UNIVERSITY OF UDINE
PHD COURSE IN BIOMEDICAL SCIENCES AND BIOTECHNOLOGY
XXVII CYCLE

PHD THESIS

NOTCH1 mutations are associated with low CD20 expression in Chronic Lymphocytic Leukemia: evidences for a *NOTCH1*-mediated epigenetic regulatory mechanism

TUTOR
Prof. Carlo Pucillo

PHD STUDENT
Federico Pozzo

SUPERVISOR
Dott. Valter Gattei
Dott. Michele Dal Bo

ACADEMIC YEAR
2014-2015

Index

Abstract

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with highly variable clinical courses and survivals ranging from months to decades. Recently, it has been reported that stabilizing mutations of *NOTCH1* are recurrently associated with CLL, being identified in about 10% of CLL at diagnosis and with higher frequencies in chemorefractory CLL, CLL in advanced disease phases, and in Richter Syndrome. All *NOTCH1* mutations disrupt the C-terminal PEST domain, causing an accumulation of a stabilized NOTCH1 isoform.

By taking advantage of a large retrospective cohort of CLL cases (n=463), for a subset of which clinical data was available, we confirm that *NOTCH1* mutations behave as independent prognosticator, identifying a high-risk subset characterized by unfavourable prognosis and poor overall survival. We further demonstrate that the presence of *NOTCH1* mutations also identifies a CLL subset which does not benefit from addition of rituximab in the context of a maintenance therapy after first-line treatment with fludarabine. These results are in keeping with recently published data, reporting that the *NOTCH1* mutated subset of CLL patients does not benefit from the addition of rituximab to chemotherapeutic treatment with fludarabine plus cyclophosphamide.

As the reasons for this different clinical behaviour remained to be elucidated, considering that the response to rituximab treatment in B cell neoplasms directly depends upon CD20 expression, we investigated whether *NOTCH1* mutations could affect CD20 expression in CLL. By taking advantage of a wide CLL series (n=692), we demonstrated that *NOTCH1* mutated CLL cells (87/692) were characterized by lower CD20 expression and lower relative lysis induced by rituximab in-vitro. Consistently, CD20 expression by CLL cells was up-regulated in-vitro by exposure to γ-secretase inhibitors, and the stable transfection of the NOTCH1 intracellular domain (NICD) into CLL-like cells resulted in a strong downregulation of both CD20 protein and transcript. By using these NICD transfectants, we investigated the protein interactions of RBPJ, a transcription factor acting either as activator or repressor of NOTCH1 pathway when respectively bound to NICD or histone deacetylases (HDACs). Compared to controls, NICD transfectants had RBPJ preferentially complexed to NICD, and showed higher levels of HDACs interacting with the promoter of the CD20 gene. Finally, treatment with the HDAC inhibitor valproic acid upregulated CD20 in both NICD transfectants and primary CLL cells. In conclusion, *NOTCH1* mutations are associated with low CD20 levels in CLL and are responsible for a dysregulation of HDAC-mediated epigenetic repression of CD20 expression. In conclusion, in the present thesis we i) confirm that *NOTCH1* mutations are an independent prognosticator of overall survival in CLL; ii) identify a CLL subset, characterized by the presence of *NOTCH1* mutations, that do not benefit from addition of rituximab to chemotherapeutic treatment; iii) provide a proof of concept that *NOTCH1* mutations responsible for a truncated NOTCH1 protein are associated with low CD20 expression levels in CLL by a dysregulated HDAC-dependent repression mechanism. This low CD20 levels may be, in turn, responsible for the specific immunoresistance to rituximab-based treatments, such as FCR, of *NOTCH1* mutated CLL.

- 1 -
Chronic Lymphocytic Leukemia

1.1 – General Features

Chronic Lymphocytic Leukemia (CLL) is a clonal lymphoproliferative disorder characterized by the accumulation of mature B-lymphocytes in the peripheral blood, bone marrow and lymphoid organs. It is the most common form of leukemia in western countries, representing approximately 16% to 30% of all leukemias. Incidence rate is 2-6/100,000 persons/year, increasing with age to 12-13/100,000 at 65 years, the mean age of diagnosis.[1;2]

CLL was initially considered a homogeneous disease of immunologically incompetent and resting B cells, which accumulated because of faulty apoptotic mechanisms. At present, this view has dramatically changed: CLL is now considered a highly heterogeneous disease, deriving from antigen-stimulated B cells, which either avoid death through the intercession of external signals or die by apoptosis, only to be replenished by slowly proliferating precursor cells.[1;3-5]

CLL clinical course is therefore extremely variable, with survival ranging from months to decades. Many CLL patients can live with a non-progressive disease for up to 15 years after initial diagnosis, and never require therapy; a 20% will, despite therapy, rapidly succumb to the disease in a few years. Median overall survival of CLL patients is currently 7.5 years; 50% of patients are alive after 5 years and almost 30% have a 10-years survival.[6] Because of this heterogeneity in clinical course and outcome, many studies focused on finding clinical and biological prognosticators capable to identify which patients may benefit from an early or more aggressive treatment strategy.[7] However, there is general agreement that CLL remains an incurable disease.[2;7]

1.2 - Etiology and Clinical manifestations

The etiology of CLL is currently unknown, yet the monoclonal nature of the proliferating B lymphocytes implies that inducing lesions must exist in the progenitor clone.[1] Some genetic lesions seem to appear very early in the leukemic transformation, but whether these lesions are causative or sequential to B cell maturation through the germinal centers (i.e., related to a mature B cell receptor signaling, see paragraph 1.4.3) is still under debate.[8;9] Significant familial aggregation of CLL has been demonstrated, but the mode of inheritance is unknown.[9]

Clinical features at diagnosis are variable, with more than a quarter of patients being asymptomatic. Unlike acute leukemias, signs and symptoms of CLL develop gradually, making disease onset difficult to pinpoint. Patients are often diagnosed of CLL because of accidental discover of an unexplained absolute lymphocytosis or a nontender lymphadenopathy during routine exams. Otherwise, patients may have only mild symptoms of night sweats, fevers and asthenia. Tissue involvement is usually limited to peripheral blood, bone marrow and lymphoid organs, with an initial mild infiltration of lymph nodes (lymphadenopathy), liver (hepatomegaly) and spleen (splenomegaly), which worsen in later disease stages. CLL patients in advanced stages frequently develop autoimmune diseases such as hemolytic anemia and thrombocytopenia.[2]

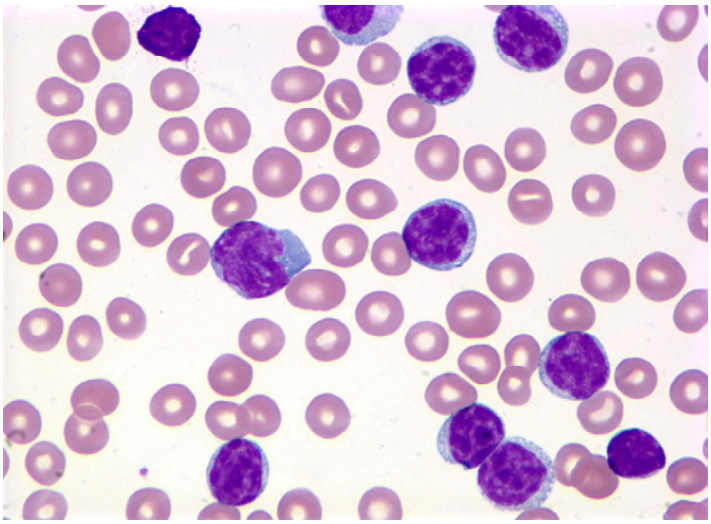

Figure1.2 Peripheral blood from a patient with CLL. *CLL lymphocytes appear as small, round, with the characteristically clumped, "soccer ball" chromatin (original magnification ×1000).*[2]

1.3 - Diagnosis

The diagnosis of chronic lymphocytic leukemia is made upon presence of an absolute lymphocytosis (>5000 lympho/ul) but has been made easier by routine immunophenotyping. CLL is phenotypically defined by three main features: (i) shared B-cell markers (CD19+, CD20+, CD23+) with CD5+ antigen and no other pan-T-cell markers; (ii) monoclonal expression of either κ or λ light surface membrane immunoglobulin (smIg) chain; (iii) smIg, CD79b, CD20 and CD22 expressed with low density.[1] These characteristics are generally adequate for a precise diagnosis of CLL and they distinguish CLL from other disorders such as prolymphocytic leukemia, hairy-cell leukemia, mantle-cell lymphoma, and other lymphomas that can mimic CLL.[10]

1.4 - Clinical and molecular prognostic tools

The first clinical prognostic systems considered clinical symptoms, physical signs and laboratory values. However, in the recent years there has been considerable progress in the identification of molecular and cellular makers that may predict the disease progression tendency in patients with CLL.[7]

1.4.1 - Staging and Clinical markers

The initial prognostic tool in CLL was a clinical staging system developed in 1975 by Rai et al. and based on disease burden (lymphoadenopathy, organomegaly- spleen and liver, cytopenias)[11] followed by Binet in 1978.[12] Over the years, the pattern of CLL diagnosis shifted from patients presenting bulky nodes and cytopenias, to the detection of latent lymphocytosis in asymptomatic individuals. Moreover, it is now recognized that a subset of patients with early stage CLL have a disease that will rapidly evolve to a more advanced, refractory, and fatal disease. What Rai and Binet staging lack is the ability to prospectively identify the rapidly evolving patient from patients destined to remain with early stage for decades.

A number of clinical and biological factors have been evaluated and shown to be of prognostic relevance. These include age, gender, lymphocyte count, bone marrow infiltration time, lymphocyte doubling time[13], soluble CD23 levels and serum β-2 microglobulin levels.[14] Several models or clinical trials evaluated the effectiveness of different, independent and combined, markers for prognostic grouping or overall survival (OS).[15-17]

Most CLL patients present low-stage disease at the time of diagnosis and accurate disease progression evaluation is a major challenge. Therefore, several new molecular and cellular markers have been evaluated for predicting prognosis and are now taken into account to better refine prediction of disease progression.

1.4.2 - Chromosomal aberrations

Chromosomal abnormalities can be detected in up to 50% of patients with CLL using conventional cytogenetic analysis and are, in general, deletions and/or amplifications of involved chromosomal regions; translocations are rare.[18] Interphase fluorescence in situ hybridization (FISH) has emerged as the standard method for detecting chromosomal deletions in CLL.[19] The method is based on sequence-specific hybridization to the gene(s) of interest by a large DNA probe, which is tagged with a fluorochrome to allow visualization by fluorescent microscopy.

The most common cytogenetic aberrations are represented by deletion 13q14, trisomy 12, deletion 11q22.q23 and deletion 17p13.

Deletion 13q14 (del13) is the most frequent alteration, occurring in 50-60% of cases. It encompasses the DLEU1/2 locus, the miR15-16 cluster and can extend up to the RB1 locus.[20] miR-15-16 appear to play a role in cell quiescence, inhibiting cyclin-dependant kinase 6 and BCL2, and TP53 regulation.[21] It is the only

abnormality that seems to confer a favorable prognosis, even when compared to cases harboring no apparent alteration.[22]

Trisomy 12 (tris12) occurs in approximately 15% of CLL cases and is thought to alter the gene dosage of one or more proto-oncogenes, although the precise molecular mechanism of this alteration is not currently understood[1;19], even though there is increasing evidence for a role of driver mutation for CLL.[8] It associates with *NOTCH1* mutations and increased integrin signaling.[23;24] It may also play a role in Richter transformation.[25]

Deletion 11q22.q23 (del11) affects the ATM gene (ataxia telangiectasia mutated), which is responsible for TP53 activation upon DNA damage pathway activation. ATM haploinsufficiency impairs cellular response to chemotherapy.[26] In a fraction of cases, the deletion only targets the BIRC3 gene, which is in close proximity to ATM.[27]

Deletion 17p13 (del17) affects the *TP53* gene and is found in 5-10% of CLL cases at diagnosis, usually with a concomitant *TP53* point mutation on the other allele. Altered *TP53* function is the most relevant predictor of outcome, making del17 the most important marker to test before entering any therapy regimen.

Multivariate analyses revealed an independent prognostic relevance of genomic aberrations, and a hierarchical model was developed: del17>del11 > tris12 > no aberration >del13 (in descending order of adversity).[19]

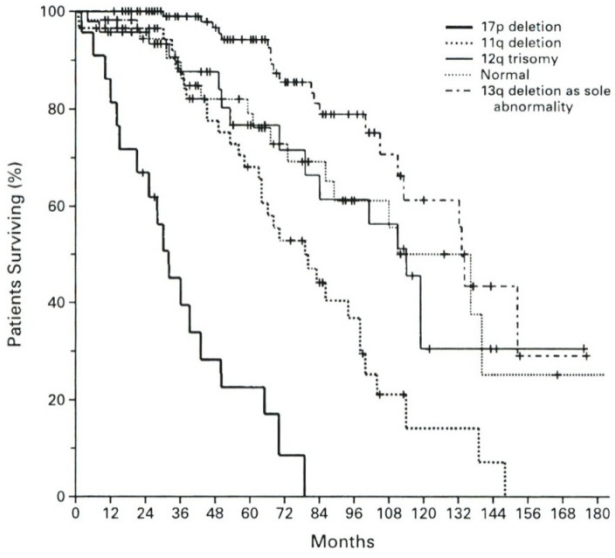

Figure 1.4.2 Probability of survival from diagnosis in the five genetic categories as reported by Döhner et al.[19] *The median survival times for the groups with del17, del11, tris12, normal karyotype, del13 as sole deletion were 32,79,114,111, and 133 months respectively.*

1.4.3 - B-Cell Receptor and Immunoglobulin mutational status

The B-Cell Receptor (BCR) is a multimeric complex constituted of a membrane-bound IG glycoprotein and a heterodimer IGα/IGβ(CD79A/CD79B), located on the surface of B cell. The IG glycoprotein is composed by two identical heavy chains, α, β, γ, δ or ε and two identical light chains, κ or λ.

The B-Cell Receptor mediates immunological interactions between the B cell and the microenvironment. BCR activation can be induced by antigen or can be ligand-independent, and triggers a cascade of signaling events that normally cause B cell selection, proliferation, differentiation, and antibody production.[28] Thereby, BCR signaling allows for the expansion of selected, foreign antigen-specific B cells, and deletion of unwanted, self-reactive B cells.[29]

The antigen-binding sites of immunoglobulins (Ig) derive from recombination of V-D-J gene segments for the heavy chains and V-J segments for the light chains. *V(D)J* recombination confers diversity by random and imprecise rearrangement, especially of the complementary determining region 3 (CDR3) during B-cell development in the bone marrow. Further maturation of the B lymphocyte, taking place in the lymphoid organs, requires antigen engagement, cooperation of T lymphocytes and other cells, and involves somatic hypermutation (SHM) and class-switch recombination (CSR). SHM introduces point mutations in variable regions of rearranged immunoglobulin heavy and light chains, increasing antigen affinity by several orders of magnitude. CSR modifies the constant region of heavy chains, altering the effector functions of Igs.[30]

The BCR has always been a key molecule to understand CLL. For long time it was given that CLL cancer cells stemmed from a population of naïve B-cells because of their morphology: small size, high nucleus/cytoplasm ratio and membrane co-expression of IgM-IgD, which is the typical phenotype of virgin B-cells. Later studies[31-33] on Ig genes rearrangements highlighted that more than 50% of the analyzed patients carried somatic mutations in heavy variable region, thus suggesting that B-cells may have already gone through the germinal centers. CLL subgroup with very unfavourable clinical outcome presents none or few (<2%) mutations (UM CLL) in *IGHV* genes, in respect to the closest germ line sequence. CLL cells of this particular subgroup seem to receive continuous anti-apoptotic and/or proliferating microenvironmental stimuli via BCR leading to a more aggressive disease than the subgroup with M configuration of *IGHV* genes (≥2%; M CLL), compared to the closest germ line sequence.

The expression of M IGHV genes correlates with a longer time to clinical progression and time to treatment compared with cases expressing UM genes, especially among patients with early stage of disease. This indicated for the first time that prognosis could be predicted at the time of initial diagnosis regardless of the actual tumor burden. Nowadays, mutational status of IGHV genes has become the golden standard for prognostication in CLL, especially since the marker does not change during the clinical course.[34;35]

BCRs in CLL patients are characterized by a biased usage of IGHV and IGLV genes. It is not uncommon that specific V-genes associate with specific (D)/J-genes, leading to remarkably similar, stereotyped heavy chain CDR3 and smIgs.[36;37] These data support a model of antigen-driven selection and expansion of CLL clones, suggesting recurrent binding of restricted sets of antigenic epitopes.[1;38-40]

The importance of BCR signaling in CLL is further stressed by comparative Gene Expression profiling data that revealed BCR signaling as the most prominent pathway activated in CLL cells isolated from lymphatic tissues.[41] Thus, targeting BCR signaling has become a prominent therapeutic strategy in the recent years (see paragraph 1.5.3).

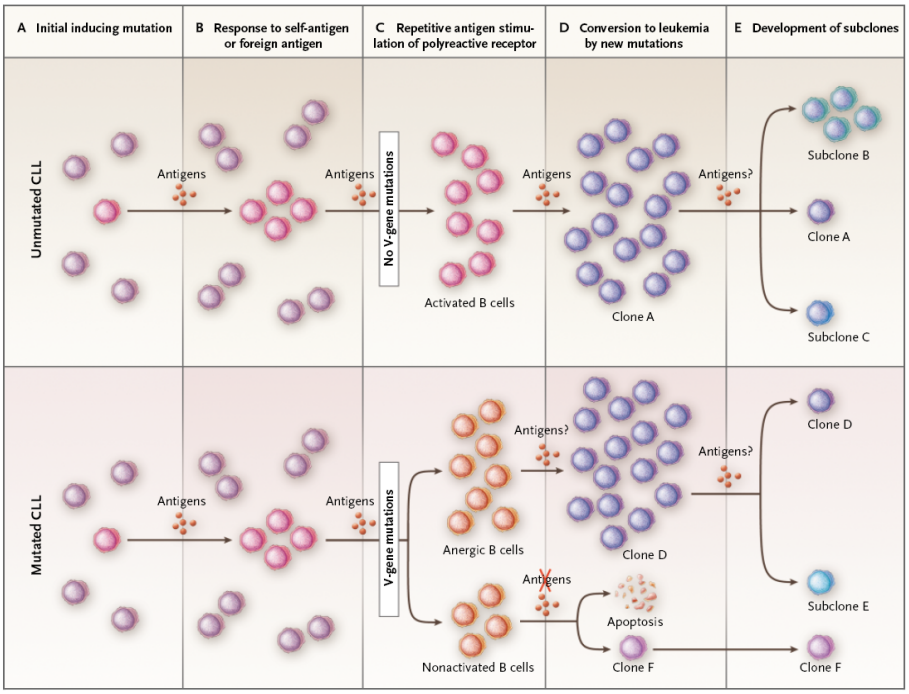

Figure 1.4.3 Model of development and evolution of CLL cells. *An initial inducing lesion occurs in a single B lymphocyte. providing a growth advantage to the cell over other clones. In unmutated CLL clones, repetitive interactions between antigens and polyreactive BCR promote clonal growth. In mutated CLL cells, V-gene mutations develop and abrogate BCR's polyreactivity ("clonal ignorance"). Additional DNA mutations cause the leukemic transformation. Differences in the signals received through the BCR and other receptors determine the level of clonal expansion. Continued cycling leads to other genetic changes that determine the course of the disease. Adapted from Chiorazzi et al.*[1]

1.4.4 - Immunophenotypic markers: ZAP70, CD38, CD49d

ZAP-70 (zeta-associated protein-70) is a member of the Syk family of tyrosine kinases and is associated with the CD3 complex.[41;42] ZAP-70 was described as the most differentially expressed gene between M and UM CLL subtypes, highlighting a strong correlation with IGHV mutational status.[43] ZAP-70 intra-cytoplasmic expression is currently investigated by flow cytometry[41;44-46], although without a common standardized protocol.[47;48] Using a cut-off set at 20% of positive cells, ZAP-70 expression was demonstrated to have an independent negative prognostic impact in CLL.[44;45;47]

CD38 is a 45-kDa type II membrane glycoprotein that acts as a cell surface receptor and as an enzyme, regulating cytoplasmic Ca^{++} levels.[49] CLL cell growth and survival are favored by the interactions with the microenvironment[50], more likely to occur in peripheral lymphoid organs and/or bone marrow: residential CLL cells express CD38 at higher levels[51-53] while endothelial, stromal, and nurse-like cells[54-56] express its ligand CD31. CD38 exerts its functions upon translocation into lipid rafts and association with other molecules, including CD19 and ß1 integrins such as CD49d.[57;58] CD38 expression is heterogeneous among CLL cases and by using a 30% cut-off of positive cells, significant prognostic differences were found by investigating both chemotherapy requirements and overall survival.[34]

CD49d, a.k.a. α4 integrin, is an adhesion molecule capable of mediating cell-to-cell interactions, binding to vascular-cell adhesion molecule-1 (VCAM-1), fibronectin (FN) and Emilin-1.[59;60] CD49d-expressing CLL cells were shown to have a high propensity to adhere to fibronectin substrates, and an increased CD49d protein expression was demonstrated in CLL cells from advanced Rai stage patients.[61] CD38 triggering in CD38+/CD49d+ CLL was demonstrated to lead to an overproduction of specific chemokines (CCL3 and CCL4), recruiting TNFα-producing macrophages, which in turn are responsible for VCAM-1 upregulation by stromal/endothelial cells. This interaction resulted in an increased survival of CD49d-expressing CLL cells.[62]

CD49d has been identified as an independent negative prognosticator for CLL.[63-66] Recently, a multicentric analysis validated the robustness of CD49d as a prognostic biomarker and its relative prognostic power in comparison with other markers: CD49d emerged as the strongest flow cytometry–based prognostic marker, with greater prognostic value than CD38 and ZAP-70.[67]

1.4.5 - Genetic lesions: TP53, BIRC3, SF3B1

Somatic genetic mutations played a limited role in CLL prognostication until recent times. *TP53* mutations were the first genetic lesion to be intensively investigated. The advent of Next Generation Sequencing has greatly elucidated the genomic complexity of CLL, discovering a somatic mutation rate much higher than previously thought.[9;68] The most recurrently mutated genes identified are *TP53*, *BIRC3*, *SF3B1* and *NOTCH1*. Mutations of *NOTCH1* and its role in CLL will be described in Chapter 1.6.

