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Hypomethylation of Alu Elements in Chronic Lymphocytic Leukaemia

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ABSTRACT

CLL is a malignant disease characterized by the uncontrolled clonal proliferation of small, mature-appearing neoplastic dysfunctional CD5+ B lymphocytes in which is driven by changes in DNA methylation. Genetic alterations and signalling via surface immunoglobulin which constitutes a major part of the B cell receptor also contribute to CLL pathogenesis. The clinical outcome of CLL is heterogeneous with some patients requiring therapy immediately after diagnosis due to the rapid progression of the disease in contrast to others who do not require treatment for many years. IGHV mutational status, genomic changes and patient age should always be considered when choosing the right therapy. Retrotransposons such as Alus comprise more than 25% of the human genome and their hypomethylation has been shown in solid tumours. However, there have been scant considerations of their role in CLL carcinogenesis. Therefore, in this study, there has been a broad overview of the relationship between major cytogenetic aberrations and hypomethylation of retrotransposons while mentioning novel treatments that can be used. 13q.14.3, 17p13.1 and 11q22-q23 were the most found deletions in CLL patients. All deletions were found to be associated with DNA hypomethylation of retrotransposons at CpG sites influencing the function of DLEU1&2, RB1, TP53, and ATM. While various specific genomic events are seemed to be directly related to a differential clinical outcome and frequency between CLL patients that bear mutated-IGHV or unmutated-IGHV genes, until today very few studies have tried to link these genomic events to hypomethylation of retrotransposons, therefore future studies are necessary so to identify the exact mechanisms which subsequently can lead to new novel therapeutic approaches.

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INTRODUCTION

Chronic Lymphocytic Leukaemia (CLL) is a chronic lymphoproliferative disorder which it's characterized by the uncontrolled clonal proliferation of small, mature-appearing neoplastic dysfunctional CD5+ B lymphocytes. CLL sites mostly in peripheral blood, in lymph nodes, spleen and in the bone marrow which as a result induces serious events such as lymphocytosis, infiltration of leukemic cells into the marrow, lymphadenopathy and splenomegaly. While several studies suggested that genetic alterations and signalling via surface immunoglobulin (highly connected with B cell receptors) are the main causes for CLL genesis the exact aetiology until today remains unknown. As there are no treatments yet and the mortality rates are increasing year by year the importance to uncover the exact aetiology of CLL is imperative (Kipps et al., 2017) (Bosch and Dalla-Favera, 2019) (Rozman and Montserrat, 1995). Epidemiologically CLL accounts for ~300,000 newly cases each year and it's the most frequent form of cancer with 30% while Chronic Myeloid Leukaemia and Acute Myeloid Leukaemia is estimated at 20% respectively (REDAELLI et al., 2004) (Lihteh and Foster, 2021). Population-based studies in the Black-African populations indicated that CLL for males is about 4.9 and 2.4 for females per 100,000. In contrast, CLL in the white populations is 6.8 for males and 3.5 for females per 100,000 (Li et al., 2015) (Kipps et al., 2017). Therefore, the risk for the white populations in relation to the Black-African populations is significantly higher and that males are at a higher risk to develop CLL in contrast to females while the median age of diagnosis for CLL has been estimated at 64-70 years (Siegel et al., 2012) (Nabhan et al., 2014) (Li et al., 2015). This study by Kerber and O'Brien, 2005 has shown that the risk to develop CLL due to inherited factors is ~35% making the family history to be one of the best-characterized risk factors. Throughout the years is been introduced by the researchers that CLL can be distinguished into two subgroups which varies according to the clinical behaviour of the presence or absence of mutations in the immunoglobulin heavy chain variable region (IGHV) genes (Damle et al., 1999) (Hamblin et al., 1999). IGHV genes are specialized to encode a part of the B-cell receptor (Efremov, Turkalj and Laurenti, 2020) (Hengeveld, Levin, Kolijn and Langerak, 2021). CLL cells that express an unmutated IGHV are derived from B cells that haven't experienced differentiation in the germinal centres (Kipps et al., 2017). The germinal centres are transiently formed sites in the lymph nodes in which the B cells are experiencing somatic hypermutations in

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their IGHV genes while under an immune response (De Silva and Klein, 2015) (Kipps et al., 2017). The clinical outcomes and behaviour of CLL patients along with transcriptomic analysis have shown that those patients that express an unmutated IGHV will experience a more aggressive disease in relation to those patients with a mutated IGHV (Figure 1) (Darwiche, Gubler, Marolleau and Ghamlouch, 2018). This is because CLL cells are derived from B cells that have experienced differentiation in the germinal centres, express immunoglobulin and have undergone somatic hypermutation and/or class-switch recombination. Class-switch recombination is a process that is often found in B cells under an immune response to antigen (Kipps et al., 2017) (Rozovski, Keating and Estrov, 2018).

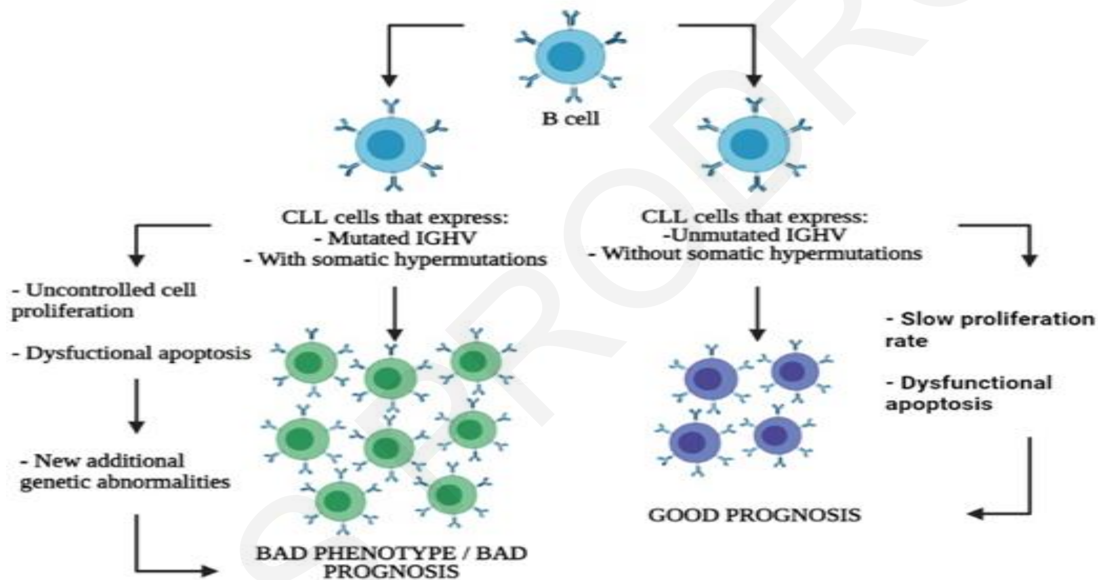

Figure 1: A clinical phenotype and behaviour model for CLL. This model describes the clinical phenotype for CLL patients whether they express a mutated IGHV or unmutated IGHV.

Both mutated and unmutated IGHV subtypes under comparative gene expression profile analysis seem to originate from antigen-experienced CD27+ B cells derived from germinal centres (Chiorazzi and Ferrarini, 2011) (Seifert et al., 2012). Conversely, this study by Seifert et al., 2012 has suggested that unmutated IGHV CLL cells resemble CD5+CD27− naive B cells whereas mutated IGHV cells resemble CD27+CD5+ B cells. Therefore, unmutated IGHV genes seem to originate from pre-germinal centre CD5+CD27− B cells and arise from naive B cells while mutated IGHV genes seem to originate from post-germinal centre CD27+CD5+ B cells

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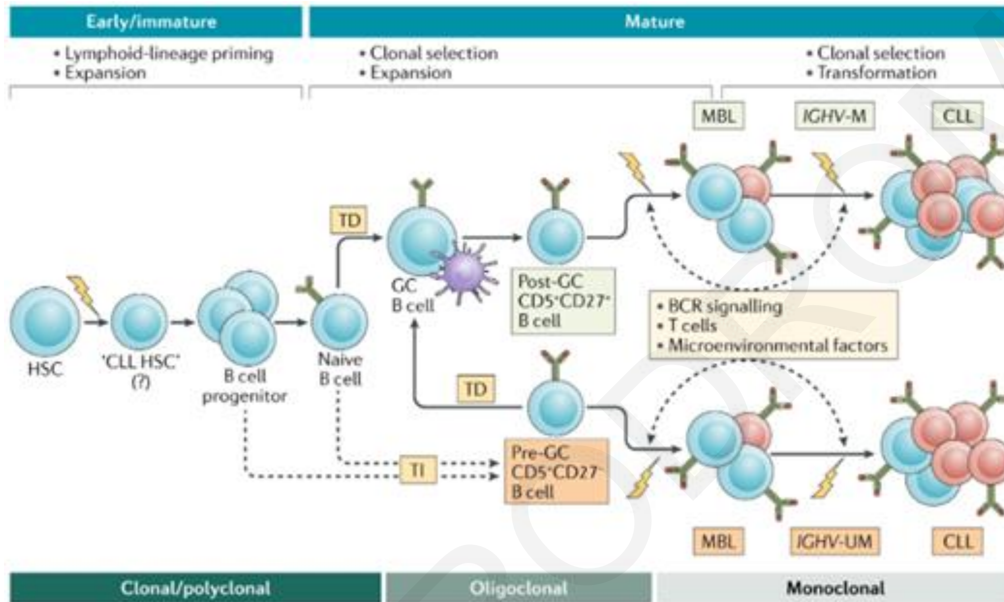
which undergo the germinal centre reaction. The cellular origin of CLL as explained above is not yet fully determined but the evolution from these progenitors to monoclonal B lymphocytosis can eventually cause CLL which is dictated by additional genetic and epigenetic abnormalities, B cell receptor stimulation, and microenvironmental factors (Figure 2) (Fabbri and Dalla-Favera, 2016).



<u>Figure 2:</u> The cellular origins of CLL cells. It's been suggested that all start from haematopoietic stem cells (HSCs) that acquire genetic and epigenetic alterations (lightning strikes). While this hypothesis is still under conflict (Damm et al., 2014) (Marsilio et al., 2017) it's been suggested that the cellular output is a polyclonal expansion of B cell progenitors. CLL cells with mutated IGHV originate from post-germinal centres - CD27+CD5+ B cells and CLL cells with unmutated IGHV originate from the pre-germinal centre - CD5+CD27− B cells. Unmutated IGHV CLL cells might arise from naïve B cells or by a T-cell independent process while mutated IGHV CLL cells might arise from CD5+CD27+ B cells that have undergone the germinal centre reaction. By additional genetic and epigenetic alterations (lightning strikes), the evolution from these progenitors to monoclonal B lymphocytosis (MBL) leads to CLL. The dashed arrows are hypothetical mechanisms of T cell-dependent antigen (TD) and T cell-independent antigen (TI) that could play a role in the rise of precursors CLL cells. The figure is taken by Bosch and Dalla-Favera, 2019.

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All these suggest that the mutational status of IGHV in leukemic cells is driven by distinctive epigenetic programming pathways, especially, the pre-germinal centres cells that express an unmutated IGHV in relation to the post-germinal centre of memory B cells that express a mutated IGHV (Tobin et al., 2002) (Ghia et al., 2008). This is further supported in recent years by new studies showing that many genes are epigenetically silenced in CLL. Therefore, continuous advances related to the molecular mechanism of DNA methylation in CLL cells can be very promising for discovering new novel diagnostic markers or innovative therapies (RAVAL, BYRD and PLASS, 2006). It is remarkable that back in 1999, there were two pioneer papers which have showed that CLL is driven by somatic mutations in IGHV genes and therefore they can be used as a predictive genetic tool for CLL while highlighting the importance of epigenetic programming in the progression of CLL (Damle et al., 1999) (Hamblin et al., 1999).

Hereditary Factors and Environmental Factors

As it's been already mentioned the genetic factors are not only playing a role in CLL pathogenesis but also contribute to the susceptibility of the disease. There are pieces of evidence from the CLL Research Consortium that 9% of the patients do have a relative with CLL. This percentage increases even more by another 8.5% in first-degree relatives (Cerhan and Slager, 2015). Several studies have identified by genome-wide association studies that single nucleotide polymorphisms (SNPs) might contribute to familial CLL pathogenesis. SNPs in CLL are involved in numerous genetic alterations and nearly 30 loci are been observed in familial CLL (Di Bernardo et al., 2008) (Crowther-Swanepoel et al., 2010) (Slager et al., 2011). Such an example of altered genes in CLL-associated SNPs was observed in IRF4 in which it's under-expressed in mice. These mice subsequently suffered from CLL due to hyperactivation of the Notch signalling (Shukla et al., 2013) (Shukla, Shukla, Joshi and Lu, 2016). Also, in BCL2 which encodes an anti-apoptotic protein in CLL patients, it was found to be overexpressed. BCL2 was followed with reduced expressions of mir-15a and mir-16-1 which both are involved in familial CLL (Calin et al., 2005) (Veronese et al., 2014). These two miRNAs can repress the ability of BCL2 even at low expression levels disrupting apoptosis and enhancing the B cell receptor signalling which contributes to B cells survival (Cimmino et al., 2005) (Chen et al., 2005). Also, mice that have an allele at the mir-16-1 locus have suffered from B cell lymphoproliferative disorders and subsequently with CLL

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(Raveche et al., 2007). Therefore, common genetic variations of familial CLL and altered expression of genes that are located close to SNPs can enhance the heritable risk and disease susceptibility.

The environmental factors is another aspect that needs to be fully determined on how it's associated with CLL. According to the National Cancer Institute, the environmental factors that are linked with the everyday lifestyle of individuals are thought to be linked in about 80-90% of the total cancer cases (Lindsey, 2005). In 2014, the US Department of Veterans Affairs concluded that exposure to agent orange (a herbicide) is a significant risk factor for CLL pathogenesis (Baumann Kreuziger, Tarchand and Morrison, 2013). Insecticide chemicals were also linked with CLL pathogenesis (Schinasi et al., 2015). In contrast, there was little evidence that viral infections, blood transfusions and dietary factors can increase the risk of CLL (Hjalgrim et al., 2015) (Kipps et al., 2017).

DNA Methylation

DNA methylation is an epigenetic mechanism that regulates gene expression by recruiting proteins involved in gene suppression or by inhibiting the binding of transcription factors to the DNA. DNA methylation was discovered closely to the year when DNA was firstly identified as a genetic material (Moore, Le and Fan, 2012). In 1948 a study that was performed by Rollin Hotchkiss showed a modified cytosine called 5-methylcytosine (5mC) that was separated from cytosine. He introduced that this process might happen naturally in DNA regulation (D. Hotchkiss, 1948). This observation was not fully supported by other scientists until 1981 in which further studies demonstrated that DNA methylation was involved naturally in gene regulation and cell differentiation (Holliday and Pugh, 1975) (Compere and Palmiter, 1981).

DNA methylation principally occurs at the cytosine bases of eukaryotic DNA in dinucleotide CpG sites. CpG sites are regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5' → 3' direction in which statistically are low-expressed throughout the genome but are concentrated in high densities in C + G rich

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regions (CpG islands) at gene promoters (De Smet et al., 1999) (Wilson, Power and Molloy, 2007). Interestingly, CpG dinucleotides have also been found within the intergenic and intronic regions of the DNA in which in these regions CpG sites are ~70-90% methylated and are estimated to be 0.75-1% of the total amount of bases in the DNA (Ehrlich et al., 1982) (Tuck-Muller et al., 2000). Today, researchers know that DNA methylation is the transfer of a methyl group (CH_3) from S-adenyl methionine to the carbon 5 of cytosine residue in CpG sites which subsequently forms 5mC. This epigenetic mechanism is catalyzed by DNA methyltransferase enzymes (DNMTs) which their main function is to regulate gene transcription, expression and suppress retrotransposition (Moore, Le and Fan, 2012). Some of the DNMTs can act either as de novo DNMTs in which they transfer CH_3 onto a DNA sequence or they can function as maintenance DNMTs by copying a CH_3 from an existing DNA sequence to a new after replication (Hervouet et al., 2018) (Jin and Robertson, 2012). The most important DNMTs are considered to be Dnmt3a, Dnmt3b and Dnmt1 in which are all important for the normal function of DNA methylation during cell differentiation, embryonic development, and gene expression regulation (Moore, Le and Fan, 2012) (Upchurch, Haney and Opavsky, 2016). This is further supported by this study by Suzuki & Bird, 2008 in which they showed that mice that lack DNMTs were followed with reduced methylation levels and died early in development.

Alterations in gene expression and cell differentiation can be linked with epigenetic changes. More specifically, when a disruption occurs in DNA methylation patterns then the development of any form of cancer is significantly increased (Figure 3) (Wilson, Power and Molloy, 2007). In recent years a large amount of research about DNA methylation on cancer and tumour suppressor genes is performed. This is because tumour suppressor genes are often silenced in cancer because of hypermethylation and/or because genomes of the cancer cells are often hypomethylated (Moore, Le and Fan, 2012).

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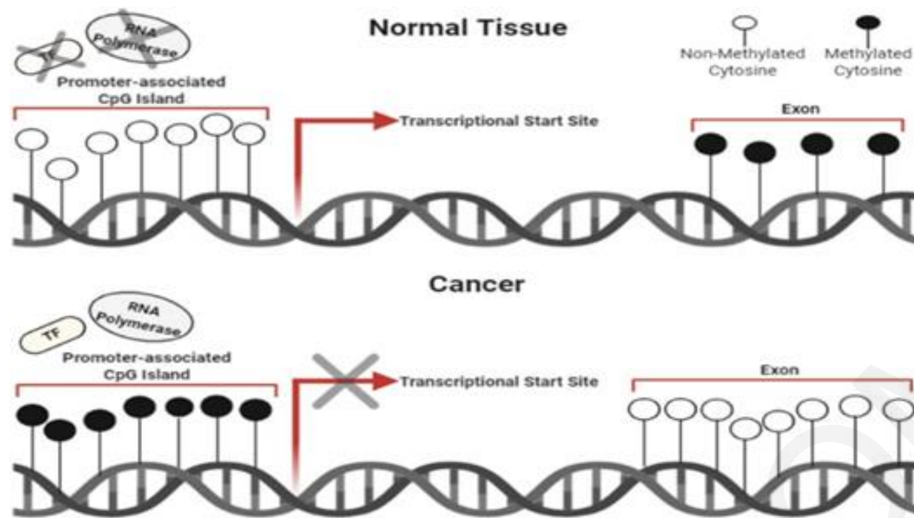

Figure 3: DNA methylation in healthy tissue and cancer. This diagram shows a genomic region of DNA in normal tissue and cancer. In cancer, global hypomethylation induces transcriptional re-activation of DNA sequences which are integrated into the genome contributing to mutagenesis and carcinogenesis. In a normal tissue DNA methylation prevents transcription factors (TF) and RNA polymerase from binding at the promoter regulating gene expression and suppress retrotransposition.

Hypomethylation of Alu elements

Retrotransposons, including LINE (long interspersed elements) and SINE (short interspersed elements), comprise a large portion of the mammalian genome. The ones that are mostly studied from LINE and SINE are LINE-1 and Alu elements (Mighell, Markham and Robinson, 1997) (Price, 2004). Alu elements (Alus) are primate-specific repeats which are ~300 nucleotides in length and comprise more than 25% of the human genome that influence genome evolution, regulation and disease (Figure 4) (Kim, Cho, Han and Lee, 2016) (Barrow et al., 2020). As it's been already introduced, they belong to the family of retroelements called SINEs and are considered as mobile elements because they have a copy number that exceeds more than 1 million copies in all the human genome (Lander et al., 2001) (Dewannieux, Esnault and Heidmann, 2003).

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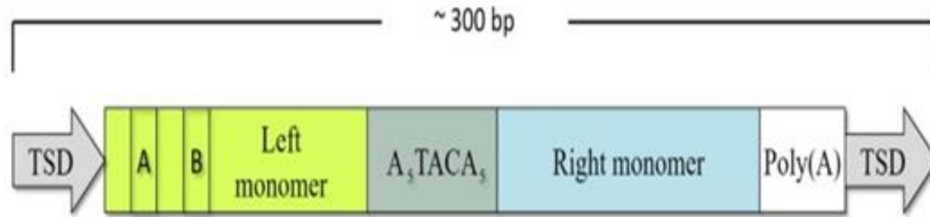

Figure 4: Schematic representation of an Alu element. Alu is composed with two monomers (left and right), a poly(A) tail and two boxes (A and B) in which they contain the promoter RNA polymerase III (binding sites) and through the target site duplication (TSD) it can insert into the human genome. The figure is taken by Kim, Cho, Han and Lee, 2016.

Although, while Alus are considered as retrotransposons and mobile elements only a small fraction of Alus is capable of generating new copies. This was mainly attributed to the fact that the oldest subfamilies of Alus such as AluJ have lost their ability to retrotranspose because of sequence changes over time whilst the newest members of AluS and AluY families have retained their ability to ‘’jump’’ and therefore generate new copies (Mills et al., 2006) (Wang et al., 2006) (Mills, Bennett, Iskow and Devine, 2007). Also, Alus are non-autonomous elements which means that when they replicate, they need trans-acting factors from the only activated family the autonomous human retroelements LINE-1 (Dewannieux, Esnault and Heidmann, 2003).

Alus are still activated throughout the human genome. Any somatic insertion events that may occur can eventually increase the progression of diseases through insertional mutagenesis and increase the genetic diversity. This happens because of the internal RNA polymerase III which potentially initiates transcription and develops RNAs that are responsible for the amplification of Alus (Deininger, 2011) (Deininger, Batzer, Hutchison and Edgell, 1992) (Roy-Engel et al., 2001). In contrast, RNA polymerase III is observed in very low levels because they are epigenetically silenced and therefore has been considered that a high amount of dispersed loci incapable to retrotranspose appear to be involved in the mechanism which as a result enhance the transcription of Alus (Ullu and Tschudi, 1984).

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Alus can be distinguished into subfamilies according to their sequence variants, their evolutionary heritage and their diagnostic mutations (Jurka and Milosavljevic, 1991). As it's been already introduced currently there are three subfamilies of Alus which are AluJ (oldest), AluS (intermediate) and AluY (youngest) (Mills, Bennett, Iskow and Devine, 2007). Of these, the AluY subfamily is considered the most active one in the human genome in contrast to the other two subfamilies. Interestingly, the Alu-Y subfamily has undergone tremendous expansions throughout the years developing further subgroups such as the AluYa5 and AluYb8 which are guided by "master" Alu copies and secondary "source" elements and therefore are responsible for the bulk of insertions in humans. Also, this ability by AluY elements to expand and evolve has been thought to be due to their stealth driven abilities making them able to escape from the negative selection which as a result produce new subfamilies that have high transposition activity (Han, 2005) (Ehrlich, 2009) (Ahmed, Li and Liang, 2013).

Also, de novo retrotransposition in Alus has been estimated to occur ~1 in every 21 births. These somatic retrotransposition are largely suppressed by epigenetic mechanisms that silence the expression of these elements. Also, hypomethylation of Alus has been observed in a range of environmental exposures such as benzene (Bollati et al., 2007) and tobacco (Breton et al., 2009) which this comes in accordance with the National Cancer Institute which stated that the environmental factors and the everyday lifestyle of individuals are highly connected with cancer cases (Lindsey, 2005).

Retrotransposition of Alus is very often and normally been prevented by DNA methylation. Genome-wide hypomethylation was one of the first-observed epigenetic abnormalities in human cancer genomes that was described, including hypomethylation of Alus (Feinberg and Vogelstein, 1983) (Ehrlich, 2009). In CLL and in other forms of cancers, deregulation of methylated retrotransposon is very common and helpful for patient prognosis (Benard et al., 2013) (Xiao-Jie et al., 2015). This is because hypomethylation is related to the re-activation of Alus that can eventually cause retrotransposition actions and activation of various oncogenes through locus-specific hypomethylation events and transcription within the retrotransposon elements from alternative transcriptional sites (Daskalos et al., 2009). At a genome-wide level, it can also induce chromosomal instability and further genetic defects which as a result can

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negatively alter patient prognosis (Sheaffer, Elliott and Kaestner, 2016). Therefore, hypomethylation is strictly related to the re-activation of Alus causing various retrotransposition events and subsequently carcinogenesis. Interestingly, the above events can eventually cause mutagenesis causing gene evolution for ~0.3% of all the human diseases (Ayarpadikannan and Kim, 2014) and subsequently carcinogenesis in epithelial tissues (Helman et al., 2014).

DNA hypomethylation can be either active or passive. Active DNA hypomethylation occurs in both dividing and non-dividing cells and it's a process that requires the Ten-Eleven Translocation enzyme family to process 5mC (Mayer et al., 2000) (Zhang et al., 2007) (Tahiliani et al., 2009). In contrast, passive DNA hypomethylation occurs only in dividing cells and it's a process that requires Dnmt1 which helps to maintain the methylation levels stable during cell replication (Bhutani, Burns and Blau, 2011) (Moore, Le and Fan, 2012). Its inhibition or dysfunction causes the newly formed cytosines to remain unmethylated and therefore the methylation sites in each cell division are reducing (Fan et al., 2001).

The active DNA hypomethylation pathway in CLL B cells has a critical impact on disease progression and therefore it is believed that it may be the key for causing CLL and an in-depth analysis could provide critical information that can be used for therapies (Bagacean et al., 2017). Various studies identified the significance of DNA hypomethylation in carcinogenesis. A study using DNA methylation inhibitors has introduced that hypomethylation can indeed promote the progression of tumours and tumour metastasis in cancer (Ateeq, Unterberger, Szyf and Rabbani, 2008). Another study by using mice has suggested that hypomethylation of transposable elements can alter the normal function of the genes and that by under ectopic induction of retrotransposons in various genes the incidence of cancer tumours was significantly increased (Waterland and Jirtle, 2003).

In CLL's epigenome, there is extensive evidence that hypomethylation under the progression of time it can auto-modify itself inducing genetic evolution and expression during the progression of the disease (Oakes et al., 2013). This is significant because treatments that exist to slow down the progression of the disease or treatments that are under development to treat CLL may not be functional for all patients. Although, until now there aren't any detailed research papers about the

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effect of global epigenetic changes on hypomethylation of Alus and how this causes the progression of CLL. Thus, this paper aims to unveil the epigenetic mechanisms of hypomethylated Alus that might be involved in CLL and mention possible novel treatments.

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Overview

The clinical outcome of CLL is heterogeneous with some patients being asymptomatic for decades whilst other patients need therapy immediately after diagnosis due to the rapid progression of the disease (Kipps et al., 2017). The need to develop treatments that can fully cure CLL patients is undeniable. There are novel therapies that have been discovered but their ability is limited as, they only moderately increase the overall survival of patients (Bagacean et al., 2016). Using these treatments, also known as treatment-free survival (TFS) as an endpoint, it helped to develop a list of highly significant prognostic factors for early diagnosis. These include the mutational status of IGHV, cytogenetic alterations, CD38 and epigenetic modifications (Nabhan, Raca and Wang, 2015) (Queirós et al., 2014) (Nabhan, Raca and Wang, 2015).

The epigenome in CLL is followed by high levels of widespread hypomethylation at the point of diagnosis which continues to evolve and mutate during the progression of the disease which highlights that epigenetic changes are associated with genetic evolution (Kulis et al., 2012) (Oakes et al., 2013). Also, deregulation of retrotransposon methylation is a very common observation in many cancers. Hypomethylation of Alu elements is associated with their re-activation and can lead to retrotransposition events which are most common in colorectal cancer, prostate, lung, and breast cancers (47-93%) and are biased towards hypomethylated regions of the genome (Tubio et al., 2014) (Xiao-Jie et al., 2015). CLL however is not only caused by epigenetic alterations but by genetic mutations as well. Studies by Oakes et al., 2013 and Landau et al., 2014 suggest that genome and epigenome are co-evolving during the progression of the disease which may be in part be the result of hypomethylation of retrotransposons leading to genomic instability. In contrast, while global hypomethylation of Alu elements has been widely reported in solid tumours, their epigenetic dysregulation is yet to be characterized therefore in this review we will focus on specific-primary cytogenetic aberrations that are linked with hypomethylation of retrotransposons of CLL patients.

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Cytogenetic aberrations and Hypomethylation of retrotransposons

As already mentioned, CLL shows global DNA hypomethylation. From these abnormalities, a huge fraction involves unbalanced translocations suggesting gains or losses of genomic material (Pfeifer et al., 2006) (Mayr et al., 2006) (Haferlach et al., 2007). This is further validated by other studies showing that by performing a comprehensive DNA methylation profiling there is a substantial intra-tumoral methylation heterogeneity. Methylation heterogeneity has been increasingly observed in CLL and it's been linked with genomic and methylome evolution (Kulis et al., 2012) (Pei et al., 2012) (Landau et al., 2014) (Puente et al., 2015). Also, CLL patients which have unmutated IGHV versus mutated IGHV generally have distinctive methylation patterns suggesting that epigenetic programming is crucial for the progression of the disease (Kipps et al., 2017). This also highlights that studying in-depth how hypomethylation of retrotransposons and key genes are interacting it can provide new therapeutic approaches.

Various specific genomic events are related to a differential clinical outcome and frequency between CLL patients that bear mutated-IGHV or unmutated-IGHV genes (Kröber et al., 2002) (Haferlach et al., 2007). Through the years recurrent chromosomal aberrations have been observed that are associated with CLL pathogenesis. Interestingly, chromosomal abnormalities can now be detected in more than 90% of the patients through the use of new improved protocols such as array comparative genomic hybridization. Some of the most important chromosomal aberrations that were detected are 13q14.3 deletion, 17p13.1 deletion and 11q22-q23 deletion. Remarkably, 80% of the total cases of CLL patients will carry at least one of the three chromosomal alterations (Döhner et al., 2000) in which somatic mutations such as TP53 and ATM were consistently observed in genes that have a role in DNA damage (Pleasance et al., 2009) (Fabbri et al., 2011) (Puente et al., 2011). Also, CLL was the first-ever human disease that was correlated with alteration in miRNAs such as mir-15a and mir-16-1 in which when they are repressed, they increase the development of CLL (Calin et al., 2002) (Bichi et al., 2002).

