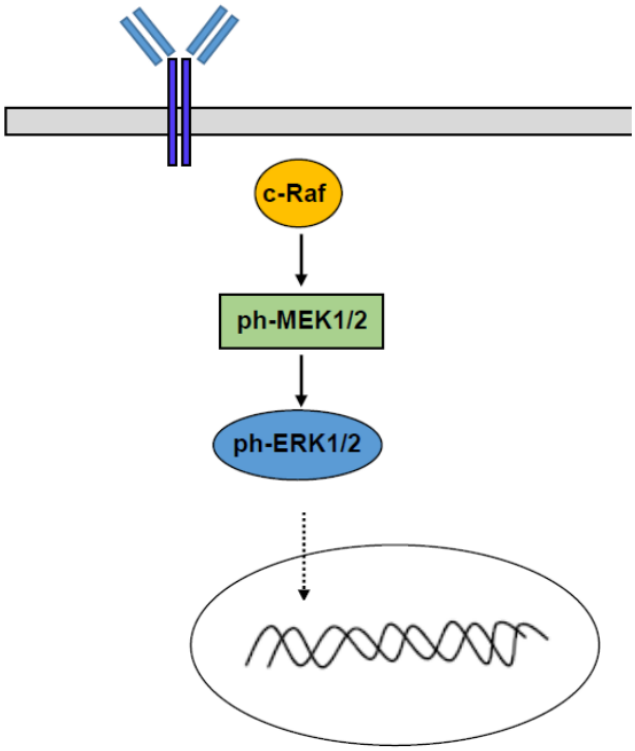


Figure 12. *Schematic representation of MAPK signaling pathway and the prototypic MEK1/2 and ERK1/2 interaction.*

MEK1/2 inhibitors

MEK kinases have been mostly studied as possible therapeutic targets in cancers and MEK inhibitors are under evaluation for the treatment of solid tumors (***Fremin & Meloche, 2010***). The rationale for targeting MEK1/2 is that it has a central role in the MAPK pathway and in transducing proliferative stimuli in a broad spectrum of human cancer cells. Oncogenic mutations in RAS or B-RAF are responsible for a large proportion of human cancers and causes constitutive MEK kinase activation. MEK1/2 inhibitors have also been

analyzed in several preclinical studies for the treatment of haematological malignancies (***Steelman et al, 2011***).

The high degree of sequence homology between MEK1 and MEK2 makes it likely that a small-molecule MEK inhibitor would target both homologues. The development of pharmacological inhibitors of MEK initiated with the discovery of PD098059 (***Dudley et al, 1995***). U0126, a MEK inhibitor shown to be more potent than PD98059, has been widely used for investigating the role of this pathway in cellular events (***Favata et al, 1998***). The first MEK inhibitor reported to inhibit tumor growth *in vivo* was PD184352 (***Sebolt-Leopold et al, 1999***). It was shown to significantly inhibit the growth of colon carcinomas in animal models (***Sebolt-Leopold et al, 1999***). Based on a promising preclinical profile that encompassed effects on proliferation, survival, invasion and secretion of pro-angiogenic growth factors, this agent was moved into the clinical setting into clinical oncology (***Allen et al, 2003***).

In addition, anticancer activity of a novel MEK1/2 inhibitor, Trametinib has been demonstrated for a broad spectrum of human tumor. Trametinib is a reversible and highly selective allosteric inhibitor of MEK1 and MEK2 with anticancer activity against metastatic melanoma. Trametinib activity has been evaluated in the treatment of variety of cancers, and is currently approved as a monotherapy for subjects with metastatic melanoma with BRAF V600E/K mutation (***Rissmann et al, 2015***). In xenografted tumor models, trametinib showed sustained inhibition of ERK phosphorylation, suppression of Ki67, and growth inhibition in tumor lines with mutant BRAF or RAS (***Yamaguchi et al, 2011***). Trametinib also decreases cell proliferation, causes G1 cell cycle arrest, and induces apoptosis (***Gilmartin et al, 2011***).

Small-molecule inhibitors of BCR signaling

Small molecule inhibitors can target several kinases in the BCR pathway. Most advanced in their clinical development are inhibitors of BTK, PI3K, and SYK inhibitors. Preclinical *in vitro* and *in vivo* studies identified these kinases as promising targets for the treatment of CLL and other B-cell malignancies (***Wiestner et al, 2012***), and their inhibition effectively interrupts BCR signaling, leading to reduced activation and proliferation of tumor cells (***Herman et al, 2010***).

Bruton's tyrosine kinase (BTK) is a cytoplasmic tyrosine kinase that is essential for BCR signaling and couples BCR induced calcium release to activation of the NF-κB pathway and cellular proliferation. (***Honigberg et al, 2010***). Ibrutinib (PCI-32765) is an orally bioavailable BTK inhibitor that irreversibly inactivates the kinase by covalently binding to a cysteine residue Cys481 (***Honigberg et al, 2010; Pan et al, 2007***). It has been shown that, Ibrutinib significantly decreases the CLL cell survival and proliferation, cell migration, and production of the chemokines (***Ponader et al, 2012; Rooij et al, 2012***). In the TCL1 mouse model of CLL, BTK inhibition leads to a profound inhibition of CLL progression and survival (***Ponader et al, 2012***). Several combination studies of Ibrutinib with frontline settings are currently ongoing.

B Cell Anergy

Anergy was described as a state of lethargy of B lymphocytes, that persist in the periphery but are unresponsive to their antigen. The molecular mechanisms underlying anergic B cells lack of responsiveness reside both in BCR proximal and distal signaling (***O'Neill et al, 2011***).

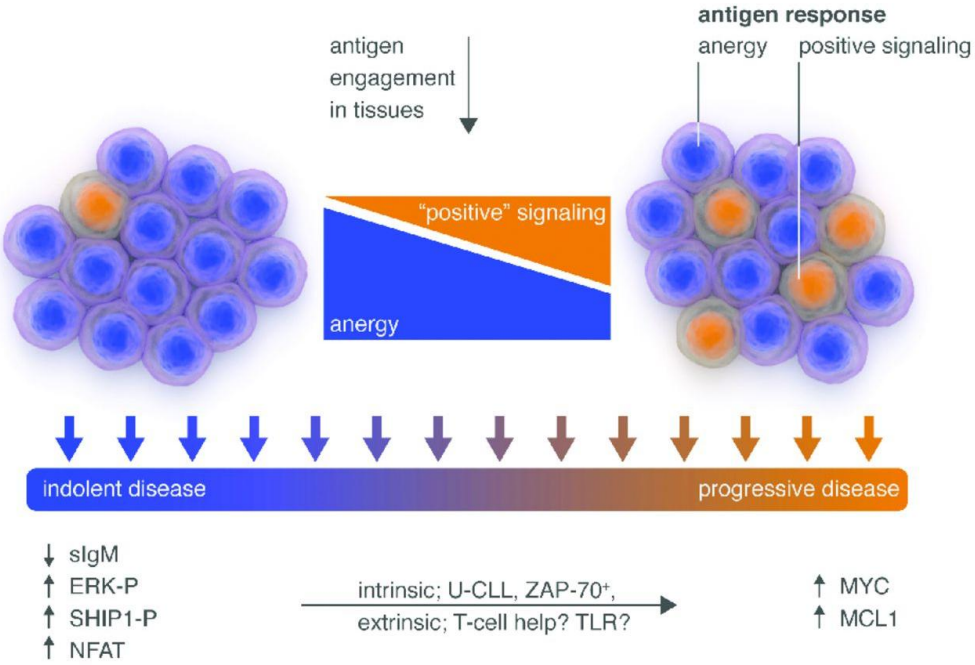


Figure 13. *Variable BCR signaling responses influence clinical outcome via differential effects on anergy and positive signaling. Antigen engagement appears to be ongoing in all CLL with anergy being the predominant outcome. Thus, markers of anergy (sIgM downmodulation, raised basal ERK phosphorylation and NFAT) are more prominent in good prognosis subsets.*

The chronic basal activation of anergic B lymphocytes, results in constitutive phosphorylation of ERK1/2 kinases and nuclear translocation of nuclear factor of activated T cells c1 (NF.ATc1) (***Dolmetsch et al, 1997***; ***Healy et al, 1997***) and constant BCR engagement induces sustained low level intracellular calcium levels (***Benschop et al, 2001***; ***Healy et al, 1997***).

In addition, anergic biochemical program is not permanent, but it requires continuous antigen binding, since many features of B cell anergy can be rapidly reversed by the termination of self antigen–induced signalling. These include recovery of BCR-mediated intracellular calcium mobilization, loss of ERK1/2 phosphorylation and expression of activation markers (***Gauld et al, 2005***). Anergic B cells display significantly weaker proliferation, impaired differentiation, and poorer Ab production after BCR stimulation (***Isnardi et al, 2010***).

It has been suggested that, in at least a proportion of patients, CLL cells might represent anergic B cells abnormally expanded (***Muzio et al, 2008; Mockridge et al, 2007***). Previously it has been reported that in approximately one-half of CLL cases, leukemic cells constitutively express phosphorylated ERK1/2 and MEK1/2 together with activated NF-ATc1 transcription factor (***Apollonio et al, 2013***). In addition, anergic CLL cells are characterized by cellular unresponsiveness to surface Ig ligation in terms of phosphotyrosine induction (***Muzio et al, 2008)***.

2. AIMS

Several evidences indicate that **B cell receptor** (BCR) stimulation is involved in the natural history of disease of CLL. Relevant proportion of CLL patients show biochemical and functional features of **anergic B cells**, characterized by the lack of signalling capacity and **constitutive activation of ERK1/2** and NF-ATc1. **Anergy** is the mechanisms that the immune system adopts to silence autoreactive B lymphocytes. Therefore, it is reasonable to suggest that reversing the anergic state of these CLL might be beneficial in terms of clinical responses. Recently, several small molecule **inhibitors of MEK1/2** have been developed. During the second part of my PhD studies, we tested the effect of the MEK inhibitor **Trametinib** in CLL, both ***in vitro*** and ***in vivo***. In addition, Trametinib was combined with the **BTK inhibitor, Ibrutinib** to assess if blocking of BCR signalling would increase its cytotoxic effect.