TP53 (Tumor Protein 53) is a tumor suppressor gene that plays a fundamental role in the DNA damage response pathway, inducing cell cycle arrest, DNA repair, senescence and apoptosis through both transcription-dependent and -independent pathways.[69] In CLL, *TP53* aberrations (deletion 17p13 or mutation) are found in 10-15% cases, with 70% of these cases harboring both deletion and mutation, 20% with deletion only and 10% with mutation only, consistently with a double-hit mechanism.[70;71] In

chemorefractory CLL, up to 40% cases display TP53 disruption. TP53-disrupted patients have a very poor clinical outcome, with progressive disease and clinical symptoms, and poor chemo/radiotherapy response, as cells are unable to activate DNA-damage pathways after treatment.[70;72] *TP53* aberrations (mutation and deletion) are currently the sole genetic defect validated as both a prognosticator and a predictor of clinical outcome, recommended for testing prior to therapy.[70] Interestingly, recent studies suggest that small *TP53*-mutated subclones might not expand in absence of therapy, suggesting a major role in chemoresistance rather than causing a proliferative advantage.[73]

BIRC3 (Baculoviral IAP Repeat Containing 3) is a negative regulator of the NF-κB non-canonical pathway[74], which plays a major role in mediating survival signals from the microenvironment.[74;75] In CLL, all BIRC3 mutations disrupt the catalytic RING domain, enabling a constitutively active NF-κB signaling. Although rare at diagnosis (<10%), BIRC3 mutations are frequent in chemorefractory cases (>30%) and, consistently, it was validated as an independent predictor of poor outcome.[22]

SF3B1 (splicing factor 3b, subunit 1) is a major component of U2 and U2-like spliceosomes, responsible for intron excision and mRNA maturation.[76] The consequences of *SF3B1* mutations aren't completely defined, but seem to alter the splicing program of genes controlling cell cycle progression and apoptosis, modulating tumor cell proliferation and survival.[9] At diagnosis *SF3B1* is mutated in 5-10% of CLL cases, increasing to 19% in chemorefractory CLL.[77;78] *SF3B1* mutations predict reduced survival independent of other clinical and biological risk factors[9] but stratify in the intermediate risk category in an integrated analysis.[22;79]

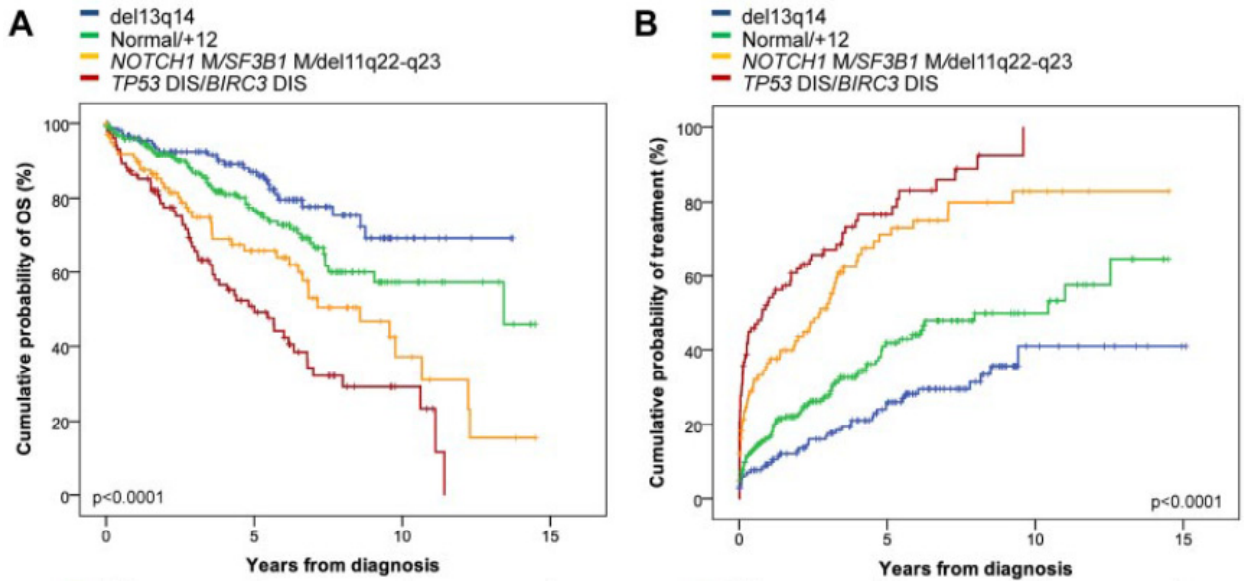

Figure 1.4.5 Kaplan-Meier estimates of OS and treatment-free survival according to the integrated mutational and cytogenetic model in the training series. *A) OS. B) Treatment-free interval. Cases harboring TP53 and/or BIRC3 disruption (TP53 DIS/BIRC3 DIS) independent of co-occurring genetic lesions are represented by the red line. Patients harboring NOTCH1 mutations (NOTCH1 M) and/or SF3B1 mutations (SF3B1 M) and/or del11 in the absence of TP53 and BIRC3 disruption are represented by the yellow line. Patients harboring +12 in the absence of the TP53 disruption, BIRC3 disruption, NOTCH1 mutations, SF3B1 mutations, and del11 and patients wild-type for all genetic lesions (normal) are represented by the green line. Cases harboring del13 as the sole genetic lesion are represented by the blue line. Adapted from Rossi et al.*[22]

1.5 - Therapeutic strategies

For decades, the standard of care for patients with early stage CLL was the "watchful waiting" because of two main reasons: the advanced age of patients and the ineffectiveness of most treatments, associated with a very low frequency of complete remission.[80] Treatment options evolved over time from chemotherapy with alkylating agents introduced in 1960s, to purine analogs in the 1980s, to integrated immunotherapy in 2000s, to targeted molecular therapy in 2010s. New drugs allowed a marked reduction of side effects and collateral toxicity, while increasing efficacy and leading to an always-higher rate of remission, either partial or complete. However, despite the advancements, CLL full eradication is still rarely achieved.[81]

1.5.1 - Chemotherapy

In the past, CLL treatment was based on alkylating agents (chlorambucil, cyclophosphamide) and corticosteroids: combinations of chlorambucil with prednisone became the benchmark of therapy in CLL.[82] The discovery of purine analogs like fludarabine monophosphate changed the paradigm, being now the building block of subsequent therapies, alone or in combination with other agents.[83-85] Nowadays fludarabine is the standard front-line therapy.[86;87]

1.5.2 - Immunotherapy

The immunotherapic approach is based on targeting surface antigens with monoclonal antibodies (mAb), alone or in combination with chemotherapeutic agents (chemo-immunotherapy) with synergistic activity. Antitumoral activity is based on Complement Dependant Cytotoxicity (CDC) or Antibody Dependant Cellular Toxicity (ADCC). The first agent entering a CLL clinical trial was Rituximab, an anti-CD20 recombinant antibody[88;89], followed by Alemtuzumab (anti-CD52).[90] At present, Rituximab is widely employed both as a single agent and in combination with Fludarabine (F+R), Chlorambucil (C+R and F+C+R), Bendamustine and other drugs, with FCR being the standard first line therapy from 2010.[81] Most recent advances led to the development of second and third generation anti-CD20 antibodies like Ofatumumab (a fully humanized antibody, directed against a different epitope than Rituximab) and Obinutuzumab (a humanized and glycoengineered antibody, with increased ADCC and reduced CDC).

1.5.3 - Molecular therapy

The recent studies have clearly demonstrated how survival of CLL cells is not a cell-autonomous process but depends on a permissive microenvironment, composed of cellular components such as macrophages, T cells, stromal, follicular and dendritic cells[3], which provide essential factors for activation of proliferative signaling. The BCR signaling plays a key role in this context and recently a number of molecules were synthesized, aiming at inhibiting different component of this pathway. The most promising drugs include

Ibrutinib (against Bruton's tyrosine kinase, inhibiting downstream NF-κB and MAPK pathways) and Idelalisib (against PI3Kδ, inhibiting AKT and MAPK pathways).[91] Other novel promising agents include BCL2 inhibitors like ABT-263 and ABT-199, the latter having less platelet toxicity, which impair the antiapoptotic capabilities of the tumoral cell and inducing efficient tumor lysis.[92]

1.5.4 - Stem cell transplantation

Allogenic SCT is increasingly considered in the management of chemotherapy-resistant patients, even though not widely used because of a number of reasons: difficulty to obtain uncontaminated cells from the bone marrow, high incidence of opportunistic infections, regimen-related toxicity, graft-versus-host disease. The major advantage of SCT is the graft-versus-leukemia effect, in a context of non-myeloablative conditioning. The question for treatment of young, high-risk patients (e.g. deletion 17p13, TP53 mutations, insufficient response to therapy) might not be if, but rather when allogenic transplantation should be considered.[93]

- 2 -
NOTCH1

Notch receptors are single-pass transmembrane proteins that participate an highly conserved signaling pathway that regulates many aspects of cellular differentiation and tissue homeostasis in metazoans.[94] The studies that led to the discovery of Notch genes started when genetics was born, as the name derives from a *Drosophila Melanogaster* phenotype described by Thomas Hunt Morgan in 1911 as characterized "notched" wings. Further studies linked a translocation affecting the Notch locus to human T-cell Acute Lymphoblastic leukemia, providing the first evidence of an association between Notch genes and cancer.[95] To date, Notch1 alterations have been reported in a number of haematological malignancies, including multiple myeloma, acute myeloid leukemia, lymphoma and chronic lymphocytic leukemia.[95]

1.6.1 - Structure and activation mechanism

The Notch pathway plays different roles during cell development in different cell types; this is possible because Notch signaling is tightly modulated by four receptors, NOTCH1/2/3/4, which share sequence and structure homology, and five ligands: Jagged 1/2 and Delta-like 1/3/4, all of which are also single-pass transmembrane proteins.[94]

The Notch receptor is synthesized as a single polypeptide, then translocated to the trans-Golgi system, where it is glycosylated and cleaved by a furin-like protease at site S1 in its two subunits. The resulting mature Notch1 receptor is an heterodimer of an extracellular (N^{EC}) and transmembrane (N^{TM}) domains. The subunits are held together by the intrinsic stability of the heterodimerization domain (HD), through a non-covalent Ca^{++}-dependant bond.[95;96]

The structure of Notch receptors consists in a series of juxtaposed functional domains, which can slightly vary among the family members. The N^{EC} domain of NOTCH1 is essentially composed by 36 EGF-like repeats, followed by a negative regulatory region of three Notch-LIN12 repeats, the HD domain and the S2 cleavage site. The N^{TM} domain is enriched with protein-protein interaction domains that mediate the effector functions: the RAM domain is responsible for interaction with RBPJ, further enhanced by six ankyrin repeats (ANK domain); the transactivation domain (TAD) is involved in transcription regulation; The c-terminal PEST domain is responsible for protein stability and is targeted by the E3 ubiquitin ligase FBXW7.[97-99]

Signaling is initiated when a Notch receptor binds its ligand, causing a conformational change that leads to receptor endocytosis and exposure of the buried S2 site, target of the transmembrane metalloproteinase ADAM10. Cleavage of N^{TM} at S2 creates a short-living transmembrane intermediate, immediately cleaved at

the intracytoplasmic site S3 by γ-secretase, a multisubunit protease consisting of presenilin 1 or 2, PEN2, APH1 and nicastrin. The S3 cleavage frees the Notch IntraCellular Domain (NICD), allowing it to translocate into the nucleus.[94]

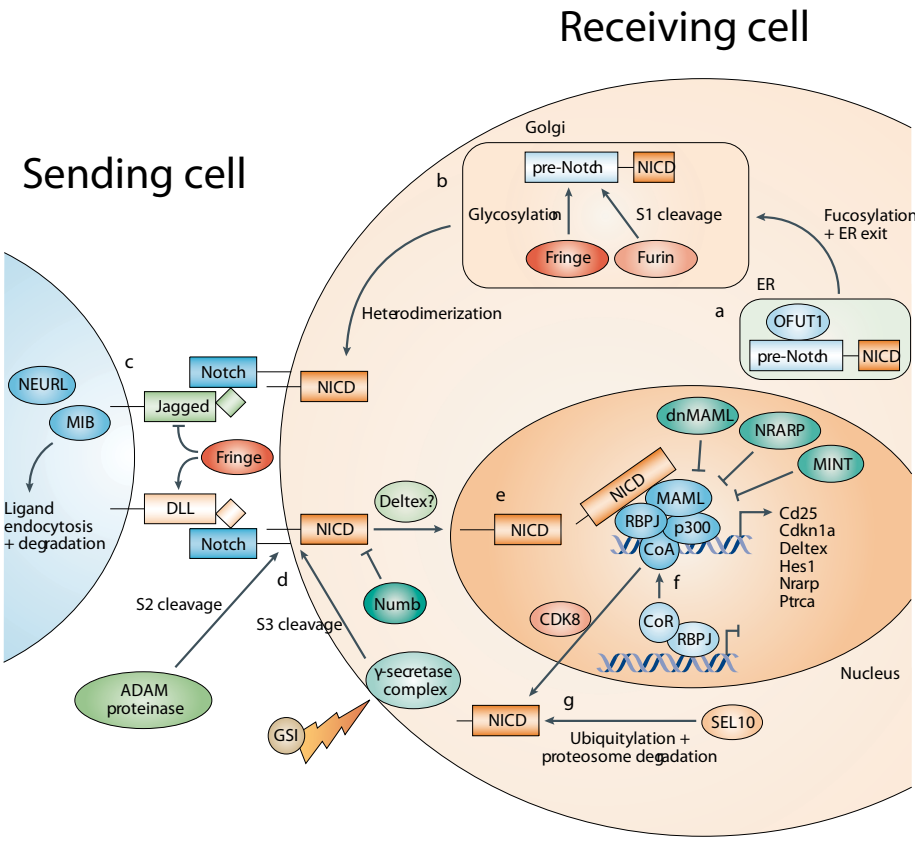

Figure 2.1 The Notch signaling pathway *(adapted from Grabher et al.[100]).*

1.6.2 - The Notch Transcriptional Complex

In the nucleus, the NICD is able to interact with its main interactor, the DNA binding factor RBPJ (also known as RBP-Jκ, CBF1 or CSL, short for CBF1/SU(H)/LAG-1), via RAM/ANK domains. Binding to RBPJ induces a conformational change that recruits other scaffold proteins like MAML, the third component of the core Notch Transcriptional Complex (NTC). The NTC in turn interacts with a number of chromatin modifiers, such as p300 and PCAF, and other components to initiate transcription of genes containing RBPJ binding site.[94;101;102] Among direct or indirect Notch effectors, many of them are involved in proliferation (Myc, p21, p27, CycD1, NF-κB), migration (CCR4, CCr8, CXCR6, CCR6, CCR7) and signaling (IL-6, IL-8, VEGFR-2, IL7R).[94]

In absence of NICD, RBPJ associates with multiple proteins that suppress transcription, including multiple complexes with hystone deacetylase[103-109] and demethylase activity.[110] For this reason, RBPJ has been described as a "molecular switch", capable to activate or inhibit transcription, depending on the Notch

activation status in the cell.[103] It is believed that normal Notch transcription complexes have an half-life in the order of minutes, in part because of ubiquitination of the PEST domain, which leads to NICD degradation and signaling inhibition.[94;97;98;105;106]

1.6.3 - Notch as an oncogene

The Notch signaling pathway is highly conserved among species and regulates cell-fate decisions throughout embryonic and adult life. In the majority of tissues, Notch maintains an undifferentiated state, but there are cases in which it induces differentiation[111] Notch signaling is highly context- and cell type-dependant, although certain genes seem to be common targets (e.g. HES, HEY families, responsible for linage commitment decisions).

The oncogenic role of the *NOTCH1* gene was discovered in 1991 through analysis of t(7;9)(q34;q34.3) translocation in T-ALL.[95;111] The translocation breakpoint fuses the 3' end of *NOTCH1* with T-Cell Receptor β enhancer/promoter elements, driving the expression of aberrant, constitutively nuclear NOTCH1 peptides.[112] Subsequently, aberrant NOTCH1 activation was found in more than 50% of T-ALL cases [113] but also progenitor cells, with mutations of the HD and PEST domains.[114] In keeping with its putative role in leukemogenesis, MYC and NF-κB were identified as downstream targets of aberrant Notch signaling, while interactions with WNT, SHH and AKT/PI3K pathways were reported.[111;115] Several studies also linked aberrant Notch signaling with breast cancer, lung cancer, glioma, multiple myeloma, Burkitt's lymphoma, Hodgkin lymphoma, DLBCL and B-CLL.[95;111]

1.6.4 - The role of *NOTCH1* in chronic lymphocytic leukemia

The first clues of an involvement of Notch signaling in CLL came from the observation that NOTCH2 induced CD23 expression, which was closely related with cell survival.[116] Later, the attention focused on NOTCH1, which was found constitutively expressed in CLL, along with NOTCH2 and their ligands DLL1-2.[117] Therefore, the authors hypothesized a paracrine/autocrine loop which could lead to a constitutive activation of Notch signaling, inducing apoptosis resistance. *NOTCH1* mutations were subsequently discovered in CLL cases[118] and suggested to be of clinical relevance.[119]

These data were validated by two studies aimed at identifying recurrent somatic mutations in CLL by next generation sequencing[120;121]: in both studies, *NOTCH1* was found to be mutated in ~10% of CLL at diagnosis and correlated with a worse prognosis.[120-122] Surprisingly, all *NOTCH1* mutations clustered in the exon 34, coding for the PEST domain, 80% of those being a dinucleotide CT deletion (c.7541-7542delCT): the deletion causes the shift of the open reading frame and the generation of a truncated protein (p.P2514Rfs*4) at the level of the PESP peptide, resulting in the disruption of the ubiquitination signal.

NOTCH1 mutations have been found associated with specific stereotyped BCR configurations[123;124] and trisomy 12[23;125-127]; a recent study described downregulation of β2-integrin expression in CLL cases harboring both *NOTCH1* mutations and trisomy 12.[24] A role for *NOTCH1* mutations in chemoresistance has also been described, although not yet comprehensively investigated.[128]

The negative impact of *NOTCH1* mutations in CLL has been validated by several studies[129-132] and confirmed to be associated with chemo/immune-refractoriness and Richter transformation.[78;120;129-134] Currently, *NOTCH1* mutations stratify in the intermediate risk category when included in an integrated analysis.[17;22] Of note, recent reports provided evidence of an association between *NOTCH1* mutations and resistance to immunotherapy with the anti-CD20 mAb rituximab: CLL patients carrying *NOTCH1* mutations treated with an FCR protocol (fludarabine+cyclophosphamide+rituximab) did not benefit of the inclusion of rituximab, displaying a significantly lower progression-free survival than *NOTCH1*-wild-type patients (median PFS FCR: *NOTCH1*-wt 57.3 months; *NOTCH1*-wt 34.2 months), and comparable to those treated with an FC protocol (Median PFS FC: *NOTCH1*-wt 32.8 months; *NOTCH1*-wt 33.9 months; Figure 2.4.1).[133]

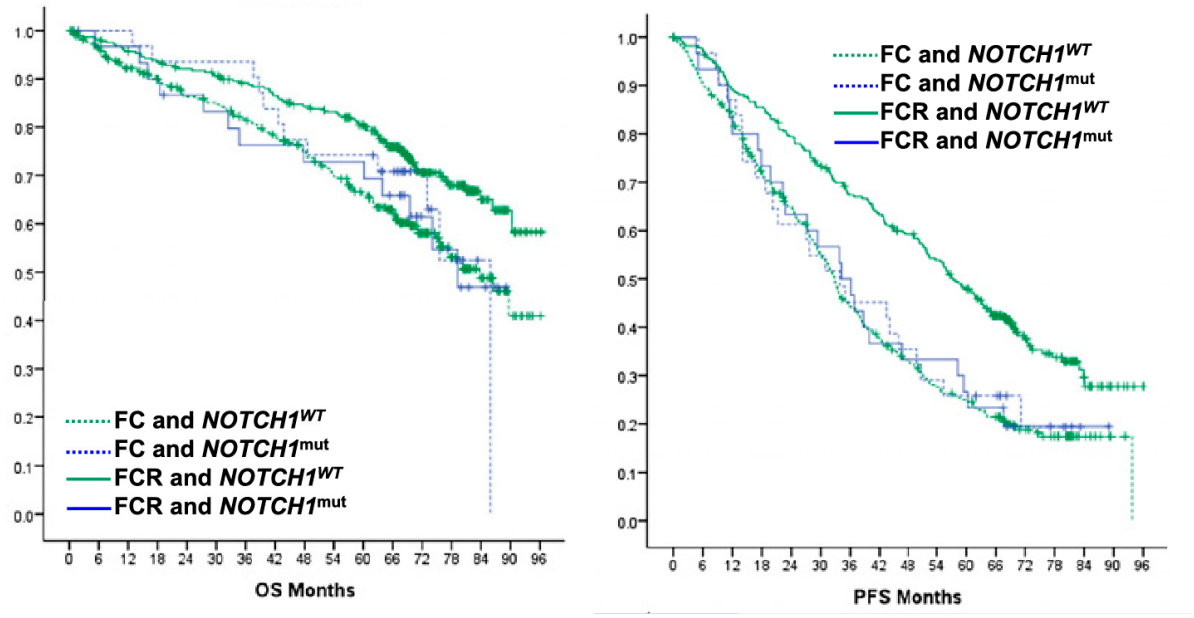

Figure 2.4.1 Kaplan-Meier curves for Overall Survival and Progression Free Survival from the CLL8 trial *NOTCH1-mut patients seem not to benefit from the addition of rituximab to the protocol. Adapted from Stilgenbauer et al.*[133]

- 3 -
Aim of the study

NOTCH1 mutations are found in about 10% of chronic lymphocytic leukemia (CLL) cases at diagnosis, and turned out to be an independent predictor of overall survival, identifying a subset of high risk patients with unfavourable prognosis. In CLL, *NOTCH1* mutations have been recently associated with clinical resistance to immunotherapy, specifically to the anti-CD20 recombinant antibody rituximab, although the mechanisms behind this peculiar behavior remain to be clarified. In lymphoproliferative disorders, susceptibility to rituximab is determined by CD20 levels, which are in turn epigenetically modulated via HDAC.

By taking advantage of a wide CLL series, we aimed to investigate the prognostic relevance of *NOTCH1* mutations, also in relationship with the main tumor burden and biologic markers for CLL, as well as its predictive relevance in the context of a rituximab-based maintenance therapy. Furthermore, to determine the mechanisms through which *NOTCH1* mutations could affect CLL susceptibility to rituximab-mediated immunotherapy, we investigated the role of Notch pathway in both primary CLL cases and cell line models of NOTCH1 mutation. In particular, we analyzed the correlation between CD20 expression and *NOTCH1* mutational status in primary CLL cases, in terms of (i) surface expression by flow cytometry, (ii) susceptibility to in-vitro CDC mediated by rituximab, (iii) capability of primary CLL lymphocytes to up-regulate CD20 upon exposure to Notch pathway inhibitors.

Literature data report that, in lymphoproliferative disorders, susceptibility to rituximab is determined by CD20 levels, which are in turn epigenetically modulated via histone deacetylases (HDAC). We therefore investigated the protein interactions of RBPJ, a transcription factor acting either as activator or repressor of the Notch pathway, when respectively bound to NICD or HDACs, by taking advantage of co-immunoprecipitation and chromatin immunoprecipitation techniques.

The results reported in the present thesis provide evidence that the presence of truncating *NOTCH1* mutations in CLL is associated with low CD20 expression levels, and with a relative resistance to rituximab treatment in-vitro. The low CD20 expression in *NOTCH1*- mut CLL can be ascribed to a *NOTCH1* mutation-driven epigenetic dysregulation of a transcriptional repression mechanism involving HDACs.

- 4 -
Materials and Methods

CLL patients

The study was approved by the Internal Review Board of the Centro di Riferimento Oncologico di Aviano (Approval n. IRB-05-2010), included peripheral blood samples from CLL patients diagnosed according to the current guidelines[135] Informed consent was obtained from the participants in accordance with the declaration of Helsinki. CLL cases were characterized for the main cytogenetic abnormalities, CD38, CD49d and ZAP70 expression, *IGHV* mutational status, as previously described.[64]

Primary CLL cells were obtained from peripheral blood samples by Ficoll-Hypaque (Pharmacia) density gradient centrifugation and used either directly or cryopreserved until use. All studies were performed on highly purified (>95% CLL cells),[58] or after purification by immunomagnetic negative selection, as described.[58]. In-vitro studies were performed in CLL cells from NOTCH1 mutated cases with relevant NOTCH1 mutational burden, i.e >25% of total DNA, or in NOTCH1 wild type cases, as control.

For a subset of 463 CLL cases, provided by the Department of Haematology of the S. Eugenio Hospital (University of Tor Vergata, Rome, Italy), clinical data were available.

DNA/RNA extraction and amplification

Nucleic acids were purified using DNA Mini/Micro kit (Qiagen), DNA/RNA AllPrep Mini/Micro kit (Qiagen), RNA Mini/Micro kit(Qiagen) or TRIZOL reagent (Invitrogen), according to manufacturer's instructions. cDNA was synthesized using up to 300 ng of RNA, either OligodT or Random Primers (Promega) and Improm-II Reverse Transcriptase (Promega), according to manufacturer's instructions.

Evaluation of *NOTCH1* mutational status

The presence of*c.*7541-7542delCT *NOTCH1* mutation was investigated by amplification refractory mutation system (ARMS) PCR, utilizing a "control" primer pair (Control Sense and Antisense) and a competing mutation-specific forward primer (delCT sense, Table 4.1), as previously described.[120;122;136] PCR was performed with G2 Taq Polymerase (Promega).

Evaluation of the *NOTCH1* mutational load by Next Generation Sequencing was performed amplifying genomic DNA with ad-hoc primers (Table 4.1) modified according to Illumina protocol, by using a high fidelity Taq polymerase (Phusion High-Fidelity DNA Polymerase, Thermo Scientific). The obtained PCR

products were subjected to next generation sequencing on a MiSeq sequencer (Illumina) to obtain a ~1000 coverage-fold for amplicons.

The presence of *NOTCH1* mutations other than the c.7541-7542delCT was investigated by Sanger sequencing in the entire *NOTCH1* PEST domain, using the BigDye 3.1 terminator kit (Life Technologies) on a 3130 Genetic Analyzer (Life Technologies), as reported.[123] The mutational load was roughly determined (about 50%, 25-50%, about 25%, <25% of mutated DNA) by visual inspection of sequence electropherograms, as reported.[125]

Quantitative real-time PCR (qRT-PCR)

Hydrolysis probes for *MS4A1* (Hs.PT.56a.24784282), *HDAC1* (Hs.PT.58.39528456) and *HDAC2* (Hs.PT.58.3484574) were from Integrated DNA Technologies (IDT). Taqman Gene Expression assays for *B2M* (Hs00984230_m1), *HES1* (Hs00172878_m1) were from Life Technologies. Reactions were done in triplicate from the same cDNA reaction (technical replicates) with FastStart Universal Probe Master (Roche) on a CFX96 (Bio-Rad) instrument; the relative amount of each gene was calculated utilizing the expression of *B2M* as internal control using the equation $2^{-\Delta Ct}$ where $\Delta Ct=(Ct^{gene}-Ct^{B2M})$. Fold changes between classes were calculated as reported.[137] All qRT-PCR experiments were performed on a CFX96 Thermal Cycler (Bio-Rad).

To evaluate *NOTCH1* mutational load by qPCR, genomic DNA from *NOTCH1* mutated cases was amplified in two separate reactions: a "control" amplification using the control primers for ARMS-PCR, and a "delCT mutation-specific" amplification using the delCT-specific primer with the antisense control primer (Table 4.1). Real-time PCR was performed using the SYBR Green Core Reagents kit (Life Technologies). The "control" amplification was used as a loading control and relative quantity of delCT mutation was calculated using the $2^{-\Delta Ct}$ equation.

