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THE PIVOTAL ROLE OF NOTCH SIGNALING IN B-CELL PRECURSOR ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL) CHEMOSENSITIVITY

S.S.D. MED/15

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"What we have done for ourselves alone dies with us;
what we have done for others and the world
remains and is immortal"

Albert Pike, 1809-1891

TO WHOM IT MAY CONCERN

Sommario

Gli studi che suggeriscono che la via di segnalazione di Notch potrebbe essere coinvolta nella modulazione della risposta farmacologica nelle neoplasie ematologiche, T-LLA, B-LLC e LAM, sono in continuo aumento. Precedentemente, nel nostro gruppo abbiamo dimostrato che Notch3 e Notch4 supportavano la sopravvivenza delle cellule primarie di B-LLA, suggerendo quindi un coinvolgimento della segnalazione di Notch nella risposta ai farmaci. In questo lavoro, abbiamo adottato approcci *in vitro*, *in silico* ed *in vivo* per comprendere appieno il ruolo della via di Notch nella patogenesi della B-LLA, in termini di prognosi, proliferazione, sopravvivenza e risposta ai farmaci. Le linee cellulari di B-LLA sono state acquistate dall'ATCC, mentre le cellule primarie sono state isolate dal midollo osseo o dal sangue periferico di 51 pazienti di B-LLA. Esperimenti di citofluorimetria e western-blot hanno dimostrato che le cellule primarie, derivanti da pazienti ad alto rischio di B-LLA, presentavano una sovraespressione di Notch3, Notch4 e Jagged2, mentre si osservava una riduzione dei livelli di espressione di Notch1-4 al termine del trattamento chemioterapico, suggerendo un fondamentale ruolo della segnalazione di Notch nella B-LLA in risposta al farmaco. Abbiamo quindi analizzato la sopravvivenza cellulare *in vitro* di cellule di B-LLA trattate con agenti chemioterapici convenzionali (Citarabina, Ara-C; Desametasone, Dexa; Doxorubicina, Doxo) in singolo o in combinazione con diversi modulatori della via di segnalazione Notch, di cui anticorpi anti-Notch, gamma secretase inibitori (GSI), l'inibitore del fattore di trascrizione di Notch (SAHM1) o anticorpi bloccanti. Le GSIs e l'anti-Notch4 potenziavano la morte cellulare indotta da farmaci nelle cellule B-LLA, regolando i livelli intracellulari di specie reattive dell'ossigeno (ROS), che a loro volta erano in grado di modulare i livelli di espressione di proteine implicate nella sopravvivenza, come mTor, Akt, NFκ-B ed Erk. Dopodiché, le osservazioni *in vitro* sono state riproposte con successo in modelli di xenotrapianto murini di B-LLA, mediante l'iniezione della linea cellulare di B-LLA RS4;11 in topi NOG. La co-somministrazione *in vivo* dell'inibitore GSI-XII o dell'anti-Notch4 con l'Ara-C riduceva il carico leucemico nel midollo osseo, prolungando così la sopravvivenza dei modelli murini, rispetto al solo DMSO o all'Ara-C. Nel complesso, i nostri risultati evidenziano il valore

prognostico dell'espressione di Notch nella B-LLA, nonché il suo ruolo critico nella sopravvivenza delle cellule di B-LLA e nella risposta alla chemioterapia sia *in vitro* che *in vivo*. Abbiamo quindi dimostrato che l'inibizione della segnalazione di Notch migliora la chemiosensibilità delle cellule di B-LLA, migliorando la riduzione del carico leucemico nel midollo osseo mediata dall'Ara-C, suggerendo dunque che la segnalazione di Notch potrebbe essere una possibile strategia terapeutica per sradicare la malattia residua minima nella B-LLA.

Abstract

Growing evidence suggests that Notch signaling pathway can modulate drug response in hematological malignancies including T-ALL, B-CLL and AML. In B-ALL we have previously demonstrated that Notch3 and Notch4 support survival of primary B-ALL cells, suggesting a role for Notch signaling in drug response. Here, we used *in vitro, in silico,* and *in vivo* approaches to comprehensively the role of Notch pathway in B-ALL pathogenesis in terms of prognosis, proliferation, survival and drug response. B-ALL cell lines were obtained from ATCC, while B-ALL primary cells were isolated from bone marrow or peripheral blood of 51 B-ALL patients. Flow cytometry and western immunoblotting analyses showed that primary leukemia cells from high-risk patients overexpressed Notch3, Notch4, and Jagged2 while displaying a reduction in expression levels of Notch1-4 following chemotherapy, suggesting that Notch signaling may be critical to drug response in B-ALL. We then analyzed *in vitro* cell survival of B-ALL cells treated with conventional chemotherapeutic agents (Cytarabine, Ara-C; Dexamethasone, Dexa; Doxorubicin, Doxo) alone or in combination with Notch signaling modulators, including anti-Notch blocking antibodies, gamma secretase inhibitors (GSIs), and Notch transcription factor inhibitor (SAHM1). GSIs and anti-Notch4 were all capable of potentiating drug-induced cell death in B-ALL cells, up-regulating intracellular levels of reactive oxygen species (ROS) that were then capable to modulate pro-survival protein levels such as mTor, Akt, NFκ-B and Erk. *In vitro* observations were successfully translated in mouse-based xenograft models of B-ALL, obtained by injecting the B-ALL line RS4;11 in NOG mice. The *in vivo* co-administration of Notch inhibitor GSI-XII or anti-Notch4 with the chemotherapeutic agent Ara-C lowered bone marrow leukemic burden, thus prolonging mouse survival, compared with DMSO or Ara-C alone. Overall, our results highlighted the prognostic value of Notch expression in B-ALL as well as its critical role in B-ALL cell survival and response to chemotherapy *in vitro* and *in vivo*. We demonstrated that inhibition of Notch signaling enhances the chemosensitivity of B-ALL cells, improving Ara-C-mediated reduction of blast cells in bone marrow, suggesting that Notch signaling is a possible therapeutic strategy to eradicate the minimal residual disease in B-ALL.

Preface

The works presented in this thesis were published and reproduced/adapted with the permission of:

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Characterization of a new B-ALL cell line with constitutional defect of the Notch signaling pathway. DOI: 10.18632/oncotarget.24836

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List of Abbreviation

ABL1: Abelson murine leukemia viral oncogene homolog 1

ADAM: A Disintegrin and Metalloprotease

AKT: AKT8 virus oncogene cellular homolog

ALL: Acute Lymphoblastic Leukemia

AML: Acute Myeloid Leukemia

ANK1: Ankyrin 1

APH-1: Anterior Pharynx-defective 1

BCR: Breakpoint Cluster Region protein

Bhlh: basic helix-loop-helix protein

BM: Bone Marrow

BME: β-Mercaptoethanol

BCP: Precursor B cell

CDK: Cyclin-dependent kinases

CDKN2A/CDK2B: Cyclin-Dependent Kinase Inhibitor 2A/2B

CLL: Chronic Lymphoblastic Leukemia

CLP: Common Lymphoid Progenitor

c-MYC: Avian myelocytomatosis virus oncogene cellular homolog

CML: Chronic Myeloid Leukemia

CMP: Common Myeloid Progenitor

CNA: Copy Number Alteration

CNS: Central nervous system

CRLF2: Cytokine Receptor Like Factor 2

DNA: Deoxyribonucleic Acid

DSL: Delta/Serrate/lag-2

DUX4: Double Homeobox 4

EGF: Epidermal Growth Factor Receptor

EMT: Epithelial–Mesenchymal Transition

ER: Endoplasmic Reticulum

ERG: ETS-related gene

ERK: Extracellular signal-Regulated Kinase

ETS: Erythroblast Transformation-Specific

ETV6: Translocation-Ets-leukemia virus

FAB: French-American-British

FAK: Focal Adhesion Kinase

FAT1: FAT Atypical Cadherin 1

FISH: Fluorescence in situ hybridization

FOXO: Forkhead box

GPX3: Glutathione peroxidase 3

HIF: Hypoxia-inducible factors

HSCs: Hematopoietic stem cells

iAMP21: Intrachromosomal amplification of chromosome 21

IKZF1: Ikaros Zinc Finger Family group

ILK: Integrin-linked kinase

HDAC: Histone deacetylase

HEY: Hairy/enhancer-of-split related with YRPW motif protein

HES: Hairy and enhancer of split-1

HL: Hodgkin's lymphoma

HLF: Hepatic leukemia factor

JAK1/JAK2: Janus-family tyrosine kinase1/2

KEAP1: Kelch-like ECH-associated protein 1

LFNG: LFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase

LNK: Lymphocyte adapter protein

LNR: Lin-12/Notch Repeats

NCSTN: Nicastrin

NECD: Notch extracellular domain

NHL: Non-Hodgkin lymphoma

NLS: Nuclear localization signal or sequence

MALM1-3: Mastermind-like1-3

MAPK: Mitogen-activated protein kinase

MDM2: Mouse double minute 2 homolog

MEIS1: Meis Homeobox 1

MFNG: Mitofusin-1

MLL: Mixed lineage leukemia

MNP: Multi Nucleotide Polymorphism

MRD: Minimal residual disease

mTOR: Mammalian target of rapamycin

mTORC1: Mammalian target of rapamycin complex 1

NAC: N-Acetylcysteine

NGS: Next Generation Sequencing

NICD: Notch intracellular domain

NK: Natural killer

NRF2: Nuclear Factor, Erythroid 2 Like 2

NRR: Negative regulatory region

NR3C1: Nuclear receptor subfamily 3, group C, member 1

NOX: NADPH oxidase

PAX5: Paired box protein

PBX1: Pre-B-cell leukemia transcription factor 1

PEN-2: Presenilin enhancer 2

PI: Propidium iodide

PI3K: Phosphoinositide 3 kinase

PTEN: Phosphatase and tensin homologue deleted on chromosome 10

PTPN1: Tyrosine-protein phosphatase non-receptor type 1

p16INK4A: cyclin-dependent kinase inhibitor 2A

RAM: RBP-J-associated molecule

RAS: Retrovirus-associated DNA sequences

RB1: Retinoblastoma protein

RFNG: Radical fringe

ROS: Reactive Oxygen Species

RTK: Receptor tyrosine kinases

RUNX1: Runt-related transcription factor 1

SAHM1: Stapled α-helical peptide derived from mastermind-like 1

SF1: Splicing Factor 1

SNPs: Single nucleotide polymorphisms

TACE: Tumor necrosis factor-α-converting enzyme

TAD: Transactivation domain

TCF3: Transcription factor 3

TET2: Tet Methylcytosine Dioxygenase 2

TYK2: Tyrosine kinase 2

TXNIP: Thioredoxin-interacting protein

WHO: World Health Organization

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I. Introduction

1. Hematopoiesis and Leukemogenesis

Physiologically, the hematopoiesis is the process that define the production, proliferation, self-renewal and differentiation of blood cells, which all derived from multipotent hematopoietic stem cells (HSCs), residing at the apex of the hematopoietic system [1, 2] (**Figure I**). In response to growth factors, such as stem cell factor glycoproteins (Interleukins 1 to 7) and colony-stimulating factors, HSCs can generate and maintain in the blood, bone marrow, spleen and thymus, two differentiated lineages: the lymphoid lineage, including T-, B-, and natural killer-(NK) cells, and the myeloid lineage, consisting of erythrocytes, megakaryocytes, granulocytes, and monocyte/macrophage [3]. The lymphoid cells are part of the immune system and have the key role of controlling infection [2], while the myeloid cells have roles more heterogeneous, e.g. erythrocytes are responsible for carrying and delivering oxygen to the body organs and tissues; the megakaryocytes produce the platelets or thrombocytes, responsible for blood clotting; the myeloblast cells differentiate into four types of cells, such as neutrophils, eosinophils, basophils and monocytes, which have the capability of defending the body against infection and toxins, [4]. Conversely, in a pathological situation, leukemic transformation of an HSCs involves a disruption in the course of normal proliferation and differentiation process, the resistance to apoptotic signals, and increased self-renewal [5]. The prevalent theory of leukemogenesis is that a single hematopoietic cell, vulnerable of a mutation, that goes into an unlimited process of self-renewal resulting in malignant, poorly differentiated hematopoietic cells, called clonal origin of leukemia cell [6, 7] (**Figure I**). Leukemia cells behave oppositely than normal hematopoietic precursors, having slower cell division and longer time to produce DNA, collecting persistently in the bone marrow of leukemic patients and progressively replace hematopoietic cells [7, 8]. Eventually, this process can result in bone marrow failure, characterized by severe anemia, bleeding, and infections [7, 9].

Having to classify the different type of leukemia, firstly leukemia can be characterized as acute or chronic disease. In acute leukemia, the abnormal clonal proliferation contains very immature cells (blasts) that do not function properly. The blasts multiply quickly and the disease progresses rapidly [10]. Instead, in chronic leukemia, the blasts tend to proliferate more slowly than in acute leukemia, the abnormal cells show various levels of differentiation beyond the blast stage and may even function normally [11]. Moreover, leukemia can be divided into lymphocytic or myeloid lineage. Lymphocytic leukemia (also known as lymphoid or lymphoblastic leukemia) develops in the white blood cells called lymphocytes in the bone marrow. Myeloid (also known as myelogenous) leukemia occurs in the early stages of myeloid cells, like red blood cells, platelets, and most of the white blood cells [12]. In our case, in the next chapters we're going to pay attention on the topic of B-acute lymphoblastic Leukemia (B-ALL), an acute and lymphoid type of leukemia.

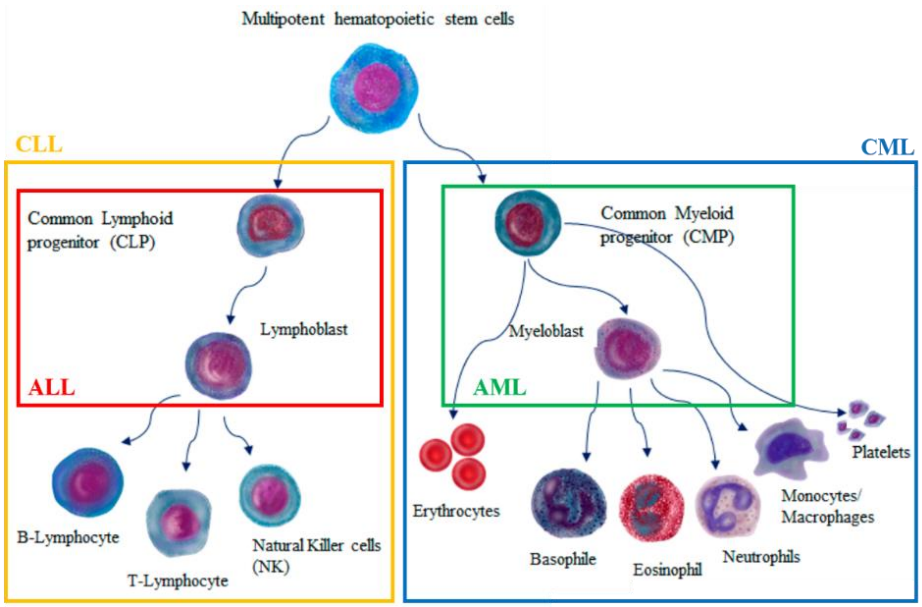

Figure I. Hematopoiesis and Leukemogenesis processes. This process leads to the formation of highly specialized circulating blood cells from HSCs in the bone marrow. Multipotent hematopoietic stem cells in BM differentiate into myeloid or lymphoid progenitor cells. Myeloid cells differentiate into red blood cells, platelets, and myeloblasts, which differentiate into basophils, neutrophils, eosinophils, and macrophages, while lymphoid cells differentiate into B and T-lymphocytes and natural killer cells (NK). Moreover, lymphoid neoplasms, i.e. Chronic Lymphoblastic Leukemia (CLL), yellow panel, and Acute Lymphoblastic Leukemia (ALL), red panel, derived from B- and T-lineage Common Lymphoid Progenitor (CLP), while myeloid neoplasms, i.e. Chronic Myeloid Leukemia (CML), blue panel, and Acute Myeloid Leukemia (AML), green panel, derived from Common Myeloid Progenitor (CMP). Modified from [13].

1.2. B-Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a hematologic malignancy in which uncontrolled proliferation of lymphoblasts (of B- or T- cell origin) occurs in the bone marrow, peripheral blood, and/or tissues [14]. Notably, B-ALL is characterized by the clonal expansion of $CD19^{+}$, neoplastic B-cell precursors at different developmental stages [15]. ALL has a bimodal incidence distribution with roughly 60% of cases diagnosed in patients younger than 20 years of age, accounting for nearly 80% of childhood leukemias [16]. About 3,000 children in the United States and 5,000 children in Europe are diagnosed with ALL each year, with the peak incidence of ALL occurs between age 2 and 5 years [14]. The estimated global incidence of B-ALL is around one to 5 per 100,000 persons per year [17] (**Figure II**). Nowadays, more than 90% of all patients obtained disease complete remission following treatment, but the prognostic for relapsed/refractory patients or adult B-ALL patients is still poor [18]. Likewise, the precise pathogenetic events leading to the development of B-ALL are unknown [7]. Only a few cases (<5%) are associated with inherited, predisposing genetic syndromes, such as Down's syndrome, Bloom's syndrome, ataxia-telangiectasia, and Nijmegen breakage syndrome, or with ionizing radiation or exposure to specific chemotherapeutic drugs [19, 20]. There are further increasing evidence on the association between overweight at birth and risk for childhood B-ALL [21]. In the last years, many other risk factors have been suggested, including parental occupation, maternal reproductive history, parental tobacco or alcohol use, maternal diet, prenatal vitamin use, exposure to pesticides or solvents, and exposure to high levels (>0.3 or 0.4 μT) of residential, power-frequency magnetic field [22, 23].

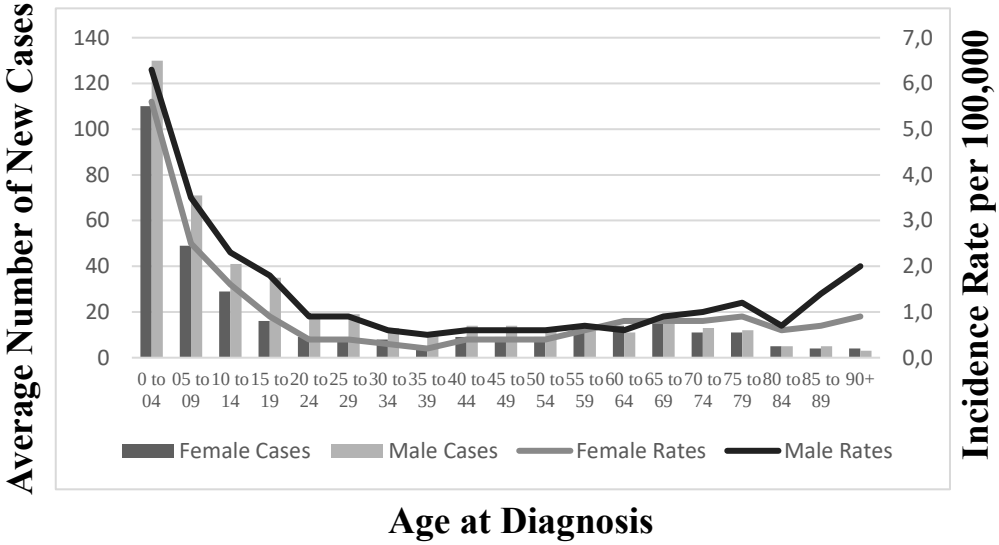

Figure II. Average Number of New Cases Per Year and Age-Specific Incidence Rates per 100,000 Population of ALL, in UK between 2014 and 2016. In the UK in 2014-2016, on average each year around 5 in 100 (6%) new cases were in people aged 75 and over. In contrast to most cancer types, acute lymphoblastic leukemia occurs most frequently at younger ages. Age-specific incidence rates are highest in infants aged 0-4 and drop sharply through childhood, adolescence and young adulthood, reaching their lowest point at age 30-34 in males and 35-39 in females, and increasing slightly thereafter. The highest rates are in the 0 to 04 age group for females and males. Incidence rates are significantly lower in females than males in a few (mainly younger) age groups. The gap is widest at age 35 to 39, when the age-specific incidence rate is 2.8 times lower in females than males.