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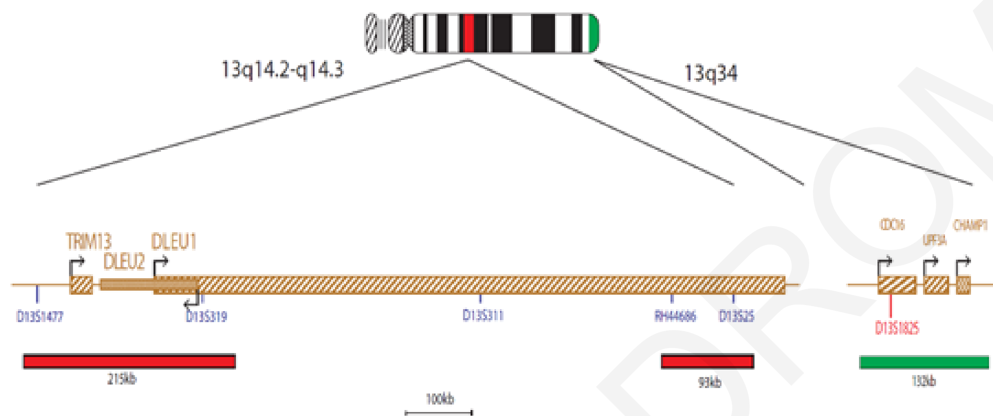
13q14.3 deletion

The most common chromosomal aberration in CLL is the deletion of 13q14.3 which is observed in 50% of the total cases (Figure 5).



<u>Figure 5:</u> Location of 17p13.1 deletion. In red colour, you can observe the location of the 13q14.2-q14.3 probe deletion and with green colour the 13qter sub-telomere. DLEU1 and DLEU2, both are located within the region of 13q14. RB1 is located between the genetic markers D13S319 and D13S25. The figure is taken by Intergenetics, 2021.

Often this deletion is interstitial and homozygous in almost 15% of the total cases (Döhner et al., 2000). Although, while it is observed in about half of the total cases, it's followed by the presence of mutated-IGHV and with good clinical outcome (>90% 5-year overall survival) (Döhner et al., 2000) (Kröber et al., 2002) (Haferlach et al., 2007). This study by Calin et al., 2002 has shown that many regions between 130 and 550 kb are followed by a minimal deleted region of 29 kb between the exons 2 and 5 of DLEU2 (act as tumour suppressor gene) which is strictly related to lymphoma. Interestingly, DLEU1 is considered to be the most likely CLL-associated candidate tumour suppressor gene within the 13q14 region. The 13q14 deleted region comprises two miRNAs (hsa-mir-16-1 and hsa-mir-15a), including the coding-region retinoblastoma gene (RB1) (Figure 5).

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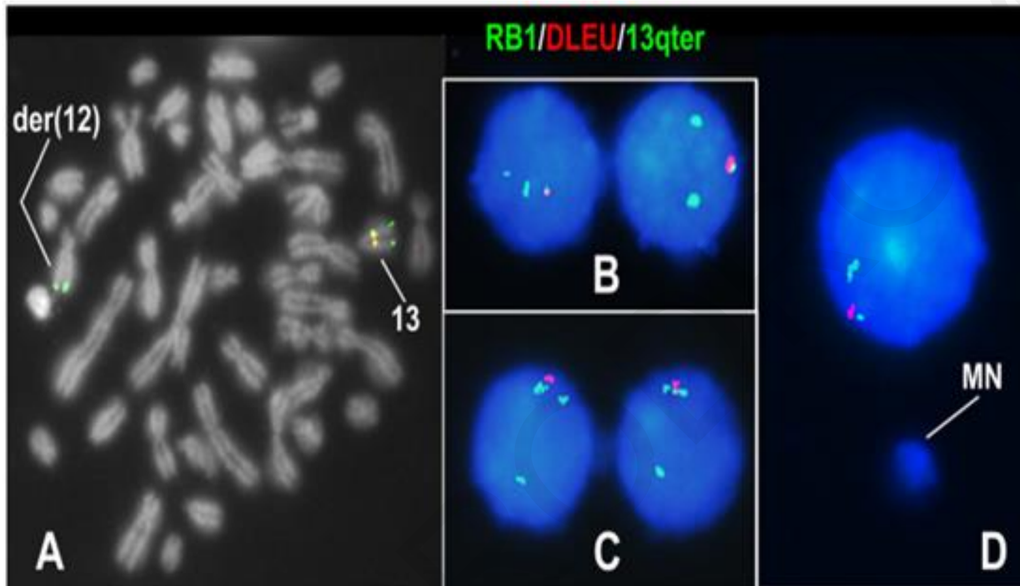
Hsa-mir-16-1 and hsa-mir-15a deleted regions are found in almost 70% of CLL cases. miRNAs are very important for the human body due to their ability to bind messenger RNAs and determine their degradation or inhibition of translation (Valencia-Sanchez, 2006). This is further supported by this study from Nava-Rodríguez et al., 2019 in which they showed a heterozygous deletion of approximately 2.6 megabase pair on chromosome 13 which included both DLEU1 &2 and RB1 in CLL patients (Figure 6).



<u>Figure 6:</u> Fluorescence in situ hybridization (FISH) to study DLEU, 13q sub-telomere and RB1. A, normal chromosome 13 shows the signals DLEU (red), 13q sub-telomere (green) and RB1 (green) whilst it's observed a 13q sub-telomere on the der (12) chromosome. B-C, binucleated cells and D, micronucleated cells shows deletion of RB1-DLEU. The figure is taken by Nava-Rodríguez et al., 2019.

This cytogenetic aberration on chromosome 13 is linked with hypomethylation of retrotransposons at CpG sites. It's have been suggested that deletion of both hsa-mir-16-1 and hsa-mir-15a can promote activation of tumour-promoting genes which might be an important consequence of DNA hypomethylation and oncogenesis. This study by Benetti et al, 2008 has shown that miRNAs can regulate the expression of DNMTs and histone modifications which as a result contributes to the regulation of DNA methylation. Alterations in the expression of miRNAs have been proposed to play a role in the downregulation of the Dicer gene and

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subsequently in the rapid progression of CLL. Dicer is an RNase III family nuclease that is involved in the initiation of RNA interference by generating small RNAs and which is involved in many silencing pathways (Reza Farzaneh et al., 2016). Therefore, this study examined Dicer by performing chromatin immunoprecipitation (ChIP)–immunoblot assays and they showed that there is a significant reduction in chromatin domains suggesting a loss of DNA methylation in the absence of Dicer1 (Figure 7). The loss of DNA methylation in Dicer1 null cells was the direct consequence of decreased Dnmt1, Dnmt3a and Dnmt3b mRNA levels (Figure 8) indicating that miRNAs might regulate the expression of DNMTs and histone modifications which as a result contributes to the regulation of DNA methylation.

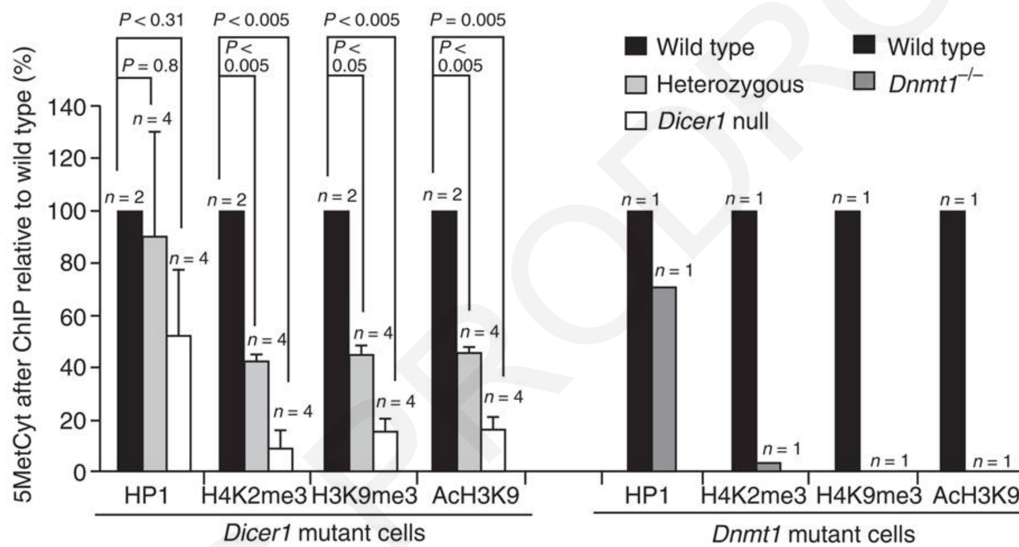

Figure 7: **DNA methylation levels at chromatin regions H4K2me3, AcH3K9 and H3K9me3.** A Chromatin immunoprecipitation (ChIP)–immunoblot assay revealed significantly decreased levels at H3K9me3, H4K2me3 and AcH3K9 bound chromatin, in Dicer1-null cells compared to the wild type controls. Interestingly HP1 bound chromatin which binds to the methylated H3K9me was not significantly decreased in Dicer1-null cells. Dnmt1−/− cells were used as a control. The figure is taken by Benetti et al, 2008.

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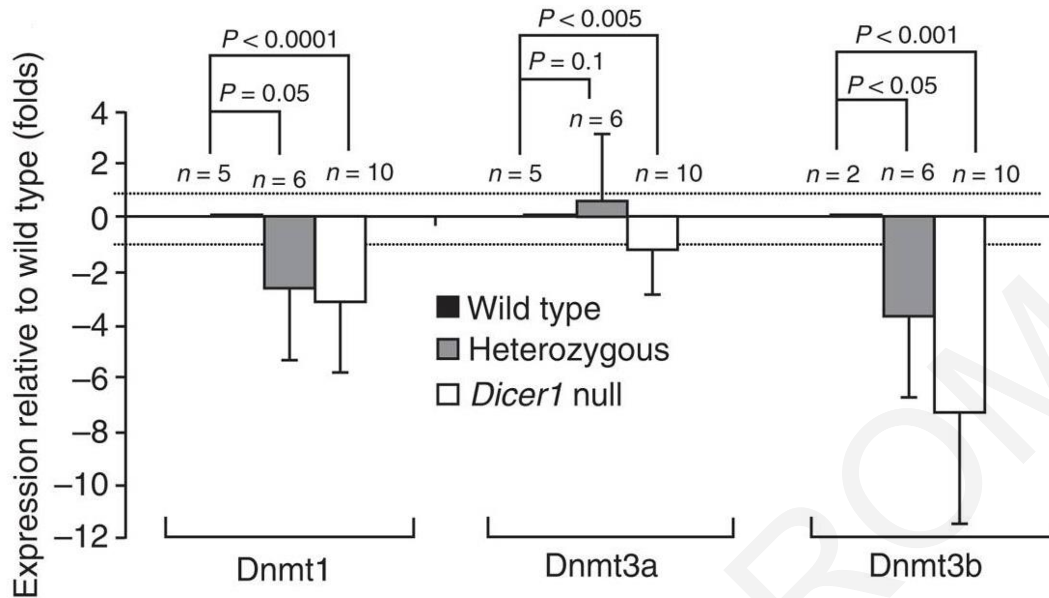

Figure 8: Decreased DNMTs mRNA levels in Dicer1-null cells. A real-time quantitative RT-PCR analysis revealed a significant decrease in the expression of Dnmt1, Dnmt3a and Dnmt3b mRNA levels in Dicer1 null cells in relation to the wild type controls. The figure is taken by Benetti et al, 2008.

They also demonstrated that by overexpression of Dnmt1, or both Dnmt3a and Dnmt3b, in Dicer1-null cell's DNA methylation can be rescued. (Figure 9). These cells under overexpression were able to recover sub-telomeric DNA methylation as well as global DNA methylation indicating that loss of DNA methylation is the consequence of the altered DNMTs expression. Also, increased expression of Retinoblastoma family proteins in Dicer1 null cells has been found when Dnmt3a and Dnmt3b were downregulated (Figure 10). Increased levels of retinoblastoma proteins such as RB1 is responsible for repressing promoters by associating with histone deacetylases and decreasing promoter acetylation which subsequently causes loss of DNA methylation (Brehm et al., 1998).

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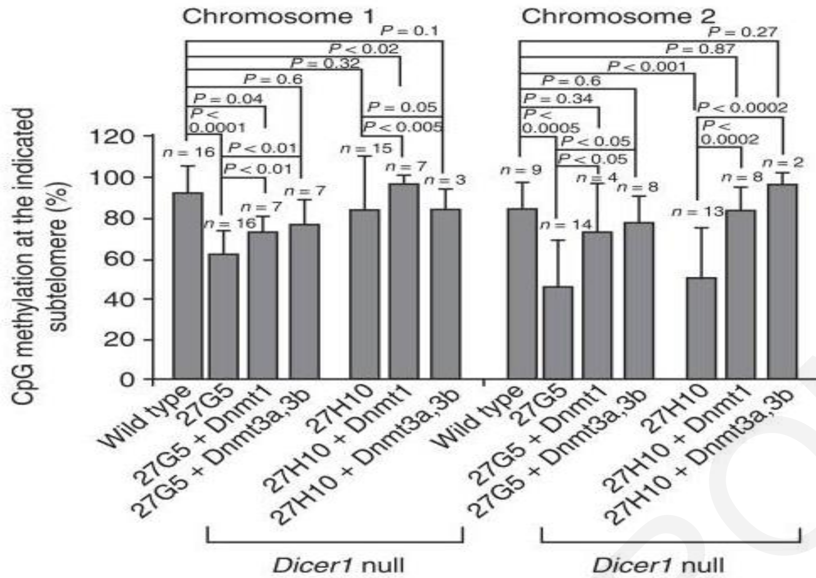
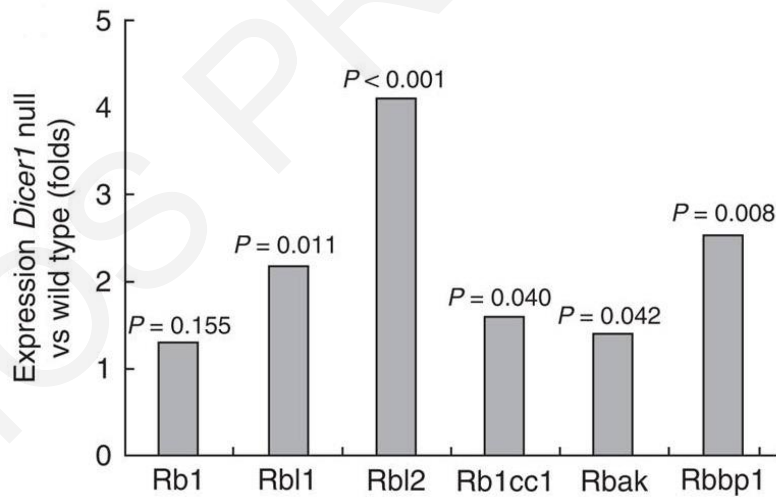


<u>Figure 9:</u> DNMTs can rescue DNA methylation. Quantitative RT-PCR analysis revealed that Dicer1-null cells under overexpression of Dnmt1, or both Dnmt3a and Dnmt3b enzymes can largely recover CpG methylation at the indicated sub-telomeres. The figure is taken by Benetti et al, 2008.



<u>Figure 10:</u> Downregulation of Dnmt3a,3b induces the induction of retinoblastoma family proteins. Reduced gene expression of Dnmt3a and Dnmt3b in Dicer1 null cells induced increased expression of retinoblastoma family genes Rbl1 and Rbl2, Rb1cc1, Rbak and Rbbp1. Rbak encodes a putative mediator of E2f-mediated transcriptional activation which is crucial for

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the control of cell division. Rbbp1 mediates histone deacetylases (HDACs) which brings transcriptional repression of E2f-dependent promoters. The figure is taken by Benetti et al, 2008.

Also, this study by Sinkkonen et al, 2008 has shown in mouse embryonic stem cells under Dicer knockout a disruption of miRNAs such as miRNA-290. miRNA-290 is significant because it regulates the expression of Dnmt3a and Dnmt3b. The miRNAs disruption induced DNA hypomethylation and repressed levels of histone methylation H3K9 causing the embryonic stem cells to grow very slowly. Although, under ectopic expression of de novo DNMTs or by transfection of the miR-290 cluster miRNAs into the embryonic stem cells the growth phenotype was rescued and DNA methylation was increased indicating that promoter methylation defects are due to repressed DNMTs (Figure 11). Both studies come in accordance with what we explained earlier about miRNAs, DNMTs and DNA methylation.

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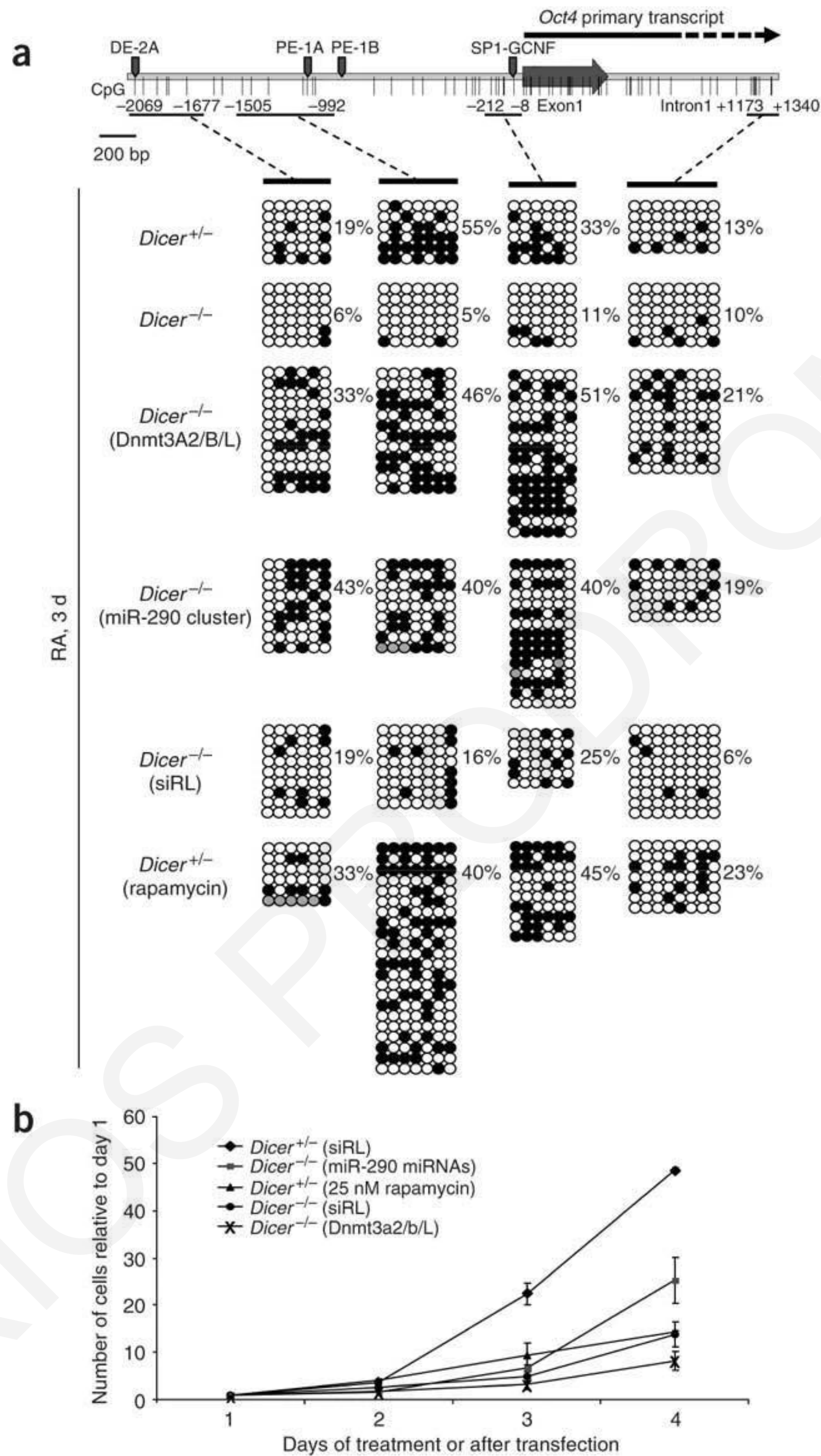

<u>Figure 11:</u> Dicer-null embryonic stem cells growth phenotype and DNA methylation was rescued under ectopic expression on the Oct4 promoter. (a) CpG methylation analysis in four different regions (SP1-GCNF, PE-1A, PE-1B and DE-2A) of Oct4. The scheme identifies positions of bisulfite-sequenced regions relative to the Oct4 transcription initiation site. Under

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ectopic expression with plasmids expressing EGFP-Dnmt3a2, EGFP-Dnmt3b and EGFP-Dnmt3L, Dicer-null embryonic stem cells (Dicer−/− cells) showed an increased de novo DNA methylation when treated with retinoic acid for 3 days. Transfection with miR-290 cluster miRNAs Dicer-null embryonic stem cells (Dicer−/− cells) had also increased levels of de novo DNA methylation. Dicer-null embryonic stem cells (Dicer−/− cells) treated with siRL (small interfering RNA against Renilla luciferase mRNA) showed no significant DNA methylation increase. (b) Proliferation of Dicer +/− cells and Dicer−/− cells under different treatments. Same numbers of Dicer +/− and Dicer−/− cells were transfected with plasmids expressing Dnmt3a2, Dnmt3b and Dnmt3l, miR-290 cluster miRNAs, siRL or rapamycin. Dicer−/− cells transfected with miR-290 cluster miRNAs rescues the growth phenotype more than the other ones. The figure is taken by Sinkkonen et al, 2008.

Therefore, 13q deletion and the mutational status of IGHV are crucial factors for the progression and the clinical outcome of CLL (Ehrlich, 2002). In contrast, this study by Rossi et al., 2013 explored the expression profile of the entire miR-15a and miR-16-1 clusters evaluating possible clinicopathological correlation and they found that 13q deletion does not strictly correspond to miRNAs downregulation in CLL suggesting that further studies are necessary.

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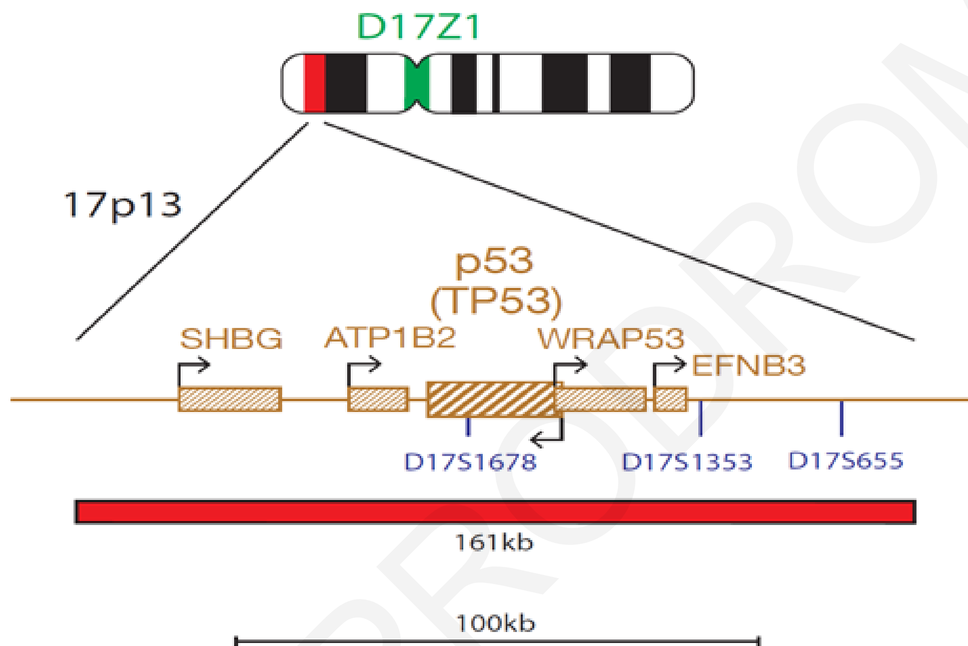
17p13.1 deletion

Another recurrent chromosomal aberration is the deletion of 17p13.1 which is observed in a small fraction of CLL patients (7% of the total cases) (Dijkstra et al., 2008) (Figure 12).



<u>Figure 12:</u> Location of 17p13.1 deletion. In red colour, you can observe the location of the 17p13.1 probe deletion along with TP53. With green colour you can observe the genetic markers D13S319 and D13S25. The figure is taken by Intergenetics, 2021**.**

While it is very low observed it has the worst prognosis (35% 5-year overall survival) in contrast to all the genetic lesions together, signifying how serious this aberration is (Döhner et al., 2000). This deletion is followed by the presence of unmutated IGHV (Tam et al., 2009) (Delgado et al., 2014). The 17p13.1 deletion affects TP53 which it's a known transcription factor and it's involved in various processes such as triggering cell apoptosis and/or cell-cycle arrest maintaining that way genome integrity and preventing clonal evolution (Figure 13) (Harris and Levine, 2005) (Chang, Jiang and Qi, 2010) (Delgado et al., 2014).

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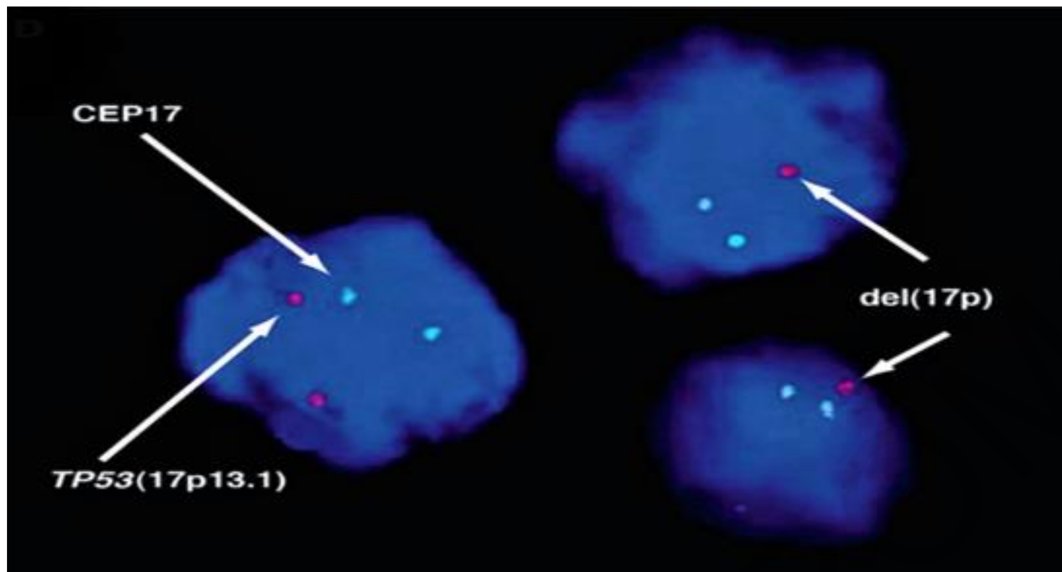
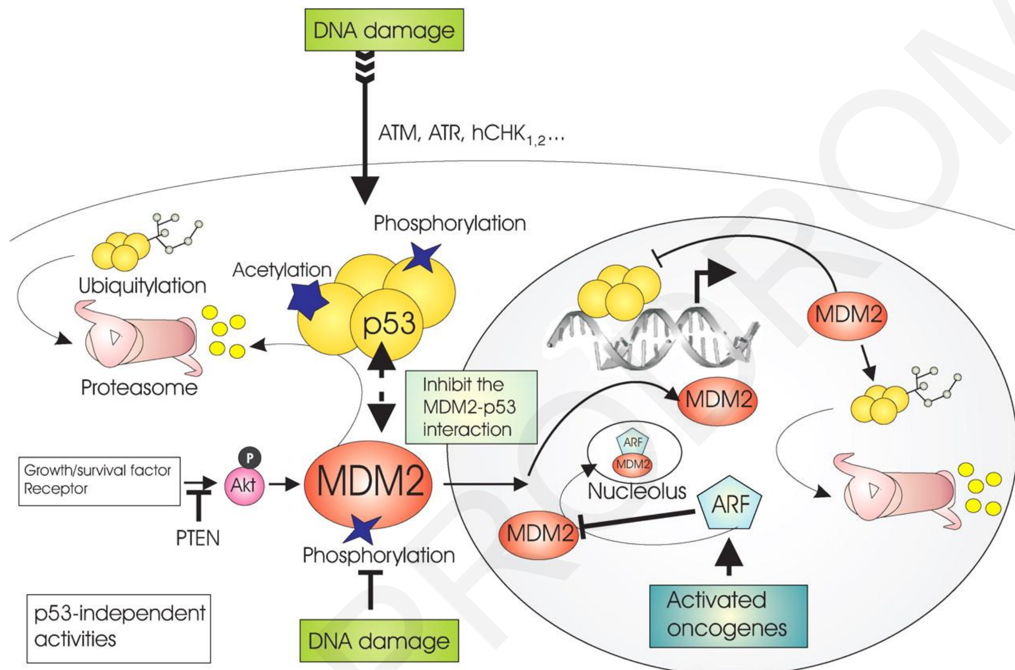


<u>Figure 13:</u> Detection of 17p13.1 deletion by fluorescence in situ hybridization (FISH). CLL cells present a hemizygous deletion of the 17p13 locus which hosts several genes with important regulatory functions, including the TP53 which becomes deleted as of a consequence of the aberration. Magnification: 400x. The figure is taken by Chang, Jiang and Qi, 2010.

TP53 is regulated by the murine double minute-2 gene (MDM2). MDM2 is regulated in part by a TP53 responsive promoter. In cells that have a functional TP53, the MDM2 inhibits by direct and tonic interaction its activity (Vassilev et al., 2004). Therefore, by using inhibitors of the MDM2-TP53 interaction in tumour cells it can promote the levels of TP53 and eventually the induction of cell cycle arrest and apoptosis (Figure 14) (Stommel and Wahl, 2004). Such a treatment that can inhibit the MDM2-TP53 interaction is called nutlin and it might be a new therapeutic strategy for CLL patients (Secchiero et al., 2006). This study by Secchiero et al., 2006 has shown that Nutlin 3 triggers apoptotic pathways in B-CLL cell samples. From the 29 B-CLL patient samples 28 showed to be vulnerable to nutlin 3 cytotoxicities. Further experiments showed that 10 B-CLL samples expressed p53 due to nutlin 3, although only a minority expressed MDM2 (Figure 15). This phenomenon was attributed to the B-CLL cells since all control samples displayed normal induction of MDM2. Also, cytotoxicity on B-CLL cells which was induced by nutlin 3 was tested whether it was caused by apoptosis. They exposed B-CLL cells to nutlin 3 (10 μM) for 24 hours. They found a significant increase in the annexin-V–positive cells (indicate cells in late apoptosis) suggesting that nutlin 3 causes

cytotoxicity through apoptosis (Figure 16). Interestingly, the cytotoxic activity of nutlin-3 in B-CLL cells is enhanced by combinations of treatments such as fludarabine or chlorambucil in concentrations that are minimally toxic toward normal B lymphocytes which are very important. Although, while these data are promising their observations require further preclinical evaluation for the treatment of CLL.