3. MATERIALS AND METHODS

Tissue samples and cell purification

Leukemic lymphocytes were obtained from peripheral blood of CLL patients, diagnosed according to National Cancer Institute Working Group (NCIWG) guidelines (***Hallek et al, 2008***). All patients were either untreated or off therapy for at least 6 months before the beginning of the study. The following parameters were analyzed for each patient: age, sex, disease stage at diagnosis according to Binet or modified Rai (***Rai et al, 1975***) criteria, CD38 expression, IGHV gene mutational status (***Hamblin et al, 1999***), ZAP70 expression (***Wiestner et al, 2003***), characterization of disease as progressive or stable as defined by the NCIWG and survival time. Leukemic cells were purified immediately after blood withdrawal using a B-lymphocyte enrichment kit (RosetteSep, StemCell Technologies). Purity of all preparations was always more than 99%, and the cells coexpressed CD19 and CD5 on their cell surfaces as checked by flow cytometry (FC500; Beckman Coulter); preparations were virtually devoid of natural killer (NK) cells, T lymphocytes, and monocytes.

CD19+ B lymphocytes were purified from PB of healthy donors (buffy coat) by negative selection with the use of a B lymphocyte enrichment kit (RosetteSep; StemCell Technologies) following the manufacturer's instructions and further purified with the use of EasySep™ Human B Cell Enrichment Kit (Stem Cell Technologies). Purity of $CD19^+$ lymphocytes was greater than 98%. All tissue samples were obtained with the approval of the institutional Ethics Committee of San Raffaele Scientific Institute. Informed consent was obtained according to the Declaration of Helsinki.

Cell lines and Cell culture and inhibitors

The stable cell lines JURKAT (T cell leukemia), DAUDI (Burkitt lymphoma), RPMI-8226 (multiple myeloma), RIVA (diffuse large B cell lymphoma), THP1 (acute monocytic leukemia), Ly-3 (diffuse large B cell lymphoma) and MEC1 (CLL) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DsMZ, Germany).

Purified primary leukemic cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 15 ug/mL gentamicin (complete RPMI;

Invitrogen), at the concentration of 5 x 10^6 cells/mL in the presence or the absence of different stimuli or inhibitors as indicated. All cells lines were maintained at a density of $2x10^5$ – $1x10^5$ cells per mL in 25 cm^2 filtered flasks. For *in vitro* studies Trametinib and Ibrutinib were purchased from Selleck Chemicals and for *in vivo* experiments were purchased from MedChem Express.

Western blot analysis and antibodies

Cells (10 million cells per sample) were pelleted, resuspended in cold lysis buffer (150 mM NaCl, 1% NP40, 50 mM Tris HCl, 1 mM EDTA, protease inhibitors, phosphatase inhibitors) and spin in microcentrifuge at 4°C for 20 minutes at 16 000g; lysate was recovered from supernatant. Whole protein extracts (30 μg) were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDSPAGE), and proteins from gel were electron-transferred onto nitrocellulose membranes and incubated with anti phospho-ERK1/2, anti phopsho-MEK1/2, anti phopsho-PD1, anti TCL1 (Cell Signaling Technology), anti ERK1/2, anti BTK (from Santa Cruz). antibodies were diluted in a solution of PBS+0,1% Tween (PBST) containing 5% of non fat dry milk according to manufacturers' instructions. Specific secondary antibodies HRP-conjugated were used for the detection of corresponding primary antibodies used for Western blot analysis: goat anti-rabbit Ig, goat anti mouse Ig (Sigma). Immunoreactivity was revealed by incubation with secondary antibodies conjugated with Horseradish Peroxidise (HRP). Densitometric analysis of ERK-specific bands was performed using ImageQuant Software (GE Healthcare). The values of individual patients were calculated as percentage of the positive control (MEC1 cell line) and determined as the ratio of the OD of phospho-ERK and OD of total ERK.

Analysis of cell viability and apoptosis

After each different treatment at indicated doses and time points, the percentage of viable cells was determined using the CellTiter-Glo Luminescent Cell Viability Assay kit, according to the manufacturer's instructions (Promega). All measurements were performed in duplicate and the viability of treated cells was normalized to the relative untreated control.

Induction of apoptosis was evaluated by double staining with FITC (fuorescein isothiocyanate)-conjugated Annexin V and PI (propidium iodide), using the Annexin V-FITC Apoptosis Detection Kit (eBioscence), according to the manifacturer's instructions. Samples were analyzed on a FC500 flow cytometer (Beckman Coulter).

Nucleofection of MEC1 cell line

With the use of the Nucleofector system (Amaxa Biosystems), specific siRNA's were introduced into MEC1 cells (6×10^6 cells) after resuspension in 100 μL of Cell Line Nucleofector Solution L. Then cells were mixed with 1 μg of the siMek1, siMek2 or scramble siRNA (Origene) used as a negative control. Nucleofections were done with the use of the Amaxa Nucleofector device with the C-05 program. Transfection efficiency was assessed by mRNA quantifications. Nucleofected MEC1 cells were collected and processed for quantification analysis at 24 and 48 hours after nucleofection.

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from cells with the use of ReliaPrep™ RNA Cell Miniprep System (Promega) according to the manufacturer's protocol. Quantification of Mek1 and Mek2 mRNA levels were achieved by real-time polymerase chain reaction (PCR) using Fast SYBR® Green Master Mix (ThermoFisher Scientific) and ABI-7500 (Applied Biosystems) according to manufacturer's instruction. Briefly, 500 ng of total RNA was reverse-transcribed (RT), subsequently RT product with specific primers was used as template for real-time PCR. All real-time experiments were performed in triplicate. Data were analyzed with the SDS2 software (Applied Biosystems) following the manufacturer-suggested comparative method. Data were normalized on the expression of Actin and expressed either as fold change relative to control ($2^{-\Delta Ct}$).

Cell synchronization

MEC1 cells were synchronized by the addition of 2mM thymidine (Sigma-Aldrich) for 18 hours, washed, and resuspended in complete RPMI, cultured for an additional 9 hours, and treated a second time with 2mM thymidine for an additional 12 hours. After the second incubation in thymidine, cells were washed twice in PBS to release from thymidine block

and resuspended in complete RPMI. Cells were analyzed for cell-cycle distribution at the time of release and 6 and 24 hours after the release.

Cell-cycle analysis

Transduced MEC1 cells were washed in PBS, resuspended in DNA staining buffer (PBS, 1% NP-40, RNAse and 50 μg/mL propidium iodide), incubated for 10 minutes at room temperature and analyzed for DNA content on a FC500 cytometer (Beckman Coulter). Data were analyzed with CXP analysis software (Beckman Coulter). Cell doublets and debris were excluded from further analysis by gating individual cells in peak area versus peak width plot.

Purification, adoptive transfer and culture of TCL1 leukemias

The TCL1 leukemia cells that were used in the adoptive transfer experiments which have been obtained from the Eμ-TCL1 transgenic mouse spleen, which had a C57BL/6N background. For this reason, the adoptive-transfer experiments with these leukemias were done in C57BL/6N recipients (8-week-old male mice; Charles River Laboratories). TCL1 leukemia cells (splenic lymphocytes) used in the adoptive-transfer experiments, were not purified because they represented more than 80% of the CD5/CD19 positive mononuclear cell population isolated from the spleens of leukemic animals. The purity of the selected populations was evaluated by staining with anti-CD5 phycoerythrin (PE)-conjugated and anti-CD19 PE-Cyanine7 (PC7)-conjugated antibodies (Affymetrix, eBioscience), followed by flow cytometric analysis on a FC500 cytometer (Beckman Coulter). For adoptive transfer, 10×10^6 TCL1 leukemia cells were resuspended in sterile 300 μL PBS, and injected intraperitoneally into the syngeneic recipients.

Statistical analysis

Student's paired or unpaired t-tests were used for statistical comparisons. Comparison of survival curves was performed with the use of the log-rank test. Analyses were performed using GraphPad Prism 4 software (GraphPad Software, San Diego, CA, USA). A P-value <0.05 was considered as statistically significant.

4. RESULTS

Investigating the MEK/ERK inhibitor Trametinib in leukemia and lymphoma cell lines

Considering that, in a proportion of cases, CLL is a disease characterized by anergic B cell, showing constitutive phosphorylation of ERK, we sought to investigate whether the MEK1/2 inhibitor Trametinib could be effective in CLL cells *in vitro* and *in vivo*. To this purpose, we initially planned to study the effect of Trametinib in terms of growth inhibition and cytotoxic effect on stable cell lines.

In order to determine the effective Trametinib concentration, experiments were performed by challenging exponentially growing CLL cell line MEC1 with a serial dilution of drug concentration and estimating growth inhibition between the treated and untreated condition. To identify a dose range, MEC1 cells were treated with Trametinib for 72 hours in cell culture. A concentration dependent decrease in viability was observed in our *in vitro* working conditions and we determined the effective Trametinib concentrations as 3μM (Figure14, left panel).

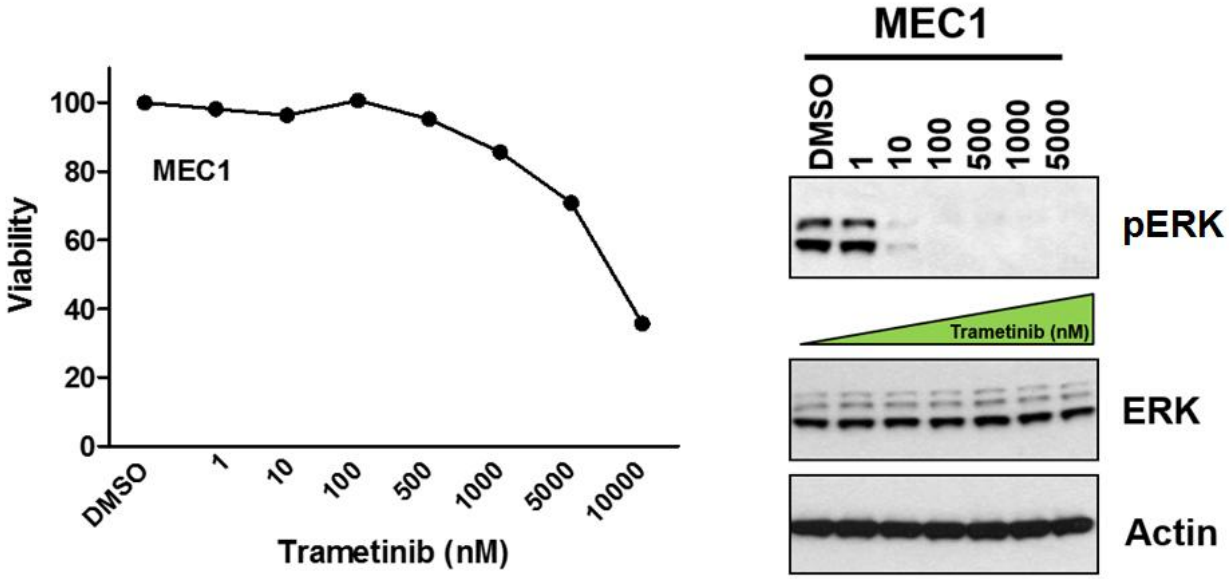


Figure 14. Left panel: *Viability of MEC1 cells after exposure to increasing concentration of Trametinib for 72 hours.* ***Right panel:*** *Western blotting analysis for phosphorylated ERK (pERK) in MEC1 cells after exposure to increasing concentration of Trametinib for 1 hour.*