1.2.1. Classification and Biology of B-ALL

B-ALL is a biologically heterogeneous disorder characterized by various clinical features and different cancer cells [24]. Notably, childhood B-ALL often displays significant heterogeneity in both its morphology, in the immunophenotype, in the genetic aberrations, and even in the clinical symptoms and response to therapy [24-26]. Consequently, the recent World Health Organization (WHO) on ALL recommends that the French-American-British (FAB) morphologic classification (L1, L2, L3) be abandoned (**Table I**), since this classification has no clinical or prognostic relevance [27, 28]. Contrary, WHO advocates the use of the immunophenotypic classification (**Table II**), that is an independent prognostic parameter [27]. In this case, B-ALL can be subdivided into: early pre B-ALL (also known as pro B-ALL), common B-ALL, pre B-ALL and mature B-ALL [27, 29]. Furthermore, the innovative use of cytogenetic analysis and molecular cytogenetic studies, such as fluorescence in situ hybridization (FISH), revealed recurring

chromosome abnormalities in approximately 80% of ALL, including numerical and structural changes, such as translocations, inversions, or deletions [30]. These cytogenetic abnormalities (**Table III**) are incorporated in the WHO classification of ALL revised in 2016 [27]. In this classification were further included 2 new provisional entities of B-ALL: BCR-ABL1-like B-ALL, originally reported as a subtype of poor prognosis childhood ALL with a gene expression profile similar to Philadelphia chromosome-positive ALL, and B-ALL with intrachromosomal amplification of chromosome 21 (iAMP21), detected by FISH with a probe for RUNX1, showing 5 or more copies of the gene [27]. The first type is observed in about 2-5% of children compared with about 30% of adults [31], while the second type is checked about 1.5-2% of children, although extremely rare in adults [32]. Recently, the screening of DNA sequence of specific areas of interest within the genome (targeted sequencing) has identified also recurrent mutations in B-ALL [33], demonstrating a lack large genomic instability, including tens of recurrent copy number alterations (CNAs), involving genes serving for lymphoid growth or tumorigenesis [34], such as the B-lymphoid development regulators *PAX5* and *IKZF1*, tumor suppressors such as *CDKN2A/CDKN2B* and *RB1*, and drug response-related genes like *NR3C1* [35]. Moreover, microarray analyses found alterations involving the *CRLF2* gene, demonstrating its overexpression in one-third of BCR-ABL1-like cases and more than half of ALL patients with Down syndrome. Frequently, this alteration appears concomitantly with gain-of-function mutations of *JAK1* or *JAK2* genes [36, 37]. Another common mutation in B-ALL is the MLL-rearranged (Mixed-lineage leukemia–rearranged) leukemia [38]. It is a unique entity notable for initiation in utero, myeloid and lymphoid features, and poor responsiveness to therapy [38]. Fortunately, nowadays most laboratories around the world are being equipped with next-generation sequencing (NGS), favoring the increase in the number of studies involving large patient cohorts and leading to the discovery of new targets and new molecular entities [34]. The most recent main studies concerning of NGS applications in B-ALL demonstrated: about 90% of 93 cases harbored at least one mutation among *FAT1*, *SF1*, *CRLF2*, *TET2*, and *PTPN1* genes correlated to a better survival [39]; the deregulation of *DUX4* and *ERG* genes in up to 7% of B-ALL, associated with a favorable outcome [40]; Fischer et al.

found an association of *TCF3-HLF*−fusion with *PAX5* haploinsufficiency and enrichment in stem cell and myeloid expression signatures [41]; about one-third of infant BCP-ALL presents high hyperdiploidy with more than 50 chromosomes, demonstrating the involvement of the *RTK-RAS* pathway and histone modifiers, with no observation of recurrent fusion genes [42].

Here below we will discuss some of these molecular abnormalities and their contribution to B-ALL.

Morphologic Classification	
FAB Type	**Feature of Blasts**
L1	Small cells with scant cytoplasm; nucleoli indistinct and not visible
L2	Large heterogeneous cells with moderately abundant cytoplasm; clefting and indentation of nucleus; large and prominent nucleoli
L3	Large cells with moderately abundant cytoplasm; regular, oval-to-round nucleus; prominent nucleoli; prominent cytoplasmic basophilia and cytoplasmic vacuoles

Table I. The French-American-British (FAB) classification. The older, traditional classification of acute lymphoblastic leukemia (ALL) is the French-American-British (FAB) classification. This has now been replaced by the newer World Health Organization.

Markers	**Pro B-ALL**	**Common B-ALL**	**Pre B-ALL**	**Mature B-ALL**
TdT	++	++	++	++
CD10	-	++	++	++
CD19	++	++	++	++
CD20	-	+	+	+
CD22	++	++	++	++
CyCD79	++	++	++	++
CyIgm	-	-	++	++
SmVpre-B/15	-	-	-	++
SmIg-CD79	-	-	-	++
CD34	+	+	+	+
HLA-DR	++	++	++	++

Table II. Immunophenotypic classification of precursor B-ALL. The morphological appearance taken into consideration, surface markers on the leukemic cells determine the phenotype of leukemia. Modified from [48].

WHO classification of B-acute lymphoblastic leukemia with recurrent genetic abnormalities	
Genetic abnormalities	**Involved-Genes**
Hypodiploidy	-
Hyperdiploidy	-
t(9;22)(q34;q11.2)	[BCR-ABL1]
t(v;11q23)	[MLL rearranged]
t(12;21)(p13;q22)	[ETV6-RUNX1]
t(1;19)(q23;p13.3)	[TCF3-PBX1]
t(5;14)(q31;q32)	[IL3-IGH]
Intrachromosomal amplification of chromosome 21	iAMP21
Translocations involving tyrosine kinases or cytokine receptors	[BCR-ABL1]

Table III. World Health Organization (WHO) classification of B-ALL from revision of 2016. In 1997, the World Health Organization proposed a composite classification in attempt to account for morphology and cytogenetic profile of the leukemic blasts and identified three types of ALL: B lymphoblastic, T lymphoblastic and Burkitt-cell Leukemia.[19] Later revised in 2008, Burkitt-cell Leukemia was eliminated as it is no longer seen as a separate entity from Burkitt Lymphoma, and B-lymphoblastic leukemia was divided into two subtypes: B-ALL with recurrent genetic abnormalities and B-ALL not otherwise specified. B-ALL with recurrent genetic abnormalities is further delineated based on the specific chromosomal rearrangement present. In 2016, two new provisional entities were added to the list of recurrent genetic abnormalities and the hypodiploid was redefined as either low hypodiploid or hypodiploid with TP53 mutations. [27]

1.2.1.1. Mutations in signaling pathway components

Genetic events that give rise to leukemic transformation occur inside components of tyrosine kinase (*RTK*) signaling pathways or/and other signaling pathway components. In fact, several numbers of genes encoding signaling pathway components including *RAS*, *JAK1/JAK2*, *CRLF2*, *PTPN1*, *FAT1* are recurrent mutation targets in B-ALL (**Table IV, Figure III**).

- **RAS**

In physiological conditions of bone marrow, RAS signaling pathway is fundamentally involved and controls the essential steps of primitive and definitive blood-cell production, regulating hematopoiesis, erythropoiesis, myelopoiesis, thrombopoiesis, the formation of monocytic and lymphocytic lineages [43]. Whereas, the expression RAS signaling receptors is modified in the bone marrow following cellular maturation or an injury, ligation of RAS signaling receptors has been shown to modify the status of the bone marrow resulting in accelerated hematopoiesis after injury [44]. Consequently, this mechanism involves that activating mutations of RAS signaling are among the most frequent mutations in cancer, initially reported in about 15% of pediatric BCP-ALL [43, 44].

Nevertheless, the clinical significance of RAS mutations is still debated, and it is unknown whether the recent inclusion of minimal residual disease (MRD) levels as risk criterion influences the prognostic effect of RAS pathway mutations in contemporary protocols [45]. Therefore, recent studies found that even mutations in *NRAS*, *KRAS*, *FLT3* and *PTPN11* are more frequently observed at relapse (34–38%), and in part confer a poor prognosis [46-48]. Recently, Jerchel IS and co-workers demonstrated that given that clonal mutations at initial diagnosis were retained at relapse and that subclonal mutations often expanded at relapse, RAS pathway mutations may serve as a biomarker to identify patients eligible for *MEK/ERK* targeted therapy [45]. Furthermore, they concluded by suggesting that the synergistic effect between MEK inhibition and prednisolone may be of additional advantage in the treatment of B-ALL [45].

- **JAK1/JAK2**

JAK1 is a critical effector of proinflammatory cytokine signaling and plays important roles in immune function, so that abnormal JAK1 activity has been linked to immunological and neoplastic diseases, while specific functions of JAK1 in the context of hematopoiesis, and specifically within HSCs, have not clearly been delineated [49]. Nevertheless, one study suggests that mice with conditional deletion of *JAK1* in the hematopoietic system are characterized by leukocytosis, reduced spleen and thymus weights [50]. *JAK1* and *JAK2* gain of function mutations are found also in human hematological malignancies, favoring the validation of the JAK-inhibitors, used clinically to treat various types of leukemia and myeloproliferative neoplasms (MNPs) [51]. However, as these studies highlight, unexpected side effects from long term profound JAK1 inhibition may occur and could even result in stem cell failure if these results in mice were extrapolated to humans [52-54]. A recent clinical trial of Phase 2 Study is systematically investigating key questions of whether the addition of a JAK-inhibitor Ruxolitinib with chemotherapy in children, adolescents, and young adults BCR-ABL1-like can decrease relapse and improve survival of these patients.

- **CRLF2**

CRLF2 is a member of type I cytokine receptor family, that forms a functional complex with IL-7 receptor α chain and thymic stromal lymphopoietin, inducing the activation of signal transducers and activators of transcription proteins [55]. *CRLF2* rearrangement is found in approximately 50% of pediatric Philadelphia-like B-cell acute lymphoblastic leukemia (Ph-like B-ALL), and around 50% of *CRLF2+* cases harbor *JAK* mutations [55]. Having said that, *CRLF2* rearrangement in B-ALL could further facilitate the *JAK2* mutation assay for targetable therapy [55].

- **PTPN1**

PTPN1 is a tyrosine phosphatase implicated as a negative regulator of the insulin pathway and removes phosphate molecules from activated insulin receptor kinase [56]. Besides, PTPN1 regulates the activity of a variety of other kinases implicated in a variety of cellular contexts including EGFR, JAK2, TYK2, and FAK, and it has been associated with the regulation of several cellular processes including invasion, cytokine sensitivity, cell adhesion, and proliferation [56]. Contrary, loss of *PTPN1* in preclinical studies leads to increased JAK-STAT pathway activity in hematopoietic cells *in vitro*, while a deletion of *PTPN1* in mice results in a hematopoietic malignancy [57]. As inactivation mutations in *PTPN1* are restricted to distinct subsets of leukemia and lymphoma, a future challenge will be to identify in which cellular contexts do they contributing to the initiation or maintenance of leukemogenesis or lymphomagenesis.

- **FAT1**

The cadherin gene *FAT1*, located on chromosome 4q34-35 within a region frequently deleted in human cancers, encodes a large protein with 34 extracellular cadherin repeats [58]. Although the gene was originally cloned from a human T-cell acute lymphoblastic leukemia (T-ALL) cell line, *FAT1* just recently gained

interest owing to its altered gene expression levels and the detection of somatic mutations identified by next-generation sequencing (NGS) even in B-ALL [39]. Recently, de Bock CE and colleagues identified *FAT1* cadherin as a unique and independent prognostic factor for relapse-free and overall survival in pediatric pre-B-ALL, underlining *FAT1* in future studies as a new MRD marker [59].

1.2.1.2. Mutations in transcription factors

Lymphopoiesis is orchestrated by a small number of transcription factors, which are subjected to mutation or molecular rearrangement in B-ALL [39, 41]. Among them *IKZF1*, *ETV6* and Runt-related transcription factor 1 (*RUNX1***),** steroidogenic factor 1 (*SF-1*), Double Homeobox 4 (*DUX4*) and *ERG*, *TCF3* and *PAX5* (**Table IV, Figure III**).

- **IKZF1 alterations**

IKZF1 is a member of the Ikaros family and is mainly expressed in the hematopoietic and lymphopoietic system [60]. IKZF1 is required for differentiation and maturation of B- and T-cells, possibly by involvement in the rearrangement of immunoglobulins and immune receptor genes [60]. Given the diverse roles of Ikaros in hematopoiesis and the growing list of key developmental genes that are co-regulated by Ikaros, it is not surprising that loss of Ikaros function has been observed in human hematopoietic malignancies [61]. The first description of alterations in Ikaros function in human disease was reported by Lei Sun and colleagues, showing aberrant expression of Ikaros isoforms in infant B-ALL [62]. Besides, deletions in *IKZF1* with the consequent expression of DN protein isoforms found in B-cell precursor ALL correlate with an inherent resistance to current therapeutic strategies and increased risk of poor outcomes, in about a quarter of high-risk pediatric B-ALL cases [63]. Therefore, several groups have described the increased DN Ikaros isoforms in B-ALL to coincide with expression of the Philadelphia chromosome, a translocation of chromosomes 9 and 22 resulting in constitutive activation of the ABL tyrosine kinase [64-66]. Additionally, Iacobucci

I and colleagues suggested that the increased expression of the DN Ikaros isoforms in 49% of adult Philadelphia chromosome-positive (Ph+) ALL cases is linked with resistance to targeted therapy with tyrosine kinase inhibitors [64]. Some research groups are focusing to understand the mechanisms involved downstream the DN Ikaros isoforms, such as experimental models of Ikzf1 mutated pre-B cells that favor the upregulation of pro-survival signaling pathways such as JAK-STAT, as well as increased expression of anti-apoptotic proteins such as Bcl-xL and Bcl-2 [67, 68]. Overall, the clarification of Ikaros function should provide an in-depth view of its role in normal hematopoietic regulation, as well as guide therapeutic choices in malignant tumors characterized by loss of Ikaros function.

- ETV6/RUNX1 fusion gene

RUNX1 is a Runt family transcription factor critical for normal hematopoiesis [69]. Consequently, it is easy to understand that one of the most frequent chromosomal lesions on pediatric B-ALL is t(12; 21)(p13; q22), which results in its molecular genetic counterpart, the *ETV6/RUNX1* (also known as *TEL/AML1*) fusion gene [69]. This alteration occurs in approximately 25% of childhood ALL diagnosed between the ages of 2 and 10 years, with a median age of 4 years [70]. The *ETV6/RUNX1* fusion gene results in the generation of a persistent pre-leukemic clone, which postnatally converts, at low frequency, to ALL after the acquisition of necessary secondary genetic abnormalities [70]. Although multiple studies have identified a variety of second genetic hits, their correlation with the *ETV6/RUNX1* fusion gene and their effects on the pathogenesis of leukemia need to be further explored [70, 71]. Given that the *ETV6/RUNX1* fusion gene drives not only the leukemic transformation process but also the maintenance and propagation of leukemia cells, the specific components of affected signaling pathways and epigenetic regulators, constitute ideal therapeutic targets. For instance, inhibitions of the aberrantly activated PI3K/AKT/mTOR pathway, STAT3 signaling and/or MDM2/P53 interaction represent promising therapeutic strategies [72, 73].

- DUX4 rearrangement and ERG deregulation

DUX4 is a powerful transcriptional regulator with an unknown still physiological role in normal cells [74]. *In vitro*, full-length *DUX4* is expressed in human embryonic, mesenchymal stromal cells and induced pluripotent stem cells [74]. Whereas, *ERG* is a member of the ETS family of transcription factors, with a role in hematopoiesis stem cell maintenance [75]. Several studies demonstrated that *ERG* has an impact on the characteristics of leukemia development/maintenance in several settings [76-78], it is frequently overexpressed in human AML and T-ALL and it is associated with poor outcome in these types of leukemia [79]. Indeed in 2007, Charles G. Mullighan and co-worker demonstrated that the *ERG* deletions resulting in enhanced transcriptional activity, defining a novel subtype of B-Progenitor Acute Lymphoblastic Leukemia [80]. Recently, Jinghui Zhang and colleagues performed expression profiling and DNA copy-number analysis on 1,913 patients with B-ALL combined with whole-genome, whole-exome and RNA sequencing in a subset, demonstrating that transcriptional deregulation of *ERG* was observed in all *DUX4*-rearranged cases with identification of a noncanonical aberrant ERG transcript resulting in a truncated protein [81]. Seeing as, some studies suggested that *ERG* transcript retained DNA binding activity and acted as a competitive inhibitor of wild-type ERG, promoting lymphoid leukemogenesis in mice [81], this finding may allow for improved risk stratification and guide therapy in patients with B-ALL, as patients with this form of leukemia have an excellent prognosis.

- PAX5 rearrangement in TCF3-PBX1

TCF3 is a transcriptional activator widely expressed and influential in diverse cellular processes, as well as critical in lymphocyte development, precisely in the normal B-cell hematopoiesis is essential [82]. The oncogenic TCF3-PBX1, also known as E2A-PBX1, fusion gene results from a translocation between chromosomes 1 and 19 in pre-B-cell acute leukemia (pre-B-ALL) [83] . Whereas, *PAX5* is a transcription factor that is expressed exclusively in cells of the B-cell lineage [84]. It plays hence a crucial role in B-cell development and commitment

of the bone marrow multipotent progenitor cells to the B-lymphoid lineage [84]. Especially since, antibodies to PAX5 are used for the diagnosis of lymphoid malignancies, particularly precursor B-ALL [85]. Nevertheless, a genome-wide analysis using oligo SNP arrays recently demonstrated that *PAX5* (paired-box domain 5) is the main target of somatic mutations in childhood BCP-ALL being altered in 38.9% of the cases [86]. *PAX5* alterations are heterogeneous consisting of complete loss in 17%, focal deletions in 10%, point mutations in 7% and translocations in 1% of the cases [86]. *PAX5* complete loss and *PAX5* point mutations differ [86]. Familiades J et colleagues published "a GRAALL study", showing that *PAX5* deletions are highly skewed toward BCR-ABL1 and *TCF3-PBX1* fusion genes, occurring in 71% of patients in deleted *PAX5* compared to 22% in normal *PAX5* and 29% in mutant *PAX5* [87]. It suggests a very important role of *PAX5* dosage during the transformation process of these two oncogenes [87]. Furthermore, in 2018 Thayana Conceição Barbosa and colleagues reported a case report of a 16-year-old boy identified a novel *PAX5* rearrangement in *TCF3-PBX1* in B-ALL [88].

Disease	Abnormality	Gene involved	Incidence
B-ALL	t(9;22)(q34;q11)	BCR ABL	Adult: 30% Children: 3%
	t(12;21)(p13;q22)	TEL AML1	Adult: <1% Children: 20%
	t(4;11)(q21;q23)	MLL AF4	Adults: 5% Infants: 60%
	t(1;19)(q23;p13)	E2A PBX1	5%
	t(8;14)(q24;q32)	c-MYC IgH	1%
	t(17;19)(q22;p13)	E2A HLF	<1%
	t(11;19)(q23;p13)	MLL ENL	<1%
		JAK1/2/3 mutations	10%

Table IV. The main genetic abnormalities in ALL. Identification of recurring cytogenetic abnormalities and molecular alterations in ALL has had a major impact on risk assessment and several structural and chromosomal changes have been incorporated into existing classification systems. Modified from [29].

1.2.1.3. Epigenetics modifier mutations

Aberrant promoter methylation and histone modifications have been shown to play a role in B-ALL pathophysiology. Moreover, some studies have established a relationship between the epigenetics signatures with defined genomic rearrangement in B-ALL [89, 90]. In B-ALL mutations have been found in epigenetics actors such as *MLL*, *DNMT3A*, *TET2* genes etc [89]. It is proposed that these genes could be in pre-leukemic hematopoietic stem cells and occur early in the evolution of B-ALL (**Table IV, Figure III**).

- MLL mutations

The *MLL* (Mixed Lineage Leukemia) gene encodes a protein that plays an essential role in early development and hematopoiesis by acting as a histone methyltransferase and transcriptional co-activator, but also a common target for chromosomal translocations associated with human acute leukemia [91]. Rearrangements of the *MLL* gene at 11q23 occurs in at least two-thirds of infants with ALL, less frequent than in older individuals [92, 93]. This rearrangement known also as t(4;11)(q21;q23)/MLL-AFF1(AF4), occurs where physiologically *MLL* gene regulates hematopoiesis through the maintenance of normal homeotic gene expression [92, 93]. Of all patients treated with topoisomerase II inhibitors, between 2 and 12% go on to develop secondary leukemia, reporting a smaller number of cases of ALL compared to AML [94, 95]. This therapy-related leukemia secondary also harbors *MLL* translocations in at least 70% of cases [94, 95].

- TET2

The Tet methylcytosine dioxygenase enzymes (TET1-3) mediate active DNA demethylation of cytosines in CG dinucleotides [96]. Besides, *TET2* is the most expressed *TET* gene in the hematopoietic tissue, especially in hematopoietic stem cells [96]. Loss-of-function mutations of *TET2* have been found in patients with a wide range of hematological diseases, including mature B-ALL (2%) [97]. More

recently, *TET2* mutations were also identified in mantle cell lymphomas and in diffuse large B-cell lymphoma (12%) in which they were associated with an altered DNA methylation pattern of genes involved in hematopoietic development [98]. Clinically, targeting aberrant DNA methylation using hypomethylating agents is an alternative to conventional chemotherapy, modulating the decreased TET2 activity in the transformed cells.