<u>Figure 14:</u> Molecular interaction between MDM2-TP53. MDM2 is expressed through the interaction with p53. MDM2, as a result, inhibits p53 activity because it stimulates its degradation in the nucleus and the cytoplasm which as a result blocks its transcriptional activity. Various DNA damaging agents or altered oncogenes can induce the p53 activation due to the promotion of p53 and MDM2 phosphorylation. Also, activated oncogenes cause the activation of ARF proteins which interact with MDM2 sequestering it into the nucleolus preventing p53 degradation and promoting apoptosis, growth arrest, DNA repair, and senescence. Conversely, Akt activation enables the nuclear import of MDM2 which destabilizes p53. The figure is taken by M Moll and Petrenko, 2003.

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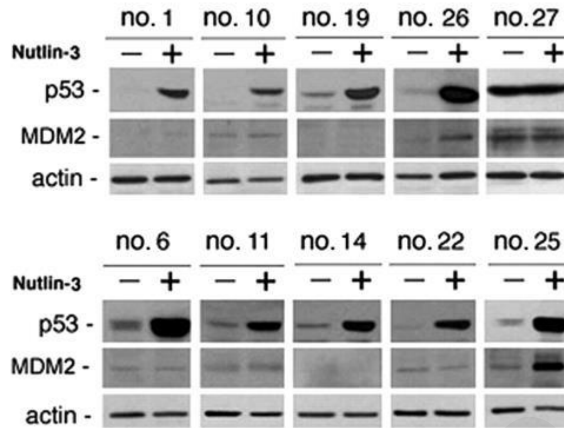

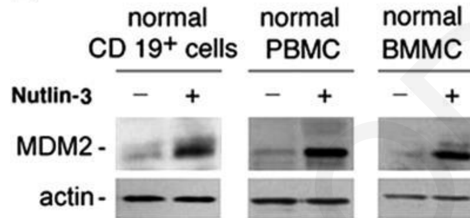

<u>Figure 15:</u> MDM2-p53 interaction induction under nutlin 3 treatment. (A) Western Blot analysis for 10 patient samples showed a variable expression of p53 and low expression of MDM2 (nos 25, 26). (B) Investigation of normal cells (CD19+ B cells, PBMCs, and BMMCs) indicated normal expression of MDM2. High constitutive and unmodified level of patient number 27 indicates p53 mutation. The figure is taken by Secchiero et al., 2006.

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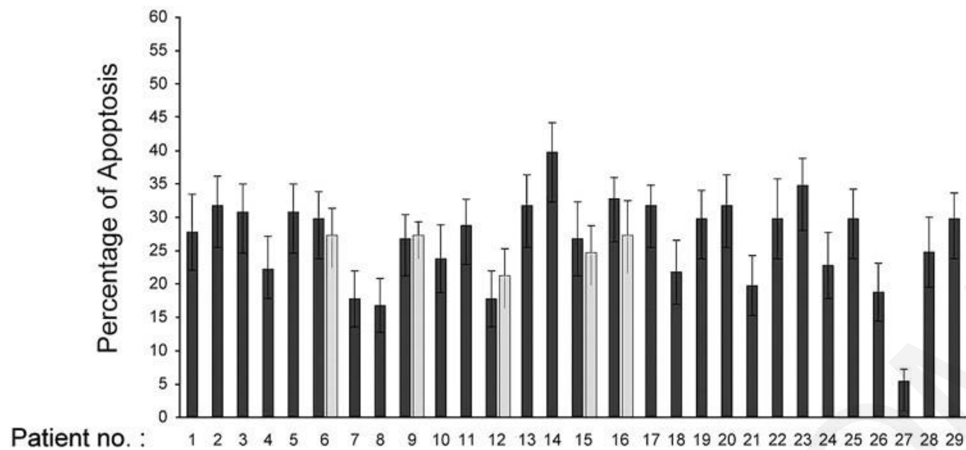

Figure 16: Nutlin 3 causes cytotoxicity through apoptosis. B-CLL cells were exposed to nutlin 3 for 24 hours stained with V–FITC/PI and subsequently analyzed by flow cytometry. Induction of apoptosis in nutlin 3 treated B-CLL samples, calculated in a significant variable ($P < .05$) increase in the percentage of annexin-V–positive cells. Gray bars indicate 5 B-CLL patient samples retested for response to nutlin-3 showing similar results. The figure is taken by Secchiero et al., 2006.

About 75% of CLL cases with 17p13.1 deletion are followed by TP53 mutations. Except for TP53 deletions and mutations, there are other mechanisms that might be involved in the disruption of TP53 (Shanafelt et al., 2006) (Stilgenbauer et al., 2007). Interestingly, in 10% of CLL patients, TP53 is mutated, which can be inactivated by the insertion of somatic mutations which can occur either by the presence or absence of genomic loss (Dal-Bo et al., 2009). CLL patients with inactive TP53 are followed by a poor response to different chemotherapies such as alkylating agents and purine analogues (Hewamana and Dearden, 2011).

These studies by Murphy et al., 1999, Blobel, 2000, Fuks et al., 2001 and Nasr et al., 2003 have shown that TP53 can influence DNA methylation through short-range interactions, as both reside in complexes with histone acetylases or deacetylases. In fibroblasts from Dnmt1 knockout mice, they found that it triggers loss of genomic DNA methylation, apoptotic and epigenetic deregulation, and mobilization of retroelements such as Alus (Jackson-Grusby et al., 2001). Also, Jackson-Grusby et al., 2001 has shown that when Dnmt1 is inactive it causes hypomethylation and p53-dependent apoptosis (Figure 17). Loss of Dnmt1 blocks DNA

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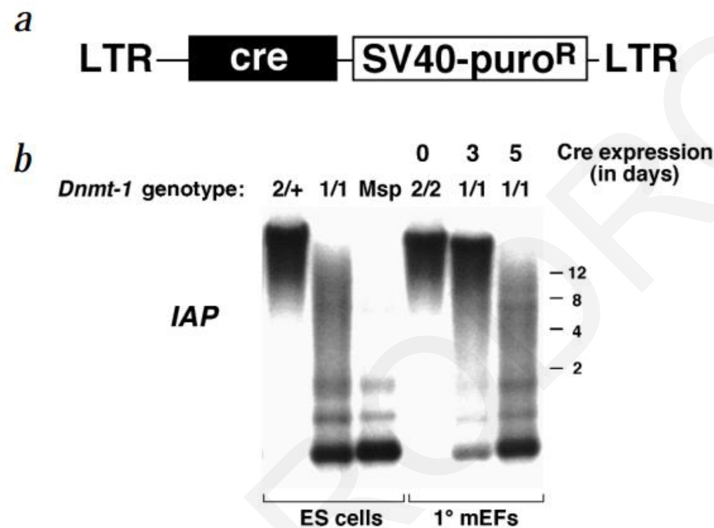
replication and apoptosis through p53 leading to genetic instability. Genetic instability was observed by the deregulation of several genes such as Tdag, Xist, H19, Igf2, Grb10, Peg3 and repetitive elements (IAP, L1, G7e) (Table 1). Hypomethylation can cause DNA damage and subsequent cell death. Although this study didn't identify how hypomethylation causes p53-mediated cell death in which it would be very important for understanding how tumour cells acquire global hypomethylation. It's worth mentioning that global hypomethylation and p53 mutational inactivation were also observed in human tumours by other studies.



<u>Figure 17:</u> Somatic cell cultures for conditional inactivation of Dnmt1. (a) A schematic of the cre retroviral vector that was used. Primary embryonic fibroblasts from mouse strains were obtained to study hypomethylation. A cre retroviral vector was used in vitro for cre-mediated recombination by infection of fibroblast cultures. Cre gene subcloned in the retroviral vector pMX-puro allowing selection of the retrovirally infected cells. (b) Methylation status by western blot analysis of Dnmt1. Genomic DNAs were probed with an IAP probe. The methylation status of Dnmt1 fibroblasts infected with pMXpuro vectors is shown. Cre-expressing fibroblasts showed progressive hypomethylation when at 3- to 5- days post-infection because of Dnmt1 inactivation. The figure is taken by Jackson-Grusby et al., 2001.

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<u>Table 1:</u> Deregulation of several imprinted genes by hypomethylation in fibroblast cultures. GeneCHIP assay on Dnmt11lox/1lox (mutant cells) and Dnmt12lox/2lox (control cells) was compared to each other (infected with pMXCP and pMXpuro, respectively) as both SV40-transformed and Trp53-mutant fibroblasts. Genes induced more than two-fold with a p-value ≤0.05 were added and categorized based on function. The green colour indicates the deregulated genes and the red colour indicates overexpression.

Gene	Accession Number	Fold Δ	Description	Gene	Accession Number	Fold Δ	Description
Methylation				**Apoptosis/Stress response**			
Ahcy	L32836	3.0	SAH hydrolase	Hif1a	X95580	3.9	hypoxia inducible factor
Dnmt1	X14805	-39	DNA methyltransferase	Traf3	U33840	3.7	Tnf receptor-associated
Imprinted				Hsp25	I07577	3.2	heat shock protein
Tdag	U44088	4.4	TDAG51	Hsp105	D67016	2.7	heat shock protein
Xist	L04961	3.5	Xist	Traf4	X92346	2.5	Tnf receptor-associated
Gene	Accession Number	Fold Δ	Description	Gene	Accession Number	Fold Δ	Description
Peg3	U48804	-2.4	Zn-finger protein Pw1 gene	Adprp1	aa119245	2.4	pADP-ribose polymerase
Grb10	U18996	-3.1	growth factor receptor-b.p.	Bag1	AF022223	2.1	bcl-2 binding protein
Igf2	U71085	-4.5	insulin-like growth factor 2	**Cell cycle**			
H19	X58196	-8.9	H19	Cdkn1a	U09507	3.7	cyclin kinase inhibitor
Retroviral/Repetitive elements				Chek1	AF016583	3.5	checkpoint kinase
IAP	C78676	47.4	intracisternal A particle	Rpa2	D00812	3	Rad51 homolog
G7e	U69488	4.6	viral envelope protein	Ccng2	af005885	2.6	cyclin G2
L1Md-Tf14	D84391	3.1	L1 repeat	Cdc25a	U27323	2.5	cell division cycle 25A
IAP	AF003867	-2.3	intracisternal A particle	Gas2	M21828	-3	growth arrest specific
Chromatin/silencing				Cks1	AA032836	-4.4	cyclin kinase regulation
Slbp	U75680	3.7	histone stem-loop b. p.	**Tumor Suppressor/oncogene**			
Hmgi	J04179	2.4	nonhistone HMG protein	Junb	U20735	13.4	Jun-B oncogene
Sin3a	u22394	2.4	SIN3 yeast homolog A	Ect2	L11316	4.1	Ect2 oncogene
Tif1b	U67303	2.3	TIF1B	Csk	U05247	2.6	c-Src kinase
Cenpa	AA060808	-2.0	centromere autoantigen A	Catnb	M90364	2.3	b-Catenin
Hmg1	Z11997	-2.1	HMG protein 1	Dp1	U28168	2	adenomatosis polyposis coli
DNA recombination/Repair				Rela	M61909	2	v-rel oncogene homolog A
Tdg	aa407018	3.9	Thymine-DNA glycosylase	tsg101	U52945	2	tumor susceptibility gene
Rad52	U06837	2.3	RAD52 homolog	Nmyc1	M29211	-251	Nmyc1
Mre11a	U58987	2.2	putative endo/exonuclease	**Growth Factor/receptor/signaling**			
Pold1	Z21848	-3.8	DNA polymerase delta 1	Gro1	J04596	77	GRO1 oncogene
Transcription factor				Scya2	M19681	26.4	small inducible cytokine
Pea3	X63190	10.3	polyoma enhancer activator	Ereg	D30782	11.1	epiregulin
Nfe2l2	U70475	4.8	erythroid nuclear factor2	Jak2	L16956	6.4	janus kinase 2
Zfp147	D63902	4.1	zinc finger protein	Sema3c	X85994	5.2	semaphorin E
Zfp36	M58691	3.8	zinc finger protein	Pdgfa	M29464	5.2	PDGFa
Klf4	U20344	3.3	gut, Kruppel-like factor	Sfrp1	U88566	4.4	frizzled related
Zfp64	U49046	3.1	zinc finger protein	Cmkor1	AF000236	4.3	Chemokine orphan receptor
Gata2	AB000096	2.7	GATA2	Cish3	U88328	3.9	SOCS-3
Cebpa-rs1	U19891	2.1	C/EBP, related sequence 1	Epha2	U07634	3.3	Eph receptor A2
Crabp1	X51715	-11.7	cellular retinoic acid-b. p.	Gpcr12	D21061	3.2	G-protein coupled receptor
IFN pathway				Csf1	M21952	3	M-CSF
Ifi202a	M31418	6.3	interferon activated gene	Il10rb	U53696	3	IL10 receptor beta
Scyb10	M33266	6.0	interferon activated gene	Rgs16	U94828	2.8	regulator of G proteins
Ifi203	af022371	5.8	interferon activated gene	Thbs2	I07803	2.6	thrombospondin 2
Ifit1	aa616578	5.7	interferon activated gene	Map2k3	D87115	2.5	MAP kinase kinase 3b
Irf7	U73037	4.7	interferon regulatory factor	Bin1	U86405	2.4	brain amphiphysin 2
Mx2	J03368	4.5	myxovirus resistance 2	Fgfr1	M33760	2.3	FGFR1
Isg15	X56602	3.2	interferon activated gene	Il1rap	X85999	2.1	IL-1 receptor accessory
Eif2ak2	M65029	3.1	ds RNA dep. protein kinase	Efnb1	Z48781	-2	ephrin B1
Ifit3	I32974	3.0	interferon activated gene	Ldlr	X64414	-2.2	LDL receptor
Ifngr	J05265	2.2	Interferon g-receptor	Fzd8	U43321	-3.7	frizzled 8
MHC				**Insulin-like growth factor pathway**			
LOC56628	M69070	24.8	MHC class I antigen	Igfbp6	X81584	2.6	Igf binding protein 6
H2-T10	M35244	8.5	Histocompatibility 2	Cyr61	M32490	2.5	Igf binding protein 10
H2-T23	M11284	5	Histocompatibility 2	**Collagens**			
H2-D	M69068	4.6	Histocompatibility 2	Col6a2	X65582	-2	procollagen VI, a-2
H2-Q7	M29881	3.8	Histocompatibility 2	Col5a1	aa030649	-2.1	collagen a1(V)
H2-K	U47329	3.8	Histocompatibility 2	Col1a1	D38162	-2.2	procollagen XI, a-1
H2-M3	U18797	2.4	MHC class I antigen	Col6a1	X66405	-2.6	procollagen VI, a-1
Tissue Specific				Col9a1	M32136	-12.2	a-1 typeIX collagen
Slpi	U73004	35.7	leukoprotease inhibitor	**Other**			
Pem	M32484	14.5	placental homeobox	Ttgn2	D50032	3.6	TGN38A
Eng	X77952	6.9	endoglin	Gtf2h1	AJ002366	2.8	TFIIH, 62 kD subunit
Ptgs2	M64291	6.1	prostaglandin synthase 2	Eif3	U14172	2.8	translation initiation factor
Dazl	X95724	5.8	DAZ-like autosomal	Rpo2-1	U37500	2.6	RNA polymerase II 1
Xlr3b	L22977	4.5	B-cell surface antigen 3b	Tk1	M19438	2.5	thymidine kinase 1
Procr	I39017	4.2	endothelial receptor	Cyp2e1	L11650	2	cytochrome P450
Pl1	M35662	4.0	placental lactogen 1	U2af2	X64587	-2.0	splicing factor
Adfp	M93275	2.9	adipose differentiation	Mor1	X07295	-2.5	malate dehydrogenase
Crya2	M73741	2.7	a-B2-crystallin gene	Scd2	M26270	-3.4	stearoyl-coA desaturase 2
Tcte3	M26332	2.2	T-complex testis gene	Jup	M90365	-4	plakoglobin
Ctla2a	X15591	-4.4	cytotoxic T lymphocyte	Gjb2	M81445	-21.4	connexin (Cx26) gene

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Experimental findings by using gene amplification assays have introduced that loss of TP53 induces loss of DNA methylation which as a result induced uncontrolled amplification of the TROP1 gene which is a determinant of cell growth at the early stages of tumour development (Nasr et al., 2003) (Zanna et al., 2007). This study by Nasr et al., 2003 has shown by using gene amplification assay in vivo that p53 plays a significant role in the regulation of DNA methylation. When TP53 was suppressed, it caused hypomethylation and amplification of the TROP1 gene. TROP1 gene (38 kDa transmembrane type I glycoprotein) is a cell-cell adhesion regulatory molecule that is often found overexpressed in a large fraction of human cancers including leukaemia and is found in the 2p21 band in the human karyotype (Figure 18) (Zanna et al., 2007) (Klein et al., 1990) (Momburg et al., 1978).

Transfected cells (Figure 19) are clonal descendants of a single gene-transfection event and therefore the methylation pattern of each TROP1 transfectant reveals information on the DNA methylation pattern of the original, single transfected gene. Southern blot analysis revealed that TROP1 transfectants had TROP1 gene amplification with frequent hypomethylation. Also, complete hypomethylation was observed at CpG sites (Figure 20, arrowhead). They further investigated this by treating DNA from TP53-null cell lines (mut/mut B) with S-adenosyl-methionine and SssI methylase (used to study cytosine methylation). They observed in vitro DNA methylation at CpG sites of the Li–Fraumeni cell lines (Figure 21). Subsequently, the Li–Fraumeni cells were transfected with the methylated DNA and the TROP1 transfectants were selected for gene amplification. Notably, they observed that the in vitro methylation annihilated the amplification of the TROP1 gene from the Li–Fraumeni cells (Figure 22). This suggested that the uncontrolled amplification of the TROP1 gene was due to hypomethylation of the gene and due to the absence of TP53, therefore, understanding molecularly how this is achieved it could provide critical information about the progression of tumours.

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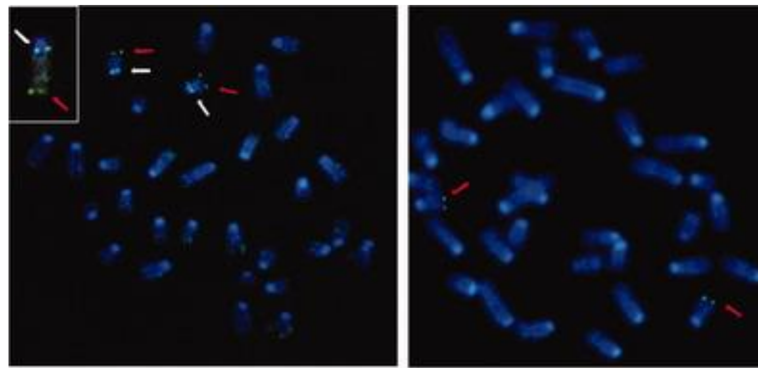

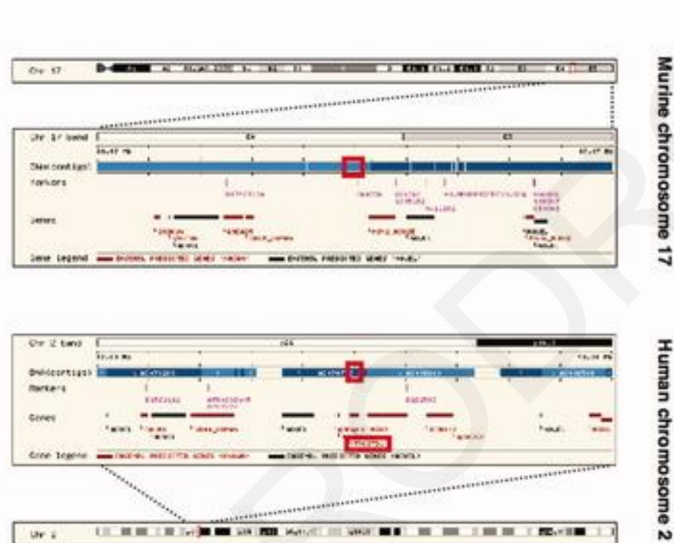

<u>Figure 18:</u> The location and chromosome region of TROP1 gene. (a) Fluorescence in situ hybridization revealed the exact location of the mTROP1 gene in the mouse embryo. In the mouse embryo, it was found at chromosome 17, band 17E5 which corresponds to the 2p21 band in the human karyotype where the TROP1 gene maps (Calabrese et al., 2001). Left upper image represents co-hybridization with a chromosome 17 specific probe (white arrows) and λ phage probes having the mTROP1 gene (red arrows). The right upper image shows hybridization with only the mTROP1 probe (red arrows). (b) The chromosome regions for both murine chromosome 17 and human chromosome 2 while indicating the with red rectangles the map location. The figure is taken by Zanna et al., 2007.

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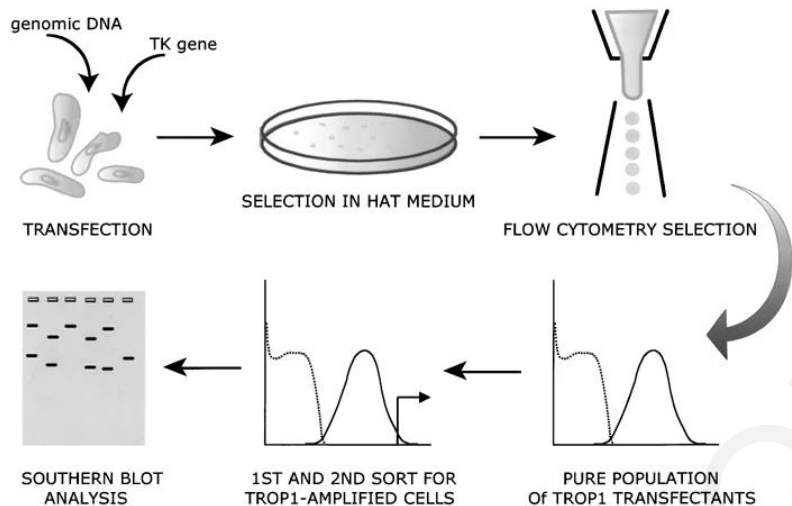
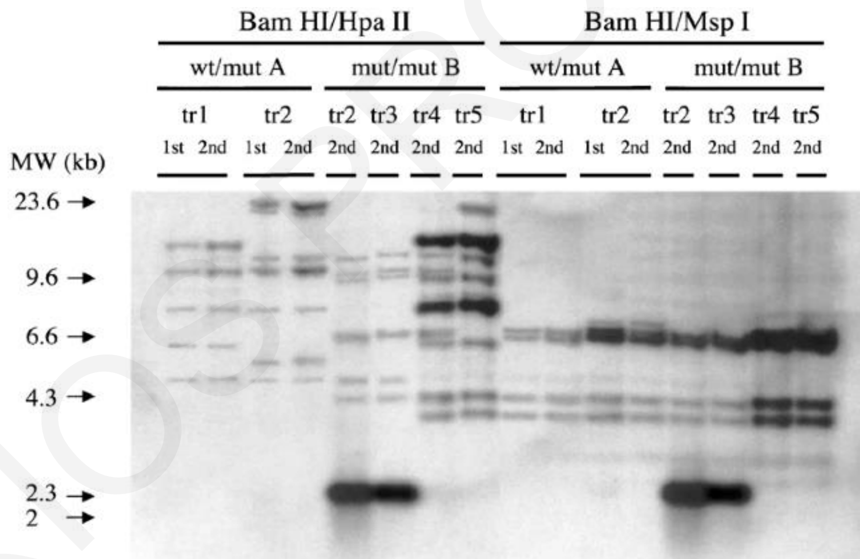


<u>Figure 19:</u> Selection of Trop-1-expressing cells were selected by immunofluorescence flow cytometry. DNA was extracted from Li–Fraumeni cell strains or control cells and transfected in L cells. HAT medium was used to obtain the transfected L cells. The cells that expressed the TROP-1 gene were selected by flow cytometry and further analyzed by Southern blotting. The figure is taken by Nasr et al., 2003.



<u>Figure 20:</u> Hypomethylation causes TROP1 gene amplification in transfectant cells. The DNA samples were digested with BamHI and with HpaII or MspI. 1st: first sort and 2nd: second and final sort indicate the successive cycles of selection for transfectants. In tr 2 and tr 3 there is a marked hypomethylation of the amplified DNA on the TROP1 transfectants (mut/mut B) (arrowhead). The figure is taken by Nasr et al., 2003.

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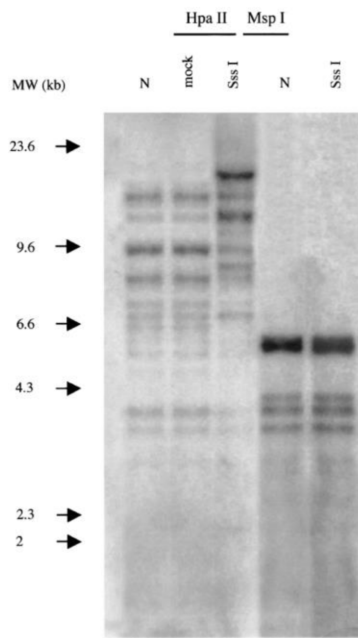
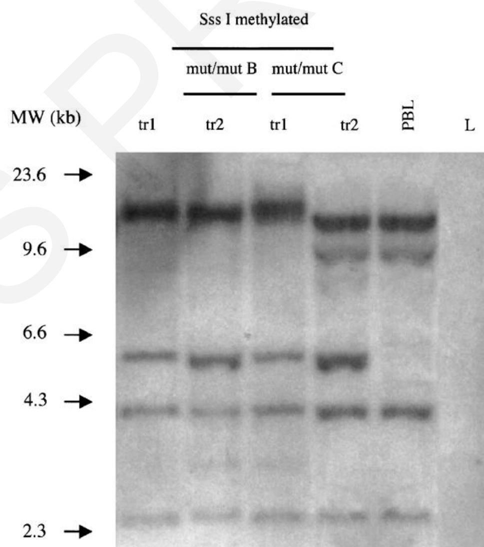


<u>Figure 21:</u> DNA methylation of the Li–Fraumeni cells. Southern blot analysis revealed DNA methylation at CpG sites with S-adenosyl-methionine as a methyl donor. DNA from TP53-null cell lines (mut/mut B) were treated with S-adenosyl-methionine and SssI methylase. Also, the DNA samples were digested with HpaII or MspI. The figure is taken by Nasr et al., 2003.



<u>Figure 22:</u> Lack of amplification of DNA TROP1 transfectants. Southern blot analysis revealed no gene amplification of DNA TROP1 transfectants while indicating the status of TP53. The donor DNA was methylated in vitro with the SssI methylase. Untreated DNA was obtained at the same frequency as DNA TROP1 transfectants. PBL indicates human peripheral blood leukocytes and L: untransfected L cells. The figure is taken by Nasr et al., 2003.

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Also, this study by Zanna et al., 2007 has shown the expression of TROP1 under tumour progression. They analyzed fresh keratinocytes, immortalized keratinocytes, and cell lines at different stages of skin carcinogenesis. They observed in fresh murine tissues a high expression of mTROP1 in the colon (Ileum colon) and duodenum (first part of the small intestine) whereas a clear expression was observed in the kidney and lung. Low expression was observed in the liver and spleen and no expression was observed in the brain, heart, and muscle (Figure 23. a). Consequently, they analyzed different cell lines from different stages of skin carcinogenesis (Figure 23, b) and they observed expression in squamous carcinomas cells (SN161) and in immortalized keratinocytes (C5n), low expression was observed in papilloma cells (2P6) and in B9 (squamous carcinomas) whereas no expression was observed in spindle carcinoma cells (Carc-C, Carc-B, A5). All these indicated that the expression of mTROP1 at the early stages of tumour progression is followed by a potential growth advantage characterized by a rapid growth proliferation of the cells. This was validated when both mTROP1 and hTROP1 transfectants were followed by a markedly stimulated growth in relation to the vector-transfected cells (Figure 24). They also visualized in vivo the expression of TROP1 in mouse and men at early stages of tumour development (Figure 25) (Figure 26). Altogether, these two studies suggest that TP53 induces loss of DNA methylation which as a result induces the uncontrolled amplification of the TROP1 gene which is a determinant of cell growth at the early stages of tumour development. Therefore, loss of TP53 can cause DNA hypomethylation and DNA methylation-dependent gene amplification. Further analysis between TP53 and DNA methylation and TROP1 may shed light on the mechanism and subsequently on new therapeutic approaches in chronic lymphocytic leukaemia.

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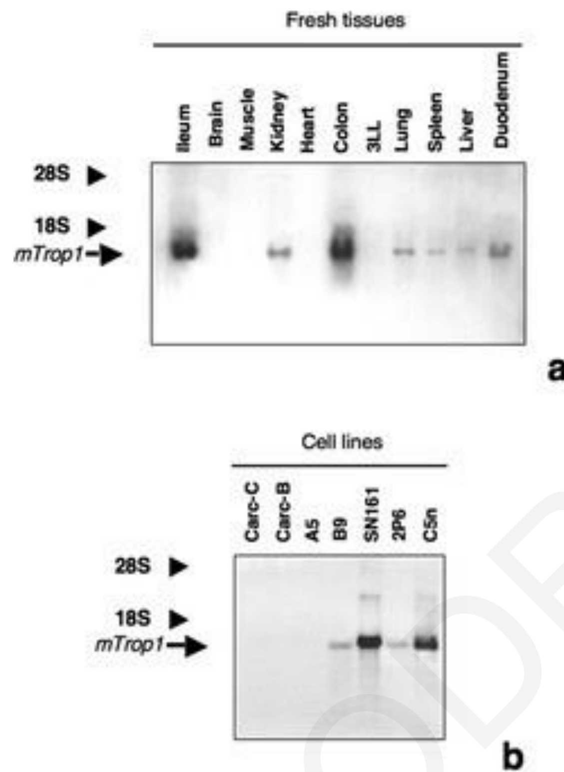

Figure 23: mTROP1 expression during tumor progression. (a) Northern blot analysis revealed in fresh murine tissues mTROP1 expression. (b) Cell lines representative of different stages of skin carcinogenesis were also analyzed. 3LL stands for lung carcinoma cell line, Carc-C, Carc-B, A5 are spindle cell carcinomas, B9 and SN161 are squamous carcinomas, 2P6 are papilloma and C5n immortalized keratinocytes cell lines. The filters applied were hybridized by the murine TROP1 (EGP-314) cDNA probe (arrow). 8S and 18S rRNA are also indicated (arrowheads). The figure is taken by Zanna et al., 2007.