Western blot

Total proteins were extracted in RIPA lysis buffer (Santa Cruz) from cultured cells, quantified with Bradford assay (Bio-Rad), ran in 4-15% Criterion SDS-PAGE gels (Bio-Rad) and transferred to nitrocellulose membranes with Trans-blot turbo kit (Bio-Rad). Cleaved NOTCH1 (clone D3B8, Cell Signaling), total NOTCH1 (clone D1E11, Cell Signaling), anti-HDAC1 (clone 10E2, Cell Signaling), anti-HDAC2 (clone 3F3, Cell Signaling), anti-CD20 (clone L26, Abcam) antibodies were used for protein detection. Mouse-anti-β-actin antibody (clone AC-74, Sigma-Aldrich) was used as loading control. Ladder is the ProteinC Precision Plus molecular weight standard (Bio-Rad). Immunodetection was performed with HRP-conjugated antibodies (Amersham) with ClarityECL (Bio-Rad) and Hyperfilm ECL films (Amersham). Films were digitally acquired with an Epson Perfection V330 Photo desktop scanner (Epson).

Flow cytometry and cell sorting

CD20 expression was investigated in 692 CLL cases by flow cytometry, using an anti-CD20 antibody (clone L27, BD Biosciences) either FITC (495 cases) or PE-Cy7 (197 cases), as described.[58;64] CD20 expression was evaluated in the neoplastic (i.e. CD19+, CD5+, κ/λ clonal) and residual normal B cell (i.e. CD19+ CD5-) components. CD20 expression after in-vitro experiments was evaluated with the PE conjugated anti-CD20 antibody. CD20 levels were expressed as Mean Fluorescence Intensity (MFI) in log10 mode.

Viability was assessed with Annexin V/7-amino-actinomycin-D (7AAD) staining (BD Pharmingen). Quantitative flow cytometry was performed using QuantiBrite PE Beads (BD QuantiBrite). Irrelevant isotype-matched antibodies were used to determine background fluorescence. Quality controls were performed using Rainbow Multicheck beads and Cytometer Setup&Tracking beads (CST; BD Biosciences). All experiments were performed on FACSCanto, FACSCanto II and LSR Fortessa instruments (BD Biosciences) and analyzed with FACSDiva (BD Biosciences) or FlowJo software (FlowJo).

Cell sorting experiments were performed on a FACSAriaIII (BD Biosciences) sorter. CLL cells from *NOTCH1* mutated cases were sorted with a using a PE-conjugated anti-CD20 antibody for the detection of $CD20^{low}$ and $CD20^{high}$ fractions, as previously described.[23]

Complement-dependent cytotoxicity (CDC) assay

For CDC assay, $2x10^5$ primary CLL cells or NICD-transfected MEC-1 cells were incubated with rituximab (5µg/ml) in a final volume of 150 µl for 10 min at room temperature prior to the addition of pooled normal human AB serum (25%). After a further incubation at 37° C for 1 hour, the number of residual viable cells was estimated by staining cells with 7-amino-actinomycin-D (7-AAD, Becton-Dickinson), as previously described.[138]

Cell cultures conditions

MEC1 cells were purchased from DSMZ and maintained at a concentration of 0.5-2x106 cells/ml in RPMI-1640 (Biochrom) supplemented with 10% heat inactivated fetal bovine serum (Biochrom), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-glutamine(Invitrogen).

NICD Plasmids

Plasmids containing the NICD coding sequence were engineered cloning the NICD coding sequence (derived from the EF.hICN1.CMV.GFP, gift from Linzhao Cheng, Addgene plasmid #17623) in a pcDNA3.1-NT-GFP-TOPO scaffold using the TOPO-TA cloning kit (Invitrogen). Site-directed mutagenesis was performed with the Quikchange II XL Mutagenesis kit (Agilent). The NICD-mut vector was modified by inserting the c.7541-7542delCT mutation, while the NICD-null was modified by inserting the c.5304G>A nonsense mutation. The plasmids were purified with QiaAmp MIDIprep kit (Qiagen).

Transfection with vectors encoding for NICD

MEC-1 cells were transfected with the Amaxa Nucleofector L kit (Lonza). Briefly, $6x10^6$ cells were washed twice in PBS, resuspended in 100µl of supplemented transfection medium with 3µg of linearized vector and electroporated (program C-005). Cells were readily resuspended in 2.5ml of pre-warmed RPMI+20%FBS and left growing for four days. Single cell cloning was performed with a FACSAria III cell sorter (BD Biosciences) for GFP-positive cells. One cell was seeded in 100µl RPMI+20%FBS and incubated at 37°C. After a week, Geneticin (G418, Invitrogen) was added at a concentration of 500µg/ml for antibiotic selection. Positively transfected clones were evaluated by direct sequencing and western blotting.

In-vitro treatment with pharmaceutical compounds

Primary CLL cell samples and NICD transfected MEC-1 cells were treated with 10µM of γ-secretase inhibitor (GSI) L-685,458 (Sigma) at a concentration of $2x10^6$ cells/ml for 6-24 hours before evaluation of MS4A1 transcript levels by QRT-PCR (at 6 hours) and evaluation of CD20 expression by flow cytometry (at 24 hours). GSI treatment was performed in *NOTCH1* mutated cases with relevant *NOTCH1* mutational burden, i.e.>10% of mutated DNA. In control experiments, equal volume of the appropriate solution compound was added.

NICD transfected MEC-1 cells were treated with 3mM of HDAC inhibitor 2-propylpentanoic acid (VPA, Depakin, Sanofi) at a concentration of $2x10^6$ cells/ml for 48 hours before evaluation of MS4A1 transcript levels by QRT-PCR and evaluation of CD20 expression by flow cytometry. In control experiments, equal volume of the appropriate solution compound was added.

Co-Immunoprecipitation

Nuclear extracts were obtained as following: 20x10^6 cells were collected, resuspended in Nuclear Extract buffer #1 (25mM HEPES, 5mM KCl, 0.5 mM $MgCl_2$, protease inhibitors); one volume of Nuclear Extract buffer #2(25mM HEPES, 5mM KCl, 0.5 mM $MgCl_2$, protease inhibitors, 1% NP-40) was added and left rotating at 4°C for 15'. After centrifuging, supernatant was removed and nuclei were washed once with Nuclear Extract buffer #3 (25mM HEPES, 5mM KCl, 0.5 mM $MgCl_2$, protease inhibitors, 0.5% NP-40). Nuclear pellet was then lysed in Nuclear Extract buffer #4 (25mM HEPES, 10% (w/v) Sucrose, 350 mM NaCl, protease inhibitors, 0.01% NP-40) and sonicated with 3 cycles of 30 seconds in a Biorupture sonicator (Diagenode). Lysates were quantified by Bradfors assay (Bio-Rad).

Co-immunoprecipitation was performed with Protein G-Mag Sepharose beads (GE healthcare) according to manufacturer's protocol using anti-RBPJ (clone ab25949, Abcam) and isotype (Millipore) antibodies. Eluates were loaded on SDS-PAGE and western blotting was performed as described above using the following antibodies: NOTCH1 (D1E11, Cell Signaling), HDAC1 (10E2, Abcam), HDAC2 (HDAC2-62, Abcam). Anti-ERK 1/2 (Millipore) and Anti-BRG1 (Santa Cruz) were used as loading controls for cytoplasmic and nuclear lysates. Image acquisition was performed using ImageQuant LAS4000 and TL Version 7.0 software (GE Healthcare).

Chromatin Immunoprecipitation assay (ChIP)

ChIP assays were performed with SimpleChIP enzymatic Chromatin IP kit using Magnetic Beads (Cell Signaling), according to standard manufacturer's protocol. Cells ($40x10^6$) were cross-linked with 1% formaldehyde and lysed according to the protocol. DNA was digested with 1µl Micrococcal nuclease to a fragment size from 150 to 900 base pairs. Seven µg of cross-linked chromatin were used to perform immunoprecipitation with either HDAC1 (10E2, Abcam), HDAC2 (HDAC2-62, Abcam), Hystone H3 (kit provided) or isotype (kit provided) antibodies. Each reported ChIP is an average of three independent experiments. Quantification of *MS4A1* and *HES1* promoter DNA bound to immunoprecipitated HDAC1 or HDAC2 was determined by quantitative real-time PCR using SsoFast Evagreen Supermix (Biorad) (Table 4.1) using the Percent Input Method according to the equation: Percent input= 2% x $2^{(Ct\ 2\%\ Input\ Sample - Ct\ IP\ Sample)}$.

Statistical analysis

All statistical analyses were performed using MedCalc software (MedCalc Software).

OS and RD were estimated using the Kaplan-Meier plots and comparisons between groups were made by means of log-rank test. The Cox proportional hazard regression model was chosen to assess the independent effect of covariates, treated as dichotomous, on OS and RD. The association between presence of *NOTCH1* mutations and other variables was calculated using the χ^2 test with a Yates' correction for continuity.

Data were compared using Student's t-test for independent or paired samples, eventually adjusted for unequal variances (Welch-test). Reported values for experiments using NICD transfected cells including CDC assay, treatment with pharmaceutical compounds (i.e. GSI and VPA) and ChIP assy were an average of three independent experiments. Data are presented as Tukey box-and-whiskers plots: the box represents the 25th (bottom) and 75th (top) percentiles, the band inside the box is the median and the whiskers span from the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile. Outliers exceeding this range are reported as circles. Alternatively, data are presented as histograms, indicating the mean ± standard error mean (SEM).

Table 4.1 PCR Primers used in the study

***NOTCH1* delCT ARMS-PCR and qPCR**	
Control S	GTGACCGCAGCCCAGTT
Control AS	AAGGCTTGGGAAAGGAAGC
delCT S	TCCTCACCCCGTCCCGA
delCT NGS	
NGS-S	CCTGGCGGTGCACACACTATTC
NGS-AS	TGGGAAAGGAAGCCGGGGTCT
***NOTCH1* exon 34 Sequencing**	
PEST 1 S	GTGACCGCAGCCCAGTT
PEST 1 AS	AAGGCTTGGGAAAGGAAGC
Pest 2 S	GAGGAGTAGCTGTGCTGCGA
Pest 2 AS	ATCCAGCAGCAGCAAAGCCT
Pest 3 S	CTCGGCTCTCCACTCAGGAA
Pest 3 AS	ACCAATACAACCCTCTGCGG
Pest 4 S	GGTAGCTCATCATCTGGGACA
Pest 4 AS	CTCAACCACCTGCCTGGGAT
Pest 5 S	CATCCCAGGCAGGTGGTTGA
Pest 5 AS	ACATCGTGAGGCTGCTGGAC
Pest 6 S	GCTGCTTCCTCTGGTGATGG
Pest 6 AS	CTGGGCTTGCGGACCTTCTT
5693 AS	CTCCTCTTCCTCGCTGTTGC
5681 AS	TGGGGCGCGCCGTTTTCTTGA
Chromatin Immunoprecipitation	
MS4A1 S	GGTCTTTTTCAAGAAGTGAAACCT
MS4A1 AS	CTACTGATTTCCTGTCACCTGATG
HES1 S	GCGTGTCTCCTCCTCCCATT
HES1 AS	CCTGGCGGCCTCTATATATA

- 5 -
Results

5.1 - *NOTCH1*delCT mutation induces accumulation of mutant protein

A cohort of 1608 CLL cases was screened for the presence of *NOTCH1* c.7541-7542delCT mutation (delCT hereafter) by ARMS-PCR[122]; 200 out of 1608 cases (12.4%) were positively identified and marked as *NOTCH1* mutated (*NOTCH1*-mut, Figure 5.1.1A-B). ARMS-PCR-negative cases were marked as wild-type (*NOTCH1*-wt). In a subset of 803 patients, *NOTCH1* exon 34 was also analyzed by Sanger sequencing, to rule out the presence of other mutations than delCT. Of 803 cases, 12 harboured nonsense or frameshift mutations, while 5 harboured missense mutations. For the purpose of our analyses, these cases were considered separately from delCT-positive cases.

As ARMS-PCR is an end-point assay, quantification of mutational burden can only be roughly estimated from the intensity of the lower delCT-specific band (Figure 5.1.1B). To overcome this issue, we developed a quantitative Real-Time PCR assay, in which the control amplicon from ARMS-PCR was separately amplified and used as normalizer for the overall *NOTCH1* quantity (Figure 5.1.1C). The approach was initially validated with a plasmid dilution curve (range 1-99% of mutated *NOTCH1*, r=0.993, Figure 5.1.1D), and later with a Next-Generation Sequencing (NGS) approach, with primary CLL cases (r=0.917, Figure 5.1.1E).

In accordance with the presence of a truncating mutation, i.e. c.7541-7542delCT, which leads to an incomplete NOTCH1 protein with impaired degradation[128;139], immunoblotting analysis of whole cell lysates from *NOTCH1*-mut CLL cases showed high transmembrane NOTCH1 and NICD levels, both with molecular weights consistent with the truncation of the *NOTCH1* mutated protein. On the contrary, *NOTCH1*-wt CLL, although eventually expressing significant amount of transmembrane NOTCH1 in some instances, usually expressed less NICD protein than *NOTCH1*-mut cases (Figure 5.1.2).[117;121;128]

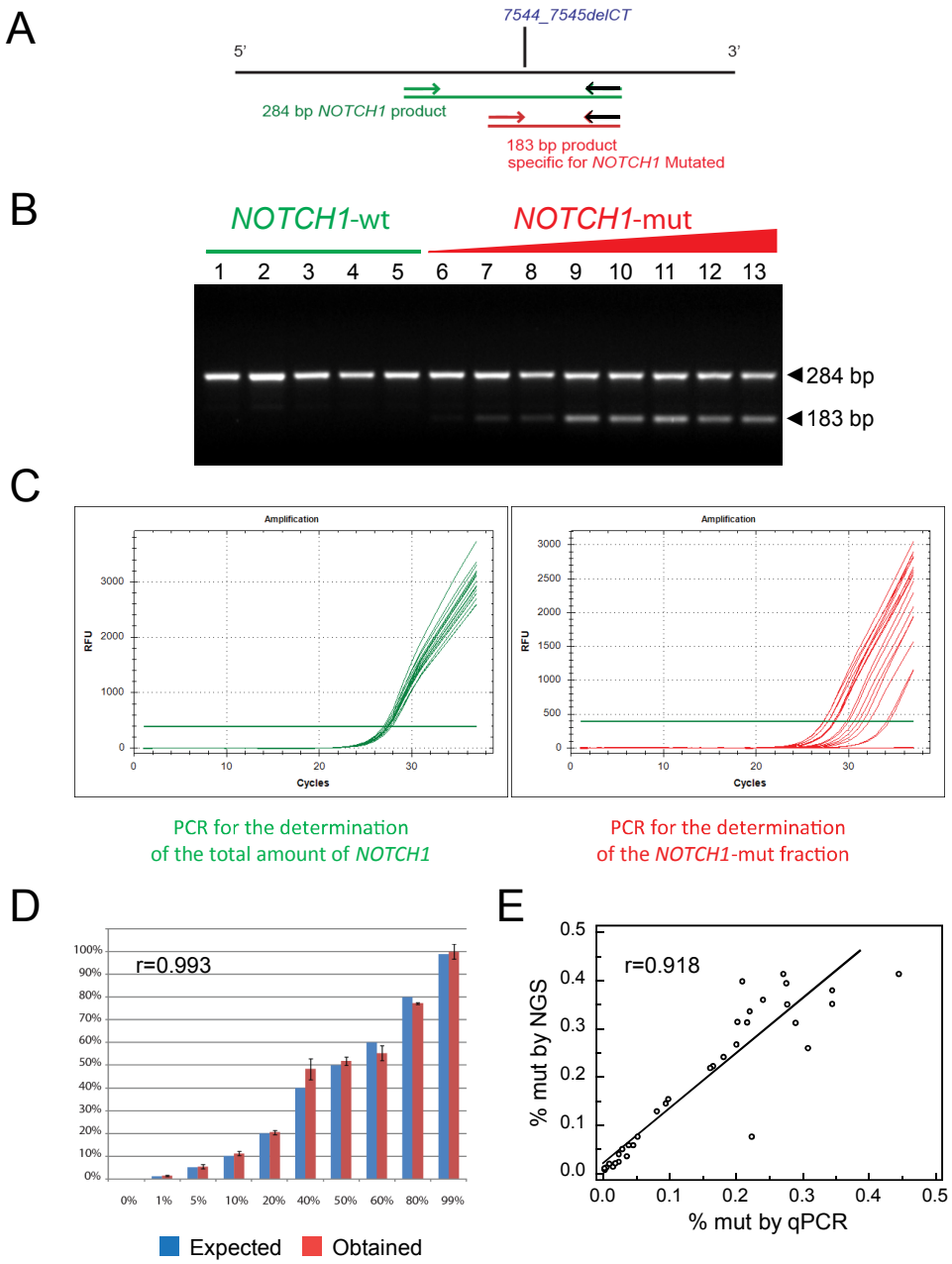

Figure 5.1.1 Evaluation and quantification of NOTCH1 mutational status in CLL cases. *A) Diagram illustrating the ARMS-PCR approach. In green, the sense control primer and amplicon for total NOTCH1; in red, the c.7541-7542delCT-specific primer and amplicon; in black, the shared antisense primer. B) Representative results of the ARMS PCR assay showing 5 CLL samples that scored negative (samples 1-5) and 8 that scored positive for NOTCH1 delCT mutation (samples 6-13). Negative samples shows a normal band of 284bp. Positive samples showed an additional mutant band of 183bp. C)Set-up of quantitative real time PCR assay. Control amplification (green) is used as reference. D) Validation of the qPCR assay using a plasmid dilution curve (r= Pearson correlation coefficient). E) Validation of the qPCR assay by Next Generation Sequencing.*

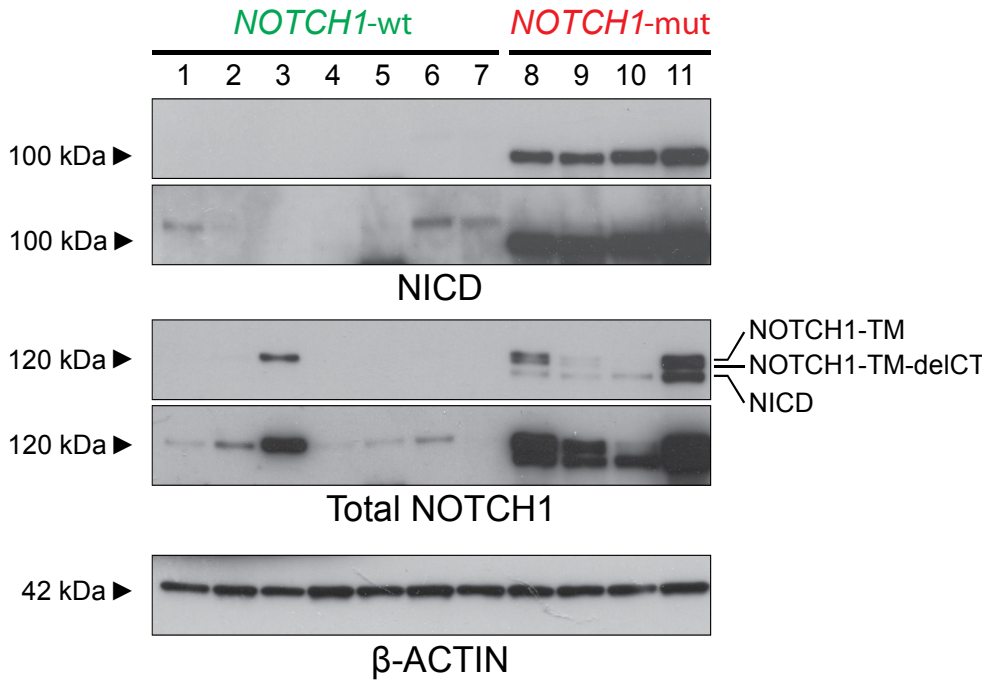

Figure 5.1.2 *NOTCH1* delCT mutation leads to NICD accumulation in CLL cases. *Representative immunoblot of NOTCH1 in 11 CLL cases (4 NOTCH1-mut cases) for cleaved NICD (Val1754; upper panel). The blot was then re-probed with total NOTCH1 (middle panel). A second high-exposure blot is reported. The mutated form of transmembrane NOTCH1 is visible as a lower band (NOTCH1-TM-delCT). NOTCH1-mut cases show a strong accumulation of NICD compared to wild type cases. NOTCH-TM: transmembrane NOTCH1; NICD: cleaved NOTCH Intracellular Domain. B-actin was used as loading control (lower panel). Reference molecular weights are reported.*

5.2 - Clinical impact of *NOTCH1* mutations

For a subset of 463 CLL cases, clinical data of Overall Survival (OS) were available. We therefore aimed to verify the clinical impact of *NOTCH1* delCT mutation in our cohort.

Out of 463 cases, 41 cases (8.9%) carried a delCT mutation, as detected by ARMS-PCR. CLL at diagnosis was significantly associated with all the investigated prognostic markers, utilized as dichotomous variables as reported[10], including the markers of tumor burden modified Rai (mod-Rai) staging (p=0.0031), β2M (p=0.0270), lymphocyte doubling time (LDT, p<0.0001), and soluble CD23 (sCD23, p<0.0001), and the biologic markers *IGHV* status (p<0.0001), ZAP70 (p<0.0001), CD38 (p<0.0001), and CD49d (p=0.0053; Table 5.2.1). Regarding specific chromosomal abnormalities, a significant association was found between *NOTCH1* delCT mutation and trisomy 12 (p=0.0003), as previously reported[23;125-127], whereas *NOTCH1* delCT mutation and del11 or del17 resulted not associated (p>0.05 in both cases).

OS data were available for all the 463 patients entering the study. Median follow-up was 76 months (range 3-271 months), with 56 deaths and 407 censored patients. All the investigated prognostic markers were proven to maintain their prognostic impact as OS predictors also in our CLL series (Table 5.2.2). When

testing the prognostic relevance of *NOTCH1* delCT mutation, a significantly shorter survival was found in *NOTCH1*-mut patients compared to *NOTCH1*-wt patients ($p<0.0001$, Figure 5.2.1A and Table 5.2.2). Of note, the presence of*NOTCH1* delCT mutation behaved as negative prognosticator also in the context of patients characterized by an unfavorable configuration of all the investigated prognosticators, either markers of tumor burden (Figure 5.2.1B), or biologic markers (Figure 5.2.1C) and, in some instances, in the context of patient subgroups with a favorable configuration (Figure 5.2.2).