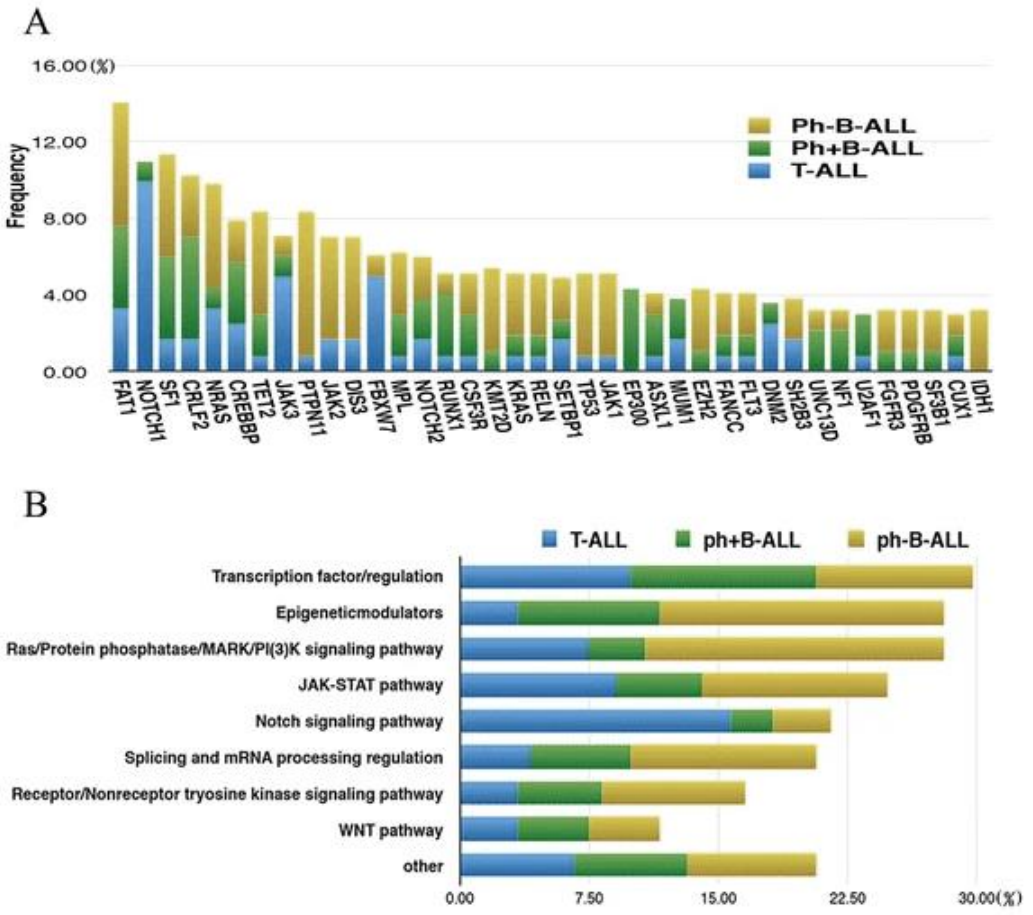

Figure III. Frequency of gene mutations and related signal pathways in ALL subtypes. A) Frequency of the top 38 gene mutations in different ALL subtypes, which are shown in indicated colors. B) Frequency of gene mutations involved in different functional pathways. Figure modified from [39].

1.2.2. Sign and symptoms in B-ALL

Leukemia may present in various ways. Many times, the symptoms are non-specific, including fever, weight loss and loss of appetite [10, 26, 27]. Occasionally, there are also symptoms that are related to bone marrow insufficiency, i.e. pallor and bruising [14, 17, 99]. Clinically, patients with ALL typically present constitutional symptoms, fatigue, bleeding, infections, and/or bone pain, with less than 10% of individuals having symptomatic central nervous system (CNS) involvement at diagnosis [26, 29]. Mature B-cell ALL can also present as extramedullary (gastrointestinal or testicular involvement) disease [14]. Age at diagnosis has a strong prognostic effect, but also leucocyte count is a continuous prognostic variable, with increasing counts conferring a poorer outcome [10, 24]. Indeed, patients with extreme hyperleukocytosis ($>400\times10^9$/L) are at high risk for early complications, such as central nervous system involvement, hemorrhage and pulmonary and neurological events due to leukocytosis [24].

1.2.3. B-ALL treatment and emerging approaches

The treatment options of B-ALL are dependent on several important factors, of which the most significant is the type or subtype of B-ALL [14]. Additional to this, other factors should be taken into consideration to establish a treatment plan, including cytogenetic abnormalities of the blasts as well as clinical features, the patient's age and involvement of the central nervous system (CNS) [100]. The mainstay of treatment is chemotherapy and, in some protocols, children with CNS diseases are given cranial irradiation [100]. Except for patients with mature B-cell ALL, who are treated with short-term intensive chemotherapy (including high-dose methotrexate, cytarabine, and cyclophosphamide), treatment for B-ALL typically consists of a remission-induction phase, an intensification (or consolidation) phase, and continuation therapy (long-term maintenance) [14, 28, 100]. The goal of induction treatment for B-ALL is to clear the blood and bone marrow of immature white blood cells (blasts) and bring about a complete remission, or complete response [28]. Otherwise, the consolidation treatment for B-ALL is given to prevent leukemia cells from coming back, indeed begins when the person goes into

remission after induction treatment, while the long-term maintenance is used to prevent or delay the cancer's return if it is in complete remission after the initial treatment or to slow the growth of advanced cancer after the initial treatment [28]. Finally, allogeneic hematopoietic stem cell transplantation may be indicated for the treatment of several subgroups of B-ALL patients with poor prognostic factors, such as those with Ph-positive disease (even when treated with a tyrosine kinase inhibitor) or with a poor initial response to treatment, as shown by minimal residual disease monitoring with different techniques, such as flow-cytometry or molecular biology [14, 28, 100]. Here below we will discuss some of the new emerging approaches to treated specific B-ALL cases.

- Tyrosine Kinase inhibitors

Historically, Ph-positive ALL presented 5-year survival ~5–20% and the Allo-SCT has been the only chance for cure, as long as the advent of TKIs made a turning point in the treatment of Ph-positive ALL [31]. The second-generation ABL kinase inhibitor, called Dasatinib, was developed as a dual Src/Abl kinase inhibitor, penetrating the blood-brain barrier and was effective at treating CNS disease [101]. Besides, Dasatinib was shown to be effective in inducing complete remission when used in combination with prednisone and intrathecal methotrexate [101]. Ponatinib, a third-generation TKI with the ability to inhibit most BCR-ABL1 kinase domain mutations, has recently gained approval for resistant Ph-positive ALL [102]. Progressively, the PACE trial demonstrated the ability of Ponatinib to generate a cytogenetic response in 47% of Ph-positive ALL patients after Dasatinib failure [103]. Moreover, clinical trial studies demonstrated that Ibrutinib, the inhibitors of pre-BCR+ B-cell by targeting Bruton’s tyrosine kinase (BTK) and B lymphocyte kinase (BLK), is tolerance in malignant B-cells and has progressed into phase III trials [104]. Furthermore, the combination treatment of Ibrutinib with Vincristine or Dexamethasone demonstrated valid activity during the therapy of B-ALL [100]. Other studies tested the combination of Ruxolitinib with Nilotinib demonstrating that it usually inhibits the proliferation of leukemia cells especially in Ph + ALL [100].

- Signaling pathway inhibitors

The screening of new targets and seeking novel effective inhibitors of signaling pathway involved in hematopoiesis are necessary and will provide more options for B-ALL treatment. For example, one group demonstrated that the combination of Temsirolimus, a specific inhibitor of mTOR, with Ibrutinib resulted in the cell growth reduction during the B-cell receptor pathway *in vivo* [105], others found that *in vitro* the Rapamycin (Sirolimus) inhibits cell growth and even promotes cell death in B-precursor ALL [106], while T315I, an integrin-linked kinase (ILK) inhibitor, which downregulates protein kinase B (Akt) and p-Akt, a unique mutation because of its resistance to the approved BCR-ABL inhibitors [107]. Otherwise, Idelalisib is a promising treatment option for BCP-ALL patients with TCF3-PBX1 (E2A-PBX1), whereas other drugs could be useful depending on the genetic context of individual patients [83], ABL001, also named Asciminib, could bind to the Myristoyl pocket of ABL1 and induces the formation of kinase conformation [108]. Besides, ABL001 is a potent and selective ABL1 inhibitor that is undergoing clinical development testing in patients with CML and Ph + ALL [108].

- Epigenetic therapies

Given that pediatric leukemia represents a heterogeneous group of diseases characterized by germline and somatic mutations that manifest within the context of disturbances in the epigenetic machinery and genetic regulation, current efforts are focused on drug candidates targeting histone deacetylases (HDACs) or methyltransferases as possible therapeutic agents, such as EPZ-5676, Decitabine, Vorinostat, Panobinostat etc [14, 100]. Fortunately, DNA methyltransferase inhibitors or hypomethylating agents, including Azacitidine and Decitabile, are already approved for use in MDS [109]. Also, clinical trials in phase 1 and phase 2 for Decitabile and Vorinostat, respectively, demonstrated their ability to alter the abnormal cellular pathways of leukemic blasts and essentially turn off anti-apoptotic proteins, while the leukemic cells have become primed for cytotoxic cell kill via chemotherapeutic agents [110]. EPZ-5676 is used for ALL harbor rearrangements of the MLL gene, because it blocks the activity of DOT1L, a protein

that plays an important role in the malignant process in these type of leukemia [111]. Many other classes of epigenetic modulators are under investigation for B-ALL treatment. These classes include: IDH inhibitors, BET inhibitors, DOT1L inhibitors etc [100, 112].

- **Other target therapies**

There are many other emerging therapies under investigation for B-ALL treatment such as antibody therapy [113]. A deep description of emerging therapies in B-ALL is beyond the scope of this study. However, it is worthy to mention that a good strategy consists in the use of the combination of two therapies than one [100, 110]. It may be the consequence of the existence of many clones in B-ALL developments, each dependent on one or two molecular abnormalities with are the target of pharmacological agents.

2. The Notch signaling pathway

2.1. The Notch receptors and ligands

The canonical Notch signaling pathway was first identified in the context of lateral inhibition of the peripheral nervous system of insects in 1917, when Thomas Hunt Morgan and colleagues described a strain of Drosophila with notches at the end of their wing blades [114, 115]. This curious trait was attributed to a partial loss of function (haploinsufficiency) of what would be later identified as the *NOTCH* gene [115]. Later, Notch signaling was described as an evolutionarily conserved pathway, being crucially involved in cell fate decision, proliferation, development, adult homeostasis and stem cell maintenance [114]. Notch proteins (**Figure IV**) are single-pass transmembrane receptors that transduce extracellular signals into cells and mediate cell-cell interactions [115]. There are four Notch receptors in mammals (namely Notch1–Notch4), of which Notch1 and Notch2 have each 36 epidermal growth factor (EGF)-like repeats, while Notch3 and Notch4 have 34 and 29 repeats, respectively, followed by three Lin-NOTCH repeats (LNR) [115, 116]. The

intracellular domain called Notch intracellular domain (NICD) presents the RBP-J-associated molecule (RAM) domain, six ankyrin repeats (ANK), nuclear localization sequences (NLS), a transactivation domain (TAD) required for activating transcription and a proline-, glutamate-, serine- and threonine-rich (PEST) domain which regulates NOTCH degradation [115, 116]. RAM domain and ANK repeats have been identified as regions involved in the interaction with CSL transcription factors [114-116]. The hydrophobic region or heterodimerization domain, together with the LNR repeats, form the negative regulatory region (NRR), adjacent to the cell membrane involved in the prevention of ligand-independent activation of Notch [114-116]. Furthermore, there are five canonical Notch ligands in mammals belonging to Delta (Dll1, Dll3 and Dll4) and Jagged (Jag 1 and Jag 2) families [115, 116]. Like the Notch receptors, these ligands are single-pass transmembrane proteins having multiple EGF-like repeats and cysteine-rich sequences known as the Delta-Serrate-Lag2 (DSL) motif [115, 117]. The EGF-like repeats and the DSL motifs on the ligands are required for them to bind and activate the Notch receptors on the neighboring cells, moreover these ligands may display or specificity or redundancy for different Notch receptors [114, 115].

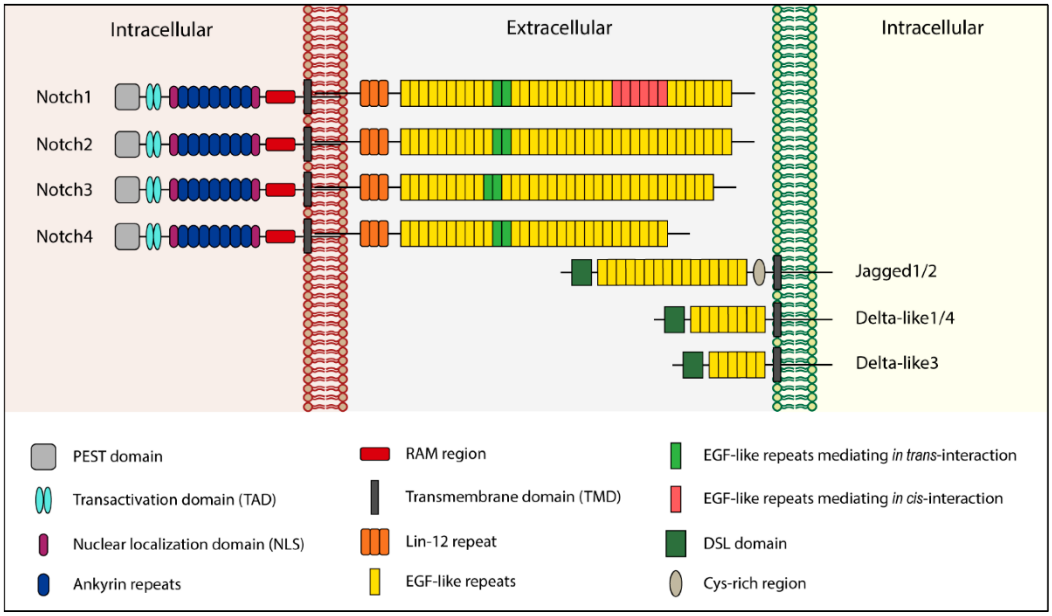

Figure IV. Structure of human Notch receptors and ligands. Figure adapted from [116].

2.2. Mechanism of Notch signaling

Initially, as illustrated in **Figure V**, the Notch receptors are transcribed and translated as 210-300 kDa large precursor molecules. A series of post-translational modifications are required for the precursors to acquire their active forms [118]. The intact precursor molecules are first glycosylated in the endoplasmic reticulum (ER) by O-fucosyletransferase (Pofut-1 in mammals), which adds fucose to serine or threonine sites on specific EGF-like repeats [118]. The glycosylated precursors are then cleaved in the trans-Golgi network into two subunits by furin-like convertases (S1-cleavage) [118]. This cleavage converts the precursor molecule into the noncovalently linked Notch extracellular domain (NECD) and Notch transmembrane-Notch intracellular domain (TM–NICD) complex, which is then further glycosylated by enzymes of the Fringe family [118, 119]. In mammals, three Fringe genes have been identified: Lunatic fringe (Lfng), Radical fringe (Rfng) and Maniac fringe (Mfng) [114, 118, 119]. Fringe proteins add N-acetylglucosamines moieties to already existing O-fucose molecules on the EGF-repeats [116, 118, 119]. This modification in the Notch ligand-binding domain seems to alter the responsiveness of the receptor to different ligand interactions or enhance S2 mediated cleavage of the receptor [116, 118, 119]. The effects of Fringe dependent modification of Notch are complex and the outcome of the signaling largely seems dependent on the combination of receptor, fringe family member and ligand [116, 118, 119]. The mature Notch receptor is then translocated to the cell surface and is, via its EGF-like repeats, activated upon binding to one of its ligands, which are expressed on neighboring cells. The receptor-ligand binding results in a conformational change of the receptor and the exposure of an extracellular metalloprotease site (S2). S2 cleavage of the NECD is controlled by the ADAM/TACE (a desintegrin and metallopeptidase/tumor necrosis factor α converting enzyme) family of transmembrane proteases resulting in an active membrane anchored Notch [116, 118, 119]. This Notch form is subsequently cleaved within the TD close to the cytoplasmic border by the presenilin-γ-secretase complex (S3), between gly1743 and val1744 (termed

site 3 or S3), which is composed of a four-protein complex consisting of the catalytic component presenilin and the three co-factors, nicastrin (NCSTN), anterior pharynx-defective 1 (APH-1) and presenilin enhancer 2 (PEN-2). Following the multi-step cleavage of Notch and liberation of NICD from the inner membrane, activated NICD is translocated into the nucleus via endocytosis and endosomal trafficking [118, 120, 121]. In the nucleus NICD normally is a transcriptional activator, consisting of ankyrin repeats domain, a RAM (RBP-Jκ associated molecule) domain, a transactivation domain (TAD), a nuclear localization signal (NLS), and a PEST domain that regulates protein stability, and binds to the transcriptional repressor RBP-Jκ, which together with co-activators belonging to the 1-3 Mastermind-like family (MAML1-3) of proteins [115, 118, 120, 121]. Notch ligands are also cleaved by γ-secretase and ADAM/TACE metalloprotease complexes, thus providing an additional level of regulation of the pathway. The complex further recruits different co-regulators e.g. the histone acetyltransferase p300 and other chromatin remodeling factors together with the cyclin-dependent kinase (CDK) 8 [114, 119, 120]. Recruitment of CDK8 leads to phosphorylation of NICD and thereby subsequent proteasomal degradation of the complex through E3-ligase FBW7 (Cdc4) mediated ubiquitination of the TAD and PEST domains (polypeptide rich in proline, glutamate, serine and threonine), thereby terminating active Notch signaling. Other important regulators of Notch signaling are the Numb and Numb-like proteins, which act upstream of S3 cleavage to antagonize Notch signaling through direct interaction via Notch ankyrin (ANK)-repeats [114, 119, 120]. It is also likely that NICD can act in a RBP-Jκ independent non-canonical manner and interact with several other components in the nucleus e.g. Hif-1α, NFκB and β-catenin [122]. Some of the best-known target genes belong to the *HES/HEY* (Hairy Enhancer of Split/Hairy Enhancer of Split related) family, which are basic helix-loop-helix (*bHLH*) transcriptional repressors important for development, proliferation, differentiation and cell fate decision [120, 122]. Besides *bHLH* transcription factors, several other genes like the protooncogene *c-Myc* and the cell cycle regulators *p27KIP1* and *cyclin D1* have been identified as Notch targets [119, 120, 122].

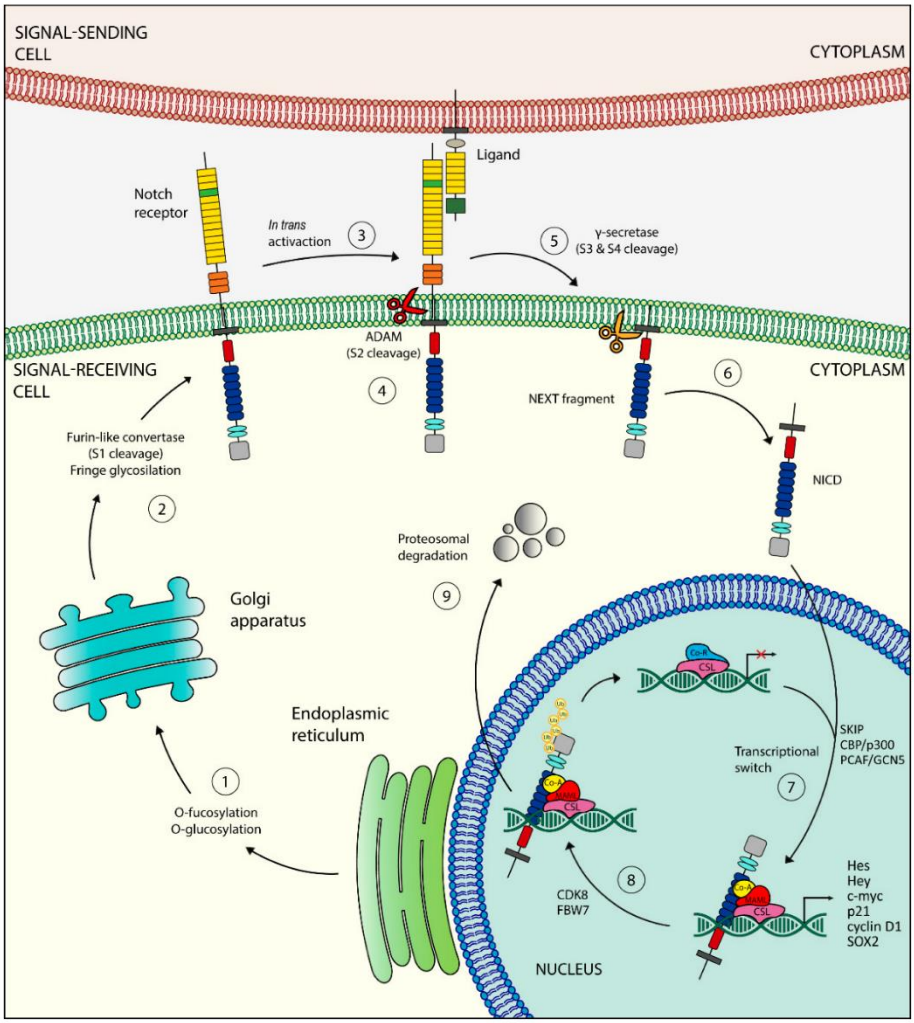

Figure V. Schematic representation of the Notch signaling pathway. Once synthesized in the endoplasmic reticulum (①), the inactive single peptide precursor moves to the Golgi where it is cleaved by a furin-like convertase (S1 cleavage) (②) and translocate into the cell membrane. The binding with a Notch ligand (③) induces the second cleavage (S2) by a member of the disintegrin and metalloproteinases (ADAM) family (④), resulting in the formation of a membrane-tethered Notch truncated (NEXT) fragment, which is further processed in two sites (S3 and S4) by a presenilin-dependent γ-secretase complex (⑤), generating the Notch intracellular domain (NICD), the active form of the Notch receptor (⑥). The NICD can now enter into the nucleus, where it exerts its transcriptional activity (⑦). The ubiquitination of the NICD (⑧) leads to its proteasome degradation (⑨). Figure adapted from [116].

2.3. Role of Notch in development

As already mentioned, Notch was first identified in Drosophila as a mutation associated to the wing-Notching phenotype [114, 115]. Subsequently, it was identified as a highly conserved and a master developmental gene involved in the embryonic development and tissue homeostasis from flies to mammals [114, 115]. In human, Notch participates in the control of cell proliferation, self-renewal,

apoptosis, migration and differentiation regulating a wide variety of developmental processes, such as neurogenesis, heart and endocrine development [114]. It has been proposed that Notch regulates developmental processes through three models, i.e. lateral inhibition, lateral induction and lineage decisions [121] (**Figure VI**). Lateral induction occurs among cells with equal capability to undergo different fates. In this case, Notch activation maintains cells in undifferentiated state, while neighbor cells undergo the differentiation [121]. During lateral induction, Notch receptor-expressing cells undergo differentiation, while adjacent cells are maintained in the undifferentiated state. Over lineage induction means at each cell division, Notch is activated in one daughter cell, but not in the other cell, which results in the adoption of distinct cell fates [114, 121].