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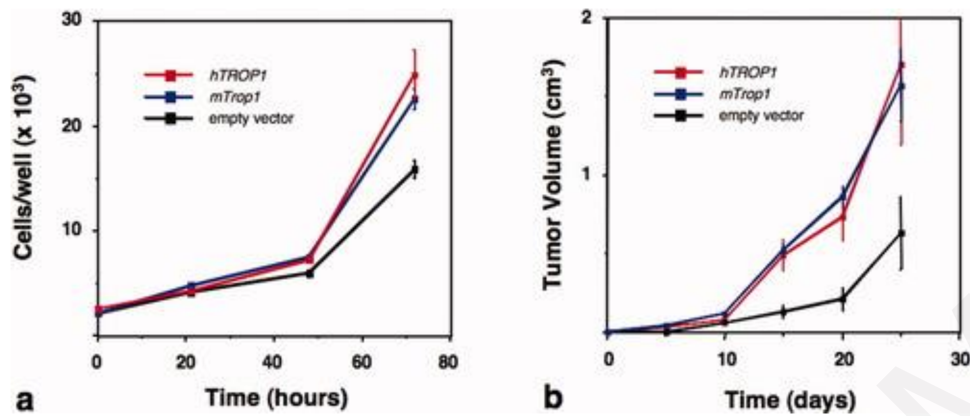
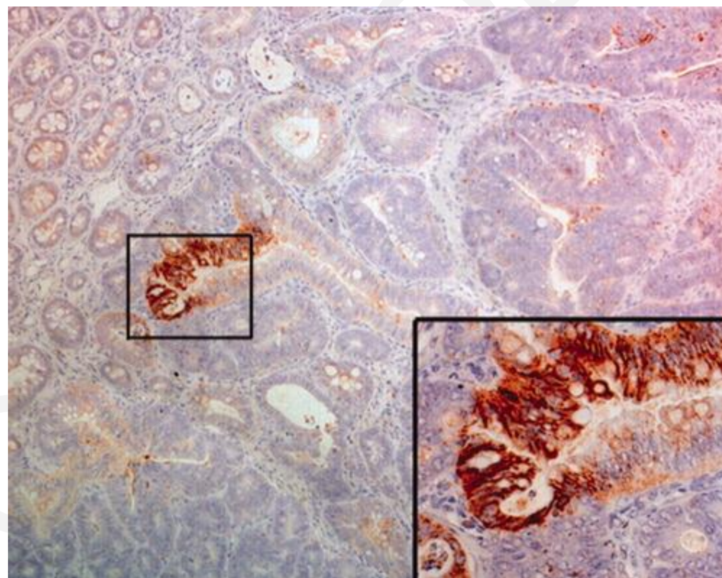


<u>Figure 24:</u> Growth of mTROP1 and hTROP1 transfectants. ((a) In vitro: the growth rate of the murine immortalized, nontumorigenic epithelial cell line of thymic origin (MTE 4–14) transfected with hTROP1, mTROP1 and vector alone. (b) In vivo: the growth rate of the L cell (tumorigenic cell line) transfected with hTROP1, mTROP1 and vector alone. Red colour indicates human TROP1 transfectants, the blue colour indicates murine TROP1 transfectants and black colour indicates transfection with vector only. The figure is taken by Zanna et al., 2007.



<u>Figure 25:</u> Marked induction of TROP1 in dysplastic crypt foci of ApcMin mice. Immunohistochemical staining for mTROP1 in the small intestine of murine shows TROP1 induction, an early focal hyperplasia/low-grade dysplasia (Magnification: 20x). The right bottom image shows a strong immunoreactivity for TROP1 to a focal area in the context of dysplastic glands (Magnification: 40x). The figure is taken by Zanna et al., 2007.

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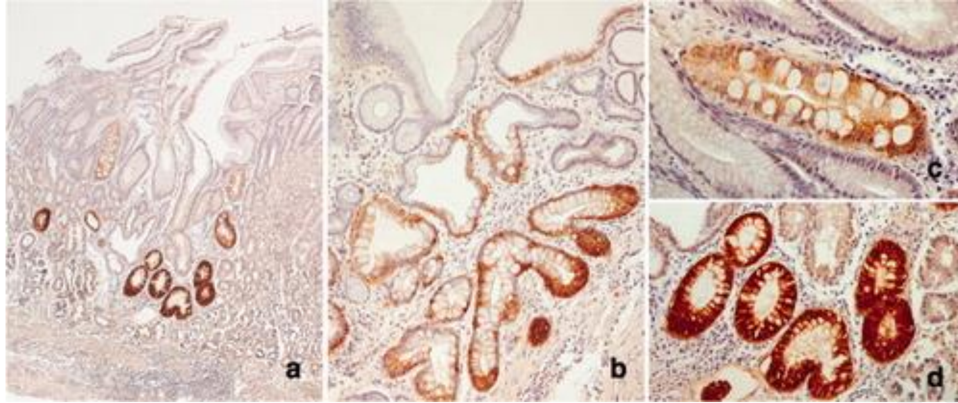

<u>Figure 26:</u> Marked induction of TROP1 in the human stomach. (a) Immunohistochemical staining for hTROP1 in the human fundic gastric mucosa shows no immunoreactivity in the normal gastric mucosa. hTROP1 positivity is presented in the glands showing intestinal metaplasia (magnification: 10x). (b) The gastric mucosa shows well-developed goblet cells and enterocytes with high expression of TROP1 (magnification: 20x). (c and d) TROP-1 is found highly expressed in hyperplastic areas of intestinal metaplasia (Magnification: 40x). The figure is taken by Zanna et al., 2007.

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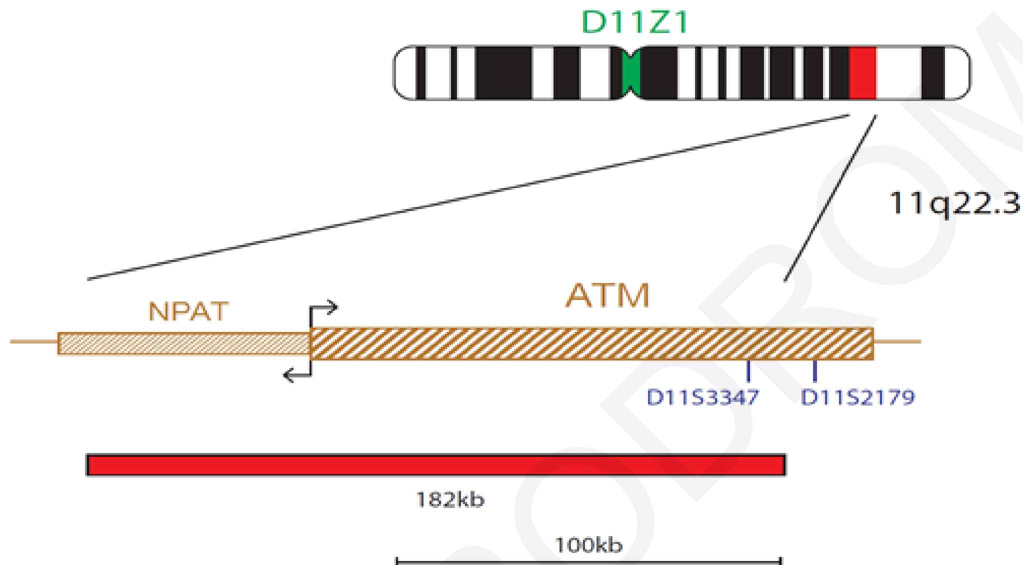
11q22-q23 deletion

Another chromosomal aberration that was observed in CLL patients is the deletion of 11q22-q23 in which it's observed in ~35% of the total cases of CLL (Figure 27).



<u>Figure 27:</u> Location of 11q22.3 deletion. The ATM probe is 182 kb (red colour) and covers the end of the NPAT gene and the centromeric end of the ATM gene to just beyond the D11S3347 marker. NPAT gene encodes the nuclear protein NPAT of the ATM locus. The D11Z1 is a control probe for the 11 centromere (green). The figure is taken by CytoCell ATM Deletion, 2021.

This deletion makes the progression of CLL evolve rapidly which as a result causes a shorter overall survival (68% 5-year overall survival) (Döhner et al., 2000) (Grossmann et al., 2012). This aberration targets the gene coding for ataxia-telangiectasia mutated (ATM) (Figure 28) (Austen et al., 2005).

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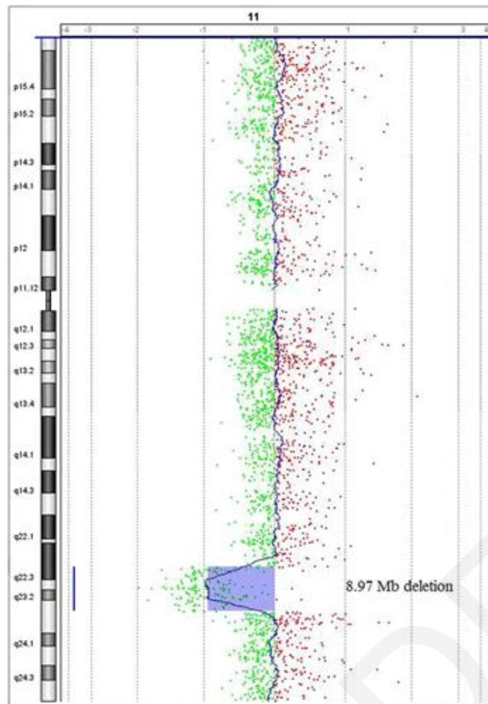

<u>Figure 28:</u> Location of 11q22.3-11q23.3 deletion by using Array comparative genomic hybridization (Array CGH). This analysis revealed an 8.97 Mb deletion at chromosome 11q22.3-11q23.3 which include the ATM gene by using classic G-band karyotyping with array CGH methods. The figure is taken by Liu et al., 2014.

ATM is a kinase that acts upstream of TP53 and controls the DNA damage response (DDR) which is a very important pathway for resolving double-stranded DNA breaks (DSBs) (Grossmann et al., 2012) (Kwok and Stankovic, 2019). DDR pathway contains sensors that detect damaged DNA and activate a cascade of signal transducers which causes the activation of DDR effectors such as cell cycle arrest, DNA repair and apoptosis (Figure 29) (Ray and Fry, 2015) (Sulli, Di Micco and di Fagagna, 2012). Therefore, this deletion can disrupt the DNA-repair mechanisms, causes genomic instability and a more-aggressive disease (Cagnetta et al., 2017).

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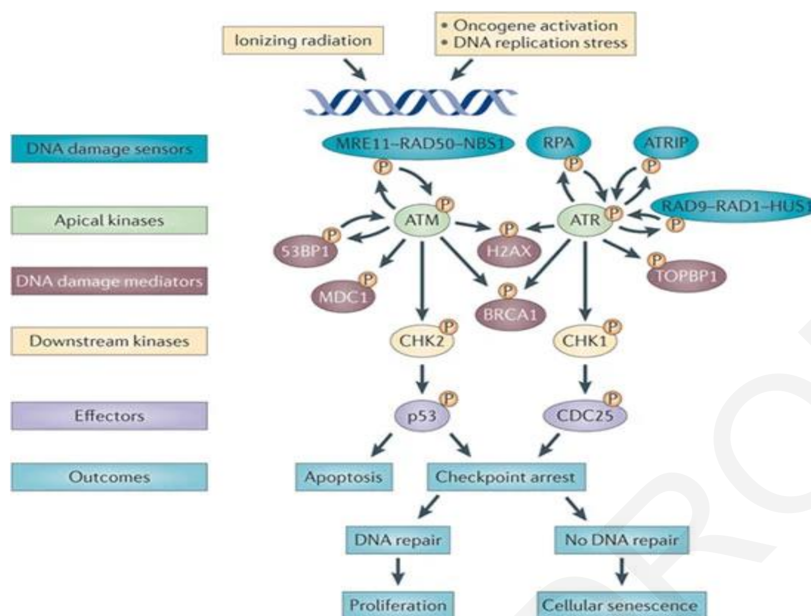

<u>Figure 29:</u> Molecular Pathway of DDR. DDR pathway is comprised of two DNA damage sensors, the MRE11–RAD50–NBS1 (MRN) complex which is the first sensor that detects DNA double-strand breaks (DSBs), the replication protein A (RPA) and the toroidal damage checkpoint complex RAD9–RAD1–HUS1 (9-1-1). These sensors recruit ATM through the MRN complex and ataxia telangiectasia and Rad3-related (ATR) through RPA and the 9-1-1 complex. This causes phosphorylation of H2AX on Ser139 or γH2AX in the proximal region of the damaged DNA. ATM is mainly activated by DSBs in which is helped by ATR under activation of various oncogenes. γH2AX acts as a recruit mediator of DNA damage checkpoint 1 (MDC1) which enhances DDR signalling through the high accumulation of the MRN complex and activation of ATM. BRCA1 acts at sites of damaged DNA on phosphorylation by ATM and ATR. ATM activation sustains DDR signalling through the p53-binding protein 1 (53BP1) which then leaves so the downstream kinases CHK2 (which is mainly phosphorylated by ATM) and CHK1 (which is mainly phosphorylated by ATR) can activate the effectors p53 and the cell division cycle 25 (CDC25) phosphatases. This results in apoptosis, cell cycle arrest and subsequently to DNA repair or cellular senescence. The figure is taken by Sulli, Di Micco and di Fagagna, 2012.

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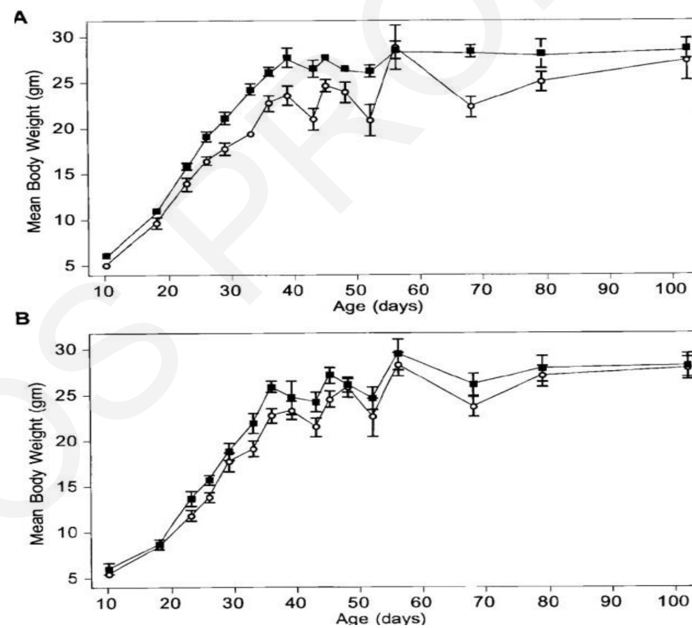
This study by Lavin & Shiloh, 1997 has introduced that humans that lack ATM are followed by ataxia-telangiectasia, which is a multisystem disease that causes genomic instability, mild immunodeficiency, and a predisposition to developing lymphoid tumours with translocations involving antigen receptor loci. Also, further studies from Barlow et al., 1996 Elson et al., 1996 and Xu et al., 1996 have shown that mice deficient to ATM recapitulate the ataxia-telangiectasia phenotype in humans which are also followed by excessive lymphoid hallmarks. This study by Barlow et al.,1996 has shown that mutant female and male (ATM locus knockout) mice weighed significantly less in relation to their wild type littermates and couldn't grow substantially under normal culture conditions (Figure 30). They further investigated potential immunological abnormalities in these mice, and they observed that between the age of 2 to 4 months the mutant mice developed thymic lymphoblastic lymphomas (Figure 31). Their tumours were widely metastatic and grew rapidly causing heart and lung compression and metastasis and consequently death at the age of 4.5 months. This study by Xu et al., 1996 also observed with histological analysis thymic lymphomas in ATM knockout mice (Figure 32).



<u>Figure 30:</u> ATM knockout mice exhibit growth abnormalities. (A) ATM knockout female mice and (B) ATM knockout male mice weighed significantly less in contrast to their wild type littermates from age 8 days to 3 months. Embryonic fibroblasts captured from these mutant mice were unable to grow substantially under normal conditions. Closed squares indicate the wild type animals or cells and open circles the ATM knockout mice or cells. The figure is taken by Barlow et al.,1996.

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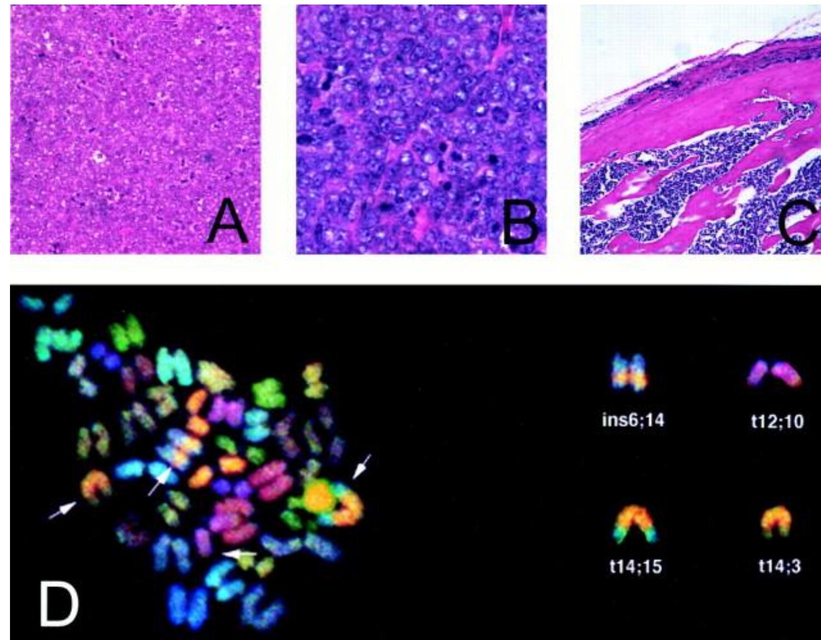

<u>Figure 31:</u> ATM knockout mice develop thymic lymphoblastic lymphomas. (A) Hematoxylin and eosin staining in tissues of ATM knockout mice revealed primary tumours with monomorphic lymphoblastic cells (B) and cells that are ready to divide and create two new cells. (C) The tumour grew in a way that invaded the subperiosteal hematoma and the bone marrow. (D) Chromosomal aberrations using SKY demonstrated these translocations t(12;10), t(14;13), and t(14;15) and an insertion ins(6;14) (white arrows) on early passage cells which might reflect karyotypic abnormalities intrinsic to the tumour. The figure is taken by Barlow et al.,1996.

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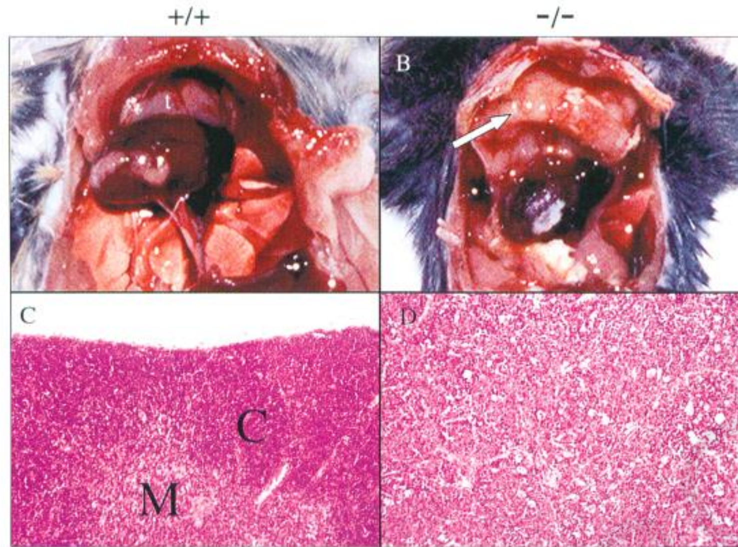

<u>Figure 32:</u> Histological analysis of ATM knockout mice present thymic lymphomas. (A) Physical appearance images of normal mice showed a normal appearance of thymus (B) whereas ATM knockout mice showed thymic lymphomas (white arrow) when at 3-month-old. (C) Hematoxylin and eosin staining showed normal thymus sections and (D) thymic lymphomas. C indicates the cortex and M the medulla. The figure is taken by Xu et al., 1996.

The deletion of 11q22-q23 is commonly found in CLL cases with unmutated IGHV (Dal-Bo et al., 2009). A genome-wide analysis of DNA methylation by HpaII tiny fragment Enrichment by Ligation-mediated PCR Assay (HELP Assay) which is a technique used for determining whether DNA has been methylated has shown that CLL patients with 11q22-q23 abnormalities or deletion are displaying significant hypomethylation levels (Figueroa et al., 2010). Also, this study by Morenos et al., 2014 identified as well that leukemic patients with 11q22-q23 abnormalities or deletions are displaying significant hypomethylation levels. While there were studies that showed that leukemic patients are displaying hypomethylation levels there are limited studies about how these deletions of 11q22-q23 and 17p13.1 are connected with retrotransposon methylation in CLL. More specifically there are two studies in which they have introduced genetic instability along with retrotransposon methylation. The first study is from Fabris et al., 2011 reporting that there are significant hypomethylation levels of LINE-1 and Alus which is strictly related to 17p13.1 deletions. The second study is from Hoxha et al., 2014 in which they introduced that there are associations between LINE-1 and Alu methylation with telomere length. These observations are further confirmed by showing that telomere length is

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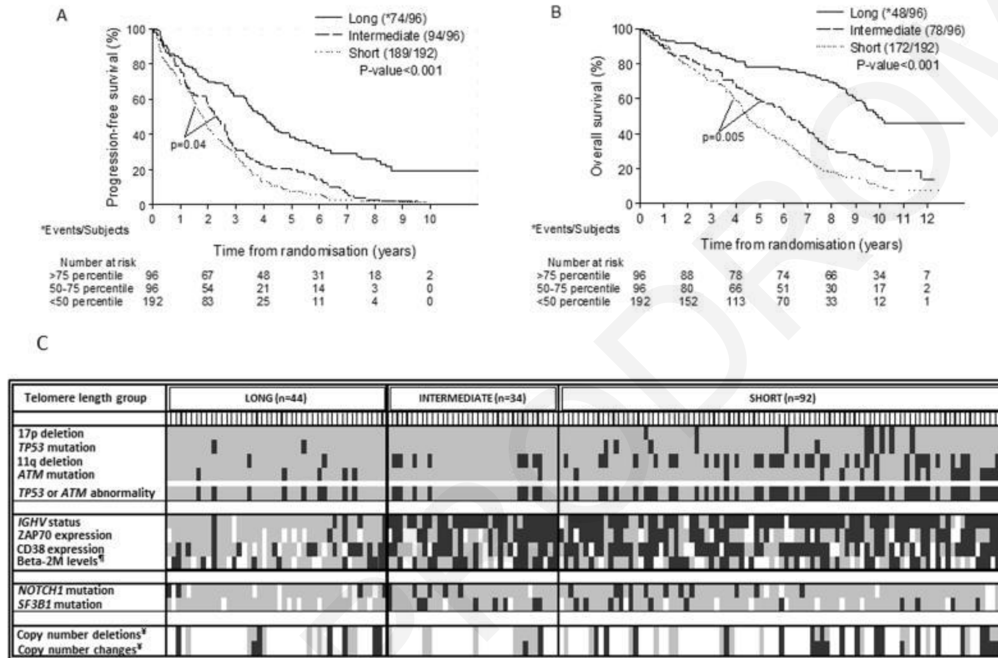
connected with both 11q22-q23 and 17p13.1 deletions and mutations in ATM which together link methylation of retrotransposons, telomere length and generation of genetic abnormalities in CLL (Figure 33) (Strefford et al., 2015). Further extensive evidence has also linked loss of retrotransposon methylation to their activation and genetic instability in solid tumours (Symer et al., 2002) (Daskalos et al., 2009).



<u>**Figure 33:**</u> **Telomere length is connected with both 11q22-q23 and 17p13.1 deletions and ATM mutations.** Kaplan-Meier plots of mean telomere length for (A) progression free-survival, and for (B) overall survival in three groups ((telomere length - short (<50 percentile), intermediate (50-75 percentile) and long (>75 percentile)). 96 patients in the long telomere length group had 4.0 and 9.9 years of progression-free survival and overall survival respectively. (C) The presence and absence of each biomarker according to the telomere length groups in 170 CLL patients with complete TP53 and ATM deletion. Each short vertical line under the telomere length group name corresponds to a single patient. Black boxes indicate the presence of the biomarker, grey boxes indicate the absence of the biomarker and white boxes indicate missing data on the particular biomarker. The intermediate and the short telomere length groups were followed by unmutated IGHV genes, TP53 abnormalities, biallelic ATM inactivation, and

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genomic complexity at a higher frequency in relation to the long telomere length group. The figure is taken by Strefford et al., 2015.

It's worth mentioning that these studies by Fabris et al., 2011 and Hoxha et al., 2014 have used a pyrosequencing-based approach that was only focused on specific subfamilies (L1HS and AluSx) because of their relative abundance in the genome and as surrogate markers of DNA methylation. The pyrosequencing-based approach is a DNA sequencing technique that is based on the chain of enzymatic reactions during the DNA synthesis that relies on the detection of pyrophosphate release into the complementary strand and in the light which is generated as a result of luciferin oxidation by luciferase on nucleotide incorporation (Ramon et al., 2003). This approach has provided novel information about the disease, but they were unable to study the complex system of retrotransposon DNA methylation in CLL. These studies have suggested that DNMTs are thought to be crucial for DNA methylation although, they didn't observe any significant correlation between the expression levels of DNA hypomethylation and DNMTs which contradicts to what is been already introduced in this paper. Also, while they suggested that DNMTs expression might be independent of TP53 deficiency in CLL tumours they revealed a novel prognostic marker for CLL called SAT-a repetitive element.

In contrast, this study by Barrow et al., 2020 has demonstrated a novel way to analyze locus- and evolutionary subfamily-specific retrotransposons using the Illumina Infinium Human Methylation 450K microarray platform. They analyzed publicly available data from the International Cancer Genome Consortium (European Genome-phenome Archive accession number: EGAS00001000272). It's important that they obtained approval from the Northeast - Newcastle & North Tyneside 1 Research Ethics in which all donors provided written informed consent. Consequently, HM450K probes mapping to CpG sites within retrotransposons were identified using the Data Integrator function of the UCSC Genome Browser34 in which they identified 13,130 microarray probes mapping to 37 Alu subfamilies. From these 10.782 probes were differentially methylated only in CLL individuals. From the 10.782 hypomethylated probes, nine were highly frequent in CLL patients (Table 2). This was achieved by t-test, with correction for false discovery rate (FDR) by the Benjamini-Hochberg method in R platform

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(version3.4.0) using ggplot2, heatmap2, qqman and corrplot packages, and GraphPad (version 7.0b).

<u>Table 2:</u> The top nine differentially methylated retrotransposon loci in CLL. The top differentially methylated loci in CLL patients in comparison with the normal CD19$^+$ B cells from healthy individuals. Mean methylation levels (β) for both are presented along with mean methylation change (Δβ), FDR-adjusted P value, genomic location ('chr.') and feature, relation to enhance region and CpG island. Table taken by Barrow et al., 2020.

Probe ID	Element	chr	Gene	Genomic feature	Island status	Enhancer	Healthy	CLL	Δβ	PFDR
cg20820557	*AluSx*	6:53453216				TRUE	0.86	0.16	0.70	1.88×10^{-104}
cg04258086	L1ME3B	16:85966544					0.84	0.26	0.58	3.83×10^{-87}
cg05922591	*AluY*	19:55174624	*LILRB4*	Body			0.81	0.22	0.59	1.44×10^{-78}
cg17505852	L1M4b	22:30003602	*NF2*	Body	S_Shelf		0.87	0.18	0.69	3.81×10^{-78}
cg11665613	FRAM	1:12268883	*TNFRSF1B*	3'UTR		TRUE	0.86	0.28	0.58	6.35×10^{-73}
cg00981250	HAL1b	6:144330345	*PLAGL1*	TSS1500	S_Shore		0.88	0.32	0.56	2.09×10^{-72}
cg16564946	L1PA16	6:32304275	*C6orf10*	Body			0.84	0.29	0.55	2.97×10^{-70}
cg10795552	L1MB7	5:138605898			N_Shelf		0.86	0.26	0.60	1.89×10^{-69}
cg22894805	L1MB8	15:41983772	*MGA; MIR626*	Body; TSS200			0.79	0.25	0.54	3.28×10^{-68}

The potential of this approach may be very beneficial if it is widely used as because identifying the exact retrotransposon loci that are significantly hypomethylated it could provide new novel therapies for CLL. Also, a summary of the most frequent cytogenetic aberrations that are observed in CLL that can be used as individual biomarkers along with their prognostic value can be observed in Table 3.

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Table 3: **Most frequent cytogenetic aberrations that are observed in CLL.** In this table you can see the frequency, the 5-year overall survival, the features and deletions that are associated with each specific lesion of all the explained cytogenetic aberrations in CLL.

Genetic Lesion	Frequency	5-Year OS	Features
13q14.3 del. **(DLEU1&2 and RB1 del.)**	50%	>90%	-Enriched in early stages -Enriched in IGHV-Mutated
17p13.1 del. **(TP53 del.)**	7%	35%	-Enriched in advanced stages -Enriched in IGHV-Unmutated
11q22.3 del. **(ATM del.)**	35%	68%	-Enriched in advanced stages -Enriched in IGHV-Unmutated

The influence on different treatment strategies

As technology advances our understanding of CLL is year by year increasing. Recent advances have enabled scientists to uncover and dissect cytogenetic abnormalities that underpin CLL. Along with the emergence of new drugs, the treatment strategy for CLL patients has shifted from universal chemoimmunotherapy to a more specialized individualized approach. When at diagnosis, the assessment of the mutational status of IGHV, molecular cytogenetics (FISH) and the mutational status of TP53 are highly recommended in the guidelines of the International Workshop on Chronic Lymphocytic Leukemia (iwCLL). This is to ensure that a patient with CLL will have a proper diagnosis, indications for treatment, response assessment, and supportive management. Also, the already mentioned molecular tests are considered highly mandatory due to their ability to provide data that can help the patient to receive the best therapeutic strategy. Therefore, treatment decisions for CLL patients lie on symptoms and classification into low, medium, and high-risk categories (Rai staging or Binet staging system).