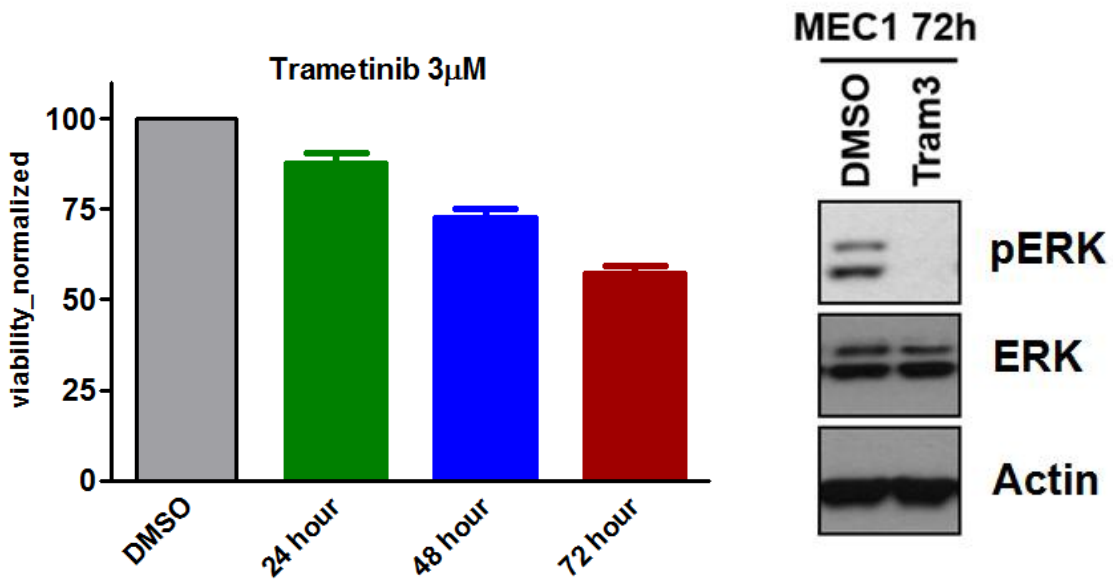


Figure 15. Left panel: *Time course analysis of Trametinib. Viability of human CLL cell line, MEC1, treated with 3uM of Trametinib for 24, 48 and 72 hours in vitro.* ***Right panel:*** *Western blotting analysis for phosphorylated ERK in MEC1 cells after exposure to 3uM of Trametinib for 72 hours.*

We then checked the ERK phosphorylation status in MEC1 cell line and found that ERK is constitutively active. To study whether Trametinib is able to decrease ERK phosphorylarion *in vitro*, we cultured MEC1 cells with Trametinib at different concentrations up to 3μM for 1 hour and studied ERK phosphorylation status by western blot analysis. We observed that, starting from 0.1uM, Trametinib was able to decrease ERK phosphorylation *in vitro* (Figure 15).

To further validate the Trametinib effect *in vitro*, we employed a panel of six cell lines consisting of various leukemia (PCL12, THP1 and Jurkat), lymphoma (Ly3, Riva and Daudi) and multiple myeloma lines (RPMI-8226).

We initially profiled their ERK phosphorylation status and found that all lines expressed constitutively phosphorylated [pERK(+),] (Figure 16). We then analyzed the proliferative response after 72 hour of culture with 3μM of Trametinib. At this concentration, Trametinib was able to reduce cell viability in all cell lines tested in our *in vitro* assay (Figure 3). In addition, the cells were treated in the same conditions with 3μM of Trametinib for 1 hour, and were then lysed and subjected to Western blot analysis for cellular ERK status. A decrease in ERK phosphorylation was observed in all cell lines we have tested.

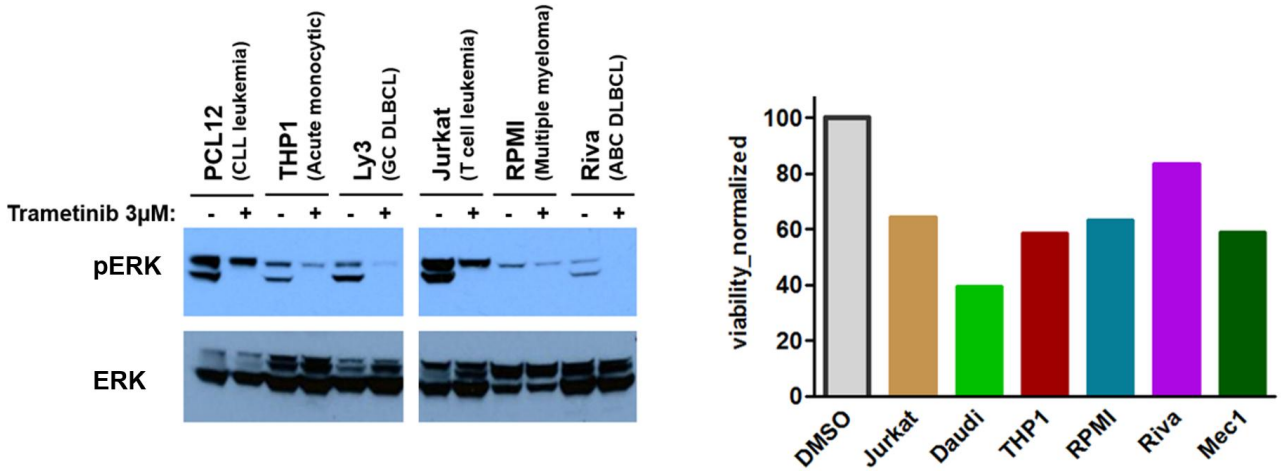


Figure 16. Left: *Inhibition of ERK phosphorylarion in vitro in various human leukemia and lymphoma cell lines. Cell were incubated with 3uM of Trametinib for 24 hour and then lysed for western blot analysis.* ***Right:*** *Growth of different human cell lines incubated with 3uM of Trametinib for 72 hours. Cell lines: MEC1 and PCL12 (CLL Leukemia), THP1 (Acute Monocytic Leukemia), Ly-3 (Activated B-cell Diffuse Large B-cell Lymphoma), Jurkat (T-cell Leukemia), RPMI-8226 (Multiple Myeloma), Daudi (Burkitt's lymphoma) and Riva (Activated B-cell Diffuse Large B-cell Lymphoma) cells.*

Transfection experiments with siMEK1 and siMEK2

To further confirm that Mek1/2 is an appropriate therapeutic target in CLL, we evaluated the functional consequences of Mek1 and Mek2 knockdown in the CLL cell lines MEC1. As shown in Figure 17, both Mek1 and Mek2 were efficiently down-regulated by RNA interference, with an approximately 70–80% reduction in mRNA levels as compared to cells transfected with control siRNA.

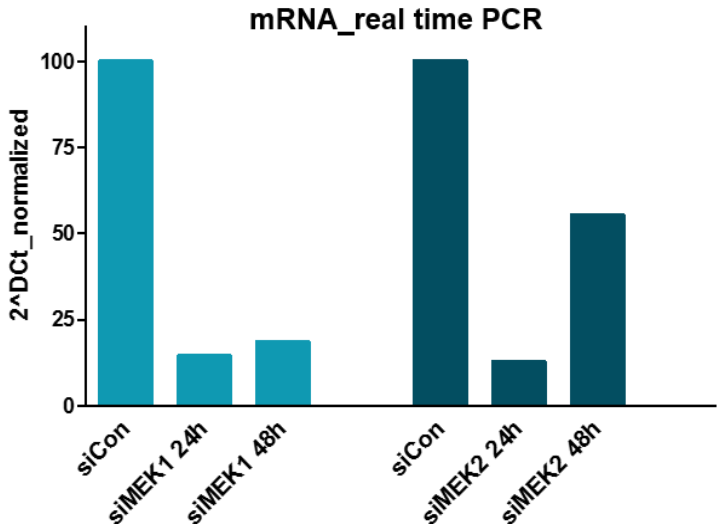


Figure 17. *Efficient siRNA mediated knockdown of Mek1 and Mek2 in MEC1 cells. mRNA levels were measured by qRT-PCR, 24 and 48 hours after transfection.*

We then evaluated the viability of the transfected cell lines after 48 hour and 72 hour of siRNA transfection as well as induction of apoptosis after 72 hour of siRNA transfection. Down-regulation of Mek1 or Mek2 associated with a reduction in the viability of MEC1 cells up to 50 % (Figure 18). Silencing Mek2 was more effective compared to Mek1 with inhibition of (>15%) and no further enhancement of this effect was seen when both Mek1 and Mek2 were simultaneously silenced (Figure 18).

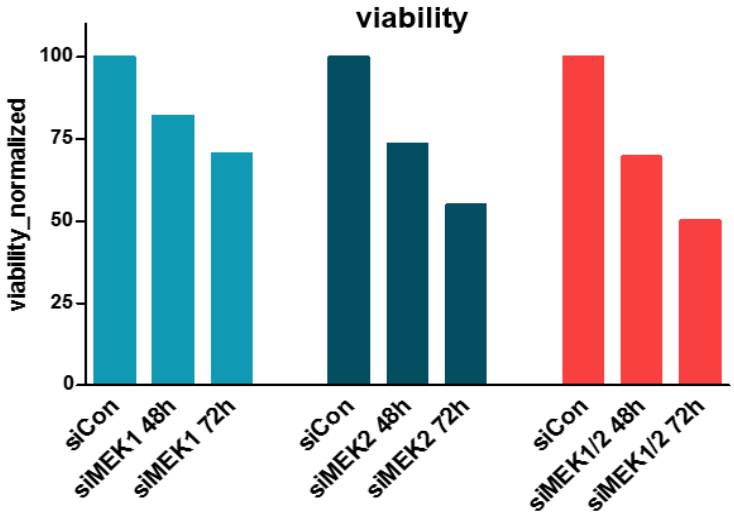


Figure 18. *Cells transfected with appropriate targeting or scrambled control siRNAs were harvested for 48 and 72 hours after transfection.*

Furthermore, induction of apoptosis was determined by Annexin V-FITC/propidium iodide staining, followed by flow cytometric analysis. Downregulation of Mek1 or Mek2 induced the apoptosis of MEC1 cells and silencing Mek2 was more effective compared to Mek1 (Figure 19).