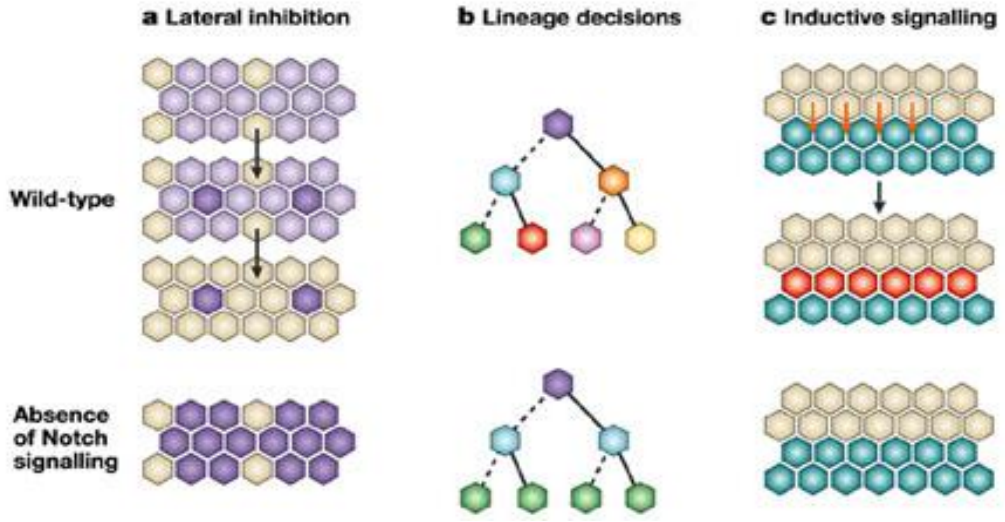

Figure VI. The Notch signaling pathway is used for a wide range of cell-fate decisions. The top panels show how cell fates are specified in wild-type cells; the bottom panels show the consequences of no Notch signaling. a) Lateral inhibition: a pair or a group of equivalent precursor cells (light purple) signal through the Notch pathway to inhibit each other's ability to adopt a distinct fate. In a sequential process (shown by the arrows), which is amplified by feedback loops, one cell in each group (dark purple) 'wins' by lacking Notch activation. Notch activation in the other cells results in an alternative cell fate (yellow). b) Asymmetric cell divisions: at each cell division, Notch is activated in one daughter cell (solid lines) but not in the other cell (dashed lines), which results in the adoption of distinct cell fates (indicated by different colors). c) Inductive signaling: one group of cells (yellow) signals (orange arrows) to a distinct neighboring group of cells (green) to induce a new cell fate along the interface between them (red). Figure modified from [121].

2.3.1. Role of Notch signaling in organ development

Since Notch pathway is a signaling network essential for proper organ development in an embryo and is indispensable for tissue regeneration in the adult, the dysfunctions in Notch signaling pathway have been linked to the pathogenesis of several inherited human diseases, including Alagille Syndrome, Spondylocostal dysostosis, Hajdu Cheney Syndrome, mucoepidermoid carcinoma, Aortic valve disease, Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CASASIL) and T-acute lymphoblastic leukemia (T-ALL) [123-132] (**Table V**). For Alagille Syndrome, most cases (94%) are associated with a mutation in the Notch ligand *JAGGED1*, including some cases with deletions in chromosome 20 where the ligand gene is located [130]; the remained patients present a mutation in other genes of Notch family, mostly (1-2%) *NOTCH2* [129]. Alagille patients are characterized by bile duct malformation, a combination of heart defects, broad and prominent forehead due to bone defect, and central nervous system impairment [129, 130]. Spondylocostal dysostosis, however, is caused by a mutation in one of at least five different genes, specifically *DLL3*, *MESP2*, *LFNG*, *HES7* and *TBX6*, though an altered *DLL3* gene is still the most common cause [128]. The Notch gene mutations cause malformations affecting the spine and ribs, bringing accordingly severe breathing [128]. Another rare disease, characterized by mutations in Notch signaling, is the Hajdu Cheney Syndrome, after mutations in the *NOTCH2* gene, which primary manifestations are focal bone destruction and osteoporosis, while secondary are craniofacial abnormalities, renal cysts, cleft palate and cardiac defects [132]. Diversely, CADASIL is a neurological disorder involving mutations in Notch signaling, which genetic risk factors have been mapped to chromosome 19q13 and many mutations on *NOTCH3* gene [124]. The most common clinical manifestations are migraine headaches and transient ischemic attacks or strokes, which usually occurs between 40 and 50 years of age. Mutations in the *NOTCH3* gene cause an abnormal accumulation of Notch receptors at the cytoplasmic membrane of vascular smooth muscle cells both in cerebral and extracerebral vessels, following leukoencephalopathy [124]. All these diseases Notch-associated reflected the broad requirement for Notch signaling in embryogenic and adult development [114, 116].

Gene	Disease associated to mutated gene	Ref.
DLL3	Spondylocostal dysostosis (axial skeleton segmentation disorder)	[131]
JAG1	Alagille Syndrome	[130]
LFNG	Spondylocostal dysostosis (axial skeleton segmentation and growth disorder)	[128]
NOTCH1	T-ALL, Aortic valve disease	[125]
NOCTH2	Alagille Syndrome, Hajdu-Cheney Syndrome	[129, 132]
NOCTH3	CADASIL	[124]
NOCTH4	Schizophrenia	[123]

Table V. Mutations in Notch signaling components result in developmental defects and human diseases. Summary of some mutations involved in the Notch signaling, cause of human diseases.

2.3.2. Role of Notch signaling in lymphocyte development

Since Notch signaling plays a major role in hematopoiesis and lymphocyte development, Notch receptors and ligands are widely expressed in the whole hematopoietic system [114] (**Figure VII**). Some studies suggested that the different Notch receptors and their specific ligand interactions may have distinct roles in HSC self-renewal and differentiation [133, 134]. Notably, accumulating evidence suggest that Notch1 plays a significant role in T-cell versus B-cell fate determination [134]. Radtke et al. even demonstrated that the inhibition of RBP-J dependent Notch1 signaling completely blocks T-cell development and causes incremental development of B-cells in the thymus and vice versa, while activation of Notch signaling increases the frequency of multipotent progenitor cells and drives T-cell differentiation in a dose-dependent manner [134, 135]. They demonstrated also that the inactivation of Notch2 does not affect T-cell development, indicating any redundancy for Notch1 and Notch2 in T:B lineage commitment [134, 135]. However, the Notch2 receptor is predominantly expressed in B-cells and a Dll1/Notch2 interaction seems necessary for marginal zone B (MZB) cell differentiation [133, 134]. Once again, loss or gain of function approaches were useful strategies to investigate the role of Notch signaling in hematopoiesis and lymphocyte development. The retroviral expression of active forms of Notch1 (NICD1), Notch4 (NICD4) or Hes1 in stem/progenitors cell-enriched populations, increases the pool of cells with repopulating capabilities, such as mouse KLS (c-Kit+Scal1+Lin-) and Lin- cord blood cells [136]. Delaney et al. gave an important insight to Notch requirement in hematopoiesis, as they observed

that treatment of $CD34^{+}CD38^{-}$ cord blood progenitors with a low density of DLL1 enhanced generation of NOD/SCID repopulating cells, while an high density of the ligand-induced a switch in lymphoid lineage [137]. In this term, we can say that though Notch is involved in the maintenance of HSCs, Notch could then act as a decisional specification stimulating the common lymphoid to differentiate into T-lineage, while silencing of Notch activity leads to the onset of B-cell lineage [133-135]. In conclusion, the different role of each receptors and ligands during hematopoiesis or lymphoid differentiation is still under investigation.

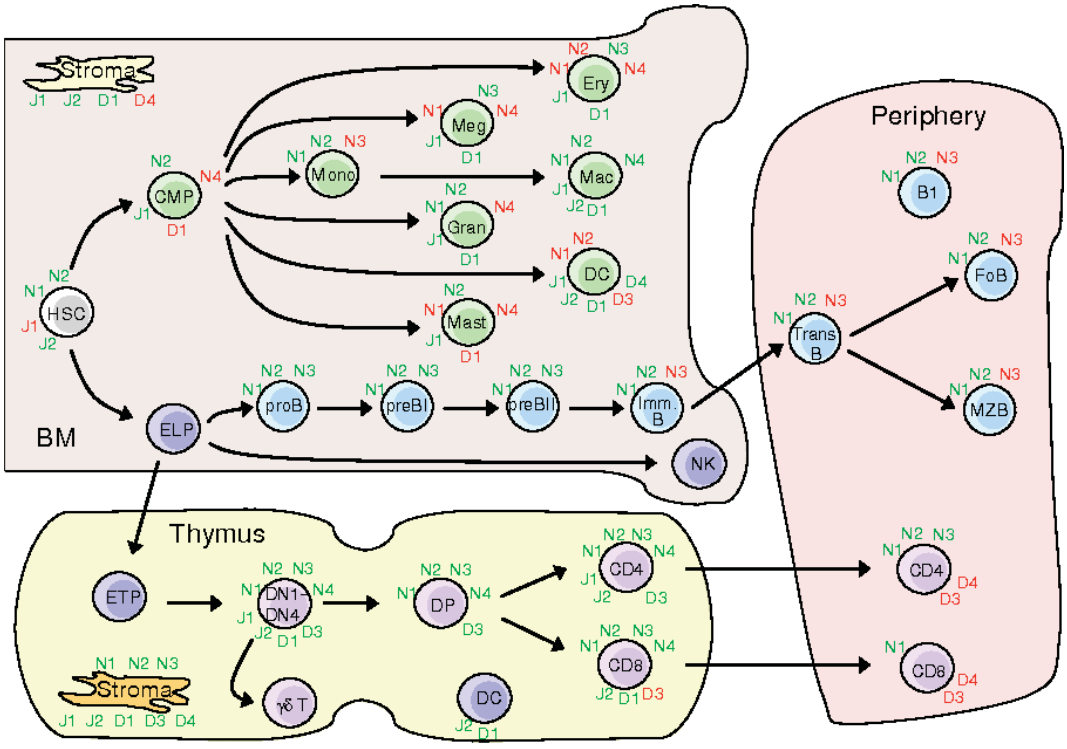

Figure VII. Expression pattern of Notch receptors (above cells) and ligands (below cells) in different hematopoietic lineages in the organ of developmental origin. In green, expressed genes, red, absence of detectable expression. Figure adapted from [134].

2.4. Role of Notch signaling in cancer

While Notch signaling is crucially involved in normal regulation of cell differentiation, proliferation, development, its dysregulation often has even a profound effect on the cellular fate and may lead to tumor formation [114, 134].

Indeed, the role of Notch in cancer is largely documented both in solid tumors and in liquid malignancies, leading to either activation or inhibition of the pathway [114, 117, 126]. Even though Notch pathway was early recognized as an oncogene, it can act as oncogene or tumor suppressor depending on the cell context [114]. Generally, Notch may act as an oncogene in tissues where it is involved in stem cell self-renewal or in cell fate decisions, while it may have a tumor suppressive role in tissues where Notch promotes terminal differentiation [138]. The first report of the oncogenic role of Notch has been described in T-ALL, an aggressive neoplasm of immature T-cells, as more than 50% of patients display an activating mutation in Notch1 gene [84, 125]. Since then, overexpression or downregulation of Notch components have been associated to cancer initiation, growth, epithelial to mesenchymal transition (EMT), angiogenesis and microenvironment-promoted cancer growth and chemo-resistance [139]. Some evidence suggest that in a few tumor types, including human hepatocellular carcinoma, skin and small lung cancer, expression of Notch1, Notch2, Jagged1 and Hes1 are reduce, while the speculatively activation of Notch signaling may function in a tumor suppressive manner [138, 140]. For example, during angiogenesis, Notch1 as well as the ligand DLL4 have been shown to interact with vascular endothelial growth factor (VEGF) and Hif-1α which are key controllers of both normal and tumor-related angiogenesis [141]. Other Notch components, such as Notch1 and Jagged1 were besides mostly studied for their role in cancer, representing good therapeutic targets [133, 134, 138, 140]. Furthermore, Notch knock-down using siRNA, shRNA, blocking antibodies, reduces cancer cell proliferation, growth, migration/invasion and sensitizes cells to chemotherapy in a several cancer, increasing the critical role of Notch signaling in cancer and favoring its role as target therapy [133, 134, 138, 140].

2.5. Role of Notch signaling in leukemia

The human *NOTCH1* gene was first identified through its involvement in a t(7:9)(q34;q34.3) chromosomal translocation found in approximately 1% of T-ALL, while later activating mutations in the HD and PEST domains were

discovered in 55-60% of human T-ALL, indicating a broader role for Notch1 in cancer formation [125, 126]. Then, an oncogenic role for Notch signaling has also been discovered in others hematological disease, including in Hodgkin's lymphoma (HL), anaplastic large-cell non-Hodgin's lymphoma (NHL), acute myeloid leukemias (AML) and B-cell chronic lymphoid leukemias (CLL) [117, 142-144]. The prevalence of Notch mutation in AML is probably less than 5%, and Notch ligation in AML cells has diverse or only minor effects, while seem that the AML express mostly JAG1, Notch1 and Notch2 [145]. In AML, the role of Notch remains controversial, although our group has recently demonstrated that the protective role of the microenvironment can be abrogate mediated the inhibition of Notch, reducing the chemoresistance of AML cells [144]. Otherwise, in CML Notch emerges as tumor suppressor gene rather than oncogene, although still poorly investigated [138]. The importance of Notch activation was instead discovered in T-ALL from the use of GSIs for *in vitro* experiments, demonstrating that these inhibitors sensitized the T-ALL cell survival [125, 126]. Subsequently, other Notch inhibitors showed the same profile, like Notch transcription factors inhibitors (e.g. SAMH1) and Notch blocking antibodies [16, 113]. Besides Notch1, higher levels of Notch3 were found in T-ALL cells, and its genetic inhibition through siRNA led to growth inhibition and apoptosis [16]. In CLL, however, constitutive activation mutations of Notch are a hallmark, especially in the PEST domain of Notch receptors and are associated with a shorter overall survival [16, 146]. Rosati et al. found high expression of Notch1, Notch2, Jagged1 and Jagged2 in CLL correlated with higher activation of the pathway [146]. Accordingly, our group demonstrated that Notch inhibition induces CLL apoptosis and sensitizes leukemia cells to treatment with chemotherapeutic agents [147]. Expect in T-ALL, Notch mutation is very rare in other leukemia types. In B-ALL, *NOTCH1* mutation was not observed, but a tumor suppressor role of the pathway was suggested. Notch seems to be epigenetically silenced in B-ALL since *NOTCH3*, *JAGGED1*, *HES2*, *HES4* and *HES5* are frequently hypermethylated in B leukemia cell lines and in primary B-ALL [148]. Restoration of *HES5* expression by lentivirus transduction resulted in growth arrest and apoptosis in *HES5* negative B-ALL cells [148]. In contrast, our

group has recently shown that microenvironment of bone marrow protects B-ALL cells from apoptosis induced by chemo agents through Notch activation [117].

2.6. Notch signaling-related therapeutic strategies

Therapeutic strategies targeting Notch are designed to interfere with specific steps of the pathway, such as ligand-receptor interactions and receptor processing, agonistic and antagonistic antibodies, stimulatory fusion proteins, inhibitors of intracellular signaling and transcription complex formation [113, 145, 149, 150] (**Figure VIII**). For example, inhibition of the γ-secretase activity, with general downregulation of Notch signaling, has been used in experimental *in vitro* studies, displaying in some cases a strong antineoplastic capacity [149-151]. Indeed, as already cited, unless NICD is translocated to the nucleus, the NICD form of Notch is ubiquitinated and thereafter degraded by the proteasomes [118-121]. In this instance, contrarily and if necessary, the use of proteasomal inhibitors may thus enhance Notch signaling [113]. Indeed, various proteasomal inhibitors are now used in the treatment of hematologic malignancies, and they are also tried as immunosuppressive agents, but it is not still known whether inhibition of Notch signaling contributes to their clinical effects [149, 150]. Coming back to inhibition of Notch, several GSIs have been developed for cancer therapy, including BMS906024, MK0752, PF03084014, R04929097 and are already used in clinical trials [149, 150]. The more serious problem is that physiologically, as already announced, some adult tissues required Notch activity for their function, such as gastric epithelium, thus explaining the gastrointestinal toxicity observed during GSIs treatment [114, 119, 152]. Furthermore, to by-pass to this issue, some researchers started to introduce specific small molecules against Notch targets, such as receptor or ligand decoy or blocking monoclonal antibodies [145, 149]. This alternative therapeutic approach is mostly interesting, as a specific and unique Notch component could be responsible for the cancer phenotype, i.e. NOTCH1 mutation for T-ALL [125, 126]. Several Notch receptors blocking antibodies were also used in clinical trials, like OMP-59R5/Tarextumab, OMP-52M51 and OMP-21M18/Demcizumab [150], while others are under investigation.

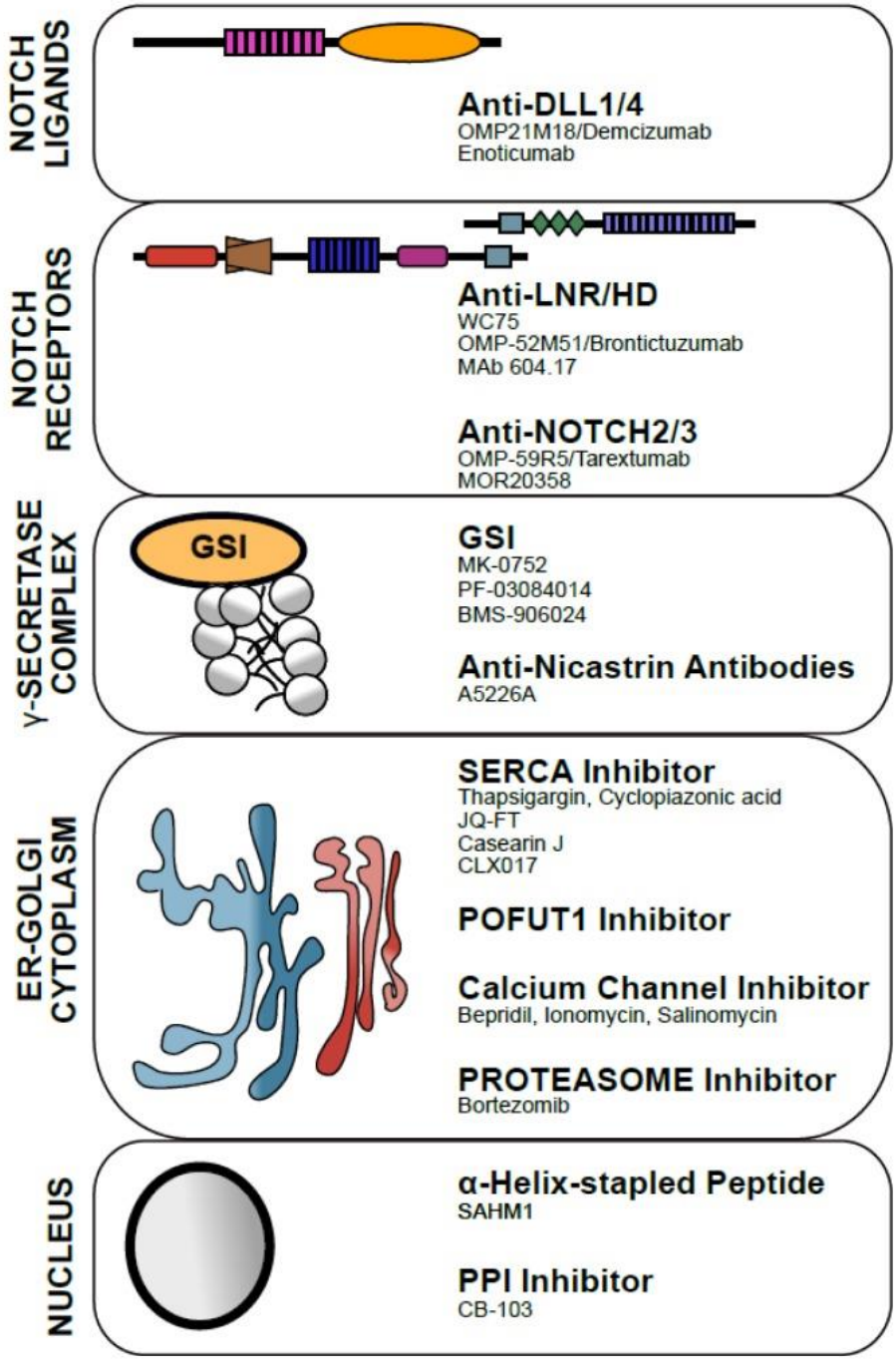

Figure VIII. Latest strategies to target Notch in hematological malignancies. The figure shows an overview of therapeutic targeting of Notch signaling, with emphasizing innovative approaches or experiences that translated pre-clinical observations into clinical trials. Figure adapted from [150].