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First-line treatment-naive CLL patients

BTK or also known as Bruton's tyrosine kinase is encoded by the BTK gene in humans and it's important for the B cell receptors pathway because it mediates the development, function, and survival of the B cells (Küppers, 2005). BTK has been associated with B cell malignancies such as CLL and in recent years has been thought to be an important therapeutic strategy (Akinleye et al., 2013). B cells that become dysfunctional it's because they become overexpressed and continuously activated which as a result causes the cells to thrive in the peripheral blood, bone marrow and lymph nodes (Wiestner, 2014). By inhibiting BTK can reduce the growth and survival signalling of malignant B cells (Figure 33) (Xue et al., 2020). This statement is further supported with this study by Woyach et al., 2014 in which they selectively downregulated the expression of BTK protein by small interfering RNA (siRNA) in primary tumour cells from 31 CLL patients. They observed a significant reduction in the survival of CLL tumour cells and stimulation of apoptosis in relation to the control ones (Figure 34).

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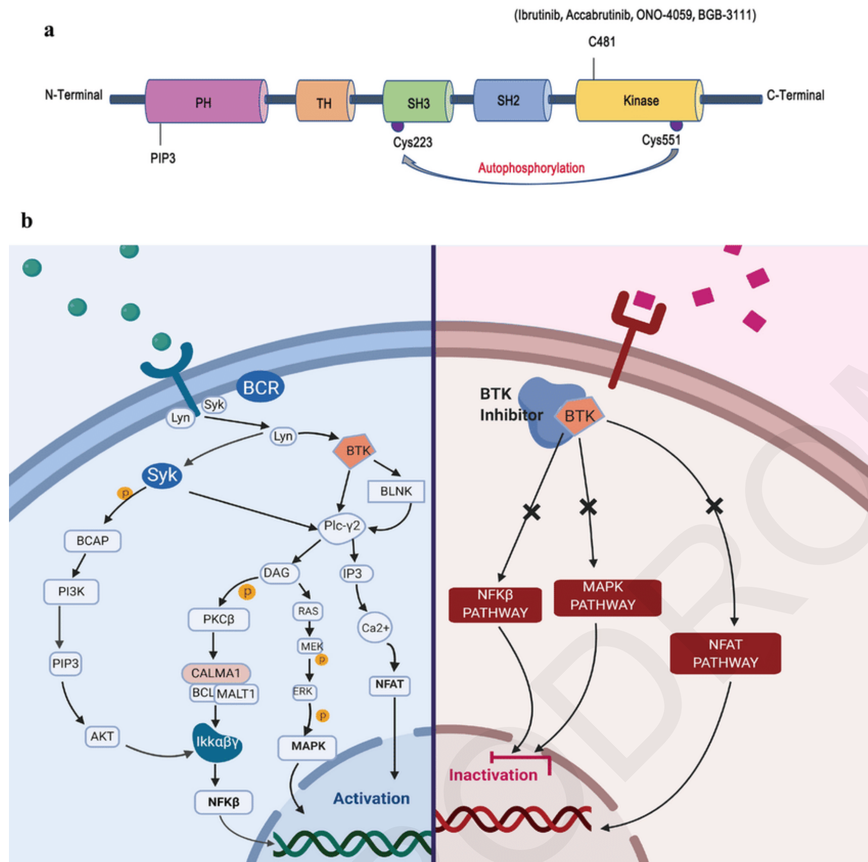

<u>Figure 33:</u> Structure of BTK and associated signaling pathways. (a) BTK has five structural domains, the PH domain is involved in the recruitment of BTK on the cell membrane, TH along with SH3 and SH2 are involved in the binding with multiple proteins whereas the kinase domain activates BTK and the C481 (binding site of ibrutinib). (b) BTK activation and inactivation by BTK inhibitors. The left figure represents the extracellular antigen bond BCR in which BTK can mediate biological processes by activating several pathways such as NF-kB, MAPK, NFAT, and mTOR pathways. The right figure represents small-molecule BTK inhibitors that inhibit downstream pathways of the BCR pathway such as NF-kB, MAPK, and NFAT which as a result causes anti-tumour activity in B cell lymphoma. The figure is taken by Xue et al., 2020.

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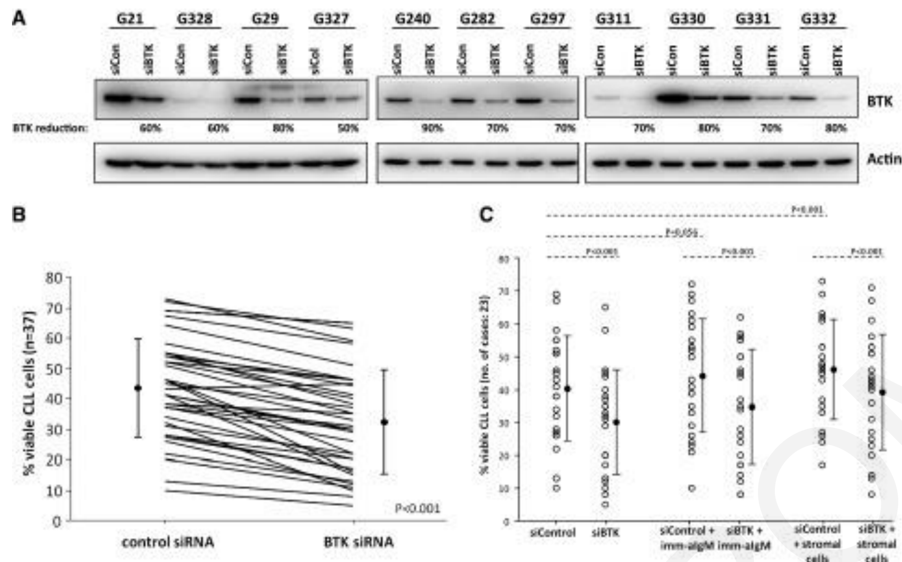

<u>Figure 34:</u> Knockdown of BTK reduces the survival of CLL tumor cells. (A) Under knockdown of BTK by siRNA a western blot analysis was performed 72 hours later after nucleofection with BTK-specific siRNA and control. BTK reduction levels are observed (%). (B) CLL tumour cells survival after 72 hours after nucleofection with BTK-specific siRNA and control. Viable CLL cells were determined based on the percentage of cells negative for PI and Annexin V (healthy cells). BTK siRNA cells had a significantly reduced cell viability in contrast to the control siRNAs. (C) CLL tumour cells after nucleofection with BTK-specific siRNA and control and co-incubation with stromal cells and stimulation with IgM were tested for cell viability. CLL tumour cells viability was significantly reduced indicating induction of apoptosis under BCR activation and stromal protection. The figure is taken by Woyach et al., 2014.

A treatment called ibrutinib it's highly recommended for patients that have TP53 dysfunction/mutations. Ibrutinib was the first BTK inhibitor treatment to be examined for patients with CLL. In February 2014, FDA approved ibrutinib for patients with relapsed or refractory CLL (FDA Approves Imbruvica (Ibrutinib for CLL), 2014) and two years later in March 2016 it was further approved for treatment-naïve patients (FDA Approves Imbruvica (ibrutinib) for the First-Line Treatment of CLL, 2016). According to the National Comprehensive Cancer Network guidelines, this treatment is recommended as a first-line treatment for all CLL patients (Treatment by Cancer Type, 2019).

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Several studies have shown that ibrutinib is followed by improved progression-free survival, overall survival, and overall response rate for CLL patients (Byrd et al., 2014) (Burger et al., 2015) (Kipps et al., 2019). In contrast, this study by Ahn et al., 2017, which was published a year after the approval has warned that 17p13.1 deletion and TP53 dysfunction/mutation remains a risk factor for disease progression on BTK-inhibitor therapy. The mechanism of this treatment lies in the active substance ibrutinib which works against the dysfunctional B lymphocytes. This is achieved by the blockage of the enzyme BTK which it's an enzyme that promotes the survival of B lymphocytes and their migration to the organs (Imbruvica (ibrutinib), 2021).

In patients that do not have a TP53 dysfunction/mutation, the assessment of IGHV mutational status is vital. Long-term studies about CLL patients with mutated IGHV had a durable response of more than 10 years when treated with Fludarabine, Cyclophosphamide, and Rituximab (FCR) (Fischer et al., 2016) (Thompson et al., 2016). Therefore, for CLL patients with mutated IGHV has been suggested that FCR or bendamustine plus rituximab (BR) can be used as the most preferred chemoimmunotherapy treatments. To those patients that will probably not tolerate FCR or BR, there are other less intense drug combinations. Combination of chlorambucil with a CD20 monoclonal antibody such as obinutuzumab, ofatumumab and rituximab are some of the examples (Hallek et al., 2010) (Goede et al., 2014) (Thompson et al., 2016) (Fischer et al., 2016). It's worth mentioning that when ibrutinib with or without CD20 monoclonal antibodies when treated in low-risk patients the outcomes are similar to those observed under chemoimmunotherapy treatments (Woyach et al., 2018) (Shanafelt et al., 2018) (Moreno et al., 2019).

Treatments that are based on BTK inhibitors must be administered continuously because minimal residual disease-negative responses are not achievable with these drugs. Therefore, this prompts us to new issues which are related to long-term toxicities, compliance, drug interactions and cost. Indeed, in the last decades, the outcome for CLL patients is much improved in which the overall survival for patients is dependent on the course of different treatments during the course of the disease (Brown et al., 2017). Another aspect that is important for the course of the disease is the patients with relapsed/refractory CLL. Historically, these patients had restricted

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treatment options and the majority of them were unsatisfactory, although, this situation has changed and now there are some very good options for these patients.

Treatment of relapsed or refractory chronic lymphocytic leukemia

In patients with relapsed or refractory CLL, the chemotherapy induces short-lasting responses in which the progression-free survival is less than 2 years (Badoux et al., 2011) (Fischer et al., 2011). This is attributed to the acquisition of resistant clones selected by previous therapies and to the difficult completion of the treatment because of their excessive toxicity (Bosch and Dalla-Favera, 2019). The median progression-free survival in already treated patients with ibrutinib is ~51 months (O'Brien et al., 2018). FDA in 2018 has approved a combination of rituximab and venetoclax as an early option for these patients (Seymour et al., 2018).

Another BTK inhibitor that can be used is acalabrutinib in which was first approved to treat mantle cell lymphoma in 2018. Acalabrutinib belongs to the class of imidazopyrazines and when administered it can inhibit BTK activity which as a result will prevent the activation of the B cell antigen receptor signalling pathways. Consequently, it inhibits the activation of both B cells and BTK-associated downstream signalling survival pathways which can ultimately decrease the malignant B cells that overexpress BTK (Acalabrutinib, 2021). Taken together, both ibrutinib and acalabrutinib can inhibit the BTK signalling pathways which as a result can reduce the production of tumour cells (Figure 35) (Zhu et al., 2021).

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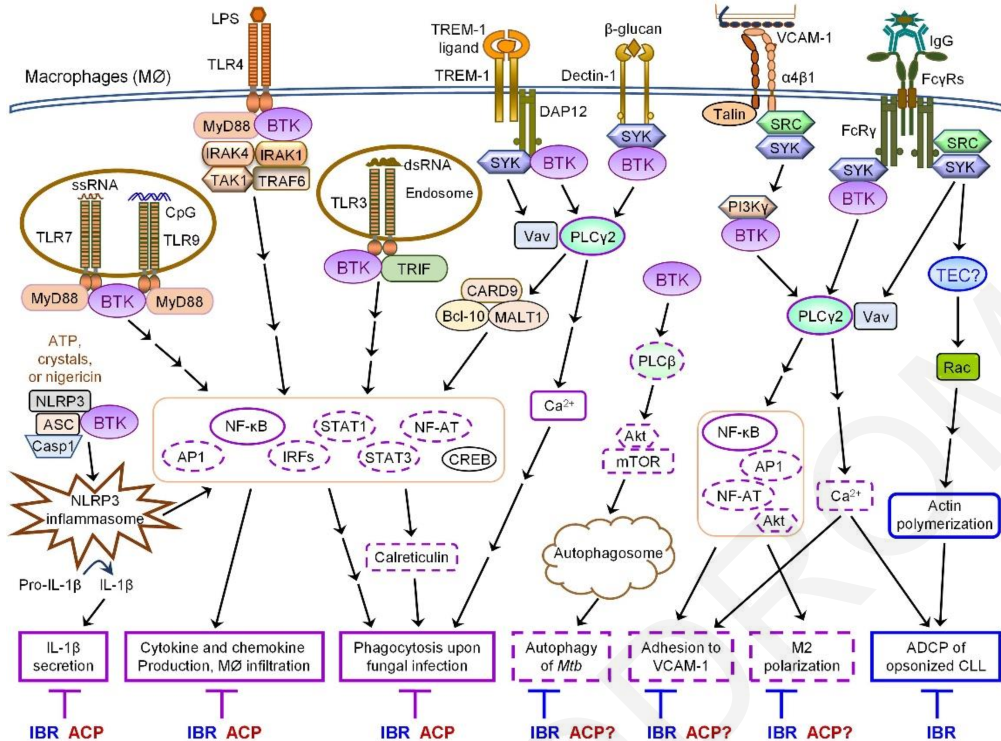

<u>Figure 35:</u> Effects of Ibrutinib and Acalabrutinib and BTK signaling pathways in macrophages. Both ibrutinib (IBR) and acalabrutinib (ACB) can inhibit major proximal events and downstream effectors of TLR-, NLRP3-, TREM- 1-, and Dectin-1 through BTK-dependent mechanisms (indicated as purple boxes) suggesting their potential in reducing the production of tumour cells. Ibrutinib can alter the function of α4β1-, FcγR and AKT-Mtor through BTK-dependent mechanisms (indicated as dashed purple boxes). Also, in contrast to acalabrutinib, ibrutinib can alter the function of FcγR-mediated ADCP of opsonized CLL cells by macrophages via BTK-independent mechanisms through off-target inhibition of TEC (indicated as blue boxes), suggesting that both drugs have a differential potential in combination therapies. The figure is taken by Zhu et al., 2021.

This treatment has a higher toxicity profile than ibrutinib because of its high specificity resulting in a 95% objective response rate in patients with relapsed or refractory CLL (Byrd et al., 2016). Idelalisib, which is a PI3Kδ inhibitor is another approved treatment for these patients. This drug is specific for use after treatment with ibrutinib and venetoclax because it's been associated with high toxicity causing autoimmune complications and an increased risk of opportunistic infections

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such as diarrhoea 42%, pneumonitis 20% and hepatotoxicity 16% (Furman et al., 2014) (de Weerdt, Koopmans, Kater and van Gelder, 2017).

There are many novel treatments that are under investigation in early phase clinical trials and there are also novel targeted therapies that are used. Although, we need to comprehend that hypomethylation of key genes can induce therapy resistance in cancer tumours. This study by Luzhna et al., 2010 indicated that diminished radiation responsiveness was strictly associated with DNA hypomethylation in radiation-resistant cells. However, radiation resistance was efficiently reversed under SAM (methyl donor) treatment (Figure 36) (Figure 37). Also, the researchers concluded that we have to find a perfect balance of DNA methylation in order to ensure a better efficacy for both drug and radiation responsiveness. In contrast, this study by Yosifov et al., 2020 indicated that they couldn't correlate chemoresistance with epigenetic changes such as DNA methylation. The role of DNA methylation in the resistance to therapy in solid tumours is understudied. Recent advances in applied genetic engineering and genome editing can reinforce the already existing ones to provide new ones that could target DNA methylation in CLL patients (Romero-Garcia, Prado-Garcia and Carlos-Reyes, 2020).

Such an example is the clustered regularly interspaced short palindromic repeats/associated protein 9 (CRISPR/Cas9) which is a specific system that enables the addition or removal of DNA from the genome. The evolvement of genetic engineering has given us a new version of CRISPR called Nuclease-dead Cas9 (Cas9). Cas9 is an enzymatically inactive mutant of Cas9 in which the endonuclease activity is been removed but the DNA binding activity is preserved. Cas9 was also associated with DNMTs or ten-eleven translocation-1 enzymes. This as a result generated new systems such as the dCas9-DNMT3A and the dCas9-TET1 in which they have the ability to generate methylation and hypomethylation on specific genes in a specific-sequence manner. Therefore, CRISPR is a new powerful tool that is very promising in the fight against CLL or other cancers and in the acquisition of therapy resistance (Vojta et al., 2016) (Liu et al., 2016).

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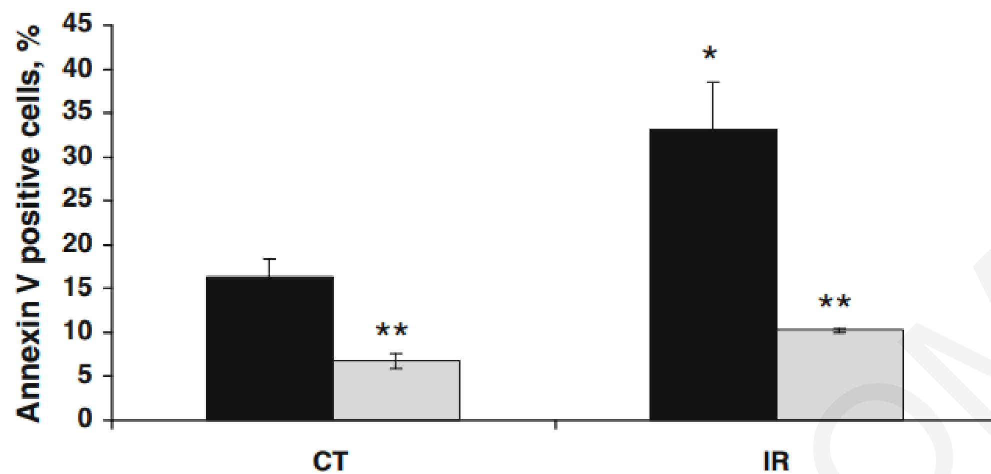
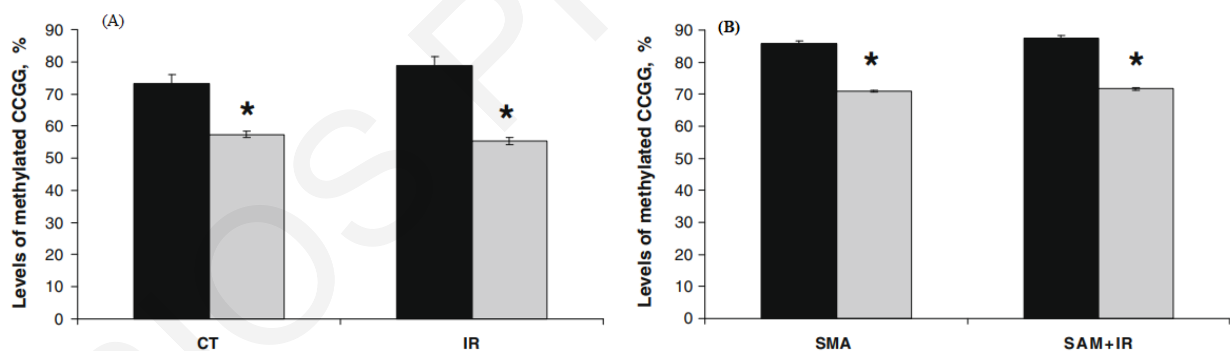


<u>Figure 36:</u> Radiation-induced apoptosis in MCF-7 breast adenocarcinoma cells and their doxorubicin-resistant variant MCF-7/DOX cell. An Annexin V-FITC assay for control non-irradiated (CT) and irradiated with 5 Gy of X-rays cells (IR) lower levels of apoptosis in MCF-7/DOX cells in contrast to MCF-7 cells. IR (ionizing radiation) can induce DNA damage and apoptosis while MCF-7/DOX cells were resistant to radiation-induced apoptosis. Black bars indicate the MCF-7 cells and grey bars – MCF-7/DOX cells. The figure is taken by Luzhna et al., 2010.



<u>Figure 37:</u> DNA methylation levels for both MCF-7 and MCF-7/DOX cells. (A) A cytosine extension assay for control (CT) and irradiated cells (IR) with 5 Gy of X-rays. (B) A cytosine extension assay for control (CT) and irradiated cells (IR) for 100 mM SAM treated (SAM) and irradiated with 5 Gy of X-rays after SAM treatment cells (SAM + IR). Black bars indicate the MCF-7 and grey bars the MCF-7/DOX cells. SAM-treated and IR cells caused an increase in methylation levels (CCGG sites) MCF-7 (up to 90%) and MCF-7/DOX cell lines (up to 70%). The figure is taken by Luzhna et al., 2010.

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Quality of Life

CLL patients are very commonly followed by progressive defects in both cell-mediated and antibody-mediated immunity including hypogammaglobulinemia and B cell and T cell quantitative and functional defects. The progression of CLL causes a degree of immune impairment which gets worse very fast. Also, under immunosuppressive treatments such as purine analogue chemotherapies, anti-CD20 monoclonal antibodies or drugs that inhibit kinases involved in immune receptor signalling then these patients are at higher risk to develop infectious complications (Forconi and Moss, 2015) (Kipps et al., 2017).

The most observed infection in CLL patients involves bacterial infections in the respiratory tract (Forconi and Moss, 2015) (Kipps et al., 2017). Usually, it is treated with intravenous immunoglobulin replacement therapies which they can mitigate the risks of infections, particularly in patients with hypogammaglobulinemia who have a more life-threatening infection (Albin and Cunningham-Rundles, 2014) (Forconi and Moss, 2015) (Katragkou, Roilides and Walsh, 2018). Another way to treat these kinds of infections are immunoglobulin formulations which are usually administered subcutaneously and prophylactic antibiotics which are not so widely used but they respond well to opportunistic infections, especially in CLL patients that undergo treatments that alter the immune function. In contrast, there are no conducted studies about assessing the benefits of intravenous immunoglobulin replacement therapies against prophylactic antibiotics in CLL patients (Albin and Cunningham-Rundles, 2014) (Dhalla et al., 2014) (Spadaro et al., 2016) (Perez et al., 2017).

CLL patients may also suffer from autoimmune complications which are found in 25% of the total cases. Autoimmune complications usually target haematological lineages which as a result causes autoimmune haemolytic anaemia, immune thrombocytopenic purpura, pure red cell aplasia or autoimmune granulocytopenia (Visco et al., 2014). Autoimmune haemolytic anaemia is the most common complication that is found in CLL patients which accounts for more than 12% of the total cases, and about 5% involves patients with immune thrombocytopenic purpura. These two complications together in CLL are considered the most commonly found diseases in adults. Pure red aplasia accounts for <1% of the total cases and it's a disorder in which the bone

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marrow stops producing erythrocytes causing reticulocytopenia. Pure red aplasia in order to be diagnosed it requires a bone marrow biopsy showing the absence of erythroid precursor cells without myelodysplasia, as well as the exclusion of viral infections that can impair erythropoiesis, such as Parvovirus B19, Epstein–Barr virus, viral hepatitis B or hepatitis C and HIV infections (Visco et al., 2014) (Means, 2016).

Lastly, the rarest of all is autoimmune granulocytopenia which is found in <0.2% of CLL patients and it can be diagnosed by a bone marrow biopsy that shows maturation arrest at a late stage in granulocyte differentiation and exclusion of other causes of isolated acquired neutropenia (Farruggia and Dufour, 2014) (Visco et al., 2014) (Fujita et al., 2017). Again, there are no controlled trials for patients with autoimmune complications in CLL patients, therefore, the main treatment that is usually provided are corticosteroids (Hodgson et al., 2011) (Taylor et al., 2015).

Several retrospective analyses were performed in which they showed that CLL patients are followed by a higher-incidence ratio to develop secondary primary malignancies such as melanoma, sarcomas, and lung, renal and prostate cancers. The main cause for this incidence is attributed to immune deficiencies which are usually found in CLL patients (Flynn, Andritsos, Lucas and Byrd, 2010) (Tadmor, Aviv and Polliack, 2011) (Hisada, 2001). While there were studies that suggested that CLL patients that undergo chemotherapy are at higher risk to develop secondary cancers, this study by Maurer et al., 2016 has shown that the risk is similar for both treated and untreated patients.

Richter syndrome is a syndrome in which malignant B cells cause the transformation of CLL to a much more aggressive lymphoma such as diffuse large B-cell lymphoma Hodgkin lymphoma or Hodgkin-Reed-Sternberg-like cells surrounded by CLL cells. The incidence ratio for CLL patients that are most likely to develop Richter syndrome ranges from 2-7% and occurs mostly in patients with NOTCH1 mutations and in patients with certain stereotypical immunoglobulin molecules encoded by the IGHV genes (Bockorny, Codreanu and Dasanu, 2011) (Rossi and Gaidano, 2016). In terms of clinical suspicion, it can be observed by late-night sweats, fatigue, weight loss, enlarged lymph nodes and increased serum lactic dehydrogenase. In contrast, the

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prognosis for these patients is very low, especially for those that are pretreated or are under treatment for CLL and for those who have clonally lymphocyte transformation related to CLL (Rossi et al., 2011) (Mauro et al., 2015) (Kipps et al., 2017).

Overall, through research CLL patients can now be distinguished into subgroups with distinct clinical features which as a result improved substantially prognosis and therapies. In contrast, our understanding of the mechanisms that are associated with immune dysfunction and how it contributes to various autoimmune diseases such as autoimmune haemolytic anaemia, therapy resistance or therapy complications is currently understudied.

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Conclusion and Future Perspectives

This review has been built to evaluate the effect of global epigenetic changes on the hypomethylation of Alu elements in CLL. Indeed, while global hypomethylation of Alu elements has been widely reported in solid tumours, there hasn't been a fully detailed account of specific-primary cytogenetic aberrations that are linked with hypomethylation of retrotransposons and how this contributes to CLL. This could be very helpful to elucidate its complex epigenetic mechanisms and how to regulate the disease. Thus, the aim of this review was to collect research papers from the literature and therefore identify specific-primary cytogenetic aberrations that are linked with hypomethylation of Alu elements that might be involved in driving the disease and mention treatments that are ready to use for patients with distinct CLL phenotypes.

CLL is a chronic lymphoproliferative disorder which it's characterized by the uncontrolled clonal proliferation of dysfunctional CD5+ B lymphocytes. Importantly, CLL accounts for ~300,000 newly cases each year. The mortality rates are increasing year by year due to the fact that the median age of diagnosis for CLL patients is 64-70 years and therefore they are diagnosed too late while the disease is significantly progressed. The underlying mechanisms of this disorder are not fully elucidated, signifying the need to understand the biology behind CLL.

Although, through the years by studying CLL we know that it might be originating from antigen-experienced CD5-CD27 B cells and that it can be distinguished into CLL cells that express mutated IGHV or unmutated IGHV. The clinical outcomes for patients that express an unmutated IGHV will experience a more aggressive disease in contrast to those patients with a mutated IGHV. This is because CLL cells are derived from B cells that have experienced differentiation in the germinal centres, express immunoglobulin and have undergone somatic hypermutation and/or class-switch recombination. Also, the mutational status of IGHV is driven by distinctive epigenetic programming pathways especially the patients that express unmutated IGHV. While in recent years more and more research papers publish evidence of epigenetically silenced genes, we still struggle to understand how DNA methylation is involved.

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DNA methylation is a very crucial epigenetic mechanism whose main function is to regulate gene expression through the recruitments of proteins that are necessary for gene suppression or to inhibit transcription factors. DNA methylation is catalyzed by DNMTs which are specific enzymes that regulate gene transcription, expression and suppress retrotransposition events. The development of cancer is mostly caused by alterations in DNA methylation patterns which as a result will alter the function of gene expression and cell differentiation. A significant alteration in DNA methylation and in cancer is called hypomethylation of Alu elements which as a result can induce transcriptional re-activation of DNA sequences that are integrated into the human genome causing mutagenesis and carcinogenesis. AluY subfamily is considered the most active one in the human genome right now with huge expansions and high evolvements throughout the years due to its stealth driven abilities. Hypomethylation induces re-activation of Alu elements which subsequently causes various retrotransposition events and activation of several oncogenes through locus-specific hypomethylation and transcription within the retrotransposon elements. Also, these events at a genome-wide level can induce chromosomal instability and mutagenesis causing gene evolution of all the human diseases and subsequently carcinogenesis. It is believed that active DNA hypomethylation in CLL B cells which requires the function of the Ten-Eleven translocation enzyme family it may be the key to causing CLL.

While various specific genomic events are seemed to be directly related to a differential clinical outcome and frequency between CLL patients that bear mutated-IGHV or unmutated-IGHV genes, to the best of our knowledge very few studies have tried to link these genomic events to hypomethylation of retrotransposons. Some of the most important cytogenetic aberrations in which about 80% of CLL patients will carry at least one of them are 13q14.3 deletion, 17p13.1 deletion and 11q22-q23 deletion. 13q14.3 deletion has been found to be strictly associated with hypomethylation of retrotransposons of CpG sites. DNA hypomethylation at CpG sites on chromosome 13 can cause the deletion of both has-mir-16-1 and has-mir-15a which as a result induces the uncontrolled activation of tumour-promoting genes and subsequently the progression of CLL. 17p13.1 deletion can affect TP53 which is a known transcription factor. TP53 is regulated by MDM2 in which this relationship is disrupted when TP53 influences DNA methylation through short-range interactions. Also, loss of DNA methylation causes uncontrolled amplification of the TROP1 gene causing CLL tumour development. 11q22-q23 deletion has

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been shown to have significant hypomethylation levels at Line-1 and Alus. Also, there were associations of hypomethylation of LINE-1 and Alus with telomere length. This causes re-activation of retrotransposons and genetic instability and consequently a more-aggressive disease through the disruption of the ATM gene. While CLL's epigenome is been found to be followed with high levels of widespread hypomethylation so far it hasn't been widely studied, so this would be something for future work. Here it's been given a thorough view of our current understandings about epigenetic and genetic alterations in CLL and how hypomethylation of retrotransposons might be implicated in this situation. Advances in the genomic, epigenomic and transcriptomic landscape of CLL can provide new insights about the disease and new novel treatments.

Also, while there have been introduced several treatments that are approved and currently in use for patients with distinct CLL phenotypes there are also other treatments under preclinical and clinical investigation such as therapies that can modulate the immune system and cell transplantation.