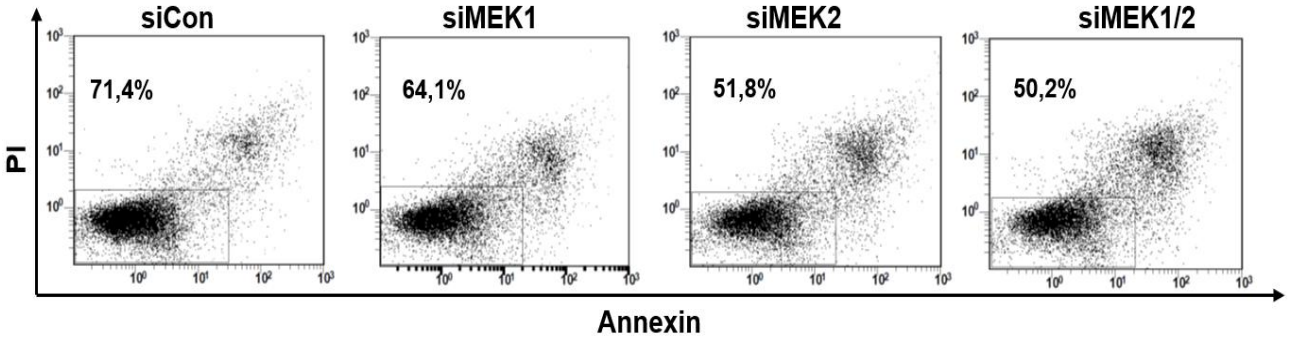


Figure 19. *Induction of apoptosis. Annexin V binding was carried out with the Annexin V-FITC Detection Kit. The percentage values refer to live (annexin V and PI negative) cells present in the rectanguilar gate in each dot plotcells.*

Relationship between ERK and MEK activation in human primary CLL cells

Anergy has been described as a state of lethargy of B lymphocytes, that persist in the periphery and are unresponsive to antigenic stimulation. The molecular mechanisms underlying anergic B cells lack of responsiveness in BCR signaling in CLL is associated also with constitutive activation of ERK. These feature is considered a molecular signature of *in vivo* cellular anergization in patients with CLL (**Mockridge et al, 2007; Muzio et al, 2008; Apollonio et al 2013**).

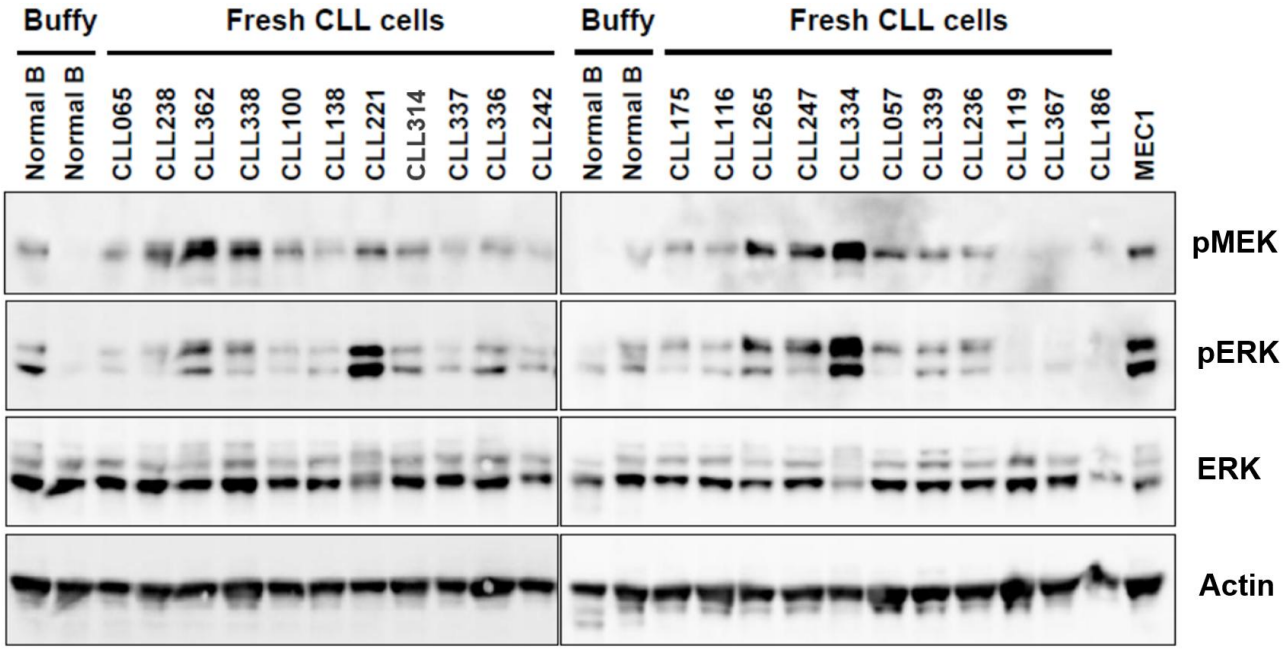


Figure 20. *MEK and ERK phosphorylation status in purified primary (fresh) CLL cells and healthy donors (normal) CD19+ B cells. In all immunoblot analyses, MEC1 cell line were loaded together as positive control.*

To determine whether ERK is activated in human primary CLL cells, we examined the activity of MAPK pathway by western blot. The increase in MAPK activity was confirmed by immunoblot analysis using phospho-specific ERK1/2 and MEK1/2 antibody in 22 CLL samples and in 4 normal B cells obtained from healthy donors (buffy coat). We observed significant MEK and ERK phosphorylation activity in 12 out of 22 (54%) CLL samples analyzed (Figure 20). ERK phosphorylation was considered positive when the cases where higher than 10% of the value measured in the MEC1 cells. Normal B cells purified by immunomagnetic methods from buffy coats of healthy donors, were also heterogeneous in terms of MAPK activity.

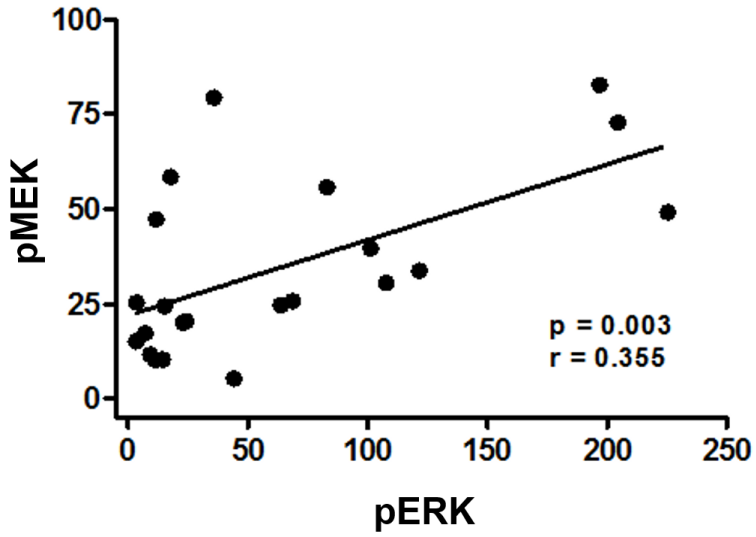


Figure 21. *Correlation between MEK and ERK phosphorylation status in CLL cells. ERK1/2 constitutive phosphorylation significantly correlated with MEK1/2 constitutive activation.*

In addition to ERK activation in CLL, we also examined the phosphorylation of MEK1/2 using phospho-specific MEK antibody and determined whether the activation of ERK is accompanied by the activation of MEK. Activation of MEK in CLL cells was detected in 13 of the 23 cases analyzed (59%) (Figure 20).

ERK activation was positively associated with MEK activation, and this relationship was statistically significant (Figure 21, P = 0.003).

Impact of ERK inhibition on human primary CLL cells: relationship with ERK phosphorylation

To verify whether the inhibition of MEK might affect CLL cell survival *in vitro*, we treated freshly purified CLL cells with 3uM of Trametinib. By AnnexinV/PI staining analysis, we found that the use of Trametinib induced a decrease in cell viability and an induction of apoptosis in primary CLL cells from patients (Figure 22). We observed a significant reduction in viability in 18 CLL patients, when cultured with 3uM Trametinib (Figure 9), though at variable levels.

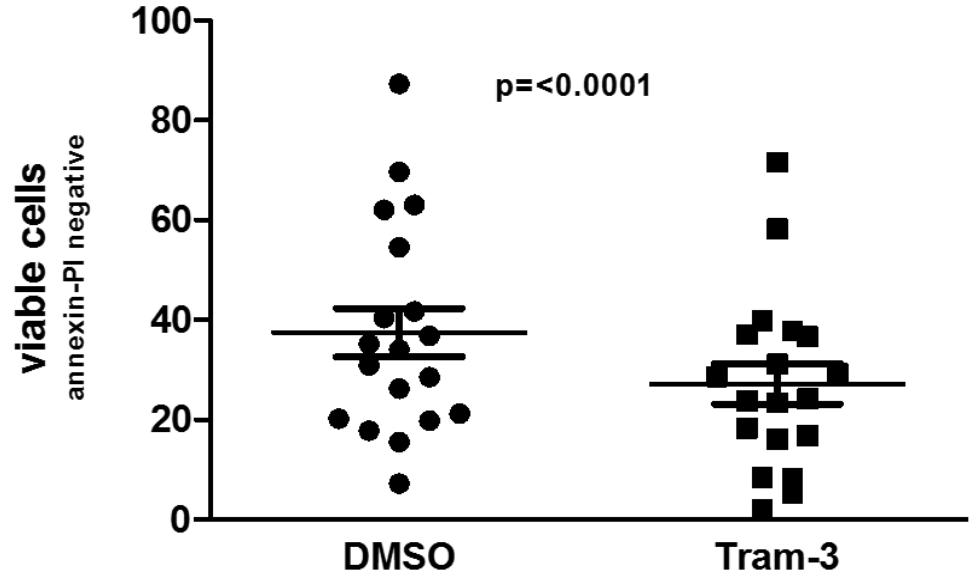


Figure 22. *Freshly purified primary CLL cells were treated for 48 hours with 3uM of Trametinib and induction of apaoptosis was analyzed by AnnexiV and PI staining.*

Interestingly, we observed that MEK/ERK pathway activity (the anergic status of the CLL cells) was associated with the relative sensitivity to Trametinib in CLL cells *in vitro*. In detail, CLL samples that had constitutive ERK phosphorylation (9 pERK(+) samples) had a mean percentage of survival of 55.6% ± 6.9 compared with 83.8% ± 4.5 survival of the 9 pERK(-) cases (Figure 23, P = 0.003).