Consistently, *NOTCH1* delCT mutation retained its independent prognostic value in bivariate Cox regression analyses (Table 5.2.3), without interaction effect with all the investigated prognosticators (Table 5.2.4). The impact of *NOTCH1* delCT mutation as independent prognostic factor for OS was also demonstrated by multivariate Cox proportional hazards analysis in a 4-variable model which included IGHV status (UM IGHV vs. M IGHV) and FISH subgroups (del11/del17 vs. tris12/del13/normal) along with mod-Rai staging (intermediate/high vs. low mod-Rai stages, Table 5.2.5).[10]

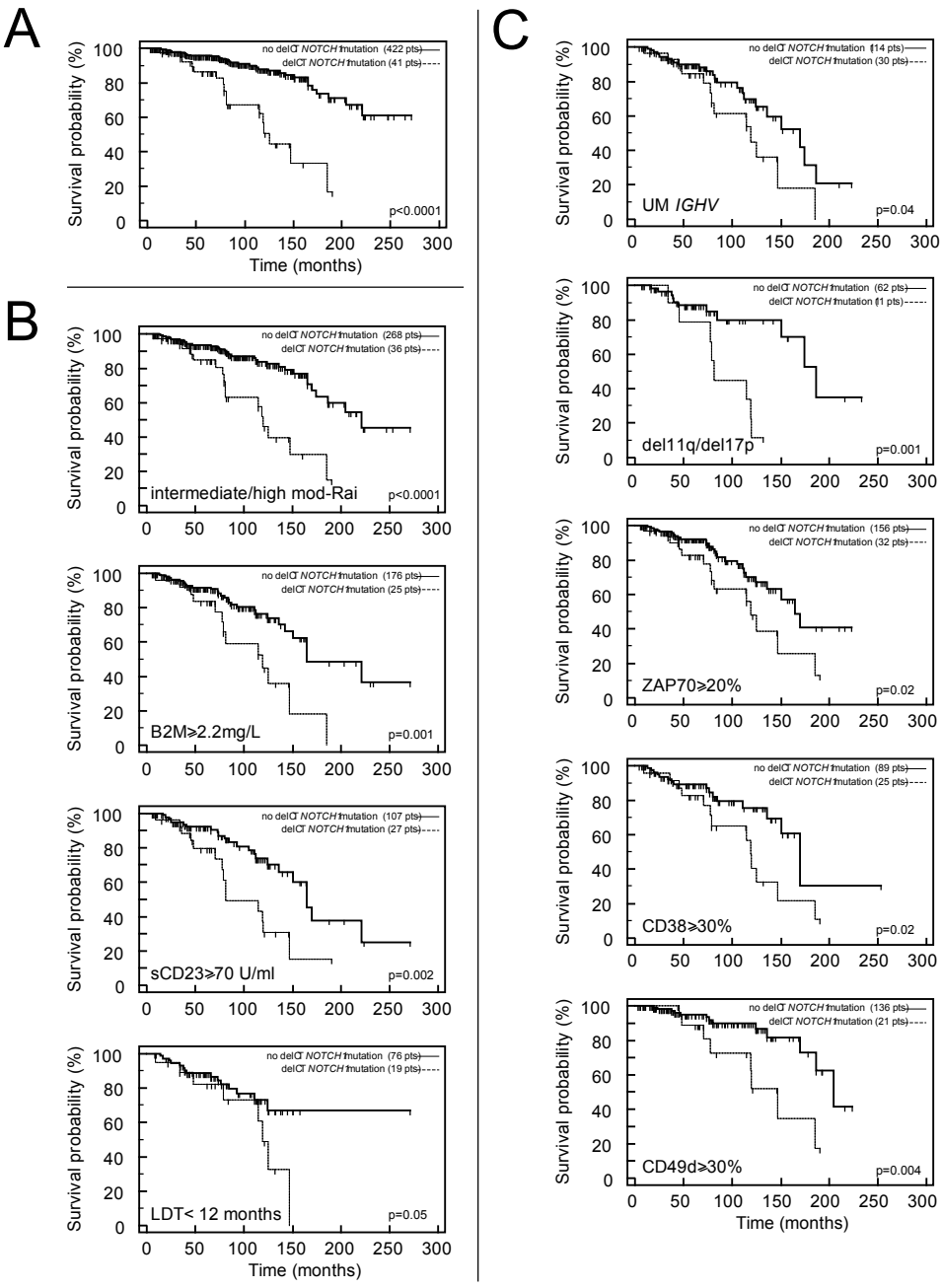

Figure 5.2.1 Clinical significance of c.7541-7542delCT *NOTCH1* mutation in CLL. *A) Kaplan-Meier curves obtained by comparing OS intervals of CLL cases with NOTCH1 delCT mutation (median survival 125 months) and without NOTCH1 delCT mutation (median survival not reached). B) Kaplan-Meier curves obtained by comparing OS intervals of CLL cases with and without NOTCH1 delCT mutation in the context of an unfavorable configuration of markers of tumor burden. C) Kaplan-Meier curves obtained by comparing OS intervals of CLL cases with and without NOTCH1 delCT mutation in the context of an unfavorable configuration of biological markers.*

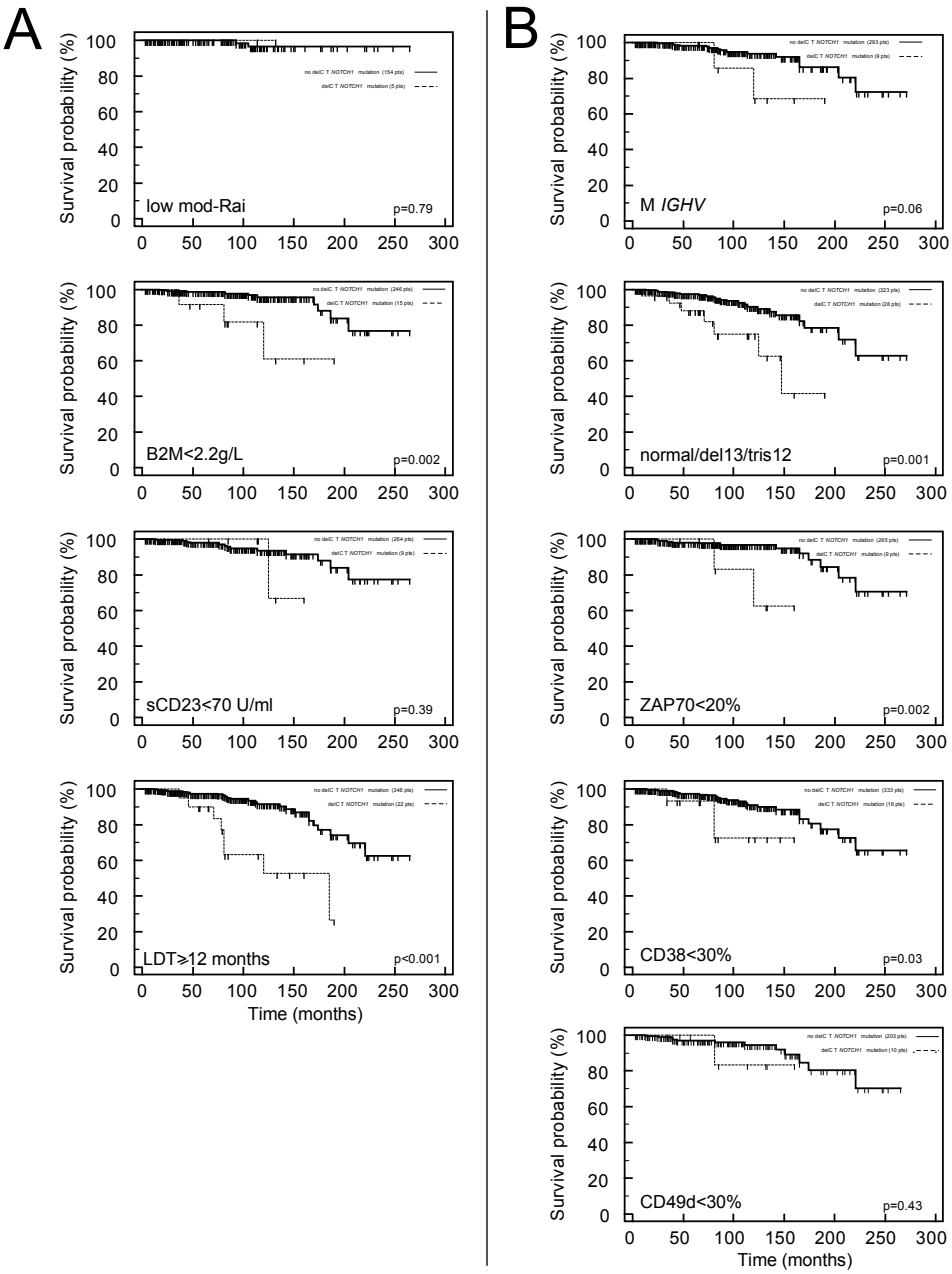

Figure 5.2.2 Clinical significance of NOTCH1 delCT mutation in the context of a favorable configuration of the main clinical and biological prognosticators in CLL. *A) Kaplan-Meier curves obtained by comparing OS intervals of CLL cases with NOTCH1 delCT mutation and without NOTCH1 delCT mutation in the context of a favorable configuration of markers of tumor burden: mod-Rai staging, β2M, sCD23, LDT. B) Kaplan-Meier curves obtained by comparing OS intervals of CLL cases with NOTCH1 delCT mutation and without NOTCH1 delCT mutation in the context of a favorable configuration of biological markers: IGHV status, FISH subgroups, and ZAP70, CD38, CD49d expression. The number of patients (pts) included in each group is reported in parenthesis; the reported p value refer to log-rank test.*

Table 5.2.1 Association between *NOTCH1*delCT mutation and the main tumor burden and biological prognosticators for CLL.

	no*NOTCH1*delCT	*NOTCH1*delCT	sample size	p value
mod-RAI				
Low mod-RAI	154	5	463	0.0031
Intermediate/High mod-Rai	268	36		
β2M				
< 2.2 g/L	246	16	463	0.0270
≥ 2.2 g/L	176	25		
LDT				
≥ 12 months	346	22	463	<0.0001
< 12 months	76	19		
sCD23				
< 70 U/ml	264	9	407	<0.0001
≥ 70 U/ml	107	27		
***IGHV* status**				
M *IGHV*	293	9	446	<0.0001
UM *IGHV*	114	30		
ZAP70				
< 20%	265	9	462	<0.0001
≥ 20%	156	32		
CD38				
< 30%	333	16	463	<0.0001
≥ 30%	89	25		
CD49				
< 30%	203	10	370	0.0053
≥ 30%	136	21		
tris12				
no tris12	342	26	424	0.0003
tris12	43	13		
del11				
no del11	349	31	424	0.0571
del11	36	8		
del17				
no del17	365	37	424	0.7181
del17	20	2		

The association between presence of NOTCH1 mutation and the main tumor burden and biological prognosticators for CLL was calculated using the χ2 test with a Yates' correction for continuity.

Table 5.2.2 Univariate Cox regression analyses of OS.

	Sample size	HR (95% CI)	p value
intermediate and high Mod-Rai stage	463	13.41 (3.29-54.65)	0.0003
β2M more than or equal to 2.2 g/L	463	5.83 (3.13-10.86)	<0.0001
<12 months LDT	463	3.65 (2.11-6.33)	<0.0001
sCD23 more than or equal to 70 U/ml	407	6.52 (3.53-12.04)	<0.0001
UM *IGHV* mutational status	446	7.11 (3.97-12.71)	<0.0001
del11 and del17 FISH subgroups	424	3.56 (2.01-6.32)	<0.0001
ZAP70 more than or equal to 20%	462	6.49 (3.51-11.98)	<0.0001
CD38 more than or equal to 30%	463	4.46 (2.63-7.59)	<0.0001
CD49d more than or equal to 30%	370	2.86 (1.42-5.75)	0.0033
NOTCH1 delCT mutation	463	4.52 (2.52-8.11)	<0.0001

OS, Overall Survival; HR, hazard ratio; CI, confidence interval

Table 5.2.3 Bivariate Cox regression analyses of OS.

	Sample size	HR (95% CI)	p value
NOTCH1 delCT mutation	463	3.43 (1.91-6.15)	<0.0001
intermediate and high Mod-Rai stage		11.33 (2.77-46.42)	0.0008
NOTCH1 delCT mutation	463	3.18 (1.76-5.76)	0.0001
β2M more than or equal to 2.2 g/L		5.04 (2.68-9.47)	<0.0001
NOTCH1 delCT mutation	463	3.41 (1.85-6.25)	0.0001
<12 months LDT		2.89 (1.62-5.14)	0.0003
NOTCH1 delCT mutation	407	2.69 (1.43-5.08)	0.0023
sCD23 more than or equal to 70 U/ml		5.28 (2.79-10.03)	<0.0001
NOTCH1 delCT mutation	447	2.14 (1.14-4.00)	0.018
UM *IGHV* mutational status		6.01 (3.27-11.03)	<0.0001
NOTCH1 delCT mutation	424	4.19 (2.24-7.87)	<0.0001
del11 and del17 FISH subgroups		3.05 (1.71-5.46)	0.0002
NOTCH1 delCT mutation	462	2.46 (1.34-4.52)	0.0040
ZAP70 more than or equal to 20%		5.30 (2.80-10.05)	<0.0001
NOTCH1 delCT mutation	463	2.49 (1.31-4.74)	0.0055
CD38 more than or equal to 30%		3.37 (1.88-6.03)	<0.0001
NOTCH1 delCT mutation	370	3.03 (1.36-6.74)	0.0068
CD49d more than or equal to 30%		2.34 (1.13-4.84)	0.0227

OS, Overall Survival; HR, hazard ratio; CI, confidence interval

Table 5.2.4 Bivariate Cox regression model of OS with interaction term.

	p value
Mod-Rai stage	n.c.
β2M	0.332
LDT	0.159
sCD23	0.840
IGHV mutational status	0.504
FISH subgroups	0.811
ZAP70	0.234
CD38	0.569
CD49d	0.752

n.c., not converging data; p value, p of interaction effect

Table 5.2.5 Multivariate Cox regression analysis of OS.

	Sample	HR (95% CI)	p value
intermediate and high Mod-Rai stage	413	5.48 (1.32-22.73)	0.0197
UM *IGHV* mutational status		4.13 (2.13-8.01)	<0.0001
del11 and del17 FISH subgroups		2.26 (1.25-4.11)	0.0075
NOTCH1 delCT mutation		2.47 (1.29-4.75)	0.0067

A multivariate Cox regression analysis of OS was performed by including the following covariates: modified Rai stage (Mod-Rai; intermediate/high vs. low), IGHV gene mutational status (UM IGHV vs. M IGHV), FISH subgroups (del11 and del17 vs. tris12/del13/normal), NOTCH1 delCT mutation. The sample of 413 cases included 48 events and 365 censored patients. OS, Overall Survival; HR, hazard ratio; CI, confidence interval.

5.3 - Impact of *NOTCH1* mutations on Immunotherapy

Recently, the German CLL Study Group CLL8 Trial[133] (see par. 1.6.4) reported a negative association between *NOTCH1* mutations and response to rituximab immunotherapy; CLL patients bearing *NOTCH1* mutations seemed not to benefit from the addition of rituximab to the fludarabine/cyclophosphamide protocol in terms of both progression free survival and overall survival.

Therefore, we aimed to assess the clinical impact of *NOTCH1* mutations in a cohort of 123 progressively and previously untreated CLL who underwent first-line treatment with fludarabine plus rituximab followed by consolidation/maintenance immunotherapy with rituximab.

Out of 123 cases, 20 cases (16.26%) carried a *NOTCH1* delCT mutation, as detected by ARMS-PCR. In 91 out of 123 cases, *NOTCH1* exon 34 was sequenced by Sanger sequencing. All patients were homogeneously assigned to first-line induction treatment with six months courses of intravenous (25 mg/m^2) or oral fludarabine (30-40 mg/m^2) followed by four weekly doses (375 mg/m^2) of rituximab.[140;141] Regarding response to the first-line therapy, 43 out of 123 patients achieved complete remission. Among the 80 remaining patients, 46 achieved complete remission but remaining positive for minimal residual disease (MRD) detection by flow cytometry, whereas the other 34 patients showed partial remission/stable disease. Among these 80 patients with incomplete first-line therapy response, 59 underwent to a consolidation/maintenance phase with rituximab (four monthly cycles of rituximab at 375 mg/m^2 followed by twelve months doses of rituximab at 150 mg/m^2) whereas the other 21 patients remained not consolidated (Figure 5.3.1A). The clinical and biological data, reported in Table 5.3.2, were all collected at the time of first-line treatment.

Of note, 18 out of 20 *NOTCH1*-mut cases had less than MRD- complete response to first-line therapy whereas only 2 *NOTCH1*-mut cases reached complete remission (p=0.0213). The association between *NOTCH1* mutations and the other parameters evaluated as prognosticators in the study are reported for the whole cohort of patients (Table 5.3.2), and for the sub cohort of patients with less than MRD- complete response to first-line therapy (Table 5.3.3) either undergoing or not consolidation/maintenance therapy (Table 5.3.4-6).

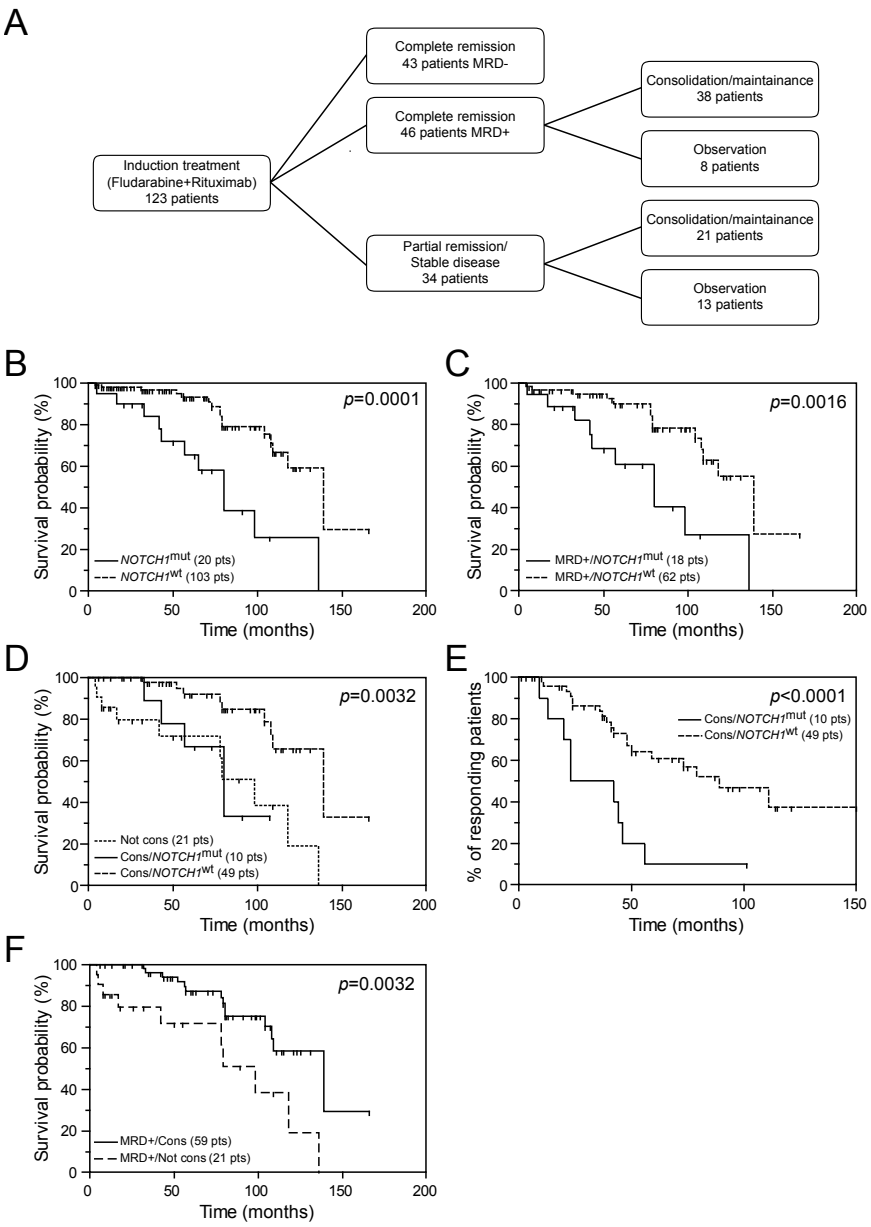

Figure 5.3.1 *NOTCH* mutated CLL patients do not benefit from rituximab-based immunotherapy. *A) Consort diagram for CLL patients included in the study. B) Kaplan-Meier curves comparing OS intervals of NOTCH1-mut cases (median survival 80 months) and NOTCH1-wt cases (median survival 139 months) of the whole cohort. C) Kaplan-Meier curves comparing OS intervals of NOTCH1-mut cases with incomplete response to first-line therapy (MRD+/NOTCH1^{mut}) cases (median survival 80 months) and NOTCH1-wt with incomplete response to first-line therapy (MRD+/NOTCH1^{wt}) cases (median survival 139 months). D) Kaplan-Meier curves comparing OS intervals of consolidated NOTCH1-mut (Cons/NOTCH1^{mut}) cases (median survival 80 months), consolidated NOTCH1-wt (Cons/NOTCH1^{wt}) cases (median survival 139 months), and not consolidated (Not cons) cases (median survival 98 months). E) Kaplan-Meier curves comparing RD intervals of consolidated NOTCH1-mut (Cons/NOTCH1^{mut}) cases (median RD 33 months) and consolidated NOTCH1-wt (Cons/NOTCH1^{wt}) cases (median RD 89 months). F) Kaplan-Meier curves comparing OS intervals of CLL cases that underwent consolidation/maintenance therapy (Cons, median survival 139 months) and not consolidated (Not cons) CLL cases (median survival 98 months) in the cohort of patients with incomplete response to first-line therapy. The number of patients (pts) included in each group is reported in parenthesis; the reported p value refer to log-rank test.*

When testing the prognostic relevance of *NOTCH1* mutations, a significantly shorter Overall Survival (OS) after treatment was observed in patients carrying *NOTCH1* mutations compared to patients lacking *NOTCH1* mutations (66% versus 93% at five years; log-rank test p=0.0001, Figure 5.3.1B). Similarly, in the context of the 80 CLL patients with less than MRD- complete response, a significantly shorter OS was observed in patients carrying *NOTCH1* mutations compared to patients lacking *NOTCH1* mutations (55% versus 90% at five years; log-rank test p=0.0016, Figure 5.3.1C and Table 5.3.7). This observation confirms the prognostic relevance of *NOTCH1* mutations also in partially responding/not responding CLL patients. Consistently, *NOTCH1* mutations retained its independent prognostic value in bivariate Cox regression analyses when corrected for the main tumor burden and biological prognosticators, which were proven to maintain their prognostic impact as OS predictors in univariate analyses (Table 5.3.8 and Table 5.3.7). The impact of *NOTCH1* mutations as independent prognostic factor for OS (p=0.0098, Hazard Ratio, HR=3.61, 95% Confidence Interval, CI=1.37-9.53) was also documented by multivariate Cox proportional hazards analysis in a 3-variable model which included *TP53* disruption (presence of *TP53* mutations and/or 17p deletion) and consolidation/maintenance immunotherapy (absence of therapy, Table 5.3.1). In keeping with previously reported data[140;141], consolidated patients showed longer OS than not consolidated patients (71% versus 87% at five years; log-rank test p=0.0032, Figure 5.3.1F). In the cohort of the 59 patients that underwent consolidation/maintenance phase with rituximab, the presence of *NOTCH1* mutations was found in 10 out of 59 cases whereas 8 out of 21 not consolidated patients were *NOTCH1* mutated. Clinically, in the context of consolidated patients, *NOTCH1*-mut cases experienced OS intervals shorter than those of *NOTCH1*-wt cases (67% versus 92% at five years; log-rank test p=0.0053) and not dissimilar to those of not consolidated cases (67% versus 72% at five years; log-rank test p=0.93, Figure 5.3.1D and Table 5.3.7).

Response Duration (RD) intervals, defined as time from the end of first-line treatment to relapse (event) or last follow-up, were also evaluated in the cohort of the 59 homogeneously consolidated patients. As shown in Figure 5.3.1E and Table 5.3.7, consolidated *NOTCH1*-mut patients experienced significantly shorter RD intervals than consolidated *NOTCH1*-wt patients (11% at 60% at five years; log-rank test p=0.0009).

Table 5.3.1 Multivariate Cox regression analyses of OS in the cohort of patients with incomplete first-line therapy response

	OS		
Parameter	**Sample size**	**HR (95% CI)**	**P value**
TP53 mutations/17p deletion	79	3.82 (1.43-10.21)	0.0079
Consolidation/maintenance		2.40 (0.97-5.92)	0.0593
NOTCH1 mutations		3.61 (1.37-9.53)	0.0098

OS. overall survival; HR. hazard ratio; CI. confidence interval

Table 5.3.2 Association between *NOTCH1* mutations and the main tumor burden and biological prognosticators for CLL in the whole analyzed cohort.

	No *NOTCH1* mutations	*NOTCH1* mutations	Sample size	p value
Age				
<63 years	50	7	123	0.3862
≥ 63 years	53	13		
Modified (mod-) RAI				
Low mod-RAI	21	2	123	0.4372
Intermediate mod-RAI	82	18		
β2M				
< 2.2 mg/L	42	5	123	0.2813
≥ 2.2 mg/L	61	15		
LDT				
≥ 12 months	48	9	123	0.9096
< 12 months	55	11		
sCD23				
< 70 U/ml	60	3	122	0.0008
≥ 70 U/ml	42	17		
tris12				
no tris12	88	13	121	0.1125
tris12	14	6		
del11				
no del11	88	14	121	0.2976
del11	14	5		
***TP53*mutations/del17**				
no *TP53* mutations/del17	88	17	121	0.9927
*TP53*mutations/del17	14	2		
***IGHV* status**				
M *IGHV*	61	4	121	0.0022
UM *IGHV*	40	16		
CD38				
< 30%	81	2	123	<0.0001
≥ 30%	22	18		
CD49d				
< 30%	51	2	118	0.0014
≥ 30%	47	18		
ZAP70				
< 20%	55	0	123	<0.0001
≥ 20%	48	20		

The association between presence of NOTCH1 mutations and the main tumor burden and biological prognosticators for CLL cases was calculated using the χ2 test with a Yates' correction for continuity.

Table 5.3.3 Association between *NOTCH1* mutations and the main tumor burden and biological prognosticators for CLL patients with less than MRD- complete response to first-line therapy.

	No *NOTCH1* mutations	*NOTCH1* mutations	Sample size	p value
Age				
<63 years	34	5	80	0.0794
≥ 63 years	28	13		
Modified (mod-) RAI				
Low mod-RAI	12	2	80	0.6469
Intermediate mod-RAI	50	16		
β2M				
< 2.2 mg/L	28	5	80	0.2951
≥ 2.2 mg/L	34	13		
LDT				
≥ 12 months	32	9	80	0.8829
< 12 months	30	9		
sCD23				
< 70 U/ml	31	3	80	0.0246
≥ 70 U/ml	31	15		
tris12				
no tris12	52	12	79	0.1544
tris12	9	6		
del11				
no del11	50	13	79	0.5685
del11	11	5		
***TP53* mutations/del17**				
no *TP53* mutations/del17	53	16	79	0.8582
TP53 mutations/del17	8	2		
***IGHV* status**				
M *IGHV*	29	4	78	0.0901
UM *IGHV*	31	14		
CD38				
< 30%	49	2	80	<0.0001
≥ 30%	13	16		
CD49d				
< 30%	29	1	79	0.0032
≥ 30%	32	17		
ZAP70				
< 20%	31	0	80	0.0004
≥ 20%	31	18		
Consolidation/maintenance				
yes	49	10	80	0.0913
no	13	8		

The association between presence of NOTCH1 mutations and the main tumor burden and biological prognosticators for CLL cases was calculated using the χ2 test with a Yates' correction for continuity.