3. Reactive Oxygen Species (ROS)

The reactive oxygen species (ROS) is a collective term used to describe many free oxygen radicals and reactive molecules derived from molecular oxygen, including superoxide anion (O_2-), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2) and singlet oxygen (O_2), while the NOX family are the critical determinants of the redox state [153, 154]. For a long time, ROS were only recognized as a kind of toxic by-products from aerobic metabolism, which caused damage to cells by deleteriously modifying biological molecules, including lipids, proteins and nuclear acids [154, 155]. Furthermore, ROS are usually linked with aging and many distinct human diseases, such as cardiovascular disease, neurological disorders, cancers and chronic inflammation [153-156]. However, as research progressed, another voice of ROS emerged. Indeed, some researchers claim that ROS can also function as second messengers, fine-tuning the complicated network of signal transduction, thus triggering or modulating various cellular activities, such as cell proliferation, cell differentiation and cell death [157, 158]. It is currently unknown the specific role of ROS in both physiological and pathological development, but it was demonstrated that any alterations of intracellular ROS levels may induce a cellular signal and determine the behavior of cells in both situations [156-158].

3.1. Role of ROS in biological processes

As already announced, ROS participate in various cellular processes in response to extracellular and intracellular signals [156-158]. It follows that, any alterations of intracellular ROS levels will induce a cellular signal, which can either damage cells or be used as intracellular messengers to regulate the activities of several proteins, including several transcription factors [157, 158] (**Figure IX**). In term of cell proliferation, ROS exhibit a dual effect, i.e. low levels are required for cell growth, while high levels lead to apoptosis or necrosis [159, 160]. Another role in which the ROS are involved is to determinate cell differentiation. Indeed, a report described the role of ROS in human embryonic stem cells (hESCs) differentiation, suggesting that the continuous exposure to ROS could diminish the pluripotency of

hESCs and consequently induce hESCs to differentiate into bi-potent mesendoderm, which could be further specified to mesoderm or endoderm [161]. Some studies also demonstrated the role of ROS in the apoptosis, induced by H_2O2, in several cell types including rat neural AF5, glioma, colon cancer and human cervical carcinoma HeLa cells [154, 156, 160]. Here, we can conclude saying that the role of ROS in the physiological development is largely studied and demonstrated, now it is necessary to better understand its role in term of cancer, disease and pathological development.

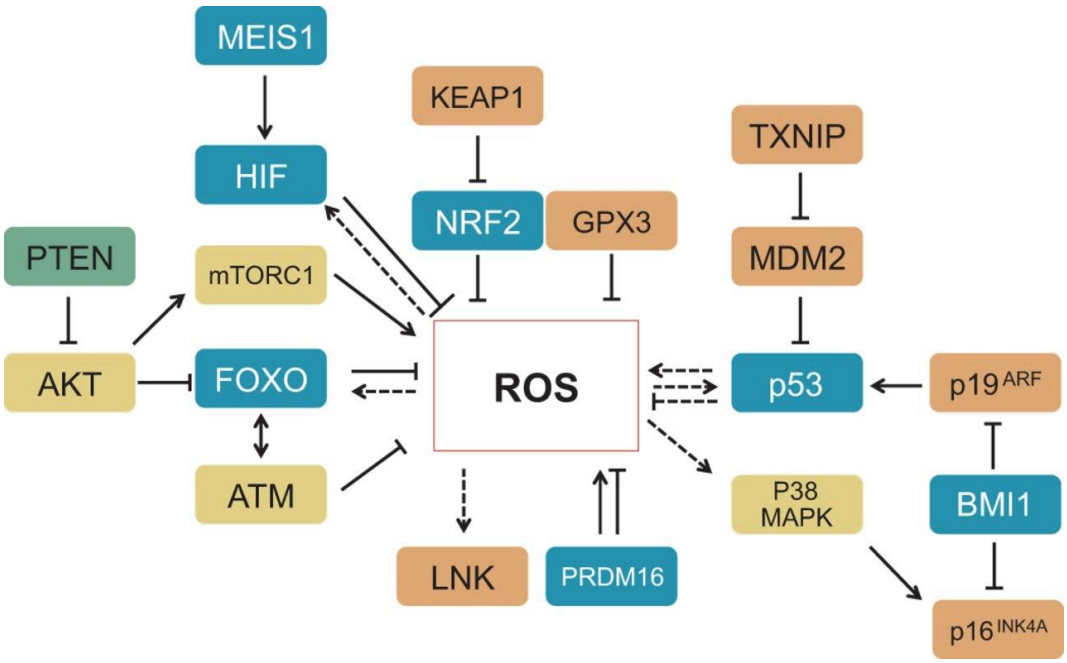

Figure IX. ROS mediated-cellular signaling. Intricate control of ROS can be either directly or indirectly mediated by several transcription factors (blue), as well as by kinases (yellow) and phosphatases (green). Other regulators, such as the cytokine signaling inhibitor LNK, the modulator KEAP1, the E3 ubiquitin ligase MDM2, the cell cycle inhibitors p16INK4A and p19ARF (which are negatively modulated by the polycomb group member BMI1), the complex mTORC1, TXNIP, and the antioxidant enzyme GPX3 (all shown in orange) can also control ROS levels. Dashed arrows and lines indicate regulations that have not been explicitly shown to occur in stem cells; unbroken lines represent interactions that have been shown in stem cells. p53 has both antioxidant and pro-oxidant functions (shown in somatic cells). Figure adapted from [157].

3.2. Role of ROS in cancer

Elevated levels of ROS, alteration in redox balance, and deregulated redox signaling are common hallmarks of cancer progression and resistance to treatment [156-158]. These are due to since high metabolic activity, cellular signaling, peroxisomal activity, mitochondrial dysfunction, activation of oncogene, and increased enzymatic activity of oxidases, cyclooxygenases, lipoxygenases, and thymidine phosphorylases [153-156]. It was demonstrated ROS-mediated signaling pathways activate pro-oncogenic signaling which eases in cancer progression, angiogenesis, and survival [159-162]. Therefore, ROS play a vital role in every stage of cancer development, including initiation, promotion, and progression [155]. An increase in intracellular ROS levels may result in the activation of oncogenes and oncogenic signals, including constitutively active mutant *RAS*, *BCR-ABL*, and *c-MYC* which are involved in cell proliferation and inactivation of tumor suppressor genes, angiogenesis, and mitochondrial dysfunction [153, 156-158]. Studies have demonstrated that H_2O_2 can promote the activation of Ras protein and growth factor signaling which in turn activates PI3K/Akt/mTOR, MAPK/ERK and inactivates PTEN signaling cascades [163, 164]. Recently, it has been also demonstrated that breast cancer-associated mitochondrial DNA haplogroup promotes neoplastic growth via ROS-mediated AKT activation [165]. Furthermore, clinically, chemotherapy and radiotherapy are designed to exuberantly-increase cellular ROS levels to induce irreparable damages subsequently resulting in tumor cell apoptosis [157, 158, 160]. Anthracyclines, such as Doxorubicin, Daunorubicin and Epirubicin, generate in effect the highest levels of cellular ROS [154, 155].

3.3. Crosstalk between Notch Signaling pathway and ROS

The development and regulation of physiological activities require orchestrated activities, such as Notch signaling pathways but also the modulation of ROS levels [114, 157, 158]. In effect, studies conducted in the last several years have provided clear evidence that Notch signaling pathway is regulated at least in part by NOX-derived ROS [153, 154]. In bone marrow-derived mesenchymal stem cells

Boopathy et al. observed the upregulation of Notch1 after a myocardial infarction, which induced an increased level of H_2O_2 [166]. Another research group demonstrated that Notch signaling lowers the levels of ROS in cancer cells, improving cancer cell survival and chemoresistance [167]. In 2015, Sankaranarayanan Kannan and colleagues published that Notch activation regulated the antioxidant response via suppression of the master regulator NRF2, suggesting a pivotal role of Notch/NRF2 in the regulation of antioxidant responses in AML and a potential clinical significance for ROS-inducing chemotherapies [168]. A clear distinction between Notch robust cell-fate decisions and ROS modulation may provide clues for clinical strategies and drug discovery targeting several diseases and cancer.

II. Rational and aims

The pivotal influence of the several different signaling pathways on tumor cells, as a basis for chemosensitivity and treatment failure, has been clearly shown [144, 168]. Consequently, many of the most recent therapeutic approaches have shifted from classic single chemotherapies to the combination between chemo agents and inhibitors, which interfere with the activation of signaling pathways involved in the development of tumor cells, i.e. Notch signaling, or by targeting other signaling molecules at post-translational level including NFkB, Akt, mTOR, MAPK/ERK [105, 110, 112]. It has recently been shown that Notch ligands protect B-cells from apoptosis in germinal centers [169], while our group extended the knowledge on the protective role of Notch signaling in B-cell malignancies, i.e. B-ALL and C-LL [117, 147]. Furthermore, some evidence suggest that Notch mutation is very rare in B-ALL, but it seems to be epigenetically silenced [148]. Recently, we observed high expression levels of Notch receptors and ligands in a subset of 12 B-ALL patients, whose abrogation through Notch inhibitors, such as GSIs, resulted in B-ALL cells apoptosis [15]. By literature, we knew that Notch signaling pathway controls not only proliferation, survival, differentiation, but also lowers the levels of ROS in cancer cells, improving cancer cell survival and chemoresistance [167]. These previous observations suggested the involvement of Notch signaling not only in B-ALL cell survival, but also in chemosensitivity.

The aims of this study are:

- to study the epigenetics patterns of Notch genes in B-ALL and to investigate if Notch may be a marker of risk for B-ALL patients;
- to assess of the effects of inhibitors of Notch signaling in combination with some chemo-agents on B-ALL chemosensitivity and modulation of intracellular levels of ROS;
- to validate a *in vivo* xenograft model of NOG, confirming the *in vitro* observations and to study the preclinical effect of combination treatments.

Overall, we demonstrated through *in vitro*, *in silico* and *in vivo* approaches the contribution of Notch signaling pathway to B-ALL cell response and chemotherapy.

III. Materials and Methods

Chemicals and antibodies

For flow cytometry analysis, mouse IgG2b-FITC (IC0041F), goat IgG-PE (IC108P), anti-Jagged1-4 FITC (FAB1277F) and anti-Dll3-PE (FAB4315P) were from R&D System (Minneapolis, MN). Mouse IgG2a-PE (400214), mouse IgG1κ-PE (400114), anti-Notch1-PE (352106), anti-Notch2-PE (348304), anti-Notch3-PE (354406), anti-Notch4-PE (349004), anti-Dll1-PE (346404), and anti-Dll4 (346506) were from Biolegend (San Diego, CA). For B-ALL cell identification we used anti-CD19-PerCp/-APC and anti-CD45-PerCp/-APC/-V450/V500 antibodies all from MiltenyiBiotec, (Bergisch Gladbach). Rabbit anti-Deltex1 (ab135730), rabbit anti-HES5 [EPR15578] (ab194111), rabbit anti-HEY1 antibody (ab154077) were from Abcam (Cambridge, UK). Rabbit anti-c-Myc (D84C12) was from Cell Signaling (Leiden). For western blot analysis anti-Notch2 (sc-5545) was from Santa Cruz Biotechnology (Dallas, TX), anti-GAPDH (G9545) and HRP conjugated secondary antibodies against mouse, rabbit or goat were from Sigma Aldrich (Darmstadt). The rest of antibodies used for Western blot including anti-Notch1 (4380), anti-Notch3 (5276), anti-Notch4 (2423), anti-Hes1(11988), anti-Jagged1(2155), anti-Jagged2(2620), anti-DLL-4 (4147), anti-mTOR (2983), anti-AKT/(Thr308)AKT (9272/2965), anti-NF-κB/(Ser536)NF-κB (8242/4764) and anti-ERK1/2/(Thr202/Tyr204) ERK1/2 (4695/4370) were from Cell Signaling (Leiden). For cell culture, recombinant human Jagged1 (1277-JG), Jagged2 (1726-JG), DLL-4 (1506-D4) and blocking Notch-3 (AF1559) were from R&D Systems (Minneapolis, MN). Neutralizing Notch4 (sc-8643) was from Santa Cruz Biotechnology (Dallas, TX). Recombinant human Jagged1 and Jagged2 were from R&D System. GSI-IX (DAPT) was purchased from Stemgent (Cambridge, MA), while GSI-XII and SAHM1 were from Merck Millipore (Darmstadt). Cytarabine (Ara-C), Dexamethasone (Dexa), Doxorubicin (Doxo) were provided by Pharmacy Unit of the University Hospital of Verona.

Patients and samples

The characteristics of patients involved in this study are presented in **Table 3**. This study was conducted in accordance with the declaration of Helsinki, all primary cell samples being collected from B-ALL patients after written informed consent as approved by the Ethical Committee of Azienda Ospedaliera Universitaria Integrata Verona Italia (N. Prog. 1828, May 12, 2010 - '*Institution of cell and tissue collection for biomedical research in Onco-Hematology*'). Primary B-ALL samples were used equally fresh or after freezing.

Cell line culture and validation

B-ALL cell lines including VR-ALL, RS4;11, and SUP-B15 were cultured in complete RPMI (RPMI supplemented with 10% fetal bovine serum (FBS), 1% L-Glutamine and 1% Penicillin/Streptomycin). VR-ALL cells were isolated, characterized and validated in our laboratory, as previously described [151], while RS4;11, and SUP-B15 cell lines were acquired from American Type Culture Collection (ATCC). Stability and identity of all B-ALL cell lines were controlled during and at the end of the current study using flow cytometry of membrane marker, morphological analysis after Giemsa staining and Short Tandem Repeat (STR). The stability and identity of all other cell lines (including HEK 293, HEK293T, CEM and Ramos) used as control in western immunoblot were validated only through flow cytometry of membrane marker and morphological analysis after Giemsa staining. All cell lines were routinely tested to be Mycoplasma-free using the Mycoplasma PCR detection kit from Sigma Aldrich (Darmstadt). Once thawed cells were passaged every week and were not used for more than 3 months. This period corresponding to 8-12 cell passages.

Western blotting

Cells were lysed with appropriate amount of RIPA buffer (25 nM Tris pH 7.6, 150 mM NaCl, 1% NP40, 1% Na-deoxycholate, 0.1% SDS) supplemented with complete Protease Inhibitor (Roche) and 1 mM Na3VO4. Proteins were separated

on a 10% polyacrylamide gel. Subsequently, proteins were transferred onto nitrocellulose membrane (GE Healthcare), labeled with appropriate antibody and acquired by LAS4000 (GE Healthcare) instrument. GAPDH was used as loading control. All samples subjected to immunoblotting contained more than 80% of leukemia cells. The specificity of all Notch antibodies was validated on three control lysates, as previously described (5,14,15): HEK 293, HS27A stromal cell line and CEM T-ALL cell line.

MTT viability assay

To study the specific relative basal sensitivity of B-ALL cells to different agents, cells were seeded in 96 well plates and cultured for 48 hours in presence of increasing concentrations of each modulator or drug. To determinate IC50 dose for each drug and modulator, we performed the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) metabolic activity assay. B-ALL cell lines (10^4 cells per wells) or B-ALL primary cells (10^5 cells per wells) were seeded in 96 wells plates, with increasing concentrations of different drugs. Cell viability was then assessed by adding 10 µL of MTT into each well and keeping in incubator for 3 hours. Metabolically active, viable cells converted MTT into a colored formazan, which was solubilized with a volume of acid isopropanol equal to the volume of cell suspension. The product was then measured at 570 nm in a spectrophotometric microplate reader (PerkinElmer VICTORX4). The viability was expressed as the percentage of optical density of treated cells compared to optical density of cells treated with specific vehicles. The effective concentration to induce 50% reduction of B-ALL cell viability (IC50) derived from the equations that best fit the linear range of the dose-response curve. Each experimental condition was done in sextuplicate and repeated at least twice.

Cell proliferation and TOPRO-3 viability assay

Cell proliferation was evaluated by carboxy fluorescein succinimidyl ester (CFSE) (Life Technologies) staining. Briefly, cells were washed twice with PBS and

resuspended in 0.1% PBS-BSA, stained with CFSE (5mM) for 10 minutes in the dark at 37°C and incubate 5 minutes on ice. Then, cells were pelleted by centrifugation and washed three times with fresh media and cultured from 1 to 4 days. At the end of the experiment, cells were harvested and stained with anti-CD19-PerCP-Vio700. In order to discriminate live cell population, samples were stained with TO-PRO-3 (1μM) and analyzed by FACS. Relative cell proliferation was expressed as percentage of CFSE median fluorescence of treated cells compared to cells treated with the specific vehicle.

Measurement of ROS levels

Immediately prior to use, we prepare a fresh stock solution of carboxy-H2DCFDA (Life Technologies) in sterile dimethylsulfoxide (DMSO). Briefly, 50.000 cells were stained with 5μM cm-H2DCFDA for 30 minutes in the dark, in a conventional incubator (37°C, 5% CO2). Then we washed twice the cells with PBS and immediately measured ROS levels by flow cytometry.

Apoptosis

Apoptotic rate of B-cells was assessed using FITC-Annexin V/ Propidium Iodide (PI) staining. Briefly, cells were washed twice with PBS and then stained with APC-conjugated anti-CD19 for 15 minutes in the dark at room temperature. Cells were re-suspended in binding buffer (MiltenyiBiotec) and stained for 15 minutes with FITC-conjugated Annexin V (MiltenyiBiotec) at 1 μg/mL concentration. Cells were then analyzed by flow cytometry.

Xenograft mouse model

NOD/Shi-scid/IL-2Rγnull (NOG) mice were purchased from Taconic (Germantown, NY) and kept in pathogen-free conditions in the animal facility of the Interdepartmental Centre of Experimental Research Service of the University of Verona. B-ALL cells ($5x10^6$) were injected into the tail vein of totally irradiated

(1.2 Gy, 137173 Cesium source), 8-12-week-old mice. At day 14 post-injection, mice were assigned to one of the following treatment arms: DMSO (GSIs vehicles) or IgG isotype (0.5 mg/kg), Ara-C (100 mg/kg) + DMSO or Ara-C + IgG isotype, Ara-C + GSI-XII mg/kg) or Ara-C + anti-Notch4 (0.5 mg/kg), all administered through intraperitoneal daily injection for 3 days. In case of combined treatment, mice were firstly treated for 3 days with Ara-C followed by GSI-XII or anti-Notch4 for 3 days. Animals were sacrificed after 4 weeks from injection of cell lines, and bone marrow leukemic burden was evaluated as percentage of human CD19+ cells. To assess the effect of N-acetylcysteine (NAC) on animal outcome, mice were treated for six days with NAC (150mg/kg), starting from the first day of Ara-C administration. To exclude any toxic effect of NAC, non-transplanted mice were also treated with NAC only. Animal experiments presented here were approved by the review board of the Italian Health Ministry.

RNA silencing

RNA interference was performed using FlexiTube SiRNA (Quiagen), sequences of the siRNAs used in this work are listed here below. Transfections were performed using MACSfectin (transfection solution (MiltenyiBiotec) according to manufacturer instructions.

GENE symbol (ID)	**SiRNA ID**	**Target sequence**
NOTCH 4 (4855)	Hs_NOTCH4_6	TCGGGACTTCTGTTCAGCCAA
	Hs_NOTCH4_5	CAGATATGTAAGGACCAGAAA
	Hs_NOTCH4_3	CACAACGGGCAGTGTGAGAAA
	Hs_NOTCH4_1	TCGCTATTTAAGAACCCTAAA
NOTCH 3 (4854)	Hs_NOTCH3_5	CTGCGAGATTAATGAGGATGA
	Hs_NOCTH3_3	AAGGAATAGTTAACATCAAA
	Hs_NOTCH3_2	ATGCCTAGACCTGGTGGACAA
	Hs_NOTCH3_1	CAGCGTGACCGAGATAGGTCA

Genomic sequencing and analysis

Cell samples were collected after written informed consent, as approved by the Ethics Committee of Azienda Ospedaliera Universitaria Integrata Verona (N. Prog. 1828, May 12, 2010 - '*Institution of cell and tissue collection for biomedical research in Onco-Hematology'*). Mononuclear cells were purified by Ficoll-Paque centrifugation (Lymphoprep, Fresenius Kabi Norge AS for Axis-Shield Poc AS, Oslo, Norway), washed in phosphate-buffered saline solution (PBS) and resuspended at $10x10^6$/mL concentration in PBS. DNA was obtained from 10^7 cells by GentraPuregene cell kit (Qiagen, Hilden, Germany). Genomic DNA was quantified using the Quant-iTPicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA, USA) on a NanoDrop 3300 fluorospectrometric (Thermo Scientific, Wilmington, DE, USA). Then, 1.3 µg of DNA was sheared to 100-450 bp on a Covaris S220 instrument (Covaris, Woburn, Massachusetts, USA). Fragmentation was verified on the Agilent 2100 Bioanalyzer using a DNA 1000 assay (Agilent, Santa Clara, CA, USA). Sheared DNA was subjected to Illumina paired-end DNA library preparation using the TruSeq DNA Sample preparation kit (Illumina, San Diego, CA, USA). The quality of the library was evaluated with an Agilent High Sensitivity DNA assay (Agilent, Santa Clara, CA, USA) and then quantified using the Quant-iTPicoGreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA, USA) on a NanoDrop 3300 fluorospectrometric (Thermo Scientific, Wilmington, DE, USA). Exome capture was performed starting from 500 ng of library as input material with TruSeq Exome Enrichment Kit (Illumina, San Diego, CA, USA) following manufacturer's instruction. The quality of the whole exome library was checked with an Agilent High Sensitivity DNA assay and quantified by qPCR on a Stratagene MX3000P (Agilent, Santa Clara, CA, USA) using Kapa Library Quant kit (Kapa Biosystems, Woburn, MA).Whole exome library was sequenced with an Illumina HiSeq 1000 sequencer (Illumina Inc., San Diego, CA, USA) and 100-bp paired-end sequences were generated. Putative pathogenic variants identified in Notch genes were confirmed by Sanger sequencing. Raw reads were processed by Knome Inc. using hg19 human genome assembly as a reference. Called annotated variants were filtered using knomeVARIANTS software. "Intron", "synonymous", "non-genic" and "untranslated" options were deselected and variants passing the

quality filter was selected ("Passes quality filter" option). Only variants with a phred scaled call confidence > 50 were considered and a threshold on allele frequency < 0.05 was set. Potentially damaging variants were selected by setting a minimum effect score threshold of 0.469 and evolutionary conserved variants were selected by setting a conservation score ≥ 0.8. Next, 2 systems biology–ranking algorithms, Endeavor (http://homes.esat.kuleuven.be/≈bioiuser/endeavour/tool/endeavourweb/php), and ToppGene (http://toppgene.cchmc.org/prioritization.jsp), were used to rank the putative disease-causative genes. The softwares were trained with a custom panel of 44 genes known to be involved or related to ALL. Default prioritization parameters were used for each algorithm.