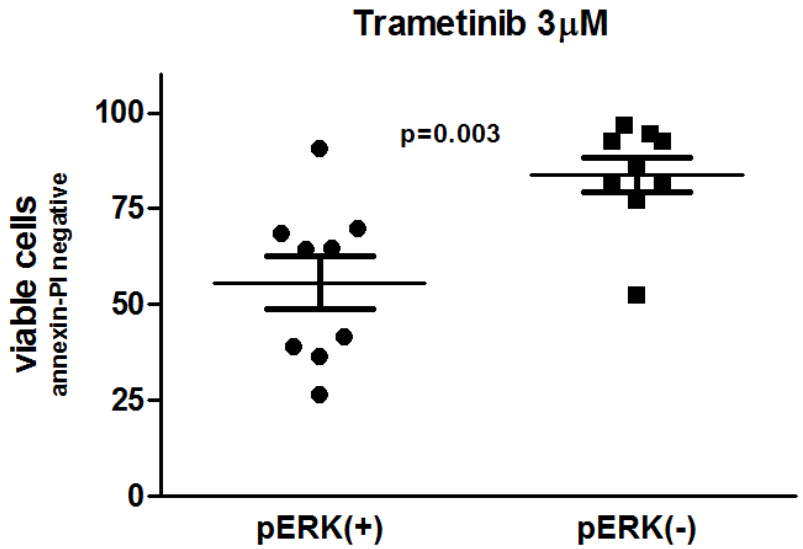


Figure 23. *ERK pathway activity (the anergic status of the CLL cells) is a predictive marker for the relative sensitivity to Trametinib in CLL.*

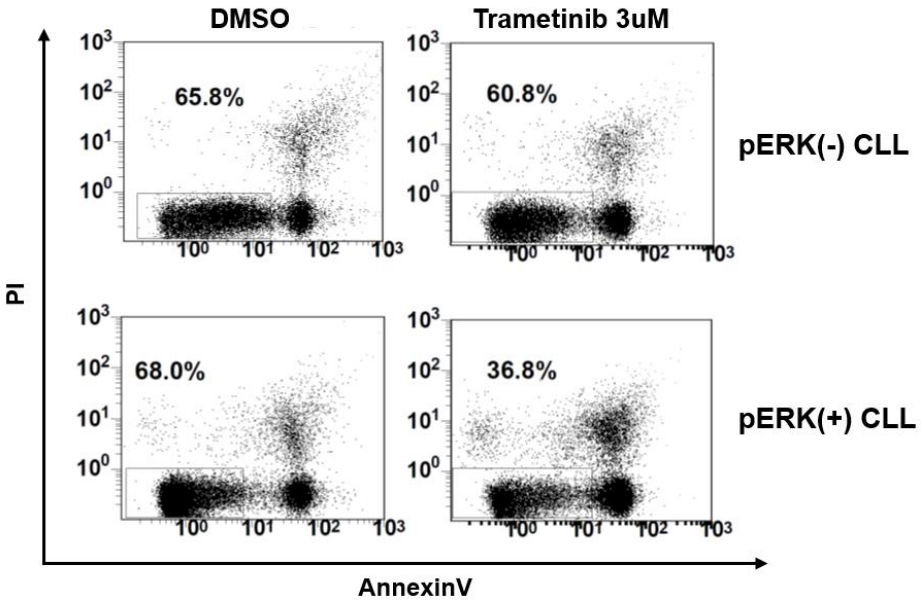


Figure 24. *Representative plots showing the induction of apoptosis in primary pERK(-) and pERK(+) CLL cells. Annexin V binding was carried out with the Annexin V-FITC Detection Kit. The percentage values refer to live (annexin V and PI negative) cells present in the rectanguilar gate in each dot plot.*

In addition to viability experiments, ERK phosphorylation was examined after incubating the primary CLL cells with increased concentration of Trametinib. Treatment of the CLL with the Trametinib greatly reduced the phosphorylation levels of ERK, suggesting efficient and specific inhibition of the MEK/ERK pathway in leukemic cell. Trametinib was able to inhibit constitutive ERK1/2 phosphorylation in pERK(+) CLL samples, starting from low concentrations (Figure 25).

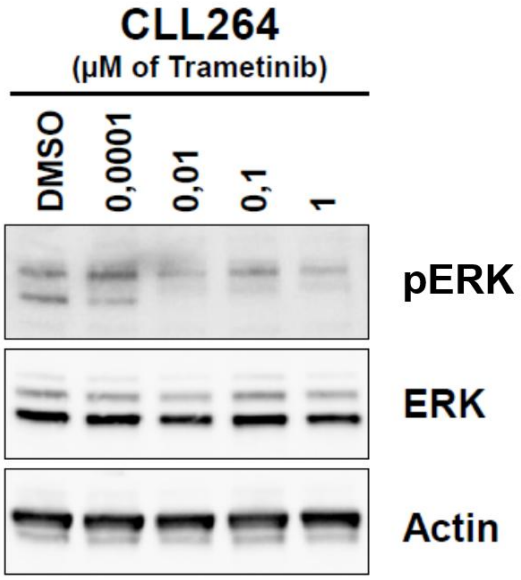


Figure 25. *The levels of ERK1/2 inhibition in CLL cells treated for 1 hour with increasing concentration of Trametinib in vitro.*

Furthermore, to verify potential differences among patients on the basis of ERK1/2 phosphorylation levels, we have correlated the Trametinib mediated reduction in viability to the levels of ERK phosphorylarion in 9 CLL cases. We found significant positive correlation between the ERK1/2 phosphorylation levels of CLL cell (calculated by western analysis) and the Trametinib mediated reduction in viability ($p = 0.0423$). CLL cases with the highest relative ERK phosphorylation level were the ones that were more sensitive to Tranetinib treatment (Figure 26).

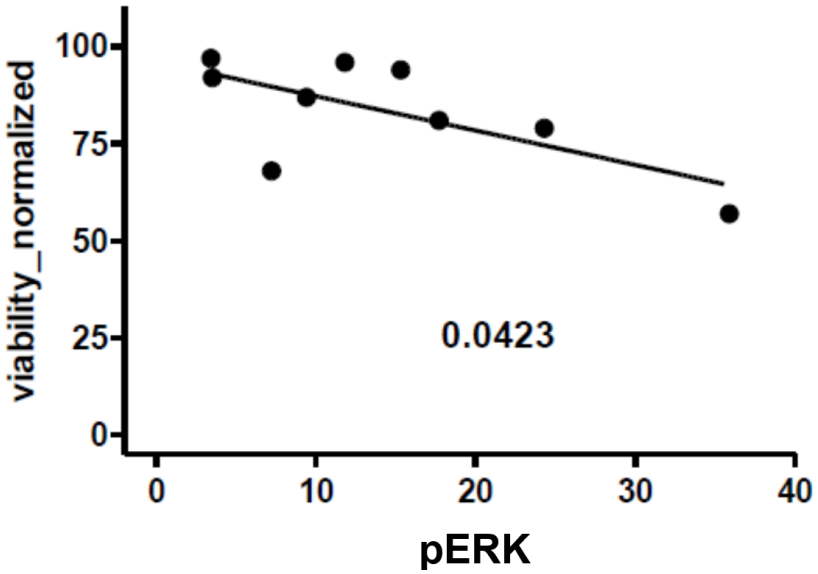


Figure 26. *Correlation between ERK1/2 phosphorylation levels of CLL cell and Trametinib mediated reduction in viability.*

Conversely, we did not observe any significant associations between the IGHV mutational status, CD38 positivity or progression status and Trametinib effect in our CLL cohort.

MEK inhibition in purified normal B cells

Normal B cells, isolated from 4 buffy coats (Figure 14) obtained from healthy donors were cultured with 3uM of Trametinib for 48 hours. We observed that Trametinib is able also to decrease the viability of purified CD19+ B cells (31%) though to a lesser extent as compared to CLL cells (45%) (Figure 28).

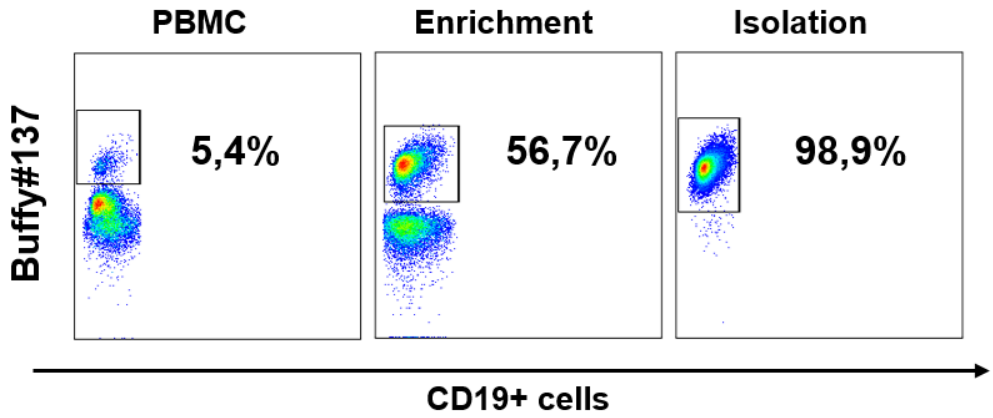


Figure 27. *Representative plots showing the purity of the CD19+ normal B cells, isolated from the buffy coat of healthy donors.*

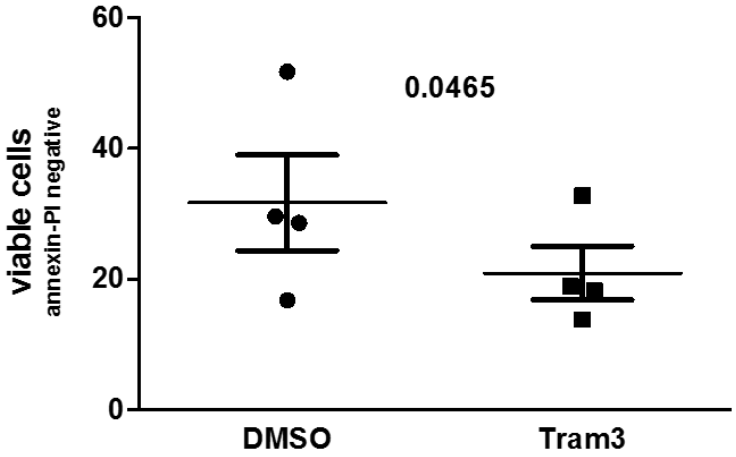


Figure 28. *Isolated normal B cells were treated for 48 hours with 3uM of Trametinib and induction of apaoptosis was analyzed by AnnexinV and PI staining.*

Combined ERK and BTK inhibition *in vitro*

After demonstrating the efficacy of Trametinib as single agent, we further evaluated its effect in combination with the BTK inhibitor Ibrutinib on leukemia cell lines and primary CLL lymphocytes. The efficacy was assessed in terms of cytotoxicity and induction of apoptosis. Changes in protein expression were assessed by western blotting analysis. We first exposed CLL cell lines MEC1 and PCL12 to 3μM Trametinib, 1μM Ibrutinib and to the combination of both drugs *in vitro*, for 72 hours (Figure 29).