Table 5.3.4 Association between *NOTCH1* mutations and the main tumor burden and biological prognosticators for CLL patients that underwent consolidated/maintenance therapy.

	No *NOTCH1* mutations	*NOTCH1* mutations	Sample size	p value
Age				
<63 years	30	3	59	0.1435
≥ 63 years	19	7		
Modified (mod-) RAI				
Low mod-RAI	10	1	59	0.7454
Intermediate mod-RAI	39	9		
β2M				
< 2.2 mg/L	26	3	59	0.3259
≥ 2.2 mg/L	23	7		
LDT				
≥ 12 months	26	5	59	0.8644
< 12 months	23	5		
sCD23				
< 70 U/ml	27	2	59	0.0937
≥ 70 U/ml	22	8		
tris12				
no tris12	42	7	58	0.3626
tris12	6	3		
del11				
no del11	40	7	58	0.5926
del11	8	3		
***TP53* mutations/del17**				
no *TP53* mutations/del17	42	10	58	0.5418
TP53 mutations/del17	6	0		
***IGHV* status**				
M *IGHV*	24	3	57	0.3883
UM *IGHV*	23	7		
CD38				
< 30%	40	2	59	0.0004
≥ 30%	9	8		
CD49d				
< 30%	27	1	58	0.0206
≥ 30%	21	9		
ZAP70				
< 20%	27	0	59	0.0045
≥ 20%	22	10		

The association between presence of NOTCH1 mutations and the main tumor burden and biological prognosticators for consolidated CLL cases was calculated using the χ2 test with a Yates' correction for continuity.

Table 5.3.5 Association between *NOTCH1* mutations and the main tumor burden and biological prognosticators for not consolidated CLL patients.

	No *NOTCH1* mutations	*NOTCH1* mutations	Sample size	p value
Age				
<63 years	4	2	21	0.8312
≥ 63 years	9	6		
Modified (mod-) RAI				
Low mod-RAI	2	1	21	0.6465
Intermediate mod-RAI	11	7		
β2M				
< 2.2 mg/L	2	2	21	0.9783
≥ 2.2 mg/L	11	6		
LDT				
≥ 12 months	6	4	21	0.7806
< 12 months	7	4		
sCD23				
< 70 U/ml	4	1	21	0.6694
≥ 70 U/ml	9	7		
tris12				
no tris12	10	5	21	0.8312
tris12	3	3		
del11				
no del11	10	6	21	0.6694
del11	3	2		
***TP53* mutations/del17**				
no *TP53* mutations/del17	11	6	21	0.9783
TP53 mutations/del17	2	2		
***IGHV* status**				
M *IGHV*	5	1	21	0.4345
UM *IGHV*	8	7		
CD38				
< 30%	9	0	21	0.0078
≥ 30%	4	8		
CD49d				
< 30%	2	0	21	0.6885
≥ 30%	11	8		
ZAP70				
< 20%	4	0	21	0.2414
≥ 20%	9	8		

The association between presence of NOTCH1 mutations and the main tumor burden and biological prognosticators for not consolidated CLL cases was calculated using the χ2 test with a Yates' correction for continuity.

Table 5.3.6 Distribution of the main tumor burden and biological prognosticators in consolidated or in not consolidated CLL patients.

	Not consolidated	Consolidated	Sample Size	p value
Age				
<63 years	6	33	80	0.0574
≥ 63 years	15	26		
Modified (mod-) RAI				
Low mod-RAI	3	11	80	0.9068
Intermediate mod-RAI	18	48		
β2M				
< 2.2 mg/L	4	29	80	0.0317
≥ 2.2 mg/L	17	30		
LDT				
≥ 12 months	10	39	80	0.8938
< 12 months	11	28		
sCD23				
< 70 U/ml	5	29	80	0.0783
≥ 70 U/ml	16	30		
tris12				
no tris12	15	49	79	0.3260
tris12	6	9		
del11				
no del11	16	47	79	0.8757
del11	5	11		
***TP53* mutations/del17**				
no *TP53* mutations/del17	17	52	79	0.5191
TP53 mutations/del17	4	6		
CD38				
< 30%	9	42	80	0.0399
≥ 30%	12	17		
CD49d				
< 30%	2	28	80	0.0041
≥ 30%	19	30		
ZAP70				
< 20%	4	27	80	0.0578
≥ 20%	17	32		
***IGHV* status**				
M *IGHV*	6	27	78	0.2179
UM *IGHV*	15	30		
***NOTCH1* mutations**				
no *NOTCH1* mutations	13	49	80	0.2179
NOTCH1 mutations	8	10		

The association between presence of NOTCH1 mutations and the main tumor burden and biological prognosticators for not consolidated CLL cases was calculated using the □2 test with a Yates' correction for continuity..

Table 5.3.7 Univariate Cox regression analyses.

Parameter	OS (incomplete first-line response)			OS (consolidated)			RD (consolidated)		
	Sample size	HR (95% CI)	p value	Sample size	HR (95% CI)	p value	Sample size	HR (95% CI)	p value
Age ≥63 years	80	3.72 (1.53-9.019)	0.0037	59	2.93 (0.98-8.73)	0.0554	59	2.87 (1.33-6.19)	0.0073
Intermediate Mod-Rai stage	80	1.91 (0.44-8.158)	0.3846	59	1.26 (0.27-5.76)	0.7589	59	1.53 (0.53-4.40)	0.4264
β2M ≥2.2 mg/L	80	3.66 (1.36-9.875)	0.0105	59	4.28 (1.18-15.51)	0.0272	59	1.90 (0.88-4.09)	0.0996
LDT <12 months	80	0.86 (0.36-2.051)	0.7396	59	1.65 (0.50-5.40)	0.4105	59	0.67 (0.31-1.44)	0.3134
sCD23 ≥70 U/ml	80	4.78 (1.62-14.07)	0.0047	59	5.96 (1.32-26.82)	0.0205	59	2.16 (0.98-4.77)	0.0559
Trisomy 12	79	2.60 (0.90-7.49)	0.0765	58	3.32 (0.85-12.89)	0.0836	58	1.70 (0.58-4.98)	0.3335
11q deletion	79	1.45 (0.53-3.97)	0.4691	58	1.15 (0.25-5.23)	0.8485	58	1.97 (0.79-4.88)	0.1442
TP53 mutations/17p deletion	79	2.36(0.96-5.82)	0.0632	58	2.07 (0.62-6.87)	0.2366	58	0.97 (0.34-2.82)	0.9693
UM *IGHV* mutational status	78	1.50 (0.63-3.58)	0.3592	57	2.34 (0.72-7.60)	0.1576	57	3.34 (1.46-7.66)	0.0044
CD38 ≥30%	80	1.87 (0.82-4.23)	0.1329	59	1.50 (0.50-4.47)	0.4633	59	2.44 (1.15-5.16)	0.0203
CD49d ≥30%	79	1.84 (0.73-4.63)	0.1962	58	1.08 (0.37-3.10)	0.8865	58	1.76 (0.81-3.80)	0.1520
ZAP70 ≥20%	80	2.10 (0.83-5.28)	0.1156	59	1.40 (0.47-4.18)	0.5439	59	1.72 (0.79-3.72)	0.1677
NOTCH1 mutation	80	3.56 (1.54-8.23)	0.0031	59	4.69 (1.42-15.47)	0.0115	59	3.58 (1.59-8.05)	0.0020
Consolidation/maintenance	80	3.24 (1.42-7.43)	0.0055	-	-	-	-	-	-

OS, overall survival; RD, response duration; HR, hazard ratio; CI, confidence interval

Table 5.3.8 Bivariate Cox regression analyses.

OS (incomplete first-line response)	Sample size	HR (95% CI)	pvalue
*NOTCH1*mutations	80	3.74 (1.57-8.92)	0.0030
Age ≥63 years		3.88 (1.57-9.58)	0.0033
*NOTCH1*mutations	80	2.86 (1.21-6.75)	0.0165
B2M ≥2.2 mg/L		3.00 (1.09-8.23)	0.0328
*NOTCH1*mutations	80	2.22 (0.91-5.42)	0.0495
sCD23 ≥70 U/ml		3.57 (1.14-11.20)	0.0293
*NOTCH1*mutations	79	4.98 (1.98-12.53)	0.0007
TP53 mutations/17p deletion		3.86 (1.42-10.47)	0.0083
NOTCH1 mutations	80	2.66 (1.08-6.50)	0.0326
Consolidation/maintenance therapy		2.38 (0.97-5.82)	0.0581

OS, overall survival; HR, hazard ratio; CI, confidence interval

5.4 - Negative correlation between CD20 expression and *NOTCH1* mutations

Previously published data report a direct correlation between response to rituximab treatment CD20 expression and to in B cell neoplasms[142]; to investigate whether CD20 expression could be affected by the presence of *NOTCH1* mutations, we evaluated the surface CD20 expression in a large retrospective, unselected, consecutive cohort of 495 CLL cases. CD20 expression was determined by flow cytometry with a FITC-conjugated antibody, and mean fluorescence intensity (MFI) was calculated on CD19+/5+ lymphocytes. Out of 495 cases, 60 cases carried a *NOTCH1* mutation.

CD20 expression was generally lower than in the normal non-neoplastic residual B cell component,[135] although variable when stratified according to the main cytogenetics aberrations[19]; in particular cases with trisomy 12 displayed the highest levels, in accordance with previous observations (Figure 5.4.1A).[143]

When the expression of CD20 was evaluated with respect to *NOTCH1* mutational status, *NOTCH1*-mut showed a lower Mean Fluorescent Intensity (MFI) of CD20 expression than *NOTCH1*-wt cases both in trisomy 12 cases (mean MFI in 6 *NOTCH1*-mut cases = 12926±3676; mean MFI in 17 *NOTCH1*-wt cases = 28216±5228; p=0.027) and in non-trisomy 12 (mean MFI in 21 *NOTCH1*-mut cases = 10207±1310; mean MFI in 153 *NOTCH1*-wt cases = 15208±1578; p=0.017) cases (Figure 5.4.1C).

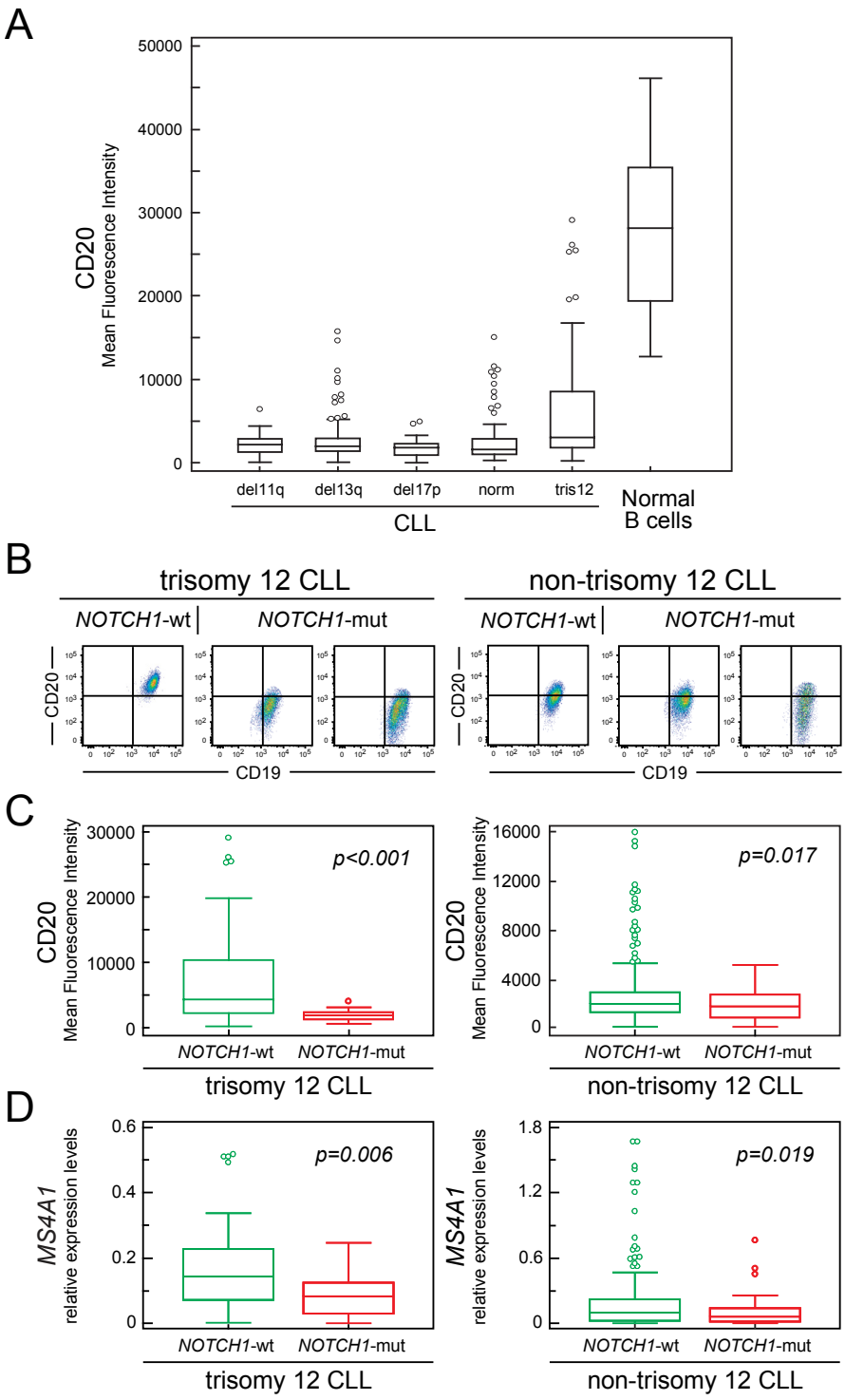

Figure 5.4.1 Correlation between *NOTCH1* mutations and CD20 expression in CLL (first series). *A) Box-and-whiskers plots showing CD20 protein expression levels, in CLL cells from a series of 495 CLL cases, according to cytogenetic abnormalities, and in the residual normal B cell (i.e. CD19+/5-) components, as evaluated by flow cytometry using a FITC-conjugated anti-CD20 antibody. B) Dot plot showing CD20 expression, as evaluated by flow cytometry using a FITC-conjugated anti-CD20 antibody, in prototypic NOTCH1-mut and NOTCH1-wt cases of trisomy 12 and non-trisomy 12 CLL categories. C) Box-and-whiskers plots showing CD20 protein expression levels, evaluated as above, in 89 trisomy 12 CLL cases (20 NOTCH1-mut cases, 69 NOTCH1-wt cases) and 406 non-trisomy 12 CLL*

cases (40 NOTCH1-mut cases, 366 NOTCH1-wt cases). The corresponding p values are reported. D) Box-and-whiskers plots showing MS4A1 transcript expression levels, as evaluated by QRT-PCR, in 52 trisomy 12 CLL cases (15 NOTCH1-mut cases, 37 NOTCH1-wt cases) and 223 non-trisomy 12 CLL cases (31 NOTCH1-mut cases, 192 NOTCH1-wt cases). The corresponding p values are reported.

To validate our results, we identified a second retrospective, unselected cohort of 197 CLL cases (27 *NOTCH1* mutated cases). CD20 expression was again determined by flow cytometry using an anti-CD20 PE-Cy7-conjugated antibody on CD19+/5+ B lymphocytes. In the validation series, the same results as above were confirmed: *NOTCH1*-mut cases were characterized by a lower CD20 expression, both in trisomy 12 CLL (mean MFI in 6 *NOTCH1*-mut cases = 12926±3676; mean MFI in 17 *NOTCH1*-wt cases = 28216±5228; p=0.027) and non-trisomy 12 CLL (mean MFI in 21 *NOTCH1*-mut cases = 10207±1310; mean MFI in 153 *NOTCH1*-wt cases = 15208±1578; p=0.017,) categories (Figure 5.4.2).

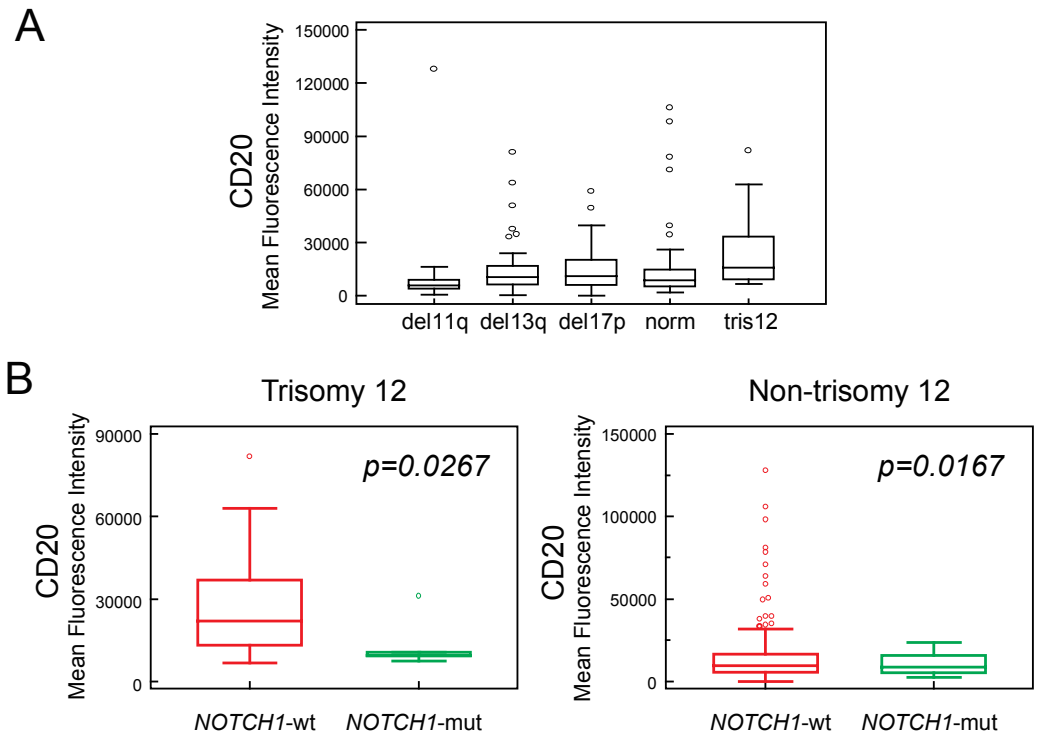

Figure 5.4.2 Correlation between *NOTCH1* mutations and CD20 expression in CLL (second series). *A) Box-and-whiskers plots showing CD20 protein expression levels in CLL cells from a series of 197 CLL cases, according to cytogenetic abnormalities, as evaluated by flow cytometry using a PE-Cy7-conjugated anti-CD20 antibody. B) Box-and-whiskers plots showing CD20 protein expression levels, as evaluated by flow cytometry with a PE-Cy7-conjugated anti-CD20 antibody, in 23 trisomy 12 CLL cases (6 NOTCH1-mut cases, 17 NOTCH1-wt cases) and in 174 non-trisomy 12 CLL cases (21 NOTCH1-mut cases, 153 NOTCH1-wt cases). The corresponding p values are reported.*

Consistently, *MS4A1* transcript levels, as evaluated in 275 cases (46 *NOTCH1*-mut), were lower in *NOTCH1*-mut than in *NOTCH1*-wt cases both in the trisomy 12 (p=0.006) and in the non-trisomy 12 (p=0.019) CLL categories (Figure 5.4.1D). In keeping with the concept of a generally common and share low CD20 expression in CLL, no difference where detected in term of number of CD20 positive cells between NOTCH1 mutated and NOTCH1 unmutated cases when this expression was evaluated by flow cytometry analysis (data not shown).

To corroborate the correlation between CD20 expression and *NOTCH1* mutations, we had the chance to perform exploratory cell sorting to isolate $CD20^{Low}$ and $CD20^{High}$ subpopulations in 3 cases with different *NOTCH1* mutational load, as determined by both qPCR and NGS, i.e. 26%, 45%, 35% of total DNA, respectively (Figure 5.4.3A). In these experiments, the $CD20^{Low}$ subpopulation showed an enrichment in *NOTCH1* mutational burden when compared to the burden of the $CD20^{High}$ subpopulation (32% vs. 15%, 38% vs. 32%, 48% vs. 39%). Consistently, expression of the *MS4A1* transcript was always significantly lower in the $CD20^{Low}$ than in the $CD20^{High}$ subpopulations (Figure 5.4.3B).

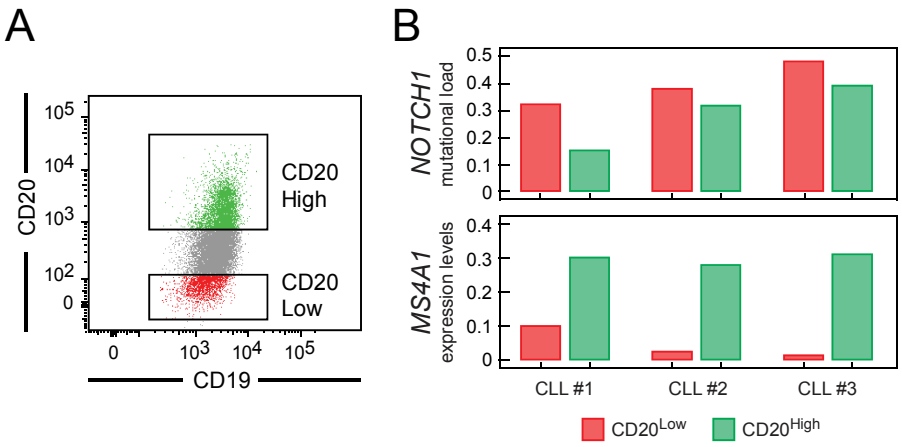

Figure 5.4.3 Correlation between *NOTCH1* mutational load and CD20 expression in CLL. *A) Illustrative dot plot of CD19 vs. CD20 identifying $CD20^{high}$ and $CD20^{low}$ sorted sub-populations according to cell sorting experiments. B) Histograms showing NOTCH1 mutational load (upper panel), as determined by NGS and expressed in percentage of NOTCH1-mut DNA, and MS4A1 transcript levels, as determined by QRT-PCR, in the $CD20^{low}$ and $CD20^{high}$ subpopulations, as obtained by performing a cell sorting in 3 NOTCH1-mut CLL cases.*

CD20-mediated immunotherapy with rituximab is strongly dependant on the surface expression levels of CD20 molecules on target cells, which affects the susceptibility of CD20+ cells to CDC and/or ADCC.[142] To evaluate the capability of the humanized anti-CD20 antibody rituximab to kill *in-vitro* CLL cells bearing or not *NOTCH1* mutations, CDC assay was performed utilizing purified CLL cells from 6 *NOTCH1*-mut cases (mutational load >10% of *NOTCH1* mutated DNA) and 7 *NOTCH1*-wt cases, randomly selected from the second CLL series. *NOTCH1*-mut CLL cells showed significantly lower relative lysis induced by rituximab than *NOTCH1*-wt CLL cells (mean % of relative lysis = 2.5±0.7 vs. 29.4±11.1, p=0.045, p=0.0449, Figure 5.4.4A), and the killing capacity of rituximab in vitro directly correlated with the levels of CD20 on the surface of the CLL cells (Figure 5.4.4B).

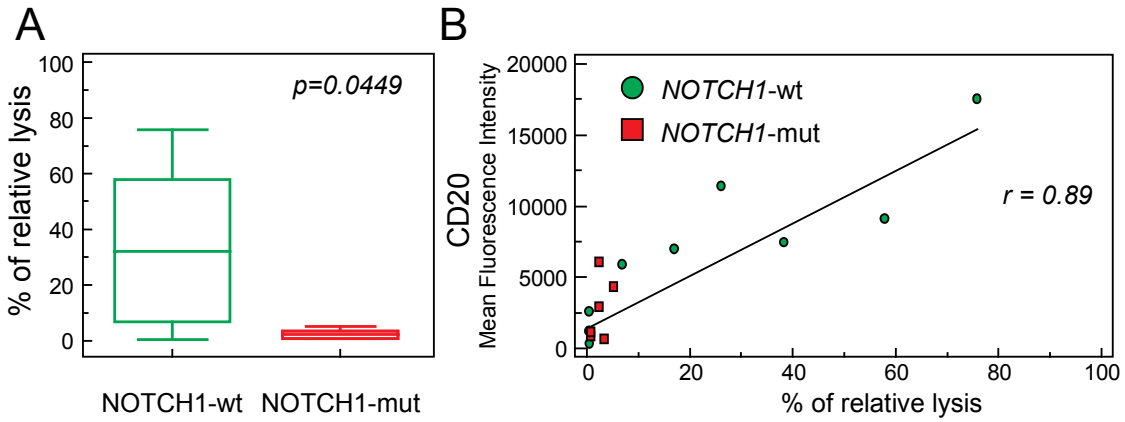

Figure 5.4.4 Correlation between *NOTCH1* mutations, CD20 expression, and susceptibility to rituximab in CLL. *A) Box-and-whiskers plots showing the percentage of relative lysis of CLL cells, from NOTCH1-mut and NOTCH1-wt CLL cases, treated with rituximab in a standard CDC assay. The corresponding p value is reported. B) Correlation graph showing CD20 expression versus percentage of relative lysis in NOTCH1-mut and NOTCH1-wt CLL cases, as evaluated by CDC assay (r= Pearson correlation coefficient).*

5.5 - Correlation between Notch signaling and CD20 expression

To verify to what extent the CD20 expression may be dependent or influenced by the Notch signaling pathway, we treated primary CLL samples with γ-secretase inhibitors (GSI), pharmacologic inhibitors of γ-secretases (see par. 1.6.2), which block proteolysis of NICD upon activation of the NOTCH1 receptor.[117;144;145]

CLL cell samples from 6 *NOTCH1* mutated and 5 *NOTCH1* wild type cases were treated with GSI, for different time points. Upon GSI treatment, NOTCH1 signaling was consistently impaired, as defined by a reduction of *HES1* expression in both *NOTCH1*-wt and *NOTCH1*-mut CLL (not shown). *MS4A1* transcript levels resulted augmented at 6h in *NOTCH1*-wt cases and in a lesser extent in *NOTCH1*-mut cases (Figure 5.5.1A). Consistently, at 24 hours, CD20 protein expression levels, as defined by flow cytometry evaluation, was augmented upon GSI treatment both in *NOTCH1*-wt and, in a lesser extent, in *NOTCH1*-mut cases, in keeping with the presence of higher levels of NICD in the latter compared to the former cases (Figure 5.5.1B-C and Figure 5.1.2).