Notch gene methylation pattern

Processed methylation data publicly available were downloaded from NCBI GEO under accession number GSE49031[170]. These data represent genome wide DNA methylation levels measured using the InfiniumHumanMethylation 450k BeadChip assay (Illumina, San Diego, CA, USA). Using custom R scripts only the normalized β-values corresponding to the 10 genes of interest were retrieved (Notch1, Notch2, Notch3, Notch4, Jagged1, Jagged2, DLL-1, DLL-3, DLL-4, Hes1). The average β-values for each probe matching the genes across individuals grouped by condition was calculated and plotted using the R package ggplot2. According to the averaged β-value a single probe was classified as hypomethylated ($\beta \leq 0.2$), intermediate-methylated ($0.2 < \beta < 0.8$) or hypermethylated ($\beta \geq 0.8$).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (La Jolla, CA). Data were expressed as mean ± standard error means (SEM). Mann-Whitney and Kruskal-Wallis were used to compare 2 groups or more than 2 groups, respectively. Pearson Chi-square analysis was used to test association between variables.

IV. Results

1. Mutational and epigenetic patterns of Notch genes in human B-ALL

The pathologic role of Notch signaling in T-ALL and CLL is often linked to the mutational status of the pathway. More than 50% of patients with T-ALL present an activating mutation of *NOTCH1* [125, 126], whereas in CLL the occurrence of *NOTCH1* mutations in a subset of patients may explain the activation and therefore the oncogenic function of Notch signaling [146, 147]. To assess the presence of mutations in Notch family genes in B-ALL, we performed whole genome sequencing in samples derived from six patients. Consistently with other studies, we identified many mutations usually found in B-ALL, including those affecting NCOR1 and PAX5 genes (**Table 1**). None of the activating Notch point mutations observed in T-ALL and CLL was present in B-ALL samples. Considering the small number of patients analyzed, we compared our results with those published by other groups regarding genomic characterization of cytogenetic alterations in ALL samples [30]. We found no evidence of Notch activating mutations in B-ALL. In one patient (Patient 14) [151], we found missense variants both in *NOTCH2* (E38K) and *JAGGED1* (P871R) genes as well as a putative mutation in *NOTCH1* (K1821N); this patient with multiple aberrations in Notch components was also affected by Alagille syndrome [129, 130], a genetic disease characterized by multiple aberrations in JAGGED1 and/or NOTCH2 genes (**Table 1**). Therefore, to exclude mutations associated only with ALGS, we compared a whole genome sequencing of other patients suffering from B-ALL only. None of these patients presented any mutation associated with ALGS, such as aberrations in JAGGED1 or/and NOTCH2 (data not shown). However, none of the aberrations found in this patient led to the activation of the pathway. Aberrant DNA methylation is one of the main features of B-ALL cells and may influence the course of the treatment [98, 171]. Epigenetic status of Notch genes has been described elsewhere (GSE49031) [171]. Using bioinformatics tools, like Endeavour and ToppGene, we analyzed the change in β-value of probes corresponding to Notch genes in B-ALL cells, as

compared to normal B-cells. We observed a variation in several probes when leukemia cells at diagnosis were compared with normal B-cells (**Table 2**), thus depicting a possible epigenetic regulation of Notch genes in B-ALL. Overall, the emerging picture is that each components of Notch signaling is characterized by a processed methylation, therefore no specific receptors or ligands cannot be used as markers that can guide therapeutic decisions, whereas epigenetic deregulations may correlate with patient's prognosis, leukemia cell survival and drug resistance.

Global prioritization	Patient 40	Patient 41	Patient 42	Patient 43	Patient 44	Alagille/ Patient 14 (ALGS-PT)
1	CDH2	EPHA2	TNC	MCM2	JAG1	NOTCH3
2	ITGA1	TYK2	VAV2	CNTN1	BCR	NOTCH1
3	NCOR1	LAMA4	FLNB	AGRN	TSC2	JAG1
4	DLG1	COL18A1	HSPG2	CENTD2| STARD10	KDR	EPHA2
5	PTPRB	HSPG2	KRT18	NUMB	NRCAM	PAX5
6	KRT18	KRT18	LTBP1	PLK4	LAMA4	PTPN11
7	STK36	LAMA3	TLE2	LAMA1	C6	CHUK|ERLIN1
8	SLC25A5	NFASC	RECQL5	HABP2	LRP2	STAT1
9	IFNAR2	NCOR1	NCOR1	KRT18	KRT18	HD
10	MAPK13	BCLAF1	BCLAF1	PTPRM	LAMB2	MST1
11	DPYD	COL6A2	KRT1	CDC25C	LRP5	LAMA3
12	MAP3K12	MYH9	SVEP1	NBEA	BCLAF1	KRT18
13	ADAMTS1	LY75	CTBP2	NCOR1	NCOR1	TF
14	TNN	AKAP13	ERCC5	BCAN	CTBP2	NUMA1
15	CRIM1	STK36	MKNK2	BCLAF1	PABPC1	LTBP1
16	RBL2	PABPC1	USH2A	PAK2	CFTR	NCOR1
17	DSP	HMCN1	DLG5	STK36	RAD54B	BCLAF1
18	DUSP5	C1S	CFTR	CTBP2	C8A	SERPINA5
19	CAP1	PLXND1	USP16	PTPRB	TFR2	PABPC1
20	SETD2	RECQL	F5	PABPC1	HERC2	LIG1
>20						PIK3C2B

Table 1. List of prioritized genes. The rank of genes with potentially damaging mutations in different disease prediction algorithms and the combined results.

	Number of Probes	B-ALL VS B cell		
Genes		Up	Down	Equal
NOTCH1	63	4	25	34
NOTCH2	6	0	3	3
NOTCH3	42	11	1	40
NOTCH4	200	125	37	38
JAGGED1	24	9	1	14
JAGGED2	41	14	7	20
DLL-1	35	3	5	27
DLL-3	19	12	1	6
DLL-4	24	3	6	15

Table 2. Changes in probe β-value between B-ALL cells and normal donor B-cells. Processed methylation data publicly available were downloaded from NCBI GEO under accession 115 number GSE49031. The average β-values for each probe matching the genes across 116 individuals were calculated using the R package ggplot2. According to the averaged β-value a 117 single probe was classified as hypomethylated ($\beta \leq 0.2$), intermediate-methylated ($0.2 < \beta < 0.8$) or 118 hypermethylated ($\beta \geq 0.8$).

2. Notch components are highly expressed in B-ALL samples

We have described elsewhere the expression of Notch components in B-ALL samples. Here, we analyzed by flow cytometry the Notch expression pattern in a larger cohort of patients (n=51) (**Table 3**) and in B-ALL cell lines. We found the expression of Notch1, Notch3, Notch4, Jagged2, DLL-3, and DLL-4, in B-ALL primary cells (**Figure 1A**). Through Western blot analysis, we observed that Notch expression pattern was similar both in primary cells and B-ALL cell lines (**Figure 1A-B**). Lobry C and colleagues [172] and Kannan S and colleagues [173] previously reported that the presence of Notch receptors and ligands does not always correlate with the activation of the pathway. We then analyzed the expression of the Notch target gene *HES1* in B-ALL cell lines and primary B-ALL cells from randomly chosen patients (n=21). We observed the expression of Hes1 in B-ALL cell lines as well as in 13/21 primary B-ALL cell samples (61.9%) (**Figure 1C, 2A-B**). In addition, proteins corresponding to other Notch target genes such as *HES5*, *HEY1*, *DELTEX1*, and *C-MYC*, were also expressed in primary samples (**Figure 1C, 2A**). Treatment of cells lines with Notch inhibitors, such as SAHM1, GSI-IX, and GSI-XII, reduced the band corresponding to Hes1, thus

confirming that Hes1 expression in B-ALL cells arises at least in part from Notch activation (**Figure 2C**). We have previously shown that Notch3 and Notch4 are the main active receptors in B-ALL samples [117]. B-ALL cell line lysates were probed with anti-Notch3 (D11B8) and -Notch4 (L5C5) antibodies, which recognize full length, and the cleaved form of corresponding receptors according to manufacturer's instruction and confirmed using specific siRNA against Notch3 and Notch4 (**Figure 2D**). B-ALL cell lines expressed the active forms of Notch3 and Notch4, as confirmed by their protein level sensitivity to Notch inhibitors (**Figure 2C**). However, some samples lacking Hes1 expression still showed the presence of Notch4 active form, suggesting a non-CSL coupling of Notch4 in B-ALL cells.

	Sex	Age	Subtype and Cytogenetics	MRD after 42 days
Patient 1	F	4	Pre-B ALL	No
Patient 2	F	4	Pre-B ALL	Yes
Patient 3	M	18	Common ALL, hyperdiploidy	Yes
Patient 4	M	71	Mature B-cell ALL,t(4;11)	No
Patient 5	F	37	Common ALL	Yes
Patient 6	F	51	Common ALL, hyperdiploidy	No
Patient 7	M	71	Common ALL, NOS: 47XXY/46XY	No
Patient 8	F	13	Pre-B ALL	Yes
Patient 9	F	49	Common ALL	Yes
Patient 10	M	89	Pre-B ALL	Yes
Patient 11	M	28	Common ALL with t(9;22)	Yes
Patient 12	F	1	Common ALL	NA
Patient 13	M	43	Common ALL	NA
Patient 14	M	20	Common ALL/Alagille syndrome	Yes
Patient 15	F	3	Common ALL	No
Patient 16	M	10	Common ALL	Yes
Patient 17	M	1	Pre-B with t(19;11)	Yes
Patient 18	F	3	Common ALL/ Down syndrome	No
Patient 19	F	2	Common ALL	NA
Patient 20	M	16	Common ALL	Yes
Patient 21	F	53	CML, Blast crisis	Excluded
Patient 22	M	13	Common ALL with t(9;22)	No
Patient 23	M	62	Common ALL, NOS: 45,XY,7,t(8;12)(q13;p13)/46,XY	Yes
Patient 24	M	2	Pre-B ALL	NA
Patient 25	M	81	Common ALL with t(9;22)	No
Patient 26	F	63	Common ALL with t(9;22)	Yes
Patient 27	F	3	Pre-B ALL	Yes
Patient 28	M	25	Common ALL	Yes
Patient 29	F	52	Common ALL with t(9;22)	No
Patient 30	F	43	Common ALL	Yes
Patient 31	M	3	Common ALL	No
Patient 32	M	69	B-ALL, hyperdiploidy	Yes
Patient 33	F	2	Pro-B ALL	NA
Patient 34	F	39	Common ALL, NOS :45XY, t(3;9)(q13;p24),-20	NA

Patient 35	M	6	Common ALL	No
Patient 36	F	4	Common ALL	Yes
Patient 37	M	74	Pro-B ALL	NA
Patient 38	F	77	Common ALL with t(9;22)	Yes
Patient 39	F	7	Pre-B ALL	Yes
Patient 40	M	3	Common ALL	No
Patient 41	M	22	Common ALL	Yes
Patient 42	M	28	Common ALL	No
Patient 43	M	24	Common ALL	Yes
Patient 44	M	23	Pre-B ALL	No
Patient 45	F	3	Common ALL	Yes
Patient 46	F	3	Common ALL	No
Patient 47	M	15	Common ALL	yes
Patient 48	M	72	Pro-B ALL t (4;11)	yes
Patient 49	M	19	Pre-B ALL	yes
Patient 50	M	24	Common ALL	yes
Patient 51	M	41	Common ALL	no
Summary				
Samples	**Sex**	**Median Age**	**B-ALL Subtypes**	**Molecular Features**
51	Females = 22 Males = 29	Children (23) 6[1-18] Adults (28) 48 [19-89]	B-ALL common (37) Pre-B (10) Pro-B(3) Mature B (1)	BCR-ABL (7) Hyperdiploidy (3) Hypodiploidy (2) NOS (4) Trisomy 21(1) t(4;11) (2) Alagille Syndrome (1)

Table 3. Characteristics of B-ALL patients. B-ALL cells were obtained after informed consent from bone marrow or peripheral blood samples of 51 patients with B-ALL at diagnosis, including 23 pediatric cases and 28 adults. MRD: minimal residual diseases. NOS: No otherwise specified, NA: data not available.

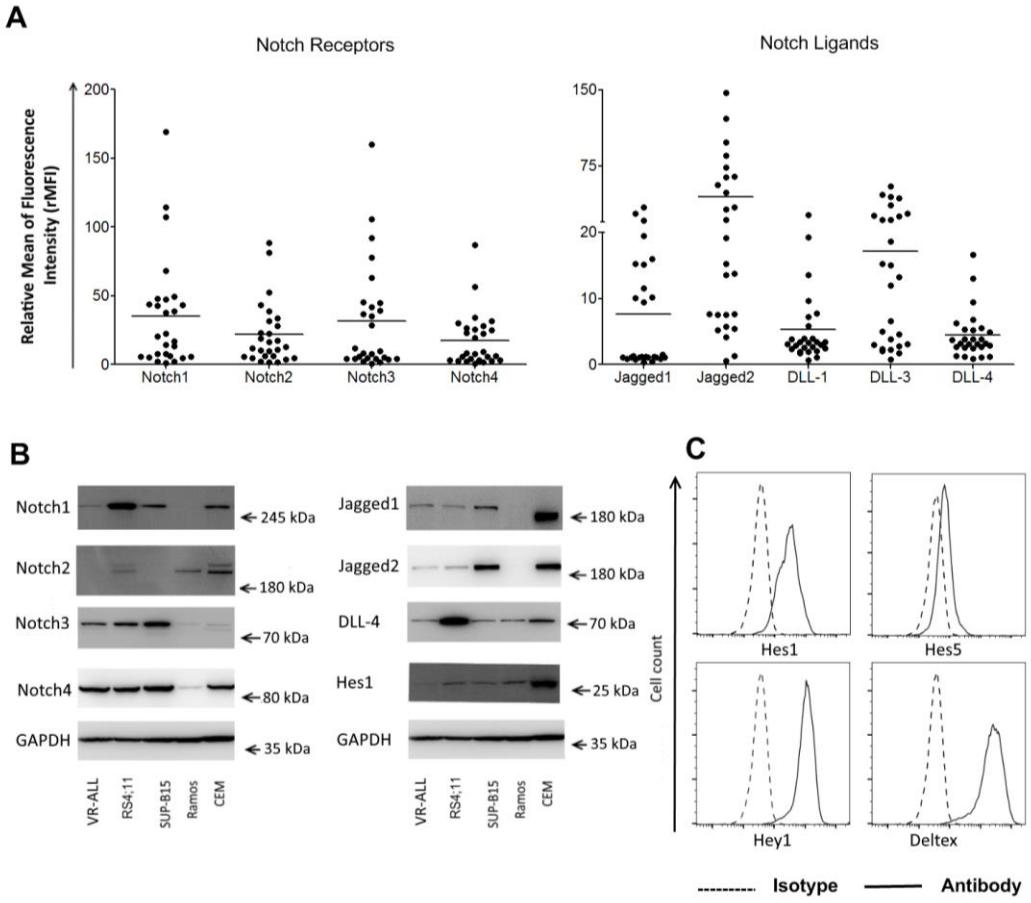

Figure 1. **Notch expression and activation in B-ALL samples.** A) Flow cytometric analysis of B-ALL cells (n = 45) using fluorochrome-conjugated antibodies specific for extracellular Notch receptors and ligands; data are represented as mean fluorescence intensity of each antibody normalized to specific fluorochrome-conjugated controls. B) Immunoblot of B-ALL cell lines (VR-ALL, RS4;11, and SUP-B15), the lymphoma cell line Ramos, and the T-ALL cell line CEM probed for Notch1-4, Jagged1-2, DLL-4, and Hes1 expression. Data are representative of six independent experiments. C) Representative expression of Notch targets (Hes1, Hes5, Hey1, and Deltex1) in primary B-ALL sample analyzed by flow cytometry using fluorochrome-conjugated antibodies Data are representative of 5 B-ALL cell samples.

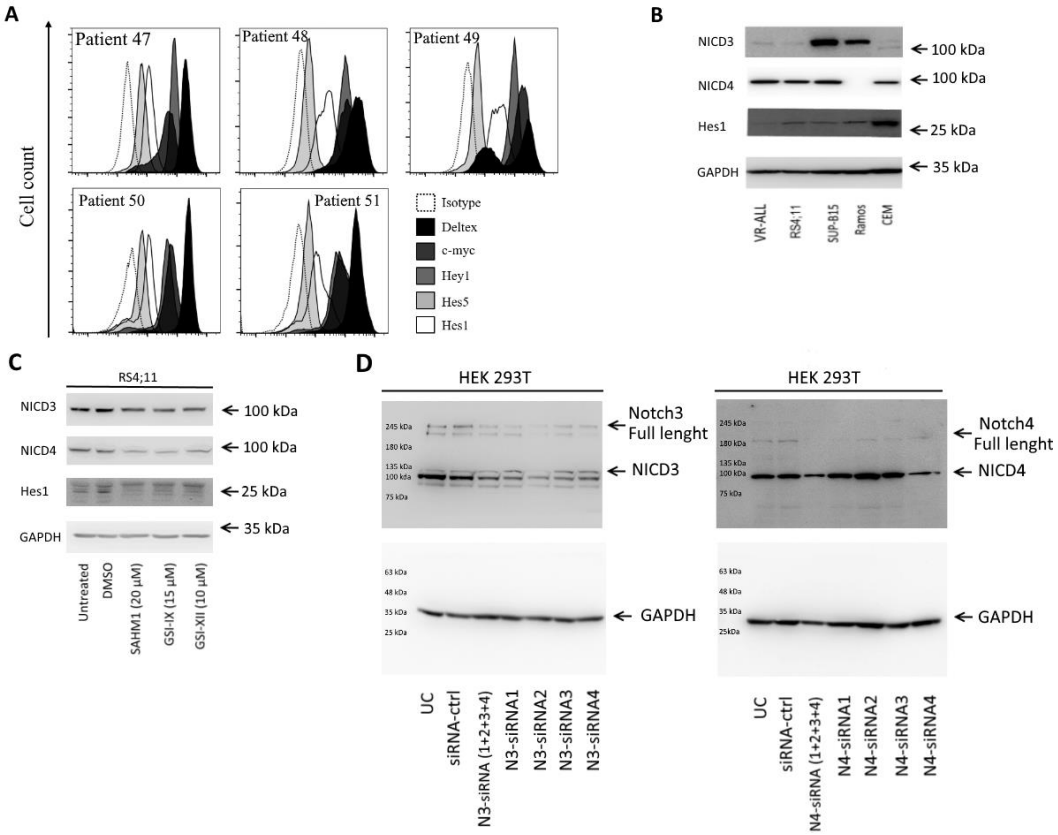

Figure 2. Notch activation in B-ALL cell lines. A) Flow cytometric analysis of Notch target gene in primary B-ALL cells (n=5) using fluorochrome-conjugated antibodies specific for each target gene. B) Immunoblot of B-ALL cell lines probed for Hes1 (D6P2U), active forms of Notch 3 (D11B8) and Notch4 (L5C5). C) Bands corresponding to Hes1 and active forms of Notch3 and Notch4 were all sensitive to treatment with Notch inhibitors. D) Immunoblot analysis of Notch 3 (D11B8) and 162 Notch4 (L5C5) in HEK 293T cells silenced for Notch3 and Notch4.

3. Notch signaling overexpression in high-risk patient group

Higher levels of Notch signaling have been described as prognostic factor in many hematologic malignancies, including, CLL and AML [144-147]. We asked whether Notch expression could correlate with response to treatment or patient's overall survival. B-ALL patient risk stratification is based on many prognostic factors, including cytogenetics, age, and white blood cell count (WBC) [18]. We did not find any correlation between Notch expression levels and cytogenetics or WBC, nor difference in Notch expression between pediatric and adult patients. A research group of St. Jude Children's Research Hospital classified as high-risk group, patients with blast cell persistence in bone marrow more than 42 days after treatment [174]. Thus, we classified cell samples into two cohorts, that is low-risk

group including patients achieving complete remission within 42 days following induction, and high-risk group, including patients with persistent leukemia cells in the bone marrow after 42 days of treatment (refractory) (**Table 3**). The analysis of Notch pathway expression in the samples collected from patients at diagnosis revealed that the high-risk group presented higher levels of Notch3, Notch4, and Jagged2 at diagnosis, as compared with the low-risk group (**Figure 3**). In addition, using Pearson Chi square analysis to seek association between Notch protein levels and drug response, we found a dependent association between Jagged1, Jagged2, and drug refractoriness (**Table 4**). These observations suggested that Notch levels at diagnosis could play a role in chemosensitivity and therefore have a predictive value for the response to treatment.