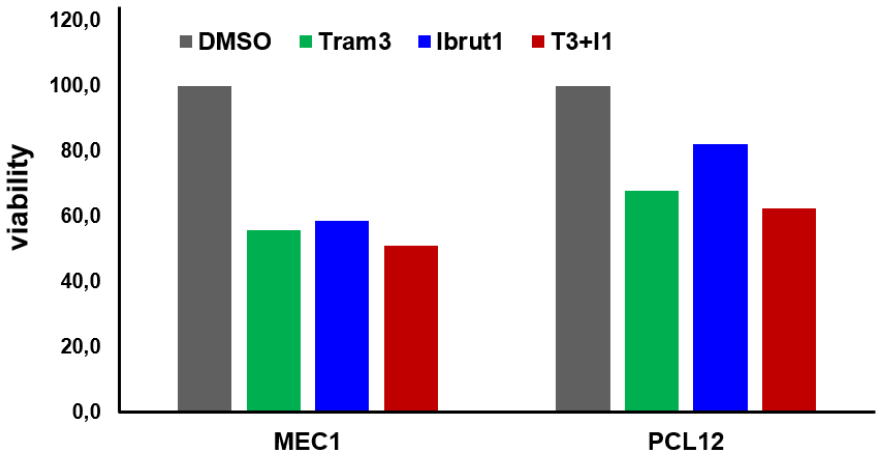


Figure 29. *Impact of combined ERK and BTK inhibition in cells lines*

The same sets of experimetns were also repeated on human primary CLL cells. The capacity to potentiate induction of apoptosis was assessed by annexin-V staining. As expected, Ibrutinib alone was able to reduce viability of CLL cells but no synergistic effects was apparent between Trametinib and Ibrutinib when compared to either drug alone (Figure 30).

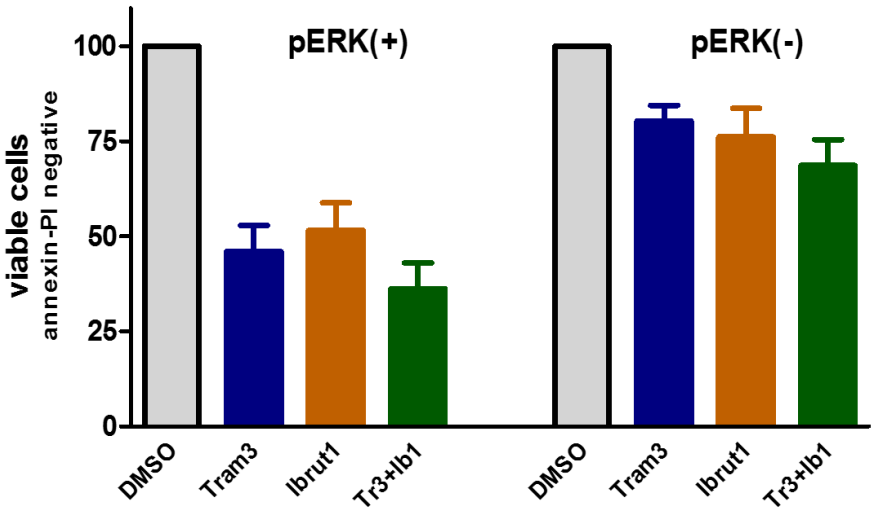


Figure 30. *Combined ERK and BTK inhibition in human CLL cells: effects on viability measured as Annexin/PI negative cells in culture in the presence of Trametinib 3uM alone, Ibrutinib 1uM alone and the combination of both drugs*

Interestingly, as for Trametinib, also Ibrutinib was less effective in decreasing the viability of pERK(-) CLL cells when compared to pERK(+) cells (Figure 31).

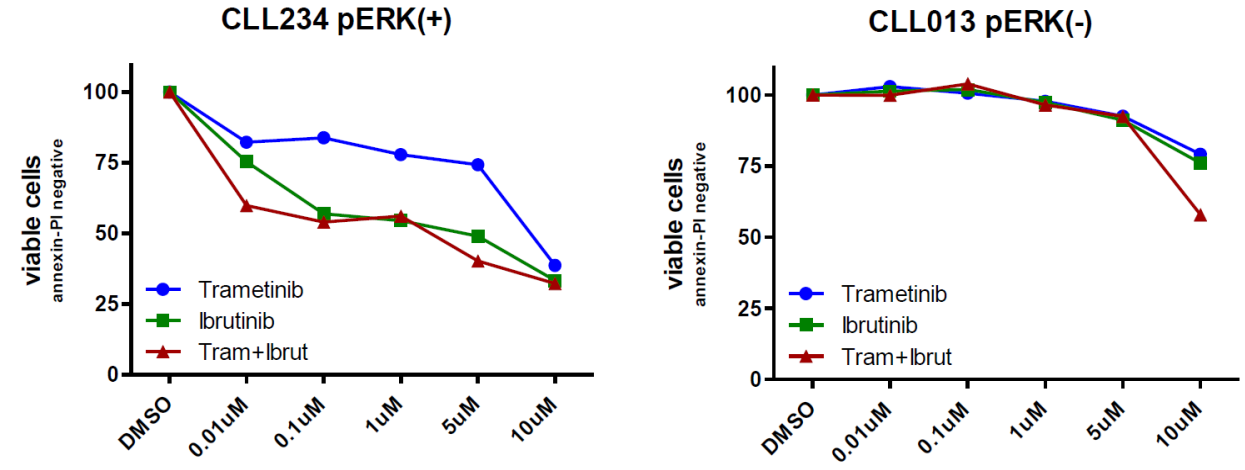
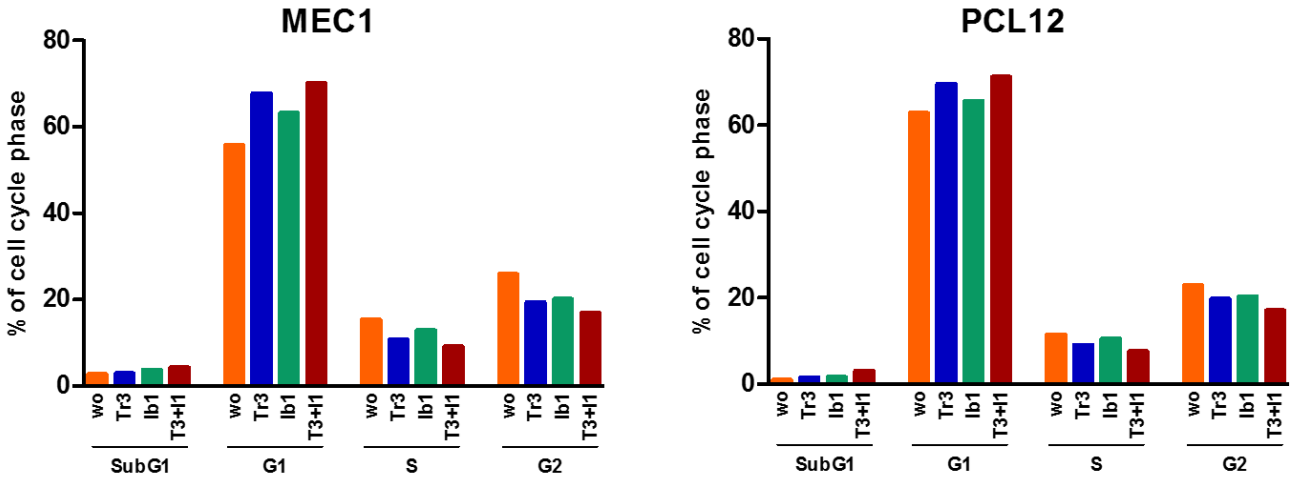


Figure 31. *Trametinib and Ibrutinib dose response in pERK(+) and pERK(-) CLL cells.*

Cell cycle analysis

To further clarify the role of Trametinib and Ibrutinib in regulating proliferation, we synchronized CLL MEC1 and PCL12 cell lines with thymidine for 48 hour and evaluated their cell cycle distribution in 24 hour after release from thymidine block. After release from thymidine block, MEC1 cells were treated with Trametinib 3μM, Ibrutinib 1μM or with combination of both Trametinib and Ibrutinib. Cells treated with Trametinib showed increased percentage of cells in G1 and reduced percentage of cells in S and G2 phase, indicating a block at G1 phase of the cell cycle (Figure 32). Cells treated with Ibrutinib also had reduced percentage of G1 but at a lesser extent compared to Trametinib. Again, no differences were seen when both Trametinib and Ibrutinib was used together.