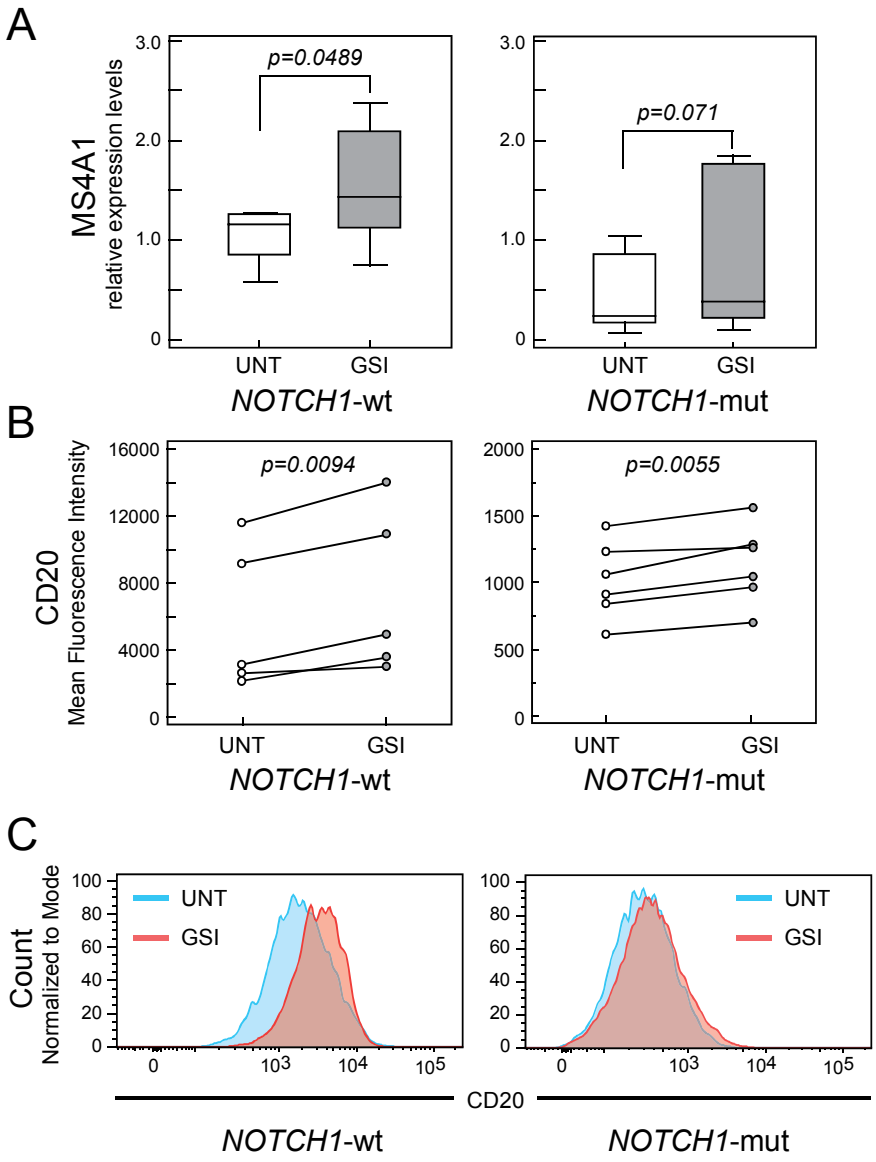

Figure 5.5.1 Induction of CD20 expression by GSI treatment in *NOTCH1* mutated and *NOTCH1* wild type CLL cases. A*) Box-and-whiskers plots showing MS4A1 transcript expression levels of CLL cell samples, untreated (UNT) and GSI treated (GSI) for 6 hours, of NOTCH1-mut and NOTCH1-wt cases, as evaluated by QRT-PCR. The corresponding p values are reported. B) Dot-and-line diagrams showing CD20 expression levels of CLL cell samples, untreated (UNT) and GSI treated (GSI) for 24 hours, of NOTCH1-mut and NOTCH1-wt cases, as evaluated by flow cytometry. The corresponding p values are reported. C) Histograms showing CD20 expression levels of CLL cell samples, untreated and GSI treated for 24 hours, of prototypic NOTCH1-mut and NOTCH1-wt cases, as evaluated by flow cytometry.*

5.6 - Establishment of an in-vitro model of NICD-transfected CLL-like cells

Chronic lymphocytic leukemia is a very heterogeneous disease (see par. 1.2 and 1.4) with a number of biological markers that might influence the behavior of the different primary samples in an ex-vivo context. To overcome this heterogeneity and investigate in detail the mechanisms trough which *NOTCH1* mutations may affect CD20 expression in CLL, we devised an in-vitro model, overexpressing the NICD to mimic protein accumulation.

To achieve this, we employed the CLL-like MEC1 cell line, which constitutively express a wild-type form of NOTCH1. MEC1 cells were stably transfected with three different GFP-fusion constructs: i) a vector encoding for the wild-type NICD fragment of NOTCH1 (NICD-wt); ii) a vector modified by inserting the c.7541-7542delCT mutation (NICD-mut); iii) as a control, a vector containing a nonsense mutation, i.e. c.5304G>A, at the beginning of the NICD coding sequence (NICD-null). After transfection, we performed single-cell sorting to obtain clonal populations.

Expression of exogenous vector was variable among the transfected clones, with an overexpression of the NICD-wt and a more discrete expression of NICD-mut and NICD-null compared to endogenous constitutive *NOTCH1* levels (Figure 5.6.1A-B). Consistently, both NICD-transfected clones were characterized by constitutive higher transcript levels of *HES1* and *HES5*, two well-characterized NOTCH1 target genes, than the null control (Figure 5.6.1C).

NICD-transfected cells were characterized by a constitutively reduced CD20 expression than NICD-null cells, at both RNA and protein level (Figure 5.6.1D). As a consequence, NICD-wt and NICD-mut cells resulted to be less sentitive to rituximab in an *in-vitro* CDC assay, showing lower relative lysis compared to NICD-null cells ($p=0.011$ and $p=0.043$ respectively, Figure 5.6.1E). Moreover, upon GSI treatment, CD20 protein and transcript expression was up-regulated both in NICD-wt and NICD-mut cells, although with increments lower than in NICD-null cells. (Figure 5.6.1F).

According to these validations, we assumed both wild type NICD-transfected and mutated NICD-transfected MEC-1 cells as useful *in-vitro* models of *NOTCH1* mutated CLL, in which the NICD accumulation due to a truncated PEST domain for the presence of the delCT mutation is mimicked by the exogenous wild type or mutated NICD overexpression.

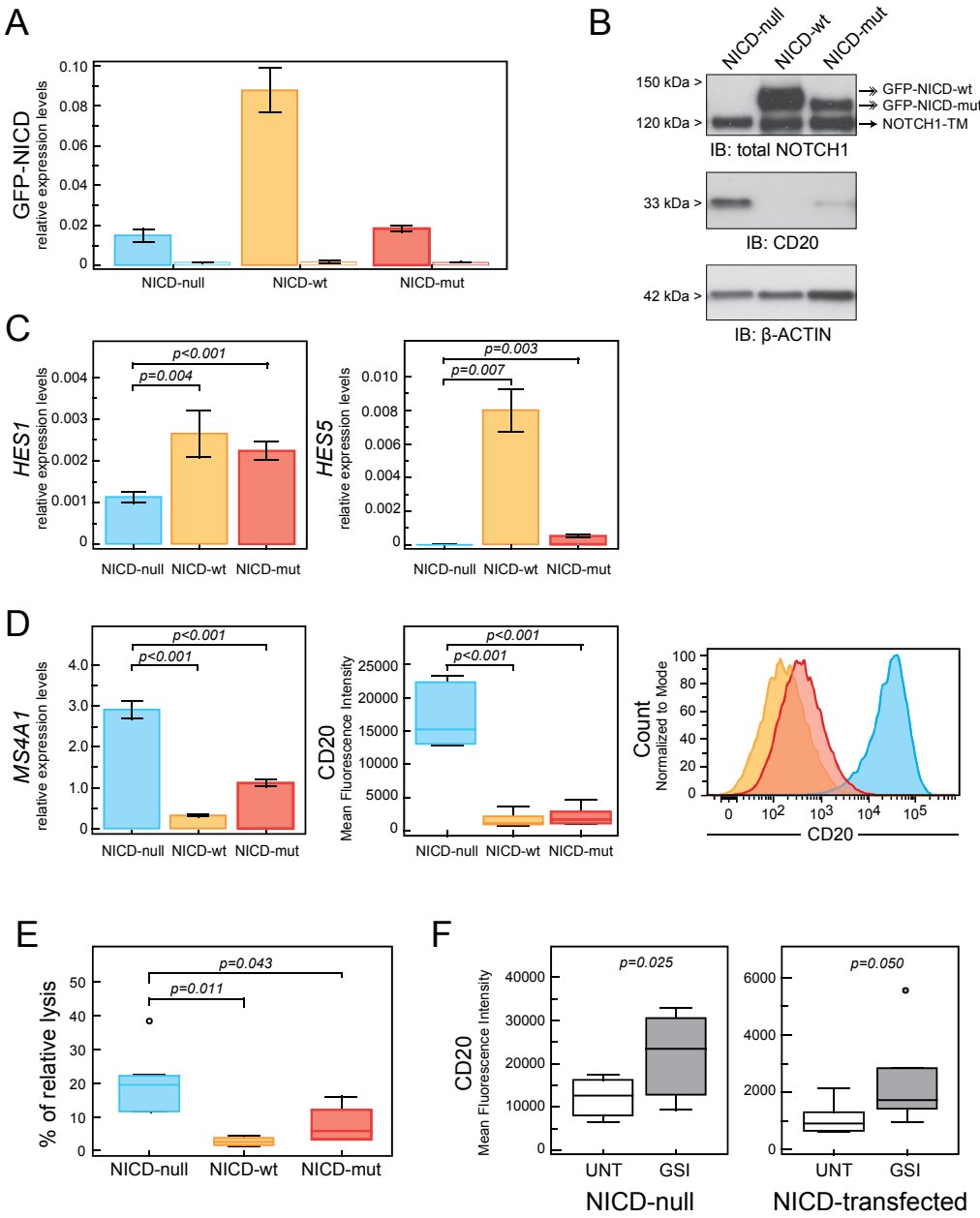

Figure 5.6.1 Establishment of an in-vitro model of NICD-transfected MEC-1 cells. *A) Constitutive transcript levels of transfected NICD (filled boxes) and endogenous NOTCH1 (empty boxes) in NICD-transfected cells, as evaluated by QRT-PCR. B) NOTCH1 and CD20 protein expression levels of NICD-transfected cells, as evaluated by WB. β-actin was used as loading control. Exogenous transfected NICD is indicated as GFP-NICD, endogenous NICD is indicated as NICD. C) Histograms showing constitutive expression levels of HES1 and HES5, as evaluated by QRT-PCR. D) Histograms (left panel) and box-and-whiskers plots (middle panel) showing constitutive MS4A1 transcript and CD20 protein expression levels, as evaluated by QRT-PCR and flow cytometry, respectively. Right panel reports a representative overlay histogram of CD20 expression in NICD-null (blue histogram), NICD-wt (yellow histogram) and NICD-mut (red histogram). E) Box-and-whiskers plots showing the percentage of relative lysis upon rituximab treatment, as evaluated by CDC assay. F) Box-and-whiskers plots showing CD20 protein expression levels in untreated (UNT) and upon GSI treatment (GSI) for 24 hours, as evaluated by flow cytometry. Results of three independent experiments are reported. The corresponding p values are reported.*

5.7 - Identification of a *NOTCH1* mutation-dependent mechanism of CD20 down-regulation via HDAC1/HDAC2 epigenetic repression

In Notch signaling, the NOTCH1 receptor, upon ligand engagement, is cleaved by γ-secretases and the NICD translocates to the nucleus (see par. 1.6.1), where it binds to the transcription factor RBPJ, which is converted from a transcriptional repressor to a transcriptional activator, displacing HDAC-containing co-repressor complexes.[103;139;146] In this context, RBPJ represents the major transcriptional regulator of Notch pathway and also a functional link between NICD and HDACs. Therefore, we aimed to investigate if NICD accumulation, as occurring upon *NOTCH1* mutations in CLL, in particular the c.7541-7542delCT mutation, could affect the balancing of two functions of RBPJ, namely the alternative formation of RBPJ/NICD and RBPJ/HDAC complexes.[103;104]

To address this issue, we performed co-immunoprecipitation experiments to detect the alternative presence of NICD or HDACs (specifically, HDAC1 and HDAC2) bound to RBPJ in NICD-transfected with respect to null-transfected cells. Interestingly, both NICD-wt and NICD-mut cells showed higher levels of NICD bound with RBPJ; on the contrary, they showed lower levels of HDAC1/2 co-immunoprecipitated with RBPJ than MEC1 cells transfected with the control vector (Figure 5.7.1A). Of note, no signficant difference in HDAC1/2 and RBPJ expression was detected between transfectants at protein levels (Figure 5.7.1B). Consistently, comparable constitutive *HDAC1/HDAC2* transcript levels were found in NOTCH1-mut versus NOTCH1-wt primary CLL (Figure 5.7.2).

This un-balancing of the transcriptional activation/repression equilibrium of RBPJ turned in favor of the activation of Notch signaling, was also in keeping with the higher transcript levels of *HES1* and *HES5* detected in NICD-producing transfectants, as described above (Figure 5.6.1C).

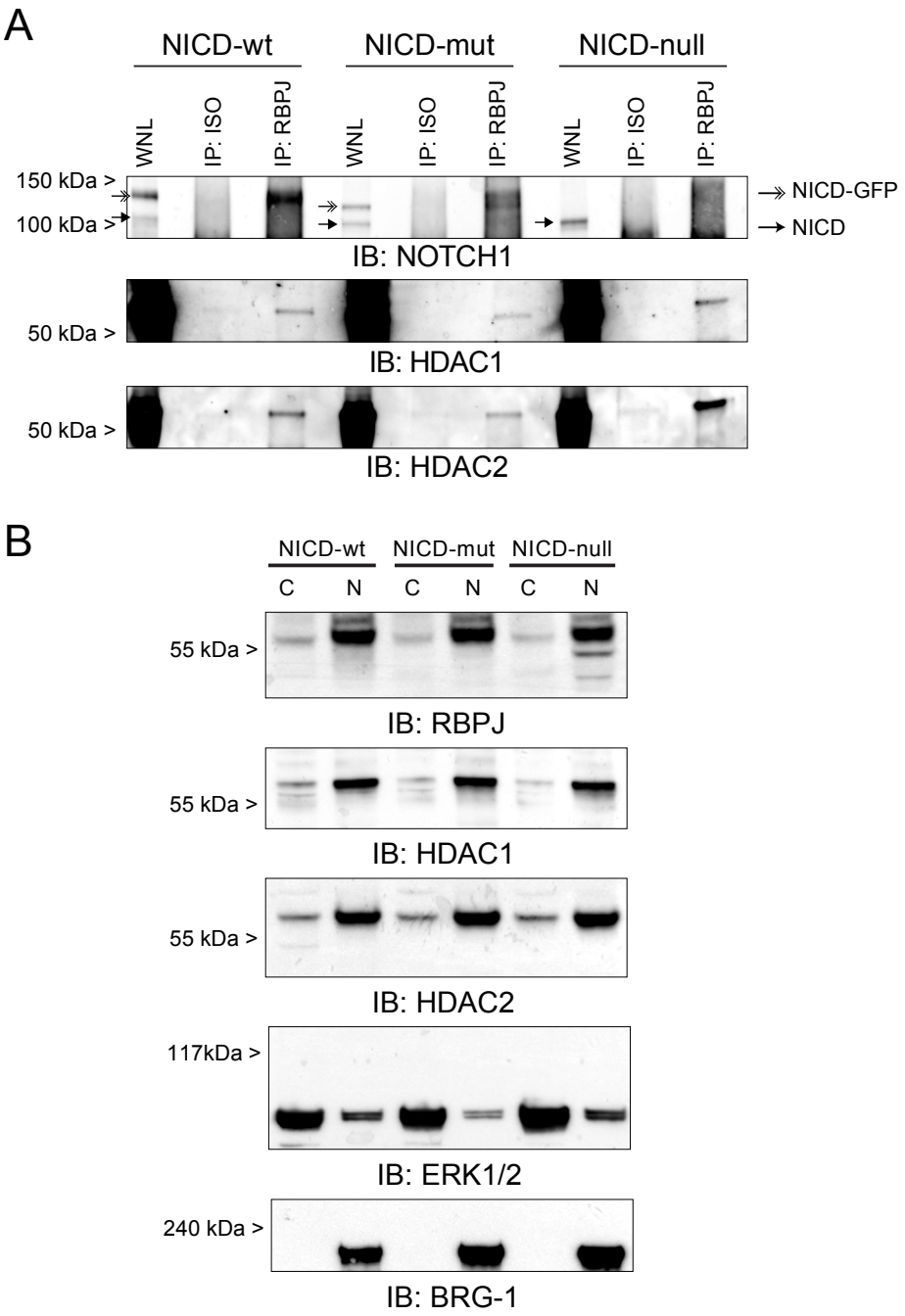

Figure 5.7.1 Co-immunoprecipitation of RBPJ with NOTCH1 or HDAC1/2. *A) Immunoblotting with antibodies recognizing the total NOTCH1 (upper panel), HDAC1 (middle panel), and HDAC2 (lower panel) in whole nuclear lysates (WNL), immunoprecipitates with isotypic control (ISO) and immunoprecipitated with RBPJ (RBPJ) derived from NICD-wt, NICD-mut and NICD-null cells. Exogenous transfected NICD is indicated as GFP-NICD, endogenous NICD is indicated as NICD. B) Immunoblotting for NOTCH1, RBPJ, HDAC1 and HDAC2 of cytoplasmic (C) and nuclear (N) lysates from NICD-wt, NICD-mut and NICD-null transfectants. ERK1/2 was used as cytoplasmic control. BRG-1 was used as nuclear control. HDAC1 and HDAC2 are equally expressed among the NICD transfectants.*

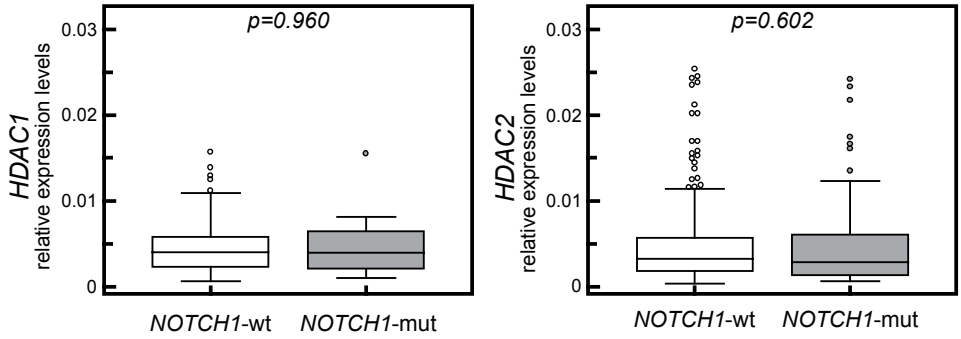

Figure 5.7.2. Constitutive HDAC1 and HDAC2 expression levels in NOTCH1-mut and NOTCH1-wt CLL cases. *Box-and-whiskers plots showing constitutive HDAC1 (left panel) and HDAC2 (right panel) transcript expression levels of 275 CLL cases (46 NOTCH1-mut and 229 NOTCH1-wt cases), as evaluated by QRT-PCR. The corresponding p values are reported.*

Previous studies identified epigenetic silencing of CD20 expression via HDACs as a mechanism confering resistance to rituximab in lymphomas.[147-149] We therefore evaluated if the preferential interaction of RBPJ with NICD could result in an increased availability of HDAC1/HDAC2 for the transcriptional repression of *MS4A1*[103;146]; we performed chromatin immunoprecipitation (ChIP) assays on nuclear lysates from NICD transfectants, to quantitatively evaluate the HDAC1/HDAC2 binding at the *MS4A1* promoter. Higher levels of DNA corresponding to the *MS4A1* promoter were found in HDAC1 and HDAC2 chromatin immunoprecipitates from NICD-producing cells compared to NICD-null cells (Figure 5.7.3A). Of note, a higher involvement of HDAC2 with respect to HDAC1 was evidenced by both co-immunoprecipitation and ChIP experiments (Figure 5.7.1A, 5.7.3). Consistent with the higher expression of *HES1* in NICD transfectants, lower levels of DNA corresponding to the *HES1* promoter were found in HDAC1/2 immunoprecipitates from NICD-producing cells (Figure 5.7.3B).

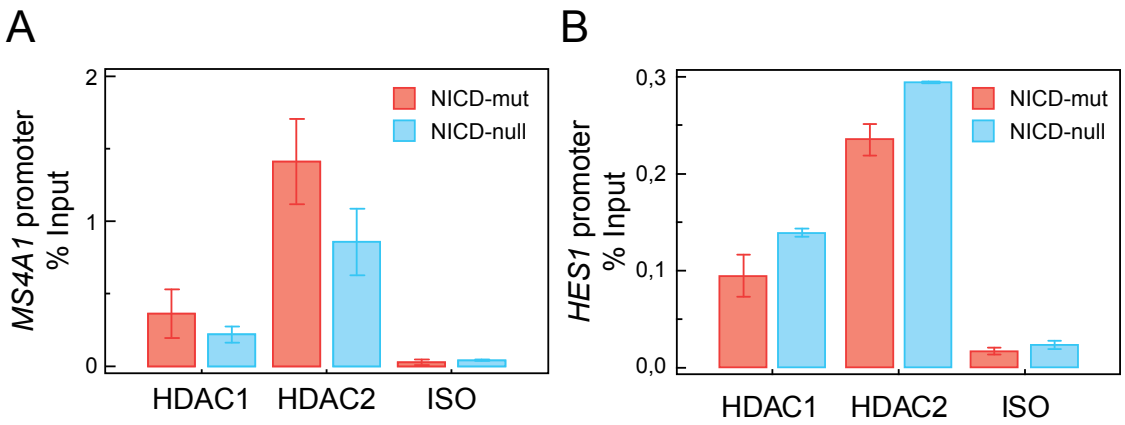

Figure 5.7.3 Chromatin immunoprecipitation of HDAC1/2. *A) Analysis of the MS4A1 promoter (upper panel) and of the HES1 promoter (lower panel) in total chromatin preparation (INPUT), and ChIP with isotypic control (ISO), antibodies recognizing HDAC1 and HDAC2, as evaluated by QRT-PCR. Results from a representative out of three experiments is reported.*

These results suggest that increased NICD levels, as occurring in NICD-producing transfectants, may trigger a NICD-dependant dislodgement of HDAC-containing repression complexes from RBPJ. This phenomenon is associated with an increased availability of HDACs to repress transcription of the *MS4A1* gene.

5.8 - Influence of HDAC1/HDAC2 activity on CD20 expression in *NOTCH1* mutated CLL

We then evaluated if the higher levels of HDAC1/HDAC2 bound to the MS4A1 promoter could effectively affect CD20 expression.