Such treatments are called immune-modulatory drugs such as thalidomide and lenalidomide. Although, while these drugs are approved, they were only used in combination with anti-CD20 monoclonal antibodies, rituximab, or other treatments (James et al., 2014) (Chavez et al., 2016). Mechanistically, lenalidomide causes the expression of p21cip1/waf1 which is a well-known cyclin-dependent kinase inhibitor that belongs to the CIP/Kip family of cyclin-dependent kinases inhibitors. This expression causes the inhibition of cyclin-dependent kinase and CLL cell proliferation during G1 and S phases resulting in an enhanced immune synapse formation, enhancing immune function, and alleviating the severity of hypogammaglobulinaemia (Fecteau et al., 2014) (Badoux et al., 2011) (Ramsay et al., 2008). In contrast, this drug has been associated with high toxicity causing tumour flare, tumour lysis syndrome and myelosuppression. These conditions were highly found in CLL patients when treated with lenalidomide in contrast to other patients with other haematological symptoms indicating why it is not so-preferred for these patients (Kips et al., 2017). Also, when in use with other therapies such as anti-CD20 monoclonal antibodies or rituximab the efficacy of this drug is increasingly enhanced (Giannopoulos, Mertens and Stilgenbauer, 2011). The overall response rate of

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lenalidomide as first-line therapy is 60% whereas as a salvage treatment 40% (Ferrajoli et al., 2008) (Badoux et al., 2011). A 7-month treatment with rituximab, lenalidomide was followed with increased response rates at 95% for patients over 65 years of age and 78% for patients below 65 years of age in which progression free-survival for both groups was ~20 months (James et al., 2014).

Another potential therapy for patients with relapsed or refractory CLL it's allogeneic stem cell transplantation. Allogeneic stem cell transplantation is the transfer of stem cells from a healthy individual to a CLL patient after chemotherapy or radiation (Giralt and Bishop, 2008). This therapy is restricted because donor availability, the high patient's age, myelosuppression toxicities and impaired resistance to infections limits its application. Also, its efficacy is another issue (Kips et al., 2017). Two clinical studies revealed that CLL patients without serious medical comorbidities and a median age between 53-60 the progression-free survival was 40-50% with overall survival of 50-70%. The deaths without recurrent or progressive disease or non-relapse mortality between 3-5 years were calculated at 25-40% (Guasch and Fuchs, 2005) (Dreger et al., 2013). This therapy can have a high potential and therefore, research studies are in development so to design a better tolerated cell-based therapy with high curative potential.

Chimeric antigen receptors (CARs) are T cells modified ex vivo to express new surface receptors, then are expanded in vitro and then inserted back into the patient's body (Dai, Wang, Lu and Han, 2016). CARs contain an antigen-binding domain, a stalk and transmembrane region, an intracellular co-stimulatory signalling domain and a CD3ζ protein complex (Jackson, Rafiq and Brentjens, 2016). In order for the CAR gene to be inserted into the T cells, retroviral vectors are used. Subsequently, when the CAR T cells are subjected to the corresponding antigen then the antigen triggers activation and growth of the CAR T cells which eliminate the target antigens. In CLL cells that express antigens such as CD19 are specifically targeted using CAR technology (Kips et al., 2017). This study by Porter et al., 2015 has shown that by using CAR technology to target B-lymphocyte antigen CD19 in 14 patients with relapsed or refractory CLL the outcome was very promising. Of the 14 patients, four had a complete remission and four partial remissions. Interestingly in two patients with complete remission, the CAR T cell remained functional beyond 4 years. In contrast, the efficacy for patients with acute lymphoblastic

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leukaemia was significantly increased. This was attributed to the fact that CLL patients have augmented qualitative defects in T cells and are much older than in patients with acute lymphoblastic leukaemia. Also, this therapy has some negative effects for CLL patients such as cytokine release syndrome which is an inflammatory syndrome causing high fever and multiple organ dysfunction. Although still, this therapy remains a promising one and currently there are large phase trials at stage II that assess the use of CAR T cell therapy in CLL patients.

The development of immune checkpoint inhibitors is a revolutionary milestone in the field right now. Immune checkpoint inhibitors are proteins that are found on the surface of antigen-presenting cells which are responsible for the regulation of the immune system. As cancer cells have the ability to evade the immune system detection and destruction by inhibiting T cells, this brought the idea of the development of immune checkpoint inhibitors (Sharma and Allison, 2015) (Heinzerling et al., 2019). In CLL cells there are high levels of immune checkpoint receptors such as PD-1 and its ligands PD-L1 and PD-L2. CLL cells that express PD-L1 and PD-L2 can suppress PD-1-expressing effector T cells causing exhausted and non-functional T cells (Ramsay, Clear, Fatah and Gribben, 2012). A preclinical study by McClanahan et al., 2015, indicated that PD-L1 blockade prevents immune dysfunction and leukaemia development in a mouse model. Although, it's early to consider this treatment because clinical trials of immune checkpoint inhibitors are currently in phase I/II in which CLL patients are yet to show much clinical activity.

All these novel treatments are most likely to be very expensive for the general public although it is exciting to have them under development. These treatments are a result of our understanding of the biology of CLL. Also, all these leukaemia-based studies indicated that through the use of combination therapies we can target distinctive phenotypes of CLL. Combination therapy can target more efficiently various B cell survival signalling pathways which as a result will eradicate CLL cells. Therefore, a combination between different agents that have a synergistic activity can result in better and effective treatments. In contrast, CLL still remains an incurable disease and these emerging therapies are showing great promise for the near future.

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There is no doubt that scientific advances depend not only on new ideas, conceptual leaps, and paradigm shifts but also to a large extent on technological advances. The current technology while it is advanced still limits our ability to distinguish specific methylation patterns in cells. In contrast, the discovery of next-generation sequencing has enabled us to examine genome-wide DNA methylation patterns with single-nucleotide resolution. Through the years it's expected that technology will improve significantly which as a result will reduce the costs to perform sequencing analysis, making the technology more accessible for the scientists, thus more scientific works will be performed.

To study DNA methylation there are a variety of tools that can be used more frequently in the near future such as bisulfite sequencing of chromatin immunoprecipitated DNA. By using the current technologies, it is very difficult to capture the relationship between DNA methylation, chromatin modification and DNA sequence. Although, this tool is a novel cost-effective approach that enables the investigation of genetic and epigenetic processes between allele-specific DNA methylation, histone modification and other epigenetic regulators. This is achieved by the direct bisulfite sequencing of Polycomb H3K27me3 (histone)-enriched DNA which as a result captures DNA methylation (Statham et al., 2012). Another method is methyl-seq which it's a method that can analyze DNA methylation in more than 90,000 regions in the whole genome by performing alignment, methyl calling and subsequently calculating alignment and methylation metrics. This is accomplished by the use of methyl sensitive restriction enzymes along with next-generation DNA sequencing technology (Liu, Lin, Park and Tseng, 2019) (MethylSeq, 2021).

Reduced representation bisulfite sequencing is another tool that analyzes and compares genomic methylation patterns. This tool is an efficient high-throughput technique that sequences genome-wide methylation profiles in high-CpG genomic DNA by combining restriction enzymes and bisulfite sequencing (Meissner, 2005). There are also other tools that can be used to study DNA methylation such as oxidative BiSulfite sequencing, BiSulfite Padlock Probes, Tet-Assisted Bisulfite sequencing, Methylated DNA ImmunoPrecipitation sequencing and other.

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This study by Popp et al, 2010 has performed an analysis about genome-wide DNA methylation with a very small sample as low as 150ng indicating how important the development of technology is. Another study developed an ultra-low-input, tagmentation-based whole-genome bisulfite sequencing to analyze the DNA methylation profiling method. This method needs only ~10 ng of input DNA, which is 100-fold less than required by the conventional whole-genome bisulfite sequencing. They indicated that ~10 ng of input DNA can provide high-quality coverage of each strand so to study DNA methylation (Adey and Shendure, 2012). In contrast, while the advances of technology enabled the scientists to make new protocols in order to precisely quantify DNA methylation at single-nucleotide resolution using smaller samples many of these methods such as bisulfite sequencing and methylation-sensitive enzyme-based assays, are not accurate/precise enough (Tahiliani et al., 2009) (Huang et al., 2010). As there are other high-throughput techniques such as RNA and chromatin immunoprecipitation (ChIP) sequencing that are becoming more accessible to researchers the need to integrate high throughput data is important so as to understand how gene expression is regulated in health and disease.

To conclude, this review has identified primary cytogenetic aberrations in CLL patients and how hypomethylation of retrotransposons might be implicated in this complex disorder. It's been also introduced various treatments that are currently approved and ready to use and future novel treatments that could extend the quality of life for CLL patients. Advances in technology might be the key for CLL such as CRISPR which is a new powerful tool that is very promising in the field right now. In contrast, this study has raised many questions thus, future work is needed so to unveil accurately how these epigenetic dysregulations of Alus are affecting the genome and their role as potential drivers in CLL.

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ABBREVIATIONS

ABBREVIATION	MEANING
CLL	Chronic lymphocytic leukemia
IGHV	Immunoglobulin heavy chain variable region
SNPs	Single nucleotide polymorphisms
5mC	5-methylcytosine
DNMTs	DNA methyltransferase enzymes
LINE	Long interspersed elements
SINE	Short-interspersed elements
ALUs	Alu elements
TFS	Treatment-free survival
ATM	Ataxia-telangiectasia mutated
DDR	DNA damage response
DSBS	Double-stranded DNA breaks
MRN	MRE11–RAD50–NBS1
RPA	Replication protein A
9-1-1	RAD9–RAD1–HUS1
ATR	Ataxia telangiectasia and Rad3-related
MDC1	DNA damage checkpoint 1
53BP1	p53-binding protein 1
MRN	MRE11–RAD50–NBS1 complex
9-1-1	Toroidal damage checkpoint complex RAD9–RAD1–HUS1
BTK	Bruton’s tyrosine kinase

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FCR	Fludarabine, Cyclophosphamide, and Rituximab
siRNA	Small interfering RNA
CAR	Chimeric antigen receptor

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BIBLIOGRAPHY

Adey, A. and Shendure, J., 2012. Ultra-low-input, tagmentation-based whole-genome bisulfite sequencing. Genome Research, 22(6), pp.1139-1143.

Ahmed, M., Li, W. and Liang, P., 2013. Identification of three new Alu Yb subfamilies by source tracking of recently integrated Alu Yb elements. *Mobile DNA*, 4(1).

Ahn, I., Underbayev, C., Albitar, A., Herman, S., Tian, X., Maric, I., Arthur, D., Wake, L., Pittaluga, S., Yuan, C., Stetler-Stevenson, M., Soto, S., Valdez, J., Nierman, P., Lotter, J., Xi, L., Raffeld, M., Farooqui, M., Albitar, M. and Wiestner, A., 2017. Clonal evolution leading to ibrutinib resistance in chronic lymphocytic leukemia. Blood, 129(11), pp.1469-1479.

Akinleye, A., Chen, Y., Mukhi, N., Song, Y. and Liu, D., 2013. Ibrutinib and novel BTK inhibitors in clinical development. Journal of Hematology & Oncology, 6(1).

Albin, S. and Cunningham-Rundles, C., 2014. An update on the use of immunoglobulin for the treatment of immunodeficiency disorders. *Immunotherapy*, 6(10), pp.1113-1126.

Ateeq, B., Unterberger, A., Szyf, M. and Rabbani, S., 2008. Pharmacological Inhibition of DNA Methylation Induces Proinvasive and Prometastatic Genes In Vitro and In Vivo. *Neoplasia*, 10(3), pp.266-278.

Austen, B., Powell, J., Alvi, A., Edwards, I., Hooper, L., Starczynski, J., Taylor, A., Fegan, C., Moss, P. and Stankovic, T., 2005. Mutations in the ATM gene lead to impaired overall and treatment-free survival that is independent of IGVH mutation status in patients with B-CLL. *Blood*, 106(9), pp.3175-3182.

Ayarpadikannan, S. and Kim, H., 2014. The Impact of Transposable Elements in Genome Evolution and Genetic Instability and Their Implications in Various Diseases. *Genomics & Informatics*, 12(3), p.98.

Badoux, X., Keating, M., Wang, X., O'Brien, S., Ferrajoli, A., Faderl, S., Burger, J., Koller, C., Lerner, S., Kantarjian, H. and Wierda, W., 2011. Fludarabine, cyclophosphamide, and rituximab chemoimmunotherapy is highly effective treatment for relapsed patients with CLL. Blood, 117(11), pp.3016-3024.

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Badoux, X., Keating, M., Wen, S., Lee, B., Sivina, M., Reuben, J., Wierda, W., O'Brien, S., Faderl, S., Kornblau, S., Burger, J. and Ferrajoli, A., 2011. Lenalidomide as initial therapy of elderly patients with chronic lymphocytic leukemia. Blood, 118(13), pp.3489-3498.

Bagacean, C., Tempescul, A., Le Dantec, C., Bordron, A., Mohr, A., Saad, H., Olivier, V., Zdrenghea, M., Cristea, V., Cartron, P., Douet-Guilbert, N., Berthou, C. and Renaudineau, Y., 2017. Alterations in DNA methylation/demethylation intermediates predict clinical outcome in chronic lymphocytic leukemia. *Oncotarget*, 8(39), pp.65699-65716.

Bagacean, C., Zdrenghea, M., Tempescul, A., Cristea, V. and Renaudineau, Y., 2016. Anti-CD20 monoclonal antibodies in chronic lymphocytic leukemia: from uncertainties to promises. *Immunotherapy*, 8(5), pp.569-581.

Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J., Ried, T., Tagle, D. and Wynshaw-Boris, A., 1996. Atm-Deficient Mice: A Paradigm of Ataxia Telangiectasia. *Cell*, 86(1), pp.159-171.

Barrow, T., Wong Doo, N., Milne, R., Giles, G., Willmore, E., Strathdee, G. and Byun, H., 2020. Analysis of retrotransposon subfamily DNA methylation reveals novel early epigenetic changes in chronic lymphocytic leukemia. *Haematologica*, 106(1), pp.98-110.

Baumann Kreuziger, L., Tarchand, G. and Morrison, V., 2013. The impact of Agent Orange exposure on presentation and prognosis of patients with chronic lymphocytic leukemia. *Leukemia & Lymphoma*, 55(1), pp.63-66.

Benard, A., van de Velde, C., Lessard, L., Putter, H., Takeshima, L., Kuppen, P. and Hoon, D., 2013. Epigenetic status of LINE-1 predicts clinical outcome in early-stage rectal cancer. *British Journal of Cancer*, 109(12), pp.3073-3083.

Benetti R, Gonzalo S, Jaco I, Munoz P, Gonzalez S, Schoeftner S et al (2008). A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol 15: 268–279.

Bhutani, N., Burns, D. and Blau, H., 2011. DNA Demethylation Dynamics. *Cell*, 146(6), pp.866-872.

MARIOS PRODROMOU

Bichi, R., Shinton, S., Martin, E., Koval, A., Calin, G., Cesari, R., Russo, G., Hardy, R. and Croce, C., 2002. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. Proceedings of the National Academy of Sciences, 99(10), pp.6955-6960.

Bockorny, B., Codreanu, I. and Dasanu, C., 2011. Hodgkin lymphoma as Richter transformation in chronic lymphocytic leukaemia: a retrospective analysis of world literature. British Journal of Haematology, 156(1), pp.50-66.

Bollati, V., Baccarelli, A., Hou, L., Bonzini, M., Fustinoni, S., Cavallo, D., Byun, H., Jiang, J., Marinelli, B., Pesatori, A., Bertazzi, P. and Yang, A., 2007. Changes in DNA Methylation Patterns in Subjects Exposed to Low-Dose Benzene. *Cancer Research*, 67(3), pp.876-880.

Bosch, F. and Dalla-Favera, R., 2019. Chronic lymphocytic leukaemia: from genetics to treatment. *Nature Reviews Clinical Oncology*, 16(11), pp.684-701.

Brehm, A., Miska, E., McCance, D., Reid, J., Bannister, A. and Kouzarides, T., 1998. Retinoblastoma protein recruits histone deacetylase to repress transcription. Nature, 391(6667), pp.597-601.

Breton, C., Byun, H., Wenten, M., Pan, F., Yang, A. and Gilliland, F., 2009. Prenatal Tobacco Smoke Exposure Affects Global and Gene-specific DNA Methylation. *American Journal of Respiratory and Critical Care Medicine*, 180(5), pp.462-467.

Brown, J., Hillmen, P., O'Brien, S., Barrientos, J., Reddy, N., Coutre, S., Tam, C., Mulligan, S., Jaeger, U., Barr, P., Furman, R., Kipps, T., Cymbalista, F., Thornton, P., Caligaris-Cappio, F., Delgado, J., Montillo, M., DeVos, S., Moreno, C., Pagel, J., Munir, T., Burger, J., Chung, D., Lin, J., Gau, L., Chang, B., Cole, G., Hsu, E., James, D. and Byrd, J., 2017. Extended follow-up and impact of high-risk prognostic factors from the phase 3 RESONATE study in patients with previously treated CLL/SLL. Leukemia, 32(1), pp.83-91.

Burger, J., Tedeschi, A., Barr, P., Robak, T., Owen, C., Ghia, P., Bairey, O., Hillmen, P., Bartlett, N., Li, J., Simpson, D., Grosicki, S., Devereux, S., McCarthy, H., Coutre, S., Quach, H., Gaidano, G., Maslyak, Z., Stevens, D., Janssens, A., Offner, F., Mayer, J., O'Dwyer, M., Hellmann, A., Schuh, A., Siddiqi, T., Polliack, A., Tam, C., Suri, D., Cheng, M., Clow, F.,

MARIOS PRODROMOU

Styles, L., James, D. and Kipps, T., 2015. Ibrutinib as Initial Therapy for Patients with Chronic Lymphocytic Leukemia. New England Journal of Medicine, 373(25), pp.2425-2437.

Byrd, J., Brown, J., O'Brien, S., Barrientos, J., Kay, N., Reddy, N., Coutre, S., Tam, C., Mulligan, S., Jaeger, U., Devereux, S., Barr, P., Furman, R., Kipps, T., Cymbalista, F., Pocock, C., Thornton, P., Caligaris-Cappio, F., Robak, T., Delgado, J., Schuster, S., Montillo, M., Schuh, A., de Vos, S., Gill, D., Bloor, A., Dearden, C., Moreno, C., Jones, J., Chu, A., Fardis, M., McGreivy, J., Clow, F., James, D. and Hillmen, P., 2014. Ibrutinib versus Ofatumumab in Previously Treated Chronic Lymphoid Leukemia. New England Journal of Medicine, 371(3), pp.213-223.

Byrd, J., Harrington, B., O'Brien, S., Jones, J., Schuh, A., Devereux, S., Chaves, J., Wierda, W., Awan, F., Brown, J., Hillmen, P., Stephens, D., Ghia, P., Barrientos, J., Pagel, J., Woyach, J., Johnson, D., Huang, J., Wang, X., Kaptein, A., Lannutti, B., Covey, T., Fardis, M., McGreivy, J., Hamdy, A., Rothbaum, W., Izumi, R., Diacovo, T., Johnson, A. and Furman, R., 2016. Acalabrutinib (ACP-196) in Relapsed Chronic Lymphocytic Leukemia. New England Journal of Medicine, 374(4), pp.323-332.

Cagnetta, A., Soncini, D., Orecchioni, S., Talarico, G., Minetto, P., Guolo, F., Retali, V., Colombo, N., Carminati, E., Clavio, M., Miglino, M., Bergamaschi, M., Nahimana, A., Duchosal, M., Todoerti, K., Neri, A., Passalacqua, M., Bruzzone, S., Nencioni, A., Bertolini, F., Gobbi, M., Lemoli, R. and Cea, M., 2017. Depletion of SIRT6 enzymatic activity increases acute myeloid leukemia cells' vulnerability to DNA-damaging agents. *Haematologica*, 103(1), pp.80-90.

Calabrese, G., Crescenzi, C., Morizio, E., Palka, G., Guerra, E. and Alberti, S., 2001. Assignment<footref rid="foot01">¹</footref> of TACSTD1 (alias TROP1, M4S1) to human chromosome 2p21 and refinement of mapping of TACSTD2 (alias TROP2, M1S1) to human chromosome 1p32 by in situ hybridization. Cytogenetic and Genome Research, 92(1-2), pp.164-165.

Calin, G., Dumitru, C., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F. and Croce, C., 2002. Nonlinear partial differential equations and applications: Frequent deletions and down-

MARIOS PRODROMOU

regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia. Proceedings of the National Academy of Sciences, 99(24), pp.15524-15529.

Calin, G., Ferracin, M., Cimmino, A., Di Leva, G., Shimizu, M., Wojcik, S., Iorio, M., Visone, R., Sever, N., Fabbri, M., Iuliano, R., Palumbo, T., Pichiorri, F., Roldo, C., Garzon, R., Sevignani, C., Rassenti, L., Alder, H., Volinia, S., Liu, C., Kipps, T., Negrini, M. and Croce, C., 2005. A MicroRNA Signature Associated with Prognosis and Progression in Chronic Lymphocytic Leukemia. *New England Journal of Medicine*, 353(17), pp.1793-1801.

Cerhan, J. and Slager, S., 2015. Familial predisposition and genetic risk factors for lymphoma. *Blood*, 126(20), pp.2265-2273.

Chang, H., Jiang, A. and Qi, C., 2010. Aberrant Nuclear p53 Expression Predicts Hemizygous 17p (TP53)Deletion in Chronic Lymphocytic Leukemia. *American Journal of Clinical Pathology*, 133(1), pp.70-74.

Chavez, J., Piris-Villaespesa, M., Dalia, S., Powers, J., Turba, E., Nodzon, L., Komrokji, R., Sokol, L., Locke, F., Lancet, J., Sotomayor, E., Kharfan-Dabaja, M. and Pinilla-Ibarz, J., 2016. Results of a phase II study of lenalidomide and rituximab for refractory/relapsed chronic lymphocytic leukemia. Leukemia Research, 47, pp.78-83.

Chen, L., Apgar, J., Huynh, L., Dicker, F., Giago-McGahan, T., Rassenti, L., Weiss, A. and Kipps, T., 2005. ZAP-70 directly enhances IgM signaling in chronic lymphocytic leukemia. *Blood*, 105(5), pp.2036-2041.

Chiorazzi, N. and Ferrarini, M., 2011. Cellular origin(s) of chronic lymphocytic leukemia: cautionary notes and additional considerations and possibilities. *Blood*, 117(6), pp.1781-1791.

Cimmino, A., Calin, G., Fabbri, M., Iorio, M., Ferracin, M., Shimizu, M., Wojcik, S., Aqeilan, R., Zupo, S., Dono, M., Rassenti, L., Alder, H., Volinia, S., Liu, C., Kipps, T., Negrini, M. and Croce, C., 2005. miR-15 and miR-16 induce apoptosis by targeting BCL2. *Proceedings of the National Academy of Sciences*, 102(39), pp.13944-13949.

Compere, S. and Palmiter, R., 1981. DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. *Cell*, 25(1), pp.233-240.

MARIOS PRODROMOU

Crowther-Swanepoel, D., Broderick, P., Di Bernardo, M., Dobbins, S., Torres, M., Mansouri, M., Ruiz-Ponte, C., Enjuanes, A., Rosenquist, R., Carracedo, A., Jurlander, J., Campo, E., Juliusson, G., Montserrat, E., Smedby, K., Dyer, M., Matutes, E., Dearden, C., Sunter, N., Hall, A., Mainou-Fowler, T., Jackson, G., Summerfield, G., Harris, R., Pettitt, A., Allsup, D., Bailey, J., Pratt, G., Pepper, C., Fegan, C., Parker, A., Oscier, D., Allan, J., Catovsky, D. and Houlston, R., 2010. Common variants at 2q37.3, 8q24.21, 15q21.3 and 16q24.1 influence chronic lymphocytic leukemia risk. *Nature Genetics*, 42(2), pp.132-136.

Cytoenetics. 2021. CytoCell ATM Deletion. [online] Available at: <https://www.ogt.com/ca/products/product-search/cytocell-atm-deletion/7> [Accessed 24 October 2021].

Dai, H., Wang, Y., Lu, X. and Han, W., 2016. Chimeric Antigen Receptors Modified T-Cells for Cancer Therapy. JNCI: Journal of the National Cancer Institute, 108(7).

Dal-Bo, M., Bertoni, F., Forconi, F., Zucchetto, A., Bomben, R., Marasca, R., Deaglio, S., Laurenti, L., Efremov, D., Gaidano, G., Del Poeta, G. and Gattei, V., 2009. Intrinsic and extrinsic factors influencing the clinical course of B-cell chronic lymphocytic leukemia: prognostic markers with pathogenetic relevance. Journal of Translational Medicine, 7(1).

Damle, R., Wasil, T., Fais, F., Ghiotto, F., Valetto, A., Allen, S., Buchbinder, A., Budman, D., Dittmar, K., Kolitz, J., Lichtman, S., Schulman, P., Vinciguerra, V., Rai, K., Ferrarini, M. and Chiorazzi, N., 1999. Ig V Gene Mutation Status and CD38 Expression As Novel Prognostic Indicators in Chronic Lymphocytic Leukemia. *Blood*, 94(6), pp.1840-1847.

Damm, F., Mylonas, E., Cosson, A., Yoshida, K., Della Valle, V., Mouly, E., Diop, M., Scourzic, L., Shiraishi, Y., Chiba, K., Tanaka, H., Miyano, S., Kikushige, Y., Davi, F., Lambert, J., Gautheret, D., Merle-Béral, H., Sutton, L., Dessen, P., Solary, E., Akashi, K., Vainchenker, W., Mercher, T., Droin, N., Ogawa, S., Nguyen-Khac, F. and Bernard, O., 2014. Acquired Initiating Mutations in Early Hematopoietic Cells of CLL Patients. *Cancer Discovery*, 4(9), pp.1088-1101.

Darwiche, W., Gubler, B., Marolleau, J. and Ghamlouch, H., 2018. Chronic Lymphocytic Leukemia B-Cell Normal Cellular Counterpart: Clues From a Functional Perspective. *Frontiers in Immunology*, 9.

MARIOS PRODROMOU

Daskalos, A., Nikolaidis, G., Xinarianos, G., Savvari, P., Cassidy, A., Zakopoulou, R., Kotsinas, A., Gorgoulis, V., Field, J. and Liloglou, T., 2009. Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. *International Journal of Cancer*, 124(1), pp.81-87.

De Silva, N. and Klein, U., 2015. Dynamics of B cells in germinal centres. *Nature Reviews Immunology*, 15(3), pp.137-148.

De Smet, C., Lurquin, C., Lethé, B., Martelange, V. and Boon, T., 1999. DNA Methylation Is the Primary Silencing Mechanism for a Set of Germ Line- and Tumor-Specific Genes with a CpG-Rich Promoter. *Molecular and Cellular Biology*, 19(11), pp.7327-7335.

de Weerdt, I., Koopmans, S., Kater, A. and van Gelder, M., 2017. Incidence and management of toxicity associated with ibrutinib and idelalisib: a practical approach. Haematologica, 102(10), pp.1629-1639.

Deininger, P., 2011. Alu elements: know the SINEs. *Genome Biology*, 12(12), p.236.

Deininger, P., Batzer, M., Hutchison, C. and Edgell, M., 1992. Master genes in mammalian repetitive DNA amplification. *Trends in Genetics*, 8(9), pp.307-311.

Delgado, J., Salaverria, I., Baumann, T., Martinez-Trillos, A., Lee, E., Jimenez, L., Navarro, A., Royo, C., Santacruz, R., Lopez, C., Payer, A., Colado, E., Gonzalez, M., Armengol, L., Colomer, D., Pinyol, M., Villamor, N., Aymerich, M., Carrio, A., Costa, D., Clot, G., Gine, E., Lopez-Guillermo, A., Campo, E. and Bea, S., 2014. Genomic complexity and IGHV mutational status are key predictors of outcome of chronic lymphocytic leukemia patients with TP53 disruption. Haematologica, 99(11), pp.e231-e234.

Dewannieux, M., Esnault, C. and Heidmann, T., 2003. LINE-mediated retrotransposition of marked Alu sequences. *Nature Genetics*, 35(1), pp.41-48.

Dhalla, F., Lucas, M., Schuh, A., Bhole, M., Jain, R., Patel, S., Misbah, S. and Chapel, H., 2014. Antibody Deficiency Secondary to Chronic Lymphocytic Leukemia: Should Patients be Treated with Prophylactic Replacement Immunoglobulin?. Journal of Clinical Immunology, 34(3), pp.277-282.

MARIOS PRODROMOU

Di Bernardo, M., Crowther-Swanepoel, D., Broderick, P., Webb, E., Sellick, G., Wild, R., Sullivan, K., Vijayakrishnan, J., Wang, Y., Pittman, A., Sunter, N., Hall, A., Dyer, M., Matutes, E., Dearden, C., Mainou-Fowler, T., Jackson, G., Summerfield, G., Harris, R., Pettitt, A., Hillmen, P., Allsup, D., Bailey, J., Pratt, G., Pepper, C., Fegan, C., Allan, J., Catovsky, D. and Houlston, R., 2008. A genome-wide association study identifies six susceptibility loci for chronic lymphocytic leukemia. *Nature Genetics*, 40(10), pp.1204-1210.

Dijkstra, M., van Lom, K., Tielemans, D., Elstrodt, F., Langerak, A., van 't Veer, M. and Jongen-Lavrencic, M., 2008. 17p13/TP53 deletion in B-CLL patients is associated with microRNA-34a downregulation. Leukemia, 23(3), pp.625-627.

Döhner, H., Stilgenbauer, S., Benner, A., Leupolt, E., Kröber, A., Bullinger, L., Döhner, K., Bentz, M. and Lichter, P., 2000. Genomic Aberrations and Survival in Chronic Lymphocytic Leukemia. *New England Journal of Medicine*, 343(26), pp.1910-1916.

Dreger, P., Schnaiter, A., Zenz, T., Böttcher, S., Rossi, M., Paschka, P., Bühler, A., Dietrich, S., Busch, R., Ritgen, M., Bunjes, D., Zeis, M., Stadler, M., Uharek, L., Scheid, C., Hegenbart, U., Hallek, M., Kneba, M., Schmitz, N., Döhner, H. and Stilgenbauer, S., 2013. TP53, SF3B1, and NOTCH1 mutations and outcome of allotransplantation for chronic lymphocytic leukemia: six-year follow-up of the GCLLSG CLL3X trial. Blood, 121(16), pp.3284-3288.