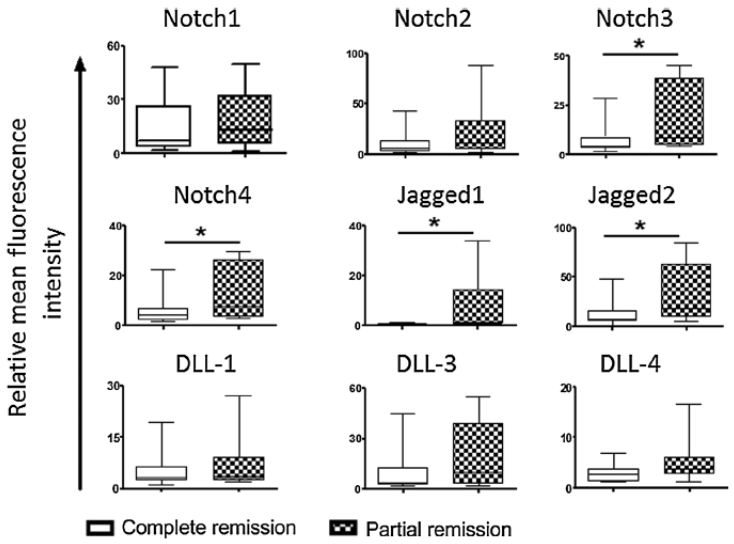

Figure 3. Notch expression as prognostic marker. Samples collected from patients at diagnosis were later classified according to treatment outcome, that is, patients responsive (11) to treatment and refractory patients (11). Then Notch expression levels were analyzed in each B-ALL sample through flow cytometric analysis. *, $p < 0.05$.

Data analyzed	Notch1			Data analyzed	Jagged2		
	Responsive	Refractory	Total		Responsive	Refractory	Total
High	23	14	37	High	9	18	27
Low	27	36	63	Low	41	32	73
Total	50	50	100	Total	50	50	100
p-value	0.0312			p-value	0.0213		
Data analyzed	Notch2			Data analyzed	DLL1		
High	9	14	23	High	9	9	18
Low	41	36	77	Low	41	41	82
Total	50	50	100	Total	50	50	100
p-value	0.2348			p-value	1		
Data analyzed	Notch3			Data analyzed	DLL3		
High	9	14	23	High	14	18	32
Low	41	36	77	Low	36	32	68
Total	50	50	100	Total	50	50	100
p-value	0,1174			p-value	0.45		
Data analyzed	Notch4			Data analyzed	DLL4		
High	9	14	23	High	14	18	32
Low	41	36	77	Low	36	32	68
Total	50	50	100	Total	50	50	100
p-value	0,1174			p-value	0.2		
Data analyzed	Jagged1						
High	0	14	14				
Low	50	36	86				
Total	50	50	100				
p-value	<0.0001						

Table 4. Relationship between Notch expression level and therapy outcome after 42 days. According to the average expression level of each specific receptor and ligand, sample were classified as high expression level and low expression level. Chi Square analysis were performed to test the association of Notch expression level and response to therapy. Data are expressed as percentage or frequencies.

4. Notch expression pattern is modulated by chemotherapy

To address a possible role for Notch signaling in B-ALL cell response to chemotherapy, we investigated the changes in the expression pattern of Notch components after chemotherapy. We have previously observed that Notch expression pattern in B-ALL cells correlates with its expression in bone marrow stroma cells [15]. Thus, considering the variability among patients, our aim was to assess whether, at any time point after treatment, bone marrow cells (including stromal cells) were characterized by reduced levels of Notch proteins. Patients achieving complete remission following chemotherapy display no or a few blast

cells in bone marrow [18, 28]. Consequently, B-ALL blast cells ($CD19^+$) cannot be used to follow the general Notch downregulation in the bone marrow following the treatment. Therefore, we focused the assessment of Notch expression by focusing on the entire non-leukemic bone marrow cell population ($CD19^+$ cells) before and after the treatment, thus showing the reduction of Notch expression because of chemotherapy (**Figure 4A**). Retrieving GSE49031 data, we observed that the epigenetic pattern of Notch genes changes from diagnosis to follow-up period, whereas patients at diagnosis and relapsed patients had the same methylation pattern (**Figure 4B**). This change in Notch expression according to the treatment status may reflect a possible role for Notch signaling in the response to chemotherapy. To clarify this issue, we treated B-ALL cell lines for 72 hours with chemotherapeutic agents, including Ara-C and Dexa. Increasing concentrations of these pharmacological agents determined significant and dose-dependent decrease in our B-ALL cell line viability. The IC50 values for each drug are shown in **Table 5.** At 24 hours of treatment, although MTT and Annexin V/ Propidium Iodide (PI) assays revealed no apoptosis in treated cells, immunoblots revealed a decrease of Notch1, Notch2, and Notch3 protein levels in samples treated with Ara-C and Dexa. Notch4 levels did not change regardless the chemotherapeutic agents (**Figure 4C**), suggesting that Notch4 could be irrelevant for drug response or, by contrast, it could be the main Notch receptor involved in chemoresistance. To assess the contribution of each pathway component to chemosensitivity, we decided to interfere with Notch molecules expression in B-ALL cells upon drug treatment**.**

Drugs	VR-ALL	RS4 ;11	SUP-B15
Ara-C	5,321e-007	8,445e-007	6,380e-007
Dexamethasone	1,193e-008	3,071e-009	4,201e-009
Doxorubicin	1,184e-008	1,648e-008	1,025e-008

Table 5. Sensitivity of B-ALL cell lines to drugs. Cells were cultured for 48h with increasing concentration of each drugs. Then cell viability was assessed by MTT assay. The viability was expressed as the percentage of optical density of treated cells compared to optical density of cells treated with the specific vehicle. The effective concentration to induce 50% reduction of B-ALL cells viability (IC50) derived from the equations that best fit the linear range of the dose-response curve. Each experimental condition was done in sextuplicate and repeated at least twice.

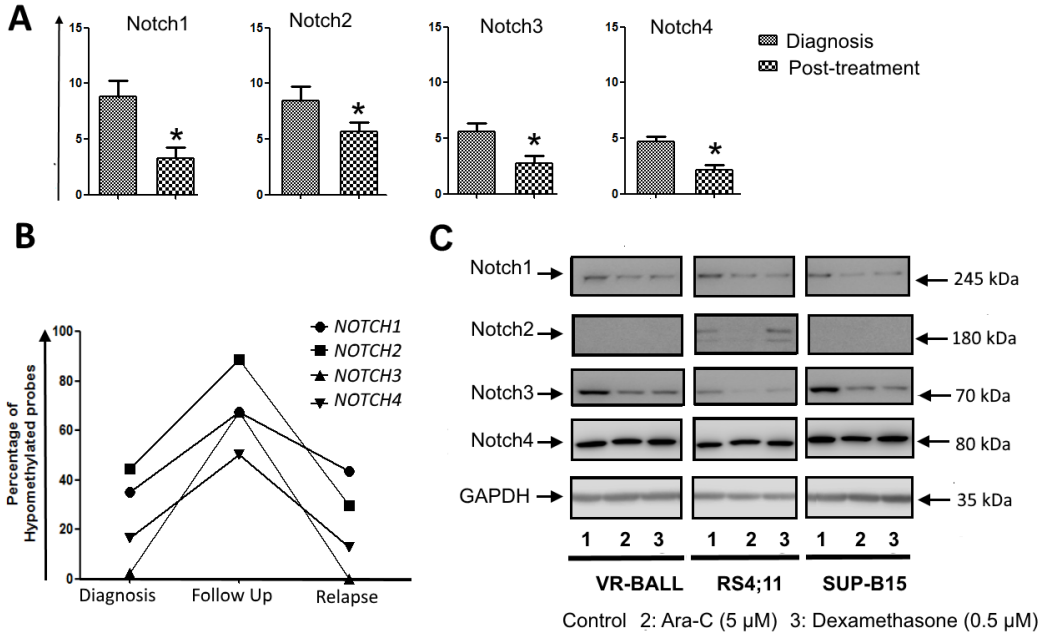

Figure 4. Treatment-induced changes in the expression of Notch receptors in B-ALL cells. A) Flow cytometric analysis of Notch expression in cell population from patient samples collected before and after the treatment. Data are represented as mean fluorescence intensity of each antibody normalized to specific fluorochrome conjugated control. B) Percentage of hypomethylated probes in samples from patients collected at diagnosis, after the treatment, or from relapsed patients. Relative hypomethylation was defined as probes with low β-value as compared to normal B cells. C) Immunoblot analysis for Notch1-4 in B-ALL cell lines treated for 24 hours with Ara-C (5 mmol/L) and dexamethasone (0.5 mmol/L). Data are representative of four independent experiments. *, $p < 0.05$.

5. Notch inhibition affects B-ALL cell survival and proliferation

We tested three categories of Notch inhibitors for their effects on B-ALL cell survival, proliferation, and drug response, that is Notch blocking antibodies, GSI-IX/GSI-XII, and a Notch transcription factor inhibitor (SAHM1). We previously demonstrated, using Western blot analysis of active form of Notch receptors as well as *RBP-Jk* reporter genes, that all these inhibitors were capable of inhibiting Notch signaling in leukemic cells [144, 151]. In this study, the concentration used for each inhibitor was enough to reduce the levels of Hes1 in B-ALL cells (**Figure 2C**). We observed that GSI-IX and GSI-XII significantly reduced B-ALL primary cell viability *in vitro* (**Figure 5A**). Although a reduction in cell viability following SAHM1 addition was observed, the effect was not statically significant (**Figure 5A**). We have previously shown that 5 mg/mL of anti-Notch3 or anti-Notch4 antibodies are enough to inhibit Notch signaling in B-ALL cell samples [15, 144].

Accordingly, anti-Notch3 (5 mg/mL) or anti-Notch4 (5 mg/mL) blocking antibodies decreased B-ALL leukemia cell survival *in vitro* (**Table 6**). We also observed a reduction in cell proliferation of B-ALL cell lines cultured in media supplemented with the Notch inhibitors (**Figure 5B**). We then generated a xenograft model of B-ALL by injecting the B-ALL cell line RS4;11 into the tail vein of NOG mice; after 4 weeks, mice showed 69% to 80% of human $CD19^+$ cells in the bone marrow (**Figure 5C**). At week 2 following injection, mice received intraperitoneal injection of GSI-IX (10 mg/kg) or GSI-XII (10 mg/kg) or their vehicle (DMSO) daily for 3 days. Only a slight, and not significant reduction in levels of human $CD19^+$ cells was observed in the bone marrow of mice treated with GSI-IX and GSI-XII (**Figure 5C**).

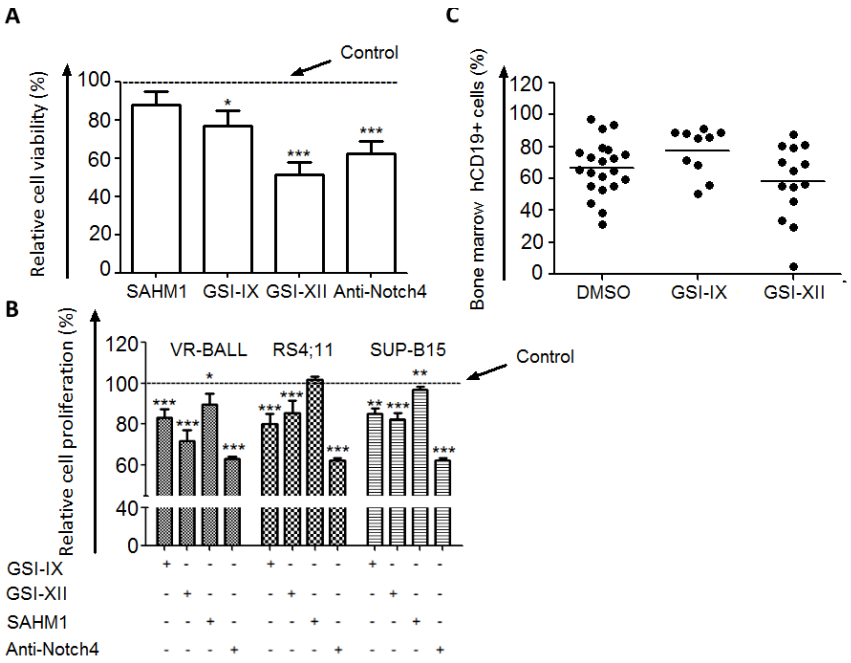

Figure 5. Notch inhibition reduces B-ALL cell proliferation and survival. A) Relative viability of primary B-ALL cell samples cultured for 4 days in presence of Notch inhibitors, that is, SAHM1 (20 mmol/L), GSI IX (15 mmol/L), and GSI-XII (10 mmol/L). Cells were collected, stained with Topro-3, and analyzed by flow cytometry; viable cells were identified as Topro-3-negative cells. Data are expressed as the mean _ SEM of four independent experiments involving 12 patient samples. B) Relative proliferation of B-ALL cell lines stained with CFSE and treated for 4 days with Notch inhibitors; CFSE dilution was analyzed by flow cytometry and is expressed as relative proliferation. Data are reported as mean ±SEM of four independent experiments performed in duplicate. C) Flow cytometric analysis of human $CD19^+$ cells in bone marrow samples obtained from mice transplanted with the B-ALL cell line RS4;11. Starting from day 14 post-engraftment, mice were treated for 3 days with either GSI-IX (10 mg/kg) or GSI-XII (10 mg/kg) or their vehicle (DMSO). The assay was performed with at least five mice in each group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

	Viable cell population (%)			
	VR-ALL	RS4;11	SUP-B15	Patients
Anti-Notch3	110.0 ± 4.624	96.62 ± 1.049 p=0.0020	99.02 ± 4.805	72.53 ± 8.107 p=0.0363
Anti-Notch4	99.68 ± 4.881 ns	81.51 ± 3.848 p=0.0020	41.37 ± 7.008 p=0.0020	70.55 ± 9.27 p=0.0467
rJagged1	110.7 ± 4.794	97.80 ± 0.9163	95.56 ± 6.708	85.40 ± 9.48
rJagged2	111.7 ± 3.213	97.40 ± 1.513	103.5 ± 4.309	84.50 ± 12.74
rDLL-4	112.8 ± 4.413	96.32 ± 1.599	112.8 ± 10.05	99.28 ± 12.11

Table 6. Notch inhibition reduces B-ALL cell survival. Relative cell viability of B-ALL primary cells or B-ALL cell lines cultured for 4 days in the presence of Notch modulators including, rJagged1 (5 µg/ml), rJagged2 (5 µg/ml), rDLL-4 (5 µg/ml), anti-Notch3 (5 µg/ml) and anti-Notch4 (5 µg/ml). Cells were collected, stained with Topro-3 and analyzed by flow cytometry; viable cells were identified as Topro-3-negative cells; relative viability was obtained as the percentage of viable cells in treated samples compared to samples treated with IgG (5 µg/ml). Data are reported as mean ± SEM of 3 independents experiments performed in duplicate and involving 12 patient samples.

6. Notch Inhibition potentiates B-ALL cell chemosensitivity

Considering the lack of significant reduction of leukemic cell burden in mouse bone marrow, we investigated the effect of the association of Notch inhibitors with chemotherapeutic agents on B-ALL cell *in vitro* survival and *in vivo* clearance. GSI-IX or GSI-XII were all capable of sensitizing B-ALL cells to Ara-C, dexamethasone, and doxorubicin treatments (**Figure 6A**), whereas SAHM1 was not (**Figure 7**). These effects were mostly mediated by Notch4, as only its blockade reproduced all the effects observed with GSI-IX and GSI-XII, thus promoting B-ALL cell apoptosis induced by all the drugs (**Figure 6A, 8**). We next investigated whether the synergy between Notch inhibition and chemotherapy could also occur in the mouse xenograft model of B-ALL. To this aim, we assessed *in vitro* the most effective combination schedule of drugs with Notch inhibitors. B-ALL cell lines were treated with Ara-C, Dexa, or Doxo associated with one of the Notch inhibitors. Results clearly highlighted that the strongest antileukemic effect was obtained *in vitro* with Ara-C plus GSI-XII combination (**Figure 7**). Therefore, 2 weeks after injection of RS4;11 in NOG mice, animals were treated for 3 days with Ara-C alone followed by 3 days of treatment with Notch inhibitors (GSI-XII, anti-Notch4) or their respective controls (DMSO, IgG isotype). Analysis of mouse bone marrow at 4 weeks showed that Ara-C significantly reduced the levels of human $CD19^+$ cells, particularly when Ara-C treatment was followed by GSI-XII or anti-Notch4

treatment (**Figure 6B**). Reduction of leukemic burden in the bone marrow correlated with increased mouse survival, with a median survival of 42, 51, and 55 days for vehicle (DMSO), Ara-C, and Ara-C+GSI-XII, respectively (**Figure 6C**).

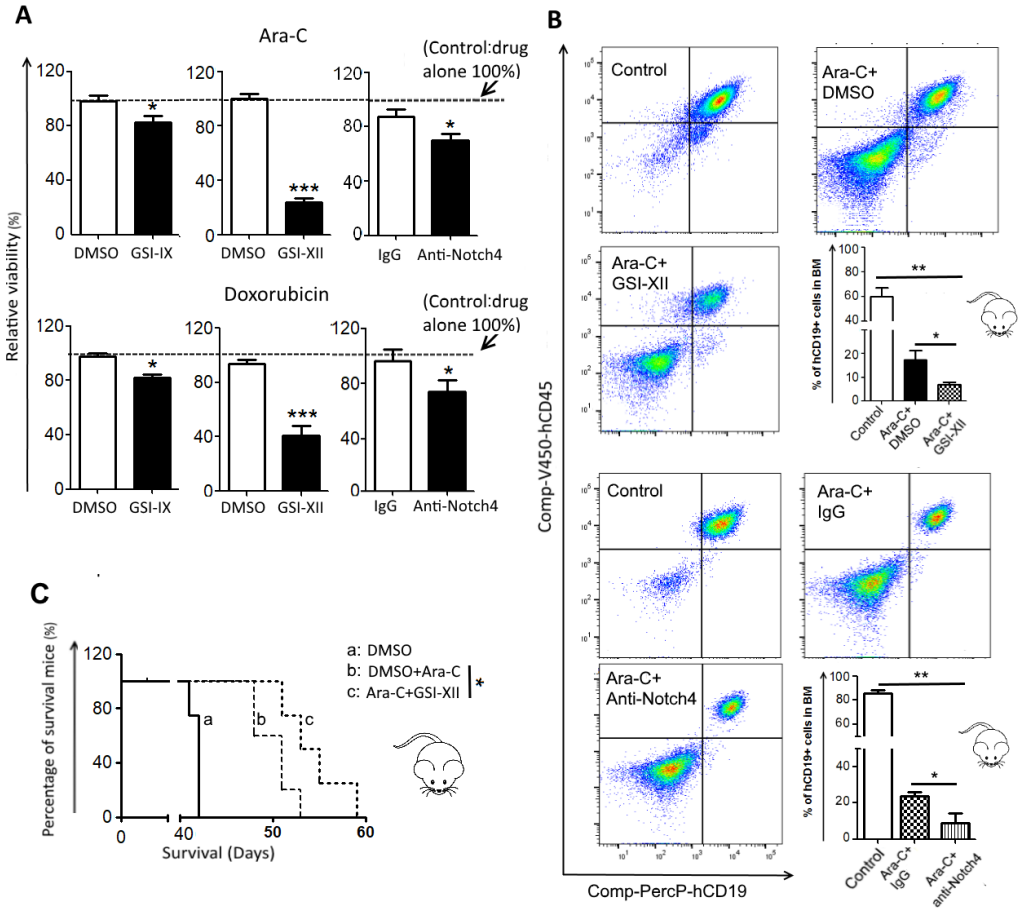

Figure 6. Notch inhibition enhances drug effects *in vitro* and in *vivo*. A) Primary B-ALL cells were cultured for 2 days in presence of Ara-C (5 mmol/L) or doxorubicin alone or in combination with Notch inhibitors, that is, GSI IX (15 mmol/L), GSI-XII (10 mmol/L), and anti-Notch4–blocking antibodies (5 mg/mL). Cells were then collected, stained with Annexin V/PI, and analyzed by flow cytometry. Data are expressed as the mean ± SEM of three to five independent experiments involving eight patient samples. B) Representative and quantitative flow cytometric analysis of human $CD19^+$ cells in bone marrow samples obtained from mice transplanted with the B-ALL cell line RS4;11. Starting from day 14 post-engraftment mice were treated for 3 days with Ara-C, followed by 3 days of Notch inhibitors (GSI-XII 10 mg/kg, anti-Notch4 0.5 mg/kg) or their respective controls (DMSO and the control isotype IgG1 0.5 mg/kg). Data are reported as mean ± SEM of values obtained from at least six mice marrow. C) Mice survival as analyzed with the log-rank (Mantel–Cox) test. The assay was performed with at least five mice in each group. *, $p < 0.05$; **, $p < 0.01$.