We treated NICD-transfected cells with the HDAC inhibitor Valproic Acid (VPA), which was shown to be able to augment CD20 expression in lymphomas.[149] In both NICD-mut and NICD-null cells, treatment with VPA for 48 hours was able to significantly increase *MS4A1* transcript (NICD-mut, mean fold increase =1.7, p=0.001; NICD-null, mean fold increase =1.5 p=0.003, Figure 5.8.1A) and CD20 protein expression (NICD-mut, mean fold increase =1.3, p=0.041; NICD-null, mean fold increase =1.4, p=0.029, Figure 5.8.1B) expression. Of note, in keeping with the higher HDAC1/HDAC2 levels bound on the *MS4A1* promoter, that may theoretically confer to cells higher resistance to the HDAC inhibitor treatment, NICD-producing transfectants had lower CD20 protein increases than the null control (mean ΔMFI 407, NICD-mut vs. mean ΔMFI 2202, NICD-null cells, p=0.05).

Similar results were obtained by treating with VPA primary CLL cells of 7 *NOTCH1*-mut and 7 *NOTCH1*-wt cases. In both categories, VPA treatment was able to significantly increase *MS4A1* transcripts (*NOTCH1*-mut, mean fold increase =1.5, p=0.05; *NOTCH1*-wt, mean fold increase = 1.8, p=0.02, Figure 5.8.2A) and CD20 protein (*NOTCH1*-mut, mean fold increase = 1.3, p=0.05; *NOTCH1*-wt, mean fold increase = 1.3, p=0.005, Figure 5.8.2B).

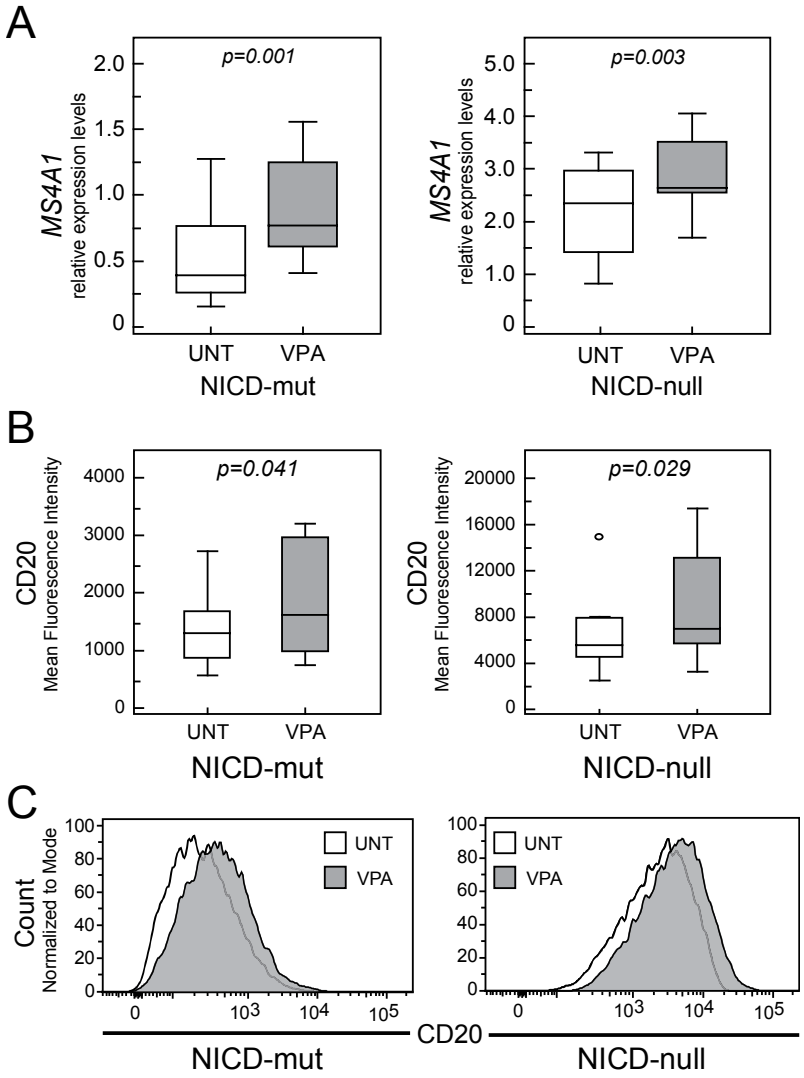

Figure 5.8.1 Induction of CD20 expression by HDAC inhibition in NICD-transfected cells. *A) Box-and-whiskers plots showing MS4A1 transcript expression levels of untreated (UNT) and VPA treated (VPA) cell samples for 48 hours of NICD-mut and NICD-null cells, as evaluated by QRT-PCR. B) Box-and-whiskers plots showing CD20 protein expression levels of NICD-mut and NICD-null cells, untreated (UNT) and VPA treated (VPA) for 48 hours, as evaluated by flow cytometry. The corresponding p values are reported. Results of three independent experiments are showed. C) Representative overlay histograms showing CD20 expression levels of NICD-mut and NICD-null cells, untreated (UNT) and VPA treated (VPA) for 48 hours, as evaluated by flow cytometry.*

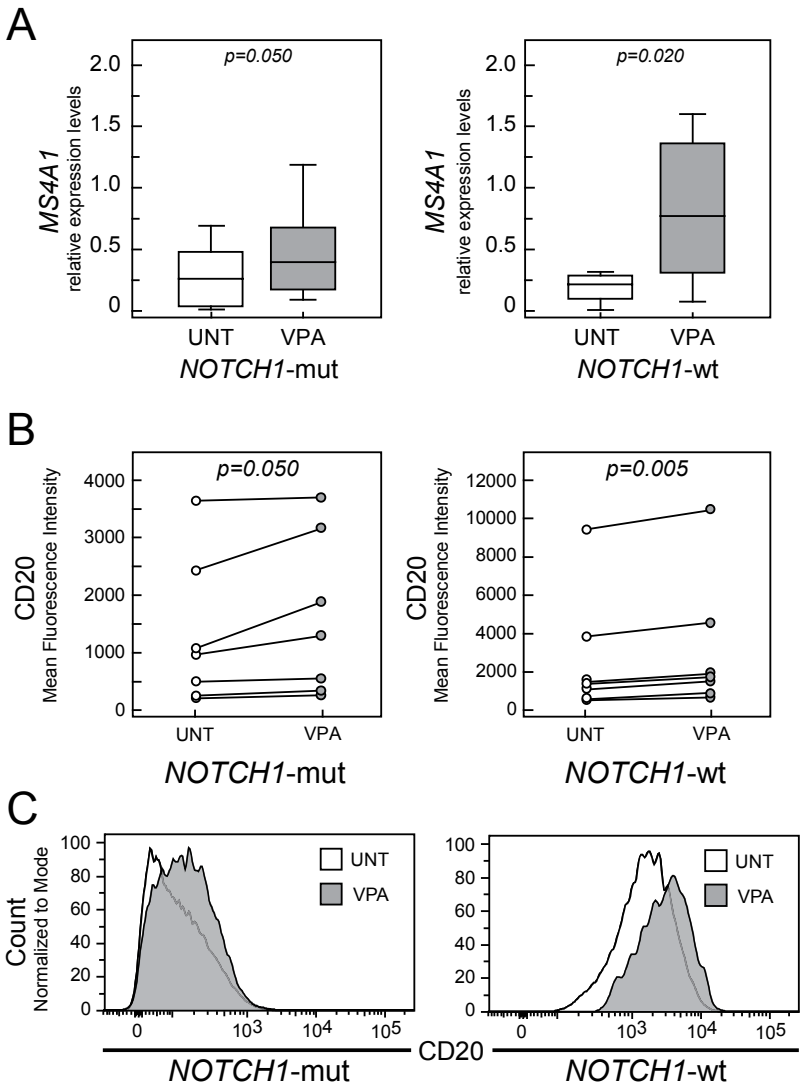

Figure 5.8.2 Induction of CD20 expression by HDAC inhibition in primary CLL cells. *A) Box-and-whiskers plots showing MS4A1 transcript expression levels of CLL cell samples, untreated (UNT) and VPA treated (VPA) for 48 hours, of NOTCH1-mut and NOTCH1-wt cases, as evaluated by QRT-PCR. The corresponding p values are reported. B) Dot-and-line diagrams showing CD20 expression levels in primary CLL cells, untreated (UNT) and VPA treated (VPA) for 48 hours, from NOTCH1-mut and NOTCH1-wt cases, as evaluated by flow cytometry. C) Representative overlay histograms showing CD20 expression levels of CLL cell samples, untreated and VPA treated for 48 hours, of prototypic NOTCH1-mut and NOTCH1-wt cases, as evaluated by flow cytometry.*

- 6 -
Discussion

Chronic lymphocytic leukemia (CLL) is a heterogeneous disease with highly variable clinical courses and survivals ranging from months to decades. Recently, it has been reported that stabilizing mutations of *NOTCH1* are recurrently associated with CLL, being identified in about 10% of CLL at diagnosis and with higher frequencies in chemorefractory CLL, CLL in advanced disease phases, and in Richter Syndrome.[119-122;150] All *NOTCH1* mutations disrupt the C-terminal PEST domain, causing an accumulation of a stabilized NOTCH1 isoform.[151]

Clinically, in consecutive CLL series, *NOTCH1* mutations turned out to be an independent predictor of overall survival, identifying a subset of high risk patients with an unfavourable prognosis similar to that associated with *TP53* disruption.[122;129-132]

Although there is an increasing number of interesting new compounds in clinical development, such as agents targeting BCR signaling or BCL2 inhibitors, that may yield high response rates even in relapsed and refractory CLL patients, immuno-chemotherapy combinations of fludarabine, cyclophosphamide and rituximab still represent the canonical regimen as frontline treatment for patients in good physical condition,[10;87] despite the relative low levels of CD20 usually expressed on the surface of CLL cells.[133;135]

By taking advantage of a large retrospective cohort of CLL cases, for a subset of which clinical data was available, we initially confirm that *NOTCH1* mutations behave as independent prognosticator, identifying a high-risk subset characterized by unfavourable prognosis and poor overall survival (par. 5.2). This observation holds true in univariate, bivariate and multivariate models, which included the main biological/molecular prognostic markers in CLL (Figure 5.2.1).[10]

We further demonstrate that the presence of *NOTCH1* mutations also identifies a CLL subset which does not benefit from addition of rituximab to chemotherapeutic treatment in the context of a maintenance therapy (par. 5.3). Specifically, in the context of consolidated patients, *NOTCH1* mutated cases experienced shorter OS and RD intervals than *NOTCH1* wild type cases, not dissimilar to those of not consolidated cases (Figure 5.3.1). These results are in keeping with those recently emerged from the CLL8 Trial,[133] where the *NOTCH1* mutated subset of CLL patients does not benefit from the addition of rituximab to chemotherapeutic treatment with fludarabine plus cyclophosphamide.

As the reasons for this different clinical behaviour remain to be elucidated, considering that the response to rituximab treatment in B cell neoplasms directly depends upon CD20 expression, [142] we investigated whether NOTCH1 mutations could affect CD20 expression in CLL.

Here we provide evidence that the presence of *NOTCH1* mutations identifies a CLL subset characterized by particularly low levels of CD20 protein and transcript, this phenomenon holding true both in non-trisomy 12 CLL, and in the trisomy 12 CLL category that usually has relatively higher CD20 levels and a higher frequency of NOTCH1 mutations (Figure 5.4.1-2).[122;126;143] This finding is consistent with a lower sensitivity of CLL cells bearing NOTCH mutations to rituximab exposure in-vitro (Figure 5.4.4).

The inverse correlation between *NOTCH1* mutations and CD20 expression was also demonstrated by cell sorting experiments of CLL cases with different *NOTCH1* mutation levels, in which higher percentages of *NOTCH1* mutated DNA were found in the sorted $CD20^{Low}$ component compared to the $CD20^{High}$ counterpart. (Figure 5.4.3). This could be also reflected in lower sensitivities to rituximab treatment in patients with higher *NOTCH1* mutational load.

Deregulated Notch signaling in the hematopoietic system has been linked to leukemias and lymphomas, from the initial evidence of an involvement of *NOTCH1* in T-ALL, to chromosomal translocations and mutations that lead to enhanced receptor cleavage or to more long-living NICD,[113] that result in an enhanced transcription of NOTCH1 target genes.[152] Although a direct association between Notch signaling and CD20 expression in CLL has not yet been documented, we defined a correlation by treating CLL cells with γ-secretase inhibitors. In particular, GSI treatment was able to substantially augment CD20 expression in *NOTCH1* wild type CLL cells, whereas increments appeared of smaller extent in *NOTCH1* mutated CLL cells that were characterized by higher NICD protein expression levels (Figure 5.1.2). These results were in keeping with the concept of an accumulation of NICD due to the presence of truncated mutations of the PEST domain in *NOTCH1* mutated CLL, for this reason less susceptible to Notch signaling perturbation. Of note, these results are relevant considering that various clinical trials are underway to investigate the possibility of targeting NOTCH1 using GSI. In particular, several GSI compounds have been already tested in Phase I studies for the treatment of several solid tumors and of other pathologies such as Alzhaimer.[144;145]

Notch signaling requires recruitment of histone acetylase complexes and exchange of histone variants to activate transcription. Moreover, NOTCH1 activity is highly sensitive to chromatin modifications and histone rearrangements that could contribute to target gene specificity.[139] The balance of histone acetylation, induced by hystone acetyl transferases (HATs), and deacetylation, induced by HDACs, represents the epigenetic mechanism of regulation of gene expression. Histone hypoacetilation by HDAC activity is associated with gene silencing. Specifically, by removal of acetyl groups from histones, HDACs create a non-permissive chromatin confirmation that prevents the transcription of genes.[153;154]

CSL proteins, represented by RBPJ/CBF-1 in humans, are DNA-binding proteins that are the main effectors of the Notch pathway. In particular, RBPJ forms a trimeric complex with the co-activator MAML and the NICD, which is essential for NICD-dependent transcription in-vitro and in-vivo. The assembly of the co-activator complex not only promotes transcription, but also results in turnover of NICD. In *NOTCH1* wild type cells, the rapidly changing levels of Notch pathway activity require that the nuclear effectors do not have a long half-life. In particular, the cyclin-dependent kinase-8 (CDK8), by phosphorilatying NICD, enables the binding with the nuclear ubiquitin ligase SEL10 which leads to degradation via ubiquitination. This interaction requires the complete C-terminal PEST region.

Destruction of NICD results in the dissociation among RBPJ, MAML and the other co-activators. In the absence of NICD, RBPJ recruits co-repressors, including SMRT and CIR, which in turn specifically recruit HDAC1 and HDAC2. The obtained complex represses Notch signaling (Figure 6.1A).[103;139;146]

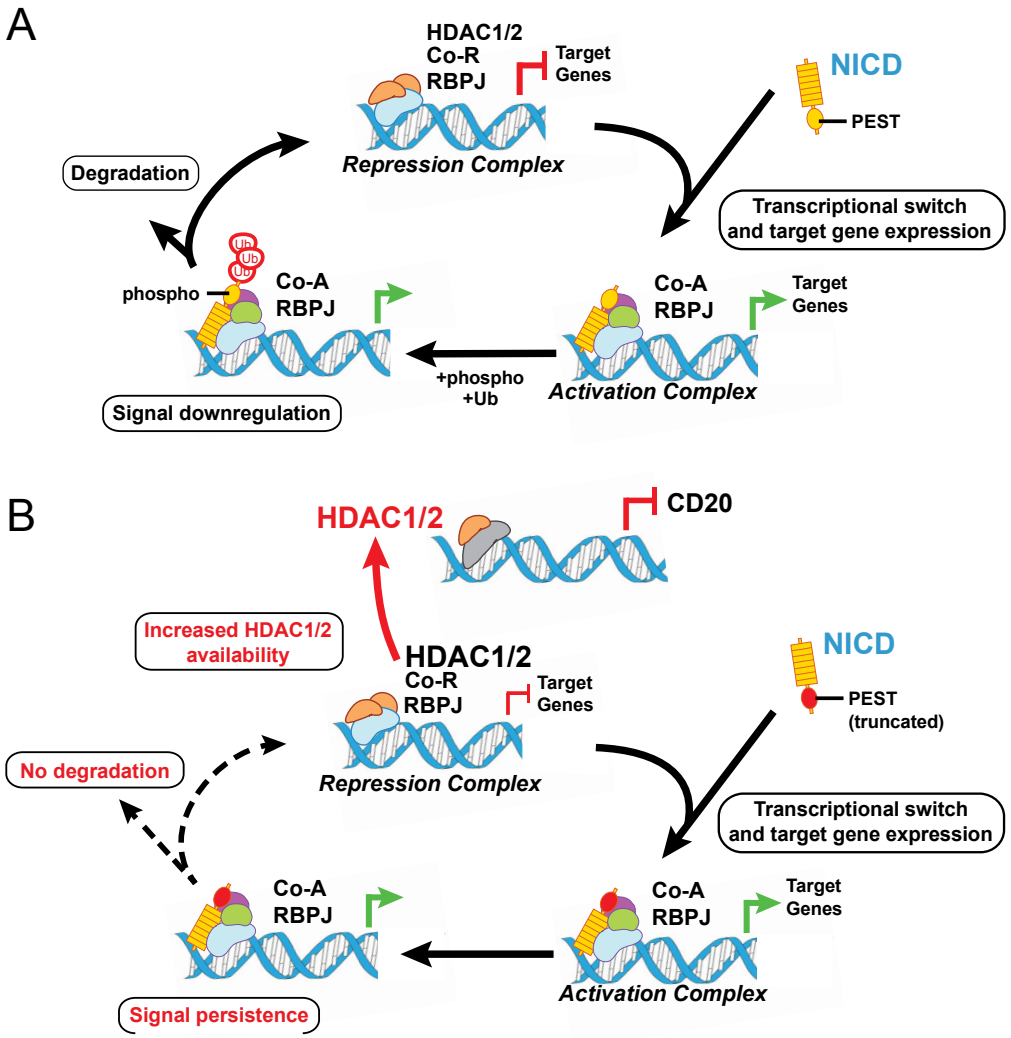

Figure 6.1 Putative model of a *NOTCH1* mutation-dependent mechanism of CD20 down-regulation via HDAC1/HDAC2 epigenetic repression in CLL. *A) Regulated balancing in NOTCH1-wt CLL. NOTCH signaling relies upon binding of NICD to RBPJ. Complex half-life is determined by the NICD degradation via ubiquitination which requires the entire PEST domain. In the absence of NICD, RBPJ recruits several corepressors, including HDACs, inhibiting NOTCH signaling. B) Dysregulated balancing in NOTCH1-mut CLL. Impaired degradation of NICD determines a more stable RBPJ/NICD complex, allowing unbound HDACs to target the CD20 promoter and impair MS4A1 transcription.*

In the present study, we showed evidences that *NOTCH1* with C-terminal truncations with impaired NICD ubiquitination and degradation, as those determined by the presence of the c.7541-7542delCT, could behave as gain-of-function alleles and influence epigenetic regulation of CD20 expression by HDAC1/HDAC2, via an un-balanced Notch signaling regulation (Figure 6.1B). In particular, wild type

NICD-transfected and mutated NICD-transfected MEC-1 cells were characterized by higher levels of NICD bound to RBPJ and lower levels of HDAC1/HDAC2 bound to RBPJ than cells transfected with the null control vector, as defined by co-immunoprecipitation experiments (Figure 5.7.1A). On the contrary, they were characterized by higher levels of HDAC1/HDAC2 bound to the MS4A1 promoter than the null control, as defined by ChIP assay (Figure 5.7.2A).

Altered expression of HDACs could play an active role in tumor onset and progression.[153;154] To restore epigenetic regulation, a wide range of compounds able to inhibit HDAC functionality have been identified, most of them commonly employed as drugs in anticancer therapies.[153;154] In particular, it has been previously shown that HDAC inhibitors augment the cytotoxic activity of rituximab by increasing CD20 expression both at transcript and protein levels, in turn enhancing the surface expression of CD20 antigen on lymphoma cells.[148] In this context, in the present study, by treating cells with the HDAC inhibitor VPA, we were able to augment CD20 expression at transcript and protein levels in *NOTCH1* wild type CLL cases. Of note, NICD-producing transfectants appeared less susceptible to the treatment, showing lower CD20 increases (Figure 5.8.1). This evidence could be related with the higher levels of HDAC1/HDAC2 bound to the MS4A1 promoter associated to the presence of a truncated *NOTCH1* mutated protein, as evidenced by ChIP experiments (Figure 5.7.2), and that may confer resistance to the HDAC inhibitor treatment.

In conclusion, in the present thesis we i) confirm that *NOTCH1* mutations are an independent prognosticator of overall survival in CLL; ii) identify a CLL subset, characterized by the presence of *NOTCH1* mutations, that do not benefit from addition of rituximab to chemotherapeutic treatment; iii) provide a proof of concept that *NOTCH1* mutations responsible for a truncated NOTCH1 protein are associated with low CD20 expression levels in CLL by a dysregulated HDAC-dependent repression mechanism. This low CD20 levels may be, in turn, responsible for the specific immunoresistance to rituximab-based treatments, such as FCR, of *NOTCH1* mutated CLL.

The data here reported confirm the role of *NOTCH1* mutations as a novel important prognostic parameter to be considered in drawing prognostic scores for CLL, as well as a putative role as an outcome predictor of anti-CD20 immunotherapy and in the perspective of identifying *NOTCH1* mutated CLL patients for specific target therapies.[151]

References

1. Chiorazzi N, Rai KR, Ferrarini M. Chronic Lymphocytic Leukemia. N Engl J Med 2005;352:804-815.
2. Inamdar KV, Bueso-Ramos CE. Pathology of chronic lymphocytic leukemia: an update. Ann.Diagn.Pathol. 2007;11:363-389.
3. Burger JA, Ghia P, Rosenwald A, Caligaris-Cappio F. The microenvironment in mature B-cell malignancies: a target for new treatment strategies. Blood 2009;114:3367-3375.
4. Messmer BT, Messmer D, Allen SL et al. In vivo measurements document the dynamic cellular kinetics of chronic lymphocytic leukemia B cells. J.Clin.Invest 2005;115:755-764.
5. Ponzoni M, Doglioni C, Caligaris-Cappio F. Chronic lymphocytic leukemia: the pathologist's view of lymph node microenvironment. Semin.Diagn.Pathol. 2011;28:161-166.
6. Hamblin T. Chronic lymphocytic leukaemia: one disease or two? Ann.Hematol. 2002;81:299-303.
7. Shanafelt TD, Geyer SM, Kay NE. Prognosis at diagnosis: integrating molecular biologic insights into clinical practice for patients with CLL. Blood 2004;103:1202-1210.
8. Landau DA, Wu CJ. Chronic lymphocytic leukemia: molecular heterogeneity revealed by high-throughput genomics. Genome Med. 2013;5:47.
9. Gaidano G, Foa R, Ia-Favera R. Molecular pathogenesis of chronic lymphocytic leukemia. J.Clin.Invest 2012;122:3432-3438.
10. Hallek M, Cheson BD, Catovsky D et al. Guidelines for the diagnosis and treatment of chronic lymphocytic leukemia: a report from the International Workshop on Chronic Lymphocytic Leukemia updating the National Cancer Institute-Working Group 1996 guidelines. Blood 2008;111:5446-5456.
11. Rai KR, Sawitsky A, Cronkite EP et al. Clinical staging of chronic lymphocytic leukemia. Blood 1975;46:219-234.
12. Binet JL, Auquier A, Dighiero G et al. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer 1981;48:198-206.
13. Montserrat E, Sanchez-Bisono J, Vinolas N, Rozman C. Lymphocyte doubling time in chronic lymphocytic leukaemia: analysis of its prognostic significance. Br.J.Haematol. 1986;62:567-575.
14. Hallek M, Wanders L, Ostwald M et al. Serum beta(2)-microglobulin and serum thymidine kinase are independent predictors of progression-free survival in chronic lymphocytic leukemia and immunocytoma. Leuk.Lymphoma 1996;22:439-447.
15. Wierda WG, O'Brien S, Wang X et al. Characteristics associated with important clinical end points in patients with chronic lymphocytic leukemia at initial treatment. J.Clin.Oncol. 2009;27:1637-1643.
16. Eichhorst B. Frontline therapy for chronic lymphocytic leukemia patients. Clin.Adv.Hematol.Oncol. 2009;7:638-641.
17. Pflug N, Bahlo J, Shanafelt TD et al. Development of a comprehensive prognostic index for patients with chronic lymphocytic leukemia. Blood 2014;124:49-62.

18. Dyer MJ, Zani VJ, Lu WZ et al. BCL2 translocations in leukemias of mature B cells. Blood 1994;83:3682-3688.

19. Dohner H, Stilgenbauer S, Benner A et al. Genomic aberrations and survival in chronic lymphocytic leukemia. N.Engl.J.Med. 2000;343:1910-1916.

20. Dal Bo M, Rossi FM, Rossi D et al. 13q14 deletion size and number of deleted cells both influence prognosis in chronic lymphocytic leukemia. Genes Chromosomes.Cancer 2011;50:633-643.

21. Pekarsky Y, Croce CM. Role of miR-15/16 in CLL. Cell Death.Differ. 2015;22:6-11.

22. Rossi D, Rasi S, Spina V et al. Integrated mutational and cytogenetic analysis identifies new prognostic subgroups in chronic lymphocytic leukemia. Blood 2013;121:1403-1412.

23. Zucchetto A, Caldana C, Benedetti D et al. CD49d is overexpressed by trisomy 12 chronic lymphocytic leukemia cells: evidence for a methylation-dependent regulation mechanism. Blood 2013;122:3317-3321.