Drugs.com. 2016. FDA Approves Imbruvica (ibrutinib) for the First-Line Treatment of Chronic Lymphocytic Leukemia. [online] Available at: <https://www.drugs.com/newdrugs/fda-approves-imbruvica-ibrutinib-first-line-chronic-lymphocytic-leukemia-4353.html> [Accessed 20 October 2021].

Efremov, D., Turkalj, S. and Laurenti, L., 2020. Mechanisms of B Cell Receptor Activation and Responses to B Cell Receptor Inhibitors in B Cell Malignancies. *Cancers*, 12(6), p.1396.

Ehrlich, M., 2002. DNA methylation in cancer: too much, but also too little. *Oncogene*, 21(35), pp.5400-5413.

Ehrlich, M., 2009. DNA hypomethylation in cancer cells. *Epigenomics*, 1(2), pp.239-259.

MARIOS PRODROMOU

Ehrlich, M., Gama-Sosa, M., Huang, L., Midgett, R., Kuo, K., McCune, R. and Gehrke, C., 1982. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic Acids Research*, 10(8), pp.2709-2721.

Elson, A., Wang, Y., Daugherty, C., Morton, C., Zhou, F., Campos-Torres, J. and Leder, P., 1996. Pleiotropic defects in ataxia-telangiectasia protein-deficient mice. *Proceedings of the National Academy of Sciences*, 93(23), pp.13084-13089.

Ema.europa.eu. 2021. Imbruvica (ibrutinib). [online] Available at: <https://www.ema.europa.eu/en/documents/overview/imbruvica-epar-medicine-overview_en.pdf> [Accessed 20 October 2021].

Fabbri, G. and Dalla-Favera, R., 2016. The molecular pathogenesis of chronic lymphocytic leukaemia. *Nature Reviews Cancer*, 16(3), pp.145-162.

Fabbri, G., Rasi, S., Rossi, D., Trifonov, V., Khiabanian, H., Ma, J., Grunn, A., Fangazio, M., Capello, D., Monti, S., Cresta, S., Gargiulo, E., Forconi, F., Guarini, A., Arcaini, L., Paulli, M., Laurenti, L., Larocca, L., Marasca, R., Gattei, V., Oscier, D., Bertoni, F., Mullighan, C., Foá, R., Pasqualucci, L., Rabadan, R., Dalla-Favera, R. and Gaidano, G., 2011. Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. Journal of Experimental Medicine, 208(7), pp.1389-1401.

Fabris, S., Bollati, V., Agnelli, L., Morabito, F., Motta, V., Cutrona, G., Matis, S., Grazia Recchia, A., Gigliotti, V., Gentile, M., Deliliers, G., Bertazzi, P., Ferrarini, M., Neri, A. and Baccarelli, A., 2011. Biological and clinical relevance of quantitative global methylation of repetitive DNA sequences in chronic lymphocytic leukemia. Epigenetics, 6(2), pp.188-194.

Fan, G., Beard, C., Chen, R., Csankovszki, G., Sun, Y., Siniaia, M., Biniszkiewicz, D., Bates, B., Lee, P., Kühn, R., Trumpp, A., Poon, C., Wilson, C. and Jaenisch, R., 2001. DNA Hypomethylation Perturbs the Function and Survival of CNS Neurons in Postnatal Animals. *The Journal of Neuroscience*, 21(3), pp.788-797.

Farruggia, P. and Dufour, C., 2014. Diagnosis and management of primary autoimmune neutropenia in children: insights for clinicians. Therapeutic Advances in Hematology, 6(1), pp.15-24.

MARIOS PRODROMOU

Fecteau, J., Corral, L., Ghia, E., Gaidarova, S., Futalan, D., Bharati, I., Cathers, B., Schwaederlé, M., Cui, B., Lopez-Girona, A., Messmer, D. and Kipps, T., 2014. Lenalidomide inhibits the proliferation of CLL cells via a cereblon/p21WAF1/Cip1-dependent mechanism independent of functional p53. Blood, 124(10), pp.1637-1644.

Feinberg, A. and Vogelstein, B., 1983. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, 301(5895), pp.89-92.

Ferrajoli, A., Lee, B., Schlette, E., O'Brien, S., Gao, H., Wen, S., Wierda, W., Estrov, Z., Faderl, S., Cohen, E., Li, C., Reuben, J. and Keating, M., 2008. Lenalidomide induces complete and partial remissions in patients with relapsed and refractory chronic lymphocytic leukemia. Blood, 111(11), pp.5291-5297.

Figueroa, M., Lugthart, S., Li, Y., Erpelinck-Verschueren, C., Deng, X., Christos, P., Schifano, E., Booth, J., van Putten, W., Skrabanek, L., Campagne, F., Mazumdar, M., Greally, J., Valk, P., Löwenberg, B., Delwel, R. and Melnick, A., 2010. DNA Methylation Signatures Identify Biologically Distinct Subtypes in Acute Myeloid Leukemia. Cancer Cell, 17(1), pp.13-27.

Fischer, K., Bahlo, J., Fink, A., Goede, V., Herling, C., Cramer, P., Langerbeins, P., von Tresckow, J., Engelke, A., Maurer, C., Kovacs, G., Herling, M., Tausch, E., Kreuzer, K., Eichhorst, B., Böttcher, S., Seymour, J., Ghia, P., Marlton, P., Kneba, M., Wendtner, C., Döhner, H., Stilgenbauer, S. and Hallek, M., 2016. Long-term remissions after FCR chemoimmunotherapy in previously untreated patients with CLL: updated results of the CLL8 trial. Blood, 127(2), pp.208-215.

Fischer, K., Cramer, P., Busch, R., Stilgenbauer, S., Bahlo, J., Schweighofer, C., Böttcher, S., Staib, P., Kiehl, M., Eckart, M., Kranz, G., Goede, V., Elter, T., Bühler, A., Winkler, D., Kneba, M., Döhner, H., Eichhorst, B., Hallek, M. and Wendtner, C., 2011. Bendamustine Combined With Rituximab in Patients With Relapsed and/or Refractory Chronic Lymphocytic Leukemia: A Multicenter Phase II Trial of the German Chronic Lymphocytic Leukemia Study Group. Journal of Clinical Oncology, 29(26), pp.3559-3566.

MARIOS PRODROMOU

Flynn, J., Andritsos, L., Lucas, D. and Byrd, J., 2010. Second malignancies in B-cell chronic lymphocytic leukaemia: possible association with human papilloma virus. British Journal of Haematology, 149(3), pp.388-390.

Forconi, F. and Moss, P., 2015. Perturbation of the normal immune system in patients with CLL. *Blood*, 126(5), pp.573-581.

Fujita, M., Kawabata, H., Oka, T., Hishizawa, M., Kitano, T., Kondo, T., Yamashita, K., Yurugi, K., Hirai, H., Maekawa, T. and Takaori-Kondo, A., 2017. A Rare Case of Adult Autoimmune Neutropenia Successfully Treated with Prednisolone. Internal Medicine, 56(11), pp.1415-1419.

Fuks, F., 2001. Dnmt3a binds deacetylases and is recruited by a sequence-specific repressor to silence transcription. The EMBO Journal, 20(10), pp.2536-2544.

Furman, R., Sharman, J., Coutre, S., Cheson, B., Pagel, J., Hillmen, P., Barrientos, J., Zelenetz, A., Kipps, T., Flinn, I., Ghia, P., Eradat, H., Ervin, T., Lamanna, N., Coiffier, B., Pettitt, A., Ma, S., Stilgenbauer, S., Cramer, P., Aiello, M., Johnson, D., Miller, L., Li, D., Jahn, T., Dansey, R., Hallek, M. and O'Brien, S., 2014. Idelalisib and Rituximab in Relapsed Chronic Lymphocytic Leukemia. New England Journal of Medicine, 370(11), pp.997-1007.

Ghia, E., Jain, S., Widhopf, G., Rassenti, L., Keating, M., Wierda, W., Gribben, J., Brown, J., Rai, K., Byrd, J., Kay, N., Greaves, A. and Kipps, T., 2008. Use of IGHV3–21 in chronic lymphocytic leukemia is associated with high-risk disease and reflects antigen-driven, post–germinal center leukemogenic selection. *Blood*, 111(10), pp.5101-5108.

Giannopoulos, K., Mertens, D. and Stilgenbauer, S., 2011. Treating chronic lymphocytic leukemia with thalidomide and lenalidomide. Expert Opinion on Pharmacotherapy, 12(18), pp.2857-2864.

Giralt, S. and Bishop, M., 2008. Principles and Overview of Allogeneic Hematopoietic Stem Cell Transplantation. Cancer Treatment and Research, pp.1-21.

Goede, V., Fischer, K., Busch, R., Engelke, A., Eichhorst, B., Wendtner, C., Chagorova, T., de la Serna, J., Dilhuydy, M., Illmer, T., Opat, S., Owen, C., Samoylova, O., Kreuzer, K., Stilgenbauer, S., Döhner, H., Langerak, A., Ritgen, M., Kneba, M., Asikanius, E.,

MARIOS PRODROMOU

Humphrey, K., Wenger, M. and Hallek, M., 2014. Obinutuzumab plus Chlorambucil in Patients with CLL and Coexisting Conditions. New England Journal of Medicine, 370(12), pp.1101-1110.

Grossmann, V., Kohlmann, A., Schnittger, S., Weissmann, S., Jeromin, S., Kienast, J., Kern, W., Haferlach, T. and Haferlach, C., 2012. Recurrent ATM and BIRC3 Mutations in Patients with Chronic Lymphocytic Leukemia (CLL) and Deletion 11q22-q23. *Blood*, 120(21), pp.1771-1771.

Guasch, G. and Fuchs, E., 2005. Mice in the world of stem cell biology. Nature Genetics, 37(11), pp.1201-1206.

Guasch, G. and Fuchs, E., 2005. Mice in the world of stem cell biology. Nature Genetics, 37(11), pp.1201-1206.

Haferlach, C., Dicker, F., Schnittger, S., Kern, W. and Haferlach, T., 2007. Comprehensive genetic characterization of CLL: a study on 506 cases analysed with chromosome banding analysis, interphase FISH, IgVH status and immunophenotyping. *Leukemia*, 21(12), pp.2442-2451.

Hallek, M., Fischer, K., Fingerle-Rowson, G., Fink, A., Busch, R., Mayer, J., Hensel, M., Hopfinger, G., Hess, G., von Grünhagen, U., Bergmann, M., Catalano, J., Zinzani, P., Caligaris-Cappio, F., Seymour, J., Berrebi, A., Jäger, U., Cazin, B., Trneny, M., Westermann, A., Wendtner, C., Eichhorst, B., Staib, P., Bühler, A., Winkler, D., Zenz, T., Böttcher, S., Ritgen, M., Mendila, M., Kneba, M., Döhner, H. and Stilgenbauer, S., 2010. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. The Lancet, 376(9747), pp.1164-1174.

Hamblin, T., Davis, Z., Gardiner, A., Oscier, D. and Stevenson, F., 1999. Unmutated Ig VH Genes Are Associated With a More Aggressive Form of Chronic Lymphocytic Leukemia. *Blood*, 94(6), pp.1848-1854.

Han, K., 2005. Under the genomic radar: The Stealth model of Alu amplification. *Genome Research*, 15(5), pp.655-664.

MARIOS PRODROMOU

Harris, S. and Levine, A., 2005. The p53 pathway: positive and negative feedback loops. Oncogene, 24(17), pp.2899-2908.

Heinzerling, L., de Toni, E., Schett, G., Hundorfean, G. and Zimmer, L., 2019. Checkpoint Inhibitors. Deutsches Aerzteblatt Online,.

Helman, E., Lawrence, M., Stewart, C., Sougnez, C., Getz, G. and Meyerson, M., 2014. Somatic retrotransposition in human cancer revealed by whole-genome and exome sequencing. *Genome Research*, 24(7), pp.1053-1063.

Hengeveld, P., Levin, M., Kolijn, P. and Langerak, A., 2021. Reading the B-cell receptor immunome in chronic lymphocytic leukemia: revelations and applications. *Experimental Hematology*, 93, pp.14-24.

Hervouet, E., Peixoto, P., Delage-Mourroux, R., Boyer-Guittaut, M. and Cartron, P., 2018. Specific or not specific recruitment of DNMTs for DNA methylation, an epigenetic dilemma. *Clinical Epigenetics*, 10(1).

Hewamana, S. and Dearden, C., 2011. Treatment options for high-risk chronic lymphocytic leukaemia. Therapeutic Advances in Hematology, 2(3), pp.147-159.

Hisada, M., 2001. Solid tumors after chronic lymphocytic leukemia. Blood, 98(6), pp.1979-1981.

Hjalgrim, H., Rostgaard, K., Vasan, S., Ullum, H., Erikstrup, C., Pedersen, O., Nielsen, K., Titlestad, K., Melbye, M., Nyrén, O. and Edgren, G., 2015. No evidence of transmission of chronic lymphocytic leukemia through blood transfusion. *Blood*, 126(17), pp.2059-2061.

Hodgson, K., Ferrer, G., Pereira, A., Moreno, C. and Montserrat, E., 2011. Autoimmune cytopenia in chronic lymphocytic leukaemia: diagnosis and treatment. *British Journal of Haematology*, 154(1), pp.14-22.

Holliday, R. and Pugh, J., 1975. DNA modification mechanisms and gene activity during development. *Science*, 187(4173), pp.226-232.

MARIOS PRODROMOU

Hotchkiss RD (1948). The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. J Biol Chem 175: 315–332. Discovered 5-methylcytosine in the mammalian genome.

Hoxha, M., Fabris, S., Agnelli, L., Bollati, V., Cutrona, G., Matis, S., Recchia, A., Gentile, M., Cortelezzi, A., Morabito, F., Bertazzi, P., Ferrarini, M. and Neri, A., 2014. Relevance of telomere/telomerase system impairment in early stage chronic lymphocytic leukemia. Genes, Chromosomes and Cancer, 53(7), pp.612-621.

Huang, Y., Pastor, W., Shen, Y., Tahiliani, M., Liu, D. and Rao, A., 2010. The Behaviour of 5-Hydroxymethylcytosine in Bisulfite Sequencing. PLoS ONE, 5(1), p.e8888.

Illumina.com. 2021. MethylSeq. [online] Available at: <https://www.illumina.com/products/by-type/informatics-products/basespace-sequence-hub/apps/methylseq.html> [Accessed 20 November 2021].

Intergenetics. 2021. Deletion 13q14 detected by MLPA analysis - Intergenetics. [online] Available at: <https://intergenetics.eu/en/exam/deletion-13q14-detected-by-mlpa-analysis/> [Accessed 22 October 2021].

Jackson, H., Rafiq, S. and Brentjens, R., 2016. Driving CAR T-cells forward. Nature Reviews Clinical Oncology, 13(6), pp.370-383.

Jackson-Grusby, L., Beard, C., Possemato, R., Tudor, M., Fambrough, D., Csankovszki, G., Dausman, J., Lee, P., Wilson, C., Lander, E. and Jaenisch, R., 2001. Loss of genomic methylation causes p53-dependent apoptosis and epigenetic deregulation. Nature Genetics, 27(1), pp.31-39.

James, D., Werner, L., Brown, J., Wierda, W., Barrientos, J., Castro, J., Greaves, A., Johnson, A., Rassenti, L., Rai, K., Neuberg, D. and Kipps, T., 2014. Lenalidomide and Rituximab for the Initial Treatment of Patients With Chronic Lymphocytic Leukemia: A Multicenter Clinical-Translational Study From the Chronic Lymphocytic Leukemia Research Consortium. Journal of Clinical Oncology, 32(19), pp.2067-2073.

Jin, B. and Robertson, K., 2012. DNA Methyltransferases, DNA Damage Repair, and Cancer. *Advances in Experimental Medicine and Biology*, pp.3-29.

MARIOS PRODROMOU

Jurka, J. and Milosavljevic, A., 1991. Reconstruction and analysis of human alu genes. *Journal of Molecular Evolution*, 32(2), pp.105-121.

Katragkou, A., Roilides, E. and Walsh, T., 2018. Role of Immunoglobulin Therapy to Prevent and Treat Infections. Management of Infections in the Immunocompromised Host, pp.339-358.

Kerber, R. and O'Brien, E., 2005. A cohort study of cancer risk in relation to family histories of cancer in the Utah population database. *Cancer*, 103(9), pp.1906-1915.

Kim, S., Cho, C., Han, K. and Lee, J., 2016. Structural Variation ofAluElement and Human Disease. *Genomics & Informatics*, 14(3), p.70.

Kipps, T., Fraser, G., Coutre, S., Brown, J., Barrientos, J., Barr, P., Byrd, J., O'Brien, S., Dilhuydy, M., Hillmen, P., Jaeger, U., Moreno, C., Cramer, P., Stilgenbauer, S., Chanan-Khan, A., Mahler, M., Salman, M., Eckert, K., Solman, I., Balasubramanian, S., Cheng, M., Londhe, A., Ninomoto, J., Howes, A., James, D. and Hallek, M., 2019. Long-Term Studies Assessing Outcomes of Ibrutinib Therapy in Patients With Del(11q) Chronic Lymphocytic Leukemia. Clinical Lymphoma Myeloma and Leukemia, 19(11), pp.715-722.e6.

Kipps, T., Stevenson, F., Wu, C., Croce, C., Packham, G., Wierda, W., O'Brien, S., Gribben, J. and Rai, K., 2017. Chronic lymphocytic leukaemia. *Nature Reviews Disease Primers*, 3(1).

Klein, C., Hartmann, B., Schön, M., Weber, L. and Alberti, S., 1990. Expression of 38-kD Cell-Surface Glycoprotein in Transformed Keratinocyte Cell Lines, Basal Cell Carcinomas, and Epithelial Germs. Journal of Investigative Dermatology, 95(1), pp.74-82.

Kröber, A., Seiler, T., Benner, A., Bullinger, L., Brückle, E., Lichter, P., Döhner, H. and Stilgenbauer, S., 2002. V H mutation status, CD38 expression level, genomic aberrations, and survival in chronic lymphocytic leukemia. *Blood*, 100(4), pp.1410-1416.

Kulis, M., Heath, S., Bibikova, M., Queirós, A., Navarro, A., Clot, G., Martínez-Trillos, A., Castellano, G., Brun-Heath, I., Pinyol, M., Barberán-Soler, S., Papasaikas, P., Jares, P., Beà, S., Rico, D., Ecker, S., Rubio, M., Royo, R., Ho, V., Klotzle, B., Hernández, L., Conde, L., López-Guerra, M., Colomer, D., Villamor, N., Aymerich, M., Rozman, M., Bayes, M., Gut, M., Gelpí, J., Orozco, M., Fan, J., Quesada, V., Puente, X., Pisano, D., Valencia, A., López-

MARIOS PRODROMOU

Guillermo, A., Gut, I., López-Otín, C., Campo, E. and Martín-Subero, J., 2012. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. *Nature Genetics*, 44(11), pp.1236-1242.

Kulis, M., Heath, S., Bibikova, M., Queirós, A., Navarro, A., Clot, G., Martínez-Trillos, A., Castellano, G., Brun-Heath, I., Pinyol, M., Barberán-Soler, S., Papasaikas, P., Jares, P., Beà, S., Rico, D., Ecker, S., Rubio, M., Royo, R., Ho, V., Klotzle, B., Hernández, L., Conde, L., López-Guerra, M., Colomer, D., Villamor, N., Aymerich, M., Rozman, M., Bayes, M., Gut, M., Gelpí, J., Orozco, M., Fan, J., Quesada, V., Puente, X., Pisano, D., Valencia, A., López-Guillermo, A., Gut, I., López-Otín, C., Campo, E. and Martín-Subero, J., 2012. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic leukemia. Nature Genetics, 44(11), pp.1236-1242.

Küppers, R., 2005. Mechanisms of B-cell lymphoma pathogenesis. Nature Reviews Cancer, 5(4), pp.251-262.

Kwok, M. and Stankovic, T., 2019. Targeting the Ataxia Telangiectasia and Rad3 Signaling Pathway to Overcome Chemoresistance in Cancer. *Targeting Cell Survival Pathways to Enhance Response to Chemotherapy*, pp.203-230.

Landau, D., Clement, K., Ziller, M., Boyle, P., Fan, J., Gu, H., Stevenson, K., Sougnez, C., Wang, L., Li, S., Kotliar, D., Zhang, W., Ghandi, M., Garraway, L., Fernandes, S., Livak, K., Gabriel, S., Gnirke, A., Lander, E., Brown, J., Neuberg, D., Kharchenko, P., Hacohen, N., Getz, G., Meissner, A. and Wu, C., 2014. Locally Disordered Methylation Forms the Basis of Intratumor Methylome Variation in Chronic Lymphocytic Leukemia. *Cancer Cell*, 26(6), pp.813-825.

Lander, E., Linton, L., Birren, B., Nusbaum, C., Zody, M., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham,

MARIOS PRODROMOU

D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R., Wilson, R., Hillier, L., McPherson, J., Marra, M., Mardis, E., Fulton, L., Chinwalla, A., Pepin, K., Gish, W., Chissoe, S., Wendl, M., Delehaunty, K., Miner, T., Delehaunty, A., Kramer, J., Cook, L., Fulton, R., Johnson, D., Minx, P., Clifton, S., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R., Muzny, D., Scherer, S., Bouck, J., Sodergren, E., Worley, K., Rives, C., Gorrell, J., Metzker, M., Naylor, S., Kucherlapati, R., Nelson, D., Weinstock, G., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Smith, D., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H., Dubois, J., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R., Federspiel, N., Abola, A., Proctor, M., Roe, B., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W., de la Bastide, M., Dedhia, N., Blöcker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D., Burge, C., Cerutti, L., Chen, H., Church, D., Clamp, M., Copley, R., Doerks, T., Eddy, S., Eichler, E., Furey, T., Galagan, J., Gilbert, J., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L., Jones, T., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W., Kitts, P., Koonin, E., Korf, I., Kulp, D., Lancet, D., Lowe, T., McLysaght, A., Mikkelsen, T., Moran, J., Mulder, N., Pollara, V., Ponting, C., Schuler, G., Schultz, J., Slater, G., Smit, A., Stupka, E., Szustakowki, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y., Wolfe, K., Yang, S., Yeh, R., Collins, F., Guyer, M., Peterson, J., Felsenfeld, A., Wetterstrand, K., Myers, R., Schmutz, J., Dickson, M., Grimwood, J., Cox, D., Olson, M., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G., Athanasiou, M., Schultz, R., Patrinos, A. and Morgan, M., 2001. Initial sequencing and analysis of the human genome. *Nature*, 409(6822), pp.860-921.

Lavin, M. and Shiloh, Y., 1997. THE GENETIC DEFECT IN ATAXIA-TELANGIECTASIA. *Annual Review of Immunology*, 15(1), pp.177-202.

MARIOS PRODROMOU

Li, Y., Wang, Y., Wang, Z., Yi, D. and Ma, S., 2015. Racial differences in three major NHL subtypes: Descriptive epidemiology. *Cancer Epidemiology*, 39(1), pp.8-13.

Li, Y., Wang, Y., Wang, Z., Yi, D. and Ma, S., 2015. Racial differences in three major NHL subtypes: Descriptive epidemiology. *Cancer Epidemiology*, 39(1), pp.8-13.

Lihteh, W. and Foster, S., 2021. *What is the global prevalence of leukemias?*. [online] Medscape.com. Available at: <https://www.medscape.com/answers/1201870-175991/what-is-the-global-prevalence-of-leukemias#:~:text=An%20estimated%20300%2C000%20new%20cases,AML%20represents%2020%25%20of%20cases.> [Accessed 1 October 2021].

Lindsey, H., 2005. Environmental Factors & Cancer. *Oncology Times*, 27(4), p.8.

Liu, N., Yan, J., Chen, X., Song, J., Wang, B. and Yao, Y., 2014. Prenatal diagnosis of a de novo interstitial deletion of 11q (11q22.3 → q23.3) associated with abnormal ultrasound findings by array comparative genomic hybridization. Molecular Cytogenetics, 7(1).

Liu, P., Lin, C., Park, Y. and Tseng, G., 2019. MethylSeqDesign: a framework for Methyl-Seq genome-wide power calculation and study design issues. Biostatistics, 22(1), pp.35-50.

Liu, X., Wu, H., Ji, X., Stelzer, Y., Wu, X., Czauderna, S., Shu, J., Dadon, D., Young, R. and Jaenisch, R., 2016. Editing DNA Methylation in the Mammalian Genome. Cell, 167(1), pp.233-247.e17.

Luzhna L, Kovalchuk O. Modulation of DNA methylation levels sensitizes doxorubicin-resistant breast adenocarcinoma cells to radiation-induced apoptosis. Biochem Biophys Res Commun. (2010) 392:113–7. doi: 10.1016/j.bbrc.2009.12.093

M Moll, U. and Petrenko, O., 2003. The MDM2-p53 interaction. National Library of Medicine, Volume 1(Issue 14).

Marsilio, S., Khiabanian, H., Fabbri, G., Vergani, S., Scuoppo, C., Montserrat, E., Shpall, E., Hadigol, M., Marin, P., Rai, K., Rabadan, R., Devereux, S., Pasqualucci, L. and Chiorazzi, N., 2017. Somatic CLL mutations occur at multiple distinct hematopoietic maturation stages: documentation and cautionary note regarding cell fraction purity. *Leukemia*, 32(4), pp.1040-1043.

MARIOS PRODROMOU

Maurer, C., Langerbeins, P., Bahlo, J., Cramer, P., Fink, A., Pflug, N., Engelke, A., von Tresckow, J., Kovacs, G., Stilgenbauer, S., Wendtner, C., Müller, L., Ritgen, M., Seiler, T., Fischer, K., Hallek, M. and Eichhorst, B., 2016. Effect of first-line treatment on second primary malignancies and Richter's transformation in patients with CLL. *Leukemia*, 30(10), pp.2019-2025.

Mauro, F., Chauvie, S., Paoloni, F., Biggi, A., Cimino, G., Rago, A., Gentile, M., Morabito, F., Coscia, M., Bellò, M., Sacchetti, G., Rossi, D., Laurenti, L., Autore, F., Campanelli, M., Trastulli, F., Nicolai, E., Riminucci, M., Gaidano, G., Guarini, A., Gallamini, A. and Foà, R., 2015. Diagnostic and prognostic role of PET/CT in patients with chronic lymphocytic leukemia and progressive disease. Leukemia, 29(6), pp.1360-1365.

Mayer, W., Niveleau, A., Walter, J., Fundele, R. and Haaf, T., 2000. Demethylation of the zygotic paternal genome. *Nature*, 403(6769), pp.501-502.

Mayr, C., Speicher, M., Kofler, D., Buhmann, R., Strehl, J., Busch, R., Hallek, M. and Wendtner, C., 2006. Chromosomal translocations are associated with poor prognosis in chronic lymphocytic leukemia. Blood, 107(2), pp.742-751.

Mayr, C., Speicher, M., Kofler, D., Buhmann, R., Strehl, J., Busch, R., Hallek, M. and Wendtner, C., 2006. Chromosomal translocations are associated with poor prognosis in chronic lymphocytic leukemia. Blood, 107(2), pp.742-751.

McClanahan, F., Hanna, B., Miller, S., Clear, A., Lichter, P., Gribben, J. and Seiffert, M., 2015. PD-L1 checkpoint blockade prevents immune dysfunction and leukemia development in a mouse model of chronic lymphocytic leukemia. Blood, 126(2), pp.203-211.

Means, R., 2016. Pure red cell aplasia. Hematology, 2016(1), pp.51-56.

Meissner, A., 2005. Reduced representation bisulfite sequencing for comparative high-resolution DNA methylation analysis. Nucleic Acids Research, 33(18), pp.5868-5877.

Mighell, A., Markham, A. and Robinson, P., 1997. Alusequences. *FEBS Letters*, 417(1), pp.1-5.

Mills, R., Bennett, E., Iskow, R. and Devine, S., 2007. Which transposable elements are active in the human genome?. *Trends in Genetics*, 23(4), pp.183-191.

MARIOS PRODROMOU

Mills, R., Bennett, E., Iskow, R., Luttig, C., Tsui, C., Pittard, W. and Devine, S., 2006. Recently Mobilized Transposons in the Human and Chimpanzee Genomes. *The American Journal of Human Genetics*, 78(4), pp.671-679.

Momburg, F., Moldenhauer, G., Hämmerling, G. J., & Möller, P. (1987). Immunohistochemical study of the expression of a Mr 34,000 human epithelium-specific surface glycoprotein in normal and malignant tissues. Cancer research, 47(11), 2883–2891.

Moore, L., Le, T. and Fan, G., 2012. DNA Methylation and Its Basic Function. *Neuropsychopharmacology*, 38(1), pp.23-38.

Moreno, C., Greil, R., Demirkan, F., Tedeschi, A., Anz, B., Larratt, L., Simkovic, M., Samoilova, O., Novak, J., Ben-Yehuda, D., Strugov, V., Gill, D., Gribben, J., Hsu, E., Lih, C., Zhou, C., Clow, F., James, D., Styles, L. and Flinn, I., 2019. Ibrutinib plus obinutuzumab versus chlorambucil plus obinutuzumab in first-line treatment of chronic lymphocytic leukaemia (iLLUMINATE): a multicentre, randomised, open-label, phase 3 trial. The Lancet Oncology, 20(1), pp.43-56.

Morenos, L., Chatterton, Z., Ng, J., Halemba, M., Parkinson-Bates, M., Mechinaud, F., Elwood, N., Saffery, R. and Wong, N., 2014. Hypermethylation and down-regulation of DLEU2 in paediatric acute myeloid leukaemia independent of embedded tumour suppressor miR-15a/16-1. Molecular Cancer, 13(1), p.123.

Murphy M, Ahn J, Walker KK, Hoffman WH, Evans RM, Levine AJ and George DL . (1999). Genes Dev., 13, 2490–2501.

Nabhan, C., Aschebrook-Kilfoy, B., Chiu, B., Smith, S., Shanafelt, T., Evens, A. and Kay, N., 2014. The impact of race, ethnicity, age and sex on clinical outcome in chronic lymphocytic leukemia: a comprehensive Surveillance, Epidemiology, and End Results analysis in the modern era. *Leukemia & Lymphoma*, 55(12), pp.2778-2784.

Nabhan, C., Raca, G. and Wang, Y., 2015. Predicting Prognosis in Chronic Lymphocytic Leukemia in the Contemporary Era. *JAMA Oncology*, 1(7), p.965.

Nabhan, C., Raca, G. and Wang, Y., 2015. Prognosis in Chronic Lymphocytic Leukemia—Reply. *JAMA Oncology*, 1(7), p.988.