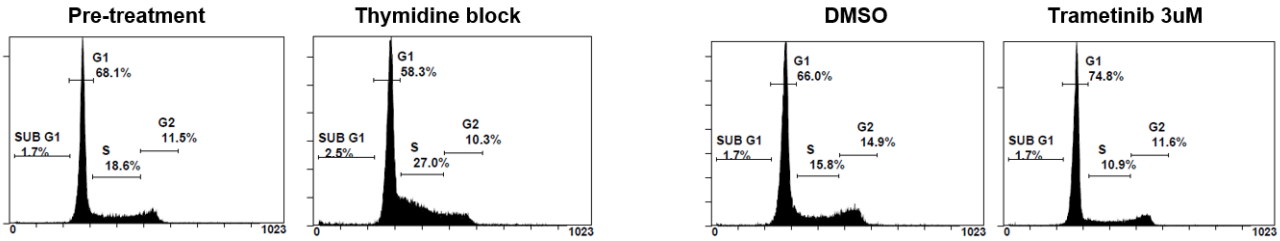


Figure 32. *Cells were synchronized with thymidine for 48 hour, treated with Trametinib 3 μM or Ibrutinib 1 μM, and analyzed for cell-cycle distribution by PI staining and flow cytometry 24 hours after release from thymidine block.* ***Upper graph****: Cell cycle analysis of MEC1 and PCL12 cell lines.* ***Lower graph****: Representative cell cycle analysis plots of cells in pre treatment, Thymidine block release and after 24 hour of treatment.*

In vivo activity of Trametinib in TCL1 transgenic mouse model of CLL

Due to the similarity with human CLL, TCL1-transgenic mice are extensively used in the dissection of the pathogenic mechanisms leading to CLL.

In order to study Trametinib effect *in vivo*, we started investigating whether TCL1 leukemias express constitutively active ERK, as described for human CLL. We have isolated the splenocytes from leukemic TCL1-tg spleen, and observed significant phosphorylation of ERK in all investigated TCL1 mice we have analyzed (Figure 33, left). Then, TCL1 splenocytes, isolated from TCL1-tg spleen, were placed in culture with Trametinib alone or in combination with Ibrutinib to evaluate a possible cytotoxic effect. Trametinib effectively decreased the viability (59% ± 4.5) of four different TCL1 leukemias that we analyzed (Figure 33, right).

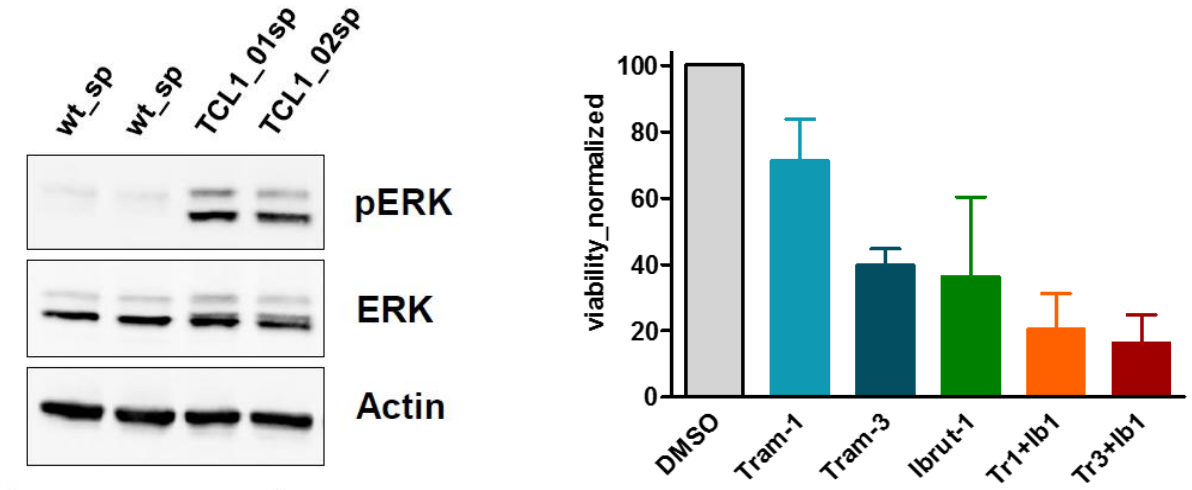


Figure 33. Left: *ERK phosphorylation status in 2 representative saples of normal and TCL1-tg leukemic mice cells.* ***Right:*** *Isolated TCL1 cells from a representative animal were cultured in vitro to study the effect of the ERK and BTK inhibitors on viability*

In addition to viability experiments, we checked if Trametinib was able to decrease the ERK phosphorylation of TCL1 cells *in vitro*. Freshly isolated splenocytes were cultures *in vitro* for 48 hours with MEK inhibitor. Similarly, to human anergic CLL cells, Trametinib was able to inhibit the ERK1/2 phosphorylation level in all TCL1 leukemias that we have analyzed (Figure 34).

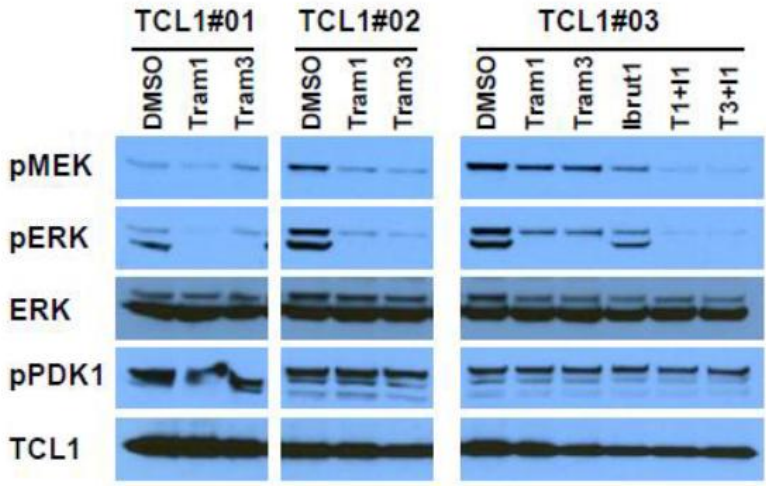


Figure 34. *Western blot analysis of TCL1 cells cultured with MEK and BTK inhibitors for 1 hour to study the reduction in phosphorylation.*

We then checked if Trametinib is able to reduce ERK phosphorylation *in vivo*. To this purpose, 3 mg/kg of Trametinib was administered to mice by oral gavage, and ERK status was followed 6 hour and 24 hours post administration. Single Trametinib dose completely inhibited ERK phosphorylation in 6 hours and inhibition lasted for at least for 24 hours in TCL1-tg mouse *in vivo* (Figure 35).

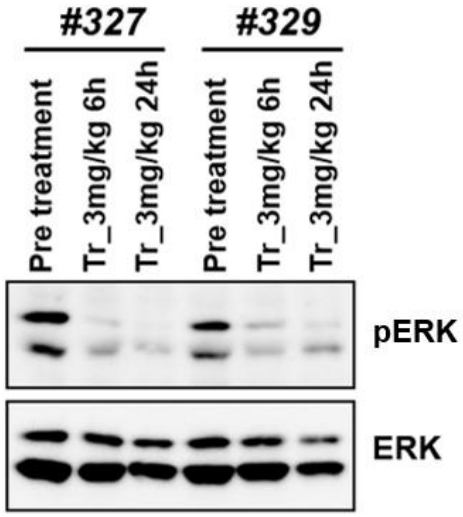


Figure 35. *Western blot analysis of ERK phosphrylation status of mice treated with Trametinib in vivo for 6 hours and 24 hours.*

To study a possible effect on survival by Trametinib *in vivo*, we used the TCL1 transgenic mouse spleen cells for adoptive transfer experiments. TCL1 leukemia cells represent more than 80% of the CD5/CD19 positive mononuclear cell population of the spleens of leukemic animals (Figure 36). For adoptive transfer, 10×10^6 TCL1 leukemia cells were resuspended in sterile 300 µL PBS, and injected intraperitoneally into syngeneic recipients.

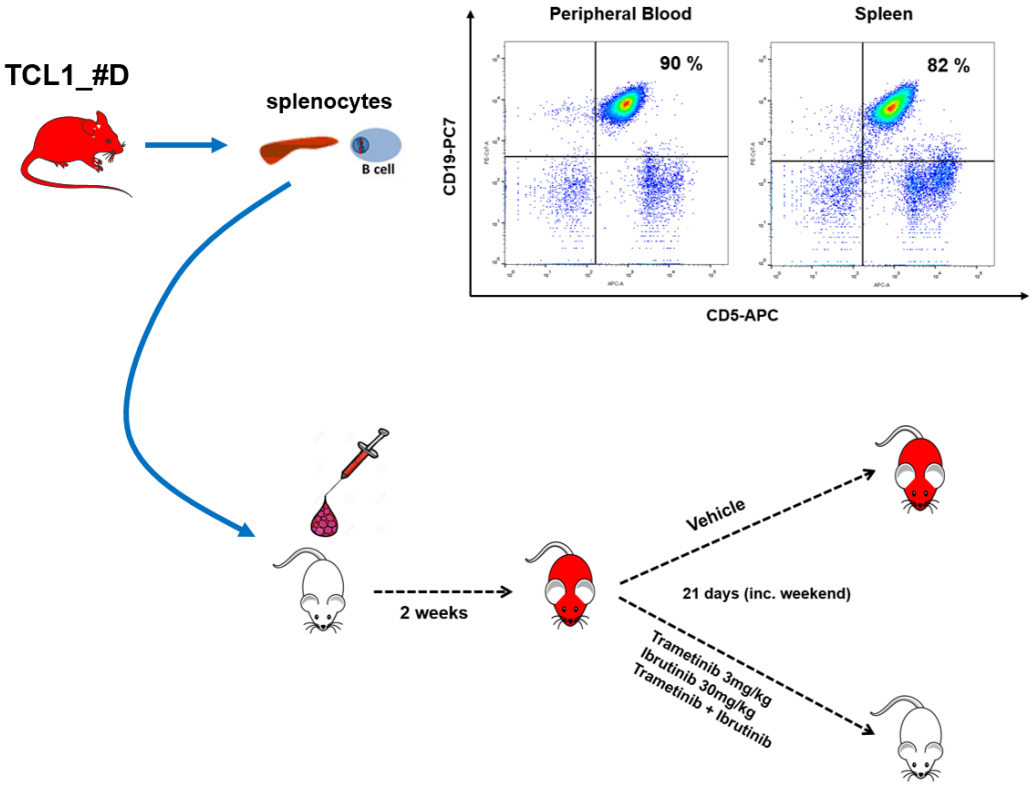


Figure 36 *The purity of the selected populations was evaluated by staining with anti-CD5-PE and anti-CD19-PE, followed by flow cytometric analysis. For adoptive transfer, 10 × 10^6 TCL1 leukemia cells were injected intraperitoneally into the syngeneic recipients. Two weeks after injection, mice were treated by oral gavage with the inhibitors for three weeks, as indicated.*

Two weeks later, on Day 15th of post TCL1 adoptive transfer, 3 mg/kg/day Trametinib, 30mg/kg/day Ibrutinib or both inhibitors were administered by oral gavage for 21 days. Animals were treated daily with the inhibitors, diluted in 200 µL of carrier solution.