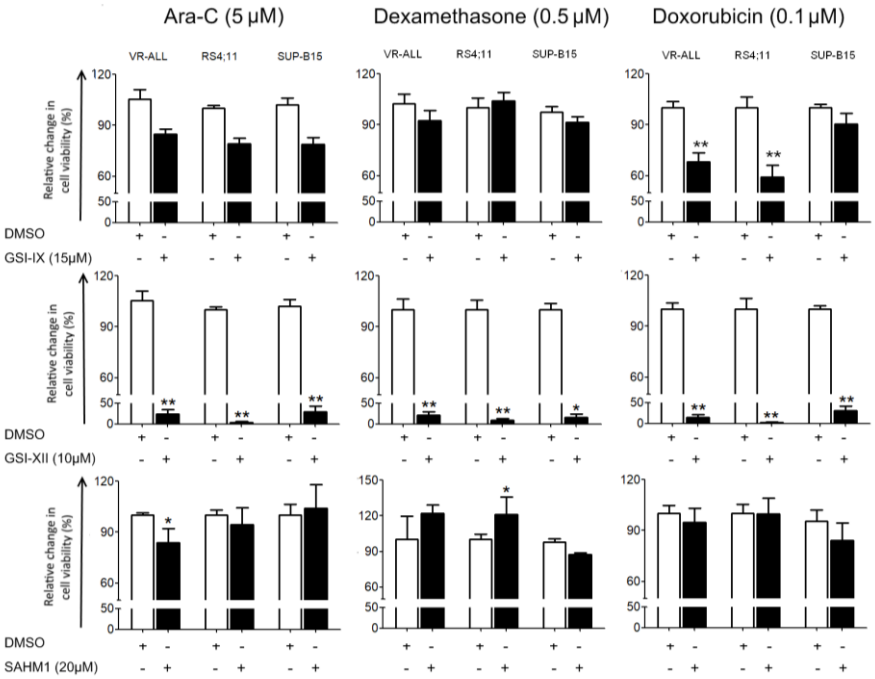

Figure 7. Notch inhibition potentiates drug efficacy. Primary B-ALL cells were cultured for two days in presence of Ara-C (5 μM), Dexamethasone (0.5 μM) or Doxorubicin (0.1 μM) alone or in association with Notch inhibitors, i.e. SAHM1 (20 μM), GSI IX (15 μM), GSI-XII (10 μM), and anti-Notch4 (5 μg/ml). Cells were then collected, stained with Annexin V/PI and analyzed by flow cytometry. Data are expressed as mean ± SEM of 5 independent experiments. *p<0.05, **p<0.01.

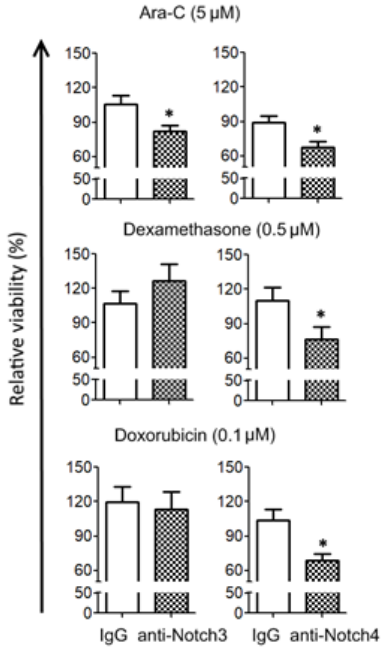

Figure 8. Contribution of each Notch receptor to drug efficacy. Primary B-ALL cells were cultured for two days in the presence of Ara-C (5 μM), Dexamethasone (0.5 μM) or Doxorubicin (0.1μM) alone or in association with Notch receptor blocking antibodies. All antibodies were used at a final concentration of 5 μg/ml. Data are reported as mean ± SEM of 4 independent experiments performed in triplicate, using samples from 8 patients: *p<0.05.

7. Notch signaling modulates drug resistance by controlling production of ROS species

Many anticancer drugs trigger cell death through the induction of ROS species [154]. We observed that pre-treatment of B-ALL cell lines with anti-oxidants, such as NAC or β-mercaptoetanol (BME), prevented cell death induced by Ara-C or Doxo (**Figure 9A**). It has been demonstrated that Notch inhibition can potentiate cell death by increasing the levels of intracellular ROS [167]. Accordingly, we found that NAC administration to B-ALL xeno-transplanted mice suppressed the survival advantage conferred by the co-administration of GSI-XII with Ara-C (**Figure 10A**). NAC administration had no effect on survival of non-transplanted mouse, excluding any toxic effect of NAC alone (**Figure 9B**). To assess whether Notch inhibition favored Ara-C- and doxorubicin-induced cell death by enhancing intracellular ROS levels, we used oxidation of c-H2DCFDA as surrogate for ROS production. We observed that cells treated with Ara-C or Doxo in association with Notch inhibitors showed increased levels of ROS, as compared with cells treated with Ara-C or doxorubicin only (**Figure 10B**). To determine whether pro-oxidant effects of Notch inhibitors were responsible for the sensitization of B-ALL cells to Ara-C and Doxo, we incubated cells with NAC or BME two hours before combined treatment with Ara-C/Doxo and Notch inhibitors. Cell death was analyzed at 48 hours using Annexin V/PI staining. Our results showed that NAC or BME treatment abrogated cell death induced by Ara-C or doxorubicin, used either as single agents or in combination with Notch inhibitors (**Figure 11A**, **9C**). To confirm this critical role for ROS in Notch inhibition-mediated cell death, we assessed through Western immunoblotting the levels of proteins capable to support the pro-survival role of Notch towards cancer cells, that is mTOR, AKT, NF-kB, and ERK1/2 [163-165]. We observed that Ara-C treatment associated with Notch inhibition through either GSIs or anti-Notch4 reduced the levels of mTOR, pNF-kB/NF-kB, and pERK1/2/ERK. Interestingly, NAC pre-treatment prevented the reduction of expression levels of pro-survival proteins in B-ALL cells incubated with Ara-C associated with Notch inhibitors. However, considering mTOR and pNF-kB/NF-kB proteins, NAC-induced rescue was different according to the type of Notch inhibitor used (GSIs or anti-Notch4), whereas a common pattern of modulation

between GSIs and anti-Notch4 was found for pERK1/2 and ERK1/2, thus suggesting that ERK1/2 may act as the main downstream target involved in ROS dependent Notch-mediated survival and chemosensitivity in B-ALL cells (**Figure 11B**).

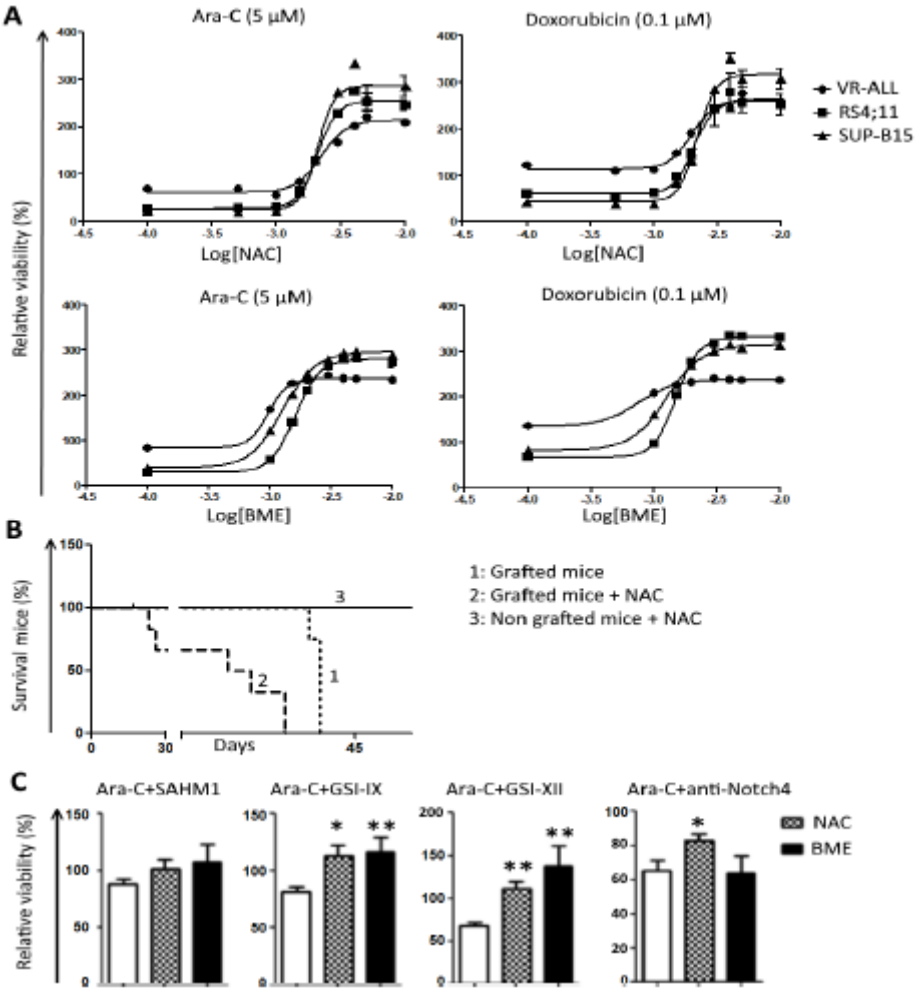

Figure 9. Drug-mediated cell death is sensitive to antioxidants. A) Relative viability, as assessed by MTT assay, of B-ALL cell lines treated for 48 hours with either Ara-C or Doxorubicin and increasing concentration of NAC or BME. Data are reported as mean ± SEM of 3 independent experiments performed in sexplicate. B) Effects of NAC (150 mg/kg) treatment on mice transplanted (grafted mice) or not 'non-grafted mice) with RS4;11 cell line and treated or not daily with NAC for 6 days starting at day 14 post engraftment. Differences in survival curves were analyzed with the Log-rank (Mantel-Cox) Test. C) Primary B-ALL cells were cultured for two days in presence of Ara-C alone or with Notch inhibitors, i.e. SAHM1 (20 µM), GSI IX (15 µM) or GSI-XII (10 µM), and with or without NAC (2 mM) and BME (2 mM). Data are expressed as mean ± SEM of 5 independent experiments. *p<0.05, **p<0.01.

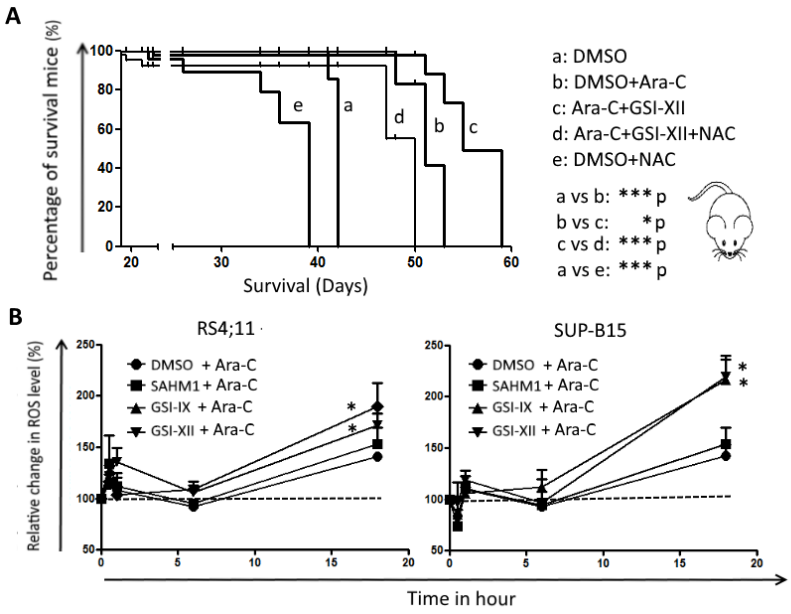

Figure 10. Notch inhibition controls redox system. A) Survival of mice transplanted with RS4;11 cell line and subdivided in five treatment groups. NAC treatment started at day 14 and lasted for 6 days. Differences in survival curves were analyzed with the log-rank (Mantel– Cox) test. B) ROS levels in B-ALL cell lines treated with Notch inhibitors (SAHM1 20 mmol/L, GSI IX 15 mmol/L, or GSI-XII 10 mmol/L) in association with Ara-C (5 mmol/L). Cells were collected at each time point and stained with 5 mmol/L of cm-H2DCFDA for 30 minutes. Fluorescence of oxidized cm- H2DCFDA was measured by flow cytometry. Data are representative of three independent experiments performed in duplicate. The control group used for statistics was Ara-C+DMSO. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

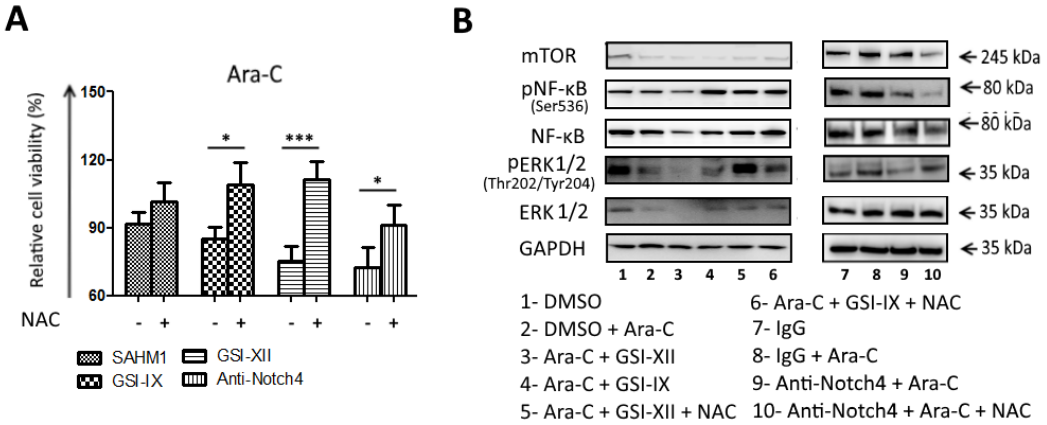

Figure 11. Notch inhibition-induced cell death is sensitive to the oxidative state. A) Primary B-ALL cell samples were cultured for 2 days in presence of Ara-C (5 mmol/L) alone or with Notch inhibitors, that is, SAHM1 (20 mmol/L), GSI IX (15 mmol/L), GSI-XII (10 mmol/L), and with or without NAC (2 mmol/L). Cells were collected, stained with Annexin V/PI, and analyzed by flow cytometry. Data are expressed as mean SEM of four independent experiments using eight different patient samples. B) Representative Immunoblot analysis of pro-survival proteins in B-ALL primary cells treated with Ara-C (5 mmol/L) alone or with Notch inhibitors, that is, GSI IX (15 mmol/L), GSI-XII (10 mmol/L), anti-Notch4 (5 mg/mL), and with or without NAC (2 mmol/L). Experiments were performed with samples from at least four patients. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

V. Discussion and future directions

The pathogenetic role of Notch signaling in leukemic diseases, such as T-ALL and CLL, has been ascribed to the presence of activating mutations in genes coding for components of the Notch pathway [125, 147]. Mutations status of Notch in these pathologies is correlated with patient's prognosis, leukemia cell survival and drug resistance [144-145]. However, Notch pathway overexpression due to paracrine signals between leukemic cells and stromal cell microenvironment may result in enhanced leukemia cell survival, as shown both in B-ALL [15] and AML cells [144, 172, 173] that normally lack activating Notch mutations [90, 112]. The reason for this abnormal expression of the Notch pathway is unclear, but Notch receptor and ligand overexpression can be a consequence of side mutations or cytogenetic abnormalities affecting other signaling systems, as it occurs in AML for β-catenin [175], Flt3 [136], and PML-RAR [176]. In AML, even in absence of Notch gene mutations, a significant expression of Notch pathway correlated with shorter patient survival [173]. Recently, some publications reported the oncogenic role of Notch to inhibit a selective growth advantage to precursors B-ALL cells, where Notch pathway genes ted to be epigenetically silenced. Briefly, *JAG1*, *HES2* and *HES4* are methylated, while *NOTCH3* and *HES5* were found preferentially hypermethylated both in B-ALL cell lines and primary B-ALL [177-178].

Accordingly, gene sequencing of six patients with B-ALL revealed the lack of any activating mutation in Notch components; according to previous studies, we found some mutations in genes associated to the Notch pathway regulation, including NCOR1, NUMB, and TCF3 [73, 171], which could be related to patient outcomes and involved in drug response and survival of leukemia cells [145]. Despite the numerosity limitations, our genomic profiling of B-ALL provides useful data to confirm some molecular vulnerabilities that could be exploited in the design of more effective targeted therapies [179, 180]. However, deeper studies are needed to pinpoint the contribution of NOTCH in the hierarchy of events driving B-cells.

In this study, we observed that B-ALL cells expressed high levels of different components of the Notch pathway. Notch1, Notch3, Notch4, Jagged2, DLL-3, and DLL-4 resulted upregulated both in B-ALL primary cells and B-ALL cell lines and

the pathway was functional, as shown by the expression of Notch target genes *HES1*, *HES5*, *HEY1*, *DELTEX1*, and *c-MYC* in B-ALL cells. The functionality of the pathway was also revealed by the sensitivity of target genes to Notch inhibitors, such as SAHM1, GSI-IX, and GSI-XII. However, an important finding is that Notch pathway promotes B-ALL cell chemosensitivity *in vitro* and *in vivo*, potentiating B-ALL cell survival, proliferation, and modulating drug resistance through the control of ROS species. We observed when analyzing samples from patients, that expression levels of all Notch receptors decreased upon chemotherapy. Consistently, changes in expression levels of Notch1-3 in B-ALL cells were associated *in vitro* to drug treatments. However, all the drugs tested *in vitro* were unable to modulate Notch4 levels in B-ALL cell lines at 24 hours, thus suggesting a critical role for Notch4 in B-ALL cell survival. To assess the specific contribution of Notch4 and other Notch receptors to B-ALL cell chemosensitivity, we studied the effects of either pan-Notch inhibitors, such as GSI-IX and GSI-XII, or specific receptor blockade in terms of cell survival, proliferation, and drug response. Even if anti-Notch4 and anti-Notch3 antibodies were all capable of lowering B-ALL cell viability, only anti-Notch4 reproduced the inhibitory effects of GSI-IX and GSI-XII by sensitizing B-ALL cells to apoptosis induced by Ara-C, dexamethasone or doxorubicin. These results raised two issues; being the only receptor whose blockade reproduces effect of pan Notch inhibitors, Notch4 appears as be the main mechanism of Notch-mediated drug resistance in B-ALL. Secondly a poor result with the Notch transcription factors SAHM1 could depict an involvement of non-canonical Notch signaling in B-ALL cell response to chemotherapy. This latter hypothesis was supported by the fact that the presence of active form of Notch4 in B-ALL cell was not always associated to the expression of Notch target genes *HES1*. Accordingly, we have recently characterized a new B-ALL cell line (VR-ALL) displaying a non-CSL dependent Notch signaling; VR-ALL cell line is sensitive to GSIs and anti-Notch4, but poorly to SAHM1 [151]. Consequently, Notch4 seemed to be the main driving mechanism of Notch-mediated drug resistance in B-ALL cells.

The challenge of frontline therapy in B-ALL is to completely eradicate leukemia cells in bone marrow after induction. Patients with no evidence of minimal residual

disease following induction show a better outcome as compared to patients with persistent leukemic clones, requiring new therapeutic strategies [18, 28]. The association between clinical status and Notch expression suggested the involvement of the Notch pathway in chemoresistance. But the validation on a larger B-ALL cohort of patients would further strengthen the prognostic value of Notch signaling monitoring. Importantly, through *in vitro* approaches, we identified Ara-C+GSI-XII as the most active combination of chemotherapeutic agents and Notch inhibitors. The effectiveness of this combination was confirmed in the xenograft model of B-ALL. We observed that GSI-XII potentiates antileukemic effects of Ara-C by reducing the bone marrow leukemic burden and prolonging the overall survival of mice undergoing xenotransplantation with the B-ALL cell line RS4;11. Preclinical and clinical use of GSIs are hampered by their high toxicity, likely due to off-target effects and the inhibition of all Notch receptors, including those not involved in the disease [28]. In this study, we showed the central role of Notch4 in B-ALL chemosensitivity and we demonstrated that association of Ara-C to anti-Notch4 blocking could lead to an antileukemic effect equivalent to the one observed with GSIs treatment. These findings may pave the way to the association of Notch inhibitors to chemotherapy in the induction regimen or during consolidation therapy with the aim of eradicating minimal residual disease. In fact, Notch inhibitors act either by potentiating the effect of drugs on specific pathways or by interfering with pathways that sustain resistance to anticancer drugs [145, 150]. The three chemotherapeutic agents used in our study have as common feature, the capability of inducing the production of high levels ROS in cancer cells, leading to apoptosis [156-158]. Emerging data revealed that leukemic cells in general present so high level of ROS that any little increase in ROS levels may induce cell death. That is why many proteins associated to ROS production such as P53 and its targets including BAX, PUMA are down regulated in cancer cells [165-166]. Data from many studies revealed that Notch signaling protects cancer cells from apoptosis by keeping low level of ROS. Inhibition of Notch being sufficient to increase levels of ROS in leukemic cells, even when P53 is mutated [167].

In our study, we demonstrated that Notch inhibitors potentiate drug-induced apoptosis by increasing ROS production and decreasing mTOR, NF-kB, and ERK

levels. Abrogation of these effects by NAC clearly showed that Notch inhibitors improve antileukemic activity of drugs through the induction of ROS.

Taken together, our data provide preclinical data in favor of the potential usefulness of the association of Notch inhibitors with conventional chemotherapy to reduce the relapse rate.

However, prior to think about clinical study, the next step consists in the generation of Notch knockdown mice model by infecting RS4;11 cell line with lentivirus particles carrying shRNA corresponding to following targets RBP-jk, MALM1 and Notch4. The engraftment efficiently of silences cells will be compared also to *in vivo* after injection in NOG mouse model. In parallel, we plan to generate some recombinant monoclonal antibodies against Notch4 to reduce the toxicity derived from Notch inhibitors *in vivo*. The recombinant monoclonal antibodies will be used to treat our xenograft model of B-ALL in combination with chemotherapeutic agents, and the progression of the disease will be monitored weekly.

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