24. Riches JC, O'Donovan CJ, Kingdon SJ et al. Trisomy 12 chronic lymphocytic leukemia cells exhibit upregulation of integrin signaling that is modulated by NOTCH1 mutations. Blood 2014;123:4101-4110.

25. Chigrinova E, Rinaldi A, Kwee I et al. Two main genetic pathways lead to the transformation of chronic lymphocytic leukemia to Richter syndrome. Blood 2013;122:2673-2682.

26. Ouillette P, Li J, Shaknovich R et al. Incidence and clinical implications of ATM aberrations in chronic lymphocytic leukemia. Genes Chromosomes.Cancer 2012;51:1125-1132.

27. Rossi D, Fangazio M, Rasi S et al. Disruption of BIRC3 associates with fludarabine chemorefractoriness in TP53 wild-type chronic lymphocytic leukemia. Blood 2012;119:2854-2862.

28. LeBien TW, Tedder TF. B lymphocytes: how they develop and function. Blood 2008;112:1570-1580.

29. Goodnow CC, Sprent J, Fazekas de St GB, Vinuesa CG. Cellular and genetic mechanisms of self tolerance and autoimmunity. Nature 2005;435:590-597.

30. Odegard VH, Schatz DG. Targeting of somatic hypermutation. Nat Rev Immunol 2006;6:573-583.

31. Fais F, Ghiotto F, Hashimoto S et al. Chronic lymphocytic leukemia B cells express restricted sets of mutated and unmutated antigen receptors. J Clin.Invest 1998;102:1515-1525.

32. Oscier DG, Thompsett A, Zhu D, Stevenson FK. Differential rates of somatic hypermutation in V(H) genes among subsets of chronic lymphocytic leukemia defined by chromosomal abnormalities. Blood 1997;89:4153-4160.

33. Schroeder HW, Jr., Dighiero G. The pathogenesis of chronic lymphocytic leukemia: analysis of the antibody repertoire. Immunol Today 1994;15:288-294.

34. Damle RN, Wasil T, Fais F et al. Ig V gene mutation status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukemia. Blood 1999;94:1840-1847.

35. Hamblin TJ, Davis Z, Gardiner A, Oscier DG, Stevenson FK. Unmutated Ig V(H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. Blood 1999;94:1848-1854.

36. Messmer BT, Albesiano E, Efremov DG et al. Multiple distinct sets of stereotyped antigen receptors indicate a role for antigen in promoting chronic lymphocytic leukemia. J.Exp.Med. 2004;200:519-525.

37. Stamatopoulos K, Belessi C, Moreno C et al. Over 20% of patients with chronic lymphocytic leukemia carry stereotyped receptors: Pathogenetic implications and clinical correlations. Blood 2007;109:259-270.

38. Chiorazzi N, Ferrarini M. B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. Annu Rev Immunol 2003;21:841-894.

39. Stevenson FK, Caligaris-Cappio F. Chronic lymphocytic leukemia: revelations from the B-cell receptor. Blood 2004;103:4389-4395.

40. Stevenson FK, Krysov S, Davies AJ, Steele AJ, Packham G. B-cell receptor signaling in chronic lymphocytic leukemia. Blood 2011;118:4313-4320.

41. Herishanu Y, Perez-Galan P, Liu D et al. The lymph node microenvironment promotes B-cell receptor signaling, NF-kappaB activation, and tumor proliferation in chronic lymphocytic leukemia. Blood 2011;117:563-574.

42. Chan AC, van Oers NS, Tran A et al. Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. J Immunol 1994;152:4758-4766.

43. Wiestner A, Rosenwald A, Barry TS et al. ZAP-70 expression identifies a chronic lymphocytic leukemia subtype with unmutated immunoglobulin genes, inferior clinical outcome, and distinct gene expression profile. Blood 2003;101:4944-4951.

44. Del Principe MI, Del Poeta G, Buccisano F et al. Clinical significance of ZAP-70 protein expression in B-cell chronic lymphocytic leukemia. Blood 2006;108:853-861.

45. Orchard JA, Ibbotson RE, Davis Z et al. ZAP-70 expression and prognosis in chronic lymphocytic leukaemia. Lancet 2004;363:105-111.

46. Zucchetto A, Bomben R, Dal Bo M et al. ZAP-70 expression in B-cell chronic lymphocytic leukemia: evaluation by external (isotypic) or internal (T/NK cells) controls and correlation with IgV(H) mutations. Cytometry B Clin.Cytom. 2006;70:284-292.

47. Rassenti LZ, Huynh L, Toy TL et al. ZAP-70 compared with immunoglobulin heavy-chain gene mutation status as a predictor of disease progression in chronic lymphocytic leukemia. N Engl J Med 2004;351:893-901.

48. Marti G, Orfao A, Goolsby C. ZAP-70 in CLL: towards standardization of a biomarker for patient management: history of clinical cytometry special issue. Cytometry B Clin.Cytom. 2006;70:197-200.

49. Howard M, Grimaldi JC, Bazan JF et al. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. Science 1993;262:1056-1059.

50. Deaglio S, Vaisitti T, Bergui L et al. CD38 and CD100 lead a network of surface receptors relaying positive signals for B-CLL growth and survival. Blood 2005;105:3042-3050.

51. Ghia P, Guida G, Stella S et al. The pattern of CD38 expression defines a distinct subset of chronic lymphocytic leukemia (CLL) patients at risk of disease progression. Blood 2003;101:1262-1269.

52. Jaksic O, Paro MM, Kardum S, I et al. CD38 on B-cell chronic lymphocytic leukemia cells has higher expression in lymph nodes than in peripheral blood or bone marrow. Blood 2004;103:1968-1969.

53. Patten PE, Buggins AG, Richards J et al. CD38 expression in chronic lymphocytic leukemia is regulated by the tumor microenvironment. Blood 2008;111:5173-5181.

54. Burger JA, Tsukada N, Burger M et al. Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1. Blood 2000;96:2655-2663.

55. Caligaris-Cappio F. Role of the microenvironment in chronic lymphocytic leukaemia. Br J Haematol 2003;123:380-388.

56. Tsukada N, Burger JA, Zvaifler NJ, Kipps TJ. Distinctive features of "nurselike" cells that differentiate in the context of chronic lymphocytic leukemia. Blood 2002;99:1030-1037.

57. Deaglio S, Vaisitti T, Billington R et al. CD38/CD19: a lipid raft-dependent signaling complex in human B cells. Blood 2007;109:5390-5398.

58. Zucchetto A, Vaisitti T, Benedetti D et al. The CD49d/CD29 complex is physically and functionally associated with CD38 in B-cell chronic lymphocytic leukemia cells. Leukemia 2012;26:1301-1312.

59. de la Fuente MT, Casanova B, Moyano JV et al. Engagement of alpha4beta1 integrin by fibronectin induces in vitro resistance of B chronic lymphocytic leukemia cells to fludarabine. J Leukoc.Biol. 2002;71:495-502.

60. Spessotto P, Cervi M, Mucignat MT et al. beta 1 Integrin-dependent Cell Adhesion to EMILIN-1 Is Mediated by the gC1q Domain. J.Biol.Chem. 2003;278:6160-6167.

61. Eksioglu-Demiralp E, Alpdogan O, Aktan M et al. Variable expression of CD49d antigen in B cell chronic lymphocytic leukemia is related to disease stages. Leukemia 1996;10:1331-1339.

62. Zucchetto A, Benedetti D, Tripodo C et al. CD38/CD31, the CCL3 and CCL4 chemokines, and CD49d/vascular cell adhesion molecule-1 are interchained by sequential events sustaining chronic lymphocytic leukemia cell survival. Cancer Res 2009;69:4001-4009.

63. Bulian P, Gaidano G, Del PG, Gattei V. CD49d expression in chronic lymphocytic leukemia: a prognostic parameter and a therapeutic target. Future.Oncol 2008;4:355-358.

64. Gattei V, Bulian P, Del Principe MI et al. Relevance of CD49d protein expression as overall survival and progressive disease prognosticator in chronic lymphocytic leukemia. Blood 2008;111:865-873.

65. Rossi D, Zucchetto A, Rossi FM et al. CD49d expression is an independent risk factor of progressive disease in early stage chronic lymphocytic leukemia. Haematologica 2008;93:1575-1579.

66. Shanafelt TD, Geyer SM, Bone ND et al. CD49d expression is an independent predictor of overall survival in patients with chronic lymphocytic leukaemia: a prognostic parameter with therapeutic potential. Br J Haematol 2008;140:537-546.

67. Bulian P, Shanafelt TD, Fegan C et al. CD49d is the strongest flow cytometry-based predictor of overall survival in chronic lymphocytic leukemia. J.Clin.Oncol. 2014

68. Villamor N, Lopez-Guillermo A, Lopez-Otin C, Campo E. Next-generation sequencing in chronic lymphocytic leukemia. Semin.Hematol. 2013;50:286-295.

69. Xu-Monette ZY, Medeiros LJ, Li Y et al. Dysfunction of the TP53 tumor suppressor gene in lymphoid malignancies. Blood 2012;119:3668-3683.

70. Pospisilova S, Gonzalez D, Malcikova J et al. ERIC recommendations on TP53 mutation analysis in chronic lymphocytic leukemia. Leukemia 2012;26:1458-1461.

71. Zenz T, Vollmer D, Trbusek M et al. TP53 mutation profile in chronic lymphocytic leukemia: evidence for a disease specific profile from a comprehensive analysis of 268 mutations. Leukemia 2010;24:2072-2079.

72. Rossi D, Fangazio M, Gaidano G. The spectrum of genetic defects in chronic lymphocytic leukemia. Mediterr.J.Hematol.Infect.Dis. 2012;4:e2012076.

73. Rossi D, Khiabanian H, Spina V et al. Clinical impact of small TP53 mutated subclones in chronic lymphocytic leukemia. Blood 2014;123:2139-2147.

74. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu.Rev.Immunol. 2009;27:693-733.

75. Hertlein E, Byrd JC. Signalling to drug resistance in CLL. Best.Pract.Res.Clin.Haematol. 2010;23:121-131.

76. Wahl MC, Will CL, Luhrmann R. The spliceosome: design principles of a dynamic RNP machine. Cell 2009;136:701-718.

77. Rossi D, Bruscaggin A, Spina V et al. Mutations of the SF3B1 splicing factor in chronic lymphocytic leukemia: association with progression and fludarabine-refractoriness. Blood 2011;118:6904-6908.

78. Messina M, Del G, I, Khiabanian H et al. Genetic lesions associated with chronic lymphocytic leukemia chemo-refractoriness. Blood 2014;123:2378-2388.

79. Cortese D, Sutton LA, Cahill N et al. On the way towards a 'CLL prognostic index': focus on TP53, BIRC3, SF3B1, NOTCH1 and MYD88 in a population-based cohort. Leukemia 2014;28:710-713.

80. Raphael B, Andersen JW, Silber R et al. Comparison of chlorambucil and prednisone versus cyclophosphamide, vincristine, and prednisone as initial treatment for chronic lymphocytic leukemia: long-term follow-up of an Eastern Cooperative Oncology Group randomized clinical trial. J.Clin.Oncol. 1991;9:770-776.

81. Lad DP, Malhotra P, Varma S. Chronic lymphocytic leukemia: inception to cure: are we there? Indian J.Hematol.Blood Transfus. 2013;29:1-10.

82. Hansen MM, Andersen E, Christensen BE et al. CHOP versus prednisolone + chlorambucil in chronic lymphocytic leukemia (CLL): preliminary results of a randomized multicenter study. Nouv.Rev.Fr.Hematol. 1988;30:433-436.

83. Flinn IW, Byrd JC, Morrison C et al. Fludarabine and cyclophosphamide with filgrastim support in patients with previously untreated indolent lymphoid malignancies. Blood 2000;96:71-75.

84. O'Brien SM, Kantarjian HM, Cortes J et al. Results of the fludarabine and cyclophosphamide combination regimen in chronic lymphocytic leukemia. J.Clin.Oncol. 2001;19:1414-1420.

85. Bosch F, Ferrer A, Lopez-Guillermo A et al. Fludarabine, cyclophosphamide and mitoxantrone in the treatment of resistant or relapsed chronic lymphocytic leukaemia. Br.J.Haematol. 2002;119:976-984.

86. Eichhorst BF, Busch R, Hopfinger G et al. Fludarabine plus cyclophosphamide versus fludarabine alone in first-line therapy of younger patients with chronic lymphocytic leukemia. Blood 2006;107:885-891.

87. Hallek M. Chronic lymphocytic leukemia: 2013 update on diagnosis, risk stratification and treatment. Am.J.Hematol. 2013;88:803-816.

88. McLaughlin P, Grillo-Lopez AJ, Link BK et al. Rituximab chimeric anti-CD20 monoclonal antibody therapy for relapsed indolent lymphoma: half of patients respond to a four-dose treatment program. J.Clin.Oncol. 1998;16:2825-2833.

89. Itala M, Geisler CH, Kimby E et al. Standard-dose anti-CD20 antibody rituximab has efficacy in chronic lymphocytic leukaemia: results from a Nordic multicentre study. Eur.J.Haematol. 2002;69:129-134.

90. Osterborg A, Dyer MJ, Bunjes D et al. Phase II multicenter study of human CD52 antibody in previously treated chronic lymphocytic leukemia. European Study Group of CAMPATH-1H Treatment in Chronic Lymphocytic Leukemia. J.Clin.Oncol. 1997;15:1567-1574.

91. Hallek M. Signaling the end of chronic lymphocytic leukemia: new frontline treatment strategies. Blood 2013;122:3723-3734.

92. Anderson MA, Huang D, Roberts A. Targeting BCL2 for the treatment of lymphoid malignancies. Semin.Hematol. 2014;51:219-227.

93. Dreger P. The evolving role of stem cell transplantation in chronic lymphocytic leukemia. Hematol.Oncol.Clin.North Am. 2013;27:355-369.

94. Aster JC, Blacklow SC, Pear WS. Notch signalling in T-cell lymphoblastic leukaemia/lymphoma and other haematological malignancies. J.Pathol. 2011;223:262-273.

95. Mirandola L, Comi P, Cobos E et al. Notch-ing from T-cell to B-cell lymphoid malignancies. Cancer Lett. 2011;308:1-13.

96. Rand MD, Grimm LM, rtavanis-Tsakonas S et al. Calcium depletion dissociates and activates heterodimeric notch receptors. Mol.Cell Biol. 2000;20:1825-1835.

97. Gupta-Rossi N, Le BO, Gonen H et al. Functional interaction between SEL-10, an F-box protein, and the nuclear form of activated Notch1 receptor. J.Biol.Chem. 2001;276:34371-34378.

98. O'Neil J, Grim J, Strack P et al. FBW7 mutations in leukemic cells mediate NOTCH pathway activation and resistance to gamma-secretase inhibitors. J.Exp.Med. 2007;204:1813-1824.

99. Artavanis-Tsakonas S, Matsuno K, Fortini ME. Notch signaling. Science 1995;268:225-232.

100. Grabher C, von BH, Look AT. Notch 1 activation in the molecular pathogenesis of T-cell acute lymphoblastic leukaemia. Nat.Rev.Cancer 2006;6:347-359.

101. Wallberg AE, Pedersen K, Lendahl U, Roeder RG. p300 and PCAF act cooperatively to mediate transcriptional activation from chromatin templates by notch intracellular domains in vitro. Mol.Cell Biol. 2002;22:7812-7819.

102. Fryer CJ, White JB, Jones KA. Mastermind recruits CycC:CDK8 to phosphorylate the Notch ICD and coordinate activation with turnover. Mol.Cell 2004;16:509-520.

103. Castel D, Mourikis P, Bartels SJ et al. Dynamic binding of RBPJ is determined by Notch signaling status. Genes Dev. 2013;27:1059-1071.

104. Hsieh JJ, Zhou S, Chen L, Young DB, Hayward SD. CIR, a corepressor linking the DNA binding factor CBF1 to the histone deacetylase complex. Proc.Natl.Acad.Sci.U.S.A 1999;96:23-28.

105. Kao HY, Ordentlich P, Koyano-Nakagawa N et al. A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. Genes Dev. 1998;12:2269-2277.

106. Oswald F, Winkler M, Cao Y et al. RBP-Jkappa/SHARP recruits CtIP/CtBP corepressors to silence Notch target genes. Mol.Cell Biol. 2005;25:10379-10390.

107. Kuroda K, Han H, Tani S et al. Regulation of marginal zone B cell development by MINT, a suppressor of Notch/RBP-J signaling pathway. Immunity. 2003;18:301-312.

108. Zhou S, Hayward SD. Nuclear localization of CBF1 is regulated by interactions with the SMRT corepressor complex. Mol.Cell Biol. 2001;21:6222-6232.

109. Sakano D, Kato A, Parikh N et al. BCL6 canalizes Notch-dependent transcription, excluding Mastermind-like1 from selected target genes during left-right patterning. Dev.Cell 2010;18:450-462.

110. Liefke R, Oswald F, Alvarado C et al. Histone demethylase KDM5A is an integral part of the core Notch-RBP-J repressor complex. Genes Dev. 2010;24:590-601.

111. Capaccione KM, Pine SR. The Notch signaling pathway as a mediator of tumor survival. Carcinogenesis 2013;34:1420-1430.

112. Ellisen LW, Bird J, West DC et al. TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. Cell 1991;66:649-661.

113. Weng AP, Ferrando AA, Lee W et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004;306:269-271.

114. Lin YW, Nichols RA, Letterio JJ, Aplan PD. Notch1 mutations are important for leukemic transformation in murine models of precursor-T leukemia/lymphoma. Blood 2006;107:2540-2543.

115. Palomero T, Lim WK, Odom DT et al. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. Proc.Natl.Acad.Sci.U.S.A 2006;103:18261-18266.

116. Hubmann R, Schwarzmeier JD, Shehata M et al. Notch2 is involved in the overexpression of CD23 in B-cell chronic lymphocytic leukemia. Blood 2002;99:3742-3747.

117. Rosati E, Sabatini R, Rampino G et al. Constitutively activated Notch signaling is involved in survival and apoptosis resistance of B-CLL cells. Blood 2009;113:856-865.

118. Di Ianni M, Baldoni S, Rosati E et al. A new genetic lesion in B-CLL: a NOTCH1 PEST domain mutation. Br.J.Haematol. 2009;146:689-691.

119. Sportoletti P, Baldoni S, Cavalli L et al. NOTCH1 PEST domain mutation is an adverse prognostic factor in B-CLL. Br.J.Haematol. 2010;151:404-406.

120. Fabbri G, Rasi S, Rossi D et al. Analysis of the chronic lymphocytic leukemia coding genome: role of NOTCH1 mutational activation. J.Exp.Med. 2011;208:1389-1401.

121. Puente XS, Pinyol M, Quesada V et al. Whole-genome sequencing identifies recurrent mutations in chronic lymphocytic leukaemia. Nature 2011;475:101-105.

122. Rossi D, Rasi S, Fabbri G et al. Mutations of NOTCH1 are an independent predictor of survival in chronic lymphocytic leukemia. Blood 2012;119:521-529.

123. Rossi D, Spina V, Bomben R et al. Association between molecular lesions and specific B-cell receptor subsets in chronic lymphocytic leukemia. Blood 2013;121:4902-4905.

124. Strefford JC, Sutton LA, Baliakas P et al. Distinct patterns of novel gene mutations in poor-prognostic stereotyped subsets of chronic lymphocytic leukemia: the case of SF3B1 and subset #2. Leukemia 2013;27:2196-2199.

125. Balatti V, Bottoni A, Palamarchuk A et al. NOTCH1 mutations in CLL associated with trisomy 12. Blood 2012;119:329-331.

126. Del Giudice I, Rossi D, Chiaretti S et al. NOTCH1 mutations in +12 chronic lymphocytic leukemia (CLL) confer an unfavorable prognosis, induce a distinctive transcriptional profiling and refine the intermediate prognosis of +12 CLL. Haematologica 2012;97:437-441.

127. Lopez C, Delgado J, Costa D et al. NOTCH1 mutations in chronic lymphocytic leukemia with trisomy 12. Genes Chromosomes.Cancer 2012

128. Arruga F, Gizdic B, Serra S et al. Functional impact of NOTCH1 mutations in chronic lymphocytic leukemia. Leukemia 2013

129. Villamor N, Conde L, Martinez-Trillos A et al. NOTCH1 mutations identify a genetic subgroup of chronic lymphocytic leukemia patients with high risk of transformation and poor outcome. Leukemia 2012

130. Willander K, Dutta RK, Ungerback J et al. NOTCH1 mutations influence survival in chronic lymphocytic leukemia patients. BMC.Cancer 2013;13:274.

131. Weissmann S, Roller A, Jeromin S et al. Prognostic impact and landscape of NOTCH1 mutations in chronic lymphocytic leukemia (CLL): a study on 852 patients. Leukemia 2013;27:2393-2396.

132. Oscier DG, Rose-Zerilli MJ, Winkelmann N et al. The clinical significance of NOTCH1 and SF3B1 mutations in the UK LRF CLL4 trial. Blood 2013;121:468-475.

133. Stilgenbauer S, Schnaiter A, Paschka P et al. Gene mutations and treatment outcome in chronic lymphocytic leukemia: results from the CLL8 trial. Blood 2014;123:3247-3254.

134. Schnaiter A, Paschka P, Rossi M et al. NOTCH1, SF3B1, and TP53 mutations in fludarabine-refractory CLL patients treated with alemtuzumab: results from the CLL2H trial of the GCLLSG. Blood 2013;122:1266-1270.

135. Matutes E, Owusu-Ankomah K, Morilla R et al. The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. Leukemia 1994;8:1640-1645.

136. Del Poeta G, Dal Bo M, Del Principe MI et al. Clinical significance of c.7544-7545 delCT NOTCH1 mutation in chronic lymphocytic leukaemia. Br.J.Haematol. 2013;160:415-418.

137. Bomben R, Dal-Bo M, Benedetti D et al. Expression of mutated IGHV3-23 genes in chronic lymphocytic leukemia identifies a disease subset with peculiar clinical and biological features. Clin.Cancer Res. 2010;16:620-628.

138. Macor P, Tripodo C, Zorzet S et al. In vivo targeting of human neutralizing antibodies against CD55 and CD59 to lymphoma cells increases the antitumor activity of rituximab. Cancer Res. 2007;67:10556-10563.

139. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat.Rev.Mol.Cell Biol. 2006;7:678-689.

140. Del Poeta G., Del Principe MI, Consalvo MA et al. The addition of rituximab to fludarabine improves clinical outcome in untreated patients with ZAP-70-negative chronic lymphocytic leukemia. Cancer 2005;104:2743-2752.

141. Del Poeta G., Del Principe MI, Buccisano F et al. Consolidation and maintenance immunotherapy with rituximab improve clinical outcome in patients with B-cell chronic lymphocytic leukemia. Cancer 2008;112:119-128.

142. Golay J, Lazzari M, Facchinetti V et al. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. Blood 2001;98:3383-3389.

143. Tam CS, Otero-Palacios J, Abruzzo LV et al. Chronic lymphocytic leukaemia CD20 expression is dependent on the genetic subtype: a study of quantitative flow cytometry and fluorescent in-situ hybridization in 510 patients. Br.J.Haematol. 2008;141:36-40.

144. Josien H. Recent advances in the development of gamma-secretase inhibitors. Curr.Opin.Drug Discov.Devel. 2002;5:513-525.

145. Wolfe MS. Secretase targets for Alzheimer's disease: identification and therapeutic potential. J.Med.Chem. 2001;44:2039-2060.

146. Hsieh JJ, Hayward SD. Masking of the CBF1/RBPJ kappa transcriptional repression domain by Epstein-Barr virus EBNA2. Science 1995;268:560-563.

147. Hiraga J, Tomita A, Sugimoto T et al. Down-regulation of CD20 expression in B-cell lymphoma cells after treatment with rituximab-containing combination chemotherapies: its prevalence and clinical significance. Blood 2009;113:4885-4893.

148. Shimizu R, Kikuchi J, Wada T et al. HDAC inhibitors augment cytotoxic activity of rituximab by upregulating CD20 expression on lymphoma cells. Leukemia 2010;24:1760-1768.

149. Sugimoto T, Tomita A, Hiraga J et al. Escape mechanisms from antibody therapy to lymphoma cells: downregulation of CD20 mRNA by recruitment of the HDAC complex and not by DNA methylation. Biochem.Biophys.Res.Commun. 2009;390:48-53.

150. Wang L, Lawrence MS, Wan Y et al. SF3B1 and other novel cancer genes in chronic lymphocytic leukemia. N.Engl.J.Med. 2011;365:2497-2506.

151. Paganin M, Ferrando A. Molecular pathogenesis and targeted therapies for NOTCH1-induced T-cell acute lymphoblastic leukemia. Blood Rev. 2011;25:83-90.

152. Weng AP, Millholland JM, Yashiro-Ohtani Y et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. Genes Dev. 2006;20:2096-2109.

153. Ropero S, Esteller M. The role of histone deacetylases (HDACs) in human cancer. Mol.Oncol. 2007;1:19-25.

154. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat.Rev.Cancer 2006;6:38-51.