Flow cytometric analysis were performed on peripheral blood samples of the TCL1 mice, withdrawn from the tail vein. Analysis were performed during and on the last day of treatment. We observed that Trametinib was able to delay the expansion of leukemic cells both after 2 and 3 weeks of treatment, though not reaching a significant difference (p=0.0683). Ibrutinib alone was very efficient to delay the leukemia (p=0.0002) though no synergistic effects were observed when both inhibitors were used together (Figure 37).

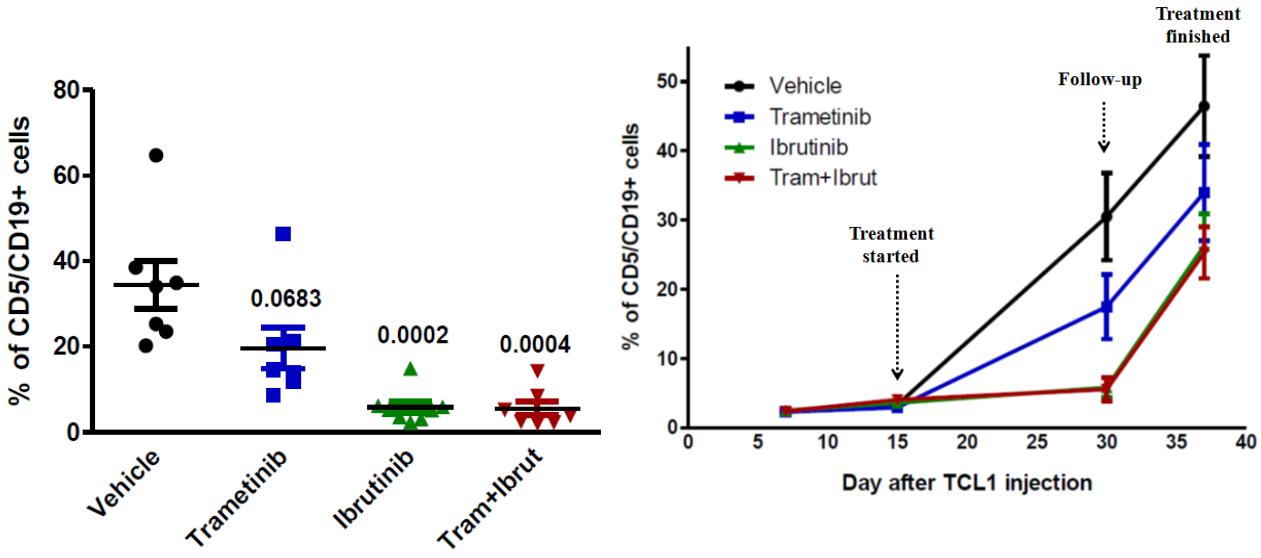


Figure 37. *TCL1 leukemic mice were treated for 21 days with 3 mg/kg/day Trametinib, 30mg/kg/day Ibrutinib or both. Inhibitors were administered daily by oral gavage for 21 days. Left:CD5/CD19 positive B cells among each mouse group, during treatment at day 15. Right: Leukemia follow-up before, during and after treatment completion.*

5. DISCUSSION

The ERK1/2 MAP kinase pathway is a central regulator of cell proliferation and controls both cell growth and cell cycle progression.

Synthetic MEK1/2 inhibitors, which prevents activation of ERK1/ERK2, were reported to inhibit the proliferation of various cell types, including fibroblasts, T lymphocytes, smooth muscle cells, hepatocytes and epithelial cell lines (***Dudley et al., 1995; Williams et al., 1998; Sebolt-Leopold et al., 1999; Talarmin et al., 1999***). Conversely, expression of constitutively active forms of MEK1 (***Brunet et al., 1994; Seger et al., 1994***) enhances the rate of cell proliferation. Small molecule inhibitors of MEK1/2 have been proposed that show activity in cell-based assays. Among these, Trametinib was recently approved as a single-agent by the FDA for the treatment of patients with BRAF mutated metastatic melanoma, in combination with BRAF inhibitors.

CLL is a disease with anergic B cell characteristics, that is characterized by constitutive phosphorylation of ERK (***Muzio et al, 2008; Apollonio et al, 2013***). In this thesis, we sought to investigate whether MEK1/2 inhibitor Trametinib could be exploited to efficiently target CLL cells *in vitro* and *in vivo*. Indeed we were able to show that trametinib is able to induce growth inhibition and cytotoxic effect in a number of leukemia and lymphoma cell lines which showed to be phosphor ERK positive. In aprticular Trametinib induced inhibition of cell growth, an increased G1 phase and a decreased S phase.

To prove the specificity of the inhibition and the relevance of the MAPK pathway in sustaining proliferation and viability of leukemic cells, we were also able to show substantial cytotoxicity and decrease in proliferation, when siMEK1 and siMEK2 were used in MEC1 cells line to deplete Mek1 and Mek2.

More importantly, we cultured primary human CLL cells expressing constitutive levels of pERK with effective dose of Trametinib, and observed a reduction in viability. Nevertheless, Trametinib efficacy was vey heterogeneous and interstingly it correlated with functional anergy of the cells and expression of phospho-ERK. Conversely, phospho-ERK negative CLL cells responded to a lesser degree to MEK inhibition. Positive and significant correlation was observed between ERK positivity and viability of the CLL cells, when

cultured with Trametinib. Higher levels of pERK positivity were associated with elevated cytotoxicity. This observation opens the interesting possibility of exploiting Trametinib as a new therapeutic option in CLL, at least in a subset of cases of the disease. To this end, the correlation with the levels of pERK suggest the possibility that a pre-therapy screening of the activation of the MAPK pathway may be proposed as a companion diagnostics for the use of MEK inhibitors in clinical trials exploring the use of such inhibitors.

To further strengthen this observation, we were also able to evaluate the efficacy of Trametinib *in vivo* in the TCL1 transgenic mouse model of CLL. Our preliminary data suggests that MEK inhibition is able to delay the leukemia expansion *in vivo,* though not reaching significance, probably due to a limited number of animals utilized in this pilot study. At present, we are following the impact of Trametinib treatment on TCL1 mouse survival.

In conclusion, during my stay at San Raffaele Institute, we preliminarily showed that in preclinical models (both *in vitro* and *in vivo*) a therapeutic effect can be observed when targeting the MAPK pathway, constitutively active in anergic B cells and in a subset of CLL patients. In particular, we showed that treatment with Trametinib significantly reduces the survival of a series of *pERK(+)* cell lines, human primary CLL and TCL1 mouse leukemic cells *in vitro*. We have also studied if Trametinib would potentiate the cytotoxic activity of BTK inhibitor Ibrutinib, both *in vitro* and *in vivo*, though with no success.

These data further underscore the relevance of the anergic pathway as a potential therapeutic target in CLL. As these new therapeutic option will progressively be evaluated, the challenge going forward will be to find effective combinations of MEK1/2 inhibition with other inhibitors in order to improve the quality and the depth of the clinical responses, aiming for a final cure of the disease.

SUMMARY

Chronic lymphocytic leukemia (CLL) represents the most common leukemia in the Western world. It is characterized by a neoplastic clonal expansion of CD5 B lymphocytes.

Deletion of the chromosomal 13q14 is the most frequent chromosomal aberration in CLL, but how it contributes to CLL pathogenesis and outcome of disease is still unclear. *miR-15a* and *miR-16-1*, located in the 13q14 region, are important for regulating levels of TP53 transcript and protein, As TP53 is a negative regulator of TERT, the catalytic component of telomerase, we hypotized that levels of TERT, are correlated with levels of *miR-15a/16-1* and TP53. During first part of my PhD thesis, we characterized CLL cases, with the sole 13q14 deletion and with no chromosomal abnormalities, for *miR-15a/16-1*, TP53 and TERT expression. We found that levels of *miR-15a* and *miR-16-1* were significantly lower in 13q14del CLL than in CLL with no chromosomal abnormalities and both miRNAs levels inversely correlated with TP53 expression. TERT levels inversely correlated with TP53 levels in 13q14del CLL, but not in CLL with normal cytogenetic profile and were significantly lower in the former group of CLL than in the latter one. Notably, disease progression was more rapid in both group of CLL with high TERT levels (>median), whereas high TP53 levels (>median) conferred better clinical outcome only in the subgroup of 13q14del CLL. Within the 13q14del CLL, TERT/ TP53 level profile identified subgroups of patients with different clinical outcomes. Within the 13q14del CLL, high TERT/low TP53 level profile is an independent marker of worse clinical outcome. Overall, these data indicate that in 13q14del CLL, the *miR-15a/miR-16-1* and TP53 axis may be an important pathway regulating telomerase expression, and TERT/TP53 profile may be particularly useful in refining the prognosis of patients with 13q14del CLL.

Relevant proportion of CLL patients show biochemical and functional features of anergic B cells, characterized by the lack of signalling capacity and constitutive activation of ERK1/2. Anergy is the mechanisms that the immune system adopts to silence autoreactive B lymphocytes. Therefore, it is reasonable to suggest that reversing the anergic state of these CLL might be beneficial in terms of clinical responses.

The aim of my PhD studies, regarding search for new therapeutic strategies, was to evaluate the therapeutic potential of agents that target ERK activity in CLL, *in vitro* and *in vivo*. MEK1/2 inhibitor Trametinib was recently approved as a single-agent for the treatment of metastatic melanoma. During my stay at San Raffaele Institute, we preliminarily showed that in preclinical models (both *in vitro* and *in vivo*) a therapeutic effect can be observed when targeting the MAPK pathway, constitutively active in anergic B cells and in a subset of CLL patients. In particular, we showed that treatment with Trametinib significantly reduces the survival of a series of *pERK(+)* cell lines, human primary CLL and TCL1 mouse leukemic cells *in vitro*. We have also studied if Trametinib would potentiate the cytotoxic activity of BTK inhibitor Ibrutinib, both *in vitro* and *in vivo*, though with no success. Our data underscored the relevance of the anergic pathway as a potential therapeutic target in CLL.

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