MARIOS PRODROMOU

Nadeu, F., Delgado, J., Royo, C., Baumann, T., Stankovic, T., Pinyol, M., Jares, P., Navarro, A., Martín-García, D., Beà, S., Salaverria, I., Oldreive, C., Aymerich, M., Suárez-Cisneros, H., Rozman, M., Villamor, N., Colomer, D., López-Guillermo, A., González, M., Alcoceba, M., Terol, M., Colado, E., Puente, X., López-Otín, C., Enjuanes, A. and Campo, E., 2016. Clinical impact of clonal and subclonal TP53, SF3B1, BIRC3, NOTCH1, and ATM mutations in chronic lymphocytic leukemia. Blood, 127(17), pp.2122-2130.

Nasr, A., Nutini, M., Palombo, B., Guerra, E. and Alberti, S., 2003. Mutations of TP53 induce loss of DNA methylation and amplification of the TROP1 gene. Oncogene, 22(11), pp.1668-1677.

Nava-Rodríguez, M., Domínguez-Cruz, M., Aguilar-López, L., Borjas-Gutiérrez, C., Magaña-Torres, M. and González-García, J., 2019. Genomic instability in a chronic lymphocytic leukemia patient with mono-allelic deletion of the DLEU and RB1 genes. *Molecular Cytogenetics*, 12(1).

NCCN. 2019. Treatment by Cancer Type. [online] Available at: <https://www.nccn.org/professionals/physician_gls/default.aspx> [Accessed 20 October 2021].

O'Brien, S., Furman, R., Coutre, S., Flinn, I., Burger, J., Blum, K., Sharman, J., Wierda, W., Jones, J., Zhao, W., Heerema, N., Johnson, A., Luan, Y., James, D., Chu, A. and Byrd, J., 2018. Single-agent ibrutinib in treatment-naïve and relapsed/refractory chronic lymphocytic leukemia: a 5-year experience. Blood, 131(17), pp.1910-1919.

Oakes, C., Claus, R., Gu, L., Assenov, Y., Hüllein, J., Zucknick, M., Bieg, M., Brocks, D., Bogatyrova, O., Schmidt, C., Rassenti, L., Kipps, T., Mertens, D., Lichter, P., Döhner, H., Stilgenbauer, S., Byrd, J., Zenz, T. and Plass, C., 2013. Evolution of DNA Methylation Is Linked to Genetic Aberrations in Chronic Lymphocytic Leukemia. *Cancer Discovery*, 4(3), pp.348-361.

Oncology Times, 2016. FDA Approves Imbruvica (Ibrutinib for CLL). [online] 38(7), p.34. Available at: <https://www.drugs.com/newdrugs/fda-approves-imbruvica-ibrutinib-chronic-lymphocytic-leukemia-4007.html>.

MARIOS PRODROMOU

Patel, K. and Pagel, J., 2021. Current and future treatment strategies in chronic lymphocytic leukemia. Journal of Hematology & Oncology, 14(1).

Pei, L., Choi, J., Liu, J., Lee, E., McCarthy, B., Wilson, J., Speir, E., Awan, F., Tae, H., Arthur, G., Schnabel, J., Taylor, K., Wang, X., Xu, D., Ding, H., Munn, D., Caldwell, C. and Shi, H., 2012. Genome-wide DNA methylation analysis reveals novel epigenetic changes in chronic lymphocytic leukemia. Epigenetics, 7(6), pp.567-578.

Perez, E., Orange, J., Bonilla, F., Chinen, J., Chinn, I., Dorsey, M., El-Gamal, Y., Harville, T., Hossny, E., Mazer, B., Nelson, R., Secord, E., Jordan, S., Stiehm, E., Vo, A. and Ballow, M., 2017. Update on the use of immunoglobulin in human disease: A review of evidence. Journal of Allergy and Clinical Immunology, 139(3), pp.S1-S46.

Pfeifer, D., Pantic, M., Skatulla, I., Rawluk, J., Kreutz, C., Martens, U., Fisch, P., Timmer, J. and Veelken, H., 2006. Genome-wide analysis of DNA copy number changes and LOH in CLL using high-density SNP arrays. Blood, 109(3), pp.1202-1210.

Pleasance, E., Cheetham, R., Stephens, P., McBride, D., Humphray, S., Greenman, C., Varela, I., Lin, M., Ordóñez, G., Bignell, G., Ye, K., Alipaz, J., Bauer, M., Beare, D., Butler, A., Carter, R., Chen, L., Cox, A., Edkins, S., Kokko-Gonzales, P., Gormley, N., Grocock, R., Haudenschild, C., Hims, M., James, T., Jia, M., Kingsbury, Z., Leroy, C., Marshall, J., Menzies, A., Mudie, L., Ning, Z., Royce, T., Schulz-Trieglaff, O., Spiridou, A., Stebbings, L., Szajkowski, L., Teague, J., Williamson, D., Chin, L., Ross, M., Campbell, P., Bentley, D., Futreal, P. and Stratton, M., 2009. A comprehensive catalogue of somatic mutations from a human cancer genome. *Nature*, 463(7278), pp.191-196.

Popp, C., Dean, W., Feng, S., Cokus, S., Andrews, S., Pellegrini, M., Jacobsen, S. and Reik, W., 2010. Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature, 463(7284), pp.1101-1105.

Porter, D., Hwang, W., Frey, N., Lacey, S., Shaw, P., Loren, A., Bagg, A., Marcucci, K., Shen, A., Gonzalez, V., Ambrose, D., Grupp, S., Chew, A., Zheng, Z., Milone, M., Levine, B., Melenhorst, J. and June, C., 2015. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. Science Translational Medicine, 7(303).

MARIOS PRODROMOU

Price, A., 2004. Whole-genome analysis of Alu repeat elements reveals complex evolutionary history. *Genome Research*, 14(11), pp.2245-2252.

Pubchem.ncbi.nlm.nih.gov. 2021. Acalabrutinib. [online] Available at: <https://pubchem.ncbi.nlm.nih.gov/compound/Acalabrutinib> [Accessed 20 November 2021].

Puente, X., Beà, S., Valdés-Mas, R., Villamor, N., Gutiérrez-Abril, J., Martín-Subero, J., Munar, M., Rubio-Pérez, C., Jares, P., Aymerich, M., Baumann, T., Beekman, R., Belver, L., Carrio, A., Castellano, G., Clot, G., Colado, E., Colomer, D., Costa, D., Delgado, J., Enjuanes, A., Estivill, X., Ferrando, A., Gelpí, J., González, B., González, S., González, M., Gut, M., Hernández-Rivas, J., López-Guerra, M., Martín-García, D., Navarro, A., Nicolás, P., Orozco, M., Payer, Á., Pinyol, M., Pisano, D., Puente, D., Queirós, A., Quesada, V., Romeo-Casabona, C., Royo, C., Royo, R., Rozman, M., Russiñol, N., Salaverría, I., Stamatopoulos, K., Stunnenberg, H., Tamborero, D., Terol, M., Valencia, A., López-Bigas, N., Torrents, D., Gut, I., López-Guillermo, A., López-Otín, C. and Campo, E., 2015. Non-coding recurrent mutations in chronic lymphocytic leukaemia. Nature, 526(7574), pp.519-524.

Puente, X., Pinyol, M., Quesada, V., Conde, L., Ordóñez, G., Villamor, N., Escaramis, G., Jares, P., Beà, S., González-Díaz, M., Bassaganyas, L., Baumann, T., Juan, M., López-Guerra, M., Colomer, D., Tubío, J., López, C., Navarro, A., Tornador, C., Aymerich, M., Rozman, M., Hernández, J., Puente, D., Freije, J., Velasco, G., Gutiérrez-Fernández, A., Costa, D., Carrió, A., Guijarro, S., Enjuanes, A., Hernández, L., Yagüe, J., Nicolás, P., Romeo-Casabona, C., Himmelbauer, H., Castillo, E., Dohm, J., de Sanjosé, S., Piris, M., de Alava, E., Miguel, J., Royo, R., Gelpí, J., Torrents, D., Orozco, M., Pisano, D., Valencia, A., Guigó, R., Bayés, M., Heath, S., Gut, M., Klatt, P., Marshall, J., Raine, K., Stebbings, L., Futreal, P., Stratton, M., Campbell, P., Gut, I., López-Guillermo, A., Estivill, X., Montserrat, E., López-Otín, C. and Campo, E., 2011. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature, 475(7354), pp.101-105.

Queirós, A., Villamor, N., Clot, G., Martinez-Trillos, A., Kulis, M., Navarro, A., Penas, E., Jayne, S., Majid, A., Richter, J., Bergmann, A., Kolarova, J., Royo, C., Russiñol, N., Castellano, G., Pinyol, M., Bea, S., Salaverria, I., López-Guerra, M., Colomer, D., Aymerich,

MARIOS PRODROMOU

M., Rozman, M., Delgado, J., Giné, E., González-Díaz, M., Puente, X., Siebert, R., Dyer, M., López-Otín, C., Rozman, C., Campo, E., López-Guillermo, A. and Martín-Subero, J., 2014. A B-cell epigenetic signature defines three biologic subgroups of chronic lymphocytic leukemia with clinical impact. *Leukemia*, 29(3), pp.598-605.

Ramon, D., Braden, M., Adams, S., Marincola, F. and Wang, L., 2003. Journal of Translational Medicine, 1(1), p.9.

Ramsay, A., Clear, A., Fatah, R. and Gribben, J., 2012. Multiple inhibitory ligands induce impaired T-cell immunologic synapse function in chronic lymphocytic leukemia that can be blocked with lenalidomide: establishing a reversible immune evasion mechanism in human cancer. Blood, 120(7), pp.1412-1421.

Ramsay, A., Johnson, A., Lee, A., Gorgün, G., Le Dieu, R., Blum, W., Byrd, J. and Gribben, J., 2008. Chronic lymphocytic leukemia T cells show impaired immunological synapse formation that can be reversed with an immunomodulating drug. Journal of Clinical Investigation,.

RAVAL, A., BYRD, J. and PLASS, C., 2006. Epigenetics in Chronic Lymphocytic Leukemia. *Seminars in Oncology*, 33(2), pp.157-166.

Raveche, E., Salerno, E., Scaglione, B., Manohar, V., Abbasi, F., Lin, Y., Fredrickson, T., Landgraf, P., Ramachandra, S., Huppi, K., Toro, J., Zenger, V., Metcalf, R. and Marti, G., 2007. Abnormal microRNA-16 locus with synteny to human 13q14 linked to CLL in NZB mice. *Blood*, 109(12), pp.5079-5086.

Ray, P. and Fry, R., 2015. The Cell. *Systems Biology in Toxicology and Environmental Health*, pp.11-42.

REDAELLI, A., LASKIN, B., STEPHENS, J., BOTTEMAN, M. and PASHOS, C., 2004. The clinical and epidemiological burden of chronic lymphocytic leukaemia. *European Journal of Cancer Care*, 13(3), pp.279-287.

Reza Farzaneh, M., Shahryari, J., Safaei, A., Valibeigi, B., Karimi Davani, S. and Tabibi, N., 2021. Dicer Gene Expression as a Prognostic Factor in Acute Lymphoblastic Leukemia and Chronic Lymphocytic Leukemia in Fars Province.

MARIOS PRODROMOU

Romero-Garcia, S., Prado-Garcia, H. and Carlos-Reyes, A., 2020. Role of DNA Methylation in the Resistance to Therapy in Solid Tumors. Frontiers in Oncology, 10.

Rossi, D. and Gaidano, G., 2016. Richter syndrome: pathogenesis and management. Seminars in Oncology, 43(2), pp.311-319.

Rossi, D., Khiabanian, H., Spina, V., Ciardullo, C., Bruscaggin, A., Famà, R., Rasi, S., Monti, S., Deambrogi, C., De Paoli, L., Wang, J., Gattei, V., Guarini, A., Foà, R., Rabadan, R. and Gaidano, G., 2014. Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. Blood, 123(14), pp.2139-2147.

Rossi, D., Spina, V., Deambrogi, C., Rasi, S., Laurenti, L., Stamatopoulos, K., Arcaini, L., Lucioni, M., Rocque, G., Xu-Monette, Z., Visco, C., Chang, J., Chigrinova, E., Forconi, F., Marasca, R., Besson, C., Papadaki, T., Paulli, M., Larocca, L., Pileri, S., Gattei, V., Bertoni, F., Foà, R., Young, K. and Gaidano, G., 2011. The genetics of Richter syndrome reveals disease heterogeneity and predicts survival after transformation. Blood, 117(12), pp.3391-3401.

Rossi, M., Fuligni, F., Ciccone, M., Agostinelli, C., Righi, S., Luciani, M., Laginestra, M., Rigolin, G., Sapienza, M., Gazzola, A., Mannu, C., Cuneo, A., Pileri, S. and Piccaluga, P., 2013. Hsa-miR-15a and Hsa-miR-16-1 Expression Is Not Related to Proliferation Centers Abundance and Other Prognostic Factors in Chronic Lymphocytic Leukemia. *BioMed Research International*, 2013, pp.1-13.

Roy-Engel, A., Carroll, M., Vogel, E., Garber, R., Nguyen, S., Salem, A., Batzer, M. and Deininger, P., 2001. Alu Insertion Polymorphisms for the Study of Human Genomic Diversity. *Genetics*, 159(1), pp.279-290.

Rozman, C. and Montserrat, E., 1995. Chronic Lymphocytic Leukemia. *New England Journal of Medicine*, 333(16), pp.1052-1057.

Schinasi, L., De Roos, A., Ray, R., Edlefsen, K., Parks, C., Howard, B., Meliker, J., Bonner, M., Wallace, R. and LaCroix, A., 2015. Insecticide exposure and farm history in relation to risk of lymphomas and leukemias in the Women's Health Initiative observational study cohort. *Annals of Epidemiology*, 25(11), pp.803-810.e4.

MARIOS PRODROMOU

Secchiero, P., Barbarotto, E., Tiribelli, M., Zerbinati, C., di Iasio, M., Gonelli, A., Cavazzini, F., Campioni, D., Fanin, R., Cuneo, A. and Zauli, G., 2006. Functional integrity of the p53-mediated apoptotic pathway induced by the nongenotoxic agent nutlin-3 in B-cell chronic lymphocytic leukemia (B-CLL). Blood, 107(10), pp.4122-4129.

Seifert, M., Sellmann, L., Bloehdorn, J., Wein, F., Stilgenbauer, S., Dürig, J. and Küppers, R., 2012. Cellular origin and pathophysiology of chronic lymphocytic leukemia. *Journal of Experimental Medicine*, 209(12), pp.2183-2198.

Seymour, J., Kipps, T., Eichhorst, B., Hillmen, P., D'Rozario, J., Assouline, S., Owen, C., Gerecitano, J., Robak, T., De la Serna, J., Jaeger, U., Cartron, G., Montillo, M., Humerickhouse, R., Punnoose, E., Li, Y., Boyer, M., Humphrey, K., Mobasher, M. and Kater, A., 2018. Venetoclax–Rituximab in Relapsed or Refractory Chronic Lymphocytic Leukemia. New England Journal of Medicine, 378(12), pp.1107-1120.

Shanafelt, T., Wang, V., Kay, N., Hanson, C., O'Brien, S., Barrientos, J., Erba, H., Stone, R., Litzow, M. and Tallman, M., 2018. A Randomized Phase III Study of Ibrutinib (PCI-32765)-Based Therapy Vs. Standard Fludarabine, Cyclophosphamide, and Rituximab (FCR) Chemoimmunotherapy in Untreated Younger Patients with Chronic Lymphocytic Leukemia (CLL): A Trial of the ECOG-ACRIN Cancer Research Group (E1912). Blood, 132(Supplement 1), pp.LBA-4-LBA-4.

Shanafelt, T., Witzig, T., Fink, S., Jenkins, R., Paternoster, S., Smoley, S., Stockero, K., Nast, D., Flynn, H., Tschumper, R., Geyer, S., Zent, C., Call, T., Jelinek, D., Kay, N. and Dewald, G., 2006. Prospective Evaluation of Clonal Evolution During Long-Term Follow-Up of Patients With Untreated Early-Stage Chronic Lymphocytic Leukemia. Journal of Clinical Oncology, 24(28), pp.4634-4641.

Sheaffer, K., Elliott, E. and Kaestner, K., 2016. DNA Hypomethylation Contributes to Genomic Instability and Intestinal Cancer Initiation. *Cancer Prevention Research*, 9(7), pp.534-546.

Shukla, V., Ma, S., Hardy, R., Joshi, S. and Lu, R., 2013. A role for IRF4 in the development of CLL. *Blood*, 122(16), pp.2848-2855.

MARIOS PRODROMOU

Shukla, V., Shukla, A., Joshi, S. and Lu, R., 2016. Interferon regulatory factor 4 attenuates Notch signaling to suppress the development of chronic lymphocytic leukemia. *Oncotarget*, 7(27), pp.41081-41094.

Siegel, R., DeSantis, C., Virgo, K., Stein, K., Mariotto, A., Smith, T., Cooper, D., Gansler, T., Lerro, C., Fedewa, S., Lin, C., Leach, C., Cannady, R., Cho, H., Scoppa, S., Hachey, M., Kirch, R., Jemal, A. and Ward, E., 2012. Cancer treatment and survivorship statistics, 2012. *CA: A Cancer Journal for Clinicians*, 62(4), pp.220-241.

Sinkkonen L, Hugenschmidt T, Berninger P, Gaidatzis D, Mohn F, Artus-Revel CG et al (2008). MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nat Struct Mol Biol 15: 259–267.

Slager, S., Rabe, K., Achenbach, S., Vachon, C., Goldin, L., Strom, S., Lanasa, M., Spector, L., Rassenti, L., Leis, J., Camp, N., Glenn, M., Kay, N., Cunningham, J., Hanson, C., Marti, G., Weinberg, J., Morrison, V., Link, B., Call, T., Caporaso, N. and Cerhan, J., 2011. Genome-wide association study identifies a novel susceptibility locus at 6p21.3 among familial CLL. *Blood*, 117(6), pp.1911-1916.

Spadaro, G., Pecoraro, A., De Renzo, A., Della Pepa, R. and Genovese, A., 2016. Intravenous versus subcutaneous immunoglobulin replacement in secondary hypogammaglobulinemia. Clinical Immunology, 166-167, pp.103-104.

Statham, A., Robinson, M., Song, J., Coolen, M., Stirzaker, C. and Clark, S., 2012. Bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq) directly informs methylation status of histone-modified DNA. Genome Research, 22(6), pp.1120-1127.

Stilgenbauer, S., Sander, S., Bullinger, L., Benner, A., Leupolt, E., Winkler, D., Krober, A., Kienle, D., Lichter, P. and Dohner, H., 2007. Clonal evolution in chronic lymphocytic leukemia: acquisition of high-risk genomic aberrations associated with unmutated VH, resistance to therapy, and short survival. Haematologica, 92(9), pp.1242-1245.

Stommel, J. and Wahl, G., 2004. Accelerated MDM2 auto-degradation induced by DNA-damage kinases is required for p53 activation. The EMBO Journal, 23(7), pp.1547-1556.

MARIOS PRODROMOU

Strefford, J., Kadalayil, L., Forster, J., Rose-Zerilli, M., Parker, A., Lin, T., Heppel, N., Norris, K., Gardiner, A., Davies, Z., Gonzalez de Castro, D., Else, M., Steele, A., Parker, H., Stankovic, T., Pepper, C., Fegan, C., Baird, D., Collins, A., Catovsky, D. and Oscier, D., 2015. Telomere length predicts progression and overall survival in chronic lymphocytic leukemia: data from the UK LRF CLL4 trial. Leukemia, 29(12), pp.2411-2414.

Sulli, G., Di Micco, R. and di Fagagna, F., 2012. Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. Nature Reviews Cancer, 12(10), pp.709-720.

Suzuki, M., & Bird, A. DNA methylation landscapes: Provocative insights from epigenomics. Nature Reviews Genetics 9, 465–476 (2008) doi:10.1038/nrg2341.

Symer, D., Connelly, C., Szak, S., Caputo, E., Cost, G., Parmigiani, G. and Boeke, J., 2002. Human L1 Retrotransposition Is Associated with Genetic Instability In Vivo. Cell, 110(3), pp.327-338.

Tadmor, T., Aviv, A. and Polliack, A., 2011. Merkel cell carcinoma, chronic lymphocytic leukemia and other lymphoproliferative disorders: an old bond with possible new viral ties. Annals of Oncology, 22(2), pp.250-256.

Tahiliani, M., Koh, K., Shen, Y., Pastor, W., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L., Liu, D., Aravind, L. and Rao, A., 2009. Conversion of 5-Methylcytosine to 5-Hydroxymethylcytosine in Mammalian DNA by MLL Partner TET1. *Science*, 324(5929), pp.930-935.

Tam, C., Shanafelt, T., Wierda, W., Abruzzo, L., Van Dyke, D., O'Brien, S., Ferrajoli, A., Lerner, S., Lynn, A., Kay, N. and Keating, M., 2009. De novo deletion 17p13.1 chronic lymphocytic leukemia shows significant clinical heterogeneity: the M. D. Anderson and Mayo Clinic experience. *Blood*, 114(5), pp.957-964.

Taylor, A., Neave, L., Solanki, S., Westwood, J., Terrinonive, I., McGuckin, S., Kothari, J., Cooper, N., Stasi, R. and Scully, M., 2015. Mycophenolate mofetil therapy for severe immune thrombocytopenia. British Journal of Haematology, 171(4), pp.625-630.

MARIOS PRODROMOU

Thompson, P., Tam, C., O'Brien, S., Wierda, W., Stingo, F., Plunkett, W., Smith, S., Kantarjian, H., Freireich, E. and Keating, M., 2016. Fludarabine, cyclophosphamide, and rituximab treatment achieves long-term disease-free survival in IGHV-mutated chronic lymphocytic leukemia. Blood, 127(3), pp.303-309.

Tobin, G., Thunberg, U., Johnson, A., Thörn, I., Söderberg, O., Hultdin, M., Botling, J., Enblad, G., Sällström, J., Sundström, C., Roos, G. and Rosenquist, R., 2002. Somatically mutated Ig VH3-21 genes characterize a new subset of chronic lymphocytic leukemia. *Blood*, 99(6), pp.2262-2264.

Tubio, J., Li, Y., Ju, Y., Martincorena, I., Cooke, S., Tojo, M., Gundem, G., Pipinikas, C., Zamora, J., Raine, K., Menzies, A., Roman-Garcia, P., Fullam, A., Gerstung, M., Shlien, A., Tarpey, P., Papaemmanuil, E., Knappskog, S., Van Loo, P., Ramakrishna, M., Davies, H., Marshall, J., Wedge, D., Teague, J., Butler, A., Nik-Zainal, S., Alexandrov, L., Behjati, S., Yates, L., Bolli, N., Mudie, L., Hardy, C., Martin, S., McLaren, S., O'Meara, S., Anderson, E., Maddison, M., Gamble, S., Foster, C., Warren, A., Whitaker, H., Brewer, D., Eeles, R., Cooper, C., Neal, D., Lynch, A., Visakorpi, T., Isaacs, W., van't Veer, L., Caldas, C., Desmedt, C., Sotiriou, C., Aparicio, S., Foekens, J., Eyfjörd, J., Lakhani, S., Thomas, G., Myklebost, O., Span, P., Børresen-Dale, A., Richardson, A., Van de Vijver, M., Vincent-Salomon, A., Van den Eynden, G., Flanagan, A., Futreal, P., Janes, S., Bova, G., Stratton, M., McDermott, U. and Campbell, P., 2014. Extensive transduction of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes. *Science*, 345(6196).

Tuck-Muller, C., Narayan, A., Tsien, F., Smeets, D., Sawyer, J., Fiala, E., Sohn, O. and Ehrlich, M., 2000. DNA hypomethylation and unusual chromosome instability in cell lines fromICF syndrome patients. *Cytogenetic and Genome Research*, 89(1-2), pp.121-128.

Ullu, E. and Tschudi, C., 1984. Alu sequences are processed 7SL RNA genes. *Nature*, 312(5990), pp.171-172.

Upchurch, G., Haney, S. and Opavsky, R., 2016. Aberrant Promoter Hypomethylation in CLL: Does It Matter for Disease Development?. *Frontiers in Oncology*, 6.

Valencia-Sanchez, M., 2006. Control of translation and mRNA degradation by miRNAs and siRNAs. *Genes & Development*, 20(5), pp.515-524.

MARIOS PRODROMOU

Vassilev, L., Vu, B., Graves, B., Carvajal, D., Podlaski, F., Filipovic, Z., Kong, N., Kammlott, U., Lukacs, C., Klein, C., Fotouhi, N. and Liu, E., 2004. In Vivo Activation of the p53 Pathway by Small-Molecule Antagonists of MDM2. Science, 303(5659), pp.844-848.

Veronese, A., Pepe, F., Chiacchia, J., Pagotto, S., Lanuti, P., Veschi, S., Di Marco, M., D'Argenio, A., Innocenti, I., Vannata, B., Autore, F., Marchisio, M., Wernicke, D., Verginelli, F., Leone, G., Rassenti, L., Kipps, T., Mariani-Costantini, R., Laurenti, L., Croce, C. and Visone, R., 2014. Allele-specific loss and transcription of the miR-15a/16-1 cluster in chronic lymphocytic leukemia. *Leukemia*, 29(1), pp.86-95.

Visco, C., Barcellini, W., Maura, F., Neri, A., Cortelezzi, A. and Rodeghiero, F., 2014. Autoimmune cytopenias in chronic lymphocytic leukemia. American Journal of Hematology, 89(11), pp.1055-1062.

Vojta, A., Dobrinić, P., Tadić, V., Bočkor, L., Korać, P., Julg, B., Klasić, M. and Zoldoš, V., 2016. Repurposing the CRISPR-Cas9 system for targeted DNA methylation. Nucleic Acids Research, 44(12), pp.5615-5628.

Wang, J., Song, L., Grover, D., Azrak, S., Batzer, M. and Liang, P., 2006. dbRIP: A highly integrated database of retrotransposon insertion polymorphisms in humans. *Human Mutation*, 27(4), pp.323-329.

Waterland, R. and Jirtle, R., 2003. Transposable Elements: Targets for Early Nutritional Effects on Epigenetic Gene Regulation. *Molecular and Cellular Biology*, 23(15), pp.5293-5300.

Wiestner, A., 2014. BCR pathway inhibition as therapy for chronic lymphocytic leukemia and lymphoplasmacytic lymphoma. Hematology, 2014(1), pp.125-134.

Wilson, A., Power, B. and Molloy, P., 2007. DNA hypomethylation and human diseases. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1775(1), pp.138-162.

Wilson, A., Power, B. and Molloy, P., 2007. DNA hypomethylation and human diseases. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1775(1), pp.138-162.

Woyach, J., Bojnik, E., Ruppert, A., Stefanovski, M., Goettl, V., Smucker, K., Smith, L., Dubovsky, J., Towns, W., MacMurray, J., Harrington, B., Davis, M., Gobessi, S., Laurenti,

MARIOS PRODROMOU

L., Chang, B., Buggy, J., Efremov, D., Byrd, J. and Johnson, A., 2014. Bruton's tyrosine kinase (BTK) function is important to the development and expansion of chronic lymphocytic leukemia (CLL). Blood, 123(8), pp.1207-1213.

Woyach, J., Ruppert, A., Heerema, N., Zhao, W., Booth, A., Ding, W., Bartlett, N., Brander, D., Barr, P., Rogers, K., Parikh, S., Coutre, S., Hurria, A., Brown, J., Lozanski, G., Blachly, J., Ozer, H., Major-Elechi, B., Fruth, B., Nattam, S., Larson, R., Erba, H., Litzow, M., Owen, C., Kuzma, C., Abramson, J., Little, R., Smith, S., Stone, R., Mandrekar, S. and Byrd, J., 2018. Ibrutinib Regimens versus Chemoimmunotherapy in Older Patients with Untreated CLL. New England Journal of Medicine, 379(26), pp.2517-2528.

Xiao-Jie, L., Hui-Ying, X., Qi, X., Jiang, X. and Shi-Jie, M., 2015. LINE-1 in cancer: multifaceted functions and potential clinical implications. *Genetics in Medicine*, 18(5), pp.431-439.

Xu, Y., Ashley, T., Brainerd, E., Bronson, R., Meyn, M. and Baltimore, D., 1996. Targeted disruption of ATM leads to growth retardation, chromosomal fragmentation during meiosis, immune defects, and thymic lymphoma. *Genes & Development*, 10(19), pp.2411-2422.

Xue, C., Wang, X., Zhang, L., Qu, Q., Zhang, Q. and Jiang, Y., 2020. Ibrutinib in B-cell lymphoma: single fighter might be enough?. Cancer Cell International, 20(1).

Yosifov, D., Bloehdorn, J., Döhner, H., Lichter, P., Stilgenbauer, S. and Mertens, D., 2020. DNA methylation of chronic lymphocytic leukemia with differential response to chemotherapy. Scientific Data, 7(1).

Zanna, P., Trerotola, M., Vacca, G., Bonasera, V., Palombo, B., Guerra, E., Rossi, C., Lattanzio, R., Piantelli, M. and Alberti, S., 2007. Trop-1 are conserved growth stimulatory molecules that mark early stages of tumor progression. Cancer, 110(2), pp.452-464.

Zenz, T., Kröber, A., Scherer, K., Häbe, S., Bühler, A., Benner, A., Denzel, T., Winkler, D., Edelmann, J., Schwänen, C., Döhner, H. and Stilgenbauer, S., 2008. Monoallelic TP53 inactivation is associated with poor prognosis in chronic lymphocytic leukemia: results from a detailed genetic characterization with long-term follow-up. Blood, 112(8), pp.3322-3329.

MARIOS PRODROMOU

Zhang, F., Pomerantz, J., Sen, G., Palermo, A. and Blau, H., 2007. Active tissue-specific DNA demethylation conferred by somatic cell nuclei in stable heterokaryons. *Proceedings of the National Academy of Sciences*, 104(11), pp.4395-4400.

Zhu, S., Gokhale, S., Jung, J., Spirollari, E., Tsai, J., Arceo, J., Wu, B., Victor, E. and Xie, P., 2021. Multifaceted Immunomodulatory Effects of the BTK Inhibitors Ibrutinib and Acalabrutinib on Different Immune Cell Subsets – Beyond B Lymphocytes. Frontiers in Cell and Developmental Biology, 9.

MARIOS PRODROMOU